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Chemical Constituents and Phytotoxicity of Solvent Extracted Fractions of Stem Bark of *Grewia optiva* Drummond ex Burret

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Abstract: Studies on the chemical constituents of the stem bark of *Grewia optiva* have led to the isolation of four triterpenes; betulin (1), betulinic acid (2), oleanolic acid (3), ursolic acid (4) one sterol; daucosterol (5). The structures of these compounds have been elucidated through spectral studies including 1D and 2D-NMR experiments (HMQC, HMBC, COSY, NOESY and *J*-resolved) and EI and HRMS. They were isolated for the first time from this genus. Crude ethanolic extract of the stem bark of *Grewia optiva* were tested for their *in Vitro Phototoxic* Bioassay against *Lemna minor* plant. This paper is based on the isolation, characterization of chemical constituents as well as Phytotoxicity of crude extract and different solvent extracted fractions.

Key words: *Grewia optiva* · Triterpenes · Phytoxicity · *Lemna minor* · Daucosterol

INTRODUCTION

Grewia belongs to family Tiliaceae, comprises approximately 150 species, small trees or shrubs is distributed in subtropical and tropical regions, represented in Pakistan by ten species [1]. It is one of the most important ingredients of many medical prescriptions in traditional medicines and has been successfully developed into a medicine to treat cough and sore throats. The root and stem bark of these plants have been used in folk medicine for the treatment of malaria, diarrhoea, dysentery, typhoid fever, small pox, cough, irritable condition of intestine and bladder, eczema and rheumatism [2]. *In-vitro* studies indicate that they have anti-oxidant, anti-bacterial, hepatoprotective [3] and antimalarial activities [4]. In the course of studying compounds from plants of the genus *Grewia*, steroids, glycosides, flavones [5], triterpenes and lignans [6] have been isolated and characterized.

MATERIALS AND METHODS

General Experimental Procedures: Column chromatography: silica gel 60, mesh size 70-230 (Merck, 0.063-0.200 mm), Preparative TLC: silica gel 60 PF254 (Merck); visualized under UV and detection with I_2 and $CeSO_4$ spray. UV spectra (MeOH): Hitachi-U-3200 spectrophotometer; λ_{max} (ε) in nm. IR spectra (KBr): Jasco-A-302 spectrophotometer; υ in cm^{-1}. ^{1}H- and ^{13}C-NMR, COSY, NOESY, *J*-resolved, HSQC and HMBC spectra: Bruker spectrometers, Avance Av 500, 600/150 MHz); chemical shifts (δ) in ppm, coupling constants (*J*) in Hz. HR-EI-MS: Jeol-JMS-HX-110 mass spectrometer; EI, source at 250° and 70 eV. Optical rotations were measured on *Jasco-DIP-360* digital polarimeter and CD spectra on *Jasco-J-810* spectro-polarimeter.

Plant Material: The stem bark (7 kg) of *Grewia optiva* were collected from lower Dir region (Khyber Pakhtunkhwa), Pakistan during October 2009 and the plant was identified by Dr. Jehandar Shah, Vice Chancellor Benazir University, Upper Dir (Khyber Pakhtunkhwa) and a voucher (specimen No. 9105) has been deposited in the Herbarium of the Department of Botany, University of Peshawar, Peshawar, Pakistan.

Extraction and Isolation: The stem bark (7 kg) of *G. optiva* was extracted repeatedly (3 times) with ethanol at room temperature. The combined ethanolic extracts were freed of the solvent in vacuo to thick syrup, which was

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partitioned between ethyl acetate (EtOAc) and water. The EtOAc layer was washed with H_2O, dried (anhydrous Na_2SO_4) and evaporated under reduce pressure to give a gummy residue which was further fractionated into petroleum ether soluble and insoluble fractions. The petroleum ether insoluble part (20 g) was re-fractionated into ethyl acetate soluble and insoluble fractions. The EtOAc soluble fraction was dried over Na_2SO_4 (anhydrous) and further divided it into n-hexane-EtOAc (1:1) soluble and insoluble fractions. The soluble fraction was separated through filtration, evaporated under vacuum to get powder residue (12.0g) and subjected to gravity column chromatography (Merck Kieselgel 60, 70–230 mesh, 350 g), eluted with petroleum ether, petroleum ether-EtOAc, $CHCl_3$–MeOH in increasing order of polarity. As a result various fractions were obtained and combined on the basis of TLC to ultimately afford 20 fractions. On combining the elutes on the basis of TLC, twenty fractions (F-1-F-20) were ultimately obtained. Of these, fraction (F-6) afforded on column chromatography afforded betulin (1, 10mg) and betulinic acid (2, 12mg). Fraction F-7 on column chromatography followed by prep. TLC ($CHCl_3$-MeOH (9.5:0.5) afforded oleanolic acid (3; 6.5mg) and ursolic acid (4; 7.0mg). Fraction F-8 on column chromatography fractionated into three sub-fractions B1-B3. B1 and B2 were not pursued in the present study while sub-fraction B3 gave rise to a daucosterol (5; 6mg) as white amorphous powder through prep.TLC ($CHCl_3$-MeOH (9.6:0.4).

Phytotoxicity: Different fractions and crude ethanolic extract of the stem bark of *grewia optiva* were tested for their *In vitro Phototoxic Bioassay.* They were applied on *Lemna minor* plant. Some samples showed low activity.

RESULTS AND DISCUSSION

All the chemical constituents were isolated from petroleum ether-ethyl acetate insoluble fractions. Betulin (1) was obtained as white amorphous powder. Its molecular formula, $C_{30}H_{50}O_2$ was determined on the basis of EI-MS. (calcd. 442 for $C_{30}H_{50}O_2$). The IR spectrum indicated characteristic absorption bands caused by hydroxyl group at 3400 and olefinic carbons at 1603.4 cm^{-1}. The ^1H and ^{13}C NMR spectral data (Table 1) of the Betulin were assigned using values reported for 3β, 28-dihydroxylup-20(29)-ene (Betulin). The ^{13}C NMR indicated thirty carbon signals in the broad band decoupled spectrum; six methyl, twelve methylene, six methine and six quaternary carbon atoms from DEPT experiments [7].

Betulinic acid (2) was obtained as white amorphous powder. Its molecular formula, $C_{30}H_{48}O_3$ was determined on the basis of EI-MS. (calcd. 456 for $C_{30}H_{50}O_2$). The IR absorption at 3500 cm^{-1} for OH st.; 1700 cm^{-1} for carboxylic acid carbonyl carbon and1625 cm^{-1} for C=C olifinic. The UV spectrum displayed a maximum at 195.0 nm. The ^1H and ^{13}C NMR spectral data (Table 2) of the Betulinic acid were assigned using values reported for 3 β,-dihydroxylup-20(29)-ene-28-oic acid (Betulinic acid). The ^{13}C-NMR indicated thirty carbon signals in the broad band decoupled spectrum; six methyl, twelve methylene, six methine and six quaternary carbon atoms from DEPT experiments. The chemical shifts at δ_C 180.3, 150.4 and 109.6 were the characteristic peaks for betulinic type of skeleton, assigned to C-28, C-20 and C-29 respectively [8, 9].

Oleanolic acid (3) was obtained as white amorphous powder. Its molecular formula, $C_{30}H_{48}O_3$ was determined on the basis of EI-MS. (calcd. 456 for $C_{30}H_{50}O_2$). The ^1H and ^{13}C NMR spectral data (Table 3) of the Oleanolic Acid were assigned using values reported for 3β-hydroxyolean-12-en-28-oic acid (oleanolic acid). The ^{13}C NMR indicated thirty carbon signals in the broad band decoupled spectrum; six methyl, twelve methylene, six methine and six quaternary carbon atoms from DEPT experiments. The chemical shifts at δ_C 178.9, 122.6 and 144.8 were the characteristic peaks for oleanolic type of skeleton, assigned to C-28, C-12 and C-13 respectively. The oxygen deshielding chemical shift at δ 79.2 was assigned to C-3 [10].

Ursolic acid (4) was obtained as white amorphous powder. Its molecular formula, $C_{30}H_{48}O_3$ was determined on the basis of EI-MS. (calcd. 456 for $C_{30}H_{50}O_2$).The ^1H and ^{13}C NMR spectral data (Table 4) of the Betulinic acid were assigned using values reported for 3β-hydroxyurs-12-en-28-oic acid (oleanolic acid). The ^{13}C NMR indicated thirty carbon signals in the broad band decoupled spectrum; six methyl, twelve methylene, six methine and six quaternary carbon atoms from DEPT experiments. The chemical shifts at δ_C 180.0, 125.6 and 139.7 were the characteristic peaks for ursolic type of skeleton, assigned to C-28, C-12 and C-13 respectively [11].

Daucosterol (5) was obtained as white amorphous powder. Its molecular formula, $C_{35}H_{60}O_6$ was determined on the basis of EI-MS. (calcd. 575 for $C_{35}H_{60}O_6$) [12].

Crude extract as well as solvent extracted fractions of the stem bark exhibited Phytotoxicity against *Lemna minor* plant.

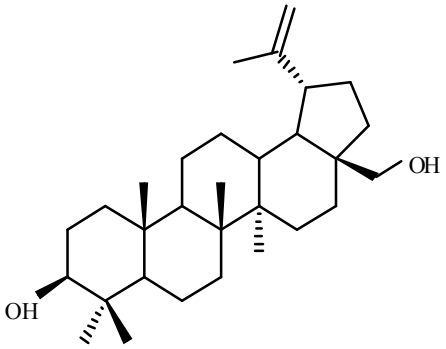

Fig. 1: 3β, 28-dihydroxylup-20(29)-ene (Betulin) (1)

Table 1: ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR Data of betulin (1)

No. C	^{1}H	Multiplicity (*J* in Hz)	^{13}C	Type C
1			38.87	CH_2
2			20.84	CH_2
3	3.79 Hz, H-3	dd, 10.8, 4.8	78.98	CH
4			38.72	C
5			55.5	CH
6			18.31	CH_2
7			33.98	CH_2
8			40.93	C
9			55.37	CH
10			37.32	C
11			27.41	CH_2
12			25.22	CH_2
13			37.32	CH
14			42.72	C
15			27.06	CH_2
16			29.3	CH_2
17			46.47	C
18			50.41	CH
19	2.39	m	48.77	CH
20			150.4	C
21			29.76	CH_2
22			34.09	CH_2
23	0.94	s	27.99	CH_3
24	0.80	s	15.98	CH_3
25	0.74	s	15.36	CH_3
26	0.96	s	16.1	CH_3
27	1.00	s	14.77	CH_3
28			60.8	CH_2
29	4.66	s	109.6	CH_3
30	1.66	s	19.09	CH_3

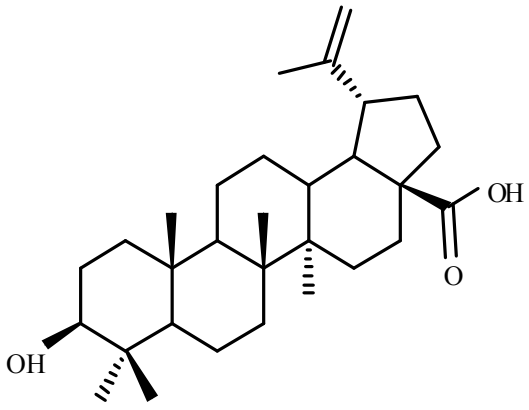

Fig. 2: 3β, 28-dihydroxylup-20(29)-ene-28-oic acid (Betulinic acid) (2)

Table 2: ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR Data of betulinic acid (2)

No. C	δ_H	Multiplicity (*J* in Hz)	δ_C	Type C
1			38.87	CH_2
2			27.9	CH_2
3	4.47Hz, H-3	dd, 10.2, 5.5	79.02	CH
4			38.72	C
5			55.5	CH
6			18.31	CH_2
7			34.3	CH_2
8			40.93	C
9			50.50	CH
10			37.2	C
11			20.8	CH_2
12			25.22	CH_2
13			38.40	CH
14			42.40	C
15			30.60	CH_2
16			32.10	CH_2
17			56.30	C
18			46.80	CH
19	3.0,	t, 10.2, 5.5	49.20	CH
20			150.40	C
21			29.76	CH_2
22			34.09	CH_2
23	0.82	s	27.99	CH_3
24	0.91	s	15.30	CH_3
25	0.81	s	16.00	CH_3
26	0.95	s	16.10	CH_3
27	0.93	s	14.77	CH_3
28			180.3	C
29	4.66	s	109.6	CH_3
30	1.68	s	19.40	CH_3

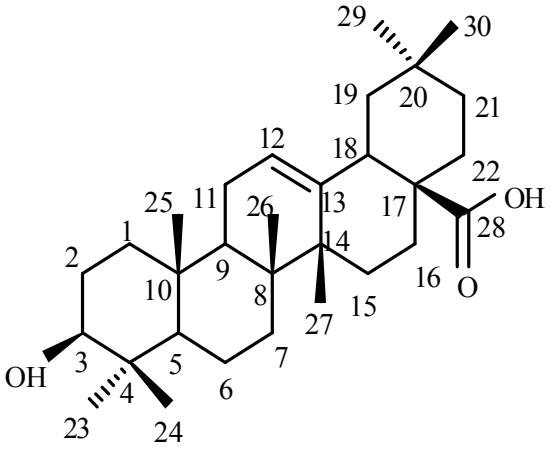

Fig. 3: 3β, hydroxyolean-12en-28-oic acid (oleanolic acid) (3)

Table 3: ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR Data of oleanolic acid (3)

No. C	δH	Multiplicity J in Hz	Type C	δC
1			CH_2	39.0
2			CH_2	28.1
3	3.44,	dd,10.2/5.5	CH	78.2
4			C	39.4
5			CH	55.9
6			CH_2	18.8
7			CH_2	33.4
8			C	39.8
9			CH	48.2
10			C	37.4
11			CH_2	23.8
12	5.49	Brs	CH	122.6
13			C	144.8
14			C	42.2
15			CH_2	28.4
16			CH_2	23.8
17			C	46.7
18	2.52	d, 11.0	CH	42.1
19			CH_2	46.6
20			C	31.0
21			CH_2	34.3
22			CH_2	33.2
23	1.24	s	CH_3	28.8
24	1.02	s	CH_3	16.5
25	0.88	s	CH_3	15.6
26	1.04	s	CH_3	17.5
27	1.30	s	CH_3	26.2
28			C	180.0
29	0.94	d, 6.2	CH_3	33.4
30	1.02	s	CH_3	23.8

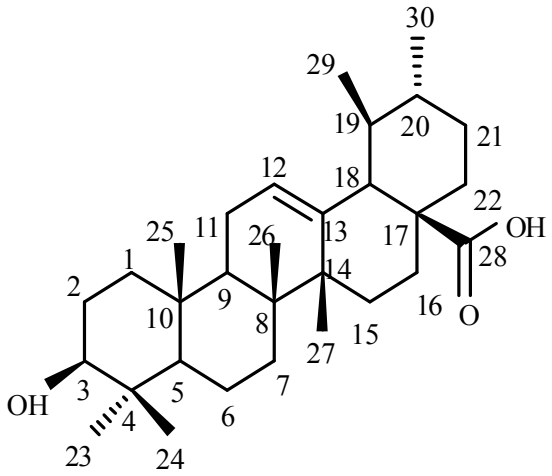

Fig. 4: ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR Data of ursolic acid (4)

Table 4: ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR Data of ursolic acid (4)

No. C	δH	Multiplicity J in Hz	Type C	δC
1			CH_2	38.4
2			CH_2	28.1
3	3.43	Brs	CH	78.1
4			C	38.4
5			CH	55.8
6			CH_2	18.8
7			CH_2	33.6
8			C	40.0
9			CH	48.3
10			C	37.4
11			CH_2	23.6
12	5.50	Brs	CH	125.6
13			C	139.7
14			C	42.5
15			CH_2	28.7
16			CH_2	24.9
17			C	48.0
18	2.52	d, 11.0	CH	53.5
19			CH	39.5
20			CH	39.1
21			CH_2	31.1
22			CH_2	37.3
23	1.24	s	CH_3	28.8
24	1.02	s	CH_3	15.7
25	0.93	s	CH_3	16.6
26	1.05	s	CH_3	17.4
27	1.22	s	CH_3	23.8
28			C	180.0
29	0.97	s	CH_3	17.5
30	0.99	d, 6.1	CH_3	21.4

Table 5: *In vitro* Phytotoxicity of crude extract and fractions of stem bark of *G. optiva*

		No. of Fronds			
Fraction	Conc. of Compooud (µg/mL)	Sample	Control	% growth Regulation	Conc. of Std. Drug (µg/mL)
Crude ethanolic extract	1000	14	19	26.3	0.015
	100	18		5.26	
	10	19		0	
Pet. Ether fraction	1000	15	19	21	0.015
	100	17		10.5	
	10	18		5.26	
Pet.ether+EtOAc fraction	1000	12	19	37	0.015
	100	17		10.5	
	10	18		5.26	
n-BuOH fraction	1000	14	19	26.3	0.015
	100	17		10.5	
	10	18		5.26	

CONCLUSION

In summary, the phytochemical study on the chemical constituents of the stem bark of *G. optiva* has led to the isolation of four triterpenes; betulin (1), betulinic acid (2), oleanolic acid (3), ursolic acid (4) and one sterol; daucosterol (5). All these compounds were for the first time reported from this species. Phytotoxic bioassay of crude extract along with solvent extracted fractions exhibits some activity against *Lemna minor* plant.

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