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Research article

Phytochemical and bioactivity investigation of Thai pigmented-upland rice: Dam-Mong and Ma-led-Fy varieties

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Abstract

Importance of the work: This is the first data on the bioactive compounds and biological activities of two Thai pigmented-upland rice varieties (Dam-Mong and Ma-led-Fy).

Objectives: To examine the bioactive compounds and biological activities of the glutinous Dam-Mong and non-glutinous Ma-led-Fy Thai pigmented-upland rice varieties.

Materials & Methods: Dam-Mong and Ma-led-Fy rice varieties were extracted using 50% ethanol to obtain Dam-Mong extract (DME) and Ma-led-Fy extract (MFE). The extracts were evaluated for their bioactive compounds (total phenolic, total flavonoid, anthocyanins and cyanidine-3-O-glucoside contents) and biological activities (anti-oxidant, anti-glucosidase, immunomodulatory and anti-inflammatory).

Results: MFE and DME had antioxidant and anti-glucosidase enzyme activities. They both stimulated mouse splenocyte proliferation that revealed an immune-enhancing effect without cytotoxicity. In addition, in lipopolysaccharide-induced inflammatory macrophage cells, both rice extracts inhibited the up-regulated expression of inflammatory-related genes (cyclooxygenase-2, interleukin-1β, inducible nitric oxide synthase and tumor necrosis factor-α). The extracts inhibited the production of prostaglandin E2 and nitric oxide. Furthermore, they had anti-edematic action on rat's paws in a dose-dependent manner. In general, MFE had greater bioactivity than DME, which was related to their respective amounts of phytochemical contents.

Main finding: Overall, for the first time, this study indicated that Thai pigmented-upland rice extracts could be developed as potential natural substances for novel dietary supplements or cosmeceutical products, especially the Ma-led-Fy variety.

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Introduction

Rice (*Oryza sativa* L.) is considered not only as a basic macronutrient source of carbohydrate to provide energy to populations worldwide, it also is an important source from which to deliver other micronutrients, including minerals, vitamins and phytochemicals (Reddy et al., 2017). Besides the well-known constituents, such as oryzanols, vitamin E, amino acids and essential fatty acids, several phenolic compounds from rice have been shown to confer antioxidant, anti-inflammatory, anti-allergic and anticancer effects (Paiva et al., 2014). For health-related purposes, eating pigmented rice is acknowledged as a better source of bioactive compounds than white rice (Shao et al., 2018. Anthocyanin as a flavonoid is most commonly found in the pericarp of black rice, which has given rise to a range of purple to black colors of rice. Several phytochemical studies of pigmented rice showed differences in the phenolic compounds and anthocyanin content, such as cyanidin-3-O-glucoside and peonidin-3-O-glucoside, in Japanese black-purple rice pericarp (Pereira-Caro et al., 2013), while a different amount of total anthocyanin was found in Indonesian pigmented rice (Fatchiyah et al., 2020). In terms of bioactivity, black rice has reported high antioxidant capacity (Irakli et al., 2012), anti-inflammatory (Zhao et al., 2021) and anticancer (Ghasemzadeh et al., 2018) effects that are attributed to the immune response. In addition, the supportive data on the immunomodulatory effect of rice have been reported, such as rice milk modulated the acquired immune response by increasing IL-2 production (Bub et al., 2003) and rice extract stimulated the production of IL-10 (Yamazaki et al., 2008), which is a secreted cytokine from monocytes in response to inflammation, allergies and autoimmune disorder (Cominelli, 2004).

Indigenous upland rice has been selected for tolerance to particular constraints, such as drought, poor soil quality and water supply, all of which ultimately affect yield performance and nutritional values (Phapumma et al., 2020. Research has reported on the development of upland rice cultivation to improve the yield potential and grain quality (Phapumma et al., 2020) and functional properties constituents, such as high anthocyanin and phenolic compounds (Sutharut and Sudarat, 2012; Vichapong et al., 2010). Nowadays, upland rice has been increasingly grown intercropped with newly planted rubber and oil palm to help increase a farmer's income (Somsana et al., 2013; Phapumma et al., 2020). There are many kinds of rice, including lowland, deep-water and upland rice, for different growing ecologies. For example, upland rice is grown on dry soil without surface water accumulation (Phapumma et al., 2020. Therefore, upland rice varieties were the focus of the current study due to their adaptability to a wide range of unfavorable environments and their resistance to diseases, insects, and their high tolerance to drought (Narenoot et al., 2017). In contrast lowland rice varieties are grown under waterlogged paddy conditions and need sufficient water throughout the growing season (Zaman et al., 2018. 'Dam-Mong' is an glutinous pigmented-upland rice which has a black seed coat, light-sensitive behavior, short life span and generally it is cultivated around August. 'Ma-led-Fy' is a non-glutinous pigmented-upland rice which originated from southern Thailand. It has brown-purple seed and its cultivation period is from the beginning of June to the end of October (Table 1). Both rice varieties have been developed at the Agronomy Station, Khon Kaen University and are widely distributed to the community according to their good flavor; however, data are limited on their health benefits. The current study investigated the phytochemical content, antioxidant, alpha-glucosidase inhibitory, immunomodulatory and anti-inflammatory activities of these two rice varieties. The results should provide essential data to support promotion for their health benefits.

Materials and Methods

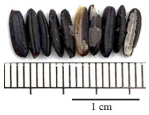
Preparation of rice extracts

The Dam-Mong and Ma-led-Fy rice samples were obtained from the Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Thailand. Whole grain rice without hulls was ground into powder and macerated using 50%ethanol (1 g: 6 mL ratio) for 6 d and filtered. Using a rotary evaporator (ETERA, Japan), the filtrate was concentrated at 45–50 °C and then processed in a freeze-dryer (Christ, Germany). The ethanolic extracts of Ma-led-Fy (MFE) and Dam Mong (DME) were obtained with yields of 4.8% and 3.5%, respectively.

Phytochemical analysis

The total phenolic content was determined using the modified Folin-Ciocalteu method and tannic acid as a standard solution (Sripanidkulchai and Fangkrathok, 2014). After dissolving a 20 µL sample in 50% ethanol, it was mixed with 100 µL of 1N Folin-Ciocalteau reagent and 80 µL of 7% sodium carbonate solution in a 96-well plate. After incubation at room temperature for 30 min, the optical density was measured at 760 nm and the total phenolic content was expressed as milligrams of tannic acid equivalent (TAE) per gram of extract.

Table 1 General features and physical characteristics of Dam-Mong and Ma-led-Fy rice samples

Parameter	Rice variety	
	Dam-Mong (ULR017)	Ma-led-Fy (ULR291)
General features		
Type	Glutinous	Non-glutinous
Growing season	August–December	June–November
Productivity (kg/ha)	2,652	2,180
Amylose content (%)	4.64	21.92
Grain shape and color		
Physical characteristics of seed		
Length (mm)	8.42±0.54[a]	7.96±0.60[a]
Width (mm)	2.81±0.10[a]	2.74±0.34[a]
Weight (mg)	32.48±2.44[a]	25.32±2.24[b]
L*(Brightness) *	38.45±0.22[a]	47.06±1.16[b]
a*(Red-green) *	0.55±0.21[a]	4.91±0.80[b]
b*(Yellow-blue) *	1.33±0.20[a]	3.49±0.42[b]
Morphological image **		

Mean ± SD (n = 3) in each row superscripted with different lowercase letters indicate significant ($p < 0.05$) differences.
*Rice color conducted using L*a*b* system with an intensity meter (Hunterlab, USA);
**Rice grains photographed using a digital camera (D5300; Nikon, Japan)

The total flavonoid content was determined using the modified colorimetric method and quercetin as a standard solution (Zhao et al., 2018). After dissolving the sample in 50% ethanol (100 µL), it was mixed with 5% sodium nitrite (20 µL) in a 96-well plate and incubated at room temperature for 6 min; then, 10% aluminum chloride (35 µL) was added and kept for another 6 min. The mixture was measured at 430 nm and the total flavonoid content was expressed as milligrams of quercetin equivalent (QE) per gram of extract.

The total anthocyanin content was determined using the modified pH differential spectrophotometric method (Giusti and Wrolstad, 2005). The sample was separately dissolved in 0.025 M potassium chloride buffer (pH 1.0) and 4 M sodium acetate (pH 4.5) and the absorbance was measured at wavelengths of 520 and 700 nm. The total anthocyanin content was calculated using a molecular weight of cyanidin-3-glucoside at 449.2 and extinction coefficient of 26,900 and expressed as milligrams per gram of extract.

The content of cyanidin-3-glucodide was quantitated using a high-performance liquid chromatography (HPLC) method, as modified from Chaiyasut et al. (2018). The sample, dissolved in methanol, was filtered through a 0.45 µm syringe and 20 µL of filtrate was injected in triplicate. The analytical system (Agilent 1109 series) consisted of a reverse phase C18 Agilent hypersil OSD column (5 um, 4.6×250 mm) and a gradient mobile phase (A: 0.5% acetic acid in acetonitrile, B: 0.5% acetic acid in deionized water with A: B from 5:95 to 10:90 for 5 min and 10:90 to 60:40 for 35 min) at a flow rate of 1 mL/min. The detection was carried out at 254 nm and 30 °C. The peak area and retention time were compared with the standard cyanidin-3-glucodide.

In vitro bioactivity studies

The antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method as modified from (Likhitwitayawuid et al., 2006). Briefly, 100 µL of various concentrations of sample dissolved in ethanol were mixed with 100 uL of 1M DPPH solution in a 96-well plate and incubated for 30 min at room temperature when an absorbance of 517 nm was recorded. The radical scavenging inhibition was determined out and defined as 50% effective concentration (EC_{50}). Ascorbic acid was used as positive controls and ferric reducing antioxidant power (FRAP) assay (Famurewa et al., 2019) was carried out. Briefly, the FRAP reagent, containing 300 mM acetate buffer (pH3.6), 20 mM ferric chloride and 10 mM 2,4,6-tripyridyl-s-triazine, was freshly prepared. The reaction mixture, composed of a sample dissolved in 50% ethanol (6 µL), deionized water (18 µL) and FRAP reagent (180 µL), was incubated in

a 96-well plate for 4 min and the absorbance was measured at 600 nm. Ferrous sulfate was used to construct the standard curve and the value was expressed as millimoles per milligram of extract.

The alpha-glucosidase inhibitory activity was performed using p-nitrophenyl-D-glucopyranoside (PNPG) as a substrate (Matsui et al., 1996). The reaction mixture containing 20 µL of each of sample, glucosidase enzyme (1 unit/mL) and 0.1 M sodium phosphate buffer (pH 6.8) was pre-incubated in a 96-well plate at 37 °C for 20 min; then, the 2 mM of the PNPG substrate (20 µL) was added and further incubated for 30 min. Finally, 1 mM sodium carbonate (40 µL) was added and the absorbance of released p-nitrophenol was measured at 405 nm. Acarbose and dimethyl sulfoxide were used as the positive and negative controls, respectively. The value was expressed as IC_{50} in terms of milligrams per milliliter.

Cell-based studies

The immunomodulatory effect was carried out in mouse splenocytes, as previously described (Fangkrathok et al., 2014). Briefly, splenocytes were freshly prepared from Balb/cMlac mouse spleen and cultured in RPMI 640 medium supplemented with 10%fetal bovine serum and 1%antibiotic-antimycotic solution (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL Gibco Amphotericin B). The splenocytes (1×10^7 cells/mL) were incubated with the test sample (50 µL) and allowed to proliferate in the absence and presence of 50 µL of Phytohemagglutinin (PHA, 25 µl/mL) or poke weed mitogen (PWM, 1 µg/mL). Incubation was done in a humidified atmosphere with 5% CO_2 at 37°C for 48 h. The cells were stained with Almar blue for 3 h and measured under the spectrofluorometer (Excitation: 480 nm, Emission 520 nm); then, the % cell viability was calculated.

The anti-inflammatory effect was determined in the murine macrophage cell line, RAW 264.7 cells (Promo Cell, Germany).

(1) For cytotoxicity testing, the cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated calf serum (HyClone, USA) and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml), followed by incubation in a humidified atmosphere with 5% CO_2. Various concentrations of the tested samples were incubated with the cells for 24 h and the cell viability was analyzed using MTT assay (Mosmann, 1983). The absorbance was measured at 570 nm and the results were expressed as 50% inhibitory concentration (IC_{50}).

(2) Nitric oxide production was performed with slight modification of the method of Yang et al. (2009). To induce inflammation, the cells (1.5×10^5 cells/mL) were pre-incubated with *Escherichia coli* lipopolysaccharide (LPS) at a concentration of 1 µg/mL and the tested sample for 24 h, then nitrite as a stable metabolite of nitric oxide in the culture media was measured using Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid). Next, 100 mL of each of cell culture medium and Griess reagent were mixed and incubated at room temperature for 10 min and the absorbance was measured at 540 nm. Aminoguanidine and fresh culture medium were used as the positive control and blank, respectively. The results were expressed as 50% inhibitory concentration (IC_{50}).

(3) Prostaglandin E2 (PGE2) production was measured using an immunoassay (Prostaglandin E2 ELISA Kit, MyBioSource, USA) according to the manufacturer's specifications. The cells were cultured overnight in a 12-well plate and treated with various concentrations of tested samples at 37 °C for 24 h; then, LPS was added and further incubated for 24 h. The culture supernates (100 µL) were mixed with primary antibody (50 µL) and PGE2 conjugate (50 µL) and incubated at room temperature. After washing five times with wash buffer, the substrate (100 µL) was added and incubated at room temperature for 20 min; then, the absorbance was measured at 450 nm and compared with the standard curve. The results were expressed as 50% inhibitory concentration (IC_{50}).

(4) The reverse transcriptase-polymerase chain reaction (PCR) method was used to determine the expression of three inflammatory-related genes: COX-2, IL-1β, iNOS and TNF-**α**. The cells were cultured overnight in a 12-well plate and treated with different concentrations of the tested samples. LPS was added after incubation at 37 °C in a humidified atmosphere with 5% CO_2 for 22 h and then incubated for another 2 h. Using an extraction kit (GE Healthcare, United Kingdom, total RNA was collected from the treated cells. A reverse transcriptase kit (Omniscript, Germany was used to synthesize the first-strand cDNA from total RNA (40 ng). The primers were used to amplify the respective fragments. PCR was accomplished by incubating every cDNA sample with the primers, Taq polymerase and deoxynucleotide mix. Amplification was carried out for 30 cycles and the conditions aligned with prior reports (Won et al., 2006; Sripanidkulchai et al., 2009; Sato et al., 2011). The primer sequences were: β-actin, 5'-TCATGAAGTGTGACGTTGACATCCGT-3' (forward) and 5'-CCTAGAAGCATTTGCGGT GCACGATG-3' (backward); COX-2, 5'-GGAGAGACTATCAAGATAGT-3' (forward) and 5'-ATG GTCAGTAGACTTTTACA-3' (backward); IL-1β, 5'-CAGGAT GAGGACATGAGCACC-3' (forward) and 5'-CTCTGCAGACTCAAACTCCAC-3' (backward); iNOS, 5'-AATGGCAACATCAGGTCGGCC ATCACT-3' (forward) and 5'- GCTGTGTGTCACAGAAGTCTCGAACTC-3'

(backward); TNF-α, 5'-ATGAGCACAGAAAGCATGATC-3' (forward) and 5'-TACAGGCTTGTCACTCGAATT-3' (backward). The RT-PCR product densities were acquired using a Gel Documentation and System Analysis machine (Syngene, United Kingdom, while the PCR products were inspected on 1.5% agarose gel and visualized using Novel Juice (Sigma-Aldrich/supplier, Germany staining. The inflammatory-related gene expressions were presented as the relative expression level of β-actin.

Animal studies

Male Sprague-Dawley rats, each aged 7–8-wk and weighing 280–320 g, were obtained from the National Animal Center, Mahidol University, Thailand. In each hanging cage, 3–5 rats were housed and kept in an air-conditioned room with a 12 hr light-dark cycle. Commercial pellet food and tap water were provided *ad libitum*. Throughout the experiment, the rats were anesthetized using intraperitoneal injection with 100 µL thiopental sodium (50 mg/kg body weight). The rats were arbitrarily separated into eight groups of five so that there were ten paws in each group. Both left and right paws were injected with 0.15 mL of 0.1% carrageenan into the sub plantar region just below the lateral malleolus to induce the paw edema (Sripanidkulchai et al., 2009). The animals were intra-peritoneal injected as follows: Group1, normal saline as a negative control; Groups 2–4, MFE treated with three different doses; Groups 5–7, DME treated with three different doses; and Group 8, diclofenac as a positive control. The treatment lasted 7 hr and paw swelling was expressed as the edema rate (ER (%) = $(V_t - V_0)/V_0 \times 100$, where V_t is the volume at time t after treatment and V_0 is the volume at time 0).

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± SD. The data were compared between treated groups and control groups. One-way ANOVA and multiple comparisons (LSD) were used to analyze significant differences at $p < 0.05$.

Ethics statements

Animal care procedures were approved by the Animal Ethics Committee of Khon Kaen University, Thailand (Record No. IACUC-KKU-27/61 / Reference No. 0514.1.75/14 and Record No. IACUC-KKU-59/61 / Reference No. 0514.1.75/47 for the examination of immunomodulatory and anti-inflammatory activities, respectively).

Results and Discussion

Pigmented rice is reportedly more nutritious than regular white rice and nowadays it is commonly consumed due to its health-promoting effects (Shao et al., 2018. Multiple bioactivities of black rice were associated with its phytochemical contents (Ito and Lacerda, 2019). Two varieties of pigmented-upland rice, glutinous Dom-Mong and non-glutinous Ma-led-Fy, were studied because they are commonly known as an intercropped plant for which there is limited health-benefits data. The 50% ethanol extracts of rice grain were investigated in this study based on preliminary data that demonstrated Ma-led-Fy contained the highest yield and contents of total phenolics and total flavonoids compared to rice aqueous and 99% ethanol extracts (yield of the extracts were 5.49 ± 0.64%, 3.20 ± 0.16% and 2.36 ± 0.32%, respectively; of total phenolics were 537.67 ± 0.38 mg/g, 357.79 ± 0.26 mg/g and 253.41 ± 0.18 mg/g, respectively; of total flavonoids were 352.21 ± 0.25 mg/g, 163.92 ± 0.12 mg/g and 133.67 ± 0.10 mg/g, respectively). In addition, the current study focused on the rice pericarp due to reporting of upland rice varieties, most of which involved the study of active substances and their pharmacological activity in the rice pericarp part (Vichapong et al., 2010; Sutharut and Sudarat, 2012).

Physical characteristics and phytochemical contents

In general, both pigmented-upland rice varieties had similar physical properties (Table 1). However, Ma-led-Fy had slightly greater L*, a* and b* values than Dam-Mong with a smaller seed size (Table 1). In terms of phytochemical contents, the rice extracts had high contents of bioactive compounds (Table 2). However, MFE had relatively higher total phenolic, total flavonoid, total anthocyanin and cyanidine-3-O-glucoside contents than those of DME. These findings indicated the high phytochemical levels of these two rice varieties and supported the reported high nutritional values of pigmented rice varieties (Ito and Lacerda, 2019).

In vitro studies

Although both Ma-led-Fy and Dam-Mong are indigenous up-land rice varieties, their genotypes are different (Phapumma et al., 2020). The current results indicated that their phenotypic characteristics were different in terms of the contents of amylose and bioactive compounds (total phenolics, total flavonoids and anthocyanin contents), which was in agreement with another report (Somsana et al., 2013). Therefore,

Table 2 Phytochemical contents, antioxidant and alpha-glucosidase inhibitory activities of Dam-Mong (DME) and Ma-led-Fy (MFE) rice extracts

Parameter	Rice variety extract	
	DME	MFE
Phytochemical contents		
Total phenolic (mg TAE/g)	259.18±0.57[a]	537.67±0.38[b]
Total flavonoid (mg QE/g)	242.04±1.00[a]	352.21±0.25[b]
Anthocyanins (mg/g)	26.43±0.51[a]	27.16±0.92[a]
Cyanidine-3-O-glucoside (mg/g)	0.50±0.01[a]	0.62±0.07[b]
Antioxidative activity		
DPPH (EC_{50}, µg/mL)*	69.56±0.05[a]	61.67±0.05[b]
FRAP (mM/mg extract)	9.83±0.87[a]	19.48±1.68[b]
Anti-glucosidase enzyme activity (IC_{50}, mg/mL)**	1.00±0.02[a]	0.70±0.02[b]

Mean ± SD (n = 3) in each row superscripted with different lowercase letters indicate significant ($p < 0.05$) differences.

TAE = Tannic acid equivalent; QE = Quercetin equivalent; DPPH = 2,2-Diphenyl-1-picrylhydrazyl; EC_{50} = Half-maximal effective concentration; IC_{50} = Half-maximal inhibitory concentration; FRAP = Ferric reducing antioxidant power

*Ascorbic acid used as positive control (EC_{50} = 4.11 ± 0.01 µg/mL);

**Acarbose used as positive control (IC_{50} = 0.62 ± 0.01 mg/mL).

the relationship of these phytochemical characteristics and the bioactivity of both rice extracts were further studied. The antioxidant activity investigated using DPPH assay revealed that MFE had a lower EC_{50} value (61.67 ± 0.05 µg/mL) than DME (69.56 ± 0.95 µg/mL), which was consistent with the results of the FRAP assay, where the value of MFE (19.48 ± 1.68 nM/mg) was higher than for DME (9.73 ± 0.87 mM/mg), as shown in Table 2. These findings were associated with the higher phytochemical contents of MFE than DME. Irakli et al. (2012) reported on the phytochemical analysis of rice and suggested that the phenolics and flavonoids contributed to the beneficial health effects. Several anthocyanins, such as cyanidin-3-O-glucoside and peonidin-3-O-glucoside, were reported to be secondary metabolites that were localized in the pericarp and aleurone layers of the black-purple rice seed (Pereira-Caro et al., 2013). The correlation has been reported of the anthocyanins content and the high antioxidant activity of black glutinous and non-glutinous rice, with the wide range of hydroxyl groups in their molecular structure perhaps being responsible for the activity (Sun et al., 2015; Pedro et al., 2016). The results of the alpha-glucosidase inhibitory effect demonstrated that both rice extracts decreased glucosidase enzyme activity in a dose-dependent manner. The inhibitory effect of MFE was greater than for DME, which were 63.84%, 73.29% and 77.23% versus 25.31%, 46.54% and 57.01% at extract concentrations of 0.1, 0.5 and 1.0 mg/mL, respectively. MFE produced strong and comparable inhibitory effect as the positive control Acarbose with an IC_{50} value of 0.70 ± 0.02 mg/mL versus 0.62 ± 0.01mg/mL, respectively, whereas DME had slightly a greater IC_{50} value of 1.00 ± 0.02 mg/mL (Table 2). This finding was in accordance with the reports on the anti-diabetic potential of purple and red rice extracts (Boue et al., 2016) and the enhancing effect on glucose metabolism of germinated pigmented rice (Chung et al., 2019) that suggested the potential of both rice extracts for diabetic patients. However, further studies are required on the effects of the current two rice extracts on glucose metabolism in animals based on clinical trials. Considering the rice phytochemicals conferring this inhibitory effect, phenolic compounds may play roles as it has been reported that dietary polyphenols and anthocyanins from blueberries had high alpha-glucosidase inhibitory effects (Wu et al., 2017; Zhang et al., 2019).

Cell-based studies

Immunomodulatory activity

Mouse splenocytes were isolated and cultured to determine the effect of rice extracts on cell proliferation. In the absence of mitogen, both rice extracts were not toxic to splenocytes at concentrations of 12.5–200 µg/mL. MFE had lower cytotoxicity than DME with IC_{50} values of 748.56±4.99 µg/mL and 411.08±32.97 µg/mL, respectively. Notably, both extracts induced cell proliferation and in particular, MFE could induce splenocyte proliferation up to 400 µg/mL concentration (Fig 1). In the presence of mitogens, the rice extracts also induced splenocyte proliferation. DME slightly increased cell proliferation in the presence of PHA or PWM (Fig. 1A), whereas MFE only significantly enhanced the cell proliferation in the presence of PHA (Fig. 1B). The strong mitogenic effect of MFE was notable in the current study. Many rice phytochemicals may have mitogenic properties. Lectins are carbohydrate-binding proteins, known as potent immunomodulatory agents, having both innate and adaptive immune system effects, such as stimulating immune cell proliferation, migration, differentiation and activation, and activating phagocytosis and cytokine production (Jandú et al., 2017). Rice lectin had been reported to confer immunomodulatory effects

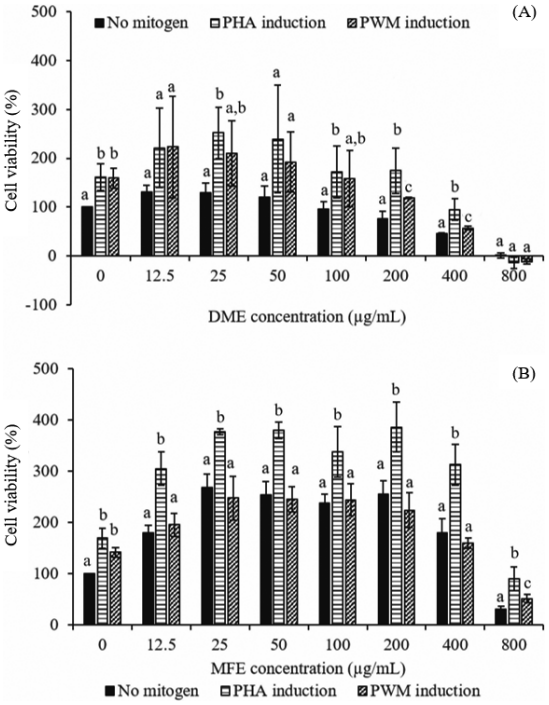


Fig. 1 Mean percentage of effect of DME (A); MFE (B) on cell viability of mouse splenocyte in no mitogen and PHA- or PWM-induced groups, where different lowercase letters above histograms indicate significant ($p < 0.05$) differences within each concentration and error bars represent ±SD

(Peumans et al., 1983; Souza et al., 2013; Nakata et al., 2017). Many constituents in rice bran have shown immunomodulatory effects, including phytosterols (Park et al., 2017). Anthocyanins had been reported to increase human lymphocyte proliferation both in the absence and presence of PHA (Joshi et al., 2017). However, the mitotic constituents of the two studied rice extracts need further investigation. Furthermore, the mitotic effects of rice extracts in the presence of mitogens are of interest and should be encouraged for use in future studies, since PHA induces T-cell proliferation, whereas PWM induces T-cell dependent B-cell proliferation (Kovanen and Knuutila, 1989. The current findings have provided the first report on the immunostimulatory effect of the 50% ethanolic extracts of these two rice varieties and the result support another report on the immunomodulatory effect of rice bran hydrolysate (Phusrisom et al., 2020).

Anti-inflammatory activity

The anti-inflammatory effect of the rice extracts was investigated in LPS-induced RAW264.7 macrophage cells. The cytotoxicity test was conducted for 24 h incubation with various concentrations of the rice extracts up to 1,000 µg/mL. Both rice extracts were not toxic to the cells. The anti-inflammatory effect was evaluated at 50–200 µg/mL concentrations of the extracts. As shown in Fig. 2, by themselves, both DME

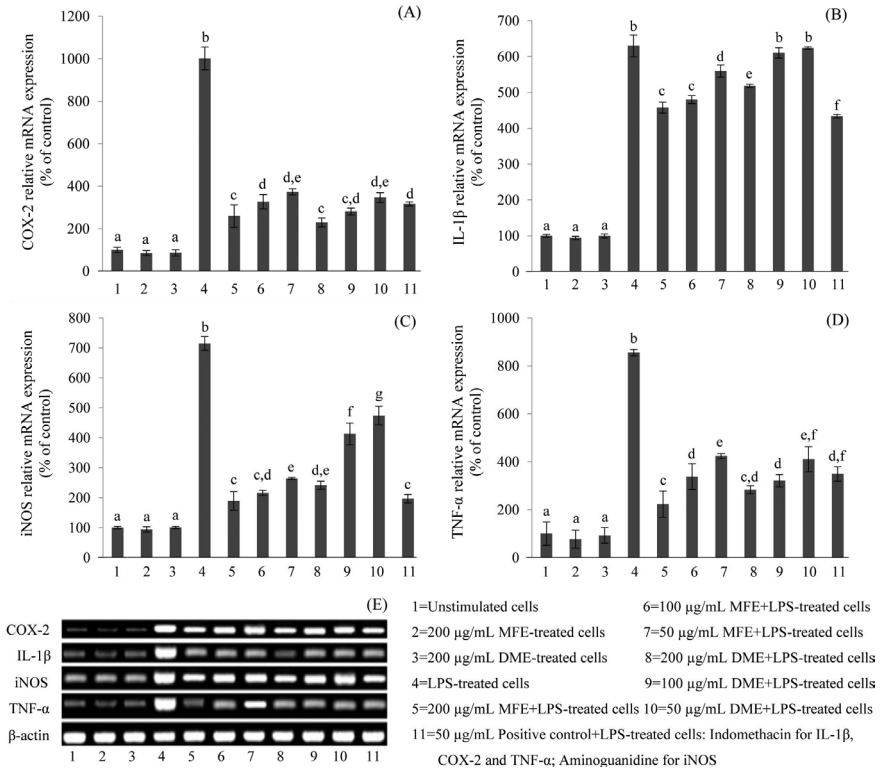


Fig. 2 Mean percentage of effects of Dam-Mong extract (DME) and Ma-led-Fy extract (MFE) on mRNA expression of COX-2 (A); IL-1β (B); iNOS (C); TNF-α (D); amplified bands of these genes (E), where β-actin is a house-keeping gene and different lowercase letters above histograms indicate significant ($p < 0.05$) differences; error bars represent ±SD

and MFE did not affect the genetic expression of the studied pro-inflammatory mediators (COX-2, IL-1β, iNOS and TNF-α), whereas LPS up-regulated the expressions of these genes. Both DME and MFE inhibited the LPS-induced up-regulation of these genes in dose-dependent manners. MFE had stronger inhibitory effects on the expression of IL-1β, iNOS and TNF-α than DME, as indicated by MFE at a concentration of 200 µg/mL having inhibitory levels of 27.33±0.90%, 73.61±12.17% and 73.91 ± 18.10 %, respectively. However, both DME and MFE produced similar inhibition to COX-2 expression. Notably, the inhibition of some genetic expression was comparable to the effects of the positive controls. Considering the IC_{50} values, both the rice extracts had strong inhibitory effects on COX-2 and TNF-α genetic expression, as did indomethacin (Table 3). The inhibitory effects of these rice extracts on the production of nitric oxide and prostaglandin E2 were demonstrated, with both rice extracts similarly inhibiting the production of nitric oxide, with a weaker effect than for the standard drug aminoguanidine. Of interest was that MFE had a stronger inhibitory effect on prostaglandin E2 production than did the standard drug indomethacin.

Animal studies

The anti-inflammatory effects were demonstrated of the rice extracts in carrageenan-induced edema in rat paws (Table 4). Both rice extracts inhibited the paw edema in dose-dependent manners with comparable effect to the standard drug Diclofenac. MFE produced a stronger inhibitory effect than DME. Half an hour after the administration of the high dose (100 mg/kg.bw), MFE continuously decreased the paw swelling, which was superior to the effect of Diclofenac (10 mg/kg.bw) and the rat fully recovered faster than in the other groups. All animals survived throughout the experiment, suggesting the safety of the rice extracts. The current results supported a recent report on the anti-inflammatory effect of pigmented rice (Zhao et al., 2021). The findings of the high phytochemical contents and multiple bioactivities of these two rice varieties indicated their high potential for health promotion. With the more pronounced effects from MFE than DME, further studies on the specific effects of each group and their constituents may be important to identify active principal ingredients for future development of functional foods and nutraceutical products.

Table 3 50% inhibition concentration (IC_{50}) of the rice extracts on the inhibition of nitric oxide and prostaglandin E2 production and inflammatory-related genetic expression

Parameter	DME	MFE	Positive control
Nitric oxide (mg/mL)*	294.49±11.15^{a}	273.78±10.54^{a}	45.32±0.72^{b}
Prostaglandin E2 (µg/mL)**	111.06±13.33^{a}	31.39±2.50^{b}	71.43±4.29^{c}
Genetic expression (µg/mL) **			
COX-2	39.68±6.15^{a}	38.23±7.56^{a}	36.54±2.10^{a}
IL-1β	564.01±15.69^{a}	370.37±12.78^{b}	80.33±4.69^{c}
iNOS	74.32±9.17^{a}	40.12±8.24^{b}	34.51±5.13^{b}
TNF-α	43.18±5.26^{a}	40.86±7.56^{a}	42.23±5.12^{a}

Mean ± SD (n = 3) in each row superscripted with different lowercase letters indicate significant ($p < 0.05$) differences. DME = Dam-Mong rice extract; MFE = Ma-led-Fy rice extract;

*, **Aminoguanidine and Indomethacin, respectively, used as positive controls

Table 4 Inhibitory effect of rice extracts on carrageenan-induced edema of rat paws

Time (h)	Edema rate (%)							
	Normal saline	DME 10 mg/ kg.bw	DME 50 mg/ kg.bw	DME 100 mg/ kg.bw	MFE 10 mg/ kg.bw	MFE 50 mg/ kg.bw	MFE 100 mg/ kg.bw	Diclofenac 10 mg/kg.bw
0	7.11±0.13^{a}	7.12±0.06^{a}	7.13±0.06^{a}	7.20±0.07^{a}	7.22±0.30^{a}	7.25±0.33^{a}	6.88±0.17^{a}	6.92±0.22^{a}
0.25	18.00±0.16^{a}	19.14±0.04^{b,i}	19.11±0.13^{c,i}	16.63±0.10^{d}	10.12±0.29^{e,j}	9.83±0.44^{f,j}	8.43±0.24^{g,k}	8.60±0.10^{h,k}
0.50	44.00±0.47^{a}	36.92±0.98^{b}	32.29±0.80^{e}	27.50±0.86^{d}	20.34±1.04^{e}	15.57±1.07^{f,i}	8.52±1.23^{g}	15.36±0.94^{h,i}
0.75	46.66±1.05^{a}	36.34±0.92^{b,i}	35.25±0.97^{c,i}	32.70±0.90^{d}	30.66±0.89^{e}	26.73±0.95^{f}	10.64±0.75^{g}	21.31±0.20^{h}
1	40.80±0.98^{a}	32.00±0.55^{b}	30.27±0.93^{c}	21.68±0.89^{d,i}	38.79±0.83^{e}	27.46±0.89^{f}	8.73±0.62^{g}	21.79±1.11^{h,i}
2	35.78±0.92^{a}	23.10±1.05^{b}	20.38±0.87^{c,i}	16.27±1.02^{d}	27.45±0.97^{e}	20.58±1.30^{f,i}	6.00±0.50^{g}	13.44±0.72^{h}
3	31.74±0.94^{a}	24.34±0.75^{b}	14.53±0.89^{c,i}	7.23±0.77^{d,j}	26.35±0.70^{e}	15.07±1.04^{f,i}	4.09±0.09^{g}	7.99±0.18^{h,j}
4	25.93±0.73^{a}	14.90±0.87^{b}	5.09±0.49^{c,i}	5.27±0.37^{d,i}	21.00±0.95^{e}	11.70±0.70^{f}	2.06±0.10^{g}	4.87±0.90^{h,i}
5	15.35±0.85^{a}	8.13±0.91^{b,i}	1.50±0.25^{c,j}	0.64±0.10^{d,j}	10.34±0.34^{e}	8.51±0.49^{f,i}	0.95±0.07^{g,j}	2.36±0.22^{h,j}
6	8.50±0.22^{a}	4.22±0.33^{b,i}	0.56±0.07^{c,j}	0.35±0.07^{d,j}	6.24±0.24^{e}	4.44±0.44^{f,i}	0.43±0.11^{g,j}	0.73±0.06^{h,j}
7	4.51±0.20^{a}	0.98±0.09^{b}	0.00^{c,i}	0.00^{d,i}	3.11±0.11^{e}	1.34±0.13^{f}	0.00^{g,i}	0.00^{h,i}

Values expressed as mean ± SD of both right and left paws (5 animals or 10 paws/group);

Different lowercase superscripts for each time indicate significant ($p < 0.05$) differences.

DME = Dam-Mong rice extract; MFE = Ma-led-Fy rice extract; mg/kg.bw = Amount of extract (mg) per rat's body weight (kg).

10, 50, 100 in column headings for DME or FME indicate amount at 10 µg/mL, 50 µg/mL and 100 µg/mL, respectively

In conclusion, this study provided data on the phytochemical constituents of two pigmented-upland rice varieties, namely glutinous Mong-Dam rice and non-glutinous Ma-led-Fy rice, which are very rich in phenolic and anthocyanin contents. The ethanolic extracts of both rice varieties demonstrated bioactivities regarding antioxidant, alpha glucosidase inhibitory, immunomodulatory and anti-inflammatory activities. In addition, the overall data indicated that Ma-led-Fy rice extract had stronger bioactivities than Dam-Mong rice extract. However, further investigation is needed on the molecular relationships and genotypic differences that reflect the bioactive compounds conferring specific bioactivities of both rice varieties.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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