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Phytochemical screening, total phenolics, total flavonoids, total tannins, antioxidant activities, and α-glucosidase inhibition of ethanolic leaf extracts from 37 cultivars of *Antidesma puncticulatum* Miq.

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ABSTRACT

Introduction: *Antidesma puncticulatum* Miq., commonly known as Mao-Luang in Thailand, is a medicinal plant widely renowned for its abundance of bioactive compounds with diverse pharmacological properties. This study investigated the phytochemical components, antioxidant potentials, and α-glucosidase inhibitory activity of ethanolic leaf extracts from 37 cultivars of *A. puncticulatum*.

Methods: The leaves of 37 different varieties of *A. puncticulatum* were dried, finely ground, and then extracted using ethanol. The phytochemical screening, total amounts of phenolics, flavonoids, and tannins of the extracts were assessed. To evaluate their antioxidant potential, the extracts were subjected to the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and the ferric reducing antioxidant power (FRAP) Assays. In addition, standard *in vitro* methods were used to determine the α-glucosidase-inhibitory activity of the extracts.

Results: Five phytochemical classes were detected in high concentrations in all of the extracts. Total phenolics, flavonoids, and tannins contents were in the range of 9.86-26.75 mg gallic acid equivalent (GAE)/g dry extract, 8.10-16.18 mg catechin equivalent (CE)/g dry extract, and 14.83-33.67 mg CE/g dry extract, respectively. According to DPPH, ABTS, and FRAP assay results, the antioxidant activities of the extracts were 5.10-9.07, 0.73-2.12, and 2.06-4.01 mg ascorbic acid equivalent antioxidative capacity (AEAC)/g crude extract, respectively. Interestingly, the crude extracts from *A. puncticulatum* were more effective as α-glucosidase inhibitors than acarbose, with IC_{50} values ranging from 1.26 to 25.07 µg/mL.

Conclusion: The ethanolic leaf extracts of *A. puncticulatum* were potent sources of phytochemicals with antioxidant and antidiabetic properties. Therefore, more research is needed to substantiate their potential applications as therapeutic remedies for diabetes.

Implication for health policy/practice/research/medical education:
The extract of *Antidesma puncticulatum* leaves contained phytochemicals that possessed strong antidiabetic activity as confirmed by the low IC_{50} value tested by α-glucosidase inhibition assay compared to acarbose. Thus, they could be served as promising natural treatments for diabetic mellitus.

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Introduction

Diabetes mellitus (DM) refers to a chronic disease, which is diagnosed by a high level of blood glucose. Long-term hyperglycemia can lead to cardiovascular disease, retinopathy, neuropathy, and nephropathy. Based on research on pancreatic cell cultures, it is now known that Sars-Cov-2 causes diabetes by damaging the cells in the pancreas and liver that control blood glucose (1). By 2050, the World Health Organization (WHO) predicts that 22% of the world's population will have diabetes (2). There are various forms of DM, one of which is type 2 diabetes mellitus (T2DM), which is characterized by both impaired glucose-mediated insulin production and resistance to insulin action (3,4). The vital enzyme α-glucosidase accelerates the breakdown of α-glycosidic linkages in oligosaccharides and disaccharides into monosaccharides through intestinal absorption, raising blood glucose levels. Inhibiting α-glucosidase continues to be the foundation of treatment for post-prandial hyperglycemia (5). In order to lower hyperglycemia in diabetic patients, a number of synthetic drugs, including acarbose and other α-glucosidase inhibitors are used; however, they may have adverse reactions, including fatal hypoglycemia, lactic acidosis, idiosyncratic liver cell injury, persistent neurological impairment, and even death (6). Plants have long been used for diabetes treatment by blocking α-glucosidase. These plants have built-in antioxidants that fight against the body's excessive levels of reactive oxygen species (ROS) in order to reduce the dangers of T2DM pathogenesis and progression (7). In order to treat T2DM effectively, cheaply, and with minimal side effects, it is crucial to investigate α-glucosidase inhibitors produced by several plant species. Phytochemicals such as phenolic and flavonoid substances have gained attention as alternatives to synthetic compounds according to their health benefits such as antidiabetic and antioxidant properties. Phenolic compounds present in plant materials have been reported to be safer and some of them show stronger antioxidant activity compared to synthetic substances. Thus, there are great potentials for these phytochemicals to be applied for pharmacological uses (8).

Antidesma puncticulatum Miq. (also called in Thai as Mao-Luang) is a fruit tree that is a member of the Phyllanthaceae family (synonyms, *A. bunius* (L.) Spreng. var. *thwaitesianum* (Mull Arg.) Trim. and *A. thwaitesianum* Mull.Arg.). It is widely distributed in tropical Asia, Africa, Australia, and Pacific Island countries (9). High levels of nutritional components, including anthocyanins, flavonoids, phenolic acids, carbohydrates, organic acids, proteins, and minerals, can be found in *A. puncticulatum* fruits (10). These bioactive substances exhibit several pharmacological characteristics, including antioxidant, antibacterial, anti-inflammatory, antidiabetic, and anticancer properties (11-13). *A. puncticulatum* seeds and marcs, which are residues from processing food products, revealed antioxidant activity and had an antihypertensive impact in hypertensive rats (14). An extract of *A. puncticulatum* wood induced apoptosis of breast cancer cell lines (MDA-MB-435) (15). According to literature, *A. puncticulatum* leaves have been used to treat syphilis, snakebites, and skin conditions. In addition, they can help with indigestion, coughing, and stomachaches; they also exhibit hepatotoxic and hepatoprotective effects (16,17). According to reports, the leaves of *A. puncticulatum* from the Philippines helped control blood sugar levels (18). The methanolic extracts of *A. puncticulatum* leaves demonstrated antidiabetic activity and significant suppression of NO generation in BV2 cells and LPS-stimulated RAW264.7 macrophages (19,20). Additionally, the ethanolic leaf extract of *A. puncticulatum* contains sources of antioxidants that have been proven to exhibit α-glucosidase inhibitory activity (18,21,22).

In the northeast of Thailand, *A. puncticulatum* is widely grown, particularly in the Phu Phan National Park, Sakon Nakhon province, which is recognized for its variety of natural resources, including the diversity of *A. puncticulatum* cultivars (23). Numerous studies have been conducted on *A. puncticulatum* cultivars in various plant parts, including fruits, seeds, and pomace (14,24-27). Among other parts, *A. puncticulatum* leaves are still underutilized and under-explored. Research on the biological activities of *A. puncticulatum* leaves could provide a primary information for their further utilizations and help enhance agricultural sustainability for the fruit farmers. So far, there is little information available regarding the phytochemical compounds, antioxidants, and anti-glucosidase inhibitory activities in the leaves of the various *A. puncticulatum* cultivars (21,28). Knowledge of the leaves of some *A. puncticulatum* cultivars is still neglected and needs to be fulfilled. Therefore, the purpose of the current study was to investigate the phytochemical components, total phenolics, total flavonoids, and total tannin levels in the ethanolic leaf extracts of 37 *A. puncticulatum* cultivars harvested in northeastern Thailand. The antioxidant and α-glucosidase inhibition capacities of these extracts were also studied. The information from this study could serve as the basis for understanding the phytochemicals present in various *A. puncticulatum* leaf cultivars as well as their potential as natural antioxidant and antidiabetic agents.

Materials and Methods

Chemicals

Acarbose, aluminum chloride, chloroform, 2,2-diphenyl-1-picrylhydrazyl, ethanol, Folin-Ciocalteu reagent, ferric chloride, methanol, potassium iodide, 2,4,6-Tri(2-pyridyl)-*s*-triazine, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), *p*-nitrophenolglucopyranoside (pNPG), sodium carbonate, and sodium hydroxide were purchased from Sigma Aldrich (USA).

Plant materials

Fresh leaves of 37 *A. puncticulatum* cultivars were collected from Phu Phan National Park, Sakon Nakhon province, Thailand. With the support of Assistant Professor Dr. Sudarath Sakhunkhu, a botanist in the Department of Plant Science at the Rajamangala University of Technology Isan, Thailand, the samples were confirmed using taxonomic features. These plant cultivars were placed for future use in the Rajamangala University of Technology Isan Herbarium, Faculty of Natural Resources, with herbarium numbers ranging from M01 to M037. The leaves were thoroughly cleaned, then dried for 48 hours at 50 ˚C in an oven (Binder FD53, USA). Then, they were finely ground at 22000 rpm using an electrical grinder (Waring 8011BU, USA) and kept in sealed containers for the next experiments.

Extraction procedure

The crude plant extracts of *A. puncticulatum* were prepared by the maceration method according to the method modified from Dechayont et al (21). Dried leaf powder from each cultivar (200 g) was macerated for seven days in 95% ethanol (800 mL) at ambient temperature. The filtration was performed using Whatman's No. 1 filter paper. Then, the extracts were dried at 50 ^{0}C using a rotary evaporator (Buchi R205, Switzerland). The viscous extract was further dried for 48 hours at 50 ^{0}C in an oven. The dried extracts were collected in air-tight containers and kept at 0 ˚C for the next experiments.

Phytochemical screening

Test for alkaloids (Wagner's test)

Each crude extract (0.1 g) was dissolved in 1% (v/v) hydrochloric acid (5 mL) and warmed in a water bath for 5 min. The extract solution was subsequently filtered. Wagner's solution was made by combining iodine (1.27 g) and potassium iodide (2 g) in 100 mL of distilled water. The Wagner's solution was added to the filtrate (2-3 drops). The presence of alkaloids was identified by brown precipitation (29).

Test for flavonoids (Shibita's reaction test)

The ethanolic extract (0.1 g) was dissolved in 50% (v/v) methanol (2 mL) and warmed in a water bath (5 minutes). A little magnesium metal was added to the solution. Then, a few drops of concentrated HCl were added. The mixture was observed for red as indicative of flavonols (30).

Test for anthraquinones (Borntrager's reaction)

Ten mL of chloroform was used to dissolve 0.1 g of the extract. The solution was heated in a water bath for 5 minutes and immediately filtered. The filtrate was allowed to cool, whereupon 10% (v/v) ammonia solution was added. Anthraquinones were identified by the presence of pink color in the upper aqueous layer (30).

Test for coumarins (NaOH test)

The ethanolic extract (0.1 g) was added to a test tube. A filter paper soaked in a 1 M sodium hydroxide solution was placed over the test tube. The test tube was submerged in a water bath for a couple of minutes. After that, the filter paper was examined under the UV light at 366 nm. The presence of coumarin in the sample was identified by the observation of yellow fluorescence (31).

Test for saponins (Frothing test)

The extract (0.1 g) was added to a test tube containing distilled water (10 mL). The mixture was mixed and warmed for five minutes in a water bath. Saponins were assumed to be present if frothing remained after warming (29).

Test for tannins (Ferric chloride test)

The sample solution was prepared by dissolving the extract (0.1 g) in distilled water (10 mL). After filtration, 5% (w/v) ferric chloride was dropped into the filtrate. Tannins could be detected by the appearance of black or blue-green color (29).

Test for steroids (Liebermann-Burchard test)

The crude extract (0.1 g) was dissolved in 10 mL of chloroform and filtered, whereupon 1 mL of acetic anhydride was added and mixed. The concentrated sulfuric acid (1 mL) was then gently filled into the test tube. The presence of green color indicated the existence of steroids (30).

Test for terpenoids (Salkowski test)

The sample solution containing 0.1 g of the extract in 10 mL of chloroform was prepared and filtered into a test tube. Then, concentrated sulfuric acid (3 mL) was added slowly. The interface of the solution was observed to be a reddish-brown color, thus confirming the presence of terpenoids (31).

Test for cardiac glycoside (Keller-Kiliani test)

The amount of 0.1 g of each crude extract was dissolved in distilled water (5 mL). Then, 2 mL of glacial acetic acid and a few drops of 1% (v/v) ferric chloride were added, respectively, whereupon concentrated sulfuric acid (1 mL) was added gently. The presence of a brown ring at the interface indicated the existence of cardiac glycoside (29).

Determination of total phenolic content

The Folin-Ciocalteu method was performed to determine the total phenolic content (32). First, 0.4 mL of plant extract solution in ethanol (4 mg/mL) and 2 mL of Folin-Ciocalteu reagent (10%) were added to test tubes and stirred for 5 minutes. Then, 1.6 mL of sodium carbonate (7.5%) was added to terminate the reaction. The solution was held for 30 minutes at ambient temperature in the

dark. A UV-visible spectrophotometer (Biochrom, Libra S12, USA) was used to determine the absorbance of the samples at 725 nm. Gallic acid standards in the concentration range of 10-100 µg/mL were used to create the calibration curve. From the standard curve, y = 0.0056x + 0.0799; R^2 = 0.9964, the phenolic content of each extract was calculated and reported as mg GAE/ g dry extract.

Determination of total flavonoid content

The total flavonoid content was examined using the aluminum chloride colorimetric method by following the method of Medini et al (33). A mixture of 1.25 mL of distilled water, 0.25 mL of sample extract solution in methanol (4 mg/mL), and 75 µL of sodium nitrite solution were combined and incubated for 6 minutes. Then, 0.15 mL of 10% aluminum chloride was added and left to react at room temperature for 5 minutes. The amount of 0.85 mL of NaOH was added to the solutions of which the final volume was adjusted to 3.0 mL. A UV-visible spectrophotometer was used to measure the absorbance at 510 nm. The experiment was carried out in triplicate. A standard of catechin with a concentration range of 10-500 µg/mL was used to create the calibration curve. From the standard curve, y = 0.0002x + 0.0289; R^2 = 0.9936, the flavonoid content was calculated and expressed as mg CE/g dry extract.

Determination of total condensed tannin contents

The method of Sun et al (34), was used to determine the total condensed tannin contents. The 50 µL of the extract solution in methanol (4 mg/mL) was mixed with 4% vanillin solution in methanol (3 mL) in test tubes covered with aluminum foil. The concentrated HCl (1.5 mL) was added to the test tube, which was kept for 15 minutes at ambient temperature. The absorbance was then measured using a UV-visible spectrophotometer at 500 nm. The experiment was carried out three times. A standard of catechin with a concentration range of 1-3.5 mg/mL was used to create the calibration curve. From the standard curve, y = 0.1476x - 0.0239; R^2 = 0.9972, the amount of tannin in each extract was calculated and expressed as mg CE/g dry extract.

DPPH radical scavenging activity

The methods described by Jiangseubchatveera et al (35) and Tan and Chan (36) were followed. The extract (75 µL) and DPPH (0.2 mM) in methanol (150 µL) were added in a 96-well microplate and kept for 30 minutes in the dark. A microplate reader (BMG Labtech, Switzerland) was used to measure the absorbance (at 520 nm). The calibration curve was prepared using ascorbic acid as a standard (concentration range 0.5-40 µg/mL). The scavenging activity of the extracts against DPPH was calculated using the following equation y = -0.0088x + 0.4582 (R^2 = 0.9982). The results were expressed in mg AEAC/ g crude extract.

2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS+) radical scavenging capacity

In accordance with the procedures outlined by Re et al (37) and Iqbal et al (29), the antioxidant activity of the extracts was determined using the ABTS radical scavenging examination. A 1:1 mixture of ABTS (7.4 mM) and potassium persulfate (2.6 mM) was used to produce a stock solution of $ABTS^+$, which was then allowed to react for 12 hours at room temperature in the dark. By adding methanol to 3 mL of stock solution in a 100 mL volumetric flask, the fresh $ABTS^+$ working solution was generated. Then, 20 µL of the extract solutions were combined with 180 µL of the $ABTS^+$ working radical solution, which was then incubated for 6 minutes at ambient temperature. The 96-well microplate reader (BMG Labtech, Switzerland) was used to measure the absorbance at 734 nm. The ABTS radical scavenging values of the extracts were calculated from the calibration curve of ascorbic acid (concentration range 1-60 µg/mL) using the following equation y = -0.0044x + 0.3124 (R^2 = 0.9978). The results were expressed in mg AEAC/ g crude extract.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to the method of Dechayont et al (38). The FRAP solution was obtained by mixing acetate buffer pH 3.6 (300 mmol/L), ferric chloride hexahydrate (20 mmol/L), and 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) (10 mmol/L) in HCl (40 mmol/L) at a ratio of 10:1:1 (v/v), respectively. After that, 180 µL of the FRAP solution was mixed with 20 µL of each extract and allowed to incubate for 8 minutes at ambient temperature. The absorbance was read at 593 nm using a microplate reader (BMG Labtech, Switzerland). The extracts were measured in triplicate. The FRAP value of the extracts was determined from the ascorbic acid standard curve (y = 0.0021x + 0.1933, R^2 = 0.9965) in the linearity range of various ascorbic acid concentrations (20, 40, 60, 80, and 100 µg/mL). The results were expressed in mg AEAC/ g crude extract.

α-Glucosidase inhibition assay

The method described by Matsui *et al.* (39) was followed; 20 µL of various concentrations of extracts (2.0 mg/mL) was added to phosphate buffer, pH 6.8 (120 µL of 10 mM) followed by the addition of 20 µL α-glucosidase (0.2 U/mL). The mixtures were incubated at 37 °C for 15 minutes. Then, 20 µL of 5mM pNPG was added to the reaction mixture and allowed to incubate at 25 °C for 15 minutes. The reaction was terminated by adding 40 µL of 1 mM sodium carbonate solution. The absorbance of the mixture was measured at 405 nm. Acarbose solutions with a concentration range of 0.03-2 mg/mL were used as a positive control. The following equation was used to calculate the percentage inhibition:

% Inhibition = {[(A-B)-(C-D)]/(A-B)}x100

where A is the absorbance of the mixture of the buffer instead of the sample, pNPG and enzyme solution; B is the absorbance of the mixture of buffer and pPNG solution without enzyme; C is the absorbance of the mixture of sample, pNPG and enzyme solution, and D is the absorbance of the mixture of sample and pPNG solution without enzyme.

Statistical analysis

Data were shown as the means and standard deviation of measurements made in triplicate. Utilizing IBM SPSS STATISICS 25.0, the ANOVA test was used to evaluate the analysis of variance. Duncan's New Multiple Range Test was performed to determine differences between the means with a level of significance of $P \leq 0.05$.

Results

Phytochemical screening

The phytochemical screening of the ethanolic leaf extracts from different cultivars of *A. puncticulatum* revealed that tannins and steroids were present in all the ethanolic leaf extracts. Terpenoids, flavonoids, and saponins were found in the extracts from some cultivars. However, alkaloids, anthraquinones, coumarin, and cardiac glycosides were not detected in the studied extracts as shown in Table 1. Moreover, the extraction yield (%) is summarized in Table 1. The % yield of the ethanolic leaf extracts ranged from 2.52% to 11.15%. M27 "Khaoyai No.2" had the highest values while M28 "Punna No.120" had the lowest % extraction yields.

Table 1. Phytochemical screening and extraction yields (%) of different ethanolic extracts of *Antidesma puncticulatum*

Sample codes	Sample names	Extraction yields (%)	Phytochemical compounds*								
			Alkaloids	Flavonoids	Anthraquinones	Coumarins	Saponins	Tannins	Steroids	Terpenoids	Cardiac glycosides
M01	Sahatsakhan	6.34	-	++	-	-	+	++	+	+	-
M02	Lompad	7.65	-	-	-	-	-	+	++	-	-
M03	DongLuang	5.24	-	-	-	-	-	++	++	-	-
M04	Sanhome	8.23	-	+	-	-	-	+	+	++	-
M05	Sanphan	4.13	-	-	-	-	-	++	++	+	-
M06	Worasarn	5.52	-	++	-	-	-	++	++	-	-
M07	Wangkhumpoon	5.23	-	-	-	-	-	++	++	-	-
M08	Sorwannarsung	6.78	-	-	-	-	-	++	+	-	-
M09	Nongtaokhum	2.15	-	-	-	-	-	++	++	-	-
M10	Kumlai	5.47	-	+	-	-	-	++	++	-	-
M11	Kru-in	5.88	-	-	-	-	-	++	++	-	-
M12	Naichan	5.54	-	-	-	-	-	+	++	+	-
M13	Tongdee	6.27	-	-	-	-	-	++	+	-	-
M14	Phuphanthong	6.65	-	-	-	-	-	++	++	-	-
M15	Phuzong	4.98	-	-	-	-	-	++	++	-	-
M16	Petnongzang	3.93	-	+	-	-	++	++	++	-	-
M17	Kangmoddang	8.53	-	+	-	-	+	+	+	++	-
M18	Faprathan	4.81	-	-	-	-	-	++	++	-	-
M19	Kalasin No.1	5.14	-	++	-	-	+	+	++	-	-
M20	Wannawong No.1	3.57	-	-	-	-	-	+	+	-	-
M21	Wannawong No.2	6.28	-	-	-	-	-	++	++	-	-
M22	Wannawong No.3	3.90	-	-	-	-	-	+	+	-	-
M23	Sang krow No.1	5.82	-	-	-	-	-	++	++	-	-
M24	Sang krow No.2	10.67	-	-	-	-	-	++	++	-	-
M25	Sang krow No.3	4.95	-	+	-	-	-	++	++	-	-
M26	Khaoyai No.1	4.02	-	+	-	-	++	++	+	-	-
M27	Khaoyai No.2	11.15	-	++	-	-	-	+	+	++	-
M28	Punna No.120	2.52	-	-	-	-	-	+	++	-	-
M29	Sangsawang	9.20	-	+	-	-	-	++	+	++	-
M30	Thepnimit	6.56	-	-	-	-	-	++	++	-	-
M31	Nongwai	5.59	-	+	-	-	-	++	+	++	-
M32	Lungkumlar	3.20	-	-	-	-	-	+	++	-	-
M33	Lungplain	4.88	-	-	-	-	-	+	++	-	-
M34	Huoibang	4.03	-	-	-	-	-	+	++	-	-
M35	Yaikumtar	4.48	-	-	-	-	-	+	++	-	-
M36	Takwai	6.56	-	-	-	-	-	+	+	-	-
M37	Chankasem	6.06	-	+	-	-	-	++	+	++	-

*Note**: The presence of chemical constituents is: (-) absent, (+) low concentration, (++) high concentration.

Total phenolic, flavonoid, and condensed tannin contents

The amounts of total phenolics, total flavonoids, and total tannins in the different ethanolic extracts of *A. puncticulatum* are presented in Table 2. M19 "Kalasin No.1" gave the highest values of total phenolic content at 26.75 ± 0.23 mg GAE/g dry extract and M31 "Nongwai" showed the lowest value of total phenolic content at 9.86 ± 0.20 mg GAE/g dry extract. Meanwhile, the highest content of total flavonoids was observed in M10 "Kumlai" (16.18 ± 0.62 mg CE/g dry extract) and the lowest level of total flavonoids was observed in M04 "Sanhome" (8.10±0.06 mg CE/g dry extract). For total condensed tannins detection, M09 "Nongtaokhum" gave the highest value at 33.67 ± 1.43 mg CE/g dry extract and M10 "Kumlai" showed the lowest value at 12.05 ± 0.78 mg CE/g dry extract. The averages of total phenolic, flavonoid, and

Table 2. Total phenolic, flavonoid, and tannin contents in different ethanolic extracts of *Antidesma puncticulatum* cultivars

Sample codes	Sample names	Total phenolic content (mg GAE/g dry extract)	Total flavonoid content (mg CE/g dry extract)	Total tannin content (mg CE/g dry extract)
M01	Sahatsakhan	10.30±0.30^{o}	9.98±0.18^{q}	26.17±1.43^{cdfg}
M02	Lompad	12.80±0.23^{m}	12.78±0.68^{efghi}	27.50±1.80^{cdef}
M03	DongLuang	14.35±0.11^{l}	8.62±0.08^{r}	25.40±1.78^{fghi}
M04	Sanhome	23.04±0.28^{c}	8.10±0.06^{r}	23.73±0.89^{ghijkl}
M05	Sanphan	12.86±0.10^{m}	11.34±0.98^{jklmnop}	21.29±2.05^{klmn}
M06	Worasarn	18.25±0.37^{h}	14.96±0.74^{bc}	30.41±2.00^{b}
M07	Wangkhumpoon	13.11±0.70^{m}	12.46±0.52^{fghijk}	21.13±1.64^{lmn}
M08	Sorwannarsung	15.54±0.15^{k}	10.46±0.96^{pq}	17.29±1.17^{o}
M09	Nongtaokhum	24.90±0.05^{b}	11.00±0.70^{klmnopq}	33.67±1.43^{a}
M10	Kumlai	18.01±0.36^{h}	16.18±0.62^{a}	12.05±0.78^{q}
M11	Kru-in	23.44±0.78^{c}	15.72±0.38^{ab}	18.22±0.59^{o}
M12	Naichan	20.97±0.26^{e}	11.54±0.52^{ijklmnop}	18.13±0.89^{o}
M13	Tongdee	14.99±0.30^{k}	10.68±0.54^{opq}	23.46±0.98^{hijklm}
M14	Phuphanthong	10.91±0.26^{n}	12.14±1.06 ^{fghijklm}	25.11±2.21^{fghij}
M15	Phuzong	19.00±0.19^{g}	13.78±0.20^{de}	24.50±0.88^{ghijk}
M16	Petnongzang	17.09±0.15^{i}	12.86±0.40^{efgh}	29.53±0.59^{abc}
M17	Kangmoddang	18.14±0.21^{h}	13.12±0.16^{efg}	25.74±0.38^{dfgh}
M18	Faprathan	19.35±0.19^{g}	13.00±0.40^{efg}	14.83±0.78^{p}
M19	Kalasin No.1	26.75±0.23^{a}	11.22±0.46^{jklmnopq}	23.71±0.38^{ghijkl}
M20	Wannawong No.1	23.28±0.31^{c}	11.92±0.20^{ghijklmno}	22.01±1.92^{jklmn}
M21	Wannawong No.2	16.30±0.13^{j}	13.38±0.32^{def}	25.99±0.75^{cdfgh}
M22	Wannawong No.3	24.86±0.54^{b}	10.76±0.48^{mnopq}	17.92±1.15^{o}
M23	Sang krow No.1	16.26±0.01^{j}	12.00±0.68^{ghijklmn}	24.32±0.98^{ghijkl}
M24	Sang krow No.2	25.32±0.26^{b}	14.92±0.92^{bc}	28.25±1.01^{abcd}
M25	Sang krow No.3	23.52±0.20^{c}	15.72±0.34^{ab}	29.69±0.81^{ab}
M26	Khaoyai No.1	23.21±0.10^{c}	11.58±0.94^{hijklmnop}	26.0.3±2.35^{cdfg}
M27	Khaoyai No.2	25.19±0.15^{b}	10.78±0.26^{mnopq}	19.69±0.89^{mno}
M28	Punna No.120	17.24±0.15^{i}	11.34±0.42^{jklmnop}	22.85±0.51^{jklmn}
M29	Sangsawang	15.17±0.11^{k}	10.88±0.60^{lmnopq}	21.29±1.17 ^{klmn}
M30	Thepnimit	19.23±0.29^{g}	16.10±1.40^{ab}	19.21±0.75^{no}
M31	Nongwai	9.86±0.20^{o}	10.36±0.78^{pq}	23.66±0.61^{ghijkl}
M32	Lungkumlar	21.60±0.47^{d}	12.30±0.60 ^{fghijk}	19.69±1.45^{mno}
M33	Lungplain	20.29±0.25^{f}	11.90±0.2 ^{ghijklmno}	23.03±1.09^{ijklm}
M34	Huoibang	19.51±0.78^{g}	12.62±0.32^{efghij}	27.95±2.39^{bcde}
M35	Yaikumtar	15.06±0.24^{k}	14.38±1.02^{cd}	21.02±1.77^{lmn}
M36	Takwai	18.34±0.23^{h}	12.24±1.38 ^{fghijklm}	18.62±0.78^{o}
M37	Chankasem	23.15±0.19^{c}	11.20±0.8 ^{jklmnopq}	21.83±1.30^{klmn}

Note: Data values are expressed as means ± standard deviation. Vertically different letters mean that the values are significantly different, $P \leq 0.05$. Abbreviations: mg GAE/g dry extract, milligram gallic acid equivalent per gram of dry extract; mg CE/g dry extract, milligram catechin equivalent per gram of dry extract.

tannin contents in the different ethanolic extracts of *A. puncticulatum* cultivars were 18.68 mg GAE/g dry extract, 12.28 mg CE/g dry extract, and 23.11 mg CE/g dry extract, respectively.

Antioxidant activities

The antioxidant activities of the ethanolic extracts of *A. puncticulatum* are presented in Table 3. Ascorbic acids were used as reference standards for making a calibration curve for their antioxidant activities. In the case of the DPPH method, maximum antioxidant activity among extracts was exhibited in M35 "Yaikumtar' with 9.07 ± 0.13 mg AEAC/ g crude extract. The ABTS method confirmed that M35 "Yaikumtar" exhibited maximum antioxidant power with a value of 2.12 ± 0.04 mg AEAC/ g crude extract. However, for FRAP measurement, M25 "Sang krow No.3"

Table 3. Antioxidant activities and α-glucosidase inhibition of different ethanolic extracts of *Antidesma puncticulatum*

Sample codes	Sample names	Antioxidant activities (mg AEAC/g crude extract)			α-glucosidase inhibition IC_{50} (μg/mL)
		DPPH	ABTS	FRAP	
M01	Sahatsakhan	5.47±0.08^{k}	1.90 ±0.06^{cd}	2.06±0.11^{n}	4.22±0.35^{klmn}
M02	Lompad	5.10±0.07^{k}	1.81 ±0.04^{de}	3.06±0.06^{jkl}	1.47±0.09^{o}
M03	DongLuang	6.74±0.02^{j}	1.71 ±0.07^{efg}	2.88±0.78^{lm}	4.43±0.27^{klmn}
M04	Sanhome	5.29±0.08^{k}	1.23 ±0.09 ^{op}	3.62±0.07^{cdef}	2.40±0.27^{no}
M05	Sanphan	6.10±0.08^{j}	1.26 ±0.07 ^{op}	3.24±0.03^{hijk}	8.53±0.63^{gh}
M06	Worasarn	7.52±0.42^{h}	0.73 ±0.04 ^{r}	3.64±0.10^{bcdef}	3.64±0.18^{lmno}
M07	Wangkhumpoon	8.49±0.06^{bcdef}	1.29 ±0.04 ^{nop}	3.54±0.03^{cdefg}	13.24±0.76^{cde}
M08	Sorwannarsung	8.53±0.25 ^{bcdef}	1.58 ±0.07^{hijk}	3.32±0.04^{ghij}	14.01±0.76^{cd}
M09	Nongtaokhum	8.94±0.12^{ab}	1.82 ±0.08^{de}	3.14±0.05^{jkl}	4.84±0.15 ^{klmn}
M10	Kumlai	8.75±0.12^{abcde}	1.40 ±0.07^{lmn}	3.62±0.18^{cdef}	5.14±0.82^{jklm}
M11	Kru-in	8.90±0.24^{abc}	1.51 ±0.08^{ijkl}	3.50±0.09^{cdefgh}	25.07±1.36^{a}
M12	Naichan	8.71±0.08^{abcde}	1.53 ±0.05^{ijkl}	3.33±0.15^{ghij}	3.81±0.17 ^{lmno}
M13	Tongdee	8.85±0.07^{abc}	2.04 ±0.06^{ab}	3.01±0.07^{kl}	15.05±0.84^{cd}
M14	Phuphanthong	8.18±0.27^{fg}	1.47 ±0.02^{klm}	3.13±0.04^{jkl}	7.91±3.51^{ghi}
M15	Phuzong	8.32±0.26^{efg}	1.50 ±0.04^{jkl}	3.60±0.05^{cdefg}	5.38±0.31^{ijklm}
M16	Petnongzang	8.47±0.04^{bcdefg}	1.50 ±0.05^{jkl}	3.66±0.07^{bcde}	1.26±0.21^{o}
M17	Kangmoddang	8.19±0.16^{fg}	1.53 ±0.05^{ijk}	3.92±0.18^{ab}	2.78±0.51^{mno}
M18	Faprathan	8.33±0.19^{defg}	1.62 ±0.02^{ghij}	3.51±0.02^{cdefgh}	4.90±0.21$^{j\ klmn}$
M19	Kalasin No.1	8.56±0.31 ^{bcdef}	1.80 ±0.06^{def}	3.40±0.08^{efghi}	5.81±0.31^{ijkl}
M20	Wannawong No.1	8.76±0.09^{abcde}	1.68 ±0.05^{gh}	3.65±0.03^{bcdef}	2.75±0.25^{mno}
M21	Wannawong No.2	8.61±0.21^{abcdef}	1.24 ±0.07 ^{op}	3.67±0.01^{bcde}	4.77±0.59 ^{klmn}
M22	Wannawong No.3	8.53±0.25 ^{bcdef}	1.61 ±0.04^{ghij}	3.33±0.05^{ghij}	3.71±0.81 ^{lmno}
M23	Sang krow No.1	8.54±0.19 ^{bcdef}	1.38 ±0.08^{mn}	3.71±0.02^{bcd}	10.11±0.66^{fg}
M24	Sang krow No.2	8.02±0.77^{g}	1.22 ±0.06 ^{p}	3.77±0.02^{abc}	12.72±0.30^{de}
M25	Sang krow No.3	8.42±0.13^{cdefg}	1.01 ±0.09^{q}	4.01±0.01^{a}	6.68±1.00^{hijk}
M26	Khaoyai No.1	8.70±0.12^{abcde}	1.47 ±0.11^{klm}	3.39±0.04^{efghi}	4.71±0.42 ^{klmn}
M27	Khaoyai No.2	8.80±0.11^{abcde}	1.66 ±0.09^{gh}	3.75±0.03^{bc}	9.11±0.63^{fgh}
M28	Punna No.120	8.81±0.06^{abcd}	1.94 ±0.04^{bc}	2.90±0.01^{lm}	15.48±2.14^{c}
M29	Sangsawang	8.58±0.13 ^{bcdef}	1.52 ±0.06^{ijkl}	3.37±0.05^{fghi}	15.42±0.86^{c}
M30	Thepnimit	8.58±0.21 ^{bcdef}	1.63 ±0.10^{ghi}	3.73±0.05^{bc}	7.53±1.50^{hij}
M31	Nongwai	8.79±0.15^{abcde}	1.86 ±0.06^{cd}	3.63±0.08^{cdef}	5.77±0.46^{ijkl}
M32	Lungkumlar	8.14±0.79^{fg}	1.59 ±0.09^{ghijk}	3.15±0.07^{jkl}	7.50±0.44^{hij}
M33	Lungplain	8.75±0.07^{abcde}	1.53 ±0.08^{ijk}	3.44±0.05^{defgh}	19.03±0.09^{b}
M34	Huoibang	8.90±0.07^{abc}	1.69 ±0.03^{fgh}	3.13±0.01^{jkl}	8.52±1.22^{ghi}
M35	Yaikumtar	9.07±0.13^{a}	2.12 ±0.04^{a}	2.74±0.03^{m}	20.90±1.42^{b}
M36	Takwai	8.91±0.14^{abc}	1.34 ±0.04^{no}	3.33±0.05^{ghij}	5.03±0.46^{jklmn}
M37	Chankasem	8.94±0.06^{ab}	1.51 ±0.08^{ijkl}	3.74±0.02^{bc}	11.07±6.18^{ef}
Positive control (Acarbose)	-	-	-	-	2,739.78±37.80

Note: Data values are expressed as means ± standard deviation. Vertically different letters mean that the values are significantly different, $P \leq 0.05$
Abbreviations: mg AEAC/g crude extract, milligram ascorbic acid equivalent antioxidative capacity per gram of crude extract

exhibited the highest antioxidant activity with a value of 4.01 ± 0.01 mg AEAC/ g crude extract. The average values of antioxidant power in various *A. puncticulatum* cultivars evaluated by the DPPH, ABTS, and FRAP method were 8.20, 1.55 and 3.40 mg AEAC/g crude extract, respectively. Thus, various ethanolic extracts of *A. puncticulatum* influenced antioxidant activities.

α-Glucosidase inhibition activity

In this assay, the anti α-glucosidase activity of ethanolic extracts from *A. puncticulatum* was compared to that of acarbose, a standard reagent of α-glucosidase inhibition. The IC_{50} values of various crude extracts ranged from 1.26 μg/mL to 25.07 μg/mL (Table 3). Among the extracts from different cultivars, the extract of M02 "Lompad" and M16 "Petnongzang" possessed maximum α-glucosidase inhibition. It is worth noting that the standard reagent acarbose exhibited the highest IC_{50} value of 2739.78 μg/mL indicating the lowest α-glucosidase inhibitory effect. The results suggest that all ethanolic extracts from *A. puncticulatum* were capable of inhibiting α-glucosidase.

Discussion

Ethanol is a medium polar solvent generally used for the extraction of phytochemical constituents. According to the "like dissolves like" theory, the structures of compounds determine their solubility in the solvent polarity (40). The percentage extraction yields of 37 cultivars of *A. puncticulatum* (2.52%-11.15%) were comparable to those reported in the literature for various medicinal plants. Also, from previous research, the ethanolic leaf extracts of *A. puncticulatum* were abundant sources of hydroxycinnamic acids and flavonoids (21).

From the phytochemical screening, tannins, steroids, terpenoids, flavonoids, and saponins were found in the ethanolic leaf extracts from 37 cultivars of *A. puncticulatum*. Tannins and steroids were discovered in all of the extracts in this study, while terpenoids, flavonoids, and saponins were distributed differently in some cultivars. These phytochemical compounds detected are known to be of biological and medical importance. For example, tannins can precipitate microbial proteins thus making nutritional protein unavailable for them, so it has been reported to have antidiarrheal, antibacterial, antitumor, and antiviral activities (41-43). Steroids are a group of secondary metabolites synthesized from cholesterol. A wide range of medical and pharmaceutical activities of steroids have already been reported, such as antidiabetic, antitumor, immunosuppressive, antibacterial, and antihelminthic properties, along with plant growth hormone regulation and sex hormone and cardiotonic activity (44-47). Terpenoids, also known as isoprenoids or terpenes, are the most widespread group of natural products in all classes of living things. In the present study, they were found in high concentrations in the extracts from M04, M17, M27, M29, M31, and M37. Terpenoids display a wide range of biological activity and are effective in treating inflammation, postherpetic neuralgia, migraines, dysmenorrhea, and discomfort associated with surgery joints (48,49). Moreover, flavonoids which are a group of phenolic substances were found in the extracts from M01, M06, M19, and M27. They have many biological and pharmaceutical properties, especially antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and anti-tumor effects (50-53). In this study, saponins, which are a large family of amphiphilic glycosides of steroids and triterpenes, were found in high amounts only in the extracts from M16 and M26. Saponins show an enormous range of bioactivities such as anti-diabetic, anti-allergic, anti-inflammatory anti-nociceptive, and anti-cancer activities (54,55). According to these qualitative phytochemical screening results, the ethanolic leaf extracts of different *A. puncticulatum* cultivars could be exploited as natural pharmacological agents.

The variations of phenolic, flavonoid, and condensed tannin contents depend on different parameters such as geographical and weather conditions during sample collection, extraction method, solubility, and classification of solvent (56). Phenolic compounds are the most prevalent bioactive constituents with free radical scavenging activity in medicinal plants (57). From Table 2, 37 ethanolic extracts of *A. puncticulatum* contained total amounts of phenolic in the range of 9.86-26.75 mg GAE/g dry extract. Previously, there were reports revealing the total phenolic content of various *A. puncticulatum* leaf extracts in Thailand. In a study by Dechayont et al (21), the total phenolic content of the "Fapatan" cultivar was 231.14 mg GAE/g dry extract, which was relatively high compared to our study (19.35 mg GAE/g dry extract). On the other hand, Jorjong et al (28) determined the total phenolic content of fifteen Mao-Luang (*A. thwaitesianum* Mull. Agr.) cultivars and found they varied from 1.02 to 2.05 mg GAE/g DW. The variations in total phenolic content from different reports could be partly due to different solvents and conditions used for the extraction. Compared to other plant materials, the total phenolic contents of leaf extracts from *A. puncticulatum* in the present study were relatively higher than black mulberry (1.836-2.483 mg GAE/g DW) (58). Dechayont et al (21) revealed that hydroxycinnamic acids including caffeic acid were the main phenolic compounds in the ethanolic leaf extracts from *A. puncticulatum*. Moreover, gallic acid was found in ethanolic leaf extract from *A. puncticulatum* (22).

Flavonoids, which are secondary metabolite components, have been related to a number of medicinal properties, including antioxidant, anti-aging, antiviral and anti-inflammatory effects (59). In this experiment, the total flavonoid contents of 37 ethanolic extracts of *A. puncticulatum* were calculated as the equivalent of catechin

and were in the range of 8.10-16.18 mg CE/g dry extract (Table 2). The total flavonoid contents of the extracts in this study were higher than those of the leaf extracts from fifteen Mao-Luang cultivars reported earlier by Jorjong et al (28). Dechayont et al revealed that flavonoid compounds such as naringenin and flavone glucuronide were found in *A. thwaitesianum* Mull. Agr. ethanolic leaf extract. Moreover, flavonoid derivatives such as catechin and procyanidin B2 were present in ethanolic male leaf extract from *A. thwaitesianum* Mull. Agr. (22).

Tannins are reported as primary antioxidant or free radical scavengers that possess beneficial biological activities, including cardio-protective, anti-cancer, anti-nutritional, as well as, antimicrobial properties (60,61). They can be classified into hydrolysable tannins, such as gallotannins and ellagitannins and condensed tannins, including polyhydroxyflavan-3-ols or proanthocyanidins (62). From this experiment, the total condensed tannin content of the 37 ethanolic extracts of *A. puncticulatum* was found to be in the range of 14.83-33.67 mg CE/g dry extract (Table 2). The types of condensed tannin of *A. puncticulatum* have been until now little studied. From the previous study by Sakhunkhu et al, procyanidin B2, which is a proanthocyanidin, was found in ethanolic leaf extract from *A. thwaitesianum* Mull. Agr. It was documented that the consumption of procyanidin can reduce the risk of cardiovascular disease, T2DM, and cancers (63).

Antioxidants play significant roles in scavenging free radicals and terminating the chain reaction before the biological cells are damaged. Thus, antioxidants from various resources have been progressively investigated on their activities and their associations in reducing the risk of degenerative and chronic diseases such as cancer, cardiovascular diseases, DM, and so forth (64). Among others, DPPH, ABTS, and FRAP assays have been widely used to investigate antioxidant activities because these assays could be performed by using general laboratory equipment and detected with spectrophotometry. The DPPH method is a simple method for measuring free radicals by using electron transfer. The color of the DPPH free radical will change when it is reduced by antioxidants turned from violet to colorless (65). For the ABTS assay, the ABTS radical cations were scavenged by antioxidants to change the color from blue to colorless (66). In the FRAP test, this method is based on the reduction of ferric complexes. After reacting with the antioxidant, Fe^{3+} colorless solution was reduced to Fe^{2+} blue solution (67). In this study, the antioxidant activities of 37 ethanolic extracts of *A. puncticulatum* showed high total antioxidant activity in the DPPH assay and exerted moderate activity in the ABTS and FRAP assays. These experimental results were consistent with an earlier report which found strong DPPH scavenging activity of the leaf extracts of *A. thwaitesianum* Mull. Agr. (18). The DPPH free radicals are more suitable for both lipophilic (such as triterpenoids aglycone) and hydrophilic (such as sugar) antioxidants while the ABTS and FRAP assays usually react with hydrophilic antioxidants (68). Antioxidant properties may be associated with phenolics, flavonoids, and tannins content. For example, M19 "Kalasin No.1", which contained the highest phenolic content also showed relatively high DPPH (8.56 mg AEAC/g extract) and ABTS (1.80 mg AEAC/g extract) antioxidant activity. In addition, M10 "Kumlai" contained high flavonoid content with relatively high DPPH (8.75 mg AEAC/g extract) and FRAP (3.62 mg AEAC/g extract) antioxidant activity. Moreover, M09 "Nongtaokhum" which possessed the highest tannin content showed strong DPPH (8.94 mg AEAC/g extract) and ABTS (1.82 mg AEAC/g extract) antioxidant activity. The antioxidant activity of phenol molecules is due to the hydroxyl substituents on the aromatic ring, which have the capacity to chelate metal ions and scavenge free radicals (33,69). The compositions of phenolic compounds in the ethanolic leaf extract from *A. puncticulatum* that were previously investigated include hydroxycinnamic acids such as caffeic derivatives and flavonoids (21). Additionally, six polyphenols, including corilagin acid, gallic acid, ferulic acid, ellagic acids, vicinin II, and amentoflavone, were found in the methanolic extracts of *Antidesma bunius* leaves by separation using column chromatography. Compared to quercetin, these phenolic compounds exhibited greater *in vitro* antioxidant activities by promoting lactate dehydrogenase leakage from cell cytosol and glutathione reductase, and depressing nitric oxide levels (70).

The antidiabetic activity of the ethanolic leaf extract from *A. puncticulatum* was investigated by α-glucosidase inhibitory assay. Theoretically, blood glucose levels rise as a result of oligosaccharides in the colon being broken down into glucose by the acidic enzyme α-glucosidase. By delaying the digestion of carbohydrates when this enzyme's activity is inhibited, blood glucose elevation can be effectively delayed (71). According to recent studies, plant-bioactive compounds with antioxidant properties may function as α-glucosidase activity inhibitors (72-74). From our results, all 37 ethanolic extracts of *A. puncticulatum* cultivars exhibited stronger α-glucosidase inhibition activity than the positive control, acarbose. This research is also consistent with Elya et al results (4) where IC_{50} values of ethanolic leaf extract of *Antidesma bunius* (L.) Spreng were lower than acarbose. The inhibitory activity of enzyme α-glucosidase in *A. puncticulatum* leaves was probably related to saponins, tannins, and flavonoids presented in these ethanolic extracts (75). In our study, we found that M02 "Lompad" possessed strong α-glucosidase inhibitory activity (IC_{50} 1.47 μg/mL), which is correlated with the relatively high ABTS antioxidant activity (1.81 mg AEAC/g extract). Among other cultivars, M16 "Petnongzang" possessed the strongest α-glucosidase inhibitory activity (IC_{50} 1.26 μg/mL) with relatively

strong DPPH (8.47 mg AEAC/g extract) and FRAP (3.66 mg AEAC/g extract) antioxidant activity. In the previous investigation of Dechayont et al (21), phenolic components were identified in the ethanolic leaf extract of *A. puncticulatum*, including caffeic acid, naringenin, and flavone glucuronide. Among these compounds, caffeic acid was reported to lower the hepatic outflow of glucose and boost adipocyte glucose absorption through increasing expression of the hepatic glucose 4 transporters (76). In addition, naringenin can lower plasma glucose levels and raise insulin sensitivity in animal studies (77). Consistent with our study, *Antidesma bunius* L. (Mao Luang) methanolic extract has anti-diabetic properties through the improvement of hepatic glycogen storage and islet of Langerhans regeneration (19). Overall, the extracts of 37 cultivars of *A. puncticulatum* demonstrated exceptional antidiabetic efficacy as shown by excellent α-glucosidase inhibitory activity, making this plant a prospective source of bioactive chemicals for pharmacological uses.

Conclusion

The qualitative screening revealed that the extracts of *A. puncticulatum* leaves could be a reliable source of crucial phytochemicals, including tannins, steroids, terpenoids, flavonoids, and saponins. The 37 ethanolic extracts of *A. puncticulatum* leaves showed significant amounts of phenolics, flavonoids, and condensed tannins, which contributed to their high antioxidant activity. Interestingly, these ethanolic extracts demonstrated higher α-glucosidase inhibitory activity compared to the acarbose used as a positive control. The findings from this study could inspire additional *in vivo* research determining whether the extracts of *A. puncticulatum* leaves could be served as a promising natural treatment for diabetic mellitus.

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Conflict of interests

The authors declare that they have no conflict of interest.

Ethical considerations

The authors have paid close attention to ethical considerations concerning authorship, data collection, review, and analysis.

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