Characterization of phenolic compounds from *Phyllanthus emblica* fruits using HPLC-ESI-TOF-MS as affected by an optimized microwave-assisted extraction

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**ABSTRACT**

A simple microwave-assisted method for extracting antioxidant phenolic compounds from emblic (*Phyllanthus emblica* L.) fruits was developed and optimized with response surface methodology (RSM). The influence of process conditions including microwave power, irradiation time, ethanol concentration, and solvent-to-solid ratio on the extraction of total phenolic content was analyzed using a second-order regression equation. The optimum extraction parameters were as follows: microwave power, 480 W; irradiation time, 29 s; ethanol concentration, 66%; liquid-to-solid ratio, 25 mL/g. Under these conditions, the recovery of total phenolic content was 133.58 ± 15.61 mg gallic acid equivalent/g dry weight (DW). The extracts were also analyzed using HPLC-ESI-TOF-MS to identify eight compounds including hamamelitannin, mucic acid-1,4-lactone-3-O-gallate, ellagic acid, and isocorilagin. To our knowledge, this is the first report of hamamelitannin in emblic fruit. The extracts exhibited strong antioxidant capacities in DPPH and ABTS\(^+\) radical scavenging assays and showed an excellent inhibition of lipid peroxidation and reducing property. These findings illustrated that the optimized microwave-assisted extraction method was efficient in extracting phenolic compounds with high antioxidant activity from emblic fruits. It was suggested that emblic fruits may be a new potential source as natural antioxidant agents applied in food industry.

**INTRODUCTION**

Reactive oxygen species (ROS) play important roles in either the initiation or progression of carcinogenesis by oxidative stress in the aerobic metabolism.\(^1\) The strong oxidative stress causes lipid peroxidation, DNA damage, and denaturation of proteins. Our body has developed sophisticated antioxidant systems against oxidative stress through the antioxidant nutrients.\(^1\) Antioxidants are a series of bioactive compounds that are able to capture free radicals to reduce risks of aging and chronic cardiovascular diseases.\(^2\) Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ) have been used for reducing oxidative stress.\(^1\) However, due to the potential toxicity and carcinogenicity, the uses of these synthetic antioxidants have raised significant concerns about the safety problems.\(^3\) As
a consequence, more attention has been placed on exploiting natural and safe antioxidants from plant and agricultural by-products as health-promoting additives.\textsuperscript{[4]} There has been an increasing demand for phytochemicals as ingredients in food, pharmaceuticals, dietary supplements, and cosmetics.\textsuperscript{[5]} However, extraction methods and conditions can significantly affect the yield, quality, and bioactivity of phytochemicals from plant.\textsuperscript{[2]} To identify phytochemicals, simple, efficient and environment-friendly methods for extraction are needed.

\textit{Phyllanthus emblica} L., commonly known as emblic, is one of the most important traditional medicinal plants from the family Euphorbiaceae. This species grows widely in tropical and subtropical areas of China, India, Indonesia, and the Malay Peninsula.\textsuperscript{[6,7]} Emblic fruit is well accepted by consumers for its special taste and used extensively as an antioxidant, antiaging, anticancer, anti-inflammatory, antimicrobial, and immunomodulatory agent in many traditional medicinal systems, such as Chinese herbal medicine, Tibetan medicine, and Ayurvedic medicine.\textsuperscript{[8–12]} Recent studies show that the fruit is rich in polyphenols, ascorbic acid, tannins, flavones, polysaccharides, and other bioactive substances.\textsuperscript{[10–20]} Emblic fruit could be considered as plant source for natural antioxidants and nutraceutical or pharmaceutical ingredients due to the abundant existence of phenolic compounds. Previous studies of \textit{P. emblica} were focused on phenolic identification, methods for the facile and high effective extraction and maintaining strong antioxidant activities from \textit{P. emblica} fruits have not been reported.

Methods for extraction of phenolic compounds include microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE) from herbal medicine.\textsuperscript{[4,21,22]} Among them, MAE is a relatively simple and efficient alternative to conventional heating, boiling, or refluxing extraction techniques. MAE can offer shorter operation times, reduced both extraction time and solvent consumption, simplified manipulation and lower energy input.\textsuperscript{[23]} It also produces higher extraction rates of the target species and better results with lower costs.\textsuperscript{[4]} The mechanism of microwave-assisted extraction is attributed to heating polar solvents with microwave energy while in contact with solid samples, and to partition interest compounds between the sample and the solvent.\textsuperscript{[24]} Furthermore, the thermal degradation effects using microwave irradiation can be avoided while favoring the rapid desorption from matrices.\textsuperscript{[25]} The efficiency of the MAE extraction is influenced by many factors including duration of microwave irradiation, microwave power, solvent to solid ratio, and their interaction with each other. Thus, optimizing these parameters is required to extract a large amount of quality phenolic compounds.\textsuperscript{[4,22]}

In order to study the potential industrial application of \textit{P. emblica} as an antioxidant source, an MAE method for extracting polyphenols from \textit{P. emblica} was developed, and the extraction conditions were optimized according to the enhancement of extraction yield along with maintaining antioxidant capacity by using response surface methodology (RSM). This MAE method was also compared with a conventional method for phenolics extraction. Moreover, the extracts were investigated using high-resolution high-performance liquid chromatography with mass spectrometry detection (HPLC-ESI-TOF-MS) to determine phenolic compositions. \textit{In vitro} assays were designated to evaluate the antioxidant activity.

\textbf{Materials and methods}

\textit{Plant materials}

Fruits were harvested from \textit{P. emblica} trees grown in an orchard in Huian county, Fujian province of China (Southeast of China; north latitude 24°49′-25°15′, east longitude 118°38′-119°05′, altitude 29 m). Harvested fruits were immediately transported to the laboratory at Fujian Agriculture and Forestry University and dried in a forced-air oven at 40°C to constant weight, and then ground using an electric grinder (IKA model-A11, Staufen, Baden-Württemberg, Germany). The ground powders were passed through a standard 425 μm sieve and were collected and stored at 4°C in airtight bags until further use.
**Reagents**

All chemicals and solvents used in this study were of analytical grade. Gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and potassium persulfate were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Na$_2$CO$_3$, FeSO$_4$, Folin–Ciocalteu’s phenol reagent, Na$_2$HPO$_4$, and HCl were purchased from China National Pharmaceutical Group Co. (Beijing, China).

**Microwave-assisted extraction of total phenolic content**

Phenolic compounds from powders of emblic fruits were extracted using a domestic microwave oven system (2450 MHz, Galanz Model G80F20CN2L-B8, Foshan, Guangdong, China). The apparatus was equipped with a digital control system for microwave power and irradiation time. The microwave oven was modified in order to condense the vapors generated during extraction from the sample. Different concentrations of ethanol in water were used as an efficient and safe solvent for the extraction of phenolic compounds.[4]

The ground emblic fruit sample (2.0 g each) were used to test the following parameters in the microwave oven: extraction time (10–50 s), microwave power (80–720 W), ethanol concentration (10–90%), and liquid-to-solid ratio (10–40 mL/g). The influence of each parameter was analyzed in single-factor experiments. After MAE treatment, each extracted slurry was centrifuged at 8000 rpm for 10 min (5810R, Eppendorf AG., Germany), and supernatant was collected to a volumetric flask. Each trial was carried out five times. All extracts were stored at 4°C until further use.

**Conventional solvent extraction of total phenolic content**

Phenolic compounds in emblic fruits were also extracted using a conventional solvent extraction method described by Nampoothiri et al.[10] Briefly, 2.000 g of powder was mixed with 70% ethanol (v/v) in a conical centrifugal tube and the mixture was kept at a temperature (27 ± 2°C) with stirring for 8 h to extract phenolic compounds. The extraction process was repeated till the solvent became colorless. After the conventional solvent extraction treatment, the supernatant was collected and stored at 4°C until further use.

**Determination of Total Phenolic Content (TPC)**

The amount of total phenolics in all extracts was determined by the Folin–Ciocalteu method with slight modifications.[24] The TPC was expressed as mg of gallic acid equivalents (GAE) per gram of powder on dry weight (DW) basis.

**Central composite design and optimization**

On the basis of the single-factor experimental results, major influence factors and their levels were confirmed. A five-level (−2, −1, 0, +1, and +2) and four-variable (X$_1$, X$_2$, X$_3$, and X$_4$) central composite design (CCD) was performed to obtain a second-order model (Eq. 1) to predict the content of total phenolics.[21] The independent variables and their levels were microwave power (X$_1$, 240–560 W), irradiation time (X$_2$, 20–40 s), ethanol concentration (X$_3$, 60–80% v/v), solvent to solid ratio (X$_4$, 10–30 mL/g).

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j
\]  

(1)
where $Y$ represents the response function (in this case the TPC yield); $\beta_0$ is a constant coefficient; $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are regression coefficients for linear, quadratic, and interactive terms, respectively; and $X_i$ and $X_j$ represent the coded independent variables while $k$ equals to the number of the tested factors ($k = 4$). All experiments were performed randomly in triplicate. Statistical analysis was performed using Design-Expert 8.0.6 software and fitted to a second-order polynomial regression model containing the coefficient of individual linear, quadratic and interactive terms. An analysis of variance (ANOVA) with 95% confidence level was then carried out for each response variable in order to test the model significance and suitability.

**Identification of phenolic compounds by HPLC-ESI-TOF-MS**

Separation of phenolic compounds was performed with Agilent 1200 series high-performance liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) consisting of a vacuum degasser, autosampler, and a binary pump equipped with a Phenomenex Luna C18(2) analytical column (4.6 × 250 mm 5µm, Phenomenex Inc., Torrance, CA, USA) based on a method described by Taamalli et al.\textsuperscript{[25]} with some modifications. The mobile phases were water with acetic acid (0.5%) (phase A) and methanol (phase B), and the solvent gradient was changed according to the following conditions: 0–10 min, 5–30%B; 10–60 min, 30–45% B; 60–90 min, 45–60% B; 90–120 min, 60–90% B. The flow rate was set at 0.80 mL/min throughout the gradient. The column temperature was maintained at 25°C, and the injection volume was 10 µL.

To identify phenolic compounds, the HPLC system was coupled to an Agilent 6520 accurate-mass quadrupole time-of-flight (Q-TOF) LC/MS (Agilent Technologies, Santa Clara, CA, USA), electro-spray ionization (ESI) was operated in the negative ion mode in the mass range 50 to 1000 (m/z). The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 350°C; drying gas flow, 11.0 L/min; and nebulizing gas pressure, 40psi.

**Determination of antioxidant activity**

The antioxidative properties of the sample were evaluated by the scavenging activity against DPPH and ABTS radicals and the determination of anti-lipid peroxidation assay and reducing power according to the methods described by Chen et al.\textsuperscript{[26]}, Dahmoune et al.\textsuperscript{[8]}, and Huang et al.\textsuperscript{[27]}, respectively.

**Statistical analysis**

All the experiments were carried out in triplicate, and the results were expressed as means ± SE (standard error). Statistical analyses were performed using the Statistical Product and Service Solutions version 19.0 software (IBM Corporation, Armonk, NY, USA). When significance occurred, means were separated by Fisher’s Least Significant Difference (LSD) at $P< 0.05$ or $P< 0.01$ level.

**Results and discussion**

**Optimizing the TPC yield by microwave-assisted extraction using RSM**

Based on the single-factor experimental results (Figure 1), major influence factors and their levels were confirmed on the MAE process. The MAE of phenolic compounds from emblic fruits was optimized using response surface methodology (RSM). The experimental design is presented in Table 1 where there were six replicates.

The regression coefficients of the intercept, linear, quadratic, and interaction terms of the model were calculated using a Design Expert program (version 8.0.6), and the factors and interactions were subjected to analysis of variance (ANOVA) for testing significance. Regression coefficients and
ANOVA of the second-order polynomial models for the yield of TPC are summarized in Table 2. Two linear parameters, microwave power ($X_1$), and liquid solvent to solid ratio ($X_4$) were significant ($P < 0.05$), whereas irradiation time ($X_2$) and ethanol concentration ($X_3$) were not significant. All the quadratic parameters were highly significant at $P < 0.01$ level. The interactions $X_1X_2$, $X_1X_3$, $X_1X_4$, and $X_3X_4$ were also significant ($P < 0.05$). According to the yield of TPC, the model of final predictive equation was found to be as follows (Eq. 2):

$$Y(\text{TPC}) = 129.38 + 2.38X_1 + 0.47X_2 - 0.9X_3 + 1.84X_4 - 2.51X_1X_2 - 1.86X_1X_3 + 4.25X_1X_4 - 1.23X_2X_3 - 0.15X_2X_4 - 5.09X_3X_4 - 2.72X_1^2 - 3.19X_2^2 - 4.82X_3^2 - 4.81X_4^2$$

(2)

The ANOVA in Table 2 showed that the quadratic polynomial model was highly significant ($P < 0.0001$) and sufficient to represent the actual relationship between the response and significant parameters. The model also showed statistically insignificant lack of fit at 95% confidence level, which indicated that the fitted models were considered adequate. Furthermore, the determination coefficient ($R^2$) of 0.9352 and the adjusted determination coefficient ($R^2_{Adj}$) of 0.8748 for the model did not differ greatly in a good statistical model.\[4,28\] The low value of pure error suggesting that the model was reliable and reproducible which agrees with the previous data obtained from the ANOVA. The results indicated that the model should work well for the prediction of TPC yield in this MAE from the fruit of $P. emblica$.

To investigate the interactive effects of the operational parameters and their mutual interaction on TPC yield, this study generated the two- and three-dimensional response surface profiles of multiple

![Figure 1. The effect of microwave power (a), extraction/irradiation time (b), ethanol concentration (c), and liquid-to-solid ratio (d) on the total phenolic yield extracted from $Phyllanthus emblica$ fruits ($n = 3$).](image-url)
non-linear regression models (Figure 2), respectively. The interactive effects of microwave power ($X_1$) and irradiation time ($X_2$) had a significant influence on the acquired ratio of TPC ($P < 0.01$). The microwave power ($X_1$) and ethanol concentration ($X_3$) had significant interactive effects ($P < 0.05$) on the extraction yield of TPC. The interactive effect of microwave power ($X_1$) and liquid-to-solid ratio ($X_4$) had a significant influence on the recovery of TPC ($P < 0.01$). The extraction yield of TPC gradually increased with the increase of irradiation time up to 32.5 s and ethanol concentration up to 69.5% and decreased slowly after reaching the critical values. When microwave power and ethanol concentration are at a certain value, the extraction of TPC increased with irradiation time increase. The recovery of TPC using microwave energy appears a highly significant function of the synergistic effect of ethanol concentration ($X_3$) and liquid to solid ratio ($X_4$) ($P < 0.01$).

Using the Design Expert 8.0.6 software, we were able to identify optimal conditions for MAE of TPC from emblic fruit: microwave power, 480 W; irradiation time, 29.06 s; ethanol concentration, 66.06%; and liquid-to-solid ratio, 25mL/g. The predicted extraction yield of TPC was 133.62 mg GAE/g DW at those conditions. To test the reliability of the model, emblic fruit was extracted using the MAE with the established parameters. A mean value of TPC from this experiment was 133.58 ± 15.61 mg GAE/g DW ($n = 5$), which was in close agreement with the predicted value and were not significantly different ($P > 0.05$) using a paired t-test. This experiment showed that the RSM model was valid. The confirmation suggested that a high yield of TPC can be extracted from emblic fruit using this model. The TPC yield from this study was considerably higher than it obtained using conventional solvent extractions (92 ± 2.66 mg GAE/g DW). Additionally, this extraction method was simple and fast.

Table 1. Experimental design with the response surface methodology for total phenolic compounds (TPC) yield extracted from Phyllanthus emblica fruit using microwave-assisted extraction (MAE) method.

| Run number | Microwave power (W), $X_1$ | Irradiation time (s), $X_2$ | Ethanol concentration (%), $X_3$ | Solvent-to-solid ratio (mL/g), $X_4$ | Recovery of TPC (mg GAE/g) |
|------------|-----------------------------|-----------------------------|---------------------------------|-----------------------------------|-----------------------------|
| 1          | 0(400)                      | 0(30)                       | 0(70)                           | 0(20)                             | 132.151                     |
| 2          | −1(320)                     | 1(35)                       | 1(75)                           | 1(25)                             | 104.524                     |
| 3          | 0(400)                      | 0(30)                       | 0(70)                           | 2(30)                             | 114.090                     |
| 4          | 0(400)                      | 0(30)                       | −2(60)                          | 0(20)                             | 112.816                     |
| 5          | 0(400)                      | −2(20)                      | 0(70)                           | 0(20)                             | 115.239                     |
| 6          | −1(320)                     | 1(35)                       | −1(65)                          | 1(25)                             | 119.452                     |
| 7          | 0(400)                      | 0(30)                       | 0(70)                           | 0(20)                             | 121.239                     |
| 8          | 0(400)                      | 0(30)                       | 0(70)                           | 0(20)                             | 126.063                     |
| 9          | 0(400)                      | 2(40)                       | 0(70)                           | 0(20)                             | 117.483                     |
| 10         | 0(400)                      | 0(30)                       | 0(70)                           | 0(20)                             | 132.174                     |
| 11         | 1(480)                      | −1(25)                      | 1(75)                           | −1(15)                            | 115.417                     |
| 12         | 1(480)                      | 1(35)                       | 1(75)                           | −1(15)                            | 111.063                     |
| 13         | 0(400)                      | 0(30)                       | 2(80)                           | 0(20)                             | 106.841                     |
| 14         | 1(480)                      | −1(25)                      | −1(65)                          | −1(15)                            | 108.905                     |
| 15         | 0(400)                      | 0(30)                       | 0(70)                           | 0(20)                             | 132.435                     |
| 16         | −1(320)                     | −1(25)                      | 1(75)                           | 1(25)                             | 106.764                     |
| 17         | 1(480)                      | −1(25)                      | −1(65)                          | 1(25)                             | 133.021                     |
| 18         | 1(480)                      | 1(35)                       | −1(65)                          | −1(15)                            | 105.734                     |
| 19         | −1(320)                     | −1(25)                      | 1(75)                           | −1(15)                            | 118.551                     |
| 20         | −1(320)                     | 1(35)                       | 1(75)                           | −1(15)                            | 121.957                     |
| 21         | −1(320)                     | 1(35)                       | −1(65)                          | −1(15)                            | 112.532                     |
| 22         | −1(320)                     | −1(25)                      | −1(65)                          | 1(25)                             | 105.829                     |
| 23         | −1(320)                     | −1(25)                      | −1(65)                          | −1(15)                            | 103.868                     |
| 24         | 2(560)                      | 0(30)                       | 0(70)                           | 0(20)                             | 123.418                     |
| 25         | 0(400)                      | 0(30)                       | 0(70)                           | 0(20)                             | 132.151                     |
| 26         | 1(480)                      | 1(35)                       | −1(65)                          | 1(25)                             | 127.112                     |
| 27         | 1(480)                      | 1(35)                       | 1(75)                           | 1(25)                             | 112.667                     |
| 28         | −2(240)                     | 0(30)                       | 0(70)                           | 0(20)                             | 113.036                     |
| 29         | 0(400)                      | 0(30)                       | 0(70)                           | −2(10)                            | 105.676                     |
| 30         | 1(480)                      | −1(25)                      | 1(75)                           | 1(25)                             | 115.923                     |
Analysis of emblic fruits extracts by HPLC-ESI-TOF-MS

The HPLC has been commonly used for identifying the composition of plant extracts. However, the HPLC method was not satisfactory for complete characterization of compounds with high sensitivity. LC-MS had emerged as a more powerful tool for the determination of many compositions of extracts.\[10\] The TOF-MS instrumentation had been shown to provide excellent mass resolution and mass accuracy and was the perfect choice for molecular formula determination.\[25\] Thus, the HPLC-ESI-TOF-MS method was used to analyze the extracts derived from emblic fruits. A total of eight peaks were observed as shown in Figure 3. The compositions were identified by their retention times and mass spectra as compared with reported data in the literature, which are summarized in Table 3. Among them, the peak area of compound 2 was the largest (7.2% of the total), followed by compounds 1 (5.3%), 8 (3.4%), 6 (2.8%), 5 (2.2%), 7 (2.1%), 3 (1.2%), and 4 (1.1%). By analyzing the MS data of the eight compounds, including retention time ($T_R$), experimental $m/z$, molecular formula, error of the experimental $m/z$, and the MS2 fragments of the individual compounds, these compounds were successfully identified through reference to the Mass Bank MS database (https://massbank.eu/MassBank/index.html) and available literatures.

The mass spectra of compound 1 ($T_R = 24.790$ min) showed a [$M-H]$ at $m/z$ 343 and fragment anions at $m/z$169 and 191. According to the previous reports (Luo et al.\[15\] and Zhang et al.\[16\]), compound 1, producing the anion at $m/z$ 169, which was a characteristic fragment of galloyl group formed by the loss of a mucic acid group (191 Da), was identified as mucic acid-1,4-lactone-3-O-gallate.

The mass spectra of compounds 2 ($T_R = 32.367$ min) showed [$M-H]$ at $m/z$483, and produced MS2 characteristic fragment anions at $m/z$313, 271, 211, 169, and 125. The ion $m/z$313 arises from the loss of a gallate unit (170 Da) from the [$M-H]$ at $m/z$483. The fragment ion at $m/z$271 is explained by the loss of a C$_2$H$_2$O from the latter fragment, while the fragment at $m/z$211 derives from the loss of C$_2$H$_4$O$_2$ from the fragment at $m/z$271. The fragment ion at $m/z$169, typical mass in the negative mode of galloyl group, indicates the loss of a – C$_2$H$_5$Ogroup ($m/z$42) from the fragment.
at m/z 211, and the fragment ion at m/z 169 showed a loss of a carboxyl group (44 Da) producing the fragment ion at m/z 125. Thus, the peak was identified as 2',5'-di-O-galloyl-D-hamamelose (hamameltannin), which was previously reported by Touriño et al.\[29\] To our knowledge, this is the first time that this compound has been identified in *P. emblica* fruit. Quantification of the hamameltannin content was calculated to 36 mg/100 ml in *P. emblica* fruit extracts by HPLC-DAD according to the methods described by Duckstein et al.\[30\]

Compound 3 (T<sub>R</sub> = 41.720 min) showed a precursor anion at m/z 633 with four fragment anions at m/z 463, 301, and 169. The anion at m/z 169 showed the existence of galloyl group in Compound 3. The ion at m/z 463 arises from the loss of a gallate unit (170 Da) from the [M−H]<sup>−</sup> at m/z 633. According to Nampoothiriet al.\[10\], the anion at m/z 301 was a characteristic fragment of ellagic acid, produced

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Figure 2. Response surface plots of the total phenolic yield extracted from *Phyllanthus emblica* fruits using microwave-assisted extraction as a function of significant interactions among factors: (a) microwave power and extraction/irradiation time; (b) microwave power and ethanol concentration; (c) microwave power and liquid-to-solid ratio; (d) extraction/irradiation time and ethanol concentration; (e) extraction/irradiation time and liquid-to-solid ratio; and (f) ethanol concentration and liquid-to-solid ratio.
through the loss of a $-\text{C}_6\text{H}_10\text{O}_5$ group ($m/z$162) from the fragment at $m/z$463. Therefore, the peak was identified as isocorilagin, which was previously reported by Liu et al.\textsuperscript{[14]} and Luo et al.\textsuperscript{[15]}

The mass spectra of compounds 4 ($T_R=45.055$ min) and 5 ($T_R=54.944$ min) showed precursor anions ([M−H]−) at $m/z$ 197 and 183, and both showed two major fragment anions at $m/z$124 and 78. Through comparison of the MS2 fragmentation data with other studies (Touriño et al.\textsuperscript{[29]}; Zhang et al.\textsuperscript{[31]} and the Mass Bank MS database, compounds 4 and 5 were identified as ethyl gallate and methyl gallate. Of these compounds, ethyl gallate has been identified in leaves of *Juglans regia* by Zhang et al.\textsuperscript{[31]} and methyl gallate has been identified in leaves of *Hamamelis virginiana* by Touriño et al.\textsuperscript{[29]}

The mass spectra of compound 6 ($T_R=71.966$ min) showed a precursor anion [M−H]− at $m/z$301 and fragment anions at $m/z$257(by the loss of one CO$_2$, i.e.,$-\text{CO}_2$), and 229 (−CO), respectively. Through comparison of the MS2 fragmentation data from *Terminalia bellerica* and *Emblica officinalis*.\textsuperscript{[10]} and the Mass Bank MS database, compounds 6 was identified as ellagic acid.

The mass spectra of compound 7 ($T_R=75.742$ min) showed a [M−H]− at $m/z$447 with fragment anions (301, 271, and 151 $m/z$). Based on the Retro–Diels–Alder reaction (Chang and Wu\textsuperscript{[32]}), the fragment at $m/z$ 151 which corresponds to the fragment of flavone aglycones, resulted from the separated heterocyclic ring (ring C) at positions C-2 and C-4. According to the previous reports (Taamalli et al.\textsuperscript{[25]}; Huang et al.\textsuperscript{[33]}; Yamazaki et al.\textsuperscript{[34]}), quercetin showing a [M−H]− at $m/z$301 produced a fragment of a $-\text{C}_6\text{H}_4\text{O}_4$ group at $m/z$ 151 through the loss of a $-\text{C}_6\text{H}_4\text{O}_4$ group via the Retro-Diels-Alder reaction, and the ion at $m/z$301 is diagnostic of quercetin derivatives resulting from the loss of a rhamnoside unit ($m/z$146) from the [M−H]− at $m/z$483. Therefore, compound 7 was identified as quercetin-3-O-rhamnoside.
The mass spectra of compounds 8 (T_R = 78.86 min) exhibited precursor anion ([M−H]^−) at m/z 233 and fragment anions at m/z 217, 163, and 75. The molecular formula was established to be C_{15}H_{22}O_2 from El-MS based on mass spectra [M^+/m/z 234] data. The fragment ion at m/z 217 is explained by the loss of a hydroxyl unit from the [M−H]^− at m/z 233. However, due to lack of the effective information in MS^2 fragments and too many isomers of molecular formula C_{15}H_{22}O_2, the compound in peak 8 was undefined.

We identified seven phenolic compounds in P. emblica fruit, such as mucic acid-1,4-lactone-3-O-gallate, hamamelitannin, isocorilagin, ethyl gallate, methyl gallate, ellagic acid, quercetin-3-O-rhamnoside. One of the original contributions of our work to the emblic fruits extraction is the identification of hamamelitannin, which was isolated from Hamamelis virginiana[29] but not reported in the fruit of P. emblica.

**Determination of antioxidant capacity**

Different in vitro antioxidant methods had been used to analyzing antioxidant activities of plant extracts.[27] The polyphenols extracted by the optimized MAE method exhibited strong dose-dependent scavenging activities based on the assay methods of DPPH radical (R^2 = 0.99), ABTS radical cation (R^2 = 0.98), its inhibition of lipid peroxidation capacity (R^2 = 0.99), and reducing property (R^2 = 0.99) (Figure 4). Furthermore, the extract had antioxidant activity greater than either the control BHT or ascorbic acid (Table 4). Our result demonstrated that the emblic fruit extracts had strong antioxidant activity.

The HPLC-ESI-TOF-MS analysis of ethanolic extracts identified eight compounds as the main phenolics. The high antioxidant activity of these phenolics is likely related to their high degree of hydroxylation of aromatic rings, the arrangement of the hydroxyl group, as well as the number of ortho-hydroxyl groups and galloyl group, on benzene nucleus structure. More importantly, we identified hamamelitannin as a major compound in emblic fruit. It showed strong free radical scavengers against ABTS, DPPH and electron transfer, which could be due to the fact that there are seven hydroxyl groups bonded to the aromatic ring.[29] The structure of phenolics possesses a number of hydroxyl groups which can lose a protonated hydrogen and then form strong coordination oxygen ion complexes with metal ion.

Additionally, these phenolics may be able to directly trap free radicals and/or act through metal chelation.[17] It was reported that mucic acid-1,4-lactone-3-O-gallate, ellagic acid, and isocorilagin in methanolic extracts of emblic fruit could chelate ferrous ion and inhibit linoleic acid peroxidation.[17] Furthermore, ethyl gallate,[31] methyl gallate[29] and quercetin-3-O-rhamnoside[34] were reported to be responsible for the antioxidant activity of several extracts. The ortho-hydroxyl structures could also increase the antioxidant activity of phenolics because the ortho-quinone was easily formed.[31,35] As a result, the remarkable antioxidant activity of ethanolic extracts of P. emblica could be attributed to the presence of the high percentage of phenolic compounds, such as hamamelitannin, mucic acid-1,4-lactone-3-O-gallate, and ellagic acid, which have a large number of hydroxyl groups presented in their aromatic rings and ortho-hydroxyl structures of these phenolic compounds as well as possible synergistic effects of these compounds.

**Conclusion**

This study established an MAE method for extracting polyphenols with high antioxidant activities from P. emblica fruit using RSM. An optimal polyphenol yield of 133.58 mg GAE/g DW was achieved under the following optimum operating conditions: microwave power of 480 W, irradiation time of 29 s, ethanol concentration of 66%, and liquid-to-solid ratio of 25mL/g. HPLC-ESI-TOF-MS analysis identified eight phenolic compounds including hamamelitannin, which was a prominent compound that has not been reported before in emblic fruits. Moreover, the extract had significantly
high antioxidant activities. The presence of hydroxylation and ortho-hydroxyl groups in their aromatic rings of phenolic compounds from emblic fruits were the most important factor contributing to antioxidant activities, nevertheless, the further study to identify the phenolic compounds from *P. emblica* and elucidate their antioxidant mechanisms are in progress. Results from this study showed that the optimized MAE method is simple and easy for use to effectively extract antioxidant phenolic compounds from emblic fruits. The information will contribute to the future application of *P. emblica* as raw materials in health-care food industry.

**Table 4.** The half maximal inhibitory concentration (IC$_{50}$, mg/mL) of sample, BHT, and ascorbic acid in antioxidant activities assays.

| Assays                        | Sample       | BHT          | Ascorbic acid |
|-------------------------------|--------------|--------------|---------------|
| DPPH                          | 0.030 ± 0.002| 0.045 ± 0.002| 0.024 ± 0.001 |
| ABTS*                         | 0.426 ± 0.06 | 0.215 ± 0.05 | 0.562 ± 0.08  |
| Inhibition of peroxidation    | 0.578 ± 0.04 | 0.851 ± 0.11 | 0.617 ± 0.06  |
| Reducing power                | 0.173 ± 0.02 | 0.065 ± 0.001| 0.131 ± 0.003 |

IC$_{50}$: The concentration for a 50% inhibition and in the reducing power assay that means the concentration for a 0.5 absorbance at 700 nm.

Data are represented as means ± SE (n = 3).
Funding

This research was financially supported by the National Natural Science Foundation of China [Grant No. 31501694 and No. 81673542], Specialized Research Fund for the Doctoral Program of Higher Education [Grant No. 2011351520016], Natural Science Foundation of Fujian Province [Grant No. 2011J05050], and Scientific Research Fund of Fujian Provincial Education Department [Grant No. J2012016]. Yongyu Li was also funded by the China Scholarship Council.

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