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Identification of phenolic compounds isolated from pigmented rices and their aldose reductase inhibitory activities

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Abstract

Two anthocyanins (cyanidin-3-*O*-β-glucoside and peonidin-3-*O*-β-glucoside) and other phenolic (ferulic acid) were, respectively isolated from black and pigmented brown rices (*Oryza sativa* L. *japonica*) and their complete structures were determined by spectroscopic analyses (H NMR, C NMR and MALDI MASS). The HPLC profile of anthocyanins extracted from black rice showed cyanidin-3-*O*-β-glucoside as the first peak (85%) and peonidin 3-*O*-β-D-glucoside as the second (15%), while that of pigmented brown rice showed ferulic acid as the first peak (85.7%) and tocols as the second (14.3%). Several tocols were isolated and identified from the unsaponifiable fractions of both rices having some difference on their structures and amounts. The aldose reductase inhibitory activity of isolated compounds was in the following decreasing order: cyanidin-3-glucoside > quercetin > ferulic acid > peonidin-3-glucoside > tocopherol.

All isolated compounds showed significant inhibitory activity against aldose reductase suggesting that both pigmented rices might contribute significantly in combating diabetic complications as health-promoting food ingredients in food processing.

Keywords: Aldose reductase inhibitory activity; Phenolics; Black rice; Pigmented brown rice; Health-promoting food ingredients

1. Introduction

A great deal of interest is given to the association between the consumption of pigmented rices and the improvement of human health due to the great antioxidant potency of phenolic compounds they contain. Pigmented rices have been reported to contain acetylated procyanidin, anthocyanins, and other phenolics with significant free radical scavenging activity. Hu, Zawistowski, Wenhua, and Kitts (2003) reported that black rice (belonging to *Oryza sativa* L. *indica*) contains pigments, which are located in the aleurone layer as a mixture of anthocyanins. Those colorings are naturally occurring compounds that belong to the family of flavonoids in which pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin represent the most commonly occurring anthocyanin aglycons. Anthocyanins often occur in foods with a sugar moiety (glycoside), such as glucose, galactose, xylose, rhamnose, and rutinoside, which are located at the positions 3 and less frequently 7 of the chromane rings (Miller, 1996).

The health benefits of flavonoids are usually linked to two properties: (i) inhibition of certain enzymes such as xanthine oxidase, aldose reductase (AR); and (ii) antioxidant activity (Cotelle, 2001). The antioxidant activity of isolated compounds are well known, thus, their AR inhibitory activity have been the core of the present work. Useful contributions in the area have been made by Hu et al.

Abbreviations: AR, aldose reductase; ARI, aldose reductase inhibitor; EtOH, ethanol; HPLC, high performance liquid chromatography; MALDI-MASS, matrix assisted laser desorption/ionization; NADPH, nicotinamide adenine dinucleotide phosphate; TMS, trimethylsilane.

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(2003), Ichikawa et al. (2001), Ryu, Park, and Ho (1998) who have demonstrated the structure–biological activity relationship of phenolic compounds, hence, the present work has focused on the structure elucidation of phenolic compounds from pigmented rices and anti-enzymatic activity. Various works were done on pigmented rices (belonging to the variety *Oryza sativa* L. *indica*) in order to elucidate both their phenolics profile and their related antioxidant activities, however, studies on pigmented rice grains belonging to *Oryza sativa* L. *japonica* are seldom in the literature. Further, most published reports on the antioxidant activity of pigmented rices related this property exclusively to their anthocyanin structures and content, however, other phytochemicals such as tocols contained in the lipophilic fraction of the same plant material possess potent antioxidative capacity and could be also taken into consideration. This work tried to contribute to cover this unsufficiency.

AR catalyzes NADPH-dependent reductions of various sugar-derived carbonyl compounds to their respective sugar alcohols (e.g. sorbitol) acting in the development of diabetic complications such as retinopathy and neuropathy (Van Zandt et al., 2004). Through various clinical studies, the development and progression of these complications in type 1 and type 2 patients have been clearly linked to elevated blood glucose levels. Although glucose is preferentially metabolized through the glycolytic pathway, during conditions of hyperglycemia, as observed in diabetes mellitus, elevated blood glucose levels saturate the normal pathways of glucose metabolism and a dramatic increase in flux through the polyol pathway results (Scheme 1). For this reason, this enzyme is considered as a drug target in many research works related to diabetic complications. Isolated compounds from both studied rices belong to phenol derivates, one of the main classes of aldose reductase inhibitors (ARIs). Among ARIs, which have been developed with promising results in the past years, almost all have demonstrated undesired side effects during human clinical trials (Van Zandt et al., 2004). Therefore, evaluating further natural sources for potential AR inhibitory activity may lead to discover safer and more effective phytochemicals.

The purpose of the present study was to elucidate the molecular structure of phenolic compounds isolated from black and pigmented brown rices (belonging to the cultivar *Oryza sativa* L. *japonica*) and to assess their health benefits, in particular their AR inhibitory activities.

2. Materials and methods

2.1. Material

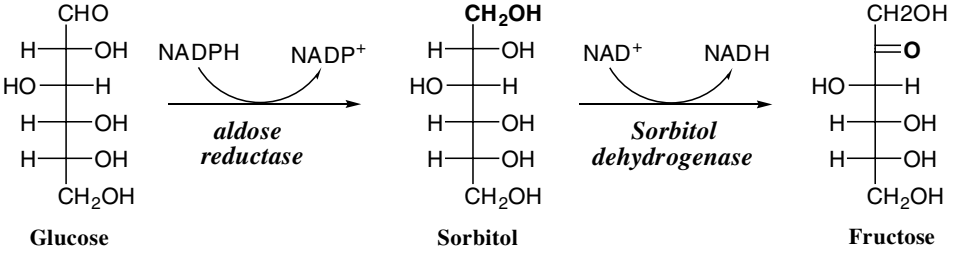
Plant materials used in this study include black rice and pigmented brown rices that were harvested from Osaka Prefecture University rice field and milled using Retsch™ miller (Nihonseiki Kaisha Ltd., Osaka, Japan). After milling, powders of milled rices were kept at cold (6 °C) until further use.

2.2. Preparation of black and pigmented brown rices extracts

Black and pigmented brown rices were extracted overnight at room temperature with 1% HCl/methanol and 1% HCl/ethanol (v/v), respectively to fully release compounds of interest from the vacuolar inclusions in which they are located and break some linkages between those phenolics with proteins, polysaccharides, and other components of pigmented rices to help obtain those compounds in their free states. This was followed by filtration through Whatman filter paper. Solvent was removed at 35 °C using a rotary evaporator and resultant residues were used for further study.

2.3. HPLC analysis

The profile of anthocyanins and ferulic acid, respectively from black rice and pigmented brown rice was determined using an HPLC system (Hitachi, Ltd., Tokyo, Japan) consisting of a L-4200 UV–Vis detector, D-2500 chromatogram integrator, and a L-6200 intelligent pump, equipped with a solvent delivery unit, an online vacuum membrane degasser, in conjunction with an LC organizer. Analytical separation of anthocyanin compounds was carried out on a ERC-ODS-1161 column (100 mm × 6 mm), that of ferulic acid was done using a Nova-pack C 18 column (150 × 4.6 mm, i.d. 5 μm). The samples were introduced onto the column via a nonmetal injection valve with a peak Sample of 10 μL volume and elution under isocratic conditions was performed with 2.5% formic acid in 75% aqueous methanol as mobile phase. The solvent flow rate was fixed at 0.5 mL/min and the chromatogram was recorded at 530 nm and 280 nm, respectively for black and pigmented brown rices.



Scheme 1.

2.4. Separation of anthocyanins, ferulic acid and tocopherols

2.4.1. Anthocyanins from black rice

Crude extract of black rice was purified using the method of Hu et al. (2003) with a slight modification, by means, filtration through a Sephadex G-15 column using increasing methanol in water, followed by gel filtration through a Bio-Gel P-2 column with aqueous acetic acid, pH 2.5 as mobile phase. Anthocyanin-rich fractions were identified and concentrated under vacuum prior to their purification. The isolated anthocyanins were further purified using a Sephadex LH-20 column with increasing methanol in water (up to 50% v/v).

2.4.2. Ferulic acid from pigmented brown rice

The extract of pigmented brown rice was primarily separated using a silica gel column with hexane/ethylacetate (4:1, v/v), followed by 100% ethylacetate. The ethylacetate fraction was further purified by a Sephadex LH-20 column with increasing methanol in water.

2.4.3. Tocols from both pigmented rices

Crude fatty matters from pigmented rices were extracted from rice flours using hexane under shaking (125 rpm) at 30 °C overnight, followed by the removal of the extracting solvent under vacuum. Extracted oils (14.98 g for black rice and 7.55 g for pigmented brown rice) were poured into an Erlen-meyer flask with 20 mg of BHA to avoid the oxidation of oil, then 50 mL of 80% ethanolic KOH were added to saponify oil.

After 10 min of saponification, 10 mL of absolute alcohol were added while saponification was being done under reflux. After saponification, the solution was immediately cooled in ice, then 100 mL of hexane were added to the mixture under shaking. The saponified solution was transferred into a separating funnel where the lower aqueous layer was discarded. The remained upper layer containing unsaponifiables were washed twice with distilled water, followed by the removal of the lower aqueous phase.

The unsaponifiable fraction was concentrated under vacuum and later used for further identification. The resulting unsaponifiable fractions (247.4 mg for black rice and 59.5 mg for pigmented rice) were kept at 0 °C overnight to remove sterols and unsaponified triglycerides as precipitates. The hexane-soluble fraction was concentrated using rotary evaporator at 30 °C prior to the separation of individual tocols. Various tocols were eluted on a silica gel 60 column with the use of sequential concentrations of diethyl ether (from 5% to 30% v/v) in hexane as previously reported (Qureshi, Salser, Parmar, & Emerson, 2001).

2.5. Identification of anthocyanins, ferulic acid, and tocols

To identify isolated compounds, ^{1}H, ^{13}C, and ^{1}H–^{1}H correlation spectroscopy (COSY) were measured using CD_3OD and TMS as a solvent and an internal standard, respectively. Mass analysis was performed with a MALDI Mass spectrometer (Shimadzu Corp., Japan) using electrospray ionization in positive (for anthocyanins and tocols) and negative (for ferulic acid) ion modes and the identification of isolated compounds was done by comparing obtained data to those found in the literature. Tocols were exclusively identified from their UV–Vis λ_{max} and mass spectral characteristics.

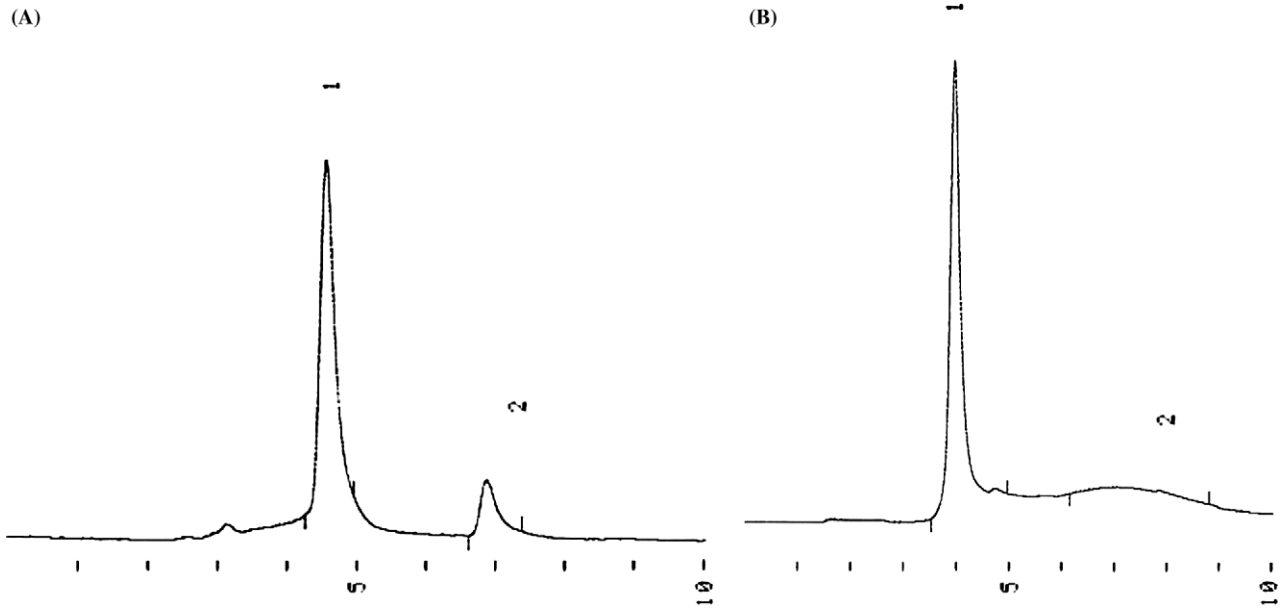
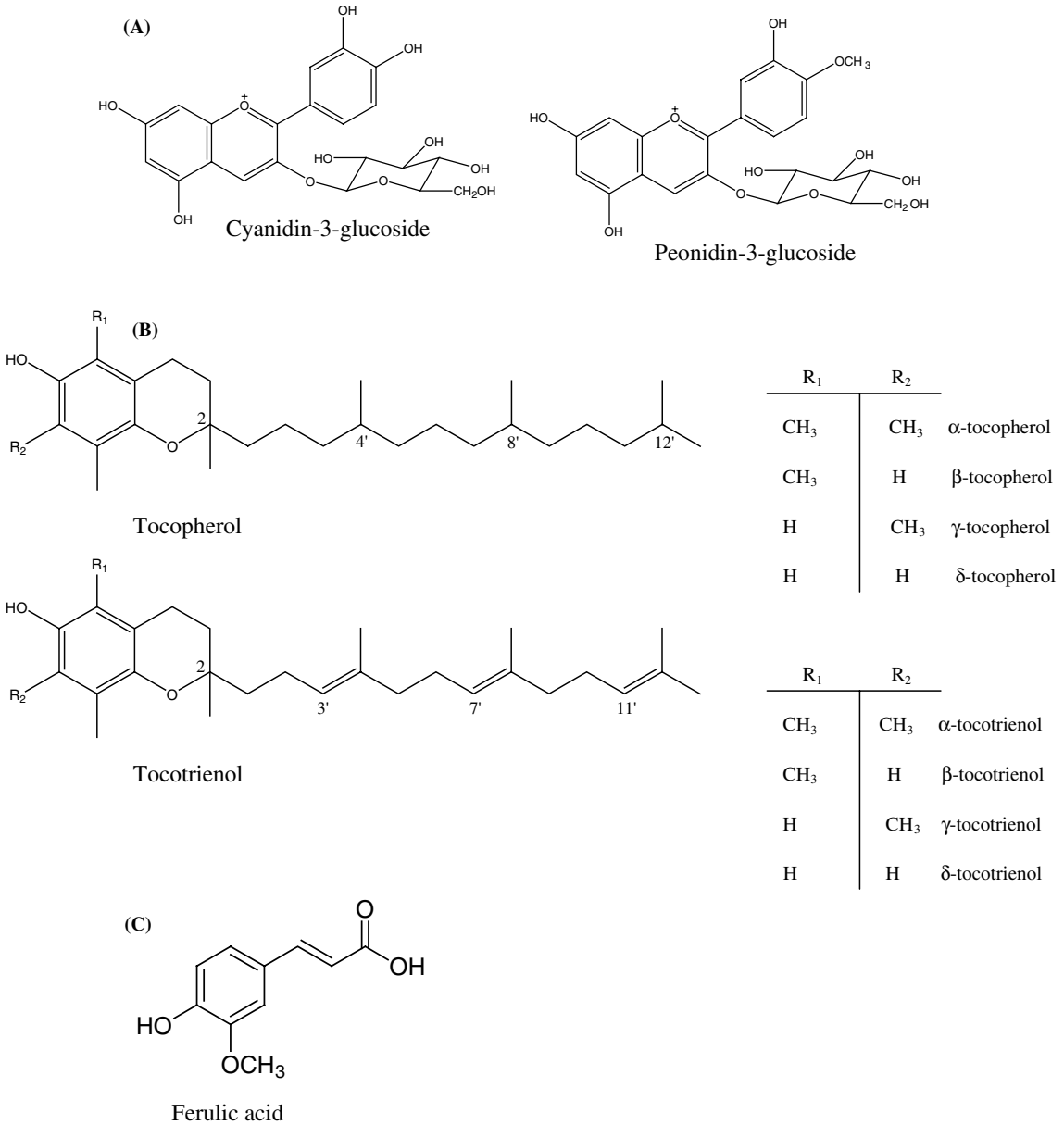


Fig. 1. HPLC-UV–Vis profile of the crude extracts of black (A) and pigmented brown (B) rices. The chromatogram of (A) was recorded at 530 nm and that of (B) at 280 nm columns: ERC-ODS for A and Nova pack C-18 for B; mobile phase: 2.5% formic acid in 75% aqueous MeOH; flow rate: 0.5 mL/min; detection: VIS-530 nm for A and UV-280 nm for B; injection volume: 10 μL. The retention times of (A): peak 1, cyanidin-3-glucoside and peak 2, peonidin-3-glucoside are, respectively 4.6 and 6.9 min; (B): peak 1, ferulic acid and peak 2, attributed to tocols mixture are 3.9 and 7 min, respectively.

2.6. Aldose reductase inhibition (ARI) assay

AR activity was assayed according to the method of Hayman and Kinoshita (1965) as modified by Fujita, Funako, and Hayashi (2004). The crude porcine aldose reductase used in this work was supplied by a slaughterhouse located in Sakai, Osaka, Japan. The extraction of the crude porcine AR was done according to the method described by Fujita et al. (2004) from the supernatant fraction obtained by homogenizing 48 lenses (20 g) with 100 ml of 0.1 M potassium phosphate buffer (pH 7.09 at 5 °C). The homogenate was centrifuged at 10,000*g* for 20 min (at 4 °C) and the supernatant (crude AR enzyme solution) was collected without any purification for screening and stored at −20 °C until use. The human recombinant AR was supplied by Wako chemicals, use with the purity above 95% (enzyme 1 μg; SDS–PAGE and silver strains). The assay mixture (1 mL) containing 50 μM sodium phosphate buffer (pH 6.2), 10 mM DL-glyceraldehyde, and 150 μM NADPH. Ten microliters of various concentrations of test compounds were added to the assay mixture and preincubated at 37 °C during 10 min and the enzymatic reaction was initiated by adding 5 μL of enzyme solution. The incubation was performed during 20 min at the same temperature. The decay of absorbance (due to the NADPH oxidation) was measured at 340 nm using a UV-160 A Shimadzu spectrophotometer. The percent inhibition of test compounds was calculated by substracting the AR activity



R_1	R_2	
CH_3	CH_3	α-tocopherol
CH_3	H	β-tocopherol
H	CH_3	γ-tocopherol
H	H	δ-tocopherol

R_1	R_2	
CH_3	CH_3	α-tocotrienol
CH_3	H	β-tocotrienol
H	CH_3	γ-tocotrienol
H	H	δ-tocotrienol

Fig. 2. Structures of isolated compounds: (A) anthocyanins from black rice; (B) tocols from both pigmented rices, and (C) ferulic acid from pigmented brown rice.

measured in the absence of inhibitors to that with assessed with the use of inhibitors. The concentration of each test compound giving 50% inhibition (IC_{50}) was then calculated by using the GraFit software.

2.7. Statistical analysis

All experiments were conducted in triplicate and statistical analysis was done using the Statistical Package for Social Science (SPSS) programme.

3. Results and discussion

3.1. Identification of phenolic compounds isolated from black and pigmented brown rices

The HPLC chromatogram recorded at 530 nm from the crude extract of black rice showed two peaks related to two anthocyanins at the retention time 4.56 min (peak 1) and 6.88 min (peak 2) as shown in Fig. 1A. The ^{1}H and ^{13}C NMR of anthocyanins isolated from black rice are shown in Figs. 3 and 4 and their related processed NMR data are listed below. Structures of isolated compounds from both pigmented rices are shown in Fig. 2.

Data for peonidin-3-glucoside. Dark red, Vis λ_{max} (MeOH, nm), 519; ^{1}H NMR (CD_3OD, 25 °C); *Aglycon*: H_{-4} 8.072 (s), H_{-6} 6.76 (s), H_{-8} 6.99 (s), $H_{-2'}$ 8.22 (d), $H_{-5'}$. 7.12 (d), $H_{-6'}$, 8.30 (dd), OCH_3: 3.96 (s); *Glucose moiety*: H_{-1} 5.41 (s), H_{-2} 3.49 (m), H_{-3} 3.43 (t), H_{-4} 3.28 (t), H_{-5} 3.53 (m), H_{-6a} 3.54 (m), H_{-6b} 3.77 (m) and ^{13}C NMR (CD_3OD, 25 °C); *Aglycon*: C_{-2} 161.8, C_{-3} 144.3, C_{-4} 135.9, C_{-5} 157.8, C_{-6} 102.5, C_{-7} 168.8, C_{-8} 94.6, C_{-9} 156.1, C_{-10} 112.3, $C_{-1'}$ 119.7, $C_{-2'}$ 114.5, $C_{-3'}$ 148.3, $C_{-4'}$ 155.1, $C_{-5'}$ 116.8, $C_{-6'}$ 127.9, OCH_3: 56.2; *Glucose moiety*: C_{-1} 102.6, C_{-2} 73.4, C_{-3} 76.7, C_{-4} 69.8, C_{-5} 77.9, C_{-6} 61.0 ^{1}H NMR showed a phenyl ring system (8.22 ppm [2H, d], 7.12 ppm [2H, d] and 8.30 ppm [2H, dd]), three aromatic or olefinic protons (8.072, 6.76, and 6.99 ppm) that appeared as singlets and a methoxy group at 3.96 ppm as a singlet. An anomeric proton was observed at 5.41 ppm, d, $J = 7.5$ Hz followed by a multiplet between 3 and 4 ppm related to a glucose moiety. The ^{13}C NMR data showed 15 aromatic or olefinic carbons indicative of an anthocyanidin aglycon and one methoxyl carbon (56.2 ppm), 6 carbons indicative of glucose with the anomeric carbon at 102.6 ppm and 5 other carbons of the sugar moiety between 60 and 80 ppm.

Data for cyanidin-3-glucoside. Light red, Vis λ_{max} (MeOH, nm), 519; ^{1}H NMR (CD_3OD, 25 °C); *Aglycon*: H_{-4} 8.85 (s), H_{-6} 6.66 (d), H_{-8} 6.86 (d), $H_{-2'}$, 7.97 (d), $H_{-5'}$,7.0 (d), $H_{-6'}$, 8.19 (dd); *Glucose moiety*: H_{-1} 5.32 (s), H_{-2} 3.49 (m), H_{-3} 3.37 (t), H_{-4} 3.23 (t), H_{-5} 3.49 (m), H_{-6a} 3.49 (m), H_{-6b} 3.70 (m) and ^{13}C NMR (CD_3OD, 25 °C); *Aglycon*: C_{-2} 161.9, C_{-3} 144.4, C_{-4} 135.1, C_{-5} 157.9, C_{-6} 102.5, C_{-7} 168.7, C_{-8} 94.3, C_{-9} 156.2, C_{-10} 111.1, $C_{-1'}$ 119.7, $C_{-2'}$ 117.7, $C_{-3'}$ 146.3, $C_{-4'}$ 154.6, $C_{-5'}$ 116.9, $C_{-6'}$ 127.1. *Glucose moiety*: C_{-1} 102.3, C_{-2} 73.3, C_{-3} 76.7, C_{-4} 69.9, C_{-5} 77.9, C_{-6} 61.0.

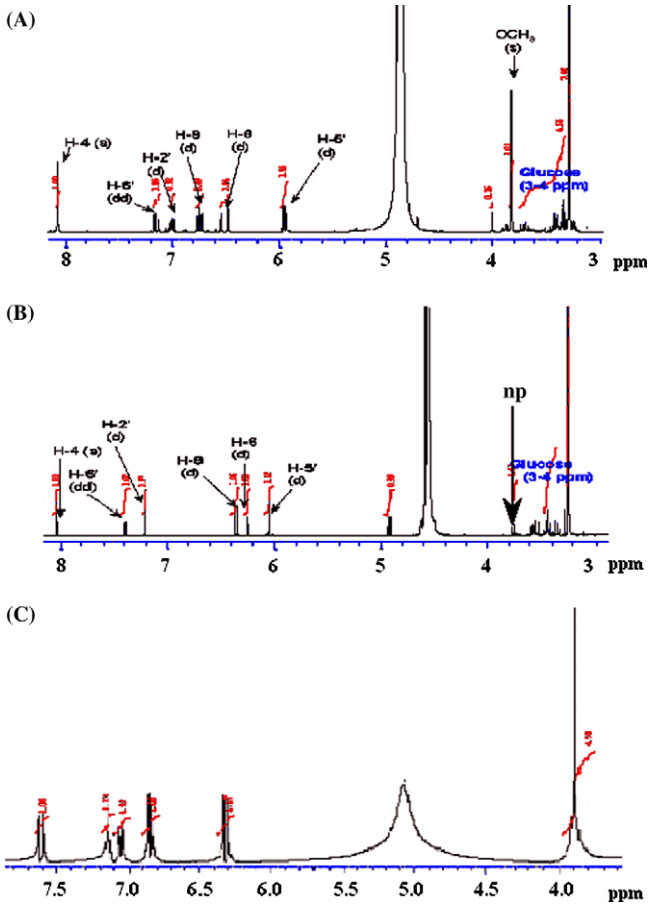


Fig. 3. ^{1}H NMR of isolated anthocyanins from black rice: peonidin-3-glucoside (A), cyanidin-3-glucoside (B), and ferulic acid from pigmented brown rice (C). In all ^{1}H NMR, chemical shifts (δ 6–8.5 ppm) are related to the aromatic ring. The absence of methyl group peak (δ 3.96 ppm) in (B) is clearly visible as compared to (A), np: no peak.

^{1}H NMR showed a phenyl ring system (7.97 ppm [2H, d], 7.0 ppm [2H, d] and 8.19 ppm [2H, dd]), three aromatic or olefinic protons [8.85 (s), 6.66 (d), and 6.86 (d) ppm]. An anomeric proton was observed at 5.32 ppm, d, $J = 7.5$ Hz followed by a multiplet between 3 and 4 ppm related to a glucose moiety. The ^{13}C NMR data showed 15 aromatic or olefinic carbons indicative of an anthocyanidin aglycon, 6 carbons indicative of glucose with the anomeric carbon at 102.3 ppm and 5 other carbons of the sugar moiety between 60 and 80 ppm. Peak assignments were done with the aid of H–H COSY as mentioned before. The anomeric cross-peaks of the two anthocyanins at 5.41/102.6 ppm and 5.32/102.3 ppm, respectively for peonidin-3-glucoside and cyanidin-3-glucoside indicated the presence of one monosaccharides into their respective structure and those values were in accordance with a β-glucopyranosyl moiety. The ^{1}H and ^{13}C NMR spectra of the two isolated anthocyanins showed many similarities with their corresponding spectra as shown in Figs. 3(A and B) and 4(A and B). In contrast, both the ^{1}H and ^{13}C NMR spectra of compound A showed

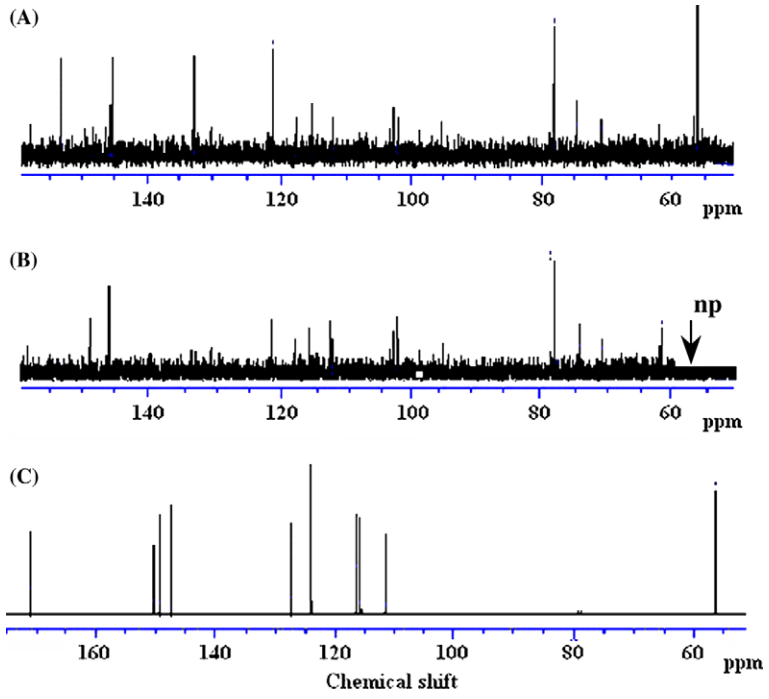


Fig. 4. ^{13}C NMR of isolated anthocyanins from black rice: peonidin-3-glucoside (A), cyanidin-3-glucoside (B), and ferulic acid from pigmented brown rice (C). The absence of methyl group in (B) is clearly visible by comparing (A) and (B). In all ^{13}C NMR, shifts comprised in the region (δ 100–170 ppm) are related to the benzenic ring. The absence of shift at δ: 56.2 ppm in (B) attests the absence of methoxy group in that structure as compared to compound (A), np: no peak.

Table 1
HPLC characteristics and relative phenolic content in black and pigmented brown rices

Compound	RT (min)	Relative amount (%)
Black rice		
Cy-3-glc	4.6	85.0
Pn-3-glc	6.9	15.0
Pigmented brown rice		
Ferulic acid	3.9	85.7
Tocols mixture	7.0	14.3

RT, retention time; Cy-3-glc, cyanidin-3-glucoside; Pn-3-glc, peonidin-3-glucoside.

Table 2
MS spectral data of phenolics from black and pigmented brown rices

Compound	M	*m*/*z*
Peonidin-3-glucoside	463.1	301.1 (M−162:M−Glc)
Cyanidin-3-glucoside	449.0	287.0 (M−162:M−Glc)
Ferulic acid	193.7	147.9 (M−45:M−COOH)
		96.0 (M−97:M−COOH + OCH_3 + OH + $3H^+$)

Glc, glucose; M−Glc, molecular ion minus glucose; M−COOH, molecular ion minus carboxylic group.

characteristic peaks at 3.96/56.2 ppm related to the presence of a methoxy group into that molecule, which were absent in both ^{1}H and ^{13}C NMR spectra of compound B. The MALDI-MS data of isolated anthocyanins are summarized in Table 2 and their fragmentation patterns are shown in Fig. 5, both matched previously published data (Aoki, Kuze, & Kato, 2002). The fragment ion peaks corresponding to two anthocyanins, peonidin with *m*/*z* at 301 and cyanidin with *m*/*z* at 287 were detected in their respective mass spectrogram and the difference of 14 between both values attests the presence of a methoxy group in compound A in spite of a hydroxyl group in B. The neutral loss of 162 (*m*/*z*) from their respective molecular ions indicates the existence of a glucose moiety in the structures of both identified anthocyanins. Mass spectral characteristics of isolated anthocyanins were consistent with those found in the literature (Aoki et al., 2002). On the basis of the above considerations, the two anthocyanins related to two peaks found in the HPLC chromatogram were finally identified as peonidin-3-glucoside and cyanidin-3-glucoside. These anthocyanins elucidated from black rice (*Oryza sativa* L. *japonica*) were also identified in black rice belonging to *Oryza sativa* L. *indica* (Hu et al., 2003), thus, in spite of the difference in the rice cultivar, the color of rice grain seems to be more determinant to the molecular structure of phenolic compounds it contains.

From the HPLC chromatogram of the crude extract of pigmented brown rice recorded at 280 nm, two peaks were detected at the retention times 3.93 min (peak 1) and 7.05 min (peak 2) as shown in Fig. 1B, which were attributed to ferulic acid and tocol mixtures, respectively. The ^{1}H and ^{13}C NMR of this compound are shown in Figs. 3 and 4, respectively and related chemical shifts of isolated ferulic acid are listed below.

Data for ferulic acid. Yellowish, UV λ_{max} (EtOH, nm), 242, 293 (sh), 323; ^{1}H NMR (CD_3OD, 25°C); *Benzenic*

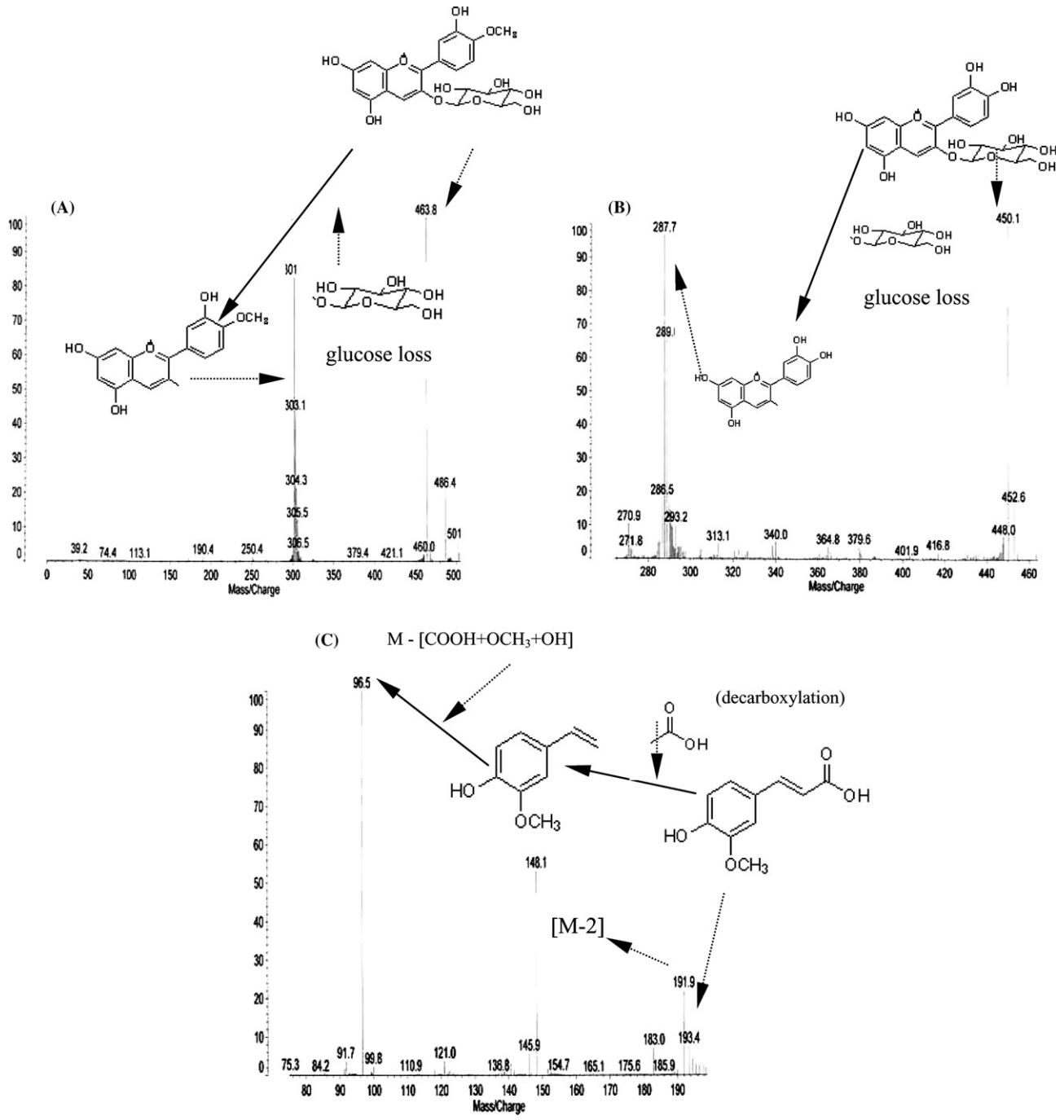


Fig. 5. MALDI-MS fragmentation patterns of isolated compounds from both pigmented rices. Panels (A)–(C) show the MALDI-MS of peonidin-3-glucoside, cyanidin-3-glucoside, and ferulic acid, respectively. The MS spectrograms were obtained with positive electrospray ionization mode for (A) and (B), with the negative mode for (C). The insets in each panel show the structure of each compound, their respective molecular and fragment ions.

cycle: H_{-2} 7.59 (s), H_{-4} 6.0 (s), H_{-5} 7.6 (d), H_{-6} 7.14 (d), H_{-7} (OCH_3) 3.89 (s); *Alkyl chain:* H_{-8} 6.34 (d), H_{-9} 6.29 (d) ^{13}C NMR (CD_3OD, 25 °C); *Benzenic cycle*: C_{-1} 127.6, C_{-2} 111.6, C_{-3} 150.1, C_{-4} 146.9, C_{-5} 116.4, C_{-6} 123.8, OCH_3 56.3; *Alkyl chain*: C_{-8} 149.1, C_{-9} 115.6, C_{-10} 171.1.

^1H NMR showed a benzenic with chemical shifts between 6 and 8 ppm, a methoxyl group at 3.89 ppm as a singlet. The *trans* nature of the double bond (of the alkyl chain) for ferulic acid was evident from both vinyl protons ($H_{-1'}$, and $H_{2'}$) to the aromatic H-2 and H-6 signals as well as the distinct chemical shifts at 6.34 and 6.29 ppm and vicinal coupling constant (15.9 Hz) of these vinyl protons in comparison to those analogous *cis*-isomers. ^{13}C NMR showed 6 aromatic carbons and a methoxyl group, with two olefinic carbons at 149.1 and 115.6 and a carboxyl group at 171.1 ppm. The mass spectral data of ferulic acid isolated from pigmented brown rice

are shown in Table 2 and its fragmentation pattern is illustrated in Fig. 5, both were consistent with the literature data (Baderschneider & Winterhalter, 2001), by means, ferulic acid with a molecular ion at 193 (M−H), fragment ions at 147.9 (related to a loss of the carboxylic acid group, M−45), and at 96 (related to a loss of carboxylic, methoxy, and hydroxyl groups and 3 hydrogens, M−97).

Data of tocols. The identification of isolated tocols was done from their UV and Mass spectral characteristics and the respective amount of tocol fractions obtained from black rice was: α-tocopherol (33 mg) with (UV λ_{max}: 294 nm and *m*/*z*: 431); γ-tocopherol (21 mg) with (UV λ_{max}: 298 nm and *m*/*z*: 417); β-tocotrienol (4 mg) with (UV λ_{max}: 296 nm and *m*/*z*: 411); δ-tocotrienol (19 mg) with (UV λ_{max}: 292 nm and *m*/*z*: 399) and the following composition from pigmented brown rice: α-tocopherol (11 mg); γ-tocopherol (5 mg); α-tocotrienol (7 mg) with (UV λ_{max}: 292 nm and *m*/*z*: 426); δ-tocotrienol (6 mg). UV and mass characteristics of tocols were consistent with published data (Cahoon et al., 2003). The above amounts of various isolated tocols are related to the described method of isolation used in the present study. The difference in the side chains and methyl substitution on the chromanol ring of tocols in both cultivars might be due to the difference in enzymes involved in the methylation. Thus, as tocols are well known to possess potent antioxidant activity, their contribution to the global antioxidant potency of the crude extracts of both studied pigmented rices might be considered.

Relative quantitative anthocyanin content in pigmented rices. The relative proportion of isolated phenolics from black and pigmented brown rices are shown in Table 1 and was estimated by extracting three times a sample which was analyzed by HPLC method. Cyanidin-3-glucoside predominated in black rice with 85% against 15% for peonidin-3-glucoside. In pigmented brown rice, ferulic acid had the higher relative amount (85.7%) than tocol mixtures (14.3%).

3.2. Inhibition effect of isolated compounds on AR

ARI test was done using the isolated compounds (cyanidin-3-glucoside, peonidin-3-glucoside, ferulic acid, and α-tocopherol) using quercetin as a control. As shown in Figs. 6 and 7, all isolated compounds showed AR inhibitory abilities with various degrees of effectiveness. The inhibitory effect of the isolated compounds increased with the increasing in the concentration. Among all isolated compounds, cyanidin-3-glucoside showed the highest inhibitory activity against AR. The isolated compounds exhibited AR inhibitory activity with IC_{50} values ranged from 8.7 to 27.5 μg/mL against human recombinant AR with the respective values (in μg/mL) for each isolated compounds: cyanidin-3-glucoside (8.7), peonidin-3-glucoside (13.7), ferulic acid (11.9), α-tocopherol (27.5) and quercetin, the control (11.4). Interestingly, cyanidin-3-glucoside showed lower IC_{50} value than that of quercetin (the ARI higher than the control), while ferulic acid had similar IC_{50} value to that of the well-known AR inhibitor used in this assay.

α-Tocopherol, which among all isolated compounds, showed the highest DPPH free radical scavenging activity (data not shown) had the lowest efficiency to inhibit the key enzyme of the polyol pathway suggesting that this lipophilic compound could not dissolve in the aqueous buffer system used in this work, hence, there was no significant contact between studied enzyme and this phytochemical. Ruiz et al. (2004) studying the crystallographic structure of the aldose reductase-IDD552 complex, concluded that the inhibition of that enzyme was done by binding with the active site, making hydrogen-bond interactions between the H atom of the functional group of the inhibitor and the His 110 and Tyr 48 side-chains of the O atom, with further electrostatic interactions with $NADP^+$. Structurally, the difference in the inhibitory potency of isolated compounds can be explained by their hydrogen-donating ability, which is higher in ferulic acid (being a carboxylic acid) than other isolated compounds. The difference of hydroxyl groups linked to the chromane ring between cyanidin-3-glucoside (4) and peonidin-3-glucoside (3) may explain the difference in the aldose reductase inhibitory effect of both isolated anthocyanins, with the higher inhib-

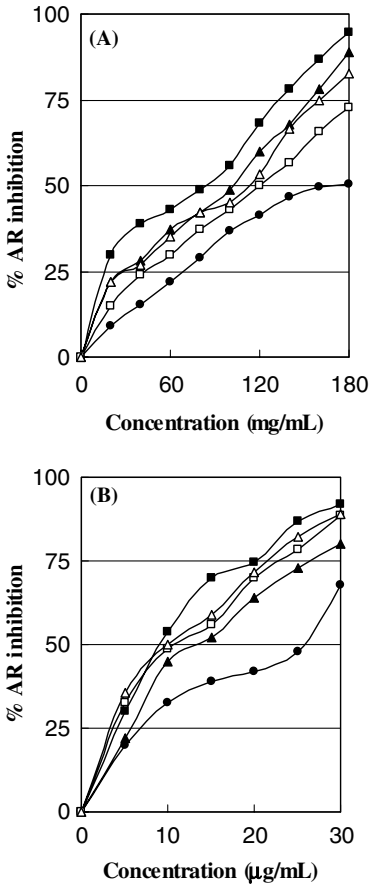


Fig. 6. Inhibition of (A) crude porcine and (B) human recombinant aldose reductase by isolated compounds: (▲) peonidin-3-glucoside; (■) cyanidin-3-glucoside; (●) α-tocopherol; (□) ferulic acid; (△) quercetin.

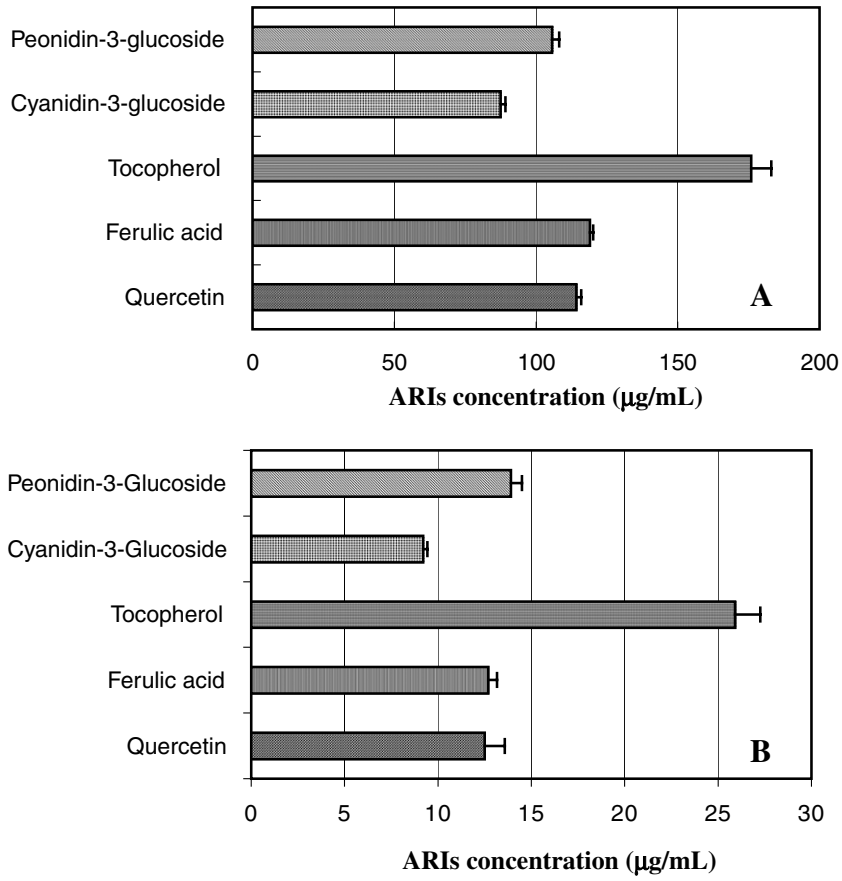


Fig. 7. Half-inhibition (IC_{50}) of isolated compounds on crude porcine AR (A) and human recombinant AR (B) activity. The values represent the means ± SD. Horizontal bars indicate the standard deviation.

itory activity for cyanidin-3-glucoside. Tocopherol, which has a long alkyl-chain side linked to the aromatic ring (having a hydroxyl group with or without methyl groups) have their structure stabilized both by the resonance of the π-electrons system of the chromanol ring and the inductive effect due to the methyl group and the alkyl chain side might have an important hydrogen-donating ability involved to inhibit the studied enzyme. Suryanarayana, Kumar, Saraswat, Petrash, and Bhanuprakash (2004) reported that tannoids isolated from *Emblica officinalis* possess an inhibition efficiency slightly higher than that of quercetin and the IC_{50} values obtained were 6.1 and 9.8 μg/mL for *Emblica* tannoids and 9.2 and 13.5 μg/mL for quercetin, respectively on rat lens AR and human recombinant AR. The latter value related to the same enzyme studied in our work is higher than the IC_{50} value obtained in the present study (11.4 μg/mL). The concentrations range in which a compound is appointed as an AR inhibitor is very wide in the literature, from 0.0013 to 1800 μM (Angel de la Fuente & Manzanaro, 2003). Interestingly, the IC_{50} values of all isolated compounds (converted to μM) are included in the defined range suggesting that all those compounds are potent AR inhibitors. Structurally, distinct compounds such as flavonoids, benzopyrans, spirohydantoins, alkaloids, nonsteroidal anti-inflammatory agents, and quinines have all been shown to inhibit the studied enzyme with various degrees of efficacy and specificity.

Sorbinil, statil, tolrestat, alrestatin, epalrestat, and ALO1576 are some of the well-studied inhibitors that have also been clinically tested. However, most of synthetically available ARIs have not clinically proved their effectiveness and some have demonstrated side effects. In contrast, most of dietary components (including compounds isolated from pigmented rices) are often free from adverse effects and their continuous screening can lead to the discovery of more efficient and safer ARIs against diabetic complications. The study of kinetic parameters and the type of inhibition mechanism of the AR were not taken into consideration in the present experiment. Also, the synergistic effect and the acidic constant (to establish the relevant protonation state) of the isolated phenolics, if evaluated, could be for some contribution on understanding the real mechanism of action of isolated phenolic compounds. Finally, the study of the selectivity of isolated compounds on aldehyde reductase could help to elucidate whether or not they have some deleterious side effects on human body.

4. Conclusion

Our present experimental results revealed the presence of phenolic compounds in both pigmented rices, in partic-

ular, cyanidin-3-glucoside and peonidin-3-glucoside in black rice, and ferulic acid in pigmented brown rice, with further tocols in both rices. Furthermore, all isolated compounds demonstrated AR inhibitory activities. These data suggest that black rice and pigmented brown rice possess marked health benefits in preventing diabetic complications by inhibiting the key enzyme (aldose reductase) involved in their development.

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