



Comparative analysis of bioactive-phytochemical characteristics, antioxidants activities, and anti-inflammatory properties of selected black rice germ and bran (*Oryza sativa* L.) varieties

Sariya Mapoung^{1,2} · Warathit Semmarath^{1,2,5} · Punnida Arjsri^{1,2} · Pilaiporn Thippraphan^{1,2} · Kamonwan Srisawad^{1,3} · Sonthaya Umsumarn^{2,4} · Kanokkarn Phromnoi⁶ · Sansanee Jamjod⁷ · Chanakan Prom-u-Thai⁷ · Pornngarm Dejkriengkraikul^{1,2,3,7}

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Abstract

Black rice has numerous health benefits and one of the well-known functional foods throughout the world. To encourage the increasing trend of the consumer interest in health-promoting functional foods, special varieties of rice have been developed offering greater nutrient values and exhibiting biological activities that are beneficial to the consumer. In this study, we aimed to evaluate the associations of the phytochemical contents, antioxidants, and anti-inflammatory properties among eight selected black rice germ and bran extracts (BR extracts) from 4 non-glutinous and 4 glutinous rice varieties. Accordingly, glutinous BR extracts possessed higher degree of Cyanidin-3-O-glucoside (C3G), Peonidin-3-O-glucoside (P3G) contents, antioxidant and anti-inflammatory properties than the non-glutinous BR extracts. Pearson's correlation indicated that the amount of C3G in the BR extracts had a strong positive association with the antioxidant properties (DPPH; $r=0.846$, ABTS; $r=0.923$, and FRAP; $r=0.958$, $p<0.01$). While P3G exhibited a strong positive association with the anti-inflammatory properties (r value = 0.717 and 0.797 for IL-6 and TNF- α inhibition, respectively, $p<0.05$). Lastly, the principal component analysis (PCA) categorized the black rice varieties into three groups: Group A with high C3G content and superior antioxidant properties, Groups B with a high amount of P3G and potent anti-inflammatory properties, and Group C with a lower amount of phytochemical contents and less potent bioactivities. Overall, the outcomes of this study could provide vital information to food industries in selecting the variety of black rice for the functional food based on the anthocyanin contents that could benefit to consumers for new normal healthy lifestyle.

Keywords Black rice · Anthocyanins · Antioxidants · Anti-inflammatory · Cyanidin-3-O-glucoside

✉ Pornngarm Dejkriengkraikul
pornngarm.d@cmu.ac.th

¹ Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

² Center for Research and Development of Natural Products for Health, Chiang Mai University, Chiang Mai, Thailand

³ Anticarcinogenesis and Apoptosis Research Cluster, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

⁴ Division of Veterinary Preclinical Sciences, Department of Veterinary Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand

⁵ Akkratchkumari Veterinary College, Walailak University, Nakhon Si Thammarat 80160, Thailand

⁶ Division of Biochemistry, School of Medical Sciences, University of Phayao, Phayao, Thailand

⁷ Lanna Rice Research Center, Chiang Mai University, Chiang Mai, Thailand

Introduction

Rice (*Oryza sativa* L.) is an important staple crops that provides nutritional substance for half of the world's population [1, 2]. Pigmented rice has been reported to contain a higher amount of phytochemical compounds than white rice [2]. Black rice is a special rice cultivar that is mainly cultivated in Asian countries such as China, Sri Lanka, Indonesia, India, the Philippines, Bangladesh, Malaysia, Thailand and Myanmar [3]. Black rice has been recognized for the rich natural anthocyanin compounds that accumulate in both the rice germ and bran.

Rice bran (contained bran layer and rice germ) was removed from the whole grain by using rice mulling process [4, 5]. Previously studies reported that, rice germ and bran are good source of proteins, fatty acid, minerals, dietary fiber and phytochemical compounds which play an important role in biological activities such as anti-inflammation and antioxidants [6]. Black rice is known to have a higher protein content than white rice, particularly in terms of the essential amino acids, crude fiber, and health-promoting phytochemicals such as phenolics, flavonoids, and vitamin E [7, 8]. Moreover, black rice contains greater amount of essential micronutrients such as Zn, Mg, Ca, K, N, Fe, Cu, and Mn than with white rice [2]. Researchers worldwide had reported the health promotion effects of black rice such as improvement of learning and memory in cerebral-ischaemic mice [9], prevention of non-communicable diseases such as cardiovascular diseases, diabetics and inflammatory bowel diseases by its antioxidants and anti-inflammation properties [10–12]. Therefore, Black rice has gained increasing amount of attention for its higher nutrient values and wide-ranging of health benefits.

Cyanidin-3-glucoside (C3G) is the major anthocyanin found in black rice followed by peonidin-3-glucoside (P3G) [13]. Anthocyanins has been reported for a range of health benefits such as antioxidant, anti-inflammatory [14], and anti-diabetic capabilities [15], as well as the ability to inhibit cancer progression in certain cancer types in both in vitro and in vivo experiments [16, 17]. Moreover, the black rice germ and bran contain phenolics, flavonoids, tocopherols and γ -oryzanol that have been identified for their health-promoting benefits such as antioxidants and anti-inflammatory properties [18].

Balanced levels of free radicals, reactive oxygen species (ROS), and/or reactive nitrogen species (RNS) are required for the human body and its organs. Oxidative stress and systemic chronic inflammation can lead to several diseases that have been associated with an increase in mortality rates worldwide [19]. These include cardiovascular disease, cancer, diabetes mellitus, chronic kidney disease, and non-alcoholic fatty liver disease, as well as

autoimmune and neurodegenerative disorders [20, 21]. Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, could induce an inflammatory induction via increasing pro-inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factors- α (TNF- α) secretions from macrophages [14]. Moreover, the oxidative stress environment is recognized as a key element of the pathogenesis of a wide range of chronic inflammation conditions [22]. Therefore, a balance of oxidative stress levels and inflammation conditions is essential for human physiological functions. The health-promoting benefits of black rice include its antioxidant and anti-inflammatory properties. A study involving healthy volunteers aged 65–74 years group found that the inflammatory marker, IL-6, and C-reactive protein levels were decreased after the consumption of black rice germ and bran drinking powder for 24 weeks when compared to baseline data [23]. The antioxidant and inflammation markers in coronary heart disease patients aged 45–75 years were decreased after received black rice supplement from 6 months when compared to baseline data [12].

At present, black rice has begun to attract greater amount of attention in Thailand and in international markets [13]. Black rice is consumed in daily meals by a significant proportion of the world's population. It is now being consumed as a different functional food products such as anthocyanin-rich black rice bread, anthocyanin-rich rice beverages, fresh germinated purple rice noodles, and anthocyanin-rich black rice extracts supplements [24, 25]. The nutrient values of food and/or these supplements is a key factor in consumer decision-making. To meet the growing consumer demand for black rice, special varieties of rice have been developed that utilize novel rice varieties containing more nutrient values and could offering a broader range of biological activity. In this study, we aimed to evaluate the associations between the phytochemical contents of eight selected Thai black rice varieties and their antioxidants and anti-inflammatory properties. Accordingly, eight Thai black rice varieties, four non-glutinous rice varieties (BKU 5, K4, K2, and CMU 107) and four glutinous rice varieties (KAK 1, PES 1, SNG 5, and KDK) were collected from the same agricultural fields located in northern, Thailand.

Materials and methods

Rice cultures

The eight rice varieties, Kum Chao CMU 107 (CMU 107), Sang 5 CMU (SNG 5), Pieisu 1 CMU (PES 1), Kum Akha 1 CMU (KAK 1), Bien Koo 5 CMU (BKU 5), K2 CMU (K2), K4 CMU (K 4), and Kum Doi Saket (KDK) with purple pericarp color (Fig. 1A), were provided by Lanna Rice

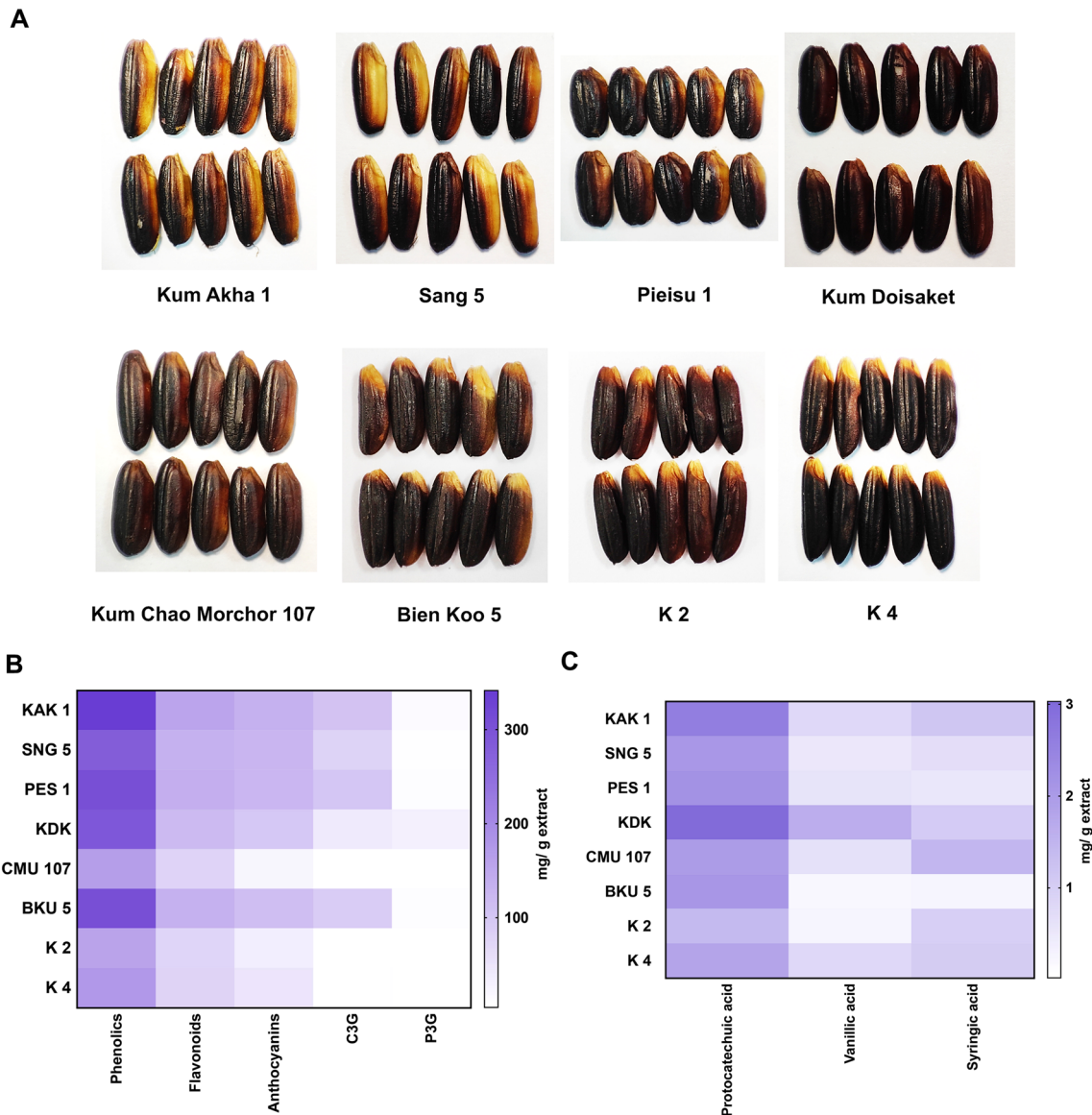


Fig. 1 The characteristic of black rice grain and their phytochemical contents. The characteristics of rice grain (unpolished) of the eight selected black rice varieties in this study (**A**). The heat map of the

chemical composition of BR extracts (**B, C**). The data are demonstrated in mean \pm S.D. from three independent experiments

Research Center, Chiang Mai University. The rice varieties were grown in the same paddy field during the wet season (from June to November of 2020) on the sandy loam textured soil of the Sansai series with pH = 5.8 and 5.5 (1:1, soil:water), respectively. The average temperatures during the cropping seasons in 2020 were 27.5 °C, with 80.2% relative humidity. The average sunshine duration was 5.2 h, while the average precipitation recorded during the grain filling was 4.0 mm (Northern Meteorological Center, 2018). Seedlings (14 days old) were planted using a mechanical transplanting machine, with spacing between hills of 20 × 25 cm, in 20 × 40 m² plots for each variety. All plants received basal fertilizer to the soil similarly with

a total of 85 kg NH₂CONH₂ ha⁻¹, 35 kg P₂O₅ ha⁻¹, and 15 kg K₂O ha⁻¹ which were four splits equally at 7 days after planting, tillering, booting, and flowering stages [26]. The fields were permanently flooded under 0.1–0.2 m of water until maturity. The fungicide isoprothiolane and insecticide fipronil were applied at recommended rates to control pests. Weeds were manually removed. The samples of all rice varieties were harvested at maturity by the combine harvesting machine and the moisture content of the samples was reduced to reach 14%. The samples were processed to prepare unpolished rice samples, the caryopsis, by dehusking paddy rice with a rice testing machine (model P-1, Ngek Seng Huat Company, Thailand).

Chemicals and reagents

RPMI-1640, trypsin and penicillin–streptomycin were supplied by Gibco BRL Company (Waltham, MA, USA). Cyanidin-3-O-glucoside, peonidin-3-O-glucoside, Folin–Ciocalteu phenol reagent, gallic acid, anhydrous sodium carbonate (Na₂CO₃), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Griess reagent, Trichloroacetic acid (TCA) and Sulforhodamine B sodium salt were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Thermo Scientific (Waltham, MA, USA). Trifluoroacetic acid (TFA) was obtained from Panreac Applichem ITW Reagents (Darmstadt, Germany). Butanol (HPLC grade), Ethanol (HPLC grade), Methanol (HPLC grade) and water (HPLC grade) were purchased from RCL Labscan Limited (Bangkok, Thailand).

Sample preparation and extraction

The black rice germ and bran extract (BR extract) were prepared by following the previously reported protocol [27]. Briefly, black rice germ and bran were removed from the whole grains using a rice milling machine (Kinetic (Hubei) Energy Equipment Engineering Co., Ltd., Wuhan, Hubei, China). The whole grains black rice was milled with rice milling machine for 2 cycles and passed through an 80-mesh sieve to obtain black rice germ and bran [10, 28]. The freshly prepared black rice germ and bran (150 g) were then extracted with 50% ethanol by shaking at room temperature overnight and filtered through filter paper to separate the residue. The filtrated samples were evaporated using a rotary vacuum evaporator (Büchi, Flawil, Switzerland) to obtain concentrated ethanolic fractions. The ethanolic fractions were further partitioned with saturated butanol as at ratio 1:2 using a separating funnel. The upper layer part in the butanol fraction was collected to obtain a medium polar fraction, while the residual fraction or lower part was discarded. The medium polar fraction was then evaporated to remove the butanol and it was freeze-dried to obtain the black rice germ and bran extract. The BR extract was kept at -20 °C for further experiments.

Quantification of total phenolic, total flavonoids and total anthocyanin contents

Total phenolic assay

Total phenolic contents of the BR extracts were determined by Folin-Ciocalteu assay with modifications as has been previously described [14]. Briefly, each dilution of BR extracts (0.4 mL) was mixed with the 10% Folin–Ciocalteu reagent

(0.3 mL) and incubated at room temperature for 3 min. Then, the 7.5% sodium carbonate (Na₂CO₃) solution (0.3 mL) was added to the mixture and incubated in the dark at room temperature for 30 min. The absorbance of the blue complex was evaluated at 765 nm using a UV–visible spectrophotometer (Shimadzu, Kyoto, Japan). The standard curve of gallic acid (GA) was then determined, while total phenolic content of BR extract was calculated as milligrams of GA per g extract (mg GA/g extract).

Total flavonoid assay

The total flavonoid content of the BR extracts were evaluated using the modified aluminum chloride (AlCl₃) colorimetric assay, as has been previously described [14]. Briefly, each dilution of the BR extract (0.25 mL) was mixed with the 5% sodium nitrite (NaNO₂) (0.125 mL) and incubated at room temperature for 5 min. Subsequently, 10% AlCl₃ (0.125 mL) was added to the mixture, which was then incubated for 5 min. After that, 1.0 mL of sodium hydroxide (NaOH) was added and incubated in dark at room temperature for 15 min. The absorbance of the mixture was determined at 510 nm using a UV–visible spectrophotometer. The standard graph of catechin (CE) was carried out, the total flavonoid content of BR extract was calculated as milligrams of CE per g extract (mg CE/g extract).

Total anthocyanin assay

Total anthocyanin content of the BR extracts were measured using the pH differential method by following the previously reported protocol [14]. The BR extracts were dissolved in 0.1% HCl in 80% methanol and incubated for 12 h. The BR extracts were then diluted to various concentrations using two different solvents, 0.025 M potassium chloride (KCl) buffer at a pH of 1.0 and 0.45 M sodium acetate (CH₃COONa) buffer at a pH of 4.5. BR extract samples were then incubated at room temperature for 15 min. The absorbance of each sample was measured at 520 and 700 nm using a UV–visible spectrophotometer. Total anthocyanin content in the extract was expressed as cyanidin-3-glucoside, which was the most sufficient anthocyanin component. Total anthocyanin content was calculated using the following formula:

$$\text{Total anthocyanin content} = \frac{A \times M_w \times DF \times 103}{\epsilon \times L}$$

$$A = (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 1.0}} - (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 4.5}}$$

MW = Molecular weight of cyanidin-3-glucoside (449.2 g/mol)

DF = Dilution factor

ϵ = Molar extinction coefficient, L × mol⁻¹ × cm⁻¹

L = Cell path length (1 cm).

Quantification of phenolic acids, flavonoids and anthocyanin by high-performance liquid chromatography (HPLC)

Quantification of phenolic acids by HPLC

The phenolic compounds present in eight BR extracts were analyzed on an Agilent Infinity 1260 HPLC system equipped with a reversed-phase C18 column (Zorbax Eclipse Plus C18, 250 mm × 4.6 mm, 5 μm) and a pre-column (Zorbax Eclipse Plus-C18, 12.5 mm × 4.6 mm, 5 μm). The mobile phases were 0.1% TFA (mobile phase A) and methanol (mobile phase B). The gradient elution was used as follows: 0–49 min, from 100 to 0% mobile phase A, 50–100 min, from 0 to 100% mobile phase A. The flow was set as 1 mL/min. The injection volume was at 10 μL and the measurements were carried out at the wavelength of 260 nm.

Quantification of anthocyanin by HPLC

The anthocyanin, C3G and P3G contents of all the BR extracts were measured as following HPLC conditions. The mixture of 0.4% TFA in water and 0.45% TFA in acetonitrile (82:18, v/v) was used as the mobile phase in an isocratic elution system. The flow was set as 1 mL/min for 30 min. The injection volume was at 20 μL and the measurements were carried out at the wavelength of 530 nm [27].

Determination of antioxidant actives

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of the BR extracts was measured according to the previously described method [29]. Briefly, the BR extracts were diluted to various concentrations in methanol. The various concentration of BR extracts (20 μL) was added to DPPH methanolic solution (180 μL) and incubated in dark at room temperature for 30 min. The absorbance was measured at 540 nm. The DPPH scavenging activity was calculated using following formula:

$$\text{DPPH scavenging activity} = \frac{(A_{\text{Control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

Radical cation ABTS⁺ scavenging activity

The ABTS⁺ scavenging activity of the BR extracts were determined using the ABTS radical cation method with slight modifications, as has been previously reported [29].

The ABTS reagent was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (K₂S₂O₈) (1/1, v/v) and incubated in the dark for 12–16 h to allow free radical generation. The ABTS⁺ solution (990 μL) was then mixed with various concentrations of BR extract (10 μL) and incubated at room temperature for 6 min. The absorbance was measured immediately at 734 nm. The ABTS⁺ scavenging activity was then calculated using the following formula:

$$\text{ABTS scavenging activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 10$$

Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was performed as has been previously described [29]. Various concentrations of the BR extracts (40 μL) were mixed with the FRAP reagent (3 mL) and incubated at 37 °C for 4 min. Absorbance was measured immediately at 593 nm using a UV–visible spectrophotometer. The standard solutions consisted of FeSO₄·7H₂O at different concentrations. The positive control was ascorbic acid. Results were expressed as milligrams of Fe (II) per 1 g of fraction (mg FeE/g fraction).

Cells cultures

The mouse macrophage cell line, RAW264.7, was obtained from the American Type Culture Collection (ATCC). A low cell-binding dish (Thermo Scientific, USA) was used to culture RAW264.7 cells. The cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin. Cells were then maintained in a 5% CO₂ humidified incubator at 37 °C.

The Human monocytes cell line, THP-1 cells (TIB-202TM) were purchased from the ATCC. The cells were grown in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/mL streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. Before an experiment the THP-1 monocytes were activated by phorbol 12-myristate 13-acetate (PMA) for 24 h to differentiate to THP-1 macrophages. After 24 h incubation, the culture medium was changed to PMA-free RPMI for the resting stage for 24 h in a 5% CO₂ humidified incubator at 37 °C before the experiments.

Cytotoxicity assay

The effects of BR extracts on the macrophage, RAW264.7 or THP-1 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [14]. RAW264.7 cells (1.5 × 10³ cells/well) or THP-1 cells (3.0 × 10³ cells/well) were plated in

96-well plate incubated in a 5% CO₂ humidified incubator at 37 °C overnight. The cells were then treated with an increasing concentrations of BR extract for 24 h. The MTT dye solution (15 µL) was added to each well and incubated for a further 4 h. After incubation, the culture medium was removed, and formazan crystals were dissolved with DMSO (200 µL). The absorbance for the MTT assay was measured at 510 nm using a microplate reader. Cell viability was calculated compared to the control and interpreted as the % of the control.

Determination of nitric oxide (NO) production

The level of LPS-induced NO production from RAW264.7 cells was determined of the nitrite concentration in the cultured medium using the Griess reagent system as has been previously reported with slightly modifications [14]. Briefly, RAW264.7 cells (3.5×10^5 cells/well) were plated into 24-well plates and incubated in a 5% CO₂ humidified incubator at 37 °C overnight. The cells were then treated with or without BR extracts (200 µg/mL) for 2 h. After that, the cells were treated with LPS (1 µg/mL) and incubated for a further 24 h. After incubation, culture supernatant was mixed with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) at a ratio 1:1. The absorbance of the mixture was determined at 510 nm using a microplate reader. The nitrite concentration in the cultured medium was calculated and compared with standard sodium nitrite (NaNO₂).

Determination of IL-6 and TNF-α production

To determine the IL-6 or TNF-α secretion, LPS-induced THP-1 macrophages were determined by ELISA kit (Biolegend, San Diego, CA, USA) following the manufacturer's instructions. Briefly, THP-1 cells (3.5×10^5 cells/well) were treated with or without BR extracts (200 µg/mL) for 2 h. After that, LPS (1 µg/mL) was added and incubated for a further 24 h. After incubation, culture supernatant was kept and measured IL-6 and TNF-α levels using ELISA kit, and the absorbance was measured at 450 and 570 nm using a microplate reader. The IL-6 or TNF-α secretions in the culture medium was calculated and compared with the standard curve.

Statistical analysis

The principal component analysis (PCA) was applied to standardize the results obtained from a total of 14 parameters and to associate the phytochemical contents (eight parameters) in the BR extracts along with their antioxidant properties (three parameters) and anti-inflammatory properties (three parameters). All data are presented as

mean \pm standard deviation (S.D.) values. The statistical analysis was performed with Prism version 9.0 software using an independent *t* test and a one-way ANOVA with Dunnett's test. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 vs. control. The Pearson's coefficient correlation test and principal Component Analysis (PCA) were performed to correlate the phytochemical components with the antioxidative and anti-inflammatory properties. The Pearson's coefficient correlation test (0.5–0.7 = moderated correlation; 0.7–0.9 = high correlation; 0.9–1 = very high correlation) [30, 31] and principal Component Analysis (PCA) were performed to correlate the phytochemical components with the antioxidative and anti-inflammatory properties.

Results

Chemical characterization and phytochemical contents of eight selected BR extracts

The black rice varieties used in this study were comprised of four glutinous varieties and four non-glutinous varieties. Representative samples of the selected black rice varieties are presented in Fig. 1A. In this study, the germ and brans were removed from each variety using a rice milling machine and extracted by solvent-partitioned extraction technique. The yield of germ and bran extracts of the black rice varieties are shown in Table 1. The total phenolic and total flavonoids contents of eight BR extracts were different among black rice varieties. The total phenolic contents in the BR extracts varied from 157.12 (K2) to 341.31 (KAK 1) mg GAE/ g extract as is shown in Table 1 and Fig. 1B. The flavonoid contents in the BR extracts varied from 75.76 (K2) to 155.21 (KAK 1) mg CAE/g extract as is shown in Table 1 and Fig. 1B. It was found that the glutinous rice varieties contained a significantly higher amounts of total phenolic and flavonoid contents than the non-glutinous rice varieties, with the exception of BKU 5 (*p* < 0.05).

The phenolic bioactive compounds in the BR extracts were then investigated using HPLC. Protocatechuic acid, vanillic acid, and syringic acid were found in all eight black rice germ and bran extracts at different concentrations. Protocatechuic acid and vanillic acid were found in the highest amounts in KDK, while syringic acid was found in the highest amount in CMU 107 as is shown in Fig. 1C.

Anthocyanin contents of eight selected black rice germ and bran extracts

Black rice is known to be rich in anthocyanins. Therefore, the total anthocyanin contents of the BR extracts were determined. The total anthocyanin contents in the BR extracts varied from 22.32 (CMU 107) to 132.43 (KAK 1) mg C3G

Table 1 Characterization and phytochemical composition of BR extracts

Rice variety name	Abbreviation	Endosperm type	Yield (%: w/w)	Total phenolic (mg GAE/g extract)	Total flavonoid (mg CAE/g extract)
Kum Akha 1	KAK 1	Glutinous	6.0	341.31 ± 6.88 ^a	155.21 ± 3.53 ^a
Sang 5	SNG 5	Glutinous	4.6	280.56 ± 4.49 ^c	132.39 ± 5.79 ^b
Pieisu 1	PES 1	Glutinous	4.8	306.25 ± 2.05 ^b	138.43 ± 3.62 ^b
Kum Doi Saket	KDK	Glutinous	6.0	290.12 ± 1.23 ^c	119.39 ± 3.53 ^b
Kum Chao Morchor 107	CMU 107	Non-glutinous	6.0	166.19 ± 3.75 ^d	79.76 ± 2.95 ^c
Bien Koo 5	BKU 5	Non-glutinous	6.2	306.34 ± 11.15 ^b	133.35 ± 6.94 ^b
K2	K2	Non-glutinous	5.1	157.12 ± 5.67 ^d	75.76 ± 1.72 ^c
K4	K4	Non-glutinous	4.8	174.42 ± 1.64 ^d	77.33 ± 7.18 ^c

The data are demonstrated in mean ± S.D. from three independent experiments

^{a,b,c,d}Difference letters indicated statistic difference among black rice varieties ($p < 0.05$, One way ANOVA with Tukey's test)

Table 2 Anthocyanin composition of BR extracts

BR extracts	Total anthocyanins (mg/g extract)	C3G (mg/g extract)	P3G (mg/g extract)
KAK 1	132.43 ± 1.69 ^a	106.76 ± 2.94 ^a	14.48 ± 0.40 ^b
SNG 5	126.19 ± 3.36 ^a	80.47 ± 5.49 ^b	11.42 ± 0.69 ^c
PES 1	124.61 ± 1.43 ^a	100.68 ± 2.17 ^a	13.07 ± 0.80 ^b
KDK	98.63 ± 9.86 ^b	40.53 ± 3.12 ^c	32.37 ± 1.10 ^a
CMU 107	22.32 ± 3.82 ^c	4.61 ± 0.10 ^d	6.40 ± 0.22 ^d
BKU 5	114.19 ± 2.04 ^a	89.68 ± 6.60 ^b	13.21 ± 0.72 ^b
K 2	35.49 ± 5.09 ^c	6.84 ± 0.07 ^d	6.67 ± 0.25 ^d
K 4	50.57 ± 3.55 ^c	8.40 ± 0.19 ^d	7.83 ± 0.15 ^d

The data are demonstrated in mean ± S.D. from three independent experiments

^{a,b,c,d}Difference letters indicated statistic difference among black rice varieties ($p < 0.05$, One way ANOVA with Tukey's test)

equivalents/ g extract. Furthermore, the total anthocyanins contents were found to be significantly higher amount in the glutinous rice varieties than in the non-glutinous rice varieties, with the exception of BKU 5 ($p < 0.05$). A previous study determined that C3G and P3G were the major anthocyanins found in black rice [13, 23]. Therefore, the C3G and P3G concentrations in BR extracts were determined using HPLC technique. The results indicated that the concentrations of C3G varied from 4.61 (CMU 107) to 106.76 (KAK 1) mg/g extract, while the P3G concentrations in the BR extracts varied from 6.40 (CMU 107) to 32.37 (KDK) mg/g extract, as is shown in Table 2. The results could summarize that the KAK 1 contained significantly the highest amount of anthocyanins, while C3G was the major active compound as is shown in Fig. 1B. C3G was found in a considerably greater amount than P3G in the KAK 1, SNG 5, PIS 1, and BKU 5 varieties, while C3G and P3G concentrations in the KDK, CMU 107, K2 and K4 varieties were determined to be similar.

Table 3 Antioxidant properties as IC₅₀ and ferric reducing/antioxidant power (FRAP) of BR extracts by different assays using vitamin E as the positive control

BR extracts	Radical scavenging assay IC ₅₀ (µg/mL)		Reducing power assay (mg Fe/g extract)
	DPPH	ABTS	FRAP
KAK 1	25.11 ± 1.98 ^{**a}	7.30 ± 1.39 ^a	633.48 ± 17.28 ^a
SNG 5	34.31 ± 0.79 ^b	10.05 ± 2.64 ^a	591.99 ± 8.64 ^a
PES 1	29.49 ± 1.47 ^a	8.92 ± 2.22 ^a	614.42 ± 1.81 ^a
KDK	35.26 ± 2.31 ^b	10.12 ± 2.13 ^a	509.08 ± 14.58 ^b
CMU 107	77.63 ± 5.14 ^c	15.84 ± 2.78 ^b	280.86 ± 18.51 ^c
BKU 5	42.51 ± 5.86 ^b	8.56 ± 1.15 ^a	544.33 ± 22.84 ^a
K 2	97.37 ± 8.29 ^c	20.29 ± 2.13 ^b	240.90 ± 7.90 ^c
K 4	87.65 ± 2.70 ^c	19.53 ± 1.41 ^b	287.27 ± 7.66 ^c
Vitamin E	32.28 ± 3.04	2.52 ± 0.44	2168 ± 75.24

The data are demonstrated in mean ± S.D. from three independent experiments

^{**} $p < 0.01$ (Independent t test) vs. vitamin E (Positive control)

^{a,b,c,d}Difference letters indicated statistic difference among black rice varieties ($p < 0.05$, One way ANOVA with Tukey's test)

Antioxidant properties of eight selected BR extracts

The human health-promoting potential of black rice is its antioxidant properties exists within [13]. Therefore, the antioxidant properties of BR extracts were investigated through the free-radical-scavenging activities of the DPPH and ABTS radicals. A 50% inhibitory concentration (IC₅₀) of the BR extracts is shown in Table 3. The DPPH assay demonstrated that the IC₅₀ value of vitamin E, as the positive control, was 32.28 ± 3.04 µg/mL. Surprisingly, the IC₅₀ value of KAK 1 was 25.11 ± 1.98 µg/mL, which was significantly lower than that of vitamin E ($p < 0.01$). The SNG 5, PES 1 and KDK varieties exhibited similar levels of antioxidant

activity with vitamin E. The ABTS assay revealed that, the KAK 1, SNG 5, PIS 1, KDK, and BKU 5 varieties exhibited higher levels of antioxidant activity than CMU 107, K2 and K4 ($p < 0.001$), as is shown in Table 3. Lastly, to confirm another antioxidant activity of BR extracts, we employed the FRAP assay, which is a study of electron transfer. The FRAP assay demonstrated that KAK 1, SNG 5, PES 1 and BKU 5 varieties exhibited higher levels of antioxidant activity than the other black rice extracts ($p < 0.001$). Taken together, it can be concluded that, KAK 1 exhibited the strongest antioxidant activity followed by PES 1, SNG 5, KDK, and BKU 5.

Potent anti-inflammatory effects of eight selected BR extracts in LPS- induced macrophages, RAW 264.7 cells, and THP-1 cells

The anti-inflammatory properties of black rice is considered one of its most beneficial health-promoting properties [24]. The eight selected black rice varieties had not previously been investigated for their anti-inflammatory properties. Thus, their potential anti-inflammatory properties were investigated in this study. The selected eight BR extracts at the concentrations up to 200 $\mu\text{g/mL}$ had no cytotoxicity effect on macrophages (RAW264.7 and THP-1 cells) after 24 h incubation (data not shown). Therefore, a concentration of 200 $\mu\text{g/mL}$ of the BR extracts was used in this experiment to investigate the anti-inflammatory property of BR extracts.

The NO production was determined by the Griess reagent system. NO production significantly increased in LPS-induced RAW267.4 cells when compared with the non-LPS group ($p < 0.001$). Remarkably, pretreating the cells with C3G, P3G (20 $\mu\text{g/mL}$) and BR extracts (200 $\mu\text{g/mL}$) significantly decreased the LPS-induced NO production in the macrophages when compared with the control ($p < 0.001$, Fig. 2A). The CMU 107, K2 and K4 treatments inhibited NO production from 100% in the control group to 37.8% (62.2% inhibition), 38.9% (61.1% inhibition), and 41.8% (58.2% inhibition), respectively. The other varieties (KAK 1, SNG 5, PIS 1, KDK and BKU 5) displayed lower inhibitory effect on NO production but significantly different from the control group. Moreover, C3G and P3G treatments decreased NO production from 100% in the control group to 40.3% (59.7% inhibition) and 48.2% (51.8% inhibition), respectively as is shown in Fig. 2A. Conclusively, the CMU 107, K2, and K4 varieties exhibited the most potent NO production inhibitory effects ($p < 0.05$).

Importantly, the pro-inflammatory cytokines, such as IL-6 and TNF- α are secreted from immune cells during the inflammatory cascade. Therefore, the effects of BR extracts on IL-6 and TNF- α secretion from LPS-induced THP-1 cells were then determined. The IL-6 and TNF- α secretions were significantly increased in LPS- induced THP-1 cells when compared to non-LPS cells ($p < 0.001$). The C3G and P3G

at 20 $\mu\text{g/mL}$ significantly decreased IL-6 and TNF- α secretion from LPS-induced THP-1 cells when compared with the control ($p < 0.001$) as is shown in Fig. 2B, C. The BR extracts (200 $\mu\text{g/mL}$) treatments dramatically decreased IL-6 and TNF- α secretions in LPS-stimulated THP-1 cells when compared with the control, as is shown in Fig. 2B, C. KDK displayed the highest effects on inhibited cytokines, IL-6, and TNF- α secretion from LPS-induced THP-1 cells when compared with the other BR extracts ($p < 0.05$). Remarkably, the KDK treatment displayed highest reduction in IL-6 secretion from 100% in the control to 21.2% (78.8% inhibition, $p < 0.001$) as shown in Fig. 2B. While KAK 1, SNG 5 and CMU 107 showed subsequently reduction in IL-6 secretion from 100 to 31.5% (68.5% inhibition), 41.1% (58.9% inhibition) and 42.0% (58.0% inhibition), respectively. The other varieties (PES 1, BKU 5, K 2 and K 4) displayed less inhibitory effect on IL-6 secretion but significantly different ($p < 0.001$) from the control group. The C3G and P3G treatment significantly decreased IL-6 secretion from 100% in control group to 61.2% (38.8% inhibition) and 73.2% (26.8% inhibition), respectively (Fig. 2B). With regard to TNF- α secretions, the KDK treatment also inhibited TNF- α secretions from 100% in the control to 39.4% (60.6% inhibition) as shown in Fig. 2C. The other varieties (KAK 1, SNG 5, PES 1, CMU 107, BKU 5, K 2 and K 4) displayed lower inhibitory effect on TNF- α secretion but PES 1, CMU 107 and K 4 were significantly inhibited TNF- α secretions when compared with control group ($p < 0.05$). The C3G and P3G treatment significantly decreased TNF- α secretion from 100% in control group to 64.0% (36.0% inhibition) and 61.8% (38.2% inhibition), respectively (Fig. 2C).

Relationship between phytochemical contents and their biological activities among eight selected BR extracts

Previous studies have reported that the total phenolic content in an extract is related to its antioxidant properties [32]. Therefore, the correlation between the phytochemical contents and their biological activities of the BR extracts was evaluated using Pearson's correlation analysis [30, 31]. The correlation coefficient (r value) and statistical differences are presented as a heat map which is shown in Fig. 3A. The total phenolic and flavonoid contents in the BR extracts indicated a significantly very high positive correlation with the antioxidant (DPPH assay $r = 0.9110$; $p < 0.01$, ABTS assay $r = 0.9739$; $p < 0.001$ and FRAP assay $r = 0.9760$; $p < 0.001$) and anti-inflammatory properties (NO production; $r = 0.9029$; $p < 0.01$) but not inhibitory effects on cytokine production (IL-6 and TNF- α) [30, 31]. The protocatechuic acid content in the extracts had a significantly strongly positive association with the antioxidant properties (DPPH assay $r = 0.7680$; $p < 0.05$ and

Fig. 2 The Anti-inflammation of BR extracts. Macrophages, RAW264.7 or THP-1 cells were pretreated with BR extracts at concentration 200 µg/mL for 2 h, followed by stimulation with 1 µg/mL of LPS for 24 h. After incubation, the culture medium was collected and determined NO (A), IL-6 (B) and TNF-α (C) production. The data are demonstrated in mean ± S.D. from three independents experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Independent t test) vs. LPS-stimulated macrophages (Control). a,b,c,d Difference letters indicated statistic difference among black rice varieties ($p < 0.05$, One way ANOVA with Tukey’s test)

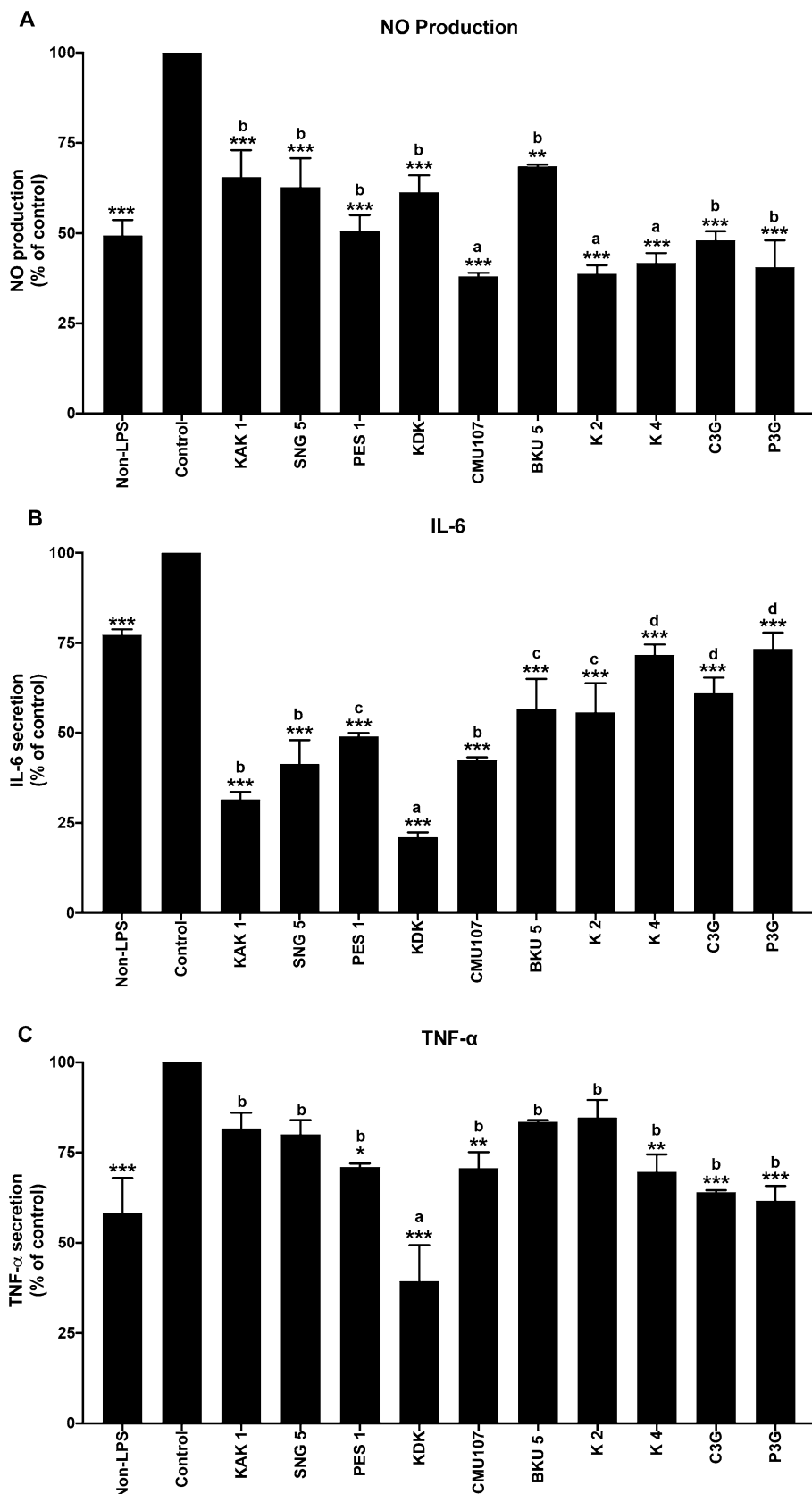
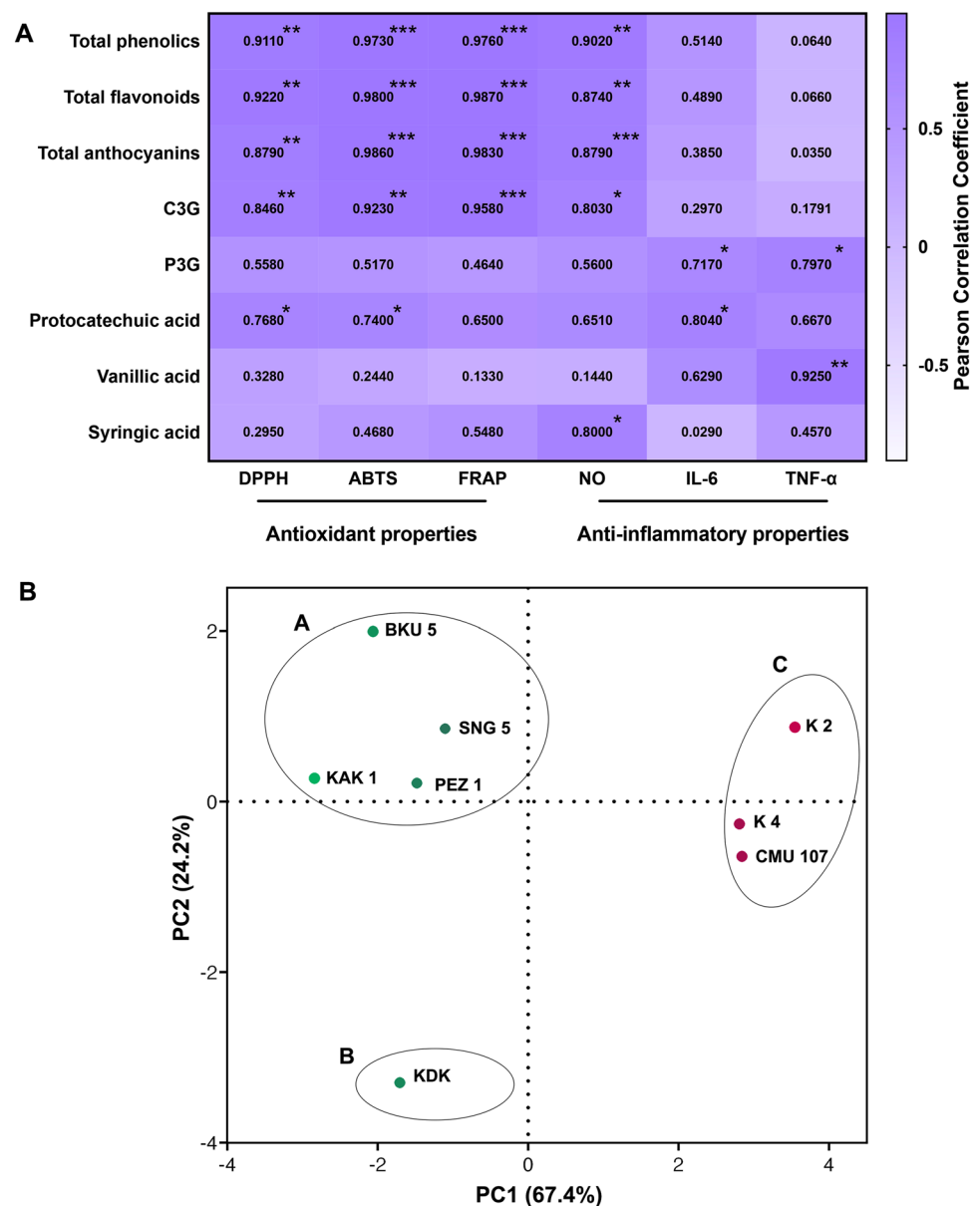


Fig. 3 The correlation of black rice phytochemical, antioxidant activities and anti-inflammation properties. The heat map of the Pearson correlation coefficients for the relationship between phytochemicals contents and their bioactivities (A). The principal component analysis showing associations between phytochemical components and antioxidant and anti-inflammation properties (B). *Significant at the level 0.05 ($p < 0.05$, independent t test), **significant at the level 0.01 ($p < 0.01$, independent t test) and ***significant at the level 0.001 ($p < 0.001$, independent t test)



ABTS assay $r = 0.7400$; $p < 0.05$), while syringic acid exhibited a significantly strong positive association with the inhibitory NO production ($r = 0.8000$; $p < 0.05$). The total anthocyanin content in the BR extracts had a significantly very high positive association with their antioxidant properties (DPPH assay $r = 0.8790$; $p < 0.01$, ABTS assay $r = 0.9860$; $p < 0.001$ and FRAP assay $r = 0.9830$; $p < 0.001$) and significantly strong association with their anti-inflammatory activities (NO production; $r = 0.8790$; $p < 0.001$). Interestingly, the C3G concentrations in the BR extracts had a very high positive association with their antioxidant properties (DPPH assay $r = 0.8460$; $p < 0.01$, ABTS assay $r = 0.9230$; $p < 0.01$ and FRAP assay $r = 0.9580$; $p < 0.001$). While P3G showed a significantly strongly positive association ($p < 0.05$) with the inhibitory

of cytokines secretion, IL-6 ($r = 0.7170$; $p < 0.05$) and TNF- α ($r = 0.7970$; $p < 0.05$).

Categorization of eight selected black rice varieties using the principal component analysis (PCA)

The first principal component (PC1) accounted for 67.4%, while the second principal component (PC2) accounted for 24.2% of the total variations in the results. Figure 3B displayed the distinct groups of the black rice varieties. Group A is comprised of the varieties BKU 5, SNG 5, KAK 1, and PEZ 1. The distinct characteristics of this group are that these rice varieties contain high amounts of total phenolic, total flavonoid, total anthocyanins, and C3G concentrations in conjunction with their strong antioxidant properties. In

this group, most of the rice varieties were glutinous type with the exception of BKU 5, which was the only non-glutinous rice variety in this group. The second group contained only KDK (glutinous type). The rice variety contained high amount of P3G concentrations and exhibited strong anti-inflammatory properties. The third group contained the varieties K2, K4 and CMU 107. The characteristics of this group include lower amounts in phytochemical profiles and less potent bioactivities than the other two groups. Interestingly, all the rice varieties in this group were of the non-glutinous type. The results indicated that each variety of black rice possessed different phytochemical contents and exhibited differing degrees of biological activity. Notably, the glutinous type of rice displayed remarkable phytochemical profile and potent biological properties than the non-glutinous rice. Interestingly, BKU 5 was the only non-glutinous rice varieties that exhibited remarkable phytochemical profiles and strong biological activities when compared with the other non-glutinous rice varieties.

Discussion

At present, black rice is being consumed across the globe as functional food and indirectly as an ingredient in a range of supplementary or alternative health products. Black rice is an anthocyanins-enriched type of food that is increasing in research interest due to its potent nutritional value and health-promoting effects. Coronavirus Disease 2019 (COVID-19) pandemic caused by a coronavirus SARS-CoV-2 infection and resulted in severe acute respiratory distress syndrome (ARDS) or severe acute respiratory syndrome (SARS) [33, 34]. Unfortunately, nowadays there are no specific treatment for COVID-19. Therefore, natural supplementary products could be supportive prevention against SAR-CoV-2 infection [35]. Black rice consumption is popular in the northern region of Thailand as well as other part of Thailand for new normal lifestyle after COVID-19 pandemic. More than 37 black rice varieties have been found in the northern region of Thailand with variations in total anthocyanin content (0.5–16.0 mg/100 g), γ -oryzanol concentration (52.0–111.4 mg/100 g), Zn concentration (16.9–28.8 mg/kg) and Fe concentration (7.8–15.2 mg/kg) [36]. The local black rice varieties cultivated in the northern region varied in terms of nutrient quality. However, the new black rice varieties with improved nutrients and health benefits could be developed to meet the consumers demand for functional food. Therefore, the phytochemical contents, the anthocyanins concentration (C3G and P3G), the antioxidant and anti-inflammation properties of eight selected black rice varieties were investigated in this study.

The results indicate that the total phenolic and flavonoid contents in the BR extracts varied for the different

rice varieties, wherein KAK 1 possessed the greatest total phenolic (341.31 ± 6.88 mg GAE/g extract) and flavonoids (155.21 ± 3.53 mg CAE/g extract) contents ($p < 0.05$). The active compounds found in the BR extracts were investigated using HPLC. Protocatechuic acid, vanillic acid, and syringic acid were found in all eight BR extracts at various concentrations. A previous study reported that protocatechuic acid, vanillic acid, and syringic acid were major phenolic compounds present in black rice [2, 3]. Similarly, our data demonstrated that KDK rice variety possessed the highest amounts of protocatechuic acid, and vanillic acid, while CMU 107 possessed the highest syringic acid concentrations. Remarkably, the KAK 1 variety was considerably high in all these of these phenolic compounds. Taken together, the glutinous rice varieties (KAK 1, PES 1, SNG 5, and KDK) possessed higher total phenolic and flavonoid contents than the non-glutinous rice varieties (CMU 107, K2 and K4), with the exception of BKU 5.

Anthocyanin is a pigmented flavonoid which is predominantly found in black rice varieties [2]. The total anthocyanin contents in the BR extracts in our study varied from 22.32–132.43 mg/g germ and bran extract as measured by pH differential method. The KAK 1 variety possessed the highest total anthocyanins content (132.43 ± 3.34 mg/g germ and bran extract), while the CMU 107 variety contained the lowest amount of total anthocyanin content. The HPLC results showed that, the KAK 1 variety contained the highest C3G content (106.76 ± 2.94 mg/g germ and bran extract) ($p < 0.05$), while the CMU 107 variety possessed the lowest C3G content (4.61 ± 0.10 mg/g germ and bran extract). Interestingly, P3G was found at the highest concentration in the KDK variety (32.37 ± 1.10 mg/g germ and bran extract) ($p < 0.05$), while CMU 107 showed the lowest P3G content (6.40 ± 0.22 mg/g germ and bran extract) as was determined by HPLC. Similar to the results of previous studies, C3G was determined to be the major anthocyanin present in black rice followed by P3G [13]. The glutinous rice varieties (KAK 1, PES 1, SNG 5, and KDK) and non-glutinous variety, BKU 5, possessed higher total anthocyanins content ($p < 0.05$), C3G and P3G. Another non-glutinous rice varieties (CMU 107, K2 and K4) contained much lower amounts of anthocyanins. Our findings are similar to those of other reports, which found that glutinous black rice varieties contained higher amounts of anthocyanins than non-glutinous rice varieties [8, 13, 37].

The results from three independent antioxidant assays point indicate KAK 1 exhibited the greatest antioxidant properties when compared with the other black rice varieties. According to the results, KAK 1 contained the highest phenolics, flavonoids, anthocyanin, and C3G contents and exhibited the strongest antioxidant properties. The Pearson correlation coefficient showed that, the amounts of phenolics, flavonoids, anthocyanins and C3G contents

were positively correlated with their antioxidant properties. This finding was coincided with previous study that the total phenolic contents in the black rice extracts were highly positively correlated with their antioxidant activities [18]. ROS is essential for normal cellular function, but excessive ROS levels can also be harmful. Oxidative stress can be the cause of inflammation via activation of the NF- κ B signaling pathway leading to pro-inflammatory cytokines, including IL-6, and TNF- α secretion [19]. This study found that KDK, which contained the highest amount of P3G, exhibited the most potent anti-inflammatory properties via the inhibition of IL-6 and TNF- α secretions from LPS-induced THP-1 cells. Interestingly, with regard to the P3G, protocatechuic acid, and vanillic acid concentration in the BR extracts showed a strongly positive correlation with their anti-inflammation via the inhibition of IL-6 and TNF- α secretions. Moreover, a previous study involved the purple maize (*Zea mays* L.) as P3G, protocatechuic acid and vanillic acid in the plant were significantly positively correlated with the inhibition of TNF- α secretions [32]. The positively correlation effects of anthocyanin, total phenolic, total flavonoid with their biological properties might be from synergistic effects. Previous study reported that the combination between phenolic compounds and anthocyanin showed synergistic anti-inflammation effects [38]. Moreover, the combination of anthocyanin and phenolic extract showed the synergistic effects on inhibited tumor growth and lung metastasis in breast cancer, MDA-MB-231 cells xenograft mice model [39]. However, the combination between two or more phytochemical compounds not always enhancing biological activities. This combination could be additive, synergistic and antagonism [40]. The combination of black rice anthocyanin-rich fraction and gallic acid or caffeic acid had synergistic anti-inflammation on LPS-induced RAW 246.7 cells. While the combination of black rice anthocyanin-rich fraction and p-hydroxybenzoic acid, vanillic acid and ferulic acid had no evidence for synergistic effects [10]. The combination between black rice anthocyanin-rich extract and rosmarinus acid exhibited additive protective effects against DSS-induced colitis in mice via inhibited inflammation [11].

PCA is a useful statistical tool for identifying variations between samples and visualizing the cluster of samples depending on variation [41, 42]. In this study, PCA was used to classify black rice varieties according to their phytochemical components, antioxidant and anti-inflammatory properties to finally categorized the black rice varieties into three groups. According to the PCA results, a high levels of phytochemicals, total phenolics, flavonoids, anthocyanins, and C3G contents, along with high antioxidant properties were categorized into group A. A previous study reported that KAK 1, PES 1, SNG 5 and BKU 5 possessed high protein contents, crude fiber, and mineral contents [42]. Our findings provide evidence that the black rice varieties, KAK 1,

PES 1, SNG 5 and BKU 5, might be the appropriate choices for the consumers who looking for the functional food with high antioxidant as well as high protein and fibers. In Group B, KDK possessed higher P3G, protocatechuic acid, and vanillic acid concentration, which correlated with its anti-inflammation properties. This black rice variety might be suitable for consumers who suffer from the chronic low-grade inflammation disease. A chronic low-grade inflammatory state is an altered intercellular communication of innate immune systems due to the accumulation of damage and oxidative stress in the body [20, 43]. Oxidative stress elicits an amplification of the release of cytokines and can lead to the hyperactivation of macrophages resulting in an increase in circulatory levels of proinflammatory cytokines. In aging perspective, this phenomenon is referred to as inflamm-aging [44]. A chronic low-grade inflammation can result in the increased threshold of pro-inflammatory cytokines including, IL-6, IL-1 β , and TNF- α in the blood circulation, in which the individuals with this condition are prone to many chronic systemic diseases such as diabetes, frailty syndrome, inflammatory bowel syndrome and cardiovascular diseases [43, 45]. Since coronavirus disease 2019 (COVID-19) has spread across the world, and the number of people with Long-COVID symptoms has been increasing. Long COVID is associated with ongoing symptoms extend beyond 4–12 weeks after COVID-19 infection [46, 47]. Chronic systemic inflammation with increasing cytokine levels, especially IL-6 level, is one of the prevailing Long-COVID symptoms [48]. Since no specific treatments for Long-COVID has been developed, the use of anti-inflammation natural compounds might be a preventive strategy to prevent inflammation-related symptoms long-COVID patients. A recent study has reported that anthocyanins-rich black rice extract inhibit the inflammation via the inhibition of cytokines, IL-6, IL-1 β , and IL-18 secretions in SAR-CoV-2 spike glycoprotein induced A549 lung cells and THP-1 macrophages [27]. Therefore, black rice could be considered as a functional food or used to develop a supplement for Long-COVID prevention. The last group of black rice varieties, CMU 107, K2 and K4 contained lower amounts of phytochemicals compounds, antioxidant and anti-inflammatory properties. However, in previous study, the rice bran extract of CMU 107 had been reported to contain high amount of γ -oryzanol and tocopherol (vitamin E) [42]. These compounds contain health-promoting benefits such as antioxidant, anti-diabetic, anti-hypertensive and cholesterol-lowering effect [49]. The outcomes of this study can provide necessary scientific data for food industries as well as rice cultivator in selecting the variety of black rice for the functional food based on the anthocyanin contents that could benefit to the health of consumers.

The consumption of black rice has been increasing in recent years due to its health promoting benefits. To further

encourage the growing trend for the consumption of health-promoting functional foods, special varieties of rice have been developed with the novel rice varieties that contained greater nutrient values and exhibited strong biological activities that suitable for new normal healthy lifestyle of the consumer. Therefore, the total phenolics, total flavonoids, anthocyanins, C3G and P3G contents in eight black rice varieties as well as their antioxidant, and anti-inflammatory properties have been investigated in this study. PCA was used to categorize the black rice varieties into three groups depending upon their phytochemicals content, as well as their antioxidant, and anti-inflammatory properties. our findings could provide vital information for food industries as well as rice cultivator in selecting the variety of black rice for the functional food based on the anthocyanin contents that could benefit to the health of consumers.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirement This study does not involve any human or animal testing.

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