Agarwood Induced Laxative Effects via Acetylcholine Receptors on Loperamide-Induced Constipation in Mice

Mamoru Kakino, Hiroshi Izuta, Tetsuro Ito, Kazuhiro Tsuruma, Yoko Araki, Masamitsu Shimazawa, Masayoshi Oyama, Munekazu Inuma, and Hideaki Hara

1Department of Biofunctional Evaluation, Molecular Pharmacology, Gifu Pharmaceutical University, Gifu 501-1196, Japan
2Department of Bioactive Molecules, Pharmacognosy, Gifu Pharmaceutical University, Gifu 501-1196, Japan
3Nagara Research Center, API Co., Ltd., 692-3 Nagarayamasaki, Gifu 502-0071, Japan

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Agarwood (Aquilaria sinensis, Aquilaria crasna) is well known as an incense in the oriental region such as Thailand, Taiwan, and Cambodia, and is used as a digestive in traditional medicine. We investigated the laxative effects and mechanism of agarwood leaves extracted with ethanol (EEA-1, Aquilaria sinensis; EEA-2, Aquilaria crasna). EEA-1, EEA-2, the main constituents of EEA (mangiferin, and genkwanin-5-O-primeveroside), and senna increased the frequency and weight of stools in loperamide-induced constipation model mice. EEA-1 and EEA-2 did not induce diarrhea as a side effect, but senna induced severe diarrhea. EEA-1 and senna increased gastro-intestinal (GI) transit in the model mice. EEA-1, but not senna, also increased the intestinal tension of isolated jejunum and ileum in guinea pigs, and the tension increase was blocked by atropine, a muscarinic receptor antagonist, but not by other inhibitors (granisetron, pyrilamine, or bradykinin-antagonist peptide). Furthermore, the increase in frequency and weight of stools induced by EEA-1 was blocked by pre-administration of atropine in the model mice. These findings indicate that EEA exerted a laxative effect via acetylcholine receptors in the mouse constipation model.

Key words: agarwood; Aquilaria crasna; Aquilaria sinensis; constipation; laxative effect

Constipation is a common public health problem with a well-recognized tendency to cause discomfort and affect the quality of life. It increases during aging and can be a chronic condition leading to the taking of laxatives in the long term. Constipation is not only discomforting but can cause abdominal distension, vomiting, restlessness, gut obstruction, and perforation, and can cause aspiration or fatal pulmonary embolism. Nowadays, 20 to 30% of people over the age of 60 use a laxative more than once a week. Magnesium oxide or sennoside, this being the main constituent of senna-containing drugs, is typically administered for treatment of constipation for its powerful purgative/laxative activities, but these drugs induce severe diarrhea as a side effect. Furthermore, repeated use of senna or another anthraquinoids-containing drug can induce melanosini (colic melanosini), a risk factor for colorectal neoplasm.

Agarwood leaves are drunk as a health tea in Thailand and Taiwan. Characteristic sesquiterpenes and chro-mone derivatives have been isolated from agarwood, and some of these have sedative analgesic effects. Phytochemical research has been carried out on the trunk and resin of agarwood, but little is known about the pharmacological effects of agarwood leaves. Our previous study indicated that an acetone extract of agarwood and its main content, genkwanin-5-O-beta-primeveroside, increases the frequency and weight of stools in normal mice.

The purpose of present study was to investigate the laxative effects of agarwood leaves extracted with ethanol (EEA) and to clarify the mechanism. First, we evaluated the laxative activity of EEA and their main constituents in the constipation model with male and female mice, and then identified the effect of EEA-1 on the gastrointestinal tract to determine the mechanism of EEA, and finally evaluated intestinal tension by the magnus method with or without various receptor antagonists.

Materials and Methods

Materials. Agarwood (Aquilaria sinensis) gathered in Thailand; Aquilaria crasna (Aquilaria crasna) gathered in Taiwan) and senna leaves were supplied by API (Gifu, Japan). The species of these botanical materials were identified by Professor Munekazu Inuma (Department of Bioactive Molecules, Pharmacognosy, Gifu Pharmaceutical University). Loperamide hydrochloride, acetylcysteine chloride, atropine sulfate standard, 5-hydroxytryptamine, granisetron hydrochloride, histamine dihydrochloride, pyrilamine maleate, charcoal, and dymethyl sulfoxide were purchased from Wako (Osaka, Japan). A bradykinin-peptide was purchased from the Peptide Institute (Osaka, Japan). A bradykinin antagonist was purchased from Anaspec (San Jose, CA).

Extraction procedure. Dried agarwood leaves (50 g) were extracted with 60% ethanol (1,000 ml) at room temperature for 24 h. We got 8.24 g of EEA-1 from Aquilaria sinensis, and 8.99 g of EEA-2 from Aquilaria crasna.

Isolation procedure. The process of isolation was performed as described in a previous report. Dried agarwood leaves (1.5 kg) were successively extracted with acetone (8 liters) 4 times. Evaporation of the solvents gave acetone extract (70 g). The acetone extract was...
suspended in CHCl₃-methanol (1:1), and the insoluble part was isolated from CHCl₃-methanol solvent by filtration to yield mangiferin (2.45 g). The filtrate was subjected to chromatography on silica gel eluted with a CHCl₃-methanol solvent system (10:1–1:1, linear gradient) to prepare six crude fractions. The 5:1 crude fraction (4 g) was separated in a Sephadex LH-20 column eluted with methanol to give iriflophenone-2-O-alfa-rhamnose (1.5 g). The (3:1) polar crude fraction (6 g) was separated by chromatography in a silica gel column eluted with a benzene-methanol-H₂O solvent system (8:2:0.1), and then re-crystallized from methanol-H₂O (9:1) to give genkwanin-5-O-beta-primeveroside (0.63 g).

Animals. All animal experiments were carried out following the “Principles of Laboratory Animal Care” (NIH publication number 85-23, revised 1985), and “Guidelines of the Animal Investigation Committee of Gifu Pharmaceutical University,” with the approval of that committee.

Male and female ddY mice (7–10 weeks old) and male Hartley guinea pigs (5–6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The animals were housed under a controlled room temperature (24.5–25.0 °C) at 60 ± 10% humidity under a 12/12 h light/dark cycle. Food pellets and tap water were provided ad libitum.

Frequency and weight of stools. The frequency and weight of stools were measured as the frequency and total wet weight for each mouse over 8 h in loperamide-induced constipation model mice. EEA-1 (1,000 mg/kg), EEA-2 (1,000 mg/kg), mangiferin (10 mg/kg), genkwanin-5-O-beta-primeveroside (10 mg/kg), iriflophenone-2-O-alfa-rhamnose (10 mg/kg), and senna (500 mg/kg) were administrated orally, and then loperamide hydrochloride (5 mg/kg) or distilled water (as control) was orally administrated to the mice 1 h after sample administration, and we measured the frequency and weight of stools for each mouse during consecutive 2-h periods (0–2 h, 2–4 h, 4–6 h, 6–8 h). The frequency of stools was measured as the number of stool beads. The mice were separated in small transparent cages (11 cm height, 17.5 cm width, and 11 cm depth, one mouse to a cage).

Gastrointestinal (GI) transit. The mice were fasted for 14 h with water available ad libitum before the experiment. EEA-1 (300, 500, 1,000 mg/kg) and senna (500 mg/kg) were administrated orally, then loperamide hydrochloride (5 mg/kg) or distilled water (as control) was administrated subcutaneously after 30 min, then charcoal meal (5% charcoal/10% gum arabic) was administrated orally (at a volume of 0.1 ml/10 g body weight) after 30 min, and then the mice were sacrificed by cervical dislocation under anesthesia with diethyl ether. They were dissected, and the small intestine from the pylorus to the blind intestine was carefully removed. For each animal, GI transit was calculated as the percentage of the distance traveled by the charcoal meal relative to the total length of the small intestine. The equation below was used to calculate GI transit (%).

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\text{GI transit (\%)} = \frac{\text{distance traveled by the charcoal}}{\text{(total length of the small intestine)}} \times 100
\]

Contractions of the isolated jejunum and ileum by the magnus method. The guinea pigs were killed by cervical dislocation. Segments of guinea pig intestine were suspended at a maximum tension of 0.5 g in Automatic Organ Bath (Panlab Technology for Bioresearch, Barcelona, Spain) containing Tyrode’s solution (137 mm NaCl, 5 mm KCl, 2.5 mm CaCl₂·2H₂O, 0.1 mm MgCl₂·6H₂O, 0.3 mm NaH₂PO₄·2H₂O, 11.9 mm NaHCO₃, and 5.6 mm glucose, pH 7.4). The minimum resting tension of the suspended intestine was determined as the basal. Spontaneous movement was monitored with a recorder (Occlal Bridge Amp; AD Instruments, Castle Hill, Australia) via an isotonic transducer (PowerLab 8/30; AD Instruments). EEA-1 and senna extract were cumulatively added to the organ bath at final concentrations of 10, 40, and 100 μg/ml. Distilled water supplemented with dimethyl sulfoxide (DMSO) was added as a control at final concentrations of 0.001, 0.004, and 0.014%. EEA-1 or senna solution was prepared in 1% DMSO/distilled water at a concentration of 10 mg/ml. Various receptor antagonists (1 μg/ml of atropine sulfate, 100 μg/ml of granisetron hydrochloride, 1 μg/ml of pyrilamine maleate, and 10 mg/ml of bradykinin antagonist) were separately added to the organ bath 15 min before sample administration. Average tensions were determined 5 min after administration of the samples.

Statistical analysis. Data are presented as mean ± SEM. Statistical comparisons were made by Student’s t-test, paired t-test, Tukey’s multiple comparison test, or two-way repeated measures analysis of variance (ANOVA), followed by t-test (JSTAT for Windows; Vector, Tokyo).

Results

Laxative effects of EEA-1, EEA-2, and senna in the model mice

As for the laxative effects of EEAs on the frequency and wet weight of stools in loperamide-induced constipation model of male mice, loperamide hydrochloride (5 mg/kg) reduced the frequency and the wet weight of stools to 15.6 ± 6.9% (n = 6) and 19.4 ± 13.3% (n = 6) of control, respectively (Fig. 1A and B). First we compared the two kinds of EEA (EEA-1, Aquilaria sinensis gathered in Taiwan; EEA-2, Aquilaria crassa gathered in Thailand). EEA-1 restored the stool frequency and stool weight in the mice to 91.1 ± 10.1% and 99.8 ± 12.9% of control, respectively. Similarly, EEA-2 restored the stool frequency and the stool weight to 111.6 ± 18.2% and 116.7 ± 11.0% of control, respectively. There was no significant difference between EEA-1 and EEA-2 as to stool frequency and stool weight. Next we investigated the effects of the main constituents of the EEAs (mangiferin, genkwanin-5-O-beta-primeveroside, and iriflophenone-2-O-alfa-rhamnose). Mangiferin restored the stool frequency and the stool weight to 95.8 ± 14.5% and 100.3 ± 7.6% of control respectively. Genkwanin-5-O-Primeveroside restored the stool frequency and the stool weight to 67.2 ± 9.4% and 68.1 ± 5.7% of control respectively, but iriflophenone-2-O-alfa-rhamnose did not. On the other hand, senna, a positive control, increased the stool frequency and the stool weight to 78.4 ± 16.2% and 373.0 ± 62.3% of control respectively.

To investigate sexual differences in the laxative effects of EEAs, we also examined the effects of EEA-1 on the frequency and wet weight of stools in loperamide-induced constipation model female mice. Loperamide hydrochloride reduced the frequency and the wet weight of stools to 17.2 ± 10.2% and 19.4 ± 10.9% of control respectively (Fig. 1C and D). EEA-1 restored the stool frequency and the stool weight in the mice to 91.2 ± 3.4% and 73.7 ± 10.4% of control respectively. Similarly, ethanol extract of senna increased the stool frequency and the stool weight to 78.7 ± 16.2% and 206.4 ± 34.5% of control respectively.

Diarrhea frequencies of the EEs and senna

EEA-1 and EEA-2 did not induce diarrhea at any time during the observation period (Fig. 2A and C). On the other hand, senna (500 mg/kg) induced diarrhea at 0–8 h (Fig. 2B and C), and the rate of diarrhea was 100% at 0–2 h, 83% at 2–4 h, and 50% at 4–8 h.

Effects of EE-1 and senna on gastrointestinal (GI) transit

To determine the effects of EEA-1 and senna on the GI tract, we measured GI transit in the mice (Fig. 3).
Loperamide hydrochloride at 5 mg/kg reduced the GI transit time; value was 26.4 ± 1.8% of control. EEA-1 at 1,000 mg/kg (p.o.) significantly accelerated GI transit (p < 0.01); value was 51.7 ± 2.0% of control. However, lower doses of EEA-1 (300 and 500 mg/kg) failed to induce a significant acceleration in GI transit (Fig. 3). Senna at 500 mg/kg (p.o.) significantly accelerated GI transit; value was 49.8 ± 2.0% of control.

Effects of EEA-1 and senna on the tension of isolated guinea pig intestine
To determine the effects of EEA-1 and senna on intestinal tension, we measured the tension of the isolated jejunum and ileum in guinea pig (Fig. 4). EEA-1 and senna were applied to the isolated jejunum, ileum, and colon at final concentrations of 10, 40, and 140 μg/ml. Representative examples of EEA-1 at 10, 40, and 140 μg/ml and acetylcholine at 100 ng/ml are shown in Fig. 4A (effect on ileum) and Fig. 4B (effect on colon). EEA-1 at 40 and 140 μg/ml significantly increased the tension of jejunum (by approximately 1.2 and 1.3 times basal level) (Fig. 4C). EEA-1 at 40 and 140 μg/ml significantly increased the tension of ileum (by approximately 1.6 and 2.5 times basal level). EEA-1 did not affect the colon statistically (data not shown). Senna at 140 μg/ml tended to decrease the tension of the jejunum and ileum, but not significantly.

Effects of various receptor antagonists on the increment of intestinal tension induced by EEA-1 in the guinea pig
To determine the mechanism of the EEA-1-induced increment of intestinal tension, we evaluated the effects of various receptor antagonists on intestinal tension.
Fig. 2. Diarrhea Frequency of EEA-1 and Senna in Loperamide-Induced Constipation Model Mice.

Stool shape of the mice 2 h after administration of EEA-1 (1,000 mg/kg) and senna (500 mg/kg) (A and B). (C) Diarrhea frequency every 2 h (0–2 h, 2–4 h, 4–6 h, 6–8 h) until 8 h after oral administration of EEA-1 and senna to the mice.

Fig. 3. Effects of EEA-1 and Senna on Gastrointestinal (GI) Transit in Loperamide-Induced Constipation Model Mice.

EEA-1 (300, 500, 1,000 mg/kg) and senna (500 mg/kg) were administrated (p.o.). Constipation was induced by injection of loperamide (5 mg/kg, s.c.) 30 min after administration of EEA-1 or senna. Charcoal was administrated (p.o.) 30 min after administration of loperamide. The mice were subjected to laparotomy, and the length of charcoal from stomach in the intestine was measured 20 min after the administration of charcoal. GI transit (%) = (distance travelled by the charcoal)/(total length of the small intestine) × 100. a, b, c, p < 0.05, among different characters (n = 5 or 6, one-way ANOVA by Tukey’s multiple comparison test).

Fig. 4. Effects of EEA-1 and Senna on the Tension of the Jejunum and Ileum.

Intestinal tension was measured by the magnus method. EEA-1 (white circle) and senna (black circle) were added at concentrations of 10, 40, and 140 µg/ml. A representative example of the intestinal tension of jejunum (A) and colon (B) after EEA-1 administration. The intestinal tension of the jejunum and ileum was calculated as the average for 5 min after administration of each sample (C). Data are shown as mean ± SEM. "p < 0.01, *p < 0.05 vs. vehicle (n = 5 or 6, paired Tukey’s multiple comparison test).
treatment with atropine, an acetylcholine receptor antagonist, at a concentration of 1 μg/ml significantly suppressed the contraction induced by EEA-1 (p < 0.01). However, pre-treatment with granisetron, a 5-HT receptor antagonist, at 100 μg/ml, with pyrilamine, a histamine receptor antagonist, 1 μg/ml, and with a bradykinin-antagonist peptide, a bradykinin receptor antagonist, at a concentration of 10 ng/ml failed to suppress the contraction induced by EEA-1 (Fig. 5B, C, and D).

Effect of pretreatment with atropine on the laxative effect induced by EEA in loperamide-induced constipation model mice

To determine the effect of atropine on EEA-1-induced laxative activity, atropine was administrated orally 10 min before administration of EEA-1. The laxative effect of EEA-1 at 1,000 mg/kg was significantly inhibited by pre-administration of atropine at 5 mg/kg in the mice (Fig. 6). Atropine at 5 mg/kg did not affect on the frequency or the wet weight of stools in normal mice (Fig. 6).

Discussion

In the present study, EEAs at 1,000 mg/kg and senna at 500 mg/kg exhibited laxative activity in loperamide-induced constipation model mice. We have reported that oral administration of acetone extract of agarwood, but not methanol extract, had a laxative effect in normal mice. The components of the EEAs were similar to those of the acetone extract of agarwood and both EEAs and the acetone extract of agarwood contained genkwanin 5-O-beta-primeveroside. This study is the first of EEAs possessing a laxative effect that evaluated laxative activity in loperamide-induced constipation model mice.

We measured GI transit in the mice. EEA-1 at 1,000 mg/kg and senna at 500 mg/kg significantly accelerated GI transit. These results indicate that the laxative effects of EEA-1 and senna are at least partly dependent on acceleration in the GI tract.

Senna contains anthraquinone derivatives, sennosides A, B, C, and D, which have powerful laxative/purgative effects. In the present study, the laxative potency of the EEAs at 1,000 mg/kg was the same as that of senna at 500 mg/kg in vivo, but senna induced severe diarrhea at the same dose (500 mg/kg). On the other hand, the EEAs at 1,000 mg/kg did not induce diarrhea as a side effect. This indicates that EEAs are suitable for daily use.
in patients suffering from chronic constipation and are better than senna.

To investigate the pharmacological mechanism underlying the laxative effects of EEA-1 and senna, we measured the motility tension of isolated ileum or jejunum of guinea pigs by a magnus method. EEA-1 at concentrations of 40 to 140 µg/ml significantly increased the tension of jejunum and ileum, but senna did not.

Next, to investigate the biochemical mechanism underlying the laxative effect of EEA-1, we measured the tension of the jejunum by a magnus method with four kinds of receptor antagonists (atropine, granisetron, pyrilamine, and bradykinin-antagonist peptide). The increment of intestinal tension due to EEA-1 was decreased by atropine, an acetylcholine receptor antagonist, but not by the other antagonists (granisetron, a 5-HT antagonist; pyrilamine, a histamine antagonist; and bradykinin-antagonist peptide, a bradykinin antagonist). Then we investigated to determine whether atropine inhibits the in vivo laxative activity of EEA-1 by measuring the stool frequency and stool weight of mice with pretreatment by atropine in loperamide-induced constipation model mice, as well as the increments of intestinal tension induced by EEA-1 ex vivo. Although administration of atropine at 5 mg/kg did not affect stool frequency or stool weight, atropine at the same dose suppressed the laxative effect of EEA-1 in the model mice. Taken together, these results indicate that administration of atropine inhibited the laxative effect of EEA-1 without aggravating constipation via acetylcholine receptors, and that the laxative effect of EEA-1 acts via acetylcholine receptors, as well as EEA-1-induced increment of intestinal tension.

Senna at the same dose as EEA-1 did not affect the tension of the ileum or jejunum by the magnus method. The main constituents of senna, sennosides, are decomposed to the corresponding laxative aglycones, rhein-9-anthrones, by intestinal flora, and this decomposition is essential for the laxative effect of senna. This suggests that some constituents of EEA-1 have a laxative effect without modification by intestinal flora.

Generally, the national census in Japan shows that women are more vulnerable to constipation than men (Japan Ministry of Health). Moreover, clinical instances of gastrointestinal motility disorders in pregnancy have been reported. This indicates that women are more vulnerable to constipation than men. We evaluated the laxative activity of EEA-1 in both male and female mice using a loperamide-induced constipation model. EEA-1 at 1,000 mg/kg induced laxative activity in female mice as much as in male mice. This indicates that the laxative effect of EEA-1 is not markedly different between male and female.

Finally, we investigated effects of constituents in EEA (genkwain-5-O-beta-primeveroside, genkwain, iriflophenone-2-O-alfa-rhamnoside, and mangiferin) on stool frequency and stool weight in loperamide-induced constipation model mice. Our previous study showed that genkwain-5-O-beta-primeveroside has a laxative effect in normal mice, and the present study indicates that genkwain-5-O-beta-primeveroside has a laxative effect in loperamide-induced constipation model mice, while iriflophenone-2-O-alfa-rhamnoside does not. Not only genkwain-5-O-beta-primeveroside but mangiferin showed a laxative effect. Collectively, these two constituents, genkwain-5-O-beta-primeveroside and mangiferin, may be the pharmacologically active constituents of EEA-1 and EEA-2 as for laxative effect. There are few reports on decomposition or absorption as to genkwain-5-O-primeveroside. We found that genkwain (aglycon) did not affect loperamide-induced constipation model mice in the same examination (data not shown). In oral administration, the residue of primeveroside is essential to the laxative effect of genkwain-5-O-beta-primeveroside. Mangiferin is known as the pharmacologically active constituent of common anemarrhena (Anemarrhena asphodeloides Bunge), and a blood glucose-lowering effect has been reported. There is no previous report that indicates the laxative effect of mangiferin.

In conclusion, EEA-1 and EEA-2 had a laxative effect on loperamide-induced constipation model mice without causing diarrhea. The laxative effect of EEA-1s may be partly due to acceleration of gastrointestinal transit, via acetylcholine receptors.

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