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VALIDATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY DENSITOMETRY METHOD FOR QUANTITATIVE DETERMINATION OF USNIC ACID IN ETHYL ACETATE EXTRACTS OF *USNEA SP*

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ABSTRACT

This research aims to determine usnic acid content in ethyl acetate extracts of six usnea sp (*Usnea mekista* (Stirt.) G. Awasthi, *Usnea baileyi* (Stirt.) Zahlbr. *Usnea articulata* (L.) Hoffm, *Usnea longissima* Ach. *Usnea perplexans* Stirt., *Usnea molliuscula* Stirt.) using validated High Performance Thin Layer Chromatography Densitometric Method. The thallus of lichen was extracted by maceration with ethyl acetate. Ethyl acetate extracts were evaporated by rotary evaporator. Separation was carried out on HPTLC silica gel 60 F_{254} plate as a stationary phase using a mixture of ethyl acetate-hexane (7:3 v/v) as the mobile phase. Detection was performed densitometrically in absorbance mode at 282 nm. Usnic acid standard solution in the concentration range of 50-500 µg/mL shows linearity with a correlation coefficient (r) = 0.9999. Limit of detection and limit of quantitation obtained were 5.20 µg/mL and 17.32 µg/mL, respectively. Precision of the method was evaluated by intraday and interday relative standard deviation (RSD). The results were intraday RSD of peak response 0.06-0.70% and interday RSD of peak response 0.25-1.16%. Accuracy was determined by calculating the percentage recovery at three concentration levels. The results were 98.37%, 97.54% and 99.22% respectively. Usnic acid contents found in *Usnea mekista* (Stirt.) G. Awasthi, *Usnea baileyi* (Stirt.) Zahlbr. *Usnea articulata* (L.) Hoffm, *Usnea longissima* Ach. *Usnea perplexans* Stirt., *Usnea molliuscula* Stirt. were 51.26%, 10.40%, 16.56%, 38.64%, 12.13%, and 50.92% respectively. The highest usnic acid content was found in the *Usnea mekista* species with a level of 51.26%. This HPTLC method was validated in accordance with

International Conference on Harmonization(ICH) guideline requirements and proven to provide appropriate results.

KEYWORDS: Usnic Acid; *Usnea* sp; Validation; HPTLC Densitometry.

INTRODUCTION

Usnic acid (Figure 1) is a compound that has many biological activities such as antimicrobial, antifungal, antiviral, antiprotozoa, antiproliferative, anti-inflammatory, analgesic, antipyretic, anti-herbivore and anti-insect effect.[1,2,3,4] One of the lichen containing usnic acid is *Usnea sp*. Based on empirical experience, *Usnea* sp, which in its local language is called kayu angin, has properties as antidiarrheal, treatment of bloody stools, seizures, abdominal pain, difficulty urinating, menstrual disorders, hemorrhoids and headaches.[5]

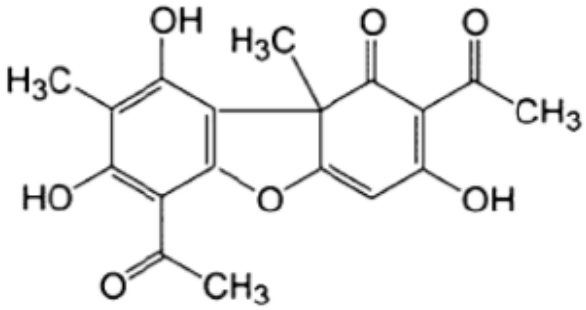


Fig. 1: Structure of usnic acid.[7]

Previous research on the determination of usnic acid contents from *Usnea subflorida, Usnea florida, Usnea barbata, Usnea longissima, Usnea hirta,* and *Usnea rigida* by HPLC method have been carried out by Cansaran et al., 2006.[6] Meanwhile, TLC densitometry method was used for determining usnic acid content in *Usnea siamensis* by Chaowuttikul et al., 2014.[7]

High performance thin-layer chromatography (HPTLC) is mainly applied for the identification, qualitative and quantitative estimation of herbal and animal extracts, fermentation mixtures, drugs and excipients and formulated herbal products.[8] HPTLC is one of the useful methods which doesn't need tedious clean up and by using appropriate mobile phases and reagents, all interfering agents will be omitted.

The High Performance Thin Layer Chromatography (HPTLC) densitometry method was used for the determination of six Usnea sp species because this method is a very rapid, accurate and precise chromatographic technique for many herbal components assay and can be used

for routine quality control of herbal products.[9] In this research, quantitative analysis of usnic acid in ethyl acetate extracts of usnea sp by means of HPTLC-densitometry method has been developed and validated for selectivity, linearity, accuracy, intra and interday precision.

MATERIALS AND METHODS

Lichen material

Six lichens species were collected in the highlands and mountains in the regions of West Sumatra, Indonesia. We confirmed our identification by sending all samples to Harrie Sipman (Berlin Museum) and voucher specimen numbers were deposited at the Biota Sumatran Laboratory, Andalas University, West Sumatra (Indonesia) with the reference numbers cited in Table 1 (FFJT 22, FFJT 23, FFJT 25, FFJT 26, FFJT 27, FFJT 28).

Table 1: *Usnea* sp species were analyzed.

Sample code	Species	Voucher specimen numbers
E22	*Usnea mekista* (Stirt.) G. Awasthi	FFJT 22
E23	*Usnea baileyi* (Stirt.) Zahlbr.	FFJT 23
E25	*Usnea articulata* (L.) Hoffm	FFJT 25
E26	*Usnea longissima* Ach..	FFJT 26
E27	*Usnea perplexans* Stirt.	FFJT 27
E28	*Usnea molliuscula* Stirt.,	FFJT 28

Equipment

The equipment used in this study were the analytical balance (Kern), HPTLC silica gel 60 F_{254} plates (20 x 20 cm) (Merck, Germany), Camag TLC Scanner 4 with vision CATS (CAMAG), UV-Vis spectrophotometers (Shimadzu 7000 Pharmaspec) and laboratory equipment that is common for chemical analysis.

Chemicals

Pro analytic solvent, ethyl acetate and methanol were purchased from Merck. The usnic acid was obtained from Facility for Developing and Improving Production Capacity of Medicine and Traditional Medicine 2015, Indonesian Ministry of Health, Faculty of Pharmacy, Andalas University.

PROCEDURES

Preparation of samples

The thallus of lichens was dried and grinded. The amount of 50 grams of thallus powder was dissolved in 100 ml ethyl acetate for three days. Thus, it was filtered. Maceration was conducted twice in this process.

Preparation of standard solution

Standard stock solutions were prepared by dissolving 10 mg of usnic acid in 10 mL ethyl acetate to get a concentration of 1000 µg/mL and it was diluted to get solution with the following concentrations of 50 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL and 500 µg/mL. These solutions were perfectly sealed and kept out of sun light.

Determination of maximum absorption wavelength

The standard stock solution of usnic acid with a concentration of 1000 µg/mL was diluted to get a concentration of 10µg/mL. This solution was analyzed by UV spectrophotometer at a wavelength of 200-400 nm to determine its maximum absorption wavelength.

Optimized chromatographic conditions for estimation of usnic acid from *Usnea sp* samples

The HPTLC system used consisted of CAMAG TLC Scanner 4 supported by win CATS software version 1.4.7 equipped with CAMAG Linomat 5 sample spotter and CAMAG Reprostar 3 system for photo-documentation. Chromatographic separation of the phytochemical constituents was achieved on TLC plate (E. Merck) pre-coated with silica gel 60 F254 (0.2 mm thickness) on aluminium sheet support. Plate was developed up to a distance of 80.0 mm in CAMAG twin trough glass chamber pre-saturated with mobile phase of ethyl acetate-hexane by ratio1:1; 2:8; 3:7; 4:6; 6:4; 7:3; and 8:2 for 15 minutes. The plates were dried at room temperature and then heated to identify compact bands. Quantitative analysis was performed at wavelength 254 nm in reflectance mode with CATS 4 operated by Win CATS software (Version 1.4.7).

Validation of analytical method

The described method was validated according to the ICH guidelines.[10] The following validation characteristics were evaluated: linearity, precision, accuracy, the limits of detection (LOD) and the limit of quantification (LOQ).

Linearity. Each standard solution with the concentrations of 50 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL and 500 µg/mL was applied on HPTLC plate. Plate was developed by using selected mobile phase and analysed by using *HPTLC scanner*.

Limit of Detection and Limit of Quatitation were calculated through the slope and standard deviation method using the following formula: LOD = (3.3 × δ) / S, and LOQ = (10 × δ) / S, Where: δ: is the standard deviation of the Y intercept of the linear regression equations. S: is the slope of the linear regression equations.

Precision. The standard solution was made as three different concentrations such as 100 µg/mL, 300 µg/mL, and 500 µg/mL. The intra-day assay involved three replicates per day and inter-day assay were performed on three following days.

Accuracy. Standard solution of usnic acid was added at three different concentration (40%, 80%, and 120%) to the sample.Three determinations were performed for each concentration level.

$$\text{Recovery (\%)} = \frac{Cobs - Cs}{Cst} x100\%$$

Where: C_{obs} is the observed concentration of usnic acid detected in the sample solution after added standard usnic acid solution (µg.mL^{-1}). C_s is the concentration of usnic acid detected in mangosteen peel extract sample solution without added standard usnic acid solution (µg.mL^{-1}). C_{st} is the actual concentrations of standard usnic acid solution (µg.mL^{-1})

Estimation of usnic acid from ethyl acetate extracts of six *Usnea sp.*

Ethyl acetate extracts of six *Usnea sp* were dissolved in mixed solvent of ethyl acetate and methanol (7:3 v/v), then it was applied on HPTLC silica gel 60 F_{254} plate. The content of usnic acid in each samples was determined by plotting data of its area under curve (AUC) in the linear regression equation of calibration curve.

RESULTS AND DISCUSSSION

The standard solution of usnic acid in ethyl acetate at a concentration of 20 µg/mL was analyzed by UV spectrophotometer at a wavelength of 200-400 nm, giving a maximum absorption wavelength of 282 nm with an absorbance value of 0.636 (Figure. 2).

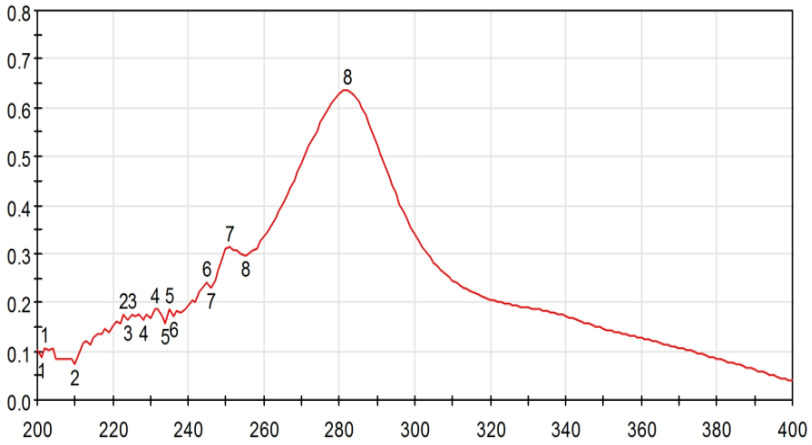
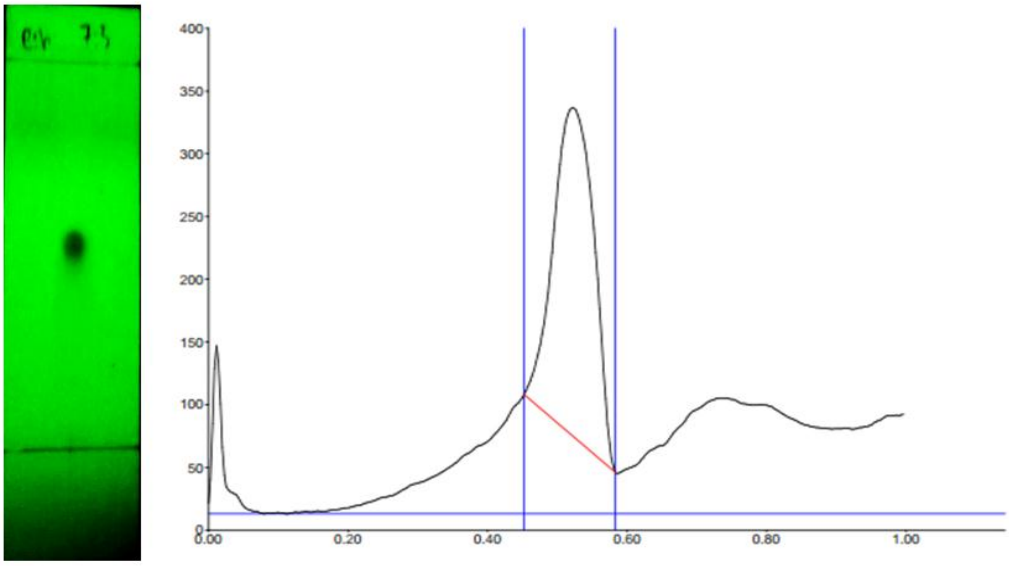


Fig. 2: UV spectrum of 10 µg/mL usnic acid in ethyl acetate solvent (λmax = 282 nm, A = 0.636).

In this research, HPTLC densitometry method was validated for determining usnic acid levels in ethyl acetate extracts from six usnea species, namely *Usnea mekista* (Stirt.) G. Awasthi, *Usnea baileyi* (Stirt.) Zahlbr., *Usnea articulata* (L.) Hoffm, *Usnea longissima* Ach., *Usnea perplexans* Stirt, and *Usnea molliuscula* Stirt. The plates were developed with different mobile phases than those used in previous studies. Chromatographic separation was performed using silica gel high performance thin-layer chromatography (HPTLC) plates with ethyl acetate - hexane (7:3 v/v) as mobile phase. Usnic acid Rf-value was about 0.51 (Figure 3a) and spots were scanned at 282 nm through a mercury lamp (Figure 3b).



(a) **(b)**

Fig. 3: (a) HPTLC plate of usnic acid with ethyl acetate – hexane (7:3 v/v) as mobile phase, (b) densitogram of usnic acid (300 µg/mL).

Polonia et. al.,[11] validated the densitometry HPTLC method for determining usnic acid levels in chloroform extract of *Usnea florida* (L.) and the mobile phase used was toluene-acetic acid glacial (9:1). Whereas, Sing et al.,[12] validated the densitometry HPTLC method for determining usnic acid levels in 4 species, namely *Usnea aciculifera* (UA), *U. ghattensis* (UG), *U. longissima* (UL) and *U. stigmatoides* (US) from acetone extract. Separation and determination of usnic acid using the mobile phase of toluene-1,4-dioxane-formic acid (18:4.5:0.2, v/v).

Validation of the method of analysis was carried out based on ICH (International Conference on Harmonization) in 1995. The results showed linear relationship between the peak areas and the usnic acid concentrations. The plot was linear in the range of 50-500 µg/mL ($y = 85.249 + 20.022 X$, $r = 0.9999$). A method can be said to have good linearity if it has a correlation coefficient of $0.98 \leq r < 1$.[13]

This analysis method shows sensitivity in the limit of detection (LOD) of 5.20 µg/mL and limit of quantitation (LOQ) of 17.32 µg/mL. The determining value of LOD was conducted to determine the lowest concentration levels of sample with significant response toward blank specimen. Meanwhile, the LOQ was conducted to determine the lowest analytical concentration of the sample which can be calculated precisely and accurately matched with the operational condition of applied method.[14]

Both intraday and interday precision was determined in terms of % coefficient of variation (CV). Intraday and interday precisions (n=6) for usnic acid were found to be 0.06 – 0.70% and 0.25 – 1.16%, respectively, which demonstrated the good precision of proposed method. Precision values meet requirements because of the value of CV<2%.[13]

Analysis of determining level was conducted toward the ethyl acetate extract of six *Usnea sp* with following sample codes E22, E23, E25, E26, E27, and E28. The level was established by dissolving the extract into ethyl acetate and methanol solution. Average concentration in E22, E23, E25, E26, E27 and E28 were 51.26, 10.40, 16.56, 38.64, 12.13, and 50.92% respectively. Data of usnic acid level of each species can be seen in the Table 3.

Table 3: Percentage of usnic acid content in *Usnea sp.*

Sample code	Usnic acid content (%)
E22	51.26±0.90
E23	10.40±1.27
E25	16.56±1.29
E26	38.64±7.00
E27	12.13±3.64
E28	50.92±2.57

The highest level of usnic acid was found in the E22 sample with the name of *Usnea mekista*, followed by the E28 sample with the species name of *Usnea molliuscula* Stirt. The different level of usnic acid in the six species can be caused by different growth condition of each species. Secondary metabolic was produced by certain organism in specific condition such as height, raindrops, and land fertilizer.[15] Differences in growing regions result in different compounds and pharmacological activities.[16]

Accuracy is the closeness of agreement between the values found expressed by % recovery. In this research, a standard addition method was applied to the test solution whose concentration was known. The percentage of recovery obtained at the addition of 40%, 80% and 120% of usnic acid standard were 98.35%; 97.53%; and 99.22% respectively. Percentage of recovery obtained meets the requirements in the range of 80-110%.[13] The data can be seen in Table 2.

Table 2: **Validation of HPTLC densitometry analytical method.**

Parameters	Results
Linearity	
Linearity range	50-500µg/mL
Regression equation	Y = 85,249 + 20,022X
Correlation coefficient (r)	0,9999
Limitof Detection and Limit of Quantitation (µg/mL)	5,20 and 17,32
Precision (%)	
Interday	0,25 – 1,16
Intraday	0,06 – 0,70
Accuracy (%)	
Addition of 40%	98,37
Addition of 80%	97,54
Addition of 120%	99,22

Table 3: Percentage of usnic acid content in *Usnea sp*.

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E28	50.92±2.57

CONCLUSION

Determination of usnic acid levels in ethyl acetate extract from six Usnea sp species using the HPTLC-Densitometry method met the validation requirements which included linearity with a correlation coefficient of 0.9999, detection limit of 5.20 µg/mL and quantitation limit of 17.32 µg/mL, precision intraday and interday which produces % CV <2 and accuracy that gives recovery in the range of 80-110%. The highest usnic acid content were found in *Usnea mekista* with a level of 51.29% and *Usnea molliuscula* Stirt with a level of 50.92%.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

REFERENCES

1. Yamamoto Y, Miura Y, Kinoshita Y, Higuchi M, Yamada Y, Murakami A, Ohigashi H, Koshimizu K. Screening of tissue cultures and thalli of lichens and some of their active constituents for inhibition of tumor promoter-induced epstein-barr virus activation. Chem. Pharm. Bull, 1995; 43: 1388–1390.
2. Fournet A, Ferreira ME, Rojas De Arias A, Torres De Ortiz S, Inchausti A, Yaluff G, Quilhot W, Fernandez E, Hidalgo ME. Activity of compounds isolated from chilean lichens against experimental cutaneous leishmaniasis. Comp Biochem Physiol, 1997; 111: 69–74.
3. Ingo´lfsdo´ttir, K. Usnic acid. Phytochemistry, 2002; 61(7): 729–736.

4. Cocchietto M, Skert N, Nimis PL, Sava G. A review on usnic acid, an interesting natural compound.Naturwissenschaften, 2002; 89: 137–146.
5. Maulidiyah, CahyanaAH,Suwarso WP, Nurdin M. Isolation and structure elucidation of eumitrin A1 from lichen usnea blepharea motyka and its cytotoxic activity. International Journal of PharmTech Research, 2015; 8(4): 782-789.
6. Cansaran D, Kahya D, Yurdakulol E, Atakol O. Identification and quantitation of usnic acid from the lichen usnea species of anatolia and antimicrobial activity. Verlag der Zeitschrift für Naturforschung, 2006; 61c: 773-776.
7. Chaowuttikul C, Thitikornpong W, PalanuvejC, Ruangrungsi N. Quantitative determination of usnic acid content in Usnea siamensisby TLC densitometry and tlc image analysis. Res. J. Pharm., Biol. Chem. Sci., 2014; 5(1): 118-125.
8. Biringanine G, Chiarelli MT, Faes M, Duez P. A validation protocol for the HPTLC standardization of herbal products: application to the determination of acteoside in leaves of Plantago palmata Hook. f.s. Talanta, 2006; 69: 418-424.
9. Dhalwal K, Biradar YS, Shinde VM, Mahadik KR and Rajani M. Phytochemical evaluation and validation of a polyherbal formulation using HPTLC. Phcog Mag, 2008; 4: 89-95.
10. ICH guidelines. International conference on harmonization of note for guidance on validation of analytical procedures: text and methodology, Step 5 of the ICH Process, 1995.
11. Polónia MA, Carneiro AF&Pinto MMM. Quantification of Usnic Acid in Usnea Florida by Densitometric HighPerformance thin Layer Chromatography. Journal of Liquid Chromatography, 1991; 14(8): 1447-1452.
12. Singh S, Khatoon S, JoshiY, Prgyadeep S, Upreti DK, & Rawat AKS. A validated HPTLC densitometric method for simultaneous determination of evernic and usnic acids in four Usnea species and comparison of their antioxidant potential. Journal of chromatographic science, 2016; 54(9): 1670-1677.
13. Harmita. Petunjuk pelaksanaan validasi metode dan cara perhitungannya. Majalah Ilmu Kefarmasian, 2004; 1(3).
14. Ermer J, Miller JH, editors. Method validation in pharmaceutical analysis: A guide to best practice. John Wiley & Sons, 2006 Mar 6.
15. Sudibyo RS. Metabolit Sekunder : Manfaat dan Perkembangannya dalam Dunia Farmasi.Pidato Pengukuhan Guru Besar Univ. Gadjah Mada. Yogyakarta, 2002.

Availableat:https://repository.ugm.ac.id/digitasi/download.php?file=2573_pp110600014.pdf[Accessed 15 January 2019].

16. Widyastuti YE and Regina K. 2000. Duku, Jenis Dan Budaya. Jakarta: Penebar Swadaya.