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CHEMICAL CONSTITUENTS OF THE LICHENS *CLADONIA MULTIFORMIS* AND *CRYPTOTHECIA* SP.

(Sebatian kimia liken *Cladonia multiformis* dan *Cryptothecia* sp.)

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Abstract

Two depsides (atranorin **1** and evernic acid **4**), an aromatic compound (methyl β-orcinolcarboxylate **2**) and one cleavage product of depside (everninic acid **3**) were isolated from two lichens, *Cladonia multiformis* (**1** and **2**) and *Cryptothecia* sp. (**3** and **4**). The identification of the four compounds was carried out by comparison of the recorded NMR data with that of the reported. Compounds **1** and **4** showed antibacterial activity against *Bacillus subtilis* and *Enterobacter aerogenes*.

Keywords: lichen, *Cladonia*, *Cryptothecia*, antibacterial activity

Abstrak

Dua depsida (atranorin **1** dan asid evernik **4**), satu sebatian aromatik (metil β-orcinolkarboksilat **2**) dan satu hasil pemutusan depsida **4** (asid everninik **3**) telah dipencilkan daripada dua liken, *Cladonia multiformis* (**1** dan **2**) dan *Cryptothecia* sp. (**3** dan **4**). Pengecaman keempat empat sebatian dilakukan secara membandingkan data RMN yang direkodkan dengan data yang dilaporkan. Sebatian **1** dan **4** telah menunjukkan aktiviti antibakteria terhadap *Bacillus subtilis* dan *Enterobacter aerogenes*.

Kata kunci: liken, *Cladonia*, *Cryptothecia*, aktiviti antibakteria

Introduction

Lichen is a composite organism consisting of symbiotic association of fungus and photosynthetic partner, usually a green algae or crynobacterium [1, 2]. The lichen produces many unusual secondary products which are not found in other plants such as depsides, depsidones, diphenyl ethers, dibenzofurans and pulvinic acid derivatives [1, 2, 3]. They are found growing in almost everywhere from the poles to the tropics, from the intertidal zones to the peaks of mountains and on every kind of surfaces from soil, rocks and tree bark to the backs of living insects [3, 4].

Cladonia multiformis Merr. (Fig. 1). Primary squamules persistent or disappearing; middle-sized, 1-4 mm long, up to 0.5 mm broad; digitately lobed; ascending; flat to involute; the upper side glaucescent to olive-green or brownish; the underside white, darkening toward the base; esorediate. Podetia from the upper side of the primary squamules; up to 45 mm tall and about 1-2 mm in diameter; cup-bearing. Apothecia on the apices of the branches or proliferations, dark brown. Habitat on thin, mineral soil in sun to partial shade [4, 5]. However, no chemical study

on this lichen has been reported. Previous studies on the taxa of this genus led to the isolation of usnic acid [6], α-D-glucan [7] and fatty acids [8].

Crustose's *Cryptothecia* (Fig. 2) with thick, almost zoned thalli with a conspicuous prothallus developing at the periphery. Clearly defined fruiting bodies are not formed. Asci simply arise (sometimes in groups) within the thallus; spores colorless to pale brown, very large, muriform, 1-2 per ascus. Habitat on bark in subtropical woodlands [4]. But, no chemical study on this lichen has been reported.

Figure 1. *Cladonia multiformis*

Figure 2. *Cryptothecia* sp.

Materials and Methods

Plant Material

Cladonia multiformis (GHR2) and *Cryptothecia* sp. (LDS1) were collected in February 2012 from Genting Highlands, Pahang and in April 2013 from Lembah Danum, Sabah, respectively. Both voucher specimen are deposited at the School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti

Kebangsaan Malaysia. All samples were cleared of clinging debris such as soil, mosses and leaves using small paintbrush and forceps.

Extraction and Isolation

Cladonia multiformis (52.4 g) was extracted with acetone by using Soxhlet (3 x 8 hours) to give 1.10 g (2.1 %) of acetone extract. The extract was subjected to column chromatography (CC) eluted with $CHCl_3$-MeOH in the order of increasing polarity, to give ten fractions (A-J). Purification of fractions B (28.9 mg) and G (5.3 mg) by using Sephadex LH-20 column eluted with methanol gave compound **1** (4.1 mg) and **2** (1.9 mg), respectively.

Cryptothecia sp. (7.4 g) was extracted with acetone at room temperature (3 x 3 days) to yield 0.28 g (3.8 %) of acetone extract. The extracts was subjected to CC eluted with $CHCl_3$-MeOH in the order of increasing polarity, to give four fractions (A-D). Fraction B (17.1 mg) was purified by preparative thin layer chromatography (PTLC) (7H:3EA) to yield compound **3** (2.0 mg). Purification of fraction D (86.4 mg) by using Sephadex LH-20 column eluted by methanol gave compound **4** (6.3 mg).

Atranorin **1** (4.1 mg): pale orange fine needles. R_f = 0.76 (standard solvent C; toluene:acetic acid; 170:30). $C_{19}H_{18}O_8$. ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H: 12.56 (1H, *s*, 4-OH), 12.52 (1H, *s*, 2-OH), 11.96 (1H, *s*, 2′-OH), 10.36 (1H, *s*, 8′-CHO), 6.52 (1H, *s*, H-5′), 6.41 (1H, *s*, H-5), 3.99 (3H, *s*, 7′-COOC$\underline{H}_3$), 2.69 (3H, *s*, 9′-CH_3), 2.55 (3H, *s*, 9-CH_3), 2.10 (3H, *s*, 8′-CH_3). ^{13}C-APT NMR ($CDCl_3$, 125 MHz) δ_C: 102.8 (C-1), 169.1 (C-2), 108.5 (C-3), 167.5 (C-4), 112.9 (C-5), 152.5 (C-6), 169.7 (C-7), 193.9 (C-8), 25.6 (C-9), 116.8 (C-1′), 162.9 (C-2′), 110.2 (C-3′), 152.0 (C-4′), 116.0 (C-5′), 139.9 (C-6′), 172.2 (C-7′), 24.1 (C-8′), 9.4 (C-9′), 52.4 (COO$\underline{C}H_3$) [2].

Methyl β-orcinolcarboxylate **2** (1.9 mg): pale orange amorphous. R_f = 0.53 (standard solvent C). $C_{10}H_{12}O_4$. ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H: 12.02 (1H, *s*, 2-OH), 6.21 (1H, *s*, H-5), 5.41 (1H, *s*, 4-OH), 3.93 (3H, *s*, COOC$\underline{H}_3$), 2.46 (3H, *s*, 6-CH_3), 2.11 (3H, *s*, 3-CH_3). ^{13}C-APT NMR ($CDCl_3$, 150 MHz) δ_C: 105.2 (C-1), 158.1 (C-2), 108.6 (C-3), 163.1 (C-4), 110.6 (C-5), 140.1 (C-6), 51.8 (COO$\underline{C}H_3$), 24.1 (6-CH_3), 7.6 (3-CH_3), 172.6 ($\underline{C}OOCH_3$) [2].

Everninic acid **3** (2.0 mg): colourless fine needles. R_f = 0.47 (standard solvent C). $C_9H_{10}O_4$. ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H: 11.76 (1H, *s*, 2-OH), 6.29 (1H, *s*, H-5), 6.24 (1H, *s*, H-3), 3.99 (3H, *s*, 4-OCH_3), 2.49 (3H, *s*, 6-CH_3). ^{13}C-APT NMR ($CDCl_3$, 150 MHz) δ_C: 105.5 (C-1), 160.5 (C-2), 101.3 (C-3), 165.3 (C-4), 111.4 (C-5), 144.0 (C-6), 51.9 (4-OCH_3), 24.3 (CH_3), 172.2 (COOH) [2].

Evernic acid **4** (6.3 mg): pale orange amorphous. R_f = 0.24 (standard solvent C). $C_{17}H_{16}O_7$. ^{1}H NMR (acetone-d_6, 600 MHz) δ_H: 11.24 (1H, *br s*, COOH), 7.05 (1H, *d*, *J* = 1.2 Hz, H-5′), 6.89 (1H, *d*, *J* = 1.2 Hz, H-3′), 6.71 (1H, *br s*, 2-OH/2′-OH), 6.67 (1H, *br s*, 2-OH/2′-OH), 6.40 (1H, *d*, *J* = 1.8 Hz, H-3), 6.31 (1H, *d*, *J* = 1.8 Hz, H-5), 3.96 (3H, *s*, 4-OCH_3), 2.63 (3H, *s*, 8′-CH_3), 2.46 (3H, *s*, 8-CH_3). ^{13}C-APT NMR (acetone-d_6, 125 MHz) δ_C: 103.8 (C-1), 163.4 (C-2), 100.9 (C-3), 165.0 (C-4), 112.0 (C-5), 144.0 (C-6), 166.0 (C-7), 23.6 (C-8), 120.7 (C-1′), 158.0 (C-2′), 103.9 (C-3′), 152.2 (C-4′), 115.8 (C-5′), 137.8 (C-6′), 169.9 (C-7′), 18.5 (C-8′) [2, 9].

Bioassay

The pure compounds **1** and **4** were assayed for antibacterial activity against *Bacillus subtilis* (ATCC 11774), *Staphylococcus aureus* (ATCC 11632), *S. epidermidis* (ATCC 12228), *Enterobacter aerogenes* (ATCC 13048) and *Escherichia coli* (ATCC 10536). Pure compounds were freshly prepared at 1 mg/mL in DMSO. 10 µL of these solutions were applied on MHA plates. Antibiotic sample (chloramphenicol) and the solvent used were also tested as positive and negative controls, respectively. The plates were incubated at 37^{o}C for 24 h [10].

Results and Discussion

Atranorin **1** (Fig. 3) is a depside and was isolated as white fine needles. The ^{1}H NMR spectrum showed the presence of three intramolecular hydrogen-bonded six-membered ring hydroxyl groups (δ_H 11.96, 12.52 and 12.56), three methyl groups (δ_H 2.10, 2.55 and 2.69), one methoxyl group (δ_H 3.99), two aromatic protons (δ_H 6.41 and 6.52) and one aldehyde proton (δ_H 10.36). The ^{13}C-APT NMR spectrum displayed 19 signals consistent with 19 carbons. The

presence of two carbonyl ester groups were shown at δ_C 169.1 and 169.7. It also showed a carbon of aldehyde group at δ_C 193.9.

Methyl β-orcinolcarboxylate **2** (Fig. 3) is an aromatic compound and was obtained as a pale orange amorphous. The ^{1}H NMR spectrum showed a simple skeleton, which has only one benzene ring. Six proton signals appeared to represent two hydroxyl groups (δ_H 5.41 and 12.01) with the latter is in the form of six-membered ring due to intramolecular hydrogen bonding, an aromatic proton (δ_H 6.21), a methoxyl group (δ_H 3.93) and two methyl groups (δ_H 2.11 and 2.46). The ^{13}C-APT NMR spectrum showed a total of ten carbon signals assigned to six quaternary carbons (δ_C 105.2, 108.6, 140.1, 158.1, 163.1 and 172.6), an aromatic carbon (δ_C 110.6), a methoxyl group (δ_C 51.8) and two methyl groups (δ_C 7.6 and 24.1).

Everninic acid **3** (Fig. 3) is a cleavage product of the depside **4** appeared as a colourless fine needles. The ^{1}H NMR spectrum showed only five signals dedicated to a hydroxyl group (δ_H 11.76), two aromatic protons (δ_H 6.24 and 6.29), a methoxyl group (δ_H 3.93) and a methyl group (δ_H 2.49). The ^{13}C-APT NMR spectrum showed nine carbon signals consisting of five quaternary carbons (δ_C 105.5, 143.9, 160.5, 165.3 and 172.2), two aromatic carbons (δ_C 101.3 and 111.4), one methyl carbon (δ_C 24.3) and one methoxyl carbon (δ_C 51.9).

Evernic acid **4** (Fig. 3) is a depside and was isolated as a pale orange amorphous. The ^{1}H NMR spectrum indicated the presence of two hydroxyl group signals appeared at δ_H 6.67 and 6.71. The aromatic protons H-3, H-5, H-3′ and H-5′ appeared as a doublet at δ_H 6.40, 6.31, 6.89 and 7.05, respectively. A signal at δ_H 11.24 (br *s*) is dedicated to carboxylic acid. The ^{13}C-APT NMR spectrum showed 17 signals correspond to 17 carbons. The signal at δ_C 166.0 and 169.9 assigned to carbonyl ester (C-7) and COOH (C-7′), respectively.

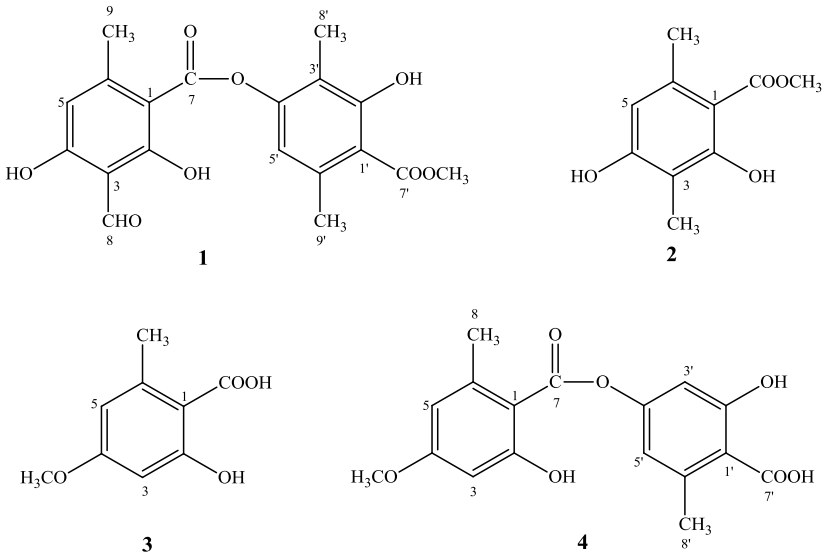

Figure 3. Structure of compounds **1**, **2**, **3** and **4**

Compounds **1** and **4** were evaluated for their antibacterial activity. Both compounds showed inhibitory against *Bacillus subtilis* and *Enterobacter aerogenes* which is comparable with the activity of Chloramphenicol. The inhibitory values are shown in Table 1.

Table 1. Antibacterial activity of compounds **1** and **4**

Compound	**Inhibitory zone (mm)**				
	B. subtilis	***S. aureus***	***S. epidermidis***	***E. aerogenes***	***E. coli***
1	8±0.5	-	-	10.0±0.0	-
4	10±0.0	-	-	12±0.0	-
Chloramphenicol	23±0.0	20.5±0.2	19.0±0.5	26±0.7	26±0.3

Conclusion

Chromatographic separation of acetone extract of *Cladonia multiformis* yielded two compounds which are identified as atranorin and methyl β-orcinolcarboxylate. This is the first reported phytochemical studies of this lichen. Everninic and evernic acids, on the other hand, were successfully isolated from acetone extract of *Cryptothecia* sp. and this is also the first reported chemical investigation of this lichen.

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