Extraction Optimization, Purification and Immunostimulatory Activity in vitro of Polyphenols from Apple (Malus domestica) Peel

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ABSTRACT

Apple peel polyphenols were extracted by ultrasound-assisted extraction (UAE) and conditions for the extraction of polyphenols were optimized by the response surface methodology. The optimized conditions determined as 32 min of extraction time, 29 °C of extraction temperature and ethanol concentration of 56% with a yield of 35.08 ± 0.26 mg gallic acid equivalent/g dry weight. Six resins were used for the purification and D101 resin showed the highest ratio of desorption for polyphenols and further applied for dynamic adsorption/desorption test. Catechin, procyandin B, quercitin-3-galactoside and quercitin xyloside were identified in purified polyphenols by HPLC-MS/MS. Finally, the immunostimulatory activity in vitro of purified polyphenols on RAW264.7 cell lines was evaluated. The apple peel polyphenols exhibited a dose-dependent effect (p < 0.05) on the strongest propagation of RAW264.7 cells. The results demonstrate that apple peel is a valuable source of polyphenols and could be used as a natural immunostimulating agent for application in functional foods.

Keywords: Apple peel; extraction; immunostimulating activity; polyphenols; purification; RAW264.7

INRODUCTION

In recent years, the disposal of agro-industrial wastes has become an enormous challenge for the food processing industries. Utilization of plant byproducts from agro-food industry is very important from the environmental viewpoint (Machado et al. 2014). These tendencies increase the efforts of byproducts valorization especially press residues and peeling wastes to increase the yield and make it cost-efficient (Kammerer et al. 2010; Weisz et al. 2009). The phenolic compounds, obtained from waste materials, have gained much interest because of their bio-functional properties like antioxidant ability, coloring potential and health-stimulating effects (Idris & Donnelly 2009; Kammerer et al. 2010).

Apple is a very good source of polyphenols possessing strong antioxidant activity (Vayndorf et al. 2013). Apple peel which is an agro-industrial byproduct contains high amounts of polyphenols and antioxidants (Massini et al. 2013), but their industrial use has only focused on pectin and fiber (Shalini & Gupta 2010). The main bioactive compounds present in the apple peel are phenolic acids, flavonols, flavon-3-ols, anthocyanins, and dihydrochalcones (Boyer & Liu 2004). Procyanidins like (+)-catechin, (−)-epicatechin, chlorogenic acid, phloridzin, and quercetin conjugates are most commonly found compounds in apple peel (Karaman et al. 2013).

To date, for the extraction of polyphenols from plants, numerous novel extraction procedures comprising supercritical fluid extraction, ultrasound-assisted
extraction (UAE) and microwave-assisted extraction have been reported. UAE is comparatively cheap, simple and needs lesser instrumental requirements than other extraction techniques. The factors affecting the quality extraction of bioactive compounds are extraction time, temperature, solvent concentration, frequency, and power of ultrasound (Feng et al. 2015). Furthermore, response surface methodology (RSM) is a very suitable statistical approach for the optimization of different processing conditions (Zhu & Liu 2013). Macroporous adsorption resins are frequently used for separation and purification of medicinal and natural products. These resins are handy, cheap, and convenient. Moreover, these are eco-friendly and have good chemical consistency (Buran et al. 2014). Previously, various natural extracts containing functional compounds have been purified by macroporous adsorption resins such as phenolics and rosmarinic acid (Lin et al. 2012), red pigments (Zhang et al. 2011), and lycopene (Liu et al. 2010). Therefore, in the present study, the optimization for the extraction of polyphenols from apple peel by UAE was investigated. Then, the purification and identification of apple peel polyphenols were carried out. Finally, the immunostimulatory activity in vitro of apple peel polyphenols was evaluated.

MATERIALS AND METHODS

CHEMICALS AND RAW264.7 CELL

Folin-Ciocalteau reagent was procured from Kayon Biological Technology Co. Ltd. (Shanghai, China). Gallic acid (GA) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Resins of AB-8, D-101, S-8, HP-20, and NKA-II were procured from Hefei Sanxing Resin Technology Co. Ltd. (Anhui, China), and polyamide was the product of Wuxi Linjiang Resin Technology Co. Ltd. (Jiangsu, China). RAW264.7 murine macrophage cell line was purchased from Nanjing University of Chinese Medicine (Nanjing, China).

PREPARATION OF APPLE PEEL POLYPHENOLS

Fresh apple fruits (cv. Fuji) were obtained from local market in Nanjing, China. After washing, the peel was removed manually by stainless steel knife and immediately frozen at -18 °C overnight. The frozen peel was then freeze-dried at -40 °C (LyoQuest-55, TelStar, Spain) for 48 h and grounded into powder by using a kitchen-type grinder (MJ-M176P, Panasonic, Japan). Polyphenols were extracted with ethanol solvent using ultrasonic bath (KQ5200DE, Kunshan Co. Jiangsu, China) with fixed frequency at 40 kHz, 150W power and 60% amplitude level. In all experimental runs, 2 g sample of apple peel powder was mixed with 50 mL of ethanol solvent in 250 mL flask and the extractions were completed with different extraction time and temperature (Table 1). Filtration of the extract with filter paper under vacuum was done and ethanol solvent was removed under reduced pressure and temperature of 45±5 °C using a rotary vacuum evaporator (EYELA DSB-2100, Tokyo, Japan).

| Experiment | Independent variables | Investigated response |
|------------|-----------------------|-----------------------|
|            | Extraction time $X_1$ (min) | Temperature $X_2$ (°C) | Ethanol conc. $X_3$ (%) | Total phenols (mg GAE/g) |
| 1          | 25                    | 20                    | 40                | 32.91            |
| 2          | 45                    | 20                    | 40                | 31.81            |
| 3          | 25                    | 40                    | 40                | 31.52            |
| 4          | 45                    | 40                    | 40                | 28.82            |
| 5          | 25                    | 20                    | 80                | 30.98            |
| 6          | 45                    | 20                    | 80                | 28.62            |
| 7          | 25                    | 40                    | 80                | 30.39            |
| 8          | 45                    | 40                    | 80                | 26.32            |
| 9          | 18                    | 30                    | 60                | 32.36            |
| 10         | 52                    | 30                    | 60                | 27.86            |
| 11         | 35                    | 13                    | 60                | 32.83            |
| 12         | 35                    | 47                    | 60                | 29.71            |
| 13         | 35                    | 30                    | 26                | 31.23            |
| 14         | 35                    | 30                    | 94                | 29.35            |
| 15         | 35                    | 30                    | 60                | 35.62            |
| 16         | 35                    | 30                    | 60                | 34.2             |
| 17         | 35                    | 30                    | 60                | 33.88            |
DESIGN OF OPTIMIZATION EXPERIMENTS

In this study, RSM was used for the determination of optimum values of independent variables for extraction of polyphenols. A Box-Behnken design (BBD) based on seventeen randomized runs with three replicates at the central point was used. A second-order polynomial model with multiple regression equation was used to investigate the experimental data. The equation used for this model is given as follows:

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

(1)

where \( Y \) is measured response; \( \beta_0 \) shows the intercept; \( n \) belongs to the number of factors analyzed; \( \beta_i, \beta_{ii}, \) and \( \beta_{ij} \) representing the linear (main effect); quadratic and cross product model coefficients, respectively, whereas, \( X_i \) and \( X_j \) are the levels of the independent parameters.

DETERMINATION OF TOTAL PHENOLIC CONTENT

The content of total polyphenols was determined by the Folin-Ciocalteu method described by Jabbar et al. (2015) using GA as a standard. Absorbance (Abs) was measured at 750 nm using a spectrophotometer (UV-6300, Mapada, China). Total polyphenols content was expressed as mg of GA equivalent (GAE) on dry weigh of apple peel powder (mg GAE/g DW).

PURIFICATION OF POLYPHENOLS BY MACROPOROUS ADSORPTION RESIN STATIC ADSORPTION AND STATIC DESORPTION TESTS

Resins of AB-8, D-101, HP-20, S-8, NKA-II, and polyamide were treated with adequate quantity of distilled water. All six resins were pre-weighed and washed with ethanol and carefully replaced with deionized water before the adsorption experiment (Zhang et al. 2011). Resins (1.0 g) were placed into a 250 mL conical flask for static adsorption test then each 25 mL crude apple peel polyphenols extract was introduced into each flask and shaken using an orbital shaker (SLK-O3000-S, Scilogex, China) at speed of 100 rpm for 24 h. Sample was taken at different time and the phenolic concentration was measured by the method of Jabbar et al. (2015). After adsorption experiment, filtration and washing of resin was done by distilled water and then desorption was performed with 25 mL of 80% ethanol solution in the 250 mL air-tight conical flask with shaking at speed of 100 rpm for 24 h at room temperature followed by phenolic concentration determination. Adsorption capacity was determined by the following equations (Lin et al. 2012; Liu et al. 2010):

\[ Ac (mg/g) = (Co - C1) \times V / M \]  

(2)

Adsorption rate was calculated by the following equation:

\[ Ar (\%) = 100\% \times (Co - C1) / Co \]  

(3)

Desorption rate was calculated by the following equation:

\[ Dr (\%) = 100\% \times Cd x Vd / ((Co - C1) \times V) \]  

(4)

where \( Ac \) represents the adsorption capacity (mg/g); \( Co \) represents the initial concentration of polyphenols (mg/mL); \( C_1 \) represents the concentration of polyphenols at equilibrium (mg/mL); \( M \) denotes as weight of hydrated resin; \( V \) is the polyphenols volume (mL); \( Ar \) is rate of adsorption (%); \( Dr \) is the rate of desorption (%), \( Cd \) is concentration of polyphenols in desorption solution (mg/mL); and \( Vd \) is desorption solution volume (mL).

DYNAMIC ADSORPTION AND DESORPTION EXPERIMENTS

A glass column (16 × 600 mm) wet-packed with D101 resin and the bed volume (BV) of 120 mL were utilized for dynamic adsorption and desorption experiments. For the investigation of relationship between adsorption ratio and flow rate, total polyphenols solution with different concentrations were flowed through glass column. Elution by different concentration of ethanol solution at different flow rates was performed at adsorption equilibrium, to investigate the relationship between desorption ratio and flow rate. The content of total polyphenols was determined as described by Jabbar et al. (2015).

IDENTIFICATION OF APPLE PEEL POLYPHENOLS BY HIGH-RESOLUTION MASS SPECTROMETRY

The samples were analyzed by liquid chromatography-mass spectrometry (LC-MS) system (G2-XS QToF, Waters). Sample of 2 μL solution was injected into the UPLC column (2.1 × 100 mm ACQUITY UPLC BEH C18 column, 1.7 μm particle) with a flow rate of 0.4 mL/min. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The gradient was 5% mobile phase B for 2 min, 5-40% mobile phase B for 12 min, 40-95% mobile phase B for 2 min, 95% mobile phase B for 2 min. MS was performed using electrospray source in positive ion mode with MS² acquisition mode, with a selected mass range of 50-1200 m/z. The lock mass option was enabled using leucine-enkephalin (m/z 556.2771) for recalibration. The ionisation parameters were as follow: Capillary voltage 2.5 kV, collision energy 40 eV, source temperature 120 °C, and desolvation gas temperature 400 °C. Data acquisition and processing were performed using Masslynx 4.1.
ASSAY OF IMMUNOSTIMULATING ACTIVITY in vitro CELL CULTURE

The RAW264.7 cells cryopreserved in liquid nitrogen were rapidly thawed in a water bath at 37 °C, transferred to sterile tissue culture dishes, suspended in DMEM added with 10% newborn calf serum, 100 IU/mL penicillin and 100 IU/mL streptomycin, and incubated in a humidified 5% CO₂ incubator at 37 °C. When the cells were grown over 80% of the bottom of the culture dishes, the adherent cells were used for further culture.

ASSAY OF CELL VIABILITY

The viability of RAW264.7 cells was measured by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT)-based colorimetric assay (Yuan et al. 2015). A 100 μL/well of RAW264.7 cell suspension was added in a 96-well culture plate and incubated (37 °C, 5% CO₂) for 12 h. The adherent RAW264.7 cells were washed twice by phosphate buffered saline (PBS) and then incubated with medium containing various concentrations of sample (50, 100, 200, 400, and 800 μg/mL) as well as complete medium alone (blank control) or LPS (10 μg/mL, positive control) for 48 h. The stimulated cells were washed twice by PBS, and 200 μL of MTT solution (0.5 mg/mL) was added to each well. After further incubation for 4 h, 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was determined by using a microplate reader. The cell viability was calculated by using the following equation:

\[
\text{Cell viability} = \frac{Abs_{\text{sample}}}{Abs_{\text{control}}} \tag{5}
\]

ASSAY OF PHAGOCYTOSIS

The influence of apple peel polyphenols on phagocytic activity was investigated by using the neutral red phagocytosis assay. As described above, RAW264.7 cells suspension was added in 96-well plates with medium alone or different concentrations of sample or LPS solution and incubated for 48 h at 37 °C. After removal of supernatant, cells were washed with PBS twice to remove non-adherent cells and in each well of neutral red solution (100 μL) was added. After incubation for 1 h, supernatant was removed. Cells were washed with PBS twice to remove the excess neutral red solution and cell lysate (1.0 M acetic acid/ethanol = 1:1, v/v) (100 μL) was added to each well and incubated overnight at room temperature. Each well was measured by a microplate reader at Abs of 540 nm. The phagocytosis index of macrophages was calculated by using the following equation:

\[
\text{Phagocytosis index} = \frac{Abs_{\text{sample}}}{Abs_{\text{control}}} \tag{6}
\]

ASSAY OF ACID PHOSPHATASE ACTIVITY

The effect of apple peel polyphenols on acid phosphatase activity of RAW264.7 cells was determined according to the reported method (Wang et al. 2013). The cultured RAW264.7 cells were treated with complete medium alone or apple peel polyphenols solution (50, 100, 200, 400, and 800 μg/mL) or LPS (10 μg/mL). After incubation for 48 h at 37 °C, the supernatant was aspirated from the wells, 25 μg of 1% Triton X-100 and 150 μL of 1.0 mg/mL p-nitrophenyl phosphate (substrate of acid phosphatase) were added to each well, and the plate was incubated at 37 °C for 1 h. A 50 μL of 3.0 M NaOH solution was added to terminate the reaction and the Abs of the culture at 405 nm was measured by spectrophotometer. The index of acid phosphatase activity was calculated by using the following equation:

\[
\text{Index of acid phosphatase activity} = \frac{Abs_{\text{sample}}}{Abs_{\text{control}}} \tag{7}
\]

STATISTICAL ANALYSIS

The Design-Expert software (version 8.0.6) was used for the experimental design and data analysis of RSM. The data were statistically analyzed by one-way analysis of variance (ANOVA) procedure with SPSS software, version 19.0, (Chicago, IL, USA), followed by the Duncan test.

RESULTS AND DISCUSSION

OPTIMIZATION OF EXTRACTION OF POLYPHENOLS BY RSM FITTING OF RESPONSE SURFACE MODEL

BB design was adopted to study the interaction between three independent variables. The individual parameters and their relevant response outcomes obtained in different experimental runs are listed in Table 1. The extraction yield of polyphenolic compounds ranged from 26.32 to 35.62 mg/g DW. Considering only the significant factors, the obtained model which showed the correlations between the total polyphenols and the extraction variables is given below:

\[
Y = 5.88-0.12X_1-0.083X_2-0.080X_3-0.14X_1^2-0.11X_2^2-0.14X_3^2 \tag{8}
\]

The F-value of 27.99 obtained by ANOVA model showed that the model was highly significant (p < 0.0001). A good correlation between the observed and predicted values is shown when the R² value is near to one (Gangadharan et al. 2008). The (R²) value was 0.9730, suggesting that for phenolic compounds extraction rate 97.3% of results could be explained by regression model. The significance of each factor on extraction rate of phenolic compounds could be explained by analysis of p-values of model. As listed in Table 2, the extraction
rate of phenolic compounds was significantly affected \((p < 0.05)\) by independent variables \((X_1, X_2, \text{and } X_3)\) and quadratic terms of \(X_1^2, X_2^2\) and \(X_3^2\). The results showed that extraction time was the most significant single factor for the polyphenols extraction rate which is followed by temperature of extraction and ethanol concentration.

### TABLE 2. ANOVA results of experimental model

| Source | Sum of squares | df | Mean square | F value | P-value |
|--------|----------------|----|-------------|---------|---------|
| Model  | 0.76           | 9  | 0.085       | 27.99   | 0.0001*** |
| \(X_1\) | 0.19           | 1  | 0.19        | 64.53   | <0.0001*** |
| \(X_2\) | 0.095          | 1  | 0.095       | 31.30   | 0.0008*** |
| \(X_3\) | 0.087          | 1  | 0.087       | 28.71   | 0.0011**  |
| \(X_1X_2\) | 0.012         | 1  | 0.012       | 4.12    | 0.0820   |
| \(X_1X_3\) | 0.0081         | 1  | 0.0081      | 2.72    | 0.1434   |
| \(X_2X_3\) | 0.0018         | 1  | 0.0018      | 0.61    | 0.4605   |
| \(X_1^2\) | 0.23           | 1  | 0.23        | 76.39   | <0.0001*** |
| \(X_2^2\) | 0.12           | 1  | 0.12        | 41.38   | 0.0004*** |
| \(X_3^2\) | 0.21           | 1  | 0.21        | 69.19   | <0.0001*** |
| Residual | 0.021          | 7  | 0.003       |         |         |
| Cor total | 0.78          | 16 |             |         |         |

\(R^2 = 0.9730, \text{Adj } R^2 = 0.9382, **p < 0.01, ***p < 0.001\)

**EFFECT OF INDEPENDENT VARIABLES ON POLYPHENOLS EXTRACTION**

Polyphenols in obtained apple peel extracts varied from 26.32 to 35.62 mg GAE/g (Table 1). Following conditions (35 min, 30 °C and 60% ethanol concentration) for extraction gave the maximum yield of total polyphenols. Three-dimensional response surfaces were plotted for the results of polyphenols extraction (Figure 1), showing the interaction between all the significant combined factors. It is evident from Figure 1(A) that by keeping the ethanol concentration at a fix rate of 60%, both time and temperature exhibited slightly negative effect on the yield of polyphenols. It means that by increasing time and temperature the yield of polyphenols also increased to a certain level then it decreased. Cujic et al. (2016) also reported similar findings with dried chokeberry. This could be explained by the fact that polyphenols are present in the outer region and, therefore, more readily accessible to the solvent (Da-Porto et al. 2013). Also, higher extraction time provided longer contact of solids with solvent and thus improved the diffusion of polyphenols (Odabas & Ilkay 2016). The increasing trend of the yield during UAE is because of the mechanical effect resulted in breakdown of cavitation bubbles which causes a disturbance of plant cell walls at solid matrix surface. Resultantly, mass transfer was increased which further expanded the contact between matrix and material. Ghafoor et al. (2009) also reported similar findings for grape seeds. On the other hand, increase in temperature resulted in higher extraction of polyphenols and thus increased the solubility of analytes in the solvent. Similar results are reported by Ramic et al. (2015) who extracted polyphenols and anthocyanins from A. melanocarpa by-products. Figure 1(B) shows the similar trend that increase in ethanol concentration and time above a certain level decreased the yield of polyphenols by keeping the temperature fix at 30 °C. Slightly negative significant effects showed that the extraction yield of polyphenols increased with increase in all the independent variables tested, but after a certain point it tended to decrease by further increasing the independent variables. This behavior could be explained in a way that an increase in time of ultrasonic extraction might induce the degradation of polyphenol by exerting more mechanical effects on the matrix. Furthermore, increasing temperature also has degrading effect on the extraction of polyphenols (Dranca & Oroian 2016). Similarly, a high ethanol concentration above a certain point changed its polarity and resulted in decreased extraction of polyphenols. Figure 1(C) shows the combined influence of solvent concentration and temperature on the observed response of polyphenols.
The basic objective of this study was to enhance the yield of polyphenols with more efficient way while keeping the extraction process more economical with energy conservation (Gan & Latiff 2011). As results, the extraction process was optimized with following conditions: extraction time of 32 min, extraction temperature of 29 °C and ethanol concentration of 56%. For the purpose of validation of the model, a control experiment was performed under the optimized conditions. A mean value of 35.08 ± 0.26 mg GAE/g DW showed the compliance

FIGURE 1. Response surface plots showing combined effects of independent variables (extraction time, temperature, and ethanol concentration) on the extraction of polyphenols from apple peel. (A) Extraction temperature and time, (B) extraction time and ethanol concentration, (C) extraction temperature and ethanol concentration.
of the results with the predicted values and showed a good correlation of the RSM model. It can be seen from the results that optimized results are obtained at the slightly lower side from the central point.

SCREENING OF MACROPOROUS ADSORPTION RESIN BY STATIC ADSORPTION/DESORPTION METHOD

Chemical features and physical properties of resins play an important role for the determination of adsorption characteristics of macroporous adsorption resin. As shown in Figure 2(A), the adsorption capacities of apple peel polyphenols on HP-20, polyamide, AB-8, and NKA-II resins were significantly more than those of other resins \( (p < 0.05) \). But, the desorption ratio of these resins was low due to decreased desorption capacities of these resins. The reason behind this fact could be the polarity of resins through surface electrical property and hydrogen bonding interactions which resulted in strong affinity towards polyphenols. In contrast to the adsorption capacities, non-polar D101 resin possessed the highest desorption capacities and desorption ratio towards polyphenols than other five tested resins. This fact could be explained by the difference in surface area and pore diameter of D101 resin. Similar finding of polyphenols purification through D101 is reported by Yi et al. (2016).

DYNAMIC ADSORPTION AND DESORPTION OF APPLE PEEL POLYPHENOLS EFFECT OF INITIAL CONCENTRATION OF POLYPHENOLS

Figure 2(B) shows the interaction between initial concentration of polyphenols and dynamic adsorption. It is evident from the Figure 2(B) that adsorption capacity was highest when the polyphenols initial concentration was 2.14 mg/mL. A gradual increase in adsorption capacity was observed with the increasing concentration of polyphenols due to the availability of more and more active spots relative to polyphenols. This phenomenon is in agreement with that reported by Feng et al. (2015). However, the adsorption capacity decreased above a certain level of increasing concentration of polyphenols. This is because impurities were also adsorbed on D101 and as a result active sites were occupied by the impurities, so resulted in minor decrease in adsorption capacity.

EFFECT OF FLOW RATE

Figure 2(C) shows the interaction of desorption capacity with flow rate. When the flow speed was lower than 1 BV/h the adsorption capacity was high. But when the speed of flow was more than 1 BV/h, the adsorption capacity was decreased. This fact could be explained that at high speed some of the polyphenols were not absorbed by the resin and leaked (Xi et al. 2015). Production efficiency was very low at flow speeds of 0.5 and 1.0 BV/h and did not show any significant differences, therefore, 1.0 BV/h was chosen as optimum flow speed. On the other hand, when the speed of elution was lower than 1.5 BV/h, the rate of desorption was higher than 90% (Figure 2(D)). Similar observations are reported in a previous study of purification of tea polyphenols (Liu et al. 2014). However, when the elution speed was higher than 1.5 BV/h, the desorption ratio was decreased. This showed that at low elution speed a complete desorption of polyphenols was occurred. The possible reason could be that ethanol might be entered the micro pores of the resin and the polyphenols at low elution speed get dissolved and eluted more meticulously. Optimum elution speed was 1.0 BV/h because there was no significant difference in desorption ratios at elution speeds of 0.5, 1.0 and 1.5 BV/h.
As shown in Figure 2(E), as the concentration of ethanol was increased from 20% (v/v) to 100%, the ratio of desorption also increased. At ethanol concentration of 80% (v/v), the desorption ratio of 90.8% was shown. There was no significant effect on the desorption ratio when the concentration of ethanol increased continuously, and similar result was reported by Yin et al. (2010). Polyphenols cannot be dissolved in lowest concentration of ethanol. However, at highest ethanol concentration some impurities could be desorbed along with the polyphenols. Therefore, the optimum concentration of ethanol for desorption experiment was 80% (v/v).

Figure 3 shows the phenolic compounds identified by HPLC-MS along with their retention time and the MS/MS fragment ions used for identification. Four phenolic compounds viz. procyanidin B, catechin, quercetin 3-galactoside and quercetin xyloside, mainly flavan-3-ols and flavonoid derivatives in apple peel, were identified by comparing the [M-H] ion, fragment ions and the wavelengths of maximum absorption with those reported in the literature. It can be observed from the Figure 3 that the molecular mass of the peaks obtained during the mass spectrum are equal to the reported values (Giomaro et al. 2014; Grigoras et al. 2013).
FIGURE 3. Mass spectrum of identified polyphenols in apple peel through HPLC-MS/MS
IMMUNOSTIMULATING ACTIVITY OF APPLE PEEL POLYPHENOLS

It has been reported in several studies of in vitro and in vivo that polyphenols, such as flavonoids, possess antioxidant as well as immunomodulatory activities (Benavente-Garcia et al. 2008; Salman et al. 2008). In the present study, therefore, some parameters were detected to evaluate the immunostimulating activity of apple peel polyphenols by using RAW264.7 cells.

EFFECT OF APPLE PEEL POLYPHENOLS ON RAW264.7 CELL VIABILITY

Prior to evaluating the immunostimulating activity of apple peel polyphenols, the effect of apple peel polyphenols on cell viability was investigated to ensure noncytotoxic to RAW264.7 cells using MTT assay. After treating with various concentrations of apple peel polyphenols (50, 100, 200, 400, and 800 μg/mL), it was found that all concentrations of apple peel polyphenols were non-cytotoxic to RAW264.7 and the cell viability increased in dose dependent manner from 50 to 400 μg/mL (Figure 4(A)). Macrophages play an important role in host defense as part of the nonspecific defense (innate immunity) as well as specific defense (adaptive immunity). Macrophages not only maintain homeostasis but also provide a defense against pathogens and invading cells, such as cancer cells (Gamal-Eldeen et al. 2007). Similar activity of purified polyphenols of pinecones has been reported by Yi et al. (2017).

EFFECT OF APPLE PEEL POLYPHENOLS ON PHAGOCYTIC ACTIVITY OF RAW264.7 CELL

The phagocytic activity, one of the most important parameters of macrophage, represents the first and imperative defense in immune response (Bai et al. 2012). Therefore, the effect of apple peel polyphenols on phagocytic activity of RAW264.7 cell was investigated by the neutral red phagocytosis assay and the results are presented in Figure 4(B). The phagocytosis indices of different concentrations of apple peel polyphenols on devouring neutral red of peritoneal macrophages were all more than 1.0. The phagocytosis index for treatment at 50 or 800 μg/mL of apple peel polyphenols was significantly increased compared to that of blank group \((p < 0.01)\).

EFFECT OF APPLE PEEL POLYPHENOLS ON ACID PHOSPHATASE ACTIVITY

As a marker enzyme of lysosome, acid phosphatase is a signal enzyme for macrophage activation (Ke et al. 2013). Significant change in the acid phosphatase activity is associated with the activation or inhibition of macrophages (Shin et al. 2003). The immunocompetence of macrophages can, therefore, be represented by the activity of acid phosphatase. In this study, the effect of apple peel polyphenols on acid phosphatase activity of RAW264.7 cells is depicted in Figure 4(C). The acid phosphatase activity increased with the increase of concentration of apple peel polyphenols from 50 to 400 μg/mL, whereas it tended to decline at a concentration of 800 μg/mL.
CONCLUSION
In this study, UAE was successfully applied for the extraction of polyphenols from apple peel and BBD was used to optimize the extraction variables. As results, the optimal conditions determined were as follows: extraction time 32 min, extraction temperature 29 °C and ethanol concentration 56%. Under these optimal conditions, the maximum extraction yield of 35.08 ± 0.26 mg GAE/g DW was achieved. Six resins were examined for the purification of apple peel polyphenols with adsorption/desorption capacities, and it was found that D101 resin had good adsorption and desorption ratios for the purification of apple peel polyphenols. Dynamic absorption was optimized for initial concentration of 2.14 mg/mL, feeding speed 1 BV, elution speed 1.5 BV and 80% ethanol for desorption. In addition, catechin, procyanidin B, quercetin-3-galactoside, and quercetin xyloside were identified as main phenolics compounds for apple peel by LC-MS. Finally, in vitro immunostimulatory activity of apple peel polyphenols on RAW264.7 cell lines was evaluated. The results showed that apple peel polyphenols exhibited strong propagation on the proliferation of RAW264.7 in a dose-dependent manner. This result also indicated that apple peel polyphenols possessed good immunostimulatory activity towards RAW264.7 cell, suggesting apple peel a valuable source of polyphenols.

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