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Effects of Bromelain and Trypsin Hydrolysis on the Phytochemical Content, Antioxidant Activity, and Antibacterial Activity of Roasted Butterfly Pea Seeds

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Abstract: Butterfly pea (*Clitoria ternatea* L.) is a traditional medicinal and edible herb, whose health-promoting benefits have been attributed to its phenolic constituents. In this study, the effects of enzymatic hydrolysis on total phenolic content (TPC) and total flavonoid content (TFC), antioxidant (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP)) and antibacterial activities of raw and roasted (200 °C, 10–20 min) butterfly pea seeds were investigated. Roasting reduced the yield of seed aqueous extracts, but it increased the TPC and FRAP values, hence the reducing ability, of the extracts. Bromelain hydrolysis enhanced the TPC and TFC of the roasted seeds up to 2- and 18-fold higher, respectively. Trypsin hydrolysis drastically increased the TPC, but not TFC, of roasted seeds; trypsin-hydrolyzed, 20 min roasted sample had the highest TPC (54.07 μg gallic acid equivalent (GAE)/mg) among all samples. Bromelain hydrolysis significantly improved the antioxidant activity of the roasted seed samples, where the antioxidant activity of bromelain-hydrolyzed, 20 min roasted sample was about 50% greater than the non-hydrolyzed 20 min roasted sample. Trypsin hydrolysis raised the FRAP values of the 20 min roasted sample to 70.28 mg Fe(II) equivalent/g, the highest among all samples. Nevertheless, trypsin only weakly elevated the ABTS scavenging activity of the roasted samples, showing no enhancement of the DPPH scavenging activity. On the other hand, only bromelain hydrolysates of raw and 10 min roasted seeds were active against *Staphylococcus aureus*. Taken together, bromelain hydrolysis can be used to enhance the extractable phytochemical contents and antioxidant activity of roasted butterfly pea seeds.

Keywords: roasted butterfly pea; enzymatic hydrolysis; phenolic compounds; antioxidant activity; antibacterial activity

1. Introduction

Clitoria ternatea L., commonly known as butterfly pea, is a perennial, tropical leguminous twiner which belongs to the Fabaceae family. The plant of *C. ternatea* is one of the four traditional herbs that has been used in Ayurvedic medicine [1], and the blue flowers of the butterfly pea have been used as a natural food colorant in many Asian cuisines for centuries [2]. The extracts from different parts of the plant have been claimed to possess numerous pharmacological activities, including antioxidant, antidiabetic, and hepatoprotective activities [3]. These health benefits of the butterfly pea are mainly

attributed to the naturally-occurring phenolic compounds, which are widely distributed and ubiquitous in the plant [4,5]. Although many of these minor compounds are recognized as anti-nutrients, they provide health benefits when consumed in moderate amounts [6].

Phenolic compounds are the biggest group of phytocompounds which account for majority of antioxidant activity found in plants and plant products [7]. Their redox properties play vital roles in decomposing peroxides, quenching singlet and triplet oxygens, besides adsorbing and neutralizing free radicals [8,9]. The position and number of hydroxyl group govern the antioxidant activity of phenolic compounds [5]. Flavonoids are the biggest cluster of phenolic compounds in the forms of glycosides or free state [7]. They have been reported to delay or prevent several severe and degenerative ailments—including aging, arthritis, memory loss, cataract, stroke, cancer, cardiovascular diseases and Alzheimer's disease—due to their potent antioxidant activities [10]. In addition, the production of flavonoids in response to microbial infection has been established as an effective defense strategy in plants. The antimicrobial activity of these substances relies on their capability of forming complexes with soluble and extracellular proteins, bacterial cell walls, and membranes [11,12].

In addition to phytocompounds, plant peptides are gaining attention as candidates of functional ingredients. Plant peptides are specific protein fragments that exert beneficial effects in different applications. These bioactive peptides possess many physiological functions, including antioxidative [13,14], antimicrobial [12,15,16], and antihypertensive [17] activities. They may already be present naturally or can be derived from chemical or biological treatments. Process treatments, such as heat treatment [18] and enzymatic hydrolysis [13,15,19], have been reported to enhance the release of phytocompounds, proteins, and peptides. Moderate heat treatments, such as boiling and roasting, may break down complex structures, increase the availability of bioactive components, and produce new compounds [13,20,21], consequently affecting their functional properties [18]. Furthermore, bromelain and trypsin hydrolysis are commonly practiced on legume seed extracts [22]. Bromelain has a wide specificity for protein cleavage and is stable over a broad pH range (pH 4–8). It generally cleaves the protein sites with arginine, lysine, tyrosine, glutamic acid, glycine, ornithine, methionine sulfoxide, and alanine, but the specificity of cleavage may be altered by a change in pH [23,24]. Trypsin hydrolyses C-terminal amide bond specifically to arginine and lysine. The catalytic pocket of trypsin has a negatively-charged aspartate, which is responsible for binding basic amino acids with positive charges [25]. Enzyme-hydrolyzed, roasted butterfly pea seeds could be used as value-adding ingredients to improve the overall quality and functionality of food products, but experimental evidence justifying this hypothesis is lacking. With this gap of knowledge in mind, this study was conducted to determine the total phenolic and flavonoid contents in the non-hydrolyzed and the enzymatically hydrolyzed extracts of raw and roasted butterfly pea seeds, as well as to evaluate their antioxidant and antibacterial activities.

2. Materials and Methods

2.1. Materials

Butterfly pea (*Clitoria terantea*) seeds were collected from local growers in Kampar, Perak, Malaysia. All reagents and chemicals were of analytical grade and purchased from Merck, Germany; Sigma Aldrich, USA; QRëC®, Singapore; and Nacalai Tesque, Japan, unless stated otherwise.

2.2. Sample Preparation

The mature seeds of butterfly pea were separated from the seed pods manually. They were spread evenly on an aluminum tray and roasted at 200 °C using a fan-force oven (Roller Grill, Bonneval, France) for 10 min and 20 min. After that, the seeds were cooled to room temperature and ground into flour using an IKA®A11 basic analytical mill (IKA®-Werke GmbH & Co. KG, Staufen, Germany).

2.3. Extraction and Yield Determination

Extraction of raw and roasted seed flours was carried out in 10 volumes of distilled water with constant stirring at room temperature for 2 h. The crude extract was then centrifuged at 5000 rpm (2935 × g) for 10 min using a tabletop centrifuge G16-C (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany). The supernatant was collected and freeze-dried using a ScanVac CoolSafe 4-15L Freeze Dryer (LabogeneTM, Lillerød, Denmark). Then, the total yield of the sample was obtained by weighing the dried extract and calculated by using the equation below. The freeze-dried sample was then stored at −20 °C prior to analysis.

$$Yield\ (\%) = \frac{Mass\ (\mathrm{g})\ of\ dry\ extract}{Total\ mass\ (\mathrm{g})\ of\ ground\ flour} \times 100\%$$

2.4. Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out using a procedure [13] with slight modification. The freeze-dried sample was suspended in 5 volumes of distilled water and mixed thoroughly. To prepare bromelain hydrolysates, the pH of the suspension was adjusted to pH 7.0 with 1 M NaOH or 1 M HCl, added with 8 milli Anson Unit (mAU)/g of bromelain (2 mAU/mg for biochemistry, Enzyme Commission (EC) 3.4.22.32, Chemical Abstracts Service (CAS) 9001-00-7, Merck, Germany), and followed by incubation in a shaking water bath at 37 °C for 2 h. After incubation, the hydrolysis reaction was terminated by heating the solution in a boiling water bath for 10 min. The hydrolysate was collected after centrifugation at 7000 rpm (4109 × g) for 30 min, freeze-dried and stored at −20 °C prior to analysis. These procedures were repeated for trypsin hydrolysis by adding 8 Proteinase K Unit (PU)/g of trypsin (250 PU/mg trypsin, EC 3.4.21.4, CAS 9002-07-7, Nacalai Tesque, Japan) at an optimum pH of 8.0.

2.5. Determination of Total Phenolic Content

Total phenolic content (TPC) in the crude extract and hydrolysates was determined using the Folin-Ciocalteu method [26], with minor modification. Briefly, 250 µL of the sample (1 mg/mL of dry extract in distilled water) was mixed with 500 µL of Folin-Ciocalteu's phenol reagent and 2.5 mL of distilled water. The mixture was vortexed and left in the dark for 5 min at room temperature (25 °C). Next, 5 mL 7% (*w/v*) of sodium carbonate solution and 4.25 mL of distilled water were pipetted into the mixture, which was then kept in the dark for 2 h. The absorbance of the mixture was read at 750 nm. A standard curve was prepared using gallic acid solution (20–100 µg/mL 80% (*v/v*) aqueous ethanol). TPC was expressed as microgram of gallic acid equivalent (GAE) per milligram of dry extract.

2.6. Determination of Total Flavonoid Content

Total flavonoid content (TFC) in crude extract and hydrolysates was determined using a colorimetric method [27] with slight modification. Briefly, 250 µL of the sample (1 mg/mL of dry extract in distilled water) was mixed with 1.25 mL of distilled water and 75 µL 5% (*w/v*) of sodium nitrite solution. The mixture was swirled and left in the dark for 6 min at room temperature (25 °C). After that, 0.15 mL 10% (*w/v*) of aluminum chloride hexahydrate solution was added into the mixture, which was allowed to react for another 5 min. Next, 0.5 mL of 1 M sodium hydroxide solution was added into the mixture and the absorbance was read at 510 nm. A standard curve was prepared using quercetin solution (20–100 µg/mL 80% (*v/v*) aqueous methanol). TFC was expressed as microgram of quercetin equivalent (QE) per milligram of dry extract.

2.7. Determination of Antioxidant Activity

2.7.1. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) Free Radical Scavenging Assay

ABTS free radical scavenging activity was determined using a procedure [26] with slight modification. A solution of 7 mM ABTS diammonium salt solution was mixed with equal volume of 2.45 mM potassium persulfate solution. The solution was kept in the dark for 18 h at room temperature (25 °C) to produce a stable dark green solution. This ABTS stock solution was then diluted with distilled water to obtain an absorbance of 0.7 ± 0.02 at 734 nm. The resulting ABTS solution (1 mL) was added into 100 µL of the sample (1.5 mg/mL of dry extract in distilled water). The mixture was incubated for 5 min at room temperature before the absorbance was read at 734 nm. Control was assayed by replacing the sample with distilled water. Trolox solution (0–25 µg/mL 80% (*v/v*) aqueous ethanol) was prepared and assayed under the same conditions. Antioxidant activity was expressed as milligram of Trolox equivalent (TE) per gram of dry extract.

2.7.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Assay

DPPH free radical scavenging activity was measured according to a procedure [26] with slight modification. The DPPH radical solution was prepared freshly by dissolving 4 mg of DPPH in 100 mL of 80% (*v/v*) aqueous methanol. For assay, an aliquot of 100 µL sample (2 mg/mL of dry extract in distilled water) was added to 250 µL of DPPH radical solution and 2 mL of 80% (*v/v*) aqueous methanol. The resulting solution was mixed vigorously and left in the dark for 20 min at room temperature (25 °C) prior to measurement of absorbance at 515 nm. Control was assayed by replacing the sample with distilled water. The Trolox solution (0–25 µg/mL 80% (*v/v*) aqueous ethanol) was prepared and assayed under the same conditions. Antioxidant activity was expressed as milligram of Trolox equivalent (TE) per gram of dry extract.

2.7.3. Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay was carried out according to a method [28] with slight modification. The FRAP reagent was freshly prepared by mixing 50 mL of 0.3 M sodium acetate buffer (pH 3.6), 5 mL of 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) solution (in 40 mM HCl solution), and 5 mL of 20 mM ferric (III) chloride hexahydrate solution; then, it was incubated in a water bath at 37 °C. For assay, a mixture of 1 mL of FRAP reagent, 30 µL of crude extracts or hydrolysates (1 mg/mL of dry extract in distilled water), and 1 mL of distilled water was prepared. Control was assayed by replacing the sample with distilled water. The mixture was then incubated in a water bath at 37 °C for 30 min prior the measurement of absorbance at 593 nm. Ferrous sulfate solution (0–1.0 mg/mL) was prepared and assayed under the same conditions. Reducing power was reported as milligram of Fe (II) equivalent per gram of dry extract.

2.8. Well-Diffusion Antibacterial Assay

The antibacterial activity was evaluated against human pathogenic bacteria using the well diffusion method [29]. Firstly, Gram-negative bacteria *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (clinical isolate), and Gram-positive bacteria *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 33591) were cultured for 18 h on Muller Hinton agar (MHA) using the streak plate method. The cultured bacteria were then inoculated into 2 mL 0.85% (*w/v*) normal saline to achieve an optical density reading in the range of 0.08–0.1 at 625 nm. The culture suspension was then streaked evenly on MHA (25 mL) using a sterile cotton swab. After that, 6 mm diameter wells were prepared by using a sterile cork borer. Then, 30 µL of sample (12.5–100 mg/mL), equivalent to 0.375–3.000 mg, was inoculated into the wells individually. Distilled water was used as sterility control and a 10 µg ampicillin disc was used as positive control. The diameter of the inhibition zone (if any) was measured after incubation at 37 °C for 24 h.

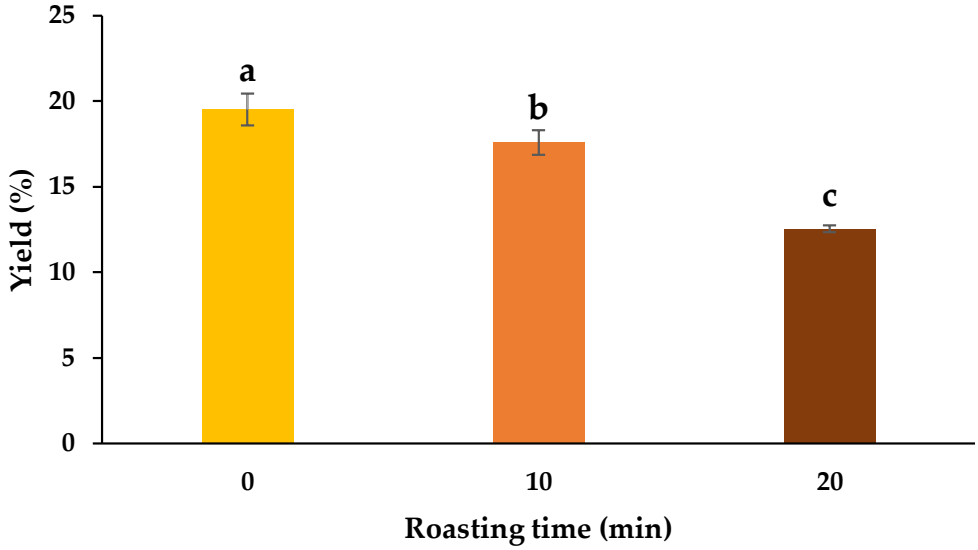
2.9. Statistical Analysis

All extractions and assays were conducted in triplicate. Data are expressed as mean ± standard deviation. Data were subjected to variance analysis (ANOVA) and *t*-test using SPSS Statistics (IBM Corporation, Armonk, NY, US). Significant differences were reported at $p < 0.05$.

3. Results and Discussion

3.1. Yield, TPC, and TFC

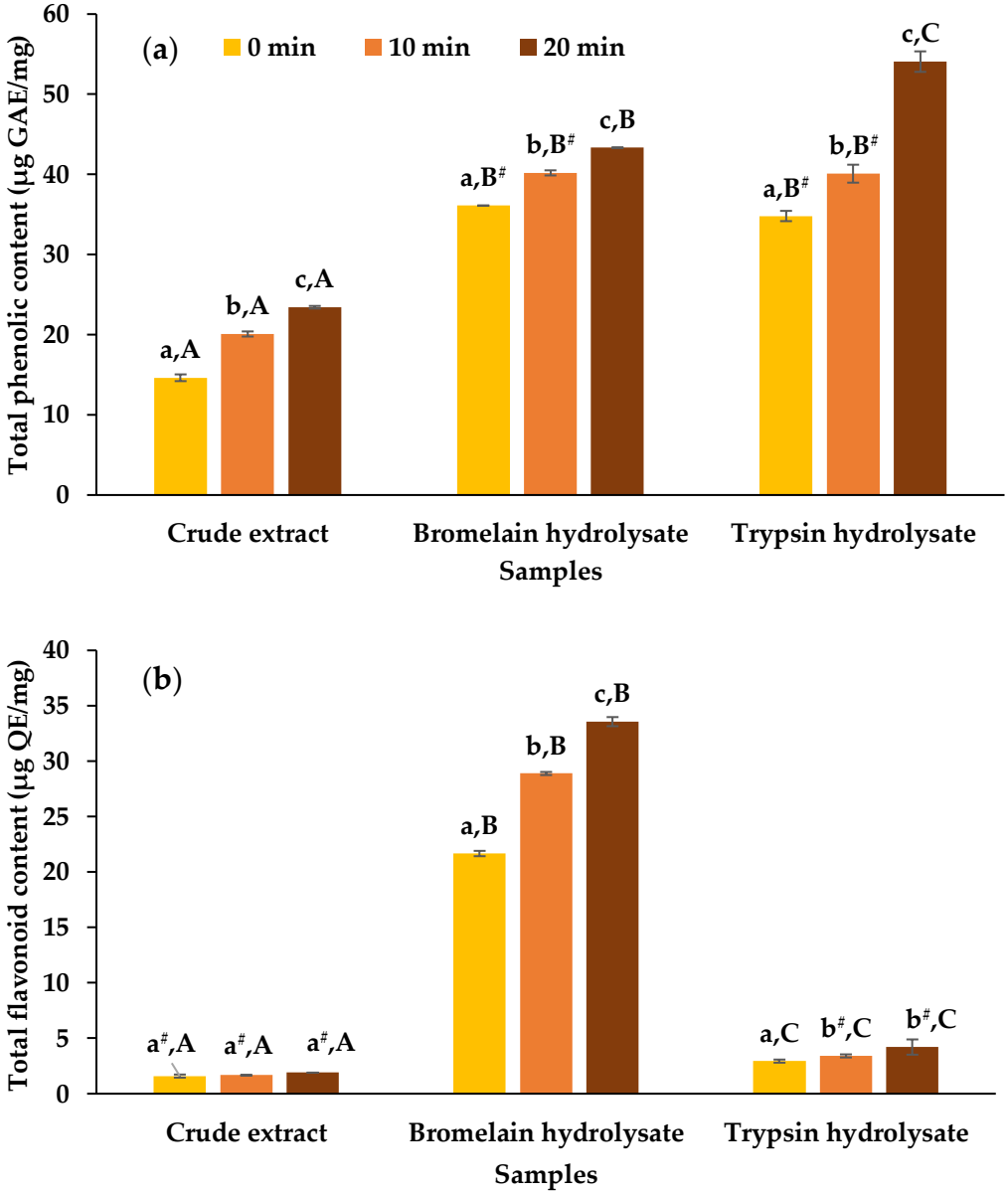
Mixed solvents, such as water and organic solvents, are commonly used to extract plant phenolics in various studies. However, the use of organic solvents will limit the application of such extracts in food processing due to safety concerns. Thus, in this study, we used water to extract water-soluble components from raw and roasted butterfly pea seeds. The yield of the aqueous extracts of raw and roasted seeds of butterfly pea is shown in Figure 1. The highest yield (19.52 ± 0.93%) was observed in the 0 min roasted seed extract, followed by the 10 min (17.59 ± 0.72%) and the 20 min roasted seed extract (12.55 ± 0.20%). The decrease in extraction yield after roasting is generally due to the occurrence of thermal denaturation of cellular components in the seeds and formation of water-insoluble materials through browning reaction [18]. Besides, excessive roasting can further lead to the breakdown of products and the reduction of volatile components [20,21].



Note: Different letters (a–c) denote significant difference ($p < 0.05$).

Figure 1. Extraction yield (%) of 0 min, 10 min, and 20 min roasted seeds of butterfly pea.

Figure 2a,b shows the TPC and TFC, respectively, in raw and roasted butterfly pea seed extracts before and after enzymatic hydrolysis using bromelain and trypsin. Non-hydrolyzed extracts showed the lowest amount of TPC (14.63–23.43 μg GAE/mg) among all samples. In contrast, both bromelain (36.11–43.33 μg GAE/mg) and trypsin (34.81–54.07 μg GAE/mg) hydrolysates showed an average of two-fold increase in TPC. Similarly, the non-hydrolyzed extracts had the lowest amount of TFC (1.59–1.90 μg QE/mg) among all samples. In contrast, bromelain hydrolysis increased the TFC up to about 18-fold, with the highest (33.57 ± 0.41 μg QE/mg) found in 20 min roasted seed extract. Although slight increase of TFC in trypsin hydrolysates was detected, it was significantly ($p < 0.05$) lower than that in the bromelain hydrolysates. To our knowledge, no previous studies have documented a connection between protease treatments and the additional release of phytochemicals from butterfly pea seeds.



Note: Different letters (a–c) in the same sample with different roasting time and different letters (A–C) among different samples with the same roasting time denote significant difference ($p < 0.05$). Bars with hash ($^{\#}$) denote insignificant difference ($p > 0.05$).

Figure 2. Total phenolic content (**a**) and total flavonoid content (**b**) in crude extracts, bromelain hydrolysates, and trypsin hydrolysates of raw and roasted butterfly pea seeds.

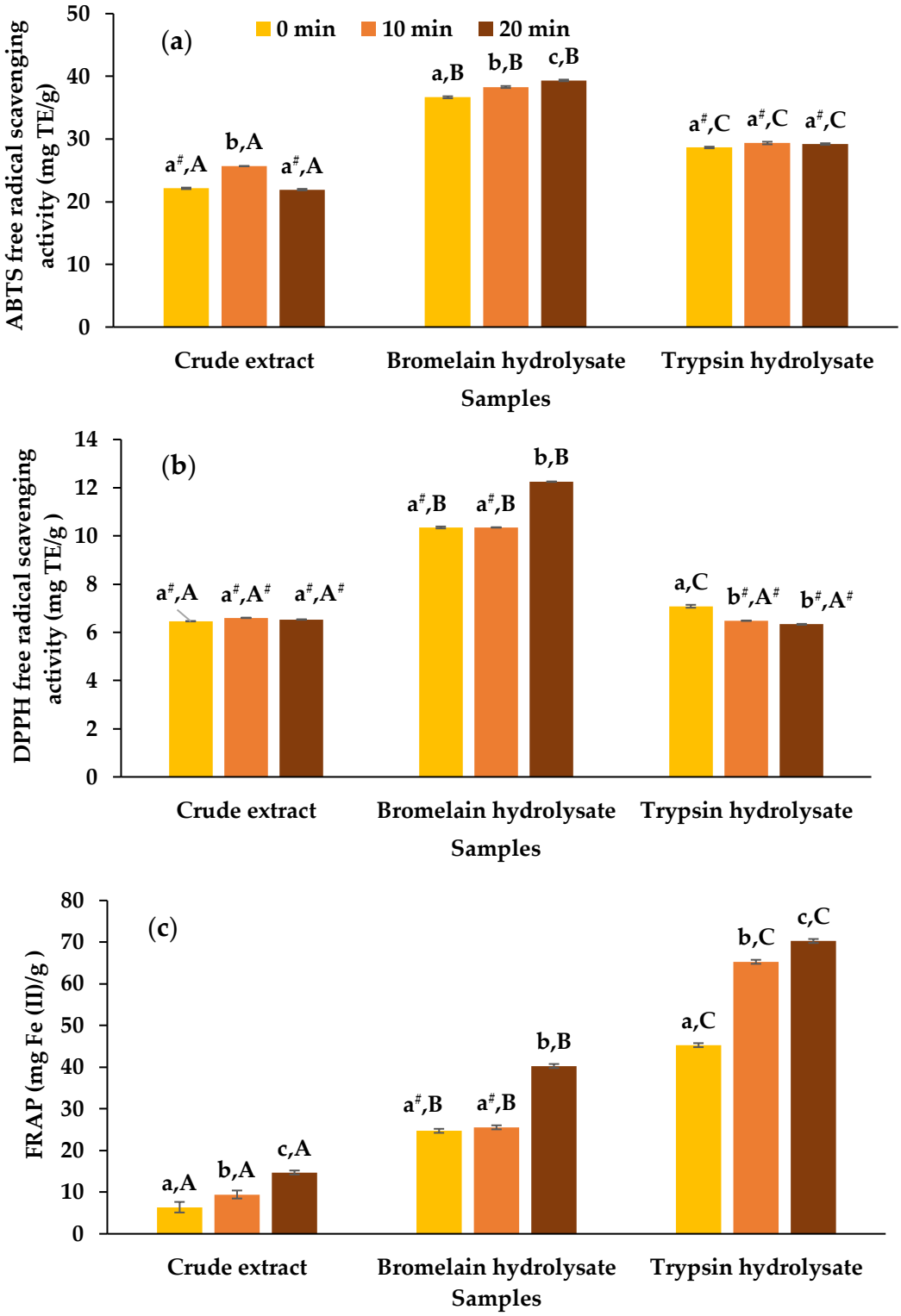
The roasting process is normally carried out on legume seeds to denature anti-nutritional factors and to develop unique flavor before incorporating them into food [6,18,21]. It could cause the disruption of cell structure and allow the TPC and TFC in the complex tissue matrix to be readily extractable [6,18,20,21]. This phenomenon has been demonstrated as well in the present study, where the slight increasing trend of TPC was observed before enzymatic hydrolysis was carried out. Samples were further processed by using enzymatic hydrolysis to enhance the extraction of TPC and TFC. Enzymatic hydrolysis has been reported to enhance the release of phenolic compounds from flaxseed meal by using neutrase, alcalase, flavourzyme, papain, pepsin, and pancreatin [19]; and other cereals and legumes [15], such as rice bran, by using α-amylase followed by a combination of glucoamylase, protease, and cellulose [14].

Enzymes exert hydrolysis and disrupt the bonding between cell components, conjugated compounds, and phenolics, thus enhancing the release of phenolic compounds [14]. Increase of non-proteinogenic constituents after the proteolytic treatment of plant matrix implies the release of bound and soluble conjugated phytochemicals. Quercetin is an example of one such compound which is known to bind to plasma protein and plant matrix [30]. The rate and the extent of hydrolysis are attributed to the types of enzyme used, the available substrate, hydrolysis conditions, as well to the resistance of hydrolysis and the effect of other constituents during the hydrolysis process [22]. Legumes are known to have anti-nutritional factors, including trypsin and chymotrypsin inhibitors [31]. It has been reported that the seeds of butterfly pea contain at least four different types of trypsin inhibitors with different sizes [32]. These inhibitors could have weakened the trypsin activity during enzymatic hydrolysis in raw butterfly pea seeds. In contrast, the elevated TPC levels in the trypsin hydrolysates of the roasted seeds may be attributed, at least in part, to the inactivation or degradation of trypsin inhibitors during roasting.

3.2. Antioxidant Activities of Crude Extracts and Hydrolysates

Antioxidant activities, including ABTS, DPPH free radical scavenging activities, and ferrous reducing antioxidant power (FRAP), in crude extracts and enzymatic hydrolysates of raw and roasted butterfly pea seeds are shown in Figure 3. These free radical scavenging and metal reducing assays were employed in this study as they have been commonly used to characterize the antioxidant activity of various phytochemicals, including phenolic compounds of edible seeds [5,6,8]. Crude extracts of raw and roasted seed extracts possessed the lowest antioxidant activities among all samples. The antioxidant activities were ranged at 21.93–25.68 mg TE/g for ABTS assay, 6.63–6.74 mg TE/g for DPPH assay, and 6.39–14.72 mg Fe(II)/g for FRAP assay. In general, roasting contributed minor effects on the ABTS and DPPH antioxidant activities, but more significant increments were observed in FRAP assay. Furthermore, enzymatic hydrolysis increased the antioxidant activities in all samples, where the highest ABTS (36.66–39.35 mg TE/g) and DPPH (12.58–13.39 mg TE/g) free radical scavenging activities were found in bromelain hydrolysates; the highest FRAP values (45.28–70.28 mg Fe(II)/g) were observed in trypsin hydrolysates. In this study, we report, for the first time, the effectiveness of bromelain and trypsin treatments in improving the antioxidant activities of roasted butterfly pea seeds.

Both ABTS and DPPH free radical scavenging assays showed positive correlation to the TFC in butterfly pea seed extracts, especially the bromelain hydrolysates with the highest TFC exhibited the greatest free radical scavenging activities. The results are in agreement with a similar study [4], which demonstrated that flavonoids are major phytochemicals in contributing antioxidant activity due to their free radical scavenging ability, positive reducing power, and capability of donating H-atom to peroxy radical. In addition, the potency of antioxidant activity of hydrolysates is dependent on the duration of hydrolysis and the enzyme specificity. It has been reported that lower molecular weight hydrolysates (1000–3000 Da) may result in higher functional properties, such as scavenging activity and antibacterial activity, compared to those with high molecular weight hydrolysates [12,33]. Since water was used as a medium for extraction in the present study, soluble proteins and their conjugates were the targets for bromelain and trypsin. A similar study [34] on enzymatic hydrolysis of rice (*Oryza sativa* L.) extracts reported that the bromelain hydrolysates showed higher ABTS free radical scavenging activities than the non-hydrolyzed extract. Moreover, different yield of soluble peptides, amino acids, especially aromatic phenylalanine and tyrosine, and other phytochemical-conjugates obtained by using different proteolytic enzymes resulted in varying biological, chemical, and functional properties [34,35].



Note: Different letters (a–c) in the same sample with different roasting time and different letters (A–C) among different samples with the same roasting time denote significant difference ($p < 0.05$). Bars with hash ($^{\#}$) denote insignificant difference ($p > 0.05$).

Figure 3. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) free radical scavenging activity (**a**), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (**b**), and FRAP values (**c**) of crude extracts, bromelain hydrolysates, and trypsin hydrolysates of raw and roasted butterfly pea seeds.

The results of FRAP assay revealed a similar trend to the TPC, where trypsin hydrolysate showed the highest TPC with the greatest FRAP values. This suggests that non-flavonoid phenolics may account for the reducing power of butterfly pea seed extracts and hydrolysates. This correlation was also reported in a similar study on yellow peas, green peas, lentils, common beans, and soybeans [36]. Furthermore, the reducing power could be contributed by different sizes of peptides, types of amino acid, amount of hydrophobic, sulfur-containing and acidic amino acids available in the hydrolysates. For example, the presence of certain amino acids, such as lysine, leucine, methionine, tyrosine, isoleucine, histidine, and tryptophan, has been reported to increase the reducing power of hydrolysates [37]. In this study, the specific active site of trypsin might have increased the amount of these peptides and amino acids with high reducing power in the hydrolysates. These findings are also in agreement with a study [38] on flaxseed protein hydrolysates, where trypsin-hydrolyzed extracts exhibited higher FRAP values compared to papain-hydrolyzed extracts. On the basis of our experimental data, we are not able to define a single compound mainly responsible for the antioxidant activity we detected. On the other hand, we cannot exclude that the detected activity is related to a single metabolite rather that to a group of them, belonging to the same or to different chemical classes. Future studies using component-specific analytical methods, such as HPLC, will allow the detection of specific phytochemicals resulting from the bromelain and trypsin treatments, particularly the specific compounds that are responsible for the observed bioactivities.

3.3. Antibacterial Activity

Both Gram-negative *Escherichia coli* (ATCC 35218) and *Klebsiella pneumoniae* (clinical isolate) were resistant to ampicillin, and both Gram-positive *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 33591) were ampicillin susceptible. All samples, except bromelain hydrolysates, did not show antibacterial activity (Table 1) regardless of their high levels of TPC and TFC (Figure 2). An antibacterial effect was observed in bromelain hydrolysates with a decreasing trend from raw to 20 min roasted seeds against *S. aureus*.

Insignificant antibacterial activity of butterfly pea seed crude extract and hydrolysates was observed in this study. This could be owing to the low amount of antibacterial phytochemicals in the aqueous extract and the treatments of sample, which were roasting and enzymatic hydrolysis. Although roasting enhanced TPC and TFC, it could have reduced the biological versatility of the potential antibacterial properties by altering their molecular structures [6,18]. Besides, antibacterial protein or peptide are amphiphilic with the presence of positive motifs. These hydrophobic structures could be denatured during the heat treatment, thus leading to ineffective interaction with the bacterial membrane [12,16]. Apart from the concern of sample type, using different enzymes and different duration of hydrolysis are crucial in producing antibacterial peptides. It has been reported that alcalase hydrolysate of lupine (*Lupinus*) possessed inhibitory activity against both *E. coli* and *S. aureus* [39], while protease hydrolysates of flaxseed (*Linum usitatissimum*) protein inhibited only *E. coli* but did not inhibit *S. aureus* [40]; however, protease hydrolysates of *Oryza sativa* bran protein did not possess antimicrobial activity against either *E. coli* and *S. aureus* [41]. Furthermore, due to the permeability properties of the outer membrane barrier in Gram-negative bacteria, the susceptibility of the bacteria to antibacterial agents is reduced [42]. In comparison to *S. aureus*, *E. faecalis* has an effective strategy to deactivate antibacterial agents, such as the production of different proteases that can degrade the antibacterial peptides [43–45].

Table 1. Zone of inhibition of ampicillin disc, crude extracts, and hydrolysates of raw and roasted seeds.

Sample	Roasting Time (min)	Amount (mg)	Zone of Inhibition (mm)[#]			
			E. c[a]	*K. p*[b]	*E. f*[c]	*S. a*[d]
Ampicillin disc	na	0.01	-	-	26	31
Crude extract	0	0.375–3	-	-	-	-
	10	0.375–3	-	-	-	-
	20	0.375–3	-	-	-	-
Bromelain hydrolysate	0	0.375	-	-	-	10
		0.75	-	-	-	8
		1.5	-	-	-	10
		3	-	-	-	7
	10	0.375	-	-	-	11
		0.75	-	-	-	8
		1.5	-	-	-	8
		3	-	-	-	8
	20	0.375	-	-	-	8
		0.75	-	-	-	-
		1.5	-	-	-	-
		3	-	-	-	-
Trypsin hydrolysate	0	0.375–3	-	-	-	-
	10	0.375–3	-	-	-	-
	20	0.375–3	-	-	-	-

[#] Data are means of three determinations. na, not applicable; [a] *Escherichia coli* (ATCC 35218); [b] *Klebsiella pneumoniae* (clinical isolate); [c] *Enterococcus faecalis* (ATCC 29212); [d] *Staphylococcus aureus* (ATCC 33591); -, no zone of inhibition.

Based on our observations in this study, it can be proposed that the water extract of butterfly pea seeds is an inferior source of antibacterial compounds. In addition, roasting and/or protease treatments did not effectively enhance antibacterial effects or antibacterial spectrum, despite additional release of phytochemicals from the seeds. This implies that water-soluble phenolics/flavonoids of butterfly pea seeds are weak antibacterial agents. Thus, our findings suggest that future research aiming for antibacterial compound discovery from these seeds should perform extraction with non-water solvents.

4. Conclusions

Roasting of butterfly pea seeds reduced the yield of aqueous extracts. Roasting had slightly increased antioxidant activities but did not enhance antibacterial activity of the seed extract. With the aid of enzymatic hydrolysis, TPC and TFC in the raw and roasted seed extracts increased significantly, with enhanced antioxidant activities. Trypsin hydrolysates showed the highest level of FRAP values. Positive correlation was found between the TFC and the ABTS and DPPH free radical scavenging activities of bromelain hydrolysates. Only bromelain hydrolysates of raw and 10 min roasted seeds were active against *Staphylococcus aureus*, but trypsin hydrolysates did not show any antibacterial activity. In short, we have established, for the first time, that bromelain treatment can enhance the extractable phenolic and flavonoid contents and antioxidant activities of roasted butterfly pea seeds. As an extension to the current study, future investigations using component-specific analytical methods will enhance knowledge of the specific phytochemical compounds extractable from roasted butterfly pea seeds through bromelain hydrolysis.

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Conflicts of Interest: The authors declare no conflict of interest.

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