

การวิเคราะห์องค์ประกอบแอนโทไซยานินในรำข้าวสี

Analysis of Anthocyanin Composition in Pigmented Rice Brans

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บทคัดย่อ

ข้าวสีมีเมล็ดเป็นสีแดง ม่วง หรือน้ำเงินของสารสีแอนโทไซยานินในรำข้าว แอนโทไซยานินจากข้าวสายพันธุ์ต่าง ๆ ที่มีสีดำ น้ำเงิน และม่วงและข้าวขาวได้มีการตรวจวิเคราะห์โดยสเปกโตรโฟโตมิเตอร์และโครมาโตกราฟีสมรรถนะสูงและนำมาตรวจวัดปริมาณเพื่อประเมินศักยภาพในการเป็นสีหรืออาหารเสริม นำรำข้าวมาละลายในเมทานอลและน้ำ (ในอัตราส่วน 70 ต่อ 30 ปริมาตรต่อปริมาตร) ที่มีกรดไฮโดรคลอริกความเข้มข้น 0.1 เปอร์เซ็นต์เป็นเวลา 24 ชั่วโมง นำสารละลายสกัดเมทานอลและน้ำมากรองและละลายภายใต้สุญญากาศโดยเครื่องระเหยสุญญากาศ พบว่ามีสารสีแอนโทไซยานินในข้าวทุกสายพันธุ์ที่ทำการศึกษาได้แก่ข้าวสายพันธุ์หอมนิล ข้าวไรซ์เบอร์รี่ ข้าวดำสุโขทัย ข้าวหอมนิลจักรพรรดิ และข้าวหอมแม่พญาทองคำ แอนโทไซยานินทั้งหมดมีปริมาณอยู่ในช่วง 2-3.419 มิลลิกรัมต่อกรัมน้ำหนักแห้ง ข้าวบางสายพันธุ์มีแอนโทไซยานินหลายชนิด โดยใช้แอนโทไซยานินมาตรฐาน 5 ชนิด พบว่ามี 3 ชนิดมีคุณสมบัติทางสเปกโตรสโกปีและโครมาโตกราฟี แอนโทไซยานินที่พบมากที่สุดได้แก่ cyanidin-3-glucoside ในข้าวสีดำ, pelargonidin-3-glucoside ในข้าวสีน้ำเงิน และ delphinidin-3-glucoside ในข้าวสีม่วง รำข้าวสีดำมี cyanidin-3-glucoside ที่มีคุณสมบัติต้านอนุมูลอิสระสูงสุด การศึกษานี้แสดงให้เห็นว่าโครงสร้างแอนโทไซยานินมีความสัมพันธ์กับกิจกรรมของแอนโทไซยานินทั้งด้านการใช้เป็นสารให้สีอาหารและคุณค่าทางโภชนาการ

คำสำคัญ: แอนโทไซยานิน ข้าวสี รำข้าว

Abstract

Colored rices are hulled grains containing red, purple or blue pigments of anthocyanins in bran. Anthocyanin pigments from a wide variety of black, blue, and purple rice and white rice were identified by spectrophotometric and HPLC methods and they were quantified to evaluate their potential as natural colorants or functional food ingredients. Rice bran was soaked in methanol:water

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(70:30, v/v) containing 0.1% hydrochloric acid for 24 hours. The methanol:water extract was prefiltered and then evaporated using a vacuum rotary evaporator. The following pigmented cultivars had the anthocyanins in all tests Hom-nin rice, Riceberry rice, Homdum-sukothai rice, Homnin-jakkapat rice and Hommae-payathongdum. The total anthocyanin contents varied significantly and exhibited a range of 2-3.419 mg/g d.w. Some grains had complex anthocyanin profiles. Of the 5 anthocyanin compounds used as standards, 3 were characterized by comparison of the spectroscopic and chromatographic properties with those of authentic standards. The most abundant anthocyanins were cyanidin-3-glucoside in black rice, pelargonidin-3-glucoside in blue rice, and delphinidin-3-glucoside in purple rice. The black rice bran which contained cyanidin-3-glucoside had the highest antioxidative activities. These findings support the relevance of structure related activities of anthocyanins both for understanding food colour and their particular nutritional value.

Keywords: anthocyanin, pigmented rice, rice bran

Introduction

Rice is the principle cereal food and the staple food of the world's population.¹ Colored rice is a hulled grain containing red or purple color in addition to light gray on its bran, especially black rice has long been consumed and considered to be a healthy food. It is reported that colored rices contain a lot of anthocyanin pigments.² Anthocyanins are belong to water-soluble plant pigments and representatives of flavonoids which are responsible for the blue, purple and red color of many plant tissues. The colors range from red to purple or blue, depending on pH. They occur primarily as glycosides of their respective molecule, the position of this attachment, aglycone anthocyanidin chromophores. The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached and number of aliphatic or aromatic acids attached to sugars in the molecule.³ Reactive free radicals have been

postulated to contribute to the causes of chronic inflammatory proliferative diseases (CIPD), especially arteriosclerosis and cancer, through oxidative damage of essential enzymes, cells, and tissues.⁴ Aerobic respiratory organisms use oxygen to produce energy for living, but reactive oxygen species (ROSs) are generated during the oxidative metabolisms. ROSs are atoms with, at least, an unpaired electron in the most outside shell; they are highly reactive. ROSs are such as superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide. ROSs are often associated with damage to cellular functions and the kind of damage depends on nature of ROSs; furthermore damages induced by oxidative stress have cumulative effects connected with various diseases. There is therefore widespread interest in defining the possible role of the diet in preventing and reversing reactive oxygen species (ROS)-induced chronic diseases.⁵

Anthocyanins may reduce the risks of cardiovascular diseases and cancer with antiinflammatory, antioxidant and chemoprotective properties and they were found to inhibit low density lipoprotein oxidation *in vitro*. Delphinidin could inhibit cell invasion of human fibrosarcoma HT-1080 cell *in vitro*.⁶ Studies with pelargonidin 3-glucoside has suggested that the compound is associated with a health benefits, including decreased risk of cardiovascular disease,⁷ reduced risk of cancer,⁸ reversal of age-related neurodegenerative declines,⁹ improved gluco-regulation,¹⁰ and protection of brain tissue from hypoxia.¹¹ Cyanidin-3-O- β -D-glucoside has antioxidant, anti-inflammatory, and ironchelating properties that have been demonstrated in numerous studies using several methods, both *in vivo* and *in vitro*.^{12,13} it might be very useful both in prevention and therapy of postischemic reperfusion brain damage.¹⁴ Delphinidin-3-glucoside, one of the predominantly bioactive compounds of anthocyanin preparations, is a natural colorant found in fruits and flowers¹⁵ has been shown that it has beneficial effects on human low-density lipoprotein cholesterol oxidation.¹⁶ Delphinidin-3-glucoside directly inhibited platelet aggregation and it has roles in the inhibition of inflammatory cytokines¹⁷ and elevation of high-density lipoprotein.¹⁸ Malvidin contributes to the antioxidant and it has a potential preventive role against chronic inflammatory diseases such as inflammation-mediated chronic maladies such as obesity, diabetes, hypertension and cardiovascular disease.¹⁹⁻²¹ Peonidin is a powerful

anthocyanin and phytonutrient which may act as an antioxidant and anti-inflammatory. Research has shown that peonidin is a powerful antioxidant which damages free radicals which are harmful by-products that are released during oxygen related reactions and have been linked with , damaging your body's cells, an increased risk of developing cancer, an increased risk of developing diabetes and increased signs of aging. It is preferable as nutritional supplements and possible therapeutic agents against cancer and cardiovascular diseases.²² The present study aimed to characterize anthocyanin composition in a diverse colored rice brans to identify anthocyanin-rich grains and their antioxidative potential for the development of functional foods and/or functional food colorants.

Materials and Methods

Cereal grains

A diverse array of black, blue, purple, red, and white rice were used in the present study. These included black rice (cvs. Homnin, Homdum-sukothai, Homnin-jakkapat, Hommae-payathongdum), blue rice (cv. Riceberry), purple rice (cv. Khawklum), red rice (cv. Homdang-sukothai) and white rice (cv. Saohai). Rice samples were purchased or obtained from private grain producers and/or the retail market.

Anthocyanin extraction

Anthocyanins in rice brans were extracted according to the method described by Abdel-Aal and Hucl⁷ with slight modifications. Three grams of the ground materials were extracted twice by mixing with 24 mL of methanol

acidified with 1.0 N HCl (70:30, v/v) and shaken on rotatory shaker at 200 rpm for 24 h. The apparent pH of the mixture was adjusted to 1.0 before shaking and was checked and readjusted if necessary after 15 and 30 min of shaking. The crude extracts were centrifuged at 10,000 g and 4°C for 20 min and then refrigerated for 2 days to precipitate large molecules. The extracts were recentrifuged at 10,000 g and 4°C for 20 min.

Quantification of total anthocyanin

Total anthocyanin content determination was based on a pH-differential method and expressed as delphinidin-3-glucoside equivalents⁸ according to the following formula: $c \text{ [mg/L]} = A \cdot M \cdot DF / \epsilon M \cdot d$, with A = absorption value, M = molecular weight of delphinidin-3-glucoside (465 g/mol), DF = dilution factor, ϵM = molar extinction coefficient of delphinidin-3-glucoside at pH 1 (29,000 L/mol·cm), and d = path length of the cuvette (1 cm).

DPPH (1-1 Diphenyl-2-picryl hydrazyl) assay

The free radical scavenging activity of different fractions was measured by the DPPH scavenging method proposed by Shimada, et al.⁹ DPPH concentration of 2.5×10^{-4} M in methanol was prepared and 2.0 ml of this solution were added to 2.0 ml of different rice extracts obtained in different storage conditions. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. The DPPH radical-scavenging activity was calculated according to the following: % of DPPH

scavenging activity = $\{1 - (AbS/AbC)\} \times 100$, where AbC was the absorbance of the control and AbS was the absorbance in the presence of the test compound. EC_{50} is the effective concentration in mg extract/ml which inhibits the DPPH activity by 50%. BHT was used for comparison.

Analysis of anthocyanins

Anthocyanins in the extracts were separated and quantified with an 1100 series chromatograph (Agilent, Mississauga, ON, Canada) equipped with a G1311A quaternary pump, G1329A temperature controlled injector, G1316A temperature-controlled column thermostat, G1322A degasser, G1315B UV-visible detector. A 150 × 2.00 mm, 5 μm particle size Phenomenex Luna C18 (2) rapid resolution column was employed for separation. Separation of anthocyanins was conducted at 60°C, to improve separation efficiency, particularly for those grains exhibiting complex anthocyanin composition. The column was eluted with a gradient mobile phase consisting of (A) 6% formic acid and (B) absolute methanol at 1 mL/min. The gradient was programmed as follows: 0-7 min, 82-80% A; 7-10 min, 80-75% A; 10-25 min, 75-40% A; 25-26 min, 40-82% A; and 26-28 min, hold at 82% A. The separated anthocyanins were detected and measured at 520 nm. Five selected pure anthocyanin compounds including cyanidin 3-glucoside, delphinidin 3-glucoside, malvidin, peonidin, pelargonidin 3-glucoside were purchased from Polyphenols Laboratories (Sandens, Norway). All experiments were carried out in triplicate.

Results

UV-vis data provide some means of confirming some of the aglycones. Table 1 shows that UV-vis maxima for delphinidin-3-glucoside at 524-529 nm, for cyanidin-3-glucoside at 514-517 nm, pelargonidin-3-glucoside observed at 500-504 nm, for peonidin at 522-526 nm, for malvidin at 544-549 nm.

Table 1 Maximum absorption wavelength of anthocyanins in pH 1 buffer

Anthocyanin	λ_{\max}
Delphinidin-3-glucoside	527
Cyanidin-3-glucoside	516
Pelargonidin-3-glucoside	502
Peonidin	524
Malvidin	547

Black rice brans had a wide range of total anthocyanins depending upon cultivar.¹⁰ In the present study, black rice brans, with an average of 2,400 $\mu\text{g/g}$ of rice bran, was found to possess the highest TAC among all of the studied colored rice bran, which is 24 times higher than that of red rice bran (102 $\mu\text{g/g}$) (Table 2). On the other hand, white rice bran had a very small concentration of TAC (12 $\mu\text{g/g}$), which may belong to one or more other groups of pigments because no anthocyanin peaks were detected by HPLC analyses in the white rice bran extract (Figure 1). The results on free radical quenching capability of rice bran antioxidants obtained by solvent extractions are presented in Table 2. Total antioxidant activity of the rice bran extracts increased with the increasing concentration of

the extracts and a significant was determined for of the extract (Table 2). However, the total antioxidant activities of all the rice bran extracts were less than that of the positive control BHT (EC_{50} of 27.30 mg/L).

Anthocyanins were extracted from pigmented rice brans. The anthocyanin was obtained from 70:30 (v/v) methanol:water containing 1% HCl fraction. Total anthocyanin contents in the pigmented rice brans and non-pigmented rice bran were 102-3,594 $\mu\text{g/g}$ and 12 $\mu\text{g/g}$, respectively (Table 2). Also, anthocyanin extract was analyzed with HPLC method using gradient system was also analyzed. There were three peaks of cyanidin-3-glucoside, pelargonidin-3-glucoside and peonidin-3-glucoside in HPLC chromatogram of the anthocyanin extracts. Cyanidin-3-glucoside was found in all pigmented rice brans in this study; and it was the major anthocyanin in black and red rice bran. In addition, delphinidin-3-glucoside and pelargonidin-3-glucoside were also found in black rice brans. Delphinidin-3-glucoside was the major anthocyanin found in purple rice bran while pelargonidin-3-glucoside was the major anthocyanin in blue rice bran. However, it was also found in small amount in red rice bran.

The colored rices exhibited diverse anthocyanin compositions. From Table 3, cyanidin-3-glucoside was the most abundant anthocyanin in black rice and red rice, accounting for 81-95% and 77% of the total anthocyanins, respectively.

Table 2 Total Anthocyanin contents (TAC) determined by pH-differential method and antioxidant capacity of pigmented rice brans

Rice cultivar	Pigmented rice	TAC ($\mu\text{g/g}$)	DPPH (EC_{50}) ^a (mg/L)
Hom-nin	Black rice	1,937	72.62
Homdum-sukothai	Black rice	2,025	92.17
Homnin-jakkapat	Black rice	3,594	106.44
Hommae-payathongdum	Black rice	2,042	61.32
Riceberry	Blue rice	1,719	152.46
Khawklum	Purple rice	1,261	94.40
Homdang-sukothai	Red rice	102	177.94
Saohai	White rice	12	673.69
BHT ^b		0	27.30

^a EC_{50} value, the effective concentration at which the antioxidant activity was 50%; the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was scavenged by 50%. EC_{50} value was obtained by interpolation from linear regression analysis.

^bTreatment with the synthetic antioxidant butylated hydroxytoluene, BHT.

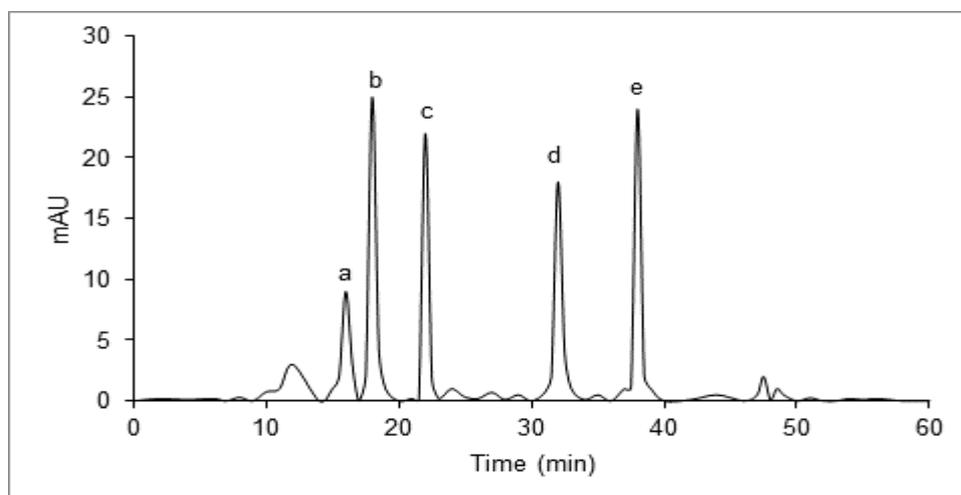


Figure 1 HPLC profiles of acidified methanol (1% HCl) anthocyanin on a C18 column. Detection was at 520 nm: (a) delphinidin-3-glucoside; (b) cyanidin-3-glucoside; (c) pelargonidin-3-glucoside; (d) peonidin; (e) malvidin

Table 3 Average concentration of anthocyanins (micrograms per gram) in black, blue, purple and red rice brans

Rice cultivar	Pigment of rice	Anthocyanin ^a			
		Dp-3-Glu	Cy-3-Glu	Pg-3-Glu	Total
Hom-nin	Black	43 ± 2.7	1,170 ± 14.6	220 ± 6.2	1,433 ± 22.4
Homdum-sukothai	Black	36 ± 6.4	1,490 ± 23.3	46 ± 7.3	1,572 ± 16.2
Homnin-jakkapat	Black	170 ± 4.4	2,710 ± 12.8	239 ± 8.6	3,119 ± 15.8
Hommae-payathongdum	Black	75 ± 8.9	1,760 ± 15.3	227 ± 6.9	1,835 ± 23.6
Riceberry	Blue		236 ± 6.3	1,242 ± 14.7	1,478 ± 16.3
Khawklum	Purple	862 ± 12.7	124 ± 8.2		986 ± 9.2
Homdang-sukothai	Red		47 ± 5.8	16 ± 1.4	63 ± 5.7
Saohai	White				nd ^b

Each value is expressed as mean ± SE.

^aCy, cyanidin; Dp, delphinidin; Pg, pelargonidin; Glu, glucoside.

^bNot detected.

Pelargonidin-3-glucoside came second in black and red rice, whereas delphinidin-3-glucoside was the third major anthocyanin in black rice brans. Ryu *et al.* (1998)¹⁰ found two main anthocyanins in 10 black rice varieties in which Cyanidin-3-glucoside is the most common (0.0-470 mg/100 g), whereas peonidin-3-glucoside (0.0-40 mg/100 g) is the second. The anthocyanin composition of blue rice bran differed from that of black rice brans. This study identified the main anthocyanin in blue rice bran as pelargonidin-3-glucoside, being 84% of the total anthocyanins (Table 3). Cyanidin-3-glucoside was the second dominant anthocyanin at 16% of the total anthocyanins. In purple rice bran, 2 anthocyanin compounds were observed with delphinidin-3-glucoside and cyanidin-3-glucoside (Table 3). Delphinidin-3-glucoside was the predominant anthocyanin in purple rice bran. Anthocyanin was not detected in white rice bran. This indicates that

no other pigment contributed to the color of white rice grain.

Discussion

The TAC results indicated that colored rice bran such as black, blue and purple rice bran may hold promise for the development of functional foods and/or natural colorants. The availability and agronomic performance of these rice brans will determine their potential market. Blue rice bran had an average TAC of 1,719 µg/g (Table 2), which is higher than that red rice bran (102 µg/g). Additionally, anthocyanin concentrations were significantly influenced by growing conditions and environment in pigmented rice due to the pigment location in the outer pericarp or fruit coat.⁷ Thus, anthocyanins in pigmented rices are more prone to environmental effects. Purple rice bran used in the present study contained lower TAC compared to blue rice bran (Table 2). Red and white rice bran

exhibited small concentrations of TAC. These data were in agreement with previous results.⁶ The HPLC analysis of white rice bran extracts showed an absence of anthocyanin compounds, which indicated that the small amount of TAC might be contributed by one or more other groups of pigment. Fractionation of rice kernels into bran by abrasive or roller milling was able to concentrate anthocyanin pigments in the bran fractions. In black, brown, and red the bran fraction contained 3-4 times higher anthocyanin content than the whole grains. The addition of time of abrasion might increase the pigment recovery in the combined bran fractions obtained from pigmented rices.¹¹

In general, the colored grains studied showed substantial differences in their TAC, and some of them, such as black rice, purple corn, and blue wheat, had remarkable levels of anthocyanins.¹²

When the relationship between the total anthocyanin content determined by colorimetry (Table 2) versus that determined by HPLC (Tables 3) was examined, a significant positive correlation was obtained with a correlation coefficient (r) of 0.987 and a slope of 1.1889. This shows that the colorimetric method overestimated the HPLC values by 19%. The difference may be due to the contribution of other pigments present in the grains that have an absorbance at 535 nm. This overestimation was consistent from one grain to another. Anthocyanins have been recognized as health-enhancing substances and have been found in many

types of grains. The present study showed a diversity of anthocyanins in a selection of black, blue, pink, purple, and red rice brans. It also shows substantial differences in anthocyanin content and composition among the rice brans studied. Some of the rice brans exhibited a three pigments such as black rice brans; others had two pigments such as blue, purple and red rice brans. Such diversity in anthocyanin composition would help in the selection process for the development of anthocyanin-rich grain products. In addition, the anthocyanin pigments in rice brans can be concentrated by dry milling and fractionation processes to produce fractions that are high in anthocyanin contents, even much higher than those found in fruits and vegetables. The data suggest that some of the colored rice brans such as black rice brans, may hold promise for the development of grain-based functional foods or natural colorants on the basis of their anthocyanin content and composition.

The main anthocyanin pigments of black rice have been reported to be Cy-3-Glu, Pn-3-Glu and petunidin-3-glucoside (Pt-3-Glu).¹² In this study, it was found that black rice (Homnin-jakkapat) contained higher TAC than the others, especially Cy-3-Glu which had strong antioxidant activities *in vitro* system. From the results, it could be concluded that Homnin-jakkapat contained high amount of anthocyanin contents on its bran and anthocyanins could be isolated from the Homnin-jakkapat by chromatography successively. In red rice, it was not so clear: Abdel-Aal et al.¹³ reported that Cy-3-Glu was

the main anthocyanin, whereas Kim et al. for black, blue, pink, purple and red cereal grain.¹⁴ concluded that red rice did not contain anthocyanin pigments. However, in this study, Homdang-sukothai rice bran was found that it contained Pg-3-Glu and Cy-3-Glu that corresponded to the previous report of Abdel-Aal et al.¹³ Anthocyanins are the most prominent pigments in rice bran and they are strong antioxidants. Their double bond conjugate systems allow electron delocalization, resulting in very stable structures and a powerful antioxidant activity. Furthermore, the extent and position of hydroxylation and methoxylation in the B ring modulates their stability and reactivity. Differences in antioxidant activities between various anthocyanins have been noted in several studies. Although Homnin-jakkapat contains higher TAC than the others, however; Hommae-payathongdum has highest antioxidant capacity. The increase in anthocyanin content in rice bran was not always associated with a similar proportional increase in antioxidant capacity. It has been reported that pH differences have a major influence on scavenging capacity of anthocyanins and that the presence of acid in the solvent has a negative influence on the antioxidant capacity of samples.

ROSs plays a crucial role in a wide range of common diseases and age-related degenerative conditions including cardiovascular disease, inflammatory conditions, and neurodegenerative diseases

such as Alzheimer's disease, mutations and cancer.¹ So antioxidant capacity is widely used as a parameter to characterize food or medicinal plants and their bioactive components. In this study, the antioxidant activity of the anthocyanin extract was evaluated and it showed very strong antioxidant activity. Thus, these results suggest that anthocyanin extract from black rice can be used as antioxidant material, food additives. This suggests that use of anthocyanin extract from black rice may offer an attractive new antioxidant agent against ROS. In Table 3, the anthocyanins for which structures are noted as confirmed on the basis of congruence of properties with authentic standards are completely named because glucose has been reported as the most common hexose in grain anthocyanins.¹⁵

It is known that the anthocyanin biosynthesis pathway is controlled in response to different developmental and environmental cues. The colour of rice results from the accumulation of the corresponding pigments, the orange to red pelargonidin, the red to magenta cyanidin and the violet to blue delphinidin. Each variety of pigment rice has a unique set of anthocyanins.¹⁶ In the present work, the TAC was calculated value and identified six anthocyanins and in contrast to pigment rice brans considering the contribution of individual anthocyanins to the TAC on the basis of their concentration and antioxidant capacity.

Conclusions

This study suggests that medicinal rice plants can be promising sources of potential black rice bran antioxidants and anticancer activity. The present results will form the basis for selection of black rice species for further investigation in the potential drug discovery of new natural bioactive compounds. Homnin-jakkapat and Hommae-payathongdum are good choices for the plant scientists to develop new rice cultivars with high bioactive compounds with high nutritive value.

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