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INHIBITORY EFFECT OF GERMINATED PIGMENTED RICE ON KEY METABOLIC ENZYMES ASSOCIATED WITH DIABETES AND HYPERGLYCEMIA

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The inhibitory effects of germinated pigmented rice on the key metabolic enzymes linked to diabetes and hyperglycemia were investigated. Four kinds of pigmented rice, Heukjinjubyeo (dark purple), Keunnunjami (blackish purple), Superjami (blackish purple), and Superhongmi (reddish brown), and normal brown rice were germinated for 3 days and their ethanolic extracts were analyzed for phenol content and inhibitory activities against the enzymes α-glucosidase, α-amylase, dipeptidyl peptidase-4, lipase, and xanthine oxidase. The pigmented rice showed significantly higher total phenol content and enzyme inhibitory activities compared with the normal brown rice. The inhibition of enzymes markedly increased during germination which may have been due to the substantial increase in the phenolic content of rice. The results suggest that germinated pigmented rice, particularly Keunnunjami and Superjami, may be potentially useful as a functional food in the management of diabetes and hyperglycemia.

Keywords: Pigmented rice, Germination, Enzyme inhibition, Phenol

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world and the staple food in many countries, especially in Asia. It contains high amount of carbohydrates and is a good source of protein, minerals, and vitamins (Fresco, 2005). Brown or unpolished rice, with its bran and embryo intact, is considered a healthier alternative to white rice due to its high contents of dietary fiber, antioxidants, and bioactive compounds, such as g-oryzanol and g-aminobutyric acid, and it has been found to lower the risk of diabetes and hyperglycemia (Dinesh Babu *et al*., 2009). However, despite its superior nutritional value over white rice, brown rice is seldom consumed as staple food because of its hard texture. Various processes such as soaking, fermentation, and germination have been used to improve the nutritional and eating qualities of brown rice. Germination, in particular, is regarded as an effective and inexpensive method of improving the texture, palatability, and nutrient content of brown rice (Wu *et al*., 2013). It was reported that germinated brown rice has greater amount of bioactive compounds than brown rice and possesses strong anti-diabetic and antioxidative effects (Dinesh Babu *et al*., 2009; and Patil and Khan, 2011). Germination of brown rice involves the soaking of rice grain in water for 1-2 days to induce a slight germination, during which time complex biochemical changes occur, resulting in the softening of the texture and increased nutrient bioavailability and absorption (Patil and Khan, 2011; and Wu *et al*., 2013).

Rice with colored pericarp, known as pigmented variety, has higher nutritional content and greater antioxidant capacity than ordinary non-pigmented rice and its consumption has been found to reduce the risk of

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developing diabetes, hyperlipidemia, and cardiovascular disease (Ling *et al*., 2001). In recent years, new lines of pigmented rice have been developed in Korea through conventional breeding. The present study focuses on four kinds of pigmented rice, namely Keunnunjami, Superjami, Superhongmi, and Heukjijubyeo. Keunnunjami, a giant embryo rice mutant, and Superjami are both blackish purple rice with Heukjinjubyeo (dark purple rice) as one of their parental cultivars. They both contain high amounts of cyanidin-3-glucoside, an anthocyanin that has strong antioxidant capacity (Kwon *et al*., 2011; and Han *et al*., 2012). Superhongmi, on the other hand, is a newly-developed reddish brown rice.

Diabetes, a metabolic disorder characterized by hyperglycemia, is a growing health problem and one of the leading causes of death worldwide. One of the treatments of diabetes is to inhibit the key digestive enzymes associated with starch and lipid digestion. The inhibition of enzymes involved in carbohydrate and lipid digestion such as α-glucosidase, á-amylase, and lipase would delay the digestion and absorption of lipids and carbohydrates and reduce plasma glucose level, resulting in the suppression of postprandial hyperglycemia (Hamden *et al*., 2013; and Dey *et al*., 2014). The inhibition of other enzymes like dipeptidyl peptidase-4 (DPP-4) and xanthine oxidase could also serve as a therapeutic approach for the management of diabetes and hyperglycemia (Desco *et al*., 2002; and Deacon and Holst, 2006). There are limited studies on the inhibitory effect of pigmented rice and germinated brown rice against carbohydrate hydrolyzing enzymes (Yao *et al*., 2010; and Kim *et al*., 2013) and, to the best of our knowledge, none on the effect of germination on the enzyme inhibitory activity of pigmented rice. This study was carried out to determine the inhibitory effects of germinated pigmented rice Heukjinjubyeo, Keunnunjami, Superjami, and Superhongmi, in comparison with that of the germinated normal brown rice, on the key metabolic enzymes (α-glucosidase, α-amylase, DPP-4, lipase, and xanthine oxidase) associated with hyperglycemia and diabetes. The enzyme inhibition activities in relation to the phenolic content in rice were also assessed.

MATERIALS AND METHODS

Materials

Four whole grain pigmented rice, dark purple Heukjinjubyeo (HJ), blackish purple Keunnunjami (KJ), blackish purple Superjami (SJ), reddish brown Superhongmi (SH), and ordinary Normal Brown rice (NB) were obtained from the Department of Agricultural Science, Korea National Open University. They were grown from May to October 2014 under the same cultivation conditions. All chemicals and standards used in the study are of analytical grade and were purchased from Sigma-Aldrich, Inc. (Steinhein, Germany).

Rice Germination

Four sets of 50 g rice grains from each sample were washed twice with distilled water to remove any dirt. The first set, which served as the non-germinated rice sample, was placed in tray topped with paper towel and dried in an oven at 50 °C for 2 h to lower the moisture content. The dried rice samples were then ground and pulverized, packed in hermetically sealed Ziploc plastic bags, and stored at -20 °C until further analysis. The remaining 3 sets of rice samples were placed evenly in a tray overlaid with cotton pads and cheesecloth and enough water was added until the rice grains were soaked. The whole tray was covered with a clean wrap with holes to accommodate proper moisture condition and incubated at 30 °C. After 24, 48, and 72 h of germination, one set of each rice sample was collected. The germinated rice were dried and stored using the same method described above for the non-germinated rice.

Preparation of Ethanolic Extracts from Germinated and Non-Germinated Rice

The pulverized rice samples (10 g) were mixed with 80% ethanol (100 ml) and subjected to 1-h shaking at 25 ºC, followed by filtration using a Whatman filter paper No. 4. The extraction process was repeated 3 times and the filtrate was evaporated to 5 ml at 40 °C using a rotary evaporator (Eyela SB-1200, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The extracts were then freeze-dried and stored at -20 ºC until further analysis. They were dissolved in 10 mg/ml dimethyl sulfoxide prior to use.

Enzyme Inhibition Assays

~-Glucosidase

The α-glucosidase inhibitory activity of the rice extract was determined using the method of Hong *et al*. (2013) with slight modifications. The extract (50 µl) was mixed with α-glucosidase solution (100 µl) and incubated at 37 °C for 10 min. The mixture was added with 50 µl of p-nitrophenyl-α-D-glucopyranoside and further incubated at 37 °C for 20 min. A 1 ml of 1 M Na_2Co_3 was added to stop the reaction and the absorbance of the reaction mixture was measured at

450 nm using a UV/visible spectrophotometer (DU 800 series, Beckman Coulter, USA).

~-Amylase

The α-amylase inhibitory activity was measured according to the method described by Kazeem *et al*. (2013). The sample extract (125 µl) was added with á-amylase (62.5 µl) and 200 mM potassium phosphate buffer (62.5 µl, pH 6.8) and incubated at 37 °C for 10 min. A 125 µl of 1% starch was added to the mixture and incubated for another 5 min. To produce a colored solution, a 125 µl of 48 mM 3,5-dinitrosalicylic acid was added. The colored solution was incubated at 100 °C for 15 min and then cooled in ice. Finally, a 1.5 ml of distilled water was added to the solution and the absorbance was measured at 405 nm.

Dipeptidyl Peptidase-4

The DPP-4 inhibitory activity was determined using a DPP-4 inhibitor screening kit (Cayman Chemical Co., MI, USA) following the instruction manual. Briefly, three types of wells were prepared: 100% initial activity well (30 µl assay buffer, 10 µl diluted DPP, and 10 µl HPLC-grade water), background well (40 µl assay buffer and 10 µl HPLC grade water), and inhibitor well (30 µl assay buffer, 10 µl diluted DPP, and 10 µl sample extract). To initiate the reaction, 50 ìl of the diluted substrate solution was added into each well and the whole plate was covered and incubated at 37 °C for 30 min. The plate cover was then removed and the fluorescence was read using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The enzyme inhibition was calculated by subtracting the fluorescence of the background well from the fluorescence of the 100% initial activity and inhibitor wells and the value obtained was used in the following equation:

Enzyme inhibitory activity (%) = [(initial activity – inhibitor)/initial activity] x 100

Lipase

The sample extract (100 µl) was mixed with 860 µl enzyme buffer [porcine pancreatic lipase, 10 mM 3-(N-morpholino) propabelsulfonic acid, 4-morpholinepropanesulfonic acid, 100 nM tris-HCl/5 mM $CaCl_2$ (pH 6.8)] and the mixture was incubated at 37 ºC for 5 min (Lee *et al*., 2010). A 40 µl of 10 mM p-nitrophenyl butyrate was added and the reaction mixture was further incubated for 15 min. The absorbance was measured at 400 nm.

Xanthine Oxidase

The sample extract (100 µl) was mixed with 0.1 M potassium phosphate buffer (600 µl, pH 7.5) (Sahgal *et al*., 2009). A 200 µl of 2 mM xanthine was added, followed by 100 µl of 0.2 unit/ml of xanthine oxidase solution. The reaction mixture was incubated at 37 ºC for 5 min and then added with 1 ml of 1 N HCl. The absorbance was measured at 292 nm. The enzyme (α-glucosidase, α-amylase, lipase, and xanthine oxidase) inhibitory activities were calculated as follows:

Enzyme inhibitory activity (%) = $[(1 - A_{sample})/A_{blank}] \times 100$

Determination of Total Phenolic Content

The total phenolic content was measured according to the method of Muntana *et al*. (2010). The sample extract (100 µL) was mixed with 2 mL of 20% sodium carbonate and the mixture was shaken vigorously for 3 min. A 2 mL of 20% Folin-Ciocalteu reagent was added and the mixture was shaken for 1 h to allow complete formation of the blue complex. The absorbance was measured at 750 nm. The total phenolic content was expressed as mg/g Gallic Acid Equivalent (GAE).

Statistical Analysis

All data presented are mean ± SE (n = 3). The data were evaluated by one-way ANOVA using Statistical Package for Social Sciences software program version 22 (SPSS Inc., Chicago, IL) and the differences between the means were assessed by Tukey's test. Statistical significance was considered at $p<0.05$. The correlations between enzyme inhibitory activities and phenolic contents were determined using Pearson correlation analysis.

RESULTS AND DISCUSSION

~-Glucosidase Inhibitory Activity

Germination markedly increased the α-glucosidase inhibitory activity of the pigmented rice samples, but did significantly affect the enzyme inhibition activity of NB rice (Figure 1). Prior to germination, the NB, HJ, KJ, and SJ rice exhibited similar inhibitory activity. However, from the first to third day of germination, the inhibition of α-glucosidase enzyme was highest in KJ (50-76%) and lowest in NB (28-32%). The acarbose, an oral α-glucosidase inhibitor used for treating type 2 diabetes mellitus, showed significantly higher inhibitory activity (85%) than the germinated rice samples. The α-glucosidase, a key enzyme in carbohydrate digestion, catalyzes the breakdown of starch and disaccharides into glucose that enters the bloodstream

(Aloulou *et al*., 2012). The inhibition of digestive enzymes is considered an effective therapeutic approach in the management of diabetes. An inhibitor of α-glucosidase can slow the release of glucose from dietary complex carbohydrates and delay glucose absorption, resulting in the suppression of postprandial hyperglycemia (Kumar *et al*., 2011). It has been previously shown that pigmented grains such as red, purple, and black rice, purple corn, and black soybean could inhibit the α-glucosidase enzyme and that black rice possessed the highest α-glucosidase inhibitory among the pigmented grains analyzed (Yao *et al*., 2010). In the present study, germination substantially increased the α-glucosidase inhibitory activity of pigmented rice, particularly KJ, suggesting that it may be a useful process in enhancing the anti-diabetic property of pigmented rice.

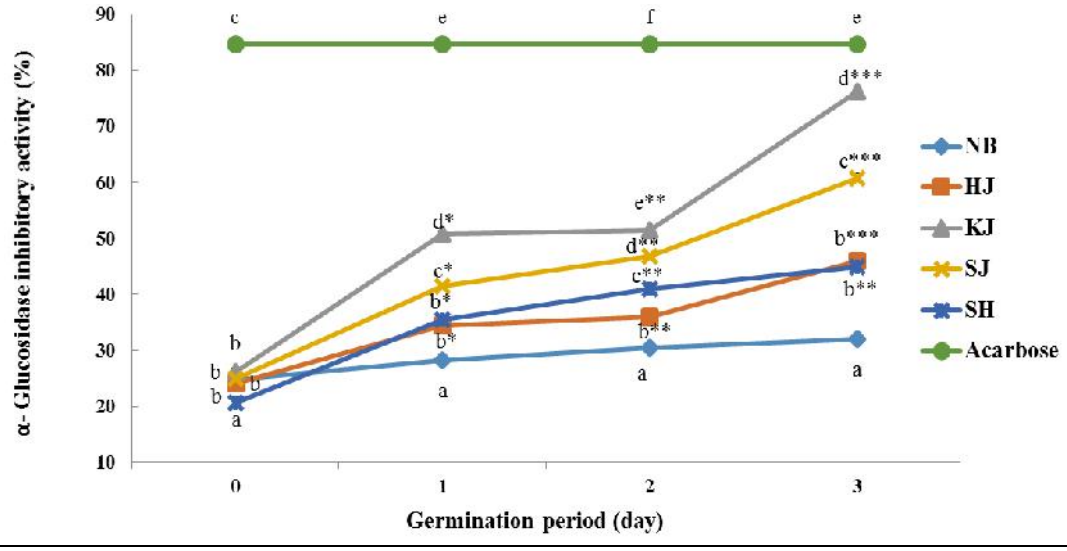
~-Amylase inhibitory activity

The α-amylase inhibitory activity was highest in KJ and SJ rice (60%) and lowest in NB and HJ rice (47%) prior to germination (Fig. 2). The activity markedly increased in all rice samples during germination. On the last day of germination, the enzyme inhibitory activity was highest in KJ (84%) and lowest in NB (59%). The α-amylase, secreted by the pancreas to the small intestine, is a key digestive enzyme that breaks down the carbohydrates into oligosaccharides, which are then hydrolyzed by α-glucosidase into monosaccharides such as glucose (Barrett and Udani 2011). The inhibition of the amylase enzyme would decrease the rate of carbohydrate absorption, thereby reducing the postprandial increase of blood glucose (Tundis *et al*., 2010). A study in the past revealed that germination improved the enzyme inhibitory activity of rough rice (Kim *et al*., 2013). The present study showed that pigmented rice has greater inhibitory activity than brown rice and that germination significantly improved the enzyme inhibition activity of rice. Extracts from germinated pigmented rice, especially KJ, may be potentially beneficial in the management of postprandial hyperglycemia.

DPP-4 Inhibitory Activity

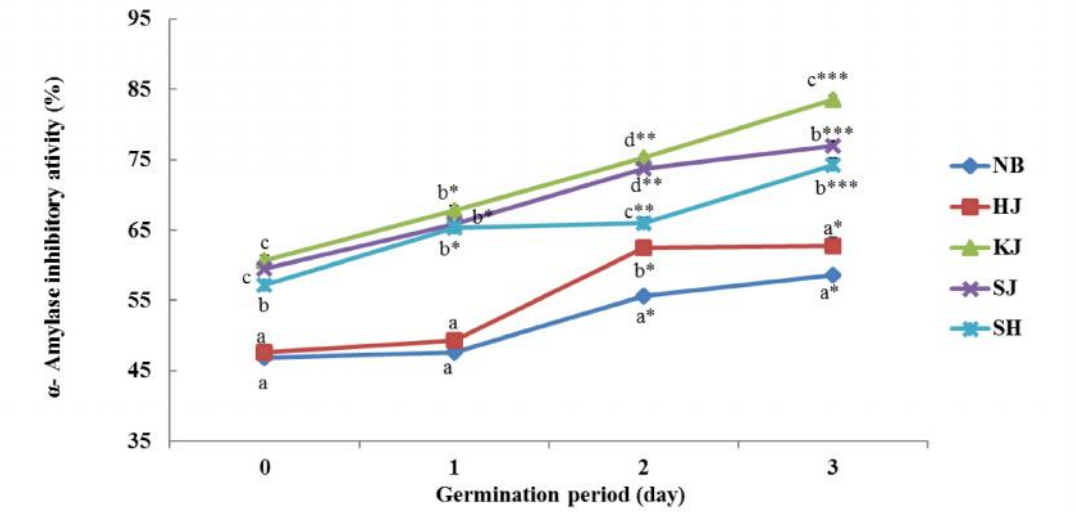
Before germination, the KJ and SJ rice exhibited the highest DPP-4 inhibitory activity (4%) while the NB rice showed the lowest activity (3%) (Figure 3). On the 2nd and 3rd day of germination, the inhibition of DPP-4 enzyme significantly increased in all samples. The KJ and SJ rice showed the highest inhibitory activity (13%), followed by HJ and SH (10%), then NB rice (7%). DPP-4 is the chief enzyme responsible for degrading the incretins, such as Glucagon-Like Peptide-1 (GLP) and glucose-dependent insulinotropic polypeptide (GIP), hormones that are released in response to food ingestion and stimulate insulin secretion and inhibit glucagon secretion (Wani *et al*., 2008). Studies have shown that inhibition of DPP-4 enzyme resulted in increased serum

Figure 1: ~-Glucosidase Inhibitory Activity of Germinated Pigmented Rice, Means with Different Letters are Significantly Different (p<0.05) Across Rice Samples



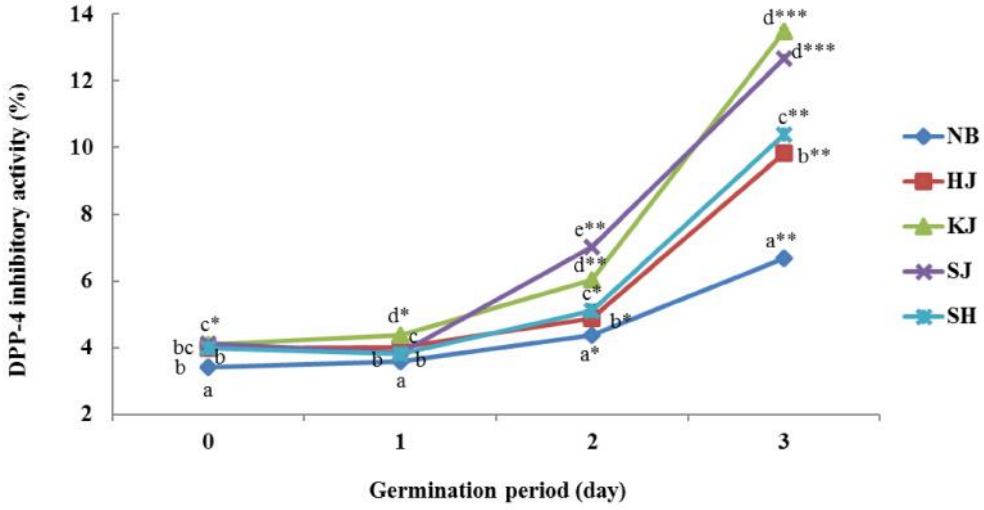
Note: * Indicates significant difference (p<0.05) across germination time, NB: Normal brown rice; HJ: Heukjinjubyeo, KJ: Keunnunjami, SJ: Superjami, SH: Superhongmi.

Figure 2: ~-Amylase Inhibitory Activity of Germinated Pigmented Rice, Means with Different Letters are Significantly Different (p<0.05) Across Rice Samples



Note: * Indicates significant difference (p<0.05) across germination time, NB: Normal brown rice; HJ: Heukjinjubyeo, KJ: Keunnunjami, SJ: Superjami, SH: Superhongmi.

Figure 3: Dipeptidyl Peptidase-4 Inhibitory Activity of Germinated Pigmented Rice, Means with Different Letters are Significantly Different (p<0.05) Across Rice Samples



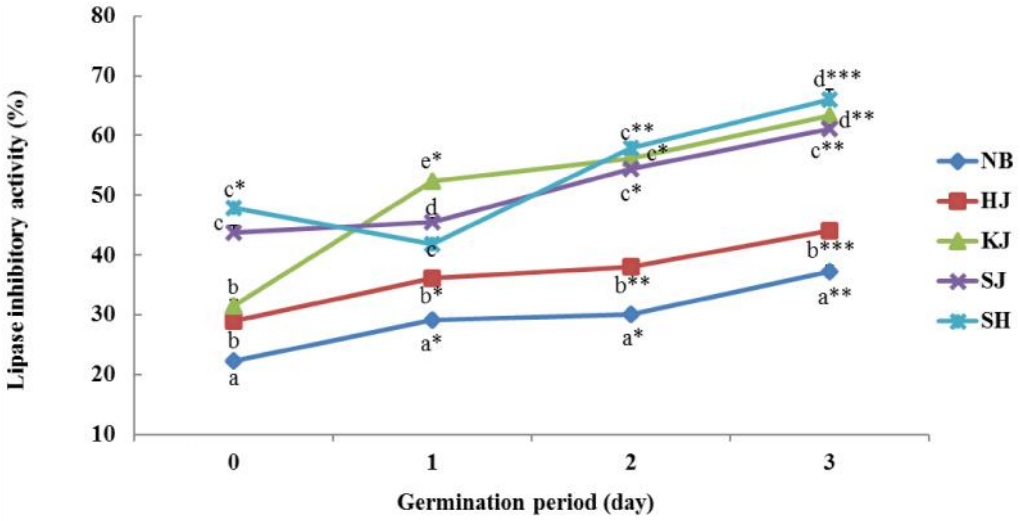
Note: * Indicates significant difference (p<0.05) across germination time, NB: Normal brown rice; HJ: Heukjinjubyeo, KJ: Keunnunjami, SJ: Superjami, SH: Superhongmi.

levels of GLP and GIP, leading to improved glucose tolerance and reduced postprandial hyperglycemia in diabetic patients and animal models (Deacon and Holst, 2006). It was previously reported that germination could increase the DPP-4 inhibitory activity in rough rice (Kim *et al*., 2013). The enhanced DPP-4 inhibitory activity of pigmented rice after germination further illustrates that germination could be an effective method in improving the antihyperglycemic properties of pigmented rice.

Lipase Inhibitory Activity

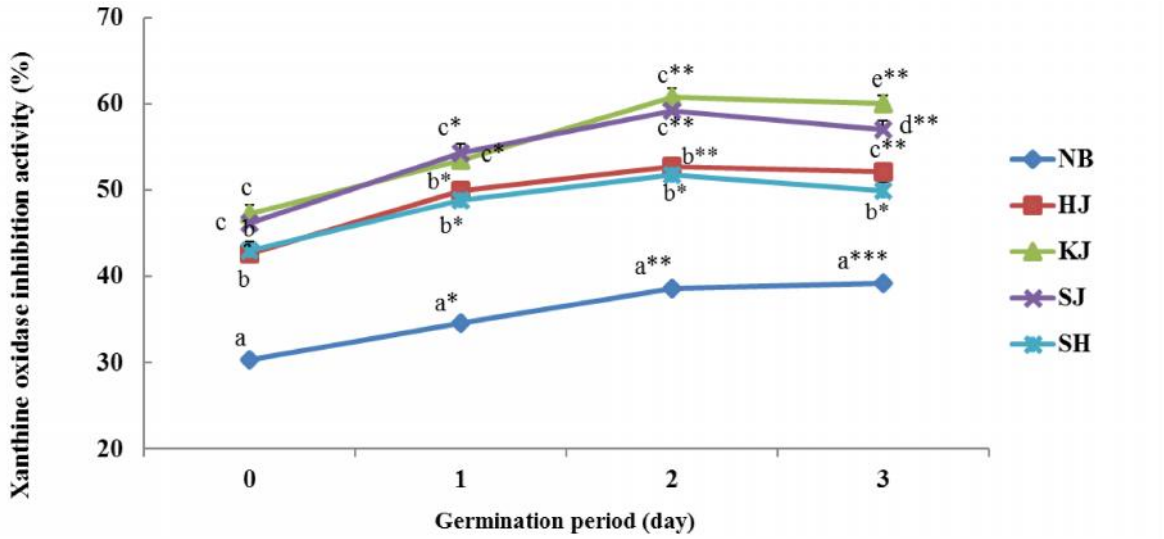
The lipase inhibitory activity was highest in SJ and SH rice (44-48%) and lowest in NB rice (22%) prior to germination (Figure 4). A significant increase in the enzyme inhibitory

Figure 4: Lipase Inhibitory Activity of Germinated Pigmented Rice, Means with Different Letters are Significantly Different (p<0.05) Across Rice Samples



Note: * Indicates significant difference (p<0.05) across germination time, NB: Normal brown rice; HJ: Heukjinjubyeo, KJ: Keunnunjami, SJ: Superjami, SH: Superhongmi.

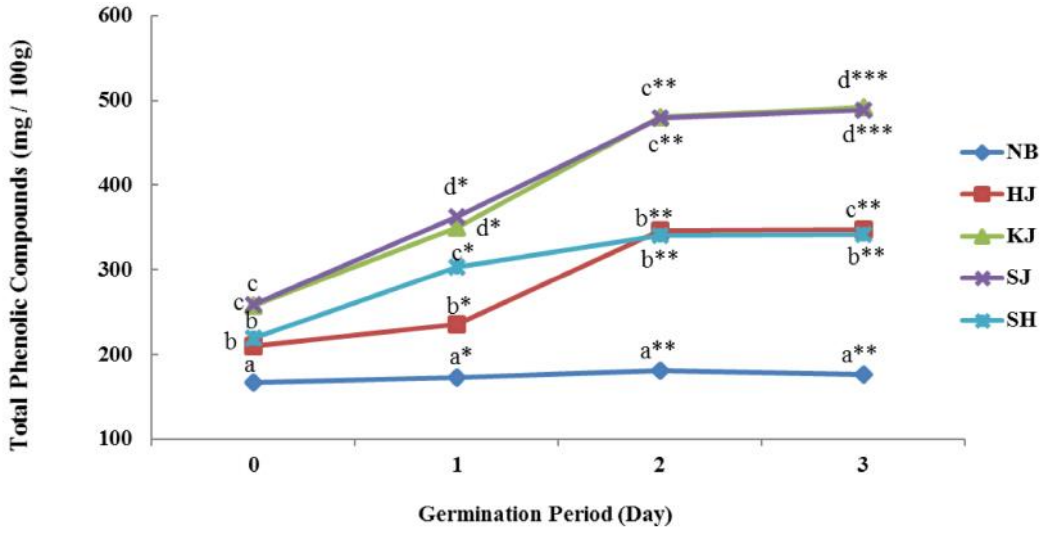
Figure 5: Xanthine Oxidase Inhibitory Activity of Germinated Pigmented Rice, Means with Different Letters are Significantly Different (p<0.05) Across Rice Samples



Note: * Indicates significant difference (p<0.05) across germination time, NB: Normal brown rice; HJ: Heukjinjubyeo, KJ: Keunnunjami, SJ: Superjami, SH: Superhongmi.

activity was observed in all the samples after 2 and 3 days of germination. On the 3rd day of germination, the highest inhibitory activity was found in KJ and SH rice (63-66%), followed by SJ rice (61%). The NB rice showed the lowest activity (37%). Kim *et al.* (2013) also reported an increase in the lipase inhibitory activity of rough rice after germination. The enzyme lipase is a key digestive enzyme that catalyzes the breakdown of non-absorbable triglycerides into fatty acids and glycerol absorbable by the small intestine (Hamden *et al.*, 2013). Inhibition of lipase has been shown

Figure 6: Total Phenolic Content of Germinated Pigmented Rice, Means with Different Letters are Significantly Different (p<0.05) Across Rice Samples



Note: * Indicates significant difference (p<0.05) across germination time, NB: Normal brown rice; HJ: Heukjinjubyeo, KJ: Keunnunjami, SJ: Superjami, SH: Superhongmi.

to inhibit fat digestion and absorption, leading to a decrease in plasma cholesterol and triglyceride levels in diabetic rats (Aloulou *et al*., 2012).

Xanthine Oxidase Inhibitory Activity

Prior to germination, the KJ and SJ rice showed the highest xanthine oxidase inhibitory activity (46-47%), followed by HJ and SH rice (43%), then NB rice (30%) (Figure 5). All rice samples showed a considerable increase in their inhibitory activities during germination. However, from the 2nd to 3rd day of germination, no significant change was observed in the inhibitory activities in all rice samples. At this stage, the activities were highest in KJ and SJ rice (60-61%), followed by HJ and SJ (52-53%). The NB rice showed the lowest activity (19%). Xanthine oxidase, a superoxide-generating enzyme, plays a major role in the generation of free radicals in diabetes (Desco *et al*., 2002). Its inhibition has been found to decrease the hyperglycemia-induced oxidative stress in diabetic mice (Bravard *et al*., 2011). The increased xanthine oxidase inhibitory activity observed in pigmented rice, especially KJ and SJ, after germination suggests that germination could improve the antioxidative properties of rice. Germinated pigmented rice may be beneficial in the suppression of oxidative stress under hyperglycemic condition.

Total Phenolic Content

The total phenolic content of all the rice samples significantly increased during germination (Figure 6). The HJ and SH showed a 55-65% increase of phenolic content on the 2nd and 3rd day of germination. The KJ and SJ exhibited the highest phenolic content (488-492 mg GAE/100 g rice), which increased by 88-91% after 3 days of germination. On the other hand, the phenolic content of NB rice (175 mg GAE/100 g rice) only increased by 4-8% during germination. Past investigations revealed that the phenolic contents in rice are related mainly to the pericarp color; grains with darker pericarp color such as black rice has higher phenolic content than those with lighter color like red and brown rice (Walter *et al*., 2013). Germination has been previously shown to significantly improve the total phenolic content of brown rice (Tian *et al*., 2004; and Wu *et al*., 2013). It was reported that during germination, the cell walls surrounding many compounds are broken down and the free and bound phenolics are released, resulting in an increase in total phenolic content (Kaukovirta-Norja *et al*., 2004).

Correlations Between the Enzyme Inhibitory Activities and Phenolic Content

Significant positive correlations were found between the α-glucosidase (r = 0.88), α-amylase (r = 0.93), DPP-4 (r = 0.99), and xanthine oxidase (r = 0.98) inhibitory activities and total phenolic content. On the other hand, no significant correlation was observed between the lipase inhibitory

activity and phenolic content. These findings indicate that the phenolics are probably the main components responsible for the inhibitory activity of the germinated rice. A number of studies have shown that the enzyme inhibitory activities of various plant extracts were dependent of their phenolic content (Dey *et al*., 2014; and Mohamed, 2014). Yao *et al*. (2010) reported that the total phenolic content was positively correlated with the α-glucosidase inhibitory activity of pigmented rice. Pigmented rice is rich in polyphenol anthocyanins (Kim *et al*., 2008), which has been shown to possess inhibitory properties against α-glucosidase and α-amylase (Mohamed, 2014; and Xiao and Hogger, 2015). Hence, the substantial increase in the phenolic content of pigmented rice samples, particularly in KJ and SJ, may have been responsible for the marked increase in their inhibitory activities against the enzymes analyzed.

CONCLUSION

Pigmented rice showed greater inhibitory activities against α-glucosidase, α-amylase, DPP-4, lipase, and xanthine oxidase compared with the normal brown rice. In general, inhibition of the enzymes significantly increased with germination time. Taken together, the inhibitory activities of the rice samples analyzed were in the order: Keunnunjami > Superjami, > Heukjinjubyeo > Superhongmi > normal brown rice. The improved enzyme inhibition activities of pigmented rice samples is possibly due to the substantial increase in their total phenolic content during germination. These findings demonstrate that extracts from germinated pigmented rice, particularly the blackish purple Kennunjami and Superjami rice, may be useful as potential inhibitor for the treatment and management of postprandial hyperglycemia and diabetes.

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