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Laxative Effect of Agarwood Leaves and Its Mechanism

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We investigated the laxative activity of an extract of agarwood leaves from *Aquilaria sinensis*. The laxative activity was measured in mice by counting the stool frequency and stool weight, and the drugs were orally administered. An acetone extract of agarwood leaves and senna (a representative laxative drug) both increased the stool frequency and weight, but a methanol extract did not. The laxative effect of the acetone extract was milder than that of the anthraquinoid laxative, senna, and the former did not induce diarrhea as a severe side effect. We identified the main constituent contributing to the laxative effect of the acetone extract as genkwanin 5-O-*β*-primeveroside (compound 4). Compound 4 strengthened the spontaneous motility and induced contraction in the ileum. This ileal contraction induced by compound 4 was inhibited by atropine, but not by azasetron, suggesting that the effect of compound 4 was mediated by acetylcholine receptors, and not by serotonin. The laxative mechanism for compound 4 may in part involve stimulation of intestinal motility *via* acetylcholine receptors.

Key words: laxative effect; agarwood leaf; *Aquilaria sinensis*

Constipation is a life-style problem and may increase during aging. It can be a chronic condition for which patients may need to take laxatives in the long term. Nowadays, 20 to 30% of people over the age of 60 use laxatives more than once a week,[1)] the major laxatives used being anthraquinoids based on a crude extract of senna (*Cassia angustifolia*, *C. acutifolia*, Leguminoceae) or rhubarb (*Rheum palmatum*, *R. tanguticum*, *etc.*, Polygonaceae).[2)] However, the powerful purgative activity of anthraquinoids makes them unsuitable for regular use, and even periodic use of these laxatives can induce pseudomelanosis coli, a risk factor for colorectal neoplasma.[3)] Other crude purgative drugs include rose fruit (*Rosa multiflora*, Rosaceae), genka (*Daphne genkwa*, Thymelaeaceae), and pharbitis seed (*Pharbitis nil*, Convolvulaceae). However, their purgative activities are also too powerful, and they are not now in common use as laxatives. Against this background, we searched for other naturally occurring laxatives. We describe here a new laxative source, namely, agarwood leaves (*Aquilaria sinensis*, Thymelaeceae; "Jinko" in Japanese). Agarwood is well known as incense in the Oriental region, and has also been used as a sedative, analgesic, and digestive in traditional medicine. Characteristic sesquiterpenes[4–6)] and chromone derivatives[7–9)] have been isolated from agarwood, and some of these sesquiterpenes have sedative and analgesic effects.[10,11)] Although thorough phytochemical research has been carried out on the trunk and resin of agarwood, little is known about the pharmacological effect of agarwood leaves. The purpose of the present study was to elucidate this effect. We first evaluated the laxative activities of an acetone extract of agarwood leaves (EAL) and methanol EAL, then identified the major laxative constituents of EAL, and finally investigated its mechanism of action.

Materials and Methods

Materials. Agarwood (*Aquilaria sinensis*) and senna leaves were purchased from Kaya Co., Ltd. (Osaka, Japan) and Matsuura Yakugyo Co., Ltd. (Aichi, Japan), respectively. Acetylcholine chloride, serotonin, atropine sulfate, azasetron hydrochloride, and dimethyl sulfoxide were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Extraction and isolation procedures. The process of extraction and isolation is shown in Fig. 1. Ground agarwood leaves (1.5 kg) were successively extracted with acetone (8-liter) four times, and then six times with methanol for two days. Evaporation of the solvents *in vacuo* gave acetone (70 g) and methanol (150 g) extracts

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Abbreviations: EAL, extract of agarwood leaf

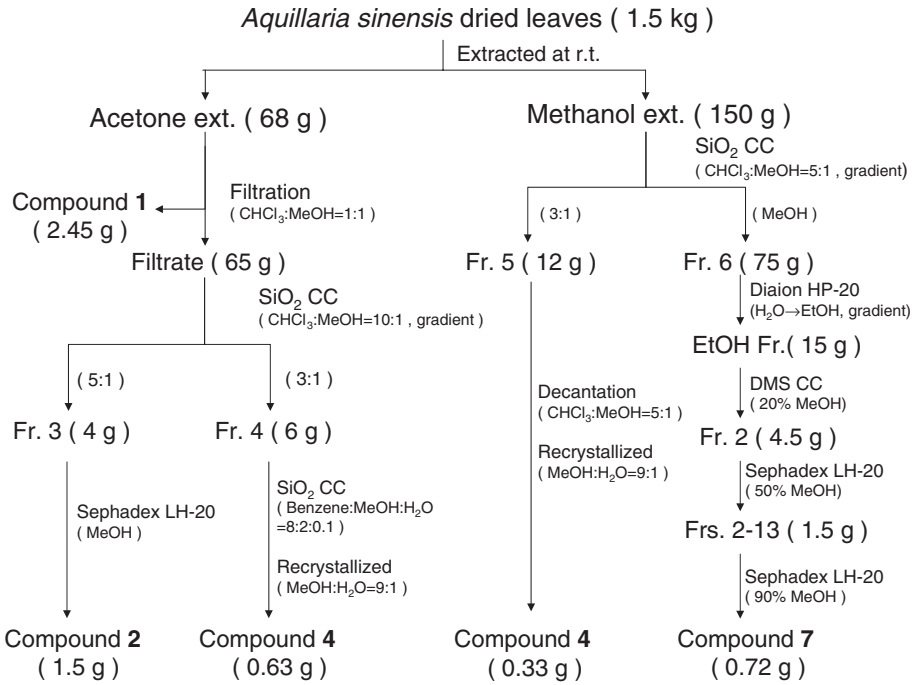

Fig. 1. Flowchart of the Isolation Process for the Acetone and MeOH Extracts of Leaves of *Aquillaria sinensis.*

which were used in the laxative assay and for identification of the constituents. Ground senna leaves (0.5 kg) were extracted with 50% aqueous ethanol (3-liter) for one day. Evaporation of the solvent *in vacuo* gave an aqueous ethanol extract (32.4 g) which was used in the laxative assay.

The acetone extract of agarwood leaves was suspended in $CHCl_3$-methanol (1:1), and the insoluble part was recrystallized from methanol to yield compound 1 (2.45 g). The filtrate was subjected to chromatography on silica gel eluted with a $CHCl_3$-methanol solvent system (10:1–1:1, linear gradient) to prepare six crude fractions. The 5:1 crude fraction was separated in a Sephadex LH-20 column eluted with methanol to give compound 2 (1.5 g). The polar crude fraction (3:1) was separated by chromatography in a silica gel column eluted with a benzene-methanol-H_2O solvent system (8:2:0.1), and then recrystallized from methanol-H_2O (9:1) to give compound 4 (genkwanin 5-*O*-β-primeveroside) (0.63 g).

The methanol extract of agarwood leaves was subjected to chromatography in a silica gel column eluted with a $CHCl_3$-methanol solvent system (5:1–1:1, finally methanol, linear gradient) to prepare six crude fractions. The 2:1 crude fraction was purified by decantation with $CHCl_3$-methanol (5:1), and then recrystallized with methanol-H_2O (9:1) to yield compound 4 (0.33 g). The crude methanol fraction was separated in a Diaion HP-20 column (H_2O-ethanol, linear gradient), DMS column (20% methanol, linear gradient) and Sephadex LH-20 column (50% and 90% methanol) to give compound 7 (0.72 g).

Animals. The experimental design and all procedures were in accordance with the Animal Care Guidelines issued by the Animal Experimental Committee of Gifu Pharmaceutical University. Male ddY mice (7–10 weeks old), 20–35 g body weight, were purchased from Japan SLC (Hamamatsu, Japan). Adult JW rabbits and adult Hartley guinea pigs were purchased from Kitayama Labes Co. (Nagano, Japan) and Nippon SLC Co. (Shizuoka, Japan), respectively. The animals were housed at a controlled room temperature (24.5–25.0 °C) with a 12/12 h light/dark cycle. Animal food pellets and tap water were provided *ad libitum*.

Frequency and weight of stools. Test samples were dissolved or suspended in distilled water and administered orally at 0.1 ml/10 g of body weight to mice by an oral tube (FG6202, Fuchigami Kikai Co., Kyoto, Japan). The frequency and weight of the stools were measured as the frequency and total stool wet weight produced by each mouse during four consecutive 2-h periods (0–2 h, 2–4 h, 4–6 h, and 6–8 h) after the administration of a test sample or distilled water (control). Each mouse was observed in a cage (11 cm height, 17.5 cm width, and 11 cm depth).

Diarrhea frequency. The diarrhea frequency was determined by counting the number of episodes of diarrhea in each mouse during the four 2-h periods (0–2 h, 2–4 h, 4–6 h, and 6–8 h) after the administration of a test sample or distilled water (control). The criterium for diarrhea was whether ordinary or non-ordinary stools were produced. An ordinary stool means in pellet form,

brown color, and rugby ball shape. A non-ordinary stool means loose, muddy, and watery. The judgment was made by a masked observer (Y.I.).

Spontaneous movement of the isolated ileum. The rabbits and guinea pigs were killed by exsanguination under deep anesthesia (pentobarbital and diethyl ether, respectively). Segments of the rabbit ileum were suspended at 1 g tension in an organ bath containing Tyrode's solution (137 mm NaCl, 5 mm KCl, 2.5 mm $CaCl_2 \cdot 2H_2O$, 0.1 mm $MgCl_2$-$6H_2O$, 0.3 mm $NaHPO_4$-$2H_2O$, and 5.6 mm glucose; pH 7.4). Spontaneous movement was monitored by a recorder (U-228; Pantos Co., Kyoto, Japan) *via* an isotonic transducer (EG-650H; Nihon Kohden, Tokyo, Japan). Segments of the guinea pig ileum were suspended at 0.5 g tension in an organ bath containing Tyrode's solution, and recordings were made as just described. Each test drug was cumulatively added to the bath. Various receptor agonists and antagonists (acetylcholine chloride, serotonin, atropine sulfate, and azasetron hydrochloride) were also separately added to the bath.

Statistical analysis. Data are presented as the mean ± S.E.M. Statistical comparisons were made with one-way ANOVA followed by Dunnet's multiple-comparison test, paired *t*-test, or Student's *t*-test (StatView software version 5.0; SAS Institute, Cary, NC, USA). A value of $p < 0.05$ is considered to indicate statistical significance.

Results

Laxative effects of acetone EAL and methanol EAL

To examine the laxative effects of EAL on the bowel movement in mice, acetone EAL and methanol EAL were orally administered (Fig. 2). Acetone EAL (at a dose of 1000 mg/kg, p.o.) induced significant increases (up to 2–3 times the control value) in stool frequency and stool weight both 2–4 h and 6–8 h after its oral administration (Fig. 2C and D). However, a lower dose of acetone EAL (100 or 300 mg/kg, p.o.) and any dose of methanol EAL (100, 300 or 1000 mg/kg, p.o.) failed to induce a significant effect (Fig. 2A, B, E and F).

Laxative effect of senna

To examine the laxative effect of senna leaves (used as a positive control) on the bowel movement in mice, the aqueous ethanol extract was orally administered (Fig. 3). At a dose of 300 mg/kg, p.o., the senna extract significantly increased the stool frequency and stool weight both 2–4 h and 6–8 h after its administration (Fig. 3A and B). At 1000 mg/kg, p.o., it significantly increased (by approximately 10 times) both the stool frequency and stool weight 2–4 h after its administration (Fig. 3C and D), while it induced no changes at 30 mg/kg, p.o.

Major constituents of EAL and their spectral data

The structures of the major constituents of EAL are shown in Fig. 4. Two new benzophenone derivatives [iriflophenone 2-*O*-α-rhamnoside (compound 2) and iriflophenone 3, 5-*C*-β-diglucoside (compound 7)] and two known compounds [mangiferin (compound 1) and genkwanin 5-*O*-β-primeveroside (compound 4)] were isolated, together with three minor compounds [genkwanin 5-*O*-β-glucoside (compound 3), genkwanin (compound 5), and genkwanin 4′-methyl ether 5-*O*-β-primeveroside (compound 6)].

Mangiferin (compound 1): a pale yellow powder; negative ion HR-FAB-MS *m/z*: 421.0779 $[M-H]^-$ (calcd. 421.0771 for $C_{19}H_{17}O_{11}$); ^{1}H-NMR (DMSO-d_6) δ: 3.14 (1H, dd, $J = 8.2$, 7.8 Hz, glc-H-4), 3.22 (1H, dd, $J = 8.2$, 5.6 Hz, glc-H-5), 3.22 (1H, dd, $J = 9.0$, 7.8 Hz, glc-H-3), 3.34 (1H, dd, $J = 11.2$, 5.6 Hz, glc-H-6), 3.67 (1H, d, $J = 11.2$ Hz, glc-H-6), 4.03 (1H, dd, $J = 9.8$, 9.0 Hz, glc-H-2), 4.58 (1H, d, $J = 9.8$ Hz, glc-H-1), 6.36 (1H, s, H-4), 6.85 (1H, s, H-5), 7.37 (1H, s, H-8), 9.68 (1H, s, C-7-OH), 10.51 (1H, s, C-3-OH), 10.58 (1H, s, C-6-OH), 13.77 (1H, s, C-1-OH); ^{13}C-NMR (DMSO-d_6) δ: 61.5 (glc-6), 70.2 (glc-4), 70.6 (glc-2), 73.1 (glc-1), 79.0 (glc-3), 81.6 (glc-5), 93.3 (C-4), 101.3 (C-9a), 102.6 (C-5), 107.6 (C-2), 108.1 (C-8), 111.7 (C-8a), 143.7 (C-7), 150.8 (C-10a), 154.0 (C-6), 156.2 (C-4a), 161.8 (C-1), 163.8 (C-3), 179.1 (C-9).

Iriflophenone 2-*O*-α-rhamnoside (compound 2): a pale yellow powder; negative ion HR-FAB-MS *m/z*: 391.1019 $[M-H]^-$ (calcd. 391.1029 for $C_{19}H_{19}O_9$); EIMS *m/z* (rel. int.): 392 (M+, 4), 246 (M-rhamnose, 96), 245 (100), 153 (54), 121 (28), 118 (6); ^{1}H-NMR (methanol-d_4) δ: 1.19 (3H, d, $J = 6.4$ Hz, rha-H-6), 3.10 (1H, dd, $J = 9.6$, 3.0 Hz, rha-H-3), 3.29 (1H, dd, $J =$ 9.6, 3.6 Hz, rha-H-4), 3.41 (1H, dd, $J = 3.0$, 0.8 Hz, rha-H-2), 3.44 (1H, dd, $J = 6.2$, 3.6 Hz, rha-H-5), 5.22 (1H, d, $J = 0.8$ Hz, rha-H-1), 6.07 (1H, d, $J = 2.0$ Hz, H-5), 6.30 (1H, d, $J = 2.0$ Hz, H-3), 6.81 (2H, d, $J = 8.6$ Hz, H-3′,5′), 7.61 (2H, d, $J = 8.6$ Hz, H-2′,6′); ^{13}C-NMR (methanol-d_4) δ: 17.9 (rha-6), 70.7 (rha-5), 71.4 (rha-2), 71.7 (rha-3), 73.5 (rha-4), 100.2 (rha-1), 95.5 (C-3), 97.9 (C-5), 109.2 (C-1), 115.9 (C-3′,5′), 132.6 (C-1′), 132.8 (C-2′,6′), 158.3 (C-6), 158.4 (C-2), 163.0 (C-4,4′), 197.6 (C-7).

To hydrolyze compound 2, a solution of 2% H_2SO_4 (5 ml) containing compound 2 (3.0 mg) was refluxed for 2 h. After cooling the reactant, the precipitate was filtered to give an aglycone (compound 2a). Compound 2a (iriflophenone): ^{1}H-NMR (acetone-d_6) δ: 5.97 (2H, s, H-3,5), 6.85 (2H, d, $J = 8.4$ Hz, H-3′,5′), 7.61 (2H, d, $J = 8.4$ Hz, H-2′,6′); ^{13}C-NMR (acetone-d_6) δ: 95.9 (C-3,5), 106.2 (C-1), 115.1 (C-3′,5′), 132.3 (C-2′,6′), 133.3 (C-1′), 161.8 (C-4′), 163.1 (C-2,6), 163.1 (C-2), 164.8 (C-4), 197.9 (C-7).

Genkwanin 5-*O*-β-primeveroside (compound 4): a pale yellow powder; negative ion HR-FAB-MS *m/z*: 577.1547 $[M-H]^-$ (calcd. 577.1557 for $C_{27}H_{29}O_{14}$); EIMS *m/z* (rel. int.): 314 (16), 284 (M-primeverose,

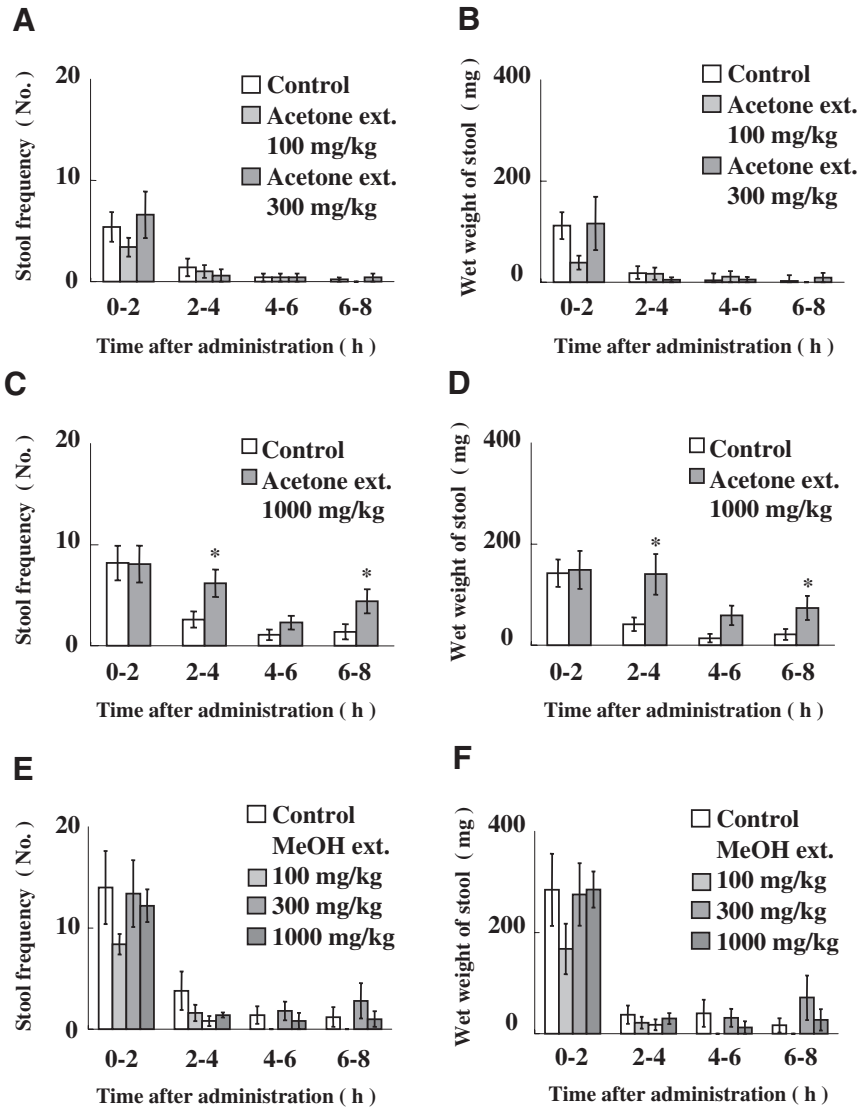

Fig. 2. Effects of EAL (the extract of agarwood leaves) on the Stool Frequency and Stool Weight in Mice.

The tool frequency (number) and weight (mg) were measured during four consecutive 2-h periods (0–2 h, 2–4 h, 4–6, and 6–8 h) after the oral administration of acetone EAL or methanol EAL to mice. Data are shown as the mean ± SE for 5–9 mice. *, $p < 0.05$ *vs.* Control at the same time.

100), 255 (22), 241 (10), 166 (8), 118 (6); ^{1}H-NMR (DMSO-*d*6) δ: [sugar moiety] 3.00 (1H, dd, $J = 8.1$, 7.6 Hz, xyl-H-2), 3.03 (1H, dd, $J = 10.8$, 5.5 Hz, xyl-H-5), 3.10 (1H, dd, $J = 8.8$, 8.7 Hz, xyl-H-3), 3.24 (1H, dd, $J = 8.8$, 5.5 Hz, xyl-H-4), 3.28 (1H, dd, $J = 9.6$, 9.2 Hz, glc-H-4), 3.34 (1H, dd, $J = 9.6$, 8.8 Hz, glc-H-3), 3.39 (1H, dd, $J = 8.8$, 7.6 Hz, glc-H-2), 3.56 (1H, dd, $J = 9.2$, 5.2 Hz, glc-H-5), 3.67 (1H, dd, $J = 10.6$, 1.2 Hz, glc-H-6), 3.69 (1H, dd, $J = 10.8$, 1.5 Hz, xyl-H-5), 3.97 (1H, dd, $J = 10.6$, 5.2 Hz, glc-H-6), 4.18 (1H, d, $J = 7.6$ Hz, xyl-H-1), 4.79 (1H, d, $J = 7.6$ Hz, glc-H-1), [aglycone moiety] 3.89 (3H, s, OMe), 6.69 (1H, s, H-3), 6.85 (1H, d, $J = 2.4$ Hz, H-6), 6.91 (2H, d, $J = 8.8$ Hz, H-3′,5′), 7.02 (1H, d, $J = 2.4$ Hz, H-8), 7.91(2H, d, $J = 8.8$ Hz, H-2′,6′), 10.31 (1H, s, C-4′-OH); ^{13}C-NMR (DMSO-*d*6) δ: [sugar moiety] 67.1 (xyl-5), 68.8 (glc-6), 69.5 (glc-4), 71.1 (xyl-4), 73.5 (glc-2), 75.0 (xyl-3), 75.7 (glc-3,5), 75.9 (glc-5), 78.2 (xyl-3), 103.7 (glc-1), 106.0 (xyl-1), [aglycone moiety] 56.2 (OMe), 96.9 (C-8), 102.9 (C-6), 105.9 (C-3), 109.2 (C-10), 115.9 (C-3′,5′), 121.2 (C-1′), 128.1 (C-2′,6′), 158.1 (C-5), 158.5 (C-9), 160.9 (C-4′), 161.4 (C-2), 163.6 (C-7), 176.9 (C-4).

Iriflophenone 3, 5-*C*-β-diglucoside (compound 7): brown amorphous gum; negative ion HR-FAB-MS, *m/z*: 570.1585 $[M-H]^-$ (calcd.: 570.1574 for $C_{25}H_{30}O_{15}$); ^{1}H-NMR (methanol-*d*4) δ: [sugar moiety] 3.42 (2H, dd, $J = 12.8$, 6.6 Hz, glc-H-5a,5b), 3.50 (2H, t, $J = 8.6$, glc-H-5a,5b), 3.52 (2H, t, $J = 8.6$ Hz, glc-H-2a,2b), 3.68 (2H, dd, $J = 10.0$, 8.6 Hz, glc-H-2a,2b), 4.92 (2H, d, $J = 10.0$ Hz, glc-H-1a,1b), [aglycone moiety] 6.78 (2H, d, $J = 8.8$ Hz, H-3′,5′), 7.64 (2H, d, $J = 8.8$ Hz, H-

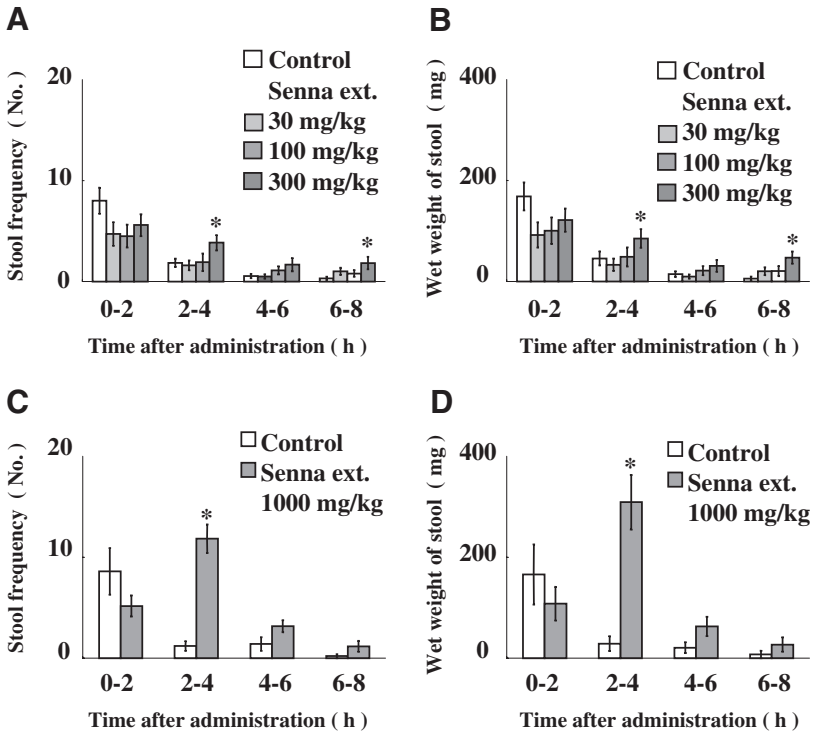

Fig. 3. Effects of the Extract of Senna Leaves on the Stool Frequency and Stool Weight in Mice.
The stool frequency (number) and weight (mg) were measured during four consecutive 2-h periods (0–2 h, 2–4 h, 4–6, and 6–8 h) after the oral administration to mice. Data are shown as the mean ± SE for 5–13 mice. *, $p < 0.05$ *vs.* Control at the same time.

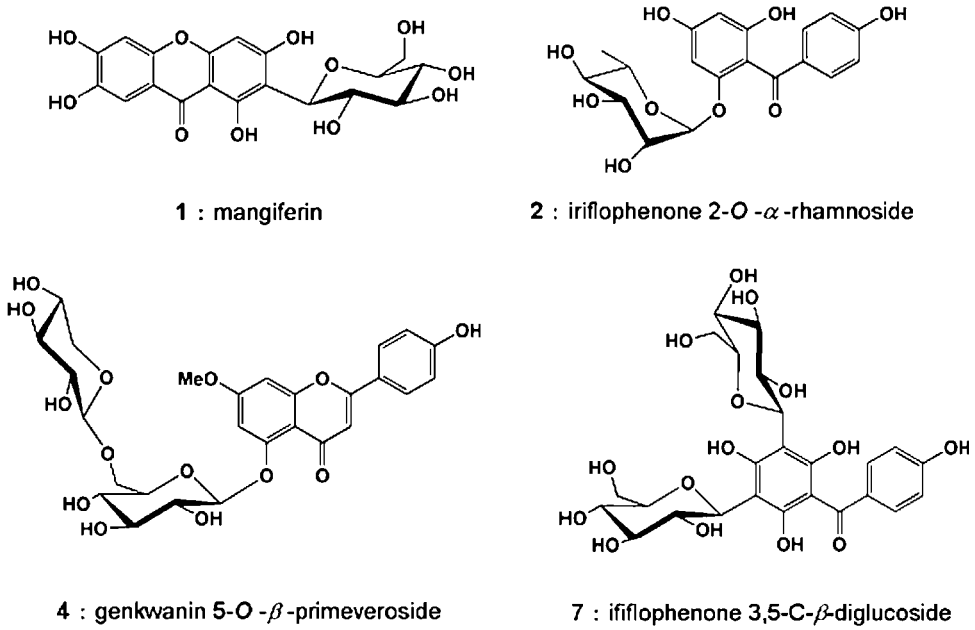

Fig. 4. Major Constituents Iisolated from Acetone EAL and Methanol EAL.
Two new benzophenone derivatives [iriflophenone 2-*O*-α-rhamnoside (compound 2) and iriflophenone 3, 5-*C*-β-diglucoside (compound 7)] and two known compounds [mangiferin (compound 1) and genkwanin 5-*O*-β-primeveroside (compound 4)] were isolated.

2′,6′); ^{13}C-NMR (methanol-*d*4) δ: [sugar moiety] 61.9 (glc-6), 70.9 (glc-4), 74.1 (glc-2), 76.9 (glc-1), 79.1 (glc-3), 82.5 (glc-5), [aglycone moiety] 105.3 (C-3,5), 115.6 (C-3′,5′), 132.6 (C-1′), 132.7 (C-1), 133.0 (C-2′,6′), 158.9 (C-2,6), 160.1 (C-4), 162.9 (C-4′), 198.6 (C-7).

Structural determination

^{1}H- and ^{13}C-NMR spectra were measured with a JEOL AL-400 instrument operated at 400 MHz and 100 MHz in acetone-d_6, methanol-d_4, or DMSO-d_6, with tetramethylsilane (TMS) used as an internal standard.

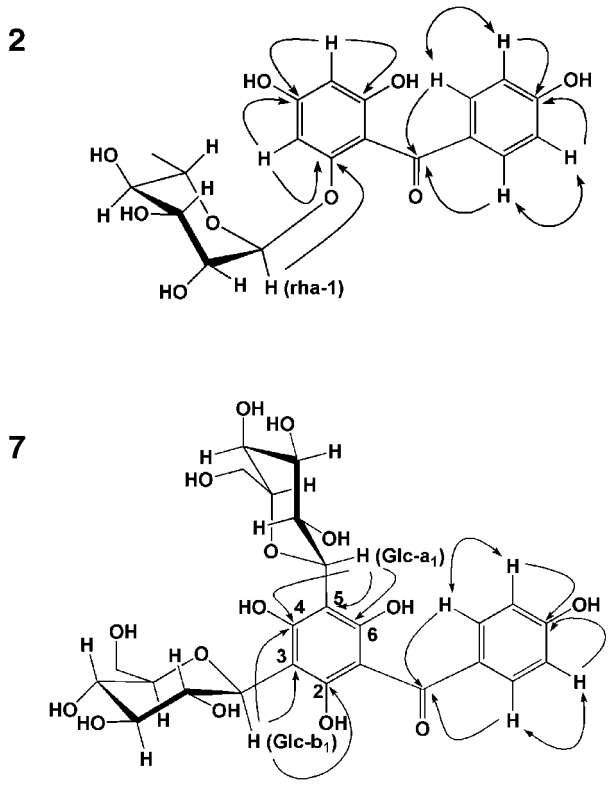

Fig. 5. HMBC Correlations (→) and ^{1}H-^{1}H Correlations (↔) for Compounds 2 and 7.

Compound 2, iriflophenone 2-*O*-α-rhamnoside; compound 7, Iriflophenone 3, 5-*C*-β-diglucoside.

The molecular formula $C_{19}H_{19}O_9$ of compound 2 was established from the negative ion HR FAB-MS and EI-MS data, together with ^{1}H- and ^{13}C-NMR spectra. The ^{1}H- and ^{13}C-NMR spectra suggested the presence of a rhamnose unit which was supported by an ion peak at m/z 246 ([M-146]) in the EI-MS data. The ^{13}C-NMR spectrum displayed a signal at δ 197.6 attributable to a carbonyl group. The rest of the signals, corresponding to twelve carbons, suggested the presence of two aromatic benzene rings. In the ^{1}H-NMR spectrum, the presence of a *para*-substituted benzene ring was shown by proton signals δ 6.81 (1H, d, $J = 8.6$ Hz) and 7.61 (1H, d, $J = 8.6$ Hz), in addition to a *meta*-substituted benzene ring due to two doublets at δ 6.07 (1H, d, $J = 2.0$ Hz) and 6.30 (1H, d, $J = 2.0$ Hz). The HMBC and COSY spectra allowed complete assignment and identification of the aglycone as 4, 2′, 4′, 6′-tetrahydroxybenzophenone (iriflophenone) (18, 19). The usual acidhydrolysis of compound 2 gave iriflophenone. The presence of *O*-α-rhamnose was confirmed by the characteristic carbon signal of a methyl group at δ 17.9 and by a proton signal for an anomeric proton at δ 5.22 (1H, d, $J = 0.8$ Hz). Its linkage position at C-2 was determined by HMBC (Fig. 5). The structure of compound 2 was finally concluded to be iriflophenone 2-*O*-α-rhamnoside.

The molecular formula $C_{25}H_{30}O_{15}$ of compound 7 was established from the negative ion HR FAB-MS data. The ^{1}H- and ^{13}C-NMR spectra were similar to those of compound 2, suggesting that the aglycone of compound 7 was an iriflophenone. In the ^{1}H-NMR spectrum, no aromatic protons based on the *meta*-substituted benzene ring found in compound 2 were apparent. In the ^{13}C-NMR spectrum, 13 aromatic carbons were detected, and the carbons (C-1′–C-6′) were superimposed, while the others (C-1–C-6) were shifted. These results suggested that the aromatic ring was fully substituted and had a symmetrical structure such as a 2, 4, 6-trihydroxybenzene ring (phloroglucinol unit). Multiplicity was revealed by the DEPT experiment, and complete assignment was performed by HMBC and COSY experiments. The ^{1}H-NMR spectrum showed a signal at δ 4.92 (d, $J = 10.0$ Hz) attributable to an anomeric proton of a glucose moiety, and the carbon at δ 76.9 was found to be C-1 of *C*-β-glucoside. The ^{13}C-NMR spectrum showed that the two glucosyl carbons were completely superimposed, indicating that two *C*-β-glucose moieties were symmetrically substituted at C-3 and C-5. Clear HMBC correlations were observed between the anomeric proton of *C*-β-glucose and C-3,5 through J_3 correlation, and C-4 through J_4 correlation in the aglycone moiety of iriflophenone (Fig. 5), this confirming that the linkage positions of the *C*-β-glucose moieties were at C-3 and C-5. The structure of compound 7 was thus determined to be iriflophenone 3,5-*C*-β-diglucoside. The structure of genkwanin 5-*O*-β-primeveroside (compound 4) was completely characterized after an analysis of the above-mentioned spectral data together with 2D NMR experiments.

Laxative activity of the major compounds in EAL

As shown in Fig. 6, compound 2 (at 1000 mg/kg, p.o.) significantly increased both the stool frequency and stool weight 6–8 h after its administration (Fig. 6C and D), but compounds 1 and 7 (at 1000 mg/kg, p.o.) had no effects (Fig. 6A, B, E and F).

Compound 4 (at doses of 100–1000 mg/kg, p.o.) had significant laxative activity (Fig. 7 C-H). At 1000 mg/kg, p.o., it significantly increased both the stool frequency and stool weight 2–4 h and 6–8 h after its administration (Fig. 7G and H), whereas at 10 and 30 mg/kg, p.o. it had no significant laxative activity (Fig. 7A and B). The smallest effective dose of compound 4 was 100 mg/kg. The time-course characteristics of the laxative effect of compound 4 were similar to those of acetone EAL, suggesting that it was one of the main laxative constituents of agarwood leaves.

Diarrhea frequency

Table 1 shows the diarrhea frequency for each 2-h period. The senna extract at 300 mg/kg, p.o. (but not at 30 or 100 mg/kg) induced diarrhea. Methanol EAL and acetone EAL (at 100–1000 mg/kg, p.o.) failed to induce diarrhea. Likewise, the major constituents of EAL (compounds 1, 2, 4 and 7) did not induce diarrhea, even at 1000 mg/kg, p.o.

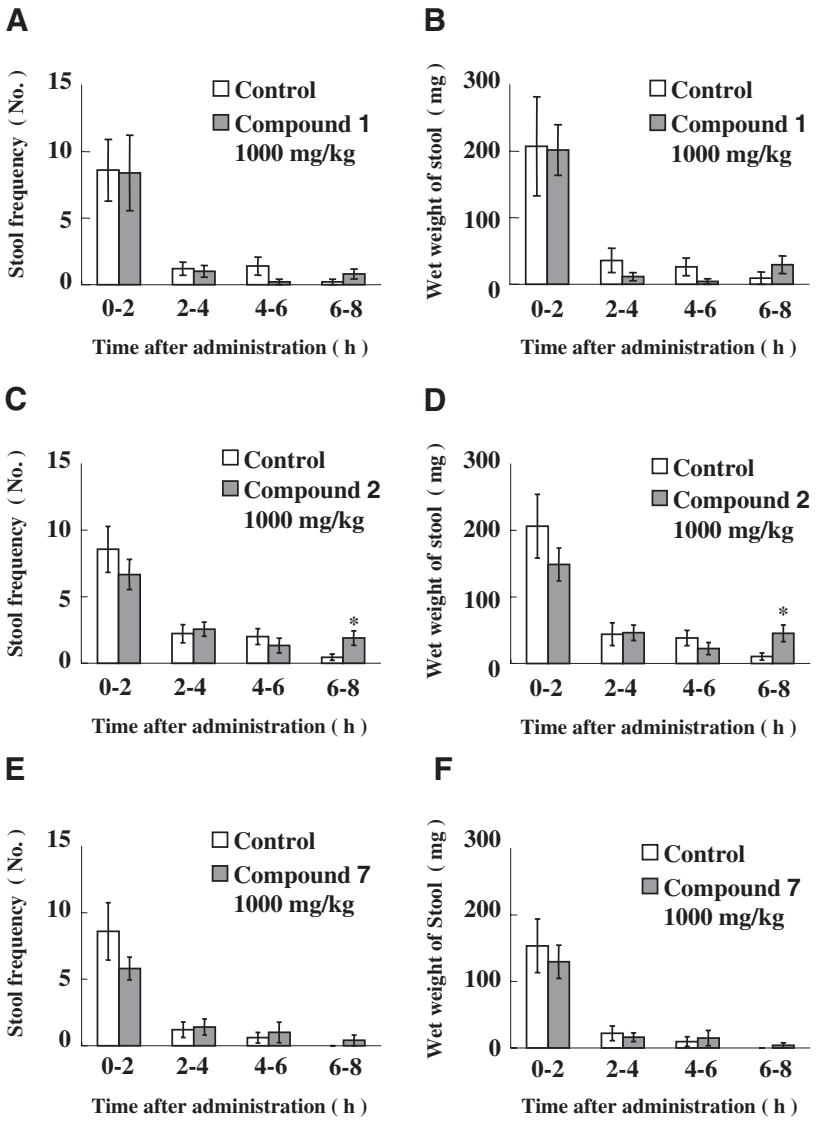

Fig. 6. Effects of the Major Constituents (compounds 1, 2 and 7) of EAL on the Stool Frequency and Stool Weight in Mice.
The stool frequency (number) and weight (mg) were measured during four consecutive 2-h periods (0–2 h, 2–4 h, 4–6, and 6–8 h) after the oral administration to mice. Data are shown as the mean ± SE for 5–9 mice. *, $p < 0.05$ *vs.* Control at the same time.

Effects of compound 4 on the spontaneous motility in an isolated rabbit ileum

Figure 8 shows a typical trace obtained from the rabbit ileum after the application of compound 4 which induced a contraction when applied at 1×10^{-3} g/ml. The amplitudes of the contractions observed before the application of compound 4 and after its application at 1.0×10^{-5}, 1.0×10^{-4}, and 1.0×10^{-3} g/ml were 3.72 ± 0.11, 3.74 ± 0.20, 3.76 ± 0.23, and 4.66 ± 0.35 cm, respectively (mean ± SE, $n = 4$) (equivalent to 100, 100, 101 and 125% of the "before" value). At 1.0×10^{-3} g/ml, compound 4 induced a significant contraction.

Effect of a pretreatment with atropine or azasetron on the contraction induced by compound 4 in an isolated guinea pig ileum

The contraction by acetylcholine in an isolated guinea pig ileum was antagonized by a pre-treatment with atropine. The pretreatment with atropine, an acetylcholine-receptor antagonist, at 1×10^{-7} g/ml significantly suppressed the contraction by compound 4. Compound 4 at a concentration of 1×10^{-3} g/ml induced a contraction in the guinea pig ileum, this contraction being antagonized by atropine (Fig. 9). The contraction by serotonin was antagonized by a pre-treatment with azasetron. Compound 4 at a concentration of 1×10^{-3} g/ml induced a contraction. However, a pre-treatment with azasetron, a 5-HT_3 receptor antagonist, at 1×10^{-5} g/ml failed to suppress this contraction (Fig. 10).

Discussion

In the present study, an oral administration of acetone EAL, but not of methanol EAL, had a laxative effect on mice, the effect induced by 1000 mg/kg, p.o. being

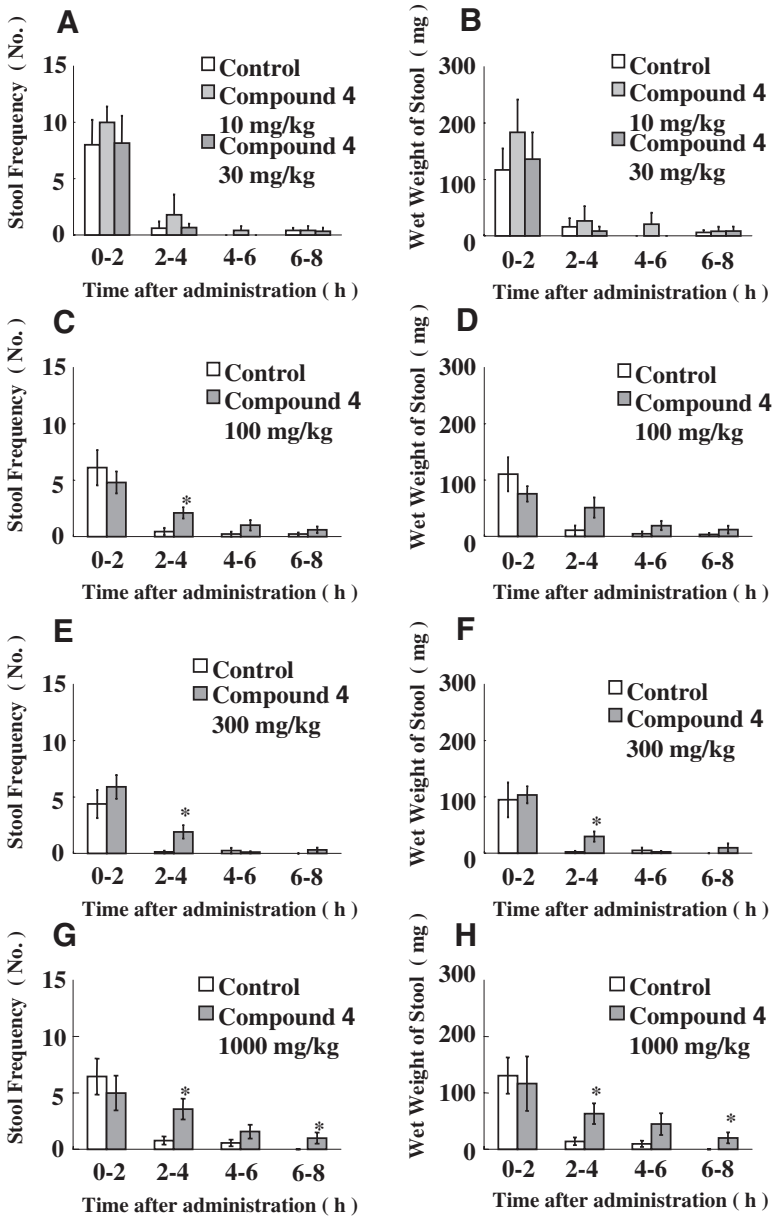

Fig. 7. Effects of Compound 4 (genkwanin 5-*O*-β-primeveroside) on the Stool Frequency and Stool Weight in Mice.
The stool frequency (number) and weight (mg) were measured during four consecutive 2-h periods (0–2 h, 2–4 h, 4–6, and 6–8 h) after the oral administration to mice. Data are shown as the mean ± SE for 5–10 mice. *, $p < 0.05$ *vs.* Control at the same time.

evident in two phases (2–4 h and 6–8 h after its administration). This is the first report of EAL possessing laxative activity in mice. An extract of senna, a representative drug, exhibited laxative activity at 300 and 1000 mg/kg, p.o. The potency of acetone EAL at 1000 mg/kg, p.o. was the same as that of the senna extract at 300 mg/kg, p.o., and the biphasic pattern of the EAL effect was similar to that seen with the extract of senna. The laxative effect of the senna extract at 1000 mg/kg, p.o. was intense but transient, being evident only 2–4 h after its administration. These findings indicate that, like senna, EAL had a laxative effect.

To identify the major laxative constituents of EAL, we repeatedly separated and purified by chromatography to isolate four compounds. These were two new benzophenones [iriflophenone 2-*O*-α-rhamnoside (Fig. 4, compound 2) and iriflophenone 3,5-*C*-β-diglucoside (compound 7)] and two known compounds [mangiferin (compound 1) and genkwanin 5-*O*-β-primeveroside (compound 4)]. Compound 4 (0.63 g) and compound 2 (1.5 g) were purified from EAL (68 g), the yield constant being approximately 1% and 2.2%, respectively (Fig. 1). The rate of EAL and compound 4 was 100. On the other hand, the effective doses of EAL and compound 4 to affect stool frequency and weight were 1000 and 100 mg/kg, respectively. The rate was 10. This discrepancy may have been due to the difference in effect between a mixture (the extract) and a

Table 1. Diarrhea Frequency of EAL (extract of agarwood leaf), 50% Ethanol Extract of Senna Leaf, and Major Components (compounds 2 and 4) Isolated from EAL

Treatment p.o.	mg/kg	n	Diarrea frequency (no.)			
			0–2 h	2–4 h	4–6 h	6–8 h
Control		10	0/10*	0/10	0/10	0/10
Agarwood	100	5	0/5	0/5	0/5	0/5
	300	5	0/5	0/5	0/5	0/5
	1000	9	0/9	0/9	0/9	0/9
Control		10	0/10	0/10	0/10	0/10
Senna	30	10	0/10	0/10	0/10	0/10
	100	10	0/10	0/10	0/10	0/10
	300	12	0/12	7/12	7/12	4/12
	1000	6	0/6	6/6	4/6	0/6
Control		10	0/10	0/10	0/10	0/10
Compound 2	100	5	0/5	0/5	0/5	0/5
	300	5	0/5	0/5	0/5	0/5
	1000	9	0/9	0/9	0/9	0/9
Control		10	0/10	0/10	0/10	0/10
Compound 4	10	4	0/4	0/4	0/4	0/4
	30	5	0/5	0/5	0/5	0/5
	100	10	0/10	0/10	0/10	0/10
	300	10	0/10	0/10	0/10	0/10
	1000	8	0/8	0/8	0/8	0/8

Control mice were administered with each vehicle. Diarrhea frequency was measured as the total number in each 2 h. *Values are presented as no. of diarrhea incidents/no. tested.

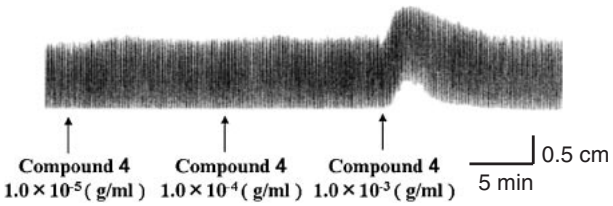

Fig. 8. Effect of Compound 4 (genkwanin 5-*O*-*β*-primeveroside) on the Spontaneous Motility in an Isolated Rabbit Ileum.

Segments of the rabbit ileum were suspended at 1 g tension in an organ bath containing Tyrode's solution. Their spontaneous movements were monitored on a recorder *via* an isotonic transducer. The amplitude of contraction was observed before the application of compound 4. Compound 4 induced significant contraction at 1.0×10^{-3} g/ml.

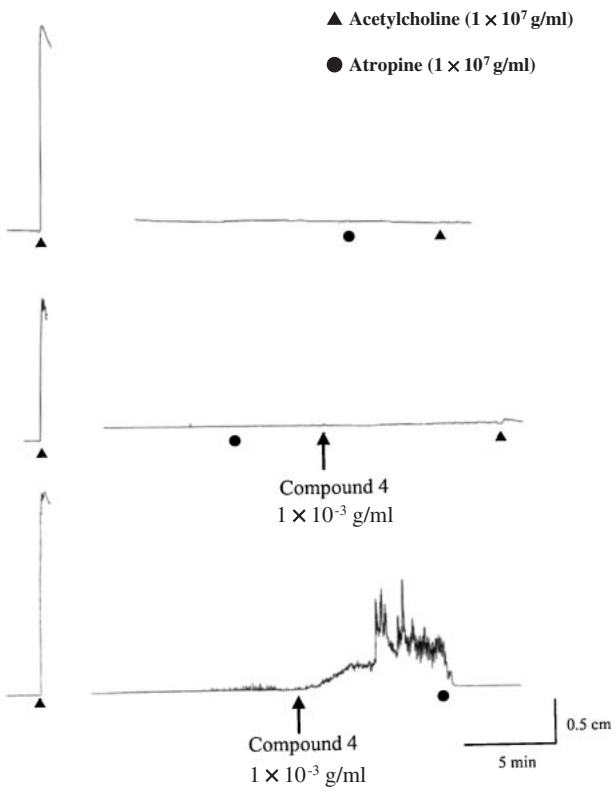

Fig. 9. Effect of a Pretreatment with Atropine on the Contraction of an Isolated Guinea Pig Ileum Induced by Compound 4 (genkwanin 5-*O*-*β*-primeveroside at 1.0×10^{-3} g/ml).

Segments of a guinea pig ileum were suspended at 0.5 g tension in an organ bath containing Tyrode's solution. Acetylcholine chloride and atropine were also added to the bath.

single compound; for example, EAL contained such compounds as 4 and 2 which each had a laxative effect.

Compound 4 at 100, 300, and 1000 mg/kg, p.o. had the strongest laxative effects among these compounds and was fast-acting (the laxative activity was first evident 2–4 h after its administration). Furthermore, at 1000 mg/kg, p.o. it showed lasting laxative activity, (the effect being still evident after 6–8 h). Its effect was milder and longer lasting than that of the senna extract. In contrast, compound 2 at 1000 mg/kg, p.o. displayed slow-acting laxative activity, its effect only being evident 6–8 h after the its administration). The metabolites of compound 2 may possibly have exerted laxative effects in this late phase. Meanwhile, compounds 1 and 7 had no effects on bowel movement in mice. Genkwanin, one of the flavones, has been reported to have a variety of pharmacological effects such as antioxidative,[12] radical scavenging,[13] and antimicrobial activities.[14] However, there are no reports of genkwanin having a laxative effect.

Senna is a traditional medicine containing anthraquinone derivatives, its effective constituents being sennosides A, B, C and D. Senna is a major and popular laxative, and it has been reported to accelerate spontaneous ileal contractions to induce a purgative effect.[15,16] The mechanism involves the sennosides and their active derivatives acting on the intestinal mucosal epithelium and submucosal nerve bundles, thereby stimulating prostaglandin (PG) synthesis and endogenous acetylcholine release, and consequently enhancing the colonic smooth muscle contraction.[17–19] Unfortunately, the purgative activities of anthraquinoids (including senna) are too powerful for them to be regularly used, and even periodic use of these laxatives can induce pseudomelanosis coli, a risk factor for colorectal neoplasma.[3] Indeed, in the present study, the senna extract at 300 and 1000 mg/kg, p.o., had a strong laxative effect, and it induced severe diarrhea at the same doses as those

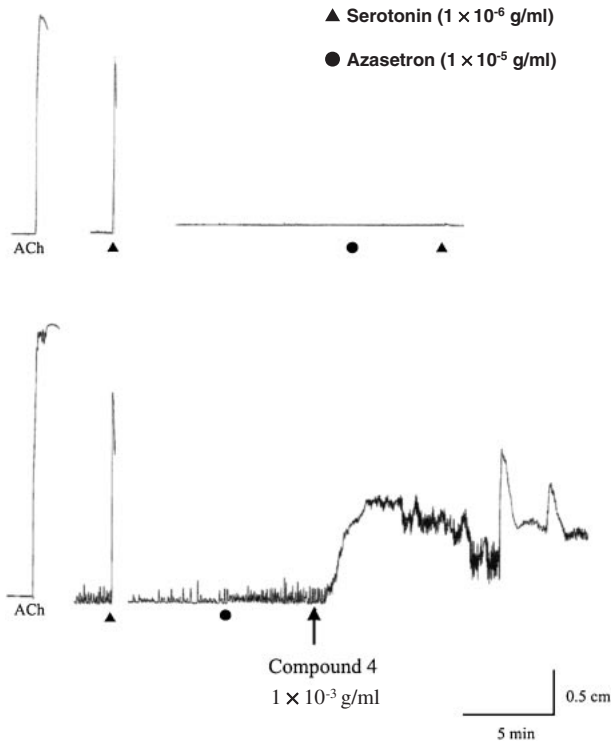

Fig. 10. Effect of a Pretreatment with Azasetron on the Contraction of an Isolated Guinea Pig Ileum Induced by Compound 4 (genkwanin 5-*O*-β-primeveroside at 1.0×10^{-3} g/ml).

Segments of a guinea pig ileum were suspended at 0.5 g tension in an organ bath containing Tyrode's solution. Serotonin and azasetron hydrochloride were also added to the bath.

inducing a purgative effect. The active constituents of acetone EAL, compounds 2 and 4 (even at the highest dose of 1000 mg/kg, p.o.), did not produce diarrhea as a side effect. These findings indicate that the safety margin for senna is narrow or non-existent, whereas that of EAL is relatively wide.

We propose for two reasons that the major EAL constituent with laxative activity was compound 4 (genkwanin 5-*O*-β-primeveroside): first, the effective dose of compound 4 (100 mg/kg) was smaller than that of compound 2 (1000 mg/kg); second, the time-course characteristics of the laxative effect of compound 4 were similar to those of acetone EAL, unlike those of compound 2.

To clarify the mechanism underlying the laxative effect of EAL, we evaluated the effects of compound 4 on the spontaneous motility of an isolated rabbit ileum and on the contraction of an isolated guinea pig ileum. Compound 4 induced contractions at a concentration of 1.0×10^{-3} g/ml, suggesting that its purgative activity may have been due to acceleration of the spontaneous ileal movement. Further, the contraction it induced was inhibited by the acetylcholine-receptor antagonist, atropine, but not by the 5-HT_3 receptor antagonist, azasetron. It therefore seems likely that the contractile effect of compound 4 may at least in part have been exerted *via* acetylcholine receptors. Taken together, these results indicate that compound 4 acted *via* a mechanism different from that of senna.

In summary, EAL had a laxative effect on mice that was milder than that of senna, and it did not produce diarrhea as a severe side effect, unlike senna. Furthermore, we identified the main constituent contributing to the laxative effect of EAL as genkwanin 5-*O*-β-primeveroside (compound 4). Compound 4 may act as a laxative partly by contracting the ileum *via* acetylcholine receptors.

Acknowledgments

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