Isolation and identification of cytotoxic compounds from the rhizomes of *Paris quadrifolia* L.

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ABSTRACT

Background: *Paris quadrifolia* L. is a medicinal plant which contains steroidal saponins. The present study reports isolation and structural identification of six pennogenyl saponins obtained from *P. quadrifolia* rhizomes. The four spirostan saponins were obtained from *P. quadrifolia* for the first time. The cytotoxic effects of the sub-fractions and six compounds isolated from the plant extract were evaluated on tumour cells. **Materials and Methods:** Ethanol extract from the rhizomes of *P. quadrifolia* were partinioned using column chromatography. The saponins were isolated from the obtained sub-fractions by isocratic RP HPLC and their structures were determined by means of 1D and 2D NMR spectroscopy and MALDI TOF MS. The cytotoxic effects of the sub-fractions and the isolated compounds were tested against human promyelocytic leukaemia cells (HL-60), human cervical adenocarcinoma cells (HeLa) and human breast cancer cells (MCF-7) using the [(3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide (MTT) assay. **Results:** Six pennogenyl saponins were isolated from *P. quadrifolia* rhizomes: pennogenin 3-*O*-β-D-glucopyranoside (1), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (2), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (3), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (4), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (5), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (6). Pennogenyl saponins 5 and 6 exhibited cytotoxic activity against HL-60, HeLa and MCF-7 tumour cells with IC_{50} values of 1.0 ± 0.04 μg/ml, 1.8 ± 0.072 μg/ml and 2.4 ± 0.096 μg/ml respectively, and 2.0 ± 0.08 μg/ml, 2.5 ± 0.125 μg/ml and 3.2 ± 0.128 μg/ml respectively. **Conclusion:** Compounds **1**-4 were isolated from this species for the first time.

Key words: Cytotoxicity, *Paris quadrifolia*, pennogenin, saponins, structure elucidation

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INTRODUCTION

The genus *Paris* (*Melanthiaceae*) includes 24 species of plants, which grow in an extensive area from Europe to Asia. Apart from the European *Paris quadrifolia* and the Caucasian *P. incompleta*, the other 22 species of *Paris* grow in western Asia, western Siberia and the Himalayas.[1] The plant occurs in deciduous forests in Poland and studies of this species are conducted at present.[2,3]

The *Paris* species contain a wide range of steroidal compounds which are potential cytotoxic agents. Many articles describe species of *Paris* widespread in the Far East as plants of traditional Chinese medicine. The objects of those studies were mainly *P. polyphylla* var. *chinensis* and *P. polyphylla* var. *yunnanensis*.[4-8]

In this work, we present isolation of six pennogenyl saponins from *P. quadrifolia* rhizomes and determination of their structures: pennogenin 3-*O*-β-D-glucopyranoside (1), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (2), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (3), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (4), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (5), pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-

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β-D-glucopyranoside (6). Earlier, these substances had been identified in other species of *Paris*[9-11] and other genera/species: *Trillium*,[12-18] *Heloniopsis orientalis*,[19,20] *Polygonatum kingianum*,[21] *Trachycarpus wagnerianus*,[22] *Dioscorea*,[23,24] *Triteleia lactea*,[25] *Majanthemum dilatatum*,[26] *Ophiopogon japonicus*,[27] *Dracaena mannii*,[28] *Ypsilandra thibetica*.[29] But the six saponins mentioned above have not been isolated at the same time from one species of *Paris*. Except saponins 5 and 6,[30,31] the spirostan saponins 1, 2, 3, 4 from *P. quadrifolia* rhizomes were obtained from this plant for the first time.

The cytotoxic effects of fractions obtained from *P. quadrifolia* extract and the six mentioned compounds were examined *in vitro* on HL-60, HeLa and MCF-7 tumour cells. Saponins 5 and 6 showed significant cytotoxic activity against the cells.

MATERIALS AND METHODS

General experimental procedures

Column chromatography was performed on a column (35 × 4.8 cm) with silica gel (Kieselgel 60; 0.05-0.2 mm; Macherey-Nagel). Thin layer chromatography (TLC) was carried out on precoated Kieselgel 60 (Merck). Semi-preparative high-performance liquid chromatography (HPLC), isocratic separations were run with the use of a Gradient Pump (Pharmacia LKB), Econosil C18 column (250 × 10 mm; 10 μm; Alltech) connected to a VYDAC C18 guard column; KNAUER injector with a loop of 200 μl. The elution profile was monitored with a differential refraction detector RIDK 102 (Laboratorni Pristroje Praha).

D and L configurations of sugar components were assigned as previously described.[32-34]

Gas liquid chromatography (GLC) analyses were performed on a TOP GC 8000 (CE Instruments) gas chromatograph equipped with a flame ionization detector (FID) and a DB-23 fused-silica capillary column (60 m, 0.3 mm I.D., 0.15 μm film thickness, JandW scientific).

Mass spectra of all saponins were recorded in ethanol solutions on a Bruker BIFLEX III MALDI TOF mass spectrometer equipped with a nitrogen laser (λ = 337 nm) in a DHB matrix (2,5-dihydroxybenzoic acid). The spectra were recorded in positive mode in range of m/z 400-2500 amu (averages of 250 to 450 acquisitions) with a pulse width of 3ns, and an energy density of 10^6 to 10^7 W cm^{-2}. A mixture of peptides was used as the calibration standard. All nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance III 700 MHz spectrometer at 27°C in C_6D_5N, and calibrated according to Transcranial magnetic stimulation tetramethylsilane (TMS).

Plant material

P. quadrifolia L. was collected at Gdańsk (Poland, 54°21'69"N, 18°33'24"E). The fresh rhizomes of the plant were dried at room temperature. A voucher specimen has been deposited in the Herbarium of the Medical University of Gdańsk (GDMA herbarium).

Extraction and isolation

Dried plant rhizomes (410 g) were incubated with distilled water at 40°C for 24 h, extracted with 96% ethanol for 25 h at room temperature, then ethanol was evaporated using a vacuum evaporator (40°C). Next, water was added to the residue and the whole was frozen and lyophilised yielding 80.1 g of extract. Obtained sample was defatted by petroleum ether yielding 78.2 g of degreased material. n-Butanol/water extraction was performed for part of this material (71 g). Each 10 g of lyophilisate were mixed with 250 ml of distilled water and extracted with 80 ml of n-butanol three times. Butanol layers were collected and n-butanol was removed at low pressure using a rotatory evaporator at 35°C. The extract was placed in water, frozen, and lyophilised giving 7.5 g of material. A solution of the extract (40 ml) in a mixture of $CHCl_3/CH_3OH/H_2O$ (72 v: 18 v:1.8 v) was transferred to the silica gel Kieselgel 60 column and separated into fractions by gradient flash chromatography. Elution was conducted with a mixture of $CHCl_3/CH_3OH/H_2O$ with gradually increasing volumes of methanol (72v: 18v: 1.8v; 63v: 27v: 2v; 54v: 36v: 2.5v; 45v: 45V: 3v; 27v: 63v: 4v; 0v: 100v: 0v respectively) with the flow rate of the mobile phase 15-18 ml/min.

The presence of saponins in the eluents was monitored by TLC on precoated Kieselgel 60 plates developed with $CHCl_3$/MeOH/H_2O (7v/3v/0.5v). The chromatograms were visualised with Liebermann-Burchard reagent (Ac_2O/$CHCl_3$/H_2SO_4 at 20v/50v/1v) and heated at 90°C for 10 min.

Single eluents of 10 ml from the column chromatography of similar composition were combined, which resulted in 11 sub-fractions. Organic solvents were removed at low pressure in a rotatory evaporator at 35°C. Distilled water was added to each sub-fraction and the aqueous suspensions obtained were frozen and freeze-dried, which yielded the following: 7-25-954.3 mg, 26-71-1934.1 mg, 72-85-247 mg, 86-92-129.7 mg, 93-103-415.8 mg, 104-109-149.1 mg, 110-117-86.5 mg, 118-136-15.2 mg, 137-159-100.5 mg, 160-186-288.3 mg, 187-198-96.8 mg. The saponins from each fraction were isolated by isocratic reversed-phase high-performance liquid chromatography (RP HPLC) using mobile phase MeOH/CH_3CN/H_2O 32v: 25v: 25v and flow rate 3.8 ml/min. The mobile phase was removed (low pressure evaporation at 40°C), then the saponins were suspended in water, frozen and lyophilised. No pennogenyl saponins were found in the last four sub-fractions.

MALDI TOF and NMR spectra of all saponins were recorded [Figures A.1. and B.1. Appendix].

Pennogenin 3-O-β-D-glucopyranoside (1): White powder; MALDI TOF MS *m/z*: 593 $[M + 1]^+$, 575 $[M + 1\text{-}18]^+$, 615 $[M + 23]^+$, 631 $[M + 39]^+$, molecular formula $C_{33}H_{52}O_9$.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (2): White powder; MALDI TOF MS *m/z*: 739 $[M + 1]^+$, 721 $[M + 1\text{-}18]^+$, 761 $[M + 23]^+$, 777 $[M + 39]^+$, molecular formula $C_{39}H_{62}O_{13}$.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (3): White powder; MALDI TOF MS

Table 1: ^1H and ^{13}C NMR data of compounds 1-6 (700 MHz, C_6D_5N)

Residue/ atom	1		2		3		4		5		6	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
	Glc		→4-Glc		→2-Glc		→4-Glc		→2,4-Glc		→2,4-Glc	
1	5.041	102.68	4.947	102.64	5.059	100.97	4.949	102.64	4.944	100.31	4.946	100.38
2	4.064	75.39	3.992	75.72	4.297	78.43	3.990	75.82	4.232	77.98	4.226	77.95
3	4.305	78.64	4.235	76.87	4.302	80.33	4.230	76.65	4.224	77.98	4.226	77.95
4	4.282	71.74	4.485	78.45	4.198	72.43	4.483	77.79	4.399	78.76	4.418	77.82
5	3.984	78.46	3.722	77.36	3.903	78.97	3.683	77.47	3.647	77.11	3.604	77.14
6	4.565, 4.416	62.92	4.267, 4.148	61.77	4.525, 4.379	63.32	4.223, 4.097	61.61	4.214, 4.094	61.38	4.183, 4.041	61.36
Rha (I)	-		Rha		-		→4-Rha		Rha		→4-Rha	
1			5.911	102.81			5.890	102.32	5.872	103.36	5.848	102.28
2			4.721	72.78			4.587	73.22	4.688	72.56	4.566	73.38
3			4.589	73.06			4.624	73.57	4.548	72.89	4.558	73.06
4			4.349	74.24			4.483	80.53	4.343	74.06	4.457	80.59
5			5.031	70.54			5.063	68.45	4.941	70.57	4.947	68.48
6			1.737	18.81			1.700	91.51	1.645	18.57	1.606	19.33
Rha (III)	-		-		-		Rha		-		Rha	
1							6.336	103.33			6.297	103.28
2							4.906	72.74			4.903	72.79
3							4.553	73.09			4.510	72.99
4							4.324	74.18			4.306	74.10
5							4.406	70.55			4.382	70.56
6							1.615	18.60			1.613	18.57
Rha (II)	-		-		Rha		-		Rha		Rha	
1					6.412	102.69			6.415	102.25	6.413	102.15
2					4.824	73.21			4.839	72.67	4.857	72.59
3					4.654	73.52			4.639	72.89	4.642	72.95
4					4.371	74.88			4.371	74.25	4.371	74.27
5					5.029	70.16			4.974	69.61	4.964	69.70
6					1.796	18.78			1.779	18.76	1.781	19.00
Pennogenin												
1	1.712, 0.948	37.35	1.725, 0.958	37.63	1.756, 0.946	37.61	1.722, 0.954	37.56	1.769, 0.979	37.67	1.766, 0.975	37.70
2	2.123, 1.737	30.38	2.069, 1.705	30.27	2.137, 1.899	30.33	2.054, 1.704	30.23	2.075, 1.897	30.19	2.072, 1.872	30.26
3	3.919	78.19	3.863	78.28	3.946	78.60	3.854	78.37	3.861	78.06	3.858	78.21
4	2.708, 2.464	39.44	2.697, 2.458	39.30	2.812	39.67	2.693, 2.455	39.48	2.796, 2.741	39.31	2.799, 2.746	39.24
5	-	140.86	-	140.91	-	141.52	-	140.98	-	140.94	-	140.99
6	5.304	121.81	5.316	121.93	5.310	121.39	5.312	121.94	5.314	122.04	5.319	121.85
7	1.909, 1.508	32.05	1.970, 1.527	32.63	1.920, 1.538	32.91	1.918, 1.529	32.51	1.927, 1.536	32.75	1.936, 1.531	32.59
8	1.616	32.28	1.620	32.58	1.651	32.39	1.624	32.42	1.644	32.52	1.642	32.54
9	0.961	50.21	0.968	50.51	0.981	50.31	0.971	50.41	0.989	50.49	0.988	50.42
10	-	37.31	-	37.29	-	37.68	-	37.31	-	37.29	-	37.28
11	1.591, 1.497	20.82	1.614, 1.547	21.14	1.603, 1.523	21.14	1.604, 1.503	21.22	1.605, 1.530	21.29	1.595, 1.523	21.24
12	2.186, 1.531	32.24	2.172, 1.527	32.38	2.171, 1.538	32.67	2.174, 1.554	32.27	2.174, 1.539	32.44	2.176, 1.561	32.30
13	-	45.24	-	45.25	-	45.69	-	45.17	-	45.17	-	45.18
14	2.084	53.07	2.082	53.41	2.107	53.10	2.085	53.29	2.097	53.50	2.095	53.22
15	2.218, 1.505	31.86	2.203, 1.513	31.96	2.238, 1.561	32.07	2.205, 1.517	32.00	2.232, 1.564	31.92	2.229, 1.526	31.97
16	4.463	90.06	4.464	90.18	4.478	90.66	4.461	90.21	4.471	90.08	4.468	90.06
17	-	90.17	-	90.26	-	90.82	-	90.22	-	90.29	-	90.22
18	0.970	17.03	0.965	17.34	0.977	17.61	0.982	17.26	0.972	17.18	0.972	17.20

Contd...

Table 1: Contd...

Residue/ atom	1		2		3		4		5		6	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
19	0.948	19.72	0.947	19.53	1.112	19.90	0.952	19.51	1.099	19.46	1.099	19.50
20	2.284	44.72	2.286	44.98	2.288	44.86	2.285	44.90	2.286	45.00	2.286	44.96
21	1.235	9.89	1.236	9.90	1.248	9.96	1.235	9.88	1.240	9.73	1.237	10.18
22	-	109.83	-	109.86	-	110.41	-	109.98	-	109.95	-	109.96
23	1.739, 1.697	32.24	1.726, 1.685	32.29	1.737, 1.696	32.39	1.748, 1.694	32.27	1.739, 1.693	32.27	1.730, 1.691	32.26
24	1.588, 1.588	28.98	1.578, 1.578	29.04	1.607, 1.607	28.94	1.598, 1.598	29.00	1.620, 1.620	28.77	1.605, 1.605	28.96
25	1.589	30.43	1.586	30.75	1.613	30.76	1.598	30.64	1.622	30.57	1.607	30.67
26	3.514, 3.514	66.71	3.516, 3.516	66.92	3.534, 3.534	66.73	3.510, 3.510	66.86	3.516, 3.516	66.92	3.517, 3.517	66.82
27	0.687	17.03	0.705	17.43	0.702	17.81	0.701	17.30	0.699	17.35	0.703	17.37

NMR: Nuclear magnetic resonance

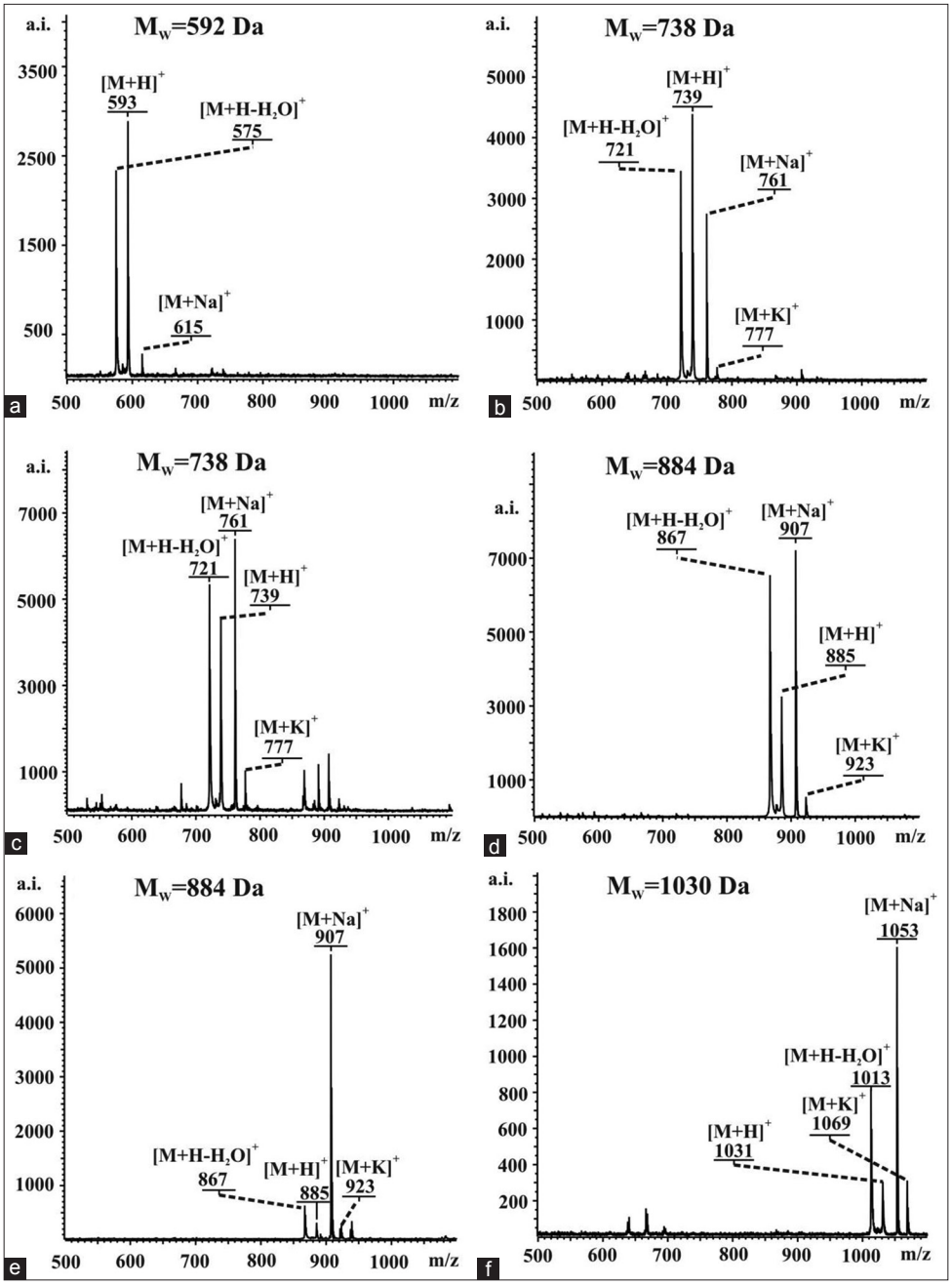

Figure A.1: Maldi toff ms spectra of the saponins isolated from rhizomes of *Paris quadrifolia L*.: a)-1, b)-2, c)-3, d)-4, e)-5, f)-6

m/z: 739 $[M + 1]^+$, 721 $[M + 1\text{-}18]^+$, 761 $[M + 23]^+$, 777 $[M + 39]^+$, molecular formula $C_{39}H_{62}O_{13}$.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (4): White powder; MALDI TOF MS *m/z*: 885 $[M + 1]^+$, 867 $[M + 1\text{-}18]^+$, 907 $[M + 23]^+$, 923 $[M + 39]^+$, molecular formula $C_{45}H_{72}O_{17}$.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (5): White powder; MALDI TOF MS *m/z*: 885 $[M + 1]^+$, 867 $[M + 1\text{-}18]^+$, 907 $[M + 23]^+$, 923 $[M + 39]^+$, molecular formula $C_{45}H_{72}O_{17}$.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→

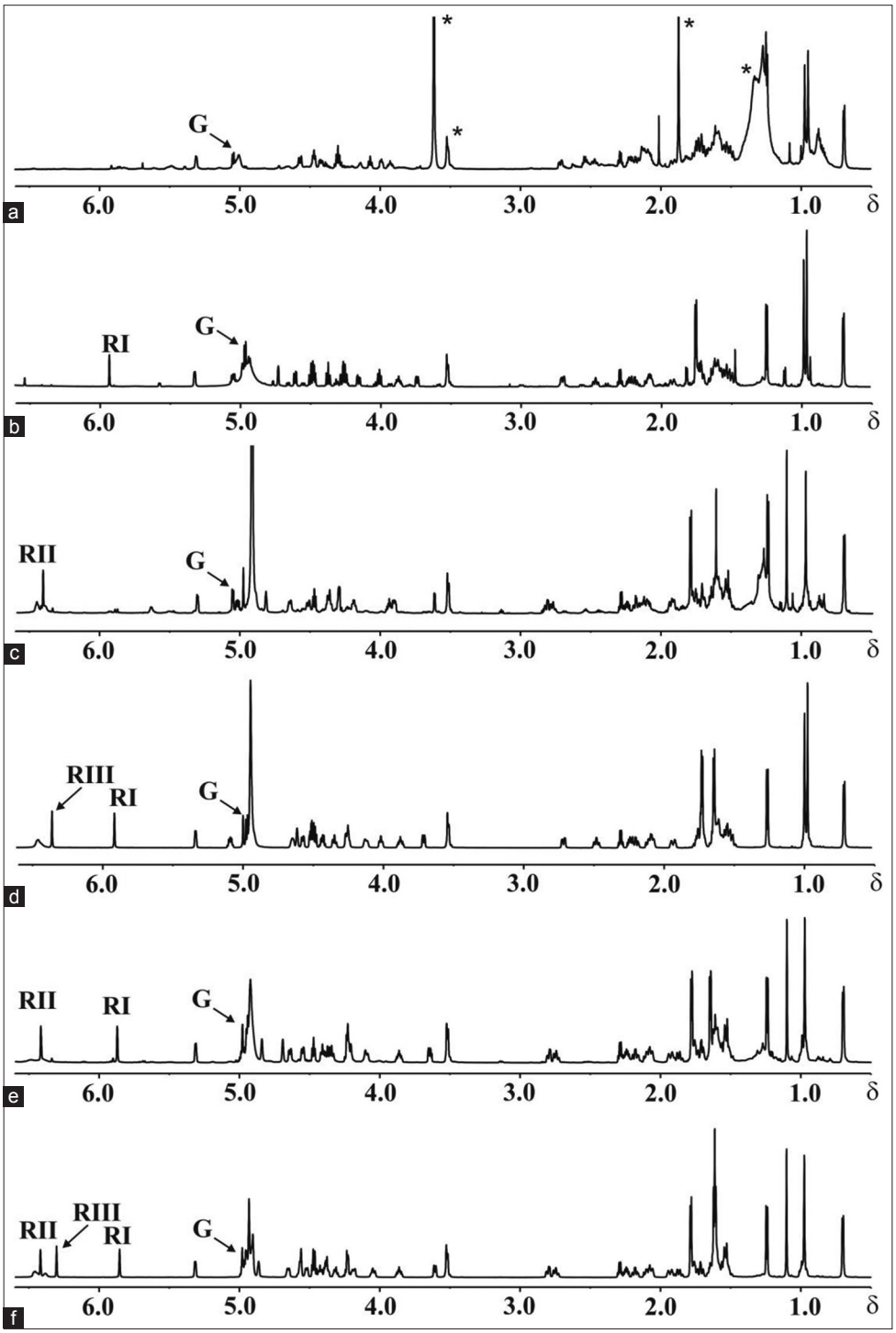

Figure B.1: 1H NMR spectra of the saponins isolated from rhizomes of *Paris quadrifolia L*.: a)-1, b)-2, c)-3, d)-4, e)-5, f)-6

2)]-β-D-glucopyranoside (6): White powder; MALDI TOF MS *m/z*: 1031 $[M + 1]^+$, 1013 $[M + 1\text{-}18]^+$, 1053 $[M + 23]^+$, 1069 $[M + 39]^+$, molecular formula $C_{51}H_{82}O_{21}$.

Cytotoxicity assay

Cell culture

The HL-60 human promyelocytic leukaemia cell line was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The HeLa human cervical adenocarcinoma and MCF-7 human breast cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in an incubator in a humidified atmosphere with 5% of CO_2 at 37°C (Heraceus, Hera cell).

Evaluation of cytotoxicity

Cell viability was determined using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[35-37] Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and treated for 24 h with the isolated pennogenyl saponins in the concentration range 0-50 μg/ml. Next, MTT (0.5 mg/ml) was added directly to the medium and cells were further incubated for 3 h at 37°C. The optical density of the formazan solution was measured at 550 nm with a plate reader (Victor, 1420 multilabel counter). Results are expressed as IC_{50} values. ± SD was calculated from at least three independent experiments.

RESULTS AND DISCUSSION

The isolation of pennogenyl saponins from *P. quadrifolia* rhizomes was completed in a few steps described in the experimental part. The procedure involved drying, ethanol extraction, degreasing and n-butanol/water extraction. The n-Bu-OH soluble fraction was subjected to column chromatography on silica gel in the eluent mixture ingredients gradient mode concentration (with growing concentration of MeOH). Eluates were monitored by TLC and 11 sub-fractions were obtained. Six pennogenyl saponins 1-6 (0.002, 0.019, 0.005, 0.194, 0.017 and 0.158% of the rhizome dry mass respectively) were isolated from 1-7 sub-fractions by semi-preparative isocratic RP HPLC. An especially large amount of saponin **6** was found in three sub-fractions: 93-103 (74.17%), 104-109 (62.42%) and 86-92 (61.27%) [Table 2].

Structural studies

The structures of pennogenyl saponins 1-6 [Figure 1] were elucidated by analyses of their molecular mass (MALDI TOF MS) and 1D and 2D NMR spectra (^{1}H, COSY, TOCSY, ROESY, HMQC, HMBC).

The following cationised ions of the saponins under study were observed in the MALDI TOF MS spectra: $[M + H]^+$ (quasimolecular), $[M + Na]^+$, $[M + K]^+$ and $[M + H\text{-}H_2O]^+$, which are consistent with their molecular formulae and molecular weights for the following compounds: 1-$C_{33}H_{52}O_9$, 592 Da, 2-$C_{39}H_{62}O_{13}$, 738 Da,

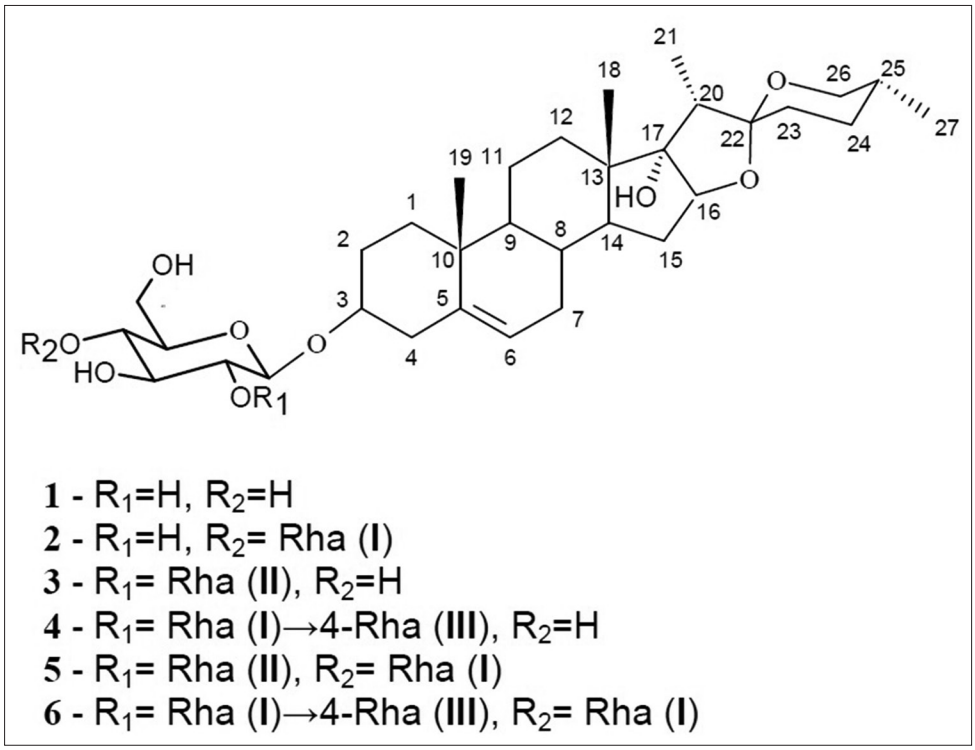

Figure 1: The structures of the saponins isolated from rhizomes of *P. quadrifolia L.*

3-$C_{39}H_{62}O_{13}$, 738 Da, 4-$C_{45}H_{72}O_{17}$, 884 Da, 5-$C_{45}H_{72}O_{17}$, 884 Da, 6-$C_{51}H_{82}O_{21}$, 1030 Da [Figure 1 and Figure A.1-Appendix].

All ^{1}H and ^{13}C chemical shifts of compounds 1-6 were assigned using ^{1}H, DQF-COSY, TOCSY, and HMQC experiments [Table 1]. Detailed analysis of the ^{1}H and ^{13}C NMR data of all the isolated compounds [Table 1] enabled the saponins to be identified: they consisted of a pennogenin residue as an aglycone part and sugar moieties.[4,30,31,38] Since our elucidations of all the aglycone parts were in very good agreement with the literature data, only the analysis of the sugar part of the saponins is described in detail here. The constituent monosaccharides of all six saponins possessed six-membered rings: They were assigned by the lack of carbon atom signals in the δ ~83-88 region of the ^{13}C NMR spectrum [Table 1].[39,40]

L configurations of all rhamnose residues and D configuration of glucose were identified by GLC of their (*S*)-(+)- and (±)-2-butyl glycosides.

Pennogenin **3-**O-β-D-glucopyranoside (1): Examination of the ^{1}H NMR spectrum [Figure B.1.A- Appendix] revealed the presence of only one anomeric proton signal at δ 5.041, which was identified on the basis of the HMQC cross peak at δ 5.041/102.68. The *gluco* configuration of this residue was assigned on the basis of the $^{3}J_{H,\,H}$ coupling constant pattern. Moreover, $^{3}J_{H\text{-}1,\,H\text{-}2}$ = 8.2 Hz clearly revealed a β-configured Glc residue. The HMBC experiment (not shown) identified the linkage between Glc and pennogenin residues as 1→3. Strong cross-peaks H-1 of Glc/C-3 of pennogenin (δ 5.041/78.19), as well as of H-3 of pennogenin/C-1 of Glc (δ 3.919/102.68) were observed in the spectrum.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (2): The ^{1}H NMR spectrum of compound 2 [Figure B.1.B-Appendix] was very similar to one described above**.** The spectrum contained two anomeric proton signals at δ 4.947 and 5.911. The first residue was identified as β-Glc ($^{3}J_{H\text{-}1,\,H\text{-}2}$ = 8.1 Hz) substituted at C-4 (δ 78.45), and the second one as terminal α-Rha (I). The α configuration of the rhamnopyranosyl unit ($^{3}J_{H1,\,H2}$< = 1.5Hz) was deduced from the absence of strong intraresidual ROESY correlations between protons H-1 and H-3/H-5. This was also confirmed by $^{1}J_{H1,\,C1}$ = 169 Hz, measured from the residual direct correlation observed in the HMBC spectrum, which is in agreement with that reported for the alpha anomer of rhamnopyranose.[41] The HMBC experiment confirmed the 1→3 linkage of Glc to pennogenin (H-1 of Glc/C-3 of pennogenin at δ 4.947/78.28, and H-3 of pennogenin/C-1 of Glc at δ 3.863/102.64). Furthermore, the substitution of Glc by the Rha (I) residue at C-4 (H-1 of Rha (I)/C-4 of Glc at δ 5.911/78.45, and H-4 of Glc/C-1 of Rha (I) at δ 4.485/102.81) was demonstrated.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (3): The ^{1}H NMR spectrum of compound 3 [Figure B.1.C- Appendix] also showed two anomeric proton signals (δ 5.059, and 6.412). The first one belonged to β-Glc ($^{3}J_{H\text{-}1,\,H\text{-}2}$ = 7.3 Hz) substituted at C-2 (δ 78.43), and the second to a terminal α-Rha (II) ($^{1}J_{H1,\,C1}$ = 171 Hz). The HMBC spectrum revealed the 1→3 linkage of Glc to pennogenin (H-1 of Glc/C-3 of pennogenin at δ 5.059/78.60, and H-3 of pennogenin/C-1 of Glc at δ 3.946/100.97), as well as the substitution of Glc by the Rha (II) residue at C-2 (H-1 of Rha (II)/C-2 of Glc at δ 6.412/78.43, and H-2 of Glc/C-1 of Rha (II) at δ 4.297/102.69).

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (4): Three anomeric signals were identified in the anomeric region of the ^{1}H NMR spectrum of compound 4 [Figure B.1.D - Appendix]. The signal with the lowest chemical shift (δ 4.949) was identified as H-1 of β-Glc ($^{3}J_{H\text{-}1,\,H\text{-}2}$ = 7.7Hz) substituted at C-4 (δ 77.79), then the signal at δ 5.890 belonging to α-Rha (I) ($^{1}J_{H1,\,C1}$ = 170Hz) substituted at C-4 (δ 80.53), and finally the signal at δ 6.336 represented the terminal α-Rha (III) ($^{1}J_{H1,\,C1}$ = 171Hz). In the HMBC spectrum, the cross-peaks at δ 4.949/78.37 (H-1 of Glc/C-3 of pennogenin) and at δ 3.854/102.64 (H-3 of pennogenin/C-1 of Glc) indicated glycosylation of the

Table 2: Contents of pennogenyl saponins in *P. quadrifolia* L. fractions

Saponin	Fraction number	T_R (min)	Yield (mg/100g)
Pennogenin 3-O-β-D-glucopyranoside (1)	7-25	33.74	0.64
Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (2)	7-25 26-71	30.78	1.93 2.70
Pennogenin 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (3)	26-71	21.02	0.90
Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (4)	7-25 26-71 72-85	27.44	0.68 36.68 3.06
Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (5)	26-71 72-85 86-92	19.10	2.70 4.08 1.40
Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (6)	72-85 86-92 93-103 104-109 110-117	18.29	36.73 61.27 74.17 62.42 19.21

aglycone at the C-3 position. Another cross-peak between the second anomeric proton at δ 5.890 and the carbon at δ 77.79 (H-1 of Rha (I)/C-4 of Glc), and the cross-peak at δ 4.483/102.32 (H-4 of Glc/C-1 of Rha (I)) revealed the 1→4 linkage of Rha (I) to Glc. Finally, the cross-peak between the third anomeric proton at δ 6.336 and the carbon at δ 80.53 (H-1 of Rha (III)/C-4 of Rha (I)), and the cross-peak at δ 4.483/103.33 (H-4 of Rha (I)/C-1 of Rha (III)) revealed the 1→4 linkage of Rha (III) to Rha (I).

Pennogenin 3-*O*-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (5): The ^{1}H NMR spectrum of compound 5 [Figure B.1.E - Appendix] also showed three anomeric proton signals (δ 4.944, 5.872, and 6.415). The first one belonged to β-Glc ($^3J_{H\text{-}1,\ H\text{-}2}$ = 6.9 Hz) substituted at C-2 (δ 77.98) and C-4 (δ 78.76), as well as two other signals of two terminal α-Rha residues ($^1J_{H1,\ C1}$ = 169 Hz, and $^1J_{H1,\ C1}$ = 171 Hz respectively). The HMBC spectrum confirmed the 1→3 linkage of Glc to pennogenin (H-1 of Glc/C-3 of pennogenin at δ 4.944/78.06, and H-3 of pennogenin/C-1 of Glc at δ 3.861/100.31). Moreover, the cross-peaks at δ 5.872/78.76 (H-1 of Rha (I)/C-4 of Glc) and at δ 4.399/103.36 (H-4 of Glc/C-1 of Rha (I)) revealed the linkage 1→4 between Rha (I) and Glc, and the cross-peaks at δ 6.415/77.98 (H-1 of Rha (II)/C-2 of Glc) and at δ 4.399/102.25 (H-2 of Glc/C-1 of Rha (II)) revealed the linkage 1→2 between Rha (II) and Glc.

Pennogenin 3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (6): Four anomeric signals were identified in the anomeric region of the ^{1}H NMR spectrum of compound 6 [Figure B.1.F - Appendix]. The signal with the lowest chemical shift (δ 4.946) was identified as H-1 of β-Glc ($^3J_{H\text{-}1,\ H\text{-}2}$ = 7.0 Hz) substituted at C-2 (δ 77.95) and at C-4 (δ 77.82), then the signal at δ 5.848 belonging to α-Rha ($^1J_{H1,\ C1}$ = 169 Hz) substituted at C-4 (δ 80.59), and finally two signals at δ 6.297 and 6.413 representing terminal α-Rha residues ($^1J_{H1,C1}$ = 171 Hz, and $^1J_{H1,C1}$ = 172 respectively).

In the HMBC spectrum, the cross-peaks at δ 4.946/78.21 (H-1 of Glc/C-3 of pennogenin) and at δ 3.858/100.38 (H-3 of pennogenin/C-1 of Glc) indicated glycosylation of the aglycone at C-3. Another cross-peak at δ 5.848/77.82 (H-1 of Rha (I)/C-4 of Glc) and the cross-peak at δ 4.418/102.28 (H-4 of Glc/C-1 of Rha (I)) revealed the 1→4 linkage of Rha (I) to Glc. Finally, the position of glycosylation by the two remaining terminal Rha residues was identified. The cross-peak at δ 6.297/80.59 (H-1 of Rha (III)/C-4 of Rha (I)) and the cross-peak at δ 4.457/103.28 (H-4 of Rha (I)/C-1 of Rha (III)) revealed the 1→4 linkage of Rha (III) to Rha (I), while the cross-peak at δ 6.413/77.95 (H-1 of Rha (II)/C-2 of Glc) and the cross-peak at δ 4.226/102.15 (H-2 of Glc/C-1 of Rha (II)) revealed the 1→2 linkage of Rha (II) to Glc.

Bioassay studies

The isolated eleven sub-fractions were evaluated for their cytotoxic activity against HL-60 and HeLa cells. The seven sub-fractions (7-25, 26-71, 72-85, 86-92, 93-103, 104-109, 110-117) showed cytotoxicity below 100 μg/ml [Table 3]. The fractions 86-92, 93-103, 104-109 with large amount of compound 6 [Table 2] were the most potent [Table 3].

The six compounds isolated from the all sub-fractions were tested on HL-60, HeLa and MCF-7 cells. Pennogenyl saponins 5 and 6 exhibited cytotoxic activity against HL-60, HeLa and MCF-7 tumour cells with IC_{50} values of 1.0 ± 0.04 μg/ml, 1.8 ± 0.072 μg/ml and 2.4 ± 0.096 μg/ml respectively, and 2.0 ± 0.08 μg/ml, 2.5 ± 0.125 μg/ml and 3.2 ± 0.128 μg/ml respectively. Saponins 1, 2 and 4 or without the ramnosyl residue or without the terminal ramnosyl residue linked to C-2 of the glucosyl group did not show any cytotoxic activity [Table 4].

CONCLUSION

The six saponins studied in this paper have been isolated at the same time from rhizomes of one species of *Paris*

Table 3: Cytotoxic activity of sub-fractions from *P. quadrifolia* L. extract

Sub-fraction	IC_{50} value (μg/ml)	
	HL-60	HeLa
7-25	75±7.5	n.t.
26-71	68±6	82±4.9
72-85	9±0.4	20±1.0
86-92	7±0.4	7±0.2
93-103	8±0.2	8±0.2
104-109	8±0.3	10±0.5
110-117	26±2.6	45±2.7

n.t. : Not tested

Table 4: Cytotoxic activity of pennogenyl saponins from *P. quadrifolia* L. rhizomes

Compound	IC_{50} value (μg/ml)		
	HL-60	HeLa	MCF-7
1	>50	>50	n.t.
2	47±2.8	>50	n.t.
3	16±0.8	18±0.9	25±1.5
4	>50	>50	n.t.
5	1.0±0.04	1.8±0.072	2.4±0.096
6	2.0±0.08	2.5±0.125	3.2±0.128
Etoposide*	0.45±0.022	>50	>50
Mitoxantrone*	0.06±0.004	0.4±0.012	0.2±0.008

*Control compounds; n.t. : Not tested

and fully structurally characterised using spectroscopic and chemical methods. The spirostan saponins 1, 2, 3 and 4 were obtained from *P. quadrifolia* rhizomes for the first time. The isolation and the identification of six pennogenyl saponins from *P. quadrifolia* rhizomes constitute significant contribution into the general knowledge of chemical composition of the *Paris* family, particularly in the field of saponin substances and *P. quadrifolia* L. itself.

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