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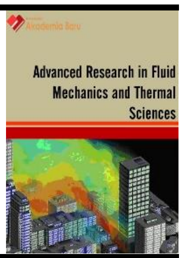

Optimization Studies By Ultrasound Assisted Solvent Extraction And Screening Of Phenolic Compounds In Papaya Seeds

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Hui Min Kee[1], Adilah Anuar[1,*] , Amira Farzana Samat[1] , Noor Hasyierah Mohd Salleh[1] , Alina Rahayu Mohamed[1]

[1] Department of Chemical Engineering Technology, Faculty of Chemical Engineering, Universiti Malaysia Perlis, 02100 Padang Besar, Perlis, Malaysia

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ABSTRACT

Phenolics are widely distributed in papaya which exhibit strong antioxidant properties and have antibacteria effect. This study was conducted to study the effect of parameters condition on yield of the total antioxidant activity (TAA), total phenolic content (TPC) and total flavonoid content (TFC) from papaya seeds by employing ultrasound assisted solvent extraction (UAE) method. Folin-Ciocalteu's method was used to determine TPC, whereas TFC by Aluminium Trichloride (AlCl3) method, while DPPH radical scavenging activity was used to determine the TAA. Solvents screening: Distilled water, Ethanol, Methanol were screened, and distilled water had been found as the best solvent in obtaining highest yield of phenolics (32 %), TAA (62.75 ± 0.0865 %), TPC (195 ± 0.0251 mg GAE/g) and TFC (71.858 ± 0.0562 mg QE/g). Phytochemicals screening: Papaya seeds revealed the presence saponins, terpenoids, flavonoids, glycosides and cardiac glycosides. Optimization study: Parameter screening method was then used to obtain the best range of extraction condition, extraction time (50 - 70min), temperature (40 - 60 °C) and solid-to-liquid ratio (1:40 - 1:60, g/mL). Response Surface Methodology (RSM) was employed to optimise extraction parameters to obtain the highest TPC (106.4 ± 0.0522 mg GAE/g) and TFC (99.831 ± 0.0474 mg QE/g) where obtained at 60 min, 1: 50 solid-to-liquid ratio and temperature 50 °C. Three parameters used in this research have the significant effect on the extraction efficiency TFC ($p<0.05$) while extraction time showed insignificant effect on the extraction of TPC. Extraction temperature was proved to be the most significant effective on the yields of TPC and TFC.

Keywords:
Carica papaya Linn, ultrasound assisted extraction, phenolic compounds

1. Introduction

Carica papaya involved in many food processing industry and consumption of papaya fruits, seeds as agriculture biomass which approximately 22% of fresh papaya weight is produced. This

* *Corresponding author.*
E-mail address: adilahanuar@unimap.edu.my (Adilah Anuar)

usually polluted our habitat and raised some environment issues over the year, could actually be utilized**.** In Chile, papaya widely used in production of different papaya products, such as marmalade, papaya syrup and candied papaya [1]. The production of papaya fruit in Taiwan is about 91.4 kilotons in 2009 [2]. Bioactive compounds extraction in papaya seeds have high potential in the food or pharmaceutical industries. Papaya seed extracts have anthelmintic effect and anti-inflammatory properties against *Staphylococcus aureus, Esherischia coli, Pseudomanas aeruginosa and Shigella flexneri* [3].

Saponin and terpenoid have antitumor activity which avoid the growth of cancer cells prevent the infections of wounds [3]. Flavonoids as anti-oxidants which stimulate the immune system and inhibit platelets aggregation [4]. Papaya seeds contain a broad spectrum of phytochemicals such as phenolic acids and flavonoids [5]. Ultrasound assisted solvent extraction is a type of non-conventional method in which utilises the principle of sound waves, produced by a continuous set of compression and expansion cycles to produce acoustic cavitation [6]. The choice of solvent based on its toxicity and polarity plays major role in extraction process which will affect the quality and yield of particular compounds obtained. Commonly extraction solvents that used included water, ethanol, methanol and acetone. Phenolic compounds in papaya seeds were hydrophilic rather than lipophilic.

2. Methodology

2.1 Ultrasound Assisted Solvent Extraction (UAE)

UAE was carried out by placing 1 g of papaya seeds and 50 ml distilled water into beaker. Then, the mixture was placed onto vortex for 5 min. After that, conical flask was placed into an ultrasonic water bath with ultrasonic power of 120 W and 40 kHz at 60°C for 60 min. Subsequently, filtration of seed extracts and samples residues was re-extract with respective extraction solvent by Ultrasound bath. After that, the filtrate was concentrated by Rotary evaporator at 45°C for 60 min and dried at 45°C in oven for overnight [4]. The yield of final extract was determined by using Equation 1 below:

$$\text{Yield (\%)} = \frac{\text{Mass of extract}}{\text{Mass of sample}} \times 100\% \qquad (1)$$

2.2 Phytochemicals Screening

Alkaline Test for Flavonoids: Two millilitre of sample was added with 5 ml of 10% ammonia solution followed by 2 ml of concentrated H_2SO_4 [2].

Ferric Chloride Test for Tannins: Two millilitre of papaya seed extract was treated by few drops of 1% $FeCl_3$ solution [28].

Salkowski Test for Glycosides: Two millilitre of sample was dissolved in 2 ml of chloroform and added 2 ml of acetic acid. 2 ml of concentrated H_2SO_4 was then added carefully [7].

Chloroform Test for Terpenoids: Two millilitre of sample was dissolved in 2 ml of chloroform followed by 2 ml of concentrated sulphuric acid was then added and heated for about 2 min [7].

Froth Test for Saponins: Two millilitre of papaya seed extract was added with 20 ml of distilled water and swirled thoroughly in flask for 15 min [8].

Keller-Killiani Test for Cardiac Glycosides: Two millilitre of sample extract was mixed with 2 ml of acetic acid and few drops of 1% solution of $FeCl_3$. 2 ml of concentrated H_2SO_4 was added carefully into it [6].

Test for Proteins: Biuret Test was carried out by adding 2 mL of sample with 2 ml Biuret reagent and observed for colour changes. Xanthoproteic test was carried out by adding 2 mL of sample with 2 ml of conc. nitric acid followed by 2 min heating. After cooled with tap water and 1 ml of NaOH [9].

Liebermann's Test for Steroids: Two millilitre of papaya seed extract mixed with 2 ml of chloroform followed by 2 ml of concentrated H_2SO_4 was added.

Fehling's Test for Carbohydrates: Few drops of Fehling reagent was added to 2mL of sample [5].

Acid Test for Phlobtannins: Two millilitre of sample was added with 1% of HCl [5].

2.3 Folin-Ciocalteu Method

TPC of papaya seed was determined by spectrophotometry using Folin-Ciocalteu reagent method as described in [15]. First, 2 mL of diluted papaya seed extract were added to a 2.5 ml of Folin-Ciocalteau reagent. Subsequently, 2.5 ml of 7% sodium carbonate was added in flask. After incubation at dark (30 min), the mixture's absorbance was measured at 765 nm. Distilled water was used as blank. The standard calibration (0.02 – 0.10 mg/ml) curve of Gallic acid in 100% methanol was plotted. Results were expressed as Gallic Acid Equivalent (GAE), in milligrams (mg) per millilitre (mL) of sample extract which was calculated using the Equation 2 below:

$$T = \frac{C \times V \times D}{M} \tag{2}$$

Where T represent total phenolic content (mg GAE/g); C represent the concentration of Gallic (mg/mL); V represent volume extract solution (mL); D represent dilution factor and M represent the weight of papaya seed.

2.4 Aluminium Chloride ($AlCl_3$) Colorimetry Assay

TFC of papaya seeds was evaluated using Aluminium Chloride Colorimetry Assay as describe in [4,16]. First of all, 0.5 mL of papaya seed extract were added with 0.5 mL of distilled water. Next, 0.15 mL of 5% aqueous sodium nitrate solution and 0.15 mL of 10% ($AlCl_3$) solution were added into the flask after 5 min. 2 mL of 1 M sodium hydroxide was added into the mixture after 6 min. The mixture's absorbance was measured at 415 nm with distilled water as blank. The standard calibration curve of Quercetin was plotted.

2.5 (DPPH) 2, 2-diphenyl-2-picrylhydrazyl hydrate assay

TAA of papaya seeds was measured using (DPPH) assay as describe in [9]. 3 ml of the sample extracts were added with 1 ml of 0.3 mM DPPH in methanol. After incubation at dark (30 min), the absorbance was measured at 517 nm with methanol as blank. The DPPH scavenging percentage was calculated according to the Equation 3 as follows:

$$\text{DPPH scavenging percentage \%} = \frac{\text{A control-A sample}}{\text{A control}} \times 100 \tag{3}$$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound.

2.6 Design of Experiment (DOE)

To evaluate the influence of the experimental variables (time, temperature and solid-to-liquid ratio) and determine the optimum levels to maximize the TPC and TFC yield, a Central Composite Design with three levels was applied, including three runs at the central point, generated by Design Expert Ver. 10 statistical package.

The experimental data for the response created by Design Expert (version 10.0.1) and the independent variables were fitted into quadratic model as in the following Equation 4:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

Where Y is the response; X represents parameters and β represents regression coefficients [17].

3. Results

3.1 Solvent Screening

As shown in Table 1, the highest yield of phenolic extract obtained in distilled water extraction was 32% while the lowest yield obtained by methanol extraction was 12%. 100 mL of hexane for extraction *Carica papaya* seed, the yield of total phenolic in papaya seeds contribute 17.8% as compared to papaya peels which only 13.5% [15]. Therefore, papaya seeds which another by-product of fruit were proved that with high antioxidant contents. the TPC was found to be maximum when extracted by distilled water (195 ± 0.0251 mg GAE/g) and minimum in the methanol extraction (106 ± 0.0044 mg GAE/g). The TFC was found to be highest in the papaya seed extract by distilled water (71.858 ± 0.0562 mg QE /g) and lowest in the methanol extraction (54.901 ± 0.0342 mg QE/g). Although methanol had higher polarity on phenolic compounds in precious study contributed different findings, different result from earlier study due to different growing condition and maturity stage of papaya at harvest as well as the extraction process (centrifugation instead of using sonicator).

The highest radical-scavenging of the papaya seeds which extracted by distilled water was (62.75 ± 0.0865%) while the lowest was extracted by methanol (27.23 ± 0.1413%) which similar to result of water extract for *Carica papaya* seed by microwave-assisted extraction [10]. Water which had high number of hydrogen able to donate hydroxyl group with antioxidants to involve in scavenging the radicals. Distilled water extracts of papaya seed show highest TPC. Distilled water was the highest polarity universal solvent. The result of methanol extract had the lowest TPC indicated that phenolic compounds in papaya seed were hydrophilic rather than lipophilic [15].

3.2 Phytochemicals Screening

From Table 1, in ripe papaya seeds extracts showed that the presence of cardiac glycosides and flavonoids whereas absence of tannins indicated the factors that affect the phytochemical screening of constituents in papaya seeds extracts which were the polarity of the solvent used for extraction and the ripeness of papaya [6].

Table 1
Phytochemical screening for *Carica papaya* seed extract

Phytochemicals screening	Test	Carica Papaya Seeds
Saponins	Froth Test	+
Tannins	Ferric Chloride Test	-
Terpenoids	Chloroform Test	+
Flavonoids	Alkaline Test	+
Proteins	Biuret / Xanthoproteic test	-
Carbohydrate	Fehling's test	-
Glycosides	Salkowski test	+
Cardiac glycosides	Keller-Killiani test	+
Steroids	Liebermann's test	-
Phlobatannins	Acid Test	-

Table 2
Yield determination and antioxidant properties in papaya seeds correspond with different type of solvents.

Solvent	Distilled water	Methanol	Ethanol
Yield	32%	12%	13%
Total Phenolic content (TPC)	195 ± 0.0251 mg GAE/g	106 ± 0.0044 mg GAE/g	148 ± 0.0331 mg GAE/g
Total Flavonoid content (TFC)	71.858 ± 0.0562 mg QE/g	54.901 ± 0.0342 mg QE/g	63.802 ± 0.0500 mg QE/g
Total Antioxidant Activity (TAA)	62.75 ± 0.0865%	27.23 ± 0.1413 %	52.85 ± 0.0604%

3.3 Parameter Screening

The optimized range of extraction parameter were: extraction time (50 min – 70 min), extraction temperature (40°C – 60°C) and solid-to-liquid ratio (1:40 – 1:60).

3.4 Optimization Study

In this study, the optimized extraction condition was designed by applying Response Surface Methodology (RSM). The TPC ranged from 87.2 to 106.4 mg GAE/g, and the ratio of maximum to minimum was 1.22018 which observed while the TFC ranged from 77.6338 to 99.831 mg QE/g, and the ratio of maximum to minimum was 1.28592 which observed in run No.11 under experimental parameters (60 min, 1:50 g/mL, and 60°C).

3.5 ANOVA Analysis

The quadratic interaction model of three parameters for TPC was evaluated. The quadratic parameters X_2, X_3, X_1X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant since $p < 0.05$ while the parameters X_1 and X_1X_2 were not significant since $p > 0.10$. As seen in Table 6, extraction temperature (X_3) is the most significant variable on the response. Higher Fisher F-value of 65.91 and the 'F-value' of 'lack of fit' shows that there was no significance which was 0.3299 ($p > 0.05$) [7]. There was only a 0.01% chance that an F-value this large could occur due to noise.

The effect of extraction time on yield of TPC was similar to previous studies for pomegranate seed oil with the total phenolic in papaya seed [15]. Longer extraction time increased the extraction yield because cell walls of papaya seed were cracked completely at first 60 min by cavitation which enhance the penetration rate of solvent into solute matrix to facilitate the diffusion of TPC out of cell walls [19]. However, longer extraction was unnecessary once the maximum yield of TPC was obtained due to possible undesirable changes occur in phenolic compound [20]. Each phenolic compound had a different solubility rate to dissolve into solvent and hence resulted in different of extraction time [15].

The observation was similar to principle of mass transfer coefficient in studies of polyphenol from papaya leaf [24].When higher solid-to-liquid ratio was used, the viscosity and density of solvent increased and reduced occurrence of cavitation phenomenon, hence, result in minimum extraction yield of TPC with highly soluble in distilled water. Further decreased of solid-to-liquid ratio caused an increased in yield due to an increasing of solubility rate and penetration rate between constituents and extraction solvent [15].

As similar to present study of TPC, yield of TFC was increased as high temperature increased the mass transfer between solvent and phenolics as well as enhanced solvent penetration through cell wall by disrupting the hydrophobic bond in cell membrane. Therefore, extraction time and temperature must be optimized to avoid degradation due to oxidative pyrolysis caused by hydroxyl (OH-) radicals during ascoutic cavitation [3].

Table 3
Analysis of variance (ANOVA) table for TPC

Source	Sum of squares	Mean Square	F-value	p-value	
Model	584.74	64.97	65.91	< 0.0001	significant
X_1-Time	0.46	0.46	0.47	0.5149	
X_2-Solid-to-liquid ratio	32.21	32.21	32.68	0.0007	
X_3-Temperature	48.08	48.08	48.77	0.0002	
X_1X_2	4.47	4.47	4.53	0.0708	
X_1X_3	121.84	121.84	123.59	< 0.0001	
X_2X_3	41.71	41.71	42.31	0.0003	
X_1^2	9.02	9.02	9.15	0.0193	
X_2^2	62.98	62.98	63.88	< 0.0001	
X_3^2	45.88	45.88	46.54	0.0002	
Residual	6.90	0.99			
Lack of Fit	5.88	1.18	2.30	0.3299	not significant
Pure Error	1.02	0.51			
Cor Total	591.64				

Table 4
Analysis of variance (ANOVA) table for TFC

Source	Sum of Squares	Mean Square	F- value	p-value	
Model	758.76	84.31	24.24	0.0002	significant
X_1-Time	25.04	25.04	7.20	0.0314	
X_2-Solid-to-liquid ratio	24.99	24.99	7.19	0.0315	
X_3-Temperature	55.62	55.62	15.99	0.0052	
X_1X_2	2.60	2.60	0.75	0.4161	
X_1X_3	163.47	163.47	47.01	0.0002	
X_2X_3	96.98	96.98	27.89	0.0011	
X_1^2	32.09	32.09	9.23	0.0189	
X_2^2	19.74	19.74	5.68	0.0487	
X_3^2	81.97	81.97	23.57	0.0018	
Residual	24.34	3.48			
Pure Error	0.29	0.15			
Cor Total	783.10				

3.6 Model Graphs

2D contour plot predicted that TPC (104.897 mg GAE/g) and TFC (99.8898 mg QE/g) at different level of parameters. 3D surface plot showed that the optimum condition in order to obtained highest yields of phenolic compounds was 60 min, 50°C and 1:50 solid-to-liquid ratio.

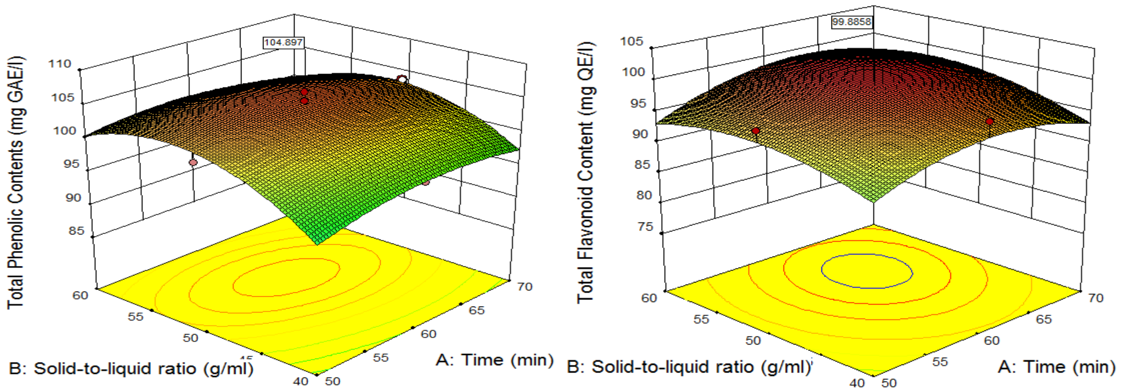

Fig. 1. Interaction of solid-to-liquid ratio and extraction time on TPC and TFC yields.

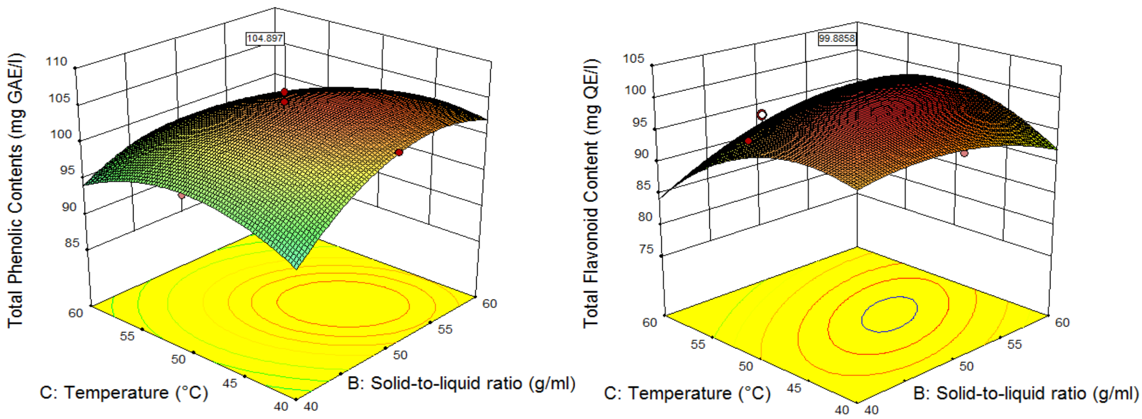

Fig. 2. Interaction of temperature and solid-to-liquid ratio on TPC and TFC yields.

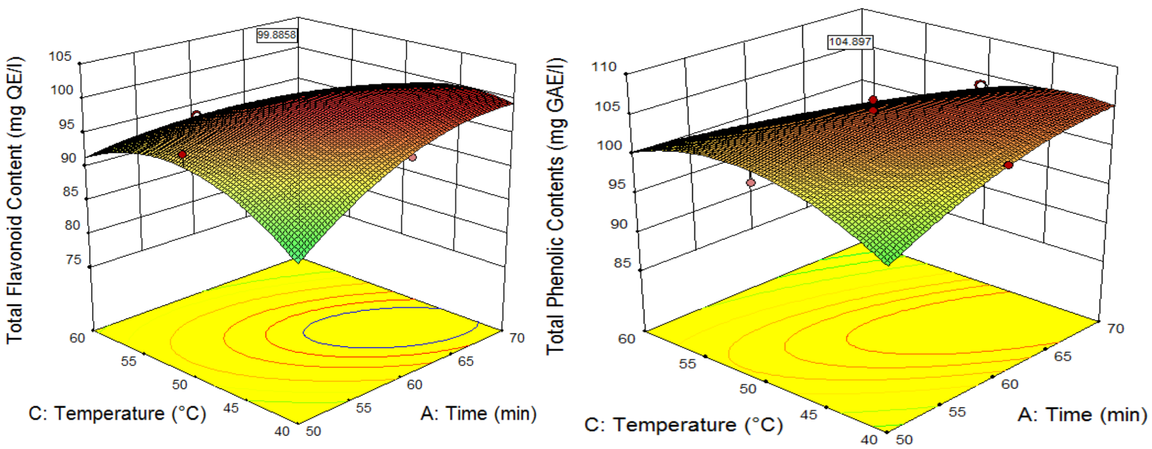

Fig. 3. Interaction of extraction temperature and extraction time on TPC and TFC yield

4. Conclusions

In conclusion, the phenolic compounds were extracted by ultrasound assisted solvent extraction (UAE) which obtained the highest TPC (195 ± 0.0251 mg GAE/g), TFC (71.858 ± 0.0562 mg QE/g) and TAA (62.75 ± 0.0865%) with distilled water extraction. Furthermore, papaya seeds extract were determined that contained saponin, terpenoids, flavonoids, glycosides and cardiac glycosides through phytochemicals screening. In addition, the maximum TPC and TFC were obtained in the optimal condition of extraction time 60 min, 1:50 solid-to-liquid ratio and extraction temperature 50 °C which were determined through parameters screening method. The maximum yield of TPC obtained at Run 16 was 106.4 ± 0.0822 mg GAE/g while TFC obtained at Run 11 was 99.83 ±0.0474 mg QE/g at optimum condition. As compared to optimum value suggested by DOE software were 67.345 min, 1:52.919 g/mL and 43.081 °C with the corresponding yield 105.997 mg GAE/g of TPC and 100.11 mg QE/g of TFC. These results showed that three parameters had a significant effect on antioxidant properties (TPC and TFC) with p-value less than 0.05 indicated that exist of significant model. Validation of this study was determined and showed the repeatability of data which the percentage error were varied from 1.44% to 3.59% for TPC and from 1.12% to 3.01% for TFC. These results indicated that the Central Composite Design (CCD) of Response Surface Methodology (RSM) was efficient and suitable software for the optimization of the extraction of phenolic compounds in papaya seeds.

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