Original paper

Study on Stability and Antioxidant Activity of Red Anthocyanidin Glucoside Rich Hybrid Rice, its Nutritional and Physicochemical Characteristics

Yuanke LIANG¹, Muhammad Umer FAROOQ¹, Yongjun HU², Zhicheng TANG¹, Yujie ZHANG¹, Rui ZENG¹, Tengda ZHENG¹, Hla Hla Ei¹, Xiaoying YE¹, Xiaomei JIA¹ and Jianqing ZHU^{1*}

¹Rice Research Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

²Yibin Products Quality Supervision and Inspection Institute, Yibin, Sichuan 644000, China

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Using HPLC, it was demonstrated that the major free phenolic compound in the pericarp and seed coat of red rice was cyanidin, which belong to anthocyanidin glucoside, exist only in the episperm of red rice as content of 1.64 ± 0.10 mg/g. Anthocyanidin glucoside play a significant role in eliminating free radicals and reacting oxygen species (ROS) to prevent cellular oxidative stress. However, the chemical stability of anthocyanidin glucoside has been one of the major drawbacks for health applications. For longer storage, our results showed that red rice anthocyanidin glucoside could be stored with 0.3 % Vitamin C or 0.5 % NaHSO₃, and be protected from light, high temperature, high pH and metal ions in environment. Antioxidant activity verified that anthocyanidin glucoside performed better than Vitamin C in ferrous ions chelating activity, scavenging activities for hydroxyl and DPPH radicals. Overall, the red rice might be used as a natural antioxidant supplement applying medical treatments in future days.

Keywords: red rice, anthocyanidin glucoside, antioxidant, episperm, stability.

Introduction

Anthocyanidin glucoside, water-soluble natural pigments, belong to the group of flavonoids and are responsible for the red, purple and blue colours of many flowers and fruits, expected to be alternatives to synthetic colourants, and attract pollinators and seed dispersers (Zhang *et al.*, 2014). Since ancient times, they have been utilized as medicines or tanning agents by humans, whose chemistry and synthesis studied over a long period. Studies have demonstrated that anthocyanidin glucoside exhibit various pharmacological effects including antioxidation (Zafra-stone *et al.*, 2007), anti-inflammation (Lin *et al.*, 2008), antineoplastic (Wang *et al.*, 2008) and induction of leukaemia cell apoptosis (Chang *et al.*, 2005). Various neuro-protective effects (Kang *et al.*, 2006), immunomodulatory (Taverniti *et al.*, 2014), the prevention of amyloid-mediated neurodysfunction (PingHsiao *et al.*, 2011) and amelioration of dyslipidemia (Li *et al.*, 2015) also have

been reported. Moreover, as for plants themselves, anthocyanidin glucoside act as ROS cleaners to maintain normal metabolic processes in response to various abiotic adversities in plants at the cellular level (Zhou *et al.*, 2017). Because of their potential beneficial effects on human health, considerable attention has drawn to anthocyanidin glucoside and its antioxidant properties. Many botanists devoted themselves to breed with high anthocyanidin glucoside crop cultivars, such as colorful spring wheat (Kniewel *et al.*, 2009), blueberry (Li X *et al.*, 2017), purple sweet potato (Tensiska *et al.*, 2017), purple endosperm rice (Zhu Q *et al.*, 2017) and red cabbage (Tong *et al.*, 2017).

Rice (*Oryza sativa* L.) is one of the most principal food crops in the world, serving as the staple food source for more than half of the world's population (Pachau *et al.*, 2017), which contributes 55 %–80 % to the total calories intake of people (Farooq *et al.*, 2009). Hybrid rice that has a yield

*To whom correspondence should be addressed.

E-mail: zhujiangqing1963@163.com

advantage of 10–20 % over conventional varieties was developed and commercially grown (Cheng *et al.*, 2007). During previous era of advancement and technology, scientist focused on improvement of production and quality of rice but still, there is a vast range of area to improve the nutritious and quality features in rice. Hence, our main focus was to breed rice for high anthocyanidin glucoside. The most common types of rice (>85 %) are white-hulled while other types have coloured hulls, mainly purple, black, and red (Goufo and Trindade, 2014). Red rice grows taller, produces more tillers than domestic rice and shatters most of its seeds early, regarded as a major weed in many rice-growing areas of the world (Oki *et al.*, 2002). Because the major functional components in pigments rice were anthocyanidin glucoside, traditional Chinese medicine used red rice for strengthening kidney function, treating anemia, promoting blood circulation, removing blood stasis, treating diabetes, and ameliorating sight (Deng *et al.*, 2013). Thus, making full use of red rice has good potential prospects for nutraceutical and functional food application (Min B *et al.*, 2011).

Organic molecules, which protect the body's cells from damage initiated by reactive oxygen species and free radicals that might otherwise exert harmful metabolic effects thus promoting health are termed as antioxidants (Goufo and Trindade, 2014). Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation. There are many methods currently used for determining antioxidant activity (Mei X *et al.*, 2017), which shown great diversity such as Ferrous ions (Fe^{2+}) chelating activity (FICA) (Patel, 2013), scavenging hydroxyl, DPPH, ABTS radical (Wang *et al.*, 2018), peroxy radical scavenging capacity (PSC) assay (Morita *et al.*, 2017) and oxygen radical absorbance capacity (ORAC) assay (Chen *et al.*, 2018).

Anthocyanidin glucoside degradation, in all coloured juices and nectars, followed first-order reaction kinetics (Kirca *et al.*, 2006). While, the change in the color of red rice during storage is attributed to the oxidative degradation of procyanidine pigments (Hayashi and Yanase, 2016). Thus, making anthocyanidin glucoside storage for a longer time has a very high application value. The objective of our work was to breed high anthocyanidin glucoside red rice, determine the anthocyanidin glucoside content of red rice and evaluate the effect of light, temperature, pH, metal ions, Vitamin C and NaHSO_3 to influence the anthocyanidin glucoside stability. Besides, in this study, anthocyanidin glucoside and antioxidant activities were determined by various assays, including FICA, scavenging activities for hydroxyl and DPPH radicals from red rice extracts.

Materials and Methods

2.1 Plant material and Chemicals The ruby red rice (*Oryza sativa* L.) germplasm was picked from Sichuan

Agricultural University Rice Research Institute since 2007. After 10 years cross breeding in Hainan and Chengdu, anthocyanidin glucoside rich red hybrid rice sterile line (Z3055A) and maintainer line (Z3055B) were picked out. The hybrid red rice Z3055A/R363 was used in this study. The reagents for HPLC have following standards: Anhydrous ethanol (99 %), Cyanidin standard (98 % purity), Peonidin standard (98 % purity), Delphinidin standard (96 % purity), Malvidin standard (96 % purity), Petunidin chloride standard (96 % purity) and Pelargonidin standard (96 % purity). Other chemical components used in this study were formic acid, methyl alcohol, ascorbic acid (Vitamin C), sodium bisulfite, DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and so on. All these reagents were analytically pure and purchased from Ruijingte Chemistry Company (Chengdu, China).

2.2 Extraction, fractionation and purification of red rice epispem The fresh red rice Z3055A/R363 samples (cleaned with distilled water) were put into the drying oven (DHG-9240B, Shen Xian Co., Ltd, Shanghai, P. R. China) 80 °C for 3 hours. Then the brown rice machine (JLG-II, Da Ji Co., Ltd, Hangzhou, P. R. China) was used to separate hulls and brown rice (with epispem, embryo, aleurone layer and endosperm). Then, we used tweezers and dissecting needles to fetch the epispem (red surface). Complete rice epispem washed with deionized water and dried at 45 °C until constant weight obtained, samples meshed into powder and passed through 60 mm sieve. Extraction of epispem powder was completed by using HCl and ethanol solution 0.1 mol/L (1:15, w/v) at 60 °C for four hours inside the ultrasonic cleaners (WD-9415B, LiuYi Co., Ltd, Beijing, China). Recovered supernatants were concentrated by using the rotavapor at 45 °C (R-210, BUCHI Co., Ltd, Switzerland). Ultrasonic cleaners used to dissolve the remaining solid residues. Final extract reconstituted with deionized water on ultrasonic cleaner before storage. Then samples were stored at –20 °C for further analysis.

2.3 Anthocyanidin glucoside contents determination The absorbance of the solution was measured at 530, 620, and 650 nm using a Shimadzu UV-visible spectrophotometer (T6S, Puxi, Co., Ltd, Beijing, China). The anthocyanidin glucoside content calculated according to the following formula:

$$\text{OD} = (A_{530} - A_{620}) - 0.1(\text{OD}_{650} - \text{OD}_{620})$$

One unit of anthocyanidin glucoside content was expressed as a change of 0.1 OD (unit $\times$ ne un $\cdot$ FW $^{-1}$). Each sample was replicated thrice to get stable value, all performed by An *et al.* (2016) with a little modification.

2.4 Identification of anthocyanidin glucoside by High performance liquid chromatography Anthocyanidin glucoside extracts in red rice were examined by Agilent ZORBAX SB (AGILENT LC1260, USA) High performance liquid chromatography (HPLC) analysis done according to the method of Beye *et al.* (2013) and Zhu Q *et al.* (2017) with a little modification.

2.5 External conditions influence on stability of red rice

Fig. 1. Red rice Z3055A, Z3055B and grain of hybrid red rice Z3055A/R363 (A) Z3055A, sterile line; Z3055B, maintainer line. The scale bar represents 10 cm. (B) Red hybrid brown and polished rice of Z3055A/R363. The scale bar represents 2 mm.

episperm anthocyanidin glucoside

2.5.1 Light conditions Red rice episperm extracts divided into two groups to evaluate light effect on anthocyanidin glucoside stability. One was under dark (covered) and second was under the fluorescent light placed in room temperature. The samples tested for anthocyanidin glucoside content at 0, 2, 4, 6 and 8 hr.

2.5.2 Temperature conditions Red rice episperm extracts were covered and placed at 10, 30, 50, 70 and 90 °C temperatures under incubators. The anthocyanidin glucoside contents measured at 0, 2, 4, 6 and 8 hr in samples.

2. 6 Inherent conditions influence on stability of red rice episperm anthocyanidin glucoside

2.6.1 The pH effect The effect of pH on the stability of red rice anthocyanidin glucoside was studied in citrate phosphate buffer solutions at five different pH *i.e.*, 1.0, 3.0, 5.0, 7.0 and 9.0. The solution (25 mL portion) then transferred to pyrex tube and examined for 8 hr with 2 hr interval in order to determine the anthocyanidin glucoside contents.

2.6.2 Metal ions on the stability The influence of metal ions in red rice episperm anthocyanidin glucoside stability were studied at FeCl₂, ZnCl₂, MoCl₅ and CdCl₂ salt solution (5 mg/L) and pH was adjusted to the 3 by HCl. Salt solution mixed with red rice episperm anthocyanidin glucoside (200 mg/L) to make a total of 50 mL solution, then heated at 30 °C in thermostatic water bath for 8 hr to determine the content of anthocyanidin glucoside every 2 hr.

2. 7 Vitamin C and NaHSO₃ utilization for extract anthocyanidin glucoside stability 1 mL of Vitamin C and NaHSO₃ (concentration 0 %, 0.3 %, 0.5 %, 0.7 % and 0.9 %) were mixed with 49 mL of anthocyanidin glucoside into 50 mL Pyrex tubes, heated under the constant temperature of 60 °C for

30 min, finally anthocyanidin glucoside contents were determined.

2. 8 Antioxidant Activity Fe²⁺ chelating activity (FICA) was measured according to Patel (2013). The hydroxyl radical scavenging activity was performed by the hydroxyl radical chelating assay (Fenton reaction) Zhuang *et al.* (2013) and Morales (2005) to assess the antioxidant activity of the refined components from red rice episperm. The DPPH radical scavenging activity was performed by the DPPH assay according to Li *et al.* (2018) and Manivannan *et al.* (2018).

2.9 Statistical Analysis The experiment was replicated thrice under the same conditions. Data were expressed as the mean ± standard error (SEM). One-way ANOVA was carried out with multiple comparisons using Duncan's test to compare the means of different groups at $p \leq 0.05$ or 0.01. All statistical analyses were performed using the SPSS 24.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results and Discussion

3. 1 Quality characteristics After 10 years of multi-locational trail plantation and demonstrations, anthocyanidin glucoside rich red hybrid rice was featured with high quality. With 100 days' growth period, the plant height of Z3055A and Z3055B hits about 102 cm (Figure 1A). The hybrid red rice Z3055A/R363 quality features can be observed in Table 1.

3. 2 Anthocyanidin glucoside contents in different plant parts As can be seen from Figure 1B, most of the anthocyanidin glucoside observed in the episperm (outer surface layer of brown red rice), while there were no anthocyanidin glucoside in white rice (Figure 2). Results indicated that the anthocyanidin glucoside contents exist only in red rice episperm as content of 1.64 ± 0.10 mg/g. Thus, to

Table 1. Quality characteristics of hybrid red rice Z3055A/R363

Quality characteristics	Result
brown rice rate	80 %
white rice rate	71.2 %
head rice rate	60.8 %
grain length	6.1 mm
length-width ratio	2.6
Chalkiness grain rate	34 %
chalkiness degree	4.4 %
transparency	Level 2*
alkali spreading value	Grade 4.8**
gel consistency	52 mm
amylose content	27.1 %
protein content	9 %

The results were tested by rice and products quality supervision inspection and testing center of Ministry of agriculture of China.

* Level 2 means transparency is between 0.61~0.70.

** Grade 4.8 means gelatinization temperature range 70~74 °C

maximize the intake of antioxidant compounds, rice should be preferentially consumed in the form of brown rice (Goufo and Trindade, 2014). The total anthocyanidin glucoside content varied greatly among different black rice cultivars (79.5–473.7 mg/100 g) and red rice (7.9–34.4 mg/100 g) (Chen *et al.*, 2012, Chen *et al.*, 2016). Previous studies reported that anthocyanidin glucoside were enriched in apple skin, whose content can be significantly enhanced by the increased expression of the *MdMYB1* and *MdLOB52* genes responding to light and nitrogen environment respectively (Tako *et al.*, 2006; Wang *et al.*, 2017). About rice, Kawahigashi *et al.* (2007) reported that *OsPR1.1* promoter was isolated and the promoter activity was monitored using a reporter gene OSB2, which encodes a transcription factor for anthocyanidin glucoside synthesis. Most of anthocyanidin glucoside enriched in the skin in most species, but Zhu *et al.* (2017) made a high-efficiency vector system for transgene stacking and used it to engineer anthocyanidin glucoside biosynthesis in the endosperm (lining part of rice) named purple endosperm rice. All of these researches provide us a new direction to search for the molecular mechanism of anthocyanidin glucoside synthesis, transportation and accumulation in natural anthocyanidin glucoside rich red rice in the future.

3.3 Effect of external conditions on anthocyanidin glucoside stability Since red rice episperm color is mainly produced by anthocyanidin glucoside, it is reasonable to examine the light and thermal stability of anthocyanidin glucoside so that this information can be used in the production process. Figure 3A represented the total anthocyanidin glucoside content from the red rice episperm extracts, which contains the contents as high as 200 mg/L at the first time. In the early 2 hours, anthocyanidin glucoside exposed the light

Fig. 2. Anthocyanidin glucoside content of different part of rice. The location of Anthocyanidin glucoside in rice. Error bars show the SEM (n = 3). Brown rice with episperm, embryo and endosperm; polished rice contains endosperm and little embryo; red rice, Z3055A/R363; white rice, G-725 (*Oryza sativa* L.).

decreased a little more than darkness group, but the differences were not significant. But after 4 hours, there were remarkable differences between 2 groups. Besides, the total anthocyanidin glucoside contents decreased considerably after exposure to fluorescence light for 8 hr at room temperature. Hence, extract disclosure to light has negative correlation with anthocyanidin glucoside contents. Bononi and Tateo (2007) and Tonon *et al.* (2010) investigated that desiccated extract's anthocyanidin glucoside were significantly sensitive to natural light, which support current study. Furthermore, plants protect them against high irradiance is to utilise anthocyanidin glucoside as a light attenuator, due to their strong light absorption (Nichelmann and Bilger 2017; Zhu *et al.*, 2017). Thus, anthocyanidin glucoside are sensitive to light when extracting from plants during storage (Weber *et al.*, 2017).

Temperature affects the stability of anthocyanidin glucoside of red rice episperm extracts, as shown in Figure 3B. Storage temperature had a significant effect on the degradation of anthocyanidin glucoside. Total anthocyanidin glucoside contents were measured for different time intervals after incubation at various temperatures. Total extract anthocyanidin glucoside contents reduced considerably, with a corresponding elevation in temperature, after incubation at 70–90 °C for 8 h, as expected. Similar findings reported by Hellström *et al.* (2013) disclosed anthocyanidin glucoside degradation was faster when stored at room temperature than in the smoothie stored at 4 °C. Hence, the anthocyanidin glucoside contents degradation and disruption in antioxidant activity are prone to elevated temperature and the degradation of anthocyanidin glucoside during storage under different temperatures showed

Fig. 3. External and Internal conditions influenced Anthocyanidin glucoside stability

(A) The light influenced the content of red rice epidermal Anthocyanidin glucoside, error bars show the SEM ($n = 3$). Statistically significant differences **($P < 0.01$), * ($P < 0.05$) and "ns" (non-significant).

(B) Temperature influenced the content of red rice epidermal Anthocyanidin glucoside, error bars show the SEM ($n = 3$). Lowercase letters indicate the statistical significance ($P \leq 0.05$).

(C) The pH influenced the content of red rice epidermal Anthocyanidin glucoside, error bars show the SEM ($n = 3$). Lowercase letters indicate the statistical significance ($P \leq 0.05$).

(D) Metal ions influenced the content of red rice epidermal Anthocyanidin glucoside, error bars show the SEM ($n = 3$). Lowercase letters indicate the statistical significance ($P \leq 0.05$).

first order kinetics (Mourtzinou *et al.*, 2008; Weber *et al.*, 2017). Mori *et al.* (2007) believed that high temperature (35 °C) reduced the total anthocyanidin glucoside content to less than half compared to the control berries group (25 °C) due to the factor of methoxylation, glycosylation, and acylation leading to an increase in the thermal stability of anthocyanidin glucoside. Yamane *et al.* (2006) found that the expression of the main and other related anthocyanidin glucoside biosynthesis genes were more in lower temperature (20 °C). Not only on anthocyanidin glucoside, temperature also have inhibitory effects on other antioxidant compounds: the decline in phenolic acids being greater at 37 °C than at 4 °C (Zhou *et al.*, 2004), while an 18 % loss of γ -oryzanol and 70 % loss of vitamin E was quite prominent when exposed to room temperature (25 °C) for 6 months (Pascual *et al.*, 2013).

3.4 pH and metal ions scavenging anthocyanidin glucoside stability The effect of pH on the stability of red rice anthocyanidin glucoside was studied in citrate phosphate buffer solutions at five different pH *i.e.*, 1.0, 3.0, 5.0, 7.0 and 9.0. As seen in Figure 3C, red rice epidermal anthocyanidin glucoside showed two distinct stability profiles: (1) Lower pH (1.0 and 3.0), and (2) Higher pH (5.0, 7.0 and 9.0). Significant decline in anthocyanidin glucoside stability was observed at pH 5.0

and above, every 2 hours. Alkali environment has significant impact on the stability of epidermal anthocyanidin glucoside, when compared with the acidic environment of pH 3.0 anthocyanidin glucoside contents were more stable at pH 9.0. Hence, internal pH 3.0 have efficient stability to store red rice epidermal anthocyanidin glucoside, the current study is in accordance with Torskangerpoll (2005) who believed that alkaline solutions could have drastic effects in storage and stability of the anthocyanidin glucoside. In contrast, Babaloo and Jamei (2018) believed that highest stability of anthocyanidin glucoside was observed in pH 1 and by increasing pH from 1 to 4, absorption is decreased, which was a little different from our results. All pigments experienced dramatic colour changes when kept in the alkaline storage period, as similar findings reported by Choi *et al.* (2017). A previous study by Williams and Hrazdina (2010) demonstrated that maximum complex formation occurs with the 3,5-diglucoside and 3-acylglucoside-glucosides at around pH 3.1, so that it could be suitable for extracting and storing anthocyanidin glucoside from red rice epidermal at pH 3.

The magnitude of hyperchromic effect was strongly affected by the concentrations and species of metal ions. Among these metal ions, all of them have a strong influence to

decrease the content of red rice episperm anthocyanidin glucoside in the following ascending order: $\text{Zn}^{2+} > \text{Cd}^{2+} > \text{Fe}^{2+} > \text{Mo}^{5+}$ (Figure 3B). Anthocyanidin glucoside have an ability to bind metal as complexation for maintaining colour stability in various anthocyanidin glucoside-rich products (Fedenko *et al.*, 2017). Araceli *et al.* (2009) believed that the blue colour was due to a complexation between anthocyanidin glucoside and some metals such as Al, Fe and Mo accounting for the B ring (with ortho-dihydroxyl group). Previous study reported that trivalent ions are more stable than divalent ions (Araceli *et al.*, 2009; Fedenko *et al.*, 2017). Therefore, metal free environment could be helpful in storage and stability of anthocyanidin glucoside in red rice.

3.5 Phytochemical characteristic of major anthocyanidin glucoside from red rice The chemical compositions in the anthocyanidin glucoside were analyzed by HPLC. The results of HPLC and tentative identification are shown in Figure 4A. Six phenolic compounds were identified in the present study, which includes delphinidin, cyanidin, petunidin chloride, pelargonidin, peonidin and malvidin. HPLC analysis revealed that the major free phenolic compound in the episperm of red rice was cyanidin. Compared to purple potato, red cabbage and color wheat (Kniewel *et al.*, 2009; Takayanagi *et al.*, 2005; Tong *et al.*, 2017) which contained at least two or three other compositions of their main anthocyanidin glucoside. According to the results of Saigusa *et al.* (2014), phenolic compounds, such as malvidin and peonidin, are the main active components of black rice, and black rice bran contained anthocyanidin glucoside 18–26 % (Laokuldilok *et al.*, 2010). But there are significant differences in phytochemical content and antioxidant activity among the different black rice varieties (Zhang *et al.*, 2010). Bell *et al.* (2014) reported that the major compositions of anthocyanidin glucoside in cherry was cyanidin-3-o-glucosiderutinoside which is similar as red rice. Cyanidin acts as antioxidant and anti-carcinogenesis properties and an important role in osteoclastic bone resorption and osteoporosis (Cheng *et al.*, 2017), which implies the potential medical function about the red rice.

3.6 Vitamin C and NaHSO_3 additives on anthocyanidin glucoside stability protection Shelf life is a major consideration in developing, producing, and marketing many food products. In current study, anthocyanidin glucoside decomposed all the time. Hence, we use Vitamin C and NaHSO_3 additives to explore how they protect the stability of red rice episperm anthocyanidin glucoside. The results (Figure 4 B and C) showed that adding both Vitamin C and NaHSO_3 could increase the content of anthocyanidin glucoside after 30 min at 60 °C water baths compared to control. Besides, the contents of anthocyanidin glucoside were different with different concentration of Vitamin C and NaHSO_3 . Vitamin C concentration of 0.3 % had the highest content of anthocyanidin glucoside with the preserving rate as high as 98.41 %, while, with the concentration of 0.5 %–0.9 % Vitamin C, the content

of anthocyanidin glucoside declined significantly from 98.41 % to 90.04 %, anthocyanidin glucoside contents were 88.96 % without vitamin C. At the same time, NaHSO_3 had the similar influence to maintain the degradation rate of anthocyanidin glucoside, NaHSO_3 concentration of 0.5 % had the highest content of anthocyanidin glucoside with the preserving rate 96.61 % and the degradation rate of anthocyanidin glucoside without NaHSO_3 was 85.19 %. Consequently, both of Vitamin C and NaHSO_3 were effective to extend anthocyanidin glucoside shelf life compared to other internal and external factors such as light, temperature, pH and metal ions. The results of Zhang *et al.* (2013) were in accordance with current study that enrichment of anthocyanidin glucoside in tomatoes could significantly extend shelf life. Therefore, it is universal to use food additives in food such as Vitamin C (Shabbar *et al.*, 2012) and NaHSO_3 as the antioxidants to retard the deterioration.

3.7 Antioxidant activities of the extract It is widely believed that oxidation is one of the necessary factors for developing many chronic degenerative processes, including aging (Teixeira *et al.*, 2013). Reducing radicals power has been used as one of the antioxidant capability indicators of medicinal herbs. The methods of FICA and scavenging activities for hydroxyl and DPPH radicals are usually used to measure the antioxidant capacity of plant extracts, which can effectively indicate the changes of antioxidant capacity (Zielinska and Michalska, 2016; Feng *et al.*, 2017). The results can be observed in Figure 4 D, E and F.

Fe^{2+} , commonly found in food systems, is well known as the most powerful pro-oxidant among the various species of metal ions (Revilija *et al.*, 2005). The purpose of ferrous ion chelating activity test was to determine the capability of testing material to bind the oxidation catalytic ferrous ion (Hsu *et al.*, 2003). In this assay, the red rice episperm extracts and Vitamin C interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine. As we can see from the Figure 4D, both the red rice episperm extracts and Vitamin C showed relatively similar trend in high chelation activity, but Vitamin C had a lower ferrous ion chelating capability compared to the red rice episperm extracts at all concentrations. It is noteworthy that the red rice episperm extracts has a better capability in chelating iron. M. Elmastaş *et al.* (2006) exposed that the iron chelating capacity test indicated a gradual decrease in the absorbance of the red Fe^{2+} /ferrozine complex as antioxidants compete with ferrozine in chelating ferrous ion.

Hydroxyl among all the oxygen radicals is the most reactive radical, a source of cancer, aging and several diseases. Oxidation of lipid is primarily initiated by hydroxyl radical resulting hydrogen atoms extraction from un-saturated fatty acids. Therefore, its chelation provides protection against severe damages. The scavenging of hydroxyl free radical in red

Fig. 4. HPLC analysis, protectiveness and antioxidant capacity

(A) HPLC chromatogram (530 nm) of episperm of red rice Anthocyanidin glucoside. The peak assignments are: (1) delphinidin, (2) cyanidin, (3) petunidin chloride, (4) pelargonidin, (5) peonidin and (6) malvidin

(B) Vitamin C additives on red rice episperm Anthocyanidin glucoside stability protection at 60 °C for 30 min. Error bars show the SEM (n = 3). Lowercase letters on the top of column indicate the statistical significance ($P \leq 0.01$).

(C) NaHSO₃ additives on red rice episperm Anthocyanidin glucoside stability protection at 60 °C for 30 min. Error bars show the SEM (n = 3). Lowercase letters on the top of column indicate the statistical significance ($P \leq 0.01$).

(D) FICA of the extracts, error bars show the SEM (n = 3). Statistically significant differences **($P < 0.01$), * ($P < 0.05$)

(E) Hydroxyl radical scavenging activity of the extracts, error bars show the SEM (n = 3). Statistically significant differences **($P < 0.01$)

(F) DPPH radical scavenging activity of the extracts, error bars show the SEM (n = 3). Statistically significant differences **($P < 0.01$), * ($P < 0.05$), "ns" (non-significant).

rice episperm extracts was found to be higher than Vitamin C (Figure 4E). Besides, both of them with the lower concentration of 2–10 mg/L had a very high speed to enhance the scavenging rate. The concentration of 10–40 mg/L gently enhanced the scavenging rate. Slightly stable scavenging rate was observed above the concentration of 40 mg/L. Biomolecules (DNA, Proteins and Nucleic acids) are prone to reactive hydroxyl radical as it degrades them quickly (Jin and Wu, 2015; Huang *et al.*, 2010). Reactive free radical formation (Fenton reaction, $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\cdot$) is accelerated with the utilization of ferrous state of iron. Hydrogen and lipid peroxides oxidized by ferrous ions (Oke and Aslim, 2010). Hydrogen peroxide and hydrogen peroxide free radicals, derived from alkyl free radicals, are the major products during the propagation period (Hsu *et al.*, 2003). Therefore, its removal leads to the increased immunity in living body against several diseases. The red rice anthocyanidin glucoside utilization could be helpful in elevating the hydroxyl scavenging activity. Hence, red rice serves as an effective source to provide immunity against

several diseases.

DPPH radical scavenging activity was increased in the red rice episperm extracts and Vitamin C (Figure 4F) with the concentration between 1 to 6 mg/L and the scavenging rate was 88 % and 70 % respectively. Besides, the increasing rate was not so regular between 6–10 mg/L, while the steepness of slope becomes nearly stable after 10 mg/L and the differences between extracts and Vitamin C was not significant. DPPH is a free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Omp and Tejk, 2009). The reaction between antioxidant molecules and radical, grades in decreased absorbance of DPPH, which ultimately results in scavenging activity of the radical by the donation of hydrogen (Elmastaş *et al.*, 2006). Dang *et al.*, (2017) reported that the fermented rice bran extracts had a strong antioxidant activity in scavenging DPPH which is accordance with our study. Kim *et al.*, (2017) found a kind of yellow grain rice mutant which was enriched flavonoids can eliminate DPPH as well as red rice anthocyanidin glucoside. The oxygen radical absorbing

capacity was ranked as follows: red > black > green > white rice (Chen XQ *et al.*, 2012), which indicated the high potential medicine value of red rice.

Conclusions

Using heterosis, we bred anthocyanidin glucoside rich red rice, and the results revealed that anthocyanidin glucoside exist only in the red rice outer layer surface. Based on the results observed in present study and the aforementioned discussion, it is concluded that the red rice anthocyanidin glucoside have a better ability than Vitamin C in FICA, scavenging activities for hydroxyl and DPPH radicals. It can be stored with 0.3 %Vitamin C or 0.3 %–0.5 %NaHSO₃, and be protected from light, high temperature, high pH and metal ions. However, still there is a vast range of area to discover the molecular mechanisms and pathways for the synthesis of anthocyanidin glucoside and its accumulation in red rice episperm. Meanwhile, the bio-fortified red rice obtained through genetic improvement by breeding is a natural source of anthocyanidin glucoside and can be used as a natural antioxidant, which could be applied in medical treatment as supplements in the near future.

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