Original Research Article

Anti-Inflammatory Activities of *Christia vespertilionis* Protease and Palm Tocotrienol-Rich Fraction in Carrageenan-induced BALB/c Paw Oedema

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ABSTRACT

Inflammation is a common ailment that could develop into a chronic disease such as cancer, diabetes and rheumatoid if not cured early. Commonly, synthetic drugs such as aspirin are used to cure inflammation. However, synthetic drugs could have adverse side effects with long-term consumption. This study investigates the anti-inflammatory activities of *Christia vespertilionis* protease (CVP) and palm tocotrienol-rich fraction (TRF) in carrageenan-induced BALB/c paw oedema. Carrageenan was injected into the left hind paw of BALB/c mice, generating an acute inflammatory response due to the induction of neutrophils. Inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and free radical nitric oxide (NO) were determined by enzyme-linked immunosorbent assay (ELISA). All parameters of inflammation such as paw size (%), amount of TNF-α (pg/mg), amount of IL-6 (pg/mg) and level of nitric oxide (µM), decreased through the treatments of CVP, TRF, a combination of CVP and TRF (CVP+TRF) and aspirin. The treatments were shown to be effective against inflammation, particularly CVP+TRF compared to aspirin, which could provide a novel therapeutic approach to inflammatory diseases.

Keywords: anti-inflammatory activities, carrageenan-induced BALB/c paw oedema, *Christia vespertilionis*, palm tocotrienol-rich fraction.

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1.0 Introduction

Inflammation is a common disease but can cause death at a chronic stage. Three out of five people worldwide die from chronic inflammatory diseases such as stroke, chronic respiratory disease, heart disorders, cancer, obesity and diabetes (1-3). Inflammation can be described as an infection or the irritation of living cells or tissues produced by an injury and their immune response (4). The body automatically releases cytokines, chemokines, prostaglandins and histamine as a natural defence called the chemotaxis mechanism (5). The injured tissue increases vascular permeability, enhances protein denaturation and builds membrane alteration. As blood vessels become dilate and permeable, neutrophils and macrophages migrate to the affected area to engulf and destroy foreign particles like pathogens, as well as to repair the damaged tissues (6).

Non-steroidal anti-inflammatory drugs (NSAIDs) and steroids are two types of drugs currently being used clinically to treat inflammation. However, these drugs produce adverse side effects such as gastric intestinal mucosa, heart illness and kidney disorders (7). Furthermore, drugs such as aspirin and steroids are only effective for one-time consumption in relieving symptoms (8). Thus, plant-derived drugs used as medicine since time immemorial can be alternative treatments. *Christia vespertilionis*, or butterfly wing, or 'daun rama-rama' as it is commonly called in Malaysia, has been reported for its anti-cancer and anti-inflammatory properties (9). 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 (10). This plant believes to having various medicinal values and emerging well to this date which the significant reasons for choosing this plant (11).

Numerous investigations have demonstrated that *C. vespertilionis* contains phytochemical substances such as phenols, alkaloids, triterpenes, fatty acids, alkanes and long-chained alcohols (12, 13). Flavonoid also most of the common compound found in *C. vespertilionis* extract that has been proven its anti-inflammatory properties that can decrease the activity of cytokines, chemokines and inflammatory enzymes (10, 14). 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 by catalysing the hydrolysis of peptide bonds. Protease is important for biological functions, including infection and physiological processes. It has proven from previous study that protease decreased paw oedema size significantly in carrageenan-induced acute inflammation model, which success to treat inflammation (15). In extracting protease from *C. vespertilionis*, 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 (16). Subsequently 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 (17).

Purified compounds are often study because they can interact synergistically with other compound to produce therapeutic effects (18). 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. Tocotrienol and tocopherol are vitamins E, which consist of four different isomers. These isomers are primarily found in vegetable oils, palm oil, rice bran oil, wheat germ, barley, seeds, nuts and grains. However, tocotrienol with a complete isoform, known as a tocotrienol-rich fraction (TRF), can only be found in palm oil initially extracted from the fruit of *Elaeis guineensis* (19). Studies have shown that TRF possesses antioxidant (20), anti-cancer (21) and anti-inflammatory (22, 23) activities. TRF has been explored for their biological activities including anti-inflammation and anti-oxidant (22, 24), and the outcome promises that more can be discovered about its therapeutic mechanisms.

Proteases derived from plants, such as bromelain from pineapples and papain from papayas, have been widely researched for their anti-inflammatory effects. These plant proteases reduce inflammation by breaking down pro-inflammatory molecules and modulating immune responses, effectively inhibiting pro-inflammatory cytokines and mediators like NF-κB and TNF-α. They have been used to treat conditions such as arthritis, muscle pain and inflammatory bowel diseases, offering a natural method for managing inflammation (17). Similarly, protease extracted from *Christia vespertilionis* (CVP) has shown notable anti-inflammatory activity. The CVP was optimised for purification and tested on LPS-stimulated RAW264.7 cells, where it demonstrated a 45.6% reduction in inflammation with an IC_{50} value of 19.24 μg/mL. This confirms its potential as an anti-inflammatory agent (25). Comparatively, tocotrienol-rich fraction (TRF) also exhibits significant antioxidant and anti-inflammatory properties. It highlights their roles in preventing and treating chronic diseases by reducing inflammation and oxidative stress (26). Both CVP and TRF represent promising candidates for developing natural anti-inflammatory treatments, given their ability to modulate inflammatory mediators effectively.

Inflammatory mediators can be a signal of the severity level of the inflammation. Tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and free radicals of nitric oxide (NO) are part of these mediators in pathogenesis inflammation (27). These inflammatory responses against pathogens such as bacteria, viruses, fungi and parasites during inflammation involve various pathophysiological processes such as neuronal communication, vasodilatation and neurotoxicity (28-31). Therefore, the level of these inflammatory mediators was also examined in inflammation models, the carrageenan-induced BALB/c paw oedema. The reason for using BALB/c is because this mouse model is believed to have similar immune response as humans, and it is widely used for animal inflammation testing (32, 33). Carrageenan-induced BALB/c paw oedema is a simple method of determining anti-inflammatory activities and evaluating inflamed paws (34). Carrageenan is a polymer derived from red seaweeds, particularly *Chondrus crispus*, and was used in this study as an activator of neutrophils, which produce acute inflammatory mediators. Previous study show that atrial natriuretic peptide (ANP) inhibits inflammation in the carrageenan-induced paw oedema model by reducing pro-inflammatory cytokines (IL-1, IL-6, TNF-α and PGE2) through the GC-A/NPRA modulated signalling pathway (6). *C. vespertilionis* has been confirmed to be an

anti-inflammatory agent when it has an inhibitory effect on monocyte adherence to endothelial cells (23, 35). Tocotrienol-rich fraction also suppressed pro-inflammatory cytokines, nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in human monocytic (THP-1) cells (22, 36). Thus, the present work aims to examine the anti-inflammatory activities of *C. vespertilionis* protease and palm TRF in carrageenan-induced BALB/c paw oedema.

2.0 Materials and methods

2.1 Plant sample

Christia vespertilionis was bought from Floranika Nursery Sungai Buloh, Selangor, Malaysia. Its voucher specimen was certified by Dr. Yong Kien Thai from the Plant Taxonomy Department, Rimba Ilmu, Universiti Malaya. The voucher specimen (KLU 50026) was placed at the herbarium of Universiti Malaya.

2.2 Chemicals and reagents

Analytical grade ammonium sulphate and sodium phosphate were acquired from R&M Chemicals (Malaysia). TRF was obtained from Gold Tri.E 70 (Sime Darby, Malaysia) 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 (*Elaeis Guineensis*). Carrageenan and aspirin were purchased from Sigma-Aldrich (Germany). A radioimmunoprecipitation buffer was acquired from Solarbio Life Sciences (China). TNF-α and IL-6 were obtained from R&D Systems (USA). Chemicals and reagents for nitric oxide (NO) determination were purchased from Sigma-Aldrich.

2.3 Protease extraction and purification from C. vespertilionis leaf extract

Based on Sharmila *et al*. (2012) (37) with some modifications, fresh leaves (20 g) were washed thoroughly with tap water and ground with 500 ml of pre-chilled 0.1 M sodium phosphate buffer at pH 7. The crude extracts were filtered through cheesecloth to remove the suspension. Then it was centrifuged at 9000 rpm at 4℃ for 15 min to remove impurities. The supernatant was collected and stored at 4℃ while awaiting further tests.

With some modifications, the ammonium sulphate precipitation method was performed using the method of Park *et al*. (2015) (38). Ammonium sulphate precipitation was performed as follows. In total, 34.85 g of ammonium sulphate and 50 ml of crude extract were stirred together overnight at 4°C until homogenous. Then centrifugation was done at 9000 rpm for 15 min at 4°C. The pellet was collected and resuspended in 10 ml pre-chilled 0.1 M sodium phosphate buffer at pH 7.

Next, dialysis was performed by pipetting the mixture into a dialysis membrane placed in a beaker containing 300 ml of pre-chilled 0.1 M sodium phosphate buffer (pH 7) which was stirred by a magnetic stirrer. The buffer was changed every 2 hr for 4 hr and dialysis was run overnight to ensure complete removal of ammonium sulphate salt. The purified sample was centrifuged at 9000 rpm for 15 min at 4°C and the supernatant was collected and kept at 4°C.

2.4 Preparation of palm TRF and aspirin

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. Aspirin (0.1g) was diluted with 10 ml of distilled water.

2.5 Experimental animals

The experiments were conducted according to the ethical norms approved by the Institutional Animal Care and Use Committee, Universiti Malaya (UM IACUC) (Approval no: S/19052022/07122021-02/R). The animals used in the experiments were female BALB/c mice (n=18) supplied by the Laboratory Animal Centre, Animal House, Ladang Mini, Institute of Biological Sciences, Universiti Malaya, Kuala Lumpur. A total of 18 female BALB/c mice were equally divided into six groups. According to new research by Rutherford (39), female mice have higher levels of the pain receptor Tlr7, which may have distinct immune responses of inflammation. The animals were acclimatized for two weeks in a controlled environment of 20-25ºC and 12-hr light/12-hr dark cycle and provided with commercial pellets (Altromin, Germany) and water. The groups were presented as non-carrageenan, carrageenan (control), aspirin, *C. vespertilionis* protease (CVP), palm TRF and a combination of CVP and TRF (CVP+TRF).

2.6 Acute toxicity study

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 (2008) (40), 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. The chosen dose range of 100 to 200 mg/kg body weight was selected to assess the acute toxicity of aspirin, CVP, TRF and CVP+TRF, ensuring a broad spectrum that covers potentially therapeutic to higher, potentially toxic levels, allowing for a comprehensive evaluation of safety margins. 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.

2.7 Carrageenan-induced paw oedema

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) (41), 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, 200 mg/kg CVP, 200 mg/kg TRF and 100 mg/kg CVP+TRF were administered through oral gavage one hour before the carrageenan

injection. The carrageenan group was given 100 µl of distilled water as a control because it has no active effects. This ensures that any changes seen in the treatment groups are caused by the substances being tested, not the water itself. Additionally, a separate group without carrageenan injection was included to assess the effects of the treatments under non-inflammatory conditions. 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:

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

a = thickness of left hind paw after carrageenan injection
b = thickness of left hind paw before carrageenan injection

The animals were then euthanised with ketamine (240 mg/kg) and xylazine (30 mg/kg) through intraperitoneal (IP) injection. The carrageenan-induced left hind 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.

2.8 Extraction of paw tissue

The paw tissue was hardened with liquid nitrogen and crushed into pieces using mortar and pestle. A total of 1g of tissue was homogenized with 10 ml of the radioimmunoprecipitation buffer. The mixture was mixed well with a vortex and incubated on ice for 30 min. The samples were then centrifuged at 3000 rpm for 20 min at 4ºC. The supernatant was collected and kept at -20ºC.

2.9 Measurement of TNF-α and IL-6

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.

2.10 Measurement of nitric oxide

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 (42, 43). 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 37ºC 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 min. 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.

2.11 Statistical analysis

The Graph Pad Prism (version 8.0.2 for Windows, GraphPad Software, San Diego, California, USA) was used for the data analysis. All data were expressed as mean ± standard deviation. The statistical significance/comparative analysis between control and treated groups was analysed using a one-way analysis of variance (ANOVA) followed by the Holm-Sidak multiple t-test and Dunnett's test. $P < 0.05$ was considered statistically significant.

3.0 Results

3.1 Protease purification from Christia vespertilionis leaf extract

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 1, the crude extract served as the baseline for comparison, with initial measurements of protein concentration, activity and purity. At 100% saturation, there was a notable increase in total protein and enzyme activity, leading to a higher specific activity, fold purity, and a decrease in activity yield. The final dialysis step further concentrated the protease, significantly increasing its purity and activity, demonstrating successful purification and concentration of the enzyme. 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.

3.2 Acute toxicity and body weights of all treatments of BALB/c mice

BALB/c mouse was used for this study due to similarity of immunological response to human being and commonly used to testing treatments for various diseases. BALB/c was used to test anti-inflammatory effects of *Christia vespertilionis* protease (CVP), palm tocotrienol-rich fraction (TRF) and combination (CVP+TRF) by induced the carrageenan in the paw oedema. First, BALB/c mice were administered orally with the respective treatment each day for 2 weeks along the measurement of body weight each week in 2 weeks observation. Based on Table 2, six groups of treatment were non-carrageenan, carrageenan (control), aspirin, CVP, TRF and CVP+TRF. The weight for each group increased by week. During week 1, the weights varied slightly across the different groups. By week 2, an overall increase in weight was observed across all groups. 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 1: 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 (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

Table 2: 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.

3.3 The paw oedema size after carrageenan injection

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 1 shows the comparison of the paw images before and after carrageenan injection, and also after the treatment with CVP and TRF. It 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. 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. The treatment with CVP and TRF (CVP+TRF) resulted in a reduction in swelling and redness compared to Figure 1(b), suggesting that the treatment effectively mitigated the inflammatory response. Reduction in oedema is an indication of the anti-inflammatory effects of CVP and TRF, which may act by modulating key inflammatory pathways involved in carrageenan-induced inflammation.

Figure 2 and Table 3 show 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 hour after the carrageenan injection. The carrageenan control group exhibited a gradual increase in paw oedema over time. In contrast, treatment with CVP resulted in a notable reduction in paw oedema, showing a consistent anti-inflammatory effect. TRF treatment also demonstrated a reduction in oedema, particularly at later time points. The combination of CVP and TRF (CVP+TRF) produced significant anti-inflammatory effects, reducing oedema further. Aspirin, used as a positive control, consistently reduced paw oedema throughout the study, comparable to the treatment groups. 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, showing a faster decline in oedema size. Both CVP and TRF treatments also reduce the paw oedema size like 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 percentage and fastest decline in oedema size. The result indicates that all treatments (aspirin, CVP, TRF and CVP+TRF) reduced paw oedema to varying degrees, with CVP+TRF being the most effective. This suggests that combining these compounds could be a promising synergistic interaction and better approach for managing inflammation.

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

Tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels are essential to compare each treatment group to the non-carrageenan control (baseline) and the carrageenan group (inflammation benchmark). As shown in Figure 3, TNF-α and IL-6 were measured as indicators of inflammation, with significant increases observed in the carrageenan group, confirming successful induction of inflammation. Treatments with aspirin, CVP, TRF and the combination of CVP+TRF effectively reduced TNF-α and IL-6 levels, demonstrating their anti-inflammatory effects. Notably, the combination of CVP and TRF resulted in the most substantial reduction, particularly in TNF-α, suggesting a synergistic effect between the two treatments. These findings

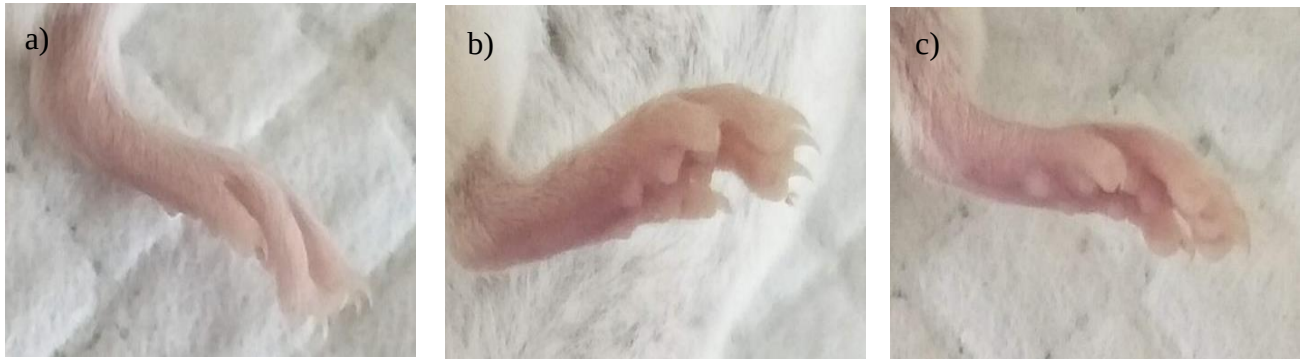

Figure 1: BALB/c's paw a) before carrageenan injection, b) after carrageenan injection, and c) after the treatment with CVP and TRF (CVP+TRF)

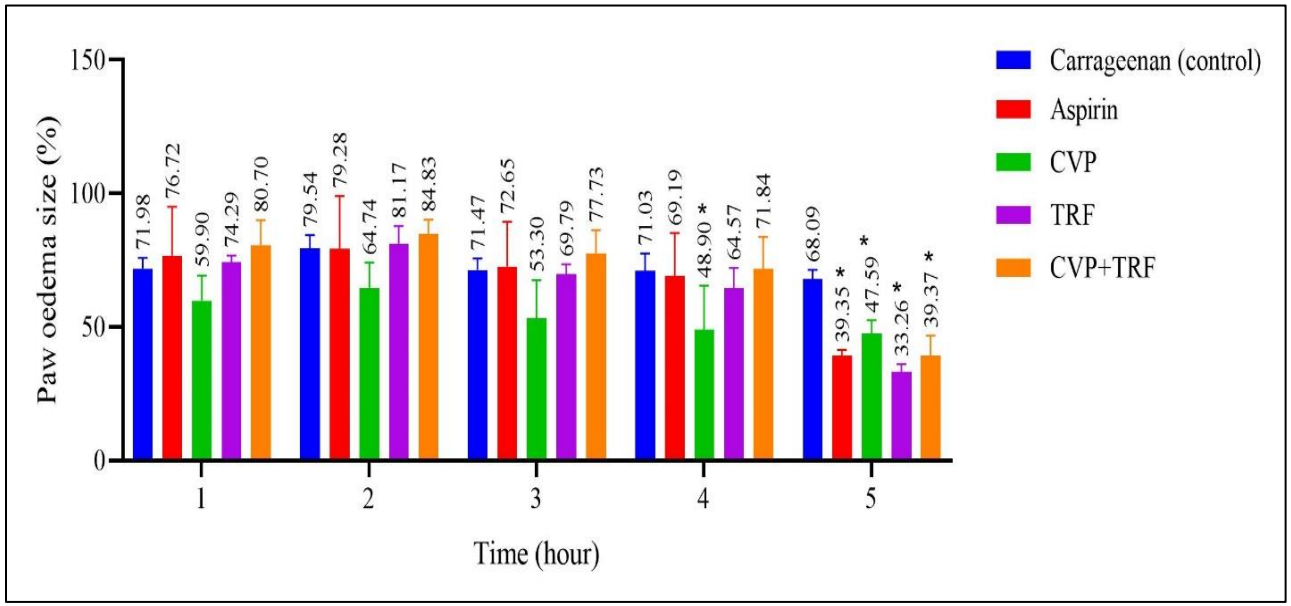

Figure 2: Effect of treatments in carrageenan-induced paw oedema BALB/c mice in terms of the paw oedema size within 5 hours after carrageenan injection. Values are expressed in mean ± SD (n=3); it indicates the paw oedema size of treated groups compared to control and was statistically analysed by one-way analysis of variance (ANOVA) followed by Holm-Sidak multiple t-test, *P < 0.05 considered as significant.

Table 3: Effect of treatments on paw oedema size (%) over time in carrageenan-induced inflammation

Group	Replicate	Paw oedema size (%)				
		1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**
Carrageenan	A	70.59	77.57	67.03	63.53	69.54
	B	76.47	85.05	75.38	74.60	70.53
	C	68.88	76.00	72.01	74.96	64.20
Average ± SD		71.98 ± 3.98	79.54 ± 4.83	71.48 ± 4.20	71.03 ± 6.50	68.09 ± 3.41
Aspirin	A	56.97	56.97	56.88	58.66	40.56
	B	80.11	86.18	70.68	61.37	40.56
	C	93.09	94.68	90.38	87.54	36.94
Average ± SD		76.72 ± 18.30	79.27 ± 19.78	72.65 ± 16.84	69.19 ± 15.94	39.35 ± 2.09*
CVP	A	69.97	75.31	69.79	67.21	44.75
	B	51.31	57.02	45.05	34.43	44.75
	C	58.42	61.90	45.05	45.05	53.27
Average ± SD		59.90 ± 9.42	64.75 ± 9.47	53.30 ± 14.28	48.90 ± 16.73*	47.59 ± 4.92*
TRF	A	72.29	73.56	66.46	59.71	30.82
	B	77.09	86.07	73.85	73.35	32.54
	C	73.48	83.87	69.06	60.66	36.42
Average ± SD		74.29 ± 2.50	81.16 ± 6.68	69.79 ± 3.74	54.80 ± 22.08	33.26 ± 2.87*
CVP+TRF	A	71.98	83.00	72.21	62.22	44.57
	B	90.70	90.85	87.64	85.20	30.92
	C	79.41	80.63	73.35	68.09	42.62
Average ± SD		80.70 ± 9.43	84.83 ± 5.35	77.73 ± 8.60	71.83 ± 11.94	39.37 ± 7.38*

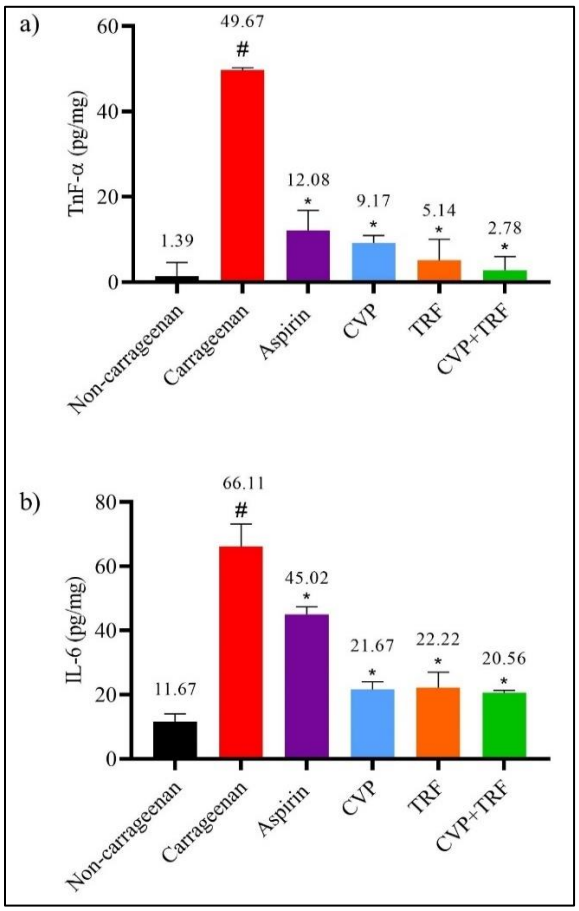

Figure 3: Effect of treatments in carrageenan-induced paw oedema at 5 hours on a) tumour necrosis factor-alpha (TNF-α) and b) interleukin-6 (IL-6). Each value represents mean ± SD, *P < 0.05 considered as a significant compared to control, #. The difference between groups were statistically analysed by one-way ANOVA, Dunnett's test.

highlight the relative effectiveness of each treatment in reducing inflammation.

3.5 Effect of 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, a marker of inflammation, was measured to assess the

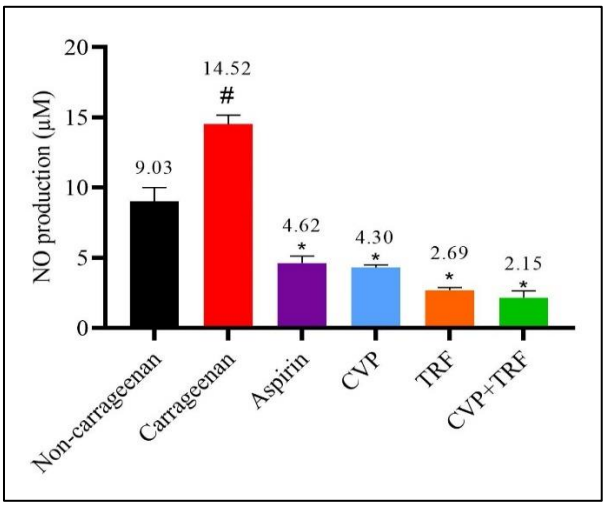

Figure 4: Effect of treatments in carrageenan-induced paw oedema on nitric oxide (NO) production in paw tissue at 5 hours. Each value represents mean ± SD, *P < 0.05 considered as a significant compared to control, #. The difference between groups were statistically analysed by one-way ANOVA, Dunnett's test.

effectiveness of anti-inflammatory treatments. Based on the Figure 4, the carrageenan group showed elevated NO levels, indicating inflammation, while the non-carrageenan control represented baseline conditions. Treatments with aspirin, CVP, TRF and their combination significantly reduced NO levels, demonstrating anti-inflammatory effects. TRF showed greater effectiveness individually compared to aspirin and CVP, and the combination of CVP and TRF resulted in the most significant reduction, suggesting a synergistic effect in reducing NO production and inflammation.

4.0 Discussion

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. Additionally, the mice showed no signs of distress, abnormal behaviour, or visible physical symptoms such as fur loss, lethargy, or swelling, further supporting the safety of the administered doses. These observations, alongside the increase in body weight, suggest that the treatments were well-tolerated and that the lethal dose (LD50) for all compounds exceeded 100 mg/kg, as reported by Ahmad Sayuti *et al*. (2021).

Upon injecting carrageenan into the paw, pro-inflammatory mediators such as histamine, nitric oxide, prostaglandins and cytokines are released. These mediators influence inflammation, pain, and the overall inflammatory condition (44). Administration of carrageenan into the left hind paw can induce a painful response through acute inflammation. The process of inflammatory response starts once carrageenan is inducted into the left hind paw, which leads to hyperalgesia (response to painful stimuli) and allodynia (a painful response to non-painful stimuli) through the production of histamine, serotonin and bradykinins (44, 45-47). Prostaglandins are then released to maintain the inflammatory process and account for the effect of the inflammatory progress (44, 47). Subsequently, pro-inflammatory cytokines such as TNF-α, IL-6 and nitric oxide are released. These mediators were also previously studied in the carrageenan-induced paw oedema with various treatments (48, 49). The results highlight the significant anti-inflammatory effects of aspirin, CVP, TRF and CVP+TRF 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 (44). Both CVP and TRF also demonstrated notable reductions in inflammation, particularly after 5-hour of the experiment. However, the combination of CVP and TRF (CVP+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, after 5-hour of the experiment. Several studies, such as those by Brox and Hackstein (2021) (50), have shown that aspirin significantly reduces inflammatory markers, therefore support aspirin's well-established anti-inflammatory effects across various models.

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 (44). Tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are cytokines that shows a significant increased indicating inflammation in the carrageenan group. Treatments like aspirin, CVP, TRF and in combination (CVP+TRF) reduced cytokines levels, with CVP+TRF showing the greatest reduction. 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 (51). 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 be effectively reduced inflammation, providing valuable insights into potential therapeutic strategies (52).

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 (47). The significant reduction of NO levels by CVP suggests its potent anti-inflammatory activity. Previous studies on proteases from medicinal plants indicate that protease can modulate inflammatory pathways, potentially by inhibiting the activity of pro-inflammatory cytokines or by directly degrading inflammatory mediators (53). TRF, a rich source of tocotrienols, demonstrates an even more substantial reduction in NO levels. 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 (54). The potent effect of TRF may be attributed to its ability to modulate these pathways more effectively than CVP alone. CVP+TRF results in the lowest NO level, indicating a possible synergistic effect. Synergy in anti-inflammatory treatments often arises from the complementary mechanisms of action of different agents (55). 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 (56). Aspirin, a well-known non-steroidal anti-inflammatory drug (NSAID), reduces NO levels and other mediators by inhibiting cyclooxygenase (COX) enzymes and reducing the production of prostaglandins. 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 plausible mechanism of action for CVP+TRF in this study likely involves the combined anti-inflammatory and antioxidant properties of both components. *Christia vespertilionis* protease (CVP) may reduce inflammation by modulating cytokine production, such as lowering TNF-α and IL-6 levels, while also inhibiting pro-inflammatory pathways. Palm tocotrienol-rich fraction (TRF) likely enhances this effect by providing potent antioxidant activity, reducing oxidative stress, and further suppressing inflammatory mediators like nitric oxide (NO). Together, CVP and TRF may act synergistically to dampen inflammation more effectively than either compound alone, targeting both cytokine and oxidative stress pathways.

5.0 Conclusion

The present study demonstrates 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 effectiveness and safety in humans. In conclusion, the treatments significantly reduced TNF-α, IL-6 and nitric oxide levels in the carrageenan-induced paw oedema, with the combination of CVP and TRF showing greater effectiveness than aspirin. These findings align with previous studies on the anti-inflammatory mechanisms of proteases and tocotrienols and suggest that the combination may offer enhanced therapeutic benefits through synergistic interactions.

Authorship contribution statement

IFZ: Data analysis, Methodology, Formal analysis, Writing–original draft. **NAM**: Writing–review & editing. **SRAH**: Resources, Draft corrections. **MAMA, IN, NI**: Supervision.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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