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Augmentation of α-amylase and α-glucosidase inhibitory properties of *Callerya speciosa* root extracts: An exploration through simulated human gastrointestinal digestion

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Abstract

The present work represents the first effort to evaluate the effect of an *in vitro* simulated human digestion model on the α-amylase and α-glucosidase inhibitory activities of *Callerya speciosa* root extracts. Through bioguided fractionation, a series of fractions were obtained from hexane (CSH) and ethyl acetate (CSE) extracts. Initial screening identified active fractions (CSH2, CSH3, CSE2, CSE3, and CSE4) for further *in vitro* digestion evaluation. The results showed a decrease in antioxidant activity across all samples after digestion, while a simultaneous increase in α-amylase and α-glucosidase inhibitory activities was observed during the small intestinal phase. Notably, compared with the undigested fraction, CSE3 demonstrated a 1.7-fold increase in α-amylase inhibition (IC_{50} = 0.86 mg/mL). Moreover, the α-glucosidase inhibitory activities of CSH3, CSE3, and CSE4 were significantly greater after digestion, with IC_{50} values of 0.18, 0.05, and 0.02 mg/mL, respectively. Impressively, during the small intestinal phase, the α-glucosidase inhibitory activities of CSH3, CSE3, and CSE4 were substantially greater than those of acarbose by 13.2-, 47.6-, and 119.0-fold, respectively, and greater than those of palmitic acid by 1.7-, 6.0-, and 15.0-fold, respectively. GC-MS and LC-ESI-MS/MS analyses identified isoflavonoids, fatty acids, and triterpenoids as key bioactive compounds in these fractions. The present work provided foundational insights for the future exploration of *C. speciosa* root extracts for diabetes management.

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Introduction

Diabetes mellitus is a chronic disease characterised by an excess of blood glucose (hyperglycaemia), which over time results in various complications, including cardiovascular disorders, atherosclerosis, myocardial infarction, stroke, blindness, renal failure, and nerve damage (Brownlee, 2001). According to the International Diabetes Federation (IDF), 537 million adults aged 20 to 79 years worldwide were diagnosed with diabetes in 2021, and 6.7 million deaths (one every five seconds) were reported (IDF, 2023). Type 2 diabetes (type 2 diabetes mellitus, T2DM) is the most common, accounting for 90% of all diabetes cases. T2DM patients cannot lower glucose levels efficiently because of a defect in insulin secretion or insulin resistance. The pathogenesis of T2DM is mainly attributed to obesity, physical inactivity, and impaired glucose tolerance; however, oxidative stress has

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recently been suggested to be one of the determinants of T2DM (Giacco and Brownlee, 2010). Therefore, therapeutic strategies for T2DM principally focus on reducing blood glucose levels, and regulating oxygen reactive species (ROS) production and accumulation in the body. A cohort study revealed that the dietary intake of antioxidants might reduce the risk of T2DM (Montonen *et al.*, 2004). On the other hand, pharmacological studies indicate that inhibiting α-amylase and α-glucosidase involved in carbohydrate digestion is the most prevalent approach for reducing blood glucose levels. α-Amylase and α-glucosidase inhibitors decelerate the hydrolysis of polysaccharides, which consequently attenuates free glucose, a cause of postprandial hyperglycaemia. Although the present synthetic drugs are effective in treating T2DM, they can potentially cause side effects, including gastrointestinal disorders (Drucker *et al.*, 2010) and cardiovascular abnormalities (Wijnen *et al.*, 2022). Hence, seeking alternative agents that have simultaneous potent antioxidant, anti-α-amylase and anti-α-glucosidase properties, but are safe for humans, is the leading task in developing current T2DM treatments. Among promising sources, natural products, especially those derived from traditional medicinal plants, are recommended due to their long history of use as treatments, and their safety for human health.

Callerya speciosa (Champ.) Schot (syn. *Millettia speciosa*) is a well-known medicinal plant belonging to the Leguminosae family, and widely distributed in southern China and Southeast Asian countries (Yin *et al.*, 2008). *C. speciosa* has been traditionally used for treating rheumatoid arthritis; bone and joint diseases; anaemia; increased blood circulation; irregular menstruation; hepatitis; and chronic bronchitis (Yin *et al.*, 2008; Chen *et al.*, 2015). In addition, its roots have been used for food and the healing of respiratory, liver, and kidney problems (Xu *et al.*, 2016). In Vietnam, *C. speciosa* has been widely grown in northern provinces, and used as a folk medicine to treat rheumatism, chronic bronchitis, and hepatitis (Thien *et al.*, 2020). Scientific studies have reported the increased pharmaceutical potential of *C. speciosa*, such as its immunoregulatory, immunostimulant (Chen *et al.*, 2021), antioxidative (Huang *et al.*, 2022), antifatigue (Zhao *et al.*, 2015), anti-obesity (Wang *et al.*, 2022), and anti-blood cancer (Lam *et al.*, 2022) activities. Despite possessing a wide range of pharmaceutical properties, comprehensive studies on the antidiabetic potential of phytocompounds derived from *C. speciosa* are still rather modest. In a recent study, pedunculoside, uvaol, ursolic acid, and rutin from the roots of *C. speciosa* plants grown in Vietnam were reported to have potent anti-α-glucosidase effects (Tuan *et al.*, 2022). In another study, an ethanolic extract of *C. speciosa* collected in China was shown to ameliorate glycolipid metabolism, which is associated with its antidiabetic properties (Zhang *et al.*, 2021). However, to the best of our knowledge, no research has investigated the effect of digestion on the antioxidant and antidiabetic activities of *C. speciosa*. Therefore, the present work aimed to evaluate the effects of *in vitro* simulated gastrointestinal digestion on the antioxidant, α-amylase, and α-glucosidase activities of *C. speciosa* root extracts.

Materials and methods

Chemicals

The solvents used for the isolation and extraction processes were purchased from Junsei Chemical Co., Ltd., Tokyo, Japan. 2,2-Diphenyl-1-picrylhydrazine (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), and butylated hydroxytoluene (BHT) were purchased from Kanto Chemical Co., Inc., Tokyo, Japan. Porcine pancreas-derived α-amylase, *Saccharomyces cerevisiae*-derived α-glucosidase, soluble starch, *p*-nitrophenyl-α-D-glucopyranoside (pNPG), silica gel, and all buffer constituents were purchased from Sigma-Aldrich, St. Louis, MO, USA. Acarbose, palmitic acid, and iodine solutions were purchased from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan. Thin-layer chromatography (TLC) plates (0.25 mm thick) and silica gel (70 - 230 mesh) were purchased from Merck, Darmstadt, Germany.

Plant materials

The *C. speciosa* samples were collected in the mountainous area of Tan Yen district, Bac Giang Province, Vietnam, in 2019. The samples were identified based on phenotypic characteristics, and confirmed by all the authors. The specimen voucher Casp_AGI2019 was deposited at the Agricultural Genetics Institute, Hanoi, Vietnam. The *C. speciosa* roots were separated from other plant parts, and prewashed with tap water several times before drying under sunlight for 7 d.

Sample preparation and extraction

Three kilograms of the pre-dried *C. speciosa* roots were washed with distilled water, and then soaked in 0.5% sodium hypochlorite to remove contaminants. Finally, the samples were washed with distilled water before they were dried in a convection oven at 40°C for 1 w. Afterward, the obtained dried roots (1.3 kg) were ground well in a blender, and then extracted with 3 L of methanol for 1 w. The extraction was performed three times. All extracts were then combined and filtered. After filtration, the filtrate was evaporated under vacuum at 50°C to obtain the crude methanolic extract (CSM, 224 g). Subsequently, the CSM was mixed with 200 mL of distilled water, and then subjected to liquid-liquid phase extractions with hexane and ethyl acetate (EtOAc), respectively. The detailed extraction and isolation process is shown in Figure 1.

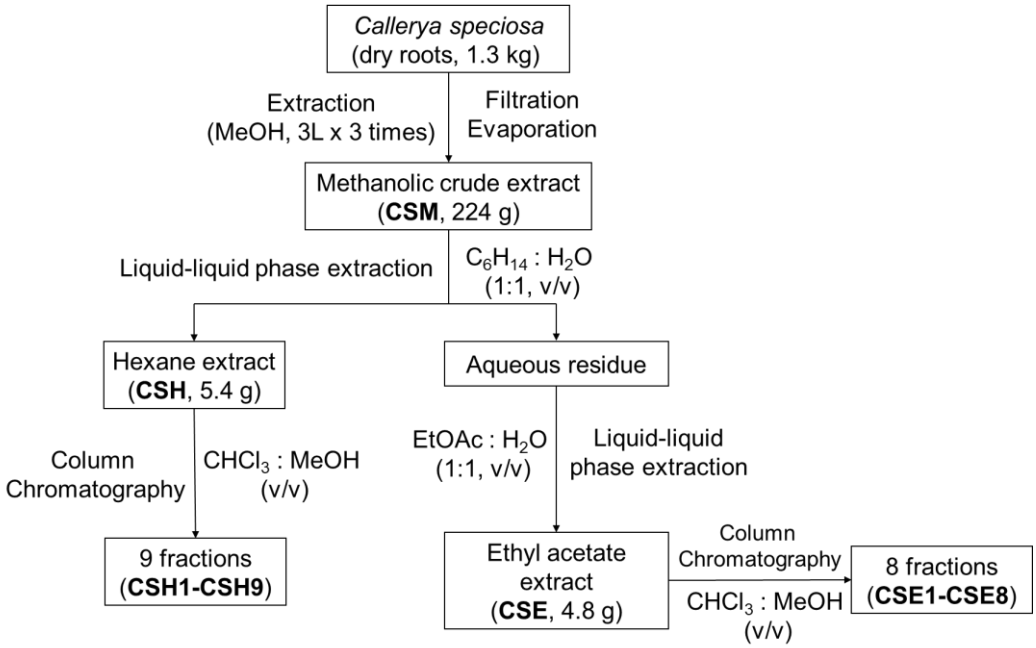

Figure 1. Extraction and fractionation procedures of bioactive constituents from *Callerya speciosa* roots.

Isolation and fractionation of bioactive components

All the obtained extracts were preliminarily tested for their biological activities, including antioxidant ability (ABTS and DPPH assays) and antidiabetic properties (α-amylase and α-glucosidase inhibition assays). The screening results showed that CSH had the best antidiabetic effect, while CSE exhibited the greatest antioxidant activity and potential antidiabetic activity. Therefore, CSH and CSE extracts were selected for subsequent bioguided fractionation to obtain the bioactive components by column chromatography over silica gel. The column chromatographs for each extract were designed separately. First, each extract (CSH extract: 5.4 g; CSE extract: 4.8 g) was subjected to a column over silica gel (70 - 230 mesh, 50 g), which was equilibrated with an initial solvent of chloroform (100%). The mobile solvents used for both columns were chloroform (C) and methanol (M) with a gradient increase in polarity. In particular, the decrease in the ratio of chloroform corresponded to an increase in the methanol portion, from C:M (100:0, v/v) to C:M (0:100, v/v). The differences among the fractions and purities of the isolates were determined by thin-layer chromatography (TLC) tests, in which the TLC staining solution contained 1% vanillin-sulfuric acid in high-grade ethanol (99.8%). The combined fractions were evaporated under vacuum to yield dry samples, which were then kept at 4°C for further analyses. The obtained fractions from CSH and CSE extracts of *C. speciosa* roots and chromatographic procedure are summarised as follows: CSH1 (0.33 g) and CSH2 (0.51 g) from C 100%, CSH3 (0.37 g) from M 0.1%, CSH4 (0.12 g) from M 0.5%, CSH5 (0.16 g) from M 2%, CSH6 (0.12 g) and CSH7 (0.11 g) from M 5%, CSH8 (0.09 g) from M 10%, CSH9 (0.20 g) from M 100%, CSE1 (0.01 g) from C 100% and M 0.1%, CSE2 (0.07 g) from M 0.1%, CSE3 (0.04 g) from M 0.2%, CSE4 (0.14 g) from M 1%, CSE5 (0.42 g) from M 2%, CSE6 (0.23 g) from M 5%, CSE7 (0.49 g) from M 20%, and CSE8 (0.93 g) from M 100%; as shown in Figure 1.

In vitro gastrointestinal digestion

The *in vitro* simulated digestion model was generated by a method adapted from Gutiérrez-Grijalva *et al.* (2017) and Un *et al.* (2022), with minor adjustments. Briefly, 1.4 mL of *C. speciosa* sample (dissolved in distilled water at a concentration of 5 mg/mL) was added to 3 mL of artificial saliva, and incubated for 5 min in a shaking water bath at 37°C. Subsequently, 3 mL of gastric juice was added to the sample tube, and the mixture was allowed to incubate for 1 h at 37°C. Subsequently, small intestinal juice (3 mL) was added to the same tube, and the mixture was incubated for an additional hour at 37°C. The sample was collected at the end of each digestive stage as follows: EtOAc was combined with the sample tube at a 1:1 ratio (v/v) to reconstitute the phytochemicals of the *C. speciosa* sample. After vigorous mixing for 2 min, the EtOAc phase was collected, filtered, evaporated, and prepared for further bioassays. The detailed components and procedures of the simulated digestion model were reported in our previous study (Un *et al.*, 2022).

Antioxidant assays

DPPH and ABTS assays were performed following reported protocols (Quan *et al.*, 2019a; 2019b). In terms of the DPPH assay, 80 µL of sample dissolved in methanol was mixed with 40 µL of 0.5 mM DPPH solution and 80 µL of 0.1 M acetate buffer (pH 5.5). Afterward, the mixture was incubated for 15 min in the dark at 25°C, and the absorbance was measured at 517 nm by a microplate spectrophotometer. For the ABTS assay, the ABTS radical solution was prepared by stirring a mixture of 7 mM ABTS and 2.45 mM $K_2S_2O_8$ (1:1, v/v). The mixture was incubated in darkness at 25°C for 16 h. In a microplate, 20 µL of each sample was mixed with 180 µL of the ABTS working solution. The plate was covered with aluminium foil, and incubated at room temperature for 15 min before the absorbance was recorded at 734 nm. For both antiradical assays, butylated hydroxytoluene (BHT) served as the positive control, while pure methanol served as the negative control. The free radical scavenging activity was calculated using Eq. 1:

$$\text{Free radical scavenging activity (\%)} = (A_{control} - A_{sample})/A_{control} \times 100 \quad \text{(Eq. 1)}$$

where, A_{sample} = absorbance of sample or positive control, while $A_{control}$ = absorbance of negative control. The antioxidant inhibitory capacity was examined in terms of the IC_{50} value (mg/mL), which signifies the concentration needed to achieve a 50% reduction in either DPPH or ABTS.

α-Amylase and α-glucosidase inhibitory assays

The α-amylase and α-glucosidase inhibitory properties of *C. speciosa* root extracts were evaluated based on the starch-iodine and pNPG methods described previously (Quan *et al.*, 2019a; 2019c). Particularly, *C. speciosa* samples and α-amylase (5 U/mL) were dissolved in 0.2 M phosphate-buffered saline (pH 6.9). In each test, 40 µL of a mixture containing α-amylase and sample (1:1, v/v) was incubated at 37°C for 10 min. To start the reaction, 30 µL of soluble starch (0.5%) was added and incubated at 37°C for 8 min. The reaction was halted by adding 20 µL of 1 M HCl, followed by 100 µL of 0.25 mM iodine solution. Finally, the absorbance of the mixture was measured at 565 nm by a microplate spectrophotometer. The inhibition percentage of *C. speciosa* samples on α-amylase was determined using Eq. 2:

$$\text{Inhibition percentage (\%)} = (A - C)/(B - C) \times 100 \quad \text{(Eq. 2)}$$

where, A = absorbance of the reaction with samples, B = absorbance of the reaction without α-amylase, and C = absorbance of the reaction without samples.

On the other hand, α-glucosidase inhibition was analysed as follows: the sample was initially diluted threefold with 40 µL of 0.1 M potassium phosphate buffer (pH 7), and then mixed with 20 µL of α-glucosidase (0.5 U/mL). After a five-minute incubation at 25°C, 20 µL of 5 mM pNPG substrate was pipetted into the mixture, and maintained at the same temperature for 10 min. Subsequently, the reaction was stopped by adding 100 µL of 0.1 M Na_2CO_3, and the absorbance was recorded at a wavelength of 405 nm. The percent inhibition of α-glucosidase by the *C. speciosa* samples was also calculated using Eq. 1. Palmitic acid and acarbose served as positive controls, while buffer served a negative control in both enzymatic assays. The IC_{50} values were calculated as earlier described.

Gas chromatography-mass spectrometry (GC-MS) analysis

The chemical compositions of the bioguided CSH fractions were identified using GC-MS analysis. A GC-MS system equipped with an autosampler was used in conjunction with a DB-5MS column 30 m × 0.25 mm i.d. × 0.25 µm film thickness (Agilent Technology, J&W Science, Folsom, CA, USA). Each sample was analysed at a concentration of 2 mg/mL. Helium was used as the carrier gas with a split ratio of 5:1. The operating conditions for gas chromatography at oven temperature were the same as those in a previous study (Anh *et al.*, 2021). Mass spectrometry scans encompassed a mass range spanning from 29 to 800 amu. The analysis of the generated peaks was performed using JEOL's GC-MS Mass Centre System version 2.65a software and the Mass Spectral Library NIST 20.

Liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) analysis

Bioactive components of the CSE fractions were analysed by LC-ESI-MS/MS technique. The analytical system consisted of an electrospray ionisation (ESI) interface in positive ionisation mode coupled with an LTQ Orbitrap XL mass spectrometer. The ESI conditions were configured as follows: an ion spray voltage of 4.5 kV, a sheath gas flow rate of 55 arbitrary units (arb), and an auxiliary gas flow rate of 15 arb. The mobile phase comprised solution A (0.1% aqueous formic acid) and solution B (100% acetonitrile), which were adjusted in a gradient program as follows: an initial composition of 5% solution B (0 - 2 min), followed by a linear increase from 5 to 70% solution B (2 - 8 min), maintaining 100% solution B from 8 to 9.5 min, then returning to 5% solution B from 9.5 to 10 min, and finally equilibrating for 2 min. The chromatographic separation was achieved using an Acquity UPLC® BEH C18 column, 1.7 µm, 50 × 2.1 mm i.d. (Waters Cooperation, Milford, MA, USA). The injection volume was 3 µL for every sample (1 mg/mL). The measurement was performed over a period of 10 min at a flow rate of 300 µL/min at room temperature. Mass spectra were acquired at a resolution of 60,000 with a scan range spanning from 100 to 1,000 m/z. Full-scan and data-dependent scan spectra for MS/MS were recorded and processed using Xcalibur software integrated with the NIST 20 database. Mass references were corroborated through the utilisation of the online database PUBCHEM, as well as references from prior publications.

Statistical analysis

All data were analysed by Minitab software, version 16.0 (Minitab Inc., State College, PA, USA). Each test was performed three times. The results were presented as mean ± standard deviation. To determine significant differences between or among the samples, one-way analysis of variance (ANOVA) was employed, and Tukey's test was utilised with a significance threshold of $p < 0.05$.

Results

Antioxidant activity

Among the fractions from the hexane extract, CSH4 and CSH5 showed the strongest antioxidant ability based on both the DPPH and ABTS assays. The IC_{50} values for the DPPH and ABTS assays of CSH4 were 0.30 mg/mL, while those of CSH5 were 0.40 and 0.50 mg/mL, respectively. Other CSH fractions exhibited moderate and negligible antiradical activities. In terms of the CSE fractions, we reported that CSE3 and CSE4 exhibited the most potent antioxidant activity, and the IC_{50} values for both the ABTS and DPPH assays of CSE3 and CSE4 were 0.10 and 0.37 mg/mL and 0.05 and 0.17 mg/mL, respectively. In general, the CSE fractions exerted stronger antioxidant effects than did the CSH fractions. Remarkably, the free radical scavenging ability of CSE4 (IC_{50} = 0.05 mg/mL) was close to that of the standard BHT (IC_{50} = 0.02 mg/mL) based on the DPPH assay.

Inhibition of α-amylase and α-glucosidase

The inhibitory activities of fractions from CSH and CSE on α-amylase and α-glucosidase are illustrated in Figure 2. Only CSH1 - CSH3 in hexane extract and CSE1 - CSE4 in EtOAc extract significantly inhibited both key enzymes linked to type 2 diabetes. Among the active fractions, the most potent inhibition on α-amylase was presented by CSE4, with an IC_{50} value of 0.22 mg/mL, followed by CSH3 (IC_{50} = 0.38 mg/mL) and CSH2 (IC_{50} = 0.59 mg/mL). Meanwhile, IC_{50} values for α-amylase inhibition by the standard compounds acarbose and palmitic acid were 0.04 and 1.57 mg/mL, respectively. On the other hand, CSE2, CSE3, and CSE4 had the strongest inhibitory effects on

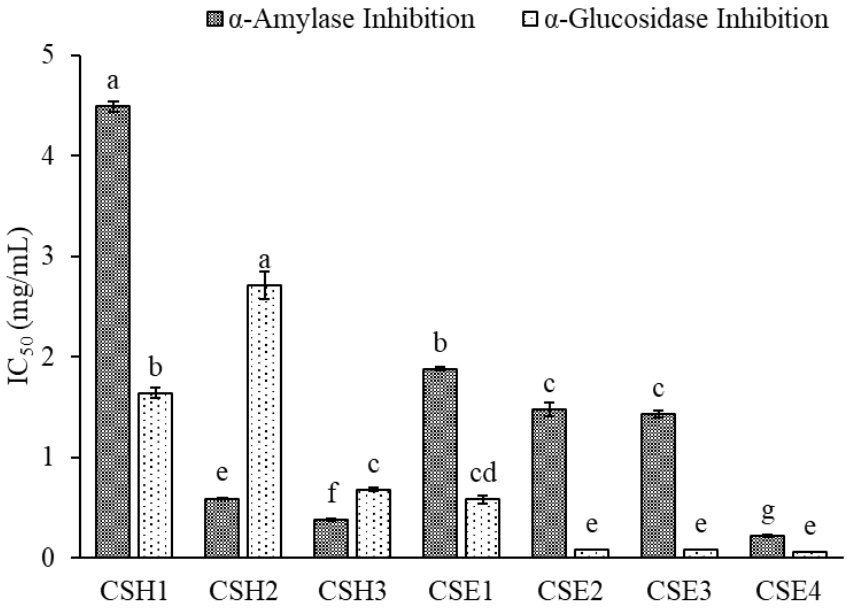

Figure 2. α-Amylase and α-glucosidase inhibitory activities of fractions isolated from CSH and CSE. Data are mean ± standard deviation. Means within a column followed by different lowercase letters are significantly different at $p < 0.05$.

α-glucosidase, with IC_{50} values of 0.08, 0.08, and 0.06 mg/mL, respectively. Notably, the α-glucosidase inhibitory activity of the three fractions CSE2, CSE3, and CSE4 was significantly greater than that of acarbose (IC_{50} = 2.38 mg/mL) and palmitic acid (IC_{50} = 0.30 mg/mL), which are well-known diabetic inhibitors.

Effects of in vitro digestion on antioxidant activity and α-amylase and α-glucosidase inhibition

Based on the results from the antioxidant and enzymatic assays, and the available content of the fractions, the CSH2 - CSH3 and CSE2 - CSE4 fractions were selected to examine the influence of the simulated digestion model on their biological activities.

The changes in the anti-radical capacity of the bioactive fractions from *C. speciosa* roots based on the simulated digestion model are presented in Table 1. All fractions derived from the hexane extract exhibited weak antioxidant activity throughout the digestion process, with a maximum tested concentration of 10 mg/mL. Hence, the calculation of IC_{50} values was exclusively performed for the fractions originating from the EtOAc extract.

In general, the antioxidant activities of all the tested fractions (CSE2 - CSE4) significantly decreased during the digestion stage, and the activities of CSE2 and CSE4 decreased by 8.1 and 7.6 times, respectively, in the DPPH assay. The simulated digestive system appeared to have a minor impact on the antioxidant activity of CSE3, reducing its activity by only 1.4 and 3.9 times in the ABTS and DPPH assays, respectively. However, a decrease in activity was markedly noted in the oral phase, after which the activity seemingly did not significantly change until the small intestinal phase. Notably, the activity of CSE2 decreased significantly during the oral phase, but relatively recovered in the subsequent phases, whereas the activity of CSE3 and CSE4 remained stable after the decrease in the oral phase. The activity of CSE4 was strongest prior to digestion, but once it entered the digestive system, it showed comparable activity to that of CSE3, with corresponding IC_{50} values of 0.35 and 0.30 mg/mL for the ABTS assay, and 0.39 and 0.38 mg/mL for the DPPH assay in the small intestinal phase, respectively.

The effects of *in vitro* digestion on the α-amylase and α-glucosidase inhibitory activities of the fractions isolated from *C. speciosa* roots are described in Table 2. Before digestion, all the samples showed significant inhibition of both α-amylase and α-glucosidase, and the recorded activity patterns were as follows: CSE4 > CSH3 > CSH3 > CSE3 and CSE2 based on the α-amylase assay, and CSE4, CSE3, CSE2 > CSH3 > CSH2 based on the α-glucosidase assay.

Table 1. Effects of *in vitro* digestion on antioxidant activity of fractions from *Callerya speciosa* root extracts.

Sample	U	O	G	I
ABTS Assay (IC_{50} mg/mL)				
CSE2	0.36 ± 0.02^{Ac}	ne	1.11 ± 0.10^{Ab}	1.29 ± 0.03^{Aa}
CSE3	0.25 ± 0.01^{Bb}	0.25 ± 0.01^{Ab}	0.32 ± 0.01^{Ba}	0.35 ± 0.03^{Ba}
CSE4	0.17 ± 0.00^{Cc}	0.18 ± 0.00^{Bc}	0.24 ± 0.01^{Bb}	0.30 ± 0.00^{Ba}
BHT	0.08	-	-	-
DPPH Assay (IC_{50} mg/mL)				
CSE2	0.18 ± 0.01^{Ac}	ne	1.61 ± 0.10^{Aa}	1.45 ± 0.02^{Ab}
CSE3	0.10 ± 0.00^{Bd}	0.34 ± 0.01^{Ac}	0.45 ± 0.03^{Ba}	0.39 ± 0.01^{Bb}
CSE4	0.05 ± 0.00^{Cd}	0.28 ± 0.02^{Bc}	0.32 ± 0.01^{Bb}	0.38 ± 0.01^{Ba}
BHT	0.02	-	-	-

Data are mean ± standard deviation. Means followed by different uppercase superscripts within a column, and lowercase superscripts within a row are significantly different at $p < 0.05$. BHT: butylated hydroxytoluene; ne: negligible effect; and (-): not determined.

Table 2. Effects of *in vitro* digestion on α-amylase and α-glucosidase inhibitions fractions from *Callerya speciosa* root extracts.

Sample	U	O	G	I
α-Amylase inhibition (IC_{50} mg/mL)				
CSH2	0.59 ± 0.01^{Bb}	0.37 ± 0.01^{Cd}	0.47 ± 0.01^{Cc}	1.06 ± 0.04^{Ca}
CSH3	0.38 ± 0.01^{Cb}	0.33 ± 0.02^{Cc}	0.39 ± 0.01^{Db}	0.51 ± 0.02^{Ea}
CSE2	1.48 ± 0.07^{Aa}	1.05 ± 0.05^{Bb}	0.46 ± 0.01^{Cc}	1.53 ± 0.04^{Aa}
CSE3	1.43 ± 0.02^{Aa}	1.18 ± 0.04^{Ab}	0.70 ± 0.02^{Bd}	0.86 ± 0.01^{Dc}
CSE4	0.22 ± 0.01^{Dc}	na	0.81 ± 0.02^{Ab}	1.18 ± 0.06^{Ba}
PA	1.57	-	-	-
Acarbose	0.04	-	-	-
α-Glucosidase inhibition (IC_{50} mg/mL)				
CSH2	2.70 ± 0.13^{A}	na	na	na
CSH3	0.68 ± 0.02^{Ba}	na	na	0.18 ± 0.00^{Ab}
CSE2	0.08 ± 0.00^{Cc}	0.37 ± 0.03^{Aa}	na	0.13 ± 0.00^{Bb}
CSE3	0.08 ± 0.00^{Cb}	0.09 ± 0.00^{Ba}	0.09 ± 0.00^{Aab}	0.05 ± 0.00^{Cc}
CSE4	0.06 ± 0.00^{Ca}	0.06 ± 0.00^{Bb}	0.02 ± 0.00^{Bc}	0.02 ± 0.00^{Dc}
PA	0.30	-	-	-
Acarbose	2.38	-	-	-

Data are mean ± standard deviation. Means followed by different uppercase superscripts within a column, and lowercase superscripts within a row are significantly different at $p < 0.05$. PA: palmitic acid; na: no activity; and (-): not determined.

In terms of the α-amylase assay, most of the test samples exhibited a significant decrease in activity over the stages of the *in vitro* digestion system, with the exception of CSE3, which exhibited a marked increase in its α-amylase inhibitory activity during the small intestinal phase (IC_{50} = 0.86 mg/mL, a 1.7-fold increase compared to that of the undigested sample). Notably, at the oral stage, all samples increased in activity, but uniquely, CSE4 exhibited a significant decrease in activity to the lowest threshold, no activity. The order of anti-α-amylase activity arrangement based on the IC_{50} values recorded in the small intestinal phase was CSH3 > CSE3 > CSH2 > CSE4 > CSE2.

With respect to α-glucosidase activity, the activities of CSH3, CSE3, and CSE4 increased significantly, with IC_{50} values recorded in the small intestinal phase of 0.18, 0.05, and 0.02 mg/mL, respectively, which corresponded to increases of 3.8, 1.6, and 3.0 times compared to the activities of undigested samples. Meanwhile, the α-glucosidase inhibitory activity of CSH2 decreased at all stages of the *in vitro* digestion system. Interestingly, CSH3 and CSE2 exhibited no activity in the gastric phase, but their activity recovered in the intestinal phase. Overall, the α-glucosidase inhibitory activities of CSH3, CSE2, and CSE3 fluctuated inconsistently across the stages of the *in vitro* digestion system, with only CSE4 showing a consistent increase in activity after digestion in the oral phase. The order of activity arrangement recorded at the small intestinal phase was CSE4 > CSE3 > CSE2 > CSH3.

Phytocompounds in bioactive extracts identified by GC-MS

Among the fractions isolated from hexane extract, CSH2 and CSH3 had the strongest inhibitory effects on α-amylase and α-glucosidase. Therefore, fractions CSH2 and CSH3 were selected for the identification of their bioactive constituents by GC-MS. Although phytosterols were predominant in CSH2, fatty acid methyl esters (FAMEs), and fatty acids, specifically palmitic acid, constituted the principal compound group in CSH3. In CSH2, γ-sitosterol was the major constituent, accounting for 70% of the peak area, followed by stigmasterol (10.93%), palmitic acid (9.17%), campesterol (5.18%), and 2-methylundecanoic acid (1.68%). Conversely, in CSH3, palmitic acid represented the primary component with a peak area of 46.97%, followed by various FAMEs (29.31%) and γ-sitosterol (3.66%).

Phytochemical components of active fractions identified by LC-ESI-MS/MS

Among the bioactive fractions, CSE2, CSE3, and CSE4 exhibited the most potent antioxidant, α-amylase, and α-glucosidase inhibition activities. In addition, GC-MS was not suitable for identifying the phytocompounds in such fractions isolated from the EtOAc extract. Therefore, liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) was used to identify the phytochemical constituents in these fractions. The tentative compounds and their profiles are listed in Table 3.

A total of 13 compound classes were characterised in the CSE2, CSE3, and CSE4 fractions using LC-ESI-MS/MS, among which isoflavonoids and fatty acids were the most abundant groups, with eight and six compounds identified, respectively. Most of the compounds were identified by positive ionisation mode, while three fatty acids, two carboxylic acids, and three unknown compounds were detected only in the fractions using negative ionisation mode. CSE2 contained four isoflavonoids (pseudobaptigenin, 7-hydroxy-6,4'-dimethoxyisoflavone, (±)-medicarpin, and 3',4'-dimethoxy-7-hydroxyisoflavone), two lignans (syringaresinol and pinoresinol), one fatty acid, one flavonoid (maackiain), one amino alcohol, one indole, and two unknown compounds. CSE3 contained five isoflavonoids (odoratin, barpisoflavone A, pseudobaptigenin, formononetin, and 3',4'-dimethoxy-7-hydroxyisoflavone), one fatty acid similar to that of CSE2, two triterpenes (bacosine and betulin), one lactone, one polyphenol, and one unknown compound. In contrast, CSE4 contained only two isoflavonoids (calycosin and odoratin), but encompassed the majority of the fatty acids, except for 13-keto-9Z,11E-octadecadienoic acid, which was characterised in CSE2 and CSE3. Furthermore, CSE4 contained one flavonoid (liquiritigenin), three triterpenes (friedelin, bacosine, and betulin), two carboxylic acids, one glycol ether, one fatty acid, ethanolamide, and one ergostane steroid.

Table 3. Phytocompounds identified from active fractions of *Callerya speciosa* ethyl acetate extract by LC-ESI-MS/MS.

Class	Identified compound	Ion mode	Precursor type	Molecular formula	Exact mass	Fragment ions (MS/MS)	Presence in fractions		
							CSE2	CSE3	CSE4
Isoflavonoids	Calycosin	pos & neg	$[M+H]^+$ $[M-H]^-$	$C_{16}H_{12}O_5$	284.07	270.04; 253.06 268.13; 239.16			+
	Odoratin	pos	$[M+H]^+$	$C_{17}H_{14}O_6$	314.08	300.08; 283.05		+	+
	Barpisoflavone A	pos	$[M+H]^+$	$C_{16}H_{12}O_6$	300.06	283.04; 163.04; 135.01		+	
	Pseudo-baptigenin	pos & neg	$[M+H]^+$ $[M-H]^-$	$C_{16}H_{10}O_5$	282.05	253.03; 225.05 253.10; 251.14; 225.15	+	+	
	Formononetin	pos & neg	$[M+H]^+$ $[M-H]^-$	$C_{16}H_{12}O_4$	268.07	254.08; 213.06; 237.04 252.12; 267.13		+	
	7-Hydroxy-6,4′-dimethoxyisoflavone	pos	$[M+H]^+$	$C_{17}H_{14}O_5$	298.08	284.05; 267.11; 166.00	+		
	(±)-Medicarpin	pos	$[M+H]^+$	$C_{16}H_{14}O_4$	270.09	137.03; 161.04; 123.00	+		
	3',4'-Dimethoxy-7-hydroxyisoflavone	pos & neg	$[M+H]^+$ $[M-H]^-$	$C_{17}H_{14}O_5$	298.08	284.05; 267.11; 165.99 282.14; 269.19; 183.20	+	+	
	9Z,11E,13E-Octadecatrienoic acid	pos	$[M+H]^+$	$C_{18}H_{30}O_2$	278.22	261.20; 243.22; 95.04			+
	13-Keto-9Z,11E-octadecadienoic acid	pos	$[M+H]^+$	$C_{18}H_{30}O_3$	294.22	277.21	+	+	
	9-Oxo-11-(3-pentyl-2-oxiranyl)-10E-undecenoic acid	neg	$[M-H]^-$	$C_{18}H_{30}O_4$	310.21	291.24; 209.21; 185.22			+

	(12Z,15Z)-7,9,10-Trihydroxyoctadeca-12,15-dienoic acid	neg	$[M-H]^-$	$C_{18}H_{32}O_5$	328.22	309.23; 291.24; 197.15			+
	(+/-)-9(10)-Epoxy-12Z-octadecenoic acid	neg	$[M-H]^-$	$C_{18}H_{32}O_3$	296.24	277.26; 171.17; 183.22			+
Flavonoids	Liquiritigenin	pos & neg	$[M+H]^+$ $[M-H]^-$	$C_{15}H_{12}O_4$	256.07	136.99; 147.01; 239.02 135.13; 119.12; 91.07			+
	Maackiain	pos	$[M+H]^+$	$C_{16}H_{12}O_5$	284.07	123.00; 175.00; 151.02	+		
Triterpenes	Friedelin	pos	$[M+H]^+$	$C_{30}H_{50}O$	426.39	409.38; 391.38; 408.42			+
	Bacosine	pos	$[M+H-H_2O]^+$	$C_{30}H_{48}O_3$	456.36	393.36; 191.21; 259.14		+	+
	Betulin	pos	$[M+H-H_2O]^+$	$C_{30}H_{50}O_2$	442.38	407.39; 191.17; 397.39		+	+
Lignans	Syringaresinol	pos	$[M+H-H_2O]^+$	$C_{22}H_{26}O_8$	418.16	383.18; 369.11; 351.15	+		
	Pinoresinol	pos	$[M+H-H_2O]^+$	$C_{20}H_{22}O_6$	358.14	323.13; 271.10; 291.15	+		
Carboxylic acids	Sebacic acid	pos	$[M+H]^+$	$C_{10}H_{18}O_4$	202.12	185.19			+
	Azelaic acid	neg	$[M-H]^-$	$C_9H_{16}O_4$	188.10	125.10; 169.06; 97.15			+
	4-Oxododecanedioic acid	neg	$[M-H-H2O]^-$	$C_{12}H_{20}O_5$	244.13	207.14; 181.17; 163.22			+
Amino alcohols	2S-Amino-4E-pentadecene-1,3R-diol	pos	$[M+H]^+$	$C_{15}H_{31}NO_2$	257.27	240.27	+		
Lactones	5-(1-Hydroxyethyl)oxolan-2-one	pos	$[M+H-H_2O]^+$	$C_6H_{10}O_3$	130.06	85.11		+	
Glycol ethers	Tri(propylene glycol) methyl ether	pos	$[M+H]^+$	$C_{10}H_{22}O_4$	206.15	131.10; 73.05; 117.09			+

Polyphenols	5-(4-Hydroxypentyl)benzene-1,3-diol	pos	$[M+H]^+$	$C_{11}H_{16}O_3$	196.11	179.06; 135.09; 161.05		+
Indoles	Indole-7-carboxaldehyde	pos & neg	$[M+H]^+$ $[M-H]^-$	C_9H_7NO	145.05	118.01	+	
Fatty acid amides	Linoleoyl ethanolamide	pos	$[M+H]^+$	$C_{20}H_{37}NO_2$	323.28	306.31, 307.30; 245.24		+
Ergostane steroids	Ergosterol peroxide	pos	$[M+H]^+$	$C_{28}H_{44}O_3$	428.33	411.37; 393.39; 253.26		+
Unknown compounds	Unknown compound 1	neg	$[M-H]^-$	$C_{20}H_{22}O_7$	374.14	313.18; 343.18; 358.18	+	
	Unknown compound 2	neg	$[M-H]^-$	$C_{17}H_{14}O_6$	314.08	298.14; 161.08; 297.16	+	
	Unknown compound 3	neg	$[M-H]^-$	$C_{17}H_{28}O_5$	312.19	267.25; 223.28; 293.24		+

pos: positive ionisation mode; neg: negative ionisation mode; and (+): present.

Discussion

In our previous work, we reported the antioxidant activity and potential antimyeloma and antilymphoma effects of fractions obtained from the ethyl acetate extract of *C. speciosa* roots (Lam *et al.*, 2022). Building upon this topic, in the present work, we investigated the influence of a simulated gastrointestinal system on the antioxidant activity and the inhibitory effects against α-amylase and α-glucosidase, two key enzymes associated with diabetes. To date, there have been limited studies on the antidiabetic potential of *C. speciosa*. In a recent study, Tuan *et al.* (2022) reported the α-glucosidase inhibitory activity of compounds isolated from *C. speciosa* roots. Their findings revealed that rutin (IC_{50} = 2.20 µg/mL), uvaol (IC_{50} = 1.96 µg/mL), and ursolic acid (IC_{50} = 1.10 µg/mL) exhibited α-glucosidase inhibition levels 77.18, 86.63, and 154.36 times greater than those observed with the standard drug acarbose (IC_{50} = 169.80 µg/mL), respectively. However, this study focused solely on investigating the activities and mechanisms of individual compounds against α-glucosidase. To the best of our knowledge, our study represents the first investigation into the α-amylase inhibitory activity of *C. speciosa*, and assessment of the impact of an *in vitro* simulated gastrointestinal model on the potential antidiabetic activities of extracts from *C. speciosa* roots.

The results revealed that the α-amylase inhibitory activity of the fractions significantly decreased under the influence of the simulated gastrointestinal system, except for that of CSE3, which was isolated from the EtOAc extract of *C. speciosa* roots. The α-amylase inhibitory activity of CSE3 in the duodenal digestion phase increased by 1.7-fold compared to that of the non-digested CSE3 sample. This could be explained by the presence of specific phytocompounds unique to CSE3, such as 5-(1-hydroxyethyl)oxolan-2-one, 5-(4-hydroxypentyl)benzene-1,3-diol, barpisoflavone A, and formononetin, and the synergistic interaction between these compounds and other bioactive compounds. Although studies on the biological activity of 5-(1-hydroxyethyl)oxolan-2-one and 5-(4-hydroxypentyl)benzene-1,3-diol are limited, formononetin is known as a signature compound of *C. speciosa* roots with potential antidiabetic properties reported in previous studies (Oza and Kulkarni, 2018). Additionally, the α-amylase inhibitory activity of the fractions from the hexane

extract seemed to be less affected by the *in vitro* gastrointestinal system than that of the fractions from the EtOAc extract. Specifically, CSH3 exerted the highest anti-α-amylase activity in the intestinal phase, with an IC_{50} of 0.51 mg/mL. This could have been due to the high content of palmitic acid and fatty acid methyl esters (FAMEs), which may play a significant role in the α-amylase inhibitory activity. Our previous study demonstrated a strong correlation between variations in α-amylase inhibitory activity and changes in the palmitic acid content of bioactive fractions isolated from *Sargassum* spp. through *in vitro* simulated gastrointestinal digestion (Un *et al.*, 2022).

In contrast, the α-glucosidase inhibitory activity of the fractions from the EtOAc extract remained stable under the influence of the simulated gastrointestinal digestion, unlike that of the fractions from the hexane extract. Except for CSH2, all other fractions showed increased activity during the *in vitro* digestion process. The activities of CSE2, CSE3, and CSE4 in the small intestinal phase were significantly greater than those of the standard compounds acarbose and palmitic acid. A plausible explanation for this could be the synergistic effects of the isoflavonoid group and other antidiabetic compounds present in the bioactive fractions. Among the compounds identified by LC-ESI-MS/MS, the reported compounds with antidiabetic activity were mainly isoflavonoids, triterpenes, flavones, and lignans. Specifically, calycosin has been reported to inhibit early cell proliferation induced by high glucose, and reduce AGE-mediated cell apoptosis in renal mesangial cells, which has significant implications for controlling the progression and/or development of diabetic nephropathy (Tang *et al.*, 2011). Odoratin and pseudobaptigenin significantly transactivate the activity of PPARγ, which has the potential to counteract hyperglycaemia due to metabolic disorders and type 2 diabetes (Zhang *et al.*, 2012; Matin *et al.*, 2013). Barpisoflavone A has been demonstrated to exhibit α-glucosidase inhibitory activity (IC_{50} = 100 μM) (Kaneta *et al.*, 2016). Additionally, medicarpin, formononetin, calycosin, and a flavone named liquiritigenin have been studied for their potent α-glucosidase inhibitory activities, with IC_{50} values of 2.93, 0.51, 12.70, and 3.61 mM, respectively (Choi *et al.*, 2010). Maackiain, a unique flavone identified from *C. speciosa*, exhibited moderate activity against α-glucosidase with an IC_{50} value of 185 μM (Kim *et al.*, 2006). Notably, the triterpenes friedelin and betulin have been recognised as potent α-glucosidase inhibitors with IC_{50} values of 45.72 and 14.90 μM, respectively. On the other hand, another identified triterpene, bacosine, has been shown to prevent the glycosylation increase of haemoglobin *in vitro*, with an IC_{50} value of 7.44 μg/mL, and to increase liver glycogen content in alloxan-induced diabetic mice, providing strong evidence of its protective effects against oxidative damage in diabetes (Ghosh *et al.*, 2011). Additionally, two lignans, syringaresinol and pinoresinol, have also been reported to exhibit strong α-glucosidase inhibitory activities, with IC_{50} values of 54.00 and 34.3 μM, respectively (Wikul *et al.*, 2012; Vi *et al.*, 2022). Although the antioxidant activities of the test samples significantly decreased through simulated gastrointestinal digestion, the recorded IC_{50} values at the duodenal phase ranging from 0.30 to 0.39 μg/mL (for CSE3 and CSE4) are still considered promising. In fact, the correlation between antioxidant activity and α-amylase and α-glucosidase inhibitory activities is an area of research interest, especially in the field of food science and medical biology, as these activities have been implicated in managing diabetes and oxidative stress (Bahadoran *et al.*, 2013; Moein *et al.*, 2017). There is a growing body of evidence that plant-derived compounds demonstrating both antioxidant and α-amylase/α-glucosidase inhibitory activities have potential for development into functional foods or therapeutic agents.

The investigation of the effects of *in vitro* digestion on biological activity plays a crucial role in the field of drug discovery and development, particularly in the utilisation of natural compounds. This investigation allows for the estimation and prediction of the potential bioavailability, stability, and interrelationship between drug components. Understanding the behaviour of natural compounds during digestion is essential for guiding modifications that can enhance their stability, absorption, and efficacy. Therefore, the present work significantly contributed to the development of new therapeutic approaches for supporting diabetes treatment by utilising highly active extracts derived from the roots of *C. speciosa*. While previous studies have elucidated the correlation between variations in the content and antidiabetic activity of fractions containing high levels of palmitic acid (CSH3) through simulated gastrointestinal digestion, the understanding of this relationship remains unclear for

isoflavonoids, flavones, triterpenes, and lignans. The preceding investigation demonstrated the high stability of isoflavonoids during simulated digestion (Walsh *et al.*, 2003), which may provide an explanation for the increased activity observed in the CSE3 and CSE4 fractions. However, the bioavailability of flavonoids poses a challenge, as most of these compounds undergo extensive metabolism in human plasma, resulting in a decrease in their activity (Lotito and Frei, 2006). Notably, the present work revealed a noteworthy increase in the activity of fractions that contained several flavonoids, specifically the CSE3 and CSE4 fractions. Another possible explanation for this finding could be the presence of fatty acids (18 carbons) and carboxylic acids unique to the CSE4 fraction, which may provide protection for highly active components against enzymatic degradation and pH changes in the simulated gastrointestinal system. In the case of CSE3, the presence of 13-keto-9Z,11E-octadecadienoic acid (a polyunsaturated fatty acid, PUFA) and 5-(1-hydroxyethyl)oxolan-2-one (a lactone) may contribute to the increased stability of the highly active components. This was consistent with previous studies demonstrating the growing evidence and widespread applications of PUFAs in enhancing the bioavailability of novel drugs (Maki and Dicklin, 2019; Aguilera *et al.*, 2022). However, further experiments on bioavailability and bioaccessibility are warranted to provide a more accurate assessment of the role of these compound groups in the antidiabetic activity of *C. speciosa* roots. Therefore, extensive investigations on variations in compound content at each digestion stage, the influence of gastric and intestinal pH, bile and pancreatic secretion, intestinal absorption capacity, and potential toxicity should be conducted using animal models before proceeding with preclinical and clinical trials in humans.

Conclusion

In the present work, the impact of simulated gastrointestinal digestion on the inhibitory activity of α-amylase and α-glucosidase by fractions isolated from *Callerya speciosa* roots was investigated for the first time. The results demonstrated that fractions containing high levels of fatty acids, FAMEs, isoflavonoids, flavones, and triterpenes (CSH3, CSE2, CSE3, and CSE4) exhibited potent inhibitory effects on these two key enzymes associated with diabetes. In particular, the α-glucosidase inhibitory activity of these fractions was significantly greater than that of the standard compounds acarbose and palmitic acid. Furthermore, the activity of these bioactive fractions was stable, and increased in the simulated gastrointestinal model, especially during the intestinal phase, wherein most compounds were absorbed. The present work would have significant implications for laying the foundation for the research and development of novel α-amylase and α-glucosidase inhibitors to support the treatment and control of blood glucose elevation using components derived from *C. speciosa* roots. However, further in-depth investigations should be conducted to comprehensively evaluate the bioavailability and bioaccessibility of these naturally sourced compounds.

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