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Biologically active anthracenones from roots of *Karwinskia parvifolia*

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Abstract

Tullidinol, a neurotoxin isolated from fruits and roots of several *Karwinskia* species, was resolved for the first time into two stereoisomers. This was achieved by means of preparative HPLC from roots of *Karwinskia parvifolia*. Structural assignments were made on the basis of spectroscopic data, including long range correlations as well as geometry optimization by means of semi-empirical methods. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Karwinskia parvifolia*; Rhamnaceae; Isolation; Anthracenones; Tullidinol; Neurotoxicity.

1. Introduction

The genus *Karwinskia* (Rhamnaceae) includes several species. In Mexico 11 species have been reported up to now (Fernández Nava 1992). The most widespread and studied of these is *Karwinskia humboldtiana*. The toxic effects originating from the ingestion of its fruit are well documented (Weller, Mitchell, & Daves 1980; Bermúdez, Lozano, Salazar, Waksman, & Piñeyro 1995). One of the anthracenones isolated from its fruit, called T544 or tullidinol (Dreyer et al. 1975) (**1**) is the responsible agent for the neuropathy associated with ingestion (Bermúdez, González-Spencer, Guerrero, Waksman, & Piñeyro 1986; Muñoz Martínez, Cuellar Pedroza, Rubio Franchini, Jáuregui Rincón, & Nathan 1994). Also, from the fruits of another species, *K. parvifolia*, several 9,10-dihidroxy anthracenonic compounds were isolated (Waksman & Ramírez 1992). One of these, called T 514 or peroxisomicine A_1 (**2**), showed selective toxicity to several human cell lines (Piñeyro, Martínez, & González 1994). These results have been corroborated by The Development and Therapeutical Program of the National Cancer Institute, where this compound is being tested in vivo. Previous phytochemical studies of the roots of *K. humboldtiana* have led to the isolation of several flavonoids (Domínguez, Temblador, & Cedillo 1976) and karwinaphtols (Mitscher, Gollapudi, Oburn, & Drake 1985) and more recently the isolation and the chemotaxonomical relevance of 7′ desmethoxitullidinol from roots of *K. umbellata*, *K. subcordata*, *K. humboldtiana*, *K. johnstonii* and *K. mollis* were established and published (Yussim et al. 1995).

2. Results and discussion

The air-dried ground roots of *K. parvifolia* were successively extracted with petrol (b.p. 60–80°C), EtOAc and MeOH at room temperature. Only the first two extracts showed biological activity (brine shrimp test (Anderson, Goetz, Suffness, & McLaughin 1991)) and were further fractionated (LD_{50} values of 310.73 and 272.36 ppm were obtained for the petrol ether and EtOAc extract, respectively).

From the petrol extract, and after repeated column and preparative TLC chromatography, two active anthraquinones were isolated (**3** and **4**). Both were reported previously from the roots of other plants belonging to this genus. They were identified by comparing spectral and physical data with those reported in the literature (Yussim et al. 1995). Biological testing gave LD_{50} values of 49.99 and 4.44 ppm for **3** and **4**, respectively (brine shrimp test).

From the EtOAc extract, the main component was a compound with Rf and spectroscopic properties identical to those previously reported for tullidinol (Dreyer et al. 1975; Muñoz Martínez, Cueva, & Joseph-Nathan 1983). Further analysis of this compound by HPLC dem-

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onstrated the presence of two components with close Rt and identical spectra (DAD detector, Fig. 1). By means of preparative HPLC small amounts of each component of the mixture were isolated. The purity of each compound was assessed by HPLC; NMR spectral data (^{1}HNMR and ^{13}CNMR) for both compounds were found to be very similar. Through mass spectrometry the molecular formula $C_{32}H_{32}O_8$ was calculated for both compounds. Careful examination of the ^{1}H and ^{13}C spectra (including HMQC and HMBC) led to unambiguous assignments for all signals (Table 1); both compounds are isomers with the same planar structure as that previously proposed for T 544 or tullidinol (Dreyer et al. 1975; Muñoz Martínez et al. 1983). Tullidinol is optically active due to the combined effects of three centers and one axis of chirality; the latter being the same for both compounds isolated when comparing the CD spectra. CD curves for both isomers (Fig. 2) exhibit strong positive Cotton effects to longer λ and strong negative Cotton effects to shorter λ, which show that the two long axes are twisted in the same sense. CD curves are opposite to that obtained with peroxisomicine A_1. Peroxisomicine A_1 was recently crystallized and X-ray analysis showed an *R* axial stereochemistry for it (Rodríguez et al., 1998). Considering that the CD spectra of these dimers are dominated by interactions between the naphthalene moieties with no significant influence from the rest of the molecule (Gill 1994), the biaryl linkage in the two isomers of tullidinol must be opposite (S) to that found in peroxisomicine A_1. On account of the stereochemistry of the A ring, NOE difference spectra demonstrated an interaction between H-1′ and H-3′ in both isomers (6% enhancement); 1′*R* 3′*S* as well as 1′*S* 3′*R* isomers have these hydrogens in a pseudoaxial position and at a distance at which this effect can be observed (2.5–2.7 Å); the structures for both isomers were optimized by means of semi-empirical molecular models (AM1 method); eight local minima were obtained; the characteristic of each one is summarized in Table 2. The computer generated minimum-energy conformations were used as a basis for calculation of the vicinal coupling constants. The calculated conformations also provided the interproton distances which were compared with the NOE results. The

Table 1
^{13}C NMR spectral data for tullidinol B_1 and B_2 (100 MHz, δ values in $CDCl_3$)

C, δ (ppm)	Tullidinol B_1, δ (ppm)	Tullidinol B_2, δ (ppm)
1	202.97	202.90
2	51.28	51.28
3	71.10	71.19
Me-3	29.20	29.26
4	43.30	43.25
4a	134.50	134.49
5	118.51	118.50
6	136.58	136.61
7	121.56	121.60
8	154.86	154.88
8a	112.92	112.93
9	165.81	165.87
9a	109.38	109.39
10	118.65	118.65
10a	139.17	139.21
1′	71.20	71.22
Me-1′	21.84	21.87
3′	69.39	69.39
Me-3′	21.72	21.74
4′	36.71	36.78
4a′	135.19	135.11
5′	122.99	123.04
5a′	134.30	134.32
6′	97.68	97.56
7′	157.13	157.20
MeO-7′	55.10	55.16
8′	96.93	97.11
9′	157.46	157.44
MeO-9′	56.28	56.28
9a′	109.52	109.51
10′	150.25	150.26
10a′	119.71	119.69

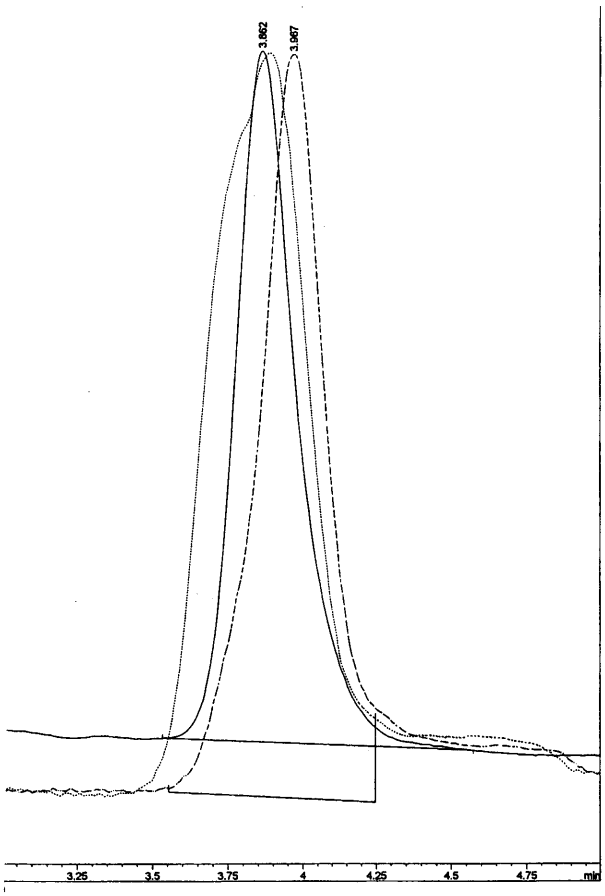


Fig. 1. HPLC chromatogram: ———, tullidinol B_1 (Rt: 3.86); · · ·, mixture of tullidinol B_1 and B_2; - - -, tullidinol B_2 (Rt 3.97). Chromatographic conditions as published (Waksman, Santoyo, Ramírez, Fernandez-Nava, & Piñeyro-López, 1997).

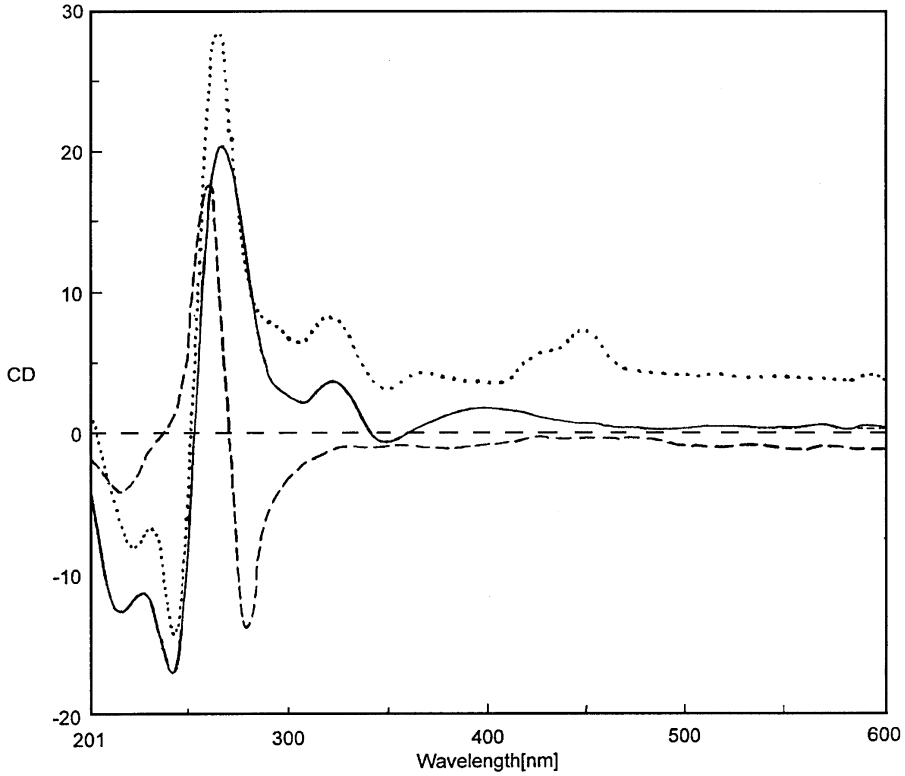


Fig. 2. CD spectra of tullidinol B_1, · · ·; tullidinol B_2, ———, and peroxisomicine A_1, - - -.

Table 2
Local minima found for tullidinol (AM1 method)

Isomer	Compound No.	Heat of formation	Torsional angle C4a′–C5′–C7–C8	Dihedral angle H4′–C4′–C3′–H3′
1′*R* 3′*S*	1	−260.46	+94	178 and 60
1′*R* 3′*S*	2	−254.70	+76	95 and −21
1′*R* 3′*S*	3	−260.62	−80	178 and 58
1′*R* 3′*S*	4	−254.40	−108	−86 and 30
1′*S* 3′*R*	5	−260.65	+83	177 and −60
1′*S* 3′*R*	6	−256.91	+89	−83 and 33
1′*S* 3′*R*	7	−260.48	−70	−177 and −70
1′*S* 3′*R*	8	−256.90	−77	−85 and 32

A ring can adopt two low energy conformations, *J* found for the coupling between H-4′ and H-3′ (11 Hz) in both compounds agree with those conformations in which H-4′ is pseudoaxial to the plane formed by O2′–C1′–C10a′–C4a′–C4′ atoms, as seen in Fig. 3. According to these results, structures 2, 4, 6 and 8 in Table 2 were discarded; furthermore, signals at 2.37 and 2.38 ppm in tullidinol B_1 and B_2, respectively, were assigned as having arisen from H-4′ axial. Quantitative values obtained from NOE diff. experiments for both isomers were not significantly different (Fig. 4); NOE enhancements observed between H-4′ axial and H-6, agree with the internuclear distances calculated for compounds **3** and **5** in Table 2; compound **3** is the negative rotamer with 1′*R* 3′*S* stereochemistry (H-4′ ax up, H-6 up and OH-8 behind the plane formed by the upper unit); on the other hand, compound **5** is the positive rotamer with stereochemistry 1′*S* 3′*R* (H-4′ ax down, H-6 down and OH-8 over the plane formed by the upper unit). Taking into account the results obtained from CD curves, the stereochemistry for both isomers should be that displayed by compound **3** in Table 2 (*S* rotamer, 1′*R* 3′*S*). The stereochemistry at the remaining chiral center (C-3) could not be defined, but since the molecules are diastereoisomers, it follows that it must be opposite in both compounds.

Both isomers of tullidinol are type B according to the classification proposed by Steglich (Gill & Steglich 1987). In the future these compounds will be recognized as tul-

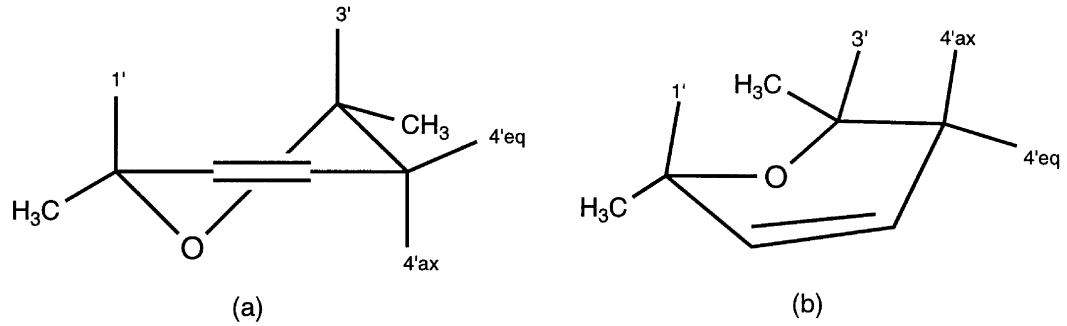


Fig. 3. Conformation of A ring in tullidinol B_1 and B_2.

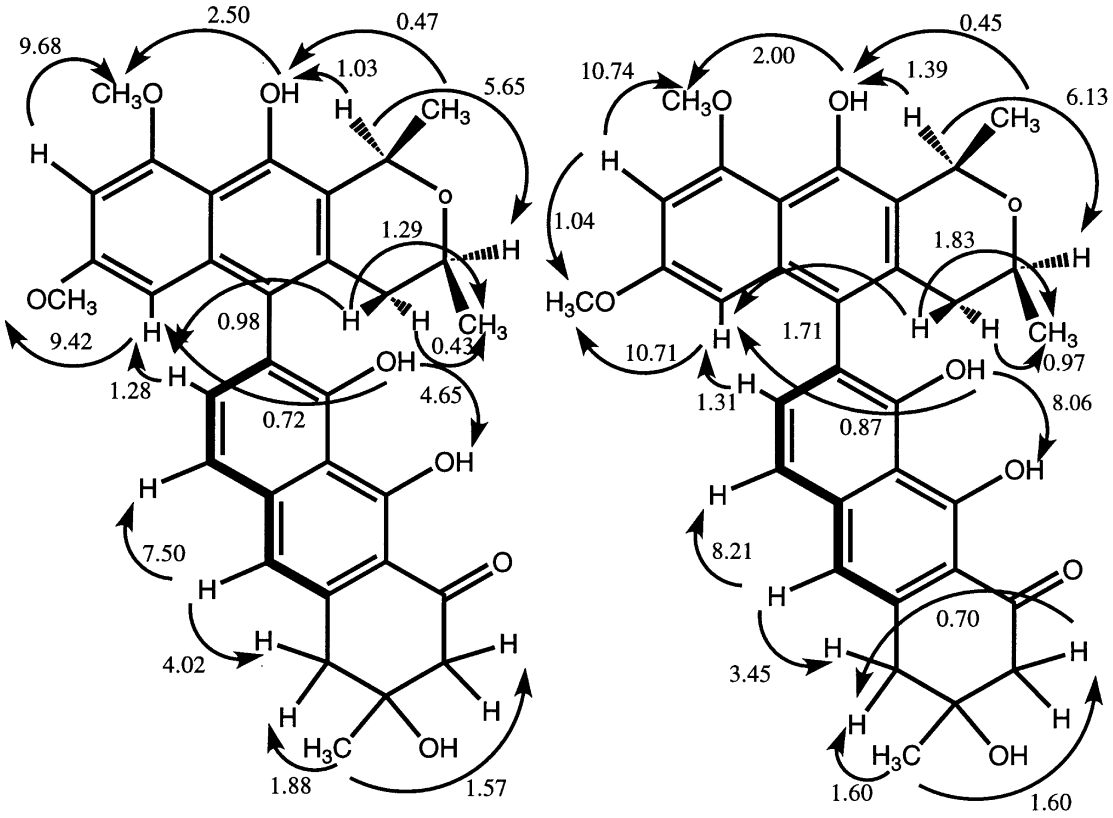


Fig. 4. Representative NOEs found in tullidinol B_1 (Left) and tullidinol B_2 (right). Numbers represent the % enhancement in the indicated direction.

lidinol B_1 and B_2. The subindices are according to the order of Rt in our system.

The presence of a pair of atropoisomers in the tullidinol isolated from *K. humboldtiana* has been previously suggested (Dreyer et al. 1975; Yussim et al. 1995), but up to now they have been inseparable. Our experimental results demonstrate that these compounds are not atropoisomers, but diastereoisomers around the configuration of C-3.

It is also important to point out that these anthracenones are not present in the fruits of *K. parvifolia* and the anthracenones which are more abundant in the fruit (T514 and T496) are absent in roots (Waksman & Ramírez 1992; Waksman, Santoyo, Ramírez, Fernandez-Nava, & Piñeyro-López 1997). The establishment of the metabolic pathway in this species might be of interest because it appears different from the other species of this genus. Tullidinol B_1 and B_2 were biologically active with LD_{50} of 12.30 and 9.88 ppm, respectively (*A. salina* test).

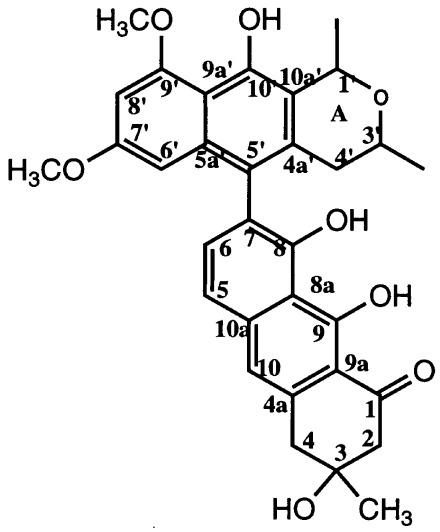
Using HPLC to examine tullidinol samples previously reported from fruits of *K. humboldtiana*, we could verify that they are also a mixture of the two isomers described in this work. Considering that tullidinol has been reported (Muñoz Martínez et al. 1983; Bermúdez et al. 1986) as the responsible agent for neurotoxic effects of fruits from *K. humboldtiana*, it will be necessary to repeat in the future the toxicological test with both isomers.

Two known anthraquinones and two new anthra-

cenones were isolated from roots of *K. parvifolia*. All the compounds were active in the brine shrimp bioassay.

3. Experimental

^{1}H and ^{13}C NMR spectra were obtained on a Bruker DPX-400 at 400.13 and 100.62 MHz, respectively, using $CDCl_3$, C_6D_6 and DMSO-d6 as solvent. NOE differential experiments were carried out with 100% deuterated solvents and in argon deareated samples both in $CDCl_3$ and C_6D_6. In order to assign all the signals observed, HMBC experiments were optimized for $J=7$ and 9 Hz Mps uncorrected were obtained in an Electrothermal 9100 TLC in precoated Kiesel gel 60 F-254 and RP18 F-254 from Merck. Analytical HPLC was carried out on a HP-1090 (DAD detector) with a C-18 column 100×2.1 mm, 5 μm. Elution was accomplished with a mixture of AcCN, MeOH, H_2O, HOAc according to the methodology previously described (Salazar, Piñeyro, & Waksman 1996). Preparative HPLC was accomplished on a Water Prep LC 200, column 80×100 mm Radial Pak C-18, 10 μm with a program adapted from the analytical separation. Semi-empirical calculations were made by means of AM1 method (MacSpartan-plus program).



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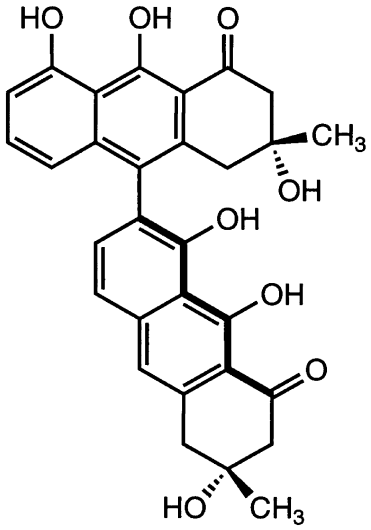
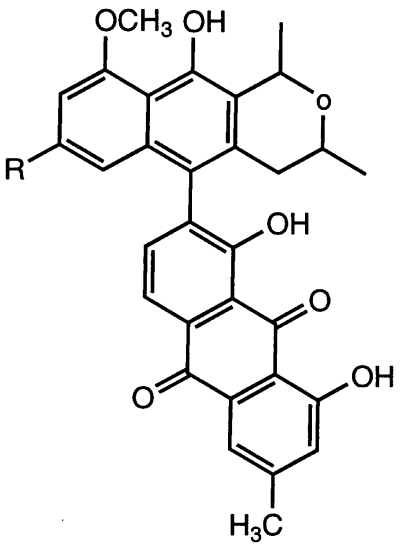
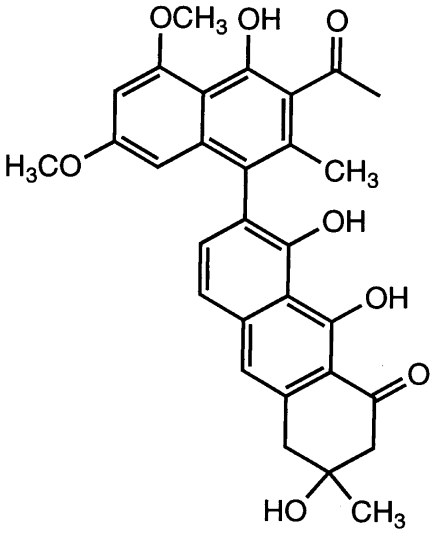


2



3 R=OCH3
4 R=H



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3.1. Plant material

The species was collected in Mexico (Choix, Sinaloa, December 1992). The plant was identified and classified by Dr. Rafael Fernández Nava (IPN, Mexico).

3.2. Isolation

The dried, powdered roots (997.0 g) were extracted repeatedly with hexane (2 l), AcOEt (2 l) and MeOH (2 l) at room temperature. The hexane extract was evaporated to dryness under reduced pressure at 40°C. The residue (2.32 g) was subjected to flash chromatography using silica gel (hexane–AcOEt, 9:1) and further preparative TLC developed with hexane–acetone (3:1) and recovered with AcOEt 0.1% in HOAc, furnished **3** and **4** (50.5 and 24.0 mg, respectively). The AcOEt extract (2.97 g) was fractionated by means of flash chromatography using silica gel (C_6H_6–AcOEt, 9:1), followed by Lobar low pressure liquid chromatography on RP-18 bonded phase, eluted with MeOH–H_2O (7:3) to obtain tullidinol **1** and compound **5** in a smaller quantity. Further separation of the two isomers from the tullidinol mixture was accomplished by HPLC to give tullidinol B_1 (25.6 mg) and B_2 (30 mg).

3.3. Tullidinol B_1

Yellow powder, m.p. 175°C dec., $[\alpha]_D$ +274° (MeOH; 0.10); ^{1}HNMR: δ 16.04 (s, 1H, 9-HO), 9.82 (s, 1H, 8-HO), 9.61 (s, 1H, 10′-HO), 7.40 (d, 1H, J=8.2, H-6), 7.28 (d, 1H, J=8.2, H5), 7.11 (s, 1H, H-10), 6.41 (d, 1H, J=1.5, H-8′), 6.25 (d, 1H, J=1.5, H-6′), 5.24 (c, 1H, J=6.19, H-1′), 4.02 (s, 3H, 9′-OMe), 3.69 (m, 1H, H-3′), 3.56 (s, 3H, 7′-OMe), 3.15 (AB system, 2H, H-4), 2.93 (d, 1H, J=18, H-2), 2.87 (d, 1H, J=18, H-2), 2.37 (dd, 1H, J=15 and 10.5, H4′-ax), 2.28 (dd, 1H, J=15 and 2, H-4′-ec), 1.65 (d, 3H, J=6.1, Me-1′), 1.50 (s, 3H, Me-3), 1.20 (d, 3H, J=6.1, Me-3′); ^{13}CNMR (see Table 1), MS (EI) m/z (rel. int): 544[M^+] (8), 526[M–H_2O] (13), 511[M–H_2O–Me] (70), 240[monomer] (42).

3.4. Tullidinol B_2

Yellow powder, m.p. 175°C dec., $[\alpha]_D$ +260° (MeOH;0.10); ^{1}HNMR: δ 16.03 (s, 1H, 9-HO), 9.82 (s, 1H, 8-HO), 9.63 (s, 1H, 10′-HO), 7.40 (d, 1H, J=8.2 Hz, H6), 7.28 (d, 1H, J=8.2 Hz, H5), 7.12 (s, 1H, H-10), 6.40 (d, 1H, J=1.5, H-8′), 6.21 (d, 1H, J=1.5, H-6′), 5.24 (c, 1H, J=6.19, H-1′), 4.0 (s, 3H, 9′-OMe), 3.71 (m, 1H, H-3′), 3.56 (s, 3H, 7′-OMe), 3.15 (AB system, 2H, H-4), 2.89 (AB system, 2H, H-2), 2.38 (dd, 1H, J=16 and 11, H-4′ax), 2.29 (dd, 1H, J=16 and 1.2, H-4′ec), 1.68 (d, 3H, J=6.1, Me-1′), 1.50 (s, 3H, Me-3), 1.21 (d, 3H, J=6.1, Me-3′); ^{13}CNMR (see Table 1), MS (EI) m/z [rel. int]: 544[M^+] (6), 526[M–H_2O] (14), 511[M–H_2O–Me] (60), 240[monomer] (38).

3.5. Bioassay

The brine shrimp (brine shrimp leach) test was routinely employed for evaluating extracts and the isolated compounds.

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