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Phytochemical Screening and Biological Activities of a Remedy from A-thi-sa-ra-wak Scripture as a Folkloric Diabetic Medicine

Piyapong Yupparach[1], Adisak Sumalee[2], Ampa Konsue[1]*

[1]*Thai Traditional Medicinal Research Unit, Division of Applied Thai Traditional Medicine, Faculty of Medicine, Mahasarakham University, Maha Sarakham, 44000, Thailand*
[2]*Health Science Program, Sukhothai Thammatirat Open University, Nonthaburi, 11120, Thailand*

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ABSTRACT

The A-thi-sa-ra-wak scripture is a folkloric Thai traditional medicinal literature containing 30 remedies for a variety of diseases. The sixth remedy in the scripture consists of *Ficus religiosa, Smilax china, S. glabra,* and *Tectona grandis* (FST), which are designated specifically for the treatment of diabetes. This study was conducted to investigate the phytochemical contents and biological activities of a remedy from the thi-sa-ra-wak scripture. Constituent plants of the remedy were extracted with aqueous (FSTA), 50% ethanol (FSTHE), and 95% ethanol (FSTE). The phytochemical screening was performed to determine total phenolic (TPC) and flavonoid (TFC) contents. The antioxidant activity were examined using the 2,2-diphenyl-1-picrylhydrazy (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{+}$) radical scavenging, and ferric reducing antioxidant power (FRAP) assays. Based on the inhibition of the α-glucosidase and α-amylase enzymes, the hypoglycemic mechanism was identified. The results of the study showed that the FSTHE had a significantly ($p < 0.05$) higher concentration of TPC, while the FSTE had a higher concentration of TFC. The FSTHE was more potent on DPPH and ABTS, but the FSTA had reducing power on FRAP. The FSTA had more potent α-glucosidase inhibition, while the FSTHE showed an effect on α-amylase inhibitory activity. Furthermore, the remedy had a significant ($p < 0.05$) effect on the suppression of the α-glucosidase and α-amylase enzymes than acarbose, a common anti-diabetic medication. The findings of this study reveal that the biological activities of the remedy contributed to the antioxidation, α-glucosidase, and α-amylase enzyme inhibitions, thereby confirming the use of the remedy as a diabetic drug.

Keywords: Antioxidation, Diabetes, Flavonoid, Phenolic compound, α-glucosidase, α-amylase

Introduction

Diabetes mellitus (DM), more commonly known as diabetes, is a serious, long-term condition that develops when the body cannot produce any or enough of insulin or cannot effectively use the insulin it makes, resulting in elevated blood glucose levels. The 10th edition of the International Diabetic Federation's report confirms that diabetes is one of the 21st century's fastest-growing global health emergencies. Diabetes is estimated to affect 537 million people worldwide in 2021 and is projected to reach 643 million by 2030 and 783 million by 2045. Additionally, it is anticipated that 541 million individuals will have reduced glucose tolerance by 2021. Over 6.7 million people between the ages of 20 and 79 are anticipated to die in 2021 as a result of diabetes-related diseases.[1] The majority of the causes of DM are associated with carbohydrate metabolism and imbalance in insulin function due to insufficiency or defective receptors. It is the most prevalent non-communicable disease, primarily characterized by high blood glucose levels, polyphagia, polydipsia, polyuria, and others.[2] One of the main causes of DM is oxidative stress.

*Corresponding author. E mail: ampa.k@msu.ac.th
Tel: +66 43 712992

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It is a characteristic of many human diseases and is the result of the uncontrolled generation and accumulation of reactive oxygen (ROS) and nitrogen species (RNS). The management of different physiological and pathological processes that occur in the human body is linked to oxidation, which can also aid in the defense against pathogens and pathogen-induced infections. Given the cytotoxicity of excessive ROS/RNS, this oxidation process is often strictly controlled by the cellular antioxidant defense. Antioxidants and free radical scavengers are chemical components of plants that can prevent or reduce oxidative organ damage.[3]

Starch and carbohydrates are the most important food sources for humans worldwide, while α-amylase and α-glucosidase are the two primary enzymes involved in the digestive system. The pancreatic α-amylases and α-glucosidase are digestive enzymes that hydrolyze carbohydrates to oligosaccharides, disaccharides, and glucose. A high blood glucose level will result following glucose absorption into the blood vessel system.[4] Increasing research suggests that structural characteristics of the particular molecules involved may more adequately explain the inhibition, even though secondary metabolites may suggest a higher potential inhibition against α-amylase and α-glucosidase. This effect reduces the metabolism of carbohydrates, which lowers blood glucose levels.[5]

Traditional Thai medicine (TTM) is folk medicine that was passed down from Thai ancestors. Medications used for healing in the past and present situation include a variety of plants, dosages, herbal components, and indications for illness treatment.[6] The A-thi-sa-ra-wak scripture, which contains 30 formulas for disease remedies, was composed in 1915 by Prince Abhakara Kiartivongse (Prince of Chumphon). The sixth recipe from the scripture consists of *Ficus religiosa* leaf, *Smilax china* rhizome, *S. glabra* rhizome, and *Tectona*

grandis wood, particularly for diabetic treatment. However, each plant in this recipe had certain phytochemical constituents and biological activities that had been reported, but none of these have been scientifically proven.

Thus, the present study was conducted to screen for phytochemicals and evaluate the antioxidant activities of the remedy extracts from A-thi-sa-ra-wak scripture. Also, the *in vitro* inhibitory effects of the extracts on *α*-glucosidase and *α*-amylase enzymes were investigated.

Materials and Methods

Plant collection

The plants in the recipe were collected from the local areas in Thailand. *Ficus religiosa* leaf and *Tectona grandis* wood were obtained from Maha Sarakham Province from March to June 2020. Rhizomes of *Smilax china* and *S. glabra* were collected from Lum Phun province. The plant materials were identified and deposited at the Faculty of Medicine, Mahasarakham University, Thailand. They were assigned voucher specimen codes (*F. religiosa* MSU.MED-FR0001/AK, *T. grandis* MSU.MED-TG0001/AK, *S. china* MSU.MED-SC0001/AK, and *S. glabra* MSU.MED0001/AK). The whole plant materials were cleaned and dried at 60°C for 48 h in a hot air oven, then pulverized.

Preparation of plant extracts

The aqueous extract (FSTA) of the recipe was prepared by boiling 100 g of *F. religiosa, S. china, S. glabra,* and *T. grandis* (1.2:1:1:1.13 w/w) twice in 1 L of distilled water for 10 min. The hydro-ethanolic (FSTHE) and ethanolic extracts (FSTE) were macerated with 50% ethanol and 95% ethanol, respectively, for 7 days (100 g per 400 mL). Using filter papers, the powder from each residue was eliminated (Whatman, Germany). The filtrate was evaporated using a rotary evaporator (Heidolph Laborota 4000, Germany) and freeze-dried to obtain a dark red-brown extract, which was stored at -20°C until it was needed.[7]

Total phenolic content assay

Total phenolic content was determined according to a modified procedure. The sample (100 µL) was oxidized with 500 µL of 0.2 N Folin-Ciocalteu's reagent and neutralized by adding 400 µL of 7.5% Na_2CO_3. The absorbance was measured at 765 nm after being mixed and incubated at room temperature for 30 min. The results were expressed as gallic acid equivalents (mgGE/gExt).[7]

Total flavonoid content assay

Flavonoid content was estimated using the aluminum chloride colorimetric method. The extract from the recipe was mixed with 100 µL of 5% aluminum chloride (w/v), and 400 µL of 2.5% Na_2NO_3. After 5 min, 500 µL of 5% $AlCl_3$ was added. The solution was allowed to stand at room temperature for 10 min and then mixed with 2,000 µL distilled water. At 415 nm, the absorbance was measured. The TFC was calculated from a standard quercetin equivalent (mgQE/gExt).[7]

DPPH radical scavenging assay

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacities of wheat extracts were calculated using the reduction of the reaction color between the DPPH solution and the sample extracts, as previously indicated by the prior approach.[6] In ethanol, 0.039 mg/mL of DPPH was dissolved. The extracts were diluted with distilled water at various concentrations to obtain sample solutions. One hundred milliliters (100 mL) of the sample solution was added to 900 mL of the working DPPH (0.1 mM) solution and then incubated in the dark for 30 minutes at room temperature. Then, the absorbance of the solution was measured at 515 nm. Ascorbic acid and Trolox® were employed as reference materials in this investigation. In each experiment, blanks were used. The DPPH radical ability was expressed as IC_{50} (mg/mL) and the inhibition percentage was calculated using the following formula:

% radical scavenging activity of DPPH = [$(A_0-A_1)/ A_0$] x 100

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.[6]*ABTS*$^+$

Radical scavenging assay

In the ABTS assay, the recipe extract was allowed to react with $ABTS^+$, a model stable free radical derived from 2,2-azinobis (3-ethylvenzothiazolin-6-sulphonic acid) ($ABTS^+$). The $ABTS^+$ (900 µL) was added to the extract (100 µL) and thoroughly mixed. After 6 minutes at room temperature, the absorbance was immediately measured at 734 nm. The same procedures were used to prepare and analyze the Trolox® and ascorbic acid solutions in 80% ethanol. The ABTS scavenging ability was expressed as IC_{50} (mg/mL) and the inhibition percentage was calculated using the following formula:

% radical scavenging activity of ABTS = [$(A_0-A_1)/ A_0$] x 100

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.[6]

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity of the recipe was estimated spectrophotometrically following the procedure described by Puangpornpitag *et al*.[8] The ferric reducing ability of the extract was measured at low pH. A sample (100 µL) was oxidized with 900 µL of FRAP reagent (300 mM acetate buffer [pH 3.6]; 10 mM tripyridyl triazine [tptz] solution; and 20 mM ferric chloride solution in ratio 10:1:1). An intense blue-colored complex was formed when the Fe^{3+}-TPTZ complex was reduced to the ferrous (Fe^{3+}) form. The absorbance at 593 nm was recorded. The reducing power of the samples increased with the absorbance values. The concentrations of $FeSO_4$ were then plotted against the concentration of the standard antioxidant, Trolox®. The FRAP values were obtained by comparing the absorbance change in the test solution with those obtained from increasing concentrations of Fe^{3+} and were expressed as mg of Trolox® equivalent per gram of sample.

α-glucosidase inhibitory assay

Using an *in vitro* assay, all extracts were tested for their ability to inhibit *α*-glucosidase. The assay method was assessed with slight modifications.[9] In brief, a volume of 120 µL of the sample solution and 100 µL of 0.1 M phosphate buffer (pH 6.8) containing *α*-glucosidase solution (0.2 U/mL) were incubated at 37ºC for 20 min. After pre-incubation, 100 µL of 5 mM *p*-nitrophenyl-*α*-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37°C for 20 min. Then, the reaction was stopped by adding 320 µL of 0.2 M Na_2CO_3 into each well, and absorbance was measured at 405 nm using a UV-Vis spectrophotometer and compared to a control that had 120 µL of buffer solution in place of the extracts. The system without *α*-glucosidase was used as a blank, and acarbose was used as a positive control. The *α*-glucosidase inhibitory activity was expressed as % inhibition and was calculated as follows:

% inhibition of *α*-glucosidase = [$(A_0–A_1)/A_0$] x 100

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. IC_{50} values were calculated by the graphical method.

α-amylase inhibitory assay

The *α*-amylase inhibitory activity was carried out following the standard protocol with slight modifications.[9] The 20 µL of each recipe extract was mixed with 180 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing *α*-amylase solution (3 units/mL). After incubation at 25°C in a water bath for 10 min, 500 µL of 1% (w/v) starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added, and the mixture was reincubated at 25°C in a water bath for 10 min. The reaction was terminated with 500 µL of color reagent (1% w/v of 3,5-dinitrosalicylic acid). Then, the mixture was incubated in a boiling water bath for 10 min. Subsequently, 500 µL of 40% tartrate solution was added to each tube after boiling, and before cooling to room temperature. The mixture was diluted with 100 mL of distilled water. Absorbance was measured at 540 nm with a UV-visible spectrophotometer. The absorbance reading was compared to the control, which contained 500 µL of buffer solution, instead of the extract (A540 control). The *α*-amylase

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inhibition was expressed as % inhibition and was calculated as follows:

% inhibition of α-glucosidase = $[(A_0–A_1)/A_0] \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. IC_{50} values were calculated by the graphical method.

Statistical analysis
All data were expressed as mean ± standard deviation of the mean (SD) from five separate experiments (n = 5). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences at $p < 0.05$ were considered to be significant.

Results and Discussion

In this study, the sixth remedy was selected to confirm its use as a diabetic treatment based on some phytochemical composition and biological activities. The results revealed that all of the extracts from the extraction processes had a dark red-brown color. The percentage yield was significantly ($p < 0.05$) higher for the FSTHE and FSTE than for the FSTA (Table 1). The level of TPC in the FSTHE was significantly ($p < 0.05$) higher than that in the FSTE and FSTA, while the FSTE demonstrated significantly ($p < 0.05$) higher TFC content than the FSTHE and FSTA as shown in Table 2. In the past, people brought natural remedies to every region to treat a variety of diseases. Natural resources, geography, development, way of life, and culture vary in each region of the world. As a result, each region may have a different approach to treating illnesses. Traditional Thai medicine is a type of healing method derived from ancient Thai wisdom that has been used since antiquity.[9] The sixth remedy is in the A-thi-sa-ra-wak scripture that listed the components of medicinal plants, as well as their uses and dosages for treating diabetes. The author, the 28th child of King Chulalongkorn of Thailand was Prince of Chumphon (Dec 19, 1880-May 19, 1923). He was commonly revered as Doctor Phon and The Father of the Thai Navy in 1993. The recipe described that the plant composition consisted of *F. religiosa* leaf (19 leaves), *S. china* rhizome (15 g), *S. glabra* rhizome (15 g), and *T.grandis* wood (15 g). All plants were boiled for approximately 30 min with drinking water.

Table 1: The percentage yield and color of various extracts from the remedy

Sample	% yield	Color
FSTA	0.84 ± 0.004^a	dark red-brown
FSTHE	3.05 ± 0.021^b	dark red-brown
FSTE	2.97 ± 0.012^b	dark red-brown

FSTA: Aqueous extract; FSTHE: 50% ethanolic extract; FSTE: 95% ethanolic extract; n = 5; Different letter in each column indicates a significant difference at *p*-value less than 0.05.

Table 2: The total phenolic (TPC) and flavonoid (TFC) contents of various extracts from the remedy

Sample	TPC (mgGE/gExt)	TFC (mgQE/gExt)
FSTA	2.027 ± 0.335^c	116.160 ± 0.511^c
FSTHE	11.218 ± 0.076^a	132.248 ± 0.749^b
FSTE	6.461 ± 0.071^b	150.870 ± 0.956^a

FSTA: Aqueous extract; FSTHE: 50% ethanolic extract; FSTE: 95% ethanolic extract; n = 5; TPC was measured with gallic acid equivalents (mgGE/gExt); TFC was measured with quercetin equivalent (mgQE/gExt); Different letter in each column indicates a significant difference at *p*-values less than 0.05.

A teacup (75 mL) of the prescribed medication for diabetics should be consumed three times daily before meals.
Common plants were found to produce secondary metabolites such as phenolic compounds or flavonoids, which act as the body's defense against infections, pests, and UV radiation.[10] The results of the phytochemical analysis showed that the remedy also contained TPC and TFC, especially TFC originating from a mixture of plants used in the recipe that possess flavonoid-like phytochemical components. The *S. china* was isolated with some phenolic acid, chlorogenic acids, flavonoids, stilbene, and phenylpropanoid glycoside. Their structures were elucidated using nuclear magnetic resonance and mass spectrometry, according to the review articles on each plant in the recipe.[11] A study that suggested *S. china* had phenolic compounds later proved it through the use of high-performance liquid chromatography analysis, with some of the phenolic compounds acting as free radical scavengers. It is made up of phenolic substances such as tannins, flavonoids, and derivatives. There are many positions where substances could be antioxidizing hydroxyl groups, especially flavonoids, one of the classes of phenolic chemicals present in plants, which were widely distributed throughout the plant kingdom.[12] The secondary metabolites TPC and TFC have molecular structures that could chelate electrons donated to free radicals, promoting, protecting, healing, and rehabilitating healthy people. The chemical structure of the phenolic and flavonoid components is optimal for their ability to block free radical activity. They can chelate metal ions and have high reactivity as hydrogen or electron donors.[13]
In comparing all the extraction techniques, the FSTHE from the recipe had much better antioxidant activity on DPPH and ABTS. The recipe was still not as effective as the beneficial components when compared to the standard substances (ascorbic acid and Trolox®). When compared to other extraction techniques, the FSTA exhibited significantly ($p < 0.05$) higher Trolox® equivalent in the FRAP method (Table 3). The antioxidant activities of the remedy might be related to its flavonoid and phenolic contents.[14] The phenolic compounds changed the color of these stable radicals into colorless or pale yellow in the DPPH radical scavenging assay. This color change was caused by the conversion of DPPH radicals into diamagnetic molecules by receiving electrons or hydrogen radicals.[15]
Phenolic compounds possess antioxidant properties, and their mechanism of action may be described as single electron transfer (SET) or hydrogen atom transfer (HAT). The phenolic and polyphenolic compounds can scavenge radicals, including reactive oxygen species (ROS). Moreover, phenolic compounds continue to inhibit the oxidative stress of LDL-cholesterol and DNA damage, which are related to cardiovascular diseases and cancer initiation.[16]
The antioxidant activity and protective effects of *S. china* root against oxidative DNA damage were confirmed in some study reports including plant combinations, and a link between such activities and the presence of phenolic chemicals in plants was established. DPPH and ABT radicals have been successfully eliminated by *S. china* root. The antioxidant activities were associated with some phenolic compounds and flavonoids.[12]
As shown in Table 4, the FSTA had a more inhibitory effect on α-glucosidase enzyme, while the FSTHE affected *α*-amylase inhibitory activity. Additionally, all techniques of extraction from the remedy had higher levels of α-glucosidase and *α*-amylase enzyme inhibition than a diabetic drug, Acarbose®. The two main enzymes in the management and prevention of diabetes are α-glucosidase and *α*-amylase. Natural compounds, such as phenolic or flavonoid compounds, had fewer side effects than medications.[16] The present study demonstrated that the phenolic and flavonoid components from the remedy can inhibit the two digestive enzymes (*α*-glucosidase and *α*-amylase). Due to their possible anti-hyperglycemic or anti-obesity effects, these enzymes should be taken into consideration as potential functional components in recipes.
A significant correlation may have existed between TPC and TFC in the inhibition of α-glucosidase and *α*-amylase enzymes. Oral administration of *T. Grandis*, one of the plants in the remedy, at doses of 2.5 and 5 g/kg for 30 days resulted in a significant ($p < 0.05$) reduction in blood glucose levels.

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Table 3: The antioxidant activities of various extracts from the remedy

Sample	DPPH (IC_{50} in mg/mL)	ABTS (IC_{50} in mg/mL)	FRAP (mgTE/gExt)
FSTA	0.304 ± 0.037^{e}	0.058 ± 0.002^{d}	2615.498 ± 13.177^{a}
FSTHE	0.031 ± 0.001^{b}	0.052 ± 0.003^{c}	1060.129 ± 8.922^{c}
FSTE	0.134 ± 0.002^{d}	0.099 ± 0.001^{e}	1227.172 ± 11.250^{b}
Ascorbic acid	0.0002 ± 0.0007^{a}	0.007 ± 0.0001^{a}	-
Trolox®	0.0516 ± 0.0003^{c}	0.016 ± 0.0003^{b}	-

FSTA: Aqueous extract; FSTHE: 50% ethanolic extract; FSTE: 95% ethanolic extract; n = 5; DPPH: 2,2-diphenyl-1-picrylhydrazy; ABTS: 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate); FRAP: Ferric reducing antioxidant power; The DPPH radical scavenging, $ABTS^{+}$, and FRAP assays were performed using Trolox® and ascorbic acid as standard substances; Different letter in each column indicates a significant difference at *p*-values less than 0.05.

Table 4: The *α*-glucosidase and *α*-amylase inhibitory activities of various extracts from the remedy

Sample	*α*-glucosidase IC_{50} (mg/mL)	*α*-amylase IC_{50} (mg/mL)
FSTA	0.113 ± 0.003^{a}	0.049 ± 0.013^{b}
FSTHE	0.159 ± 0.010^{b}	0.017 ± 0.006^{a}
FSTE	0.271 ± 0.032^{d}	0.056 ± 0.011^{c}
Acarbose®	0.195 ± 0.018^{c}	0.173 ± 0.026^{d}

FSTA: Aqueous extract; FSTHE: 50% ethanolic extract; FSTE: 95% ethanolic extract; n = 5; Acarbose® was used as a standrad substance; Different letter in each column indicates a significant difference at *p*-values less than 0.05.

The anti-hyperglycemic mechanism of *T.grandis* extract may be due to the regeneration of islets' β-cells following destruction by alloxan, as the extract shows a significant reduction of blood glucose levels in 15 and 30 days, an effect similar to that of the diabetic drug glibenclamide.[17] Natural products containing phenols or flavonoids may help to lower postprandial hyperglycemia. Before being absorbed in the intestine, they must be broken down into monosaccharides. This is mainly attributed to the action of carbohydrate hydrolyzing enzymes, including α-glucosidase and *α*-amylase enzymes.[18] The mechanism underlying the antidiabetic effect of *S. china* produces antidiabetic effects due to *α*-glucosidase inhibition by examining glucose uptake in HepG2 cell culture. In addition, the plant's chemical composition, which includes TPC and TFC, may inhibit *α*-glucosidase and *α*-amylase, improving the enzymes of the digestive system and preventing diabetes.[16]

Conclusion

The findings of this study reveal that TPC and TFC are the main components of the remedy. The assays on biological activities demonstrate the potent antioxidant activities, *α*-glucosidase, and *α*-amylase enzyme inhibition properties of the remedy. It was confirmed that diabetes could be remedied by hypoglycemia activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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