A novel approach for the efficient extraction of silybin from milk thistle fruits

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ABSTRACT

Background: Milk Thistle fruit is an important herb popularly consumed worldwide for a very long time. Silybin is the main bioactive constituent of the herb, and it has been approved by US Food and Drug Administration (FDA) as a medicine to treat liver diseases. Presently, using conventional technology, the meal of Milk Thistle fruit is used as the raw material to extract silybin. **Objective:** To investigate the necessity of detaching husk from kernel of the herb and also to propose a novel approach to enhance the extraction technology in pharmaceutical practices. **Materials and Methods:** The husk of Milk Thistle fruit was detached from the kernel of the herb using an automatic huller specially designed for this application. The husk and the meal of Milk Thistle fruit was subsequently refluxed, separately, with production rate of silybin as index for comparison of their extraction effect. **Results:** The highest production rate was achieved under optimized condition. The husk was extracted 2 times (3 hrs each) using ethyl acetate, and the ratio of solvent to raw material was 8:1. The extract was allowed to be crystallized out. **Conclusion:** The separation of kernel from the husk of Milk Thistle fruit and using only the husk as raw material can largely enhance the extraction of silybin.

Key words: Husk of milk thistle fruits, milk thistle fruits, silybin

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INTRODUCTION

Silybum marianum (L.) Gaertn (*S. marianum*), well-known as Milk Thistle, is an important herbal plant of *Silybum* genus (Asteraceae family). It is well documented in Chinese Pharmacopoeia[1] that Milk Thistle fruit had been extensively formulated into medicine, health supplements, cosmetics, animal feed, etc. Previous studies had shown that the various applications of the fruit, mentioned above, could be attributed to silymarin (SLM), fatty oils, and proteins, which are abundant in the fruit. In addition, silybin (SLB, Figure 1) in SLM has exhibited an effective capacity on recovering liver damage.[2,3] And, for this reason, SLB has a great consumption over the world as a hepato-protective drug.

Currently, SLB is being offered in the form of tablet, capsule, liposome, etc. The industrial production chain had already been constructed from the cultivation of this herbal plant to the final manufacturing of drug in China, the biggest country of the export of this product.

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The husk and the kernel of Milk Thistle fruits weigh very similarly but differ greatly in chemical constituents and biological activity. SLB for the treatment of hepatitis, liver fibrosis, and cirrhosis in clinical practices is most abundant in the husk but just found in trace levels in the kernel of Milk Thistle fruits.[4-6] On the other hand, fatty oil and proteins which were formulated into animal feedstuff and food are found mainly in the kernel.[7-9] Despite the great differences in chemical constituents and biological activity between the husk and kernel, the meal of Milk Thistle fruits with fatty oil squeezed partially is still used as raw material for the extraction in industry[10] due to the difficulties to detach the stiff and tightly wrapped husk from the kernel. As a consequence, the production rate and the purity of final product of SLB were lower than expectation and the energy consumption for its production was at an undesirable high level. Moreover, the kernel which is rich in fatty oil and protein has not been satisfactorily utilized.[11] In order to achieve an efficient and integrated utilization of Milk Thistle fruits, a novel approach and technology for the production of SLB, based on the of separation of kernel and husk of Milk Thistle fruits by an automatic huller, was established in this study [Figure 2].

MATERIALS AND METHODS

Chemicals and plant material

SLB standard (B/N: 0856-201203) was purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. MeOH of HPLC grade was obtained from Hanbang Co. Ltd (China). All the others reagents were of analytical grade from Shanghai Chemical Reagents Company (China).

Milk Thistle fruits were presented by Jiangsu Zhongxing Pharmaceutical Co. Ltd (China). The voucher specimen (No.: SP20121101) had been authenticated by Prof. Jun Chen (Department of Chinese Materia Medica and Pharmacy, School of Pharmacy, Jiangsu University, Zhenjiang, Jiangsu, China) and was deposited at the Pharmacognosy Research Facility in Jiangsu University.

Separation of husk and kernel

The husk and the kernel of Milk Thistle fruits were separated using an in house automatic huller. The huller was made specifically by our laboratory for this application and designed according to the physical properties of Milk Thistle fruits.[12] The mechanic structure of the huller was illustrated in [Figure 3]. The fruits of Milk Thistle were poured into the hulling cavity from feed hopper and were ejected by shifting teeth of internal rotating cone onto the internal surface of external cone for collision. Subsequently, the material was bounced back to the internal rotating cone where it was accelerated and ejected again.

Extraction and purification of SLB

The husk, smashed and passed over 40-mesh sieve, was used as raw material for the extraction of SLB. The fine powder of husk, 15.0 g, was refluxed with EtOAc. The suspension was then subjected to suction filtration, and the extracts were collected and pooled together. EtOAc was removed under reduced pressure using rotor evaporator (Büchi R-200, Germany) under 45°C, and the resulting sticky fluid was collected. Based on single factor experiment, the extraction technology was optimized

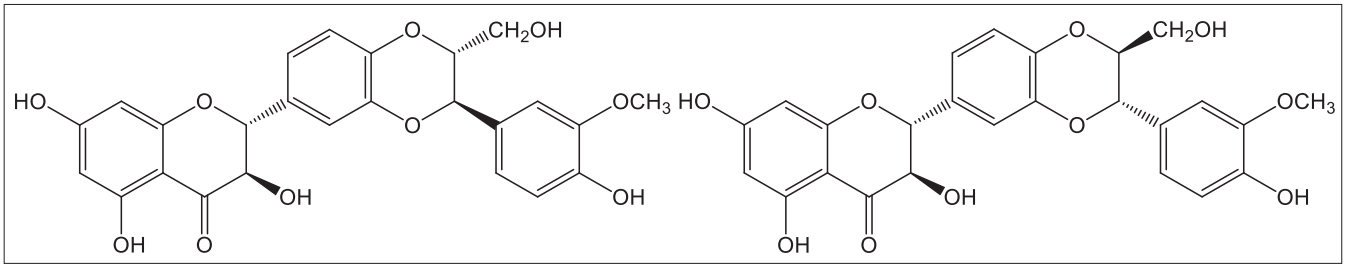

Figure 1: Chemical structures of SLB (two stereoisomers)

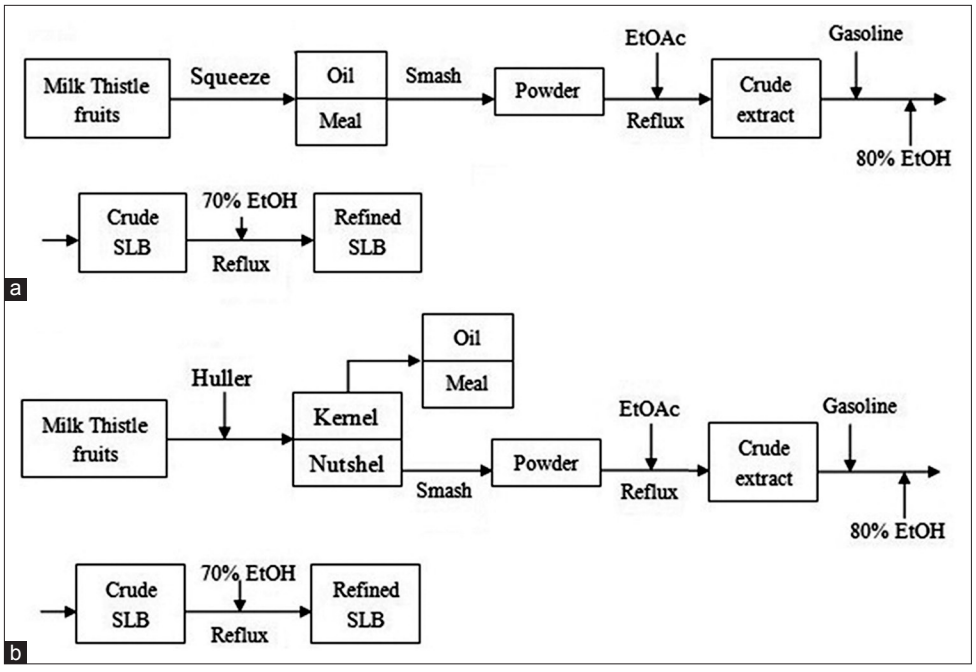

Figure 2: Flow charts of traditional (a) and new (b) producing process of SLB

by orthogonal design with production ratio of SLB as index [Table 1].

The 120# gasoline was used to remove grease from the obtained crude extract, which was then washed with 80% ethanol (*v*/*v*) for 4 times to further purify the extract. Subsequently, 70% EtOH (*v*/*v*) was chosen as the crystallization solvent. Five g of dried extract were refluxed and dissolved in 200 mL of EtOH for 3 hrs before 86 mL of H_2O was added. The reflux was continued for one more hour thereafter. After cooling the vessel to room temperature, the solution was stored at 4°C overnight and the purified SLB was collected as a pellet by centrifugation at 4000 rpm and drying at 60°C.

Determination of SLB by HPLC

SLB was determined by HPLC[1] (Waters, USA), and the analysis was performed using a Venusil ASB C_{18} column (4.6 × 250 mm, 5 μm; USA) maintained at 25°C. The mobile phase used was a mixture of MeOH and H_2O containing 2.0% HAc (45:55, *v*/*v*). UV detection wavelength was set at 288 nm to monitor the analyte. Flow rate was 1.0 mL·min^{-1} throughout the entire analysis. Calibration curve was constructed over the concentration range of 25.6 ~ 153.6 μg/mL using SLB solutions diluted from its stock solution using the mobile phases. Linear regression line of peak area (*A*) to SLB concentration (*C*) was plotted, and the linear relationship was determined by linear regression analysis and expressed as correlation coefficient (r^2).

Table 1: Orthogonal experimental design for the extraction of SLB from the husk

Level	Temperature (A, °C)	Extraction duration (B, hrs)	Ratio of solvent to material (C, g/mL)	Extraction times (D)
1	60	1	4:1	1
2	70	2	6:1	2
3	80	3	8:1	3

SLB: Silybin

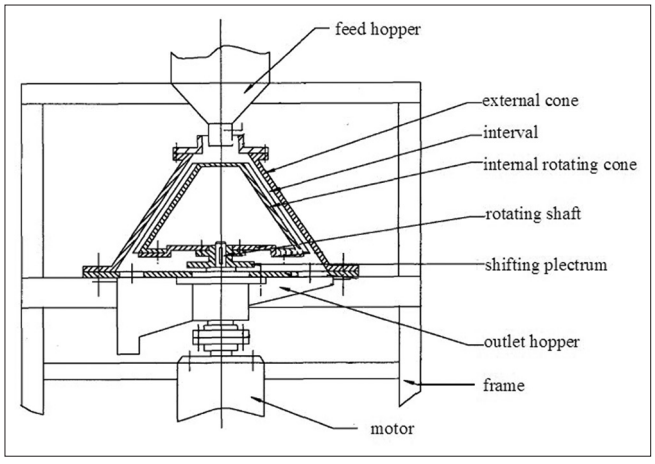

Figure 3: Mechanic structure of the automatic huller

Comparison of the novel technology with conventional technology

With production rate of SLB as index, this novel technology developed in current study was compared with conventional one, in which SLB was prepared from the meal of Milk Thistle fruits instead of the husk.

Calculation of production rate of SLB

The production rate (*Z*) was calculated by the following equation:

Z = Weight of SLB obtained/Original amount of SLB in raw material × 100%.

Original amount of SLB was determined by using the husk or the meal as raw material individually. Fine powder of the husk or the meal, 0.5 g, was refluxed with 100 mL of MeOH for 3 hrs. The suspension was filtered by suction, and the solution collected was topped up with a few MeOH to the original weight. This solution was filtered through 0.45 μm membrane syringe prior to HPLC analysis.

RESULTS

Separation of husk and kernel

After such collisions for several times in the automatic huller, stiff husks of Milk Thistle fruits had been mostly detached from the kernel. In this experiment, hulling degree above 95% was achieved.

Linearity

A high correlation coefficient, r^2 = 0.9994, was obtained from the regression line, $A = 60492C - 19621$. This demonstrates a good linear relationship between the analyte peak area and analyte concentration in the range. Typical HPLC chromatograms of SLB standard and SLB product were illustrated in [Figure 4].

Optimization of extraction conditions by orthogonal experimental design

According to the results obtained from orthogonal experiments [Tables 2 and 3], the significance of the factors decreased in the order, A > D > C > B, and the best extraction condition of SLB from Milk Thistle husk was $A_3B_3C_2D_2$ by verification test.

Production rate of SLB

The contents of SLB in the husk and the meal were 4.05% and 2.34%, respectively. Under the optimum extraction conditions, the production rate of SLB was

69.6% from Milk Thistle husk and only 60.5% from the meal. The purity of SLB in final product obtained from Milk Thistle husk was tested to be 97.3% by HPLC-UV analysis.

DISCUSSIONS

In conventional technology for the production of SLB, the meal of Milk Thistle fruits was used as raw material after fatty oil was squeezed partially. Since some of the oil and all proteins were still remained in the meal, a huge amount of non-polar solvent had to be consumed in order to remove the oil, and also the consumption of extracting solvent such as ethyl acetate was in large amount. In addition, the natural proteins in the fruits couldn't be utilized as food supplement or additives as it had been denatured and it was almost impossible to separate them from the remaining husk. Considering these factors, an automatic huller was designed and made to detach the husk from the kernel. In this study, a novel technology was developed to prepare SLB from the husk. The results also suggested that the production rate can be increased by using this novel technology.

To optimize the extraction of SLB from Milk Thistle husk, four solvents including ethyl acetate, absolute ethanol, 80% ethanol, and acetone were compared. Acetone gave the highest production rate of SLB among all solvents. However, as acetone is more toxic, expensive, and its use was strictly controlled by government, ethyl acetate was chosen as the next best extraction solvent.

Extraction methods such as maceration at room temperature, ultrasonic-assisted extraction, and reflux were compared. Even though extraction using maceration gave the highest content of SLB in the extract, the lowest production rate was unfavorable, and a longer time was needed due to the poor mass transfer. The ultrasonic-assisted extraction while applied in industrial scale by large equipments would lead to expensive operation cost as well as creating noise pollution. Therefore, the reflux method was finally chosen as the extraction method.

Finally, solvents for crystallization of SLB-rich extract were compared. Usually, the solvent for crystallization was methanol, ethanol, ethyl acetate, acetone or other mix solvents, and the production rate of SLB was higher with methanol and ethanol used than others.[9] In view of high toxicity of methanol, ethanol was the more favorable solvent for SLB crystallization in industry. Moreover, according to our previous study, the solubility of impurities including isosilybin, silydianin, silychristin etc., were higher in absolute ethanol than that of SLB. In addition, the solubility of SLB in 70% ethanol (13.23 mg/mL) was much lower than those impurities (70.71 mg/mL). Therefore, the crystallization process as mentioned above was employed. In addition, the residual solution after removing SLB could be used to prepare some by-products such as isosilybin, silydianin, and silychristin.

Table 2: Results of orthogonal experiments

No.	A	B	C	D	Production rate (%)
1	1	1	1	1	35.9
2	1	2	2	2	57.2
3	1	3	3	3	55.7
4	2	1	2	3	60.4
5	2	2	3	1	45.7
6	2	3	1	2	60.6
7	3	1	3	2	68.5
8	3	2	1	3	66.9
9	3	3	2	1	62.0
K_1	49.600	54.933	54.467	47.867	
K_2	55.567	56.600	59.867	62.100	
K_3	65.800	59.433	56.633	61.000	
R	16.200	4.500	5.400	14.233	

Table 3: Analysis of variance of orthogonal experiments

Factors	Sum of squared deviations	Freedom	F	The critical value of F	Significance
A	402.767	2	1.886	4.460	*
B	31.056	2	0.145	4.460	
C	44.309	2	0.207	4.460	
D	376.282	2	1.762	4.460	
Error	854.41	8			

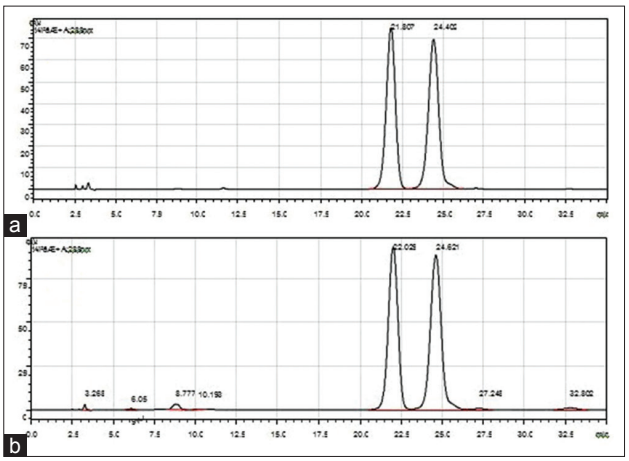

Figure 4: Typical HPLC chromatograms of SLB standard and SLB product

CONCLUSION

In this study, SLB with the purity of 97.3% was obtained by an improved extraction technology of detaching husk from kernel using an automatic huller designed according to the physical properties of Milk Thistle fruits. The technology was superior to conventional technology in production rate, operation, utilization of kernel etc., which is a promising technology in pharmaceutical practices of SLB.

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