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Anticancer diarylheptanoid glycosides from the inner bark of *Betula papyrifera*

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Abstract

Phytochemical investigations of the MeOH extract of *Betula papyrifera* inner bark led to the isolation of ten phenolic compounds of the following types: diarylheptanoid glycosides (**1**–**4**), a diarylheptanoid (**5**), a lignan (**6**), flavonoids (**7**–**8**) and chavicol glycosides (**9**–**10**). Among them, the diarylheptanoid glycoside, (*S*)-1,7-bis-(4-hydroxyphenyl)-heptan-3-one-5-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside, papyriferoside A (**1**), was isolated and its structure was determined on the basis of 1D and 2D NMR, HPLC-MS, as well as high resolution mass spectroscopic data. Platyphylloside (**4**) exerted the strongest cytotoxic activity of all isolated compounds with IC_{50} values ranging from 10.3 to 13.8 μM.

Keywords: *Betula papyrifera*; Betulaceae; Birch; Bark; Diarylheptanoid; Chavicol; Glycoside; NMR; Cytotoxic; Anticancer

1. Introduction

White birch, commonly named "paper birch", is widely distributed in the boreal forest of North America (Marie-Victorin, 2002). The important industrial exploitation of birch yields a high amount of waste bark, which prompted us to investigate this residue for possible natural bioactive compounds. Pentacyclic triterpenoids, mostly of the lupane and oleanane types, were isolated from the outer bark of various white birch species including *Betula papyrifera* Marsh. (Betulaceae) (Ekman, 1983; O'Connell et al., 1988; Cole et al., 1991; Hua et al., 1991; Fuchino et al., 1996a,b, 1998; Habiyaremye et al., 2002; Cichewicz and Kouzi, 2004; Gauthier et al., 2006). For example, betulinic acid, a promising anticancer agent (Cichewicz and Kouzi, 2004), is found in appreciable quantities (>1%) in the bark of *B. papyrifera* (O'Connell et al., 1988). Previous reports on the phytochemical investigations of the inner bark of different *Betula* species described the isolation of arylbutanoids, diarylheptanoids, lignans and phenolic glycosides (Smite et al., 1993, 1995; Pan and Lundgren, 1994; Frank et al., 1996, 1997). High antioxidant, cytotoxic, antitumor and antiviral activities for several of these natural compounds were reported (Baglin et al., 2003a,b; Setzer and Setzer, 2003; Cichewicz and Kouzi, 2004; Ju et al., 2004). We present in this work the isolation and identification of ten phenolic compounds isolated for the first time from *Betula papyrifera*. To the best of our knowledge, (*S*)-1,7-bis-(4-hydroxyphenyl)-heptan-3-one-5-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside, papyriferoside A (**1**), is a new diarylheptanoid glycoside not reported until now. 5-*O*-β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranosyl-1,7-bis-(4-hydroxyphenyl)-heptan-3-one (**2**) (Smite et al., 1993), aceroside VII (**3**) (Fuchino et al., 1996a), platyphylloside (**4**) (Smite et al., 1993), 1,7-bis-(4-hydroxyphenyl)-4-hepten-3-one (**5**) (Fuchino et al., 1996), (−)-lyoniresinol 3-*O*-β-D-xylopyranoside (**6**) (Smite et al., 1995), (+)-catechin (**7**) (Hua et al., 1991) and (+)-catechin-7-*O*-β-D-xylopyranoside (**8**) (Fuchino et al., 1998) were previ-

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ously isolated from different species of *Betula*. Chavicol 4-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside (**9**) (Higuchi et al., 1977) and chavicol 4-*O*-β-D-apiofuranoside-(1 → 6)-β-D-glucopyranoside (**10**) (Hase and Iwagawa, 1982) were previously isolated from *Pinus contorta* and *Viburnum furcatum*, respectively. The *in vitro* cytotoxicities of all isolated compounds were assessed against lung carcinoma (A-549) and colorectal adenocarcinoma (DLD-1) human cell lines, as well as against human normal skin fibroblasts (WS1).

2. Results and discussion

In the course of our search for biologically active species in the northern boreal forest of Quebec, the methanolic extract of the inner bark of *B. papyrifera* was investigated *in vitro* and found to exert a significant cytotoxic activity against A-549 (IC_{50}, 104 ± 3 μg/mL) and DLD-1 (IC_{50}, 79 ± 2 μg/mL) cell lines. After bioassay-guided fractionation of the crude extract on Diaion®, Silica gel and Polyamide columns, ten pure compounds were isolated. Structure determination of new compound **1** was accomplished on the base of 1D and 2D NMR experiments, HPLC-MS and HR-ESI-MS. Known compounds **2**–**10** were identified by comparison of their spectroscopic data with values found in the literature. Complete ^1H and ^{13}C NMR spectroscopic data for chavicol glycosides **9** and **10** are presented in Table 2.

The molecular formula ($C_{30}H_{40}O_{13}$) of Papyriferoside A (**1**), a white amorphous powder, was determined from its HR-ESI-MS spectrum (positive ion mode) on the basis of a quasimolecular ion peak at *m/z* 631.2366 $[M+Na]^+$ (calcd 631.2367). Based on ^1H, ^{13}C, DEPT and HSQC spectra, seven methylene, 18 methine and five quaternary carbon atoms were deduced (Table 1). Also, evidence of a carbonyl (δ_C 211.9) and two sugar moieties (δ_H/δ_C 4.28/103.5 and 4.97/109.7) were observed from these spectra. The presence of the sugar moieties was supported by negative ion APCI-MS analysis with the loss of fragment *m/z* 295 corresponding to hexose-pentose. Acid hydrolysis of **1** gave, after purification, D-glucose and L-arabinose and its aglycone platyphyllonol (**1a**). Identity of **1a** was confirmed by NMR (Terazawa et al., 1984) and $[\alpha]_D$ (Chen et al., 2000) spectroscopic data in comparison with those previously reported. Absolute configuration of the glucose as D and the arabinose as L was determined by optical rotations in comparison with those of authentic standards. Complete assignment of chemical shifts of aliphatic, aromatic and sugar systems were achieved by considering ^1H–^1H COSY, TOCSY and HMBC spectra. The HMBC correlations between δ_H 4.28 (Glc-1) and δ_C 76.4 (Agly-5), between δ_H 4.97 (Ara-1) and δ_C 67.9 (Glc-6) and between δ_H 4.97 (Ara-1) and δ_C 85.8 (Ara-4) showed that the diglycosidic chain α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranosyl was linked to aglycone at C-5. Finally, the absolute configuration at the C-5 position of **1a** has been previously established as *S* (Ohta et al., 1985). Thus, the structure of papyriferoside A (**1**) was confirmed as (*S*)-1,7-bis-(4-hydroxyphenyl)-heptan-3-one-5-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside.

Table 1
NMR spectroscopic data (400 MHz, CD_3OD) for papyriferoside A (**1**)

Position	δ_C, mult.	δ_H (*J* in Hz)
1	29.8, CH_2	2.72, *s*
2	46.4, CH_2	2.72, *s*
3	211.9, qC	
4	48.7, CH_2	2.79, *dd* (16.4, 6.4)
		2.56, *m*
5	76.4, CH	4.12, *m*
6	38.6, CH_2	1.75, *m*
7	31.5, CH_2	2.57, *m*
1	133.2, qC	
2, 6	130.3, CH	6.97, *d* (7.1)
3, 5	116.2, CH	6.67, *d* (7.1)
4	156.5, qC	
1	134.3, qC	
2, 6	130.5, CH	7.0, *d* (7.1)
3, 5	116.1, CH	6.69, *d* (7.1)
4	156.2, qC	
Glc*p*		
1	103.5, CH	4.28, *d* (7.6)
2	75.1, CH	3.14, *t* (8.2)
3	77.9, CH	3.34, *m*
4	71.8, CH	3.34, *m*
5	76.4, CH	3.39, *m*
6	67.9, CH_2	4.03, *m*
		3.62, *dd* (11.1, 5.3)
Ara*f*		
1	109.7, CH	4.97, brs
2	83.0, CH	4.02, *m*
3	78.7, CH	3.85, *dd* (5.8, 2.9)
4	85.8, CH	3.96, *m*
5	62.8, CH_2	3.69, *dd* (12.0, 2.9)
		3.59, *dd* (12.0, 5.3)

In vitro cytotoxic activities of the isolated compounds were assessed against human lung cancer (A-549), human colorectal cancer (DLD-1) and normal skin fibroblasts (WS1) using the resazurin reduction test as previously described in the literature (O'Brien et al., 2000). Fluorescence measurements were carried out after 48 continuous hours of contact between compounds and cells. Results presented in Table 3 are expressed as the concentration of product inhibiting cell growth by 50% (IC_{50}). Etoposide and 5-fluorouracil were used as positive controls. Among the isolated compounds, diarylheptanoid glycoside **4** exerted the most potent cytotoxic activity (IC_{50}, 10.3–13.8 μM), showing stronger activity than 5-fluorouracil towards the DLD-1 cell line. In addition, diarylheptanoid **5** (IC_{50}, 20–21 μM) and diarylheptanoid glycosides **1** (IC_{50}, 20.6–30 μM) and **2** (IC_{50}, 17–52 μM) displayed moderate cytotoxicities while **3** was inactive ($IC_{50} > 100$ μM) against all tested cell lines. In terms of structure-activity relationships, these *in vitro* results suggest that the ketone function at the C-3 position is important for the cytotoxic activity of the isolated diarylheptanoids. Moreover, the

β-D-glucopyranose moiety at the C-5 position of diarylheptanoid **4** seems to have beneficial effect on the activity in comparison with the disaccharide moieties of diarylheptanoids **1** and **2**. It is important to note that cytotoxic diarylheptanoids **1**, **2**, **4** and **5** are not selective towards cancer cell lines as they also inhibit the growth of the healthy cells. On the other hand, IC_{50} values of compounds **6**–**10** indicate that they do not exhibit any significant *in vitro* cytotoxicity against tested cancer cell lines.

3. Concluding remarks

Although the inner bark of *Betula* species have been reported to contain diarylheptanoid glycosides, lignans and phenolic glycosides (Smite et al., 1993, 1995; Pan and Lundgren, 1994; Frank et al., 1996, 1997), this is the first report of these compounds (**1**–**10**) from the white birch *Betula papyrifera*. To the best of our knowledge, papyriferoside A (**1**) is a new diarylheptanoid glycoside not reported until now which exhibited a potent cytotoxicity against lung carcinoma (A-549) and colon adenocarcinoma (DLD-1) human cell lines. Our study indicates that platyphylloside (**4**) exerted the strongest *in vitro* anticancer activity among the isolated compounds. Investigations are now in progress in our laboratory to evaluate the bioactive potential of others *Betula* species from the boreal forest of North America.

4. Experimental

4.1. General

Optical rotations were obtained on a Jasco DIP-360 digital polarimeter. Absorption UV spectra were recorded with an Agilent 8453 diode-array spectrophotometer. FTIR spectra were conducted on a Perkin–Elmer SpectrumOne. The 1D and 2D NMR spectra (^1H–^1H COSY, TOCSY, HSQC and HMBC) were performed using an Avance 400 Bruker spectrometer (400.13 MHz for ^1H, 100.61 MHz for ^{13}C spectra) equipped with a 5 mm QNP-probe. All spectra were acquired in CD_3OD and chemical shifts are reported in ppm (δ) relative to residual solvent peaks (δ_H 3.31 and δ_C 49.0). High resolution electrospray ionization mass spectrum was conducted in positive mode on an Applied Biosystems/MDS Sciex QSTAR XL QqTOF MS system. HPLC-APCI MS (negative mode) were obtained from an Agilent 1100 series system consisting of a degasser, a quatpump, an automatic injector, a temperature-controlled column compartment, a diode array detector and a mass selective detector Agilent G1946 VL model equipped with an APCI source. Analytical separations were performed on a 4.6 × 150 mm Zorbax Eclipse C18 reversed-phase column using H_2O:CH_3CN system, with pH water adjusted to 4 with formic acid (HPLC grade) to improve peak sharpness. Chromatographic conditions were the following: isocratic elution with H_2O pH 4:CH_3CN (4:1) at flow rate 0.8 mL/min and column oven 25 °C. Preparative HPLC separation (Agilent 1100) was carried out on a 9.4 × 250 mm Zorbax Eclipse C18 column using multiple wavelength detector and an automatic fraction collector. The lyophilizator was acquired from Labconco (USA). The solvents were purchased from VWR (Canada). The TLC plates (aluminium sheets of silica gel ultra pure, with indicator F_{254}) and silica gel ultra pure (40–63 μm with indicator F_{254}) were supplied by Silicycle (Canada). Polyamide CC-6 was purchased from Macherey-Nagel (Germany) and Diaion HP-20 from Supelco. Solvent systems for TLC analyses of phenolic constituents: (a) $CHCl_3$–CH_3OH–H_2O (50:15:1), (b) EtOAc–MeOH–H_2O (100:16.5:13.5), developing with H_2SO_4 20% in MeOH followed by heating at 100 °C. TLC identification of monosaccharides were carried out in CH_2Cl_2–CH_3OH–H_2O (50:25:5) solvent system, with further developing using an orthophosphoric acid solution of naphtorezorsinol 5% in EtOH, followed by heating at 110 °C. The yields were calculated from the weight of the dry plant material.

4.2. Plant material

Bark of *B. papyrifera* Marsh. was collected in October 2003 at Ferland, Quebéc, Canada. The plant was identified by Mr. Patrick Nadeau (Universtité du Québec à Chicoutimi) and a voucher specimen (No. 492114) was deposited at the Louis-Marie Herbarium of Laval University, Quebec City, Canada.

4.3. Extraction and isolation

Powdered inner bark (500 g) of *B. papyrifera* was exhaustively extracted with MeOH (3 L, 60 °C, 5 times, 1.5 h each time) followed by MeOH–H_2O 80:20 with heating. The extracts were filtered and pooled. After evaporation of MeOH in vacuo, to the aqueous phase was added EtOAc (1, 25 L) at the whole extracted. The organic phase was decanted, evaporated in vacuo and the residue lyophilized. The partially purified extract (72 g, 14%) was obtained. Further purification of this extract was carried out on an open Diaion® column by eluting with H_2O–MeOH with increasing percentages of MeOH (0–100%). The fractions collected from MeOH 40% to MeOH 100% were pooled.

The purified extract (26 g) was separated using silica gel CC by eluting with $CHCl_3$–CH_3OH–H_2O (50:15:1) and five fractions were obtained. Purification was then carried out on the fractions that showed greatest *in vitro* cytotoxic activities against the cancer cell lines: fractions **III** (8.0 g, 1.6%), **IV** (7.0 g, 1.4%) and **V** (6.0 g, 1.2%) (data not shown).

Fraction **III** was subjected to silica gel CC by eluting with EtOAc–MeOH–H_2O (100:16.5:13.5). Repeat chromatography of the obtained prepurified fraction was performed on a polyamide column with MeOH–H_2O

(0–70% of MeOH) and yielded products **6** (25 mg, 0.0050%), **4** (1.5 g, 0.30%) and **7** (80 mg, 0.016%).

Separation of fraction **IV** was performed on a Polyamid flash column using MeOH–H_2O (100% H_2O to 100% MeOH) as the eluant. After repeated chromatography, compounds **1** (45 mg, 0.0090%), **2** (6.9 mg, 0.0014%), **3** (100 mg, 0.020%), **4** (7.0 mg, 0.0014%) and **5** (10 mg, 0.0020%) were obtained. Compounds **9** (28 mg, 0.0056%) and **10** (46 mg, 0.0092%) were isolated by preparative HPLC (MeOH–H_2O 10% to 100% MeOH).

Fraction **V** was separated by silica gel CC using $CHCl_3$–CH_3OH–H_2O (26:14:3) as the eluant. The isolated major compound, with traces of two polar substances, was repurified on Polyamid flash column using MeOH–H_2O (60:40) to afford pure **8** (600 mg, 0.120%).

The purity and molecular weight of all isolated compounds were determined by an HPLC-MS system.

4.4. Papyriferoside A (*1*)

White amorphous powder; $[\alpha]_D^{25}$ − 77.9° (MeOH, *c* 0.1); UV λ_{max}^{MeOH} nm (log ε) 224 (4.2), 279 (3.6); IR ν_{max}^{neat} cm^{-1} 3344, 2925, 1703, 1613, 1515, 1447, 1365, 1229, 1066, 827; for ^1H, ^{13}C and HMBC NMR spectroscopic data, see Table 1 and Fig. 1; HR-ESI-MS *m/z* 631.2149 $[M+Na]^+$ (calcd for $C_{30}H_{40}O_{13}Na$, 631.2366) (see Chart 1).

4.5. Chavicol 4-O-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside (*9*)

White amorphous powder; $[\alpha]_D^{25}$ − 73.0° (*c* 0.75, MeOH); UV (ACN) λ_{max} 195 (4.6), 221 (4.0) nm; IR (neat) ν_{max} 3361, 2924, 1508, 1230, 1068, 667 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; HR-ESI-MS *m/z* 451.1594 $[M+Na]^+$ (calcd for $C_{20}H_{28}O_{10}Na$, 451.1580) (see Table 3).

4.6. Chavicol 4-O-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl (*10*)

White amorphous powder; $[\alpha]_D^{25}$ − 56.6° (*c* 1.4, MeOH); UV (ACN) λ_{max} 195 (4.4), 222 (4.1) nm; IR (neat) ν_{max} 3361, 2924, 1508, 1230, 1068, 667 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; HR-ESI-MS *m/z* 451.1573 $[M+Na]^+$ (calcd for $C_{20}H_{28}O_{10}Na$, 451.1580).

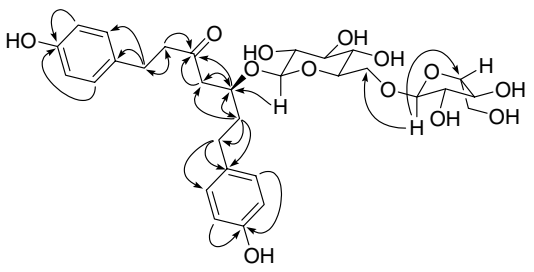
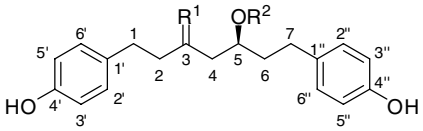


Fig. 1. Selected HMBC Correlations for papyriferoside A (**1**).



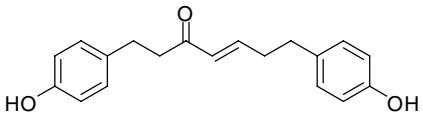
1 R^1 = O; R^2 = α-L-Ara*f*-[1→6]-β-D-Glc*p*

1a R^1 = O; R^2 = H_2

2 R^1 = O; R^2 = β-D-Api-[1→2]-β-D-Glc*p*

3 R^1 = H_2; R^2 = β-D-Glc*p*

4 R^1 = O; R^2 = β-D-Glc*p*



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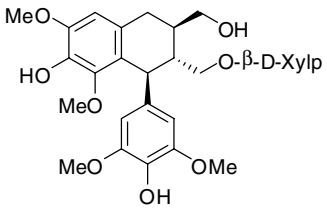

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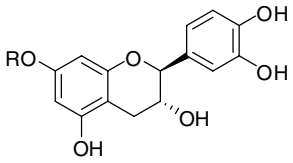
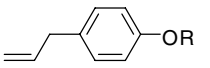


7 R = H

8 R = β-D-Glc*p*



9 α-L-Ara*f*-[1→6]-β-D-Glc*p*

10 β-D-Api-[1→6]-β-D-Glc*p*

Chart 1.

4.7. Acid hydrolysis of papyriferoside A (*1*)

Compound **1** (35 mg) in 0.5 N HCl (10 mL) was heated at 100 °C for 4 h, with the resulting hydrolysate extracted with EtOAc (3 × 5 mL), dried ($MgSO_4$) with the solvent evaporated under reduced pressure. The residue containing the aglycone was purified on silica gel open column using $CHCl_3$–CH_3OH (20:1) to afford pure **1a** (10 mg). Identity of **1a** was confirmed by NMR (Terazawa et al., 1984) and specific optical rotation (Chen et al., 2000) data in comparison with those previously reported: $[\alpha]_D^{25}$ − 3.0° (*c* 1.0, MeOH), lit. $[\alpha]_D^{25}$ − 3.3° (*c* 0.4, MeOH). The aqueous phase was neutralized with *N*,*N*-dioctylmethylamine (10% in $CHCl_3$), dried over $MgSO_4$ and the solvents were evaporated under reduced pressure. The residue containing the monosaccharide residues was purified by silica gel CC using CH_2Cl_2–CH_3OH–H_2O (10:5:1) to afford pure D-glucose (3 mg) and L-arabinose (2 mg). Absolute configuration

Table 2
NMR spectroscopic data (400 MHz, CD_3OD) for chavicol glycosides **9** and **10**

Position	**9**		**10**	
	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
1	157.5, qC		157.5, qC	
2, 6	117.8, CH	7.02, *d* (8.6)	117.8, CH	7.02, *d* (8.6)
3, 5	130.5, CH	7.11, *d* (8.6)	130.5, CH	7.11, *d* (8.6)
4	135.3, qC		135.3, qC	
7	40.4, CH_2	3.32, *m*	40.3, CH_2	3.34, *m*
8	139.2, CH	5.94, *ddt* (16.8, 10.1, 6.6)	139.2, CH	5.94, *ddt* (16.8, 10.1, 6.7)
9	115.7, CH_2	5.02, *m*	115.7, CH_2	5.02, *m*
		Glc*p*		Glc*p*
1	102.5, CH	4.85, *d* (7.7)	102.5, CH	4.84, *d* (7.6)
2	74.9, CH	3.44, *m*	74.9, CH	3.44, *m*
3	77.9, CH	3.45, *m*	77.9, CH	3.46, *m*
4	71.5, CH	3.37, *m*	71.5, CH	3.36, *m*
5	76.8, CH	3.61, *m*	76.9, CH	3.57, *m*
6	68.1, CH_2	4.06, 3.62, *m*	68.7, CH_2	4.02, 3.61, *m*
		Ara*f*		Api*f*
1	110.0, CH	4.93, *d* (1.5)	111.0, CH	4.99, *d* (2.5)
2	83.3, CH	4.01, *dd* (3.4, 1.5)	78.0, CH	3.92, *d* (2.5)
3	78.8, CH	3.83, *dd* (6.3, 3.4)	80.5, qC	
4	85.8, CH	3.97, *m*	74.9, CH_2	3.76, *d* (9.7), 3.97, *d* (9.7)
5	63.0, CH_2	3.72, *dd* (11.9, 3.4), 3.62, *m*	65.5, CH_2	3.59, *m*

Table 3
In vitro cytotoxicity results for isolated compounds **1**–**10**

Compounds	IC_{50} (μM ± SD)[a]		
	A-549[b]	DLD-1[c]	WS1[d]
1	30 ± 2	20.8 ± 0.8	20.6 ± 0.7
2	52 ± 4	30 ± 1	17 ± 2
3	>100	>100	>100
4	13.8 ± 0.5	12.6 ± 0.3	10.3 ± 0.3
5	22 ± 1	21.2 ± 0.9	20 ± 1
6	>100	>100	>100
7	>100	>100	>100
8	>100	>100	>100
9	>100	>100	>100
10	>100	>100	>100
Etoposide	1.0 ± 0.3	4 ± 1	n.d
5-Fluorouracil	24 ± 10	23 ± 6	n.d.

n.d.: not determined.
[a] Data represent mean values (± standard deviation) for three independent assays.
[b] Human lung carcinoma.
[c] Human colorectal adenocarcinoma.
[d] Human normal skin fibroblasts.

of the sugars was determined by optical rotations in comparison with those of authentic standards.

4.8. Cell lines and culture conditions

Lung carcinoma (A-549), colorectal adenocarcinoma (DLD-1) and normal skin fibroblast (WS1) human cell lines were purchased from the American Type Culture Collection (ATCC). All cell lines were cultured in minimum essential medium containing Earle's salts and L-glutamine (Mediatech Cellgro, VA), to which were added 10% fetal bovine serum (Hyclone), vitamins (1X), penicillin (100 I.U./ml) and streptomycin (100 μg/ml), essential amino acids (1X) and sodium pyruvate (1X) (Mediatech Cellgro, VA). Cells were kept at 37 °C in a humidified environment containing 5% CO_2.

4.9. Cytotoxic activity bioassay

Exponentially growing cells were plated in 96-well microplates (Costar, Corning Inc.) at a density of 5×10^3 cells per well in 100 μL of culture medium and were allowed to adhere for 16 h before treatment. Increasing concentrations of each compound in MeOH (Sigma–Aldrich) were then added (100 μL per well) and the cells were incubated for 48 h. The final concentration of MeOH in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cytotoxicity was assessed using resazurin (O'Brien et al., 2000) on an automated 96-well Fluoroskan Ascent F1™ plate reader (Labsystems) using excitation and emission wavelengths of 530 nm and 590 nm, respectively. Fluorescence was proportional to the cellular metabolic activity in each well. Survival percentages were defined as the fluorescence in experimental wells compared to that in control wells after subtraction of blank values. Cytotoxicity results were expressed as means ± standard deviation and represent the concentration inhibiting 50% of cell growth (IC_{50}). Each experiment was carried out three times in triplicate.

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