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

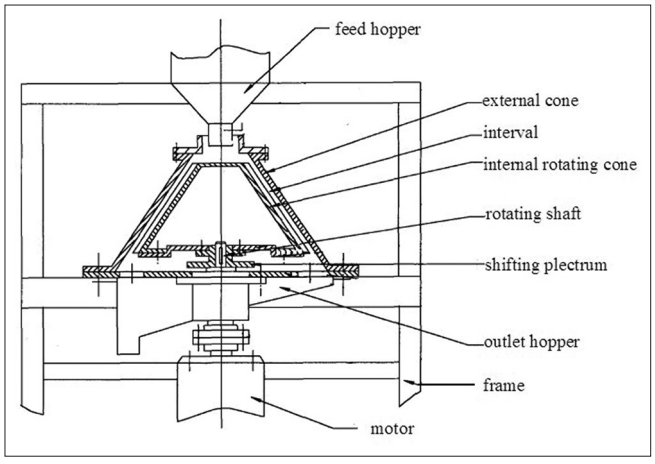


**Figure 3:** Mechanic structure of the automatic huller

### 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 | | | |

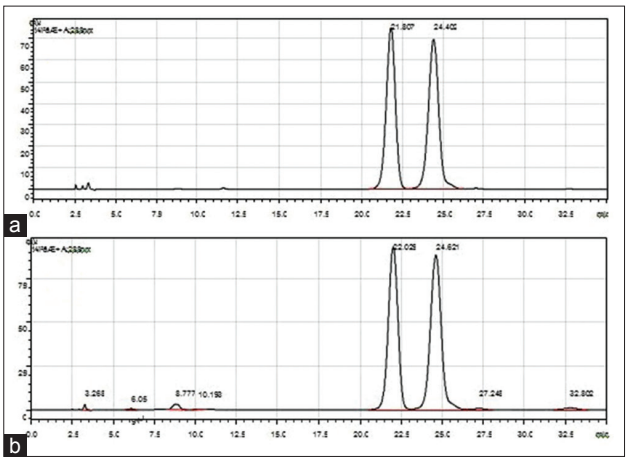


**Figure 4:** Typical HPLC chromatograms of SLB standard and SLB product

## 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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