Antibacterial Compounds and other Constituents of *Evernia divaricata* (L.) Ach.

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Summary: An activity-directed fractionation and purification process was used to isolate the antibacterial compounds from *Evernia divaricata* (L.) Ach. Six lichen substances were obtained from the active petroleum ether and ethyl acetate fractions. Antibacterial activity assay showed that (+)-(12*R*)-Usnic acid (2) and Divaric acid (6) strongly inhibited bacterial growth of *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. However, four other compounds of Atranorin (1), 2,4-Di-*O*-methyldivaric acid (3), Divaricatinic acid (4) and 2-*O*-Methylnordivaricatic acid (5) had no apparent antibacterial activities against the four bacteria. Compounds 3, 4, 5 and 6 are reported here for the first time from *E. divaricata* (L.) Ach. Furthermore, this is also the first time to report Divaric acid exhibits strong anti-bacterial properties.

Introduction

Widespread use of antibiotics causes significant increase in resistance of bacteria, particularly *Gram*- positive organisms. For example, the rapid emergence of vancomycin- resistant *Enterococcus* (VRE) is believed to be caused by the excessive use of vancomycin [1]. Furthermore, the spoilage and poisoning of foods by microorganisms are problems that have not yet been brought under adequate control despite the range of robust preservation techniques available. In nature, there are a large number of different types of antimicrobial compounds that play an important role in the natural defense of all strains of living organisms [2]

Lichen is a special symbiotic organism in nature. It consists of mycobiont (fungus) and photobiont (alga). The algal cells are located in the thalli-shaped fungal tissue, so it can grow phytosynthetically [3]. Lichen produces a diverse range of secondary metabolites, including depsides, depsidones, dibenzofurans and depsones, which are unique to lichen symbioses. These compounds have attracted much attention in investigations because of their antiviral, antibiotic, antioxidant, antitumor, allergenic and plant growth inhibitory activities [4]. However, lichen has been ignored by the modern pharmaceutical industry for their slow growing nature.

As far as our literature survey could ascertain, studies on the antibacterial constitutes of *Evernia divaricata* (L.) Ach. from Qinghai Province have not been previously published except the work of Ali Aslan *et al.* [7]. These authors investigated the antimicrobial activities only with the crude methanol extracts of this material. There was no systemic study reported about the chemical constitutes of *Evernia divaricata* (L.) Ach. Based on this information, the antibacterially active constitutes of *Evernia divaricata* (L.) Ach. were investigated for the first time in this study.

Results and Discussion

Non-polar and polar extracts from the *E. divaricata* belonging to the Parmeliaccae family were assayed for their ability to reduce or suppress the growth of four strains of bacteria *in vitro*. Antibacterial activities of the investigated extracts are shown in Table-1. The petroleum ether and ethyl acetate fractions showed significant inhibitory values against the four strains of tested bacteria, especially on *Gram*-positive ones (the inhibitory zone of A: 24 mm and the inhibitory zone of B: 28 mm on *Bacillus subtilis*, respectively. A stands for petroleum ether fraction; B stands for ethyl acetate fraction), more robust than positive control.

Further isolation and purification was employed for the two active fractions and afford us six compounds. They were confirmed to be Atranorin (1), (+)-(12*R*)-Usnic acid (2), 2,4-Di-*O*-methyldivaric

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Table-1: The bacteria inhibitory effect of the fractions of the petroleum ether extract, ethyl acetate extract, ethyl acetate extract and controls.

	Inhibitory Zone (mm)			
	Gram-positive bacteria		*Gram*-negative bacteria	
Sample	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli* DH5α	*Pseudomonas aeruginosa*
A	24	17	-	10
B	28	20	-	13
C	16	-	-	10
Positive Control	20	22	20	29

A: petroleum ether fraction; B: ethyl acetate fraction; C: methanol fraction
The concentrations of A, B and C all are 10 mg/ml, respectively.
Positive control: tetracycline hydrochloride, concentration: 0.1 mg/ml
Negative control was used with acetone (diam. = 6 mm).
-: No growth inhibition

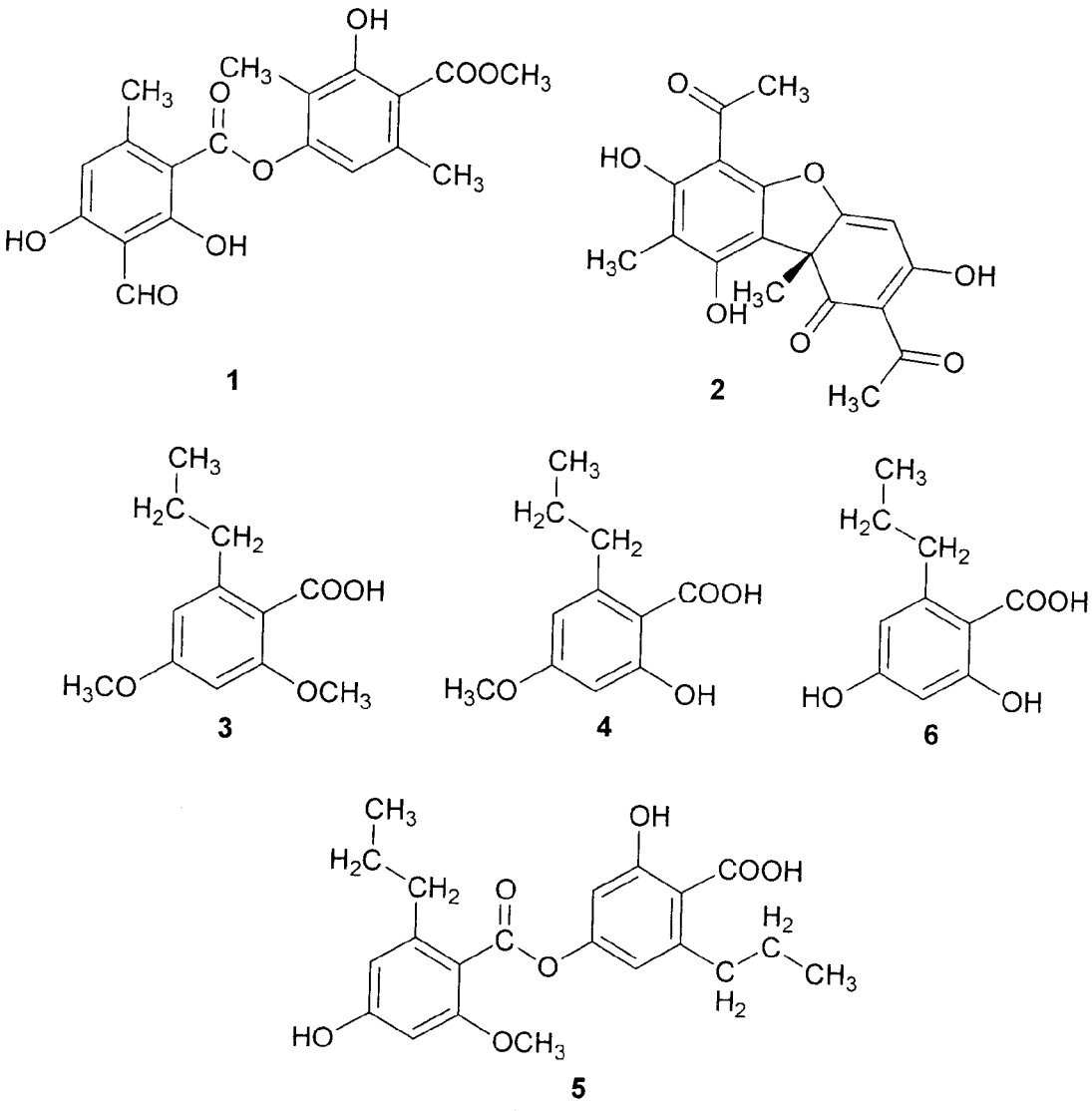

Fig. 1: Structures of compounds **1-6**.

acid (3), Divaricatinic acid (4), 2-*O*-Methyl-nordivaricatic acid (5) and Divaric acid (6) by the comparison of their spectral data with those in the references [5, 6]. The structures of compounds **1–6** were shown in Fig. 1.

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values of the six isolated compounds against bacteria are summarized in Table-2. (+)-(12*R*)-Usnic acid (2), and Divaric acid (6) showed very potent inhibitory activity against four strains of bacteria: compound 2 with MIC values of 0.0078 to 0.0156 mg/ml, and compound 6 with MIC values of 0.0625 to 0.25 mg/ml, respectively. (+)-(12*R*)-Usnic acid (2) shows significant inhibitory activity against *B. subtilis* with MIC value of 0.0078mg/ml, but its MBC value is more than 1 mg/ml. This indicates (+)-(12*R*)-Usnic acid can neither kill the spore to death, nor destroy the germination ability of endospore.

The data in the current study confirmed the evidence in a previous study demonstrating the antimicrobial properties of *E. divaricata* [7], however, this is the first study reporting the chemical constitutes of *E. divaricata*. Four compounds: 2, 4-Di-*O*-methyldivaric acid (3), Divaricatinic acid (4), 2-*O*-Methylnordivaricatic acid (5) and Divaric acid (6) were reported in *E. divaricata* species for the first time. Beljanski, V. *et al.* uncovered the excellent antimicrobial activity of Usnic acid [8-10]. Vertika Shukla reported the chemical structure of Divaric acid from Ramalinaceae [5]; Y. Ma reported that Divaric acid exhibits moderate inhibition to human leukocyte elastase [11]; However, our research is the first report to show Divaric acid with so significant antibacterial activity.

These results indicated that lichen substances can be exploited as effective inhibitors of bacterial growth. By comparison of the structures of compound **3**, **4** and **6**, we found that the both existence of 2-OH and 4-OH is essential to Divaric acid's antibacterial activity and this will be helpful for the synthesis of active ramifications which will be applied wildly in pharmaceutical industry.

Because the antibacterial intensity depends on not only the compound's structure but also its content, there might be more potent compounds of antibacterial activity in the methanol fraction. The investigation of these compounds is now undergoing.

Experimental

Plant Material

Evernia divaricata (L.) Ach. was collected in July 2007 from Niuxin Mountain (altitude: 3050 m) in Qilian County, Qinghai Province, People's Republic of China. This plant was identified and authenticated by Dr. Hai-ying Wang at the Institute of Microbiology, Chinese Academy of Sciences [12, 13]. A voucher specimen (No. 20071469-1) has been deposited at the College of Life Science, Shandong Normal University, People's Republic of China.

Extraction and Isolation of Lichen Substances

The air-dried and powdered plant material (860 g) was extracted exhaustively with methanol three times by ultrasonification at 35 °C for 30 min. After removing the solvent in vacuo, a residue (57 g) was obtained, which was mixed with silica, packed in a column and eluted with petroleum ether, EtOAc and MeOH, successively. The three fractions (8.8 g, 18.2 g and 25.6 g, respectively) were applied to anti-

Table-2: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the six lichen substances.

	Minimum inhibitory concentrations (mg·ml-1)				Minimum bactericidal concentrations (mg·ml-1)			
Sample	Bacillus subtilis	Staphylococcus aureus	Escherichia coli DH5α	Pseudomonas aeruginosa	Bacillus subtilis	Staphylococcus aureus	Escherichia coli DH5α	Pseudomonas aeruginosa
1	>1	>1	>1	>1	--	--	--	--
2	0.0078	0.0156	>1	0.0125	>1	0.25	--	0.5
3	>1	>1	>1	>1	--	--	--	--
4	>2	>2	>2	>2	--	--	--	--
5	>2	>2	>2	>2	--	--	--	--
6	0.125	0.0625	0.25	0.25	>2	>2	>2	>2

bacterial experiment. On the basis of antibacterial experiment results, the active petroleum ether fraction was subjected to repeated silica gel column chromatography eluting with Chloroform: Benzene = 1:1 to afford two fractions. From which, two pure compounds (compound 1 and 2) were obtained after recrystallization in diethyl ether.

The active EtOAc fraction was eluted with standard solvent B (n-hexane: diethyl ether: formic acid = 130:100:20) [14], followed by acetone, resulted in four fractions. The four factions were subjected to column chromatography on Sephadex LH-20 eluting with MeOH to yield compounds 3 (5 mg), 4 (15 mg), 5(50 mg) and 6 (18 mg), respectively.

All of the six isolated compounds were tested for antibacterial activity. The structures of the six compounds were determined using spectroscopic techniques including IR, ^{1}H and ^{13}C NMR, MS and X-ray.

Preparation of bacterial inoculums

All of the bacteria strains: *Bacillus subtilis* , *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* were obtained from China General Microbiological Culture Collection Center, and were maintained in our laboratory under 4℃ on LB-plates until the test. All inoculums were cultured on LB-plates and the plates were incubated for 24 h at 37 °C. After incubation, they were diluted with physical salt watcr to give a concentration of approximately 10^{8} colony forming units per ml (CFU/mL).

Evaluation of Antibacterial Activity

All six substances were separately dissolved in acetone. The sterile filter-paper (6 mm in diameter) was soaked for 30 min, and then was placed on the plates. All plates were incubated for 24 h at 37 °C. After overnight's incubation, bacterial inhibitory activity was evaluated by measuring the zone of inhibition against the tested organisms. All tests were performed in triplicate. A filter-paper containing 0.1mol/L tetracycline hydrochloride served as positive control.

The minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) were determined using the agar dilution method. We regarded the minimum concentration of thorough inhibitation of bacteria growth in holes as MIC. Ten μl containment from thorough inhibitory holes was sprayed on LB-plate; we regarded the 99.9 % inhibitation concentration as MBC [15].

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