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XANTHINE OXIDASE INHIBITORS FROM THE LEAVES OF *ALSOPHILA SPINULOSA* (HOOK) TRYON

HSÜCH-CHING CHIANG[1]*, YING-JUI LO[1] AND FUNG-JOU LU[2]

School of Pharmacy[1] and Department of Biochemistry[2], College of Medicine, National Taiwan University, 1, 1st Section, Jen-ei Road, Taipei, Taiwan, ROC

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Diploptene(**1**), β-sitosterol(**2**), a mixture of 6′-*O*-(E-P-coumaroyl)-α-glucopyranose and 6′-*O*-(E-P-coumaroyl)-β-glucopyranose(**3**), a mixture of 6′-*O*-(E-P-caffeoyl)-α-glucopyranose and 6′-*O*-(E-P-caffeoyl)-β-glucopyranose(**4**), caffeic acid(**5**) and astragalin(**6**) were isolated from an ethanolic extract of the leaves of *Alsophila spinulosa* Hook Tryon (Cyatheaceae). The plant has been used in folk medicine for hepatitis, gout, rheumatism and tumor and these compounds were tested for their inhibitory effect on xanthine oxidase. Caffeic acid was the most potent constituent (IC_{50} = 39.21 μM; K_i = 28.2 μM) and was an uncompetitive inhibitor of the enzyme with respect to the substrate xanthine.

KEY WORDS: *Alsophila spinulosa* H.T. (Cyatheaceae), leaves, phenolic compounds, flavonoid, xanthine oxidase inhibitors, hepatitis, gout, tumor.

INTRODUCTION

Alsophila spinulosa (Hook) Tryon (Cyatheaceae) is a perennial bush widely grown in Taiwan. The dried and sliced stems of the plant have been used in folk medicine for helminthic infections, rheumatism, gout, influenza with cough and hepatitis[1]; the parts of the younger stage of the plant have been used for the treatment of tumours[2].

Since the plant has been used in folk medicine for rheumatism, gout, hepatitis and tumor, we have isolated the active principles from the leaves and tested these purified constituents for their inhibitory effects on xanthine oxidase. Xanthine oxidase catalyses the hydroxylation of many purine substrates and converts hypoxanthine to xanthine and then uric acid. Gout is caused by the deposition in the joints of uric acid and is associated with painful inflammation. Inhibition of the enzyme leads to increased excretion of these purines and remission of gout[3,4]. Many xanthine oxidase inhibitors are known, e.g. allopurinol which is a clinically useful drug in the treatment of gout[5]. The enzyme also serves as a source of oxygen-derived free radicals which induce both cellular injury and edema as well as changes in vascular permeability[6]. Furthermore, xanthine oxidase serum levels are increased in hepatitis and mild hepatotoxicity[3] and also significantly increased in tumoral brain tissues compared to normal brain tissues but there was no significant difference between

*Correspondence

the enzyme levels in meningioma and astrocytoma[7]. Therefore, xanthine oxidase inhibitors could prove useful for the treatment of hepatitis, edema or brain tumor.

MATERIALS AND METHODS

Isolation and characterization of constituents from the leaves of Alsophila spinulosa H.T.:

The dried leaves of *Alsophila spinulosa* H.T. (Cyatheaceae) collected in Taiwan (7.5 kg) were extracted with ethanol thrice at room temperature to give (A) (465 g) which was further partitioned with ethyl acetate and water to give (B) (225 g) in the ethyl acetate layer and (C) (195 g) in the water layer. The fraction (B)(225 g) was then extracted successively with n-hexane, chloroform and water to give (D) (71 g), (E) (40 g) and (F) (35 g) respectively. (Chart I).

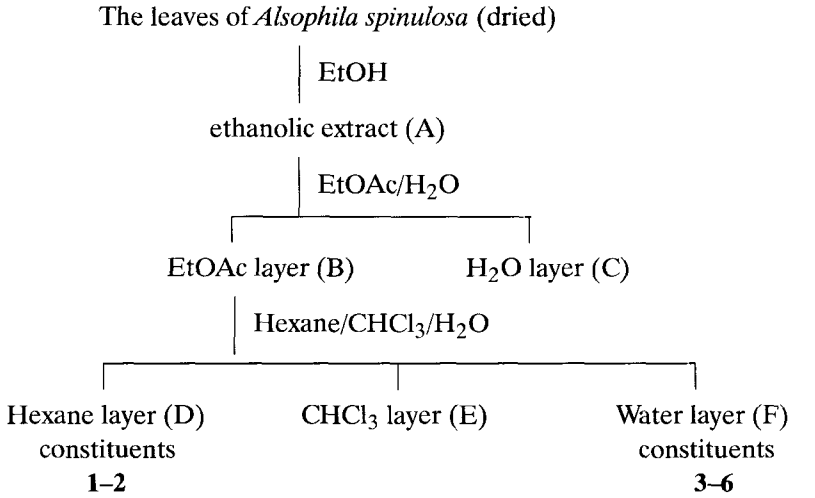


Chart I. Fractionation of the ethanolic extract of the leaves of *Alsophila spinulosa* H. T.

The hexane layer (D) (35 g) was chromatographed over a silica gel column (1050 g, #70–230, E. Merck) and eluted successively with n-hexane to give **1** (47 mg); n-hexane:$CHCl_3$(3.5:6.5) to yield **2** (153 mg). The water layer (F) (35 g) was chromatographed over a polyamide column (700 g, Sigma) and eluded with $CHCl_3$:MeOH: acetone (10:8:5) to give four fractions: Fraction (a) (170 mg) was dissolved in methanol and then percipitated by chloroform to give **3** (148 mg). Fraction (b) (1.3 g) was rechromatographed over a polyamide column and eluted with MeOH:H_2O (6:4) to yield **5** (33 mg). Fraction (c) (250 mg) was treated in the same way as Fraction (a) to afford **4** (227 mg). Fraction (d) (1.1 g) was also rechromatographed over a polyamide column and eluted with MeOH:H_2O (7:3) to give **6** (58 mg).

diploptene (**1**), m.p. 216°–217°C (acetone, colourless powder); brown to violet coloration with 10% H_2SO_4. EI-MS(m/z): 410(M^+, $C_{30}H_{50}$), 395, 218, 204, 191, 189; ^{1}H-NMR ($CDCl_3$)δ: 0.707 (3H, s, 28-Me), 0.777 (3H, s, 24-Me), 0.800 (3H, s, 25-Me), 0.828 (3H, s, 23-Me), 0.929 (3H, s, 27-Me), 0.947 (3H, s, 26-Me), 1.733 (3H, s, 30-Me),

4.763(2H, br, 29-CH_2); ^{13}C-NMR ($CDCl_3$)δ: see Table I. The spectroscopic data for **1** were identical with those in the literature[8,9,10,11] for Hop-22(29)-ene and diploptene.

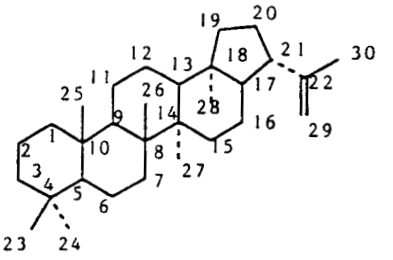
β-sitosterol (**2**), m.p. 131°–132°C (MeOH, colorless plates); purple coloration with 10% H_2SO_4. EI-MS(m/z): 414(M^+, $C_{29}H_{50}O$), 399, 396, 381, 329, 255; ^{1}H-NMR ($CDCl_3$)δ: 0.658(3H, s, 18-Me), 0.824(d, 9H, 21, 26, 27-Me), 0.986(3H, s, 19-Me), 3.499(m, CHOH), 5.335(d, C=CH); ^{13}C-NMR ($CDCl_3$)δ: see Table I. The spectroscopic data for **2** were identical with those in the literature[12,13,14] for β-sitosterol.

6′-O-(E-P-coumaroyl)-α-glucopyranose and 6′-O-(E-P-coumaroyl)-β-gluco pyranose (**3**), m.p. 201°–202°C (MeOH, deep yellow powder); purple to dark violet coloration with 10% H_2SO_4. FAB-MS(m/z): 325(M-1$^-$, $C_{15}H_{18}O_8$) 326; EI-MS(m/z): 164, 147, 120, ^{1}H-NMR (CD_3OD)δ: sugar moiety, 3.460–4.014(m, 2′, 3′, 4′, 5′-H), 4.564(1H, d, J=7.7Hz, 6'-H), 5.172(1H, d, J=3.6Hz, 1'-H), aglycone moiety, 6.341(1H, d, J=15.9Hz, α-H), 6.795(2H, d, J=8.4Hz, 3,5-H), 7.454(2H, d, J=8.0Hz, 2,6-H), 7.654(1H, d, J=15.8Hz, β-H). ^{13}C-NMR(CD_3OD)[15]δ: see Table I. Alkaline methanolysis of **3** with NaOAc: 100 mg of **3** was dissolved in methanolic 3% NaOAc (5 ml) and the solution was allowed to stand 1 h at room temperature to give methyl coumarate and D-glucose[16,17].

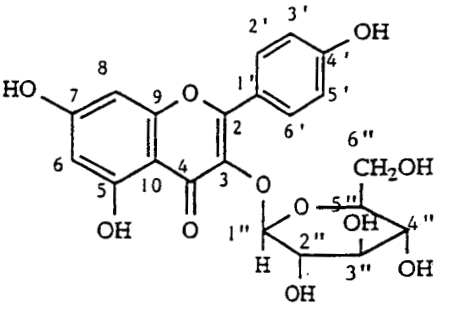
6′-O-(E-P-caffeoyl)-α-glucopyranose and 6′-O-(E-P-caffeoyl)-β-gluco pyranose (**4**), m.p. 85°–87°C (MeOH, brown gum); yellow to purple coloration with 10% H_2SO_4. FAB-MS(m/z):341(M-1$^-$, $C_{15}H_{18}O_9$), 342; EI-MS(m/z): 180, 163, 136. ^{1}H-NMR(CD_3OD)δ: aglycone moiety, 6.317 (1H, d, J=15.8Hz, α-H), 6.770(1H, d, J=8.2Hz, 5-H), 6.964(1H, d, J=8.2Hz, 6-H), 7.055(1H, s, 2-H), 7.615(1H, d, J=15.8Hz, β-H); sugar moiety[16,18]. 5.179(d, J=3.6, 1'-H), 4.57(d, J=7.8, 6'-H), 3.46-4.014(m, 2', 3', 4', 5'-H); ^{13}C-NMR (CD_3OD)δ: see Table 1. Alkaline methanolysis of **4** as previously described for **3** gave methyl caffeate and D-glucose[16,17]. The spectroscopic data for **4** were identical with those described in the literature[16,18] for a mixture of 6′-O-(E-P-caffeoyl)-α-glucopyranose and 6′-O-(E-P-caffeoyl)-β-glucopyranose.

caffeic acid (**5**), m.p. 215°-216.5°C (MeOH, yellow powder)[19]; purple coloration with 10% H_2SO_4. EI-MS(m/z): 180(M^+, $C_9H_8O_4$), 163, 136, 89, 44: ^{1}H-NMR(CD_3OD)δ: 6.248(1H, d. J=15.8Hz, α-H), 6.755(1H, d, J=8.0Hz, 5-H), 6.925(1H, d, J=8.1Hz, 1.9Hz, 6-H), 7.03(1H, d, J=2.0Hz, 2-H), 7.529(1H, d, J=15.8Hz, β-H); ^{13}C-NMR (CD_3OD)δ: see Table 1. Identical with an authentic sample of caffeic acid (Sigma, C-0625, Lot. 109F3733) as shown by mixed m.p.

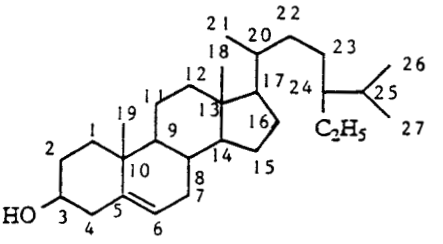
astragal in (kaempferol-3-0-glucoside) (**6**), m.p. 192. 5°-194°C (MeOH, yellow powder)[20]; yellow coloration with 10% H_2SO_4. FAB-MS(m/z): 449(M+1$^+$, $C_{21}H_{20}O_{11}$), 448; EI-MS(m/z); 286, 269, 257, 241, 229, 213, 153, 121. ^{1}H-NMR(CD_3OD)δ: 3.214-3.437(4H, m, 2″, 3″, 4″, 5″-H), 3.536(1H, dd, J=11.7Hz, 5.1Hz, 6″-H, geminal, vicinal coupling), 3.70(1H, d, J=10.8Hz, 6″-H), 5.208(1H, d, J=6.0Hz, 1″-H), 6.172(1H, br. s, 6-H). 6.362(1H, br. s, 8-H), 6.882(2H, d, J=8.0Hz, 3′, 5′-H), 8.039(2H, d, J=8.0Hz, 2′, 6′-H). ^{13}C-NMR(CD_3OD)δ: see Table 1. The spectroscopic data of **6** were identical with those described in the literature[17,20] for astragalin(kaempferol-3-0-glucoside).



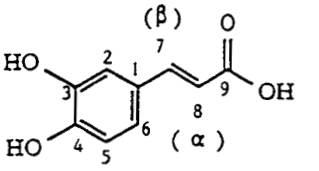
1: Diploptene



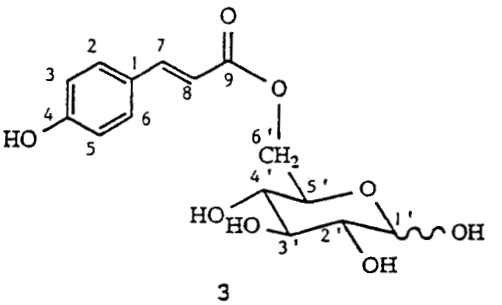
6: Astragalin



2: β-sitosterol



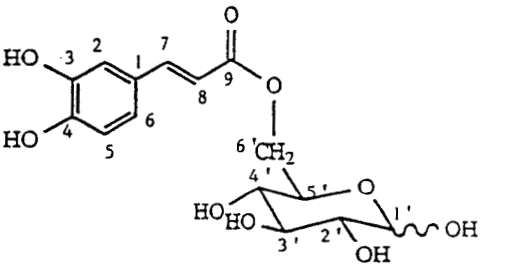
5: Caffeic acid



3

6'-O-(E-P-Coumaroyl)-α-glucopyranose

6'-O-(E-P-Coumaroyl)-β-glucopyranose



4

6'-O-(E-P-Caffeoyl)-α-glucopyranose

6'-O-(E-P-Caffeoyl)-β-glucopyranose

FIGURE 1 Structures of compounds **1** to **6**

TABLE 1
^{13}C-NMR chemical shift of compounds 1-6 (ppm, TMS)

Solvent	$CDCl_3$		CD_3OD			
Carbon	1	2	3	4	5	6
1	40.3	37.2	127.1	127.7	127.9	
2	18.7	31.6	131.2	116.5	116.4	159.1
3	42.1	71.8	116.8	146.7	146.4	135.5
4	33.2	42.3	161.3	149.6	149.2	179.4
5	56.1	140.7	116.8	114.8	115.0	162.9
6	18.7	121.6	131.2	123.0	122.7	100.3
7	33.3	31.6	β147.2	147.5	146.7	166.1
8	41.9	31.9	α114.8	115.2	116.3	94.8
9	50.4	50.1	168.7	168.6	171.5	158.4
10	37.4	36.5				105.7
11	20.9	21.1				122.7 (1′)
12	24.0	39.8				132.2 (2′,6′)
13	49.4	42.3				116 (3′,5′)
14	41.9	56.8				161.5 (4′)
15	33.6	24.3				
16	21.6	28.2				
17	54.9	56.1				
18	44.8	11.9				
19	41.9	19.3				
20	27.4	36.1				
21	46.5	18.7				
22	148.7	33.9				
23	33.4	26.1				
24	21.6	45.8				
25	15.8	29.2				
26	16.7	19.0				
27	16.7	19.8				
28	16.0	23.1				
29	110.0	12.0				
30	24.9					

Table 1 Cont'd

Solvent	$CDCl_3$		CD_3OD			
Carbon	1	2	3	4	5	6
Sugar (glucose)						
$1'$-β			98.2	98.2		104.3 ($1''$)
$2'$			75.8	75.8		75.7 ($2''$)
$3'$			76.1	76.4		78.3 ($3''$)
$4'$			71.1	71.1		71.3 ($4''$)
$5'$			76.4	76.1		78.0 ($5''$)
$6'$			62.4	62.5		62.6 ($6''$)
$1'$-α			93.8	93.8		
$2'$			72.6	72.6		
$3'$			73.8	73.8		
$4'$			71.1	71.1		
$5'$			72.9	72.9		
$6'$			62.4	62.4		

Assay for xanthine oxidase inhibition[21,22,23] .

A. 0.2 M NaH_2PO_4 solution: $NaH_2PO_4 \cdot H_2O$(E. Merck, 556 mg) was dissolved in distilled water to make 20 ml solution, B. 0.2 M Na_2HPO_4 solution: $Na_2HPO_4 \cdot 12H_2O$ (E. Marck, 1.43 g) 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 100 ml of distilled water to make 0.1 M phosphate buffer solution, pH=7.8.

Xanthine (6.084 mg, E. merck) was dissolved in 200 ml of 0.1 M phosphate buffer pH 7.8 with gentle heating and was shaken well until completely dissolved to make a 200 uM xanthine buffer solution. Five different concentrations (25, 50, 100, 200 and 400 uM) of each compound **3-6** were prepared for tests in 150 ul methanol.

control: A 200 uM solution of xanthine buffer pH=7.8(984 ul) with 6 ul of xanthine oxidase (25 units/1.3 ml, Sigma) and 10 ul of methanol was incubated for 3 min at room temperature and the uric acid formed was estimated at 295 nm against a blank sample which did not contain the enzyme but 6 ul of 0.1 M phosphate buffer solution pH=7.8 instead. The tests were performed in quadruplicate.

sample test: Aliquots (10 ul) of various concentrations of **3 – 6** were added to xanthine buffer solution (984 ul) and phosphate buffer solution (6 ul) as a blank tests. 6 ul of xanthine oxidase (25 units/1.3 ml, Sigma) was added to each 10 ul of various concentrations of test samples in 984 ul of xanthine buffer solution and treated in the same way as the control. Their inhibitory effects on xanthine oxidase were measured by a decrease in uric acid generation. The IC_{50} values were calculated from regression lines of a plot of % inhibition *vs* compound concentration. Quercetin (Sigma)[21,22] was used as a positive control for comparison. The tests were conducted in quadruplicate and the results are shown in Figure 2.

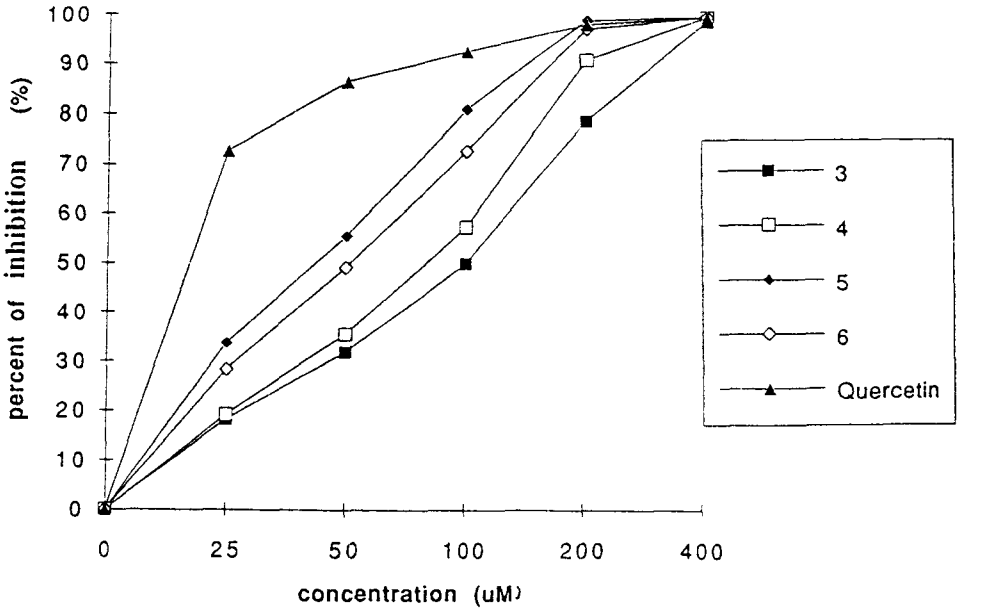


FIGURE 2 The inhibitory effect of compounds **3 – 6** and quercetin on the xanthine-xanthine oxidase system

The Lineweaver-Burk[24] plots for xanthine oxidase inhibition by compounds **3-6** are shown in Figure 3.

K_i values for compounds **3-6** were calculated by Dixon plots[25] for uncompetitive inhibitor by plotting the slope of each double reciprocal plot versus the corresponding inhibitor concentration as shown in Figure 4.

RESULTS AND DISCUSSION

Diploptene(**1**), β-sitosterol(**2**), 6′-*O*-(E-P-coumaroyl)-α-glucopyranose and 6′-*O*-(E-P-coumaryl)-β-glucopyranose(**3**), 6′-*O*-(E-P-caffeoyl)-α-glucopyranose and 6′-*O*-(E-P-caffeoyl)-β-glucopyranose(**4**), caffeic acid(**5**) and astragalin(**6**) were isolated from an ethanolic extract of the leaves of *ALsophila spinulosa* (Hook) Tryon (Cyatheaceae) as shown in Chart I. These compounds have not been previously isolated from this plant except for diploptene(**1**) and β-sitosterol(**2**)[8,9,26,27,28]. Compounds **3-6** have been tested for their inhibitory effect on xanthine oxidase.

Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and of xanthine to uric acid which has λ_{max} 295 nm which forms the basis for a spectrophotometric assay of the activity of xanthine oxidase[23].

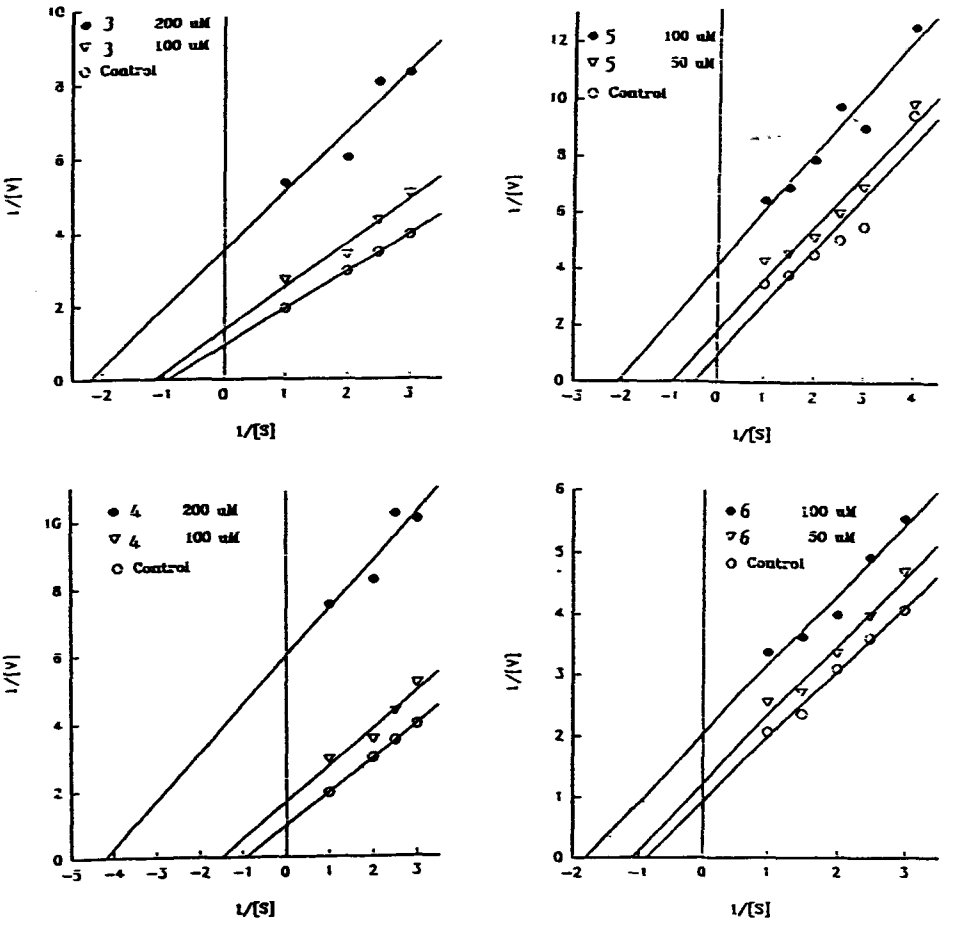
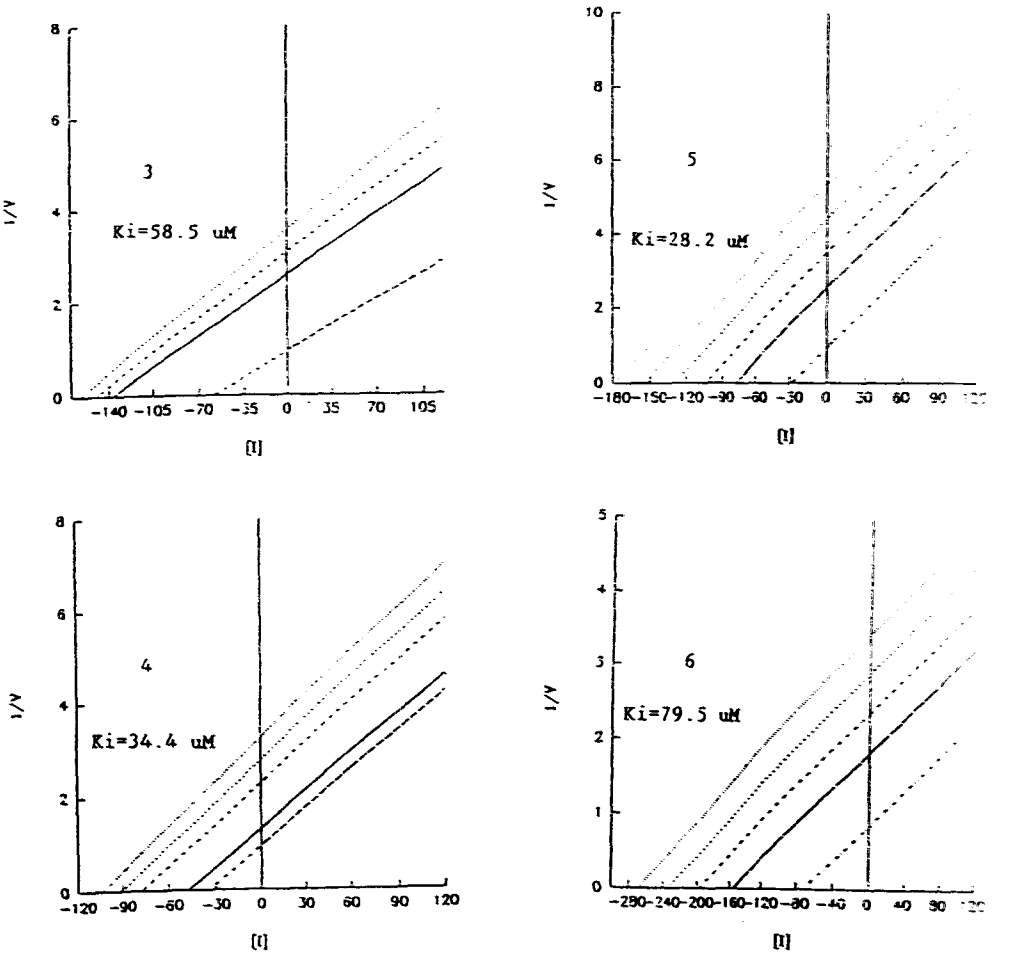


FIGURE 3 Inhibitory effects of compounds **3 – 6** on xanthine oxidase. Lineweaver-Burk plots in the absence (control, o-o) and in the presence of **3 – 6** with xanthine as the substrate. V, uM substrate metabolized/unit enzyme/min, S, substrate, uM.

The tests showed that the purified compounds **3 – 6** supressed the activity of xanthine oxidase in a dose-dependent manner as measured by a decrease in uric acid formation. The order of inhibitory activity was quercetin > 5 > 6 > 4 > 3 with IC_{50} values of 15.9, 39.21, 49.46, 71.43 and 83.31 uM respectively. (Figure 2). Although the inhibitory effects of compounds **3 – 6** on xanthine oxidase was less than that of quercetin, they indicated why the plant has been used as a folk medicine for hepatitis, gout, rheumatism and tumor in Taiwan[1,2]. One of the active principles, caffeic acid has been reported to have an inhibitory effect on tumor promotion in mouse skin caused by 12-*O*-tetradecanoyl phorbol-13-acetate with an increase in the release of active oxygen species[29,30,31], and it has been reported that reactive oxygen species and other free radicals play an important role in tumor promotion[30,31,32,33].



In this study, caffeic acid (**5**) had a greater inhibitory potency than its glucoside (**4**) which indicated that the potency was reduced by glycosylation of the COOH group in (**5**) to form the ester glucoside. On the other hand, the scavenging effects of these phenolic compounds on the superoxide anion radical decreased with decrease in the number of phenolic hydroxyl group in the aglycone moiety of the structure, e.g. (**3**). Evidently for the same reason, the flavonoid quercetin with five phenolic OH groups in the molecule was a much stronger inhibitor of xanthine oxidase than the flavonoid glycoside, astragalin (**6**) with three phenolic OH groups. It is implied that the phenolic OH group present in the structures makes an important contribution to their activity.

Kinetic studies were done on the effects of compounds **3** – **6** on the oxidation of xanthine by xanthine oxidase under our assay conditions. The results are shown in the form of Lineweaver-Burk plots[24] (Figure 3). The mode of inhibition by **3-6** was of an

umcompetitive type with respect to the substrate xanthine and it is assumed that the binding site of xanthine oxidase with compounds **3-6** is not the molybdenum site of the enzyme molecule. The apparent inhibition constants (K_i) of **3** – **6** were 58.5, 34.4, 28.2 and 79.5 uM respectively as shown by Dixon plots for uncompetitive inhibition[25]. (Figure 4).

ACKNOWLEDGEMENT

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