PHILIPP AGRIC SCIENTIST
Vol. 103 No. 4, 293-302
December 2020

ISSN 0031-7454

Phytochemical Properties, Antioxidant Activities, and Cytotoxicity of Ethanolic Bran Extracts from Philippine Pigmented Rice Cultivars

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Antioxidants are widely recognized for their immune-enhancing and disease-preventing properties. However, there are some reports that certain synthetic antioxidants can pose carcinogenic effects on human cells. Therefore, it is vital to look for an alternative source of natural, effective, and safe antioxidants for human consumption. In this study, ethanolic extracts of six pigmented rice bran samples were evaluated for their phytochemical properties, antioxidant activities, and cytotoxicity against normal human blood lymphocytes (NHBL). Results showed that the pigmented rice bran extracts had high total phenolic (70.1–178.4 mg.g^{-1} gallic acid equivalent), flavonoid (123.3–378.0 mg.g^{-1} rutin hydrate equivalent), and anthocyanin (0.8–152.5 m.g^{-1} cyanidin-3-glucoside) content. They also had strong antioxidant activities that ranged from 116.4 to 461.7 mg.g^{-1} trolox equivalent (TE) for ferric reducing antioxidant power (FRAP) and 85.4–367.7 mg.g^{-1} TE for 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity (ABTS-RCSA). All sample extracts showed low levels of effective concentration (EC) at 50 (EC_{50}) (11.6–30.3 mg.L^{-1}) and 25 (EC_{25}) (1.2–10.1 mg.L^{-1}), which is a good indication of high antioxidant activities. Pearson's correlation analysis revealed that there was a moderate to very strong positive correlation (R value: 0.623–0.993) among the phytochemical properties and antioxidant activities tested. Furthermore, pigmented rice bran extracts (100–1000 ppm) showed no toxic effect against NHBL, which implies that all samples are safe for human consumption. Therefore, pigmented rice bran extracts can be used as key ingredient in the development of safe and effective functional food and pharmaceutical products.

Key Words: antioxidant activities, cytotoxicity, correlation analysis, free radicals, human blood lymphocytes, phytochemical properties, pigmented rice bran

Abbreviations: ABTS – 2,2′ – Azinobis-(3-ethylbenzothiazoline-6-Sulphonic acid) diammonium salt, DPPH – 1,1-diphenyl-2-picrylhydrazyl, EC – effective concentration, FRAP – ferric reducing antioxidant power, GAE – gallic acid equivalent, NCS – newborn calf serum, NHBL – normal human blood lymphocytes, NSIC – National Seed Industry Council, RCSA – radical cation scavenging activity, RHE – rutin hyrdrate equivalent, TAC – total anthocyanin content, TE – trolox equivalent, TFC – total flavonoid content, TPC – total phenolic content, TPTZ – ferric tripyridyltriazine

INTRODUCTION

Reactive free radicals are one of the primary culprits in damaging biomolecules including proteins, membrane lipid, and DNA, thereby contributing to the progression of various chronic diseases such as cancer, cardiovascular diseases, neurodegenerative disorders, diabetes, and even aging (Ighodaro and Akinloye 2017). At present, antioxidants are receiving significant attention because of their immune-enhancing and disease-preventing properties. Antioxidants have the ability to neutralize excess free radicals in the body, thus protecting the cells against oxidative damage (Sen et al. 2010; Alam and Bristi 2013). In the food industry, synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tertbutylhydroquinone are being used as additives. Unfortunately, these ingredients have been reported to pose carcinogenic effects on human cells

(Sindhi et al. 2013). In view of this, researchers are finding ways to replace these synthetic antioxidants with natural ones from medicinal plant sources (Jin et al. 2012; Shebis et al. 2013).

Pigmented rice is a traditional food staple in many parts of the country. It is now gaining huge interest in the food and biomedical industries due to its enormous health and nutritional benefits (Gunaratne et al. 2013). Pigmented rice is known to contain high amounts of phytochemicals such as sterols, tocopherols, γ-oryzanols, polyphenols, amino acids, flavones, tannins, essential oils, and several anthocyanins (Deng et al. 2013). These phytochemicals, which are mostly found in the bran layer, have been shown to decrease hyperlipidemia (Guo et al. 2012), reduce atherosclerosis (Deng et al. 2013), and suppress cancer cell proliferation (Rao et al. 2010). Animal studies also showed that dietary supplementation of pigmented rice reduced atherosclerotic lesions in hypercholesterolemic rabbits (Samyor et al. 2017).

The use of plant extracts as source of antioxidants is currently at its mainstream in therapeutic treatments (Laokuldilok et al. 2010). Numerous studies have claimed that antioxidants in pigmented rice bran can be an effective chemopreventive agent due to its cytotoxic potential against various human cancer cell lines such as H28 mesothelioma (Nakashima et al. 2010), lung and colon (Dapar et al. 2013), breast, prostate, ovarian, and liver (Norhaizan et al. 2011; Banjerdpongchai et al. 2013) cancer cells. However, antioxidants reportedly have poor selectivity reactions in the cells, thereby damaging even the healthy cells during its consumption. Although plant-based antioxidants are generally safe, they can still produce reactive species with undesirable pro-oxidant effects especially when consumed at high concentrations (Rani 2017). It is therefore important to assess the cytotoxicity of the antioxidant-rich plant extracts for safe consumption. Thus, this study evaluated the phytochemical properties, antioxidant activities, and cytotoxicity against normal human blood lymphocytes of six ethanolic bran extracts from Philippine pigmented rice cultivars.

MATERIALS AND METHODS

Chemicals and Reagents

Folin-Ciocalteu reagent, gallic acid, sodium nitrite, aluminum chloride hexahydrate, sodium hydroxide, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferric tripyridyltriazine (TPTZ), sodium carbonate, acetate buffer, potassium persulfate, and ferric chloride hexahydrate were purchased from Sigma-Aldrich (Sigma-Aldrich Pte. Ltd., Singapore). All chemicals used in this experiment were of analytical reagent grade.

Rice Samples and Processing

Six pigmented rice samples composed of three red (Gomiki, Minaangan, and Red Blondie) and three black (Galo, Kotinaw, and Ominio) rice cultivars were collected during the 2015 wet season (WS) in Aurora, Albay, Masbate, Ifugao, Benguet, and Mt. Province, Philippines. NSIC Rc160, a popular white rice variety, was employed as a control in this study. It was harvested during the 2015 WS at the Central Experimental Station of the Philippine Rice Research Institute, Maligaya, Science City of Muñoz, Nueva Ecija, Philippines.

After collection, the rice samples were cleaned and dehulled using a rice dehuller (THU-35A, Satake, Japan) and were further milled using a polisher (McGill Grainman Polisher, USA) to collect the bran. The bran samples were sieved using a laboratory strainer (1.18 mm aperture and 0.634 mm diameter) to obtain uniform fine particles and remove other impurities. The recovered rice bran was placed in a polyethylene plastic bag, sealed, and refrigerated at 4°C prior to analysis.

Extraction of Rice Bran Samples

Ethanolic bran extract was prepared based on the modified method of Sultana et al. (2009). Rice bran samples (100 g) were extracted with 500 mL of 80% ethanol. The mixtures were magnetically stirred for 4 h and then allowed to stand for 48 h in a sealed Erlenmeyer flask for maximum extraction of bioactive compounds. Afterwards, the mixtures were filtered using an ordinary filter paper (10 μm pore size and 205 μm thickness) to separate the bran residue, which was oven-dried at 50°C for 2 h. The filtrate was concentrated using a rotary vacuum evaporator at 50°C under reduced pressure of 150 mbar. The bran extracts were then freeze-dried and stored in a desiccator prior to analysis.

Determination of Phytochemical Properties of Pigmented Rice Bran Extracts

Total Anthocyanin Content (TAC)

The TAC of rice bran extracts was determined using the method of Abdel-Aal and Hucl (1999) with minor modifications. About 500 $mg.L^{-1}$ of extracts were prepared using acidified ethanol (85:15 v/v absolute ethanol and 1 N HCl) in a 50-mL volumetric flask. The absorbance was measured against the blank (acidified ethanol) at 535 nm

using UV-Vis spectrophotometer (U-3210, Hitachi, Japan). The TAC was computed in terms of cyanidin-3-glucoside content using the equation

$$C = [(A \times V \times MW) / (1000 \times \varepsilon \times mass)] \times 10^3$$

where C is the concentration of anthocyanin, A is absorbance, ε is molar absorptivity of cyanidin-3-glucoside (25,965 $J.cm^{-1}$ mol^{-1}), V is total volume of anthocyanin extract, and MW is molecular weight of cyanidin-3-glucoside (449.2 $g.mol^{-1}$).

Total Phenolic Content (TPC)

The TPC of rice bran extracts was determined based on the method of Singleton et al. (1999) with minor modifications. About 500 µL of ethanolic bran extract (500 $mg.L^{-1}$) was mixed with 2.5 mL of freshly prepared Folin-Ciocalteu reagent. The solutions were allowed to stand for 15 min and added with 2 mL of 7.5% sodium carbonate. After an hour, the absorbance of the solutions was measured at 765 nm against the blank (distilled water) using UV-Vis Spectrophotometer (U-3210, Hitachi, Japan). Gallic acid was used as a reference standard to estimate the TPC of the samples. The results were expressed in mg gallic acid equivalent (GAE).g^{-1} extract.

Total Flavonoid Content (TFC)

The method described by Bao et al. (2005) with slight modifications was used to determine the TFC of ethanolic bran extracts. The assay measured the amount of carbonyl and hydroxyl groups of flavones and flavanols that were reacted with aluminum ion (Al^{3+}) forming a yellowish aluminum-flavonoid complex (Popova et al. 2004). Briefly, 500 µL of diluted bran extracts (500 $mg.L^{-1}$) were transferred into a test tube containing 2 mL of distilled water and 0.15 mL of 5% $NaNO_2$. After 5 min, 0.15 mL of 10% $AlCl_3.6H_2O$ was added to the mixture then 1 mL of 1N NaOH was incorporated after 5 min. The solutions were allowed to stand for 15 min for maximum color development. The absorbance of the solutions was measured at 415 nm against the blank (distilled water) using UV-Vis Spectrophotometer (U-3210, Hitachi, Japan). The TFC was computed using standard rutin hydrate curve and was expressed in mg rutin hydrate equivalent (RHE).g^{-1} extract.

Determination of Antioxidant Activities of Pigmented Rice Bran Extracts

Ferric Reducing Antioxidant Power (FRAP)

The modified method of Benzie and Strain (1999) was used to determine the FRAP of the rice bran extracts. The FRAP solutions were prepared by mixing 0.3 M acetate buffer, 0.01 M ferric tripyridyltriazine (TPTZ), and 0.02 M $FeCl_3.6H_2O$ at a 10:1:1 ratio. About 500 µL of diluted extract (500 $mg.L^{-1}$) and Trolox standards were transferred into a test tube containing 5 mL of FRAP working solutions and incubated for 1 h under dark condition to prevent degradation of antioxidants. The absorbance of the resulting blue color was measured at 595 nm against the blank (methanol). The results were expressed in mg trolox equivalent (TE).g^{-1} extract.

2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulphonic Acid) Diammonium Salt (ABTS) Radical Cation Scavenging Assay

The ABTS radical cation scavenging activity (ABTS-RCSA) of the rice bran extracts was performed based on the method described by Pellegrini et al. (2003) with some modifications. For the preparation of stock solutions, 0.0024 M potassium persulfate was reacted with an equal amount of 0.007 M ABTS for 12–16 h before use. Light exposure was avoided as much as possible; thus, incubation of sample was done in the dark area at room temperature. Working solutions were prepared at 1:45 stock solution. The standard, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was prepared at different concentrations (0.02–0.28 mM) from 0.8 mM stock solution. About 500 µL of diluted bran extracts (500 $mg.L^{-1}$) and standards were transferred into test tubes containing 5 mL of ABTS working solution and stood for 1 min at room temperature. The absorbance of the solutions was measured at 734 nm against the blank (methanol) using UV-Vis Spectrophotometer (U-3210, Hitachi, Japan). The results were expressed in mg TE.g^{-1} extract.

2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity

The DPPH radical scavenging activity (DPPH-RSA) of the rice bran extracts was determined based on the method developed by Muntana and Prasong (2010) with some modifications. Approximately 500 µL of various concentrations of each bran extract (0.1, 1, 10, 100, and 1000 $mg.L^{-1}$) was added into a freshly prepared 5 mL of 0.1M DPPH working solution. The mixtures were incubated for 1 h for maximum color development. The absorbance was measured against the blank (methanol) at 517 nm using UV-Vis spectrophotometer (U-3210, Hitachi, Japan). Ascorbic acid was used as external reference standard for commercial antioxidant. The DPPH-RSA of bran extracts was calculated using the formula:

$$\text{DPPH-RSA (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

The DPPH-RSA of ethanolic bran extract was expressed

as effective concentration at 50 (EC_{50}, $mg.L^{-1}$) and 25 (EC_{25}, $mg.L^{-1}$).

Evaluation of Cytotoxicity of Pigmented Rice Bran Extracts

The freeze-dried extracts of three black, three red, and one white rice cultivars of known concentrations were subjected to cytotoxicity testing against normal human blood lymphocytes (NHBL). The cytotoxicity assay of rice bran extracts was conducted at the Biological Research and Services Laboratory, Natural Science Research Institute, University of the Philippines in Diliman, Quezon City, Philippines.

The cytotoxicity test of pigmented rice bran extracts was carried out based on the method developed by Verma and Babu (1995). The blood sample was collected from a healthy individual and transferred in a green-top vacutainer tube. Whole blood sample was mixed with an equal volume of Dulbecco's phosphate buffered saline (PBS). About 4 mL of the diluted blood was carefully overlaid into 3 mL of Ficol-Paque and then centrifuged at 2,000 x g for 30 min at room temperature. The clear plasma on top layer of each tube was discarded and the cloudy buffy coat layers were transferred into a clean centrifuge tube. Afterwards, three volumes of Dulbecco's PBS were added to the cells and the solution was mixed to wash the lymphocytes. The tubes were centrifuged at 1000 x g for 5 min at room temperature. The supernatants were discarded, leaving about 0.5 mL of supernatant with the pellet. About 7 mL of Dulbecco's PBS was added to the resuspended pellet. The tubes were inverted several times to wash the cells and centrifuged at 1,000 x g for 10 min at room temperature. Finally, the supernatants were removed and supplemented RPMI (Roswell Park Memorial Institute) medium (RPMI with fetal bovine serum, penicillin-streptomycin, amphotericin B) was added into the resuspended pellet such that the final cell density was about 2 x 10^6 $cells.mL^{-1}$. T-cell cultures were incubated at 37°C for 1–4.5 h.

About 270 μL of blood lymphocyte culture was dispensed into microcentrifuge tubes. Thirty microliters of supplemented RPMI, Dulbecco's PBS/distilled water, phenol, and rice bran extracts were added to three microcentrifuge tubes containing the cell cultures. Afterwards, treated cultures were mixed and incubated at 37°C with 5% CO_2 for about 24 h. After incubation, treated cultures were obtained for cell counting. Seven microliters of each incubated cell cultures, trypan blue, and mixed solutions were placed in a hemocytometer. The number of live and dead lymphocytes were counted in all squares within the 1-mm center grid. The cell density (number of cells per mL) was computed using the formula:

$$\text{Cell density (no. of cells.mL}^{-1}) = [(\text{total no. of cells})/(\text{no. of cells in 1-mm}^2 \text{ squares})] \times 10^4 \times \text{dilution factor}$$

Statistical Analysis

All laboratory experiments were carried out in triplicates and the results were expressed as mean ± SD. Statistical Package for the Social Sciences (SPSS) version 20.0 software for windows (SPSS Inc., Chicago, Illinois, USA) was used to analyze data for antioxidant assays. Significant difference among means was established using one-way ANOVA. Tukey's test was employed to compare treatment means at 5% level of significance ($p \leq 0.05$). Pearson's correlation analysis was used to determine the strength of linear relationship among phytochemical properties and antioxidant activities tested. Dunnett's test was employed to compare the means of the control and the sample for cytotoxicity analysis at $p<0.0001$.

RESULTS AND DISCUSSION

Phytochemical Properties of Pigmented Rice Bran Extracts

The TAC of the pigmented rice bran extracts differed significantly from each other (Table 1). In general, ethanolic bran extracts of black rice samples (24.7 ± 0.1–152.5 ± 2.6 $mg.g^{-1}$ C3G) had higher TAC than those of red rice samples (0.8 ± 0.0–1.3 ± 0.0 $mg.g^{-1}$ C3G). Among the bran extracts, Ominio had the highest TAC while the three red rice bran samples and the control variety (0.2 ± 0.0 $mg.g^{-1}$ C3G) had the lowest. The result confirms our previous findings that black rice varieties contained a higher amount of anthocyanin than the red and white rice varieties (Bulatao et al. 2015). The data obtained in this study also agrees with the findings of Abdel-Aal et al. (2006) who reported that the TAC of black rice varieties was about 35-fold higher than that of red rice, except in

Table 1. Pericarp color of pigmented rice samples and total anthocyanin content (TAC) of their ethanolic bran extracts.

Rice Bran Sample	Pericarp Color	Total Anthocyanin Content ($mg.g^{-1}$ C3G)
NSIC Rc160	Light brown	0.2 ± 0.0^c
Gomiki	Red	0.8 ± 0.0^c
Minaangan	Red	1.4 ± 0.0^c
Red Blondie	Red	1.3 ± 0.0^c
Galo	Black	24.7 ± 0.1^b
Kotinaw	Black	26.1 ± 0.1^b
Ominio	Black	152.5 ± 2.6^a

Means followed by different superscript within a column are significantly different ($p \leq 0.05$) using Tukey's test.

the case of Ominio bran extract, which contained an exceedingly high amount of anthocyanin. Black rice is now being explored in various functional and biomedical applications due to its strong capability to reduce hyperlipidemia, atherosclerotic plaque formation, and cancer (Banjerdpongchai et al. 2013; Hui et al. 2010).

The phytochemical content in terms of TPC and TFC of ethanolic bran extracts varied significantly from each other (Table 2). As expected, ethanolic bran extracts of pigmented rice samples had higher TPC and TFC than the control variety NSIC Rc160. Among the samples, ethanolic bran extracts of Ominio (TPC - 178.4 ± 2.4 mg.g^{-1} GAE; TFC - 378.0 ± 7.9 mg.g^{-1} RHE) and Gomiki (TPC - 137.4 ± 2.1 mg.g^{-1} GAE; TFC - 294.3 ± 3.4 mg.g^{-1} RHE) had the highest TPC and TFC in all black and red rice samples, respectively, while NSIC Rc160 had the lowest (TPC-21.5 ± 0.1 mg.g^{-1} GAE; TFC-27.1 ± 1.5 mg.g^{-1} RHE).

In this study, no correlation was obtained between the color and phytochemical properties of pigmented rice samples. This means that aside from the anthocyanins, which are the major flavonoid present in black rice, there are also other colorless phenolic compounds like phenolic acids that are abundantly present in red rice samples (Gomiki and Minaangan) that contributed to their high phenolic content. Nevertheless, our findings are still consistent with the results reported by Walter and Marchesan (2011), which stated that pigmented rice varieties have significantly higher TPC than non-pigmented pericarp varieties. Our results are also similar with the TFC obtained by Finocchiaro et al. (2010) for the set of Italian pigmented rice varieties. According to Rodrigo et al. (2011), regular consumption of phenolic-rich foods could reduce the risk of cardiovascular diseases and aging. Several researchers also conveyed the efficacy of flavonoids and other phenolic compounds against cancer cells, which was attributed mainly to their protective mechanism against DNA damage and tumor cell proliferation (Dai and Mumper 2010; Kim et al. 2010).

Table 2. TPC and TFC of rice bran extracts.

Rice Bran Sample	Total Phenolic Content (mg.g^{-1} GAE)	Total Flavonoid Content (mg.g^{-1} RE)
NSIC Rc160	21.5 ± 0.1^{g}	27.1 ± 1.5^{f}
Gomiki	137.4 ± 2.1^{c}	294.3 ± 3.4^{b}
Minaangan	112.0 ± 1.7^{d}	244.7 ± 10.1^{c}
Red Blondie	70.1 ± 1.5^{f}	123.3 ± 3.1^{e}
Galo	147.0 ± 2.9^{b}	286.3 ± 21.2^{b}
Kotinaw	90.9 ± 2.1^{e}	161.4 ± 11.2^{d}
Ominio	178.4 ± 2.4^{a}	378.0 ± 7.9^{a}

Means followed by different superscript within a column are significantly different ($p \leq 0.05$) using Tukey's test.

Antioxidant Activities of Pigmented Rice Bran Extracts

Trolox standard calibration curve (FRAP: R^2=0.9991; ABTS: R^2=0.9950) was established to calculate the antioxidant activities of the sample extract, which was expressed in mg.g^{-1} TE. The reducing power of the ethanolic bran extracts was determined using the FRAP assay, which involves the reduction of ferric-tripyridyltriazine complex [Fe(TPTZ)$^{3+}$] into Fe^{2+} complex [Fe(TPTZ)$^{2+}$] in the presence of antioxidants. Results showed that the FRAP values of pigmented rice bran extracts varied significantly from each other (Table 3). Among the sample extracts, black rice Ominio (461.7 ± 23.4 mg.g^{-1} TE), and red rice Gomiki (207.7 ± 4.3 mg.g^{-1} TE) and Minaangan (207.0 ± 3.4 mg.g^{-1} TE) showed the strongest antioxidant activity among the pigmented rice samples. The lowest FRAP value was obtained in the control variety NSIC Rc160 (36.7 ± 0.2 mg.g^{-1} TE).

Almost similar trend was observed in the ABTS-RCSA of the ethanolic bran extracts wherein Ominio (367.9 ± 14.4 mg.g^{-1} TE) and Gomiki (266.2 ± 12.6 mg.g^{-1} TE) had the highest antioxidant activity among the black and red rice varieties, respectively. All pigmented rice bran extracts (96.2 ± 2.3-367.7 ± 14.4 mg.g^{-1} TE) had significantly higher ABTS-RCSA values than the control variety (25.4 ± 1.4 mg.g^{-1} TE).

The results obtained are consistent with the findings of Saewan and Vichit (2015) wherein they reported the strong correlation between the TPC and antioxidant activities of pigmented rice varieties, which was also evident in this study. Our result suggests that the major contributory factor of antioxidant activity in pigmented rice are the phenolic compounds such as the anthocyanins, which are known to be powerful reducing agents and strong radical cation scavengers.

The effective dosage of pigmented rice bran extracts that can significantly reduce the concentration of free radicals in the body was estimated using DPPH radical

Table 3. Ferric reducing antioxidant powder (FRAP) value and ABTS radical cation scavenging activity (ABTS-RCSA) of rice bran extracts.

Rice Bran Sample	FRAP (mg.g^{-1} TE)	ABTS-RCSA (mg.g^{-1} TE)
NSIC Rc160	36.7 ± 0.2^{f}	25.4 ± 1.4^{e}
Gomiki	207.7 ± 4.3^{c}	266.2 ± 12.6^{b}
Minaangan	207.1 ± 3.4^{c}	231.4 ± 4.2^{c}
Red Blondie	116.4 ± 1.4^{e}	85.4 ± 4.9^{d}
Galo	249.0 ± 9.3^{b}	226.7 ± 6.6^{c}
Kotinaw	157.6 ± 3.1^{d}	96.2 ± 2.3^{d}
Ominio	461.7 ± 23.4^{a}	367.7 ± 14.4^{a}

Means followed by different superscript within a column are significantly different ($p \leq 0.05$) using Tukey's test.

scavenging activity assay. This assay determined the capacity of the sample extracts to donate electrons to form more stable products, hence terminating the free-radical initiated stresses in the body that may lead to various diseases. The assay involved decolorization of the purple DPPH solution into a yellowish solution in the presence of antioxidants. Results showed that the concentrations of pigmented rice bran extracts had direct correlation with their DPPH-RSA based on the sigmoidal concentration response curve (Fig. 1). That is, as the concentration of the sample extracts increases, its DPPH-RSA also increases.

Table 4 summarizes the computed effective concentration (EC) at 50 (EC_{50}) and 25 (EC_{25}) of ethanolic bran extracts from different pigmented rice samples. EC_{50} and EC_{25} values are defined as the concentrations that cause 50% and 25% decrease in DPPH absorbance, respectively. The EC is inversely proportional to the DPPH-RSA of the extract (Arienzo et al. 2009). Thus, a lower EC value indicates higher antioxidant activity.

Among the ethanolic bran extracts, Ominio (EC_{50} - 11.6 $mg.L^{-1}$; EC_{25} - 3.9 $mg.L^{-1}$) had the highest antioxidant activity, followed by Galo (EC_{50} - 20.9 $mg.L_{-1}$; EC_{25} - 7.0 $mg.L^{-1}$), Gomiki (EC_{50} - 21.2 $mg.L^{-1}$; EC_{25} - 7.1 $mg.L^{-1}$), and Minaangan (EC_{50} - 22.0 $mg.L^{-1}$; EC_{25} - 7.3 $mg.L^{-1}$). The trend was similar to the results obtained from TPC confirming that phenolic compounds are the main responsible for the strong antioxidant activities of pigmented rice bran extracts. Meanwhile, Kotinaw (EC_{50} - 30.3 $mg.L^{-1}$; EC_{25} - 10.1 $mg.L^{-1}$) had the lowest antioxidant activity among the samples, but still showed significantly higher values than the control rice variety NSIC Rc 160. Our findings validated the importance of phenolic compounds in assessing the antioxidant power of pigmented rice bran extracts. Fujita et al. (2010) reported that the strong antioxidant activity of pigmented rice varieties is mostly attributed to the synergistic action of the hydrophilic and lipophilic antioxidants, which are effective free radical scavengers. They further stated that only lipophilic antioxidants are present in non-pigmented varieties, which explains the low antioxidant activity of

Table 4. EC_{25} and EC_{50} values of rice bran extracts and ascorbic acid.

Rice Bran Sample	EC25 Value ($mg.L^{-1}$)	EC50Value ($mg.L^{-1}$)
Ascorbic acid	1.2	3.7
NSIC Rc160	70.8	No date*
Gomiki	7.1	21.2
Minaangan	7.3	22.0
Red Blondie	8.1	24.2
Galo	7.0	20.9
Kotinaw	10.1	30.3
Ominio	3.9	11.6

*The EC_{50} of NSIC Rc160 could not be estimated from the data.

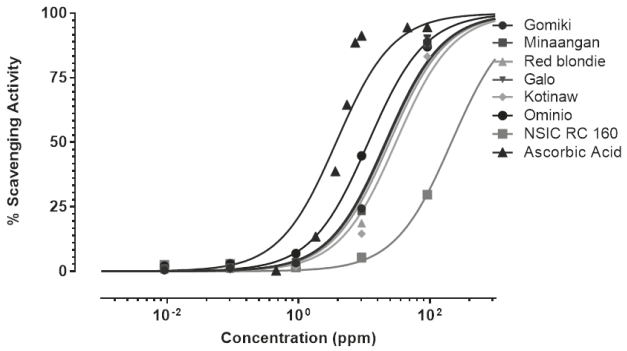

Fig. 1. Sigmoidal concentration response curve of ascorbic acid and rice bran extracts.

the control rice variety. However, the reported antioxidant activities of pigmented rice bran extracts were lower than that of the commercial antioxidant ascorbic acid (EC_{50} - 3.7 $mg.L^{-1}$; EC_{25} - 1.2 $mg.L^{-1}$). This is due to the fact that ascorbic acid was already in its pure state. Since it is a pure compound, its capacity to scavenge oxidizing species is predicted to be significantly higher than the crude rice bran extracts. The antioxidant activity of the ethanolic bran extracts could be further enhanced if the phenolic compounds or other bioactive compounds responsible for the reported activity were isolated. This study proved the strong antioxidant activities of pigmented rice bran extracts, which could be used as ingredient in developing functional food and pharmaceutical products.

Correlation Analysis of Phytochemical Properties and Antioxidant Activities

Table 5 shows the correlation coefficient (R) of the phytochemical properties (TAC, TPC and TFC) and antioxidant activities (ABTS-RCSA and FRAP) of ethanolic bran extracts. Correlation coefficient was used to determine the strength of linear relationship among the phytochemical properties and antioxidant activities tested. Interpretation of the Pearson's correlation coefficient was based on Chan (2004) and Akoglu (2018). The strength of linear relationship was classified as very

Table 5. Correlation coefficient of phytochemical properties and antioxidant activities of rice bran extracts.

Properties	Correlation Coefficient (R)				
	TPC	TFC	TAC	ABTS-RCSA	FRAP
TPC	1.000	-	-	-	-
TFC	0.993***	1.000	-	-	-
TAC	0.640**	0.627**	1.000	-	-
ABTS-RCSA	0.952***	0.979***	0.659**	1.000	-
FRAP	0.920***	0.919***	0.877***	0.924***	1.000

Classification: no * - poor (less than 0.3); * - fair (0.3-0.5); ** - moderately strong (0.6-0.8); *** - very strong (at least 0.8)

strong (at least 0.8), moderately strong (0.6–0.8), fair (0.3–0.5), and poor (less than 0.3). Correlation analysis revealed that there was a very strong positive correlation between TPC and TFC (R=0.993), TAC and FRAP (R= 0.977), TPC and ABTS-RCSA (R=0.952), ABTS-RCSA and FRAP (R=0.943), and TPC and FRAP (R= 0.920) of pigmented rice bran extracts. This implies that when the amount of TPC increases, there is a high probability that the concentrations of ABTS-RCSA, TFC, and FRAP also increases, and vice- versa. A similar trend was also noticed in the relationship of FRAP with TAC and ABTS-RCSA. Meanwhile, a moderately positive correlation was obtained between TAC and TPC (R=0.640), TAC and TFC (R=0.627), and TAC and ABTS-RCSA (R=0.659).

In general, all the phytochemical properties and antioxidant activities tested showed a very strong correlation with each other, except for the relationship of TAC with TPC, TFC, and ABTS-RCSA, which had a moderately strong correlation.

Cytotoxicity of Pigmented Rice Bran Extracts

Trypan blue exclusion assay was used to determine the cytotoxicity of the ethanolic bran extracts against normal human blood lymphocytes (NHBL) (Verma and Babu 1995). This assay is based on the ability of the viable cells to be impermeable to trypan blue. The cell would uptake the color of the dye once the membrane integrity of the cell is compromised. Different concentrations (100, 250, 500, 750, and 1000 mg.L^{-1}) were initially prepared for Gomiki and Ominio since these two varieties consistently contained the highest amount of phytochemicals and antioxidant activities all throughout the analysis. Meanwhile, a concentration of 500 mg.L^{-1} was prepared for Minaangan, Red Blondie, Galo, Kotinaw, and NSIC Rc160. Distilled water and phenol (640 mg.L^{-1}) were used as negative and positive control, respectively.

Table 6 presents the mean live cells of ethanolic bran extracts and the negative control. Results showed that the negative control (distilled water: 89.3 ± 2.2%) had comparable mean live cells with those of the ethanolic bran extracts (86.3 ± 2.9–93.4 ± 3.5%). This implies that the pigmented rice bran extracts at a concentration of 500 mg.L^{-1} had no cytotoxic effect on NHBL; hence, it is safe for human consumption.

Table 7 shows the mean live cells of the ethanolic bran extract and positive control. Results revealed that the positive control (phenol: 4.6 ± 0.8%) was extremely incomparable with the ethanolic bran extracts. Thus, it can be inferred that the ethanolic bran extracts at a concentration of 500 mg.L^{-1} had no toxic effect against NHBL.

Table 8 shows the mean live cells of the ethanolic bran extracts from Gomiki and Ominio. Results indicated that the ethanolic bran extracts of these two varieties at varying concentrations from 100 to 1000 mg.L^{-1} had comparable live cell count with that of the negative control (distilled water). On the other hand, highly significant differences were observed between the live cell count of the ethanolic bran extracts and the positive control (Table 9).

Overall, the ethanolic bran extracts at concentrations ranging from 100 to 1000 mg.L^{-1} showed no adverse effect against NHBL. Thus, pigmented rice bran extracts, when used at these concentrations, are safe for human consumption and at the same time are ideal sources of natural antioxidants that are highly beneficial to human health.

CONCLUSION

This study evaluated the phytochemical properties (TAC, TPC, and TFC), antioxidant activities (DPPH-RSA, ABTS-RCSA, and FRAP), and cytotoxicity against NHBL of ethanolic bran extracts from six pigmented rice cultivars. All pigmented rice bran extracts were found to contain a remarkable amount of TAC, TPC, TFC, DPPH-RSA, ABTS -RCSA, and FRAP values, wherein the ethanolic bran extracts of Ominio and Gomiki consistently obtained the

Table 6. Comparison between the mean live cells (%) of the rice bran extracts and negative control.

	Ethanolic Bran Extract (500 mg.L^{-1})					
	Distilled Water	NSIC Rc160	Minaangan	Red Blondie	Galo	Kotinaw
Mean live cells (%)	89.3 ± 2.2	89.2 ± 1.2	86.3 ± 2.9	89.0 ± 1.7	93.3 ± 2.4	93.4 ± 3.5

Means followed by asterisk superscript indicates significant difference ($p \leq 0.0001$) from the negative control using Dunnett's test.

Table 7. Comparison between the mean live cells (%) of the rice bran extracts and positive control.

	Ethanolic Bran Extract (500 mg.L^{-1})					
	Phenol	NSIC Rc160	Minaangan	Red Blondie	Galo	Kotinaw
Mean live cells (%)	4.6 ± 0.8	89.2 ± 1.2 ****	86.3 ± 2.9 ****	89.0 ± 1.7 ****	93.3 ± 2.4 ****	93.4 ± 3.5 ****

Means followed by asterisk superscript indicate significant difference ($p \leq 0.0001$) from the positive control using Dunnett's test.

Table 8. Comparison between the mean live cells (%) of the different concentrations of bran extracts from Gomiki and Ominio and negative control.

Concentration ($mg.L^{-1}$)	Gomiki	Ominio
1000	86.2 ± 2.6	92.1 ± 1.4
750	86.7 ± 2.1	88.9 ± 0.5
500	90.2 ± 1.1	91.0 ± 1.0
250	84.9 ± 7.0	86.2 ± 4.4
100	90.7 ± 5.5	89.2 ± 3.0
Distilled water	89.3 ± 2.2	

Means followed by asterisk superscript within a column indicate significant difference ($p \leq 0.0001$) from the positive control using Dunnett's test.

Table 9. Comparison between the mean live cells (%) of the different concentrations of bran extracts from Gomiki and Ominio and positive control.

Concentration ($mg.L^{-1}$)	Gomiki	Ominio
1000	86.2 ± 2.6****	92.1 ± 1.4****
750	86.7 ± 2.1****	88.9 ± 0.5****
500	90.2 ± 1.1****	91.0 ± 1.0****
250	84.9 ± 7.0****	86.2 ± 4.4****
100	90.7 ± 5.6****	89.2 ± 3.0****
Phenol (640 mg/L)	4.6 ± 0.8	

Means followed by asterisk superscript within a column indicate significant difference ($p \leq 0.0001$) from the positive control using Dunnett's test.

highest values in all black and red rice samples, respectively. Pearson's correlation analysis revealed that there was a moderate to very strong positive correlation among the phytochemical properties and antioxidant activities tested. Furthermore, the pigmented rice bran extracts at concentrations ranging from 100 to 1000 $mg.L^{-1}$ were found to be nontoxic against NHBL. Therefore, this study concluded that the ethanolic bran extracts from pigmented rice cultivars could be an excellent source of natural, safe, and effective antioxidants which can be used as key ingredient in the development of functional food and pharmaceutical products.

ACKNOWLEDGMENT

This research is supported by a grant from the Philippine Rice Research Institute.

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