ANTI-INFLAMMATORY EFFECT OF SINGLE AND COMBINED *Christia vespertilionis* LEAF EXTRACT AND PALM TOCOTRIENOL-RICH FRACTION

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ANTI-INFLAMMATORY EFFECT OF SINGLE AND COMBINED OF THE *Christia vespertilionis* LEAF EXTRACT AND PALM TOCOTRIENOL-RICH FRACTION

ABSTRACT

Christia vespertilionis were used traditionally to treat inflammation. Non-Steroidal Drugs (NSAIDs) are commonly prescribed to treat inflammation. However, long-term prescription of these NSAIDs will lead to adverse side-effect. This study was performed to evaluate anti-inflammatory properties of *C. vespertilionis* leaves besides purifying its protease and to determining its anti-inflammatory effect through *in vitro* and *in vivo* studies of extract combination with palm tocotrienol-rich fraction (TRF). Result showed that *C. vespertilionis* extract analysed using GC-MS contain anti-inflammatory compounds. Enzymatic protease was successfully extracted and purified through ammonium sulphate precipitation with further dialysis increases its yield by 53 % with specific activity 14.33 U/mg. The combination between *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) were tested synergistically to reduce inflammation were done through *in vitro* and *in vivo* studies and it shows that the combination of CVP and TRF (CVP+TRF) at the ratio of 14.10:8.48 µg/mL, gives 50 % inhibition (IC_{50}) of nitric oxide (NO). In conclusion, this study managed to show the synergistic effect of the combination of CVP and TRF against LPS-stimulated RAW264.7 cells and carrageenan-induced BALB/c paw oedema. The treatment could be potentially effective in reducing inflammation, and thus could be an alternative approach to NSAIDs drugs.

Keywords: anti-inflammatory, *C. vespertilionis*, protease, palm tocotrienol-rich fraction, inflammation, RAW264.7 cells and carrageenan.

KESAN ANTI-RADANG TUNGGAL DAN GABUNGAN EKSTRAK DAUN *Christia vespertilionis* DAN PECAHAN KAYA TOKOTRIENOL SAWIT

ABSTRAK

Christia vespertilionis digunakan secara tradisional untuk merawat keradangan. Ubat Bukan Steroid (NSAIDs) biasanya ditetapkan untuk merawat keradangan. Walau bagaimanapun, preskripsi jangka panjang NSAIDs ini akan membawa kepada kesan sampingan yang buruk. Kajian ini dilakukan untuk menilai sifat anti-radang daun *C. vespertilionis*, untuk menulenkan protease dan untuk menentukan kesan anti-radang melalui kajian *in vitro* dan *in vivo* dalam gabungan ekstrak. Keputusan menunjukkan bahawa ekstrak *C. vespertilionis* mengandungi sebatian anti-radang apabila dianalisis menggunakan GC-MS. Protease enzimatik berjaya diekstrak dan ditulenkan melalui pemendakan ammonium sulfat dengan langkah dialisis selanjutnya meningkatkan hasilnya sebanyak 53 % dengan aktiviti khusus 14.33 U/mg. Gabungan antara *C. vespertilionis* protease (CVP) dan pecahan kaya tokotrienol sawit (TRF) telah diuji secara sinergi untuk mengurangkan keradangan melalui *in vitro* dan *in vivo* dan ia menunjukkan bahawa gabungan CVP dan TRF (CVP+TRF) pada nisbah 14.10:8.48 µg/mL, memberikan perencatan 50 % (IC_{50}) nitrik oksida (NO). Kesimpulannya, kajian ini berjaya menunjukkan kesan sinergistik gabungan CVP dan TRF terhadap sel RAW264.7 yang dirangsang LPS dan pembengkakan tapak kaki BALB/c yang disebabkan oleh karagenan. Rawatan ini berpotensi dalam mengurangkan keradangan secara berekesan, dan dengan itu boleh menjadi pendekatan alternatif kepada ubat NSAIDs.

Kata kunci: anti-radang, *C. vespertilionis*, protease, pecahan kaya tokotrienol sawit, keradangan, sel RAW264.7 dan karagenan.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
γ	:	Gamma
δ	:	Delta
μ	:	Micro
eV	:	Electronvolt
g	:	gram
m	:	Milli
m/z	:	Mass per charge number
n	:	nano
p	:	pico
rpm	:	Revolutions per minute
s	:	seconds
%	:	Percentage
°C	:	Celcius
$(NH_4)_2SO_4$	:	Ammonium sulphate
AD	:	Alzheimer's disease
AED	:	Animal equivalent dose
AIDS	:	Acquired immunodeficiency syndrome
AIM2	:	Absent in melanoma 2
ANOVA	:	Analysis of variance
AP-1	:	Activator protein 1
APs	:	Aspartic proteases
ATCC	:	American type culture collection
ATP	:	Adenosine triphosphate

Bax	:	Bcl-2-associated X protein
BALB	:	Bagg albino (inbred research mouse strain)
cAMP	:	Cyclic adenosine monophosphate
cGMP	:	Cyclic guanosine monophosphate
CAGR	:	A compound annual growth rate
CD40	:	Cluster of differentiation 40
CI	:	Combination-index
cm	:	centimetre
CO2	:	Carbon dioxide
COX	:	Cyclooxygenase
COVID	:	Coronavirus disease
CPs	:	Cysteine proteases
CRP	:	C-reactive protein
CVP	:	*C. vespertilionis* protease
DAMPs	:	Damage-associated molecular patterns
DMEM	:	Dulbecco's modified Eagle's medium
DMSO	:	Dimethyl sulfoxide
EAM	:	Elite advanced materials
ED	:	Effective dose
EI	:	Electron ionisation
ELISA	:	Enzyme-linked immunosorbent assay
EMA	:	European medicines agency
FBS	:	Fetal bovine serum
FDA	:	Food and drug administration
GAE	:	Gallic acid equivalent
GC	:	Gas chromatography

GC-MS	:	Gas chromatography-mass spectrometry
Gibco-BRL	:	Grand island biological company
GPx	:	Glutathione peroxidase
HD	:	Human dose
HDGF	:	Hepatoma-derived growth factor
HED	:	Human equivalent dose
HMGB1	:	High-mobility group box 1
HPLC	:	High performance liquid chromatography
IACUC	:	Institutional animal care and use committee
IC_{50}	:	Half maximal inhibitory concentration
ICAM-1	:	Intercellular adhesion molecule-1
I$k\beta$-α	:	Inhibitor of nuclear factor kappa-β alpha
IL	:	Interleukin
iNOS	:	Inducible nitric oxide synthase
IP	:	Intraperitoneal
IRF	:	Interferon Regulatory Factors
JAK-STAT	:	Janus kinase-signal transducer and activator of transcription
L	:	Litre
LC/MS	:	Liquid chromatography–mass spectrometry
LD_{50}	:	Median lethal dose
LOX	:	Lipoxygenase
LPS	:	Lipopolysaccharide
LT	:	Leukotrienes
LTB4	:	Leukotriene B4
mAU	:	Milli-absorbance unit

MAPKs	:	Mitogen-activated protein kinases
MCF-7	:	Michigan cancer foundation-7
MDA-MB-231	:	Anderson - Metastatic breast 231
MS	:	Mass spectrometry
MTC	:	Medullary thyroid carcinoma
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NF-κB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIST	:	National institute of standards and technology
NK	:	Natural killer
NLRP3	:	NOD-like receptor pyrin domain-containing protein 3
NO	:	Nitric oxide
NOX	:	NADPH oxidase
Nrf-2	:	Nuclear factor erythroid 2-related factor 2
NSAIDs	:	Non-steroidal anti-inflammatory drugs
OD	:	Optical density
OECD	:	Organisation for economic co-operation and development
OH	:	Hydroxyl radicals
ONOO	:	Peroxynitrogen
PGs	:	Prostaglandins
PGE2	:	Prostaglandin E2
PRRs	:	Pattern recognition receptors
QE	:	Quercetin acid equivalent

R2	:	Rapid response
RANKL	:	Receptor activator of nuclear factor kappa-β ligand
RIPA	:	Radioimmunoprecipitation
ROS	:	Reactive oxygen species
RT	:	Real time
SD	:	Standard deviation
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SI-NET	:	Small intestinal neuroendocrine tumours
SOD	:	Superoxide dismutase
SPs	:	Serine proteases
STAT3	:	Signal transducer and activator of transcription 3
TAE	:	Tannic acid equivalent
TCM	:	Traditional Chinese medicine
TFC	:	Total flavonoid content
TGF-β	:	Transforming Growth Factor-beta
Th1	:	Type 1 T helper
THP	:	Human monocytic cells
TLR	:	Toll-like receptor
TNF-α	:	Tumour necrosis factor-alpha
TPC	:	Total phenolic content
TRF	:	Tocotrienol-rich fraction
UHPLC	:	Ultra-high-performance liquid chromatography
UK	:	United Kingdom
UKM	:	Universiti Kebangsaan Malaysia
UM	:	Universiti Malaya

USA	:	United States of America
UV	:	Ultraviolet
VCAM-1	:	Vascular cell adhesion molecule-1
WHO	:	The world health organisation

CHAPTER 1: INTRODUCTION

1.1 Background study

Inflammation is a common disease and it has been the most cause of death if not treated at an early stage. It has been shown that inflammation is intimately linked to all stages of the onset and spread of malignancy in the majority of cancers (Zhao *et al*., 2021). Inflammation can be described as an infection or irritation of living cells or tissue produced by injury and the response towards it (Amazu *et al*., 2010). Inflammation shows various symptoms such as redness, swelling, heat, pain and lack of tissue function, as response to the injury. The body will then instinctively activate its defence systems, starting with the immune system's reaction by release inflammatory mediators such as cytokines and chemokines. As a result, the damaged tissue develops membrane modification, increases protein denaturation and increases vascular permeability. As a form of natural defence known as the chemotaxis mechanism, the body instinctively releases kinins, prostaglandins and histamine (Cotran *et al*., 1994; Ruiz-Ruiz *et al*., 2017).

Inflammation can develop into acute or chronic depending on the inflammatory reactions in different organs like the heart, pancreas, liver, kidney, lung, brain, digestive tract and reproductive systems when pathogens present. There are two phases of inflammation which is acute and chronic. Acute inflammation is the first stage of inflammation, during which the immune system attempts to eliminate damaging stimuli immediately. Usually, this stage ends with the restoration of normal body and cell activities. However, chronic inflammation develops when the acute phase does not heal completely leading to a prolonged inflammatory response (de Almeida & Dufour, 2022). This ongoing inflammation can cause various health issues and is often linked to diseases such as rheumatoid arthritis, heart disease, stroke, obesity, diabetes and certain cancers. Managing chronic inflammation typically involves lifestyle changes, medications and

addressing the underlying causes to reduce the inflammatory response and promote healing. Three out of five people in the world die due to chronic inflammatory diseases (Ghasemian *et al*., 2016; Barcelos *et al*., 2019; Deepak *et al*., 2019; Tsai *et al*., 2019).

Therefore, addressing inflammation promptly during the acute phase is the best course of action as lipopolysaccharides (LPS), strong activators of the immune system detected by pattern recognition receptors (PRRs) on macrophage surfaces, can exacerbate the inflammatory response (Linlin *et al*., 2018). The main receptor responsible for this detection is Toll-like receptor 4 (TLR4), in conjunction with its co-receptor MD-2 (Gauthier *et al*., 2022). LPS also initiate signalling pathway upon binding to TLR4-MD-2 complex. This pathway involves various adaptor molecules and protein kinases, leading to the activation of transcription factors like nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1). Activation of NF-κB and AP-1 leads to the transcription of genes encoding pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and others which are potent mediators of inflammation (Ciesielska *et al*., 2021; Kim *et al*., 2023). The cytokines are then released from the macrophages into the surrounding tissue and exhibit inflammatory response by increase vascular permeability and also recruiting additional immune cells such as neutrophils, monocytes, dendritic cells and lymphocytes to the site of infection (Soliman & Barreda, 2023).

The cytokines serve as biomarkers and can be used to evaluate how well therapeutic interventions, particularly those targeting inflammation (Carrero *et al*., 2008; Machowska *et al*., 2016; Tu *et al*., 2022). Cytokines can either promote or reduce inflammation, depending on the signalling pathways they activate. They act in regulating and modulating the inflammatory response in order to maintain immune homeostasis and prevent excessive tissue damage (Al-Qahtani *et al*., 2024). The examples of pro-inflammatory cytokines are TNF-α, IL-1 and IL-6 which promote immune cells, induces

fever and stimulates the production of inflammatory mediators (Aggarwal, 2003; Dinarello, 2011; Scheller *et al*., 2011). While, anti-inflammatory cytokines such as IL-10, IL-4 and Transforming Growth Factor-beta (TGF-β) suppresses the production of pro-inflammatory cytokines, promotes wound healing and inhibits excessive inflammation (Li & Flavell, 2008; Saraiva & O'Garra, 2010; Kaplan, 2019). Additionally, high-mobility group box 1 (HMGB1), cyclooxygenase (COX), and inducible nitric oxide synthase (iNOS) are enzymes that play key roles in inflammation. By inhibiting these enzymes, the production of inflammatory mediators such as prostaglandins and nitric oxide can be reduced (Nisar *et al*., 2023).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been used commonly to suppressing inflammation in clinical settings. However, these medications have negative side effects such gastric intestinal mucosa, heart diseases and kidney problems (De Groot & Scott, 2007). Furthermore, it only effective at one-time consumption in relieving symptoms (Debnath *et al*., 2013). In fact, NSAIDs, including aspirin, ibuprofen and diclofenac are able to relieve fever, pain and inflammation in short term by inhibit inflammatory mediators, particularly enzymes cyclooxygenase (COX) (Praveen *et al*., 2010). About 8% of hospital admissions in the US have adverse side effects using synthetic drugs and Malaysia is not an exception (Philomena, 2011; Atanasov *et al*., 2021). Side effects can vary depending on factors such as individual differences in metabolism, underlying health conditions, dosage, duration of use and interactions with other medications or substances. The Malaysian government regulates through agencies like the National Pharmaceutical Regulatory Agency (NPRA) to ensure safety, efficacy and quality of pharmaceuticals (Park *et al*., 2022). However, despite regulatory efforts, adverse reactions to synthetic drugs can still occur due to prolonged exposure. Previous studies have also demonstrated that synthetic drugs frequently cause patients to develop various disorders and chronic health issues including arthritis, diabetes, obesity, cancer

and cardiovascular diseases (Nisar *et al*., 2023). Because of this, the clinical usage of these drugs has been restricted, and focus has shifted to natural substances (Shafiq *et al*., 2005; Nesaretnam & Meganathan, 2011). Plant-derived drugs that have been prescribed since the century can thus be an option. The World Health Organisation (WHO) has also recommended this alternative to address the lack of safe synthetic medication options (Pan *et al*., 2010).

Plants have been used medicinally for thousands of years due to their pharmacologically active phytochemicals, which belong to a variety of chemical classes and have significant anti-inflammatory properties (Nunes *et al*., 2020). Some such examples are colchicine (alkaloid), escin (triterpenoid saponin), capsaicin (methoxy phenol), bicyclol (lignan), borneol (monoterpene) and quercetin (flavonoid) (Hartmann, 2007; Hounsome *et al*., 2008; Patil *et al*., 2019). Over 250,000 to 1,000,000,000 plant species have been studied for their phytochemical characteristics globally over the years (Habila *et al*., 2011; Corlett, 2016). The bioactive substances present in plants are thought to be crucial for human health and capable of preventing and treating diseases and up until now, the scientific community has shown a lot of interest in them (Dahanukar *et al*., 2000). Researchers have recently focused a lot of their study on medicinal drugs derived from natural sources. Since they are more readily available, more convenient, more safe than synthetic treatments, many pharmaceutical companies are now concentrating on producing plant-derived medications (Burke *et al.*, 2005; Apu *et al*., 2012). In accordance with data provided by the World Health Organisation (WHO), estimated at 65 to 80 % of individuals residing in developing nations, including Malaysia rely predominantly on medicinal plant as their primary healthcare needs, demonstrating the crucial role of natural sources including plant in modern medicine (Calixto, 2005; Gao *et al*., 2019).

Plants have been found to possess phytochemicals that can regulate anti-inflammatory pathways, either by enhancing the production of anti-inflammatory cytokines or by

inhibiting the production of pro-inflammatory cytokines, thereby offering therapeutic targets for inflammation treatment (Liu *et al*., 2017). Anti-inflammatory properties have been detected and documented in various of plant such as *Curcuma longa* (turmeric), *Zingiber officinale* (ginger), *Rosmarinus officinalis* (rosemary) and *Olea europaea* (olive) (Ghasemian *et al*., 2016). There are many studies involving anti-inflammatory properties tested in medicinal plants, and their mechanisms of pharmacological intervention have been proven reduce inflammation-associated diseases through many experimental studies and clinical trial (Allegra, 2019; Nisar *et al*., 2023). Latest study shows that Apigenin (APG) is found in *Chamaemelum nobile* (L.) possessed anti-inflammatory effect by inhibiting activation of transcription factors like nuclear factor kappa B (NF-κB) and reducing the production of cytokines like IL-1β, IL-6, TNF-α including inducible nitric oxide synthase (iNOS expression) and malondialdehyde (MDA) (Jung *et al*., 2016; Gentile *et al*., 2018). Other study also showed quercetin from *Malus domestica* successfully to reduced iNOS including cytokines such as IL-6 and IL-12 (Bhaskar *et al*., 2016; Lu *et al*., 2018). There are some phytochemicals that have been through clinical test and effective as anti-inflammatory drugs such as colchicine, escin, capsaicin, bicyclol, borneol and bromelain. These anti-inflammatory drugs able to target the specific molecular to modulate the mechanisms so that it can reduced inflamed tissues and preventing swelling (Nisar *et al*., 2023).

People have been reported that *Christia vespertilionis*, often known as butterfly wing and "Daun rama-rama" in Malaysia for having anti-inflammatory and anti-cancer qualities (Osman *et al*., 2017). It was discovered by Joao de Loureiro and the species was recognised as *Hedysarum vespertilionis*, and now known as *Christia vespertilionis* (Whiting, 2007). This plant has widely used as medicinal purposes for a number of diseases. By crushing the leaves, it is reported to treat tuberculosis, scabies, snake bites and bone fissures. The plant also can be consumed by mixing the leaves with water to

treat bronchitis, cold muscle weakness, tonsils inflammation and to enhance blood circulation. These practices have been applied by local community or cultural group in decades for treating various health conditions (Bunawan *et al*., 2015; Das, 2016; Ariff *et al*., 2019; Chen *et al*., 2019; Ibrahim *et al*., 2022). This plant believes to having various medicinal values and emerging well to this date which the significant reasons for choosing this plant (Dar *et al*., 2017). This plant has two distinct leaf colours, one with green leaves and the other with red-coloured foliage (Ravindran *et al*., 2019). Green leaves, however, are consumed for therapeutic purposes which is the application of medical treatments to improve health outcomes in individuals with diseases. In Malaysia few years back until now, the green leaf type has increasingly gained popularity and demand due to testimonial reports by local community of its effectiveness to cure inflammation and cancer (Norazhar *et al*., 2021). Additionally, green leaves demonstrate diverse biological activities such as antioxidant, anti-inflammatory, antimicrobial, antidiabetic and anticancer properties, attributed to the presence of various compounds including alkaloids, flavonoids, terpenoids and phenolics (Zambari *et al*., 2023). In contrast, red leaves are primarily used for decorative purposes and lack substantial research evidence regarding their pharmacological activities (Ravindran *et al*., 2019). Therefore, green *C. vespertilionis* leaves hold significant promise for therapeutic applications due to its potential as a source of bioactive compounds with medicinal properties compared to red leaves.

Numerous investigations have demonstrated that *C. vespertilionis* contains phytochemical substances such as phenols, alkaloids, triterpenes, fatty acids, alkanes and long-chained alcohols (Bunawan *et al*, 2015; Dash, 2016; Smitha & Jain, 2019; Ariff *et al*., 2019). There is a study that discovered compound such as linoleate in *C. vespertilionis* extract and detected anti-inflammatory activities, which inhibit LPS-induced iNOS and COX-2 expression including cytokines (Osman *et al*., 2017). Flavonoid also most of the

common compound found in *C. vespertilionis* extract that has been proven its anti-inflammatory properties which it can decrease the activity of cytokines, chemokines and inflammatory enzymes (Al-Khayri *et al*., 2022; Ibrahim *et al*., 2022). In addition to these compounds, protease enzymes found in *C. vespertilionis* may further contribute to reducing inflammation by breaking down pro-inflammatory proteins and modulating immune responses. Proteases are enzymes that break down proteins into smaller peptides or amino acids by catalysing the hydrolysis of peptide bonds. In extracting compounds from natural sources, protease enzymes help degrade proteins in the mixture and release non-protein compounds (Banik *et al*., 2018; Lemes *et al*., 2021). Protease is important for biological functions, including infection and physiological processes. It also widely utilised in pharmacology, biotechnology and industrial operations. To determine a purified fraction contains only protease, the common technique to use is SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) by separates proteins based on their size alongside known protein standard (Nowakowski *et al*., 2014). Other method is by measure activity assay of the protease and determine if the observed activity can be attributed solely to the protease of interest. By employing these techniques, the purity of a purified protease would ascertain and obtain as protease enzymes are the target product to combat inflammatory diseases (Troncoso *et al*., 2022). It has proven from previous study that protease decreased paw oedema size significantly in carrageenan-induced acute inflammation model, which success to treat inflammation (Viswanatha Swamy & Patil, 2008).

Purified compounds are often study because they can interact synergistically with other compounds to produce therapeutic effects (Vaou *et al*., 2022). Thus, another plant that used for this study is palm tocotrienol-rich fraction. This plant's phytochemical components have been researched and it contains vitamin E which is comprises of tocotrienols and tocopherols (Qureshi *et al*., 1986). It is commonly found in vegetable

oils, nuts, seeds, and whole grains, with tocotrienols being particularly abundant in *Elais guineensis* palm fruit (Sen *et al*., 2006). Tocotrienols is vitamin E consist of alpha (α), beta (β), gamma (γ) and delta (δ) that grouping by the numbering and location of methyl substitutions on the chromanol ring (Atia *et al*., 2013). This mixture of Vitamin E called as tocotrienol-rich fraction (TRF) and mostly can be found in palm oil for almost 75 % and the rest of it is tocopherols (Nesaretnam *et al*., 2007; Zaini *et al*., 2016). TRF is obtained from palm oil through esterification, distillation, crystallisation and chromatography (Sundram & Gapor, 1992). In 2010, palm tocotrienol-rich fraction (TRF) which consists of all the isomers has been granted the GRAS (Generally Regarded as Safe) status by the US Food and Drug Administration (US FDA), which indicating palm TRF safe for human consumption (Ramanathan *et al*., 2018). TRF scientifically has potent antioxidant, anti-inflammation and anti-cancer based on previous studies. Traditionally it used to reduce wrinkles, improving skin elasticity and treating sun burn (Ghazali *et al*., 2022). It also can be used to promote hair growth and treat alopecia by inhibit oxidative stress, as tocotrienol is strong antioxidant (Beoy *et al*., 2010). Tocotrienols have since attracted interest due to their higher antioxidant activity than tocopherols. This has led to extensive research into its potential health advantages and increased interest in its therapeutic uses, particularly for the treatment of inflammation. In other investigations, tocotrienols were found to have strong anti-inflammatory effects via blocking major inflammatory pathways that involve nuclear factor-κB (NF-κB), and signal transducer and activator of transcription 3 (STAT3) (Kwang *et al*., 2007; Bachawal *et al*., 2010). Pre-clinical studies show tocotrienols suppress pro-inflammatory mediators, suggesting that they may be effective against disorders linked to inflammation (Khor *et al*., 2021).

There also study combination TRF with other compounds has increase the efficacy with other inflammatory compounds. Then, the combination treatment of TRF and

protease enzymes could potentially enhance anti-inflammatory effects, offering a more effective approach to managing inflammation-related disorders. Protease plant extract has also demonstrated anti-inflammatory properties. Both plants may contain hundreds of phytochemicals with various of biological activities (Borges *et al*., 2018; Zhang *et al*., 2019). These compounds may interact to produce more effective positive effects than those produced by each one alone. In fact, combinations of compounds reduce obesity, oxidative stress and ameliorate osteoporosis (Rayalam *et al*., 2011). Most combination treatments of different plants have synergistic anti-inflammatory effects. Cheung et al. (2009) reported that curcumin inhibited LPS-induced inflammation in RAW 264.7 cells when combined with sulforaphane or phenethyl isothiocyanate in a synergistic manner. This was demonstrated by a reduction in nitric oxide (NO), TNF-α, IL-1, inducible nitric oxide (iNOS) and COX-2 protein expression. Therefore, combining two treatments could improve the effectiveness of the inflammatory treatment. There are several potential benefits to combining two treatments, particularly plants. First, it can enhance therapeutic effect due to each plant has different mechanism pathway, which make them complement together and increase the effectiveness in treating inflammation. Second, using different plant combination increases interaction and reducing the risk of treatment resistance. Third, the plant combination become safer and less side effects. Fourth, it increases bioavailability, where the dosage of treatment in combination plant is faster to enters systemic circulation blood to accessing the site of inflammation.

Treatment combinations widely used in most critical diseases such as cancer, tuberculosis, malaria and acquired immunodeficiency syndrome (AIDS) (Chou, 2010). Treatment combinations may have additive, antagonistic or synergistic effects. However, the creation of medication combination aims to create synergistic effect that increase efficacy (Chait *et al*., 2007). The Chou-Talalay (1984), is a method for treatment combination based on the median-effect equation, using a combination index (CI)

theorem that comes with quantitative definition of synergism (CI < 1), additive (CI = 1) and antagonism (CI > 1). Numerous studies have shown that using pharmacological combinations is a viable strategy for treating a number of illnesses including cancer, inflammation and type 2 diabetes (Keith *et al*., 2005; Fitzgerald *et al*., 2006; Feala *et al*., 2010). The effectiveness of natural product combinations against inflammation and other disorders has been investigated in a number of research employing molecular and pharmacological processes. The biological substances that having anti-inflammatory effects are mediated by important regulator molecules such cytokines, nitric oxide and other mediators. Tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and neutrophil-derived free radicals, nitric oxide (NO) acts as a part of inflammatory mediators in pathogenesis inflammation (Salvatore *et al*., 2001; Volpe *et al*., 2014). TNF-α is a cytokine produces from monocytes, macrophages and T cells which triggered in the presence of pathogens and at the same time induces IL-6. IL-6 important to eliminate pathogens and restore damaged tissues through the activation of immune responses. Nitric oxide inhibits the pathogens as well. However, the excessive of mediator's production could lead to development of various inflammatory diseases and involves in various pathophysiological processes such as neuronal communication, dilatation of blood vessels and neurotoxicity (Moncada *et al*., 1991; Bogdan *et al*., 2000; Nakagawa & Yokozawa, 2002; Joo *et al*., 2014).

Complex processes set off by microbial pathogens are what cause inflammation to become pathological. Cytokines, chemokines and inflammatory mediators are expressed as a result of the activation of mitogen-activated protein kinases (MAPKs), nuclear factor-*k*B (NF-*k*B) and interferon regulatory factors (IRFs) during inflammation (Gordon & Taylor, 2005). Reducing activation signals in active macrophages has been suggested as a treatment approach for a variety of inflammatory illnesses. The lipopolysaccharide (LPS) may potently activate macrophages and generate a variety of pro-inflammatory

mediators including IL-1, TNF-α, PGE2 and nitric oxide (NO) (Chen *et al*., 2016; Khan *et al*., 2017). Recent years have seen a rise in the use of LPS-stimulated RAW264.7, a murine macrophage cell line as an *in vitro* inflammatory model for the screening of potent anti-inflammatory treatments and the exploration of the anti-inflammatory mechanisms (Zhou *et al*., 2022). A study by Trinh et al. (2020), has been investigated the immune-stimulating potential of herbal mixture by using RAW 264.7 macrophages (Guillermo & Albert, 2014; Linnerz & Hall, 2020). When RAW264.7 macrophages are exposed to LPS, it triggers the activation of various signalling pathways, including the NF-κB pathway. This activation leads to the upregulation of cytokines and chemokines. In an *in vitro* study, RAW264.7 macrophages are exposed to LPS to trigger activation of signalling pathways like NF-κB, leading to the upregulation of cytokines (e.g., TNF, IL-6) and chemokines (e.g., CCL2, CXCL8), which are analysed to understand their roles in immune response.

The significance of transitioning to an *in vivo* animal study lies in the ability to observe the complex interactions and systemic effects of cytokines and chemokines within a living organism, providing a more comprehensive understanding of their roles in inflammation and disease, which is essential for developing potential therapeutic strategies. The carrageenan-induced paw oedema model is a well-established and widely utilised experimental approach for studying acute inflammation *in vivo*. Animal inflammatory tests usually utilise BALB/c mouse because it has an immunological response that resembles a human being (Xu *et al*., 2012; Wu *et al*., 2016). The carrageenan-induced paw oedema model has been shown to share similarities with certain aspects of human inflammatory conditions, such as acute inflammation and oedema. Even though animal models cannot perfectly replicate human disease, this model provides valuable insights into the initial stages of inflammation and the effectiveness of potential anti-inflammatory treatments. It helps bridge the gap between pre-clinical research and potential clinical

applications. Inflammatory reactions after carrageenan injection into mice's paw have recently been shown to be linked to the creation of prostaglandins (created by the action of COX-1 and COX-2), and nitric oxide (NO) derived from eNOS and iNOS (Rocha *et al*., 2006; Mansouri *et al*., 2015). The inflammatory response induced by carrageenan is consistent and reproducible, making it a reliable model for investigating inflammation. It involves a series of complex cellular and molecular events, including the release of inflammatory mediators, infiltration of immune cells and increased vascular permeability.

In conclusion, finding new compounds or combinations of compounds with anti-inflammatory properties is a great significance in developing potential therapeutic inflammation interventions. Understanding the effects of *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) on inflammation can contribute to the development of novel plant-based medical treatments for inflammatory conditions as an alternative to synthetic drugs. The effects of CVP and TRF, both individually and in combination, can be assessed through *in vitro* and *in vivo* studies to determine potential synergistic effects. This comprehensive approach helps validate the compound benefits and enhances the understanding of their anti-inflammatory properties.

1.2 Research problems

Inflammation is when the body's defence mechanisms play a role in the healing process. If inflammation goes unchecked, it can progress to chronic inflammation, leading to various diseases such as diabetes, arthritis and cardiovascular diseases. Therefore, it is important to manage inflammation at early stage of acute. The most common prescribed anti-inflammatory medication is non-steroidal anti-inflammatory medications (NSAIDs) such as aspirin. However, utilising these drugs has been associated with a number of damaging effects, particularly on the gastrointestinal, haematological and kidney systems. Therefore, medicinal plants are become the alternative due to their effectiveness in

reducing inflammation with fewer side effects compared to NSAIDs. There are many choices of plants with anti-inflammatory properties, but in this study, *Christia vespertilionis* was chosen. Traditionally, locals have used this plant for centuries, often crushing its leaves into a paste to apply on injuries to reduce inflammation and prevent fever (Hofer *et al*., 2013). Most studies focus on extracts of *Christia vespertilionis*, yet the protease enzymes from this plant are also has more ability to reduce inflammation (Jadhav *et al*., 2020). Proteases have demonstrated effectiveness in reducing symptoms of inflammatory conditions, often using aspirin as a positive control in studies (Viswanatha Swamy & Patil, 2008). Therefore, the purified protease from *C. vespertilionis* holds significant potential as a plant-derived anti-inflammatory treatment, offering a promising alternative to conventional therapies.

There are also study combination of protease with other compounds that increase the efficacy of inflammation treatment (Déciga-Campos *et al*., 2021). Therefore, palm tocotrienol-rich fraction (TRF), the strong isoforms of vitamin E derived from palm fruit which is well-known for its antioxidant and anti-inflammatory properties has been selected to combine with purified *C. vespertilionis* protease (CVP). There have been numerous published investigations on the anti-inflammatory of the palm tocotrienol-rich fraction (Wu *et al*., 2008; Yam *et al*., 2009; Malavolta *et al*., 2018; Khor *et al*., 2021). However, no studies have investigated the anti-inflammatory effects of CVP or its potential synergistic effects when combined with TRF. Using LPS-stimulated RAW264.7 macrophages and carrageenan-induced BALB/c paw oedema, the aimed is to analyse the anti-inflammatory effects of CVP, and whether the combination with TRF has achieved synergistic effect and increased the effectiveness to reduce inflammation. This work is intriguing to investigate in order to develop novel therapeutic drugs from natural sources as an alternative to NSAIDs.

1.3 Research questions

1. Does *C. vespertilionis* leaf extract possess anti-inflammatory properties?
2. Does protease purified from *C. vespertilionis* leaf reduce inflammation?
3. Could *C. vespertilionis* protease combined with palm tocotrienol-rich fraction enhance the potential to reduce inflammation?

1.4 Objectives

1. To identify anti-inflammatory properties of *C. vespertilionis* leaf extract.
2. To characterise protease from *C. vespertilionis* leaf extract.
3. To investigate the anti-inflammatory effects of *C. vespertilionis* protease (CVP) and palm tocotrienol rich fraction (TRF) in single and combination treatment in LPS-stimulated RAW264.7 cells.
4. To evaluate the selected potential anti-inflammatory treatments of single and combination treatment of *C. vespertilionis* protease (CVP) and palm tocotrienol rich fraction (TRF) in BALB/c mouse model.

1.5 Significance study

The effects of combination treatment on inflammation, particularly acute level is occasionally studied. No studies have been found about protease purification of *Christia vespertilionis* leaves, including the combination of it to palm tocotrienol-rich fraction. This plant-derived treatment can be acted as an alternative to synthetic drug. Synthetic drugs usually could have negative side effects when used over an extended period. In this study, the effects of purified *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) to be compared, in single and combination treatment on treating inflammation through cell culture and animal studies. The findings of this study will help to improve the therapeutic development to reduce inflammation in a safest way as an

alternative to synthetic drugs. The results offer significant knowledge regarding the phytochemical compounds and biological effects of *C. vespertilionis*. The benefits of isolating a compound through protease purification are highlighted. The study aims to explore whether combining two plant-derived treatments can achieve synergistic effect and enhance their ability to reduce inflammation.

CHAPTER 2: LITERATURE REVIEW

2.1 Inflammation

Inflammation is a mechanism the body uses to defend itself against many harmful agents such as germs, infections and toxins (Mitchell & Cotran, 2003; Chen *et al*., 2019). The role of inflammation in the body's defence and healing mechanisms was recognised from the studies of Metchnikoff and others in the 19th century (Cavaillon, 2021). In addition, inflammation is seen to be the foundation of pathology because the alterations it causes are a sign of illness and injury (Hurley, 1972). Acute and chronic are the two categories under inflammation. In particular, acute inflammation brought on by *Staphylococcus aureus* infection of the skin (the common boil), while chronic inflammation leading to remodelling of the artery wall in atherosclerosis, the bronchial wall in asthma and chronic bronchitis, and crippling destruction of the joints in rheumatoid arthritis (Punchard *et al*., 2004).

2.1.1 Acute inflammation

Acute inflammation is when the body rapidly responds to tissue injury, infection or other harmful stimuli in order to protect the body from further damage or to eliminate the cause of injury or infection immediately and initiate the healing process. Acute inflammation is typically short duration and resolves once the underlying cause is addressed. However, in some cases, acute inflammation may not completely resolve where other triggering factors may persist leading to a prolonged or recurring inflammatory response (Akiyama *et al*., 2000; Libby *et al*., 2002). When inflammation becomes chronic and unresolved, it can then lead to tissue damage and contribute to disease progression. Disease that is linked to the chronic level includes cancer, Alzheimer's disease, arthritis, atherosclerosis, diabetes, autoimmune disorders and

cardiovascular conditions (Balkwill & Mantovani, 2001; Patil *et al*., 2019; Wang *et al*., 2020).

Therefore, acute inflammation should subside once the healing process is complete and the threat has been eliminated. However, this condition could lead to chronic inflammation due to a few reasons. First, the resolution mechanisms work efficiently, but in the end failed to resolve. Second, the initial cause of acute inflammation persists cause the immune system's response may remain active. For example, in chronic respiratory conditions like chronic bronchitis or asthma, repeated exposure to irritants or allergens can perpetuate the inflammatory response. Meanwhile, in certain autoimmune diseases, the immune system mistakenly attacks healthy tissues, leading to acute inflammation. If the autoimmune response is not adequately controlled, it can become chronic, causing sustained inflammation and tissue damage.

On top of that, during acute inflammation, immune cells generate reactive oxygen species (ROS) to combat pathogens. However, if ROS production is excessive or not adequately controlled, it can cause cellular damage and contribute to chronic inflammation. Immune cells like macrophages, lymphocytes and neutrophils can accumulate at the site of inflammation. These cells release pro-inflammatory mediators that can sustain the inflammatory process, and the persistent presence of immune cells will lead to chronic inflammation. There also can be an imbalance in the production and regulation of cytokines. Excessive production of pro-inflammatory cytokines can promote chronic inflammation.

2.1.2 Chronic inflammation

Chronic inflammation is often linked with various steps of tumorigenesis and recognised as the existence of various types of cancer (Aggarwal *et al*., 2006). In fact, the primary cause of the onset and development of COVID-19 pneumonia, which can have

fatal consequences, is likewise inflammation (Zingaropoli *et al*., 2021; Zhou *et al*., 2022). Managing chronic inflammation is crucial for preventing disease progression and promoting overall health and well-being. It is a persistent and prolonged inflammatory response that can occur in various tissues and organs in the body, lasting for a long time typically weeks, months or even years. Chronic inflammation can be harmful to the body and is linked to many diseases, in contrast to acute inflammation, which is a temporary and natural response to damage or infection.

Chronic inflammation has been linked to an increased risk of certain cancers. The continuous inflammatory state can promote genetic mutations and cell proliferation that contribute to cancer development (Greten & Grivennikov, 2019). It usually can affect the systemic system of the body, contributing to systemic conditions such as atherosclerosis (hardening of arteries), and neurological impacts such as Alzheimer's and Parkinson's. Chronic inflammation is also associated with cardiovascular complications which is an increased risk of heart disease and stroke due to its role in atherosclerosis and plaque formation.

2.1.3 Epidemiology of inflammation

Inflammatory diseases are the most significant cause of death in the world, particularly in the level of chronic. The World Health Organisation (WHO) ranks chronic diseases as the greatest threat to human health. In the United States, 60% of Americans had at least one chronic condition, 42% had more than one and 12% of adults had 5 or more chronic conditions in 2014 (Kumar, 2021). According to the Ministry of Health Malaysia (2020), it was estimated that 20% of infected women in Malaysia develop chronic pelvic inflammatory disease (PID). Of these cases, 50% were mild, 40% were moderate and 10% were severe. Meanwhile, psoriasis which is a chronic inflammatory skin condition, affects 2-3% of the population in Malaysia. Women are more likely to develop the disease

at a younger age compared to men (Affandi *et al*., 2018). The World Health Organization (WHO) reports that rheumatoid arthritis, a chronic inflammatory autoimmune disorder mainly targeting the joints, affects approximately 0.3-1% of the world's population, with a higher prevalence in developed countries. In recent years, inflammatory bowel disease increasingly becoming a concern mainly in developing regions due to it affecting the quality of life (Li *et al*., 2023). The inflammatory lung disease called as chronic obstructive pulmonary disease (COPD) is resulting from exposure to harmful gases or particles, like cigarette smoke. It ranks among the leading causes of morbidity and mortality globally, contributing to around 3.2 million deaths in 2015 (Agarwal *et al*., 2023). Asthma, one of the chronic inflammatory disorders of the airways, affects over 300 million people across all ethnicities and age groups (Soriano *et al*., 2015; Ferrante & La Grutta, 2018). These statistics highlight the significant global impact of chronic inflammatory diseases, underlining the urgent need for effective management and treatment strategies.

2.1.4 Immune cells

Immune cells are known as leukocytes or white blood cells. It acts a crucial part in the body's immune system by defending against infections, diseases and foreign invaders (Marshall *et al*., 2018). Various immune cells are involved in the inflammatory process, enhancing the immune response and contributing to tissue damage. Immune cells involved in inflammation include macrophages, lymphocytes, neutrophils and mast cells. Macrophages are large white blood cells that engulf and digest cellular debris, foreign substances and microbes. Macrophages can release pro-inflammatory cytokines and reactive oxygen species, leading to tissue damage. Lymphocytes which include T and B cells are responsible for adaptive immunity and play a critical role in the immune response against specific pathogens and antigens. Neutrophils are a type of white blood cell that

play a vital role in the initial response to infection. The prolonged presence of neutrophils can contribute to tissue damage through the release of toxic molecules. Mast cells are immune cells present in connective tissues to release histamines and other chemicals that promote inflammation and contribute to allergic responses.

2.1.5 Mechanisms of cell death in the immune system

Cell death is a complex process contributing to the immune system that can occur through various mechanisms and it plays a critical role in inflammation (Loftus *et al*., 2022). The two main forms of cell death associated with inflammation are apoptosis and necrosis. Additionally, a recently characterised form of cell death called pyroptosis has emerged as an important mechanism in inflammation.

2.1.5.1 Apoptosis

Apoptosis is a highly regulated process that occurs for various reasons throughout the life of a cell. Apoptosis plays an important role during development and tissue remodelling by eliminating unwanted or unnecessary cells. Apoptosis helps maintain the appropriate balance of cell populations within tissues and organs by eliminating excess or surplus cells (Riwaldt *et al*., 2021). Cells may undergo apoptosis in response to severe deoxyribonucleic acid (DNA) damage, cellular stress or exposure to harmful agents such as radiation or toxins. Apoptosis serves as a protective mechanism to prevent the propagation of damaged or potentially harmful cells that could pose a risk to the organism. By eliminating these cells, apoptosis helps maintain genomic stability and reduces the risk of cancer development. Apoptosis plays a role in regulating immune responses by eliminating immune cells at the end of an immune reaction or in response to signals indicating that the immune response is no longer needed. This helps prevent excessive or prolonged immune activation, which can lead to tissue damage and autoimmune diseases. In some cases, apoptosis can occur as part of pathological processes, such as in response

to infection, inflammation, or as a mechanism of cell death in certain diseases. Dysregulation of apoptosis can contribute to the pathogenesis of various conditions including neurodegenerative diseases, autoimmune disorders and cancer. The morphological characteristics of apoptosis are membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, loss of adhesion to neighbour cells and extracellular matrix followed by phagocytosis of the fragments by nearby cells (Kandouz, 2024). Meanwhile, the biochemical changes of apoptosis are involving activation of proteases (caspases), chromosomal DNA cleavage into internucleosomal fragments, phosphatidylserine externalisation and intracellular substrate cleavage by proteolysis (Green, 2022).

2.1.5.2 Necrosis

Necrosis is a form of cell death characterised by cell swelling, membrane rupture and release of cellular contents into the extracellular space, leading to inflammation and tissue damage. Unlike apoptosis, necrosis is often associated with pathological conditions, such as ischemia, trauma and infection. Necrosis is intricately linked to inflammation and plays a significant role in the pathogenesis and progression of many inflammatory diseases. The uncontrolled release of intracellular contents during necrosis triggers immune responses, leading to acute and chronic inflammation, tissue damage and the perpetuation of disease processes. Understanding the mechanisms of necrosis and its inflammatory consequences is crucial for developing therapeutic strategies to reduce inflammation and tissue injury in various diseases. Necrosis occurs due to several factors such as physicochemical trauma and also during viral and bacterial infection (Khalid & Azimpouran, 2023). Necrosis also associated with activated angiogenesis, reduced vascular maturation and presence of vascular invasion. Necrosis is considered the primary form of cell death caused by inflammation. However, the inflammatory reaction produced

from the necrotic cell was reported to promote cancer cell growth because the immune cells which react to the inflammation produced essential cytokines to nurture the surviving cancer cells (Gong *et al*., 2019).

When cells undergo necrosis, they release various intracellular molecules known as damage-associated molecular patterns (DAMPs) into the extracellular space. DAMPs include proteins, nucleic acids and other cellular components that are normally sequestered within healthy cells (Murao *et al*., 2021). Once released, these molecules are recognised by pattern recognition receptors (PRRs) on immune cells, such as macrophages and dendritic cells, leading to the activation of inflammatory signalling pathways. The release of DAMPs and other pro-inflammatory molecules from necrotic cells activates immune cells, which then produce and release cytokines, chemokines and other inflammatory mediators (Roh & Sohn, 2018).

Persistent or recurrent necrosis can lead to chronic inflammation. Chronic inflammation occurs when the inflammatory response is prolonged or unresolved, leading to ongoing tissue damage and repair. This can result in fibrosis (scarring), loss of tissue function and the development of chronic inflammatory conditions. Examples include chronic obstructive pulmonary disease, atherosclerosis and rheumatoid arthritis (Pahwa *et al*., 2023). Necrosis-induced inflammation can cause secondary tissue damage as a result of the release of proteolytic enzymes, reactive oxygen species (ROS) and other cytotoxic substances by activated immune cells. This secondary damage can exacerbate the original injury, create a cycle of cell death and inflammation and contribute to the progression of inflammatory diseases. Infections by certain pathogens such as bacteria, viruses and fungi can induce necrosis as a part of their pathogenicity.

Necrotic death is associated with an inflammatory response. Necrotic cells release factors like high mobility group box 1 protein (HMGB1) and hepatoma-derived growth

factor (HDGF) (Nikoletopoulou *et al*., 2013; Khalid & Azimpouran, 2023). These factors are sensed by a nod-like receptor protein 3 (NLRP3), which is a core protein of the inflammasome (Fu *et al*., 2020). This results in inflammasome activation and causes the release of the pro-inflammatory cytokine interleukin-1β (IL-1β). NLRP3 inflammasome activation is triggered mainly through adenosine triphosphate (ATP) produced by mitochondria released from damaged cells (Kelley *et al*., 2019).

2.1.5.3 Pyroptosis

While necrosis is an uncontrolled, non-programmed form of cell death caused by severe cellular injury, the pyroptosis is a form of programmed cell death specifically designed to combat infections, characterised by the activation of inflammasomes and caspases, leading to the release of pro-inflammatory cytokines and inflammatory cell death. The term "pyroptosis" was first proposed in 2001, which comes from the Greek root's "pyro" and "ptosis", meaning fever and fall, respectively, to describe this novel cell death in macrophages infected by *Salmonella* (Cookson & Brennan, 2001). Pyroptosis primarily mediated by inflammasome activation and gasdermin-mediated pore formation. It plays a crucial role in the immune response to infections but can also contribute to the pathology of various inflammatory diseases when dysregulated (Wei *et al*., 2022).

Pyroptosis is characterised by the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18). This release promotes an intense inflammatory response, attracting immune cells to the site of infection or injury (Rao *et al*., 2022). The process involves the activation of gasdermin proteins particularly gasdermin D, which form pores in the cell membrane. These pores disrupt the membrane integrity, causing cell swelling and lysis, and ultimately leading to cell death. Pyroptosis is typically triggered by the activation of inflammasomes, multi-protein complexes that

sense pathogenic microorganisms or danger signals. Inflammasomes such as NOD-like receptor pyrin domain-containing protein 3 (NLRP3), absent in melanoma 2 (AIM2), activate cysteine-aspartic acid protease-1 (caspase-1) and sometimes caspase-4, -5, or -11, which then cleave gasdermin D to initiate pyroptosis. The rupture of the cell membrane during pyroptosis leads to the release of intracellular contents including DAMPs and inflammatory cytokines into the extracellular space. This release further amplifies the inflammatory response (Wang *et al*., 2022).

2.1.5.4 Induction of apoptosis by medicinal plants

Immune cells, such as macrophages and dendritic cells, frequently undergo apoptosis in response to infection or cellular damage, contributing to the immune response to the site of infection or injury. Targeting apoptosis in inflammatory cells, can be a part of the mechanisms through which phytochemicals from medicinal plant act as anti-inflammatory agents. Apoptosis plays a crucial role in regulating the immune response and inflammation (Pumiputavon *et al*., 2017).

Several studies have explored the role of apoptosis in regulating inflammation and have investigated the potential of inducing apoptosis in inflammatory cells as a therapeutic approach for inflammatory diseases. The search for a therapeutic agent that can induce apoptosis mode of cell death to treat inflammation has been an indispensable approach by many studies as apoptosis is one of the important markers of potential anti-inflammation drugs. In this regard, researchers have identified that some medicinal plants and their bioactive compounds capable of inducing apoptosis. Necrosis is an alternative cellular suicide pathway if the normal apoptosis is blocked or defected. However, the inflammatory reaction produced from the necrotic cell was reported to promote cancer cell growth because the immune cells produced essential cytokines to nurture the surviving cancer cells (Vakkila & Lotze, 2004). Meanwhile, the inflammatory reaction

produced from the pyroptosis cell particularly in chronic inflammation may not be suitable due to its potential to worsen inflammation and tissue damage. Instead, therapeutic strategies that promote apoptosis or other non-inflammatory forms of cell death might be more appropriate. For general inflammation, particularly chronic and systemic inflammatory diseases, apoptosis is often more suitable as a therapeutic target. Apoptosis provides a way to resolve inflammation without triggering further inflammatory responses and helps maintain tissue homeostasis (Bock & Riley, 2022).

Recent findings suggested that natural compounds have the capability to stimulate apoptosis in inflammation cells. A few examples of the phytochemicals that are capable of inducing apoptosis are epigallocatechin gallate (EGCG) from green tea, resveratrol from the skin of red grapes and curcumin from the rhizome of *Curcuma* species (Mohammad *et al*., 2015). EGCG was reported to induce apoptosis by inhibiting proteasome activity thus led to the accumulation of inhibitor of nuclear factor kappa-β alpha (NF-*k*B*α*) and cyclin-dependent kinase inhibitor 1B (p27) protein that eventually cause growth arrest (Kazi *et al*., 2003). Meanwhile, resveratrol and curcumin induce apoptosis by increasing the sensitivity of resistant cancer cells and elevate pro-apoptotic factor, Bcl-2-associated X protein (Bax) respectively (Cotino-Nájera *et al*., 2023). In combination therapy, where phytochemicals are used alongside conventional drugs or other phytochemicals, the targeting apoptosis can enhance the overall therapeutic efficacy. For example, a combination of phytochemicals such as quercetin and sulforaphane with chemotherapeutic drugs which is cisplatin and 5-fluorouracil against cervical cancer cells (HeLa) were more effective in inducing apoptosis compared to the individual compounds, as seen by the increased sub-G0 population in cell cycle analysis (Sundaram *et al*., 2019).

2.1.6 The role of immune cells in inflammation

The main immune cells such as neutrophils, basophils, mast cells, T-cells, B-cells and others are involved in inflammation processes. Numerous extracellular mediators and regulators such as cytokines, growth factors, eicosanoids (prostaglandins and leukotrienes), complement and peptides, all play a role in regulating these activities. When there is inflammation, the body react due to imbalance inflammatory mediators by releasing nitric oxide (NO), prostaglandin E2 (PGE2) and cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) which lead to macrophages activation to kill foreign invaders like bacteria and pathogens and this process is repetitive until injury cure (Choy & Panayi, 2001; Cheon *et al*., 2006).

Macrophages are referred to as an immediate defence against foreign substances, release immune responses and it induces the production of various biological mediators to fight inflammation (Moncada *et al*., 1991; Joo *et al*., 2014). Macrophages can kill pathogens directly by phagocytosis and indirectly via the secretion of pro-inflammatory factors such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). They are responsible for processes such as antigen processing and presentation to antigen-specific T cells and this indicates that the activation of macrophages might be a hopeful strategy to resist diseases (Sun *et al*., 2016). Nitric oxide (NO) acts as biological mediators and as an agent in the immune system for body defence mechanisms. It is also important for blood vessels to function well in the vascular system (Rao *et al*., 2016). Nevertheless, it is however a free oxygen radical and toxic in pathological mechanisms, particularly in inflammatory disorders (Dawn & Bolli, 2002). The activation of immune cells is often triggered by various biological mediators released during infection or injury. Lipopolysaccharide (LPS) is one of the biological mediators and major pathogenic component of the cell wall of Gram-negative bacteria that activate macrophages and induced dendrites (Hedger *et al*., 2005; Winnall *et al*., 2011; Palladino *et al*., 2018). By

using LPS to induce macrophages, scientists can gain valuable insights into immune system functioning, inflammation mechanisms and potential treatments for inflammatory and infectious diseases.

Today, more people are aware that inflammation plays as an aggressive process and as a restorative, healing process. Inflammation is the whole cycle of events that begins with the activation of a response and ends with healing and the return of the tissue or organ to its normal state of appearance and function. A chronic inflammatory state, which could last the entirety of the person's life, develops in some diseases, yet there seems to be no resolution. This includes the inflammatory diseases retinitis, multiple sclerosis, psoriasis, osteoarthritis, inflammatory bowel diseases and atherosclerosis. It takes an interdisciplinary approach to investigate inflammation. To understand the processes involved in triggering and maintaining inflammatory states, traditionally it has been necessary to research the immune system. Today, it is acknowledged that the genetic and molecular biological basis of cellular responses are crucial for identifying genetic predisposition to inflammatory diseases, while pharmacological studies are required to identify targets and create novel therapies to mitigate chronic and life-threatening inflammatory conditions. As a result, research into inflammation also examines the immunological and cellular responses that are involved, as well as the pharmacological procedure involved in medication development.

2.1.7 Mechanisms and mediators of inflammation

Inflammation involves the release of various inflammatory mediators, which are molecules that regulate and amplify the inflammatory response. Some key inflammatory mediators include cytokines, chemokines, prostaglandins and leukotrienes (Radovanović *et al*., 2023). Cytokines are small proteins that act as chemical messengers between cells. They can promote or inhibit inflammation. Examples of pro-inflammatory cytokines

include interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). Chemokines are a subgroup of cytokines that attract immune cells to the site of inflammation. They play a crucial role in directing the movement of immune cells to areas of tissue damage or infection. Cytokines are critical in modulating the immune response and inflammation. In addition to cytokines, other important inflammatory mediators include prostaglandins and leukotrienes. Prostaglandins are lipid molecules, derived from fatty acids and play a significant role in promoting inflammation and pain. Similarly, leukotrienes also are lipid mediators that contribute to inflammation, particularly in allergic and asthma-related inflammatory responses.

Pro-inflammatory mediators like prostaglandins are produced from arachidonic acid by the action of cyclooxygenase (COX) isoenzymes. Prostaglandins improve vascular permeability, allow leukocytes to infiltrate and then promote the creation of granulomas and tissue healing during inflammation. Meanwhile, arachidonic acid metabolites, adhesion molecules, cytokines, chemokines and platelet-activating factors are all produced by cells and encourage chemotaxis (Eddouks *et al*., 2012). Chemotaxis, a process known to cause leukocytes to go in the direction of inflammatory regions. The presence of other biochemical mediators such as cytokines (Interleukin-1, Interleukin-6 and tumour necrosis factor-alpha), kinases (p38 kinase, JNKs and MAP kinase), transcription factors (NF-κB) and matrix metalloproteinases (MMPs) as well as activated cellular components leads to inflammation (Kulkarni *et al*., 2006). Plasma-derived complement proteins, kinins and the coagulation system cause the excretion of inflammatory mediators. Leukotrienes (LTs), prostaglandins (PGs) and 12-hydroxyeicosatetraenoic acid (12-HETE) are arachidonic acid metabolites that have a significant role in the development of inflammatory disorders such as asthma, arthritis and cancer (Roome *et al*., 2008).

The production of the inflammatory response is significantly influenced by prostaglandins. They aid in the development of the primary symptoms of acute inflammation and their production is markedly elevated in inflamed tissue. Cyclooxygenase (COX) was activated by arachidonic acid to produce prostaglandins (PG), whereas lipoxygenase (LOX) produced leukotrienes (LT). Interleukin-6 (IL-6) and other cytokines that control immune responses and the inflammatory process, such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), are produced as a result of leukotriene B4 (LTB4), neutrophil activation and superoxide generation. Proteolytic enzymes are released by pro-inflammatory cells that have been stimulated by mediators like cytokines, acting as protein breakdown on the skin or inside the body when there is inflammation. Leukocytes are released by elastin, a component of proteins like collagen, proteoglycans, and immunoglobulins, and it acts as a stimulus for pro-inflammatory mediators. Additionally, oxidative stress and inflammation are intimately linked, and both have a pathophysiological role in a number of disorders. Reactive oxygen species (ROS) play a crucial part in the defence systems of cells. ROS causes oxidative stress by initiating intracellular signalling pathways and elevating the expression of genes that promote inflammation (Kumar *et al*., 2014; Biswas, 2016). The activation of pro-inflammatory genes has been considered to be driven by inflammatory stimuli through a transcription factor such as nuclear factor-kappa beta (NF-κB). NF-κB acts to regulate the transcription of inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α), along with the genes encoding cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), cell adhesion molecules, immune-receptors and growth factor receptors (Patil *et al*., 2019).

2.1.8 Models used in inflammation research

Anti-inflammatory activities involve developing a model that mimics, or induces the production or exhibits the release of the biochemical mediators of inflammation, and monitoring the response of these biochemicals to the test drugs. Today, the rationale for the usage of medicinal plants has been validated through *in vitro* and *in vivo* experiments (Radovanović *et al*., 2023). The models of *in vitro* and *in vivo* can be used to assess anti-inflammatory activity of plant extracts and synthetic drugs.

2.1.8.1 *In vitro* assay

In vitro, anti-inflammatory activity study involves cell culture techniques in which the enzymes and the inflammatory mediators are directly exposed to the test drugs. Lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages derived from BALB/c mice is *in vitro* test carry out phagocytosis and pinocytosis. It is regarded as a suitable in-vitro model for macrophages (Taciak *et al*., 2018). The RAW264.7 cell line displays semi-adherent growth properties, with adherent cells that have a spindle or cuboidal shape and floating viable cells that have a round or spherical shape. RAW264.7 cells typically have a diameter that falls in range of 10 to 20 μm. RAW264.7 macrophages model is an extensively utilised for studying cellular reactions. For example, the cytotoxicity of the treatments through MTT assay, determination of nitric oxide (NO) production through Griess assay and the amount of pro-inflammatory mediators such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) through enzyme linked immunosorbent assay (ELISA).

Bioassay-guided fractionation, pharmacological and biological characterisation interaction investigations, and identification of mode activities are all components of *in vitro* testing (Agarwal *et al*., 2014). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide colorimetric reagent is a sensitive and reliable indicator that used to

measure cellular metabolic activity, including to determine the cell viability. In cytotoxicity studies of treatments and commonly using MTT assay, cultured cells are frequently used. In recent years, interest in the application of biochemical tests has grown significantly as it is quick, affordable and does not involve the usage of animals. Tests for cell viability is based on a variety of cell processes, including nucleotide absorption activity, adenosine triphosphate (ATP) synthesis, coenzyme formation, cell adhesion and membrane permeability (Ishiyama *et al*., 1996). Nitrite (NO^{2-}), is stable and non-volatile byproducts of nitric oxide (NO) breakdown, can be measured using the Griess colorimetric reagent. In numerous biological systems, NO functions as a vital physiological messenger and effector molecule. It is synthesised from L-arginine by nitric oxide synthase (NOS), which exists in three isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). While eNOS and nNOS, produced by NOS, play crucial roles in maintaining physiological homeostasis, the high levels of NO generated by iNOS are significantly associated with the pathophysiology of various diseases and inflammatory processes (Salvemini *et al*., 1996).

Plate-based assay method known as ELISA (enzyme-linked immunosorbent assay) is used to identify and measure soluble molecules such as peptides, proteins, antibodies and hormones in biological samples. ELISA involves antigen-antibody reaction with high sensitivity that can measure nitric oxide (NO) and cytokines production to test various infection. These cytokines are often produced most abundantly by the immune system in response to inflammation. Cytokines are a diverse form of protein that play an important role in the induction and suppression of inflammation. TNF-α, or tumour necrosis factor-alpha, is a strong pro-inflammatory cytokine that can activate inflammatory cells, cause the production of several inflammatory proteins, cytotoxicity and more (Rocha *et al*., 2006). While, inflammatory cytokine of interleukin-6 (IL-6) is crucial for controlling metabolism, brain activity and the coordination of the innate and acquired immune

systems (Rose-John, 2022). These pro-inflammatory cytokines are responsible for initiating inflammation in response to tissue injury, including other cytokines such as interleukin-1β (IL-1β), interleukin-8 (IL-8) and interleukin-1 (IL-1).

Lipopolysaccharide (LPS) stimulated RAW264.7 macrophages usually used to assess the anti-inflammatory effects of drug tests. Stimulation of RAW264.7 macrophages with LPS produces pro-inflammatory mediators such as cytokines and complement systems. The cells are directly pre-treated with drug tests or treatments followed by LPS stimulation (Butterweck & Nahrstedt, 2012). The severity of inflammation can be measured through the level of pro-inflammatory mediators in cell supernatants. The enzyme-linked immunosorbent assay (ELISA) is a plate-based assay technique designed to detect and quantify the pro-inflammatory mediators. Expression of pro-inflammatory mediators is controlled by several signalling pathways and transcription factors. For example, mitogen-activated protein kinases (MAPKs) that regulate the cell growth, apoptosis, cellular response to cytokines and generate inducible nitric oxide synthase (iNOS) expression.

In vitro tests usually involve a limited number of experimental variables and make collection of significant data. *In vitro* test is easy and simple to run and requires less time to accomplish. However, it is costly mainly because of cell-subculturing and maintenance, which requires expensive materials and reagents. Therefore, animal model tests (*in vivo*) can be used as an alternative and recommended particularly for developing countries. Eventually, there are cultural restrictions on animal use and strict procedures to obtain ethical approval, which is a major drawback using animals for testing. Carrageenan-induced paw oedema is the most commonly used method for acute inflammation in order to find safe and effective drugs to control inflammation.

2.1.8.2 *In vivo* assay

Carrageenan-induced paw oedema is highly sensitive, reproducible and can be used orally for active drugs (Dzem *et al*., 2017; Patil *et al*., 2019). Proper preparation is needed as this technique could cause stress in animals and it is hard to measure the paw oedema. Carrageenan-induced paw oedema has been used widely by researchers to assess the anti-inflammatory activities of natural and synthetic compounds (Boominathan *et al*., 2004). This model is mostly used for acute inflammation and the carrageenan itself is a phlogistic agent which induces inflammation. In carrageenan, sulphated sugars activate inflammatory mediators and stimulate phospholipase A2 (Fernandez *et al*., 2001; Osadebe & Okoye, 2003). In the initial stage of inflammation, carrageenan causes the postcapillary venules to widen, which leads to the slow seepage of inflammatory mediators, fluids and cells from the blood vessels into the surrounding tissue. The pro-inflammatory mediators then act to inhibit the inflammatory process (Duwiejua *et al*., 2002). In the method of carrageenan-induced paw oedema, acute inflammation is triggered by the injection trauma, during which carrageenan releases acute phase mediators like serotonin and histamine (Perianayagam *et al*., 2006). Then, prostaglandins exhibit after three hours of carrageenan injection (Patil & Patil, 2017). This model is able to predict the biological target of a test drug in inflammation and is suitable to test NSAIDs as it is sensitive to cyclooxygenase inhibitors (Sarkhel, 2016).

Today, in pharmacology, new therapeutic drugs are developed by using physiologically and chemically appropriate models. The purpose of experimental models to be applied is to predict the intended therapeutic indication based on pharmacological principles. Butterweck & Nahrstedt (2012) suggested that preclinical screening of natural products should begin with the *in vivo* evaluation in the appropriate animal models to validate its traditional use, followed by bioassay-guided fractionation processes through the use of *in vitro* models. This pre-clinical assessment of anti-inflammatory drugs should

be organised in accordance with sample size, statistical methodologies, drug delivery via specified routes and use of positive controls. Future studies should focus on exploring the mechanisms of action, pharmacokinetics and safety of phytochemical compounds. This research is essential for developing new therapeutic agents and advancing drug discovery and development.

2.2 Current challenges in inflammation treatment

Current challenges in inflammation treatment include the need for more effective and targeted therapies with fewer side effects. Many anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, can cause significant adverse effects with long-term use, including gastrointestinal issues, cardiovascular risks and immunosuppression. There is also a need for better understanding of chronic inflammation and its underlying mechanisms to develop treatments that can address the root causes rather than just managing symptoms. These challenges necessitate ongoing research to identify novel therapeutic targets and develop safer and more precise anti-inflammatory agents. Many of the medications used to treat inflammatory diseases were developed before our current understanding of the molecular mechanisms underlying the disease. Today, the underlying cause and the degree of the inflammatory condition determine how to manage inflammation. Currently, steroidal and non-steroidal anti-inflammatory drugs are widely used for anti-inflammatory agents. Other than that, Disease-Modifying Antirheumatic Drugs (DMARDs), biologic therapies, immunosuppressants, physical therapies, lifestyle modifications, topical treatments, and complementary and alternative therapies are also employed to manage and treat inflammatory diseases (Radu & Bungau, 2021).

2.2.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) commonly known a drug class of Food and Drug Administration (FDA)-approved for use as antipyretic, anti-inflammatory and analgesic agents. It is being used clinically in treating inflammation, muscle pain, arthritic, pyrexia, gout, migraines and non-chronic cases (Ghlichloo & Gerriets, 2022). Ibuprofen, naproxen and aspirin are NSAIDs that are frequently used to treat pain and reduce inflammation. They function by preventing the synthesis of prostaglandins. NSAIDs consists of groups of different chemical structure which is acetylated salicylates (aspirin), non-acetylated salicylates (diflunisal, salsalate), propionic acids (naproxen, ibuprofen, acetic acids (diclofenac, indomethacin), enolic acids (meloxicam, piroxicam) anthranilic acids (meclofenamate, mefenamic acid), naphthylalanine (nabumetone), and selective COX-2 inhibitors (celecoxib, etoricoxib). NSAIDs act by inhibiting the presence of the enzyme cyclooxygenase (COX). Arachidonic acid is transformed into thromboxanes and prostaglandins by the COX enzyme. These substances, which cause platelet adhesion and vasodilation as inflammatory reactions to damage.

Pharmaceutical companies worked for a long time to create NSAIDs that had aspirin's therapeutic effects without its principal side effect, stomach ulcers. However, NSAIDs are a diverse class of cyclooxygenase (COX) inhibitors and have interactions with cell membranes that cause a variety of inflammatory events and toxic side effects (Adegbaju *et al*., 2020). Aspirin for example has the ability to trigger an inflammatory response in some asthmatic patients giving rise to the idea of pharmacogenomics, which seeks to understand individual medication sensitivity in order to develop individualised treatment plans (Punchard *et al*., 2004). Aspirin is a non-steroidal anti-inflammatory medicine (NSAID) that can be used to relieve pain in rheumatoid arthritis, while corticosteroids also can be used to treat arthritis and various diseases as well. All of these medications did, however, damage the stomach mucosa even though they were clinically useful.

Additionally, this study contributed to the creation of a few animal models that are being employed in inflammation research today, such carrageenan oedema and adjuvant arthritis (Winter *et al*., 1962; Newbould, 1963).

Around the world, synthetic pharmaceuticals are frequently used. However, as herbal medicine has a lesser risk of side effects than synthetic drugs, it is frequently used by individuals (Nisar *et al*., 2017). Synthetic or so-called pharmaceutical medications are made to react in a specific way with the risk-related negative side effects. Meanwhile, herbal medicine offers broad complementary, adaptable and synergistic benefits to combat all disorders. Each year, over 100,000 people died as a result of synthetic drug inflexibility (Karimi *et al*., 2015). On top of that, 16,500 deaths in the USA were attributed to NSAID-related gastrointestinal side events in 1997, showing the seriousness of these issues since decades (Wolfe *et al*., 1999; Kim *et al*., 2005). Aspirin, clopidogrel, diclofenac, enoxaparin, ibuprofen, naproxen and warfarin are just a few of the synthetic medications that are frequently used by most individuals. Most of these medications are effective at treating pain and headaches. For example, paracetamol and ibuprofen, two antipyretic drugs, have been shown in prior research to have side effects such as liver damage and kidney failure when used for an extended period of time (Mann *et al*., 1993; Lesko & Mitchell, 1995; Tanne, 2006). The need to create novel therapeutics is driven by the fact that a significant number of patients with severe chronic inflammatory illness do not respond well to synthetic drugs, posing a significant clinical and socioeconomic burden.

2.2.2 Corticosteroid

A class of synthetic medications known as corticosteroids, also referred to as steroids, imitate the actions of the natural steroid hormones generated by the adrenal glands, such as cortisol (Samuel *et al*., 2017). Corticosteroids, like prednisone and dexamethasone, are

powerful anti-inflammatory medications. They are prescribed for more severe inflammatory conditions and work by suppressing the immune response and reducing inflammation. These medications are useful for treating a variety of inflammatory and immune-related disorders because they have strong anti-inflammatory and immunosuppressive characteristics.

The two categories of corticosteroids are glucocorticoids and mineralocorticoids. The most widely used corticosteroids are glucocorticoids, and they significantly reduce inflammation (Samuel *et al*., 2017). They function by attaching to glucocorticoid receptors in cells, which causes some immune and inflammatory response-related genes to be either activated or repressed. As a result, the immune system's numerous components are suppressed, and inflammation is decreased. Meanwhile, mineralocorticoids are used to treat disorders like mineralocorticoid deficit since they primarily influence the body's salt and water balance. Corticosteroids are commonly used to treat a variety of inflammatory and immune-related disorders, such as rheumatoid arthritis, atopic dermatitis, asthma, bowel inflammation (including Crohn's disease and ulcerative colitis), skin conditions like psoriasis and eczema, chronic obstructive pulmonary disease (COPD), ocular inflammation conditions and a few cancers (such as lymphoma and leukaemia) combined with other treatments.

Corticosteroids are particularly helpful for acute flare-ups or severe illnesses since they can quickly relieve inflammation and associated symptoms. However, they have adverse effects, particularly when used frequently or in high quantities. Weight gain, fluid retention, elevated blood sugar, mood swings and a weaker immune system, which may increase susceptibility to infections, are typical adverse effects. Corticosteroids are frequently recommended at the lowest effective dose and for the shortest term possible to reduce the risk of side effects. After prolonged use, abruptly ceasing corticosteroid therapy might cause adrenal insufficiency, a condition where the body's normal cortisol

synthesis is inhibited (Radu & Bungau, 2021). As a result, under medical supervision, corticosteroid therapy is normally weaned off gradually. Overall, corticosteroids are essential for treating a range of immunological and inflammatory disorders, but their usage needs to be carefully considered, monitored, and controlled to balance the advantages and hazards involved.

2.2.3 Disease-modifying antirheumatic drugs (DMARDs)

Disease-modifying antirheumatic drugs (DMARDs) are used to treat autoimmune and inflammatory conditions like rheumatoid arthritis (Sarkar *et al*., 2018). They work by targeting the underlying disease process and modifying the immune response. Aiming to slow down or alter the course of the disease, DMARDs target the underlying disease process in contrast to painkillers and anti-inflammatory medications, which largely treat symptoms. Thus, they can aid in maintaining joint functionality, lessen joint degeneration and enhance the general quality of life for those with these disorders (Benjamin *et al*., 2023).

DMARDs come in a variety of forms including methotrexate, sulfasalazine, hydroxychloroquine, leflunomide, Janus Kinase (JAK) inhibitors and biological DMARDs targets certain immune proteins implicated in the inflammatory process such as interleukin-6 (IL-6) inhibitors (tocilizumab) and tumour necrosis factor (TNF) inhibitors (adalimumab, infliximab). DMARDs are frequently recommended alone or in conjunction with other medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroids. The long-term prognosis for people with autoimmune and inflammatory disorders can be considerably improved by DMARD therapy, which can also lessen joint deterioration and enhance general quality of life. However, DMARDs associated with several adverse effects such as gastrointestinal issues and liver toxicity (Benjamin *et al*., 2023). Additionally, some DMARDs, like methotrexate, can cause

pulmonary toxicity and interstitial lung disease. Patients may also experience an increased risk of infections due to immunosuppression and long-term use can lead to renal impairment. Regular monitoring and early detection of these adverse effects are essential to manage and mitigate the risks associated with DMARD therapy.

2.2.4 Biologic therapies

A class of medications known as biologic therapeutics, commonly referred to as biologics or biological drugs, is produced utilising biotechnology or from living organisms. These medications are made to specifically target chemicals or immune and inflammatory response-related cells.

Biologics are huge and complex molecules that are often made up of proteins or antibodies, in contrast to ordinary chemical medications, which are frequently tiny molecules. They are made to interact with particular proteins, cells or receptors in the body in order to suppress or regulate specific immune responses that in autoimmune illnesses cause inflammation or tissue damage. Biologics have proven revolutionary in treating many disorders, but they can be costly and come with dangers, such as an increased risk of infection and uncommon adverse effects (Raychaudhuri & Raychaudhuri, 2009).

2.2.5 Immunosuppressants

A group of drugs known as immunosuppressants are used to reduce or suppress immune system function (Kalariya *et al*., 2023). These medications are used to treat illnesses when an overreactive or malfunctioning immune system is harming the body's own tissues or organs. Immunosuppressants are frequently used for organ transplantation, autoimmune diseases, inflammatory bowel disease (IBD), dermatological conditions, severe allergic reactions and the treatment of graft-vs-host disease. It can be given orally

as tablets, intravenously as an infusion, or intramuscularly as an injection. Corticosteroids, calcineurin inhibitors, antimetabolites and biologics that target certain immune pathways are common kinds of immunosuppressants.

Immunosuppressants, while essential for managing autoimmune diseases and preventing organ transplant rejection, can cause a range of adverse effects due to their impact on the immune system including an increased susceptibility to infections, as the immune system's ability to fight off pathogens is diminished. Patients may also experience an increased risk of malignancies, particularly lymphomas and skin cancers, due to prolonged immunosuppression. Other potential side effects include hypertension, hyperglycaemia and lipid abnormalities. Close monitoring and management of these adverse effects are critical to ensure the safe use of immunosuppressants.

2.2.6 Physical therapies

Physical procedures and exercises are used in physical therapies, sometimes referred to as physical interventions or physiotherapies, to enhance and restore movement, function and general well-being (Radu & Bungau, 2021). This treatment used to treat a variety of musculoskeletal, neurological and other health issues. They are often delivered by physical therapists or physiotherapists. Physical therapy is intended to lessen discomfort, increase range of motion, increase strength and flexibility, and speed up the healing process after accidents or illnesses. Physical therapy approaches include physical exercises, manual therapy, electrotherapy, heat and cold therapy, ultrasound therapy, traction, aquatic therapy, balance and coordination training, gait training, and assistive devices and mobility aids.

Physical therapies, while generally beneficial for managing pain, improving mobility, and promoting overall health, can sometimes have adverse effects, particularly if not properly administered such as muscle soreness and fatigue, especially after initial sessions

or when new exercises are introduced. Overuse or improper technique can lead to musculoskeletal injuries such as strains, sprains, and tendonitis. Patients with osteoporosis or other bone conditions may be at risk for fractures if the therapy is too intense or not appropriately modified. Therefore, it is necessary for physical therapy to be tailored to the individual's condition and tolerance levels, with close monitoring by a trained professional to minimise these risks.

2.2.7 Lifestyle modifications

The management of current medical issues as well as the promotion of good health depend on modifying one's way of life. Type of lifestyle changes such as healthy diet, regular physical activity, adequate sleep, stress management, limit alcohol consumption, avoid smoking and substance abuse, maintain a healthy weight, regular health check-up, practice safe sun exposure and social connections can help manage and avoid chronic illnesses including heart disease, diabetes, hypertension and obesity (Wadden *et al*., 2020).

Lifestyle modifications, while generally promoting better health and well-being, can sometimes have adverse effects if not properly managed or if taken to extremes. Changes in diet, for instance, might lead to nutritional deficiencies if certain food groups are overly restricted without appropriate substitutes. Drastic changes in physical activity can result in overuse injuries, such as muscle strains, joint pain, or stress fractures, particularly if exercise is increased too rapidly without proper conditioning. Additionally, smoking cessation can lead to withdrawal symptoms like anxiety and depression. The lifestyle modifications must be approached gradually and under the guidance of healthcare professionals to ensure they are safe and effective for the individual.

2.2.8 Topical treatments

Topical therapies are pharmaceuticals or chemicals that are applied directly to the skin or mucous membranes to cure a variety of skin disorders, reduce localised discomfort, or deliver medication to the affected area (Radu & Bungau, 2021). There are many different types of topical therapies including creams, ointments, lotions, gels, foams, patches and sprays. They are frequently applied to treat localised ailments, pain alleviation and skin issues.

However, it can cause adverse effects such as irritation, allergic reactions, contact dermatitis, photosensitivity, skin atrophy, pigmentation changes, acneiform eruptions, abnormal hair growth or loss, systemic absorption issues, infections and perioral dermatitis. These effects are influenced by the active ingredients, usage duration and individual sensitivities. Adhering to usage guidelines, using sun protection and monitoring the skin for adverse changes are essential, along with consulting healthcare professionals for any concerning reactions.

2.2.9 Complementary and alternative therapies

The term “complementary and alternative therapies” (CAM) refers to a broad range of medical procedures, supplies and frameworks that are not seen as being a part of traditional medicine (Koithan, 2009). Some typical subcategories of complementary and alternative medicine including acupuncture, herbal medicine, mind-body technique, chiropractic care, massage therapy, homoeopathy, ayurveda, naturopathy, energy healing and aromatherapy. However, it's essential to discuss these options with a healthcare professional to ensure safety and effectiveness.

Complementary and alternative therapies (CAM), can have adverse effects that vary depending on the type of therapy and individual patient factors. Herbal remedies, for

example, may cause allergic reactions, gastrointestinal issues, or interact with prescription medications, leading to potentially dangerous effects such as bleeding or liver damage. Acupuncture, though generally safe when performed by trained practitioners, can result in infections, punctured organs, or nerve damage if done improperly. Chiropractic manipulation carries risks like vertebral artery dissection or worsening of spinal issues. Mind-body practices like yoga and meditation can sometimes cause psychological distress or physical injuries, especially in those with preexisting conditions or when not performed correctly. To minimise these risks, it is crucial for individuals to consult healthcare professionals before starting any CAM therapies.

2.3 Medicinal plant as an alternative in inflammation treatment

Synthetic drugs produce adverse side effects such as gastric intestinal mucosa, heart illness and kidney disorders and are only effective at one-time consumption in relieving symptoms (De Groot & Scott, 2007; Debnath *et al*., 2013). As an alternative, there are medications made from plants that have been used for centuries. Alkaloids and phenols are two examples of the many active substances found in plants. Researchers have devoted a great deal of time to studying the substances contained in plants since they are vital to human health. Since they are more accessible, practical and secure than synthetic treatments, several pharmaceutical companies are now concentrating on producing plant-derived medications (Burke *et al*., 2005; Apu *et al*., 2012). Secondary chemicals contain unique biological properties that can be used to develop plants into medicines and treatments for many diseases. For example, taxol, which fights cancer; digitalis, which treats heart failure; and opiates, which are used to relieve pain (Rosenblum *et al*., 2008; Reddy, 2010; Zhang *et al*., 2014). The technique of extraction is necessary to separate the medicinally active components of plant tissues with selected solvent. Method of extraction can be chosen according to suitability from either maceration, Soxhlet

extraction, infusion, digestion, decoction, percolation, sonication or supercritical fluid extraction (SFE). The plant extract produces relatively impure liquids, semisolids or powders that are only fit for external or oral consumption for medicinal purposes.

Medicinal plants have phytochemical compounds that are responsible for anti-inflammatory activity. The capability of the compounds to interact with cellular targets which are involved in pathophysiological processes to act as anti-inflammatory agents can act on one or more mechanisms (Safayhi & Sailer, 1997). There are various targets that compounds that can act during inflammation and the major are pro-inflammatory mediators. Drugs obtained from natural sources contain various inflammatory mediators (arachidonic acid metabolites, peptides, cytokines and excitatory amino acid), second messengers (cGMP, cAMP, protein kinases and calcium), transcription factors expression (AP-1, NF-κB, and proto-oncogenes) and pro-inflammatory molecules (inducible NO synthase (iNOS) and cyclooxygenase (COX-2), cytokines (IL-1β, TNF-α), neuropeptides and proteases) (Kim *et al*., 2009; Rao *et al*., 2016).

Plant or herbal remedies have been employed since the dawn of civilisation. It all began when Imhotep, an Egyptian doctor learned that some plants may be used for therapeutic purposes. Since then, people have been acquiring new knowledge over the centuries, largely through cultural adaptation. There are currently three different types of systems, including TCM (Traditional Chinese Medicine), Ayurveda and Unani. These herbal medical systems, which have their roots in China and the Indian subcontinent, are well-organised and well-documented. As an alternative to synthetic medications, herbal medicine has grown dramatically during the past several years.

There are limitations of drug development from natural sources including the complex nature of extracts, isolation of compounds with considerable purity and the lowest yield of active compounds from plants (Patil *et al*., 2019). Despite the obstacles in

phytochemical compounds drug discovery, plants are interesting targets for the novel anti-inflammatory agents. There are many accessibilities of various data on anti-inflammatory plants, but newer ones still relevant. Many phytochemical compounds have been studied extensively and reached up to clinical trials. The current drug discovery scenario suggests the re-evaluation of promising plant products (preferably isolated compounds) with the help of extensive biochemical and molecular techniques to explore the therapeutic potential and safer anti-inflammatory leads. In the framework of anti-inflammatory drug development, it is logical to investigate the possible biochemical mechanisms underlying the activities of phytochemical compounds to inculcate their mechanisms of action (Benito *et al*., 2000).

2.3.1 Classification and nature of bioactive compounds

Bioactive compounds are chemical substances found in nature that have the potential to affect biological processes within living organisms. These compounds interact with biological systems such as cells, tissues and organs to produce physiological effects. Bioactive compounds can be derived from various sources, including plants, animals, fungi and microorganisms. They encompass a wide range of chemical classes including alkaloids, flavonoids, terpenoids, phenolic, peptides and fatty acids. Examples of bioactive activity include antioxidants, antimicrobials, anti-inflammatory and anticancer. These compounds have garnered significant interest due to their potential health benefits and therapeutic applications (Sorrenti *et al*., 2023). They play a crucial role in promoting health, preventing diseases and improving overall well-being. The study of bioactive compounds involves their identification, isolation, characterisation and investigation of their biological activities and mechanisms of action.

Bioactive compounds, which exert physiological effects on living organisms can be broadly classified into two main categories: metabolites and phytochemicals (Mendoza

& Silva, 2018). Metabolites are chemical compounds produced as part of metabolic processes within organisms. It encompassing primary metabolites essential for basic cellular functions such as amino acids, carbohydrates and nucleotides, and secondary metabolites with diverse biological activities such as alkaloids, flavonoids and terpenoids. In contrast, phytochemicals are bioactive compounds naturally occurring in plants, serving various ecological roles such as defence against pathogens, pests and environmental stressors. Phytochemicals include a wide range of compounds like flavonoids, phenolic acids, alkaloids, terpenoids, and many of which have been studied for their potential health benefits and therapeutic applications in humans. This classification distinguishes between compounds derived from metabolic processes within organisms (metabolites) and those specifically originating from plant sources (phytochemicals), reflecting their distinct origins and biological roles.

2.3.2 Metabolites and phytochemicals

Metabolites are small molecules that are produced and involved in various metabolic processes within living organisms. These processes include the conversion of nutrients into energy, the building and maintenance of cellular structures, and the regulation of biochemical reactions. Metabolites play a crucial role in the overall functioning and survival of organisms. There are two main categories of metabolites including primary metabolites and secondary metabolites. While metabolites as chemical compounds produced by metabolic processes within living organisms, phytochemicals are bioactive compounds naturally occurring in plants. Both metabolites and phytochemicals can exhibit diverse biological activities and contribute to the physiological functions of organisms.

Primary metabolites are essential for the basic life functions of an organism. They are involved in fundamental metabolic pathways and are required for growth, development

and reproduction. All living things have primary metabolism, which creates complex compounds like carbohydrates, proteins, lipids, cell membranes, hormones and nucleic acids (DNA and RNA), as well as glucose, amino acids, fatty acids and nucleotides (Bocso & Butnariu, 2022). Primary metabolic processes include photosynthesis, respiration, protein synthesis, solute transport and nutritional assimilation (Dhaniaputri *et al*., 2021). Amino acids are the building blocks of proteins, which play a vital role in various cellular functions, including enzymatic reactions, cell structure and signalling. Glucose is a simple sugar and a primary source of energy in most organisms. It serves as a key fuel for cellular processes through glycolysis and cellular respiration. Nucleotides are the basic units of DNA and RNA, essential for storing and transmitting genetic information. They also play a critical role in cellular signalling and energy transfer like ATP. Fatty acids are the building blocks of lipids, including triglycerides, phospholipids and cholesterol. They are essential for cell membrane structure and serve as an energy source. Organic acids such as citric acid, participate in the tricarboxylic acid cycle (TCA cycle or Krebs cycle) which is an important pathway in cellular respiration. Vitamins are essential organic compounds required in small amounts for various biochemical processes and enzyme functions. Amines such as serotonin and dopamine which are neurotransmitters involved in nerve signalling and regulation of mood and behaviour. Primary metabolites important for both physiological and neurological functions, emphasizing the necessity for their balanced presence in the body to maintain overall health and stability (Qiu *et al*., 2023).

Secondary metabolites are organic compounds produced by various organisms such as plants, fungi and bacteria. Unlike primary metabolites which are essential for growth, secondary metabolites often play a role in the organism's defence mechanisms, communication with other organisms and interactions with their environment. Secondary metabolites are not directly involved in the core metabolic processes but often have

specific ecological or biological functions. They are typically produced in response to environmental stimuli and are involved in defence, communication or other interactions with the organism's surroundings. Examples of secondary metabolites are alkaloids, terpenoids, flavonoids, tannins, phenolics, glycosides, cyanogenic glycosides, antibiotics and saponins (Dhaniaputri *et al*., 2021).

Phytochemicals derived from plants may have potential health benefits when consumed as part of the diet. These compounds are often found in fruits, vegetables, herbs and other plant-based foods and are responsible for their colour, flavour and aroma. Phytochemicals include a wide variety of compounds such as flavonoids, phenolic acids, alkaloids, glucosinolates and saponins. Phytochemicals share similarities with secondary metabolites in terms of their ecological roles such as defence mechanism and signalling, and also chemical diversity (Ahmad *et al*., 2021). Their primary role is to protect the plant from environmental stressors and pathogens. The term phytochemicals specifically emphasize their presence in edible plants and their potential health-promoting properties when consumed by humans. In humans, they are studied for their potential health benefits including anti-inflammatory, antioxidant and anticancer properties. They are typically secondary metabolites, meaning they are not directly involved in the normal growth, development or reproduction of the plant.

2.3.3 Chemical and biological activities

As secondary metabolites are important for the survival and ecological interactions of organisms, it is essential to explore their various classes and functions. There are several classes, including alkaloids, terpenoids, flavonoids, tannins, phenolic and glycoside. Each class exhibits distinct properties and serves specific functions, from defence mechanisms and attracting pollinators to medicinal applications and human health benefits. Alkaloids are a large and diverse group of nitrogen-containing compounds commonly found in

plants. They often have pharmacological effects and can act as toxins or medicines (Wu *et al*., 2022). Examples include caffeine, nicotine, morphine and quinine. Terpenoids are a class of compounds derived from isoprene units and are abundant in essential oils of plants with ability to serving as defence chemicals, attracting pollinators and acting as signalling molecules. Examples include menthol, limonene and carotenoids. Flavonoids are a group of polyphenolic compounds found in plants. They have antioxidant properties and can help protect plants from UV radiation and pathogens. Some flavonoids also have potential health benefits for humans such as quercetin, kaempferol and catechins. Tannins are a type of polyphenol that can bind to and precipitate proteins that usually found in plants including tea and red wine. Phenolic compounds are aromatic compounds with one or more hydroxyl groups with diverse functions including antioxidant activity, UV protection and defence against pathogens. Examples include resveratrol, found in grapes and red wine, and curcumin, found in turmeric.

Glycosides are compounds formed by attaching a sugar molecule to another compound, often increasing the compound's stability and solubility. They have various roles including defence, attraction of pollinators and seed dispersal. Cyanogenic glycosides are compounds that can release toxic cyanide when enzymatically hydrolysed. They are found in certain plants as a defence mechanism against herbivores. Examples include amygdalin found in bitter almonds and cassava plants. Some microorganisms produce secondary metabolites with antimicrobial properties which is can inhibit the growth of competing microorganisms and have been harnessed for human use as antibiotics such as penicillin, streptomycin and tetracycline. Saponins are glycosides with foaming properties and also can be found in various plants and have been used historically as detergents and health benefits. Examples include glycyrrhizin in liquorice and ginsenosides in ginseng.

Secondary metabolites have also been of significant interest to humans for various applications, including pharmaceuticals, as many of these compounds exhibit bioactive properties and can be used to develop medicines, herbicides, insecticides and other useful products (Seca & Pinto, 2019). However, it is essential to note that not all secondary metabolites are beneficial because some can be toxic to humans and animals. Overall, secondary metabolites represent a diverse group of compounds with various biological processes and functions, which called as metabolomics continues to be an active area of research in biology, chemistry and pharmacology.

2.3.4 Mechanism of action associated to inflammation

Secondary metabolites can play a role in inflammation, both by promoting and reducing inflammatory responses in living organisms. Different classes of secondary metabolites including flavonoids, phenolic acids, steroids, terpenoids, sesquiterpenes and tannins are responsible for the biological activity and anti-inflammatory effects of particular plants (Radovanović *et al*., 2023). The effects of secondary metabolites on inflammation also can vary depending on the specific compound, concentration and the context of the inflammatory process.

Secondary metabolites can trigger or enhance pro-inflammatory responses. For example, certain alkaloids and glycosides found in plants may act as irritants, causing local inflammation when they come into contact with tissues. Inflammatory responses may also be induced by the release of toxins from microorganisms or the activation of immune cells by specific secondary metabolites. On the other hand, several secondary metabolites exhibit anti-inflammatory properties and effects. They can help to reduce the intensity of the inflammatory response or regulate the immune system. For example, some flavonoids, terpenoids and polyphenols have been shown to possess anti-inflammatory

effects by inhibiting pro-inflammatory signalling pathways or neutralising free radicals that contribute to inflammation (Mucha *et al*., 2021).

Some metabolites can promote the release of cytokines and chemokines, which are signalling molecules that contribute to inflammation. Others can regulate immune cell activity, leading to the suppression of inflammatory responses. Some secondary metabolites may have both pro-inflammatory and anti-inflammatory effects (dual-effects), depending on the concentration and the specific cellular context. At low concentrations, a compound may exhibit anti-inflammatory properties, but at higher concentrations, it might induce inflammation or vice versa (Saleh *et al*., 2021). Secondary metabolites on inflammation are highly complex and context-dependent. The outcome of their interactions with the immune systems can vary depending on the overall physiological state of the organism, the presence of other compounds and the specific pathways involved.

When secondary metabolites function as anti-inflammatory agents, they exert their effects through several mechanisms that modulate the body's inflammatory response. Secondary metabolites can regulate the production and release of inflammatory mediators, including cytokines such as TNF-α, IL-1β and IL-6, as well as chemokines and prostaglandins to reduce inflammation (Nisar *et al*., 2023). Additionally, secondary metabolites also possess antioxidant properties, enabling them to neutralise reactive oxygen species (ROS), diminish oxidative stress and then decreasing inflammation. Secondary metabolites also target and modulate key signalling pathways involved in inflammation such NF-κB, MAPKs, JAK-STAT and Nrf-2. Through these pathways, secondary metabolites can regulate and promoting the expression of anti-inflammatory genes. Moreover, some secondary metabolites induce epigenetic changes that impact gene expression related to inflammation by altering the inflammatory response at the molecular level (Saleh *et al*., 2021). Furthermore, secondary metabolites can modulate

immune cell function and activity by enhance the activity of regulatory T cells or suppress the activation of pro-inflammatory immune cells, leading to a balanced immune response and reduced inflammation (Paudel *et al*., 2023).

Research in this field is ongoing, and scientists continue to investigate the potential therapeutic applications of secondary metabolites for managing inflammatory disorders and diseases. However, it is essential to exercise caution when considering the use of secondary metabolites for medical purposes, as their effects can be complex and may vary between individuals. Certain secondary metabolites derived from plants and natural sources have shown promising anti-inflammatory properties and are being studied for their potential therapeutic use in managing inflammation for example quercetin and curcumin (Saeedi-Boroujeni & Mahmoudian-Sani, 2021; Peng *et al*., 2021). These compounds often act through various mechanisms to modulate inflammatory responses in the body.

Alkaloid is a diverse group of nitrogen-containing compounds found in plants. Some alkaloids have been identified as having anti-inflammatory effects for example berberine, which is found in plants like *Berberis* species, barberry and goldenseal. Berberine exhibits anti-inflammatory properties by inhibiting NF-κB and reducing the expression of pro-inflammatory cytokines. It has been studied for its potential in managing various inflammatory conditions. Papaverine derived from the *Opium poppy* also has shown anti-inflammatory effects in experimental studies, particularly in models of inflammation-induced oedema (Reddi *et al*., 2021).

Flavonoid is a class of polyphenolic compounds found in fruits, vegetables and certain herbs. Most flavonoid possess antioxidant and anti-inflammatory properties. Some examples of flavonoid that is anti-inflammatory include quercetin and kaempferol. Flavonoid class including compounds such as flavone, flavanol, isoflavone, flavonol and flavanone have been linked to modulating inflammatory signalling pathways, including

NF-κB, STAT and Nrf2. They have been demonstrated to modulate the levels of pro-inflammatory cytokines, COX-2, iNOS and ROS through *in vivo* disease model systems, demonstrating their promise as innovative anti-inflammatory medications (Shin *et al*., 2020). Quercetin is a widely studied flavonoid having potent anti-inflammatory effects. It inhibits various inflammatory enzymes such as COX and LOX, and reduces the production of pro-inflammatory cytokines. Kaempferol exhibits anti-inflammatory effects by inhibiting NF-κB and modulating inflammatory signalling pathways (Hussain *et al*., 2022).

Tannin is a group of polyphenolic compounds found in plants. They are known for their ability to bind and precipitate proteins. Some tannins have been studied for their anti-inflammatory properties, particularly in the context of plant-based remedies. This includes ellagic acid, a tannin found in berries and pomegranates that has shown anti-inflammatory effects by inhibiting NF-κB and reducing the expression of inflammatory mediators (Harper, 2023). Epigallocatechin gallate (EGCG) is also considered a tannin due to its structural characteristics that mostly found in green tea and exhibits potent anti-inflammatory and antioxidant effects (Legeay *et al*., 2015). These compounds have demonstrated anti-inflammatory effects in experimental studies, but more research is needed to fully understand their mechanisms of action and its potential in clinical applications. Additionally, the effectiveness of these compounds may vary based on factors such as dosage, bioavailability and the specific inflammatory condition being targeted.

2.3.5 Phytochemical identification and characterisation

Phytochemical identification is a crucial step in the exploration of plant-based compounds for therapeutic use (Krishnananda *et al*., 2017). It helps in understanding the chemical composition such as alkaloids like morphine and quinine, or terpenoids like

taxol, and flavonoids. The identification process involves several steps and techniques to isolate, separate and characterise the phytochemicals. Extraction is the first step to extract the phytochemicals from the plant material. This can be done using various solvents or methods such as maceration involves soaking plant materials in a solvent at room temperature for extended periods, which is simple but time-consuming. Meanwhile, Soxhlet extraction uses continuous solvent reflux over plant materials, offering higher efficiency and suitability for a wide range of compounds, require greater amount of solvent. Supercritical fluid extraction (SFE) employs supercritical CO^2, often with modifiers like ethanol, providing high efficiency and selectivity, especially for thermolabile compounds, though it requires specialised equipment and training. The choice of method depends on the type of phytochemicals to be isolated and the plant material's characteristics. Factors influencing extraction efficiency include solvent selection, where matching solvent polarity to target compounds is crucial. Another factor is extraction time, where finding the optimal duration is crucial for maximising yield while maintaining compound stability. Additionally, temperature plays a role, as higher temperatures can improve extraction efficiency but may also degrade sensitive phytochemicals (Antony & Farid, 2022). By optimising these methods and factors, researchers can effectively isolate and study phytochemicals for their potential health benefits.

After extraction, the phytochemicals need to be separated from each other and the process called separation. Chromatographic techniques are widely used for this purpose including thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), and high-performance thin layer chromatography (HPTLC). These methods separate compounds based on their physical and chemical properties such as polarity, molecular size and solubility. Once separated, the phytochemicals are identified using various spectroscopic techniques such as Fourier-

transform infrared spectroscopy (FTIR) that provides information about the functional groups present in the compounds by analysing the absorption of infrared radiation. Another technique, nuclear magnetic resonance (NMR) which gives detailed information about the structure of the compounds by analysing the resonance of atomic nuclei in a magnetic field. Mass spectrometry (MS) also helps determine the molecular weight and structure of the compounds by measuring the mass-to-charge ratio of ions. After initial identification, further confirmation of the phytochemicals may be done using additional techniques or by comparing the results with known standards or databases. Once identified, the phytochemicals may be tested for their biological activities, such as antioxidant, antimicrobial or anticancer properties to understand their potential therapeutic uses (Abdallah *et al*., 2024).

Phytochemical characterisation is the process of identifying and quantifying the chemical compounds found in plants. These compounds are responsible for the biological activities of plants such as their medicinal properties and include a wide range of secondary metabolites like alkaloids, flavonoids, terpenoids and phenolic compounds. The characterisation process typically involves several steps. After identification, it is often necessary to quantify the amount of each phytochemical present in the extract. This can be done using the same chromatographic techniques mentioned above, coupled with detection systems that measure the concentration of the separated compounds. Then, the biological activity of the identified phytochemicals can be assessed through various *in vitro* and *in vivo* assays to determine their potential therapeutic effects, such as antioxidant, antimicrobial or anticancer activities (Aati *et al*., 2022). Phytochemical characterisation is essential for understanding the active components in plants, which can lead to the development of new drugs, dietary supplements or other products that utilise the beneficial properties of these natural compounds.

The study demonstrated that *Ginkgo biloba* leaf flavonoids exhibit potential as regulators of neuroexocytosis, suggesting promising therapeutic applications (Ban *et al*., 2020). The Soxhlet extraction process utilizes ethanol as the solvent and is followed by purification through column chromatography. The isolated compounds are subsequently analysed using high-performance liquid chromatography (HPLC). Following the identification of flavonoids such as quercetin and kaempferol from *Ginkgo biloba*, their characterisation involved mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy to confirmed their molecular weights and structural details. The optimal pH and temperature stability were assessed, revealing that the compounds remain stable within a pH range of 5.5-7.5 and at temperatures up to 60°C. Biological activity assays, including the DPPH radical scavenging assay and enzyme-linked immunosorbent assay (ELISA), demonstrated strong antioxidant and anti-inflammatory properties. Molecular docking and *in vitro* assays revealed that these flavonoids inhibit key enzymes in oxidative stress and inflammation pathways, such as COX-2 and NF-κB. Evaluation in cellular models showed increased cell viability and reduced inflammation markers, suggesting significant therapeutic potential.

2.3.6 Protease enzyme as bioactive compound

Protease enzymes can be categorised as bioactive compounds based on their biological activity and therapeutic potential. Protease, also referred to as proteinase, is a class of enzymes that breaks down the peptide bonds in proteins. Small peptides and amino acids can be delivered via protease for the body's needs. It also plays an important role in protein digestion, immune function and other vital processes. Besides protease coming from animals and microbes, plants are the common sources of protease. Papain and bromelain, two well-known plant proteases, are derived from papaya and pineapple, respectively. Papain and bromelain both help to speed up wound healing and growth of new tissue

when applied directly to the skin, according to studies on animals (Ajlia *et al*., 2010; Rosenberg *et al*., 2012). Proteases play an important role in physiology of plants throughout their entire life cycle, including seed germination, defence mechanisms and chloroplast photoinhibition. Cysteine proteases (CPs), serine proteases (SPs) and aspartic proteases are the three most prevalent types of proteases in plants (APs). Plant-derived proteases are drawing interest because of their strong proteolytic activity and affinity for certain substrates, exceptional stability under a wide variety of working settings (pH: 4-10 and temperature up to 60°C), and affordable raw material cost (Troncoso *et al*., 2022).

Protease has a wide diverse application and one of it is developing effective therapeutic agents. Proteases are extensively employed in commercial production, including pharmaceuticals, which account for over 60% of the global market for enzymes (Gonzàlez *et al*., 2011; Kim *et al*., 2016; Sun *et al*., 2016). Protease is frequently employed in industry as detergents, silver recovery, waste management, brewing, meat softening, milk clotting, food preparation, pharmaceuticals, cancer therapies, diagnostics, digestion and food production (Gupta *et al*., 2002; Naidu & Devi, 2005; Roy & Kumar, 2014; Kuddus, 2015; Sathya Prabhu *et al*., 2017). Market size for protease enzymes is expected to exceed 3 billion US dollars by 2024, with a compound annual growth rate (CAGR) of 6.1% (Feijoo-Siota & Villa, 2011).

Protease or proteolytic enzyme is known to have anti-inflammatory effects through several mechanisms. Protease can degrade various proteins in the body, including pro-inflammatory cytokines (Chakraborty & Bhattacharyya, 2013). Protease also can modulate the immune response by activating or inhibiting certain immune cells (de Almeida & Dufour, 2022). Protease can degrade extracellular matrix components and promote tissue repair. Protease was once a common medication prescribed by physicians for wound healing and tissue repair. The market's availability of non-steroidal anti-inflammatories (NSAIDs) however reduced the demand for protease. However, NSAIDs

can remove pain but could not speed up the process to repair tissue. NSAIDs also have adverse side effects such as gastric intestinal mucosa as well heart and kidney diseases in long-term consumption. Therefore, protease could become one of the alternatives to reduce inflammation as it is natural and safe to use. Protease can inhibit the production of pro-inflammatory mediators such as cytokines and nitric oxide that produce when inflammation occurs. Protease serves as a catalyst for other bodily enzymes, accelerating other enzymatic activities to aid in tissue repair. Protease successfully combats cancer, inflammation, oedema and other conditions (van der Hoorn, 2008; Gonzàlez *et al*., 2011). Protease has also been demonstrated to be useful in treating arthritis and minimising inflammation (Brien *et al*., 2004; Viswanatha Swamy & Patil, 2008; Rani *et al*., 2012).

Proteases aid in the digestion of proteins, lower inflammation, lessen muscle tension, and speed up the recovery time after surgery. Early research suggests that protease may be helpful in the fight against cancer cells (Müller *et al*., 2016). According to one study, serrapeptase, a proteolytic enzyme, reduced oedema and pain in 24 people who had just undergone dental surgery when they took a supplement containing 5 mg of it (Al-Khateeb & Nusair, 2008). Another investigation discovered that a supplement containing the bromelain was effective in reducing stiffness, oedema and pain related to osteoarthritis (Brien *et al*., 2004). Protease supplements reduce indigestion symptoms such as bloating, abdominal pain, belching, heartburn and appetite loss and a study found that using a papain supplement decreased bloating, unpleasant bowel movements and constipation (Muss *et al*., 2013). Despite the market being swamped with protease supplements, it is far preferable to consume enzymes from naturally occurring sources such as papaya, pineapple, kiwifruit and fermented foods. The natural sources are often viewed as safer and more sustainable alternatives compared to synthetic supplements.

Proteases are crucial enzymes that are important for a variety of biological functions. It is able to start and stop pro-inflammatory or anti-inflammatory responses by altering

the activity of cytokines and chemokines (de Almeida & Dufour, 2022). As a result, it can treat inflammation while also reducing discomfort and swelling. Protease can reduce acute inflammation toward the resolution phase and ultimately back to homeostasis, by playing a variety of biological activities. However, proteases can also encourage inflammatory disorders if they are not controlled. Protease enzymes are generally advantageous to humans, and new sources of it are continuously being investigated. *Christia vespertilionis* is one of the plants that has the ability to extract and purify its protease. Protease enzymes that were isolated and purified from this plant were not the subject of any investigations. This study seeks to extract and purify protease from *C. vespertilionis* leaves by ammonium sulphate precipitation to remove non-enzyme components and conduct dialysis to boost the enzyme activity. Additionally, since there is no research on the protease purification from *C. vespertilionis* leaves, the method must be optimised.

Proteases are involved in various biological processes, including inflammation and the pathogenesis of diseases such as diabetes, obesity, Alzheimer's disease and Parkinson's disease. The phosphorylation of I*κ*B*α* marks it for degradation by proteasomes, which are large protein complexes that function as proteases. Once IκBα is degraded, the NF-κB complex is free to translocate to the nucleus, where it activates the transcription of genes involved in inflammation, such as cytokines, chemokines, adhesion molecules and other pro-inflammatory factors. play a significant role in inflammatory pathways and that phytochemicals can modulate these pathways, suggesting potential therapeutic applications for managing inflammatory diseases (Shin *et al*., 2020).

2.3.7 Protease identification and characterisation

Protease is a type of bioactive molecule and an enzyme that break down proteins by cleaving peptide bonds, playing crucial roles in various biological processes (López-Otín

& Bond, 2008). Purifying proteases from plant samples is important because it allows for the isolation of these specific enzymes, ensuring their activity can be accurately studied and utilised without interference from other compounds in the plant extract. The advantages of purifying plant proteases include the potential for high specificity and potency in their biological effects, particularly in therapeutic applications. Proteases and phytochemicals both have anti-inflammatory properties, but they achieve this through distinct mechanisms. Proteases may help by degrading pro-inflammatory mediators and modulating immune responses, whereas phytochemicals often work by scavenging free radicals and inhibiting pro-inflammatory pathways (Shin *et al*., 2020; Schilrreff & Alexiev, 2022). Whether plant-derived proteases are better than phytochemicals in reducing inflammation depends on the specific context and application, as both have unique benefits and can be complementary in their effects.

Protease identification involves a comprehensive process starting with the selection and extraction of proteases from various sources such as bacteria, fungi, plants or animals (Kotb *et al*., 2023). Following extraction, activity assays using specific substrates like casein or synthetic peptides help determine the enzyme's specificity and activity levels, typically detected through colorimetric, fluorometric or spectrophotometric methods. The protease is then purified using ammonium sulphate precipitation, dialysis, chromatography techniques such as ion-exchange or affinity chromatography and analysed for purity and molecular weight via SDS-PAGE and zymography. This includes determining enzymatic activity through substrate specificity and various assay methods, identifying optimal pH and temperature conditions, and assessing stability. Kinetic parameters such as Km and Vmax are calculated to gauge enzyme efficiency and substrate affinity, while inhibition studies provide insights into the enzyme's active site. Molecular weight and purity are analysed using SDS-PAGE and mass spectrometry, and the isoelectric point is determined through isoelectric focusing. Structural details are revealed

by techniques like X-ray crystallography and NMR spectroscopy. Through these characterisations, researchers can fully understand proteases, leading to diverse applications in various fields. Previous study was successfully isolated and characterised a protease enzyme from *Dactylorhiza osmanica* tubers with optimal pH and temperature properties, substrate specificity for *α*-, *β*-, and *κ*-casein hydrolysis, and potential applications in the food industry (Yıldırım Çelik, 2021). The protease enzyme was isolated using techniques such as ammonium sulphate precipitation and size exclusion chromatography. The partially isolated protease had an optimal pH of 6.5, making it suitable for applications in cheese manufacturing where milk clotting occurs around pH 5.5 to 6.5. The isolated enzyme retained 100% activity for up to 21 hours at 40°C and up to 4 hours at 50°C, indicating good stability at elevated temperatures. The protease enzyme hydrolysed *α*-, *β*-, and *κ*-casein, forming new peptides larger than 15 kDa with molecular mass of the enzyme was determined to be 31 kDa. Therefore, it resulted that protease enzyme isolated from *Dactylorhiza osmanica* tubers, has potential in the enzyme production and can be fully explored in the industry.

2.4 *Christia vespertilionis*

One plant that has been studied by researchers that contain an anti-inflammatory agent is *Christia vespertilionis* known as butterfly wing and frequently referred to as "Daun rama-rama" in Malaysia (Figure 2.1). There have been reports and studies on its anti-inflammatory and anti-cancer effects properties (Osman *et al*., 2017). Typically, the leaf part of the plant was boiled and chopped into pieces to form a paste and consumed in the case of gastrointestinal problems or applied locally to wounds (Naidu, 2011).

Christia vespertilionis (L. f.) Bakh. F. of the family namely Fabaceae is known as ‘butterfly wing’ due to the leaf structure similar to the wings of a butterfly. It is cultivated primarily in Asian nations where the climate is hot and humid all year round and is

renowned as an ornamental plant. This plant is native to Southeast Asia, including Malaysia, Indonesia, Thailand, Cambodia, Laos, Vietnam and South-Eastern China. Genus *Christia* is commonly called the island pea and it has thirteen species that have been identified in tropical Asia and five species discovered in China (Murugesu *et al*., 2020; Whiting, 2007). *Christia* species are diffuse-herbs or sub-shrubs with alternately arranged trifoliate and simple leaves and stipule-like structures. Stipels and segmented loments folded, contained in the calyx are typical features of this genus (Chen *et al*., 1993). White axillary panicles and tiny flowers with petals that resemble wings were in bloom. Pollen grains from *Christia* are medium-sized and come in a variety of forms, including spheroidal, subprolate, elliptic and rhombic when viewed from the equator (Murugesu *et al*., 2020). This plant can be easily located beside highways in grassy meadows and sandy soils. The plant can reach a height of 60 to 120 cm, including quickly multiplied from seeds and semi-woody cuttings (Shah *et al*., 2019).

The leaves are available in shades of green that are frequently used for therapeutic use, and also red leaves which are usually employed for decorative purposes. Based on Zambari et al. (2023) study, green leaf *C. vespertilionis* has higher phytochemical compounds and activities than red leaf *C. vespertilionis*. The most part of *C. vespertilionis* used was the leaves where flavonoid, phenolic acid and steroid were found to be present (Upadhyay *et al*., 2013; Mutalib & Latip, 2019). Even though it is an ornamental plant, the locals have used it to treat a variety of diseases, including tuberculosis, scabies, snake bites, bronchitis and pure blood circulation (Garnock-Jones, 1983; Whiting, 2007). Research has shown that this plant has anti-inflammatory properties, which can help reduce inflammation by inhibiting the production of pro-inflammatory cytokines (Nguyen-Pouplin *et al*., 2007; Rayburn *et al*., 2009; Osman *et al*., 2017). It also has antioxidant effects, which may help to protect cells and tissues from oxidative damage

caused by free radicals by donating an electron to stabilise and prevent cellular damage (Lee *et al*., 2020).

Figure 2.1: ***Christia vespertilionis***

Besides that, it was used widely to study anti-cancer properties. Research shows that the presence of flavonoids, coumarins and quinones in the roots of *C. vespertilionis* has phytochemical properties that could prevent breast cancer. According to a study by Lee et al. (2020), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay performed on the root of *C. vespertilionis* showed anti-cancer effects against human breast carcinoma cell lines (MCF-7 and MDA-MB-231). Another study also stated that

C. vespertilionis has anti-proliferative properties against the cancer cell lines medullary thyroid carcinoma (MTC) and small intestinal neuroendocrine tumours (SI-NET) which means that the plant has the ability to stop the spread of the cancerous cells into tissues (Hofer *et al*., 2013). Corynoxidine and palmatine are two antibacterial compounds that are found to be present in the aerial portion of the plant (Nguyen-Pouplin *et al*., 2007; Sharma & Cannoo, 2016; Lee *et al*., 2020; Murugesu *et al*., 2020). This plant also was found to be antiplasmodial where christene and christanoate were discovered from *C. vespertilionis* (Upadhyay *et al*., 2013). Additionally, phytochemical compounds such as phenols, alkaloids, triterpenes, fatty acids and long chain alcohols were said to be present in *C. vespertilionis* (Hofer *et al*., 2013).

Christia vespertilionis was chosen because of its ability to treat a variety of disorders which has been proven by various researchers. However, although there are studies on anti-cancer, anti-diabetic, anti-oxidant and anti-proliferative activity, not many findings on anti-inflammatory activity. Protease is one of the essential components of anti-inflammatory drugs, that will be isolated from *C. vespertilionis* leaves in this study. There is no research on protease isolated from *C. vespertilionis*. Furthermore, each protease enzyme generated from different plant has a unique specificity (Winarti *et al*., 2018). Therefore, investigating the protease enzymes from *C. vespertilionis* could reveal novel specificities and potential applications in various biotechnological fields for further research in this area.

2.4.1 *Christia vespertilionis* mechanisms of inflammatory action

By targeting these mechanisms, phytochemicals effectively reduce inflammation and contribute to the management of inflammatory conditions. As of now, detailed mechanistic studies specifically on the anti-inflammatory action of *C. vespertilionis* are limited. However, some research has highlighted its potential anti-inflammatory effects

which are attributed to its bioactive chemicals including flavonoids, alkaloids, phenolic, tannins, saponins and terpenoids (Ibrahim *et al*., 2022). Studies have demonstrated that *C. vespertilionis* extract inhibits monocyte adherence to endothelial cells by reducing the production of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), both of which play critical roles in the inflammatory response (Fauzi *et al*., 2020). VCAM-1 and ICAM-1 are essential for mediating leukocyte adherence and transmigration to the endothelium, including monocytes. *C. vespertilionis* is rich in bioactive compounds such as polyphenols, flavonoids, terpenoids and alkaloids that can modulate the inflammatory response and inhibit the expression of VCAM-1 and ICAM-1 through multiple mechanisms. One significant mechanism involves the inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, a crucial regulator of inflammation, which it reduces the transcription of VCAM-1 and ICAM-1 genes. Furthermore, specific compounds in *C. vespertilionis*, particularly flavonoids like quercetin and apigenin, can directly inhibit the gene expression of VCAM-1 and ICAM-1 (Al-Khayri *et al*., 2022).

Previous study also showed serum uric acid levels in the hyperuricemic rats treated with *C. vespertilionis* extract at a concentration of 200 mg/kg were significantly decreased by 31.95% compared to the hyperuricemic control group (Endrini *et al*., 2023). Hyperuricemic refers to having an abnormally high level of uric acid in the blood and can lead to gout which is an inflammatory condition in the form of arthritis characterised by sudden, severe attacks of pain, redness and tenderness in joints. This reduction in uric acid level suggests that *C. vespertilionis* has antihyperuricemic properties and anti-inflammatory effects through the inhibition of xanthine oxidase. *C. vespertilionis* prevent the formation and deposition of urate crystals in joints and tissues by minimising the immune response and decreasing the release of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 (Zhao *et al*., 2022). This approach able to control hyperuricemia and

also reduces the inflammation, providing a comprehensive strategy for managing conditions like gout.

In the literature, a study examining the anti-inflammatory properties of another species within the Fabaceae family revealed that extracts abundant in compounds similar to those identified in *C. vespertilionis*, such as flavonoids and saponins, significantly diminished inflammation in both *in vitro* and *in vivo* settings (Usman *et al*., 2022). Using an untargeted tandem mass spectrometry-based molecular networking approach, researchers discovered 60 unique metabolites in the *C. vespertilionis* leaves, with flavonoids being the most prevalent class of compounds (Norazhar *et al*., 2021).

In the phytochemical profiling of the aqueous infusion of *C. vespertilionis* leaves that have done in the previous study, researchers employed liquid chromatography-mass spectrometry (LC-MS) to identify the phytochemicals present in the extract. The analysis indicated that phenolics, especially isoferulic acid, were the most abundant compounds detected in the aqueous extract of *C. vespertilionis* (Endrini *et al*., 2023). Isoferulic acid has demonstrated significant anti-inflammatory properties through inhibition of NF-κB signalling pathway which is crucial for the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6. By blocking the pathway, isoferulic acid reduces the production of these cytokines and reduce inflammation (Adeyi *et al*., 2023).

2.5 Palm tocotrienol-rich fraction

Antioxidants are chemicals that preventing free radical damage and oxidative stress in the body's cells. Highly reactive molecules known as free radicals are naturally created by the body as byproducts of numerous metabolic processes. They can also come from outside causes like exposure to chemicals, UV rays and environmental toxins (Noviany *et al*., 2023). When the body's natural defences such as antioxidants, are outnumbered by free radicals, oxidative stress results. Free radicals have the capacity to harm vital cellular

elements including DNA, proteins and cell membranes, resulting in cellular malfunction and perhaps causing a variety of health problems like ageing, inflammation and some chronic diseases. Antioxidants neutralise, stabilising and stopping free radicals from doing more harm by giving electrons. They are essential for preserving cellular health and preventing oxidative damage. Antioxidants can be divided into many categories including vitamin E (tocopherols and tocotrienols), ascorbic acid (vitamin C), beta-carotene, selenium, zinc and polyphenols.

Antioxidants, including the palm tocotrienol-rich fraction (TRF), have garnered significant attention for their potential health benefits and therapeutic properties. Palm TRF, derived from palm oil, is a potent source of tocotrienols, a subgroup of vitamin E, known for their superior antioxidant activity compared to tocopherols. *Elaeis guineensis*, known as the African oil palm, is a West African native and one of the most significant palm species. Since more than 7000 years ago, oil palm fruits have been used in primitive societies as a semi-wild food source (Murphy *et al*., 2021). As part of colonial endeavours to establish newly imported cash crops in the region, oil palm seeds were carried to the Dutch East Indies (now Indonesia) and the Malay States (modern Malaysia) throughout the nineteenth century. The Malay States steadily adopted more organised oil palm plantation farming over the twentieth century. According to Malaysian Palm Oil Production by year 2020, oil palm is a relatively new crop that only gained recognition worldwide later in the twentieth century. This was largely because of government programmes implemented in the 1970s and 1980s to develop Malaysia's agricultural and economy as a newly independent country (Murphy, 2014). Malaysia is one of the countries other than Indonesia that cultivates about 85% of the world’s oil palm trees production (Murphy *et al*., 2021). Palm oil is an environmentally-friendly and most efficient vegetable oil as it has less land to generate the same yield as compared to other oils. This was supported by Roda (2019) which stated each hectare of land can yield 3.8

tonnes of palm oil compared to sunflower oil that can only produce 0.7 tonnes, soybean for only 0.5 tonnes and rapeseeds for 0.8 tonnes per year.

On the tropical palm oil tree, palm fruits as shown in Figure 2.2, which are about the size of small plums, grow in bunches. Each bunch has 1,000 or more palm fruits, which are gathered annually. Palm fruits contain two main tissues including palm oil and palm kernel oil (Murphy, 2019). While palm kernel oil is a white-yellow liquid that is mostly collected from the endosperm tissue of the kernel (seed), palm oil is a deep orange-red, semi-solid fluid that is extracted from the fleshy mesocarp tissue. About 89% of the total fruit oil in palm trees is obtained from the mesocarp, and the remaining 11% is derived from the seed or kernel. Palm oil is frequently used for cooking oil, while palm kernel oil is implemented as the main functional component in numerous soaps, detergents and cosmetics. These two oils are employed for various downstream applications in a variety of industrial sectors because of their highly varied fatty acid contents (Goggin & Murphy, 2018). Over the years, different techniques of extraction such as adsorption, vacuum and molecular distillation, supercritical fluid extraction (SFE), membrane processing and solvent extraction, have been implemented to extract phytochemicals from palm oil. With increasing study and advancements in extraction methods, improvements have been made to extract effectively various phytochemicals from palm oil.

Natural palm oil is rich in nutrients and contains vitamins A, vitamin E, carotenes, phytosterols, squalene, coenzyme Q10, palm phenolics and phospholipids (Hoe *et al.*, 2020). Carotenoids and vitamin E are known for their antioxidant and anti-inflammatory properties. The tocopherols and tocotrienols in palm oil make up the vitamin E. Tocotrienols make up 70% of the vitamin E in palm oil and the rest is tocopherols. Tocotrienols and tocopherols are vitamin E with each consists of four different isomers including alpha (α), beta (β), gamma (γ) and delta (δ) that can be found mostly in vegetable oils, palm oil, rice bran oil, wheat germ, barley, seeds, nuts and grains.

However, tocotrienols with a complete isoform, known as tocotrienol-rich fraction (TRF) can only be found in palm oil that originally extracted from palm fruit of *Elaeis guineensis* (Durani *et al*., 2018). Palm TRF consists of 70% of tocotrienol and 30% of tocopherol has been studied extensively due to their potential for having many biological activities and generally possess various health benefits (Sundram *et al*., 2003). Palm TRF has been shown to have potent antioxidant activity, which may help to protect cells and tissues from oxidative damage caused by radicals (Conte *et al*., 2004; Teo *et al*., 2022). It has been found to also have anti-inflammatory effects, which may help to reduce inflammation in the body by inhibiting the production of pro-inflammatory cytokines (Wu *et al*., 2008; Yam *et al*., 2009; Malavolta *et al*., 2018). It also has been shown to have anti-cancer properties, however more research is needed to fully understand the mechanism of action (Conte *et al*., 2004).

Figure 2.2: Palm fruit of Elaeis guineensis (Photo sourced from: www.nutraingredients-asia.com).

Palm TRF has extensively explored and the outcome is very promising that we can discover more about their therapeutic mechanisms. Palm TRF stimulates and prevents the induction of several cancer cell lines by lipopolysaccharide (LPS) (Nesaretnam & Meganathan, 2011). Palm TRF has also been shown in prior research to have the ability to treat inflammation by suppressing pro-inflammatory mediators, cytokines, chemokines and other immune components (Wu *et al*., 2008). Previous studies show that palm TRF has shown suppression through various cell lines with cytokines expression of tumour necrosis factor-alpha (TNF-α) (Ahn KS *et al*., 2007; Kuhad & Chopra, 2009), interleukin-1 (IL-1) (Norazlina *et al*., 2007), interleukin-6 (IL-6) (Ahmad *et al*., 2005) and interleukin-8 (IL-8) (Shibata *et al*., 2008) as well as suppresses signal transducer and activator of transcription 3 (STAT3) cell-signalling pathway (Kashiwagi *et al*., 2008; Bachawal *et al*., 2010). It demonstrates that palm TRF can treat inflammation and is a potent anti-inflammatory.

Overall, the mechanisms of action of palm TRF are complex and more research is needed to fully understand how this natural extract works. However, based on available evidence and previous studies suggest that palm TRF has various health benefits, particularly in the areas of anti-oxidant, anti-inflammatory activities and anti-cancer properties.

2.5.1 Palm tocotrienol-rich fraction mechanisms of inflammatory action

Tocotrienols, a subgroup of the vitamin E family, are known for their potent antioxidant and anti-inflammatory properties. Tocotrienols exhibit their anti-inflammatory effects by modulating microglia responses through different mechanisms. Previous study, it was demonstrated that both δ-tocotrienol and Tocomin@50% were able to reduce the expression of cluster of differentiation 40 (CD40) receptor on microglia cells. CD40 is a co-stimulatory molecule present on antigen-presenting cells (APCs)

including microglia and its increased expression has been associated with neurodegenerative diseases. By decreasing CD40 expression, tocotrienols may prevent the full activation of microglia and their interaction with T cells, leading to reduced inflammation within the central nervous system. Furthermore, δ-tocotrienol exhibited better downregulation of nitric oxide (NO) production, while Tocomin@50% was more effective in reducing CD40 expression (Tan *et al*., 2016).

Another study showed that NF-κB is a transcription factor that regulates inflammatory response and tocotrienol has been shown to modulate this pathway. Tocotrienols suppress NF-κB signalling pathway through several mechanisms, including modulation of several regulatory proteins and genes, and increase expression of pro-apoptotic molecules and reduce proliferative molecules in cancer cells. Tocotrienol was found to downregulate NF-κB expression in various inflammatory disease models, including activity in bone diseases, showing reduced receptor activator of nuclear factor kappa-β ligand expression, improved osteoblast production and reduced inflammatory cytokines (Nasir *et al*., 2021).

2.6 Combination treatment

Combination therapy is a combination of several treatments to provide greater effects than utilising one therapy alone (Alayande *et al*., 2018). The disease can then be managed from a variety of aspects and by integrating several tactics, which will result in a more thorough and efficient treatment strategy. Combination therapy can be quite effective, but also needs to be closely monitored and managed by medical experts. To guarantee patient safety and therapeutic success, interactions between treatments, potential side effects and specific patient characteristics must be carefully taken into account. Combination therapy is a component of contemporary medicine since it enables medical experts to improve patient outcomes and optimise treatment plans. Combination therapy would involve

multiple mechanisms including synergistic effects, complementary phytochemicals, oxidative stress reduction and modulation of immune response.

2.6.1 Synergistic approaches

There are many diseases that does not have effective drug treatment such as Alzheimer's disease (AD), Parkinson's disease, congestive heart failure, pulmonary hypertension, inflammation and many more. A method for treating complicated disorders as such is combination therapy with a synergistic approach. For more than 2000 years, traditional Chinese medicine has been used mixtures of herbs to treat diseases and it has been practised until now (Yuan, 2000). Treatment combination has been explored widely and gained interest by researchers due to the fact it can increase treatment efficacy if the interaction achieves synergistic effect.

Previous studies have proven the efficacy of combination therapies to treat various chronic diseases such as cancer, hypertension, asthma and acquired immunodeficiency syndrome (AIDS) (Nelson, 2001; Glass, 2004; Humphrey *et al*., 2011). The combination of two or more drugs can help to enhance the ability of drugs to reduce inflammation synergistically. In general, the synergistic approach in a multi-component combination therapy provides the desired therapeutic outcome with greater bioactivity, multi-target behaviour and also aids in reducing side effects or toxicity with a lower dosage required from each component (Zhou *et al*., 2022).

The study conducted by Mutalib & Latip (2019) shows that *C. vespertilionis* leaf extract with chemotherapy drug cyclophosphamide interact synergistically by inhibiting cancer cells of WRL68. This demonstrates that *C. vespertilionis* could be combined with other medications, creating a synergistic effect that would eventually result in an efficient drug treatment. This plant has been shown to have many biological activities and has been found to be effective in treating a number of diseases, including cancer and inflammation.

Even while *C. vespertilionis* may be successful when used alone, combining it with another medication may increase that efficacy.

When employing an isobologram to produce a specific effect, the x and y axes reflect the amounts of two different drugs or treatments (A and B). The coordinate at A0 will represent the IC_{50} for medication A if the defined effect is 50 %, and the coordinate at 0B will represent the IC_{50} for drug B. Two IC_{50} points can be connected to create the line of additivity. The point on the graph will then indicate if there is synergy, additivity or antagonism. The combination index model, a quantitative indicator of medication interaction at a particular effect, was created as a result of the Loewe additivity approach. Based on its formula, the combination index (CI) can be determined, where $CI < 1$, $CI = 1$ and $CI > 1$ stand for synergy, additivity and antagonism, respectively. It is helpful to find qualified individuals for additional clinical research when approaches like Loewe Additivity in dose-effect approach with combination index and isobologram analysis are applied in combination.

In vitro and *in vivo* tests could more precisely demonstrate the efficacy of combination therapy in preclinical investigations. Since ancient times, plants have evolved to fight disease-causing pathogens through a mix of mechanisms. There hasn't been any research for the combination of *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), particularly on the test for inflammation-related issues. The mixtures of natural products offer opportunities for drug development, and by that, pharmacological investigations into combination therapies can be examined at bioactivity test.

In this study, *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) were combined to examine their synergistic effect using *in vitro* test on lipopolysaccharide (LPS) stimulated RAW264.7 macrophages and *in vivo* test on carrageenan-induced BALB/c paw oedema. Pharmacodynamic synergists originate from

complementary actions, in which a combination interacts with several locations along a particular pathway, resulting in positive therapy effects and reducing negative disease-related consequences (Ma *et al*., 2009). For instance, the study demonstrated that ginger and turmeric have synergistic anti-inflammatory effects by blocking the production of pro-inflammatory mediators in RAW264.7 macrophages that are triggered by lipopolysaccharide (LPS) and interferon (Zhou *et al*., 2022).

2.6.2 Complementary approach

A complementary strategy involves combining complementary or alternative therapies with standard medical care to offer a comprehensive and all-encompassing approach to healthcare (Beuth & Schierholz, 2007). Acupuncture, medicine plant, massage therapy, chiropractic treatment, meditation, yoga, aromatherapy, art therapy, music therapy and mindfulness exercises are just a few of the therapies that fall under complementary approaches. For example, in plants, it contains phytochemicals that have complementary anti-inflammatory effects such as flavonoids that inhibit cytokines, while other plants may contain different compounds that help to inhibit enzyme activity in the inflammation process. Complementary therapies are meant to enhance the patient's general health and may even improve the effectiveness of the primary therapy. They are used to address a variety of health issues such as pain management, stress reduction, emotional wellbeing and quality of life.

2.6.3 Oxidative stress reduction

Reactive oxygen species (ROS) are very reactive oxygen-containing molecules that can harm cellular components like lipids, proteins and DNA, resulting in oxidative stress, cellular malfunction and possibly causing a number of health problems (Yousaf *et al*., 2022). To sustain optimal health, oxidative stress must be reduced. Oxidative stress can be caused by a number of things, including exposure to toxins in the environment, UV

radiation, smoking, eating poorly, being inactive and having certain medical problems. The way to reduce oxidative stress is by combining two plants containing antioxidant compounds that would enhance the efficacy to cure inflammation.

2.6.4 Modulation of immune response

The term "modulation of immune response" refers to the deliberate modification or regulation of the body's immune system to produce particular therapeutic effects. In order to protect the body from infections, external invaders and aberrant cells, the immune system is essential (Beuth & Schierholz, 2007). The immune system can become either overactive (leading to autoimmune illnesses) or underactive (increasing susceptibility to infections or cancer). In order to stop the immune system from attacking healthy tissues or overreacting to harmless substances, immunosuppression involves reducing or inhibiting the immune system's activity. This strategy is frequently used to lessen inflammation and treat symptoms in autoimmune disorders such as lupus and rheumatoid arthritis.

2.6.5 Combination treatment mechanisms of inflammatory action

In addition to the individual effects of single phytochemicals, combining multiple phytochemicals can target various pathways and cellular processes, resulting in a synergistic anti-inflammatory effect. This approach offers a promising strategy for reducing inflammation and preventing chronic diseases in humans. The combination of resveratrol and luteolin, which are high in radicchio, peppers and celeries has been shown to synergistically inhibit TNF-α-induced monocyte adhesion to endothelial cells.

The combination interacts with endothelial cells and various immune cells. The phytochemicals may directly scavenge elevated reactive oxygen species (ROS), increase endogenous antioxidants/enzymes such as superoxide dismutase (SOD) and promote the

Nrf2/HO-1 system to fight oxidative stress. These reduced ROS and/or the chemicals directly further regulate AMPK/SIRT1, Nrf2/HO-1, and/or MAPK cascades to inhibit the NF-κB pathway in the cytosol. The molecules then transfer into the nucleus to regulate the transcription and translation of pro-inflammatory markers such as ICAM-1, VCAM-1, MCP-1, TNF-α, IL-8, IFNβ as well as upregulate anti-inflammatory molecules such as IL-10 level, Nrf2/HO-1 and endothelial NO (Zhang *et al*., 2019). These changed molecules, in turn, suppress the proliferation and migration of immune cells and maintain the integrity of endothelial cells to further reduce the production of pro-inflammatory markers and eventually inhibit inflammation.

Combining multiple phytochemicals can influence different pathways and cellular processes, leading to a synergistic anti-inflammatory effect. Previous study of the combination δ-tocotrienol and tart cherry anthocyanins, both separately and in combination, could reduce inflammation in 3T3-L1 adipocytes. The results found that neither compound had toxic effects on the cells and suggest that δ-tocotrienol and tart cherry anthocyanins can help reduce inflammation in adipocytes through the NF-*k*B pathway (Harlan *et al*., 2020). There are not many combinations have been previously studied using combination of *Christia vespertilionis* and other treatments or drugs. However, one study found that combination treatment of *C. vespertilionis* plant extract and the chemotherapy drug of cyclophosphamide was resulted in synergism, with combination index (CI) values less than 1. The synergistic effect of the plant extract combined with cyclophosphamide showed potential synergistic interactions at higher concentrations, which could enhance the cytotoxicity compared to using the drug or plant extract alone (Latip *et al*., 2019).

2.7 The challenges of the study

The challenge of this study is the economic restraints. Research can be costly at some point. Therefore, it is necessary to select the best choice of assay model that can give optimal results. When moving to clinical studies, it becomes more challenging as it has strong practical and ethical limitations. The agencies of US Food and Drug Administration (FDA), the European Medicines Agency (EMA) and the World Health Organisation (WHO) have agreed that combination therapies need supported by the disease of interest and preclinical studies (*in vitro* and *in vivo*), preferably using cell culture and animal test, together with evidence that the combination has greater efficiency than alone drug (Woodcock *et al*., 2011). The combination therapies not solely depend on the characteristics of the drugs, but also depend on the dose ratio. Two drugs combined at a given ratio are considered as dose-effect relation and at the same time optimise the synergy (Chou, 2010; Keith *et al*., 2005).

The discovery for a newer anti-inflammatory agent from natural products is an ongoing process and increasingly gaining interest to this date. Currently, researchers have been continuously studying the phytochemical properties and activities for anti-inflammatory. However, it will be better to extrapolate the anti-inflammatory studies reported by earlier studies using a sophisticated molecular approach. Thus, the screening of effectiveness of anti-inflammatory drugs at the preclinical drug development is required. There are studies conducted using crude extracts, purified or isolated compounds. However, most of the studies did not extend to the establishment of their molecular mechanisms and pharmacokinetics. Subsequently, the appropriate animal models with widespread involvement of biochemical and molecular assay need to be executed for better drug development from natural products.

CHAPTER 3: MATERIALS AND METHODS

3.1 Preparation of plant sample

This species, found mostly in Southeast Asia, has leaves with a shape or pattern resembling butterfly wings, similar to those of the butterfly bush (Buddleja) or some types of begonias (Smith, 2004; Brickell, 2008). *C. vespertilionis* thrives in shaded or partially shaded environments, preferring well-draining, moist and fertile soils rich in organic matter. It favours high humidity and warm temperatures, commonly found along stream and river edges. The plants were cultivated under optimal conditions to ensure the health and quality of the leaves. The optimal time for harvesting *C. vespertilionis* was identified as late spring to early summer, when the butterfly wing leaves are fully developed and at their peak in terms of bioactive compound content (Middleton, 2019).

In conducting this research, *C. vespertilionis* was bought from Floranika Nursery in Sungai Buloh, Selangor (Malaysia) that provides high-quality, disease-free specimens, ensuring optimal growth for the extraction process. The plant sample was certified by Dr. Yong Kien Thai from Rimba Ilmu, Universiti Malaya with voucher specimen (KLU 50026) and was placed at the University Malaya herbarium. The leaves of *C. vespertilionis* were used in this work as many research have published showing its potential to contain bioactive compounds and reducing inflammation.

Upon that, the *C. vespertilionis* plant were immediately transported to the laboratory for preparation. The preparation process began with the selection of healthy and undamaged plant leaves to ensure the integrity and representativeness of the samples. The leaves were gently rinsed with distilled water to remove any surface contaminants such as dust and soil. The plants were then kept for further use.

Palm tocotrienol-rich fraction or palm TRF was obtained from a commercial product, Gold Tri.E 70 (Sime Darby, Malaysia), which is in the form of α-tocopherol (209.7 mg/g), α-tocotrienol (182.3 mg/g), β-tocotrienol (18.5 mg/g), γ-tocotrienol (231.9 mg/g) and δ-tocotrienol (66.1 mg/g), extracted from palm fruit. The choice of this product was based on its high quality and consistency, ensuring reliable results in combination treatment with *C. vespertilionis*. The product was prepared according to the manufacturer's instructions. Specifically, palm TRF, an amber viscous liquid (0.072 mL), was diluted with 5 mL of soya oil to be used as a stock of 10 mg/mL. From this stock solution, further dilutions were made as necessary for the specific concentrations used in the *in vitro* and *in vivo* anti-inflammatory assay.

3.2 Chemicals and reagents

Ethanol of analytical grade for plant extraction, and all chemicals and reagents for phytochemical analysis was obtained from Sigma-Aldrich, (St. Louis, Missouri, USA). The analytical grade ammonium sulphate $(NH_4)_2SO_4$, dialysis membrane, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) components and reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and the protein marker was purchased from GeneDireX (Taoyuan, Taiwan). Papaya-sourced papain used as control was acquired from Sigma-Aldrich (St. Louis, Missouri, USA).

All media and reagents for cell culture including aspirin were acquired from Gibco-BRL (Paisley, Scotland) and Sigma-Aldrich, (St. Louis, Missouri, USA). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay kit was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Lipopolysaccharide (LPS) from *Escherichia coli* was obtained from Invitrogen by Thermo Fisher Scientific (Waltham, Massachusetts, USA). Chemicals and reagents (Griess) for nitrite oxide (NO) determination were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

A carrageenan (a total of 0.1 g freshly diluted with 10 mL of distilled water) and aspirin were purchased from Sigma-Aldrich, (St. Louis, Missouri, USA). Radioimmunoprecipitation (RIPA) buffer was required from Solarbio Life Sciences, (China). Tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were obtained from R&D Systems, (Minneapolis, USA). Chemicals and reagents for nitrite oxide (NO) determination were purchased from Sigma-Aldrich, (St. Louis, Missouri, USA).

3.3 Preparation of *Christia vespertilionis* leaf extract

The preparation of *C. vespertilionis* leaf extracts were performed using one gram of dried powdered sample of *C. vespertilionis*. It was extracted with 200 mL of ethanol using Soxhlet extraction for 8 h as Soxhlet extraction method only uses a small amount of plant sample and one gram of sample was sufficient for the extraction purposes. The extract was filtered and concentrated using a rotary evaporator and filtered again to remove any residues.

The extract was air-dried to obtain its dry weight. The extraction yield was calculated using the following formula:

$$\text{Extraction yield (\%)} = \frac{m^2}{m^1} \times 100$$

m^1 = mass of dry weight of *C. vespertilionis* leaves, (g)

m^2 = mass of crude extract of *C. vespertilionis* leaves, (g) (3.1)

3.4 Qualitative analysis of *Christia vespertilionis* leaf extract

One gram of dried plant extract was placed in a clean conical flask and mixed with 10 mL of ethanol. The flask was stirred until the extract dissolved completely. The solution was then filtered to remove any undissolved particles. Additional ethanol was added to adjust the final volume of the liquid extract to the desired concentration. Standard qualitative tests were performed to confirm the presence of phytochemical compounds, including alkaloids (Mayer's test), tannins ($FeCl_3$ test), saponins (foam test), cardenolides (sodium picrate test), phenolics ($FeCl_3$ test), cardiac glycosides (Keller-Kiliani test), flavonoids (ammonia test), steroids (Libermann-Burchard test) and terpenoids (Salkowski test) in the leaf extract of *C. vespertilionis*.

3.4.1 Test of alkaloid

One millilitre of 1 % (v/v) hydrochloric acid (HCl) was added to 3.0 mL of leaf extract. Then, the mixture was heated at 37 ºC for 20 min and left to cool and filtered using Whatmann filter paper. Two drops of Mayer's reagent were added and a creamy precipitate indicates presence of alkaloid in the extract.

3.4.2 Test of tannin

One millilitre of 10 % (v/v) ethanolic potassium hydroxide (KOH) was added to 1.0 mL of leaf extract. A white precipitate indicates presence of tannin.

3.4.3 Test of saponin

Two millilitre of leaf extract was shaken vigorously with 2.0 mL of distilled water for 2 min and then warmed afterwards. Frothing appearance indicates the presence of saponin.

3.4.4 Test of cardenolide

Two millilitre of glacial acetic acid containing a drop of 5 % (w/v) iron (III) chloride ($FeCl_3$) solution was added to 1.0 mL of leaf extract. This was followed by an addition of 2.0 mL of sulphuric acid (conc. H_2SO_4). Appearance of a brown ring at the interface indicates presence of a deoxy sugar characteristic of cardenolide.

3.4.5 Test of phenolic

Two drops of 5 % (w/v) iron (III) chloride ($FeCl_3$) were added to 1.0 mL of leaf extract. A greenish precipitate indicates presence of phenolic.

3.4.6 Test of cardiac glycoside

Two millilitres of chloroform and 2.0 mL of sulphuric acid (conc. H_2SO_4) were carefully added to 1.0 mL of leaf extract. A reddish-brown colour at the interface indicates presence of aglycone portion of cardiac glycoside.

3.4.7 Test of flavonoid

One millilitre of 10 % (w/v) sodium hydroxide (NaOH) was added to 3.0 mL of leaf extract. Yellow colouration indicates presence of flavonoid.

3.4.8 Test of steroid

Five drops of sulphuric acid (conc. H_2SO_4) were added to 1.0 mL of leaf extract. Red colouration indicates the presence of steroids.

3.4.9 Test of terpenoid

About 0.5 mL of chloroform and a few drops of sulphuric acid (conc. H_2SO_4) were added to 1.0 mL of extract. Reddish brown precipitate indicates the presence of terpenoid.

3.5 Quantitative analysis of *Christia vespertilionis* leaf extract

In phytochemical analysis, the determination of total phenolic content (TPC), total flavonoid content (TFC) and total tannin content (TTC) quantifies these specific compounds in plant extracts using calibration curves for accurate measurement and positive controls to ensure method reliability (Sharma *et al*., 2023). The TPC test involves constructing a calibration curve using known concentrations of a standard phenolic compound of gallic acid to quantitatively assess the phenolic content in plant extracts. A positive control in this test confirms the method's accuracy in detecting phenolic compounds by using gallic acid standard solution. Meanwhile, the TFC test employs a calibration curve with standard concentrations of a flavonoid compound of quercetin to measure flavonoid content, with quercetin as positive control to ensuring sensitivity to flavonoids. Lastly, the TTC test utilises a calibration curve based on tannic acid standard solutions at various known concentrations, to determine tannin content in extracts. Tannic acid solutions as positive controls in the TTC test validate the method's ability to detect tannins effectively.

Total phenolic, flavonoid and tannin contents were quantified in *C. vespertilionis* leaf extract. Gallic acid and quercetin were used as standard anti-inflammatory agents, according to Mokhtar et al. (2019), with some modifications to the Folin-Ciocalteu method. Meanwhile, tannic acid was used as a standard anti-inflammatory agent according to the method described by Folin-Ciocalteu, as modified by Mesfin & Won (2019). An absorbance was measured using Shimadzu, UV-1700 spectrophotometer (Tokyo, Japan).

3.5.1 Total phenolic content

About 600 µl of Folin's reagent with 1.0 mL of 7.5 % (w/v) sodium carbonate (Na_2CO_3) was added into 200 µL of extract. The mixture was incubated in the dark for 2 h in room temperature. The absorbance of each mixture was then measured at 765 nm against gallic acid (0.0-1.0 mg/mL) as the standard solution. The total phenolic content was expressed in terms of gallic acid in mg GAE/mL of extract.

3.5.2 Total flavonoid content

A total of 600 µL of methanol (CH_3OH), 40 µL of 10 % (w/v) aluminium chloride ($AlCl_3$), 40 µL of 1 M potassium acetate (CH_3COOK), and 1.12 mL of Milli-Q water were mixed well and added into 200 µL extract, then, incubated for 30 min at room temperature. The absorbance was measured at 420 nm and compared to the standard solution of quercetin (0.0-1.0 mg/mL). The total flavonoid content was expressed in terms of quercetin in mg QE/mL of extract.

3.5.3 Total tannin content

A total of 250 µL of Folin's reagent, 500 µL of 35 % (w/v) sodium carbonate (Na_2CO_3) and 3.75 mL of distilled water were added to 500 µL of leaf extract and incubated at room temperature for 30 min. The absorbance was measured at 725 nm and compared to the standard solution of tannic acid (0.0-1.0 mg/mL). The total tannin content was expressed in terms of tannic acid in mg TAE/mL of extract.

3.5.4 *In vitro* assay: Protease inhibition assay

Protease inhibition assay is an *in vitro* cell-free assays are often used for preliminary screening and quantitative analysis of protease inhibitors, including determining IC_{50} values and inhibition percentages. It measures the ability of a substance to inhibit the

activity of protease enzymes, providing data on the extent of inhibition often expressed as a percentage or IC_{50} value (the concentration of inhibitor needed to reduce enzyme activity by 50 %) (Sun *et al*., 2003; Tyndall *et al*., 2005; Berdyshev *et al*., 2023). This method allows the study to compare the efficacy of *C. vespertilionis* as an inhibitor against a well-characterised standard like aspirin. Using aspirin as a control helps validate the experimental results by providing a benchmark for the inhibition, ensuring that the observed effects of *C. vespertilionis* are accurately assessed against a known inhibitor.

The protease inhibition activity assay for *C. vespertilionis* leaf extract was conducted according to Sakat et al. (2010) and Gunathilake et al. (2018) with slight modifications. A total of 450 µL of 5 % (w/v) trypsin was pipetted into 50 µL of leaf extract at different concentration from 100-3000 µg/mL. The mixture was incubated at 37 °C for 10 min. Next, 250 µL of 1 % (w/v) casein was added and further incubated for 20 min. The reaction was terminated by adding 700 µL of 10 % (w/v) trichloroacetic acid and centrifuged at 3000 rpm for 5 min at 25 °C. The absorbance of the supernatant was measured at OD_{210nm} using a Shimadzu, UV-1700 spectrophotometer (Tokyo, Japan). Aspirin act as an anti-inflammatory drug, can serve as a control in a protease inhibition assay. The assay was carried out in triplicates and the results were compared to bovine serum albumin. The percentage of inhibition was measured using the formula below:

$$\text{Percentage inhibition (\%)} = \frac{(A^2 - A^1)}{A^2} \times 100$$

A^1 = sample absorbance

A^2 = control absorbance (3.2)

3.6 Gas chromatography-mass spectrometry (GC-MS) analysis of *Christia vespertilionis* leaf extract

A volume of 1.0 mL for *C. vespertilionis* leaf extract was diluted to 500 ppm with ethanol (C_2H_6O) as the solvent is effectively dissolves the extract and suitable for GC-MS analysis, ensuring that the sample can be accurately vaporised and analysed. Gas chromatography-mass spectrometry (GC-MS) analysis was done using Shimadzu, GCMS-QP2010 Ultra (Tokyo, Japan). A total of 0.5 µL of sample was auto-injected into the system. The system was equipped with a capillary column of RTX5MS with a 30.0 m in length × 0.25 mm in diameter and 0.25 µm of thickness. The injection temperature was set at 200 °C; with splitless injection mode. The initial temperature was 50 °C (3 min) with an accelerating rate of 10 °C (1 min) to 300 °C (10 min). Helium gas was used with a linear velocity of 47.8 cm/s. Electron ionisation (EI) mode was performed at 70 eV with a spectral range of 35-500 m/z for mass spectra results. The ion source temperature was fixed at 150 °C and the interface temperature was at 230 °C with a solvent cut-off time of 3 min. The start time was set at 3 min and the final time was 33 min. The total flow programmed was 21.6 mL/min with a column flow of 1.69 mL/min. All compounds were determined based on the interpretation of mass spectrum with standard reference spectral using the National Institute of Standards and Technology Mass Spectral Library 2011 (NIST 2011) version 2.0 g databases (https://www.nist.gov/srd/nist-standard-reference-database-1a).

3.7 Determination of protease from *Christia vespertilionis* leaf extract

Proteases are playing crucial roles in numerous biological processes. Meanwhile, recent studies showed *C. vespertilionis* potential as an anti-inflammatory agent (Lee *et al*., 2020). Due to that, purified protease from *C. vespertilionis* holds potential for therapeutic applications aimed at reducing inflammation. The purification of proteases

involves at isolating and characterising these enzymes for various biotechnological applications. In this study, proteases were extracted from *C. vespertilionis* leaves using a series of methods, beginning with crude extraction in sodium phosphate buffer, followed by purification steps involving ammonium sulphate precipitation and dialysis. These techniques are pivotal in separating proteases from other cellular components based on their solubility and molecular weight, thereby enriching the enzyme preparation and enhancing its specific activity. The enzyme assay method employed in this study for the measurement of protein content, enzyme activity and specific activity throughout the purification process, providing insights into the efficacy of ammonium sulphate precipitation and dialysis in enhancing enzyme functionality. The molecular weight of purified *C. vespertilionis* protease (CVP) was determined using SDS-PAGE, confirming its size relative to a protein marker. HPLC was also applied for purified extracts to confirm the presence of specific bioactive compounds, such as gallic acid and quercetin, which are important for their potential anti-inflammatory properties.

3.7.1 Protease extraction and purification

Fresh and cleaned *C. vespertilionis* leaves (20 g) were ground with 500 mL of pre-chilled 0.1 M sodium phosphate buffer at pH 7 and filtered using a cheesecloth to remove suspension. Then, it was centrifuged at 9000 rpm for 15 min at 4 °C to remove impurities and supernatant was collected and stored at 4 °C until further use.

Protease purification was done using method of Park et al. (2015) with some modifications. *C. vespertilionis* leaves crude extract was dissolved in ammonium sulphate based on the ammonium sulphate precipitation table: 20 w/v% (5.3 g/50 mL), 40 w/v% (11.3 g/50 mL), 60 w/v% (18.05 g/50 mL), 80 w/v% (25.8 g/50 mL) and 100 w/v% (34.85 g/50 mL). Samples were left overnight and stirred until homogenous at 4 °C. The samples were then centrifuged at 9000 rpm for 15 min at 4 °C. The pellet was collected and

resuspended with 10 mL pre-chilled 0.1 M sodium phosphate buffer at pH 7. Samples at different saturation were analysed for their total protein content, enzyme activity and specific activity.

Dialysis was then performed only on sample with the highest enzyme activity. Ten millilitre of the selected sample was pipetted into the dialysis membrane with a molecular weight cut-off of 14000 Da, and kept in a constantly stirred beaker with 300 mL of pre-chilled 0.1 M sodium phosphate buffer at pH 7. To ensure the ammonium sulphate salt was completely removed, the buffer was changed every 2 hours within 4 hours and left overnight. The next day, the purified protease was centrifuged at 9000 rpm for 15 min at 4 °C and the supernatant was collected and kept at 4 °C. The purified protease was analysed for total protein content, enzyme activity and specific activity according to method 3.7.2.

3.7.2 Enzyme activity of purified *Christia vespertilionis* protease (CVP)

Protease assay based on Sathya Prabhu et al. (2018) with slight modifications was performed on samples that underwent the ammonium sulphate precipitation and dialysis process to measure enzyme activity. This was assessed using a casein substrate assay, followed by protein content determination through the Bradford method using bovine serum albumin as the standard (Bradford, 1976). The mixture of purified protease (20 µL of CVP) and substrate (4980 µL of 2 % (w/v) casein) was dissolved in pre-chilled 0.1 M sodium phosphate buffer at pH 7 and incubated at 37 °C for 5 min. The reaction was stopped by adding 5000 µL of 10 % (w/v) trichloroacetic acid and left at room temperature for 15 min. Then, the mixture was centrifuged at 4400 rpm for 15 min at 25 °C. The supernatant was collected and mixed with 1 mL of Folin-Ciocalteu reagent and 5 mL of 1 M sodium carbonate. The absorbance was measured at 595 nm using a Shimadzu, UV-1700 spectrophotometer (Tokyo, Japan). Total protein, enzyme activity

and specific activity for each saturation of purified protease were calculated to determine the highest enzyme activity. The protein content, enzyme activity and specific activity of the purified protease selected for dialysis were also measured.

3.7.3 Determination of molecular weight of purified *Christia vespertilionis* protease (CVP)

The molecular weight of purified *C. vespertilionis* protease (CVP) was determined using 12 % (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer (pH 8.2) (Laemmli, 1970; Doan *et al*., 2020). The purified *C. vespertilionis* protease (CVP) in produced in method 3.7.1 was diluted with a loading sample buffer at a 2:1 ratio and a volume of 15 µL of CVP and protein marker, each were pipetted separately into wells of the Labnet International electrophoresis set (USA) and run for 5 h at 100 kV. The molecular weight of CVP was estimated by comparing it with a protein marker GeneDireX (Taiwan). The gel was then stained with Coomassie Brilliant Blue R-250 and destained with destaining solution, scanned and analysed using Bio-5000 plus Microtek (Hsinchu, Taiwan).

3.7.4 High performance liquid chromatography (HPLC) analysis of purified *Christia vespertilionis* protease (CVP)

The purified protease of C. vespertilionis was qquantitavely measured using UHPLC Agilent 1290 Infinity (California, United States). The column specifications used were as follows: Agilent Technologies Zorbax Eclipse Plus C18 (4.6 × 100 mm, 3.5 µm). Column and auto-sampler temperatures were maintained at 25 ºC and 4 ºC, respectively. The flow rate was 0.9 mL/min. Mobile phases were water: methanol (70:30 % v/v) with detection wavelength 272 nm. The injection volume of 20 µL was used for the qualitative analysis of standards (gallic acid and quercetin) as well as for the purified *C. vespertilionis*

protease. The peak areas corresponding to the purified protease were integrated and compared to standards, following the methods described by Fulzele & Satdive (2005) and Kiran et al. (2012). Gallic acid (10 mg) was dissolved in 10 mL of methanol to produce a stock solution of 1 mg/mL, and was prepared the same for quercetin. A calibration curve of gallic acid and quercetin at concentration of 5, 10, 20, 50 and 100 µg/mL was also prepared and the samples were done in triplicates.

3.8 *In vitro* studies: Anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in single and combination treatment in LPS-stimulated RAW264.7 macrophages

The *in vitro* studies could provide insights into the therapeutic potential of these compounds, both alone and in combination by using an established *in vitro* model of inflammation using LPS-stimulated RAW264.7 macrophages. The results from this *in vitro* analysis, would include cell viability, nitric oxide inhibition and synergistic effect.

3.8.1 Cell culture and preparation of RAW264.7 macrophages for anti-inflammatory studies

To investigate the anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), RAW264.7 macrophages were utilised as a model system. This cell line derived from murine macrophages is commonly used in research due to its responsiveness to inflammatory stimuli and its role in the immune response. For this study, RAW264.7 macrophages were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % (v/v) L-glutamine, 1 % (v/v) penicillin-streptomycin, 1 % (v/v) hepes and 1 % (v/v) sodium pyruvate. A total of 4×10^4 cells/ well were plated in 96 well plates and incubated at 37 ºC with 5 % carbon dioxide (CO_2) for 24 h.

3.8.2 Cell viability assay

The cell viability assay is a vital step to determine the cytotoxic effects of the purified *Christia vespertilionis* protease (CVP) as well as palm tocotrienol-rich fraction (TRF) on RAW264.7 macrophages. The viability of RAW264.7 cells after treatment with CVP, TRF and their combination was assessed using MTT assay. This assay measures metabolic activity as an indicator of cell health where viable cells would convert substrates into detectable signals. By comparing treated cells to untreated cells, the non-toxic concentrations of CVP and TRF can be determined to ensures the anti-inflammatory tests are accurate and were not influenced by cytotoxic effects.

There are four treatments for the cell viability assay. First, purified *C. vespertilionis* protease (CVP), second, palm tocotrienol-rich fraction (TRF) where 0.1 g of TRF stock solution were diluted in 10 mL of 70 % ethanol, third, a combination of purified *C. vespertilionis* protease and palm tocotrienol-rich fraction (CVP+TRF), and finally, positive control which is 0.1 g of aspirin in 10 mL of distilled water. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay with modifications adapted from Norfazlina et al. (2009), Funaro et al. (2016) and Mutalib & Latip (2019).

A total of 4×10^4 RAW264.7 macrophages were seeded per well in a 96-well plate and incubated for 24 h until reaching 80 % confluence. The following day, cells were treated with different concentrations of purified *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF). CVP was dissolved in high-glucose Dulbecco's modified Eagle's medium (DMEM) to achieve concentrations of 5, 10, 15, 25 and 30 µg/mL. Similarly, TRF from the stock solution was diluted with DMEM to achieve final concentrations of 5, 8, 10, 15 and 20 µg/mL. Aspirin was also diluted from its stock solution to concentrations of 5, 10, 25, 50 and 100 µg/mL, serving as a standard control. Cells were treated with and without lipopolysaccharide (LPS) (10 ng/mL) for an

additional 24 h. After treatment, the supernatant was removed and 50 µL of 0.5 mg/mL MTT solution was added to each well, followed by incubation in the dark for 2 h. Formazan crystals were dissolved with 80 µL of dimethyl sulfoxide (DMSO) and absorbance was measured at 540 nm using a Biotek Synergy H1 microplate reader (California, United States). Experiments were conducted in triplicate. The morphology of RAW264.7 macrophages, both unstimulated and LPS-stimulated, was observed using an Olympus CKX53 inverted microscope (Tokyo, Japan) at 100× magnification after 24 h.

3.8.3 Nitric oxide inhibition assay

The determination of nitric oxide (NO) levels is a crucial step in evaluating the anti-inflammatory properties of potential therapeutic agents. The Griess assay, a known-established method for measuring NO production, was employed with modifications based on the protocols described by Yam et al. (2009) and Chen et al. (2019). This assay provides a quantitative assessment of NO as an indicator of inflammatory responses in RAW264.7 macrophages.

RAW264.7 macrophages (4×10^4 cells/well) were seeded in a 96-well plate and incubated for 24 h to allow for adherence and growth up to 80 % confluence. Following that, the cells were then treated with 100 µL of the different concentrations samples that is prepared from purified *Christia vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF) and combination of CVP and TRF. Different concentrations were prepared for each treatment. For treatment 1 (CVP), concentrations used were 5, 10, 15, 25 and 30 µg/mL while treatment 2 (TRF) the concentrations were 5, 8, 10, 15 and 20 µg/mL. Treatment 3 (CVP+TRF) were prepared at different ratios of 5:5, 10:8, 15:10, 25:15 and 30:20 µg/mL. Aspirin was used as the positive control, diluted in distilled water and prepared at concentrations of 5, 10, 25, 50 and 100 µg/mL. All the different treatments were left incubated with lipopolysaccharide (LPS) at 10 ng/mL for 24 h to stimulate

inflammatory response. Following that, the supernatants were collected and mixed with an equal volume (1:1 ratio) of the Griess reagent, which is a combination of N-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilic acid. The mixture was again incubated for 30 min to allow formation of a chromophore which indicates the presence of nitrite (a stable NO metabolite). The absorbance of the resulting chromophore was measured at 540 nm using a Biotek Synergy H1 microplate reader (California, United States). The nitric oxide (NO) concentration was quantified by plotting the absorbance values against a nitrite standard curve. All experiments were performed in triplicates to ensure reproducibility and accuracy. The different combination treatment ratios of CVP and TRF (5:5, 10:8, 15:10, 25:15 and 30:20 μg/mL) were chosen to explore the potential of synergistic effects of these compounds at various concentrations, with each ratio representing the simultaneous application of both compounds at specific proportions to determine their combined impact on NO production.

3.8.4 Synergistic Effect Measurement Using Combination-Index (CI) of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF)

In order to understand the potential synergistic effects of combining purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), the combination-index (CI) method was utilised. This approach allows for a quantitative assessment of the pharmacological interaction between the two treatments. As nitric oxide (NO) is a key mediator of inflammation, its inhibition was used as a measure of anti-inflammatory activity. The inhibition of NO was evaluated following the administration of CVP, TRF and their combination (CVP+TRF). After measuring NO production, IC_{50} value were analysed which is the concentration at which the compound inhibits 50 % of NO production. This is calculated by plotting dose-response curves for each treatment. Following this, the Combination Index (CI) is calculated using the Chou-Talalay method

provides a quantitative measure of the interaction between the compounds. The CI values and isobologram for CVP+TRF were calculated using the median effect analysis method described by Chou & Talalay (1984), where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive and antagonistic effects, respectively. The CI calculation followed the equation below:

$$CI = \frac{(IC50_{\mathrm{CVP}} combination)}{(IC50_{\mathrm{CVP}} single)} + \frac{(IC50_{TRF} combination)}{(IC50_{TRF} single)} \quad (3.3)$$

3.9 *In vivo* studies: Anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in single and combination treatment in carrageenan-induced BALB/c paw oedema

In vivo studies were conducted to evaluate the anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), both individually and in combination. The carrageenan-induced paw oedema model in BALB/c mice was chosen for this study due to its well-established ability to mimic acute inflammation and assess the efficacy of potential anti-inflammatory agents. This model helps in understanding the therapeutic potential of CVP and TRF in reducing inflammation and provides insights into their possible synergistic effects including the mechanisms by which these compounds may interact to enhance anti-inflammatory activity.

3.9.1 Experimental animals

To investigate the anti-inflammatory effects of CVP, TRF and their combination, *in vivo*, an animal study was conducted using female BALB/c mice. The experiments were

conducted according to the ethical norms approved by the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) (Approval No: S/19052022/07122021-02/R). The animals used in the experiments were female BALB/c mice (n=18) and five weeks old supplied from Laboratory Animal Center, Animal House, Ladang Mini, Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia. Eighteen female BALB/c mice were divided into six groups, each having three animals. The animals were acclimatised for two weeks with a controlled environment of 20-25 ºC, 12 h light/ 12 h dark cycle and provided with commercial pellet (Altromin, Germany) and water. The groups were presented as non-carrageenan, carrageenan (control), aspirin, purified *Christia vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF) and a combination (CVP+TRF).

3.9.2 Acute toxicity assessment of CVP, TRF and CVP+TRF in BALB/c mice

Acute toxicity studies are essential in assessing the potential adverse effects of substances intended for therapeutic use. Following the guidelines of the Organisation for Economic Co-operation and Development (OECD) Test Guideline 425, this study evaluated the acute toxicity of purified *Christia vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF), their combination (CVP+TRF) and aspirin in BALB/c mice. Over a two-week period, mice were administered doses ranging from 100 to 200 mg/kg body weight orally, while a control group received distilled water. Throughout the study, observations focused on mortality rates, abnormal behaviour and changes in body weight. These findings contribute valuable insights into the safety profile of these substances which is crucial for further pharmacological investigations.

3.9.3 Carrageenan-induced paw oedema model and treatment administration

The carrageenan-induced paw oedema model is a widely known method for evaluating the anti-inflammatory effects of treatments in experimental animals. Based on the method

by Gokcen et al. (2021), this model involves inducing acute inflammation in the left hind paw of BALB/c mice using carrageenan. Treatments including purified *Christia vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF) and their combination (CVP+TRF) were administered orally prior to carrageenan injection to assess their inhibitory effects on paw oedema. Paw thickness measurements were taken at regular intervals post-injection to monitor inflammation progression, with decreases indicating potential anti-inflammatory activity.

In this study, the left hind paw BALB/c mice were injected by intraplantar with 50 µL of 1 % (w/v) carrageenan to induce paw oedema. The animals were anaesthetised with ketamine (80 mg/kg) and xylazine (10 mg/kg) through intraperitoneal (IP) injection. Carrageenan group act as control was given 100 µL distilled water, while other treatment groups including 100 mg/kg aspirin, 100 mg/kg CVP, 100 mg/kg TRF and 200 mg/kg CVP+TRF were administered through oral gavage one hour before the carrageenan injection. The thickness of the left hind paw was measured using a vernier calliper before the carrageenan injection and at intervals of 1, 2, 3, 4 and 5 h post-injection. The decrease in paw oedema size indicated anti-inflammatory activity. The percentage of increase in paw oedema size was calculated according to the formula below:

$$(Paw\ oedema\ size, \%) = \frac{a - b}{a} \times 100$$

a = the thickness of left hind paw after carrageenan injection

b = the thickness of left hind paw before carrageenan injection (3.4)

The animals were then euthanised with ketamine (240 mg/kg) and xylazine (30 mg/kg) through intraperitoneal (IP) injection. The carrageenan-induced paw oedema feet were dissected and stored in liquid nitrogen before the amount of pro-inflammatory cytokines, which is tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and free radicals of nitric oxide (NO), were measured.

3.9.4 Paw tissue preparation and homogenisation

To analyse the biochemical changes in the paw tissue following treatment, a methodical paw tissue preparation process was undertaken. This process involved using liquid nitrogen to harden the tissue. A small amount of liquid nitrogen was poured onto the paw to harden the tissue and make it easily crushed into pieces by using mortar and pestle. One gram of tissue sample was homogenised with 10 mL of radioimmunoprecipitation (RIPA) buffer. The mixture was mixed well with vortex and incubated on ice for 30 min. Then, the samples were centrifuged at 3000 rpm for 20 min at 4 ºC. The supernatant was collected and kept at -20 ºC.

3.9.5 Measurement of the tumour necrosis factor-alpha (TNF-*α*) and interleukin-6 (IL-6) in paw tissue

The levels of pro-inflammatory mediators, specifically tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), are critical indicators of inflammation. Evaluating the effect of treatments on these cytokines helps in understanding their potential anti-inflammatory properties. This test was done to determine the ability of CVP, TRF, a combination of CVP+TRF and aspirin to inhibit the production of TNF-*α* and IL-6 using a commercially available ELISA kit R&D Systems, (Minneapolis, USA). 100 µL capture antibodies for TNF-α and IL-6 were seeded overnight at room temperature in a 96-well plate. After 24 hours, a second set of biotinylated antibodies was incubated with paw tissues and standard antigens before adding streptavidin. The colour of the reaction

changed from purple to yellow, and the absorbance was recorded at 450 nm using a Biotek Synergy H1 microplate reader (California, United States). The concentration of TNF-α and IL-6 was expressed as picogram per milligram (pg/mg) of protein for cytokine concentration.

3.9.6 Measurement of nitric oxide in paw tissue

Nitric oxide (NO) is a key inflammatory mediator and its measurement can provide insights into the anti-inflammatory effects of various treatments. In this study, NO levels were measured using the Griess assay with some modifications (Yam *et al*., 2009; Chen *et al*., 2019). Tissue supernatants from samples treated with CVP, TRF, a combination of CVP+TRF and aspirin were analysed. A total of 100 µL tissue supernatants were plated in 96 well plate and incubated at 37 ºC with 5 % carbon dioxide (CO_2) for 24 hours. The supernatants were mixed with a 1:1 ratio of N-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilic acid with nitrite as standard and then incubated for 30 minutes. The absorbance was measured at 540 nm using a Biotek Synergy H1 microplate reader (California, United States). The nitric oxide (NO) data were plotted based on the nitrite standard curve. All experiments were performed in triplicates.

3.10 Statistical analysis

All tests were conducted in triplicates and presented as average ± standard deviation. Statistical analyses were performed using GraphPad Prism (version 8.0.2 for Windows, GraphPad Software, San Diego, California, USA). A two-way analysis of variance (ANOVA) was used to compare the effects of different treatments and their interactions. Mean values were considered statistically significant when $P < 0.05$. The correlation between protease inhibition activity assay and the total phenolic content of *C. vespertilionis* leaf extracts was determined using Pearson's correlation coefficient. Additionally, a one-way ANOVA was used to analyse the differences in enzyme activity

between the purified protease and the control. The statistical significance between control and treated groups was further assessed using a one-way ANOVA followed by Holm-Sidak multiple t-test and Dunnett's test to compare each treatment group to the control.

CHAPTER 4: RESULTS

4.1 Phytochemical analysis of *Christia vespertilionis* leaf extract

Phytochemical analysis is essential to understand the bioactive compounds present in *Christia vespertilionis* leaf extract, which contribute to its therapeutic properties. This analysis involves the qualitative and quantitative analysis and the results highlight the extraction yield and the presence of key phytochemicals in the leaf extract of *C. vespertilionis*.

4.1.1 Extraction yield of *Christia vespertilionis* leaf extract

The efficiency and reproducibility of the extraction process for *C. vespertilionis* leaf extract prepared by Soxhlet extraction using ethanol were evaluated. As shown in Table 4.1, the extraction was performed in triplicate using one gram of dry leaf material for each replicate. Each replicate resulted in an extraction yield of 6.63, 7.32 and 5.22 %, respectively. The average extraction yield was calculated to be 6.39 % with a standard deviation of 0.01, indicating a consistent extraction process. These findings demonstrate that the extraction method yields a stable and reproducible amount of crude extract from the dry leaf material of *C. vespertilionis*. These results suggest that the extraction process is relatively efficient, producing a consistent yield of extract from the dry leaf material of *C. vespertilionis*.

Table 4.1: Extraction yield of *Christia vespertilionis* leaf extract.

Sample	Replicate	Dry Weight (g)	Dry Extract Weight of 1 mL Crude Extract (g)	Extraction Yield (%)
***C. vespertilionis* leaf extract**	1	1.00	0.0663	6.63
	2	1.00	0.0732	7.32
	3	1.00	0.0522	5.22
Average ± SD				**6.39 ± 0.01**

4.1.2 Qualitative screening of phytochemicals in *Christia vespertilionis* leaf extract

The phytochemical screening of *Christia vespertilionis* leaf extract revealed the presence of several bioactive compounds. As shown in Table 4.2, the extract tested positive for a range of phytochemicals including alkaloids, tannins, saponins, cardenolides, phenolics, cardiac glycosides, flavonoids, steroids, and terpenoids. These results indicate that *C. vespertilionis* leaf extract contains nine different chemical classes of compounds known for their biological properties, particularly their anti-inflammatory effects. The presence of these compounds in the *C. vespertilionis* leaf extract suggests its potential for anti-inflammatory activity. For example, flavonoids and phenolics are known for their antioxidant and anti-inflammatory properties (Gonfa *et al*., 2023). Alkaloids and tannins have been shown to inhibit pro-inflammatory cytokines and enzymes. Saponins and steroids also contribute to reducing inflammation through various mechanisms. Thus, the identification of these phytochemicals supports that *C. vespertilionis* leaf extract may possess significant anti-inflammatory activity.

Table 4.2: Phytochemical compounds screening of *Christia vespertilionis* leaf extract.

No.	Phytochemical compound	*C. vespertilionis* leaf extract
1	Alkaloid	+
2	Tannin	+
3	Saponin	+
4	Cardenolide	+
5	Phenolic	+
6	Cardiac glycoside	+
7	Flavonoid	+
8	Steroid	+
9	Terpenoid	+

+ present, - absent

4.1.3 Quantitative screening of phytochemicals in *Christia vespertilionis* leaf extract

The quantitative analysis of phytochemicals in *Christia vespertilionis* leaf extract was performed to measure the total phenolic content, total flavonoid content and total tannin content. The results are showed in Table 4.3 with leaf extract exhibited a high total phenolic content of 29.25 ± 0.50 mg GAE/mL. Phenolic compounds are well-known for their antioxidant properties, which contribute to reducing oxidative stress and inflammation. The total flavonoid content was measured at 1.57 ± 0.03 mg QE/mL. Flavonoids are another class of antioxidants that have been shown to inhibit key inflammatory pathways, thus supporting the anti-inflammatory potential of the extract. Additionally, the total tannin content was 2.70 ± 3.15 mg TAE/mL, indicating the presence of tannins, which also possess significant anti-inflammatory properties.

These quantitative results align with the qualitative screening, reinforcing the potential anti-inflammatory activity of *C. vespertilionis* leaf extract. The substantial levels of phenolics, flavonoids and tannins suggest that the extract can modulate inflammatory responses effectively, possibly through antioxidant mechanisms and the inhibition of pro-inflammatory mediators.

Table 4.3: Quantitative screening of phytochemicals in *Christia vespertilionis* leaf extract.

Sample	Total phenolic content (mg GAE/mL)	Total flavonoid content (mg QE/mL)	Total tannin content (mg TAE/mL)
***C. vespertilionis* leaf extract**	29.25 ± 0.50	1.57 ± 0.03	2.70 ± 3.15

4.1.4 Protease inhibition activity assay of *Christia vespertilionis* leaf extract

Protease inhibition assays are *in vitro*, cell-free tests frequently employed for preliminary screening and quantitative evaluation of protease inhibitors (Berdyshev *et al*., 2023). Protease inhibition is a crucial mechanism in controlling inflammatory processes, as proteases are involved in the degradation of extracellular matrix components and the activation of pro-inflammatory cytokines (Ramos-Llorca *et al*., 2022). In this study protease inhibitors include *Christia vespertilionis* leaf extract with aspirin as a control were evaluated its potential anti-inflammatory effects by determine IC_{50} values and inhibition percentages. The IC_{50} value represents the concentration of the sample required to inhibit 50 % of the protease activity.

The protease inhibition assay revealed that both *C. vespertilionis* leaf extract and aspirin effectively inhibited protease activity across a range of concentrations as shown

in Figure 4.1(a). The range of concentrations helps identify the dose-response relationship and the optimal concentration needed for significant protease inhibition, ensuring that both low and high concentrations are tested for a comprehensive analysis of their inhibitory potential. *C. vespertilionis* leaf extract (blue circles) shows increased inhibition with rising concentrations, with notable inhibition at 1000 µg/mL and it achieved approximately 60 % protease inhibition, while aspirin (red squares) exhibited around 60-65 % inhibition.

Figure 4.1(b) showed IC_{50} values for protease inhibition by *C. vespertilionis* leaf extract and aspirin. The IC_{50} values were determined from these dose-response curves, with *C. vespertilionis* leaf extract showing an IC_{50} of 284.59 ± 10.63 µg/mL and aspirin showing an IC_{50} of 275.72 ± 6.93 µg/mL. These values indicate the concentrations at which each substance achieves 50 % protease inhibition. The similar IC_{50} values suggest comparable inhibitory potency, with no statistically significant difference due to the overlap in their standard deviations. The overlap in their standard deviations could be attributed to random variation rather than a true difference in efficacy. Despite the lack of significant difference in IC_{50} values, *C. vespertilionis* leaf extract presents several advantages over aspirin. As a natural product, it offers a plant-based alternative that may have fewer side effects. Given its potential for reduced side effects with less expensive to produce compared to aspirin, especially if the plant is readily available and easy to cultivate. Additionally, plant extracts often contain a range of bioactive compounds that could provide supplementary health benefits include *C. vespertilionis* which has potential anti-inflammatory properties through its ability to inhibit protease activity, supporting its use as a natural therapeutic agent in inflammation-related conditions.

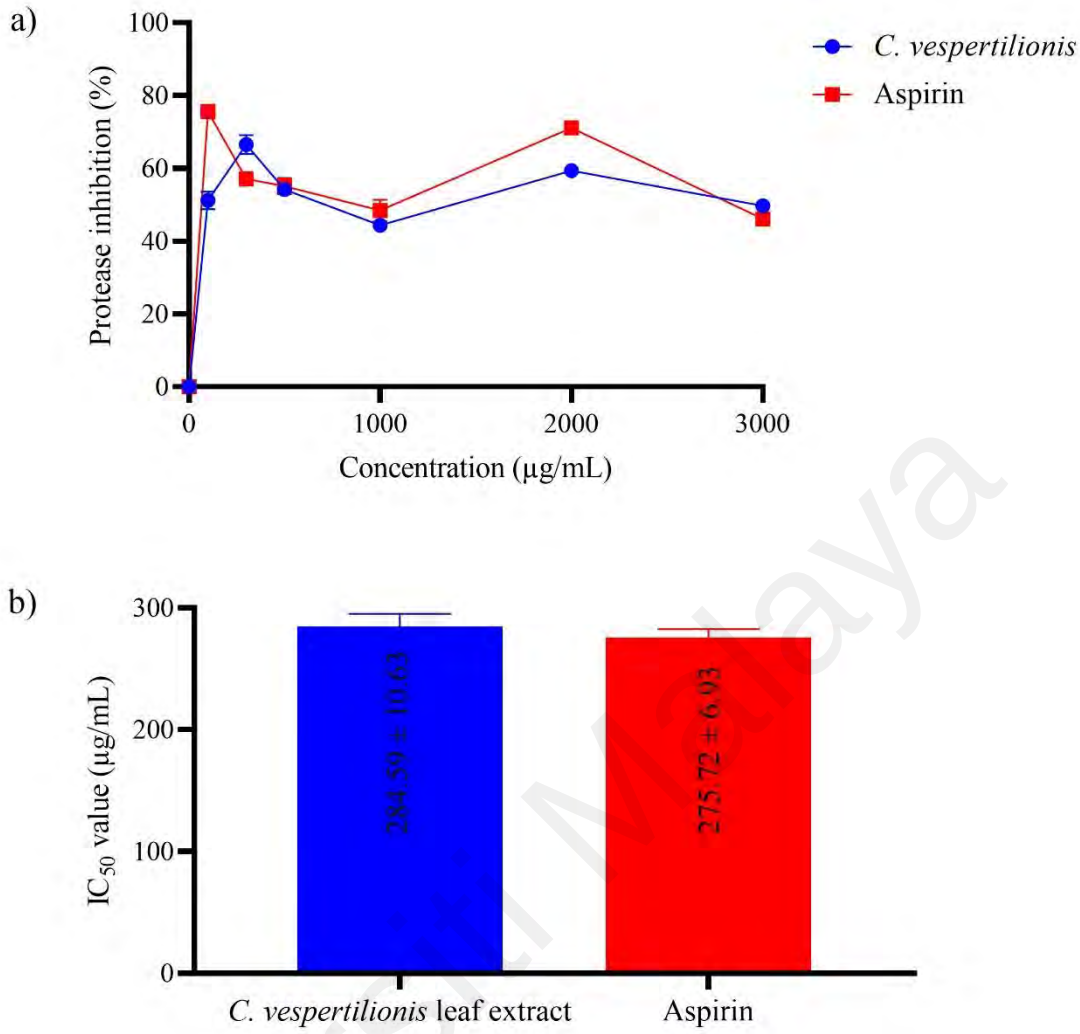


Figure 4.1: Protease inhibition activity assay of *Christia vespertilionis* leaf extract with aspirin as a control include a) Concentration-dependent protease inhibition, and b) IC_{50} values for protease inhibition.

4.1.5 Correlation between phenolic content and protease inhibition activity of *C. vespertilionis* leaf extract

The correlation between phytochemical composition and bioactivity is a fundamental aspect of exploring natural products for potential therapeutic applications. In this study, we investigate the relationship between phenolic content and protease inhibition activity of *C. vespertilionis* leaf extract. Phenolic compounds are known for their enzymatic

inhibitory properties, and by measuring phenolic content at varying concentrations and assessing the inhibitory activity against protease will explain how phenolics contribute to the biological activity of the leaf extract (Rahman *et al*., 2021).

The anti-inflammatory activity of *C. vespertilionis* leaf extract can be deduced by correlating the protease inhibition assay with the total phenolic content. The phenolic content was measured and yielding average phenolic content values of 29.25 ± 0.50 mg GAE/mL, respectively as shown in Table 4.3. Figure 4.1 shows that IC_{50} value for protease inhibition by the leaf extract was found to be 284.59 ± 10.63 µg/mL, indicating the concentration required to inhibit 50 % of the protease activity. In comparison, the reference compound aspirin exhibited an IC_{50} value of 275.72 ± 6.93 µg/mL. Based on correlation, it shows a significant positive, with an R^2 value of 0.6821, indicating that approximately 68.21 % of the variation in protease inhibition can be explained by the variation in total phenolic content. This positive correlation suggests that the phenolic compounds in the extract are likely the main contributors to its protease inhibition and, consequently, its anti-inflammatory properties. This implies that the phenolic compounds play a crucial role in the extract's ability to reduce inflammation. Additionally, the IC_{50} value of the leaf extract, which is slightly higher than that of aspirin, suggests that the phenolic compounds in the extract contribute to its protease inhibitory activity. Given the measured phenolic contents, it can be inferred that higher concentrations of phenolic compounds correlate with increased inhibitory activity (Hong *et al*., 2021). These findings highlight the potential of *C. vespertilionis* leaf extract as a natural source of protease inhibitors, with efficacy comparable to known standards like aspirin.

4.1.6 Identification of anti-inflammatory compounds in *Christia vespertilionis* leaf extract using gas chromatography-mass spectrometry (GC-MS) analysis

Christia vespertilionis is a medicinal plant that has garnered scientific interest for its potential therapeutic benefits, particularly its anti-inflammatory properties. Previous studies have suggested that this plant may contain bioactive compounds capable of modulating inflammatory responses (Samtiya *et al*., 2021). To further explore its potential, this study aims to identify and characterise the anti-inflammatory compounds present in the leaf extract of *C. vespertilionis* using gas chromatography-mass spectrometry (GC-MS) analysis.

Figure 4.2 displays the chromatogram obtained from the GC-MS analysis of the *C. vespertilionis* leaf extract. The chromatogram shows the retention times and relative intensities of the compounds detected in the extract. Each peak represents a different compound and the height and area of the peaks correspond to the concentration of the compounds present. This chromatogram provides a visual representation of the chemical profile of the leaf extract, highlighting the compounds detected during the analysis, including major compounds

Table 4.4 provides a detailed summary of the GC-MS analysis results of major compounds. The table lists detected compounds, their retention times, peak percentages, molecular formulas, molecular weights, molecular structure and known biological properties. Notably, the compounds n-hexadecanoic acid, phytol, 9,12,15-octadecatrienoic acid (Z,Z,Z)-, and squalene were identified. These compounds have been reported in the literature for their significant anti-inflammatory activities, along with other beneficial biological properties. n-hexadecanoic acid was found at retention time of 20.291 with peak area of 4.679 %, phytol was detected at 21.798 min (peak area 4.715 %), while 9,12,15-octadecatrienoic acid, (Z,Z,Z)- was detected at 22.092 min (peak area

7.243 %) and lastly, squalene presence at 27.788 min (peak area 8.814 %). The GC-MS analysis of *C. vespertilionis* leaf extract has successfully identified several compounds with notable anti-inflammatory properties. These findings support the traditional use of *C. vespertilionis* in treating inflammatory conditions and its potential as a source of natural anti-inflammatory agents.

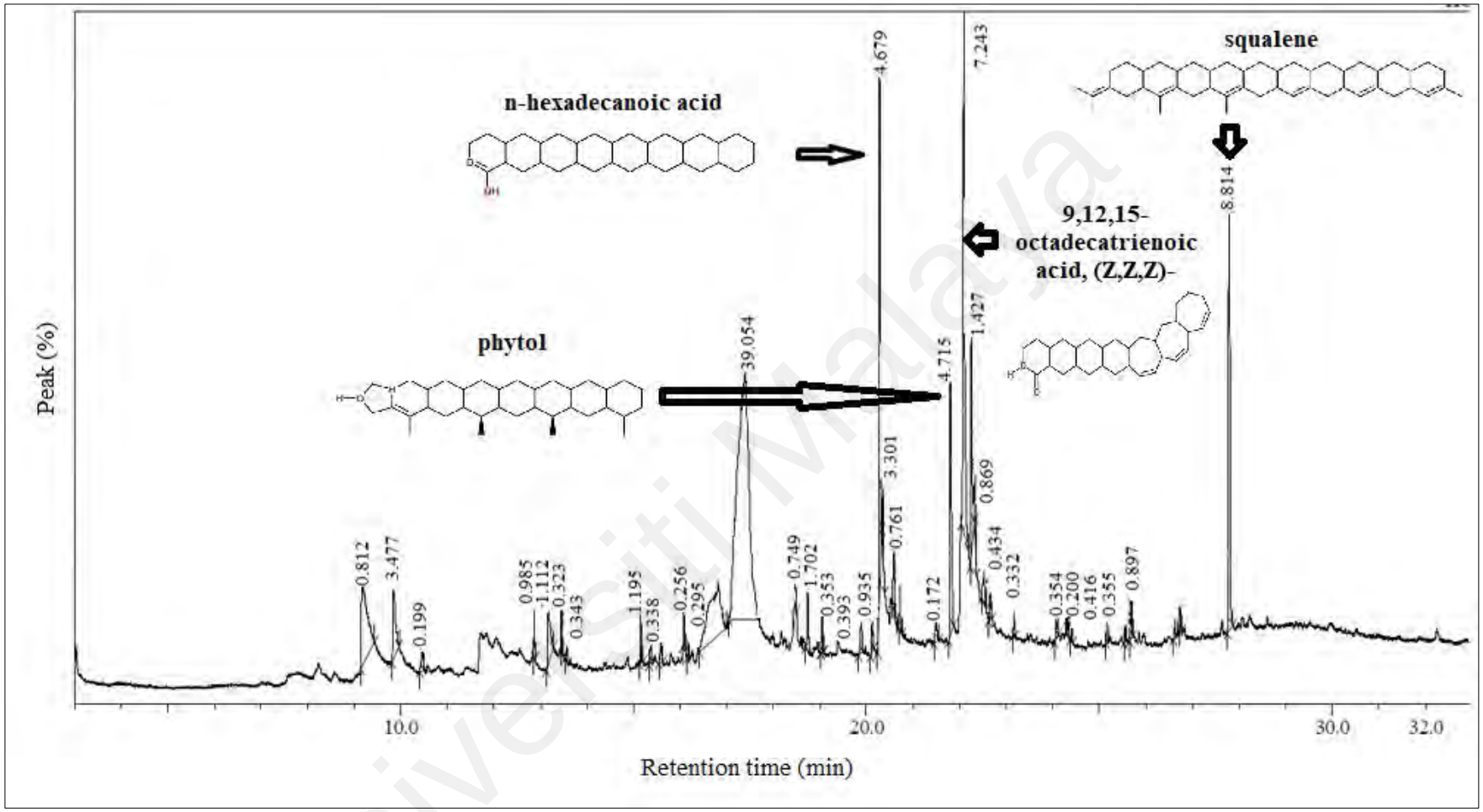
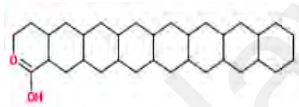
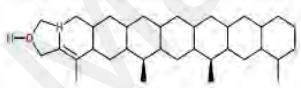
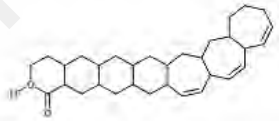
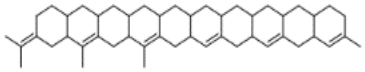


Figure 4.2: Chromatogram of *Christia vespertilionis* leaf extract (Arrow refer to the anti-inflammatory compound)

Table 4.4: GC-MS analysis and biological profiles of anti-inflammatory compounds in *Christia vespertilionis* leaf extract

No	Name of compound/ chemical classes	Retention time (min)	Peak (%)	Molecular formula	Molecular weight	Molecular structure	Biological properties
1	**n-hexadecanoic acid**	20.291	4.679	$C_{16}H_{32}O_2$	256		-anti-inflammatory (Aparna *et al*., 2012). -antibacterial (Johannes *et al*., 2016). -antiandrogenic and antioxidant (Gavamukulya *et al*., 2015).
2	**phytol**	21.798	4.715	$C_{20}H_{40}O$	296		-antimicrobial, anti-inflammatory (Silva *et al*., 2014). -anti-cancer, antinociceptive, antioxidant and antiarthritic (Gavamukulya *et al*., 2015).
3	**9,12,15-octadecatrienoic acid, (Z,Z,Z)-**	22.092	7.243	$C_{18}H_{30}O_2$	278		-anti-inflammatory, cancer preventive and antiarthritic (Sermakkani & Thangapandian, 2012; Suman *et al*., 2013).
4	**squalene**	27.788	8.814	$C_{30}H_{50}$	410		-antioxidant, antitumor and chemopreventive effect (Kelly, 1999; Kim & Karadeniz, 2012; Rao *et al*., 1998; Singab *et al*., 2015; Singariya *et al*., 2015). -anti-inflammatory (Kelly, 1999; Lacatusua *et al*., 2018).

4.2 Protease purification of *Christia vespertilionis* leaf extract

Proteases are enzymes that has capability to treat diseases. *Christia vespertilionis*, a medicinal plant known for its therapeutic potential is being explored for its protease content in this study using ammonium sulphate precipitation at various saturation levels and dialysis afterward. Papain, a widely known protease, was used as a positive control to validate the effectiveness of the purification method.

4.2.1 Protease purification from *Christia vespertilionis* leaf extract using ammonium sulphate precipitation and dialysis

Protease extracted from *C. vespertilionis* leaf extract was purified and optimised to enhance its yield and specific activity using the ammonium sulphate precipitation method at saturation levels of 20, 40, 60, 80 and 100 %. Using varying saturation levels of ammonium sulphate enables the fractionation of proteins according to their solubility. This technique is beneficial as it improves the purity of enzymes by selectively precipitating proteins at distinct saturation points. Additionally, this method optimises yield, as different proteins, including proteases, precipitate at different saturation levels. By optimising the saturation levels, maximum recovery of active protease is ensured. Furthermore, ammonium sulphate precipitation is a gentle method, which helps preserve the enzyme's activity (Wingfield, 2001).

The purification of the protease involved several steps, each marked by changes in total protein content, volume of buffer, protein concentration, total activity, specific activity, fold purity and activity yield. As shown in Table 4.5, starting with the crude extract, has contained 2.62 mg of total protein in 10 mL of buffer, the initial protein concentration was 0.262 mg/mL with a total activity of 6.2 U and a specific activity of 2.37 U/mg. This crude extract served as the baseline with a fold purity of 1.00 and an

activity yield of 100 %. The first ammonium sulphate precipitation at 20 % saturation reduced the total protein to 1.88 mg and the specific activity to 1.75 U/mg, with a fold purity of 0.74 and an activity yield of 53.23 %. As the saturation increased to 40 %, the total protein was 2.19 mg, with specific activity improving to 1.96 U/mg, fold purity to 0.83, and activity yield to 69.35 %. At 60 % saturation, the total protein increased to 2.49 mg, and the specific activity improved to 2.21 U/mg, with fold purity at 0.93 and activity yield at 88.71 %. The 80% saturation step showed a substantial increase in total protein to 4.94 mg, with a marked rise in total activity to 18.9 U and a specific activity of 3.83 U/mg. However, the activity yield dropped to 30.48 %. At 100% saturation, total protein increased drastically to 19.33 mg and total activity peaked at 23.31 U, resulting in a specific activity of 12.06 U/mg, fold purity of 5.09, and an activity yield of 37.39 %.

Dialysis is performed after ammonium sulphate precipitation to remove excess salts and other small molecules that can interfere with subsequent biochemical analyses or applications (Mohanty & Majumdar, 2020). While ammonium sulphate precipitation concentrates and partially purifies the proteins by exploiting their solubility differences, it leaves the proteins in a high-salt solution that can affect their stability and activity. Dialysis helps restore proteins to more physiological conditions, facilitates buffer exchange for enhanced stability and activity, prevents protein aggregation. Thus, dialysis is essential for ensuring the purified protein is in an optimal environment for further use. Saturation at 100 % was selected for dialysis as it yielded the highest total protein content, total activity and specific activity. The final dialysis step further concentrated the protease, with total protein reaching 23.19 mg, total activity increasing to 33.24 U, and specific activity rising to 14.33 U/mg. This final step achieved a fold purity of 6.05 and an activity yield of 53.33 %, indicating successful purification and concentration of the enzyme.

To validate the purification method and assess the quality of the purified protease, papain, a commercial protease with a specific activity of 69.78 U/mg, was used as a standard. The specific activity of the commercial papain enzyme was determined using the identical standard enzymatic assay previously employed for purified *C. vespertilionis* protease (CVP). Comparing the purified protease to papain, the commercial standard, helped confirm the validity and efficiency of the purification protocol. While the purified protease showed a significant improvement in specific activity through the purification steps, it still had a lower specific activity compared to papain. This comparison underscores the effectiveness of the purification method and highlights the potential for further optimisation to enhance the specific activity of the purified enzyme to use for further applications and studies. Overall, the process significantly increased the specific activity from 2.37 U/mg in the crude extract to 14.33 U/mg after dialysis, demonstrating effective enrichment and purification of the protease. Despite some losses in activity yield during intermediate steps, the final product exhibited high purity and activity, validating the efficacy of the purification method.

Table 4.5: Summary of total protein, total activity, specific activity, fold purity and activity yield at different purification steps of *Christia vespertilionis* protease (CVP).

Purification steps	Total protein (mg)	Volume of buffer (mL)	Protein concentration (mg/mL)	Total activity (U)	Specific activity (U/mg)	Fold purity	Activity yield (%)
Crude	2.62	10	0.262	6.2	2.37	1.00	100.00
Ammonium sulphate (20%)	1.88	10	0.188	3.3	1.75	0.74	53.23
Ammonium sulphate (40%)	2.19	10	0.219	4.3	1.96	0.83	69.35
Ammonium sulphate (60%)	2.49	10	0.249	5.5	2.21	0.93	88.71
Ammonium sulphate (80%)	4.94	10	0.494	18.9	3.83	1.62	30.48
Ammonium sulphate (100%)	19.33	10	1.933	23.31	12.06	5.09	37.39
Dialysis	23.19	10	2.319	33.24	14.33	6.05	53.33

4.2.2 Molecular weight determination of purified *Christia vespertilionis* protease (CVP)

Molecular weight determination via sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a vital test that provides confirmation of enzyme identity, assessment of purity, validation of purification methods and comparison with standards. It ensures that the purified protease is of high quality and suitable for its intended applications. The purified *C. vespertilionis* protease was subjected to SDS-PAGE to assess its purity and determine its molecular weight. The gel image as shown in Figure 4.3 displays a protein marker lane and a lane containing the purified protease (CVP). The protein marker lane from GeneDireX (Taoyuan, Taiwan), includes proteins of known molecular weights, ranging from 11 kDa to 245 kDa, serving as a reference to estimate the molecular weight of the target protein.

Based on the Figure 4.3, bands corresponding to the target protein were observed on the gel, has some degree of smearing and less distinct boundaries compared to ideal conditions. While the bands were readable and acceptable for qualitative assessment, minor imperfections in band clarity were noted, potentially due to variations in protein purity or gel running conditions. Despite these observations, the bands provided sufficient clarity to confirm the presence of the CVP. The purified *C. vespertilionis* protease (CVP) is observed as a single prominent band at approximately 48 kDa. This single band indicates that the protease purification process was successful, resulting in a high level of purity with minimal contaminating proteins. The molecular weight of the protease, approximately 48 kDa, is consistent with the expected size for this type of enzyme. This expected size is in agreement with previously reported values for protease enzyme in the literature (Park *et al*., 2013). The absence of additional bands suggests that the final product is highly pure, the CVP was effectively concentrated and isolated through the

purification steps described. This result validates the increasing specific activity and fold purity values observed in the earlier stages of the purification process, confirming the effectiveness of the protocol used. The SDS-PAGE analysis provides visual evidence of the successful purification and isolation of the protease, with a molecular weight of approximately 48 kDa, affirming the high purity and quality of the final enzyme preparation.

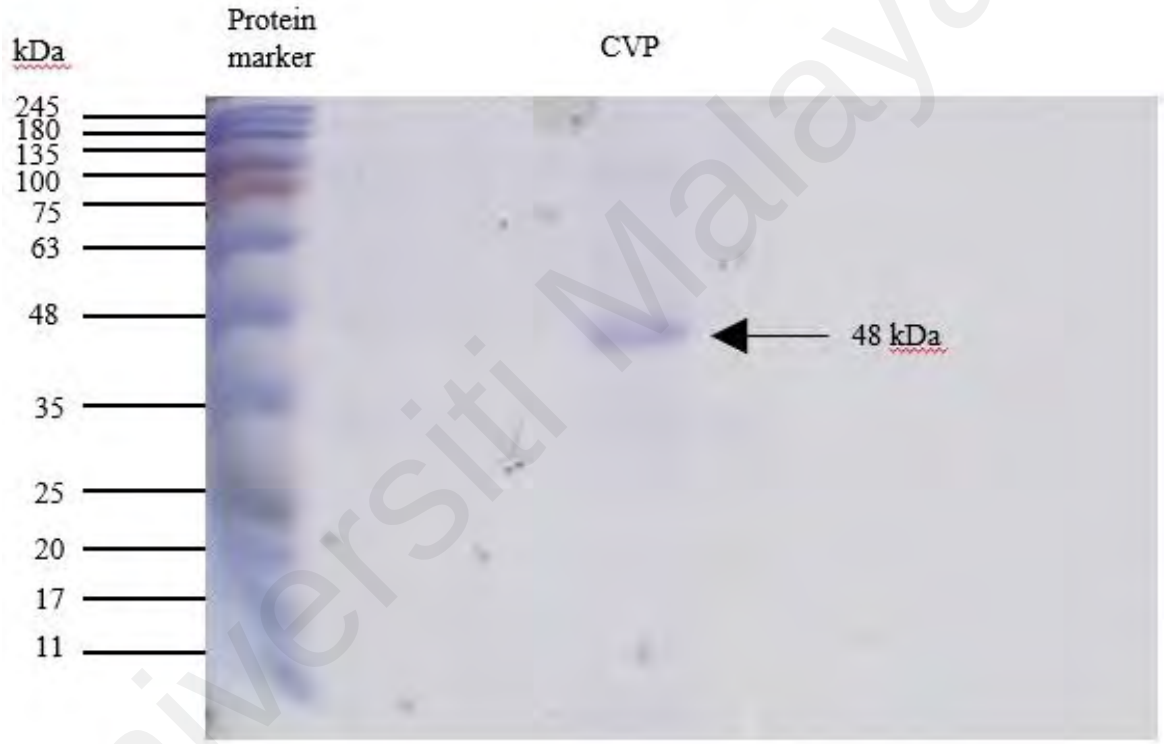


Figure 4.3: SDS-PAGE analysis of purified *Christia vespertilionis* protease (CVP). Left is the protein ladder while the right is molecular weight of CVP showed as 48kDa.

4.2.3 High Performance Liquid Chromatography (HPLC) analysis of purified *Christia vespertilionis* protease

High performance liquid chromatography (HPLC) is a versatile and powerful analytical technique that provides valuable information about the purity, quantity,

structure and activity of purified protease like CVP, making it an indispensable tool in biochemical and biotechnological research. The calibration curve in HPLC testing is essential for accurate and precise quantification of CVP. Based on the calibration curves for the two standards, gallic acid and quercetin (Figure 4.4), the results demonstrate a strong linear relationship between the concentration of each standard and their respective chromatographic responses, with high coefficients of determination (R^2 values of 0.9930 for gallic acid and 0.9134 for quercetin). This indicates that the HPLC method is both accurate and precise for these compounds. The linearity and high sensitivity of the calibration curves confirm that the method is well-suited for quantifying gallic acid and quercetin in purified *C. vespertilionis* protease (CVP), ensuring reliable and reproducible results.

The results of the HPLC analysis provide key insights into the characteristics and purity of the purified *C. vesperilionis* protease (CVP) in comparison to the standards gallic acid and quercetin. The retention times for gallic acid and quercetin are very close, at 1.073 and 1.080 minutes, respectively, indicating their elution profiles are similar and that the method can effectively separate these compounds. The retention time for CVP is slightly longer, at 1.173 minutes, suggesting it has a different elution profile compared to the standards as shown in Figure 4.5. The area under the curve (AUC) values, measured in milli-absorbance units (mAU), reflect the quantity of each compound detected. Gallic acid shows a significantly larger area (202.64 mAU) compared to quercetin (22.09 mAU), indicating a higher concentration or greater response factor for gallic acid under the same conditions. The CVP has an intermediate area of 112.48 mAU, suggesting its concentration or response factor lies between those of gallic acid and quercetin. Figure 4.5 showed the peak is sharp and well-defined, suggesting good resolution and separation from other compounds. The significant area under the peak reflects a high concentration of standards and CVP, with the maximum absorbance around 1.0 x 10^2 mAU. This data

confirms the effective detection and quantification of standards and CVP using the HPLC method. The area under the curve for CVP also shows it is present in a significant amount, which can be quantified relative to the calibration curves established. Thus, the HPLC results indicate that the CVP has a distinct retention time compared to the standards, confirming its unique elution profile. The retention time for CVP is slightly longer, at 1.173 minutes, suggesting it interacts differently with the stationary phase of the column compared to the standards. This indicates that CVP has unique chemical properties, which can be advantageous (Benmrad *et al*., 2019). Firstly, the distinct retention time confirms the purity of CVP, ensuring it is not contaminated with compounds similar to the standards. Secondly, it provides valuable information for characterising CVP, offering insights into its unique interactions and chemical behaviour. Lastly, these unique properties can be beneficial for specific applications, such as specialised protease treatments or the development of unique biochemical assays.

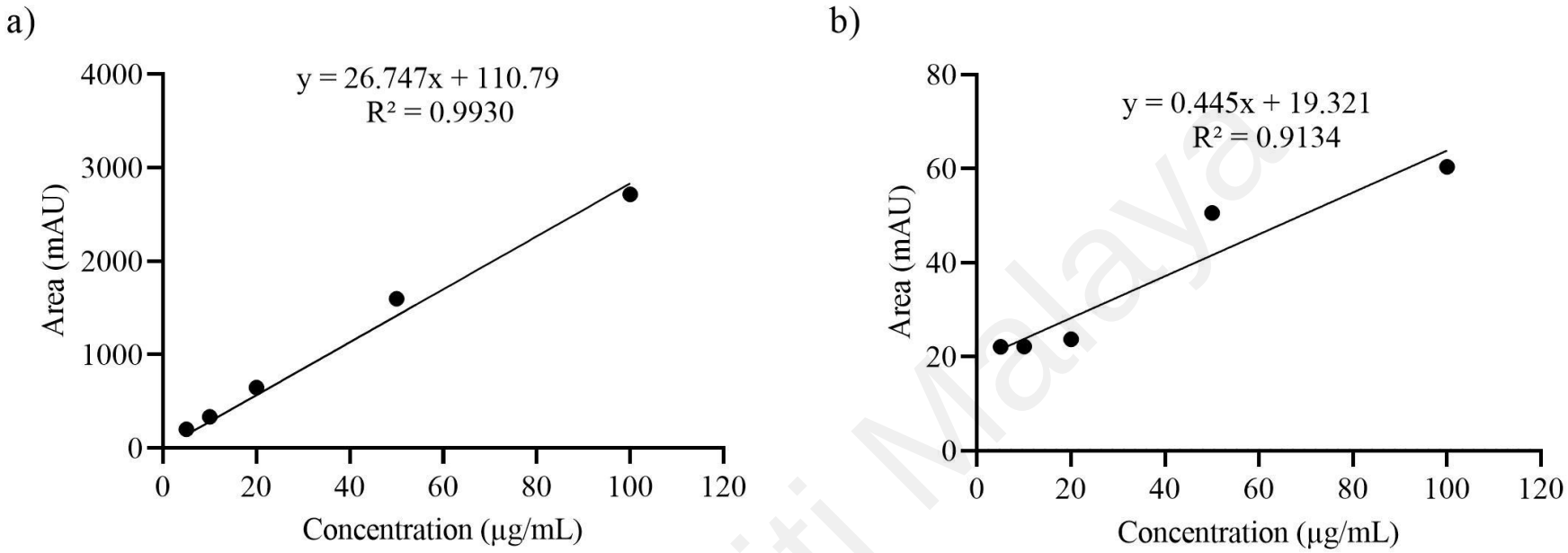
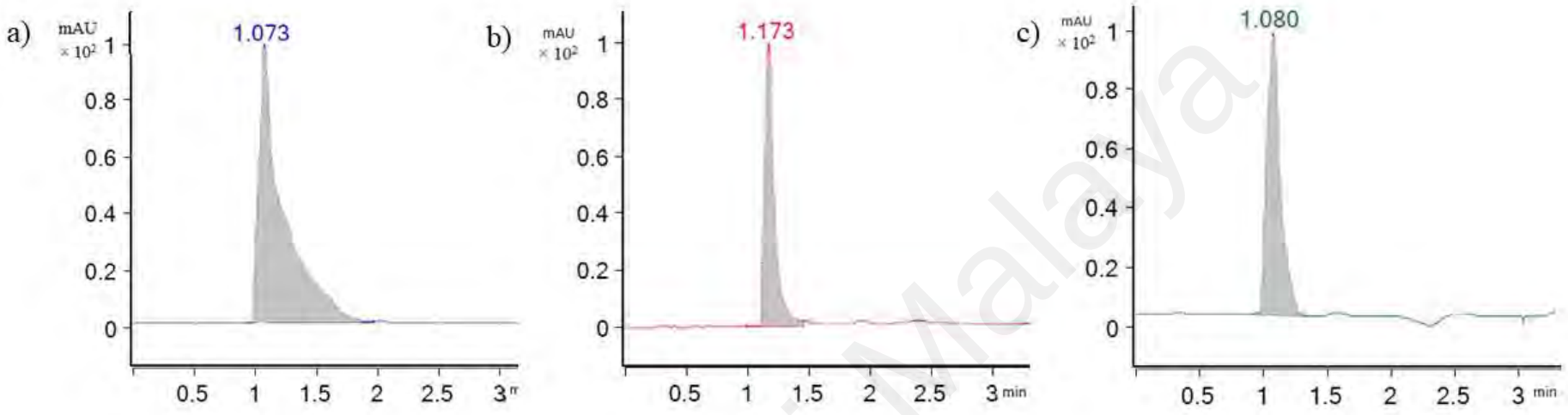


Figure 4.4: The calibration curves in HPLC for the two standards, a) gallic acid and b) quercetin.



Parameters	Gallic acid (standard)	Quercetin (standard)	Purified *C. vespertilionis* protease (CVP)
Retention time (min)	1.073	1.080	1.173
Area (mAU)	202.64	22.09	112.48

Figure 4.5: HPLC chromatogram of a) gallic acid b) quercetin and c) Purified *Christia vespertilionis* protease (CVP).

4.3 Anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in single and combination treatment in LPS-stimulated RAW264.7 macrophages

Inflammation is a critical physiological response to infection and injury, orchestrated primarily by macrophages, which play a central role in the immune system. However, excessive or chronic inflammation is implicated in a range of diseases, including arthritis, cardiovascular diseases and cancer. Therefore, understanding and controlling inflammatory responses is importance in developing therapeutic interventions. Natural products have long been investigated for their potential anti-inflammatory properties. *Christia vespertilionis* is a traditional medicinal plant reputed for its therapeutic effects that recent studies have identified purified proteases from *Christia vespertilionis* (CVP) as potential anti-inflammatory agents due to their ability to modulate immune responses (Zambari *et al*., 2023). Similarly, palm tocotrienol-rich fraction (TRF) has garnered attention for its potent antioxidant and anti-inflammatory properties (Zainal *et al*., 2019). Research has indicated that TRF can mitigate oxidative stress and modulate inflammatory pathways, making it a promising candidate for inflammation-related studies.

In this study, CVP and TRF, both individually and in combination, were investigate their anti-inflammatory effects on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. LPS, a component of the outer membrane of Gram-negative bacteria, is commonly used to induce inflammation in macrophages, mimicking the inflammatory response. The RAW264.7 cell line, derived from murine macrophages, serves as an established *in vitro* model for studying inflammatory mechanisms. By evaluating the effects of CVP and TRF on LPS-stimulated RAW264.7 macrophages, the aim is to elucidate their potential synergistic actions and underlying mechanisms in controlling

inflammation. The findings from this study could pave the way for new therapeutic strategies utilising natural compounds to combat inflammatory diseases.

4.3.1 Effect of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), single and combination treatment, on cell viability

To determine the non-toxic concentrations of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), the cell viability of RAW264.7 macrophages was assessed using an MTT assay. Cell viability assays are important for determining the effects of various compounds on cell health with presence of lipopolysaccharide (LPS), which is known to activate macrophages and inducing inflammatory responses, with comparison to cells without LPS stimulation. RAW 264.7 cells were treated with varying concentrations of CVP (5, 10, 15, 25 and 30 µg/mL), TRF (5, 8, 10, 15 and 20 µg/mL), in combination (CVP+TRF) at ratios (5:5), (10:8), (15:10), (25:15) and (30:20) µg/mL on LPS unstimulated and stimulated in order to test the treatments with the comparison of standard aspirin based on cells toxicity through MTT assay.

Results showed CVP, TRF, CVP+TRF and aspirin have no toxicity to RAW264.7 macrophages under LPS-unstimulated and stimulated conditions. Based on Figure 4.6(a), LPS-unstimulated for CVP at 5, 10, 15, 25 and 30 µg/mL were obtained cell viability percentages of 107.19 ± 2.83 %, 96.65 ± 3.22 %, 106.57 ± 1.07 %, 97.27 ± 1.07 % and 96.69 ± 1.81 %, respectively compared to control, 100.00 ± 0.00 %. Meanwhile, LPS-stimulated for CVP at 5, 10, 15, 25 and 30 µg/mL were at 87.65 ± 1.02 %, 90.00 ± 1.77 %, 97.06 ± 1.77 %, 91.77 ± 1.77 % and 88.82 ± 1.02 % of cell viability, respectively compared to control, 105.33 ± 2.84 %. Based on Figure 4.6(b), TRF without LPS stimulation at 5, 8, 10, 15 and 20 µg/mL were obtained cell viability percentages of 137.85 ± 3.53 %, 134.46 ± 3.91 %, 182.49 ± 6.85 %, 207.91 ± 9.33 % and 226.55 ± 9.33 %,

respectively compared to control, 100.00 ± 0.00 %. Meanwhile, with LPS stimulation for TRF at 5, 8, 10, 15 and 20 µg/mL, the cell viability percentages were at 122.84 ± 5.99 %, 128.60 ± 2.64 %, 138.99 ± 8.71 %, 162.05 ± 7.20 % and 179.35 ± 7.99 %, respectively compared to control, 105.33 ± 2.84 %. Figure 4.6(c) illustrates the cell viability percentages of RAW264.7 cells treated with various ratios of CVP+TRF without LPS stimulation. The ratios (5:5), (10:8), (15:10), (25:15) and (30:20) µg/mL resulted in cell viability percentages of 109.64 ± 10.29 %, 124.50 ± 2.01 %, 117.14 ± 4.18 %, 120.48 ± 2.01 % and 134.54 ± 2.01 %, respectively compared to control, 100.00 ± 0.00 %. Meanwhile, with LPS stimulation for CVP+TRF at ratio of (5:5), (10:8), (15:10), (25:15) and (30:20) µg/mL, the cell viability percentages were at 109.87 ± 2.28 %, 114.47 ± 1.97 %, 118.42 ± 3.42 %, 118.42 ± 5.22 % and 128.95 ± 3.02 %, respectively compared to control, 105.09 ± 1.16 %.

Aspirin is used in cell cytotoxicity tests due to its well-known anti-inflammatory properties and used as positive control. It serves as a reference compound to study cytotoxic effects of new compounds or treatments in comparison to aspirin. This comparison helps in understanding the safety and efficacy of new drugs or therapeutic agents, especially in research focused on anti-inflammatory mechanisms. Figure 4.6(d) indicated RAW 264.7 cells without LPS stimulation for aspirin at 5, 10, 25, 50 and 100 µg/mL were obtained cell viability percentages of 114.29 ± 1.94 %, 112.70 ± 4.05 %, 107.14 ± 1.94 %, 103.97 ± 1.12 % and 102.38 ± 0.00 %, respectively compared to control, 100.00 ± 0.00 %. Meanwhile, with stimulation of LPS for aspirin at 5, 10, 25, 50 and 100 µg/mL, the percentages of cell viability were at 101.29 ± 1.76 %, 99.86 ± 2.03 %, 96.26 ± 2.03 %, 92.67 ± 1.76 % and 94.83 ± 1.76 %, respectively compared to control, 107.94 ± 5.94 %. The results indicate that aspirin affects cell viability differently in unstimulated versus LPS-stimulated conditions. In the absence of LPS, aspirin appears to enhance cell viability, which may provide a supportive or non-toxic effect under normal conditions. In

contrast, in the presence of LPS, aspirin decreases cell viability, suggesting a potential cytotoxic effect under inflammatory conditions, which could be beneficial in reducing the number of activated inflammatory cells (Raza *et al.*, 2016).

These results demonstrate that CVP, TRF and their combination (CVP+TRF) do not exhibit cytotoxicity to RAW264.7 macrophages under both LPS-unstimulated and stimulated conditions. Interestingly, TRF and CVP+TRF significantly enhance cell viability, especially at higher concentrations, indicating potential proliferative or protective effects. The LPS-stimulated conditions generally show a decrease in cell viability compared to the unstimulated conditions, likely due to the inflammatory response induced by LPS. However, the presence of CVP, TRF and their combination mitigates this effect, suggesting their beneficial role in maintaining cell viability during inflammation. CVP and TRF (alone and combined) showed no significant cytotoxicity up to 30 μg/mL and 20 μg/mL, respectively indicating that these concentrations are safe for later experiments. TRF and the combination with CVP enhance cell viability, especially under inflammatory conditions, more effectively than aspirin. This suggests their potential for managing inflammation in therapeutic applications.

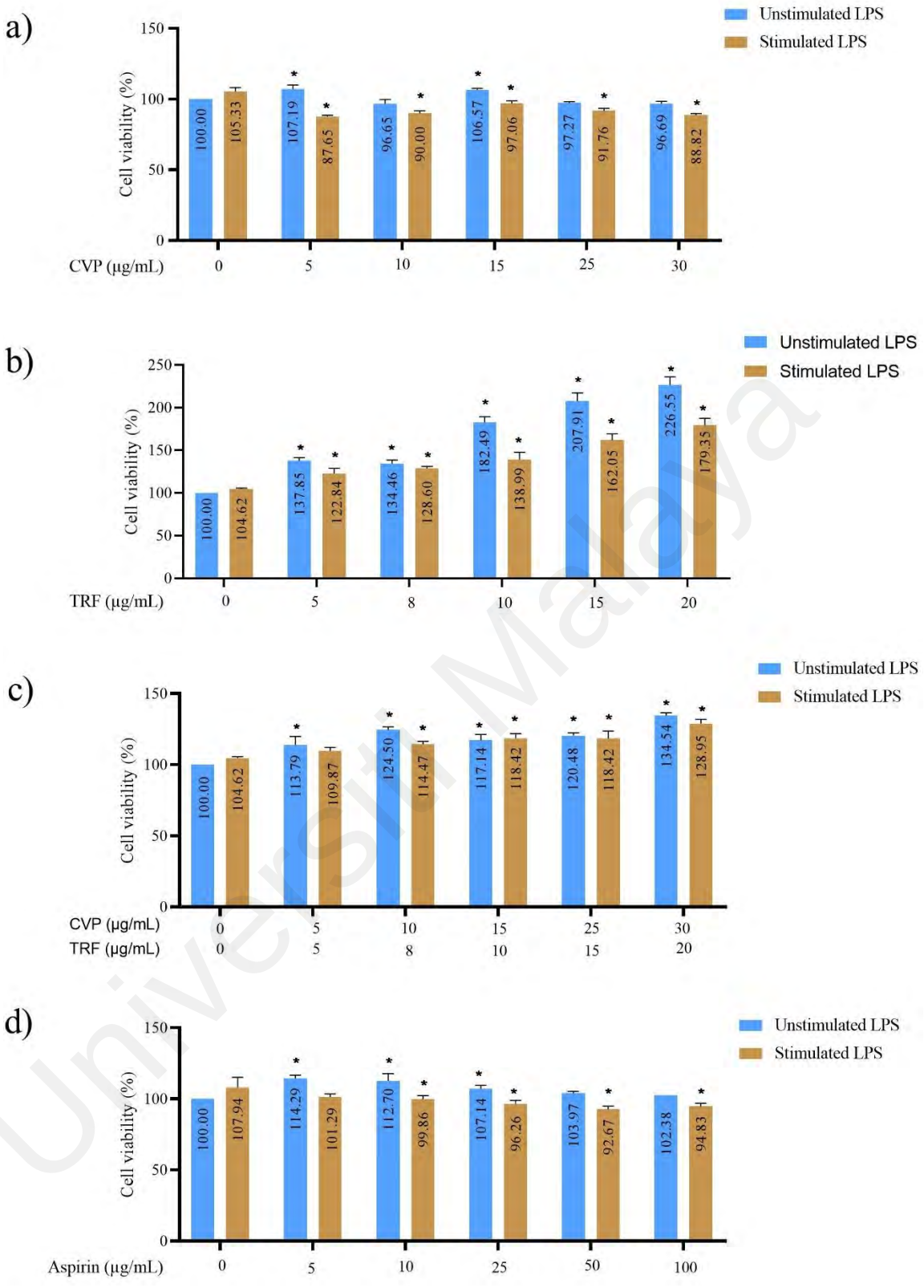


Figure 4.6: Cell viability of RAW264.7 stimulated and unstimulated LPS of a) CVP b) TRF c) CVP+TRF and d) aspirin as a control. *Indicating comparison between treatment concentrations and control group (*P < 0.05).

4.3.2 Morphology of LPS unstimulated and stimulated RAW264.7 macrophages

Studying the morphology of RAW264.7 macrophages under LPS stimulation versus their unstimulated state is essential for understanding cellular responses in inflammation (Taciak *et al*., 2018). Morphological changes reflect dynamic shifts in cell shape and structure that correlate with functional alterations, such as increased cytokine production and enhanced phagocytic activity in response to LPS. These observations provide valuable insights into macrophage activation mechanisms and help validate experimental conditions, ensuring the relevance and reliability of subsequent research findings. By visually assessing these changes, researchers gain critical information about the immune response dynamics and underlying molecular pathways involved in inflammatory processes.

Further observations were made on the morphology of LPS-unstimulated RAW264.7 cells to LPS-stimulated RAW264.7 cells, distinct differences become evident. LPS-unstimulated RAW264.7 cells typically exhibit a round or oval shape with minimal pseudopodia, indicating a quiescent state with low metabolic activity as shown in Figure 4.7(a). In contrast, LPS-stimulated RAW264.7 cells undergo significant morphological changes as illustrated in Figure 4.7(b). They become larger in size, spread out more on the culture surface and develop prominent pseudopodia. These changes reflect their activation status in response to LPS stimulation, where they upregulate, inflammatory pathways. One of the inflammatory mediators and pathways including nitric oxide (NO) production. For the purpose of testing NO production, LPS-stimulated RAW264.7 cells are preferred due to their heightened activity in producing NO in response to LPS. This activation allows researchers to accurately measure and quantify NO levels, making these cells the appropriate choice for experiments focused on understanding the inflammatory response and its mediators like nitric oxide.

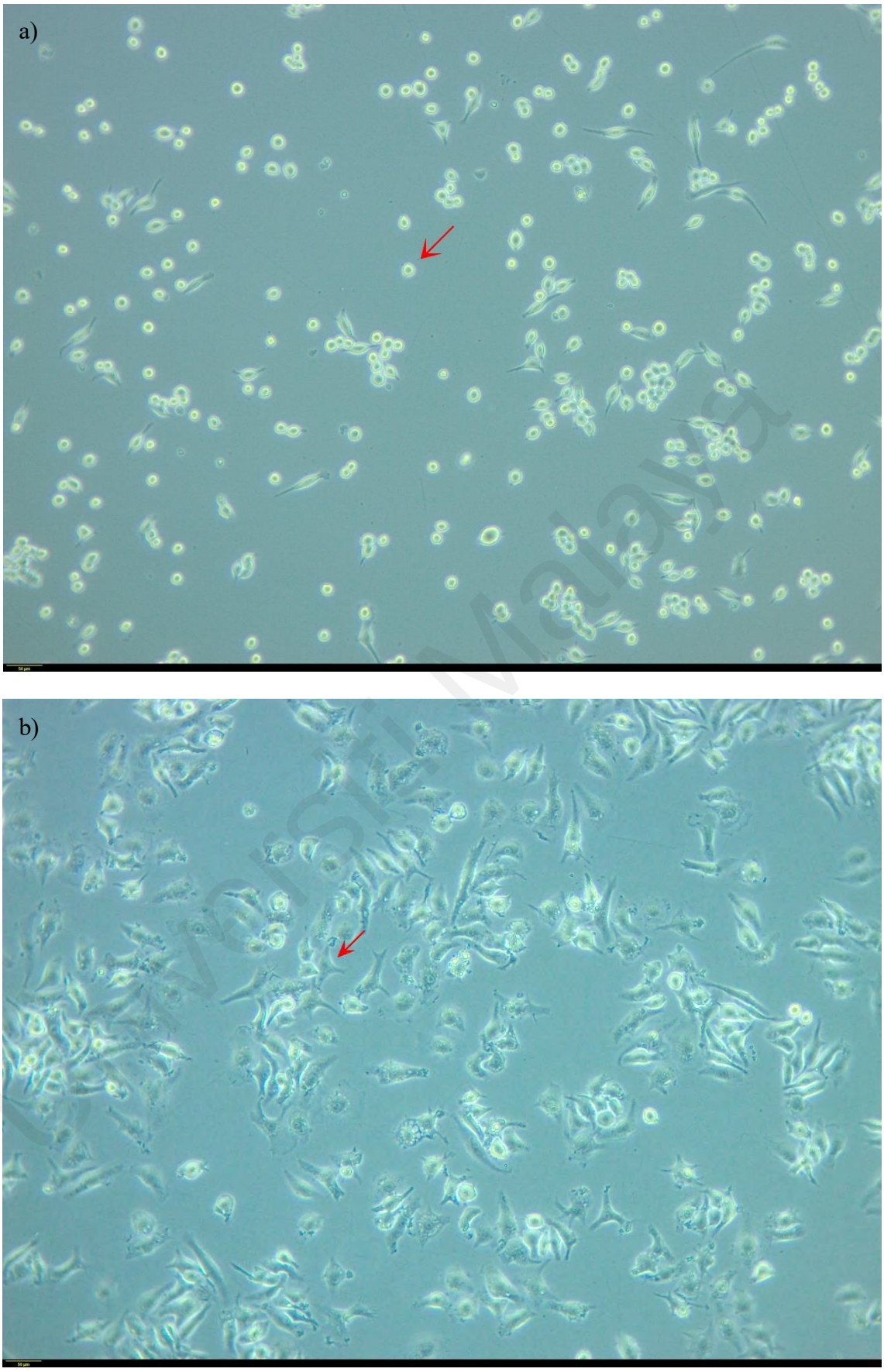


Figure 4.7: The morphology of a) RAW264.7 macrophages (unstimulated LPS) and, b) LPS-stimulated RAW264.7 macrophages (100× of magnification power under microscope observation and the scale bar is 50µm). Arrow indicates the difference of morphology.

4.3.3 Effect of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), single and combination treatment, on nitric oxide production measurement

Nitric oxide (NO) is a critical signalling molecule involved in various physiological and pathological processes, including immune response, inflammation and cellular communication. In the context of the immune system, NO is synthesized by nitric oxide synthases (NOS), with inducible NOS (iNOS) being the key enzyme responsible for NO production in activated macrophages. NO produced by iNOS plays a vital role in the defence mechanisms of macrophages against pathogens and in modulating inflammatory responses (Xu *et al*., 2018).

RAW264.7 cells are macrophage cell line that widely used as a model to study the molecular mechanisms underlying immune responses. These cells possess the ability to produce nitric oxide (NO) in response to various stimuli, making them an ideal system for investigating the regulation and function of NO in inflammation. Upon exposure to LPS, RAW264.7 cells undergo activation, characterised by morphological changes, upregulation of inflammatory mediators including significant production of NO. This process mimics the immune response during bacterial infections, providing a valuable model for studying NO production and its regulatory mechanisms. The measurement of NO production in LPS-stimulated RAW264.7 cells is a vital experimental approach for understanding the dynamics of macrophage activation and the role of NO in inflammation.

The effects of CVP, TRF, their combination (CVP+TRF) and aspirin on nitric oxide (NO) production were measured in LPS-stimulated RAW264.7 macrophages using the Griess assay. NO production, which is a marker of macrophage activation upon LPS stimulation, was significantly reduced by these compounds. As shown in Figure 4.8(a),

CVP at concentrations of 5, 10, 15, 25 and 30 µg/mL resulted in NO production percentages of 94.74 ± 5.26 %, 82.46 ± 3.04 %, 68.42 ± 5.26 %, 54.39 ± 3.04 % and 49.12 ± 2.48 %, respectively, compared to the control (100.00 ± 5.26 %). TRF, as presented in Figure 4.8(b), at concentrations of 5, 8, 10, 15 and 20 µg/mL, produced NO levels in percentage of 96.49 ± 3.04 %, 87.72 ± 3.04 %, 84.21 ± 5.26 %, 66.67 ± 2.48 % and 50.88 ± 2.48 %, respectively, compared to the control (100.00 ± 5.26 %). The combination of CVP and TRF, as illustrated in Figure 4.8(c), at ratios of (5:5), (10:8), (15:10), (25:15) and (30:20) µg/mL, resulted in NO production percentages of 91.23 ± 6.08 %, 75.44 ± 3.039 %, 54.39 ± 3.04 %, 40.35 ± 3.039 %, and 35.09 ± 3.04 %, respectively, compared to the control (100.00 ± 5.26 %). Lastly, as indicated in Figure 4.8(d), aspirin at concentrations of 5, 10, 25, 50 and 100 µg/mL resulted in NO production percentages of 95.65 ± 3.77 %, 91.30 ± 6.52 %, 71.74 ± 6.52 %, 67.39 ± 3.77 %, and 49.99 ± 3.07 %, respectively, compared to the control (100.00 ± 7.53 %).

Results showing a significantly greater reduction in nitric oxide (NO) production with the combination compared to each compound alone. At the highest tested combination ratio of CVP and TRF (30:20 µg/mL), NO production was reduced to 35.09 %, compared to 49.12 % with CVP alone at 30 µg/mL and 50.88 % with TRF alone at 20 µg/mL. These findings suggest that CVP, TRF, CVP+TRF and aspirin effectively reduce NO production in LPS-stimulated RAW264.7 macrophages, with the combination of CVP and TRF showing a particularly strong inhibitory effect, indicating a potential synergistic interaction between the two compounds. The stronger inhibitory effect of the combination of CVP and TRF on NO production suggests a synergistic interaction. This synergism likely arises from their complementary mechanisms of action, enhanced bioavailability, broad-spectrum antioxidant activity and additive inhibition of multiple inflammatory pathways. Therefore, combining CVP and TRF could be a particularly effective strategy for reducing inflammation mediated by NO production in macrophages. To further

investigate the interaction between the CVP and TRF combination, the next analysis will employ the combination index (CI) method and isobologram analysis to quantitatively determine whether the interaction is synergistic, additive or antagonistic. These tools will allow us to identify the nature of the interaction and assess the potential for a synergistic effect.

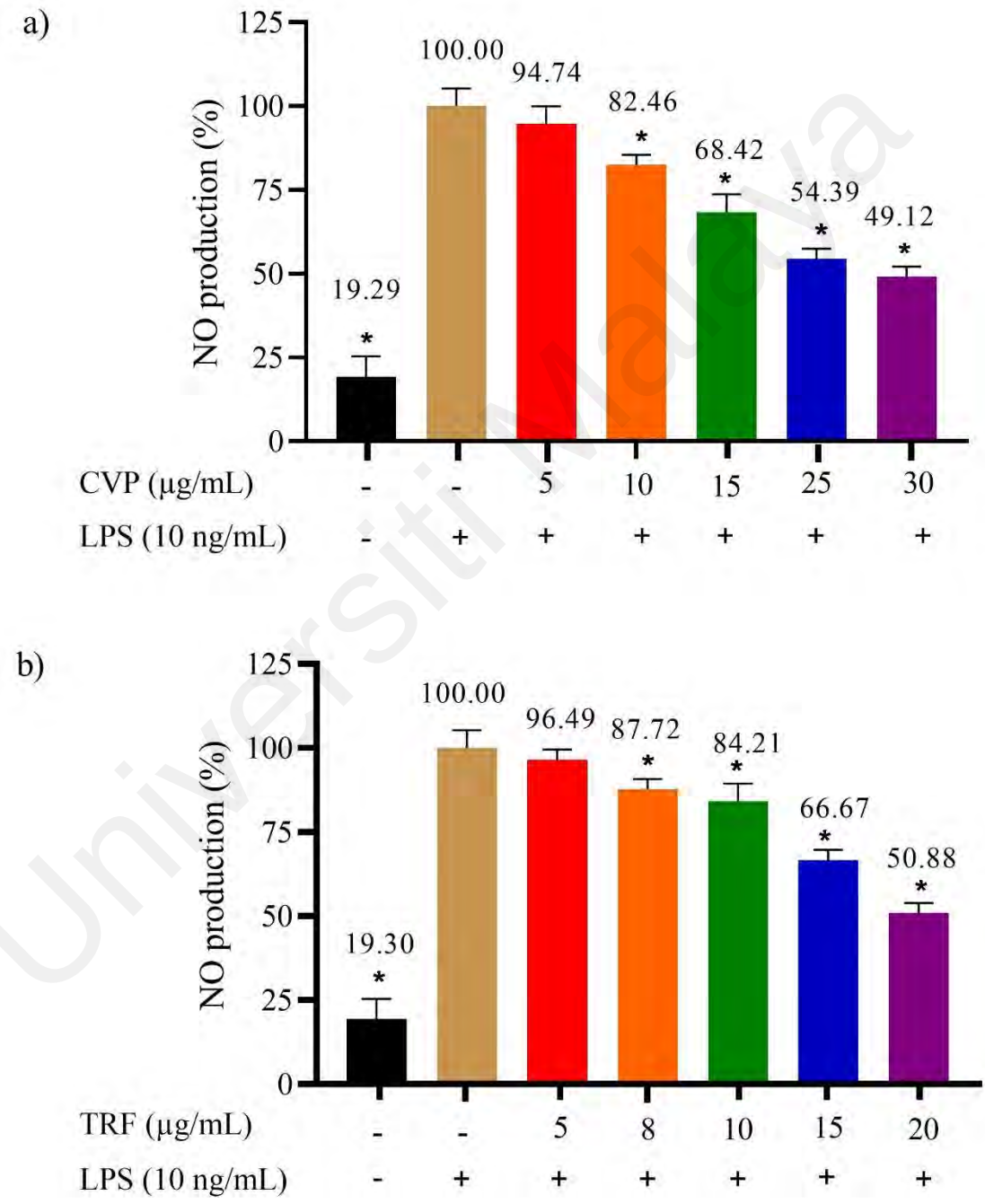
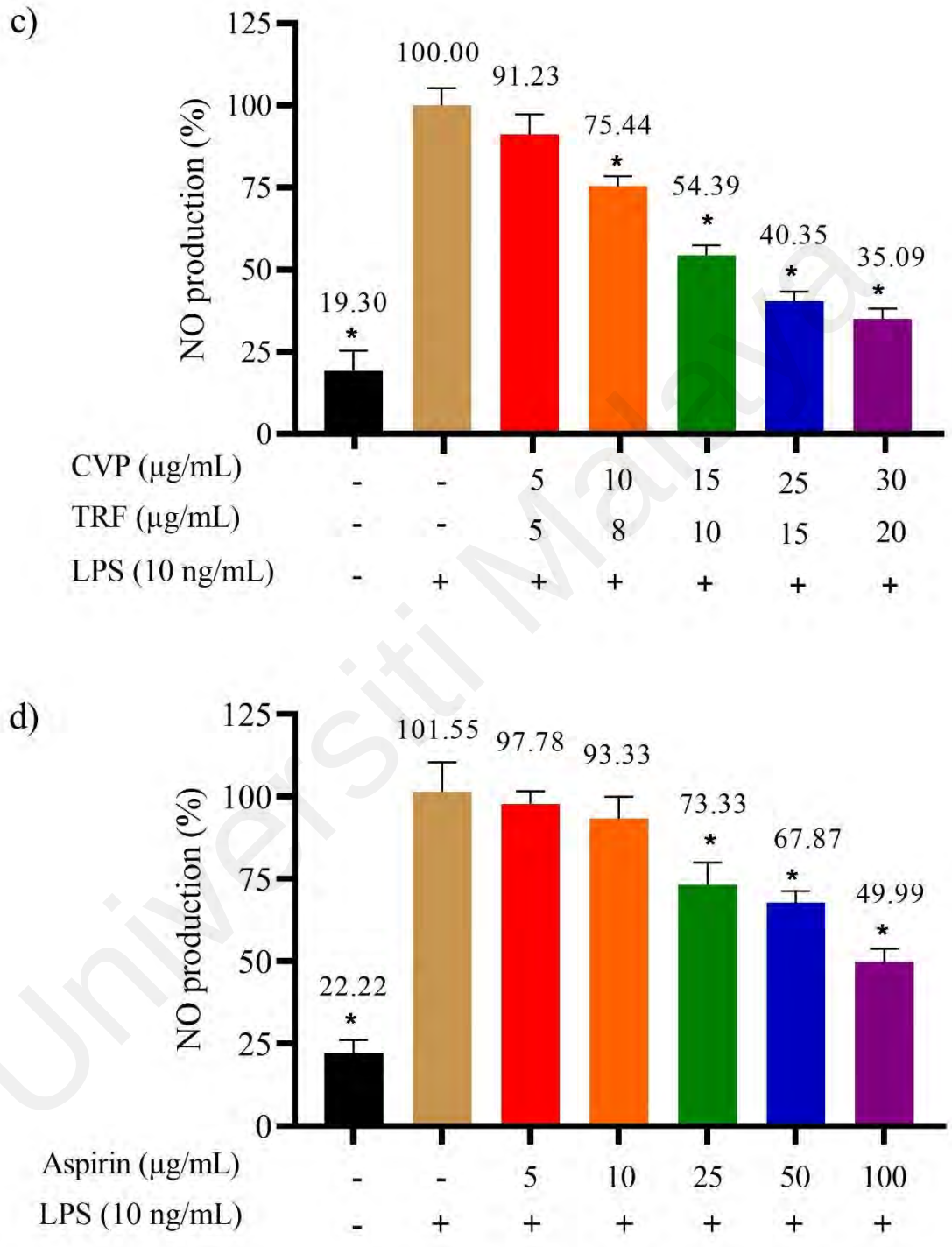


Figure 4.8: The production of nitric oxide (NO) in LPS-stimulated in RAW264.7 macrophages when treated with a) CVP, b) TRF, c) CVP+TRF, and d) aspirin as a control. * Indicates the comparison between treatment concentrations and LPS control group (*P < 0.05).

Figure 4.8, continued



4.3.4 Analysis of synergistic effect of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF)

Inflammation is a leading to chronic inflammation or cancer, cause of morbidity and mortality worldwide which driving the continuous search for more effective therapeutic strategies. Nowadays, there is a growing interest in combination therapies that can enhance treatment efficacy while minimising adverse effects. Combination therapy involves the use of multiple agents that target different pathways or mechanisms within inflammation cells, potentially leading to synergistic effects. Synergism in combination therapy occurs when the combined effect of the drugs is greater than the sum of their individual effects (Duarte & Vale, 2022). This approach can improve therapeutic outcomes, reduce the required doses of each drug, and decrease the side effects.

Purified *C. vespertilionis* protease (CVP) is a medicinal plant, known for treating various diseases including inflammation. However, its effectiveness can be limited by resistance and toxicity. Palm tocotrienol-rich fraction (TRF) on the other hand considered less toxic than conventional treatments, making it an attractive candidate for combination therapy (Zaini *et al*., 2016). Synergistic interaction between purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) was done to determine the relationship between the treatments, whether it has synergistic effect or not. The test was analysed through isobologram in the combination index (CI). The results from nitric oxide production inhibition were used to determine the relationship of CVP and TRF. The results presented in the Figure 4.9 demonstrate the effectiveness of different treatments involving CVP, TRF and their combination. Figure 4.9(a) shows the fractional response versus the concentration of CVP, TRF and their combination. The fractional response is derived from nitric oxide (NO) production measurements. As the concentration of each treatment increases, the fractional response decreases for all

treatments. This indicates that higher concentrations of CVP, TRF and their combination are more effective in inhibiting NO production, resulting in a lower fractional response. TRF alone has a higher fractional response compared to CVP and their combination at lower concentrations. However, the combination of CVP and TRF shows a synergistic effect, resulting in a more significant reduction in the fractional response, particularly at higher concentrations. The most effective concentration is marked by a black dot with a value of 0.35, indicating optimal effectiveness at this concentration.

IC_{50}, or the half-maximal inhibitory concentration, is a measure used to indicate the potency of a substance in inhibiting a specific biological function, such as nitric oxide (NO) production. NO production is measured in a controlled setting, with LPS-stimulated RAW264.7 cells exposed to various concentrations of the substances being tested: CVP, TRF, their combination and aspirin. The data from these measurements are used to create a dose-response curve, as shown in Figure 4.9(a). This curve plots the concentration of the substance against the degree of NO production, represented as fractional responses. The IC_{50} value, derived from this curve, is the concentration at which NO production is inhibited by 50 %. This value indicates the potency of the substance, with a lower IC_{50} value signifying a more potent inhibitor of NO production. Based on the Figure 4.9(b), TRF has the lowest IC_{50} value (19.71 ± 0.75 µg/mL), making it the most effective at inhibiting NO production. CVP has a higher IC_{50} value (30.87 ± 1.43 µg/mL), indicating it is less effective than TRF. The combination of CVP and TRF has an IC_{50} value of 22.58 ± 0.82 µg/mL, showing a synergistic effect and higher effectiveness than CVP alone but slightly less effective than TRF alone. Aspirin, with the highest IC_{50} value (91.45 ± 0.58 µg/mL), is the least effective treatment among those tested.

Figure 4.9(c) is an isobologram, a type of graph commonly used to illustrate the interactions between two drugs, treatments or compounds. It visually represents whether the combined effect of the compounds is additive, synergistic or antagonistic. The x-axis of the graph shows the concentration of CVP, while the y-axis shows the concentration of TRF, both in µg/mL. In this isobologram, blue points represent the effective doses of the individual compounds which includes 30.87 µg/mL for CVP and 19.71 µg/mL for TRF. The red point shows the effective doses when the compounds are used in combination in ratio of 14.10 µg/mL for CVP and 8.48 µg/mL for TRF from the total concentration of combination CVP and TRF values of 22.58 µg/mL. This point is located below the dashed additive line, which represents the theoretical additive effect, where the combined effect would equal the sum of the individual effects. The placement of the red point below this line indicates a synergistic effect, meaning that the combined effect of CVP and TRF is greater than the sum of their individual effects. This is further supported by the combination index (CI) value of 0.89, which is less than 1, confirming the synergistic interaction.

The synergy observed is likely due to the optimal combination of doses, where the ratio of 14.10 µg/mL CVP and 8.48 µg/mL TRF proves particularly effective in reducing nitric oxide (NO) levels. This effectiveness allows the two compounds to complement each other's mechanisms of action (Santana-Gálvez *et al.*, 2020). Additionally, there may be molecular or cellular interactions between the compounds that amplify their individual effects, such as enhancing uptake or activation. Moreover, this specific ratio likely results in improved pharmacokinetic profiles for both compounds, ensuring higher effective concentrations at the target site. As a result, the combination of CVP and TRF at this ratio achieves a CI of 0.89 and more than 50 % NO inhibition, demonstrating a highly effective and synergistic interaction where the compounds work better together than individually.

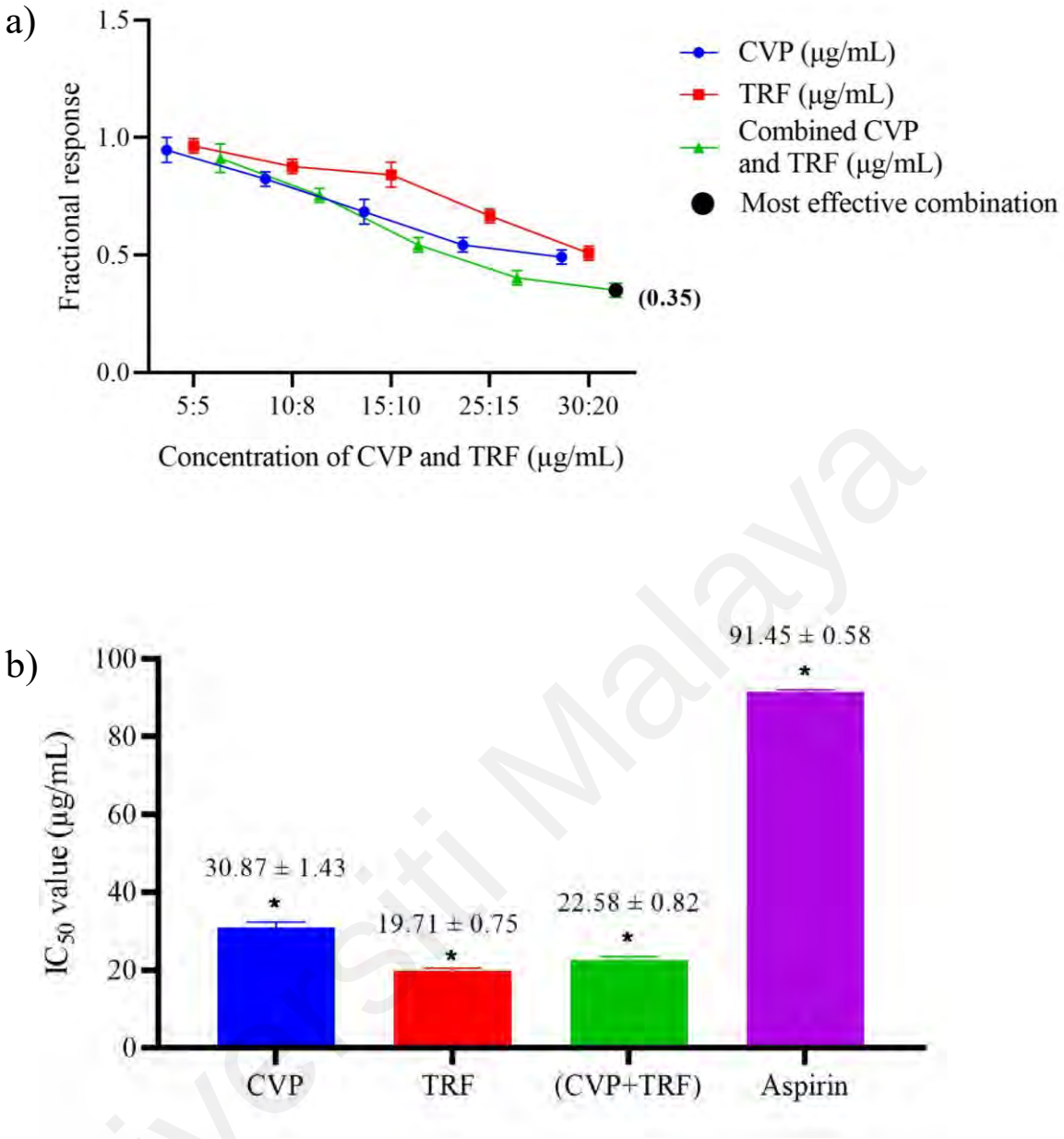
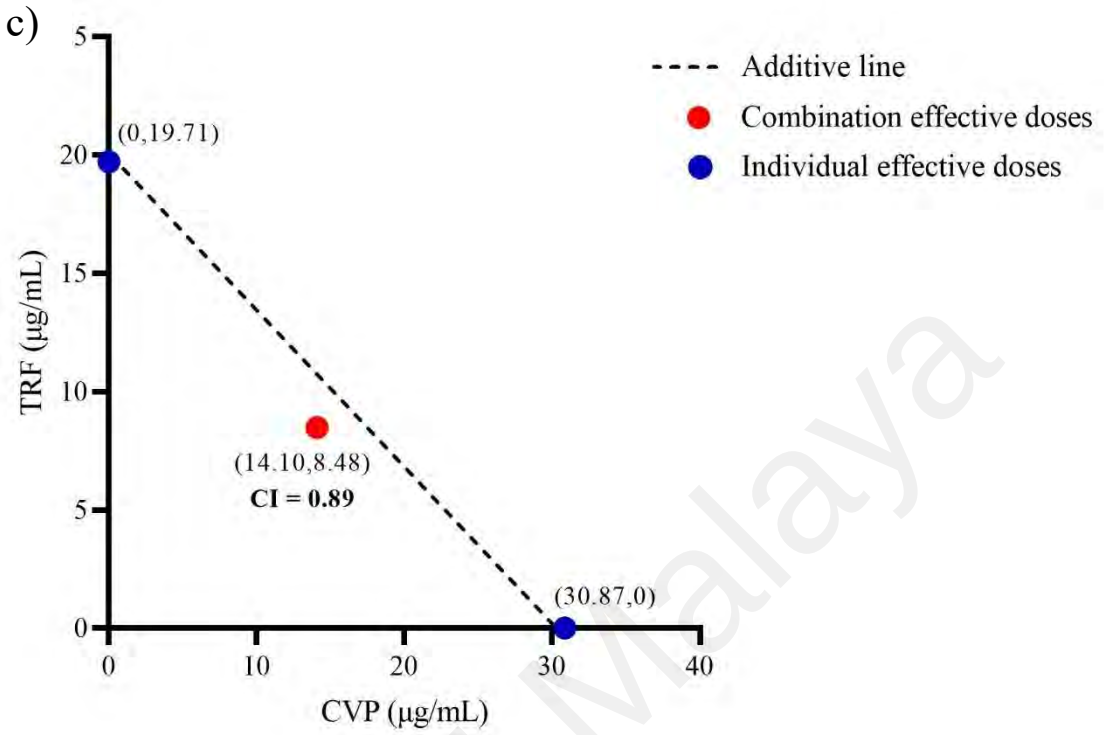


Figure 4.9: Evaluation of the synergistic interaction between CVP and TRF on LPS-stimulated RAW264.7 cells includes a) A combination dose-response curve for NO production, b) IC50 value analysis, and c) Isobologram and CI value analysis.

Figure 4.9, continued



4.4 Anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in single and combination treatment in carrageenan-induced BALB/c paw oedema

In studies examining the anti-inflammatory properties of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), both substances have shown promising individual efficacy in reducing inflammation. Carrageenan-induced paw oedema in BALB/c mice serves as a robust model to assess the therapeutic potential of these compounds. *Christia vespertilionis*, a plant indigenous to Southeast Asia, is recognised for its bioactive compounds, including proteases that exhibit anti-inflammatory properties. Similarly, palm tocotrienol-rich fraction, derived from the oil palm tree, is known for its potent antioxidant and anti-inflammatory effects, attributed to

its tocotrienol content. In this context, evaluating the effects of CVP and TRF individually and in combination through *in vivo* test provides insights into their synergistic potential against inflammation. The study aims to elucidate whether their combined application enhances therapeutic outcomes compared to their individual effects, offering implications for future therapeutic strategies in inflammatory conditions.

4.4.1 Acute toxicity assessment and body weights across treatments in BALB/c mice

The use of BALB/c mice in this study is essential for understanding disease mechanisms and evaluating the safety and efficacy of potential treatments. BALB/c mice, known for their immunological similarities to humans, are frequently employed in these studies. This study focuses on assessing the acute toxicity and body weight changes in BALB/c mice subjected to different treatments aimed at reducing inflammation. The treatments including purified *Christia vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF) and their combination (CVP+TRF) to evaluate the anti-inflammatory effects using the carrageenan-induced paw oedema model. Carrageenan is widely used to induce acute inflammation in animal models, providing a reliable platform for testing anti-inflammatory agents.

Over a period of two weeks, BALB/c mice were administered oral doses of the respective treatments daily. The body weight of the mice was measured weekly to monitor any potential adverse effects such as loss of appetite, which could indicate toxicity. The treatments included non-carrageenan, carrageenan, aspirin (as a standard anti-inflammatory agent), CVP, TRF and the combination of CVP and TRF. Based on Table 4.6, the weight for week one exhibited 16.87 ± 0.42, 17.32 ± 0.22, 17.89 ± 0.88, 17.37 ± 0.33 and 18.61 ± 0.82 g, with respective groups. In week two the weight increased to 18.38 ± 0.33, 18.64 ± 0.90, 19.34 ± 0.53, 18.71 ± 1.21 and 20.19 ± 1.17 g. The results

indicated an overall increase in body weight across all groups, suggesting that the treatments did not adversely affect the mice's appetite or health. Furthermore, the study confirmed that the lethal dose (LD50) for all treatments was greater than 100 mg/kg, highlighting their safety at the administered doses. This study not only contributes to the understanding of the anti-inflammatory properties of CVP and TRF but also establishes their safety profile in BALB/c mice, paving the way for potential therapeutic applications in humans.

Table 4.6: Body weights comparison across experimental groups of BALB/c mice

Group	Dose (mg/kg)	Weight (g)		
		Before treatment	After treatment	
			Week 1	Week 2
Non-carrageenan	-	15.97 ± 0.24	16.27 ± 0.46	17.61 ± 1.15
Carrageenan	-	16.61 ± 0.40	16.87 ± 0.42	18.38 ± 0.33
Aspirin	100	17.18 ± 0.04	17.32 ± 0.22	18.64 ± 0.90
CVP	200	17.03 ± 0.17	17.89 ± 0.88	19.34 ± 0.53
TRF	200	17.20 ± 0.06	17.37 ± 0.33	18.71 ± 1.21
CVP+TRF	100	18.07 ± 0.60	18.61 ± 0.82	20.19 ± 1.17

Values are expressed in mean ± SD (n=3); it indicates the body weight of treated groups compared to control and was statistically analysed by one-way analysis of variance (ANOVA) followed by Holm-Sidak multiple t-test, *P < 0.05 considered as a significant.

4.4.2 Paw oedema size measurement following carrageenan injection in experimental animals

Paw oedema is a common method to study inflammation and the effects of anti-inflammatory drugs. Carrageenan-induced paw oedema is a standard model for inducing acute inflammation in research. Carrageenan, when injected into the paw of a rodent, causes localised inflammation characterised by increased paw size (oedema), redness and pain. This model is widely used to evaluate the anti-inflammatory properties of various compounds.

The treatments of purified *Christia vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF) and their combination (CVP+TRF) were compared against a carrageenan control and aspirin, a standard anti-inflammatory drug in BALB/c mouse model. Figure 4.10 shows the comparison between the paw images before and after carrageenan injection shows a clear visual representation of inflammation through observable changes in swelling, redness and texture. Before the injection the paw appears normal in size, shape and colour with a smooth surface. After the injection, the paw is noticeably larger, indicating significant swelling and shows a marked increase in redness, highlighted by the arrow. This redness is due to increased blood flow, a common inflammatory response. Additionally, the post-injection paw appears more tense and possibly shinier, indicating fluid accumulation (oedema) under the skin. These visual differences effectively demonstrate the inflammatory response induced by carrageenan.

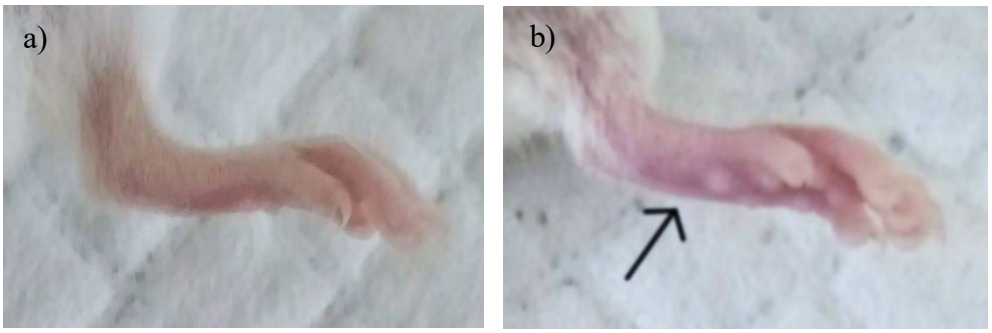


Figure 4.10: BALB/c's paw a) before carrageenan injection, and b) after carrageenan injection.

Figure 4.11 shows the paw oedema size as a percentage over time, measured in hours, for different treatment groups. The treatments include a control group (carrageenan only), aspirin, CVP, TRF and a combination of CVP and TRF. The y-axis represents the percentage increase in paw size (oedema), while the x-axis shows the time in hours after the carrageenan injection. The graph shows the paw oedema size reduced across all treatment groups, indicating the efficacy of the treatments in reducing inflammation. The carrageenan control group exhibited paw oedema sizes of 71.98 ± 3.98 %, 79.54 ± 4.83 %, 71.48 ± 4.20 %, 71.03 ± 6.50 % and 68.09 ± 3.41 % at intervals of 1, 2, 3, 4 and 5 hours, respectively. The treatment with CVP showed a marked reduction in paw oedema, with percentages of 59.90 ± 9.42 %, 64.75 ± 9.47 %, 53.30 ± 14.28 %, 48.90 ± 16.73 % and 47.59 ± 4.92 % at the respective time intervals. Similarly, TRF treatment resulted in paw oedema sizes of 74.29 ± 2.50 %, 81.16 ± 6.68 %, 69.79 ± 3.74 %, 54.80 ± 22.08 %, and 33.26 ± 2.87 % over the same periods. The combination treatment (CVP+TRF) also demonstrated significant anti-inflammatory effects, with paw oedema sizes reducing to 80.70 ± 9.43 %, 84.83 ± 5.35 %, 77.73 ± 8.60 %, 71.83 ± 11.94 %, and 39.37 ± 7.38 % at the respective time intervals. Aspirin, used as a positive control, showed a consistent reduction in paw oedema size, reaching 76.72 ± 18.30 %, 79.27 ± 19.78 %, 72.65 ± 16.84 %, 69.19 ± 15.94 % and 39.35 ± 2.09 % at the respective time intervals.

The control group (carrageenan only) shows the highest and most sustained level of paw oedema over the 5-hour period. Aspirin, a well-known anti-inflammatory drug, significantly reduces paw oedema compared to the control, showing a lower peak and a faster decline in oedema size. Both CVP and TRF treatments also reduce the paw oedema size, but not as effectively as aspirin. CVP and TRF show similar patterns, with a moderate reduction in oedema size compared to the control. The combination of CVP and TRF shows the most significant reduction in paw oedema size, even more than aspirin, suggesting a potential synergistic effect when these two compounds are used together. This combination exhibits the lowest peak and fastest decline in oedema size. The result indicates that all treatments (aspirin, CVP, TRF and CVP+TRF) reduce paw oedema to varying degrees, with the combination of CVP and TRF being the most effective. This suggests that combining these compounds could be a promising synergistic interaction and better approach for managing inflammation.

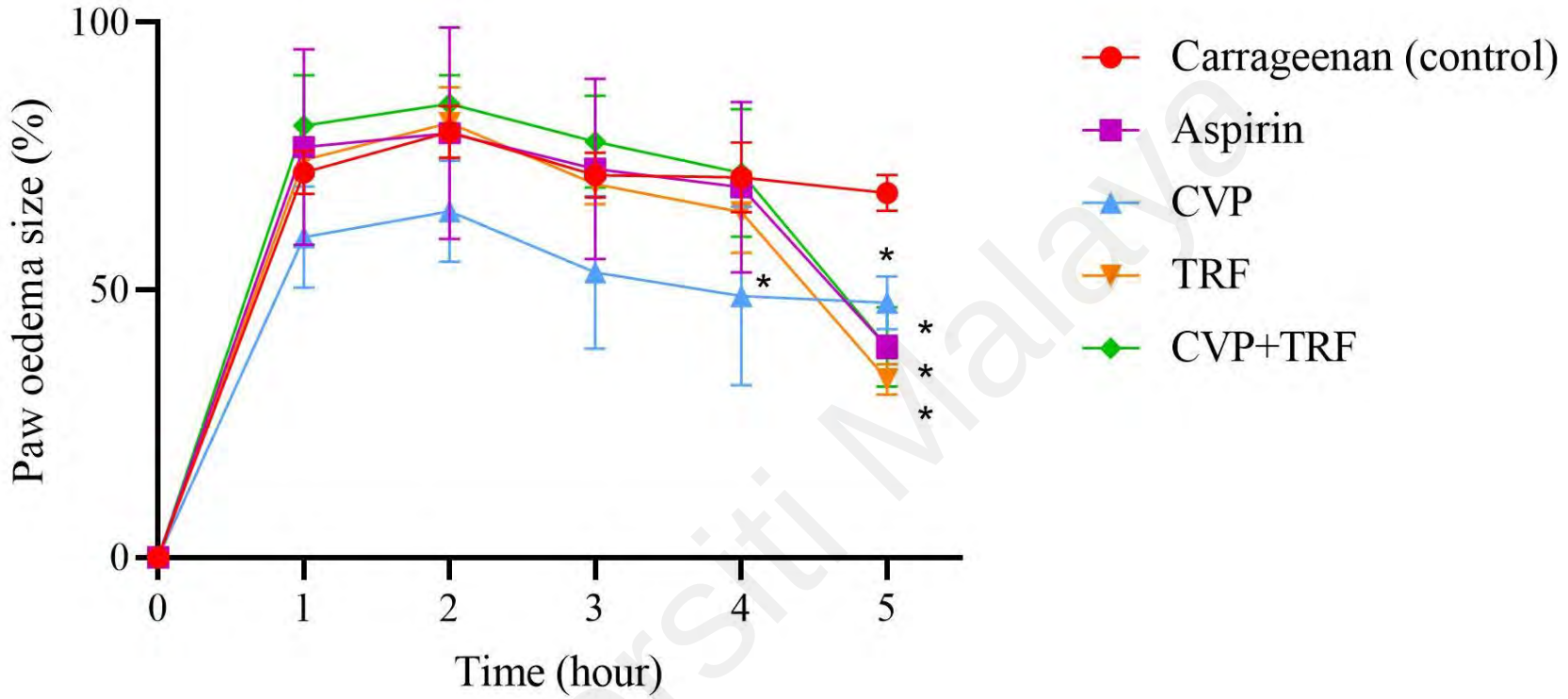


Figure 4.11: Effect of treatments in carrageenan-induced paw oedema BALB/c mice in terms of the paw oedema size within 5 hours after carrageenan injection. Values are expressed in mean ± SD (n=3); it indicates the paw oedema size of treated groups compared to control and was statistically analysed by one-way analysis of variance (ANOVA) followed by Holm-Sidak multiple t-test, *P < 0.05 considered as significant.

4.4.3 Effect of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), single and combination treatment, on tumour necrosis factor-alpha (TNF-*α*) and interleukin-6 (IL-6) in carrageenan-induced paw oedema

Carrageenan-induced paw oedema is a widely known animal model that induces local inflammation characterised by increased vascular permeability and infiltration of immune cells, leading to the release of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-*α*) and interleukin-6 (IL-6). TNF-*α* and IL-6 are key mediators of the inflammatory response, influencing various aspects of immune cell activation and tissue damage. Natural compounds such as proteases and tocotrienols have gained attention for their potential anti-inflammatory properties.

Purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) have shown promise in modulating inflammatory responses. Studies have suggested that these compounds may exert anti-inflammatory effects through various mechanisms, including inhibition of pro-inflammatory cytokine production and modulation of signalling pathways involved in inflammation (Lu *et al.*, 2022). The combination therapy of CVP and TRF presents possibility for enhancing anti-inflammatory efficacy while potentially reducing adverse effects. Understanding the synergistic effects of CVP and TRF on TNF-*α* and IL-6 levels in the context of carrageenan-induced inflammation is crucial for exploring their therapeutic potential in inflammatory conditions. This part of study aims to investigate the effects of CVP and TRF, administered singly and in combination, on TNF-α and IL-6 levels in carrageenan-induced paw oedema, and also study their impact on these key inflammatory cytokines, to understand their anti-inflammatory mechanisms and potential therapeutic applications.

Tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are cytokines and pro-inflammatory mediators which can be used as indicators to test the effectiveness of the treatments to reduce inflammation. The cytokines released from cell culture supernatants were detected by ELISA. To understand the significance of the data provided for TNF-α and IL-6 levels, it is essential to compare each treatment group to the non-carrageenan control (baseline) and the carrageenan group (inflammation benchmark). The carrageenan group shows a significant increase in TNF-α (49.67 ± 0.58 pg/mg) and IL-6 (66.11 ± 7.07 pg/mg) compared to the control, confirming successful inflammation induction. Treatments with aspirin, CVP, TRF and CVP+TRF were significantly reduce TNF-α and IL-6 levels, indicating their anti-inflammatory effects. Aspirin reduces TNF-α to 12.08 ± 4.71 pg/mg and IL-6 to 45.02 ± 2.36 pg/mg. The CVP and TRF show similar reductions with CVP reduces TNF-α to 9.17 ± 1.77 pg/mg and IL-6 to 21.67 ± 2.36 pg/mg, while TRF reduces TNF-α to 5.14 ± 4.87 pg/mg and IL-6 to 22.22 ± 4.71 pg/mg. Notably, the combination of CVP and TRF yields the lowest TNF-α level (2.78 ± 3.18 pg/mg) and a substantial IL-6 reduction (20.56 ± 0.79 pg/mg), suggesting a synergistic anti-inflammatory effect. These comparisons highlight the relative effectiveness of each treatment in reducing inflammation. In conclusion, CVP and TRF independently lower cytokine levels, indicating their anti-inflammatory potential. However, CVP and TRF combination shows the most substantial reduction, especially in TNF-α, suggesting a synergistic effect.

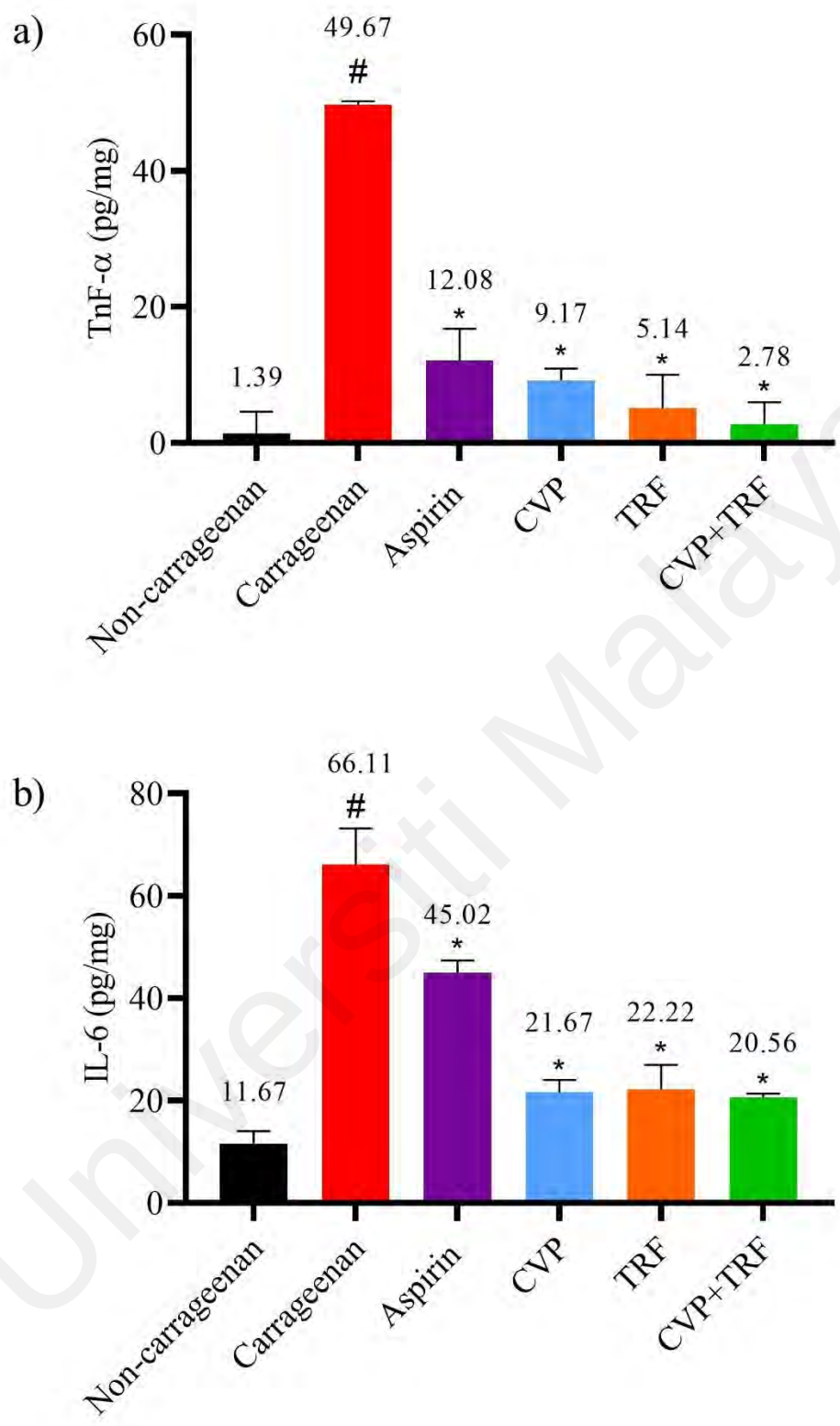


Figure 4.12: Effect of treatments in carrageenan-induced paw oedema at 5 hours on a) tumour necrosis factor-alpha (TNF-α) and b) interleukin-6 (IL-6). Each value represents mean ± SD, *P < 0.05 considered as a significant compared to control, #. The difference between groups were statistically analysed by one-way ANOVA, Dunnett's test.

4.4.4 Effect of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), single and combination treatment, on nitric oxide (NO) production in carrageenan-induced paw oedema

Nitric oxide (NO) production serves as a mediator in the inflammatory response, contributing to the regulation of blood flow, immune response and cellular signalling. Increased NO levels often indicate an inflammatory state, as seen in various pathological conditions, including carrageenan-induced paw oedema. Therefore, the analysis of NO levels can provide insight into the effectiveness of anti-inflammatory treatments includes purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), both individually and in combination. Figure 4.13 provides analysis of the relationships between the different treatments and their impact on inflammatory mediators such as nitric oxide (NO). The non-carrageenan control group shows a baseline NO level of 9.03 ± 0.97 μM, representing normal physiological conditions without inflammation while the carrageenan group exhibits a significantly increased NO level to 14.52 ± 0.65 μM, indicating strong inflammation. Aspirin, a positive control reduces NO levels to 4.62 ± 0.49 μM, confirming its anti-inflammatory properties. CVP shows a similar reduction to 4.30 ± 0.19 μM, highlighting its potential effectiveness. TRF further lowers NO levels to 2.69 ± 0.19 μM, suggesting it may be more potent than both aspirin and CVP. The combination of CVP and TRF results in the lowest NO level of 2.15 ± 0.49 μM, indicating a possible synergistic effect. These findings suggest that both CVP and TRF are effective in reducing inflammation, with the combination treatment offering the most significant reduction in NO production.

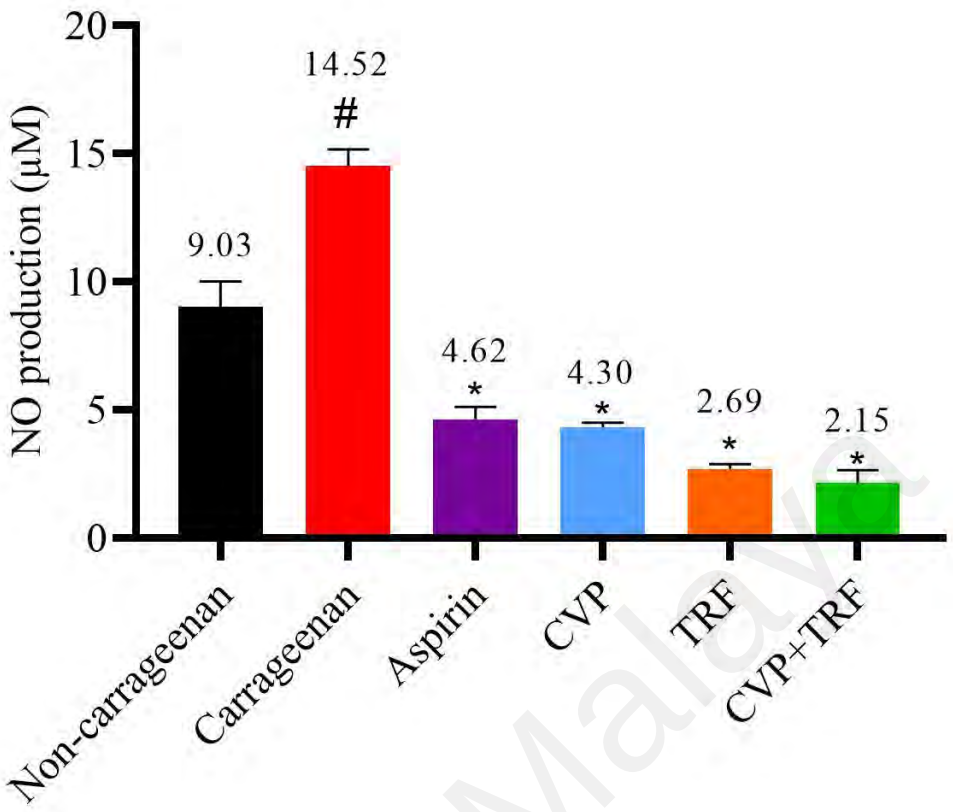


Figure 4.13: Effect of treatments in carrageenan-induced paw oedema on nitric oxide (NO) production in paw tissue at 5 hours. Each value represents mean ± SD, *P < 0.05 considered as a significant compared to control, #. The difference between groups were statistically analysed by one-way ANOVA, Dunnett's test.

CHAPTER 5: DISCUSSION

5.1 Phytochemical analysis of *Christia vespertilionis* leaf extract

C. vespertilionis is an ornamental plant and was used traditional to treat various diseases. In this study, I focus on the leaf extract as previous studies have indicated that the leaf possesses phytochemical compounds that aid the treatments. Leaf from *C. vespertilionis* was removed and extracted using ethanol as solvent. The leaves size was about 5 cm in diameter and this research focuses on *C. vespertilionis* with green leaves as green leaves were proven to be contain more phytochemical components when compared to the red leaf *C. vespertilionis* plant. Dried and grounded leaves was used in order to increase its surface area to the extraction solvent (Nobre & Raffin, 2005).

There are a number of techniques that can be used in plant extraction such as maceration, digestion, decoction, percolation and many more. However, it is important to use the right extraction techniques in order to efficiently and effectively exhibit desired phytochemical compounds (Chew *et al*., 2011). Thus, in this study, Soxhlet extraction method was used to extract phytochemical compounds from *C. vespertilionis* leaves. Soxhlet extraction method was used compared to other methods such as maceration and percolation because it has shown to have higher extraction efficiency, consumes less time and uses less sample and solvent (Zhang *et al*., 2018). This was also demonstrated by other researchers where they found that Soxhlet extraction produced more extract than cold extraction and was effective in extracting phytochemical compounds from leaves (Abah & Egwari, 2011).

Ethanol was chosen in this study as a solvent due to its lower toxicity and superior ability to extract a variety of secondary compounds that may be present in plants (Ablat *et al*., 2014). Ethanol is a polar which has different levels of electronegativity. The

melting and boiling points of ethanol as well as how it interacts with other substances during chemical processes are all influenced by its polarity. Phenolic and flavonoid can be extracted in ethanol because they are all polar solvents. Additionally, ethanol is an excellent extractor of flavonoids, catechols and tannins (Mokrani & Madani, 2016). In addition to being a virtue solvent for extracting polyphenols, ethanol is also less toxic for human consumption (Dai & Mumper, 2010). As a result, ethanol is the ideal option for extraction.

The efficiency and reproducibility of the Soxhlet extraction process for *C. vespertilionis* leaf extract using ethanol were thoroughly evaluated. It shows that the extraction yields for three replicates using one gram of dry leaf material were 6.63 %, 7.32 % and 5.22 %, respectively (Table 4.1). The average yield was 6.39 %, with a standard deviation of 0.01, indicating a high degree of consistency and reproducibility in the extraction process. These results suggest that the Soxhlet extraction method is effective in producing a stable and reproducible amount of crude extract from the dry leaf material of *C. vespertilionis*. The low standard deviation further supports the reliability of the extraction method, confirming its suitability for obtaining consistent yields in future extractions. This consistency is crucial for further experimental applications, ensuring that the variability in the amount of extract is minimised. This result is similar when compared to another study by Mutalib & Latip, 2019 which has been reported with extraction yield of 6.40 % from *C. vespertilionis* leaf ethanolic extract. Another study that uses carbon dioxide liquid and ethanol as a solvent only yielded 2.94 % (Ariff *et al*., 2018). However, the extraction yield of plants can potentially increase when utilising an appropriate extraction method. There was a study stating that it consumes time to optimise the efficient extraction method for the respective plant due to different plant requires different extraction method in order to exhibit the phytochemical compounds (Dhanani *et al*., 2017).

Quantitative analysis was done to screen presence of phytochemical compounds that is contained in the plant extract. Nine different tests were carried out focusing on nine major compounds which were phenolic, cardiac glycoside, steroid, cardenolide, flavonoid, alkaloid, saponin, tannin and terpenoid. The reason why this was done first, was to determine presence of these compounds that was reported by other researches to exhibit anti-inflammatory activities. Phenolics for instance can act as free radical scavenger, antioxidant, anti-inflammatory and anti-carcinogenic (Shahidi & Yeo, 2018) besides reducing inflammation in chronic diseases such as cardiovascular disease, cancer, diabetes and also bacterial and parasitic infections (Canini *et al*., 2007). Cardiac glycoside is important for cardiac muscle functioning (Aslam *et al*., 2009), while steroid and cardenolides are used in signalling molecules and could also potentially in alter fluidity of membranes (Sadava *et al*., 2011). Flavonoids is reported to reduce the damage caused by free radicals and oxidative reduction of macromolecules (Cazarolli *et al*., 2008). Lin et al. (1999) found that flavonoids have anti-inflammatory properties while alkaloids are efficient against fighting pathogens. Saponin on other hand has anti-fungal, antibacterial, anti-protozoal and inhibit damage to the upper digestive tract (Aslam *et al*., 2009; Ayoola & Adeyeye, 2010), tannin has antioxidant activity (Rajurkar & Gaikwad, 2012) and then terpenoids have demonstrated promising results in both preclinical and clinical applications of inflammatory illness (Ge *et al*., 2022).

Most of the compounds in *C. vespertilionis* leaf extract revealed to having anti-inflammatory properties and may effectively reduce inflammation. Previous studies have shown that flavonoids and phenolics possess potent antioxidant and anti-inflammatory activities, by reducing oxidative stress and inflammation (Joo *et al*., 2014; Gonfa *et al*., 2023). Alkaloids and tannins also have been documented to inhibit pro-inflammatory cytokines and enzymes, directly reducing inflammatory responses (Oguntibeju, 2018). Additionally, saponins and steroids contribute to anti-inflammatory effects through

various pathways, including modulation of the immune response and inhibition of inflammatory mediators (Passos *et al*., 2022). The presence of these compounds in the extract proven its potential for developing novel anti-inflammatory therapies. Therefore, further analysis was done to confirm the presents of these compounds quantitatively.

In order to further investigate the potential of this extract, total phenolic, flavonoid and tannin content were measured. Gallic acid (GAE) was used as total phenolic content equivalent to *C. vespertilionis* leaf extract which has phenolic content of 29.25 ± 0.50 mg GAE/mL. Phenolic compounds are renowned for their antioxidant properties, which play a crucial role in reducing oxidative stress and inflammation. Oxidative stress is a known contributor to chronic inflammation, and the antioxidant activity of phenolics helps neutralise free radicals, modulating immune response and inhibiting inflammatory mediators (Medina, 2011; Gonfa *et al*., 2023).

The total flavonoid content, measured at 1.57 ± 0.03 mg QE/mL, also contributes significantly to the extract's anti-inflammatory potential. Flavonoids, a subclass of phenolics, share similar antioxidant mechanisms. They not only scavenge free radicals but also enhance the body's antioxidant defence systems by upregulating the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. This amplifies the reduction of oxidative stress and decreases the associated inflammatory response (Al-Khayri *et al*., 2022). Flavonoids have been shown to inhibit key inflammatory pathways, including the downregulation of pro-inflammatory cytokines and the suppression of NF-κB signalling, which is a critical pathway in the inflammatory response (Ginwala *et al*., 2019). The presence of flavonoids in the *C. vespertilionis* extract indicates its ability to modulate these inflammatory pathways effectively, supporting its potential use as an anti-inflammatory agent.

Additionally, the total tannin content was found to be 2.70 ± 3.15 mg TAE/mL. Tannins possess notable anti-inflammatory properties, often attributed to their ability to inhibit enzymes, mediators and cytokines involved in inflammation (Park *et al*., 2013; De Melo *et al*., 2023). Tannins help reduce the overall inflammatory response by preventing the activity of pro-inflammatory mediators and inhibit pro-inflammatory enzymes like COX and LOX.

Several researchers have reported on the levels of total phenolic, flavonoid and tannin content in the *C. vespertilionis* plant in their studies. Mutalib & Latip (2019) found that the total phenolic content was higher than total flavonoid content in *C. vespertilionis* leaf ethanolic extract, which is similar to this study. Research by Smitha & Reshma (2019) also mentioned presence of flavonoid and tannin in *C. vespertilionis*, while Lee et al. (2020) have shown the presence of phenolic in *C. vespertilionis* root ethyl acetate extract. Ibrahim et al. (2022) reported that flavonoids is one of the most mentioned classes of phytochemicals in research employing *C. vespertilionis* extracts. This shows that the extract has good potential to reduce inflammation. The combined presence of high phenolic and flavonoid contents in the *C. vespertilionis* leaf extract suggests a synergistic effect in combating oxidative stress and inflammation (Hajimehdipoor *et al*., 2014). The phenolics provide a robust antioxidant capacity, directly neutralising ROS and reducing oxidative damage. Meanwhile, the flavonoids extend this protective effect by modulating inflammatory pathways and enhancing endogenous antioxidant defences (Muscolo *et al*., 2024). This synergy not only helps in reducing the immediate oxidative stress and inflammation but also contributes to long-term anti-inflammatory benefits. These quantitative findings are consistent with the qualitative screening, which confirmed the presence of phenolics, flavonoids and tannins in the *C. vespertilionis* leaf extract, highlighting their capability to effectively modulate inflammatory responses.

After determining the total phenolic content (TPC) of the *C. vespertilionis* leaf extract, the next step involves assessing its potential anti-inflammatory properties through an *in vitro*, protease inhibition assay. The phenolic compounds in the extract are likely responsible for this protease inhibition due to its ability to interact with enzymes and inhibit their activity (Gonçalves & Romano, 2017). By binding to the active sites of protease enzymes or altering their structure, phenolic compounds can prevent the enzymes from breaking down proteins, and able to reducing inflammation. In this test, high inhibition of protease means the extract has the ability to reduce inflammation. Based on the result, *C. vespertilionis* leaf extract having an IC_{50} of 284.59 ± 10.63 µg/mL and aspirin at 275.72 ± 6.93 µg/mL. These similar IC_{50} values indicate comparable potency, with aspirin being slightly more effective. The result suggests that *C. vespertilionis* leaf extract exhibits protease inhibition activity similar to that of aspirin. This finding is significant because it highlights the potential of *C. vespertilionis* as a natural alternative to synthetic drugs like aspirin for managing conditions associated with excessive protease activity, such as inflammation.

The results of total phenolic and protease inhibition can provide mechanistic insights into how *C. vespertilionis* leaf extract works. If a strong correlation is found, it suggests that the phenolic compounds might directly or indirectly interact with protease enzymes, inhibiting their activity. Oyedapo (2001) and Lee et al. (2020) also stated that anti-inflammatory activity is mainly caused by phenolic compounds. As mentioned previously, protease plays a role in the inflammation process. Therefore, the anti-inflammatory activity can be deduced by correlating the protease inhibition assay with the total phenolic content. An R^2 value of 0.6821 was observed, indicating positive correlation with the phenolic compounds in *C. vespertilionis* leaf extract are likely the primary contributors to its anti-inflammatory activities, and having ability to inhibit protease.

After determining the presence of phytochemicals compounds in the leaf extract, more work was carried out to further identify compounds that are associated with anti-inflammation by using GC-MS. Four major anti-inflammatory compounds were identified with peak area larger than 4 % similar to reported by Abd Rahim et al. (2018) and Sidek et al. (2019). The compounds includes n-hexadecanoic acid (Aparna *et al*., 2012), phytol (Silva *et al*., 2014), 9,12,15-octadecatrienoic acid, (Z,Z,Z)- (Sermakkani & Thangapandian, 2012; Suman *et al*., 2013) and squalene (Kelly, 1999; Lacatusua *et al*., 2018). The compounds mentioned above were reported to possess anti-inflammatory activity besides having other biological activities. A study by Lacatusua et al. (2018) showed that squalene was able to reduce damage to the skin using *Amaranthus cruentus* oil extract and it also showed to be chemo-preventive, as well as antioxidant and antitumor activity (Rao *et al*., 1998; Kelly, 1999; Kim & Karadeniz, 2012; Singab *et al*., 2015; Singariya *et al*., 2015). Compound of 9,12,15-octadecatrienoic acid, (Z,Z,Z)- and phytol have anti-cancer activities as reported by Sermakkani &Thangapandian (2012), Suman et al. (2013) and Gavamukulya et al. (2015). In another research, Mohd Yasin et al. (2020) also reported that phytol as a major compound in *C. vespertilionis* extract which important in reducing inflammation. Similarly, n-hexadecanoic acid possessed antibacterial and antioxidant activity including able to modulate immune response (Gavamukulya *et al*., 2015; Johannes *et al*., 2016).

The GC-MS analysis of *Christia vespertilionis* leaf extract has identified several compounds with significant anti-inflammatory properties, reinforcing findings from previous studies and highlighting the plant's therapeutic potential. Compounds such as n-hexadecanoic acid, phytol, 9,12,15-octadecatrienoic acid and squalene were detected, and each known for their ability to modulate inflammatory responses. n-hexadecanoic acid and phytol have been documented in earlier research for their roles in inhibiting pro-inflammatory mediators and modulating immune responses (Islam *et al*., 2020).

Similarly, 9,12,15-octadecatrienoic acid, an omega-3 fatty acid, has been recognised for its effectiveness in reducing inflammatory cytokines, while squalene's antioxidant properties contribute to its anti-inflammatory effects (Khoo *et al*., 2018). These findings support the anti-inflammatory potential suggested in earlier studies by Samtiya et al. (2021), demonstrating that the bioactive compounds present in *C. vespertilionis* can significantly contribute to reducing inflammation.

5.1.1 Anti-inflammatory compounds in *Christia vespertilionis* leaf extract and its mechanisms

There are 4 anti-inflammatory compounds in *C. vespertilionis* found in this study through GC-MS including phytol, squalene, 9,12,15-octadecatrienoic acid, (Z,Z,Z)- and n-hexadecanoic acid. These compounds were previously studied by many researchers and mentioned the potent of anti-inflammatory response with the mechanisms involve in the inflammatory pathways. First, phytol is a natural terpenoid alcohol and recent studies have shown that it possesses anti-inflammatory properties. Phytol mechanism is through the inhibition of pro-inflammatory mediators such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TnF-α) (Carvalho *et al*., 2020). Phytol has been shown to suppress the production of these mediators *in vitro* and *in vivo*, correspond to reducing inflammation. Phytol has antioxidant properties which can also contribute to anti-inflammatory effects (Santos *et al*., 2013). Phytol can help alleviate the inflammatory response by scavenging reactive oxygen species (ROS) and reducing oxidative stress. Phytol also has immunomodulatory effects, which can regulate the activity of immune cells such as macrophages and lymphocytes, to reduce inflammation (Islam *et al*., 2018).

Next, squalene, a natural triterpene, which is important for reducing inflammation through its modulation of the nuclear factor-kappa B (NF-κB) signalling pathway. By inhibiting the activation of NF-κB and reducing the expression of pro-inflammatory

cytokines, squalene effectively decreases inflammation. Additionally, its antioxidant properties further help reduce the inflammatory response, making it a valuable compound for controlling inflammation (Ibrahim & Mohamed, 2021). Meanwhile, 9,12,15-octadecatrienoic acid, (Z,Z,Z)- known as alpha-linolenic acid (ALA) is an omega-3 fatty acid. Research has demonstrated that ALA (alpha-linolenic acid) exhibits anti-inflammatory properties by converting into longer-chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are well-known for their strong anti-inflammatory effects, and their incorporation into cell membranes can influence cellular signalling pathways involved in inflammation. ALA can inhibit enzymes responsible for the production of pro-inflammatory eicosanoids like prostaglandins and leukotrienes. Additionally, ALA can suppress the activation of NF-κB and enhance the activity of peroxisome proliferator-activated receptor gamma (PPARγ), leading to a reduction in the expression of pro-inflammatory genes and an increase in the expression of anti-inflammatory genes (Jabeen *et al*., 2023).

Last but not least, n-hexadecanoic acid known as palmitic acid is a saturated fatty acid (Aparna *et al*., 2012). Studies have suggested that it can modulate the activity of various immune cells, including macrophages and T cells, which play key roles in the inflammatory response. This compound also inhibits NF-κB activity to decrease pro-inflammatory mediators. It has been shown to activate peroxisome proliferator-activated receptor (PPAR) to inhibit pro-inflammatory cytokines and enhance anti-inflammatory cytokines. These compounds' mechanisms align with findings from studies on other plants with similar compounds, such as green tea, olive oil, flaxseed and palmitic acid-rich oils, confirming their efficacy in reducing inflammation. This highlights the therapeutic potential of *C. vespertilionis* as a natural anti-inflammatory agent and supports further research into its therapeutic applications.

5.2 Protease extraction and purification of *Christia vespertilionis* leaf extract

The reason to purify the plant extract to isolate the protease, rather than using the crude plant extract, was because of purification allows for the identification and study of specific active compounds, which helps in understanding the precise mechanisms of action. The crude extract of *C. vespertilionis* contain multiple bioactive components as mentioned previously in this study, however the effects of individual compounds can be masked by others present in the extract, leading to difficulties in interpreting results. Previous research has shown that isolated proteases from medicinal plants can have significant therapeutic effects, including anti-inflammatory properties, due to their ability to modulate specific inflammatory pathways (Silva-López *et al*., 2019). Plant derived-protease also has increasingly been used in clinical and industrial application. Several clinical disorders have been reported treated with enzymes and combinations as supplementary therapeutic agents, particularly in trauma injuries (Viswanatha Swamy & Patil, 2008).

There are numerous studies that has supported the use of proteolytic enzymes (proteases) to treat inflammatory disorders (David & Richard, 1975; Viswanatha Swamy & Patil, 2008). Viswanatha Swamy & Patil (2008) showed that combination treatment of proteases resulted in anti-inflammatory activity and also exhibit synergistic effect in acute and subacute inflammation model. In order to understand the characteristics and function of how proteases work, proteases from *Christia vespertilionis* were isolated and purified. Recent advancements in technology have significantly improved the methods for enzyme purification, making the process more efficient and precise, allow for better isolation and characterisation of enzymes. As a result, there is a growing interest in research investigations focused on novel enzyme purification techniques to harness their potential benefits (Ullah *et al*., 2022). In contrast

to other type proteases, plant proteases have been found to be less investigated, however it is now researcher have more interest in purifying proteolytic enzyme from plant in the purpose for medicinal uses and toxicological information (Troncoso *et al*., 2022).

In this study, *C. vespertilionis* leaf extract was isolated and purified by ammonium sulphate precipitation method which is widely used as a way to purify protease (Drivdahl & Thimann, 1977; Antào & Malcata, 2005; Esposito *et al*., 2016; Nam *et al*., 2016). Ammonium sulphate, $(NH_4)_2SO_4$, is used for salting out due to its high solubility. This enables it to create solutions with extremely high ionic strengths, low cost, and availability of pure material. Additionally, NH_4^+ and SO_4^{2-} molecules has been demonstrated to stabilise protein structure (Burgess, 2009). The plant extract should be mixed with an appropriate amount of $(NH_4)_2SO_4$ to dissolve any proteins that are prone to $(NH_4)_2\ SO_4$ precipitation and can be collected after centrifugation in the form of a pellet. This study shows that the highest total protein content was obtained at 100 % ammonium sulphate saturation. Winarti et al. (2018) and Bouhlel et al. (2021) have mentioned that protease enzyme worked well with ammonium sulphate precipitation at high saturation. This occurs because salt ions compete with enzyme proteins for water molecules. As salt ions attract water, they pull water away from the proteins, causing the proteins to precipitate out of solution. This increases the specific activity of the enzyme, as the proteins become more concentrated and purified (Scopes, 1993; Wingfield, 2001; Wardani & Nindita, 2012; Winarti *et al*., 2018).

Starting with a crude extract (2.62 mg total protein, 2.37 U/mg specific activity), initial precipitation at 20 % saturation resulted in decreased protein concentration and activity, with a lower specific activity of 1.75 U/mg and 53.23 % yield. This suggests some protein loss or inactivation at lower saturation levels. At 40 % saturation, the specific activity increased to 1.96 U/mg with a higher yield (69.35 %), indicating more effective retention

of active proteins. The 60 % saturation step was optimal, yielding a specific activity of 2.21 U/mg and a high activity yield of 88.71 %. Balancing purity and yield were crucial, as higher ammonium sulphate saturation levels, like 80 % and 100 %, resulted in increased specific activity (3.83 U/mg and 12.06 U/mg, respectively) but lower activity yields (30.48 % and 37.39 %). These high saturation levels might cause non-target protein precipitation or denaturation. Ideally, maintaining high activity yield alongside increasing specific activity ensures the enzyme is both pure and active for future applications (Wingfield, 2001; Bouhlel *et al*., 2021). This balance was effectively achieved during the 100 % ammonium sulphate saturation, where specific activity significantly increased while retaining a substantial portion of the initial enzyme activity. This indicates that the purification method was efficient in producing a highly active and pure protease.

Ammonium sulphate precipitation would end up with a significant amount of salt content in the protein. Consequently, the best way to remove $(NH_4)_2SO_4$ is through dialysis. This would be an important step to take in order to remove unwanted molecules and ammonium sulphate residue that would inhibit the protease enzyme activity (Belton *et al*., 1999; Wardani & Nindita, 2012). In this study, dialysis works by using a semi-permeable membrane that has a capability of separating proteins from unwanted molecules and foreign particles. The final dialysis step resulted in the highest specific activity (14.33 U/mg) and a fold purity of 6.05, with a satisfactory activity yield of 53.33 %. This indicates effective purification while maintaining functional protease. According to Bouhlel et al. (2021), such optimised purification is crucial for bioactive enzyme recovery. The specific activity of the protease improved from 12.06 U/mg to 14.33 U/mg after dialysis, indicating successful purification. The enzymatic activity of a protease ranges from less than 1 U/mg to over 80 U/mg, depending on its purity and source as stated by Troncoso et al. (2022), thus this result correlates with the statement where the enzyme activity falls within range. However, papain has a significantly higher specific

activity of 69.78 U/mg. This gap indicates that papain is about 4.87 times more active per milligram of protein than the purified protease. While the purification process effectively increased the protease's purity and activity, the enzyme is still less efficient compared to papain. This difference could be due to inherent variations in enzyme catalytic efficiency, the presence of residual impurities or differences in enzyme stability. Despite the lower specific activity, using papain as a standard validates the purification method and highlights the efficiency of the purification steps. Nevertheless, the considerable gap suggests there is room for further optimisation to enhance the specific activity of the purified protease.

Later on, determining the molecular weight of the protease will be essential for characterising the enzyme and verifying its purity. This information can help confirm the enzyme's identity and assess its homogeneity, which is crucial for understanding its functionality and potential in therapeutic application. Identifying the molecular weight also aids in comparing the protease with known standard, ensuring its suitability for specific applications such as inflammation treatment or protein digestion. Molecular weight of the purified *C. vespertilionis* protease (CVP) was determined using the sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and has shown a weight of 48 kDa. The molecular weight of CVP falls in the range of aspartic proteases, which is the most common forms of proteases in plants besides cysteine and serine, where the molecular weight spans within 35 to 65 kDa (Troncoso *et al*., 2022). Another plant that has protease that has similar molecular weight range to the CVP is *Moringa oleifera* protease with a molecular weight of 51 kDa (Wingfield, 2001).

The molecular weight of CVP was determined to be approximately 48 kDa, which is significantly larger than the commercially available protease papain, which has a molecular weight of approximately 23.4 kDa (Kaur *et al*., 2024). This difference in

molecular size may have several implications. While papain's smaller size is associated with its well-documented efficiency and versatility in various industrial and biomedical applications, the larger size of CVP could suggest different structural and functional properties. For example, CVP's larger molecular structure may result in distinct substrate specificities or interaction capabilities, potentially offering advantages in applications where enhanced stability or unique binding characteristics are beneficial (Schauperl *et al*., 2015). This comparison emphasises the importance of molecular weight in determining the suitability of a protease for specific uses, highlighting that while papain is effective in many scenarios, CVP may be more suitable for certain uses or applications requiring its particular properties such as stability, specificity or activity under different conditions. This could offer advantages over papain in specific contexts or treatments. For example, CVP might have better stability, specificity or activity in certain environments, making it preferable for specific therapeutic or industrial applications.

For further investigation, high-performance liquid chromatography (HPLC) was performed to confirm the presence of protease in purified *C. vespertilionis* (CVP). Gallic acid and quercetin were used as standards as it was proven to possess anti-inflammatory activities and has act in mechanisms of inflammatory responses. For example, gallic acid inhibits inflammatory enzymes such as cyclooxygenase-2 (COX-2) and lipoxygenase (LOX), which are involved in the production of pro-inflammatory mediators (Karamaae *et al*., 2005; Kaur *et al*., 2005; Nikolic, 2006; Singh *et al*., 2019; Tsiogkas *et al*., 2023). Meanwhile quercetin is a flavonoid, one of the phenolic compounds responsible for curing inflammation that controlling the generation of nitric oxide (NO) and tumour necrosis factor-alpha (TNF-α) in LPS-stimulated macrophages via the NF-κB and MAPKs signalling pathways (Nakamura & Omura, 2008; Chew *et al*., 2011; Xu *et al*., 2017; Lee *et al*., 2020). The HPLC analysis shows that CVP has unique chemical properties, as evidenced by its distinct retention time and area compared to standard

compounds like quercetin and gallic acid. This combination of molecular weight, enzymatic activity and unique chemical properties supports the conclusion that CVP is a distinct and active protease enzyme, suitable for various biochemical applications. The differences in chemical properties indicated by the HPLC results suggest that CVP has unique molecular interactions, which could be explored further to understand its potential applications and interactions in biological systems.

5.3 Anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in single and combination treatment in LPS-stimulated RAW264.7 macrophages

During inflammation, RAW264.7 macrophages have the capacity to detect, engulf, and eventually destroy pathogens through phagocytosis. However, due to the fact that these phagocytic cells are mostly connected with adverse-side effects of non-steroidal anti-inflammatory drugs (NSAIDs), novel anti-inflammatory from plant sources is widely discovered to treat inflammation (Adegbaju *et al*., 2020). Various plant treatments combination has also been shown to exhibit anti-inflammatory activity. Previous study showed that combination of andrographolide and diclofenac had superior pharmacologic effects (Tandoh *et al*., 2022). Another study showed that pinitol and glucosamine have an anti-inflammatory effect on acute and subacute level of conditions (Kim *et al*., 2005). To investigate anti-inflammatory effects of treatments, RAW 264.7, a murine macrophage cell line has been used in this study for *in vitro* biological evaluation. When looking into potential novel treatments or generating new therapeutic for certain condition, cytotoxicity testing is a crucial first step. This will enable the detection of any potential cytotoxic and negative consequences before proceed to further investigations (Lee *et al*., 2009).

The viability of cells is measured calorimetrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay that change colour in response to the viability of the cells by spectrophotometer (Präbst *et al*., 2017). To achieving the desired therapeutic impact, a goal of pharmacological combination therapy is to lessen the toxicity of the drugs. Therefore, anti-inflammatory effect of purified *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in single and combination treatment were measured on RAW264.7 macrophages using MTT assay with a commercial drug, aspirin was used as positive control.

The provided bar graphs (Figure 4.6) illustrate the effects of different concentrations of CVP, TRF, their combination, and aspirin on the cell viability of LPS-stimulated and unstimulated cells. The data reveal that CVP alone, at concentrations ranging from 5 to 30 µg/mL, generally maintains cell viability above 80 % for both LPS-stimulated and unstimulated cells. This aligns with prior studies showing that phytochemicals like CVP can exhibit protective or cytotoxic effects depending on dosage and cell type, similar to findings with flavonoids known for their anti-inflammatory and antioxidant properties, yet potential cytotoxicity at higher concentrations (Al-Khayri *et al*., 2022). TRF, on the other hand, shows a significant increase in cell viability, especially at concentrations of 10 to 20 µg/mL, where viability exceeds 150 % for unstimulated cells and 120 % for stimulated cells. This is consistent with documented potent antioxidant and anti-inflammatory properties of tocotrienol-rich fractions (TRF), which protect cells from oxidative stress-induced cytotoxicity and enhance cell viability (Selvaraju *et al*., 2014).

The combination of CVP and TRF appears to improve cell viability notably, suggesting a possible synergistic effect. This observation is supported by studies on combination therapies using antioxidants and anti-inflammatory agents, which often show enhanced protective benefits and minimised potential cytotoxicity (Talib *et al*.,

2022). Aspirin shows a modest effect on cell viability, with significant increases at lower concentrations (5-10 µg/mL) but no drastic cytotoxic effects even at 100 µg/mL. The results suggest that both CVP and TRF, alone and in combination, can modulate cell viability in LPS-stimulated and unstimulated cells, with the combination therapy being particularly effective due to synergistic interactions enhancing cell protection and viability. This is consistent with previous research on the benefits of combining antioxidants and anti-inflammatory agents for better therapeutic outcomes with reduced cytotoxicity (Mucha *et al*., 2021).

Cell viability of these treatments suppressed above 80 %, which means no toxicity had occurred and the concentrations for the respective treatments can be used for the test of nitric oxide production. Plant sources of therapeutics are considered cytotoxic if they cause 50 % cell death in less than 72 hours *in vitro*, as determined by Adegbaju et al. (2020) and Vijayarathna & Sasidharan (2012). Therefore, the non-toxic nature of CVP, TRF, and their combination (CVP+TRF) suggests they are safe to use for further experiment which is nitric oxide production assay. Nitric oxide (NO) is one of the cellular mediators released by activated macrophages secreted at inflammatory sites which occurs in cardiovascular, nervous and immunological systems (Muniandy *et al*. 2018; Rao *et al*., 2016; Ren *et al*., 2019). According to Jung et al. (2014), excessive NO production by iNOS might result in harmful effects such as septic shock, arteriosclerosis, ischemic reperfusion, hypertension and other inflammatory disorders, indicating that some inflammatory disorders could be treated by reducing NO production. Therefore, inhibiting excessive nitric oxide (NO) may be a useful tactic for treating inflammatory diseases and is considered as a way to monitor the improvement of inflammatory sites (Shao *et al*., 2013; Xu *et al*., 2017).

The macrophage is stimulated by lipopolysaccharide (LPS), a component of Gram-negative bacteria's cell walls, and is widely used in inflammatory response (Xu *et al*., 2017). LPS stimulation triggers the production of pro-inflammatory mediators, including nitric oxide (NO), making it a valuable tool for assessing the anti-inflammatory potential of therapeutic agents. The purpose of using LPS in this study is to induce NO production in macrophages, allowing evaluation of the effects of purified *C. vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF) and their combination (CVP+TRF) on NO levels. By examining how these agents influence NO production in an LPS-stimulated inflammatory environment, we can better understand their potential as anti-inflammatory therapeutics. The RAW264.7 cells, a macrophage cell line, typically exhibit a spherical form with smooth edges and no pseudopodia under normal conditions. However, upon exposure to lipopolysaccharide (LPS), these cells undergo significant morphological changes to flat, elongated, cell nucleus enlarges, pseudopodia presented and irregular shape, as shown by microscope observation. These morphological changes are indicative of macrophage activation and an inflammatory response, validating the use of LPS to study NO production and the effects of potential anti-inflammatory agents like CVP, TRF and their combination. The observed changes in cell morphology confirm the successful stimulation of macrophages, which is crucial for accurately assessing the modulation of NO production by these therapeutic compounds.

NO production in LPS-stimulated cells without CVP is set at 100 %. The addition of CVP at concentrations of 5, 10, 15, 25, and 30 µg/mL reduces NO production to 94.74 %, 82.46 %, 68.42 %, 54.39 % and 49.12 % respectively. Non-stimulated cells show a baseline NO production of 19.29 %. CVP exhibits a dose-dependent inhibition of NO production in LPS-stimulated RAW264.7 cells. This suggests that CVP can effectively downregulate the inflammatory response, likely through its anti-inflammatory and antioxidant properties. The significant reduction in NO levels at higher concentrations of

CVP indicates its potential as a strong anti-inflammatory agent. This result aligns with previous findings that phytochemicals can modulate inflammatory responses by inhibiting iNOS expression and subsequent NO production (Al-Tarifi *et al*., 2022). NO production in LPS-stimulated cells without TRF is set at 100%. The addition of TRF at concentrations of 5, 8, 10, 15, and 20 µg/mL reduces NO production to 96.49 %, 87.72 %, 84.21 %, 66.67 % and 50.88 % respectively. Non-stimulated cells show a baseline NO production of 19.30 %. TRF also shows a dose-dependent reduction in NO production in LPS-stimulated RAW264.7 cells. This indicates that TRF can effectively attenuate the inflammatory response, consistent with its known antioxidant and anti-inflammatory properties. The significant reduction in NO levels at higher concentrations of TRF suggests its potential as a potent anti-inflammatory agent. The ability of TRF to lower NO production aligns with previous studies demonstrating the efficacy of palm tocotrienol-rich fractions in modulating oxidative stress and inflammation (Xu *et al*., 2017). Both CVP and TRF, individually demonstrate significant, dose-dependent reductions in NO production in LPS-stimulated RAW264.7 cells, indicating their potential as effective anti-inflammatory agents.

The combination of CVP and TRF at various concentrations (5:5, 10:8, 15:10, 25:15, 30:20 µg/mL) demonstrate a decrease in NO production, indicating a potential inhibitory effect. Similar trend occurred with aspirin at concentrations of 5, 10, 25, 50 and 100 µg/mL. The presence of LPS significantly increases NO production, but the combination and aspirin reduce NO levels in a dose-dependent manner. These findings suggest that the combination (CVP+TRF) and aspirin effectively modulate inflammatory responses by inhibiting NO production, aligning with previous research (Tsai *et al*., 2006; Xu *et al*., 2017). The combination of CVP and TRF exhibited the strongest inhibitory effect on NO production compared to individual compound due to potential synergistic interactions (Wang *et al*., 2020). CVP and TRF likely work through different biochemical pathways

to inhibit NO production, enhancing each other's efficacy. CVP's anti-inflammatory and antioxidant properties can inhibit nuclear factor-kappa B (NF-κB) activation and downregulate inducible nitric oxide synthase (iNOS), while tocotrienols also possess antioxidant properties that inhibit inflammation through other signalling pathways. Therefore, these findings highlight the therapeutic potential of CVP and TRF in combination showing a significantly greater reduction of NO production compared to each compound alone.

The synergistic effect observed between purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in inhibiting nitric oxide (NO) production suggests that these compounds complement each other's mechanisms of action, enhancing their overall efficacy. This synergy likely results from optimal combination at a ratio of 14.10 µg/mL CVP to 8.48 µg/mL TRF effectively reduces nitric oxide (NO) levels. This reduction is crucial as it signifies enhanced therapeutic efficacy due to each compound's unique properties are maximised (Singh & Yeh, 2017). CVP and TRF may interact at molecular or cellular levels, improving uptake or activation, thus amplifying their anti-inflammatory effects. Additionally, their combined use could lead to better pharmacokinetic profiles, ensuring effective concentrations at the target site. This combination not only reduces NO levels significantly but also addresses potential toxicity and resistance issues, making it a promising approach for treating inflammation. The combination of CVP and TRF demonstrates a more pronounced inhibition of NO production compared to individual treatments, as shown in the isobologram with a combination index (CI) of 0.89, indicating synergy due to CI < 1 (Chou & Talalay, 1984; Zhao *et al*., 2010).

This reinforces previous studies indicating that excessive nitric oxide (NO) production contributes to inflammatory damage, and interventions targeting NO can reduce

inflammation. Furthermore, combining diverse treatments containing various anti-inflammatory phytochemicals can synergistically modulating immune responses and reducing inflammatory mediators (Chiou & Pan, 2018; Kwak *et al*., 2020). This approach of combining complementary mechanisms can enhance therapeutic outcomes in inflammatory conditions. Thus, the observed synergy suggests that CVP and TRF may enhance each other's mechanisms of action, leading to improved efficacy and reduced toxicity, highlighting their potential as a combination therapy for inflammatory diseases. It is proven by the combination of CVP and TRF demonstrates a synergistic interaction, achieving a combination index (CI) of 0.89 and more than 50 % NO inhibition, highlighting their enhanced effectiveness when used together compared to individually. Therefore, this dose combination was selected and can be further use for *in vivo* studies to understand more about their mechanism in reducing inflammation (Pöch *et al*., 1990).

5.4 Anti-inflammatory effects of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in single and combination treatment in carrageenan-induced BALB/c paw oedema

Testing the selected dose combination *in vivo* is crucial for several reasons. Firstly, *in vitro* studies provide valuable initial insights into efficacy and mechanisms of action, but they cannot fully replicate the complexities of biological systems found in living organisms. Thus, *in vivo* studies allow to assess how the dose combination behaves in a whole organism, considering factors such as metabolism, distribution and potential side effects that may not show through *in vitro*. Secondly, *in vivo* studies provide essential data on pharmacokinetics and pharmacodynamics, helping to optimise dosing regimens for maximum therapeutic benefit while minimising adverse effects. Thirdly, these studies are pivotal for translating promising *in vitro* results into clinically relevant applications, establishing the groundwork for potential therapeutic use in human subjects. Therefore,

conducting *in vivo* studies is crucial to validate and advance the potential of the selected dose combination for practical therapeutic applications.

In vivo study involves testing carrageenan injection in paw to induce oedema. Oedema produce swelling causes by excess fluid in the tissues. From swelling to responses of inflammatory mediator's exhibitions that cause pain (Punchard *et al*., 2004). This method which is carrageenan-induced BALB/c paw oedema is a simple and common method to investigating new anti-inflammatory treatments in acute inflammation (Sini *et al*., 2010). In recent years, novel anti-inflammatory medications have frequently been evaluated using the mouse oedema model, particularly BALB/c that is albino and have pink eyes with white skin hair. Injecting carrageenan into hind paw of BALB/c caused a time-dependent development of thermal hyperalgesia (severe pain) along with oedema and neutrophil infiltration in the paw tissues (Finley *et al*., 2013). Carrageenan-induced paw oedema develops in two phases (biphasic). Histamine, serotonin and prostaglandin are the main mediators in the early stage (1-2 h) in the injured tissues of the carrageenan model. The damaged tissues is then reduced, maintained and controlled by tissue macrophages, bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins in the late phase (Brito & Antonio, 1998; Finley *et al*., 2013).

In this study, carrageenan was used to induced oedema in the left hind paw of BALB/c mice, as an activator of neutrophils that produce inflammatory mediators which can be indicator of the level severity of the inflammation. Therefore, this study revealed anti-inflammatory activity of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), single and combination treatment in carrageenan-induced BALB/c paw oedema model. Based on the results from the two-week study on BALB/c mice, the administration of oral doses of non-carrageenan, carrageenan, aspirin, CVP, TRF and the combination of CVP and TRF did not adversely affect the mice's body

weight or indicate signs of toxicity. Throughout the study period, there was a consistent increase in body weight across all treatment groups, suggesting that none of the treatments led to significant loss of appetite or adverse health effects. These findings support the safety of the administered doses, as evidenced by the body weight measurements and the confirmation that the lethal dose (LD50) for all treatments exceeded 100 mg/kg (Ahmad Sayuti *et al*., 2021).

Further research should aim to study the carrageenan-induced paw oedema model to evaluate the anti-inflammatory efficacy of CVP and TRF, both individually and in combination. This model allows for the quantification of paw swelling as a measurable endpoint, making it possible to assess the degree of inflammation and the effectiveness of the treatments in reducing it. By comparing the effects of CVP, TRF and their combination against a control group and a standard anti-inflammatory drug like aspirin, we can determine the relative efficacy of these compounds. Other than that, this study also allows to understand their mechanisms of action, and explore the potential for synergistic interactions between them. The findings from this model can serve as a foundation for further research, including chronic inflammation studies and clinical trials, aimed at developing effective treatments for human inflammatory diseases.

The results presented in Figure 4.11 highlight the significant anti-inflammatory effects of aspirin, CVP, TRF and their combination on carrageenan-induced paw oedema in a BALB/c mice model. Aspirin, a well-known anti-inflammatory drug, consistently reduced paw oedema size, confirming its efficacy (Fehrenbacher *et al*., 2012; Tandoh *et al*., 2022). Both CVP and TRF also demonstrated notable reductions in inflammation, particularly in the later hours of the experiment (Karthikeyan & Deepa, 2011). However, the combination of CVP and TRF was the most effective treatment, showing the greatest reduction in paw oedema size and suggesting a synergistic interaction between the two

compounds. This combination surpassed the efficacy of each compound alone and even outperformed aspirin, particularly in the later stages of the experiment. The anti-inflammatory mechanisms of CVP and TRF have been explored in previous studies. CVP is known to inhibit the production of pro-inflammatory cytokines such as TNF-α and IL-6. Meanwhile, TRF inhibits the production of pro-inflammatory mediators like prostaglandins by downregulating the activity of cyclooxygenase-2 (COX-2). The observed synergistic effect of CVP and TRF combination can be attributed to their complementary mechanisms of action (Rocha *et al*., 2006; Saleem *et al*., 2011). While CVP targets the inflammatory signalling pathways, TRF reduces oxidative stress and directly inhibits the pro-inflammatory mediators. This dual-action approach could result in a more comprehensive suppression of the inflammatory response, leading to a significant reduction in paw oedema (Liu *et al*., 2012). Regarding potential molecular mechanisms in carrageenan induction, there is proof that polysaccharides derived from seaweed can directly control the immune response by binding to pattern recognition receptors (PRRs), such as mannose and toll-like receptors (TLR) in macrophages (Cunha & Grenha, 2016). Lopes et al. (2020) stated that TLR4/CD14/MyD88 signalling is required for carrageenan to induce TNF production which is one of the inflammatory mediators that controlling inflammation. Myers et al. (2019) also indicated that activation of TLR2/6 and TLR4/6 are the major pathways by which carrageenan induces inflammatory responses.

Next, it is important to investigate the inflammatory mediators released after carrageenan injection in BALB/c mouse model. When mice's hind paws are injected with carrageenan, the mice develop immune response that includes paw oedema swelling, neutrophil migration, release of pro-inflammatory mediators and discomfort (Silva *et al*., 2010; Zarpelon *et al*., 2013; Tandoh *et al*., 2022). Recent studies have demonstrated that the CVP and TRF are effectively inhibit the inflammatory process by affecting different

inflammatory mediators and cytokines including inducible nitric oxide, TNF-α and IL-6. These mediators have been acted as indicator for the inflammatory effects in carrageenan-induced paw oedema and the severity of inflammation can be easily discovered (Barrot, 2012; Gregory *et al*., 2013; Necas & Bartosikova, 2013; Tsai *et al*., 2015; Zeng *et al*., 2021; Tandoh *et al*., 2022).

Tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are cytokines or inflammation markers that shows a significant increased indicating inflammation in carrageenan group. Treatments like aspirin, CVP, TRF and in combination (CVP+TRF) reduced cytokines levels, with CVP+TRF showing the greatest reduction. Aspirin, a known anti-inflammatory standard, reduces cytokine levels by inhibiting the cyclooxygenase (COX), reducing prostaglandin synthesis and lowering TNF-α and IL-6 levels (Tandoh *et al*., 2022; Arif & Aggarwal, 2023). CVP and TRF appear to have similar effects, potentially through antioxidant properties or modulation of inflammatory signalling pathways. Combining CVP and TRF may enhance their anti-inflammatory effects, suggesting a synergistic mechanism. CVP and TRF are rich in polyphenols and tocotrienols, may exert their effects by reducing oxidative stress, indirectly decreasing cytokine production through modulation of NF-κB signalling pathways (Saukkonen *et al*., 1990; Wright, 1997; Mazzon *et al*., 2008; Wu *et al*., 2008; Xu *et al*., 2012; Budin *et al.*, 2013; Lee *et al*., 2020).

The combination of CVP and TRF appears to have a synergistic effect, further enhancing these anti-inflammatory outcomes, as shown by the significantly lower cytokine levels of TNF-α and IL-6 in the combination group compared to individual treatments. This supports previous studies suggesting that combined antioxidants and anti-inflammatory compounds can more effectively reduce inflammation, providing valuable insights into potential therapeutic strategies (Mucha *et al*., 2021).

Following the analyses, nitric oxide (NO) has been identified as the next key inflammatory mediator to evaluate. Nitric oxide is a critical mediator in the inflammatory response, produced by inducible nitric oxide synthase (iNOS) in response to inflammatory stimuli such as lipopolysaccharides (LPS) and cytokines. Investigating NO levels will provide further insights into the anti-inflammatory mechanisms to treat inflammation. Nitric oxide (NO) is implicated in the early phase of the acute inflammatory response following the injection of carrageenan into the hind paw (Salvemini *et al*., 1996; Salvemini & Masferrer, 1996; Necas & Bartosivoka, 2013). In the pathophysiological condition that related to inflammation, nitric oxide which is a free radical has been involved with cell damage in the liver (Huang *et al*., 2010). Therefore, inhibiting free radicals such as hydroxyl radicals (*OH) and peroxynitrogen ($ONOO^-$) can decrease the severity of inflammation (Bogdan *et al*., 2000; Salvatore *et al*., 2001).

The significant reduction of NO levels by CVP (4.30 ± 0.19 μM) suggests its potent anti-inflammatory activity. Previous studies on proteases from medicinal plants indicate that these enzymes can modulate inflammatory pathways, potentially by inhibiting the activity of pro-inflammatory cytokines or by directly degrading inflammatory mediators (Mohd Fauzi *et al*., 2021). TRF, a rich source of tocotrienols, demonstrates an even more substantial reduction in NO levels (2.69 ± 0.19 μM). Tocotrienols are known for their antioxidant and anti-inflammatory properties can scavenge free radicals, suppress the expression of pro-inflammatory mediators, inhibit the activation NF-κB, and downregulate the expression of pro-inflammatory genes including iNOS (Ahmad *et al*., 2005; Ahn *et al*., 2007; Wu *et al*., 2008; Kuhad & Chopra, 2009). The potent effect of TRF may be attributed to its ability to modulate these pathways more effectively than CVP. The combination treatment with CVP and TRF results in the lowest NO level (2.15 ± 0.49 μM), indicating a possible synergistic effect. Synergy in anti-inflammatory treatments often arises from the complementary mechanisms of action of different agents

(Caesar & Cech, 2019). In this case, CVP's protease activity may enhance the bioavailability or effectiveness of tocotrienols from TRF, while TRF's antioxidant properties could reduce any oxidative stress induced by proteolytic activity. Additionally, the combined effects on multiple inflammatory pathways may lead to a more comprehensive suppression of inflammation (Zhao *et al.*, 2021). Aspirin, a well-known non-steroidal anti-inflammatory drug (NSAID), reduces NO levels to 4.62 ± 0.49 μM. Aspirin works primarily by inhibiting cyclooxygenase (COX) enzymes, reducing the production of prostaglandins, which are inflammatory mediators.

The greater efficacy of CVP and TRF compared to aspirin in this study suggests that targeting multiple inflammatory pathways such as proteolysis by CVP, antioxidant and gene modulation by TRF may offer superior anti-inflammatory effects than targeting a single pathway like COX inhibition by aspirin. The study's results show the potential of using natural compounds like CVP and TRF as alternatives or complements to conventional anti-inflammatory drugs. Their ability to significantly reduce inflammatory mediators and free radicals' production suggests that they could be effective in managing inflammatory conditions with fewer side effects than NSAIDs. Future research should focus on elucidating the precise molecular mechanisms of CVP and TRF, as well as conducting clinical trials to validate their efficacy and safety in humans. In conclusion, the significant reduction of TNF-*α*, IL-6 and NO levels by CVP and TRF, both individually and in combination, highlights their potential as powerful anti-inflammatory agents. These findings align with previous studies on the anti-inflammatory mechanisms of proteases and tocotrienols and suggest that their combined use may offer enhanced therapeutic benefits through synergistic interactions.

5.5 Synergistic anti-inflammation actions of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF).

The combination of purified *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) synergistically reduced inflammation, meaning their combined effect was greater than the sum of their individual effects. This synergy likely arises from their distinct phytochemicals, which have complementary anti-inflammatory properties, enhancing their overall efficacy.

Recently, the anti-inflammatory effects of *C. vespertilionis* have been studied by Lee et al. (2020). Author indicated that strong antioxidant suppresses the production of reactive oxygen species (ROS) and minimising cellular damage. Mutalib & Latip (2019) also showed that the combination of *C. vespertilionis* with other treatment could treat diseases particularly cancer, however no mechanisms have been investigated. The mechanisms underlying the anti-inflammatory effects of *C. vespertilionis* have indeed received less attention in scientific research compared to extensively studied plant-based treatments like palm tocotrienol-rich fraction (TRF). However, understanding TRF's interactions with inflammatory pathways could also uncover synergistic effects when combined with another compound like purified *C. vespertilionis* protease (CVP).

Previous study showed TRF inhibited pro-inflammatory cytokines, nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which was discovered to represent the molecular basis for its anti-inflammatory action (Wu *et al*., 2008). Combining palm TRF with astaxanthin has been studied, revealing synergistic effects in managing inflammation. Research has shown that this combination effectively inhibits inflammatory mediators such as NO, IL-6, IL-12p70 and TNF-α in RAW264.7 macrophages, surpassing the effects of palm TRF alone. These findings suggest that the synergistic anti-inflammatory mechanisms involve multiple pathways enhances the

effectiveness of treatment by targeting different aspects of the inflammatory process simultaneously (Radzun *et al*., 2022). These findings demonstrate that TRF's mechanisms of action, affecting LPS-stimulated RAW264.7 macrophages, contribute to the synergistic effects observed with the combination treatment of purified *C. vespertilionis* protease (CVP). The discovery of this synergistic action between CVP and TRF represents an innovative and successful approach for treating inflammation. In conclusion, the combination studies conducted in this research highlight the enhanced therapeutic potential achieved by combining treatments. These studies demonstrate synergistic effects that can lead to improved treatment efficacy and reduced side effects in managing inflammation. Therefore, future research should focus on investigating additional inflammatory mediators, including inflammatory genes, to further elucidate the underlying mechanisms involved in this potent combination therapy.

CHAPTER 6: CONCLUSION

In conclusion, the study on *Christia vespertilionis* leaf extract demonstrates its potential as a natural anti-inflammatory agent. Using the Soxhlet extraction method with ethanol, the extract yielded consistent and efficient results, indicating its effectiveness. Phytochemical analysis revealed significant amounts of phenolics (29.25 ± 0.50 mg GAE/mL), flavonoids (1.57 ± 0.03 mg QE/mL), and tannins (2.70 ± 3.15 mg TAE/mL), known for their antioxidant and anti-inflammatory properties. The protease inhibition assay showed an IC_{50} of 284.59 ± 10.63 µg/mL, comparable to aspirin, highlighting its potent anti-inflammatory potential. GC-MS analysis identified four key compounds which is phytol, squalene, 9,12,15-octadecatrienoic acid, (Z,Z,Z)- and n-hexadecanoic acid, each contributing to the extract's ability to reduce inflammation through various mechanisms. These findings suggest that *C. vespertilionis* has significant therapeutic potential, and has potential to further explore its use in developing treatments for inflammation-related conditions.

Based on the findings, the purification of *C. vespertilionis* of protease proved essential in isolating specific bioactive compounds, enabling a deeper understanding of their anti-inflammatory mechanisms. While the crude extract contained multiple bioactive components, purification allowed for enhanced specificity and activity of the protease, which showed promise and potential as a therapeutic agent. The ammonium sulphate precipitation method effectively concentrated the enzyme, yielding high specific activity, particularly at 100 % saturation, while subsequent dialysis further improved enzyme purity. This process highlighted the balance needed between purity and yield to retain enzyme functionality. The molecular weight of the purified protease (48 kDa) suggested it belongs to the aspartic protease family, with potential applications distinct from smaller enzymes like papain. The unique chemical properties identified via HPLC, compared to

standard anti-inflammatory compounds like quercetin and gallic acid, support the enzyme's potential in therapeutic contexts. Overall, it shows the importance of protease purification in harnessing its therapeutic potential, paving the way for further research into its applications in treating inflammation-related conditions.

The *in-vitro* study demonstrates that the combination of purified *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) effectively reduces inflammation by inhibiting nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages. Both CVP and TRF alone showed dose-dependent anti-inflammatory effects, but their combination (CVP+TRF) significantly enhanced these effects, likely due to synergistic interactions. The observed synergistic interaction, with a combination index (CI) of 0.89, supports the hypothesis that combining these compounds enhances their therapeutic efficacy and reduces potential toxicity. This combination not only maintained high cell viability but also resulted in a greater reduction of NO levels compared to individual treatments. The findings suggest that CVP and TRF could serve as promising candidates for developing novel anti-inflammatory therapies in treating conditions associated with excessive NO production and related to inflammatory diseases.

The *in-vivo* study demonstrates the significant anti-inflammatory effects of purified *C. vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF), individually and in combination, using a carrageenan-induced paw oedema model in BALB/c mice. The combination of CVP and TRF effectively reduced inflammatory markers such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and nitric oxide (NO) surpassing the effects of either agent alone and even outperforming aspirin, the standard synthetic drug in later stages. These findings suggest a synergistic interaction between CVP and TRF, where their complementary mechanisms of CVP's modulation of inflammatory signalling and TRF's antioxidant properties resulted in a comprehensive suppression of the

inflammatory response. These synergistic action of CVP and TRF enhances their therapeutic potential by targeting multiple inflammatory pathways simultaneously. This dual-action approach highlights the potential of CVP and TRF as safer alternatives to conventional non-steroidal anti-inflammatory drugs (NSAIDs), offering effective management of inflammation with reduced side effects. Future research should focus on *in vivo* pharmacokinetics, further elucidation of molecular mechanisms and clinical trials to validate the therapeutic potential of this combination in treating human inflammatory diseases. Overall, the study has proven the effectiveness of CVP and TRF in developing plant-based anti-inflammatory therapies, particularly in combination treatment. The promising results of this combination therapy provide a foundation for developing innovative treatments for inflammatory diseases.

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