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XANTHINE OXIDASE INHIBITORS FROM THE ROOTS OF EGGPLANT (*Solanum melongena* L.)

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Stigmasterol **1**, stigmasterol-β-D-glucoside **2**, β-sitosterol-β-D-glucoside **3**, dioscin **4**, protodioscin **5** and methyl protodioscin **6** were isolated and characterized from the butanol soluble part of the ethanolic extract of eggplant roots (*Solanum melongena* L. Solanaceae). Except for stigmasterol, these compounds have not been previously isolated from *Solanum melongena* L.

Since the roots of the eggplant have been used in folk medicine for rheumatism, inflammation and foot pain[1,2], these compounds were tested for their inhibitory effect on xanthine oxidase. The results showed that the phytosterols **1**, **2** and **3** displayed a stronger inhibition on xanthine oxidase than the steroidal glycosides **4**, **5** and **6**. From the structural features, the active moiety seems to be the double bond for both phytosterols and steroidal glycosides or 22-OH group in furostanol glycoside **5** as regards xanthine oxidase inhibition based on this study.

KEY WORDS: *Solanum melongena* L. (Eggplant) roots, phytosterols, steroidal glycosides, furostanol glycosides, xanthine oxidase inhibitors, gout.

INTRODUCTION

Solanum melongena L. (Solanaceae) which is an annual crop widely cultivated in Taiwan has been used in folk medicine for inflammation, warts, toothache and bloody flux[1,2], and the plant roots for rheumatism, inflammation and foot pain[1,2].

In the search for bioactive principles, the roots of eggplant were extracted and fractionated as illustrated in Chart 1. Stigmasterol **1**, stigmasterol-β-D-glucoside **2**, β-sitosterol-β-D-glucoside **3**, dioscin **4**, protodioscin **5** and methyl protodioscin **6** (see Figure 1) were isolated and characterized from the butanol layer (**IV**) of the ethanolic extract (**I**) as shown in Chart I. These compounds have not been previously isolated from *Solanum melongena* L. except stigmasterol[3,4,5,6].

Since the eggplant roots have been used in folk medicine for rheumatism, inflammation and foot pain[1,2], these purified compounds were tested for their inhibitory effects on xanthine oxidase.

The oxidation of hypoxanthine to xanthine and of xanthine to uric acid is catalysed by the same enzyme, xanthine oxidase. It transfers oxygen directly to oxidize the substrate and to form hydrogen peroxide which is in turn rapidly reduced to water by catalase. Uric acid has a low solubility and when blood levels are high, there is a

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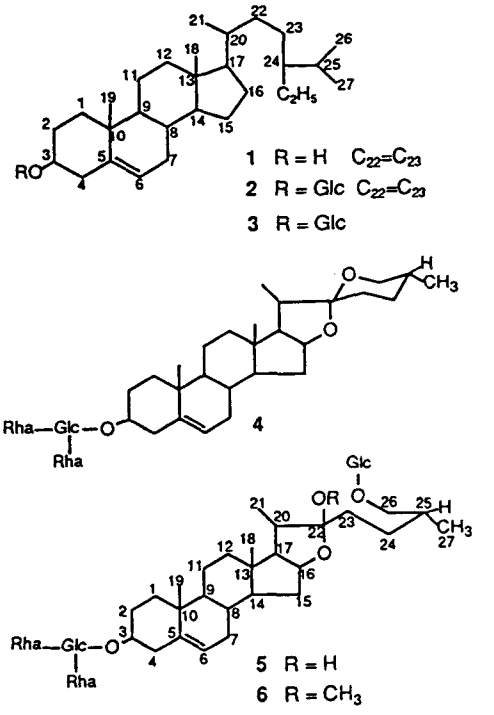

FIGURE 1 Structures of **1** to **6**.

tendency for urate crystals to be deposited in the urinary tract as "stones" and in the synovial fluid of joints giving rise to the symptoms of gout which is associated with painful inflammation[7]. Xanthine oxidase serum levels are increased in hepatitis and mild hepatotoxicity[7] so that xanthine oxidase inhibitors could be used for remission of gout or hepatitis. Many xanthine oxidase inhibitors are known[8], e.g. allopurinol which is a clinically useful drug in the treatment of gout.

MATERIALS AND METHODS

Isolation and characterization of constituents from the roots of Solanum melongena *L. (eggplant):*

The dried roots of *Solanum melongena* L.(Solanaceae) collected in Taiwan (4.5 kg) were extracted with ethanol thrice at room temperature to give **I** (294.5g) which was further treated with chloroform to give a soluble fraction **II** (74.7g) and an insoluble

fraction **III** (218.6g). The later **III** was then partitioned with n-butanol and water to obtain **IV** (56.8g) and **V** (160g) respectively. (Chart I)

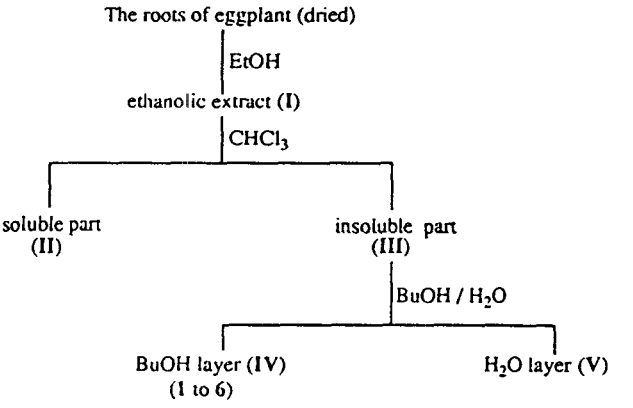

Chart I. Fractionation of the ethanolic extract of eggplant roots

The butanol layer **IV** (15g) was chromatographed over a silica gel column (600g, #70-230, E.Merck) and eluted successively with $CHCl_3$: MeOH (9:1) to give fraction 1 (**1**, 150mg); $CHCl_3$: MeOH (8:2) to yield fraction 2 (**2** and **3**, 255mg); $CHCl_3$: MeOH : H_2O (8:2:0.2) to obtain fraction 3 (**4**, 184mg) and then eluted with $CHCl_3$: MeOH : H_2O (7:3:0.5; 6:4:0.5) to afford fraction 4 (**5** and **6**, 206mg).

The fraction 2 was rechromatographed over a flash silica gel column (20g, #230-400, E. Merck) eluted with $CHCl_3$: MeOH : H_2O (8:2:0.1; flow rate, 15ml/min.) to give **2** (25mg), a mixture of **2** and **3** (150mg) and **3** (21mg). The mixture of **2** and **3** was further rechromatographed thrice in the same way to yield **2** (62mg) and **3** (53mg). The fraction 4 (205mg) was also rechromatographed by preparative MPLC on a reverse-phase column (RP-18) eluted with MeOH : H_2O (7.5:3; flow rate, 3ml/min.) to give **5** (20mg), a mixture of **5** and **6** (80mg) and **6** (25mg). The mixture of **5** and **6** (80mg) was further treated in the same way twice to yield **5** (35mg) and **6** (45mg).

1: stigmasterol, m.p. 171–172°C (MeOH, colorless plates).purple to dark green coloration with 10% H_2SO_4. IR ν_{max}^{KBr} cm^{-1} : 3426 (OH), 1650 (C=C), 1053 (C-O-C); EI-MS (m/z):, 412 (M^+), $C_{29}H_{48}O$, 397, 394, 379, 369, 351, 329, 300, 273, 271, 255, 213; ^{1}H-NMR (C_5D_5N) δ : 0.715 (3H, s, 18-Me), 0.875 (3H, d, J=6.4Hz, 27-Me), 0.906 (3H, d, J=3.4Hz, 26-Me), 1.038 (3H, d, J=6.4Hz, 21-Me), 1.079 (3H, s, 19-Me), 3.869 (1H, m, 3-H), 5.079 (1H, dd, J=8.6, 15.2Hz, 23-H), 5.419 (1H, br.s, 6-H); ^{13}C-NMR (C_5D_5N) δ : Table I. The spectroscopic data of **1** were identical with stigmasterol in the literature[9,10,11].

2: stigmasterol-β-D-glucoside, m.p. 325–326°C (MeOH, colorless plates); purple to dark green coloration with 10% H_2SO_4. IR ν_{max}^{KBr} cm^{-1} : 3424 (OH), 1637 (C=C), 1024 (C-O-C); FAB-MS (m/z) : 575 $(M+H)^+$, $C_{35}H_{58}O_6$, 574; EI-MS (m/z) : 412, 397, 394, 379, 369, 351, 329, 300, 271, 255, 213.[1] H-NMR (C_5D_5N) δ : 0.684 (3H, s, 18-Me), 0.812 (3H, d, J=8.7Hz, 27-Me), 0.862 (3H, d, J=3.6Hz, 26-Me), 1.015 (3H, s, 19-Me),

TABLE I

^{13}C-NMR chemical shift of compound **1–6** (C_5D_5N, ppm)

carbon	**1**	**2**	**3**	**4**	**5**	**6**
1	37.5	37.5	37.5	37.4	37.6	37.2
2	32.2	30.3	30.2	30.1	30.1	30.5
3	70.9	78.6	78.6	78.8	78.6	78.1
4	43.0	39.4	39.3	39.0	40.1	39.5
5	141.6	140.9	140.9	140.8	140.8	140.6
6	120.9	121.9	121.9	121.5	121.8	121.6
7	31.9	32.2	32.2	32.3	32.4	32.1
8	31.9	32.1	32.1	31.7	31.8	31.4
9	50.2	50.4	50.4	50.3	50.4	50.1
10	36.6	36.9	36.9	37.1	37.2	36.8
11	21.1	21.3	21.3	21.1	21.2	20.8
12	39.6	40.0	40.0	39.9	39.1	38.7
13	42.1	42.5	42.5	40.5	41.0	40.5
14	56.7	56.9	56.9	56.7	56.8	56.3
15	24.3	24.5	24.5	31.8	32.4	31.9
16	28.9	28.6	28.9	81.1	81.1	81.1
17	55.8	56.3	56.3	62.9	64.0	63.8
18	11.9	12.0	12.0	16.3	16.5	16.2
19	19.3	19.5	19.2	19.4	19.4	19.2
20	40.5	40.9	36.4	42.0	40.8	40.2
21	21.2	21.5	19.2	15.0	16.4	16.0
22	138.5	138.8	34.2	109.3	110.7	112.6
23	129.2	129.5	26.5	32.2	37.1	31.4
24	51.1	51.5	46.1	29.3	28.4	28.0
25	31.7	31.6	29.5	30.6	34.3	33.9
26	20.9	20.9	19.4	66.9	75.2	75.0
27	18.9	19.1	20.0	17.3	17.4	16.9
28	25.4	25.6	23.4			
29	12.2	12.2	12.1			
OCH_3						47.1
3-glc:						
1′		102.6	102.6	100.3	100.3	99.9
2′		75.4	75.3	77.9	78.9	78.6
3′		78.4	78.4	76.9	76.9	76.4
4′		71.8	71.7	78.1	78.2	78.0
5′		78.2	78.2	77.9	77.8	77.4
6′		62.9	62.9	61.4	61.4	61.1

TABLE I *Continuation*

carbon	1	2	3	4	5	6
2′-rha :						
1″				102.0	102.0	101.8
2″				72.5	72.8	72.4
3″				72.7	72.5	72.2
4″				73.9	74.1	73.6
5″				69.5	69.5	69.3
6″				18.6	18.5	18.1
4′-rha :						
1‴				102.9	102.9	102.5
2‴				72.5	72.8	72.4
3‴				72.8	72.5	72.1
4‴				74.1	73.9	73.4
5‴				70.4	70.4	70.1
6‴				18.4	18.6	18.3
26-glc :						
1⁗					104.9	104.4
2⁗					75.2	74.8
3⁗					78.4	78.1
4⁗					71.7	71.4
5⁗					78.2	78.0
6⁗					62.8	62.5

1.057 (3H, d, J=6.5Hz, 21-Me), 3.937 (1H, m, 3-H), 4.561 (1H, d, J=9.8Hz, anomeric H), 5.019 (1H, dd,J=8.7, 14.9Hz, 23-H), 5.173 (1H, dd, J=8.6, 15.2Hz, 22-H), 5.329 (1H, br.s, 6-H). ^{13}C-NMR (C_5D_5N) δ : Table I. The spectroscopic data of **2** were identical with stigmasterol-β-D-glucoside in the literature[9,10,11,12,13].

3 : β-sitosterol-β-D-glucoside, m.p. 299–301°C (EtOH, colorless granules). purple coloration with 10% H_2SO_4. IR ν_{max}^{KBr} cm^{-1} : 3397 (OH), 1637 (C=C), 1026 (C-O-C); FAB-MS (m/z) : 577 $(M+H)^+$, $C_{35}H_{60}O_6$, 576, EI-MS (m/z) : 414, 396, 382, 329, 255, 213; ^{1}H-NMR (C_5D_5N) δ : 0.666 (3H, s, 18-Me), 0.809 (3H, d, J=8.5Hz, 27-Me), 0.858 (3H, d, J=6.6Hz, 26-Me), 0.888 (3H, t, J=6.9Hz, 29-Me), 0.922 (3H, d, J=3.5Hz, 21-Me) 1.011 (3H, s, 19-Me), 4.564 (1H, d, J=9.7Hz, anomeric H), 5.329 (1H, br.s, 6-H); ^{13}C-NMR (C_5D_5N) δ : Table I. The spectroscopic data of **3** were identical with β-sitosterol-β-D-glucoside in the literature[14,15].

4 : dioscin, m.p. 286–288°C (MeOH, colorless granules). purple-yellowish green-brown coloration with 10% H_2SO_4; Ehrlich test : (-). IR ν_{max}^{KBr} cm^{-1} : 3414 (OH), 1653 (C=C), 1045 (C-O-C); 985, 920 (W), 895 (S), 865 (isospiroketal, 25R)[16,17]; FAB-MS (m/z) : 868 $(M-H)^-$, $C_{45}H_{72}O_{16}$, 868.60; EI-MS (m/z) : 414, 396, 355, 342, 326, 314, 296, 282, 271, 253, 139, 126; ^{1}H-NMR (C_5D_5N) δ: 0.814 (3H, s, 18-Me), 0.852 (3H, s, 27-Me), 1.035 (3H, s, 19-Me), 1.133 (3H, d, J=6.7Hz, 21-Me), 1.619

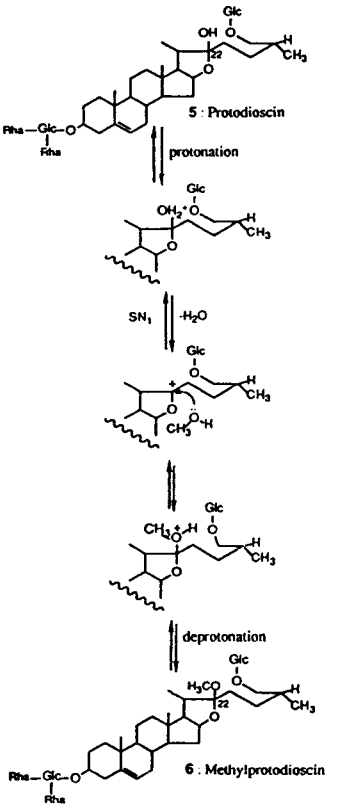

FIGURE 2 Conversion mechanism between **5** and **6**

(3H, d, J=6.1Hz, rha-Me) 1.736 (3H, d, J=6.0Hz, rha-Me), 3.637 (2H, m, 26-H), 4.344 (1H, q, J=6.5Hz, 16-H), 5.298 (1H, br.d, 6-H). ^{13}C-NMR (C_5D_5N) δ: Table I. The spectroscopic data of **4** were identical with dioscin in the literature[14,18,19,20].

5: protodioscin, m.p. 192–193°C (dil. acetone, colorless powder). Ehrlich test: (+); purple-yellowish green coloration with 10% H_2SO_4: identified as furostanol glycoside. IR ν_{max}^{KBr} cm^{-1}: 3426 (OH), 1645 (C=C), 1044 (C-O-C), no isospiroketal absorption[14,21,22]. FAB-MS (m/z): 1050 $(M+H)^+$, $C_{51}H_{84}O_{22}$, 1048.7; EI-MS (m/z): 432, 414, 396, 355, 342, 314, 296, 282, 271, 253, 139, 126, 115. ^{1}H-NMR (C_5D_5N) δ: 0.867 (3H, s, 18-Me), 0.965 (3H, d, J=6.2Hz, 27-Me), 1.012 (3H, s, 19-Me), 1.309 (3H, d, J=6.8Hz, 21-Me), 1.599 (3H, d, J=6.2Hz, rha-Me), 1.739 (3H, d, J=6.0Hz, rha-Me), 2.237 (1H, m, 20-H), 3.613 (2H, m, 26-H), 4.342 (1H, q, J=6.7Hz, 16-H), 5.307 (1H, br.d, 6-H); ^{13}C-NMR (C_5D_5N) δ: Table I. The spectroscopic data of **5** were identical with protodioscin in the literature[14,23,24,25]. Enzymatic hydrolysis of **5** (10mg) with emulsin (10mg, 500 units/mg, Sigma) in 20 ml water at 37°C for 24 h gave precipitates which

were recrystallized from methanol to afford dioscin **4**. D-glucose was detected from the water soluble portion by PPC[9,22] (see Figure 2). Boiling **5** with 20 ml methanol for 15 h converted it to **6**; while **6** (10mg) when refluxed with 20 ml acetone : water (7:3) for 15 h gave **5**.

6: methyl protodioscin, m.p. 172–174°C (MeOH, colorless powder), purple to yellowish green coloration with 10% H_2SO_4; Ehrlich test: (+), identified as furostanol glycoside. IR ν_{max}^{KBr} cm^{-1} : 3426 (OH), 1649 (C=C), 1044 (C-O-C), no isospiroketal absorption[14,21,22]. FAB-MS (m/z): 1062 $(M-H)^-$, $C_{52}H_{86}O_{22}$, 1062.72; EI-MS (m/z): 446, 414, 396, 355, 342, 326, 314, 296, 282, 271, 253, 139, 126. ^{1}H-NMR (C_5D_5N) δ : 0.807 (3H, s, 18-Me), 0.982 (3H, d, J=6.5Hz, 27-Me), 1.033 (3H, s, 19-Me), 1.169 (3H, d, J=6.8Hz, 21-Me), 1.601 (3H, d, J=6.1Hz, rha-Me), 1.739 (3H, d, J=6.0Hz, rha-Me), 2.226 (1H, m, 20-H), 3.613 (2H, m, 26-H), 4.347 (1H, q, J=6.4Hz, 16-H), 5.310 (1H, br.d, 6-H), 3.254 (3H, s, OCH_3). ^{13}C-NMR (C_5D_5N) δ : Table I. From FAB-MS and the methoxy group in ^{1}H-NMR (22-OCH_3), methyl protodioscin[24] was proposed for **6**, an artifact formed from methanol during the purification and recrystallization. The natural product was protodioscin (22-OH). The conversion mechanism between **5** and **6** was illustrated in Figure 2. The spectroscopic data of **6** were identical with methyl protodioscin in the literature[14,23,24] Enzymatic hydrolysis of **6** (10mg) with emulsin (10mg, 500 units/mg. Sigma) as described under **5** above gave dioscin **4** from the precipitates. D-glucose was detected from the water soluble portion by PPC.

Assay for xanthine oxidase inhibition[26,27]

1. *Preparation of phosphate buffer solution* : A. 0.2M NaH_2PO_4 solution : $NaH_2PO_4.H_2O$ (E.Merck, 556mg) was dissolved in distilled water to make 20 ml solution. B. 0.2M Na_2HPO_4 solution : $Na_2HPO_4.12H_2O$ (E. Merck, 1.43g) was dissolved in distilled water to make 20 ml solution. 8.5 ml of A solution and 91.5 ml of B solution was added to 100ml of distilled water to make 0.1M phosphate buffer solution, pH = 7.8.
2. *Preparation of xanthine buffer solution* : Xanthine (6.084 mg, E. Merck) was dissolved in 200 ml of 0.1M phosphate buffer pH 7.8 with gentle heating and was shaken well until completely dissolved to make a 200 μM xanthine buffer solution.
3. *Preparation of test solutions* : For different concentrations (50, 100, 200 and 400 μM) of each compounds **1** to **6** were prepared for tests, and, the following amount of each compound was dissolved in 150 μl ethanol to give a concentration of 0.01 M solution: **1** : M.W.=412, 0.618 mg; **2** : M.W.=574, 0.861 mg; **3** : M.W.=576, 0.864 mg; **4** : M.W.=869, 1.304 mg; **5** : M.W.=1049, 1.574 mg and **6** : M.W.=1063, 1.595 mg.
4. *Assay procedure :*

   A. Control : 200 μM solution of xanthine buffer pH=7.8 (984 μl) with 6 μl of xanthine oxidase (25 units/1.3ml, Sigma) and 10 μl of ethanol was incubated for 3 min. at room temperature and uric acid was estimated at 295 nm against a blank sample which did not contain the enzyme but 6 μl of 0.1M phosphate buffer solution pH=7.8 instead. Optical density (O.D.) was recorded and the tests were performed in quadruplicate.

TABLE II
The inhibitory effects of **1–6** and quercetin on xanthine oxidase

compound	percentage inhibition (%)				
	concentration (μM)				
	50	100	200	400	IC_{50}(μM)
stigmasterol (**1**)	26.3±1.48	51.2±1.80	67.9±3.16	88.5±4.67	98.7±4.54
stigmasterol-β-D-glycoside (**2**)	20.6±1.02	58.3±2.01	75.8±4.36	95.4±5.29	87.8±3.44
β-sitosterol-β-D-glycoside (**3**)	29.8±0.91	46.1±2.54	76.9±2.87	85.1±4.18	110±5.02
dioscin (**4**)	24.2±1.30	46.4±1.85	72.5±3.26	89.5±3.83	115±5.18
protodioscin (**5**)	35.3±1.66	49.1±1.78	68.6±2.93	82.4±3.24	106±4.27
methylprotodioscin (**6**)	32.1±0.80	42.5±2.31	53.6±4.06	91.5±4.13	156±5.15
quercetin	85.9±1.74	92.4±1.79	97.5±4.35	100±0.0	25.9±1.35

Each test sample (10 μl) containing 6 μl xanthine oxidase (25 units/1.3ml, Sigma) and 984 μl of 200 μM xanthine buffer solution were incubated for 3 min at room temperature. Then uric acid was estimated at 295 nm.
IC_{50} values were calculated from regression lines where : X was tested compound concentration and Y was percent inhibition of enzyme activity. Each determination represents the average mean of quadruplicate determinations.

B. Sample test : Aliquots (10 μl) of various concentrations of **1–6** were added to xanthine buffer solution (984 μl) and phosphate buffer solution (6 μl) as a blank tests. 6 μl of xanthine oxidase (25 units/1.3 ml, Sigma) was added to each 10 μl of various concentrations of test samples in 984 μl of xanthine buffer solution and treated in the same way as control (A section). Their inhibitory effects on xanthine oxidase were measured by a decrease in uric acid generation. The IC_{50} values were calculated from regression lines where : x was the tested compound concentration and y was percent inhibition of enzyme activity. Quercetin (Sigma)[26,27] was used as a positive control for comparison. The tests were conducted in quadruplicate (Table II and Figures 3 & 4).

RESULTS AND DISCUSSION

Xanthine oxidase, a flavoprotein catalyses the oxidation of hypoxanthine to xanthine and of xanthine to uric acid which shows maximal absorption at 295 nm. Therefore, xanthine oxidase activity was evaluated by the spectrophotometric measurement of the formation of uric acid from xanthine[27]. The tests showed that the purified compounds **1** to **6** suppress the activity of xanthine oxidase in a dose-dependent manner as measured by a decrease in uric acid generation. IC_{50} values (μM) of **1–6** were 98.7, 87.8, 110, 115, 105 and 156 respectively. (Table II). Simultaneous comparison with quercetin which has been reported to be active on xanthine oxidase inhibition[26,27] gave an IC_{50} value for quercetin of 25.9 μM (Table II, Figures 3 and 4).

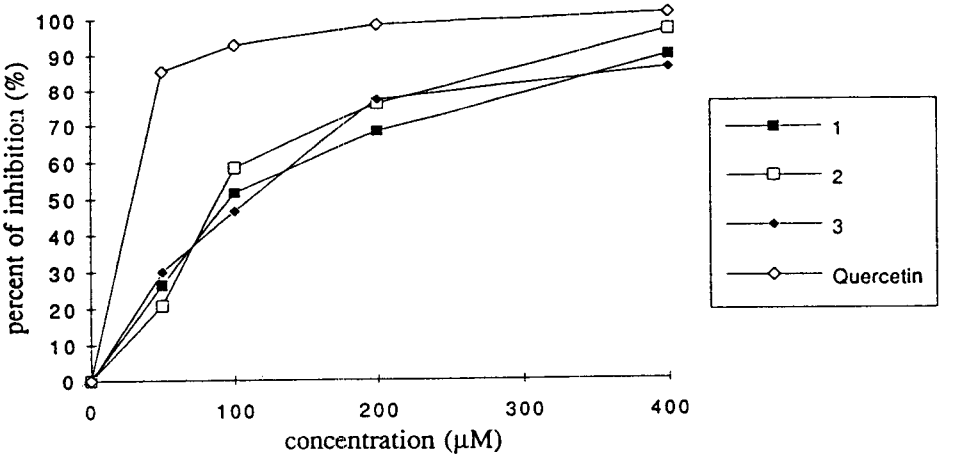

FIGURE 3 The inhibitory effecct of **1** to **3** and quercetin on xanthine-xanthine oxidase system

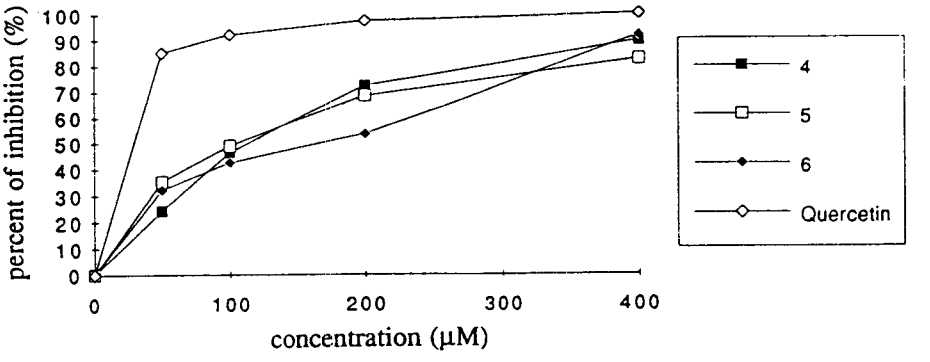

FIGURE 4 The inhibitory effect of **4** to **6** and quercetin on xanthine-xanthine oxidase system

1, **2** and **3** are phytosterols that are widely distributed in the fruits, vegetables and higher plants and the order of activity on xanthine oxidase inhibition in the tests was quercetin > 2 > 1 > 3. Although the inhibitory effects of these phytosterols on xanthine oxidase was weaker than that for quercetin (Table II, Fig. 3), it is suggested that fruits and vegetables may be used for food therapy of gout or hepatitis. From the

structural features, stigmasterol (**I**) and its glucoside (**2**) with two double bond in the aglycone moiety, as well as **2** having one sugar increasing the polarity, and β-sitosterol (**3**) glucoside with only one double bond in the aglycone moiety, the active moiety seems to be the double bond present in the structures.

Whereas **4**, **5** and **6** are steroidal glycosides in which **4** is a spirostanol glycoside with three sugars, **5** and **6** are furostanol glycosides with four sugars as bisdesmosides. **5** is the natural product, whereas **6** is an artifact formed from methanol during the purification and recrystallization which converts the 22-OH to 22-OH_3 (Figures 1 and 2). The order of activity on xanthine oxidase inhibition in this study was quercetin $> 5 > 4 > 6$ (Table II, Figure 4). Evidently, naturally occurring furostanol glycoside **5** showed a stronger activity on xanthine oxidase inhibition than the corresponding spirostanol glycoside **4** and the artifact furostanol glycoside **6**. It is implied that the 22-OH group in the structure of **5** made an important contribution to the activity.

It would seem that the affinity of the double bond and the 22-OH group in the structures of these phytosterols and steroidal glycosides for xanthine oxidase is greater than that of xanthine and consequently the formation of uric acid in xanthine-xanthine oxidase system was decreased.

In the series of antitumor research in our laboratory, each fraction in Chart **I** was also tested for anti-tumor effects in vitro on Hep-2 (laryngeal) and Colo-205 (colon) by trypan blue dye exclusion assay (DEA method)[28,29]. The butanol layer (**IV**) was found to be active and the ID_{50} values were 2.7 μg/ml (Hep-2) and 6.4 μg/ml (Colo-205).

Dioscin (**4**), protodioscin (**5**) and methyl protodioscin (**6**) from the butanol layer (**IV**) have also been isolated from *Solanum indicum* L. in our laboratory and showed anti-tumor effects on several cancer cell lines: Colo-205 (colon), KB (nasopharynx), HeLa (uterine cervix), HA22T (hepatoma), Hep-2 (laryngeal epidermoid), GBM8401/TSGH (glioma) and H1477 (melanoma)[14].

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