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Crude Bran Extracts and Fractions of Selected Traditional Red Rice (*Oryza sativa* L.) Varieties of Sri Lanka Potentiates Anti-Inflammatory Activities in Human Blood and Cell Assays

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Abstract

Crude extracts (70% ethanolic) and fractions (hexane, dichloromethane, butanol, aqueous) of brans of four Sri Lankan traditional red rice varieties (*Oryza sativa* L.), namely, Sudu Heeneti, Goda Heeneti, Masuran and Dik Wee were studied for a range of anti-inflammatory activities. The investigated activities included oxidative burst inhibitory activity in human whole blood, isolated human polymorphonuclear cells and mice peritoneal macrophages and inhibition of nitric oxide and pro-inflammatory cytokines in cell assays. Further, cytotoxicity was evaluated against normal cell line. The results showed for the first time that brans of all the rice varieties selected for the current study had anti-inflammatory activities in all the assays tested without inducing cytotoxicity on normal cells. However, significant differences were observed among different varieties and between crude extracts and fractions for investigated anti-inflammatory activities. Partitioning of crude rice bran extracts showed inhibitory activities in both non-polar and polar fractions. The non-pigmented dichloromethane fractions exhibited anti-inflammatory activities in all the assays studied. Overall, the properties observed indicate usefulness of brans of selected Sri Lankan traditional red rices in the management of inflammation associated chronic diseases.

Introduction

Inflammation is considered as a common feature in the development and progression of many chronic diseases such as diabetes, cancer, cardiovascular diseases, neurological diseases and rheumatoid arthritis [1-6]. These diseases are associated with the over production of reactive oxygen and nitrogen species [2,3,5] by Polymorphonuclear Cells (PMNs) and macrophages [3,7]. Research findings have clearly shown that the production of reactive species at the site of inflammation generates an oxidative imbalance hence contributing to tissue damage [2,3,5-7]. Further, reactive species act as modulators of protein and lipid kinases and phosphatases, membrane receptors, ion channels, and transcription factors which regulate the expression of key cytokines [2,3,6,7].

Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-1β are two pro-inflammatory cytokines implicated in the pathogenesis of many inflammatory diseases. Circulating levels of these cytokines are now used as biomarkers to access the inflammatory process [1-4,8].

Currently, many potent anti-inflammatory drugs are available with varying mode of actions to manage inflammatory diseases. However, some of these drugs are associated with serious adverse effects [9]. Further, many of these drugs are expensive, especially, in developing countries. Natural products with anti-inflammatory activity have long been used as a folk remedy for management of inflammatory diseases [9]. Rice *(Oryza sativa* L.) is a major cereal crop in the developing world and rice bran is a by-product of the rice milling industry. It is reported to have various biological activities [10-14] including anti-inflammatory activity worldwide [15-18].

In Sri Lanka rice is the dietary staple and country has many traditional rice varieties which had been in the diet for centuries [19]. Some of these varieties are claimed to possess immune boosting activities according to Sri Lankan traditional knowledge and folk medicine [20]. However, no attempts have been taken to study the anti-inflammatory potentials of any Sri Lankan rice variety. Further, although the anti-inflammatory activities of the rice bran crude extracts were reported worldwide, extremely limited research attempts have focused on exploring the anti-inflammatory activities of rice bran fractions or the isolated active ingredients. Moreover, no studies to date on oxidative burst inhibitory activity in human blood and immune cells by rice bran extracts and fractions. The present study evaluated anti-inflammatory activities of crude bran extracts and fractions of selected traditional red rice varieties of Sri Lanka in human blood and cell assays. The investigated anti-inflammatory activities included oxidative burst inhibitory activity in human whole blood, isolated human polymorphonuclear cells and mice peritoneal macrophages, nitric oxide and pro-inflammatory cytokines (TNF-α and IL-1β) inhibitory activities in cell assays.

Materials and Methods

Grain Samples

Four Sri Lankan traditional red rice varieties (*Oryza sativa* L.) namely Sudu Heeneti, Goda Heeneti, Masuran and Dik Wee were selected and obtained from Regional Rice Research and Development Centre (RRRDC), Bombuwala, Sri Lanka. The selected varieties had been cultivated in the Low Country Wet Zone (LCWZ) of Sri Lanka at RRRDC, Bombuwala.

Chemicals and Reagents

Luminol, serum opsonized zymosan, Hanks Balanced Salt Solution $(HBSS)^{++}$ (with Ca^{++} and Mg^{++}), $HBSS^{--}$ (without Ca^{++} and Mg^{++}), lymphocyte separation medium (LSM), heat inactivated fetal calf serum (FCS), Roswell Park Memorial Institute (*RPMI*)-1640 medium, trypan blue, Dulbecco's Modified Eagle's Medium (DMEM), *Fetal Bovine Serum* (*FBS*), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (*HEPES*), Ibuprofen (2-(4-Isobutylphenyl)propanoic acid), sulphanilamide, naphthyl-ethylene diamine dihydrochloride, L-glutamine, Phorbol Myristate Acetate (PMA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). *E. coli* Lipopolysaccharide (LPS) was purchased from Difco Laboratories (Michigan, USA). All the other chemicals used for the preparation of buffers and solvents were of analytical grade or cell culture tested.

Preparation of Rice Bran Crude Extracts

Rice seeds were dehulled (THU 35B, Satake, Hiroshima, Japan), polished (TM-05C, Satake, Hiroshima, Japan) and passed through a 60-mesh sieve to obtain rice bran. One gram of rice bran was extracted in 12 mL of 70% ethanol-water (v/v) overnight at room temperature (28±2˚C). Then rice bran extracts were centrifuged (825 g) for 10 min and filtered (0.45 µm nylon filters). The resulting rice bran crude extracts were evaporated using a rotary evaporator and freeze dried (Christ-Alpha 1-4 Freeze dryer, *B. Braun* Biotech International, *Melsungen*, Germany).

Partitioning of Rice Bran Crude Extracts

The freeze dried 70% ethanolic extracts of each of the selected rice varieties were suspended in water and partitioned successively with hexane (50×3 mL), dichloromethane (DCM: 50×3 mL) and butanol (BuOH: 50 × 3 mL) to obtain hexane-soluble, DCM-soluble and BuOH-soluble fractions respectively. Hexane, DCM and BuOH fractions were evaporated using a rotary evaporator and aqueous fraction was freeze dried and used in following anti-inflammatory bioassays.

Oxidative burst inhibitory activity inhuman whole blood

This experiment was conducted after obtaining ethical clearance from independent ethics committee, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan (ICCBS/IEC-008-BC-2015/Protocol/1.0.). Luminol-enhanced chemiluminescence assay was performed according to Helfand et al. [21] and Haklar et al. [22] methods with some modifications. Reaction volume of 100 µL containing 25 µL of human whole blood (1:20 dilution) and 25 µL of rice bran crude extracts/fractions (1, 10, 100 µg/mL; n =3 each) were incubated at room temperature (25±2˚C) for 15 min. Then, 25 µL of luminol (7×10^{-5} M) and 25 µL of serum opsonized zymosan were added and phagocytosis kinetic studies were monitored for 50 min using a luminometer (Luminoskan RS, Labsystem, Helsinki, Finland) with repeated scans with 30 s intervals and 1 s points measuring time.

$HBSS^+$ was served as blank and reaction mixture without rice bran crude extracts/fractions served as control. Ibuprofen was used as the reference drug. The peak and total integral chemiluminescence readings were expressed as Relative Light unit (RLU). The inhibition percentage (%) of each sample (rice bran crude extracts/ fractions) was calculated using following equation.

$$\text{Inhibition (\%)} = (RLU_{Control} - RLU_{Sample} / RLU_{Control})^* 100$$

Oxidative burst inhibitory activity in isolated human PMNs

The PMNs were isolated from blood of healthy human volunteers [23]. The ethical approval was obtained from independent ethics committee, ICCBS, University of Karachi, Pakistan (ICCBS/IEC-008-BC-2015/Protocol/1.0.). Briefly, 10 mL of venous blood was added to 50 mL falcon tubes. Equal volumes of $HBSS^{--}$ and LSM were then added and allowed erythrocytes to settle down at room temperature (25±2˚C) for 30 min. Then, plasma was laid carefully on LSM and centrifuged at 400 g at 4˚C for 20 min. The supernatant was then removed and the resulting pellet was washed twice with distilled water and added with 5-10 mL $HBSS^{--}$and centrifuged at 400 g at 4˚C for 20 min. The resulting PMNs pellet was suspended in $HBSS^{--}$ (pH 7.4). Cell viability was determined by the standard trypan blue exclusion method and cells were counted using a haemocytometer. A stock solution of 1×10^6 cells/mL was prepared and same protocol (instead of 25 µL human blood 25 µL of 1×10^6 cells/mL stock solution was used) was used as described in the previous assay [21,22] to determine the effect of rice bran crude extracts and fractions (n =3 each) on oxidative burst in human PMNs.

Oxidative burst inhibitory activity in mice peritoneal macrophages

This experiment was performed in accordance with the ethical guide lines set by the scientific advisory committee, animal care, use and standards, ICCBS, University of Karachi, Pakistan (Protocol No. 1209004). Isolation of mice peritoneal macrophages were carried out according to Bergman et al. [24] method with some modifications. Briefly, mice (n=6) were injected intraperitoneally with 10% heat-inactivated FCS. After 3 days, mice were killed by cervical dislocation and dipped whole mice in 70% ethanol. Then, peritoneal exudate was collected and centrifuged at 400 g for 20 min at 4˚C. The supernatant was removed and the resulting precipitate was washed with RPMI medium and centrifuged at 300 g for 10 min at 4°C. The supernatant was removed and the cell palette was dissolved in 1 mL of $HBSS^{--}$. Cell viability was determined by the standard trypan blue exclusion method and a stock solution of 1×10^6 cells/mL was prepared. Same protocol (instead of 25 µL human blood 25 µL of 1×10^6 cells/mL stock solution prepared was used in the assay) was followed as described in the previous assay [21,22] to determine the effect of rice bran crude extracts and fractions (n =3 each) on oxidative burst in mice peritoneal macrophages.

Nitric Oxide Inhibitory Activity

Effect of rice bran crude extracts/fractions on nitric oxide inhibitory activity was performed according to the method of Andrade et al. [25] with some modifications in 96 well micro plates (n =3 each). The mouse macrophage cell line J774.2 (European Collection of Cell Cultures, UK) was cultured in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin in 75 cm^2 culture flasks and incubated at 37˚C in a humidified 5% CO_2 incubator. Reaction volume of 200 µL containing 1×10^6 cells/mL, 30 µg/mL LPS and 25 µg/mL rice bran crude extracts/ fractions were incubated at 37˚C for 48 h in a humidified 5% CO_2 incubator. Control incubations were conducted in the same way while replacing rice bran crude extracts/fractions with DMEM medium. Then, 50 µL of cell culture supernatants were added to 50 µL of 1% sulphanilamide and 50 µL of 0.1% naphtyl-ethylene diamine dihydrochloride prepared in 2.5% phosphoric acid. The reaction mixture was incubated at room temperature (30±2˚C) for 10 min and the absorbance was measured at 550 nm using a 96-well micro plate reader (SpectraMax Plus^{384}, Molecular Devices, Sunnyvale, CA, USA). NG-Monomethyl-*L*-arginine monoacetate salt (*L-NMMA*) was used as the reference drug. The % inhibition of each rice bran crude extracts/fractions was calculated using the following equation.

$$\text{Inhibition (\%)} = [(A_c - A_b)/ A_c]^* 100$$

A_c is the absorbance of the control and A_b is the absorbance of rice bran crude extracts/fractions

Inhibitory activity against inflammatory cytokines TNF-α and IL-1 β

Inhibitory activity of rice bran crude extracts/fractions on TNF-α and IL-1 β production was performed following Singh et al. [26] with some modifications (n =3 each). THP-1 cells (Human monocytic leukemia cells) were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mML-glutamine, 5.5 mM glucose, 50 µM mercaptoethanol, 1 mM sodium pyruvate, and 10 mM HEPES in 75 cm^2 culture flasks. Flasks were incubated at 37˚C in a humidified 5% CO_2 incubator until the culture flasks attained 70% confluency. Cells were then plated in 24 well tissue culture plates (2.5×10^5 cells/mL) and 20 ng/mL PMA was added into the culture and incubated at 37˚C for 24 h in a humidified 5% CO_2 incubator. Then, 50 ng/mL LPS and 25 µg/mL rice bran crude extracts/ fractions were added and incubated at 37ºC in a humidified 5% CO_2 incubator. The cell culture supernatants collected after 4 h and 18 h of incubation periods were analyzed for TNF-α and IL-1β respectively using human TNF-α and IL-1β Duo test kits (R&D

systems, Minneapolis, USA). Control incubations were conducted in the same way while replacing rice bran crude extracts/fractions with RPMI medium. The % inhibition of each rice bran crude extracts/fractions was calculated compared to the control.

Cytotoxicity against normal cell line

Cytotoxicity of rice bran crude extracts/fractions against 3T3 cell line from mouse embryo fibroblast was evaluated according to the method of Mossman [27] with some modifications in 96-well micro plates. The 3T3 cells were cultured in DMEM supplemented with 2 mM glutamate, 5% of FBS and 1% penicillin-streptomycin in 75 cm^2culture flasks and incubated at 37°C in a humidified 5% CO_2 incubator until the culture flasks attained 70-80% confluency. Cell suspension of 6×10^4 cells/ml was prepared and cell viability was determined by the standard trypan blue exclusion method. Then, 100 µL of cell suspension was plated into each well of 96 well micro plates and incubated overnight at 37°C in a humidified 5% CO_2 incubator. Thereafter, supernatant was carefully removed and rice bran crude extracts/fractions (5, 25, 50 µg/mL; n =3 each) in 200 µL of DMEM were incubated at 37°C for 48 h in a humidified 5% CO_2 incubator. Then, 50 µL of MTT (2 mg/mL) was added to each well and incubated at 37°C for 4 h in a humidified 5% CO_2 incubator. Subsequently MTT was removed, 100 µL of DMSO was added to each well and the absorbance was measured at 540 nm using 96-well micro plate reader (SpectraMax Plus^{384}, Molecular Devices, Sunnyvale, CA, USA). Control incubations were conducted in the same way while replacing rice bran crude extracts/fractions with DMEM medium. Cyclohexamide was used as the positive control. The %inhibition (cytotoxicity) by each rice bran crude extracts/fractions were calculated using following equation.

$$\text{Inhibition (\%)} = [(A_c - A_b)/ A_c] * 100$$

A_c is the absorbance of the control and A_b is the absorbance of rice bran crude extracts/fractions

Statistical analysis

Results were analysed using SAS version 6.12 (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) and the Duncan's Multiple Range Test (DMRT) were used in statistical analysis of data. $p<0.05$ was regarded as significant.

Results

Oxidative burst inhibitory activity in human whole blood

Percent inhibition at various concentrations and IC_{50} values of rice bran crude extracts and fractions for oxidative burst inhibitory activity in human whole blood is given in Tables 1 and 4 respectively. The results demonstrated that all rice bran crude extracts and fractions (except BuOH fractions of Goda Heeneti and Dik Wee) had varying levels of oxidative burst inhibitory activity in human whole blood. The varying levels of oxidative burst inhibitory activities observed among rice bran crude extracts and fractions were significantly ($p<0.05$) different among the varieties (Table 4). Crude bran extract of Sudu Heeneti exhibited significantly ($p<0.05$) high oxidative burst inhibitory activity compared to rice bran crude extracts of other selected varieties (Table 4). The order of potency of rice bran crude extracts for oxidative burst inhibition were Sudu Heeneti>Dik Wee=Masuran=Goda Heeneti. Interestingly, rice bran crude extracts of Sudu Heeneti and Dik Wee showed significantly high ($p<0.05$) and comparable inhibitory activities respectively compared to the reference drug, ibuprofen used in the study. Among rice bran fractions hexane, DCM and aqueous fractions of selected rice showed significantly high ($p<0.05$) oxidative burst inhibitory activity compared to BuOH fractions of all selected rice. Further, BuOH fractions of Goda Heeneti and Dik Wee did not show any inhibitory activity at studied concentrations. Furthermore, results demonstrated that synergism in activity in rice bran crude extracts during the partitioning process.

Oxidative burst inhibitory activity in isolated human PMNs

Percent inhibition at various concentrations and IC_{50} values of rice bran crude extracts and fractions for oxidative burst inhibitory activity in human PMNsis given in Tables 2 and 4 respectively. Rice bran crude extracts of all selected rice showed potent oxidative burst inhibitory activity in isolated PMNs with significant differences ($p<0.05$) among the varieties. Crude rice bran extract of variety, Goda Heeneti exhibited the highest oxidative burst inhibition in human PMNs. The order of potency of oxidative burst inhibition by rice bran crude extracts were Goda Heeneti>Sudu Heeneti=Masuran>Dik Wee. Interestingly, inhibitory activities of rice bran crude extracts of Goda Heeneti, Sudu Heeneti and Masuran were significantly high ($p<0.05$) compared to the reference drug ibuprofen. However, partitioning of rice bran crude extracts showed synergism in activity in rice bran crude extracts. Among the bran fractions studied hexane, DCM and aqueous fractions of selected rice demonstrated significantly high ($p<0.05$) oxidative burst inhibitory activity compared to BuOH fractions of selected rice.

Oxidative burst inhibitory activity in peritoneal macrophages from mice

Percent inhibition at various concentrations and IC_{50} values of rice bran crude extracts and fractions for oxidative burst inhibitory activity in peritoneal macrophages from mice is given in Table 3 and 4 respectively. The results revealed that all rice bran crude extracts had significantly high ($p<0.05$) oxidative burst inhibition in mice macrophages compared to the reference drug, ibuprofen.

The order of potency was Sudu Heeneti=Goda Heeneti=Dik Wee>Masuran. Partitioning of rice bran crude extracts showed significantly high ($p<0.05$) oxidative burst inhibitory activity in hexane and aqueous fractions of all selected rice and DCM and BuOH fractions of Sudu Heeneti compared to ibuprofen.

Rice variety	Concentration (µg/mL)	% Inhibition				
		Crude	Hexane	DCM	BuOH	Aqueous
Sudu Heeneti	100	93.88±1.21	82.72±1.17	91.16±1.27	34.43±1.47	68.00±3.21
	10	55.67±1.57	9.13±4.43	19.05±2.53	1.92±7.72	11.84±5.22
	1	40.34±4.70	2.82±5.05	-3.39±5.07	-1.10±7.49	-3.54±3.33
Dik Wee	100	90.88±1.39	75.53±0.60	75.66±2.10	3.98±4.14	53.20±3.82
	10	45.83±2.54	7.16±3.59	-4.13±4.22	-10.83±12.17	-0.20±0.97
	1	30.88±1.31	-14.86±1.40	-11.13±6.45	-19.07±3.24	-11.60±3.35
Masuran	100	85.52±0.78	77.97±5.81	64.06±1.76	38.06±2.52	67.81±1.09
	10	39.53±3.53	19.12±1.42	7.98±0.39	-1.94±7.70	11.05±0.70
	1	29.68±0.71	-11.37±1.36	-12.04±4.27	-5.64±2.80	-16.25±4.45
Goda Heeneti	100	90.08±1.75	81.95±0.29	86.31±2.13	-3.58±5.21	70.09±1.49
	10	31.91±2.72	31.95±7.62	9.38±2.82	-8.95±3.18	11.85±2.65
	1	15.18±0.40	-8.73±6.27	-5.29±7.19	-5.64±0.88	8.38±1.03
Values presented as mean±SE (n = 3). DCM: Dichloromethane fraction. BuOH: Butanol fraction						

Table 1: Percent oxidative burst inhibitory activity of rice bran crude extracts and fractions in human whole blood.

Rice variety	Concentration (µg/mL)	% Inhibition				
		Crude	Hexane	DCM	BuOH	Aqueous
Goda Heeneti	100	99.65±0.04	98.87±0.21	92.18±0.51	-7.75±4.34	81.87±0.95
	10	65.72±3.57	23.59±2.24	42.38±6.88	- 11.09±3.12	46.08±1.18
	1	62.91±2.94	-13.39±0.31	- 2.23±2.80	- 13.64±0.00	10.39±1.59
Sudu Heeneti	100	99.68±0.06	98.43±0.29	97.13±0.88	54.42±1.39	78.57±1.46
	10	75.69±2.92	43.42±1.54	37.43±3.54	-7.45±3.66	46.21±1.46
	1	44.50±4.24	- 4.85±9.17	- 2.19±8.25	- 6.58±7.02	18.59±3.83
Masuran	100	99.98±0.00	99.52±0.20	90.97±1.49	17.27±1.15	80.25±0.73
	10	64.89±1.61	33.16±2.07	24.60±5.82	-13.64±3.12	49.25±3.92
	1	43.86±4.17	-12.26±1.70	5.77±6.53	-13.64±0.00	20.40±1.01
Dik Wee	100	100.00±0.01	99.62±0.03	97.07±0.27	47.22±1.84	78.90±0.12
	10	69.60±3.11	35.78±0.93	48.34±5.93	- 11.21±2.16	65.23±3.99
	1	31.05±3.51	-10.00±0.28	38.53±1.66	-13.64±0.00	23.58±1.83
Values presented as mean±SE (n = 3). DCM: Dichloromethane fraction. BuOH: Butanol fraction.						

Table 2: Percent oxidative burst inhibitory activity of rice bran crude extracts and fractions in isolated human polymorphonuclear leukocytes.

Rice variety	Concentration (µg/mL)	% Inhibition				
		Crude	Hexane	DCM	BuOH	Aqueous
Sudu Heeneti	100	95.62±10.08	99.78±3.64	84.67±6.50	81.45±7.30	97.98±1.69
	10	76.96±11.41	92.12±3.67	80.90±5.71	62.47±11.06	74.35±5.75
	1	39.13±3.28	49.97±5.51	44.74±0.98	40.79±6.46	22.49±10.21
Goda Heeneti	100	103.29±1.26	101.04±2.34	62.80±2.55	68.69±2.95	92.47±2.40
	10	64.74±2.20	71.56±10.25	29.81±1.54	31.75±9.88	60.79±5.50
	1	24.17±5.67	9.95±4.38	24.14±2.25	29.35±6.47	13.89±5.73
Dik Wee	100	101.75±0.59	100.41±0.23	63.49±2.11	54.42±4.92	95.15±2.46
	10	77.46±6.78	73.43±1.87	28.19±7.13	23.92±9.56	87.42±3.56
	1	17.76±4.48	36.31±1.10	24.31±1.18	0.44±3.44	37.68±3.38
Masuran	100	103.27±0.87	103.58±0.21	68.63±5.23	74.38±1.90	97.76±1.15
	10	74.38±8.48	58.14±7.46	20.24±2.14	32.61±1.79	76.88±4.31
	1	10.55±8.72	19.90±1.34	12.20±9.25	11.48±0.83	34.74±6.77
Values presented as mean±SE (n = 3). DCM: Dichloromethane fraction. BuOH: Butanol fraction.						

Table 3: Percent oxidative burst inhibitory activity of rice bran crude extracts and fractions in mice peritoneal macrophages.

Biological activity	Rice variety	IC_{50} values (µg/mL)				
		Crude	Hexane	DCM	BuOH	Aqueous
Oxidative burst inhibitory activity in human whole blood	Sudu Heeneti	7.14±0.73^{c}	35.88±2.79^{a}	26.86±1.31^{c}	NWR	48.12±6.25^{b}
	Dik Wee	12.33±1.43^{b}	42.52±2.26^{a}	47.94±3.46^{a}	NA	89.75±12.14^{a}
	Masuran	16.93±2.47^{ab}	34.23±3.75^{a}	59.76±6.95^{a}	NWR	47.53±8.37^{a}
	Goda Heeneti	20.39±1.15^{a}	22.71±5.07^{b}	33.82±2.46^{b}	NA	45.22±2.14^{b}
Oxidative burst inhibitory activity in isolated human polymorphonuclear leukocytes	Goda Heeneti	0.22±0.09^{c}	22.4±1.38^{a}	14.23±3.82^{b}	NA	12.85±0.86^{a}
	Sudu Heeneti	1.64±0.54^{b}	13.2±0.73^{c}	16.27±1.85^{ab}	85.9±3.97^{b}	11.11±0.98^{a}
	Masuran	1.71±0.54^{b}	17.9±0.94^{b}	24.02±2.85^{a}	NWR	9.78±0.63^{b}
	Dik Wee	3.57±0.61^{a}	16.7±0.25^{b}	11.54±2.18^{b}	112.9±10.03^{a}	9.02±0.71^{b}
Oxidative burst inhibitory activity in mice peritoneal macrophages	Sudu Heeneti	3.49±0.73^{b}	1.06±0.52^{d}	1.84±0.01^{b}	2.06±0.45^{c}	6.02±1.35^{b}
	Goda Heeneti	4.79±0.40^{ab}	7.57±0.54^{a}	41.25±6.18^{a}	31.29±12.46^{b}	8.27±0.68^{a}
	Dik Wee	5.87±0.64^{ab}	2.68±0.16^{c}	41.05±3.95^{a}	55.48±0.01^{a}	2.72±0.48^{c}
	Masuran	7.14±1.29^{a}	5.24±0.27^{b}	41.71±4.79^{a}	25.83±0.05^{b}	3.10±0.74^{bc}
Values presented as mean±SE (n = 3). Mean values in a column superscripted by different letters are significantly different at $p < 0.05$. DCM: Dichloromethane fraction. BuOH: Butanol fraction. NA: No Activity; NWR: Not Within the Range. Ibuprofen IC50 values for oxidative burst inhibition in human whole blood, human polymorphonuclear leukocytes and macrophages: 11.18±1.87 µg/mL, 2.47±0.59 µg/mL and 16.88±2.53µg/mL respectively.						

Table 4: IC_{50} Values for oxidative burst inhibitory activity of rice bran crude extracts and fractions in human whole blood, isolated human polymorphonuclear leukocytes and mice peritoneal macrophages.

Nitric oxide inhibitory activity

Nitric oxide inhibitory activity of rice bran crude extracts and fractions studied at the 25µg/mL is presented in Figure 1. The rice bran crude extract of Sudu Heeneti demonstrated the highest nitric oxide inhibitory activity compared to the rice bran crude extracts of other selected varieties. Nitric oxide inhibitory activity of bran fractions showed that DCM fractions of all the studied rice varieties had significantly high ($p<0.05$) inhibitory activities compared to hexane, BuOH and aqueous fractions and the reference drug used in this study. Further, except hexane and aqueous fractions of Sudu Heeneti and aqueous fraction of Dik Wee, all other fractions exhibited significantly high ($p<0.05$) nitric oxide inhibitory activity compared to their respective rice bran crude extracts.

TNF-α and IL-1β inhibitory activity

TNF-α and IL-1 β inhibitory activity of rice bran crude extracts and fractions studied at the 25 µg/mL is presented in Figure 2 and 3 respectively. The results revealed that rice bran crude extracts of Sudu Heeneti and Goda Heeneti exhibited both TNF-α and IL-1 β inhibitory activities at the tested concentration. However, rice bran crude extracts of Masuran and Dik Wee fail to show any inhibitory activity against TNF-α and IL-1 β at the same concentration tested. Further, TNF-α inhibitory activity of rice bran crude extracts of both Sudu Heeneti and Goda Heeneti were significantly high ($p<0.05$) compared to IL-1 β inhibition. In partitioning of rice bran crude extracts revealed that TNF-α inhibitory activity was potent with the DCM fractions followed by BuOH fractions. None of the hexane and aqueous fractions of all selected rice showed TNF-α inhibition at the studied concentration. IL-1β inhibitory activity was observed in all DCM fractions and Hexane fractions of Dik Wee and Masuran. None of the BuOH and aqueous fractions of selected rice and hexane fractions of Sudu Heeneti and Goda Heeneti showed IL-1 β inhibitory activity at the tested concentration (Figure 3).

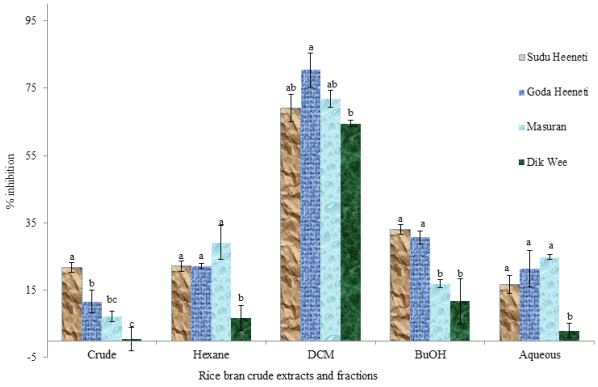

Figure 1: Nitric oxide inhibitory activity of rice bran crude extracts and fractions at 25µg/mL. Results presented as mean±SE (n = 3). Mean % inhibition values superscripted by different letters in crude bran extracts and fractions are significantly different at $p < 0.05$. DCM: Dichloromethane fraction; BuOH: Butanol fraction. Positive control: NG-Monomethyl-*L*-arginine monoacetate salt: 50% inhibition at 24.20±0.80 µg/mL.

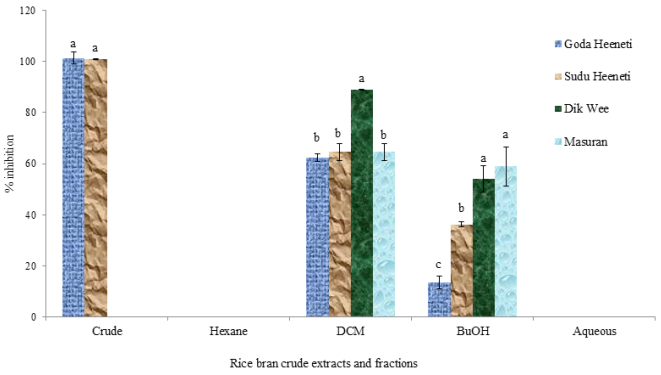

Figure 2: TNF-α inhibitory activity of rice bran crude extracts and fractions at 25µg/mL. Results presented as mean±SE (n = 3). Mean % inhibition values superscripted by different letters in crude bran extracts and fractions are significantly different at $p < 0.05$. DCM: Dichloromethane fraction; BuOH: Butanol fraction.

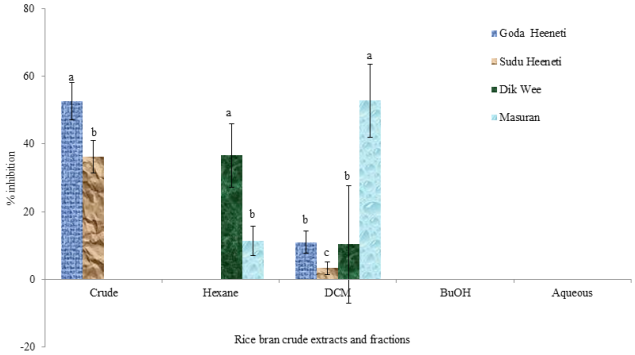

Figure 3: IL-1β inhibitory activity of rice bran crude extracts and fractions at 25µg/mL. Results presented as mean±SE (n = 3). Mean % inhibition values superscripted by different letters in crude bran extracts and fractions are significantly different at $p < 0.05$. DCM: Dichloromethane fraction; BuOH: Butanol fraction.

Cytotoxicity Against Normal Cell Line

Results revealed that there was no cytotoxicity associated with rice bran crude extracts and the fractions at the studied concentrations.

Discussion

During both acute and chronic inflammatory process PMNs and mononuclear cells in the immune system produce reactive

oxygen species [2,3,5,7]. Excess production of these radicals deregulates the cellular function and lead to many chronic diseases [2,3,5,7]. Therefore, inhibition of oxidative burst at the inflammatory site is important and demanded for prevention and management of chronic diseases. The findings of the current studies showed for the first time that rice bran crude extracts of Sri Lankan traditional rice varieties had potent oxidative burst inhibitory activities on human whole blood, isolated human PMNs and mice peritoneal macrophages. Rice bran crude extract of Sudu Heeneti showed1.6 times higher oxidative burst inhibitory activity compared to ibuprofen in human blood. Similarly, rice bran crude extracts of Goda Heeneti and Sudu Heeneti exhibited 11.2 and 1.5 times higher oxidative burst inhibition in human PMNs compared to ibuprofen. Furthermore, 4.8, 3.5, 2.8 and 2.4 times higher oxidative burst inhibitory activities were observed with rice bran crude extracts of Sudu Heeneti, Goda Heeneti, Dik Wee and Masuran respectively in macrophages compared to ibuprofen. Partitioning of rice bran crude extracts showed inhibitory activities in both non-polar and polar fractions. Further, synergism in activity in crude extracts was observed. It is reported that synergism in activity of crude extracts is a common fact for some natural products and it has been proven that some crude plant extracts are more active pharmacologically than their isolated active principles [28].

The observed oxidative burst inhibitory activity of rice bran could be attributed to the ability of bioactive compounds in crude extracts and fractions to block the complement receptors on the cell surface of phagocytes (e.g. C3b): as zymosan activates phagocytes by binding to complement receptor type 3 on the cell surface, inhibition of NADPH oxidase: a cell membrane associated enzyme of phagocytes and scavenging of reactive oxygen species produced during the oxidative burst. In this study, luminol was used as a chemiluminescent probe, which has the ability to enter the phagocytes and react with reactive oxygen species products and enhance the detection level. Therefore, in this study it is most likely that scavenging of intracellular reactive oxygen species were detected rather than extracellular reactive oxygen species by bioactive compounds in crude extracts and fractions. Further, luminol is specific for the detection of hypochlorous acid (HClO), hydroxyl (*·OH*) and superoxide (O_2^-) radicals which are primarily produced at the later phase of oxidative burst [29,30,31].

In addition to reactive oxygen species, nitric oxide has been implicated in the pathogenesis of variety of diseases including the inflammation [2,5,32]. The results demonstrated that DCM fractions of selected rice varieties had potent nitric oxide inhibitory activity compared to hexane, BuOH and aqueous fractions and rice bran crude extracts. Nitric oxide inhibitory activity of pigmented rice bran including red rice bran crude extracts is reported [18,33]. However, extremely limited research has been directed to in-depth studies on nitric oxide inhibitory activity of rice bran fractions. Hu et al. [16] reported that black rice pigmented fraction exerted 50-60% nitric oxide inhibition at 125 µg/mL in murine macrophage RAW 264.7 cells. In our study, we observed 21.33±5.41-24.82±0.74% inhibition at 25 µg/mL in pigmented red aqueous fractions of Goda Heeneti and Masuran. The concentration used was 5 times low in the present study compared to the study of Hu et al. [16]. Thus, inhibitory activity at 125 µg/mL in red aqueous fractions of Goda Heeneti and Masuran may be far superior to black rice pigmented fraction. Further, our study clearly demonstrated that DCM fractions were more potent in inhibiting nitric oxide compared to pigmented aqueous fractions showing 65-80% inhibition at 25 µg/mL. Furthermore, nitric oxide inhibitory activity of DCM fractions of selected rice were more potent than the reference drug used in this study. Therefore, further research is warranted for isolating active compounds responsible for mediating nitric oxide inhibition in DCM fractions of selected Sri Lankan traditional red rice.

Previous research findings on nitric oxide inhibition by rice bran has been explained due to the presence of γ-oryzanol, suppression of inducible nitric oxide synthase gene expression and the presence of polyphenolic anti-oxidants [16,18,34]. We have recently reported brans of the selected rice varieties in this study had high amounts of phenolic antioxidants [12]. Further, Gunaratne et al. [11] reported that brans of Sudu Heeneti has high γ-oryzanol content. Therefore, the presence of γ-oryzanol and other phenolics may be, at least partly, responsible for the observed nitric oxide inhibitory activity by brans of selected Sri Lankan traditional rice. However, exact compounds and mode of actions responsible for mediating nitric oxide inhibition by brans of selected Sri Lankan traditional rice have to be investigated further.

Cytokines are regulatory glycoproteins produced by the cells of the immune system [3,35]. TNF-α and IL-1β are two pro-inflammatory cytokines implicated in the pathogenesis of many inflammatory diseases [3,4,8,35]. The results of this study showed that TNF-α inhibition by brans of selected rice was more prominent compared to IL-1β inhibition. Further, TNF-α inhibition was most potent in DCM fractions indicating that medium polar compounds in the rice bran may be responsible in mediating this biological activity. However, IL-1β inhibitory compounds may be low polar to medium polar in the rice bran as both hexane and DCM fractions were evident for this biological activity.

Previous workers have also reported that rice bran is effective in inhibiting inflammatory cytokines including TNF-α and IL-1β and this was attributed to inhibition of gene expressions of cytokines and presence of γ-oryzanol, α-tocopherol and tocotrienols in the rice bran [36-39]. Our studies [13] on α-tocopherol contents of bran extracts of selected rice varieties showed that crude bran extracts of Sudu Heeneti and Goda Heeneti had the highest levels of α-tocopherol contents (Sudu Heeneti: 6.61±0.23; Goda Heeneti: 3.47±0.30; Masuran: 2.20±0.32; Dik Wee: 2.03±0.29 mg/100 g

bran dry weight). Further, Gunaratne et al. [11] has shown that brans of Sudu Heeneti is a rich source of γ-oryzanol, tocopherols and tocotrienols. Therefore, it is possible that γ-oryzanol, tocopherols and tocotrienols may be responsible, at least partly, for the observed TNF-α and IL-1β inhibitory activities. In cytotoxicity experiments none of the crude extracts or fractions showed cytotoxicity to normal cells.

Interesting finding of this research was that non-pigmented DCM fractions of all selected Sri Lankan traditional red rices had significant anti-inflammatory activity for all the assays tested. Earlier it was believed that colored rices have more biological activities due to the presence of pigments in the rice bran [12,33]. However, this research for the 1st time showed non-pigmented DCM fractions of colored red rice also had significant anti-inflammatory activity. Therefore, this indicates a new insight of further studying the compounds responsible for mediating anti-inflammatory activities in non-pigmented DCM fractions of colored red rices. Moreover, this is the 1st report of anti-inflammatory activities for any Sri Lankan rice variety and oxidative burst inhibitory activity by crude bran extracts and fractions of any rice variety worldwide. Findings of this study scientifically validated claims in the Sri Lankan traditional knowledge. What is more, the results also indicate that potential of using brans of Sri Lankan traditional red rices in development of novel functional foods and nutraceuticals for prevention and dietary management of inflammation associated chronic diseases.

Conclusions

Considering all, it could be concluded that brans of selected Sri Lankan traditional red rice possesses anti-inflammatory activity mediated via multiple mechanisms: inhibition of oxidative burst, nitric oxide and pro-inflammatory cytokines TNF-α and IL-1β without having cytotoxicity to normal cells. Partitioning of rice bran crude extracts showed inhibitory activities in both non-polar and polar fractions of the rice bran. Interestingly, non-pigmented DCM fractions exhibited significant anti-inflammatory activity in all the assays studied. Finally, brans of selected red rice may have the potential to use in development of plant based natural foods and nutraceuticals for prevention and dietary management of inflammation associated chronic diseases.

Conflict of Interest: There is no conflict of interest in any form between the authors.

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