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Investigating phytochemical and antioxidant activity of free and bound phenolics from brown rice bran and their correlation with enzymatic inhibitory and *in vitro* starch digestibility

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Abstract Inhibiting the activity of digestive enzymes is a highly effective approach to controlling glucose absorption and, consequently, aiding in controlling type-2 diabetes mellitus. Brown rice bran, a by-product of the rice grain polishing process, represents a natural source rich in bioactive compounds that could be explored as a potential solution to reduce postprandial hyperglycemia. The study focused on evaluating the inhibitory effects of free and bound phenolic compounds on α-amylase and α-glucosidase enzymes, which play significant roles in carbohydrate metabolism. The IC_{50} values were found to be 0.947±0.05 mg/mL for α-amylase inhibition and 2.188±0.69 mg/mL for α-glucosidase inhibition in the free phenolic compound extract. In contrast, the bound phenolic compound extract displayed higher IC_{50} values of 13.861±0.03 mg/mL and 16.883±0.15 mg/mL for α-amylase and α-glucosidase inhibition, respectively. These differences in inhibitory potential between the free and bound phenolic compounds were statistically significant ($p < 0.005$). The superior inhibition observed in the free phenolic extract can be attributed to its higher levels of phenolic, flavonoid, and anthocyanin compounds compared to the bound form. Additionally, the antioxidant content analysis using the DPPH and FRAP methods revealed that the bound phenolic compounds exhibited lower antioxidant levels compared to the free form. Furthermore, the study investigated the impact of these bioactive compounds on starch digestibility by adding 10% sample extracts, both in free and bound forms, to high amylose rice starch. The results showed that the rate of starch digestibility was reduced to 54.04% and 58.2%, respectively, compared to the control (60.52%). Phytochemical content exhibited highly positive correlations with antioxidant activity leading to stronger enzymatic inhibition and slower the digestion rate. These findings suggest that the free phenolic extract from brown rice bran holds promise as a potential antidiabetic agent due to its ability to inhibit enzymatic digestion, leading to reduced postprandial glucose levels.

Keywords: α-amylase inhibitory, α-glucosidase inhibitory, Brown rice bran, Free phenolic

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Introduction

Diabetes has become a highly prominent metabolic disorder globally, mainly due to its rising prevalence. The number of diabetes patients has seen a 10% increase from 2016 to 2019 (Kumar *et al*., 2021; Li *et al*., 2018). Managing postprandial hyperglycemia is crucial as it poses a significant risk in the development of type-2 diabetes and associated chronic diseases. To accomplish this goal, it is vital to control the absorption of carbohydrates and slow down the uptake of glucose, which in turn inhibits the conversion of starch into glucose during digestion by targeting enzymatic digestion (Boue *et al*., 2016; Kaur *et al*., 2016; Ye *et al*., 2022; Yusof *et al*., 2009). Since starch is a major source of carbohydrates and has a significant impact on elevating blood glucose levels, it is crucial to explore natural sources to impede starch digestion. Polyphenol compounds are one type of component that has been shown to interact with enzymes (Aleixandre *et al*., 2022; Deng *et al*., 2021; Sun and Miao, 2020). Additionally, due to the chemical structure of phenolic compounds, they can bind with starch molecules through non-covalent interactions. These complexes can affect the extent and rate of starch digestibility (Boue *et al.*, 2016; Li *et al.*, 2018; Miao *et al*., 2021; Mu *et al*., 2022; Zhang *et al*., 2022).

Rice is a staple food consumed worldwide, and its whole grains naturally contain pigments before undergoing the polishing process. To cater to consumer preferences for taste and visual appeal, whole rice grains are often processed into polished white rice, generating a by-product known as rice bran. Rice bran is acknowledged as a potential origin of edible oil owing to its abundant presence of bioactive components, including γ-oryzanol, tocopherols, and tocotrienols (Sohail *et al*., 2017). It also contains significant amounts of flavonoids, anthocyanins, proanthocyanidins, and various phenolic acids (Boue *et al.*, 2016; Chen *et al.*, 2022; Sivamaruthi *et al*., 2023; Wu *et al.*, 2018). Some of the polyphenols identified in brown rice bran include syringic acid, ferulic acid, cinnamic acid, catechin, myricetin, and protocatechuic acid (Ghasemzadeh *et al*., 2018). Interestingly, polyphenol compounds exist in two forms, free and bound. Many studies tend to focus on extracting free polyphenols directly using organic solvents and often underestimate a significant portion of non-extractable bound polyphenols. Rice bran contains a substantial number of phytochemicals that are covalently bound to cell wall constituents which can be treated by alkaline, acidic, or enzymatic hydrolysis, targeting the solid residues that remain after the solvent extraction process (Wang *et al*., 2020b). It can be considered a promising source of polyphenols derived from a by-product, which may exert a hypoglycemic effect by reducing postprandial blood glucose levels. Although certain studies have investigated the enzyme-inhibitory activities of rice bran

polyphenols, there is a notable gap in the available data concerning the comparison between free and bound polyphenols extracted from brown rice bran. Specifically, there is limited information on the correlation between their phytochemical properties and the inhibition capacity of enzymatic digestion. Additionally, there is limited information on their impact on *in vitro* starch digestion. Further research is warranted to elucidate the potential health benefits and mechanisms of action of these polyphenols in managing postprandial blood glucose levels.

The aims were to assess and to make a comparison of the phytochemical components of phenolics in free and bound form of brown rice bran extract, as well as the antioxidant activity. Additionally, the study is investigated the potential correlation between these phytochemicals, antioxidant capacity and both enzymatic digestibility and in vitro starch digestibility.

Materials and methods

Sample preparation

Brown rice (Hom Mali) is provided from Bangkok province in central Thailand. The whole grains of pigmented rice were milled using NW 1000 Turbo laboratory polisher (Thongtrawee, Thailand). The milled grains were then ground and sieved through a 160 μm sieve using a sieving shaker (RETSCH, AS 200) to obtain rice bran. The yield of brown bran was 17.57%, and it was kept at 4°C before the extraction process took place.

The extraction of free and bound phenolic compounds from brown rice bran

Briefly, free phenolics were extracted by dissolving the sample in acidified methanol (50 mL) using shaking waterbath (model SV 1422, Memmert, Germany) for 30 minutes. After centrifugation, the resulting supernatant was concentrated by evaporation utilizing a rotary evaporator set at 42°C and stored until further analysis (Ghasemzadeh *et al*., 2018).

In brief, to extract the bound phenolic form, the residue was hydrolyzed under alkaline conditions in a sonicator (model WUC-D10H, Wised, Daihan Scientific, Korea) for 30 minutes. The resulting hydrolysate was then neutralized and subjected to extraction with ethyl acetate. The collected supernatant was subjected to evaporation, and the resulting solution was preserved for subsequent examination (Gonzales *et al*., 2014).

Quantification of phytochemical compound

The measurement of phytochemical content was conducted the following method. Briefly, modified Folin-Ciocalteu colorimetric method, as outlined by Madaan *et al*. (2011) was applied to ascertain the total phenolic content (TPC). For 200 µL of extracts samples or gallic acid were reacted with 10-fold diluted Folin-Ciocalteu reagent. 80 µL of 1M Na_2CO_3 was added after incubated for 5 minutes and left in the absence of light at room temperature for an additional duration of 30 minutes. Absorbance was read at 760 nm. The results were quantified in mg GAE/g sample, with consideration to its dry weight (DW).

Total flavonoid content (TFC) was analyzed using a colorimetric method described by Norhazlini *et al.* (2021) with modifications. Quercetin dilutions or samples (100 µl) were mixed with 5% sodium nitrate following to stand for 5 minutes. Then, 10% aluminum chloride ($AlCl_3$) solution (50 µl) was added, followed by 1M sodium hydroxide (30 µl), the absorbance was quantified using a microplate reader (Multimode Plate Reader, PerkinElmer, Inc., Massachusetts, USA) and presented as mg QE/g dry sample.

The assessment of Total Anthocyanin Content (TAC) is followed the methodology described by Pang *et al*. (2018). For each sample of free and bound phenolic extract, a volume of 3 mL was diluted in two distinct buffer solutions: potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5). Subsequently, the absorbance was measured within the wavelength range of 520 to 700 nm. The TAC values were expressed as milligrams of *cyanidin-3-glucoside* equivalents per gram of dried sample.

Evaluation of free radical scavenging (antioxidant capacity)

The free radical scavenging was measured spectrophotometrically using the 2,2 Diphenyl 2 picrylhydrazyl (DPPH) assay. 50 µL extracts were mixed with 180 µL of 0.1 mM DPPH. The absorbance was then measured at 515 nm (Bobo-García *et al.*, 2015).

The Ferric Reducing Ability of Plasma (FRAP) assay was performed by combining 20 µL of each sample with 200 µL of freshly prepared FRAP reagent. Following an incubation period of 30 minutes at room temperature, the absorbance was measured at 595 nm. The results were quantified and expressed in µmol (Ti *et al*., 2014).

Assay of the inhibitory capacity of α-amylase and α-glucosidase

The inhibition of α-amylase capacity was performed by incubating 100 µL of porcine pancreatic amylase solution (3U/mL) in phosphate buffer with 100

µL of extracts (0.2-18mg/mL) at 37°C for 10 minutes in a waterbath. 100 µL of starch was then added to the reaction, followed by another 10 minutes of incubation. The reaction was stopped by the addition of DNSA reagent, and the samples were then subjected to heat in boiling water for 10 minutes. Afterward, the reaction mixture was allowed to cool down to room temperature. Following dilution, the absorbance of the samples was measured at 540 nm. (Li *et al*., 2018).

The inhibitory activity of α-glucosidase of the extracts were evaluated using a method similar to previous studies with minor modifications (Kazeem *et al.*, 2013). A 50 µL sample of the extract (0.2-18 mg/mL) was preincubated with 100 µL of 0.1 Units/mL enzyme solution in 0.1M phosphate buffer for 10 minutes. Subsequently, 100 µL of 10 mM pNPG was added as a substrate and then terminated using 2 mL of 0.1M Na_2CO_3 after 20 minutes of incubation. The absorbance was read at 405 nm.

In vitro starch digestibility

The effects of the sample extracts on *in vitro* rice starch digestion were examined by gelatinizing 2% rice starch solution with 10% of extracts at 90°C for 20 minutes. The starch digestion process was initiated with adding enzyme solution (α-amylase: α-glucosidase). The reaction occurred at 37°C in shaking water bath for 180 minutes. Samples (500 µL) were taken at 30, 60, 90, 120, and 180 minutes and stopped using 95% ethanol. After centrifugation, the sample was collected, and the glucose content was examined using the DNS colorimetry (Wang *et al*., 2020a).

Results

TPC, TFC, TAC, and free radical scavenging (antioxidant capacity) of free and bound phenolic extract of brown rice bran

The results of the statistical analysis were evaluated by using one way ANOVA and presented as mean ± SD. Phytochemical content is shown in Figure 1. TPC in the free phenolic extract from brown rice bran was notably higher (14.113±1.00 mg GAE/g sample) compared to the bound form, which exhibited a considerably lower level (0.235±0.01 mg GAE/g sample, $p < 0.001$). Similar trends were observed for total flavonoid and anthocyanin content, where the free form had higher concentrations (0.458±0.003 mg QE/g sample and 18.28±2.94 mg Cy-3-GE/g sample, respectively) compared to the bound extract (0.010±0.005 mg QE/g sample and 1.605±0.07 mg Cy-3-GE/g sample, respectively). The total flavonoid and anthocyanin contents of both form of phenolic extracts were significantly different ($p < 0.001$).

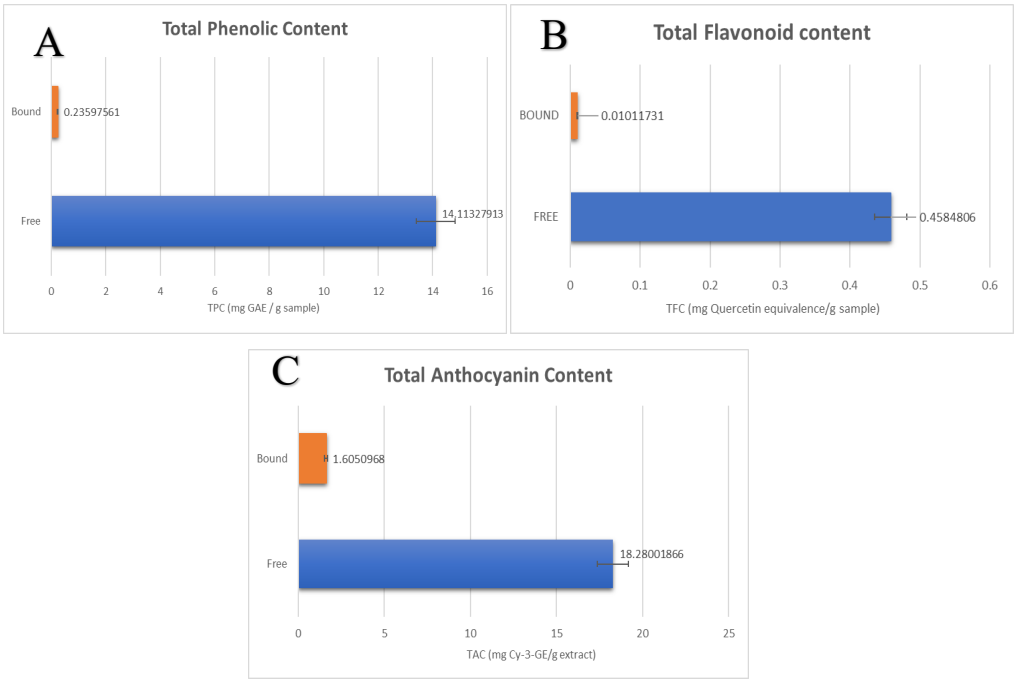

Figure 1.The comparison of TPC (A), TFC (B), TAC (C) of free and bound phenolic extract ($p < 0.001$)

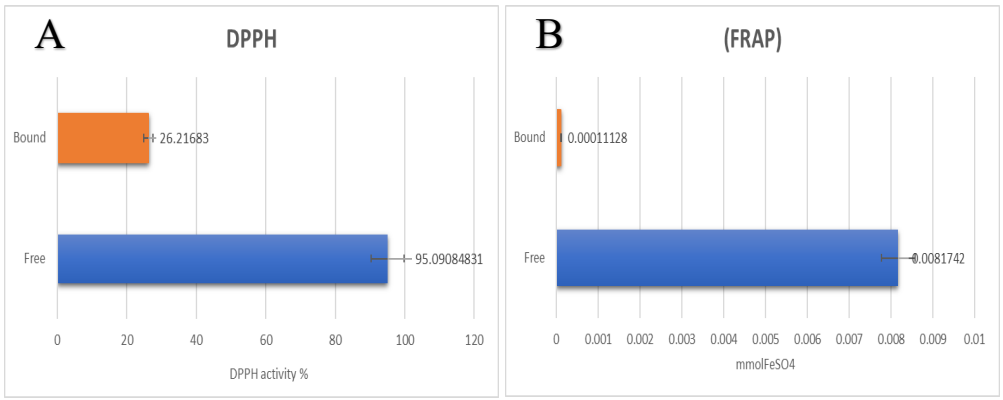

Figure 2. shows the assessment of antioxidant activities using DPPH (A) and FRAP (B) methods ($p < 0.001$)

The antioxidant activity (free radical scavenging) of the samples was assessed by evaluating DPPH and FRAP. The DPPH assay measured the scavenging activity against free radicals, while the FRAP assay expressed the value of ferric reducing ability of plasma in µmol Fe (II). The findings from both

DPPH and FRAP analyses demonstrated significantly higher results for brown rice bran extract in the free form compared to the bound form ($p<0.001$). These findings are consistent with higher levels of phytochemical content (TPC, TFC, and TAC). In the DPPH assay, the free phenolic extract from brown rice bran had an antioxidant activity of 95.09%, which was positively higher than the activity of 26.21% associated with the bound phenolic extract. Furthermore, the antioxidant capacities of the free and bound phenolic extracts were 0.0008 and 0.0001 μmol Fe (II) respectively.

The inhibition of free and bound phenolic extract against enzymatic digestion activity

The comparison of the inhibition level of free and bound phenolic extracts against α-amylase and α-glucosidase activity is shown in Figure 3. The α-glucosidase inhibition capacity exhibited a direct correlation with the rise in phenolic contents (0.2-18 mg/mL) for all samples. However, beyond a phenolic concentration of approximately 12 mg/mL, no further linear increase in inhibitory effect was observed. At the specified concentration of 18 mg/mL, the free phenolic extract demonstrated a remarkably pronounced inhibition rate of 99.63%, while the bound phenolic extract exhibited a comparatively lower inhibition rate of 49.04%. These findings indicate a notable distinction in the inhibitory activity between the two types of phenolic extracts at the given concentration. In Figure 3A, the inhibitory effect of α-amylase is depicted, with the free phenolic extract showing the highest inhibitory activity at the highest concentration of phenolic extract, reaching 85.18%. This activity was significantly different from the bound form ($p < 0.001$). The IC_{50} values for α-amylase inhibition were found to be 0.094±0.05 mg/mL for the free form and 13.861±0.03 mg/mL for the bound form.

The effect of free and bound phenolic extracts on the rice starch digestibility

The impact of rice starch digestion *in vitro* with the addition of 10% free and bound phenolic extracts is presented in Figure 4. The rapid digestion stage was observed at 60-90 minutes, followed by a slower rate thereafter. The addition of phenolic extracts significantly affected starch digestibility compared to the control. At the 180-minute mark, the control group exhibited a starch digestion rate of 60.52%. In contrast, the presence of 10% free phenolic extracts resulted in a lower starch digestion rate of 54.04%, and the presence of 10% bound phenolic extracts led to a slightly reduced rate of 58.20%. Statistical analysis revealed highly significant ($p < 0.001$) for the free form and ($p < 0.05$) for the

bound form compared to the control (rice starch only). These observations indicated that both phenolic extracts possess inhibitory properties on starch digestion, with the free form exhibiting a more prominent influence. These outcomes aligned with the discovery that the free phenolic extract that is characterized by elevated levels of TPC, TFC, TAC, and antioxidant activity, displayed superior inhibitory effects on starch digestion when compared to the bound phenolic extract.

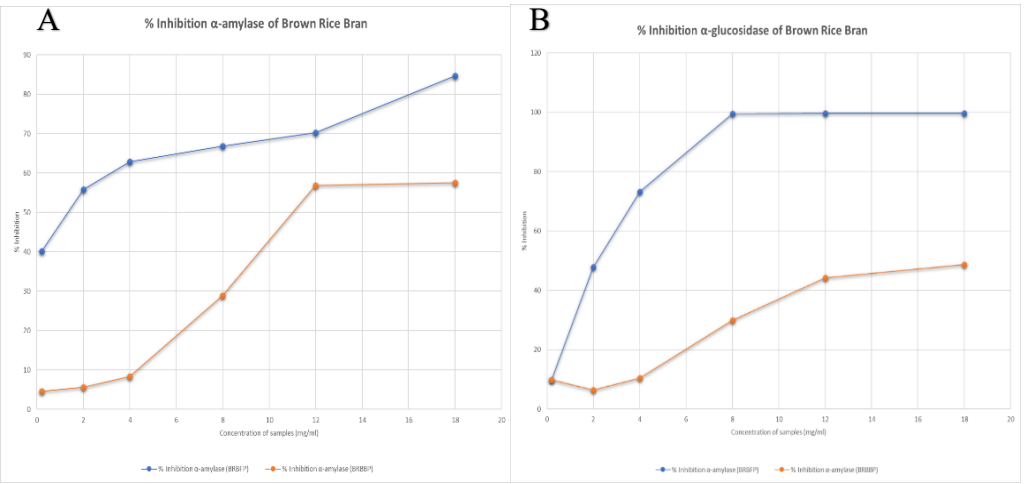

Figure 3. the α-amylase (A) and α-glucosidase (B) inhibitory capacity of free (---) and bound (---) phenolic extracts

Correlation between phytochemical, antioxidant activity and in vitro starch digestibility

Pearson correlation analysis was employed to investigate the correlations among phytochemical components, antioxidant activity, enzymatic digestibility inhibition, and *in vitro* starch digestion of both phenolic extracts of brown rice bran. The correlations were significantly differed at the 0.01 level (two-tailed) as presented in Table 1. Total phenolic, flavonoid, and anthocyanin content exhibited highly positive correlations with antioxidant activity ($r > 0.980$, for DPPH and FRAP). Moreover, the variables representing enzymatic digestibility, as indicated by IC_{50} values, showed negative correlations with phytochemical and antioxidant activity, indicating that higher levels of TPC, TFC, and TAC were associated with stronger inhibition capacity. This higher inhibitory activity resulted in a lower digestion rate of rice starch, as demonstrated in Table 1. The R value showed a strong correlation with the digestion rate ($r = 0.980$) for the inhibition of α-amylase and α-glucosidase activity.

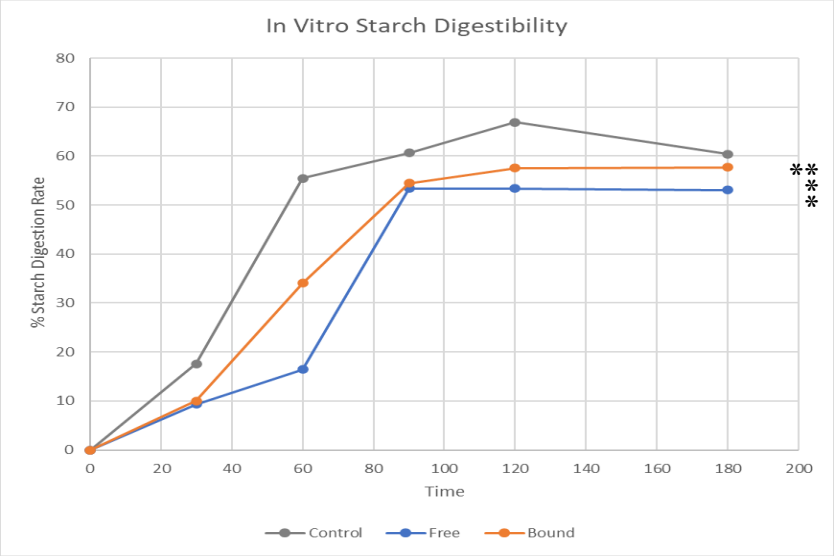

Figure 4. The rice starch digestibility with the addition of samples extracts. Data are evaluated by one-way ANOVA and followed by Tukey test (*)(***), $p < 0.05$, 0.001

Table 1. Pearson Correlations of Phytochemical content, antioxidant activity and enzymatic inhibition and *in vitro* starch digestibility (free and bound rice bran extract)

Variable	TPC	TFC	TAC	DPPH	FRAP	Inhibition of alpha amylase	Inhibition of alpha glucosidase	Digestibility rate
TPC	1							
TFC	.982**	1						
TAC	.992**	.965**	1					
DPPH	.997**	.982**	.985**	1				
FRAP	.998**	.982**	.988**	1.000**	1			
Inhibition of alpha amylase	-.997**	-.980**	-.985**	-1.000**	-1.000**	1		
Inhibition of alpha glucosidase	-.996**	-.979**	-.984**	-1.000**	-.999**	1.000**	1	
Digestibility rate	-.968**	-.968**	-.967**	-.981**	-.977**	.980**	.980**	1

** Data is significant at the 0.01 level (two-tailed).

Discussion

In this study, the form of the free phenolic extract exhibited a higher content of TPC, TFC, and TAC. These results were similar to the previous study reported with the sample of pigmented rice in whole grains or bran parts (Ghasemzadeh *et al*., 2018; Ti *et al.*, 2014; Wu *et al*., 2018), while some studies revealed different results as the bound phenolic possessed higher phytochemical compound of some natural sources extracts such as red quinoa, and pigmented rice (Pang *et al*., 2018; Zhang *et al*., 2022). The variations in the content pattern of free and bound phenolic compounds between pigmented rice grain and rice bran can be explained by the differences in their strains or varieties. Following the antioxidant activity, a previous study reported that brown rice bran showed higher levels of phenolic compounds, namely syringic acid, cinnamic acid, p-coumaric acid, and ferulic acid, distributed in the free form, which contributed to its superior free radical scavenging (Ghasemzadeh *et al.*, 2018). Additionally, rice bran with darker pigmentation exhibited higher amounts of bioactive compounds compared to regular brown rice bran (Boue *et al*., 2016; Pang *et al.*, 2018; Shao *et al*., 2014; Wu *et al*., 2018).

The inhibitory activity of enzymes is known due to the enzyme's ability to bind with phenolic compounds through non-covalent interactions or hydrophobic associations (Aleixandre *et al.,* 2022; Ali Asgar, 2013). The observed interaction forces are predominantly attributed to hydrogen bonding between hydroxyl groups and the catalytic site, as well as hydrophobic interactions involving the aromatic rings of phenolic compounds (Giuberti *et al.*, 2020). Additionally, hydroxycinnamic acids, such as caffeic, chlorogenic, ferulic, p-coumaric, and sinapic acids, possess a structural motif wherein a C=C double bond is conjugated with a carbonyl group. This specific structural arrangement is instrumental in enhancing the stability of their binding forces to the active site of α-amylase, thus contributing to their inhibitory effects on the enzyme's activity. The enzyme alpha-glucosidase plays a vital role in the last stage of carbohydrate digestion. Researchers have found that a particular flavonoid, containing two catechol groups in both A-rings and B-rings, in addition to a 3-OH group at the C-ring, is the most powerful inhibitor of a-glucosidase (Aleixandre *et al.*, 2022).

The rate of starch digestion is highly influenced by the activity of digestive enzymes that break down starch into maltooligosaccharides, and eventually glucose. Therefore, with a high inhibition of digestive enzyme activity, there is a significant decrease in the starch digestion rate, especially when 10% of bound and free extracts from brown rice bran are added compared to the control. Additionally, this could be due to the direct influence of polyphenols-starch interactions, which can also lower the rate of starch digestibility. The

previous studies showed that phenolic from natural sources such as blackcurrant, strawberry, gac fruit, persimmon reduced reduced the starch digestibility (Chusak *et al*., 2020; Li *et al*., 2018; Mu *et al*., 2021). This is also supported by a significant pearson correlation, indicating the impact of phenolic compounds and flavonoids, which are highly effective in inhibiting digestive enzyme activity and reducing the rate of starch digestion.

Nonetheless, the interaction between starch and polyphenols could potentially be impacted by factors like the varieties and molecular structure of polyphenols, the characteristics of the starch, and the conditions for complexing starch and polyphenols employed, which could have influenced the way in which the digestion enzyme inhibition occurred. Where the mode of inhibition can be classified into competitive, non-competitive, and uncompetitive. In the future, researchers plan to investigate the enzymatic inhibition kinetics and conduct a thorough characterization of phenolic compounds. This undertaking aims to gain a comprehensive understanding of the inhibition mechanisms, which, in turn, could potentially contribute to the effective reduction of starch digestion. The results of this study could have significant implications in the field of nutrition and potentially enhance the knowledge of approaches for managing starch digestion and related metabolic processes.

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