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Assessment of the anti-diabetic potential of the *Cratoxylum formosum* subsp. *formosum* extracts via carbohydrate hydrolyzing enzymes inhibitory activities

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ABSTRACT

Introduction: This research aims to evaluate the anti-diabetic activity of the extracts from different parts of *Cratoxylum formosum* subsp. *formosum*.
Methods: The *in vitro* inhibitory activities of the hexane (HEX), dichloromethane (DCM) and ethyl acetate (EtOAc) extracts from the flowers, leaves, roots and stems on pancreatic α-amylase (pAA), *Saccharomyces* α-glucosidase (SAG), rat intestinal maltase (rIM), and sucrase (rIS) were investigated.
Results: The DCM and EtOAc extracts from the flowers (IC_{50} 5.4 ± 1.5 and 10.5 ± 0.6 μg/mL) displayed the similar inhibitory activities as acarbose (IC_{50} 7.2 ± 0.4 μg/mL) in the pAA assay. The inhibitory activities of the DCM and EtOAc extracts from the flowers (IC_{50} 56.7 ± 8.9 and 20.4 ± 0.4 μg/mL), EtOAc extract from leaves (IC_{50} 45.0 ± 3.5 μg/mL), DCM and EtOAc extracts from roots (IC_{50} 35.0 ± 6.7 and 16.7± 3.6 μg/mL), and EtOAc extract from stems (IC_{50} 31.1 ± 7.3 μg/mL) were more potent than acarbose (IC_{50} 431.4 ± 16.7 μg/mL) on SAG inhibitory assay ($P<0.05$). In the rIM assay, DCM and EtOAc extracts from the flowers (IC_{50} 8.5 ± 0.2 and 12.4 ± 0.3 μg/mL) exhibited stronger inhibitory activity than acarbose (IC_{50} 38.5 ± 7.2 μg/mL) ($P<0.05$). Moreover, the inhibitory activity of DCM extract from the flowers (IC_{50} 16.9 ± 1.5 μg/mL) was comparable to the acarbose (IC_{50} 15.5 ± 1.2 μg/mL) on rIS assay.
Conclusion: The DCM and EtOAc extracts from the flowers were more active than the leaves, roots and stems in the inhibition of our defined target enzymes.

Implication for health policy/practice/research/medical education:
The flower of *C. formosum* subsp. formosum is the edible part and exert good carbohydrate hydrolyzing enzymes inhibitory activities. This part of this plant plausibly applied as a functional food to prevent and treat diabetes.
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Introduction

Diabetes mellitus is defined as a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. According to the World Health Organization (WHO) report, the global prevalence of diabetes in adults of age over 18 years has increased from 4.7% in 1980 to 8.5% in 2014. Moreover, the estimated 1.6 million deaths were directly caused by diabetes as reported in 2016 (1,2). The morbidity and mortality are not directly caused by diabetes but relatively resulted from the long-term complications in diabetes patients. Prolonged and uncontrolled hyperglycemia is a major risk, which is associated with diabetic angiopathy, neuropathy, nephropathy, and retinopathy (3). These chronic conditions definitively lead to serious clinical complications, e.g. cardiovascular and cerebrovascular disorders, chronic renal diseases, hypertension, and cataract. Thus, the glycemic control strategy is necessary for the prevention of morbidity and mortality of diabetes (4). Diabetes, especially type 2 diabetes, can be effectively managed by diet control, physical activity changes and anti-diabetic drugs (5). Several types of medications are utilized for therapeutic purposes such as biguanides,

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sulfonylureas, thiazolidinediones, α-glucosidase inhibitors and dipeptidyl peptidase-4 (DPP-4) inhibitors. However, these modern anti-diabetic drugs still produce serious adverse effects, for example, hypoglycemia, lactic acidosis, nausea, vomiting, pancreatitis, and metabolic acidosis (6). Therefore, the discovery of anti-diabetic drugs from natural origin with minimal or without any adverse effects has been extensively investigated. Different extracts from medicinal plants have been traditionally used to control diabetes globally, which are well-considered as relatively inexpensive, less toxic and minimal adverse effects (7). Therefore, the discovery of plasma glucose-lowering agents from edible plants is the priority of our research target. The Thai edible plant *Cratoxylum formosum* (Jacq.) Benth. & Hook. f. ex Dyer subsp. *formosum* belongs to the family Hypericaceae, which distributes mainly in North-eastern and Southern Thailand (8). The flowers, fresh shoots, and young leaves are served as vegetable in North-eastern and Southern Thai cuisines. Certain parts of *C. formosum* subsp. *formosum* have been used as Thai traditional medicines, for example, roots as a diuretic, leaves as for stomach pain relief and stems as a topical treatment for skin conditions. The major phytochemicals of the closest subspecies, *C. formosum* subsp. *pruniflorum*, are the prenylated xanthones, anthraquinones and phenolic compounds that have exhibited the topoisomerase I inhibitory, anti-cancer, anti-bacterial and cytotoxic activities (9-14). To date, there is no report on the inhibitory activity from the flowers of *C. formosum* subsp. *formosum* on the carbohydrate-hydrolyzing enzymes. Thus, the objective of this study was to assess the anti-diabetic potential of the extracts from the flower, leaf, stem and root parts of *C. formosum* subsp. *formosum* on the inhibition of porcine pancreatic α-amylase (pAA), *Saccharomyces* α-glucosidase (SAG), and rat intestinal maltase (rIM) and sucrase (rIS).

Materials and Methods

Chemicals and reagents

p-Nitrophenyl-α-D-glucopyranoside (NPG), α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), intestinal acetone powders from rat and porcine pancreatic α-amylase (EC 3.2.1.1) were purchased from Sigma-Aldrich, USA. Glucose LiquiColor reagent kit was purchased from HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Germany. Acarbose was purchased from Bayer Pharmaceuticals, Germany. All remaining chemicals used in this study were analytical grade.

Plant Materials

Plant samples of *C. formosum* subsp. *formosum* were collected from Roi-Et province, Thailand between October 2016 and March 2017. A plant specimen was identified and deposited at the Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham, Thailand (Voucher no. S. Sedlak 19-1). The leaf, flower, stem, and root parts of the plant were cleaned then dried in a hot-air oven at 50°C for 48 hours before the extraction process.

Extraction procedure

Ground plant samples (400 g) were extracted by Soxhlet extractor using methanol as an extracting solvent for 6 hours. Methanolic extracts from each part were concentrated under the vacuum condition by rotary evaporator. The methanol extracts were suspended in deionized water and successfully partitioned with hexane (HEX), dichloromethane (DCM), and ethyl acetate (EtOAc), respectively. The organic solvents were removed to yield HEX, DCM, and EtOAc extracts. The extracts were stored at -20°C until use. The crude extracts were dissolved in 50% DMSO/H_2O before the enzyme inhibitory assays.

Porcine pAA inhibitory assay

The inhibitory assay of the crude extract was evaluated by the amyloclastic method. The assay was modified from the previous report to a 96-well microtiter plate format (15,16). The stock solution of porcine pancreatic α–amylase was prepared by dissolving 50 mg of the enzyme in 10 mL of buffer (10mM phosphate saline buffer pH 6.9, 10mM $CaCl_2$, and 0.04% NaN_3) to yield a 65 U/mL of the enzyme. The working enzyme solution was diluted from a stock solution to a concentration of 0.1 U/mL. The reaction mixture consisted of 40 µL of working enzyme solution, 50 µL of 1.25 mg/mL of amylose and 10 µL of crude extract was dissolved in 50% DMSO/H2O and incubated for 10 minutes at 37°C. The reaction was terminated by the addition of the 100 µL of iodine reagent (0.2mM KOI_3, 4.2mM KI and 1.6mM HCl) and further incubated for 3 minutes. The starch-iodine complex was determined by measurement of absorbance at 655 nm. The inhibition percentage (IP) values of crude extracts were calculated according to the equation;

$$IP = 100 \times \left[\frac{A_{blank} - A_{test}}{A_{blank}} \right]$$

All extracts were screened at the final concentration of 100 µg/mL in triplicate. The IP values from screening assay were expressed as mean ± SD. The IP was plotted against the crude extract concentration to obtain the amount of extract necessary to inhibit porcine pAA by 50% (IC_{50}). The data were obtained from 5 independent experiments and expressed as mean ± SEM, each conducted in triplicate.

SAG inhibitory assay

The α-glucosidase inhibitory assay was performed in a 96-well microtiter plate format (17). The stock enzyme solution of α-glucosidase from *S. cerevisiae* was

prepared by dissolving enzyme in a buffer solution (0.5M phosphate buffer pH 6.5, 0.1% bovine serum albumin and 0.01% NaN_3). The working enzyme solution was diluted from a stock solution to a concentration of 3 U/mL. The reaction mixture consisted of 20 µL of working enzyme solution, 70 µL of buffer solution and 10 µL of crude extract solution was pre-incubated for 5 minutes at 37°C. The reaction mixture contained 10 µL 50% DMSO/H_2O instead of a crude extract solution that was set as the blank. The reaction was started by adding 50 µL of 20mM NPG and further incubated for 20 minutes. The reaction was terminated by adding 50 µL of 1 M Na_2CO_3. The absorbance was measured at 405 nm against a buffer blank. All extracts were screened at the final concentration of 100 µg/mL in triplicate. The IP values from screening assay were expressed as mean ± SD. The IP and IC_{50} values of crude extracts were calculated and plotted in the same manner of the porcine pAA inhibitory assay. The data were obtained from 5 independent experiments and expressed as mean ± SEM, each conducted in triplicate.

rIM and rIS inhibitory assay

The rIM and rIS inhibitory assays were performed in a 96-well microtiter plate format (18). The 0.1 g of rat intestinal acetone powder was dissolved in 10 mL of 0.1 phosphate saline buffer pH 6.8 at 4°C overnight. The enzyme suspension was centrifuged (12000 *g* at 4°C for 15 minutes) and the supernatant was kept for further enzymatic activity determination. The 30 µL of enzyme solution was incubated with 10 µL of 100mM maltose or sucrose as substrates at 37°C for 10 minutes. The reaction was terminated by heating in a hot bath at 100°C for 3 minutes and cooled down in tab water. The amount of glucose released from maltose and sucrose was assayed by the Glucose LiquiColor reagent kit. The maltase unit was defined as an amount of enzyme to convert 1µM of maltose to 2µM of glucose in 1 minute under the assay condition. The sucrase unit was defined as an amount of enzyme to convert 1µM of sucrose to 1µM of glucose in 1 minute under the assay condition. The enzyme solution was diluted to the concentration of 100 U/mL maltase and sucrose. The reaction mixture consisted of 10 µL of enzyme solution, 10 µL of crude extract dissolved in 50% DMSO/H2O and 30 µL of a substrate (10 mM maltose or 30 mM sucrose) and incubated for 10 minutes at 37°C. The reaction mixture containing 10 µL 50% DMSO/H_2O instead of crude extract solution was set as blank. The reaction was terminated by incubation in a hot water bath for 5 minutes and cooled down in tap water before the determination of liberated glucose. The IP values of crude extracts were calculated according to the equation;

$$IP = 100 \times \left[\frac{Glucose_{blank} - Glucose_{test}}{Glucose_{blank}} \right]$$

All extracts were screened at the final concentration of 100 µg/mL in triplicate. The IP values from screening assay were expressed as mean ± SD. The IP was plotted against the crude extract concentration to obtain the amount of extract necessary to rIM and rIS by 50% (IC_{50}). The data were obtained from 5 independent experiments and expressed as mean ± SEM, each conducted in triplicate.

Statistical analysis

One-way ANOVA was used to test for overall differences. Significant ANOVA was followed by Duncan's multiple comparisons for consideration of pair-wised differences between treatment groups. A *P* value of less than 0.05 was considered statistically significant.

Results

pAA inhibitory activities

According to the screening assay, the DCM and EtOAc extracts from the flowers, DCM extract from the roots, and CDM extract from the stems exhibited strong pAA inhibitory activities (Figure 1A). These extracts were obtained for further determination for the inhibitory activity. The saccharolytic inhibition of the DCM (IC_{50} 5.4 ± 1.5 µg/mL) and EtOAc (IC_{50} 10.5 ± 0.6 µg/mL) extracts from the flowers were not significantly different with acarbose (IC_{50} 7.2 ± 0.4 µg/mL) (Table 1). All extracts from the leaf part were inactive. The DCM extracts from the roots (IC_{50} 37.3 ± 1.1 µg/mL) and stems (IC_{50} 76.7 ± 12.1 µg/mL) were less potent than acarbose. By contrast, the EtOAc extracts from both parts were inactive.

SAG inhibitory activities

The result of SAG inhibitory activities demonstrated that DCM and EtOAc extracts from the flowers, EtOAc extract from the leaves, DCM and EtOAc from the roots, and EtOAc from the stems had significant inhibitory activities (Figure 1B). The EtOAc extract (IC_{50} 20.4 ± 0.4 µg/mL) was more active than the DCM extract (IC_{50} 56.7 ± 8.9 µg/mL) and acarbose (IC_{50} 431.4 ± 16.7 µg/mL), the referent compound ($P < 0.05$) (Table 1). The EtOAc extract (IC_{50} 45.0 ± 3.5 µg/mL) from the leaves was also more potent than acarbose ($P < 0.05$), whereas the DCM extract was not active at the screening concentration (100 µg/mL). The EtOAc extract (IC_{50} 16.7 ± 3.5 µg/mL) from the roots was more potent than the DCM (IC_{50} 35.0 ± 6.7 µg/mL) and acarbose ($P < 0.05$). The inhibitory activity of the EtOAc extract (IC_{50} 31.1 ± 7.3 µg/mL) form the stems was more potent than acarbose ($P < 0.05$), while the DCM extract was inactive.

rIM and rIS inhibitory activities

As the results from intestinal maltase inhibitory assay, DCM and EtOAc extracts from the flowers, DCM and EtOAc extracts from the leaves, DCM and EtOAc extracts from the roots, and DCM and EtOAc extracts from the stem were subjected to evaluation for the rIM inhibitory

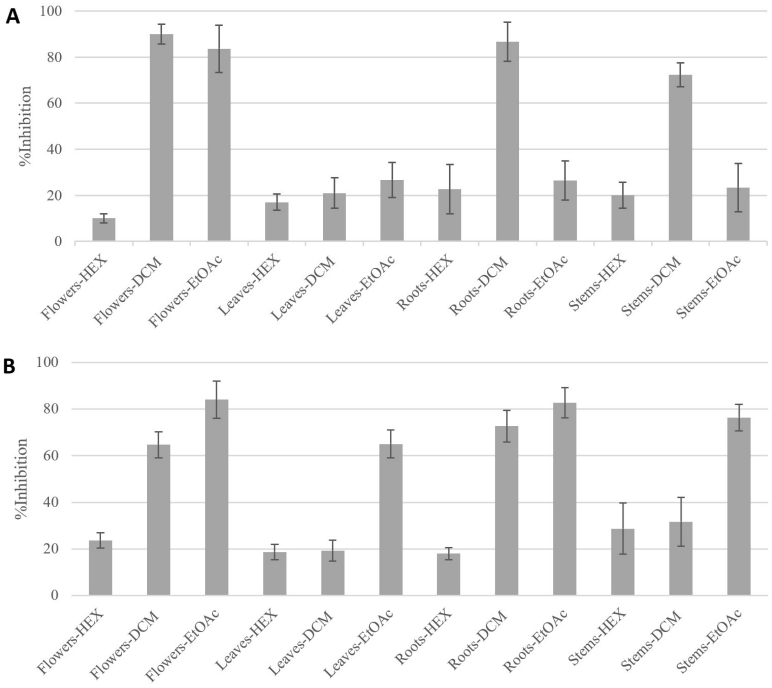

Figure 1. The inhibition percentage of the extracts from different parts of *C. formosum* subsp. *formosum* on the porcine pancreatic α-amylase (pAA) (A), and *Saccharomyces* α-glucosidase (SAG) (B) at 100 µg/mL (Mean±SD). HEX, hexane; DCM, dichloromethane; EtOAc, ethyl acetate.

activity (Figure 2A). The DCM (IC_{50} 8.5 ± 0.2 µg/mL) and EtOAc (IC_{50} 12.4 ± 0.3 µg/mL) extracts from the flowers were more potent than acarbose (IC_{50} 38.5 ± 7.2 µg/mL) ($P<0.05$) (Table 1). The DCM extract from the leaves (IC_{50} 76.8 ± 2.3 µg/mL) was less potent than the EtOAc extract (IC_{50} 45 ± 2.7µg/mL) and acarbose ($P<0.05$). The DCM and EtOAc extracts from the roots were comparable to acarbose in the maltase inhibitory capability. The inhibitory activity of DCM extract (IC_{50} 67.40 ± 2.2 µg/mL) from the stems was less active than the EtOAc one (IC_{50} 40.9 ± 1.5 µg/mL) and acarbose ($P<0.05$).

The DCM and EtOAc extracts from the flowers, EtOAc extract from the leaves, EtOAc extract from the roots, and EtOAc extract from the stems were obtained for the rIS inhibitory activity assay (Figure 2B). The effect of CDM extract (IC_{50} 16.9 ± 1.5 µg/mL) from the flowers was the

Table 1. The inhibitory activities of the extracts from different parts of *C. formosum* subsp. *formosum* on the pAA, SAG, rIM and rIS (Mean±SEM; µg/mL)

Parts	Extract	pAA	SAG	rIM	rIS
Flowers	HEX	NA	NA	NA	NA
	DCM	5.4 ± 1.5	56.7 ± 8.9*	8.5 ± 0.2*	16.9 ± 1.5
	EtOAc	10.5 ± 0.6	20.4 ± 0.4*	12.4 ± 0.3*	39.9 ± 3.1*
Leaves	HEX	NA	NA	NA	NA
	DCM	NA	NA	76.8 ± 2.3*	NA
	EtOAc	NA	45.0 ± 3.5*	45 ± 2.7	61. 8 ± 1.5*
Roots	HEX	NA	NA	NA	NA
	DCM	37.3 ± 1.1*	35.0 ± 6.7*	54.9 ± 4.9*	NA
	EtOAc	NA	16.7 ± 3.6*	37.4 ± 2.0	60.0 ± 2.5*
Stems	HEX	NA	NA	NA	NA
	DCM	76.7 ± 12.1*	NA	67.4 ± 2.2*	NA
	EtOAc	NA	31.1 ± 7.3*	40.9 ± 1.5	75.7 ± 4.1*
Acarbose		7.2 ± 0.4	431.4 ± 16.7	38.5 ± 7.2	15.5 ± 1.2

*$P<0.05$ as compared to acarbose
NA not active at the screening concentration of 100 µg/mL.
pAA, pancreatic α-amylase; SAG, *Saccharomyces* α-glucosidase; rIM, rat intestinal maltase; rIS, rat intestinal sucrase; HEX, hexane; DCM, dichloromethane; EtOAc, ethyl acetate.

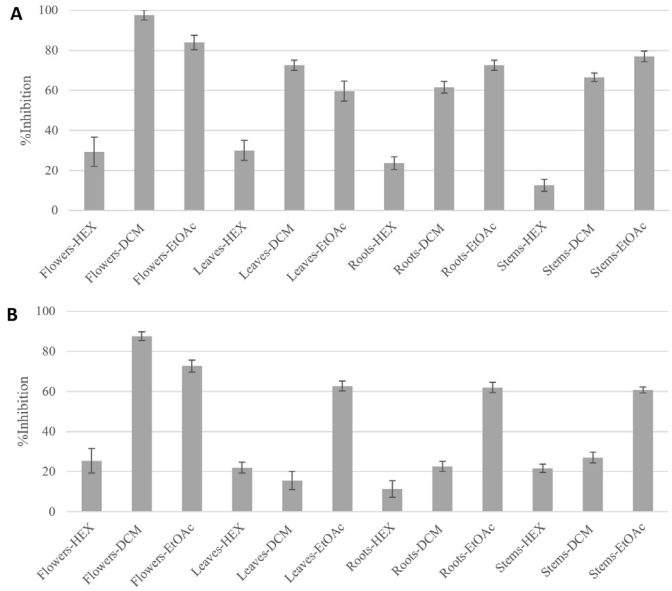

Figure 2. The inhibition percentage of the extracts from different parts of *C. formosum* subsp. *formosum* on rat intestinal maltase (rIM) (A), and rat intestinal sucrase (rIS) (B) at 100 µg/mL (Mean±SD). HEX, hexane; DCM, dichloromethane; EtOAc, ethyl acetate.

same as the acarbose (IC_{50} 15.5 ± 1.2 µg/mL). By contrast, the EtOAc extract from the flowers (IC_{50} 39.9 ± 3.1 µg/mL) was less potent than acarbose ($P<0.05$) (Table 1). The HEX and DCM extracts from the leaves were inactive under the assay condition. The EtOAc extract (IC_{50} 61. 8 ± 1.5 µg/mL) from the leaves was less active than acarbose ($P<0.05$). The HEX and DCM extracts from the roots and stems were inactive. The EtOAc extracts from the leaves (IC_{50} 60.0 ± 2.5 µg/mL) and stems (IC_{50} 75.7 ± 4.1 µg/mL) were less active than acarbose ($P<0.05$).

Discussion

Certain α-glucosidase inhibitors (acarbose, miglitol, voglibose) are one group of the diabetic drugs for the patient's glycemic control, which competitively inhibit carbohydrate-hydrolyzing enzymes to delay digestion of carbohydrates and absorption of the monosaccharides (19,20). Human pAA is the first intestinal enzyme that catalyses the hydrolysis of 1,4-glucan linkage in the ingested starch to yield maltose, maltotriose, several of α-(1,6) and α-(1,4) oligoglycans, and a small amount of glucose. The 3D structural comparison by X-ray diffraction demonstrated that human pAA had a high degree of structural identity to the porcine enzyme (21). Moreover, the enzyme kinetic characteristics and enzyme-inhibitor interaction (acarbose) between human and porcine enzymes were not significantly different (22). Thus, the porcine pAA was reasonable to use this enzyme as the experimental target in this study. These results led to the conclusion that the flowers of *C. formosum* subsp. *formosum* possessed the potent pAA inhibitory activity as compared to acarbose.

Saccharomyces cerevisiae α-glucosidase is a 1,4-α-glycosidic bond-specific hydrolase widely used as a target for the searching of anti-diabetic agents from natural origins (23). The results revealed that the EtOAc from the roots and flowers were the most active extracts among different extracts from this plant. HEX extracts from all parts were completely inactive under the assay concentration (100 µg/mL). Maltose is the disaccharide that is mainly released from the hydrolysis of starch catalysed by intestinal α-amylase. Maltase is the membrane-bound enzyme located in the intestinal brush border, and is the last enzyme to cleave maltose to release two molecules of glucose before intestinal absorption. Sucrase is another membrane-bound enzyme which hydrolyses ingested sucrose to glucose and fructose (24). The rIM and rIS were acceptable models for the human-liked maltase and sucrase inhibitory assays (25). The results demonstrated that the CDM extracts from the flowers were the most active extracts of this plant in the rIM and rIS inhibitory assays.

The investigation of porcine pAA, SAG, rIM and rIS inhibitory activities demonstrated that the flower part of *C. formosum* subsp. *formosum* displayed the strongest anti-diabetic potential. The DCM and EtOAc extracts exhibited the inhibitory activities while the HEX was completely inactive. This result indicated that the nonpolar constituents dissolved in hexane from the flowers were not the active ingredients. There were no phytochemical and biological activities reported from the flowers of this plant. The polar extract, EtOAc extract, from the leaf part of *C. formosum* subsp. *formosum* showed potent SAG, rIM and rIS inhibitory activities. Recently, the phenolic compounds from the ethanolic extract of *C. formosum* subsp. *formosum* leaves exhibited the *in vitro* protective effects against H_2O_2-induced cell death, and antioxidant activities (26). The DCM root extract exhibited moderate

pAA, rIM, and rIS inhibitory activities. Besides, the EtOAc extract from the roots showed strong α-glucosidase inhibitory activity. The phytochemical constituents have not been reported from the roots of this subspecies. Several prenylated xanthones, anthraquinones, flavonols, and phenolic compounds have been isolated and identified from the roots of *C. formosum* (27,28). Moreover, certain caged and prenylated xanthones were isolated from the roots of *C. cochinchinense* (29,30). The polar part of the stems, EtOAc extract, showed strong SAG, and potent rIM and rIS inhibitory activities. The chemical constituents from the stems of *C. formosum* ssp. *pruniflorum* and *C. cochinchinense* were prenylated xanthone, benzophenone glycosides and bisanthraquinone (31-33).

Conclusion

The inhibitory effects of the different parts of the *C. formosum* subsp. *formosum* demonstrated the anti-diabetes potential. The DCM and EtOAc extracts from the flowers and roots showed the significant saccharolytic inhibitory activities via the pAA, SAG, rIM, and rIS inhibitory activities. The DCM extracts from the leaves and stems also showed potent rIM inhibitory activity, whereas the EtOAc extracts from these parts exhibited the significant SAG, rIM, and rIS inhibitory activities. The obtained results indicated that these active extracts possibly inhibited the carbohydrate-hydrolyzing enzyme through the interferences of catalytic activity. However, the exact mechanisms of enzymatic inhibition were not revealed by the defined assay protocol. Thus, extensive enzyme kinetic studies are needed to clarify the inhibitory mechanisms. Although the *in vitro* study indicates the possibility of this plant for the prevention and treatment of diabetes, the *in vivo* study is extensively needed to assess the therapeutic effectiveness and toxicological effects for therapeutic purposes.

Authors' contribution

SS collected and identified the plant specimens. KA and BT tested the enzyme inhibition and analysed the data. SS conducted the project and wrote the paper. All authors read and confirmed the publication of the paper.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors. The authors affirm that no animal and human subjects were involved in this research.

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