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TOTAL ANTHOCYANIN CONTENT AND ANTIOXIDANT ACTIVITIES OF PIGMENTED BLACK RICE (*ORYZA SATIVA L. JAPONICA*) SUBJECTED TO SOAKING AND BOILING

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Graphical abstract

Abstract

Pigmented rice contains high value of anthocyanin and antioxidant activity. However, the different process for black rice might affect the concentration of these phytochemicals. Therefore, the present study focused on the effect of soaking and cooking on the total anthocyanin content and antioxidant activity of pigmented black rice. The raw black rice was subjected to two treatments. First, black rice was soaked for 3 hours. Second, black rice was soaked and cooked for 15, 25 and 35 minutes. The study revealed that the second treatment (soaking and cooking) caused a significant ($p<0.05$) decreased in antioxidant activities and total anthocyanin content as compared to soaking. Highest losses in total anthocyanin and antioxidant activity in cooked black rice were as follows: 35 minutes > 25 min >15 min cooking. β- Carotene degradation rate was also highest in 35 minutes cooked black rice.

Keywords: Pigmented black rice, soaking, cooking, anthocyanin, antioxidant

Abstrak

Kacang hitam mempunyai antocyanin dan antioksida yang tinggi. Namun begitu, cara penyediaan yang berbeza bagi kacang hitam boleh mempengaruhi kepekatan bahan fitokimia tersebut. Oleh sebab itu, kajian ini bertujuan untuk mengetahui kesan tempoh rendaman dan masakan ke atas kandungan antocyanin dan aktiviti antioksida dalam kacang hitam. Dua jenis kajian dikenakan ke atas kacang hitam. Yang pertama, kacang hitam direndam selama 3 jam. Kedua, kacang hitam direndam selama 3 jam dan dimasak selama 15, 25 dan 35 minit. Hasilnya menunjukkan tempoh masakan menyebabkan penurunan yang signifikasi ($p<0.05$) bagi kandungan anthocyanin dan aktiviti antioksida berbanding dengan rendaman sahaja. Kehilangan kandungan anthocyanin dan penurunan aktiviti antioksida di dalam kacang hitam adalah tinggi pada tempoh masakan yang berikut: 35 minit > 25 minit >15 minit. Penyusutan kadar β- Carotene juga adalah tinggi pada minit ke 35 semasa proses memasak.

Kata kunci: Kacang hitam, rendaman, masakan, anthocyanin, antioksida

1.0 INTRODUCTION

All rice and black rice are in the family of grasses called Gramineae. Black rice or its scientific name *Oryza sativa L. Japonica* can be distinguished easily by its dark brown to black colour. Black Rice is actually more purplish in colour than black. It is sold as unmilled rice with fiber-rich black husks. When the black rice was cooked, it will turn to deep purple colour. It was reported that black rice

(*Heugjinjubyeo*) contains many anthocyanins pigments [1]. According to [2], anthocyanin in black rice includes cyanidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, pelargonidin 3-O-glucoside and delphinidin 3-O-glucoside. HPLC analysis of this rice shows that it contained approximately 95% of cyanidin -3-O-glucoside and 5% of peonidin -3-O-glucoside. [3] reported that, total phenolic content and anthocyanin content in black rice were higher than other types of rice and it possesses powerful antioxidant properties. Due to this reason, black rice has become an alternative source of food to consume [4]. It has been reported that anthocyanin antioxidants have pathologies beneficial effects in which is able to reduce the risks of cardiovascular diseases and cancers with anti-inflammatory, antioxidant and chemo protective properties [5, 6].

Anthocyanins and vitamin C are unstable compounds since its stability are affected by several factors such as pH, storage temperature, heat, chemical structure, concentration, light, oxygen, solvents, the presence of enzymes, flavonoids, proteins and metallic ions [7]. Since black rice contains high flavonoids contents, they are not stable and can be easily destroyed by these factors. Although many factors can influence the stability of anthocyanins but the most notable factor is temperature since this compound is very sensitive to exposure to high temperature [8]. According to [9], various temperature and heating time had significant impact on total anthocyanin content and total antioxidant activity. It was reported that the level of anthocyanins in black pigmented rice decreased by about 70% resulted from cooking while the average content of phenolics in red pigmented rice decreased by 26% after cooking [3]. In addition, [10] found that cooking drastically reduced the phytochemicals and antioxidant capacities as compared to raw rice. The drastic decrease in total phenolics content and total flavonoids content could be due to thermal degradation of phenol and flavonoid compounds [11, 12] as these compounds are very sensitive to heat treatments. On exposure to heat or temperature, this compounds have tendency to breakdown into smaller stable forms, which may or/not exhibit antioxidant activity. The maximum amount of the phenolic compounds present in the rice is in the bound form. Upon cooking, the cellular breaking down facilitates the release of these bound phenolic [13].

The preparation of the black rice involves the soaking of black rice for certain time prior to cooking until the texture of cooked rice tender and soft. This processing method will incur losses to both the anthocyanin content and antioxidant activities of the cooked rice. To date, not much information on the losses of anthocyanin content and antioxidant activities subjected to different processing methods has been reported. Therefore this study was carried out to evaluate the stability of total anthocyanin and antioxidant activities when subjected to these processing methods.

2.0 METHODOLOGY

Black rice was purchased from Giant Supermarket section 7, Shah Alam, Selangor. Other chemicals were purchased from Sigma-Aldrich, United Kingdom. About 100 g of raw black rice grains was weighed and ground using a food processor (HR2027/75, Philips Compact Blender). The powdered raw black rice was sieved through a 60 mesh screen [14] and stored at 4°C for further analysis.

Processing of soaked black rice was done by soaking the raw black rice (100 g) in 400 ml of distilled water for three hours. Then, the water was discarded and the soaked black rice was freeze dried. A freeze dried sample was then ground using a food processor (HR2027/75, Philips Compact Blender) and the powder obtained was sieved through a 60 mesh screen and stored at 4°C for further analysis [15].

Processing of cooked black rice was done by placing the black rice (100 g) in three different stainless steel pans with 400 ml of distilled water and covered with a lid. It was presoaked for three hours prior to cooking. After soaking, black rice was subjected to boiling at different time, which was 15, 25 and 35 minutes respectively. After different boiling time was achieved, the pans were subsequently removed from heat and left to stand for 5 minutes. Cooked samples were frozen before freeze-drying using freeze dryer (CHRIST LCG, 121550 PMMA, Lyo Chamber Guard). Then, freeze-dried samples were ground using a food processor. The powder obtained was sieved through a 60 mesh screen and stored at 4°C for further analysis [15].

The preparation of ethanol extract from raw, soaked and cooked black rice was done by extracting the ground samples (1.5 g) with 15 ml of 850 gL^{-1} aqueous ethanol in a shaking water bath with reciprocating motion for 30 minutes at room temperature. Then it was centrifuged at 3061 × *g* for 10 minutes and the supernatant was collected. The residues were re-extracted using the same conditions. The filtrates were then combined and the final volume was adjusted to 45 ml with 850 gL^{-1} of aqueous ethanol. The extract was used for further chemical analyses [15].

Radical scavenging activity of the sample extracts were conducted based on the method developed by Brand-Williams *et al.* (1995) with slight modifications. Briefly, 600 µL of a various concentration of standard and sample ranging from 0-0.1 mg/ml were prepared. 1 mg/ml of sample extracts were mixed with DPPH solution (4.5 mL of 60 µM DPPH solution in 95% ethanol). The reaction was allowed to take place in the dark for 30 minutes and the absorbance reading at λ_{max} = 517 nm using UV-VIS spectrophotometer (UVA-160921, Helios Alpha, England) was recorded to determine the

concentration of remaining DPPH. The results were expressed as EC_{50} (Efficient Concentration at 50% scavenging activity). Ascorbic acid and the mixture of synthetic antioxidant BHA/BHT were used as standards in comparison with the extracts [16].

DPPH free radical was calculated using the following formulation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance (control–sample)}}{\text{Absorbance (control)}} \times 100$$

For Ferric Reducing Antioxidant Power (FRAP), the antioxidant capability of the extract based on ferric reducing power was determined according to the method described in [17]. The stock solutions contained 300 mM acetate buffer (pH 3.6), 10 mM of 2, 4, 6- tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM ferric chloride ($Fe_2Cl_3.6H_2O$) solution. The working solution was freshly prepared by mixing the acetate buffer, TPTZ solution and $Fe_2Cl_3.6H_2O$ solution in 10:1:1 ratio and then incubated at 37°C for 10 minutes prior to the analysis. About 100 µl of pigmented rice extracts were allowed to react with 2.9 ml of the FRAP solution for 30 minutes in the dark condition. The readings of the blue (ferrous tripyridyltriazine) complex were measured using UV-VIS spectrophotometer (UVA-160921, Helios Alpha, England) at 593 nm. The linear standard calibration curve ranging from 0-100 mM Trolox was established. The final results were expressed in mM TE/g of fresh extract weight.

For β-carotene bleaching assay (BCB) [18], about 2 ml β-Carotene solutions (0.20 mg/ml chloroform) were pipetted into a round-bottom flask containing 20µl linoleic acid and 200 µl Tween 20. The mixture was then evaporated at 40 °C for 10 minutes using a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately added with 100 ml of distilled water and was agitated vigorously to form an emulsion. Then 5 ml of aliquots were transferred into different test tubes containing 200 µl of extracts. The mixture was then gently mixed and placed in a water bath at 50 °C for 2 hours. Absorbance of the sample was measured every 15 minutes for 2 hours at 470 nm using a UV-VIS spectrophotometer (UVA-160921, Helios Alpha, England). Degradation rate (DR) was calculated according to first order kinetics by using the following equation [19]:

DR (sample or standard) = ln (a/b) X I/5, where:
a = initial absorbance (470 nm) at time 0
b = the absorbance (470 nm) at 20, 40, 60, 80, 100, 120 min
t = initial absorbance (470 nm) at time 0

Antioxidant activity (AA) was expressed as percent inhibition relative to the control, using the following formula:

$$AA = \frac{\text{DR (control)- DR (sample/standard)}}{\text{DR (control)}} \times 100$$

In addition, absorbance values (at 470 nm) versus time was plotted for the degradation of β-Carotene and the results were expressed as antioxidant activity as a function of time.

The monomeric anthocyanin pigment content of the aqueous extract was determined by using pH differential spectrophotometry [20]. A UV spectrophotometer and a 1 cm path length of disposable cell were used for spectral measurements at 510 and 700 nm. Pigment content was calculated as mg cyanidin-3-glucoside/100 g of samples weight using an extinction coefficient of 26900 L/cm/mol and molecular weight of 449.20 g/mol.

All analytical determinations were conducted in triplicates. Statistical analyses were conducted using the Statistical Analysis System (SAS) (1.3 software package). Analyses of variance were performed by ANOVA procedures. Significant differences ($P<0.05$) were determined by least square means comparison.

3.0 RESULT AND DISCUSSION

DPPH assay is a method used for screening the antioxidant activity of the sample extract since it measures the antioxidant abilities to scavenge the stable radical DPPH. Raw black rice contains significantly high DPPH radical scavenging activity (88.72%) as compared to other processed black rice (Figure 1).

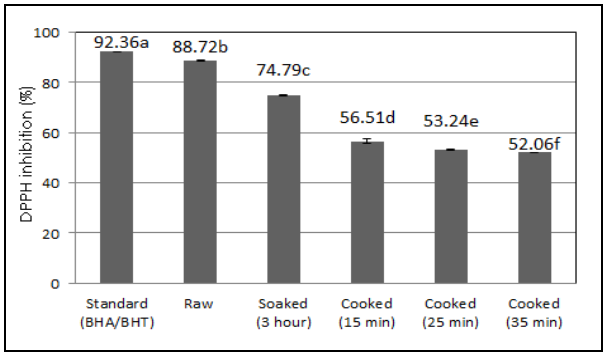

Figure 1 DPPH radical scavenging activity of BHA/BHT in raw, soaked (3 hours) and cooked (15, 25, 35 min) of black rice. Mean with different small letters indicated significant different ($p<0.05$)

[21] also conducted a study on antioxidant activity in varieties of rice and the study shows highest percentage of radical scavenging inhibition in colored variety rice (88.29%).

DPPH radical scavenging activities of black rice decreased when it was subjected to soaking (74.79%). However, further decreased of antioxidant activity was found when the pigmented rice was subjected to cooking for 15 minutes (56.51%), 25

minutes (53.24%) and 35 minutes (52.06%) respectively.

The reduction in scavenging activities of cooked black rice was comparable with the study done by [10] where pigmented rice cooked by open steaming in an autoclave showed decreased in scavenging activity from 64.40% to 26.00% after 15 minutes cooking. [22] also observed reduction in antioxidant activity of fermented black bean subjected to heat treatment at 100°C for 30 minutes. The instability of antioxidant activity in cooked black rice might be related to the degradation of the anthocyanin at high temperature since most of the antioxidant activity in purple black rice consist mainly of single anthocyanin, identified as cyanidin 3-O-beta-D-glucoside (Cy 3-Glc) [23].

Ferric reducing antioxidant power (FRAP) assay was used to measure the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex by producing intense blue colour of ferrous tripyridyltriazine (Fe^{2+}-TPTZ) [24]. The reducing properties correlated with the presence of compounds that able to exert their actions by breaking the free radical chain through donating of hydrogen atom [25].

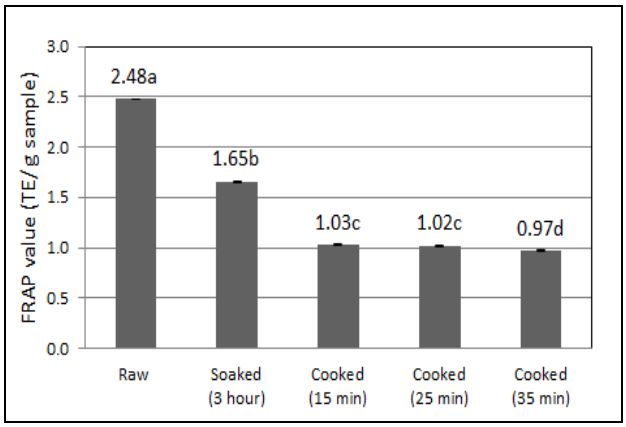

Figure 2 FRAP values of black rice in raw, soaked (3 hours) and cooked (15, 25, 35 min). Mean with different small letters indicated significant different ($p<0.05$)

Based on the results obtained (Figure 2), there was significant difference ($p<0.05$) in FRAP value for raw, soaked and cooked black rice. The FRAP values were in descending order where raw pigmented rice (2.48 TE/ g of sample)> soaked pigmented rice (1.65 TE/ g of sample) > cooked for 15 minutes (1.03 TE/ g of sample) > cooked for 25 minutes (1.02 TE/ g of sample) and > cooked for 35 minutes (0.97 TE/ g of sample). Higher FRAP assay values give higher antioxidant capacity because FRAP value is based on the reducing ferric ions, where antioxidants are the reducing agents [26]. There was no significant difference between cooked black rice for 15 minutes and 25 minutes ($p>0.05$).

Based on Figure 2, the treatments applied to raw black rice caused losses of FRAP value. Soaking for 3 hours (1.65 TE/g) causes 33.47% losses of FRAP value while cooking for 15 minutes (58.46%), 25 minutes (58.87%) and 35 minutes (60.88%) resulted in further losses of FRAP values. [28] also reported high losses of FRAP value in blueberries (40%) when it is subjected to heat treatment for 40 minutes.

High losses of FRAP values when subjected to high temperature or heat might be attributed to thermal degradation of phenolic compounds [29]. In addition, declines in antioxidant activity are accompanied by loss of other bioactive compounds such as carotenoids and polyphenols [30].

For β-carotene bleaching assay, linoleic acid produces hydroperoxides which present as free radicals during incubation at 50 °C. The presences of antioxidants in the extract are able to minimize the oxidation of β-carotene by hydro peroxides. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts. There was a correlation between degradation rate and the bleaching of β-carotene where the extract with the lowest β-carotene degradation rate exhibits the highest antioxidant activity [31].

Raw black rice exhibit lower β-carotene degradation rate compared to soaked and cooked black rice (Figure 3).

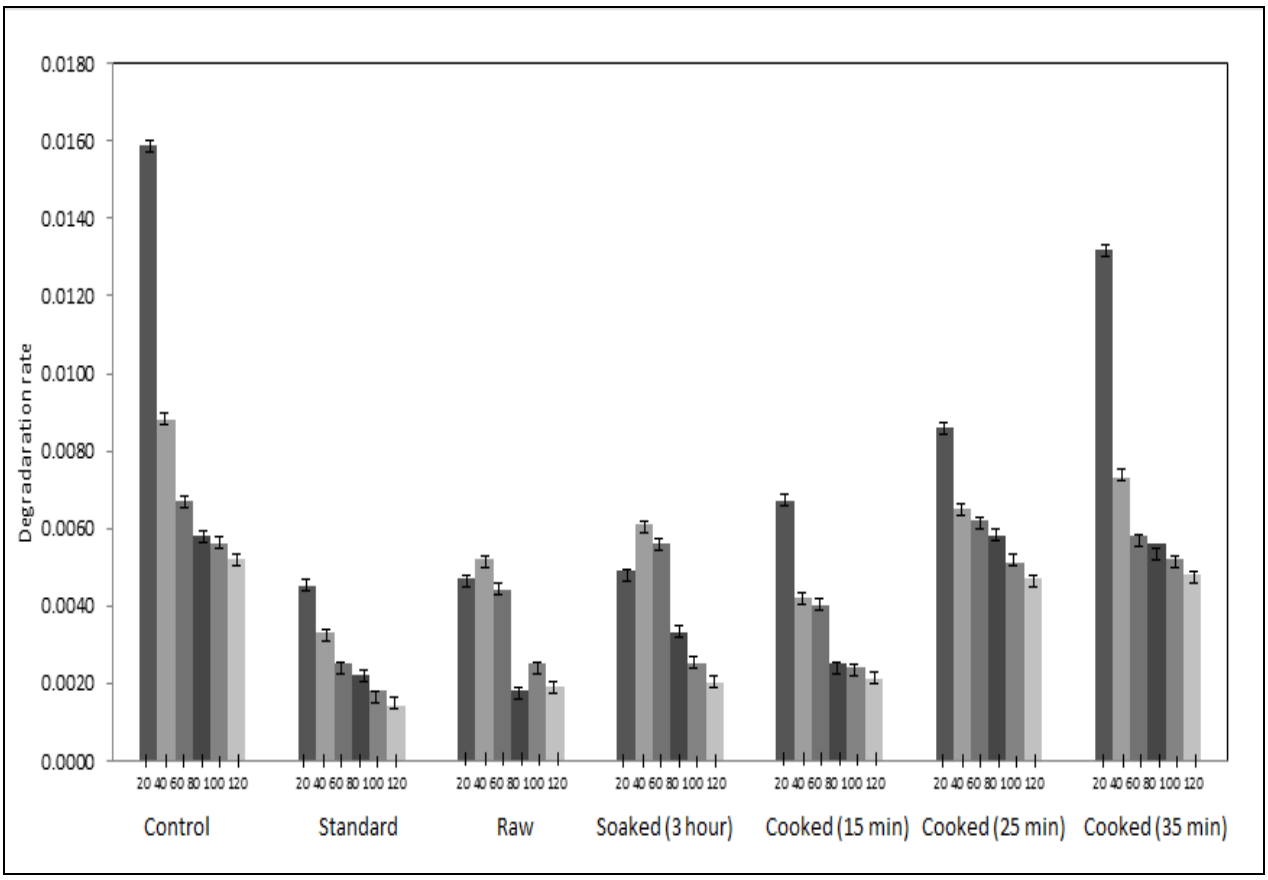

Figure 3 Degradation rate of control(chloroform), standard(BHT) and black rice samples (Raw, Soaked (3 hours) and Cooked (15, 25 and 35 min)) of ethanol extract assayed by β-carotene bleaching method (n = 3) at 20.40,60,80,100 and 120 minutes

β-carotene degradation rate in raw black rice and soaked black rice were 0.0019 and 0.0020 respectively. The β-carotene degradation rate for cooked black rice at 15, 25 and 35 minutes cooked black rice were 0.0021, 0.0047 and 0.0048 respectively. Based on the result obtained, raw black rice had the highest antioxidant activity since it exhibits low β-carotene degradation rate. In addition, all extracts had low antioxidant activity than standard (BHT). This study shows that different processing steps affect the antioxidant property of pigmented black rice as determined by the β-carotene bleaching method.

The total anthocyanin content was determined by the pH differential method [20]. Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra. The coloured oxonium form predominates at pH 1.00 and the colourless hemiketal form at pH 4.50. The pH-differential method is based on this reaction, and permits accurate and rapid measurement of the total anthocyanin, even in the presence of polymerized degraded pigments and other interfering compounds.

Based on Figure 4, total anthocyanin contents (TAC) for raw and soaked black rice were 2.00 mg/100 g and 1.45 mg/100 g of sample while cooked black rice at 15, 25 and 35 minutes contains 0.75 to 0.601mg /100 g of sample respectively. TAC of raw black rice was compared with the study done by the [32] where anthocyanin content Hom Nil rice (black non-waxy rice) was 1.89 – 3.32 mg/100 g.

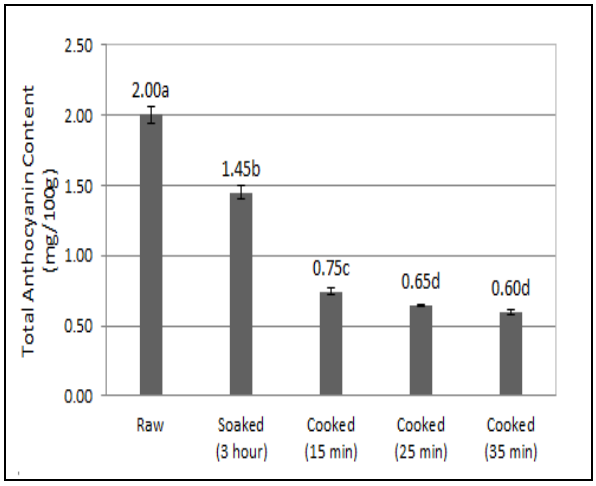

Figure 4 Total anthocyanin content of black rice in raw, soaked (3 hour) and cooked (15, 25, 35 min)). Mean with different small letters indicated significant different ($p<0.05$)

Figure 4 shows percentage losses of Total Anthocyanin Content (TAC) for black rice when subjected to soaking and cooking. Soaked black rice incurred 27.80% losses of total anthocyanin content and further losses of anthocyanin were observed in black rice subjected to cooking at different times where the losses range between 62.77 to 70.01%. According to [15] the total anthocyanin content of various cooking method in cooked rice decreased in the range of 72.00% to 88.00%. This shows that significant losses of anthocyanin during cooking might be due to the thermal degradation of anthocyanin involving hydrolysis of glycoside linkages to form chalcone or alpha diketone. Anthocyanin is known for being active compound that can readily reacting with constituent or simply degrading by the action of oxygen, light, enzyme pH modification and high temperature processing. The decrease in antioxidant activity of cooked black rice is very similar to the findings of anthocyanin. This indicates that anthocyanin plays a key role in the antioxidant activity of black rice. Recent study by [15] showed that anthocyanin had high positive correlation with total phenolic compounds (r^2=0.936) while [33] demonstrated a positive correlation between phenolic compounds and antioxidant activity.

4.0 CONCLUSION

All ethanol extracts from different treatments exhibit antioxidant activity. The ethanol extracts from cooked black rice had significant low antioxidant activity compared to soak and raw black rice. Longer cooking time (35 minutes) had significantly low antioxidant activity compared to other treatments.

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