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Full Length Research Paper

Ultrasound-assisted extraction flavonoids from Lotus (Nelumbo nuficera Gaertn) leaf and evaluation of its anti-fatigue activity

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In this study, flavonoids were obtained from Lotus (Nelumbo nuficera Gaertn) leaf using ultrasound-assisted extraction method (UAE). Optimum extraction conditions of flavonoids from Lotus leaf (FLL) were investigated through single factor test and orthogonal test. Its anti-fatigue activity was evaluated by swimming test. The results showed that the optimal extraction parameters were as followings: concentration of ethanol solution was 70% , ratio of solvent to raw material was 35, ultrasound time was 25 min. Swimming test results suggested that FLL had significant anti-fatigue activity.

Key words: Ultrasound-assisted extraction, Lotus leaf, anti-fatigue activity.

INTRODUCTION

Lotus (Nelumbo nucifera Gaertn) is a perennial aquatic crop with stout creeping yellowish white colored rhizomes (Yang et al., 2007). It is both an ornamental plant and a dietary staple in Eastern Asia, particularly in China (Hu and Skibsted, 2002). Various parts of Lotus have been employed for medicinal proposes in traditional medicina1. Also numerous studies have addressed its pharmacological actions. The rhizome extract exhibited diuretic (Mukherjee et al., 1996), anti-inflammatory (Mukherjee et al., 1997), and hypoglycemic activities (Mukherjee et al., 1997). The stalk extract showed antipyretic action in a model of yeast-induced fever in rats. The seed extract elicited hepatoprotective effect and antioxidant activity (Sohn et al., 2003). The leaf extract exhibit reducing blood pressure (Trongtorsak et al., 2007), antihyperlipidemic (Guan et al., 2003), antioxidant and hypoglycemic activities (Qie et al., 2007). Lotus leaf contains several flavonoids and alkaloids, and flavonoids are considered to be one of main components of lotus leaf (Onishi et al., 1984; Cour et al., 1995; Yang et al., 2007). A recent study has revealed that eight flavonoids and its glycosides are isolated from lotus leaf, including Isorhamnetin, kaempferol, quercetin, quercetin-3-O-β-D-xylopyranosyl (1→2)-β-D-glucopyranosyl glycosides, Astragalin, Chrysoeriol 7-O-β-D-glucoside, Isoquercitrin and hyperin (Wang et al ., 2008). Flavonoids from Lotus leaf (FLL) receive the greatest attention and are studied extensively, since they were displayed as a remarkable range of biochemical and pharmacological properties (Middleton et al., 2000; Li et al., 2009). But the anti-fatigue activity of FLL were not been reported.

The traditional techniques of solvent extraction for flavonoids from a plant materials are mostly based on the correct choice of solvents and the use of heat or/and agitation to increase the solubility of the desired compounds and improve the mass transfer. Usually the traditional technique requires longer extraction time thus running a severe risk of thermal degradation for most of the phytoconstituents (Luque de Castro and Garcia-Ayuso, 1998). The fact that one single plant can contain up to several thousand secondary metabolites makes the need for the development of high performance and rapid extraction methods an absolute necessity (Nyiredy, 2004). Keeping in pace with such requirements recent times has witnessed the use and growth of new extraction techniques with

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shortened extraction time, reduced solvent consumption, increased pollution prevention concern and with special care for thermolabile constituents. Novel extraction methods including microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SCFE), pressurized solvent extraction (PSE) drew significant research attention in the last decade (Wang et al., 2008; Mandal et al., 2007; Gao and Liu, 2005). Ultrasonic radiation is a powerful aid to accelerate of various steps of the analytical process. This energy is of great help in the pre-treatment of solid samples as it facilitates and accelerates operations such as the extraction of organic and inorganic compounds, homogenization and various others Ultrasound-assisted leaching is an effective way to extract analytes from different matrices in shorter time than other extraction techniques (Herrera and Luque de Castro, 2005). In recent years, there are technics reports for FLL extraction in some related literatures, such as classical maceration (CM) (Hong and Wang, 2001; Zhong et al., 2006), soxhlet extraction (PE) (Wang et al., 2007; Zhou et al., 2009), microwave-assistant extraction (MAE) (Jin et al., 2006; Ou, 2006; Yang et al., 2008), pressurized solvent extraction (PSE) (Chen et al., 2002), but the ultrasound-assisted extraction (UAE) was infrequent for the FLL.

This study focused on optimized ultrasound-assisted extraction parameters of flavonoids from Lotus leaf and evaluating its anti-fatigue activity.

MATERIALS AND METHODS

Plant material

Leaves of Lotus were collected in September–August in the northwest region of Zhejiang Province, China. They were dried in the air at 25 - 30℃ and then ground into powder. The plants were identified by Professor Yue Li, a biologist of Zhejiang University of Technology. A voucher specimen (No. 35784) was deposited in herbarium of Zhejiang University of Technology.

Ultrasound-assisted extraction flavonoids from lotus leaf

Powder (100 g) of Leaves of Lotus was suspended in ethanol (ethanol concentration ranged from 40 - 80 %, ratio of solvent to raw material ranged from 20 - 40) and were soaked for 4h, then under ultrasonication for certain time (ranged from 15 to 40 min) in ultrasonic cleaner (KQ2200, Rated power 300W, temperature 40℃; Kunshan Ultrasonic Instrument Co., Jiangshu, China). A deep brown extract was obtained which was filtrated by using the filter (Shoa Shong, Shanghai, China). The filtrate was absorbed through D101 macroporous absorptive resin column (4.0 × 60 cm; Fubang Chemical Science Technologies Co., Tianjin, China) at the speed of 20 ml/min. The column was eluted with dH2O up to the washed liquid colourless and then eluted with 80% ethanol (Zheng et al., 2007). The eluant was collected and evaporated by using a rotary evaporator (RE52AA; Yalong Biochemical Instrument Co., Shanghai, China) under reduced pressure at 40℃ to get the flavoniods.

Determination of total flavoniods content

The contents of flavonoids was measured using colour comparing method of KAc-$AlCl_3$ and wavelength in spectrophotometer (V-5100, Beijing Chenxiyongchuang Science and Technology Co., Beijing, China) was set at 420 nm (Jin et al., 2007; Fan et al., 2004; Liu et al., 2007).

When extraction yield of flavoniods was calculated, the following formula can be used:

$$X = \frac{C \times V \times D}{m \times 100} \times 100\ \%$$

Where; X (%) = extraction yield of flavoniods; C(mg/ml) = flavorniods content of test solution calculated by standard curve; V (ml) = volume of test solution; D = total dilution value; m(g) = mass of test sample.

Experimental animals

Kunming male mice, 2-month-old, were used for the study. Housed individually in polypropylene cages, maintained under standard conditions (12 h light and 12 h dark cycle, 25 ± 30℃, 35 - 60% humidity), the animals were fed with standard diet and water *ad libitum*. The approval of this experiment was obtained from the Institutional Animal Ethics Committee of Zhejian University of Technology.

Anti-fatigue activity of FLL

After 1 week of acclimation, 24 male mice were randomly divided into 3 equal groups: control group (CG), low dose treated group (LG) and high dose treated group (HG). The control group was given distilled water and the treated groups were given different doses of FLL (50, 150 mg/kg) by gavage once a day for 21 days. 21 day later, mice were made to swim with wire of 5% body weight tied to their tails in the pool (length: 65 cm, width: 50 cm, depth: 50 cm) filled with 30 cm depth of water at 30 - 35℃. Mice were regarded as being exhausted when they were underwater for 8 s (Chi et al., 2008), and their swimming time was immediately recorded.

Statistical analysis

The data were analyzed with SPSS 10.0 software. ANOVA was used to determine the effects of salidroside on anti-fatigue. The values were expressed as mean ± SD. The test differences were considered statistically significant when a P value was less than 0.05.

RESULTS AND DISCUSSION

Effect of concentration of ethanol solution on extraction yield of flavoniods

The result of Figure 1 showed that extraction yield of flavoniods increased with the concentration of ethanol that is, 40 - 70%. A decrease in extraction yield of flavoniods was noticed further more, that is, beyond 70%. Considering one of the aims of this work was to propose a suitable solvent for extracting the raw flavonoids, among various solvents, ethanol was selected as a right choice because it is environmentally benign and relatively safe to human health (Zhang et al., 2003; He et al., 2005). Ethanol interacted with the flavonoids probably through noncovalent interactions and promotes a rapid diffusion into the solution (Li et al., 2008; Luque de Castro and Tena, 1996). Various concentration of ethanol used exhibited

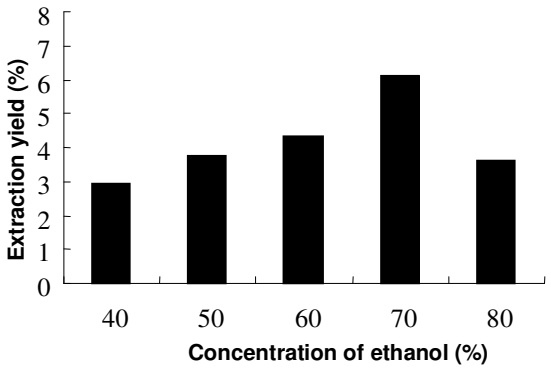

Figure 1. Effect of concentration of ethanol solution on extraction yield of flavoniods.

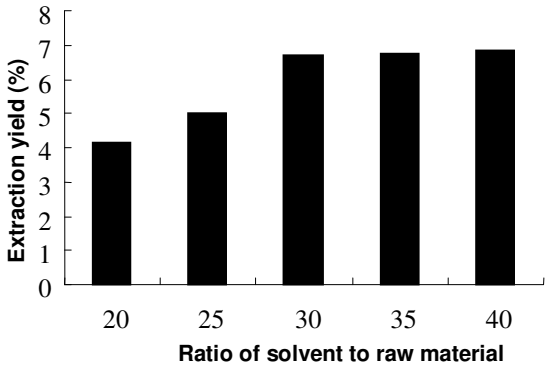

Figure 2. Effect of ratio of solvent to raw material on extraction yield of flavoniods.

different effect in changing the fluid polarity and thus had diverse effect on the solubility enhancement of the flavornoids (He et al., 2005; Sathishkumar et al., 2008). The optimal extraction yield might be fulfilled when the polarity of the fluid and its flavonoids were coincident. In this study, the results indicated that the optimal ethanol concentration for extraction flavonoids was found to be 70%.

Effect of ratio of solvent to raw material on extraction yield of flavoniods

The result of Figure 2 showed that extraction yield of flavoniods increased significantly with the ratio of solvent to raw material in a range of 20 ~ 30 and changed insignificantly when the ratio was greater than 30. This was because when the ratio of solvent to raw material was between 20 and 30 flavonoids were extracted fully with the rise of volume the extracting agent so that extraction yields increased (Sathishkumar et al., 2008). Otherwise, when the ratio of solvent to raw material reached a certain level, the extract had well soluted in the solution lead to extraction yield become steady and wouldn't increase significantly. The optimum ratio of solvent to raw material was 30.

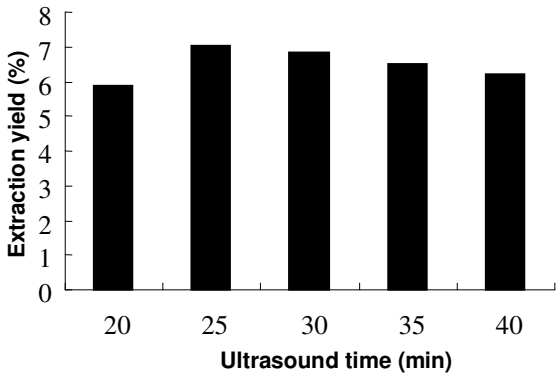

Figure 3. Effect of ultrasound time on extraction yield of flavoniods.

Effect of ultrasound time on extraction yield of flavoniods

The result of Figure 3 showed extraction yield of flavoniods Ultrasound extracted for 25 min reached its maxima and then decreased with prolonging Ultrasound time. Considering that flavonoids were located into the cytoplasm of the cells, it was clear that ultrasound led to a kind of tissue permeabilization by the disruption of important cellular structures such as cell walls and cell membranes, which are of great importance for mass transfer control. Some studies showed that, in contrast to conventional extractions, plant extracts diffused across cell walls due to ultrasound, causing cell rupture over a shorter period (Chemat et al., 2004; Schinor et al., 2004; Albu et al., 2004). In addition, this might increase amount of impurities with the rise of Ultrasound time. Therefore the optimum Ultrasound time was 25 min.

Optimum conditions for flavonoid extraction

On the basis of the effects of single factor in above chapters, the trials of tri- factor, tri-level orthogonal design were conducted and the results were given in Tables 1 and 2. According to the value of range R in Table 2, concentration of ethanol solution (factor A) exerted the most significant effect on extraction yield of flavoniods, and the order of importance that influenced extraction yield of flavoniods was found to be concentration of ethanol solution (A) > ratio of solvent to raw material (B) > ultrasound time (C). The optimal combination parameters of the extraction yield of flavoniods were $A_2B_3C_2$, namely, concentration of ethanol solution (70%), ratio of solvent to raw material (35), ultrasound time (25 min). And extraction yield of FLL was 7.15 %.

In this study, as a novel technique, ultrasound-assisted extraction has been shown to be very promising and effective for obtaining FLL, ensuring higher extraction yields of FLL at much shorter times (25 min at 40℃ , extraction yields 7.15 %) than classical maceration (8 h at 25℃, ex-

Table 1. Factors and levels of the orthogonal experiment.

No	Factor		
	A (concentration of ethanol solution) (%)	B (ratio of solvent to raw material)	C (ultrasound time) (min)
1	65	25	20
2	70	30	25
3	75	35	30

Table 2. L9 (3)3 orthogonal test result.

No.	A (concentration of ethanol solution) (%)	B (ratio of solvent to raw material)	C (Ultrasound time) (min)	Contents of flavonoids (%)
1	1	1	1	6.23
2	1	2	2	6.91
3	1	3	3	6.84
4	2	1	2	7.11
5	2	2	3	7.04
6	2	3	1	7.06
7	3	1	3	6.34
8	3	2	1	6.69
9	3	3	2	6.86
K1	6.660	6.560	6.660	
K2	7.070	6.880	6.960	
K3	6.630	6.920	6.740	
R	0.440	0.360	0.300	

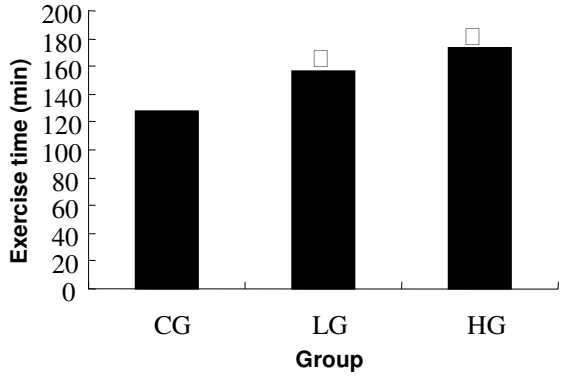

Figure 4. The effects of FLL on exhaustive exercise time in mice (mean ± SD, n=8) □P < 0.05 as compared with the control group.

traction yields 5.28%) and microwave-assistant extraction (microwave irradiation 5 min, 2.5 h at 20℃, extraction yields 6.61%) but less than soxhlet extraction (6 h at 70℃,, extraction yields 7.36%) (Zhong et al., 2006; Jin et al., 2006; Wang et al., 2007). Ultrasound-assisted extraction has many advantages: faster, more convenient operation, higher extraction yields, and so on. It's a new technic for flavonoids content from lotus leaf. But the FLL components obtained are different by different extract methods. Future studies should use HPLC method to investigate the change of the single flavonoid by ultrasound-assisted extraction.

The effects of FLL on exhaustive exercise time in mice

To explore the anti-fatigue bioactivities of FLL, mice were orally given different doses of FLL for 21days. Mice were then made to swim and the exhaustive exercise time was measured. As shown in Figure 4, exhaustive exercise time of mice of the treated groups (CG,HG) were all remarkably longer than that of the control group (CG) ($P < 0.05$). These results indicated that FLL had significant anti-fatigue activity. Further studies to clarify the detailed mechanisms involved in the anti-fatigue properties of FLL are necessary.

Conclusion

In conclusion, the ultrasound-assisted extraction conditions for FLL were optimized to find that Concentration of ethanol solution was 70%, ratio of solvent to raw material was 35, ultrasound time was 25 min and extraction yield of FLL was 7.15 %. Meanwhile, anti-fatigue activity of FLL was evaluated by swimming test. Results suggested that FLL had significant anti-fatigue activity. More research on FLL biological activity should be done in the future re-

search.

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