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Physiological Free Radicals Scavenging Potential of Brans of Selected Sri Lankan Traditional Red Rice (*Oryza sativa* L.): an *In Vitro* Antioxidant Activity Study

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Abstract

Objectives: To evaluate the effect of brans of selected Sri Lankan traditional red rice varieties on physiological free radicals scavenging potential using *in vitro* antioxidant assays.

Materials and Methods: Freeze dried 70% ethanolic extracts of brans of four traditional red rice varieties namely Sudu Heeneti, Goda Heeneti, Dik Wee and Masuran were used. Antioxidant activities were evaluated using superoxide radical scavenging activity (SRSA), nitric oxide radical scavenging activity (NORSA) and oxygen radical absorbance capacity: ORAC (measure of peroxyl radical scavenging activity) *in vitro* antioxidant assays (n=3 each).

Results and Discussion: Brans of all the selected varieties showed SRSA, NORSA and ORAC. The observed antioxidant activities varied significantly ($P<0.05$) among varieties. SRSA and NORSA were significantly high ($P<0.05$) in brans of Masuran and Sudu Heeneti respectively. For ORAC, brans of Sudu Heeneti and Goda Heeneti showed comparable and significantly high ($P<0.05$) activities compared to the rest of the varieties. The order of potency of brans of selected varieties for SRSA, NORSA and ORAC were Masuran>Sudu Heeneti=Dik Wee>Goda Heeneti, Sudu Heeneti>Dik Wee=Goda Heeneti>Masuran and Sudu Heeneti=Goda Heeneti>Dik Wee=Masuran respectively.

Conclusion: It is concluded that brans of all the selected Sri Lankan traditional red rice varieties had physiological free radicals scavenging activities with varying degrees of potentials. This is the 1st study to report such biological activities for any variety of rice in Sri Lanka and indicates its potential as a functional food ingredient in the health food industry.

Keywords: Antioxidant activities; Physiological free radicals; Sri Lankan traditional rice; Rice bran; *In vitro* antioxidant assays

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Introduction

Imbalance between formation and neutralization of free radicals causes to a condition widely known as the oxidative stress [1-4]. It is now very well-known and scientifically proven fact that this condition facilitates the formation and progression of many chronic diseases such as cancer, diabetes, atherosclerosis, cardiovascular diseases, inflammatory diseases, neurodegenerative diseases and also ageing [1-5]. Antioxidants have the ability to neutralize the free radicals generated through various mechanisms in the biological system of humans and thereby important in prevention and/or management of such chronic diseases [1-4]. Both synthetic and natural antioxidants are currently being used in the food industry [6]. The synthetic antioxidants are powerful free radical scavengers. However, the

adverse side effects associated with such synthetic compounds demand the food industry to use naturally derived antioxidants from plant foods [5-7]. To date many plants have been scientifically shown to have antioxidant activities and many more are being in the investigation process to identify more potent commercially valuable natural antioxidants [5-11].

Rice is the most widely consumed cereal in Asia and the dietary staple food for half of the world's population. Rice bran is the outer layer of the rice grain and a cheap by product of the rice milling industry [12]. It is scientifically proven to have numerous nutritional and functional health benefits including antioxidant activity [8-14]. Thus, rice bran is currently available in the international market in the form of various value added products such as stabilized rice bran, rice bran capsules, nutraceuticals, rice bran incorporated foods and rice bran oil [12,15]. Further, it is also used in the cosmaceuticals industry as it can retard the skin aging process because of the presence of powerful natural antioxidant compounds [12].

Sri Lanka is a country which has a long history of rice cultivation [16]. In early days the country was known as the granary of east and existence of many traditional or the indigenous varieties had been reported. Some of these varieties were well known to have health benefits in the Sri Lankan traditional knowledge and folklore [8,16]. Recent studies conducted in the country were able to scientifically prove such health benefits including antioxidant activities of some of the Sri Lankan traditional rice varieties [8-10,17-19]. However, the reported antioxidant properties of Sri Lankan traditional rice varieties had not been previously investigated using physiological radicals based antioxidant activity assays. Further, there are limited studies on this topic to date for other rice varieties worldwide. In the present study antioxidant activities of brans of selected Sri Lankan traditional rice varieties were evaluated using physiological radicals: superoxide, nitric oxide and peroxyl radical [measured using oxygen radical absorbance capacity (ORAC) assay] based antioxidant assays *in vitro*.

Materials and Methods

Rice samples

Four traditional red rice (*Oryza sativa* L.) varieties of Sri Lanka were selected and used in the present study. The four varieties were Sudu Heeneti, Goda Heeneti, Masuran and Dik Wee. Rice varieties were obtained from the Regional Rice Research and Development Center (RRRDC), Bombuwala, Sri Lanka which were cultivated at the experimental field conditions for the present study.

Sample preparation and extraction of rice bran

Paddy samples were dehulled (THU 35B, Satake, Hiroshima, Japan) to obtain whole grain rice. Then, whole grain rice samples were polished in a laboratory mill (TM-05C, Satake, Hiroshima, Japan) to obtain rice bran. Rice bran was sieved using a 60 mesh sieve and 1 g of rice bran was extracted overnight in 70% ethanol-water (1 g/12 ml 70% ethanol) at room temperature (30 ± 2°C). Rice extracts were then centrifuged (10 min, 825 g), filtered (0.45 µm nylon filters) and evaporated using a rotary evaporator and freeze dried (Christ-Alpha 1-4 Freeze dryer, Biotech International, Germany). The freeze dried extracts were used in evaluation of following antioxidant activity assays *in vitro*.

Chemicals and equipment

Phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), fluorescein, Quercetin and Trolox were purchased from Sigma-Aldrich (USA). Preparation of buffers and other necessary solvents were performed with the use of analytical grade chemicals from Sigma-Aldrich (USA). Absorbance (SpectraMax Plus384, Molecular Devices, USA) and florescence (SPECTRAmax- Gemini EM, Molecular Devices Inc, USA) readings were obtained using 96-well micro-plate readers.

Superoxide radical scavenging activity of rice bran

The superoxide radical scavenging activity was performed according to the method of Liu and Ng [20] with some modifications using 96-well micro plates. Reaction volume of 200 µl containing 0.2 mM NADH, 0.08 mM NBT and different concentrations of rice bran extracts (31.25, 62.5, 125, 250, 500 µg/ml) in 100 mM phosphate buffer were initially pre read at 560 nm. Then 0.008 mM PMS were added and incubated at room temperature (30 ± 2°C) for 5 min. The absorbance readings were taken at 560 nm. Quercetin was used as the positive control. Results were expressed as IC50 values, mM Quercetin equivalents (QE)/g of extract and 100 g dry weight of rice bran.

Superoxide radical scavenging activity (%)=[(Ac - As)/Ac] × 100

Where, Ac is the absorbance of the control and As is the absorbance of the sample.

Nitric oxide radical scavenging activity of rice bran

Nitric oxide radical scavenging activity was performed according to the method of Andrade et al. [21] with some modifications in 96 well micro plates. Reaction volume of 100 µl containing 20 µl of rice bran extracts (31.25, 62.5, 125, 250, 500 µg/ml), 50 µl of sodium nitroprusside solution (30 mg/ml) and 30 µl of 10 mM phosphate buffer saline were incubated at 25°C for 2.5 h. Then, 50 µl from each well was plated to a 96 well micro plate and added with 120 µl of Griess reagent and the absorbance reading was taken at 540 nm. Results were expressed as IC50 values, mM Rutin equivalents (QE)/g of extract and 100 g dry weight of rice bran.

Nitric oxide radical scavenging activity (%)=[(Ac - As)/Ac] × 100.

Where, Ac is the absorbance of the control and As is the absorbance of the sample.

Oxygen radical absorbance capacity of rice bran

The oxygen radical absorbance capacity (ORAC) of rice bran extracts was carried out according to the method described by

Ou et al. [22] with some modifications in 96-well micro plates. Trolox (0.75 and 1.5 µg/ml), fluorescein (4.8 µM) and AAPH (40 mg/ml) solutions were prepared in 75 mM phosphate buffer (pH 7.4). A reaction volume of 200 µl, containing 100 µl of fluorescein and 50 µl of sample (mg/ml) were pre-incubated at 37°C for 10 min. Then, 50 µl of AAPH was added to initiate the reaction and decay of fluorescein was measured at excitation and emission wave lengths of 494 nm and 535 nm respectively in 1 min interval for 35 min. Trolox was used as the standard antioxidant. ORAC activities of the rice bran extracts were calculated by comparing the net area under curve of fluorescein decay between the control and the rice samples using the following equation. Results were expressed as mg TE/g of extract and 100 g dry weight of rice bran.

ORAC=[(AUCs - AUCc)/(AUCt - AUCc)] × (Trolox concentration/ Sample concentration).

Where, AUCs is the area under the curve of the sample, AUCc is the area under the curve of the control and AUCt is the area under the curve of the Trolox.

Statistical analysis

Results were presented as mean ± standard error (SE). SAS software and version 6.12 was used in the statistical analysis of data. One way analysis of variance (ANOVA) and the Duncan's Multiple Range Test (DMRT) were used in measuring the differences among treatments. P<0.05 was considered as significant.

Results

Superoxide radical scavenging activity of rice bran

Superoxide radical scavenging activity of brans of selected Sri Lankan traditional rice is given in **Table 1** and the dose response relationship of bran extracts for superoxide radical scavenging activity is given in **Figure 1**. Results showed that there was a significant difference (P<0.05) among the varieties for superoxide scavenging activity. The IC50 values of bran extracts of selected varieties were ranged from 263.26 ± 5.64 to 357.09 ± 11.53 µg/ml. Superoxide scavenging activity expressed as mM QE/g extract ranged from 0.26 ± 0.01 to 0.36 ± 0.01, whereas for 100 g bran it was in the range from 2.30 ± 0.06 to 3.05 ± 0.05 mM QE. Rice bran of Masuran showed significantly high superoxide radical scavenging activity (in terms of mM QE/100 g bran) compared to brans of other selected rice varieties (3.05 ± 0.05 mM QE/100g bran). The order of potency of superoxide radical scavenging activity (in terms of mM QE/100 g bran) of rice brans were Masuran>Dik Wee=Sudu Heeneti>Goda Heeneti. The selected rice varieties showed moderate activity compared to the reference standard Quercetin used in this study. In dose response studies bran extracts of all rice varieties showed good dose response relationship **(Figure 1)**.

Nitric oxide radical scavenging activity of rice bran

Nitric oxide radical scavenging activity of brans of selected Sri Lankan traditional rice is given in **Table 2** and the dose response relationship of bran extracts for nitric oxide radical scavenging activity is given in **Figure 2**. Significant differences (P<0.05) were observed among the brans of selected rice varieties for nitric oxide radical scavenging activity. Brans of Sudu Heeneti exhibited the highest nitric oxide radical scavenging activity in terms of IC50 values, mM RE/g extract and mM RE/100 g bran compared to the other varieties tested. The order of potency of rice brans for nitric oxide radical scavenging activity was Sudu Heeneti>Goda Heeneti=Dik Wee>Masuran. Compared to the reference standard Rutin, Sudu Heeneti, Goda Heeneti and Dik Wee showed moderate nitric oxide radical scavenging activity while Masuran demonstrated a mild activity. In dose response studies bran extracts of all the selected varieties showed good dose response relationship **(Figure 2)**.

Oxygen radical absorbance capacity of rice bran

ORAC of brans of selected Sri Lankan traditional red rice is given in **Table 3**. There was a significant difference (P<0.05) among the varieties for ORAC. The ORAC of brans of selected rice varieties in terms of mM TE/g extract and mM TE/100 g bran varied from 0.77 ± 0.04 to 1.02 ± 0.04 and from 6.63 ± 0.33 to 8.89 ± 0.34 respectively. Rice brans of Sudu Heeneti and Goda Heeneti exhibited the highest ORAC. The order of potency of selected

Table 1: Superoxide radical scavenging activity of brans of selected Sri Lankan traditional rice.

Rice variety	IC_{50} (µg/ml)	mM QE/g extract	mM QE/100 g bran
Dik Wee	263.26 ± 5.64^{c}	0.36 ± 0.01^{a}	2.79 ± 0.04^{b}
Masuran	267.16 ± 6.98^{bc}	0.35 ± 0.01^{a}	3.05 ± 0.05
Sudu Heeneti	292.39 ± 10.39^{b}	0.32 ± 0.01^{b}	2.81 ± 0.10^{b}
Goda Heeneti	357.09 ± 11.53^{a}	0.26 ± 0.01^{c}	2.30 ± 0.06^{c}

Data represented as mean ± SE (n=4). Mean values in a column superscripted by different letters are significantly different at P<0.05. Results are presented on dry weight basis. QE: Quercetin equivalents; Quercetin IC_{50}: 28.44 ± 1.2 µg/ml.

Table 2: Nitric oxide radical scavenging activity of brans of selected Sri Lankan traditional rice.

Rice variety	IC_{50} (µg/ml)	mM RE/g extract	mM RE/100 g bran
Sudu Heeneti	53.89 ± 6.48^{c}	0.55 ± 0.05^{a}	4.75 ± 0.45^{a}
Dik Wee	94.31 ± 18.98^{b}	0.31 ± 0.04^{b}	2.44 ± 0.28^{b}
Goda Heeneti	105.04 ± 16.40^{b}	0.28 ± 0.03^{b}	2.42 ± 0.2^{b}
Masuran	569.67 ± 11.96^{a}	0.05 ± 0.00^{c}	0.44 ± 0.01^{c}

Data represented as mean ± SE (n=3). Mean values in a column superscripted by different letters are significantly different at P<0.05. Results are presented on dry weight basis. RE: Rutin equivalents; Rutin IC_{50}: 17.62 ± 0.01 µg/ml.

Table 3: Oxygen radical absorbance capacity of brans of selected Sri Lankan traditional rice.

Rice variety	mmol TE/g extract	mmol TE/100 g bran
Goda Heeneti	1.02 ± 0.04^{a}	8.89 ± 0.34^{a}
Sudu Heeneti	1.01 ± 0.03^{a}	8.82 ± 0.29^{a}
Dik Wee	0.89 ± 0.03^{b}	6.98 ± 0.25^{b}
Masuran	0.77 ± 0.04^{c}	6.63 ± 0.33^{b}

Data represented as mean ± SE (n=4). Mean values in a column superscripted by different letters are significantly different at P<0.05. Results are presented on dry weight basis. TE: Trolox equivalents.

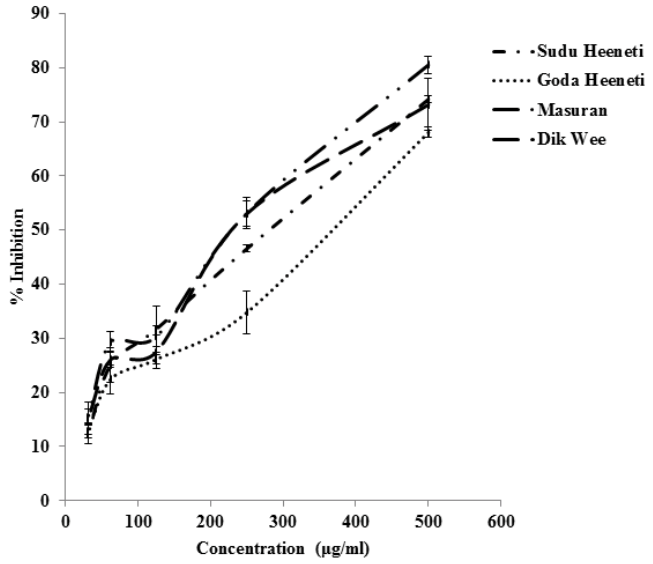

Figure 1 Dose response relationship of bran extracts of selected rice varieties for superoxide radical scavenging activity.

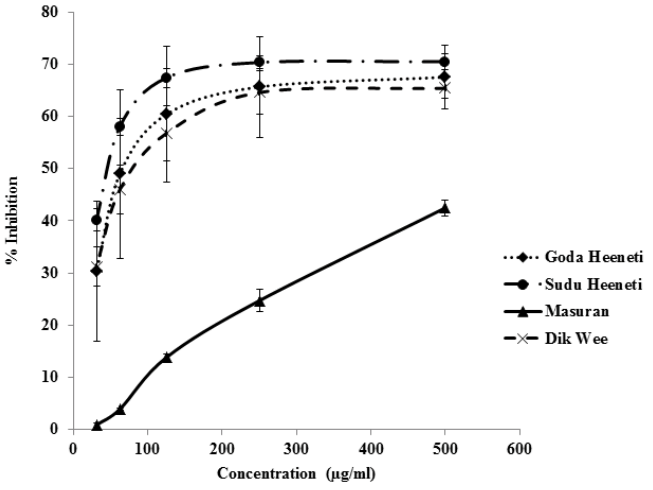

Figure 2 Dose response relationship of bran extracts of selected rice varieties for nitric oxide radical scavenging activity.

rice varieties for ORAC (in terms of mM TE/100 g bran) was Sudu Heeneti=Goda Heeneti>Masuran=Dik Wee **(Table 3)**.

Discussion

In recent years many antioxidant assays covering various mode of actions have been developed to estimate the antioxidant properties of various substrates [23,24]. The radical scavenging mechanism based *in vitro* antioxidant assays are widely used in measuring antioxidant activities of food and natural products [23,24]. Such *in vitro* antioxidant assays measure the radical scavenging abilities using both non physiological (artificial) and physiological radicals [23,24]. However, measuring antioxidant potential using physiological radicals based antioxidant activity assays may be more relevant to the biological system. Therefore, in the present study antioxidant activities of rice brans of selected Sri Lankan traditional rice varieties were studied using physiological radicals based *in vitro* antioxidant assays covering both reactive oxygen species and reactive nitrogen species. The rice varieties used in the present study and 70% ethanolic extracts of the rice bran were selected based on our previous research findings [8,9,17].

Reactive oxygen and reactive nitrogen species are generated in the human body either through metabolic processes or exposure to various hazardous chemicals, air pollutants and radiation [3,23,25]. Superoxide and peroxyl radicals are highly reactive radicals classified under reactive oxygen species. These radicals have been implicated in the pathophysiology of variety of chronic diseases such as cancers, diabetes and inflammatory diseases [3,23,26]. Functional foods having antioxidant activities via radical scavenging mechanisms are therefore important in prevention and management of such diseases [3,25]. Rice bran is well known to have antioxidant activities by numerous scientific studies [8-10,17-19]. However, the studies on superoxide radical scavenging activity and ORAC of rice bran are scare to date. According to a study by Nam et al. [27], superoxide radical scavenging activity (% inhibition) of brans of 21 rice cultivars varied from 16.7 ± 7.17 to 93.1 ± 1.24% at 0.5 mg/ml. The tested Sri Lankan rice varieties in the present study had inhibitory activity (% inhibition) in the range of 73.16 ± 4.82 to 80.51 ± 1.66% at same concentration (0.5 mg/ml). Thus, selected Sri Lankan traditional rice varieties are either more or less comparable or moderated in activity compared to the highly active rice varieties in Nam et al. [27] study. However, superoxide radical scavenging activity of rice brans of Sri Lankan traditional rice was moderate in activity compared to the reference standard Quercetin. ORAC assay is a measure of the radical scavenging activity of the physiological radical, peroxyl radical [23,24]. Results showed that bran extracts of all the rice varieties had ORAC with significantly high activities in brans of Sudu Heeneti and Goda Heeneti. However, these varieties had moderate ORAC activity compared to the ORAC of brans of recently published rice varieties by other researcher's world over [28,29]. This might be due the varietal differences and differences in the extraction procedures used.

Nitric oxide is one of the main reactive nitrogen species and serves as a mediator for many physiological functions [3,4]. However, enhanced formation of nitric oxide has been implicated in the pathogenesis of number of chronic diseases. Thus, inhibition of nitric oxide plays a beneficial role in prevention and management of various forms of chronic diseases [3,4]. Recent study by Rao et al. [30] had shown Njavara, a Indian traditional rice variety claimed to have medicinal properties in Ayurveda had high nitric oxide radical scavenging activity (IC50: 52.25 µg/ml). Interestingly, the Sri Lankan traditional red rice variety Sudu Heeneti in the present study showed comparable nitric oxide radical scavenging activity to Najvara rice variety. Further, nitric oxide radical scavenging activity of brans of Dik Wee and Goda Heeneti were comparable to nitric oxide radical scavenging activity of brans of the other important Indian rice varieties (Vasumathi, Yamini, Jyothi) studied by Rao et al. [30]. Interestingly brans Sudu Heeneti, Goda Heeneti, Dik Wee and Masuan showed nitric oxide inhibitory activities in cell assays in

our recent studies [31]. Further, dichloromethane fraction of all these varieties showed significantly high nitric oxide inhibitory activity compared the crude rice bran extracts (70% ethanolic extracts used in the present study), other rice bran fractions and the references standard NG-Monomethyl-L-arginine monoacetate salt used in the study (Unpublished data). In the present study we observed that crude 70% ethanolic extracts of all the selected rice varieties were moderated in activity compared to the reference standard Rutin indicating that most of the active compounds may concentrate in the dichloromethane fraction of the rice bran.

Reactive oxygen species and reactive nitrogen species inhibitory activities of rice bran have been explained due to the presence of phenolic antioxidants, γ-oryzanol, α-tocopherol and tocotrienols in the rice bran [30,32,33]. Recent studies conducted in Sri Lanka scientifically proved that the rice varieties tested in the present study had high amounts of phenolic antioxidants, tocopherols and γ-oryzanols [8-10,17,34]. Therefore, presence of such compounds in brans of these rice varieties may be at least partly responsible for the observed physiological radical scavenging activities. However, exact chemical compounds and mode of actions in mediating such antioxidant activities in brans of Sri Lankan traditional red rice have to be investigated further.

Rice brans of Sudu Heeneti, Goda Heeneti, Dik Wee and Masuan showed range of anti-diabetic related properties, anti-inflammatory properties and growth inhibition and cytotoxicity against various human cancer cell lines in our previous research works [8,9,18,19]. The observed radical scavenging mechanisms especially superoxide and nitric oxide radical scavenging activities in rice brans in the present study may responsible for such observed biological activities in our previous research work. Based on the findings of the present study and findings of our previous studies it can be concluded that brans of these varieties may have the potential in developing value added health foods and nutraceuticals for prevention and management of ever increasing various non communicable chronic diseases. Further, these pigmented traditional red rice varieties having high antioxidant activities may provide a source of new genes for the development of new improved varieties having high antioxidant activities and medicinal properties.

Conclusion

It is concluded that brans of all the selected Sri Lankan traditional red rice varieties had physiological free radicals scavenging activities with varying degrees of potentials. This is the 1st study to report such biological activities for any variety of rice in Sri Lanka. Therefore, consumption of these varieties with the bran may be important in prevention and dietary management of various forms of oxidative stress induced chronic diseases. Further, brans of these varieties may be a potential functional food ingredient in the health food industry.

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