ORIGINAL ARTICLE

Antioxidative Recovery Responses of Germinated Rough Rice (GRR) and Germinated Brown Rice (GBR) from Various Solvents and Their Potential towards Reactive Oxygen Species Quenching Capacity

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ABSTRACT

Introduction: Health promoting properties which generated worldwide interest in germinated rough rice (GRR) and germinated brown rice (GBR) are attributed largely by the bioactive compounds in the rice bran. Therefore, in the present study, antioxidant activities from gradient methanol and ethanol solvents followed by fractionations were evaluated. **Methods:** GRR and GBR crude extracts were successively obtained from two concentrations of methanol (80% and 100%) and ethanol (50% and 70%). They were further analyzed for 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox Equivalent Antioxidant Capacity (TEAC) and Ferric Reducing Antioxidant Power (FRAP) assays. From the potent crude extract dissolved in water, they were sequentially subjected to fractionation using solvents with increasing polarity pattern, namely hexane fraction (HF), ethyl acetate fraction (EAF) and water fractions (WF). **Results:** WF belonging to both GRR and GBR generally possessed better antioxidant characteristics, demonstrated high TPC with GBR; 101.9 ± 0.2 mg GAE/g and GRR; 63.7 ± 1.2 mg GAE/g. GRR-WF exhibited high DPPH and TEAC with 63.68 mg TEA/g and 80.30 mg TEA/g respectively. GBR exhibited high DPPH in WF with 46.17 mg TEA/g but demonstrated high in ABTS in EAF fraction with 71.60 mg TEA/g. Both GRR and GBR showed high FRAP values in WF with 45.31 and 19.68 mg GAE/g respectively. **Conclusion:** Hence, it is proven that GBR and GRR owing to their antioxidant activities possess potential benefits which in turn has increased their competence as an emerging natural and valuable health food.
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Keywords: Antioxidant activity, Germinated brown rice, Germinated rough rice, Total phenolic content

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INTRODUCTION

Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl (OH) radicals and peroxyl radicals are released into our body system as a part of the cellular oxidation process primarily involved in cell signaling pathways. However, accumulation of ROS due to excessive exposure to free radicals throughout the years renders people to be more susceptible in contracting diseases such as cancer, stroke, diabetes, and degenerative disorders (1). Oxidative stress caused by the excessive accumulation of ROS triggers deterioration and impairment of DNA, lipids and proteins (2) thereby silently inducing chronic ailments through time. Hence, the instigation of antioxidants to reverse and combat the after effect of excessive free radicals is a crucial discovery in the quest to prevent disease progression and management mechanism.

Plant based antioxidants are sought after due to their natural treatment and supplementation capacity in elucidating successful resultant of the ill effects of ROS (3). Being the secondary metabolites of plants, phenolics are proven to be good antioxidants (3) apart from contributing to vast health benefits such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects, respectively (4). Though modification of diet by the inclusion of vegetables and fruits owing to their antioxidative nature is highly regarded, consumption of grains which belong to the

first tier of the food pyramid is often neglected.

Components originated from grain complement those of fruits and vegetables benefits when taken together during meals (5). This validates the heightened benefits of phytochemicals through synergism/synergistic effect (5, 6). The genesis of brown rice as a potential health food rich in bio-actives and nutrients contributing to its notable nutraceutical and pharmaceutical ideals has drawn much interest to researches lately. Brown rice is rich in antioxidants such as tocotrienol, tocopherol, gamma oryzanol, ferulic acid and γ-aminobutyric acid (GABA) has become a notable nutraceutical and pharmaceutical ideals (7).

A recent study by Moongngarm et. al. 2010 introduced the benefit of grains to a higher notch by proving that the process of germination could further enhance and enrich brown rice as well as paddy (rough rice) with higher levels of bioactive compounds. Germinated brown rise and rough rice are favored for improvement of individual's general well-being. Because of this, they are eyed for their plausible potential as a commercial health food. Through the process of germination these bio-actives and mineral content have been proven to intensify drastically (7). In this present study, germinated rough rice was also included into this study considering the fact that the process of germination is much effective in paddy grains than brown rice itself (7). Hence, through this study it has been proven that rice apart from being the staple food and the main source of carbohydrate can also be manipulated and used for the betterment of overall health at a decent cost as compared to its rather fancy and overpriced other organic vegetable counterparts. This study mainly focuses in detecting and displaying the potential antioxidant activity of both GBR and GRR.

MATERIALS AND METHODS

Samples and Reagents

The GBR was obtained from Molecular Biomedicine Laboratory, Universiti Putra Malaysia courtesy of Prof. Maznah Ismail research team and both the brown rice and rough rice supplies were generously provided by BERNAS.SDN.BHD, Selangor, Malaysia. All chemicals used in the experiment are of analytical grade. Folin-Ciocalteu reagent, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), Trolox, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS +), and Gallic Acid were purchased from Sigma-Aldrich Deutschland, Germany. Methanol and ethanol were manufactured by HmbG chemicals, Hamburg, Germany.

Germination of Rough Rice

Rough rice germination was done according to the method described by Moongngarm et al. 2010 whereby one kg of rough rice was soaked in tap water for 48 hours until the moisture content reached up to 40 ± 2%. The water of the rice was being soaked in and changed every 8 hours up to 48 hours to ensure removals of immature grains and to inhibit cultivation of harmful microorganism which could affect the germination process. After that, the rice grains were evenly distributed on a piece of wet cheese cloth for another 48 hours to allow germination to take place after incubation at 50°C until the moisture content of the GRR grains reached approximately up to 10%. Then the GRR was sent to the dehusking process by which the hull, root and shoot were separated from the grains and the final product was grounded into fine powder and passed through a sieve (20 meshes) before extraction and stored in a chiller at 4°C before use .

Extraction and fractionation of GBR and GRR

Both GBR and GRR were extracted using varying concentrations of methanol (MeOH 80% and 100%) and ethanol (EtOH 50% and 70%) solvents. Each extraction procedure started off with 25 g of each GBR and GRR which was successively extracted with 50 mL of each solvent. The mixture of GBR or GRR and the solvent were subjected to shaking using a shaking incubator at 40°C at 180 rpm for 2 hours and at the end of the 2 hours, the mixture was filtered and the residue was added with a fresh batch of solvent and the whole process was repeated thrice. Each extract was combined and filtered through Whatman No.1 filter paper where the resultant filtrate was evaporated using rotary evaporator (Buchi, Flawil, Switzerland). Yielding extract through this process was then stored at -80°C prior to preceding analysis.

Fractionation of GBR and GRR crude extracts with high antioxidant activity and Total Phenolic Content (TPC)

The crude extracts which displayed high TPC (specified in Table I) and free radical scavenging properties were subjected to fractionation procedure which involved employment of solvents belonging to varying polarity scale in increasing order namely hexane, ethyl acetate and water. The potent crude extracts were re-dissolved in 250 mL of distilled water until it formed a completely homogeneous aqueous solution which was then serially fractionated using n-hexane, ethyl acetate and water . Each solvent was allowed to stand for 2 hours propagating the layers to separate well starting with hexane fraction (HF) followed by ethyl acetate fraction (EAF) before lastly collecting the water fraction (WF). The resultant extracts of the fractions were also evaporated using rotary evaporator and stored in -80°C prior to analysis (Fig. 1).

Total Phenolic Content Analysis

Total phenolic content analysis was performed using Folin-Ciocalteu reagent according to the method of Adedayo et al. (2012) with minor modifications. Gallic acid was using a standard for the experiment. Hence, results were reported in GAE mg/g extract. Gallic acid was prepared in 6 different concentrations

Table I: Crude extracts of GBR and GRR dissolved in distilled water were further fractionated using WF, EAF and HF

Sample	Yield (%) w/w		TPC (mg GAE/g)	
	GRR	GBR	GRR	GBR
MeOH 100%	2.1	1.2	13.9 ± 0.07	2.0 ± 0.68
MeOH 80%	2.4	1.5	19.1 ± 0.03	25.7 ± 0.2
EtOH 70%	4.1	2.0	24.9 ± 0.08	40.2 ± 0.2
EtOH 50%	3.7	1.7	56.9 ± 0.89	28.5 ± 1.2
WF	47.8	43.7	**63.7 ± 1.2**	**101.9 ± 0.2**
EAF	19.1	18.4	46.0 ± 1.0	27.4 ± 0.02
HF	32.8	37.5	27.1 ± 1.1	34.8 ± 1.7

Values are mean ± SD of three replicate analyses. TPC: total phenolics content expressed as mg gallic acid equivalent/g (mg GAE/g). MeOH; Methanol, EtOH; Ethanol, Water Fraction (WF); EAF (Ethyl acetate fraction); Hexane fraction (HF)

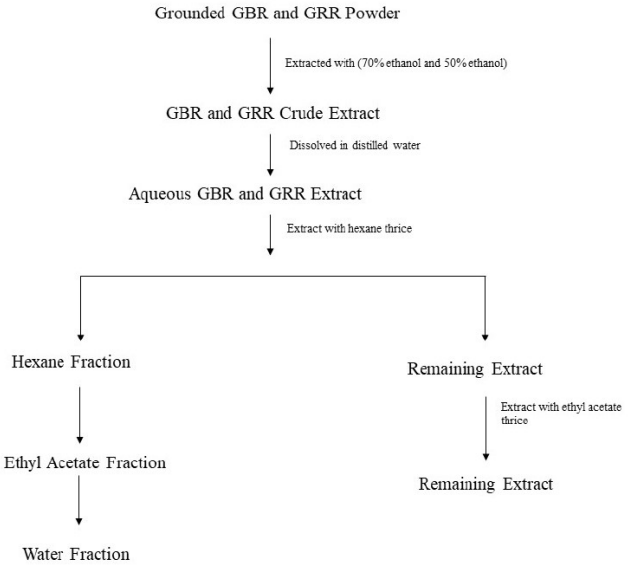

Figure 1: Extraction procedure of GBR and GRR

using dilutions of 1 mg/mL (1000 ppm) stock that was prepared into (3.125, 6.25, 12.5, 25, 50 and 100) ppm respectively. 2.5 mL of 10% Folin-Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate solution was were added to 500 µL of sample and were gently vortexed to allow the reagents to mix well. Samples were then incubated at 40°C for 1 hour before absorbance being read at 765 nm using UV-Vis spectrophotometer UV-1700 (Shimadzu Corp, Kyoto, Japan).

Free radical Scavenging Assays

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

7.0 mM ABTS and 2.45 mM potassium persulphate were prepared using deionized distilled water and were mixed together to form a dark blue solution that was allowed to stand at room temperature overnight. Prepared intense blue coloured solution is then added to distilled water until the absorbance reaches almost 734 ± 2 nm. 900 µL from the resultant ABTS solution is then added to 100 µL of sample was then allowed to stand for 2 minutes at room temperature between 23 – 26oC . Six different concentrations of Trolox (3.125, 6.25, 12.5, 25, 50 and 100) ppm were prepared for the analysis. Consequential absorbance was read using Shimadzu spectrometer UV 1700 (8).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

DPPH free radical scavenging activity was carried out according to the method reported by Chan & Ismail (2009). 195 µL of 0.1 mM of DPPH solution was prepared using 80% methanolic solution which was then added to 50 µL of sample and trolox standard that was prepared in 6 concentrations (3.125, 6.25, 12.5, 25, 50 and 100) ppm. After 1 hour of incubation in the dark at room temperature, absorbance was read at 540 nm using ELISA microplate reader (Opsys MR, Thermo Labsystems, and Franklin, MA, USA). Results were expressed in Trolox Equivalent Antioxidant mg TEA /g extract.

Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing potential of the GBR and GRR extracts were analysed referring to the method described by Berker et al. (2007). After the addition of 1.0 mL of sample, 5.0 mL of distilled water, 1.5 mL of 1 M hydrochloric acid, 1.5 mL of 1% potassium ferricyanide K3Fe(Cn)6, 0.5 mL, 1% sodium dodecyl sulphate (SDS) and lastly 0.5 mL FeCl2 were added sequentially. The mixture is then incubated at 50oC for 20 minutes and absorbance is read at 750 nm using Shimadzu spectrometer UV 1700. Gallic acid is utilized as a comparative standard and was prepared in 6 different concentrations (3.125, 6.25, 12.5, 25, 50 and 100) ppm. Results are expressed as gallic acid equivalent mg GAE /g extract.

Statistical Analysis

All the experiments were conducted in triplicate. The data were recorded as mean ± standard deviation (SD) and analyzed by Statistical Package for the Social Sciences (version 19, SPSS Inc, Chicago, IL). One-way ANOVA was conducted followed by Least Significant Difference (LSD). A value of $p < 0.05$ was deemed to be statistically significant.

RESULTS

Total phenolic contents

Among all crude extracts of GBR, crude extracts from 70% ethanol showed highest TPC value, which is 40.2 ± 0.2 mg GAE/g followed by crude extract from 50% ethanol with 28.5 ± 1.2 mg GAE/g TPC value and 25.7 ± 0.2 mg GAE/g TPC for 80% methanolic extract as well as 2.0 ± 0.68 mg GAE/g TPC for 100% methanolic extract. Compared to methanolic extracts, the ethanolic extracts exhibited high total phenolic content both in GBR and GRR. In GRR extracts from 50% ethanol showed high TPC, which is 56.9 ± 0.89 mg GAE/g. Phenolics gave a greater yield using an aqueous solution system rather than being extracted using absolute solvents (11). In all cases, the HF exhibited the lowest phenolic concentration as phenolic compounds are highly polar

and hexane is a non-polar solvent. Comparing GRR and GBR fractions , WF belonging to both GBR and GRR showed higher TPC values as compared to their HF and EAF counterparts. GBR-WF showed 101.9 ± 0.2 mg GAE /g TPC and GRR-WF 63.7 ± 1.2 mg GAE/g TPC overall (Table I).

Free radical scavenging activity

DPPH and TEA assays

Methanolic extracts for GRR possessed DPPH scavenging activity which ranged from 17.17 - 39.02 mg TEA/g extract followed by GBR with 0.53 – 16.43 mg TEA/g extract respectively. Meanwhile, both ethanolic (50 and 70%) extracts of GBR and GRR with high TPC, showed high DPPH scavenging activity with GBR 53.15 mg TEA/g activity while GRR 96.49 mg TEA/g activity respectively (Fig.2).

On the other hand, GRR-WF seems to exhibit high ABTS and DPPH scavenging activity with 80.30 mg TEA/g, and 63.68 mg TEA/g activities respectively. However, in the case of GBR, DPPH radical scavenging activity was more potent in water extract with 46.17 mg TEA/g activity while the EA extract exhibited higher ABTS scavenging activity with 98.72 mg TEA/g activity (Fig. 2 and 3).

Similarly, both ethanolic extracts of GBR and GRR with high TPC showed better ABTS scavenging activity as compared to all other extracts, GBR 102.3 mg TEA/g extract and GRR 97.20 mg TEA/g extract accordingly (Fig. 3a).

FRAP assay

Crude extracts in GBR showcased 88.87 mg GAE/g ferric reducing activity and GRR 50.31 mg GAE/g antioxidant capacity activity (Fig. 4). The methanolic extracts for both categories of plants were proven to show lower FRAP values likewise. The WF of GBR and GRR again showed high reducing value for FRAP assay, with 19.69 mg GAE /g extract and 45.32 GAE mg/g extract. Fractions of hexane and EAF showed lower reducing capacity as compared to WF generally (Fig.4).

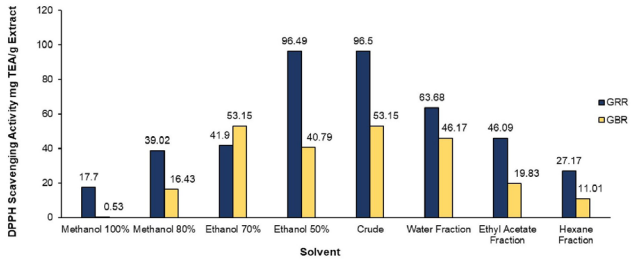

Figure 2: DPPH scavenging activity of crude extracts of GBR and GRR with their fractions

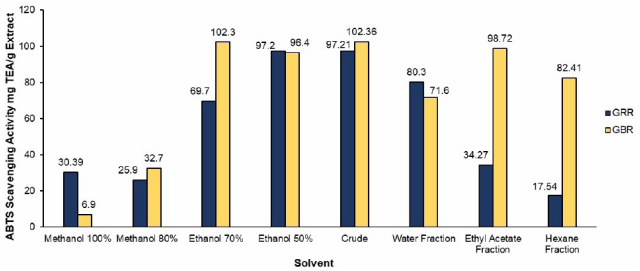

Figure 3: ABTS scavenging activity of crude extracts of GBR and GRR with their fractions

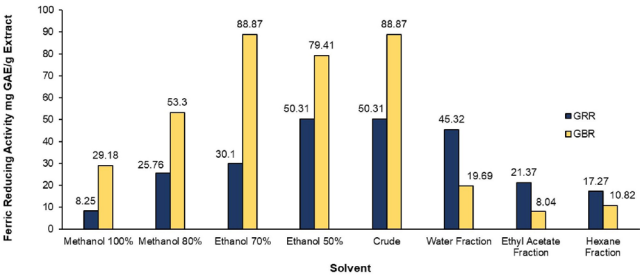

Figure 4: Ferric reducing activity properties of GBR and GRR crude extracts with their fractions

Correlation between High TPC and High Free Radical Scavenging Activity

A positive relationship was observed between levels of TPC and free radical scavenging properties of all extracts of GBR and GRR. For GRR, increased TPC level exhibited high DPPH and FRAP activity with $R^2 = 0.821$ and 0.811 for DPPH with $p < 0.05$ respectively, and $R^2 = 0.709$ for TEAC. This indicates a significant, positive, strong relationship between the phenolic content and free radical scavenging activity of extracts. As for GBR R^2 value of 0.641, 0.339 and -0.147 were obtained indicating a positive, weak correlation between TPC and DPPH, TEAC and FRAP values (Table II).

Table II:Correlation between high TPC and free radical scavenging activities (DPPH, FRAP and ABTS)

Variables	GRR		GBR	
	TPC , R^2	*p*-value	TPC, R^2	*p*-value
DPPH	0.821	< 0.05	0.641	0.121
FRAP	0.811	< 0.05	-0.147	0.753
ABTS	0.709	0.074	0.339	0.457

Statistically significant shows p-value < 0.05.

DISCUSSION

Total Phenolic Contents

Extraction of antioxidant from plant material using wide variety of solvent including methanol, ethanol and acetone are rampant because it is widely accepted that there is no single premium method for the extraction of antioxidants (12). In this study various concentrations of methanol and ethanol were used to study their effectiveness in extracting antioxidative compounds. Seventy % solvents system is more favored for its efficiency in extracting a mixture of complex and simple phenols as compared to pure solvents (13). Based on our current result of TPC activity, it is proven that TPC values between different solvent systems of GBR and GRR are

significantly different ($p < 0.05$). This could be due to the presence of highly polar compounds since water is the most polar solvent compared to other solvents being used in the experiment. Several landmark studies presented in Moongngarm & Saetung (2010), Kim et al. (2020), Vichit & Saewan (2016) and Tortayeva et al. (2014) have showed that the de-hulling of GBR and GRR as well as extracting them with different solvents can lead to biochemical contents and biological value alterations . Hence, the TPC activity showed variations in the GBR and GRR extracts. These variations depend on the solvents being used to encounter the polarity of the compounds.

Free Radical Scavenging Activity

Free radicals are the known cause of many pathological conditions (17). Each antioxidant assay is known to interpret a specific mechanism through which free radicals are combated (18). DPPH is a known to be commercially stable antioxidants assay which is simple and inexpensive (19). Likewise, TEAC establishes whether any sample is efficient enough to scavenge radicals of long life such as ABTS. Extracts are expected to decolorize or lighten the intense blue colored solution formed by ABTS radicals emanating the absorbance range of not more than 734 nm (20, 21). TEAC assay is widely accepted as a potent free radical inhibitor due to its proficiency in evaluating antioxidant capacity in food and other biological matrices (20, 21). Methanolic extracts exhibited lower activity as compared to their ethanolic counterparts. The aqueous ethanolic extracts generally possessed much better DPPH scavenging ability then methanolic GBR and GRR extracts. This can be observed in the recent results whereby both GBR and GRR possess high free radicals scavenging activity in ethanolic compared to methanolic extract. Meanwhile, WF exhibited higher antioxidants properties in DPPH and TEAC compared to ethanolic extracts for GRR . It has been reported that solvent polarity highly influences antioxidant capacity in a sample (22). Interestingly, GBR showed highest antioxidant assays in EAF, which is the second least polar solvent rank after HF. When the polarity of the solvent matches the polarity of the compounds present in the sample, antioxidant properties can be extracted more efficiently (23) . The more polar a solvent is, the better the free radical scavenging property is possessing because polar solvents are largely associated with free radical scavenging properties (24).

Ferric Reducing Antioxidant (FRAP) Assay

FRAP assay is denoted as a well-established method to determine the total antioxidant capacity of an extract (25). This method is based on the single electron transfer (SET) mechanism of a potential antioxidant (19) which is similar to the TEAC assay. GBR crude extract showed similar antioxidant properties result with 70% ethanolic extracts of GBR. Meanwhile, the GRR also showed similar antioxidant properties result in both the crude and the 50% ethanolic extracts. The ethanolic extracts seem to show high antioxidant activity which correspond with their high TPC values. Similarly, our result showed that all extracts displayed some level of electron transfer capacity such as reported by Do et al. (2014) . When an extract exhibits reducing power properties, this indicates that the antioxidant compounds in the extract are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process, allowing them to act as primary and secondary antioxidants (18).

CONCLUSION

The role of antioxidants in alleviating oxidative stress is very evidently proven by the drastic number of researches that has been on antioxidants over the past decade (19). Dietary antioxidants are heavily favored over their synthetic counterparts especially now that the paradigm shift towards natural products has taken place. The antioxidant activity of plant-based phenols is widely described for their health benefits, which are largely due to their antioxidant properties. Pertaining to the results, it is very clear that the extraction of the phenols/antioxidants largely relies on the type of solvent being used and is very crucial given the fact that it is the initial step in extraction of bio-actives out of any given plant. Owing to their antioxidant properties germinated brown rice and rough rice can be utilized as an emerging health food to help prevent and fight diseases. Further researches are necessary to investigate the properties and health benefits of GBR and GRR to enhance the existing literature on this matter and for prospective discoveries in future.

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