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Antioxidant activity and inhibitory efficacy of *Citrus grandis* peel extract against carbohydrate digestive enzymes *in vitro*

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Abstract

Citrus peel extract has been observed with many beneficial-promoting effects. Pomelo (*Citrus grandis* (L) Osbeck) peel extracts (PPE) from the flavedo and albedo of two varieties namely Long Co Co and Thanh Kieu were investigated. In this study, the qualitative analysis indicated the presence of phytochemical groups including alkaloids, flavonoids, saponins, and tannin in the PPE from the flavedo and albedo. The PPE from the Thanh Kieu flavedo was found to possess the highest antioxidant activity at the EC_{50} values of 60.89 ± 0.31 µg/mL, 60.69 ± 0.21 µg/mL, and 24.16±0.06 µg/mL for DPPH, ABTS, and FRAP assay. However, the Long Co Co albedo- PPE induced better inhibitory effect against α-amylase (EC 232-560-9) and α-glucosidase (EC 232-604-7) with respect to EC_{50} values of 3.59 ± 0.02 mg/mL and 80.77 ± 0.34 µg/mL. This result suggested potential in the anti-hyperglycemic effect of pomelo peel extract from the flavedo and albedo that can serve as a nutraceutical in food and pharmaceutical industries.

Keywords: antioxidant activity; albedo; flavedo; pomelo peel extract; α-amylase inhibition; α-glucosidase inhibition.

Practical application: Potential in anti-hyperglycemic treatment by inactivating carbohydrate digestive enzymes of PPE.

1 Introduction

Diabetes mellitus, dividing into type 1 (insulin-dependent) and type 2 (non-insulin-dependent), has seriously affected more than 170 million people around the world (Shen et al., 2012). Type 2 diabetes, which is aligned to the elevated blood glucose or insulin intolerance by insulin resistance or debilitated insulin secretion rate, contributes to 90% of diabetic cases from adults (Jia et al., 2015; Kang et al., 2014). The alleviation of hyperglycemia via inhibition of carbohydrate digestive enzymes by using α-glucosidase inhibitors has been widely used to manage the diabetes (Kang et al., 2014). Miglitol, acarbose, or voglibose as α-glucosidase inhibitors for oral anti-diabetes (Şöhretoğlu & Sari, 2019) functionally delay the glucose uptake, leading to the alleviation of hyperglycemia. However, these chemical drugs may expose many adverse side effects in long-term use such as diarrhea, stomach pain, or abdominal pain (Kang et al., 2014). Currently, it has been currently drawing attention of researchers to find natural alternatives for anti-hyperglycemia. Phytochemicals, mainly polyphenols, are plant-derived compounds that possess many health-promoting properties (Garza et al., 2013). Polyphenolic groups from plant extract have been considered active-agents in the inhibition of α-glucosidase, contributing to the control of hyperglycemia (Podsędek et al., 2014; Şöhretoğlu & Sari, 2019). Garza et al. (2013) have noted that flavonoids in plant extract are safe compounds for impairing α-amylase and α-glucosidase activities with minimal gastrointestinal side effects.

Citrus peels, an agricultural waste of citrus production, contain a valuable source of polyphenols which have been observed to reveal a potential in anti-hyperglycemia (Fayek et al., 2017). Citrus flavonoids including hesperidin, neohesperidin, and naringin extracted from the flavedo and albedo promoted anti-hyperglycemic effects in HepG2 cells (Shen et al., 2012). Orange peels extract was observed to exert the anti-hyperglycemic potential in diabetes-induced rats (Ahmed et al., 2017). The elevated blood glucose level of Streptozotocin-induced diabetic mice was attenuated by the treatment of *Citrus limetta* peel extract (Kundusen et al., 2011). Pomelo [*Citrus grandis* (L) Osbeck] peels, among citrus fruits, have been highlighted with a rich source of polyphenols and flavonoids (Tocmo et al., 2020). These compounds may also exhibit many potentials in the anti-hyperglycemic effect. Long Co Co and Thanh Kieu pomelo varieties are fruit specialty from the Mekong delta, Vietnam with relatively high production, leading to the disposal of large amounts of pomelo peels. Therefore, this study aimed to utilize the pomelo peel extract (PPE) from the flavedo and albedo of Long Co Co and Thanh Kieu varieties to investigate *in vitro* anti-hyperglycemic effect via the inhibition of α-amylase and

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α-glucosidase. The revealed finding could possibly contribute to developing functional food products or pharmaceutical drugs in anti-diabetes as well as tackling the overburden of agricultural waste from pomelo peels. The characterization of phytochemical content and antioxidant activity of PPE from the flavedo and albedo were also evaluated and compared.

2 Materials and methodologies

2.1 Materials

Pomelo fruits were selected for harvesting between April and July. The Long Co Co pomelo was yielded at Tien Giang province, Vietnam. The Thanh Kieu pomelo was supplied from the local farm in Can Tho City. Standard chemicals such as Folin-ciocalteu (F-C), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,4,6 tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, α-amylase from *Bacillus amyloliquefaciens* (EC 232-560-9, ≥250 U/g) and α-glucosidase from *Saccharomyces cerevisiae* (EC 232-604-7, ≥100 U/mg) proteinwere purchased from Merck KGaA (Darmstadt, Germany). Acarbose and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were from Fujifilm Wako Pure Chemicals Ltd., Japan. All other analytical chemicals were supported from the standard commercial supplies.

2.2 Preparation of ethanolic pomelo peel extract

Pomelo peels are constituted by two parts including the flavedo (thin greenish peel) and the albedo (thick white peel). The pomelo peel extract (PPE) was collected from the flavedo and albedo of each variety by using ethanolic extraction. These peels were dried at 50 °C for 4 h then uniformly ground to obtain the fine powder (Zarina & Tan, 2013). The fine powder from the flavedo and albedo was mixed with ethanol solution at the ratio of 1:4 (w/v) and 1:2 (w/v), respectively. The mixture was kept for 2 h then filtrated by filter paper (Whatman No 1). This filtration step was repeated at least 5 times to achieve the highest yield of PPE. The filtrate volume of 350 mL was then concentrated at 60 °C for 45 min by using a rotary evaporator (IKA RV-3V, IKA®-Werke GmbH & CO. KG, Staufen, Germany). The obtained PPE was subsequently stored and prepared for further experiments (Abudayeh et al., 2019; Ani & Abel, 2018).

2.3 Qualitative analysis of phytochemical constituents in the PPE

The qualitative analysis of phytochemical constituents including alkaloids, flavonoids, saponins, and tannin in the PPE from the flavedo and albedo was carried out following the methods from previous studies (Evans, 2002; Sofowora, 1993). For alkaloids, the Dragendorff reagent solution was dropped to the PPE solution until the appearance of brown-red precipitate, indicating the presence of alkaloids. To determine the presence of flavonoids, 2 mL of 10% NaOH was included with 3 mL of PPE. The indication of flavonoids in the PPE was observed by the yellow color of the mixture and the mixture became colorless when adding HCl solution. The formation of bubbles when stirring the mixture of PPE (2 mL) and distilled water (10 mL) for 30 sec suggested the presence of saponins in the PPE. Finally, the appearance of precipitate when adding the mixture solution of 1% gelatin and 10% NaCl to the PPE solution indicated the presence of tannin in the PPE.

2.4 Total polyphenolic content determination

The total polyphenolic content (TPC) of the PPE from the flavedo and albedo of each variety was evaluated using Folin-Ciocalteu assay. The mixture solution of PPE (0.1 mL) and 10% Foli-Ciocalteu reagent (1.5 mL) was allowed to react for 5 min. Then, 4 mL of 20% Na_2CO_3 and 4.4 mL of distilled water were included to the mixture. Prior to measuring the absorbance at 725 nm by using a 722-Visible spectrophotometer (China Yangzhou Wandong Medical Co., Ltd, China), the mixture was incubated in dark condition for 30 min. The TPC was expressed as weight of gallic acid equivalent (GAE) per weight of dry matter of the sample (mg GAE/g DM). The standard curve of gallic acid was built at the concentration range from 2 μg/mL to 14 μg/mL with R^2 of 0.9957 (Rahman et al., 2018).

2.5 Determination of DPPH radicals scavenging effect

The DPPH radicals scavenging effect of PPE was observed by the reduction in DPPH absorbance. The PPE (960 μL) at varying concentrations (100-1000 μg/mL in ethanol) was mixed with 40 μL of DPPH (1 mg/mL) for 30 min at 30 °C in the dark. The absorbance was then read at 517 nm and a control sample was the mixture of DPPH and ethanol. Vitamin C served as a positive control. The effective concentration at 50% antioxidant activity (EC_{50}) was calculated by constructing the PPE concentration versus the corresponding DPPH absorbance reduction (Abudayeh et al., 2019).

2.6 Determination of ABTS radicals scavenging effect

ABTS radicals scavenging effect was measured following the method of Nenadis et al. (2004). The $ABTS^{\cdot+}$ solution including 7 mM ABTS (2 mL) and 2.45 mM $K_2S_2O_8$ (2 mL) was allowed to react for 16 h in a dark environment and then adjusted to the absorbance value of 0.7 at 734 nm. Vitamin C served as a positive control. Then, an aliquot (10 μL) of the PPE at varying concentrations was mixed with $ABTS^{\cdot+}$ solution (990 μL) and kept for 6 min in the dark. The absorbance was recorded at 734 nm and the EC_{50} values was calculated by constructing the graph of PPE concentration versus ABTS absorbance reduction.

2.7 Measurement of ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) measurement was followed by the method of Rufino et al. (2010) with modifications. The FRAP solution was prepared by mixing 10 mM 2,4,6 tripyridyl-s-triazine (TPTZ) (0.5 mL) and 20 mM $FeCl_3.6H_2O$ (0.5 mL) in acetate buffer (300 mM, pH 3.6) at 37 °C for 5 min. The mixture of PPE (20 μL) at varying concentrations and 980 μL of FRAP solution was incubated for 5 min at 37 °C. The absorbance values were recorded at 593 nm and the positive control was Vitamin C. The standard calibration curve of PPE

concentration versus the corresponding absorbance increase was plotted to determine EC_{50} values.

2.8 Inactivation of α-amylase

The inhibitory effect of PPE against α-amylase was evaluated by a starch-iodine assay with adjustments (Sudha et al., 2011). Phosphate buffer solution (50 μL) was mixed with α-amylase solution (50 μL) at 37 °C for 5 min followed by the inclusion of 50 μL of starch (2 mg/mL). The mixture was incubated for 15 min at 37 °C and 200 μL of HCl was used to stop the reaction. Iodine solution was then added to the mixture and the absorbance was read at 660 nm. Acarbose served as a positive control. The effective concentration at 50% inhibitory effect (EC_{50}) was governed by building the standard curve of PPE concentration versus corresponding α-amylase inhibitory percentage.

2.9 Inactivation of α-glucosidase

The inhibitory effect of PPE against α-glucosidase was investigated upon the method done by Shai et al. (2011) with some changes. The mixture including α-glucosidase (20 μL), phosphate buffer (100 mM, ph 6.8) (100 μL), and PPE (40 μL) was allowed to react at 37 °C for 15 min. An aliquot (40 μL) of 5 mM p-nitro-phenyl-α-D-glucopyranoside was dropped to the mixture and incubated for 20 min at 37 °C. An aliquot of 0.1 M Na_2CO_3 (100 μL) was then used to stop reaction. The absorbance values were recorded at 405 nm and Acarbose was a positive control. The EC_{50} values were determined by constructing a standard curve of PPE concentration vs. corresponding enzyme inhibitory percentage.

2.10 Statistical analysis

Each experimental data was in three replicates. SPSS software 20.0 (IBM Corp., Armonk, Newyork, USA) was utilized to conduct one-way analysis of variance (ANOVA) and Tukey's HSD was applied to compare the mean values at the level of 5% ($p < 0.05$).

3 Result and discussion

3.1 Phytochemical groups in the PPE

The presence of phytochemical groups in the PPE from the flavedo and albedo of the two cultivars is listed in Table 1. All the tested compounds including alkaloids, flavonoids, saponins, and tannin showed positive results (+), indicating their presence in the PPE. The phytochemical compounds serve a key role in protecting plants against environmental conditions. Meanwhile, the consumption of these compounds has been observed with many health-beneficial effects. Alkaloids were reported to possess anti-inflammatory, anti-virus, anti-cancer, or antimicrobial activities (Aberoumand, 2012). Flavonoids have been well-known for antioxidant activity, anti-cancer, anti-inflammatory activities, etc. (Tocmo et al., 2020). Saponin in the PPE was also noted to alleviate the glucose uptake and cholesterol level in the bloodstream (Ani & Abel, 2018). In addition, tannin was found to contribute to the inhibition of digestible enzyme activities or the prevention of oxidative stress (Tapiero et al., 2002). The variations in extraction methods or different cultivars have been reported to exhibit different phytochemical constituents in the extract. In this study, the PPE showed the presence of saponin and flavonoids, whereas these compounds were not presented in the methanolic pomelo peel extract from a study done by Balamurugan et al. (2014). Khan (2018) indicated that alkaloids, reducing sugar, and carbohydrates were easily recognized in the PPE by the Soxhlet extraction method. The maceration method, however, showed only the presence of alkaloids and terpenoids.

3.2 Total polyphenolic content of PPE

Screening the presence of phytochemical compounds is an essential step to provide a basic understanding to researchers about the studied materials. While nutritive compounds including protein, carbohydrate, lipid, amino acids are the primary metabolites, polyphenols are considered the secondary metabolites with many health-beneficial impacts (Khan, 2018). The TPC of the flavedo and albedo PPE from two varieties are described in Figure 1. Between the two cultivars, the TPC in the Thanh Kieu variety was found to be considerably higher than the Long Co Co variety. The TPC from the flavedo PPE was noticeably higher than the albedo PPE ($p < 0.05$). In this study, the Thanh Kieu flavedo PPE was observed with the highest TPC

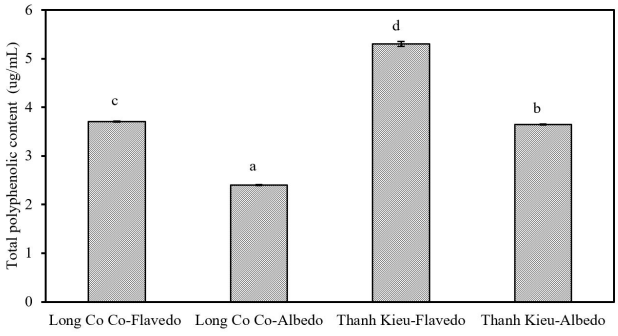

Figure 1. The total polyphenolic content of the flavedo and albedo PPE from two cultivars. Letters (a, b, c, d) present statistically significant difference between mean values ($p < 0.05$).

Table 1. The presence of phytochemical compounds in the flavedo and albedo PPE.

Pomelo variety		Alkaloids	Flavonoids	Saponins	Tannin
Long Co Co	Flavedo	+	+	+	+
	Albedo	+	+	+	+
Thanh Kieu	Flavedo	+	+	+	+
	Albedo	+	+	+	+

"+" indicates the presence of phytochemical compounds; "-" indicates the absence of phytochemical compounds.

of 5.30 ± 0.05 mg GAE/g DM. The result was consistent with past studies that the TPC from the flavedo was normally higher than the albedo (Tsai & Wong, 2019; Xiao et al., 2021). The TPC in the pomelo peels from previous reports was found to be 3-4 folds higher than our study. The TPC of PPE was observed at 17.9 ± 0.19 mg GAE/g DM in the study of Ani & Abel (2018). The pomelo peel was observed with the TPC of 18.76 ± 1.24 mg GAE/g DM (Rahman et al., 2018). However, the result in this study was comparable to other studies in the same cultivating region of Southeast Asia. The TPC in the flavedo and albedo of 7 pomelo cultivars in Thailand was in the range of 1.0-3.4 mg GAE/g DM (Pichaiyongvongdee et al., 2014). The TPC in the Da Xanh, Nam Roi, and Tan Trieu variety in Mekong Delta, Vietnam was fairly similar to our result with respective TPC in the range of 4-7 mg GAE/g DM (van Hung et al., 2020). These variations of TPC were apparently accredited by genetic factors, weather conditions, maturity, cultivation, post-harvesting, and storage conditions (Rahman et al., 2018).

3.3 Antioxidant activity of PPE

In this study, the antioxidant capacities of PPE from the flavedo and albedo were evaluated via the possibility of donating electron or hydrogen to scavenge DPPH radicals (according to the color change of DPPH solution from purple to yellow), ABTS radicals (following the color change of ABTS solution from blue to colorless) (Abudayeh et al., 2019) and reducing ferric (III) ions to ferrous (II) ions (based on the increase in blue intensity) (Castro-Vazquez et al., 2016). The effective concentrations at 50% antioxidant activity of PPE from the flavedo and albedo of two varieties are noted in Table 2. The lowest EC_{50} values at 60.89 ± 0.31 μg/mL, 60.69 ± 0.21 μg/mL, and 24.16 ± 0.06 μg/mL from DPPH, ABTS, and FRAP assay were assigned to the Thanh Kieu flavedo-PPE, indicating the highest antioxidant activity (Chang & Azrina, 2017). In this study, each evaluation method was found to result in different EC_{50} values, which could stem from the discrepancy in the mechanism of radicals scavenging for DPPH, ABTS radicals, and ferric reducing powers of PPE (Huang et al., 2005). As compared to the positive control (Vitamin C), the EC values of PPE were considerably higher than those of Vitamin C. The vitamin C required a very low dose of 1.56 – 5.67 μg/mL to exhibit antioxidant activity at 50%. Azman et al. (2019) noted that vitamin C is a strong antioxidant, exhibiting very high radicals scavenging effects. The lower efficacy of antioxidant activity from the PPE was possibly ascribed to the purity of the PPE as the PPE contained not only antioxidants (flavonoids) but also other compounds such as cellulose, hemicellulose, soluble sugar, and limonene, attenuating the antioxidant efficacy of PPE (Tocmo et al., 2020). Therefore, further purification processes should be conducted to enhance the antioxidant activity of the PPE.

In a comparison of antioxidant capacity between the flavedo and albedo PPE, the antioxidant activity of the flavedo PPE was observed with noticeably higher than that of the albedo PPE according three assays ($p < 0.05$). This observed trend was compatible with reportedly previous studies (Chang & Azrina, 2017; Tsai & Wong, 2019). The higher TPC in the flavedo PPE compared to that in the albedo PPE could associate to the higher antioxidant capacity. It was stated that the polyphenols were mainly responsible for the antioxidant activity of citrus fruits (Sun et al., 2002). Pichaiyongvongdee et al. (2014) indicated that the higher polyphenolic content could result in higher antioxidant capacity. Between the two cultivars, the Thanh Kieu PPE was recorded to have higher antioxidant activity in accordance with higher TPC compared to the Long Co Co PPE. The discrepancy in the antioxidant activity could possibly be ascribed to the genetic factors, climate conditions, cultivation, maturity stage, or post-harvesting method (Rahman et al., 2018; Toh et al., 2013). Besides, the interaction between polyphenols in PPE of each variety could promote the synergistic effects of antioxidant activity in which strong interaction between polyphenolic components could lead to better scavenging effect (Lu & Foo, 2001).

3.4 Anti-hyperglycemic effect of PPE in vitro

A broad range of therapeutic strategies has been widely used for type 2 diabetes therapy based on stimulation of endogenous insulin secretion, enhancing the sensitivity of insulin activity or the attenuation of starch metabolism via the inactivation of α-amylase and α-glucosidase (P et al., 2011). The inhibition of these digestive enzymes caused a delay in carbohydrate digestion, leading to a reduction of glucose uptake as well as lowering the elevated blood glucose levels (Sudha et al., 2011; Podsędek et al., 2014). Thus, the finding from inhibitory effects of these enzymes *in vitro* could be promising evidence for further in vivo studies in controlling type 2 diabetic syndromes. In this study, the PPE from the flavedo and albedo of the two cultivars showed a positive result in inactivating α-amylase and α-glucosidase. Effective concentrations at 50% inhibitory effect of each PPE from the flavedo and albedo of two cultivars are presented in Figure 2. Acarbose showed the highest activity of an inhibitor

Table 2. Effective concentration at 50% antioxidant activity of the flavedo PPE and albedo PPE from two cultivars corresponding to DPPH, ATBS scavenging effect and ferric reducing antioxidant power.

	Pomelo variety	EC_{50} (μg/mL)		
		DPPH	ABTS	FRAP
Flavedo	Long Co Co	71.11 ± 0.26^{d}	71.23 ± 0.20^{c}	34.86 ± 0.28^{c}
	Thanh Kieu	60.89 ± 0.31^{b}	60.69 ± 0.21^{b}	24.16 ± 0.06^{b}
Albedo	Long Co Co	81.63 ± 0.48^{e}	98.53 ± 1.06^{e}	76.51 ± 0.02^{e}
	Thanh Kieu	68.59 ± 0.29^{c}	80.51 ± 0.94^{d}	51.25 ± 0.29^{d}
Positive control (Vitamin C)		5.67 ± 0.02^{a}	3.71 ± 0.02^{a}	1.56 ± 0.00^{a}

The listed data are presented as mean ± SD. Superscripts ($^{a, b, c, d, e}$) indicate the significant difference of mean values within the same column at the level of 5% ($p < 0.05$).

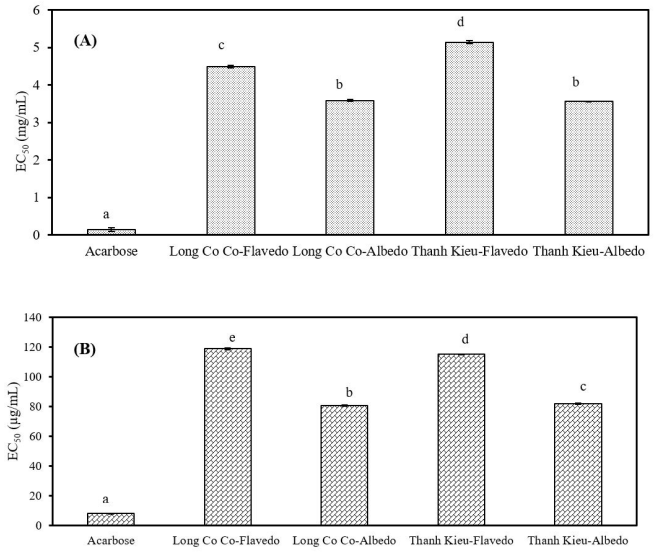

Figure 2. Effective concentration at 50% inhibitory effect of the PPE from the flavedo and albedo of two varieties against A) α-amylase and B) α-glucosidase. Superscripts (a, b, c, d) illustrate the significant difference of mean values ($p < 0.05$).

against both α-amylase and α-glucosidase at the EC_{50} values of 0.15 ± 0.05 mg/mL and 8.24 ± 0.02 µm/mL, respectively. The albedo PPE at EC_{50} values around 3.55 mg/mL was observed with higher inhibitory effect against α-amylase compared to the flavedo PPE (EC_{50}: 4.5-5.2 mg/mL). Meanwhile, α-glucosidase, which was found to susceptible to these treatments, required a lower dosage at the EC_{50} values ranging from 80 µg/mL to 120 µg/mL to show the inhibitory effect. The inhibitory effect was ascribed to the presence of phytochemical components in the extract especially flavonoids (Podsędek et al., 2014; Proença et al., 2019; Sahnoun et al., 2017). The molecular structures of these phytochemicals, composed of -OH groups at 3'- and 4'- positions of B-ring, and at 5- and 7- position of A-ring, or C2=C3 double bond, were observed to cause the inhibitory effect via the competitive inhibition (Podsędek et al., 2014; Proença et al., 2019). Muhtadi et al. (2015) also noted that the flavonoids including naringin and hesperidin in the orange peel were responsible for the antidiabetic effect.

It was stated that the inhibitory effects of these enzymes were highly associated with the TPC in the extract (Kwon et al., 2008; Tsai & Wong, 2019). However, in this study, although the TPC in the flavedo PPE was found to be higher than that in the albedo PPE, the albedo PPE appeared to induce higher inhibitory effect against α-amylase and α-glucosidase. The Long Co Co albedo-PPE showed the highest inhibitory capacity against α-amylase and α-glucosidase with the EC_{50} values of 3.59 ± 0.02 mg/mL and 80.77 ± 0.34 µg/mL, respectively. Similarly, the inhibitory activities of PPE from the Long Co Co and Thanh Kieu varieties were fairly uncorrelated to their TPC. This could possibly be due to the interaction of phenolic constituents in the polyphenolic groups rather than considering their TPC in the extract. The phenolic compositions and their variations in molecular structures between different subclasses of polyphenols, the synergistic effects of different phenolic groups or within the same phenolic group could significantly cause a high impact on the inhibitory effects against these carbohydrate digestive enzymes (Podsędek et al., 2014; Şöhretoğlu & Sari, 2019).

4 Conclusion

In this study, the Thanh Kieu flavedo-PPE showed the highest antioxidant capacity in correlation with the highest TPC compared to the others. The PPE from two varieties showed a significant effect in the inactivation of α-amylase and α-glucosidase. However, the albedo PPE was observed to promote a better inhibitory action against these enzymes compared to the flavedo PPE regardless of its lower TPC. The results confirmed the applicability of PPE from the flavedo and albedo of two cultivars (Long Co Co and Thanh Kieu) in the treatment of hyperglycemia via the inhibition of carbohydrate digestive enzymes. Further studies need to characterize the primary phytochemical constituents to give insights into the inhibitory effects of PPE against these carbohydrate enzymes.

Conflict of interest

Authors declare that there is no conflict of interest.

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