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Original Article

In vivo and *in vitro* antidiabetic effects of *Clerodendrum longisepalum*

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Abstract

This study aimed to investigate the antidiabetic properties of *Clerodendrum longisepalum.* This primary screening study focused on blood glucose levels and hematological values in diabetic rats treated with the leaf extract from *C. longisepalum* (CL). Results showed that the CL extract can lower the blood glucose level of the experimental animals from the diabetic state. In relation to this, the extract caused the hematological value in diabetic rats to drop close to normal controls. However, the *in vitro* α-glucosidase assay-guided isolation revealed that the stem extract *of C. longisepalum* has a possible connection with the inhibition of intestinal α-glucosidase. Three triterpenoids (1-3), one glycerol derivative (4) as well as six phenylpropanoid glycosides (5-10) were isolated. Compound 10 possessed good anti-α-glucosidase activity (maltase). A kinetic investigation of 10 indicated that it retarded maltase function in a noncompetitive manner. Moreover, compounds 6, 9 and 10 also showed good antioxidant activity (DPPH).

Keywords: *Clerodendrum longisepalum*, diabetic rats, α-glucosidase inhibitory activity, antioxidant activity

1. Introduction

Diabetes mellitus (DM) is a complex metabolic disorder characterized as state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated (Khan *et al.,* 2012). Type 2 diabetes mellitus (T2DM) is typically a chronic metabolic disorder characterized by hyperglycemia in the context of insulin resistance and relative lack of insulin. In addition, diabetes can cause many complications including diabetic ketoacidosis and nonketotic hyperosmolar coma (Kitabchi *et al.,* 2009). The use of medicinal plants for alternative treatment of diabetes mellitus has been increasing over the years. In many studies, the potential role of several medicinal plants as hypoglycemic agents has also been reported.

Clerodendrum longisepalum Dop (Lamiaceae) is a small shrub grown throughout Thailand, the Malaysian Peninsula, Sumatra, Java, and Borneo (throughout the island) (Leeratiwong *et al., 2011*). In Thailand, the leaves of *C. longisepalum* have been used as folklore medicine in the treatment of diabetes mellitus. There has still been no investigation of the pharmacological activities and the safety of utilization of the extract from this plant in diabetic rats. In addition, there are no reports on the chemical constituents and active compounds in this plant as potential antidiabetic inhibitors. Therefore, this work aims to study the effects of ethanolic leaf extract (CLE) on hypoglycemic properties including blood glucose levels, body weight and hematological values in streptozotocin-induced diabetic rats, as well as the first report on the chemical constituents and active components putatively responsible for α-glucosidase from the *C. longisepalum* stems.

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2. Materials and Methods

2.1 General experimental procedures

UV spectra were measured with a UV-7504 spectrophotometer. High resolution mass spectra were recorded on a Bruker Daltonics (microTOF) equipped with an electrospray ionization (ESI) ion source. The ^{1}H and ^{13}C NMR spectra were acquired by Varian Mercury+ 400 and Bruker AVANCE 400 spectrometers. Chemical shifts were reported in δ (ppm) relative to deuterated solvent residues (7.25 and 77.0 ppm for $CDCl_3$ and 3.30 and 49.0 ppm for CD_3OD). TLC was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer), and spots were visualized under UV or dipped in 3% (v/v) anisaldehyde, 9.7% (v/v) H_2SO_4, 87.3% (v/v) MeOH followed by heating. Acarbose was obtained from Bayer Vitol Leverkusen, Germany. Spectrophotometric measurements for the á-glucosidase inhibition and kinetic study were taken on a Sunrise microplate reader.

2.2 Plant material

The leaves and stems of *C. longisepalum* were obtained from Khlong Luang District, Pathum Thani Province, Thailand in January 2015 and identified by Dr. S. Khumkratok, a botanist at the Walai Rukhavej Botanical Research Institute, Mahasarakham University, where a voucher specimen (Sichaem no.1-1) is deposited.

2.3 Preparation of *C. longisepalum* leaf extract

Fresh mature leaves of *C. longisepalum* were washed, cut into small pieces and dried in a hot air oven at a temperature of 50°C and then powdered. The powder was extracted by macerating in 95% ethanol (1:10 w/v) for seven days. The mixture was filtered through a Whatman filter paper. Ethanol in the filtrate was evaporated using a rotary evaporator (Heidolph Laborota 4000, Germany). The obtained extract from *C. longisepalum* (CLE) was kept at a temperature of 4°C until being used.

2.4 Animal

Male Wistar rats weighing 150-200 g were used in the study. These rats were purchased from the National Laboratory Animal Centre (NLAC), Mahidol University, Thailand and kept in an air conditioned room at a temperature of 25± 2°C, 12-h light/12-h dark cycle and relative air humidity of 40-60%. A standard chow and water were given to the rats *ad libitum*. They were acclimatized for 7 days prior to the commencing experiments. The animal protocol followed by the researchers was approved by the Animal Ethics Committee, Pharmaceuticals and Natural Products Department, the Thailand Institute of Scientific and Technological Research (ID PS-59004) and the advice of the Institutional Animal Care and Use Committee, Mahasarakham University, Thailand.

2.5 Induction of diabetes

Rats were induced to be diabetes by a single intraperitoneal injection of 65 mg/kg streptozotocin, STZ (Sigma Chemicals, St. Louis, MO) dissolved in 20 mM citrate buffer pH 4.5. After STZ injection, the rats were provided with 2% sucrose solution as their drink for 48 hrs to alleviate the severity after initial hypoglycemic phase. Rats with blood glucose level at or above 126 mg/dl were confirmed to be diabetes and used in the study (Talubmook *et al.,* 2003).

2.6 Experimental designs

The rats were divided into four groups with six rats in each: Group I: normal control rats treated orally with 2% Tween 80; Group II: diabetic control rats treated orally with 2% Tween 80; Group III: diabetic rats administrated orally glibenclamide (0.25 mg/kg b.w.); Group IV: diabetic rats administrated orally CLE (250 mg/kg b.w.).

The CLE and 0.5%Tween 80 were administered to the rats orally (250 mg/kg of CLE) and daily for eight weeks. For normal and diabetic control rats were treated with 0.5% Tween 80 instead. The volume of administration was 2 mL for each rat. The investigation of blood glucose level, using glucometer (Accu-chek Advantage II, Roche Germany), and body weight was performed weekly. At the end of experiments, the rats were sacrificed by cervical dislocation technique. After an operation, the blood sample was then drawn from the rat hearts and centrifuged with 3,000 rpm for 10 min twice to separated blood serum for examine hematocrit (Hct). This step was determined using a microcapillary reader (Damon/IEC Division, USA). Hemoglobin concentration (Hb) was recorded on a Sahli haemometer (Superior, Germany) and red blood cell (Rbc) and white blood cell (Wbc) was also determined using a Neubauer haemocytometer.

2.7 Extraction and isolation of *C. longisepalum* stems

The air-dried stems of *C. longisepalum* (7.7 Kg) were ground and extracted with MeOH. The MeOH extract (485.7 g) of *C. longisepalum* stems was initially partition with CH_2Cl_2 and EtOAc to yield CH_2Cl_2 (101.7 g) and EtOAc (30.4 g) extracts. The CH_2Cl_2 extract was fractionated on silica gel column chromatography using the gradient systems of hexane, CH_2Cl_2, EtOAc and MeOH, respectively, yielding 1 (70 mg), 2 (22 mg) and 3 (89 mg), respectively. The EtOAc extract was fractionated on silica gel column chromatography using the gradient systems of CH_2Cl_2, EtOAc and MeOH, respectively, obtaining seven major fractions (E1-E7). The combined fraction E5 eluted by 0:100-100:0 MeOH-CH_2Cl_2 were purified by silica gel (60:40 EtOAc-hexane) to afford four subfactions (E5.1-E5.4). Subfaction E5.1 was recolumn chromatography using Sephadex LH-20 (10:90 MeOH-CH_2Cl_2 obtained 4 (8 mg), 5 (13 mg) and 7 (90 mg), respectively. Subfraction E5.3 was further purified by Sephadex LH-20 (20:80 MeOH-CH_2Cl_2) to yield 6 (16 mg) and 8 (68 mg). Finally,

fraction E7 was subsequently purified by the combination of silica gel (20:80 MeOH-CH_2Cl_2) and Sephadex LH-20 (50:50 MeOH-CH_2Cl_2) to furnish 9 (140 mg) and 10 (200 mg), respectively.

The identification of all isolated compounds was made by spectroscopic analysis, particularly NMR and MS, which coincided well with previous literature. Compounds 1-10 were assigned as 3*β*-taraxerol (1) (Ishii *et al.,* 2008), 3*β*-taraxerol acetate (2) (Li *et al.,* 2008) and (3*β*)-stigmasta-4,22,25-trien-3ol (3) (Chaves *et al.,* 2004), 1-monoacetin (4) (Nebel *et al.,* 2008), seguinoside K (5) (Wu *et al.,* 2014), trichotomoside (6) (Chae *et al.,* 2006), 2″,3″-*O*-acetylmartynoside (7), 2″,4″-*O*-acetylmartynoside (8) (Leitào & Kaplan, 1994), martynoside (9), and acteoside (10) (Liu *et al.,* 2014) (Figure 1).

2.8 α-Glucosidase inhibition assay

The evaluation of the inhibitory activity of isolated compounds (1-10) against intestinal α-glucosidases (maltase and sucrase) was also applied from previous procedure (Damsud *et al.,* 2013). Briefly, 10 µL of the test sample and substrate solution (maltose: 10 mM, 20µL; sucrose: 100 mM, 20 µL, respectively) in 0.1 M phosphate buffer (pH 6.9) were incubated at 37°C (20 min for maltase and 60 min for sucrase). The mixture was discontinued in boiling water for 10 min, and glucose released from the reaction was converted to quinoneimine using a commercial Glu Kit (Human, Germany). The absorbance of final product was determined at 503 nm, and the percent inhibition was deduced using the following equation.

$$\frac{(A_0 - A_1)}{A_0} \times 100$$

where A_1 and A_0 are absorbances with and without the sample, respectively. Acarbose was used as a positive control having inhibitory effect against maltase and sucrase with the IC_{50} values of 18.6 and 51.1 µM, respectively (Table 4).

2.9 Kinetic study

A kinetic analysis of maltase was carried out according to the above reaction except that the quantity of α-glucosidase was maintained at 0.3 U/mL while the concentrations of 10 were varied at 0, 0.064 and 1.061 mM. The type of inhibition was determined from Lineweaver–Burk plots whereas the K_i and K_i' values were deduced from the secondary plots of slope *vs* [I] and the interception *vs* [I] of the Lineweaver-Burk plots, respectively (Figures 2-5).

2.10 DPPH radical scavenging activity

Radical scavenging activity was validated by the UV absorbing method in 96-well microplate (Yen and Hsieh, 1997). Briefly, various concentrations of samples dissolved in MeOH (20 µL) were added 0.1 mM DPPH solution (100 µL). After 30 minutes incubation at room temperature in the dark, the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer.

2.11 Statistical analysis

All the data were expressed as mean standard error of mean (SEM). Statistical analysis was carried out using One-way ANOVA. The criterion for statistical significance was *p* values less than 0.05.

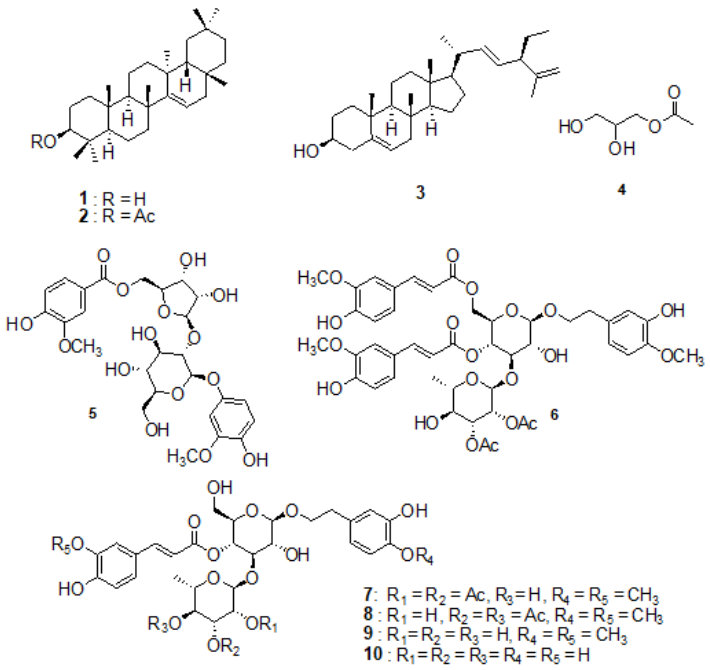

Figure 1. Structures of all isolated compounds (1-10) from the *C. longisepalum* stems.

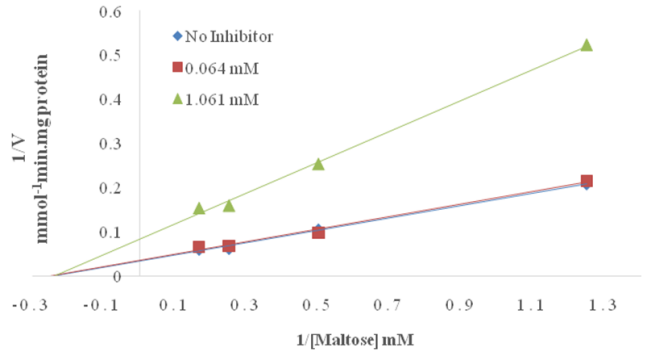

Figure 2. Lineweaver-Burk plots for inhibitory activity of 10 against maltase.

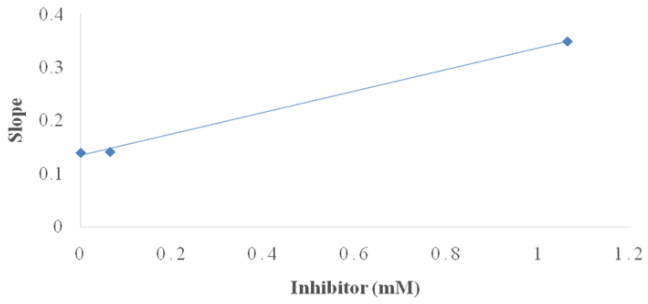

Figure 3. Secondary plot of slope *vs* [I] for deduction of K_i of 10 against maltase.

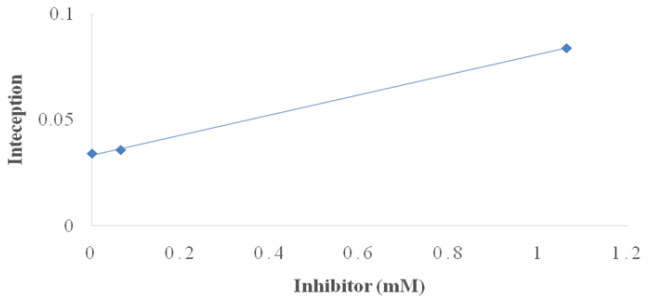

Figure 4. Secondary plot of intercept *vs* [I] for deduction of K'_i of 10 against maltase.

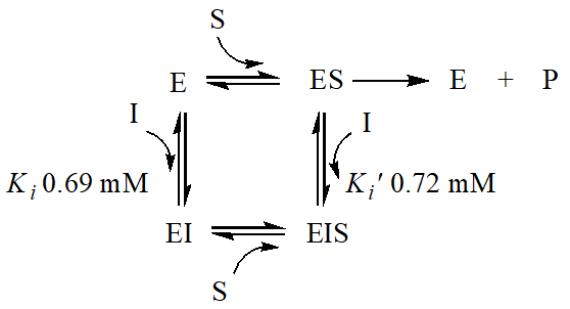

Figure 5. Proposed inhibitory mechanism of 10 against maltase (E = enzyme, S = substrate, P = product, I = inhibitor, ES = enzyme–substrate complex, EI = enzyme–inhibitor complex, EIS = enzyme-substrate-inhibitor complex).

3. Results and Discussion

3.1 Effect of CLE on blood glucose level in diabetic rats

The initial blood glucose levels in the diabetic control rats (336.50 mg/dl), diabetic rats treated with glibenclamide (366.83 mg/dl) and CLE (385.50 mg/dl) were significantly ($p<0.05$) higher than those in normal control rats (82.33 mg/dl). After eight weeks, the CLE could decrease blood glucose level (19.59%) but significantly ($p<0.05$) less than the standard agent (46.03%) in diabetic rats, while the blood glucose level in normal control rats did not decrease (Table 1).

3.2 Effect of CLE on body weight in diabetic rats

The initial body weight of all rat groups did not differ. At the end of the experiments, the extract had no effect on body weight and could increase the weight of diabetic rats (24.78%). This result was not significantly different ($p>0.05$) from diabetic control rats and diabetic rats treated with glibenclamide; however, it was still significantly ($p<0.05$) lower than normal groups with the percent values of 8.59, 25.44, and 61.12, respectively (Table 2).

3.3 Effect of CLE on hematological values in diabetic rats

Hematological values including hematocrit (Hct), hemoglobin (Hb), red blood cell (Rbc) and white blood cell (Wbc). In the diabetic control rats the Wbc value was lower than the normal control rats. The Hct value of diabetic rats treated with CLE was higher than normal control rats, while the Rbc value was slightly lower than normal control rats. In addition, the hematological values of diabetic rats treated with CLE slightly differed from glibenclamide (Table 3).

3.4 Chemical constituents and *in vitro* rat intestinal α-glucosidase activity from the stems of *C. longisepalum*

Based on the *in vitro* α-glucosidase assay-guided isolation the stem extract *of C. longisepalum* revealed a possible connection with the inhibition of intestinal α-glucosidase more than other parts (roots and leaves). Therefore, the active MeOH extract of *C. longisepalum* stems was initially partitioned with CH_2Cl_2 and EtOAc to give CH_2Cl_2 and EtOAc extracts. These extracts were isolated and purified by the combination of silica gel and Sephadex LH-20 column chromatographies using mixtures of hexane, CH_2Cl_2, EtOAc and MeOH, yielding three triterpenoids (1-3), one glycerol derivative (4) as well as six phenylpropanoid glycosides (5-10) (Figure 1). The rat intestinal α-glucosidase inhibitory activity (maltase and sucrase) of all isolated compounds, at concentrations of 0.013, 0.063 and 0.31 mg/mL, are shown in Table 4. Only acteoside (10) showed good maltase inhibitor with an IC_{50} value of 56.0 μM (Table 1) and also displayed weak activity against sucrase (IC_{50} 224.1 μM). While trichotomoside (6) exhibited weak activity toward both enzymes (maltase

Table 1. Percentage of decreasing blood glucose level in normal control, diabetic control and diabetic treated rats.

Group	Treatment and dose	% decrease in blood glucose level							
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
I	Normal control	1.39±0.69^{a}	2.02±0.53^{ab}	1.75±1.18^{a}	1.38±0.99^{a}	1.17±0.61^{ab}	1.96±1.07^{a}	1.39±0.48^{ab}	1.02±0.39^{a}
II	Diabetic control	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
III	Diabetic + glibenclamide 0.25 mg/kg	36.79±5.76^{b}	25.99±5.19^{c}	37.41±8.25^{b}	23.71±5.93^{b}	35.23±9.92^{c}	24.61±7.54^{b}	45.57±9.80^{b}	46.03±10.21^{c}
IV	Diabetic + CLE 250 mg/kg	9.88±2.72^{a}	9.33±4.01^{ab}	10.92±4.36^{a}	12.37±4.54^{ab}	15.61±3.93^{ab}	17.94±3.05^{b}	16.02±3.74^{ab}	19.59±2.86^{b}

Values representing the mean ± SEM within the same column followed by the different superscript letters (a–c) are significantly different at $p<0.05$, (n = 6).

Table 2. Percentage of increasing body weight in normal control, diabetic control and diabetic treated rats.

Group	Treatment and dose	% decrease in blood glucose level							
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
I	Normal control	8.55±1.39^{a}	17.3±2.27^{a}	25.05±1.30^{a}	34.95±1.37^{a}	44.28±0.80^{a}	49.64±0.44^{a}	55.32±1.11^{a}	61.12±1.77^{a}
II	Diabetic control	1.48±0.94^{a}	2.79±0.89^{b}	4.47±1.00^{b}	4.79±1.13^{b}	6.18±1.32^{b}	7.53±1.27^{c}	8.25±1.49^{c}	8.59±1.22^{b}
III	Diabetic + glibenclamide 0.25 mg/kg	4.36±2.19^{a}	8.89±2.68^{ab}	9.70±1.46^{b}	13.71±2.40^{b}	17.00±2.42^{b}	24.15±2.44^{b}	24.04±1.86^{b}	25.44±1.83^{b}
IV	Diabetic + CLE 250 mg/kg	4.94±2.73^{a}	7.89±5.41^{b}	9.83±7.28^{b}	12.60±6.74^{b}	14.94±7.08^{b}	18.62±7.71^{bc}	20.33±7.82^{bc}	24.78±9.25^{b}

Values representing the mean ± SEM within the same column followed by the different superscript letters (a–c) are significantly different at $p<0.05$, (n = 6).

Table 3. The hematological values in normal control, diabetic control and diabetic treated rats.

Group	Treatments and doses	Hematological value			
		Hct (%)	Hb (g/dl)	Rbc ($\times10^{6}$cell/mm^{3})	Wbc ($\times10^{3}$ cell/mm^{3})
I	Normal control	43.33±0.84^{a}	18.16±0.40^{a}	8.90±2.16^{b}	8.16±3.34^{c}
II	Diabetic control	49.50±0.76^{b}	22.16±0.90^{b}	5.50±2.62^{a}	6.06±4.75^{ab}
III	Diabetic + glibenclamide 0.25 mg/kg	46.16±1.49^{a}	23.50±0.22^{b}	8.72±2.48^{b}	5.69±1.50^{a}
IV	Diabetic + CLE 250 mg/kg	46.16±0.40^{a}	22.00±0.25^{b}	8.69±4.81^{b}	7.81±5.07^{bc}

Values representing the mean ± SEM within the same column followed by the different superscript letters (a–c) are significantly different at $p<0.05$, (n = 6).

and sucrase) with the IC_{50} values of 241.0 and 219.1 μM, respectively. To envision the mechanism underlying this inhibition, a kinetic study of 10 toward maltase was performed. In Figures 2-5, the Lineweaver-Burk plots suggest that compound 10 retarded maltase function in a noncompetitive manner.

3.5 Antioxidant of all isolated compounds (1-10)

All extracts were subjected to examination for potential free-radical scavenging on DPPH. The results indicated that compounds 6, 9 and 10 exhibited significant antioxidant activity at IC_{50} values of 0.064, 0.46 and 0.061 mM, respectively. In addition, compounds 4-7 showed moderate antioxidant activity with IC_{50} values in the range of 2.46-9.29 mM (Table 5).

4. Conclusions

In conclusion, this study confirmed the traditional use of *C. longisepalum* for treatment of diabetes. The *C. longisepalum* leaf extract (CLE) showed a beneficial hypoglycemic

Table 4. α-Glucosidase inhibitory effects of isolated compounds (1-10) and a standard inhibitor.

Compound	Percent inhibitory activity at 0.31 mg/mL[a]		IC_{50} (μM)	
	Maltase	Sucrase	Maltase	Sucrase
1	13.1±0.83	20.8±0.74	NI[b]	NI[b]
2	5.0±1.14	0.0±0.58	NI[b]	NI[b]
3	15.0±0.12	10.1±0.76	NI[b]	NI[b]
4	18.4±0.54	14.1±0.32	NI[b]	NI[b]
5	4.2±0.72	6.9±0.77	NI[b]	NI[b]
6	6.9±0.88	12.9±1.97	NI[b]	NI[b]
7	17.2±0.93	4.8±0.32	NI[b]	NI[b]
8	61.3±2.29	68.9±0.87	241.0±0.94	219.1±1.21
9	29.8±0.20	27.8±2.13	NI[b]	NI[b]
10	62.4±1.68	66.4±0.85	56.0±1.38	224.1±0.65
Acarbose	72.0±0.92	66.7±0.11	18.6±0.53	51.1±0.92

[a]Final concentration. [b]No inhibition, inhibitory effect less than 30% at final concentration of 0.31 mg/mL.

Table 5. Antioxidant activity (DPPH) of all isolated compounds 1-10 and standard agents.

Compound	IC_{50} (mM)
1	No Activity
2	No Activity
3	No Activity
4	2.46±1.26
5	9.29±0.72
6	0.064±0.35
7	2.63±0.45
8	2.93±0.92
9	0.46±1.76
10	0.061±0.57
Vitamin C	0.11±0.37
BHT	0.40±1.13

effect in diabetic rats. We also succeeded in the isolation of chemical constituents (1-10) and identifying that the active component (10) is responsible for the *in vitro* antidiabetic activity in *C. longisepalum* stems. In addition, compounds 6, 9 and 10 also showed good antioxidant activity (DPPH). Therefore, this suggests that *C. longisepalum* can be used in the treatment of diabetes without affecting hematological values.

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