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Comparison of hot water and methanol extraction combined with ultrasonic pretreatment on antioxidant properties of two pigmented rice cultivars

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Abstract

Pigmented rice is considered as the good source of phytochemicals which contains more phenolic contents and higher antioxidant activities compared with non-pigmented rice. However, those phytochemicals are normally extracted using inorganic solvent, using water was rarely found. This study was aimed to compare extraction methods on antioxidant contents and activities of two types of Thai pigmented rice (black glutinous rice and red non-glutinous rice). Pigmented rice was extracted either conventional method alone or a combination of ultrasonic plus conventional method with different solvents (methanol and hot water). Phenolic contents were analyzed by spectrophotometric assay and high-performance liquid chromatography (HPLC). Antioxidant activities were investigated by radical scavenging capacity (ABTS and DPPH). The results showed that ultrasonic pretreatment significantly enhanced the total phenolic, flavonoid, anthocyanin contents and higher antioxidant activities compared with conventional extraction irrespective of solvents and rice varieties. Ultrasonic methanol (UM) extraction proved to extract significant higher content of all the compounds analyzed in both the rice varieties. In contrast, hot water (HW) extracts exhibited the lowest amount of bioactive compounds. However, there was no significant difference between methanol extraction alone and ultrasonic pretreatment with hot water extract on antioxidant contents for black glutinous rice. The contents of individual anthocyanins and flavonoids (cyanidin 3-O-glucoside, peonidin 3-O-glucoside, and quercetin) were significantly increased with ultrasonic pretreatment compare to the conventional method in black glutinous rice while failed to detect the red rice variety. The black glutinous rice contained significantly higher contents of all analyzed compounds and antioxidant activities than red rice. In conclusions, our results demonstrated that ultrasonic pretreatment significantly enhanced antioxidant extraction with higher activities compared with conventional extraction irrespective of solvents and rice varieties. Furthermore, the efficiency of ultrasonic together with hot water extraction was almost equal to methanol extraction which was the one effective solvent for extracting antioxidant.

1. Introduction

Rice is considered as the good source of phytochemicals including phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid (Goufo and Trindade, 2014). Previous research has shown that pigmented rice contains higher phenolic content and antioxidant activities, along with greater varieties of health-promoting phytochemicals compared with non-pigmented rice (Chakuton *et al.,* 2012). Especially the black rice is well known to be rich in anthocyanin (Hou *et al.,* 2013), it contains Cyaniding 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, pelagonidin 3-O-glucoside and delphinidin 3-O-glucoside (Park *et al*., 2008). While the major flavonoid pigments in red rice were identified as procyanidins. Anthocyanin possesses strong radical scavenging activities, anti-inflammatory effects and anti-carcinogenic properties (Wang *et al.,* 2007; Radovanović and Radovanović, 2010). In addition, it has been reported that the composition of phenolic compounds is noticeably different between glutinous and non-glutinous rice grains. Notably, in non-pigmented rice, total

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phenolic in non-glutinous rice was higher than the glutinous variety.

There are several factors that affect the yield of antioxidant compounds during the extraction process. For example, solvent and extraction method used during the process. The extraction of phenolics from plant materials was often with different solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations (Dai and Mumper, 2010). Arab *et al.* (2011) reported that methanol showed significant greater yield and antioxidant activities compare with ethanol and ethyl acetate. Even though methanol is the most common and effective solvent for extracting polyphenols, it is more toxic and considered as environmental pollutant than other types of alcohol (Kapasakalidis *et al*., 2006). Furthermore, the extraction of phenolic compounds from plant materials is also influenced by the extraction protocols, which obviously have effects over the yield and antioxidant activities. In this context, due to the ineffective conventional extraction processes, it gave rise to the development of new efficient extraction techniques like ultrasonic-assisted extraction. Ultrasonic assisted extraction (UAE) involves the application of high-intensity, high-frequency sound waves and their interaction with materials. UAE is a useful technology that does not require any complex instruments and is relatively low-cost. It can be used both on a small and large scale. In most studies, phenolic compounds from pigmented rice were extracted by various solvents such as methanol, ethanol, acetone and ethyl acetate, using water as a solvent was rare. Therefore, the present study was aimed to compare extraction methods on phytochemical contents and their antioxidant capacity from Thai pigmented rice by either conventional method alone or combination of the ultrasonic plus conventional method with different solvents (methanol and hot water).

2. Materials and methods

2.1 Plant material

Black glutinous rice (Neaw dum moa37) and Red non-glutinous rice (Hom gradung-nga57) were obtained from Pattani rice research center. All rice grains were dehusked with the NW 2000 Turbo machine and removed the deformed grains manually after de-husking. All samples were stored immediately at -20°C until analysis.

2.2 Extraction methods

There are four extraction methods (1) Hot Water Extraction (HW) rice grains (20 g) were extracted with water (100 mL) for 10 mins at 100°C. The extract was filtered with nylon cloth and centrifuged at 8,000 rpm for 15 mins to obtain clear supernatant. (2) Methanol extraction (M) methanol extraction was carried out according to Choi *et al.* (2007) with slight modification. The 20 g rice was extracted with 100 mL of 80% methanol in water at room temperature for 24 hr followed by removing the grain remaining from the extract using nylon cloth and centrifuging at 8,000 rpm for 15 mins. (3) Ultrasonic pretreatment followed by hot water extraction (UHW) and (4) methanol extraction (UM) sample was carried out pretreatment with ultrasonic probe for 40 mins, 20 kHz and 40% amplitude with temperature not exceeding 35°C. Then, the extract was extracted as described in hot water and methanol extraction respectively.

2.3 Analysis of antioxidant contents

2.3.1 Determination of total phenolic contents (TPC)

Folin-Ciocalteau reagent was diluted with water 1:9 (v/v). Then 60 µL of the sample (1 mg/mL) was added to 2.5 mL of this freshly prepared reagent. The solution was incubated for 2 mins at room temperature and 2 mL of sodium carbonate (75 g/L) was subsequently added to the solution. Then, the mixture was incubated for 15 mins at 50°C and cooled with ice-water bath. The absorbance was measured at 760 nm within 15 mins. The results were expressed as mg gallic acid equivalent (GAE)/100 g grain.

2.3.2 Determination of total flavonoid contents (TFC)

The total flavonoid content was determined according to Zhishen *et al.* (1999) with slight modification. A total of 250 µL of extract (1 mg/mL) was transferred into a test tube and the mixture of 1.25 mL of distilled water and 75 µL of 5% $NaNO_2$ was added and incubated for 5 mins. After the incubation period, 150 µL of 10% of $AlCl_3$ and 1 mL of NaOH was then added respectively. When the reaction was completed, the absorbance was measured at 510 nm by using a spectrophotometer. The flavonoid content was determined by using quercetin standard and expressed as mg quercetin/100 g rice grain.

2.3.3 Determination of total anthocyanin contents (TAC)

The 400 µL of the sample (1mg/ml) was diluted with the buffer (0.025 mM KCl) at pH 1.0 and incubated for 15 mins at room temperature before the first measurement. For the second measurement, the sample was diluted with the buffer pH 4.5 (0.4 mM CH_3COONa) and incubated in the dark for 5 mins for the repeated measurement. The absorbance readings were measured against water as a blank. The concentration of Total Anthocyanin (TA) content was calculated in terms of cyanidin-3-glucoside using the following equation:

Anthocyanin contents (mg/liter) = (A× MW × DF × 1000)/ (e × 1).

Where A= (λ500 nm – λ700 nm) pH 1.0 - (λ 500 nm – λ 700 nm) pH 4.5; MW= (molecular weight) = 449.2 g/ mole for cyanidin-3-glucoside; DF = dilution factor; and e = 26900 molar extinction coefficient.

2.4 Analysis of antioxidant activities

2.4.1 Determination of DPPH

The assay was carried out according to the method of Butsat and Siriamornpun (2010). The DPPH radical was prepared by dissolving 0.0039 g of DPPH powder in the 50 mL of ethanol. A total of 3 mL of DPPH solution was added to 3 mL of rice extract (1 mg/mL) and kept in the dark at room temperature for 30 mins. The absorbance was measured at 517 nm relative to the control. The DPPH˙ solution without the added sample was measured for its absorbance as the control. The DPPH scavenging activity (%) of samples was expressed as DPPH% = (1 – A sample/A control) x 100%).

2.4.2 Determination of ABTS

The assay was carried out according to Choi *et al*. (2007). The ABTS radical cation reagent (stock solution) was prepared by adding 7 mM ABTS in ethanol with potassium persulphate (K_2S2O_8) solution to obtain the concentration of 2.45 mM and kept it in the dark for 12-14 hr at room temperature. The ABTS radical cation reagent was diluted to obtain the absorbance of 1.2-1.4 at 414 nm by adding distilled water. An aliquot (1 mL) of ABTS diluted reagent was added to 200 µL of extracts (1 mg/mL) and measured the absorbance at 414 nm after incubating in the dark against water blank after 1 hr.

2.5 HPLC analysis

Analysis of anthocyanin and flavonoid content was carried by using high-performance liquid chromatography (HPLC). Four samples were obtained from various extraction methods subsequently concentrated individually by using rotatory evaporator to reach the concentration of 1000 mg/mL.

2.5.1 Identification and quantification of anthocyanins

The anthocyanin profile was determined by using HPLC (Agilent 1100 series, DAD-FLD) according to Kim *et al.* (2010). Anthocyanin was separated on a C18 column (250 x 4.6mm, 5 µm, Luna, phenomenex) by HPLC. Elution was performed using a binary gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) according to, 0 min, 95%A/5%B; 40min, 50%A/50% B; 42 min, 0% A/100% B; 52 min, 0% A/100% B; 54 min, 95% A/5% B; and 64 min, 95%A/5%B. The flow rate was 1.0 mL/ min, and the column temperature was 30°C. The UV-vis detector wavelength was set at 520 nm. Anthocyanin (cyanidin-3-glucoside (Cy-3G) and peonidin-3-glucoside (Pe-3-G)) were used as standards to identify the compounds in the sample.

2.5.2 Identification and quantification of flavonoids

Determination of the flavonoid was performed according to the method of Kim *et al.* (2010). Flavonoid aglycones contained in rice extract were separated on a C18 column (250 × 4.6 mm, 5 µm,) by HPLC equipped with a photodiode array (PDA) detector. The elution was performed using a binary gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) according to the following gradient: 0 min, 95% A/5% B; 30 min, 60% A/40% B; 45 min, 50% A/ 50% B; 50 min, 0% A/100% B; 60 min, 0% A/100% B; 62 min, 95% A/ 5% B; and 70 min, 95% A/5% B. The flow rate was 1.0 mL/min and the column temperature was 40°C. The ultraviolet-visible (UV-vis) detector wavelength was set at 364 nm. Flavonoid (Quercetin and Apigenin) were used as standards to identify the compounds in the sample.

2.6 Statistical analysis

All determinations were carried out in triplicate, and data were subjected to analysis of variance (ANOVA), using the statistical package SPSS, Version 17. Significant differences between means were determined by Duncan's multiple range tests. P values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1 antioxidant contents

3.1.1 Total phenolic contents (TPC)

TPC from four extraction methods, the results showed that the total phenolic contents for black glutinous rice ranged from 419.56 to 568.25 mg GAE/100 g grains which were higher than red rice 248.75 – 433 mg GAE/100 g grains (Table 1). The methods with ultrasonic pretreatment significantly ($P<0.05$) increased total phenolic contents compared to HW and M extraction alone. The ultrasonic pretreatment on HW and M extraction increased the phenolic content by 7.4% and 18.9% in black glutinous rice and 25% and 18.9% in red rice. This is due to the fact that during ultrasonic treatment, ultrasonic waves increase the interaction between the molecules in the sample breaking down the cell wall and releasing the bioactive compounds (Hossain *et al.,* 2012). Similarly, Chooklin (2014) evaluated the optimum ultrasound-assisted extraction condition of brown rice extract and found that

Table 1. Effect of extraction methods on total antioxidant contents of black glutinous rice and red rice

Rice varieties/ extraction methods	TPC (mg GAE/100 g sample)	TFC (mg quercetin/100 g sample)	TAC (mg Cy-3-G/100 g sample)
Black glutinous rice			
HW	419.56±16.93^{cA}	1631.58 ± 45.43^{cA}	214.26 ± 11.32^{dA}
M	460.39 ±26.64^{bA}	1966.66 ± 43.97^{bA}	293.76 ± 11.63^{bA}
UHW	452.81± 12.16^{bA}	2003.87 ± 74.36^{bA}	237.77 ± 10.30^{cA}
UM	568.25 ± 16.06^{aA}	2534.08 ± 23.67^{aA}	358.68 ± 5.35^{aA}
Red rice			
HW	248.75 ±9.28^{dB}	855.07 ± 51.87^{dB}	1.17 ± 0.25^{dB}
M	321.60 ± 7.11^{cB}	1137.42 ± 37.47^{cB}	1.90 ± 0.31^{cB}
UHW	332.39± 6.38^{bB}	1282.87 ± 30.29^{bB}	2.58 ± 0.41^{bB}
UM	433.61 ± 12.89^{aB}	1798.19 ± 84.40^{aB}	4.82 ± 0.39^{aB}

Values are means ± SD. Values with different superscript $^{a-d}$ (lower case) letters within the same column are significantly different ($P<0.05$). Values with $^{A-B}$ different superscript (upper case letters) between rice are significantly different ($P<0.05$) respectively. HW: hot water, M: methanol, UM: ultrasonic+methanol, UHW: ultrasonic+hot water

the total phenolic content obtained by ultrasound-assisted extraction was higher than conventional extraction method by 15.31%. In addition, ultrasonic treatment prior to methanol extraction (UM) extract the highest TPC of the black glutinous rice (568.25±16.06 mg GAE/100 g grain) which was significantly higher ($P<0.05$) than other three extraction methods. The similar trend in TPC was observed in red rice variety where the samples were pre-treated with ultrasonic prior methanol extraction (433.61±12.89 mg GAE/100 g sample). The quantity TPC of the black glutinous rice and red rice by different extraction methods was UM>M=UHW>HW and UM>UHW>M>HW respectively. However, there was no significant difference between M extraction and UHW for black glutinous rice. It is possible that the efficiency of extraction solvent was also involved. Paniwnyk *et al.* (2001) reported that, especially in methanol, hydrogen peroxide and large proportions of free radicals are not formed while exposed to sonication, unlike in water that induces phenolic degradations. The ultrasound-assisted the extraction by improving the efficacy of this methodology. When compared with multiple solvents for the extraction of five isoflavone from *Iris tectorum*, methanol gave the highest extraction yield, followed by water and ethanol due to differences in the polarities and viscosities (Sun *et al.,* 2011). In another study, methanolic extract of colored and non-colored Thai rice cultivars yielded higher phenolic content than distilled water, hexane and ethyl acetate extract (Chakuton *et al.,* 2012). Usually the plant materials contain different quantities of phenolic acids, phenylpropanoids, anthocyanins and tannins and many others compounds. The chances of phenolic interaction with other plant components such as carbohydrates and proteins may lead to the formation of insoluble complexes. Therefore, the solubility of phenolic is affected by the polarity of solvent used in the extraction (Naczk and Shahidi, 2006). Though the polarity of the water is higher than the organic solvents, the polyphenols are mostly soluble in less polar organic solvents suggesting that the polar properties of the polyphenol determine its solubility (Kim and Lee, 2005). Additionally, during the ultrasonic extraction, the solvent properties affect the formation of the cavitation bubbles and cavitation is one of the main mechanisms by which ultrasound can improve extraction efficiency. Its efficiency depends on the collapse of cavitation bubbles which produces microjets that disrupt plant cell membrane and provoking the release of bioactive compounds. The formation of the cavitation bubbles is affected by the surface tension of the solvents used in the extraction. The cavitation bubbles are formed easily in liquids with lower surface tension compared to higher surface tension because the ultrasonic energy can easily overcome the surface tension forming cavitation bubbles more easily (Ghasemzadeh *et al.,* 2015). Aqueous methanol has comparatively lower surface tension compared to the water, which enabled to extract significantly higher content of flavonoids in our study.

3.1.2 Total flavonoid contents (TFC)

The present study, TFC from four extraction methods was in the range of 1631.58 – 2534.08 mg quercetin equivalent /100 g for the black glutinous rice and 855.07- 1798.19 mg quercetin equivalent /100g for the red rice (Table 1). Among four extraction methods, TFC extracted by each method had similar trend to total phenolic content. The flavonoid content of black glutinous rice and red rice extracted by ultrasonic pretreatment were significantly ($P<0.05$) increased by 18.62%, 22.3%, and 33.3%, 36.7% compared to HW and M extraction alone. The total flavonoid content of the black glutinous rice and red rice by difference extraction methods was UM>M=UHW>HW and UM>UHW>M>HW respectively. This result indicates that flavonoid extraction was enhanced by the ultrasonic treatment. In addition, ultrasonic pretreatment prior methanol extraction showed the highest content of total flavonoid with the value of 2534.08 mg quercetin

equivalent/100 g sample compared to the other three methods. Sun *et al.* (2011) reported out of three different extraction methods, ultrasonic extraction gave the highest extraction yield of flavonoids such as- tectoridin, iristectorin B, iristectorin A, tectorigenin, iris-tectorigenin A, and total isoflavones in lesser time compared to maceration and Soxhlet extraction. Therefore, ultrasonic extraction can be a potential extraction procedure to extract plant flavonoids. Paniwnyk *et al.* (2001) also reported, the extraction of rutin from the *Sophora Japonica* was improved by ultrasonic treatment (20 KHz, 27 W) carried out at room temperature compared to conventional methods (boiling and reflux) using same solvents (diluted aqueous alkali and methanol). However, the efficiency was dependent on the solvent type, where the application of the ultrasonic on methanol extraction significantly increased the yield unlike ultrasonic treatment on aqueous solvent.

3.1.3 Total anthocyanin contents (TAC)

Anthocyanin content for black glutinous rice was in the range of 214.26-358.68 mg Cyaniding 3-O-glucoside equivalents/100 g sample and 1.17- 4.82 cyanidin 3-O-glucoside equivalents/100 g sample for red rice. The combination of ultrasonic and solvent extraction resulted in increased TAC compared to solvent extraction alone in both rice varieties. This trend was similar to TPC and TFC obtained from ultrasonic pretreatment. Black glutinous rice extracted by ultrasonic treatment with methanol had highest anthocyanin content of 358.68±5.35 mg of Cy-3-G/100 g sample and this was significantly higher ($P<0.05$) than other treatments within black glutinous rice. Apparently among the red rice varieties, ultrasonic treatment with methanol indicated significantly higher anthocyanin content 4.82±0.39 cyanidin 3-O-glucoside equivalents/100 g sample than other treatments within red rice. The anthocyanin content was increased by 9.9%, 18.1% and 54.7%, 60.6% in black glutinous rice and red rice respectively compared to hot water and methanol extraction alone. As mentioned previously in TPC, this is due to the fact that ultrasonic can rupture the sample matrix releasing intracellular content and facilitating extraction procedure (Santos *et al.,* 2013). In both rice, when compared to UHW and UM, the latter is able to extract higher total anthocyanin contents. Our study result's suggests that ultrasonic pretreatment is not only superior in yielding significantly higher TPC and TFC, but also specifically increases the anthocyanin content compared to the conventional extractions. It can be due to the effect of solvents as mentioned in TPC and TFC scenario. Similarly anthocyanin accessibility can be also strongly dependent on the solvents capacity to enter rice grain structures and their ability to extract. The ultrasonic extraction is directly affected by the ultrasound frequency, intensity and sample characteristics. Sonication frequencies consists of mainly two bands, low power ultrasound (low amplitude and high frequency, 100-1000 kHz) or high power ultrasound (high amplitude and low frequency, 20-100 kHz) (Golmohamadi *et al.,* 2013). It's been well known that at lower frequency, larger cavitation bubbles are formed. Therefore the lower frequencies of high-power ultrasound (around 20 kHz) can achieve more violent bubble implosions elevating the extraction efficiency (Esclapez *et al.,* 2011). On the other hand, low intensity ultrasound which uses small power levels with lower frequencies (5 – 10 MHz) doesn't cause any physical or chemical alterations. It doesn't necessarily mean that high power ultrasonic is suitable in extraction purposes. Suitable power with lower frequencies is better than higher frequencies in extraction yield. Ravanfar *et al.* (2015) reported when high power ultrasonic is applied, the sound energy is converted into heat causing the anthocyanin degradation. Therefore exposing the materials containing anthocyanin which is very sensitive to temperature ultimately lead to low anthocyanin yield (Chigurupati *et al.,* 2002). The anthocyanin content was relatively lower in red rice varieties as to black glutinous rice. The result showed that the lowest anthocyanin content was exhibited by red rice variety extracted with hot water (1.17±0.25 cyanidin 3-O-glucoside equivalents/100 g samples) amongst all the treatments from two rice varieties. This result has agreed with Abdel-Aal *et al.* (2006) who also found that anthocyanin content in red rice (0.094 mg/g) was very much lower than black rice (3.27 mg/g) when rice was extracted twice by mixing with 24 mL of methanol acidified with 1.0 N HCl (85:15, v/v) and shaking at 1800 rpm for 30 mins. Several studies revealed that ultrasonic treatments aided the extraction of phytochemicals, especially anthocyanin like in our case. Santos *et al.* (2013) reported that ultrasound-assisted extract was more efficient in extracting anthocyanins compared to conventional methods (agitated bed and Soxhlet extraction techniques) from jambul (*Syzygium cumini*) peels with acidified ethanol as the extraction solvent. In another study, fresh red cabbage (*Brassica oleracea* L. Var. Capitata F. Rubra) extracted with ultrasonic treatment, anthocyanin content was 2 times greater compared to control extraction procedure performed in a water bath. Similarly, Sivakumar *et al.* (2009) demonstrated a significant increase in betalains (red coloured cyanins) with ultrasonic extraction from beet root in comparison with the magnet stirrer extraction method. Moreover, Cheok *et al.* (2013) reported ultrasonic treatment were able to extract total monomeric anthocyanin and total polyphenol content 45.6% and

Table 2. Effect of extraction methods on antioxidant activities of black glutinous rice and red rice

Rice varieties/ extraction methods	ABTS (% of scavenging)	DPPH (% of inhibition)
Black glutinous rice		
HW	51.31 ± 0.67^{cA}	63.59 ± 2.31^{bA}
M	55.52 ± 0.63^{bA}	68.57 ± 2.11^{aA}
UHW	54.64 ± 1.80^{bA}	68.22 ± 1.02^{aA}
UM	65.60 ± 2.49^{aA}	69.41 ± 1.06^{aA}
Red rice		
HW	46.41 ± 2.35^{cB}	47.18 ± 2.26^{cB}
M	50.32 ± 1.34^{bB}	54.30 ± 1.58^{bB}
UHW	51.51 ± 1.78^{bB}	55.61 ± 1.44^{bB}
UM	61.19 ± 1.44^{aB}	65.60 ± 3.63^{aB}

Values are means ± SD.Values with different superscript $^{a-c}$ (lower case) letters within the same column are significantly different ($P<0.05$). Values with $^{A-B}$ different superscript (upper case letters) between rice are significantly different ($P<0.05$) respectively. HW: hot water, M: methanol, UM: ultrasonic+methanol, UHW: ultrasonic+hot water

8.8% higher ($P<0.05$) compared to untreated ones (extracted with magnetic stir for 1 hr at room temperature) from mangosteen hull.

3.2 Antioxidant activities

The antioxidant activities (DPPH and ABTS) of two rice extracts obtained through different extraction methods were shown in Table 2. Significant differences ($P<0.05$) in antioxidant activities were observed in ultrasonic pretreatment methods in comparison to solvent extraction alone. Ultrasonic pretreatment increased scavenging activity of both rice extracts. This might be due to increase of TPC and TFC in the extract with ultrasonic treatment. Goffman and Bergman (2004) stated the antioxidant properties are directly correlated to the TPC. The phenolic content had a strong positive correlation with the flavonoid content and antioxidant capacity, in agreement with Shen *et al.* (2009). Especially the UM had higher antioxidant activities, followed by UHW>M>HW extraction in both the rice varieties. Representing rice extracts with higher phenolic content, flavonoid content simultaneously had higher antioxidant activities marking the significance of ultrasonic treatment.

3.3 HPLC analysis

3.3.1 Effect of extraction methods on the contents of individual anthocyanins

Anthocyanin level (Cyanidin-3-glucoside (Cy-3G) and peonidin-3- glucoside (Pe-3-G)) extracted from four extraction procedures were presented in Table 3. Chromatogram of standard was shown in Figure 1. The results show two different anthocyanins were detected in all black glutinous rice extracted with four different extraction procedures. Except in red rice variety, Cy-3G and Pe-3-G were not detected. The content of individual anthocyanins depends on extraction method, where ultrasonic pretreatment was more effective than solvent extraction alone. The trend of these compounds obtained from different extraction methods analyzed by HPLC was parallel to TPC analyzed by spectrophotometer. In addition, the black glutinous rice extracted with UM contained significantly higher ($P<0.05$) content of Cy-3G and Pe-3-G as to other extraction procedures. Cy-3G content was 24.58%, 24.63% and 54.85% higher than M, UHW and HW extracts respectively. Even in terms of Pe -3-G contents, UM was significantly higher compared to other treatments (26.1%, 25.1% and 59.6% higher than M, UHW and HW extract respectively). It was consistent with previous study report (Kim *et al.,* 2008), where three varieties of rice (black, red and wild rice) were evaluated for their anthocyanin profile and its nutritive potential. In which acidic methanol extracts of the black and wild rice marked the presence of three different pigments by HPLC analysis, characterized on the basis of UV-Vis/MS properties and the retention times of components separated by LC. While those pigments were not detected in acidic methanol extract of the red rice, out of three pigments detected, only two pigments were identified, Cyaniding-3-glucoside as the major anthocyanin and Cyaniding-fructoside. Similarly, Pengkumsri *et al.* (2015) also reported the red rice tested (Mali red rice) did not show any trace of anthocyanins. However, Laokuldilok *et al.* (2011) reported all the pigmented rice varieties contained both Cy-3G and Pe-3-G except in normal rice brans, but Cy-3G and Pe-3-G contents were relatively low in red rice brans (179.0±7.7 and 9.1±1.4 μg/g respectively). Similarly, Abdel-Aal *et al.* (2006) demonstrated even red rice varieties contained very low content of Cyanidin-3-glucoside (14.0±0.3 μg/ g) and Peonidin-3-glucoside (2.5±0.1 μg/g) in red rice. Furthermore, pH, temperature, glycosidic linkages and food matrix interactions that occurs during cooking process influences the stability of the anthocyanins. Notably, thermal in the present study, the anthocyanins obtained from hot water (HW) extraction yielding the significantly lower anthocyanins contents compared to

Table 3. Effect of extraction methods on content of Cyaniding-3-glucoside (Cy-3-G) and peonidin-3-glucoside (Pe-3-G) and their ratios of black glutinous rice

Treatment	Anthocyanin (mg/100g grain)			
	Cy-3G	Pe-3-G	Total	Cy3G:Pe3G
HW	89.00 ± 3.73 [c]	40.89 ± 4.66 [c]	129.89	69:31:00
M	184.78 ± 4.42[b]	94.67 ± 9.80 [b]	279.45	66:34:00
UHW	184.58 ± 8.02[b]	96.65 ± 7.85 [b]	281.23	66:34:00
UM	305.20 ± 7.37[a]	161.52 ± 11.98 [a]	466.72	65:35:00

Values are means ± SD. Values with [a-c] different superscript within the same column are significantly different (P<0.05; Duncan's test). HW: hot water, M: methanol, UM: ultrasonic+methanol, UHW: ultrasonic+hot water

other extractions methods. It is probably the anthocyanins could have degraded into protocatechuic acid since during heating the extract was exposed to higher temperature for a while. Hiemori *et al.* (2009) reported that the loss of anthocyanins in black glutinous rice may be attributed to the degradation or decomposition of anthocyanins resulting from thermal processing. And they also noticed that the content of total anthocyanins in the cooked rice were almost one-third lower than that in raw rice, whereas the levels of protocatechuic acid increased about three times after cooking. Their result indicates that Cyaniding-3-glucoside in black rice degraded into protocatechuic acid during cooking which could have possibly happened with our HW treatment. However, all black glutinous rice extracts (HW, UHW, M and UM) exhibited the similar individual anthocyanin ratio dominated by Cy-3G followed by Pe-3-G (Table 3) relative to previous studies, where Cyaniding-3-glucoside was identified as major anthocyanin in black glutinous rice grains with peonidin-3-glucoside as the second major anthocyanin (Abdel-Aal *et al.,* 2006). Even though composition ratio was different in our case with higher peonidin-3-glucoside content corresponding to Pengkumsri *et al.* (2015) compared to others, where peonidin-3- glucoside contributed a very low % (6-7%) of anthocyanin content (Yawadio *et al.,* 2007). The extraction procedures did not significantly affect the composition ratios of Cy-3G and Pe-3-G specifically in black glutinous rice. Still HW extract was able to illustrate higher Cy-3G ratio compared to other three black rice extracts. At the same time UM extract was able to exhibit bit higher Pe-3-G ratios compared to other extracts. The Cy-3G and Pe-3-G contents were significantly lower in HW extracts compared to other extracts while Cy-3G and Pe-3-G contents were leading in UM.

3.3.2 Effect of extraction methods on the contents of individual flavonoid

Quercetin and apigenin contents extracted from four extraction methods were presented in Table 4. Chromatogram of standard was shown in Figure 2. The extraction played a significant role in terms of quantification of quercetin and apigenin from two varieties of rice. Though red rice varieties are known to contain flavonoids, in our analysis, none of detectable content was achieved. Out of four extracts (black glutinous rice), UM extracts exhibited significantly higher (P<0.05) content of both quercetin and apigenin, almost two folds higher than UHW and M extract and six fold higher than HW extract. The present results were similar to the findings of Kim *et al.* (2010) where quercetin and apigenin was not detected in red and white rice varieties except in black rice variety. Especially, in HW black glutinous rice extract, lower content of quercetin was detected without the trace apigenin, illustrating the vulnerability of specific compounds with extraction techniques. Also the genetic, growing conditions and harvesting seasons might be also contributing towards differences in the specific flavonoid contents. The extraction procedures affected the extraction of flavonoids from the black rice varieties. Usually when black glutinous rice was extracted with HW, it was able to extract only quercetin, which was even significantly lower to other extraction methods. Although other extraction did not significantly affect the quercetin and apigenin ratios, its contents differed significantly (P<0.05) between conventional (HW and M extraction) and ultrasonic assisted extraction. However other extraction methods (M, UHW and UM) were able to extract both quercetin and apigenin, where UM had higher content of quercetin and apigenin compared to UHW and M extracts.

Table 4. Effect of extraction methods on Quercetin, and Apigenin and their ratios of black glutinous rice

Treatment	Flavonoid (mg/100 g grain)			
	Quercetin (Q)	Apigenin (A)	Total	Q: A
HW	0.06±0.02[c]	nd	0.06	100:0
M	0.25±0.02[b]	0.027±0.01[b]	0.28	90:10:00
UHW	0.20±0.02[b]	0.023±0.01[b]	0.22	90:10:00
UM	0.40±0.62[a]	0.047±0.00[a]	0.45	90:10:00

Values are means ± SD. Values with [a-c] different superscript within the same column are significantly different (P<0.05; Duncan's test), and nd = not detected. HW: hot water, M: methanol, UM: ultrasonic+methanol, UHW: ultrasonic+hot water

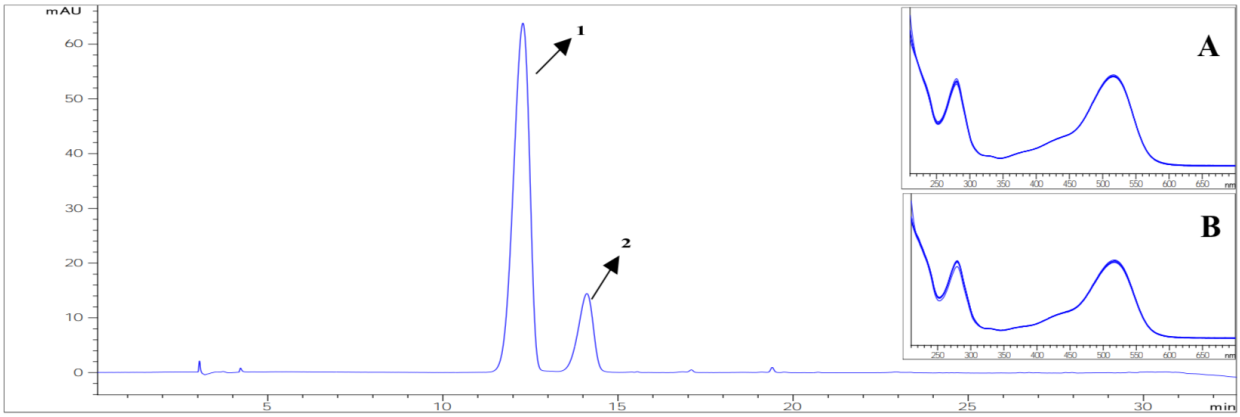

Figure 1. HPLC chromatogram of Anthocyanin standards (1, Cyaniding-3-O-glucoside; 2, peonidin-3-O-glucoside; A, Absorption spectra of Cyaniding-3-O-glucoside; B, Absorption spectra of peonidin-3-O-glucoside)

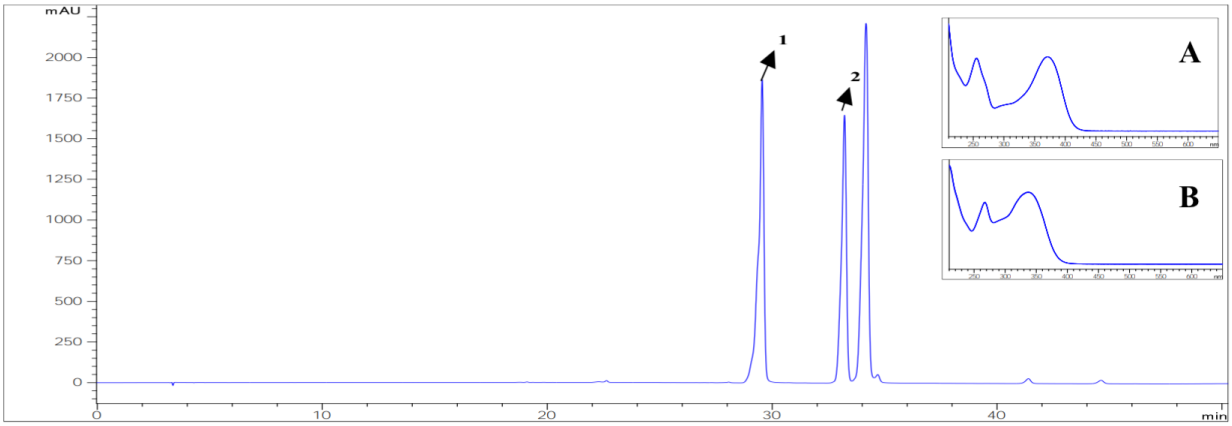

Figure 2. HPLC chromatogram of Flavonoid Standards: (1, quercetin; 2, apigenin; A, absorption spectra of Quercetin; B, absorption spectrum of Apigenin)

4. Conclusion

Current study showed, ultrasonic pretreatment on methanol (M) and hot water (HW) extraction from both rice varieties (black glutinous rice and red rice) significantly enhanced bioactive compounds analyzed in spectrophotometric and HPLC analysis of specific compounds of interest with potent antioxidant capabilities compared to conventional extractions alone. Ultrasonic methanol (UM) extraction proved to extract significant higher content of all the compounds analyzed in both the rice varieties. In contrast, hot water (HW) extracts exhibited lowest amount of bioactive compounds with significantly lower antioxidant activities. However, efficiency of ultrasonic pretreatment with hot water extraction was not less than methanol extraction alone.

Conflict of Interest

The authors declare no conflict of interest.

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