Cytotoxic New Nortriterpene from Roots of *Potentilla atrosanguinea* var. *argyrophylla* and its UPLC Quantification

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Abstract

Potentilla atrosanguinea is well known for its ethnomedicinal uses since ancient times. The present study includes the isolation of a new nortriterpene, 28-methyl-acanthochlamate (**1**), from the roots of *P. atrosanguinea*. The structure of **1** was established from HR-ESI-MS and NMR spectroscopic analysis. Its relative stereochemistry was set with the help of NOESY correlation experiment. Compound (**1**) has been quantified by a new developed UPLC-DAD method (1.1 mg/g). The *in vitro* cytotoxicity of the new compound (**1**) was evaluated by sulforhodamine B assay against three cancer cells; human cervical cancer (SiHa), epidermal carcinoma (KB), and human adenocarcinoma (Colo-205). The new isolated compound (**1**) showed a significantly higher cytotoxicity against all the cells (SiHa, IC_{50} 30.5 µg/mL; KB, IC_{50} 22.6 µg/mL and Colo-205, IC_{50} 18.8 µg/mL).

Key words

Potentilla atrosanguinea · Rosaceae · 28-methyl-acanthochlamate · quantification · cytotoxicity

Supporting information available online at http://www.thieme-connect.de/products

The therapeutic effects of more than 300 species of the *Potentilla* genus (Rosaceae) are well mentioned in traditional medicinal systems. *Potentilla* species are reported for their various pharmacological activities such as antioxidant, antiulcerogenic, antineoplastic, antidiabetic, and hepatoprotective [1–5]. Mainly polyphenols and triterpenoids have been reported from the *Potentilla* genus [6]. *Potentilla atrosanguinea* var. *argyrophylla* (Wall. ex Lehm.), is commonly known as Himalayan cinquefoil. The root decoction of *P. atrosanguinea* has been used to cure toothache and leaves paste used in cut and wound healing [7]. Its root parts are used in human food and leaves as healthful tea. There is no report available in the literature regarding the isolation of chemical constituents from *P. atrosanguinea* except few reports [8–10]. In the present study, one new nortriterpene type compound has been isolated from a combined chloroform and ethyl acetate fraction of ethanol extract of the root part of *P. atrosanguinea* (**Fig. 1**). Compound **1** was obtained as fine white crystals having a molecular formula of $C_{30}H_{46}O_4$, determined by HR-ESI-MS (obsd. $[M + H]^+$ at *m/z* 471.3465, calcd. $[M + H]^+$ 471.3474) which suggested eight degrees of unsaturation. Five methyls, elevan methylenes, five methines and eight quaternary carbons were observed from ^{13}C NMR and the DEPT spectra. NMR data showed that the molecule contains 30 carbon atoms with one methoxy and two carboxyls groups. Two terminal olefinic bonds with signals at δ_H 4.87 (2H, H-23) and δ_H 4.71, 4.58 (H-29) and five tertiary methyls singlets at δ_H 0.81(H-25), 0.94 (H-26, H-27), 1.71(H-24), 1.65 (H-30) were observed (**Table 1**). The presence of one methoxyl carbon in NMR data suggested that the basic skeleton of the molecule contains 29 carbon atoms. The interpretation of 2D NMR spectra and its comparison with a previous report [11] indicated that the molecule has a nortriterpene (lupane type) skeleton with the attachment of methyl to one of the carboxylic groups. Cross-peak correlations for H-5 to C-4, C-23, C-10 and for H-1 to C-2 and C-10 in the HMBC spectrum (**Fig. 2**) confirmed the position of one exocyclic double bond at C-4 and one carboxylic group at C-2 position. The HMBC correlations of H-18 with C-13, C-17, C-19, C-20 and H-19 with C-29 and C-18 confirmed the presence of another exocyclic double bond at C-20 position. Another carboxyl group positioned at C-17 because of cross peak correlations of H-22 with C-17 (δ_c 56.6) and C-28 (δ_c 176.7) in HMBC spectra. The attachment of a methyl group at the C-28 carboxylic group was determined by three bond correlations between the methyl proton (δ_H 3.64 s) and carbon of C-28 (δ_c 176.7) position. By comparing our interpretations with a previous report [11], the structure of compound **1** was established as 28-methyl-acanthochlamate.

The cross peak correlations of *β* oriented H-25 with H-26 and H-23 were observed. The stereochemistry was assigned at position C-5, C-10 and at C-8 as *S, S* and *R*, respectively, similar to the previous report [11]. Therefore, by literature support and NOESY interactions, the stereochemistry at C-5, C-8, C-9, C-10, C-13, C-14, C-17, C-18 and C-19 centers were assigned as *S, R, R, S, R, R, S, R* and *R*, respectively. From the observed spectroscopic data and previous literature, the final structure of compound **1** was assigned as 28-methyl-5*S*, 8*R*, 9*R*, 10*S*, 13*R*, 14*R*, 17*S*, 18*R*, 19*R*-acanthochlamate. All the spectral data are available as Supporting Information.

Compound **1** has been quantified by developing a new simple and efficient UPLC method (**Fig. 3**). The sample was analyzed at different temperatures (19, 20, 21, 23, 22, and 24 °C), and better results were observed at 22 °C. An isocratic solvent system, 0.01 % formic acid in acetonitrile (ACN; 91 %) and 0.01 % formic acid in water (9 %) was optimized. For identification of compound **1** in the sample, retention time (t_R 3.24) and UV λ_{max} 194 were compared with the standard. The calibration curve was prepared by injecting six concentrations (0.0156–0.5 mg/mL) of compound

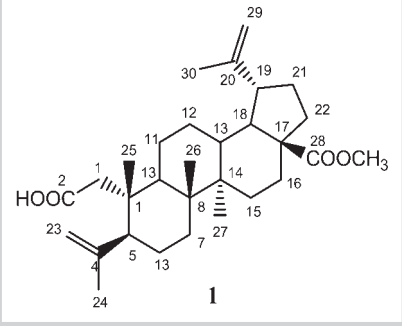

Fig. 1 Structure of 28-methyl-acanthochlamate (**1**).

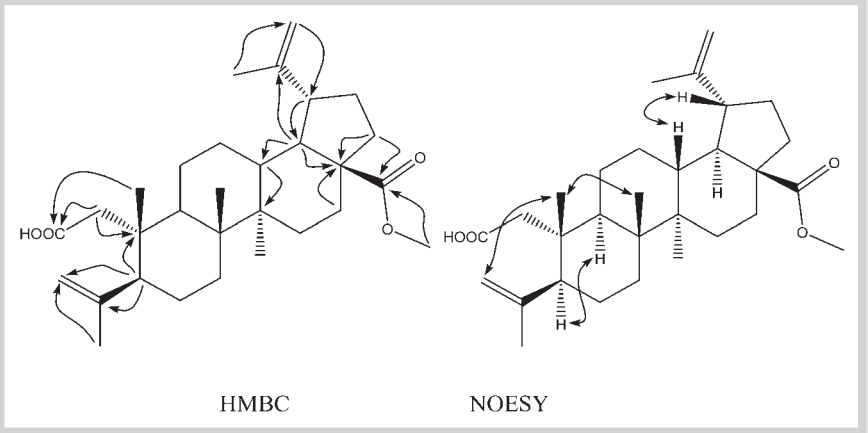

Fig. 2 Key HMBC (→) and NOESY (↔) spectral data of compound **1**.

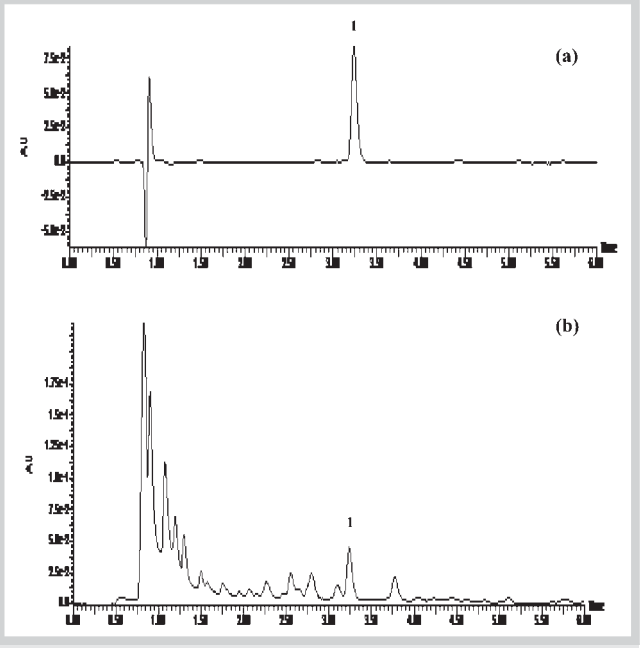

Fig. 3 UPLC-DAD chromatograms. Standard (**a**) and sample(**b**).

1. A good linearity range of the method was obtained with regression coefficients (R^2 0.999). The other parameters were obtained as limit of detection (LOD) 0.0156 mg/mL, limit of quantification (LOQ) 0.0468 mg/mL, percentage relative standard deviation (%RSD) for intra-day 0.64; inter-day precision (%RSD) 1.25, percentage recovery 80–83.5%, and %RSD for recovery 2.1% (**Table 2**). Compound **1** was quantified in the root part of *P. atrosanguinea* as 1.1 mg/g of dry plant material. Compound **1** showed significant activity on SiHa and KB cells (84.6% ± 1.3, $p < 0.001$ and 89.5 ± 1.8, $p < 0.08$, respectively) at 100 µg/mL. Whereas, against Colo-205 it showed remarkable activity (84.2 ± 2.4, $p < 0.004$) at 25 µg/mL. The IC_{50} values of Compound **1** ranges between 18.8 to 30.5 µg/mL (**Table 3**). The percentage cytotoxicity of compound **1** is provided in **Table 1 S**, Supporting Information. Results were expressed as percentage cell inhibition.

Table 1 NMR data of compound **1**.

Position	$\delta^{13}C$		$\delta^{1}H$	Key HMBC	Key NOESY
1	43.0	CH_2	2.42 (d, *J* = 14.9 Hz) 2.25 (d, *J* = 15.3 Hz)	C-2, C-10	
2	178.0	quat	–		
4	147.2	quat	–		
5	49.9	CH	2.56 (d, *J* = 12.7 Hz)	C-4, C-10, C-23	H-9
6	24.1	CH_2	1.38 (m), 1.32 (m)		
7	32.4	CH_2	1.40 (m)		
8	40.7	quat	–		
9	41.8	CH	1.95 (d, *J* = 12.4 Hz)		
10	41.3	quat	–		
11	22.6	CH_2	1.54 (m)		
12	25.3	CH_2	1.69 (m)		
13	38.3	CH	2.22 (m) (overlapped)	C-14	H-19
14	42.8	quat	–		
15	30.6	CH_2	1.88 (m), 1.34 (m) (overlapped)		
16	32.1	CH_2	2.19 (d, *J* = 13.3 Hz) 1.03 (d, *J* = 12.8 Hz)	C-17	
17	56.6	quat	–		
18	49.4	CH	1.56 (m)	C-13, C-17, C-20	
19	47.0	CH	2.97 (m)	C-18	
20	150.3	quat	–		
21	29.7	CH_2	1.17 (m), 1.29 (m)		
22	37.0	CH_2	1.37 (m), 1.86 (m) (overlapped)	C-17, C-28	
23	114.8	CH_2	4.87 (d, *J* = 13.7 Hz)		H-25
24	22.6	CH_3	1.71 (s)	C-23	
25	19.6	CH_3	0.81 (s)	C-2	H-26
26	14.5	CH_3	0.94 (s)		
27	15.8	CH_3	0.94 (s)		
28	176.7	quat	–		
29	109.8	CH_2	4.58 (s), 4.71 (s)	C-19	
30	19.3	CH_3	1.65 (s)	C-29	
OCH_3	51.3		3.64 (s)	C-28	

Table 2 Method validation data of compound **1**.

Regression Equation	Linearity*	R^2	LOD*	LOQ*	Intraday Precision (n = 6)	Interday Precision (n = 3)	Recovery % ± %RSD
y = 24958 x-92.55	0.0156–0.5	0.999	0.0156	0.0468	0.64	1.25	82.2 ± 2.1

* Data expressed in mg/mL

Materials and Methods

General

NMR spectra were recorded in $CDCl_3$, on Bruker Advance-600 spectrometer (600 MHz for ^1H and 150 MHz for ^{13}C). IR was measured by using an IRPrestige FT-IR spectrophotometer (Shimadzu). A Waters ACQUITY Ultra High Performance LC system (Waters, Milford, MA, USA) was used for the quantification analysis. The system has been equipped with autosampler, PDA detector, binary solvent delivery pump, and Masslynx v4.1 software. ACN, water and formic acid used were LC/MS/MS grade solvents and purchased from J.T. Baker (Avantor Performance Material Inc.). All the other used chemicals and solvents were of analytical grade.

Plant material

Plant material was collected from Kunjam Pass, situated at an elevation of 4590 m in Lahaul and Spiti district of Himachal Pradesh, India in the month of August 2013 and identified by taxonomist Dr. Brij Lal. A specimen was deposited in the herbarium at CSIR-IHBT, Palampur, India (voucher number-PLP 16515).

Extraction and isolation

The dried underground part (1.5 kg) of *P. atrosanguinea* was percolated with 95% EtOH at room temperature (3.5 L × 1, followed by 1.5 L × 7, 95 : 5, v/v). The extracts were combined and dried under vacuum at 45 °C with yield 67 g. A part of the extract (57 g) was suspended in distilled water followed by successive partitions with $CHCl_3$ (500 mL × 3, 8 g), EtOAc (500 mL × 3, 11.6 g) and *n*-butanol (500 mL × 3, 20 g). The $CHCl_3$ and EtOAc extracts were combined on the basis of similar TLC profiling.

16 g (pooled $CHCl_3$ and EtOAc extract) was applied to a dry column packed with silica 230–400 mesh size and eluted with step gradients EtOAc-Hexane (up to 10%), EtOAc : $CHCl_3$: MeOH : H_2O (15 : 8 : 2 : 0.5 followed by 15 : 8 : 4 : 1). Seventeen major fractions were collected. One compound (**1**) was isolated from fraction II. Compound **1** was crystallized after elution from normal phase silica gel (230–400) column by 10% EtoAc in hexane.

28-Methyl acanthoclamate (**1**): isolated as white fine needles (40 mg); $[\alpha]_D^{18.5}$ + 25.0 (*c* 0.001, MeOH); IR (neat): 2958, 2920, 1728 cm^{-1}; MP: 153–155 °C; HRMS/ESI, *m/z*: obsd. $[M + H]^+$ 471.3465, calcd. $[M + H]^+$ 471.3474.

UPLC quantification of compound 1

The stock solutions of compound **1** and sample were prepared using methanol with concentrations of 0.5 mg/mL and 5 mg/mL, respectively, and filtered through a Whatman PTFE syringe filter (size 0.2 µm). For analysis, UPLC BEH C18 (Waters Acquity; 2.1 mm × 100 mm with particle size 1.7 µm) was used. The sample was analyzed at 205 nm PDA detection wavelength with 0.5 µL of injection volume. For the validation of the developed method, all the parameters were evaluated (see Supporting Information).

Table 3 IC_{50} (µg/mL) value of compound **1**.

Sample	SiHa	KB	Colo-205
IC_{50} (µg/mL)	30.5	22.6	18.8

Sulforhodamine B assay

SiHa, KB and Colo-205 were obtained from National Centre for Cell Science, Pune, India. SiHa and KB cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Sciences) while Colo-205 cultured in F-12 HAMS medium (Invitrogen Biosciences), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Biosciences) and 1% antibiotic and antimycotic solutions (Invitrogen Biosciences). Cells were maintained at 37 °C with 5% CO_2 and 95% humidity in a CO_2 incubator [12, 13].

Cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and grown at a density of 2×10^4 cells/well in 96 well plates. Different concentrations (10, 25, 50 and 100 µg/mL) of compound **1**, in complete medium were added. Vinblastine (1 µM; Sigma-Aldrich) was used as positive control, whereas cells supplemented with complete medium only were used as negative control. Plates were incubated at 37 °C for 48 h in a CO_2 incubator. After 48 h, 50 µL of 50% trichloroacetic acid (Merck) was added and the plates were kept at 4 °C for 1 h. The plates were flicked and washed five times with water and air-dried. Subsequently, 100 µL SRB solution (in 1% GAA) was added and incubated for 30 min at room temperature. After incubation, plates were washed six times with 1% GAA, air dried and 10 mM Tris base (Sigma Aldrich) was added. The absorbance was measured using a microplate reader (BioTeK Synergy H1 Hybrid Reader) at 540 nm [14]. The growth inhibition rate was calculated as percentage of parallel negative controls. Data is the results of three independent experiments with mean ± SD. P-values were calculated with the help of GraphPad Quick-Calcs: t test calculator (http://www.graphpad.com/quickcalcs/ttest1/? Format=SD).

Supporting Information

UPLC-DAD method validation and ESI-MS analysis details as well as spectral data of the compound are available as Supporting Information.

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Conflict of Interest

None. The authors declare that they have no competing interests.

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