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Friedelin Derivatives from *Polygonum flaccidum* Showed Cytotoxic Activity on *Vero* Cell Line

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ABSTRACT

Polygonum flaccidum is an important annual Bangladeshi herb which is being used by the local populace as analgesic, anti-inflammatory and insecticidal agent. In this study, the presence of secondary metabolites in the crude methanol extract of the whole plant of *Polygonum flaccidum* as well as their cytotoxic activity was investigated. The compounds were isolated using silica gel column chromatography and purified by preparative thin layer chromatographic technique. Preliminary cytotoxic activity of the column fractions was evaluated through brine shrimp lethality bioassay and toxicity of the isolated compounds were confirmed in Vero cell line. Three triterpenoids bearing friedelin skeleton in their structures were obtained and were characterized as 19α-hydroxyfriedelin (**1**), 16*α*-hydroxyfriedelin (**2**) and 3*β*-friedelinol (**3**) by analyzing their 1D and 2D NMR spectra. Friedelin derivatives are reported for the first time from this plant among which 19α-hydroxyfriedelin (**1**) has not been previously reported from any natural sources. In the brine shrimp lethality bioassay, LC_{50} values of the column fractions F-6, F-7 and F-8 were found to be similar to that of positive control (vincristine sulfate). 16α-hydroxyfriedelin (**2**) and 3*β*-friedelinol (**3**) showed potent cytotoxic activity with IC_{50} value of 8.09 µM/mL and 7.64 µM/mL, respectively in Vero cell line. Therefore, the plant can serve as a potential source of cytotoxic compounds which may be further exploited for new drug development.

***Keywords*:** *Polygonum flaccidum*, Friedelin derivatives, NMR spectroscopy, Vero Cell line.

Introduction

Polygonum flaccidum belonging to the family Polygonaceae, is a medium-sized plant found all over the tropical countries like Bangladesh, India, Srilanka and Afghanistan. Traditionally, the whole plant has been used as diuretic, purgative, analgesic, anti-inflammatory and insecticidal agent. Many local people used the decoction of the leaves of the plant for the treatment of snake-bites.[1] Very few studies has been carried out on the different extracts of *Polygonum flaccidum* which reported several bioactive compounds including acylflavone, α-santalene, caryophyllenepoxide, borneol, sitosterin and stigmasterol.[2] α-Santalone, a potent bioactive compound has been identified by the aerial parts of *P. flaccidum* which showed significant analgesic and diuretic activity.[3,4]
In continuation of our investigation on the bioactive secondary metabolites from *Polygonum flaccidum*, herein reported are the isolation and characterization of friedelin derivatives along with their brine shrimp lethality bioassay and cytotoxic activities on *Vero* cell line. All the isolates are reported for the first time from this plant, especially compound **1** which has not been previously described in any literature.

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Materials and Methods

Plant collection
The whole plant of *P. flaccidum* Meissn. was collected from the adjacent area of the Dhaka district in January, 2014. The undesirable materials were then separated immediately. The collected plant was identified by Mr. Sardar Nasir Uddin, a chief taxonomist at Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh and a voucher specimen (DACB No. 39317) was generated for future references.

General experimental procedure
The NMR spectra were recorded on a Bruker Avance-400 NMR spectrometer operating ^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz using $CDCl_3$. The chemical shifts (δ) were reported compared with standard (TMS). The HR-MS spectrum was recorded on an Exactive Orbitrap mass spectrometer by Thermo Scientific, UK. For the column chromatographic separation, Keiselgel 60, 220-240 mesh size silica was used. Pre-coated glass plates with Keiselgel 60 were used for the preparative TLC. The plates were observed under the UV light operating at 254 and 365 nm. Oxidation of the plates were done through sprayed diluted H_2SO_4 and heated until color was developed to identify the secondary constituents.

Extraction and isolation
The powdered whole plant (1 kg) of *P. flaccidum* was soaked into 6 L of methanol at room temperature for 7 days and occasionally stirred for proper dissolving. The extract was then filtered and was taken to a round bottom flask to evaporate on rotary evaporator. 49 g of viscous mass was yielded. The whole extract was subjected to VLC using solvent system of *n*-hexane, *n*-hexane-chloroform, chloroform and

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finally with the mixture of methanol-chloroform with increasing polarities to obtain 9 major fractions.
Meticulous analysis of the TLC behavior, the fraction-6 (0.54 g), 7 (3.20 g) and 8 (2.90 g) were combined and was subjected to column chromatography with silica gel (Kieselgel 60 and mesh 220-240). Eluted of the column were done using petroleum ether, followed by mixtures of petroleum ether-EtOAc, EtOAc and finally with the mixtures of EtOAc-methanol, along with increasing degree of polarities to obtain a total of 96 fractions each with 100 ml. After TLC screening, similar fractions were mixed together and purified the compounds by washing with different solvent treatments to give **1** (5 mg), **2** (3 mg), and **3** (4 mg).

Brine Shrimp lethality bioassay (BSLB)
The cytotoxic effect of the sub-fractions obtained from the column chromatography was carried out on the brine shrimp nauplii using the protocol established earlier.[5] Different concentrations of positive standard (Vincristine sulphate) and the sub-fractions F-6 to F-8 were prepared using DMSO as solvent. Previously hatched 10 nauplii was counted and transferred in each tube containing the desired concentrations of the tested samples and kept for 24 hours incubation under the light. The number of the survivors was counted.

Cell viability assay
The cell viability studies of the pure compounds were carried out at the Centre for Advanced Research in Sciences (CARS), University of Dhaka using Vero (African Green monkey) cell line using the published protocol.[6] DMEM (Dulbecco's modified eagles' medium) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin/streptomycin/neomycin and 25 mM HEPES. 90µL of cell suspension containing 2 x 10^4 cells were implanted onto each well of 96-well plates and 10 µL of the test samples were co-treated and incubated at 37^{o}C under humid condition of 5% of CO_2 for 24 h. The test samples (31.25, 62.5, 125 and 250 µl/mL) were prepared in 2.5% DMSO where DMSO was used as negative control. After incubation using a dye named trypan blue, a non-radioactive colorimetric cytotoxicity assay kit (Sigma-Aldrich, USA) was used for measuring cell viability. Each sample was tested twice in a single experiment.

Statistical analysis
In the brine shrimp lethality bioassay, LC_{50} values were obtained from the best-fit regression line plots of the number of dead shrimps versus concentration of the column extracts drawn in MS Excel office 2010. IC_{50} values of the pure compounds were calculated from the slope of the straight line drawn in MS Excel office 2010 during cytotoxic assay in vero cell line too. The results were compared with blank and p value was set at <0.05 for statistically significance.

Results and Discussion

Compound **1**, obtained as a white amorphous powder, was isolated from the column fraction by elution with petroleum ether-EtOAc (80%-20%). It showed a dark blue spot on the TLC plate under UV light at 254 nm and also exhibited blue fluorescence at 365 nm. The compound was found to be soluble in dichloromethane, chloroform and methanol. The mass spectrum of this compound shows the peak at *m/z* 440.1 corresponding to the molecular formula $C_{30}H_{48}O_2$ (calcd. for $C_{30}H_{48}O_2$, 440.384).
The ^{1}H- and ^{13}C-NMR spectra of compound **1** were closely related to those of 3-oxo-friedelin derivatives. Friedelin is a pentacyclic triterpene consistent with a six membered ring skeleton containing a carbonyl group, eight methyl groups and C-30 nucleus. The ^{13}C-NMR spectrum of compound **1** showed 30 signals which represent the C-30 nucleus of friedelane (Table 1).[7] Among which a signal appeared at δ_C 213.2 indicating a carbonyl group (>C=O) at C-3 position. Another signal appeared at δ_C 72.8 which indicated that one of the methylene proton was hydrolyzed. The ^{1}H-NMR spectrum reveals eight methyl group protons at δ_H 0.72, 0.86, 0.87, 0.95, 0.97, 1.00, 1.01 and 1.18 all of which were in agreement with the eight signals from the methyl groups in the friedelane skeleton. The doublet methyl proton signal at δ_H 0.86 was found to be attached to C-23 (δ_C 6.8) according to HSQC spectrum and it showed correlation with the signal at C-4 (δ_C 58.3) in the HMBC spectrum. Another integrated methyl protons signal at δ_H 0.72 showed HMBC correlation with C-5 which were in close agreement with other friedelane derivatives.[8]
The ^{1}H-NMR spectra also revealed a doublet methine proton, appeared at lower field (δ_H 3.76) with a coupling constant (*J*) of 2.0 Hz. The contour plotting of HSQC spectra reveals that the methine proton was attached to δ_C 72.8 (C-19). The HMBC spectra showed that two methyl protons (δ_H 0.95, 0.97) are attached to C-20. The hydroxyl group was confirmed at position-19, because of observing an HMBC correlation from those methyl groups (δ_H 0.95, 0.97) to it. Previous study reported a synthetic derivative with 19-*β* configuration in which the methine proton (δ_H 3.76) at H-19 showed vicinal coupling with the proton of H-18 (*J* = 5.5Hz).[9] Moreover, few friedelin derivatives were reported earlier containing hydroxyl group at different positions specially C-12. The corresponding methane proton H-12 (δ_H 3.89) was placed in *β*-orientation due to the coupling constants (H-11β, *J* = 4.5 Hz) and (H-11α, *J* = 11.0 Hz), consequently hydroxyl group was placed in α-orientation in 3-oxo-12α,28-dihydroxyfriedelane.[10] According to this orientation, H-18 and H-19 should have same (*E*) orientations in compound **1** due to the coupling constant was observed (*J* = 2.0 Hz). Friedelane derivatives have β-orientation of H-18, so H-19 would have been *β*-orientation too to arise the lowest coupling constant. According to that assignment, C-19 hydroxyl group was placed into *α*-orientation, which was confirmed from the reduction of friedelan-19*α*-one in the same literature.[9] Thus, compound **1** was confirmed as 19-*α*-hydroxy-3-oxo-friedelane (Figure 1).
White amorphous powder (3 mg) of compound **2** was isolated from the column fraction by elution with PE/80-20% EtOAC that gave a dark blue spot on the TLC plate under UV light. The compound was found to be soluble in dichloromethane, chloroform and methanol. The ^{1}H and ^{13}C-NMR spectra of compound **2** were closely related to those of 3-oxo-friedelin derivatives (Table 1). The ^{1}H-NMR spectra reveals eight methyl group protons at δ 0.95, 0.75, 0.88, 0.92, 1.35, 1.38, 1.07 and 1.03 all of which were in agreement with the eight signals from the methyl groups in the friedelin skeleton. The ^{1}H-NMR spectrum of compound **2** presented a double doublet at δ_H 4.09 (*J* = 6.4 and 13.2 Hz), suggesting the presence of hydroxyl group in the structure. It also presented a signal at δ_H 2.34 that bonded to a carbon adjacent to a carbonyl group. The ^{13}C-NMR of compound **2** presented a signal at δ_C 77.14, which was attributed to a deshielded carbon.[11] The ^{13}C-NMR spectral data of compound **2** was compared to the data of 16α-hydroxyfriedelin**.** [11] This led us to establish the structure of compound **2** as being 16*α*-hydroxyfriedelin.
Compound **3**, white amorphous powder (5 mg), was isolated from the column fraction by elution with PE/75-25% EtOAC and appeared as dark blue spot on the TLC plate under UV light. The compound was found to be soluble in dichloromethane, chloroform and methanol. The ^{1}H and ^{13}C NMR spectra of compound **3** were also closely related to those of 3-oxo-friedelin derivatives (Table 1). The ^{1}H-NMR spectrum of compound **3** (Table-1) exhibited a total of eight methyl protons signals at δ 0.95 (H-23), 0.91 (H-24), 0.88 (H-25), 1.01 (H-26), 1.0 (H-27) and 1.19 (H-28), 0.98 (H-29) and 0.96 (H-30). The deshielded proton signal at δ3.76 was assigned to oxymethine proton, H-3 which was directly attached to a hydroxyl group. The proton H-3 appears in the ^{1}H-NMR spectrum as a broad singlet at δ3.76 ppm, which reflects the β-orientation of the hydroxyl group by the missing 3*J*aa coupling correspondin g to the structure of *β*-friedelinol (Figure 1).

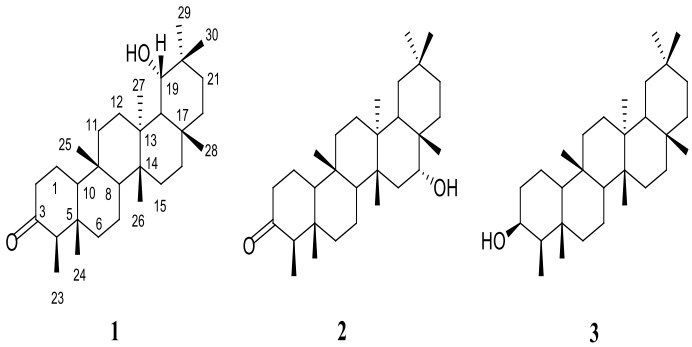

Figure 1: Friedelin derivatives (**1**-**3**) isolated from *P. flaccidum*

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Table 1: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for compound 1-3 ($CDCl_3$, δ, ppm, J/Hz

Position	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.72, m	22.3	1.63, m	22.7		15.8
2	2.41, m & 1.63, m	41.6	2.35, m & 2.43, m	41.7	1.90 (1H, *dt*, *J* = 12.4,2.0 Hz)	35.3
3		213.2		212.3	3.76 (1H, dt)	72.8
4	2.24, m	58.3	2.20, m	58.6		49.2
5		41.8		42.1		37.1
6	1.35, m & 1.67, m	41.3		41.9	1.74 (*dt*, *J* = 13.2, 10.4 Hz)	41.7
7	1.54, m	17.6		18.6		17.6
8	1.38, m	53.1		50.6		53.8
9		38.3		36.4		37.8
10	1.57, m	59.5		61.7		61.4
11	1.31, m & 1.47, m	35.6		35.6		35.3
12	1.42, m & 1.39, m	30.6		30.1		30.6
13		39.7		39.6		38.4
14		39.2		41.0		39.3
15	1.61, m & 1.32, m	37.5		43.7		32.8
16	1.59, m & 1.40, m	32.3	4.09 (1H, *dt*, *J* = 6.4; 13.2 Hz)	77.1		36.1
17		30.0		32.1		30.0
18	1.57 (m)	42.8		43.2		42.8
19	3.76 (1H, d, *J*=2)	72.8		35.4		35.6
20		32.4		28.5		28.2
21	1.30, m	35.4		32.3		32.1
22	1.39, m	29.7		32.4		39.7
23	0.86 (3H, d, *J* = 6.0 Hz)	6.8	0.95 (3H, d, *J* = 6.4 Hz)	7.3	0.95 (3H, d, *J* = 6.8 Hz)	11.6
24	0.72 (3H, s)	14.7	0.75 (3H, s)	14.5	0.91 (3H, s)	16.4
25	0.87 (3H, s)	19.5	0.88 (3H, s)	19.0	0.88 (3H, s)	18.6
26	1.03 (3H, s)	20.2	0.92 (3H, s)	17.9	1.01 (3H, s)	18.2
27	1.07 (3H, s)	19.6	1.35 (3H, s)	20.0	1.0 (3H, s)	20.1
28	1.18 (3H, s)	32.1	1.38 (3H, s)	29.7	1.19 (3H, s)	31.9
29	0.95 (3H, s)	35.0	1.07 (3H, s)	30.0	0.98 (3H, s)	35.0
30	0.97 (3H, s)	31.8	1.03 (3H, s)	35.7	0.96 (3H, s)	31.8

There were a doublet of triple signals observed at δ 1.90 (*dt*, *J*=12.4 Hz, 2.0 Hz) assigned to proton H-2 and a multiplet signal at δ 1.74 assigned to proton H-6. The remaining proton signals were assigned to methylene protons, found in the range of δ 0.88-1.57. ^{13}C-NMR spectrum indicated a total of 30 carbon signals revealing the presence of 30 carbons in the assigned compound. From these 30 carbons, there were eight methyl carbons which gave signals at δ 11.61 (C-23), 16.39 (C-24), 18.64 (C-25), 18.24 (C-26), 20.11 (C-27), 31.93 (C-28), 35.02 (C-29) and 31.79 (C-30). In addition, oxymethine carbon, C-3 gave a relatively deshielded signal at 72.79 ppm. Both the ^{1}H- and ^{13}C-NMR data of compound **3** was similar to the literature values reported for 3*β*-friedelinol.[12]

The cytotoxic effect of the sub-fractions (F-6, F-7, and F-8) of *Polygonum flaccidum* were performed by brine shrimp lethality bioassay (BSLB) against *Artemia salina* as reported by Meyer *et al*.[13] with a slight modification. In BSLB, fraction-6 (F-6) fraction-7 (F-7) and fraction-8 (F-8) showed potent cytotoxic activity with LC_{50} value of 1.67 µg/mL, 2.03 µg/mL, and 1.37 µg/mL, respectively). The results are summarized in Table 2.

Cytotoxic activity of the compound **1, 2** and **3** were also evaluated by using Vero cell line which were cultured in Dulbecco's modified eagles medium (DMEM) and incubated at 5% CO_2 incubator at 37 ^{0}C. Different concentrations of the isolated compound were added to the cell suspension and the number of viable cells was determined by using trypan blue and a hemocytometer. IC_{50} value was calculated from a regression line which was found 31.33 µM/mL for the compound **1,** 8.09 µM/mL for the compound **2** and 7.64 µM/mL for the compound **3** (Table 2). Compound **1** was not able to inhibit the proliferation of the normal Vero cells in desired concentrations. On the other hand, compound **2** and **3** were able to inhibit the proliferation of the normal Vero cells according to the guidelines of American National Cancer Institute (NCI). They have set the limit of activity for different samples like IC_{50} value of crude extracts should be less than 30 µg/ mL and for pure compounds less than 4 µg/ mL after the

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exposure time of 72 hours.[14, 15] The IC_{50} value of compound **2** and **3** was found to be lower than the specified value (4 µg/ mL) and can be recognized as potent anticancer agents. Although friedelin from ethanolic leaf extract of *Cassia tora* showed potent cytotoxic activity on HeLa and HSC-1 cell lines,[16] another study reported no significant activity of friedelin which in accordance with our findings too.[17]

Table 2: Brine shrimp lethality bioassay of different crude fractions and cytotoxic activity of the isolated compounds (**1-3**) from *P. flaccidum.*

Test sample	LC_{50} value at BSLB assay (µg/mL)	IC_{50} value at cytotoxicity assay (µM/mL)
F-6	1.67*	-
F-7	2.03*	-
F-8	1.37*	-
VS	1.76	-
1	-	31.33*
2	-	8.09 *
3	-	7.64*

VS = Vincristine sulfate (Positive standard), * Statistically significant at $p<0.05$ level

Conclusion

The present study investigated the cytotoxic activity of the isolated pure compounds from the crude methanol extract of *P. flaccidum*. A total of 3 friedelin derivatives were identified and characterized. Among them, compound **1** is a previously unreported compound from natural products and compounds **2** and **3** are being reported for the first time from this plant. The column fractions (F6-F8) showed potential cytotoxic activities in the rapid brine shrimp lethality bioassay. Further exploration of the toxicity of the isolated compounds **2** and **3** on Vero cell line exerted their cytotoxic potential roles. On the basis of the above biological findings the plant can be further exploited to identify leads for drug development.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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