Search for Naturally Occurring Substances to Prevent the Complications of Diabetes. II.[1] Inhibitory Effect of Coumarin and Flavonoid Derivatives on Bovine Lens Aldose Reductase and Rabbit Platelet Aggregation

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An EtOAc extract of Artemisiae Capillari Spica inhibited both bovine lens aldose reductase (bovine-LAR) and rabbit platelet aggregation. Two simple coumarins, scoparone (1) and scopoletin (2), and three flavonoids, capillarisin (21), cirsimaritin (22) and rhamnocitrin (23), were isolated from this extract. Scoparone (1) and scopoletin (2) exhibit a potent inhibitory effect on rabbit platelet aggregation induced by four types of agent, ADP, PAF, sodium arachidonate and/or collagen. Capillarisin (21) exhibits a potent inhibitory effect on bovine-LAR. In addition, thirteen simple coumarins, five coumarin glycosides and two flavonoids were tested for their inhibitory effect against bovine-LAR and rabbit platelet aggregation.

Key words Artemisiae Capillari Spica; bovine lens aldose reductase; diabetic complication; coumarin; flavonoid; platelet aggregation

Increased aldose reductase (AR) activity and platelet aggregation are considered as two causes of diabetic complications. Sorbitol is produced from glucose by AR through the polyol pathway and in diabetes, the substantial accumlation of sorbitol in cells causes damage. This is associated with the development of some chronic diabetic complications, such as cataracts, neuropathy and retinopathy. Also diabetes mellitus is associated with a variety of vascular complications like myocardial infarction and peripheral vessel disease.

In a previous paper, we reported results of screening tests on many plants, Chinese crude drugs and marine algae in terms of their inhibitory effect on bovine lens aldose reductase (bovine-LAR) and rabbit platelet aggregation.[1] Among these samples, an EtOAc extract of Artemisiae Capillari Spica exhibited strong inhibition of both bovine-LAR and rabbit platelet aggregation (see Table 1).

In this paper, we report the isolation of two simple coumarins, scoparone (**1**) and scopoletin (**2**), and three flavonoids, capillarisin (**21**), cirsimaritin (**22**) and rhamnocitrin (**23**), from Artemisiae Capillari Spica, *Artemisia capillaris* THUNG. (Compositae), and describe their inhibitory effects on both bovine-LAR and rabbit platelet aggregation.

Of these compounds, **1** and **2** strongly inhibited rabbit platelet aggregation, so we examined thirteen other simple coumarins and five coumarin glycosides in order to investigate structure inhibitory activity relationships with respect to bovine-LAR and rabbit platelet aggregation (see Chart 1 and Table 2).

As far as bovine-LAR inhibitory activity is concerned, 4-hydroxycoumarin (**7**) and esculetin (**9**) exhibited a strong inhibitory effect comparable with quercetin (**24**), used as positive control while capillarisin (**21**) was more highly active than the control. The other compounds did not show any significant activity.

Regarding the structure–activity relationships involving coumarin derivatives and inhibition of bovine-LAR, marked activity was associated with the presence of a hydroxyl group, especially, 4-OH, and/or 6 and 7-diOH.

As far as inhibition of platelet aggregation is concerned, simple coumarins exhibit potent activity on aggregation induced by sodium arachidonate and collagen, while coumarin glycosides were less active or completely inactive. The inhibitory effect on platelet aggregation induced by ADP and platelet-activating factor (PAF) was associated with the presence of a methoxyl group. However, having

Table 1. Inhibitory Effect of Each Extract from Artemisiae Capillari Spica on Bovine-LAR and Rabbit Platelet Aggregation

Extract	Bovine-LAR Inhibitory effect			Rabbit platelet aggregation Inhibitory effect								
	Conc.			Conc.	ADP		PAF		AA		Collagen	
	(μg/ml)	(%)	(IC_{50})	(mg/ml)	(%)	(IC_{50})	(%)	(IC_{50})	(%)	(IC_{50})	(%)	(IC_{50})
EtOAc	10	90	3.2	1	81	0.5	84	0.7	81	0.2	70	0.2
MeOH	10	95	1.2	1	49		64	0.8	26	0.1	69	0.8
H_2O	10	94	1.9	1	7		0		0		2	

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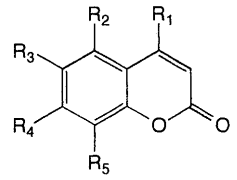
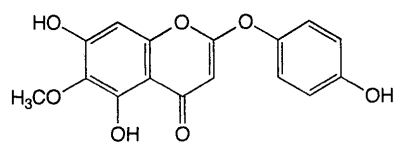


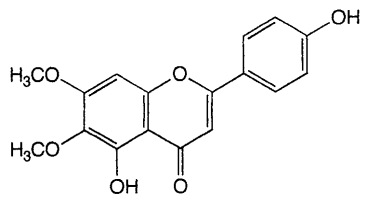
	R_1	R_2	R_3	R_4	R_5
A) simple-coumarin					
1 scoparone	H	H	OMe	OMe	H
2 scopoletin	H	H	OMe	OH	H
3 coumarin	H	H	H	H	H
4 isoscopoletin	H	H	OH	OMe	H
5 fraxinol	H	OMe	OH	OMe	H
6 isofraxidin	H	H	OMe	OH	OMe
7 4-hydroxycoumarin	OH	H	H	H	H
8 umbelliferone	H	H	H	OH	H
9 esculetin	H	H	OH	OH	H
10 4-methoxycoumarin	OMe	H	H	H	H
11 7-methoxycoumarin	H	H	H	OMe	H
12 5,7-dimethoxycoumarin	H	OMe	H	OMe	H
13 7-acetoxy-6-methoxycoumarin	H	H	OMe	OAc	H
14 7-acetoxycoumarin	H	H	H	OAc	H
15 6,7-diacetoxycoumarin	H	H	OAc	OAc	H
B) coumarin glycoside					
16 esculin	H	H	OGlc	OH	H
17 tetraacetylesculin	H	H	*	OAc	H
18 eleutheroside B	H	H	OMe	OGlc	OMe
19 fraxin	H	H	OMe	OH	OGlc
20 mandshurin	H	OMe	OGlc	OMe	H

*:O-(tetraacetylglucose)

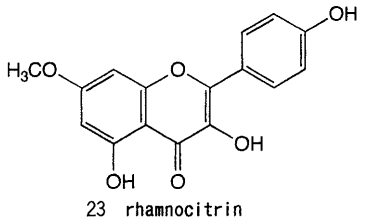
C) flavonoid



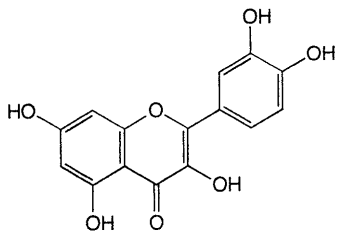
21 capillarisin



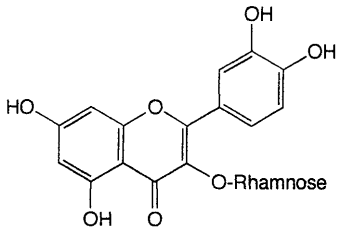
22 cirsimaritin



23 rhamnocitrin



24 quercetin



25 quercitrin

Chart 1. Structure of Test Compounds

only a methoxyl group at C-7 or an acetoxyl group means a lack of any conspicuous effect. Scoparone (**1**) and 5,7-dimethoxycoumarin (**12**) showed particularly strong inhibitory effects on rabbit platelet aggregation induced by ADP, PAF, sodium arachidonate and/or collagen.

4-Hydroxycoumarin (**7**) and esculetin (**9**) exhibited potent activity in both screening tests.

Further study of the structure inhibitory activity relationships is in progress.

Experimental

Isolation of Compounds from Artemisiae Capillari Spica Artemisiae Capillari Spica (1.5 kg) was extracted with *n*-hexane, EtOAc, MeOH and H_2O. Each extract was tested for its inhibitory effect on bovine-LAR and rabbit platelet aggregation. The EtOAc extract (110 g) exhibited strong inhibitory activity on bovine-LAR and rabbit platelet aggregation and so was subjected to silica-gel column chromatography with an *n*-hexane/EtOAc mixture. The final purification was performed by HPLC with an *n*-hexane–acetone mixture (3 : 1) as mobile phase to give five compounds, scoparone (**1**) (1.1 g), scopoletin (**2**) (7 mg), capillarisin (**21**) (25 mg), cirsimaritin (**22**) (13 mg) and rhamnocitrin (**23**) (15 mg).

Materials Coumarins **3**, **4**, **6**—**9**, **12** and **16** were commercially available. Coumarins **10** and **11** were obtained from **8** and **9** by methylation with diazomethane, and **13**—**15** and **17** were obtained from **2**, **8**, **9** and **16** by acetylation with acetic anhydride and pyridine.

Coumarins **5** and **20** were isolated from *Flaxinus japonica* Rupr. var. *japonica* Maxim. (Oleaceae).[2] Coumarin **18** was isolated from *Eleutherococcus senticosus* Maxim. (Araliaceae).[3] Coumarin **19** was isolated from *Flaxinus japonica* Blume (Oleaceae).[2] Quercetin (**24**) and quercitrin (**25**) as positive controls were isolated from *Syneilesis aconitifolia* Maxim. (Compositae).[4]

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and DL-glyceraldehyde were purchased from Wako Pure Chemicals Industries, Ltd.

Assay of Bovine-LAR Activity Each sample was tested for aldose reductase activity at concentrations of 30, 10 and/or 3 μg/ml. Bovine-LAR was prepared as previously described,[5] except that the column chromatography was carried out using DEAE-Toyopearl 650M (Tosoh, Japan). Enzyme assay was performed at room temperature in 67 mM Na-K phosphate buffer (pH 6.2) containing 0.4 M $LiSO_4$ (0.7 ml), 100 mM DL-glyceraldehyde (0.1 ml), 1.25 mM NADPH (0.1 ml) and an appropriate amount of enzyme in a total volume of 1.0 ml.

The effects of each sample on enzyme activity were determined by adding 1.0 μl of the test solution to the reaction mixture; the appropriate blank was prepared. The reaction was initiated by the addition of DL-glyceraldehyde, and the rate of NADPH oxidation was followed by recording the decrease in absorbance at 340 nm using Shimadzu UV 160A spectrophotometer. Each sample was dissolved in dimethyl sulfoxide (DMSO), and the presence of 0.1% DMSO in the final assay mixture did not affect the enzyme activity.

Determination of IC_{50} The concentration of test sample showing 50% inhibition of enzyme activity (IC_{50}) was estimated from the least squares regression line of the plots of the logarithm of the concentration *versus* remaining activity. The concentration of the test solution was expressed in micro grams in 1.0 ml of enzyme reaction mixture.

Platelet Aggregation Fresh blood, obtained from a healthy Japanese

Table 2. Inhibitory Effect of the Test Compounds on Bovine-LAR and Rabbit Platelet Aggregation

	Bovine-LAR Inhibitory effect			Rabbit platelet aggregation Inhibitory effect								
	Conc.			Conc.	ADP		PAF		AA		Collagen	
	(μg/ml)	(%)	(IC_{50})	(mg/ml)	(%)	(IC_{50})	(%)	(IC_{50})	(%)	(IC_{50})	(%)	(IC_{50})
A) Simple coumarin												
1 Scoparone	10	19		0.3	96	0.02	98	0.06	98	0.03	89	0.03
2 Scopoletin	10	60	6.2	1	100	0.3	86	0.5	94	0.1	92	0.2
3 Coumarin	10	11		1	76	0.4	90	0.6	94	0.5	91	0.5
4 Isoscopoletin	10	19		1	77	0.5	100	0.4	96	0.06	92	0.3
5 Fraxinol	10	29		1	72	0.7	100	0.4	99	0.02	94	0.2
6 Isofraxidin	10	18		0.3	14		13		100	0.22	54	0.3
7 4-Hydroxycoumarin	3	79	0.53	1	93	0.5	63	0.8	80	0.6	92	0.6
8 Umbelliferone	10	54	4.8	1	100	0.3	96	0.5	94	0.4	90	0.3
9 Esculetin	10	87	0.74	1	92	0.3	89	0.5	94	0.06	93	0.08
10 4-Methoxycoumarin	10	43		0.3	91	0.08	92	0.1	97	0.07	92	0.09
11 7-Methoxycoumarin	10	15		0.3	34		15		100	0.05	89	0.1
12 5,7-Dimethoxycoumarin	10	42		0.3	100	0.07	100	0.05	97	0.05	97	0.08
13 7-Acetoxy-6-methoxycoumarin	10	11		0.3	50	0.3	2		96	0.2	87	0.2
14 7-Acetoxycoumarin	10	0		0.3	55	0.3	26		62	0.3	77	0.2
15 6,7-Diacetoxycoumarin	10	14		0.3	−24		−24		92	0.01	71	0.2
B) Coumarin glycoside												
16 Esculin	10	28		1	20		3		14		14	
17 Tetraacetylesculin	10	6		0.3	−10		45		18		17	
18 Eleutheroside B	10	10		1	3		0		−2		−13	
19 Fraxin	10	21		1	26		10		4		6	
20 Mandshurin	10	12		1	−20		1		−9		−6	
C) Flavonoid												
21 Capillarisin	1	89	0.22	0.3	28		8		14		0	
22 Cirsimaritin	10	91	1.6	0.1	8		3		0		5	
23 Rhamnocitrin	10	21		0.1	0		5		100	0.06	11	
24 Quercetin	10	88	0.84	1	94	0.4	96	0.5	96	0.2	92	0.2
25 Quercitrin	3	81	0.5	1	13		20		6		−3	

Promotive activity is expressed by a minus sign (−).

white male rabbit, containing 0.38% sodium citrate was centrifuged at 1000 rpm for 10 min at room temperature to obtain a supernatant of platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was prepared by re-centrifugation of the remaining blood at 3000 rpm for 10 min. Each extract, the collected fractions and each compound were dissolved in DMSO or buffer and 2.5 μl of each was added to 222.5 μl PRP (5×10^5 platelets/μl). Adenosine 5′-diphosphate (ADP), PAF, sodium arachidonate and collagen were used as inducers at final concentrations of 10 μM, 0.01 μg/ml, 300 μM and 30 μg/ml, respectively. Platelet aggregation was determined by a turbidimetric method using a platelet aggregometer (Mebanix). A water soluble sample was dissolved in saline and 2.5 μl of this was added to 222.5 μl PRP, while an insoluble sample in saline was dissolved in DMSO, and 2.5 μl of this was added to 222.5 μl PRP. After incubation at 37 °C for 1 min with stirring, platelet aggregation was initiated by addition of 25 μl inducer. Saline or DMSO was used as a control.

The degree of anti-platelet aggregation (%) was calculated from the following equation: ratio of anti-platelet aggregation (%) = [1 − (platelet aggregation potency of sample/platelet aggregation potency of control)] × 100.

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References and Notes

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