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Antiproliferative and antioxidant activities of a tricin acylated glycoside from sugarcane (*Saccharum officinarum*) juice

Joaquim Mauricio Duarte-Almeida [a,*], Giuseppina Negri [b], Antonio Salatino [c], João Ernesto de Carvalho [d], Franco Maria Lajolo [a]

[a] *Laboratório de Química, Bioquímica e Biologia Molecular de Alimentos, Departamento de Alimentos e Nutrição Experimental, FCF, Universidade de São Paulo, CP 66083, CEP 05314-970, São Paulo, SP, Brazil*
[b] *Departamento de Psicobiologia, CEBRID – Universidade Federal de São Paulo, São Paulo, SP, Brazil*
[c] *Laboratório de Fitoquímica, Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, CP 11461, CEP 05422-970, São Paulo, SP, Brazil*
[d] *Divisão de Farmacologia e Toxicologia – CPQBA – Universidade Estadual de Campinas, CP 6171, CEP 13081-970, Campinas, SP, Brazil*

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Abstract

From sugarcane juice, a flavone, identified by spectroscopic methods as tricin-7-*O*-β-(6″-methoxycinnamic)-glucoside, was isolated, in addition to orientin. The tricin derivative was shown to have antioxidant activity higher than Trolox® by means of the DPPH assay and lower by the β-carotene/linoleic acid system. It showed *in vitro* antiproliferative activity against several human cancer cell lines, with higher selectivity toward cells of the breast resistant NIC/ADR line.

Keywords: *Saccharum officinarum*; Poaceae; Sugarcane; Flavonoids; Tricin; Orientin; Antioxidant; Antiproliferative

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is currently grown in many tropical countries. Brazil accounts for a large part of the world production, with current national figures exceeding 375 million ton annually, most of it intended for sugar and alcohol production (Agrianual, 2006). Sugar from sugarcane enjoys worldwide recognition due to its high quality and low price. Ethyl alcohol is an ecologically prized substitute of petroleum fuel derivatives, chiefly because it is a renewable and cleaner energy source, in addition to saving fossil fuel and giving no contribution to the greenhouse effect (Amann, 1992). In addition, sugarcane has many other uses: cattle forage and raw material for production of ''rapadura'' (a hard, dark green food consumed mainly in Brazilian rural areas), syrup and ''cachaça'' (traditional Brazilian distilled alcoholic drink). Products derived from processing of sugarcane are traditionally consumed by people of several tropical countries of America and Asia, such as Brazil and Malaysia, respectively (Yusof et al., 2000).

Phenolic compounds in sugarcane juice are partially responsible for its color. The major flavonoids in sugarcane are flavones, such as naringenin, tricin, apigenin and luteolin derivatives (Williams et al., 1974; Smith and Paton, 1985). Flavonoids are currently recognized as exerting health-beneficial effects, e.g. protecting cells from degenerative processes and reducing the development of health problems such as cancer and cardiovascular diseases (Hudson et al., 2000; Hollman, 2001). Tricin (3′,5′-dimetoxyapigenin), a flavone frequently found in monocotyledons, inhibits the growth of human malignant breast tumor cells at submicromolar concentrations (Cai et al., 2004; Cai et al., 2005). Tricin has recently been found to interfere with murine gastrointestinal carcinogenesis and may be

* Corresponding author. Tel.: +55 11 30913656; fax: +55 11 38154410.
E-mail address: maudal@usp.br (J.M. Duarte-Almeida).

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considered safe enough for clinical development as a cancer chemopreventive agent (Verschoyle et al., 2006). A bioassay-guided analysis of a Malagasy plant, *Agelaea pentagyna*, led to the isolation of tricin, which was shown to have inhibitory activity toward exocytosis from antigen-stimulated rat leukemia basophils (Kuwabara et al., 2003). The present study reports the isolation and structural determination of a new acylated tricin glucoside – from sugarcane juice, its antioxidant and antiproliferative activities.

2. Results and discussion

2.1. Characterization of the compounds

Compound **1** was isolated from sugarcane juice as a yellow powder. The IR spectrum showed peaks at 3407.48, 1643.16, 1612.38, 1593.15 and 1508.50 cm^{-1}, which indicated the presence of hydroxyl groups and carbonyls conjugated with double bonds and aromatic rings, respectively. The UV spectrum, with bands at λ_{max}^{MeOH} nm 272 (band II) and 345 (band I) in MeOH is consistent with a flavone (Mabry et al., 1970; Agrawal et al., 1989). The bathochromic shift of 48 without decreasing the intensity of band I by the addition of KOH solution indicated a free hydroxyl group at position C-4′. The glycoside and aglycone spots on paper chromatograms are purple, which necessarily implies a 5-OH group (Mabry et al., 1970). The CID (collision induced dissociation) experiments using the product ion scan mode (MS^2) allowed characterization and establishment of fragmentation pathways of tricin, with an intense (60%) tricin peak at *m/z* 329 in the negative ion mode, plus peaks corresponding to the loss of one (*m/z* 314, 30%) and two (*m/z* 299, 50%) methyl groups (Fig. 1). The positive ion mode also gave an intense tricin peak at *m/z* 331. The MS spectrum of **1** showed a deprotonated ion peak at *m/z* 651, and a product ion at *m/z* 491 indicating the presence of a hexose moiety in the molecule. The mass spectrum of the trimethylsilyl derivative of the sugar obtained upon hydrolysis of **1** showed a molecular ion at 468 (M^+) and fragments at *m/z* 437, corresponding to the loss of (CH_2O) and base peak at *m/z* 361 due to loss of $Si(CH_3)_3$.

The presence of a hexose moiety as indicated by MS analysis is also deduced from the anomeric signal in the ^1H NMR spectrum at δ 5.27 (d, $J = 7.4$ Hz, H-1″) and ^{13}C NMR resonance at δ 98.2, corresponding to the *O*-linked hexose unit. From analysis of chemical shifts of the ^1H and ^{13}C NMR spectra, and the coupling constants of the ^1H signals, the hexose was determined as beta-glucopyranose. Bathochromic shifts and MS data suggested that **1** contains a tricin moiety and had only one anomeric proton at δ 5.27, indicating the presence of a monosaccharide moiety. The B-ring signals consist of a two-proton singlet corresponding to a pair of degenerate protons (H-2′, H-6′), consistent with oxygenation at C-3′, C-4′ and C-5′ (Table 1). Signals of methoxyl groups of tricin and methoxycinnamoyl are observed in both ^1H and ^{13}C NMR spectra. Chemical shifts of both spectra, relative to the sugar moiety, are in agreement with the corresponding values of glucose. Attachment of glucose at the C-7 position of tricin is proposed based on the absence of a bathochromic shift in band II of the UV spectrum after addition of NaOAc, which is consistent with a substituted C-7 hydroxyl (Mabry et al., 1970; Colombo et al., 2005), the substituent corresponding to the acylated glucosyl group. Glucosylation at the oxygen bound to C-7 is consistent also with signals of C-3′ and C-5′ (δ 149.0), C-4′ (δ 140.9) and C-7 (δ 163.3) in the ^{13}C NMR spectrum. According to

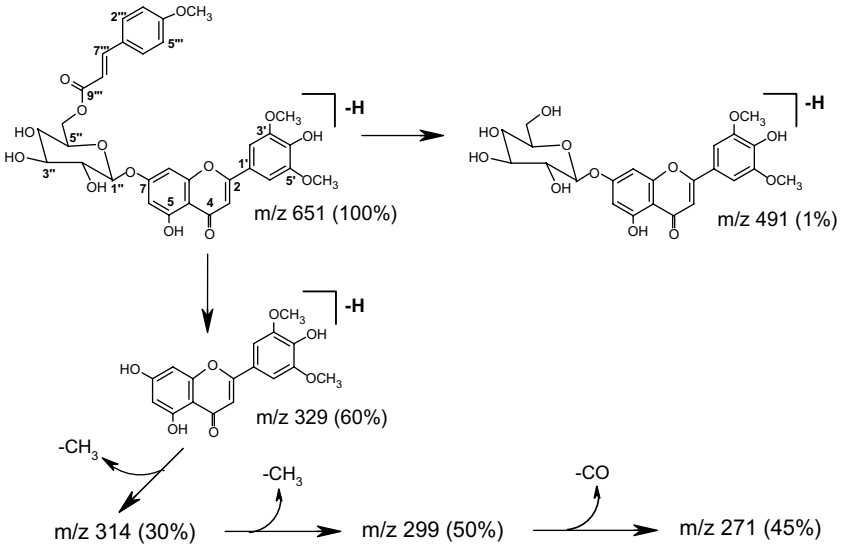

Fig. 1. Fragmentation in the MS/MS analysis, in negative mode and collision induced dissociation, proposed for tricin-7-*O*-β-(6″-*p*-methoxycinnamate)-glucoside (**1**).

Table 1
^{13}C and ^{1}H NMR chemical shift assignments and coupling constants data for tricin 7-*O*-β-(6″-methoxycinnamic)-glucoside[a]

position	^{1}H	^{13}C
2		165.0
3	7.07 – (*s*)	101.5
4		182.8
5		161.8
6	6.39 – (*d*, $J = 1.1$)	100.2
7		163.3
8	6.87-(*d*, $J = 1.1$)	95.3
9		157.6
10		105.2
1′		120.9
2″	7.37 – (*s*)	104.7
3′		149.0
4′		140.9
5′		149.0
6′	7.37 – (*s*)	104.7
3′, 5′ – OCH_3	3.72 – (*s*)	57.1
1″	5.27 – (*d*, $J = 7.4$)	98.2
2″	3.39 – (*t*, $J = 8.8$)	72.6
3″	3.57 – (*t*, $J = 8.8$)	77.7
4″	3.31 – (*t*, $J = 8.8$)	71.2
5″	3.89 – (*m*)	77.8
6″	4.13 – (*dd*, $J = 12.0$; 6.6)	68.0
	5.12 – (*dd*, $J = 12.0$; 4.8)	
1‴		120.9
2‴	7.34 – (*d*, $J = 8.4$)	129.4
3‴	6.72 – (*d*, $J = 8.4$)	104.7
4‴		157.6
5‴	6.72 – (*d*, $J = 8.4$)	104.7
6‴	7.34 – (*d*, $J = 8.4$)	129.4
7‴	7.69 – (*d*, $J = 15.6$)	132.4
8‴	6.30 – (*d*, $J = 15.6$)	129.4
9‴		182.8
4‴-OCH_3	3.72 – (s)	57.1

[a] Measuring in DMSO; coupling constants (*J*) in Hz.

Wang et al. (2004) the ^{13}C NMR signals for tricin-7-methyl-ether-5-*O*-beta-D-glucopyranoside (a compound with a free 4′-hydroxyl group) has C-3′ and C-5′ resonances at δ 148.2 whereas those for C-4′ and C-7 were at δ 139.5 and δ 163.6, respectively. By contrast, for the tricin-7-methyl-ether-4′-*O*-beta-D-glucopyranoside, the ^{13}C NMR signals for C-3′ and C-5′ appeared at δ 152.9 and those for C-4′ and C-7, at δ 137.7 and δ 165.3, respectively. In addition, in the HMBC spectrum of tricin-7-methyl-ether-4′-*O*-beta-D-glucopyranoside, correlations were observed among H-2′, H-6′ and H-1″ of glucose, indicating glucosylation at C-4′ (Wang et al., 2004). For **1**, the values corresponding to carbon atoms C-3′, C-5′, C-4′ and C-7 were similar to the tricin glucoside with a free 4′-hydroxyl group, this being consistent with a glucosylation occurring at C-7. The C-4 signal at δ 182.8 indicates the presence of a free 5-hydroxyl group, because glucosylation at C-5 implies a lower value for the C-4 signal, e.g. δ 177.1 for tricin-7-methyl-ether-5-*O*-beta-D-glucopyranoside (Wang et al., 2004).

Acylation of the glucose moiety is confirmed by analysis of the spectroscopic data. In the ^{1}H NMR spectrum, the two doublets at δ 6.72 and δ 7.34 ppm, corresponds to two hydrogen protons of a *p*-substituted phenyl moiety, suggesting the presence of a *p*-methoxycinnamic unit (Table 1). Two doublets ($J = 15.6$ Hz), each integrating for 1H at δ 6.30 and δ 7.69 ppm, were assigned to a *trans* oriented conjugated vinyl hydrogen atoms. The coupling constant between H-8‴ and H-7‴ of 15.6 Hz indicated a *trans* configuration. In the ^{13}C NMR spectrum, the signal at δ 157.6 (assigned to the carbonyl carbon atoms of *p*-methoxycinnamic ester) was observed. This spectrum showed carbon signals for two carbon atoms at δ 104.7 and δ 129.4 ppm, characteristic of a 1,4 disubstituted phenyl moiety with vinylic carbon atoms at δ 129.4 and δ 132.4 ppm. The ^{1}H NMR spectrum shows a pair of *ortho*-coupled doublets ($J = 8$ Hz), corresponding to the pairs of degenerated protons H-2‴–H-6‴ (δ 7.34) and H-3‴–H-5‴ (δ 6.72) of the aromatic ring of the methoxycinnamic acid moiety. The pronounced downfield shift of the 6′-methylene protons at δ 5.12 and 4.13 suggests a linkage between the acyl moiety and C-6″ of glucose.

Tricin and its derivatives are seemingly common in Poaceae. Some of these derivatives are unusual, e.g. tricin 4′-*O*-guaiacylglyceryl ethers (characterized as flavonolignans) from *Hyparrhenia hirta* (Bouaziz et al., 2002). Tricin 4′-*O*-coniferyl ethers, together with a more complex flavonolignan bearing an additional carbocyclic ring derived from the association of a coniferyl moiety with tricin, were obtained from *Avena sativa* (Wenzig et al., 2005). Tricin and common tricin-*O*-glycosides in sugarcane syrup mill, bagasse and leaves have also been reported (Mabry et al., 1984; Legaz et al., 1998). Colombo et al. (2005) reported the occurrence of tricin-4′-*O*-guaiacylglyceryl ethers in sugarcane leaves and bagasse, both as free aglycones and as 7-*O*-glucosides. The present paper represents the first report of an acylated tricin glycoside.

The yield of the acylated tricin glucoside obtained in the sugarcane juice (13 μg/100 g dry weight) is higher than values reported for rice (Cai et al., 2005). Considering that a correlation has been found between the ingestion of tricin and the decrease of colorectal and breast cancer (Hudson et al., 2000; Cai et al., 2004; Cai et al., 2005), the current consumption of sugarcane derivatives, such as juice, syrup and ''rapadura'' may be viewed as a contribution toward the prevention of development of such serious diseases, specially in relation to poorer people in tropical countries, who are the main consumers of these products.

Orientin (luteolin-8-*C*-glucoside) was obtained from the MeOH eluate. The corresponding mass spectrum in the negative mode gave deprotonated ion at *m*/*z* 447 (30) and products from $[M-H]^-$ ions were observed at *m*/*z* 357 (70) and peak base at *m*/*z* 327. The major fragmentation pathways concern cross-ring cleavages of the glucose and the loss of molecules of water (Cuyckens and Claeys, 2004).

2.2. Antioxidant activity

Antioxidant activity of **1** was determined using two methods, the DPPH assay (Brand-Williams et al., 1995)

Table 2
IC_{50} of tricin-7-*O*-β-(6″-methoxycinnamic)-glucoside – against human cancer cells

	UACC-62	MCF-7	NCI-ADR	786-0	NCI-460	PC-03	OVCAR-03	HT-29
Tricin-7-*O*-β-(6″-methoxycinnamic)-glucoside	ND	65.09	40.85	ND	132	116.1	459.5	31.31
Doxorubicin	6.05	1.92	37.24	2.02	6.98	21.46	6.64	2.49

ND: not determined.

and the β-carotene/linoleic acid method system (β-CLAMS) (Miller, 1971). It is assumed that DPPH scavenging liability to antioxidants is due to the hydrogen-donating ability of the latter. The antioxidant assay using the discoloration of β-carotene is widely used, because β-carotene is extremely susceptible to free-radical mediated oxidation.

In the DPPH method, **1** showed hydrogen-donating capacity of 55.4%, 23.9% above that of Trolox® in the same concentration (100 μM), in spite of possessing more methoxyl groups in its structure. In β-CLAMS, **1** presented 62.3% inhibition, which means moderate activity. Under the same condition, Trolox® showed 84.8% of efficiency in preventing oxidation of β-carotene. Solubility differences of flavonoids in a micellar water–lipid system may influence results obtained with such assays, so that the partition of the flavonoid between the two phases might influence redox results (Burda and Oleszek, 2001). The DPPH method is quantitatively more reliable than β-CLAMS; however, the latter assay provides an alternative mechanism by measuring the capability of a compound to resist peroxidation and free radical chain reaction (Tsao et al., 2005).

2.3. Antiproliferative activity

Compound **1** was shown to have antiproliferative activity against all cancer cell lines. The activity was concentration-dependent for breast (MCF-7), multidrug resistant breast (NCI-ADR), prostate (PC-03), ovary (OVCAR03), lung, non-small cell (NCI 460) and colon (HT-29) cell lines. Compound **1** was also active against melanoma (UACC-62) and kidney (786-0) cell lines, although only at high concentrations: it was not possible to determine IC50 values (Table 2). IC 50 values were lowest for the colon cell line followed by the multi-resistant breast cell line. By comparison, doxorubicin gave activity much higher for all tumoral cell lines than **1**.

Compound **1** was shown to have anticancer activity for the breast multidrug resistant and colon lines. The high specificity toward colon cell lines is in agreement with data of Verschoyle et al. (2006) based on a carcinogenesis model of the digestive system.

3. Conclusion

Sugarcane juice contains tricin-7-*O*-β-(6″-methoxycinnamic)-glucoside (**1**). This compound has considerable antioxidant activity regarding the DPPH assay, but is less active than Trolox® in the β-CLAM assay. Compound **1** possesses antitumor activity, having activity against all cell lines tested, with higher selectivity toward cells of the breast resistant NIC/ADR line.

4. Experimental

4.1. General experimental procedures

All reagents and solvents were of analytical and spectrometric grade. DPPH was purchased from Aldrich Chemical Co. (Milwaukee, WI). Trolox, Tween 40, β-carotene, linoleic acid, gentamicine, TCA, Trizma and sulforhodamine B were purchased from Sigma Aldrich Inc. (St. Louis, MO). RPMI 1640 was purchased from Gibco BRL, Life Technologies Inc. (Grand Island, NY).

UV–visible spectra were recorded with a Hewlett–Packard 8453 UV–visible spectrophotometer. IR spectra were obtained in KBr disks using a Bomem model MB 100 M series spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker Avance DPX 300 spectrometer, using DMSO-d_6 as solvent. HRESIMS was recorded on a Bruker Daltonics microTOF instrument. ESI-MSMS low resolution spectra were performed on a triplequadrupole API 4000™ system (Applied Biosystems).

4.2. Instrumentation and procedures

A triplequadrupole API 4000™ system, was used in the MS and MS^2 modes for structural determination, through electrospray ionization (ESI) by a TurboIonSpray® source at room temperature. Ultra-pure air was applied as nebulizer gas at 20 psi and N_2 as curtain gas at 12.0 psi. The capillary ESI voltage was set at 5.5 kV and −4.5 kV in positive and negative ion modes, respectively, from *m/z* 300 to 700 mass range. In MS^2 experiments, the collision cell (Q2) was filled with N_2 at 6.0 a.u. and the collision energy varied from 5 to 130 eV. Compound **1** was dissolved in CH_3OH–H_2O (1:1) containing 5.0 mmol/l of ammonium acetate. The solution was introduced into the system by a syringe pump Harvard A.S.I.P. 22 with a continuous flow of 10 μl/min. Analyses were performed by alternating positive and negative ionization modes. Analytical HPLC was carried out using an Agilent 1100 system with an autosampler and a quaternary pump coupled to a diode array detector. A Phenomenex Prodigy 5 μ ODS 3 RP-18 (250 mm × 4.6 mm; 5 μm particle size) column with a

40 min gradient of H_2O–THF–TFA (98:2:0.1) and CH_3CN at 1 ml/min were used. Column temperature was 30 °C and the solvent gradient used was based on Arabbi et al. (2004). Detection was performed at 270 and 380 nm. UV–visible spectra were recorded with a Hewlett–Packard 8453 UV–visible spectrophotometer. Spectra were recorded in MeOH solutions alone and with shift reagents (Mabry et al., 1970). Analysis of the trimethylsilylated glucose (sugar) obtained upon hydrolysis of compound **1** were performed with a Hewlett–Packard 6890 GC equipped with a 6890 Series auto-injector, coupled to a 5973 Mass Selective Detector, using a SPB-50 fused silica column (30 m × 0,25 mm × 0,25 μm – Supelco Park). The flow rate of the carrier gas (helium) was 1.0 cm^3/min with auto injections made in the splitless mode. Column temperatures varied from 70 °C, with an isothermal period of 3 min, to 310 °C at a rate of 5 °C/min, followed by an isothermal period of 5 min. The ionization potential of the mass selective detector was 70 eV. The mass limit was *m/z* 40–700. Injector and detector temperatures were at 250 °C and 230 °C, respectively. Identification was achieved by comparison with retention times and mass spectra of authentic samples. Part of the analytical data obtained were compared with results of Stochmal et al. (2001), Wang et al. (2004) and Norbaek et al. (2003), which deal with analysis of tricin glycosides. The structure of compounds **1** was further confirmed by high-resolution mass spectrometry.

4.3. Plant material

Sugarcane juice, obtained from grinding of sugarcane culm (SP813250), was purchased at a market in São Paulo city (state of São Paulo, Brazil) in September, 2004.

4.4. Extraction, isolation and identification of tricin

Elimination of sucrose from the sugarcane juice was achieved through a procedure adapted from Andrade et al. (1997). Sugarcane juice (2 L) was filtered through cheese-cloth, acidified to pH 2–3 with conc. HCl and loaded to an Amberlite XAD-2 (Supelco, Bellefonte, PA) resin column, which was washed with H_2O (1.5 L). Phenolic compounds were eluted (three times, 500 ml, at 25 °C) with MeOH–NH_4OH (99.5:0.5). The eluate was concentrated under reduced pressure at 40 °C to remove MeOH and then diluted with with H_2O to 100 ml. The latter was next passed through a Polyamide CC 6 (Macherey-Nagel Gmbh and Co., Germany) column (3.5 g/20 ml) previously conditioned in MeOH–H_2O (160 ml, 3:5, v/v). The columns were washed with H_2O (50 ml) and eluted with a MeOH–NH_4OH solution (50 ml, 99.5:0.5 v/v). The eluate was evaporated to dryness under reduced pressure at 40 °C and then redissolved in MeOH. Isolation of the flavonoid was achieved through preparative paper chromatography (Whatman 3MM - Maldstone, UK) with 30% HOAc. The compound was eluted from the paper with MeOH as eluant. The eluate was concentrated under reduced pressure at 40 °C (26 mg). The solution was filtered through a 0.25 μm poly-(tetrafluoroethylene) (PTFE) filter (Millipore Ltd., Bedford, MA) prior to analysis.

4.4.1. Tricin-7-O-β-(6″-methoxycinnamic)-glucoside

Crystalline solid; m.p. 171–173 °C; UV λ_{max}^{MeOH} nm (log ε): 272 (0.8), 345 (0.7); $\lambda_{max}^{MeOH+KOH}$ 272 (1.2), 398 (1.0); $\lambda_{max}^{MeOH+NaOAc}$ 272 (1.2), 348 (0.7), 430 (0.5); IR ν_{max}^{KBr} cm^{-1}: 3407, 1643, 1612, 1593, 1550, 1508; ^1H NMR (δ ppm) 7.69 (*d*, 1H, 15.6, H-7‴), 7.37 (*s*, 2H, H-2″, H-6′), 7.34 (*d*, 2H, 8.4, H-2‴ and H-6‴); 7.07 (*s*, 1H, H-3); 6.87 (*d*, 1H, H-8); 6.72 (*d*, 2H, 8.4, H-3‴ and H-5‴); 6.39 (*d*, 1H, H-6); 6.30 (*d*, 1H, 15.6, H-8‴); 5.27 (*d*, 1H, H-1″, 7.4); 5.12 (*dd*, 1H, H-6″a, 4.8, 12.0); 4.13 (*dd*, 1H, H-6″b, 6.6, 12.0); 3.89 – (*m*, 1H, H-5″); 3.57 (*t*, 1H, H-2″, 8.8); 3.39 (*t*, 1H, H-3″, 8.8); 3.31 (*t*, 1H, H-4″, 8.8) and 3.72 – (*s*, 9H, OCH_3). ^{13}C NMR (ppm) 182.8 C=O (C-4 and C-9‴); 165.0 (C-2); 163.3 (C-7); 161.8 (C-5); 157.6 (C-9 and C-4‴); 149.0 (C-3′ and C-5′); 140.9 (C-4′); 132.4 (C-7‴); 129.4 (C-6‴, C-2‴ and C-8‴); 120.9 (C-1′ and C-1‴); 105.2 (C-10); 104.7 (C-2″, C-6′, C-3‴ and C-5‴); 101.5 (C-3); 100.2 (C-6); 98.2 (C-1″); 95.3 (C-8); 77.8 (C-3″); 77.7 (C-5″); 72.6 (C-2″); 71.2 (C-4″); 68.0 (CH_2-6″); 57.1 (OCH_3). ESIMSMS spectra showed $[M+H]^+$ and $[M-H]^-$ peaks at *m/z* 653 and *m/z* 651, respectively. ESIMSMS gave product ions from $[M+H]^+$ at *m/z* 331 ($C_{17}O_7H_{15}$, tricin)$^+$, *m/z* 315 ($C_{16}O_7H_{11}^+$) and *m/z* 287 ($C_{15}O_6H_{11}^+$). ESIMSMS product ions from $[M-H]^-$ were observed at *m/z* 491 (M−H−*p*-methoxycinnamic acid)$^-$ ($C_{23}O_{12}H_{23}$)$^-$, *m/z* 329 ($C_{17}O_7H_{13}$, tricin)$^-$, *m/z* 315 ($C_{16}O_7H_{10}^-$), *m/z* 299 ($C_{15}O_7H_7^-$), *m/z* 271 ($C_{14}O_6H_7^-$). HRESIMS (negative ion mode) (*m/z*) 651,1704; (M−H, calcd for $C_{33}H_{32}O_{14}$: 651, 1714).

4.4.2. Hydrolysis of compound *1*

For hydrolysis of compound **1**, (1 mg) the compound was dissolved in 2 N HCl (2 ml). The mixture was heated until reflux began, this being maintained for 60 min. After cooling, the mixture was loaded on to a polyamide column, as described above. The monosaccharide was next eluted with H_2O, with the solution evaporated to dryness under reduced pressure. For methoxymation, the sugar was treated with methoxyamine hydrochloride (20 mg/ml) in pyridine at 30 °C for 180 min. TMS derivatization was carried out by addition of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (80 μl) (Sigma Chemical Co., St. Louis, MO) for 30 min at 37 °C.

4.5. Antioxidant activity

4.5.1. β-Carotenellinoleic acid method system (β-CLAMS)

The antioxidant activity by the bleaching β-carotene method was carried out according to Duarte-Almeida et al. (2006). For preparation of the reactive solution, aliquots of β-carotene (25 μL) in $CHCl_3$ (2 mg/ml) were mixed with linoleic acid (20 μg), Tween 40 (200 mg) and $CHCl_3$ (0.5 ml). $CHCl_3$ was then completely evaporated

under N_2 flow, with distilled H_2O (25 ml) saturated with oxygen was added to the mixture. The absorbance was adjusted with H_2O to 0.6. For the oxidation reaction, an aliquot of the sample (10 μL) was mixed with the β-carotene (250 μL) solution in a microplate. The samples were submitted to autoxidation at 45 °C for 120 min. The absorbance at 470 nm was measured at zero time and at 15 min intervals using the microplate spectrophotometer (Benchmark Plus, Bio-Rad). Control consisted of MeOH and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, positive control). The antioxidant activity was expressed as an inhibition percentage compared to the control (100% oxidation), and all assays were run in triplicate. Antioxidant activity of the sample was calculated as percent inhibition of oxidation versus control, according to Formula (1), where a_{sample} is the absorbance of sample at t=0 min and t=120 min, and $a_{control}$ is the absorbance of sample at $t = 0$ min and $t = 120$ min.

$$\%\ \text{Inhibition} = 100 \times \left[1 - \frac{\left(a^{0}_{\text{sample}} - a^{120}_{\text{sample}}\right)}{\left(a^{0}_{\text{control}} - a^{120}_{\text{control}}\right)}\right] \quad (1)$$

4.5.2. Radical scavenging activity (RSA)

Radical scavenging activity (RSA) of **1** was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) as a reagent. Solutions of **1** and Trolox in MeOH (100 μM) were individually added to 0.1 mM DPPH in MeOH. The mixture was incubated in the dark at 25 °C for 30 min (Duarte-Almeida et al., 2006). Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm. A lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Percent radical scavenging activity was determined by comparison with a MeOH containing Trolox as positive control. The percentage of RSA was calculated using Formula (2), where $a_{control}$ is the absorbance of the control, and a_{sample} is the absorbance of the sample.

$$\%\ \text{RSA} = \frac{a_{\text{control}} - a_{\text{sample}}}{a_{\text{control}}} \times 100 \quad (2)$$

4.6. Antiproliferative activity

Antiproliferative activities of **1** against cancer cells were evaluated with cell lines of distinct histological origins: MCF-7 (breast), NCI-ADR (breast, multidrug resistant), NCI 460 (lung, non-small cells), UACC62 (melanoma), 786-0 (kidney), OVCAR03 (ovarian), PC03 (prostate), and HT-29 (colon). The cells lines were kindly provided by Frederick Cancer Research & Development Center, National Cancer Institute (Frederick, MA, USA) and the cells were grown in vitro. Chemotherapic doxorubicin was used as positive control.

Stock cultures were grown in a medium containing RPMI 1640 (5 ml) and supplemented with 5% of fetal bovine serum. Gentamicine (50 μg/ml) was added to the experimental cultures. Cells in 96-well plates (100 μL cells/well) were exposed to various concentrations of samples in DMSO (0.25, 2.5, 25, and 250 μg/ml) at 37 °C, under an atmosphere of 5% CO_2 for 48 h. The final concentration of DMSO did not affect cell viability. A 50% solution of TCA was then added, and after incubation for 30 min at 4 °C the preparation was washed and dried. Cell proliferation was determined by spectrophotometric measurement at 540 nm of the cellular protein content using sulforhodamine B assay described by Skehan et al. (1990).

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