SHORT REPORT

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A New Trihydroxylated Fatty Acid and Phytoecdysteroids from Rhizomes of *Trillium govanianum*

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Abstract: A crude hydro-methanolic extract of *Trillium govanianum* ex D. Don (Melanthiaceae,Trilliaceae) rhizomes and its subsequent solvents soluble fractions were tested against different fungal strains i.e. *Trichophyton rubrum* ATCC 40051, *Aspergillus niger* ATCC 16888, *Candida albicans* ATCC 18804, *Microsporum canis* ATCC 32903 and *Fusarium lini* ATCC 16888. The hydro-methanolic extract showed significant activity against *T. rubrum* and *M. canis* with 80 and 75% inhibitions respectively. Among the fractions, chloroform soluble fraction showed 90% inhibition, with minimum inhibitory concentration (MIC) of 10µg/mL against *T. rubrum* followed by ethyl acetate, butanol and *n*-hexane fractions. The bio-activity guided isolation of chloroform soluble fraction leads to a new trihydroxylated fatty acid, named govanic acid (**1**) along with two known phytoecdysteroids i.e. 20-hydroxyecdysone (**2**) and 5, 20-dihydroxyecdysone (**3**). The structures of isolated compounds were elucidated through 1D, 2D-NMR spectroscopic data analysis. All compounds (**1**-**3**) in *T. govanianum* are reported herein for the first time. Compound **1** showed significant activity against *T. rubrum* with 70% inhibition and MIC value of 5 µg/mL, but lack of activity against the other test strains.

Keywords: *Trillium govanianum;* trilliaceae; govanic acid; antifungal; phytoecdysteroids.

1. Plant source

Trillium govanianum Wall ex D. Don belongs to genus *Trillium* (family: Melanthiaceae, Trilliaceae), is native to Himaliaya and distributed from Pakistan to Bhutan at an altitudinal ranges of 2500–3800 m [1]. The rhizome of this specie is commonly known as "matar zela" or "teen patra" (Pushto) in Pakistan, and "nag chhatri"(Hindi) in India [2]. In traditional medicine, the rhizomes of this plant are used for treating wounds, dysentery, skin boils, menstrual and sexual disorders and as an antiseptic [3-5].

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The plant material i.e. rhizomes of *T. govanianum* were collected from Khyber Pakhtunkhwa, Dir Upper, Kohistan in August, 2013. The plant was identified by Mr. Ghulam Jelani (Curator), Department of Botany, University of Peshawar. A voucher specimen [No. Bot. 20092 (PUP)] has been deposited in the herbarium Department of Botany, University of Peshawar, Pakistan for future reference.

As a part of our search for bioactive compounds from medicinal plants in general, and in continuation of our previous research work [6, 7], rhizomes of *T. govanianum* have been investigated for bioactive metabolites. Herein, we report bioassay guided isolation and structure elucidation of a new trihydroxylated fatty acid (**1**) and two known phytoecdysteroids (2 and 3) Figure 1. The compound 1 was evaluated for its inhibitory effects against fungal strains.

2. Previous studies

Previous phytochemical studies on *T. govanianum* have resulted in isolation of steroids and saponins with moderate antifungal and anticancer activities [7,8]. Furthermore, analgesic, anti-inflammatory, free radical scavenging, *β*-glucuronidase inhibitory and cytotoxic activities against breast, liver, lungs and urinary bladder carcinoma cells, in addition to ethnomedicinal relevance of *T. govanianum*, have also been recently reported [1, 2, 8, 9]. To our best knowledge, apart from the aforesaid literature, there are no systematic pharmacological and phytochemical studies on *T. govanianum*.

3. Present studies

The chloroform ($CHCl_3$) fraction of *T. govanianum* rhizomes was selected for isolation of compounds on the basis of their significant anticancer [7] and antifungal activities. Column chromatographic technique was used for separation of compounds [10]. A slurry prepared with silica gel was subjected to column chromatography using *n*-hexanes, chloroform, EtOAc and MeOH solvents as mobile phase in increasing order of polarity yielded eleven sub-fractions (CF_A-CF_K). The sub fraction CF_E obtained with 20-40% EtOAc in chloroform was re-chromatographed over silica gel eluting with mixture of EtOAc and *n*-hexane in increasing order of polarity which yielded compound **1** (60% EtOAc in *n*-hexane; 132 mg). The sub fraction CF_H obtained with 5% MeOH in EtOAc was re-chromatographed over silica gel eluting with mixture of MeOH and EtOAc in increasing order of polarity yielded five sub fractions. The sub fraction CF_{Hh} obtained with 5% MeOH in EtOAc was further subjected to separation process through preparative thin layer chromatography using MeOH: EtOAc (1: 9) as mobile phase resulted in compounds **2** (13 mg) and **3** (18 mg).

Compound **1** was isolated and purified as a white powder from the chloroform soluble fraction. The compound was characterized by modern spectroscopic data analysis as a trihydroxy fatty acid. In EI-MS spectrum the molecular ion peak was displayed at *m/z* 330, while FAB-MS showed molecular ion peak at *m/z* 331 $[M+H]^+$. Its molecular formula of $C_{18}H_{34}O_5$ was obtained from EI-MS at *m/z* 330.2436 (calcd; 330.2406). The molecular formula showed two degrees of unsaturation due to the presence of an olefinic and a carbonyl group in the molecule. The IR spectrum also revealed strong absorptions for acid carbonyl (C=O) and olefinic (C=C) functionalities at 1690 and 1650) cm^{-1}, respectively. The absorption at 3404 cm^{-1} showed the existence of acid hydroxyl group. The three extra oxygen atoms in molecule were placed as hydroxyl groups on the basis of ^{1}H-NMR and connectivity data.

The ^{1}H-NMR (CD_3OD, 300 MHz) spectrum revealed signals for all the protons at various chemical shift values as observed for a known compound, trihydroxy mono unsaturated fatty acid [11], except the position of double bond in chain at position C-10/C-11. The two olefinic methine protons resonated at δ 5.46 (1H, dd, J = 11.1, 6.4 Hz, H-10) and 5.56 (1H, dd, J = 11.1, 6.1 Hz, H-11), respectively. H-7 showed a complex multiplet at δ 3.37 while H-8 and H-9 exhibited doublets of doublets at δ 3.25 (6.3, 3.0 Hz) and 4.48 (8.4, 6.3) respectively.

The location of double bond was confirmed from the daughter ion peaks for these left side chain losses as given. The fragment ion at *m/z* 57, 169 (cleavage at C-8, 9), 152 (OH loss) due to $[C_4H_9]^+$, $[CH_3(CH_2)_7CH{=}CH\text{-}CHOH]^+$, $[CH_3(CH_2)_7CH{=}CH\text{-}CH]^+$ fragment losses as well as at 171 (right side chain, $[OH\text{-}CH\text{-}OH\text{-}CH(CH_2)_4COOH]^+$), 155 (O loss) and 137 (H_2O loss) which were reported due to the possible breakage between C-7 and C-8 points in the chain as depicted in **Fig. S-1**. The terminal methyl protons (H-18) resonated at δ 0.90 (t, *J* = 8 Hz), while the methylenic protons (H-3, 4, 5, 14, 15, 16 and 17) resonated in range of δ 1.34 to 2.26 respectively. A triplet was assigned to the methylenic protons of H-2 at δ 2.26 (7.1 Hz) as well as for H-12 protons at δ 2.15 (7.1 Hz) while a multiplet was observed for H-13 at δ 2.10.

The ^{13}C-NMR (CD_3OD, 150 MHz) spectrum revealed signals for almost all the carbon atoms including carbonyl quaternary carbon at δ 177.7 (C-1), olefinic carbons at δ 130 and 134.6 (C-10 and 11) along with the hydroxyl bearing carbons at δ 71.7 (C-7), 76.9 (C-8) and 69 (C-9) (Table 1). ^{1}H-^{1}H COSY was helpful in assigning the correlations among the chain protons. The coupling constant (11.1 Hz) between H-10 and H-11as well as the IR absorption at 1650 cm^{-1} indicated a *cis* geometry [12]. Consequently, compound **1** was assigned as 7, 8, 9-trihydroxy-(10Z)-10-octadecenoic acid. The compound was named as govanic acid.

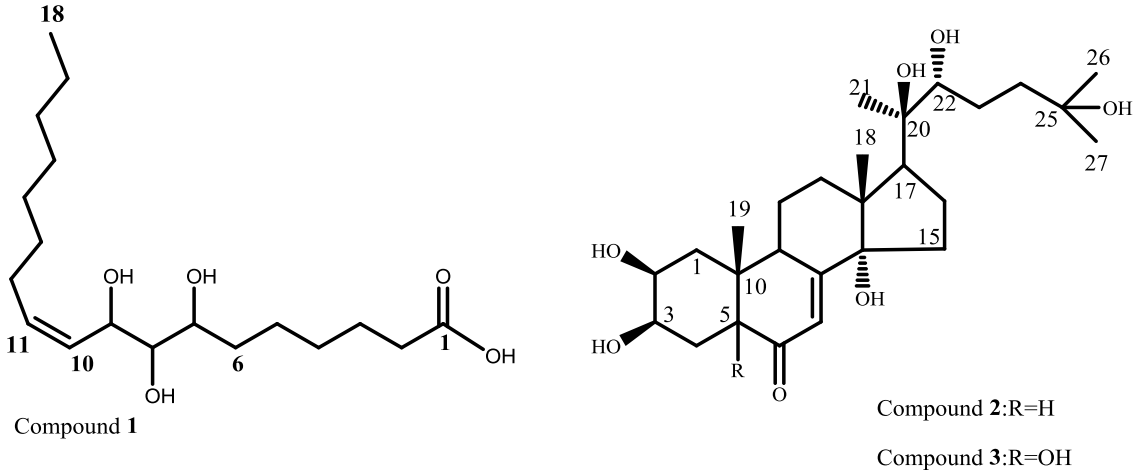

Figure 1. Chemical structures of compounds 1, 2 and 3.

The crude hydro-methanolic extract, fractions and compounds thereof were screened for antifungal activity against test strains i.e. *Trichophyton rubrum* ATCC 40051, *Aspergillus niger* ATCC 16888, *Candida albicans* ATCC 18804, *Microsporum canis* ATCC 32903 and *Fusarium lini* ATCC 16888 using previously reported method [13,14].

The results indicated (Table 2) a good activity (MIC=5 µg/mL) of govanic acid against *T. rubrum* comparable to standard drug miconazole. This activity was showed by crude hydro-methanolic extract and chloroform fraction, with both having MIC of 10 µg/mL, that got refined to 5 µg/mL in case of pure compound govanic acid.

In previous reports fatty acids have been associated with antifungal activity especially against *T. rubrum* due to their interaction with fungal cell membrane and fatty acid metabolism [15, 16]. Present study confirms earlier finding and herein report a pure compound having good activity against *T. rubrum.*

Although in bioassay guided isolation, crude hydro-methanolic extract had good activity against *M. canis* (MIC=10 µg/mL) but when isolated pure compound was tested, the activity against *M. canis* reduced, probably it is due to some other active compounds that was inhibiting the test fungi and did not got isolated or a number of compounds that were complementing each other in activity against *M. canis*, thus reduce activity upon separation.

It has been reported that the plants containing ecdysteroids possess noticeable pharmacological effects like anabolic antidiabetic, analgesic, anti-inflammatory and anthelmintic [17, 18]. Therefore due to the presence of 20-hydroxyecdysone (**2**) and 5, 20-dihydroxyecdysone (**3**), the rhizomes could also have great potential for treating the aforesaid conditions, however further detailed studies are required in this context to standardize the extract and develop effective drugs.

Table 1. ^{1}H-NMR and ^{13}C-NMR (CD_3OD, 300 and 150 MHz) chemical shift assignments in compound **1.**

C No.	δ_C	δ_H (*J*, Hz)	C No.	δ_C	δ_H (*J*, Hz)
1	177.7	-	12	32.7	2.15 t (7.1)
2	34.9	2.26, t (7.1)	13	30.2	2.10 m
3-5	26.1 - 30.6	1.34-1.61 br, m	14	28.9	1.59
6	34.6	1.59 m	15	26.9	1.54
7	71.7	3.73 m	16	30.4	1.44
8	76.9	3.25 dd (6.3, 3.0)	17	23.6	1.41
9	69.0	4.48 dd (8.4, 6.3)	18	14.4	0.90 t (8.0)
10	130.7	5.46 dd (11.1, 6.4)			
11	134.6	5.56 m (11.1, 6.1)			

Table 2. MIC values (µg/mL) of extract, fraction and compound of *T. govanianum* rhizomes.

Fungal strains	**Samples**				
	Cr. MeOH-ext	CHL- fr	Govanic acid	Amphotericin B	Miconazole
	MIC (µg/mL)				
Candida albicans	>20	>20	>20	0.6	-
Candida glabrata	>20	>20	>20	2.5	-
Aspergilllus flavus	>20	>20	>20	5.0	-
Aspergillus niger	>20	>20	>20	5.0	-
Aspergillus fumigatus	>20	>20	>20	5.0	-
Trichophyton rubrum	10	10	5.0	-	2.5
Microsporum canis	10	20	>20	-	5.0

*Concentration range 0.3125-20 µg/mL; MIC (minimum inhibitory concentration)

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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