

***Elateriospermum tapos* Seed Protein as a New Potential Therapeutic for Diabetes, Obesity and Hypertension: Extraction and Characterization of Protein**

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ABSTRACT

The *Elateriospermum tapos* seed extract is one of the major contributors to the therapeutic effects as remedy drink for chronic diseases, such as hypertension, diabetes and obesity by the locals in East Malaysia. It is believed that the component in the seed, such as protein, plays an essential role due to its nutraceutical properties. Hence, we report the extraction of protein from the seed of *E. tapos* that has the potential biological activities. The protein was initially extracted using salt solution, which was similar to the local practice and maximal values of extraction yield (44 mg/g) was performed based on the extraction parameters; pH 8, sample to buffer ratio, 1:50 (w/v), 2 h at 80 °C. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profile showed that 7S globulin was the dominant protein in the *E. tapos* seed protein isolate (ETSPI), whereas Fourier Transform Infrared (FTIR) analysis showed that the extracted protein consisted of α -helical secondary structure. This protein could be digested up to 72.5% *in vitro*. In the biological activity evaluation, the results showed that ETSPI exhibited angiotensin converting enzyme (ACE) inhibitory (56.6%/100 μ g), lipase inhibitory (26.7%/100 μ g), α -amylase inhibitory (31.2%/100 μ g), free radical scavenging (33.4%/mg) and metal reducing activities (0.97 mM FeSO₄/mg), which suggests that this protein has the potential in the aforementioned therapeutic effects.

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INTRODUCTION

Elateriospermum tapos (*E. tapos*), locally known as *kelampai* or *buah perah*, is an

indigenous palatable seed commonly found in Borneo, Peninsular Malaysia, Thailand as well in the Sumatra tropical rainforest (Van Sam & Van Welzen, 2004). This monoecious canopy species originated from the family of Euphorbiaceae and subfamily of Crotonoideae. It was reported that the fresh latex from *E. tapos* can be utilized traditionally as a wound healing agent (Corner, 1988). Apart from that, *E. tapos* was reported to be a good source of edible oil due to the extensive high amount of α -linolenic acid content (Yong & Salimon, 2006). In addition, the seed flour of the *E. tapos* was found to exhibit a good emulsifying property, which could be potentially used as a food formulation agent (Choonhahirun, 2010). The seeds were also popular among the locals as they believed that aqueous extract (as a traditional medicine drink) from *E. tapos* seeds have the ability in treating chronic diseases, such as hypertension. Even though there are many researches on the phytochemical compositions and nutritional value of *E. tapos*, however, the protein in the seed, which could also be present in the drink as aforementioned and play a major role in the therapeutic properties, has not been investigated. Storage proteins (e.g. globulin, glutemin, prolamin), α -amylase inhibitor, lectins and Bowman-Birk-inhibitors were some examples of the documented legumes derived bioactive protein, which had the potential in lowering the blood glucose and blood pressure. The importance of the pharmacological properties that implied on plant protein had lead the researchers

in further investigating its roles, notably isolated from seed in reducing the risk of certain diseases. For instance, the protein isolated from Canary seed (Valverde et al., 2017) and Amaranth seed (Fritz et al., 2011) were reported to have the ability in reducing hypertension. Besides, many researches on seed protein had proclaimed that seed protein could be an antioxidant agent, such as canary seed protein (Valverde et al., 2017), canola seed protein (Cumby et al., 2008) and watermelon seed protein (Dash & Ghosh, 2017). Hence, the scope of our research was to be mainly focusing on finding the best extraction parameter of the protein as well as evaluating the biological activities (i.e. antidiabetic, antihypertensive, antiobesity and antioxidant activities) of the isolated protein from the local medicinal drink.

MATERIALS AND METHODS

Chemicals

All the enzymes (i.e. α -amylase (A3176), lipase (L3126) and angiotensin converting enzyme, (A6778)) used in the experiment were purchased from Sigma-Aldrich Company, Malaysia. The chemicals and reagents were used in the experiment were of analytical grade and purchased from Sigma-Aldrich, Malaysia, unless stated otherwise in the method.

Materials

Elateriospermum tapos fruits were purchased from Market Satok, located in Kuching, Sarawak. The seed of the fruit was separated

and washed with deionized water before lyophilised using Labconco Freeze dryer (Fisher Scientific, USA). Subsequently, blender was used to grind the dried seed into powders, and they were then sieved (30 mesh). The seed powders were defatted by using n-hexane before being proceeded with the extraction process.

Extraction of *E. tapos* Seed Protein Isolate (ETSPI)

Extraction of ETSPI was conducted according to method by Adebawale et al. (2007) with slightly modified and the ETSPIs were extracted in three independent batches as replicates. Generally, the defatted seed powder (1 g) was suspended in 0.2 M sodium phosphate buffer (Bendosen Laboratory Chemicals, Malaysia) at different pH (i.e. 6-8) and different sample to buffer ratio (i.e. 1:10 – 1:50, w/v). Subsequently, the solution was incubated at the designated temperature (i.e. 40-80°C) and time (i.e. 0.5-2.5 h) at a constant orbital shaking speed of 250 rpm. The resulting slurry from the extraction was then centrifuged for 30 min at constant speed of 4500 rpm and the supernatant was collected and being determined the protein content by using the Bradford assay (Bradford, 1976). The extraction yield was determined based on the Bradford analysis, and expressed in mg protein per gram of sample.

Experimental Design

The single factor experiment was conducted in determining the best condition for extracting the free protein. A total of four

parameters, namely, pH, temperature, sample to buffer ratio and extraction time were investigated on which one of the parameter was changed while the other parameters were kept constant.

For the effect of pH, pH ranging from 6 to 8 with interval of 0.5 was studied, whereas the temperature, time and sample to buffer ratio were kept constant at 60 °C, 1.5 h and (1:30, w/v), respectively.

For the effect of time, extraction period ranging from 0.5 to 2.5 h with interval of 0.5 was studied, whereas the temperature, pH and sample to buffer ratio were kept constant at 60 °C, pH 7 and (1:30, w/v), respectively.

For the effect of temperature, temperature ranging from 40 to 80 °C with interval of 10 °C was studied, whereas the pH, time and sample to buffer ratio were kept constant at pH 7, 1.5 h and (1:30, w/v), respectively.

For the effect of sample to buffer ratio, the ratio at 1:10, 1:20, 1:30, 1:40 and 1:50 (w/v) were studied, whereas the temperature, time and pH were kept constant at 60 °C, 1.5 h and pH 7, respectively.

Characterization of ETSPI

The ETSPI was prepared according to aforementioned extraction procedure at the best condition and the protein was precipitated by using 0.1 M HCl through the adjustment of pH to the 4.5. The precipitated was then centrifuged at speed of 4500 rpm for 30 min, lyophilized and stored at 4°C prior to analysis.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The ETSPI was identified by performing SDS-PAGE analysis under reducing conditions with used of 12% resolving gel and 4% stacking gel based on method of Laemmli (1970). All the SDS-PAGE reagents were purchased from Bio-Rad (U.S.A). Precision plus protein standard marker (10-250 kD) was used as the standard marker for this SDS-PAGE analysis.

Fourier Transform Infrared (FTIR) Spectroscopy

ETSPI lyophilized powder was desiccated prior to FTIR analysis. The FTIR spectra of samples were recorded from 500 to 4000 cm^{-1} using Nicolet Avatar 360 FT-IR E.S.P spectrometer with attenuated total reflectance (ATR) system (Agilent Technologies, Santa Clara, USA). Background scan was conducted prior to the sample scan. Isopropanol was used to clean the sample cell before loading a new sample. The resolution was 4 cm^{-1} , and iterations were performed 32 times. The scan speed was set at 5 kHz and sensitivity of 1. The spectra were analyzed using Resolution Pro version 5.2.0 software.

***In vitro* Digestibility of ETSPI**

In vitro digestibility of ETSPI was determined according to method as described by Hsu et al. (1977). In general, ETSPI solution (6.25 mg/ml) was prepared and the pH of sample was adjusted to 8 using NaOH. The multi-enzyme mixture (i.e. trypsin, chymotrypsin

and pepsin) were immediately added to the sample and subjected to a continuous stirring at 37 °C for 10 min. The pH was recorded for every 1 min during the 10 min digestion period. The percentage of digestibility was calculated using the equation below:

$$\% \text{ Digestibility} = 210.46 - 18.10X \quad (\text{eq. 1})$$

where, X is the pH of the sample after 10 min of digestion.

Amino Acid Composition of ETSPI

The ETSPI (0.1 g) was hydrolyzed at 110 °C for 24 h with 6 M HCl under seal condition. The sample was then added with 400 μl of 50 $\mu\text{mole/ml}$ of L- α -amino-n-butyric acid (AABA, internal standard) and topped up to 100 ml using distilled deionized water. Subsequently, the sample was analyzed using a Waters-HPLC-System (U.S.A) with Waters 1525 Binary Pump, Waters 717 plus Autosampler, and Waters 2475 Multi λ Fluorescence Detector (Tan et al., 2014). Waters AccQ-Tag™ Amino Acid Analysis Column (3.9 mm \times 150 mm; packing material: silica based bonded with C_{18}) was used and maintained at 37 °C during analysis. Fluorescence was measured at wavelength of 250 nm for excitation and 395 nm for emission. Control of the apparatus and solvent mixing as well as plotting and evaluating were carried out using Breeze Workstation version 3.20. Two mobile phases were used: (a) AccQ-Tag™ Eluent A; (b) Acetonitrile/Water (60%/40%), and amino acids standard H (PIERCE, U.S.) was used as reference to calibrate the

HPLC-system. Methionine and cysteine were analyzed separately using performic acid procedure (Moore, 1963). Briefly, the sample (0.1 g) was mixed with 2 ml of performic acid solution (prepared by mixing 0.2 ml of 30% H₂O₂ with 1.8 ml of 88% formic acid) in an ice bath (0 °C) for 4 h, and subsequently, 0.3 ml of 48% hydrogen bromine (Merck, Germany) was added to the mixture in ice bath. In order to remove the bromine in the solution, 20 ml of 1 N NaOH (Mallinckrodt, U.S.A.) was added to the mixture and the resulting mixture was dried at 40 °C for 30 min. The sample was then hydrolyzed using 3 ml of 6 N HCl in an oven at 110 °C for 18 h. The HCl was then removed using rotary evaporator and the residue was dissolved in 5 ml buffer (pH 2) prior to analysis using the same setting. The amino acid composition of the ETSPI sample was presented as per 1000 residues.

***In vitro* Antioxidant Activities Determination**

The antioxidant activity of ETSPI was performed using 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) assay as described by Cai et al. (2004). The ABTS reagent was firstly incubated in the dark for 16 h at room temperature. The reagent was then diluted using ethanol to obtain an absorbance reading of 0.700 ± 0.005 at 734 nm. Subsequently, the diluted ABTS solution (3 ml) was mixed with ETSPI (5 mg) and incubated in dark for 6 min at 23 °C. The absorbance of mixtures was recorded at 734 nm. The ABTS scavenging activity was

calculated using the equation below:

$$\text{ABTS scavenging radical activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \quad (\text{eq. 2})$$

where A_{control} was referred to absorbance of control at 734 nm while A_{sample} was referred to absorbance of sample at 734 nm.

Ferric reducing antioxidant power (FRAP) analysis was conducted according to the method of Benzie and Strain (1999). The FRAP reagent (3 ml) was pre-warmed for 30 min at 37 °C before added to the ETSPI sample (5 mg). Subsequently, the sample mixture was vortexed and incubated for 1 h at 37 °C. The absorbance of resulting mixture was measured at 593 nm using a spectrophotometer (Spectamax M5, Molecular Devices, USA). Iron (II) sulphate heptahydrate (FeSO₄·7H₂O) was used as a standard, and the result was expressed as mM FeSO₄ per gram of sample.

***In vitro* α-amylase Inhibitory Assay of ETSPI**

The α-amylase inhibitory assay was carried out based on the method of Apostolidis et al. (2007). Briefly, ETSPI sample solution (100 µl at concentration of 1 mg/ml) and α-amylase (100 µl at concentration of 1 mg/ml) were mixed and incubated for 10 min at 25 °C. Subsequently, starch solution (100 µl at concentration of 1% (w/v)) was added to mixture and the incubation was continued for 10 min at 25 °C. Afterwards, dinitrosalicylic acid reagent (200 µl) was added and the resulting mixtures were heated at 95 °C for 5 min. The resulting mixtures were cooled to room temperature

and diluted with 3 ml of deionized water prior to the absorbance measurement at 540 nm. The α -amylase inhibitory percentage was calculated using the equation below:

$$\% \text{ amylase inhibitory} = \frac{(A_{\text{pos}} - A_{\text{blank}}) - (A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{pos}} - A_{\text{blank}})} \times 100\%$$

(eq. 3)

where A_{pos} was referred as absorbance of α -amylase and starch without addition of sample while A_{blank} was absorbances of blank, A_{sample} was absorbance of mixture of ETSPI solution and α -amylase solution, A_{blank} was absorbance of sample blank.

***In vitro* Angiotensin Converting Enzyme (ACE) Inhibitory Assay of ETSPI**

The angiotensin converting enzyme (ACE) inhibitory activity of ETSPI was performed according to the method by Cushman and Cheung (1971). The ETSPI solution (100 μ l at concentration of 1 mg/ml) was vigorously mixed with ACE solution (100 μ l at concentration of 50 mU/ml) and pre-incubated at 37 °C for 10 min. Consequently, histidine-histidine-leucine (HHL, 300 μ l) was added and the mixtures were incubated for another 30 min. Subsequently, 1 ml of 1 M HCl was added in order to terminate the reaction. The hippuric acid formed was then extracted using the 1.6 ml of ethyl acetate (Fisher Scientific, Malaysia). The upper fraction was collected and dried by using the Vacuum Concentrator 5301 (Eppendorf, Germany) for 1 h. The pellet obtain was then solubilized with 2 ml of deionized water and the absorbance of resulting solution was determined at 228 nm using a UV-vis spectrophotometer

(Spectamax M5, Molecular Devices, USA). The inhibitory percentage was expressed using the following equation:

$$\% \text{ ACE inhibitory} = \frac{(A_{\text{pos}} - A_{\text{blank}}) - (A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{pos}} - A_{\text{blank}})} \times 100\%$$

(eq. 4)

where A_{pos} was referred as the absorbance of ACE and HHL without addition of sample, while A_{blank} was absorbance of the blank, A_{sample} was absorbance of the mixture of ETSPI solution, ACE and HHL solution.

***In vitro* pancreatic lipase inhibiting assay of ETSPI**

The lipase inhibitory activity of ETSPI was evaluated using the method as described by Shimura et al. (1992). A mixture of ETSPI solution (100 μ l at a concentration of 1 mg/ml), phosphate buffer (0.5 ml, Bendosen Laboratory Chemicals, Malaysia) and olive oil (100 μ l, Bertolli®) were pre-incubated for 10 min at 37 °C. Subsequently, the lipase solution (100 μ l at a concentration of 0.71 mg/ml) was added into the resulting solution prior to incubation for 1 h at 37 °C. The reaction mixtures were then terminated at 95 °C for 10 min. The resulting of fatty acid released upon the reaction mixtures was determined using the titration with 0.1 N NaOH. The lipase inhibitory percentage was calculated based on the equation below.

$$\% \text{ lipase inhibitory} = \frac{V_e - V_s}{V_s} \times 100$$

(eq. 5)

where V_e was referred as the titration volume for the control (containing lipase and olive oil without ETSPI) whereas V_s

was referred as the titration volume for the sample (containing lipase, olive oil and ETSPI).

Statistical Analysis

The statistical results were performed by using SPSS version 21 (IBM, USA). One-way ANOVA was carried out and the means of results were compared by using DUNCAN multiple-range tests ($p < 0.05$). All the analyses were conducted in at least 3 replicates, and in each replicate, triplicates were used to obtain an average data.

RESULTS

Effect of pH, Sample to Buffer Ratio, Temperature and Time on Extraction Yield

As shown in Figure 1, the pH did not show a significant ($p > 0.05$) change from pH 6 to

6.5. However, the yield was significantly ($p < 0.05$) increased from pH 6.5 to 8, and the highest yield up to 21.06 mg/g was obtained. As for the effects of sample-to-buffer ratio and temperature, the trends of protein yield were significantly ($p < 0.05$) increased when both of these parameters increased. On the other hand, the time showed significant ($p < 0.05$) increase from 0.5 to 1 h. However, this parameter did not show any significant ($p > 0.05$) changes between 1 to 1.5 h. The yield was then increased from 1.5 to 2 h, and reached the highest value of protein yield (14.26 mg/g). After 2 h of extraction, the protein yield decreased. Through these findings, it was found that pH 8, 1:50 w/v, 2 h, and 80 °C were considered as the suitable parameters in obtaining the highest yield of protein. The result showed that the crude protein yield obtained was 4.4% (w/w, dry basis), as shown in Table 1.

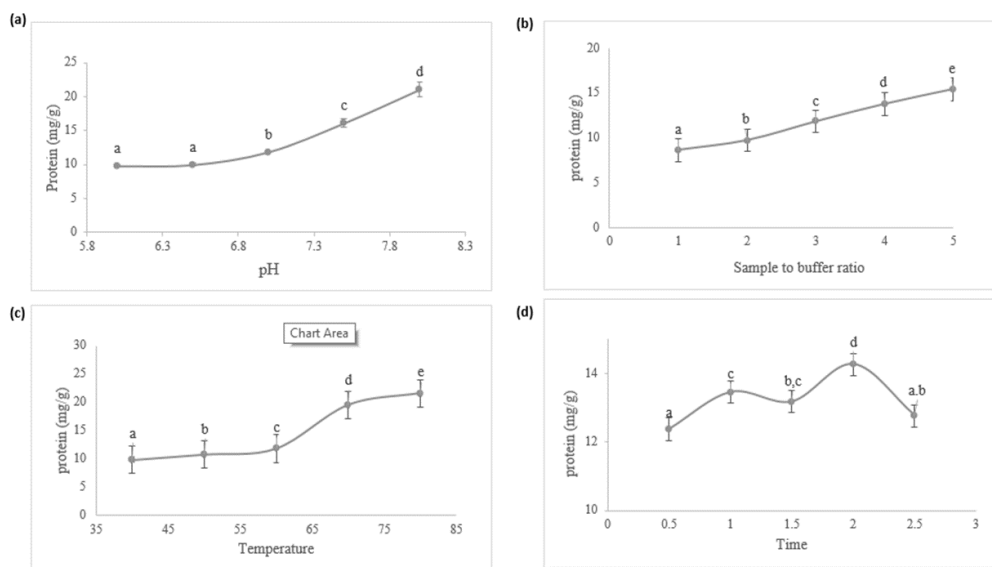


Figure 1. Effect of parameters (pH, sample to buffer ratio, temperature and time) towards the extraction yields

Table 1

Extraction yields, in vitro digestibility, antioxidant activities and biological activities of ETSPI

Extraction Yields and Activities	Values
Extraction yield, dry basis (% w/w)	4.4 ± 0.03
<i>In vitro</i> digestibility activity (%)	72.5 ± 1.2
Antioxidant activities	
(a) ABTS (%/mg)	33.4 ± 3.3
(b) FRAP (mM FeSO ₄ /mg)	0.97 ± 0.02
ACE inhibitory activity (%/100µg)	56.6 ± 3.5
α -amylase inhibitory activity (%/100µg)	31.2 ± 1.8
Lipase inhibitory activity (%/100µg)	26.7±1.2

Data points are mean ± standard deviation (*n*=3)

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Based on Figure 2, the ETSPI profile has the molecular weight bands in a range of 13 to 54 kD. The major intense band appeared at 31 kD corresponding to 7S globulin (vicilin subunit), which is one of the major storage proteins in seeds. In addition, the subunit of legumin (11S globulin) was also discerned with 2 basic bands at 21-22 kD and one minor acidic band at 44 kD (Barac et al., 2010).

Fourier Transform Infrared (FTIR) Spectroscopy

Based on Figure 3, the FTIR spectra shows the prominent bands of protein in the region of 1654 cm⁻¹, 1543 cm⁻¹ and 1454 cm⁻¹, which representing the functional groups of amide I, amide II and amide III, respectively. Other peaks at wavenumber of 520, 964, 1009, 1049, 2265, 2376, 2862, 2928, and

3445 cm⁻¹, were also found in other proteins (Chen et al., 2013; Gupta et al., 2010), which verified that extract was protein.

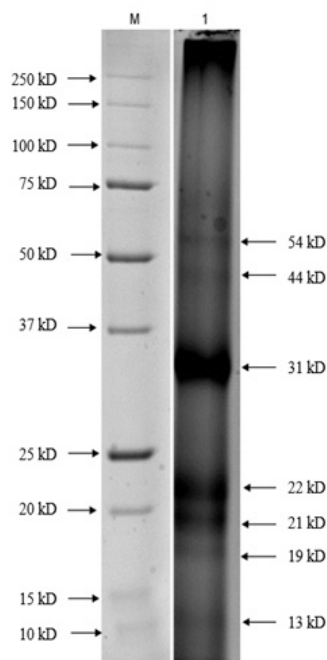


Figure 2. SDS PAGE profile of protein marker (Lane M) and ETSPI (Lane 1)

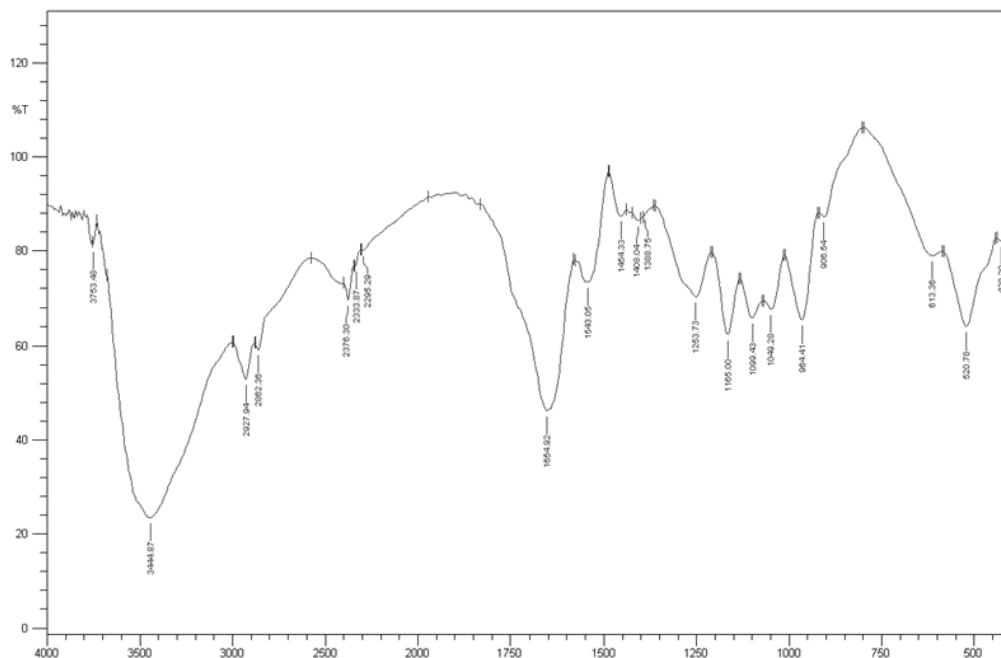


Figure 3. IR spectra of ETSPI

***In vitro* Digestibility**

As shown in Table 1, ETSPI showed slightly lower the digestibility value (72.5%) as compared with Quinoa protein (78.4%) (Elsouhaimy et al., 2015), cowpea protein isolate (78.7%) (El-jasser, 2010), and *Mucuna* bean protein (90.6%) (Adebowale et al., 2007).

Amino Acid Composition of ETSPI

The result showed that hydrophobic amino acids were dominant in ETSPI followed by acidic, uncharged polar and basic amino acids, as shown in Table 2. This characteristic amino acid profile was vital in correlation study of biological activities of ETSPI. Despite, the highest acidic amino residues such as aspartic and glutamic acid

might suggest the protein was acidic in nature. Nonetheless, ETSPI was found lack in sulphur-containing amino acids, such as cystine and methionine. This could be revealed as the methionine was commonly limiting in legumes due to its low amount in storage protein, globulin (Lam & de Lumen, 2003). In fact, the aqueous-alkaline extraction used in isolating ETSPI might give an effect in term of the destruction of these amino acids.

Biological Activities of ETSPI

Table 1 shows that ETSPI has potential in inhibiting angiotensin converting enzyme (ACE) (56.6%), lipase (26.7%) and α -amylase (31.2%). Apart from that, the protein also exhibited ABTS free radical

Table 2

Amino acid composition of ETSPI based on their chemical characteristics

Characteristic of Amino Acid	Per 1000 Amino Residues	Percentage Based on Characteristic (%)
Acidic		
Asp	117	28.2
Glu	60	
Hydrophobic		
Ala	72	38.0
Ile	43	
Gly	76	
Leu	70	
Met	19	
Phe	35	
Pro	56	
Val	83	
Basic		
Arg	67	12.9
Lys	39	
Uncharged Polar		
Cys	9	20.8
Thr	41	
Tyr	21	
Ser	60	

Asp: Aspartic acid, Glu: Glutamic Acid, Ala: Alanine, Ile: Isoleucine, Gly: Glycine, Leu: Leucine, Met: Methionine, Phe: Phenylalanine, Pro: Proline, Val: Valiune, Arg: Arginine, Lys: Lysine, Cys: Cystine, Thr:Threonine, Tyr: Tyrosine, Ser: Serine

scavenging activity of 33.4% and ferric reducing antioxidant power (FRAP value) of 0.97 mM.

DISCUSSION

Based on a single factor experiment, it showed that all the parameters (i.e. pH, sample to buffer ratio, temperature and time) had shown the effects towards the extraction yield of protein. The incremental value of protein yield across the pH could be explained by the solubility and isoelectric point (pI) of protein. The solubility of protein commonly increases when the

environmental pH of protein was above the pI value. As the phenomenon occurs, the net charge of the protein could be changed, resulting in the inducement in protein-solvent interaction (Samanta & Laskar, 2009). As a result, a better extraction yield of protein could be obtained at a higher pH condition. For the effect of sample-to- buffer ratio, the extraction yield increased as the ratio increased because higher amount of extraction buffer would encourage more proteins to be soluble. This might be due to the relaxation process of proton-transfer reaction between the buffer ions and

α -amino groups (Wijayanti et al., 2014). In other words, the increases of sample-buffer ratio will facilitate the extraction of the sample, thus increasing the protein yield. Temperature also plays an important role in the extraction procedure, as the increment of temperature will cause the protein become more porous, resulting in a higher efficiency of the protein to be solubilized in the buffer solution (Kumoro et al., 2010). For the effect of time, the longer extraction time was needed in the beginning phase of extraction as it could facilitate the solvent in diffusing the sample, which improved the protein yield. However, prolong extraction for a certain period of times could give adverse effect as the solubility of proteins could be decreased due to the formation of protein-phytate complexes (Eromosele et al., 2008). Hence, it could reduce the amount of protein yield. The pH 8, 1:50 (w/v), 80°C and 1.5 h had shown the highest values of yield in the protein extraction within the studied parameters, and these parameters were used in extracting the ETSPI.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in determining the molecular weight of the protein through the electrophoretic mobility protein separation (Luo et al., 2004). Throughout the finding, ETSPI could be classified into the group 1 vicilin as its precursor polypeptide was fragmented in the range of 12-34 kD (Döring, 2015). Moreover, the trend of SDS-PAGE profile of ETSPI was similar to the protein profiles of pea seed, which the dominant protein was the 7S globulin

subunit (Barać et al., 2015). Interestingly, this 7S globular protein was commonly delineated as an agent in inhibiting digestive enzymes, especially protease and amylase (Mundi, 2012). Despite, Lovati et al. (1998) had reported that the 7S globular protein from soybean protein could reduce the plasma cholesterol level up to 35%. This finding elucidated that the major protein fraction in this ETSPI could be part of the contributors towards its biological activities.

The FTIR analysis was performed in procuring the understanding of ETSPI protein structure. Amide I and amide II are most vital band of protein backbone as it could be used in prediction of secondary structure of proteins (Kong & Yu, 2007). From the result, it could be suggested that the secondary structure of ETSPI belongs to α -helical as the peak of amide I was located in the region of 1650 to 1660 cm^{-1} (Zhao et al., 2008).

Protein digestibility is one of the important factors in examining the nutritional quality of protein (Elsohaimy et al., 2015). The results had shown the digestibility was lower than other extract. This might attribute to the complex structure of protein such as globulin and high molecule weight of protein that present in the ETSPI, which might slow down the hydrolysis. In fact, the compact structure of protein might encumber the proteolytic enzyme in cleavage the peptide bond, and resulted a decline in digestibility.

Amino acid composition was conducted in studying its correlation with the biological activity. Throughout drug discovery research, the enzyme inhibitor was commonly worked

by interacting target enzyme through non-covalent force such as hydrophobic force, electrostatic force, hydrogen bond and Van der Waals forces. This conceptual was implemented in our research and it could be suggested that amino acid presence in ETSPI might interacted with our target enzyme (lipase, ACE, amylase) through interaction forces which resulting the inhibitory activity as shown on Table 1. Hernández-Ledesma et al. (2011) reported that ACE preferred to interact with inhibitor or substrate, which had the hydrophobic amino residue at N-terminal. Nonetheless, the bond of certain hydrophobic amino acid, such as Leu and Pro, to the active site of ACE through hydrogen and electrostatic interaction might cause the enzyme to lose its ability in binding to the substrate (Jang et al., 2011; Pan et al., 2011). In addition, Ashok and Aparna (2017) reported that bioactive peptide, which contain Val and Ala mimicked the interaction of lisinopril (ACE synthetic drug inhibitor), thus reduced the availability of the catalytic or substrate binding sites for the substrate. This similar interaction could occur when the ETSPI, which has a high abundance in Leu, Val, Ala were added to the ACE. Another possible explanation on the reduced activity was the ETSPI might also interact with the His-His-Leu (substrate), in which lowering the susceptibility of the substrate to be bound by ACE. Interestingly, ETSPI had manifest a favorable ACE inhibitory activity as compared with other seed protein extracts (e.g. flaxseed, chickpea and pea), which all of them were reported no ACE inhibitory

activity (Barbana & Boye, 2010; Marambe et al., 2008). Therefore, it was suggested that ETSPI has the potential in antihypertensive effect. On the other notes, the relative high amount of Gly, Leu, Ala and Arg in ETSPI was one of the attributions of the α -amylase inhibitory activity. These similar residues were reported to be abundant in soybean and oat, which both of these legumes were potential antidiabetic agents (Lacroix & Li-Chan, 2012). In fact, Mojica and de Mejía (2016) reported that Leu in their peptide (Leu-Ser-Lys-Ser-Val-Leu) had shown the highest interaction with α -amylase active site through the hydrophobic interaction. This hydrophobic amino acid residue of Leu is important in α -amylase inhibition as it blocks the ion channel of enzyme and thus, reduces the enzyme activity (van der Veen et al., 2001). It was also suggested that ETSPI might interact with starch and prevented the formation of glycosyl-enzyme intermediate. However, ETSPI had manifest slightly low activity as compared to barley protein isolated (51-57.3%) (Alu'Datt et al., 2012). This might be related to the compact structure of ETSPI and entrapment of active functional amino acid in the core of the protein. However, the activity could be enhanced by releasing the bioactive peptide throughout the enzymatic hydrolysis of parental chain protein (Elias et al., 2008). Thus, it could suggest that ETSPI might be a precursor in producing the bioactive peptide to enhance the inhibitory activity of α -amylase. In terms of lipase inhibitory activity, Arg was reported to contribute to the activity in lowering the cholesterol that

could relate to the antiobesity property (Oda, 2006). Despite, a protein comprising amino acid residues that are predominant with Glu and Pro, also reported to have the potential in inhibiting the lipase (Upadhyay et al., 2006). Apart from that, peptide with amino acid sequence of Cys-Gly-Pro-His-Pro-Gly-Gln-Thr-Cys, which adequately in inhibiting the pancreatic lipase was selected from phage displayed (Lunder et al., 2005). These findings supported that the result of which these amino acids were found relatively high amount in ETSPI. The olive oil, which was used as the substrate, might form emulsion with ETSPI, where the protein chain of ETSPI wraps over the oil droplets and thus, prevented the lipase from approaching them. Additionally, the inhibitory activity of lipase enzyme of ETSPI slightly similar compare to the other lipase inhibitory activity of seed extract, such as *Trigonella foenum-graecum* (30%) *Bunium persicum* (28%), and *Nigella sativa* (31%) (Birari & Bhutani, 2007). In this comparison, secondary metabolite seed extracts were used instead of protein because there were no reports on lipase inhibitor protein available. Throughout these findings and supportive literature, it could suggest that ETSPI might be nominated as a novel lipase inhibitor. The hydrophobic properties were also considered as an important factor for the accessibility of the antioxidant protein to the cellular target organ via hydrophobic interaction with the bilayer lipid membrane. In fact, the hydrophobic group of amino acids, such as Phe, Trp, Tyr and His, should be credential for the antioxidant activity due

to their chemical structure and functional group. For instance, the presence of the imidazole ring in His could act as proton donor, which neutralize the free radicals of ABTS or DPPH (Samaranayaka & Li-Chan, 2011). On the other hands, the relative high amount of Glu and Asp in ETSPI might also attribute to the antioxidant activity. It was elucidated that these amino acids were considered as metal chelating agents, which had potential in binding metal ions (Wang et al., 2009). The tendency of these amino acids in donating their excess electron towards the free radical could be one of the antioxidative abilities of ETSPI (Udenigwe & Aluko, 2012).

CONCLUSION

In summary, it can be verified that ETSPI present in the local medical drink had partially contributed to the therapeutic effects, such as antidiabetic, antihypertensive, antiobesity as well as antioxidant activities. The best extraction yield of ETSPI was obtained under the condition of extraction time of 2 h, buffer to sample ratio of 1:50 (w/v), temperature at 80 °C, and pH 8. In fact, the requisite amount of amino acids in ETSPI was the vital criterion due to its correlation with the biological activities. However, ETSPI had manifested mild activity in terms of biological activities due to the tertiary structure of the polypeptide which cause inaccessible of amino acid to the target enzyme or prooxidant. This limitation could be overcome by disrupting the tertiary structure through enzymatic hydrolysis of the isolated protein to bioactive peptide,

and this approach is suggested in our future research which will be focusing on identification of bioactive peptide.

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