Activity-Guided Isolation of Saponins from *Kalopanax pictus* with Anti-inflammatory Activity

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Received January 15, 2002; accepted March 25, 2002

By bioassay-guided separation, a known saponin, kalopanaxsaponin A (1) and a new saponin, pictoside A (2) were isolated from the stem bark of *Kalopanax pictus* as anti-inflammatory components when evaluated by vascular permeability test. Another novel saponin, pictoside B (3) was also isolated but was inactive in the test system used. The structures of pictosides A and B were elucidated as caulophyllogenin 3-*O*-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside (2) and pictogenin (3β,6β,16α,23-tetrahydroxyolean-12-ene-28-oic acid) 3-*O*-α-L-arabinopyranoside (3), respectively, by spectral analysis and by chemical degradation. Kalopanaxsaponin A and pictoside A showed significant anti-inflammatory activity at the oral doses of 50 mg/kg.

Key words *Kalopanax pictus*; Araliaceae; kalopanaxsaponin A; pictoside A; pictogenin; anti-inflammatory activity

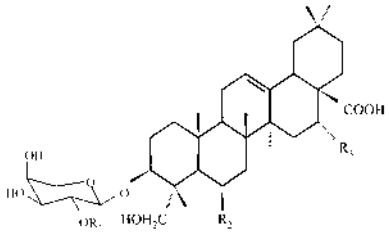
Kalopanax pictus NAKAI (Araliaceae) is a deciduous tree found in countries of the Orient. The stem bark of *Kalopanax pictus* has been used in traditional medicine to treat neuralgia, rheumatic arthritis, lumbago,[1] furuncle, carbuncle, wound,[2] diarrhea and scabies.[3] Phytochemical studies demonstrated the presence of saponins,[4] polyacetylenes,[5] phenylpropanoid glycosides,[5] lignans,[5] as well as simple phenolic glycosides.[5] During an ongoing collaborative search for novel anti-inflammatory agents originating from plants, an ethyl acetate fraction of the stem bark of *Kalopanax pictus* exhibited significant anti-inflammatory activity in a vascular permeability test.[6] Anti-rheumatic activity on adjuvant-induced arthritis in rats[7] and antinociceptive[8] and antioxidative activities[9] of EtOAc fraction and kalopanaxsaponins A and I were reported. However, this plant has not been investigated previously for systematic isolation of its anti-inflammatory components. We report herein the bioassay-guided chromatographic fractionation of an ethyl acetate soluble extract of *K. pictus*, which has led to the isolation of two anti-inflammatory saponins together with a novel inactive saponin.

Results and Discussion

Results of the activity-guided isolation from *K. pictus* extract are summarized in Chart 1. From the ethyl acetate soluble fraction (Fr.) which showed potent activity in the vascular permeability test, eleven subfractions (Frs. 1—11) were obtained and the activity was tested using doses proportional to the yields obtained in the isolation procedure. As shown in Table 1, only Fr. 8 showed significant inhibition of vascular permeability at 250 mg/kg. This fraction was further divided into 5 subfractions (Frs. 8-A—8-E) by column chromatography. Fractions 8-B and 8-D elicited inhibition of vascular permeability at doses of 120 and 100 mg/kg, respectively, while Frs. 8-A and 8-E were negative (Fig. 1). Fraction 8-C was a mixture of Frs. 8-B and 8-D. Fraction 8-B was crystallized from MeOH to give compound **1**. Repeated column chromatography of Fr. 8-D followed by recrystallization yielded compounds **2** and **3**. Compounds **1** and **2** at doses of 50 mg/kg showed potent inhibition of vascular permeability (Fig. 2), while compound **3** exhibited no activity. Compound **1** was identified as kalopanaxsaponin A [hederagenin 3-*O*-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] by direct comparison with an authentic sample.[10]

Compound **2** was obtained as colorless powder, mp 224—226 °C, $[\alpha]_D$ −1.8°. The molecular formula of **2** was determined as $C_{41}H_{66}O_{13}$ by positive high resolution (HR) fast atom bombardment (FAB)-MS measurement which showed a quasimolecular ion $[M+Na]^+$ peak at *m/z* 789.4405. The IR spectrum of **2** showed absorption bands at 1700 cm^{-1} for carboxylic acid and strong absorption bands at 3436 and 1053 cm^{-1} suggestive of an oligoglycosidic structure. On acid hydrolysis of **2** with 5% aqueous hydrochloric acid (HCl)–60% dioxane solution, **2** yielded L-arabinose and L-rhamnose, which were identified by GLC analysis of the trimethylsilyl thiazolidine derivatives as the sugars[11] and an aglycon, caulophyllogenin.[12] It showed signals for six angular methyl groups at δ 0.98, 1.04, 1.05, 1.08, 1.17 and 1.79 (all singlets for C-25, 29, 26, 24, 30 and 27 CH_3 groups), as well as signals for two anomeric protons at δ 5.13 (d, J=6.2 Hz) and 6.26 (br s) in the ^{1}H-NMR spectrum. Comparison of the ^{1}H- and ^{13}C-NMR spectra of **2** with those of **1** showed considerable structural similarity and confirmed that the disaccharide of α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl was attached at C-3 of caulophyllogenin.

The oligoglycoside structure and its connectivities to the aglycon in **2** were confirmed by heteronuclear multiple bond connectivity (HMBC) experiments. Long-range correlations



	R_1	R_2	R_3
1	α-L-rhamnopyranosyl	−H	−H
2	α-L-rhamnopyranosyl	−H	OH
3	H	OH	−OH

Chart 1. Structures of Isolated Saponins

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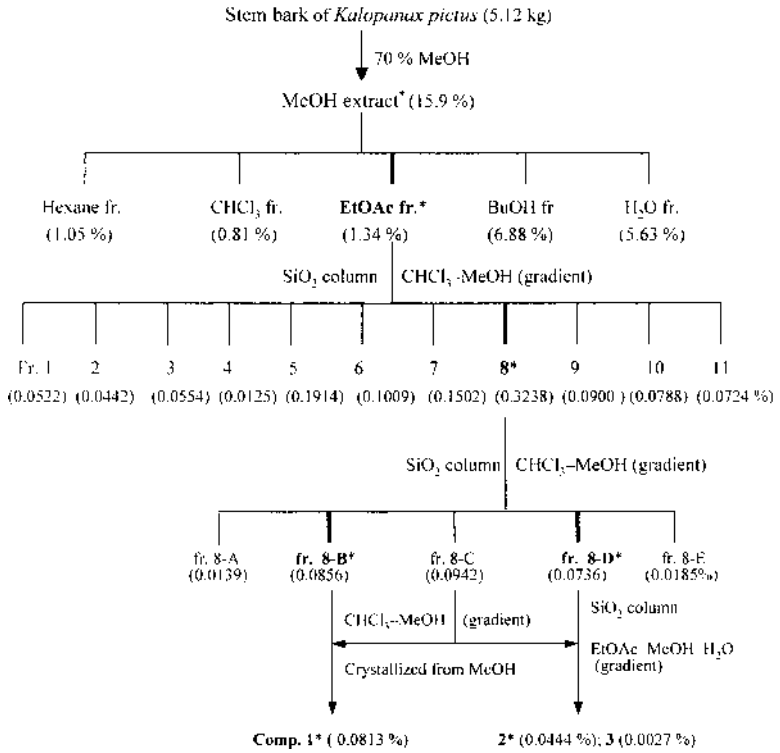

Chart 2. Isolation Procedure of the Components Having Anti-inflammatory Activity

*; Positive activity. (%); Yield from dry plant stem bark.

Table 1. Effect of Subfractions 1—11 from EtOAc Fraction on Vascular Permeability in Mice

Treatment	Yield[a] (%)	Dose (mg/kg, *p.o.*)	*n*	Evans blue (μg/mouse; M.±S.E.)	Inhibition (%)
Control	—	—	9	114.5±13.9	—
Fr. 1	0.0522	50	7	107.2±10.2	6.4
Fr. 2	0.0442	40	8	120.0±11.6	−4.8
Ibuprofen	—	200	8	74.1±7.5*	35.3
Control	—	—	8	102.7±11.8	—
Fr. 3	0.0554	50	7	105.1±8.8	−2.3
Fr. 4	0.0125	20	7	97.2±10.3	5.4
Fr. 5	0.1914	150	7	114.8±13.0	−11.8
Ibuprofen	—	200	7	69.0±7.4*	32.8
Control	—	—	8	106.1±12.0	—
Fr. 6	0.1009	80	7	95.3±7.6	10.2
Fr. 7	0.1502	110	7	87.6±9.8	17.4
Fr. 8	0.3238	250	7	71.3±9.9*	32.8
Ibuprofen	—	200	7	65.5±8.3*	38.3
Control	—	—	9	90.1±6.2	—
Fr. 9	0.0900	70	9	83.8±9.0	7.0
Fr. 10	0.0788	60	9	87.4±6.5	3.0
Fr. 11	0.0724	60	8	86.3±7.9	4.2
Ibuprofen	—	200	7	53.5±7.0*	40.6

a) Calculated from dried plant stem bark. Significantly different from the control (* $p<0.05$).

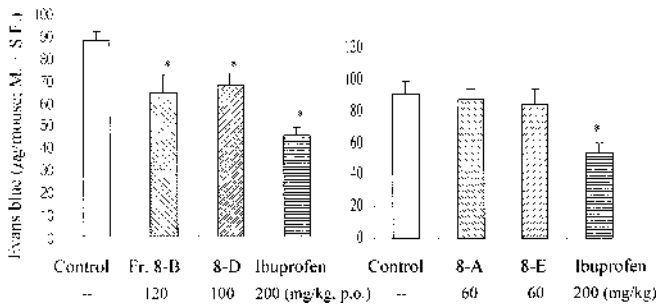

Fig. 1. Effect of Subfractions 8-A—8-E on Vascular Permeability in Mice

Significantly different from the control (* $p<0.05$).

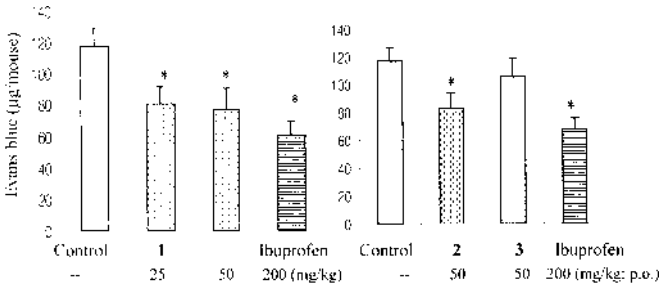

Fig. 2. Effect of Isolated Compounds from Subfraction 8 on Vascular Permeability in Mice

Significantly different from the control (* $p<0.05$).

were observed between the arabinopyranosyl H-1 (δ 5.13) and C-3 (δ 81.0) of the aglycon moiety, and between the terminal rhamnopyranosyl H-1 (δ 6.26) and the C-2 (δ 75.6) of the inner arabinopyranosyl moiety. This evidence allowed us to formulate the structure of compound **2** (pictoside A) as caulophyllogenin 3-*O*-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside. A limited number of caulophyllogenin glycosides have been isolated from *Caulophyllum robustum* (Berberidaceae),[13] *Chrysanthellum procumbens* (Compositae),[14] *Medicago polymorpha* (Leguminosae),[15] *Leontice kiangnanensis* (Berberidaceae),[16] and *Aralia elata* (Araliaceae).[17]

Compound **3** was obtained as colorless powder, mp 218—

220 °C, $[\alpha]_D$ +2.6°. The positive HRFAB-MS spectrum showed a quasimolecular ion $[M+Na]^+$ peak at *m/z* 659.3768, corresponding to the molecular formula $C_{35}H_{56}O_{10}$. The IR spectrum of **3** showed absorption bands at 1701 cm^{-1} for carboxylic acid and strong absorption bands at 3432 and 1057 cm^{-1} suggestive of an oligoglycosidic structure. The ^{1}H-NMR spectrum of **3** displayed signals corresponding to six tertiary methyls (δ 1.05, 1.19, 1.62, 1.66, 1.70, 1.84), an olefin (δ 5.75, br s), three oxygenated methines (δ 4.36, 5.12, 5.42) and an oxygenated methylene (δ 4.08, 4.52, each d, J=10.8 Hz), together with six oxygenated methine and methylene protons ascribable to a sugar unit. Acid hydrolysis of **3** with 5% HCl in 60% dioxane solution yielded L-arabinose as the sugar.[11] The aglycon moiety region in the ^{1}H- and ^{13}C-NMR spectra of **3** showed a great similarity to those of caulophyllogenin except for the resonances of position C-6 and a downfield shift of the axial methyl groups at C-4 (24-CH_3), C-10 (25-CH_3), and C-8 (26-CH_3), implying that there is an additional hydroxyl group at C-6 of **3**.[18] In the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum, a broad singlet H-6 signal at δ 5.12 displayed cross peaks with H-5α, H-7α and H-7β, further supporting that the additional secondary hydroxyl group on rings A/B was located at C-6. Hence, the aglycon of **3** was formulated as 3β,6β,16α,23-tetrahydroxyolean-12-ene-28-oic acid, a new triterpenoid sapogenin. This interpretation was unambiguously confirmed by the HMBC spectrum, which showed cross peaks between the proton resonance at δ 5.42 (br s, H-16) and three carbon signals at δ 42.1 (C-14), 48.4 (C-17) and δ 41.0 (C-18), and between the proton resonance at δ 5.12 (br s, H-6) and carbon signal at δ 36.3 (C-10). Other HMBC correlation between the arabinose anomeric proton at δ 5.07 with C-3 of the aglycon (δ 81.5) indicated that arabinose moiety was linked at C-3 of pictogenin. Thus, the structure of **3** (pictoside B) was identified as pictogenin 3-*O*-α-L-arabinopyranoside. Kalopanaxsaponin A and pictoside A showed significant anti-inflammatory activity. Both compounds showed significant inhibition of vascular permeability at the oral doses of 50 mg/kg in mice, while pictoside B showed no activity.

Experimental

Mps were measured on a Büchi B-540 apparatus, and are uncorrected. The optical rotations were determined on a JASCO P-1020 polarimeter. The IR spectra were obtained on a JASCO FT/IR-5300 spectrometer. The FAB mass spectrum was obtained in a 3-nitrobenzyl alcohol matrix in a positive ion mode on a JEOL JMS-AX505WA spectrometer. The NMR spectra were measured in pyridine-d_5 on either a Bruker Avance-600 instrument or a Varian 2000 (300 MHz), and the chemical shifts were referenced to TMS. GC analysis was performed with a Hewlett Packard 5890 Series II gas chromatograph equipped with an H_2 flame ionization detector. The column was HP-5 capillary column (30 m×0.32 mm×0.25 μm). Conditions: column temperature: 200 °C; injector and detector temperature: 290 °C, He flow rate: 3.0 ml/min. TLC was performed on silica gel 60F_{254} (Merck) and cellulose plate (Art No. 5716, Merck).

Plant Material The stem bark of *K. pictus* was collected in September 1998 in Kangwon Province, Korea and authenticated by one of us (WKW). A voucher specimen (NPRI 980130) was deposited in the herbarium of the Natural Products Research Institute, Seoul National University.

Extraction and Isolation The dried stem bark of *K. pictus* (5.12 kg) was refluxed three times with 70% MeOH in a water bath. The MeOH extract was evaporated under reduced pressure to dryness, which was partitioned in succession between H_2O and *n*-hexane, $CHCl_3$, EtOAc, and then *n*-BuOH and afforded 48.1, 37.1, 61.2, and 316.2 g of the respective extracts. A portion of the EtOAc fraction (50 g) was chromatographed over silica gel using $CHCl_3$–MeOH (gradient) as an eluent to yield 11 subfractions. A portion of the active subfraction 8 (11 g) was further purified over silica gel using $CHCl_3$ with increasing amounts of MeOH as an eluent to yield 5 subfractions (Frs. 8-A—8-E). Subfraction 8-B was crystallized from MeOH to give compound **1** (0.0813%). Subfraction 8-D was repeatedly chromatographed on silica gel 60 using EtOAc as eluent to yield saponin **2** (0.0444%) and then **3** (0.0027%). Compound **1** was identified as kalopanax saponin A [3-*O*-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl hederagenin] by comparison of spectroscopic and physical data with the reported values in the literature,[10] and confirmed by direct comparison with an authentic sample obtained from *Pulsatilla koreana* NAKAI.[10]

Pictoside A (**2**): Colorless powder, mp 224—226 °C (MeOH), $[\alpha]_D^{20}$ −1.8° (c=0.23, MeOH). Positive FAB-MS *m/z*: 789.4405 (Calcd for $C_{41}H_{66}O_{13}$+Na: 789.4401). IR (KBr) cm^{-1}: 3436 (OH), 1700 (COOH), 1053 (glycosidic C–O). ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 0.98, 1.04, 1.05, 1.08, 1.17, 1.79 (all s, CH_3-25, 29, 26, 24, 30, 27), 1.65 (3H, d, J=6.2 Hz, rha CH_3), 3.63 (1H, dd, J=4.1, 13.8 Hz, H-18), 3.75, 4.16 (1H each, d, J=10.9 Hz, H-23), 4.28 (1H, dd, J=4.1, 11.9 Hz, H-3), 5.13 (1H, d, J=6.2 Hz, ara H-1), 5.24 (1H, br s, H-16), 5.64 (1H, br s, H-12), 6.26 (1H, br s, rha H-1), ^{13}C-NMR (150.9 MHz, pyridine-d_5) δ: 39.0 (C-1), 26.1 (C-2), 81.0 (C-3), 43.4 (C-4), 47.2 (C-5), 18.0 (C-6), 33.0 (C-7), 39.8 (C-8), 47.7 (C-9), 36.8 (C-10), 23.7 (C-11), 122.3 (C-12), 144.9 (C-13), 42.0 (C-14), 36.1 (C-15, 21), 74.6 (C-16, 3′), 48.7 (C-17), 41.2 (C-18), 47.1 (C-19), 31.0 (C-20), 32.7 (C-22), 63.8 (C-23), 14.4 (C-24), 16.5 (C-25), 17.9 (C-26), 27.5 (C-27), 180.2 (C-28), 33.2 (C-29), 24.6 (C-30), 104.3 (C-1′), 75.6 (C-2′), 69.2 (C-4′), 65.6 (C-5′), 101.5 (C-1″), 72.2 (C-2″), 72.4 (C-3″), 74.0 (C-4″), 69.5 (C-5″), 18.4 (C-6″).

Pictoside B (**3**): Colorless powder, mp 218—220 °C (MeOH), $[\alpha]_D^{20}$ +2.6° (c=0.17, MeOH). Positive FAB-MS *m/z*: 659.3768 (Calcd for $C_{35}H_{56}O_{10}$+Na: 659.3771). IR (KBr) cm^{-1}: 3432 (OH), 1701 (COOH), 1057 (glycosidic C–O). ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 1.05, 1.19, 1.62, 1.66, 1.70, 1.84 (all s, CH_3-29, 30, 24, 26, 25, 27), 3.68 (1H, dd, J=4.1, 13.8 Hz, H-18), 4.08, 4.52 (1H each, d, J=10.8 Hz, H_2-23), 4.36 (1H, dd, J=4.5, 11.8 Hz, H-3), 5.07 (1H, d, J=7.2 Hz, ara H-1), 5.12 (1H, br s, H-6), 5.42 (1H, br s, H-16), 5.75 (1H, br s, H-12). ^{13}C-NMR (150.9 MHz, pyridine-d_5) δ: 39.6 (C-1), 25.9 (C-2), 81.5 (C-3), 44.2 (C-4), 47.4 (C-5), 67.0 (C-6), 40.8 (C-7), 38.9 (C-8), 48.1 (C-9), 36.3 (C-10), 23.5 (C-11), 122.3 (C-12), 144.4 (C-13), 42.1 (C-14), 35.6 (C-15), 74.5 (C-16), 48.4 (C-17), 41.0 (C-18), 46.8 (C-19), 30.6 (C-20), 35.7 (C-21), 32.4 (C-22), 63.9 (C-23), 14.9 (C-24), 17.1 (C-25), 18.3 (C-26), 26.9 (C-27), 180.0 (C-28), 32.9 (C-29), 24.3 (C-30), 106.1 (C-1′), 72.7 (C-2′), 74.3 (C-3′), 69.2 (C-4′), 66.5 (C-5′).

Acid Hydrolysis of 2 Saponin **2** (30 mg) was refluxed with 5% HCl in 60% aqueous dioxane (10 ml) for 2 h. The reaction solution was evaporated under reduced pressure, and the hydrolysate was extracted with ether. The ether extract was evaporated to yield aglycon, which was identified as caulophyllogenin by comparison of spectroscopic and physical data with the reported values in the literature.[12] The H_2O layer was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated under reduced pressure. The residue was compared with standard sugars by cellulose TLC [pyridine–EtOAc–HOAc–H_2O (36 : 36 : 7 : 21)], which showed the sugars to be arabinose and rhamnose.

Determination of the Absolute Configuration of Sugars of 2[11] A portion of the dried sugar mixture was dissolved in pyridine (0.1 ml), then the solution was added to a pyridine solution (0.1 ml) of L-cysteine methyl ester hydrochloride (2 mg) and warmed at 60 °C for 1 h. The solvent was evaporated under a N_2 stream and dried *in vacuo*. The residue was trimethylsilylated with TMS–HT (0.1 ml) at 60 °C for 30 min. After the addition of *n*-hexane and water, the *n*-hexane layer was removed and checked by GC. The retention times (t_R) of the peaks were 8.25 min (L-arabinose) and 10.5 min (L-rhamnose).

Acid Hydrolysis of 3 and Determination of the Absolute Configuration of Sugars of 3 A sample of **3** (15 mg) was treated with 5% HCl in a manner similar to that described for **2**. The aglycon was identified as 3β,16α,23-trihydroxyolean-5,12-diene-28-oic acid. mp 268—272 °C, $[\alpha]_D^{16}$ +2.6° (c=0.173, MeOH). ^{1}H-NMR (300 MHz, pyridine-d_5) δ: 1.04, 1.16, 1.17, 1.21, 1.42, 1.72 (all s, CH_3), 3.64 (1H, br dd, J=4.2, 14.4 Hz, H-18), 4.09, 4.28 (1H each, d, J=10.5 Hz, H_2-23), 4.19 (1H, dd, J=4.5, 11.4 Hz, H-3), 5.25 (1H, br s, H-16), 5.70 (1H, br s, H-6), 5.77 (1H, t, J=3.6 Hz, H-12). ^{13}C-NMR (75.5 MHz, pyridine-d_5) δ: 18.6, 20.2, 20.6, 21.6, 23.9, 24.8, 27.0, 27.2, 31.0, 32.7, 33.3, 33.5, 35.6, 36.2, 37.5, 38.0, 41.3, 42.2, 44.6, 45.7, 47.0, 48.9, 69.6, 73.9, 74.8, 120.5, 122.6, 145.3, 147.6, 179.9. MS, *m/z* (rel. int., %): 486 $[M]^+$ (8.1), 468 $[M-H_2O]^+$ (20.9), 423 $[M-(H_2O+COOH)]^+$ (7.0), 409 $[M-(H_2O+CO_2+CH_3)]^+$ (24.4), 391 $[M-(2H_2O+CO_2+CH_3)]^+$ (7.0), 264 [rings D/E]$^+$ (38.4), 246 [rings D/E−H_2O]$^+$ (93.0), 222 [rings A/B−H_2O]$^+$ (100), 201 [rings D/E−(H_2O+COOH)]$^+$ (61.6), 173 (55.8).

The sugar was identified as L-arabinose as described above.

Pharmacological Assay Male ICR mice weighing 23—30 g were used. The animals supplied from the Animal Breeding Center of Seoul National University were housed for 1 week in a temperature-controlled 12 h light-dark environment at 22±1 °C. They were fed commercial solid food (Samyang Yuji Co., Ltd., Seoul) and tap water *ad libitum*.

The vascular permeability test used was a modification of that of Whittle.[19] In brief, the male mice were fasted for 10 h prior to the experiment and the test samples were orally given in a volume of 0.1 ml/10 g [body weight (b.w.)]. Thirty minutes later, each animal was given intravenously 0.1 ml/10 g (b.w.) of 1% Evans blue solution. Sixty minutes after sample administration, 0.1 ml/10 g of 0.6% acetic acid in physiological saline was injected intraperitoneally. Twenty minutes later, the mice were killed by dislocating the neck and the abdominal cavity was washed with normal saline to make 10 ml in a test tube. To clear turbidity due to protein, 0.1 ml of 1 N NaOH solution was added to each tube and the absorbance was read at 610 nm in a spectrophotometer (Molecular Devices, U.S.A.). The vascular permeability was expressed in terms of the amount of total dye (μg/mouse) which had leaked into the abdominal cavity. The doses of the tested fractions and compounds were chosen based on the yields obtained from the MeOH extract.

Data are expressed as means±S.E.M. Unpaired Student's *t*-test was used to compare the data and $p<0.05$ was considered significant.

Acknowledgements This work was supported by a grant (project HMP-00-B-21600-0127) from the Ministry of Health and Welfare, Republic of Korea.

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