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A Spirostanol Glycoside from Wild Yam (*Dioscorea villosa*) Extract and Its Cytostatic Activity on Three Cancer Cells

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ABSTRACT

A spirostanol glycoside, (25R)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl -(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside, was isolated from wild yam (*Dioscorea villosa*) extract with reversed-phase preparative HPLC. This was the first time found in yams (*Dioscorea species*). The structure was identified by LC-MS, ^{1}H- and ^{13}C-NMR. Cytostatic activities of the isolated saponin on Hep G2, HEK293 and MCF7 cells were estimated. Growth of these cells was inhibited as higher concentration of the compound was used. The IC_{50} values of the isolated spirostanol glycoside and its aglycon, diosgenin, were 9.02 and 23.91 μM for Hep G2 cells, 13.21 and 27.31 μM for HEK293 cells, and 16.74 and 35.38 μM for MCF7 cells, respectively. The isolated compound had significantly higher active intensity than diosgenin.

Key words: *Dioscorea villosa*, diosgenin, isolation, spirostanol glycoside, yam, cytostatic activity, Hep G2 cells, HEK293 cells, MCF7 cells

INTRODUCTION

Furostanol and spirostanol glycosides, two main steroidal saponins, have been found in many types of yams (*Dioscorea species*), e.g. *Dioscorea floribund*[(1)], *D. prazeri*[(2)], *D. composita*[(3)], *D. zingiberensis*[(4)], *D. olfersiana*[(5)], *D. colletti* var. *hypoglauca*[(6,7)], and *D. pseudojaponica* Yamamoto[(8)].

Many reports indicated that steroidal saponins have hemolytic[(9,10)], hypocholesterolemic[(11,12)], hypoglycemic[(13)], anti-thrombotic[(10,14)], anti-antineoplastic[(6,7)], antiviral[(15)] and anti-cancer[(16-18)] activities. Diosgenin (the aglycon part of the yam steroidal saponin) obtained after hydrolysis of yam saponins is used as industrial starting material for the partial synthesis of steroidal drugs, e.g. progesterone and testosterone[(19-21)].

In this investigation, a major steroidal saponin was isolated by preparative HPLC from the wild yam (*Dioscorea villosa*) extract, which was usually used as an herbal dietary supplement. Collins-Burow *et al*.[(22)] indicated that flavonoid phytochemicals have estrogenic activities and could affect HEK293 and MCF7 cells proliferation. Steroidal saponin contains the estrogenic effect as well[(19-21)]. In addition, Huang *et al*.[(18)] reported that steroidal saponins of *Paris vtetnamensts* (Takht.) had cytotoxic activity for Hep G2. Therefore, we also evaluated the cytostatic activities of the isolated saponin and diosgenin on Hep G2, HEK293, and MCF7 cells.

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MATERIALS AND METHODS

I. *Chemicals and Standards*

Diosgenin (MW = 414) was purchased from Sigma Co. (St. Louis, MO, USA). Spirostanol glycoside (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (MW = 884) was isolated in our laboratory from wild yam extract (dissolved in 55-60% alcohol) of Herb Pharm Inc. (Williams, OR, USA). Acetic acid, acetonitrile (ACN), hydrochloric acid (HCl), ethanol (EtOH), isopropanol (IPA), and methanol (MeOH) were obtained from Tedia Co. (Fairfield, OH, USA). NMR solvent, *pyridine-d*$_5$, was purchased from Merck Co. (Darmstadt, Germany). Deionized water was prepared by Ultrapure^{TM} water purification system (Lotun Co., Ltd. Taipei, Taiwan) and degassed under vacuum after filtering through a 0.22-μm membrane filter (nylon) prior to use. Sodium hydroxide (NaOH) was purchased from Showa Chemical Co. (Tokyo, Japan). Minimal essential medium (MEM), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from GiBCO BRL (Gland Island, NY, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), penicillin, phosphate-buffered saline (PBS), and streptomycin were obtained from Sigma Co. (St. Louis, MO, USA).

II. *Isolation of the Spirostanol Glycoside*

The yam extract (10 mL) was filtered through a syringe

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filter (0.45 μm) (Millipore Co., Bedford, MA) followed by spirostanol glycoside purification with preparative HPLC system consisted of a *PrimeLine*™ Model 500G HPLC pump (Analytical Scientific Instruments, Inc., El Sobrante, CA, USA) and an UV/Vis detector (Schambeck SFD GmbH, Bad Honnef, Germany). Saponin separation method was based on that of our pervious report[8]. Separation was carried out on a preparative Cosmogel C18 column (250 mm × 20 mm i.d., 5 μm) (Nacalai Tesque Inc., Kyoto, Japan). Colbox column oven (Hipoint Scientific Co., Kaohsiung, Taiwan) was set at 45°C, and isocratic separation was done using a mixture of MeOH/H_2O (79/21, v/v) to yield the major spirostanol glycoside of yam extract at a flow rate of 5 mL/min with 203 nm detection. Each injection of yam extract was 0.2 mL and a total of 46 mg of the compound could be collected.

III. *Identification of the Spirostanol Glycoside*

The purified spirostanol glycoside was identified by MS and NMR. The mass spectra were conducted on a VG Platform II LC-MS (Micromass Co., Cheshire, UK) operated in the ESI positive ion mode. The source temperature was set at 200°C and cone voltage at 40 eV. The spectra of ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR, including 2-dimensional NMR [correlated spectroscopy (COSY), heteronuclear multiple bond coherence (HMBC), heteronuclear multiple-quantum coherence (HMQC), nuclear Overhauser spectroscopy (NOESY)] were recorded on a Bruker DMX-500 MHz FT-NMR (Bruker Co., Karlsruhe, Germany). The optical rotation was measured with a Jasco DIP-181 polarimeter (Jasco International Co. Ltd., Tokyo, Japan). The melting point was surveyed on a Yanaco MP-S3 micro-melting point apparatus (Yanaco Analytical Instruments Co., Kyoto, Japan).

The purified spirostanol glycoside: amorphous solid; $[\alpha]D^{24}$ –80.4° (pyridine; *c* 0.1); m.p. 285-286°C (dec.); ESI^+-MS, *m/z*, 907 $[M+Na]^+$, 885 $[M+H]^+$, 739 $[(M+H)\text{-Rha}]^+$, 723 $[(M+H)\text{-Glc}]^+$, 577 $[(M+H)\text{-Glu-Rha}]^+$, 415 $[(M+H)\text{-2}\times \text{Glc-Rha}]^+$. ^{1}H NMR (500 MHz, *pyridine-d_5*) for the aglycon moiety: δ = 0.71 (3H, d, J = 5.1 Hz, CH_3-27), 0.84 (3H, s, CH_3-18), 1.06 (3H, s, CH_3-19), 1.15 (3H, d, J = 6.9 Hz, CH_3-21), 3.88 (1H, m, H-3), 5.34 (1H, br d, J = 5.3 Hz, H-6); for the sugar moiety: δ = 1.78 [3H, d, J = 6.1 Hz, CH_3-6″ of the terminal rhamnose attached to the C-2′ of the inner glucose], 4.97 (1H, d, J = 7.1 Hz, H-1′ of the inner glucose attached to C-3 of the aglycon), 5.15 [1H, d, J = 8.1 Hz, H-1‴ of terminal glucose attached to the C-4′ of the inner glucose], 6.43 [1H, br s, H-1″ of the terminal rhamnose attached to the C-2′ of the inner glucose]. ^{13}C-NMR data (125 MHz, *pyridine-d_5*) are showed in Table 1.

IV. *Acid Hydrolysis*

The method was based on that related to Taylor *et al.*[23]. The purified compound (1 mg) was added to 2 mL of 2 N HCl (prepared with MeOH) and heated at 80°C for 2 hr in a sealed tube. After hydrolysis, neutralization with 4 N aqueous NaOH and MeOH elimination in a rotary evaporator at 35°C, the residue was extracted three times with 2 mL of hexane for 3 min each time. The aqueous fraction was exploited for sugar composition analysis by high-performance anion-exchange chromatography joined with pulsed amperometric detection (HPAEC-PAD). The analytical system was a Bioscan 817 Metrohm IC system (Metrohm, Herisau, Switzerland) including IC pump 709, injection valve unit 812 with a 20 μL loop, and an electrochemical detector (gold working electrode: E_1 = 0.05 V, 0.44 s; E_2 = 0.80 V, 0.18 s; E_3 = -0.30 V, 0.36 s). A 250 mm × 4 mm i.d. CarboPac PA1 column (Dionex, Sunnyvale, CA, USA) was used and isocratic elution was done at 1 mL/min with 10 mM NaOH mixed with 2 mM $Ba(OAc)_2$[23]. The retention times (t_R) of glucose and rhamnose were 6.75 min and 5.30 min, respectively. The hexane extracts were combined and then removed solvent in a rotary evaporator.

Table 1. ^{13}C-NMR (500 MHz) data for the isolated steroidal glycoside (in pyridine-d_5) (δ in ppm)

Carbon	Aglycon moiety	Carbon	Sugar moiety
1	38.0	3-O-Glc	
2	30.6	1′	100.5
3	78.8	2′	77.8
4	39.4	3′	76.7
5	141.3	4′	82.5
6	122.3	5′	78.2
7	32.8	6′	62.4
8	32.2		
9	50.8	Rha (1→2)	
10	37.6	1″	102.3
11	21.6	2″	72.9
12	40.3	3″	73.3
13	41.0	4″	74.6
14	57.1	5″	69.9
15	32.7	6″	19.1
16	81.6		
17	63.4	Glc (1→4)	
18	16.8	1‴	105.7
19	19.9	2‴	75.5
20	42.5	3‴	79.0
21	15.5	4‴	71.7
22	109.7	5‴	78.8
23	32.3	6‴	62.6
24	29.8		
25	31.1		
26	67.3		
27	17.8		

Afterwards, the residue was dissolved in 1 mL of MeOH for diosgenin determination (t_R = 12.1 min) with HPLC. The method was referred to that reported by Yang *et al*.[8] A *PrimeLine*™ Model 500G HPLC pump system (Analytical Scientific Instruments, Inc., El Sobrante, CA, USA) with a 250 mm × 4.6 mm i.d., 5 μm, Luna C18 column (Phenomenex, Torrance, CA) and a ELSD 2000 evaporative light-scattering detector (Alltech Associates Inc., Deerfield, Ireland) at a tube temperature of 75°C and air flow rate of 2.2 L/min were employed. The mobile phase was ACN/H_2O (95/5, v/v) at a flow rate of 1 mL/min.

V. *Cell Culture and Assay for Cytostatic Activity*

Hep G2 (human hepatocellular carcinoma, ATCC No. HB-8065), HEK293 (human embryonic kidney epithelial cell line, ATCC No. CRL-1573) and MCF7 (human breast carcinoma cell line, ATCC No. HTB-22) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HEK293 and MCF7 cells grew in MEM; Hep G2 was maintained in DMEM. Both the mediums contained 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). All cells were washed with PBS, resuspended in the above suitable mediums (2×10^4 cells/ mL) and then placed in each well of 24-well flat-bottom plates (0.98 mL/well). The cells were incubated in 5% CO_2/air for 24 hr at 37°C. After incubation, 0.02 mL of EtOH-H_2O (1/9, v/v) solution containing diosgenin or the isolated compound was added to give the final concentrations of 10, 20, 50 and 100 μM and 0.02 mL of EtOH-H_2O (1/9, v/v) was added into control wells of each kind of cells. The cells were further incubated for 72 hr in the presence of each agent, followed by evaluation of cell growth using a MTT assay[25]. That is, after termination of cell cultures, 100 μL of MTT (0.5 mg/mL) in PBS was added to each well and the plates were further incubated for another 4 hr at 37°C. The metabolized product of MTT, blue crystal formazan, was dissolved by isopropanol. The proportion of surviving cells was determined at 563 nm. A dose response curve was plotted for each compound and the concentration giving 50% inhibition (IC_{50}) was calculated. Triplicate analyses were conducted to yield the mean values. Data were subjected to Student's t-test for statistical analysis.

RESULTS AND DISCUSSION

The major spirostanol glycoside in wild yam extract was separated by reversed-phase preparative HPLC. Its peak could be resolved while 79% aqueous MeOH was used as the mobile phase (Figure 1).

I. *Identification of the Purified Compound*

The purified compound was identified with ESI^+-MS and ^{1}H-, ^{13}C-NMR. The analyses of diosgenin and sugar after acid hydrolysis were executed to identify the structure.

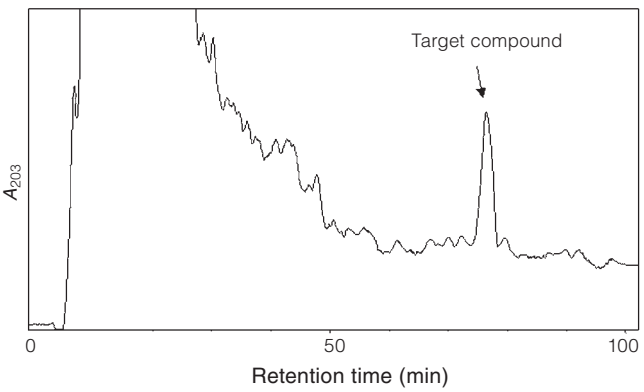

Figure 1. Separation of major spirostanol glycoside in wild yam (*Dioscorea villosa*) extract by reversed-phase preparative HPLC.

The compound crystallized from MeOH was a white powder. The molecular formula $C_{45}O_{17}H_{72}$ was estimated by the ESI^+-MS spectrum: *m/z* at 907 and 885 correspond to $[M+Na]^+$ and $[M+H]^+$ peaks, indicating that its molecular weight might be 884. Other significant peaks at 739 $[(M+H)-146]^+$, 723 $[(M+H)-162]^+$, 577 $[(M+H)-146-162]^+$ and 415 $[(M+H)-146-2\times162]^+$ conform to the losses of one deoxyhexose and one to two hexoses.

^{1}H-NMR data of the compound demonstrated diagnostic signals of two tertiary methyl groups [δ 0.84, 1.06, (s)] and two secondary methyl groups [δ 0.71 (d, J = 5.1 Hz); 1.15 (d, J = 6.9 Hz)] corresponding to the angular methyl groups of a steroidal sapogenin. The signal corresponding to an olefinic proton at δ 5.34 (br d, J = 5.3 Hz) could be ascribed to 5,6-unsaturation. In case of the sugar moiety, three anomeric hydrogen signals [δ 4.97 (d, J = 7.1 Hz), 5.15 (d, J = 8.1 Hz), 6.43 (br s)] implied the presence of three monosaccharide. The secondary methyl group signal at δ 1.78 (d, J = 6.1) might result from the deoxyhexose. The proof was supported farther by the ^{13}C-NMR data of the isolated compound (Table 1): signals of the angular methyl groups (δ 15.5, 16.7, 17.8, 19.9) corresponded to a steroidal sapogenin; olefinic carbons on the aglycon were also confirmed by signals at δ 122.3 and 141.3. ^{13}C-NMR data further indicated structure of the sugar moiety: anomeric carbons (δ 100.5, 102.3, 105.7) and a methyl group (δ 19.1) from the one deoxyhexose. The ^{13}C-NMR signal at δ 109.7 indicated the existence of a spiroketal carbon. The compound is clearly a spirostene trioligoside based on the above spectral data.

From a comparison of the ^{1}H-NMR and ^{13}C-NMR signals of the aglycon moiety of the separated compound (Table 1) with those showed in the literatures[6-8,26], the aglycon structure was affirmed as (25R)-spirost-5-en-3β-ol (diosgenin). Comparison of the ^{13}C-NMR data and the anomeric protons on ^{1}H-NMR of the sugar moiety of the compound with that presented in the reports[27,28]

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revealed the existence of a 2,4-substituted β-d-glucose, a terminal β-d-glucose and a terminal α-l-rhamnoses. The β anomeric configuration for glucose was judged by the large J_{H1-H2} value (7.1 Hz, 8.1 Hz) (> 7.0 Hz). The α anomeric configuration for rhamnose was concluded by its C_5 data (δ 69.9).

Through HMBC spectrum of the isolated compound, the anomeric proton signals at δ 4.97 (H-1′ of the 2, 4-substituted glucose), 5.15 (H-1″ of the terminal glucose) and 6.43 (H-1″ of the terminal rhamnose) displayed cross-peaks with the carbon signals at δ 78.8 (C-3 of the aglycon), 82.5 (C-4′ of the 2, 4-substituted glucose) and 77.8 (C-2′ of the 2,4-substituted glucose), respectively (Figure 2). Thus, the compound was ascertained as (25R)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl -(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside. This spirostanol glycoside is found in yams for the first time. It has been extracted from the roots of *Trillium kamtschatcense*, named trilloside A[29] and from Allium narcissiflorum[30].

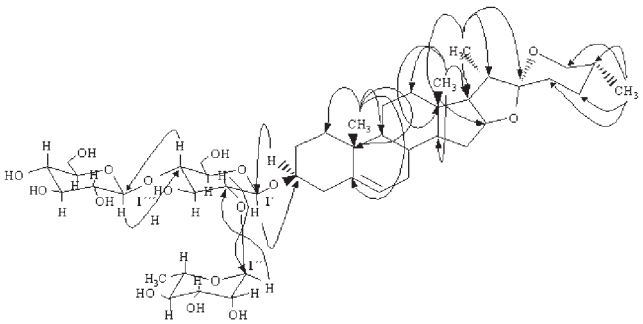

Figure 2. HMBC analysis for the isolated spirostanol glycoside.

II. *Acid Hydrolysis*

Diosgenin, glucose and rhamnose could be detected following hydrolysis of the purified spirostanol glycoside. The ratio of glucose and rhamnose was 2/1. These results confirmed the compound structure as identified above. The spirostanol glycoside (90.2%) was converted to diosgenin, which was similar to the conversions of diosin and gracillin from *D. pseudojanonica* Yamamoto, i.e. 90.4% and 90.1%, respectively[8].

III. *Assay for Cytostatic Activity*

Cytostatic activities of the isolated steroidal glycoside and its aglycon (diosgenin) on Hep G2, HEK293 and MCF7 cells were evaluated. Figure 3 indicates that the isolated saponin could inhibit growths of three kinds of cells and the activity could be raised with the increasing concentration. While 2, 5, 10, 20 and 50 μM of the isolated compound was used, the mortalities were 27.8, 45.8, 60.7, 72.1 and 87.5% for HepG2 cells, 7.8, 17.5, 59.8, 71.1 and 85.5% for HEK293 cells, and 19.8, 34.5, 44.0, 68.0 and 83.5% for MCF7 cells, respectively. Although diosgenin could also inhibit growths of these cells, it has much lower effect than the isolated compound. Comparing with the control, 2 μM of the isolated compound was sufficient to inhibit the growths of Hep G2 and MCF7 cells significantly ($p < 0.05$). However, growth of HEK 293 cells did not present significant inhibition until 5 μM of the compound was used ($p < 0.05$). Table 2 shows that the IC_{50} values of the isolated saponin and diosgenin were 9.02 and 23.91 μM for Hep G2 cells, 13.21 and 27.31 μM for HEK293 cells, and 16.74 and 35.38 μM for MCF7 cells, respectively.

Hu *et al.*[6,7] determined the bioactivities of furostanol and spirostanol glycosides of *D. colletti*i var. *hypoglauca* against K562 cells. The IC_{50} values were 1.6-6.6 μM for

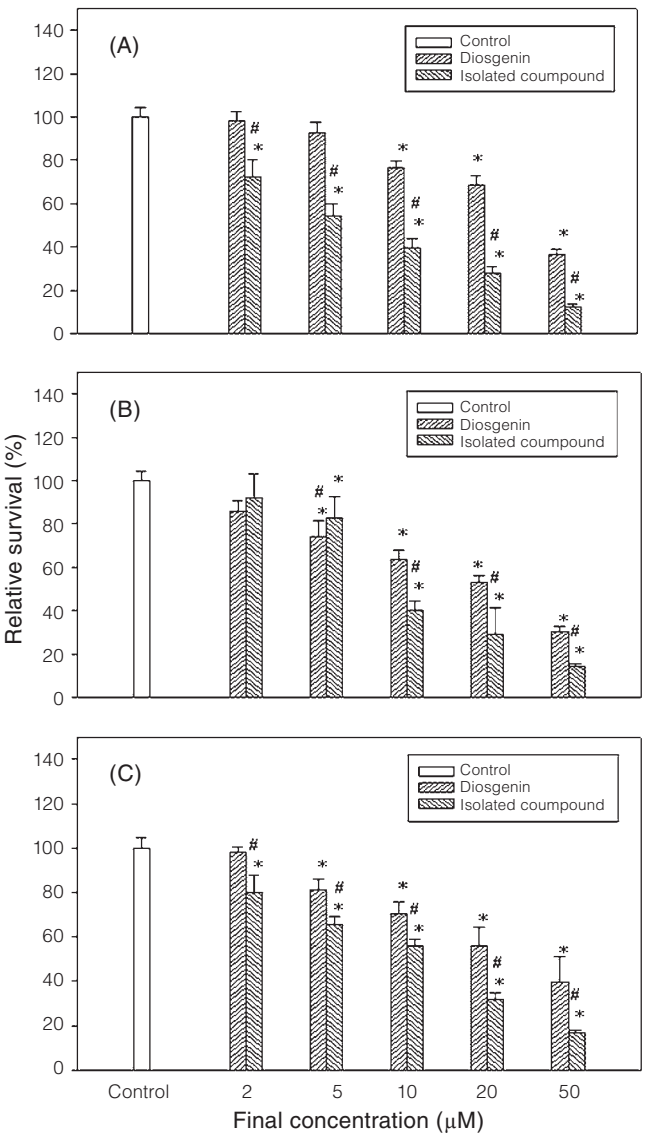

Figure 3. Effect of the isolated steroidal glycoside and diosgenin on survivals of Hep G2 (A), HEK293 (B) and MCF7 (C) cells. The survivals were determined by MTT assay at 4 hr after addition of various concentrations of these compounds. (*): Compared with control ($p < 0.05$). (#): Difference between diosgenin and isolated compound ($p < 0.05$).

Table 2. *In vitro* cytostatic activities (IC_{50}) of diosgenin and the isolated saponin

Compound	IC_{50} (μM)		
	Hep G2	HEK293	MCF7
Isolated saponin	9.02	13.21	16.74
Diosgenin	23.91	27.31	35.38

furostanol glycosides and 1.2-7.0 μM for spirostanol glycosides, respectively. Mimaki *et al*.[30] isolated furostanol and spirostanol glycosides from the rhizomes of *Hosta sieboldii* and studied their cytostatic activity on leukaemia HL-60 cells. They found that the IC_{50} values of furostanol and spirostanol glycosides were 3.0-5.9 μg/mL and 2.8-8.2 μg/mL, respectively. Many reports indicated that saponin (glycoside) had higher biological functions than sapogenin (aglycon)[11,13,30,32]. The sugar group could affect the bioactivity of functional compound[30,31,33]. Our results showed that isolated steroidal glycoside had higher cytostatic activity than diosgenin.

CONCLUSIONS

(25*R*)-Spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside, the first isolated spirostanol glycoside from yam, had higher cytostatic activity than diosgenin for Hep G2, HEK293 and MCF7 cells. Anti-inflammatory activity and induced apoptosis in these three cancer cells of the wild yam saponin and *D. pseudojaponica* Yamamoto (cultivated in Taiwan) saponins will be investigated in the near future.

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