

# Optimization of ultrasound-assisted extraction of amla (*Emblica officinalis*) fruit using response surface methodology and identification of phenolic compounds

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## Abstract

Amla (*Emblica officinalis*) fruit is rich in phenolic compounds and has antioxidant activity which is beneficial to health. The phenolic compounds of amla fruit were extracted using ultrasound-assisted extraction (UAE) method. This study aimed to optimize the extraction conditions (ethanol concentration: 48.8-91.2% and extraction time: 2.9-17.1 min) using response surface methodology to maximize the total phenolic content and antioxidant activity of amla fruit extract. Extractions at optimum conditions were compared with conventional extractions (maceration and reflux). In addition, this study also aimed to identify the phenolic compound composition of amla fruit extract using UHPLC-ESI-MS/MS. The optimum extraction conditions were obtained at an ethanol concentration of 71.00% and an extraction time of 10.39 mins. Under optimum conditions, the actual response values of total phenolic content and antioxidant activity were close to the predicted values. The predominant phenolic compounds in the amla fruit extracts were methyl gallate, mucic acid methyl ester gallate, mucic acid dimethyl ester gallate, mucic acid gallate, mucic acid 1,4-lactone-3-O-gallate, emblicanin B, gallic acid, mucic acid lactone methyl ester gallate, digalloyl glucose, and galloyl glucose. The UAE method was more efficient for extracting phenolic compounds from amla fruit with high antioxidant activity in a shorter time than conventional methods (maceration and reflux).

## 1. Introduction

Amla (*Emblica officinalis* Geartn. syn. *Phyllanthus emblica*), which is also known as malacca, is found in tropical and subtropical regions such as India, Pakistan, Bangladesh, Sri Lanka, China, and Southeast Asia (Baliga and Dsouza, 2011; Pareek and Pratap, 2011). Amla fruit has long been used in herbal medicine systems because it contains several bioactive components which is beneficial to health. The main bioactive components of amla fruit are phenolic compounds (tannins, phenolic acids and flavonoids), including ellagic acid, gallic acid, quercetin (Luo *et al.*, 2009), and tannins (emblicanin A, emblicanin B, geranin) (Yang and Liu, 2014). The phenolic content of amla fruit has antioxidant activity as free radical scavenging activity (Liu *et al.*, 2008; Luo *et al.*, 2009; Charoenteeraboon *et al.*, 2010), that have various physiological roles in the body to overcome many diseases (Yadav *et al.*, 2016). Phenolic compounds from amla fruit are reported to have a number of

pharmacological effects such as antidiabetic, antihyperlipidemic, cardioprotective, anticancer, hepatoprotective, anti-inflammatory, and antimicrobial (Variya *et al.*, 2016).

Extraction is a principal step to maximize the recovery and maintain the quality of bioactive components from natural sources. A method that has been widely used to extract bioactive components, including phenolic compounds from amla fruit, is a conventional technique for a specific time range from a few hrs to 7 days (Liu *et al.*, 2009; Luo *et al.*, 2009; Majeed *et al.*, 2009). However, conventional extraction techniques generally have many disadvantages, especially the relatively long extraction time and low efficiency. Non-conventional techniques, specifically ultrasound-assisted extraction (UAE), can increase mass transfer rates, thereby increasing yield and extract quality (antioxidant activity) with a shorter extraction time than conventional techniques (Vilkhu *et al.*, 2008; Rastogi,

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2011; Ameer et al., 2017; Soquetta et al., 2018). The advantages of the UAE method are due to the mechanical effect resulting from the acoustic cavitation of ultrasonic waves, which causes damage to the material cells, thereby increasing the solvent penetration ability (Chemat et al., 2017). Another advantage of the UAE method is that it has a relatively simple procedure and cheaper technology, compared to other non-conventional techniques (Azwanida, 2015).

The extraction equipment in the UAE method can use an ultrasonic bath or an ultrasonic probe. Several studies have used the UAE method for the extraction of bioactive components from amla fruit using an ultrasonic bath (Tsai et al., 2014; Al-Samman et al., 2019). The ultrasonic probe will provide higher energy intensity than the ultrasonic bath (Santos et al., 2009), thereby increasing the extraction efficiency of the target components (Wen et al., 2018). Therefore, the extraction is expected to take place in a shorter time while optimizing the recovery of the phenolic compound and antioxidant activity of the extract. In this study, UAE was carried out using an ultrasonic probe accompanied by optimization of process conditions and identification of phenolic compounds from Indonesia amla fruit extract which had not been carried out by previous studies. This study examined the effect of solvent concentration and extraction time on the UAE process.

Optimization of the condition of the extraction process was carried out using response surface methodology (RSM). RSM has been widely applied to optimize extraction conditions such as solvent concentration and exposure time to extract phenolic compounds from other sources in several studies (Chemat et al., 2011; Altemimi et al., 2015; Tomšik et al., 2016; Rocha et al., 2018). RSM shows the relationship between the response and some independent variables through a model (Bezerra et al., 2008). Experimental design such as the central composite design (CCD) was used to obtain complete information about the process with a minimum number of experiments (Box and Wilson, 1992). Therefore, this study aimed to optimize the extraction conditions (ethanol concentration and extraction time) using RSM to achieve maximum recovery of phenolic compounds with high antioxidant activity of amla fruit. The efficiency of the UAE method was compared with conventional extraction methods (maceration and reflux) at optimum conditions. In addition, this study also aimed to identify the phenolic compound composition of amla fruit extract.

## 2. Materials and methods

### 2.1 Materials

Amla fruit was obtained from Aceh Province, Indonesia. Amla fruit was harvested at the level of physiological maturity. The amla fruit was sorted, washed, seeded, sliced, and dried in a cabinet dryer at 40°C. The dried slices of amla were ground and sieved through a 60-mesh sieve. The dried amla fruit powder was placed in dark packaging and stored in cool and dry conditions to prevent oxidation. Folin–Ciocalteu reagent, gallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemical Co. The analytical grade was used for ethanol and other chemicals.

### 2.2 Ultrasound-assisted extraction

The UAE was carried out using an ultrasonic probe device (VCX750 Sonic and Materials Inc., USA) which operates at a constant frequency of 20 kHz, power of 750 Watts and temperature of 30°C. The extraction conditions of the UAE process were considered based on the results of preliminary experiments and earlier studies (Tsai et al., 2014). In this study, amla fruit powder (2.5 g) was placed in a 100-mL flask and mixed with 50 ml of solvent. The mixture was placed on an ultrasonic probe and the extraction was carried out at various concentrations of ethanol (48.8-91.2%) and extraction time (2.9-17.1 mins). The mixture during extraction was maintained at 30°C using a water bath with ice. The mixture was filtered using filter paper (Whatman No. 1) and the filtrate was concentrated. Then, the concentrated extract was placed in the refrigerator for further analysis.

### 2.3 Conventional extraction

#### 2.3.1 Maceration

Amla fruit powder (2.5 g) was extracted with 50 mL of ethanol solvent (using the optimum concentration of UAE) at room temperature for 24 hrs. The mixture was filtered, and the filtrate was concentrated. The concentrated extract was placed in the refrigerator.

#### 2.3.2 Reflux

Amla fruit powder (2.5 g) was extracted with 50 mL of ethanol (using the optimum concentration of UAE) in a reflux apparatus at 70°C for 3 hrs. Then, the mixture was filtered, and the filtrate was concentrated. The concentrated extract was placed in the refrigerator.

### 2.4 Experimental design

The UAE process optimization using the response surface methodology (RSM) was designed using the central composite design (CCD) (Bezerra et al., 2008). The design consisted of two independent variables (X):

ethanol concentration ( $X_1$ ) and extraction time ( $X_2$ ). The independent variables were evaluated in the following ranges: ethanol concentration 48.8 - 91.2% (w/v) and extraction time 2.9-17.1 mins. These ranges were considered based on previous experiments. The dependent variable or experimental response ( $Y$ ) was the total phenolic content, TPC ( $Y_1$ ) and antioxidant activity, DPPH ( $Y_2$ ). The unit matrix for the extraction experiment design using CCD consisted of 12 experimental runs with four replications at the centre point (Table 1). The second-order polynomial model with a single expected response ( $Y$ ) is expressed in Equation 1.

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where  $Y_i$  is the predicted response,  $X_1$ ,  $X_2$  are variable independent,  $\beta_0$  is a constant,  $\beta_1$ ,  $\beta_2$  are the linear regression coefficients;  $\beta_{11}$ ,  $\beta_{22}$  are quadratic coefficients, and  $\beta_{12}$  is interaction coefficient. The suitability of the polynomial model was assessed based on the coefficient of determination ( $R^2$ ), the p-value model, and the p-value of lack of fit.

### 2.5 Model validity test

The optimum conditions for combining extraction variables (independent variables) were calculated using a quadratic polynomial model that yielded maximum values for both response parameters (TPC and antioxidant activity). The reliability and validity of the developed model were tested by comparing the average value of the experiment with three replications under optimum conditions with the predicted value of the model.

### 2.6 Total phenolic content determination

TPC was quantified by the Folin-Ciocalteu method as described by Liu *et al.* (2008) with slight modifications. The extract (1 mL) was mixed with Folin Ciocalteu reagent (5 mL; 10% v/v). After incubation for

3 mins, the mixture was mixed with  $\text{Na}_2\text{CO}_3$  solution (4 mL; 7.5% w/v). The mixture was then incubated at room temperature and dark for 30 mins, and the absorbance was measured at 765 nm. The TPC was expressed as mg gallic acid equivalent per g amla fruit powder (mg GAE/g).

### 2.7 Antioxidant activity by DPPH assay

Antioxidant activity was calculated as a free radical scavenging activity of DPPH determined using the method described by Luo *et al.* (2009) with slight modifications. Extract (1 mL) was mixed with DPPH solution in ethanol (2 mL; 0.1 mM). The mixture was shaken strongly and incubated in the dark (30 mins). The control solution was obtained by mixing ethanol and DPPH. The absorbance was calculated at 517 nm. Antioxidant activity is calculated using equation 2.

$$\text{DPPH antioxidant activity (\%)} = \left( \frac{A_{517 \text{ control}} - A_{517 \text{ sample}}}{A_{517 \text{ control}}} \right) \times 100 \quad (2)$$

### 2.8 Liquid chromatography–mass spectrometry analysis of phenolic compound

Analysis phenolic compounds in amla fruit extract at optimum conditions were detected using LCMS type UHPLC-ESI-MS/MS with Accucore C18 column,  $100 \times 2.1$  mm, 1.5  $\mu\text{m}$  (ThermoScientific). Sample preparation was carried out by filtering the sample with a  $0.2 \mu\text{m}$  PTFE membrane. The flow rate was constant at 0.2 mL/min and the mobile phase (eluent) were  $\text{H}_2\text{O} + 0.1\%$  formic acid (A) and acetonitrile + 0.1% formic acid (B). The solvent gradient changes according to the following conditions: 0-1 min (5% B), 1-25 mins (5-95% B), 25-28 mins (95%B), 28-30 mins (5%B). The column temperature was kept constant at  $30^\circ\text{C}$  with an injection volume of  $2 \mu\text{L}$ . MS was operated in negative ion mode with a mass range of 100-1500 m/z. The optimum ESI-MS parameters were obtained at a capillary voltage of +3.8 kV and a drying gas temperature of  $320^\circ\text{C}$ .

Table 1. Experimental design for the optimization of TPC and DPPH of amla fruit extract.

Run	Independent variable		Response	
	$X_1$	$X_2$	$Y_1$	$Y_2$
	Ethanol concentration (%)	Extraction time (min)	TPC (mg GAE/g)	DPPH (%)
1	-1 (55)	-1 (5)	132.92	82.61
2	1 (85)	-1 (5)	132.31	83.62
3	-1 (55)	1 (15)	121.54	83.76
4	1 (85)	1 (15)	133.23	81.78
5	-1.41 (48.8)	0 (10)	105.54	80.18
6	1.41 (91.2)	0 (10)	127.69	81.10
7	0 (70)	-1.41 (2.9)	140.00	84.14
8	0 (70)	1.41 (17.1)	145.54	85.95
9	0 (70)	0 (10)	153.23	85.05
10	0 (70)	0 (10)	156.00	86.33
11	0 (70)	0 (10)	150.15	85.32
12	0 (70)	0 (10)	155.38	87.25

## 2.9 Statistical analysis

Design Expert-12 software was used to provide experimental design, data analysis, regression coefficients, the 3D graph and optimization on the response surface design. Paired t-test was used to test the difference between the experimental and predicted values under optimum conditions. The results of the analysis were considered statistically significant if the p-value was less than 0.05.

## 3. Results and discussion

### 3.1 Model adequacy

This experiment was designed to study the effect of the independent variables (ethanol concentration and extraction time) on the TPC and DPPH antioxidant activity. The mathematical model equation to predict the response, TPC and DPPH antioxidant activity was obtained from experimental data (Table 1) and analyzed using multiple regression. The results of the analysis of variance and the evaluation of the suitability of the second-order polynomial model are presented in Table 2.

The evaluation of the model adequacy in this study was based on some parameters, including the coefficient of determination ( $R^2$ ), p-value of the model, and p-value of lack of fit (Bezerra *et al.*, 2008). The  $R^2$  values for the TPC and DPPH models were 0.9557 and 0.9047, respectively, indicating a perfect fit. The p-value for both regression equation models was significant (p-value < 0.01), indicating the regression equation model is suitable to describe the UAE process of extracting phenolic compounds from amla fruit. Additionally, the lack of fit value of the polynomial response model in this study was not significant (p>0.05) for both responses, TPC (0.1257) and DPPH (0.6323) (Table 2). The lack of fit test showed that the mathematical model is adequate to describe the relationship between the response value

and the independent variable in extracting phenolic compounds from amla fruit.

### 3.2 Effect of extraction variable on total phenolic content

The TPC results of amla fruit extract ranged from 105.54-156.00 mg GAE/g. The highest TPC value was obtained at run 10 with 70% ethanol concentration and extraction time of 10 mins, while the lowest TPC was observed at run 5 with 48% ethanol concentration and 10 mins extraction time. Model coefficients with significant values (p<0.05) were considered to evaluate the results of multiple regression analysis. Ethanol concentration (linear and quadratic) had a significant effect (p<0.01) on TPC, and quadratic extraction time had a significant effect (p<0.05) on TPC. In contrast, linear extraction time and interaction of independent variables had no significant effect (p>0.05). The quadratic polynomial equation for the TPC of amla fruit extract, including only the significant terms, is formulated as:

Based on equation 3, the effect of the extraction variable on TPC was represented on a 3D surface plot (Figure 1a). When the extraction time was constant, the TPC of the extract increased gradually as the ethanol concentration increased from 48.8% to 70%, but the TPC decreased when the solvent concentration was increased by more than 70%. The difference in solvent concentration causes differences in the polarity of the solvent. Based on the law of similarity and miscibility, the ability of bioactive components to dissolve in a solvent is strongly influenced by the similarity (close to) of the polarity between the bioactive component and the solvent (Belwal *et al.*, 2018; Zhang *et al.*, 2018). The main bioactive components in amla are phenolic compounds such as phenolic acids, tannins, and flavonoids that belong to the hydrophilic group, but with varying degrees of polarity, which are strongly influenced by the ester, ether, or glycosylation bond.

Table 2. Regression coefficients and parameters of the suitability of the predicted second-order polynomial models for the TPC and DPPH of amla fruit extracts.

Source	TPC		DPPH	
	Coefficient	p-value	Coefficient	p-value
Intercept	-262.25	0.0005 **	18.88	0.0051 **
X <sub>1</sub>	11.43	< 0.0001 **	1.75	0.0003 **
X <sub>2</sub>	1.37	0.6002	1.10	0.0784
X <sub>1</sub> X <sub>2</sub>	0.0410	0.2083	-0.01	0.1533
X <sub>1</sub> <sup>2</sup>	-0.0820	< 0.0001 **	-0.0118	0.0003 **
X <sub>2</sub> <sup>2</sup>	-0.2153	0.0205 *	-0.0178	0.2633
R <sup>2</sup>	0.9557		0.9047	
Adjusted R <sup>2</sup>	0.9188		0.8252	
p-value model	0.0005		0.0051	
p-value lack of fit	0.1257 <sup>ns</sup>		0.6323 <sup>ns</sup>	

\* significant, \*\* very significant, <sup>ns</sup> not significant

Generally, aglycones of phenolic compounds are water-soluble glycosides, but different types of aglycones cause differences in solubility. Solubility in water increases with the increasing number of hydroxyl groups. Flavonoid compounds are generally semipolar (Belščak-Cvitanović *et al.*, 2018). Pure ethanol are suitable solvent for extracting phenolic compounds, such as flavonoids and phenolic acids, but is not suitable for dissolving tannins (Sasidharan *et al.*, 2011). Therefore, the use of a mixture of solvents (hydroalcoholic) will regulate the selectivity of the extraction because it can change the dielectric constant of the solvent. Many other studies have shown that the solvent commonly used to extract phenolic compounds from plant materials is a mixture of solvents (ethanol and water).

$$Y_{\text{TPC}} = -265.25 + 11.43 X_1 - 0.082 X_1^2 - 0.215 X_2^2 \quad (3)$$

Extraction using hydroalcoholic solvents (ethanol and water) from this study also proved a synergistic effect between ethanol and water solvents. This effect was caused by water functioning as a swelling agent for the cell-matrix material, while ethanol can cause a break in the bond between the bioactive components and the matrix. In addition, ethanol penetrates membranes more easily because it can degrade cell walls with non-polar properties and cause phenolic compounds to be extracted from plant material cells (Tiwari *et al.*, 2011).

Based on other studies, the yield of the phenolic component extract was also higher in hydroalcoholic solvents with 70% ethanol concentration compared to pure ethanol due to an increase in the polarity of the solvent due to a mixture of water into the ethanol solvent (Bimakr *et al.*, 2010; Muñiz-márquez *et al.*, 2013; Tomšik *et al.*, 2016). In addition, other studies have shown that the best phenolic compound extract results can also be achieved by using different compositions of ethanol-water solvents depending on the composition of the bioactive components of each ingredient, including using 50% ethanol concentration for extraction of olive leaves, red grape pomace fruit, and *Zizyphus lotus* (Sahin and Samli, 2013; Drosou *et al.*, 2015; Hammi *et al.*, 2015), 57-63% ethanol concentration for molasses extraction (Chen *et al.*, 2015), 60% ethanol concentration for *Cassia auriculata* leaf extraction (Sharmila *et al.*, 2016), 75% ethanol concentration for *Sparganii rhizoma* extraction (Wang *et al.*, 2013), 75-80% ethanol concentration for neem fruit extraction (Paula *et al.*, 2016), and 80% ethanol concentration for the extraction of haskap berries (Celli *et al.*, 2015).

Based on the extraction time variable, increasing the sonication time from 2.95 mins to 10 mins, the TPC value of amla fruit extract increased but showed a decreasing trend when sonication was continued (Figure

1a). At the beginning of the extraction process, sonication provides a reasonably practical effect to remove phenolic compounds contained in the cells. Ultrasonic waves can accelerate heat and mass transfer by damaging or breaking plant cell walls, making it easier to remove the desired components from the material (Roselló-soto *et al.*, 2015). However, the addition of extraction time with longer sonication is thought to cause the degradation of phenolic compounds (Tiwari, 2015). This degradation was due to the increasing number of cavitation bubbles produced by ultrasonication with increasing extraction time. Asymmetric cracking of cavitation bubbles can also damage the compounds present in the solution (Vilkhu *et al.*, 2008). Previously, Rabelo *et al.* (2016) clearly demonstrated that a sonication duration of 10 min was adequate to extract the phenolic compounds from the residual artichoke material. In addition, another study also showed that some phenolic components were extracted in the first 11 mins with UAE using direct sonication (ultrasonic probe). The short extraction time is because the energy from the sonication device with the probe system directly hits the material without an intermediary, such as in the ultrasonic bath so the cavitation effect becomes larger (Luque-García and Castro, 2003; Herceg *et al.*, 2010; Bosiljkov *et al.*, 2011).

The weakness of the ultrasonic probe was that the level of degradation of the bioactive components was higher than that of the ultrasonic bath, so it was necessary to determine the optimum time used to extract the material. Previous research to extract amla fruit using an ultrasonic bath took the best time of 15 mins (Tsai *et al.*, 2014). The use of an ultrasonic bath requires a longer optimum time to extract the phenolic components: 20 mins for the extraction of soy beverages (Rostagno *et al.*, 2007; Maran *et al.*, 2017), 30 mins for the extraction of sage (Dent *et al.*, 2015), and 60 mins for olive leaf extraction (Sahin and Samli, 2013).

### 3.3 Effect of extraction variable on antioxidant activity

The antioxidant activity value of amla fruit extract, as measured as the percentage of inhibition of DPPH, ranged from 80.18 to 87.25%. The highest DPPH value was obtained at run 12 with a combination of 70% ethanol concentration and 10 mins extraction time, while the lowest DPPH value was obtained at run 5 with 48% ethanol concentration and 10 mins extraction time. Based on multiple regression analysis (Table 2), ethanol concentration both linear and quadratic had a significant effect on antioxidant activity ( $P < 0.01$ ), whereas extraction time and interactions between process variables had no effect on the antioxidant activity

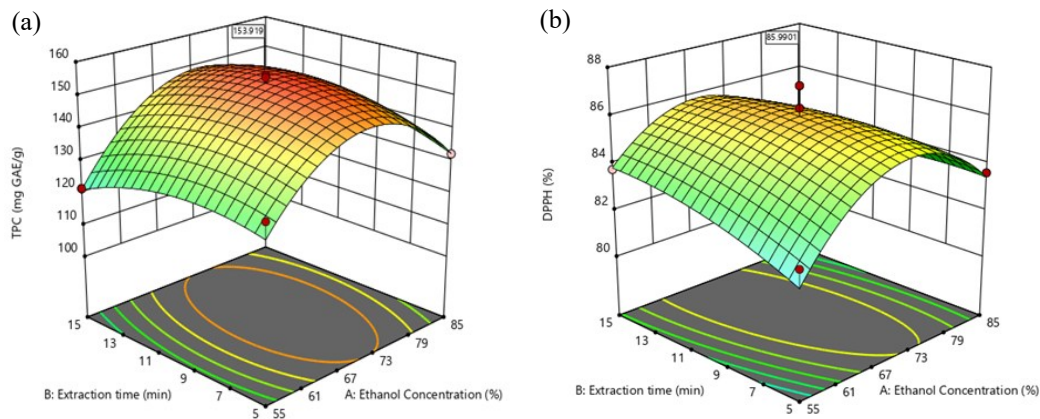


Figure 1. Response surface graphs for the effect of the extraction variable on (a) TPC (b) DPPH.

( $P > 0.05$ ). Based on the results of the analysis, the most influential factor on antioxidant activity is the concentration of ethanol. The quadratic polynomial equation for the antioxidant activity of amla fruit extract, excluding nonsignificant terms, was formulated as:

Figure 1b was a 3D surface plot that was constructed based on equation 4, showing the effect of extraction process variables on the DPPH antioxidant activity of amla fruit extract. Antioxidant activity was obtained at the highest level with 70% ethanol concentration and showed a decreasing trend when the ethanol concentration was lower or higher than 70%. This result was consistent with the effect of ethanol concentration on the TPC (Figure 1a), the difference in ethanol concentration causes differences in solvent polarity so that it affects the ability of the solvent to extract the bioactive components of the ingredients (Belwal *et al.*, 2018). The antioxidant activity was contributed by the presence of bioactive components, mainly phenolic compounds in the extract, the antioxidant activity was also influenced by the ability of the solvent to extract the phenolic compounds.

$$Y_{\text{DPPH}} = 18,88 + 1.75 X_1 - 0.0118 X_1^2 \quad (4)$$

#### 3.4 Correlation between total phenolic content and antioxidant activity

The relationship between TPC and antioxidant activity of amla fruit extract can be analyzed using Pearson correlation which was calculated based on experimental data. From the analysis results, the correlation value between the TPC and DPPH antioxidant activity of the extract was significantly positively correlated ( $R = 0.877$ ;  $p\text{-value} < 0.001$ ). This correlation value means the increase of the TPC caused the increase in the antioxidant activity of the extract because, in amla fruit extract, phenolic compounds are the main components. Phenolic compounds are one type of bioactive component that plays an antioxidant role. The phenolic structure has many hydroxyl groups,

releasing hydrogen and forming a strong coordination oxygen ion complex. Based on the literature, the phenolic compounds found in amla include hydrolyzed tannins, phenolic acids, and flavonoids. However, amla fruit also contains other types of bioactive components in small amounts, such as alkaloids, phytosterols, terpenoids, citric acid, amino acids, and ascorbic acid (Hasan *et al.*, 2016). Under previous studies, the phenolic content of amla fruit extracted using ethanol as a solvent has antioxidant activity and radical inhibitory activity (Mayachiew and Devahastin, 2008; Luo *et al.*, 2009). Another study on sage phenolic extracts also showed that there was a positive linear correlation between the total phenol and flavonoid content of the extract and its antioxidant activity, which means that the TPC of the ingredients directly affects the antioxidant capacity of the sage extract (Dent *et al.*, 2015).

#### 3.5 Optimization and validation

The optimum conditions for UAE of amla fruit aim to maximize the TPC while also maximizing the antioxidant activity of the extract. The approach to solving the optimization problem of multiple responses is the use of a multi-criteria methodology (Bezerra *et al.*, 2008). The desirability function is the most widely used multi-criteria methodology for optimizing analytical processes (Murphy *et al.*, 2005). The best compromise between two or more responses was analyzed using Design Expert statistical software with an individual desirability function scale ranging from  $d = 0$  (unwanted response) and  $d = 1$  (completely desired response). In this study, optimization calculations were carried out within the experimental area with upper and lower limits for each independent variable (ethanol concentration and extraction time) and response (TPC and DPPH) based on Table 1. The predicted optimum conditions from the model equation were ethanol concentration of 71.00% and extraction time of 10.38 mins which resulted in a TPC of 153.92 mg GAE/g and DPPH of 85.99% (Table 3). This optimal condition is obtained with a reasonably high desirability value (Bezerra *et al.*, 2008; Manohar *et*

al., 2013) approaching a value of 1 ( $d = 0.888$ ). The results of the desirability value showed that the optimum conditions obtained from the model deserve to be considered as the optimal conditions for the extraction process.

Verifying the accuracy of the empirical model equation was carried out by comparing the predicted response values with experimental results under optimal conditions. Experiments at optimum conditions were carried out in three replications with the average values shown in Table 3. Based on the t-test, there was no significant difference between the experimental and the predicted values of the TPC (t-value 0.70; p-value 0.555) and DPPH antioxidant activity (t-value -1.33; p-value 0.316). These results confirmed that the UAE process optimization of phenolic compounds from amla fruit can use a polynomial model with two process variables studied.

Previously, the best conditions for extracting amla using an ultrasonic bath were reported at 70% ethanol concentration, 15 mins extraction time, and 56 kHz frequency (Tsai *et al.*, 2014) with a TPC of 55.34 mg GAE/g. The difference in extraction time to obtain the maximum TPC from amla fruit extraction was caused by the use of different types of ultrasonic equipment. Ultrasonic probes can extract with a greater mass transfer rate because the intensity of the ultrasonic waves received by the sample becomes wider than using an ultrasonic bath. More cavitation numbers can be generated because the sample is immersed directly with the ultrasonic probe in the container (Santos *et al.*, 2009). In a sonication bath (indirect sonication), ultrasonic waves must be transferred through water and the walls of the sample container before reaching the solvent to extract the material (Kek *et al.*, 2013), thus causing a lower cavitation effect than direct ultrasonic (probe). The difference in TPC in this study with other studies can also be caused by differences in cultivars and plant growth locations. From various literature, the TPC of amla fruit extract using ethanol solvent with a different technique, extraction time, and location of plant origin ranged from 81.5-120.9 mg GAE/g (Liu *et al.*, 2008), 188.8-237.0 mg GAE/g (Poltanov *et al.*, 2009), and

104.1-304 mg GAE/g (Kumari and Khatkar, 2016).

### 3.6 Comparison with conventional extraction

To evaluate the extraction efficiency of the UAE process on the phenolic content of amla fruit, a comparison of the extraction results (TPC and DPPH antioxidant activity) using two conventional extraction methods (maceration and reflux) at optimal ethanol concentrations was carried out, but using the optimum time for each extraction method (24 hrs for the maceration and 3 hrs for the reflux). The results indicate that the UAE method can produce extracts with relatively high TPC and DPPH in 10 mins extraction time (TPC = 154.36 mg GAE/g; DPPH = 85.41%), compared to the reflux method in extraction time for 3 hrs (TPC = 120.62 mg GAE/g; DPPH = 80.90%), and the maceration method takes up to 24 hrs (TPC = 126.77 mgGAE/g; DPPH = 82.47%) (Table 4). The UAE method is very efficient for extracting phenolic compounds in a short time, with a TPC value of 21.76% a DPPH value of 3.56% higher than maceration, and a TPC value of 27.97% and a DPPH value of 5.57% higher than reflux extraction.

The recovery of phenolic components in a reasonably short time compared to conventional techniques was caused by damage to the cell wall of the material due to the assistance of ultrasonic waves during the extraction process, followed by an increase in solvent penetration into the material so that the desired component is more easily removed from the material (Medina-Torres *et al.*, 2017). However, based on the results of antioxidant activity, the use of ultrasonic waves in the amla fruit extraction process did not reduce the ability of the phenolic compounds in the extract as antioxidants, presumably because the process was carried out at a controlled temperature and short extraction time. So, it can be stated that UAE was a more appealing extraction technique to extract phenolic compounds from amla fruit in a short time, but can produce extracts with higher TPC and antioxidant activity, compared to conventional methods (maceration and reflux) which requires a longer extraction.

Table 3. Predictive and experimental value of the response under optimum conditions.

Optimum condition	Predictive value		Experimental value	
	TPC	DPPH	TPC	DPPH
Ethanol concentration: 71.00% Extraction time: 10.38 min	153.92 mg GAE/g	85.99%	154.36 mg GAE/g	85.41%

Table 4. TPC and antioxidant activity of extracts at different extraction techniques.

Method	Time	Temperature	TPC (mg GAE/g)	DPPH (%)
UAE	10 min	30°C	154.36	85.41
Reflux	3 hrs	80°C	120.62	80.9
Maceration	24 hrs	25°C	126.77	82.47

Several other studies on the extraction of phenolic compounds have also shown that ultrasonic waves can increase the effectiveness of the extraction process, which produces high extracts of bioactive components in a short time. TPC of pine seed extract from UAE increased by 30%, higher than the maceration technique (Corbin *et al.*, 2015). The TPC of the extract by the UAE method for the phenolic extract from leaf olefin (47 mg GAE/g) was higher than the conventional method (27 mg GAE/g) (Rodríguez-Pérez *et al.*, 2015). The extract of bitter melon using the UAE method yielded a higher TPC (18.73 mg GAE/g) than conventional extraction (13.53 mg GAE/g) (Lee and Yoon, 2021). In addition, determining the efficiency of the UAE process can be obtained from the comparison of the time required to extract the bioactive components. In the extraction of phenolic compounds from pomegranate peel, the extraction time using the UAE method was 87% shorter than the maceration method (Pan *et al.*, 2012). The extraction process by the UAE method of the grape extract also reduced the extraction time by ten times compared to the maceration method (Carrera *et al.*, 2012). In addition, the extraction time of green tea using UAE was 78% shorter than conventional extraction (Lee *et al.*, 2013).

### 3.7 Phenolic composition of amla fruit extract

The LCMS chromatogram identifying the phenolic compounds of amla fruit extract from the UAE method is presented in Figure 2. The composition of phenolic compounds, relative retention times (RT), relevant  $m/z$  [M-H]<sup>-</sup> data, and MS/MS fragments are presented in Table 5. Phenolic compounds were identified from the 24 peaks based on the reference in the tool and compared with some literature data. In addition, there were several other peaks but not identified as phenolic compounds. Phenolic compounds identified from the LCMS chromatogram of amla fruit extract can be categorized into three groups of compounds: phenolic acids, tannins (gallic acid esters and ellagitannins), and flavonoids.

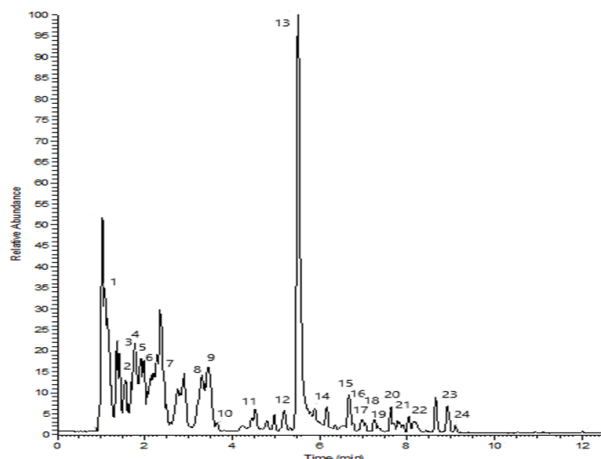


Figure 2. LCMS chromatogram of phenolic compounds in amla extract.

#### 3.7.1 Phenolic acid

Phenolic acid compounds in amla fruit extract identified were cinnamic acid (4), gallic acid (6), syringic acid (20), ellagic acid (22), and chebulic acid (23). These compounds have previously been reported in amla fruit extract (Zhang *et al.*, 2001; Luo *et al.*, 2009; Yang *et al.*, 2012; Mishra and Mahanta, 2016; Li *et al.*, 2019).

#### 3.7.2 Tannin (gallic acid ester and ellagitannin)

Gallic acid ester compounds were identified with various isomers, such as mucic acid gallate, lactone gallate, digallate, and some methyl esters (mono and di-) as also presented by Zhang *et al.* (2001) and Yang *et al.* (2012). Components of the simple ester group from the results of LCMS analysis were identified as mono gallic compounds, mucic acid gallate (1), digallate compounds, namely mucic acid digallate (7), and mucic acid lactone gallate (2). The methyl ester gallate compounds identified in the amla extract were mucic acid methyl ester gallate (5), mucic acid dimethyl ester gallate (8), mucic digallic acid methyl ester (14), and mucic acid lactone methyl ester gallate (12). Methyl ester compounds described by Zhang *et al.* (2001) occur naturally in amla fruit, but Pfundstein *et al.* (2010) suspected that this methyl ester compound was formed during the extraction process. In addition, there is a methyl gallate compound (13) which is thought to be formed during extract production due to the methanolysis reaction of compounds having galloyl groups (Yang *et al.*, 2012). The galloyl ester glucose compounds identified from the analysis were mono, di, and trigalloyl compounds. The compound galloyl glucose (3) (monogalloyl glucose), known as glucogallin (1-O-galloyl- $\beta$ D-glucose), is commonly found in amla fruit extract (Li *et al.*, 2019). In addition, digalloyl glucose (11) and trigalloyl glucose compounds (18) were also found. Many of these compounds have previously been reported in amla fruit extract (Yang *et al.*, 2012).

A number of compounds belonging to the ellagitannin group were identified in amla fruit extract. The specific ellagitannin compound found in amla fruit extract is emblicanin B (9) (2,3,4,6-bis-(S)-hexahydroxydiphenyl-2-keto-glucono-lactone). Emblicanin B has been previously identified by some researchers in amla fruit extract (Ghosal *et al.*, 1996; Liu *et al.*, 2008; Majeed *et al.*, 2009). Another identified ellagitannin compound is galloyl-HHDP (hexahydroxydiphenol)-glucose (15), known as corrilagin (1-O-galloyl-HHDP-3,6-(R)-HHDP- $\beta$ -D-glucose) and digalloyl-HHDP-glucose (19) or known as tercatain (Yang *et al.*, 2012). Other compounds identified were geraniin (16) (Liu *et al.*, 2008), ellaocarpusin (17)

(Yang *et al.*, 2012), and chebulagic acid (galloyl chebulonic acid) (21) (Zhang *et al.*, 2003).

### 3.7.3 Flavonoid

The flavonoid compounds identified in the extract were kaempferol (10) and quercetin-3-O-rhamnoside (24). Kaempferol and quercetin-3-O-rhamnoside had previously been found in amla fruit extract by many researchers (Liu *et al.*, 2008; Yang *et al.*, 2012; Li *et al.*, 2019).

Based on LC-MS results in Table 5, the main phenolic compounds that play a significant role as antioxidants in amla fruit were identified in the extract. These compounds include ellagic acid, gallic acid (Luo *et al.*, 2009), tannins (emblicanin B and geranin (Yang and Liu, 2014)), and mucic acid lactone gallate (Zhang *et al.*, 2001). Antioxidant activity is also found in phenolic compounds with ortho-hydroxyl structures that easily form ortho-quinone compounds (Choi *et al.*, 2002; Zhang *et al.*, 2009). The hydroxyl group and an ortho-hydroxyl structure on the aromatic ring of phenolic compounds such as mucic-1,4-lactone-3-O-gallic acid and ellagic acid produce a synergistic effect on the antioxidant activity of these compounds. Other compounds that act as antioxidants in some extracts are methyl gallate (Tourinho *et al.*, 2008), kaempferol (Liu *et al.*, 2008), and quercetin-3-O-rhamnoside (Yamazaki *et al.*, 2007; Luo *et al.*, 2009).

## 4. Conclusion

The RSM using the CCD was successfully used to optimize the extraction condition in UAE of phenolic compounds from amla fruit. The independent variables (especially the ethanol concentration) greatly affected the TPC and DPPH antioxidant activity of the extracts. The second-order polynomial model developed has high validity in predicting the TPC and DPPH antioxidant activity, which has been tested through some parameters. The optimum conditions to maximize both responses (TPC and DPPH) were obtained at the ethanol concentration of 71.00% and the extraction time of 10.38 mins. Under optimum conditions, no significant difference was found between the experimental values and the predicted values of the TPC and DPPH of the extract. LCMS analysis revealed that there were 24 phenolic compounds identified in amla fruit extract. In comparison to conventional extraction methods (maceration and reflux), the UAE method improved extraction efficiency and significantly decreased the extraction time without reducing the antioxidant activity of amla fruit extract.

Table 5. Phenolic compounds identified in amla fruit extract.

No	RT	Compound	[M-H] <sup>-</sup> m/z)	MS <sup>n</sup> (m/z)	Formula
1	1.10	Mucic acid gallate	361	211, 169, 89	C <sub>13</sub> H <sub>14</sub> O <sub>12</sub>
2	1.56	Mucic acid-1,4-lactone-3-O-gallate	343	191, 169, 125	C <sub>13</sub> H <sub>12</sub> O <sub>11</sub>
3	1.70	Galloyl glucose	331	169, 151, 125	C <sub>13</sub> H <sub>16</sub> O <sub>10</sub>
4	1.77	Cinamic acid	147	129, 72	C <sub>9</sub> H <sub>8</sub> O <sub>2</sub>
5	1.78	Mucic acid methyl ester gallate	375	225, 169	C <sub>14</sub> H <sub>16</sub> O <sub>12</sub>
6	1.99	Gallic acid	169	126, 125	C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>
7	2.47	Mucic acid digallate	513	361, 209, 85	C <sub>20</sub> H <sub>18</sub> O <sub>16</sub>
8	3.32	Mucic acid dimethyl ester gallate	389	357, 169, 125	C <sub>15</sub> H <sub>18</sub> O <sub>12</sub>
9	3.46	Emblicanin B	779	389, 357	C <sub>34</sub> H <sub>20</sub> O <sub>22</sub>
10	3.66	Kaempferol	285	133, 115	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>
11	4.55	Digalloyl glucose	483	313, 169, 125	C <sub>20</sub> H <sub>20</sub> O <sub>14</sub>
12	5.20	Mucic acid lactone methyl ester gallate	357	169, 143, 125	C <sub>14</sub> H <sub>14</sub> O <sub>11</sub>
13	5.51	Methyl gallate	183	151, 136, 109	C <sub>8</sub> H <sub>8</sub> O <sub>5</sub>
14	6.17	Mucic acid methyl ester digallate	527	375, 223, 191	C <sub>21</sub> H <sub>20</sub> O <sub>16</sub>
15	6.67	Galloyl HHDP glucose	633	169, 125, 123	C <sub>27</sub> H <sub>22</sub> O <sub>18</sub>
16	6.72	Geraniin	951	300	C <sub>41</sub> H <sub>28</sub> O <sub>27</sub>
17	6.97	Ellaocarpusin	1109	300, 275	C <sub>47</sub> H <sub>34</sub> O <sub>32</sub>
18	7.06	Trigalloyl glucose	635	483, 169, 125	C <sub>27</sub> H <sub>24</sub> O <sub>18</sub>
19	7.25	Digalloyl HHDP glucose	785	300, 169, 125	C <sub>34</sub> H <sub>26</sub> O <sub>22</sub>
20	7.63	Syringic acid	197	153, 138, 121	C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>
21	7.69	Chebulagic acid	953	300, 125	C <sub>41</sub> H <sub>30</sub> O <sub>27</sub>
22	8.17	Ellagic acid	301	300, 229, 223	C <sub>14</sub> H <sub>6</sub> O <sub>8</sub>
23	8.66	Chebolic acid	355	309, 147	C <sub>14</sub> H <sub>12</sub> O <sub>11</sub>
24	9.11	Quercetin-3-O-rhamnoside	447	223	C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>

## Conflict of interest

All authors declare that there is no conflict of interest.

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