HPLC Analysis of Secondary Metabolites in the Lichen *Parmotrema tinctorum* from Different Substrates

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Abstract: The main secondary metabolites of the lichen *Parmotrema tinctorum* are orsellinic acid, methyl orsellinate, lecanoric acid and atranorin. These lichen substances could vary among habitats because different environmental stress. The purpose of this study was to develop an analytical technique to determine quantities of the lichen substances grew in different substrates. The lichen *Parmotrema tinctorum* were collected from rocks, barks of trees and nylons mesh, of which the latest was the transplanted lichens, from Khao Yai National Park. Pure substances were prepared by extracting lichen with acetone and purified by column chromatography to be used as standard substances for quantitative analysis. The chemical structures were confirmed by spectroscopic method. The analysis was performed by HPLC system of HP1100 binary pump and using hypersil C18 column (250 x 4.0 mm, 5 µm). The chromatographic conditions were investigated for analyzing each substance. It was found that the optimum HPLC condition for the analysis of oresllinic acid, methyl orsellinate and lecanoric acid consisted of gradient elution using methanol as solvent A, and 1% phosphoric acid as solvent B with UV detection at wavelength 265 nm. The optimum HPLC condition for the analysis of atranorin included isocratic elution using 82:18 of methanol: 1% phosphoric acid as mobile phase and UV detection at wavelength 254 nm. Analysis samples were prepared by extracting lichen with suitable solvent overnight at room temperature followed by filtration and evaporation to dryness. The residues were then dissolved by methanol in exact volume using benzoic acid as an internal standard. The solution samples were filtered through 0.45 µm syringe membrane before injection to HPLC. The amount of orsellinic acid, methyl orsellinate, lecanoric acid and atranorin in lichen samples collected from rocks, barks of host tree and nylons mesh were evaluated and compared.

1. Introduction

Lichens received special attention in recent years because of their ability to use as bioindicator of air pollution and production of secondary metabolites[1,2] which have potential to be used commercially. Lichen produces secondary metabolic products for auto-protection against adverse environment which vary among habitats. Some of the main compounds were produced for survival in extreme environments for example lecanoric acid,
parietin, emodin, atranorin, gyrophoric acid, fumarprotocetraric acid, rhizocarpic acid, pulvinic dilactone and usnic acid, etc.[1,3]. The technique of high performance liquid chromatography (HPLC) is widely used for quantitative determination of secondary metabolites in lichen. Precise determination of lichen products is essential to enhance our understanding on production and roles of lichen novel products, which have extensive implication on sustainable utilization in several aspects.

The objective of this study were to prepare standard substances of orsellinic acid, methyl orsellinate, lecanoric acid and atranorin (Figure 1) by isolation and purification from lichen *Parmotrema tinctorum* and then quantify them from different substrates at Khao Yia National Park, which were rocks, barks of trees and nylons mesh. In order to evaluate and compare their substances on different substrate

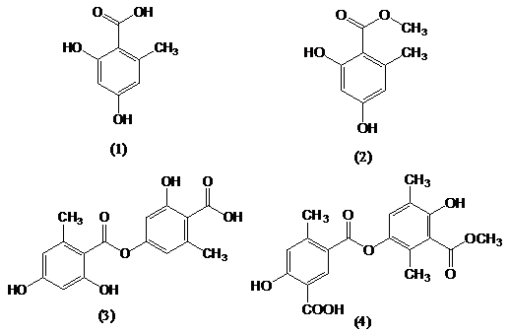

Figure 1. Chemical structures of lichen substances from *Parmotrema tinctorum*. Orsellinic acid (**1**), methyl orsellinate(**2**), lecanoric acid(**3**) and atranorin(**4**).

2. Materials and Methods

2.1 Chemicals and reagents

Methanol and acetone were HPLC grade, ortho-phosphoric acid, hexane, ethyl acetate, silica gel 60 (less than 0.063 mm) were analytical reagent grade and $CDCl_3$ and acetone-d_6 for NMR. All of them were bought from Merck.

2.2 Preparation of standard lichen substances

Standard lichen substances, orsellinic acid, methyl orsellinate, lecanoric acid and atranorin were prepared by extracting from lichen *Parmotrema tinctorum*. The crude extract was isolated and purified by column chromatography. The lichen substances

were identified by spectroscopic technique. ^{1}H and ^{13}C NMR spectra of orsellinic acid methylorsellinate, lecanoric acid and atranorin were recorded in $CDCl_3$ or acetone-d_6 on a Bruker 400 UltraShield™ (^{1}H:400 MHz,^{13}C:100MHz) then compared to those reported earlier[7,8].

Exactly 10.0 mg of each pure lichen substances, orsellinic acid, methyl orsellinate, lecanoric acid and atranorin were dissolved in 10 ml of methanol, They were used as stock standard solution for preparing standard calibration curve.

2.3 Samples preparation

Thalli of the lichen *Parmotrema tinctorum* were collected from different substrates which were rocks, barks of trees and nylons mesh in the same area at Khao Yia National Park. Five samples were collected each substrate. They were transferred to the analytical laboratory at the Chemistry Deparment, Ramkhamheang University. The samples were kept in air dried condition at room temperature and foreign debris on thallis were manually removed. Samples were ground into powder with liquid nitrogen using a ceramic mortar and pestle, and were then sieved through a 500 µm filter. The fine powder samples were kept frozen in refrigerator until analysis. A 10.0 mg of grounded samples were exactly weighted and extracted with 7 ml of pure methanol by soaking over night. The extract was then made to exact volume of 10 ml by DI water for analyzing orsellinic acid, methyl orsellinate and lecanoric acid. Another 10.0 mg of grounded samples were precisely weighed and extracted with acetone: dichloromethane (50:50) the extracted samples were filtered and evaporated to dryness. The residues were then dissolved by 70:30 of methanol: water in 10 ml, this solution was used to analyse atranorin. The solution samples were filtered through 0.45 µm syringe membrane before injection to HPLC. The analyses were three replicate and the results were average from three readings.

2.4 HPLC analysis

Lichen extracts were analyzed on a HP 1100 series consisting of HP G1312A binary pump, HP G1314A UV variable wavelength detector. Separation was achieved on an ODS Hypersil 250 x 4 mm I.D., 5 µm column. The analysis of orsellinic acid, methylorsellinate and lecanoric acid were used by gradient elution. The solvent A consisting of 1% phosphoric acid in water (pH = 2.3 - 2.7) and solvent B was 100% methanol. The run start with 35% B at flow rate 1.0 ml/min solvent B was increased to 100% within 20 min and hold for 5 min. At the end of run time, the post time was set to 10 min before a new run was started. The compounds were detected at wavelength 265 nm. The analysis of atranorin was used by isocratic elution with 82:18 (methanol: 1% phosphoric acid) at flow rate 1.0 ml/min using wavelength 254 nm. The identification of compounds was based on retention times and quantified by using external standard calibration curve.

2.5 Method validation

Linearity

Linearity of compounds were determination by using mixture of standard lichen substances in the following range: orsellinic acid and methyl orsellinate were in 1-16 µg/ml, lecanoric acid was in 100-500 µg/ml and atranorin was in 5-25 µg/ml. The calibration curves were obtained by plotting the peak area versus the concentration of the standard solutions. The linearity of calibration curve is defined in term correlation coefficient (r^2) as shown in Table1.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of each lichen substances were calculated from equations:

$$\mathrm{LOD} = \frac{3S_{y/x}}{b}$$

Whereas b is slope and $S_{y/x}$ is standard deviation of calibration curve calculated from

$$S_{y/x} = \left\{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}\right\}^{\frac{1}{2}}$$

The LOQ of each lichen substances were calculated by 10 time of $S_{y/x}$. The results of LOD and LOQ of orsellinic acid, methyl orsellinate, lecanoric acid and atranorin are shown in Table 1.

Precision

The precision was determined by seven replicates of analyses of lichen extract solution. The precision was expressed in percentage of relative standard deviation (%RSD) as shown in Table 2.

Accuracy

The accuracy of the method was tested by added (spiked) a known amount of standard lichen substances into lichen sample. Three different concentration of the standard solution of orsellinic acid (2, 4 and 6 µg/ml), methyl orsellinate (2, 4 and 6 µg/ml) lecanoric acid (5, 10 and 15 µg/ml), and atranorin (5, 10 and 15µg/ml) were added to the lichen extract solution and analyzed by the proposed HPLC method. The recovery was determined by subtraction the concentration of lichen sample from spiked lichen sample. The results are shown in Table 3.

Table1. Linearity (r^2), limit of detection (LOD) and limit of quantification (LOQ) of secondary metabolites of the lichen *Parmotrema tinctorum*

Compounds	Concentration range (µg/ml)	Linearity (r^2)	LOD (µg/ml)	LOQ (µg/ml)
orsellinic acid	1 - 16	0.9995	0.23	0.77
methyl orsellinate	1 - 16	0.9996	0.19	0.65
lecanoric acid	100 - 500	0.9992	0.68	2.28
atranorin	5 - 25	0.9993	0.14	0.47

Table 2. Precision data of amount and retention time of secondary metabolites of the lichen *Parmotrema tinctorum* (N=7)

Compounds	Amount of substances (mg/g)		Retention time	
	$\bar{x}$ ± SD	%RSD	$\bar{x}$ ± SD	%RSD
orsellinic acid	10.36 ± 0.02	0.19	6.33 ± 0.07	1.11
methyl orsellinate	15.71 ± 0.01	0.06	10.74 ± 0.07	0.65
lecanoric acid	330.24 ± 0.26	0.08	12.74 ±0.07	0.55
atranorin	13.14 ± 0.03	0.23	5.31 ± 0.02	0.38

Table 3. Accuracy/recovery data of secondary metabolites of the lichen *Parmotrema tinctorum*

Level	% Recovery ± SD			
	Orsellinic acid	Methyl orsellinate	Lecanoric acid	Atranorin
1	99.03 ± 2.92	100.35 ± 0.53	99.10 ± 1.60	102.68 ± 1.41
2	99.03 ± 2.41	99.61 ± 2.61	98.97 ± 2.67	102.26 ± 0.93
3	98.49 ± 0.65	99.84 ± 1.15	98.38 ± 1.14	99.39 ± 0.21

3. Results and Discussion

The suitable solvent for complete extraction of orsellinic acid, methyl orsellinate and lecanoric acid from *lichen Parmotrema tinctorum* is methanol whereas acetone:dichloromethane (50:50) is suitable for atranorin. Quantitative analysis was achieved by using reversed-phase high-performance liquid chromatography, gradient elution was used for separation and quantify of orsellinic acid, methyl orsellinate and lecanoric acid where as isocratic elution was used for quantify of atranorin. The chromatograms of secondary metabolites of lichen *Parmotrema tinctorum* are shown in Figure 2.

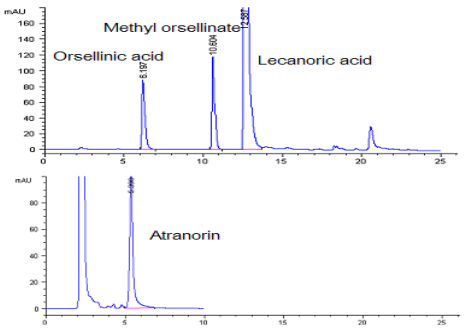

Figure 2. The HPLC chromatogram of orsellinic acid, methyl orsellinate, lecanoric acid and atranorin.

Method validation in terms of detection limit, linearity, accuracy/recovery and precision are shown in tables 1-3. It shows that the HPLC methods are efficient analytical techniques for determination secondary metabolites in the lichen *Parmotrema tinctorum*.

The amount of secondary metabolites of the lichen *Parmotrema tinctorum* from different substrates are shown in Table 4. The average amount from five sites of orsellinic acid, methyl orsellinate, lecanoric acid and atranorin in lichen on the nylon mesh were 6.3 ± 0.7, 2.5 ± 0.3, 394.6 ± 31.1 and 17.2 ± 8.0 mg/g, respectively. Those on bark of host tree were 6.7 ± 1.9, 1.9 ± 0.1, 376.9 ± 13.5 and 11.8 ± 1.8 mg/g, respectively. On rock were 7.0 ± 1.3, 2.5 ± 0.5, 387.4 ± 75.9 and 9.8 ± 2.1 mg/g, respectively. Figure 3 showed the comparison of the average amounts of these lichen substances from three substrates. The results show that atranorin has higher variation among substrate where as the other substances were not significantly differences. Sunlight could affect the amounts of atranorin [9]. Lichens grown on nylon mesh at different aspect orientation had the highest variation of atranorin, because they received different light intensity. By contrast those grew on tree and rock had less variations of this substance.

Table 4. Amount of orsellinic acid, methyl orsellinate, lecanoric acid and atranorin of the lichen *Parmotrema tinctorum* from different substrate

	Amount of orsellinic acid (mg/g)on substrate			Amount of methylorsellinate (mg /g) on substrate		
Site	Nylon	Tree	Rock	Nylon	Tree	Rock
1	6.9	5.9	5.9	2.1	2.0	2.6
2	6.8	8.0	5.8	2.6	1.8	2.5
3	5.2	5.2	8.3	2.2	1.9	2.8
4	6.5	9.4	6.5	2.6	2.1	2.9
5	6.3	4.9	8.4	2.9	1.9	1.6
mean	6.3	6.7	7.0	2.5	1.9	2.5
SD	0.7	1.9	1.3	0.3	0.1	0.5
CV	10.7	29.1	18.3	13.2	5.9	20.8

	Amount of lecanoric acid (mg /g) on substrate			Amount of atranorin (mg /g) on substrate		
Site	Nylon	Tree	Rock	Nylon	Tree	Rock
1	352.0	379.7	424.9	7.9	9.9	11.1
2	413.5	357.6	411.9	16.5	11.9	12.3
3	373.8	381.9	403.0	15.0	12.2	6.8
4	405.3	393.9	442.8	16.7	14.5	9.6
5	428.5	371.2	254.2	30.0	10.7	9.1
mean	394.6	376.9	387.4	17.2	11.8	9.8
SD	31.1	13.5	75.9	8.0	1.8	2.1
CV	7.9	3.6	19.6	46.4	14.8	21.4

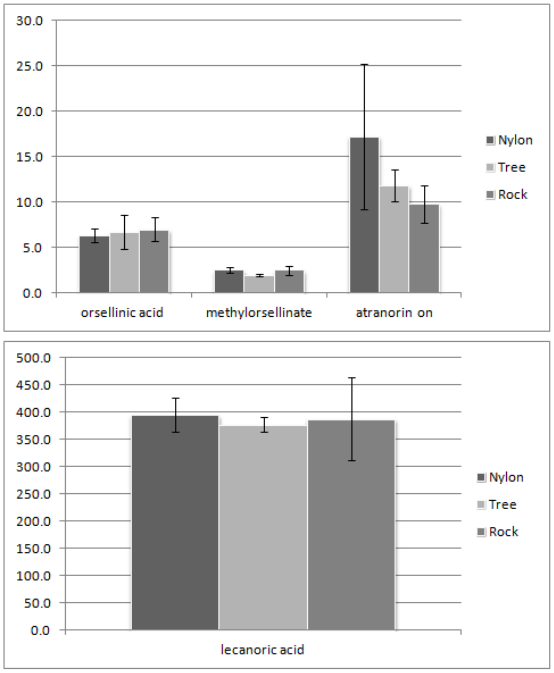

Figure 3. Comparing the amounts of orsellinic acid, methyl orsellinate, lecanoric acid and atranorin from different substrates.

4. Conclusions

The amount of four secondary metabolites**,** orsellinic acid, methyl orsellinate, lecanoric acid and atranorin were quantified by using external standard calibration curve of HPLC technique. The standard substances were prepared by extracting and purifying from the lichen *Parmotrema tinctorum***.** The results showed that the amounts of secondary metabolites from Nylon mesh, bark of host tree and rock from the same locality did not significantly differences. It can be concluded that lichens inhabited different substrates of the same locality, exposed to similar environmental factors, produced comparable amounts of secondary metabolites.

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