Bioactive Secondary Metabolites of the fungus *Noduliosporium* sp. Isolated from *Dittrichia viscose*

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Summary: The culture extract of the fungus *Nodulisporium* sp. # 9897, on chromatographic purification yielded 16-hydroxyisopimar-7-en-18-oic acid: nodulerterpene (**1**) along with cytochalasine C (**2**), D (**3**), canabiside B (**4**), ergosterol (**5**), 24-methylcholesta-7,22-*E*-diene-3β,5α-diol-6-one (**6**), (24*R*)-24-methyl-5α-cholest-7-ene-3β,5,6β-triol (**7**) and uracil (**8**). The structures of all the compounds were established with the help of spectroscopic analysis including 1D, 2D NMR data and high resolution mass spectrometry and or in comparison with the published literature. The isolates **1-4** showed mild activity against tested gram positive and gram negative pathogens. Nodulerterpene (**1**) also exhibited weak inhibitory activity against acetylcholinestrase (AChE), butrylcholinestrase (BChE) and lipoxegenase (LOX).

Introduction

The *Noduliosporium* fungal species are known to produce nodulisporic acid A as a potent, long-lasting, nontoxic systemic orally-active agent that kills fleas on dogs and is economically important as a good substitute of the existing insecticides [1, 2]. Various species of the genus *Noduliosporium* hasve also been involved in causing allergic fungal sinusitis in human [3]. This multi-functional group of the endophytic fungi has attracted the investigators all over the world to search and investigate new species of this group of organisms for their bioactive metabolites. For our ongoing project on endophytes, the ethyl acetate extract of the cultures of a *Nodulisporium* sp. isolated from the plant *Dittrichia viscosa* was investigated for its bioactive metabolites. As a result of the chromatographic purification of ethyl acetate extract of the fungal culture, we isolated 16-hydroxyisopimar-7-en-18-oic acid, nodulerterpene (**1**) along with cytochalasine C (**2**), D (**3**) [4, 5], canabiside B (**4**) [6], ergosterol (**5**) [7], 24-methylcholesta-7,22-*E*-diene-3β,5α-diol-6-one (**6**) [8], (24*R*)-24-methyl-5α-cholest-7-ene-3β,5,6β-triol (**7**) [9] and uracil (**8**) [10] were isolated from *Dittrichia viscosa*.

Results and Discussion

The EI-MS of **1** exhibited the molecular ion at *m/z* 320 $[M]^+$, whereas, the molecular formula $C_{20}H_{32}O_3$ with five DBE could be established with the help of HR-EI-MS, which showed the exact mass peak at *m/z* 320.2318. The IR spectrum showed stretching absorption bands for hydroxyl group (3465 cm^{-1}), carboxylic acid function at 3380-2415 (O-H) and 1695 (C=O) cm^{-1} and for olefinic system at 1620 cm^{-1}.

The ^{1}H-NMR spectrum of **1** displayed an olefinic methine at δ 5.34 which showed a cross-peak in COSY spectrum with a methylene resonating at δ 2.35 and 2.10. An oxygenated methylene appeared in the same spectrum at δ 3.70 (t, J = 7.5 Hz), which was found to couple in COSY spectrum with another methylene appeared at δ 1.49 (t, J = 7.5 Hz). In addition to the above data, three singlet methyls displayed their positions at δ 1.14, 0.74 and 0.72 indicating **1** to be a diterpenoid. The ^{13}C-NMR spectrum of **1** yielded well-separated 20 signals (Table 1), which were sorted out as three methyls, nine methylenes, three methines and five quaternary carbons based on the DEPT experiment. The carbonyl, olefinic and oxymethylene carbons resonated at δ 178.4, 134.6, 120.6 and 61.2 respectively, whereas, the three methyls appeared at δ 28.6, 21.2 and 13.8 in the same spectrum. The above discussed data accommodated two DBE, therefore, the remaining three DBE could be attributed to a

tricyclic system, which led to the idea that **1** must be a tricyclic diterpenoid. The ^{1}H-NMR spectrum further displayed many multiplets in up-field region due to various methylenes of cyclic systems. The observed NMR data (Table 1) was comparable with the data of hymatoxin C (**9,** Fig. 1) [11], which is a diterpenoid with sulphonate group at C-16. The larger difference in the chemical shift in NMR value of CH_2-16 (Table-1) could be attributed to the absence of sulphonate group in **1**, rather the NMR-shift of this center were comparable with the shifts [δ 3.67, t (59.0), CH_2-16] reported for hymatoxin E (**10,** Fig. 1) [11]. This comparison confirmed that compound **1** must be an alcohol instead of sulphonate.

All the carbon and hydrogen assignments were accomplished due to HSQC experiment, whereas, the structure was finally fixed by the careful analysis of COSY and HMBC spectra (Fig. 2). The singlet methyl (δ 1.14, C-19) showed HMBC correlations with carbons at δ 178.4 (C-18), 50.6 (CH-5), 42.8 (C-4) and 37.5 (CH_2-3), whereas, Me-20 (δ 0.72) displayed long-range interactions with carbons at δ 50.7 (CH-9), 50.6 (CH-5), 39.0 (CH_2-1) and 35.2 (C-10). The third methyl (δ 0.74, C-17) was found to couple with carbons at δ 43.1 (CH_2-15), 36.6 (CH_2-13) and 32.6 (C-12) in HMBC spectrum (Fig. 2). The oxymethylene protons were correlated with carbons at δ 43.1 (CH_2-15) and δ 32.6 (C-12), whereas, olefenic methine showed HMBC correlations with carbons at δ 134.6 (C-8), 50.7 (CH-9) and 50.6 (CH-5). These observations clearly demonstrated that the three rings in **1** are of six-membered each, and the information also helped to fix the positions of carboxylic acid, olefenic and oxymethylene groups. The stereochemistry at various chiral centers was confirmed through NOESY correlations and molecular model. The strong NOESY correlations of the axial (*ax*) Me-20 were observed with H-2$_{ax}$, H-6$_{ax}$, H-11$_{ax}$ and Me-19$_{ax}$ that indicated equatorial Me-18 has been oxidized to carboxylic acid. As a sum the whole data was only fit for structure **1**, which is 16-hydroxy-isopimar-7-en-18-oic acid and is named as nodulerterpene.

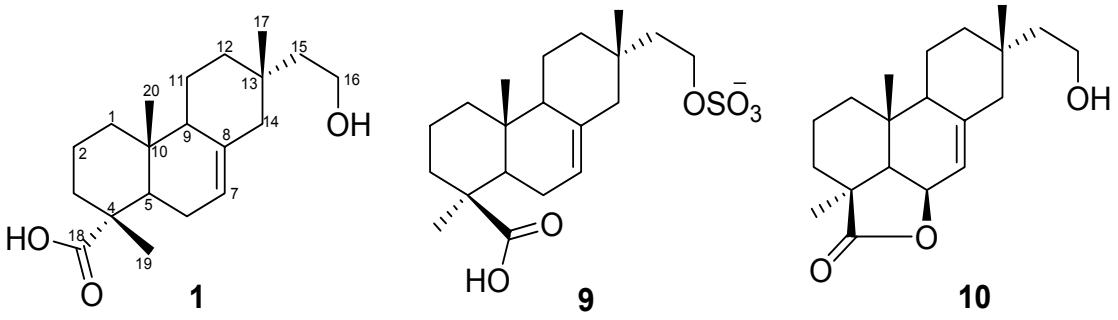

Fig. 1: Structural comparison of **1** with previously isolated diterepenoids of the same class.

Table-1: ^{1}H and ^{13}C-NMR data of compound **1** and its comparison with the reported data for **9**.

	1 (DMSO-*d6* (300 and 75 MHz)		9 (CD_3OD (400 and 100 MHz)	
Position	**^{1}H (δ, *J* = Hz)**	**^{13}C (δ)**	**^{1}H (δ, *J* = Hz)**	**^{13}C (δ)**
1	1.81 (m), 1.01 (m)	39.0	1.89 (m), 1.08 (m)	41.2
2	1.84 (m), 1.21 (m)	19.1	1.95 (m), 1.43 (m)	20.9
3	2.05 (m), 1.04 (m)	37.5	2.13 (m), 1.05 (m)	39.6
4	-	42.8	-	48.0
5	1.32 (dd, 12.3, 3.9)	50.6	1.33 (dd, 12.1, 4.3)	52.9
6	2.35 (td, 15.1, 4.8), 2.10 (br d, 15.0)	23.7	2.46 (m), 2.13 (m)	25.6
7	5.34 (br d, 5.0 Hz)	120.6	5.36 (br d, 5.9)	122.5
8	-	134.6	-	136.1
9	1.63 (overlapped signal)	50.7	1.67 (br d, 10)	52.9
10	-	35.2	-	36.9
11	1.52 (m), 1.30 (m)	20.2	1.31 (m),1.56 (m)	22.1
12	1.01 (m)	36.6	1.28 (m), 1.55 (m)	38.2
13	-	32.6	-	34.0
14	1.90 (br s)	46.5	1.91 (br s)	48.3
15	1.49 (t, 7.5)	43.1	1.58 (t, 7.4)	44.8
16	3.70 (t, 7.5)	61.2	4.09 (t, 7.4)	66.0
17	0.74 (s)	21.2	0.82 (s)	22.1
18	-	178.4	1.22 (s)	29.9
19	1.14 (s)	28.6	-	181.6
20	0.72 (s)	13.8	0.83 (s)	14.8

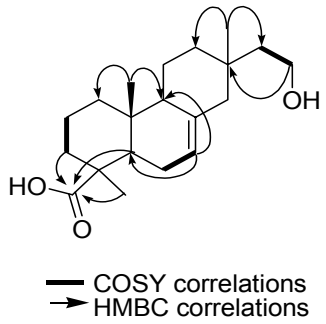

Fig. 2: Important COSY and HMBC correlations observed in the spectra of **1.**

Similar types of compounds have been reported from *Hypoxylon mammatum* fungal pathogen of *Leuce poplars*, mostly as sulphonates [11] or from the fungus *Xylaria polymorpha* as glycosides of **1** [12]. Compounds **1-8** (Fig. 3) have been isolated for the first time from any of the *Noduliosporium* sp.

Antibacterial Activity of Compounds **1-4**

The isolates **1-4** were screened for antibacterial activities and most of them were found to be weakly active against tested gram positive and gram negative pathogens (Table-2).

Enzyme Inhibitory Studies of **1**

Nodulerterpene (**1**) was also evaluated for its enzyme inhibitory properties against acetylcholinestrase (AChE), butrylcholinestrase (BChE) and lipoxegenase (LOX). Compound **1** exhibited low activity against three tested enzymes (Table-3).

Experimental

General Experimental Procedures

Open column chromatography was carried out using silica gel (GF_{254} type 60 of E. Merck, 70-230 mesh) as a stationary phase packed in glass columns with organic solvents as the mobile phase, whereas, Sephadex LH-20 was used in open glass column. HPLC was carried out at a system with a gradient pump model L-6200 A (Hitachi, Ltd, Tokyo Japan) detected with UV-VIS L-7420 Detector (Hitachi, Ltd, Tokyo Japan), using RP-18 column (250 × 10 mm) and a gradient mobile phase system of acetonitrile and water. Thin layer chromatography (TLC) was done on pre-coated aluminum TLC plates (GF_{254}, 0.25 mm E. Merck). Ceric sulphate solution was prepared in 65% sulphuric acid and used as the spraying agent. The TLC plates were visualized by spraying this reagent with subsequent heating and or under UV light at 254 and 366 nm. Optical rotation was measured on a Jasco DIP-360 digital polarimeter. IR spectra were recorded as KBr pellets on a Jasco-320-A infrared spectrophotometer. Mass spectra were recorded on Finnigan (Varian MAT) 112 and Finnigan (Varion MAT) 312 and 112S double focusing mass spectrometers connected to MASPEC data system-1 on PC base or on a Jeol JMX-HX 110 mass spectrometer. Linked scan and peak matching experiments were also performed on the same instruments. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker (300 and 75 MHz respectively) using TMS as an internal reference. Chemical shifts δ are in ppm, while coupling constants *J* are in Hz.

Table-2: Antibacterial activity of compounds **1-4.**

Compound	Zone of Inhibition (mm) against					
	Gram (-) bacteria				Gram (+) bacteria	
	E.coli	*P. aeruginosa*	*S. typhi*	*S. sonnei*	*S. aureus*	*B. subtilis*
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
1	00	6.1±00	00±00	6.5±00	6.62±0.57	7.0±20
2	7.46±0.51	6.33±00	7.33±1.12	6.5±05	8.16±0.52	7.32±0.47
3	08.66±1.12	7.33±00	9.66±0.57	8±00	8.66±0.57	7.33±1.12
4	10.5±40	9.7±51	10±53	10.41±0.45	11.31±1.15	11.3±30
Ampicilin*	12.90±0.25	13.10±0.205	13.12±1.12	13.33±1.12	12.13±0.88	12.80±0.57

Test Compound Concentration = 500μg/disc
*Standard antibiotic = 50μg/disc
No zone of inhibition (ZOI) was given by negative control (Methanol) discs.

Table-3: Enzyme inhibitory activity of **1.**

AChE (%) inhibition at 0.5 mM	AChE (IC_{50}) □M	BChE (%) inhibition at 0.5 mM	BChE (IC_{50}) □M	LOX (%) inhibition at 0.5 mM	LOX (IC_{50}) □M
72.14±0.21	123.91±0.01	84.04±0.41	219.81±0.03	86.12±0.68	230.81±0.05
Eserine	0.04±0.001	Eserine	0.85±0.001	Baicalein	22.4±1.3

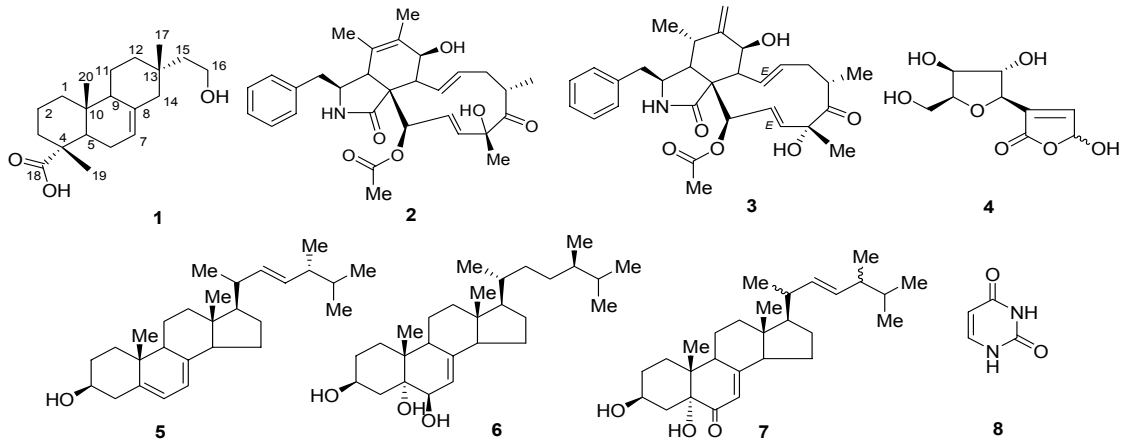

Fig. 3: Structures of the compounds **1-8** purified from the culture extract of *Noduliosporium sp.* isolated from *Dittrichia viscose.*

Isolation of Nodulisporium Sp. from Dittrichia Viscosa and its Culturing

Following surface sterilization, the endophytic fungus, internal strain no. 9897 (*Nodulisporium* sp.) was isolated from the tissues of a plant *Dittrichia viscosa* [13]. The fungus was then cultivated for 21 days at 20°C on biomalt agar medium [14].

Isolation of Metabolites from Nodulisporium Sp.

Twenty liters (20 L) of the culture medium (containing fungal mass) was extracted with ethyl acetate (EtOAc) to get 3.5 g of concentrated extract, of which 3.0 g of the extract was subjected to silica gel column chromatography eluting with *n*-hexane, *n*-hexane : EtOAc, EtOAc and EtOAc : MeOH to get 6 (M1-M6) fractions. Fraction M5 was further cleaned on sephadex LH-20 eluting with MeOH to get **1** (5 mg) and **4** (22.5 mg) as a colorless oil. Fraction M4 showed a prominent longitudinal spot on TLC and thus was purified on reversed phase HPLC eluting with gradient of acetonitrile and water on a column of 250 × 10 mm size, with a flow rate of 5 ml/min to get compounds **2** (55 mg) and **3** (15 mg). Fraction M2 showed some major with fewer minor spots on TLC and was purified on silica gel column eluted with a gradient of *n*-hexane : EtOAc (8:2 to 1:1) to get compound **5** (35 mg) and a semi-pure fraction which on further column chromatography under same conditions yielded **6** (8 mg), and **7** (6.5 mg). The fraction M3 was further purified on silica gel column eluted with 50% of EtOAc in *n*-hexane to get **8** (25 mg).

Noduloterpenoid **(1)**

White amorphous powder; $[\alpha]^{25}_{D}$ (*c* = 0.895, MeOH): +12.4; IR (KBr): 3465 (O-H), 3420-2415 and 1695 (COOH), 1620 and 1472 cm^{-1}(C=C); ^{1}H and ^{13}C NMR (See Table-1); EI-MS: *m/z* 320 $[M]^{+}$; 302 $[M-18]^{+}$, 257, 275, 167 and 153; HR-EI-MS: *m/z* 320.2318 (calcd. for $C_{20}H_{32}O_3$; 320.2351).

Enzyme Inhibitory Assays

The three enzyme inhibitory assays were performed according to the method described in literature [15-18] with slight modifications.

The percentage inhibition (%) was calculated by formula given below:

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Where:

Control=Total enzyme activity without inhibitor
Test = Activity in the presence of test compound

In all enzyme inhibitory assays, the IC_{50} values were calculated using EZ–Fit Enzyme Kinetics software (Perrella Scientific Inc. Amherst, USA). All the measurements were done in triplicate and statistical analysis was performed by Microsoft Excel 2003. Results are presented as mean ± sem.

Antibacterial Assay

The antibacterial activity was performed in sterile 96-wells microplates under aseptic environments. The method is based on the principle that microbial cell number increases as the microbial growth proceeds in the log phase of growth which results in increased absorbance of broth medium [19, 20]. The organisms were maintained on stock culture

agar. The test samples with suitable solvent and dilution were pipetted into wells (20 µg/well). Overnight maintained fresh bacterial culture after suitable dilution with fresh nutrient broth was poured into wells (180 µl). The initial absorbance of the culture was strictly maintained between 0.12-0.19 at 540 nm. The total volume in each well was kept to 200 µl. The incubation was done at 37ºC for 16-24 hours with lid on the microplate. The absorbance was measured at 540 nm using Synergy HT BioTek® USA microplate reader, before and after incubation and the difference was noted as an index of bacterial growth. The percent inhibition was calculated using the formula: Inhibition (%) = 100 (X–Y)/X where "X" is absorbance in control with bacterial culture and Y is absorbance in test sample. Results are mean of triplicate (n=3, ± sem). Ciprofloxacin, gentamycin and ampicillin were taken as standard. Minimum inhibitory concentration (MIC) was measured with suitable dilutions (5-30 µg/well) and results were calculated using EZ-Fit5 Perrella Scientific Inc. Amherst USA software, and data expressed as MIC_{50}.

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