Revista Brasileira de Farmacognosia 28 (2018) 289–293

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Validated high performance liquid chromatography for simultaneous determination of stability of madecassoside and asiaticoside in film forming polymeric dispersions

Chaowalit Monton[a,*], Chitradee Luprasong[b,*], Jirapornchai Suksaeree[c], Thanapat Songsak[d]

[a] *The Herbal Medicinal Products Research and Development Center (Cooperation between Rangsit University Harbin Institute of Technology and Heilongjiang University of Chinese Medicine), Faculty of Pharmacy, Rangsit University, Pathum Thani, Thailand*
[b] *Sun Herb Thai-Chinese Manufacturing, Faculty of Pharmacy, Rangsit University, Pathum Thani, Thailand*
[c] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Rangsit University, Pathum Thani, Thailand*
[d] *Department of Pharmacognosy, Faculty of Pharmacy, Rangsit University, Pathum Thani, Thailand*

ARTICLE INFO

Article history:
Received 8 February 2018
Accepted 6 April 2018
Available online 1 May 2018

Keywords:
Asiaticoside
Film forming polymeric dispersions
High performance liquid chromatography
Madecassoside

ABSTRACT

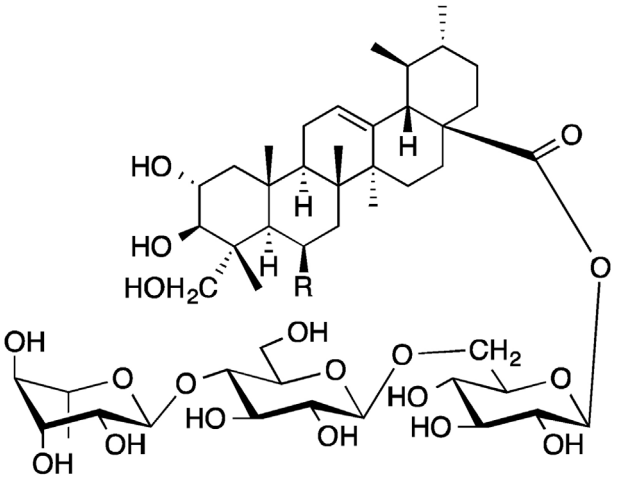
The objective of the work was to validate the high performance liquid chromatography for simultaneous determination of stability of madecassoside and asiaticoside in *Centella asiatica* (L.) Urb., Apiaceae, extract-loaded film forming polymeric dispersions. High performance liquid chromatography method was validated in five topics: linearity and range, limit of detection and limit of quantitation, specificity, precision, and accuracy. Results showed the method had a good linearity ($R^2 > 0.9990$) in the range of 5–150 μg/ml and specific. The limit of detection and limit of quantitation of madecassoside were 81 and 245 ng/ml and asiaticoside were 21 and 64 ng/ml, respectively. The percent relative standard deviation of intraday and interday precision were less than 1 and 3%, respectively. The accuracy presented as percent recovery was 101.54–103.29% for madecassoside and 100.39–102.58% for asiaticoside. This validated high performance liquid chromatography method was used to determine the stability of the formulation containing *Centella asiatica* extract. *Centella asiatica* extract-loaded film forming polymeric dispersions used Eudragit® RS 30D and Eudragit® RL 30D as film former, glycerin as plasticizer, and absolute ethanol as solvent and penetration enhancer. Three formulations with different ratio of Eudragit® RS 30D and Eudragit® RL 30D were prepared and stored for 90 days at 4 °C, 25 °C, and 40 °C. Stability results showed that almost all of the formulations were unstable at 25 °C and 40 °C. Except, two of three formulations were stable at 4 °C. However, the formulation was further developed to improve the stability of madecassoside and asiaticoside in the formulation.

Introduction

Centella asiatica (L.) Urb. is a well-known herbal medicinal plant in the family of Apiaceae. In Thailand, it is listed in National Essential Drug List; topical cream containing 70% ethanol extract of dried *C. asiatica* in a concentration of 7%w/w is used for wound healing purpose. Currently, *C. asiatica* is commonly used for the treatment of dermatological diseases including bacterial infection, psoriasis, scleroderma, and wound. Furthermore, antioxidant activity is reported as well (Bylka et al., 2014). Other biological and pharmacological effects are also reported. It is used as an adaptogen, antibiotic, blood-purifier, central nervous system relaxant, detoxifier, diuretic, emmenagogue, laxative, peripheral vasodilator, and sedative (Khare, 2007). It contains various chemical compounds including pentacyclic triterpenoids called centelloids such as madecassic acid, asiatic acid, and its glycosides; madecassoside and asiaticoside is a main chemical compound that exhibits wound healing property. The chemical structures of madecassoside (**1**) and asiaticoside (**2**). *Centella asiatica* also contains other compounds *e.g.*, asiaticoside C, asiaticoside D, asiaticoside E, asiaticoside F, centellasaponin B, centellasaponin C, isothankunic acid and oleanane-type saponins (*e.g.* terminolic acid and centellasaponin D) (Bylka et al., 2014).

* Corresponding authors.
E-mails: chaowalit@rsu.ac.th (C. Monton), Chitradee.L@rsu.ac.th (C. Luprasong).

https://doi.org/10.1016/j.bjp.2018.04.003



1 R=OH
2 R=H

The film forming polymeric dispersions (FFPD) is a new drug delivery systems. It is composed of active ingredient and inactive pharmaceutical additives including film former, plasticizer and other additives. When applying FFPD on the skin, a drug in the liquid form can permeate into the skin immediately. Subsequently, the solvent evaporates and the film is *in situ* formed on the skin and controls the drug permeation (Zurdo Schroeder et al., 2007). There are several publications which reported that FFPD can deliver some drugs such as betamethasone-17-valerate (Frederiksen et al., 2015; Garvie-Cook et al., 2015), ethinylestradiol (Zurdo Schroeder et al., 2007), and nicotine (Pichayakorn et al., 2013; Pichayakorn et al., 2015).

Stability study of herbal products is an important step in the drug approval process to assess the quality of the product at various time under the effect of environmental issues. So, the objective of the work was to validate the high performance liquid chromatography (HPLC) for simultaneous determination of stability of madecassoside and asiaticoside in *C. asiatica* extract-loaded film forming polymeric dispersions. HPLC method was validated in five topics including linearity and range, limit of detection (LOD) and limit of quantitation (LOQ), specificity, precision, and accuracy. The 90-day stability of the formulations stored at different temperatures was also investigated.

Materials and methods

Chemicals and reagents

Madecassoside (purity 95.0%, HPLC) was purchased from Sigma–Aldrich Inc., Missouri, USA. Asiaticoside (purity 99.81%, HPLC) was purchased from Chengdu Biopurify Phytochemicals Ltd., Sichuan, China. Eudragit® RS 30D and Eudragit® RL 30D (Evonik Nutrition & Care GmbH, Darmstadt, Germany) were gifted from Jebsen & Jessen Ingredients, Bangkok, Thailand. Commercial, standardized *C. asiatica* extract (*Centella asiatica* Cosmélène® containing madecassoside and asiaticoside (1%) in butylene glycol) was purchased from Greentech Biotechnologies, Saint Beauzire, France. Glycerin was purchased from Namsiang Co., Ltd., Bangkok, Thailand. Absolute ethanol was purchased from QRëC, New Zealand. Methanol and acetonitrile (HPLC grade) were purchased from Honeywell-Burdick & Jackson, Michigan, USA. Orthophosphoric acid (85%) was purchased from Carlo Erba, Val de Reuil, France. Reverse-osmosis water and ultrapure water were produced by water purifier (Mirae ST Co., Ltd., Gyeonggido, Korea).

Preparation of Centella asiatica *extract-loaded FFPD solution*

Three formulations of *Centella asiatica* (L.) Urb., Apiaceae, extract-loaded FFPD were prepared. Eudragit® RS 30D and Eudragit® RL 30D were used as film forming agent. Glycerin was used as plasticizer. Absolute ethanol was used as solvent and penetration enhancer. *C. asiatica* extract was used as an active ingredient. The 50 g of Eudragit® was used; F1, F2, and F3 composed of Eudragit® RS 30D and Eudragit® RL 30D in a ratio of 35:15 g, 25:25 g, and 15:35 g, respectively. Glycerin (20 g) was added and mixed using magnetic stirrer (CMAG HS7, Ika, North Carolina, USA). Absolute ethanol (20 g) was then added. Finally, *C. asiatica* extract (10 g) was added and mixed.

Stability test of Centella asiatica *extract-loaded FFPD*

Three formulations of *C. asiatica* extract-loaded FFPD were stored at 4 °C, 25 °C, and 40 °C. The formulations were sampled every 30 days for 90 days to analyze madecassoside and asiaticoside remaining in the formulations. The remaining madecassoside and asiaticoside were compared to an initial time. The formulation was diluted in water, sonicated, and adjusted to 100 mg/ml before analysis.

HPLC condition

Madecassoside (**1**) and asiaticoside (**2**) analysis was performed by reversed-phase HPLC (Agilent 1260 infinity, Agilent, California, USA). It was equipped with autosampler and photodiode array detector. The ACE 5 C18-PFP column (250 × 4.6 mm internal diameter, 5 μm) was used as stationary phase. It was controlled at 25 °C. Mobile phase composed of acetonitrile (A) and 0.01% orthophosphoric acid (B) was used. The gradient elution system holding 80% B for 2.5 min, decreased to 50% B in 4 min and holding for 2.5 min. Then, increased to 80% B in 1 min and holding for 2 min to equilibrate before the next injection. The flow rate of mobile phase was 1 ml/min. The injection volume was 10 μl. The detection wavelength was 210 nm.

Method validation

Linearity and range

The 1 mg/ml of stock solution of madecassoside and asiaticoside was prepared using methanol as solvent. The seven concentrations of the mixed standard were prepared: 5, 10, 25, 50, 75, 100, and 150 μg/ml. The standard solutions were filtered through 0.45 μm-pore size nylon syringe filter. They were then analyzed by the HPLC instrument ($n = 3$). The calibration curves of madecassoside and asiaticoside were constructed. The linear equation, coefficient of determination (R^2), and test range were reported.

LOD and LOQ

The blank sample was analyzed by HPLC instrument ($n = 6$). LOD and LOQ were calculated from the standard deviation of the peak area of the blank sample (σ) and the slope of the calibration curve (S) as showed below.

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{S}$$

$$\mathrm{LOQ} = \frac{10 \times \sigma}{S}$$

Table 1
Linearity parameters and limit of detection and limit of quantitation of madecassoside and asiaticoside analysis.

Topics	Madecassoside	Asiaticoside
Linear equation	$y = 192{,}231x - 389{,}939$	$y = 238{,}253x - 56{,}632$
R^2	0.9991	0.9998
Test range (μg/ml)	5–150	5–150
LOD (ng/ml)	81	21
LOQ (ng/ml)	245	64

Specificity

The specificity was achieved when the UV spectrum at the upslope, center, and downslope of the peak of the formulations in the same retention time of standard madecassoside and asiaticoside were similar to the UV spectrum of the two standards. Moreover, the specificity was also confirmed by a consideration of chromatogram of blank FFPD. The method was specific when no peak was found in the formulation at the same retention time of standard madecassoside and asiaticoside.

Precision

The three concentrations of the mixed standard were prepared; 25, 50, and 75 μg/ml. The standard solutions were filtered through 0.45 μm-pore size nylon syringe filter. They were then analyzed by the HPLC instrument ($n = 3$). The intraday and interday precisions were reported as the percent relative standard deviation (%RSD) of the analysis on the same day and the three different days, respectively.

Accuracy

Spike method was used for determination of accuracy. The three concentrations (25, 50, and 75 μg/ml) of each standard were added to *C. asiatica* extract-loaded FFPD with a known concentration of madecassoside (**1**) and asiaticoside (**2**). The concentration of madecassoside and asiaticoside in the formulation was 10 μg/ml based on final concentration. The samples were filtered through 0.45 μm-pore size nylon syringe filter. They were then analyzed by the HPLC instrument ($n = 3$). The percent recovery was reported.

Results and discussion

Stability testing result could estimate and also indicate the shelf life of the drug products. HPLC technique was used to determination of madecassoside (**1**) and asiaticoside (**2**) content remaining in the formulations. So, the HPLC method was validated in the work to assure the reliability of the analysis. The HPLC analysis in this work required short analysis time compared to other publications since we investigated only two active compounds (madecassoside and asiaticoside). Several peak and longer analysis times were found in

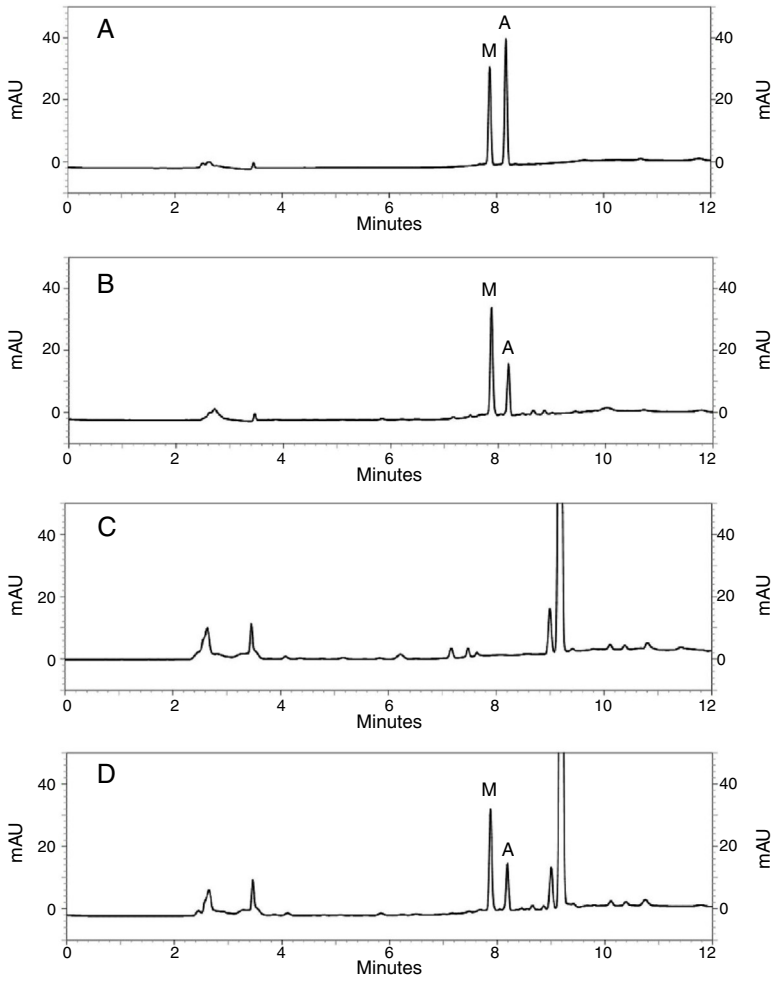


Fig. 1. HPLC chromatograms of (A) standard madecassoside (75 μg/ml) and asiaticoside (75 μg/ml), (B) *Centella asiatica* extract (10 mg/ml), (C) blank FFPD, and (D) *C. asiatica* extract-loaded FFPD (100 mg/ml). M and A stand for madecassoside and asiaticoside which were eluted at retention time of 7.9 and 8.2 min, respectively.

the analysis of active compounds in crude extract (Puttarak et al., 2016). Furthermore, HPLC method might be develop for simultaneous determination of madecassoside and asiaticoside in the formulation because some pharmaceutical ingredients interfered with the analysis. In addition, the peak of some ingredients was found in the chromatogram that might overlap with the peak of madecassoside and asiaticoside.

Table 1 shows the linearity, range, and LOD and LOQ of the analysis. HPLC method for simultaneous determination of madecassoside and asiaticoside showed a good linearity in the range of 5–150 μg/ml; R^2 of madecassoside and asiaticoside analysis were 0.9991 and 0.9998, respectively. LOD and LOQ were 81 and 245 ng/ml for madecassoside and 21 and 64 ng/ml for asiaticoside, respectively.

Fig. S1 (supplementary material) shows the UV spectra of standard madecassoside and madecassoside in the formulation. The UV spectra of madecassoside in the formulation at upslope, center, and downslope of peak had the same pattern compared to the standard madecassoside peak. The result was similar to the UV spectra of asiaticoside (Fig. S2, supplementary material). Furthermore, specificity was also confirmed by the HPLC chromatogram of the blank formulation of FFPD (Fig. 1C). No peak was found at the same retention time of standard madecassoside and asiaticoside. The result indicated that HPLC method was specific. Fig. 1A shows HPLC chromatogram of standard madecassoside and asiaticoside. They were eluted at retention time of approximately 7.9 and 8.2 min, respectively. The HPLC chromatograms in Fig. 1B and D showed madecassoside (**1**) and asiaticoside (**2**) in *C. asiatica* extract and in the formulation had the same retention time to the standard madecassoside and asiaticoside in Fig. 1A.

The HPLC method was precise and accurate, which is shown in Table 2. According to madecassoside, the %RSD of intraday and interday precision was less than 0.5% and 3%, respectively. In case of asiaticoside, the %RSD of intraday and interday precision was less than 1% and 0.6%, respectively. Moreover, the accuracy of the analysis was close to 100%. The percent recoveries of madecassoside and asiaticoside were 101.54–103.29% and 100.39–102.58%, respectively.

Table 2
Precision and accuracy of madecassoside and asiaticoside analysis.

Standards	Conc. (μg/ml)	Precision (%RSD)		Spike conc. (μg/ml)	Accuracy
		Intraday	Interday		Recovery (%)
Madecassoside	25	0.38	1.04	25	101.72 ± 2.62
	50	0.41	2.95	50	101.54 ± 4.44
	75	0.36	0.56	75	103.29 ± 4.03
Asiaticoside	25	0.40	0.32	25	102.51 ± 0.27
	50	0.82	0.52	50	100.39 ± 2.45
	75	0.05	0.40	75	102.58 ± 1.05

The contents of madecassoside and asiaticoside remaining in the formulations are shown in Fig. 2. *Centella asiatica* extract-loaded FFPD formulations were stored at different temperatures: 4 °C, 25 °C, and 40 °C for 90 days. Stability results showed that asiaticoside in the formulation was more stable than madecassoside; it was more stable at 4 °C and 25 °C than 40 °C. The high fluctuations of madecassoside and asiaticoside content were observed in the formulations stored at 40 °C. The result could be a result of the volatile property of absolute ethanol and water contained in the formulations. When temperature increased, ethanol and water evaporated, and the formulations were concentrated. So, the contents of madecassoside and asiaticoside from the HPLC analysis were high, especially at 30 days storage time, then it decreased

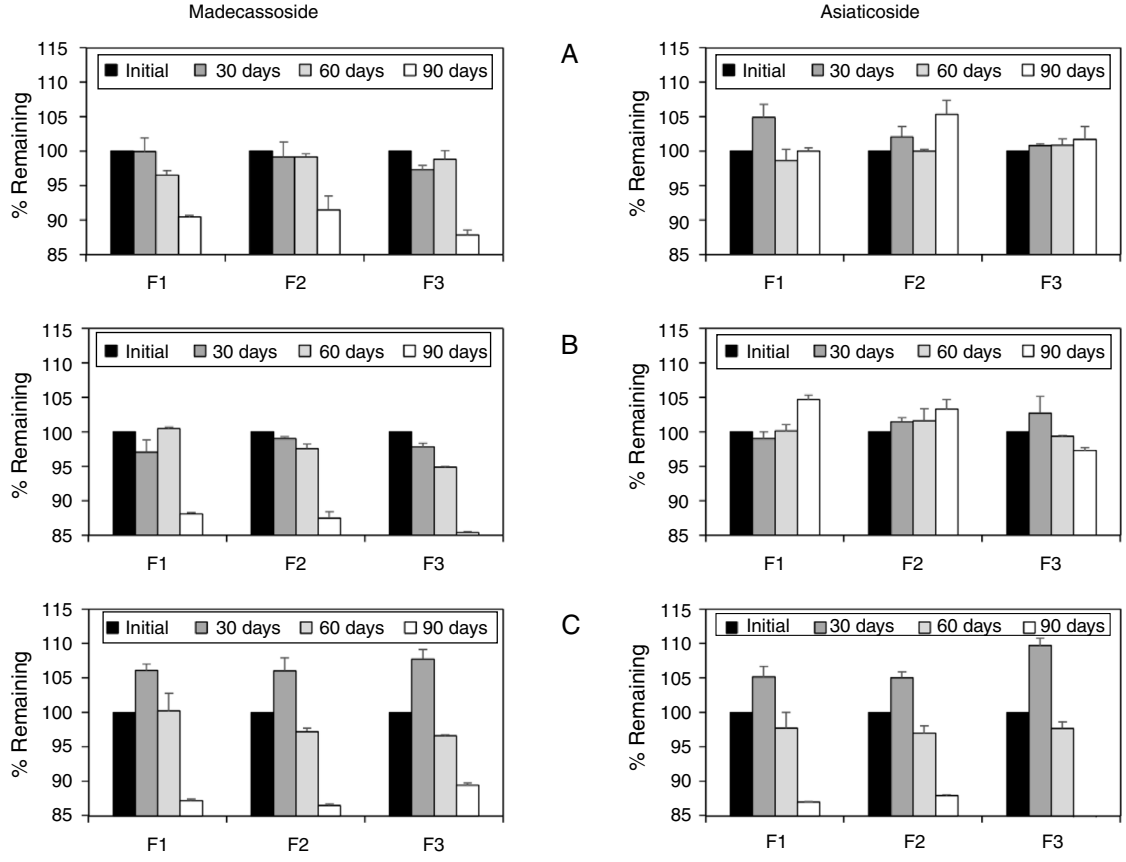


Fig. 2. Percentage remaining of madecassoside and asiaticoside in *C. asiatica* extract-loaded FFPD stored for 90 days at (A) 4 °C, (B) 25 °C, and 40 °C.

due to its degradation. Our findings are similar to the result of previous work. Puttarak et al. investigated the stability of standardized centelloids-enriched *C. asiatica* extract. They found that the glycosides madecassoside and asiaticoside degraded approximately 20% within twelve weeks under light-protected condition. Increasing storage temperature accelerated the degradation of madecassoside and asiaticoside. Furthermore, they decomposed under basic pH rather than acidic and neutral pH (Puttarak et al., 2016).

According to the stability of the formulation under different temperature conditions, all formulations were unstable for 90 days, except F1 and F2 stored at 4 °C. The content of madecassoside and asiaticoside was higher than 90%. The result indicated that the shelf life of F1 and F2 when stored at 4 °C was more than 90 days. Nevertheless, all formulations stored at any temperatures had shelf life more than 60 days but less than 90 days. High degradation of centelloids in the formulation was also reported by Inamdar et al. They investigated the stability of *C. asiatica* cream under different conditions. Results showed that when cream stored at room temperature and 40 °C for 3 months, the remaining of madecassoside in the cream was 73–100% and 65–100%, respectively, and the remaining asiaticoside was 85–91% and 73–87.5%, respectively (Inamdar et al., 1996). Glycoside compounds might be decomposed by the hydrolysis by chemical in the formulation and environmental condition or degradation naturely. The formulation will be developed in our future work to prevent the decomposition of madecassoside and asiaticoside.

Conclusions

The gradient HPLC method for simultaneous determination of madecassoside (**1**) and asiaticoside (**2**) in *C. asiatica* extract-loaded FFPD was validated. The method had a good linearity in the range of 5–150 μg/ml. HPLC method was also specific, precise, and accurate. The validated HPLC method was used to determine the 90-day stability of three formulations of *C. asiatica* extract-loaded FFPD stored at different temperatures. Almost all of the formulations were unstable. Nevertheless, two of three formulations stable at 4 °C. The formulation was further developed to improve the stability of madecassoside and asiaticoside in the formulation.

Authors' contributions

CM and CL contributed in all parts of work: running the laboratory work, analysis of the result and drafted the manuscript. JS and TS contributed in analysis of the data. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Research Institute of Rangsit University for research funding (grant no. 27/2559). We also thank Jebsen & Jessen Ingredients, Thailand for kindly support Eudragit® RS 30D and Eudragit® RL 30D. The authors are grateful to Professor Dr. J. E. Moreton, School of Pharmacy, University of Maryland at Baltimore, for his assistance in reading and editing the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.04.003.

References

Bylka, W., Znajdek-Awiżeń, P., Studzińska-Sroka, E., Dańczak-Pazdrowska, A., Brzezińska, M., 2014. *Centella asiatica* in dermatology: an overview. Phytother. Res. 28, 1117–1124.

Frederiksen, K., Guy, R.H., Petersson, K., 2015. Formulation considerations in the design of topical, polymeric film-forming systems for sustained drug delivery to the skin. Eur. J. Pharm. Biopharm. 91, 9–15.

Garvie-Cook, H., Frederiksen, K., Petersson, K., Guy, R.H., Gordeev, S.N., 2015. Biophysical elucidation of the mechanism of enhanced drug release and topical delivery from polymeric film-forming systems. J. Control Release 212, 103–112.

Inamdar, P.K., Yeole, R.D., Srivastava, M.M., Souza, N.J.D., 1996. Stability study of the active constituents in the *Centella asiatica* extract formulations. Drug Dev. Ind. Pharm. 2, 211–216.

Khare, C.P., 2007. *Centella asiatica* (Linn.) Urban. Indian Medicinal Plants: An Illustrated Dictionary. Springer New York, New York, pp. 1.

Pichayakorn, W., Suksaeree, J., Boonme, P., Amnuaikit, T., Taweepreda, W., Ritthidej, G.C., 2013. Deproteinized natural rubber film forming polymeric solutions for nicotine transdermal delivery. Pharm. Dev. Technol. 18, 1111–1121.

Pichayakorn, W., Suksaeree, J., Boonme, P., Taweepreda, W., Amnuaikit, T., Ritthidej, G.C., 2015. Transdermal nicotine mixed natural rubber-hydroxypropylmethylcellulose film forming systems for smoking cessation: *in vitro* evaluations. Pharm. Dev. Technol. 20, 966–975.

Puttarak, P., Brantner, A., Panichayupakaranant, P., 2016. Biological activities and stability of a standardized pentacyclic triterpene enriched *Centella asiatica* extract. Nat. Prod. Sci. 22, 20–24.

Zurdo Schroeder, I., Franke, P., Schaefer, U.F., Lehr, C.-M., 2007. Delivery of ethinylestradiol from film forming polymeric solutions across human epidermis *in vitro* and *in vivo* in pigs. J. Control Release 118, 196–203.