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Research Article

Extraction and Identification of Antidiabetic and Antioxidative Phytochemicals from *Pisang awak* Leaves

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ABSTRACT
Antioxidative and antidiabetic compounds were successfully extracted from *Musa* spp. ABB *cv*. (*Pisang Awak*) leaves. Effects of extraction time (0.5–4 h), solvent-to-sample ratio (10–80 ml/g), ethanol concentration (50–100 %), extraction temperature (25–60 °C) and pH (2–7) were examined. The selected key variables were optimized using response surface methodology (RSM). Quadratic models were obtained and 3D-response surfaces were plotted. An optimum condition (i.e. extraction time of 0.5 h, solvent-to-sample ratio of 48.3 ml/g and 88.5% ethanol) was obtained. This condition gave TPC of 21.54 mg GAE/g sample, DPPH scavenging activity of 6.49 mg GAE/g sample, FRAP of 9.35 mM Fe_2SO_4 and α-amylase inhibition activity of 26.62%. A total of 7 major compounds (i.e. chlorogenic acid, procyanidin, epicatechin, rutin, isoquercitrin, quercetin and kaempferol-7-neohesperidoside) were identified using liquid chromatography-quadropole time-of-flight-mass spectrometer.

Keywords: antioxidant; antidiabetic; identification; optimization; phytochemical; *Pisang Awak*

INTRODUCTION

One of the non-communicable diseases, type II diabetes mellitus or non-insulin dependent diabetes mellitus) has become a serious threat to global public health due its chronic course and complications, such as heart disease and kidney failure[1]. Different conventional therapies were introduced, for example, intake of oral hypoglycemic agents. However, modern medicines such as biguanides and thiozolidinediones showed undesired side effects. Alternative approaches (e.g. herbal drugs and control diet) were therefore gaining interests due to their effectiveness, safety and acceptability[2]. A recent study showed that inhibition of α-amylase in degradation of dietary starch works well as an important therapeutic treatment especially for postprandial hyperglycemia patients[3]. Hence, the use of an agent that could reduce oxidative stress and postprandial hyperglycemia via inhibition of the α-amylase activity may be therapeutic for diabetic patients. In this study, *Pisang Awak* leaves were used as the source of the antioxidative and antidiabetic agents. This type of banana is the most famous cultivar with triploid hybridization of ABB[4], which is the hybrid of diploids *M. acuminata* with *M. balbisiana* Colla, genomes of AA and BB, respectively. It is able to grow almost everywhere in Malaysia throughout the year. However, the values of the banana leaves are remained unknown and most people are only using the banana leaves as a lignocellulose source or as a wrapping material. For example, one of the Malaysian cuisines, *nasi lemak*, is normally wrapped with banana leaves because it is believed that banana leaves could help in preventing rancidity of the fat-containing foods. It is also used for medicinal purposes in some regions as a cool compress for burns and other skin diseases. The ash of the leaves was reported to be used in treating diarrhea, ulcers and eczema[5]. The other potential uses such as analgesic, antimalarial, antimicrobial, hemagglutination inhibitory and hemolysis inhibitory agents[6] were also reported in other hybrids of banana. Hence, it is interesting to explore the potential of banana leaves. An optimization study of the extraction parameters (i.e. extraction time, S/S ratio and ethanol concentration) was performed in order to develop a procedure that can greatly compromise the quality and productivity of the extraction. The antioxidative and antidiabetic properties of the extract were investigated. The targeted responses were total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity, ferric reducing/antioxidant power (FRAP) and antidiabetic activity. Apart from that, identification of the extracted phytochemicals was subsequently performed using liquid chromatography-quadropole time-of-flight-mass spectrometry (LC QTOF MS) approach.

MATERIALS AND METHODS

Materials

Banana leaves were collected from farms located in Penang, Malaysia. The leaves were cleaned with distilled water and cut into smaller pieces prior to freeze-drying. Subsequently, the sample was ground into powder and sieved (30-mesh size). α-Amylase with activity of 10 units/mg solid was purchased from Sigma-Aldrich, USA. All other chemicals and reagents used in this study were of analytical grade and were purchased from Sigma–Aldrich company.

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Extraction of phytochemicals

The samples (0.5 g) were added with extraction solvent with different concentrations in a 50 ml centrifuge tube at different S/S ratio, followed by incubation at different temperatures and extraction periods. During the incubation, the centrifuge tubes were covered with aluminium foil to prevent exposure to light and the mixtures were placed in an incubator with a constant shaking at 200 rpm. The mixtures were then centrifuged (4500 rpm, 30 min, 4 °C) and supernatants were transferred and made up to a total volume of 50 ml. The solutions were stored at -20 °C prior to analysis. All the extractions were performed in at least three replicates.

Experimental Design

The experimental design of this study comprised of two major parts: (A) single factor experiment and (B) optimization study using Response Surface Methelodgy (RSM). In the single factor experiment, several key variables were selected (i.e. solvent concentration, S/S ratio, extraction temperature, extraction time and pH). The steepest ascent ranges of the significant factors were then selected and examined in the optimization study. Box-Behnken design (BBD) was employed and quadratic models were developed.

Single factor experiments

Effect of extraction time

The extraction times ranging from 0.5 to 4 h were investigated. The other parameters were remained in constant (i.e. extraction temperature of 25 °C, S/S ratio of 25:1 and 100% ethanol).

Effect of solvent-to-sample ratio (S/S ratio)

The S/S ratios ranging from 10:1 to 80:1 were investigated. The other parameters were remained in constant (i.e. extraction time of 1 h, extraction temperature of 25 °C and 100% ethanol).

Effect of solvent concentration

The ethanol concentrations ranging from 50 % to 100 % with 10 % intervals were investigated. The other parameters were remained in constant (i.e. extraction time of 1 h, extraction temperature of 25 °C and S/S ratio of 25:1).

Effect of extraction temperature

The extraction temperatures ranging from 25 to 60 °C with 5 °C intervals were investigated. The other parameters were remained in constant (i.e. extraction time of 1 h, solvent concentration of 100 % and S/S ratio of 25:1).

Effect of pH

Different pHs ranging from 2 to 7 were investigated. The other parameters were remained in constant (i.e. extraction time of 1 h, solvent concentration of 100 %, extraction temperature of 25 °C and S/S ratio of 25:1).

Optimization study

The experimental design consisted of 12 different combinations of the independent variables and 5 center points. The BBD design matrix was shown in Table 1. The three factors (i.e. extraction time, S/S ratio and ethanol concentration) were chosen for this experiment and designated as X_1, X_2 and X_3, respectively. They were divided into three levels, which were high, intermediate and low levels. Data were analyzed using multiple regressions in order to fit the following quadratic polynomial model:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y was the response variable; β_o was the model constant; β_i was the linear coefficient; β_{ii} was the quadratic coefficient; β_{ij} was the interaction coefficient; and ε was the statistical error.

Determination of total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu reagent according to the method described by Shui and Leong[7]. Sample (40 µl) was mixed well with 1.8 ml of Folin-Ciocalteu reagent (prediluted at a ratio of 1:10) and incubated for 5 min at room temperature before the addition of 1.2 ml of sodium bicarbonate (7.5 %). The mixture was then incubated in dark place for 60 min at room temperature to allow color development. Absorbance was measured against the blank reagent at 765 nm using a spectrophotometer (Spectramax M5, Molecular Devices, USA). Gallic acid at a concentration range of 0.1 to 1.0 mg/ml was used as standards. TPC was expressed as mg gallic acid equivalents (GAE)/g sample. Each extract was analyzed in three replicates.

Antioxidant activity determination

DPPH assay

The free radical-scavenging ability of the extracts (pre-diluted in the ratio of 1:10) was evaluated as described by Liu et al.[8]. To 1 ml of 0.1 mM ethanolic solution of DPPH, 33.3 µl of test extracts/standard was added. The mixture was incubated at 30 °C in the dark for 30 min and the discoloration was measured at 517 nm using a spectrophotometer. Radical scavenging activity (%DPPH) was calculated using the equation below:

$$\%DPPH = \frac{A_{cont} - A_{samp}}{A_{cont}} \times 100\% \quad (2)$$

where A_{cont} was the absorbance of the control, and A_{samp} was the absorbance of the sample. DPPH• scavenging activity was then expressed as mg GAE/g sample based on the calibration curve. Each extract was analyzed in three replicates

FRAP assay

The ferric reducing antioxidant power (FRAP) of banana leaf extract was estimated using the method described by Benzie and Strain[9]. The reducing activity was determined by assessing the ability to reduce $FeCl_3$ solution. A mixture of 2.5 ml of a 10 mM tripyridyltriazine (TPTZ) solution in 40 mM hydrochloric acid, 2.5 ml of 20 mM $FeCl_3 6H_2O$ (ferric chloride hexahydrate) and 25 ml of 0.3 M acetate buffer at pH 3.6 was prepared as FRAP reagent. The freshly prepared FRAP working reagent was pre-warmed at 37 °C and then 600 µl of the FRAP reagent was added to 8.1 µl of extract. The absorbance was measured at 593 nm against the blank after 1 hour of incubation at 37 °C. FRAP value was calculated and expressed as mM Fe^{2+} equivalent (Fe(II)). All determinations were performed in three replicates.

α-Amylase inhibition assay

Prior to analysis, the alcohol was removed from the sample using a vacuum concentrator (Concentrator 5301,

Table 1 BBD with the observed responses and predicted values for TPC, DPPH, FRAP and α-amylase inhibition activity.

Run	Independent variables			Dependent variables (responses)							
	X_1 (h)	X_2 (ml/g)	X_3 (%)	TPC (mg GAE/ g sample)		DPPH (mg GAE/ g sample)		FRAP (mM FE_2SO_4)		α-amylase inhibition activity (%)	
				Expt.[a]	Pred.[b]	Expt.[a]	Pred.[b]	Expt.[a]	Pred.[b]	Expt.[a]	Pred.[b]
1	0.5	10	70	9.54	9.74	1.6	1.61	3.89	4.10	3.62	4.26
2	0.5	30	50	18.29	17.97	4.43	4.64	6.64	6.72	7.08	5.89
3	1.25	30	70	18.68	18.74	6.85	6.96	5.98	5.98	11.11	11.21
4	2	10	70	10.50	10.72	2.37	2.24	4.17	4.44	2.82	1.26
5	2	30	90	19.23	19.54	6.27	6.06	8.97	8.90	12.71	13.90
6	0.5	30	90	19.81	20.14	6.03	5.69	7.72	7.72	25.62	24.61
7	2	30	50	16.29	15.95	4.32	4.66	5.57	5.58	9.02	10.03
8	1.25	10	50	6.90	7.01	1.32	1.10	2.35	2.07	5.67	6.21
9	1.25	50	50	18.49	19.02	7.45	7.12	7.23	7.43	8.34	7.97
10	0.5	50	70	22.08	21.86	6.9	7.03	9.35	9.08	10.23	11.79
11	1.25	30	70	18.89	18.74	7.35	6.96	6.04	5.98	11.60	11.21
12	1.25	10	90	12.62	12.08	3.03	3.37	5.13	4.93	11.66	12.02
13	2	50	70	18.46	18.26	6.78	6.78	8.98	8.78	8.87	8.22
14	1.25	30	70	18.67	18.74	7.08	6.96	5.99	5.98	12.36	11.21
15	1.25	50	90	19.83	19.71	7.1	7.31	8.62	8.90	25.30	24.75
16	1.25	30	70	19.31	18.74	7.05	6.96	5.71	5.98	10.95	11.21
17	1.25	30	70	18.14	18.74	6.45	6.96	6.19	5.98	10.04	11.21

[a] Mean of triplicate determinations

[b] Predicted values

Eppendorf, Germany) and distilled deionised water was used to reconstitute the extract.

The α-amylase inhibitory assay was carried out using the method described by Ranilla et al.[10]. α-Amylase enzyme solution (1 mg/ml) was prepared using 0.02 M sodium phosphate buffer containing 6.0 mM sodium chloride at pH 6.9. A starch solution (1%, w/v) was prepared in 0.02 M sodium phosphate buffer and heated in boiling water for 30 min in order to solubilize the starch. To each tube, 100 µl of α-amylase solution and sample were mixed and incubated at 25 °C for 10 min. Starch solution (100 µl) was then added into the mixture, followed by incubation at 25 °C for 10 min. Subsequently, 200 µl of dinitrosalicylic acid was added and heated in boiling water bath for 5 min. The mixture was cooled to room temperature and diluted with 3 ml of distilled water. The absorbance was measured at 540 nm using a spectrophotometer.

The α-amylase inhibitory activity was calculated as follows:

$$\% \text{ inhibition} = \frac{A_{Control\,2} - (A_{sample} - A_{Control\,1})}{A_{Control\,2}} \times 100 \quad (3)$$

where A_{sample} was the absorbance of sample, $A_{control\ 1}$ was the absorbance of a mixture of starch solution and sample without addition of enzyme and $A_{control\ 2}$ was defined as a mixture of starch solution and enzyme without addition of sample. All tests were performed in three replicates.

Identification of phytochemicals using LC Q-TOF MS

Sample Preparation

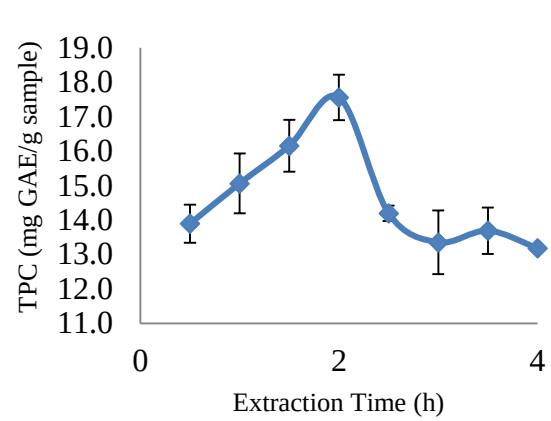
The extracted sample was pre-diluted and then filtered through a 0.45 µm filter prior to analysis using Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS (Agilent Technologies, USA).

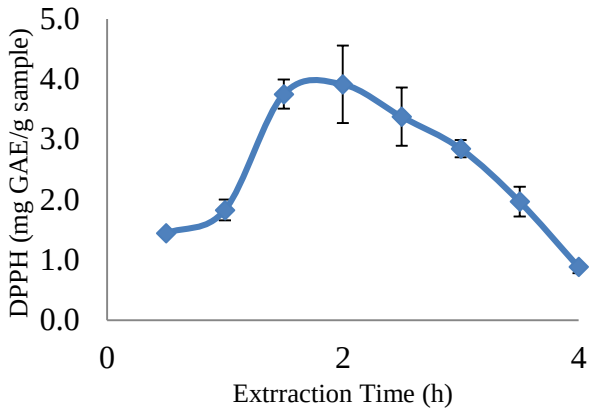
LC-Q-TOF MS and MSMS analyses

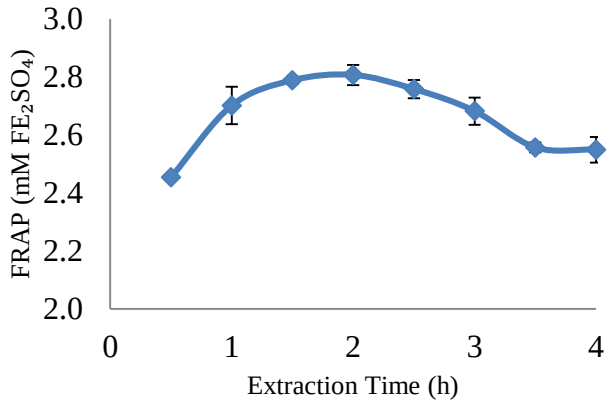
The MS and MSMS analyses were carried out using positive mode electrospray ionization (ESI) which was equipped with 1200 series (Agilent Technologies, USA) gradient pump. The separation was performed using an Agilent ZORBAX SB-C18 column with a diameter of 0.5 mm, length of 150.0 mm and particle size of 5.0 µm. The operating conditions were: injection volume: 5 µL; mobile phase: (A) distilled water with 0.1 % formic acid, (B) acetonitrile with 0.1% formic acid; flow rate: 20 µL/min. The pump gradient was: 0 min, 3 % B; 3.75 min, 20 % B; 8.75 min, 40 % B; 15-17.5 min, 90 % B; 22.5-32.5 min, 3 % B. For MS analysis, dry gas temperature of 325 °C, gas flow rate of 5 L/min, nebulizer pressure of 30 psi and capillary voltage of 3500 V were used. The acquisition mode was set at the m/z range of 25-1600, with a scan rate of 1 spectra/ms. For MSMS analysis, collision energies of 20-40 eV were used. The other conditions were the same as the MS analysis.

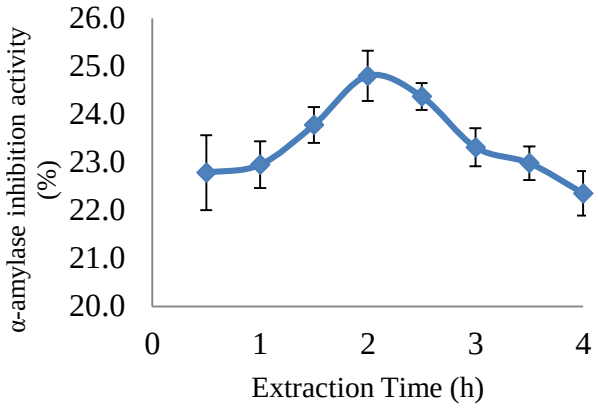
Database search of the unknown compounds

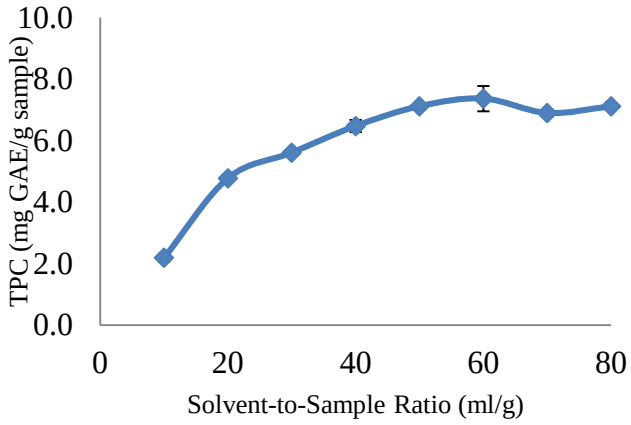
The results obtained from the Agilent MassHunter Workstation Software – Data Acquisition were processed using the MassHunter Qualitative Analysis software. Molecular feature extraction (MFE) was performed in order to determine the molecular masses. The molecular feature extraction was carried out using the following setting: target data type: small molecules (chromatographic); peak filters: above 5000 counts (to eliminate the background noise in order to obtain the high-quality fragment spectra); positive ions: +H, +Na, +K (potential cation adduct that present in the extract); isotope mode: common organic molecules and charge state: limit assigned charge states to a maximum of 1.

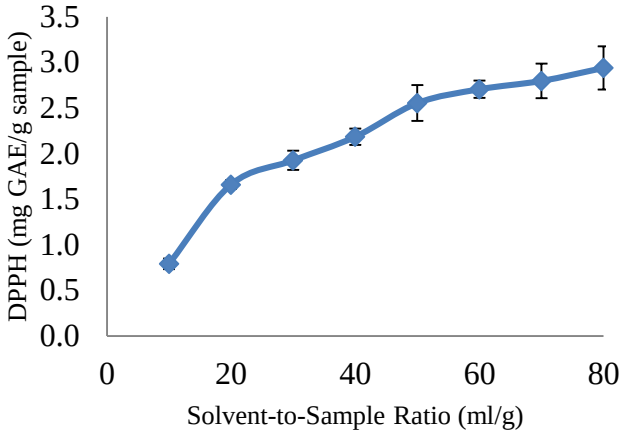

TPC (mg GAE/g sample)
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
0
2
4
Extraction Time (h)

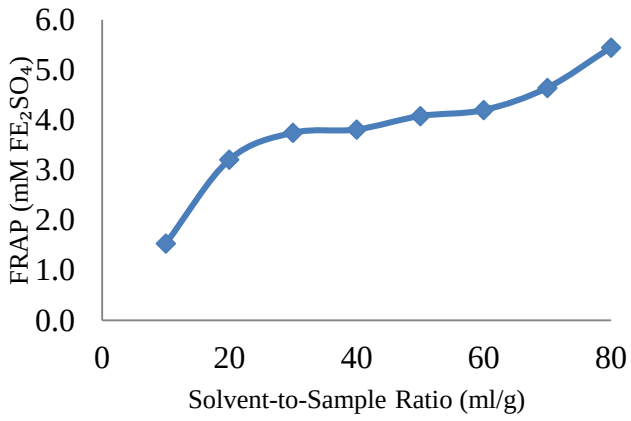

DPPH (mg GAE/g sample)
5.0
4.0
3.0
2.0
1.0
0.0
0
2
4
Extrraction Time (h)

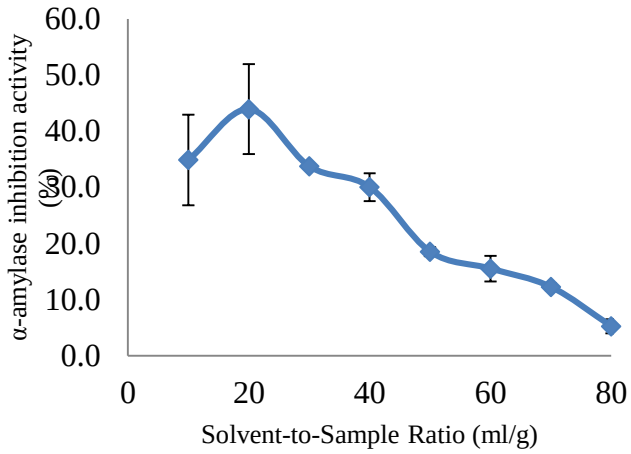

FRAP (mM FE2SO4)
3.0
2.8
2.6
2.4
2.2
2.0
0
1
2
3
4
Extraction Time (h)


α-amylase inhibition activity (%)
26.0
25.0
24.0
23.0
22.0
21.0
20.0
0
1
2
3
4
Extraction Time (h)


TPC (mg GAE/g sample)
10.0
8.0
6.0
4.0
2.0
0.0
0
20
40
60
80
Solvent-to-Sample Ratio (ml/g)


DPPH (mg GAE/g sample)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
Solvent-to-Sample Ratio (ml/g)


FRAP (mM FE2SO4)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80
Solvent-to-Sample Ratio (ml/g)


α-amylase inhibition activity (%)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
20
40
60
80
Solvent-to-Sample Ratio (ml/g)

After the MFE search, the mass list was uploaded to the metabolite search in batches using the METLIN Metabolite Database. The mass list was searched against the neutral mass in the database with accuracy less than 5 ppm of mass difference. The matched MS/MS spectra of compounds were verified using Mass Frontier™ 6.0. In Mass Frontier analysis, fragments were generated using all possible rules, including aromatic rings, the default limit of 10 000 fragments per iteration, and four iterations. These spectra were compared to the experimental MSMS spectra. The compound was predicted based on the fragment ions. Standards were also used to perform the verification of data.

Statistical analysis

Comparison of means was performed using one-way analysis of variance (ANOVA) followed by Duncan's test. This statistical analysis in the single factor experiment was performed using statistical software package SPSS for Windows, Version 12.0 (SPSS Institute, Inc., Cary, NC, USA) whereas Design-Expert software (Version 6.0, Stat-Ease, Inc., Minneapolis, MN) was used in optimization study.

RESULTS AND DISCUSSION

Single factor experiment

Type of solvent is an important variable for phytochemical extraction. Generally, extraction of phenolic components from plant materials using alcohol were applied because of its efficiency in giving a high extraction yield. Apart from that, ethanol is a better choice compared to methanol because the latter shown its cytotoxic nature. The use of ethanol is also considered as environmentally friendly and it is classified as GRAS (Generally Recognized as Safe). Thus, ethanolic extraction of antioxidant compounds from herbs were widely studied (Chan et al., 2009). In the preliminary study, several commonly used extraction solvents (i.e. acetone, n-hexane, methanol, ethanol and water) were first investigated and result showed that ethanol and methanol gave the highest score for the responses (i.e. TPC, DPPH• scavenging activity, FRAP and amylase inhibition activity)(data not shown). In regards to the advantages of ethanol aforementioned, ethanol was chosen as the extraction solvent for this study. Following, five extraction parameters (i.e. extraction time, S/S ratio, ethanol concentration, pH and extraction temperature) were investigated. The four targeted responses were TPC, DPPH• scavenging activity, FRAP and amylase inhibition activity.

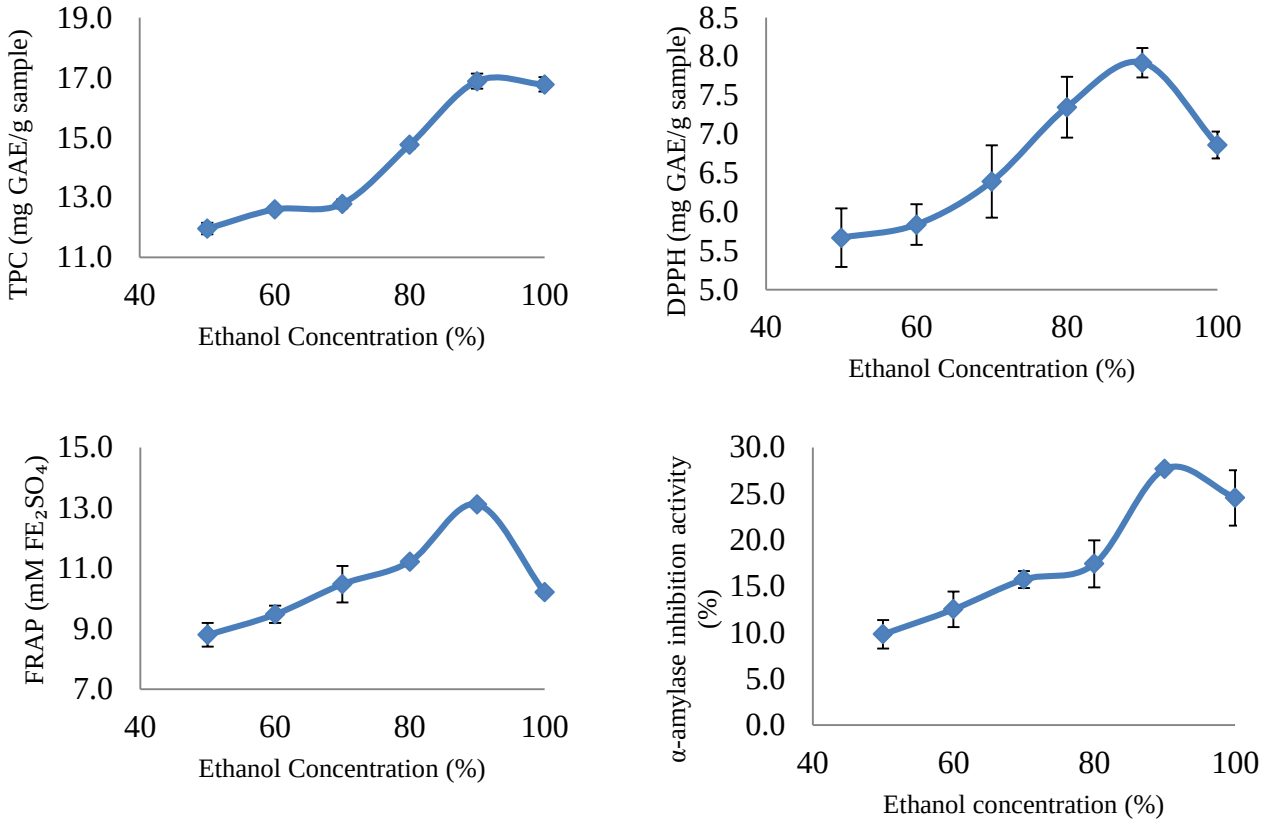


Fig. 1 Effects of (a) extraction time, (b) S/S ratio and (c) ethanol concentration on the (i) TPC, (ii) DPPH• scavenging activity, (iii) FRAP activity and (iv) amylase inhibition activity of "Pisang Awak" leaves extract.

Effect of extraction time

In this study, a range of extraction time was designed based on the practical and economical aspects. The responses as a function of extraction time were shown in Fig. 1a (i-iv). Result showed that as the extraction time increased from 0.5 to 2 h, all the responses increased. The highest values obtained at 2 h of extraction were: TPC of 16.92 mg GAE/g sample, DPPH• scavenging activity of 3.92 mg GAE/g sample, FRAP of 14.03 mM Fe_2SO_4 and amylase inhibition activity of 24.80%. However, each response showed a negative gradient after 2 h of extraction. According to Silva et al.[11], excessive extraction time was not just ineffective in extraction but also caused degradation to the extracted

Table 2 ANOVA for response surface quadratic model: the estimated regression model of relationship between dependent variables and independent variables.

Source	Sum of Squares	DF	Mean Square	F Val ue	Prob > F
TPC (mg GAE/ g sample)[a]					
Model	285.5	9	31.722	115.75	< 0.0001
X_1	3.437	1	3.4366	12.54	0.0095
X_2	193	1	193	704.25	< 0.0001
X_3	16.59	1	16.593	60.545	0.0001
X_2^2	59.78	1	59.778	218.13	< 0.0001
X_1X_2	5.252	1	5.2517	19.16	0.0032
X_2X_3	4.795	1	4.7946	17.49	0.0041
Residual	1.918	7	0.2741		
Lack of Fit	1.2	3	0.3999	2.226	0.2275
DPPH (mg GAE/ g sample)[b]					
Model	71.8	9	7.982	50.52	< 0.0001
X_2	49.6	1	49.584	313.79	< 0.0001
X_3	3.0	1	3.022	19.12	0.0033
X_1^2	1.1	1	1.068	6.76	0.0013
X_2^2	4.2	1	4.232	26.78	< 0.0001
X_3^2	10.0	1	9.986	63.19	0.0092
X_2X_3	2.0	1	2.005	12.69	0.0355
Residual	1.1	7	0.158		
Lack of Fit	0.7	3	0.220	1.98	0.2596
FRAP (mM FE_2SO_4)[c]					
Model	59.7	9	6.639	78.57	< 0.0001
X_2	43.4	1	43.41	513.77	< 0.0001
X_3	9.35	1	9.351	110.68	< 0.0001
X_1^2	4.26	1	4.26	50.42	0.0002
X_2^2	0.63	1	0.632	7.47	0.0292
X_1X_3	1.35	1	1.348	15.95	0.0052
X_2X_3	0.49	1	0.487	5.76	0.0475
Residual	0.59	7	0.084		
Lack of Fit	0.47	3	0.156	5.11	0.0746
α-amylase inhibition activity (%)[d]					
Model	591.80	9	65.76	32.07	< 0.0001
X_1	21.58	1	21.58	10.52	0.0142
X_2	104.88	1	104.88	51.16	0.0002
X_3	255.21	1	255.21	124.49	< 0.0001
X_1^2	16.50	1	16.50	8.05	0.0252
X_2^2	34.16	1	34.16	16.66	0.0047
X_3^2	80.66	1	80.66	39.34	0.0004
X_1X_3	55.15	1	55.15	26.90	0.0013
X_2X_3	30.11	1	30.11	14.69	0.0064
Residual	14.35	7	2.05		
Lack of Fit	11.44	3	3.81	5.24	0.0717

[a] The coefficient of determination (R^2) and adjusted R^2 of the model were 0.9933 and 0.9848 respectively.
[b] The coefficient of determination (R^2) and adjusted R^2 of the model were 0.9848 and 0.9653 respectively.
[c] The coefficient of determination (R^2) and adjusted R^2 of the model were 0.9902 and 0.9776 respectively.
[d] The coefficient of determination (R^2) and adjusted R^2 of the model were 0.9763 and 0.9459 respectively.

phenolic components and thus loss its activity due to oxidation after the exposure to light or oxygen[12]. Fick's second law of diffusion also stated that final equilibrium will be achieved between the plant matrix and solvent after a certain time. Hence, an excessive extraction time was not useful to extract more phytochemicals[11]. From the industrial perspective, shorter extraction time is preferred as less process time consumed and economical. Taking into account of these facts, extraction times of 0.5-2 h were selected for RSM optimization study.

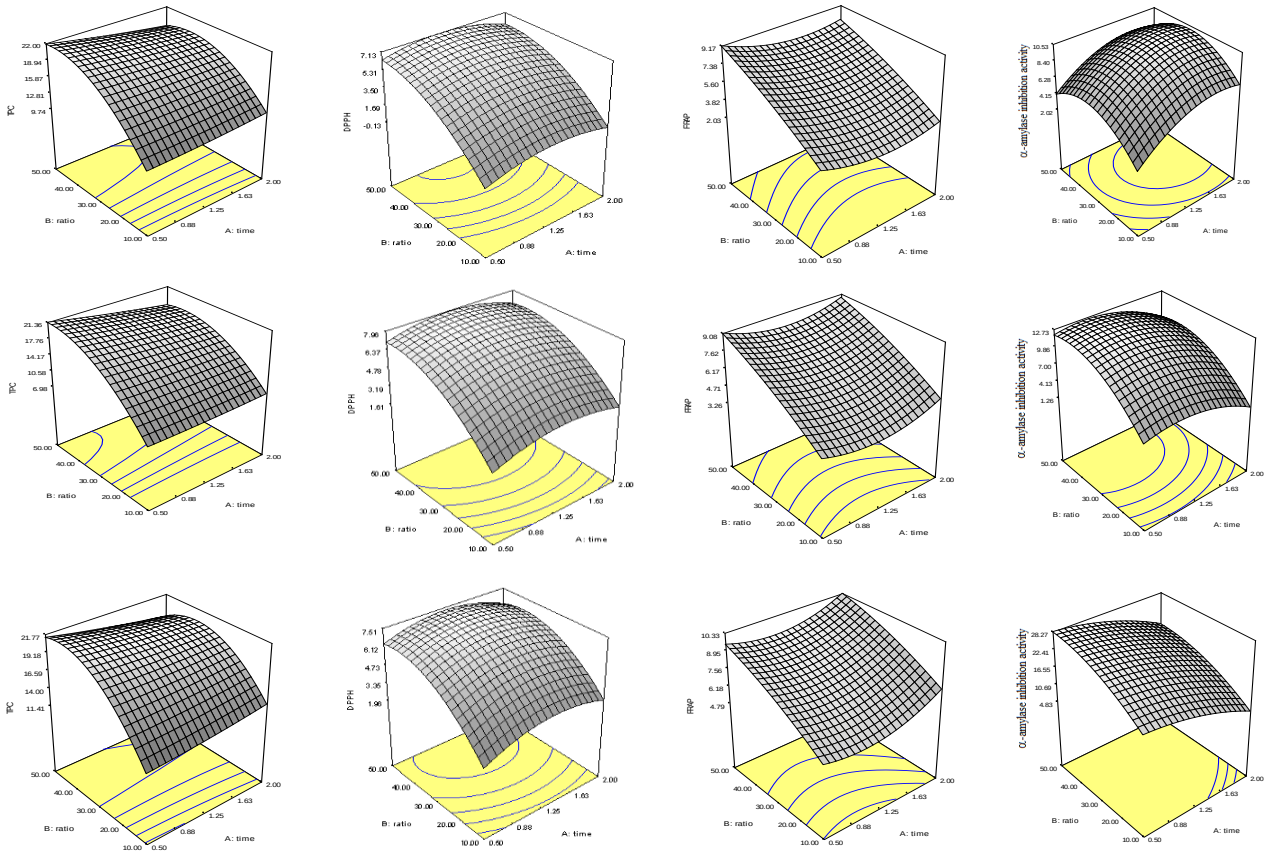


Fig. 2 Three-dimensional plots for (a) TPC, (b) DPPH radical scavenging activity, (c) FRAP and (d) amylase inhibition activity of "Pisang Awak" leaves extract as a function of S/S ratio and time at different ethanol concentrations: (i) low; (ii) middle and; (iii) high level.

Effect of solvent-to-sample ratio (S/S ratio)

The impact of the S/S ratio (i.e. 10:1; 20:1; 30:1; 40:1; 50:1, 60:1, 70:1 and 80:1) on the targeted responses were investigated at a temperature of 25 °C for a constant extraction period of 1 h using 100% ethanol. It could be observed that the TPC of the extract increased as the S/S ratio increased (Fig. 1b (i)). The result of the one-way analysis of variance (ANOVA) showed that there was a highly significant ($p<0.05$) difference between the S/S ratio of 10 and 50 ml/g. It was also found that the activities of DPPH• scavenging and FRAP increased as the ratio of S/S increased. Theoretically, high solvent volume gave better efficiency in eluting of the antioxidative components due to the excessive swelling of the material. This occurrence created a large concentration difference between the interior of the banana leaves and the external solvent, thus prompted a higher diffusion rate of solute particles and released more phytochemical molecules in the solution. However, a limited source of antioxidant material was used in this study; an excessive solvent could cause a dilution to the extracted components. Therefore, further increase the volume of solvent would not bring any significant effects to the responses. The ANOVA analysis showed that there was no statistically significant ($p>0.05$) differences when the S/S ratio was more than 50 ml/g in both TPC (Fig. 1b (i)) and DPPH• scavenging activity (Fig. 1b (ii)). α-Amylase inhibitory activity was found to exhibit a negative trend. Increase in the S/S ratio led to a decrease of antidiabetic activity. Similar result was reported by Li et al.[13]. Even though there was a significant ($p<0.05$) increase in FRAP at S/S ratio of 70 and 80 ml/g, a dramatic drop of α-amylase inhibition activity from ratio 40 to 50 ml/g was observed. Thus, the range of S/S ratio was chosen from 10:1 to 50:1.

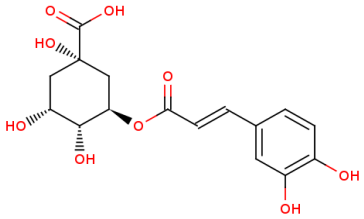
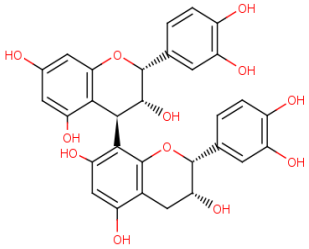
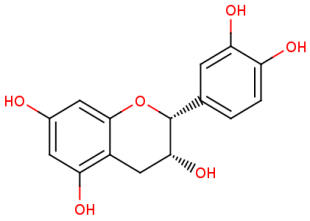
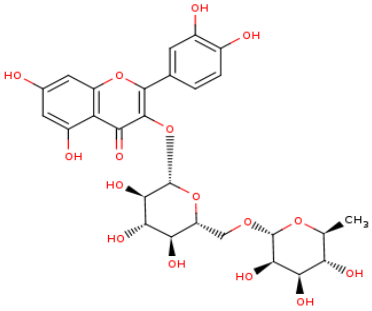
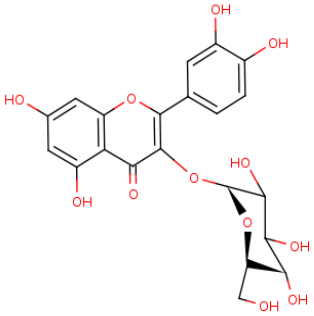
Effect of ethanol concentration

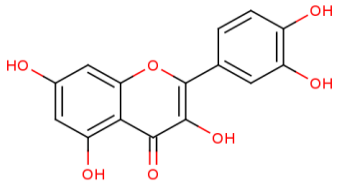
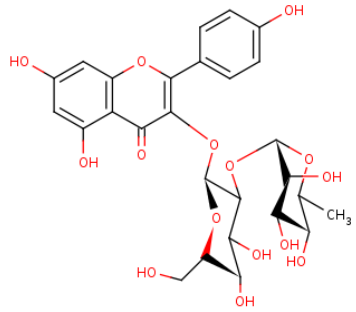
The high activity of the ethanolic extracts was exhibited as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols. Aqueous ethanol was found to be more efficient in cell walls and seeds degradation, thus led to the release of phytochemicals from leaves. Apart from that, aqueous ethanol enhanced the solubility of phytochemicals[12]. However, the ethanol concentration in this solvent has to be investigated in order to be used as a highly efficient extracting agent. The TPC, DPPH• scavenging activity, FRAP and amylase inhibitory activity of the extract as a function of the ethanol concentration were shown in Fig. 1c (i-iv).

All the dependent variables were highly dependent on the ethanol concentration employed for the extraction. As the ethanol concentration increased from 50 to 90%, a significant ($p<0.05$) increasing pattern was observed in each response whereas using an absolute ethanol caused a decrease in the responses. The phytochemicals extracted using 90% ethanol reached a maximum value in all responses and further increase in ethanol concentration showed that there was a significant decrement in the FRAP. Addition of a small quantity of water (10%) to ethanol seemed to enhance the extraction efficiency significantly

($p<0.05$). This could be explained by the increase of the polarity of the solvent, which enable the solvent system to extract phenolic substances from different polarities. Silva et al.[11] reported that the optimal yields of extraction from *Inga edulis* leaves and *Ligustrum lucidum* were found using 80-90% ethanol. Therefore, 50%, 70% and 90% were selected as the low, middle and high levels, respectively, to be employed in RSM optimization study.

Table 3 The identified antioxidant and antidiabetic compounds of ethanol extract from *Pisang Awak* leaves with the MSMS data.

No.	Compound	Mass	Fragments ions (m/z)
1	Chlorogenic Acid 	354.0931	163.039, 145.029, 135.047
2	Procyanidin 	578.142	427.104, 291.083, 411.108, 409.091, 301.069, 291.09, 289.073, 287.055, 275.055, 271.060, 259.059, 247.058, 163.039, 139.040, 127.039, 123.045
3	Epicatechin 	290.0795	207.066, 165.055, 147.043, 139.038, 123.044
4	Rutin 	610.1534	303.050, 129.058, 85.032
5	Isoquercitrin 	464.0967	303.050, 85.028

6	Quercetin 	302.0433	299.047, 285.039, 274.048, 257.041, 165.016, 153.017, 137.022, 121.027, 109.028, 95.049, 81.032, 68.997, 55.0174
7	Kaempferol-7-neohesperidoside 	594.1585	449.108, 287.055, 147.065, 145.049, 129.054, 85.028, 71.049

Effect of extraction temperature and pH

Extraction temperature and pH are also important parameters, which commonly used in optimizing the extraction condition. However, the result revealed that the responses were not significantly ($p>0.05$) affected by the temperature and pH (data not shown). Therefore, room temperature (25 °C) was chosen as incubation temperature and pH was not monitored in the optimization study.

Optimization study

To optimize the extraction parameters, BBD was employed by engaging the selected factor based on the steepest ascent ranges: incubation time (0.5 – 2 h), solvent-to-sample ratio (10 – 50 ml/g) and concentration of ethanol (50 – 90%). The experimental and predicted results of 17 trials extraction using BBD were shown in Table 1. The highest yield of TPC (22.08 mg GAE/g sample) was obtained in run No. 10 with experimental variables of extraction time of 0.5 h, S/S ratio of 50 ml/g and 70% of ethanol. The lowest TPC (6.9 mg GAE/g sample) was obtained in run No. 8 under experimental conditions of extraction time of 1.25 h, S/S ratio of 10 ml/g and 50% of ethanol. Apart from that, the experimental results showed that DPPH• scavenging activity and FRAP were ranged from 1.6 – 7.45 mg GAE/g sample and 2.35 – 9.35 mM Fe_2SO_4, respectively. The maximum DPPH• scavenging activity was obtained in run No. 9 at extraction time of 1.25 h, solvent-to-sample ratio of 50 ml/g and 50% ethanol; whereas the highest FRAP was recorded in run No. 10, which also showed the highest TPC value. A broad range of α-amylase inhibition activity were found (2.82 – 25.62%) and the highest inhibitory activity was achieved under the condition of extraction time of 0.5 h, solvent-to-sample ratio of 30 ml/g and 90% of ethanol.

Model fitting

Table 2 showed the quadratic model relating the effect of the independent variables; their interaction and regression coefficients of the response variables were generated by analysis of variance (ANOVA). The statistical significance of the model was also measured by the F-test and the residuals analysis were performed to validate the model at 95% confidence level. The regression coefficients and the response surfaces were used to investigate the effect of variables on the TPC, antioxidant and amylase inhibition activities. In order to check the significance level of the corresponding variables, p-value was determined. A smaller p-value or a greater F-value indicates a more significant of the variable. In addition, the lack-of-fit was used to check whether the estimated model fitted to the actual shape of the surface. The insignificance ($p>0.05$) of the lack–of-fit indicates that the model was well fitted to the experiment data, whereas high coefficient of determination (R^2) illustrated that the model was well adapted to the response[12].

TPC

The ANOVA showed that the model was highly significant as a very low p-value ($p<0.0001$) was obtained in TPC (Table 2). Table 2 showed that R^2 value for the regression model of TPC was 0.9933. This value suggested that the predicted model was well defined to the real behaviour of the system. The insignificance ($p>0.05$) of the lack-of-fit had also proven the reliability of the models. It could be observed in Table 2 that X_1, X_2, X_3, X_2^2, X_1X_2 and X_2X_3 were highly significant ($p<0.01$). The largest effects were exhibited by X_2 and X_2^2 followed by X_3. The relationship between the tested independent variables and TPC could be explained using Eq. (4):

$$TPC = -13.36851 - 1.02200X_1 + 1.09786X_2 + 0.30336X_3 + 0.31449X_1^2 - 0.00941983X_2^2 - 0.00127692X_3^2 - 0.076389X_1X_2 + 0.023622X_1X_3 - 0.00273708X_2X_3 \quad (4)$$

DPPH• scavenging activity

The predicted model was highly significant ($p<0.0001$) and the R^2 value of this model was 0.9848. No significance ($p>0.05$) of lack-of-fit was found and this value would give a relatively good fit to the mathematic model. Based on the p-value, it can be seen that the variable with the largest effect on the DPPH• scavenging activity was X_2 and X_2^2 followed by X_1^2, X_3, X_3^2 and X_2X_3. Eq. (5) showed the mathematic model of the DPPH• scavenging activity:

$$DPPH = -16.54819 + 4.62444X_1 + 0.46418X_2 + 0.30370X_3 - 1.78311X_1^2 - 0.00385125X_2^2 \; 0.00172625X_3^2$$

$– 0.014833X_1X_2 + 0.00583333X_1X_3 –0.00128750X_2X_3$ (5)

FRAP

Similar to TPC and DPPH• scavenging activity, the quadratic model was highly significant ($p<0.0001$) with great R^2 value (0.9902) and the lack-of-fit was not significant ($p>0.05$) in this response. The largest effect was found in X_2 and X_3, followed by X_1^2, X_1X_3, X_2^2 and X_2X_3. The predicted model for FRAP is shown in Eq. (6).

$FRAP = 4.71245– 6.84674X_1 + 0.24886X_2 – 0.052407X_3 + 1.78825X_1^2 – 0.000968188X_2^2 + 0.000601813X_3^2 – 0.010617X_1X_2 + 0.038694X_1X_3 – 0.000871875X_2X_3$ (6)

α-Amylase inhibition activity

The significance level of the model and lack-of-fit were of $p<0.0001$ and $p>0.05$, respectively. The R^2 value for the model was 0.9763 indicated a good agreement between experimental and predicted values. It could be observed from Table 2 that both the linear and quadratic terms of all parameters (X_1, X_2, X_3, X_1^2, X_2^2 and X_3^2) had significant ($p<0.05$) effects on α-amylase inhibition activity. Interaction terms of X_1X_3 and X_1X_3 also gave the significant ($p<0.01$) effect. Among all the three extraction parameters studied, ethanol concentration had the most critical role in the extraction of antidiabetic compounds from *"Pisang Awak"* leaves followed by S/S ratio and extraction time. The following equation (Eq. 7) was established to explain the α-amylase inhibition activity:

$\alpha\text{-amylase inhibition activity} = 22.84966 + 24.21678X_1 + 0.14580X_2 –1.14580X_3 – 3.51922X_1^2 – 0.00712036X_2^2 + 0.010942X_3^2 – 0.00933056X_1X_2 – 0.24755X_1X_3 + 0.00685875X_2X_3$ (7)

Interpretation of RSM

3D response surfaces of TPC, DPPH• scavenging activity, FRAP and α-amylase inhibition activity as a function of incubation time and S/S ratio were given in Fig. 2a–2d. The 3D plots were shown in (i) low, (ii) middle and (iii) high levels of ethanol concentration (i.e. 50%, 70% and 90%, respectively).

TPC

Fig. 2a(i-iii) shows the response surface plots of TPC as a function of extraction time and S/S ratio. The overall results showed that higher S/S ratio and shorter extraction time would give higher TPC, which appeared as a rising ridge, but TPC remained constant after S/S ratio of 40 ml/g at 70% of ethanol shown in Fig. 2a(ii). Besides, when at a high ethanol concentration (90%), TPC decreased when S/S ratio was more than 40 ml/g as shown in Fig. 2a(iii). The interaction of extraction time and S/S ratio showed significant effect in this experiment. At low extraction time (0.5 h), higher efficiency in the extraction of phenolic was observed when increased the S/S ratio, whereas at a low S/S ratio of 10 ml/g, lower efficiency in the extraction of phenolic was found when the extraction time increased. The interaction of S/S ratio and ethanol concentration also showed significant effects in this experiment. At a high S/S ratio (50 ml/g), lower TPC value was obtained when the ethanol concentration increased.

DPPH• scavenging activity

Fig. 2b(i-iii) shows the 3D response surface plot of DPPH• scavenging activity as a function of extraction time and S/S ratio. DPPH• scavenging activity increased gradually with the increase of S/S ratio, and achieved optimum value at around 1.6 h and S/S ratio of 40 ml/g and subsequently decreased. The result also showed that the effect of S/S ratio was more significant than extraction time. Apart from that, a negative interaction between S/S ratio and ethanol concentration was observed in Fig. 2b(iii). At low ethanol concentration (Fig. 2b(i)), higher S/S ratio gave higher efficiency in the extraction of antioxidant compounds while at S/S ratio of 10 ml/g, higher ethanol concentration achieved higher DPPH• scavenging activity.

FRAP

Fig. 2c(i-iii) shows that increase of S/S ratio had shown to increase FRAP value. A positive quadratic effect of extraction time was observed, FRAP activity was decreased gradually at the lower level of time but FRAP was raised as time increased. Conversely, a small negative quadratic effect of S/S ratio was shown where FRAP increased with the increase of S/S ratio and achieved a maximum value at 0.5 h and S/S ratio of 50 ml/g.

The interaction between extraction time and ethanol concentration showed significant effect in this experiment. At low ethanol concentration (Fig. 2d (i)), longer extraction time showed a decreased efficiency in the extraction of antioxidant compounds as it provided the time requirement for the solutes to expose to the extraction solvents. However, low extraction time caused an increase in FRAP as the ethanol concentration increased. While a negative interaction between S/S ratio and ethanol concentration was observed. Besides, at low ethanol concentration, high FRAP activity was obtained when the S/S ratio increased.

α-Amylase inhibition assay

The 3D response surfaces for α-amylase inhibition activity as a function of extraction time and S/S ratio were given in Fig. 2d (i-iii). α-Amylase inhibition activity gradually increased with the increase of extraction time and S/S ratio, and achieved optimum activity at around 1.63 h and S/S ratio of 30 ml/g at low ethanol concentrations (Fig. 2d (i)). The interaction of extraction time and ethanol concentration shows significant effect in this experiment. At low ethanol concentration (Fig. 2d (i)), long extraction time showed higher efficiency in the extraction of antidiabetic compounds. However, longer extraction time caused a decrease in inhibition ability at high ethanol concentrations (Fig. 2d (iii)). The interaction of S/S ratio and ethanol concentration showed a positive effect in this experiment. At high ethanol concentration (Fig. 2d(iii)), α-amylase inhibition activity increased as the S/S ratio increased. Overall the results above suggested that the change in extraction parameters had influenced α-amylase inhibition activity distinctly.

Verification of predictive models

The optimization study was to assess combination responses in order to achieve maximum TPC, antioxidant and antidiabetic activities, where efficiency, energy conservation, feasibility and economical aspect were taken into consideration. In order to verify the predicted model, the practical and predicted values of the responses were compared under the given optimum condition: extraction time of 0.5 h, S/S ratio of 48.3 ml/g and 88.5% ethanol. The

predicted values for TPC, DPPH• scavenging activity, FRAP and amylase inhibition activity were of 21.54 mg GAE/g sample, 6.49 mg GAE/g sample, 9.35 mM Fe_2SO_4 and 26.62%, respectively. In comparison, the experimental values were 20.15 mg GAE/g sample, 6.77 GAE/g sample, 9.64 mM Fe_2SO_4 and 22.58%, respectively. This implied that there was a fit degree between the values observed in experiment and the value predicted from the regression model. Hence, the response surface model could be applied effectively to optimize the extraction of antioxidant and antidiabetic compounds from "*Pisang Awak*" leaves. In addition, the optimal TPC, DPPH• scavenging activity and FRAP were much higher than the results reported by other researchers who also studied the optimization of antioxidant extraction from other plants[12].

Phytochemicals identification

Total of 7 potential antioxidant and antidiabetic compounds were found in the LCMS analysis. They are chlorogenic acid, procyanidin, epicatechin, rutin, isoquercitrin, quercetin and kaempferol-7-neohesperidoside. The identified compounds with their molecular structures and fragment ions were shown in Table 3. For examples, the first compound, chlorogenic acid, is an ester of caffeic acid with quinic acid that belongs to a type of hydroxycinnamic acids. It exhibited an effect on the detoxification of carcinogenic via intake of variety of fruits and vegetables, such as coffee beans, apples, blueberries, pineapple, green pepper and coffee[14-15]. It is also well-known as antioxidant as well as inhibitor of oxidation of human low-density lipoprotein (LDL), which is an early event in coronary disease[16]. The second compounds, procyanidin, is a type of proanthocyanidin (or condensed tannins) class of flavonoids[16]. It can be found in red raspberries, cocoa bean and chocolate[14] and it plays a role as an antioxidant compound[16]. On the other hand, the third compound, epicatechin, is a type of flavanols, which belongs to the class of flavonoids. It was found in many foods such as green leaves and black and green tea[17]. Schroeter et al.[18] have reported that epicatechin in cocoa and dark chocolate has an effect in improving and maintaining cardiovascular health. The present of epicatechin was also reported to prevent protein oxidation by stabilizing the membrane via hindering the approach of the peroxyl radical to erythrocyte membranes[19]. The fourth compound, rutin, is a glycosylated flavonoid. Singh et al.[20] reported that rutin from *Pteris vittata* L. exhibited inhibitory activity against *B. cereus*, *P. aeruginosa* and *K. pneumonia*. Apart from that, it has an effective range of medicinal use such as enhance of symptoms related to lymphatic and venous vessels insufficiency, hemorrhagic diseases and hypertension[21]. It could also form the amylase-resistant component via binding with fatty acids and proanthocyanidins including flavan-3-ols to buckwheat starch and resulted in decrease of glycemic indexes[22]. The fifth compound, isoquercitrin (the 3-glucoside of quercetin)[23], is a type of flavanols, which belongs to the flavonoids class[24]. It is one of the compounds that responsible for antidiabetic through inhibition of glucose transport. Rogerio et al.[25] have reported that isoquercitrin has anti-inflammatory properties which served as eosinophilic suppressors in the blood and lung parenchyma. This compound can also be found in eastern redbud, maize, *Trifolium* blossoms and tobacco[23]. The sixth compound, quercetin, is under subclass of anthoxanthins (flavonols) and class of flavonoids[15,24]. High concentration of quercetin can be found in olives[26]. It has a capability to completely prevent LDL oxidation by scavenging free radicals and chelating transition metal ions[15,16]. The elderly who consumes 26 mg/day of quercetin via vegetables and fruit can reduces the risk of getting coronary heart disease[15]. Besides, Rogerio et al.[25] reported that quercetin has anti-inflammatory properties, which served as eosinophilic suppressors in the blood and lung parenchyma. The seventh compound, kaempferol-7-neohesperidoside, is a flavonoid glycoside which was previously reported in the seeds of the *Litchi chinensis* fruit and it was shown to possess anti-proliferative effects on a variety of human cancer cell line in vitro[27]. It was also reported that kaempferol glycosides exhibit an effect in lowering blood glucose[28]. Referring to these compounds, it could be suggested that *Pisang Awak* leaves can be a good source for producing nutraceutical and pharmaceutical products.

CONCLUSION

Bioactive phytochemicals from *Pisang Awak* leaves were successfully extracted. Extraction time, S/S ratio and ethanol concentration were shown to be important extraction parameters. The optimum condition for the extraction was: extraction time of 0.5 h, S/S ratio of 48.3 ml/g and ethanol concentration of 88.5%. Quadratic models were successfully developed in predicting all the responses and the results showed that experimental values were corresponding well with the predicted values. Apart from that, chlorogenic acid, procyanidin, epicatechin, rutin, isoquercitrin, quercetin and kaempferol-7-neohesperidoside were identified. This extract showed commercial values in the nutraceutical and pharmaceutical industries.

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REFERENCES

1. Lakshmanasenthil S, Vinothkumar T, Geetharamani D, Marudhupandi T, Suja G, Sindhu NS. Fucoidan-a novel α-amylase inhibitor from Turbinaria ornate with relevance to NIDDM therapy. Biocatalysis and Agricultural Biotechnology 2014; doi: 10.1016/j.bcab.2014.02.003.
2. Bhutkar MA, Bhise SB. In vitro assay of alpha amylase inhibitory activity of some indigenous plants. International Journal of Chemical Sciences 2012; 10(1): 457-462.
3. Lakshmanasenthil S, Vinothkumar T, Geetharamani D, Maruthupandi T. Screening of seaweeds collected from southeast coastal area of India for alphaamylase inhibitory activity, antioxidant activity and biocompatibility. International Journal of Pharmacy and Pharmaceutical Sciences 2013; 5: 240–244.

4. Mohapatra D, Mishra S, Sutar N. Banana and its by-product utilization: an overview. Journal of Scientific & Industrial Research 2010; 69(5): 323-329.
5. Okoli RI, Aigbe O, Ohaju-Obodo JO, Mensah JK. Medicinal herbs used for managing some common ailments among Esan people of Edo state, Nigeria. Pakistan Journal of Nutrition 2007; 6(5): 490-496.
6. Sahaa RK, Acharyaa S, Shovon SSH, Royb P. Medicinal activities of the leaves of *Musa sapientum var. sylvesteris in vitro*. Asian Pacific Journal of Tropical Biomedicine 2013; 3(6): 476-482.
7. Shui GH, Leong LP. Residue from star fruit as valuable source for functional food ingredients and antioxidant nutraceuticals. Food Chemistry 2006; 97(2): 277-284.
8. Liu LX, Sun Y, Laura T, Liang XF, Ye H, Zeng XX. Determination of polyphenolic content and antioxidant activity of kudingcha made from *Ilex kudingcha* C.J. Tseng. Food Chemistry 2009; 112(1): 35-41.
9. Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in Enzymology 1999; 299: 15-27.
10. Ranilla LG, Apostolidis E, Genovese MI, Lajolo FM, Shetty K. Evaluation of indigenous grains from the Peruvian Andean region for antidiabetes and antihypertension potential using *in vitro* methods. Journal of Medicine Food 2009; 12(4): 704-713.
11. Silva EM, Rogez H, Larondelle Y. Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology. Separation and Purification Technology 2007; 55(3): 381-387.
12. Chan SW, Lee CY, Yap CF, Wan Aida WM, Ho CW. Optimisation of extraction conditions for phenolic compounds from limau purut (Citrus hystrix) peels. International Food Research. Journal 2009;16: 203-213.
13. Li FL, Li QW, Gao DW, Peng Y. The optimal extraction parameters and anti-diabetic activity of flavonoids from *Ipomoea batatas* leaf. African Journal of Traditional Complementary and Alternative 2009; 6(2): 195-202.
14. Giada MLR. Food phenolic compounds: main classes, sources and their antioxidant power, Chapter 4, Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants. Intech Publishing, Brazil, 2013.
15. Rao BN. Bioactive phytochemicals in Indian foods and their potential in health promotion and disease prevention. Asia Pacific Journal of Clinical Nutrition 2003; 12(1), 9-22.
16. Diallo B, Vanhaelen M, Gosselain OP. Plant constitutents involved in coating practices among traditional African potters. Experientia 1995; 51(1): 95-97.
17. Obanda M, Owuor PO, Taylor SJ. Flavanol composition and caffeine content of green leaf as quality potential indicators of Kenyan black teas. Journal of the Science of Food and Agriculture 1997; 74(2): 209-215.
18. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Kelm M. (–)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proceeding of National Academic of Sciences U.S.A. 2006; 103(4): 1024-1029.
19. Martínez V, Ugartondo V, Vinardell MP, Torres JL, Mitjans M. Grape epicatechin conjugates prevent erythrocyte membrane protein oxidation. Journal of Agriculture and. Food Chemistry 2012; 60(16): 4090-4095.
20. Singh M, Govindarajan R, Rawat AKS, Khare PB. Antimicrobial flavonoid rutin from *Pteris Vittata* L. against pathogenic gastrointestinal microflora. American Fern Journal 2008; 98(2): 98-103.
21. Pathak D, Pathak K, Singla A. Flavonoids as medical agents - recent advances. Fitoterapia 1991; 62: 371-389.
22. Takahama U, Hirota S. Fatty acids, epicatechin-dimethylgallate, and rutin interact with buckwheat starch inhibiting its digestion by amylase: Implications for the decrease in glycemic index by buckwheat flour. Journal of Agriculture and Food Chemistry 2010; 58(23): 12431-12439.
23. Douglass CD, Howard WL, Wender SH. The isolation of isoquercitrin from the seed pods of Cercis canadensis. Journal of American Chemical Society 1949; 71(8): 2658-2659.
24. Sharma G, Prakash D, Gupta C. Phytochemicals of nutraceutical importance: Do they defend against diseases? Chapter 1, Phytochemicals of Nutraceutical Importance, CABI Publishing, India, 2014, 1-20.
25. Rogerio AP, Kanashiro A, Fontanari C, Silva EVG, Lucisano-Valim YM, Soares EG, Faccioli LH. Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine allergic asthma. Inflammation Research 2007; 56(10): 402-408.
26. Vlahov G. Flavonoids in three olive (*Olea europaea*) fruit varieties during maturation. Journal of the Science of Food and Agriculture 1992; 58(1): 157-159.
27. Xu X, Xie H, Hao J, Jiang Y, Wei X. Flavonoid glycosides from the seeds of litchi chinensis. Journal of Agricuture and Food Chemistry 2011; 59(4): 1205-1209.
28. Yamasaki K, Hishiki R, Kato E, Kawabata J. Study of kaempferol glycoside as an insulin mimic reveals glycon to be the key active structure. ACS Medicinal Chemistry Letters 2011; 2(1) 17-21