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Contributed Paper

Application of Ultrasonic Assisted Extraction of Bioactive Compounds from the Fruits of *Antidesma puncticulatum* Miq. and Evaluation of Its Antityrosinase Activity

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ABSTRACT

The ripe fruits of *Antidesma puncticulatum* are used as commercial sources for phenolic compounds and anthocyanins for functional foods. The aim of this study was to optimize the process parameters for extraction of phenolic and anthocyanin compounds with good antioxidant activity from the fruits using ultrasonic assisted extraction (UAE). A four-factor, five-level, six-center point central composite design was performed. Effects of solvent-to-solid ratio (X_1: 7.5-37.5 mL/g), acidified ethanol solution (X_2: 0-80%), extraction temperature (X_3: 20-100°C), and extraction time (X_4: 0-20 min) on the recovery of total phenolic and total anthocyanin contents with good antioxidant activity were investigated. The second order polynomial models of the response variables were obtained with an R^2 of 0.9455, 0.9308 and 0.8998, respectively. The optimal extraction conditions to obtain maximum yields of targeted compounds were 30 mL/g X_1, 45% (v/v) X_2, 80°C X_3 and 12 min X_2. The experimental values were in good agreement with the predicted values under the optimized conditions. The major constituents in the optimized extract were organic acids whereas phenolic compounds were minor components. Delphinidin-3-sambubioside-5-rhamnoside and delphinidin were identified by HPLC-ESI/MS as major anthocyanins in the extract. The plant extract showed moderate inhibitory activity on mushroom tyrosinase with a non-competitive inhibitory mechanism. The UAE can be considered as an effective method for extracting the biologically active compounds from fruits of *A. puncticulatum*.

Keywords: *Antidesma puncticulatum* Miq., anthocyanins, antityrosinase activity, phenolic compounds, response surface methodology, ultrasonic assisted extraction

1. INTRODUCTION

Phenolic compounds-rich plant extracts are considered as good sources for health promoting effects in functional food industry [1-4]. One of the interesting sources for

phenolic compounds is tropical fruits. Of 18 Thai *Antidesma* species, *Antidesma puncticulatum* Miq. (family Phyllanthaceae; synonyms, *A. bunius* (L.) Spreng. var. *thwaitesianum* (Müll.Arg.) Trim. and *A. thwaitesianum* Müll.Arg.) is an indigenous plant in Southeast Asia, especially in the dipterocarp forest of the Phupan Valley in the north-eastern area of Thailand [5]. The fruits of this plant are classified to be bigger in size and grow up to 8 mm compared with other fruit plants belonging to the same genus. Its ripe fruit is of purplish-red or dark-purple which depending on the level of ripeness that occurs from August to October.

Several phenolic compounds have been reported in fruits-based wine of *A. puncticulatum*. These are caffeic acid, catechin, gallic acid and monogalloyl glucoside. The fruits-based wine also contains anthocyanin pigments, such as cyanidin-3-sophoroside, delphinin-3-sambubioside and pelargonidin-3-malonyl glucoside [6]. Not only the constituents have been identified but also a potential health promoting benefit related to the above mentioned bioactive components have been reported. The biological activities described in literature are anti-apoptosis and anti-inflammatory effects in human breast epithelial cells [7], antioxidant [6], and α-glucosidase inhibitory activity [8].

In order to use these compounds as either bioactive components or colorants in functional foods, an efficient extraction procedure should be the primary step to maximize the targeted yields. The use of ultrasonic assisted extraction (UAE) is recognized as one of the modern techniques for the extraction of bioactive compounds from plant material [9]. Compared to other techniques (solvent extraction, microwave assisted extraction and supercritical fluid extraction), the UAE offers some advantages as it is a simple method with low instrument requirements and easy to scale up for industries [10]. The principle of UAE to enhance the phenolic compounds recovery is based on the production of acoustical cavitation to disrupt the cell wall of the plant material, leading penetration of solvent to dissolve desired constituents [11]. The use of a low temperature for the extraction process protects degradation of the heat unstable compounds [9].

To achieve a maximum yield of desired bioactive compounds, a response surface methodology (RSM) was employed together with UAE. This technique has been introduced as an effective statistic method for optimizing complex processes. It offers an economically efficient procedure compared to using a one-variable-at-a-time methodology which is the classical method. This is because the RSM can be used to study several process variables simultaneously interacting with each other. A lower number of experiments are required to predict the optimal conditions of the desired system [2].

However, there were no studies undertaken up to now to maximize the content of the bioactive compounds in the ripe fruits of *A. puncticulatum*. In the present study, some extraction variables of UAE affected the extraction yields of total phenolic content (TPC) and total anthocyanin content (TAC) of *A. puncticulatum* fruits were conducted using the RSM. The quantification of organic acids in the extract obtained from the optimized extraction conditions was performed by high-performance liquid chromatography (HPLC). The major anthocyanin constituents in the extract were identified by high-performance liquid chromatography tandem electrospray ionization mass spectrometry (HPLC-ESI/MS). Additionally, *in vitro* antityrosinase activity was also tested to support the use of the

extracts for health promotion benefits.

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh fruits of *A. puncticulatum* in a fully-ripe stage were harvested in August 2013 from Ubon Ratchathani province, Thailand. The plant was identified by Dr. Bancha Yingngam and its voucher specimen was deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University (BCY UBU no. 025). The whole fresh fruits were dried in a freeze dryer (Martin Christ GmbH, Germany) at -55°C for 72 h and powdered through a 60-mesh sieve before extraction.

2.2 Chemicals

The authentic L-ascorbic acid, cyanin-3-glucoside, gallic acid, L-3, 4-dihydroxyphenylalanine (L-DOPA), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxyl-2, 5-7,8-tetramethyl-chroman-2-carboxylic acid (trolox), kojic acid, and mushroom tyrosinase (1881 units/mg) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

2.3 UAE Procedure

The UAE was performed using an ultrasonic bath (ULTRAsonika™ 57H model, C&A Sales Industrial Supplies, USA). The ultrasonic bath was set a working frequency of 45 kHz and controlled temperatures by circulating external from a temperature controller. One gram of *A. puncticulatum* fruit powder was subjected to the UAE for predetermined points of each variable. The obtained samples were then centrifuged at 3500 rpm for 10 min, followed by collecting the supernatant for further quantification of total anthocyanin and total phenolic contents.

In order to set the range of each variable into the RSM, effects of relevant independent parameters affected on the yields of the corresponding response variables were studied using a one-variable-at-a-time method. The TPC and TAC were used as indicators of bioactive compounds. Briefly, the effect of liquid-to-solid ratio was firstly studied in the range from 5 to 60 mL/g while other extraction parameters were kept constant (25% (v/v) ethanol acidified with 1% (v/v) acetic acid was fixed in all experiments, 45°C, 10 min). Second, the impact of the concentrations of acidified ethanol in water (0-100% (v/v)) on the extraction yields of the above-mentioned variables were evaluated. Other extraction parameters were performed at 20 mL/g solvent-to-solid ratio, 45°C, and 10 min. Third, influence of extraction temperatures was examined in the range starting from 30°C to 90°C. The extraction conditions were fixed constant for other variables at 20 mL/g solvent-to-solid ratio, 25% acidified ethanol solution, and 10 min extraction time. Finally, the effect of extraction times affected on extraction yields of the dependent variables was investigated. The ranges of this factor were varied between 5 and 40 min whereas other process parameters were fixed constant at 20 mL/g solvent-to-solid ratio and 25% acidified ethanol solution at 45°C.

2.4 Experimental Design

The RSM was performed using Design Expert software (version 7.0.0, Stat-Ease, Inc., Minneapolis, MN, USA) to optimize process parameter for obtaining the maximized yield of total anthocyanin content and total phenolic content. A central composite design (CCD) of RSM was employed in the experiments by setting the ranges of each independent variable based on the values obtained from a one-variable-at-a-time method described in

the section above. Liquid-to-solid ratio (X_1), acidified ethanol concentration (X_2), extraction temperature (X_3), and extraction time (X_4) was chosen as independent variables for the experiments. The TPC (Y_1) and TAC (Y_2) and antioxidant activity (Y_3) were selected as the responses for the combination of those independent variables. The CCD was performed that consisting of 2^4 factorial designs, 8 axial points, and 6 replicates as indicated in Table 1. The experimental number of factorial designs and axial points of the study was applied for estimation of curvature of the model whereas the replicate experiments used for estimation of a pure error sum of squares [12]. The experiments were randomized to minimize the effects of unexplained variability affected by extraneous factors. A second-order polynomial equation corresponding to the CCD was given as follows:

$$Y = \beta_0 + \Sigma_{i=1}^{3} \beta_i X_i + \Sigma_{i=0}^{3} \beta_{ii} X^2 + \Sigma_{i=0}^{2} \Sigma_{j=j+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

Table 1. The CCD and experimental data for extraction of the total phenolic content (Y_1), the total anthocyanin content (Y_2), and the antioxidant activity (Y_3) from *A. puncticulatum* fruits.

Experimental number	Independent variables				Dependent variables		
	X_1 (mL/g)	X_2 (%v/v)	X_3 (°C)	X_4 (min)	Y_1 (mg GAE/g dry powder)	Y_2 (mg Cy-3-glu/g dry powder)	Y_3 (mM TE/g dry powder)
1	15	20	40	5	27.04±1.09	0.92±0.03	1.24±0.03
2	30	20	40	5	30.13±1.89	1.00±0.01	1.61±0.02
3	15	60	40	5	30.67±3.33	0.98±0.02	1.44±0.02
4	30	60	40	5	30.84±1.38	1.06±0.03	1.80±0.04
5	15	20	80	5	35.60±2.07	0.98±0.01	1.46±0.02
6	30	20	80	5	43.37±5.09	1.07±0.02	1.71±0.07
7	15	60	80	5	36.51±1.81	1.05±0.06	1.72±0.03
8	30	60	80	5	38.82±5.02	1.12±0.03	1.89±0.04
9	15	20	40	15	28.33±0.82	0.96±0.02	1.51±0.02
10	30	20	40	15	27.41±2.32	0.96±0.02	1.93±0.01
11	15	60	40	15	31.06±1.16	1.07±0.05	1.68±0.02
12	30	60	40	15	31.22±2.24	1.09±0.04	2.54±0.02
13	15	20	80	15	34.65±0.87	0.93±0.03	1.92±0.01
14	30	20	80	15	32.66±0.37	1.02±0.04	2.80±0.02
15	15	60	80	15	38.97±1.35	1.06±0.03	2.01±0.02
16	30	60	80	15	39.12±0.54	1.12±0.02	2.38±0.05
17	7.5	40	60	10	33.76±0.76	0.96±0.07	1.61±0.01
18	37.5	40	60	10	41.17±2.86	1.11±0.10	2.66±0.03
19	22.5	0	60	10	18.88±0.62	0.75±0.01	1.04±0.03
20	22.5	80	60	10	25.13±2.02	1.01±0.11	1.59±0.01
21	22.5	40	20	10	32.55±0.77	1.02±0.02	2.17±0.04
22	22.5	40	100	10	53.60±1.85	1.02±0.02	2.66±0.02
23	22.5	40	60	0	40.03±0.72	1.09±0.04	2.29±0.03
24	22.5	40	60	20	39.01±0.65	1.08±0.03	2.49±0.01
25	22.5	40	60	10	39.85±1.37	1.02±0.03	2.55±0.03
26	22.5	40	60	10	42.95±1.12	1.01±0.02	2.49±0.01

Table 1. Continued.

Experimental number	Independent variables				Dependent variables		
	X_1 (mL/g)	X_2 (%v/v)	X_3 (°C)	X_4 (min)	Y_1 (mg GAE/g dry powder)	Y_2 (mg Cy-3-glu/g dry powder)	Y_3 (mM TE/g dry powder)
27	22.5	40	60	10	43.04±1.72	1.06±0.05	2.76±0.02
28	22.5	40	60	10	39.63±1.17	0.99±0.02	2.43±0.02
29	22.5	40	60	10	39.88±1.12	1.03±0.02	2.59±0.07
30	22.5	40	60	10	40.00±1.0	1.04±0.20	2.51±0.03

X_1 solvent to solid ratio; X_2 acidified ethanol concentration; X_3 extraction temperature; X_4 extraction time

where Y is the predicted response of dependent variable, b_0 is the model constant, b_i, b_{ii}, and b_{ij} are the model coefficients representing the linear, quadratic and interaction effects of the variables. Finally, the obtained predicted mathematical models giving the maximum recovery yield of Y_1, Y_2 and Y_3 were checked to verify the validity of the obtained mathematical models. For chemical and biological characterization of the extract, the ethanol of the samples obtained from the optimum extraction process was removed under vacuum by a rotary evaporator (BüCHI, Flawil, Switzerland), followed by drying under a freeze-dryer.

2.5 Determination of TPC

The TPC of the plant extracts was determined using the Folin-Ciocalteu method as described by Gong et al. [13]. The results were expressed as mg gallic acid equivalent per gram of plant powder (mg GAE/g).

2.6 Determination of TAC

The TAC in the extract was determined using a pH differential method according to the previously reported method [12]. The amount of total anthocyanins was calculated in terms of cyanidin-3-*O*-glucoside equivalents per gram of dry plant powder (mg Cy-3-glu/g).

2.7 DPPH• Free Radical Scavenging Activity Assay

The antioxidant activity (AA) of *A. puncticulatum* extract was measured using DPPH• assay according the method described by Gong et al. [13]. The antioxidant activity of the plant extract was calculated from the standard curve of trolox and the results are expressed as millimolar trolox equivalent per gram of dry plant powder (mM TE/g).

2.8 Quantification of Organic acids in the *A. puncticulatum* Fruit Extract

The amounts of the five organic acids, citric acid, L-ascorbic acid, L-(+)-tartaric acid, oxalic acid and L-malic acid, in the freeze dried extract obtained under the optimum extraction conditions were determined by HPLC-DAD (Agilent Technologies 1100 series, USA). The HPLC instruments comprised of a degasser, quaternary pump, automated sample injector, column oven and a diode array detector. Separation of the sample (5 µL) was carried out on Lichrosphere C_{18} (4.5 mm × 250 mm i.d., 4.5 µm particle size) using a 50 mM phosphate buffer solution as mobile phase. The flow rate was set at 0.7 mL/min at 35°C. The detection wavelength of each organic acid was set at 214 nm, except for L-ascorbic acid (254 nm). The results were expressed as mg/g freeze dried extract.

2.9 Identification of Phenolic Compounds and Anthocyanins

The constituents of phenolic compounds and anthocyanins in the extract were identified using a positive and a negative ionization mode of HPLC-ESI/MS (Thermo Fisher Scientific Company, USA). This instrument consisted of a Dionex UltiMate 3000 UHPLC coupled with a mass spectrometer. The sample (5 µL) was separated on a Lichrosphere C_{18} column (250 × 4.6 mm i.d., 4.5 µm particle size) by setting a flow rate of 0.6 mL/min at 25°C. The mobile phase was 0.1% formic acid in water (A) and acetonitrile (B). The gradient elution was 90%A (4 min), from 90% to 80%A (6 min), from 80% to 10%A (30 min), constant at 10%A (5 min), and from 10% to 90%A (15 min). The positive ion electrospray was used by fixing nebulizer gas (N_2) at a pressure of 6 bars and flow rate of 1.5 L/min. Heated capillary was set at 230°C with a voltage of 1.7 kV. The full scans mass spectra were measured from m/z 50 to 2000. Mass fragments of compounds in the extract were identified based on their retention times, UV-VIS spectra and mass spectra in comparison to authentic standards and to literature data.

2.10 Enzymatic Assay for Testing the Antityrosinase Activity

2.10.1 Tyrosinase inhibition assay

The tyrosinase inhibition activity was determined according to the method of Chang et al. [14]. The IC_{50} of tyrosinase activity of the samples leading to 50% loss of activity was calculated. Kojic acid was used as standard reference (0.005-1 mg/mL).

2.10.2 Kinetic analysis

The kinetic reaction of *A. puncticulatum* extracts affected on activity of mushroom tyrosinase was examined [14]. The Lineweaver-Burk plots were constructed and Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of the tyrosinase were calculated.

2.11 Statistical Analysis

All experiments were performed in triplicate and the results are expressed as means ± standard deviation (SD) (n=3). The RSM data were analyzed using Design Expert software. Analysis of variance (ANOVA) was used to determine by setting the p value lower than 0.05 as a minimum statistically significant difference. The lack of fit of the mathematical model was checked with a Fisher's statistical test (F-test). The fitness of the second order polynomial model was determined and expressed in terms of the coefficient (R^2, R_{adj}^2).

3. RESULTS AND DISCUSSION

3.1 Selection of Relevant Ranges of Tested Independent Variables

3.1.1 Effect of solvent-to-solid ratio

As shown in Figure 1A, the extraction efficiency of TPC significantly increased from 29.80±0.87 to 36.50±1.54 mg GAE/g as the ratio of solvent-to-solid increased within the ranges of 5 to 20 mL/g (p<0.05). Like extraction yields of TPC, ratio of solvent-to-solid showed similar yields of TAC (Figure 1B). These results should be attributed to the increase in driving force of the mass transfer of targeted compounds [2]. Further increasing the ratios of solvent-to-solid did not affect the yields of TPC and TAC. The mass transfer of these chemicals reached a plateau at the liquid-to-solid ratio of 20 mL/g for TPC and TAC. The solvent-to-solid ratio of 20 mL/g was chosen for the following experiments.

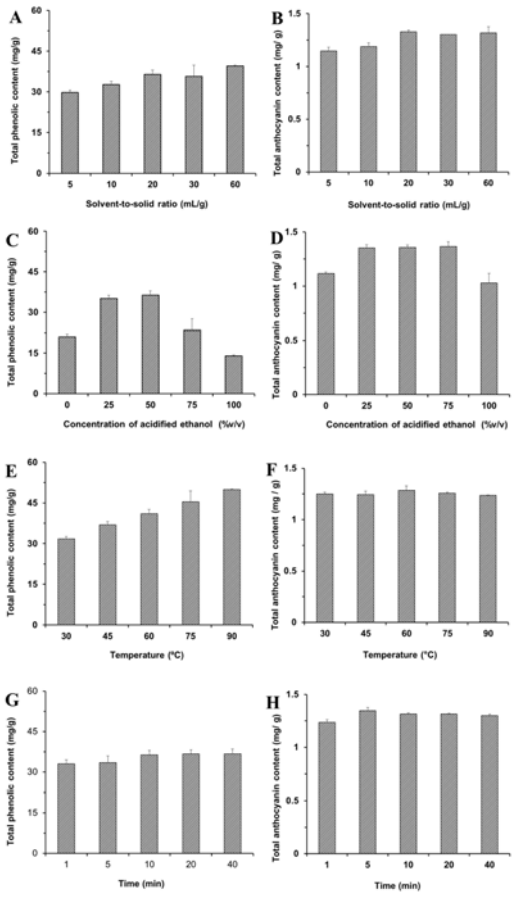

Figure 1. Effects of four independent variables on the yields of total phenolic content and total anthocyanin content from *A. puncticulatum* fruits: **A**-**B** solvent-to-solid ratios, **C-D** acidified ethanol concentrations, **E-F** extraction temperatures, and **G-H** extraction times. Values are means ± standard deviation of three separate determinations (n = 3).

3.1.2 Effect of acidified ethanol concentration

Acidified ethanol solution (1% (v/v) acetic acid) was employed as extraction solvent because anthocyanins usually are extracted under the mild conditions of acidified solvents [15]. The extraction yields of TPC increased with higher percentages of acidified ethanol solutions from 0% (21.11±1.41 mg GAE/g) to 25% (36.38±1.89 mg GAE/g) (p<0.001) (Figure 1C). Not any change the yields of TPC was observed when percentages of acidified ethanol solutions continued to increase from 25% to 50% (p>0.05). On the contrary, a sharp decrease in the yield of TPC was found with increasing concentrations of acidified ethanol from 50% (36.38±1.89 mg GAE/g) to 75% (23.49±1.16 mg GAE/g) (p<0.001). Yields of TAC showed a similar pattern like TPC yields by increasing to a maximum value at 25% acidified ethanol solution (Figure 1D). Concentrations of acidified ethanol higher than 75% sharply dropped the yields of TPC (p<0.001). Thus, 25% acidified ethanol solution was fixed constant for the following experiments.

3.1.3 Effect of extraction temperature

The extraction yields of TPC increased with rising the temperatures up to 75°C, followed by remaining constant up to 90°C (Figure 1E) whereas not any change was observed for the TAC yields (Figure 1F). This may be due to the increasing temperatures enhanced both diffusion coefficient and solubility of the desired phenolic compounds [2]. In order to avoid the degradation of some heat sensitive compounds at high temperature, 75°C was the preferable temperature for extraction of the targeted compounds.

3.1.4 Effect of extraction time

The extraction yields of dependent variables increased as the extraction times increased from 1 to 10 min (Figure 1G-H). The maximum yield of TPC was found at 10 min of extraction time whereas the highest yield of TAC was observed after 5 min. There was no increasing in the yields of TPC and TAC when rising the extraction time up to 40 min. The possible reason for this phenomenon may be caused by the very

good solubility of the phenolic compounds and anthocyanins of *A. puncticulatum* fruits in hydroalcoholic solutions resulting in a shorter extraction time.

3.2 Statistical Analysis and Fitting to the Response Surface Models

A total of 30 experimental numbers was designed and the corresponding response values are shown in Table 1. Extracted yields of TPC and TAC and measurement of the AA of the plant extract were observed in ranges between 18.88-53.60 mg GAE/g, 0.75-1.12 mg Cy-3-glu/g, and 1.00-2.80 mM TE/g, respectively. The experimental values of both independent and dependent variables were analyzed to obtain the regression equation through various models. The results indicated the quadratic model to be the best model for the extraction process by exhibiting the probability values (p) lower than 0.0001. The model summary statistics for Y_1, Y_2, and Y_3 also showed the maximum values of R^2, R_{adj}^2, and R_{pred}^2 in comparison to other models. The determination coefficient (R^2) is a coefficient of the total variation in the response predicted model. A high value of R^2 indicates a good adequacy of the model [2]. In this study, the R^2 for TPC, TAC and AA were 0.9455, 0.9308 and 0.8998, respectively. These values indicated that the obtained models could predict the responses within the range of the independent variables. The tested independent variables and the corresponding variables were related by a second-order polynomial model as given in terms of the actual factors as follows:

$$Y_1 = 40.90 + 1.07X_1 + 1.27X_2 + 4.38X_3 - 0.48X_4 - 0.32X_1X_2 + 0.36X_1X_3 - 1.00X_1X_4 - 0.23X_2X_3 + 1.04X_2X_4 - 0.52X_3X_4 - 1.19X_1^2 - 5.05X_2^2 - 0.21X_3^2 - 0.68X_4^2 \quad (2)$$

$$Y_2 = 1.03 + 0.033X_1 + 0.051X_2 + 0.013X_3 + 0.000416X_4 - 0.001875X_1X_2 + 0.0008125X_1X_3 - 0.009375X_1X_4 - 0.000625X_2X_3 + 0.014X_2X_4 - 0.013X_3X_4 + 0.005729X_1^2 - 0.033X_2^2 + 0.001979X_3^2 + 0.018X_4^2 \quad (3)$$

$$Y_3 = 2.56 + 0.24X_1 + 0.099X_2 + 0.13X_3 + 0.18X_4 - 0.01X_1X_2 - 0.021X_1X_3 + 0.086X_1X_4 - 0.066X_2X_3 - 0.024X_2X_4 + 0.48X_3X_4 - 0.14X_1^2 - 0.35X_2^2 - 0.07X_3^2 - 0.076X_4^2 \quad (4)$$

The ANOVA for those mathematical models was summarized in Table 2. Significance of each variable was evaluated by ANOVA analysis followed by an F-test. The F-values indicated the influence of each independent variable on the corresponding responses. The model of Y_1, Y_2 and Y_3 showed F-values of 18.59, 14.42, and 9.63 with their p-values less than 0.001, indicating the model significance [12]. The fitness of the models was analyzed through the lack of fit and the suitability models to predict accurately and the variable should have a p-value higher than 0.05 [2]. The lack of fit coefficients for Y_1, Y_2, Y_3 had F-values of 2.33, 1.37, and 5.10 with non-statistically significant difference (p>0.05) indicating the predicted models were not relative to the pure error. The coefficient of variation (C.V.%) was analyzed to evaluate the variation of the experimental data from the predicted models. The results found that these values were 6.31, 2.66 and 10.76 for Y_1, Y_2 and Y_3, respectively, indicating a high precision and a good reliability of the experiments [12].

The p-values were also used to check the effects of four independent variables on the responses. If the p-values are less than 0.05, the term coefficients are significant [2]. The results for analysis of the TPC yield (Y_1)

found that the linear coefficients (X_1, X_2, X_3) and the quadratic coefficients (X_1^2, X_2^2) were important factors due to their statistical significant differences (p<0.05). In cases of the yields of TAC, the linear coefficients (X_1, X_2, X_3), interaction coefficients (X_2X_4), and the quadratic coefficients (X_2^2, X_4^2) were statistically significant (p<0.05). For AA (Y_3), the coefficients that showed statistical significance were four linear coefficients (X_1, X_2, X_3, X_4) and two quadratic coefficients (X_1^2, X_2^2). The remaining coefficients were not important factors due to the fact that there was no significant statistical difference (p>0.05). Therefore, X_1, X_2, X_2 and X_4 were important factors for the extraction of target compounds from *A. puncticulatum* fruits.

Table 2. ANOVA and statistical parameters for the response surface models and the lack of fit testing of extraction of target compounds from *A. puncticulatum* fruits.

Source	Total phenolic content (Y_1)				Total anthocyanin content (Y_2)				Antioxidant activity (Y_3)			
	SS [a]	DF [b]	MS [c]	F-value	SS [a]	DF [b]	MS [c]	F-value	SS [a]	DF [b]	MS [c]	F-value
Model	1308.90	14	93.49	18.59□	0.1500	14	0.0110	14.42□	6.56	14	0.47	9.63□
Linear coefficient												
X_1	27.24	1	27.24	5.42*	0.0260	1	0.0260	35.43□	1.39	1	1.39	28.61□
X_2	38.84	1	38.84	7.72#	0.0630	1	0.0630	85.90□	0.24	1	0.24	4.85*
X_3	460.34	1	460.34	91.53□	0.0004	1	0.0004	5.46*	0.41	1	0.41	8.34#
X_4	5.62	1	5.62	1.12ns	<0.0001	1	<0.0001	<0.01ns	0.77	1	0.77	15.84□
Interaction coefficients												
X_1X_2	1.66	1	1.66	0.33ns	<0.0001	1	<0.0001	0.08ns	0.0016	1	0.0016	0.03ns
X_1X_3	2.07	1	2.07	0.41ns	0.0011	1	0.0011	1.44ns	0.0072	1	0.0072	0.15ns
X_1X_4	15.90	1	15.90	3.16ns	0.0009	1	0.0009	1.92ns	0.12	1	0.12	2.45ns
X_2X_3	0.87	1	0.87	0.17ns	<0.0001	1	<0.0001	0.01ns	0.07	1	0.07	1.44ns
X_2X_4	17.24	1	17.24	3.43ns	0.0028	1	0.0028	4.51*	0.009	1	0.009	0.19ns
X_3X_4	4.25	1	4.25	0.85ns	0.003	1	0.003	3.76ns	0.036	1	0.036	0.74ns
Quadratic coefficients												
X_1^2	38.79	1	38.79	7.71#	0.0009	1	0.0009	1.23ns	0.54	1	0.54	11.08#
X_2^2	700.68	1	700.68	139.32□	0.0300	1	0.0300	40.75□	3.27	1	3.27	67.19□
X_3^2	1.25	1	1.25	0.25ns	0.0001	1	0.0001	0.15ns	0.14	1	0.14	2.78ns
X_4^2	12.52	1	12.52	2.49ns	0.0091	1	0.0091	12.42#	0.16	1	0.16	3.30ns
Residual	75.44	15	5.03		0.0110	15	0.0007		0.73	15	0.049	
Lack of fit	62.13	10	6.21	2.33ns	0.0081	10	0.0008	1.37ns	0.66	10	0.066	5.10ns
Pure error	13.31	5	2.66		0.0029	5	0.0006		0.065	5	0.013	
Cor total	1384.34	29			0.16	29			7.29	29		
R^2	0.9455				0.9308				0.8998			
Mean	35.53				1.02				2.05			
C.V. %	6.31				2.66				10.76			
Adeq. Precision	20.42				17.64				10.46			

[a] Sum of squares, [b] Degree of freedom, [c] Mean square; R^2 = Coefficient of multiple determination; R_{adj}^2 = Adjusted R^2; C.V.% = Coefficient of variance

Statistically significant difference at * p<0.05, # p<0.01, and □ p< .001; ns: non-significant

3.3 Response Surface Analysis

In order to understand more details about the relationships between the independent variables and their interactions affected on the dependent variables, three dimensional surface and contour background plots were constructed from mathematical models given in Eq. (5)-(7). Each plot composed of two independent variables affected on the corresponding response variables by fixing the remaining variables at level zero.

3.3.1 Effect of extraction parameters on TPC

Figure 2 shows effects of four independent variables on the extraction yields of TPC (Y_1). X_2 was the most influenced

parameter affected on Y_1 yield (p<0.001), followed by X_2 (p<0.01) and X_1 (p<0.05), respectively, whereas extraction time did not significantly exhibit on such response (p>0.05). No interaction between each independent variable was observed for Y_1 yield (p>0.05).

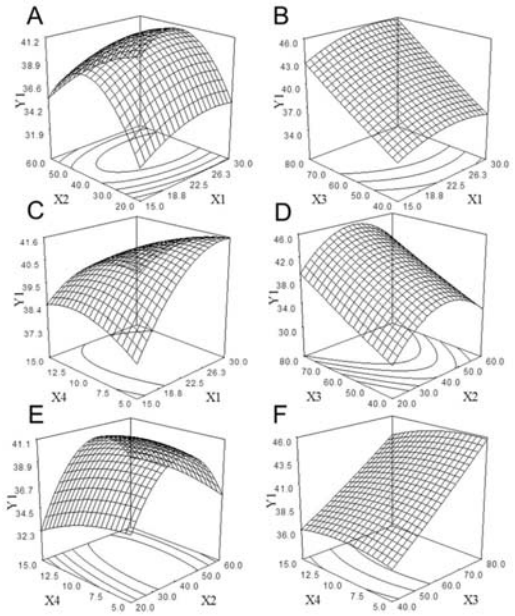

Figure 2. Response surface plot showing the effects of independent variables on the yield of total phenolic contents: **A** solvent-to-solid ratio and acidified ethanol concentration, **B** solvent-to-solid ratio and extraction temperature, **C** solvent-to-solid ratio and extraction time, **D** acidified ethanol concentration and extraction temperature, **E** acidified ethanol concentration and extraction time, **F** extraction temperature and extraction time.

Lower values of X_1 in ranges of 15-22.5 mL/g resulted in lower yields of Y_1. However, the yield of Y_1 increased when raising the values of X_1 from 22.5 to 30 mL/g, followed remaining constant up to 37.5 mL/g (Figure 2A, B, C). This is probably due to the fact that more solvent can permeate through the cell walls of the plant material to dissolve the phenolic compounds [16]. These results are in agreement with previous reports describing that the addition of ethanol into water plays a significant role for the yield of TPC in the extract [2].

The ability to extract phenolic compounds with a single solvent was much lower compared to a combined solvent system between alcohol and water [16]. As expected, increasing acidified ethanol concentration from 20 to 45% resulted in increased yields of Y_1 (Figure 2A, D, E). However, Y_1 yields decreased when extracting with acidified ethanol concentrations higher than 45%. The reason therefore might be that the addition of a certain amount of ethanol to the extraction solvent gives an appropriate polarity to phenolic compounds whereas higher amounts of ethanol in the extraction solvent give a lower polarity [2]. These findings are in good agreement with previous reports [16].

The extraction temperature showed a positive effect on Y_1 yields (Figure 2B, D, F). Yields of Y_1 rapidly increased within an extraction temperature from 40°C to 80°C. This result should be attributed to a higher temperature which show the ability to rupture the phenolic matrix bonds, decrease the dielectric constant of water and solvent viscosity, and improve the solubility and diffusion rate of phenolic compounds, resulting in improved extraction efficiency [12,17].

3.3.2 Effect of extraction parameters on TAC

Anthocyanins are water-soluble compounds that belong to a parent class of phenolic compounds. As presented in Figure 3, X_2 showed the strongest effects on Y_2 yield (p<0.001), followed by X_1 (p<0.001) and X_3 (p<0.05), but not X_4 (p>0.05). It was noticed that X_2 and X_4 exhibited positive

interactive effects by showing a positive effect on the yield of Y_1 (p<0.05) (Figure 3E). Increasing values of X_1 resulted in increased yields of TAC starting from 15 mL/g up to 30 mL/g. Like this, the pattern of Y_2 yields increased with raising X_2 values in the range of 20-45% (Figure 3A, D, E). This is probably due to the fact that more solvent with the appropriate polarity can enter into the plant cells to dissolve higher amounts of anthocyanins [18].

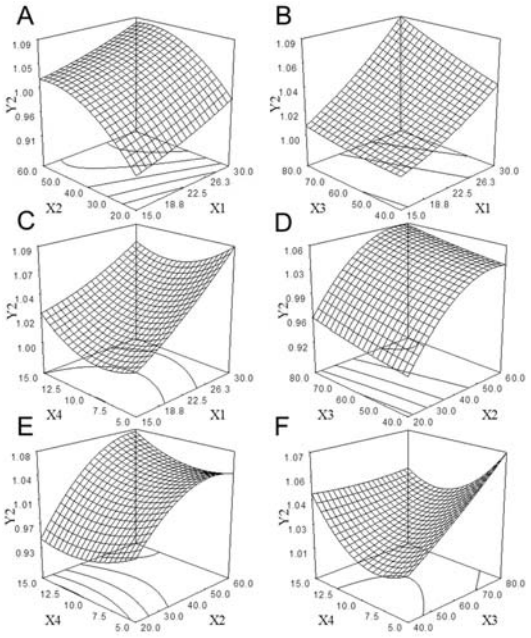

Figure 3. Response surface plot showing effects of independent variables on the yield of total anthocyanin contents (refer to Figure 3 for (**A**), (**B**), (**C**), (**D**), (**E**) and (**F**) plots).

Like above tested variables, the yields of Y_2 also increased with increased X_3 in a range between 40°C-80°C (Figure 3B, D, F). The reason to describe why raising the extraction temperature increased the TAC yield should attribute from several properties together: softening of the plant tissue, disrupting the bonding between anthocyanins and plant matrix, increasing solubility and diffusion rate of anthocyanins [12].

3.3.3 Effect of extraction parameters on AA

The results showed that *A. puncticulatum* fruit extract demonstrated good antioxidant activity. This may be due to the hydroxyl groups in the antioxidant compounds (organic acid, anthocyanins and other phenolic compounds) which act as electron donors. These molecules can terminate the radical chain reaction by converting the DPPH$^{\bullet}$ radicals into a more stable form [9,19].

With respect to effects of extraction parameters affected on antioxidant activity (AA) of the extracts, X_1 was the most influenced factor (p<0.001), followed by X_4 (p<0.001), X_3 (p<0.01), and X_2 (p<0.05). However, there was no interaction of those tested independent variables on AA (p>0.05). As shown in Figure 4A -C, higher antioxidant activity of the extract was observed when raising X_1 values from 15 mL/g up to 30 mL/g. This is probably because a higher volume of solvent can dissolve more antioxidant compounds when compared to a lower volume [16,18].

Next, the effect of acidified ethanol concentration (X_2) on the AA was evaluated and the results are shown in Figure 4A, D, and E. This variable influenced the linear and quadratic coefficient of the antioxidant activity. The AA of the extract increased with the increased X_2 value from 20% to 45%, and decreased when the proportion of such variable further increased up to 60%. These results are in agreement with our previous study due to the addition of ethanol to water has a great influence on the antioxidant activity of extracts [2]. A correlation between the AA of extracts and the extraction temperature could be observed. The AA of the extract increased with increasing temperature (Figure 4B, D and F). Although several antioxidant compounds may degrade when exposed to high

temperature [20], the antioxidant activity of the extract in this study did not decrease in the range of temperature used (40-80°C). This may be due to the rate of extraction of thermally stable antioxidants which was higher than the rate of the thermal decomposition of the unstable ones [21].

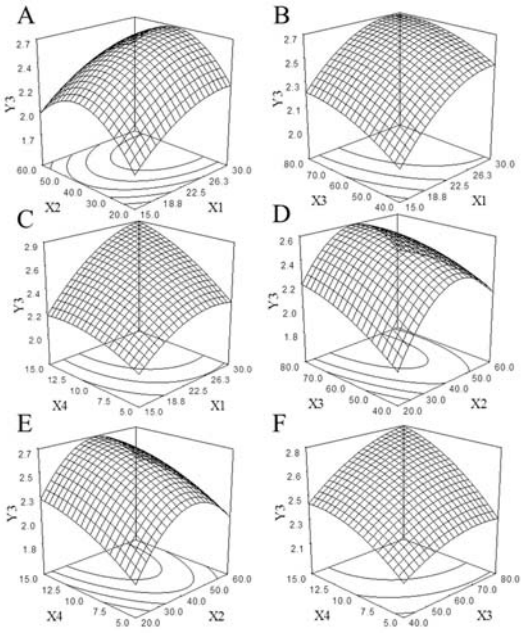

Figure 4. Response surface plot showing effects of independent variables on the antioxidant activity (refer to Figure 3 for (**A**), (**B**), (**C**), (**D**), (**E**) and (**F**) plots).

It could be also noticed that the extraction temperature exhibited a more significant effect than the acidified ethanol concentration on the AA of the extract. This may result from the fact that *A. puncticulatum* contains organic acids as main components as described in the following section. These compounds dissolve easier in the hydroalcoholic solvent and the extractability of these compounds improved with higher temperature. Finally, X_4 showed a significant effect on Y_3 of the extracts (p<0.001). The Y_3 values of the extract increased with the increasing of extraction time from 5 min to 12.20 min, but beyond this level the antioxidant activity reached the plateau region (Figure 4C, E, F).

3.4 Validation of the Optimized Conditions

The optimum conditions for extracting the target compounds from *A. puncticulatum* fruits by UAE was obtained at X_1, X_2, X_3 and X_4 of 30 mL/g, 44.83%, 79.99°C, and 12.20 min, respectively. These conditions were re-checked to verify the suitability of the obtained mathematical models and to ensure that no bias toward the experimental values using the slightly modified conditions: X_1 of 30 mL/g, X_2 of 45%, X_3 of 80°C and X_4 of 12 min. The experimental values of Y_1, Y_2 and Y_3 under such conditions were 44.89±1.03 mg GAE/g, 1.07±0.02 mg Cy-3-glu/g, and 2.73±0.19 mM TE/g dry powder, respectively. These experimental values matched well with the predicted values (p>0.05), indicating the proposed mathematical models were adequate for extracting the target compounds from *A. puncticulatum* fruits.

With respect to the advantages of UAE, similar results were also reported for other plants concerning the improvement of bioactive compounds extractability [22,23]. Applying the UAE caused structured changes by destructing the cell walls of the plant material [23]. Thus, the UAE has a higher efficiency than the conventional extraction methods, i.e., heat reflux extraction, concerning the reduction of extraction time and energy consumption.

3.5 Chemical Characterization of Freeze Dried *A. puncticulatum* Extract

3.5.1 Quantification of organic acids

The freeze dried samples of *A. puncticulatum* fruit extracts were purple in color due to anthocyanin constituents. Organic acids were the major components in such

extracts and their amounts were analyzed by a HPLC method. The plant extract contained amounts of L-(+)-tartaric acid, citric acid, L-malic acid, ascorbic acid, and oxalic acid of 71.95±0.49, 62.61±3.24, 15.24±1.92, 1.05±0.24, and 0.60±0.02 mg/g freeze dried extract, respectively.

3.5.2 Identification of phenolic compounds

The phenolic compounds-containing plant extract was identified by both negative and positive ionization modes using HPLC-ESI/MS. Compound 1 (retention time, t_R=4.03 min) showing a $[M-H]^-$ parent ion at m/z 191 was identified to be quinic acid. Compound 2 (t_R=13.20 min) exhibited $[M-H]^-$ parent ion at m/z 593 with producing a fragment ion at m/z 285 was identified as kaempferol-3-*O*-rutinoside. Compound 3 (t_R=14.10 min) with a $[M-H]^-$ parent ion at m/z 353 was identified to be 5-*O*-caffeoylquinic acid. Compound 4 (t_R =16.88 min) was identified as guaiacyl (8-5)ferulic acid hexoside. This is the first time that glycosylated lignans are reported in *A. puncticulatum* fruit extract. Compound 4 (t_R = 18.28 min) showed $[M-H]^-$ parent ion at m/z 301. This ion further produced to form characteristic fragment ion at m/z 257 and 229 whereas quercetin exhibited fragment ion at m/z 179 and 151. Thus, it was identified as ellagic acid, not quercetin. These phenolic compounds may play an important role in contributing to the antioxidant activity of the extracts.

3.5.3 Identification of anthocyanins

The anthocyanins in the freeze dried extract were identified using HPLC-ESI/MS with a positive ionization mode. Two anthocyanins were detected in the mass spectrograms regarding the data of fragmentation patterns, UV-VIS spectra, sugar moieties and retention times (t_R). The first compound (t_R =14.29 min) showed a $[M-H]^+$ parent ion at m/z 743 (Figure 5A). The MS^2 secondary peak at m/z 597 indicated the loss of a rhamnose residue (M^+-146) from a molecular ion. The MS^1 parent ion produced a MS^2 base peak at m/z 303 (delphinidin) due to fragmentation of a disaccharide residue (hexose + pentose moiety, 162+132). The MS^1 secondary peak at m/z 465 also supported the sequential loss of pentose residue (M^+-132). Presence of sambubioside in the chemical structure of this compound also produced a greater polarity. Thus, this compound was eluted before the corresponding aglycone when separating by HPLC. This result suggested that it was delphinidin-3-sambubioside-5-rhamnoside.

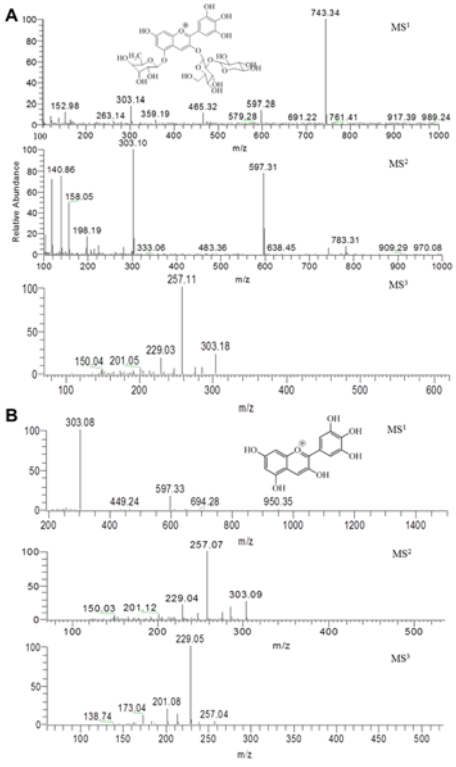

Figure 5. Tentative identification of anthocyanins in the *A. puncticulatum* extract: **A** delphinidin-3-sambubioside-5-rhamnoside and **B** delphinidin.

The latter compound (t_R =15.02 min) showed $[M-H]^+$ parent ion at m/z 303, followed by yielding MS^2 and MS^3 fragments at m/z 257 and 229, respectively (Figure 5B). Thus, this compound was identified as delphinidin. This result was confirmed by comparison with its UV-VIS spectra and mass characteristics and data from literatures [24]. However, other anthocyanins that have been reported elsewhere as components in wine-based products of this plant (cyanidin-3-sophoroside, delphinin-3-sambubioside, pelargonidin-3-malonyl glucoside) were not be detected in this study [6]. This is probably due to the different extraction process, type of sample and plant variety.

3.6 Inhibitory Activity of *A. puncticulatum* Extract on Mushroom Tyrosinase

3.6.1 Effect of *A. puncticulatum* extract on mushroom tyrosinase

The dose-response curves for the tyrosinase inhibitory effects of the *A. puncticulatum* extract and kojic acid are depicted in Figures 6A and B, respectively. The inhibitory diphenolase activities of tyrosinase increased with increase of extract concentrations. Increasing concentrations of plant extract from 0.1 to 2 mg/mL resulted in increased percentages of tyrosinase inhibition from 7.00±3.24% to 88.36±1.42%, respectively. The IC_{50} value of plant extract was calculated to be 0.65±0.03 mg/mL. On the other hand, IC_{50} value of kojic acid was 0.029±0.00 mg/mL which was found to be 22.41 times more effective than *A. puncticulatum* extract. This result revealed that *A. puncticulatum* extract had moderate inhibitory effects on mushroom tyrosinase.

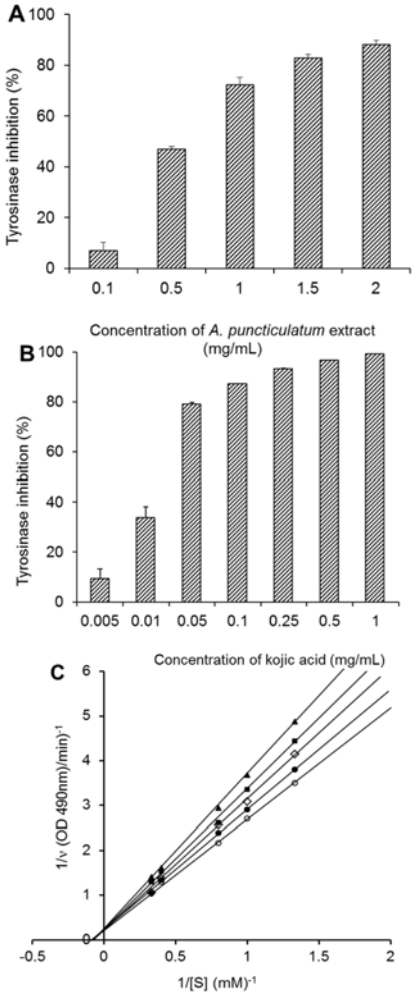

Figure 6. Dose-response curves for the tyrosinase inhibition effects: **A** *A. puncticulatum* extract and **B** kojic acid. Lineweaver-Burks plots for the inhibition of tyrosinase by different concentrations of *A. puncticulatum* extract (**C**).

3.6.2 Inhibition type of *A. puncticulatum* extract on mushroom tyrosinase

The type of inhibition of *A. puncticulatum* extract on mushroom tyrosinase during the oxidation of L-DOPA was studied using Lineweaver-Burk plot analysis. As shown in

Figure 6C, increasing concentrations of plant extract resulted in decreased values of V_{max} whereas K_m remained constant. This indicated that the test extract exhibited non-competitive-type inhibition against the diphenolase activity of mushroom tyrosinase [25]. The apparent K_m and V_{max} values of the solution mixture without plant extract were calculated to be 16.73 mM and 5.41 U whereas V_{max} kinetic parameter decreased to 4.83 U in the presence of plant extract. Thus, the mechanism of action of this plant extract that act as non-competitive inhibitor should be through the allosteric effect by binding to different sites on the tyrosinase compared with L-DOPA.

4. CONCLUSIONS

The UAE were performed for maximizing the yield of total phenolics and total anthocyanins with good antioxidant activity from *A. puncticulatum* ripe fruits. All test independent variables play an important role affecting the response values. The optimal extraction conditions to achieved maximum yields of such dependent variables were liquid-to-solid ratio of 30 mL/g, acidified ethanol concentration of 45% (v/v), extraction temperature of 80°C and extraction time of 12 min. Under these conditions, the experimental yields of total phenolics, total anthocyanins, and antioxidant activity were close to the predicted values. Organic acids were characterized as the major phenolic compounds in the extract. The mushroom tyrosinase inhibition kinetics showed that the extract displayed a non-competitive inhibition. Thus, the UAE could be a technique for rapid and maximum extraction yields of bioactive compounds from ripe fruits of *A. puncticulatum*.

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