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Original article

Terpenoids isolated from *Azadirachta indica* roots and biological activities

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The chemical study of roots from *Azadirachta indica* A. Juss., Meliaceae, led to the isolation of two new terpenoids, limonoid morenolide and diterpene 17-hydroxy-sandaracopimar-8,15-dien-11-one, in addition to the four well-known limonoids nimbinene, nimbinal, nimbandiol and salannin, and three diterpenoids nimbidiol, ferruginol, and 6,7-dehydroferruginol. Their structural elucidations were based on one and bidimensional Nuclear Magnetic Resonance and Electrospray ionization mass spectrometry spectra data which was compared to the data found in literature. The anti-inflammatory, cytotoxic and antimycobacterial activities of the identified terpenoids were evaluated.

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Introduction

Azadirachta indica A. Juss., Meliaceae, commonly known as neem, originated from South and Southeast Asia, is also found in tropical and subtropical areas of Africa, America and Australia (Puri, 1999). Neem has been widely used by humankind since prehistoric times to treat diseases, and has been known as "divine tree" (Kumar and Navaratnam, 2013). Biological activities such as antibacterial (Joy Sinha et al., 2017), antiviral (Ashfaq et al., 2016), antifungal (Osman Mohamed Ali et al., 2017), molluscicidal (Ebenso, 2004) and antihyperglycemic (Ezeigwe et al., 2015) have been attributed to different parts and extracts of neem, besides the antifeedant activity characteristic of limonoids present in this species (Mordue (Luntz) and Blackwell, 1993). The limonoids, also known as tetranortripernoids, are the main representatives of the chemical composition of neem. Azadirachtin is considered the most important antifeedant compound, which showed activity in at least 550 insect species (Mondal and Mondal, 2012).

Neem compounds it also showed inhibition of nitric oxide (NO) production, characterized in the methanolic extract of neem leaves, which evidenced a strong effect on proinflammatory cell signaling and apoptotic cell death mechanisms (Schumacher et al., 2011). Twenty limonoids from *A. indica* were also evaluated for their inhibitory activity against NO production in mouse macrophage RAW 264.7 cells induced by lipopolysaccharide (LPS) (Akihisa et al., 2017).

In Brazil, the presence of pests in agriculture and livestock are limiting to production. Works were been carried out with neem extracts in these cases. For example, neem oil hydroalcoholic extract was highly toxic to the *Iphiseiodes zuluagai* mite (coffee pests) (Mourao et al., 2004); another work presented an acaricidal effect of neem extracts against *Rhipicephalus microplus* (cattle tick) (Giglioti et al., 2011). In relation to public health, the ethanolic extract of neem oil also showed activity against larvae of *Aedes aegypti* (mosquito transmitting dengue) (Wandscheer et al., 2004).

Due to ethnopharmacological importance and few researches regarding the neem roots, the present study was based on the chemical characterization and biological investigation of the *Azadirachta indica* roots (dichloromethane and ethyl acetate extracts). It led to the identification of a new C-*seco* limonoid (**1**) and a new pimarane diterpene (**6**), along with seven known additionals terpenoids (**2–5**, **7–9**), which were characterized by spectra data, mainly obtained by ^{1}H and ^{13}C NMR (1D and 2D) and mass spectra (MS), in comparison with

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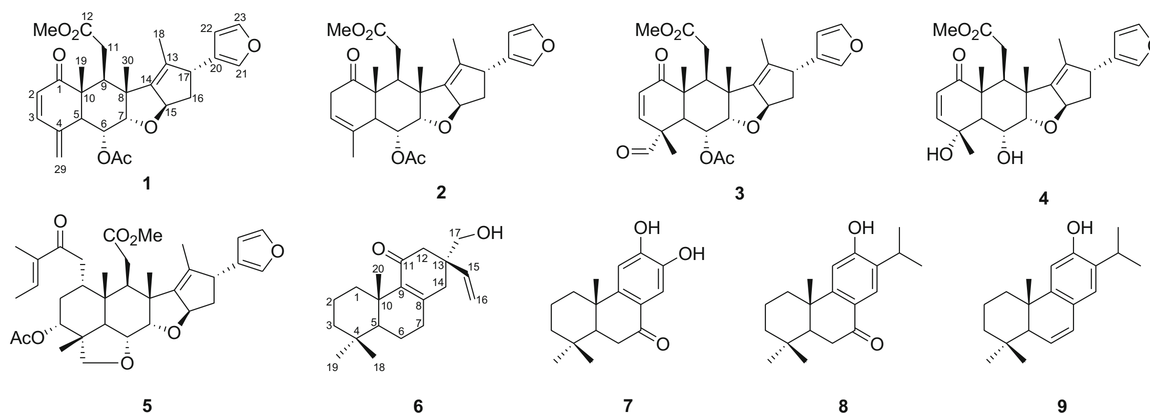

literature data. The biological investigations were assessed for their cytotoxic, inhibition of NO production, and antimycobacterial activities.

Materials and methods

General experimental procedures

The NMR analysis was carried out on a Bruker Ascend 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$, and used TMS as the internal standard. Chemical shifts (δ) in ppm and coupling constants (*J*) in Hz. HRESI-MS mass spectra were obtained on a micrOTOF-Q II BrukerDaltonics mass spectrometer, with the use of the positive ion mode of analysis. Gas chromatography coupled with low resolution mass (GC/MS) were determined on a GCMS-QP5050A Shimadzu with the use of an ionization energy of 70 eV. Column chromatography (CC) was performed on silica gel 60 (0.063–0.200 mm, MERCK). For the preparative thin layer chromatography (PTLC) silica gel 60 PF_{254} MERCK was used on glass plates. *n*-Hexane (98.5%), methanol (MetOH, 99.8%), ethyl acetate (AcOEt, 99.5%), dichloromethane (CH_2Cl_2, 99.5%) and *n*-butanol (99.4%) were purchased from Synth (São Paulo, Brazil).

Plant material

The roots of *Azadirachta indica* A. Juss., Meliaceae, were collected in Cachoeiro de Itapemirim city, Espirito Santo state, Brazil, in December 2015. A voucher specimen (H10154) was deposited in the herbarium of the Centro de Ciências e Biotecnologia of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF).

Extraction and isolation

Dried and powdered roots (2.5 kg) from *A. indica* were extracted with methanol at room temperature. After solvent evaporation, 148.3 g of crude methanol extract was suspended in a $MeOH:H_2O$ solution (1:3), and partitioned with CH_2Cl_2, EtOAc, and *n*-butanol, successively. The CH_2Cl_2 fraction (AID, 38.1 g) was chromatographed on a silica gel column (CC) and eluted with a gradient of CH_2Cl_2:MeOH, yielding nine subfractions (AID1–AID9). Compounds **8** and **9** were identified in admixture from the AID1–4 (14 mg) fraction. The fraction AID4 (14.4 g) was subjected to CC, with the use of *n*-hexane:EtOAc gradient as eluent, yielding eight fractions. The AID4-4 (764 mg) fraction was rechromatographed yielding thirteen fractions. The AID4-5 (854 mg) fraction was purified by PTLC with the use of CH_2Cl_2:MeOH (7:3) as eluent in order to obtain compounds **2** (12 mg) and **6** (2 mg). Similarly, the AID4-4.7 fraction was purified by PTLC (CH_2Cl_2:MeOH, 7:3) yielding compound **3** (8 mg). Fraction AID4-6 was fractionated by silica gel CC with a *n*-hexane:EtOAc mixture, yielding eight fractions. The AID4-6.4 and AID4-6.6 fractions yielded compound **7** (405 mg) and **4** (6 mg), respectively. The EtOAc fraction (AIA, 5.6 g) was fractionated by silica gel CC with a gradient of *n*-hexane:EtOAc to yield nine fractions (AIA1–AIA9). The AIA4 (3.5 g) fraction was rechromatographed similarly in order to yield thirteen fractions. Compound **1** (8 mg) was isolated from fraction AIA4-11. Thereafter, compound **5** (5 mg) was obtained from fraction AID4-13 (822 mg) by silica gel CC (gradient of CH_2Cl_2:MeOH).

Cell culture and treatments

The RAW 264.7 macrophage cell line was obtained from the ATCC (VA, USA) and cultured under previously described condition (Ventura et al., 2015).

Quantification of NO production

NO production by macrophages was estimated indirectly by the Griess method to measure nitrites concentration and performed as described previously (Park et al., 2009). Fifty microliters of each supernatant (obtained as above) were added to Griess reagent (Sigma Aldrich). After 10 min, the absorbance was measured at 570 nm in plate spectrophotometer (Dinatech MR5000). The nitrite concentration in the supernatant was determined in μg/ml using as reference a sodium nitrite curve decreased from the value obtained with the cell-free additives. L-NMMA (NG-methyl-L-arginine acetate) was used as reference standards.

Culture of mycobacteria and evaluation of bacterial growth

Two strains of mycobacteria, which differ in degree of virulence, were used in this study: strains of *Mycobacterium tuberculosis* (virulent laboratory strain H37Rv, ATCC 27294; and highly virulent Mtb strain Beijing M299 isolated from a TB patient in Mozambique) evaluated for virulence in a previous study (Ribeiro et al., 2014). The mycobacterial strains were grown in suspension of Middlebrook 7H9 broth, containing 10% dextrose albumin complex (ADC), 0.5% glycerol and 0.05% Tween-80 at 37 °C under conditions of containment of Biosafety 3. To study the antimycobacterial activity of the samples, we used the MTT assay to quantify bacterial growth in liquid medium (Moodley et al., 2014). Bacterial suspensions and MTT proceeding were described previously (Ventura et al., 2015). The resulting samples were measured by optical density at 570 nm. Untreated bacterial suspensions were used to control the spontaneous growth of bacteria. Rifampicin was used as a positive control.

Cytotoxicity assay

The MTT assay was used in the cytotoxicity test (Muzitano et al., 2006). Cells were plated at the above concentration and culture condition, in a final volume of 100 μl. After 2 h, for macrophage adhesion, the cells were stimulated or not with 1 μg/ml LPS (*Escherichia coli* Lipopolysaccharide) alone or in combination with the samples evaluated for 24 h. At the end of the incubation, 5 μl of MTT solution (5 mg/ml) were added to each one and 2 h later the plate supernatant was removed, the crystals formed were solubilized by HCl (4 mM) and added to isopropanol. The reading was performed on a plate spectrophotometer at 570 nm.

Statistical analyses

The analysis of variance (ANOVA) was defined, followed by the test of means of Newman–Keuls and Bonferroni, with index of reliability of 95%.

Results and discussion

The limonoid morenolide (**1**) was isolated as a yellow, amorphous powder. Its molecular formula $C_{28}H_{32}O_7$ was deduced by HR-ESI-MS (*m/z* 503.2033 $[M+Na]^+$, $C_{28}H_{32}NaO_7$, calc. *m/z* 503.2046). The ^{13}C-DEPTQ NMR spectrum of **1** displayed carbon signals attributed to five methyls [including one of carbomethoxyl (δ_C 51.7) and one of acetyl (δ_C 21.3) groups], three methylenes (including one terminal methylene sp^2 at δ_C 119.0), eleven methines [including five sp^2 at δ_C 125.4 (CH-2), 147.0 (CH-3), 139.0 (CH-21), 110.5 (CH-22) and 143.0 (CH-23) and three sp^3 monooxygenated at δ_C 68.0 (CH-6), 84.3 (CH-7) and 86.9 (CH-15)], three carbonyl [one conjugated carbonyl ketonic at δ_C 202.5 (C-1), one carbonyl carbomethoxy at δ_C 173.6 (C-12) and one carbonyl acetyl at δ_C 171.1 (C-1′) groups] and six quaternary [including seven sp^2 corresponding to four olefinics at δ_C 140.4 (C-4), 135.2 (C-13), 146.3 (C-14) and 126.8 (C-20)] carbon atoms.

^{1}H NMR spectrum showed three signals corresponding to methyl groups linked to sp^3 carbon atoms at δ_H 1.70 (3H-18, *d*, 1.5 Hz), 1.38 (3H-30, *s*) and 1.12 (3H-19, *s*), one singlet regarding to acetyl group at δ_H 2.16 (3H-2′, *s*) and one singlet at δ_H 3.64 (MeO-12, *s*) attributed to the methoxyl group. The singlet signals at δ_H 5.45 and 5.13 were attributed to a terminal methylene group and the signals at δ_H 7.05 (H-3, *d*, 10.2 Hz) and 5.77 (H-2, *d*, 10.2 Hz) were also used to characterize α,β-unsaturated carbonyl. The presence of hydrogen signals at δ_H 7.34 (H-23, *t*, 1.5 Hz), 7.27 (H-21, *m*), and 6.36 (H-22, *dd*, 1.8, 0.8 Hz) correlated, respectively, in the HSQC with carbon signals at δ_C 143.0 (CH-23), 139.0 (CH-21) and 110.5 (CH-22) were attributed to the furan ring characteristic in limonoids C-*seco* (Ara et al., 1989).

The HMBC spectrum revealed cross-peaks corresponding to heteronuclear long-range coupling ($^2J_{CH}$ and $^3J_{CH}$) of 2H-29 with both CH-3 (δ_C 147.0) and CH-5 (δ_C 44.2); H-3 with C-1 (δ_C 202.5), C-4 (δ_C 140.4) and CH-5 (δ_C 44.2); and H-2 with both C-4 (δ_C 140.4) and C-10 (δ_C 49.3) which enabled us to establish the presence of a carbonyl α,β-unsaturated and terminal methylene group into six-membered ring (ring A). The HMBC spectrum and also cross-peaks of MeO-12 (δ_H 3.64)/δ_C 173.6 (C-12); 2H-11 (δ_H 2.97, 2.31)/δ_C 173.6 (C-12), 36.7 (CH-9); 3H-19 (δ_H 1.12)/δ_C 202.5 (C-1), 49.3 (C-10), 44.2 (C-5) and 36.7 (CH-9); and 3H-30 (δ_H 1.38/δ_C 84.3 (C-7), 47.8 (C-8), 36.7 (C-9) and 146.3 (C-14) indicated ring C cleavage involving the bond C-12 and C-13 to produce the ester function (COOMe). Additional cross-peaks involving H-21/CH-22 and C-20; H-22/C-20, CH-21 and CH-23; H-23/CH-22 and C-20; and H-17/C-13, CH-15, CH_2-16, C-20, CH-21, and CH-22 were also revealed by HMBC, justifying the presence of the furan ring linked to ring D. Additional heteronuclear long-range couplings observed in the HMBC spectrum are presented in Table 1. HSQC and ^{1}H–^{1}H-COSY (Fig. 1) were also used for the complete and unambiguous ^{1}H and ^{13}C chemical shifts assignments.

The relative stereochemistry of **1** was determined through ^{1}H–^{1}H-NOESY spectrum analysis, which revealed significant dipolar–dipolar interactions (NOE) between H-9 and H-5, H-9 with H-15, 3H-19 with H-6, H-6 with 3H-30, and 3H-30 with H-7 summarized in Fig. 1.

17-Hydroxy-sandaracopimar-8,15-dien-11-one (**6**) was obtained as a white amorphous powder. Its molecular formula $C_{20}H_{30}O_2$ was deduced based on LREIMS and HRESIMS spectra which showed, respectively, molecular ion peak at *m/z* 302 $[M^{+\cdot}]$ and a sodium adduct ion at *m/z* 325.2064 $[M+Na]^+$ ($C_{20}H_{30}NaO_2$, calculated value *m/z* 325.2143). The ^{13}C-DEPTQ NMR spectrum of **6** showed carbon signals attributed to three methyl, nine methylene (including one sp^2 at δ_C 116.4 and one sp^3 carbinolic at δ_C 68.8), two methine (including one sp^2 at δ_C 140.7), one carbonyl (conjugated carbonyl ketonic at δ_C 196.8) and five quaternary (including three sp^2 corresponding to two olefinics at δ_C 154.8 and 142.8) carbon atoms. Thus, the expanded molecular formula $(CH_3)_3(CH_2)_7(=CH_2)(CH_2OH)(=CH)(CH)(C=C)(C=O)(C)_3$ is in agreement with the molecular $C_{20}H_{30}O_2$ (six degree of unsaturation), deducted by the LRIEMS e HRESIMS spectra and compatible with a tricyclic pimarane-type skeleton sustaining one conjugated carbonyl group and two double bonds.

^{1}H-NMR spectrum of **6** showed three singlet signals corresponding to methyl groups linked to sp^3 carbon atoms at δ_H 1.18 (3H-20, *s*), 0.92 (3H-18, *s*) and 0.88 (3H-19, *s*). The signals at δ_H 5.67 (H-15, *dd*, 17.5, 10.4 Hz), 5.23 (1H-16a, *d*, 10.4 Hz), and 5.06 (1H-16b, *d*, 17.5 Hz), which revealed respectively heteronuclear correlation via one bond in the HSQC spectrum with carbon signals at δ_C 140.7 (CH-15) and 116.4 (CH_2-16), were used to characterize the presence of a terminal vinyl group; the signal at δ_H 3.41–4.44 (m)/δ_C 68.8 was attributed to an oxygenated methylene (CH_2-17). The presence of hydrogens at δ_H 2.51 (1H-12a, *dd*, 16.5, 2.2 Hz) and 2.34 (1H-12b, *d*, 16.5 Hz) were used to postulate the existence of hydrogens attached to a α-carbon of carbonyl group.

The signals at δ_C 196.8 (C-11), 152.8 (C-8), and 142.8 (C-9) indicated a α,β-unsaturated carbonyl group, which was confirmed and located by HMBC spectrum through heteronuclear long-range interactions between the 2H-12 and C-11, C-13, CH-15 and CH_2-17; 3H-20 with both C-9 and C-10 (δ_C 47.3); and 2H-14 with C-8, C-9 and CH-15. The presence of a vinyl group (CH-15 and CH_2-16) attached to C-13 was confirmed by HMBC correlations of 2H-16 with C-13, CH-15 and CH_2-16. Additional heteronuclear long-range couplings observed in the HMBC spectrum are shown in Table 1.

For the complete and unambiguous ^{1}H and ^{13}C chemical shifts assignments (Table 1) were also used 2D HSQC and ^{1}H–^{1}H-COSY. The relative stereochemistry of **6** was determined through NOESY spectrum analysis in which strong NOEs between H-20 with H-19 and between H-17 with H-12 were observed (Fig. 2).

The seven known additional terpenoids, with four C-*seco* limonoids, nimbinene (**2**) (Kraus and Cramer, 1981), nimbinal (**3**) (Rojatkar et al., 1989), nimbandiol (**4**) (Kraus and Cramer, 1981), and salannin (**5**) (Henderson et al., 1964), and three abiatane diterpenes, nimbidiol (**7**) (Majumder et al., 1987), ferruginol (**8**) (Lee et al., 2005), and 6,7-dehydroferruginol (**9**) (Katoh et al., 2007) were characterized through the comparison of their NMR data with literature values.

In order to verify the biological potential of the samples, the inhibition of NO production, cytotoxic and antimycobacterial activities of compounds **1**–**5**, **7**–**9** were evaluated and the results are shown in Table 2. Compound **6** was not tested as there was no enough mass.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for **1** and **6**, including results obtained by heteronuclear 2D shift-correlated HSQC ($^1J_{HC}$) and HMBC ($^nJ_{HC}$, n = 2 and 3), in $CDCl_3$ as solvent. Chemical shifts (δ, ppm) and coupling constants (*J* in Hz, in parentheses).

	1			**6**		
Position	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC
1	202.5, C	–	3, 19	36.0, CH_2	2.76–2.80 0.82–0.88	20
2	125.0, CH	5.77, *d* (10.2)		18.1, CH_2	1.69–1.76 1.43–1.47	1
3	147.0, CH	7.05, *d* (10.2)	29	41.6, CH_2	1.41–1.48 1.15–1.20	18, 19
4	140.4, C	–	2, 3, 5	33.6, C	–	18, 19
5	44.2, CH	3.40, *d* (11.7)	3, 29, 7,19	52.6, CH	1.02, *dd* (12.1, 1.8)	7, 18, 19, 20
6	68.0, CH	5.43, *dd* (11.7, 3.3)	5, 7	19.0, CH_2	1.64–1.67 1.44–1.48	
7	84.3, CH	4.08, *d* (3.3)	30	35.0, CH_2	2.28, *dd* (19.4, 6.1) 2.18–2.22	5
8	47.8, C	–	9, 16, 30	152.8, C	–	14
9	36.7, CH	2.86, *t* (4.9)	7, 11, 19, 30	142.8, C	–	14, 20
10	49.3, C	–	2, 5, 19	37.3, C	–	20
11	34.6, CH_2	2.97, *dd* (16.1, 5.0) 2.31, *dd* (16.1, 5.0)	9	196.8, C	–	12
12	173.6, C	–	9, 11, 12	45.5, CH_2	2.51, *dd* (16.5, 2.2)2.34, *d* (16,5)	14
13	135.2, C	–	17, 16, 18	43.5, C	–	12, 14, 16
14	146.3, C	–	9, 15, 18, 30	38.3, CH_2	2.40, *d* (17.9)2.21, *dd* (17.9, 1.8)	12
15	86.9, CH	5.54, *m*	16, 17	140.7, CH	5.67, *dd* (17.5, 10.4)	14, 12
16	41.3, CH_2	2.21, *m* 2.06, *m*	17	116.4, CH_2	5.23, *d* (10.4) 5.06, *d* (17,5)	
17	49.4, CH	3.66, *m*	16, 18	68.8, CH_2	3.41–3.44, *m*	12
18	12.9, CH_3	1.70, *d* (1.5)		33.6, CH_3	0.92, *s*	19
19	14.0, CH_3	1.12, *s*	5, 9	21.9, CH_3	0.88, *s*	18
20	126.8, C	–	17, 21, 22, 23	19.3, CH_3	1.18, *s*	
21	139.0, CH	7.27, *m*	23, 22, 17			
22	110.5, CH	6.36, *dd* (1.8, 0.8)	17, 21, 23			
23	143.0, CH	7.34, *t* (1.5)	21, 22			
29	119.0, CH_2	5.45, *s* 5.13, *s*	3, 5			
30	16.8, CH_3	1.38, *s*	9			
1′	171.1, C	–	2′			
2′	21.3, CH_3	2.16, *s*				
MeO	51.7, CH_3	3.64, *s*				

Number of hydrogens bound to carbon atoms deduced by ^{13}C-DEPTQ NMR spectra. Chemical shifts and coupling constants (*J*) obtained for 1D ^{1}H NMR spectra. Superimposed ^{1}H signals are described without multiplicity and chemical shifts deduced by HSQC, HMBC and ^{1}H–^{1}H-COSY spectra. All ^{1}H and ^{13}C chemical shift assignments of **1** were also based on homonuclear ^{1}H–^{1}H-COSY and heteronuclear 2D shift-correlated HSQC ($^1J_{CH}$) and HMBC ($^nJ_{CH}$, n = 2 and 3) NMR.

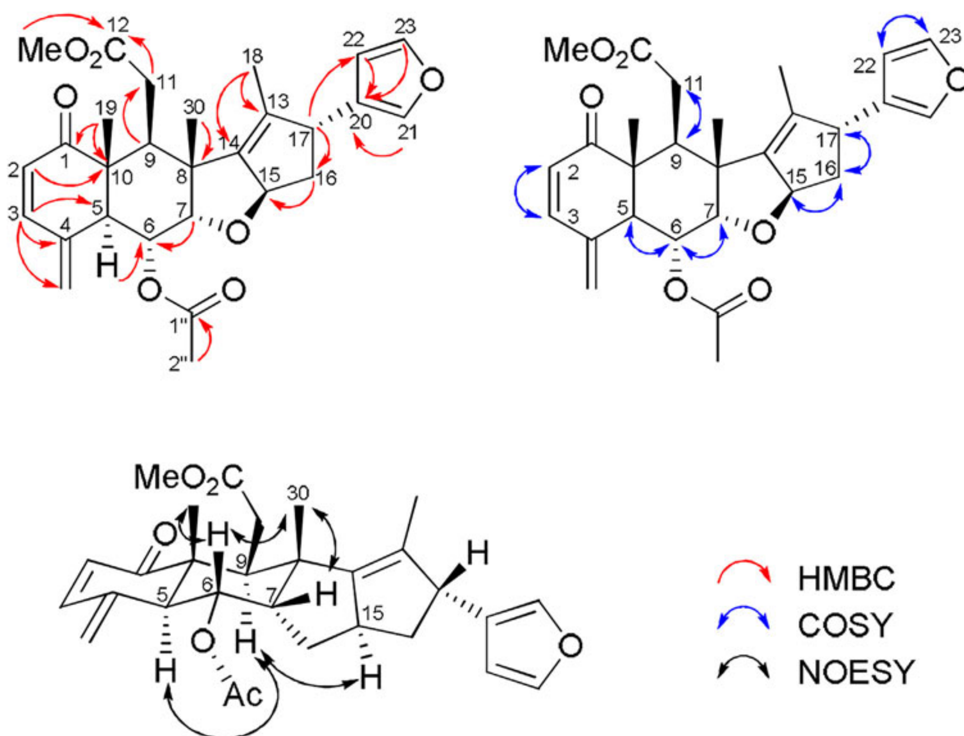

Fig. 1. Key correlations observed in the HMBC, COSY, and NOESY NMR spectra of **1** (in $CDCl_3$).

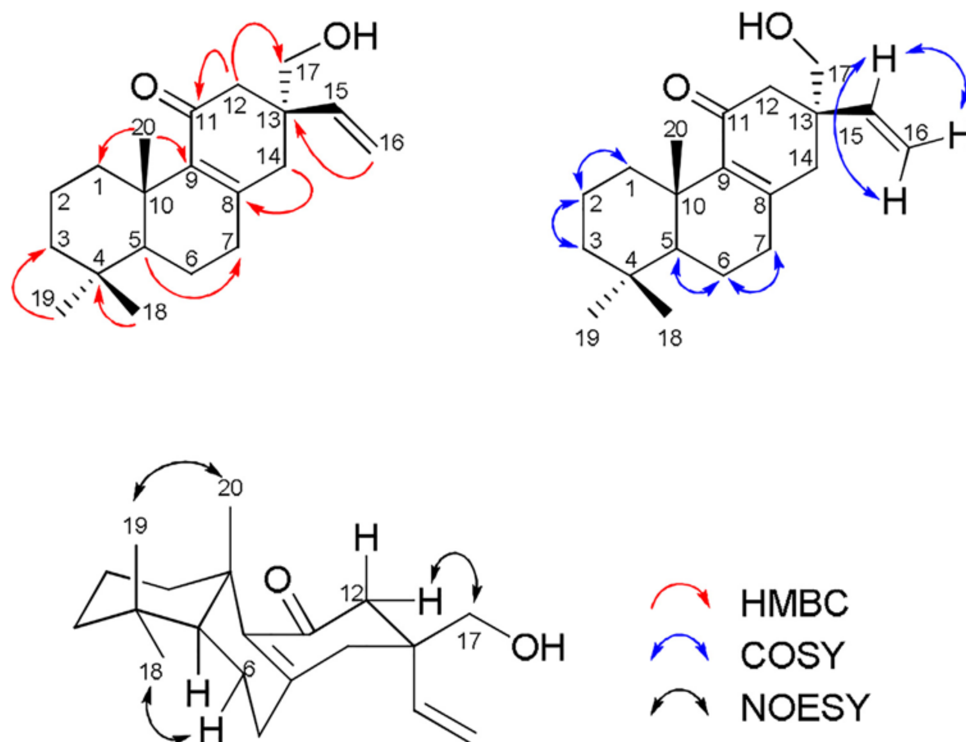

Fig. 2. Key correlations observed in the HMBC, COSY, and NOESY NMR spectra of **6** (in $CDCl_3$).

Table 2
NO inhibition, cytotoxic and antimycobacterial activities of compounds **1**–**5**, **7**–**9**.

	IC_{50} (μg/ml)		MIC_{50} (μg/ml)	
Compound	NO	MTT	Mtb H37Rv	MtbM299
1	1.42 ± 0.2	224.7 ± 0.2	48.7 ± 1.1	>100
2	4.6 ± 0.1	99.21 ± 0.1	>100	>100
3	2.42 ± 0.1	266.8 ± 0.1	>100	>100
4	4.48 ± 0.8	59.11 ± 0.1	35.3 ± 0.7	>100
5	2.74 ± 1.3	1.46 ± 0.4	2.9 ± 1.2	3.4 ± 1.0
7	1.17 ± 0.2	8.48 ± 0.9	9.8 ± 1.3	16.2 ± 0.7
8 and **9**	14.61 ± 0.05	7.63 ± 1.1	3.8 ± 1.4	5.8 ± 1.7
L-NMMA[a]	78.3 ± 6.5	>100	–	–
Rifampicin[b]	–	–	0.2 ± 0.1	1.1 ± 0.1

[a] Standard inhibitor of NO.
[b] Standard anti-TB drug.

Primarily, the inhibitory effects on nitric oxide (NO) production by LPS-stimulated macrophages were evaluated. The results showed that all the compounds studied act as potent inhibitors of NO (with IC_{50} values below 15 μg/ml), with compounds **1**, **3**, **5** and **7** showing more pronounced inhibitory activity.

To verify if the samples showed cytotoxicity, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed with the use of commercial lactate dehydrogenase (LDH) kit. The results showed that compounds **1**–**4** caused low cytotoxicity, while the others were cytotoxic. Of particular note are the compounds **1** and **3** which, besides being strong anti-inflammatory agents, due to the fact that they can significantly inhibit NO production, they also have almost no cytotoxic effect.

The compounds were further evaluated for antimycobacterial activity against the strains of *Mycobacterium tuberculosis* H37Rv (virulent laboratory strain) and M299 (isolated hypervirulent Beijing). Compound **1** was shown to be promising for growth inhibition of the Mtb strain H37Rv, showing low MIC_{50} (48.7 μg/ml) and no cytotoxicity interference, exhibiting IC_{50} of 224.7 μg/ml. Compounds **5**, **7**–**9**, despite having a good response in inhibition of both virulent and super virulent strains, were highly cytotoxic according to the MTT assay.

Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

MSP, ARCJ and SIB contributed in collecting plant sample and identification, confection of herbarium and running the laboratory work. LLGV, SDC, TLBV and EL contributed to biological studies. RBF and IJCV analysis of the NMR and MS data. All the authors have read the final manuscript and approved the submission.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2004.08.011.

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