P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol. 26 No.2 April - June 2021
Page: [138-144]
Original research article

Anti-Diabetic Potential of Cashew Nut (*Anacardium occidentale*) Shoots and Leaves Extracts under Simulated *In Vitro* Digestion

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Received 6 October 2019; Received in revised form 11 May 2020
Accepted 27 August 2020; Available online 25 June 2021

ABSTRACT

Anacardium occidentale has long been used for diabetes therapy; however, its active components remain unknown. This study aimed to investigate the phytochemical contents, especially phenolics and flavonoids, along with α-glucosidase inhibitory activity and bioaccessibility, of extracts from the shoots and leaves of *A. occidentale*. The methanolic extract from leaves showed higher total phenolics (98.21±0.35g GAE/ 100 g extract) and total flavonoids (89.53±0.65 g RE/ 100 g extract) than the shoots extract. Upon bioaccessibility testing, phenolics and flavonoids from the leaf extract were more bioaccessible than those from the shoot extract. The α-glucosidase inhibitory activity of *A. occidentale* was higher when predigested than it was after stimulated gastric digestion and pancreatic digestion. These results suggest that *A. occidentale* has the potential to be used for diabetes therapy and as a functional food component.

Keywords: *Anacardium occidentale*; Polyphenolics; *In vitro* digestion; α-Glucosidase inhibitor

1. Introduction

Diabetes mellitus (DM) is metabolic disorder identified by high blood glucose levels caused by the lack of pancreatic insulin secretion [1]. The worldwide prevalence of diabetes for all age groups has increased every year. Epidemiological studies clearly indicate that diabetes is the main cause of complications related to neurological problems, limb amputation, coronary artery disease and renal failure [2]. One therapeutic approach to decreasing postprandial hyperglycemia is to retard glucose absorption via α-glucosidase inhibition. It is widely accepted that delaying glucose absorption after a meal is

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beneficial to combating disease symptoms [3], and various α-glucosidase inhibitors are used as drugs for diabetes therapy {acarbose and voglibose). However, patients have reported side effects such as flatulence, diarrhea and abdominal discomfort [4]. Therefore, it is of interest to search for drugs, including from natural sources like Thai herbs, which can minimize side effects.

The fresh shoots and leaves of *Anacardium occidentale* (cashew nut) are widely consumed, and are rich sources of dietary compounds possessing several pharmacological properties, including antidiabetic efficacy. The polyphenol and flavonoid rich *A. occidentale* has also revealed potent antidiabetic activity by way of inhibiting α-glucosidase, a key enzyme linked to type II diabetes; its consumption has been considered to be an effective strategy to control blood glucose [5]. The *A. occidentale* leaf has been reported to contain quercetin, myricetin, catechin, epicatechin, amentoflavone,proanthocyanidin, myricetin-3- *O*- rhamnoside and quercetin- 3- *O*-rhamnoside [6]. The stability of polyphenolics including flavonoids from cashew, and their level of anti α-glucosidase activity may vary during gastric and intestinal digestion, due to food matrix, pH, temperature, presence of inhibitors or enhancers of absorption, presence of enzymes and other related factors [7]. When dietary polyphenolic compounds from food are exposed to *in vitro* digestive enzymes, they are typically transformed structurally and chemically [8]. Therefore, in this work, the bioaccessibility of the polyphenolics from shoots and leaves was studied using an *in vitro* simulated gastrointestinal digestion method. In addition, changes in the α-glucosidase inhibition activity of digestive phenolic metabolites were investigated after the digestion treatments.

2. Materials and Methods

2.1 Materials

All the chemicals used in this study were obtained from Sigma Aldrich Co. (USA). All solvents were purchased from Labscan (Thailand).

2.2 Preparation of extracts

The fresh shoots and leaves of *A. occidentale* were purchased from Thung Song Market, Nakon Si Thammarat, Thailand in May 2018. Samples (each 500 g) were separately air- dried at ambient temperature and ground into powder. The dried powders were mixed with 50 mL of methanol, CH_2Cl_2 and hexane at ambient temperature for 24 hours. The extracts were concentrated to dryness using a rotary evaporator with the water bath set at 40°C. The crude extracts were kept in the cold prior to assay.

2.3 Quantification of total phenolics and flavonoids

The total phenolic contents of plant extracts were quantified based on the method described by Hossain et al. [9]. The reaction mixture, having 100 µL of each extract, was introduced with 2 % Na_2CO_3 (2 mL). After 2 min, 50 % Folin-Cioclateu's reagent (100 µL) was added and the mixture was then left for 30 min at 25 °C. Spectrophotometric analysis was performed at 750 nm. The results were expressed as gallic acid equivalents (GAE). The flavonoid content of extracts was determined based on the method developed by Zhishen et al. [10]. The 100 µL of extract was mixed with 500 µL of distilled water and 50 µl of 5% sodium nitrate. After 6 min, 50 µl of 10% aluminum chloride and 200 µl of 1 M sodium hydroxide were added and left at room temperature for 15 min. The flavonoid contents were measured at 510 nm and the results were expressed as rutin equivalents (RE).

2.4 *In vitro* digestion by gastric and pancreatic juices

The assay was adapted from our previously described work [11]. First, 150 mg of porcine-pepsin was mixed with 90 mg of NaCl in 50 mL of distilled water to prepare the gastric juice. HCI was used to change the pH to 1.2. The methanol extract (150 mg/mL) was added to gastric juice, and the mixture was incubated at 37°C for 1 h. After stopping the reaction with boiling water, 5 mL of shoots and leaves gastric juice sample was spared for pancreatic digestion. Then, 1000 mg of pancreatin was mixed with 50 mL of phosphate buffer (20 mM, pH 8.0) to make the pancreatic juice. Later, 5 mL of pancreatic juice was added to each of the shoots and leaves methanol extracts. The reaction mixture was incubated at 37°C for 2 h. The reaction was stopped by liquid nitrogen. The samples were centrifuged at 6,000 rpm, 4°C for 30 min and filtrated by Whatman No 541. The enzyme activity was stopped in boiling water 100°C for 20 min and then stored at 4°C for further analysis.

2.5 α-Glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity was measured according to the method developed by Damsud et al. [12]. Acarbose was used as positive control. The percentage of inhibition was calculated using the following formula: % inhibition = $[(A_0-A_1)/A_0]\times100$ where A_0 is the absorbance without the sample, and A_1 is the absorbance with the sample. The type of inhibition was determined from Lineweaver– Burk plots, where the V_{max} and K_m values were determined from the plot graph of substrate concentration.

2.6 Statistical analyses

Data are presented as mean ± standard deviation (S.D.) of triplicate examinations. Microsoft Excel, 2007 was used for plotting graphs. Graph Pad Prism Version 5 for Windows was used to calculate IC_{50} values.

3. Results and Discussion

3.1 Extractability

In the extraction method, the data from extracts of shoots and leaves in various solvents are shown in Table 1. The extract yield of shoots ranged from 1.32 to 8.83±0.72 g/100 g sample, while the leaves extracts ranged from 1.43 to 9.95 g/100 g sample. The methanol extract of shoots and leaves showed higher yield, whereas methylene chloride extract yields were lower.

3.2 Total phenolics and flavonoids

The polyphenolic contents in the investigated shoot and leaf extracts are shown in Table 1. The leaf extract contained a higher amount of phenolics (98.21±0.35 g GAE/100 g extract) than the shoot extract did (20.75±0.41 g GAE/100 g extract). A similar trend was also observed in the flavonoid content of the leaf methanol extract (89.53±0.65g RE/100 g extract) and shoot (55.32±0.21 g RE/100 g extract) extracts. The polarity of the solvent also played a key role in increasing the solubility of phenolic compounds [13]. Comparing the different extracts, the methanol extract of leaves had higher amounts of total phenolics and flavonoids than the shoot extract did. Damsud et al. [5] have reported similar results, showing that leaves of *A. occidentale* have higher total phenolic content than shoots. As demonstrated in these results, leaves of *A. occidentale* can play a vital role in the inhibition of α-glucosidase activities, due to the presence of polyphenolics. Hence, methanol extracts of shoots and leaves were selected for the investigation of bioaccessibility.

3.3 Bioaccessibility

The total phenolic and flavonoid contents of *A. occidentale* methanol extracts after gastric and pancreatic digestion are shown in Table 1. The total phenolic and

Table 1. Extraction yield, total phenolic and flavonoid contents of *A. occidentale*.

Solvents	Extract yield (g/100g) sample)		Total phenolics (g GAE/extract)		Flavonoids (g RE/100 g extract)	
	Shoots	Leaves	Shoots	Leaves	Shoots	Leaves
MeOH	8.83±0.72	9.95±1.32	20.75±0.41	98.21±0.35	55.32±0.21	89.53±0.65
CH_2Cl_2	1.32±0.04	1.43±0.16	1.62±0.41	9.45±0.31	10.54±0.03	11.54±0.24
Hexane	2.16±0.17	3.48±0.07	0.55±0.18	0.73±0.48	3.74±0.71	1.23±0.06
GDM	-	-	12.43±0.31	75.32±0.43	25.28±0.21	58.45±0.35
PDM	-	-	11.86±0.75	55.65±1.81	21.12±0.32	34.21±0.57

Values are presented as means of three independent analyses ± standard deviation (n=3)
GAE – Gallic acid equivalents; RE – Rutin equivalents; GDM – Gastric digested methanol extract; PDM – Pancreatic digested methanol extract.

Table 2. α-glucosidase inhibitory activity of *A. occidentale*.

Solvents	IC_{50} [a] (mg/mL)					
	Yeast α-glucosidase		Maltase		Sucrase	
	Shoots	Leaves	Shoots	Leaves	Shoots	Leaves
MeOH	74.34±0.41	79.21±0.82	4.36±0.21	0.91±0.02	3.21±0.07	0.90±0.04
CH_2Cl_2	NI[b]	NI	25.64±0.43	10.98±0.22	14.53±0.65	1.250±0.14
Hexane	NI	NI	89.45±0.56	43.94±0.13	25.21±0.81	13.07±0.25
GDM	123.56±4.12	134.75±5.23	14.45±0.57	3.43±0.15	3.67±0.16	1.15±0.05
PDM	143.25±3.65	142.67±3.12	25.61±0.17	10.67±0.56	10.81±1.12	7.64±0.11
Acarbose	2.99±0.14		0.59±0.02		1.59±0.12	

[a]The IC_{50} value is defined as the inhibitory concentration needed to inhibit 50% of enzyme activity
[b]No inhibition, less than 60% inhibition at 5 mg/mL.

Table 3. The V_{max} and K_m values of α-glucosidase inhibition assay.

S. No	Samples mg/mL	Pre-digestion				Gastric-digestion				Pancreatic-digestion			
		Shoots		Leaves		Shoots		Leaves		Shoots		Leaves	
		Vmax (mM/ min)	Km (mM)	Vmax (mM/ min)	Km (mM)	Vmax (mM/ min)	Km (mM)	Vmax (mM/ min)	Km (mM)	Vmax (mM/ min)	Km (mM)	Vmax (mM/ min)	Km (mM)
1	0.50	6.36	24.84	5.95	22.55	5.29	21.32	5.74	25.28	6.06	22.18	6.53	25.81
2	0.75	4.65	26.74	4.90	29.36	4.67	26.86	4.85	28.98	4.62	26.48	4.67	26.66
3	Control	8.06	23.46	8.06	23.46	8.06	23.46	8.06	23.46	8.06	23.46	8.06	23.46

Vmax – maximum velocity; Km – Michaelis-Menton Constant; Control – absence of plant extracts.

flavonoid contents of shoot (20.75-11.86 g and 55.32-21.12 GAE/100 g extract) and leaf (98.21-55.65g and 89.5-34.21 GAE/100 g extract) methanol extracts decreased after the simulated digestion process. From the obtained results, the gastric digestion product had higher total phenolic and flavonoid contents than the pancreatic digestion product did. This is may be due to low pH (2.0) conditions during gastric digestion which may make these compounds become more bioaccessible. Likewise, the active compound (polyphenolic and flavonoid) contents were found in larger amounts from the gastric phase than from the pancreatic phase. A previous investigation on broccoli showed that phenolic and flavonoid contents were higher in the crude extract compared to degraded extract after gastric digestion [14].

3.4 α-Glucosidase inhibitory activity assay and their stability

The digestion of disaccharides such as sucrose and maltose into glucose is carried out by a group of α- glucosidase enzymes. Hence, inhibition of these enzymes would retard blood glucose level rise and control diabetes mellitus. The α- glucosidase inhibitory activities of shoot and leaf extracts were assessed in two different enzyme (yeast and rat intestinal) assays. In this study, the methanol extracts of shoots and leaves showed the highest activity against yeast, maltase and sucrase. In consequence, the *in vitro* simulated gastrointestinal digestion method was used to investigate the accessibility of α- glucosidase inhibitory activity. The methanol extracts of shoots and

leaves showed the most potent inhibitory activity against yeast α-glucosidase and rat intestinal (maltase and sucrase), shoots (IC_{50}- 74.34±0.41 mg/ mL), leaves (IC_{50}- 79.21±0.82), shoots (IC_{50}- 4.36±0.21 mg/mL), leaves (IC_{50}- 0.91±0.02 mg/ mL), shoots (IC_{50}- 3.21±0.07 mg/mL), and leaves (IC_{50}-0.90±0.04 mg/mL) respectively.

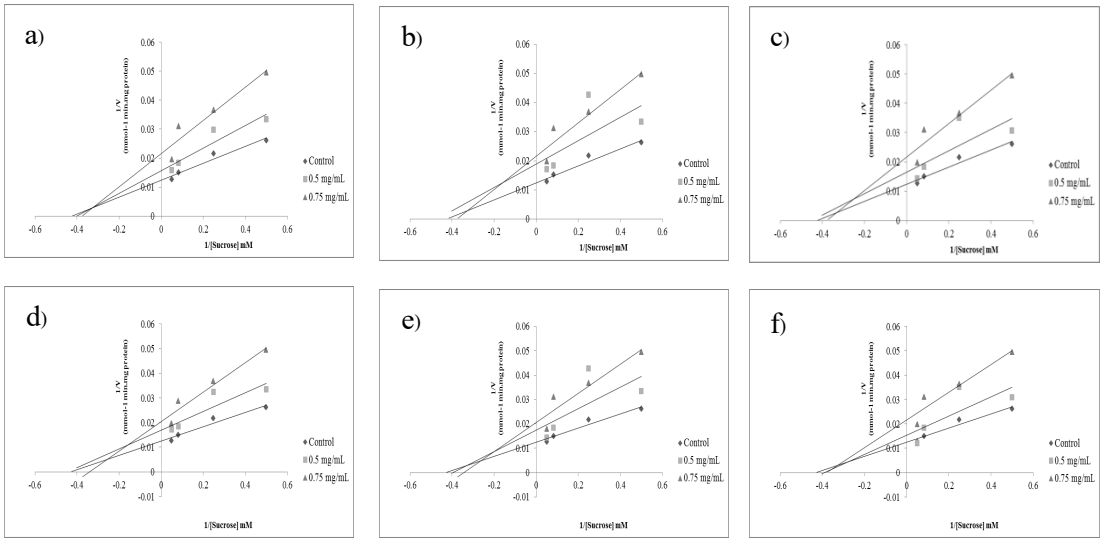

Fig. 1. Lineweaver-Burk plots of α-glucosidase inhibition a) Crude shoot extract; b) Gastric digested shoot extract c) Pancreatic digested shoot extract d) Crude leaf extract e) Gastric digested leaf extract f) Pancreatic digested leaf extract.

On the other hand, gastric digested and pancreatic digested shoot and leaf methanol extracts showed a decreased level of α-glucosidase inhibitory activity (Table 2).

According to the results, the α-glucosidase inhibitory activity was reduced in the shoots and leaves after the simulated digestion. This may be due to the decreased degradation of polyphenolics in methanol extracts after digestion. Many plant extracts and natural product compounds, especially polyphenols and flavonoids, have been reported to have α- glucosidase inhibitory activities [15]. In a previous study, Fadeyi et al. [16] reported the presence of polyphenols, tannins and several kinds of flavonoids, such as myricetin, quercetin, kaempferol, rhamentin, cyanidin, peonidin and dephinidin in *A. occidentale*.

Interestingly, the methanol shoot and leaf extracts including the gastric digested and pancreatic digested extracts selectively inhibited sucrase. As rat intestinal sucrase used in this experiment is closely similar to that found in the human intestine [17], it is likely to be an active component responsible for blood glucose lowering through inhibiting glucosidase. The kinetic assay was performed using Lineweaver-Burkplots. The inhibitory activity against the intestinal sucrase showed the same trend, non-competitive inhibition. The results showed that the shoot methanol extract displayed non-competitive inhibition of α-glucosidase; seen in the enzyme inhibition assay, the K_m value remains the same (23.46 mM) and the V_{max} value was lower (6.36 and 4.65 mM/min) at 0.50 and 0.75 mg/mL. Similarly, non-competitive inhibition of α-glucosidase was also exhibited in the gastric (21.32 mM) and pancreatic phases (22.18 mM). Moreover, the leaf extract also exhibited non-competitive inhibition of α-glucosidase; the K_m value of methanol extract was 22.55 mM, the gastric (25.28 mM) and pancreatic phases (25.81mM) were slightly varied

(Figs. 1 a- f). These results show that *A. occidentale* (shoots and leaves) has potential in diabetes treatment, by inhibition of α-glucosidase. Non- competitive inhibitors act by combining with an enzyme molecule, regardless of whether a substrate molecule is bound to the enzyme's active site or not, altering the conformation of the enzyme, thus reducing its catalytic activity. Previous studies have proved that plant extracts exhibit α- glucosidase enzyme inhibition by the non-competitive mode of inhibition [12]. Thus, polyphenolic compounds from *A. occidentale* shoot and leaf methanol extracts might bind with the enzyme or enzyme-substrate complex thus inhibiting or reducing α- glucosidase activity. α- glucosidase enzyme inhibition by methanol extracts was slightly varied after gastric and pancreatic treatments, and it was shown that the inhibitory activity of methanol extracts could reach the intestinal tract with some modifications.

4. Conclusion

In this study, the higher stability and levels of polyphenolics and flavonoids from *A. occidentale* leaves, as compared to shoots, after *in vitro* digestion, was observed for first time. Additional clinical experiments are needed to further investigate the stability, bioaccessibility and efficacy of the polyphenolic and flavonoid contents of *A. occidentale* leaves.

Acknowledgements

This study was supported by Innovation and Natural Product Research Unit, Faculty of Science and Technology, Rajamangala University of Technology Sivijaya, Nakhon Si Thammarat. We thank Professor. Preecha Phuwapraisirisan and Professor. Mary Ann Lila for valuable discussion.

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