Extraction optimization of polyphenols from fruits of *Pyracantha fortuneana* (Maxim.) Li by ultrasonic assistant method and their antibacterial activity

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Abstract: The present study was designed to utilise ultrasound assistance technology to optimize the extraction conditions of polyphenols, and identify their antibacterial activity from fruits of *Pyracantha fortuneana* (Maxim.) Li, facilitated by the use of orthogonal experiment methodology. A four factors and three levels of orthogonal design was carried out to elucidate the effect of ethanol concentration, solvent-to-solid ratio, ultrasonic temperature and time on the yields of the polyphenols. The results showed that the optimal conditions were as follows: ethanol concentration 70%, solvent-to-solid ratio 70:1, ultrasonic temperature 30°C, and ultrasonic time 40 min, the maximum polyphenol yield was 5.58mg/g under the optimum extraction condition. The extracted hydro-alcohol polyphenols showed the excellent antibacterial potential to both *Staphylococcus aureus* and *Escherichia coli* with the minimum inhibitory concentration (MIC) 10 mg/ml and 20 mg/ml, and the corresponding diameter of inhibition zone (DIZ) was 7.4 and 6.8 mm, respectively. The results indicated the ability of ultrasound assistance technology to obtain polyphenols from fruits. Furthermore, present results highlighted that fruits of *Pyracantha fortuneana* (Maxim.) Li are a potential natural source of bioactive compounds with strong antibacterial activity. These compounds could be consider for potential application in nutraceutical and functional foods ingredient or pharmaceutical applications.

Keywords: *Pyracantha fortuneana* (Maxim.) Li, Polyphenols, Orthogonal experiment, technology, antibacterial activity.

INTRODUCTION

Pyracantha fortuneana (Maxim.) Li is an evergreen shrub or small tree, it belongs to Pyracantha, Rosaceae, locally called Huo-ji, with a wide distribution in Asia and Europe. In China, it is mainly grows in the south and northwest, and usually been used as Traditional Chinese medicine, such as for treatment of dyspepsia (Deng *et al*., 1990), dysentery, and the treatment of sores by applied externally. Additionally, it has been used as hedges and landscaping materials in courtyard. In Japan, it principally been used as a skin-whitening agent in cosmetics (Van Gelder *et al*., 1997). Moreover, recent researches have indicated that *P. fortuneana* (Maxim.) Li has antioxidant activities and immunoprotective effects in mice (Yuan *et al*., 2010), and may remarkably ameliorate rats lipoprotein metabolism (Hou JJ *et al*., 2002; 2003). Usually, the fruits of *P. fortuneana* (Maxim.) Li are also marketed mainly as fresh fruit or as processed beverage.

In recent years, the physiological function of foods including fruits, vegetables, grains and food components, such as phytochemicals which possess beneficial biological properties and pharmacological potential has received much attention (Nayak *et al*., 2015; Shehata *et al*., 2015), phenolic compounds are a major class of phytochemicals found in plants. These compounds have diverse properties, such as antioxidants, anti-inflammatory, anti-allergic, and anti-carcinogenic activity, and these properties also improve quality and value of the food (Liu, 2004).

Polyphenols from the *P. fortuneana* (Maxim.) Li could add value to the medical research and processing industry when the compounds are extracted effectively by applying efficient extraction technologies. Optimization and standardization of extraction parameters for these health benefitting bioactive phytochemicals from fruits of *P. fortuneana* (Maxim.) Li are important to retain their antioxidative properties. Generally, industrial extraction from herbs is mainly being carried out using conventional methods. The "ideal" extraction method must provide high extraction efficiency and should be non-destructive and time saving (Rombaut *et al*., 2014). Recently, more rapid and automated methods including microwave-assisted extraction, enzyme-assisted extraction, and super-critical extraction, and ultrasound-assisted extraction (Krishnaswamy *et al*., 2013; Dahmoune *et al*., 2014). Amongst them, ultrasound-assisted method has been confirmed as a more economic and efficient extraction means (Chen *et al*., 2015, Wong Paz *et al*., 2015; Rodrigues *et al*., 2015). However, to our knowledge, there was no literature reporting on the optimization of phenolic compounds extraction technology from fruits of *P. fortuneana* (Maxim.) Li by ultrasound-assisted procedure. In addition, more and more attention has been paid to orthogonal experiment, due to it is a useful tool for

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estimating the effects of multiple factors and their interactions on one or more response variables.

In this study, extraction of phenolic compounds from the fruits of *P. fortuneana* (Maxim.) Li by ultrasound-assisted was optimized using single factor experiment combined orthogonal methodology. The effect of ultrasonic temperature, ultrasonic time, ethanol concentration in water (% v/v) and solvent-to-solid ratio were taken into account. The total phenolic content in optimized conditions was used to understand the most efficient extraction method. Finally, *in vitro* antibacterial activity of polyphenols from fruits of *P. fortuneana* (Maxim.) Li against *Escherichia coli* and *Staphylococcus aureus* was determined to ascertain their medicinal potentials.

MATERIAL AND METHODS

Plant material

Fresh mature *P. fortuneana* (Maxim.) Li fruits were obtained from the Municipal Market at Zunyi (Zunyi, China). The fruits were washed by hand and air dried at 60 ºC in a conventional air forced oven for 24 h. After drying, the fruits were triturated in a domestic blender, and the powder was sieved in a nylon sieve (1mm mesh), and then kept at room temperature until use.

Extraction process

Extractions were carried out in an 2.7L open rectangular ultrasonic cleaner bath (Unique Model USC, 25 kHz, 150W), with the internal dimensions 14×24×9 cm. The temperature was controlled and maintained at a designed temperature by circulating external water from a thermostatic water bath. Extractions were implemented in glass tubes (D=20 mm; h = 175 mm) using 250 mg of the dried *P. fortuneana* (Maxim.) Li fruits powder and ethanol solution. Ethanol was acidified with HCl. And the ethanol volume, concentration and the extraction time were according to the given in the experimental planning. After the extraction process, the mixture was centrifuged at 4000×g for 10min at 20ºC. The supernatant was designated as total polyphenols and stored at -20°C until the next analysis.

The experiment was carried out according to a orthogonal experimental design, based on single-factor experiments, with four independent variables: ultrasonic temperature, ultrasonic time, ethanol concentration in water (% v/v) and solvent-to-solid ratio.

Single-factor experiment

Single-factor experiment with six-level and four-variable was used for optimisation of extraction variables. The independent variables and their levels were as follows: ethanol concentration 30%, 40%, 50%, 60%, 70% and 80%, solvent-to-solid ratio 20:1, 30:1, 40:1, 50:1, 60:1 and 70:1 (mL/g), ultrasonic temperature 20, 30, 40, 50 , 60 and 70ºC, and of ultrasonic time 20, 30, 40, 50, 60 and 70 min, respectively. The polyphenol content was used as indice for optimization of total polyphenols extraction modification condition.

Orthogonal experimental design

Based on the preliminary range of the ultrasonic extraction variables using the single-factor experiment, a orthogonal experiment with four independent variables (A, ethanol concentration; B, solvent-to-solid ratio; C, ultrasonic temperature; D, ultrasonic time) at three levels was performed (Chen *et al*., 2014). According to orthogonal test as $L_9(3^4)$, nine modification conditions were carried out (table 1). The results of these nine conditions were evaluated using range and variance analysis. *k1*, *k2*, and *k3*, was the average value of level 1, 2, and 3 for each factor, respectively. Optimum conditions were determined by the highest value of k (k_{max}) of various factors. Range (R) was the difference between the maximum and minimum ($k_{max} - k_{min}$) for each factor.

Total phenolic content assay

Total polyphenols contents were measured by the Folin Ciocalteu method according to Terpinc *et al*. (2012) with some modifications. Exactly 0.1 ml extract or the standard gallic acid (10 to 100mg/ml) was measured, then they were mixed with exactly 1ml of 1:3 Folin-Ciocalteu reagent, 2 ml of 20% Na_2CO_3 solution, and 2 ml distilled water, then mixed. The mixture was read at 765 nm with a spectrometer (Shimadzu UV-1800) after 40 min at room temperature. The TPC was expressed as mg gallic acid equivalents (GAE) per g dried extract.

Antibacterial activity determination

The antibacterial activity was determined by agar-well diffusion method according to Sun *et al*. (2014), *Escherichia coli* (*E. coli*, ATCC 25322) and *Staphylococcus aureus* (*S. aureus*, ATCC25922) were used as reference bacteria, which were stored in our laboratory. And the volum of total polyphenols extraction of *P. fortuneana* (Maxim.) Li fruits was 60μL in each well, PBS solution used as negative control. All the experiments were repeated triplicate.

RESULTS

Single factor experiment analysis

Effect of ethanol concentration on the yield of polyphenol

To investigate the influence of different ethanol concentration on the yield of polyphenol when the other reaction conditions were set as follows: solvent-to-solid ratio was 50:1, ultrasonic temperature and time was 40°C and 40 min, respectively. As shown in fig. 1a, the polyphenol yield increased with the concentration of ethanol from 30 to 70%, and then declined from 70% to 80%, reaching a maximum (5.26 mg/g) at 70%. Thus, the ultrasonic power of 60% to 80% was suitable for the following orthogonal experiment.

Table 1: The factors and levels of orthogonal experimental design for the extraction of polyphenol.

Levels	Factors			
	A Ethanol concentration (%)	B Solvent-to-solid ratio	C Ultrasonic temperature (°C)	D ultrasonic time (min)
1	60	50:1	30	30
2	70	60:1	40	40
3	80	70:1	50	50

Table 2: Orthogonal experimental design test results of the polyphenol extraction process.

Experiment No.	A	B	C	D	Polyphenol content (mg/g)
1	1	1	1	1	3.36
2	1	2	2	2	3.41
3	1	3	3	3	4.12
4	2	1	2	3	3.11
5	2	2	3	1	5.57
6	2	3	1	2	5.85
7	3	1	3	2	3.81
8	3	2	1	3	4.42
9	3	3	2	1	4.04
k1	3.63	3.43	4.54	4.32	-
k2	4.84	4.47	3.52	4.36	-
k3	4.09	4.58	4.50	3.88	-
Range (R)	1.21	1.15	1.02	0.48	-
Sequence	A>B>C>D				
Optimal level	A_2	B_3	C_1	D_2	
Optimal combination	$A_2B_3C_1D_2$				

Table 3: Antibacterial activity of the extracted polyphenol in fruits of *P. fortuneana* (Maxim.) Li.

Concentration (mg/mL)	Diameter of inhibition zone (DIZ) (mm)[a]	
	E. coli	*S. aureus*
0	-	-
5	-	-
10	-	7.4±0.07
20	6.8±0.32	9.5±0.24
40	9.7±0.13	+
60	+	+

[a] Average of three replicates± SD; + indicates complete inhibition and – indicates no inhibition

Effect of solvent-to-solid ratio on the yield of polyphenol

As shown in fig. 1b, the yield of polyphenol was affected by solvent-to-solid ratio, when the ethanol concentration, ultrasonic temperature and time was fixed at 60%, 40°C and 40 min, respectively. The polyphenol yield increased rapidly with the ratio from 20:1 to 60:1 and decreased above 60:1. The result implied that the polyphenol yield reached the highest value (4.73 mg/g) when solvent-to-solid ratio was 60:1 (60mL/g). Hence, high solvent-to-solid ratio can reduce the polyphenol yield in turn. Thus, solvent-to-solid ratio ranged from 50:1 to 70:1 was selected for the following orthogonal experiment.

Effect of ultrasonic temperature on the yield of polyphenol

As evident from fig. 2a, the maximum amount of the polyphenol was 5.02 mg/g at 30°C. The extraction yield of polyphenol increased sharply with the temperature increasing from 20°C to 30°C, then decreased slowly when the temperature continued to rise but still higher than 20°C, however, there was no distinct difference with the temperature between 40°C and 70°C. Therefore, the ultrasonic temperature of 30–50°C was selected for the optimal temperature.

Effect of ultrasonic time on the yield of polyphenol

It was observed that as the ultrasonic time was changed from 20min to 70min, the yield increased at first and reached to its maximum (4.83mg/g) at 40min, then decreased (fig. 2b), which indicated that extension in the ultrasonic time may result in instability, oxidation and degradation of polyphenols. Consequently, the ultrasonic time of 30min to 50min was considered for further orthogonal experiment.

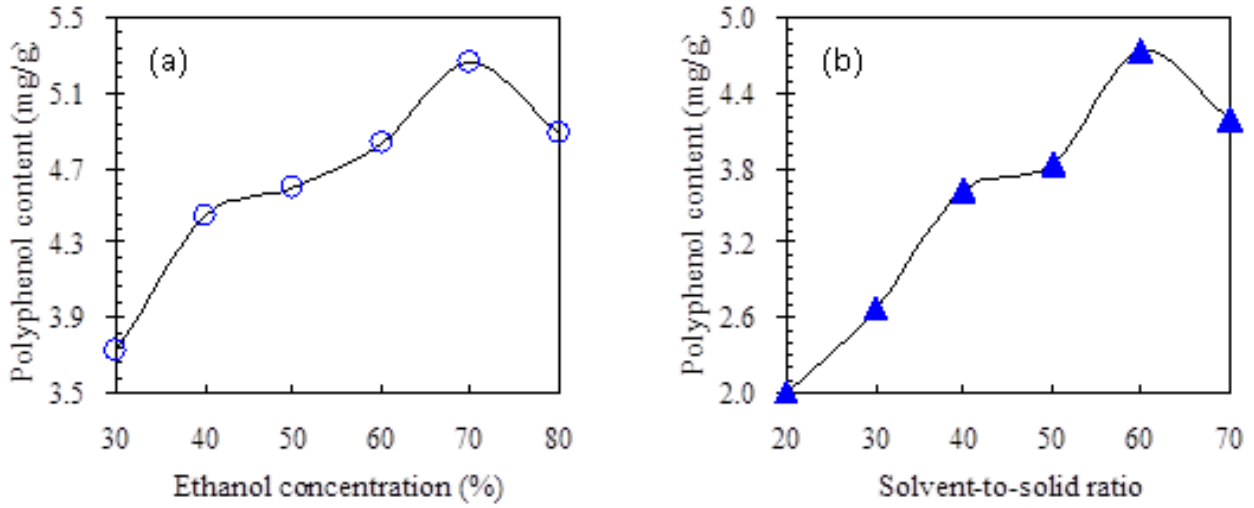

Fig. 1: Effect of ethanol concentration in water (% v/v) (a) and solvent-to-solid ratio (b) on polyphenol content in fruits of *P. fortuneana* (Maxim.) Li.

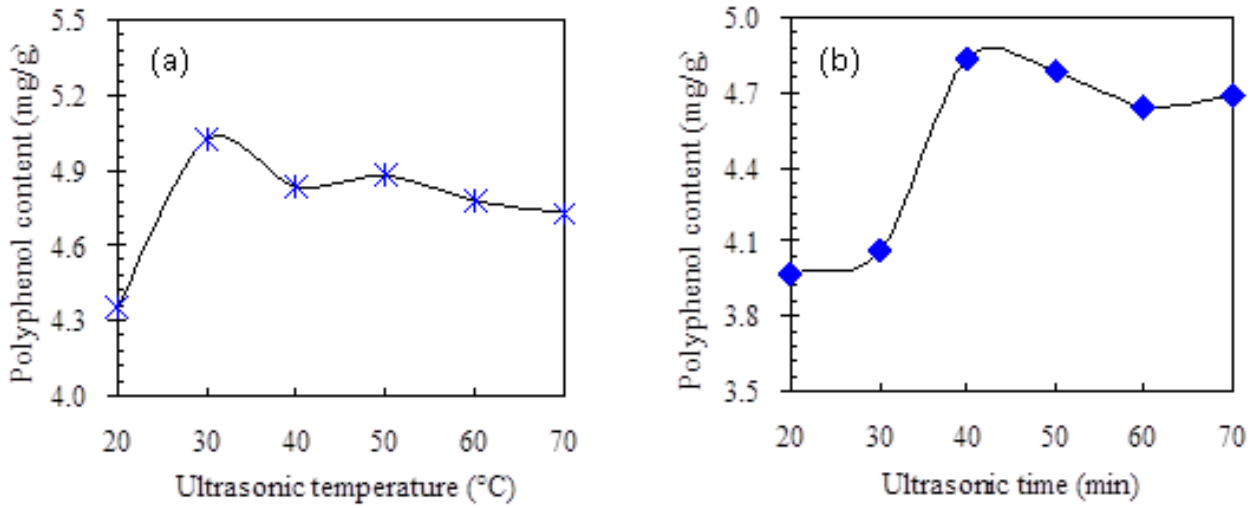

Fig. 2: Effect of ultrasonic temperature (a) and time of extraction (b) on polyphenol content in fruits of *P. fortuneana* (Maxim.) Li.

Optimization of polyphenols extraction by orthogonal experiment

The designed factors and levels of orthogonal experiment and results obtained by single factor experiments were shown in table 1. The highest content of polyphenol was presented in the experiment No. 6 (table 2). According to R values, the sequence of factors affecting the polyphenol content was A>B>C>D, namely ethanol concentration > solvent-to-solid ratio >temperature >time. Moreover, according to *k* value, the optimal level was A_2, B_3, C_1 and D_2, respectively. Therefore, the optimal conditions of orthogonal experimental design for polyphenol extraction were ethanol concentration of 70%, solvent-to-solid ratio of 70:1, ultrasonic temperature of 30°C, and ultrasonic time of 40 min ($A_2B_3C_1D_2$). Under the optimum extraction parameters, the polyphenol yield was 5.58 mg/g, which was the highest yield in all the experiments in this study. The findings verified the validation of the orthogonal model and the suitability of the optimizing ultrasonic assistant extraction conditions for polyphenols in the fruits of *P. fortuneana* (Maxim.) Li.

Antibacterial activities of extracted polyphenols

The range of the extracted polyphenols concentration in the tests was from 0 to 60mg/mL. The antibacterial activities (DIZ values) of extracted polyphenols showed remarkable bacterial and concentration difference. For example, the antibacterial activity was relatively stronger against gram-positive *S. aureus* than gram-negative *E. coli*, with a bigger DIZ value of *S. aureus* at the same polyphenol concentration (table 3). The MIC of the extracted polyphenols against *E. coli* was 20 mg/mL, with the corresponding minimum DIZ value was 6.8 mm, while at this concentration the DIZ value against *S.*

aureus was 9.5 mm. Moreover, the MIC against *S. aureus* was 10 mg/mL, with the DIZ 7.4 mm. In addition, *E. coli* and *S. aureus* was absolutely inhibited when the polyphenol concentration higher than 40 and 20 mg/mL, respectively (table 3). These results indicated that antibacterial activity of extracted polyphenols in the fruits of *P. fortuneana* (Maxim.) Li was concentration and bacterial species independent.

DISCUSSION

The polyphenol was one of the content of foods, beverages, and plants (Williamson and Carughi, 2010). Plant polyphenols were consisted in most plant parts, which played a considerable function in plant physiology (Doke and Guha, 2015). The plant polyphenols can be estimated by several methods, each with their own advantages and disadvantages. An ultrasonic assisted method has been proposed for extraction polyphenols from fruits of *P. fortuneana* (Maxim.) Li using ethanol, which was used as solvent because it was a affordable and safe solvent, altogether fitting to a green chemistry approach (Rodrigues *et al*., 2015). The effects of different parameters on the extraction of polyphenols were investigated in present study in triplicate.

The polyphenol yield increased with the increasing ethanol concentration and then declined, reaching a maximum (5.26mg/g) at 70% (fig. 1a). This may be due to the greater ethanol concentration produced high solubility of polyphenols, but too high concentration increased the ethanol solution polarity, resulting in a decline in the solubility of the polyphenols, which resulted in the decrease of polyphenol extraction yield. With the increase of the solvent-to-solid ratio, the polyphenol yield increased, however, it decreased when the ratio above 60:1, therefor the solvent-to-solid ratio ranged from 50:1 to 70:1 was selected for further experiment. This could be as a result of the fact that higher ratio of solvent to raw material lead to the lower concentration and viscosity of the extraction solvent, which facilitated dissolution of more polyphenols molecules in the solvent (Chen *et al*., 2015). However, more polyphenols increased the viscosity of the medium which was against the formation of cavitations (Xu *et al*., 2014). Ultrasonic wave propagated in liquid medium producing special cavitation effect which disrupted the material cell wall, promoted the penetration of the solvent into the cells and facilitated mass transfer. Moreover, the cavitation production was more difficult in viscous liquids where the cohesive forces were stronger (Chen *et al*., 2015). Hence, high solvent-to-solid ratio can reduce the polyphenol yield in turn. At the same time, the yield was also increased with the increase of temperature, and then decreased slowly when the temperature continued to rise. This phenomenon can be explained by the fact that high temperature increased the polyphenols solubility and facilitated their diffusion out from the cells (Braga *et al*., 2006). However, higher temperature may destroy the structure of polyphenols, and lead to oxidation and breakdown of phenolic compounds also (Doke and Guha, 2015). Therefore, the ultrasonic temperature of 30–50°C was selected for the optimal temperature.

In recent years, polyphenols have received considerable attention, which were imperative in a human diet, and they were widely distributed in various plant species (Li *et al*., 2006). Furthermore, the phenolic compounds possessed tremendous benefits, such as the antioxidant property, which was associated with the ability of free radical scavenging and broke its chain reaction, also chelated metals (Bakirel *et al*., 2008). Moreover, Padam *et al*. (2012) reported thant polyphenols of various plant species provided potential antibacterial activity. Importantly, Katalinic *et al*. (2006) observed that there was a positive relationship between antibacterial activity and total phenol contents, this phenomenon was also shown in our previous study (Sun *et al*., 2014; Li et al., 2015). The present study suggested that the hydro-alcohol polyphenols extracts in the fruits of *P. fortuneana* (Maxim.) Li had obvious antibacterial activity, moreover, the gram-positive bacterium *S. aureus* was more sensitive to the extracts than gram-negative bacterium *E. coli*, this result agreed with the previous studies (Lopez *et al*., 2005; Sun *et al*. 2014). A probable explanation for these phenomena may rest with the distinct disparities in outer layers of bacteria, there was an outer membrane on the gram-negative bacteria, and also a unique periplasmic space was observed, but these were not found in gram-positive bacteria (Duffy and Power, 2001).

CONCLUSIONS

Ultrasound technology was used for the first time to extract polyphenols from the fruits of *P. fortuneana* (Maxim.) Li. Under the most favorable extraction conditions optimized by single factor experiment model and orthogonal experiment as follows: ethanol concentration 70%, solvent-to-solid ratio 70:1, ultrasonic temperature 30°C, and ultrasonic time 40 min, the maximum polyphenol yield was 5.58 mg/g. On the other hand, the hydro-alcohol polyphenols extracts of *P. fortuneana* (Maxim.) Li fruits possessed excellent antibacterial potential, with the MIC value 10 mg/ml and 20 mg/ml to the *S. aureus* and *E. coli*, respectively. These information is useful for development of garden fruit-based nutraceutical-rich health food formulations. However, further studies on the chemical structures and other biological functions of fruit polyphenols as a new health care products are needed.

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