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Paviosides A–H, eight new oleane type saponins from *Aesculus pavia* with cytotoxic activity

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ABSTRACT

A phytochemical analysis of *Aesculus pavia* has led to the isolation of eight novel triterpenoid saponins, based on oleane type skeleton and named paviosides A–H (**1a, 1b–4a, 4b**). On the basis of chemical, and 2D NMR and mass spectrometry data, the structures of the new compounds were elucidated as 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-glucopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C (**1a**), 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-glucopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C (**1b**), 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-galactopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C (**2a**), 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-galactopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C (**2b**), 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C (**3a**), 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C (**3b**), 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl protoaescigenin (**4a**), and 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl protoaescigenin (**4b**). The compounds showed cytotoxic activity on J-774, murine monocyte/macrophage, and WEHI-164, murine fibrosarcoma, cell lines. Among them, paviosides E–H (**3a, 3b** and **4a, 4b**) showed higher activity with values ranging from 2.1 to 3.6 μg/mL. Structure–activity relationship studies indicated the positive effect on the activity of xylose unit in the place of glucose, while a little detrimental effect is observed when glucose is substituted by galactose. The aglycone structure and the presence of a tigloyl or an angeloyl group at C-21 do not affect significantly the inhibitory activity on both tested cell lines.

1. Introduction

The genus *Aesculus* (fam. *Aesculaceae*; tribe *Aesculae*) includes about a dozen of species originating from Eurasia and North America. Its wide geographical distribution, both in Old and New World, indicates a wide range of adaptation to various habitats, including harsh conditions. Usually, *Aesculus* species are grown as ornamental plants because of their beautiful flowers and large foliage. However, seeds and bark of *Aesculus* spp., mainly the horse chestnut tree *Aesculus hyppocastanum*, have been widely used in European traditional medicine since the 16th century.[1] In particular, the saponin mixture, named aescin, isolated from this plant, is commercialized and used as remedy for chronic venous insufficiency, hemorrhoids and post-operative edema.[2]

Several triterpenoid saponins based on oleane type aglycon were isolated in some *Aesculus* spp.[3] These compounds showed interesting activity such anti-inflammatory, antiedematous, capillaro-protective activity, and hypoglycaemic activity.[3]

Aesculus pavia, commonly known as red buckeyes or scarlet buckeye, is a shrubby or small tree species native of North America and Asia and later on introduced in Europe. In a search for natural compounds from ornamental plants[4–7] we have recently performed a bioassay-guided phytochemical analysis of an *A. pavia* genotype, that shows a constitutive immunity towards the leaf specific fungal parasite *Guignardia aesculi*. The analysis led to the isolation of a new coumarin, named pavietin, showing specific antifungal activity against the pathogen.[8] In addition, it was undetectable in *A. hippocastanum* trees, which bear white flowers, produce the typical chestnut fruit and are very susceptible to the *G. aesculi* attacks.[9]

Thus, we performed a phytochemical analysis of *A. pavia* L. with the aim of analyze the saponin composition. The study led to the

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isolation of eight saponin compounds, named paviosides A–H (**1a, 1b–4a, 4b**, Chart 1). Paviosides were subjected to further cytotoxicity tests using J-774 (murine monocyte/macrophage) and WEHI-164 (murine fibrosarcoma) cell lines and obtained results are reported herein.

2. Results and discussion

The butanol soluble part of the MeOH extract of the aerial parts of *A. pavia* was chromatographed on silica RP-18 by MPLC and then subjected to HPLC purification to afford paviosides A–H (**1a, 1b–4a, 4b**, Chart 1).

Pavioside A (**1a**) gave in the positive HRFABMS a pseudo-molecular ion peak at *m/z* 1101.5822 $[M+H]^+$, which indicated the molecular formula as $C_{55}H_{88}O_{22}$. The IR spectrum of **1** showed the presence of hydroxyl (3400 cm^{-1}), ester group (1750 cm^{-1}) and glycosidic linkage (1000–1100 cm^{-1}).

The ^1H NMR spectrum of **1a** showed signals for seven tertiary methyl groups [δ 0.80, 0.84, 0.89, 0.95, 0.96 1.05 and 1.36], three anomeric sugar protons [δ 4.31 (d, J = 7.5 Hz), 4.75 (d, J = 7.5 Hz), 4.95 (d, J = 7.4 Hz)]. A further feature of the ^1H NMR spectrum was a broad singlet at δ 5.31, typical of H-12 of a Δ^{12} oleane skeleton, which was also indicated by the signals at δ 123.5 and 145.0, due to C-12 and C-13, respectively, in ^{13}C NMR spectrum (Table 1),[10] indicative of the olean-12-en triterpenoid.

FAB MS spectrum (positive ion) of **1a**, besides the pseudomolecular ion peak at *m/z* 1101 $[M+H]^+$, showed diagnostic fragmentation peaks at *m/z* 969 $[M+H-132]^+$ (loss of one pentose), *m/z* 939 $[M+H-162]^+$ (loss of one hexose), and *m/z* 807 $[M+H-(132+162)]^+$ indicating a branched sugar moiety.

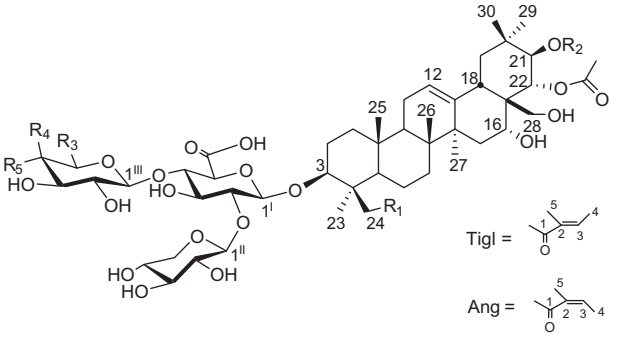
2D COSY and HOHAHA allowed to recognize the signal of H-3 at δ 3.40 (dd, J = 4.0 and 11.5 Hz) that was connected by HSQC experiment with the relative carbon C-3 (δ 89.9). The J values of H-3 clearly indicated its axial position and the chemical shift value of C-3 (+10 ppm) in comparison to models[10] indicated C-3 as glycosilation site.

Table 1
^{13}C NMR data of the aglycone portion of paviosides A–H (**1a**, **1b–4a**, **4b**)*

Position	**1** δ_C (mult.)	**4** δ_C (mult.)		**1** δ_C (mult.)	**41** δ_C (mult.)
1	39.6 (CH_2)	39.6 (CH_2)	16	74.0 (CH)	74.0 (CH)
2	27.0 (CH_2)	27.0 (CH_2)	17	48.8 (C)	48.8 (C)
3	89.9 (CH)	86.9 (CH)	18	41.0 (CH)	41.0 (CH)
4	38.9 (C)	38.9 (C)	19	47.1 (CH_2)	47.1 (CH_2)
5	55.9 (CH)	55.9 (CH)	20	31.2 (C)	31.2 (C)
6	18.7 (CH_2)	18.7 (CH_2)	21	78.3 (CH)	78.3 (CH)
7	33.6 (CH_2)	33.6 (CH_2)	22	74.5 (CH)	74.5 (CH)
8	40.5 (C)	40.5 (C)	23	28.6 (CH_3)	28.6 (CH_3)
9	47.8 (CH)	47.8 (CH)	24	16.8 (CH_3)	62.9 (CH_3)
10	37.2 (C)	37.2 (C)	25	15.6 (CH_3)	15.6 (CH_3)
11	24.8 (CH_2)	24.8 (CH_2)	26	17.0 (CH_3)	17.0 (CH_3)
12	123.5 (CH)	123.5 (CH)	27	27.3 (CH_3)	27.3 (CH_3)
13	145.0 (C)	145.0 (C)	28	64.0 (CH)	64.0 (CH)
14	42.7 (CH)	42.7 (CH)	29	33.3 (CH_3)	33.3 (CH_3)
15	36.0 (CH_2)	36.0 (CH_2)	30	20.6 (CH_3)	20.6 (CH_3)

Data extracted from pavioside A (**1a**) and pavioside G (**4a**). Data for paviosides B–F (**1b**, **2a**, **2b–3a**, **3b**) exactly agree with those of A (**1a**). Data for pavioside H (**4b**) exactly agree with those of G (**4a**).
* The spectra were measured in CD_3OD.

Analysis of 2D COSY and HOHAHA spectra indicated the presence of nine spin systems, six belonging to the aglycone and the remaining three to three sugar residues. Each proton was related to the directly bonded carbon through an HSQC spectrum. Concerning the aglycone, the first spin system included the ring A carbons from C-1 to C-3, confirming oxygenation at C-3; the second included non-oxygenated carbons 5–7; the third included C-9, C-11 and C-12, showing no oxygenation on these carbons and confirming a double bond at C-12; the fourth included the ring D carbons C-15 and C-16, showing oxygenation at the latter place; the fifth is formed by non-oxygenated C-18 and C-19; the sixth included the ring E carbons C-21 and C-22 showing oxygenation at both places. An isolated hydroxymethylene group was also



Pavioside A (1a)	R_1 = H	R_2 = Tigl	R_3 = CH_2OH	R_4 = H	R_5 = OH
Pavioside B (1b)	R_1 = H	R_2 = Ang	R_3 = CH_2OH	R_4 = H	R_5 = OH
Pavioside C (2a)	R_1 = H	R_2 = Tigl	R_3 = CH_2OH	R_4 = OH	R_5 = H
Pavioside D (2b)	R_1 = H	R_2 = Ang	R_3 = CH_2OH	R_4 = OH	R_5 = H
Pavioside E (3a)	R_1 = H	R_2 = Tigl	R_3 = H	R_4 = H	R_5 = OH
Pavioside F (3b)	R_1 = H	R_2 = Ang	R_3 = H	R_4 = H	R_5 = OH
Pavioside G (4a)	R_1 = OH	R_2 = Tigl	R_3 = H	R_4 = H	R_5 = OH
Pavioside H (4b)	R_1 = OH	R_2 = Ang	R_3 = H	R_4 = H	R_5 = OH

Chart 1. Chemical structures of paviosides A–H (**1a**, **1b–4a**, **4b**).

detected. Then, the HMBC spectrum allowed to connect these partial substructures as showed in Figure 1.

Concerning the relative configuration of the oxygenated chiral carbons of the aglycone, the stereochemistry at H-16 has been determined as α on the basis of the small *J* values of the broad doublet at δ 4.48 (H-16) in the ^{1}H NMR spectrum, while the configurations at C-21 and C-22 have been determined as β and α, respectively on the basis of the large coupling constants (10.5 Hz) in comparison to analogous compounds.[11] ROESY cross peak of H-16 with H_2-28 (Fig. 1) confirmed their cis-relationship and the stereochemistry at C-16, while dipolar interactions of H-18 with H-22 and of Me-29 with H-21 (Fig. 1) were also indicative of a cis-relationship between the two couple of substituents and thus confirmed the stereochemistry at C-21 and C-22.

Comparison of the obtained data with those reported in the literature confirmed the proposed structure including the relative stereochemistry at chiral carbons.

All these data pointed to a 3β, 16α, 21β, 22α dihydroxyolean-12-en-28-ol (skeleton), commonly known as barringtogenol C. Its 18β series have been recognized by the chemical shift values of C-12, C-13, C-18 and C-29, in comparison to model compounds.[12,13]

Further inspection of the ^{1}H and ^{13}C NMR spectrum of **1a** (Tables 1 and 2) indicated that the triterpene skeleton was diesterified with one acetyl [^{1}H: 2.10 (s); ^{13}C: δ 170.2 (s), 21.0 (q, 7 Hz)] and one tigloyl group [^{1}H: 7.05 (d, 7 Hz), 1.66 (d, 7 Hz), 1.96 (s); ^{13}C: δ 168.5 (s), 129.6 (s), 136.7 (d, 7 Hz), 14.1 (q, 7 Hz), 12.5 (q, 7 Hz)]. The next step was the elucidation of the acylation pattern, which was solved by inspection of diagnostic $^3J_{C-H}$ couplings between oxymethine protons and carbonyl ester carbons. As showed in Figure 1, the two ester carbonyls of tigloyl and acyl were correlated to oxymethine resonances H-21 and H-22, respectively, thus locating the remaining free hydroxyls at C-16 and C-28.

On acid hydrolysis, followed by trimethilsylilation and by GLC analysis of the released monosaccharides on a chiral column, compound **1a** afforded D-glucuronic acid, D-glucose and D-xylose in the ratio of 1:1:1.

The first step in the analysis of the saccharide part of **1a**, was the association of the three anomeric carbons (δ 102.2, 104.2, and 104.3) with their anomeric proton signals (δ 4.75, 4.31, and 4.95, respectively), through the HSQC experiment. Their nature was determined by combined analysis of 2D COSY, HOHAHA, and HSQC spectra. Then, HMBC (Fig. 1), together with ROESY spectra, gave key informations both for glycosilation sites on the aglycone and interglycosidic linkages.

Thus, starting from the anomeric proton at δ 4.31 (H-1^I), by analysis of 2D COSY, HOHAHA, and HSQC, the sequence of a hexopyranose unit was identified. The large coupling constants, observed in the 2D HOHAHA subspectra for H-1^I, H-2^I, H-3^I and H-5^I, and the presence of a carboxylic group at C-6 indicated a β-glucopyranosiduronic (GlcA^I).

With the same type of analysis the other two sugars were identified as β-xylopyranose^{II} (δ 4.95) and β-glucopyranose^{III} (δ 4.75) by the large coupling constant value found for all protons of each spin system.

The glucuronic residue (GlcA^I) was connected at C-3 of the aglycone on the basis of HMBC cross peaks of H-1^I (δ 4.31) with C-3 (δ 89.9), and of H-3 (δ 3.40) with C-1^I (δ 104.2) (Fig. 1), and also confirmed by the strong ROESY peak between H-1^I (δ 4.31) and H-3 (δ 3.40) (Fig. 1).

Xylose^{II} was placed at C-2 of glucuronic acid (C-2^I) because of the HMBC cross peak of H-1^{II} (δ 4.95) with C-2^I (δ 79.0) while Glc^{III} was placed at C-4 of glucuronic acid (C-4^I) because of the HMBC cross peak of H-1^{III} (δ 4.75) with C-4^I (δ 81.1). This was confirmed by the chemical shifts of both C-2^I and C-4^I of GlcA^I that showed glycosilation shifts[14,15] compared to methyl-O-glucuronic acid, thus indicating this carbons as glycosilation sites of two further sugars.

On the basis of these data, pavioside A (**1a**, Chart 1) has been established as 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-glucopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C.

Pavioside B (**1b**) had a molecular formula of $C_{55}H_{88}O_{22}$, established by HRFABMS. Its molecular weight, identical to compound **1a**, and analogy of NMR profiles indicated a close similarity between the two compounds. A comparative analysis of ^{1}H and ^{13}C NMR, and 2D COSY spectra (Tables 1 and 2 and Section 3) of **1b** with those of **1a**, showed a good coincidence in the chemical shift of both compounds. Some slight modifications were only found only in the esterification patter. Further inspection of the ^{1}H and ^{13}C NMR spectra of **1b** (Tables 1 and 2) indicated that the triterpene skeleton was diesterified with one acetyl [^{1}H: 2.10 (s); ^{13}C: δ 170.2 (s), 21.0 (q, 7 Hz)] and one angeloyl group [^{1}H: 5.95 (d, 7 Hz), 2.10 (d, 7 Hz), 2.02 (s); ^{13}C: δ 167.9 (s), 129.1 (s), 136.9 (d, 7 Hz), 15.9 (q, 7 Hz), 21.0 (q, 7 Hz)]. Key information for the locations of ester groups have been obtained by HMBC spectra that unambiguously located the angeloyl group at C-21 and the acetyl group at C-22.

Therefore, the structure of **1b** has been determined as: 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-glucopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C.

Pavioside C (**2a**) showed the same molecular formula of **1a** and **1b** by positive HRFABMS (Section 3). The presence of barringtogenol C aglycone has been confirmed by ^{1}H and ^{13}C NMR data (Tables 1 and Section 3). Signals for tigloyl and acetyl groups were also detected in the spectra (Table 2) and HBMC cross peaks located them at C-21 and C-22, respectively. Differences with **1a** and **1b** had to be related to the saccharide portion that by NMR data (Table 2) appeared to be composed of β-glucuronic acid (δ 4.32; δ 104.2), β-xylose (δ 4.95; δ 104.3) and β-galactose (δ 4.30; δ 101.2).

Acid hydrolysis of **2a**, followed by trimethilsylilation and GLC analysis of the released monosaccharides on a chiral column,

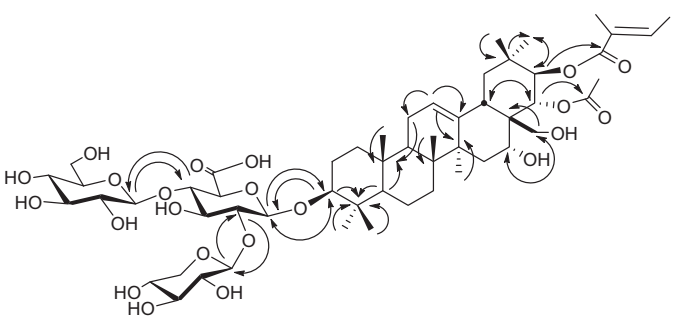


Figure 1. Selected HMBC (H→C) and ROESY (H↔H) correlations exhibited by pavioside A (**1a**).

Table 2
^{13}C and ^{1}H NMR data of the sugar portion and acyl groups of paviosides A–F (**1a**, **1b–3a**, **3b**)*

	1			**2**			**3**	
	δ_C (mult.)	δ_H (mult., *J* in Hz)		δ_C (mult.)	δ_H (mult., *J* in Hz)		δ_C (mult.)	δ_H (mult., *J* in Hz)
3-O-Sugars								
GlcA I			GlcA I			GlcA I		
1	104.2 (CH)	4.31 (d, 7.5)		104.2 (CH)	4.32 (d, 7.5)		104.2 (CH)	4.31 (d, 7.5)
2	79.0 (CH)	3.53[a]		79.0 (CH)	3.55[a]		79.1 (CH)	3.53[a]
3	76.7 (CH)	3.34[a]		76.5 (CH)	3.31[a]		76.6 (CH)	3.32[a]
4	81.1 (CH)	3.68[a]		81.1 (CH)	3.67[a]		81.0 (CH)	3.67[a]
5	75.5 (CH)	3.22[a]		75.4 (CH)	3.22[a]		75.5 (CH)	3.21[a]
6	171.5 (C)			171.5 (C)			171.5 (C)	
Xyl II			Xyl II			Xyl II		
1	104.3 (CH)	4.95 (d, 7.4)		104.3 (CH)	4.95 (d, 7.4)		104.3 (CH)	4.95 (d, 7.4)
2	75.2 (CH)	3.28[a]		75.2 (CH)	3.29[a]		75.1 (CH)	3.28[a]
3	78.1 (CH)	3.55[a]		78.2 (CH)	3.55[a]		78.1 (CH)	3.56[a]
4	71.3 (CH)	3.62[a]		71.3 (CH)	3.63[a]		71.2 (CH)	3.62[a]
5	67.5 (CH_2)	3.31[a], 3.91[a]		67.4 (CH_2)	3.30[a], 3.90[a]		67.4 (CH_2)	3.32[a], 3.91[a]
Glc III			Gal III			Xyl III		
1	102.2 (CH)	4.75 (d, 7.5)		101.2 (CH)	4.30 (d, 7.5)		104.3 (CH)	4.95 (d, 7.4)
2	74.4 (CH)	3.50[a]		71.9 (CH)	3.84[a]		75.3 (CH)	3.28[a]
3	76.2 (CH)	3.37[a]		73.8 (CH)	3.62[a]		78.1 (CH)	3.55[a]
4	71.4 (CH)	3.59[a]		69.7 (CH)	3.87 (dd, 3.2, 2.5)		71.2 (CH)	3.62[a]
5	76.4 (CH)	3.23[a]		76.2 (CH)	3.45[a]		67.3 (CH_2)	3.31[a], 3.90[a]
6	62.6 (CH_2)	3.54[a], 3.60[a]		62.0 (CH_2)	3.47[a]			
Acyls								
Ac			Ac			Ac		
1	170.2 (C)			170.2 (C)			170.2 (C)	
2	21.0 (CH_3)	2.10 (s)		21.0 (CH_3)	2.10 (s)		21.0 (CH_3)	2.10 (s)
Tigl (**1a**)			Ang (**2a**)			Ang (**3a**)		
1	168.5 (C)			167.9 (C)			167.9 (C)	
2	129.6 (C)			129.1 (C)			129.1 (C)	
3	136.7 (CH)	7.05 (dq, 7.0)		136.9 (CH)	5.95 (dq, 7.0)		136.9 (CH)	5.95 (dq, 7.0)
4	14.1 (CH_3)	1.66 (d, 7.0)		15.9 (CH_3)	2.10 (d, 7.0)		15.9 (CH_3)	2.10 (d, 7.0)
5	12.5 (CH_3)	1.96 (s)		21.0 (CH_3)	2.02 (s)		21.0 (CH_3)	2.02 (s)
Ang (**1b**)			Tigl (**2b**)			Tigl (**3b**)		
1	167.9 (C)			168.5 (C)			168.5 (C)	
2	129.1 (C)			129.6 (C)			129.6 (C)	
3	136.9 (CH)	5.95 (dq, 7.0)		136.7 (CH)	7.05 (dq, 7.0)		136.7 (CH)	7.05 (dq, 7.0)
4	15.9 (CH_3)	2.10 (d, 7.0)		14.1 (CH_3)	1.66 (d, 7.0)		14.1 (CH_3)	1.66 (d, 7.0)
5	21.0 (CH_3)	2.02 (s)		12.5 (CH_3)	1.96 (s)		12.5 (CH_3)	1.96 (s)

Data for paviosides G–H (**4a**/**4b**) exactly agree with those of paviosides E–F (**3a**, **3b**).
* The spectra were measured in CD_3OD.
[a] Superimposed by other signals.

afforded D-glucuronic acid, D-galactose and D-xylose in the ration of 1:1:1 thus confirming the identification based on NMR spectra.

HMBC spectra showed the same interglycosidic linkages of **1a** and **1b**, and indicated that in **2a** the β-D-Gal (J_{H1-H2} = 7.5 Hz) residue substitutes β-D-Glc at C-4^{I} of GlcA. Thus, the structure of **2a** has been determined as: 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-galactopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C (**2a**).

Pavioside D (**2b**) showed a close similarity to pavioside C (**2a**) having the same molecular formula, determined by positive HRFABMS (see Section 3), and analogous NMR spectra (Tables 1 and 2). Deep analysis of NMR data indicated **2b** to be composed by the following substructures: barrigtogenol C, β-GlcA, β-Gal, β-Xyl, acyl and angelate groups. HMBC spectra allowed to connect these substructures in the formula depicted in **2b**. Thus, **2b** differed from **2a** in having an angelate group at the place of a tigloyl group. Thus, the structure of **2b** has been determined as: 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-galactopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C (**2b**).

Paviosides E (**3a**) and F (**3b**) appeared very close to one from each other by MS and NMR data. In fact, both compounds had the same molecular formula ($C_{54}H_{86}O_{21}$) determined by pseudomolecular ion peaks in HRFABMS spectra (Section 3). These values were 30 amu less than those of paviosides A–D, suggesting the absence in both molecules of an oxymethylene moiety. Deeper analysis of NMR spectra (Tables 1 and 2) evidenced in both compounds **3a** and **3b** the presence of barrigtogenol C as aglycone. Acyl and tigloyl groups were detected in **3a**, while acyl and angeloyl groups were evident in the spectra of **3b**. So, differences have to be related to the sugar portion of the molecules that in agreement with MS and NMR data appeared to be composed of a β-GlcA residue, and two residues of β-Xyl. This identification has been confirmed by GLC analysis of hydrolyzed compounds (Section 3). HMBC cross peaks allowed to connect in both compounds the two xylose moieties at C-2^{I} and C-4^{I} of glucuronic acid, respectively. Thus, the structures of the compounds have been determined as: 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C (**3a**), and 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C (**3b**).

Paviosides G (**4a**) and H (**4b**) showed a close analogy in their chemical structure having the same molecular weight (see Section 3), and very similar NMR data (Tables 1 and 2). In particular, their

molecular weights were 16 amu higher than that of paviosides E–F (**3a, 3b**), indicating the presence of a further oxygen in the molecules.

The ^{1}H NMR spectra of both compounds showed six tertiary methyl groups at δ 0.80 (Me-26), 0.84 (Me-25), 0.89 (Me-29), 0.96 (Me-30), 0.98 (Me-23), and 1.36 (Me-27). The absence of the characteristic singlet at δ 1.05 attributable to Me-24 suggested that this position could be the site of further oxygenation. This has been confirmed by the presence of an hydroxymethylene carbon (δ 62.9, Table 1) that showed key HMBC correlations peaks with H-3 (δ 3.45), H-5 (δ 0.91), and Me-23 (δ 0.98). Thus, the aglycone of these saponins was based on the structure of 24-hydroxyl barrigtogenol C which is named protoaescigenin.

Investigation of NMR spectra of paviosides G (**4a**) and H (**4b**) indicated the same trisaccharide skeleton of paviosides E (**3a**) and F (**3b**) composed by β-GlcA, and two residues of β-Xyl as reported in formulas. Differences in paviosides G and H were related to the substitution of a tigloyl group in pavioside G with a angeloyl group in pavioside H, both attached at C-21.

Thus, the chemical structures of paviosides G (**4a**) and H (**4b**) have been determined as: 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl protoaescigenin (**4a**), and 3-*O*-[β-D-xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl protoaescigenin (**4b**).

The detailed phytochemical investigation of saponins from *A. pavia* L. is limited to samples located in Nacogdoches (Texas, USA)[16,17] and Hangzhou (Zhejiang, China).[18] In both cases, the isolated saponins were based on oleane type aglycone but showed minor (Texas, USA) and major (Zhejiang, China) differences with the chemical structure of saponins here reported. The difference in the chemical structure of *A. pavia* L. saponins of this and previous work could be related to different geographical distribution of the analyzed species (Italy, Texas and China).

The use of non destructive methods for the structural elucidation of paviosides A–H allowed to preserve the required amounts for biological screening.

Thus, all the eight saponins isolated from *A. pavia* (**1a, 1b–4a, 4b**) were tested for cytotoxic activity on two different cell lines (J-774, murine monocyte/macrophage, and WEHI-164, murine fibrosarcoma) in vitro. Results, expressed as IC_{50} (μg/mL, the concentration that inhibited the cell growth by 50%), against J-774 (A) and WEHI-164 (B) are: (**1a**) 5.7 (A) and 4.8 (B); (**1b**) 5.1 (A) and 4.9 (B); (**2a**) 5.7 (A) and 6.5 (B); (**2b**) 6.6 (A) and 7.1 (B); (**3a**) 2.9 (A) and 2.1 (B); (**3b**) 2.8 (A) and 2.3 (B); (**4a**) 3.6 (A) and 3.5 (B); (**4b**) 3.5 (A) and 3.2 (B) (Fig. 2).

All the isolated compounds showed significant activity against the tested cell lines and this data is in agreement with activity previously found for analogous compounds.[17,18] The most active compounds of the series are paviosides E–H (**3a, 3b** and **4a, 4b)** (values ranging from 2.1 to 3.6 μg/mL). Interestingly, these four compounds have a trisaccharide chain composed by two xyloses and a glucuronic acid while differ for the aglycone structure, barrigtogenol C in paviosides E–F (**3a**, **3b)** and protoaescigenin (24-hydroxyl barrigtogenol C) in paviosides G–H (**4a**, **4b)**. So, it seems that the structural change in the aglycone structure does not affect the activity. On the contrary, a difference in the nature of residues constituting the trisaccharide chain is a key feature for the activity. In fact, the substitution of a glucose unit ⁎⁎(as found in paviosides A–B (**1a, 1b**) with a xylose unit (as found in paviosides E−H (**3a**, **3b**, **4a**, and **4b**) increases the cytotoxic activity, while a little detrimental effect is observed when glucose is substituted by a galactose (as found for paviosides C–D (**2a, 2b**). Finally, the substitution at C-21 of a tigloyl group (**1a–4a**) with an angeloyl group (**1b–4b**) does not affect significantly the inhibitory activity on both tested cell lines.

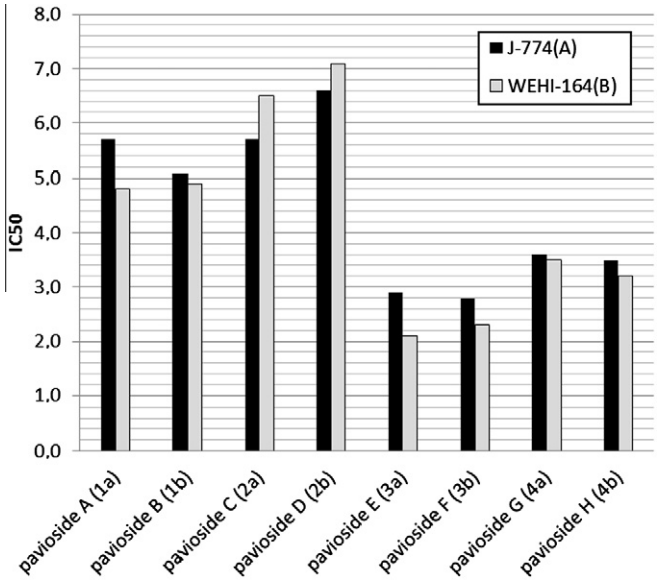


Figure 2. Cytotoxic activity of paviosides A–H (**1a**, **1b–4a**, **4b**) against two different cell lines: J-774, murine monocyte/macrophage (A), and WEHI-164, murine fibrosarcoma (B), in vitro. Results are expressed as IC_{50} (μg/mL, the concentration that inhibited the cell growth by 50%).

3. Experimental

3.1. General experimental procedures

Low and high resolution FAB mass spectra (glycerol matrix) were measured on a Prospect Fisons mass spectrometer. Optical rotations were determined on a Perkin Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and 10-cm microcell. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer. Chemical shifts were referred to the residual solvent signal (CD_3OD: δ_H 3.34, δ_C 49.0). The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. ^{1}H connectivities were determined by using COSY and HOHAHA experiments; the 2D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing. One-bond heteronuclear ^{1}H–^{13}C connectivities were determined with 2D HSQC pulse sequence with an interpulse delay set for $^1J_{CH}$ of 130 Hz. Two and three bond heteronuclear ^{1}H–^{13}C connectivities were determined with 2D HMBC experiments, optimized for $^{2\text{-}3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (NOE) measurements were performed by 2D ROESY experiments. Medium-pressure liquid chromatography (MPLC) in gradient mode was performed on a Büchi 861 apparatus on a glass column 50/460 cpl (460 × 50 mm) using Merck reversed-phase silica gel, LiChroprep RP-18 (40–63 μm), and eluting with the following linear gradient: H_2O 100% (300 ml), 9 mixtures from H_2O/MeOH (9:1) to H_2O/MeOH (1:9) (9 × 300 ml), MeOH 100% (500 ml). HPLC in isocratic mode was performed on a Varian apparatus equipped with an RI-3 refractive index detector using Waters semipreparative 300 × 7.5 mm, i.d. μ-Bondapack C_{18} column (injection amount = 180 μL, flow-rate = 2.5 mL/min) and analytical 300 × 3.9 mm, i.d. μ-Bondapack C_{18} column (injection amount = 100 μL, flow-rate = 0.8 mL/min).

3.2. Plant material, extraction and isolation

Fresh aerial parts (200 g, dry weight) of *Aesculus pavia* L. were air-dried under controlled temperature (22 °C) and without

exposure of light. They were chopped and then exhaustively extracted at room temperature with the following solvents in the order: hexane, $CHCl_3$, $CHCl_3$/MeOH (9:1), and MeOH. Each solvent extraction takes two days and was repeated three times using 2 L of solvent. The MeOH extract (15 g) was partitioned between BuOH and water and the organic layer was concentrated in vacuo to afford a crude extract (8 g), which was chromatographed by MPLC on RP-18 column using a linear gradient solvent system from H_2O to MeOH.

Fractions eluted with H_2O/MeOH 1:9 (300 mg) and MeOH (1.5 g), containing saponins, were combined and re-chromatographed on RP-18 column using a linear gradient system from H_2O/MeOH (6:4) to MeOH, obtaining 7 fractions. Fraction 6 (150 mg), eluted with H_2O/MeOH (1:9), was further purified on semipreparative C-18 HPLC column with a mobile phase of H_2O/MeOH (3:7), obtaining eight crude saponins. The isolated compounds have been purified by analytic C-18 HPLC column with a mobile phase of H_2O/MeOH (1:1), yielding eight pure new compounds: paviosides A (**1a**, 7.8 mg, t_R 18.4 min), B (**1b**, 6.6 mg, t_R 19.5 min), C (**2a**, 7.4 mg, t_R 20.2 min), D (**2b**, 6.5 mg, t_R 21.5 min), E (**3a**, 5.2 mg, t_R 22.6), F (**3b**, 4.5 mg, t_R 23.2), and G (**4a**, t_R 24.8 mg), H (**4b**, 3.9 mg, t_R 25.7 mg).

3.2.1. Pavioside A (1a)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-glucopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C: $[\alpha]_D^{25}$ −26.2 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1101.5822 $[M+H]^+$, calcd for $C_{55}H_{88}O_{22}$ *m/z* 1100.5815; FABMS (positive ion): *m/z* 1101 $[M+H]^+$, *m/z* 969 $[M+H-132]^+$, *m/z* 939 $[M+H-162]^+$, and *m/z* 807 $[M+H-(132+162)]^+$; ^{1}H NMR data: δ 0.79 (1H, m, H-5), δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), 0.95 (3H, s, Me-23), 0.96 (3H, s, Me-30), 1.05 (3H, s, Me-24), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.40 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.2.2. Pavioside B (1b)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-glucopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C: $[\alpha]_D^{25}$ −25.5 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1101.5825 $[M+H]^+$, calcd for $C_{55}H_{88}O_{22}$ *m/z* 1100.5815; FABMS (positive ion): *m/z* 1101 $[M+H]^+$, *m/z* 969 $[M+H-132]^+$, *m/z* 939 $[M+H-162]^+$, and *m/z* 807 $[M+H-(132+162)]^+$; ^{1}H NMR data: δ 0.79 (1H, m, H-5), δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), 0.95 (3H, s, Me-23), 0.96 (3H, s, Me-30), 1.05 (3H, s, Me-24), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.40 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.2.3. Pavioside C (2a)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-galactopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C: $[\alpha]_D^{25}$ −22.5 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1101.5821 $[M+H]^+$, calcd for $C_{55}H_{88}O_{22}$ *m/z* 1100.5815; FABMS (positive ion): *m/z* 1101 $[M+H]^+$, *m/z* 969 $[M+H-132]^+$, *m/z* 939 $[M+H-162]^+$, and *m/z* 807 $[M+H-(132+162)]^+$; ^{1}H NMR data: δ 0.79 (1H, m, H-5), δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), 0.95 (3H, s, Me-23), 0.96 (3H, s, Me-30), 1.05 (3H, s, Me-24), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.40 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.2.4. Pavioside D (2b)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-galactopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C: $[\alpha]_D^{25}$ −21.7 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1101.5824 $[M+H]^+$, calcd for $C_{55}H_{88}O_{22}$ *m/z* 1100.5815; FABMS (positive ion): *m/z* 1101 $[M+H]^+$, *m/z* 969 $[M+H-132]^+$, *m/z* 939 $[M+H-162]^+$, and *m/z* 807 $[M+H-(132+162)]^+$; ^{1}H NMR data: δ 0.79 (1H, m, H-5), δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), 0.95 (3H, s, Me-23), 0.96 (3H, s, Me-30), 1.05 (3H, s, Me-24), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.40 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.2.5. Pavioside E (3a)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl barringtogenol C: $[\alpha]_D^{25}$ −22.1 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1071.5735 $[M+H]^+$, calcd for $C_{54}H_{86}O_{21}$ *m/z* 1070.5728; FABMS (positive ion): *m/z* 1071 $[M+H]^+$, *m/z* 939 $[M+H-132]^+$, and *m/z* 807 $[M+H-(132+132)]^+$; ^{1}H NMR data: δ 0.79 (1H, m, H-5), δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), 0.95 (3H, s, Me-23), 0.96 (3H, s, Me-30), 1.05 (3H, s, Me-24), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.40 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.2.6. Pavioside F (3b)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-angeloyl-22-acetyl barringtogenol C: $[\alpha]_D^{25}$ −21.3 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1071.5737 $[M+H]^+$, calcd for $C_{54}H_{86}O_{21}$ *m/z* 1070.5728; FABMS (positive ion): *m/z* 1071 $[M+H]^+$, *m/z* 939 $[M+H-132]^+$, and *m/z* 807 $[M+H-(132+132)]^+$; ^{1}H NMR data: δ 0.79 (1H, m, H-5), δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), 0.95 (3H, s, Me-23), 0.96 (3H, s, Me-30), 1.05 (3H, s, Me-24), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.40 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.2.7. Pavioside G (4a)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-tigloyl-22-acetyl protoaescigenin: $[\alpha]_D^{25}$ −26.8 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1087.5635 $[M+H]^+$, calcd for $C_{54}H_{86}O_{22}$ *m/z* 1086.5635; FABMS (positive ion): *m/z* 1087 $[M+H]^+$, *m/z* 955 $[M+H-132]^+$, and *m/z* 823 $[M+H-(132+132)]^+$; ^{1}H NMR data: δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), δ 0.91 (1H, m, H-5), 0.96 (3H, s, Me-30), 0.98 (3H, s, Me-23), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.26 and 4.30 (each 1H, d, *J* = 12.0 Hz, H_2-24), 3.45 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.2.8. Pavioside H (4b)

3-*O*-[β-D-Xylopyranosyl (1→2)] [-β-D-xylopyranosyl (1→4)]-β-D-glucopyranosiduronic acid 21-ageloyl-22-acetyl protoaescigenin: $[\alpha]_D^{25}$ −25.9 (*c* 0.1 MeOH); HRFABMS (positive ion): found *m/z* 1087.5635 $[M+H]^+$, calcd for $C_{54}H_{86}O_{22}$ *m/z* 1086.5635; FABMS

(positive ion): *m/z* 1087 $[M+H]^+$, *m/z* 955 $[M+H-132]^+$, and *m/z* 823 $[M+H-(132+132)]^+$; ^1H NMR data: δ 0.80 (3H, s, Me-26), 0.84 (3H, s, Me-25), 0.89 (3H, s, Me-29), δ 0.91 (1H, m, H-5), 0.96 (3H, s, Me-30), 0.98 (3H, s, Me-23), 1.36 (3H, s, Me-27), 2.96 (1H, dd, *J* = 4.5, 13.6 Hz, H-18), 3.26 and 4.30 (each 1H, d, *J* = 12.0 Hz, H_2-24), 3.45 (1H, dd, *J* = 4.0, 11.5 Hz, H-3), 3.66 and 4.02 (each 1H, d, *J* = 12.0 Hz, H_2-28), 4.48 (1H, br d, *J* = 4.5, H-16), 4.50 (1H, d, *J* = 10.5, H-22), 4.69 (1H, d, *J* = 10.5, H-21), 5.31 (1H, br s, H-12), for sugars see Table 2; ^{13}C NMR data: Tables 1 and 2.

3.3. Determination of sugar stereostructures

A solution of each paviosides (1 mg) in 1 N HCl (0.25 ml) was heated at 80 °C for 4 h. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in 1-(trimethyl silyl) imidazole (Trisil-Z) and pyridine (0.1 mL) and the solution was stirred at 60 °C for 5 min. After drying the solution with a stream of N_2, the residue was separated by water and CH_2Cl_2 (1 mL, v:v = 1:1). The CH_2Cl_2 layer was analyzed by GC using a L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of injector and detector were 200 °C for both. A temperature gradient system was used for the oven; the initial temperature was maintained at 100 °C for 1 min and then raised to 180 °C at the rate of 5 °C/min. Peaks of the hydrolysate of **1a** were detected at 10.98 (D-xylose), 14.66 (D-glucose) and 21.07 (D-glucuronic acid) in the ratio of 1:1:1. The hydrolysate mixtures of **1b** gave the same peaks. Peaks of the hydrolysate of **2a** and **2b** were detected at 10.98 (D-xylose), 13.77 (D-galactose) and 21.07 (D-glucuronic acid) in the ratio of 1:1:1. Peaks of the idrolysate of **3a, 3b, 4a** and **4b** were detected at 10.98 (D-xylose) and 21.07 (D-glucuronic acid) in the ratio of 2:1. Retention times for authentic samples after being treated simultaneously with Trisil-Z were 10.98 (D-xylose), 11.05 (L-xylose), 13.77 (D-galactose) and 13.84 min (L-galactose), 14.66 (D-glucose) and 14.73 (L-glucose), 21.07 (D-glucuronic acid) and 21.10 (L-glucuronic acid). Co-injection of hydrolysates of **1a** and **1b** with standards D-Xyl, D-Glc and D-GlcA gave single peaks. Co-injection of hydrolysates of **2a** and **2b** with standards D-Xyl, D-Gal and D-GlcA gave single peaks. Co-injection of hydrolysates of **3a, 3b, 4a** and **4b** with standards D-Xyl and D-GlcA gave single peaks.

3.4. Cells and culture

WEHI 164 cells (murine fibrosarcoma cell line) were maintained in adhesion on Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/mL) and streptomycin (100 μg/mL). *J774* cells (murine monocyte/macrophage cell line) were grown in suspension culture, in Techne stirrer bottles, spun at 25 rpm and incubated at 37 °C in DMEM medium supplemented with 10% FBS, 25 mM Hepes, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/m).

3.5. Cytotoxicity assay

WEHI 164 and *J774* cells were plated on 96-well microliter plates and allowed to adhere at 37 °C in 5% CO_2/95% air for 2 h. Then, the medium was replaced with 50 μL of fresh medium and 75 μL aliquot of 1.2 v/v serial dilution of each pavioside was added and then the cells incubated for 72 h. The cells viability was assessed through an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] conversion assay. Briefly, 25 μL of MTT (5 mg/mL) was added and the cells were incubated for additional 3 h. Following this time the cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v:v) *N*,*N*-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The viability of each cell line in response to treatment with paviosides was calculated as: % dead cells = 100 − (OD treated/OD control) × 100.

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References and notes

1. Bisset, N. G. *Herbal Drugs and Phytopharmaceuticals*; Medpharm Scientific Publishers, CRC Press: Stuttgart, Germany, 1994. pp 268–272.
2. Sirtori, C. R. *Pharmacol. Res.* **2001**, *44*, 183.
3. Zhang, Z.; Li, S.; Lian, X.-Y. *Pharm. Crops* **2010**, *1*, 24.
4. Curir, P.; Dolci, M.; Lanzotti, V.; Taglialatela-Scafati, O. *Phytochemistry* **2001**, *56*, 717.
5. Curir, P.; Dolci, M.; Dolci, P.; Lanzotti, V.; De Cooman, L. *Phytochem. Anal.* **2003**, *14*, 8.
6. Curir, P.; Dolci, M.; Corea, G.; Galeotti, F.; Lanzotti, V. *Plant Biosyst.* **2006**, *140*, 156.
7. Galeotti, F.; Barile, E.; Curir, P.; Dolci, M.; Lanzotti, V. *Phytochem. Lett.* **2008**, *1*, 44.
8. Curir, P.; Galeotti, F.; Dolci, M.; Barile, E.; Lanzotti, V. *J. Nat. Prod.* **2007**, *70*, 1668.
9. Johne, B.; Schütz, S. In *Molecular Biology of Fungi*; Kues, U., Ed.; Wissenschaftlicher Fachverlag: Langgons, 2003; p 83.
10. Mahato, S. B.; Kundu, A. P. *Phytochemistry* **1994**, *37*, 1517.
11. Yoshikawa, M.; Murakami, T.; Matsuda, H.; Yamahara, J.; Murakami, N.; Kitagawa, I. *Chem. Pharm. Bull.* **1996**, *44*, 1454.
12. Duddeck, H.; Elgamol, M. H. A.; Ricca, G. S.; Danieli, B.; Palmisano, G. *Org. Magn. Reson.* **1978**, *11*, 130.
13. Corea, G.; Lanzotti, V.; Cammareri, C.; Conicella, C.; Laezza, C.; Bifulco, M. *Bioorg. Med. Chem.* **2004**, *12*, 4909.
14. Breitmaier, E.; Voelter, W. *Carbon-13 NMR spectroscopy*; VCH: Weinheim, Germany, 1987. p 379.
15. Pretsch, E.; Bühlmann, P.; Affolter, C. *Structure Determination of Organic Compounds: Tables of Spectral Data*; Springer Verlag: Berlin, 2000. pp 203–237.
16. Zhang, Z. Z.; Li, S. Y.; Zhang, S. M.; Gorenstein, D. *Phytochemistry* **2006**, *67*, 784.
17. Zhang, Z. Z.; Li, S. Y. *Phytochemistry* **2007**, *68*, 2075.
18. Sun, Z.-L.; Zhang, M.; Wu, Y.; Wan, A.-H.; Zhang, R. *Fitoterapia* **2011**, *82*, 106.