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ENZYME-ASSISTED WATER EXTRACTION OPTIMIZATION, ANTIOXIDANT CAPACITY AND PHENOLIC PROFILING OF EXTRACTS FROM *GARCINIA MANGOSTANA* LINN

 Poh-Lee Tan[1,2]
 Kelvin Quai-Siang Soo[2]
 Yih-Phing Khor[1]
 Faridah Abas[3]
 Chin-Ping Tan[1+]

[1]*Department of Food Technology, Faculty of Food Science and Technology, Putra University Malaysia, Serdang, Selangor, Malaysia.*
[2]*Furley Bioextracts Sdn. Bhd., Semenyih, Selangor, Malaysia.*
[3]*Department of Food Science, Faculty of Food Science and Technology, Putra University Malaysia, Serdang, Selangor, Malaysia.*
[1,2]*Email:* pollytan54@gmail.com
[2]*Email:* kelvinsoo@furleybio.com
[1]*Email:* sweet_appie@hotmail.com
[3]*Email:* faridah_abas@upm.edu.my
[1]*Email:* tancp@upm.edu.my

(+ Corresponding author)

ABSTRACT

The application of enzymes in extraction processes within the food industry has not been widely explored. In this paper, an enzyme-assisted water extraction method was employed and optimized to extract the antioxidative and phenolic compounds from the pericarp of *Garcinia mangostana* L. Fractional factorial design (FFD) was developed to screen out the significant factors from five extraction parameters, including enzyme concentration, liquid-solid ratio, reaction temperature, pH and reaction time. Next, a central composite design approach was used to optimize the significant parameters after the screening process. The optimal conditions according to statistical analysis were 1.46% w/w of enzyme (Celluclast® 1.5L & Pectinex® Ultra SP-L) concentration, 22.80 liquid-solid ratio, reaction temperature of 30 °C, pH 3 and 30 min of reaction time. Under the optimized conditions, antioxidant activities indicated by the 2,2-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) values and total phenolic content obtained were 86.51%, 86.77% and 21.222 mg gallic acid equivalent (GAE)/g, respectively.

Contribution/Originality: Phenolic compounds extracted from pericarp of *Garcinia mangostana* L. are functional compounds which contained high antioxidant activities. Response surface methodology was employed to optimize the usage of enzymes in the extraction activity of antioxidative and phenolic compounds from the *Garcinia mangostana* L.'s pericarp. Besides using as the active pharmaceutical ingredient, the pericarp extract can be developed as a potential functional food ingredient. To our knowledge, this is the first report on the enzyme-assisted water extraction of the pericarp of *Garcinia mangostana* L. and its usage as one of the food ingredients in functional product.

1. INTRODUCTION

Garcinia mangostana, commonly known as mangosteen, belongs to the Clusiaceae family. Primarily, mangosteen is consumed as a fresh fruit and it is a delicacy especially for Southeast Asian. Currently, mangosteen has become well known in Western Europe and America markets because of its properties especially from the pericarp (hull) which has been reported to enhance human health (Suksamrarn et al., 2003). The mature fruit of mangosteen has a dark purple pericarp, which is rich in secondary bioactive metabolites, such as xanthones, anthocyanins and oligomeric

proanthocyanidins (Zarena & Sankar, 2012). Extensive research of extraction of mangosteen pericarp have been conducted and published in recent years; however, organic solvents extraction such as ethanol, methanol, hexane and acetone were commonly used in those studies, whereby these kinds of extracts obtained could have several limitations as the solvents used are toxic to human and non-environmentally friendly (Machmudah et al., 2015). Besides that, solvent extraction requires further evaporation steps if the extract is to be used in food applications. Most of the natural bioactive compounds and phenolic compounds present in plant materials are protected by the plant cell walls which contain polysaccharides such as cellulose, hemicellulose and pectins (Gil-Chávez et al., 2013). Degradation of plant cell wall structure and depolymerization of plant cell wall polysaccharides could facilitate the release of these intracellular compounds. Hence, some enzymes such as cellulose, pectinase, β-glucosidase, xylanase and β-gluconase have been used to assist in the extraction of natural bioactive compounds from plant matrix (Gil-Chávez et al., 2013).

Several studies have reported on the pre-treatment of plant tissues prior to the extraction of natural bioactive compounds. Cellulase® multifect xylanase (MX), Cellulase® CL and Kleerase® alpha-fetoprotein (AFP) were incorporated in the extraction of phenolics from citrus peels study, in which total phenolic contents of five citrus peels were improved with the enzyme-assisted extraction (Li, Smith, & Hossain, 2006). Enzyme-assisted extraction was performed in a study to improve antioxidant compounds in black carrot juice. Total anthocyanin content obtained was almost doubled after applying the enzyme-assisted extraction in the black carrot juice processing (Khandare, Walia, Singh, & Kaur, 2011). In another study, enzymatic hydrolysis of rapeseed meal was performed by using Viscozyme® L, Pectinex® Ultra SP-L and Celluclast® 1.5L (Rodrigues, Carvalho, & Rocha, 2014). Their findings indicated that carbohydrate hydrolysis process was improved and protein content in the rapeseed meal was increased after the treatment of enzymes. Hence, enzyme-assisted extraction of bioactive compounds from plants for their application in food would be another potential and "greener" extraction method. Response surface methodology (RSM) is a widely used statistical tool in optimizing experimental parameters and to reduce time-consuming procedure of the one-factor-at-a-time (OFAT) optimization method, thus maximizing the targeted responses (Rajha et al., 2014). The objective of this study is to optimize enzyme-assisted water extraction technology in an attempt to maximize antioxidative compounds and total phenolic content from the pericarp of *Garcinia mangostana*. However, due to a large number of extraction variables involved, fractional factorial design (FFD) was used to identify the most important factors from a large number of parameters with a small number of runs. Further to the initial screening steps by FFD, optimization of the extraction technology was enhanced by Central Composite Design (CCD).

2. MATERIALS AND METHODS

2.1. Plant Materials

Mature fruits of *Garcinia mangostana* L. were contributed by Furley Bioextracts Sdn. Bhd. and were collected from Furley's mangosteen plantation in Jelebu, Negeri Sembilan.

2.2. Chemicals and Reagents

Celluclast® 1.5L and Pectinex® Ultra SP-L were purchased from Brenntag Asia Pacific, Selangor, Malaysia. Celluclast® 1.5L has an activity of ≥700 units/g and is an enzyme complex that can catalyze the breakdown of cellulose into glucose, cellobiose and glucose oligomers. Pectinex® Ultra SP-L has an activity of ≥3,800 units/mL and is an enzyme complex with high pectolytic activity with some hemicellulolytic activity. Folin Ciocalteu reagent, sodium carbonate, 2,2-diphenyl-2-picrylhydrazyl (DPPH), and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich (M) Sdn. Bhd. (Selangor, Malaysia). Ethanol 96% was purchased from Fisher Scientific (M) Sdn. Bhd. (Selangor, Malaysia). Suma Tab D4 tab was purchased from Pilah Syabas Marketing (Negeri Sembilan, Malaysia).

2.3. Sample Preparation

Mangosteen fruits were cleaned and immersed into food-grade disinfectant tablets, Suma Tab D4 tab (Diversey, Wadeville, South Africa) for 5 min. They were rinsed thoroughly with reverse osmosis water. The pericarps of the mangosteen were separated from the flesh and crushed into small pieces approximately 3 cm x 3 cm of dimension prior to drying.

2.4. Drying of Mangosteen Pericarp

The mangosteen pericarp was dried at temperatures of 75°C in a hot air oven (Good and Well, model YXD-4A, Selangor, Malaysia) until the moisture content of mangosteen pericarp reached around 10 % dry basis (Suvarnakuta, Chaweerungrat, & Devahastin, 2011). The moisture content of mangosteen pericarp was evaluated by using a moisture analyzer (OHAUS, Model MB 25, Shanghai, China). The dried mangosteen pericarp was crushed into powder using a dry crusher and sieved through a vibrating sifter with 60 mesh screen sieve and yielded particle size of around 250 μm. The crushed pericarp was packed into plastic bags, sealed and stored at room temperature until further extraction process.

2.5. Enzyme-Assisted Water Extraction Process

Celluclast® 1.5L and Pectinex® Ultra SP-L (ratio 1:1) was dispersed in 100 mL deionized water to obtain solutions of defined concentrations (0 − 10% w/w). The defined solid-liquid ratio values (1:10 − 1:30) of mangosteen pericarp powder were added to the enzymatic solution, which was adjusted to defined pH values (3.0 − 8.0) with 0.1 M hydrochloric acid (HCl) solution. The samples were placed in a shaking water bath at defined temperatures (30°C − 100°C) and defined reaction times (30 − 180 min). After enzymatic treatment, the samples were centrifuged at 2500 rpm for 15 min. After centrifugation, the liquid extracts were collected and stored at 10°C prior to analysis. All the experiments were performed in triplicate.

2.6. Experimental Design and Statistical Analysis

2.6.1. Preliminary Screening of Significant Factors using Two-Level Fractional Factorial Design

Fractional factorial design (FFD) was used because there were five factors to be studied and fewer runs were targeted. Initially, FFD was used to screen the most important extraction parameters of mangosteen pericarp. The real and coded values of five quantitative variables are presented in Table 1. The variables were studied at two levels, high (+1) and low (-1). If two-level full factorial design with five variables was applied, 32 runs of experiments were needed. Thus, to reduce the number of runs for economic purpose, a half fractional factorial design (25-1=16) was introduced, whereby 21 runs were conducted, including 5 center points. FFD design was based on the first-order polynomial model as the following equation:

$$Y = \beta o + \beta i X I$$

where Y is the response, βo is the model intercept, βi is the linear regression coefficient, and $X I$ is the level of the independent variable. All the experiments were performed in triplicate, and averages of the results of DPPH, ABTC and TPC analyses were taken as responses.

Table 1. The actual and coded values of five variables in two-level fractional factorial design.

Variables	Unit	Low actual	High actual
A: Enzyme concentration	% w/w	0.00	10.00
B: Liquid-solid ratio		10.00	30.00
C: Reaction time	Min	30.00	180.00
D: Temperature	°C	30.00	100.00
E: Reaction pH		3.00	8.00

2.6.2. Extraction Optimization using Central Composite Design (CCD)

After screening significant variables from two-level fractional factorial design, central composite design (CCD) was applied for further analysis and optimization of the interaction between significant variables. CCD studies five different levels [(+alpha, -alpha) (axial points); (+1, -1) (factorial points) and the center point]. The responses for CCD optimization are averages of the results of DPPH, ABTS and TPC analyses. All the experiments were carried out in triplicate.

2.7. Antioxidant Activity Evaluation

2.7.1. 2,2-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay

A concentration of 100 µM DPPH radical was prepared with ethanol and 3.9 mL of DPPH ethanolic solution was added into a 0.2 mL of mangosteen pericarp extract. The mixture was mixed vigorously for 10 s and left to stand in the dark for 60 min at room temperature. The absorbance was measured at 540 nm by using a UV-Vis spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). Pure ethanol was used as a blank to calibrate the spectrophotometer (Chari, Manasa, Srinivas, & Sowbhagya, 2013).

The radical-scavenging activity on DPPH was expressed as % inhibition as equation as below:

$$\% \text{ Inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

where $OD_{control}$ and OD_{sample} are optical densities of ethanol and mangosteen pericarp extract, respectively.

2.7.2. 2,2'-Azinobis (3-Ethylbenzothiazoline-6-Sulphonic Acid) (ABTS) Assay

ABTS (10 mg) was diluted in 2.6 mL of potassium persulfate solution (2.45 mM) to form ABTS. + radical cations with a final concentration of 7 mM. The mixture was left to stand in the dark for 12-16 h at room temperature before use. The ABTS. + was diluted with deionized water to an absorbance of 0.70 ± 0.02 at 734 nm. 1 mL of mangosteen pericarp extract was added with 3 mL of ABTS. + solution and stand in the dark at room temperature for 60 min. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer. Deionized water was used as blank to calibrate the spectrophotometer (He et al., 2012). Percentage inhibition of the ABTS radicals by mangosteen pericarp extract is calculated by using the following equation:

$$\% \text{ Inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

where $OD_{control}$ and OD_{sample} are optical densities of reverse osmosis water and mangosteen pericarp extract, respectively.

2.7.3. Total Phenolic Content (TPC)

Total phenolic content of mangosteen pericarp extract was analyzed by using Folin Ciocalteu method (Azlim et al., 2010). The extract was prepared at a concentration of 1 mg/mL. A 100 µL aliquot of extract was mixed into 750 µL Folin Ciocalteu reagent (prediluted 10-fold with reverse osmosis water). Then, 0.75 mL of 6% (w/v) sodium carbonate solution was added after the solution was incubated for 5 min at room temperature and mixed gently. The mixed solution was incubated again for 90 min at room temperature. Absorbance was measured at 725 nm with a UV-Vis spectrophotometer, and total phenolic content of the extracts was expressed in milligram of gallic acid equivalent per gram of mangosteen dry pericarp (mg GAE/g). Standard calibration curve of gallic acid ($0.01 - 0.05$ mg/mL) was plotted.

2.8. Phenolic Profiling using Liquid Chromatography Mass Spectrometry (LC-MS/MS)

Profiling of mangosteen pericarp extract after optimization process was performed with liquid chromatography mass spectrometry (LC-MS/MS) (Applied Biosystems (AB) Sciex Instruments, 3200 quadrupole ion trap (QTRAP),

Massachusetts, USA). The sample was filtered through 0.45 µm nylon syringe filter and injection volume is 20 µL. Mobile phase consisted of water with 0.1% of formic acid plus 5mM ammonium formate (A) and acetonitrile with 0.1% of formic acid plus 5mM ammonium formate (B). The gradient program was as 10% of mobile phase A to 90% of mobile phase B from 0.01 min to 8.0 min, held for 2 min and back to 10 % A in 0.1 min and re-equilibrated for 5 min. AB Sciex 3200 QTrap LC-MS/MS with Perkin Elmer (PE) Flexar FX 15 series ultra high performance liquid chromatography (UHPLC) (voltage 4.5 kV; source temperature 500°C; desolvation gas 40 psi and source gas 40 psi) was used and operated with negative polarity and controlled by software Analyst 1.5.2. The column used was Phenomenex Synergi Fusion-RP C18 (California, USA). Flow rate was in gradient flow between 250 − 400 mL/min.

2.9. Statistical Analysis

The statistical analysis was performed using Design Expert software, version 7.1.5 (STAT-EASE, Inc., Minneapolis, MN) and assessed by analysis of variance (ANOVA) at a confidence level above 95% or p-value less than 0.05.

3. RESULTS AND DISCUSSIONS

3.1. Screening of Significant Variables using FFD

Fractional factorial design was applied to screen the significant factors for extraction of mangosteen pericarp. The design of experiments with independent variables and responses are shown in Table 2.

Table 2. Fractional factorial design shows variables (coded values) and responses.

Std.	Run	Independent variables					Responses		
		A	B	C	D	E	TPC(Y_1)	DPPH(Y_2)	ABTS (Y_3)
1	4	-1	-1	-1	-1	1	10.62	86.13	82.85
2	18	1	-1	-1	-1	-1	10.18	87.02	90.07
3	2	-1	1	-1	-1	-1	25.13	90.11	89.80
4	19	1	1	-1	-1	1	19.54	89.01	91.04
5	11	-1	-1	1	-1	-1	10.58	86.65	81.51
6	13	1	-1	1	-1	1	10.18	85.66	90.57
7	15	-1	1	1	-1	1	25.86	89.07	84.65
8	6	1	1	1	-1	-1	19.07	89.26	91.17
17	7	0	0	0	0	0	19.70	90.50	90.47
18	8	0	0	0	0	0	19.67	90.77	90.37
19	9	0	0	0	0	0	18.81	91.00	90.27
20	3	0	0	0	0	0	19.15	90.40	90.67
21	10	0	0	0	0	0	18.90	90.77	90.24
9	5	-1	-1	-1	1	-1	10.53	83.94	84.15
10	17	1	-1	-1	1	1	10.30	88.99	88.67
11	1	-1	1	-1	1	1	30.75	87.11	89.27
12	12	1	1	-1	1	-1	22.40	90.83	90.00
13	21	-1	-1	1	1	1	10.61	87.96	80.94
14	20	1	-1	1	1	-1	10.19	87.38	88.83
15	16	-1	1	1	1	-1	29.98	88.24	85.59
16	14	1	1	1	1	1	24.55	90.42	89.97

Note: A is the coded value of enzyme concentration, B is the coded value of liquid-solid ratio, C is the coded value of reaction time, D is the coded value of temperature, E is the coded value of reaction pH, Y_1 is the total phenolic content (mg GAE/g of mangosteen dry pericarp), Y_2 is the DPPH value in % inhibition, Y_3 is the ABTS value in % inhibition.

3.2. Regression Models for TPC, DPPH & ABTS

The significant variables and interaction terms were employed in multiple regression analysis to obtain predicted responses by the following second-order polynomial equations:

For TPC:

Y_1 = 17.53 - 1.73A + 7.13B + 0.097C + 1.13D + 0.27E - 1.54AB + 0.099AC - 0.075AD + 0.070AE + 0.11BC + 1.12BD + 0.24BE + 0.071CD - 0.099CE + 0.12DE

For DPPH:

$Y_2 = 87.99 + 0.59A + 1.27B + 0.094C + 0.12D + 0.057E + 0.039AB - 0.49AC + 0.71AD - 0.11AE - 0.10BC - 0.23BD - 0.41BE + 0.30CD + 0.14CE + 0.45DE$

For ABTS:

$Y_3 = 87.44 + 2.60A + 1.49B - 0.79C - 0.26D - 0.20E - 0.99AB + 0.88AC - 0.41AD + 0.22AE - 0.30BC + 0.036BD - 6.250E - 003BE - 0.056CD + 0.076CE + 0.23DE$

Results of the statistical significance of regression models are shown in Table 3 with F-test and p-value by using ANOVA.

Table 3. Multiple regression analysis results for the significant variables and interactions on responses of TPC, DPPH and ABTS.

Variables	TPC (Y_1)			DPPH (Y_2)			ABTS (Y_3)		
	F value	p-value	Mean Square	F value	p-value	Mean Square	F value	p-value	Mean Square
Model	360.92	<0.0001	62.91	61.14	0.0006	3.50	417.04	<0.0001	12.63
A	274.10	<0.0001	47.78	95.61	0.0006	5.48	3565.13	<0.0001	107.95
B	4668.81	<0.0001	813.79	450.61	<0.0001	25.81	1179.02	<0.0001	35.70
C	0.87	0.4037	0.15	2.46	0.1922	0.14	328.73	<0.0001	9.95
D	118.02	0.0004	20.57	4.19	0.1100	0.24	37.11	0.0037	1.12
E	6.80	0.0595	1.19	0.92	0.3909	0.05	20.61	0.0105	0.62
AB	218.05	0.0001	38.01	0.42	0.5525	0.02	516.58	<0.0001	15.64
AC	0.89	0.3982	0.16	65.72	0.0013	3.76	412.69	<0.0001	12.50
AD	0.51	0.5128	0.09	141.33	0.0003	8.09	87.74	0.0007	2.66
AE	0.45	0.5405	0.08	3.30	0.1433	0.19	25.57	0.0072	0.77
BC	1.08	0.3578	0.19	2.94	0.1618	0.17	48.35	0.0022	1.46
BD	116.10	0.0004	20.24	14.62	0.0187	0.84	0.69	0.4516	0.02
BE	5.41	0.0806	0.94	47.25	0.0023	2.71	0.021	0.8927	0.00
CD	0.46	0.5337	0.08	24.73	0.0076	1.42	1.67	0.2656	0.05
CE	0.91	0.3950	0.16	5.48	0.0794	0.31	3.07	0.1545	0.09
DE	1.28	0.3219	0.22	57.52	0.0016	3.29	28.56	0.0059	0.86
R²			0.999			0.996			0.999
Adj R²			0.997			0.979			0.997
CV			2.33			0.27			0.20

Note: *A* is the coded value of enzymes (Celluclast® 1.5l and Pectinex® Ultra SPL) concentration (0 – 10 % w/w), *B* is the coded value of liquid-solid ratio (10.00 – 30.00), *C* is the coded value of reaction time (30 – 180 mins.), *D* is the coded value of temperature (30ºC – 100ºC), *E* is the coded value of reaction pH (3.00 – 8.00), Y_1 is the total phenolic content (mg GAE/g of mangosteen dry pericarp), Y_2 is the DPPH value in % inhibition, Y_3 is the ABTS value in % inhibition.

Fractional factorial design was used to estimate all main effects and all two-factor interactions, meanwhile higher order interactions were negligible because low-order interactions are more important than higher order interactions (Jaynes, Xianting, Hongquan, Wengkee, & Chih-Muig, 2013). Five replicated center points were added into this FFD experiment to provide an estimate of pure error and it does not affect the estimates of factorial effects.

Based on the ANOVA results, Model F-value of 360.92 for the response of TPC (Y_1) analysis implies that the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. The R-squared value is 0.9993. In this case, the significant model terms are A, B, D, AB and BD. For response Y_2, DPPH assay, Model F-value of 61.14 shows that the model is significant and there is only 0.06% chance that a "Model F-value" this large could occur due to noise. This percentage could be referred from the p-value shown in Table 3. R-squared value is 0.9957. For this model, A, B, AC, AD, BD, BE, CD and DE are significant model terms. Referring to multiple regression analysis result for the response (Y_3), Model F-value of 417.04 implies that the model is significant. The R-squared value is high, 0.9994. The significant model terms are A, B, C, D, E, AB, AC, AD, AE, BC and DE. Coefficient of determination (R²) and the adjusted determination coefficient (Adj R²) were summarized in Table 3 and confirmed the model was highly significant. Meanwhile, low values of coefficient of the variation (CV) indicated smaller residuals relative to the predicted value and a high degree of precision.

According to the p-value of all factors shown in the Table 3, it can be concluded that only two independent variables, A (enzyme concentration) and B (liquid-solid ratio) have significant influences on the total three dependent variables, total phenolic content, % inhibition of DPPH and % inhibition of ABTS. which are different from the other three factors C (reaction time), D (temperature) and E (reaction pH).

3.3. Optimization of Enzyme-Assisted Water Extraction using Central Composite Design (CCD)

After screening significant factors of the extraction process by FFD, response surface methodology was employed to optimize maximal antioxidant activities and total phenolic content obtained from enzyme-assisted water extraction method. The two most significant factors, enzyme concentration (A) and liquid-solid ratio (B), were used in the 2^2 full factorial central composite design. Results of ANOVA, goodness of fit and the adequacy of the models are summarized in Table 4.

Table 4. Analysis of response values (TPC, DPPH, ABTS) and their ANOVA results under two most significant parameters.

Source	Mean Square	F-ratio	p-value
TPC (Y_1)			
Model	69.89	24.15	<0.0001
Quadratic	95.55	24.94	<0.001
A	32.80	11.33	0.0046
B	37.90	13.09	0.0028
AB	20.40	7.05	0.0189
A²	70.58	24.39	0.0002
B²	168.05	58.06	<0.0001
A²B	2.00	0.69	0.4202
AB²	18.78	6.49	0.0232
Lack of Fit	5.66	2.11	0.1701
R²	0.924		
Adj R²	0.885		
Pred R²	0.817		
DPPH (Y_2)			
Model	44.25	37.43	<0.0001
Quadratic	4.76	17.09	0.0001
A	5.81	34.39	<0.0001
B	10.14	60.06	<0.0001
AB	1.34	7.91	0.0138
A²	4.41	26.12	0.0002
B²	7.71	45.65	<0.0001
A²B	1.10	6.52	0.0229
AB²	0.99	5.84	0.0229
Lack of Fit	0.09	0.51	0.4859
R²	0.949		
Adj R²	0.924		
Pred R²	0.841		
ABTS (Y_3)			
Model	0.25	16.18	<0.0001
Quadratic	0.29	18.78	<0.0001
A	0.25	16.33	0.0009
B	0.42	26.85	<0.0001
AB	2.450E-003	0.16	0.6973
A²	0.17	11.15	0.0042
B²	0.54	34.74	<0.0001
Lack of Fit	0.03	2.17	0.1405
R²	0.835		
Adj R²	0.783		
Pred R²	0.693		

Note: A: Enzyme concentration; B: Liquid-solid ratio; C: Reaction time; D: Temperature; E: Reaction pH; TPC: Total phenolic content; DPPH: 2,2-Diphenyl-2-picrylhydrazyl; ABTS: 2,2'-Azinobis (3-ethylbenzithiazoline-6-sulphonic acid).

The relationship between process parameters and responses is presented in equations as follows:

$$Y_1 = 21.86 - 2.02A + 0.34B - 1.60AB - 2.50A^2 - 3.86B^2 + 0.71A^2B - 2.17AB^2$$

$$Y_2 = 89.90 - 0.85A + 1.13B + 0.41AB - 0.62A^2 - 0.83B^2 - 0.52A^2B - 0.50AB^2$$

$$Y_3 = 90.91 - 0.13A + 0.16B + 0.017AB - 0.12A^2 - 0.22B^2$$

Referring to the ANOVA test results, "Lack of Fit" for the responses of TPC, DPPH and ABTS was not significant and adequate with satisfactory R^2 values. This results indicated that the quadratic model was highly significant ($p<0.0001$) for the three responses. Total phenolic content of mangosteen pericarp extract obtained by enzyme-assisted water extraction are maximized with the optimal conditions proposed and response surface analysis of data in Table 4 demonstrated that effects of enzyme concentration and liquid-solid ratio were significant ($p<0.05$) but the interaction term (A^2B) was insignificant ($p>0.05$). It can be seen that variable with the largest effect on TPC was the quadratic term of liquid-solid ratio (B^2) followed by the quadratic term of enzyme concentration (A^2), interaction term of (AB^2), linear term of enzyme concentration (A), interaction term (AB) and linear term of liquid-solid ratio (B). The coefficient of determination (R^2) of the predicted model for TPC was 0.9235 and the p-value for lack of fit was 0.1701 which suggests the model was a good fit to the mathematical model.

ANOVA results in Table 4 indicated that the linear coefficients (A, B), quadratic coefficients (A^2, B^2) and interaction coefficients (AB, A^2B, AB^2) were all significantly ($p<0.05$) correlated with the DPPH values. Variable with the largest effect on DPPH was the linear term of liquid-solid ratio (B) followed by linear term of enzyme concentration (A), quadratic term of liquid-solid ratio (B^2), quadratic effect of enzyme concentration (A^2), interaction terms (A^2B, AB^2, AB). The model was good fit to the mathematical model as coefficient of determination (R^2) for the predicted model for DPPH was 0.9493 with the p-value of lack of fit was 0.4859.
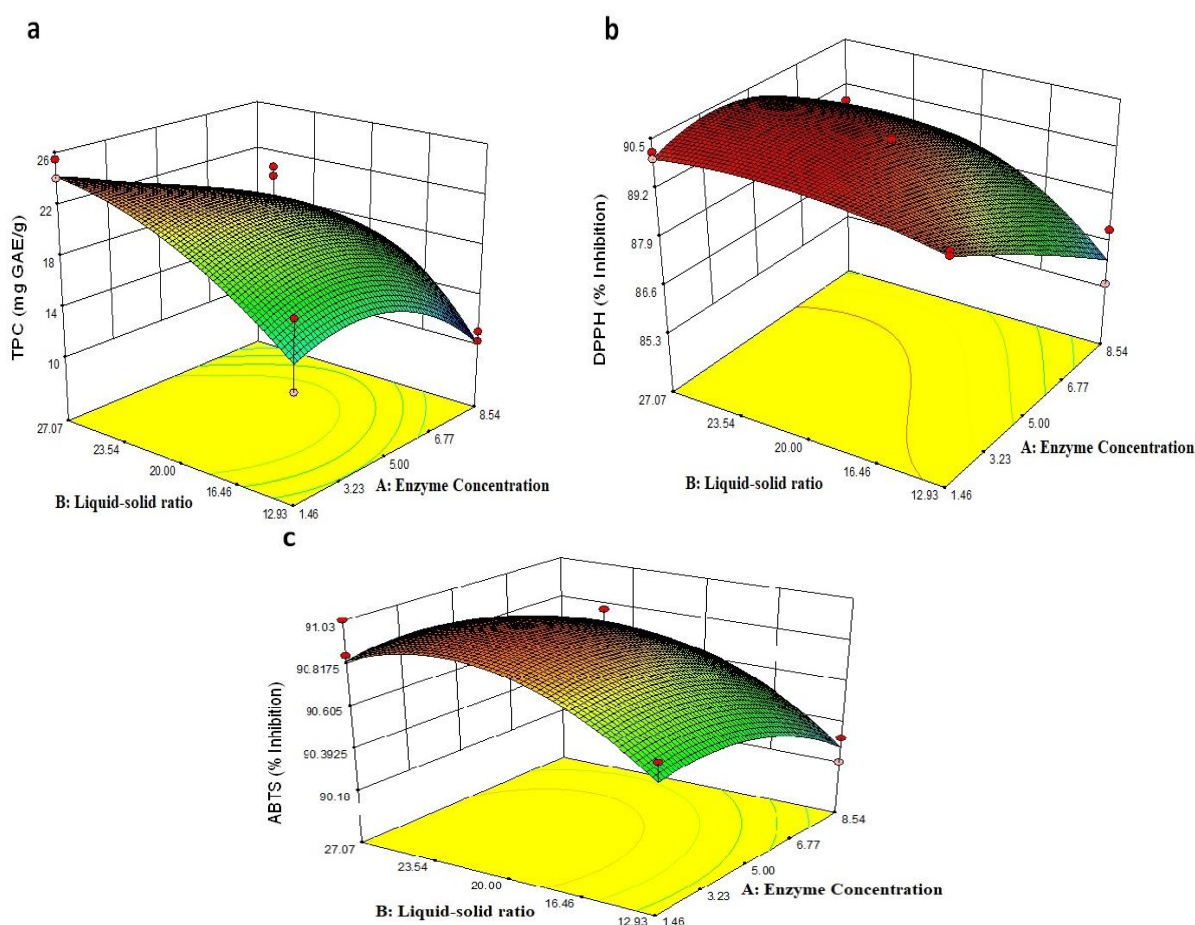

Figure 1. Response surface plot for (a) TPC (in mg GAE/g), (b) DPPH (in % inhibition) and (c) ABTS (in % inhibition) as a function of enzyme concentration and liquid-solid ratio.

It can be observed that variable with the largest effect on ABTS was quadratic term of liquid-solid ratio (B^2) followed by linear term of liquid-solid ratio (B) and quadratic term of enzyme concentration (A^2). Interaction terms (AB) were insignificant ($p>0.05$) to this contribution. The coefficient of determination (R^2) in this response was 0.8349 and p-value for Lack of Fit was 0.1405. These values imply that the model selected has good fit.

Three-dimensional (3D) response surface plot was the best way to demonstrate the effect of independent variables on the dependent responses (Zhang et al., 2012). Referring to Figure 1 (a), total phenolic content almost linearly increased with increasing liquid-solid ratio. However, for enzyme concentration factor, total phenolic content rose at first but started to decline when enzyme concentration reached 5.00 % (w/w). Influence of liquid-solid ratio on DPPH value was not significantly shown in Figure 1 (b), but the increasing enzyme concentration resulted in the decreasing of DPPH value. The ABTS values obtained showed a positive quadratic model and the response rose at the beginning with the increasing of liquid-solid ratio, but it started to decrease slightly once the liquid-solid ratio reached the high levels. This may be supported with the fact that mass transfer of soluble bioactive compounds from mangosteen pericarp to solvent was enhanced due to the increasing of the contact surface between mangosteen pericarp and the solvent at lower liquid-solid ratio (Bamba et al., 2018). A similar trend was observed on the enzyme concentration on the yield of ABTS results as shown in Figure 1 (c). The % inhibition value of ABTS started to decrease with higher concentrations of enzymes. This might be caused by end-product inhibition happening with higher loads of enzymes resulting in a faster total hydrolysis process.

Due to cost-saving considerations, the lowest levels of factors within optimum zone and yielded maximum levels of the three responses would be preferred. Therefore, the predicted maximal values of total phenolic content (20.581 mg GAE/g), DPPH assay (90.08%) and ABTS assay (90.88%) with the desirability of 0.799 were obtained at 1.50% of enzyme concentration and 18.75 of liquid-solid ratio.

3.4. Model Validation

Adequacy of the model was validated by using optimal conditions for the extraction process. The optimal conditions were enzyme concentration of 1.50% w/w, liquid-solid ratio of 18.75 at constant conditions of temperature (30°C), pH 3 and reaction time (30 min). The maximum experimental values of total phenolic content, DPPH value and ABTS values obtained under optimal conditions were 21.222 mg GAE/g, 86.51% and 86.77% ($n=3$), respectively. The values were statistically validated by one sample t-test using Minitab® Release 14 12.0 (Minitab Inc., Pennysylvania, USA). As shown in Table 5, the data obtained from experiments conducted under optimal conditions were in good agreement with the predicted values that were generated by RSM.

Table 5. Comparison of total phenolic content and antioxidant properties predicted value and experimental value from mangosteen pericarp extract by enzyme-assisted extraction.

Values	TPC (mg GAE/g)	DPPH (%)	ABTS (%)
Predicted value	20.58	90.08	90.88
Experimental value	21.22 ± 0.35	86.51 ± 0.33	26.77 ± 0.21
p-value	0.962	0.037	0.038

Note: The data were expressed as mean ± standard deviation for the experimental value. TPC: Total phenolic content; DPPH: 2,2-Diphenyl-2-picrylhydrazyl; ABTS: 2,2'-Azinobis (3-ethylbenzithiazoline-6-sulphonic acid).

3.5. Phenolic Profiling of Mangosteen Pericarp Extract using LCMS/MS

Mass spectrometry (MS) is a useful tool that ionizes chemical compounds to generate charged molecules or fragment ions and to measure mass-to-charge ratios of the compounds (Ramos-Jerz, Jerz, Villanueva-Rodriquez, & Winterhalter, 2018). Negative ionization mode is often used to identify compounds containing only carbon, oxygen and hydrogen; they are poorly detected in positive ionization (Tada et al., 2019). Identification of the compounds from LC-MS/MS analysis was determined by comparisons with Sciex internal natural product database and were tentatively characterized on the basis of previous literature data. Qualitative identification was determined by mass spectrum, retention time and presence of fragment ions.

Table 6. Phenolic compounds identified in enzyme-assisted water extracted mangosteen pericarp extract.

Compound	Retention Time (Min)	Proposed Compound	[M-H]⁻ (m/z)	MSᵃ (m/z)	Maximum Intensity (cps)	Identification Reference
A	5.04	Smeaxanthone	395	395, 257	6.7 x10⁶	Destandau, Michel, Toribio, and Elfakir (2014)
B	5.96	Apigenin-6-C-glucoside	431	431, 330, 285, 243	7.2 x 10⁶	Kang, Price, Ashton, Tapsell, and Johnson (2016)
C	7.01	Garcimangosone C	411	411, 353, 283	6.7 x 10⁶	Destandau et al. (2014)
D	7.27	Alpha- mangostin	409	409, 365, 340	3.5 x 10⁶	Sciex internal database
E	7.66	Gartanin	395	395, 377, 283	3.0 x 10⁶	Sciex internal database
F	7.92	1,5-Dihydroxy-3-methoxy-2-(3-methyl-2-buten-1-yl)-9H-xanthen-9-one	325	325, 310, 267, 223	1.2 x 10⁷	Destandau et al. (2014)
G	8.19	8-desoxygartanin	379	379, 335, 281	4.0 x 10⁷	Sciex internal database
H	8.45	Gamma-mangostin	395	395, 309, 297, 271	3.4 x 10⁷	Sciex internal database

Based on the previous studies on *Garcinia mangostana*, so far, researchers have found at least 80 types of xanthone compounds in various parts of the plant (Vemu, Nauman, Veenstra, & Johnson, 2019). Mangosteen pericarp has been proven by Ibrahim et al. (2016) and another study of Mamat, Azizan, Baharum, Mohd Noor, and Mohd Aizat (2018) to contain high content of secondary metabolites such as xanthones, flavonoids, triterpenes and benzophenones (Ibrahim et al., 2016; Mamat et al., 2018). The most abundant metabolites in the mangosteen pericarp are α-mangostin and γ-mangostin (Fabiola, Bruno, Kelly, & Alexeia, 2013), but depends on the extraction method and polarity of the solvents used. Water as the universal polar solvent is able to extract polar metabolites, while non-polar solvents are suitable to extract non-polar metabolites. Some of the most common organic solvents such as dichlorometane, acetone, methanol and ethanol were used to extract mangosteen (Aizat, Ahmad-Hashim, & Jaafar, 2019), however, these carbon-based organic solvents generally are hazardous to health if swallowed and inhaled more than the maximum limit and it may cause skin problems if long term of contact (Joshi & Adhikari, 2019). Moreover, based on the studies so far, there is no single solvent that could be suitable to extract and obtain the whole range of metabolites due to the varieties of metabolites with different polarities, chemical behavior and concentration (Kim, Choi, & Verpoorte, 2010).

Therefore, some advanced extraction techniques have been developed to extract polyphenolic compounds from the mangosteen pericarp such as microwave-assisted extraction (Mohammad et al., 2019), ultrasonic bath (Cheok, Chin, Yusof, Talib, & Law, 2013) and supercritical carbon dioxide extraction reported by Zarena and Sankar (2009) and Hamid, Bakar, Park, Ramli, and Wan (2018). However, these techniques require specific equipment to extract the bioactive compounds. Hence, on a large-scale production, a simple method such as water extraction with the enzyme-assisted method was useful on the extraction of the pericarp of *Garcinia mangostana*.

For this part of study, tentative identification of compounds from the pericarp of *Garcinia mangostana* contained smeaxanthone *m/z* 395 (tR = 5.04 min), apigenin-6-C-glucoside *m/z* 431 (tR = 5.96 min), garcimangosone-C *m/z* 411 (tR = 7.01), α-mangostin *m/z* 409 (tR = 7.27), gartanin *m/z* 395 (tR = 7.66), 1,5-dihydroxy-3-methoxy-2-(3-methyl-2-buten-1-yl)-9H-xanthen-9-one *m/z* 325 (tR = 7.92), 8-desoxygartanin *m/z* 379 (tR = 8.19) and γ-mangostin *m/z* 395 (tR = 8.45) were based on different retention times, the *m/z* ratios and fragment ions identified by LC-MS/MS. 8-desoxygartanin has the highest intensity (4.0 x 107 cps) among all the compounds identified

followed by γ-mangostin (3.4 x 107 cps) and 1,5-dihydroxy-3-methoxy-2-(3-methyl-2-buten-1-yl)-9H-xanthen-9-one (1.2 x 107 cps) as shown in Table 6. 8-desoxygartanin was first isolated and identified by Govindachari, Kalyanaraman, Muthukumaraswamy, and Pai (1971). 8-desoxygartanin was one of the prenylated xanthones found in mangosteen pericarp extract and being one of the most studied xanthones besides α-, β- and γ-mangostins (Vemu et al., 2019). To date, there were several solvents have been used for the extraction of 8-desoxygartanin such as methanol, acetone, acetonitrile and ethyl acetate (Ji, Avula, & Khan, 2007) but none of the studies found in the isolation of 8-desoxygartanin compound extracted from mangosteen pericarp were using water extraction methods. The evaluation of the biological activity of 8-desoxygartanin were mostly being studied in a group of bioactive compounds instead of single isolated compound, however, there was a study on antileptospiral activity of crude extracts and five purified xanthones (α-mangostin, γ-mangostin, garcinone C, garcinone D and 8-desoxygartanin) from *G. mangostana* (Seesom et al., 2013). The study exhibited the antimicrobial activity of the crude extracts and individual purified xanthones against non-pathogenic and pathogenic leptospira.

Based on the previous research, α- and γ-mangostins are two major bioactive compounds (Rukthong, Sereesongsang, Kulsirirat, Boonnak, & Sathirakul, 2020). More evaluations have been done on α-mangostin if compared to γ-mangostin, however, γ-mangostin was also found to have anti-inflammatory effects (Nakatani, Nakahata, Arakawa, Yasuda, & Ohizumi, 2002; Sukma, Tohda, Suksamran, & Tantisira, 2011), as an amylodogenesis inhibitor (Yokoyama, Ueda, Ando, & Mizuguchi, 2015), anti-proliferative activity on human malignant glioma cells (Chang, Huang, Chen, & Yang, 2010), human colon cancer cells (Chang & Yang, 2012) and breast cancer cells (Yeong et al., 2020), hepatoprotective effect (Husen et al., 2019) and neuroprotective (Do & Cho, 2020). From the results obtained and presented in Table 6, γ-mangostin has a relatively high intensity found in the enzyme-assisted mangosteen pericarp water extract. During the preliminary study revealed in subsection 3.4, high antioxidant activities being reported and it may due to the presence of the xanthones and its derivatives found in the mangosteen pericarp water extract.

α-mangostin is the most abundant xanthone found in *Garcinia mangostana* from most of the previous studies. However, α-mangostin identified under this enzyme-assisted water extraction of mangosteen pericarp was not relatively high (Table 6). This might be due to the poor water solubility of α-mangostin, which was a hydrophobic polyphenol compound in the pericarp of the mangosteen. In a previous study of Ghasemzadeh, Jaafar, Baghdadi, and Tayebi-Meigooni (2018), the results obtained showed that by using water extraction method exhibited a lower concentration of α-mangostin compared to other solvents such as ethanol, ethyl acetate and dichloromethane. Nevertheless, with the assistance of enzymes during water extraction, α-mangostin was able to be extracted from the mangosteen pericarp.

Apigenin-6-C-glucoside showed higher intensity in the mangosteen pericarp water extract compared to α-mangostin based on the results shown in Table 6. Apigenin, also known as isovitexin is a flavonoid isolated from plant materials such as tea (Peng et al., 2021), buckwheat (Huda et al., 2021), fenugreek (Khole et al., 2014) and some edible sprouts and seeds (Kalinová, Vrchotová, & Tříska, 2021), but so far there is no information regarding the extraction of apigenin-6-C-glucoside from mangosteen. Practically, apigenin is insoluble in polar solvents such as water and nonpolar solvents such as silicon fluid and safflower oil, however, the organic solvents like dimethylsulfoxide (DMSO) and Tween 80 were used to increase solubility of apigenin in the aqueous solution (Wang, Firrman, Liu, & Yam, 2019). Therefore, the result shown in this study exhibited the availability of apigenin-6-C-glucoside in enzyme-assisted mangosteen pericarp water extracts might be due to the usage of enzymes in the aqueous solution treated on mangosteen pericarp managed to dissolve apigenin and increase its solubility in water.

4. CONCLUSIONS

Study of using water for the extraction of pericarp of *Garcinia mangostana* L. indicated that only two factors, enzyme concentration and liquid-solid ratio were significantly important for the extraction process. Under optimal

conditions, 1.50% w/w enzyme concentration, 18.75 liquid-solid ratio, reaction temperature of 30°C, pH 3 and 30 min of reaction time, antioxidant capacities indicated by DPPH and ABTS values and total phenolic content obtained were 86.51%, 86.77% and 21.222 mg GAE/g, respectively. Results shown in the response surface plots indicated that enzyme concentration has an optimal condition in correlation with the extraction of phenolic compounds and antioxidants activities of mangosteen pericarp extract. Tentatively, pericarp of *G. mangostana* has been identified to contain smeaxanthone, apigenin-6-C-glucoside, garcimangosone-C, α-mangostin, gartanin, 1,5-Dihydroxy-3-methoxy-2-(3-methyl-2-buten-1-yl)-9H-xanthen-9-one, 8-desoxy- gartanin and γ-mangostin based on different retention times, *m/z* ratios and fragment ions identified by LC-MS/MS. The result showed that enzymatic treatment interacted with the mangosteen pericarp cell walls and enhanced the efficiency of extraction, thus increasing the releasing of secondary metabolites which contributed to antioxidant activities and high content of phenolic compounds. This method could be used for the extraction of natural products in a "greener" method.

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