The Effect of Bioactive Polyphenols from *Anacardium occidentale* Linn. Leaves on α-Amylase and Dipeptidyl Peptidase IV Activities

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Abstract: Anacardium occidentale *Linn.* (A. occidentale *L.*) *leaves possess bioactive polyphenols which are associated with antidiabetic potency for the management of type 2 diabetes mellitus (T2DM). In this study, free, soluble ester, and insoluble-bound phenolic fractions from young and mature leaves of* A. occidentale *L. were extracted. Subsequently, all fractions were investigated for their inhibitory effect on α-amylase and dipeptidyl peptidase IV (DPPIV) activities. Both free (72.45 ± 3.6%) and soluble ester (83.40 ± 4.7%) phenolic fractions in the mature leaves extracts had significantly demonstrated greater α-amylase inhibitors than the young leaves. Likewise, soluble ester (4.09 ± 0.34 μg/mL) and insoluble-bound (4.87 ± 0.32 μg/mL) phenolic fractions in the mature leaves extracts were significantly more effective in inhibiting DPPIV than the young leaves. As for fractions comparison, insoluble-bound derived from the young leaves extract was a more potent α-amylase inhibitor than free and soluble ester phenolic fractions ($p < 0.0001$). Besides, soluble ester and insoluble-bound phenolic fractions showed a stronger inhibitor of DPPIV than the free phenolic ($p < 0.001$), irrespective of the maturity of the leaves. In conclusion, this study showed that* A. occidentale *L. extracts possessed antidiabetic properties, which may potentially be used as an alternative treatment for T2DM management.*

Keywords: Anarcadium occidentale *Linn; dipeptidyl peptidase IV; α-amylase; inhibitor*

■ INTRODUCTION

Diabetes mellitus is one of the major health problems that affect millions of people worldwide. It is projected that 642 million people aged 20–79 years around the globe will suffer from diabetes by 2040 [1]. In Malaysia, since the first National Health Morbidity Survey (NHMS I) until recent NHMS V, the prevalence of diabetes mellitus among the adult population has markedly increased from 6.3% in 1986, 8.2% in 1996, 11.6% in 2006 and 15.2% in 2011 to 17.5% in 2015 [2-3]. Besides, diabetes also becomes one of the leading causes of death globally [1]. In 2012, it was reported that diabetes had caused 1.5 million (2.7%) deaths [4].

The management of diabetes involves continuous medical care with multifactorial risk reduction strategies, including lifestyle modifications and treatments with synthetic hypoglycemic drugs. Nevertheless, it is estimated that 70–80% of the world population still relies on traditional herbal medicine to meet their primary health care needs, including treatments for diabetes, especially in rural areas of developing nations [5]. For example, plants such as Aloe vera, *Andrographis paniculata*, *Centella asiatica, Curcuma longa*, and *Anacardium occidentale* have been demonstrated to possess antidiabetic properties [6-8], owing to the presence of bioactive compounds such as carotenoids and polyphenols that act as antidiabetic agents [9].

In the carbohydrate metabolism, polyphenols such as phenolic acids, flavonoids, and tannins play a role in inhibiting α-amylase, a glycoside hydrolase enzyme that, if attenuated can slow down the breakdown of long chain carbohydrates to glucose, thus preventing a sudden rise in postprandial blood glucose levels [10-12].

Polyphenols are also able to regulate postprandial glucose levels through the inhibition of dipeptidyl peptidase IV (DPPIV), a serine protease that localizes on the cell surface of various tissues, including the small intestine [13]. By inhibiting DPPIV, it prevents the rapid degradation of incretins such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) that play a vital role in blood glucose control. GLP-1 and GIP actions include stimulating insulin secretion, lowering glucagon concentration and slowing gastric emptying [14-15].

Anacardium occidentale Linn. (*A. occidentale* L.) or commonly known as cashew tree, is a popular tropical plant among various ethnics in Malaysia for its leaves, shoots, the cashew seed, and the cashew apple. Its shoots or young leaves are especially one of the commonly consumed vegetables by various ethnics in Malaysia [16]. The leaves were noted to be petiolate, elliptic-obovate, measuring 4 to 22 cm long and 2 to 15 cm broad, have a cuneate base with obtuse tip, reticulate venation, entire and smooth edges, a spiral arrangement and have a notable leathery texture [17]. When young, the leaves are pliable and reddish (Fig. 1), and when the leaves mature, they turn to dark green and leathery with prominent yellow veins (Fig. 2).

As described earlier, the leaves of *A. occidentale* L. also possess an excellent source of bioactive compounds, including phenolic acids [8,18-19]. Alkali and water extracts of *A. occidentale* L. contained predominantly gallic acid as well as protocatechuic, *p*-hydroxybenzoic, cinnamic, *p*-coumaric, and ferulic acids [20]. Besides, flavonol glycosides were also identified from the extract of *A. occidentale* L. young leaves, with the highest constituent was kaempferol-3-*O*-glucoside, followed by kaempferol-3-*O*-arabinofuranoside and quercetin-3-*O*-glucoside [16]. For this reason, this study sought to determine whether *A. occidentale* L. leaves extracts to play a role in the inhibition of α-amylase and DPPIV enzymes.

In nature, the insoluble-bound phenolics are one of the components of cell walls, while soluble ester phenolics compartmentalized within the plant cell vacuoles [21]. Therefore, the yield of extraction depended on the polarity of the compound to the solvent and method of extraction [22]. In this study, the soluble ester and insoluble-bound phenolic fractions were undergone extraction with acid-alkali treatments to release them from the bonded form. Here, we compared the enzyme-inhibitory activities of the extracts between free, soluble esters, and insoluble-bound phenolic fractions, as well as between the young and mature leaves. It is anticipated that these findings could provide some possible mechanisms by which they are used in the management and prevention of type 2 diabetes mellitus.

Fig 1. Young leaves of *A. occidentale* L.

Fig 2. Mature leaves of *A. occidentale* L.

■ EXPERIMENTAL SECTION

Materials

All chemicals used in this study were analytical grade and purchased either from Merck or Fisher Scientific Chemicals (Germany). Ultrapure water was obtained from Mili- Q system and was used in HPLC and enzyme assays. The recombinant DPPIV (human) enzyme was purchased from Enzo Life Sciences (USA). DPPIV-Glo (TM) Protease assay was purchased from Promega. α-Amylase enzyme and sodium potassium tartrate tetrahydrate were purchased from Nacalai Tesque. Acarbose (95%) and 3,5-dinitrosalicylic acid were purchased from Acros. Soluble starch, monosodium phosphate, disodium phosphate, and standards reference were purchased from Sigma Chemical Co. (Missouri, USA). Parts of young and mature leaves of *A. occidentale* L. (2.0 kg) were purchased from Taman Pertanian (Kuantan, Malaysia).

Procedure

Sample preparation for extraction

Young and mature leaves of *A. occidentale* L. (2.0 kg) were selected and washed under running water for several times. The leaves were chopped into small pieces. The leaves were then put into a freezer bag and kept frozen at -20 °C for 24 h. The frozen leaves were freeze-dried with conditions of 63 Pa vacuum pressures at 30 °C for three days. Subsequently, the samples were ground and sieved to obtain a uniform size of fine powder (< 1 mm). The powdered samples were kept in a vacuum-sealed container and stored at -20 °C for further analysis.

Sample extraction

The fine powdered of *A. occidentale* L. young and mature leaves were extracted using the maceration method with three different phenolic fractions - free, soluble ester, and insoluble-bound phenolic fraction extracts. The samples were extracted using methanol according to the procedure described by Dvořáková et al. [23] and Singh et al. [24] with slight modifications.

Free phenolic fraction. Three grams of powdered sample was extracted three times, with 30 mL of methanol (80%) on an ultrasonic bath at 60 °C for 1 h. The samples were centrifuged at 9000 ×g for 15 min at 4 °C. The supernatant was filtered using a no. 4 Whatman filter paper and evaporated until a semisolid residue was obtained using a Büchi rotary evaporator (Newcastle, DE). The semisolid residue was labeled as a crude extract. The crude extract was then extracted with diethyl ether (3 × 30 mL). The ether extracts were collected and evaporated to dryness using a Büchi rotary evaporator with 850 mbar at 35 °C. The dried sample was labeled as free phenolic and dissolved into two solvents, methanol, and DMSO. The free phenolic extract was stored at -20 °C for further analysis. The supernatant with esterified phenolic compounds was moved into a new Falcon tube.

Soluble ester phenolic fraction. The supernatant with esterified phenolic compounds was treated with 20 mL of 4 M sodium hydroxide (NaOH) for 2 h at room temperature. The treated supernatant was then acidified to pH 2 by adding 6 M hydrochloride acid (HCl) and was extracted with diethyl ether (3 × 10 mL). The ether extract was collected and evaporated to dryness using a Büchi rotary evaporator with 850 mbar at 35 °C. The dried sample was labeled as soluble ester phenolic and dissolved into two solvents, methanol, and DMSO. Soluble ester phenolic extract was stored at -20 °C for further analysis.

Insoluble-bound phenolic fraction. The residue obtained from free phenolic fraction extract was treated with 15 mL of 4 M sodium hydroxide (NaOH) for 1 h at room temperature. The treated residue was then acidified to pH 2 by adding 2 M hydrochloride acid (HCl) followed by centrifuged for 10 min at 5000 ×g. The layer of supernatant was collected and extracted with 15 mL of hexane to remove free fatty acid and other lipids contaminants. The liberated phenolic was extracted with diethyl ether (3 × 10 mL). The ether extracts were collected and evaporated to dryness using a Büchi rotary evaporator with 850 mbar at 35 °C. The dried sample was labeled as insoluble bound phenolic and dissolved into two solvents, methanol, and DMSO. The insoluble-bound phenolic extract was stored at -20 °C for further analysis.

High-performance liquid chromatography (HPLC) analysis

High-performance liquid chromatography (HPLC) analysis consists of HPLC equipped with an ultraviolet-visible (UV-Vis). Before injection, each

extracted sample was allowed to be filtered by a 0.45 µm PTFE filter (Millipore). The injection volume was 5 µL. A C-6 phenyl column (250 × 4.6 mm i.d., particle size 5 µm, C-6 Phenyl, phenomenex, Torrance, CA, USA) was used for the separation of sample components at 25 °C. For phenolic acid analysis, the mobile phase comprised of solvent A (methanol) and solvent B (a 1.5%, v/v, solution of formic acid in water). For flavonoids analysis, the mobile phase comprised of solvent A (methanol), solvent B (acetonitrile) and solvent C (0.5–1.0%, v/v, solution of formic acid in water). The flow rate was set at 1.0 mL/min for the compounds.

In vitro *α-amylase inhibition study*

α-Amylase enzyme activity was 3100 Unit/mg of protein (Nacalai Tesque). The α-Amylase enzyme was dissolved in sodium phosphate or phosphate buffer solution to give a concentration of 12 unit/mL solution. Soluble starch (1%, w/v) in water was used as a substrate solution. Briefly, 250 µL of extracted samples with DMSO were put into a screw-top Falcon tube. Then, 250 µL of the α-amylase enzyme in a phosphate buffer solution with pH 6.9 was added into the Falcon tube. The mixture was preincubated for 10 min at 25 °C. Approximately 250 µL of the starch solution was mixed in the mixture as a substrate and incubated for another 10 min at 25 °C. Next, 500 µL of DNS color reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH) was added into the Falcon tube to terminate the reaction. The Falcon tube was then placed into boiling water for 10 min. The mixture was cooled down and diluted with 5 mL of distilled water.

The α-amylase activity was determined by measuring the absorbance of the mixture in a transparent 96-well plate at 540 nm. Control incubations that represent 100% enzyme activity were conducted in an identical fashion replacing plant extract with DMSO. The absorbance (A) due to maltose generated was calculated using the following calculation:

A 540 nm control or plant extract = A 540 nm Test – A 540 nm Blank

The % of maltose generated was calculated from the linear equation obtained from the maltose standard calibration curve (0–0.25%, w/v, maltose). Percentage of inhibition = 100 - % of reaction, whereby:

$$\%\ \text{reaction} = \frac{\text{Means Maltose in sample}}{\text{Means Maltose in negative control}} \times 100$$

In vitro *dipeptidyl peptidase IV (DPPIV) inhibition study*

The *A. occidentale* L. extracts were examined for inhibition bioactivities on DPPIV using the Promega DPPIV-Glo Protease Assay kit with a white 96-well plate. Before the inhibition test was performed, each sample was allowed to pass through a 0.45 µm PTFE filter (Millipore). The purified porcine DPPIV enzyme (88% homology to the human enzyme) from Sigma-Aldrich was used to determine the activity of DPPIV inhibitor. DPPIV enzyme activity was defined as 1 Unit produces 1 µmol free pNA/min, using standard DPPIV assay condition at 37 °C. The DPPIV inhibitor activities were observed using a luminometer (Luminoskan Ascent 2.6) to detect luminescence on the white 96-well plate. IC_{50} values (µg/mL) were calculated from the quadratic formula generated from the log (concentration) and percentage inhibition. The protocols of analysis were based on the manufacturer specifications from Promega [25]. Kaempferol was used as a positive control.

Statistical analysis

Results were expressed as means ± SEM. The analysis was analyzed using SPSS statistical software (SPSS version 20; SPSS Inc., Chicago, IL). T-test analysis was used to compare the means between young and mature leaves. One-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test was used to compare means between fractions. The level of significance was set at $p < 0.05$, which corresponds to a 95% confidence level.

■ RESULTS AND DISCUSSION

High-Performance Liquid Chromatography (HPLC) Analysis

The presence of phenolic acid and flavonoids in *A. occidentale* L. leave extracts were first determined by HPLC using external standards. In this analysis, it was found that all extracts contained phenolic acid and flavonoids. Free phenolic fraction had a higher

concentration of phenolic acid but a lower concentration of flavonoids compared to soluble ester and insoluble-bound phenolic fractions. Mature leaves of *A. occidentale* L. contained higher concentration in both phenolic acid and flavonoids than the young leaves. This trend was in line with a previous study where the presence of phenolic compounds varies in different parts of leaves. The variation of phenolic compounds concentration has also been reported at different stages of maturity of plants [26].

An *in vitro* Study of α-Amylase and DPPIV Inhibitory by Maturity

Phenolic compounds in three different fractions between the young and mature leaves of *A. occidentale* L. were further analyzed for their α-amylase inhibitory effect (Table 1). Free phenolic fraction (72.45 ± 3.6%) in the mature leaves demonstrated a higher inhibition percentage of α-amylase activities than that in the young leaves ($p < 0.05$). Similarly, soluble ester phenolic fraction (83.40 ± 4.7%) in the mature leaves also exhibited a greater α-amylase inhibitor as compared to the young leaves ($p < 0.001$). However, the α-amylase inhibitory effect of the insoluble-bound phenolic fraction was not significantly different between the matured and young leaves.

Table 2 shows the concentration of phenolic compounds fractions from the young and mature leaves of *A. occidentale* L. in the inhibition of DPPIV (IC_{50}). IC_{50} is defined as the concentration of an inhibitor (extracts of *A. occidentale* L. leaves) where the DPPIV enzyme activity is reduced by half (50%). A lower concentration indicates a more potent inhibitor. Regardless of fractions, the mature leaves were generally more effective in reducing the activity of DPPIV than the young leaves. Specifically, soluble ester (4.09 ± 0.34 μg/mL) and insoluble-bound (4.87 ± 0.32 μg/mL) phenolic fractions of the mature leaves were found to be significantly more potent inhibitors of DPPIV activities than the young leaves ($p < 0.001$). Nevertheless, no statistical difference was found between the mature and young leaves for the DPPIV inhibition activity by a free phenolic fraction.

In summary, it is clear that the mature leaves of *A. occidentale* L. are more potent inhibitors of both α-amylase and DPPIV enzyme activities than the young leaves. Of relevance, our previous study had found that the concentration of phenolic acid and flavonoids were higher in *A. occidentale* L. mature leaves than in the young leaves (unpublished data). Therefore, it is believed that the higher phenolic content may proportionately increase its antioxidant capacity toward inhibiting both enzyme activity [12].

Table 1. The inhibition percentage of α-amylase activities in the presence of compounds extracted (0.6 mg/mL) from *A. occidentale* L. Results are expressed in means ± SEM

Fraction[a]	Young (%)	Mature (%)
Free	54.92 ± 2.3	72.45 ± 3.6[b]
Soluble ester	16.34 ± 1.5	83.40 ± 4.7[c]
Insoluble-bound	69.04 ± 2.8	69.65 ± 1.9

[a]Fraction of phenolic compounds extracted from *A. occidentale* L. was used in the α-amylase inhibition study; [b]Young and mature leaves were compared by t-test, p-value < 0.05; [c]Young and mature leaves were compared by t-test, p-value < 0.001

Table 2. DPPIV inhibition activity for free, soluble ester and insoluble-bound phenolic compounds are tabulated by concentration (IC_{50}) = μg/mL. Results are expressed in means ± SEM

Fractions[a]	Young	Mature
Free	156.6 ± 27.5	53.46 ± 6.49
Soluble ester	8.29 ± 0.33	4.09 ± 0.34[b]
Insoluble-bound	11.48 ± 0.48	4.87 ± 0.32[b]

[a]Fractions of phenolic compounds extracted from *A. occidentale* L. were used in the DPPIV inhibition study; [b]Young and mature leaves were compared by t-test, p-value < 0.001

An *in vitro* α-Amylase and DPPIV Inhibitory Activity Study by Fractions

Fig. 3 shows the comparison between the free, soluble ester and insoluble-bound phenolic fractions in inhibiting α-amylase activity in the young and mature leaves, respectively. In the young leaf extracts, the insoluble- bound phenolic fraction (69.04 ± 2.8%) demonstrated a significantly more potent inhibitor than free (54.92 ± 2.3%, $p < 0.05$) and soluble ester phenolic fractions (16.34 ± 1.5%, $p < 0.0001$). Likewise, free

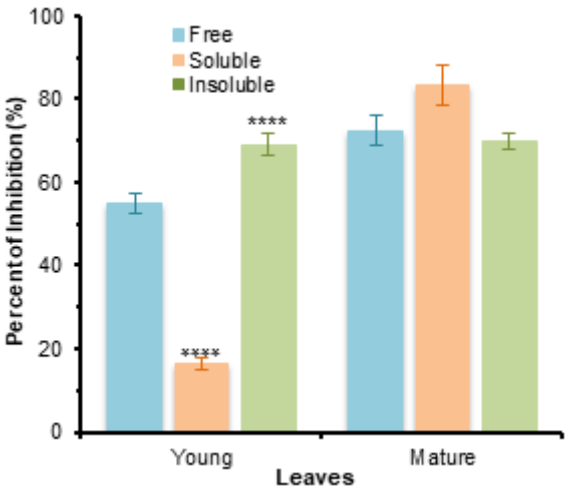

Fig 3. Comparison within phenolic fractions in inhibiting α-amylase activities using one-way ANOVA followed by Tukey's multiple comparison test analysis. Results are expressed in means ± SEM. *p < 0.05 and ****p < 0.0001

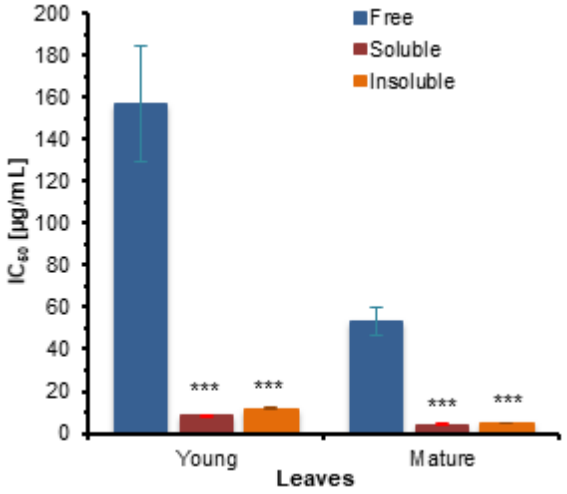

Fig 4. Comparison within phenolic fractions in DPPIV inhibition activities using one-way ANOVA followed by Tukey's multiple comparison test analysis. Results are expressed in means ± SEM, ***p < 0.001

phenolic had a greater capability in inhibiting α-amylase enzyme than soluble ester phenolic fraction ($p < 0.0001$). On the contrary, we found no significant differences in the α-amylase inhibitory effect between fractions of the mature leaves' extracts.

Fig. 4 indicates that in young leaf extracts, soluble ester (8.29 ± 0.33) and insoluble-bound (11.48 ± 0.48) phenolic fractions demonstrated a stronger inhibitor of DPPIV than free phenolic (156.6 ± 27.5, $p < 0.001$). A similar trend for the mature leaves extracts was noted where soluble ester (4.09 ± 0.34) and insoluble-bound (4.87 ± 0.32) phenolic fractions also exhibited a lower IC_{50} than free phenolic (53.46 ± 6.49, $p < 0.001$). No significant differences were found between the soluble ester and insoluble-bound phenolic fractions for both the young and mature leaves, respectively.

It appears that insoluble-bound fraction was the most potent inhibitor when compared to free and soluble ester phenolic fraction in both α-amylase and DPPIV inhibition activities. Although soluble ester phenolic fraction did not strongly inhibit the α-amylase activity, it was nevertheless effective in inhibiting DPPIV activity. We speculate that soluble ester and insoluble-bound phenolic fractions may contain a higher concentration of flavonoids than the free phenolic fraction. As indicated by a previous study, flavonoids were a strong inhibitor of α-amylase and DPPIV enzymes [27]. In another study, it was shown that the soluble ester and insoluble-bound phenolic extracts of *Moringa oleifera* seed flour were more effective with minimum inhibitory concentration against bacteria than free phenolic fractions [24].

■ CONCLUSION

Our findings indicated that the mature leaves of *A. occidentale* L. possessed better antidiabetic properties, as shown by a greater inhibition of both α-amylase and DPPIV enzymes when compared to young leaves. It was also observed that regardless of the maturity of the leaves, soluble ester and insoluble-bound phenolic fractions were a more potent inhibitor of α-amylase and DPPIV enzymes than free phenolic. Further studies of the elucidation and characterization methodologies are required in identifying specific phenolic compounds that are responsible for the inhibition of these enzymes.

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