Comparative Evaluation of Protease Extraction from Leaves of *Syzygium polyanthum*

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Received: August 3, 2018
Accepted: December 16, 2018

DOI: 10.22146/ijc.40860

Abstract: *In this study, the comparison of four extraction methods for protease from the leaves of* Syzygium polyanthum *was evaluated. The methods include sodium phosphate buffer, 1-methyl-8-oxyquinolinium betaine (QB) solvent based extraction, hydrogen-bond acceptor (HBA) solvent based extraction and Trichloroacetic-acetone (TCA) precipitation. The effectiveness of these extraction methods was measured based on the specific activity demonstrated by the crude protease extracted in this experiment. The results indicated that QB solvent-based extraction method could extract the crude protease from the leaves of* S. polyanthum *with the highest specific activity of 10.204 ± 0.329 U/mg compared to other extraction methods.*

Keywords: *plant protease; protease extraction;* Syzygium polyanthum

■ INTRODUCTION

In recent years, protease has gained recognition globally for the widespread applications in food processing, medicine, pharmaceuticals, detergents, leather tanning and chemical industries [1]. Statistically, the protease accounts for almost 60% of the total global market as an industrial enzyme [2]. Proteases are a group of enzymes that catalyze hydrolytic reactions in which the peptide bonds of protein molecules are degraded to small units of amino acids [3].

The protease can be derived from four main sources of living organisms. Namely; microbes, animals, plants and human being [4]. The protease from animals and microbes have been widely studied and currently known to be the primary producers of the protease used in the industrial sectors. However, the protease from animal has raised certain limitations especially when involving certain religious requirements. According to Boone and Verbeke [5], some food restrictions are being practised in some religions. For example, pork, none-slaughtered meat, and their derivatives could be the source of protease but prohibited among Judaism and Muslim believers while pork and beef are prohibited for Hinduism and Buddhism believers. Therefore, other alternative sources of proteases from plants are being investigated to eliminate doubtfulness and sensitivity among consumers especially when there is an involvement of protease in the food industry. Besides, plants also contain proteases which responsible for regulating their physiological and metabolic processes [6]. There is a wide range of plants that are known for their medicinal properties and being investigated for their active compounds in drug discovery [7]. The studies are not limited only to understanding the physiological functions of plant protease, but also to explore their benefits and importance for industrial applications.

Malaysia is well known as a tropical rainforest country which provides wide opportunity to study protease from unexplored plant like *Syzygium polyanthum*. *S. polyanthum* (Wight) *Walp*. is a medium-sized and evergreen herb plant known as 'salam' or 'serai kayu' among the local people in Malaysia and Indonesia which belongs to the Myrtaceae family [8]. The leaves of *S. polyanthum* are edible and aromatic and is commonly

used as a spice and flavoring agent in Southeast Asian cuisines including in Malaysia [9].

Determination of suitable protease extraction protocol is considered to be a preliminary requirement to study its properties. Nevertheless, there are many types of extraction protocols used to extract protease from the plant leaves ranging from simple solvent-based extraction method to a more complex method which is associated with purification of the protease. For instance, simple extraction methods using distilled water and buffer solution are applied to extract protease from the plant leaves of *Canavalia ensiformis* where the leaves were first treated with liquid nitrogen to form powder [10]. In addition, the new serine protease namely populnein has also been extracted from the leaves of *Thespesia populnea* using sodium phosphate buffer solution followed by ammonium sulfate precipitation [6]. Populnein presented a promising activity in the stabilization of human red blood cell membrane which is similar to the action of diclofenac, a commonly used non-steroidal anti-inflammatory drug. In addition, the protease from the leaves of *Artocarpus integer* has been successfully extracted using buffer solution followed by ammonium sulfate precipitation as partial purification [11].

To date, numerous researches have been conducted on the identification of secondary metabolites and phytochemicals present in *S. polyanthum* [9,12]. Recent findings have indicated that the phytochemicals and secondary metabolites isolated from *S. polyanthum* were nutritious and demonstrated positive effects as anti-diarrheal [9] and anti-diabetic agents [8]. Furthermore, this plant is commonly used by local people as a natural meat tenderizer and its leaves also have been extensively applied in traditional medicines by Indonesian people due to the medical importance and therapeutic activities [9,12]. However, no study has been conducted so far on this plant regarding protease extraction. This may probably be due to the fact that extraction of plant protease is complicated and there is no specific protocol that will suit all the diverse plant species [13-14].

This study, therefore aimed at evaluating different protease extraction method to isolate protease from the young leaves of *S. polyanthum.* In this study, the young leaves of *S. polyanthum* were extracted for the protease by using four different extraction protocols including sodium phosphate buffer extraction [11], QB solvent-based extraction [15], HBA solvent-based extraction [16] and Trichloroacetic acid (TCA)-acetone precipitation [17]. The crude extracts were assessed based on the specific activity. All these methods have been widely used for the extraction of protease from any part of plants especially the plant leaves [11,15,18].

■ EXPERIMENTAL SECTION

Materials

The young leaves of *S. polyanthum* were freshly plucked from the plants located at Kuantan, Pahang, Malaysia. They were cleaned using distilled water and then cut into small pieces. 150 mg of leaves sample was used in each extraction.

Instrumentation

Refrigerated centrifuge (5810R, Eppendorf, Germany) was used to separate the components of a complex mixture based on the centrifugal force of the instrument and density of the components. The absorbance of the enzyme samples was monitored by Ultraviolet-Visible Spectrophotometer (UV-Vis Spectrophotometer, Cary 50 Bio, Varian, United States).

Procedure

Sample preparation of S. polyanthum *leaves for different extraction method*

The young leaves of *S. polyanthum* were first divided into two sets; Set A and Set B. For Set A, the leaves were cut into small pieces and then treated with liquid nitrogen in pre-cooled mortar and pestle meanwhile for Set B, the leaves were only cut into small pieces and fined using mortar and pestle without liquid nitrogen. About 150 mg of *S. polyanthum* leaves samples from both sets were used for each of the following extraction methods; sodium phosphate buffer extraction, QB solvent-based extraction, HBA solvent-based extraction, and TCA-acetone precipitation. The temperature was kept constant at 4 °C throughout the experiment by keeping the crude enzyme sample in the ice box to avoid enzyme inactivation and denaturation.

The crude extract was analysed for protease activity assay and total protein. A simplified flow chart of the experimental steps is summarized in Fig. 1.

Sodium phosphate buffer solvent-based extraction

The extraction method was adapted from [11] with modification. 1 mL of 0.05 M sodium phosphate buffer, pH 7.0 was used to extract 150 mg *S. polyanthum* leaves samples for both Set A and Set B. The sodium phosphate buffer prepared contains 100 mM sodium chloride (NaCl), 10 mM ascorbic acid and 0.75 g polyvinylpolypyrrolidone (PVPP). The homogenates were filtered using Whatman No 1 filter paper before centrifugation for 20 min at 4 °C and 10000 rpm using a refrigerated centrifuge. After centrifugation, the supernatant (crude enzyme) was transferred from the 15 mL centrifuge tube and then stored at 4 °C for further investigation of protease activity assay and total protein determination.

QB solvent-based extraction

QB solvent-based extraction was conducted by following the method reported by [15] with slight modification. 150 mg leaves sample was extracted with 1 mL of extraction buffer containing 2 M potassium phosphate, pH 7.8, 1% Triton X 100, 80% glycerol, 1 M dithiothreitol (DTT) and distilled water in 15 mL centrifuge tube. The mixture was incubated and vortexed vigorously for 30 sec. Then, the lysate was centrifuged at 12000 rpm for 20 min at 4 °C using a refrigerated centrifuge. After centrifugation, the supernatant (crude enzyme) was transferred and stored at -20 °C for further analysis.

HBA solvent-based extraction

HBA method described by [16] was followed with slight modification. The extracting solvent consists of 100 Mm Tris pH 7.5 and 10% sucrose. Then, 0.28% β-mercaptoethanol was added into the solvent. 150 mg leaves sample were incubated with 1 mL of extraction buffer in 15 mL centrifuge tube. The mixture was vortexed for 30 sec before it was incubated in ice for 15 min. After incubation, the mixture was centrifuged for 10 min at 4 °C and 12000 rpm using a refrigerated centrifuge. After centrifugation had completed, the supernatant was collected and then stored at -20 °C for further investigation.

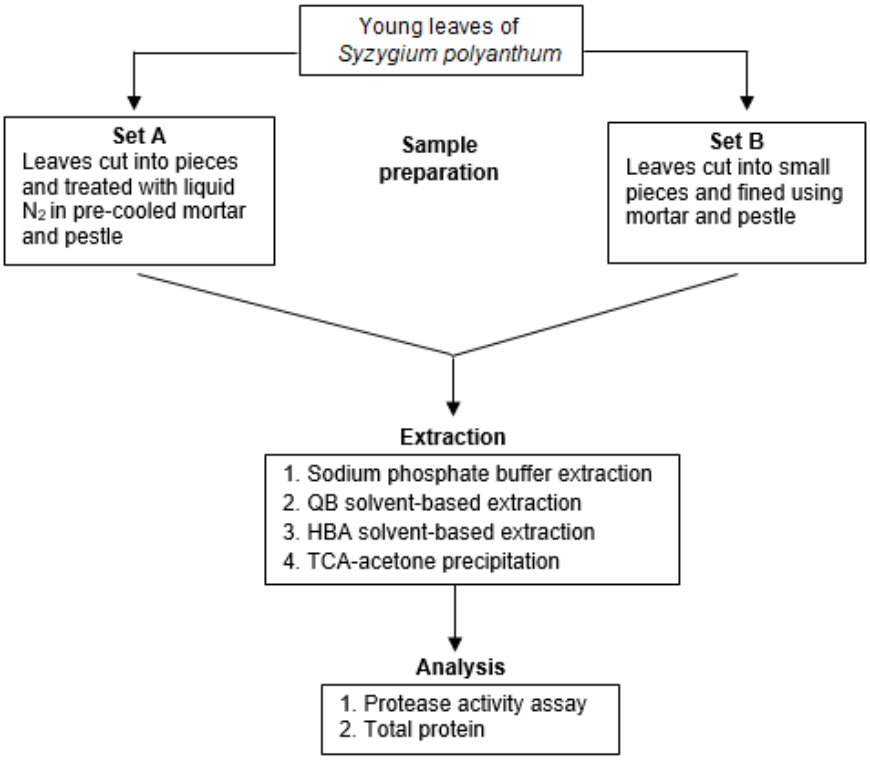

Fig 1. A simplified flow chart of the experimental steps

TCA-acetone precipitation method

TCA-acetone precipitation was conducted by following the protocol reported by [17] with slight modification. The extraction solution was freshly prepared with 10% TCA and 0.07% β-mercaptoethanol in cold acetone. 2 mL of extraction solvent was used to extract 150 mg of leaves in 15 mL centrifuge tube. The mixture was incubated for 24 h at 4 °C. Then, the mixture was centrifuged for 15 min at 10000 rpm, and 4 °C using a refrigerated centrifuge. The supernatant was transferred to other tubes to remain only the pellet inside the tube. The pellet was rinsed twice until the pellet becomes colorless using ice-cold acetone with 0.07% β-mercaptoethanol. Finally, the pellet was dried and resuspended in 0.05 M sodium phosphate buffer, pH 7.0. Both pellet and supernatant were assayed for their total protein and protease activity to confirm the presence of protease.

Total protein determination

The quantitative analysis of the total protein concentration was conducted using Bradford method [19] using Bio-Rad Protein Assay (Bio-Rad, Germany). The reaction mixture was prepared by the addition of 0.5 mL of Bradford reagent, 0.1 mL of protein sample and 0.4 mL of distilled water into a 1.5 mL microcentrifuge tube. The tube was capped and swirled slowly. The mixture inside the tube was left to react for 5 min at room temperature. The determination was carried out in triplicates. Then, the protein content was monitored by measuring the absorbance of the samples at 595 nm using UV-Vis Spectrophotometer. The amount of protein present in each of the samples was calculated from the bovine serum albumin standard curve which was constructed with the concentration of 0 to 1.20 mg/mL.

Protease activity assay

The protease activity assay was conducted by using casein as the substrate by following the protocol by Sigma Aldrich [20]. 0.5 mL of 0.65% (w/v) casein in 0.05 M potassium phosphate buffer, pH 7.5, was added into a 15 mL centrifuge tube that containing 0.1 mL of the crude enzyme extract and partially purified enzyme (for TCA-acetone precipitation method). The tube was capped and swirled slowly and the mixture was then incubated at 37 °C for 10 min. The enzymatic reaction was terminated by the addition of 0.5 mL of 110 mM trichloroacetic acid (TCA) into the tube. The mixture was further incubated at 37 °C for 30 min. Next, the mixture was filtered into 1.5 mL microcentrifuge tube by using a syringe equipped with 0.45 µL filter. 0.25 mL of filtrate was mixed with 0.65 mL of sodium carbonate solution and 0.15 mL of 0.5 M of Folin-Ciocalteau reagent. The mixture was incubated again at 37 °C for another 30 min. All the enzyme samples were assayed in triplicates. After the completion of incubation process, the absorbance of the enzyme samples were measured at 660 nm in Ultraviolet-Visible Spectrophotometer (UV-Vis Spectrophotometer). One unit of protease activity represents the amount of enzyme catalyzing the production of 1 µmol of tyrosine per minute at 37 °C in the reaction. The L-tyrosine standard curve was constructed for a concentration range from 0–0.110 µmol to determine the amount of tyrosine liberated from the enzyme reaction in this assay. The protease activity and specific activity of the protease were calculated using Eq. (1) and (2).

$$\text{Protease activity (U/mL)} = \frac{\text{Tyrosine conc.}(\mu\text{mol}) \times \text{Vol. of reaction solution}(\text{mL})}{\text{Vol. of crude enzyme}(\text{mL}) \times \text{Vol. of sample in cuvette}(\text{mL}) \times \text{Reaction time}(\text{min})} \quad (1)$$

$$\text{Specific activity (U/mg)} = \frac{\text{Protease activity } (\frac{\text{U}}{\text{mL}})}{\text{Total protein } (\frac{\text{mg}}{\text{mL}})} \quad (2)$$

■ RESULTS AND DISCUSSION

Comparison of Protease Activity and Specific Activity by Different Extraction Protocols

The young leaves of *S. polyanthum* were first divided into two sets of experiment samples; Set A, involved pre-treatment of the fresh leaves using liquid nitrogen in a pre-cooled mortar and pestle, while Set B, without pre-treatment of liquid nitrogen but the fresh leaves were cut into small pieces and ground using mortar and pestle. The leaves samples were extracted by using four extraction protocols including sodium phosphate buffer extraction [11], QB solvent-based extraction [15], HBA solvent-based extraction [16] and Trichloroacetic acid (TCA)-acetone precipitation [17].

Table 1. Protease activity and total protein of protease extracted from the leaves of *S. polyanthum* by four different extraction protocols

Extraction Protocols		Protease Activity (U/mL)		Total Protein (mg/ mL)	
		Set A (Pretreated with liquid N_2)	Set B (Non-pretreated with liquid N_2)	Set A (Pretreated with liquid N_2)	Set B (Non-pretreated with liquid N_2)
Sodium Phosphate Buffer		0.271 ± 0.023	0.361 ± 0.012	0.368 ± 0.003	0.312 ± 0.024
QB Solvent		1.510 ± 0.127	1.000 ± 0.010	0.192 ± 0.006	0.098 ± 0.003
HBA Solvent		0.012 ± 0.064	0.502 ± 0.031	5.148 ± 0.004	0.357 ± 0.011
TCA-acetone	P	n.d.	n.d.	n.d	n.d
	S	0.033 ± 0.018	0.935 ± 0.006	17.854 ± 0.002	0.557 ± 0.004

P= precipitate; S= supernatant; n.d. = not detected

Table 2. Specific activity of the protease isolated from the leaves of *S. polyanthum* by four different extraction protocols

Extraction Protocols		Specific Activity (U/mg)	
		Set A (Pretreated with liquid N_2)	Set B (Non-pretreated with liquid N_2)
Sodium Phosphate Buffer		0.736 ± 0.063	1.157 ± 0.097
QB Solvent		7.865 ± 0.706	10.204 ± 0.329
HBA Solvent		0.002 ± 0.011	1.406 ± 0.097
TCA-acetone	P	n.d.	n.d
	S	0.0005 ± 0.003	0.862 ± 0.008

P= precipitate; S= supernatant; n.d. = not detected; *p*-value = 0.0009

150 mg of the leaves samples were kept constant for each extraction method so that the results obtained are comparable. The crude enzyme extracted was measured based on the protease activity and specific activity. The results are tabulated in Table 1 and Table 2.

From 150 mg of leaves samples used, protease activity recorded is shown in Table 1. Both Set A and Set B were observed to have high contents for QB solvent-based extraction method: 1.510 ± 0.127 U/mL (Set A) and 1.000 ± 0.010 U/mL (Set B). For TCA acetone precipitation method, the protease activity of 0.033 ± 0.018 U/mL and 0.935 ± 0.006 U/mL were found only in the supernatant for both Set A and Set B, respectively but none of the protease activity was detected in precipitate for both Set A and Set B. Other than that, the total protein was also detected in all extraction methods except in the pellet of TCA-acetone precipitation for both Set A and Set B.

The results in Table 1 shows the high amount of total protein in Set A due to the pre-treatment of liquid nitrogen compared to Set B sample (without pre-treatment of liquid nitrogen). The extraction of protein from the plant leaves is quite challenging because the leaves have a cell wall which is rich in fiber content and composed of polysaccharides cellulose, pectin, and lignin. The presence of these structures may cause difficulty in crushing the plant tissues [29]. In the study, the purpose of using liquid nitrogen during sample preparation was to ensure complete disruption of the sample for easy pulverization of the young leaves into a powdery form. According to Wang et al. [21], one of the important factors that may affect the efficiency of extraction from a tissue sample was the quality of the sample disruption. This report agrees with Wu et al. [22] which stated that the extraction of plant tissue is really affected by the fineness of the tissue powder formed after pulverization since plant tissues are protected by a rigid cell wall. Besides that, the usage of liquid nitrogen associated with precooled mortar and pestle also may prevent protein degradation because it requires a shorter time to disrupt the plant tissues compared to sample without pre-treatment with liquid nitrogen [29].

Liquid nitrogen is known as cryogenic liquid that exists in a liquid state at a very low temperature of -196 °C which means, it may cause flash freezing during its contact with living tissue and has an effect on the protease activity in the leave extract [28]. Since high value of specific activity is required [25], therefore, a null hypothesis was made and the *p*-value was determined to investigate that the pre-treatment of liquid nitrogen may not affect an increase in the value of specific activity of the protease. Based on the results provided in Table 2, QB solvent for Set B (without pre-treatment of liquid nitrogen) recorded the highest specific activity (10.204 ± 0.329 U/mg). From this result, it is clear that the involvement of liquid nitrogen in the sample preparation does not affect the value of the specific activity of the protease ($p = 0.0009$).

S. polyanthum is rich in phenolic compounds [23] and may affect the extraction process because these compounds may bind and precipitate enzymes, thus resulting in the loss of protease activity. There are several factors that should be considered in choosing the best buffer for protease extraction from the plants, for example, salt, reducing agents, buffering system and stabilizing elements [31]. Each of the factors plays an important role in the efficiency of the extraction. In the extraction using a sodium phosphate buffer, sodium chloride is added as an additive into the buffer to maintain the ionic strength of the buffer and to improve the stability of the protease [32]. Other than salt, polyvinylpolypyrrolidone (PVPP) also was used as the component of the buffer. This chemical can eliminate the phenolic compound especially from the plant tissues with a high content of phenolic compounds like *S. polyanthum* [29].

For QB solvent method, the extraction buffer consists of several additives like dithiothreitol (DTT), Triton X 100 and glycerol. DTT is a thiol agent used to reduce the disulfide bonds and the usage of non-ionic detergent like Triton X-100 may be effective in trapping phenolics in the detergent phase to enable the removal of these compounds and subsequently increasing the purity of the protease [17]. Besides that, the addition of stabilizing element like glycerol also may help in increasing the solution viscosity thus preventing aggregation of the protease [31]. All of these factors may contribute to a good specific activity performed by the protease extracted by this method.

In this study, Tris buffer of pH 7.5 was used in HBA solvent-based extraction method. Tris buffer is temperature dependent and it contains reactive amines that may be reactive in enzymatic reaction [32]. To avoid complication during extraction in HBA solvent, β-mercaptoethanol, a reducing agent, was added in the extraction buffer to prevent oxidation of protease [17].

TCA-acetone precipitation method is a common method for protein. For this study, both supernatant and precipitate phases were tested to ensure the presence of protease. Theoretically, TCA-acetone precipitation method can reduce the solubility of the protein which can promote the formation of precipitate due to the isoelectric effect caused by trichloroacetic acid but somehow may be leaving the remaining protein as non-functional [30]. This could be the reason why protease activity of the protease extracted by TCA-acetone precipitation could not be detected in pellet for both Set A and Set B. Besides that, the pellets formed during the extraction were very little indicating that only a small amount of protease was precipitated. Furthermore, TCA-acetone precipitation method requires consecutive processes like homogenization and precipitation which may cause losses of protease.

In this present study, 0.05 M sodium phosphate buffer was used to dissolve the pellet. However, some portion of the pellet still remained undissolved in the buffer. This may be due to the level of solubility of the pellet in solubilizing buffer [13]. According to Carpentier et al. [24], the removal of interfering compounds in protease extraction was complicated. This probably may be due to the incompletion of foreign substances removal from the pellet which had interfered with the protease activity readings during spectrophotometric analysis. Besides, the protease remains in the supernatant due to the several factors like the speed of centrifugation and centrifugal force produced during centrifugation. Theoretically, the spinning of the samples at very high speeds, using the centrifugal force causes the density difference between

the protease and the solute which are enhanced significantly and the formation of the pellet can be observed [27]. During the extraction, only a small amount of pellet was formed in TCA-acetone precipitation method and this may cause undetectable reading of protease activity and total protein. Therefore, we can infer that TCA-acetone precipitation method is not suitable for protease extraction from the leaves of *S. polyanthum*.

■ CONCLUSION

This study has been able to evaluate different protease extraction methods namely; sodium phosphate buffer solvent-based extraction, QB solvent-based extraction, HBA solvent-based extraction, and TCA-acetone precipitation to isolate the protease from the young leaves of *S. polyanthum*. In conclusion, QB solvent-based extraction method showed the most suitable extraction method for the young leaves of *S. polyanthum* with the highest specific activity of 10.204 ± 0.329 U/mg (Set B leaves sample) obtained. Besides, the involvement of liquid nitrogen in the preparation and pre-treatment of the sample does not affect the value of the specific activity of the protease.

■ ACKNOWLEDGMENTS

We thank all the members of our laboratory for their helpful criticism and suggestions throughout this work. This work was supported by an internal grant, RDU 160334, awarded by Universiti Malaysia Pahang, Malaysia.

■ REFERENCES

[1] Jisha, V.N., Smitha, R.B., Pradeep, S., Sreedevi, S., Unni, K.N., Sajith, S., Priji, P., Josh, M.S., and Benjamin, S., 2013, Versatility of microbial proteases, *Adv. Enzyme Res.*, 1 (3), 39–51.

[2] Tomar, R., Kumar, R., and Jagannadham, M.V., 2008, A stable serine protease, wrightin, from the latex of the plant *Wrightia tinctoria* (Roxb.) R. Br: Purification and biochemical properties, *J. Agric. Food Chem.*, 56 (4), 1479–1487.

[3] Sawant, R., and Nagendran, S., 2014, Protease: An enzyme with multiple industrial applications, *World J. Pharm. Pharm. Sci.*, 3 (6), 568–579.

[4] Mótyán, J.A., Tóth, F., and Tőzsér, J., 2013, Research applications of proteolytic enzymes in molecular biology, *Biomolecules*, 3 (4), 923–942.

[5] Bonne, K., and Verbeke, W., 2008, Muslim consumer trust in halal meat status and control in Belgium, *Meat Sci.*, 79 (1), 113–123.

[6] Ishwarya, S., and Sangeetha, R., 2013, A new serine protease from the leaves of *Thespesia populnea, Prep. Biochem. Biotechnol.*, 43 (1), 95–107.

[7] Gupta, A., Shah, A.P., and Chaphalkar, S.R., 2016, Extraction of proteases from medicinal plants and their potential as anti-viral targets, *J. Biotechnol. Biomater.*, 6 (2), 1000228.

[8] Widyawati, T., Yusoff, N.A., Asmawi, M.Z., and Ahmad, M., 2015, Antihyperglycemic effect of methanol extract of *Syzygium polyanthum* (Wight.) leaf in streptozotocin-induced diabetic rats, *Nutrients*, 7 (9), 7764–7780.

[9] Malik, A., and Ahmad, A.R., 2013, Antidiarrheal activity of ethanolic extract of bay leaves (*Syzygium polyanthum* [Wight.] Walp.), *Int. Res. J. Pharm.*, 4 (4), 106–108.

[10] Gonçalves, R.N., Barbosa, S.D.G., and da Silva-López, R.E., 2016, Proteases from *Canavalia ensiformis*: Active and thermostable enzymes with potential of application in biotechnology, *Biotechnol. Res. Int.*, 2016, 3427098.

[11] Balqis, Z.S., and Rosma, A., 2011, *Artocarpus integer* leaf protease: Purification and characterisation, *Food Chem.*, 129 (4), 1523–1529.

[12] Har, L.W., and Ismail, I.S., 2012, Antioxidant activity, total phenolics and total flavonoids of *Syzygium polyanthum* (Wight) Walp leaves, *Int. J. Med. Aromat. Plants*, 2 (2), 219–228.

[13] Maldonado, A.M., Echevarría-Zomeño, S., Jean-Baptiste, S., Hernández, M., and Jorrín-Novo, J.V., 2008, Evaluation of three different protocols of protein extraction for *Arabidopsis thaliana* leaf proteome analysis by two-dimensional electrophoresis, *J. Proteomics*, 71 (4), 461–472.

[14] Isaacson, T., Damasceno, C.M.B., Saravanan, R.S., He, Y., Catala, C., and Rose, J.K.C., 2006, Sample

extraction techniques for enhanced proteomic analysis of plant tissue, *Nat. Protoc.*, 1 (2), 769–774.

[15] Ni, M., Dehesh, K., Tepperman, J.M., and Quail, P.H., 1996, GT-2: *in vivo* transcriptional activation activity and definition of novel twin DNA binding domains with reciprocal target sequence selectivity, *Plant Cell*, 8 (6), 1041–1059.

[16] Fan, Z., and Yuan, L., 2010, Production of multifunctional chimaeric enzymes in plants: A promising approach for degrading plant cell wall from within, *Plant Biotechnol. J.*, 8 (3), 308–315.

[17] Abdullah, F., Chua, L.S., and Rahmat, Z., 2017, Comparison of protein extraction methods for the leaves of *Ficus deltoidea*, *J. Fundam. Appl. Sci.*, 9 (2), 908–924.

[18] Faurobert, M., Pelpoir, E., and Chaïb, J., 2007, Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues, *Plant Proteomics*, 335 (2), 9–14.

[19] Bradford, M.M., 1976, A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*, 72 (1-2), 248–254.

[20] Cupp-Enyard, C., 2008, Sigma's non-specific protease activity assay-casein as a substrate, *J. Vis. Exp.*, 19, 899.

[21] Wang, W., Vignani, R., Scali, M., and Cresti, M., 2006, A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis, *Electrophoresis*, 27 (13), 2782–2786.

[22] Wu, X., Gong, F., and Wang, W., 2014, Protein extraction from plant tissues for 2DE and its application in proteomic analysis, *Proteomics*, 14 (6), 645–658.

[23] Rahim, E.N.A.A., Ismail, A., Omar, M.N., Rahmat, U.N., and Ahmad, W.A.N.W., 2017, GC-MS analysis of phytochemical compounds in *Syzygium polyanthum* leaves extracted using ultrasound-assisted method, *Pharmacogn. J.*, 10 (1), 110–119.

[24] Carpentier, S.C., Witters, E., Laukens, K., Dekkers, P., Swennen, R., and Panis, B., 2005, Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis, *Proteomics*, 5 (10), 2497–2507.

[25] Theresa, P., *Specific Activity and Its Importance in Protein Isolation*, https://www.thebalance.com, accessed on 27 July 2018.

[26] G-Biosciences, *Plant Protein Extraction and Protein Precipitation Technique*, https://info.gbiosciences.com/blog/plant-protein-extraction-and-protein-precipitation-techniques, accessed on 23 May 2018].

[27] JoVE Science Education Database, *General Laboratory Techniques. An Introduction to the Centrifuge*, JoVE, Cambridge, MA, https://www.jove.com/science-education/5019/an-introduction-to-the-centrifuge, accessed on 23 May 2018.

[28] Naufumi, H., 1972, Denaturation of enzyme protein by freeze-thawing and freeze-drying; I. freeze-thawing and freeze-drying of myosin and some other muscle proteins, *Institute of Low Temperature Science*, B17, 1–20.

[29] Özbek, A.G., and Bilek, S.E., 2018, Plant based protein sources and extraction, *Current Investigations in Agriculture and Current Research*, 169-171.

[30] G-Biosciences, *What protein precipitation techniques are used for concentration and clean up?*, https://info.gbiosciences.com/blog/bid/163405/what-protein-precipitation-techniques-are-used-for-concentration-and-clean-up, accessed on 26 September 2018.

[31] Cable, J., How to design the perfect protein purification buffer, https://bitesizebio.com/7893/how-to-design-the-perfect-protein-purification-buffer/, accessed on 27 September 2018.

[32] European Molecular Biology Laboratory, *Protein purification: Extraction and clarification choice of lysis buffer and additives*, https://www.embl.de/pepcore/pepcore_services/protein_purification/extraction_clarification/solubility_studies/, accessed on 27 September 2018.