HETEROCYCLES, Vol. 60, No. 7, 2003, pp. 1709 - 1715

Received, 2nd May, 2003, Accepted, 30th May, 2003, Published online, 2nd June, 2003

DIOSPOLYSAPONIN A, A NEW POLYOXYGENATED SPIROSTANOL SAPONIN FROM THE TUBERS OF *DIOSCOREA POLYGONOIDES*

Jaime Niño Osorio,[a] Oscar M. Mosquera Martinez,[a] Yaned M. Correa Navarro,[a] Yoshihiro Mimaki,[b,*] Hiroshi Sakagami,[c] and Yutaka Sashida [b]

[a] Laboratorio de Biotecnología-Productos Naturales, Escuela de Tecnología Química, Universidad Tecnológica de Pereira, La Julita, A. A. 97 Pereira, Colombia
[b] Laboratory of Medicinal Plant Science, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1, Horinouchi, Hachioji, Tokyo 192-0392, Japan
[c] Department of Dental Pharmacology, Meikai University School of Dentistry, 1-1, Keyaki-dai, Sakado, Saitama 350-0283, Japan

Abstract — Chemical investigation of the tubers of *Dioscorea polygonoides* has resulted in the isolation of a novel polyoxygenated spirostanol saponin, named diospolysaponin A (**1**), along with a known spirostanol saponin (**2**). The structure of **1** was determined on the basis of spectroscopic analysis and by enzymatic hydrolysis followed by chromatographic and spectroscopic studies to be (23*S*,25*R*)-12β,14β,17β,23-tetrahydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside. The cytotoxic activity of **1** and **2** and their aglycons against HSC-2 cells is briefly presented.

INTRODUCTION

Dioscorea plants are known as a rich source of diosgenin and its related steroidal sapogenins and saponins,[1] which are used as precursors for the commercial production of corticosteroids, contraceptive hormones, and steroidal pharmaceuticals.[2,3] *Dioscorea polygonoides* Humb. et Bonpl. (Dioscoreaceae) is distributed from Mexico to Brazil crossing by Colombia. Although *D. polygonoides* has been suggested to abundantly contain steroidal sapogenins,[4] most of which are considered to be present as glycosides in the plant, their chemical structures have not been disclosed. The present investigation on the steroidal saponins of *D. polygonoides* tubers has resulted in the isolation of a novel polyoxygenated spirostanol saponin, named diospolysaponin A (**1**), along with a known spirostanol saponin (**2**). This paper mainly reports the structural elucidation of **1** on the basis of spectroscopic analysis and by enzymatic hydrolysis followed by chromatographic and spectroscopic studies. The cytotoxic activity of **1** and **2** and their aglycons against HSC-2 human oral squamous cell carcinoma cells is briefly discussed.

RESULTS AND DISCUSSION

The MeOH extract of *D. polygonoides* was partitioned between *n*-BuOH saturated with H_2O and H_2O. A series of chromatographic separations of the *n*-BuOH-soluble portion gave diospolysaponin A (**1**) and compound (**2**). Compound (**2**) was identified as (25*R*)-spirost-5-en-3β-yl (diosgenin) *O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.[5]

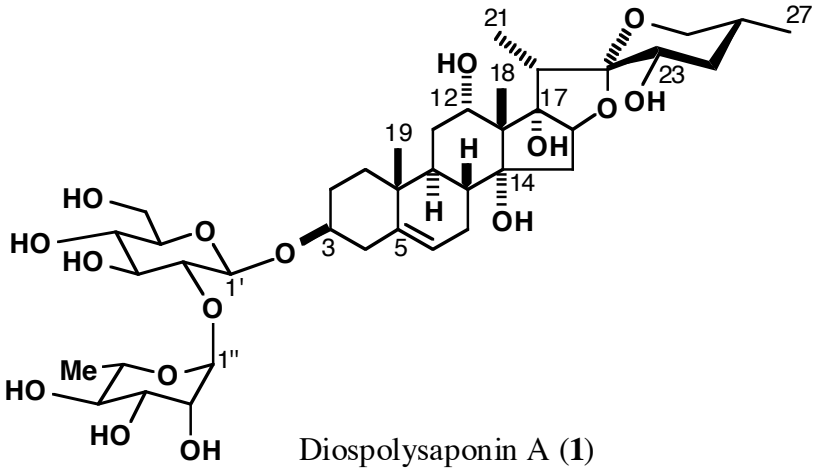
Diospolysaponin A (**1**) was obtained as an amorphous solid with a molecular formula of $C_{39}H_{62}O_{16}$, as determined by the data of the positive-ion HR-ESI-MS (*m/z* 809.3936 $[M + Na]^+$, Δ +0.6 mmu) and the ^{13}C-NMR spectrum (39 carbon signals). The ^1H-NMR spectrum of **1** contained two three-proton singlet signals at δ 1.13 and 1.05 and two three-proton doublet signals at δ 1.38 (*J* = 7.1 Hz) and 0.69 (*J* = 5.8 Hz), and the ^{13}C-NMR spectrum showed an acetal carbon signal at δ 112.2, suggesting **1** to have a spirostanol skeleton. Furthermore, the ^1H-NMR spectrum exhibited two anomeric proton signals due to monosaccharides at δ 6.38 (d, *J* = 1.4 Hz) and 5.03 (d, *J* = 7.3 Hz), as well as a three-proton doublet signal at δ 1.78 (*J* = 6.2 Hz), which was associated with a methyl carbon signal at δ 18.7 by the HMQC spectrum, indicating that one of the sugars is a 6-deoxyhexose. Enzymatic hydrolysis of **1** with naringinase gave a new steroidal sapogenin (**1a**), and D-glucose and L-rhamnose as the carbohydrate moieties. The monosaccharides, including their absolute configurations, were identified by direct HPLC analysis of the hydrolysate, which was performed on an aminopropyl-bonded silica gel column using MeCN-H_2O (17:3) as solvent system, with detection being carried out using a combination of RI and optical rotation (OR) detectors.

The sapogenin (**1a**) was analyzed for $C_{27}H_{42}O_7$ by the combined positive-ion HR-ESI-MS (*m/z* 501.2800 $[M + Na]^+$, Δ -2.8 mmu) and the ^{13}C-NMR spectrum (27 carbon signals). The deduced molecular formula of **1a** was higher by four oxygen atoms than that of **2**, and the ^1H-NMR spectrum of **1a** measured in DMSO-d_6 showed signals for five exchangeable protons at δ 6.02 (br), 5.31 (s), 4.87 (s), 4.58 (d, *J* = 4.5 Hz), and 4.50 (d, *J* = 8.0 Hz), which disappeared on the addition of the vapor of HCl. These data indicated that **1a** has two tertiary and three secondary hydroxyl groups. The gross structure of **1a** was established by the following spectroscopic analysis. The multiplet proton signal centered at δ 3.81 ($W_{1/2}$= 21.4 Hz) was shown to be coupled with two methylene groups [δ 2.03 and 1.73 (H_2-2); δ 2.64 and 2.59 (H_2-4)] and was assigned to the H-3 axial proton geminally bearing an equatorial-oriented hydroxyl group. The olefinic carbon signals at δ 140.8 (C) and 121.8 (CH) were unequivocally assigned to C-5 and C-6, respectively. In the HMBC spectrum **1a**, the three-proton singlet signal at δ 1.02 showed a long-range correlation with the C-5 carbon and was assigned to Me-19. Consequently, the other methyl singlet at δ 1.15 was assignable to Me-18, which exhibited $^3J_{C,H}$ correlations with two quaternary carbons at δ 88.3 and 94.1, each bearing a hydroxyl group. On the other hand, a methylene group and an oxymethine group adjacent to it with an ABX-spin system at δ 2.47 and 1.75 (each dd, *J* = 12.4, 7.2 Hz), and 5.14 (t, *J* = 7.2 Hz) were attributable to H_2-15 and H-16, respectively, while a methyl group and a methine group adjacent to it with an A_3M-spin system at δ 1.38 (d, *J* = 7.1 Hz) and 3.48 (q, *J* = 7.1 Hz) were assignable to Me-21 and H-20. In the HMBC spectrum, long-range correlations were observed from δ 2.47 to δ 88.3 and 94.1, δ 1.75 to δ 88.3, and from δ 5.14, 3.48, and 1.38 to δ 94.1. The above data were consistent with the presence of a hydroxyl group at C-14 and C-17. The downfield-shifted triplet signal at δ 4.52 (*J*

Table 1. ^{1}H- and ^{13}C-NMR spectral data for **1a** in C_5D_5N

position	^{1}H		J (Hz)	^{13}C
1 eq	1.79			37.8
ax	1.13	td	13.3, 3.8	
2 eq	2.03			32.5
ax	1.73			
3	3.81	br m	21.4 [a)]	71.1
4 eq	2.64	dd	12.6, 5.1	43.4
ax	2.59	t	12.6	
5	-			140.8
6	5.45	br d	4.9	121.8
7 a	2.68			25.9
b	1.82			
8	2.09			36.0
9	2.02			39.3
10	-			36.9
11 eq	1.89			29.2
ax	1.88			
12	4.52	t	2.7	76.5
13	-			49.1
14	-			88.3
15 □	2.47	dd	12.4, 7.2	39.9
□	1.75	dd	12.4, 7.2	
16	5.14	t	7.2	91.8
17	-			94.1
18	1.15	s		21.9
19	1.02	s		19.4
20	3.48	q	7.1	40.3
21	1.38	d	7.1	9.2
22	-			112.1
23	3.92	dd	11.2, 4.8	67.6
24 eq	2.07			38.2
ax	1.77			
25	1.80			31.5
26 eq	3.44	t	10.9	65.9
ax	3.41			
27	0.67	d	6.0	16.8

[a)] $W_{1/2}$



Diospolysaponin A (**1**)

= 2.7 Hz), which was coupled with methylene protons at δ 1.89 and 1.88 (H_2-11), showed HMBC correlations with the C-13 (δ 49.1) and C-14 quaternary carbon signals, confirming the presence of a hydroxyl group at C-12. The remaining one hydroxyl group was presumed to be located at the ring-F part. The three-proton doublet signal at δ 0.67 (J = 6.0 Hz) attributable to Me-27 showed a spin-coupling correlation with the broad multiplet centered at δ 1.80, which was unambiguously assigned to H-25 and exhibited correlations with oxymethylene protons at δ 3.44 and 3.41, and with methylene protons at δ 2.07 and 1.77. The methylene protons, in turn, showed correlations with the terminal hydroxymethine proton at δ 3.92 (dd, J = 11.2, 4.8 Hz). These subsequent correlations led us to confirm the locus of a hydroxyl group at C-23. Thus, the planar structure of **1a** was established.

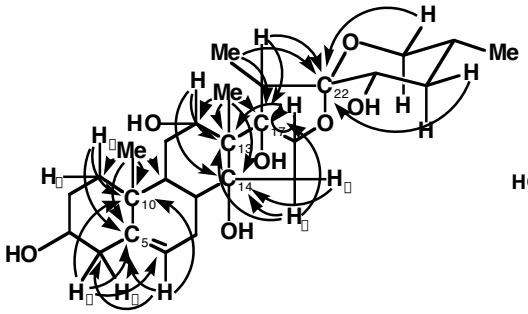


Figure 1. HMBC correlations of **1a**

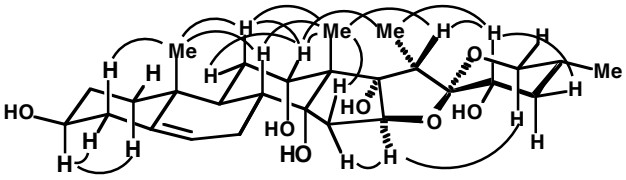


Figure 2. NOE correlations of **1a**

The NOE correlations and proton spin-coupling constants made the relative stereochemistry assignable. The 12β, 14β, and 17β configurations of the hydroxyl groups, as well as the usual spirostanol ring junctions and configurations of B/C *trans*, C/D *trans*, D/E *cis*, C-20β, and C-22β, in **1a**, were confirmed by the following NOEs: H-8 (δ 2.09)/Me-18 and Me-19, H-11ax (δ 1.88)/Me-18 and Me-19, H-12/H_2-11, Me-18 and Me-21, H-15β (δ 2.47)/H-16, H-15β (δ 1.75)/Me-18, H-16/H-26ax, and Me-18/H-20. The 12β-hydroxyl configuration was ascertained by the coupling constants between H_2-11 and H-12 ($^3J_{H\text{-}12,H\text{-}11ax}$ = 2.7 Hz and $^3J_{H\text{-}12,H\text{-}11eq}$ = 2.7 Hz). The coupling constants, $^3J_{H\text{-}23,H\text{-}24ax}$ = 11.2 Hz, $^3J_{H\text{-}23,H\text{-}24eq}$ = 4.8 Hz, and $^3J_{H\text{-}25,H\text{-}26ax}$ = 10.9 Hz, gave evidence for the C-23*S* and C-25*R* configurations. The signal assignable to H-9 (δ 2.02) was shifted downfield by more than 1.0 ppm compared with that of diosgenin, and the signal due to H-16 (δ 5.14) was also moved downfield by about 0.5 ppm compared with that of 17β-hydroxydiosgenin (pennogenin).[6] These phenomena were considered to be caused by the 1,3-diaxial effects between the H-9 proton and the two hydroxyl groups at C-12β and C-14β, and between the H-16 proton and the hydroxyl group at C-14β. The structure of **1a** was thus assigned as (23*S*,25*R*)-spirost-5-ene-3β,12β,14β,17β,23-pentol.

The ^{13}C-NMR resonances for the diglycoside moiety of **1** composed of a β-D-glucopyranosyl unit and an α-L-rhamnopyranosyl unit, which were assigned by the combined use of the ^{1}H-^{1}H COSY, TOCSY, and HMQC spectra, indicated that it was identical to that of **2**. This was ascertained by the observation of a $^3J_{C,H}$ correlation from the anomeric proton of the rhamnosyl group at δ 6.38 to C-2 of the glucosyl residue at δ 77.9. The diglycoside was confirmed to be linked to the C-3 hydroxyl group of the aglycon by a long-

range correlation between the anomeric proton of the glucosyl at □5.03 and the C-3 carbon of the aglycon at □ 77.7. Accordingly, the complete structure of **1** was characterized as (23*S*,25*R*)-12□,14□,17□,23-tetrahydroxyspirost-5-en-3□-yl *O*-□-L-rhamnopyranosyl-(1□ 2)-□-D-glucopyranoside.

Diospolysaponin A (**1**) is a newly described spirostanol saponin and unique in structure having four hydroxyl groups at C-12, C-14, C-17, and C-23 in addition to the C-3 glycosyloxy group.

The cytotoxic activity of **1** and **2**, and their aglycons (**1a** and diosgenin) against HSC-2 cells was evaluated. Compound (**2**) has been reported to show cytotoxic activities against several cultured tumor cells.[6,7] It was also cytotoxic to HSC-2 cells (IC_{50} 3.4 □g/mL) as potent as doxorubicin (IC_{50} 2.5 □g/mL) used as a positive control. Diosgenin was found to show a moderate cytotoxic activity with an IC_{50} value of 20.8 □g/mL. Compounds (**1**) and (**1a**) did not show any apparent cytotoxicity even at the sample concentration of 100 □g/mL.

EXPERIMENTAL

Optical rotations were measured using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 (500 MHz for ^{1}H-NMR, Karlsruhe, Germany) spectrometer using standard Bruker pulse programs. Chemical shifts are given as □ values with reference to tetramethylsilane (TMS) as an internal standard. ESI-MS were recorded on a Micromass LCT (Manchester, UK) mass spectrometer. Silica gel 60 (Merck, Darmstadt, Germany), octadecylsilanized (ODS) silica gel (Aldrich, Milwaukee, WI, USA), and Sephadex LH-20 (Pharmacia, Uppsala, Sweden) were used for column chromatography. TLC was carried out on precoated Silica gel 60 F_{254} (0.25 mm thick, Merck) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution in absolute EtOH, followed by heating. HPLC was performed using a system comprised of a Tosoh CCPM pump (Tokyo, Japan), a Tosoh CCP PX-8010 controller, a Tosoh RI-8010 detector, a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne injection port with a 20 mL sample loop. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA); penicillin and streptomycin sulfate (Meiji-Seika, Tokyo, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA). All other chemicals used were of biochemical reagent grade.

Plant material

Dioscorea polygonoides was collected in the fields of Aranzazu, Caldas, Colombia in October of 1998. The plant was identified by one of the authors (J. N. O.), and the plant specimen has been deposited in the herbarium of the Universidad de Antioquia, Medellín, Colombia (voucher no. HUA 132745).

Extraction and isolation

A dried powder (1.0 kg) of *D. polygonoides* tubers was extracted with MeOH (5 L) at 95 °C for 3 h twice. After removal of the solvent by evaporation, the viscous extract was partitioned between *n*-BuOH saturated with H_2O and H_2O. The *n*-BuOH-soluble portion (42.1 g) was chromatographed on silica gel and elution with $CHCl_3$-MeOH with increasing amount of MeOH to give eight fractions (Fr. I—Fr. VIII). Fr. VII

(9.1 g) was further separated by a silica gel column using CH_2Cl_2-MeOH gradients into eight subfractions (Fr. VIIa—Fr. VIIh). Subfraction VIIe (979 mg) was subjected to ODS silica gel column chromatography eluting with $CHCl_3$-MeOH-H_2O (2:7:1) to give **2** (100 mg). Subfraction VIIh (2.3 g) was chromatographed on silica gel eluting with $CHCl_3$-MeOH-H_2O (77:21:2) to give four fractions (Fr. VIIh-1—Fr. VIIh-4). Fr. VIIh-2 (67 mg) wad subjected to column chromatography on ODS silica gel eluting with MeOH-H_2O (4:3) to afford crude **1** with impurities. It was purified by Sephadex LH-20 column chromatography eluting with MeOH and ODS silica gel column chromatography with dioxane-Me_2CO-H_2O (8:2:15) and MeCN-H_2O (3:7) to furnish **1** (35 mg) in pure form.

Diospolysaponin A (1): amorphous solid; $[\alpha]_D$ -61.3° (*c* = 0.10, $CHCl_3$-MeOH (1:1)); positive-ion HR-ESI-MS *m/z* 809.3936 $[M + Na]^+$ (Calcd 809.3930 for $C_{39}H_{62}O_{16}Na$); IR (film) ν_{max} 3376 (OH), 2955, 2928 and 2870 (CH), 1053 cm^{-1}; ^{1}H-NMR (C_5D_5N) δ 6.38 (1H, d, *J* = 1.4 Hz, H-1''), 5.38 (1H, br d, *J* = 4.8 Hz, H-6), 5.15 (1H, t, *J* = 7.2 Hz, H-16), 5.03 (1H, d, *J* = 7.3 Hz, H-1'), 5.00 (1H, dq, *J* = 9.3, 6.2 Hz, H-5''), 4.81 (1H, dd, *J* = 3.4, 1.4 Hz, H-2''), 4.63 (1H, dd, *J* = 9.3, 3.4 Hz, H-3''), 4.52 (1H, dd, *J* = 11.9, 2.3 Hz, H-6'a), 4.49 (1H, br s, H-12), 4.36 (1H, t, *J* = 9.3 Hz, H-4''), 4.35 (1H, dd, *J* = 11.9, 5.4 Hz, H-6'b), 4.30 (1H, t, *J* = 9.0 Hz, H-3'), 4.27 (1H, dd, *J* = 9.0, 7.3 Hz, H-2'), 4.17 (1H, t, *J* = 9.0 Hz, H-4'), 3.92 (1H, dd, *J* = 10.9, 4.6 Hz, H-23), 3.90 (2H, overlapping, H-3 and H-5'), 3.47 (1H, q, *J* = 7.1 Hz, H-20), 3.43 (2H, br s, H_2-26), 1.78 (3H, d, *J* = 6.2 Hz, Me-6''), 1.38 (3H, d, *J* = 7.1 Hz, Me-21), 1.13 (3H, s, Me-18), 1.05 (3H, s, Me-19), 0.69 (3H, d, *J* = 5.8 Hz, Me-27); ^{13}C-NMR (C_5D_5N) δ 37.6 (C-1), 30.2 (C-2), 77.7 (C-3), 39.0 (C-4), 139.8 (C-5), 122.6 (C-6), 26.0 (C-7), 36.1 (C-8), 39.3 (C-9), 37.1 (C-10), 29.2 (C-11), 76.5 (C-12), 49.1 (C-13), 88.3 (C-14), 39.9 (C-15), 91.8 (C-16), 94.2 (C-17), 21.9 (C-18), 19.3 (C-19), 40.4 (C-20), 9.2 (C-21), 112.2 (C-22), 67.7 (C-23), 38.3 (C-24), 31.6 (C-25), 66.0 (C-26), 16.9 (C-27), 100.3 (C-1'), 77.9 (C-2'), 79.7 (C-3'), 71.9 (C-4'), 78.3 (C-5'), 62.7 (C-6'), 102.1 (C-1''), 72.6 (C-2''), 72.9 (C-3''), 74.2 (C-4''), 69.5 (C-5''), 18.7 (C-6'').

Enzymatic hydrolysis of 1

A mixture of **1** (14.6 mg) and naringinase (51.9 mg) (Sigma) was dissolved in EtOH (3 mL) and AcOH/AcOK buffer (pH 4.3, 5 mL), which was incubated at rt for 24 h. The reaction mixture was passed through a Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan) column eluting initially with MeOH-H_2O (2:3) and then with Me_2CO-EtOH (1:1) to give a sugar fraction and an aglycon fraction. The aglycon fraction was subjected to column chromatography on silica gel eluting with $CHCl_3$-MeOH (20:1) to give **1a** (7.5 mg).

Compound (1a): amorphous solid; $[\alpha]_D$ -58.0° (*c* = 0.10, $CHCl_3$-MeOH (1:1)); positive-ion HR-ESI-MS *m/z* 501.2800 $[M + Na]^+$ (Calcd 501.2828 for $C_{27}H_{42}O_7Na$); IR (film) ν_{max} 3388 (OH), 2952, 2931 and 2873 (CH), 1051 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 6.02 (br), 5.31 (s), 4.87 (s), 4.58 (d, *J* = 4.5 Hz), and 4.50 (d, *J* = 8.0 Hz); ^{1}H-NMR (C_5D_5N) and ^{13}C-NMR (C_5D_5N), see Table 1.

Identification of monosaccharides

The sugar fraction obtained by enzymatic hydrolysis of **1** was passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA), which was then analyzed by HPLC under the following conditions: column, Capcell Pak NH_2 UG80 (4.6 mm i.d. x 250 mm, 5 μm, Shiseido, Tokyo, Japan); solvent, MeCN-H_2O

(17:3); flow rate, 0.9 mL/min; detection, RI and OR. Identification of L-rhamnose and D-glucose was carried out by comparison of their retention times and optical rotations with those of authentic samples. t_R (min): 9.21 (L-rhamnose, negative optical rotation), 19.87 (D-glucose, positive optical rotation).

Cell culture and assay for cytotoxic activity

HSC-2 cells were maintained as monolayer cultures at 37 ℃ in DMEM supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate in a humidified 5% CO_2 atmosphere. Cells were trypsinized and inoculated at 6 x 10^3 per each 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, San Jose, CA, USA), and incubated for 24 h. After washing once with PBS, they were treated with 24 h without or with test compounds. They were washed once with PBS and incubated for 4 h with 0.2 mg/mL MTT in DMEM medium supplemented with 10% FBS. After the medium was removed, the cells were lysed with 0.1 mL DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using Labsystems Multiskan^R (Biochromatic, Helsinki, Finland) connected to a Star/DOT Matrix printer JL-10. The IC_{50} value, which reduces the viable cell number by 50%, was determined from the dose-response curve.[8,9]

ACKNOWLEDGMENTS

We would like to thank to COLCIENCIAS and the Universidad Tecnológica de Pereira for their financial support to the project No. 1110-05-515-96; 202-96.

REFERENCES

1. S. B. Mahato, A. N. Ganguly, and N. P. Sahu, *Phytochemistry*, 1982, **21**, 959.
2. A. Merkli, P. Christen, and I. Kapetanidis, *Plant Cell Rep.*, 1997, **16**, 632.
3. T. Ikenaga, R. Handayani, and T. Oyama, *Plant Cell Rep.*, 2000, **19**, 1240.
4. F. Martin, *Econ. Bot.*, 1969, **23**, 373.
5. Y. Mimaki, O. Nakamura, Y. Sashida, T. Nikaido, and T. Ohmoto, *Phytochemistry*, 1995, **38**, 1279.
6. Y. Mimaki, M. Kuroda, Y. Obata, Y. Sashida, M. Kitahaka, A. Yasuda, N. Naoi, Z. W. Xu, M. R. Li, and A. N. Lao, *Nat. Prod. Lett.*, 2000, **14**, 357.
7. T. Nakamura, C. Komori, Y. Y. Lee, F. Hashimoto, S. Yahara, T. Nohara, and A. Ejima, *Biol. Pharm. Bull.*, 1996, **19**, 564.
8. S. Furuya, F. Takayama, Y. Mimaki, Y. Sashida, K. Satoh, and H. Sakagami, *Anticancer Res.*,2000, **20**, 4189.
9. S. Furuya, F. Takayama, Y. Mimaki, Y. Sashida, K. Satoh, and H. Sakagami, *Anticancer Res.*, 2001, **21**, 959.