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Thai pigmented rice bran extracts inhibit production of superoxide, nitric oxide radicals and inducible nitric oxide synthase in cellular models

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ABSTRACT

Objective: To study the inhibitory effect of rice bran extracts of Thai black Kam Muang and red Hawm Dawk Mali Deang on oxidative stress factors including superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), and inducible nitric oxide synthase (iNOS).

Methods: Bran extracts (40% ethanol) of Kam Muang and Hawm Dawk Mali Deang were obtained and evaluated for *in vitro* 2-2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) and $NO^{\bullet}$ scavenging activity. Their inhibitory effects on cellular $O_2^{\bullet-}$ and $NO^{\bullet}$ were measured in phorbol 12-myristate 13-acetate-stimulated neutrophil-like HL-60 cells and lipopolysaccharide-stimulated RAW264.7 macrophages, respectively, and their viability was monitored using the MTT assay. The effect on iNOS expression was also assessed by the Western blotting assay. Total contents of phenolics, flavonoids, and subtypes were also determined.

Results: Hawm Dawk Mali Deang exhibited about 3.5-fold greater cellular $O_2^{\bullet-}$ inhibitory activity than Kam Muang [EC_{50} values of (23.57±4.54) and (81.98±1.45) μg/mL, respectively] in phorbol 12-myristate 13-acetate-stimulated HL-60 cells. Hawm Dawk Mali Deang exhibited about 2-fold higher *in vitro* $ABTS^{\bullet+}$ and $NO^{\bullet}$ scavenging activity than Kam Muang, but it exerted cellular $NO^{\bullet}$ inhibitory activity of only about 26% (undetermined EC_{50} value) in lipopolysaccharide-stimulated RAW264.7 cells. Conversely, Kam Muang exerted potent cellular $NO^{\bullet}$ inhibitory activity [EC_{50} value: (281.13±59.18) μg/mL] and dose-dependently decreased iNOS levels. No cytotoxicity of both extracts was detected in both cell types. As for corresponding contents, Hawm Dawk Mali Deang contained higher contents of phenolics and flavonoids than Kam Muang. Moreover, Kam Muang and Hawm Dawk Mali Deang had a high content of total anthocyanins [(14.73±0.52) mg C3GE/g of extract] and total proanthocyanidins [(115.13±1.47) mg CE/g of extract], respectively.

Conclusions: Based on these data, bran extracts of Thai black Kam Muang and red rice Hawm Dawk Mali Deang can help lower oxidative stress and inflammation attributed partly to $O_2^{\bullet-}$ and $NO^{\bullet}$.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are free radicals (FR) formed under physiological processes, *e.g.*, cellular respiration, various events of growth and cell death, and defensive systems. The primary ROS and RNS produced in cells are superoxide ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$), respectively[1].

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While our own body produces FR in moderate amounts to perform many useful functions, we also expose to exogenous ROS/RNS. As a highly reactive radical, $NO^{\bullet}$ itself directly damages normal functional tissues, and it can also react with $O_2^{\bullet-}$ to form an even stronger oxidant, peroxynitrite ($OONO^-$). Increased contents of FR that exceed cellular antioxidant defenses consisting of enzymatic and non-enzymatic antioxidants cause continued oxidative stress, leading to cellular oxidative damage and chronic inflammation[2]. Moreover, $NO^{\bullet}$ that is excessively produced by inducible nitric oxide synthase (iNOS) in abnormal situations, is considered as a key mediator that can induce the generation of pro-inflammatory cytokines, leading to chronic inflammation as well[3]. Therefore, the strong inhibitory activities against $NO^{\bullet}$ and $O_2^{\bullet-}$ in the body have the potential for blocking the accumulation of both radical species and their subsequent reactions, which partly cause continued oxidative stress and chronic inflammation implicated in various diseases such as cancer, diabetes, cardiovascular, neurological, and pulmonary diseases.

In recent years, plant-derived natural antioxidants have been used frequently, given that they present activity comparable with synthetic antioxidants. Among plants, black and red rice varieties are potent sources of antioxidants. Several studies have demonstrated high antioxidant capacity in red and black rice using chemical assays that do not adequately mimic *in vivo* cell environments[4–9], but surprisingly only a few studies have assessed such an antioxidant potential using cell-based assays[10–12]. Unlike the antioxidant effects, a few studies have reported the anti-inflammatory effects of pigmented rice varieties. Whole grain extracts from only some red and black rice varieties were shown to inhibit the release of $NO^{\bullet}$ and pro-inflammatory cytokines, *e.g.*, tumor necrosis factor- α , and interleukin-6 as well as suppress the expression of inflammatory enzymes, *e.g.*, iNOS and cyclooxygenase-2[13–16]. High contents of hydrophilic compounds such as phenolics, flavonoids, and their subtypes were found in water-organic solvent extracts of black and red rice, and these contents appeared to relate to their antioxidant[4,8,12,17,18] and anti-inflammatory activities[15,16].

Despite many studies on the antioxidant activity of pigmented rice varieties, the inhibitory effects of bran extracts on levels of $O_2^{\bullet-}$, $NO^{\bullet}$, and iNOS have not yet been evaluated in cellular models. Therefore, this research explored the inhibitory effect of 40% ethanolic bran extracts of Thai pigmented rice cultivars namely Kam Muang (KM) (black rice) and Hawm Dawk Mali Deang (HMD) (red rice)[12], on $O_2^{\bullet-}$ levels in differentiated neutrophil-like HL-60 cells, as well as on levels of $NO^{\bullet}$ and iNOS in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. In addition to cellular models, direct scavenging activity against artificial 2-2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) and natural $NO^{\bullet}$ was determined using *in vitro* assays. Moreover, corresponding hydrophilic contents of phenolics, flavonoids, and their subtypes were determined.

2. Materials and methods

2.1. Plant materials and preparation of rice bran extracts (RBE)

KM and HMD were collected from 2 provinces of Thailand, Udon Thani, and Chiang Mai, respectively. In this preparation, 4 kg of HMD bran and 7 kg of KM bran were separately extracted in 20 L and 35 L of ethanol-water (40:60, v/v), respectively, for 72 h (sample-solvent ratio at 1:5). Extraction was repeated 2 more times with the same sample-solvent ratio for 24 h. Then the solvents from these 3 repeated extractions were combined and dried using a rotary vacuum evaporator and a freeze dryer. The yields of KM and HMD bran extracts were 7.86% and 9.34%, respectively (g of dry extract per g of KM or HMD bran). The rice bran extracts were stored at -20 ℃ until use. Several concentrations of each extract were prepared in hot water and estimated for antioxidant activities.

2.2. Chemical antioxidant assays

2.2.1. $ABTS^{\bullet+}$ radical scavenging assay

$ABTS^{\bullet+}$ scavenging activity is an important feature of antioxidants appreciably reported in plant extracts and is accessed on the basis of the decolorization of $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ radical scavenging activity of RBE at the concentration ranging from 1 to 20 μg/mL was monitored and expressed as the percent inhibition of absorbance of $ABTS^{\bullet+}$ and half-maximal effective concentrations (EC_{50}; μg/mL) as previously described[12]. Protocatechuic acid (PCA) and catechin (CT), which have been reported to be mainly present in black and red rice, respectively[19] were used as the controls in parallel.

2.2.2. $NO^{\bullet}$ scavenging assay

Sodium nitroprusside (SNP) in aqueous solution at physiological pH spontaneously generates $NO^{\bullet}$, which reacts with oxygen to produce nitrite ions that can be determined using the Griess reagent. Thus, scavengers of $NO^{\bullet}$ compete with oxygen, leading to reduced production of nitrite ions. The nitric oxide scavenging activity of RBE at the concentrations range of 1 to 40 μg/mL was estimated using Griess Illosvoy reaction and expressed as the percent inhibition of absorbance of nitrite ions and EC_{50} (μg/mL) as previously described[20]. PCA and CT were used as the controls in parallel.

2.3. Cellular antioxidant assays

2.3.1. Superoxide anion ($O_2^{\bullet-}$) inhibition assay in differentiated neutrophil-like HL-60 cells

Human promyelocytic leukemia cell line HL-60 was purchased from the American Type Culture Collection, and cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal

bovine serum at 37 ℃ in 5% CO_2 atmosphere. HL-60 cells were induced into neutrophils with 1.3% dimethyl sulfoxide for 6-8 d. Differentiated cells (1×10^6 cells) were pretreated with or without various concentrations of RBE (10-500 μg/mL KM, 10-80 μg/mL HMD) for 15 min and then stimulated with 15 ng/mL phorbol 12-myristate 13-acetate (PMA) for 1 h. The $O_2^{\bullet-}$ inhibitory activity and relative $O_2^{\bullet-}$ levels (%) in different treatment groups were examined and presented as mean percentage and EC_{50} (μg/mL) relative to the only PMA-induced group (without extract) as previously described[12]. Also, the extracts at the same listed concentration ranges were tested alone without PMA.

2.3.2. $NO^{\bullet}$ inhibition assay in LPS-stimulated RAW264.7 macrophages

Murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection, and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum at 37 ℃ in 5% CO_2 atmosphere. RAW264.7 macrophages (1×10^6 cells/well) were seeded in 6-well plates overnight before pretreatment with or without various concentrations of RBE (100-1 000 μg/mL KM, 62.5-250 μg/mL HMD) for 2 h and then were stimulated with 10 ng/mL LPS for 24 h. Concentrations of nitrite and nitrate in the culture medium were quantified and used as an indirect measure of $NO^{\bullet}$ production. The $NO^{\bullet}$ inhibitory activity and relative $NO^{\bullet}$ levels in different treatment groups were monitored and presented as mean percentage and EC_{50} (μg/mL) relative to the only LPS-induced group (without extract) as previously described[21]. Also, the extracts at the same listed concentration ranges were tested alone without LPS.

2.3.3. MTT cell viability assay

The viability of both cell types exposed to either PMA or LPS with or without RBE at the same concentration range applied in the two inhibition assays was monitored using the MTT assay. These treated cells were incubated with 500 μg/mL MTT solution for 1 h. The precipitated purple formazan was dissolved in dimethyl sulfoxide. The absorbance was determined at 570 nm. The percentage of cell viability was calculated by the following formula:

$$\%\ \text{Cell viability} = \frac{A_{extract}\times 100}{A_{control}}$$

Where $A_{control}$ is the absorbance of the reaction without the extract and $A_{extract}$ is the absorbance of the extract.

The concentrations of RBE were considered to be non-toxic with the percentage of viable cells higher than 70%.

2.4. Western blot analysis

To detect the expression levels of iNOS, the RAW264.7 cells treated with or without the KM extract (100-1 000 μg/mL) or the HMD extract (62.5-250 μg/mL) for 2 h, followed by incubation with LPS for 24 h, and the unstimulated cells incubated with the extract alone (from Method 2.3.2) were collected. The lysate proteins were separated by 7.5% SDS-poly acrylamide gel electrophoresis and immunoblotted with the anti-iNOS polyclonal antibody (Merck Millipore, Germany). Goat anti-rabbit secondary antibody (LI-COR Biotechnologies) was used for detection of the target protein. The signals were then detected using the Odyssey CLx Imaging System (LI-COR Biotechnologies). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Following incubation with the anti-GAPDH monoclonal antibody (Cell Signalling Technology), goat anti-mouse secondary antibody (LI-COR Biotechnologies) binding was visualized by the Odyssey CLx Imaging System.

2.5. Phytochemical content analysis

2.5.1. Determination of total phenolic content

Total phenolic content was measured using the Folin–Ciocalteu method as previously described[21], and expressed as mg of gallic acid equivalent (GAE) per 1 g of RBE.

2.5.2. Determination of total flavonoid content

Total flavonoid content was determined using the aluminum chloride colorimetric method as previously described[21], and expressed as mg of catechin equivalent (CE) per 1 g of RBE.

2.5.3. Determination of total proanthocyanidin content

Total proanthocyanidin content was estimated using the vanillin-HCl method as previously described[12], and expressed as mg of catechin equivalent (CE) per 1 g of RBE.

2.5.4. Determination of total anthocyanin content

Total anthocyanin content was measured according to the pH differential method as previously described[22], and expressed as cyanidin 3-glucoside (C-3-G) equivalent (C3GE) per 1 g of RBE.

2.6. Statistical analysis

The results were presented as mean ± SD from at least three separate experiments. The EC_{50} was extrapolated from a dose-dependent curve by plotting percent inhibition values *versus* the extract concentrations using GraphPad Prism 5.0 software and cubic spine interpolation. All statistical analyses were carried out using IBM SPSS statistics 22.0 software. Statistically significant differences between treatment groups were determined by one-way analysis of variance (ANOVA) followed by Duncan or Dunnett's T3 for multiple comparisons with a significance level of 0.05 ($P < 0.05$).

3. Results

3.1. Chemical antioxidant activity of RBE

3.1.1. $ABTS^{\bullet+}$ radical scavenging activity

The KM and HMD extracts dose-dependently scavenged $ABTS^{\bullet+}$ with high potency described as a low EC_{50} value (the smaller EC_{50} value, the stronger antioxidant activity) in comparison to PCA and catechin CT (Table 1). Also, the activity of the HMD extract was 2-fold higher than that of the KM extract.

Table 1. ABTS and nitric oxide radical scavenging activity of the extracts of Kam Muang and Hawm Dawk Mali Deang.

Samples	EC_{50} (μg/mL)	
	ABTS radical scavenging activity	Nitric oxide radical scavenging activity
Kam Muang	15.26±1.05	22.10±1.05
Hawm Dawk Mali Deang	7.27±0.37	11.93±1.48
Protocatechuic acid	2.70±1.74	13.08±0.55
Catechin	2.30±0.78	7.15±4.60

Values are presented as mean ± SD (where $n \geq 3$).

3.1.2. In vitro $NO^{\bullet}$ scavenging activity

The KM and HMD extracts dose-dependently exerted potent $NO^{\bullet}$ scavenging activity with a low EC_{50} value as compared to PCA and CT (Table 1). Moreover, the HMD extract possessed 2-fold higher *in vitro* $NO^{\bullet}$ scavenging activity than the KM extract. In this context, the suppressive effect of both extracts on the generation of $NO^{\bullet}$ *in vitro* can be considered as direct scavenging activity of $NO^{\bullet}$ as no other factors, *e.g.*, iNOS, are involved.

3.2. Cellular antioxidant activity of RBE

3.2.1. $O_2^{\bullet-}$ inhibitory activity

The $O_2^{\bullet-}$ inhibition assay was conducted to assess the inhibitory activity of RBE against $O_2^{\bullet-}$ levels in neutrophil-like differentiated HL-60 cells after stimulation with PMA. The results revealed that the KM and HMD extracts at various concentrations showed a dose-dependent inhibitory effect with EC_{50} values of (81.98±1.45) and (23.57±4.54) μg/mL, respectively, and relative $O_2^{\bullet-}$ levels (%) were dose-dependently decreased (Figure 1a and 1c). It was evident that the HMD extract had 3.5-fold higher $O_2^{\bullet-}$ inhibitory activity than the KM extract. Considering that the decrease in absorbance of blue

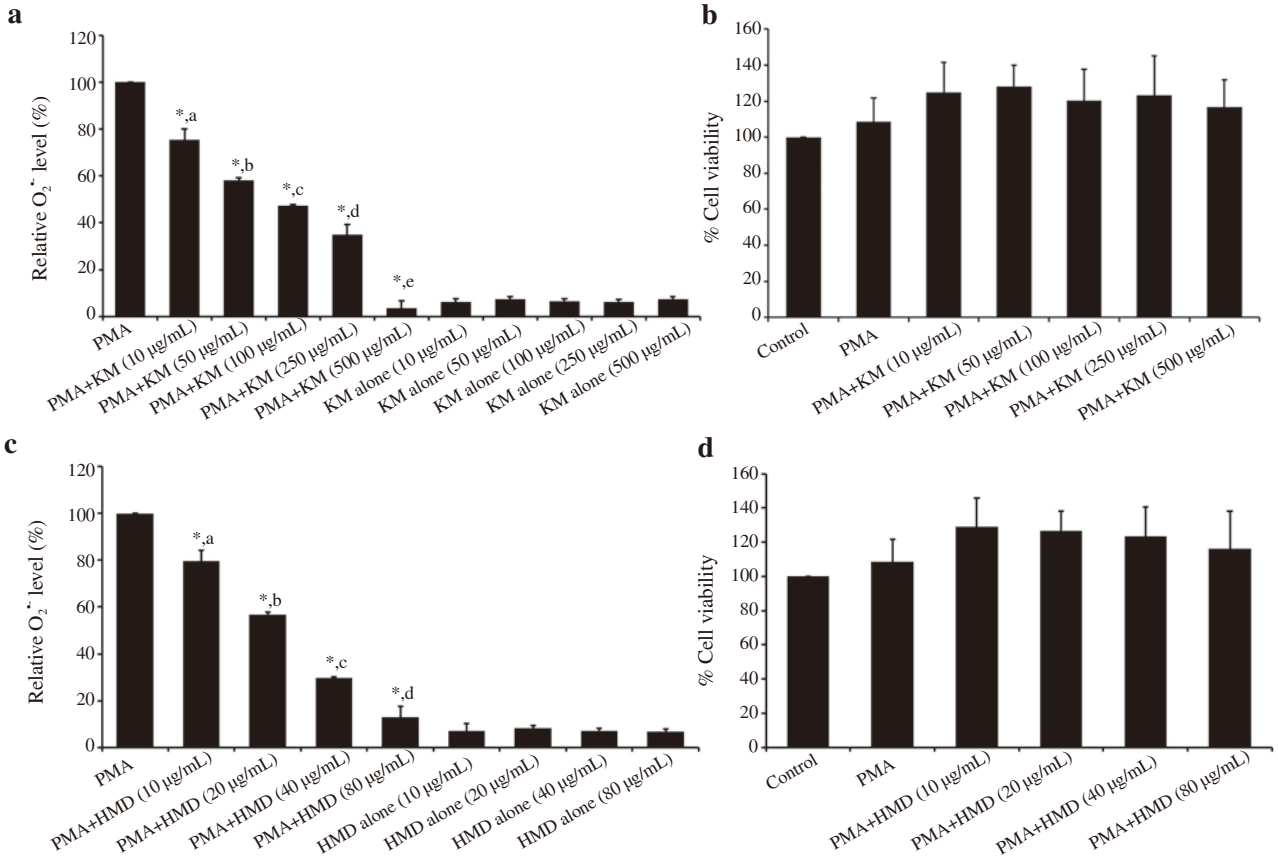

Figure 1. Effects of Kam Muang (KM) and Hawm Dawk Mali Deang (HMD) extracts on $O_2^{\bullet-}$ levels in phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophil-like HL-60 cells (a, c) and the viability of HL-60 cells (b, d). Values are presented as mean percentage ± SD ($n \geq 3$). Statisical differences ($P < 0.05$) are denoted by different letters (a-e) between treatment groups. $^{*}P < 0.05$ *vs.* PMA only-treated group.

formazan could be artificial in the presence of dead cells, and to avoid such misinterpretation, the MTT assay was consequently used in parallel to determine viable PMA-stimulated HL-60 cells treated with the same concentration range of the KM or HMD extract in comparison to the control cells (without PMA and the extract). As shown in Figure 1b and 1d, the KM and HMD extracts at all concentrations tested had no cytotoxic effect on these cells, thus indicating their actual $O_2^{\bullet-}$ inhibitory activity.

3.2.2. $NO^{\bullet}$ inhibitory activity

In addition to $O_2^{\bullet-}$ inhibitory activity, the inhibitory effect of the KM and HMD bran extracts on $NO^{\bullet}$ levels was assessed in LPS-stimulated RAW264.7 macrophages using the $NO^{\bullet}$ inhibition assay. KM exerted potent cellular $NO^{\bullet}$ inhibitory activity with an EC_{50} value of (281.13±59.18) μg/mL, while HMD had cellular $NO^{\bullet}$ inhibitory activity ranging from only 19.70% to 25.55% (an undetermined EC_{50} value) in LPS-stimulated RAW264.7 macrophages. As shown in Figure 2a and 2c, stimulation with only 10 ng/mL LPS resulted in the highest increase (taken as 100%) in nitrite/nitrate levels (unstimulated control cells were used as a baseline for relative quantification). However, LPS combined with three increasing doses of the KM extract significantly inhibited nitrite/nitrate levels in a dose-dependent manner relative to LPS alone (Figure 2a). Unexpectedly, LPS combined with three increasing doses of the HMD extract did not effectively suppress the generation of $NO^{\bullet}$ in RAW264.7 macrophages, and therefore nitrite/nitrate levels remained high (74.45%-80.30%) (Figure 2c). Moreover, the extract itself was found to stimulate low levels of $NO^{\bullet}$ in a dose-dependent manner (Figure 2a and 2c). In Figure 2b and 2d, the MTT assay detected the viability of LPS-stimulated RAW264.7 macrophages treated with RBE over 100% as compared with the control cells (without LPS and the extract). These results confirmed the actual inhibitory effect of the KM extract.

Although exerting greater direct $NO^{\bullet}$ scavenging activity than the KM extract *in vitro*, the HMD extract turned out to be ineffective in inhibiting $NO^{\bullet}$ levels in LPS-stimulated RAW264.7 cells. These

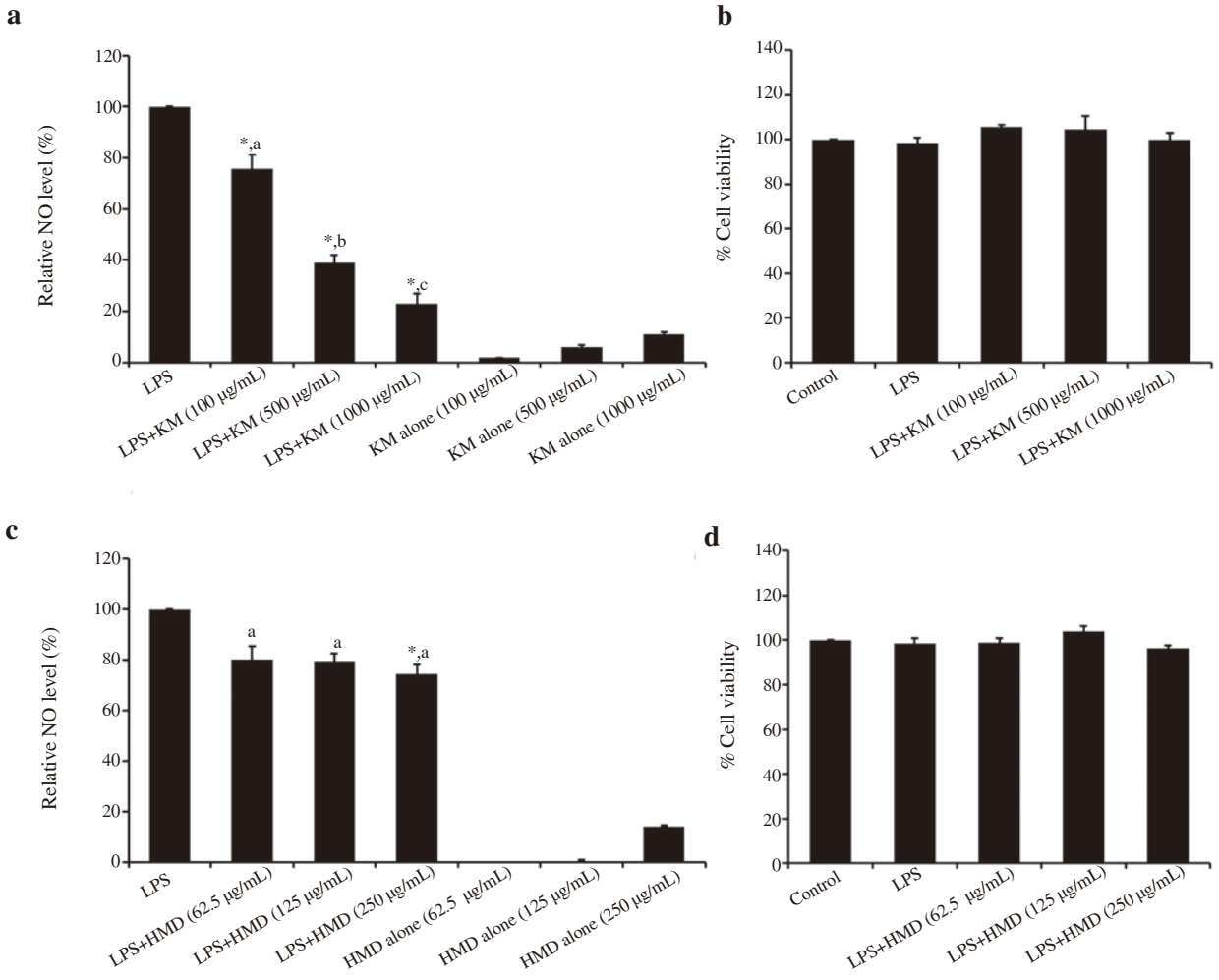

Figure 2. Effects of the Kam Muang (KM) and Hawm Dawk Mali Deang (HMD) extracts on $NO^{\bullet}$ levels in lipopolysaccharide (LPS)-stimulated RAW264.7 cells (a, c) and the viability of RAW264.7 cells (b, d) . Values are presented as mean percentage ± SD ($n \geq 3$). Statisical differences ($P < 0.05$) are denoted by different letters (a-c) between treatment groups. $^{*}P < 0.05$ *vs.* LPS only-treated group.

results indicated that the direct NO•-scavenging effect of the KM and HMD extracts was less likely to be only one mechanism to decrease the NO• level. We further speculated that both extracts might have different regulation on iNOS, a key enzyme for NO• synthesis in the macrophage inflammatory response, subsequently resulting in varying levels of cellular NO•.

3.3. Effects of RBE on LPS-induced levels of iNOS expression

To evaluate whether different suppressive effects of the KM and HMD extracts on NO• level were related to the regulation of iNOS expression, iNOS protein levels were examined in LPS-stimulated RAW264.7 macrophages. As shown in Figure 3a and 3b, the expression of the iNOS enzyme was almost undetectable in unstimulated RAW264.7 cells, but its level was significantly increased after LPS stimulation. However, the pretreatment of macrophages with the KM extract significantly reduced LPS-stimulated iNOS levels in a dose-dependent manner (Figure 3a). Therefore, marked decreases in cellular NO• in these stimulated cells were apparently due to the potent inhibitory effect of the KM extract on iNOS expression, despite its low direct NO•-scavenging effect relative to the HMD extract. In contrast, pretreatment with the HMD extract increased such iNOS dose-dependently (Figure 3b), thus hindering decreases in cellular NO• levels. Nevertheless, NO• levels were reduced by about 26% through its potent direct NO•-scavenging effect (Figure 2c), resulting in no detectable cytotoxicity caused by such elevated iNOS (Figure 2d). Furthermore, the KM and HMD extracts alone induced low levels of iNOS in RAW264.7 macrophages (Figure 3a and 3b), consistent with the detection of low levels of NO• (Figure 2a and 2c).

3.4. Contents of phenolics, flavonoids, anthocyanins, and proanthocyanidins

The total contents of phenolics, flavonoids, anthocyanins, and proanthocyanidins in 40% ethanolic bran extracts of black rice KM and red rice HMD were assessed (Table 2). The results showed that the HMD extract contained 1.4- and 2.0-fold higher total contents of phenolics and flavonoids, respectively than the KM extract did. Correspondingly, the red rice HMD extract exerted stronger scavenging activity against *in vitro* $ABTS^{\bullet+}$ and NO• radicals as well as more potent inhibitory activity against cellular $O_2^{\bullet-}$ levels.

Table 2. Total contents of phenolics, flavonoids, anthocyanins, and proanthocyanidins.

Phytochemical contents	Hawm Dawk Mali Deang	Kam Muang
Phenolics (mg GAE/g extract)	201.11±2.69	137.78±5.73
Flavonoids (mg CE/g extract)	149.69±6.50	71.56±2.24
Anthocyanins (mg C3GE/g extract)	0.62±0.03	14.73±0.52
Proanthocyanidins (mg CE/g extract)	115.13±1.47	18.79±0.61

Values are presented as mean ± SD (where $n \geq 3$).

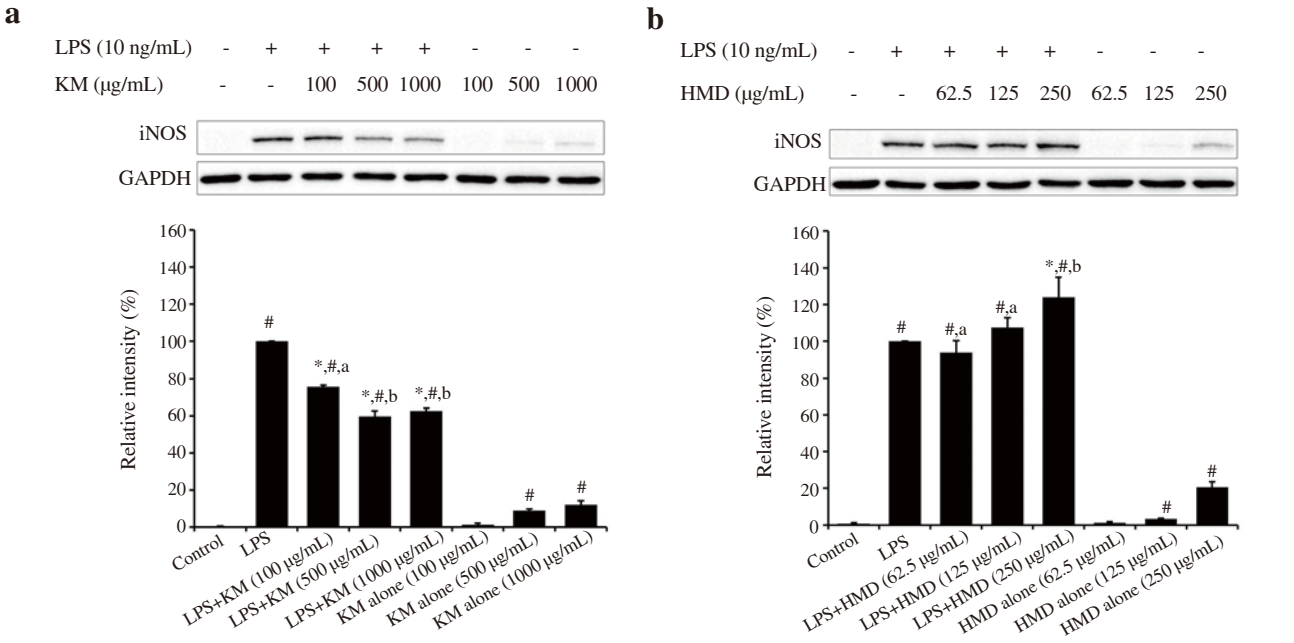

Figure 3. Effects of Kam Muang (KM) (a) and Hawm Dawk Mali Deang (HMD) (b) extracts on the iNOS expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. The expression levels of iNOS are presented as mean percentage ± SD ($n \geq 3$). Statisical differences ($P < 0.05$) are denoted by different letters (a-b) between treatment groups. $^{*}P < 0.05$ *vs*. LPS-treated group; $^{\#}P < 0.05$ *vs*. control group.

4. Discussion

Among various radical-scavenging methods, the $ABTS^{\bullet+}$ radical scavenging assay is common and suitable for measuring the antioxidant capacities of both hydrophilic and lipophilic antioxidants[21,22]. $NO^{\bullet}$ radicals formed within the cell can be produced in the *in vitro* $NO^{\bullet}$ scavenging assay for assessing the antioxidant capacity. However, the antioxidant capacity of RBE obtained from these chemical assays cannot be extrapolated to the *in vivo* situation due to some limitations. For example, ABTS radical is synthetic and not similar to radicals found in biological systems[23]. Thus cell-based assays have become an increasingly attractive alternative because they can better predict the antioxidant capacity of phytochemical compounds under the complex biological systems by taking cellular distribution and bioavailability into account[24].

As for the cellular antioxidant activity of RBE, the results showed that the HMD extract exerted inhibitory effects on cellular levels of $O_2^{\bullet-}$ (higher potency) and $NO^{\bullet}$ (lower potency) through its potent direct scavenging activity. In contrast, the KM extract showed potent inhibitory effects on cellular $NO^{\bullet}$ levels by its potent suppressive effect on iNOS. Despite differences in grain types, grain fractions, and extraction solvents, the suppressive effect of the black rice extract on levels of $NO^{\bullet}$ and iNOS in our results was consistent with a few studies described previously[13,14]. However, such effects of the red rice extract were inconsistent[16]. These findings further emphasize the importance of the modulation of $NO^{\bullet}$ activity in medicinal dietary plants[23]. Furthermore, the data in which colored rice extracts alone induced low levels of $NO^{\bullet}$ and iNOS expression in RAW264.7 macrophages have never been reported in these previous studies[14-16].

Jun *et al*. have reported that the total contents of phenolics and flavonoids were highest in an aqueous mixture of 40% ethanol rather than in greater/less than 40% or absolute ethanol[17]. These phenolics and flavonoids serve as great antioxidants because of their several hydroxyl groups bonded to the aromatic ring(s), which can neutralize oxidative stress by transferring their electrons to free radicals. However, the red rice HMD extract decreased cellular $NO^{\bullet}$ levels, but only to some extent due to its stimulating effect on iNOS levels. As for anthocyanins–a class of flavonoids, the black rice KM extract contained a markedly high content of these compounds. This result showed a positive correlation with its high potency in reducing iNOS levels and was associated with the previous study reporting that anthocyanins decreased iNOS protein and mRNA expression in activated macrophages[24]. As for proanthocyanidins–a class of polymeric phenolic compounds, the red rice HMD extract had a high content of these compounds. Therefore, anthocyanins and proanthocyanidins could be mainly responsible for potent antioxidant activities in the KM and HMD extracts, respectively. Several studies have demonstrated pigmented rice as a potential source of these bioactive antioxidant compounds[25].

In conclusion, the present study revealed for the first time (to our knowledge) that the 40% ethanolic bran extract of red rice HMD displayed inhibitory effects on cellular levels of $O_2^{\bullet-}$ (higher potency) and $NO^{\bullet}$ (lower potency) through its potent direct scavenging activity as compared to the KM extract. In contrast, the 40% ethanolic bran extract of black rice KM showed potent inhibitory effects on cellular $NO^{\bullet}$ levels by its potent suppressive effect on iNOS. These data support their potential use as dietary supplements to protect against continued oxidative stress and chronic inflammation attributed partly to $O_2^{\bullet-}$ and $NO^{\bullet}$.

Conflict of interest statement

We confirm there is no conflict of interest.

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References

[1] Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS sources in physiological and pathological conditions. *Oxid Med Cell Longev* 2016; **2016**: 1245049.

[2] Silpak B, Rintu D, Ena R. Role of free radicals in human inflammatory diseases. *AIMS Biophy* 2017; **4**(4): 596-614.

[3] Soufli I, Toumi R, Rafa H, Touil-Boukoffa C. Overview of cytokines and nitric oxide involvement in immuno-pathogenesis of inflammatory bowel diseases. *World J Gastrointest Pharmacol* 2016; **7**: 353-360.

[4] Butsat S, Siriamornpun S. Phenolic acids and antioxidant activities in husk of different Thai rice varieties. *Int J Food Sci Technol* 2010; **16**: 329-336.

[5] Daiponmak W, Senakun C, Siriamornpun S. Antiglycation capacity and antioxidant activities of different pigmented Thai rice. *Int J Food Sci Technol* 2014; **49**: 1805-1810.

[6] Min SW, Ryu SN, Kim DH. Anti-inflammatory effects of black rice, cyanidin-3-O-beta-D-glycoside, and its metabolites, cyanidin and protocatechuic acid. *Int Immunopharmacol* 2010; **10**(8): 959-966.

[7] Pang Y, Ahmed S, Xu Y, Beta T, Zhu Z, Shao Y, et al. Bound phenolic compounds and antioxidant properties of whole grain and bran of white, red and black rice. *Food Chem* 2018; **240**: 212-221.

[8] Shao Y, Hu Z, Yu Y, Mou R, Zhu Z, Beta T. Phenolic acids, anthocyanins,

proanthocyanidins, antioxidant activity, minerals and their correlations in non-pigmented, red, and black rice. *Food Chem* 2018; **239**: 733-741.

[9] Sompong R, Siebenhandl-Ehn S, Linsberger-Martin G, Berghofer E. Physicochemical and antioxidative properties of red and black rice varieties from Thailand, China and Sri Lanka. *Food Chem* 2011; **124**: 132-140.

[10]Hansakul P, Srisawat U, Itharat A, Lerdvuthisopon N. Phenolic and flavonoid contents of Thai rice extracts and their correlation with antioxidant activities using chemical and cell assays. *J Med Assoc Thai* 2011; **94**(Suppl 7): S122-130.

[11]Srisawat U, Panunto W, Kaendee N, Tanuchit S, Itharat A, Lerdvuthisopon N, et al. Determination of phenolic compounds, flavonoids, and antioxidant activities in water extracts of Thai red and white rice cultivars. *J Med Assoc Thai* 2010; **93**(Suppl 7): S83-91.

[12]Surarit W, Jansom C, Lerdvuthisopon N, Kongkham S, Hansakul P. Evaluation of antioxidant activities and phenolic subtype contents of ethanolic bran extracts of Thai pigmented rice varieties through chemical and cellular assays. *Int J Food Sci Technol* 2015; **50**: 990-998.

[13]Ichikawa H, Ichiyanagi T, Xu B, Yoshii Y, Nakajima M, Konishi T. Antioxidant activity of anthocyanin extract from purple black rice. *J Med Food* 2001; **4**: 211-218.

[14]Kitisin T, Saewan N, Luplertlop N. Potential anti-inflammatory and anti-oxidative properties of Thai colored-rice extracts. *Plant Omics* 2015; **1**: 69-77.

[15]Limtrakul P, Yodkeeree S, Pitchakarn P, Punfa W. Suppression of inflammatory responses by black rice extract in RAW 264.7 macrophage cells *via* downregulation of NF- κ B and AP-1 signaling pathways. *Asian Pac J Cancer Prev* 2015; **16**(10): 4277-4283.

[16]Limtrakul P, Yodkeeree S, Pitchakarn P, Punfa W. Anti-inflammatory effects of proanthocyanidin-rich red rice extract via suppression of MAPK, AP-1 and NF-kappaB pathways in Raw 264.7 macrophages. *Nutr Res Pract* 2016; **10**: 251-258.

[17]Jun HI, Song GS, Yang EI, Youn Y, Kim YS. Antioxidant activities and phenolic compounds of pigmented rice bran extracts. *J Food Sci* 2012; **77**: C759-764.

[18]Nam SH, Choi SP, Kang MY, Koh HJ, Kozukue N, Friedman M. Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chem* 2006; **94**: 613-620.

[19]Ciulu M, Cádiz-Gurrea M L, Segura-Carretero A. Extraction and analysis of phenolic compounds in rice: A review. *Molecules* 2018; **23**: 2890.

[20]Rozina P, Kundu SK, Pijush S. *In vitro* nitric oxide scavenging activity of methanol extracts of three bangladeshi medicinal plants. *J Pharm Innov* 2013; **1**(12): 83-88.

[21]Dechayont B, Itharat A, Phuaklee P, Chunthorng-Orn J, Juckmeta T, Prommee N, et al. Antioxidant activities and phytochemical constituents of *Antidesma thwaitesianum* Mull. Arg. leaf extracts. *J Integr Med* 2017; **15**: 310-319.

[22]Lee J, Durst RW, Wrolstad RE. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *J AOAC Int* 2005; **88**: 1269-1278.

[23]Borchard J, Mazzarella L, Spelman K. A review of medicinal plants that modulate nitric oxide activity. *Altern Med Stud* 2012; **2**: 26-35.

[24]Hämäläinen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: Genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediators Inflamm* 2007; **2007**: 45673.

[25]Samyor D, Das AB, Deka SC. Pigmented rice a potential source of bioactive compounds: A review. *Int J Food Sci Technol* 2017; **52**: 1073-1081.