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Assessment of polyphenols in purple and red rice bran: Phenolic profiles, antioxidant activities, and mechanism of inhibition against amylolytic enzymes

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ABSTRACT

Pigmented Thai rice varieties, including purple (Riceberry) and red (Hommali), are gaining popularity due to their health benefits as a source of polyphenols that may exert a hypoglycemic effect through specific inhibition of amylolytic enzymes. This study determined the free phenolic extract from purple rice bran (PFE) to exhibit notably greater content of phytochemical compounds than did phenolic extracts from red rice bran, whether free (RFE) or bound fractions. This phytochemical content correlated with increased antioxidant activity and strong inhibition capacity against amylolytic enzymes, suppressing the conversion of carbohydrates into glucose. Several polyphenol compounds were identified in pigmented rice bran extracts, including benzoic acid, chlorogenic acid, ferulic acid, apigenin, and rutin; among these, flavonoids exhibited greater effect on inhibition capacity. Mechanistically, PFE was found to act as a competitive and uncompetitive inhibitor of α-amylase and α-glucosidase respectively, while RFE showed respective uncompetitive and competitive inhibitory modes.

1. Introduction

Diabetes is one of the most significant lethal diseases worldwide that is associated with high levels of glucose in the bloodstream. According to the International Diabetes Foundation, diabetes-related deaths worldwide surpass 4 million annually, with the expectation that this global figure will surge to 700 million by the year 2045 (Kumar et al., 2021). Diabetes can lead to numerous complications including stroke, cardiovascular disease, and cancer (Maida et al., 2022; Tomic et al., 2022). Accordingly, it is imperative to apply preventative measures against risk factors contributing to diabetes. The significant elevation in blood glucose levels that leads to diabetes is influenced by complex genetic and environmental factors. Postprandial hyperglycemia has emerged as a primary instigator in individuals with type 2 diabetes, primarily influenced by dietary patterns, in which starch plays a predominant role. In those with type 2 diabetes, rapid conversion of starch into glucose during the digestive process results in a surge in blood glucose levels after ingestion (Nag and Majumder, 2023). A number of medications that effectively regulate high blood glucose levels are available for diabetes management; one such is acarbose, which works by inhibiting α-glucosidases in the small intestine, thereby slowing down the digestion and absorption of complex carbohydrates, and attenuating the post-meal increase in blood glucose levels. However, the side effects of acarbose result in decreased patient adherence (Uuh Narvaez and Segura Campos, 2022). Hence, further investigation into techniques that impede the conversion of starch into glucose is warranted, including starch modification through complexation with other compounds aimed at reinforcing its structure and thereby slowing its digestion.

Rice, being a globally consumed staple food, is a crucial contributor to elevated postprandial blood glucose levels. Pigmented rice varieties, including black, purple, red, and brown rice, have gained considerable popularity as functional food ingredients owing to their well-known health benefits, including improvements in metabolism, anti-hyperglycemic effects, and antiproliferative activity (Bhat et al., 2020; Ghasemzadeh et al., 2018; Zhang et al., 2020). They also contain a substantial quantity of dietary fiber, promoting a sense of fullness that reduces calorie consumption, leading to effective weight management (Das et al., 2023). Comprehending the nutritional benefits and health advantages of rice is crucial for motivating individuals to incorporate it into their daily dietary habits. In addition, there is a growing interest in assessing varieties of pigmented Thai rice for the potentially beneficial constituents such as lipids and bioactive compounds (Bunmusik et al., 2023; Siripattanakulkajorn et al., 2024). Pigmented rice is also potentially rich in diverse biological activities (Bhat et al., 2020; Munkong

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et al., 2023; Sangma and Parameshwari, 2023; Yamuangmorn & Prom-u-Thai, 2021), which advantageous properties are most probably linked to the wide variety of phenolic compounds derived from the bran layer. It has been suggested that polyphenols can exert a hypoglycemic effect during digestion through forming intricate bonds with amylolytic enzymes (Sun et al., 2019). In addition, research has indicated that polyphenols and starch can form inclusion and non-inclusion complexes (Ngo et al., 2022). Ultimately, the presence of polyphenols disrupts the activity of α-amylase, hindering hydrolysis of α-1,4-glucan polysaccharides (e.g., starch) and delaying glucose release (Aleixandre et al., 2022; Giuberti et al., 2020).

Interestingly, polyphenols include not only compounds soluble in polar aqueous or organic solvents, but a substantial portion of non-extractable compounds that have been consistently undervalued. This subset of phenolic compounds are covalently bound to plant cell walls and can be released under alkaline or acidic conditions as well as through enzymatic hydrolysis (Wang et al., 2020). Recent studies have investigated the distribution of phytochemical components in pigmented rice. In brown rice bran, phenolic compounds that have been identified as present in bound form include *p-coumaric* acid, ferulic acid, methyl ferulate, syringic acid, and gallic acid, (Ti et al., 2014; Ye et al., 2022). Meanwhile, free phenolics extracted from black or purple rice bran contain notable amounts of anthocyanins (Chen et al., 2022; Shao et al., 2014; Wu et al., 2018). In red rice bran, ferulic acid and *p-coumaric* acid are more abundant in bound form, whereas syringic acid, quercetin, and catechin exist predominantly in the free form (Ghasemzadeh et al., 2018). The diversity of these findings can be attributed to rice variety differences and the different methods of extraction used. It is reasonable to hypothesize that pigmented rice bran from other varieties may yield yet different results reflecting their unique characteristics. Thailand, as the second-largest rice exporter in Southeast Asia, produces many varieties of rice, according to Promkhambut et al. (2023), the pigmented purple and red rice that are popularly consumed have great potential in terms of nutritional characteristics and bioactive compounds.

Conducting a comparative analysis of the higher phytochemical content in purple and red rice bran would offer new insights into its potential health benefits. Additionally, the inhibitory effect of free and bound phenolic extracts from these rice varieties on amylolytic enzymes needs to be explored further. Therefore, this study aimed to characterize the phenolics in purple and red rice bran extracts in terms of their distribution between free and bound forms, their antioxidant activity, and their inhibitory capacity against α-amylase and α-glucosidase enzymes.

2. Materials and methods

2.1. Preparation of samples

The samples utilized comprised two varieties of pigmented rice, purple rice (Riceberry) and red rice (Hommali), obtained from the Chainat province in central Thailand. Whole grains of pigmented rice were milled using an NW 1000 Turbo laboratory polisher (Thongtrawee, Thailand) to separate the rice grain (endosperm) from the bran. The bran was then sieved with a 160 μm sieve, resulting in yields of 18.11% and 19.62% for purple and red rice bran, respectively. The samples were preserved at 4 °C for subsequent extraction processes.

2.2. Extraction of free phenolic compounds

The extraction method followed Ghasemzadeh et al. (2018), with some modifications. Purple and red rice bran (1 g) were extracted using 15% methanol acid solution (50 mL) for 30 min and centrifuged at 2500 rpm for 10 min. The supernatant was collected and concentrated at 42 °C using a Buchi Rotavapor (model R-300). Subsequently, 10 mL of 15% methanol acid solution was added, and the mixture was stored for further analysis. The samples were labeled as purple – free extract (PFE) and red – free extract (RFE).

2.3. Extraction of bound phenolic compound

The residue from free phenol extraction was further processed to obtain bound phenolics using the method of Pang et al. (2018) with modification. In brief, defatted samples were hydrolyzed under alkaline conditions (40 mL, 2 M NaOH) for 30 minutes at 60 °C in a sonicator bath (model WUC-D10H, Wised, Daihan Scientific, Korea). The solutions were acidified with 4 M HCl to pH 1.5–2.5 and transferred to a separation funnel, where they were extracted twice with ethyl acetate. The supernatant was combined and evaporated using a rotary evaporator to yield the extracted bound phenolics, which residues were dissolved in distilled water and labeled as purple – bound extract (PBE) and red – bound extract (RBE) respectively.

2.4. Evaluation of total phenolic content (TPC)

Quantification of TPC involved the establishment of a standard curve using gallic acid. For the assay, 2 mL of Folin-Ciocalteu reagent was mixed with the samples and incubated at 30 °C for 4 min. Next, 80 μL of 1 M Na_2CO_3 was added, followed by a 30-min incubation period in the dark. Subsequently, the absorbance of all samples was measured against the blank at 760 nm using a UV–vis spectrophotometer (Shimadzu UV-1800, Japan). TPC was expressed as milligrams of gallic acid equivalents per gram of dry sample (mg GAE/g), according to Madaan et al. (2011).

2.5. Total flavonoid content (TFC)

TFC was determined using a quercetin standard curve and expressed as mg quercetin equivalents per gram of sample (mg QE/g sample). The measurement method followed that of Norhazlini et al. (2021), with some modifications. Briefly, 100 μL of either quercetin solution or sample was reacted with 60 μL of 5% $NaNO_3$ for 5 min. Subsequently, 50 μL of a 10% $AlCl_3$ solution was added to each sample and incubated for 6 min. Lastly, 30 μL of 1 M NaOH was added to each well of the plate and the absorbance was read at 510 nm using a microplate reader (Multimode Plate Reader, PerkinElmer, Inc., Massachusetts, USA).

2.6. Total anthocyanin content (TAC)

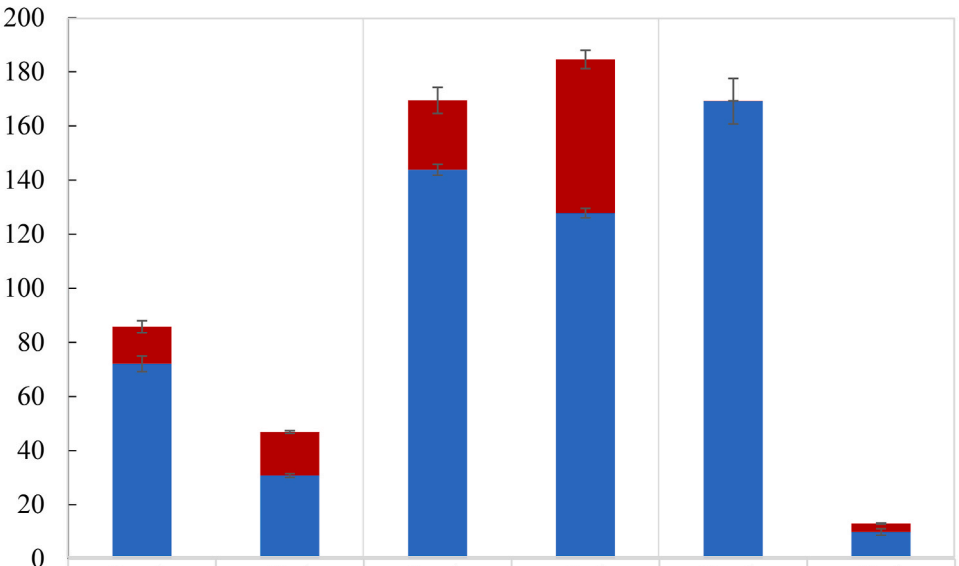
TAC was determined by diluting each sample in two different buffers (potassium chloride buffer pH 1.0 and sodium acetate buffer pH 4.5) to a final volume of 3 mL. Cyanidin-3-glucoside (Cy-3-GE) was used as a standard. The absorbance of the samples was measured against a blank (distilled water) using a UV–vis spectrophotometer at 520 nm and 700 nm (Pang et al., 2018). The TAC (expressed as mg of Cy-3-GE per gram of sample on a dry weight basis) was then calculated using the following formula:

$$A \times MW \times DF \times 1000/(\varepsilon \times L)$$

where *A is the absorbance, MW is the molecular weight of cyanidin-3-glucoside, DF is the dilution factor, ε is the molar absorbance of cyanidin-3-glucoside, L is the cell path length, and 1000 is the conversion factor from milliliters to liters.*

2.7. Antioxidative activity (2,2-diphenyl-2-picrylhydrazyl)

Assessment of free radical scavenging activity, expressed as a percentage, was carried out using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as detailed by Bobo-García et al. (2015). Briefly, 50 μL of sample was combined with 180 μL of 0.1 mM DPPH in methanol within a 96-well microplate and incubated for 30 minutes. Subsequently, the absorbance was quantified at 515 nm.



	TPC (mg GAE/g sample)		TFC (mg QE/ g sample)		TAC (mg Cy-3-GE/g sample)	
Bound	13.71[b]	16.08[b]	25.67 [b]	56.76 [c]	0.07 [b]	3.14 [b]
Free	72.09[a]	30.78[c]	143.79[a]	127.81[d]	169.17 [a]	9.92 [c]

Fig. 1. Comparison of the distribution of free and bound phenolic compounds in TPC, TFC, and TAC. The data are reported as mean ± std (n = 3). The values denoted by different letters indicate significant differences ($p < 0.05$) among various samples for each parameter (TPC, TFC, and TAC).

2.8. Ferric reducing ability of plasma (FRAP) assay

Antioxidant activity was determeined using the FRAP method, in which 20 μl of sample extracts was combined with 200 μL of freshly prepared FRAP reagent comprising 10 mM TPTZ and 20 mM ferric chloride in 300 mM acetate buffer (pH 3.6). After a 30-min incubation at room temperature, the absorbance was measured at 595 nm, using distilled water as the blank. The results were visualized by plotting alongside the Trolox standard (Ti et al., 2014).

2.9. $ABTS^{\bullet+}$ radical cation-based assay

$ABTS^{\bullet+}$ was formulated by combining a 7 mM ABTS solution with 140 mM potassium persulfate and incubating it for 16 h in the dark at room temperature. The resultant solution was diluted with ethanol to achieve a final absorbance of 0.7 at 734 nm, indicating readiness for analysis. To determine antioxidant capacity, 10 μL of sample or Trolox (used as a standard) was reacted with 290 μL of diluted ABTS, followed by a 6-min incubation (Tan et al., 2023). The absorbance was measured at 734 nm with the trolox as a standard.

2.10. Inhibitory effect against α-amylase and inhibition mode

Sample extracts were tested for inhibition of α-amylase activity by combining 100 μL of pancreatic α-amylase solution (3 U/mL), prepared in phosphate buffer (20 mM, pH 6.9) with sodium chloride (6.7 mM), with 100 μL of sample extract or acarbose as a positive control (concentration ranging from 0.2 to 18 mg/mL). This mixture was incubated at 37 °C for 10 min in a shaker bath, then combined with 100 μL of 1% starch solution (as a substrate), followed by an additional 10 min of incubation. To stop the reaction, dinitrosalicylic acid color reagent was introduced and the samples were placed in boiling water for 10 min. After cooling to room temperature, the absorbance was measured at 540 nm Li et al. (2018) and the inhibition capacity was calculated using the following formula:

$$\text{Inhibition } (\%) = 1 - \frac{Abs\ sample - Abs\ blank}{Abs\ control} \times 100$$

where *Abs sample denotes sample or acarbose with enzyme, Abs blank is the same without enzyme, and Abs control is buffer with enzyme.*

Enzymatic inhibition kinetics were further investigated by varying starch solution concentration (0.1%, 0.25%, 0.5%, 1%, and 2% w/v) while maintaining a constant concentration of PFE or RFE (as the inhibitor) and enzyme units. The analytical procedure was as described above. The mode of the inhibitory action exerted by each sample extract on α-amylase was determined by visualization in a Lineweaver-Burk plot correlating 1/[substrate] (mg/mL) with 1/[V] (reaction rate).

2.11. Inhibitory effect against α-glucosidase and inhibition mode

The analysis of α-glucosidase inhibition was based on prior studies with minor modifications (Kazeem et al., 2013). Briefly, solutions containing a sample or acarbose as a positive control (0.2–18 mg/mL) were mixed with 100 μL of 0.1 units/mL enzyme solution in phosphate buffer (0.1 M, pH 6.8) for 10 min. Subsequently, 100 μL of 4-nitrophenyl α-D-glucopyranoside (10 mM) was introduced as a substrate. After a 20-min incubation, the reaction was halted by adding 2 mL of 0.1 M Na_2CO_3. The absorbance was measured at 405 nm and the inhibition rate was determined using the same formula used to assess α-amylase activity.

The kinetics of α-glucosidase inhibitory activity for PFE and RFE were determined following a procedure similar to that described above, with slight modifications: the optimal concentrations of samples were used (as inhibitor), and the concentration of the substrate (4-nitrophenyl α-D-glucopyranoside) ranged from 0.5 to 10 mM. Absorbance was measured at 405 nm at 5-min intervals and mode of action was determined from a Lineweaver-Burk plot correlating 1/[substrate] (mm/L) with 1/[V] (reaction rate).

Table 1
The antioxidative capacity of free and bound phenolic fractions found in the bran of purple and red rice, assessed by DPPH, FRAP, and ABTS assays.

SAMPLES	DPPH (%)	FRAP (mg TE/g sample)	ABTS (mg TE/g sample)
PFE	99.81 ± 0.2 [a]	19.45 ± 0.2 [a]	9.16 ± 0.1 [a]
PBE	25.18 ± 1.1 [b]	13.50 ± 0.4 [b]	5.95 ± 0.6 [b]
RFE	94.96 ± 0.8 [c]	15.91 ± 0.8 [c]	8.49 ± 0.4 [c]
RBE	30.23 ± 2.2 [d]	13.96 ± 0.8 [b]	7.10 ± 0.2 [d]

*) Values in each column with different letter are significantly different ($p < 0.05$), $n = 3$.

2.12. Identification of polyphenol compounds using high-performance liquid chromatography (HPLC) in free and bound fractions

The compositions of free and bound phenolic fractions of pigmented Thai rice bran were determined using a method adapted from Wu et al. (2018). A Hitachi Chromaste HPLC system was utilized with a detector and an ACE 5 C 18 column (250 × 4.6 mm, 5 μm). The mobile phase was pumped at a constant flow rate of 0.8 mL/min. The injection volume was 20 μL, the column temperature was maintained at 25 °C, and measurements were conducted at 280 nm. The mobile phase consisted of a 70:30 acetonitrile/methanol (solution A) and 0.1% glacial acetic acid (solution B). Sample (1 mL) were aliquoted by pipet into 1.5 mL vials with syringe filters containing a nylon membrane size of 0.45 μm, then were subjected to HPLC. Polyphenol compounds were identified by comparing their peak retention times with the following of standards:

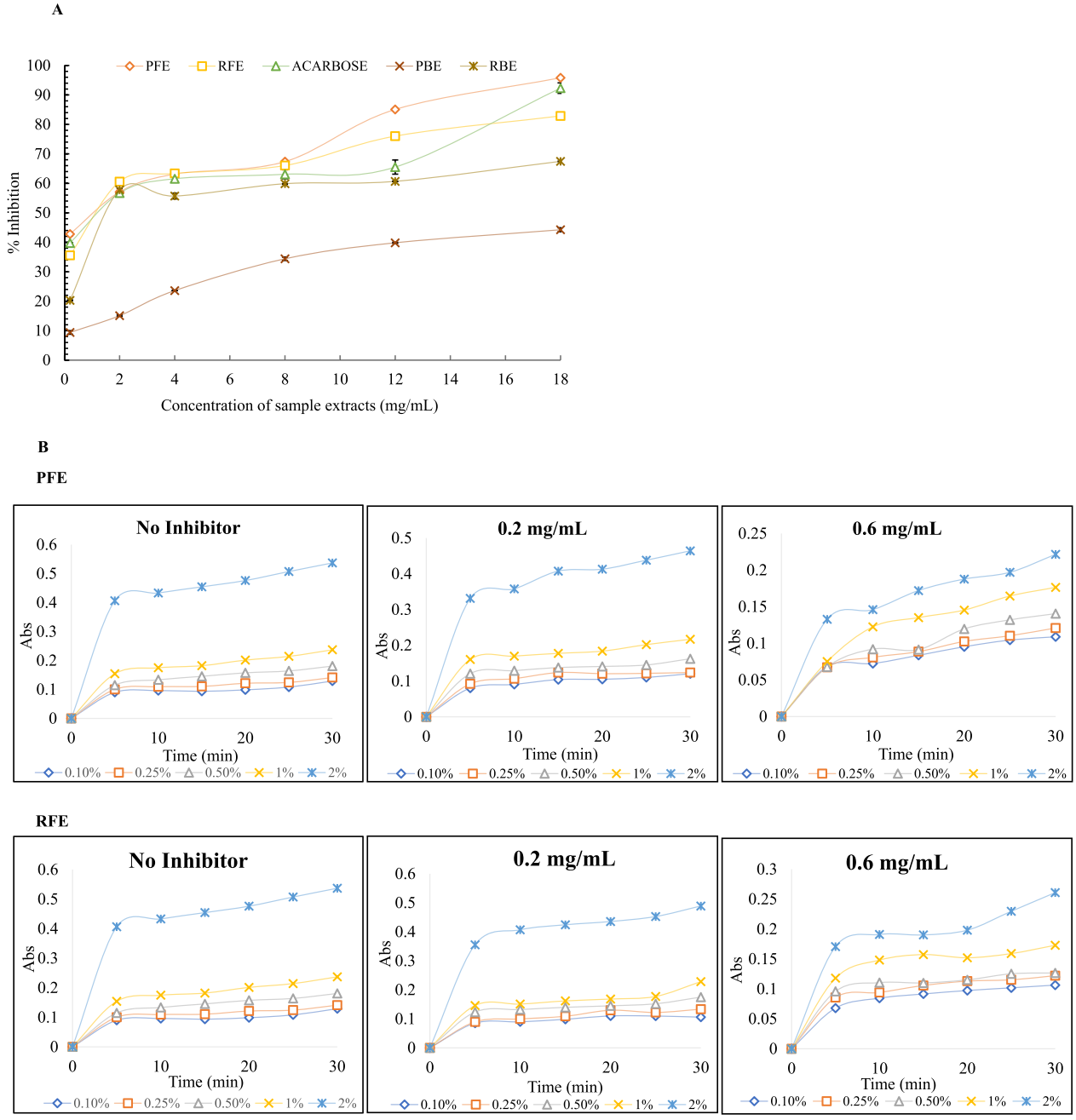
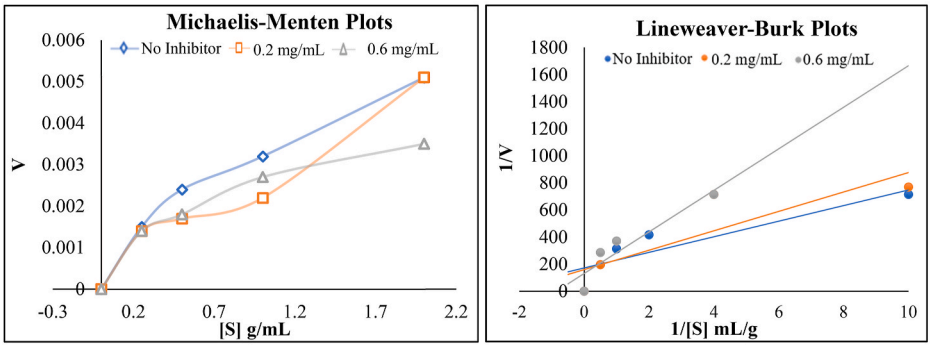


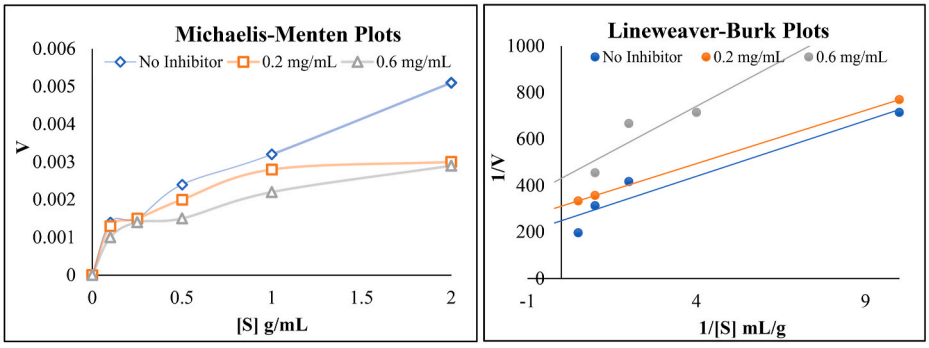
Fig. 2. Inhibitory capacity of PFE, RFE, acarbose, PBE, and RBE against α-amylase activity (A), inhibition rate of α-amylase by the presence of PFE and RFE (B), inhibition mode of PFE and α-amylase activity (C), and inhibition mode of RFE and α-amylase activity (D).

C



	0	0.2	0.6
Vmax	0.0058	0.0062	0.0076
Km	0.2787	0.4463	1.1652
COMPETITIVE			

D



	0	0.2	0.6
Vmax	0.0040	0.0032	0.0023
Km	0.1923	0.1402	0.1235
UNCOMPETITIVE			

Fig. 2. (*continued*).

benzoic acid, chlorogenic acid, ferulic acid, rutin, gallic acid, and quercetin.

2.13. Statistical analysis

Data analysis was performed using the statistical software SPSS version 29.0 (IBM SPSS, New York, USA) with analysis of variance (ANOVA) at a significance level of 5%, followed by Tukey's test for post-hoc analysis. The reported data are presented as mean ± standard deviation (triplicate measurements). Pearson's correlation analysis was used to determine the relationship between test parameters, with statistical significance set at $p < 0.05$.

3. Results and discussion

3.1. The phytochemical composition of pigmented rice bran

Fig. 1 shows the total phenolic, flavonoid, and anthocyanin contents of the free and bound fraction of purple and red rice bran extracts. The TPC of pigmented rice bran extracts varied from 13.71 to 72.09 mg GAE/g sample, with the free phenolic fraction having a significantly higher TPC than the bound fraction. The highest TPC value was obtained for the PFE at 72.09 ± 2.9 mg GAE/g sample, that of the RFE was significantly lower at 30.78 ± 0.7 mg GAE/g sample ($p < 0.05$). Interestingly, the RBE showed notably higher but statistically non-significant differences from the PBE. Specifically, the total phenolic content of the RBE measured 16.08 ± 0.5 mg GAE/g sample, whereas that for the PBE was 13.71 ± 2.2 mg GAE/g sample. There were statistically significant differences in the levels of free and bound phenolics in Thai pigmented rice bran ($p < 0.05$). Together, these results reveal fascinating distinctions in the distribution of phenolic compounds in rice bran extracts. For one, the phenolic content of pigmented rice bran from varieties such as Riceberry and Hommali is predominantly distributed in the free form, with a tendency to be easily extracted using organic solvents. The total flavonoid and anthocyanin content in extracts ranged from 25.66 to 143.79 mg QE/g sample and 0.07–169.17 mg Cy-3-GE/g sample, respectively. Red rice bran contained higher overall flavonoid content, but when considering individual fraction, PFE had the highest level (143.79 ± 2.0 mg QE/g sample), followed by RFE, RBE, and PBE (127.81 ± 1.7; 56.76 ± 3.4; and 25.67 ± 4.81 mg QE/g sample, respectively). Similar studies have confirmed higher flavonoid levels in red rice bran (Bhat and Riar, 2017; Huang and Lai, 2016). Meanwhile, anthocyanin content in the PFE was notably higher than that in RFE (169.17 ± 8.4 mg Cy-3-GE/g sample and 9.92 ± 1.2 mg Cy-3-GE/g sample, respectively, $p < 0.05$).

Taken together, these results indicate that bran from the purple rice variety Riceberry possesses a higher phytochemical content compared to bran from red rice Hommali. This substantial presence of total phenolic and total anthocyanin content contributes significantly to the phytochemical profile of rice bran, resulting in its heightened antioxidant activity. In extracts, the presence of a deep purple color consistently indicated higher levels of anthocyanins. Additionally, the anthocyanin content in both purple and red rice bran was soluble in methanol acid solution.

The variation in the distribution of phytochemical compounds observed here is similar to the reports of Ti et al. (2014) and Ghasemzadeh et al. (2018) regarding phenolic extracts of pigmented rice bran from genotypes of Southern China and Malaysia, in which phenols primarily occurred in free form. However, some cereals like red quinoa and pigmented rice from Hangzhou, China have been demonstrated to contain phenolics predominantly in bound form (Pang et al., 2018; Shao et al., 2014; Zhang et al., 2020, 2022). Differences in observed phenolic distribution may be due to the different strains and extraction methods used across studies (Wu et al., 2018). Bound phenolics are phenolic compounds that cannot be extracted by means of organic solvents or polar aqueous solutions on account of being physically trapped in plant matrices such as dietary fiber and intact cells or covalent bonds with macromolecules such as cellulose or other components of plant cell walls (Wang et al., 2020; Ye et al., 2022). A combination of alkaline and acidified treatments, followed by sonication, can effectively release

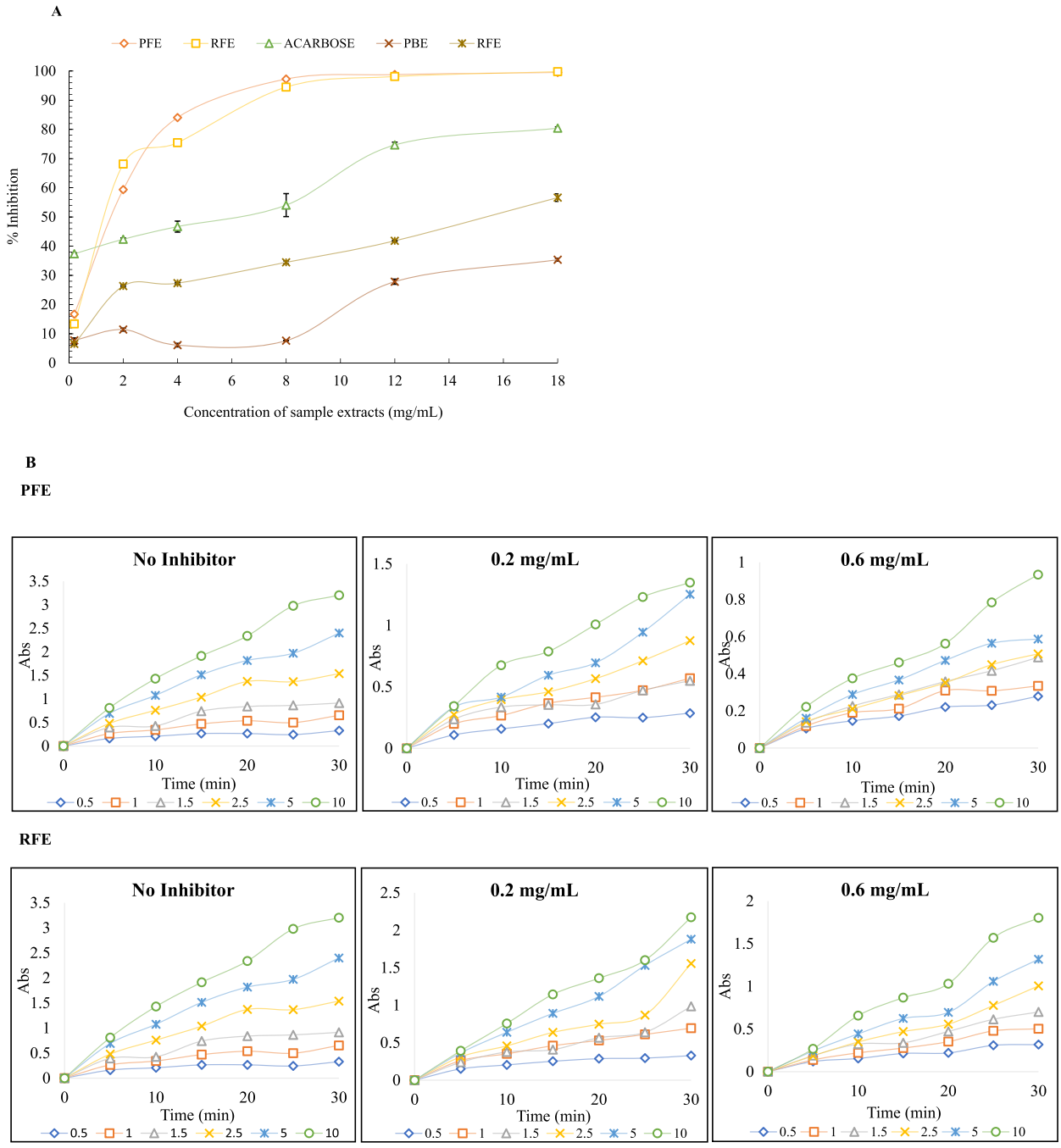


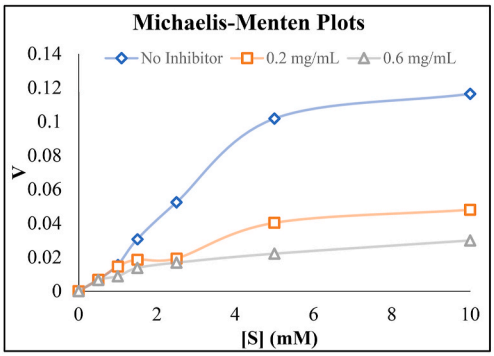
Fig. 3. Inhibitory capacity of PFE, RFE, acarbose, PBE, and RBE against α-glucosidase activity (A), inhibition rate of α-glucosidase by the presence of PFE and RFE (B), inhibition mode of PFE and α-glucosidase activity (C), and inhibition mode of RFE and α-glucosidase activity (D).

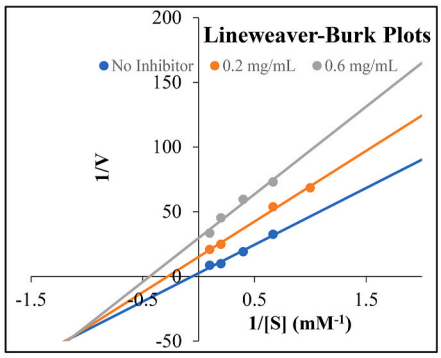
bound phenolics trapped within plant cell walls (Gonzales et al., 2014; Yusoff et al., 2022). The observed variability in phenolic distribution supports the notion that phenolic compounds within grains at the cellular and subcellular levels are not consistently or uniformly distributed (Sumczynski et al., 2017).

3.2. Free radical scavenging (antioxidative capacity)

The antioxidant capacity of phenolic fraction derived from the bran layer of purple and red rice was assessed using three distinct methods: the 2,2-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing ability of plasma (FRAP), and $ABTS^{\bullet+}$ radical cation-based assays that shown in Table 1. In all cases, the results displayed a linear correlation with phytochemical composition, indicating that higher concentrations of TPC, TFC, and TAC correspond to the higher antioxidant activity. Some studies have attributed a material's level of antioxidant activity to its polyphenol content (Ghasemzadeh et al., 2018; Pang et al., 2018; Sumczynski et al., 2017; Zhang et al., 2022). Of the fractions tested, PFE exhibited significantly higher antioxidant activity, with DPPH, FRAP, and ABTS values of 99.81 ± 0.2%, 19.45 ± 0.2 mg TE/g sample, and 9.16 ± 0.1 mg TE/g sample, respectively. The bound phenolic fractions exhibited no significant difference between purple and red rice bran in terms of FRAP (13.50 ± 0.4 and 13.96 ± 0.8 mg TE/g sample, respectively), but demostrated significant distinction in DPPH and ABTS assays

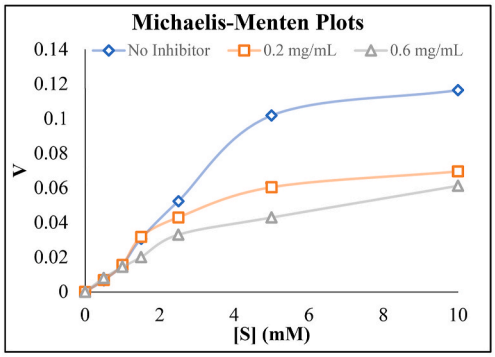
C

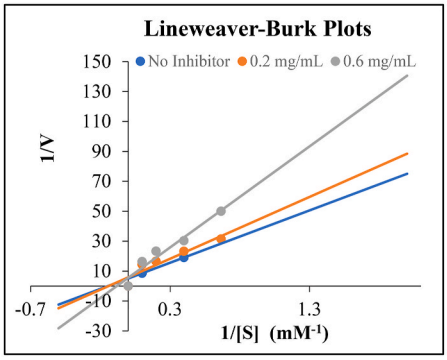




	0	0.2	0.6
Vmax	0.3972	0.0662	0.0337
Km	17.4370	3.6181	2.2843
UNCOMPETITIVE			

D





	0	0.2	0.6
Vmax	0.1963	0.1714	0.1806
Km	6.8669	7.0815	12.1922
COMPETITIVE			

Fig. 3. (*continued*).

(PBE: 25.18 ± 1.1% and 30.23 ± 2.2%; RBE: 5.95 ± 0.6, and 7.10 ± 0.2 mg TE/g sample; $p < 0.05$ for both). Previous research has indicated that polyphenols effectively donate hydrogen and act as agents for electron transfer or Fe^{3+} reduction, aligning with the fundamental principles behind the DPPH, FRAP, and ABTS assays (Aron and Kennedy, 2008). The differing antioxidant level determinations among the three assays were likely influenced by the specific types of antioxidant compounds within the sample and their molecular properties and structures. In this way, utilizing different assays enables the specific evaluation of antioxidant mechanisms or types. DPPH measures free radical scavenging, FRAP evaluates antioxidant reducing power, and ABTS assay assesses both radical scavenging and reducing capabilities. The use of multiple assays contributes to a comprehensive understanding of the overall antioxidant potential of a substance or sample, revealing its activity in multiple contexts and against different types of oxidative stress.

3.3. Inhibition of amylolytic enzymes and kinetics

3.3.1. Inhibition capacity of free and bound phenolics on α-amylase activity and mode of inhibition

The inhibitory effect of free and bound phenolic extracts from purple and red rice bran against α-amylase is presented in Fig. 2A. Acarbose, a commercial medicine for diabetes, was included to compare the effectiveness of the sample as an alternative hypoglycemic agent. At the concentration of 0.2 mg/mL, the inhibition capacity varied from 9.8% to 42.3%. Higher concentrations resulted in greater inhibitory effect, ranging from 34.39% to 67.37% at 8 mg/mL. At the highest concentration tested (18 mg/mL), the inhibition capacity increased to 67.4%–95.8%. The free phenolic extracts both demonstrated significantly greater effect than that of acarbose (IC_{50} 0.70 < 0.75 < 1.73 mg/mL, PFE > RFE > acarbose; lower IC_{50} indicates higher inhibitory capacity). This outcome aligns with the polyphenol compounds findings: as the phytochemical and antioxidant values increased, there was a corresponding increase in the inhibitory effect on α-amylase.

The phenolic compounds found in pigmented cereals and various other plants, such as cocoa, pomegranates, cranberries, grape seed, tea extracts, sea buckthorn, persimmon, and Chinese berry leaves have demonstrated ability to exert antioxidant effects and inhibit enzymes, including α-amylase, thereby hindering the hydrolysis of α-1,4-glucan polysaccharides (Barrett et al., 2013; Li et al., 2018; Mu et al., 2022; Yilmazer-Musa et al., 2012; Zheng et al., 2021). However, the bound phenolics of purple and red rice bran extract exhibited lower enzyme inhibition activity compared to acarbose (IC_{50} 1.73 < 5.28 < 18.58 mg/mL, acarbose > RBE > PBE; $p < 0.05$). At the highest concentration of 18 mg/mL, PBE and RBE respectively showed 67.5% and 43.7% inhibition, notably lower than acarbose, which achieved 92% inhibition, and also lower than the inhibitory effects of PFE and RFE at 95.8% and 83.4% inhibition, respectively. In contrast, the bound phenolic extracts of red quinoa, brown rice bran, and mung bean skin are reported to more strongly inhibit α-amylase than corresponding free extracts because the bound extracts have higher antioxidant content (Ye et al., 2022; Zhang et al., 2022; Zheng et al., 2020). As inhibition of amylolytic enzymes by polyphenol compounds depends on chemical structure, variation in composition could explain the differing ability of different extracts to inhibit digestive enzymes. Chlorogenic acid and ferulic acid have double C=C bonds in their structures and may be conjugated with a carbonyl group, which is capable of stabilizing their binding to the enzyme active site. Additionally, hydroxyl groups on the B ring play an important role in enzyme inhibition by facilitating the formation of hydrogen bonds between -OH groups and enzymes (Li et al., 2018; Sun and Miao, 2020; Sun et al., 2019).

Polyphenols inhibit α-amylase through binding interaction with the enzyme's active site, allosteric site, or enzyme-substrate complexes, which are potentially facilitated by hydrogen bonding and hydrophobic forces (Selvaraj et al., 2022). On the enzyme side, specific amino acid residues within the active site, namely Asp^{300}, Asp^{197}, and Glu^{233},

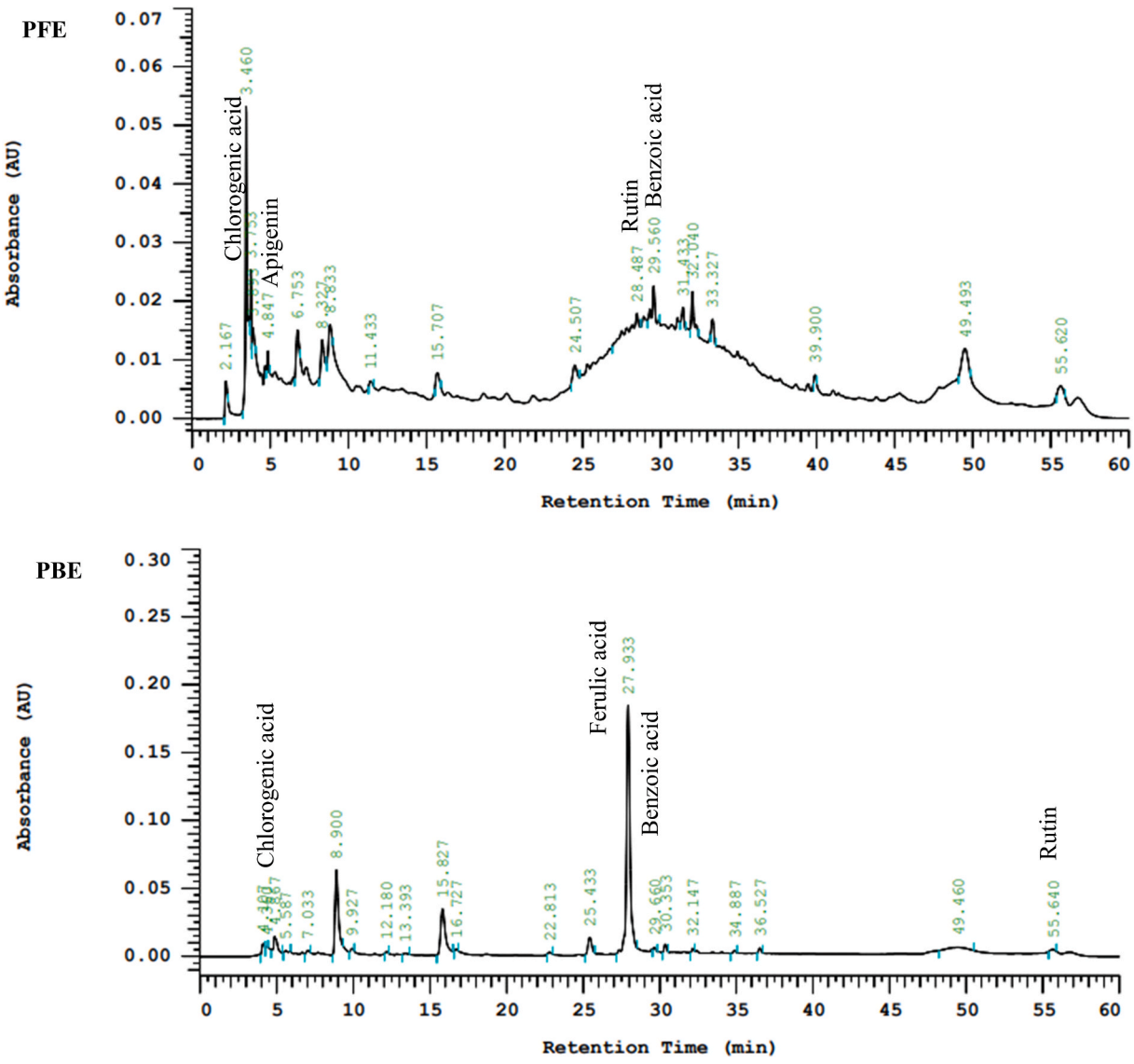


Fig. 4. Model chromatogram of polyphenol compounds in PFE, PBE, RFE, and RBE.

interact with the polyphenol aromatic ring. Meanwhile, for polyphenols, the arrangement of hydroxyl groups and overall molecular structure are a crucial in enzyme binding. Some polyphenols have structures resembling the enzyme substrate, enabling them to competitively inhibit the enzyme by competing for its active site and interfering with the enzyme-substrate interaction (He et al., 2023).

To further investigate the mechanism of α-amylase inhibition by free phenolic extracts of purple and red bran extracts, inhibition rate was measured at 5-min intervals with range of starch concentrations as the substrate. Increasing the substrate concentration and extending the reaction time led to higher absorbance, indicating more product formation. However, higher extract concentrations significantly reduced the enzyme's ability to hydrolyze starch (as shown in Fig. 2B). After calculating the inhibition rate, the outcomes were used to create Michaelis-Menten plots (showing the initial reaction velocity with varying concentrations of starch) and Lineweaver-Burk plots, which are depicted in Fig. 2C (PFE) and 2D (RFE). The Michaelis-Menten plots illustrate the relationship between observed rate of reaction and substrate concentration, and indicate that the catalyst reaches saturation at high substrate concentrations (Chrisman et al., 2023). The Lineweaver-Burk plot revealed the extract's perspective mode of α-amylase inhibition: PFE displayed a competitive mode, whereas RFE exhibited an uncompetitive mode. The competitive mode of PFE was characterized by an increase in K_m as sample concentration increased. However, there was also a slight increase in $V_{max,}$ which might be due to the substrate itself acting in a manner that changes the enzyme's behavior under certain conditions. It can be also explained by the fact that PFE, as an inhibitor, possesses a strong ability to compete with the substrate for binding to the α-amylase active site of, resulting in the disruption of its catalytic activity. In uncompetitive inhibition, the inhibitor (RFE) forms an enzyme-inhibitor-substrate complex that leads to slower formation of products. This mode of inhibition is distinct because the binding site of the inhibitor is different from that of the substrate, and is often observed with polyphenols that have a high affinity for the enzyme-substrate complex or possess specific structural elements that facilitate their binding to the complex. The exact structural characteristics that determine polyphenol propensity for uncompetitive inhibition can differ depending on the enzyme and polyphenol involved, but generally, uncompetitive inhibition may be exhibited by polyphenols having a large number of hydroxyl groups and galloyl moieties, or high polymerization degree (Moreno-Córdova et al., 2020).

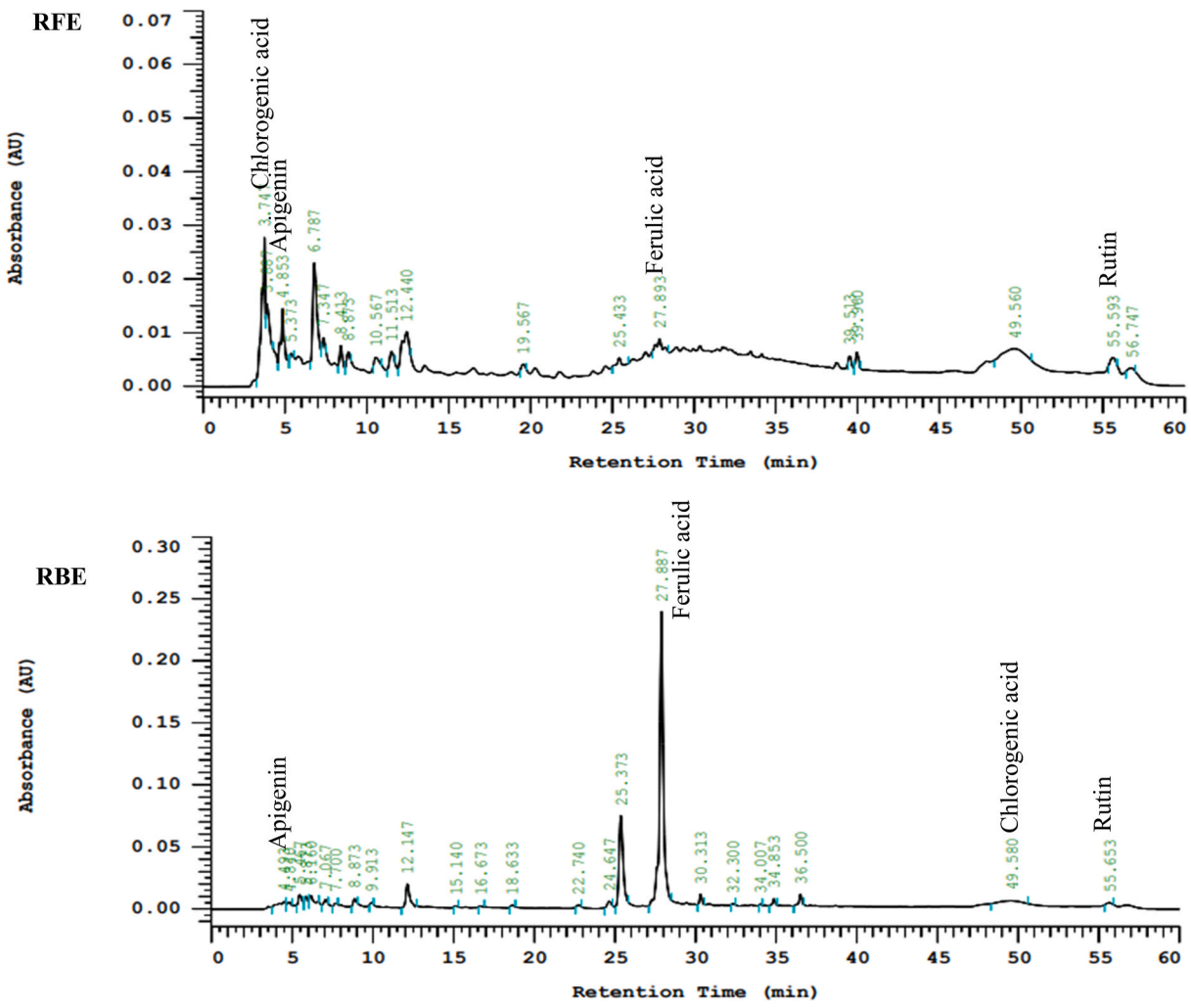


Fig. 4. (*continued*).

3.3.2. *Inhibition capacity of free and bound phenolic on α-glucosidase activity and mode of inhibition*

Fig. 3A illustrates the inhibitory effects of PFE, RFE, PBE, and RBE on α-glucosidase, which consistently aligned with the extract's phytochemical composition and antioxidant effects. Broadly, high content of phenolics, flavonoids, and anthocyanins contributed to superior inhibition of α-glucosidase activity. Among extracts, the highest inhibition was observed for PFE, with TPC, TFC, and TAC of 72.09 mg GAE/g sample, 143.79 mg QE/g sample, and 169.17 mg Cy-3-GE/g sample, respectively, and an IC_{50} of 0.5 mg/mL. This was followed by RFE, which had an IC_{50} of 0.8 mg/mL. Meanwhile, both bound extracts exhibited a lower inhibition capacity than acarbose (IC_{50} 5.0 < 14.87 < 28.15 mg/mL, acarbose > RBE > PBE, respectively, $p < 0.05$). The correlation between free phenolic extract concentration and α-glucosidase inhibition aligned with the trend observed for α-amylase inhibition, with inhibition rate reaching a maximum of over 95%; however, the disruptive effect on α-glucosidase was notably greater than on α-amylase. This result aligns with the findings of previous studies (Li et al., 2018; Quan et al., 2019; Zheng et al., 2020). Wu et al. (2018) highlighted anthocyanins in black rice bran as contributors to superior inhibition, but flavonoids have also been reported to play a significant role (Aleixandre et al., 2022; Gutiérrez-Grijalva et al., 2019). Especially, flavonoids contained in purple and red rice bran extract, including apigenin and rutin, are reported to be more effective in inhibiting α-glucosidase activity due to having multiple hydroxyl groups (-OH). This characteristic could explain the comparatively elevated inhibitory potential (Aleixandre et al., 2022; Li et al., 2019).

The enzyme α-glucosidase is essential in the final stage of carbohydrate hydrolysis. Its role differs from that of α-amylase in that it converts dextrin, maltose, and other oligosaccharides into glucose within the small intestine. When α-glucosidase is functioning effectively, glucose release can lead to postprandial hyperglycemia in patients with type 2 diabetes. Inhibiting of these amylolytic enzymes disrupts the catalytic activity involved in carbohydrate hydrolysis and delays glucose uptake, thereby inducing hypoglycemic effects and reducing the risk of developing type 2 diabetes.

Investigating the kinetics of inhibition is a valuable approach for differentiating the various ways in which an inhibitor may interact with an enzyme. This study investigated the inhibitory mechanisms of PFE (3C) and RFE (3D) on α-glucosidase using the substrate 4-nitrophenyl α-D-glucopyranoside, with concentrations ranging from 0.5 to 10 mM. This revealed that higher substrate concentrations led to increased product formation, but the presence of PFE and RFE at concentrations of 0.2 mg/mL and 0.6 mg/mL inhibited the catalytic activity, resulting in lower product formation (shown in Fig. 3B). Michaelis-Menten plots indicated a decrease in initial velocity (v) as the extract concentration increased, a trend further illustrated in the Lineweaver-Burk (Fig. 3C and D). Subsequent determination of V_{max} and K_m values showed PFE and RFE to inhibit α-glucosidase through different modes of action. In Lineweaver-Burk kinetic plot, the K_m value of PFE decreased at concentrations of 0.2 and 0.6 mg/mL, and the initial V_{max} of 0.3917 decreased to 0.0662 and 0.0337 respectively. Thus, this reaction was

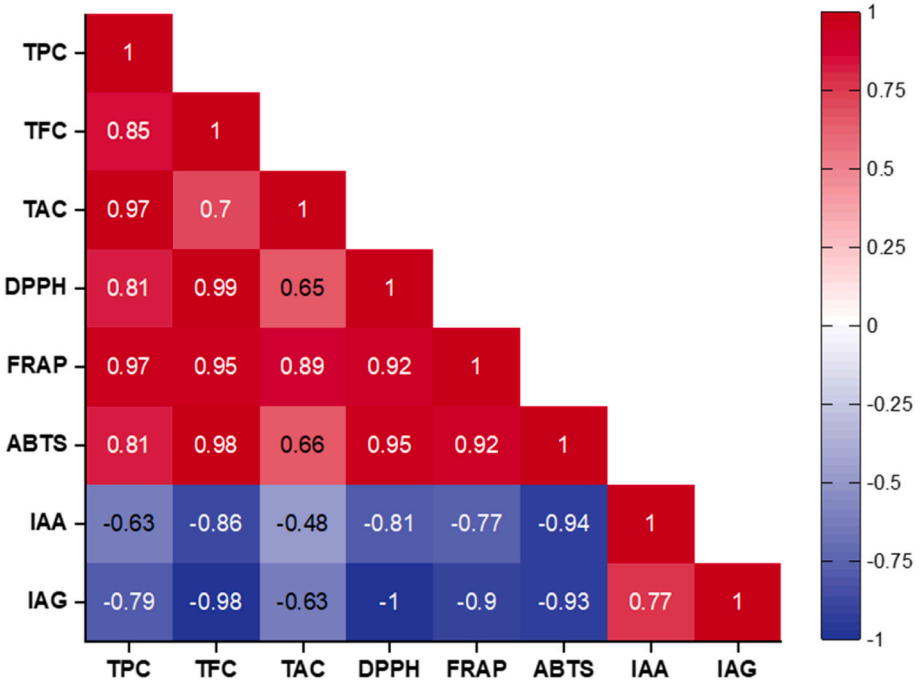


Fig. 5. Pearson Correlation analysis among all parameters
Note: TPC is total phenolic content; TFC is total flavonoid content; TAC is total anthocyanin content; DPPH, FRAP, ABTS are antioxidant activity by different assessments; IAA is inhibition of α-amylase activity (IC_{50}); IAG is inhibition of α-glucosidase activity (IC_{50}).

uncompetitive. RFE conversely demonstrated a consistent V_{max} despite increasing K_m at higher concentrations, characteristic of competitive binding and contention with the substrate for occupancy of the active site.

3.4. HPLC of polyphenol compounds in free and bound fractions of pigmented Thai rice bran

The polyphenol composition of free and bound fractions from purple rice bran (Riceberry) and red rice bran (Hommali) is illustrated in Fig. 4. For purple rice bran, benzoic acid, chlorogenic acid, apigenin, and rutin were identified in the PFE, of which apigenin was not detected in the PBE. Meanwhile, both free (RFE) and bound (RBE) of red rice bran extracts exhibited similar composition, particularly in the presence of chlorogenic acid, ferulic acid, apigenin, and rutin. According to a prior report, black rice bran from multiple rice varieties in Beijing, China, does not contain free chlorogenic acid, whereas indica and japonica varieties exhibited high content levels; meanwhile, the flavonoid apigenin was absent in the free form but detected in the bound form (Wu et al., 2018). Additionally, bound fractions from other varieties of indica rice in Southern China were found to lack chlorogenic acid (Ti et al., 2014).

Regarding flavonoid compounds, apigenin in bound form was not detected in purple rice bran extract, while all samples featured rutin. Other flavonoids such as quercetin are known to be abundant in bound form within rice bran extracts (Ghasemzadeh et al., 2018; Wu et al., 2018; Zhang et al., 2020). Some of the flavonoids present in purple and red rice bran extracts, such as apigenin and rutin, have been reported more effective in inhibiting α-glucosidase activity due to their multiple hydroxyl groups (-OH) (Aleixandre et al., 2022). Moreover, apigenin lacks the substitution of $–OCH_3$ in its molecular structure, which makes it more efficient inhibitor of α-amylase (Sun et al., 2019). Notably, the strongest inhibitory effect was observed for PFE because it possesed the greatest total amount of phytochemical compounds among the four tested extracts. The differing distribution of polyphenol compounds in each sample contributed to the differences observed in their inhibition capacities against amylolytic enzymes.

3.5. Correlations between phytochemical content, antioxidant activity, and the inhibition of amylolytic enzymes

Pearson correlation was performed to identify the relationships among all parameters (Fig. 5). This revealed that phytochemical compounds, including TPC, TFC, and TAC, having a highly significant relationship with antioxidant activity ($r > 0.80$, significant $p < 0.05$). However, TAC demonstrated only moderate correlation with DPPH and ABTS ($r = 0.65$ and 0.66, respectively). The ability of pigmented rice bran to decrease amylolytic enzyme activity is consistent with its greater radical scavenging ability. TPC, TFC, and TAC all exhibited negative correlations with amylolytic inhibitory effect. The correlation between anthocyanin content and inhibitory capacity was not particularly significant ($R = -0.485$ and -0.632 for α-amylase and α-glucosidase, respectively), whereas the correation for flavonoid content was strong correlation. The flavonoid content in purple and red rice bran has previously been reported to reach $r = -0.86$ and -0.98, for inhibition of α-amylase (IAA) and inhibition of α-glucosidase (IAG), respectively. This result is supported by the findings of Johnson et al. (2011) that a blueberry fraction containing proanthocyanidins featured a lower IC_{50} value for α-amylase compared to the fraction enriched with anthocyanins. Moreover, a flavonoid with two catechol groups in its A- and B-rings, accompanied by a hydroxyl group at the C-ring, exhibited the highest activity against α-glucosidase. Among any compounds classes as various flavonoids, flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanidins, have been identified as the most potent inhibitors of α-glucosidase (Giuberti et al., 2020).

4. Conclusion

Ths study systematically investigated the distribution of free and bound phenolics in purple and red rice bran extracts and the inhibitory effects of phenolic fractions on amylolytic enzymes. Significant differences between free and bound fractions were observed in total phenolic,

flavonoid, and anthocyanin contents; specifically, the free phenolic extract exhibited higher levels of all three, which contibuted to its superior free radical scavenging abilities as assessed by DPPH, FRAP, and ABTS assays. Red rice bran extract showed high flavonoid content, while purple rice bran extract was rich in anthocyanins. Flavonoid compounds extracted from red rice bran exhibited a strong positive correlation with antioxidant activity but a significant negative correlation with amylolytic enzymes inhibition. Flavonoid compounds identified in the samples included apigenin and rutin; the phenolic compounds such as benzoic acid, ferulic acid, and chlorogenic acid were also observed. Assessment of the mode of inhibition revealed purple and red rice bran extracts to have different modes, which could contribute to their respective inhibitory capacities. High inhibition capacity for α-amylase and α-glucosidase supports a hypoglycemic effect. Taken together, these findings enhance our understanding of how the distribution of phenolics in the bran of Thai purple and red rice bran of varieties differs from other varieties, providing valuable information about phytochemical compounds and antioxidant activity that could be utilized in developing functional food ingredients, especially for promoting health among diabetics.

CRediT authorship contribution statement

Sandra Kusumawardani: Writing – original draft, Methodology, Investigation, Formal analysis. **Naphatrapi Luangsakul:** Conceptualization, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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