Studies on gastric lesion protective substances in crude drugs: Isolation of active principle from the leaves of *Ginkgo biloba* L.

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Abstract

Free radicals have been reported to participate in formation of gastric lesions, and thus this scavenging action shows gastric lesion protective activity. It was known that *Ginkgo biloba* L. exhibits the effect of scavenging free radicals. In the present study, we attempted to isolate an active principle from *Ginkgo biloba* L.: *Ginkgo biloba* L. inhibited the formation of gastric lesions induced by ethanol, aspirin and indomethacin. An ethyl acetate and hexane layer of *Ginkgo biloba* L. gave sciadopitysin and 2-(12-heptadecenyl)-6-hydroxy-benzoic acid when separated by using the inhibitory activity on ethanol-induced gastric lesions in rats as an index. And these compounds had a positive effect on gastric lesion protection similarly to sucralfate.

Key words *Ginkgo biloba* L., sciadopitysin, 2-(12-heptadecenyl)-6-hydroxy-benzoic acid, gastric lesion protection.

Introduction

*Ginkgo biloba* L. is a deciduous tree originating in China, the sole surviving species of the family Ginkgoaceae. Its ancestry has been traced back more than 200 million years. Extracts of *Ginkgo biloba* L. and ginkgolide derivatives which were its constituents have been used to attenuate post-ischemic brain injury and Alzheimer’s disease,¹ and it was reported that some flavonoids such as kaempferol and luteolin isolated from *Ginkgo biloba* L. exhibits the effect of scavenging free radicals.² Free radicals have been reported to participate in formation of gastric lesions, and thus this scavenging action shows gastric lesion protective activity.³

We have previously reported gastric lesion protective principles isolated from crude drugs.⁴ In the present study, we attempted to isolate an active principle from *Ginkgo biloba* L., and to measure its gastric lesion protective activity.

Experimental

General: Dried leaves of *Ginkgo biloba* L. (Chinese) were purchased from Tochimoto Tenkaido Co., Ltd, Osaka, Japan in 1996. The following drugs were used: ethanol (Wako, Osaka, Japan), aspirin and indomethacin (Nacalai tesque, Kyoto, Japan).

IR spectra were recorded with a Hitachi (Tokyo, Japan) IR-260-30 spectrometer. ¹H- and ¹³C-NMR spectra were recorded with a Bruker (Germany) ARX-500 (500.0 and 125.8 MHz) or DPX-400 (400.1 and 100.6 MHz) spectrometer. EI-MS were recorded with a Hitachi M-2000 mass spectrometer. Flash column chromatography was carried out on a column packed with Kieselgel 60 G (Merck, Germany) silica gel. Preparative thin layer

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chromatography (PTLC) was carried out with Kieselgel 60 PF254 (Merck).

All animal tests went according to the animal welfare guidelines of Kobegakuin University and the experimental protocol was approved by the Animal studies Committee of Kobegakuin University.

**Ethanol-induced gastric lesions test:** Male Wistar ST rats (SLC. Co., Ltd. Shizuoka), weighing 170-200 g, were used. The animals were fasted overnight, but allowed free access to water until the start of the experiment. Gastric mucosal lesions were produced according to the method of Robert et al. Test samples (below) were suspended in 10% gum arabic solution and provided at a volume of 5 ml/kg of body weight. Each sample or vehicle (10% gum arabic solution) was given to rats orally 30 min prior to oral administration of 1 ml of 99.5% ethanol (EtOH). One hr after the EtOH administration, the animals were sacrificed under ether anesthesia by decapitation. The stomach was removed and inflated by injecting 10 ml of 1% formalin to the gastric lumen for 10 min. Subsequently, the stomach was incised along the greater curvature and examined for lesions. The length (mm) of each lesion was measured under a dissecting microscope (x 10) with a square grid, and the sum of the lengths per stomach was used as a lesion index.

The test samples used were as follows: hot water extraction of *Ginkgo biloba* L., fractions of *Ginkgo biloba* L., sciadopitysin, 2-(12-heptadecenyl)-6-hydroxy-benzoic acid, sucralfate, and cimetidine.

**Aspirin-induced gastric lesions test:** Gastric mucosal lesions were produced according to the method of Bridie et al. Each sample or vehicle (10% gum arabic solution) was given to rats orally 30 min prior to oral administration of 200 mg/kg, p.o. of aspirin. Four hr after the aspirin administration, the animals were sacrificed under ether anesthesia by decapitation.

**Indomethacin-induced gastric lesions test:** Gastric mucosal lesions were produced according to the method of Urushidani et al. Each sample or vehicle (10% gum arabic solution) was given to rats orally 30 min prior to Indomethacin administration. Indomethacin, suspended with a trace of Tween 80 and then in a 10% gum arabic solution, was given s.c. at 20 mg/kg in a volume of 0.2 ml/100 g. One hr after the indomethacin administration, the animals were sacrificed under ether anesthesia by decapitation.

**Extraction and isolation from *Ginkgo biloba* L.:** Dried leaves of *Ginkgo biloba* L. (10 kg) were powdered and extracted three times with methanol (MeOH, 20 L) at room temperature, for one day per extraction. The solution was lyophilized after concentration to give methanol extract (yield: 10.5%).

The methanol extract was suspended in 1.5 L of water. This suspension was extracted three times with hexane (1.5 L). The hexane layer was concentrated under reduced pressure to give a residue (3.8%) of leaves. The aqueous layer was extracted three times with ethyl acetate (AcOEt, 1 L). The AcOEt layer was concentrated to give a residue (1.3%). The aqueous layer was filtered to give a precipitate (5.4%).

The AcOEt layer was subjected to silica gel flash column chromatography using hexane-AcOEt (1:1 to 0:10) and AcOEt - MeOH (10:0 to 2:8) to give fractions 1 - 5 (Fr. 1, 0.28%; Fr. 2, 0.28%; Fr. 3, 0.39%; Fr. 4, 0.05%; Fr. 5, 0.28%). Fr. 4 was purified using PTLC with CHCl₃-MeOH (95:5) to give an active compound, Compound A (0.003%) (below).

Compound A (sciadopitysin (1)): pale yellow crystals. m.p. 286-287. IR (nujol) cm⁻¹: 2950, 2850, 1660. El-MS m/z: 580 [M⁺]. The compound was identified by the NMR spectra and by direct comparison of its physical properties with that of respective authenticated samples.

The hexane layer was chromatographed on silica gel flash column by using hexane-AcOEt (10:0 to 0:10) to give 6 fractions, 6-11 (Fr. 6, 0.40%; Fr. 7, 1.37%; Fr. 8, 0.64%; Fr. 9, 0.51%; Fr. 10, 0.53%; Fr. 11, 0.39%).

Fr. 7 was rechromatographed on silica gel flash column using hexane-AcOEt (9:1 to 2:8) to give 5 fractions, 12-16 (Fr. 12, 0.003%; Fr. 13, 0.05%; Fr. 14, 0.47%; Fr. 15, 0.64%; Fr. 16, 0.20%).

Fr. 14 was chromatographed on silica gel flash column using hexane-AcOEt (7:3 to 2:8) to give 6 fractions 17-22 (Fr. 17, 0.003%; Fr. 18, 0.01%; Fr. 19, 0.16%; Fr. 20, 0.09%; Fr. 21, 0.15%; Fr. 22, 0.003%).

Fr. 21 was purified by reversed-phase column chromatography (Cosmosil 75C₁₈ - OPN, Nacalai Tesque
Inc., Kyoto, Japan, EtOH-water 6:4 to 8:2) to give Compound B (0.01%) (below), and 4 fractions, 23-26 (Fr. 23, 0.07%; Fr. 24, 0.01%; Fr. 25, 0.01%; Fr. 26, 0.04%).

Compound B (2-(12-heptadecenyl)-6-hydroxy-benzoic acid (2))$^{14}$: colorless powder. IR (nujol) cm$^{-1}$: 3000, 2950, 2860, 1650, 1450. EI-MS m/z: 374 [M]$^+$. $^1$H-NMR (CDCl$_3$) $\delta$ : 0.89 (3H, t, 7.5 Hz), 1.32 (2H, m), 1.61 (2H, m), 2.01 (4H, m), 2.98 (2H, t, 7.5 Hz), 5.34 (2H, m), 6.77 (1H, dd, 1.2, 7.5 Hz), 6.87 (1H, dd, 1.2, 7.5 Hz), 7.35 (1H, t, 7.5 Hz). 13$^C$-NMR (CDCl$_3$) $\delta$ : 14.0 (q), 22.4 (t), 26.9 (t), 27.2 (t), 29.35 (t), 29.52 (t), 29.59 (t), 29.66 (t), 29.69 (t), 29.70 (t), 29.81 (t), 29.84 (t), 31.99 (t), 32.02 (t), 36.5 (t), 110.5 (s), 115.9 (d), 122.8 (d), 129.84 (d), 129.93 (d), 135.5 (d), 147.9 (s), 163.4 (s), 176.4 (s).

**Dimethylation of Compound B:** Compound B (100 mg) dissolved in ether (30 ml) was methylated with CH$_3$N$_2$ at room temperature for 6 hr. The product was purified by PTLC (hexane-AcOEt 2:8) to give dimethyl Compound B (100 mg, 93.0%).

**Oxidation of Compound B:** Water (10 ml), acetone (10 ml), Compound B (300 mg), trimethylamin-N-oxide dehydrat (19 mg), and OsO$_4$ (11 mg) were stirred for 4 hr. Powdered NaIO$_4$ (2.2 g) was added over 30 min and the slurry stirred for 2 hr. The mixture was extracted with ether, and purified by PTLC (hexane-AcOEt 3:7) to give Compound C (150 mg, 58.4%) (below).

Compound C (2-(11-formylundecyl)-6-hydroxy-benzoic acid (3)) : colorless oil. IR (nujol) cm$^{-1}$: 2950, 2850, 2250, 1730, 1660, 1620, 1460. EI-MS m/z: 320 [M]$^+$. 1$^H$-NMR (CDCl$_3$) $\delta$ : 1.27 (14H, m), 1.61 (4H, m), 2.42 (2H, dt, 1.9, 7.5 Hz), 2.97 (2H, t, 7.5 Hz), 6.77 (1H, br. d, 7.5 Hz), 6.86 (1H, br. d, 7.5 Hz), 7.35 (1H, t, 7.5 Hz), 9.76 (1H, t, 1.9 Hz). 13$^C$-NMR (CDCl$_3$) $\delta$ : 22.1 (t), 29.12 (t), 29.28 (t), 29.34 (t), 29.39 (t), 29.48 (t), 29.49 (t), 29.76 (t), 32.0 (t), 36.5 (t), 43.9 (t), 110.5 (s), 115.8 (d), 122.7 (d), 135.2 (d), 147.9 (s), 163.4 (s), 176.4 (s), 205.3 (s).

### Results and Discussion

**Gastric lesion protective activity of Ginkgo biloba L.:**

The purpose of the present study was to examine gastric lesion protective activity of Ginkgo biloba L. in ethanol- and aspirin- and indomethacin-induced gastric lesions as index. Ginkgo biloba L. at doses ranging from 50 to 250 mg/kg inhibited the formation of gastric lesions induced by ethanol (Fig. 1), aspirin (Fig. 2), and indomethacin

![Fig. 1](image1.png) **Fig. 1** Effects of G. biloba L., sulcrate and cimetidine on ethanol-induced gastric lesions in rats. Gastric lesions were produced by giving ethanol (99.5%, 1 ml/rat, p.o.). Each drug was given 30 min before ethanol administration. Animals were sacrificed 1 hr after ethanol treatment. Each value represents the mean ± S.E. of 5 rats per group. * : p < 0.05 , ** : p < 0.01, *** : p < 0.001 (Student’s t-test).

![Fig. 2](image2.png) **Fig. 2** Effects of G. biloba L., sulcrate and cimetidine on aspirin-induced gastric lesions in rats. Gastric lesions were produced by giving aspirin (100 ml/kg, p.o.). Each drug was given 30 min before aspirin administration. Animals were sacrificed 4 hr after aspirin treatment. Each value represents the mean ± S.E. of 5 rats per group. * : p < 0.05, ** : p < 0.01, *** : p < 0.001 (Student’s t-test).
induced by ethanol and aspirin in a dose-dependent manner, but did not prevent the lesions induced by indomethacin. *Ginkgo biloba* L. showed gastric lesion protective activity similarly to sucralfate, and inhibited the formation of gastric lesions by secreted gastric acid similarly to cimetidine.

**Separation of extracts using inhibitory effect on ethanol-induced gastric lesions as index:** The leaves of *Ginkgo biloba* L. were extracted with methanol at room temperature. The solvents were evaporated *in vacuo* at room temperature to give the respective methanol extracts. The MeOH extract was suspended in water. This suspension was extracted with hexane and AcOEt. The hexane and AcOEt layer was concentrated under reduced pressure to give a residue. The aqueous layer was filtered to give a precipitate (Fig. 4). Activity was evaluated using the ethanol-induced gastric lesions in rats. The hexane and AcOEt extract showed a significant inhibitory activity against gastric lesions (250 mg/kg, p.o.).

The AcOEt layer was divided into five fractions (Fr. 1-5) by silica gel flash column chromatography. The gastric lesion protective activity was detected in Fr. 4. This fraction showed a significant protection at a dose of 100 mg/kg, p.o., respectively. Fr. 4 was further purified by silica gel preparative thin layer chromatography (PTLC) to give Compound A (1, sciadopitysin) (Fig. 4).

The hexane layer was divided into six fractions (Fr. 5).

(Fig. 3) in a dose-dependent manner. Cimetidine that is a type of antihistamine doses ranging from 10 to 50 mg/kg inhibited the formation of gastric lesions induced by aspirin and indomethacin in a dose dependent manner, but did not prevent the lesions induced by ethanol.

On the other hand, sucralfate, that binds to the hydrochloric acid in the stomach and acts like an acid buffer with cytoprotective properties, at doses ranging from 50 to 250 mg/kg inhibited the formation of gastric lesions...
6-11) by silica gel flash column chromatography (Fig. 5). The gastric lesion protective activities were detected in Fr. 7, 8, 9, and 10. These showed significant inhibition at doses of 50 mg/kg, p.o., respectively.

Fr. 7 was again subjected to chromatography, and gave an active fraction, Fr. 14 (50 mg/kg, p.o.). Fr. 14 was purified by chromatography and gave an active fraction 21, Fr. 21 (25 mg/kg, p.o.). Fr. 21 was further purified by reversed-phase chromatography to give Compound B (2) (Fig. 5).

Compound B appeared as a colorless powder. The MS spectrum showed m/z 374 [M]+. The 1H-NMR spectrum showed long-chain olefin and 1, 2, 3-trisubstituent aromatic ring, and the 13C-NMR spectrum showed 24 carbons. The IR spectrum showed carboxyl and hydroxyl groups. Ring 2 was observed to have a carboxyl group at C-1, long-chain olefin at C-2, and hydroxyl group at C-6 positions by the long-range C-H COSY spectrum of dimethyl derivative of ring 2.

Compound B was oxidized by Lemieux-Johnson reaction10 for the purpose of clarifying a position of the olefine, and gave Compound C (3) (scheme 1).

Compound C appeared as a colorless oil. The MS spectrum showed m/z 320 [M]+. The 1H-NMR spectrum showed long-chain hydrocarbon, aldehyde proton, and 1, 2, 3-tri substituent aromatic rings, and the 13C-NMR spectrum showed 19 carbons. Ring 3 was determined as 2-(11-formylundecyl)-6-hydroxy-benzoic acid. These observations suggest that Compound B was 2-(12-heptadecenyl)-6-hydroxy-benzoic acid (m = 3, n = 11).

**Gastric lesion protection by 1 and 2**: Sciadopitysin at doses ranging from 5 to 25 mg/kg inhibited the formation of gastric lesions induced by ethanol in a dose-dependent manner. The 50% effective dose (ED50) was 22.3 mg/kg, p.o. (Fig. 6).

2-(12-heptadecenyl)-6-hydroxy-benzoic acid at doses ranging from 5 to 25 mg/kg inhibited the formation of gastric lesions induced by ethanol in a dose-dependent manner. The ED50 was 8.6 mg/kg, p.o. (Fig. 6).

Sciadopitysin and 2-(12-heptadecenyl)-6-hydroxy-benzoic acid showed gastric lesion protective activity similarly to sucralfate. These compounds may act like

![Scheme 1](Image 123x512 to 488x744)  
**Scheme 1** Oxidation of Compound B
an acid buffer with cytoprotective properties.

The structure of 2-(12-heptadecenyl)-6-hydroxybenzoic acid resembles urushiol known by that inflammation was caused. It was interesting that 2-(12-heptadecenyl)-6-hydroxybenzoic acid showed gastric lesion protective action.

Conclusion

Ginkgo biloba L. inhibited the formation of gastric lesions induced by ethanol, aspirin and indomethacin. An ethyl acetate and hexane layer of Ginkgo biloba L. gave sciadopitysin and 2-(12-heptadecenyl)-6-hydroxybenzoic acid when separated by using the inhibitory activity on ethanol-induced gastric lesions in rats as an index. These compounds had a positive effect on gastric lesion protection similarly to sucralfate. They may act like an acid buffer with cytoprotective properties.

References