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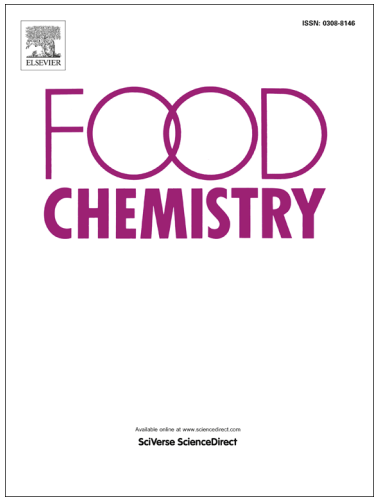
Sritongtae, B, Sangsukiam, T, Morgan, MRA et al. (1 more author) (2017) Effect of acid pretreatment and the germination period on the composition and antioxidant activity of rice bean (Vigna umbellata). Food Chemistry, 227. pp. 280-288. ISSN 0308-8146

https://doi.org/10.1016/j.foodchem.2017.01.103



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PII: S0308-8146(17)30114-0
DOI: http://dx.doi.org/10.1016/j.foodchem.2017.01.103
Reference: FOCH 20495

To appear in: *Food Chemistry*

Received Date: 9 February 2016
Revised Date: 18 January 2017
Accepted Date: 18 January 2017

Please cite this article as: Sritongtae, B., Sangsukiam, T., Morgan, M.R.A., Duangmal, K., Effect of acid pretreatment and the germination period on the composition and antioxidant activity of rice bean (*Vigna umbellata*), *Food Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.foodchem.2017.01.103

Effect of acid pretreatment and the germination period on the composition and antioxidant activity of rice bean (*Vigna umbellata*)

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Abstract

This research evaluated effect of germination period and acid pretreatment on chemical composition and antioxidant activity of rice bean sprouts. Moisture, total phenolics, reducing sugar and B vitamins (thiamine, riboflavin, and niacin) content of steamed sprouts increased with increasing germination time ($p \leq 0.05$). Pretreatment with 1% (w/v) citric acid for 6 hours significantly increased the total phenolic content. The 18-hour-germinated rice beans showed the highest crude protein content, as determined using the Kjeldahl method. During germination, acid pretreatment led to a significant decrease in the intensity of the 76-kDa band. Germination caused a significant increase in radical scavenging activity and ferric reducing antioxidant power, especially in sprouts from citric acid-treated seeds. The antioxidant activities of the ethanolic extracts from both pretreated beans and the control were 1.3–1.6 times higher than those obtained from the water extracts. Major phenolics found in both 0-hour and 18-hour-germinated rice beans were catechin and rutin.

Keywords: germinated bean; rice bean; sprouts; amino acid profile; antioxidant activity; *Vigna* spp.

1. Introduction

Rice bean (*Vigna umbellata*) is in the same genus as mung bean (*Vigna radiata*), azuki bean (*Vigna angularis*) and black bean (*Vigna mungo*). Rice bean is one of the most economically important agricultural products of Thailand and has been used for making pastry filling, bean sprouts, and traditional Thai

desserts. Rice bean seeds are a good source of protein, with a well-balanced amino acid composition for human consumption. Rice bean is particularly rich in methionine compared to black bean and mung bean. The contents of lysine, tyrosine and valine are approximately equal to those of comparable bean species. Katoch (2013) reported the chemical composition of 16 rice bean genotypes collected from India as follows: crude protein (23.17–25.57 %), lipids (1.92–3.42 %), dietary fibre (4.11–5.56 %), carbohydrate (52.23–55.65 %), ascorbic acid (15.14–29.19 mg/100 g) and niacin (3.48–4.28 mg/100 g).

Rice bean also contains phenolic compounds that exhibit antioxidant activity. Yao, Cheng, Wang, Wang, and Ren (2012) reported that different varieties of rice beans from China had significant differences in their total phenolic content (TPC) and flavonoid contents with values ranging from 3.27 to 6.43 mg gallic acid equivalents/g and 55.95 to 320.39 µg catechin equivalents/g, respectively. In most varieties, vitexin was the dominant phenolic, followed by catechin and isovitexin. However, these compounds were not found in some varieties. The rice bean varieties tested showed DPPH radical scavenging activities of 39.87–46.40 µM Trolox® equivalent/g. The effect of solvent on TPC extractability and the antioxidant activity of green pea, yellow pea, chickpea, lentil, yellow soybean, black soybean, red kidney and black bean extracts was reported by Xu and Chang (2007). Annegowda, Bhat, Tze, Karim, and Mansor (2013) also found that water, 96% (v/v) methanol and 96% (v/v) ethanol had different extraction efficiencies for phenolic content and antioxidant capacity measurement. The highest TPC and antioxidant capacity (DPPH radical scavenging, ABTS radical scavenging activity and FRAP) were found in methanolic extracts from *Clitoria fairchildiana* seed, while the highest total flavonoid content was found in ethanolic extracts.

The first stage of seed germination is imbibition, resulting in 1) absorption of water, 2) rehydration of cellular constituents and 3) activation of proteolytic and amylolytic enzymes. Germination also causes biochemical changes that affect nutritional value. The germination process leads to increased levels of amino acids, peptides and structural proteins, as well as the breakdown of some seed storage compounds. This process also leads to decreased level of antinutrients (Bewley, 1997). Ghavidel and Prakash (2007) reported that 24-hour germination caused a significant increase in the protein and thiamine levels of all legume samples tested (*Phaseolus aureus*, *Vigna catjang*, *Lens culinaris* and *Cicer arietinum*), while phytic acid and tannin, were reduced by 18–21% and 20–38%, respectively. An increase in total amino acids and phenylalanine was also found in 24-hour germinated mung bean (Wongsiri, Ohshima, & Duangmal,

2015)

Moreover, germination usually causes an increase in beneficial bioactive compounds that exhibit antioxidant activity such as phenolics, amino acids and peptides. The increase in phenolic content during germination might be due to a release of cell wall-bound phenolics or phenolic biosynthesis (Randhir, Lin & Shetty, 2004). The total phenolic acids and total flavonoid content of 5-day-germinated mung beans were 10.7 and 2.3 times higher than those of non-germinated mung bean seeds, respectively (Pająk, Socha, Gałkowska, Rożnowski, & Fortuna 2014). Significant increases in TPC and DPPH radical scavenging activities were found in 7-day-germinated mung bean, alfalfa and fava bean compared to those of non-germinated seeds (Cevallos-Casals & Cisneros-Zevallos, 2010). However, the TPC of germinated mah (*Vigna mungo*) and masur (*Lens esculantus*) decreased significantly after germination for 12 and 24 hours, respectively ($p \leq 0.05$) (Gujral, Angurala, Sharma & Singh, 2011). Peptides and free amino acids have been reported to contribute to antioxidant activity in bean seeds. Cysteine is the most reactive amino acid, containing a nucleophilic sulphur-containing side chain. Tyrosine and phenylalanine contain aromatic side chains from which hydrogen is easily removed. The imidazole-containing side chain of histidine is also oxidatively labile (Elias, Kellerby, & Decker, 2008).

Pretreatment of seeds with acid can cause acidic stress. However, some acids at the right concentrations enhance the germination of seeds. The effect of seed germination under stress conditions is different from the changes observed under normal conditions. Plants demonstrate various adaptive strategies in response to different stresses such as high osmotic pressure, water deficit, cold, heat and high salt, which ultimately affect plant growth and productivity. The reactions of plants towards environmental stress involve various types of physiological and biochemical responses depending on the genetic and biochemical make-up of the plant. One defence mechanism in plants is phenolic biosynthesis. Pretreatment with acid before germination affects the physiological and biochemical responses of seeds during germination. The acid-growth hypothesis postulates that cell wall loosening induced by acidification is a prerequisite of growth promotion (Rayle & Cleland, 1992). McCue, Zheng, Pinkham, and Shetty (2000) reported that pretreatment of green pea seeds (*Pisum sativum*) with acidified water (pH 3) and 50 µM salicylic acid (pH 3) for 24 hours before germination caused a 2.31 % and 11.02 % increase in TPC, respectively, after two

days of germination compared to those of the control. They hypothesized that a low pH environment stimulated phenolic synthesis by acting as a signal to activate defence response genes.

Many studies have reported composition and nutritional changes in different beans during the first few days of germination. However, there have been no reported studies on the changes in the chemical composition of rice beans during germination. To investigate the potential use of germinated rice bean as an alternative for beans in a composite meal, the change in the composition, B vitamins, and phytate content during first 24 hours of germination in rice beans needs to be investigated. Therefore, the objectives of this research were to study the effect of citric acid pretreatment and germination on the chemical composition, free amino acids and total phenolic compounds and to characterise the phenolics and antioxidant activities of the rice bean sprouts.

2. Materials and methods

2.1 Materials

Dried rice bean seeds (*Vigna umbellata*) were obtained from Choomsin Food Industry Co., Ltd (Nonthaburi, Thailand). Folin-Ciocalteu reagent and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO). An AccQ-Fluor™ reagent kit and AccQ Tag Eluent A solution were purchased from Waters (Milford, MA). Amino acid standard H was purchased from Thermo Scientific (Rockford, IL). All other reagents were of analytical grade.

2.2 Pretreatment and germination

Dried rice bean seeds were soaked in excess tap water (1:10, w:v) containing 1 % (w/v) citric acid as a pretreatment at room temperature (30±1 °C) for 6 h. After being drained in a sieve for 5 min, all seeds were allowed to germinate under a wet muslin cloth at room temperature (30±1 °C with 73±5 % relative humidity) in the dark with nebulization of tap water every 6 h. Rice bean seeds soaked in tap water without citric acid and germinated under the same condition were used as the control. Both pretreated seeds and control seeds were randomly selected after 0, 6, 12, 18 and 24 hours of germination, then immediately

transferred into a steamer set over boiling water and steamed for 10 minutes. After steaming, the sprouts were cooled until the surface was dried at room temperature for 20 min. The steamed bean sprouts were ground using a Waring blender (Waring Commercial, Cumbria, UK) at high speed for 3 min before further analysis.

2.3 Chemical composition and nutritional analysis

2.3.1 Chemical composition analysis

The moisture content was determined using the oven-dry method at 105 ºC (AOAC, 2006). Crude protein was calculated from total nitrogen content determined using the Kjeldahl method (AOAC, 2006) with a nitrogen-to-protein conversion factor of 6.25. The crude fat content was determined using a Soxhlet extraction method with petroleum ether as the solvent (AOAC, 2006). The ash content was determined according to AOAC (2006). The carbohydrate content was determined by subtracting the total percentage compositions of moisture, protein, fat, and ash contents from 100. The thiamine, riboflavin and niacin content were determined according to AOAC (2006). The phytate content was carried out according to the method described by Wheeler and Ferrel (1971).

2.3.2 Total phenolic and reducing sugar content

The ground bean sprouts were extracted with 95 % methanol (1:10, w:v) at 30 °C in a shaking water bath (Gesellschaft für Labortechnik, model GFL 1092) for 2 h. After centrifugation at 10,000 × g for 10 min, the supernatant was subjected to total phenolic content (TPC) determination, expressed as mg of gallic acid equivalents per gram of rice bean (dry basis, d.b.), using the Folin-Ciocalteu reagent (Singleton & Rossi, 1965).

The ground bean sprouts were also extracted with water using the same procedures as in the TPC extraction. The determination of reducing sugar content, expressed in mg glucose per gram of rice bean (d.b.), was carried out according to the method described by Fournier (2005).

2.3.3 Nitrogen content, TCA-soluble peptides, total free amino acid content and protein pattern

The samples was prepared according to the method of Wongsiri et al. (2015). Protein nitrogen in all samples was determined using the Kjeldahl method (AOAC, 2006). The non-protein nitrogen in all samples was estimated as the difference between the amount of total nitrogen and the true protein nitrogen content. To determine the trichloroacetic acid (TCA)-soluble peptide and free amino acid contents, ground samples were extracted using the method of Kudre and Benjakul (2013) with a slight modification. The ground bean sprouts (3 g) were mixed with 27 mL of cold 5 % (w/v) TCA. The mixture was continuously stirred for 2 min before being allowed to stand on ice for 1 h. After centrifugation at 10,000 × g for 10 min, the supernatant was divided into two portions. The first portion was for the determination of TCA-soluble peptide, expressed as µmol tyrosine per gram of rice bean (d.b.), using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The second portion was used in the determination of free amino acid content using high performance liquid chromatography (HPLC) using the AccQ.Tag method (Astephen, 1993) as modified by Wongsiri et al. (2015). The external standards, excluding tryptophan, were used. Identification of amino acid in the sample was done on the comparison with standard amino acid running under the same condition.

The protein pattern of rice bean sprouts was analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The sample was prepared according to the method of Duangmal and Taluengphol (2010). The rice bean protein was mixed at a 1:1 (v:v) ratio with the sample treatment buffer (60 mM Tris-HCL, pH 6.8, containing 40 g/L SDS, 200 g/L glycerol and 1 g/L bromophenol blue) and boiled for 2 min. The molecular weight markers (Sigma, S8445) and protein samples (7 µg), determined using a modified Lowry method (Peterson, 1983), were loaded onto the polyacrylamide gel consisting of a 10 % (w/v) separating gel and 4 % (w/v) stacking gel and subjected to electrophoresis at a constant current of 20 mA per gel using a Hoefer mini VE gel electrophoresis unit (Amersham Pharmacia Biotech, Uppsala, Sweden). After separation, the proteins were exposed to Coomassie Brilliant Blue R-250 solution and de-stained. The protein band intensity was determined using GeneTools software (Syngene, Frederick, MD).

2.4 Nutraceutical analysis

2.4.1 Effect of extraction solvents on antioxidant activities of rice bean sprouts

Ground rice bean sprouts at different germination stages were extracted with water or ethanol. To control the pH, the water extract was prepared by adding 100 mL of 0.1 M phosphate buffer (pH 6.0) to 10 g of ground sample. The mixture was shaken and incubated at 30 °C in a shaking water bath at 200 rpm for 2 h. The water extract supernatant was obtained after centrifugation at 10,000 × g for 10 min. For the ethanolic extract of the sprouts, the same protocol was repeated with 80 % (v/v) ethanol.

The DPPH radical scavenging activity of both extracts was evaluated according to the method of Brand-Williams, Cuvelier, and Berset (1995) as modified by Wongsiri et al. (2015). FRAP of both extracts was determined as described by Benzie and Strain (1996). The results were calculated and expressed as μmol Trolox® equivalent per gram of rice bean sprouts (d.b.).

2.4.2 Phenolic acid and flavonoid composition of rice bean sprouts

The water and ethanolic extracts obtained from both pretreated rice bean and the control at 0 and 18 hours of germination from Section 2.4.1 were used for phenolic composition determination. The phenolic acid and flavonoid composition were determined using liquid chromatography and mass spectrometry (LC-MS; Bruker amaZon SL, Chicago, IL) equipped with an electrospray ionization (ESI) source. Separation was achieved on a Luna C18 column (2.1 x 150 mm, 3 μm) at 35 °C. The identification of the phenolic acids and flavonoids was carried out by comparing MS spectra of the unknown peaks with MS data of candidate molecules registered in the MassBank database (http://www.massbank.jp/).

The phenolic acid and flavonoid compositions in water and ethanolic extracts were also determined using HPLC (Waters, Milford, MA) equipped with a UV/VIS detector (Waters model 2487, Milford, MA). Separation was carried out with a Luna C18 column (4.6 × 250 mm, 5 μm), with a flow rate of 0.70 mL/min at 35 °C. The mobile phase was a gradient of 0.1 % formic acid (mobile phase A) and 70 % methanol (mobile phase B). The gradient conditions were

as follows: the initial condition started with 0 % B and was increased to 40 % B over 15 min, then 42 % B at 35 min, 55 % B at 50 min, 70 % B at 60 min, and 100 % B at 70 min. The gradient was then decreased to 0 % B at 85 min. The identification and integration of peaks were performed by Empower software (Waters, Milford, MA). A known mixture of 14 phenolic compounds was applied as an external standard.

2.5 Statistical Analysis

All experiments were run in triplicate. Statistical data analysis was performed using analysis of variance (ANOVA). Comparison of means was carried out with Duncan's multiple range tests. Analysis was performed using an SPSS package (SPSS 20.0 for Windows; SPSS Inc., Chicago, IL.).

3. Results and discussion

3.1 Effect of pretreatment and the germination period on the chemical composition of rice bean sprouts

Table 1 shows that the chemical composition of the sprouts was affected by the length of the germination period. The bean sprout moisture content increased significantly during the 24-hour germination period ($p<0.05$). This may be due to the absorption of water by the seeds during imbibition. The TPC and reducing sugar content of the control increased with increasing germination time. The result showed that a significant reduction in crude fat content was observed after 12 hours of germination ($p\leq0.05$) compared with the amount of crude fat in raw seed (0.93±0.02 g/100 g d.b.). This decrease could be due to the use of crude fat as an energy source during germination. The carbohydrate and ash content were not altered over the 24-hour germination period. The crude protein content of the control, calculated by multiplying the amount of total nitrogen content (obtaining from Kjeldahl method) by a factor of 6.25, reached its highest value after 18 hours of germination ($p\leq0.05$) (Table 2). The changes in the chemical composition of pretreated rice bean during germination showed the same trends as the control (Table 1). Kaur (2015) also found that crude protein levels in germinated rice bean significantly increased with increasing germination period from 0-48 hours ($p\leq0.01$). Kim et al. (2013) reported that during germination of soya bean (*Glycine max* L. Merr.), the protein content

increased from 35.1±0.32 % to 38.7±0.30 % (w/w), while the lipid content decreased from 10.1±0.21 % to 9.7±0.15 % (w/w) after germination over 24 hours. Germination for 0–5 days also caused a significant increase in the crude protein content of germinated mung bean (*Vigna radiate* L.) and germinated chickpea (*Cicer arietinum* L.) (Masood, Shah & Zeb, 2014).

During soaking, a small amount of phenolics might be leached into the soaking water (Gujral, Angurala, Sharma & Singh, 2011). The germination period strongly affected the TPC of rice bean sprouts for all treatments. At 24 hours of germination, the TPC of the control and the pretreated rice bean were 2.06 and 2.55 times higher than that of the 0-hour-germinated rice bean, respectively. The increase in TPC was presumably due to phenolics biosynthesis during seed germination and a release of cell wall-bound phenolics (Randhir, Lin & Shetty, 2004). Wongsiri et al. (2015) reported that the TPC of germinated mung bean increased with increasing germination time over 0–24 hours ($p \leq 0.05$), with germination for 24 hours, resulting in a 3.48-fold increase of TPC in germinated mung beans. In germinated green peas (*Pisum sativum*), lentils (*Lens culinaris*) and mung beans (*Vigna radiata*), a significant increase in TPC was observed after 3 days of germination (Świeca & Gawlik-Dziki, 2015).

Pretreatment before germination significantly increased the TPC compared to the control. Pretreatment with 1% (w/v) citric acid (pH 2.38) may cause environmental stress to the bean seeds and/or a release of cell wall-bound phenolics. According to the acid growth theory, external acidification can cause cell wall loosening resulting in the release of cell wall- bound phenolic compounds during germination (Rayle & Cleland, 1992). Hao, Wu, Li, Wang, and Liu (2016) found that pretreatment of buckwheat with acidified water at pH 5.83 promoted the accumulation of rutin during 8 days of germination.

The thiamin, riboflavin and niacin content in rice bean sprouts significantly increased during 24-hour germination period (Table 1). Raw rice bean seeds contained considerable amounts of phytic acid (7.84±0.08 mg/g d.b.). As show in Table 1, a significant decrease in phytate content was observed after 18 hours of germination ($p \leq 0.05$). Similar results in the changes of thiamine and phytate content were also found in 24-hour germinated green gram, cowpea, lentil and chickpea (Ghavidel & Prakash, 2007). In our study, the acid pretreatment before germination led to a significant increase in thiamin, riboflavin, and niacin content ($p \leq 0.05$). However, it had no significant effect on the change of phytate content during germination.

3.2. Effect of the germination period on the nitrogen content and protein pattern

Table 2 shows that the amount of total nitrogen of bean sprouts from both treatments tended to increase over 24 hours of germination. The non-protein nitrogen content was taken as the difference between the amount of total nitrogen and true protein nitrogen contents. A significant increase in the true protein nitrogen content was observed during 24 hours of germination, but there was no significant change in the amount of non-protein nitrogen. Thus, an increase in the amount of total nitrogen of both treatments might be mainly due to an increase in true protein nitrogen.

TCA-soluble peptides in beans with different periods of germination for both the control and the pretreated rice bean, shown in Table 2, significantly decreased during 24 hours of germination ($p \leq 0.05$), whereas the amount of total free amino acid tended to increase with increasing germination period (Table 2). The increase in total free amino acids over longer germination periods was more pronounced in pre-treated beans. Histidine, methionine, and valine were the major essential amino acid, while alanine was the major non-essential amino acid found in rice bean sprout, followed by glutamic acid and serine (Fig. 1). The increasing of essential and non-essential amino acid was more pronounced during the first 12 hours of germination in the acid-pretreated seed. Tryptophan was not quantified due to its loss during acid hydrolysis. Although no analysis concerning tryptophan was carried out, tryptophan commonly accounts for only about 1% of the total amino acid content in rice bean (Anderson, 2007). Acid pretreatment and the germination period significantly affected the amount of antioxidant amino acids (histidine, cysteine, tyrosine, methionine and phenylalanine) of the rice bean sprouts during the first 12 hours of germination. This increase is most likely due to the hydrolysis of polypeptides into free amino acids by proteolytic enzymes during the germination process (Ghavidel & Prakash, 2007). The threonine, proline and methionine contents were reported to be increased during 1–5 days of germination in black beans (*Phaseolus vulgaris* L.) (López-Barrios, Antunes-Ricardo & Gutiérrez-Uribe, 2016). Lou, Jin, Hao, Wang, Zhu, and He (2014) reported that after 72 hours of germination, the free amino acid contents of green and white faba beans (*Vicia faba* L.) were 11.3 and 8.0 times higher, than that of 0-hour-germinated faba bean, respectively. According to our results, acid pretreatment caused an increase in the levels of free amino acids such as alanine, arginine, aspartic, and glutamic, as well as the antioxidant amino

acids cysteine, methionine, tyrosine, phenylalanine and histidine, during the first 12 hours of germination (Fig. 1). Many researchers reported that environmental stress led to an induction of genes coding for proteolytic enzymes, which hydrolyse peptides into free amino acids, resulting in an increase in free amino acid content during germination. Shen et al. (2015) reported that the free amino acid composition of 10-hour-germinated rice (*Oryza sativa* L.) after oxygen-deficit stress was higher than that of untreated germinated rice.

Fig. 2 shows the SDS-PAGE patterns of proteins extracted from rice beans after different periods of germination. The results showed that the germination period did not cause a change in the protein band pattern in the control (Fig. 2a). Pretreatment before germination led to a 20.96–30.67% and 56.95–82.28% decrease in the intensity of protein bands with MW of 50 and 76 kDa, respectively, in the samples at 18 and 24 hours of germination (Fig. 2b). The decrease in intensity of this band might be due to acidic stress from the citric acid pretreatment causing degradation of storage proteins during the germination of rice bean. In this experiment, the protein bands with MW 20–24 kDa and 27–73 kDa are likely to be the basic and acidic subunits of the 11S globulin. The protein bands with MW of 50 and 76 kDa are likely to be β and α-subunits of 7S globulin (Raut, Kharat, & Mendhulkar, 2015). Katoch (2013) reported that the protein content in different genotypes of rice bean were ranged from 20.35–22.75 %. Globulins (13.11–15.56 g/100 g sample), and albumins (6.13–7.47 g/100 g sample) were major components, followed by glutelins (1.72–2.29 g/100 g sample) and prolamins (1.45–1.97 g/100 g sample).

3.3 Effect of extraction solvents on antioxidant activities of rice bean sprouts

Table 3 presents the antioxidant activity of different extracts of rice bean sprouts. The results showed that the length of the germination period affects the antioxidant activity of rice bean extracts. The DPPH radical scavenging activity and FRAP of the extracts from both treatments increased significantly as the germination period increased ($p \leq 0.05$). This trend was found in both water and ethanolic extracts. However, the citric acid pretreatment resulted in a greater increase in the antioxidant activities of both extracts. The increasing antioxidant activity of sprout extracts from both treatments can be explained by the increased TPC. The results showed a high correlation coefficient (r) between the DPPH activity and the TPC in both pretreated rice bean and the control during

germination (Table 2).

Higher antioxidant activities (DPPH radical scavenging activity and FRAP) and TPC of germinated seeds compared those of raw seeds were also observed in germinated alfalfa seeds (*Medicago sativa* L.), as well as in lentil (*Lens culenta* L.), mung bean (*Vigna radiate* L.), onion (*Allium cepa* L.), broccoli (*Brassica oleraceae* L.), red cabbage (*Brassica oleraceae capitata rubra* L.) and radish (*Raphanus sativus japonicum* L., *Raphanus sativus rambo* L., *Raphanus sativus sinicum rosae* L.) (Aguilera et al., 2015). Fouad and Rehab (2015) also found that germination caused a significant increase in DPPH radical scavenging activity, total phenolic content and free amino acid content in germinated lentils ($p \leq 0.05$). Similar results were also found in germinated mung bean (Wongsiri et al., 2015)

These results also demonstrated that the influence of extraction solvent on measured antioxidant activities of rice bean extracts was remarkable. DPPH radical scavenging activity and FRAP of the ethanolic extracts at each germination period from both treatments were 1.2–1.6 and 1.4–1.6 times higher, respectively, than those of the water extracts due to the different solubility of phenolic compounds in the extraction solvents. Oomah, Caspar, Malcolmson, and Bellido (2011) also reported that the antioxidant activity of red lentil (*Lens culinaris* L.) and yellow pea (*Lathyrus aphaca* L.) extracts obtained from 80 % (v/v) ethanol were 2.17 and 1.24 times higher, respectively, than those of water extracts, as determined using an ORAC assay.

3.4 Phenolic acid and flavonoid composition of rice bean sprouts

The amount of each phenolic compound in rice bean is presented in Table 4. The HPLC chromatogram of mixed standard solution is shown in Supplementary Fig. 1a, while samples of the HPLC chromatogram from water and ethanolic extracts of rice bean sprouts are shown in Supplementary Fig. 1b and 1c, respectively. The chromatograms of phenolic compounds identified using LC-MS are shown in Supplementary Fig. 2. Table 4 shows that the main phenolics found in both 0-hour and 18-hour-germinated rice bean were catechin and rutin. The amount of each phenolic compound in rice bean sprouts tended to increase after 18-hour germination period. The higher amount of each phenolic compound was also found in acid pretreated rice bean compared to the

control (Table 4). The amount of each phenolic compound varied among the different varieties of rice bean. Some of the phenolic compounds found in the present study were similar to those found in rice beans from China. Yao et al. (2012) reported that three phenolic acids, *p*-coumaric acid (5.67-39.72 µg/g), ferulic acid (11.57-78.32 µg/g), and sinapic acid (8.16-27.35 µg/g), and five flavonoids, catechin (24.76-182.64 µg/g), epicatechin (1.35-11.24 µg/g), vitexin (26.46-401.84 µg/g), isovitexin (0.43-271.97 µg/g) and quercetin (10.77-35.46 µg/g), were found in the ethanolic extracts of rice beans from China.

The number of phenolic compounds found in ethanolic extract was higher than that of water extract (Table 4 and Supplementary Fig. 1). The results from HPLC showed that ten phenolic compound, gallic acid, catechin, 4-hydroxybenzoic acid, vanillic acid, epicatechin, *p*-coumaric acid, ferulic acid, vitexin, rutin, and *trans*-cinnamic acid, were found in water extracts of both 0-hour and 18-hour-germinatied rice beans. Fourteen phenolic compounds, gallic acid, catechin, 4-hydroxybenzoic acid, vanillic acid, epicatechin, *p*-coumaric acid, sinapic acid, ferulic acid, vitexin, rutin, *trans*-cinnamic acid, quercetin, naringenin, and genistein, were found in ethanolic extract of both 0-hour and 18-hour-germinated rice beans. These results showed that the extraction solvent strongly affected the number of identified phenolics.

Unidentified peaks at retention time of 19.02, 19.60, 20.17, 21.58, 21.79, and 23.73 min in the water extract and at retention time of 19.57, 20.07, 20.43, 21.89, 22.65, and 23.71 min in ethanolic extract were observed (Supplementary Fig. 1). The separation of phenolics on C18 column is based on the polarity of each compound, these unidentified phenolic compounds might be phenolics that exhibit polarities between gallic acid (retention time at 15.53 min) and catechin (retention time at 25.01 min).

The results from HPLC were supported by the results from LC-MS. It was found that the variety of phenolic compounds in ethanolic extracts identified using LC-MS was higher than that in water extract (Supplementary Fig. 2). In water extract, five phenolic compounds were found in both pretreated rice bean and the control at both 0-hour and 18-hour germination periods: 4-hydroxybenzoic acid and four flavonoids (robinin, rutin, astragalin and 7-methylquercetin). The number of phenolics found in the ethanolic extracts was higher than that of water extracts because the extraction of each phenolic acid and flavonoid depended on solvent polarity. In the ethanolic extract, seven phenolic compounds were found: two phenolic acids (4-hydroxybenzoic acid and sinapic acid) and

five flavonoids (rutin, astragalin, epicatechin, 7-methylquercetin and genistein). Flavonoids in plants can be found as flavonoid aglycones (i.e., hydrophobic flavonoid or hydrophobic antioxidant) and flavonoid glycosides (i.e., hydrophilic flavonoid or hydrophilic antioxidant) in which one or more hydroxyl groups of the aglycones are bound to a sugar (Kumar & Pandey, 2013). In germinated rice bean extract, epicatechin, 7-methylquercetin and genistein are hydrophobic antioxidants, while robinin, rutin, astragalin are hydrophilic antioxidants. Thus, using 80 % (v/v) ethanol as the extraction solvent showed higher extraction efficiencies for epicatechin, 7-methylquercetin and genistein compared to water. Xu and Chang (2007) also reported that the polarity of the extracting solvent affected the TPC and antioxidant activities of green pea, yellow pea, chickpea, lentil, yellow soybean, black soybean, red kidney and black bean extracts.

4. Conclusions

Germination led to an increase in the crude protein, TPC, reducing sugar, and B vitamins (thiamine, riboflavin, and niacin) content of rice bean and a decrease in phytate content in rice bean. Pretreatment with 1% (w/v) citric acid resulted in an increase in both TPC and B vitamins content of rice bean and a change in some storage proteins during germination. Acid pretreatment and longer germination periods also caused an increase in DPPH radical scavenging activity and FRAP. The ethanolic extract of the rice bean sprouts possessed higher antioxidant activities than the water extract. In all samples, the number of identified phenolics found in the ethanolic extracts, as identified using HPLC and LC-MS, was higher than that in the water extracts. Rice bean sprouts might be a potent source of functional ingredients due to their phytochemical and antioxidant activity.

Acknowledgement

This research has been supported by the Thailand Research Fund (Royal Golden Jubilee Ph.D. Program, Grant No. PHD/0154/2553) co-funded by Newton Fund (Ph.D. placement), UK.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

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Fig. 2 The SDS-PAGE patterns of protein extracted from rice bean sprouts. Std: standard molecular weight marker; (a) The rice bean sprout under no pretreatment; and (b) The rice bean sprout with pretreatment.

Supplementary Fig. 1 Typical HPLC chromatograms of phenolic compounds in mixed standard solution (a) water extract of pretreated rice bean at 18 h of germination (b), ethanolic extract of pretreated rice bean at 18 h of germination (c). Peak identifications: (1) gallic acid, (2) catechin, (3) 4-hydroxybenzoic acid, (4) vanillic acid, (5) epicatechin, (6) *p*-coumaric acid, (7) sinapic acid, (8) ferulic acid, (9) vitexin, (10) rutin, (11) *trans*-cinnamic acid, (12) quercetin, (13) naringenin, and (14) genistein

Supplementary Fig. 2 Typical LC-MS chromatograms of phenolic compounds in rice bean sprouts extract: ethanolic extract of pretreated rice bean at 0 h of germination (a), water extract of pretreated rice bean at 18 h of germination (b), ethanolic extract of pretreated rice bean at 18 h of germination (c). Peak identifications: (1) 4-hydroxybenzoic acid, (2) robinin, (3) rutin, (4) astragalin, (5) epicatechin, (6) sinapic acid, (7) 7-methylquercetin, and (8) genistein

Table 1 Effects of pretreatment and the germination period on the chemical composition of rice bean sprouts

Pretreatment/ Germination period (h)	Moisture (g/100 g)	Crude protein (g/100 g d.b.)	Carbohydrate (g/100 g d.b.)	Crude fat (g/100 g d.b.)	Ash (g/100 g d.b.)	Total phenolic (mg of GAE/ g d.b.)	Reducing sugar (mg glucose/ g d.b.)	Thiamin (mg/100 g d.b.)	Riboflavin (mg/100 g d.b.)	Niacin (mg/100 g d.b.)	Phytate (mg/g d.b.)
No pretreatment (water)											
0	46.94^{a}±0.74	21.62^{a}±1.09	72.37^{b}±1.36	0.55^{b}±0.08	5.46^{ab}±0.23	1.17^{a}±0.09	1.52^{a}±0.15	0.10^{a}±0.01	0.08^{a}±0.00	0.36^{a}±0.03	7.71^{d}±0.15
6	56.72^{b}±0.70	22.84^{ab}±1.69	69.68^{ab}±1.82	1.30^{h}±0.07	6.17^{d}±0.08	1.71^{b}±0.07	1.90^{bc}±0.20	0.16^{b}±0.00	0.12^{c}±0.00	0.54^{b}±0.05	7.44^{d}±0.11
12	57.94^{b}±1.28	23.30^{ab}±1.01	69.57^{a}±0.79	0.94^{f}±0.04	6.19^{d}±0.24	1.74^{b}±0.04	2.20^{c}±0.12	0.17^{c}±0.00	0.14^{d}±0.00	0.57^{b}±0.00	7.33^{d}±0.28
18	57.72^{b}±0.92	24.57^{b}±0.92	68.74^{a}±0.88	0.89^{ef}±0.08	5.80^{bc}±0.20	2.20^{de}±0.12	2.04^{bc}±0.11	0.21^{e}±0.00	0.14^{d}±0.00	0.83^{d}±0.00	6.59^{bc}±0.23
24	61.21^{c}±1.53	24.02^{ab}±0.76	69.77^{ab}±0.81	0.77^{d}±0.06	5.44^{ab}±0.11	2.41^{e}±0.21	2.95^{ef}±0.21	0.31^{g}±0.00	0.15^{d}±0.00	1.08^{f}±0.00	6.22^{ab}±0.26
1% (w/v) citric acid											
0	45.57^{a}±1.08	23.04^{ab}±1.71	71.02^{ab}±1.93	0.66^{c}±0.05	5.28^{a}±0.27	1.22^{a}±0.07	1.72^{ab}±0.16	0.18^{d}±0.00	0.11^{b}±0.00	0.39^{a}±0.00	7.68^{d}±0.23
6	59.70^{c}±1.11	22.35^{ab}±0.39	70.28^{ab}±0.54	1.04^{g}±0.04	6.32^{d}±0.27	2.04^{cd}±0.22	2.61^{de}±0.14	0.27^{f}±0.00	0.17^{e}±0.00	0.55^{b}±0.04	7.49^{d}±0.36
12	59.88^{c}±0.42	23.32^{ab}±1.44	69.80^{ab}±1.30	0.88^{ef}±0.06	6.01^{cd}±0.22	1.94^{bc}±0.08	2.59^{d}±0.08	0.30^{g}±0.00	0.26^{f}±0.02	0.71^{c}±0.05	7.31^{d}±0.35
18	60.91^{c}±0.30	24.89^{b}±1.46	68.81^{a}±1.46	0.79^{de}±0.03	5.51^{ab}±0.11	2.83^{f}±0.16	2.85^{def}±0.32	0.31^{g}±0.00	0.28^{g}±0.00	0.91^{e}±0.05	6.71^{c}±0.37
24	60.12^{c}±0.22	24.35^{b}±1.96	69.79^{ab}±2.17	0.20^{a}±0.04	5.53^{ab}±0.25	3.11^{g}±0.24	3.08^{f}±0.34	0.30^{g}±0.00	0.28^{g}±0.00	1.25^{g}±0.00	6.02^{a}±0.23

Mean value followed by different superscript letters in the same column are significantly different ($p \leq 0.05$)

d.b., dry basis

Table 2 Effects of pretreatment and the germination period on the nitrogen and TCA-soluble peptide content of rice bean sprouts

Pretreatment	Germination period (h)	Amount of total nitrogen (g /100 g d.b.)	True protein nitrogen (g/100 g d.b.)	Non-protein nitrogen[ns] (g/100 g d.b.)	TCA-soluble peptide (g/100 g d.b.)	Total free amino acid (mg/100 g d.b.)
No pretreatment (water)	0	3.46^{a}±0.17	2.21^{a}±0.22	1.25±0.27	0.40^{d}±0.02	413.54^{b}±27.64
	6	3.65^{ab}±0.27	2.51^{abcd}±0.04	1.14±0.24	0.32^{b}±0.01	335.35^{a}±34.03
	12	3.73^{ab}±0.16	2.54^{abcd} ±0.14	1.19±0.30	0.37^{c}±0.02	468.31^{bc} ±14.31
	18	3.93^{b}±0.15	2.62^{bcd}±0.23	1.31±0.10	0.24^{a}±0.01	744.3^{ef}±17.22
	24	3.84^{ab}±0.12	2.47^{abc}±0.16	1.37±0.11	0.25^{a}±0.02	1764.10^{g}±7.66
1% (w/v) citric acid	0	3.69^{ab}±0.27	2.30^{ab}±0.26	1.38±0.07	0.40^{cd}±0.02	580.79^{d}±26.31
	6	3.58^{ab}±0.06	2.54^{abcd}±0.20	1.04±0.17	0.38^{cd}±0.01	723.89^{e}±19.10
	12	3.73^{ab}±0.23	2.59^{bcd}±0.18	1.14±0.32	0.37^{c}±0.01	799.99^{f}±60.34
	18	3.98^{b}±0.23	2.83^{cd}±0.26	1.15±0.18	0.30^{b}±0.01	562.06^{d}±41.15
	24	3.90^{b}±0.31	2.86^{d}±0.04	1.04±0.28	0.31^{b}±0.02	526.85^{cd}±19.11

Mean values followed by different superscript letters in the same column are significantly different ($p \leq 0.05$).

[ns] Mean values not significantly different ($p > 0.05$) in the same column.

d.b., dry basis.

Table 3 Effects of extraction solvent on the antioxidant activity of rice bean sprouts under no pretreatment and with pretreatment as well as correlation coefficient (*r*) between the antioxidant activity and TPC

Pretreatment	Germination period (h)	Antioxidant activity			
		DPPH assay (μmol TE/g d.b.)		FRAP assay (μmol TE/g d.b.)	
		Water extract	Ethanol extract	Buffer extract	Ethanol extract
No Pretreatment (water)	0	6.65^{a}±0.35	9.88^{a}±0.28	2.69^{a}±0.18	3.99^{a}±0.07
	6	9.68^{b}±0.53	12.85^{b}±0.42	4.08^{b}±0.51	6.55^{b}±0.18
	12	12.45^{d}±0.74	17.53^{d}±0.82	4.50^{bc}±0.14	6.55^{b}±0.29
	18	13.49^{d}±0.54	18.38^{de}±0.46	4.68^{c}±0.24	6.67^{b}±0.23
	24	15.00^{e}±0.47	20.08^{f}±1.14	4.89^{cd}±0.38	7.08^{c}±0.27
r between antioxidant activity and TPC during germination		0.944	0.856	0.924	0.868
1% (w/v) citric acid	0	6.57^{a}±0.38	9.32^{a}±0.22	2.65^{a}±0.18	4.01^{a}±0.19
	6	11.44^{c}±0.56	14.81^{c}±0.71	4.64^{c}±0.26	6.87^{bc}±0.07
	12	13.35^{d}±0.84	19.01^{e}±0.36	5.03^{cd}±0.30	6.91^{bc}±0.32
	18	15.72^{e}±0.89	20.60^{f}±0.27	5.32^{d}±0.24	7.21^{c}±0.18
	24	15.47^{e}±0.24	20.62^{f}±0.25	5.34^{d}±0.18	7.22^{c}±0.16
r between antioxidant activity and TPC during germination		0.952	0.874	0.861	0.819

Mean values followed by different superscript letters in the same column are significantly different ($p \leq 0.05$).

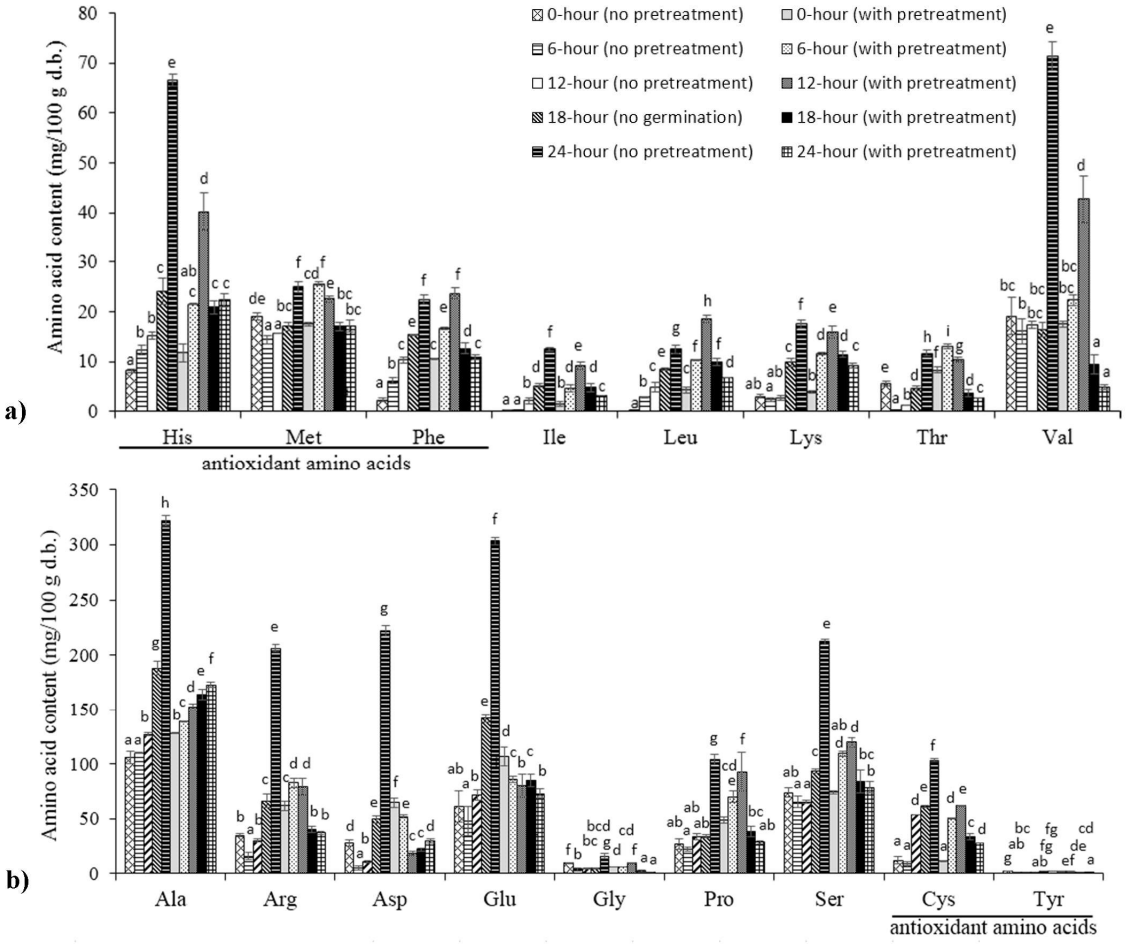
d.b., dry basis.

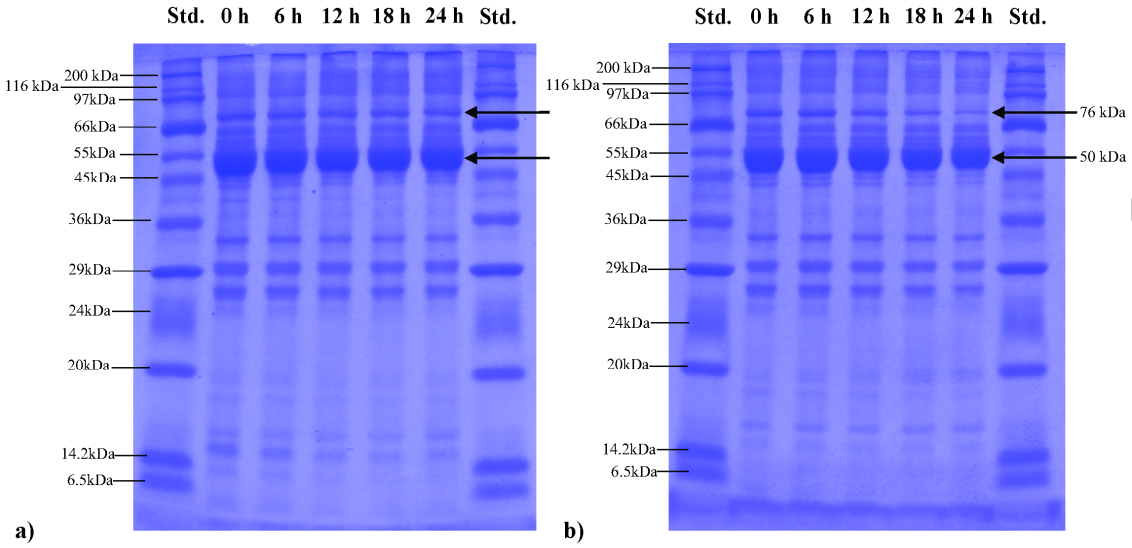
mpounds	Retention time (min)	λmax (nm)	0-hour rice bean sprouts, no pretreatment		0-hour rice bean sprouts, with pretreatment		18-hour rice bean sprouts, no pretreatment		18-hour rice bean sprou with pretreatment	
			Water extract	Ethanolic extract	Water extract	Ethanolic extract	Water extract	Ethanolic extract	Water extract	Ethanoli extract
llic acid	15.529	265	13.07±0.07	8.74±0.17	13.36±0.05	13.59±0.46	15.90±0.16	12.75±0.48	13.88±0.65	11.68±0
techin	25.012	280	35.96±0.20	108.72±5.21	39.33±0.22	103.52±5.17	48.60±2.28	102.70±8.25	47.50±0.08	150.67±10
Hydroxybenzoic d	28.196	265	2.51±0.05	7.82 ±0.25	5.15±0.74	9.60±0.20	3.16±0.02	9.14±0.34	3.15±3.65	10.09±1
illic acid	32.112	265	2.60±0.03	1.01±0.02	2.76±0.03	1.07±0.02	2.78±0.01	0.96±0.01	2.84±0.03	1.03±0
icatechin	32.253	280	14.96±1.03	12.86±3.19	16.95±0.88	14.26±0.11	20.62±0.56	17.14±0.38	11.34±1.50	15.02±1
oumaric acid	46.309	280	4.42±0.11	5.08±0.14	7.66±0.10	6.30±0.44	4.17±0.19	5.19±0.53	7.32±0.05	7.55±0
apic acid	49.803	280	nd	1.74±0.02	nd	2.08±0.01	nd	5.70±0.03	nd	12.15±0
rulic acid	50.006	280	22.88±0.05	22.27±0.99	22.82±0.13	23.71±0.38	24.60±0.42	22.97±1.46	25.22±0.04	24.16±1

Table 4 The content of individual phenolic compound in rice beans (μg/g dry sample)

texin	56.281	265	4.06±0.17	2.13±0.01	3.92±0.01	2.02±0.07	4.52±0.01	2.58±0.02	4.71±0.04	2.85±0
tin	63.640	265	30.47±0.47	154.74±2.29	46.88±3.04	208.18±0.52	31.57±0.80	170.07±8.31	49.48±1.09	206.69±0
ns-Cinnamic d	73.343	280	1.03±0.03	5.10±0.26	0.92±0.02	2.97±0.01	1.09±0.03	7.01±0.52	1.24±0.02	4.94±0
ercetin	74.336	265	nd	35.94±0.35	nd	30.05±1.04	nd	48.92±0.20	nd	44.62±3
ringenin	74.860	280	nd	12.51±1.87	nd	11.54±0.52	nd	11.27±0.49	nd	15.14±0
nistein	76.173	265	nd	3.91±0.02	nd	3.54±0.03	nd	4.89±0.22	nd	5.12±0

nd: not detected





Highlights (maximum 85 characters, including spaces, per bullet point).

- Germination increases crude protein and total phenolic content of rice bean
- Acid pretreatment and germination improve antioxidant activities of rice bean
- Germination strongly affects an increase in alanine, glutamic acid and serine
- Acid pretreatment leads to a significant decrease in intensity of the 76 kDa band
- Ethanolic extract contains more phenolics and antioxidant than water extract