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Research article

Homogenizer-Assisted Extraction of Antioxidative Compounds from Whole Riceberry Flour and Quality Changes After Freeze-Drying Process

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Abstract

Keywords

antioxidative compounds;
extraction;
freeze-drying;
homogenization;
riceberry

The objectives of this study were to determine the optimum conditions for the extraction of whole riceberry flour, and to evaluate the effects of freeze-drying on the phenolic content and antioxidant activity of extracts during 6 weeks of storage. Response surface methodology and factorial experimental design were employed to study the effects of extraction time (10-30 s) and ethanol concentration (40-60 % v/v) on homogenizer-assisted extraction (HAE) of phenolic content and antioxidant activity. The HAE under optimum conditions (20 s and 60%) was able to extract a high phenolic content (76.4 ± 2.0 mg GAE/g dw) and antioxidant activity (88.97 ± 0.97 µmol Trolox/g dw). The accuracy and reliability of the extraction was confirmed and the optimal conditions were determined using response surface methodology and central composite rotatable design. In the case of the freeze-drying process, phenolic content and antioxidant activity decreased by 21.42 ± 0.11 and 32.16 ± 0.87% after 6 weeks storage, respectively. The regression model for the extraction of total phenolic content from riceberry flour that was developed might contribute to large-scale industrial applications aimed at obtaining the optimum extraction conditions by using homogenization methods. This study is a valuable contribution to the fields of adding value to riceberry flour and finding new sources of functional ingredients.

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1. Introduction

Many pigmented rice cultivars have been reported to contain high levels of nutrients with high potential to promote human health due to the great antioxidant potency of their phytochemicals such as phenolics, anthocyanidins, flavonoids, folate, vitamin E, and β-carotene [1]. Thai riceberry rice has been developed to provide nutritional benefits to consumers and to be a medical supplement for type 2 diabetes and anaemia patients because it contains a high amount of iron and has a low glycaemic index [2]. Leardkamolkarn *et al.* [3] reported that bran extract of riceberry had potential anticancer activity, as well as hypoglycaemic, hypolipidemic, antioxidant, and anti-inflammation properties. Despite the dietary benefits of pigments in the rice, it was not ideal for food consumption due to its limitation in terms of the hard texture of cooked pigmented rice. Previous researchers have attempted to investigate methods of extraction of antioxidant compounds from riceberry and their application in diets containing bioactive compounds [4]. High-pressure homogenization is one of the most commonly used processes which does not involve thermal food processing. This technology uses the combined forces of high-velocity impact, intense shear, high vibrational frequency, cavitation, and instant controlled pressure-drop [5]. Homogenization is a method that has been applied widely in the pharmaceuticals, food, and other material manufacturing industries. High-pressure homogenization was not only shown to help significantly with emulsification and sterilization; the method was also used to decrease the particle size and release bioactive compounds. Li *et al.* [6] reported that high-pressure homogenization disrupted cellular structures so that high impact shear could damage cell walls, mitigate particle size, and cause release of a significant ($P \leq 0.05$) level of cellular products. The preservation of heat-sensitive biological substances by freeze-drying offers benefits such as retention of biochemical properties and long shelf life for storage. Tangtua *et al.* [7] also reported that freeze-drying could maintain the stability of bioactive compounds from microbial cells. This study aimed to optimize the homogenizer-assisted extraction of phenolic content with antioxidant properties from whole riceberry flour using response surface methodology. Extraction time and concentration level of ethanol were factors that were examined. In addition, the crude extracts obtained were also investigated for the effects of freeze-drying on total phenolic content and antioxidant capacity over a 6 weeks storage period.

2. Materials and Methods

2.1 Riceberry

Thai dark purple rice, *cv*. Riceberry, was obtained from Wattana Rice Trade Community Enterprise Groups, Phichit Province, Thailand. Whole riceberry grains were ground into flour using a kitchen mill (Otto, OT-122G, Thailand) at 25,000 rpm, and passed through a 60-mesh screen sieve to ensure the homogeneity of particle size. The rice flour was stored at 4°C until extraction. All chemicals and reagents used for spectrophotometric analysis were analytical grade. Folin-Ciocalteu's phenol reagent and ethanol were obtained from Merck (Darmstadt, Germany). The other compounds, namely, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picryl hydrazyl (DPPH), and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Experimental design

Response surface methodology (RSM) and a face-centred central composite design (CCRD) were employed to optimize the effects of ethanol concentration (40-60 % v/v) and extraction time (10-30 s) on total phenolic content and antioxidant capacity from whole riceberry powder. The experiments

consisted of 9 treatments, for which triplicate extractions were performed (Table 1). The effects of unexplained variation in the observed responses due to extraneous variables factors were minimized by randomizing the experimental order. The design variables were the ethanol concentration (X_1, % v/v) and extraction time (X_2, s), while the response variables were the content of total phenolic compounds and antioxidant activity [8].

Table 1. Experimental design with actual and predicted response values

Treatment	Independent variables		Total phenolic content (mg GAE eq./g db)		DPPH (µmol Trolox eq./g db)	
	Ethanol (% v/v)	Time (s)	Actual*	Predicted	Actual*	Predicted
1	40	10	61.07 ± 1.15	57.36	41.54 ± 1.02	41.26
2	40	20	61.73 ± 1.15	64.47	63.46 ± 2.77	64.17
3	40	30	49.07 ± 2.31	50.03	60.38 ± 0.77	58.48
4	50	10	63.07 ± 1.15	65.81	52.56 ± 1.18	51.32
5	50	20	75.07 ± 1.15	72.92	70.26 ± 2.56	74.23
6	50	30	59.07 ± 2.31	58.47	68.33 ± 0.97	68.55
7	60	10	67.73 ± 2.31	68.70	59.86 ± 1.26	61.38
8	60	20	76.40 ± 2.00	75.81	88.97 ± 0.97	84.29
9	60	30	61.73 ± 1.15	61.36	76.92 ± 0.38	78.61

* Mean ± standard deviation of triplicate analyses

2.3 Homogenizer-assisted extraction

Homogenizer-assisted extraction (HAE) was used for the antioxidant compound extraction from the whole riceberry grain flour. The optimized extraction conditions were determined according to the experimental design which is shown in Table 1. For the extraction, the riceberry flour was extracted with aqueous ethanol (40, 50, and 60% v/v). For each trial, 50 g of flour sample was mixed with 500 ml of the solvent. All extractions were done in a homogenizer at 11,000 rpm (ESR-200 Handlhead Emulsifier, China) by varying the extraction time for 10, 20, and 30 s. The extract was filtered through Whatman No. 42 filter paper and then centrifuged at 3000 × g at 5°C for 10 min (Universal 32 R, Hettich, Germany). The extract solvent was evaporated using a rotary (N-1300E·V·S Series, Eyela, Japan) under the vacuum of 50 mm Hg at 45ºC to obtain the dry extract, which was stored at 4°C in a refrigerator (HITACHI, R-64V-3, Thailand) until use for quantitative analysis of total phenolic content and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging.

2.4 Freeze-drying process and storage stability of riceberry extract powder

The optimum conditions for antioxidative compound extraction from riceberry flour were selected for subsequent freeze-dried extract preparation. For the freeze-drying process, crude extract was poured into an aluminium tray and frozen at -20°C in a freezer for 24 h. The frozen samples were put in a freeze-drier (CHRIST, Alpha 1-4 LDplus, Germany) for 24 h. After completion of the freeze-drying process, each extract was ground into a powder form and stored in a transparent low-density polyethylene zip lock bag at ambient room temperature of 27 ± 5°C in order to evaluate the water activity (a_w), total phenolic content, and DPPH radical scavenging activity. Samples were collected at 0, 2, 4, and 6 weeks of the storage for analysis of storage stability.

2.5 Analytical measurements

The total phenolic content of the samples was determined by Folin-Ciocalteau reagent as described by Singleton *et al.* [9]. In a test tube, 200 µl of rice extract (previously diluted ten-fold with distilled water) was mixed with 1.5 ml of Folin-Ciocalteau reagent and allowed to stand for 5 min at ambient temperature. A 1.5 ml of 7.5% (w/v) sodium carbonate solution was added to the mixture and agitated with a vortex mixer (Scientific industries, G-560E, U.S.A.). The final concentration of rice extract was 6.25% (v/v). After 1 h incubation at ambient room temperature, the absorbance was measured at 750 nm using a UV-Vis spectrophotometer (Thermo, Genessys 10 Series, U.S.A.). The content of phenolics was calculated from the regression equation of the calibration curve of standard gallic acid (20-200 μg/ml in water). The results were calculated according to the standard curve of gallic acid and expressed as milligrams gallic acid equivalents (GAE) per gram of dry weight (dw). The free radical scavenging activity was analysed by the DPPH method according to Altemimi *et al*. [10]. A 10 ml aliquot of DPPH solution (dissolving 2 mg DPPH with 100 ml of methanol) was mixed with 100 µl of the sample extracts. All mixture samples were kept at room temperature (25-28°C) in the dark for 30 min, and then a UV-spectrophotometer was used to measure the absorbance of the solution at 517 nm. The calibration curve was performed with Trolox solution. The result of total antioxidant activity was expressed as equivalent of micromoles of Trolox per gram of riceberry flour (µmol Trolox /g dw). The water activity of rice extract powder was measured at 25°C using a water activity meter (AQUA LAB, Series 3 TE, U.S.A.). After equilibration, the a_w value was recorded. The measurements were taken in triplicate.

2.6 Statistical analysis

All analyses were performed in triplicate. Response surface methodology was performed by R version 3.2.2. Analysis of variance (ANOVA) and Duncan's New Multiple Range Test were used to evaluate the difference between means at the 95% confidence interval.

3. Results and Discussion

3.1 Total phenolic content

Various ethanol concentration levels and the HAE method were applied for the extraction of total phenolic content, which were determined by colorimetric method with the Folin-Ciocalteu reagent. The highest concentration level of total phenolic content (76.4 ± 2.0 mg GAE/g extract) was obtained from extraction with aqueous ethanol at 60% concentration with HAE for 20 s; whereas, the lowest total phenolic content (49.07 ± 2.31 mg GAE/g extract) was obtained from extraction with 40% ethanol for 30 s (Table 1). Compared with traditional methods (soxhlet extraction, ultrasound-assisted extraction, and microwave-assisted extraction), the high-speed shearing homogenization extraction method has the advantages of faster extraction times, less solvent waste, higher yields, simpler device, and easier operation [11]. When comparing the results from previous research and our study, it was found that total phenolic content of 76.4 ± 2.0 mg GAE/g extracted from riceberry flour using the HAE method for 20 min was much higher than solvent extraction using 70:30 ethanol-water for 6 h (53.4 ± 1.4 mg GAE/g extract) [4], ultrasound-assisted extraction (48.5 ± 0.5 mg GAE/g extract), soxhlet extraction (18.1 ± 0.5 mg GAE/g extract), and maceration method (21.7 ± 0.3 mg GAE/g extract) [12]. This result showed that HAE had potential application in the extraction of total phenolic content from riceberry flour. Therefore, response surface methodology was used to optimize the HAE method in order to maximize the concentration of

phenolic compounds extracted. Two independent variables including the concentration level of ethanol (%) and extraction time (s) were studied. The regression model for total phenolic content is shown in equation 1.

$$TPC = -60.64 + 3.94Time + 3.34Ethanol - 0.108Time^2 - 0.028Ethanol^2 \quad (1)$$

The model was significant ($P \leq 0.05$) with an adjusted R^2 of 0.83 (Table 2). The Shapiro-Wilk test indicated that standardized residuals were normally distributed ($p = 0.443$). These values indicated that the model can be used to predict the response value within the study range.

Table 2. Coefficients estimated and *p*-value of regression models

Terms	Total phenolic content		DPPH	
	Estimated coefficients	***p*-value**	**Estimated coefficients**	***p*-value**
(Intercept)	-60.637	0.0375*	-50.501	<0.0001**
Time	3.944	<0.0001**	6.580	<0.0001**
Ethanol	3.344	0.0063**	1.006	<0.0001**
$Time^2$	-0.108	<0.0001**	-0.143	<0.0001**
$Ethanol^2$	-0.028	0.0199*		
Model		<0.0001**		<0.0001**
Lack of fit		0.0003**		<0.0001**
R^2		0.9064		0.9589
Adjusted R^2		0.8894		0.9536

Note: ** $p < 0.01$ extremely significant; * $p < 0.05$ significant

The effects of the ethanol concentration (40 and 60%) on the total phenolic content were significantly different ($P \leq 0.05$) against each other at the same extraction time (10, 20, and 30 s) which obtained total phenolic contents of 61.07 ± 1.2 and 67.73 ± 2.3, 61.73 ± 1.2 and 76.4 ± 2.0, and 49.07 ± 2.3 and 61.73 ± 1.2 mg GAE/g extract, respectively). Increasing the concentration of ethanol from 40 to 60% tended to improve the extractability of total phenolic content regardless of the extraction time used (Figure 1). Ethanol was a suitable solvent for manufacturing industry. The excessive diffusion of solvent caused swelling of plant cells creating pores so that more phenolic content could be extracted out from the cells. The appropriate concentration levels of ethanol provided a suitable polarity for the target compounds. This was because there was an increase in the concentration difference inside the plant cells and external solvent, which allowed more solvent to diffuse into the cells causing maximum phenol extraction [13]. The low solubility of phenolic content in absolute solvents might be owing to the stronger interactions between polyphenols and proteins than that between non-covalent polyphenol and macromolecule. However, the solubility could increase depending on the addition of water to organic solvents, which could result in weakening of the hydrogen bonds.

In a related study, Onwuka *et al.* [14] compared the extraction of phenolic content in musk tree seed (*Buchholzia coriacea*) with different ethanol and water mixtures at 20, 40, 60, 80 and 100% aqueous solutions. The most suitable concentration level was found to be a 40% aqueous solution followed by 60%. A further increase in the ethanol concentration to 80% caused a significant ($P \leq 0.05$) decrement in the extracted mass. In addition, the phenolic compositions of peanut skin were investigated by Nepote *et al.* [15] with different concentration levels of ethanol. They reported that ethanol at the concentration level of 50% (v/v) contributed the highest polyphenol content, a result which decreased with increasing concentration levels of ethanol higher than 70% (v/v) [16].

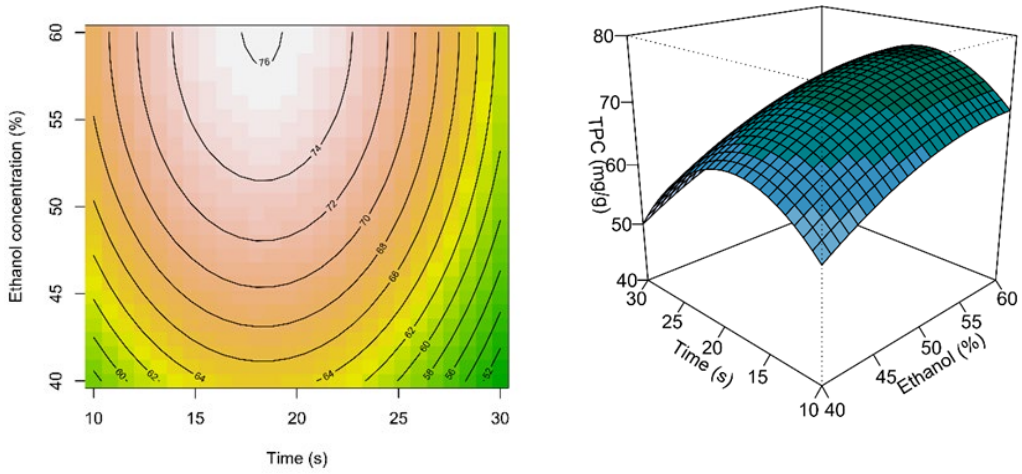

Figure 1. Contour (A) and surface plot (B) of total phenolic content as a function of extraction

The influence of different extraction times (10, 20 and 30 s) on the concentration level of total phenolic content was examined. Total phenolic content increased with longer extraction times, and a maximum value was achieved at extraction times between 15 to 20 s and decreased thereafter (Figure 1). The results indicated that higher temperature with longer extraction times may increase the chance of oxidation of the phenolic compounds, which decreases the content of phenolics in the extracts. Cao *et al*. [17] investigated the effect of extraction time (2.0-25 min) on the total phenolic content from rattan (*Calamoideae faberii*) after homogenate extraction. The total phenolic content initially increased with increasing extraction time during 0.5-2 min. However, there were no further increases in the total phenolic content after 2 min extraction. Hence, the optimum extraction time using the homogenate method of phenolic content from rattan was 2 min. The homogenization of samples at the optimum extraction time enhanced the release of bioactive compounds to the solution by rupturing the plant [18].

3.2 Antioxidant capacity (DPPH radical scavenging activity)

The free radical scavenging activity of various extracts from plant were evaluated by DPPH assay. DPPH is a stable free radical that dissolves in organic solvents such as ethanol and methanol. The reduction capacity of DPPH free radical is calculated directly from the decrease in its absorbance at 517 nm, when the colour of the DPPH assay solution turns to light yellow from purple. The degree of discoloration indicates the scavenging potentials of plant extract antioxidants [19]. The effect of different ethanol concentration levels and extraction time on the DPPH radical scavenging activity of riceberry flour extracts (Table 1) revealed more than 29 µmol Trolox/g in all sample extracts. However, the highest DPPH scavenging activity (88.97 ± 0.97 µmol Trolox/g dw) was detected in samples extracted with 60% concentration level of ethanol solvent for 20 s. A low DPPH radical scavenging activity (29-36 µmol Trolox/g dw) was found in solvent extraction without using the homogenization process. The effect of ethanol concentration levels and extraction time on DPPH radical scavenging activity was described by equation 2. The model was statistically significant

($P \leq 0.05$) with an adjusted R^2 of 0.9536 and a normal distribution of standardized residuals ($p = 0.7709$), which indicated the validity of the model.

$$DPPH = -50.50 + 6.58Time + 1.01Ethanol - 0.143Time^2 \quad (2)$$

The antioxidant capacities of crude extract were found to be sensitive to ethanol concentration. The DPPH radical scavenging activity of crude extract exhibited higher levels with increasing concentration levels of ethanol (Figure 2), and these correlated with the total phenolic content (Figure 3). Based on these experimental results, it could have been that highly active phenolic compounds presented in riceberry powder extracts were moderately polar. The effects of extracting solvent concentration on DPPH radical scavenging activity was investigated by Safdar *et al.* [20]. They reported that the value of the DPPH radical scavenging activity of kinnow (*Citrus reticulate* L.) peel extract with 50% aqueous ethanol (v/v) was higher than 20% ethanolic extracts. Similarly, Do *et al.* [21] reported the effect of solvent extraction on the antioxidant activity of *Limnophila aromatic*, a medicinal herb in Southeast Asia, and observed that 100% ethanolic extract produced the highest DPPH radical scavenging activity of 180 µg/ml. Moreover, Zuorro *et al.* [22] stated that raising the ethanol concentration level from 60 to 100% was associated with decreasing the antioxidant capacity (DPPH) of brewers' spent grain crude extract from 0.043 ± 0.005 to 0.019 ± 0.003 mg Trolox/g dw.

Although there was a strong correlation between antioxidant activity and total phenolic content of the extract, the optimal conditions for extraction varied based on the criteria used in the calculation. If the highest total phenolic content was the only criteria, the optimal condition was extraction with 60% ethanol for 18.5 s. If DPPH was the only criteria, the optimal condition was extraction with 60% ethanol for 23 s. When both total phenolic content and DPPH were criteria, the optimal condition was extraction using 60% ethanol for 20.5 s (Table 3). This condition was further compared with the freeze-drying method and analysed for the antioxidant activity of freeze-dried riceberry powder extracts during 6 weeks storage at 27 ± 5°C.

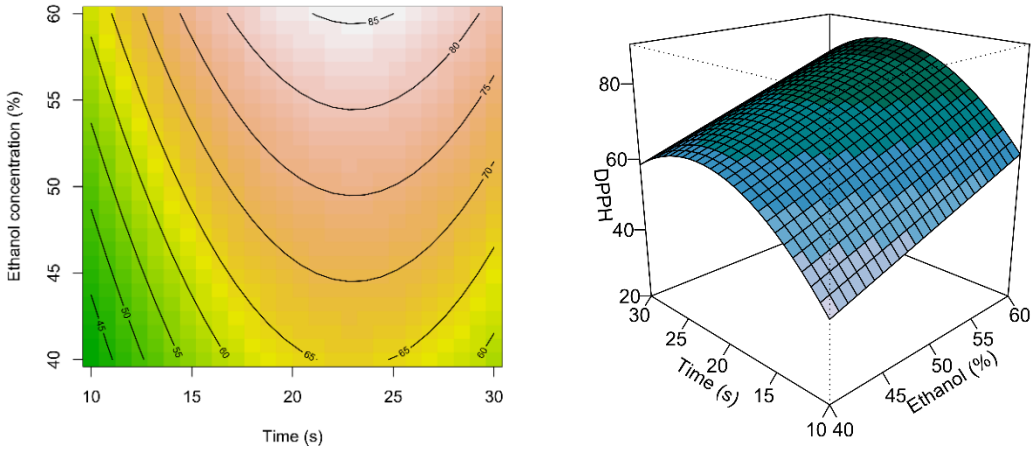

Figure 2. Contour (A) and surface plot (B) of DPPH radical scavenging activity as a function of extraction time and ethanol concentration

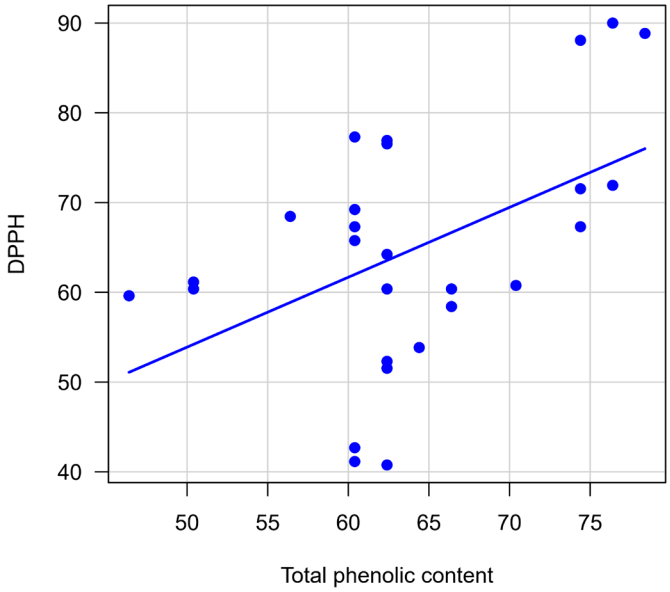

Figure 3. Correlation between total phenolic content and DPPH radical scavenging activity (Pearson's correlation coefficient = 0.478, *p*-value = 0.0116)

Table 3. Optimal condition for extraction of total phenolics and antioxidant from riceberry bran

Criteria		Extraction conditions		Predicted response values	
TPC	**DPPH**	**Time (s)**	**Ethanol (%)**	**TPC**	**DPPH**
Maximum	–	18.5	60.0	76.11	82.68
–	Maximum	23.0	60.0	73.74	85.59
Maximum	Maximum	20.5	60.0	75.60	84.69

3.3 Effect of storage on antioxidant activity of freeze-dried riceberry powder extracts

Freeze-drying is a suitable technique for preserving heat-sensitive compounds. It has been widely used to preserve nutritive quality by preventing oxidation, reducing the loss of volatile substances, and minimizing the degradation of thermolabile compounds during industrial processes [23]. The results of this study (Figure 4) showed that the total phenolic content and DPPH radical scavenging activity of freeze-dried riceberry extract ranged from 60 to 78 mg GAE/g dw and from 59 to 88 mg µmol Trolox/g dw during 6 weeks of storage, respectively.

Both the total phenolic content and the activity of DPPH radical scavenging in dried samples decreased by 21.42 ± 0.11 and 32.16 ± 0.87% after 6 weeks storage, respectively. The reduction in total phenolic content and antioxidant activity might have been caused by the high a_w (ranging from 0.4 to 0.6) of the dried samples. Water activity correlated with the stability of foods in terms of chemical reactions. Usually an a_w value higher than 0.4 enhances the deterioration of oxidation ability [24]. Hence, the a_w increased because the moisture content could penetrate through the transparent low-density polyethylene zip lock bag used during storage. Another main cause of phenolic degradation in riceberry powder extracts was oxidation. The presence of oxygen inside the packaging was associated with enzymatic oxidation of the antioxidants. A low-density polyethylene

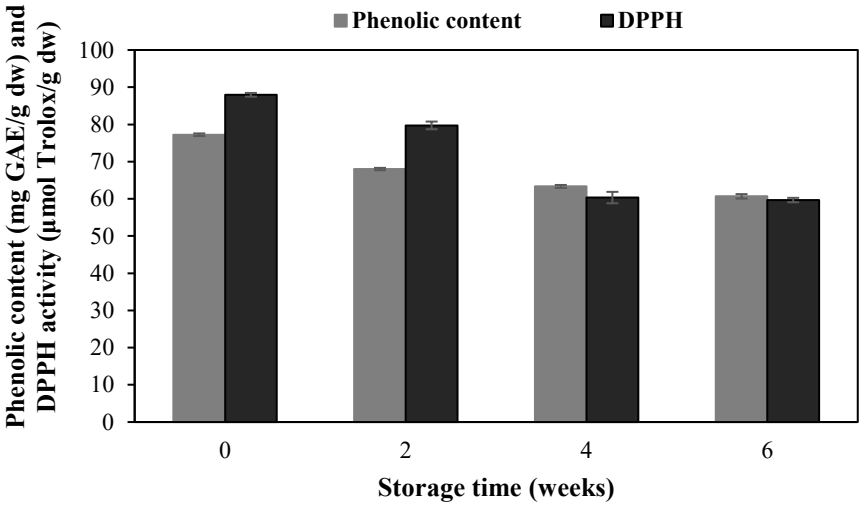

Figure 4. Total phenolic content and DPPH radical scavenging activity of riceberry extract during storage

film allowed high permeability to oxygen and moisture transfer from the external environment into the product as compared with high-density polyethylene, polyethylene terephthalate, and metalized films [25]. According to Niazmand and Yeganehzad [26], vacuum packaging gave more stable total phenolic content and antioxidant capacity from barberries than low-density polyethylene packaging during 4 weeks storage at 25°C. Moreover, the riceberry powder extracts from the present study were stored in transparent bags at relatively high temperatures (more than 25°C). This was another possible reason for the degradation of the phenolics present in the dried extracts. Ali *et al*. [27] also observed that dried *Piper betle* extracts stored at low temperature of 5ºC in the dark condition retained total phenolic content and antioxidant activity more than 95% after 180 days of storage. Therefore, degradation of total phenolic content and antioxidant activity from riceberry powder extracts occurred throughout the storage time.

4. Conclusions

The experimental design approach using RSM was successfully applied to optimize the homogenizer-assisted extraction of antioxidative compounds from whole riceberry flour. The most efficient extraction conditions were established using 60% (v/v) ethanol concentration with an extraction time of 20 s, conditions which produced the highest total phenolic content and a DPPH radical scavenging activity of 76.4 ± 2.0 mg GAE/g and 88.97 ± 0.97 µmol Trolox/g, respectively. The freeze-dried crude extract results suggested that the overall trends of total phenolic content and antioxidant capacity in dried samples was to decrease, whereas a_w slightly increased during 6 weeks of storage. The regression model from the present study can be adapted for industrial solvent extraction process optimization of total phenolic content from riceberry flour, which was much more efficient when used with the homogenization process. Furthermore, the extract can potentially be applied as an active ingredient for the functional food industry

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