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Oligomeric proanthocyanidins are the active compounds in *Abelmoschus esculentus* Moench for its α -amylase and α -glucosidase inhibition activity

Yuyun Lu ^{a,1}, Manuela Franziska Demleitner ^{b,1}, Lixia Song ^c,
Michael Rychlik ^b, Dejian Huang ^{a,c,*}

^a Food Science and Technology Programme, Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543

^b Chair of Analytical Food Chemistry, Technische Universität München, Alte Akademie 10, D-85350 Freising, Germany

^c National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Jiangsu 215123, China

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ABSTRACT

We found that *Abelmoschus esculentus* Moench (okra, or lady's finger) exhibits significant α -amylase and α -glucosidase inhibitory activity. Assay-guided fractionation of the okra extracts resulted in identification of oligomeric proanthocyanidins in unripe okra seeds as the compounds responsible to the activity. High-performance liquid chromatography (HPLC) analysis using a diol column revealed oligomers are a dominant composition with higher degree (>20) of polymerisation appearing as one large peak in the chromatogram. Thiolytic treatment of the proanthocyanidins using methyl thioglycolate produced thioether derivatives of (epi)gallocatechins and (epi)catechins as major products. Based on the ratio of terminal and extension units, the mean degree of polymerisation was estimated to be 21.97. B-type gallocatechin oligomers up to pentadecamer could be detected using matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectra.

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1. Introduction

As type II diabetes mellitus is becoming a pandemic disease, insensitive research has been on finding new ways to treat and prevent hyperglycaemia. Fruits and vegetables are rich in

phytochemicals that are believed to reduce starch digestion rate and hence are beneficial to prevent post-prandial hyperglycaemia (Cheplick, Kwon, Bhowmik, & Shetty, 2010; Fu et al., 2015; O'Keefe & Bell, 2007; Ou & Gu, 2014; Pei, Yu, Bruno, & Bolling, 2015). Tropical region has great diversity of vegetables and fruits that yet their bioactive constituents in starch hydrolase inhibition are

* Corresponding author. Food Science and Technology Programme, Department of Chemistry, National University of Singapore, 117543 Singapore. Tel.: +65 6516 8821; fax: +65 6775 7895.

E-mail address: chmhdj@nus.edu.sg (D. Huang).

¹ These authors contributed equally to the work.

Chemical compounds: Quercetin 3-rutinoside (PubChem CID: 5280805); Quercetin-3-glucoside (PubChem CID: 25203368); Gallocatechin (PubChem CID: 65084); Epigallocatechin (PubChem CID: 72277); Epicatechin (PubChem CID: 72276); (+)-Catechin (PubChem CID: 9064). <http://dx.doi.org/10.1016/j.jff.2015.10.037>

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largely unknown. To rapidly discover vegetables that are of potential in reducing the digestibility of starch, high throughput assay method should be applied.

There are a number of high throughput assays reported for quantification of starch hydrolase inhibitory activity and the common ones include pNPG hydrolysis, coupled glucose oxidase and peroxidase assay on *o*-dianisidine oxidation, and turbidity assay. The potential problem for pNPG assay is that it is a synthetic substrate, *p*-nitrophenyl- α -pyranoglucoside (pNPG) can be carried out in a 96-well microplate format yet pNPG is a synthetic substrate and its affinity to α -amylase and α -glucosidase is different from that of natural substrate (i.e. starch and oligosaccharides) of the enzyme. The coupled glucose oxidase and peroxidase assay suffers severe interference from polyphenolic compounds and is not suitable for screening α -amylase inhibition activity of fruits and vegetables (Wong & Huang, 2014). The turbidity assay is by far the most convenient and ideal assay for botanical materials because it applies starch as the natural substrate and the reaction kinetics solely relies on the starch digestion progress instead of artificial probes as indicator. Therefore, this method provides the most reliable results and was selected to screen a large number of medicinal plants in the attempt to discover a new type of naturally occurring starch hydrolase inhibitors as functional food ingredients.

Abelmoschus esculentus Moench (*A. esculentus*) belongs to mallow family and is an edible flowering plant also known as lady's finger or okra. It is widely distributed globally but most common in tropical region such as Southeast Asia (Camciuc, Deplagne, Vilarem, & Gaset, 1998; Sabitha, Panneerselvam, & Ramachandran, 2012). Noticeable amount of work has been carried out on the health promoting benefits of okra. Huynh, Nguyen, Tran, and Vo (2008) reported that okra could decrease the absorption of cholesterol and triglyceride in hyperlipidaemia mice. Besides, previous works had shown that the alcoholic fruit extract of okra possesses antidepressant activity (Jimmy Devi, Ganesh, Rajeshwari, & Srinivas, 2009), hypoglycaemic activity (Sabitha, Ramachandran, Naveen, & Panneerselvam, 2011) and anti-diabetic activity (Subrahmanyam et al., 2011). Yet there was no characterisation of the active compound structures. It is therefore our goal to elucidate the chemical structures of the starch hydrolase inhibitors in okra. We reported here that the oligomeric (epi)gallocatechin with high mean degree of polymerisation of 22 in okra seeds is responsible for its starch hydrolase inhibitory activity with an IC_{50} value of $2.30 \pm 0.14 \mu\text{g/mL}$ (α -amylase) and $16.88 \pm 0.26 \mu\text{g/mL}$ (α -glucosidase).

2. Materials and methods

2.1. Materials

Corn starch (S4126), α -amylase (A 3176, type VI-B, from porcine pancreas), acarbose, α -glucosidase in the form of rat intestine acetone powder, and methyl thioglycolate were obtained from Sigma-Aldrich Chemical Co. (Singapore, Singapore). Calcium chloride and anhydrous sodium carbonate were purchased from Thermo Fisher (Singapore, Singapore). Phosphate buffered saline (ultra-pure grade) was purchased from Vivantis Technologies (Selangor Darul Ehsan, Malaysia). Sephadex LH-20

was purchased from GE Healthcare Bio Sciences AB (Uppsala, Sweden). Bio-Gel® P 2 Gel was purchased from Bio-Rad laboratories (Hercules, CA, USA). Unripe okra was purchased from local supermarket in Singapore. All other reagents and chemicals were of analytical or HPLC grade.

Vegetables were purchased from local wholesale market and treated as previously reported before extraction was carried out using methanol acetone and water mixture (2:2:1). The high throughput assay of the resulting solution was carried out according to our previous procedure (Liu, Song, Wang, & Huang, 2011).

2.2. Extraction of proanthocyanidins from okra seeds

One kilogram of fresh okra was separated into seeds and skins manually; the seeds were freeze-dried for 5 days, and then crushed by blender and stored at -20°C . The freeze-dried okra seed powders (40 g) were extracted by an acetone-ethanol-water-acetic acid mixture (200:200:95:5, by volume, $3 \times 0.4 \text{ L}$) for 2 h at room temperature. The mixture was filtered and the solvent was evaporated by a rotary evaporator at 40°C to yield slurry. Liquid-liquid extraction was chosen to further process the crude extract. The slurry was dissolved in water (200 mL) and extracted 3 times with equal volume of *n*-hexane, chloroform, ethyl acetate and *n*-butanol, respectively. The water phase was collected, evaporated, and dried to give a crude extract with yield of 27.4% (based on dried seed powders). The crude proanthocyanidin fraction (8 mL, 0.5 g/mL) was filtered through a Sartorius Ministar 0.45 μm porosity filter before loaded on a gel column chromatography (40 cm \times 30 mm inner diameter) filled with Sephadex LH-20. The column was washed with water at a flow rate of 1.0 mL/min, and the eluents were collected with 10 min collection span per tube for 40 tubes. The fractions were screened for their α -amylase inhibition activity and fractions that showed activity (tubes 6–13) were pooled. The solvents were removed by rotary evaporator and the residue was freeze-dried to give a light brown powder (0.45 g), which was reconstituted in a 2 mL aqueous solution. The solution was filtered through a Sartorius Ministar 0.45 μm porosity filter and loaded onto a Bio-Gel P 2 gel column (120 cm \times 15 mm inner diameter). The column was washed with water at a flow rate of 5 mL/40 min with collection portion of 5 mL per tube for 14 tubes. The fractions collected were screened for α -amylase inhibition activity. The ones with activity (5–7) were pooled and volatiles were removed. The residue was freeze-dried to give a light brown powder (50 mg) for further characterisation.

2.3. HPLC method for separation of polyphenolic compounds

The dried sample was diluted in acetonitrile (10 mg/mL) and filtered through a 0.45 μm PTFE membrane filter prior to injection. Twenty μL of sample was injected into the HPLC column (250 mm \times 4.6 mm \times 4.6 mm i.d. 3 μm Atlantis C-18) and eluted with mobile phase A (0.2% acetic acid adjusted to pH 3.2) and phase B (acetonitrile). The flow rate was 0.2 mL/min and the column temperature was 30°C . A gradient elution at 10 min intervals was performed starting as follows: 0–10 min: 97–90% A, 11–20 min: 90–70% A, 21–30 min: 70–60% A, 31–40 min: 60–40% A, 41–50 min: 100% B.

2.4. HPLC and tandem mass spectral analysis

This analysis was performed using a Develosil® diol (250 mm × 4.6 mm i.d., 5 µm) column protected with a guard column made of the same stationary materials (Seto, Japan). Samples were dissolved in deionised water and the solutions were filtered through a Sartorius Ministar 0.45 µm membrane filter. Five µL solution (10 mg/mL) was injected into the column and eluted with flow rate of 1.0 mL/min under the column temperature of 35 °C. The mobile phase A is 2% acetic acid in acetonitrile while the mobile phase B is a solution of acidic aqueous methanol (CH₃OH:H₂O:HOAc, 95:3:2 v/v/v). The starting mobile phase condition was 7% B holding isocratic for 3 min before ramping solvent B to 37.6% over 57 min and then to 100% B in 3 min and held for 7 min prior to returning to starting conditions (7% B) in 6 min. The column was equilibrated with 7% B for 5 min prior to the next run. Detector wavelength was set at 280 nm.

2.5. Identification of proanthocyanidins

Liquid chromatography/mass spectra (LC/MS) were acquired using a Bruker Amazon ion trap mass spectrometer (BillERICA, MA, USA) equipped with a Dionex ultimate 3000 quaternary rapid separation HPLC system (Bannockburn, IL, USA). The heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was operated at 80 psi for sheath gas flow rate and 20 psi for auxiliary gas flow rate. The full scan mass spectra from *m/z* 50–2000 were acquired in both positive and negative ion modes with a scan speed at one scan per second. The MS/MS collision gas was helium with collision energy of 30% of the 5 V end-cap maximum tickling voltage.

MALDI-TOF mass spectra were collected on a Bruker micro TOF-QII mass spectrometer (BillERICA, MA, USA) equipped with delayed extraction and a dinitrogen laser set at 337 nm. The length of one laser pulse was 3 ns. The measurements were carried out using the following conditions: positive polarity, linear flight path with 21 kV acceleration voltage, and 100 pulses per spectrum. The samples were dissolved in deionised water (10 mg/mL). Sodium chloride and 2,5-dihydroxybenzoic acid as the matrix were used to enhance ion formation. Sodium chloride solution (1.0 µL, 0.1 M) was added to the sample solution (1.0 mL) followed by addition of 2,5-dihydroxybenzoic acid (20 mg/mL) at a volume ratio of 1:5. The resulting solution (6.0 µL) was evaporated and introduced into the spectrophotometer.

2.6. Thiolytic reaction of proanthocyanidins for HPLC analysis

Thiolytic reaction was conducted following a reported method with minor modification (Fu, Loo, Chia, & Huang, 2007). In a small glass vial, proanthocyanidin solution (250 µL, 2 mg/mL in methanol) was mixed with methanol acidified with concentrated HCl (250 µL, 3.3%, v/v) and 500 µL of methyl thioglycolate (5% v/v in methanol). The vial was sealed with an inert Teflon cap, heated in 45 °C oil bath for 30 min and then kept in the freezer (–20 °C) until analysis. The HPLC separation was conducted on an Atlantis C18 column (250 mm × 4.6 mm, 3 µm) fitted with a guard column of the same materials (Waters, MA, USA). The binary mobile phases consisted of A (2% acetic acid in water, v/v) and

B (methanol), which were delivered in a linear gradient of B from 15 to 80% (v/v) in 45 min in room temperature. The flow rate was set at 1.0 mL/min. Detector wavelength was set at 280 nm.

2.7. Determination of α-amylase and α-glucosidase inhibition activity

Turbidity change was determined by a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) by following a literature procedure (Liu et al., 2011). The assay solution consisted of enzyme solution (20 µL), inhibitor solution (20 µL), and 2% gelatinised starch solution (60 µL). The enzyme concentrations used for this assay were 3 U/mL for porcine pancreatic α-amylase and 1 × 10^{–2} U/mL for rat intestine α-glucosidase. Okra seed proanthocyanidin was first dissolved in DI-water and then diluted 1.5 times sequentially by sodium phosphate buffer to appropriate concentrations.

In a 96-well micro plate, 20 µL of enzyme solution was pre-incubated with 20 µL of proanthocyanidin solution with series of concentrations (or 20 µL of buffer solution as negative control, 20 µL of acarbose solution for positive control) in a microplate reader for 15 min at 37 °C. The reaction was started by injecting 60 µL starch solution using a 12-channel pipette. The turbidity change was immediately monitored at 660 nm for 2 h. To ensure sufficient mixing and avoid starch sedimentation, the change of optical density (OD) was used as an indication of turbidity level. The percentage of inhibition was calculated by Eq. (1):

$$\% \text{inhibition} = \frac{\text{AUC}_{\text{sample}} - \text{AUC}_{\text{control}}}{\text{AUC}_{\text{sample}}} \times 100 \quad (1)$$

in which AUC_{sample} is the area under the curve (AUC) of inhibitor; AUC_{control} is the area under the curve without inhibitors. The IC₅₀ can be defined as the concentration of inhibitor that produces 50% inhibition of enzyme activity under the specified assay condition. It was obtained from interpolation of percentage of inhibition against inhibitor concentration curve.

2.8. Statistical analysis

Statistical analysis was performed using SPSS® 17.0 for Windows® (SPSS Inc., Chicago, IL, USA). The data were expressed as mean of quintuple runs ± SD with replicate analysis.

3. Results and discussion

Southeastern Asia is abundant in wide variety of fruits and vegetables, which are rich sources of micronutrients including polyphenolic antioxidants, tocopherols, and carotenoids (Isabelle et al., 2010). It was suggested that the phytochemicals in vegetables may help control hyperglycaemia through blocking the glucose absorption and reduce the digestion rates of starch in our staple foods (Zhang & Hamaker, 2009). Therefore, we screened the starch α-amylase and α-glucosidase inhibition activity of the vegetable samples we collected. However, to our disappointment, among the vegetables, only the okra extracts (2:2:1 mixture of methanol, acetone, and water) exhibited

significant inhibitory activity. Therefore, we applied the assay-guided fractionation to elucidate the compounds responsible for the activity.

To further locate the part of okra tissue that contains active compounds, the okra seeds and the flesh were separated manually and extracted separately. Activity assay shows that the active compounds are mostly found in the seeds while the flesh shows very little activity. The crude extract of okra seeds was hence dried, reconstituted in water and subjected to sequential extraction of solvents of increasing polarity from *n*-hexane, chloroform, ethyl acetate to *n*-butanol. The active compounds are retained in aqueous phase and in *n*-butanol extracts.

3.1. Characterisation of polyphenolic compounds of okra seeds in *n*-butanol fraction

Polyphenolic compounds were separated with Atlantis C-18 column and four major peaks were detected at 254 nm (Fig. 1). All peaks resulted in characteristic MS and MS² signals as shown in Table 1. Structures of these compounds were assigned based on the *m/z* of their parent ions and their corresponding fragmentation ions from MS².

Peak 1 gave the positive and negative *m/z* values of 627.6 and 625.6, respectively. The MS⁻ fragmentation gives mass losses of 324.7 and 122.1 resulting in a product ion with *m/z* 300.9 and second product ion with *m/z* 178.8. The first product ion *m/z* 300.9 is deprotonated quercetin [M – H]⁻, whereas the second product ion *m/z* 178.8 results from a complicated fragmentation. A previous study quantified polyphenolic compounds in okra seed and skin and revealed that quercetin-3-*O*-β-glucopyranosyl-(1"→6")glucoside is the flavonol with the highest concentration in okra seed (Arapitsas, 2008). The UV chromatogram shows the maxima of the chromophoric system of quercetin with 255 and

352 nm. After comparison of the MS and UV data it can be concluded that peak 1 is quercetin-3-*O*-β-glucopyranosyl-(1"→6")glucoside. Similar to the compound in peak 1, peak 2 gave the positive and negative *m/z* values of 611.6 and 609.6, respectively. A previous study (Arapitsas, 2008) revealed that rutin is a minor compound in okra seed. The characteristic MS² fragmentation pattern in ESI-MS positive mode is an ion with a loss of 146.6 from the molecular ion and has *m/z* of 465. The daughter ion further loses a mass of 162.2 resulting in a second daughter of 302.8. The first daughter ion is isoquercetin, which is obtained by the loss of the rhamnose part, whereas the second daughter ion is the quercetin moiety after the loss of the glucosyl group. The UV chromatogram shows the maxima of the chromophoric system of quercetin with 257 and 355 nm. This compound can thereby be characterised tentatively as quercetin-3-*O*-rutinoside, also known as rutin. Peak 3 gave the positive and negative *m/z* values of 463.3 and 465.3, respectively. Additional MS² fragmentation experiments resulted in a product ion of 302.8 (quercetin) through a mass loss of 162.5. The UV chromatogram shows the maxima of the chromophoric system of quercetin at 256 and 356 nm. Therefore, peak 3 is identified as quercetin-3-*O*-glucoside. Besides, a previous study (Arapitsas, 2008) also characterised isoquercetin as one of the major polyphenolic components in okra seed extract. Finally, peak 4 gave the positive and negative *m/z* values of 551.4 and 549.4, respectively. Unlike the previous compounds, the fragmentation pattern is different. The [M – H]⁻ ion first fragments by losing 44.1 to give a daughter ion at *m/z* 505.3. This daughter ion is further fragmented into 300.7 with a loss of 204.6. The loss of 44 is significant for CO₂ release, which is characteristic for malonic acid moieties. The product ion with an *m/z* of 300.7 was already characterised as quercetin. The UV chromatogram shows the maxima of the chromophoric system of quercetin with 257 and

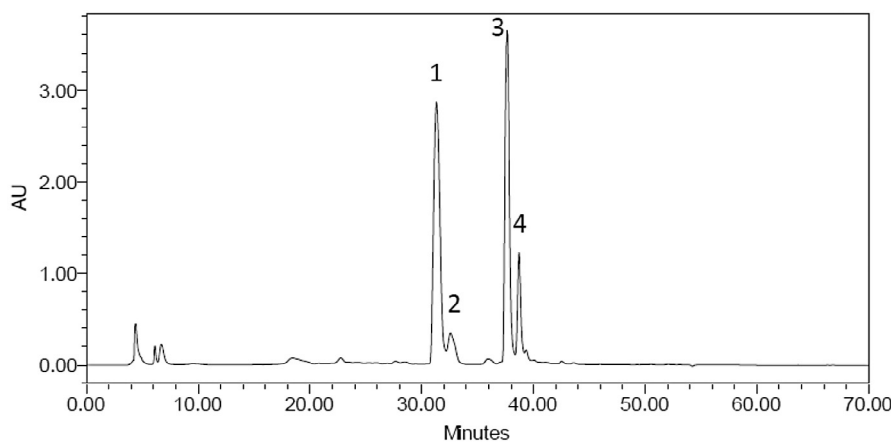


Fig. 1 – HPLC chromatogram of polyphenolic compounds in butanol fraction at 254 nm.

Table 1 – ESI-MS of flavonols in okra seeds.

Peak number	Retention time (min)	<i>m/z</i> [M – H] ⁻	MS ²	<i>m/z</i> [M + H] ⁺	MS ²	UV λ _{max} ACN/H ₂ O
1	31.3	625.6	300.9, 178.8	627.6	302.8	255, 352
2	32.6	609.6	300.9	611.6	465.0, 302.8	257, 355
3	37.6	463.3	300.9	465.3	302.8	256, 356
4	38.7	549.4	505.3, 300.7	551.4	302.8	257, 356

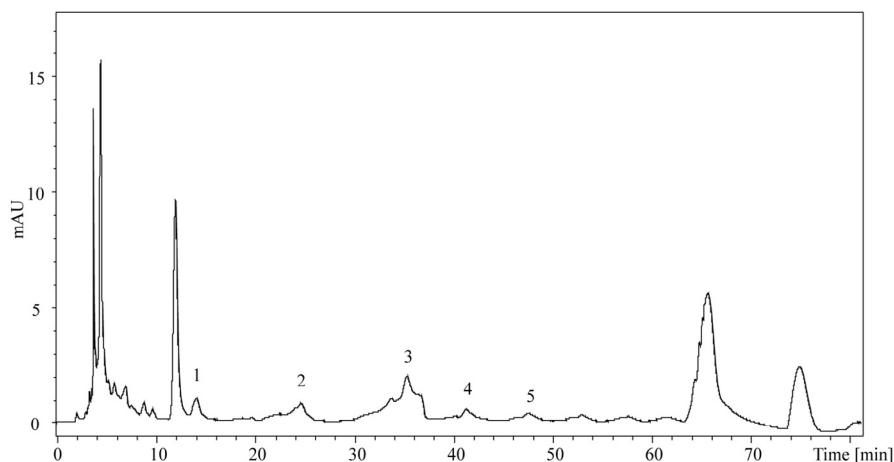


Fig. 2 – HPLC chromatogram of oligomeric proanthocyanidins in okra seeds.

356 nm. Thereby, compound 4 can be characterised as quercetin-3-O-(malonyl) glucoside. However, independent testing found that these flavanols and their glycosides did not show any inhibitory activity on α -amylase.

3.2. Chemical characterisation of the active components of okra seeds by HPLC/MSⁿ

Proanthocyanidins from Okra seeds were characterised using the Develosil[®] diol HPLC column because of its established good performance for such separation (Wang, Liu, Song, & Huang, 2012) with good baseline-resolved separation of up to decamers (Buendía et al., 2010; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013).

Fig. 2 shows the HPLC separation of okra seed proanthocyanidins. HPLC-ESI-MSⁿ at anionic mode was performed to further characterise these peaks. Results are summarised in Table 2. Structures of these compounds were assigned based on the m/z of their parent ions and their corresponding fragment ions from MS².

Peak 1 gave the m/z value of 609 and it fitted well with a dimer of gallicocatechin [$306 \times 2 - (2 - 1) \times 2 - 1$]. The main daughter ion peaks of anionic MS² are m/z at 591, 423, and 305. The daughter ion peak at m/z 591 [$M - H - 18$]⁻ occurs from the loss of one water molecule and the peak at m/z 423 [$M - H - 18 - 168$]⁻ is the result from retro-Diels–Alder (RDA) fragmentation of the

C rings after loss of one molecule of water. Finally, peak at m/z 305 [$M - H - 304$]⁻ was assigned to be (epi)gallicocatechin and might arise from quinone-methide cleavage of the interflavanyl bond (Wang et al., 2012). Peaks 2, 3, 4, 5 gave the parent ion peaks at m/z [$M - H$]⁻ 913, 1217, 1521, and 1825, respectively. They have been characterised by previous reports, and our results were consistent with reference data (Wang et al., 2012). Peak 2 was assigned to be a trimer of (epi)gallicocatechin based on the m/z value of 913 [$306 \times 3 - (3 - 1) \times 2 - 1$]. Its main fragment ions at 895 [$913 - H - 18$]⁻ might occur from the loss of one water molecule from the parent ion, Peak at m/z 727 [$M - H - 18 - 168$]⁻ might occur from loss of one water molecule and together with the retro-Diels–Alder fission of the heterocyclic rings. Peak at m/z 559 [$M - H - 18 - 168 \times 2$]⁻ might occur from two retro-Diels–Alder fissions and together with loss of a water molecule. Peak at m/z 423 [$M - H - 18 - 168 - 304$]⁻ might occur from loss of one water and (epi)gallicocatechin moiety and together with the Retro-Diels–Alder fission of the heterocyclic rings. Peaks 3, 4, and 5 gave the m/z values of 1217 [$306 \times 4 - (4 - 1) \times 2 - 1$], 1521 [$306 \times 5 - (5 - 1) \times 2 - 1$], and 1825 [$306 \times 6 - (6 - 1) \times 2 - 1$], and showed similar fragmentation patterns with peak 2 tabulating in Table 2. Therefore, peaks 3, 4, and 5 were assigned to be (epi)gallicocatechin's tetramer, pentamer, and hexamer, respectively.

3.3. MALDI-TOF mass spectra analysis of okra seed proanthocyanidins

A complementary and effective spectroscopic technique for multiply charged species is MALDI-TOF mass spectra, which could be used for the detection of high mass molecules or analysis of polymers, and also could reveal information about their chain lengths due to the production of only a singly charged molecular ion for each parent molecule (Fu et al., 2015; Mateos-Martín, Fuguet, Quero, Pérez-Jiménez, & Torres, 2012).

Fig. 3 shows a series of peaks arising from okra seed proanthocyanidins at m/z [$M + K$]⁺ or [$M + Na$]⁺. The major peak assignments are listed in Table 3. The most frequent mass difference between the major peaks was at m/z 304, which coincides with the mass of (epi)gallicocatechin. Besides, because of the occurrence of 1 A-type linkage in the two oligomers,

Table 2 – ESI-MS (negative mode) of okra seed oligomeric proanthocyanidins.

Peak number	m/z [$M - H$] ⁻	Polymer	Basic unit ^a	MS ² fragmentation ions
1	609	Dimer	(GC) ₂	591, 423, 305
2	913	Trimer	(GC) ₃	895, 727, 559, 423, 305
3	1217	Tetramer	(GC) ₄	1199, 1031, 913, 607, 423
4	1521	Pentamer	(GC) ₅	1396, 1215, 1091, 911, 305
5	1825	Hexamer	(GC) ₆	1701, 1521, 1396, 1217, 1091, 912, 726, 609, 423, 305

^a GC is the abbreviation for (epi)gallicocatechin; the stereochemistry of the chiral carbons on the C ring of flavanol units is not defined.

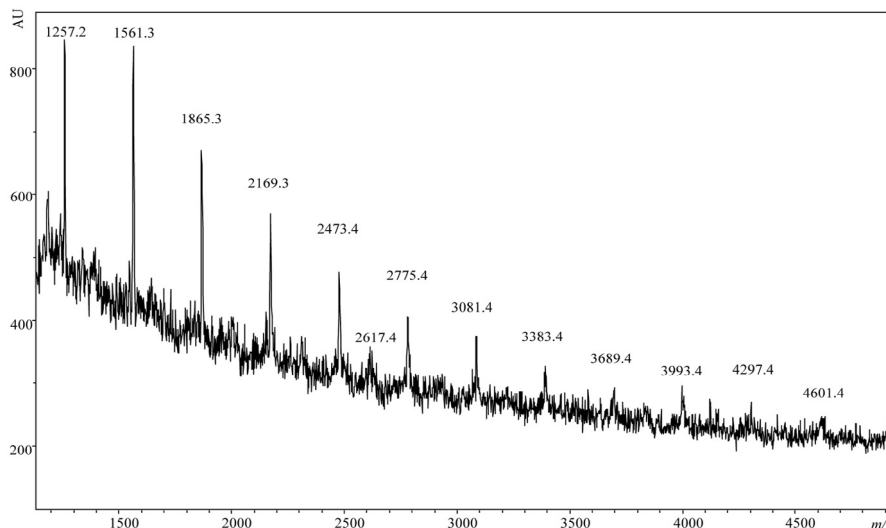


Fig. 3 – MALDI-TOF mass spectrum of okra proanthocyanidins.

Table 3 – Observed masses of okra oligomeric proanthocyanidins by MALDI-TOF mass spectra.

m/z $[M + K]^+$	Polymer	Basic unit ^a	Interflavan bond
1257.2	Tetramer	(GC) ₄	B type
1561.3	Pentamer	(GC) ₅	B type
1865.3	Hexamer	(GC) ₆	B type
2169.3	Heptamer	(GC) ₇	B type
2473.3	Octamer	(GC) ₈	B type
2775.4	Nonamer	(GC) ₉	1 A type
3081.4	Decamer	(GC) ₁₀	B type
3383.4	Undecamer	(GC) ₁₁	1 A type
3689.4	Dodecamer	(GC) ₁₂	B type
3993.4	Tridecamer	(GC) ₁₃	B type
4297.4	Tetradecamer	(GC) ₁₄	B type
4601.4	Pentadecamer	(GC) ₁₅	B type

^a GC is the abbreviation for (epi)gallocatechin; the stereochemistry of the chiral carbons on the C ring of flavanol units is not defined.

nonamer and undecamer, the difference compared with decamer was at m/z 302 and 306, respectively. In addition, several small peaks were also detected, the molecular weight at 2617.4 (m/z $[M + Na]^+$) is octamer of (epi)catechin.

3.4. Thiolysis of proanthocyanidins from okra seeds

To further investigate the main composition and determine if the sequence of the okra seed proanthocyanidins is composed of (epi)gallocatechin and (epi)catechin, depolymerisation through thiolysis reaction was carried out by following the standard conditions using methyl thioglycolate (Chen, Fu, Qin, & Huang, 2009). The reaction mixture was analysed by HPLC (Fig. 4) and also identified using LC-ESI-MS. The major product observed was the expected 4β -(carboxymethyl)sulphonyl-(epi)gallocatechin methyl ester, peak 5, along with significant amounts of 4β -(carboxymethyl)sulphonyl-(epi)catechin methyl ester, peak 6, and much smaller peaks for gallocatechin,

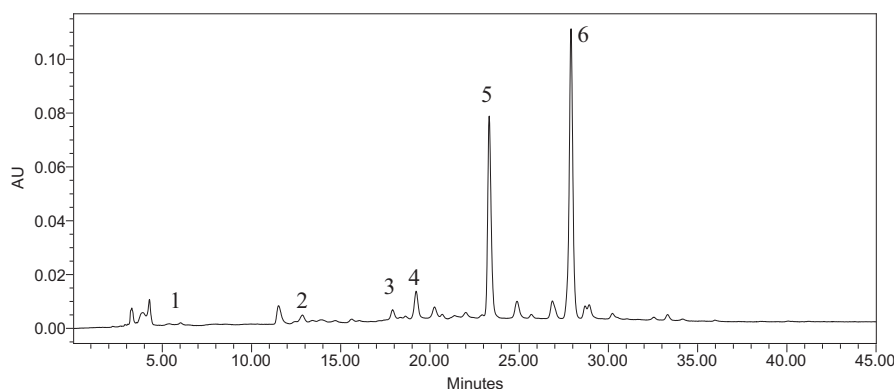


Fig. 4 – HPLC chromatogram (UV 280 nm) of thiolytic products of proanthocyanidins by methyl thioglycolate: gallocatechin, 1; (epi)gallocatechin, 2; (epi)gallocatechin thioether, 3; (epi)catechin, 4; (epi)gallocatechin thioether, 5; (epi)catechin thioether, 6.

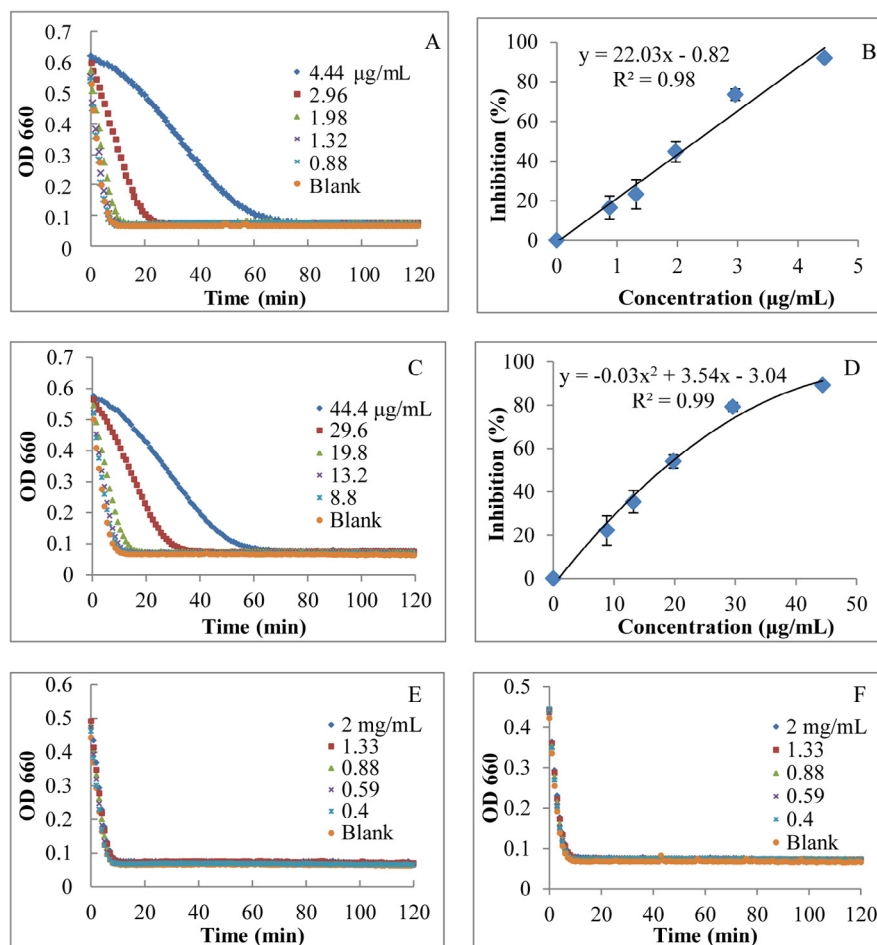


Fig. 5 – (A) Turbidity change in the presence of different concentrations of okra seeds extract that reflects the inhibitory activity of okra proanthocyanidins on starch hydrolysis by α -amylase. **(B)** The relationship between α -amylase inhibition activity and the okra seed proanthocyanidin concentration. **(C)** Turbidity change in the presence of different concentrations of okra seeds extract that reflects the inhibitory activity of okra proanthocyanidins on starch hydrolysis by α -glucosidase. **(D)** The relationship between α -glucosidase inhibition activity and the okra seed proanthocyanidin concentration. **(E)** Turbidity change in the presence of different concentration of sample that reflects the inhibitory effect of thiolysed okra proanthocyanidins on starch hydrolysis by α -amylase. **(F)** Turbidity change in the presence of different concentration of sample that reflects the inhibitory effect of thiolysed okra proanthocyanidins on starch hydrolysis by α -glucosidase.

(epi)gallocatechin, and (epi)catechin. This result suggests that there are significant amounts of epigallocatechin extension units, and also small amount of catechin extension units in the proanthocyanidins. By comparing the peak areas of the individual curves obtained from HPLC chromatogram, the mean degree of polymerisation (mDP) of okra seed proanthocyanidins was estimated to be 21.97 based on Eq. (2).

$$\text{mDP} = 1 + \frac{\sum[\text{extension units of thiolated gallocatechin}]}{\sum[\text{terminal units of gallocatechin}]} \quad (2)$$

Characteristics of monomeric units and mDPs are different in proanthocyanidins isolated from different plants. Procyanidins exist most widely in nature plant compared with prodelphinidins. However, prodelphinidins are most heterogeneous in constituent units and could coexist with procyanidins (Zhou, Lin, Wei, & Tam, 2011). Other than okra seed proanthocyanidins, chiku

and blackcurrants were all observed to have high contents of (epi)gallocatechin subunits within the mixture of proanthocyanidins (Törrönen et al., 2012; Wang et al., 2012).

Thiolysis reaction cannot degrade the A-type linkage in proanthocyanidins. The terminal units containing A-type linkage are released as an A-type dimer or as an A-type dimer thioether. Although small amounts of A-type linkage proanthocyanidins were detected (Fig. 4, Table 3), in our thiolysis result, no A-type dimer derivatives were observed.

3.5. Inhibition activity of okra seed proanthocyanidins on α -amylase and α -glucosidase

Fig. 5 shows the inhibitory effects of different concentrations of proanthocyanidins against α -amylase and α -glucosidase. The result showed that a very low concentration of proanthocyanidins (2 $\mu\text{g/mL}$) could cause significant α -amylase inhibition

(Fig. 5A). In addition, there is an increased linearly relationship between the inhibition activity and the concentrations of proanthocyanidins (Fig. 5B). Based on the dose–response curve, the IC_{50} for α -amylase inhibition was calculated to be $2.30 \pm 0.14 \mu\text{g/mL}$. This is more than one order of magnitude lower than that of acarbose (IC_{50} of $31.20 \pm 2.60 \mu\text{g/mL}$). In addition, the IC_{50} for α -glucosidase inhibition was measured to be $16.88 \pm 0.26 \mu\text{g/mL}$, which is also lower than acarbose (IC_{50} of $35.60 \pm 1.47 \mu\text{g/mL}$). Therefore, the PACs from okra seeds are much more potent than acarbose on equal weight basis.

To investigate if proanthocyanidin is solely responsible for α -amylase and α -glucosidase inhibition activity of okra seeds, the inhibition activity of the thiolysed product mixtures was tested. The result showed that there is no detectable α -amylase and α -glucosidase inhibition activity (Fig. 5E and F), indicating that the proanthocyanidins are the inhibitors. However, we cannot completely rule out the possibility that other type of inhibitors may react with thiols and lose activity as well.

The proanthocyanidins in unripe *Manilkara zapota* (chiku), which is mainly composed of galliccatechin as monomeric unit, have comparable α -amylase inhibition activity with an IC_{50} value of $4.2 \mu\text{g/mL}$ and α -glucosidase inhibition with an IC_{50} of $16.6 \mu\text{g/mL}$ (Wang et al., 2012). Proanthocyanidins in stems of *Fagopyrum dibotrys* rhizome (wild buckwheat) that is mainly composed of B-type catechin derivatives have a significant lower α -amylase inhibitory activity with an IC_{50} value of $10.7 \mu\text{g/mL}$ and much weaker glucosidase inhibitory activity (Liu et al., 2013). Added hydroxyl group in the B ring in galliccatechin oligomer may promote binding with enzymes.

4. Conclusions

Among wide range of commonly consumed vegetables, lady's finger stands out because it exhibits a significant inhibitory activity of starch hydrolases, which may be of potential for its use as a functional ingredient for controlling hyperglycaemia. Furthermore, assay-guided fractionation revealed that the oligomeric (epi)galliccatechin with high mean degree of polymerisation of 22 is the active compound responsible for its activity. This is the first report on characterisation of proanthocyanidins from okra and the active compound with IC_{50} values of $2.30 \pm 0.14 \mu\text{g/mL}$ (α -amylase) and $16.88 \pm 0.26 \mu\text{g/mL}$ (α -glucosidase). Proanthocyanidins are known to bind proteins unselectively because of their multiple phenol groups that can interact with protein through hydrogen bonds. This may be a problem when proanthocyanidins are applied to food matrix such as wheat flour. The potential for proanthocyanidins as active ingredients in low GI noodle can only be realised if it can preserve the enzyme inhibitory activity in grain flours.

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REFERENCES

- Arapitsas, P. (2008). Identification and quantification of polyphenolic compounds from okra seeds and skins. *Food Chemistry*, 110(4), 1041–1045.
- Buendía, B., Gil, M. I., Tudela, J. A., Gady, A. L., Medina, J. J., Soria, C., López, J. M., & Tomás-Barberán, F. A. (2010). HPLC-MS analysis of proanthocyanidin oligomers and other phenolics in 15 strawberry cultivars. *Journal of Agriculture and Food Chemistry*, 58(7), 3916–3926.
- Camciuc, M., Deplagne, M., Vilarem, G., & Gaset, A. (1998). Okra-*Abelmoschus esculentus* L. (Moench.) a crop with economic potential for set aside acreage in France. *Industrial Crops and Products*, 7(2), 257–264.
- Chen, W., Fu, C., Qin, Y., & Huang, D. (2009). One-pot depolymerizative extraction of proanthocyanidins from mangosteen pericarps. *Food Chemistry*, 114(3), 874–880.
- Cheplick, S., Kwon, Y. I., Bhowmik, P., & Shetty, K. (2010). Phenolic-linked variation in strawberry cultivars for potential dietary management of hyperglycemia and related complications of hypertension. *Bioresource Technology*, 101(1), 404–413.
- Fu, C., Loo, A. E. K., Chia, F. P. P., & Huang, D. (2007). Oligomeric proanthocyanidins from mangosteen pericarps. *Journal of Agriculture and Food Chemistry*, 55(19), 7689–7694.
- Fu, C., Yang, X., Lai, S., Liu, C., Huang, S., & Yang, H. (2015). Structure, antioxidant and α -amylase inhibitory activities of longan pericarp proanthocyanidins. *Journal of Functional Foods*, 14, 23–32.
- Huynh, N. T., Nguyen, N. Q., Tran, T. V. A., & Vo, P. N. (2008). Hypolipidemic effect of extracts from *Abelmoschus esculentus* L. (Malvaceae) on tyloxapol- induced hyperlipidemia on mice. *Mahidol University Journal of Pharmaceutical Sciences*, 35, 42–46.
- Isabelle, M., Lee, B. L., Lim, M. T., Koh, W. P., Huang, D., & Ong, C. N. (2010). Antioxidant activity and profiles of common fruits in Singapore. *Food Chemistry*, 123(1), 77–84.
- Jimmy Devi, O., Ganesh, P., Rajeshwari, D., & Srinivas, K. (2009). Antidepressant activity of *Abelmoschus esculentus* alcoholic fruit extract. *International Journal of Pharmaceutical Sciences and Research*, 1(2), 302–306.
- Liu, T., Song, L., Wang, H., & Huang, D. (2011). A high-throughput assay for quantification of starch hydrolase inhibition based on turbidity measurement. *Journal of Agriculture and Food Chemistry*, 59(18), 9756–9762.
- Liu, T., Yip, Y. M., Song, L., Feng, S., Liu, Y., Lai, F., Zhang, D., & Huang, D. (2013). Inhibiting enzymatic starch digestion by the phenolic compound diboside A: A mechanistic and in silico study. *Food Research International*, 54(1), 595–600.
- Mateos-Martín, M. L., Fuguet, E., Quero, C., Pérez-Jiménez, J., & Torres, J. L. (2012). New identification of proanthocyanidins in cinnamon (*Cinnamomum zeylanicum* L.) using MALDI-TOF/TOF mass spectrometry. *Analytical and Bioanalytical Chemistry*, 402(3), 1327–1336.
- Montero, L., Herrero, M., Prodanov, M., Ibáñez, E., & Cifuentes, A. (2013). Characterization of grape seed procyanidins by comprehensive two-dimensional hydrophilic interaction \times reversed phase liquid chromatography coupled to diode array detection and tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 405(13), 4627–4638.
- O'Keefe, J. H., & Bell, D. S. (2007). Postprandial hyperglycemia/hyperlipidemia (postprandial dysmetabolism) is a cardiovascular risk factor. *The American Journal of Cardiology*, 100(5), 899–904.
- Ou, K., & Gu, L. (2014). Absorption and metabolism of proanthocyanidins. *Journal of Functional Foods*, 7, 43–53.

- Pei, R., Yu, M., Bruno, R., & Bolling, B. W. (2015). Phenolic and tocopherol content of autumn olive (*Elaeagnus umbellata*) berries. *Journal of Functional Foods*, 16, 305–314.
- Sabitha, V., Panneerselvam, K., & Ramachandran, S. (2012). In vitro α -glucosidase and α -amylase enzyme inhibitory effects in aqueous extracts of *Abelmoschus esculentus* (L.) Moench. *Asian Pacific Journal of Tropical Biomedicine*, 2(1), 162–164.
- Sabitha, V., Ramachandran, S., Naveen, K. R., & Panneerselvam, K. (2011). Antidiabetic and antihyperlipidemic potential of *Abelmoschus esculentus* (L.) Moench in streptozotocin-induced diabetic rats. *Journal of Pharmacy and Bioallied Sciences*, 3(3), 397–402.
- Subrahmanyam, G. V., Sushma, M., Alekya, A., Neeraja, C. H., Harsha, H. S., & Ravindra, J. (2011). Antidiabetic activity of *abelmoschus esculentus* fruit extract. *International Journal of Research Pharmacy and Chemistry*, 1, 17–20.
- Törrönen, R., McDougall, G. J., Dobson, G., Stewart, D., Hellström, J., Mattila, P., Pihlava, J. M., Koskela, A., & Karjalainen, R. (2012). Fortification of blackcurrant juice with crowberry: Impact on polyphenol composition, urinary phenolic metabolites, and postprandial glycemic response in healthy subjects. *Journal of Functional Foods*, 4, 746–756.
- Wang, H., Liu, T., Song, L., & Huang, D. (2012). Profiles and α -amylase inhibition activity of proanthocyanidins in unripe *Manilkara zapota* (chiku). *Journal of Agriculture and Food Chemistry*, 60(12), 3098–3104.
- Wong, A. I. C., & Huang, D. (2014). Assessment of the Degree of Interference of Polyphenolic Compounds on Glucose Oxidation/Peroxidase Assay. *Journal of Agriculture and Food Chemistry*, 62(20), 4571–4576.
- Zhang, G., & Hamaker, B. R. (2009). Slowly digestible starch: Concept, mechanism, and proposed extended glycemic index. *Critical Reviews in Food Science and Nutrition*, 49(10), 852–867.
- Zhou, H., Lin, Y., Wei, S., & Tam, N. (2011). Structural diversity and antioxidant activity of condensed tannins fractionated from mangosteen pericarp. *Food Chemistry*, 129(4), 1710–1720.