Arch. Pharm. Res. **14**(2), 147-159 (1991)

Pharmacological Activities of Flavonoids (III)
Structure-Activity Relationships of Flavonoids in Immunosuppression

Chang-Johng Kim and Seung Kil Cho
Department of Pathophysiology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea
(Received March 20, 1991)

Abstract □ Effects of twenty-one different flavonoids and their related compounds on the phagocytosis of colloidal carbon by macrophages in liver and spleen, humoral immune responses against bacterial α-amylase and cellular immune responses against oxazolone and dinitrofluorobenzene were studied *in vivo* and *in vitro*. It was shown that most of the flavonoids accelerated significantly the phagocytosis, and they suppressed significantly not only humoral and cellular immune responses but also the development of immunological memory after the antigenic stimulation. Especially, malvin was the most active in phagocysis, and disodium cromoglycate and morin were the most active in humoral and cellular immunosuppression, respectively. Daidzein had the most potent inhibitory activity in the development of memory cells. The structure-activity relationships of the flavonoids in immunosuppression became apparant from these results: 1. The presence of $C_{2\text{-}3}$ double bond and C_4 ketone group in C-ring was important for their immunosuppressive activity. 2. Flavonoids with benzene ring at 2 or 3 position in C-ring showed the almost same activities. 3. The opening of C-ring did not affect their immunosuppressive activity. 4. The glycosylated flavonoids at 3 position in C-ring were less potent than their aglycones. 5. Di- or tri-hydroxylated flavonoids in B-ring were more potent than mono-hydroxylated. 6. Chromanochromanone also had the immunosuppressive activity.

Keywords □ Structure-activity relationships, flavonoids, phagocytosis, carbon clearance, immune responses, immunological memory, bacterial α-amylase, oxazolone, dinitrofluorobenzene.

Flavonoids comprise a large group of naturally occurring low molecular weight substances widely distributed in the vegetable kingdom[1]. They have been shown to possess anti-inflammatory, antiallergic, antiviral, spasmolytic and anti-carcinogenic activities[2-10]. Recently, the isolation of immunomodulating constituents from medicinal plants such as flavonoids[11-18], polysaccharides[19,20], saponins[21,22] and triterpenes[23,24] has been studied. All flavonoids are derived from the basic structure flavone (2-phenylchromone or 2-phenylbenzopyrone) which is structurally related to the antiallergic drug cromolyn. Quercetin and other flavonoids inhibit histamine release from rat mast cells, basophils and neutrophils induced by antigens, concanavalin A and Ca^{2+} ionophore A23187[25-27].

In the previous studies, we reported that structure-activity relationships of twenty-one flavonoids and related compounds in anti-hypersensitivities: most of the flavonoids have the inhibitory activities of type I, II, III and IV hypersensitivities induced by antigenic stimulation[28], and also most of the flavonoids have anti-inflammatory activities, but they inhibited the wound healing in accordance with anti-inflammatory activity[29].

In the present study, we examined effects of several flavonoids of different chemical classes on phagocytosis, immune responses and development of immunological memory against antigenic stimulation. The data indicate that flavonoids inhibited humoral and cellular immune responses depending on the antigenic stimuli and particular structures of the flavonoids.

EXPERIMENTAL METHODS

Animals

Male Sprague-Dawley rats weighing about 120 g and male ICR mice weighing about 16 g were used. They were supplied with the laboratory pellet and tap water *ad libitum*.

Conditions of irradiation

Mice used as recipients were irradiated in a plastic container placed on a revolving turntable. The dose rate was 71.9 r/min. The source of X-rays was Philips MG-320D operated under the following conditions: 180 kVp at 15 mA, target distance of 50 cm, and added filtration was 1.0 mm Al. The preliminary experiments showed that the exposure of 600 r X-irradiation to the recipients just before the cell transfer was most suitable to the survival of animals and the antibody titer obtained in the recipients. A fixed radiation dose of 600 r was given to mice 5 hours before cell transfer throughout the experiment.

Materials

Twenty-one flavonoids and their related compounds were purchased from Sigma Chemical Co., Carl Roth, Fison plc Pharmaceutical Division, Wako Pure Chemical Co. and Aldrich Chemical Co. (Table I) and purified by column chromatography with the mixture of chloroform-methanol in silica gel. Bacterial α-amylase, oxazolone, dinitrofluorobenzene, cyclophosphamide and zymosan were also purchased from Sigma Chemical Co. Freund's incomplete adjuvant was obtained from Difco Co., Pelikan drawing ink 17 black was from Pelikan AG. and prednisolone acetate was from Roussel Uclaf Co. Other reagents were of first grade. Flavonoids were suspended in 5% arabia gum solution and orally administered at a dose of 50 and 100 mg per kg body weight.

Determination of carbon clearance

Flavonoids were administered 1 hour before i. v. injection of colloidal carbon (Pelikan drawing ink 17 black 3 m*l*: physiological saline solution 8 m*l*) 0.1 m*l* per 10 g body weight. In 5, 10, and 15 minutes after carbon black injection, blood was obtained by retro-orbital venous puncture with a heparinized capillary tube, and suspended in 2 m*l* of 0.1% Na_2CO_3 solution. Optical density (O.D.) was determined at 675 nm wave length[30]. The carbon clearance was calculated according to the formula;

$$\text{Carbon clearance } (t_{1/2}) = \frac{(t_2 - t_1)_{1/2}\ ODt_2}{ODt_1 - ODt_2}$$

where, t_1 is 5 mins, t_2 is 10 or 15 mins, ODt_1 and ODt_2 are optical densities at time t_1 and t_2 respectively.

Hematoxylin-eosin staining of liver and spleen

Liver and spleen were fixed in 10% formalin solution and stained with hematoxylin and eosin by Harris method[31].

Phagocytosis of Kupffer cells

Numbers of Kupffer cells that phagocytized colloidal carbon were counted microscopically by hematoxylin-eosin staining according to the method of Kubo *et al.*[30].

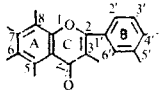
Primary humoral immune response

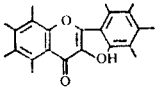
Mice were immunized by a intraperitoneal and subcutaneous injections of 100 μg bacterial α-amylase (BαA) with Freund's incomplete adjuvant, respectively[32] and then antibody titer[33] was measured from 5 to 60 days after antigenic stimulation. Drugs were given once a day for 13 days from 3 days before immunization.

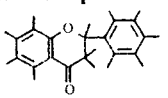
Development of immunological memory after humoral antigenic stimulation

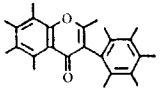
Lymphocytes suspensions were prepared from spleen and lymph node (LN) of mice 4 weeks after primary BαA immunization. The organs were gently chopped with fine scissors in chilled Hank's balanced salt solution and forced through a stainless steel screen. The tissue was pipetted vigorously to free cells and then passed through a 80 mesh/cm stainless steel cytosieve. The collected free cells were washed once by centrifugation for 10 minutes at 1,000 rev/min. More than 90% of spleen and lymph node cells were viable as judged by Tryphan blue exclusion test[34]. The cell suspensions were adjusted to appropriate cell concentration and mixed with BαA (100 μg/m*l*). One m*l* of this single cell suspension (7.1×10^6 cells/m*l*) was transferred intravenously into the tail vein of the previously X-irradiated recipient mice and the size of immunological memory was

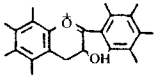
Table I. Nomenclature and source of the flavonoids and related compounds studied.

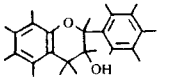

Flavone


Flavonol


Flavanone


Isoflavone


Anthocyanidin


Catechin

Flavonoids		Substituents[1]								Source
		3	5	6	7	2′	3′	4′	5′	
Flavones	Flavone	H	H	H	H	H	H	H	H	Sigma Co.
	Chrysin	H	OH	H	OH	H	H	H	H	Sigma Co.
	Baicalein	H	OH	OH	OH	H	H	H	H	Carl Roth
	Apigenin	H	OH	H	OH	H	H	OH	H	Aldrich Chemical Co., Inc.
Flavonols	Fisetin	OH	H	H	OH	H	OH	OH	H	Sigma Co.
	Kaempferol	OH	OH	H	OH	H	H	OH	H	Sigma Co.
	Morin	OH	OH	H	OH	OH	H	OH	H	Wako Pure Chemical Co.
	Myricetin	OH	OH	H	OH	H	OH	OH	OH	Sigma Co.
	Taxifolin	OH	OH	H	OH	H	OH	OH	H	Sigma Co.
	Quercetin	OH	OH	H	OH	H	OH	OH	H	Sigma Co.
	Rutin	O Rutinose	OH	H	OH	H	OH	OH	H	Tokyo Kasei Inc., Co.
Flavanones	Hesperetin	H	OH	H	OH	H	OH	OMe	H	Sigma Co.
	Naringin	H	OH	H	O Rhamnose	H	H	OH	H	Sigma Co.
	Hesperidin	H	OH	H	O Rhamnose	H	OH	OMe	H	Sigma Co.
Isoflavones	Daidzein	H	H	H	OH	H	H	OH	H	Carl. Roth
Anthocyanins	Cyanin	O Glucose	O Glucose	H	OH	H	OH	OH	H	Sigma Co.
	Malvin	O Glucose	O Glucose	H	OH	H	OCH_3	OH	OCH_3	Sigma Co.
Catechins	Catechin	OH	OH	H	OH	H	OH	OH	H	Sigma Co.
Chalcones	Neohesperidin				Rhamnose					
Chromano-chromanones	Rotenone									Aldrich Chemical Co., Inc.
	Disodium cromoglycate									Fison plc pharmaceutical division

[1]These substituents are for the positions of the above structural formula.

estimated by the determination of antibody production in the recipients 7 and 11 days after the same antigenic stimulation. Drugs were given orally once a day for 9 days from 3 days before cell transfer.

Measurement of antibody titer

Blood was collected from the retro-orbital venous plexus with a heparinized capillary tube. Serum was diluted with physiological saline solution two-fold serially and inactivated by heating at 58℃ for 40 mins. This serum (0.5 m*l*) was mixed with 0.5 m*l* of BαA solution (1 μg/m*l*) dissolved with physiological saline containing 0.01% of bovine serum albumin and 1 mM of calcium acetate. After incubation of them at 37℃ for 50 mins, 1 m*l* of 0.4 M acetate buffer (pH 6.0) and 2.0 m*l* of 1% starch solution were added and further incubated for 15 mins. After this incubation, 5 m*l* of 1 M acetic acid was added. The residual amylase activity was measured by determining amount of reducing sugar[35]. This mixture 0.5 m*l* was transferred to another test tube and mixed with 10 m*l* of 0.1% I_2/0.1% KI solution. Optical density was determined at 660 nm wave length.

Antibody titer[33] was calculated according to the formula :

$$\text{Enzyme units} = \frac{D_b - D}{D_b} \times 40$$

D : O.D. after enzyme reaction
D_b : O.D. of blank solution

$$\text{Antibody titer} = (\text{E.U.}_{\cdot B\alpha A} - \text{E.U.}_{\cdot \text{antiserum}}) \times \frac{N}{10}$$

$\text{E.U.}_{\cdot B\alpha A}$: Enzyme units of control group
$\text{E.U.}_{\cdot \text{antiserum}}$: Residual enzyme units after neutralization with antiserum
N : Dilution number

Secondary cellular immune response

Oxazolone-induced dermatitis-Rats were sensitized by painting 50 μ*l* of a 10% oxazolone in acetone to the shaved right flank. Nine days after the sensitization, 10 μ*l* of 1% oxazolone in acetone was applied to the dorsal surface of the right ear[36] and cellular immune responses were elicited by the swelling of ear by means of cell-mediated hypersensitivity. Baseline measurements of ear thickness were made immediately prior to challenge with an engineer's micrometer. Reaction to oxazolone was measured 24, 48, 72 and 120 hours after challenge as an increase in ear thickness. Drugs were given orally once a day for 9 days after the sensitization.

Development of immunological memory after cellular antigenic stimulation

Dinitrofluorobenzene-induced dermatitis-mice, given flavonoids once a day for 2 days, were sensitized twice a day with 25 μ*l* of 0.5% dinitrofluorobenzene (DNFB) in acetone by painting on the shaved abdomen, and challenged with 5 μ*l* of 0.5% DNFB on the footpads and ears. Single cell suspensions of lymph node cells (4.0×10^7 cells/m*l*) were prepared 72 hours after first sensitization as same as the above development of immunological memory in humoral immune response, and 1 m*l* of this single cell suspension was injected intravenously into the tail vein of the recipients. The recipients were challenged 1 hour after cell transfer by applying with 20 μ*l* of 0.2% DNFB in acetone on the dorsal surface of each ear, and cellular immune responses were elucidated by the swelling of ear by means of cell-mediated hypersensitivity[37]. Baseline measurements of ear thickness were made immediately prior to challenge. Ear swelling was measured with an engineer's micrometer 24 hours after cell transfer.

RESULTS

Acceleration of carbon clearance

Generally flavonoids accelerated significantly the carbon clearance as compared as the control group ($t_{1/2} = 13.24$ min) as shown in Table II. Malvin was the most active flavonoid. Disodium cromoglycate, baicalein, daidzein, quercetin, flavone and hesperetin at doses of 50 and 100 mg/kg also shortened the half-life of colloidal carbon in blood dose-dependently, and their activities were the same as zymosan which is known as a stimulating agent of macrophages[30].

Enhancing activity of phagocytosis by macrophages of liver and spleen

Colloidal carbons were phagocytized by macrophages of liver and spleen (Fig. 1-1 and 2) and so we could elucidate microscopically the number of colloidal carbon-phagocytized Kupffer cells of the liver with the hematoxylin-eosin staining. It was shown that flavonoids increased significantly the

Table II. Effects of flavonoids on the clearance and Kupffer cell uptake of carbon black.

Drugs[1]	Dose (mg/kg)	No. of animal	Carbon clearance[2] ($t_{1/2}$, min.)	Cell no. per 100 mm^2 of Kupffer cells phagocytized carbon[3]
Control	–	9	13.24± 2.99	118.5± 6.4
Flavone	50	6	6.68± 2.69*	133.2± 4.7*
	100	6	4.57± 1.11*	142.5± 5.3*
Chrysin	50	6	4.29± 1.12**	143.4± 5.2*
	100	6	7.55± 1.68	129.2± 3.6*
Baicalein	50	6	4.50± 1.02**	142.1± 4.1*
	100	6	3.82± 0.92**	157.6± 5.1**
Apigenin	50	6	12.22± 3.65	120.6± 2.8
	100	6	14.13± 2.34	104.7± 2.1
Fisetin	50	6	3.52± 1.08**	161.1± 5.7**
	100	6	4.34± 2.69**	142.7± 4.6*
Kaempferol	50	6	9.47± 1.57	125.6± 3.6
	100	6	6.85± 1.08*	131.3± 2.9*
Morin	50	6	7.76± 2.34	126.7± 5.1
	100	6	6.17± 2.69*	137.2± 2.9*
Myricetin	50	6	8.58± 4.46	129.3± 3.3*
	100	6	3.32± 0.35**	162.6± 4.0**
Taxifolin	50	6	13.41± 1.16	119.0± 2.4
	100	6	10.87± 1.78	120.3± 2.5
Quercetin	50	6	6.67± 3.25*	131.7± 3.6*
	100	6	2.12± 0.23**	168.6± 4.3**
Rutin	50	6	12.43± 0.98	119.7± 3.6
	100	6	10.38± 1.35	120.8± 1.9
Hesperetin	50	6	6.84± 1.16*	131.4± 3.8*
	100	6	4.50± 2.42**	142.5± 5.0*
Naringin	50	6	8.38± 2.62	131.2± 6.3*
	100	6	4.09± 0.85**	143.9± 2.5*
Hesperidin	50	6	6.64± 1.08*	134.1± 3.1*
	100	6	7.15± 1.42*	129.3± 4.0*
Daidzein	50	6	5.11± 1.25*	139.2± 3.3*
	100	6	4.17± 0.97**	148.5± 2.8*
Cyanin	50	6	4.06± 0.43**	145.5± 4.3*
	100	6	3.77± 0.63**	156.4± 3.9**
Malvin	50	6	3.24± 0.57**	163.7± 4.5**
	100	6	3.98± 0.68**	146.5± 5.7*
Catechin	50	6	7.94± 0.95	131.4± 3.7
	100	6	9.49± 1.49	124.7± 2.8
Neohesperidin	50	6	4.82± 1.17*	138.7± 3.5*
	100	6	6.04± 4.11*	135.9± 3.0*
Rotenone	50	6	3.26± 0.36**	159.1± 3.9**
	100	6	5.22± 1.40*	136.5± 2.7*
Disodium cromoglycate	50	6	4.85± 1.20*	137.9± 3.3*
	100	6	4.00± 1.11**	145.1± 2.8*
Cyclophosphamide	50	6	5.54± 1.21*	136.0± 1.9*
	100	6	4.87± 1.15*	137.1± 4.3*
Prednisolone acetate	10	6	17.23± 1.75	98.7± 3.6
	20	6	19.45± 2.11	91.6± 2.5
Zymosan	50	6	4.81± 0.62*	138.7± 2.3*
	100	6	4.05± 0.72**	144.7± 2.8*

[1] Mice were orally treated (but zymosan, i.p.) with drugs 1 hours before the i.v. injection of carbon suspension (Pelican drawing ink 17 black 3 m*l*; saline 8 m*l*) at a dose of 0.1 m*l*/10 g.

[2] Carbon clearance calculated as follows:

$$\text{Carbon clearance}(t_{1/2}) = \frac{(t_2 - t_1)\ 1/2\ OD_{t_2}}{OD_{t_1} - OD_{t_2}}$$

t_1 and t_2 represent 5 and 10 or 15 minutes, respectively, after the injection of carbon suspension and OD_{t_1} and OD_{t_2} are their optical density at that time.

[3] Carbon-phagocytized Kupffer cells were microscopically counted by H & E staining.

Each value represents the mean± S.E.; Significantly different from control (*$p<0.05$ and **$p<0.01$).

Table III. Inhibitory activities of flavonoids on the antibody production in primary humoral immune response against bacterial α-amylase[1].

Drugs[2]	Dose (mg/kg)	No. of animal	Antibody titer[3] Days after injection of antigen 35	40	45	50	55	60
Control	–	6	152.6± 11.4	230.1± 19.6	248.2± 17.5	269.7± 18.4	293.5± 27.5	313.1± 17.5
Flavone	50	6	109.1± 10.6	137.8± 15.4*	170.3± 15.6*	186.7± 16.4*	215.7± 19.6	252.1± 23.6
	100	6	–	–	–	–	–	–
Chrysin	50	6	78.3± 14.5*	180.1± 14.2	201.3± 10.7	235.2± 16.2	285.2± 15.3	301.1± 19.6
	100	6	109.9± 17.4	166.8± 19.6	194.2± 19.5	221.8± 23.5	263.5± 19.2	296.0± 31.5
Baicalein	50	6	97.2± 7.4*	171.7± 15.4	196.7± 7.5	204.5± 19.7	215.6± 19.7	246.7± 24.3*
	100	6	85.7± 11.4*	150.3± 16.9*	170.7± 15.7*	191.5± 16.4*	199.7± 20.6*	211.5± 20.4*
Apigenin	50	6	92.1± 16.7*	156.7± 23.4	182.9± 21.5	175.6± 26.7*	211.4± 18.7*	224.9± 27.4*
	100	6	103.9± 15.6	171.4± 21.3	228.3± 16.7	216.5± 21.4	239.0± 28.4	253.4± 25.4
Fisetin	50	6	130.6± 14.2	197.5± 16.2	205.3± 14.2	235.4± 19.4	261.5± 23.6	294.7± 29.7
	100	6	105.3± 13.3	130.5± 14.3*	187.0± 21.5	205.4± 20.4	230.5± 19.7	241.5± 39.6*
Kaempferol	50	6	115.6± 12.6	196.7± 14.3	210.5± 14.3	225.4± 26.7	245.3± 31.4	278.7± 31.5
	100	6	96.5± 11.5*	127.5± 16.4*	184.5± 19.6	199.5± 21.5	216.7± 24.3	230.5± 19.7*
Morin	50	6	130.5± 11.4	190.5± 17.5	196.4± 14.3	201.5± 25.4	224.5± 19.6	233.4± 31.2*
	100	6	121.4± 15.6	172.5± 19.7	194.3± 16.5	199.7± 20.7	216.4± 18.7	230.5± 27.4*
Myricetin	50	6	148.7± 15.1	230.5± 15.6	237.2± 10.5	240.1± 19.7	260.5± 24.3	270.6± 18.4
	100	6	121.5± 17.3	180.5± 25.4	196.4± 16.4	208.5± 17.6	221.9± 21.5	230.5± 23.2*
Taxifolin	50	6	130.6± 14.2	194.7± 14.3	211.5± 9.7	220.6± 15.1	230.7± 31.4	241.5± 29.6*
	100	6	98.4± 15.3*	135.7± 15.6*	184.2± 10.5	205.7± 19.6	210.5± 21.4*	215.3± 23.4*
Quercetin	50	6	120.3± 14.3	165.1± 15.7	177.1± 19.6	190.5± 21.4*	209.7± 20.6*	234.4± 28.5*
	100	6	77.7± 16.4*	134.4± 19.7*	157.0± 18.4*	175.0± 20.5*	195.2± 23.6*	213.4± 31.4*
Rutin	50	6	131.2± 17.2	196.4± 15.1	220.6± 16.4	229.6± 18.4	243.5± 31.6	245.6± 36.7*
	100	6	104.3± 15.2	143.5± 21.5*	187.7± 20.9	196.5± 19.6*	215.4± 24.3	230.5± 28.7*
Hesperetin	50	6	102.3± 11.6*	191.8± 13.5	214.8± 21.4	232.6± 19.4	285.0± 29.4	308.1± 31.4
	100	6	123.8± 19.6	165.2± 14.6	191.1± 30.5	214.1± 27.4	267.9± 25.4	294.4± 27.5
Naringin	50	6	110.9± 15.2	146.7± 11.5*	171.0± 14.7*	195.9± 21.4*	208.7± 19.6*	247.7± 30.5*
	100	6	151.1± 16.7	156.7± 14.2	195.1± 14.5	225.7± 16.4	231.0± 18.4	246.4± 35.7*
Hesperidin	50	6	127.8± 15.7	206.4± 19.4	186.9± 15.7	221.8± 19.4	225.0± 15.7	250.6± 27.4*
	100	6	85.5± 17.3*	147.2± 21.4*	169.0± 21.3*	193.6± 18.4*	201.5± 19.7*	236.0± 31.4*
Daidzein	50	6	110.4± 7.6	143.5± 18.4*	174.3± 10.2*	194.3± 16.7*	210.5± 19.6*	230.5± 17.4*
	100	6	90.4± 11.4*	115.6± 17.2*	135.6± 15.7*	161.7± 18.4*	190.1± 21.4*	205.7± 20.5*
Cyanin	50	6	149.6± 11.5	219.6± 21.4	235.7± 23.4	265.5± 30.5	275.4± 29.6	285.4± 19.6
	100	4	133.6± 9.7	199.4± 15.4	224.7± 24.3	251.7± 21.5	263.4± 19.7	267.7± 23.3
Malvin	50	6	144.7± 15.3	196.7± 18.4	224.7± 30.5	240.5± 16.4	275.4± 7.4	279.4± 24.7
	100	6	130.5± 14.3	178.5± 16.4	199.4± 19.7	230.2± 17.9	239.4± 11.9	244.5± 21.6*
Catechin	50	6	143.6± 21.4	215.7± 19.6	220.7± 33.5	254.5± 29.7	301.4± 37.5	315.7± 40.1
	100	6	150.6± 19.7	208.7± 21.4	221.4± 19.6	240.4± 30.5	285.7± 19.6	297.5± 28.7
Neohesperidin	50	6	126.7± 19.6	186.4± 21.4	221.1± 35.6	236.4± 31.5	258.9± 29.3	303.1± 35.7
	100	6	129.3± 21.3	174.0± 19.7	205.8± 18.5	223.8± 27.4	264.3± 29.3	278.8± 31.4
Rotenone	50	6	123.7± 19.6	167.7± 21.7	188.0± 19.4	188.4± 18.6*	210.6± 27.6*	229.2± 29.4*
	100	6	–	–	–	–	–	–
Disodium cromoglycate	50	6	94.2± 14.3*	145.7± 19.6*	176.4± 11.4	196.3± 15.7*	215.7± 14.4	220.4± 29.3*
	100	6	69.4± 9.5**	98.7± 13.4**	115.7± 5.8**	131.4± 19.6*	164.6± 15.1*	187.9± 15.7**
Cyclophosphamide	50	6	84.2± 19.3*	123.5± 21.5*	150.7± 24.3*	177.9± 29.7*	193.5± 31.4*	211.7± 29.8*
	100	6	77.6± 12.5*	98.7± 18.7**	115.6± 15.1*	140.7± 19.6*	175.4± 23.6*	198.7± 31.4*
Prednisolone acetate	10	6	88.7± 9.7*	120.5± 15.5*	165.7± 15.4	183.5± 18.7*	201.5± 18.4*	225.7± 19.6*
	20	6	80.5± 11.5*	104.7± 19.6*	123.5± 16.7*	164.5± 19.4*	188.9± 16.7*	210.1± 21.5*

[1]Mice were sensitized with i.p. and s.c. injection of bacterial α-amylase at a dose of 100 μg, respectively.
[2]Drugs were orally given once daily for 13 days from 3 days before sensitization of antigen.
[3]Antibody titer was measured by assay the amount of reduced sugar according to the Nakashima method. Each value represents the mean± S.E.; Significantly different from control (*$p<0.05$ and **$p<0.01$).

Fig. 1-1. Liver of control mouse given carbon black ink. Carbon particles were diffusely phagocytized by Kupffer cells (H & E, ×400).

Fig. 1-2. Liver of mouse treated by malvin at a dose of 50 mg/kg. Flavonoids enhanced phagocytosis of carbon particles (H & E, ×400).

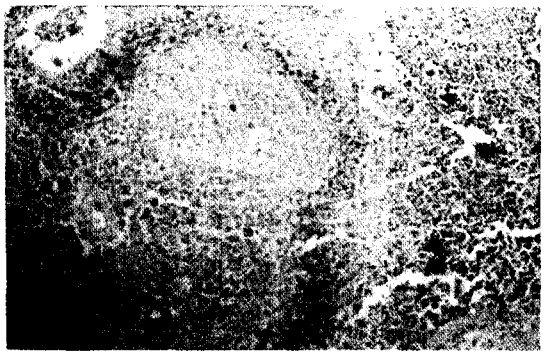


Fig. 2-1. Spleen of control mouse given carbon black ink. Carbon particles were phagocytized by the macrophages on marginal zone in spleen (H & E , ×100).

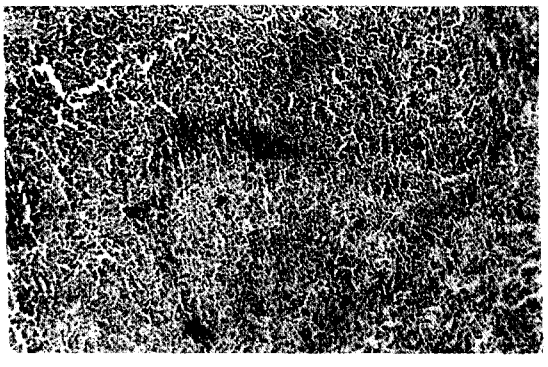


Fig. 2-2. Spleen of mouse treated by malvin at a dose of 50 mg/kg. Flavonoids potentiated markedly phagocytosis (H & E, ×100).

phagocytosis of Kupffer cells as the same pattern as carbon clearance (Table II). It can be shown that flavonoids act as stimulating agents of macrophages such as zymosan[30]. Simultaneously, the phagocytosis of colloidal carbon by macrophages in the marginal zone of spleen was also potentiated as same pattern as that of Kupffer cells in liver (Fig. 2-1 and 2).

Suppressive activity of humoral immune response

The time course of the immune response in mice immunized with BαA in Freund's incomplete adjuvant was shown in Fig. 3. The detectable anti-BαA activities in the serum appeared about 7 days after the primary antigenic stimulation and increased sharply from 35 days after the primary antigenic stimulation.

Most of flavonoids were found to act as immunosuppressive agents in humoral immune response against BαA as shown in Tables III and IV. Generally flavonoids inhibited the primary humoral immune response. Disodium cromoglycate was more suppressive in the antibody production against BαA dose-dependently than others, and daidzein, quercetin and baicalein also significantly suppressed the antibody production 35 days after immunization of BαA in Freund's incomplete adjuvant as shown in Table III. Their activities at doses of 50 and 100 mg/kg were less than that of cyclophosphamide, but were the same as prednisolone acetate at doses of 10 and 20 mg/kg.

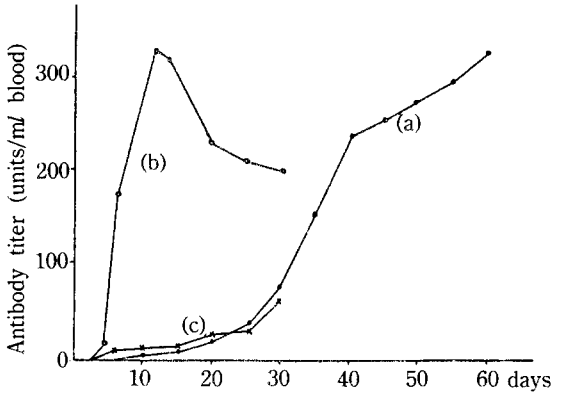


Fig. 3. Antibody response against bacterial α-amylase (BαA) in mice.

(a) non-irradiated mice primed with BαA
(b) X-irradiated mice challenged with BαA after transfer of primed cells
(c) X-irradiated mice challenged with BαA after transfer of non-primed cells

Inhibitory activity of the development of immunological memory after humoral antigenic stimulation

The BαA and single cell suspension from the spleen and lymph node cells of mice primed 4 weeks before were injected intravenously into X-irradiated mice. It was shown that all of the recipients gave the secondary type of antibody response as shown as Fig. 3. The anti-BαA activity could be detected on day 5, increased sharply, reached the maximum on day 10-12 and then decreased gradually. The results of this experiment suggest that immunological memory cells developed gradually with the lapse of time after the primary antigenic stimulation up to 6 weeks.

Disodium cromoglycate, baicalein, quercetin, daidzein and flavone also suppressed significantly the antibody production of the X-irradiated recipient on day 7 and 11 after challenge with BαA after cell transfer as shown as Table IV. It was shown that flavonoids inhibited the development of immunological memory and their activities were less than those of cyclophosphamide and prednisolone acetate.

Suppressive activity of cellular immune response

Cell mediated immune (CMI) response was evaluated by assessing the ability of rats to respond to oxazolone, which is the thymus cell (T-cell) dependent contact sensitizing agent[36]. In 24 hours after challenge of oxazolone, ear thickness increased sharply, reached the maximum on day 2 and then decreased gradually.

Most of the flavonoids significantly suppressed the cellular immune response (Tables V and VI). Morin at a dose of 100 mg/kg suppressed the ear swelling at 24, 48, 72 and 120 hours after challenge of oxazolone. Quercetin and disodium cromoglycate at a dose of 100 mg/kg also suppressed significantly the oxazolone-mediated ear swelling as shown as Table V.

Inhibitory activity of the development of immunological memory after cellular antigenic stimulation

The lymph node cell suspensions from mice previously sensitized DNFB were injected intravenously into the recipients and challenged 1 hour after cell transfer by painting of DNFB on each ear. The ear of the recipients swollen 24 hours after the challenge with DNFB because of immunological memory after antigenic stimulation[37]. Flavonoids significantly suppressed the ear swelling as shown as Table VI. These results suggest that flavonoids inhibited the development of memory cell against DNFB. Morin, quercetin, myricetin, baicalein, flavone, daidzein and chrysin at doses of 50 and 100 mg/kg suppressed significantly the ear swelling dose dependently as compared as the control group. They have the same activity as cyclophosphamide.

DISCUSSION

Flavonoids, benzo-γ-pyrone derivatives, structurally resemble necleosides, isoalloxazine and folic acid[38], and this similarity is the basis of many of the current hypothesis of their physiological action. Our studies have shown that on the whole flavonoids had the immunosuppressive activities as same as Lx (polysaccharides) from *Licorice root*[32]. Most of the flavonoids we tested potentiated the phagocytic activity of Kupffer cells in liver and macrophages in spleen. On the other hand, they suppressed not only the humoral and cellular immune responses but also the development of immunological memory.

We reported that flavonoids inhibited fibroblast proliferation[29] and hypersensitivity types I-IV which were mediated by B or T-lymphocytes[28]. On the basis of our results, it is possible to suggest that flavonoids inhibited the proliferation and the devel-

Table IV. Inhibitory activities of flavonoids on the antibody production through the development of immunological memory in humoral immune response against bacterial α-amylase[1]

Drugs[2]	Dose (mg/kg)	No. of animal	Antibody titer[3] Days after challenge 7	11
Control	–	6	190.4± 15.4	316.0± 39.4
Flavone	50	6	134.4± 21.5*	243.6± 34.7*
	100	6	130.3± 24.7*	221.6± 28.5*
Chrysin	50	6	163.3± 30.5	299.7± 29.5
	100	6	148.1± 29.1	274.3± 31.4
Baicalein	50	6	126.7± 21.4*	235.7± 23.1*
	100	6	119.5± 19.6*	215.4± 26.5*
Apigenin	50	6	125.4± 30.7*	241.3± 39.7*
	100	6	154.3± 19.6	288.6± 31.5
Fisetin	50	6	154.4± 23.3	288.9± 24.5
	100	6	157.2± 18.7	270.4± 21.1
Kaempferol	50	6	157.6± 23.7	285.4± 22.3
	100	6	141.6± 30.1	272.4± 19.1
Morin	50	6	147.9± 19.7	314.3± 31.9
	100	6	135.4± 20.5	311.3± 34.7
Myricetin	50	6	181.5± 15.7	247.9± 30.6
	100	6	172.6± 11.5	235.3± 21.4
Taxifolin	50	6	175.8± 20.5	284.6± 18.4
	100	6	145.3± 21.5	274.6± 21.4
Quercetin	50	6	132.5± 19.6*	243.4± 24.4*
	100	6	122.3± 15.4*	214.9± 23.2*
Rutin	50	6	156.7± 19.6	294.7± 16.7
	100	6	179.6± 14.3	298.5± 15.4
Hesperetin	50	6	190.7± 18.8	304.5± 34.6
	100	6	159.0± 16.7	290.2± 29.7
Naringin	50	6	128.5± 21.4*	241.5± 40.5*
	100	6	167.3± 19.7	290.3± 37.4
Hesperidin	50	6	135.6± 21.4	253.7± 35.7
	100	6	129.6± 19.6*	242.7± 37.9*
Daidzein	50	6	134.3± 17.6*	223.6± 21.3*
	100	6	105.7± 15.4*	184.7± 25.7*
Cyanin	50	6	169.4± 15.4	301.4± 20.0
	100	4	155.8± 19.5	295.7± 22.5
Malvin	50	6	174.5± 23.5	253.7± 19.6
	100	6	136.6± 26.7	216.4± 30.4*
Catechin	50	6	184.7± 16.7	317.5± 36.5
	100	6	124.1± 15.4*	315.4± 31.4
Neohesperidin	50	6	176.7± 17.9	300.4± 37.5
	100	6	148.3± 14.3	296.5± 41.4
Rotenone	50	6	122.7± 13.7*	243.5± 17.6*
	100	6	–	–
Disodium cromoglycate	50	6	121.4± 17.5*	230.6± 28.7*
	100	6	83.2± 16.4*	201.4± 24.6*
Cyclophosphamide	50	6	105.7± 10.5*	184.5± 27.6*
	100	6	67.8± 9.4**	164.9± 25.4*
Prednisolone acetate	10	6	169.7± 17.5	215.7± 21.7*
	20	6	101.5± 15.4*	198.5± 15.4*

[1] Recipient mice were sensitized with a i.v. injection of 7.1×10^6 spleen and lymph node cells from donor mice.
[2] Drugs were given orally to recipients from 3 days before cell transfer once daily for 9 days.
[3] Antibody titer was measured by assay the amount of reduced sugar according to the Nakashima method. Each value represents the mean± S.E.; Significantly different from control (*$p<0.05$ and **$p<0.01$).

Table V. Inhibitory activities of flavonoids on the secondary cellular immune response in oxazolone-induced dermatitis

Drugs[1]	Dose (mg/kg)	No. of animal	Swelling (%)[2] 24 hr	48 hr	72 hr	120 hr
Control	–	6	5.84± 1.97	10.21± 2.35	7.75± 1.72	8.84± 1.75
Flavone	50	6	4.72± 1.25	9.24± 2.04	7.24± 1.44	7.45± 2.14
	100	4	4.45± 1.04	8.27± 1.97	7.07± 1.75	7.31± 1.54
Chrysin	50	6	5.32± 1.09	10.05± 1.84	7.51± 1.74	8.43± 2.36
	100	6	5.07± 2.10	8.97± 2.10	7.12± 1.46	7.49± 1.72
Baicalein	50	6	4.72± 0.75	9.25± 1.76	7.15± 1.87	7.31± 1.36
	100	6	4.35± 1.43	8.75± 1.43	6.43± 1.76	6.91± 1.75
Apigenin	50	6	5.44± 1.87	10.12± 1.74	7.37± 1.37	7.75± 1.44
	100	6	4.99± 1.35	9.84± 1.56	7.21± 1.28	7.57± 1.69
Fisetin	50	6	5.65± 1.27	10.18± 1.94	7.45± 1.54	8.12± 1.66
	100	6	5.27± 2.15	8.94± 1.69	7.15± 1.35	7.54± 1.71
Kaempferol	50	6	5.45± 1.88	10.04± 1.78	7.64± 1.64	7.95± 1.45
	100	6	5.17± 1.75	9.88± 1.51	6.93± 1.37	7.14± 1.65
Morin	50	6	4.18± 1.36	7.27± 1.37	6.09± 1.27	6.51± 1.88
	100	6	3.25± 1.27*	6.75± 1.46*	5.43± 1.14*	5.92± 1.75*
Myricetin	50	6	4.71± 1.51	8.89± 2.31	6.97± 1.69	7.36± 1.44
	100	6	4.24± 1.13	7.84± 1.98	6.43± 1.61	6.88± 1.36
Taxifolin	50	6	5.36± 1.92	9.89± 2.54	7.83± 1.75	8.12± 1.54
	100	6	5.07± 1.57	9.79± 1.74	7.45± 1.47	7.80± 1.47
Quercetin	50	6	4.65± 1.43	7.95± 1.76	6.15± 1.44	6.78± 1.48
	100	6	4.09± 1.17	6.90± 1.64*	4.04± 1.35*	6.13± 1.79*
Rutin	50	6	5.35± 1.36	10.13± 1.96	7.54± 1.36	8.12± 1.36
	100	6	4.95± 1.73	9.45± 2.31	7.11± 1.27	7.97± 1.75
Hesperetin	50	6	4.70± 1.64	8.51± 1.87	6.96± 1.27	7.31± 2.36
	100	6	4.36± 1.57	7.14± 1.54*	6.40± 1.54	6.85± 1.75
Naringin	50	6	4.87± 1.31	9.15± 1.48	6.35± 1.75	6.98± 2.14
	100	6	4.71± 1.27	8.85± 1.99	6.11± 1.36	6.41± 1.75*
Hesperidin	50	6	4.62± 1.51	8.41± 1.36	7.17± 1.64	7.94± 1.46
	100	6	4.19± 1.36	7.75± 1.27	6.95± 1.07	7.43± 1.73
Daidzein	50	6	4.81± 0.75	9.67± 3.11	6.47± 1.43	6.95± 1.54
	100	6	3.84± 0.98*	7.41± 1.97	5.75± 1.05*	6.03± 1.56*
Cyanin	50	6	4.98± 1.72	9.57± 1.69	7.57± 1.33	8.36± 2.10
	100	4	4.41± 1.31	8.75± 1.75	6.98± 1.57	7.45± 1.96
Malvin	50	6	5.05± 2.07	9.37± 1.76	7.53± 1.64	8.12± 2.14
	100	6	4.37± 1.46	8.25± 1.51	6.41± 1.77	7.09± 1.85
Catechin	50	6	4.81± 1.32	8.41± 1.36	6.28± 1.54	7.12± 1.41
	100	6	4.27± 1.54	8.05± 1.44	5.84± 1.36	6.87± 1.35
Neohesperidin	50	6	5.64± 1.27	10.11± 1.94	7.31± 2.10	7.75± 1.64
	100	6	5.07± 1.44	8.49± 1.75	6.84± 1.75	7.43± 1.97
Rotenone	50	6	5.75± 1.47	10.14± 1.44	7.57± 1.36	8.52± 1.36
	100	4	5.64± 1.69	10.05± 1.57	7.12± 1.97	8.09± 1.57
Disodium cromoglycate	50	6	4.41± 1.44	7.21± 1.64	6.37± 1.54	6.72± 1.94
	100	6	4.05± 1.64	6.89± 1.75*	5.45± 1.14*	6.15± 1.87*
Cyclophosphamide	50	6	4.64± 1.36	8.17± 1.88	6.91± 1.36	7.21± 1.36
	100	6	4.18± 1.54	7.05± 1.91*	6.31± 1.27	6.75± 1.75
Prednisolone acetate	10	6	4.97± 1.88	8.63± 1.74	7.25± 1.64	7.45± 1.46
	20	6	4.43± 0.97	8.05± 1.55	6.89± 1.31	7.04± 1.14

[1] Drugs were orally administered for 9 days.
[2] Rats were sensitized by applying 50 μl of 10% oxazolone in acetone solution to shaved right flank and at Day 9, given a challenge by applying 10 μl of 1% oxazolone in acetone solution to the dorsal surface of the right ear and the thickness of ear was measured with engineer's micrometer.
Each value represents the mean± S.E.; Significantly different from control (*$p<0.05$).

Table VI. Inhibitory activities of flavonoids on the development of immunological memory in cellular immune response after transfer of dinitrofluorobenzene-primed lymph node cells[1]

Drugs[2]	Dose (mg/kg)	No. of animal	Ear swelling(%)[3]	Drugs[2]	Dose (mg/kg)	No. of animal	Ear swelling(%)[3]
Control	–	12	36.2± 2.6	Naringin	50	12	20.9± 3.4*
Flavone	50	12	17.6± 2.0*		100	12	19.4± 4.7*
	100	12	11.8± 1.5**	Hesperidin	50	12	16.9± 3.1*
Chrysin	50	12	21.5± 1.3*		100	12	14.1± 1.8**
	100	12	19.6± 2.1*	Daidzein	50	12	19.3± 2.4*
Baicalein	50	12	15.7± 2.3**		100	12	17.7± 2.0*
	100	12	14.2± 1.9**	Cyanin	50	12	20.8± 0.5*
Apigenin	50	12	24.3± 2.1		100	12	16.2± 2.0*
	100	12	21.8± 2.4*	Malvin	50	12	23.8± 1.8
Fisetin	50	12	27.3± 1.5		100	12	13.1± 2.0**
	100	12	35.8± 2.1	Catechin	50	12	16.3± 1.1*
Kaempferol	50	12	25.8± 3.0		100	12	12.5± 0.6**
	100	12	25.7± 4.0	Neohesperidin	50	12	28.0± 4.9
Morin	50	12	13.6± 4.3**		100	12	16.6± 1.9*
	100	12	9.3± 1.4**	Rotenone	50	12	30.3± 1.9
Myricetin	50	12	15.6± 1.7**		100	12	31.4± 1.4
	100	12	14.1± 2.0**	Disodium cromoglycate	50	12	13.5± 1.4**
Taxifolin	50	12	23.9± 3.4		100	12	11.4± 1.6**
	100	12	30.2± 5.8	Cyclophosphamide	50	12	14.2± 1.7**
Quercetin	50	12	13.5± 1.5**		100	12	12.5± 0.6**
	100	12	11.1± 1.3**	Prednisolone acetate	10	12	16.2± 2.3*
Rutin	50	12	23.3± 1.2		20	12	12.5± 1.9**
	100	12	19.0± 1.3*				
Hesperetin	50	12	15.6± 3.1**				
	100	12	13.7± 1.3**				

[1] Mice as donor of DNFB-immune LN cells were sensitized by twice daily with paintings of 25 μl of 0.5% DNFB on the clipped abdomen and 5 μl on the food pads and ears, and DNFB-immune LN cells were taken 3 days after the last painting. Single cell suspensions (4×10^7 cells) were injected i.v. into normal syngeneic recipients. The recipients and controls were challenged within 1 hour after cell transfer by applying 20 μl of 0.2% DNFB on the dorsal side of ech ear.

[2] Thickness of ear was measured 24 hour later with engineer's micrometer.

[3] Drugs was orally treated once daily 2 days before antigen painting in donor mice.

Each value represents the mean± S.E.; Significantly different control (*$p<0.05$ and **$p<0.01$).

opment of memory cells of B-and T-lymphocytes and also inhibited the releasing of lymphocyte-activating factor from macrophage. These results were in agreement with the reports of Suzuki *et al.*[11], Ito *et al.*,[13] and Schwartz *et al.*[14].

On the other hand, the potency of flavonoids varied depending on their structure. Based on our results, the following observations can be made : 1. The abolition of the double bond between C_2 and C_3 leads to decreased inhibition. This is apparent from the lower potency of 2,3-dihydroquercetin, known as taxifolin, than that of quercetin itself. This is in agreement with the results of Varma[39], Middleton[40], Landolfi[41], Hsu[42], Mora[43] and Kim *et al.*,[28] 2. Flavones (flavone, baicalein) and flavonols (morin, quercetin), which have a ketone group at C_4, were more potent than anthocyanins (cyanin, malvin) and catechins (catechin) in humoral and cellular immunosuppression. 3. Isoflavones (daidzein) which have a benzene ring at C_3, have the almost same activity as those of the flavones (baicalein) and flavonols (morin, quercetin, myricetin), which have a benzene ring at C_2. 4. The extent of inhibition by hesperidin and neohesperidin are similar,

pointing out that the activity is not lost when the C-ring is opened. 5. Rutin, which is glycosylated at C_3 with rutinose in C-ring, was significantly less potent than the parent aglycone, quercetin, 6. The inhibitory activity of hesperidin (hesperetin-7-*O*-rhamnose) was lower than that of its aglycone in cellular immune reaction, but not in the humoral immune reaction. 7. The inhibitory activity of flavones (flavone, baicalein) and flavonols (quercetin, morin, myricetin, fisetin) were silimilar. This fact suggests that the hydroxy group at C_3 dose not affect the activity. 8. Chromanochromanones (disodium cromoglycate) also have the immunosuppressive activity. 9. Increaing the number of -OH group in B-ring from one (kaempferol) to two or three (quercetin, myricetin) enhanced the inhibitory activity. 10. In the flavonols, quercetin, an orthohydroxylated in B-ring (3′,4′-dihydroxy) was more potent than morin, which is metahydroxylated (2′,4′-dihydroxy) in humoral immune response, but the potency was similar in cellular immune response.

LITERATURE CITED

1. Middleton, E. Jr.: The flavonoids. *Trends in Pharmacol. Sci.* **5**, 335 (1984).
2. Gupta, M. B., Bhalla, T. N., Gupta, G. P., Mitra, C. R. and Bhargava, K. P.: Anti-inflammatory activity of taxifolin. *Japan. J. Pharmacol.* **21**, 377 (1971).
3. Hikino, H., Taguchi, T., Fujimura, H. and Hiramatsu, Y.: anti-inflammatory principles of *Caesalpinia sappan* wood and of *Haematoxylon compechianum* wood. *Planta Med.* **31**, 216 (1977).
4. Parmar, N. S. and Ghosh, M. N.: Anti-inflammatory activity of gossypin, a bioflavonoid isolated from *Hibiscus vitifolius* L. *Ind. J. Pharmac.* **10**, 277 (1978).
5. Fourie, T. G. and Snyckers, F. O.: A flavone with anti-inflammatory activity from the roots of *Rhus undulata*. *J. Nat. Prod.* **47**, 1057 (1984).
6. Villar, A., Gasco, M. A. and Alcaraz, M. J.: Anti-inflammatory and antiulcer properties of hypolaetin-8-glycoside, a novel plant flavonoid. *J. Pharm. Pharmacol.* **36**, 820 (1984).
7. Al-Meshal, I. A., Tariq, M., Parmar, N. S. and Ageel, A. M.: Anti-inflammatory activity of the flavonoid fraction of hat. *Agents and Actions* **17**, 379 (1985).
8. Suganda, A. G., Amoros, M., Fauconnier, B. and Girre, L.: Antiherpetic and antipoliomyeletic effects of *Matricaria inodora* L. *Planta. Med. Phytother.* **18**, 215 (1984).
9. Cazzulani, P., Panzarasa, R., De Stefani, C. and Graziani, G.: Mechanism of flavoxate antispasmodic activity comparative *in vitro* studies. *Arch. Int. Pharmacodyn.* **274**, 189 (1985).
10. Horinchi, T., Fujuki, H., Hakii, H., Suganuma, M., Yamashita, K. and Sugimura, T.: Modulation of phorbol ester receptors in mouse skin by application of quercetin. *Jpn. J. Can. Res.* **77**, 526 (1986).
11. Suzuki, K. and Hosokawa, T.: Immunosuppressants containing flavonoids. *Jpn. Kokai Tokyo Koho.* Pat. No. 88233917 (1988).
12. Berg, A. U. J., Baron, D. P. and Berg, P. A.: Immunomodulating properties of cianidanol on responsiveness and function of human peripheral blood T-cells, and K-cells. *Int. J. Immunopharmacol.* **10**, 387 (1988).
13. Ito, N., Ogawara, H. and Watanabe, S.: Preparation of isoflavone derivatives and salts thereof as oncostic and immunosuppressive agents and pharmaceutical composition containing them. *PCT. Int. Appl.* Pat. No. 8702982 (1987).
14. Schwartz, A., Sandra, L. S. and Middleton, E. Jr.: Quercetin inhibition of the induction and function of cytotoxic T lymphocyte. *Immunopharmacol.* **4**, 125 (1982).
15. Zhong, F., Jiang, Y., Wu, F., Shu, R., Cai, X. and Tan, J.: Effect of the total flavone of *Hippophae rhamnoides* fruits on mouse cell immune function. *Zhongcaoyao* **20**, 331 (1989).
16. Yasukawa, K., Takido, M., Takeuchi, M. and Nitta, K.: Effect of flavonoids on tumor promotor's activity. *Prog. Clin. Biol. Med.* **280**, 247 (1988).
17. Nagai, H., Osuga, K. and Koda, A.: Inhibition of hypersensitivity reactions by soluble derivatives of baicalein. *Japan. J. Pharmacol.* **25**, 763 (1975).
18. Sankawa, U. and Chun, Y. T.: Anti-allergic substances from Chinese medicinal plant. Advances in Chinese medicinal material research. World Scientific Publ. Co., Singapore, p. 171 (1985).
19. Kinoshita, G., Nakamura, F. and Maruyama, T.: Immunological studies on polysaccharide fraction from crude drugs. *Shoyakugaku Zasshi* **40**, 325 (1986).

20. Kim, C. J., Choi, C. S. and Cho, S. K.: The effects of Cantharis polysaccharide fraction on the inflammation and immune reaction. in press (1991).
21. Siena, S., Bregni, M., Formosa, A., Brando, B., Marenco, P., Lappi, D. A., Bonadonna, G. and Gianni, A. M.: Immunotoxin-mediated inhibition of chronic lymphocytic leukemia cell proliferation in human. *Cancer Res.* **49**, 3328 (1989).
22. Xue, W. L. and Matthew, R. W.: Radix *Tripterygium wilfordii*-a Chinese herbal medicine with potent immunosuppressive properties. *Transplantation* **50**, 82 (1990).
23. Hemi, H., Kitame, F., Ishida, N., Kusano, G. and Kondo, Y.: Inhibitory effects on lymphocyte proliferation of some naturally occurring compounds obtained from medicinal plant. *Proc. Symp. Wakan-Yaku* **11**, 61 (1978).
24. Altmann, W. A., Elson, C. E. and Cook, M. E.: The immunosuppressive activity of the plant metabolite geraniol in chicks. *Fed. Am. Soc. Expt. Biol.* **4**, A935 (1990).
25. Ammellal M., Bronner, C., Briancon, F., Haag, M., Anton, R. and Landry, Y.: Inhibition of mast cell histamine release by flavonoids and biflavonoids. *Planta Med.* **51**, 16 (1985).
26. Middleton, E. Jr. and Drzewiecki, G.: Effects of flavonoids and transient metal cations on antigen-induced histamine release from human basophils. *Biochem. Pharmacol.* **31**, 1449 (1982).
27. Bennett, J. P., Gompert, B. D. and Wollenweber, E.: Inhibitory effects of natural flavonoids on secretion from mast cells and neutrophils. *Arzneim-Forsch.* **31**, 433 (1981).
28. Kim, C. J. and Chung, J. M.: Pharmacological activities of flavonoids (I). Relationships of chemical structure of flavonoids and their inhibitory activity of hypersensitivities. *Yakhak Hoeji* **34**, 348 (1990).
29. Kim, C. J., Su, S. K., Joo, J. H. and Cho, S. K.: Pharmacological activities of flavonoids (II). Relationships of anti-inflammatory and actigranulomatous actions. *Yakhak Hoeji* **34**, 407 (1990).
30. Kubo, M., Nagao, T., Matsuda, H., Tani, T., Arichi, S. and Kitagawa, I.: Studies on moutan cortex IX. On the phagocytosis of the mouse reticuloendothelial system (1). *Yakugaku Zasshi* **105**, 26 (1985).
31. Harris, H. F.: On the rapid conversion of haematoxylin into haematein in staining reactions. *J. Appl. Microsc. Lab. Meth.* **3**, 778 (1900).
32. Takata, M., Kumagai, A. and Kitagawa, M.: Mechanism of immunosuppression induced by Licorice root extract (LX). *Proc. Symp. Wakan-Yaku* **6**, 19 (1972).
33. Hamaoka, T., Kitagawa, M., Matsuoka, Y. and Yamamura, Y.: Antibody production in mice. I. The analysis of immunological memory. *Immunol.* **17**, 55 (1969).
34. Ramellini, G. and Meldolesi, J.: Liver protection by silymarin: *In vitro* effect of dissociated rat hepatocyte. *Arzneim-Forsch.* **26**, 69 (1976).
35. Somogyi, M.: Notes on sugar determination. *J. Biol. Chem.* **195**, 19 (1952).
36. Kishore, V. and Latman, N.: Effect of nutritional copper deficiency on adjuvant arthritis and immuno-competence in the rats. *Agents and Actions* **140**, 274 (1984).
37. Moorhead, J. W.: Soluble factors in tolerance and contact sensitivity to 2,4-dinitrofluorobenzene in mice. *J. Exp. Med.* **150**, 1432 (1979).
38. Havsteen, B.: Flavonoids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol.* **32**, 1141 (1983).
39. Varma, S. D.: Inhibition of aldose reductase by flavonoids. *Proc. Clin. Biol. Res.* **213**, 343 (1986).
40. Middleton, E. Jr. and Drzewiecki, G.: Flavonoid inhibition of human basophil histamine release stimulated by various agents. *Biochem. Pharmacol.* **33**, 3333 (1984).
41. Landolfi, R., Mower, R. L. and Steiner, M.: Modification of platelet function and arachidonic acid metabolism by bioflavonoids. *ibid.* **33**, 1525 (1984).
42. Hus, K. K. and Chen, F. C.:Structure-activity relationship of substitued flavonoids. *Tai-Wan Ko Hsueh* **27**, 23 (1973).
43. Mora, A., Paya, M., Rios, L. and Alcaraz, M. J.: Structure-activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymatic lipid peroxidation. *Biochem. Pharmacol.* **40**, 793 (1990).