Poster Session | Food Function/Nutrition

[6-1130-P] Functional/Wellness Foods &Nutrition (6th)

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[6-1130-P-03] Evaluation of Total Anthocyanins and Antioxidant Activity of Thai Rice Cultivars for Phenotypic Selection in Rice Breeding

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Keywords: Extraction, Anthocyanins, Antioxidant activity, Thai black rice

Black rice has been gained increasing interest for consumers and rice breeders due to its high nutritional values of anthocyanin contents and antioxidative properties. The objective of this study was to determine the optimal solvents for anthocyanin extraction and quantification of antioxidant activity for selection of Thai rice cultivars with high anthocyanins and antioxidant activity to be used in rice breeding program. The dehulled mature seeds of Thai black rice cv. Hom Nin were extracted by different solvent types and the extracts were evaluated for total anthocyanin contents and antioxidant activity by spectrophotometry and DPPH assay, respectively. The results demonstrated that the extract with 1% HCl in 80% methanol gave the highest total anthocyanins and antioxidant activity. This solvent was subsequently used for extraction of seeds from eight rice cultivars, which consisted of four non-pigmented (white) and four black rice cultivars. It was found that the extracts from black rice cultivars showed no significantly different levels of antioxidant activity, possibly due to interference by hydrochloric acid in DPPH assay. Therefore, 80% methanol was used for anthocyanin extraction of rice cultivars. The results showed that antioxidant activity had positive correlation with amount of total anthocyanin contents and phenotypic traits of pericarp colors. In this study, Thai black rice cv. Mali Dum (MLD) gave the highest total anthocyanin contents and antioxidant activity which were correlated with coloration of extracted sample and pericarp color. Our study suggested that MLD would be a good source of high anthocyanins and antioxidant activity for use as parental line in rice breeding program for improvement of rice with health promoting properties. Moreover, advanced breeding lines with high anthocyanin contents and antioxidant activity could be identified by methanolic extraction method followed by spectrophotometric measurements and DPPH assay.

Evaluation of Total Anthocyanins and Antioxidant Activity of Thai Rice Cultivars for Phenotypic Selection in Rice Breeding

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ABSTRACT

Black rice has been gained increasing interest for consumers and rice breeders due to its high nutritional values of anthocyanin contents and antioxidative properties. The objective of this study was to determine the optimal solvents for anthocyanin extraction and quantification of antioxidant activity for selection of Thai rice cultivars with high anthocyanins and antioxidant activity to be used in rice breeding program. The dehulled mature seeds of Thai black rice cv. Hom Nin were extracted by different solvent types and the extracts were evaluated for total anthocyanin contents and antioxidant activity by spectrophotometry and DPPH assay, respectively. The results demonstrated that the extract with 1% HCl in 80% methanol gave the highest total anthocyanins and antioxidant activity. This solvent was subsequently used for extraction of seeds from eight rice cultivars, which consisted of four non-pigmented (white) and four black rice cultivars. It was found that the extracts from black rice cultivars showed no significantly different levels of antioxidant activity, possibly due to interference by hydrochloric acid in DPPH assay. Therefore, 80% methanol was used for anthocyanin extraction of rice cultivars. The results showed that antioxidant activity had positive correlation with amount of total anthocyanin contents and phenotypic traits of pericarp colors. In this study, Thai black rice cv. Mali Dum (MLD) gave the highest total anthocyanin contents and antioxidant activity which were correlated with coloration of extracted sample and pericarp color. Our study suggested that MLD would be a good source of high anthocyanins and antioxidant activity for use as parental line in rice breeding program for improvement of rice with health promoting properties. Moreover, advanced breeding lines with high anthocyanin contents and antioxidant activity could be identified by methanolic extraction method followed by spectrophotometric measurements and DPPH assay.

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1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereals, serving as a staple food consumed by people in many countries, especially in Asia (Liu et al., 2017; Pang et al., 2018). Pigmented rice has been popular as a healthy food because it contains more nutrients beneficial for human health (Alves et al., 2016; Maulani et al., 2019). Rice can be classified by grain colors which are white, brown, red and black. Black rice has high accumulation of anthocyanins in its pericarp tissues (Reddy et al., 1994; Goufo and Trindade, 2014). Recently, black rice has received more attention from consumers and rice breeders since it contains several nutrients and antioxidant compounds (Lim and Ha, 2013; Rahman et al., 2016).

Anthocyanins which belong to a major class of water-soluble flavonoids, are the primary pigments in colored rice grains (Abdel-Aal et al., 2006). Several health benefits of anthocyains as health-promoting substances due to their antioxidant activity have been recognized (Nam et al., 2006). They include anti-inflammatory activity, anti-cancer activity, prevention of cardiovascular diseases and obesity, control of diabetes, mitigation of oxidative stress, vision improvement and anti-microbial activity (He and Giusti, 2010; Kruger et al., 2014; Sompong et al., 2011).

Extraction of anthocyanins has been conducted using different extracting solvents, including water, methanol, and ethanol, combined with some acids such as citric acid, hydrochloric acid, and acetic acid (Halee et al, 2018; Jansom et al., 2016). In rice, extraction of anthocyanins with acidified methanol/ethanol followed by spectrophotometric measurement has been used extensively (Chin et al., 2016; Jansom et al., 2016; Jiamyangyuen et al., 2017; Na Rachasima et al., 2017). DPPH (2,2-Diphenyl-1-picryhydrazyl) assay is commonly used to evaluate antioxidant activity of foods and plant extracts because it is simple, rapid, inexpensive and reproducible. There are factors that may influence the reaction in DPPH method such as extracting solvents, sample concentration, pH, reaction time, different antioxidant standard and assay conditions (Ferri et al., 2013; Mishra et al, 2012).

Due to the increased demand for black rice as health-promoting foods for human, it is important to develop rice varieties with enhanced anthocyanin contents, high yield and other good agronomic characteristics (Rahman et al., 2016). In rice breeding, selection of black rice with high anthocyanins and antioxidant activity is needed to be used as parental lines. The methods to evaluate anthocyanin contents and antioxidant activity are necessary to analyze the phenotypic traits of parental and progeny lines. These methods should be conducted simply and less costly. Moreover, they should be able to distinguish different rice lines in the steps of phenotypic selection.

The objective of this study was to investigate the effects of extracting solvents on anthocyanin contents and antioxidant activity of Hom Nin black rice. The appropriate solvent was subsequently used to evaluate total anthocyanin contents and antioxidant activity against DPPH from different rice cultivars, including white and black rice. The results will provide the simple and reliable method for analyzing phenotypic traits of rice cultivars with high anthocyanins and antioxidant activity for selection of parent and progeny lines in breeding program to improve rice varieties with more nutritional value.

2. MATERIALS AND METHODS

2.1 Plant materials

Mature seeds of eight rice cultivars were used in this study and collected from different sources. Four non-pigmented rice cultivars, which were simply called white rice, were Taichung 65 (T65), Kitaake (Kit), RD-MAEJO 2 (RDMJU 2) and Pathumthani 1 (PTT1). Four pigmented rice cultivars, which were simply called black rice, were Kham Noi (KNO), Kham Yai (KY), Hom Nin (HN) and Mali Dum (MLD) (Figure 1). T65, RDMJU2, PTT1 and HN were kindly provided by Maejo University, Chiang Mai province, Thailand. KNO, KY and MLD were kindly provides by Center of community rice production, Kudchum, Yasothon province, Thailand. Finally, Kit was kindly provided by Prof.Dr.Thomas W. Okita, Institute of Biological Chemistry, Washington State University, USA.

Figure 1 Phenotypic traits of mature seeds of eight rice cultivars. Non-pigmented rice cultivars, which were simply called white rice, were Taichung 65 (T65), Kitaake (Kit), RD-MAEJO 2 (RDMJU2) and Pathumthani 1 (PTT1). Pigmented rice cultivars, which were simply called black rice, were Kham Noi (KNO), Kham Yai (KY), Hom Nin (HN) and Mali Dum (MLD).

2.2 Extraction of rice seeds with various solvent types

The mature rice seeds were dehulled and grounded into fine powder. Seed powder of 100 mg from HN black rice cultivar were extracted by 1 ml of six different solvent types which were water, 50% methanol, 80% methanol, 1% HCl in water (V/V), 1% HCl in 50% methanol (V/V) and 1% HCl in 80% methanol (V/V). The seed extracts were mixed by vortexing and incubated at room temperature for 30 min. The supernatants were collected by centrifugation at 12,000 rpm for 10 min. Each extraction was performed with three replicates. Each extract from different solvents types was diluted using the same extracting solvent type with the sample extract / solvent volume ratio at 1/4. The diluted extracts with different solvent types were subjected to measurement of total anthocyanin contents and antioxidant activity. The optimal solvents which were found to be 80 % methanol and 1% HCl in 80% methanol were selected and used to extract the seeds of eight rice cultivars.

2.3 Determination of total anthocyanin contents

One hundred milligrams of mature seeds of eight rice cultivars, including four white rice (T65, Kit, RDMJU2, and PTT1) and four black rice (KNO, KY, HN and MLD) were grounded into fine powder followed by the extraction with 1 ml of two solvent types which were 1% HCl in 80% methanol and 80 % methanol. The seed extracts were mixed by vortexing and incubated at room temperature for 30 min. The supernatants were collected by centrifugation at 12,000 rpm for 10 min. Each extraction was performed with three replicates. Each extract from different solvents types was diluted using the same extracting solvent type with the sample extract / solvent volume ratio at 1/6. The rice seed extracts were used for determination of total anthocyanin contents by the method modified from Chin et al. (2016). The absorbance was measured at 530 and 675 nm using microplate reader (SPECTROstar® Nano, Germany). The anthocyanin contents were determined as follow: monomeric anthocyanin = (A x MW x DF x 1000) / ε x 1 (Na Rachasima et al., 2017). Three replicates were analyzed for each sample.

2.4 Determination of antioxidant activity by DPPH assay

The anthocyanin extracts of eight rice cultivars with 1% HCl in 80% methanol and 80 % methanol were analyzed for antioxidant activity by DPPH assay. Each extract from different solvents types was diluted using the same extracting solvent type with the sample extract / solvent volume ratio at 1/4. The Trolox equivalent antioxidant capacity (TEAC) assay using Trolox as a standard was used to measure total antioxidant activities against 2,2-diphenyl-1-picrylhydrazyl (DPPH) among seed anthocyanin extracts of eight rice cultivars, according to the described method (Shao et al., 2014; Zhu et al., 2017). The 100 µmol/l DPPH solution was prepared in methanol. The diluted seed extract solution of 20 µl was mixed with 180 µl DPPH solution for the reaction. After incubating the reactions at room temperature for 30 min in the dark, the absorbance at 516 nm was measured by microplate reader (SPECTROstar® Nano, Germany). The DPPH scavenging activity was calculated as follows: %DPPH inhibition = $[(A_{control} - A_{sample}) / A_{control}] \times 100$. A_{sample} was absorbance value of the extract in DPPH solution and $A_{control}$ was absorbance value of DPPH solution with methanol instead of the extract. The antioxidant activity value was calculated by using different concentration of Trolox standard (10, 15, 20, 25, 50, 75, 100 and 125 mg/l) as a standard curve. The results were expressed as TEAC in µmol Trolox equivalents per gram of powdered rice seeds. Three replicates were analyzed for each sample.

2.5 Statistical analysis

The results were presented as means ± standard deviation (SD) of triplicate determinations. Statistical analysis was performed using R3.6.0 program (http://www.r-project.org). The data were analyzed of variance and the significant differences among means were determined using the Duncan test at a level $P<0.01$.

3. RESULTS AND DISCUSSION

3.1 The optimal solvents for seed extract of black rice cv. Hom Nin

The black rice cultivar cv. Hom Nin was used to study the appropriate solvent types for rice seed extracts to be used for evaluation of anthocyanin contents and antioxidant activity. The six different solvent types which were water, 50% methanol, 80% methanol, 1% HCl in water (V/V), 1% HCl in 50% methanol (V/V) and 1% HCl in 80% methanol (V/V), were used for the extraction of Hom Nin seeds. The result showed that the extracts exhibited statistically significant differences (P<0.01) in total anthocyanin contents and antioxidant activity by DPPH assay (Table 1). The extracts with solvents containing 1 % HCl gave higher total anthocyanin content than the solvents without 1% HCl. The extract with 1 % HCl in 80% methanol gave highest anthocyanin contents of 1.41

μmol/gDW, followed by the extracts with 1 % HCl in 50% methanol and 1% HCl in water which exhibited the total anthocyanins of 1.36 and 0.49 μmol/gDW, respectively.

Table 1 Total anthocyanin contents and antioxidant activities by DPPH assays of Hom Nin rice powder crude extracts obtained from different extraction solvents.

Solvent types	Total anthocyanin contents (μmol/gDW)	Antioxidant activity / TEAC (μmol/gDW)
water	0.17 ± 0.01^{c}	7.72 ± 0.01^{d}
50% methanol	0.20 ± 0.02^{c}	11.32 ± 0.33^{b}
80% methanol	0.12 ± 0.01^{c}	9.23 ± 0.03^{c}
1% HCl in water	0.49 ± 0.01^{b}	13.70 ± 0.03^{a}
1% HCl in 50% methanol	1.36 ± 0.04^{a}	13.64 ± 0.13^{a}
1% HCl in 80% methanol	1.41 ± 0.10^{a}	13.94 ± 0.06^{a}

Mean ± standard deviation of triplicate analyses.
Values in each row (small letter) bearing different superscripted letters are statistically different ($P< 0.01$).

The antioxidant activities of the extracts by 1% HCl in 80% methanol, 1% HCl in 50% methanol and 1% HCl in water were higher than those of water and methanol. The extract with 1% HCl in 80% methanol gave the highest value of antioxidant activity of 13.94 μmol/gDW (Table 1). The values of antioxidant activity using 1% HCl with water and 1% HCl in 50% methanol were 13.70 and 13.64 μmol/gDW, respectively, and showed no significant differences (P≥0.01). The previous report showed that the solvents of ethanol or methanol acidified with 1% HCl were optimal for the extraction of rice seeds for anthocyanin content determination (Chin et al., 2016; Krongsuksirichai et al., 2016). In this study, we found that 1% HCl in 80% methanol gave highest values of anthocyanin content and antioxidant activity; therefore, we selected this solvent type for the seed extraction of eight rice cultivars for further studies.

3.2 Total anthocyanin contents and antioxidant activities of eight rice cultivars

The selected solvent of 1%HCl in 80% methanol was used to extract the seed powder of eight rice cultivars. White rice cultivars which were T65, Kit, RDMJU2 and PTT1 as well as black rice cultivars which were KNO, KY, HN and MLD were extracted with 1%HCl in 80% methanol. The extracts were subsequently analyzed for total anthocyanin contents and antioxidant activities. The results showed that the amounts of anthocyanins in black rice were higher than white rice (Figure 2). The level of total anthocyanins of 1.374 μmol/gDW in black rice cv. MLD was highest and consistent with dark black color in it pericarp (Figure 1). Following MLD, there were HN, KY, and KNO which had total anthocyanin contents of 0.945, 0.776, and 0.502 μmol/gDW, respectively, corresponding to their pericarp color intensity. On the other hand, all white rice cultivars which were T65, Kit, RDMJU2 and PTT1, showed little detectable anthocyanin contents of 0.002 μmol/gDW. The results were consistent with the previous study which demonstrated that grain anthocyanin content of black rice was much higher than those of brown and white rice (Rahman et al., 2016).

The antioxidant activity by DPPH-radical scavenging activity assay of the extracts by 1%HCl in 80% methanol was evaluated. The results showed that the antioxidant activity of black rice were higher than white rice. The black rice cv. MLD had highest TEAC against DPPH of 17.37 μmol/gDW. This result was consistent with highest amount of anthocyanin contents (1.374 μmol/gDW) and darkest black color in it pericarp (Figure 2 and 1). Other black rice cv. HN, KY and KNO had the antioxidant activity of 17.09, 16.42 and 16.71 μmol/gDW, respectively. The white rice cv. T65, Kit, RDMJU2 and PTT1 had not significantly different values of TEAC which were 3.71, 3.69, 3.70 and 3.70 μmol/gDW, respectively (P≥0.01). Although black rice cultivars cv. HN, KY and KNO with significantly different anthocyanin contents (P<0.01) corresponding to their pericarp colors, the values of TEAC could not be clearly distinguished among these black rice (Figure 2). Therefore, it might be difficult to evaluate relative antioxidant activities among different black rice cultivars for selection of parental lines and progeny lines derived from the crosses between white and black rice in our breeding programs.

The previous study showed that the acidity of sample extracts had the effect on DPPH assay, leading to different estimation of their antioxidant activity (Pekal and Pyrzynska, 2015). To determine whether 1% HCl affected the DPPH assay of rice seed extracts, we extracted the rice seeds from eight cultivars with 80% methanol and used for analysis of anthocyanins and antioxidant activity. The results showed that the extract by 80% methanol gave the significantly difference ($P<0.01$) of total anthocyanin contents among black rice cultivars (Figure 3). The black rice cv. MLD had the highest TEAC of 8.89 μmol/gDW which was consistent with highest total anthocyanins of 0.121 μmol/gDW and darkest color of its pericarp (Figure 3 and 1). The black rice cv. KNO showed the lowest TEAC of 4.48 μmol/gDW which was consistent with lowest total anthocyanins of 0.051 μmol/gDW and less dark color of its pericarp (Figure 1).

The present study indicated that the seed extracts with methanol would be appropriate for antioxidant activity by DPPH method. Several studies reported the factors affecting DPPH assay including reaction time, solvent types, and acidity (Mishra et al., 2012; Pekal and Pyrzynska, 2015). For rice seeds, the extracts with methanol were performed for evaluation of antioxidant activity by TEAC assay (Walter et al., 2013; Huang and Lai, 2016; Jiamyangyuen et al., 2017).

However, the extracts with 80% methanol gave about 10-fold lower amount of total anthocyanin content than those extracted by 1% HCl in 80% methanol. Thus, for the evaluation of total anthocyanins of rice seeds, the extraction with 1% HCl in 80 % methanol might be more appropriate. Several studies on the extraction of black rice seeds for analysis of anthocyanin contents using extraction buffer with acidified methanol have been reported (Chundet et al., 2012; Chin et al., 2016; Jiamyangyuen et al., 2017; Halee et al., 2018).

In this study, the seed extracts with 1% HCl in 80% methanol might be appropriate for evaluation of total anthocyanin contents by spectrophotometry. However, 80 % methanol with no acidity could be suitable for assessment to antioxidant activity by DPPH assay.

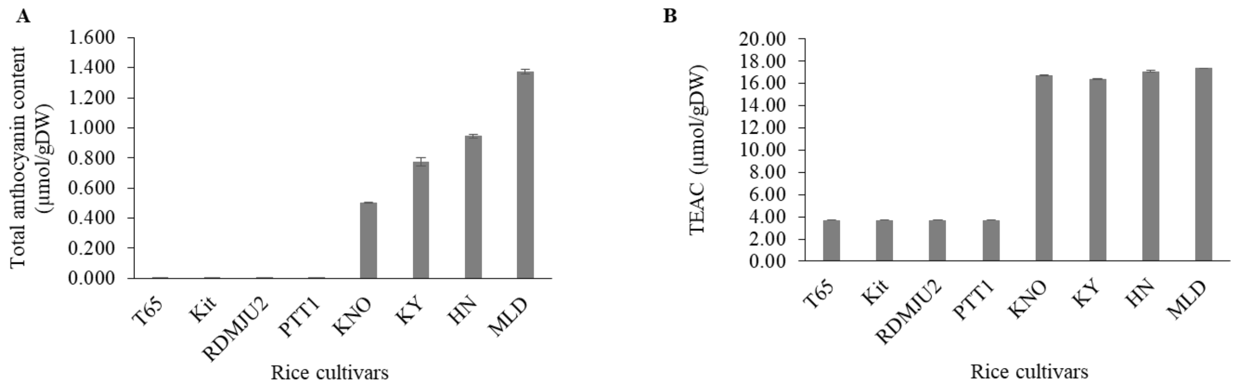

Figure 2 Assessments of total anthocyanin contents (a) and antioxidant activity against DPPH (b) of the rice seeds extracts with 1%HCl in 80% methanol. White rice cultivars were T65, Kit, RDMJU2 and PPT1. Black rice cultivars were KNO, KY, HN and MLD. All the values were represented as mean ± SD.

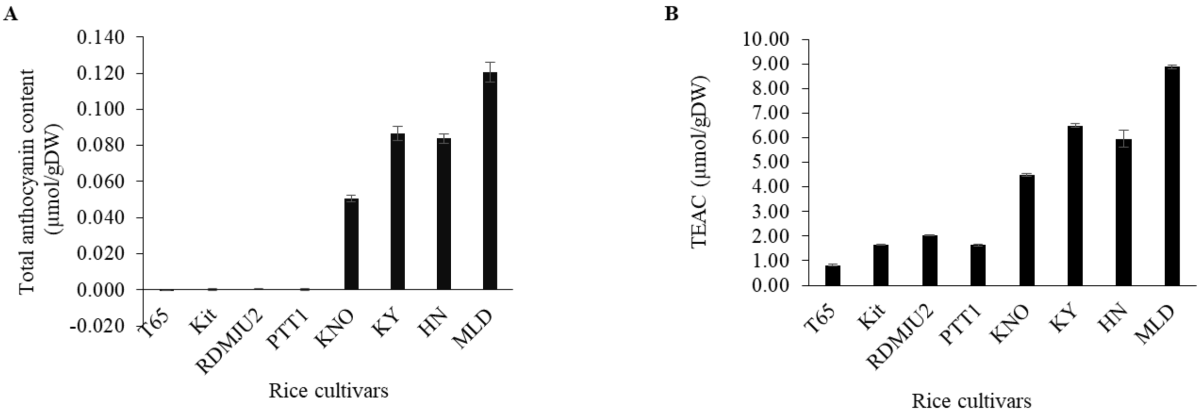

Figure 3 Assessments of total anthocyanin contents (a) and antioxidant activity against DPPH (b) of the rice seeds extracts with 80% methanol. White rice cultivars were T65, Kit, RDMJU2 and PPT1. Black rice cultivars were KNO, KY, HN and MLD. All the values were represented as mean ± SD.

3.3 The optimal incubation time for antioxidant activity in DPPH assays

To determine the appropriate incubation time for evaluation of antioxidant activity of the extracts by DPPH assay, the extracts with 1% HCl in 80% methanol and 80% methanol were measured for antioxidant activity after incubation time of 10, 20, 30, 40, 50 and 60 min. The extracts with 1% HCl in 80% methanol showed that the antioxidant activity of all white rice had no change throughout the time of 10-60 min. Moreover, all black rice showed similar values of antioxidant activity throughout 60 min (Figure 4A). The result demonstrated that the solvent of 1% HCl in 80% methanol might not be suitable for seed extraction for DPPH method probably due to interference of the acidified condition in the assay. The previous study showed that DPPH method for measurement of antioxidant activity of foods and plant extracts required a pH range between 4-8 (Ferri et al., 2013).

On the other hand, the extracts with 80% methanol showed increase in antioxidant activity of all eight rice cultivars when the time increased from 10-30 min (Figure 4B). During incubation period of 10-30 min, the antioxidant activity of all rice cultivars increased at the same pattern and the different values of antioxidant activity among different cultivars could be observed. Thus, the incubation time of reaction at 30 min would be optimal for all rice cultivars to assess antioxidant activity by DPPH method. In addition, at 30 min, the different amounts of antioxidant activity among black rice cultivars could be clearly distinguished (Figure 4B). The result was consistent with several studies on assessment of antioxidant activity by DPPH assay of sample extracts by methanol and incubation time of reaction for 30 min (Ferri et al, 2013; Pekal and Pyrzynska, 2015; Patil et al., 2016; Jiamyangyuen et al., 2017; Halee et al., 2018).

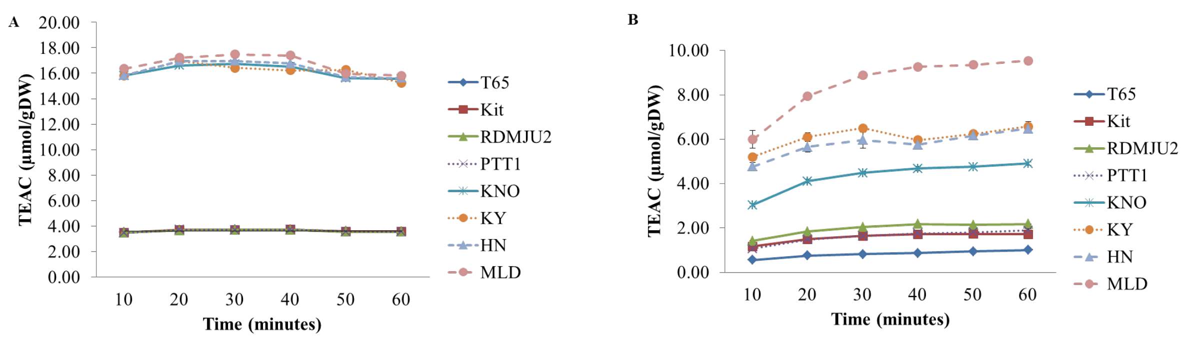

Figure 4 Assessment of incubation time in antioxidant activity by DPPH assay of extracts with 1% HCl in 80% methanol (a) and 80% methanol (b). White rice cultivars were T65, Kit, RDMJU2 and PPT1. Black rice cultivars were KNO, KY, HN and MLD. All the values were represented as mean ± SD.

The results revealed that white rice also had antioxidant activity but much lower than black rice. RDMJU2 which is glutinous rice cultivar made from rice breeders at Maejo University, Chiang Mai, Thailand showed highest antioxidant activity among white rice. In addition, RDMJU2 has high yield and good agronomic characteristics, including semi-dwarf and non-photoperiod sensitivity. The black rice, MLD which is landrace rice in the northeastern region of Thailand gave highest anthocyanin contents and antioxidant activity, consistent with the previous report (Kongkachuichai and Charoensiri, 2010). Hence, both RDMJU2 and MLD would be good sources for use as parental lines in improvement of rice with high quality traits and increased nutritional values.

4. CONCLUSION

Extracting solvents and assay conditions could affect the measurement of total anthocyanin contents and antioxidant activity. The results demonstrated that the appropriate solvent for rice seed extraction to analyze total anthocyanin contents was 1 % HCl in 80 % methanol followed by the spectrophotometric measurement. For antioxidant activity, the extraction with 80 % methanol could be suitable for DPPH assay, because the acidity of 1 % HCl might interfere with the reactions. These extraction solvents and the methods for determination of anthocyanins and antioxidant activity will be applied for screening and selection of rice cultivars with high anthocyanins and antioxidant activity to be used as parental lines and for selection of progeny lines in rice breeding program. In rice breeding, it is needed to determine the correlation between genotype and phenotype of rice populations such as F_2 progeny. The simple method for evaluating phenotypes of relative total anthocyanins and antioxidant activity of many lines will be very useful, less time-consuming and less costs. This study also suggested that RDMJU2 (white rice) and MLD (black rice) would be good candidates for use in rice breeding to provide health-promoting foods.

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