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Effects of different storage conditions on steroidal saponins in yam (*Dioscorea pseudojaponica* Yamamoto) tubers

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Abstract

Effect of storage on steroidal saponins, furostanol and spirostanol glycosides, in yam (*D. pseudojaponica* Yamamoto) tubers was determined. Unpeeled and vacuum-sealed peeled tubers were stored at −18, 4, 17 and 25 °C from 5 to 80 days separately. Furostanol glycosides in unpeeled tubers could be converted by furostanol glycoside 26-*O*-*β*-glucosidase (F26G) to spirostanol glycosides after 4 °C storage for 35 days (chilling injury could be found), or 17 and 25 °C storage for 50 days. The conversion increased with storage times. Peeled tubers stored at 17 and 25 °C for 5 days could experience organoleptic injury, which would enlarge with increasing storage period. After 35 days of storage, large part of vacuum-sealed tubers transformed to juice with stench. In the early 20 days storage, saponins in these tubers were lost rapidly. F26G activity in decreasing order was 25 °C > 17 °C > 4 °C and could be inhibited under vacuum.

Keywords: Furostanol glycoside; Furostanol glycoside 26-*O*-*β*-glucosidase; Spirostanol glycoside; Steroidal saponin; Yam (*Dioscorea* spp.); Storage

1. Introduction

Yam (*Dioscorea* spp.) is one of the principal staple foods in numerous tropical countries (Hariprakash & Nambisan, 1996). It is also widely used in traditional Chinese medicine to promote health and longevity (Liu, Wang, Shyu, & Song, 1995). Because of its health profit, yam has been a popular food in Taiwan for many years. Steroidal saponins, furostanol and spirostanol glycosides, exist in many kinds of yams, for instance, *Dioscorea floribunda* (Hoyer, Sucrow, & Winkler, 1975), *D. composita* (Espejo, Campos, Jung, & Giral, 1982), *D. zingiberensis* (Tang & Jiang, 1987), *D. olfersiana* (Haraguchi, Zaccharias, Young, & Chu, 1994), *D. colletti* var. *hypoglauca* (Hu, Dong, Yao, Kobayashi, & Iwasaki, 1996; Hu, Yao, Kobayashi, & Iwasaki, 1997), *D. pseudojaponica* Yamamoto (Yang, Lu, & Hwang, 2003a) and *D. polygonoides* (Osorio et al., 2005). They were considered to be the major functional compounds in the crops (Hu et al., 1996, 1997; Liu et al., 1995). Many literatures reported that steroidal saponins had notable anti-carcinogenic (Hu, Lin, Liu, & Yang, 2007), anti-thrombotic (Zhang et al., 1999), anti-viral (Aquino et al., 1991), hemolytic (Zhang et al., 1999), hypocholesterolemic (Sauvaire, Ribes, Baccou, & Loubatieŕes-Mariani, 1991) and hypoglycemic (Kato, Miura, & Fukunaga, 1995) capacities. The aglycon part (sapogenin) of the yam steroidal saponins namely diosgenin has been used as starting material for semi-synthesis of steroidal hormones such as progesterone and testosterone (Chen & Wu, 1994).

Afoakwa and Sefa-Dedeh (2001) investigated chemical compositions and quality changes occurring in trifoliate yam (*D. dumetorum* pax) tubers after harvesting. The harvested tubers were stored under cold room condition (4 °C) and tropical ambient condition (28 °C) for 24–72 h. Their results showed that moisture content decreased about 6–10%, starch levels declined from 70.5 to 66.5 g/100 g, and sugar and fibre contents slightly increased within 72 h after harvesting. Moreover, textural properties, namely hardness

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and adhesiveness, also increased considerably during storage. Under the same storage conditions, Afoakwa and Sefa-Dedeh (2002) pointed out that storage could cause decreases in the rheological properties (paste characteristics) of the starch in trifoliate yam tuber within 36 h of harvesting; similarly, α- and β-amylase activities in the tubers more than doubled within 24 h after harvesting. Samples stored at 4 °C showed smaller changes in both rheological properties and amylase activities than those stored at 28 °C in all experiments. Medoua, Mbome, Agbor-Egbe, and Mbofung (2005) investigated the effect of storage under tropical ambient conditions (19–28 °C, RH 60 ~ 85%) for 56 days on the physicochemical characteristics of flours produced from trifoliate yam tubers, and found that water absorption capacity, oil absorption capacity, water-soluble index, hydrophilic–lipophilic index, swelling capacity and least gelatinizing concentration were significantly influenced by tuber storage time.

Dinan, Harmatha, and Lafont (2001) reported that the age, the cultivar or the geographic locality of a plant could significantly affect its saponin content. The storage condition after plant harvesting might influence the saponin level as well. There was, however, no thorough report concerning the effects of various storage conditions on saponins in yam. Yang et al. (2003a) determined three furostanol glycosides and three spirostanol glycosides in *D. pseudojaponica* Yamamoto, which is the Taiwanese native yam cultivar. Wang and Liu (1992) reported that the quality of yam tubers could be maintained well for 26 weeks when they were stored at 17 °C.

In general, there are two selling forms of yam tubers in the Taiwanese market, whole tubers (at ambient temperature, 25 °C under atmosphere condition) and vacuum-sealed peeled tubers (at 4 or −18 °C). In this study, we investigated the changes of steroidal saponins in yam (*D. pseudojaponica* Yamamoto) tubers under different storage conditions. The unpeeled tubers (under atmosphere condition) and vacuum-sealed peeled tubers were stored at −18, 4, 17 and 25 °C from 5 to 80 days, respectively.

2. Materials and methods

2.1. Yam samples and their preparation

Yam (*D. pseudojaponica* Yamamoto) tubers (white flesh and cortex), cylindrical shape (ca. 4 cm in diameter and ~120 cm long), were randomly harvested (matured) on January 15, 2007 from a farm in Keelung City, Taiwan and divided into two main groups. All tubers were cleaned with distilled water, wiped with sterilized dry cloth and then cut into sticks of 25 cm long and ca. 4 cm in diameter. The sticks in each group were also randomly divided into 87 portions of about 300 g each. A total of 84 portions was used for storage (21 portions of the tuber sticks were randomly sampled for each storage condition) while the other 3 portions were used for control. One group of tuber sticks was further peeled and vacuum-sealed (using polyethylene bags) with a vacuum packaging machine (model: TH-250, Dah Yeou Industrial Co., Ltd., Taichung, Taiwan). The other group was preserved as whole tuber sticks in a normal atmosphere. The cortex was about 2.2% of total tuber weight. After these tubers were stored at −18, 4, 17 and 25 °C for 5, 10, 20, 35, 50, 65 and 80 days separately, 3 portions in each condition were took out randomly and cut the tuber sticks into 4 mm thick slices using a Salad Shooter (National Presto Industries, Eau Claire, WI, USA) (the sticks with cortices were peeled in advance). These slices were lyophilized with a freeze-dryer (Vastech Scientific Co., Ltd., Taipei, Taiwan) and ground to 40 mesh or below for saponin analysis.

2.2. Chemicals and materials

Three furostanol glycosides including 26-*O*-β-D-glucopyranosyl 3β, 22, 26-trihydroxy-(25*R*)-furost-5-ene-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside (**1**) (MW = 1194), protodioscin (**2**) (MW = 1048) and protogracillin (**3**) (MW = 1064) were obtained through heating 26-*O*-β-D-glucopyranosyl-22α-methoxyl-(25*R*)-furost-5-en-3β, 26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside (**4**) (MW = 1208), methyl protodioscin (**5**) (MW = 1062) and methyl protogracillin (**6**) (MW = 1078) in 30% aqueous acetone at 95 °C for 4 h, separately (these solutions were then concentrated to dryness) with the method of Matsurra, Ushiroguchi, Itakura, and Fuwa (1989). Compounds **4**~**6** (furostanol glycosides), and three spirostanol glycosides including (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*- {[α-L-rhamnopyranosy-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside (**7**) (MW = 1014), dioscin (**8**) (MW = 868) and gracillin (**9**) (MW = 884), were isolated from the yam tubers with our previous reported method (Yang et al., 2003a). Diosgenin standard was purchased from Sigma (St. Louis, MO, USA). Fig. 1 shows the structures of these steroidal saponins and diosgenins. Methanol and *n*-butanol were obtained from Tedia Co. (Fairfield, OH, USA). Deionized water was prepared by Ultrapure™ water purification system (Lotun Co., LTD. Taipei, Taiwan). Crude furostanol glycoside 26-*O*-β-glucosidase (F26G) was prepared from the fresh yam tuber using the method reported by Inoue and Ebizuka (1996).

2.3. Hydrolysis of furostanol glycosides with yam crude F26G in the model system

The method was based on that reported by Inoue and Ebizuka (1996). Sample vials (25 ml) contained 10 mL of 200 μM compound **1**, **2** or **3** (prepared with 50 mM acetate buffer solution, pH 5.0) were added 3 mL of crude F26G followed by reaction at 4, 17 and 25 °C for 1, 2, 3 and 5 days, respectively. These reactions progressed under atmosphere and vacuum (with vacuum sealing) conditions separately. In order to reduce the interference of dissolved

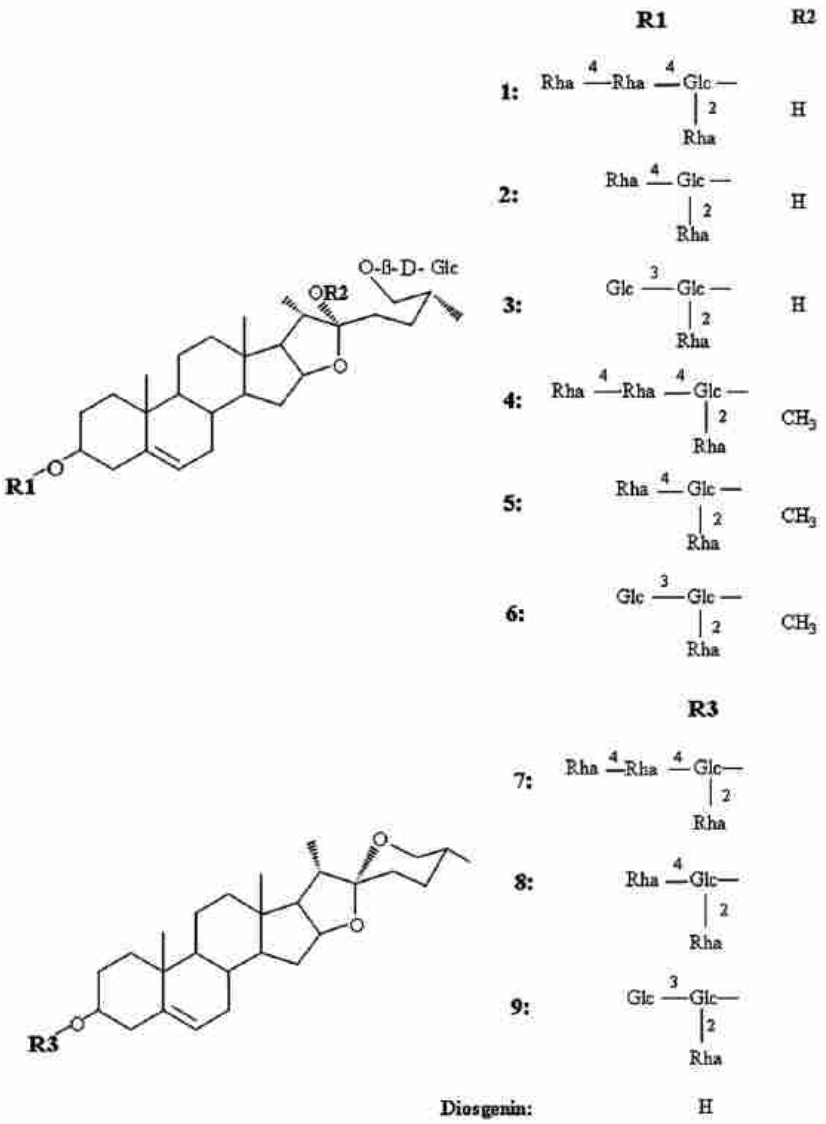

Fig. 1. Structures of furostanol and spirostanol glycosides of the yam tuber (*D. pseudojaponica*).

oxygen, the deionized water was degassed rigorously before use. As we could see, these two reaction conditions would result in different enzyme activities to the furostanol glycosides conversion.

2.4. Extraction of yam saponins

Saponins in yam tuber powder were extracted with the method of Yang, Lu, and Hwang (2003b). A 40 g of the powder was extracted with 1 L of methanol at ambient temperature (25 °C) for 24 h followed by filtration and concentration in a rotary evaporator at 30 °C. The extracted residue was suspended in 25 mL of distilled water and then partitioned against 25 mL of *n*-butanol 3 times to yield saponin extract. After washing the extract with 50 mL of distilled water for 3 times, *n*-butanol was removed using a rotary evaporator at 45 °C. Yam saponins in model system were extracted as following: 10 mL of *n*-butanol was added to each vial to extract the saponins for 3 times after enzyme hydrolysis. The combined extract (30 mL) was washed with 30 mL of distilled water 3 times and then concentrated to dryness. Each dried extract was dissolved in 1 mL of methanol for HPLC analysis.

2.5. HPLC analysis of yam saponins

Yam saponins were analyzed with a *PrimeLine*™ Gradient Model 500G HPLC pump system (Analytical Scientific Instruments, Inc., El Sobrante, CA, USA) and an Alltech 3300 evaporative light scattering detector (ELSD) (Alltech

Associates Inc., Deerfield, Ireland) (tube temperature, 75 °C; air flow rate, 2.8 L/min). The analytical condition was similar to that reported by Yang et al. (2003b). A Luna C18 column (4.6 mm i.d. × 250 mm, 5 μm particle size) (Phenomenex, Torrance, CA, USA) kept at 45 °C in a Colbox column oven (Hipoint Scientific Co., Kaohsiung, Taiwan) and a step gradient solvent system mixing methanol and deionized H_2O, 62/38 (v/v) from 0 to 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min, were used to separate saponins. A Chem-Win computer software system (Shuen-Hua Co., Taipei, Taiwan) was employed to process data.

2.6. Statistical analysis

The quantitative analyses of the yam saponins were carried out in triplicate and the mean values were calculated. Statistical analyses of the data were executed by the analysis of variance and least significant difference test (LSD) procedure was used to determine significance between means, at a level of $p < 0.05$.

3. Results and discussion

3.1. Changes of steroidal saponins in yam tubers under various storage conditions

Compounds **1-3** were the natural furostanol glycosides in yams. They could be methylated at position C-22 to form compounds **4-6** while treated with methanol during the procedures of extraction and isolation (Inoue & Ebizuka 1996; Hu et al., 1997). The original contents of compounds **1-3** and **7-9** in the tuber fleshes were 50.42, 53.32, 27.79 μg/g dw and 31.14, 43.13, 20.12 μg/g dw, respectively (see footnote a in Tables 1 and 2). In the investigation, unpeeled and peeled yam tubers were stored under atmospheric and vacuum-sealed conditions separately. During 80 days of storage, the content and composition of furostanol and spirostanol glycosides in peeled and unpeeled tubers at −18 °C, and peeled tubers at 4 °C did not show significant differences in level. The unpeeled tubers stored at 17 and 25 °C could also maintain saponin content and composition until 35 days later. After 50–80 days of storage, all furostanol glycosides in these tubers were reduced significantly as storage period increased, whereas the spirostanol glycosides showed opposite results. The changes of steroidal saponins in the unpeeled tubers stored at 25 °C were larger than those stored at 17 °C. There were no pronounced changes in saponin content and composition when the tubers (with cortices) were stored at 4 °C within 20 days. These tubers presented obvious chilling injury after they were stored for 35 days. The injury became more and more serious as storage time increased. Three furostanol glycosides and three spirostanol glycosides in these tubers also showed significant decreases and increases, respectively after 35–80 days storage. The longer the tubers were stored, the more the changes of steroidal saponins took place.

Table 1
The contents of furostanol glycosides in yam tubers after storing at various temperatures from 5 to 80 days

Compound[a]	Storage day	Content of saponins (μg/g dw)[b] Storage temperature of yam tuber (°C)								LSD[c] at 0.05 probability
		Tuber with cortex & atmosphere storage				Tuber without cortex & vacuum storage				
		−18	4	17	25	−18	4	17	25	
	5	50.51 ± 3.41	50.31 ± 2.72	51.20 ± 3.08	50.65 ± 1.94	50.31 ± 2.58	50.07 ± 2.58	48.34 ± 3.57	49.03 ± 4.12	
	10	49.32 ± 2.54	49.84 ± 2.80	50.11 ± 2.54	50.28 ± 3.16	49.84 ± 1.93	50.28 ± 2.15	40.32 ± 2.25	38.68 ± 3.08	
	20	51.13 ± 3.01	49.50 ± 2.63	49.99 ± 3.31	49.76 ± 2.37	51.13 ± 2.87	49.87 ± 2.87	17.44 ± 2.08	13.65 ± 1.84	
1	35	50.24 ± 3.25	47.03 ± 3.02	50.20 ± 2.06	49.09 ± 2.94	50.24 ± 2.98	50.10 ± 2.98	–	–	1.03
	50	49.83 ± 4.04	43.42 ± 3.22	49.31 ± 4.01	47.29 ± 1.35	50.06 ± 3.61	50.06 ± 3.61	–	–	
	65	49.89 ± 3.32	37.13 ± 4.12	48.53 ± 2.89	46.82 ± 4.11	49.83 ± 3.32	49.83 ± 3.32	–	–	
	80	49.77 ± 3.54	30.41 ± 3.71	47.66 ± 3.42	44.86 ± 3.46	49.68 ± 3.02	49.91 ± 3.02	–	–	
	5	53.41 ± 3.72	53.30 ± 3.65	52.86 ± 3.72	53.28 ± 1.92	53.22 ± 2.86	53.41 ± 1.42	51.82 ± 3.92	51.37 ± 4.04	
	10	54.09 ± 2.77	53.28 ± 2.80	53.37 ± 2.77	54.09 ± 2.77	52.99 ± 3.61	53.25 ± 3.01	42.85 ± 2.25	38.44 ± 3.34	
	20	53.07 ± 4.12	52.91 ± 2.63	54.02 ± 4.12	53.37 ± 3.42	53.10 ± 2.64	53.35 ± 2.82	20.04 ± 1.76	15.15 ± 1.31	
2	35	53.26 ± 2.94	49.25 ± 3.02	53.19 ± 2.98	52.67 ± 5.02	53.41 ± 3.72	54.01 ± 3.53	–	–	1.41
	50	52.96 ± 1.99	44.80 ± 3.22	53.36 ± 1.79	50.92 ± 4.31	53.16 ± 4.52	52.90 ± 3.22	–	–	
	65	53.06 ± 3.68	37.44 ± 4.12	50.36 ± 1.61	48.86 ± 2.90	52.87 ± 3.06	53.41 ± 1.99	–	–	
	80	52.89 ± 4.21	31.51 ± 3.71	49.93 ± 4.09	46.72 ± 4.04	53.18 ± 3.39	53.15 ± 2.76	–	–	
	5	28.04 ± 2.16	27.27 ± 2.42	27.55 ± 1.88	27.60 ± 2.29	28.10 ± 2.14	27.73 ± 3.26	26.19 ± 2.58	25.75 ± 3.07	
	10	27.57 ± 3.24	28.02 ± 3.34	27.60 ± 1.63	27.73 ± 3.18	27.61 ± 3.83	27.94 ± 1.71	19.37 ± 1.75	18.04 ± 1.39	
	20	27.38 ± 1.94	27.47 ± 2.55	27.83 ± 3.46	27.81 ± 1.64	28.02 ± 4.21	27.55 ± 2.09	1.45 ± 0.18	1.01 ± 0.16	
3	35	27.71 ± 1.33	24.65 ± 2.81	27.45 ± 4.05	27.69 ± 3.11	27.66 ± 2.56	27.62 ± 3.20	–	–	1.63
	50	27.27 ± 4.15	19.69 ± 1.94	26.51 ± 3.40	25.53 ± 2.84	27.27 ± 3.75	27.48 ± 2.66	–	–	
	65	27.55 ± 2.42	13.29 ± 1.38	25.72 ± 2.76	24.42 ± 4.13	27.52 ± 3.38	27.55 ± 4.00	–	–	
	80	27.20 ± 3.38	7.94 ± 0.82	24.33 ± 3.61	22.11 ± 3.52	27.39 ± 2.52	27.35 ± 1.57	–	–	

[a] The original contents of compounds **1**~**3** in yam tuber flesh (control) were 50.42 ± 2.34, 53.32 ± 3.72, 27.79 ± 1.57 μg/g dw, respectively.
[b] Values are mean ± S. D. obtained by trizplicate analyses; – = no determined.
[c] Least significant difference: Difference of two means between treatments including controls exceeding this value is significant ($p < 0.05$).

Table 2
The contents of spirostanol glycosides in yam tubers after storing at various temperatures from 5 to 80 days

Compound[a]	Storage day	Content of saponins (μg/g dw)[b] Storage temperature of yam tuber (°C)								LSD[c] at 0.05 probability
		Tuber with cortex & atmosphere storage				Tuber without cortex & vacuum storage				
		−18	4	17	25	−18	4	17	25	
	5	31.23 ± 3.04	31.20 ± 3.15	30.41 ± 1.68	31.25 ± 2.44	31.09 ± 3.24	31.24 ± 2.99	30.05 ± 3.01	29.22 ± 2.43	
	10	31.05 ± 2.52	31.47 ± 2.64	31.22 ± 2.26	30.90 ± 3.87	31.26 ± 2.52	31.09 ± 3.26	22.88 ± 2.14	20.59 ± 2.04	
	20	31.19 ± 2.83	31.11 ± 2.06	31.13 ± 3.48	31.61 ± 2.57	30.94 ± 2.83	31.14 ± 2.76	5.34 ± 0.21	2.82 ± 0.10	
7	35	31.21 ± 3.39	33.90 ± 3.37	31.22 ± 3.04	32.28 ± 3.32	30.91 ± 3.39	31.25 ± 3.02	–	–	1.21
	50	30.98 ± 2.81	37.06 ± 2.92	32.45 ± 4.02	33.77 ± 3.58	31.17 ± 2.81	30.84 ± 2.79	–	–	
	65	31.08 ± 2.60	42.46 ± 2.57	32.42 ± 2.51	34.26 ± 2.99	31.09 ± 2.60	31.21 ± 2.33	–	–	
	80	31.06 ± 2.18	47.97 ± 2.49	33.65 ± 2.46	35.84 ± 3.41	31.11 ± 2.18	31.03 ± 3.82	–	–	
	5	43.02 ± 2.86	43.10 ± 3.13	43.40 ± 4.02	42.99 ± 3.11	43.25 ± 1.84	43.19 ± 3.33	41.32 ± 4.10	40.68 ± 2.09	
	10	43.35 ± 3.30	43.17 ± 2.67	43.08 ± 3.62	42.23 ± 2.06	43.07 ± 2.95	42.89 ± 2.92	33.10 ± 3.55	31.73 ± 3.71	
	20	43.09 ± 4.16	43.26 ± 3.71	42.51 ± 2.97	43.08 ± 3.47	42.91 ± 3.70	43.32 ± 2.90	15.32 ± 1.28	11.44 ± 1.04	
8	35	42.96 ± 2.25	46.41 ± 3.84	43.26 ± 3.00	43.69 ± 2.40	43.10 ± 2.90	43.03 ± 2.90	–	–	1.32
	50	42.86 ± 3.81	50.02 ± 2.99	43.09 ± 4.04	45.27 ± 3.28	43.31 ± 2.90	43.21 ± 2.90	–	–	
	65	43.02 ± 2.53	56.16 ± 5.12	45.51 ± 2.66	46.76 ± 4.21	43.07 ± 2.90	43.01 ± 2.90	–	–	
	80	43.18 ± 1.59	60.98 ± 4.81	45.87 ± 2.75	48.58 ± 3.82	43.00 ± 2.90	43.09 ± 2.90	–	–	
	5	19.85 ± 2.53	20.30 ± 2.20	20.22 ± 3.00	20.27 ± 1.63	20.01 ± 3.52	20.19 ± 3.14	19.27 ± 1.88	18.10 ± 1.75	
	10	20.27 ± 1.55	19.95 ± 3.02	20.23 ± 2.43	20.16 ± 1.84	19.90 ± 2.44	20.05 ± 2.67	12.34 ± 1.30	10.03 ± 0.99	
	20	20.15 ± 1.61	20.36 ± 2.42	20.03 ± 1.99	20.10 ± 2.53	20.21 ± 2.52	19.99 ± 1.31	ND	ND	
9	35	20.31 ± 2.49	22.63 ± 1.60	20.35 ± 2.17	20.20 ± 3.41	20.07 ± 1.99	20.34 ± 1.65	–	–	1.06
	50	19.93 ± 1.76	26.71 ± 1.74	21.12 ± 2.54	21.97 ± 2.65	20.18 ± 3.24	20.11 ± 2.88	–	–	
	65	20.09 ± 2.30	32.00 ± 2.81	21.86 ± 1.05	22.88 ± 1.78	19.89 ± 2.27	20.02 ± 2.73	–	–	
	80	20.14 ± 2.17	36.33 ± 3.05	22.90 ± 1.38	24.77 ± 2.35	20.10 ± 2.46	19.88 ± 3.56	–	–	

[a] The original contents of compounds 7~9 in yam tuber flesh (control) were 31.14 ± 2.19, 43.13 ± 2.90, 20.12 ± 1.73 μg/g dw, respectively.
[b] Values are mean ± S. D. obtained by trizplicate analyses; ND = not detected; – = no determined.
[c] Least significant difference: Difference of two means between treatments including controls exceeding this value is significant ($p < 0.05$).

Fig. 2 shows some chromatograms of yam tuber extracts for different storage time at 4 °C under atmosphere condition. All data are showed in Tables 1 and 2.

Inoue and Ebizuka (1996) reported that glucoses at position C-26 on furostanol glycoside structures could be excided by F26G (the number, type and bond form of carbohydrate group at position C-3 could be kept) and converted to their corresponding spirostanol glycosides in the rhizomes of crape ginger (*Cotus speciosus*). Kalinowska, Zimowski, Pączkowski, and Zdzislaw (2005) illustrated

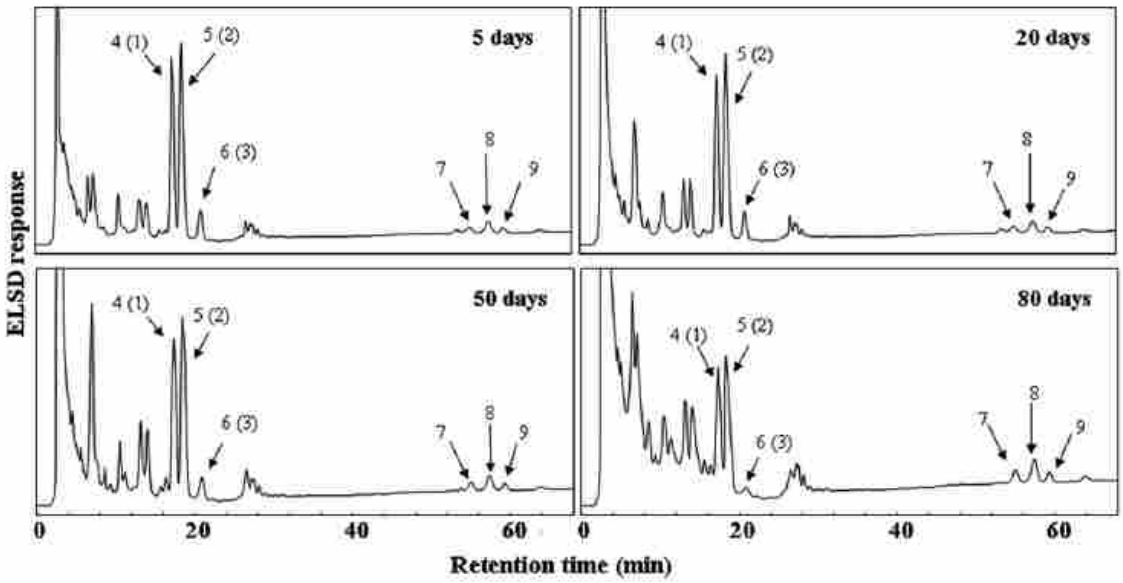

Fig. 2. HPLC chromatograms of saponins extracts of yam tubers after 4 °C storage for various times under atmosphere condition. HPLC conditions: column, Luna C-18 (4.6 mm i.d. × 250 mm, 5 μm); mobile phase, MeOH/ H_2O = 62/38 (v/v) from 0 to 20 min and 71/29 (v/v) from 21 to 65 min; flow rate, 1 ml/min; detection, evaporative light scattering detector (ELSD) (tube temperature, 75 °C; gas flow rate, 2.8 mL/min).

that bidesmosidic furostanol glycosides, contained in freshly harvested rhizomes of crape ginger, could be converted to monodesmosidic spirostanol glycosides in stored rhizomes. We found that furostanol and spirostanol glycosides coexisted in freshly harvested yam tubers. The furostanol glycosides in the long-term stored yam tubers could also be converted to spirostanol glycosides regardless of 4, 17 or 25 °C storage under atmospheric condition (Tables 1 and 2). The chilling injured tubers (4 °C storage) presented the highest conversion. The decreased moles of furostanol glycosides were nearly equal to the increased moles of spirostanol glycosides in these tubers. Scandalios (1993) indicated that the production of activated oxygen species (AOS) might contribute to the formation of chilling injury. Wise and Naylor (1987) illustrated that AOS would damage membrane lipids, proteins and nucleic acid and disorder homeostasis of the organism further. Moreover, enzyme activities (e.g. superoxide dismutase, catalase, duaiacol peroxidase and so on) and chemical compositions (e.g. carotenoids, flavonols and phenolic compounds, etc) could be changed in the chilling injured plants (Asada, 1994; Walker & Mckersie, 1993). Yam crude F26G still had enzyme activity (in the chilling injured yam tubers) at low temperature (4 °C); nevertheless, this enzyme activity seemed to be inhibited in vacuum-sealed peeled tubers.

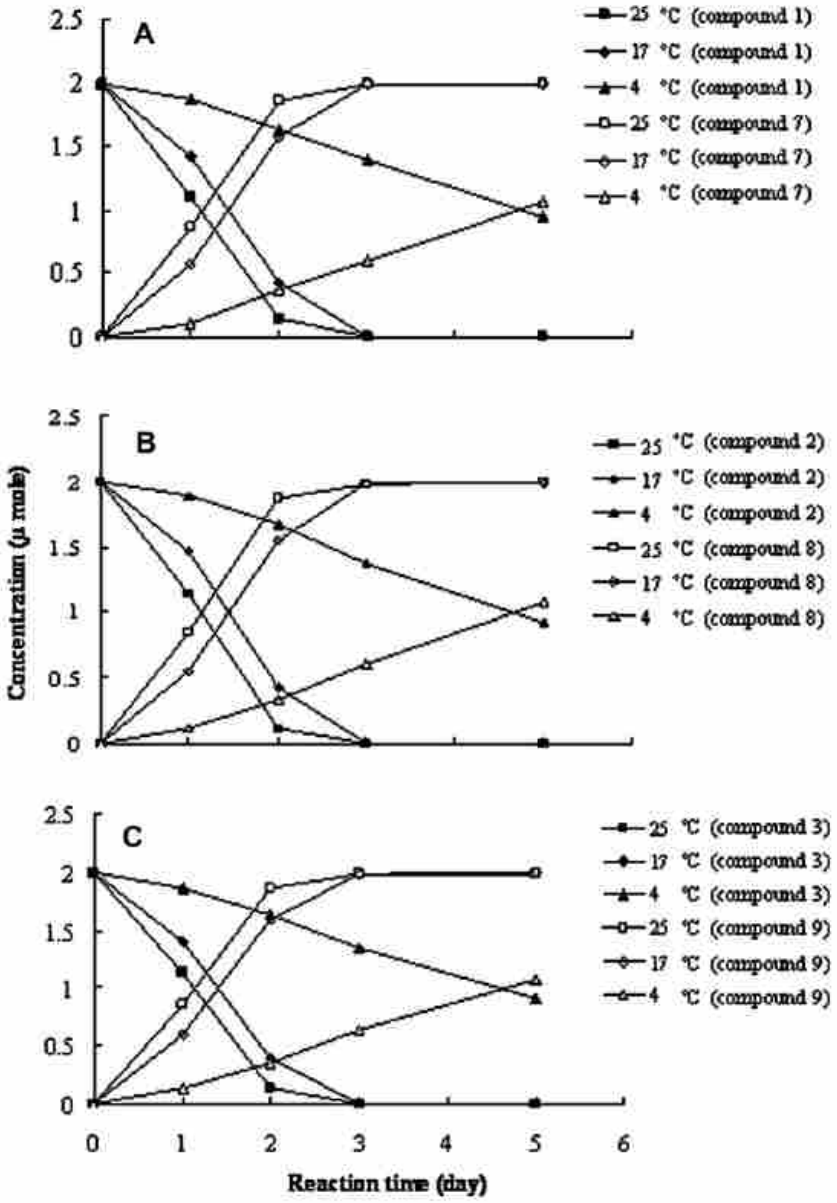

Fig. 3. Changes of saponins after yam crude F26G hydrolysis at different temperature from 1 to 5 days under atmosphere condition (A) compounds 1 and 7; (B) compounds 2 and 8; (C) compounds 3 and 9.

After 5 days of storage at 17 and 25 °C, the vacuum-sealed peeled tubers appeared organoleptic injury. The injury of these tubers was too serious to extract saponins and determine their contents quantitatively after 20 days of storage (the textures of tubers almost lost and transformed to juice with stench). Platenius (1943) evidenced that anaerobic respiration at low level of oxygen resulted in the accumulation of toxic end products of incomplete oxidation that would injure tissues of vegetables. All furostanol and spirostanol glycosides showed significant reduction when the vacuum-sealed peeled tubers were stored at 17 and 25 °C for 5 days. The reduction was aggravated with increasing storage period. Compared to 25 °C stored tuber, the reduction of steroidal saponins in the 17 °C stored one was lower (Tables 1 and 2). These saponins would be dissolved into the transformed juice and flowed away. These furostanol and spirostanol glycosides could be determined definitely in the juice (data not showed).

3.2. Changes of steroidal saponins of yam tubers in model systems under varied conditions

In order to understand the yam F26G activity under atmosphere and vacuum-sealed conditions, we used a model system referred to the method of Inoue and Ebizuka (1996) for evaluation. Fig. 3 shows that compounds **1**, **2** and **3** under atmosphere condition could be converted to compounds **7**, **8** and **9** in all test temperatures. Furostanol glycosides could be converted to their corresponding spirostanol glycosides completely when they were incubated at 17 and 25 °C for 3 days. Yam F26G also showed conversion activity in the model system at 4 °C, though its conversion ability was lower than those at 17 and 25 °C. It is interesting to notice that all furostanol glycosides in the model systems could maintain their original configurations under vacuum-sealed condition, regardless of reaction temperature (data not showed). Therefore, the yam F26G activity could be inhibited in the vacuum condition. Cabezas (1985) mentioned that deficiency in oxygen supply could influence β-glucosidase activity.

4. Conclusions

The content and composition of steroidal saponins in yam tuber seemed to closely relate with their qualities. The larger changes were occurred in the injured yam tubers. The changes were caused by F26G which could convert furostanol glycosides to their corresponding spirotanol glycosides. Its activity was influenced by temperature and could be inhibited in the vacuum condition. A detailed study of the role of air/oxygen to the conversion activity will be explored in the future work.

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Anti-proliferative activities of furostanol and spirostanol glycosides from yam (*Dioscorea pseudojaponica* Yamamoto) and diosgenin on six cancer cell lines

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Running title: Anti-proliferative activities of yam saponins and diosgenin on cancer cell lines

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Abstract

The anti-proliferative activities of furostanol and spirostanol glycosides from yam (*Dioscorea pseudojaponica* Yamamoto) and diosgenin on A 549, Hep G2, KB, MDA-MB-231, SK-Hep 1, and TGSH 9021 cancer cell lines through MTT assay were estimated. Results show that all samples had cytotoxic activities on these cell lines and presented dose dependent pattern. These samples also demonstrated higher cytotoxicity on these cell lines incubated for 48 hr than those incubated for 24 hr except Hep G2. The spirostanol glycosides had the highest cytotoxic activity in all cell lines, especially for A 549, MDA-MB-231, SK-Hep-1 and TSGH-9201 (all of the IC_{50} values were around 1 μM). Furthermore, diosgenin was more active than furostanol glycosides. The spirostanol glycosides presented higher potential for cancer treatment.

Keyword: Dioscorea pseudojaponica Yamamoto; diosgenin; furostanol glycoside; spirostanol glycoside; cytotoxic activity

1. Introduction

Saponins are glycosides with steroid or triterpene aglycon [1]. Many literatures revealed that yams (*Dioscorea* species) such as *Dioscorea colletti* var. *hypoglauca* [2, 3], *D. composite* [4], *D. floribunda* [5], *D. olfersiana* [6], *D. prazeri* [7], *D. pseudojaponica* Yamamoto [8], *D. zingiberensis* [9] and *Dioscorea villosa* [10] contained steroidal saponins including furostanol glycosides with two sugar chains attached at the C-3 and C-26 position (bidemoside saponins) and spirostanol glycosides with one sugar chain attached at the C-3 position (monodemoside saponins). Reports indicated that steroidal saponins could provide anti-neoplastic [3], anti-carcinogenic [11], anti-thrombotic [12], anti-viral [13], hemolytic [14], hypocholesterolemic [15] and hypoglycemic [16] capacities. The aglycon of yam steroidal saponins namely diosgenin have also been used to produce steroidal drugs in the pharmaceutical industry [17, 18]. Diosgenin could induce human 1547 osteosacroma cell cycle arrest in G_0/G_1 phase [19].

There were few reports about the anti-proliferative activities of yam saponins. The cytostatic activity of a spirostanol glycoside isolated from wild yam (*Dioscorea villosa*) has been studied with HEK293 (human embryonic kidney epithelial cell line) and MCF-7 (human breast carcinoma cell line) cells [10]. The cytotoxic effects of furostanol and spirostanol glycosides from *D. collettii* var. *hypoglauca* have also been determined using K562 (chronic myeloid leukemia cell line) cells [3]. In this investigation, we evaluated the cytostatic effects of the purified furostanol glycoside sample composed of 26-*O*-ß-D-glucopyranosyl-22*α*-methoxyl-(25*R*)-furost-5-en-3 ß, 26-diol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*O*-{[*α*-L-rhamnopyranosyl-(1 → 4)]-*O*-[*α*-L-rhamnopyranosyl -(1 → 4)]}-ß-D-glucopyranoside (compound 1), methyl protodioscin (compound 2) and methyl protogracillin (compound 3), the purified spirostanol glycoside sample composed of (25*R*)-spirost-5-en-3 ß-ol 3-*O*-*α*-L-rhamno- pyranosyl-(1→ 2)-*O*-{[*α*-L- rhamnopyranosy-(1→4)]-*O*-[*α*-L-rhamnopyranosyl-(1→4)]}-ß-D-glucopyranoside (compound 4), dioscin (compound 5) and gracillin (compound 6), and diosgenin on six different cancer cell lines using the MTT assay. The steroidal saponins were prepared from tubers of *D. pseudojaponica* Yamamoto; their structures were showed in Fig. 1. These cell lines used were A 549 (human lung adenocarcinoma cell line), Hep G2 (human hepatocellular carcinoma cell line), KB (human mouth epidermal carcinoma cell line), MDA-MB-231 (human breast adenocarcinoma cell lines), SK-Hep-1 (human hepatic adenocarcinoma cell line), and TSGH 9201 (human gastric carcinoma cell line).

2. Materials and methods

2.1. Yam samples.

Yam (*D. pseudojaponica* Yamamoto) tubers (white cortex and flesh, cylindrical shape, ca. 4 cm in diameter and ~110 cm long) were harvested randomly from a field in Keelung City, Taiwan. These tubers were cleaned, cut into 4 mm thick slices with a Cucina^{TM} slicer (model: HR7633) (Koninklijke Philips Electronics Co., Suzhou, Jiangsu, China), and then lyophilized in the freeze-drying system (Vestech Scientific Co. Ltd., Taipei, Taiwan). These dried slices were further ground in the RT08 grinder (Rong-Tsong Co., Taipei, Taiwan) to 40 mesh or below for furostanol and spirostanol glycosides preparation.

2.2. Chemicals and reagents.

Solvents used for steroidal saponins preparation, *n*-butanol and methanol, were purchased from Tedia Co. (Fairfield, OH, USA). Deionized water (dd H_2O) was prepared by Ultrapure^{TM} water purification system (Lotun Co., LTD. Taipei, Taiwan). Diosgenin standard was purchased from Sigma (St. Louis, MO, USA). Ethanol (EtOH, 95%), isopropanol and hydrochloride (HCl) were purchased from Merck Co. (Darmstadt, Germany). The furostanol glycoside sample mixing compounds 1 (20.36 mg, MW = 1208), 2 (20.95 mg, MW = 1062) and 3 (19.58 mg, MW = 1078), and the spirostanol glycoside sample mixing compounds 4 (15.51 mg, MW = 1014), 5 (20.09 mg, MW = 868) and 6 (15.40 mg, MW = 884) were separated through XAD-2 (Sigma Co., St. Louis, MO, USA) column chromatography after methanol extraction from the yam powder and purified with reversed-phase preparative high-performance liquid chromatography (HPLC) as described in our previous paper [8]. Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS), trypsin-EDTA and sodium bicarbonate were purchased from Hyclone (Logan, UT, U.S.A.). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT), L-glutamic acid, penicillin, phosphate-buffered saline (PBS) and streptomycin were obtained from Sigma Co. (St. Louis, MO, USA).

2.3. Cell cultures

The A 549 (ATCC No.: CCL-185), Hep G2 (ATCC No.: HB-8065), KB (ATCC No.: CCL-17), MDA-MB-231 (ATCC No.: HTB-26) and SK-Hep-1 (ATCC No.: HTB-52) cell lines purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were incubated in DMEM and the TSGH 9201 cell lines (BCRC No.: 60146) obtained from Bioresource Collection and Research Centre (BCRC, Hsinchu, Taiwan) were incubated in RPMI-1640 medium. All the media contained 10% heat inactivated FBS, penicillin (100 units/mL), streptomycin (100 μg/mL) and 0.25 ng/ml amphotericin, and were maintained in a humidified atmosphere of 5% CO_2 at 37 ˚C. The six kinds of cell lines were trypsinized, resuspended in the above suitable media (5×10^4 cells/ mL) and then plated in each well of 24-well flat-bottom tissue culture plates (0.99 mL/ well), individually, for cytotoxicity test. These cell lines were maintained overnight to let cell stabilize.

2.4. Experimental design

Each sample (furostanol glycoside, spirostanol glycoside and diosgenin) was dissolved in EtOH-H_2O (1/1, v/v) followed by dilution to prepare the series concentrations of 200, 500, 1000, 2000, 5000 and 10000 μM in advance. The wells of plates (as described above) were then added 0.01 mL of these sample solutions to give the final concentrations of 2, 5, 10, 20, 50 and 100 μM for each kind of cells, and 0.01 mL of EtOH-H_2O (1/1, v/v) was added into control wells. The cells were further incubated in a humidified atmosphere of 5% CO_2 at 37 ˚C for 24 and 48 hr, respectively. Each experiment was carried out in eight replicates (n=8).

2.5. Anti-proliferative activity assay

The inhibition of cell growth was determined using the MTT assay [20]. After termination of cell incubation, the media were removed and the cells were washed with 2 mL of PBS. Each well of plates was then added 0.5 mL of suitable media (DMEM or RPMI-1640) and 50 μL of MTT in PBS (5 mg/mL), and incubated at 37 ˚C for 4 hr again in 5% CO_2 incubator. After media removing, the MTT metabolic product (formazan crystal) was dissolved by adding 200 μL/well of isopropanol (with 4% HCl). The surviving cells were measured at 570 nm against background at 630 nm and the results were expressed as percent cytotoxicity index (%) = [1 - (A_{570}-A_{630} sample / A_{570}-A_{630} control)] × 100 %. A dose response curve was

plotted for each sample, and the concentration resulting in 50% inhibition (IC_{50}) was determined. The mean values of experimental results were calculated. The data were subjected to Student's *t*-test for statistical analysis.

3. Results

MTT assay is a simple and reliable technique to measure cell viability for screening of anti-proliferative agents [21]. Fig. 2 and Fig. 3 showed the plots of cytotoxicity (%) versus concentration (μM) of each sample (the furostanol and spirostanol glycosides from *D. pseudojaponica* Yamamoto, and diosgenin) after cell lines were incubated for 24 and 48 hr, respectively. Table 1 illustrated the IC_{50} values of these samples calculated from the graphs. All samples had cytotoxic effects on the six cell lines and presented dose dependent pattern substantially. The three samples, furostanol glycoside especially, in these cell lines except Hep G2 incubated for 48 hr showed higher anti-proliferative activities than those incubated for 24 hr. For the six cell lines, the IC_{50} values of the spirostanol glycoside sample, diosgenin and the furostanol glycoside sample were in the ranges of 1.52~23.61, 16.76~34.01 and 49.67~74.43 μM after 24 hr incubation, and 0.54~10.19, 9.82~25.22 and 11.68~104.34 μM after 48 hr incubation, respectively. The spirostanol glycoside sample presented the highest cytotoxicity in all tests; diosgenin also had higher cytotoxic effect than the furostanol glycoside sample. Compared with other cell lines, the furostanol and spirostanol glycosides in Hep G2, and diosgnin in TSGH-9201 showed the lowest cytotoxic effect after 48 hr incubation.

4. Discussion

D. pseudojaponica Yamamoto (a Taiwanese native variety of yam) is a popular crop in Taiwan, which is used in Taiwanese traditional medicine and many dishes. Steroidal saponin is the important functional compound in yam. Sugar group could affect the bioactivities of functional compounds including saponins [22]. Many reports demonstrated that saponin (glycoside) had higher bioactive effects such as anti-cancer [10], hypocholesterolemic [15], hypoglycemic [16], and hemolytic [23] than sapogenin (aglycon). The numbers of glycosidic chains of saponins would influence bioactivity as well [22]. For triterpene saponins, the monodesmosidic compounds (with one sugar chain attached at the C-3 position) showed higher hemolytic effect than the bidesmosidic ones (with two sugar chains attached at the C-3 and C-22 position) [24].

Many researches employed HL-60 human promyelocytic leukemia cells to evaluate the cytostoxic activities of steroidal saponins from *Dracanena draco* [24], *Hosta sieboldii* [1], *Ruscus aculeatus* [25], *Slimax china* [26], *Triteleia lacteal* [26] an so on. Some furostanol glycosides from these plants had higher activities than spirostanol glycosides, whereas others showed opposite results. Structure of steroidal saponins would influence cytotoxic effect on cancer cells [26]. The furostanol and spirostanol glycosides separated form *Slimax china* and *Triteleia lacteal* had higher cytostoxic activities on HL-60 cancer cells than diosgenin [26]. (25*R*)-Spirost-5-en-3*β*-ol 3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*O*-[*β*-D-glucopyranosyl- (1 → 4)]-*β*-D-glucopyranoside, a spirostanol glycoside from wild yam (*Dioscorea villosa*), also presented higher cytostatic activity on HEK293 and MCF-7 cells than diosgenin [10]. However, compared with diosgenin, the furostanol glycosides from *Dracaena afromontana* were less cytotoxic on KB cells [27]. The furostanol and spirostanol glycosides from *D. collettii* var. *hypoglauca* showed similar cytotoxic activity on K562 cells [3]. Saponins in different cancer cell lines would also present different cytotoxic activities.

In our results (the steroidal saponins from *D. pseudojaponica* Yamamoto), spirostanol glycosides had higher cytotoxic effect on A 549, Hep G2, KB, MDA-MB-231, SK-Hep-1 and TSGH-9201 cell lines than furostanol glycosides and diosgenin. Besides, the furostanol glycosides had less activity on these cell lines than diosgenin. Except Hep G2 cells, the spirostanol glycosides exhibited notable anti-proliferative activities on the others after 48 hr incubation, especially for A 549, MDA-MB-231, SK-Hep-1 and TSGH-9201 cells (all of the IC_{50} values were around 1 μM). The sample has the potential to be exploited as anticancer agent; its effects on cell cycle arrest and apotosis in A 549, MDA-MB-231, SK-Hep-1 and TSGH-9201 cells will be investigated in the near future.

Acknowledgments

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Figure captions

Fig. 1. The structures of steroidal saponins from *D. pseudojaponica* Yamamoto and diosgenin.

Fig. 2. Anti-proliferative activity of the furostanol glycoside sample (Fug) and spirostanol glycoside sample (Spg) from *D. pseudojaponica* Yamamoto, and diosgenin (Dio) on A 549 (a), MDA-MB-231 (b), Hep G2 (c), KB (d), SK-Hep-1 (e) and TSGH 9201 (f) cell lines after 24 hr incubation.

Fig. 3. Anti-proliferative activity of the furostanol glycoside sample (Fug) and spirostanol glycoside sample (Spg) from *D. pseudojaponica* Yamamoto, and diosgenin (Dio) on A 549 (a), MDA-MB-231 (b), Hep G2 (c), KB (d), SK-Hep-1 (e) and TSGH 9201 (f) cell lines after 48 hr incubation.

Table 1. The IC_{50} values determined through the plot of percent cyttoxicity of cell lines versus the concentrations of Dio, Fug and Spg after 24 or 48 hr incubation.

Hour	Sample	IC_{50} (μM)					
		A549	MDA-MB-231	Hep G2	KB	SK-Hep1	TSGH-9201
24	Dio	20.38 ± 5.11 efghij	22.53 ± 4.17 defghi	29.67 ± 17.21 de	26.40 ± 1.92 def	16.76 ± 7.21 fghijk	34.01 ± 8.11 d
	Fug	50.88 ± 12.01 c	49.67 ± 9.18 c	74.21 ± 36.56 b	96.21 ± 5.65 a	61.57 ± 15.42 c	74.43 ± 21.90 b
	Spg	2.08 ± 1.32 mn	4.55 ± 1.20 klmn	23.61 ± 6.29 defgh	5.06 ± 1.20 klmn	1.52 ± 1.11 mn	2.16 ± 1.52 mn
48	Dio	12.75 ± 2.09 ghijklmn	13.73 ± 8.43 ghijklm	22.04 ± 11.37 defghij	9.82 ± 5.02 jklmn	16.71 ± 8.32 fghijk	25.22 ± 12.71 defg
	Fug	29.43 ± 7.81 de	21.59 ± 14.73 defghij	104.34 ± 22.31 a	11.68 ± 6.86 hijklmn	18.22 ± 10.33 efghij	15.73 ± 7.41 fghijkl
	Spg	1.13 ± 1.19 n	1.07 ± 1.27 n	10.19 ± 0.72 ijklmn	3.84 ± 1.90 lmn	0.54 ± 1.01 n	1.01 ± 1.05 n

[a.] All values are mean ± SD obtained by eight replicate analyses.

[b.] Values bearing different letters in the table are significantly different ($p<0.05$).

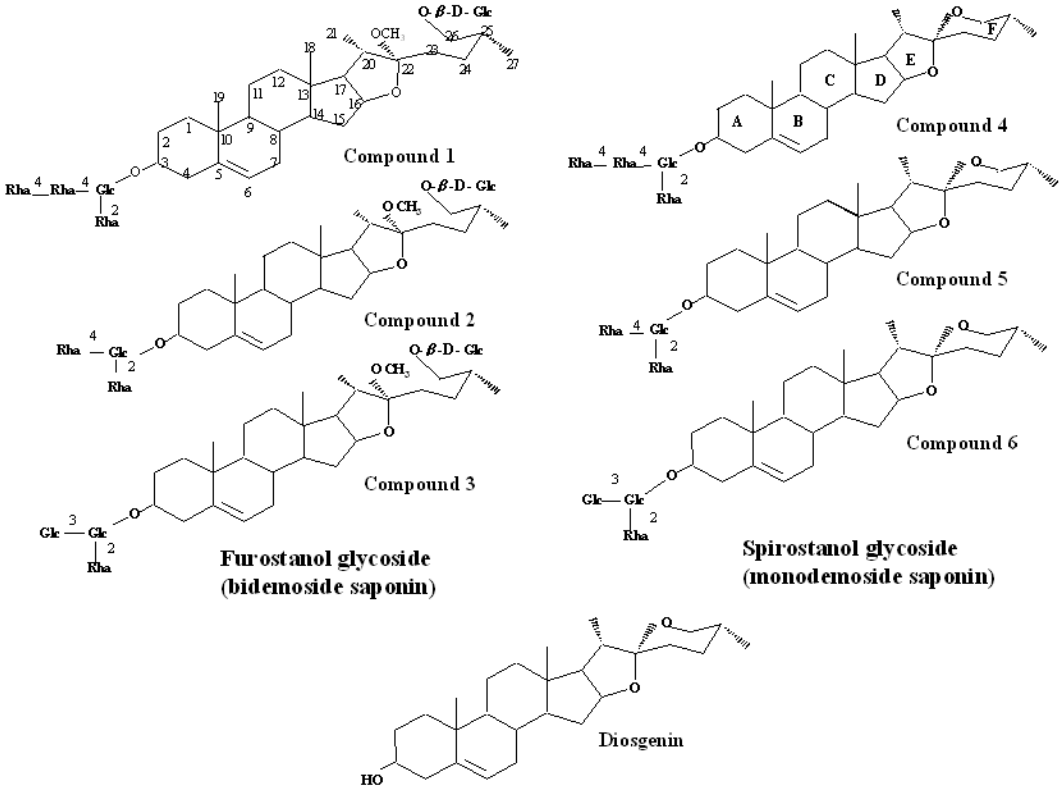
Compound 1
Compound 2
Compound 3
Furostanol glycoside
(bidemoside saponin)
Compound 4
Compound 5
Compound 6
Spirostanol glycoside
(monodemoside saponin)
Diosgenin

Fig. 1

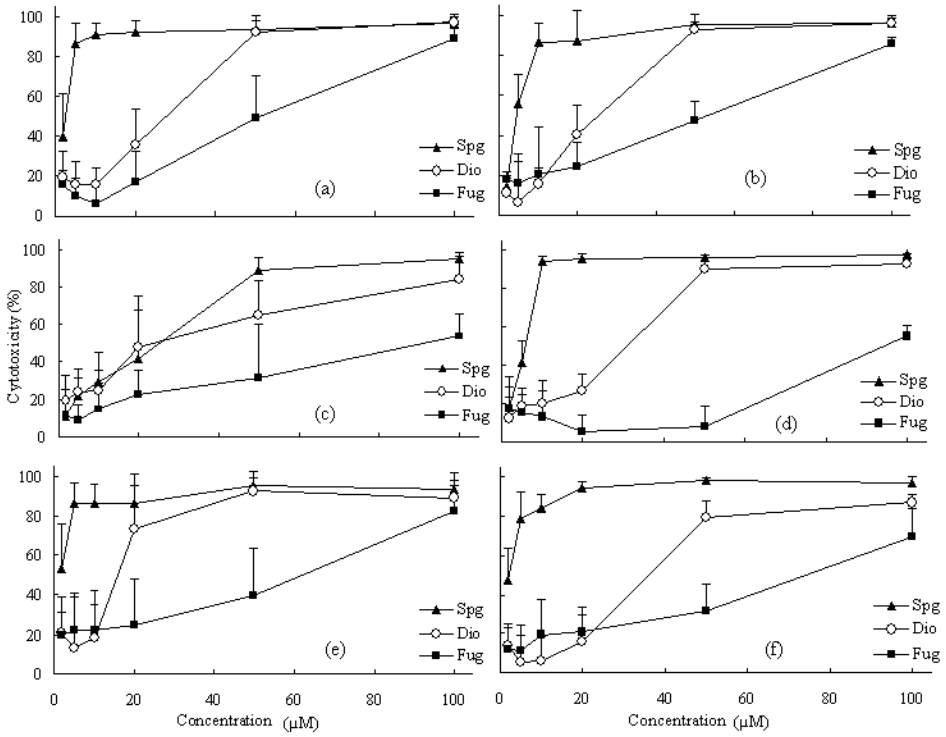

Fig. 2

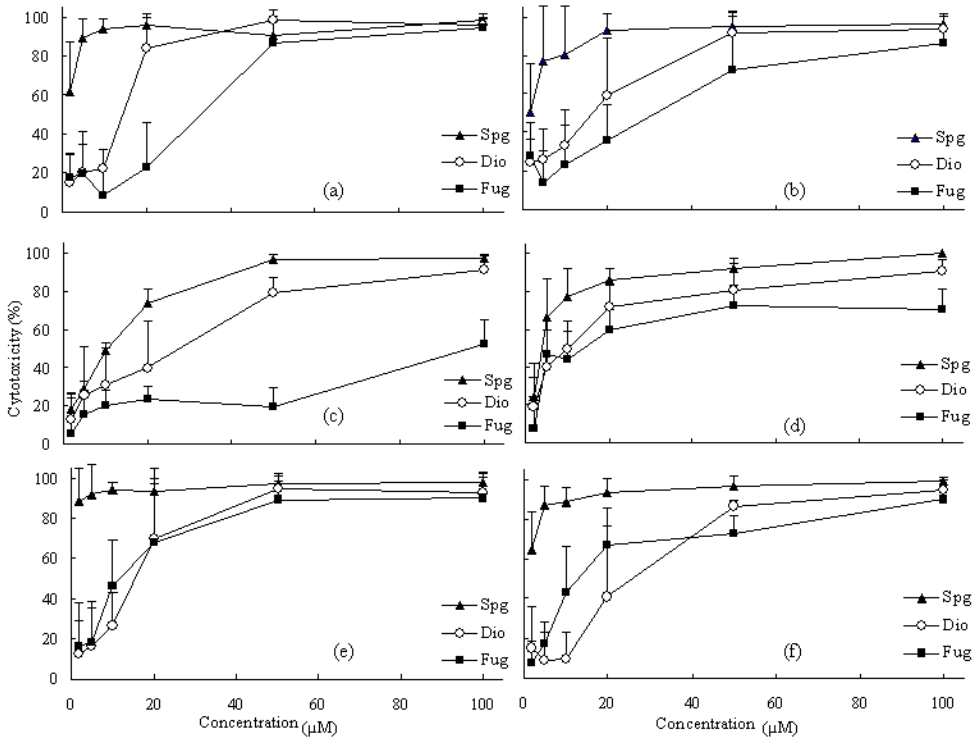
Cytotoxicity (%)
Concentration (μM)
Concentration (μM)
(a)
(b)
(c)
(d)
(e)
(f)
Spg
Dio
Fug

Fig. 3

出席國際學術會議心得報告

計畫編號	96-2313-B-040-002-
計畫名稱	基隆山藥皂苷抗發炎和抗炎功效與其皂苷結構關係之探討
出國人員姓名 服務機關及職稱	楊登傑助理教授 中山醫學大學
會議時間地點	2008/05/10~05/16 Baltimore (USA)
會議名稱	32th International Symposium on High Performance Liquid Phase Separation and Related Techniques
發表論文題目	An improved HPLC method for determination of *cis*- and *trans*- carotenoids in *Dunanleilla salina* cultivated in Taiwan

一、參加會議經過

於去年十二月初收到主辦單位以 e-mail 寄送之邀請函，邀請能寄發論文摘要。因本人自從 2003 年以來每年參加此研討會均獲益良多，此研討會之發表的論文和探討的內容，都為當今世界上頂尖之高效能液相分離和其相關技術之理論及應用探討，同時亦包含發展中之最新技術和未來可能之發展層面和願景，因此本人接受大會之邀請，將自己的研究成果摘要寄至於大會主辦單位，並有幸得到大會的接受函，能參加「第 32 屆國際高效能液相分離與相關技術研討會」，並發表論文。

二、與會心得

此研討會是國際公認在高效能液相層析領域方面最具重要性的國際會議，自1973年來每年由北美和歐洲各國輪流主辦。探討領域廣泛，包含所有尖端的高效能液相層析技術、尖端分離理論的基礎研究和所有相關的應用領域，特別強調於各研究領域之快速及革命性的發展。此次大會共有599篇論文發表，分為30大領域，從Chemicals and polymers、Chiral、Environmental、Food/beverages and Food safety、Natural products and traditional Chinese medicines、Other applications、Pharmaceutical analysis、Column methodologies、High-speed and high-throughput separations、Instrumentation design and automation、High-temperature、Monoliths、Multidimensional separations、New packing material、Small particles/high pressure、Theory/retention mechanism、Biomarkers/proteomics、Drug/metabolites in biological matrices、Endogeneous compounds in biological matrices、Genomics and metabolomics、Protein/drug binding、Protein and peptides、Electrodriven separations、Hydrophilic interaction chromatography(HILIC)、Detection and hyphenated methods、Method development、Microfluidics and nano-technology、Preparative preparation、Sample preparation及Supercritical Fluid Chromatography。內容可謂包羅萬象，分為口頭宣讀、論文壁報以及特別演講等，可謂精采絕倫，更體會出世界各國的科學家們在高效能

液相分離與相關技術之努力，使其進展是如此一日千里，參加此次之研討會真是獲益良多，相信更能使自己擅用此些最新之高效能液相分離與相關技術，強化日後之研究工作，使自己之研究能更得心應手。

三、建議

高效能液相分離與相關技術，應用的範圍相當廣泛，舉凡蛋白質體分析，動物代謝產物分析、天然物分析與分離，食品分析、藥物分離檢測、環境檢測分析…等等，都需要利用此些技術，含蓋的領域有生物學、化學、食品學、環境科學、營養學、藥學、醫學…等等相當廣範，建議國內專家學者可以多參加此研討會，除可與世界的專家學者互動外，更可獲知最新的科技發展，定有利於自己的研究工作發展。

四、攜回資料名稱及內容

帶回許多作者於此次大會發表之壁報論文之 reprint，大會之論文摘要集共計 599 篇以上，以及參展廠商之相關儀器設備資料包括：monolithic column， micro-fluid separation 設備，evaporated light scattering detector (ELSD)設備，液相層析質譜儀(LC-MS)等等。