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Method Validation and Skin Permeation Studies of Microemulsion Containing *Carthamus tinctorius* Flower Extract as a Functional Ingredient to inhibit 5α-reductase activity.

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ABSTARCT

The purpose of this study was to develop method validation for evaluation of skin permeation and proportion of safflomin A of *Carthamus tinctorius* floret extract (CT) loaded microemulsion as a functional ingredient to inhibit 5α-reductase activity. CT was extracted using a simple maceration technique. 2% CT was loaded into suitable microemulsion (CTME). The method for determination of safflomin A as an active ingredient of CT was validated in terms of the analytical parameters such as specificity, accuracy, precision, linearity, range, limit of detection (LOD), limit of quantification (LOQ) and determined based on the International Conference on Harmonization (ICH) guidelines. The linearity ranges of safflomin A was gained over 5- 25 ppm. Good linearity was observed over the above-mentioned range with linear regression equation Y = 36447x + 26600 (x is concentration of analytes in µg/ml and Y is peak area). The value of correlation coefficient accounted for 0.9954. The limit of detection (LOD) and limit of quantification (LOQ) was 1.8644 ppm and 6.2148 ppm. The recovery range was found to be 92.34 to 99.43 % for all three spiked levels. The RSD values from repeated extractions was 1.79%. The validation of developed method on precision, accuracy, specificity, linearity, and range were also performed with well-accepted results. The method validation of CTME was further study to determine skin permeation of safflomin A. The skin permeation was tested using franz diffusion cells. The result showed that the skin permeation rate of CTME was better than that of CT in aqueous formulation, making up 0.6321 and 0.0985 µg/cm^2.h. To sum up, the validation of developed method for analysis of safflomin A in CTMEs on precision, accuracy, specificity, linearity, and range were also performed with well-accepted results and can predicted the skin permeation rate of safflomin A in *Carthamus tinctorius* floret extract loaded microemulsion.

Keywords: skin permeation, microemulsion, *Carthamus tinctorius*, safflomin A, Method validation

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INTRODUCTION

Safflower, or *Carthamus tinctorius* L., is a plant grown in northern Thailand which is known for its medicinal properties which have resulted in its use for hair treatment. *C. tinctorius* floret ethanolic extract (CTE) could stimulate the hair growth-related gene and suppress the hair loss-associated gene expression in dermal papilla cells [1]. The phytochemical constituents of this plant contained 2-hydroxyarctiin, 2-methylbutyric acid, 3-methylbutyric acid, anhydrosafflor yellow B, carthamin, carthamidin, carthamone, caryophyllene, hydroxysafflor yellow A, isocarthamin, isocarthamidin, kaempferol, neocarthamin, safflower yellow, safflomin A and B etc., [2]. Safflomin A is the most phytochemical compound, finding in the floret of *Carthamus tinctorius* L. The method was used to determine qualitative and quantitative of safflomin A in ethanolic extracts from safflower was high performance liquid chromatography(HPLC). The ethanolic extracts from safflower have effectively inhibited the 5α-reductase enzyme, while providing finasteride 5α-reductase inhibitory activity equivalent to 24.30 mg for each gram of the extract, whereas in contrast the typical finasteride dose required for the treatment of hair loss is 1 mg daily. Furthermore, the safflower extract was shown to generate superior hair growth when compared to minoxidil [3]. These findings concur with those of Junlatat and Sripanidkulchai [4] whose work observed the stimulation of hair follicle proliferation, the synthesis of markers promoting hair growth, the presence of VEGF (vascular endothelial growth factor), and keratinocyte growth factor (KGF) through the application of safflower extract. In addition, the expression of transforming growth factor-β1 (TGFβ1) which is a signaling molecule capable of restricting epithelial cell growth was shown to be reduced [4-6]. For an enhancement in bioavailability of the active compound in this plant, micromeulsion was used as a potential carrier. microemulsion may be described as a transparent and optically isotropic oil or water dispersion for which the size of the particles marginally exceeds that of swollen micelles by around 20-200 nm. The components of microemulsions can be optimized through the use of pseudoternary phase diagrams which indicate the appropriate quantities of water, oil, and surfactant to be mixed. It is possible to identify specific microemulsion regions in the form of a phase diagram. There are three main types of microemulsions: water in oil (W/O), oil in water (O/W), and bicontinuous microemulsions [7]. In order to assess the quality and evaluate the formulated products, the method for quantitative determination of the main active constituent in the florets is needed. Since safflomin A is the most abundant chemical found in the florets, it is used as a chemical marker in our experiment. Therefore, the purpose of this study was to develop method validation for evaluation of skin permeation and proportion of safflomin A of *Carthamus tinctorius* floret extract (CT) loaded microemulsion as a bioactive ingredient to inhibit 5α-reductase activity.

MATERIALS AND METHOD

Materials

C. tinctorius florets were purchased from local market in Pathum Thani, Thailand. Safflomin A was purchased from Sigma-Aldrich, Germany. Acetonitrile and methanol were of HPLC grade from Merck, Germany. All the water used in this study was Ultrapure, obtained from a Milli-Q RO system (Milipore Corporation, France).

CT extraction

In this process, 200 g of powder from the CT underwent maceration in 1 L of 95% (w/w) ethanol at a temperature of 27 ± 2 ^{0}C for a period of 8 hours. It was regularly stirred at two-hour intervals. The extract was subsequently filtered through Whatman No. 1 filter paper with the aid of a vacuum pump. This process was repeated on two further occasions to re-extract the residues. The filtrates were then gathered, combined, and dried using a rotary evaporator (Rotavapor R210, Buchi, Switzerland) at a temperature of 40 ^{0}C. CT extracts underwent analysis for safflomin A with the assistance of HPLC before being placed in storage at the temperature of -80 ^{0}C.

CT loaded in ME preparation

The composition chosen for the ME was derived from the pseudoternary phase diagram which provided the greatest ME area. The chosen ME was prepared by mixing the various components, which comprised IPM (10-20%), propylene glycol (3-40%), polysorbate 80 (30-40%), and water (10-30%). The CTME (CT-loaded microemulsion) was produced by injecting CT extract into the aqueous phase prior to the addition of the other components. The final CT concentration for the microemulsion was 2% w/w.

Extraction of CTME

Unentrapped safflower was extracted from the wax matrix of ME by using cold methanol precipitation technique, as followed, 1 g of CT-ME was dissolved in 9 g of methanol, and then it was thoroughly mixed using vortex mixer. After that, it was centrifuged at 6000 rpm, 0° C for 30 min. All the oil compartment will be solidified and crystallized; the supernatant was filtered through 0.45 µM nylon syringe membrane filter and kept for HPLC analysis. ME base was also extracted by the same procedure.

Method validation for analysis of safflomin A in CTME

The analytical method was validated on specificity, precision, accuracy, linearity, range, and limits of detection and quantification as followed from Kajsongkram *et al.* [8] and Kumar *et al.* [5].

Apparatus

The analytical method of two markers was performed on a Waters Alliance 2695 LC system connected with a Waters model 2996 photodiode-array detector. Data collection and processing were carried out using an Empower workstation. The optimum HPLC system was comprised of a C18 reverse phase column (Luna C18, 150x4.6 mm i.d., 5 µm particle size). The gradient was eluted with acetonitrile and water at a flow rate of 1.0 ml/min and PDA detection at 401 nm. The mobile phase consisted of two different solutions, solution A and solution B. The temperature of the column was controlled at 40°C. An injection volume was 20 µl. The detection wavelength was fixed at 401 nm. Total run time was 10 min per sample.

Accuracy

Percentage recovery of safflomin A was defined by spiking known amount of standard safflomin A into extracted ME base. Each concentration was analyzed in triplicate to calculate the accuracy.

Precision

Repeatability and intermediate precision were determined by using reflex sympathetic dystrophy (RSD). For repeatability, three concentration of safflomin A was each analyzed separately in triplicate in the same day. For intermediate precision, the same experiment was conducted in another day.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurity, degradation products, and matrix components. In this study, the specificity was demonstrated by running a procedural blank, standard, and sample and placebo solutions.

Linearity and range

Six concentrations over the range of 5 to 25 ppm of standard safflomin A were analyzed by our described method. Calibration curve was constructed from concentration of safflomin A and peak area. The range was obtained from standard linear curve.

Detection limit and quantitation limit

The HPLC signal of the safflomin A was compared to the extracted ME base. Detection limit and quantitation limit were the concentration that gave signal three times and ten times greater than the noise, respectively.

Skin permeation evaluation

Evaluation of skin permeation. The sample of CTME were determined for skin permeation compared with CT in aqueous solution as adapted from Jimenez [9]. Strat-M®Membrane for transdermal diffusion testing

(Merck Millipore, Germany) was used as a model membrane for the skin permeation study because of its similarity to human skin in lipid content and permeability. The skin samples were mounted between the two half-cells of a diffusion chamber with a 37^0C water jacket to control the temperature. The surface of the skin was placed in contact with the donor chamber, which was filled with the micromeulsion formulation. The receptor chamber was filled with 0.1 M PBS (pH 7.4) and stirred with a star-head Teflon magnetic bar driven by a synchronous motor. At time intervals of 0.5, 1, 2, 4, 8, 12, 24 and 48 h, a 1 mL aliquot of receptor was withdrawn, and the same volume of fresh medium was added back into the chamber. The concentration of safflomin A in the samples was analyzed by HPLC. The concentration of permeants in the samples was analyzed by HPLC, and the cumulative amount was plotted against time. The flux was determined as the slope of linear portion of the plot. Lag time was also obtained by extrapolating the linear portion of the penetration profile to the abscissa.

Statistical Analysis

The statistical analysis results were shown in the form of mean ± S.D for no fewer than three independent experiments. One-way ANOVA was used for the analysis of the data to determine the extent of any differences between treatments. The level set for statistical significance was $p < 0.05$.

RESULTS AND DISSCUSSION

Physical appearance of MEs were a yellowish transparent liquid. CT was dissolved in the aqueous phase of ME at a final concentration of 2.0 % w/w. CTMEs with appropriate formulation was produced as light orange transparent liquids. Cold methanol precipitation method was used to extract unentrapped safflomin A from the matrix of the ME. The resulting supernatant from ME base and CT-ME were the clear liquid and pale yellow liquid, respectively. After filtration with 0.45 µm membrane filter, there was no waxy material precipitate from the solution. The specificity of this method was determined by analysis of the blank, placebo, safflomin A standard and sample solution chromatograms (Figs. 1-4). Good separation of the peak of safflomin A was obtained, accounted for 13.776 min of the retention times for safflomin A .The comparison of chromatograms among blank, placebo, standard and sample, there was no interference observed from the peaks of the blank, placebo. It shows that the method is high specificity. For linearity studied, five solutions in the range of 5-25 ppm of safflomin A standard were analyzed (Table 1). Each concentration was made and analyzed in triplicate. The peak areas obtained against each concentration of the analytes were used to build a linear regression equation as well as determined value of correlation coefficient. Good linearity was observed over the above - mentioned range with linear regression equation Y = 36447x + 26600 (x is concentration of analytes in µg/ml and Y is peak area). The value of correlation coefficient was 0.9954 as shown in Fig. 5**.** This study was performed by adding known amounts of safflomin A to the placebo samples. Three level of solutions were made and having concentrations at 10, 15, 20 ppm (Table 2). The recovery ranges for safflomin A were 92.34 to 99.43 % respectively (limit 80 to 110%). The relative standard deviation of safflomin A ranged from 0.4519 to 0.7577 %. Repeatability was studied by calculating the relative standard deviation (RSD) from six determinations of the 100% concentration of sample. The studied was performed on the same day and under same experimental conditions. The concentrations of safflomin A determinations in the sample solution with the relative standard deviation were calculated (Table III). The RSD values obtained for safflomin A was 1.79% (limit not less than 3.7%) as displayed in Table 3. The result showed that the developed method was precise. LOD were calculated by using the following equations. LOD = 3.3 x SD/S and LOQ = 10 x SD/S, where SD = the standard deviation of the response, S = Slope of the calibration curve. The LOD value was 1.8644 ppm and the LOQ value was 6.2148 ppm for the simultaneous estimation of safflomin A. Method validation following ICH guidelines indicated that the developed method had high sensitivity.

The permeability of CTME from frans diffusion cell method over 48 h was measured with high performance liquid chromatography as shown in Fig. 6 and table 4. The permeation rate of safflomin A in aqueous formulation, a chemical component of this extract, was 0.0985 µg/cm^2/ h. When CT was formulated in form of microemulsion, the permeation rate rose to 0.6321 µg/cm^2/ h. Hence, the skin permeation rate of CTME was better than that of CT in aqueous formulation. It was due to the construction of micromeulsion containing water, oil, surfactant and co-surfactant content. This could be attributed to skin permeation enhancement capacity of the used surfactant, as surfactants loosen or fluidize the lipid matrix of the stratum corneum which is the principal diffusion barrier of the skin and act as skin permeation enhancer.

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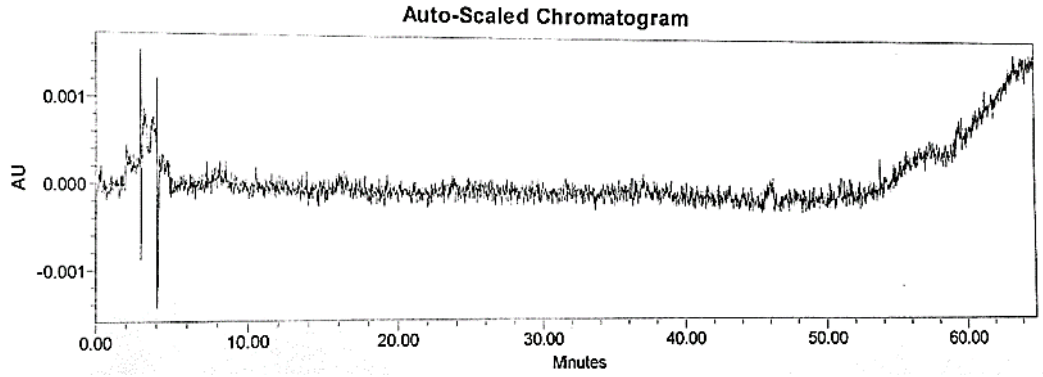

Figure 1: HPLC Chromatogram of Blank Solutions.

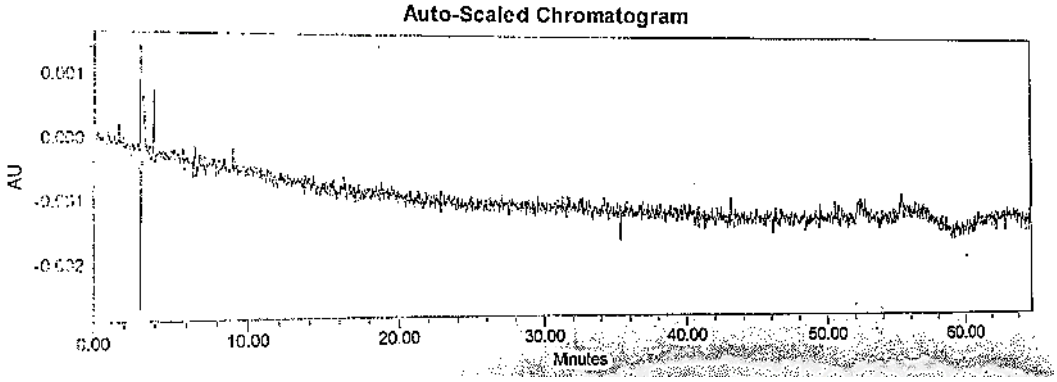

Figure 2: HPLC Chromatogram of Placebo Solutions.

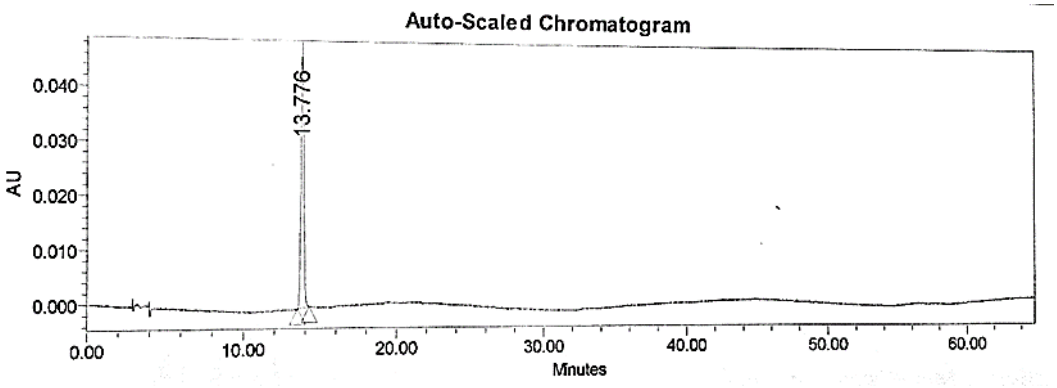

Figure 3: HPLC Chromatogram of safflomin A Standard Solutions.

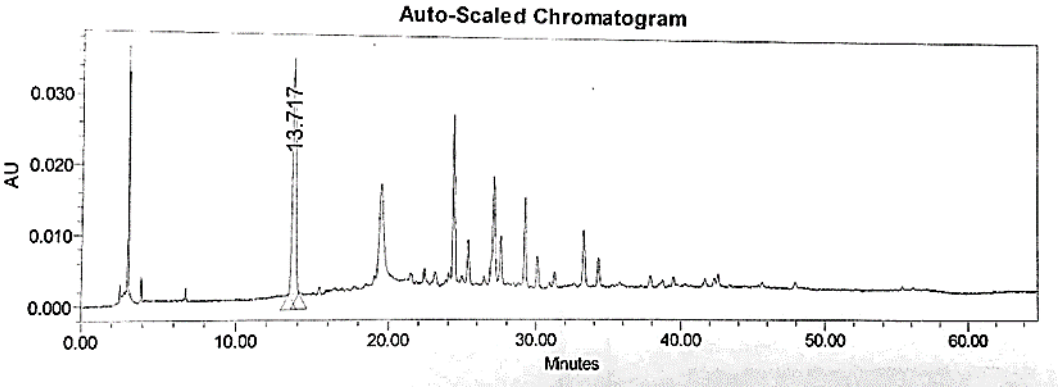

Figure 4: HPLC Chromatogram of Sample Solutions.

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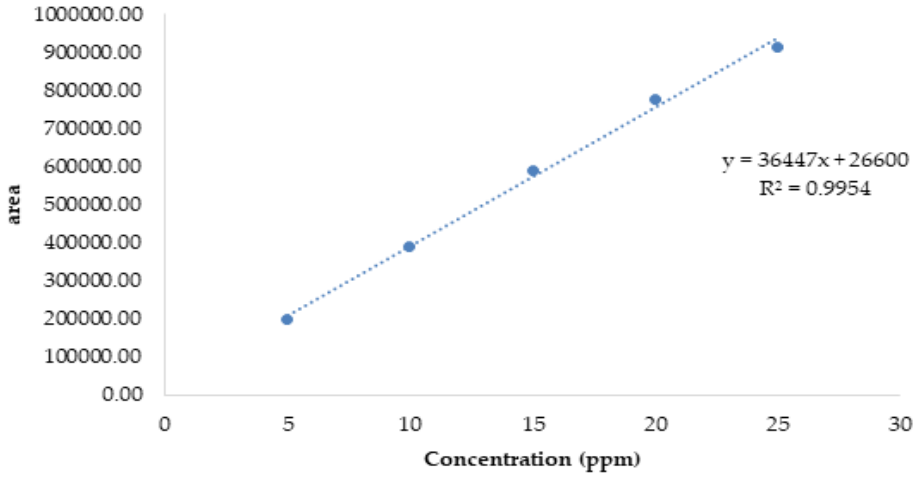
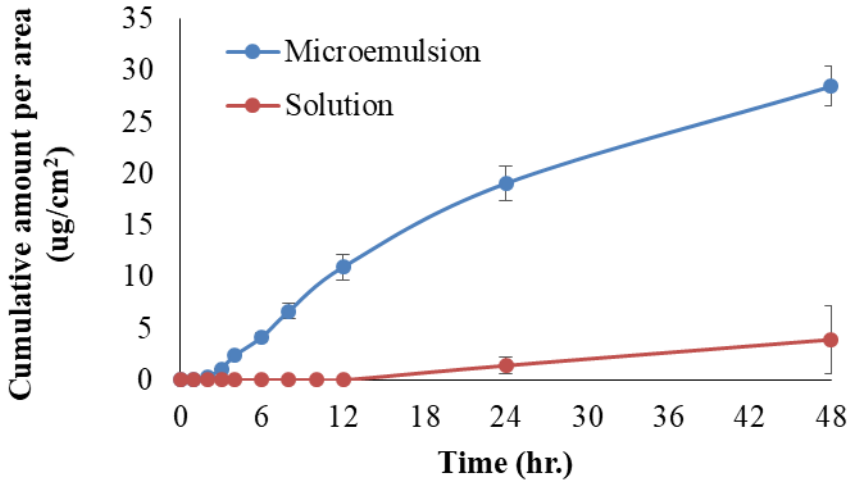


Figure 5: Calibration Curve of safflomin A Standard by HPLC.



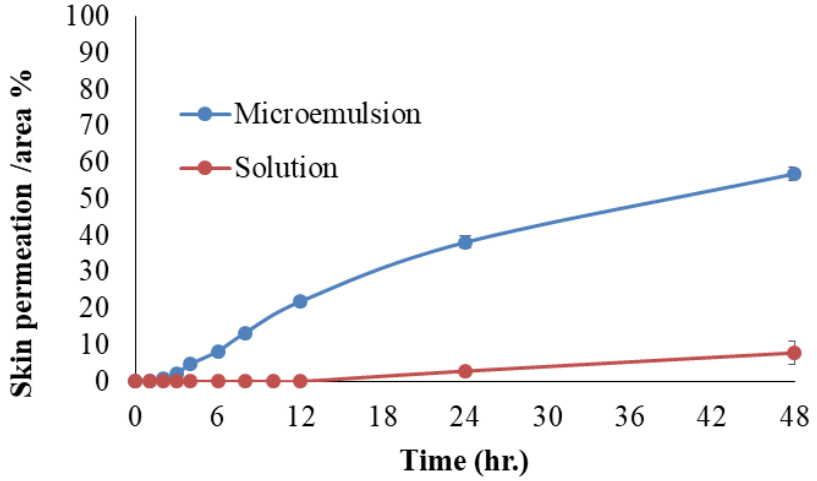


Figure 6: Skin permeation rate of CTME compared with CT in aqueous formulation.

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Table 1: Linearity and range of safflomin A by HPLC.

Sample number	Concentration of (ppm)	Peak area
1	5	195,797.67
2	10	390,074.67
3	15	589,778.33
4	20	777,692.67
5	25	913,155.00

Table 2: Acculacy data of safflomin A by HPLC.

Amounted (ppm)	% Recovery	% RSD
10	92.34	0.7002
15	96.42	0.4519
20	99.43	0.7577

Table 3: Precision studies of safflomin A by HPLC.

Number	safflomin A content in ME (%w/w)
1	0.002485
2	0.002525
3	0.002512
4	0.002531
5	0.002506
6	0.002410
% RSD	1.795080

Table 4: Permeation coefficient of CTME compared with CT in aqueous formulation.

Samples	Permeation coefficient (μg/cm^2.h)
CTME	0.6321±0.05
CT in aqueous solution	0.0985±0.01

CONCLUSION

Safflomin A as the main active ingredient of *Carthamus tinctorius* Flower Extract loaded in microemulsion could be determined simultaneously, and the validity of the method was also verified. The proposed analytical method for simultaneous estimation of safflomin A in the microemulsion is accurate, precise, linear, robust, reproducible and within the range. A stable CTME was successfully formulated to improve permeation in comparison with the extract solution. The skin permeation rate of CTME was better than that of CT in aqueous formulation. Therefore, use of nanotechnology for stable transdermal delivery systems of bioactive compounds from Thai medicinal plants is one approach to improve skin and hair follicle permeation.

ACKNOWLEDGMENTS

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REFERENCES

[1] Tian Y., Yang ZF., Li Y., Qiao Y., Yang J., and Jia YY. J Ethnopharmacol. 2010; 129 : 1-4.

[2] Kumar N., Rungseevijitprapa W., Narkkhong NA., Suttajit M., and Chaiyasut C. J Ethnopharmacol. 2012; 139: 765-71.
[3] Sinclair RD. Male androgenetic alopecia. J Men Health Gender. 2004;1(4):319-27.
[4] Junlatat J., and Sripanidkulchai, B. Phytother Res. 2014; 28:1030 - 1036.
[5] Kumar N., Tharatha S., and Chaiyasut C. African Journal of Pharmacy and Pharmacology. 2011; 5(20) :2335-2341.
[6] Yue S., Tang Y., Li S., and Duan J. Molecules. 2013; 18: 15220-15254.
[7] Soradech S., Kusolkumbot P., and Thubthimthed S. JAPS. 2018; 8(3): 046-054.
[8] Kajsongkram T., Rotamporn S., Limbunruang S., and Thubthimthed S. International Journal of Medical and Health Sciences. 2015; 9(12): 813-817.
[9] Jimenez MM. Int. J. Pharm. 2004; 27245–27255.