Optimization of Ultrasound-Assisted Extraction of Polyphenols from *Xanthoceras sorbifolia* Husks and their Determination using HPLC

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(Received on 26th October 2018, accepted in revised form 2 August 2019)

Summary: *X. sorbifolia* can be used as both food material and medicinal plant. In this study, ultrasound-assisted extraction (UAE) was firstly used to optimize the extraction process of total polyphenols from *X. sorbifolia* husks. Ultrasonic time, ultrasonic temperature, ratio of solvent to material and volume fraction of ethanol were investigated and optimized by orthogonal experiments. Then, High Performance Liquid Chromatography (HPLC) was applied to analyze the polyphenols of the extract under the optimal extraction process. The optimized results for UAE was 40 min of ultrasonic time, 40 °C of ultrasonic temperature, 100:1 mL/g of solvent to material ratio and 60% aqueous ethanol. Under the optimal extraction process, the total polyphenol content (TPC) of the extract was 23.16 mg GAE/g DW. Furthermore, the most abundant polyphenols in *X. sorbifolia* husks extract, such as gallic acid, protocatechuic acid, epicatechin, myricetin-3-O-*β*-D-rutinoside, rutin and quercetin, were analysed using HPLC. In addition, UAE extract showed antioxidant activity in a concentration-dependent manner. Accordingly, *X. sorbifolia* husks can be used as a rich source of natural antioxidants. To sum up, these results provided a solid chemical basis underlying the future research and application of *Xanthoceras sorbifolia* husks.

Keywords: *Xanthoceras sorbifolia*, Ultrasonic-assisted extraction, Orthogonal design, High Performance Liquid Chromatography, antioxidant activity

Introduction

Xanthoceras sorbifolia Bunge. (Sapindaceae family), indigenous to North China, is a kind of widely cultivated woody oil-bearing crop with special medicinal values [1, 2]. *X. sorbifolia* seeds were rich in oil (55%-70%), which included unsaturated fatty acids (85%-93%) [3]. Traditionally, *X. sorbifolia* was used to fight against rheumatism, arterial sclerosis, hyperpiesia, hyperlipemia, chronic hepatitis, and enuresis of children [4]. Previous research reported that *X. sorbifolia* mainly contained triterpenoid saponins, phenolic compounds and flavonoids [4-6], which exhibited some pharmacological effects including vascular relaxation, antioxidant activity, anti-tumor, inhibitory effects on HIV-1 protease [3-5, 7].

X. sorbifolia husks were a kind of agricultural waste, which can be prepared as activated carbon [8]. The phytochemical constituents of *X. sorbifolia* husks included triterpenoid saponins, isoxazoline, coumarins and alkaloids [9-12]. Furthermore, our previous study showed that *X. sorbifolia* husks were rich in antioxidant polyphenols [2, 13]. Pharmacological researches demonstrated that *X. sorbifolia* husks exhibited some biological activities, including cytotoxicity [9, 14], improving learning and memory impairment [1, 15, 16], antioxidant, immunomodulatory effects [17] and tyrosinase inhibitory effects [18].

Reflux extraction (RE), maceration extraction (ME), heating extraction (HE), microwave-assisted extraction (MAE), supercritical fluid carbon dioxide (SF-CO_2) extraction (SFE) and UAE were used to extract the active constituents from the agricultural waste material [19]. However, conventional solvent extraction (RE, ME and HE) processes showed certain limitations such as high extraction temperature, lower efficiency, low extraction yield, use of large quantity of solvents, mass transfer resistance, and health hazards [19, 20]. Through comparison MAE and HE, Rodsamran *et al*.

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[21] discovered that MAE can be a short processing time for pectin extraction from *Citrus aurantifolia* peel with suitable pectin properties. Using SFE, ZHANG *et al*. [10] founded that the content of unsaturated fatty acids of *X. sorbifolia* seeds was approximately 90%.

UAE, as an efficient, economical, and environment friendly method, could induce the cavitation of the solvent and further generate cavitation effect [19]. The cavitation force could continuously accelerate the heat and mass transfer rate, and then caused discruption of plant cells [22, 23]. Safdar *et al*. [19] demonstrated that UAE was a more efficient technique and yielded comparatively higher polyphenol contents of *Citrus reticulate* peel than ME. Using reflux extraction, Li *et al*. [24] reported TPC of *X. sorbifolia* husks by single-factor design. However, there was no literature that reported TPC of *X. sorbifolia* husks extract obtained using UAE combined with orthogonal design to optimize the extraction process based on the publications we searched from 2000 to 2019.

In this study, the main objective was to investigate the UAE variables (ultrasonic time, ultrasonic temperature, ratio of solvent to material and volume fraction of ethanol), and optimize these variables values by orthogonal design for the yield of total polyphenol maximization from *X. sorbifolia* husks. Furthermore, the polyphenol compositions of UAE extract under the optimal extraction process was quantified by HPLC with standards. Meanwhile, the antioxidant activity of the UAE extract was evaluated.

Experimental

Materials

Rutin, gallic acid, quercetin (Chengdu Must Biotechnology Co., Ltd.), DPPH (Sigma), ABTS (Aladdin), Sodium tungstate (Shanghai Zhongqin Chemical Reagent Co., Ltd.), Sodium molybdate (Tianjin chemical reagent four plant), Lithium sulfate (Tianjin BASF Chemical Co., Ltd.). Acetonitrile (Fisher Scientific). myricetin-3-O-*β*-D-rutinoside, epicatechin and protocatechuic acid were prepared as described previously [2]. All other chemicals were of the analytical grade commercially available.

Xanthoceras sorbifolia husks samples

X. sorbifolia husks (Sapindaceae), collected in Gansu Province, China, in 2017, were possessed as described previously [2].

Preparation of Folin-Ciocalteu

The Folin-Ciocalteu solution was prepared according to the literature [25]. Then this solution was kept at 4 ℃ for further use.

Ultrasonic-assisted extraction (UAE)

As far as UAE was concerned, ultrasonic machine (KQ-300DE, Dongguan Keqiao Ultrasonic Instrument Co. LTD, China) with fixed frequency at 100 kHz was used. The pulverized *X. sorbifolia* husks (10.0002 g) were extracted with the Ultrasonic machine using 1000 mL (solvent to material ratio, 100:1 mL/g), 60% aqueous ethanol. The ultrasonic temperature was 40 ℃, and the ultrasonic time was 40 min. The extract was filtered and then concentrated under reduced pressure to yield the ethanol extract (XSH, 2.080 g). The ethanol extract was kept at 4 ℃ for determination of the polyphenol content, HPLC analysis and antioxidant activity. Then, 140 mg of the UAE extract was dissolved in purified water to 100 mL for the determination of TPC after filtering.

Preparation of standard curve

The standard curve was adopted by our previous results [13]. Briefly, 0.3, 0.5, 0.7, 0.9, 1.1 and 1.25 mL of the diluted solutions were separately put into 5 mL flasks, and 1 mL water was individually added, followed by 0.5 mL Folin-Ciocalteu with fully shaking. Then, 1 mL 10% Na_2CO_3 was added, and diluted to 5 mL using purified water and mixed well. After keeping at 60 ℃ for 60 min, the absorbance values were examined at 762 nm with distilled water as control by using UV-1750 UV-Vis spectrophotometer (Shimadzu, Japan). The regression equation was $Y=0.1298x+0.0583$ ($R^2=0.9981$), linear range was 1.2-5.0 mg/L.

Determination of total polyphenolic contents (TPC)

The determination of total polyphenolic content (TPC) of *X. sorbifolia* husks was tested according to the results of our research previously [24]. The optimal chromogenic condition was as follows, chromogenic time (100 min), chromogenic temperature (60 ℃), volume of Folin-ciocalteu reagent (0.6 mL) and volume of 10% Na_2CO_3 solution (0.75 mL). Briefly, 0.2 mL of the extract solution (as

described in Fraction 2.4) was separately put into 5 mL flasks, and 1 mL water was individually added, followed by 0.6 mL Folin-Ciocalteu with fully shaking. Secondly, 0.75 mL 10% Na_2CO_3 was added, and diluted to 5 mL using purified water with mixing well. After keeping at 60 ℃ for 100 min, the absorbance values were examined at 762 nm with purified water as control by using UV-1750 UV-Vis spectrophotometer (Shimadzu, Japan).

Antioxidant activity

The antioxidant capacity of the extract was measured by their ability to scavenge the ABTS radical cation and DPPH radical using previously reported methods [26], with some modifications. Briefly, a solution (20 uL) of each sample was added to the ABTS (DPPH) radical solution (180 uL). After reacting with the ABTS radical solution for 10 min (DPPH radical solution for 30 min), the absorbance value (A_i) of ABTS at 734 nm (or Ai of DPPH at 517 nm) was measured using an Enzyme-linked Immunosorbent Assay Reader (Spectramax 190-Molecular Devices). The blank absorbance (A_0) was measured using ethanol. The ABTS and DPPH radical solutions were prepared daily. The antioxidant activity was expressed as the percentage inhibition of the ABTS/DPPH radical and was determined by the following equation:

$$AA(\%) = [1 - Ai/Ao] \times 100\%$$

Identification of polyphenols

Preparation of standard solution

Appropriate amount of gallic acid, protocatechuic acid, epicatechin, myricetin-3-O-β-D-rutinoside, rutin and quercetin were accurately weighed, using methanol as solvent, to prepare a reference solution including 0.213 mg/mL gallic acid, 0.605 mg/mL of protocatechuic acid, 9.600 mg/mL epicatechin, 0.915 mg/mL of myricetin-3-O-*β*-D-rutinoside, 0.410 mg/mL of rutin and 1.110 mg/mL of quercetin, respectively. Then 0.2 mL of each of the above reference solutions were put into the tube, mixed thoroughly, and then accurately draw 1 mL to dilute to a 2 mL volumetric flask with methanol. The solution was passed through a 0.22 μm microporous membrane, and kept at 4 °C before use.

Preparation of the sample solution

Appropriate amount of *X. sorbifolia* husks polyphenol extract (XSH, 4.808 g/g DW) as accurately weighed to prepare a solution of 100.72 mg/mL of XSH, with methanol as the solvent. The solution was filtered using 0.22 μm nylon microporous membrane, and then used for HPLC analysis.

Chromatographic conditions

The standards and samples were analyzed by an Agilent-1220 high performance liquid chromatograph (HPLC) system (Agilent, American), comprised of a solvent delivery unit (G1311C) and a UV detector (G1314F). The column was Shimadzu Inertsil ODS-C18 (250 mm×4.6 mm, 5 μm). 0.1% formic acid was used as mobile phase A. Chromatographic acetonitrile was used as mobile phase B. Then the mobile phase was filtered by passing through a 0.45 μm filter membrane. The column loaded with these compounds were run gradiently with a mobile phase consisting of 0.1% formic acid and acetonitrile (Table-1) for the determination of the polyphenols from *X. sorbifolia* husks. The detection wavelength was 260 nm, and the flow rate was 0.8 mL/min. The column temperature was 25 ℃, and a sample of 20 μL of this solution was directly injected.

Table-1: Liquid Chromatography Conditions.

Time（min）	Phase A（%）	Phase B（%）
0～5	80	20
5～20	80→60	20→40

Experiment design

Single-factor design

The optimal extraction process was firstly studied using the single-factor optimization method, as far as ultrasonic time (30, 40, 50, 60 and 70 min), ultrasonic temperature (30, 35, 40, 45, 50, 55 and 60 ℃), ratio of solvent to material (25, 50, 75, 100, 125 mL/g) and volume fraction of ethanol (50%, 60%, 70%, 80% and 90%) were concerned. Briefly, as described in Fraction 2.4, about 1.0 g powders of *X. sorbifolia* husks were accurately weighed and put into tubes with plugs to get the UAE extract. Then, 0.2 mL of the extract solutions were separately put into 5 mL flasks, and 1 mL water was individually added, followed by 0.6 mL Folin-Ciocalteu with fully shaking. Secondly, 0.75 mL 10% Na_2CO_3 was added, and

diluted to 5 mL using water, and mixed well. After keeping at 60 ℃ for 100 min, the absorbance values were examined at 762 nm with distilled water as control by using UV-1750 UV-Vis spectrophotometer (Shimadzu, Japan).

Orthogonal experiments

On the basis of the single-factor test result, a three-level-four-factor $L_9(3^4)$ was employed to determine the best combination of extraction variables for the maximization of polyphenolic content, As can be seen from Table-2.

Table-2: Factors and levels of orthogonal test.

	A	B	C	D
levels	Extraction time (min)	Extraction temperature (℃)	Ratio of solvent to material (mL/g)	Ethanol volume ratio (%)
1	40	40	50	60
2	50	45	75	70
3	60	50	100	80

Statistical analysis

All experiments were carried out in three times to ensure the repeatability. Sample concentrations providing 50% inhibitory ability (IC_{50}) were obtained by fitting dose-response data to a four-parametric logistic nonlinear regression model, using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA).

Results and discussion

Single-factor experimental analysis

In this study, the efficiency of different extraction time on the extraction rate of total polyphenols from *Xanthoceras sorbifolia* husks were studied, and the results were listed in Fig. 1. Firstly, the other extraction conditions of polyphenols from *Xanthoceras sorbifolia* husks, such as ethanol concentration, ration of solvent to raw material, and extraction temperature were fixed at 70%, 25:1 and 50 ℃. As can be seen from Fig. 1, the extraction rate of polyphenols continued to increase from 30 to 50 min, and showed the highest (13.701 mg GAE/g DW) at 50 min. Then the extraction rate of polyphenols from *Xanthoceras sorbifolia* husks decreased from 50 to 70 min. Therefore, 50 min was choosed as the optimal extraction time to continue the orthogonal test.

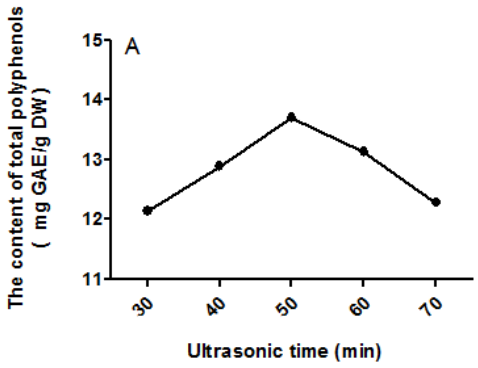

Fig. 1: Effects of time on the extraction rate of polyphenols from *X. sorbifolia* husks

The effect of different extraction temperatures on the extraction rate of total polyphenols from *Xanthoceras sorbifolia* husks (Fig. 2) were also studied. First, the other extraction conditions of polyphenols from *X. sorbifolia* husks, such as ethanol concentration, ration of solvent to raw material, and extraction time were fixed at 70%, 25:1 and 50 min. As can be seen from Fig. 2, the extraction rate of polyphenols continued to elevate from 30 to 45 ℃, and reached the highest (17.746 mg GAE/g DW) at 45 ℃. Then the extraction rate of polyphenols from *X. sorbifolia* husks lowered from 45 to 60 ℃. Therefore, 45 ℃ was choosed as the optimal extraction temperature to undergo the orthogonal test.

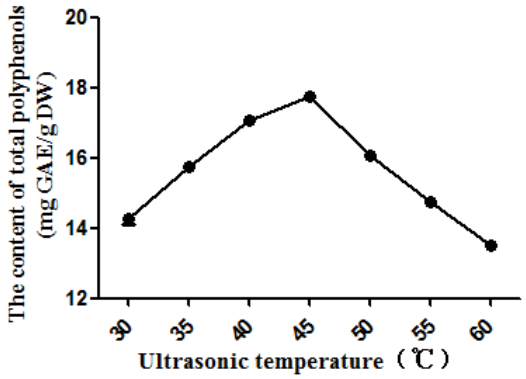

Fig. 2: Effects of temperature on the extraction rate of polyphenols from *X. sorbifolia* husks.

The effects of ethanol concentration on extraction rate of polyphenols from *X. sorbifolia* husks were investigated, and the results were listed in

Fig. 3. Firstly, the other extraction conditions of polyphenols from *Xanthoceras sorbifolia* husks, such as extraction temperature, ration of solvent to raw material, and extraction time were fixed at 50 °C, 25:1 and 50 min. Above all, as can be seen from Fig. 3, the extraction rate of polyphenols from *X. sorbifolia* husks increased with the ethanol concentration changed from 50% to 70%, and reached the highest (18.808 mg GAE/g DW) with the ethanol concentration of 70%. Then, the extraction rate of polyphenols reduced with the ethanol concentration changed from 70% to 90%. Correspondingly, 70% ethanol was choosed as the best extraction solvent to do the orthogonal test.

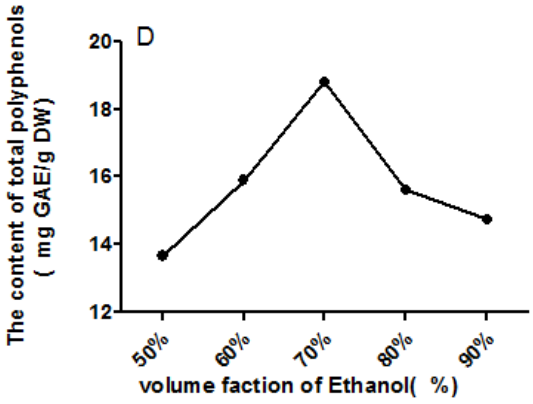

Fig. 3: Effects of ethanol concentration on the extraction rate of polyphenols from *X. sorbifolia* husks

The effects of liquid-solid ration on extraction rate of polyphenols from *X. sorbifolia* husks were studied. Firstly, the other extraction conditions of polyphenols from *X. sorbifolia* husks, such as ethanol concentration, extraction temperature, and extraction time were fixed at 70%, 50 °C and 50 min. As can be seen from Fig. 4, the extraction rate of polyphenols from *Xanthoceras sorbifolia* husks elevated with the liquid-solid ratio changed from 25:1 to 75:1, and exhibited the highest (14.213 mg GAE/g DW) with the liquid-solid ratio of 75:1. Then, the extraction rate of polyphenols diminished with the solid-liquid ratio changed from 75:1 to 125:1. Accordingly, 75:1 was choosed as the best liquid-solid ratio to continue the orthogonal test.

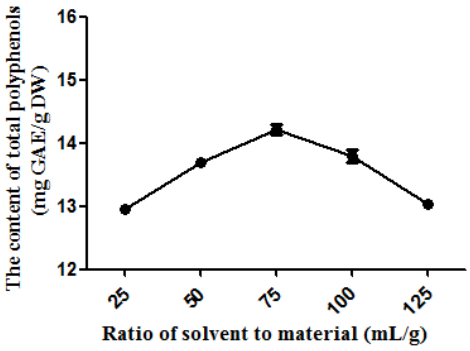

Fig. 4: Effects of solid-liquid ratio on the extraction rate of polyphenols from *X. sorbifolia* husks

Orthogonal experimental analysis

As it was shown in Table-3 and Table-4, the optimal UAE process was 40 min of ultrasonic time, 40 °C of ultrasonic temperature, 100:1 mL / g of solvent to material ratio and 60% aqueous ethanol using orthogonal design.

Table-3: Result of orthogonal test.

Number	Factors				Absorbance
	A	B	C	D	
1	1	1	1	1	0.460
2	1	2	2	2	0.426
3	1	3	3	3	0.374
4	2	1	2	3	0.451
5	2	2	3	1	0.347
6	2	3	1	2	0.434
7	3	1	3	2	0.371
8	3	2	1	3	0.449
9	3	3	2	1	0.433
k1	0.420	0.427	0.447	0.413	
k2	0.411	0.407	0.436	0.410	
k3	0.417	0.414	0.364	0.425	
R	0.009	0.020	0.083	0.015	

Table-4: Variance analysis results.

Variation sources	SS	df	MS	*F*	*P*
A	0.000	2	0.000	0.000	1.000
B	0.001	2	0.001	1.417	0.414
C	0.012	2	0.006	16.983	0.056
D	0.000	2	0.000	0.000	1.000
Error	0.01	8			

The total polyphenol content

Using the optimal UAE extraction process, the total polyphenol content of the extract was 23.16 mg GAE/g DW with SEM 0.70%. Li *et al.* [24] reported that the content of total polyphenol from *X.*

sorbifolia husks was 2.14 mg GAE/g DW, using reflux extraction. Compared these two results, we undoubtedly discovered that the content of total polyphenol from *X. sorbifolia* husks using UAE combined with orthogonal design was much higher than that using reflux extraction combined with single-factor test.

Polyphenols of UAE extract

Under the optimal liquid chromatography conditions, the separation between gallic acid, protocatechuic acid, epicatechin, myricetin-3-O-*β*-D-rutinoside, rutin, quercetin and adjacent chromatographic peaks was excellent. The reference substance and the test sample liquid chromatography pictures were shown in Fig. 6. The sample solution Fig. 6 (A) and the reference solution Fig. 6 (B) have corresponding chromatographic peaks at the same retention time. Fig. 6 (B) present the chromatograms obtained from ethanolic extract of *X. sorbifolia* husks. As can be seen from Fig. 6 and Table-5, the main polyphenols from *X. sorbifolia* husks were gallic acid, protocatechuic acid, epicatechin, myricetin-3-O-β-D-rutinoside, rutin and quercetin, which was the same with the literatures [2, 13, 27].

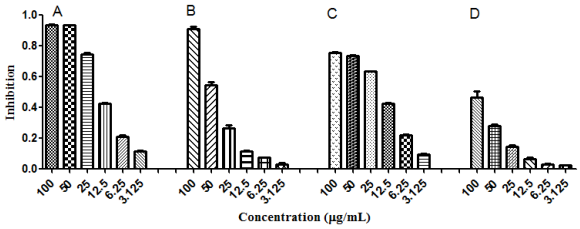

Fig. 5: Antioxidant activity of the extract from *X. sorbifolia* husks (A: The inhibition of rutin against ABTS; B: The inhibition of extract against ABTS; C: The inhibition of rutin against DPPH; D: The inhibition of extract against DPPH.)

Table-5: HPLC quantification of polyphenols present in the sample.

Polyphenols	Content (mg/100 g DW)
gallic acid	**11.93 ± 0.14**
protocatechuic acid	**20.41 ± 0.39**
epicatechin	**63.57 ± 1.78**
myricetin-3-O-*β*-D-rutinoside	**16.78 ± 0.36**
rutin	**40.01 ± 1.09**
quercetin	**20.61 ± 0.52**

Data was Presented as $\bar{x} \pm s$ (n=3)

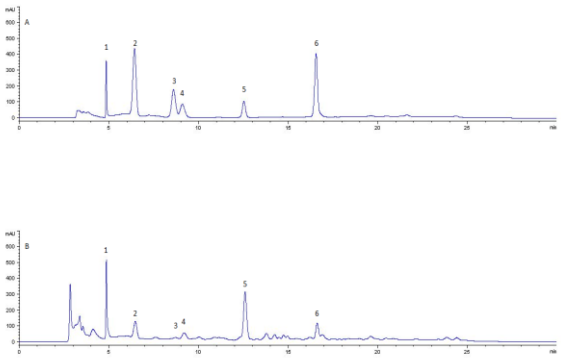

Fig. 6: HPLC chromatograms of reference substances (A)and the ethanol extract of X. sorbifolia husks (B) (1 gallic acid, 2 protocatechuic acid, 3 epicatechin, 4 myricetin-3-O-β-D-rutinoside, 5 rutin, 6 quercetin).

Antioxidant activity

According to the results in Fig. 5, both the positive control rutin and the extract exhibited concentration-dependent inhibitory effect against ABTS and DPPH radicals. It was reported that some of these polyphenols exhibited potent antioxidant activity [2]. In this study, the IC_{50} values of rutin against ABTS and DPPH radicals were 12.87 ± 0.21 μg/ml and10.29 ± 0.17 μg/mL separately. Whereas, the IC_{50} value of the extract against ABTS radical was 250.1 ± 0.16 μg/mL.

Conclusion

In summary, the ultrasonic-assisted extraction (UAE) process was firstly optimized using orthogonal design to extract the total polyphenols from *X. sorbifolia* husks. The optimal UAE process was 40 min of ultrasonic time, 40 ℃ of ultrasonic temperature, 100:1 mL/g of solvent to material ratio and 60% aqueous ethanol. With this optimal process, the total polyphenol content was 23.16 mg GAE/g DW, of which the main polyphenols were gallic acid, protocatechuic acid, epicatechin, myricetin-3-O-*β*-D-rutinoside, rutin and quercetin using HPLC analysis. The results showed that the optimal UAE extract was a rich source of natural antioxidants. Because oxidative stress showed great influences on chronic diseases such as cardiovascular and metabolic diseases and cancer [28], *X. sorbifolia* husks may be used as a candidate agent for preventing and curing

these chronic illnesses.

Acknowledgement

This work was supported by 2018 Sichuan college students' innovation and entrepreneurship training project (grant number: 201810634037), Key projects of the Sichuan Provincial Education Department (grant number: 18ZA0208), The cooperative research project between Nanchong City and North Sichuan Medical College (grant number: NSMC20170446), Doctoral research fund of North Sichuan Medical College (grant number: CBY16-QD09), Construction project of the first class subject group in Pharmacy of North Sichuan Medical College (2018).

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