

Anti-Inflammatory Activities of Constituents in Sang Yod Rice Extracts, γ -Oryzanol, Vitamins E, B1, B2 and B3, Using Inhibitory Effects on Nitric Oxide (NO) Production in Lipopolysaccharide (LPS) Activated RAW 264.7 Murine Macrophage Cells

Itharat A^{1,3*}, Uttama S² and Makchuchit S^{1,3}

¹Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Khlong Luang, Pathumthani 12120, Thailand

²Graduate School, Faculty of Medicine, Thammasat University, Khlong Luang, Pathumthani 12120, Thailand

³Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University, Khlong Luang, Pathumthani 12120, Thailand

Abstract

Background: Sang Yod rice is red-violet pigmented rice grown in the south of Thailand (Phattalung province) for over a century. It has been highly valued in the province over decades for its unique taste, nutritious value, and health improving properties. In very recent years, particularly with Royal approval, it has attracted the attention of scientists who have described anti-oxidant properties of some of the constituents in the bran and in bran oil of Sang Yod rice. The objectives of our study were to develop optimum extraction and quantitative procedures for some of the main bioactive compounds in Sang Yod rice (γ -oryzanol, vitamin E, vitamins B1, B2 and B3), and to study the anti-inflammatory activity of extracts using inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS) - induced murine macrophage RAW 264.7 cell line.

Results: Sang Yod rice bran crude extracted by maceration in 95% ethanol exhibited the highest inhibitory effects on NO production with IC_{50} values of 15.59 ± 1.23 μ g/ml; this extract also had high content of γ -oryzanol (317 mg %w/w) and vitamin E (211 mg %w/w). Rice bran extracted using soxhlet extraction using chloroform showed the highest contents of γ -oryzanol (344 mg %w/w). Rice bran extracts exhibited higher γ -oryzanol content than whole grain rice. The highest content of vitamin B1 and vitamin B2 were in Sang Yod rice bran extracted by boiling in water (91 and 88 mg %w/w of extract, respectively). Pure γ -oryzanol and vitamin B2 showed the highest NO inhibitory effects (IC_{50} =1.27 and 7.78 μ g/ml respectively).

Conclusions: The anti-inflammatory of Sang Yod rice properties, appear to correlate to content of essential compounds, especially content of γ -oryzanol and vitamin E in Sang Yod rice bran extracts. The simple procedure of maceration with 95% ethanol of rice bran provides an extracts with high anti-inflammatory activity, with high content of anti-inflammatory and antioxidant compounds. This extract, easily produced from Sang Yod rice bran, is suggested as suitable for commercial preparation and marketing of formulation, containing high levels of essential compounds as functional health improving food products of this pigmented rice.

Keywords: Nitric oxide inhibitory effect; RAW 264.7 cells; Sang Yod rice; γ -Oryzanol; Vitamin E; Vitamin B1; Vitamin B2; Vitamin B3

Introduction

Rice (*Oryza sativa* L., Family Poaceae) is the main staple food crop in almost all Asian countries and is of particular economic value to Thailand. Whereas white and brown rice are the crops that are mostly consumed world-wide, there are a large variety of pigmented wild rice species, and rice cultivars, grown in Asia [1]. These varieties produces rice, with colors ranging from red, purple and black, are now increasingly popular. They demand higher prices in domestic and export markets due to their purported health improving properties. The colors of these pigmented rice varieties are due to a range of anthocyanines in the outer layers [2].

The major by-products during milling of rice are rice husk (20%), rice bran (8%) and rice germ (2%) [3]. Whereas most of these by-products have previously been used as animal feeds, or in the brewery industry, their importance and economic value as functional health improving foods has now been recognized, particularly for the pigmented-rice varieties. Therefore, research in the industrial use of pigmented rice by-products, such as rice bran (RB) and rice bran oil (RBO) (and bioactive constituents therein), that might have positive health effects have attracted increased attention in the last 5-10 years.

In addition to fiber and nutritive elements of the whole grain, pigmented rice bran is rich in minerals, antioxidants, ligands and a plethora of phytochemicals. Some of these include water-soluble, fat-soluble and insoluble antioxidants, such as γ -oryzanol, vitamin E, vitamins B1, B2, B3 and B6, tocopherol, tocotrienols, selenium, phenolic acids, and phytic acid [4-8]. There is some research indicating

that consumption of rice bran or rice bran oil of brown rice is beneficial in treating a variety of diseases, including: type 2 diabetes; hypertension; hyperlipidemia; cardiovascular diseases; stomach and colon cancer; improvement of immune system and liver function. Colored rice, broadly known as enriched rice with improved health properties [9], is highly nutritious [10] and it has been used for: reducing blood cholesterol [11]; for its antioxidative properties [12,13]; to prevent Alzheimer's disease [14]; as a natural antioxidant to improve the stability of foods [15]; decreasing the incidence of atherosclerosis disease [16]; proposed as a UV-A filter in sunscreen [17]; and natural treatment of inflammatory diseases [18,19].

Relatively fewer data are available for pigmented varieties of rice, and most are *in vitro* studies that demonstrate that several constituents of such rice species and cultivars exhibit strong antioxidant activities [1,4-8]. There is only one *in vitro* report of antibacterial activities of

***Corresponding author:** Itharat A, Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani 12120, Thailand, Tel: +6629269749; Fax: +6629269705; E-mail: iarunporn@yahoo.com

Received February 26, 2016; **Accepted** March 21, 2016; **Published** March 25, 2016

Citation: Itharat A, Uttama S, Makchuchit S (2016) Anti-Inflammatory Activities of Constituents in Sang Yod Rice Extracts, γ -Oryzanol, Vitamins E, B1, B2 and B3, Using Inhibitory Effects on Nitric Oxide (NO) Production in Lipopolysaccharide (LPS) Activated RAW 264.7 Murine Macrophage Cells. Med Aromat Plants 5: 244. doi:10.4172/2167-0412.1000244

Copyright: © 2016 Itharat A, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

pigmented-rice crude extracts from four different types of rice [20]. Recent *in vivo* studies in animal models has demonstrated the positive effects of black rice bran against chemically-induced inflammation, indicating that pigmented rice bran may contain anti-inflammatory and anti-allergic constituents [19,21,22]. Only two *in vitro* studies to date have demonstrated the anti-inflammatory effects of purple and black rice [9,18].

Sang Yod rice is the dark red-violet pigmented rice from the south of Thailand (Phatthalung province) that has been supported by Her Majesty Queen Regent Sirikit [23] of Thailand. Sang Yod rice contains various nutritive substances, including vitamin B1, B2, B6, carbohydrate, fibers and protein. It is also rich in various minerals such as iron, calcium and phosphorus [24]. Despite the historic use of Sang Yod rice in southern Thailand as a health improving food, there is a paucity of scientific data supporting the rational for such use. The three main aims of this study were: (a) to develop optimum extraction procedures that afford the highest yield of the crude extract from Sang Yod de-husked whole grain rice, and from Sang Yod rice bran, (b) to use quantitative assays to determine yields of γ -oryzanol and vitamins E, B1, B2 and B3 in the extracts, and finally (c) to study the anti-inflammatory activities of each of the extracts using inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-induced murine macrophage leukemia RAW 264.7 cell line [25,26].

Methods

Materials and reagents

Raw 264.7 murine macrophage leukemia cell lines were kindly provided by Assoc Prof Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical sciences, Prince of Songkla University, Thailand. RPMI Medium 1,640 (RPMI 1,640), L-glutamine, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (P/S), Trypsin-EDTA and trypanblue were purchased from Gibco, NY, USA. Phosphate Buffer Saline (PBS) was from Amresco, Ohio, USA. Sodium bicarbonate was from BHD, Poole, England, lipopolysaccharide (LPS, from *Escherichia coli*), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were from Sigma, MO, USA. Ninety six well micro plates were also purchased from Costar Corning, NY, USA. Other chemicals were from Sigma, MO, USA and Merck, Darmstadt, Germany. Solvents purchased from Merck (Darmstadt, Germany), standard γ -oryzanol, vitamins E, B1, B2, B3 from Sigma-Aldrich (Seelze, Germany). Supercritical fluid extraction (24L) (Guangzhou, Masson New Separation Technology Co. LTD, China) The HPLC system consisted of an Agilent 1100 series, including an auto sampler, column oven, and a variable wavelength UV-VIS detector (model G1379A). The column used for γ -oryzanol analyses was Hypersil ODS (4.0 x 250 mm, 5 μ m) from Agilent Technologies, Palo Alto, CA, USA. The column used for analyses of vitamins E, B1, B2 and B3 was Phenomenex C18 (4.6 x 100 mm, 3 μ m) from Phenomenex, CA, USA.

Plant materials and extraction methods

De-husked whole grain Sang Yod rice and Sang Yod rice bran used in this study were collected from the Phatthalung province, Thailand (organic rice) on October 2011. This rice was identified and confirmed by Rice Department of Phattalung province. The rice bran was placed on a large tray and any extraneous matter removed before use. The whole grain rice was dried (50°C) and stored at the room temperature before extraction. Six different extraction methods utilized were as follows:

Maceration by 95% ethanol

The plant material (100 g) was macerated in 95% ethanol (1 L) for 3 days at room temperature, repeated 2 times, filtered and solvent (3 L) evaporated to dryness using a rotary film at 45 °C then dried to constant weight by using vacuum desiccator. The extract was stored at -20°C.

Supercritical fluid extraction

The plant material (100 g) was extracted with CO₂ at a critical temperature of 31°C for 21 hours, critical pressure of 74 bars and kept it in the collector tank, and filtered before used. The extract was stored at -20°C.

Boiling in water

The plant material (100 g) was boiled in distilled water (1 L) at 70°C for 30 minutes, filtered and dried by using freeze dryer. The extract was stored at -20°C.

Water from washing rice

The plant material (100 g) was washed with sterile water three times at ratio of rice : sterile water as 1 : 2 and dried by using a freeze dryer. The extract was stored at -20°C.

Expression methods

The plant material (100 g) was put into expression tool, to separate oil and residue. The oil was kept at room temperature for 24 hours and filtered. The extract was stored at -20°C.

Soxhlet extraction method

Using plant material: solvent ratios of 30 g : 1 L, the samples were extracted in a Soxhlet apparatus using solvents of increasing polarity; hexane, chloroform and then methanol at temperatures 40, 50 and 60°C, respectively. The samples were extracted in each solvent for 30 minutes, filtered and evaporated to dryness using a rotary film evaporator at 45°C then dried to constant weight in vacuum desiccator. The extract was stored at -20°C.

Rice bran was extracted by all methods above except the extraction method as washing rice with water. Whole grain rice was extracted only two methods (maceration in 95% ethanol and washing rice with water). The nine different extracts from Sang Yod rice were listed in Table 1.

Determination of γ -oryzanol content in extracts

γ -oryzanol content in rice bran extract was qualified using reverse phase high performance liquid chromatography (RP-HPLC) according to the method described in the literature [5]. Each extract (10 mg) was dissolved in 1.0 ml of methanol, and filtered through a syringe filter with PTFE (0.2 μ m; Ascorbic syringe filter). Aliquots (10 μ l) of prepared samples were injected on to the Hypersil ODS column using a rheodyne injector (or autosampler), using a mixture of methanol: acetonitrile: dichloromethane: acetic acid (50:44:3:3, v/v/v/v) as a mobile phase at a flow rate of 1.0 ml/min with UV detection at 330 nm. The content of γ -oryzanol was calculated from calibration graphs obtained by using different concentrations of standard γ -oryzanol. Calibration data was triplicate analyses were performed in three different days. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area and correlation coefficients (r^2) of 0.9998. All sample analyses, and cell calibration experiments, were repeated on three separate occasions, with triplicates assays for each sample on any one day. Data for extracts are reported as mg per 100 g of extract.

Abbreviation [*]	Part of rice ^{**}	Extraction Method	Yield (% w/w) ^{***}
MEB	Rice bran	Maceration with ethanol 95%	6.53
MEWR	Whole bran	Maceration with ethanol 95%	6.00
SFB	Rice bran	Supercritical fluid extraction	10.34
BWB	Rice bran	Boiling in water, and freeze drying	38.20
WWWWR	Whole bran	Washing rice with water and freeze drying	0.07
EXB	Rice bran	Expression method	2.16
SXHB	Rice bran	Soxhlet extraction using hexane	15.23
SXCB	Rice bran	Soxhlet extraction using chloroform	0.73
SXMB	Rice bran	Soxhlet extraction using methanol	5.67

The 1st letter(s) in the abbreviations refers to the method of extraction. The 2nd letter(s) refers to the solvent used. The last letter(s) refers to sample used (Whole rice (WR) or rice bran (B)); *M=Maceration, SF=Supercritical fluid extraction, B=Boiling, W=Washing, E=Expression, SX=Soxhlet extraction, E=Ethanol, W=Water, H=Hexane, C=Chloroform, M=Methanol; **B=Rice bran, WR=Whole rice bran; ***% W/W=grams of extracts per 100 grams of starting sample.

Table 1: Percentage yields of various crude extracts from whole Sang Yod rice and rice bran using various extraction methods.

Determination of Tocopherol (Vitamin E) content in rice bran extract

Tocopherol (Vitamin E) content in each extract was measured by using reverse phase high performance liquid chromatography (RP-HPLC) according to the method described in the literature [27] with some modification. Rice extract (10.0 mg) was dissolved in 1.0 ml of methanol and filtered through a syringe filter with PTFE (0.2 μ m; ascorbic syringe filter). Aliquots (10 μ l) of the samples were injected onto the Phenomenex C18 column using a rheodyne injector (or autosampler), with UV-detection at 292 nm. A mixture of methanol : water (98 : 2 v/v) was used as a mobile phase at a flow rate 1.5 ml/min in isocratic mode. The content of Tocopherol was calculated from calibration graphs obtained by using different concentrations of standard Tocopherol, calibration data Calibration data was triplicate analyses were performed in three different days. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area and correlation coefficients (r^2) of 0.9996. All sample analyses, and cell calibration experiments, were repeated on three separate occasions, with triplicates assays for each sample on any one day. Data for extracts are reported as mg per 100 g of extract.

Determination of Vitamin B1, Vitamin B2, and Vitamin B3, content in rice extracts

Vitamin B1, Vitamin B2 and Vitamin B3 content in extracts were measured by using reverse phase high performance liquid chromatography according to a published method [28], with some modification. Extracts 10.0 mg were dissolved in 1.0 ml of methanol and filtered through a syringe filter with PTFE (0.2 μ m; ascorbic syringe filter). Aliquots of the samples (10 μ l) were injected onto the Phenomenex C18 column using a rheodyne injector (or autosampler), using a mixture of 0.1 Mol KH_2PO_4 (pH 7) : methanol (90:10 v/v) as a mobile phase at flow rate 0.7 ml/min in isocratic mode. A variable-wavelength detector set at 234, 266 and 261, respectively, was used for detection. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area and correlation coefficients (r^2) of standard Vitamin B1, Vitamin B2 and Vitamin B3 ($r^2=0.997, 0.989$ and 0.999 respectively). All sample analyses, and cell calibration experiments, were repeated on three separate occasions, with triplicates assays for each sample on any one day. Data for extracts are reported as mg per 100 g of extract.

Anti-inflammatory activity of extracts using the inhibition of NO production in LPS-induced RAW 264.7 cell line

Inhibitory effects on NO production by murine macrophages-like

RAW 264.7 cells were evaluated by the following the method described by Tewtrakul and Itharat [29]. The RAW 264.7 cells were washed with phosphate buffer saline (PBS) free of magnesium and calcium. The PBS was decanted and cells were harvested with 0.25% trypsin-EDTA and fresh medium was added. The cell pellet, was obtained by centrifugation (1000 rpm, 6 min), was resuspended in 10 ml of medium to make a single cell suspension. The viable cells were counted by trypan blue exclusion in hemacytometer and diluted with medium to give a final concentration of 1×10^6 cells/ml for RAW 264.7 cells. One hundred microlitres per well of these cells suspension were seeded in each 96-well microplates with 1×10^5 cells/well and allowed to adhere for 1 hour at 37°C in 5% CO_2 . After that the medium was replaced with fresh medium containing 10 μ g/ml of lipopolysaccharide (LPS) together with test samples at various concentrations and then incubated for 48 hours. The extracts were dissolved in methyl sulfoxide (DMSO) and the water extracts were dissolved in sterile water, sterilized by filtration (pore size, 0.2 μ m) before testing. Stock solutions (10 mg/ml) of the extracts were stored at -20°C until use. The extracts were diluted in medium to produce required concentrations. A hundred microlitres of each concentration was added to each well of plate to obtain final concentrations of 1-100 μ g/ml. The final dilution used for treating the cells was contain not more than 1% of the initial solvent; this concentration was used in the solvent control wells. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method. Briefly, after 48 hours incubation with test samples, MTT solution (10 μ l, 5 mg/ml in PBS) was added to the wells. After 2 hours incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 70% of that in the control (vehicle-treated) group. Indomethacin was used as positive controls. Inhibition (%) was calculated using the following equation and IC_{50} values were calculated from the Prism program.

$$\% \text{ Inhibition} = \frac{A - B}{A - C} \times 100$$

Where, A-C: NO_2^- Concentration (μM) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)].

Statistical analysis

The experimental data were expressed as mean \pm SEM (n=3) and analyzed using SPSS and Student paired t-test.

Results and Discussion

From the various extraction methods of rice bran, whole grain and de-husk whole grain of Sang Yod rice were comparison studied on yield, essential chemical content in rice and their anti-inflammatory activity and they were also discussed for useful of Sang Yod rice extraction for health.

The percentage yields of crude extracts from de-husked whole grain Sang Yod rice or rice bran by different methods are shown in Table 1. Boiling rice bran afforded the highest yield (38.2 %w/w) followed by soxhlet extraction using hexane as the solvent (15.23 %w/w). Washing whole grain rice with water, and soxhlet extraction using chloroform as solvent afforded the lowest yields of crude extracts (0.07 and 0.73 %w/w respectively).

The mg %w/w (%w/w) yields of γ -oryzanol and vitamins E, B1, B2 and B3 in all the nine crude extracts are shown in Table 2. The highest yields of γ -oryzanol were in rice bran extracts using soxhlet using chloroform as solvent (344 mg %w/w), followed by expression (309 mg %w/w), and maceration with 95% ethanol (317 mg %w/w). The results are in agreement with previous studies which have reported that pigmented rice bran has the highest content of γ -oryzanol [1,5,10,30,31]. Rice bran extracts, obtained by the maceration (95% ethanol) and soxhlet extraction using chloroform contained the highest levels of vitamin E (211 and 203 mg %w/w). As expected, the water soluble vitamins B1, B2 and B3 were only present in extracts obtained by boiling with water of rice bran or whole de-husked rice grains, there levels being below the detection levels in the organic solvent extracts.

Sang Yod rice and rice bran extracts were investigated for their inhibitory activities against LPS induced NO production in RAW 264.7 cell lines. Measurement of nitrite accumulation in the culture medium, by the Griess reaction, was used to determine NO production. The inhibitory effects of all the nine rice extracts on production of NO are shown in Table 3. Rice bran extract using the 95% ethanolic maceration method (MEB) exhibited the highest inhibitory activity, with an IC_{50} value of 15.59 ± 1.23 μ g/ml (Table 3). The soxhlet extract using chloroform (SXCB) showed the second strongest anti-inflammatory activity with an IC_{50} value of 16.52 ± 3.36 μ g/ml. However pure γ -oryzanol showed the highest inhibitory active of the pure vitamins (IC_{50} value 1.27 ± 0.71 μ g/ml). There is only one previous study on anti-inflammatory activities of γ -oryzanol-rich extracts from GAM BOUNG Thai glutinous purple rice bran from northern part of Thailand [18]. Using soxhlet extraction with a mixture of hexane: ethyl acetate (7:3), followed by semi-purification using silica column chromatography they obtained semi-pure extracts which exhibited the high inhibitory effects on NO production ($IC_{50} = 29.32 \pm 2.21$ μ g/ml). A further purification step employed by Saenjum and coworker's clearly leads to enrichment of γ -oryzanol, with a consequent high anti-inflammatory effect our crude extract, IC_{50} value 58.45 μ g/ml, Saenjum and coworker's enriched extracts, IC_{50} value 29.32 μ g/ml. However, Sang Yod rice bran extract obtained by maceration with 95% ethanol (MEB) showed a higher inhibitory effect on NO production than the colored rice from Saenjum and coworker's report ($IC_{50} = 15.59 \pm 1.23$ μ g/ml).

None of the extracts exhibited any toxic effects against the RAW 264.7 cell line (% Cytotoxicity <20 μ g/ml for MTT assay). The extracts obtained by MEB and SXCB need to be further purified to obtain enriched-semi-purified extracts with even a higher anti-inflammatory effect. Already these two crude extracts have a higher activity than indomethacin (IC_{50} value: MEB= 15.59 ± 1.23 ; SXCB= $16.52 \pm$

3.36 ; indomethacin= 21.01 ± 1.21 μ g/ml) which is a positive anti-inflammatory compound.

Vitamin E levels in pigmented rice have been reported to be in the range 460-770 mg/100 g of extract [1,5]. The Sang Yod rice and rice bran extracts used in this study contained lower levels of vitamin E (range 93-211 mg %wt), because the type of rice and the extraction methods were different. Our study is the first report on Sang Yod rice and rice bran extracts. The high content of γ -oryzanol and vitamin E in SXCH and MEB extracts account for the strong anti-inflammatory effects of these extracts.

It has been reported that the content of vitamins B1, B2 and B3 in Sang Yod whole rice are in the range of 0.32, 0.01 and 6.40 mg/100 g of Sang Yod whole rice grains, but there are no reports on vitamin content of vitamins in rice extracts. In our study the content of all vitamins B were higher than reported by the Thai Rice Department, Phatthalung province [24]. This is obviously because their data relates to content in Sang Yod whole rice grains, whereas our data is for content in extracts of Sang Yod rice and rice bran. Our study shows that Sang Yod rice bran extracts contain high γ -oryzanol and vitamins E, B1, B2, and B3, suggesting that these extracts would be a good source of natural antioxidants. The extracts obtained by boiling Sang Yod rice bran showed moderate anti-inflammatory activity compared to pure vitamins B1, B2 and B3. The extract obtained from washing rice grain with water showed high content of vitamins B, especially B2, suggesting that washing Sang Yod rice grain during commercial processing, or prior to cooking will result in the loss of vitamins B1, B2, and B3. This extract also had highest content of vitamin B2, and exhibited higher anti-inflammatory effect ($IC_{50} = 7.78 \pm 0.52$ μ g/ml) than vitamins B1 and B3. The expression of rice bran yield an extract that had a high content of γ -oryzanol and vitamin E, and this extract had higher anti-inflammatory activity than extract obtained by boiling rice. The expression method can therefore be used as a simple process to obtain an oily product rich in vitamins from Sang Yod rice bran, that can be formulated as oily capsules for sale for the treatment of inflammatory diseases. Supercritical fluid extract had a high content of γ -oryzanol, but did not show any anti-inflammatory activity. This method is complicated and expensive, and therefore not recommended in the production of health products from Sang Yod rice bran.

A few reports have recently been published on the anti-inflammatory effects of rice extracts from pigmented rice. Ethanolic extracts of rice bran of colored rice were shown to have an inhibitory effect on edema induced by carragenan in Speague-Dawley rat's paw [19,22]. Black rice bran extract had inhibitory effected against 12-O-tetradecanolyphorbol-13-acetate (TPA)-induced skin edema and 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD) in inflammatory mouse models [21], and it also showed the inhibition of nitric oxide by lipopolysaccharide (LPS)-induced with RAW 264.7 cells [9]. However, there are no previous reports in animal models of anti-inflammatory activities of extract of Sang Yod rice bran.

MEB and BWB is that maceration of rice bran with 95% ethanol is better than boiling of rice bran with water. However, result from washing with water of whole rice given a very high value (164 mg) for γ -oryzanol. In each case, starting material with rice bran or whole rice were 100 g. γ -oryzanol in whole rice is 164 mg/100 g compared to rice bran (118 mg/100 g) but whole rice only contains about 8% bran which gave γ -oryzanol 9.44 mg in 100 g of whole rice. However, γ -oryzanol obtained from washing with water of whole rice show high yield than rice bran. Thus, washing with water of whole rice which is preparing boiled rice as diary food, should be concern loss on γ -oryzanol.

Rice extract ^a	γ -oryzanol	Vitamin E	Vitamin B1	Vitamin B2	Vitamin B3
MEB	317 \pm 1.01 (20.7 \pm 0.07)	211 \pm 5.66 (13.8 \pm 0.37)	ND	ND	ND
MEWR	183 \pm 2.02 (11.0 \pm 0.12)	113 \pm 4.64 (6.8 \pm 0.28)	ND	ND	ND
SFB	184 \pm 3.00 (19.0 \pm 0.31)	95 \pm 1.25 (9.8 \pm 0.13)	ND	ND	ND
BWB	118 \pm 2.11 (45.1 \pm 0.81)	ND	91 \pm 1.06 (34.8 \pm 0.40)	35 \pm 1.03 (13.4 \pm 0.39)	88 \pm 1.04 (33.6 \pm 0.40)
WWWR	164 \pm 3.26 (0.11 \pm 0.00)	ND	68 \pm 1.04 (0.05 \pm 0.00)	87 \pm 1.06 (0.06 \pm 0.00)	62 \pm 1.02 (0.04 \pm 0.00)
EXB	309 \pm 1.04 (6.67 \pm 0.02)	156 \pm 3.04 (3.4 \pm 0.07)	ND	ND	ND
SXHB	135 \pm 1.56 (20.6 \pm 0.24)	109 \pm 3.28 (16.6 \pm 0.50)	ND	ND	ND
SXCB	344 \pm 3.04 (2.5 \pm 0.02)	203 \pm 4.29 (1.5 \pm 0.03)	ND	ND	ND
SXMB	107 \pm 3.44 (6.1 \pm 0.20)	107 \pm 2.66 (6.1 \pm 0.15)	ND	ND	ND

^aAbbreviations used for extraction method/solvent/sample are as given in footnote to Table 1; Data are mg %w/w \pm S.E.M (mg of pure compound per 100 g of extract); ND=not detected; Values in bold are %w/w yields as mg per 100 g of starting material (whole rice or rice bran)

Table 2: Yields of γ -oryzanol and vitamins E, B1, B2, and B3 in various crude extracts of whole Sang Yod rice and rice bran expressed as mg %w/w.

Rice extract/ Pure compound	% Inhibition at concentration 50 μ g/ml (Mean \pm S.E.M)	IC ₅₀ (μ g/ml)	% Cytotoxicity by MTT	Data from previous studies
MEB	58.45 \pm 2.11	15.59 \pm 1.23	4.15 \pm 1.21	NPS [~]
MEWR	9.20 \pm 2.89	NT	10.23 \pm 1.06	NPS
SFB	2.40 \pm 1.44	NT	10.23 \pm 1.06	NPS
BWB	63.42 \pm 1.23	36.33 \pm 2.99 [*]	3.26 \pm 1.03	NPS
WWWR	11.06 \pm 2.15	NT	15.67 \pm 2.13	NPS
EXB	67.74 \pm 1.22	30.09 \pm 3.12 [*]	4.98 \pm 1.22	NPS
SXHB	51.48 \pm 5.28	58.45 \pm 1.36 [*]	2.15 \pm 2.39	29.32 μ g/ml [18]
SXCB	59.10 \pm 3.78	16.52 \pm 3.36	5.88 \pm 2.56	NPS
SXMB	7.57 \pm 3.33	NT	16.12 \pm 2.99	NPS
γ -oryzanol	89.98 \pm 2.55	1.27 \pm 0.71 [*]	3.65 \pm 2.43	NPS
Vitamin B1	71.55 \pm 2.55	23.41 \pm 1.13	12.45 \pm 1.20	NPS
Vitamin B2	80.98 \pm 2.16	7.78 \pm 0.52	11.45 \pm 0.23	NPS
Vitamin B3	69.25 \pm 1.89	45.14 \pm 0.44	2.30 \pm 0.45	NPS
Vitamin E	88.32 \pm 2.55	2.45 \pm 0.45 (5.69 μ M)	13.02 \pm 0.45	1.72 μ g/ml (4 μ M) [31]
Indomethacin	81.20 \pm 1.02	20.01 \pm 1.21	16.50 \pm 0.25	

NT: Not tested; ^{*}Asterisk (*) indicated a significant difference at the level $p < 0.05$, compared with Indomethacin; [~]NPS: No previous studies

Table 3: Anti-inflammatory activities of various crude extracts and isolated pure γ -oryzanol and vitamins E, B1, B2 and B3 from Sang Yod whole rice bran or rice bran.

Conclusions

In summary, Sang Yod rice bran extracted by maceration in 95% ethanol exhibited the highest inhibitory effects on NO production with IC₅₀ value of 15.59 \pm 1.23 μ g/ml. This extract can be good choice for preparation of commercial products of Sang Yod rice for treatment of inflammatory diseases. However further studies are required to confirm anti-inflammatory activity is mediated by inhibition of COX-1 and COX-2 enzymes. The NO inhibitory activity appears to correlate to content of essential compounds in Sang Yod rice, especially content of γ -oryzanol. Our data provides some rationale for the historic use of this rice in southern Thailand as a health enhancing food product, and suggests procedures that might be used for the commercial preparation of marketable functional products of Sang Yod rice.

Acknowledgements

The authors wish to thank the Nation Research Council of Thailand, National Research University Project of Thailand Office of Higher Education Commission, Center of Excellence on Applied Thai Traditional Medicine Research (CEATMR) and Thammasat University, Thailand, for financial support. In addition, we would like to thank Professor L. A. Damani who is visiting Professor in CEATMR for his critical comments and careful scientific editing of this manuscript.

References

1. Aguilar-Garcia C, Gavino G, Mosqueda MB, Hevia P, Gavino VC (2007) Correlation of tocopherol, tocotrienol, -oryzanol and total polyphenol content in rice bran with different antioxidant capacity assays. *Food Chem* 102: 1228-1232.
2. Oki T, Masuda M, Nagai S, Takeichi M, Kobayashi M, et al. (2005) Radical-scavenging activity of red and black rice. Rice is life: scientific perspectives for the 21st century. Proceedings of the World Rice Research Conference held in Tsukuba, Japan 256-259.

3. Esa NM, Ling TB, Peng LS (2013) By-products of rice processing: an overview of health benefits and applications. *J Rice Res* 1: 107.
4. Sompong R, Siebenhandl-Ehn S, Linsberger-Martin G, Berghofer E (2011) Physicochemical and antioxidative properties of red and black rice varieties from Thailand, China and Sri Lanka. *Food Chem* 124: 132-140.
5. Chotimakorn C, Benjakul S, Silalai N (2008) Antioxidant components and properties of five long-grained rice bran extracts from commercial available cultivars in Thailand. *Food Chem* 111: 636-641.
6. Butsat S, Siriamornpun S (2010) Phenolic acids and antioxidant activities in husk of different Thai rice varieties. *Food Sci Technol Int* 16: 329-336.
7. Vichapong J, Sookserm M, Srijesdaruk V, Swatsitang P, Srijaranai S (2010) High performance liquid chromatographic analysis of phenolic compounds and their antioxidant activities in rice varieties. *Food Science and Technology* 43: 1325-1330.
8. Huang SH, Ng LT (2011) Quantification of tocopherols, tocotrienols, and γ -oryzanol contents and their distribution in some commercial rice varieties in Taiwan. *J Agric Food Chem* 59: 11150-11159.
9. Min SW, Ryu SN, Kim DH (2010) Anti-inflammatory effects of black rice, cyanidin-3-O-beta-D-glycoside, and its metabolites, cyanidin and protocatechuic acid. *Int Immunopharmacol* 10: 959-966.
10. Boonsit P, Pongpiachan P, Julsrigrival S, Karladee D (2010) Gamma oryzanol content in glutinous purple rice landrace varieties. *CMU J Nat Sci* 9: 151-157.
11. Qureshi AA, Sami SA, Salsler WA, Khan FA (2001) Synergistic effect of tocotrienol-rich fraction (TRF(25)) of rice bran and lovastatin on lipid parameters in hypercholesterolemic humans. *J Nutr Biochem* 12: 318-329.
12. Xu Z, Godber JS (2001) Antioxidant activities of major components of γ -oryzanol from rice bran using a linoleic acid model. *Journal of the American Oil Chemist's Society* 78: 645-649.
13. Shokrzadeh M, Ebadi AG (2006) Study of antioxidant activity in four kinds of cultivated rice grains of Muzandaran province (Iran). *Pak J Biol Sci* 9: 2723-2725.
14. Alloul K, Sauriol L, Kennedy W, Laurier C, Tessier G, et al. (1998) Alzheimer's disease: a review of the disease, its epidemiology and economic impact. *Arch Gerontol Geriatr* 27: 189-221.
15. Nanua JN, McGregor JU, Godber JS (2000) Influence of high-oryzanol rice bran oil on the oxidative stability of whole milk powder. *J Dairy Sci* 83: 2426-2431.
16. Saunders RM (1985) Rice bran: Composition and potential food uses. *Food Reviews International* 1: 465-495.
17. Coppini DP, Paganizzi P, Santi P, Ghirardini A (2001) Capacity protective against solar radiation of vegetable origin derivatives. *Cosmetic News* 136: 15-20.
18. Saenjum C, Chaiyasut C, Chansakaow S, Suttajit M, Sirithunyalug B (2012) Antioxidant and anti-inflammatory activities of gamma-oryzanol rich extracts from Thai purple rice bran. *J Med Plants Res* 6: 1070-1077.
19. Schussheim Y, Aschner M, Brodsky B, Proscura E, Erlanger-Rosengarten A, et al. (2011) Anti-inflammatory effects of peptide fragments of H2A histone and *Oryza Sativa Japonica* protein. *Peptides* 32: 125-130.
20. Pumirat P, Luplertlop N (2013) The in-vitro antibacterial effect of colored rice crude extracts against *Staphylococcus aureus* associated with skin and soft-tissue infection. *Journal of Agricultural Science* 5: 102-109.
21. Choi SP, Kim SP, Kang MY, Nam SH, Friedman M (2010) Protective effects of black rice bran against chemically-induced inflammation of mouse skin. *J Agric Food Chem* 58: 10007-10015.
22. Lerdvutisophon N, Jansom C, Jansom V, Sireeatawong S (2012) The anti-inflammatory effect of various Thai rice. *Planta Med* 78: P1120.
23. Bureau of Rice Research and Development (2006) Rice knowledge bank.
24. Thai Food Composition Organization (1999) Thai Food Composition (Rice nutrition facts). Institute of Nutrition, Mahidol University.
25. Ischiropoulos H, Zhu L, Beckman JS (1992) Peroxynitrite formation from macrophage-derived nitric oxide. *Arch Biochem Biophys* 298: 446-451.
26. Kim NY, Kang TH, Song EK, Pae HO, Chung HT, et al. (2000) Inhibitory effects of butanol fraction of the aqueous extract of *Forsythia koreana* on the nitric oxide production by murine macrophage-like RAW 264.7 cells. *J Ethnopharmacol* 73: 323-327.
27. Edison B (2009) Analysis of Tocopherols by High Performance Liquid Chromatography. *E-Journal of Chemistry* 6: 395-398.
28. Ekinci R, Kadakal C (2005) Determination of seven water-soluble vitamins in Tarhana, a traditional Turkish cereal food, by High-Performance Liquid Chromatography. *ACTA Chromatographic* 15: 289-297.
29. Tewtrakul S, Itharat A (2007) Nitric oxide inhibitory substances from the rhizomes of *Dioscorea membranacea*. *J Ethnopharmacol* 109: 412-416.
30. Nam SH, Choi SP, Kang MY, Koh HJ, Kozukue N, et al. (2006) Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chem* 9: 613-620.
31. Gitto E, Tan DX, Reiter RJ, Karbownik MK, Manchester LC, et al. (2010) Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrioxamine (desferoxamine) in rat liver homogenates. *J Pharm Pharmacol* 53: 1393-1401.