Research Paper

Alpha-Amylase Inhibitory and Antioxidant Activities in Aqueous Acetone Extract and its Fractions from *Ampelocissus martini* Root

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This study aimed to evaluate and correlate phytochemical content, antioxidant activity and alpha-amylase inhibitory activity in aqueous acetone extract and fractions derived from it (ethyl acetate fraction, water-soluble fraction, aqueous methanol fraction and aqueous acetone fraction) which are obtained from *Ampelocissus martini* Planch. root. Ethyl acetate fraction and aqueous acetone fraction had the highest total phenolic content and total proanthocyanidin content, respectively. 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), cupric reducing antioxidant capacity and ferric reducing antioxidant power assays showed that aqueous acetone fraction had higher antioxidant activity compared to other samples and standards. Acarbose and all samples except aqueous methanol fraction inhibited alpha-amylase, a key enzyme linked to type 2 diabetes, in a dose-dependent manner and aqueous acetone fraction showed the strongest inhibition (half-maximal inhibitory concentration=8.77±0.28 µg/ml). Aqueous acetone fraction was a mixed noncompetitive inhibitor of the enzyme. Correlation analysis showed strong positive correlations among proanthocyanidin content, antioxidant activity and alpha-amylase inhibitory activity. These results suggest that aqueous acetone fraction, proanthocyanidins-rich fraction from *Ampelocissus martini* root, may be used for effective diabetes management because of its high potential antioxidant activity and alpha-amylase inhibitory activity.

Key words: *Ampelocissus martini*, proanthocyanidins, alpha-amylase inhibitory activity, antioxidant activity, antidiabetic activity

Diabetes mellitus is a group of disorders generally characterized by hyperglycemia resulting from a deficiency in insulin secretion and/or insulin action[1]. The disease is rapidly growing worldwide with an increasing morbidity and mortality[2]. The most common type of diabetes in adults is type 2 diabetes[3]. Hyperglycemia can induce the excessive generation of free radicals resulting in oxidative stress, which further increases the progression of many diseases including diabetes[4,5]. The chronic hyperglycemia of diabetes causes damage and failure of many organs including the eyes, heart and kidneys[1].

Alpha (α)-amylase (EC 3.2.1.1) or 4-α-D-glucan glucanohydrolase is one of two key enzymes linked to type 2 diabetes and is found in saliva and small intestine of the human body. It hydrolyzes complex polysaccharides to oligosaccharides which are then hydrolyzed by intestinal α-glucosidase to liberate glucose before entering the bloodstream[6]. Inhibition of α-amylase by inhibitors will reduce postprandial blood glucose levels. Acarbose, inhibitor of α-amylase has been used for the management of hyperglycemia and type 2 diabetes, but with undesirable side effects including diarrhea and flatulence[7]. Finding natural and safer enzyme inhibitors with strong antioxidant activity has been proposed for the treatment of diabetes since it can control hyperglycemia and diabetes complications caused from oxidative stress[8-10].

Phenolic compounds are widely distributed in the plant kingdom and classified into several groups based on the number of phenol rings and structural elements that bind these rings to one another[11,12]. Their diverse structures greatly influence their solubility in water and organic solvents[13]. Phenolic compounds including proanthocyanidins which contain antioxidant activity and α-amylase inhibitory activity have been considered

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as a potential antidiabetic agents in management of type 2 diabetes[3,6,14,15]. *Ampelocissus martini* (*A. martini*) Planch. is a wild grape commonly found in Thailand. Leaves, roots and bark of the plant have been used as ingredients in Thai traditional medicine to provide relief of symptoms. Many parts of the plant such as the fruits, vine, rhizome, leaves contain high concentrations of phenolic compounds with antioxidant activity[16,17]. Phenolic compounds such as gallic acid, caffeic acid, resveratrol, catechin, epicatechin, rutin and quercetin are present in seeds[18]. Antibacterial activity has also been found in fruits of this plant[19]. However, limited information is available on the α-amylase inhibitory activity of extracts from root of *A. martini* as well as the relationship among content of phenolics and proanthocyanidins, antioxidant and antidiabetic activities. In this study, phenolic and proanthocyanidin content, *in vitro* antioxidant activity and α-amylase inhibitory activity from *A. martini* root extract and its fractions were determined and correlated.

MATERIALS AND METHODS

Chemicals and reagents:

All chemicals and reagents were of analytical grade. Hydrochloric acid (HCl), petroleum ether, ethyl acetate, methanol, ethanol and acetic acid were purchased from QRec (Auckland, New Zealand). Gallic acid, vanillin, (+)-catechin, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), 2,2' Azino Bis (3-Ethylbenzthiazoline-6-Sulphonic Acid) diammonium salt (ABTS), 2,9-dimethyl-1,10-phenanthroline (Neocuproine), sephadex-LH 20, potato starch, α-amylase from porcine pancreas type VI-B, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (Missouri, United States of America (USA)). Sodium carbonate (Na_2CO_3), Potassium persulfate ($K_2S_2O_8$), Ferric chloride ($FeCl_3$), Copper (II) chloride dihydrate ($CuCl_2.2H_2O$), Iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) and Sodium acetate trihydrate ($CH_3COONa.3H_2O$) were obtained from Ajax Finechem (Auckland, New Zealand). Ascorbic acid, Sodium chloride (NaCl), Dimethyl Sulfoxide (DMSO), 3,5-dinitrosalicylic acid and Folin-Ciocalteu's reagent were obtained from Carlo Erba (Milan, Italy). Quercetin, Butylated Hydroxytoluene (BHT) and acarbose were purchased from Acros Organic (New Jersey, USA).

Plant material and sample preparation:

A. martini was collected from the wild in Roi-Et Province, Northeastern Thailand in December 2019. A dry specimen of the plant number Siripipatthana 1 was deposited at the Khon Kaen University (KKU) herbarium, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand. Roots of the plant were used in the current study. The roots were washed and dried in shade. An electronic grinder were used to grind the dried roots into a fine powder which was then stored in an air-tight container at 25° in darkness.

Different solvent extractions and gel chromatographic fractionation:

Method of Chen *et al.*[20] with some modifications was used. Dried root powder (20 g) was extracted for 1.5 h with 700 ml of 70 % aqueous acetone at 25° using a magnetic stirrer (Clifton® Ceraplate; Nickel-Electro, United Kingdom (UK)). Centrifugation at 2200 g for 20 min (Rotanta 46R; Andreas Hettich GmbH and Co. KG, Germany) yielded a supernatant and pellet. The pellet was re-extracted 2 times using the same procedure and the three resulting supernatants were pooled and then filtered using Whatman no.1 paper (GE Healthcare, UK). Acetone was removed using a rotary vacuum evaporator (Hei-VAP g3; Heldolph instrument Gmbh and Co. KG, Germany) at 40° to obtain an aqueous extract. The aqueous extract was divided into 2 parts. The first part was freeze-dried to yield crude extract (Aqueous Acetone Extract (AAE)). The second one was extracted with hexane (300 ml) and then with petroleum ether (300 ml) to remove lipophilic compounds prior to fractionation using the modified methods[21,22]. The remaining aqueous extract was extracted with ethyl acetate (3×300 ml). Two fractions were obtained; Ethyl Acetate Fraction (EF) and the Water-Soluble Fraction (WF). The EF was dried using a rotary vacuum evaporator at 40° while the WF was freeze-dried. Next, 7 ml of 20 mg/ml WF was prepared in 50 % aqueous methanol and chromatographed on a Sephadex LH-20 column (2×50 cm). Elution was performed at a flow rate of 0.5 ml/min with 50 % aqueous methanol and then with 70 % aqueous acetone and two fractions retained; Aqueous Methanol Fraction (AMF) and Aqueous Acetone Fraction (AAF). Finally, the AMF and AAF were dried using vacuum evaporation and freeze-drying. For freeze-drying, each fraction was kept at -40° in a deep freezer for 24 h, then was lyophilized under vacuum (0.4 mbar) using a freeze-dryer (Alpha 3-4 LSCbasic; Martin Christ GmbH, Germany). 50 % DMSO was prepared in distilled water. AAE and all fractions were separately dissolved in 50 %

DMSO to yield stock solution (20 mg/ml).

Analysis of Total Phenolic Content (TPC):

TPC was evaluated according to the method of Farhadi *et al.*[23] with slight modification. Briefly, the sample was diluted to appropriate concentration in 50 % DMSO. An aliquot of 0.4 ml of sample was mixed with 2 ml of 10 % Folin-Ciocalteu's reagent and incubated for 5 min at 25° prior to adding 1.6 ml of 7.5 % (w/v) Na_2CO_3. The mixture was allowed to stand for 30 min at 25°, followed by measurement of absorbance at 765 nm (Genesys 20 4001/4; ThermoFisher Scientific, USA). A blank was prepared as above but 50 % DMSO was used instead of sample. The TPC of samples was reported as milligram of Gallic Acid Equivalent per gram of Dry Weight (mg GAE/g DW).

Analysis of Total Proanthocyanidin Content (TPAC):

TPAC assay was based on a procedure reported by Li *et al.*[24]. Each sample was diluted to appropriate concentration in distilled water. The sample solution (0.25 ml) was mixed with 1.5 ml of 4 % (w/v) vanillin in ethanol (freshly prepared) in a test tube, followed by addition of 0.75 ml of 37 % (w/w) HCl. The tube was capped and left for 15 min at 25° prior to the measurement of absorbance at 500 nm (Genesys 20 4001/4; ThermoFisher Scientific, USA). A blank was prepared as above but 50 % DMSO was used instead of sample. The TPAC of each sample was reported as milligram of Catechin Equivalent per gram of Dry Weight (mg CE/g DW).

DPPH assay:

The ability of samples to scavenge DPPH radicals ($DPPH^{\bullet}$) was evaluated based on a modified method[23]. Briefly, 0.1 mM of $DPPH^{\bullet}$ was freshly prepared by dissolving DPPH in methanol and 1 ml of this solution was mixed with 0.5 ml of various concentrations of sample (5-30 µg/ml of AAE, 5-50 µg/ml of EF, 5-25 µg/ml of WF, 10-60 µg/ml of AMF, 2.5-12.5 µg/ml of AAF, 12.5-100 µg/ml of BHT, 1-10 µg/ml of ascorbic acid and 1-16 µg/ml of Trolox) or 50 % DMSO (negative control). The mixture was allowed to stand for 30 min at 25° in the dark and then the absorbance was measured at 517 nm (Genesys 20 4001/4; ThermoFisher Scientific, USA) using 50 % DMSO as the blank. Percentage (%) of DPPH inhibition was calculated based on equation (1).

$$\text{DPPH inhibition (\%)} = [(A_0 - A_S)/A_0] \times 100 \quad (1)$$

Where A_0=Absorbance of mixture in the absence of sample (negative control) and A_S=Absorbance of mixture in the presence of sample.

A graph was constructed by plotting % inhibition against sample concentration. The antioxidant activity was expressed as the half maximal Inhibitory Concentration (IC_{50}) value which is the concentration required to cause 50 % inhibition. BHT, ascorbic acid and Trolox were employed as positive controls.

ABTS assay:

The ABTS assay described by Re *et al.*[25] was used to measure ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity of samples. To produce $ABTS^{\bullet+}$ solution, 7 mM of ABTS diammonium salt solution was mixed with 2.45 mM $K_2S_2O_8$ at the volume ratio of 1:1, the mixture was left to stand in the dark at 25° for 16 h and then diluted with distilled water to absorbance at 0.700±0.020 at 734 nm. A volume of 0.5 ml of sample or 50 % DMSO (negative control) was mixed with 1 ml of the $ABTS^{\bullet+}$ solution. After 6 min of incubation at 25° in the dark, the absorbance of the reaction was measured at 734 nm (Genesys 20 4001/4; ThermoFisher Scientific, USA), using 50 % DMSO as the blank. The percent inhibition was calculated similarly to DPPH inhibition and antioxidant activity was expressed as IC_{50} value. BHT, ascorbic acid and Trolox were also used as positive controls.

Cupric Reducing Antioxidant Capacity (CUPRAC) assay:

The method of Apak *et al.*[26] with some modifications was performed to evaluate CUPRAC value. Briefly, 0.5 ml of 10 mM $CuCl_2$ solution was mixed with 0.5 ml of 7.5 mM Neocuproine in 96 % ethanol. The sample was diluted to appropriate concentration in 50 % DMSO and 0.55 ml of the diluted sample or 50 % DMSO (for blank) was added to the previous mixture solution. The reaction solution was allowed to stand for 30 min at 25°, followed by measurement of absorbance at 450 nm (Genesys 20 4001/4; ThermoFisher Scientific, USA). The CUPRAC values of samples was calculated using a standard curve of Trolox and reported as milligram of Trolox Equivalent per gram of Dry Weight (mg TE/g DW).

Ferric Reducing Antioxidant Power (FRAP) assay:

The method of Zhang *et al.*[27] was used with slight modification. Firstly, FRAP reagent was prepared by mixing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$ and 300 mM sodium acetate buffer (pH 3.6) in the ratio of 10:10:100 (v/v/v). Then 3 ml of the reagent was mixed with 0.1 ml of sample or 50 % DMSO (for blank). After

incubation for 15 min at 25°, the absorbance of the mixture at 593 nm was measured (Genesys 20 4001/4; ThermoFisher Scientific, USA). The FRAP value of samples was expressed as micromole Ferrous ion (Fe^{2+}) equivalent/g Dry Weight (μmol Fe^{2+}/g DW).

α-Amylase inhibition assay:

The methods of Wickramarantne *et al.*[28] and Olaokun *et al.*[29] were performed with minor modifications. AAE and its fractions at varying concentrations, 2 units/ml of α-amylase, and 1 % (w/v) soluble potato starch were prepared in 20 mM sodium phosphate buffer (pH 6.9) containing 6.7 mM NaCl. A volume of 0.2 ml of the sample was mixed with 0.2 ml of α-amylase and then incubated for 10 min at 37°. Soluble potato starch (0.2 ml) was added and incubated for 5 min at 37°. To terminate the reaction, 0.2 ml of 3,5-dinitrosalicylic acid reagent was added to the mixture. The mixture was boiled for 10 min at 90° and then cooled to 25°. Distilled water (1.5 ml) was added to dilute the mixture prior to absorbance measurement at 540 nm (Genesys 20 4001/4; ThermoFisher Scientific, USA). For the control, 0.2 ml of the sample solvent was used instead of sample. For the blank, the enzyme solution was replaced by the buffer. Acarbose was used as a positive control. The α-amylase inhibitory activity was calculated as percentage (%) inhibition, based on equation (2).

$$\% \text{ Inhibition} = [(A_{control} - A_{blank}) - (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100 \quad (2)$$

Where $A_{control}$=Absorbance of the control, A_{blank}=Absorbance of the blank and A_{sample}=Absorbance of the sample.

The concentration of the sample resulting in a 50 % inhibition of α-amylase activity (IC_{50}) was calculated from a graph plot of concentration against % inhibition.

Mode of α-amylase inhibition:

The method of Kazeem *et al.*[30] with some modifications was used to evaluate mode (type) of α-amylase inhibition. Two set of tubes were prepared. In the former set, 0.2 ml of AAF (6 mg/l and 9 mg/l) or standard acarbose (25 mg/l) was pre-incubated with 0.2 ml of α-amylase (2 units/ml) for 10 min at 37°. In the latter set, 0.2 ml of 20 mM sodium phosphate buffer (pH 6.9) containing 6.7 mM NaCl was pre-incubated with 0.2 ml of α-amylase. Then, 0.2 ml of soluble potato starch (1, 2, 4 and 6 mg/ml) was added to both sets and incubated for 5 min at 37°, followed by addition of 0.2 ml of 3,5-dinitrosalicylic acid reagent and boiling for 10 min at 90°. The reaction mixture was cooled to 25° and 1.5 ml of distilled water was added to the mixture before measurement of the absorbance at 540 nm (Genesys 20 4001/4; ThermoFisher Scientific, USA). Using a maltose standard curve and absorbance of sample, the amount of reducing sugars released was calculated and converted to reaction velocity. The double reciprocal plot or Lineweaver-Burk plot of 1/[S] against 1/V where V is reaction Velocity and [S] is Substrate (starch) concentration was constructed. Mode of inhibition can be obtained from the graph analysis.

Statistical analysis:

All experiments were performed in triplicates. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software ver. 25.0 (IBM, Armonk, New York, USA). Data were subjected to Analysis of Variance (ANOVA). Duncan's new multiple range test was used to evaluate the significant differences between means and a value of $p<0.05$ was considered as statistically significant. Values of $1/IC_{50}$ and other data were used in correlation analysis and the results were reported as the Pearson's correlation coefficients (r).

RESULTS AND DISCUSSION

Phytochemical contents among AAE and its fractions (EF, WF, AMF and AAF) were significantly different (Table 1). TPC in AAE and its fractions expressed as mg GAE/g DW significantly decreased in the order of EF>AAF>AAE>WF>AMF while TPAC expressed as mg CE/g DW decreased in the order of AAF>AAE>WF>EF>AMF.

AAE and its fractions and standards (BHT, ascorbic acid and Trolox) showed a linear relationship between percentage of free radical inhibition and sample concentrations in DPPH and ABTS assays (data not shown). Each IC_{50} value calculated using the linear equation is shown in Table 2. IC_{50} is the sample concentration required to cause 50 % inhibition; therefore, lower IC_{50} values indicate greater radical scavenging activity[23,27]. For the DPPH assay, calculated IC_{50} values revealed that AAE and its fractions had significantly higher inhibition of DPPH radicals compared to BHT. AAF had the highest antioxidant activity as revealed by the lowest IC_{50} and antioxidant activity of AAF was much higher than that of Trolox, but similar to that of ascorbic acid. For the ABTS assay, according to their IC_{50} values, the scavenging effect on the ABTS radicals decreased in the order of AAF>EF>AAE>WF>AMF. All extracts were determined to have weaker antioxidant activities than ascorbic acid. However, AAF had more efficient antioxidant activity than standards (BHT and Trolox).

TABLE 1: TPC AND TPAC OF AAE AND ITS FRACTIONS FROM *A. martini* ROOT

Sample	TPC (mg GAE/g DW)	TPAC (mg CE/g DW)
AAE	288.10±0.73[c]	333.35±0.96[d]
EF	495.68±0.00[e]	121.92±0.24[b]
WF	268.17±0.24[b]	329.41±2.08[c]
AMF	86.82±0.12[a]	22.02±0.06[a]
AAF	475.05±0.47[d]	536.11±4.15[e]

Note: The results are expressed as mean±Standard Deviation (SD) (n=3). [(a-e)]Different letters in the same column are significantly different at p<0.05; AAE: Aqueous Acetone Extract; EF: Ethyl Acetate Fraction; WF: Water-Soluble Fraction; AMF: Aqueous Methanol Fraction and AAF: Aqueous Acetone Fraction

TABLE 2: ANTIOXIDANT ACTIVITIES OF AAE AND ITS FRACTIONS FROM *A. martini* ROOT

Sample	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (mg/ml)	CUPRAC (mg TE/g DW)	FRAP (mmol Fe^{2+}/g DW)
AAE	17.80±0.02[d]	11.34±0.03[f]	994.79±1.73[c]	311.42±0.39[d]
EF	35.00±0.06[e]	10.53±0.02[e]	685.73±0.65[b]	162.38±0.34[c]
WF	16.36±0.02[c]	11.46±0.04[g]	1023.03±1.73[d]	388.66±0.39[e]
AMF	50.58±0.12[f]	45.22±0.06[h]	220.88±0.54[a]	101.67±0.32[a]
AAF	7.12±0.00[a]	6.27±0.01[b]	2027.95±8.81[f]	773.50±4.67[g]
BHT	83.46±0.80[g]	7.52±0.03[c]	685.38±2.62[b]	132.73±0.69[b]
Ascorbic acid	6.91±0.02[a]	5.64±0.01[a]	1757.58±4.34[e]	1039.25±6.44[h]
Trolox	13.79±0.05[b]	8.29±0.01[d]	ND	728.38±3.58[f]

Note: The results are expressed as mean±SD (n=3) which, with [(a-h)]different letters in the same column are significantly different at p<0.05. IC_{50} is the Inhibitory Concentration of sample to scavenge 50 % radicals. ND: Not Determined; AAE: Aqueous Acetone Extract; EF: Ethyl Acetate Fraction; WF: Water-Soluble Fraction; AMF: Aqueous Methanol Fraction; AAF: Aqueous Acetone Fraction; DW: Dry Weight; DPPH: 2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate; ABTS: 2,2'-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic acid); CUPRAC: Cupric Reducing Antioxidant Capacity and FRAP: Ferric Reducing Antioxidant Power

The antioxidant activity can also be expressed through measurement of the reduction of metal ions such as Cupric ion (Cu^{2+}) and Ferric ion (Fe^{3+}) by antioxidants in the sample. For the CUPRAC assay, copper (II)-neocuproine [Cu(II)-Nc] complex was reduced to form the Cu(I)-Nc[26] while for the FRAP assay, ferric tripyridyltriazine [Fe(III)-TPTZ] complex was reduced to form the ferrous tripyridyltriazine [Fe(II)-TPTZ] complex[27]. High CUPRAC and FRAP values indicate high antioxidant activity. As shown in Table 2, AAF demonstrated the highest antioxidant activity followed by WF>AAE>EF>AMF, respectively in both assays. In addition, AAF showed much higher antioxidant activity compared to ascorbic acid and BHT in CUPRAC assay and had significantly higher antioxidant activity compared to BHT and Trolox in FRAP assay.

Acarbose and all samples except AMF inhibited α-amylase in a dose-dependent manner (data not shown). IC_{50} (sample concentration causing 50 % enzyme inhibition) of AAE and its fractions was found to range from 8.77±0.28 µg/ml to 21.36±0.30 µg/ml while IC_{50} of acarbose was found to be 21.19±0.79 µg/ml (Table 3). AAF showed the lowest IC_{50} value; therefore, AAF had much higher α-amylase inhibitory activity compared to other samples and standard acarbose. There was no detectable activity in AMF, therefore, its IC_{50} value could not be calculated.

Kinetic study employed the Lineweaver-Burk plot to identify the mode of α-amylase inhibition by inhibitor. AAF showing the lowest IC_{50} value for α-amylase inhibition which was tested in this study. AAF displayed a mixed noncompetitive mode of inhibition towards α-amylase (fig. 1A) while acarbose showed noncompetitive inhibition on the enzyme (fig. 1B).

Pearson's correlation analysis was performed to test the possible relationship between phytochemicals, antioxidant activity and α-amylase inhibitory activity in *A. martini* roots. The results were reported as Pearson's correlation coefficient (r) and shown in Table 4. α-Amylase inhibitory activity was significantly correlated with TPC (r=0.748), TPAC (r=0.878) and all antioxidant activities (r≥0.804). In addition, TPAC showed higher correlation to all antioxidant activities compared to TPC.

TABLE 3: LINEAR REGRESSION EQUATION, R^2 AND IC_{50} FOR α-AMYLASE INHIBITION OF AAE AND ITS FRACTIONS FROM *A. martini* ROOT

Sample	Linear regression equation	R^2	IC_{50} (µg/ml)
AAE	y=5.1689x-8.9881	0.9898	11.41±0.1[b]
EF	y=1.1158x+30.819	0.9849	17.18±0.49[c]
WF	y=2.5401x-4.2417	0.9777	21.36±0.30[d]
AMF	-	-	n. i.
AAF	y=5.9287x-1.9497	0.9860	8.77±0.28[a]
Acarbose	y=1.1409x+25.867	0.9866	21.19±0.79[d]

Note: IC_{50}: Amount required for a 50 % inhibition of α-amylase activity. The results are expressed as mean±SD (n=3) which, with [(a-d)]different letters in the same column are significantly different at $p<0.05$. n. i.: No inhibition observed up to 100 µg/ml, -: No data; AAE: Aqueous Acetone Extract; EF: Ethyl Acetate Fraction; WF: Water-Soluble Fraction; AMF: Aqueous Methanol Fraction and AAF: Aqueous Acetone Fraction

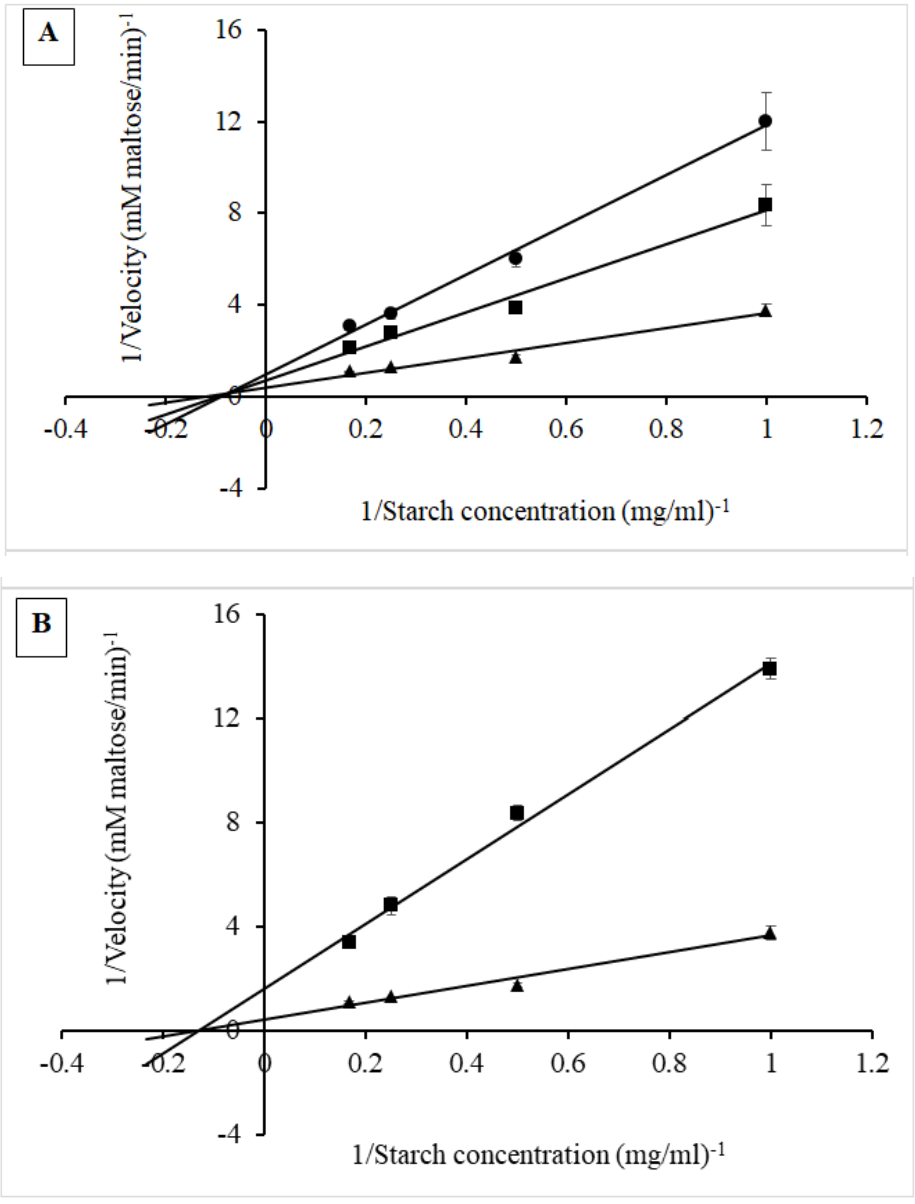

Fig. 1: Lineweaver-Burk plots of α-amylase inhibition at various concentrations of starch in the presence of inhibitor, (A) In the presence of AAF at 0 (▲); 6 (■) and 9 (●) mg/l and (B) In the presence of acarbose at 0 (▲) and 25 (■) mg/l

Using natural phenolic compounds including proanthocyanidins which has α-amylase inhibitory activity and strong antioxidant activity is an effective treatment of type 2 diabetes[3,6,14,15]. Therefore, TPC, TPAC, antioxidant activity, α-amylase inhibitory activity and their relation were evaluated in AAE and its fractions (EF, WF, AMF and AAF) from *A. martini* root.

EF and AAF showed the highest TPC and TPAC, respectively. Difference in TPC and TPAC found in AAE and its fractions may have resulted from many factors such as chemical structures of phenolic compounds including proanthocyanidins, polarity of different solvents used, extraction methods and the presence of interfering substances[31]. Phenolic compounds including proanthocyanidins have diverse structures resulting in different solubilities of the compounds in water and organic solvents[13]. AAE was obtained from 70 % aqueous acetone, which is a commonly used solvent system for extraction of phenolic compounds and proanthocyanidins[32,33]. Using ethyl acetate (which has lower polarity compared to water) helps to remove low molecular weight phenolics including monomeric flavonoids and partly oligomeric proanthocyanidins from the aqueous fraction (WF)[34] and yields EF. In fractionation of WF by Sephadex LH-20 column chromatography, aqueous methanol (50 % v/v) has been used to remove polysaccharides and some monomeric flavonoids from the column before elution with aqueous acetone (70 % v/v) was performed to obtain concentrated polymeric proanthocyanidins[22,34].

Several methods such as DPPH, ABTS, CUPRAC and FRAP were performed to evaluate the antioxidant activity of substances by the reduction of certain compounds including metals and radicals because they are simple and widely used techniques[10,13]. AAF showed higher antioxidant activity compared to all samples in all assays. Correlation analysis confirmed that the strongest antioxidant activity of AAF was related to high proanthocyanidin content (Table 4). The results were consistent with the report of Lin *et al.*[35] demonstrating that antioxidant activity had high correlation coefficient with proanthocyanidin content. Phenolic compounds especially proanthocyanidins are structurally diverse. The number and positions of the hydroxyl groups, and the nature of substitutions on the aromatic rings give rise to the scavenging free radicals, donating hydrogen atoms or electrons, or chelating metal cations[36]. AAF, proanthocyanidin-rich fraction showed stronger antioxidant activity compared to two standards (BHT, trolox) in both free radical scavenging activity and metal reducing power; therefore, it might be used as a source of alternative antioxidant.

TABLE 4: PEARSON'S CORRELATION COEFFICIENT OF PHYTOCHEMICAL CONTENTS, ANTIOXIDANT ACTIVITY AND α-AMYLASE INHIBITION

Assay	TPC	TPAC	DPPH	ABTS	CUPRAC	FRAP	Amylase inhibition
TPC	1	0.503	0.511	0.846**	0.639*	0.506	0.748**
TPAC		1	0.935**	0.877**	0.963**	0.950**	0.878**
DPPH			1	0.880**	0.980**	0.996**	0.815**
ABTS				1	0.950**	0.881**	0.927**
CUPRAC					1	0.982**	0.895**
FRAP						1	0.804**
Amylase inhibition							1

Note: *Correlation is significant at the 0.05 level and **Correlation is significant at the 0.01 level

Using inhibition of α-amylase has been a therapeutic approach for controlling postprandial hyperglycemia in type 2 diabetes because it decreases the rate of starch degradation, which would in turn causes decreasing glucose absorption and concentration of postprandial blood glucose[6,37]. AAF showed higher α-amylase inhibitory activity compared to all samples. This might have resulted from high content of TPC and TPAC because correlation analysis showed that α-amylase inhibitory activity was significantly correlated with TPC (r=0.748), TPAC (r=0.878) (Table 4). No detectable activity in AMF may have resulted from the low values of both TPC and TPAC (Table 1). The results were consistent with the report of Lin *et al.*[35] demonstrating that the α-amylase inhibitory activity had a high correlation coefficient with proanthocyanidin content. Also, a significant correlation between the phenolic content and amylase inhibitory activity has been observed in other reports[38,39]. Phenolic compounds including proanthocyanidins can inhibit α-amylase by cooperative effects of hydrophobic interaction and hydrogen bond formation between the substances and the enzyme[40]. In addition, AAF acted as mixed noncompetitive inhibitor of the enzyme. Mixed noncompetitive inhibitor can bind to either the free enzyme or the Enzyme-Substrate (ES) complex and a single inhibitor can prevent the binding of substrate and decreases the turnover number of the enzyme[41].

In conclusion, this study confirms that *A. martini* root can be a good source of natural phytochemicals and that there were correlations among contents of phenolics and proanthocyanidins, antioxidant and α-amylase inhibitory activities in AAE and its fractions from the plant root. Proanthocyanidins had strong correlation with both α-amylase inhibitory and antioxidant activities; therefore, AAF, proanthocyanidins-rich fraction, might be used for effective diabetes management. Further *in vivo* assays for both activities as well as proanthocyanidin structure identification in AAF may be required to advance the study.

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Conflict of interests:

The authors declared no conflict of interest.

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