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STUDY ON CHEMICAL CONSTITUENTS OF THE LICHEN *PARMOTREMA TINCTORUM* (NYL.) HALE

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Abstract. A phytochemical investigation was conducted on foliose lichen, *Parmotrema tinctorum* (Nyl.) Hale, collected in Lam Dong province, Vietnam. Color reactions for identification of lichen substances (+K deep yellow, +C red, +KC red, + P pale yellow) suggested the presence of atranorin, lecanoric acid, quinones, depsides, and xanthones containing two free hydroxyl groups in *meta*-position. In fact, six compounds, including atranol (**1**), methyl haematomate (**2**), divaricatinic acid (**3**), methyl divaricatinate (**4**), atranorin (**5**) and lecanoric acid (**6**) were isolated in the acetone extract, in which isolates (**3**) and (**4**) were reported for the first time in such species. Their structures were elucidated by X-ray diffraction or spectroscopic data and compared with those in references.

Keywords: atranorin, lecanoric acid, lichen, *Parmotrema tinctorum*, X-ray diffraction.

Classification numbers: 1.1.1; 1.1.6

1. INTRODUCTION

Lichens are symbiotic products of a mycobiont (fungal partner) and photobiont (algal partner) and are known to produce a range of unique secondary metabolites [1]. Characteristic compounds of lichens are depsides, depsidones, diphenyl ethers, benzofuran, usnic acid, and anthraquinone derivatives, presumably of fungal origin and their biological activities remain largely underexplored. However, a few have been shown to possess antibiotic, anti-mycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic and anti-proliferative activities [2, 3]. *Parmotrema* is a large genus in the Parmeliaceae with approximately 350 species of foliose lichens and a high level of diversity in the tropical areas of the world. In Vietnam, investigation on chemical constituents of *Parmotrema* has not been noticed so far as only few studies have paid attention on it, especially *Parmotrema tinctorum* (Nyl.) Hale. Previous studies

on its chemical constituents reported the presence of flavoxanthin, *β*-citraurin, atranorin, lecanorol, lecanoric acid, isolecanoric acid, salazinic acid, and some monocyclic aromatic compounds such as orsellinic acid, ethyl orsellinate and methyl β-orsellinate [4-10]. Moreover, *P. tinctorum* extracts showed presence of carbohydrates, phenols, flavanoids, tannins, terpenoids, coumarins and saponins which may be basis of its biological effects [11]. In fact, the extracts of *P. tinctorum* exhibited significant antioxidant, antibacterial, antifungal activities as well as inhibitory potential against carbohydrate digestive enzymes and aldose redutase [12-15]. Our preliminary study on cytotoxic activity of the *P. tinctorum* extracts showed that the acetone extract (at a concentration of 100 µg/mL) inhibited more than 50 % of MCF-7 and NCI-H460 cancer cell lines [9]. As part of our studies on bioactive secondary metabolites, we continue to show the isolation and identification of six compounds from the acetone extract of *P. tinctorum* collected in Lam Dong province, Vietnam. Spot tests on upper cortex with useful lichen reagents (K, C, KC, and P) were also conducted to suggest general identification of lichen substances.

2. MATERIALS AND METHODS

2.1. Lichen material

Parmotrema tinctorum (Nyl.) Hale was collected in Lam Dong province, Vietnam on December 2015. The scientific name was identified by Dr. Kawinnat Buaruang (Lichen herbarium of Ramkhamhaeng University, Department of Biology, Faculty of Science, Ramkhamhaeng University, Thailand). A voucher specimen (No Par-0117) was deposited in the herbarium of the Department of Chemistry, Faculty of Science, Can Tho University of Medicine and Pharmacy, Can Tho City, Viet Nam.

2.2. General experimental procedures

The NMR experiments were performed on a Bruker DMX 300 and 500 spectrometers. HRMS-ESI was carried out on a MICROMASS ZABspecTOF spectrometer for electrospray ionization. Melting points were measured on a Melting Point Meter M5000 Krüss.

The crystal data was collected on a Enraf-Nonius FR590-kappa diffractometer with a CCD area detector and graphite monochromated MoKα radiation. The structure was solved using direct methods, refined with the Shelx software package and expanded using Fourier techniques. Computing software programs for: Data Collection, Cell Refinement and Data Reduction: COLLECT/HKL2000. Structure solution: SHELX-S97. Structure Refinement: SHELXL2012; CRYSTALBUILDER. Molecular Graphics: ORTEP-III; MERCURY.

Spot tests were carried out with reagents K (10% KOH), C (30 % potassium hypochlorite) and K followed by C (KC), P (5 % *p*-phenylenediamine in ethanol). Column chromatography was performed on normal phase silica gel (40-63 μm, Keselgel 60, Merck 7667). Thin layer chromatography (TLC) was performed on Kieselgel 60F254 plates (Merck) and spots were visualized under UV light or sprayed with vanillin (0.5 g vanillin in 80 mL sulfuric acid and 20 mL ethanol), then heated. All solvents used were purchased from Chemsol, purity ≥ 99.0 %.

2.3. Extraction and isolation

Air-dried crushed thallus of the lichen *P. tinctorum* (300 g) were successively and exhaustively extracted with 3 liters of acetone by a hot Soxhlet to give acetone extract (80.0 g,

ρ = 26.7 %). When the acetone extract was evaporated under reduced pressure, a precipitate occurred and was filtered off (2.3 g). The precipitate after re-crystallized was subjected to a silica gel column and eluted with *n*-hexane- ethyl acetate 95:5 to yield atranorin (**5**, 8.2 mg). The rest of acetone extract was then subjected to silica gel column chromatography and eluted by the solvent system of petroleum ether–ethyl acetate with increasing ethyl acetate ratios to obtain seven fractions from Ac1 to Ac7. The fraction Ac2 was subjected to preparative TLC using *n*-hexane–chloroform 8:2 as eluent to afford atranol (**1**, 3.5 mg) and methyl haematomate (**2**, 4.9 mg). The fraction Ac3 was silica gel re-chromatographed, eluting with *n*-hexane–ethyl acetate–acetic acid (95:5:0.5) to give methyl divaricatinate (**4**, 4.2 mg). The fraction Ac6 was subjected to a silica gel column and eluted with *n*-hexane: ethyl acetate (85:15) to yield divaricatinic acid (**3**, 5.4 mg) and lecanoric acid (**6**, 7.3 mg).

Atranol (**1**): yellow solid; M.p 124-125°C; ^{1}H NMR (acetone-d_6, 500 MHz) δ_H: 10.69 (2H, *s*, 2-OH, 4-OH), 10.27 (1H, *s*, H-7), 6.26 (2H, *s*, H-1, H-5), 2.23 (3H, *s*, H-8); ^{13}C NMR (acetone-d_6, 125 MHz) δ_C: 108.4 (C-1), 163.0 (C-2), 109.2 (C-3), 163.0 (C-4), 108.4 (C-5), 151.5 (C-6), 194.1 (C-7), 22.2 (C-8); ESI-HRMS *m/z* 175.0373 $[M+Na]^+$ (calcd. for $C_8H_8O_3Na$).

Methyl haematomate (**2**): white needles (acetone); M.p 146-147 °C.

Divaricatinic acid (**3**): white needles (acetone).

Methyl divaricatinate (**4**): white solid; ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H: 11.69 (1H, *s*, 2-OH), 6.34 (1H, *s*, H-3), 6.29 (1H, *s*, H-5), 3.92 (3H, *s*, 7-OCH_3), 3.80 (3H, *s*, 4-OCH_3), 2.83 (2H, *m*, H-1'), 1.55 (2H, *m*, H-2'), 0.96 (3H, *t*, 7.5, H-3'); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C: 104.7 (C-1), 165.5 (C-2), 98.8 (C-3), 163.9 (C-4), 110.7 (C-5), 147.7 (C-6), 171.9 (C-7), 55.2 (4-OCH_3), 51.8 (7-OCH_3), 38.9 (C-1'), 24.9 (C-2'), 14.2 (C-3').

Atranorin (**5**): colorless powder; ^{1}H NMR (DMSO-d_6, 500 MHz) δ_H: 2.04 (3H, *s*, H-8'), 2.35 (3H, *s*, H-9), 2.39 (3H, *s*, H-9'), 3.88 (3H, *s*, 7'-OCH_3), 6.41 (1H, *s*, H-5), 6.65 (1H, *s*, H-5'), 10.21 (1H, *s*, H-8), 10.52 (1H, *s*, 2-OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ_C: 107.9 (C-1), 163.6 (C-2), 109.0 (C-3), 161.7 (C-4), 115.2 (C-5), 151.4 (C-6), 164.5 (C-7), 193.8 (C-8), 20.1 (C-9), 110.6 (C-1'), 157.4 (C-2'), 116.3 (C-3'), 148.8 (C-4'), 115.7 (C-5'), 136.5 (C-6'), 169.7 (C-7'), 9.3 (C-8'), 21.1 (C-9'), 52.3 (7'-OCH_3); ESI-HRMS *m/z* 397.0890 $[M+Na]^+$ (calcd. for $C_{19}H_{18}O_8Na$).

Lecanoric acid (**6**): pale yellow needles (acetone); ^{1}H NMR (DMSO-d_6, 300 MHz) δ_H: 10.31 (1H, *s*, 2-OH), 9.99 (1H, *s*, 2'-OH), 6.62 (1H, *d*, 2.1, H-3'), 6.59 (1H, *d*, 2.1, H-5'), 6.22 (2H, *s*, H-3, H-5), 2.37 (3H, *s*, 8-CH_3), 2.35 (3H, *s*, 8'-CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ_C: 108.1 (C- 1), 160.0 (C-2), 100.4 (C-3), 161.0 (C-4), 109.8 (C-5), 139.4 (C-6), 166.6 (C-7), 21.37 (C-8), 116.3 (C-1'), 158.8 (C-2'), 107.3 (C-3'), 152.1 (C-4'), 114.6 (C-5'), 140.2 (C-6'), 170.4 (C-7'), 21.4 (C-8').

3. RESULTS AND DISCUSSION

Spot tests on upper cortex showed a deep yellow with K, pale yellow with P, red with C (Figure 1). The results suggested the presence of atranorin and related compounds due to a deep yellow color with K. A pale yellow with P should be involved in the occurrence of atranol, ethyl haematommate, and salazinic acid. The thallus gave red with C and red when +KC suggesting the presence of quinones, depsides and xanthones containing two free hydroxyl groups in *meta*-position [16]. Finally, by a rapid step analysis, color reactions gave useful hints for the presence of certain functional groups of a lichen substance and also for classification of lichens. However, color reactions on upper cortex can only provide general information of lichen substances. As a part of searching bioactive compounds from lichens, the extraction and isolation were then

performed to confirm the occurrence of the suggested compounds and also for evaluation bioactivities of isolated compounds in the future.

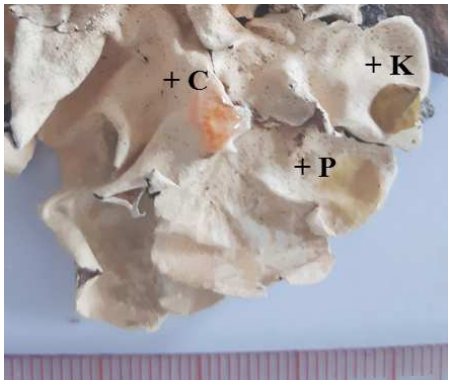

Figure 1. The result spot tests on the thallus *P. tinctorum.*

Air-dried crushed thallus of *P. tinctorum* were extracted with acetone by a hot Soxhlet to give acetone extract. Chromatographic purification of the acetone extract led to the isolation of six compounds (**1**–**6**) (Figure 2).

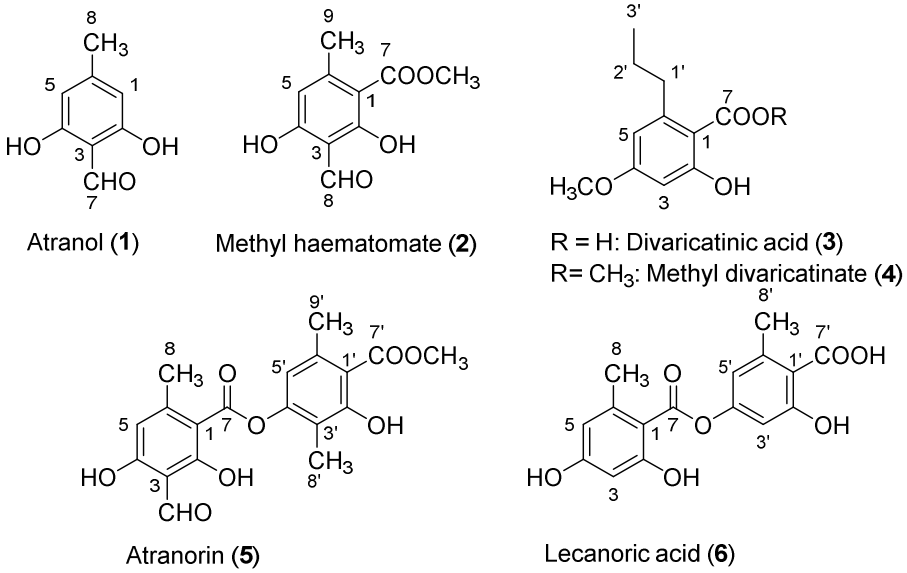

Figure 2. Structures of compounds **1**-**6** isolated from *P. tinctorum.*

Compound **1** appeared as yellow solids and the ESI-HRMS showed an ion peak at *m/z* 175.0373 $[M+Na]^+$ corresponding the molecular formula of $C_8H_8O_3$. The ^{1}H-NMR spectrum exhibited six singlet protons for two chelated hydroxyl groups at δ_H 10.69 (2-OH, 4-OH), a formyl proton at δ_H 10.27 (H-7), two aromatic protons at δ_H 6.26 (H-1, H-5) and a methyl group at δ_H 2.23 (H-8). The ^{13}C NMR spectrum showed eight carbon signals including a methyl group [(δ_C 22.2 (C-8)], two aromatic methines δ_C 108.4 (C-1, C-5), a formyl group δ_C 194.1 (C-7), and four quaternary aromatic carbon signals at δ_C 163.0, 163.0, 109.2 and 151.5. The obtained spectroscopic data were suitable with the published ones [16]. Therefore compound **1** was atranol.

The structures of **2** and **3** were determined by X-ray diffraction as methyl haematomate and divaricatinic acid, respectively. CCDC 1811395 and 1811394 (compounds **2** and **3**, respectively) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

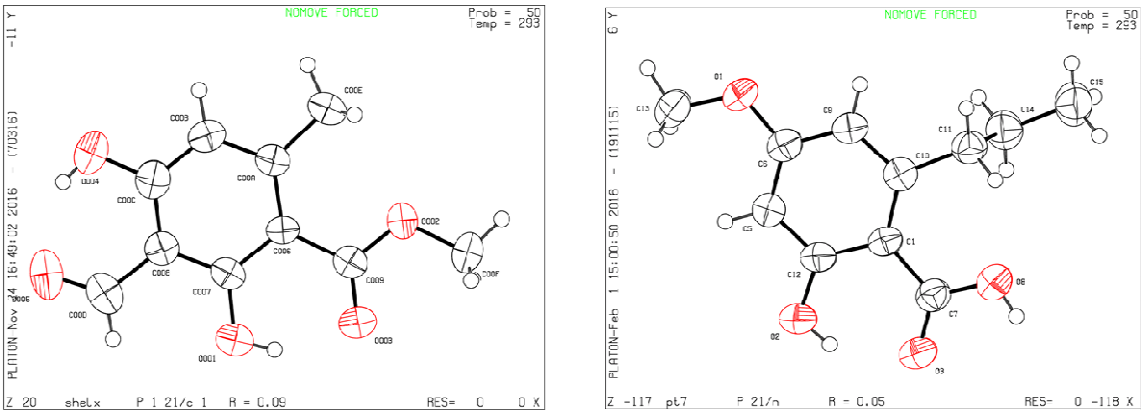

Figure 3. Crystal structures of methyl haematomate (**2**) and divaricatinic acid (**3**).

Compound **4** obtained as a white solid. The ^{1}H-NMR spectrum displayed signals of one hydroxyphenyl group at δ_H 11.69 (1H, *s*, 2-OH), two aromatic methine protons at δ_H 6.34 (1H, *d*, *J* = 2.5 Hz, H-3) and 6.29 ppm (1H, *d*, *J* = 2.5 Hz, H-5), two methoxy groups at δ_H 3.92 (3H, *s*, 7-OCH_3) and 3.80 ppm (3H, *s*, 4-OCH_3), one *n*-propyl group [δ_H 2.83 (2H, *m*, H-1'), 1.55 (2H, *m*, H-2') and 0.96 (3H, *t*, *J* = 7.5 Hz, H-3')]. The ^{13}C-NMR spectrum showed the resonances of 12 carbons including one carbonyl ester group at δ_C 171.9 (C-7), two methoxy groups [δ_C 55.2 (4-OCH_3) and 51.8 (7-OCH_3)], one *n*-propyl group [(δ_C 38.9 (C-1'), 24.9 (C-2') and 14.2 (C-3')] and six aromatic carbons. Comparison with previously reported data [16] confirmed the structure of **4** as methyl divaricatinate.

Compound **5** was isolated as colorless powder. The ^{1}H-NMR spectrum of **5** displayed one methoxy group at δ_H 3.88 (3H, *s*, 7'-OCH_3), one formyl group at δ_H 10.21 (1H, *s*, 8-CHO), three methyl groups [δ_H 2.04 (3H, *s*, H-8'), 2.35 (3H, *s*, H-9) and 2.39 (3H, *s*, H-9')], two aromatic methine protons [δ_H 6.41 (1H, *s*, H-5) and 6.65 (1H, *s*, H-5') and one chelated hydroxyl proton at δ_H 10.52 (1H, *s*, 2-OH). The ^{13}C- and DEPT-NMR spectra displayed two carboxyl groups at δ_C 164.5 (C-7) and 169.7 (C-7'), an aldehyde carbon at δ_C 193.8 (C-8), a methoxy group at δ_C 52.3 (7'-OCH_3), three methyl groups [δ_C 9.3 (C-8'), 20.1 (C-9) and 21.1 (C-9')] and twelve aromatic carbon signals. All these properties suggested that the structure of **5** was atranorin. These spectroscopic data were compatible with the published ones [9].

Structure of compound **6** was confirmed by the ^{1}H-NMR spectrum as two chelated hydroxyl groups at δ_H 10.31 (2-OH) and 9.99 (2'-OH), four aromatic methine protons [δ_H 6.62 (1H, *d*, *J* = 2.1 Hz, H-3'), 6.59 (1H, *d*, *J* = 2.1 Hz, H-5') and 6.22 (2H, *s*, H-3, H-5), two methyl groups at δ_H 2.37 (8-CH_3) and 2.35 (8'-CH_3). The ^{13}C NMR spectrum of **6** showed signals due to 16 carbons corresponding to two methyls, four aromatic methines, eight quaternary carbons and two carboxyl carbons. Comparison of these data with the ones in literature [16], suggested that compound **6** was lecanoric acid.

4. CONCLUSIONS

Spot tests on upper cortex of the lichen *Parmotrema tinctorum* (Nyl.) Hale in Vietnam suggested the presence of atranorin, lecanoric acid, quinones, depsides, and xanthones containing two free hydroxyl groups in *meta*-position. Actually, in present study, six compounds were isolated in the acetone extract, including atranol (**1**), methyl haematomate (**2**), divaricatinic acid (**3**), methyl divaricatinate (**4**), atranorin (**5**) and lecanoric acid (**6**). The compounds (**3**) and (**4**) were reported for the first time in such species. It would be of interest for further chemical investigations and evaluation cytotoxic effects of isolated compounds to discover a new source of bioactive substances from lichens in Vietnam.

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