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IN VITRO ALPHA-GLUCOSIDASE AND ALPHA-AMYLASE ENZYME-INHIBITED ACTIVITIES BY WATER-LILY (*Nymphaea* genus) EXTRACTS

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ABSTRACT

Chalong Kwan (C), Khao Mongkol (K) and Chompoo Mameaw (P) are the water-lily in the family of Nymphaeaceae which were planted in Thailand. In this study, leaves (1), flowers (2), tuber (3), pollen (4), branches (5) of the three water-lily were boiled with distilled water (W) and macerated on 95% ethanol (E). The extracts from water-lily including C3-E, P1-E, P3-E, C1-W, P1-W, P3-W, and P5-W were showed the highest level of free radical scavenging activities measured by (DPPH) assay (SC_{50} range of 0.003-0.007), which is more than L-ascorbic acid (SC_{50} of 0.013 ± 0.0001 mg/mL) about four times. The effect of anti-diabetic was determined inhibitory activities of α-glucosidase and α-amylase by enzymatic assay. P4-E extract was the highest exhibited the α-amylase activities (AC_{50} of 0.02 ± 0.001 mg/mL) with higher than acarbose for ten times (AC_{50} of 0.23 ± 0.14 mg/mL). Whereas, the C2-E, C3-E, and C1-W extracts inhibited the activity of α-glucosidase (GC_{50} range of 0.08-0.11), which higher than acarbose for 6 times (GC_{50} of 0.63 ± 0.04 mg/mL). The ethanol extracts from Chompoo Mameaw (P1-E and P3-E) which demonstrated the highest antioxidant and anti-diabetic activity, might be possible for further development to natural products for diabetic patients.

Key words: Water-lily, Nymphaeaceae, anti-diabetic, free radical scavenging

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia or raised blood sugar. The causes are from that the pancreas didn't produce enough insulin, or the body cannot effectively use the insulin it produces. In 2016, DM was direct caused of 1.6 million deaths and may be increased double in 2030 (Mitra *et al*., 2012; Telagari & Hullatti, 2015). DM have been two types including Type 1 (T1DM) diabetes which characterized by deficient insulin production and Type 2 diabetes (T2DM) is unbalanced of the absorption of blood sugar and insulin secretion. Whereas, T2DM is more common than T1DM about 90% of total diabetic patients (Telagari & Hullatti, 2015). One of the major management therapies in delaying or preventing T2DM were regulated the plasma glucose level by controlling production or absorption of glucose via suppression of α-amylase and α-glucosidase activities which carbohydrate-hydrolyzed enzymes. The α-amylase is involved in the breakdown of long chain carbohydrates by hydrolyzing the α-D-(1-4)-glycosidic linkages, whereas α-glucosidase breaks down starch and disaccharides to glucose hydrolyzing terminal non-reducing (1-4)-linked α-D-glucose residues with release of D-glucose. Acarbose is a leading inhibitor of α-amylase and α-glucosidase in the gastro-intestinal tract, but it has been many side effects such as abdominal pain, diarrhea, bloating, and flatulence (Telagari & Hullatti, 2015; Ademiluyi *et al*., 2015; Poovitha & Madasamy, 2016). Moreover, the oxidative stress was contributed to the pathogenesis

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of T2DM by mitochondrial dysfunction leading to impair the insulin signaling pathway and increased the insulin resistance state (Shen & Pierce, 2015). Several researches show medicinal plants such as *Asystasia gangetica* and *Canarium tramdenum* the good relationship between the antioxidant activity and the inhibition of α-amylase and α-glucosidase (Salehi1 *et al.,* 2013; Reddy *et al*., 2010; Quan *et al*., 2019). Therefore, screening of inhibitory α-amylase, α-glucosidase and free radical scavenging activity in herbal medicinal plants has been much attention.

Chalong Kwan, Khao Mongkol and Chompoo Mameaw are a tropical water-lily in *Nymphaea* genus (subgenus *Brachyceras*) of Nymphaeaceae family in Thailand. It was characterised by deep violet blooms with a yellow center and the stamens have modified themselves into petals, which more than 100 petals. Chompoo Mameaw was red pink petals with a yellow center and have the size of flower appears very full about diameter 8-12 cm as well as smell very fragrant. Khao Mongkol was white petals and purple line on the back with a yellow center. The flower appears very full of diameter 8-15 cm and smell very fragrant (Akkarakultron *et al*., 2014). The leaves, stamens, seeds, and roots of plants in Nymphaeaceae family are used as a food ingredient and traditional medicine in Thailand such as hematemesis, hematuria, hyperlipidemia and obesity. Also, the extract from *Nelumbo nucifera* Gaertn, lotus in Nymphaeaceae family have many pharmacological and biological properties such as antioxidant, antidiabetic, anti-hyperlipidemic, and anti-obesity (Bhardwaj & Modi, 2016). Additionally, the extracts from *Nymphaea nouchali* Burm. f., *Nymphaea stellata* Willd and *Nymphaea rubra*, they are one of the water-lily, and have been the anti-diabetic activities in the animal model (Rajagopal & Sasikala, 2008; Rahuja *et al*., 2013; Parimala & Shoba, 2014). However, there is no significant report on the antioxidant and anti-diabetic activity of the water-lily including Chalong Kwan, Khao Mongkol, and Chompoo Mameaw. Thus, the aim of this study extracts from water-lily were investigated on free radical scavenging activity and screening of α-amylase and α-glucosidase inhibition activities for anti-diabetic activity.

MATERIALS AND METHODS

Preparation and extraction

The leaves (1), flowers (2), tuber (3), pollen (4), and branches (5) of water-lily including Chalong Kwan (C), Khao Mongkol (K) and Chompoo Mameaw (P) was supported from RMUTT Lotus Museum, Thanyaburi District, Pathumthani, Thailand. The plants extraction was modified as described by Boonpisuttinant *et al*., 2019. The plants were washed and dried in an incubator at 50–55°C and ground by blender machine. One hundred g of the water-lily powders were extracted by boiled on the distilled water (W) and macerated on 95% (v/v) ethanol with occasionally shaking (E). Subsequently, the all extracts were filtered with paper Whatman no.1. The pooled filtrates were evaporated and dried by a rotary evaporator and freeze-dryer, respectively. Finally, the crude extracts were weighed for % yields-calculated, and stored at 4°C.

Free radical scavenging activity

Free radical scavenging activity was performed using DPPH radical scavenging as described by Boonpisuttinant *et al*. (2019). Briefly, DPPH solution were prepared with 0.5 mg of DPPH dissolved in 1 mL ethanol. After that, 50 µL of DPPH solution and 50 µL of the extracts were added in 96-well plate, and incubated at room temperature (RT) for 30 min. Then, the absorbances of percentages DPPH radical scavenging were determined by microplate reader at 515 nm. Finally, the percentages of the DPPH scavenging of extracts and ascorbic acid (positive control) were evaluated as the following:

$$\% \text{ Inhibitory activity} = [(A_{cont.} - A_{test})/A_{cont}] \times 100$$

Where, $A_{cont.}$ and A_{test} are the absorbance of the DPPH solution and extracts DPPH-plus, respectively. The 50% DPPH scavenging of each extract (SC_{50} [mg/mL]) was obtained from the plotted graph with percentages inhibition and the extracts concentrations.

Phytochemical analysis

The extracts were determined the phytochemicals constituents as described by Boonpisuttinant *et al.* (2019).

Inhibition of α-amylase activity

Alpha-amylase inhibitory activities was modified as described by Telagari and Hullatti (2015). Briefly, for 96 well plate, the mixture reaction was prepared with 50 µL of 100 mM phosphate buffer (pH 6.8), 20 µL of soluble starch (1%), 10 µL of 2 U/mL α-amylase, and followed 20 µL of the extracts as well as incubated at RT for 30 min. After that, the 50 µL of DNS reagent was mixed and boiled at 60°C for 10 min. The absorbance of enzyme inhibitory activity was examined at 560 nm by Microplate Reader. The percentage inhibition was calculated as the following equation:

$$\% \text{ Inhibition} = [(A_{cont.} - A_{test})/A_{cont}] \times 100$$

Where, $A_{cont.}$ and A_{test} are the absorbance of the control and the samples, respectively. The concentrations providing 50% inhibition (AC_{50} [mg/mL]) of extract was determined by the graph plotted of percentage inhibition and the extract concentrations.

Inhibition of α-glucosidase activity

The inhibition of α-glucosidase activities of extract was determined by using P-NPG (P-nitrophenyl-α-D-glucopyranoside) as previously described Telagari & Hullatti, 2015. Briefly, in a 96 well plate, the 50 µL of phosphate buffer (pH 6.8), 10 µL of 1 U/mL α-glucosidase, 20 µL of 5 mM P-NPG, and the extract at the various concentrations (20 µL) were added and incubated at RT for 30 min. After that, the 50 µL of 0.1 M Na_2CO_3 was added for stop reactions. Finally, the absorbance of the reaction was measured at 405 nm using Microplate Reader. Acarbose is used as a positive control. The percentage inhibition was calculated as the following:

$$\% \text{ Inhibition} = [(A_{cont.} - A_{test})/A_{cont}] \times 100$$

Where, $A_{cont.}$ and A_{test} are the absorbance of the control and the samples, respectively. The concentrations providing 50% inhibition (GC_{50} [mg/mL]) of extract was calculated from the plotted graph with percentage inhibition and the sample concentrations.

Statistical analysis

The data were presented as the means ± standard deviation (SD) of at least three independent experiments. Statistical significance was determined by using one-way analysis of variance (ANOVA), followed for multiple comparison by Tukey's HSD test at the significance level ($p < 0.05$).

RESULTS AND DISCUSSION

The extraction yields and free radical scavenging activity

In this study, the *Nymphaea* "Chalong Kwan (C)", "Khao Mongkol (K)", "Chompoo Mameaw (P)" are water-lily in *Brachyceras* sub-genus of Nymphaeaceae family. The percentage of yield (% yield), the leaves (1), flowers (2), tuber (3), pollen (4), branches (5) of the three water-lilies were extracted by 95% ethanol maceration (E) and boiling with distilled water, were range of 3.76 – 19.86%, as shown in Table 1. After that, the all of extracts were determined the free radical scavenging, anti-diabetic activities, and investigated on phytochemical constituent.

For DPPH radical scavenging activity, the antioxidant compounds could give electrons or free radicals to hydrogen of DPPH with reduced violet color to yellow color (Ho *et al*., 2012). Table 1 shows the antioxidant activity by DPPH free radical scavenging of the extracts from water-lily *Nymphaea* "Chalong Kwan (C)", "Khao Mongkol (K)", "Chompoo Mameaw (P)". It was found that all water-lily extracts exhibited the DPPH radical scavenging activities. Moreover, the ethanol extract from the tubers of Chalong Kwan (C3-E), the ethanol extracts from the leaves and the tuber of Chompoo Mameaw (P1-E and P3-E), the water extract from the leaves of Chalong Kwan (C1-W) and the water extracts from leaves, tuber and branches of Chompoo Mameaw (P1-W, P3-W and P5-W) gave the highest of DPPH radical scavenging activities (SC_{50}) of 0.004 ± 0.0001 mg/mL, 0.003 ± 0.0002 mg/mL, 0.004 ± 0.0002 mg/mL, 0.004 ± 0.0001 mg/mL, 0.004 ± 0.0003 mg/mL, 0.007 ± 0.0004 mg/mL and 0.007 ± 0.0001 mg/mL, respectively, which was superior than L-ascorbic acid (SC_{50} value as 0.013 ± 0.0001 mg/mL) about 2-4 times (p <0.05). Wongklang *et al*. (2014) previously reported that the effect of the ethanol extract from flower of *N. nucifera*, which is the lotus in Nymphaeaceae family showed the antioxidant activity with the SC_{50} of 0.0163 mg/mL following by flower stalk and torus (SC_{50} = 0.0180 and 0.0382 mg/mL. respectively). Moreover, the extracts from *Nymphaea* spp. showed the antioxidant activities by inhibiting the DPPH radical (Madhusudhanan *et al*., 2011; Zhao *et al*., 2011; Parimala & Shoba, 2013). Additionally, major of phytochemicals in the extracts were contained the anthraquinone, flavonoid and carotenoid (Table 2), which may affect on DPPH radical scavenging of the extracts from water-lily. Thus, the difference of extraction process such as solvents and temperature might to shown difference phytochemicals and effect of biological activities (Selvakumari *et al*., 2016).

Alpha-amylase and alpha-glucosidase inhibitory activity

Generally, starch digestion in the human is typically observed two steps beginning with α-amylase and followed by α-glucosidase to produce glucose before permeation at small intestine by insulin. The deficiency and malfunction of insulin causes high glucose levels in the blood. All extracts from the three water-lilies were investigated anti-diabetic by inhibition of the α-amylase and α-glucosidase activities. The results demonstrated that all extracts could inhibit both α-amylase and α-glucosidase (Figure 1 & Figure 2). Ethanol extract from pollen of Chompoo Mameaw (P4-E) was the highest suppressed the α-amylase activities (AC_{50} = 0.02 ± 0.001 mg/mL), which was higher than acarbose (positive control) about ten times (AC_{50} value as 0.23 ± 0.14 mg/mL as shown in Figure 1. Some phytochemical constituents and their

Table 1. The extraction yields and free radical scavenging activity of the water-lily extracts

Sample	% yield		Free radical scavenging activity [SC_{50} (mg/mL)]	
	E	*W*	*E*	*W*
C1	6.03	6.80	0.450 ± 0.0112[g]	0.004 ± 0.0001[a]
C2	11.70	12.26	0.010 ± 0.0001[b]	0.020 ± 0.0005[c]
C3	4.66	5.90	0.004 ± 0.0001[a]	0.010 ± 0.0004[b]
C4	7.33	8.00	0.030 ± 0.0007[d]	0.012 ± 0.0007[b]
C5	4.16	4.80	0.034 ± 0.0023[d]	1.120 ± 0.1212[h]
K1	3.76	15.53	0.040 ± 0.0007[e]	0.020 ± 0.0014[c]
K2	17.8	19.86	0.030 ± 0.0009[d]	0.020 ± 0.0011[c]
K3	11.20	4.33	0.020 ± 0.0006[c]	0.010 ± 0.0006[b]
K4	7.20	8.43	0.030 ± 0.0015[d]	0.012 ± 0.0008[b]
K5	12.46	4.06	0.430 ± 0.0125[g]	0.430 ± 0.1809[g]
P1	7.60	8.63	0.003 ± 0.0002[a]	0.004 ± 0.0003[a]
P2	8.93	10.50	0.011 ± 0.0010[b]	0.020 ± 0.0011[c]
P3	5.10	6.46	0.004 ± 0.0002[a]	0.007 ± 0.0004[a]
P4	5.96	7.36	0.230 ± 0.0006[f]	0.030 ± 0.0009[d]
P5	4.16	4.53	0.270 ± 0.0102[f]	0.007 ± 0.0001[a]
L-ascorbic acid			0.013 ± 0.0005[c]	

Note: C is water-lily "Chalong Kwan", K is water-lily "Khao Mongkol" and P is water-lily "Chompoo Mameaw"; 1 is the part of leaves, 2 is the part of flowers, 3 is the part of tuber, 4 is the part of pollen, and 5 is the part of branches; W was boiling with distil water for 2 hr.; E was maceration with 95% ethanol for seven days.

Table 2. The phytochemical constituents of the water-lily extracts

Sample	Phytochemical													
	Alkaloids		Anthraquinone		Carotenoids		Flavonoids		Tannins		Xanthones		Glycosides	
	E	*W*	*E*	*W*	*E*	*W*	*E*	*W*	*E*	*W*	*E*	*W*	*E*	*W*
C1	–	–	+	+	+	+	+	+	–	+	–	–	–	+
C2	–	–	+	+	+	+	+	+	–	–	–	–	+	–
C3	–	–	+	+	+	+	–	+	+	–	–	–	–	+
C4	–	–	+	+	-	+	–	+	+	–	–	–	–	+
C5	–	–	+	+	+	+	+	+	+	–	–	–	+	+
K1	–	–	+	+	+	+	+	+	+	–	–	–	+	–
K2	–	–	+	+	-	+	+	+	+	–	–	–	–	–
K3	–	–	+	+	+	+	–	+	–	+	–	–	–	–
K4	–	–	+	+	+	+	–	+	+	–	–	–	–	–
K5	–	–	+	+	+	+	+	+	+	–	–	–	+	–
P1	–	–	+	+	+	+	+	+	+	+	–	–	+	+
P2	–	–	+	+	+	+	+	+	+	+	–	–	–	+
P3	–	–	+	+	+	+	–	+	+	–	–	–	+	+
P4	–	–	+	+	+	+	–	+	–	–	–	–	–	–
P5	–	–	+	+	+	+	+	+	–	+	–	–	–	+

Note: C is water-lily "Chalong Kwan", K is water-lily "Khao Mongkol" and P is water-lily "Chompoo Mameaw"; 1 is the part of leaves, 2 is the part of flowers, 3 is the part of tuber, 4 is the part of pollen, and 5 is the part of branches; W was boiling with distil water for 2 hr.; E was maceration with 95% ethanol for seven days.

quantities in the P4-E extract such as anthraquinone and carotenoids might be response for this activity.

From the previously report, the flavonoids from *N. nucifera* leaf (NLF) showed high inhibitory activity against α-amylase with AC_{50} values of 2.20 ± 0.18 mg/mL (Selvakumari *et al*., 2016). Subsequently, the ethanol extracts from pollen and branches of Chalong Kwan (C4-E and C5-E) and ethanol extract from pollen of Khao Mongkol (K4-E) can also inhibit α-amylase activities (AC_{50} value as 0.44 ± 0.03 mg/mL, 0.62 ± 0.003, and 0.34 ± 0.02, respectively, which was comparable to Acarbose.

In the Figure 2 exhibited the effect of water-lily extracts on inhibition of α-glucosidase activity. The ethanol extracts from flowers, tuber, and branches of Chalong Kwan (C2-E, C3-E and C5-E) gave the highest inhibition of α-glucosidase activity with

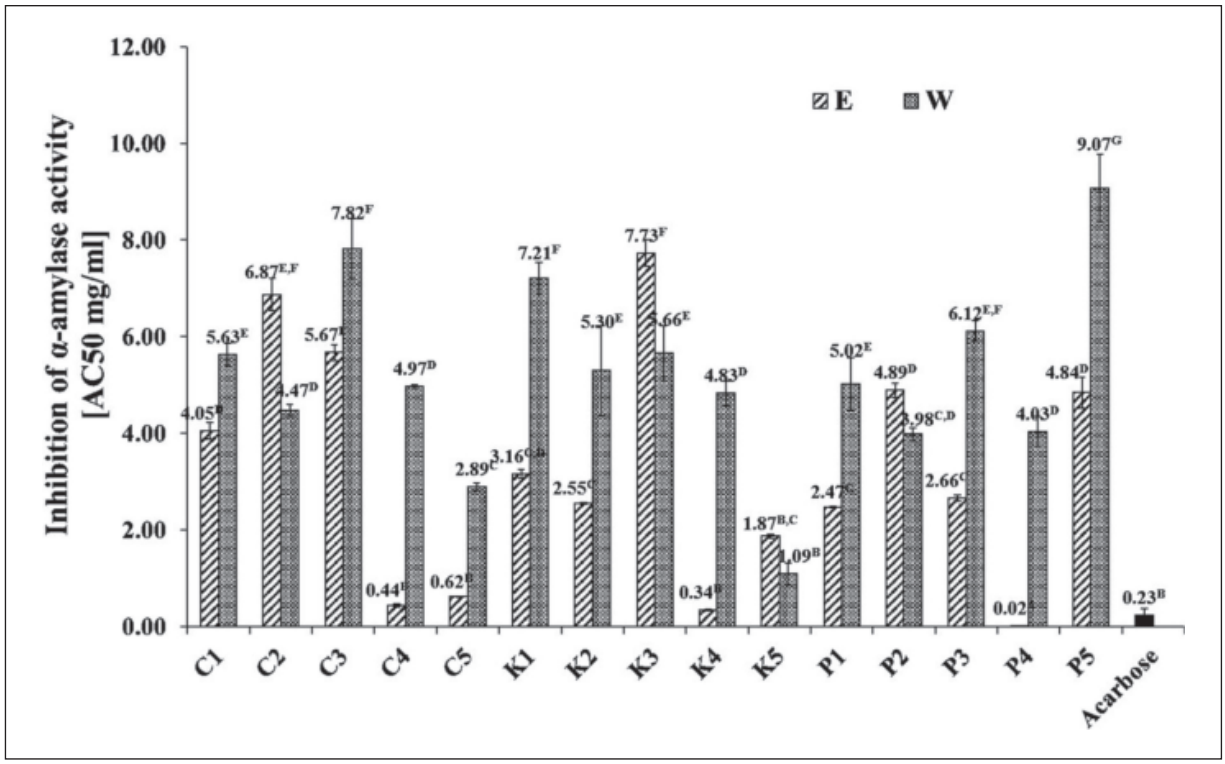

Fig. 1. Inhibition of α-amylase activity of water-lily extracts. C = water-lily "Chalong Kwan", K = water-lily "Khao Mongkol" and P = water-lily "Chompoo Mameaw"; 1= leaves, 2 = flowers, 3 = tuber, 4 = pollen, and 5 = branches; W was boiling with distil water for 2 hr.; E was maceration with 95% ethanol for seven days.

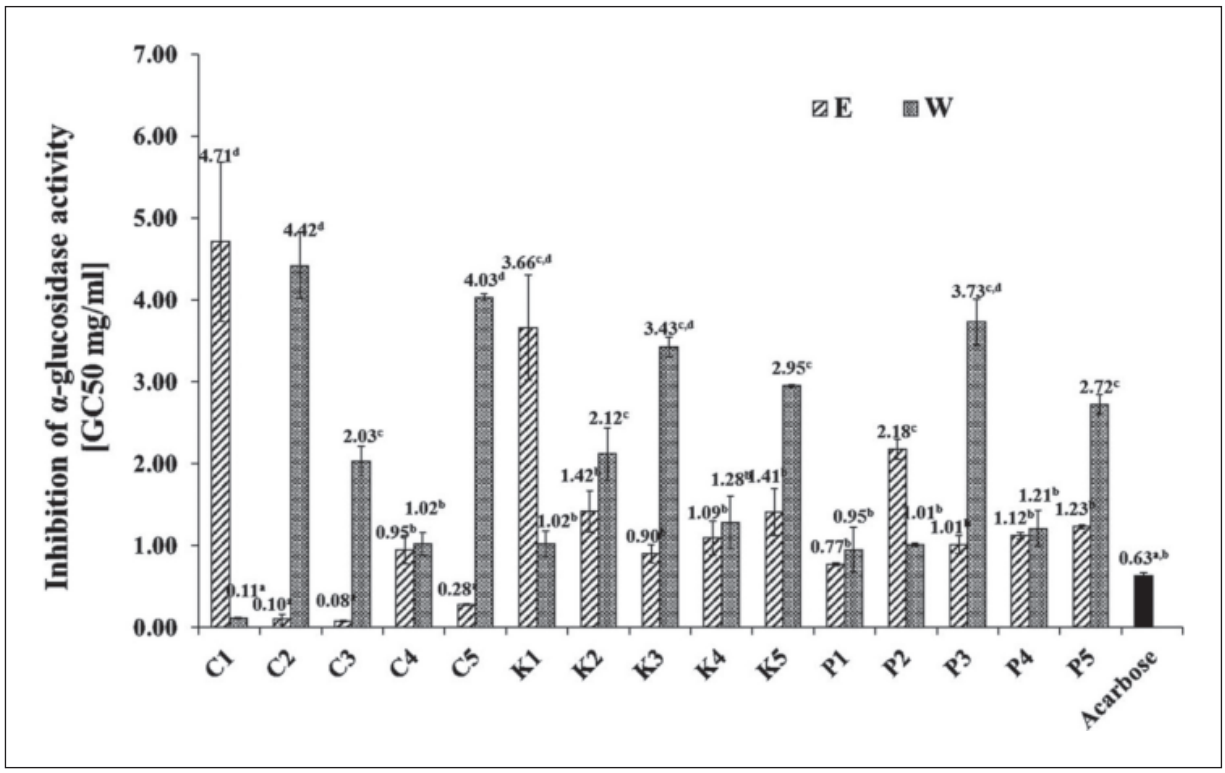

Fig. 2. Inhibition of α-glucosidase activity of water-lily extracts. C = water-lily "Chalong Kwan", K = water-lily "Khao Mongkol" and P = water-lily "Chompoo Mameaw"; 1= leaves, 2 = flowers, 3 = tuber, 4 = pollen, and 5 = branches; W was boiling with distil water for 2 hr.; E was maceration with 95% ethanol for seven days.

GC_{50} values of 0.10 ± 0.06 mg/mL, 0.08 ± 0.01 mg/mL and 0.28 ± 0.01 mg/mL, respectively as well as the water extract from leaves of Chalong Kwan (C1-W) (GC_{50} = 0.11 ± 0.01 mg/mL), which were higher than Acarbose (GC_{50} value as 0.63 ± 0.04 mg/mL) about 6 times. Some phytochemical constituents and their quantities in the Chalong Kwan extracts such as anthraquinone, Carotenoids and carotenoids might be response for this activity. Liu *et al*. (2013) was found that the flavonoids from *N. nucifera* leaf (NLF) shown the high inhibitory activity of α-glucosidase with GC_{50} values of 1.86 ± 0.018 mg/mL. Moreover, the corrrelation (R) of the inhibition of alpha glucosidase activity and antioxidant activity of Chalong Kwan, and Chompoo Mameaw extracts was 0.817 and 0.650, which can be classified as moderate relationship, whereas the inhibition of alpha amylase showed no relationship to antioxidant activity.

CONCLUSION

All extracts from water-lily (Chalong Kwan, Khao Mongkol and Chompoo Mameaw) prepared by ethanol extraction, presented the significant antioxidant and in vitro anti-diabetic activity by controlling the digestion of carbohydrate via suppressed the hydrolysis of α-D-(1-4)-glycosidic linkages of starch and terminal non-reducing (1-4)-linked of α-D-glucose residues leading to release of D-glucose through α-amylase and α-glucosidase activity, respectively. Antioxidant activity of all extracts also related to the inhibition of alpha glucosidase. Thus, all extracts that exhibited the inhibition of α-amylase and α-glucosidase activities, as well as antioxidant activity, might be a valuable novel therapeutic diabetic agent. The ethanol extracts from Chompoo Mameaw (P1-E and P3-E) showed the highest antioxidant via DPPH free radical scavenging activities and anti-diabetic activity by inhibition of α-amylase and α-glucosidase activities, which might be further developed to natural product for diabetic patients.

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