

Evaluating the Hypoglycemic Efficacy and Quality Assurance of Ya That Opchoei Mixture

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Abstract

Diabetes mellitus, diagnosed as Madhumeho (sweet urine) in Thai traditional medicine, is believed to stem from imbalanced life elements. Ya That Opchoei mixture (YTO), a polyherbal mixture, is used to treat its symptoms. This study assessed the hypoglycemic potential of YTO and conducted qualitative and quantitative analyses of its bioactive constituents. YTO significantly inhibited α -glucosidase at IC_{50} 0.05 ± 0.00 mg/mL and α -amylase activities at IC_{50} 0.04 ± 0.01 mg/mL. YTO at concentrations of 2.22 mg/mL significantly increased C-peptide secretion and stimulated glucose uptake. Liquid chromatography-mass spectrometry revealed constituents from *Amomum testaceum*, *Cinnamomum bejolghota*, *C. burmanii*, *Syzygium aromaticum*, and *Glycyrrhiza glabra*. High-performance liquid chromatography enabled quantification of bioactive compounds, including glycyrrhizic acid, eugenol, cinnamic acid, cinnamaldehyde, and coumarin. The coumarin content in 100 mL YTO was within the tolerable daily intake set by the European Food Safety Authority. This study confirms the hypoglycemic potential of YTO and presents its quality control process for safety assurance.

Keywords

Ya That Opchoei mixture, diabetes mellitus, Madhumeho, hypoglycemic activity, quality assessment

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Introduction

Diabetes mellitus poses significant health and economic challenges. The worldwide occurrence of this disease among adults is predicted to reach 783.2 million or 12.2% by 2045 and costs associated with managing diabetes are expected to reach 1054 billion USD in the same year.¹ The primary characteristic of diabetes mellitus is hyperglycemia, which subsequently leads to macrovascular and microvascular complications.² In Thai traditional medicine, diabetes mellitus, diagnosed as Madhumeho (sweet urine), is attributed to imbalanced phlegm leading to bile dysfunction. Treatment involves careful elemental diagnosis based on the four elements (earth, water, wind, and fire), utilizing bitter, spicy, and hot-tasting medicines, tailored dietary adjustments, adequate hydration, sleep, and exercise.³ Ya That Opchoei mixture (YTO) is formulated from a blend of spicy-hot tasting herbs such as the barks of *Cinnamomum burmannii* (Nees.) Blume. and *C. bejolghota* (Buch.-Ham.) Sweet, the young flowers of *Syzygium aromaticum* (L.) Merr. et Perry, a spicy-cool herb similar in taste to the *Amomum testaceum* Ridl. fruit, and the rhizome of the bitter-sweet tasting herb *Glycyrrhiza glabra* L. The YTO mixture is used in Thai community hospitals to treat symptoms associated with elemental

imbalances.⁴ Despite its widespread use, YTO faces two primary challenges: limited scientific validation of its claimed biological activity and batch-to-batch quality variability owing to inconsistencies in raw materials and preparation processes. Therefore, this study aimed to evaluate the hypoglycemic activity of YTO and to standardize its bioactive compounds to ensure safety through consistent quality.

Materials and Methods

Chemical Materials and Cells

Reagents and standards, including acarbose, acetonitrile, α -amylase, α -glucosidase, camphor, cinnamaldehyde, cinnamic acid, dexamethasone, dimethyl sulfoxide (DMSO), eugenol, formic acid, glucose, glutaMAX, insulin, 1-isobutyl-3-methylxanthine, methanol,

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Figure 1. Morphological characteristics of the starting materials.

First row, from left to right: *A. testaceum* fruit, *S. aromaticum* flower, *C. burmannii* bark.

Second row, from left to right: *G. glabra* rhizome, *C. bejolghota* bark.

menthol, *p*-nitrophenyl- α -D-glucopyranoside (PNPG), hydroxyethyl piperazine ethane sulfonic acid (HEPES), and resazurin sodium were bought from Sigma-Aldrich, USA. Glycyrrhizic acid monoammonium salt was obtained from Carlo Erba (Italy). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), antibiotic/antimycotic solution, Roswell Park Memorial Institute 1640 medium (RPMI 1640), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), and Qubit dsDNA broad range (BR) assay kits were obtained from Thermo Fisher Scientific, USA. A C-peptide 2 (Rat/Mouse) ELISA kit and phosphate-buffered saline solution (PBS) were bought from Merck-Millipore. A Glucose Uptake Assay kit (cell-Based) was acquired from Cayman Chemical (USA). The cell lines 3T3 J2 fibroblasts, 3T3-L1 fibroblasts, and RIN-m5F cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Herbal Materials Identification

The starting plant materials for YTO, including fruits of *A. testaceum*, dry barks of *C. burmannii*, dry barks of *C. bejolghota*, dry rhizomes of *G. glabra*, and dry buds of *S. aromaticum* were purchased from local traditional drugstores in Thailand (Figure 1). The plant species were authenticated using macroscopic and microscopic characters.

Quality Assessment of the Starting Herbal Ingredients

Before YTO formulation, limit tests were performed on its herbal ingredients. These assessments included an evaluation of foreign matters, total ash, acid-insoluble ash, and water contents. The extractive values for each plant were determined.⁵⁻⁹

Formulation and Quality Assessment of YTO

After quality assessment, the plant materials were cleaned, milled, and sieved through a No. 40 sieve. YTO was formulated from 24 g each of

C. burmannii bark, clove bud, *C. bejolghota* bark, *A. testaceum* fruit, and *G. glabra* root, and then boiled in 3 l of distilled water at $80 \pm 5^\circ\text{C}$ for 15 min. The mixture was filtered to obtain a red-brown solution. The flavoring agents, camphor and menthol, were added when the solution cooled to 50°C . Before further experimentation, YTO was tested to meet the physicochemical requirements (pH, viscosity, and refractive index). Screening for microbial contamination and pesticide residues was also performed.¹⁰ YTO was freeze-dried to yield a homogeneous powder, which was preserved at 4°C until further experiment.

Hypoglycemic Activity Assays

Evaluation of α -Glucosidase-Inhibitory Activity

YTO extract was examined for α -glucosidase inhibition activity with some modification from the method described by Bhatia.¹¹ Briefly, α -glucosidase (0.2 U/mL, 50 μL) was mixed with YTO at varying concentrations (0.01-2 mg/mL). The reactant was kept at 37°C for 5 min. Next, 1 mM PNPG (50 μL) and 50 μL of 50 mM phosphate buffer (pH 6.9) were added. The reaction was then developed at 37°C for 10 min. The concentration of free *p*-nitrophenol was determined by a microplate reader at 405 nm. Experiments were performed in triplicate.

Evaluation of α -Amylase-Inhibitory Activity

YTO extract was tested for α -amylase-inhibitory activity using a method adapted from Luyen et al¹² The combination of YTO at varying concentrations (0.01-2 mg/mL) along with 50 μL potato starch in phosphate buffer solution (pH 7.0). The reaction was then incubated at 37°C for 5 min. Next, 20 μL of 5 $\mu\text{g/mL}$ α -amylase enzyme was added and incubated at 37°C for 15 min. To terminate the reaction, 50 μL of 1 N

hydrochloric acid was added. Iodine solution was added to produce the color. The mixture was then measured by a microplate reader at 650 nm. Experiments were conducted with three independent replicates.

Evaluation of % Cell Viability

To assess cell viability before investigating C-peptide secretion and glucose uptake, the cytotoxicity of the extract of YTO was assessed in 3T3-J2 cells. Briefly, 3T3-J2 cells were cultured at 5×10^3 cells/well in a 96-well plate containing DMEM in an incubator (5% CO₂), 37 °C, 24 h. The cells were then incubated with YTO at varying concentrations for an additional 24 h. Subsequently, cells were rinsed 2 times with sterile PBS, treated with 0.15 mg/mL resazurin solution (20 µL) along with 100 µL of fresh medium, and kept in a 5% CO₂ incubator, 37 °C, 2 h. The viability of cultured cells was then assessed by measuring the fluorescence intensity of the resazurin reduction product at 560 nm excitation and 600 nm emission wavelengths. Three replicate experiments were performed.¹³

$$\% \text{ Cell Viability} = \frac{(\text{Change in OD of tested cells})}{(\text{Change in OD of control cells})} \times 100$$

Evaluation of C-peptide secretion activity from RIN-m5F cells

Based on the % cell viability, the IC₅₀ value (2.22 mg/mL) and its tenfold dilution (0.22 mg/mL) were chosen as the concentrations for determining C-peptide secretion and for the glucose uptake experiments. RIN-m5F cells were used for evaluating C-peptide secretion. Briefly, the RIN-m5F cells were cultured at 5×10^4 cells/well in 48-well plates containing RPMI 1640 medium in an incubator (5% CO₂, 37 °C, 24 h). The cells were then incubated with YTO at concentrations of 0.22 mg/mL and 2.22 mg/mL for 24 h. A vehicle was used as the negative control. After treatment, the cells were collected in centrifuge tubes, washed 2 times with sterile Krebs-Ringer bicarbonate-HEPES buffer (KRBH), and then suspended in 100 µL aliquots of either glucose-free KRBH, KRBH supplemented with 5.5 mM glucose, or KRBH containing 22 mM glucose. Each reaction was kept in an incubator (5% CO₂), 37 °C, 30 min. After 30 min, the supernatants were collected for C-peptide measurement using an ELISA kit. Total cellular DNA was extracted from the remaining cell pellets and quantified using a Qubit assay. C-peptide secretion data were adjusted based on the total DNA content. Three independent experiments were performed.¹⁴

Evaluation of Glucose Uptake Activity of 3T3-L1 Adipose Cells

YTO extract was evaluated for its cellular glucose uptake inducing potential using a 2-NBDG fluorescence probe. For cell culture, 3T3-L1 adipose cells were differentiated in both transparent and black 96-well plates and maintained in an incubator (5% CO₂) at 37 °C. The test groups were pre-incubated with YTO (0.22 and 2.22 mg/mL, concentrations) for 24 h.

The positive control group received a 10 min treatment with 50 nM insulin before the uptake study. All groups were then incubated with 100 µg/mL 2-NBDG (80 µL) for 25 min at 37 °C in a 5% CO₂ incubator. Then, cells were cleaned with cold buffer (200 µL, 2-3 times). A confocal microscope with 485 nm and 535 nm was used to determine the cellular uptake of 2-NBDG. Additionally, a microplate reader was used to measure the fluorescence intensity of 2-NBDG at the same excitation and emission wavelengths. The rate of glucose uptake was calculated as previously described.¹⁵

% Glucose uptake increase rate

$$= \frac{(\text{F sample} - \text{F Blank})}{(\text{F Control} - \text{F Blank})} \times 100$$

F: fluorescent signal.

Chemical Compound Identification Using LC-MS

The chemical constituents of YTO were analyzed using a Thermo Ultimate 3000 HPLC combined with a Bruker Amazon SL mass spectrometer. YTO (1 mg/mL) was separated on an ACE C-18 column (5 µm, 4.6 × 250 mm I.D.) with a mobile phase gradient system. The mobile phase was composed of solvent A (0.1% aqueous acetic acid) and solvent B (1% acetonitrile). The gradient eluted from 100% A to 0% A in 60 min. The flow rate was maintained at 0.7 mL/min, 35 °C. The wavelength of the UV detector was set at 270 nm. The EIMS instrument employed a quadrupole ion trap. The MS condition included a capillary voltage of 4500 V, the pressure of nebulizer gas was 2 bar, and the drying gas temperature was 220 °C with a flow rate of 7.0 l/min. The mass spectrometer was conducted in a positive ion mode with full-scan detection ranging from 70 to 1000 m/z. Characterization of phytochemical compounds in YTO was based on a combination of UV spectra, retention times, and MS fragmentation patterns.

Quantitative Analysis of Bioactive Markers Using HPLC

The experiment was performed following the ICH Q2 (R1) guidelines.¹⁶ Freeze-dried YTO powder (50 mg) was homogeneously mixed with methanol 10 mL and sonicated in an ultrasonicator at 40 °C for 30 min. Standard stock solutions (50 mL) of coumarin (100 µg/mL), cinnamic acid (50 µg/mL), glycyrrhizic acid (500 µg/mL), cinnamaldehyde (50 µg/mL), and eugenol (500 µg/mL) were prepared in methanol. Working solutions were then prepared by diluting the standard solutions to generate calibration curves with at least five different concentrations of each compound. All prepared solutions were stored at 4 °C until further experiments.

Data Analysis

IC₅₀ values were determined by GraphPad Prism software (v.6.0). The three independent experimental data were reported as mean ± standard error of the mean. One-way analysis of

Table 1. Limit Tests and Physicochemical Properties of the Herbal Materials Used.

Plant materials	%Water content	%Foreign matters	%Ash content		%Extractive values	
			Total ash	Acid-insoluble ash	Ethanol-soluble extraction	Water-soluble extraction
<i>C. bejolghota</i> ⁴	10.72 ± 1.10	0.02 ± 0.00	1.28 ± 0.10	0.07 ± 0.00	31.80 ± 0.32	25.21 ± 0.08
<i>G. glabra</i> ⁵	[11.80%] 9.44 ± 0.12	- 0.01 ± 0.00	[11.70%] 5.39 ± 0.00	[3.76%] 0.22 ± 0.00	[14.33%] 14.80 ± 0.17	[4.10%] 33.87 ± 0.74
<i>S. aromaticum</i> ⁶	- 4.86 ± 0.06	- 0.43 ± 0.12	[< 7%] 5.50 ± 0.03	[< 7%] 0.27 ± 0.02	[> 25%] 15.46 ± 0.17	[> 20%] 25.09 ± 0.52
<i>A. testaceum</i> ⁷	[8.43%] 7.53 ± 0.17	- 0.10 ± 0.06	[< 0.62%] 8.28 ± 0.06	[1.27%] 2.38 ± 0.06	[9.42%] 3.01 ± 0.12	[25.7%] 9.90 ± 0.32
<i>C. burmannii</i> ⁸	[< 11%] 11.40 ± 0.30	[< 2%] 0.01 ± 0.00	[< 10%] 4.56 ± 0.02	[< 3%] 0.23 ± 0.01	- 22.86 ± 1.67	[< 6%] 17.63 ± 1.15
	[≤ 10%]	-	[≤ 10.5%]	[≤ 0.3%]	[≥ 4%]	[≥ 16%]

[Reference Values].

variance (ANOVA) followed by the Tukey's post-hoc test or the Games-Howell post-hoc test were used to evaluate statistically significant differences (SPSS software, version 2020). A *P*-value ≤ 0.05 was considered statistically significant.

Results

Quality Assessment of the Staring Herbal Materials and YTO

The physical appearance of the material is shown in Figure 1. The microscopic characteristics of the raw materials are presented in Supplemental Data 1. Five herbal materials were analyzed for their limit tests and physicochemical properties. The water content ranged from 4.86–11.40%, and foreign matter contamination ranged from 0.01–0.43%. The materials showed ethanol extractive values of 3.01–31.80% and water-soluble extractive values of 9.90–33.87% (Table 1). The physicochemical parameters of the YTO mixture were determined. The pH of YTO was 7.29, the viscosity was 3.3130 mPa, and the refractive index was 1.0030. The microbial tests exhibited the absence of *C. perfringens*, *E. coli*, *S. aureus*, and *Salmonella* spp. No organophosphate and carbamate residues were found in the pesticide analysis of YTO (Table 2).

Inhibition of α -Amylase and α -Glucosidase by YTO

At concentrations of 2, 1, 0.5, 0.25, 0.13, 0.06, and 0.03 mg/mL, YTO exhibited α -amylase activity by 83.77 ± 0.81%, 75.85 ± 1.04%, 71.70 ± 1.18%, 66.41 ± 3.37%, 62.61 ± 1.02%, 57.67 ± 4.50%, and 46.10 ± 0.52%, respectively (Figure 2). YTO exhibited a lower IC₅₀ (0.05 ± 0.00 mg/mL) compared to that of the positive control acarbose (0.01 ± 0.00 mg/mL). Similarly, the extracts at concentrations of 2, 1, 0.5, 0.25, 0.13,

Table 2. Physicochemical Parameters and Contamination Test Results of YTO.

Parameters ⁹	YTO
pH	7.29 ± 0.00 [6.5–7.5]
Viscosity (mPa)	3.3130 ± 0.6
Relative density	1.003 ± 0.00 [1.010–1.030]
Refractive index, at 25 °C	1.334 ± 0.00 [1.333–1.334]
Microbial limit tests	
Total viable aerobic count	< 5 × 10 ⁵ CFU/g [< 5 × 10 ⁵ CFU/g]
<i>C. perfringens</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>Salmonella</i> spp.	Not detectable [Not detectable]
Heavy metals	
Arsenic, mercury, lead, cadmium	Not detectable [Not detectable]
Pesticide residues	
Dicrotophos, chlorpyrifos, malathion, prothiofos, profenofos, aldicarb sulfoxide, oxamyl, methiomyl, aldicarb, carbaryl, methiocarb	Not detectable [Not detectable]
% Freeze drying yield	0.46 ± 0.01 %w/v

[Reference values].

0.06, and 0.03 mg/mL inhibited α -glucosidase activity by 97.61 ± 1.38%, 97.30 ± 0.85%, 97.23 ± 0.45%, 97.13 ± 0.82%, 91.97 ± 1.02%, 59.50 ± 6.79%, and 33.39 ± 0.84%, respectively (Figure 3). The IC₅₀ for α -glucosidase inhibition was 0.04 ± 0.00 mg/mL, which was higher than that of acarbose (0.24 ± 0.01 mg/mL).

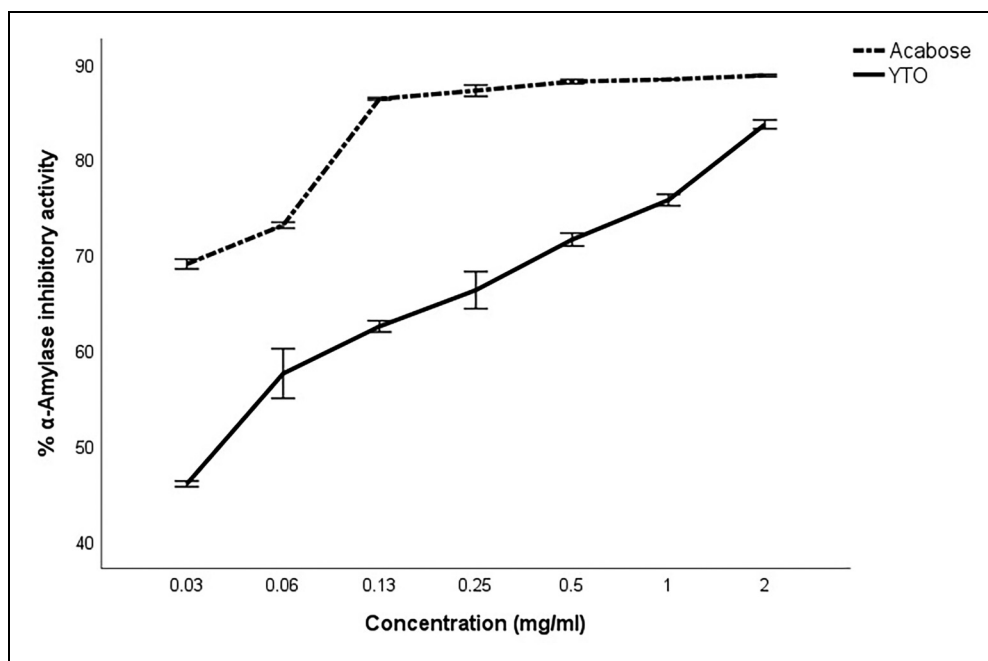


Figure 2. % α -Amylase inhibitory activity of YTO.

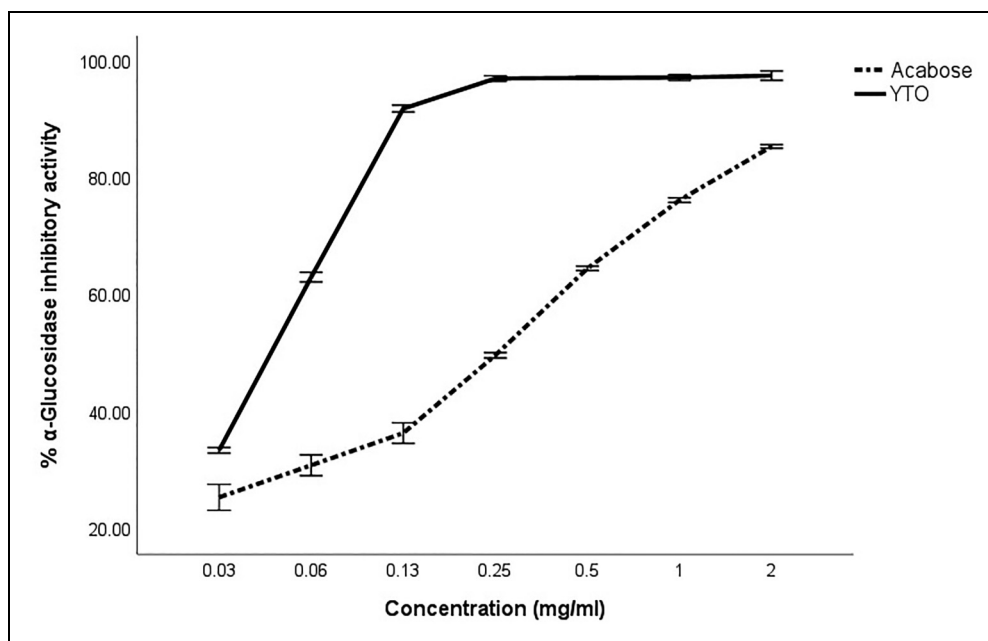


Figure 3. % α -Glucosidase inhibitory activity of YTO.

% Cell Viability of YTO

Treatment with YTO at concentrations of 0.00025–2.5 mg/mL displayed a dose-dependent effect on cell viability (Table 3). Only the highest concentration, 2.5 mg/mL, showed a significant difference in viability from that in the control. The IC_{50} of YTO was 2.22 mg/mL.

C-Peptide Secretion Activity of YTO

This study evaluated the antidiabetic potential of YTO by determining its effects on C-peptide secretion from insulin-secreting RIN-m5F cells. C-peptide, a co-secreted product of insulin, is an indirect marker of insulin release. Thus, C-peptide levels can be used to indirectly assess pancreatic β -cell function and

insulin secretion ability. YTO at concentrations of 0.22 and 2.22 mg/mL significantly increased C-peptide secretion from RIN-m5F cells compared to that from the control group ($P < .05$) in the presence of various glucose concentrations. However, these two concentrations did not differ significantly in their ability to stimulate C-peptide release (Figure 4).

Glucose Uptake Ability of YTO

A glucose uptake assay with 2-NBDG was used to investigate the capability of YTO to improve insulin sensitivity by assessing its activity on glucose transport into 3T3-L1 cells (Figure 5). While YTO induced 2-NBDG uptake in 3T3-L1 cells at both concentrations of 0.22 and 2.22 mg/mL compared to that in the control group, only the higher concentration exhibited a significant difference compared to that of insulin (Figure 6). This suggests that YTO may possess insulin-sensitizing properties, particularly at the higher concentration, by enhancing glucose transport into adipocytes.

Identification of Phytochemical Compounds by LC-MS

The LC-MS chromatogram (Figure 7) showed various compounds in YTO. The constituents were characterized by comparing their mass fragmentation profiles and retention times with those described in previous publications and available online databases, including SDBS and PubChem (Table 4).

Table 3. % Cell Availability of Control and YTO.

Concentration (mg/mL)	Control $\pm$ S.E.M	YTO $\pm$ S.E.M
0.000025	99.67 $\pm$ 1.76	101.44 $\pm$ 1.93
0.00025	96.09 $\pm$ 1.37	101.54 $\pm$ 0.87
0.0025	91.60 $\pm$ 4.69	93.30 $\pm$ 0.53
0.025	92.53 $\pm$ 2.01	87.77 $\pm$ 4.05
2.5	91.26 $\pm$ 1.57*	31.00 $\pm$ 3.92*

* Significantly different compared to the control at $P \leq .05$.

Quantitative Analysis of Bioactive Markers Using HPLC

Bioactive markers of YTO were analyzed using a validated HPLC method. The HPLC chromatogram revealed peaks corresponding to coumarin, cinnamic acid, glycyrrhizic acid, cinnamaldehyde, and eugenol (Figure 8). Among the compounds analyzed, glycyrrhizic acid exhibited the highest yield, whereas cinnamaldehyde showed the lowest yield (Table 5).

Discussion

α -Amylase in saliva and the pancreas, along with α -glucosidase in the small intestine, hydrolyze carbohydrates into glucose, impacting blood sugar levels.²⁴ Based on the results, YTO significantly inhibited α -amylase and α -glucosidase activities, which could be associated with its herbal composition. A study by Ervina (2019) showed that an aqueous extract of *C. burmannii* exhibited α -glucosidase inhibitory and antioxidant activities.²⁵ Another study reported that *C. burmannii* and *C. bejolghota* extract effectively inhibited the activities of both pancreatic α -amylase and intestinal α -glucosidase.²⁶ *G. glabra* extract was found to inhibit both enzymes.²⁷ Compounds from *S. aromaticum*, including eugenol, maslinic acid, and oleanolic acid, were found to decrease the activities of carbohydrate-digestion enzymes in diabetic rats.^{28,29} Besides inhibiting carbohydrate-digestion enzymes, YTO exhibited the abilities of stimulating insulin release and enhancing cellular glucose uptake. Considering the herbal ingredients of YTO, *C. burmannii* extract has been shown to modulate insulin signaling, glucose transport, and adiponectin secretion.^{30,31} *C. burmannii* bark, similar to that of other *Cinnamomum* species, contains cinnamic acid, cinnamaldehyde, and coumarin. Cinnamic acid protects against diabetes-induced DNA damage, regulates the expression of glucose uptake transporters in L6 myotubes, and induces insulin release.^{32–34} Cinnamaldehyde exhibits both hypoglycemic and hypolipidemic effects *in vivo* and shows α -amylase inhibitory activity.^{35–37} Coumarin possesses antidiabetic properties by

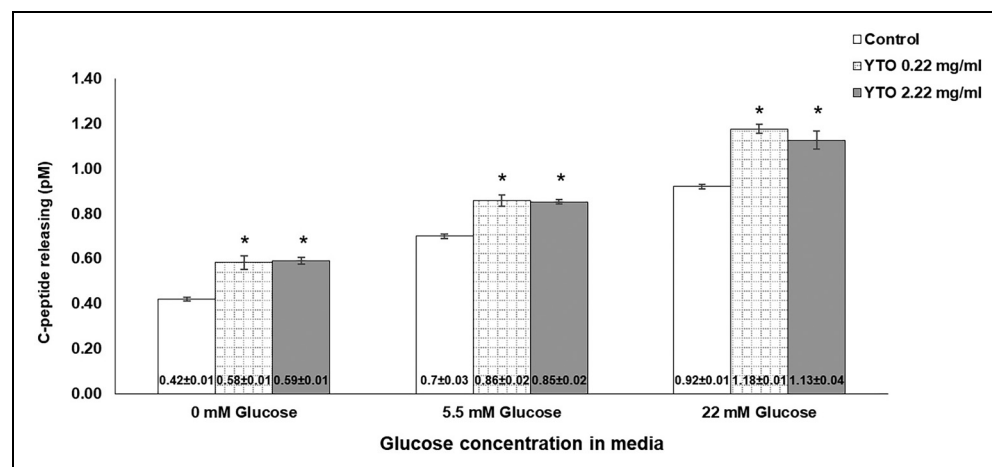


Figure 4. Effect of YTO on stimulating insulin release from RIN m5F cell.

* Statistically significant difference from the control group ($P \leq .05$).

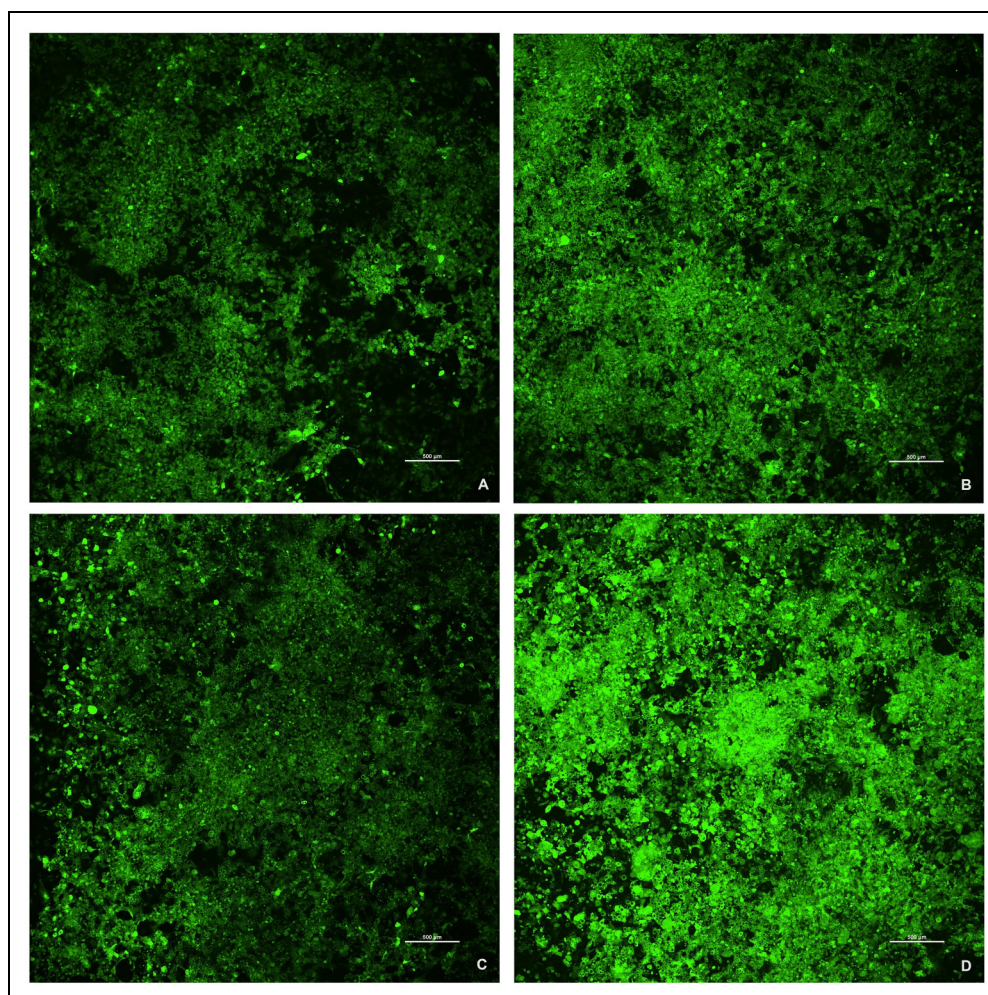


Figure 5. Effects of YTO in stimulating 2-NBDG uptake by 3LT3 adipocytes.
A = Control, B = Insulin, C = YTO 0.22 mg/mL, D = YTO 2.22 mg/mL.

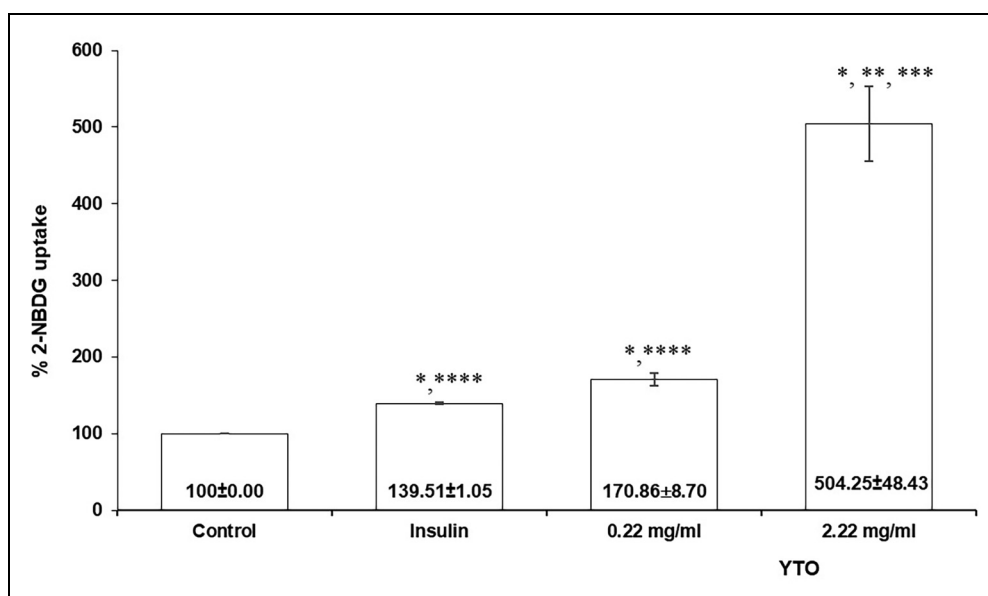


Figure 6. % 2-NBDG uptake in 3T3 adipocytes induced by YTO.

* Statistically significant difference from the control group, ** Statistically significant difference from the insulin group, *** Statistically significant difference from the YTO 0.22 mg/mL group, **** Statistically significant difference from the YTO 2.22 mg/mL group ($P \leq .05$).

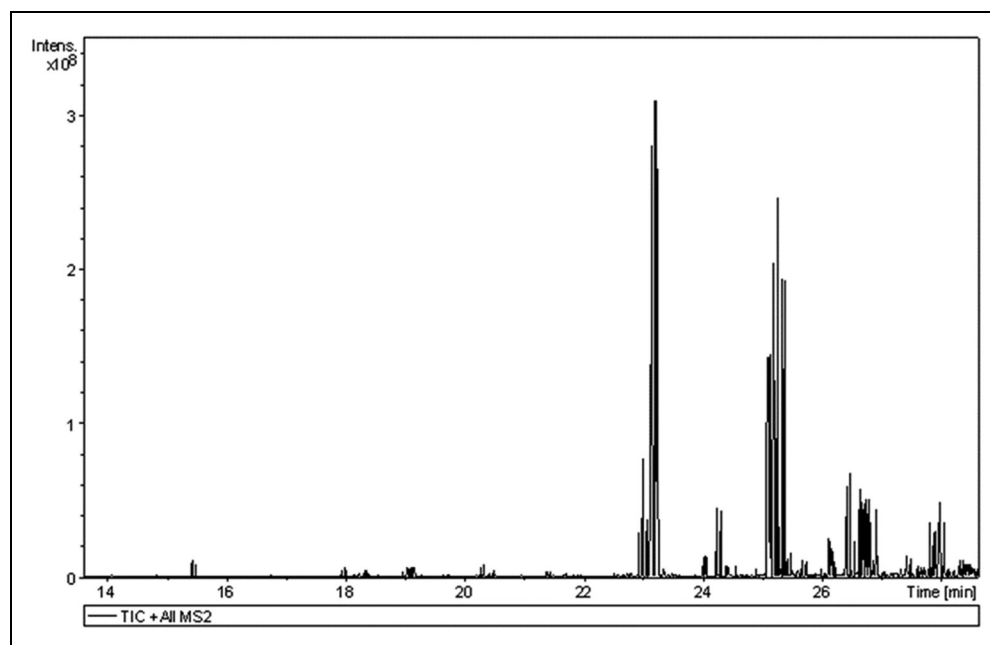


Figure 7. Total ion chromatogram based on the MS² chromatogram of YTO.

stimulating insulin secretion, lowering blood glucose, and inhibiting protein glycation.³⁸ Additionally, a methanolic extract of *C. burmannii* containing 0.07% w/w of coumarin demonstrated no significant acute or subchronic toxicity *in vivo* (500, 1000, and 2000 mg/kg, doses).³⁹ Type A proanthocyanidins from *C. burmannii*, *C. cassia*, and *C. verum*, have been shown to exhibit insulin-like effects.⁴⁰ The extract of *C. bejolghota* bark was reported to improve glucose tolerance, decrease blood sugar levels in rats, enhance antioxidation enzymes, and reduce the malondialdehyde content in streptozotocin-induced diabetic animals.⁴¹ *G. glabra* extract and its isolated isoflavones, genistein, daidzein, and formononetin, displayed various mechanisms of hypoglycemic action, including PPAR- γ receptor binding, which led to decreased blood sugar levels, stimulated insulin secretion, and elevated glucose tolerance.^{19,42,43} Moreover, glycyrrhizic acid possessed the ability to protect cells from diabetic complications.⁴⁴ *S. aromaticum* powder was shown to lower blood glucose levels in diabetic rats.⁴⁵ Its isolated compounds, dehydrodieugenol, dehydrodieugenol B, and oleanolic acid, showed hypoglycemic activity in KK-A^y type 2 diabetic mice.⁴⁶ Ellagic and gallic acids were also detected in this plant.¹⁷ Ellagic acid has been reported to reduce fasting blood glucose levels, stimulate insulin release, modulate RS1 expression, regulate GLUT4 gene expression, suppress lipid accumulation, and decrease inflammation in the adipose tissue.⁴⁷ Gallic acid showed antidiabetic activity in Db/db mice by upregulating pAkt, PPAR- γ , and Glut4.⁴⁸ A meta-analysis further highlighted the benefits of eugenol for antihyperglycemic activity, including increased glucose uptake and prevention of late diabetic complications.⁴⁹ These findings imply that the synergistic effects of plant constituents in YTO may contribute to its antihyperglycemic effect through various mechanisms.

Quality assurance of YTO was performed according to the WHO guidelines.^{6,7} The starting materials were authenticated by their morphological and microscopic characteristics. Sclereids of different sizes and shapes were found in the fruits of *A. testaceum*, the barks of *C. bejolghota*, and *C. burmannii*. Pollen grains were used to characterize the *S. aromaticum* flowers. The rhizome of *G. glabra* contained vessels and fibers (Supplementary Data 1). The limit tests of the raw materials did not exceed the previously reported limits.^{4–8} The physicochemical and contamination test results of YTO met the requirements of the Thai herbal preparation pharmacopoeia standards.⁹

LC-MS analysis of YTO revealed 21 compounds.^{17,18,20–23} The result indicated that YTO contained compounds in the following order: *G. glabra* > *C. burmannii* > *S. aromaticum* > *C. bejolghota* = *A. testaceum*. Hydrophilic compounds, such as proanthocyanidins, phenolic acids, triterpenoid glycosides, and flavonoid glycosides, were preferentially extracted over lipophilic compounds because of the preparation method for YTO (decoction). Five easily detected and quantified compounds with hypoglycemic activity were chosen as the bioactive markers of YTO. The quantification result demonstrated that glycyrrhizic acid was the most abundant compound ($6.80 \pm 0.15\%$ w/w), followed by eugenol, coumarin, cinnamic acid, and cinnamaldehyde (Table 4). Although coumarins show hypoglycemic activity, their potential hepatotoxicity requires careful consideration. A tolerable daily intake (TDI) of 0.1 mg/kg bodyweight for coumarin has been recommended by the European Food Safety Authority (EFSA).^{50,51} The coumarin content in YTO was 0.052% w/v (based on the freeze-dried weight of 0.46 ± 0.1 g/100 mL). Therefore, the consumption of 100 mL YTO/day by adults remains within the TDI established by the EFSA.

Table 4. Identification of the Compounds in YTO.

Peak	t_R (min)	MS^2	Fragment	Molecular formula	Compound identification	Source
1	5.8	171.0 (M+H) ⁺	124	C ₇ H ₆ O ₅	Gallic acid	<i>S. aromaticum</i> ¹⁷
2	8.6	322.4 (M) ⁺	176	C ₂₀ H ₁₈ O ₄	Glabrene	<i>G. glabra</i> ¹⁸
3	9.9	432.7 (M) ⁺	271 (Genistein)	C ₂₁ H ₂₀ O ₁₀	Genistin	<i>G. glabra</i> ¹⁹
4	10.4	302.0 (M) ⁺	294, 214	C ₁₄ H ₆ O ₈	Ellagic acid	<i>S. aromaticum</i> ¹⁷
5	11.6	416.3 (M) ⁺	254.8 (Daidzein)	C ₂₁ H ₂₀ O ₉	Daidzin	<i>G. glabra</i> ¹⁹
6	13.0	269.7 (M+H) ⁺	243.8, 179.2	C ₁₆ H ₁₂ O ₄	Formononetin	<i>G. glabra</i> ¹⁹
7	15.5	355.2 (M+H) ⁺	293.8	C ₁₆ H ₁₈ O ₉	Biflorin	<i>S. aromaticum</i> ¹⁷
8	15.5	579.9 (M+H) ⁺		C ₂₇ H ₃₀ O ₁₄	Isoviolanthin	<i>G. glabra</i> ²⁰
9	18.3	377.8 (M+H) ⁺	334.7, 310.3, 248.9	C ₂₀ H ₂₄ O ₇	(7S/R,8R)-1-(4-hydroxy-3-methoxyphenyl)-2,4-[(E)-3-hydroxy-1-propenyl]-2-methoxyphenoxyl-1,3-propanediol	<i>C. bejolghota</i> ²¹
10	19.2	146.6 (M) ⁺	-	C ₉ H ₆ O ₂	Coumarin	<i>C. burmanni</i> ²²
11	19.2	148.7 (M) ⁺	-	C ₉ H ₈ O ₂	Cinnamic acid	<i>C. burmanni</i> ²²
12	19.6	471.9 (M+H) ⁺	453	C ₃₀ H ₄₆ O ₄	Glycyrrhetic acid	<i>G. glabra</i> ¹⁸
13	19.6	132.4 (M) ⁺	-	C ₉ H ₈ O	Cinnamaldehyde	<i>C. burmanni</i> ²²
14	26.7	468.8 (M) ⁺	449.4	C ₃₀ H ₄₄ O	Glabrolide	<i>G. glabra</i> ¹⁸
15	28.2	418 (M) ⁺	401.7, 331.8	C ₂₂ H ₂₆ O ₈	Syringaresinol	<i>C. burmanni</i> ²²
16	28.2	164.9 (M) ⁺	111.8	C ₁₀ H ₁₂ O ₂	Eugenol	<i>S. aromaticum</i> ¹⁷
17	34.4	339.7 (M+H) ⁺	297.9	C ₂₁ H ₂₂ O ₄	Licochalcone A	<i>G. glabra</i> ¹⁸
18	35.9	369.4 (M+H) ⁺	330.3, 285.4, 129.2	C ₂₁ H ₂₀ O ₆	Licoaryl coumarin	<i>G. glabra</i> ¹⁸
19	40.2	162.9 (M) ⁺	134.3	C ₁₀ H ₁₀ O ₂	Methoxycinnamaldehyde	<i>C. burmanni</i> ²²
20	41.9	426.8 (M) ⁺	402.5, 286.3	C ₂₉ H ₄₆ O ₂	Stigmas-4-ene-3,6-dione	<i>A. testaceum</i> ²³
21	51.8		575.5, 533.3, 287.6		A type proanthocyanidin	<i>C. burmanni</i> ²²

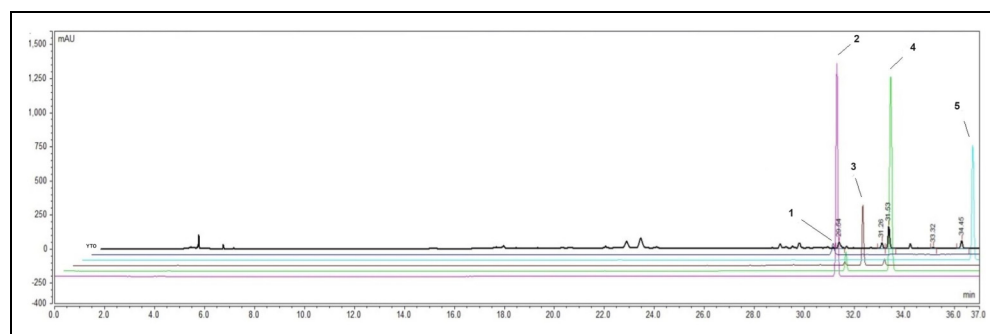


Figure 8. HPLC chromatogram of YTO compared to the standard peaks.

1 coumarin at 29.6 min, 2 cinnamic acid at 31.2 min, 3 glycyrrhizic acid at 31.5 min, 4 cinnamaldehyde at 33.3 min, and 5 eugenol at 34.4 min.

Table 5. Linearity, Range, LOD, LOQ, and Contents of the Bioactive Markers in YTO.

Compound	Slope (A)	Intercept (B)	R ²	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Content (% w/w)
Coumarin	0.9286	0.1548	0.9992	0.6-30.0	0.22	0.68	0.11 ± 0.00
Cinnamic acid	2.5612	0.4471	0.9964	0.1-3.6	0.11	0.33	0.03 ± 0.00
Glycyrrhizic acid	0.0432	0.0306	0.9998	10.0-150.0	0.37	1.12	6.80 ± 0.15
Cinnamaldehyde	1.0614	0.0909	0.9995	0.1-8.0	0.55	1.66	0.02 ± 0.00
Eugenol	0.1466	0.0713	0.9995	1.0-120.0	0.02	0.07	0.81 ± 0.01

Abbreviations: LOD, Limit of detection; LOQ, Limit of quantitation.

Conclusion

In the present study, YTO was prepared using standardized raw materials, and the mixture was subsequently evaluated for its hypoglycemic activity and quality. The hypoglycemic results indicated that this mixture inhibited the activity of α -amylase and α -glucosidase. YTO induced insulin release and increased glucose uptake in cell-based diabetes mellitus assays. LC-MS analysis demonstrated that YTO contained flavonoids, phenolic compounds, lignans, proanthocyanidins, and terpenoids. The bioactive markers glycyrrhizic acid, eugenol, coumarin, cinnamic acid, and cinnamaldehyde were analyzed using HPLC. The coumarin content in 100 mL of YTO complied with the TDI set by the EFSA for use in adults. These findings indicated the therapeutic potential of YTO as a safe and effective herbal medicine for managing hyperglycemia, a critical factor in diabetes mellitus and its associated complications. Nonetheless, this study was based on cell culture analyses, limiting its applicability to humans. Future work should include *in vivo* studies to verify the efficacy and safety of YTO. If positive results are obtained, clinical trials can be used to assess its effectiveness and adverse effects in human patients. Altogether, this research provides a significant foundation for further studies on YTO use in diabetes mellitus management.

Authors' Contributions

Conceptualization: Thaweesak Juengwatanatrakul (TJ), Kusuma Jitsaeng (KJ), Rawiun Kaewamatawong (RK). Investigation: TJ, KJ, RK. Formal analysis: KJ, RK. Project administration: TJ. Writing—original draft: TJ, KJ, RK. Writing—review and editing: RK.

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Data Availability Statement

Voucher specimens with reference numbers TOB-UBU-1-10 are deposited at the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University. All data generated in the current research work have been included in the manuscript.

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Supplemental Material

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