Sains Malaysiana 52(5)(2023): 1371-1381
http://doi.org/10.17576/jsm-2023-5205-04

Optimisation of Protease Purification from *Christia vespertilionis* Leaves and Its Anti-Inflammatory Activity

(Pengoptimuman Penulenan Protease daripada Daun *Christia vespertilionis* dan Aktiviti Anti-Keradangan)

Izzah Farhah Zambari[1], Sitti Rahma Abdul Hafid[2] & Nur Airina Muhamad[1,*]

[1]*Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Federal Territory, Malaysia*
[2]*Nutrition Unit, Product Development and Advisory Services Division, Malaysian Palm Oil Board, 43000 Kajang, Selangor Darul Ehsan, Malaysia*

Received: 30 July 2022/Accepted: 10 May 2023

ABSTRACT

Christia vespertilionis (L. f.) Bakh. f. is widely known for its anti-inflammatory and anti-cancer properties. However, there are no previous studies about extracting and purifying protease enzymes from *C. vespertilionis* leaves. Therefore, this study was conducted to extract and optimise the purification of protease from *C. vespertilionis* leaves and characterise its anti-inflammatory properties. The optimisation was performed using different levels of ammonium sulphate saturation (20, 40, 60, 80 and 100%). Next, dialysis was carried out for the sample with the highest specific activity (16.88 U/mg), achieved with 100% ammonium sulphate saturation. At 100% saturation, *C. vespertilionis* leaves showed an increase in the specific activity to 20.06 U/mg after dialysis. The findings demonstrate the successful extraction and purification of *C. vespertilionis* protease (CVP) with a molecular weight of 48 kDa, as proven by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. CVP also exhibited anti-inflammatory activity, with an inhibition of 45.6% in lipopolysaccharide (LPS)-stimulated RAW264.7 cells and IC_{50} of 19.24 µg/mL. The HPLC test further confirmed the presence of gallic acid and quercetin compounds in *C. vespertilionis*, which cure inflammation. The results indicate that optimised CVP purification was achieved and its anti-inflammatory ability was proven.

Keywords: Ammonium sulphate; *C. vespertilionis*; purification; protease; SDS-PAGE

ABSTRAK

Christia vespertilionis (L. f.) Bakh. f. terkenal dengan sifat anti-radang dan anti-kansernya. Walau bagaimanapun, tiada kajian tentang pengekstrakan dan penulenan enzim protease daripada daun *C. vespertilionis*. Oleh itu, kajian ini dijalankan untuk mengekstrak dan mengoptimumkan penulenan enzim protease daripada daun *C. vespertilionis* dan untuk mencirikan keupayaannya dalam menyembuhkan keradangan. Pengoptimuman dilakukan menggunakan tahap ketepuan amonium sulfat yang berbeza (20, 40, 60, 80 dan 100 %). Kemudian, dialisis dijalankan ke atas sampel yang mempunyai aktiviti khusus tertinggi (16.88 U/mg) yang diperoleh pada ketepuan ammonium sulfat 100%. Pada ketepuan 100%, daun *C. vespertilionis* menunjukkan peningkatan aktiviti khusus kepada 20.06 U/mg selepas dialisis. Hasil menunjukkan pengekstrakan dan penulenan protease *C. vespertilionis* (CVP) dengan berat molekul 48 kDa telah berjaya diperoleh, seperti yang dibuktikan melalui ujian elektroforesis gel natrium dodesil sulfat-poliakrilamida (SDS-PAGE). CVP juga menunjukkan aktiviti anti-radang dengan perencatan sebanyak 45.6% dalam sel RAW264.7 yang dirangsang oleh lipopolisakarida (LPS) dengan IC_{50} pada 19.24 µg/mL. Ujian HPLC juga mengesahkan kehadiran asid galik dan kuersetin dalam *C. vespertilionis* yang mempunyai keupayaan untuk menyembuhkan keradangan. Ini menunjukkan CVP telah melalui kaedah penulenan yang optimum dan membuktikan keupayaannya untuk menyembuhkan keradangan.

Kata kunci: Amonium sulfat; *C. vespertillonis*; penulenan; protease; SDS-PAGE

Introduction

Inflammation is closely associated with the body's response to injuries. Upon inflammation, the body responds to the imbalance in inflammatory mediators, such as nitric oxide (NO), prostaglandin E2 (PGE2) and cytokines, including interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α). The responses lead to macrophage activation, killing foreign invaders like bacteria and pathogens, which occurs repetitively until the injury is cured (Cheon et al. 2006; Choy & Panayi 2001). Previously, doctors prescribed protease for healing and tissue repair following an injury. However, the emergence of non-steroidal anti-inflammatories (NSAIDs) in the market has suppressed the benefits of protease. Although NSAIDs can reduce pain, their application cannot speed up tissue repair. NSAIDs have also been associated with adverse side effects such as gastric intestinal mucosa, and heart and kidney diseases. Therefore, protease could become one of the alternatives to cure inflammation, as it is natural and safe to use.

Proteases can be found primarily in plants, animals, and organisms in certain conditions and environments. Research has shown that papaya and pineapple contain protease enzymes called papain and bromelain, respectively. Protease can inhibit the production of proinflammatory mediators such as cytokines and nitric oxide during inflammation. Protease also acts as a catalyst that supports enzymes in the body and facilitates enzymatic reactions involved in tissue repair. Currently, protease is widely used in the manufacturing industry, contributing to about 60% of the enzyme market worldwide (Gonzàlez et al. 2011; Kim et al. 2016; Sun et al. 2016). The common applications include detergent, leather manufacturing, waste management, brewing, meat softening, milk-clotting, food production, pharmaceutical products, cancer treatment, diagnostics, digestion, viral disorders treatment, and silver recovery industries (Gupta, Beg & Lorenz 2002; Kuddus 2015; Naidu & Devi 2005; Roy & Kumar 2014; Sathya Prabhu et al. 2017). The role of protease as an anti-cancer, anti-inflammatory, and anti-oedema agent, its procoagulant activity, and its role in the digestive system and other metabolisms have been reported (Gonzàlez et al. 2011; van der Hoorn 2008). The ability of protease to reduce inflammation and treat arthritis has also been proven (Brien et al. 2004; Viswanatha Swamy & Patil, 2008). Proteases can modulate inflammation through the cleavage of adhesion molecules that can have pro- or anti-inflammatory effects by regulating the expression and activity of different proinflammatory cytokines, chemokines, and other immune components. Generally, protease enzymes benefit humans, and research has continued to identify new sources of protease enzymes.

In this study, *C. vespertilionis* (L. f.) Bakh. f. (Family: Fabaceae) was selected for protease purification. Commonly known as the butterfly wing or 'daun rama-rama', it is typically consumed by cancer patients in Malaysia, and researchers are keen to explore the true potential of this plant. The plant originates in South-eastern China, India, Thailand, Cambodia, Laos, Vietnam, Indonesia and Malaysia. It is used traditionally by the locals to cure diseases such as tuberculosis, scabies, snake bites, bronchitis and poor blood circulation (Garnock-Jones 1983; Whiting 2007). Findings have demonstrated the anti-inflammatory (Nguyen-Pouplin et al. 2007; Osman et al. 2017; Rayburn, Ezell & Zhang 2009), antioxidant (Lee et al. 2020), anti-cancer, antidiabetic (Murugesu et al. 2020), antiplasmodial (Upadhyay et al. 2013) and antiproliferative (Hofer et al. 2013) properties of this plant. Despite extensive research on *C. vesspertilionis*, no study was found regarding protease purification from this plant. Therefore, this study aimed to extract and optimise the purification of protease from *C. vespertilionis* leaves through ammonium sulphate precipitation to remove unwanted components and dialysis to increase the enzyme activity, with detection of anti-inflammatory activities through *in vitro* test and HPLC tests.

Materials and Methods

Plant materials

Leaves of *C. vespertillonis* were collected from Floranika Nursery Sungai Buloh in Selangor, Malaysia. Dr. Yong Kien Thai from the Institute of Biological Sciences, University of Malaya, authenticated the voucher specimen. The specimen voucher for *C. vespertilionis* (KLU 50026) was deposited at the herbarium of Universiti Malaya.

Extraction and purification of protease

C. vespertilionis leaves weighing 20 g were washed thoroughly with tap water and ground in 500 mL of pre-chilled 0.1 M sodium phosphate buffer at pH 7. The crude extract was filtered through cheesecloth to remove the pulp. The filtrate was centrifuged at 9000 rpm for 15 min at 4 °C to remove impurities. The supernatant was collected and stored at 4 °C until further tests.

AMMONIUM SULPHATE PRECIPITATION AND DIALYSIS

The ammonium sulphate precipitation method was performed using the 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), 100 w/v% (34.85 g/50 mL). All mixtures were stirred overnight at 4 °C until homogeneous and centrifuged at 9000 rpm for 15 min at the same temperature. The pellet was collected for each saturation and resuspended with 10 mL of pre-chilled 0.1 M sodium phosphate buffer at pH 7. All samples were analysed for their total protein content, enzyme activity and specific activity.

Next, dialysis was performed on the sample with the highest enzyme activity, i.e., *C. vespertilionis* at 100% saturation. The sample was pipetted into the dialysis membrane with a molecular weight cut-off of 14 000 Da and left in a beaker containing 300 mL of pre-chilled 0.1 M sodium phosphate buffer at pH 7. The dialysis solution was stirred with a magnetic bar on a magnetic stirrer plate. The buffer was changed every 2 h for 4 h and left overnight to ensure the complete removal of ammonium sulphate. Subsequently, the purified protease was centrifuged at 9000 rpm for 15 min at 4 °C, and the supernatant was collected and kept at 4 °C. Further analyses of the total protein content, enzyme activity and specific activity were carried out on the collected supernatant.

PROTEASE ASSAY

Protease assay was performed using the method of Sathya Prabhu et al. (2017) with some modifications. This method measured the protein content, enzyme activity, and specific activity in all samples during the ammonium sulphate precipitation and dialysis. The 20 µL of *C. vespertilionis* protease (CVP) and 4980 µL of 2% casein as substrate were dissolved in the pre-chilled 0.1 M sodium phosphate buffer at pH 7. Afterwards, the mixture was incubated at 37 °C for 5 min, and the reaction was stopped by adding 5000 µL of 10% (w/v) trichloroacetic acid and left at room temperature for 15 min. The mixture was subsequently 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).

DETERMINATION OF MOLECULAR WEIGHT

The molecular weight of *C. vespertilionis* protease (CVP) was determined via 12% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer (pH 8.2) (Laemmli 1970). *C. vespertilionis* protease (CVP) was diluted with loading sample buffer at 2:1 dilution. A total of 15 µL of CVP and protein marker (GeneDireX, Taiwan) were separately pipetted into the well and ran for 5 h at 100 kV. The molecular weight of CVP was estimated by comparing it with the protein marker. The gel was stained with Coomassie Brilliant Blue R-250 and destained with a destaining solution. The gel band was scanned and analysed using Bio-5000 plus Microtek (Hsinchu, Taiwan).

CYTOTOXICITY TEST

The cytotoxicity of *C. vespertilionis* protease (CVP) in RAW264.7 cells was tested via MTT assay with some modifications (Funaro et al. 2016; Mutalib & Latip 2019; Sakdarat et al. 2009). Cells with a count of 4×10^4 were seeded per well, followed by incubation for 24 h. The next day, 80% confluence cells in the 96-well plate were treated with 100 µL of the sample (CVP) at 5, 10, 15, 25 and 30 µg/mL, with aspirin used as a control (5, 10, 25, 50 and 100 µg/mL). The cells were incubated with and without LPS (10 ng/mL) for 24 h. After 24 h of incubation, the supernatant was discarded and 50 µL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added into each well followed by incubation in the dark for 2 h. An 80 µL of dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals, and the absorbance was measured at 540 nm using a Biotek Synergy H1 (United States) microplate reader. All experiments were performed in triplicate.

RAW264.7 CELLS INHIBITION

Cell inhibition was determined by the Griess assay with some modifications (Chen et al. 2019; Yam et al. 2009). RAW264.7 cells were seeded in the 96-well plate and incubated for 24 hours. The cells were treated with 100 µL of the sample (CVP), with aspirin as a control. The cells were incubated with LPS (Invitrogen, USA) for 24 h. After 24 h of incubation, the supernatants were mixed with N-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilic acid at a 1:1 ratio against a nitrite standard and incubated in the dark for 30 min. The absorbance

was measured at 540 nm using Biotek Synergy H1 (United States) microplate reader. Inhibition of LPS-stimulated RAW264.7 cells was determined based on the nitrite standard curve. All experiments were performed in triplicate.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Gallic acid and quercetin in *C. vespertilionis* extract were analysed qualitatively using high-performance liquid chromatography (HPLC). The chromatography was performed using UHPLC Agilent 1290 Infinity (California, United States). Column specifications 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 applied was 0.9 mL/min. Water: methanol (70:30 %v/v) were used as mobile phases, with a detection wavelength of 272 nm. The injection volume of 20 µL was applied for the qualitative analysis of the standard (gallic acid and quercetin) and sample (*C. vespertilionis* extract). The peak area corresponds to the sample integrated by comparison with the standard (Fulzele & Satdive 2005; Kiran, Venkata & Nagoji 2012). An accurately weighed amount of gallic acid (10 mg) was dissolved in 10 mL of methanol to obtain a standard stock solution of 1 mg/mL, and the same process was repeated for quercetin. A calibration curve of gallic acid and quercetin was established separately with five dilutions at concentrations of 5, 10, 20, 50, and 100 µg/mL. Each concentration of standard and sample was measured in triplicate.

STATISTICAL ANALYSIS

All the analyses were performed using GraphPad Prism (version 8.00 for Windows, GraphPad Software, San Diego, California, USA). A one-way ANOVA statistical analysis was applied for the purified sample versus enzyme activity.

Results and Discussion

In this study, *C. vespertilionis* leaf extract was prepared for the purification of protease using the ammonium sulphate precipitation method, which is widely used for protease purification from other sources (Antào & Malcata 2005; Drivdahl & Thimann 1977; Esposito et al. 2016; Nam, Walsh & Yang 2016). The ammonium sulphate accumulates protein at the bottom, which can be pelleted by centrifugation. Figure 1(a) shows the highest total protein content of 19.33 mg obtained at 100% ammonium sulphate saturation compared with other saturation percentages. Further analysis showed a higher total activity of *C. vespertilionis* protease (CVP) (23.31 U/mL) at 100% ammonium sulphate saturation than at other saturation percentages (Figure 1(b)). Findings have shown that ammonium sulphate addition can enhance the specific activity of the enzyme. These findings explain the high specific activity (16.88 U/mg) of CVP at 100% saturation (Figure 1(c)). Protease enzyme is known to work well with ammonium sulphate precipitation at high saturation due to the ability of salt to attract enzyme proteins from water molecules, thus enhancing the specific activity of the enzyme (Wardani & Nindita 2012; Winarti et al. 2018).

Following ammonium sulphate precipitation, dialysis was performed to remove unwanted molecules and ammonium sulphate residue that could inhibit the protease enzyme activity (Belton et al. 1999; Wardani & Nindita 2012). The dialysis was performed using a semi-permeable membrane that can separate the proteins from unwanted molecules and foreign objects. CVP exhibited enhanced specific activity up to 20.06 U/mg after the dialysis (Figure 2). Papain, a well-known protease enzyme that originated from papaya, was used as a positive control, showing a specific activity of 69.78 U/mg. The difference in specific activity of CVP compared with papain was 49.72. CVP purification via dialysis resulted in 53.33% protein recovery with 6.52-fold purification, indicating a successful purification from crude extract (Table 1). The results show that the additional purification step using dialysis is more effective for purifying protease from *C. vespertilionis* than using ammonium sulphate precipitation alone. The SDS-PAGE analysis showed that the molecular weight of the purified CVP is 48 kDa (Figure 3).

The anti-inflammatory activity of CVP was measured in RAW264.7 cells using the MTT assay. A commercial drug, aspirin, was used as a positive control. Lipopolysaccharide (LPS)-stimulated RAW264.7 cells were treated with different concentrations of CVP at 5, 10, 15, 25, and 30 µg/mL and the control group received aspirin at 5, 10, 25, 50, and 100 µg/mL for 24 h. Cell viability was maintained above 80% in all treatments, indicating no toxicity, as shown in Figure 4(a). The concentrations for the respective treatments can be used for the subsequent *in vitro* analysis of anti-inflammatory activity, measuring the inhibition of LPS-stimulated RAW264.7 cells with nitric oxide induction using the Griess assay.

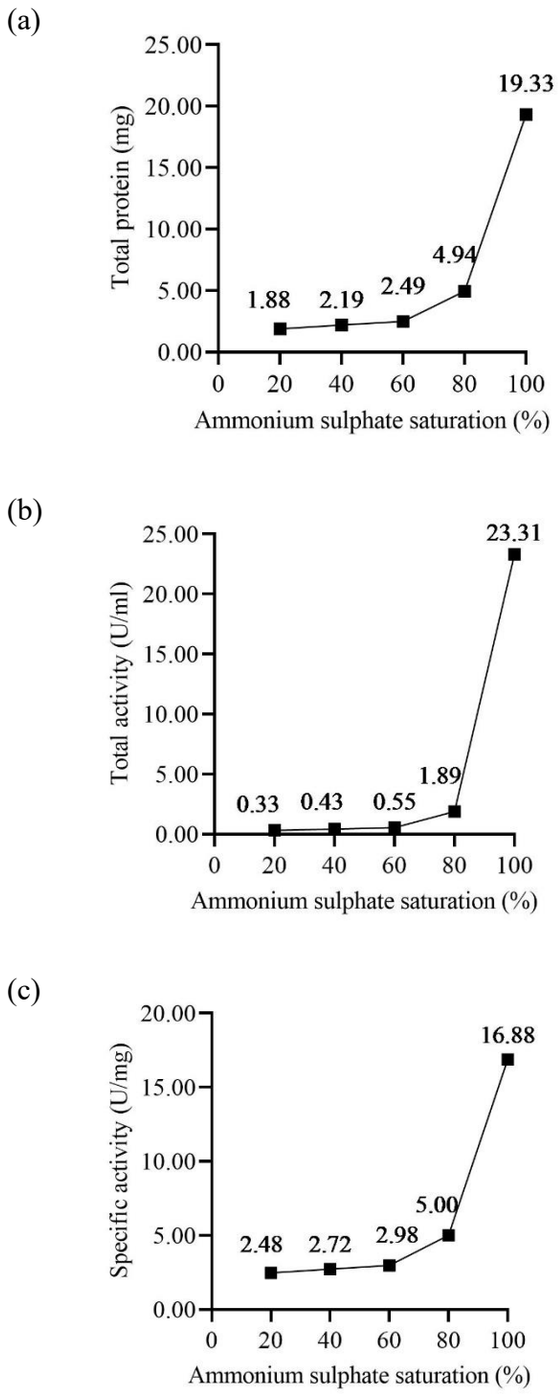

FIGURE 1. a) Total protein, b) total activity and c) specific activity of *C. vespertilionis* protease (CVP) at different saturation of ammonium sulphate

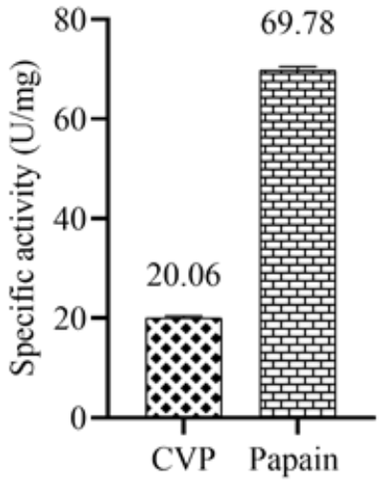

FIGURE 2. The specific activity of *C. vespertilionis* protease (CVP) after dialysis and papain (control)

TABLE 1. Summary of total protein, total activity, specific activity, fold purity and activity yield at different purification steps of *C. vespertilionis* protease (CVP)

Purification steps	Total protein (mg)	Total activity (U/mL)	Specific activity (U/mg)	Fold purity	Activity yield (%)
Crude of *C. vespertilionis*	2.62	0.62	3.07	1.00	100.00
Ammonium sulphate saturation (100%)	19.33	23.31	16.88	5.48	37.39
Dialysis	23.19	33.24	20.06	6.52	53.33

Nitric oxide (NO) is one of the cellular mediators secreted at inflammatory sites in cardiovascular, nervous and immunological systems (Rao, Ahmad & Mohd 2016; Ren et al. 2019). Thus, the inhibition of nitric oxide has been used to monitor the healing process at the inflammation sites. RAW264.7 cells stimulated with 10 ng/mL LPS were typically characterised by increased nitric oxide production. Effective treatments should reduce this nitric oxide production in the cells. Therefore, the inhibitory effects of the treatments with CVP and aspirin (control) on nitric oxide production in LPS-stimulated RAW264.7 cells were measured. The results show the anti-inflammatory activity of CVP and aspirin at 45.61 and 36.96% of cell inhibition at 30 and 100 µg/mL concentrations, respectively, as shown in Figure 4(b). Additionally, treatment with CVP recorded an IC_{50} value of 19.24 µg/mL compared with aspirin, 58.39 µg/mL. The results demonstrate the effectiveness of CVP in curing inflammation, which is comparable to that of aspirin.

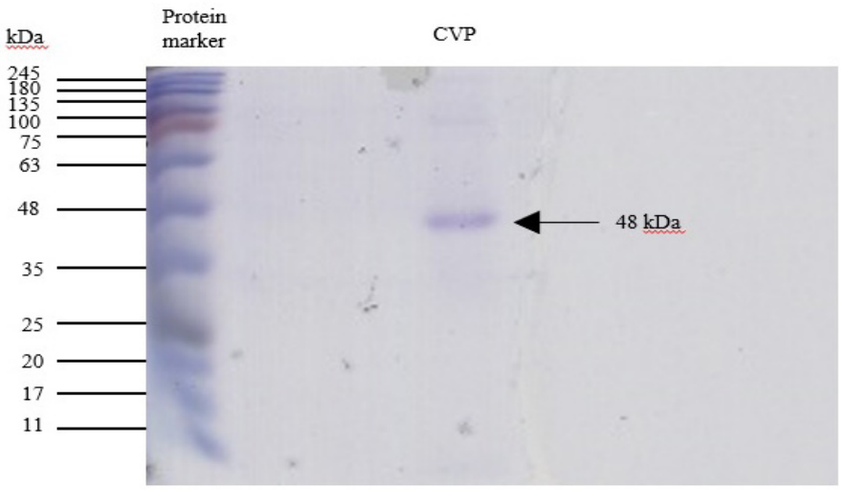

FIGURE 3. SDS-PAGE analysis of *C. vespertilionis* protease (CVP). On the left is the ladder and on the right is the band corresponding to CVP at the molecular weight of 48 kDa

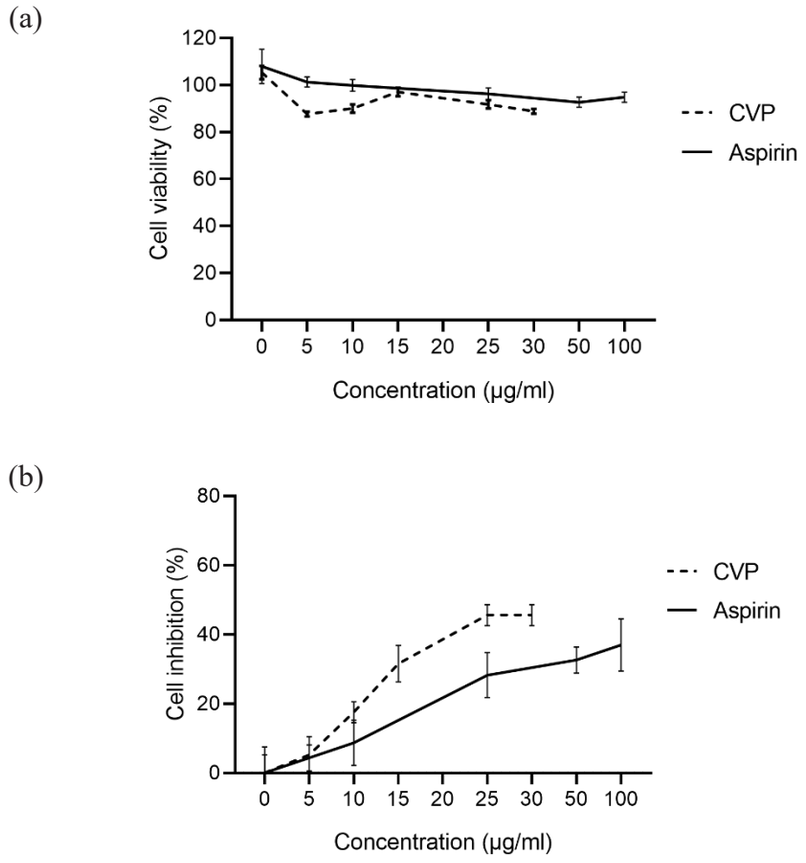

FIGURE 4. Anti-inflammatory activity of *C. vespertilionis* protease (CVP) and aspirin (control) in LPS-stimulated RAW264.7 cells measured in terms of a) cell viability (%) and b) cell inhibition (%) when induced with nitric oxide

Previous studies have reported the presence of anti-inflammatory compounds in *C. vespertilionis* and their anti-inflammatory activities (Nguyen-Pouplin et al. 2007; Osman et al. 2017; Rayburn, Ezell & Zhang 2009). High-performance liquid chromatography (HPLC) was performed to prove the presence of anti-inflammatory compounds in *C. vespertilionis*. Gallic acid and quercetin were used as standards as previous studies had proven their anti-inflammatory activities. Gallic acid has therapeutic effects on diseases such as cardiovascular disease, cancer, inflammation and neurodegenerative disorders (Karamaae, Kosinska & Pegg 2005; Kaur et al. 2005; Nikolic 2006; Singh et al. 2019). Meanwhile, quercetin is a flavonoid, one of the phenolic compounds responsible for curing inflammation (Chew et al. 2011; Lee et al. 2020).

The qualitative HPLC chromatogram of *C. vespertilionis* was examined. The calibration curve for standards (gallic acid and quercetin) is shown in Figure 5. The peaks, retention time (min) and area (mAU) determined from the chromatograms of the standards and *C. vespertilionis* are summarised in Table 2 and Figure 6. A peak corresponding to gallic acid was identified at the retention time of 1.073 min, shown in Figure 6(a) and a sharp peak denoting quercetin at the retention time of 1.080 min, shown in Figure 6(b). The authentic sharp peaks of *C. vespertilionis* at the retention time of 1.173 min are shown in Figure 6(c). The analysis showed the presence of peaks around the retention time similar to those of the standards (gallic acid and quercetin) in the HPLC chromatogram of *C. vespertilionis*. Therefore, the HPLC test confirmed the presence of gallic acid and quercetin in *C. vespertilionis,* indicating the potential of *C. vespertilionis* as an anti-inflammatory agent.

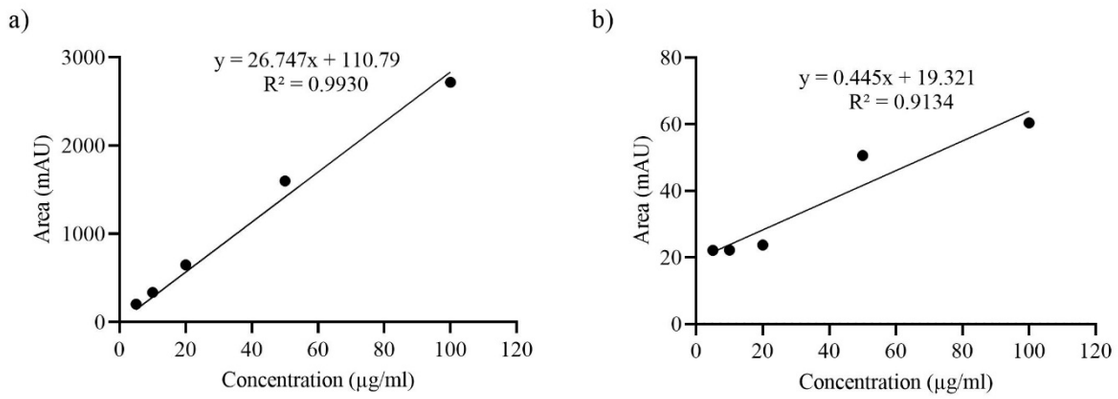

FIGURE 5. HPLC calibration curve of a) gallic acid and b) quercetin

TABLE 2. HPLC analysis of gallic acid, quercetin and *C. vespertilionis*

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

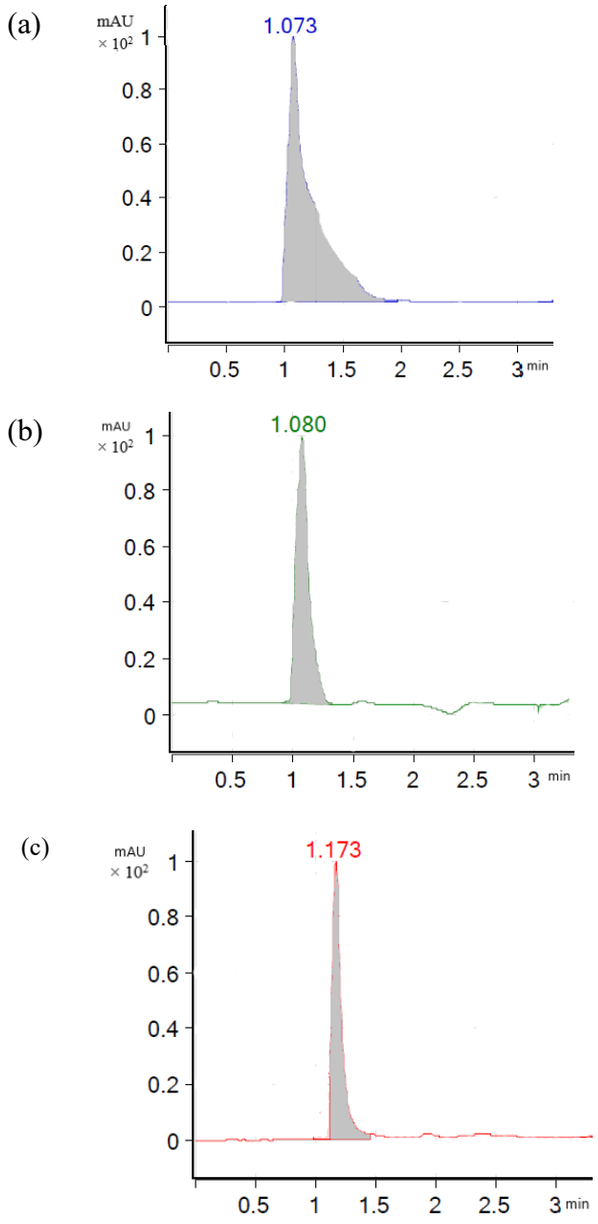

FIGURE 6. HPLC chromatogram of a) gallic acid, b) quercetin and c) *C. vespertilionis*

CONCLUSIONS

In conclusion, *C. vespertilionis* protease (CVP) showed the highest specific activity of 16.88 U/mg at 100% ammonium sulphate saturation. The value was further increased to 20.06 U/mg after the second-step purification via dialysis, showing that the molecular weight of the protein was 48 kDa. Additionally, CVP exhibited 45.6% inhibition in LPS-stimulated nitric oxide RAW264.7 cells, demonstrating a potent anti-inflammatory activity compared with aspirin at 36.96% inhibition. CVP and

aspirin also showed no cytotoxicity according to their concentrations. HPLC analysis showed the presence of anti-inflammatory standards, gallic acid and quercetin in *C. vespertilionis*, further supporting its potential use as a potent anti-inflammatory agent.

ACKNOWLEDGEMENTS

The authors would like to express gratitude to Universiti Malaya for providing financial assistance for this research project under the Research University of Faculty Programme Grant Scheme (GPF006B-2018). Appreciation is also given to the Institute of Biological Sciences and Infra Analysis Laboratory for providing the laboratories and infrastructures needed to perform this research.

REFERENCES

Antào, C.M. & Malcata, F.X. 2005. Plant serine proteases: Biochemical, physiological and molecular features. *Plant Physiology and Biochemistry* 43(7): 637-650.

Belton, P.S., Sorensen, H., Sorensen, S., Bjergegaard, C. & Michelson, S. 1999. *Chromatography and Capillary Electrophoresis in Food Analysis*. Cambridge: The Royal Society of Chemistry.

Brien, S., Lewith, G., Walker, A., Hicks, S.M. & Middleton, D. 2004. Bromelain as a treatment for osteoarthritis: A review of clinical studies. *Evidence-Based Complementary and Alternative Medicine* 1(3): 251-257.

Chen, M., Qin, Y., Ma, H., Zheng, X., Zhou, R., Sun, S., Huang, Y., Duan, Q., Liu, W., Wu, P., Xu, X., Sheng, Z., Zhang, K. & Li, D. 2019. Downregulating NF-kappaB signaling pathway with triterpenoids for attenuating inflammation: *in vitro* and *in vivo* studies. *Food Function* 10: 5080-5090.

Chew, K.K., Ng, S.Y., Thoo, Y.Y., Khoo, M.Z., Wan Aida, W.M. & Ho, C.W. 2011. Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of *Centella asiatica* extract. *International Food Research Journal* 18: 571-578.

Cheon, H., Rho, Y.H., Choi, S.J., Lee, Y.H., Song, G.G., Sohn, J., Won, N.H. & Ji, J.D. 2006. Prostaglandin E2 augments IL-10 signaling and function. *Journal of Immunology* 177: 1092-2100.

Choy, E.H.S. & Panayi, G.S. 2001. Cytokine pathways and joint inflammation in rheumatoid arthritis. *New England Journal of Medicine* 344(12): 907-916.

Drivdahl, R.H. & Thimann, K.V. 1977. Proteases of senescing oat leaves I. Purification and general properties. *Journal of Plant Physiology* 59(6): 1059-1063.

Esposito, M., Di-Pierro, P., Dejonghe, W., Mariniello, L. & Porta, R. 2016. Enzymatic milk clotting activity in artichoke (*Cynara scolymus*) leaves and alpine thistle (*Carduus defloratus*) flowers. Immobilization of alpine thistle aspartic protease. *Food Chemistry* 204(2): 115-121.

Fulzele, D.P. & Satdive, R.K. 2005. Comparison of techniques for the extraction of the anti-cancer drug camptothecin from *Nothapodytes foetid*. *Journal of Chromatography A* 1063(1-2): 9-13.

Funaro, A., Wu, X., Song, M., Zheng, J., Guo, S., Rakariyatham, K., Rodriguez-Estrada, M.T. & Xiao, H. 2016. Enhanced anti-inflammatory activities by the combination of luteolin and tangeretin. *Journal of Food Science* 81(5): 1320-1327.

Garnock-Jones, P.J. 1983. Note on some folklore drugs and remedies of the Lau Group, Fiji. *South Pacific Journal of Natural Science* 4: 4-8.

Gonzàlez, R.N., Badillo, J.A., Aranda, A.J.S. & Carmen, O.S.M. 2011. Production of plant proteases *in vivo* and *in vitro*-a review. *Biotechnology Advances* 29(6): 983-996.

Gupta, R., Beg, Q. & Lorenz, P. 2002. Bacterial alkaline proteases: Molecular approaches and industrial applications. *Applied Microbiology Journal* 59(1): 15-32.

Hofer, D., Schwach, G., Tabrizi-Wizsy, N.G., Sadjak, A., Sturm, S., Stuppner, H. & Pfragner, R. 2013. *Christia vespertilionis* plant extracts as novel antiproliferative agent against human neuroendocrine tumor cells. *Oncology Reports* 29(6): 2219-2226.

Karamaae, M.A., Kosinska, A. & Pegg, R.B. 2005. Comparison of radical-scavenging activities of selected phenolic acids. *Polish Journal of Food and Nutrition Sciences* 14: 165-170.

Kaur, S., Michael, H., Arora, S., Harkonen, P.L. & Kumar, S. 2005. The *in vitro* cytotoxic and apoptotic activity of Triphala-an Indian herbal drug. *Journal of Ethnopharmacology* 97: 15-20.

Kim, M., Si, J.B., Reddy, L.V. & Wee, Y.J. 2016. Enhanced production of extracellular proteolytic enzyme excreted by a newly isolated *Bacillus subtilis* FBL-1 through combined utilization of statistical designs and response surface methodology. *RSC Advances* 6(56): 51270-51278.

Kiran, K.K., Venkata, N.R. & Nagoji, K.E.V. 2012. A validated RP-HPLC method for the determination of Bedamustine hydrochloride in tablet dosage form using gemcitabine hydrochloride as internal standard. *FABAD Journal of Pharmaceutical Sciences* 37(3): 133-139.

Kuddus, M. 2015. Cold-active microbial enzymes. *Biochemistry and Physiology* 4(1): 1-2.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259): 680-685.

Lee, J.J., Yazan, L.S., Kassim, N.K., Che Abdullah, C.A., Esa, N., Lim, P.C. & Tan, D.C. 2020. Cytotoxic activity of *Christia vespertilionis* root and leaf extracts and fractions against breast cancer cell lines. *Molecules* 25(11): 2610.

Murugesu, S., Perumal, V., Balan, T., Fatinanthan, S., Khatib, A., Arifin, N.J., Mohd Shukri, N.S.S., Saleh, M.S.M. & Hin, L.W. 2020. The investigation of antioxidant and antidiabetic activities of *Christia vespertilionis* leaves extracts. *South African Journal of Botany* 133: 227-235.

Mutalib, N.A. & Latip, N.A. 2019. Synergistic interactions between *Christia vespertilionis* leaves extract and chemotherapy drug cyclophosphamide on WRL-68 cell line. *Asian Journal of Pharmaceutical Research and Development* 7(3): 109-113.

Nam, S.H., Walsh, M.K. & Yang, K.Y. 2016. Comparison of four purification methods to purify cysteine protease from Asian pear fruit (*Pyrus pyrifolia*). *Biocatalysis and Agricultural Biotechnology* 5: 86-93.

Naidu, K.S.B. & Devi, K.L. 2005. Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. *African Journal of Biotechnology* 4(7): 724-726.

Nikolic, K.M. 2006. Theoretical study of phenolic antioxidants properties in reaction with oxygen-centered radicals. *Journal of Molecular Structure: Theochem* 774: 95-105.

Nguyen-Pouplin, J., Tran, H., Tran, H., Phan, T.A., Dolecek, C., Farrar, J., Tran, T.H., Caron, P., Bodo, B. & Grellier, P. 2007. Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam. *Journal of Ethnopharmacology* 109(3): 417-427.

Osman, M.S., Ghani, Z.A., Ismail, N.F., Razak, N.A., Jaapar, J. & Ariff, M.A. 2017. Qualitative comparison of active compounds between red and green Mariposa *Christia vespertillionis* leaves extracts. *Proceedings of the American Institute of Physics Conference* 1885: 020282.

Park, S.R., Lim, C.Y., Kim, D.S. & Ko, K. 2015. Optimization of ammonium sulfate concentration for purification of colorectal cancer vaccine candidate recombinant protein GA733-FcK isolated from plants. *Frontiers in Plant Science Frontiers* 6: 1040.

Rao, U.S.M., Ahmad, B.A. & Mohd, K.S. 2016. *In vitro* nitric oxide scavenging and anti-inflammatory activities of different solvent extracts of various parts of *Musa paradisiaca*. *Malaysian Journal of Analytical Sciences* 20(5): 1191-1202.

Rayburn, E.R., Ezell, S.J. & Zhang, R. 2009. Anti-inflammatory agents for cancer therapy. *Molecular and Cellular Pharmacology* 1: 29-43.

Ren, J., Li, L., Wang, Y., Zhai, J., Chen, G. & Hu, K. 2019. Gambogic acid induces hemeoxygenase-1 through Nrf2 signaling pathway and inhibits NF kappaB and MAPK activation to reduce inflammation in LPS-activated RAW264.7 cells. *Biomedicine & Pharmacotherapy* 109: 555-562.

Roy, S. & Kumar, V. 2014. A practical approach on SDS PAGE for separation of protein. *International Journal of Science and Research* 3(8): 955-960.

Sakdarat, S., Shuyprom, A., Pientong, C., Ekalaksananan, T. & Thongchai, S. 2009. Bioactive constituents from the leaves of *Clinacanthus nutans* Lindau. *Bioorganic & Medicinal Chemistry* 17(5): 1857-1860.

Sathya Prabhu, D., Apoorva, S., Nandita, J., Palani, C. & Devi Rajeswari, V. 2017. Purification of protease enzyme from the leaf, seed and pod samples of *Vicia faba* L. *International Food Research Journal* 25(5): 1904-1911.

Singh, M.P., Gupta, A. & Singh Sisodia, S. 2019. Qualitative analysis of gallic acid by HPLC method in different extracts of *Terminalia bellerica* Roxb. fruit. *FABAD Journal of Pharmaceutical Sciences* 44(2): 101-106.

Sun, Q., Zhang, B., Yan, Q.J. & Jiang, Z.Q. 2016. Comparative analysis on the distribution of protease activities among fruits and vegetable resources. *Food Chemistry* 2(13): 708-713.

Upadhyay, H.C., Sisodia, B.S., Cheema, H.S., Agrawalb, J., Pal, A., Darokar, M.P. & Srivastava, S.K. 2013. Novel antiplasmodial agents from *Christia vespertilionis*. *Natural Product Communications* 8(11): 1-4.

van der Hoorn, R.A. 2008. Plant proteases: From phenotypes to molecular mechanisms. *Annual Review of Plant Biology* 59: 191-223.

Viswanatha Swamy, A.H.M. & Patil, P.A. 2008. Effect of some clinically used proteolytic enzymes on inflammation in rats. *Indian Journal of Pharmaceutical Sciences* 70(1): 114-117.

Wardani, A.K. & Nindita, I.O. 2012. Purification and characterization of protease from protease-producing bacteria isolated from tofu whey. *Jurnal Teknologi Pertanian* 13(3): 149-156.

Winarti, A., Fitriyanto, N.A., Jamhari, Pertiwiningrum, A., Bachruddin, Z., Pranoto, Y. & Erwanto, Y. 2018. Optimizing of protease purification from *Bacillus cereus* TD5B by ammonium sulfate precipitation. *Chemical Engineering Transactions* 63: 709-714.

Whiting, P.A. 2007. Commercial production of *Christia subcordata* Moench by establishing cultural practices and by applying plant growth regulators. Dissertation. United States: The University of Georgia (Unpublished).

Yam, M.L., Abdul Hafid, S.R., Cheng, H.M. & Nesaretnam, K. 2009. Tocotrienols supress proinflammatory markers and cyclooxygenase-2 expression in RAW264.7 macrophages. *Lipids* 44(9): 787-797.

*Corresponding author; email: nurairina@um.edu.my