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Potential anti-inflammatory and anti-oxidative properties of Thai colored-rice extracts

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Abstract

In Thailand, there has been growing interest in the use of colored rice extracts as a new source of anti-oxidative and anti-inflammatory effects. This study investigates the effects of different colored rice extracts in terms of their biological content, anti-oxidative activity, and their ability to reduce pro-inflammatory cytokines and matrix metalloproteinase (MMP) expression. Various colored rice from different rice cultivating areas in Thailand were used to obtain ethanolic extracts. The biological compounds in colored-rice extracts were determined by Folin-Ciocalteu colorimetric and pH-differential methods. To determine the anti-oxidative properties of colored-rice extract, DPPH radical scavenging, ferrous reducing power, and lipid peroxidation assays were used. The cytotoxicity of colored rice extracts was determined by MTT assay on a human promyelocytic leukemia (HL-60) cell line *in vitro*. The inhibition of pro-inflammatory cytokines (IL-6, TNF-α, NF-κB) and MMP expression in LPS-induced HL-60 cells was determined by ELISA assay. Moreover, MMP activity was determined by gelatinolytic zymography. The results found that red (Mun Poo, MP) rice exhibited high anti-oxidative activity and reduced pro-inflammatory cytokines and MMP-2 expression in LPS-induced HL-60 cells. This study provides new insights into the potential use of Thai colored rice extracts, especially red rice, as a source of anti-oxidants and anti-inflammation.

Keywords: *Oryza sativa* L., Colored-rice extract, Anti-inflammatory activity, Anti-oxidative activity, Pro-inflammatory cytokines, Matrix metalloproteinases.
Abbreviations: DPPH_ 2,2-diphenyl-1-picryl hydrazyl; ELISA_ enzyme-linked immunosorbent assay; HL-60_ human promyelocytic leukemia cell line; IL-6_ interlukin-6; LPS_ lipopolysaccharide; MMP_ matrix metalloproteinase; NF-κB_ nuclear factor kappa B; TNF-α _tumor necrosis factor alpha.

Introduction

Colored rice (*Oryza sativa* L.) is one of the staple foods in Asia. Many different colored rice varieties have been cultivated and are widely distributed in Thailand, including red, brown, and black rice. The variety of rice colors are caused by different biological compositions of the bran layer. Previous studies have also found biologically active compounds in colored rice grains, such as anthocyanins and polyphenols (Jang and Xu, 2009; Fujita et al., 2010; Gunaratne et al., 2013). Oxidative stress, resulting from the accumulation of reactive oxygen species (ROS), can cause cellular damage and inflammation. Oxidative stress can induce inflammatory cells to produce inflammatory mediators, such as cytokines and chemokines, which further enhance tissue damage from the recruitment of more inflammatory cells, eventually resulting in more oxidative stress (Reuter et al., 2010). Pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB) play an important role in signal transduction cascades during the progression of inflammation. Moreover, the induction of pro-inflammatory cytokines can induce nitric oxide production, which plays an important role in oxidative stress-induced inflammation (Hussain and Harris, 2007). Another inflammatory process is the production of matrix metalloproteinases (MMPs). MMPs are extracellular matrix degrading enzymes that can be secreted by the induction of many pro-inflammatory cytokines, such as TNF-α and interleukins (Parks et al., 2004). Recent studies have reported that rice extracts exhibit the potential to reduce disease risk due to their anti-oxidative activities – reducing oxidative stress, ameliorating hyperglycemia and hyperlipidemia, and anti-cancer activity (Liao et al., 2006; Hansakul et al., 2011; Prangthip et al., 2013; Niu et al., 2013). However, little is known about the anti-oxidative and anti-inflammatory properties of Thai colored rice extracts. This study investigated the potential anti-oxidative activities of Thai colored rice extracts. Biological compounds in colored rice extracts were identified, quantified, and compared. The anti-inflammatory properties of the extracts were studied in inflammatory-induced human promyelocytic leukemia (HL-60) cells, *in vitro*. The results of this study will provide important information about the potential use of Thai colored rice extracts as anti-oxidative and anti-inflammatory agents to improve human health.

Results

Total phenolic content

The total phenolic content of the colored rice extracts were 0.234 – 2.849 mg mL^{-1} (Table 2). However, colored rice extracts from the four different rice varieties did not exhibit significantly different average total phenolic concentrations by Folin-Ciocalteu colorimetric method. In the Mun Poo (MP) group, the extracts exhibited the highest average total phenolic content, at 1.428 mg GAE mL^{-1}. The average total phenolic content in the Sang Yod (SY) group contained 1.297 mg GAE mL^{-1}, with Niew Dam (ND) containing 1.097 mg GAE mL^{-1}, and Hom Nil (HN) the lowest, at 0.636 mg GAE mL^{-1}. Moreover, it was found that within-group variations in the rice source affected total phenolic content. In the MP group, the MP-Pink extracts had the highest total phenolic content (2.849 mg GAE mL^{-1}); In the SY group, the SY-Songkla extracts had the highest (1.944 mg GAE mL^{-1}); in the ND group, the ND-Doi Hang extracts had the highest level (1.688 mg GAE mL^{-1}); and in the HN group, the HN-Tai Tai extracts had the highest total phenolic content (1.319 mg GAE mL^{-1}).

Total anthocyanin content

Different rice varieties had significantly different average levels of total anthocyanin; the highest level was found in the ND group (2.817 mg mL^{-1} extract) (Table 2), followed by the HN group with 0.782 mg mL^{-1}, MP group 0.144 mg mL^{-1}, and SY group 0.092 mg mL^{-1}. Moreover, varied rice sources in the same group, such as the SY and HN groups, had similar total anthocyanin contents. However, variations by source were found in the MP and ND groups. In the MP group, the MP-Phayao extract had the significantly highest total anthocyanin content (0.309 mg mL^{-1} extract). In the ND group, the ND-Doi Hang extract had the significantly highest total anthocyanin content (5.172 mg mL^{-1}).

Anti-oxidative activities of colored rice extracts

The anti-oxidative activities of colored rice extracts were determined using three different methods (DPPH radical scavenging, ferrous reducing power, and lipid peroxidation assays). In the DPPH radical scavenging assay, highest average scavenging activity ranged from MP, HN, ND, and SY extracts (SC_{50} = 0.016, 0.048, 0.057, 0.068 mg mL^{-1}, respectively) (Table 2). Variations in rice source did not significantly affect scavenging activity. However, SY-Phattalung HPH and SY-Songkla extracts had the highest scavenging activity in the SY group (SC_{50} = 0.041, 0.034 mg mL^{-1}, respectively). The highest average ferrous reducing power ranged from MP, ND, SY, to HN extract (0.592, 0.416, 0.407, and 0.222 mg AAE mL^{-1}, respectively) (Table 2). In the MP group, MP-Pink extract had the highest ferrous reducing power, at 1.137 mg AAE mL^{-1}. In the SY group, SY-Songkla extract had the highest power, at 0.660 mg AAE mL^{-1}. In the ND group, ND-Doi Hang extract was 0.565 mg AAE mL^{-1}, and in the HN group, HN-Tai Tai was 0.411 mg AAE mL^{-1}.Highest average % lipid peroxidation inhibition activity ranged from SY, MP, ND, to HN (46.00%, 36.51%, 29.45%, and 26.47%, respectively) (Table 2). Only SY and HN extracts had significantly different lipid peroxidation inhibition activity among the varied rice sources. In the SY group, SY-Phattalung had the highest lipid peroxidation inhibition activity (62.42%). In the HN group, HN-Phayao had the highest lipid peroxidation inhibition activity (32.96%). After HL-60 cells were treated with LPS *in vitro*, the MTT assay was used to determine % cell viability. The lethal dose (LD_{50}) of LPS treatment for HL-60 was 1 µg mL^{-1}, the indicated concentration of LPS treatment for subsequent analysis.

Inhibition of pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB)

Pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB) were detected using commercial ELISA kits. All of the pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB) exhibited significantly higher production in the LPS-treated group than the control (Fig. 1). Four mg mL^{-1} dexamethasone inhibited all determined pro-inflammatory cytokine expression. The highest IL-6 inhibitory activity, determined post-treatment of LPS-induced HL-60 cells, was seen in MP, SY, and ND extracts, with similar inhibition ability to dexamethasone. HN extracts failed to inhibit IL-6 expression in LPS-induced HL-60 cells. The highest TNF-α and NF-κB inhibitory activities were found in MP and SY extracts, but failed in HN and ND.

Inhibition of nitric oxide (NO) production

Griess assay showed that NO production was lowest, ranging from dexamethasone, MP, and SY post-treated groups. Nevertheless, ND and HN post-treated groups failed to inhibit NO production compared with the LPS-induced HL-60 cells (pre-treated) group (P>0.05) (Fig. 2).

Inhibition of gelatinolytic activities (MMP-2 and MMP-9)

The highest MMP-2 activities were found in the PC (PMA-induced HL-60 cells used as positive control) group, followed by HN, ND, SY, to MP post-treated groups. After LPS-induced HL-60 cells, the dexamethasone (PD) post-treated group exhibited the lowest MMP-2 activity (Fig. 3). Gelatin zymography failed to detect MMP-9 in any experimental condition (data not shown). Qualitative analysis of gelatinolytic activities (MMP-2 and MMP-9), determined using gelatin zymography, was further investigated for MMP-2 and MMP-9 activity in each experimental condition using an ELISA assay as a semi-quantitative measure. MMP-2 and MMP-9 ELISA results showed highest expression in LPS-induced HL-60 cells (Fig. 4), with the lowest MMP-2 activity from dexamethasone, MP, and SY post-treated groups. The ND and HN post-treated group failed to inhibit MMP-2 activity to a significant level. In addition, the lowest MMP-9 activities were found in the MP group, followed by SY, then ND post-treated groups. However these were not significantly different from the LPS-induced HL-60 cells post-treated with dexamethasone. The HN post-treated group failed to inhibit MMP-9 activity compared with the LPS-induced HL-60 cells (pre-treated) group (P<0.05).

Discussion

Rice extract, obtained from the rice production process in the form of crude rice-bran oil, may become an alternative source of anti-oxidative agents. The anti-oxidative properties of rice-bran oil have been studied previously (Kitisin et al., 2013; Xu and Godber, 2001), and it has been found that anti-oxidative agents found in rice are stored as phytochemical secondary metabolites (Namdeo, 2007). Phenolic compounds are major phytochemicals found in rice grain (Deng et al., 2013). In this study, total phenolic content (TPC) was highest in Mun Poo (MP) rice extract (Table 2.) – consistent with a previous

Table 1. Colored-Rice Grain Samples Obtained from Different Rice Cultivation Areas in Thailand.

Colored-Rice Varieties	Name of Colored Grain	Description*
Red rice (Mun Poo, MP)	Mun Poo Phayao (MP-Phayao) Mun Poo Pink (half polished by hand) (MP-Pink) Mun Poo (half polished by machine) (MP-HPM)	- Sensitive to photoperiod - Cultivated in Phayao Province, Thailand
Brown rice (Sang Yod, SY)	Sang Yod Phattalung (half polished by machine) (SY-Phattalung HPM) Sang Yod Phattalung (half polished by hand) (SY-Phattalung HPH) Sang Yod Song Kla (SY-Songkla)	- Sensitive to photoperiod - Cultivated in Phattalung Province, Thailand - Cultivated in Songkla Province, Thailand
Black glutinous rice (Niew Dam, ND)	Niew Dam Phayao (ND-Phayao) Niew Doi Hang (ND-Doi Hang)	- Sensitive to photoperiod - Cultivated in Phayao Province, and Doi Hang District, Chang Rai Province, Thailand
Black non-glutinous rice (Hom Nil, HN)	Hom Nin Germinated (HN-Ngok) Hom Nin Tai Tai (HN-Tai Tai) Hom Nin Thinnakorn (HN-Thinnakorn) Hom Nin Phayao (HN-Phayao)	- Non-sensitive to photoperiod - Generally cultivated in every part of Thailand

*(BRRD, 2014; Rujirapisit et al., 2012)

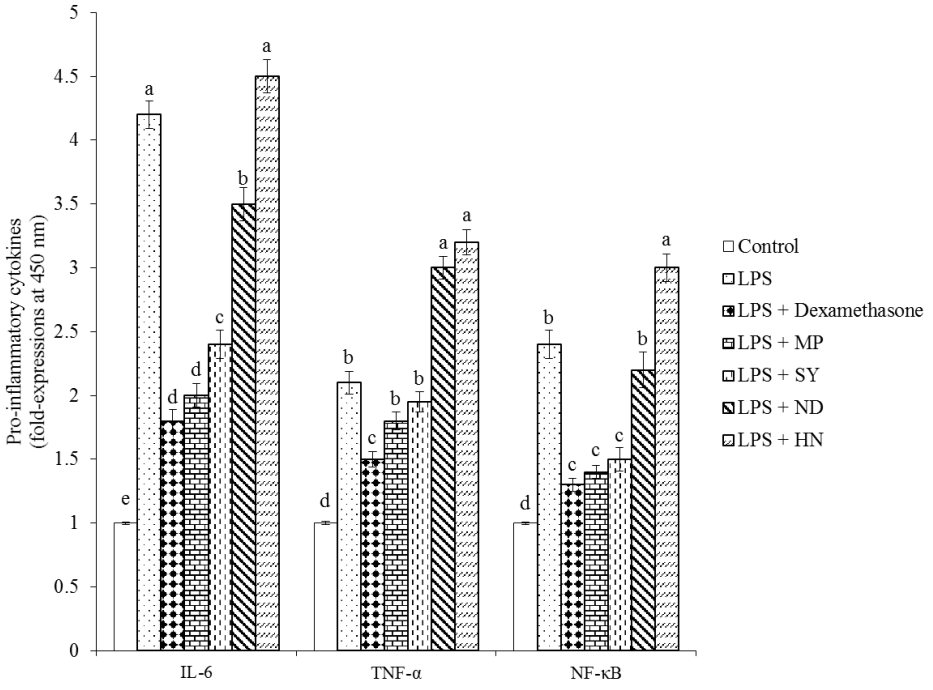

Fig 1. Relative fold-expression of pro-inflammatory cytokine (IL-6, TNF-α, and NF-κB) expression in HL-60-cell culture supernatants after pre-treatment with LPS (1 μg mL^{-1}) for 24 h, and post-treatment with 0.5 mg mL^{-1} of colored-rice extract (MP, SY, ND, and HN) or 4 mg mL^{-1} of dexamethasone. The effect of each treatment on pro-inflammatory cytokine expression assigned as fold-expression of pro-inflammatory cytokine from the treated group to those in the control, determined by ELISA. Results represented as means ± standard deviation of three independent determinations. Different letters indicate significant differences (P<0.05).

report from Shao et al. (2014), which showed that red rice had the highest TPC. Differences in TPC levels in each type of colored rice may result from the progressive metabolization of lignin during grain development (Boudet, 1998). However, we found no significant difference in TPC among the colored rice extracts tested (Table 2). Water soluble pigments, anthocyanins, are other phytochemicals found in the rice. This study revealed that black glutinous rice (ND) and brown rice (SY) accumulated significant amounts of anthocyanins as total anthocyanin content (TAC), compared with other rice varieties (Table 2). The results were similar to Zhang et al. (2010), who found that high amounts of anthocyanins in black rice may result from the richer pigmentation of the bran than white rice, and the distinctive development of outer bran layers and the endosperm of black rice. Moreover, cyaniding-3-glucoside (Cy-3-G), a prominent anthocyanin found most in black rice, followed by peonidin-3-glucoside (Pe-3-G), were a result of high TAC (Saikia et al., 2012). Furthermore, polishing methods in rice have changed from manual (retaining some bran and germ components) to machine in order to produce white rice without bran, germ components, and which contains only starchy endosperm (Shobana et al., 2001). This machine production technology may affect the total phenolic and anthocyanin contents of the extracts suggested in the study (Table 2). Previous studies have determined that colored rice exhibits greater anti-oxidative activity than white rice (Fujita et al., 2010; Nagai et al., 2002). The anti-oxidative properties of red rice (MP) showed the highest capacity to scavenge the DPPH radical (Table 2). The scavenging ability of the DPPH radical in the MP groups correlated with that observed with the TPC of MP. Like DPPH scavenging activity, MP extracts may act as an electron-donating substance, showing the highest ferrous reducing power activity (Table 2). Thus, the reducing capacity against DPPH radicals, and the ferrous reducing power of red rice extract can result from proanthocyanidins and condensed tannins, which are the most prevalent phenolics found in red rice, and which serve as the major anti-oxidative agents in red rice extract (Min et al., 2011; Saikia et al., 2012). Moreover, brown rice (SY) and red rice (MP) extracts showed high percentages of lipid peroxidation inhibition; both also contained high TAC (Table 2). Previous studies have suggested that anti-lipid peroxidation activities are correlated with high TAC, as the major free-radical scavenging activity in brown and red rice (Hansakul et al., 2011; Muntana and Prasong, 2010). An imbalance of free radicals can cause oxidative stress as well as induce inflammation and cancer (Reuter et al., 2010). Regarding the results of biological-compound analysis and anti-oxidative activity, the potential anti-inflammatory effects of colored rice extracts were evaluated with human promyelocytic leukemia (HL-60) cells, *in vitro*. After LPS-induced inflammation of HL-60 cells, post-treatment with MP extract exhibited the highest reduction in pro-inflammatory cytokine (IL-6, TNF-α, and NF-κB) production (Fig. 1). This result was similar to the study by Niu et al. (2013), which reported the anti-inflammatory (IL-1β, IL-6, and *COX-2* mRNA expressions) properties of red rice in RAW 264.7 mouse macrophage cells. The authors suggested that the anti-oxidative activities of the high phytochemical composition of red rice might be a major factor for its anti-inflammatory potential (Niu et al., 2013). Moreover, Prangthip et al. (2013) found that riceberry bran extract could reduce TNF- α and IL-6 in streptozotocin (stz)-induced diabetes rats, which may result from feruloyl esters, a component of γ-oryzanol. In this study, MP extracts had the highest capacity to reduce NO production in LPS-induced HL-60 cells (Fig. 2), which may result from high levels of anti-oxidative compounds and the ability to reduce pro-inflammatory cytokines, especially NF-κB (Fig. 1). In addition, γ-oryzanol in rice bran also exhibited the ability to reduce NO production in macrophage cells by inhibiting NF-κB activation (Nagasaka et al., 2007). Taken together, this revealed that the components of rice bran, especially from red rice, including phenolics, anthocyanins and others, support the finding that γ-oryzanol, α-tocopherol, and ferulic acid, synergize anti-oxidative activities to reduce inflammation *in vitro* and *in vivo* (Prangthip et al., 2013; Gunaratne et al., 2013; Niu et al., 2013). Matrix metalloproteinases (MMPs) comprise a family of extracellular matrix degrading enzymes that degrade various components of the extracellular matrix (ECM), and are believed to play an important role in tissue remodeling during inflammation (Chen et al., 2013). Previous studies have shown that coordinated up-regulation of pro-inflammatory cytokines (such as TNF-α and interleukins) stimulated MMP expression in immune cells (Siasos et al., 2012; Sprague and Khalil, 2009). However, imbalanced MMP expression can cause a progression of inflammation and lead to greater disease severity (Birrell et al., 2006). In this study, MP, SY, and ND significantly reduced MMP-2 expression in LPS-induced HL-60 cells (Fig. 3 and 4). A previous report found that anti-oxidative compounds (anthocyanins and polyphenol) can be used to inhibit HT1080 cell invasion and MMP-2 and MMP-9 expression *in vitro* (Nagase et al., 1998). Moreover, gelatinases (MMP-2 and MMP-9) can be activated through the MAP-Kinase, NF-κB, and AP-1 signaling pathways (Chandrasekar et al., 2006; Sun et al., 2007; Chen et al., 2013). Thus, this study suggests that colored rice extracts, especially those high in TPC and TAC, such as red rice (MP), can be used to reduce MMP-2 expression in HL-60 cells via their anti-oxidative activities.

Materials and Methods

Colored rice samples

The samples used in this study comprised of four varieties of colored rice (*Oryza sativa* L.) – red rice (Mun Poo, MP), brown rice (Sung Yod, SY), black glutinous rice (Niew Dam, ND), and black non-glutinous rice (Hom Nin, HN). Each colored rice variety was obtained from a different rice cultivation area (Table 1). The rice samples were kindly provided by Dr. Nisakorn Saewan, School of Cosmetic Science, Mae Fah Luang University, Muang, Chiang Rai, Thailand.

Preparation of colored rice extracts

Each colored rice grain sample was prepared according to the method described by Jang and Xu (2009), with some modifications. Each grain sample was weighed and extracted with absolute ethanol (Merck) at a ratio of 2:1 w v^{-1}. The mixture was vortex mixed for 1 hr. The supernatant was separated using paper filtration (Whatman no. 42 filter paper). The colored rice extracts were stored at 0ºC for later analysis.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) of each extract was evaluated by Folin-Ciocalteu colorimetric method, with slight

Table 2. Total Phenolic, Anthocyanin, and Anti-Oxidative Activities of Colored-Rice Extracts

Name of Colored-Rice Extract	Biological Compounds		Anti-Oxidative Activities		
	Total Phenolics (mg GAE mL^{-1} extract)	Total Anthocyanins (mg mL^{-1} extract)	DPPH Activity (SC_{50}) (mg mL^{-1})	Reducing Power (mg AAE mL^{-1} extract)	Inhibition of Lipid Peroxidation (%)
MP-Phayao	1.044 ± 0.031^{b}	0.309 ± 0.028^{a}	0.019 ± 0.002	0.361 ± 0.013^{b}	34.294 ± 0.852
MP-Pink	2.849 ± 0.104^{a}	0.028 ± 0.008^{b}	0.019 ± 0.006	1.137 ± 0.119^{a}	36.206 ± 0.951
MP-HPM	0.736 ± 0.145^{b}	0.111 ± 0.077^{b}	0.013 ± 0.003	0.384 ± 0.039^{b}	39.032 ± 0.554
Total Average	1.428 ± 0.985	0.144 ± 0.127^{B}	0.016 ± 0.005^{A}	0.592 ± 0.376^{A}	36.510 ± 2.380^{B}
SY-Phattalung HPM	0.806 ± 0.107^{b}	0.128 ± 0.031	0.147 ± 0.000^{b}	0.226 ± 0.031^{b}	50.352 ± 0.498^{b}
SY-Phattalung HPH	0.817 ± 0.054^{b}	0.051 ± 0.008	0.041 ± 0.000^{a}	0.209 ± 0.015^{b}	62.422 ± 0.747^{a}
SY-Songkla	1.944 ± 0.144^{a}	0.097 ± 0.034	0.034 ± 0.003^{a}	0.660 ± 0.046^{a}	25.227 ± 0.711^{c}
Total Average	1.297 ± 0.614	0.092 ± 0.040^{A}	0.068 ± 0.054^{B}	0.407 ± 0.239^{AB}	46.000 ± 18.967^{A}
ND-Phayao	0.703 ± 0.181^{b}	1.247 ± 0.312^{b}	0.057 ± 0.000	0.317 ± 0.090^{b}	29.976 ± 0.908
ND-Doi Hang	1.688 ± 0.102^{a}	5.172 ± 0.568^{a}	0.058 ± 0.000	0.565 ± 0.004^{a}	28.918 ± 0.782
Total Average	1.097 ± 0.557	2.817 ± 2.179^{A}	0.057 ± 0.000^{AB}	0.416 ± 0.150^{AB}	29.447 ± 0.748^{C}
HN-Ngok	0.234 ± 0.040^{b}	0.262 ± 0.252	0.027 ± 0.014^{a}	0.041 ± 0.008^{c}	12.425 ± 0.778^{c}
HN-Tai Tai	1.319 ± 0.153^{a}	1.038 ± 1.397	0.058 ± 0.000^{b}	0.411 ± 0.093^{a}	19.055 ± 0.641^{c}
HN-Thinnakorn	0.572 ± 0.147^{b}	1.365 ± 0.944	0.060 ± 0.000^{b}	0.192 ± 0.024^{b}	27.382 ± 0.040^{b}
HN-Phayao	0.512 ± 0.052^{b}	0.375 ± 0.028	0.043 ± 0.010^{b}	0.247 ± 0.034^{b}	32.960 ± 0.038^{a}
Total Average	0.636 ± 0.393	0.782 ± 0.816^{B}	0.048 ± 0.015^{AB}	0.222 ± 0.130^{C}	26.466 ± 6.998^{C}

Results expressed as means ± SD (n = 3). ANOVA was followed by nonparametric Kruskal-Wallis test and Mann-Whitney U test to compare groups. Different letters indicate significant differences (P<0.05).

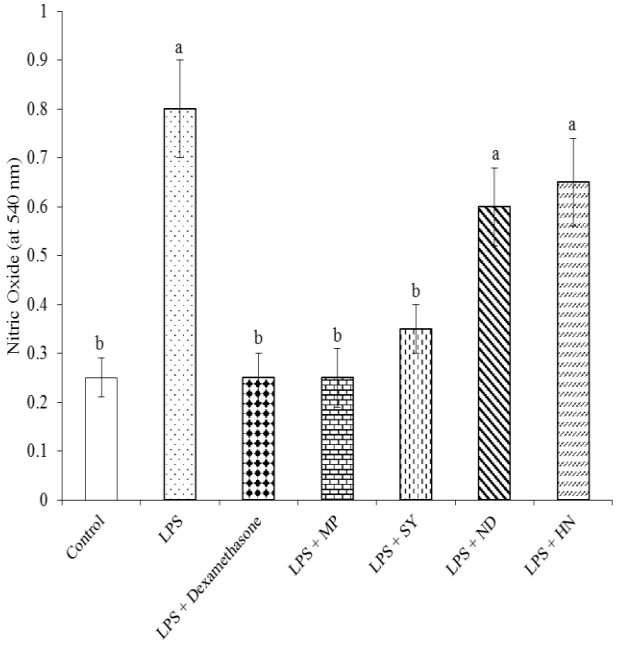

Fig 2. Nitric oxide (NO) production in the HL-60 cell culture supernatants, after pre-treatment with LPS (1 µg mL^{-1}) for 24 h and post-treatment with 0.5 mg mL^{-1} colored-rice extract (MP, SY, ND, and HN) or 4 mg mL^{-1} dexamethasone. The effect of each treatment on NO production was determined by Griess assay compared with control. Results represented as means ± standard deviation of three independent determinations. Different letters indicate significant differences (P<0.05).

modification (Gajula et al., 2009). 12.5 µl of Folin-Ciocalteu's phenol reagent was added to 12.5 µl of extract sample and diluted with 50 µl distilled water. After 5 min, 125 µl of 7% $NaNO_3$ solution was added to the mixture and incubated for 90 min at 25ºC. The absorbance of the sample was then measured at 750 nm against a blank using a microplate reader (Sunrise^{TM} TECAN, Switzerland). A standard curve of total phenolics was constructed using gallic acid standard solution. The experiments were conducted in triplicate. The results are expressed as mg gallic acid equivalents g^{-1} dry colored rice grain sample.

Determination of total anthocyanin content (TAC)

The monomeric anthocyanin pigment content of each colored rice extract was evaluated by pH-differential method, modified from Giusti and Wrolstad (2001). 100 µl of potassium chloride buffer solution (pH 1.0) was mixed with 100 µl of colored-rice extract sample, and 100 µl of sodium acetate buffer solution (pH 4.5) was mixed with 100 µl of colored-rice extract sample. Both solutions were left to equilibrate for 30 min. The absorbance of each solution was measured at 520 nm and 700 nm. The pigment content was

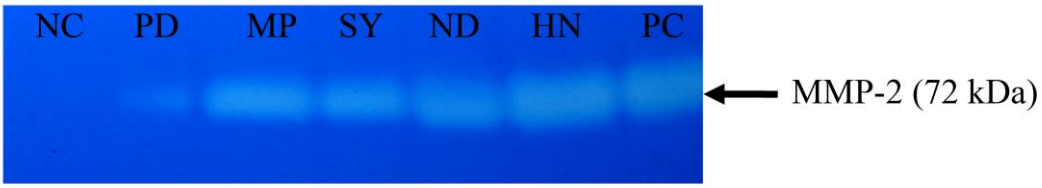

Fig 3. MMP-2 expression in HL-60 cell culture supernatants after pre-treatment with LPS (1 µg mL^{-1}) for 24 h, and post-treatment with 0.5 mg mL^{-1} of colored-rice extract (MP, SY, ND, and HN) or 4 mg mL^{-1} of dexamethasone (PD), determined by gelatin zymography, compared with culture medium (negative control, NC). The supernatant from HL-60 cells induced by PMA was used as a positive expression of MMP-2 (PC). The results represent three independent experiments.

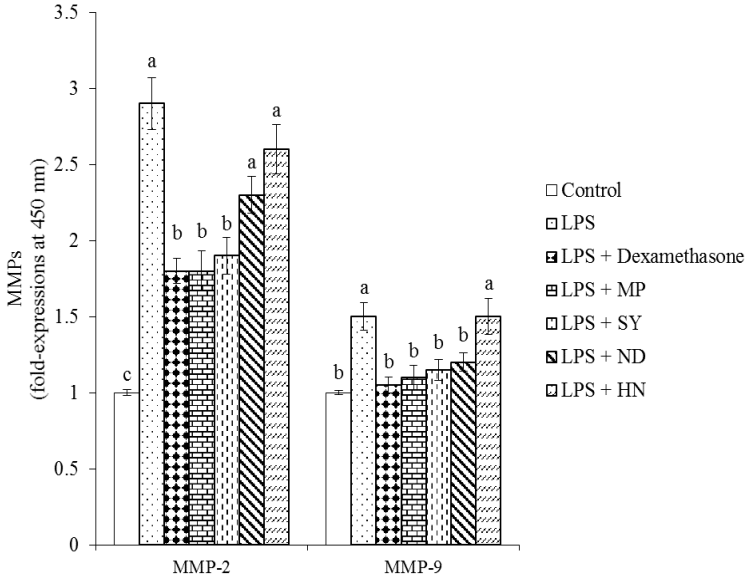

Fig 4. Relative fold-expression of MMP-2 and MMP-9 expression in HL-60 cell culture supernatants after pre-treatment with LPS (1 µg mL^{-1}) for 24 h, and post-treatment with 0.5 mg mL^{-1} colored-rice extract (MP, SY, ND, and HN) or 4 mg mL^{-1} dexamethasone. The effect of each treatment on MMP-2 and MMP-9 expression, assigned as fold-expression of MMP-2 and MMP-9 from the treated group to those in the control, determined by ELISA. Results represented as means ± standard deviation of three independent determinations. Different letters indicate significant differences (P<0.05).

represented as milligrams cyanidin-3-glucoside 100 g^{-1} fresh weight, using an extinction coefficient (ε) of 26,900 l cm^{-1} mol^{-1} and molecular weight (MW) of 449.2 g mol^{-1}. The absorbance equation for the diluted solution was calculated, as follows: $A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$.
The monomeric anthocyanin pigment concentration in the original sample was calculated as follows: Monomeric anthocyanin (mg/L) = (A x MW x dilution factor (DF) x 1000) / (ε x 1)

Determination of diphenyl picryl hydrazyl (DPPH) radical scavenging activity

The free-radical scavenging activity of each colored-rice extract was evaluated according to Kitisin et al. (2013), with some modifications. The study was used to determine the anti-oxidative activity of colored rice extract samples. DPPH (2,2-diphenyl-1-picryl hydrazyl) was purchased from Sigma-Aldrich (USA). An aliquot of 5 µl of each sample or 100% DMSO (as a negative control) was reacted with 195 µl of 100 µM DPPH ethanolic solution in a 96-well microplate. The mixture was kept at room temperature in a dark room for 30 min. Absorbance was measured at 517 nm against a blank, using a microplate reader. Percentage scavenging effect was calculated using the equation below: % radical scavenging activity = $[(A_{control} - A_{sample}) / A_{control}] \times 100$
The scavenging activities of colored rice extract samples were expressed as 50% scavenging concentration, SC_{50} (mg mL^{-1}), obtained by interpolation from the linear regression analysis.

Determination of ferrous reducing power

Ferrous-reducing power was used to measure the reduction of $[Fe(CN)_6]^{3-}$ to $[Fe(CN)_6]^{4-}$ by anti-oxidants, a blue complex formed by excess Fe^{3+} ions, expressed as ascorbic acid equivalents (AAE). Potassium ferricyanide and trichloroacetic acid (TCA) were purchased from Wako Chemicals (Osaka, Japan). The anti-oxidative activity of colored rice extracts, determined by ferrous reducing power method, was determined using the method of Kuda and Yano (2009), with slight modification. 25 µl of each sample solution were mixed with 25 µl of 0.1 mol L^{-1} phosphate buffers (pH 7.2) and 5 µl of 1 g 100 mL^{-1} potassium ferricyanide in a 96-well microplate. After incubation at 37°C for 60 min, 25 µl of 10% TCA were added; absorbance was measured at 700 nm (A1). Then 25 µl of 0.1% ferric chloride ($FeCl_3$) was added to the mixture, and the absorbance was

measured again (A2). Ferrous reducing power was calculated as follows: Reducing power (OD) = ($A2_{sample}$ – $A1_{sample}$) - ($A2_{control}$ – $A1_{control}$)

Determination of lipid peroxidation

Lipid peroxidation inhibition activity was determined according to the thiobarbituric acid reactive substances (TBARS) method (Armstrong and Browne, 1994), with some modifications. 100 µl of colored rice extract was mixed with 900 µl linoleic acid, then incubated at 100°C for 20 min. One ml of saline buffer solution (pH 3.5) and 1 ml of the mixture of 20 mM of TBA in 10% TCA were added to the final mixed solution, then incubated at 100°C for 30 min, to generate a pink-colored product. Absorbance was measured at 532 nm using a microplate reader. Percent inhibition of lipid peroxidation was calculated as follows:
% Inhibition of lipid peroxidation = [($A_{control}$ – A_{sample})/$A_{control}$ x 100]

In vitro HL-60 culture preparation and differentiation

A human promyelocytic leukemia (HL-60) cell line was obtained from the American Type Culture Collection (ATCC); it was maintained in RPMI 1640 (Gibco-Invitrogen, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, USA), and 1% antibiotics (200 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin, Gibco, USA) at 37ºC with 5% concentration of CO_2 incubator. The cells were detached from the surface using trypsin/EDTA (PAA Laboratories GmbH, Austria). HL-60 cells were subcultured every 2-3 days. HL-60 cells underwent differentiation in the granulocytic pathway when the cells reached $3x10^5$ to $4x10^5$ cells mL^{-1}, in RPMI 1640 supplemented with 0.7% dimethylformamide (Sigma-Aldrich, Canada) for four days (Mullick et al., 2004). The HL-60 cells, cultured in a 96-well microplate, were divided into three groups to determine the anti-inflammatory activities of the colored rice extracts. In Group 1, normal HL-60 cells were supplemented only with growth medium. In Group 2, inflammatory induced HL-60 cells, pre-treated 24 hr with 1 µg mL^{-1} of lipopolysaccharide (LPS; Sigma-Aldrich, USA) (Liao et al., 2006). Group 3, 24 hr pre-treated HL-60 cells with LPS were post-treated with 0.5 mg mL^{-1} of colored rice extract from four varieties (MP, SY, ND, and HN) for 24 hr to investigate the anti-inflammatory activity of the extracts. The supernatant from each treatment group was used to determine the levels of pro-inflammatory cytokines, including interlukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and nuclear factor kappa B (NF-κB), and the levels of matrix metalloproteinase (MMP) expression.

In vitro colorimetric MTT (tetrazolium) assay for cell viability

The colorimetric MTT assay used was similar to that described by Mosmann (1983). A stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich, USA) was prepared by dissolving 5 mg MTT mL^{-1} in phosphate buffered saline (PBS) solution at pH 7.5 (Sigma-Aldrich, USA) and filtered through a 0.2 µm filter. After 24 hr incubation in each experimental group, the supernatant was removed and collected for further pro-inflammatory cytokine detection by enzyme-linked immunosorbent assay (ELISA). The HL-60 cells were washed twice with PBS. MTT solution was added at 20 µL of MTT in each well and incubated at 37ºC in the dark for 3 hr. The MTT solvent from each well was removed and washed with PBS. Then, 150 µl of dimethyl sulfoxide (DMSO; Amresco, USA) were added to each well. Plate absorbance was measured at 570 nm on a microplate reader, with a reference wavelength of 630 nm. Plates were normally read within 1 hr after adding DMSO. Percent cell viability was calculated using the equation below:
% cellular mitochondrial activity = [(A_{570}–A_{630}) / (A^o_{570}-A^o_{630})] × 100
Whereas; A_{570} = $Absorbance_{570nm}$ of sample
A_{630} = $Absorbance_{630nm}$ of sample background
A^o_{570} = $Absorbance_{570nm}$ of control
A^o_{630} = $Absorbance_{630nm}$ of control background

In vitro pro-inflammatory cytokines (IL-6, TNF-α, and NF-κB) detection

The pro-inflammatory cytokine concentrations in the supernatant sample from each group were determined using commercially available kits (IL-6: R&D Quantikine® ELISA Cat.No. D-6050, TNF-α: R&D Quantikine® ELISA Cat.No. D-TA00C, and NF-κB: Invitrogen ELISA Cat.No. KH-O0371) following the manufacturer's instructions. Absorbance was measured at 450 nm by microplate reader. Dexamethasone, at 4 mg mL^{-1}, was used as positive treatment for pro-inflammatory cytokine inhibitory effects.

In vitro nitric oxide scavenging activity

The supernatant from each group treatment was used to determine the nitric oxide (NO) scavenging activity by using Griess reagent, following Promega Code TB299. 500 µl of cell supernatant was added to a solution (500 µl) of Griess reagent (1% sulfanilamide, 0.1% naphythyl ethylenediamine dihydrochloride in 5% H_2PO_4). Absorbance was read at 546 nm. NO production was determined and calculated from the nitrate standard curve.

In vitro matrix metalloproteinase (MMP) detection

The gelatinolytic activity of MMP-2 and MMP-9 in each supernatant sample was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with some modification (Luplertlop and Missé, 2008). The supernatant from HL-60 cells, induced by 10 ng mL^{-1} of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, USA), was used as a positive control. Briefly, 20 µl of supernatant sample was run without prior denaturation on an 8% SDS-PAGE embedded with 0.1% gelatin. Subsequently, the gel was stained with Coomassie Brilliant Blue R-250 and de-stained in 30% methanol/10% acetic acid (v v^{-1}). The areas of protease activity appeared as clear bands against a dark blue background, where the protease had digested the substrate. Gels were scanned and the density of each band was semi-quantified with a computer assisted image analysis program (1D Image Analysis Software, Kodak Digital Science v.3.0, Eastman Kodak, Rochester, NY, USA). Gelatin zymography is useful for the detection of pro-MMP-2 (72 kDa), active-MMP-2 (64 kDa), pro-MMP-9 (92 kDa), and active-MMP-9 (67 kDa) in cell culture supernatants.
The gelatinolytic MMPs were also determined in the supernatant sample from each treatment by using R&D Quantikine® ELISA Kits (Cat.No. DMP2F0 for MMP-2 and Cat.No. DMP900 for MMP-9) as following the manufacturer's instructions. Absorbance was measured at 570 nm by microplate reader.

Statistical analyses

Each independent experiment was carried out in triplicate. The results were expressed as means ± standard deviation (SD). SPSS (statistical package version 16, SPSS Inc., Chicago, IL, USA) software program was used to conduct the analyses. The results of each experiment were analyzed using one-way analysis of variance (ANOVA) followed by a nonparametric Kruskal-Wallis test. The Mann-Whitney U test was used to compare groups. Differences were considered statistically significant at $P<0.05$.

Conclusion

The findings from this comparison of different colored rice varieties in Thailand provide new information on their varying potential anti-inflammatory properties. Further in-depth studies of the effects of these anti-oxidative activities in an *in vivo* model need to be conducted. This study may provide a factual basis for the alternative use of colored rice extracts as potential anti-oxidative sources to reduce inflammation, which can be exploited to produce high-value products for cosmetic and pharmaceutical purposes.

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