Rapid and Sensitive HPLC Method for the Determination of Polyphenols in Various Lichen Species of Himalayan Origin

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Abstract: A reversed-phase high performance liquid chromatograph (HPLC) with photodiode array detection method was developed to determine bioactive polyphenolic substances, methyl β-orcinolcarboxylate (MBO) and ethyl haematommate (EH) in thalli of various lichen species. The MBO and EH were separated by RP-HPLC (C_{18} column, 150 mm × 4.6 mm, 5 μm) using isocratic elution systems of acetonitrile:water (0.1% acetic acid). Base line separation of the compounds was obtained in less than 20 min. The method was validated for linearity, repeatability, limits of detection (LOD), and limits of quantification (LOQ). Repeatability (inter- and intra-day, $n = 6$) showed less than 1.5% relative standard deviation (RSD). The LOD and LOQ were found to be 5.97 and 15.51 ng for MBO and 42.63 and 69.03 ng for EH, respectively. The validated HPLC method was employed to quantify MBO and EH in eleven lichen species, used in folklore/traditional systems of medicine collected from the Himalayan region of India.

Keywords: Lichen, Polyphenol, Methyl β-orcinolcarboxylate (MBO), Ethyl haematommate (EH), RP-HPLC

INTRODUCTION

Lichens are complex plants consisting of a slow growing symbiotic association of fungi and algae. They produce characteristic secondary metabolites that are unique with respect to those of higher plants.[1] Several lichen

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extracts have been used for various remedies in folk medicine, indicating the frequent occurrence of metabolites with various properties such as anti-biotic,[2] antimycobacterial,[3] antiviral,[4,5] analgesic and antipyretic,[6] anti-oxidant,[7,8] anticoagulant and antithrombotic.[9] There are about 13,500 lichen species growing through out the world and they produce a wide range of chemical compounds. Among these, approximately 350 secondary metabolites are produced through polyketide, shikimic acid, and mevalonic acid pathways.[10] Various phenolic compounds are extensively studied for a range of bioactivities such as antibacterial, antifungal, antiviral, anticancer, antioxidant, and preservatives from various lichen species.[11−16] In various systems of traditional medicine worldwide, including the Indian system of medicine, these lichen species are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders, spermatorrhoea, and many disorders of the blood and heart.[17,18]

Isolation of the phenolic compounds, atranorin, methyl orsenillate, orse-nillic acid, and lecanoric acid from the lichen *Parmotrema tinctorum* (Nyl.) Hale (*P. tinctorum*) using thin-layer chromatography (TLC) and column chromatography, respectively, was described.[19] HPLC methods have been reported for the analysis of secondary metabolites of lichen such as benzoic and solorinic acids,[20] sekikaic acid.[21] Several other chemicals such as atranol, chloroatranol, hematommic acid, chlorohematommic acid, methyl haematommate, methyl chlorohaematommate, ethyl haematommate, ethyl chlorohaematommate, methyl *β*-orsellinate, atranorin, chloroatranorin, and (+)-usnic acid have been quantified in *Lethariella canariensis* using the HPLC method.[16] This method, however, lacks good base line separation, validation, and sensitivity limits, and has a longer analysis time. To the best of our knowledge, no validated analytical method has so far been reported for the quantitative estimation of polyphenolic compounds, specifically, Methyl *β*-orcinolcarboxylate and Ethyl haematommate in lichen species.

In this paper, we report an accurate and precise, RP-HPLC-PDA method for analysis of biological active polyphenolic metabolites, methyl *β*-orcinolcarboxylate (MBO) and ethyl haematommate (EH) in various lichen species of Himalayan origin. The present validated HPLC method is suitable and specific for rapid screening of these metabolites in complex biological samples of lichen species widely used in folklore and traditional medicine systems, particularly of the Asian regions of Tibet, Bhutan, Nepal, China, and India.

EXPERIMENTAL

Solvents and Standards

Acetonitrile (Merck, India), methanol (Merck, India), and glacial acetic acid (Merck, India), and Milli-Q water used were of HPLC grade. Analytical

grade ethanol was used for extraction of methyl β-orcinolcarboxylate (MBO) and ethyl haematommate (EH) from lichens. MBO and EH were isolated from lichen (*Everniastrum cirrhatum*) in our laboratory and authenticity confirmed by their spectral analysis.[11,22] The structure of MBO and EH are presented in Figure 1.

HPLC System and Chromatographic Conditions

The Waters HPLC apparatus (Milford, MA, USA) consisted of a 1515 solvent delivery system, 717 auto-injector equipped with 2996 photodiode array detector. Data obtained were processed using Waters EmpowerPro chromatographic software. The injector, solvent delivery controller, and Empower Pro chromatographic manager were integrated to give precise and reproducible results. Column Symmetry C_{18} (150 × 4.6 mm id, 5 μm, Waters) was used for method development. In addition, a Mettler Toledo AG-245 electronic balance, and Millipore filtration assembly were used in the study. A mobile phase consisting of a mixture of (A) Acetonitrile - (B) H_2O(0.1% AcOH) (50:50 v/v) was filtered, degassed by sonication, and used at a column temperature of 28°C and a flow rate of 1.0 mL/min. The scanning acquisition of the column effluent was monitored over the range 210–400 nm at 1.2 nm intervals of the absorption spectrum, and components were quantified at 265 nm. Stock solution (1 mg/mL) of MBO and EH were prepared in methanol. The targeted polyphenols standards gave responses in the range of 0.02–1.0 AUFS, depending on the concentration of the component.

Lichen Material and Sample Preparation

Various lichens species, *Everniastrum cirrhatum*, *Flavoparmelia caperata*, *Heterodermia leucomela*, *Lecanora flavidorufa*, *Leptogium pedicellatum*,
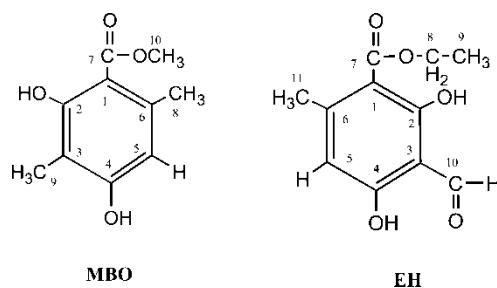

Figure 1. Chemical structures of ployphenols methyl β-orcinolcarboxylate (MBO) and ethyl haematommate (EH).

Lobaria isidiosa, *Phaeophyscia hispidula*, *Ramalina*, *Rimelia reticulate*, *Stereocaulon foliolosum*, and *Usnea* were collected from the Himalayan region of Narayan Ashram, situated on Kailash Mansarowar Yatra Marg, District of Pitthoragarh, Uttaranchal, India, which is a cold climatic region about 1800 meters above sea level. The lichen species were identified and the specimen vouchers have been deposited in the Institute's repository for the record. The selection of lichen species was based on their use in folk medicine and, also, some species were taken randomly. Lichens were air dried at room temperature under shade. After air drying, they were ground to a fine powder in a grinder. The powdered material (1.0 g) was extracted with 50 mL of 95% ethanol by cold percolation overnight, at room temperature. The extracts were filtered using Whatman filter paper No. 1 and concentrated at 40°C under reduced pressure with a rotary evaporator. The concentrated extract was lyophilized to obtain a fine crude extract. This lyophilized extract was dissolved in 1 mL HPLC grade methanol. Prior to injection, all samples were filtered through 0.45 μm PTFE filters.

Method Validation

The method was validated according to the ICH guidelines[23] on the validation of analytical methods, which covers various parameters like linearity range, precision, reproducibility, specificity, accuracy, recovery, and system suitability. All results were expressed as percentages (dry weight basis), and n represents the number of replicates. For the statistical analysis, SPSS and Excel 2000® (Microsoft) software were used.

Linearity

A stock solution of MBO and EH 1 mg/mL was prepared by dissolving in methanol. Various dilutions were prepared using stock solutions in the concentration range of 6.25–100 μg/mL in methanol. The samples were filtered through 0.45 μm nylon filters, and 20 μL of each were injected, in triplicate, on the column. Peak areas for five injections were used for the computation of slope; intercept and correlation coefficient (r^2) were estimated.

Repeatability

Repeatability of the method was checked by analyzing six replicate samples of three concentration levels of metabolites (12.5, 25, and 50 μg/mL of MBO and EH) and calculating percent relative standard deviation (% R.S.D.) of retention time and peak area.

Intermediate Precision

Intermediate precision of the method was checked by repeating the procedure for 3 consecutive days and calculating the R.S.D. between 3 days for retention time and peak area. The standard deviation and coefficient of variation were calculated for each day. In order to check whether the results obtained on the different days were significantly different, the results were analyzed by means of an ANOVA. Within- and between-days variation coefficients were calculated.

Accuracy and Recovery of MBO and EH from Thallus (Assay by Spiking)

The developed analytical method was validated for its accuracy in determining the metabolites content in lichen extracts. For demonstrating accuracy of an analytical method, three concentration levels of metabolite solution (12.5, 25, and 50 μg/mL concentrations of MBO and EH) were prepared in triplicate and analyzed. Recovery studies from lichen extract (*Everniastrum cirrhatum*) was carried out by spiking a specified amount of metabolites (12.5, 25, and 50 μg/mL concentrations of MBO and EH) in 10 mg of extract matrix in a small vial. Solutions were filtered through 0.45 μm nylon filters and 10 μL of this solution was injected in triplicate for obtaining the percentage recovery on the same day.

System Suitability

Data from 10 μL of six injections of 50 μg/mL was utilized for calculating system suitability parameters like capacity factor, USP tailing factor, and number of theoretical plates.[24]

Robustness

Overall appearance of the chromatogram depends on a wide range of factors covering dynamic properties of the device used for mixing of solvents, dwell volume of the system, and efficiencies of various components of the instrument. These factors play a very critical role in the case of multi-step gradients. In the present study, we have developed a method with isocratic elution; therefore, robustness of the method is not as critical as in the gradient mode. However, efforts were made to evaluate the robustness of the developed method. The influence of small deliberate variations of the chromatographic condition in the analysis of MBO and EH were examined throughout. The factors examined were flow rate (mL min^{-1}), mobile phase (percentage of solvent A and solvent B), and temperature (°C). One parameter at a time was changed to estimate the effect. In each assay, six injections of 10 μL working standard solutions were studied.

RESULTS AND DISCUSSION

HPLC chromatographs of a mixture of two reference compound solutions (MBO and EH) and a representative lichen thallus (*Everniastrum cirrhatum*) extract are presented in Figure 2. MBO and EH elute at 4.916 and 14.969, respectively.

HPLC Method Development

The coelution of other components of a complex biological sample matrix with the targeted compound can be checked by means of PDA spectral data acquisition. Separation and photodiode array spectrum of MBO and EH (A) iso-absorbance map (contour plot) and (B) 3D view and (C) UV-spectra of reference compounds (MBO and EH) and methanol extract of lichen thallus (*Everniastrum cirrhatum*) are presented in Figure 3. The reliability of the
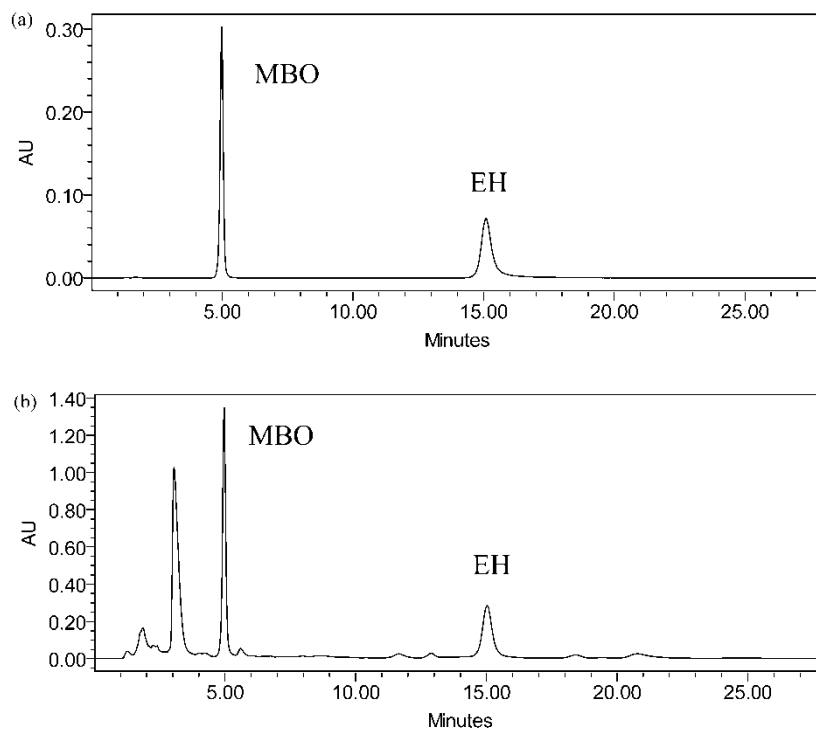
Figure 2. Representative chromatograms (a) reference compounds MBO (Methyl β-orcinolcarboxylate) and EH (ethyl haematommate) and (b) Methanol extract of lichen (*Everniastrum cirrhatum*). Chromatographic conditions are described in experimental section.
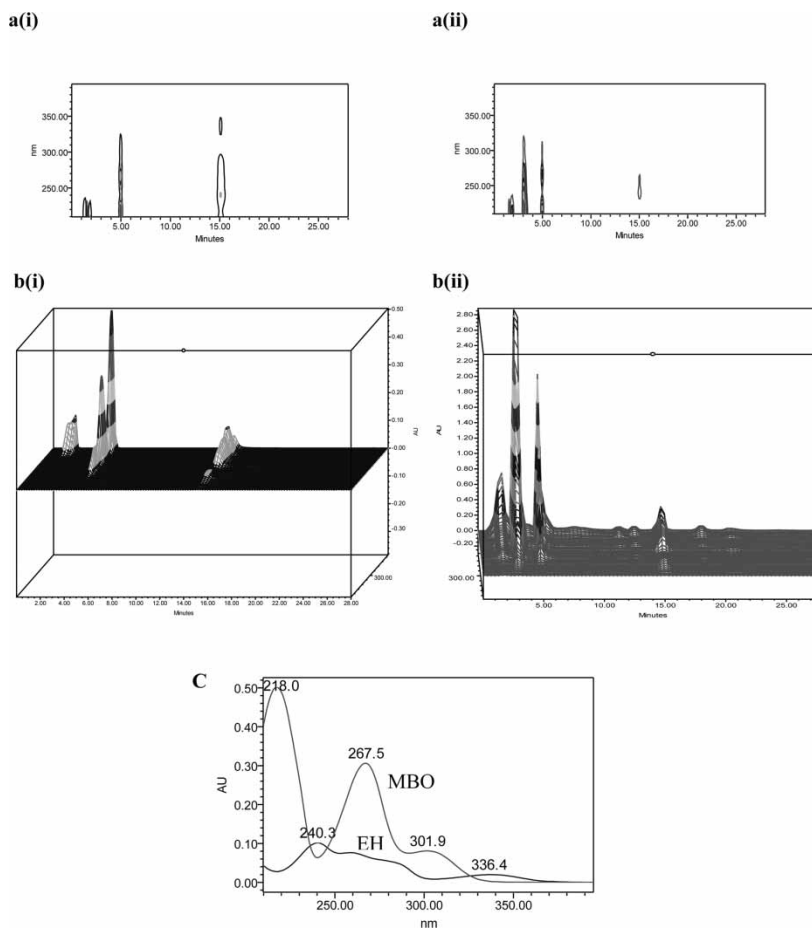
a(i)   a(ii)

b(i)   b(ii)

C

Figure 3.   Separation and photodiode array spectrum of MBO and EH (a) iso-
absorbance map (contour plot), (b) 3D view and (c) UV-spectra of compounds obtained
in chromatographic run of (i) standards and (ii) methanol extract of *Everniastrum
cirrhatum.*

peak purity test can further be enhanced by carrying out the test at various
wavelengths. It is interesting to note, that the results of peak purity tests
slightly vary according to the wavelength used in both standard and real
sample analysis (Table 1). Typically, when using PDA software (Waters
Empower®), a component with a peak purity angle lower than its purity
threshold would be considered most likely to be a pure substance and the
separation would be acceptable. The absorption spectra of both peaks were
stored in the software. At 265 nm, the degree of peak purity is higher and,
also, the absorption spectrum of both compounds is optimum for simultaneous
quantitation. Therefore, quantitation of both compounds, MBO and EH, was

Table 1. Effect of the wavelength selection on the results of peak purity test in the simultaneous analysis of targeted compounds in lichens

Wavelength (nm)	Methyl β-orcinolcarboxylate		Ethyl haematommate	
	Purity angle	Purity threshold	Purity angle	Purity threshold
Standard (50 μg/mL)				
230	0.055	0.266	0.099	0.280
254	0.054	0.266	0.101	0.281
265	0.055	0.267	0.104	0.281
280	0.053	0.265	0.096	0.277
Sample (Lichen extract)				
230	0.861	0.811	0.056	0.234
254	0.849	0.865	0.096	0.236
265	0.564	0.892	0.110	0.236
280	0.829	0.865	0.095	0.236

performed at 265 nm with peak area data acquisition for both reference and sample solutions.

Linearity

Regression statistics computed for MBO and EH analytical tests are summarized in Table 2. The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to concentration of substance within a working concentration range (6.25 μg/mL-50 μg/mL). The linearity of the method was observed in the expected concentration range, demonstrating its suitability for analysis. The goodness of fit (r^2), i.e., correlation coefficient, was found to be 0.9990 and 0.9992 for MBO and EH, respectively. Slopes and intercepts of these

Table 2. Regression statistics for methyl β-orcinolcarboxylate and ethyl haematommate analysis

Compounds	Concentration range[a]	Linear regression equation	Goodness for fit (r^2)	Limit of detection (LOD) ng	Limit of quantitation (LOQ) ng
MBO	6.25–50 μg/mL	Y = 6439.8 X − 12086	0.9990	5.97	15.51
EH	6.25–50 μg/mL	Y = 2328.2 X − 72896	0.9992	42.63	69.03

[a]Number of replicate three.

equations of relationship were used for the computation of limit of detection (LOD) and quantitation (LOD) of MBO and EH, considering the three and ten times signal to noise ratio, respectively. Regression equation and sensitivity values (LOD and LOQ) for MBO and EH are depicted in Table 2.

Precision

The precision of an analytical method represents the degree of agreement among the individual analytical results, when the method is applied repeatedly to multiple samples. Repeatability in HPLC refers to the use of chromatographic conditions within a laboratory, over a short period of time, using the same compound with the same equipment, and is expressed as the percent R.S.D. The present method agreed with the test for repeatability, as determined by percent R.S.D (%) of the area of the peaks and retention time of six replicates injection at 100% assay concentration, i.e., (50 µg/mL) were in the range of 0.245–1.140%.

Intermediate precision was estimated by quantifying the variations (peak area and retention time) in analysis when a method is used within same laboratory, on different days, by different analysts, and on same equipment. The intermediate precision was studied by preparing the standard curve for 3 different days, and the results of inter-day variation are given in Table 3. The method passed the test for intermediate precision, as percent R.S.D. of the retention time and peak area obtained within 3 different days were within the range of 0.458–1.341%. No significant difference was observed in intra and inter-day estimation of peak area and retention time of both MBO and EH, when calculated by one way ANOVA.

Specificity

Specificity is the ability of an analytical method to assess, unequivocally, the substance in the presence of components that are present in the sample matrix. The representative chromatogram (Figure 2) of thallus extract of lichen shows

Table 3. Intermediate precision of methyl β-orcinolcarboxylate (MBO) and ethyl haematommate (EH) analysis

| Concentration (µg/mL) | Intra-day variation (%RSD) | | | | Inter-day variation (%RSD) | | | |
| | MBO | | EH | | MBO | | EH | |
	Retention time	Peak area	Retention time	Peak area	Retention time	Peak area	Retention time	Peak area
12.5	0.892	1.025	0.635	0.968	1.042	1.251	0.985	0.643
25.0	0.728	0.958	0.522	1.140	0.880	1.341	0.867	0.693
50.0	0.625	0.245	0.828	0.338	0.458	0.984	0.667	0.554

Table 4. Accuracy/recovery data for methyl β-orcinolcarboxylate (MBO) and ethyl haematommate (EH) analysis of lichen *Everniastrum cirrhatum*

Parameters	Concentration	% Recovery (Mean $\pm$ RSD)	
		MBO	EH
Assay	12.5	97.58 ± 0.78	98.24 ± 0.65
	25.0	98.24 ± 0.68	99.12 ± 0.59
	50.0	98.01 ± 0.54	97.46 ± 0.41
Assay (Spiking)	12.5	97.25 ± 0.77	98.54 ± 0.62
	25.0	98.33 ± 0.38	100.89 ± 0.56
	50.0	101.25 ± 0.12	99.68 ± 0.49

that other components or metabolites do not interfere with the peak elution, indicating specificity of the method for MBO and EH. The variation in retention time (%RSD) and peak purity and its homogeneity, was evaluated by comparing the PDA data with those of the reference compounds, using library matching. All peaks were found to be pure at upslope, apex, and down slope of the peak. Empower software defines the peak purity when in the purity angle (a measure of spectral homogeneity; the weighted average of all spectral contrast angles calculated by comparing the spectra from each data point in the integrated peak against the peak apex spectrum) is lower than the peak threshold angle (sum of purity noise angle and solvent angle). The confirmation of MBO and EH peaks was also evaluated by comparing the spectral at apex of each peak in the sample to that of the library maintained for reference compounds. In this case, the match angle was less than the match threshold, which confirms the single component elution.

Accuracy

The accuracy of an HPLC method is the closeness of test results to the true value of the reference substance obtained by the optimized chromatographic conditions. It was determined by application of an analytical procedure to an MBO and EH of known purity (for a drug substance), as well as by recovery

Table 5. System suitability report of the separation of MBO and EH from lichens

Compounds	Retention time (R_t)	USP resolution	USP tailing factor	Capacity factor	Theoretical plates N (USP tangent method)
MBO	4.916	5.52	0.909	1.60	4296
EH	14.969	9.32	0.916	6.78	5399

Table 6. Different parameters studied for robustness.

Chromatographic parameters	Peak area count (% RSD)		Retention time (% RSD)	
	MBO	EH	MBO	EH
Mobile phase concentration (A:B)				
50:50	0.245	0.338	0.625	0.828
49:51	0.787	0.658	1.023	0.789
51:49	0.443	0.334	1.108	0.583
Flow rate				
1.0	0.283	0.237	0.598	0.794
1.2	0.588	0.621	0.481	0.584
1.5	0.556	0.448	0.524	0.623
Temperature				
26	0.891	0.785	0.926	0.942
28	0.824	0.679	1.254	0.987
30	0.669	0.882	1.128	1.234

studies, in which a known amount of a standard is spiked in the methanol extracts of one of the lichen species, i.e., *Everniastrum cirrhatum*. The results of accuracy studies from solution and lichen extract are summarized in Table 4. It is clearly evident that method is accurate within the working range.

System Suitability Testing

System suitability tests are an integral part of chromatographic methods development.[23,24] The results of system suitability are given in Table 5, which verify that resolution and reproducibility of the system are adequate and the method is acceptable for the analysis.

Table 7. MBO and EH contents (%) in various lichen species

S. No.	*Lichen sp.*	MBO (%)	EH (%)
1	*Everniastrum cirrhatum*	0.1911	0.1497
2	*Flavoparmelia caperata*	0.0083	0.0110
3	*Heterodermia leucomela*	0.5905	0.8039
4	*Lecanora flavidorufa*	0.0544	0.0020
5	*Leptogium pedicellatum*	0.0042	ND
6	*Lobaria isidiosa*	0.0077	ND
7	*Phaeophyscia hispidula*	0.0120	ND
8	*Ramalina sp.*	0.0041	ND
9	*Rimelia reticulata*	0.4290	0.2654
10	*Stereocaulon foliolosum*	0.1472	0.0561
11	*Usnea sp*	0.0024	ND

Robustness

Results obtained in this study are presented in Table 6. The % RSD of peak area and retention time of MBO and EH in lichen species were calculated ($n = 6$) for every level and factor studied. The perusal of the data indicates that the studied factors remained unaffected by small variations of these
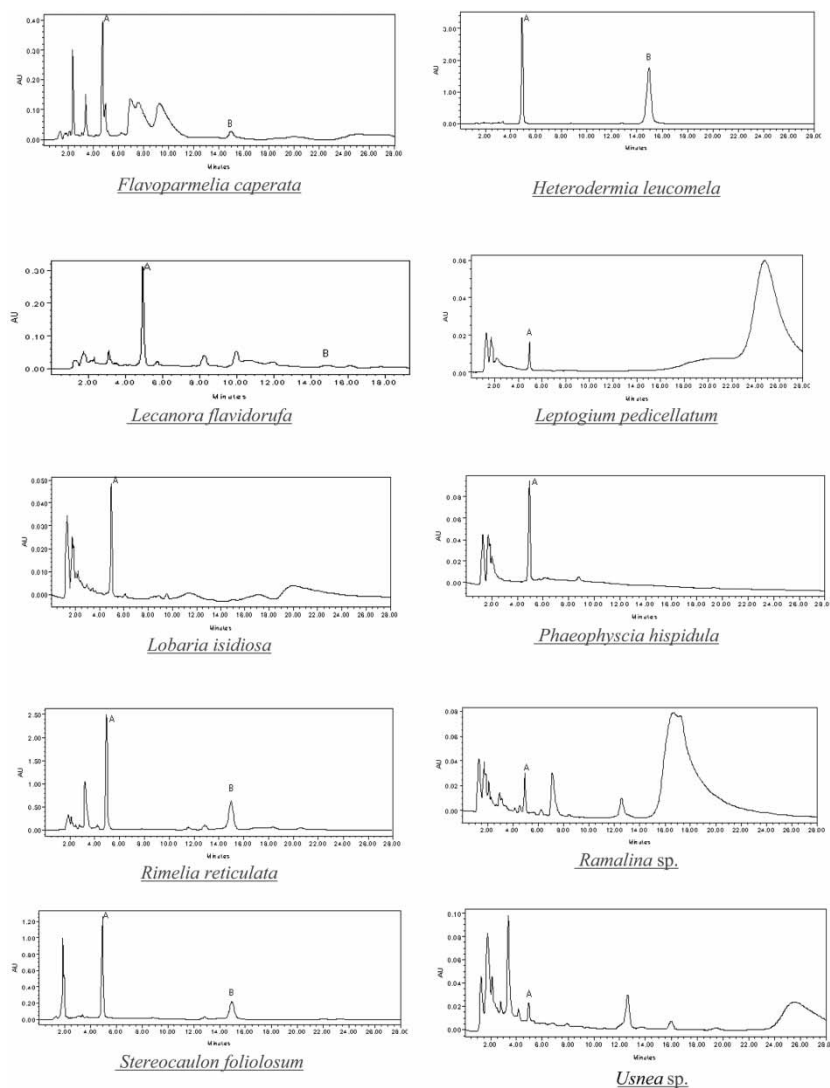

Figure 4. HPLC fingerprint of various lichen species. (For extraction and chromatographic protocol conditions see experimental section).

parameters, because the %RSD for MBO and EH are within the recommended range, i.e., 1.0% established according to the ICH. Therefore, it can be concluded that the method is consistent in front of the mobile phase, flow rate, and temperature.

Estimation of MBO and EH in Various Lichen Species

The developed method was successfully applied for assaying polyphenolic metabolites, methyl β-orcinolcarboxylate (MBO) and ethyl haematommate (EH), in different lichen species. The results are depicted in Table 7. The chromatographic conditions of the method are found suitable to monitor chemical variability in various lichen species. HPLC profiles of various Lichen species have been presented in Figure 4. The HPLC profiles of lichen species indicate the chemical variability between the lichen species. The content of MBO and EH present in different lichen samples could also be correlated with the antifungal activity (data not shown), further confirming the fact that these two compounds are responsible for their biological activity.

CONCLUSION

This is the first report on the analysis of important biologically active polyphenolic metabolites of lichen species, namely methyl β-orcinolcarboxylate (MBO) and ethyl haematommate (EH). The analytical parameters calculated from calibration curves were found to be satisfactory. This method proposes a simple, accurate, and precise determination of MBO and EH in various lichen species. Additionally, the LC method can easily be utilized as a suitable analytical procedure for quality control and quantification of these metabolites in lichens, commonly used for various purposes in folklore and traditional systems of medicine of the Asian region.

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REFERENCES

1. Muller, K. Pharmaceutically relevant metabolites from lichens. Appl. Microbiol. Biotechnol. **2001**, *56*, 9–16.
2. Lawery, J.D. Biological role of lichen substances. Bryologist **1986**, *89*, 111–122.

3. Ingolfsdottir, K.; Chung, G.A.C.; Skulason, V.G.; Gissurarson, S.R.; Vilhelmsdottir, M. Antimycobacterial activity of lichen metabolites *in vitro*. Euro. J. Pharma. Sci. **1998**, *6*, 141–144.

4. Pengsuparp, T.; Cai, L.; Constant, H.; Fong, H.H.S.; Lin, L.Z.; Kinghorn, A.D.; Pezzuto, J.M.; Cordell, G.A.; Ingolfsdottir, K.; Wagner, H.; Hughes, S.H. Mechanistic evaluation of new plant-derived compounds that inhibit HIV-1 reverse transcriptase. J. Nat. Prod. **1995**, *58*, 1024–1031.

5. Neamati, N.; Hong, H.; Mazumder, A.; Wang, S.; Sunder, S.; Nicklaus, M.C.; Milne, G.W.A.; Proksa, B.; Pommier, Y. Depsides and depsidones as inhibitors of HIV-I integrase: discovery of novel inhibitors through 3D database searching. J. Med. Chem. **1997**, *40*, 942–951.

6. Okuyama, E.; Umeyama, K.; Yamazaki, M.; Kinoshita, Y.; Yamamoto, Y. Usnic acid and diffractaic acid as analgesic and antipyretic components of *Usnea diffracta*. Planta Med. **1995**, *61*, 113–115.

7. Hidalgo, M.E.; Fernandez, E.; Quilhot, E.; Lissi, E. Antioxidant activity of depsides and depsidones. Phytochemistry **1994**, *37*, 1585–1587.

8. Halici, M.; Odabasoglu, F.; Suleyman, H.; Cakir, A.; Aslan, A.; Bayir, Y. Effects of water extract of *Usnea longissima* on antioxidant enzyme activity and mucosal damage caused by indomethacin in rats. Phytomedicine **2005**, *12*, 656–662.

9. Herrero, J.C.M.; Carbonero, E.R.; Gorin, P.A.J.; Iacomini, M. Anticoagulant and antithrombotic activity of a sulfate obtained from a glucan component of the lichen Parmotrema mantiqueirense Hale. Carbohydr. Polym. **2005**, *60*, 7–13.

10. Elix, J.A. Biochemistry and secondary metabolites. In *Lichen: Biology*; Nash, T.H., Ed.; Cambridge University Press: 1996, 154.

11. Caccamese, S.; Toscano, R.M.; Bellesia, F.; Pinetti, A. Methyl β-orcinolcarboxylate and depside from *Parmelia furfuracea*. J. Nat. Prod. **1985**, *48*, 157–158.

12. Ingolfsdottir, K.; Bloomfield, S.F.; Hylands, P.J. *In-vitro* evaluation of the antimicrobial activity of lichen metabolites as potential preservatives. Antimicrobial. Agents Chemother. **1985**, *28*, 289–292.

13. Kumar, K.C.S.; Müller, K. Lichen metabolites. I. Inhibitory action against leukotriene B4 biosynthesis by a non-redox mechanism. J. Nat. Prod. **1999**, *62*, 817–820.

14. Piovano, M.; Garbarino, J.A.; Giannini, F.A.; Correche, E.R.; Feresin, E.R.; Tapia, A.; Zacchino, S.; Enriz, Z. Evaluation of antifungal and antibacterial activities of aromatic metabolites of Lichens. Bol. Soc. Chil. Quím. **2002**, *47*, 235–240.

15. Bezivin, C.; Tomasi, S.; Devehat, F.L.; Boustie, J. Cytotoxicity activity of some lichen extracts on murine and human cancer cell lines. Phytomedicine **2003**, *10*, 499–503.

16. Marante, F.J.T.; Castellano, A.G.; Rosas, F.E.; Aguiar, J.Q.; Barrera, J.B. Identification and quantitation of allelochemicals from the lichen *Lethariella canariensis*: phytotoxicity and antioxidative activity. J. Chem. Ecol. **2003**, *29*, 2049–2071.

17. Sakalni, A.; Upreti, D.K. Folk uses of some lichens in Sikkim. J. Ethnopharmacol. **1992**, *37*, 229–233.

18. Sochting, U. *Lichens of Bhutan: Biderversity and Use*; Botanical Institute, Department of Mycology, University of Copenhagen: Denmark, 1999.

19. Jayaprakasha, G.K.; Rao, L.J.; Singh, R.P.; Sakariah, K.K. Improved chromatographic method for the purification of phenolic constituents of the lichen parmotrema tinctorum (Nyl.) Hale. J. Chromatogr. Sci. **1998**, *36*, 8–10.

20. Feige, G.B.; Lumbsch, H.T.; Huneck, S.; Elix, J.A. Identification of lichen substances by a standardized high-performance liquid chromatographic method. J. Chromatogr. A. **1993**, *646*, 417–427.

21. Cordeiro, L.M.; Iacomini, M.; Stocker-Worgotter, E. Culture studies and secondary compounds of six Ramalina species. Mycol. Res. **2004**, *108*, 489–497.
22. Bedrinana, G.; Hammond, G.B. Constituents of the lichen *Anaptychia leucome-laena*. Fitoterapia **1996**, *67*, 287.
23. ICH. International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) Q2B: Guide lines on validation of analytical procedure-methodology. 1996.
24. *USP. U.S. Pharmacopea 23, supplement 4 U.S*; Pharmacopeal Convention, Inc.: Rockville, MD, 1996.