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Two new phenolic compounds from the lichen *Parmotrema cristiferum* growing in Vietnam

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ABSTRACT

Two new phenolic compounds, cristiferides A-B (**1-2**) together with six known compounds, 2,4-dihydroxyphthalide (**3**), lecanoric acid (**4**), orsellinic acid (**5**), 5-chloroorsellinic acid (**6**), methyl haematommate (**7**), and methyl β-orsellinate (**8**) were isolated from the lichen *Parmotrema cristiferum* (Taylor) Hale (Parmeliaceae). The structures of isolated compounds were identified from its spectroscopic data and by comparison with the literatures. Compounds **1-3** and **6-8** were evaluated for alpha-glucosidase inhibition. Compounds **2** and **7** revealed potent activity with IC_{50} values of 72.66 μM and 48.73 μM, respectively.

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1. Introduction

Vietnamese lichens have possessed the wealth of the chemical diversity, which sometimes comprised unprecedented scaffolds (Duong et al. 2015, 2017, 2018a, 2018b, Nguyen et al. 2018; Nguyen et al. 2020). As a few examples, *Parmotrema dilatatum* and *Parmotrema tsavoense* produced dozens of novel and bioactive compounds (Duong et al. 2018b; Devi et al. 2020). The lichen *Parmotrema cristiferum* (Taylor) Hale occurred popularly in high-altitude Vietnamese forests. Previous studies of this lichen

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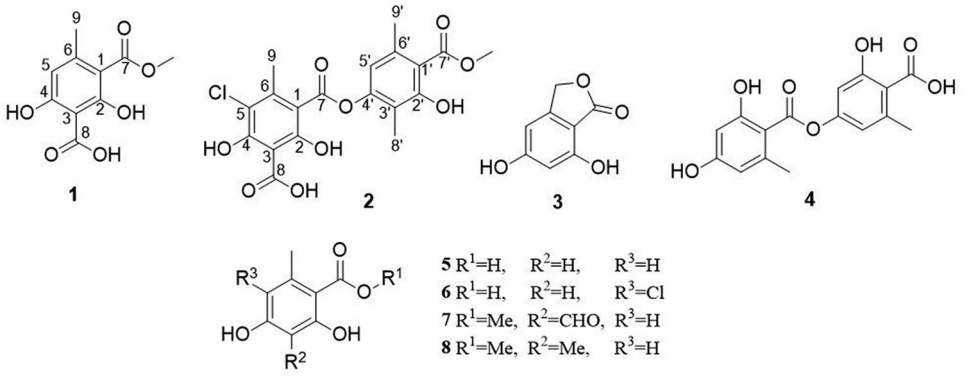

Figure 1. Chemical structures of **1**–**8**.

reported that its methanolic extract had various pharmacological activities such as antifungal (Rajan et al. 2016), antimicrobial, antioxidant and insecticidal (Kekuda et al. 2015) activities. However, no chemical data of this lichen are reported. As part of a continuing investigation of Vietnamese lichens, the lichen *P. cristiferum* was investigated. In this paper, the isolation and structural elucidation of two new phenolic compounds, cristiferides A-B (**1-2**) together with six known compounds, 2,4-dihydroxyphthalide (**3**) (Duong and Bui 2019), lecanoric acid (**4**) (Ahmann and Mathey 1967), orsellinic acid (**5**) (O'Donovan et al. 1980), 5-chloroorsellinic acid (**6**) (Jayaprakasha and Rao 2000), methyl haematommate (**7**) (Bézivin et al. 2003), and methyl β-orsellinate (**8**) (Tram et al. 2020) from the lichen *P. cristiferum* are reported. Their structures were elucidated from spectroscopic data and comparison with literature data. Compounds **1**-**3** and **6**-**8** were evaluated for alpha-glucosidase inhibition.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. Its molecular formula was determined as $C_{10}H_{10}O_6$ by a deprotonated ion peak at *m/z* 225.0407 on HRESI mass spectrum (calcd. for $C_{10}H_9O_6$, 225.04046). The ^{1}H-NMR and HSQC spectra revealed two hydrogen-bond phenolic groups (δ_H 14.88 and 14.25), one methine proton (δ_H 6.02, 1H, s), one methoxy group (δ_H 3.74, 3H, s), one methyl group (δ_H 2.16, 3H, s). The JMOD spectrum, in accordance with HSQC spectrum, exhibited two carboxyl carbons (δ_C 177.2 and 169.1), one methine carbon (δ_C 107.7), one methoxy carbon (δ_C 51.2), one methyl (δ_C 20.5), and five quaternary carbons (δ_C 164.7, 162.4, 141.5, 112.2, and 102.5, the two former oxygenated). These spectroscopic data suggested that compound **1** was a monoaromatic compound (Figure 1). HMBC correlations of H_3-9 (δ_H 2.16, s) to C-1 (δ_C 112.2), C-5 (δ_C 107.7), and C-6 (δ_C 141.5), of H-5 (δ_H 6.02) to C-1, C-4 (δ_C 164.7), and C-6 defined the connectivity arising through C-1-C-6-C-5-C-4 (Figure S1). Further, the ^4J long range HMBC correlation of H_3-9 and ^3J HMBC correlation of 7-OCH_3 indicated the presence of the methoxy group at C-7. At last, HMBC cross-peaks of the hydroxy group at δ_H 14.88 to C-1 (δ_C 112.2), C-2 (δ_C 162.4), and C-3 (δ_C 102.5) indicated that this hydroxy group was attached at C-2. Likewise, HMBC correlations of

the second hydroxy group at δ_H 14.25 to C-3, C-4 (δ_C 164.7), and C-5 (δ_C 107.7) defined the position of this group at C-4. All above functionalities accounted for five degrees of unsaturation and a $C_9H_9O_4$ moiety. Thus, the remaining COOH moiety was anchored at C-3. Combined, the chemical structure of **1** was elucidated as shown, namely cristiferide A (Figure 1). The chemical structure of **1** was highly similar to methyl haematomate (**7**), a co-isolate from this lichen, except for the replacement of the carboxylic acid group in **1** for the aldehyde group at C-3 in **7**. The 3-COOH group is quite rare among lichen monoaromatic compounds.

Compound **2**, white amorphous powder had the molecular formula as $C_{19}H_{17}O_9Cl$ by a deprotonated ion peak at *m/z* 423.0482 on HRESI mass spectrum (calcd. for $C_{19}H_{15}O_9Cl$, 423.04883). The ^{1}H-NMR and HSQC spectra revealed three hydrogen-bond phenolic groups (δ_H 15.73, 15.11, and 11.82), one methine proton (δ_H 6.66, 1H, s), one methoxy group (δ_H 4.00, 3H, s), three methyl groups (δ_H 2.53, 2.41, 2.15, each 3H, s). HMBC correlation of the methoxy group at δ_H 4.00 with carbon at δ_C 173.2 indicated the presence of an ester group in **2**. The occurrence of two downfield hydroxy groups at δ_H 15.73 and 15.11 was very uncommon and similar to those of **1**. This might be indicative of a carboxyl group at C-3 in the A-ring. In the B-ring, HMBC correlations of H_3-9′ to C-1′ (δ_C 109.7), C-5′ (δ_C 117.8), C-6′ (δ_C 140.1), of H-5′ to C-1′, C-3′ (δ_C 118.0), C-4′ (δ_C 154.9), and C-6′, of H_3-8′ to C-2′ (δ_C 163.2), C-3′, and C-4′ defined the chemical structure of B-ring (Figure 1). The ^{13}C chemical shift of C-4′ (δ_C 154.9) indicated that **2** was a depside with the ester linkage at C-7 and C-4′ (Figure 1) (Huneck and Yoshimura 1996).

NMR comparison of **2** and **1** gave the structural similarities in the A-ring. The difference is the replacement of H-5 in **1** by a chlorine atom in **2**, supported by HMBC correlations of H_3-9 (δ_H 2.41, s) to C-1 (δ_C 111.8), C-5 (δ_C 112.3), and C-6 (δ_C 138.8). Careful comparison of NMR data of **2** with **1**, chloroatranorin, and cladonioidesin provided full NMR assignments of the A-ring (Ahmann and Mathey 1967; Jiang et al. 2001; Vu et al. 2015). The position of the ester group of **2** could be attached to either C-8 or C-7′, giving two possibilities of **2** (**2 A** and **2B**) (Figure S2). If the ester group was anchored at C-8 (in **2 A**, Figure S2), the ^{13}C chemical shift of C-7′ would be 176.6 ppm. Literature data (Huneck and Yoshimura 1996) indicated that the ^{13}C chemical shift of 7′-COOH is around 172-174 ppm in $CDCl_3$, DMSO-d_6, and acetone-d_6. Thus, the structure **2 A** was precluded. NMR comparison of **2** and elatinic acid (Culberson et al. 1987) in the same deuterated solvent (acetone-d_6) gave the similarity, supporting the possibility **2B** (Table S1). In **2B**, the ^{13}C chemical shift of 8-COOH in **1** and **2** was identical, confirming the previous finding. Consequently, compound **2** was elucidated as shown, namely cristiferide B.

As the methanolysis of lichen metabolites is frequently occurred when using methanol as a solvent, the crude extract was prepared by maceration in EtOAc to avoid the artifactual formation. In order to confirm that the new metabolites reported for *P. cristiferum* occurred naturally in the original lichen, an extraction process using EtOAc was conducted again at room temperature on a new lichen sample (Venditti 2020). The extract and isolated compounds **1** and **2** were then analyzed by HPLC-DAD (Figure S15 and S16). The results indicated the native occurrence of new compounds. The stability of cristiferide B in methanol when reproducing the purification process and

storing the compound in methanol for a week, supports its genuine lichen production. Compounds having methyl ester groups are quite common among lichen metabolites (Duong et al. 2015, 2017, 2018a, 2018b). Cristiferide B shared the similar A-ring with those of natural elatinic acid derivatives, i.e., cladonioidesin and 2-*O*-methylsquamatic acid reported previously (Garbarino et al. 1987; Vu et al. 2015).

Compounds **1**-**3** and **6**-**8** were evaluated for the alpha-glucosidase inhibition. Compounds **2** and **7** revealed potent inhibition toward alpha-glucosidase (IC_{50} 72.66 μM and 48.73 μM, respectively, compared to the positive control, acarbose with the IC_{50} value of 332.5 μM) while other compounds failed to reveal any activity (Table S2). Compounds **4** and **5** were reported their weak alpha-glucosidase inhibition (Lopes et al. 2008; Rama Krishna et al. 2019) (Table S3).

3. Experimental

3.1. General experimental procedures

The NMR spectra were recorded on a Bruker Avance III spectrometer (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR) using residual solvent signals as internal references: acetone-d_6 at δ_H 2.05, δ_C 29.84, 206.26. The HR–ESI–MS was recorded on a HR–ESI–MS MicrOTOF–Q mass spectrometer on a LC-Agilent 1100 LC-MSD Trap spectrometer. Thin layer chromatography (TLC) was carried out on precoated silica gel 60 F_{254} or silica gel 60 RP–18 F_{254S} (Merck) and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. Gravity column chromatography was performed on silica gel 60 (0.040–0.063 mm, Himedia). The HPLC analysis was performed with the Agilent 1260 HPLC-UV/PDA system (Santa Clara, CA, U.S.A) using a Phenomenex Luna C18 column (150 × 4.6 mm. i.d., 5 μm) at 25 ◦C

3.2. Lichen material

The thallus of lichen *P. cristiferum* were collected in Duc Trong district, Lam Dong province, Vietnam in March 2020. The scientific name of the lichen was determined by Dr. Thi-Phi-Giao Vo, Faculty of Biology, Ho Chi Minh University of Science, National University – Ho Chi Minh City. A voucher specimen (UE-L006) was deposited in the herbarium of the Department of Organic Chemistry, Ho Chi Minh University of Education.

3.3. Extraction and isolation

The clean, air-dried and ground material (1.1 kg) was macerated in EtOAc at room temperature (10 L x 5 times, each 12 hours) and the filtrated solution was concentrated under reduced pressure to afford the crude EtOAc extract (330 g). The crude extract was suspended in water and successively liquid-liquid partitioned into *n*-hexane (**H**, 29.2 g), *n*-hexane: EtOAc (5:5) (**HEA**, 48.7 g) and EtOAc (**EA**, 157.5 g) to afford corresponding extracts.

The **HEA** extract (48.7 g) was applied to silica gel column chromatography using a mobile phase of *n*-hexane-EtOAc (stepwise, 3:1, 2:1, 1:1, v/v) to give 10 fractions

(**HEA1**- **HEA10**). Fraction **HEA9** (1.9 g) was applied to Sephadex LH-20 gel chromatography, eluted with MeOH to obtain four subfractions **HEA9.1-4**. Subfraction **HEA9.4** (187 mg) was subjected several times to preparative TLC using an eluent as $CHCl_3$-EtOAc-acetone-acetic acid (2:2:2:0.01) to afford compounds **1** (2.1 mg), **3** (21 mg), **4** (3.4 mg), **5** (15 mg), **6** (1.4 mg), **7** (11 mg), and **8** (21 mg). Subfraction **HEA9.2** (96 mg) was loaded onto Sephadex LH-20 gel chromatography using MeOH as an eluent, yielding compound **2** (1.8 mg).

3.4. HPLC experiments proving the natural occurrence of compound 1–2

An EtOAc extract of the studied lichen and isolated compounds were analyzed through a HPLC-DAD using the modified method (Duong et al. 2017). Samples were injected separately. The mobile phase consisted of (ACN + 0.1% HCOOH) as solvent A and (H_2O + 0.1% HCOOH) as solvent B with a gradient of 5% − 10% A over 5 min, 10 →30% A over 15 min, 30 →80% A over 10 min, 80 →100% A over 5 min, then 100% A for 5 min. The flow rate was 1 mL/min, and 10 µL of each sample was injected. HPLC analysis was conducted individually using the EtOAc extract of the lichen and two compounds **1**-**2**. The HPLC chromatograms were showed in Figures S15 and S16.

3.4.1. Cristiferide A (1)

White amorphous powder; ^{1}H NMR (Acetone-d_6, 500 MHz) δ_H 14.88 (1H, s, 2-OH), 14.25 (1H, s, 4-OH), 6.02 (1H, s, H-5), 3.74 (1H, s, H-10), 2.16 (1H, s, H-9). ^{13}C NMR (Acetone-d_6, 125 MHz) δ_C 177.2 (C-8), 169.1 (C-7), 164.7 (C-4), 162.4 (C-2), 141.5 (C-6), 112.2 (C-1), 107.7 (C-5), 102.5 (C-3), 51.2 (C-10), 20.5 (C-9). HRESIMS *m/z*: $[M-H]^-$ 225.0407 for $C_{10}H_9O_6$ (calcd. 225.04046).

3.4.2. Cristiferide B (2)

White amorphous powder;; ^{1}H NMR (Acetone-d_6, 500 MHz) δ_H 15.73 (1H, brs, 2-OH or 4-OH), 15.11 (1H, brs, 2-OH or 4-OH), 11.82 (1H, s, 2′-OH), 6.66 (1H, s, H-5′), 4.00 (1H, s, H-10′), 2.53 (1H, s, H-9′), 2.41 (1H, s, H-9), 2.15 (1H, s, H-8′).^{13}C NMR (Acetone-d_6, 125 MHz) δ_C 176.6 (C-8), 173.2 (C-7′), 169.8 (C-7), 166.2 (C-2), 163.2 (C-2′/C-4), 154.9 (C-4′), 140.1 (C-6′), 138.8 (C-6), 117.8 (C-3′), 117.7 (C-5′), 114.6 (C-3), 112.3 (C-5), 111.9 (C-1), 109.7 (C-1′), 52.7 (7′-OCH_3), 23.8 (C-9′), 18.0 (C-9), 9.5 (C-8′). HRESIMS *m/z*: $[M-H]^-$ 423.0479 (100%), 425.0456 (32.0%), 424.0517 (20.5%), 426.0489 (6.6%) for $C_{19}H_{16}O_9Cl$ (calcd. 423.0483).

3.5. Alpha-glucosidase inhibition assay

The alpha-glucosidase inhibitory activity of compounds **1-3** and **6-8** was determined using a method adapted from a previous method (Devi et al. 2020). All samples were analyzed in triplicate at five different concentrations around the IC_{50} values, and the mean values were retained. The inhibition percentage (%) was calculated by the following equation: Inhibition (%) = [1 − (Asample/Acontrol)] × 100.

4. Conclusions

From the lichen *Parmotrema cristiferum* (Taylor) Hale, eight compounds were isolated and elucidated. These are cristiferides A-B (**1-2**), 2,4-dihydroxyphthalide (**3**), lecanoric acid (**4**), orsellinic acid (**5**), 5-chloroorsellinic acid (**6**), methyl haematommate (**7**), and methyl β-orsellinate (**8**). All isolated compounds were reported for the first time in the title lichen. Cristiferide B and methyl haematommate revealed potent inhibition toward alpha-glucosidase with IC_{50} values of 72.66 μM and 48.73 μM, respectively.

Disclosure statement

No potential conflict of interest was reported by the authors.

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