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# Extraction optimization and antioxidant activity of *Phyllanthus urinaria* polysaccharides

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**Abstract**

Three extraction parameters including extraction time, material to solvent ratio, and extraction temperature for the extraction yield of *Phyllanthus urinaria* polysaccharides were optimized by response surface methodology. The results revealed that the optimal extraction process was extraction time of 5.3 h, extraction temperature of 93 °C, and material to solvent ratio of 1:47 g/mL, under which the polysaccharide yield was 6.40%; The three parameters had significant effect on the polysaccharide yield, and the influence of extraction time was the greatest and that of extraction temperature was the lowest. The antioxidant activity test indicated that the equivalent scavenging ability of *Phyllanthus urinaria* polysaccharides on DPPH and ABTS free radicals was 0.88 and 0.84 μg TBHQ/μg of polysaccharides, respectively; while the reducing power and total antioxidant capacity of *Phyllanthus urinaria* polysaccharides was equivalent to 93.62% and 93.55% of TBHQ, respectively. The *Phyllanthus urinaria* polysaccharides had good antioxidant activity.

**Keywords:** *Phyllanthus urinaria*; polysaccharides; antioxidant activity; response surface methodology.

**Practical Application:** The optimal extraction conditions for *Phyllanthus urinaria* polysaccharides are desirable and practical, and the extracted polysaccharides can be used as a promising nutraceutical and food ingredient.

## 1 Introduction

Active oxygen free radicals are intermediate metabolites produced in life activities (Tahmouzi & Ghodsi, 2014). Excessive reactive oxygen free radicals can cause damage to macromolecular substances in organisms, induce various diseases, accelerate the aging of body, and thus seriously affect human health (Al-Reza et al., 2016; Han et al., 2019; Mo et al., 2017; Ru et al., 2019). In order to reduce the damage of active oxygen radicals, antioxidants are commonly used. At present, there are two main types of antioxidants, one is synthetic and the other is natural. Synthetic antioxidants have limited therapeutic effects due to their significant toxicity, so the search for safe and effective natural antioxidants from natural resources is receiving more and more attention (Carocho et al., 2018). Many studies have shown that the natural polysaccharides isolated from plants have a good antioxidant activity (Chen et al., 2019a; Zhao et al., 2019), which can effectively scavenge free radicals in organisms and have a good health care effect (Chen et al., 2014). Therefore, it is of great significance to develop them into a new type of natural antioxidant.

*Phyllanthus urinaria* is an annual herbaceous plant of the genus Euphorbiaceae, which is widely distributed in tropical and subtropical countries (Liu et al., 2018, 2019). Historically, *Phyllanthus urinaria* is used as a folk medicine for its good biological activities such as antiviral, antioxidant, anti-inflammatory, and antimicrobial activities (Hau et al., 2009; Lin et al., 2008). *Phyllanthus urinaria* contains a variety of active ingredients, mainly including lignans, tannins, flavonoids, phenolics, and terpenoids (Geethangili & Ding, 2018; Mediani et al., 2015). At present, the research of *Phyllanthus urinaria* is mainly focused on the extraction of bioactive substances (Liu et al., 2018), the microencapsulation of bioactive substances (Lam et al., 2013; Liu et al., 2019), and the study of antiviral, antitumor, hepatoprotective, antidiabetic, antioxidative, antihypertensive, thrombolytic and antimicrobial effects of phenolic compounds (Du et al., 2018; Geethangili & Ding, 2018). In recent years, functional polysaccharides have become a research hotspot due to their good biological activity (Jiang et al., 2019). *Phyllanthus urinaria* is rich in polysaccharides, however, little is known about the extraction optimization and antioxidant activity of *Phyllanthus urinaria* polysaccharides.

In this paper, three parameters for the extraction of *Phyllanthus urinaria* polysaccharides were optimized for the highest polysaccharide yield by response surface methodology (RSM), and its antioxidant activity was also evaluated. The aim was to provide a theoretical basis for the extraction of *Phyllanthus urinaria* polysaccharides and its development and utilization in the field of food and medicine.

## 2 Experimental

### *2.1 Chemicals*

Ferric chloride, glucose, phenol, DPPH, tert-butylhydroquinone (TBHQ), trichloroacetic acid, ABTS, ammonium molybdate, sodium phosphate, and potassium ferricyanide were bought from Shanghai Macklin Co. Ltd.

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### *2.2 Extraction of Phyllanthus urinaria polysaccharides*

*Phyllanthus urinaria* was dried (50 °C, 48 h), pulverized into powder and passed through a 100-mesh sieve. The resulting powder was subjected to extract *Phyllanthus urinaria* polysaccharides at different extraction time, material to solvent ratio and extraction temperature. After the extraction was completed, the extracted slurry was centrifuged (12000 rpm, 15 min), and the polysaccharide content of the resulting supernatant was measured by the phenol-sulfuric acid method using glucose as a standard (DuBois et al., 1956). The polysaccharide yield of *Phyllanthus urinaria* was determined as following (Equation 1):

$$Yield\ (\%)=\frac{V\times C}{M}\times 100 \quad (1)$$

where $V$ was the supernatant volume (mL); $C$ was the polysaccharide content (mg/mL) obtained from the glucose standard curve; and $M$ was the weight (mg) of powder.

### *2.3 Extraction optimization design*

Based on the evaluation of the effects of extraction parameters on the polysaccharide yield by single-factor experiment, a Box-Behnken design was used to optimize the extraction parameters of extraction time (*A*, 4-6 h), extraction temperature (*B*, 80-100 °C) and material to solvent ratio (*C*, 1:40-1:60 g/mL) on the polysaccharide yield (*Y*, %) by the Design-Expert software. The Box-Behnken design and results were shown in Table 1.

### *2.4 Antioxidant activity test*

The polysaccharide supernatant obtained under the optimal extraction process was concentrated to a third volume at 50 °C and then subjected to remove proteins using Sevag reagent. Then, the polysaccharides were precipitated by adding 4-fold volume of absolute ethanol at 4 °C for 24 h. The precipitates were collected by centrifugation at 5000 rpm for 20 min and then dialyzed against deionized water at 4 °C for 48 h, and followed by lyophilization to achieve the powder of *Phyllanthus urinaria* polysaccharides. The powder was dissolved to prepare various concentrations of polysaccharide solution for the antioxidant activity test using TBHQ as a control. The reducing power (RP), ABTS, and DPPH assays were performed by the method of Liu et al. (2018). The total antioxidant capacity (TOAC) was also measured according to Zhang et al. (2014).

**Table 1**. Box-Behnken design and test results.

| Run | Independent variables | | | *Y* (%) |
|---|---|---|---|---|
| | *A* (h) | *B* (°C) | *C* (g/mL) | |
| 1 | 4 (-1) | 90 (0) | 1:60 (1) | 4.98 |
| 2 | 6 (1) | 90 (0) | 1:40 (-1) | 5.97 |
| 3 | 5 (0) | 80 (-1) | 1:60 (1) | 5.46 |
| 4 | 5 (0) | 90 (0) | 1:50 (0) | 6.38 |
| 5 | 6 (1) | 90 (0) | 1:60 (1) | 5.82 |
| 6 | 5 (0) | 100 (1) | 1:60 (1) | 5.39 |
| 7 | 5 (0) | 100 (1) | 1:40 (-1) | 6.15 |
| 8 | 6 (1) | 100 (1) | 1:50 (0) | 6.01 |
| 9 | 5 (0) | 90 (0) | 1:50 (0) | 6.31 |
| 10 | 5 (0) | 80 (-1) | 1:40 (-1) | 5.49 |
| 11 | 5 (0) | 90 (0) | 1:50 (0) | 6.35 |
| 12 | 4 (-1) | 90 (0) | 1:40 (-1) | 5.46 |
| 13 | 4 (-1) | 100 (1) | 1:50 (0) | 5.49 |
| 14 | 4 (-1) | 80 (-1) | 1:50 (0) | 5.23 |
| 15 | 6 (1) | 80 (-1) | 1:50 (0) | 5.61 |

Notes: extraction time (*A*), extraction temperature (*B*), material to solvent ratio (*C*) and polysaccharide yield (*Y*).

### *2.5 Statistical analysis*

All the test was conducted in triplicate. Design-Expert software was used to design, analyze, and optimize the extraction yield of *Phyllanthus urinaria* polysaccharides. A p-value less than 0.05 was significant difference.

## 3 Results and discussion

### *3.1 Single-factor test analysis*

*Effect of extraction time on polysaccharide yield*

It can be seen from Figure 1A that when the extraction time increased, the polysaccharide yield increased and reached a maximum at 4 h, but decreased after 4h. This is because the increase in extraction time makes it easier and faster to dissolve the polysaccharides into the solvent (Lin et al., 2017), but too long extraction time will result in the destruction of polysaccharide

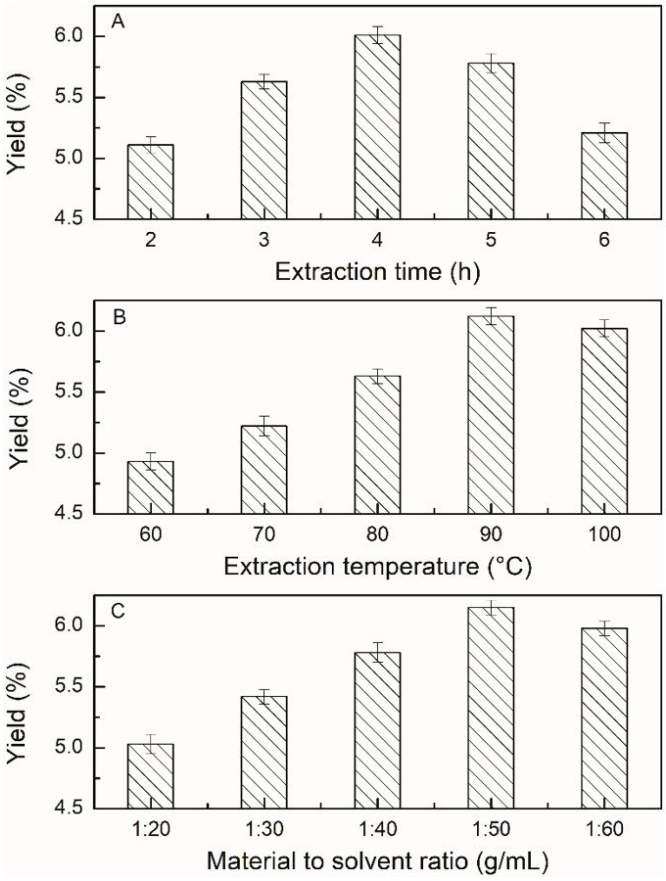


**Figure 1**. Effect of (A) extraction time, (B) extraction temperature and (C) material to solvent ratio on polysaccharide yield.

structure (Chen et al., 2019b; Zheng et al., 2019) and competitive dissolution of other impurities (Nuerxiati et al., 2019), leading in a decrease in yield. This phenomenon was consistent with the extraction of polysaccharides in other literatures (Wang et al., 2018b; Yang et al., 2017). Therefore, the extraction time should be chosen around 4 h.

*Effect of extraction temperature on polysaccharide yield*

As shown in Figure 1B, with the increase of extraction temperature from 60 to 90 °C, the polysaccharide yield increased and reached a maximum at 90 °C, but decreased over 90 °C. As the extraction temperature continues to increase, the diffusion coefficient of polysaccharides increases, and consequently the content of polysaccharides in the aqueous solution increases. However, excessive temperature can cause the hydrolyzation or degradation of polysaccharides (Hu et al., 2018; Li et al., 2019; Lin et al., 2017), resulting in a decrease in polysaccharide yield and an increase in energy consumption. This trend was in correspondence with the reports of polysaccharide extraction by other authors (Xu et al., 2019; Zhang et al., 2017). Therefore, the extraction temperature should be around 90 °C.

*Effect of material to solvent ratio on polysaccharide yield*

As can be observed in Figure 1C, when material to solvent ratio increased in the range of 1:20 to 1:60 g/mL, the polysaccharide yield had a maximum at 1:50 g/mL. When the amount of material to solvent ratio is low, the polysaccharides in the powder cannot be completely transferred to the solvent, resulting in an incomplete extraction and the low polysaccharide yield. Increasing material to solvent ratio can cause a greater concentration difference between the internal plant cells and the external solvent, which can be beneficial for the rapid diffusion of polysaccharides (Ying et al., 2011); however, too large material to solvent ratio can also cause other impurities in the powder to competitively diffuse and dissolve with the polysaccharides, resulting in a decrease in the polysaccharide yield. Therefore, the material to solvent ratio should be around 1:50 g/mL.

### *3.2 Extraction optimization of polysaccharide yield*

According to the single-factor test analysis and the Box-Benhnken design principle, taking extraction time (*A*, h), extraction temperature (*B*, °C) and material to solvent ratio (*C*, g/mL) as independent variables and polysaccharide yield (*Y*, %) as response value, RSM was used to analyze the three parameters and investigate the significance of each parameter on the polysaccharide yield and the optimal combination of various parameters.

*Establishment of fitted model and analysis of variance*

The statistical analysis software (Design-Expert 8.05) was used to perform the fitting analysis of regression model on the data in Table 1. A quadratic response surface regression model was established, and the quadratic multiple regression equation for the polysaccharide yield was obtained as follows (Equation 2):

$$Y = 6.35 + 0.28A + 0.16B - 0.18C + 0.035AB + 0.083AC - 0.18BC - 0.41A^2 - 0.35B^2 - 0.38C^2 \quad (2)$$

In order to verify the feasibility of the regression equation for the extraction process of *Phyllanthus urinaria* polysaccharides, the variance analysis and the significance test were performed on the regression model. The results were presented in Table 2. As shown in Table 2, the p-value of the regression model was less than 0.01, showing the regression model was extremely significant; the p-value of the lack-of-fit was 0.1148 > 0.05, indicating the loss was not significant; the correlation coefficient of the model was $R^2$ = 0.9881, which revealed that 98.81% of the test data can be explained by this regression equation, and can better reflect the change of the polysaccharide yield; the adjustment correlation coefficient of the model was *R*2 adj = 0.9668, which considered that the model was reliable and had high credibility. In summary, the regression model had a good fitting with the test data, and the test error was small. Therefore, the model can be used to predict and analyze the extraction parameters of *Phyllanthus urinaria* polysaccharides.

The results of variance analysis of the regression model in Table 2 revealed that the p-values of extraction time (*A*), extraction temperature (*B*) and material to solvent ratio (*C*), the interaction between *B* and *C*, and the quadratic terms of $A^2$, $B^2$ and $C^2$ were less than 0.01, which indicated that they had an extremely significant effect on the polysaccharide yield. However, the others were all more than 0.05, showing there was no significant influence. It can be concluded from the p-values that the influence of extraction time on the polysaccharide yield was the greatest and that of extraction temperature was the lowest.

*Response surface analysis*

The response surface plots of the regression model were shown in Figure 2. According to the design principle and analysis of the response surface experiment, the greater the curvature of three-dimensional response surface diagrams, the more obvious the difference between the two parameters was, which

**Table 2**. Analysis of variance for Box-Behnken test.

| Source | Sum of squares | Degree of freedom | Mean square | *F*-value | *p*-value |
|---|---|---|---|---|---|
| Model | 2.63 | 9 | 2.63 | 46.32 | 0.0003 |
| *A* | 0.63 | 1 | 0.63 | 100.15 | 0.0002 |
| *B* | 0.20 | 1 | 0.20 | 30.91 | 0.0026 |
| *C* | 0.25 | 1 | 0.25 | 39.89 | 0.0015 |
| *AB* | 0.0049 | 1 | 0.0049 | 0.78 | 0.4188 |
| *AC* | 0.027 | 1 | 0.027 | 4.31 | 0.0926 |
| *BC* | 0.13 | 1 | 0.13 | 21.09 | 0.0059 |
| $A^2$ | 0.63 | 1 | 0.63 | 99.84 | 0.0002 |
| $B^2$ | 0.45 | 1 | 0.45 | 70.91 | 0.0004 |
| $C^2$ | 0.52 | 1 | 0.52 | 82.54 | 0.0003 |
| Residual | 0.032 | 5 | 0.0063 | | |
| Lack of fit | 0.029 | 3 | 0.0097 | 7.87 | 0.1148 |
| Pure error | 0.0025 | 2 | 0.0012 | | |
| Cor. total | 2.67 | 14 | | | |
| $R^2$ = 0.9881 | *R*2 adj = 0.9668 | | | | |

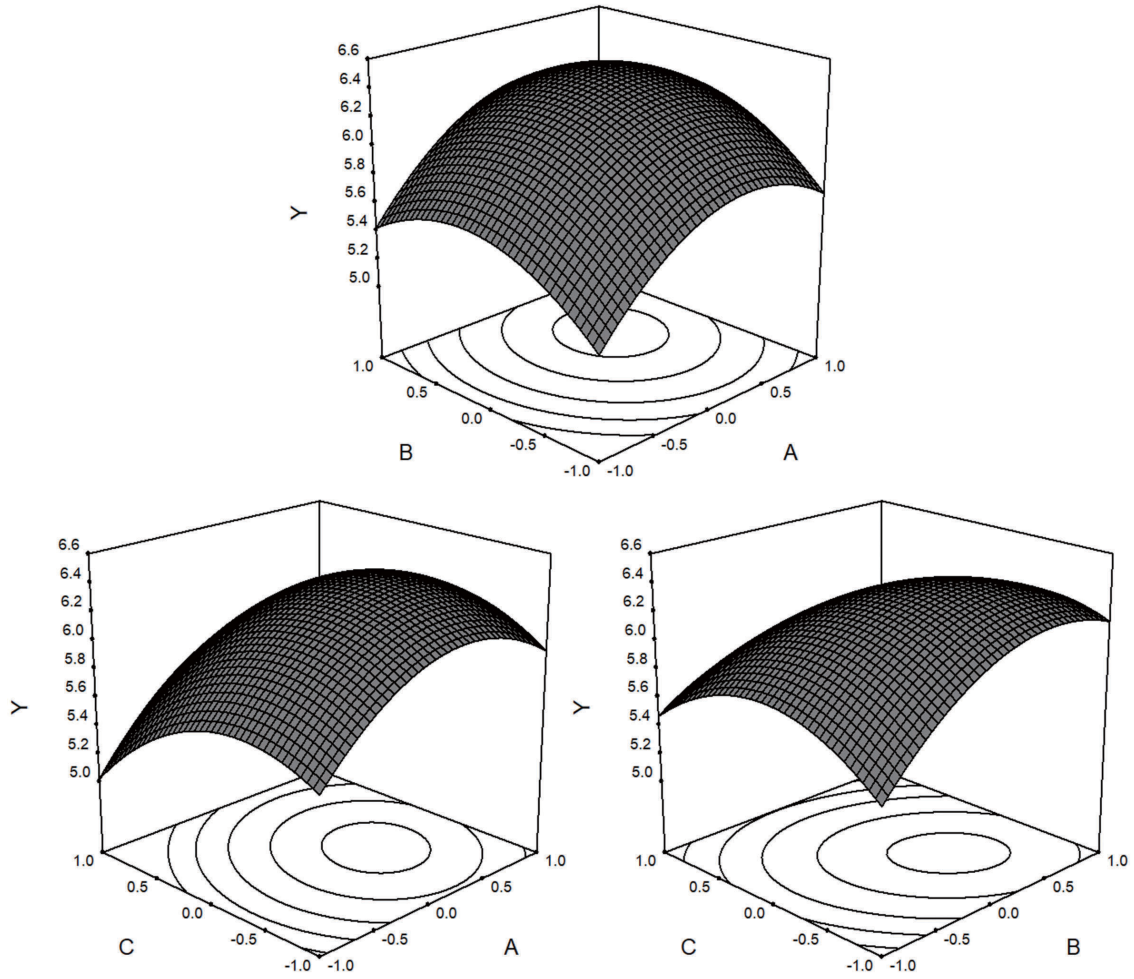


**Figure 2**. Plots of contours and response surfaces for extraction time (*A*), extraction temperature (*B*) and material to solvent ratio (*C*) on polysaccharide yield (*Y*).

can effectively reflect the interaction between the parameters on the polysaccharide yield. It can be observed in Figure 2 that the influence of interaction between extraction temperature (*B*) and material to solvent ratio (*C*) was significant, but that of other interaction was not significant.

*Verification of optimal extraction process*

It can be seen from Figure 2 that the three three-dimensional response surface diagrams had the highest points, and the polysaccharide yield (*Y*) increased first and then decreased with the increase of extraction time (*A*), extraction temperature (*B*) and material to solvent ratio (*C*), indicating that there must be an optimal extraction process for the highest polysaccharide yield. By solving the model equation, the optimal extraction process of *Phyllanthus urinaria* polysaccharides was: extraction time of 5.3 h, extraction temperature of 93 °C and material to solvent ratio of 1:47 g/mL. Under this process, the polysaccharide yield was 6.40%, which was 0.62% relative error with the predicted yield (6.44%). The results indicated that the established model can effectively and reliably explain the impact of various parameters on the polysaccharide yield.

### *3.3 Antioxidant activity analysis*

The ability of *Phyllanthus urinaria* polysaccharides to scavenge free radicals of DPPH and ABTS presented in Figure 3A and 3B showed that the polysaccharides had good scavenging ability to DPPH and ABTS free radicals. As sample concentration increased, the scavenging effect of the polysaccharides increased linearly. The scavenging effect of the polysaccharides on DPPH and ABTS free radicals was slightly lower than that of TBHQ. The $IC_{50}$ of polysaccharides for scavenging DPPH and ABTS free radicals was 6.54 μg/mL and 114.13 μg/mL, respectively; correspondingly, that of TBHQ was 5.78 μg/mL and 95.30 μg/mL, respectively. It can be concluded from their $IC_{50}$ that the equivalent scavenging ability of the polysaccharides on DPPH and ABTS free radicals can be expressed as 0.88 and 0.84 μg TBHQ/μg of polysaccharides, respectively. In comparison with the IC50 of other polysaccharides, the ability of *Phyllanthus urinaria* polysaccharides to scavenge DPPH free radicals was close to that of *Rosa roxburghii Tratt*. Polysaccharides (8.38 μg/mL) (Wang et al., 2018a), but higher than that of Chestnut rose polysaccharides (100 μg/mL) (Chen & Kan, 2018) and *Lentinus edodes* polysaccharides from tobacco waste (560 μg/mL) (Lin et al., 2019); the ability of *Phyllanthus urinaria* polysaccharides to scavenge ABTS free radicals was higher

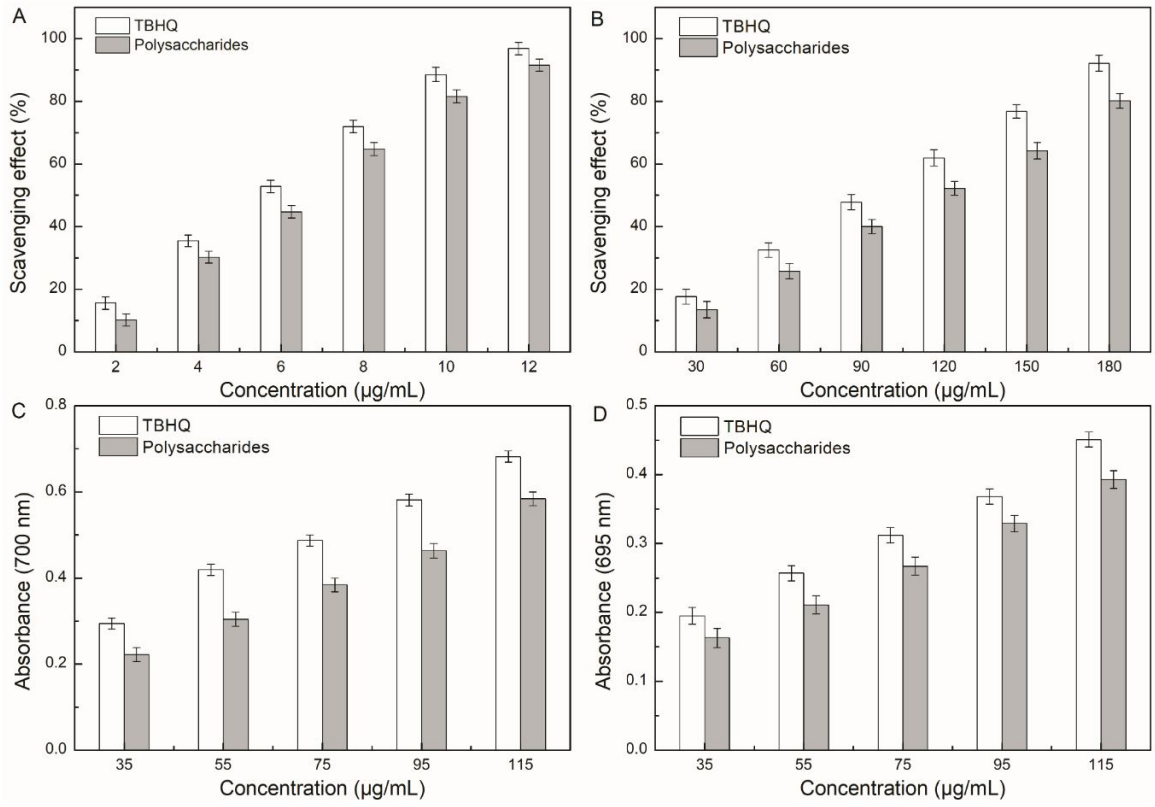


**Figure 3**. Scavenging effect of polysaccharides and TBHQ on DPPH (A) and ABTS free radicals (B); reducing power (C) and total antioxidant capacity (D) of polysaccharides and TBHQ.

than that of *Chimonobambusa quadrangularis* Polysaccharides (452 μg/mL) (Chen et al., 2019a) and Kiwifruit Polysaccharides (1980 μg/mL) (Han et al., 2019), but lower than that of *Pouteria campechiana* seed Polysaccharides (29.9 μg/mL) (Ma et al., 2020).

According to the experimental principle of RP and TAOC, the larger an absorbance was, the greater RP and TAOC were. Figure 3C and 3D showed that the absorbance had a linear increase with increasing sample concentration, and the absorbance of TBHQ was slightly higher than that of polysaccharides. The linear regression equations of the polysaccharides for RP and TAOC were $y = 0.0044x + 0.0603$ ($R^2$=0.9818), $y = 0.0029x + 0.0059$ ($R^2$=0.9882), respectively; correspondingly, those of TBHQ were $y = 0.0047x + 0.1409$ ($R^2$=0.9933), $y = 0.0031x + 0.083$ ($R^2$=0.9915), respectively. According to the slopes of the regression equations, it can be calculated that the RP and TAOC of the polysaccharides were equivalent to 93.62% and 93.55% of TBHQ, respectively. Compared with other polysaccharides, the RP of *Phyllanthus urinaria* polysaccharides (absorption of 0.463 at 95 μg/mL) was higher than that of litchi polysaccharides (absorption of 0.320 at 1000 μg/mL) (Gao et al., 2017) and polysaccharides from tobacco waste (absorption of 0.377 at 1000 μg/mL) (Jing et al., 2016); meanwhile, the TAOC of *Phyllanthus urinaria* polysaccharides (absorption of 0.451 at 115 μg/mL) was higher than that of *Trapa quadrispinosa* polysaccharides (absorption of 0.510 at 400 μg/mL) (Raza et al. 2017).

In general, *Phyllanthus urinaria* polysaccharides possessed good antioxidant activity by the comparison with the control (TBHQ) and other polysaccharides. It was reported that the molecular mass, uronic acid content, and monosaccharide composition of polysaccharides are related to their antioxidant capacity (Wang et al., 2016). So, the antioxidant activity of *Phyllanthus urinaria* polysaccharides was affected not by only one factor but by the combination of multiple factors (Shang et al., 2019).

## 4 Conclusions

The extraction parameters, including extraction time, material to solvent ratio and extraction temperature, for the extraction of *Phyllanthus urinaria* polysaccharides were optimized by RSM. The optimal extraction process was: extraction time of 5.3 h, extraction temperature of 93 °C, and material to solvent ratio of 1:47 g/mL, under which the polysaccharide yield (6.40%) was basically consistent with the predicted yield (6.44%). The antioxidant activity test showed that the polysaccharides had the strong ability to scavenge DPPH and ABTS free radicals, and had good reducing power and total antioxidant capacity.

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