

## Anti-inflammatory Action of Phenolic Compounds from *Gastrodia elata* Root

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(Received August 23, 2006)

Previous screening of the pharmacological action of *Gastrodia elata* (GE) root (Orchidaceae) showed that methanol (MeOH) extracts have significant anti-inflammatory properties. The anti-inflammatory agents of GE, however, remain unclear. In this experiment, MeOH extracts of GE were fractionated with organic solvents for the anti-inflammatory activity-guided separation of GE. Eight phenolic compounds from the ether (EtOEt) and ethyl acetate (EtOAc) fractions were isolated by column chromatography: 4-hydroxybenzaldehyde (I), 4-hydroxybenzyl alcohol (II), benzyl alcohol (III), bis-(4-hydroxyphenyl) methane (IV), 4-(4'-hydroxybenzyloxy)benzyl methylether (V), 4-hydroxy-3-methoxybenzyl alcohol (VI), 4-hydroxy-3-methoxybenzaldehyde (VII), and 4-hydroxy-3-methoxybenzoic acid (VIII). To investigate the anti-inflammatory and anti-oxidant activity of these compounds, their effects on carrageenan-induced paw edema, arachidonic acid (AA)-induced ear edema and analgesic activity in acetic acid (HAc)-induced writhing response were carried out *in vivo*; cyclooxygenase (COX) activity, reactive oxygen species (ROS) generation in rat basophilic leukemia (RBL 2H3) cells and 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity were determined *in vitro*. These phenolic compounds not only had anti-inflammatory and analgesic properties *in vivo*, but also inhibited COX activity and silica-induced ROS generation in a dose-dependent manner. Among these phenolic compounds, compound VII was the most potent anti-inflammatory and analgesic. Compound VII significantly inhibited silica-induced ROS generation and compound VI significantly increased DPPH radical scavenging activity. Compounds I, II and III significantly inhibited the activity of COX-I and II. These results indicate that phenolic compounds of GE are anti-inflammatory, which may be related to inhibition of COX activity and to anti-oxidant activity. Consideration of the structure-activity relationship of the phenolic derivatives from GE on the anti-inflammatory action revealed that both C-4 hydroxy and C-3 methoxy radicals of benzyl aldehyde play an important role in anti-inflammatory activities.

**Key words:** *Gastrodia elata*, Phenolic compounds, Anti-inflammatory activity, Anti-oxidant activity

### INTRODUCTION

*Gastrodia* rhizome, the dried tuber of *Gastrodia elata* Blume (GE, Orchidaceae; Korean name: Chun-ma) is a very important traditional herbal medicine used to treat headache, migraine, dizziness, epilepsy, rheumatism, neuralgia, paralysis and other disorders (Tang and Eisenbrand, 1992). However, the components of GE that are the biologically active agents in this medical treatment of the various inflammatory diseases remain unclear.

Phytochemical studies of this plant have revealed the presence of several phenolic compounds (Hayashi et al., 2002). An earlier study showed that GE can reduce lipid peroxide levels and acts as a free radical scavenger in rats with ferric chloride-induced seizure (Liu and Mori, 1992, 1993). In the present study, using structure-activity guided separation of GE based on anti-inflammatory action, eight compounds, 4-hydroxy benzyl alcohol (I), 4-hydroxy benzaldehyde (II), benzyl alcohol (III), bis(4-hydroxyphenyl) methane (IV), 4-(4'-hydroxybenzyloxy)benzyl methyl ether (V), 4-hydroxy-3-methoxy benzyl alcohol (VI), 4-hydroxy-3-methoxy benzaldehyde (VII) and 4-hydroxy-3-methoxy benzoic acid (VIII), were isolated chromatographically. In addition, the anti-inflammatory and analgesic activities of these phenolic compounds were studied using

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the models of carrageenan-induced paw edema, arachidonic acid (AA)-induced ear edema and acetic acid (HAc)-induced writhing response *in vivo*. In order to study the mechanism of their anti-inflammatory activity, inflammatory mediators such as reactive oxygen species (ROS), scavenger activity of 1,1-diphenyl-2-picrylhydrozyl (DPPH) free radical, and cyclooxygenase (COX) activity were determined *in vitro*.

## MATERIALS AND METHODS

### Drugs and reagents

Dried rhizomes of *G. elata* (10.0 kg) were purchased from Bumhan Pharmaceutical Ltd. Co. (Korea, Kyung-Dong Marketing). Reference phenol derivatives, ibuprofen, carrageenan and arachidonic acid (AA) were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Ovine cyclooxygenase (COX)-1 and human recombinant COX-2 were purchased from Cayman Chemical Co. (Ann Arbor, MI., U.S.A.). The chemiluminescent cyclooxygenase activity kit was purchased from Stressgen Bioreagents Co. (Michigan 48108, U.S.A.).

### Animals

Pathogen-free male Sprague-Dawley rats weighing 180 to 200 g and male mice weighing 18 to 20 g were purchased from HanLim Animal (Hwa Sung-Gun, Kyung Ki-Do, Korea), and were housed for one week in a normal environmentally controlled animal room (temperature 25°C, humidity 55%, illumination 300-500 Lux) with pathogen-free food (Samyang Co.) and water *ad libitum*.

### Instrumental analysis and general experimental procedure

Melting points were determined on a BUCHI melting point B-540 (Switzerland). High resolution mass spectrometry was performed using Micromass Autospec (England). The UV spectra were recorded with a Hitachi 624 digital spectrophotometer and the IR spectra with a Bruker 48 (Germany). The <sup>1</sup>H NMR spectra were recorded with a Varian Gemini 2000, 200 MHz (USA) and Bruker AM-500, 500 MHz (Germany). The mass spectra were measured with Hitachi double-focusing and JEOL JMS-01-SG-2 mass spectrometers. The specific rotations were measured with a JASCO DIP-SL unit. Silica gel (Kieselgel 60, Merck) was used for column chromatography. Thin-layer chromatography (TLC) was carried out on Merck plates precoated with Kieselgel 60 F<sub>254</sub> and preparative layer chromatography (PLC) on plates (20×20 cm, 0.75 mm thick) coated with Kieselgel G F<sub>254</sub> (Merck).

### Extraction and Isolation

The dried and powdered rhizomes of *G. elata* (10.0 kg)

were extracted with EtOEt (3 L×3, 34.7 g), and then with MeOH (3 L×5, 1155 g) under reflux at 45°C. The MeOH extract was suspended in distilled water (3 L) and then successively partitioned with n-hexane, EtOAc and n-BuOH to give hexane (8.9 g), EtOAc (47.9 g), BuOH (295.4 g) and H<sub>2</sub>O (278.9 g) soluble fractions.

The EtOEt extract (34.7 g) was subjected to silica gel column chromatography, and eluted with benzene-ether solvent system (19:1 to 17:3) to give 13 fractions. The fractions were re-chromatographed on silica gel with a CHCl<sub>3</sub>-MeOH solvent system resulting in compounds I (125 mg), II (165 mg) and III (134 mg).

The EtOAc soluble fraction (47.9 g) was subjected to a silica gel column chromatography using CHCl<sub>3</sub>-MeOH solvent system (10:0 to 0:10) to give 240 fractions. Fractions 10-33 (1.74 g) were re-chromatographed on a silica gel column with benzene-EtOAc (10:0 to 80:20) to give compound IV (199 mg). Fraction 4 (1.1 g) was applied to a silica gel column by the same method to give compounds V (195 mg), VI (282 mg) and VII (195 mg). Compound VIII (1.1 g) was isolated from fraction 7 (1.71 g) by the same method.

### 4-Hydroxybenzaldehyde (I)

Pale brown needles (from ether-hexane), mp 117-118°C, IR cm<sup>-1</sup>: 3150 (OH), 1650 (CHO), 1600, 1595, 1515 (aromatic). <sup>1</sup>H-NMR (δ in acetone-d<sub>6</sub>): 7.01, 7.80 (each 2H, d, J=9Hz, arom.-H), 9.86 (1H, s, CHO). This compound was identified as 4-hydroxybenzaldehyde by direct comparison with an authentic sample (IR and mixed mp) and was bluish-green with FeCl<sub>3</sub>-K<sub>3</sub>[Fe(CN)<sub>6</sub>].

### 4-Hydroxybenzyl Alcohol (II)

Colorless prisms (from MeOH-H<sub>2</sub>O), mp 116-117°C. UV nm (log<sub>e</sub>): 225 (3.93), 277 (3.19), 283 (3.12). IR cm<sup>-1</sup>: 3360 (OH), 1595, 1515 (aromatic). <sup>1</sup>H-NMR (δ in methanol-d<sub>4</sub>): 4.46 (2H, s, PhCH<sub>2</sub>O-), 6.86, 7.16 (each 2H, d, J=9 Hz, arom.-H). This compound was identified as 4-hydroxybenzyl alcohol by direct comparison with an authentic sample (mixed mp and IR) and was bluish-green with FeCl<sub>3</sub>-K<sub>3</sub>[Fe(CN)<sub>6</sub>].

### Benzyl alcohol (III)

Colorless liquid from MeOH-H<sub>2</sub>O, bp<sub>760</sub> 204.7°C, mol wt 108.13, This compound was identified as 4-hydroxybenzaldehyde by direct comparison with an authentic sample (IR and mixed bp). Mass spectrum m/e : 108 (M<sup>+</sup>), IR ν<sub>max</sub><sup>CCl<sub>4</sub></sup> cm<sup>-1</sup>: 3620, 3500-3250, 1020 (OH), 3090, 3070, 3040, 2000-1600, 1500, 700 (mono-substituted benzene), <sup>1</sup>H-NMR (δ 15% solution in CDCl<sub>3</sub>-d<sub>6</sub>): 7.95 (1H, OH), 5.35 (2H, singlet, -CH<sub>2</sub>-), 2.66 (5H, singlet, aromatic proton -CH<sub>2</sub>-).

**Bis(4-hydroxyphenyl)methane (IV)**

Colorless liquid from MeOH-H<sub>2</sub>O, mp 155°C, mol wt 200.24, and gave a bluish-green color with FeCl<sub>3</sub>-K<sub>3</sub>[Fe(CN)<sub>6</sub>]. This compound was identified as bis(4-hydroxyphenyl)methane by direct comparison with an authentic sample on TLC (R<sub>f</sub>, 0.78 in CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O=65:35:10, 0.26 in CHCl<sub>3</sub>:MeOH=9:1, 0.88 in BuOH:HAc:H<sub>2</sub>O=4:1:1) and mixed mp. IR ν<sub>max</sub><sup>Cl<sub>4</sub></sup> cm<sup>-1</sup>: 3620, 3500-3250, 1020 (OH), 3090, 3070, 3040, 2000-1600, 1500, 700 (mono-substituted benzene).

**4-(4'-Hydroxybenzyloxy) benzyl methyl ether (V)**

Colorless needles (from ether-hexane), mp 120-123°C, and was bluish-green in FeCl<sub>3</sub>-K<sub>3</sub>[Fe(CN)<sub>6</sub>] in EtOH. UV nm (log $\epsilon$ ): 231 (4.17), 277 (3.35), 280 (sh 3.32). IR cm<sup>-1</sup>: 3300 (OH), 1610, 1595, 1580, 1515 (aromatic). <sup>1</sup>H-NMR ( $\delta$  in acetone-d<sub>6</sub>): 3.30 (3H, s, OCH<sub>3</sub>), 4.37 (2H, s, PhCH<sub>2</sub>OCH<sub>3</sub>), 5.00 (2H, s, PhCH<sub>2</sub>OPh), 6.81, 6.92, 7.25, 7.31 (each 2H, d, J=9.5Hz, 8 $\times$ arom.-H), 8.38 (1H, s, OH, D<sub>2</sub>O-exchangeable). MS m/z (%): 244 (M<sup>+</sup>, 2.2), 137 (OC<sub>6</sub>H<sub>4</sub>CH<sub>2</sub>OCH<sub>3</sub>, 17.5), 107 (HO=C<sub>6</sub>H<sub>4</sub>=CH<sub>2</sub>, 100). High resolution MS, calculated for C<sub>15</sub>H<sub>16</sub>O<sub>3</sub> (M<sup>+</sup>) m/z: 244.1099; actual: 244.1093.

**4-Hydroxy-3-methoxy benzyl alcohol (vanillyl alcohol) (VI)**

Colorless liquid from MeOH-H<sub>2</sub>O, mp 116-117°C, mol wt 155. This compound was identified as 4-hydroxy-3-methoxy benzyl alcohol by direct comparison with an authentic sample on TLC (R<sub>f</sub>, 0.84 in CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O=65:35:10, 0.57 in CHCl<sub>3</sub>:MeOH=9:1, 0.84 in BuOH:HAc:H<sub>2</sub>O=4:1:1) and mixed mp, and was bluish-green with FeCl<sub>3</sub>-K<sub>3</sub>[Fe(CN)<sub>6</sub>], MS m/z (%): 244 (M<sup>+</sup>, 2.2), 137 (OC<sub>6</sub>H<sub>4</sub>CH<sub>2</sub>OCH<sub>3</sub>, 17.5), 107 (HO=C<sub>6</sub>H<sub>4</sub>=CH<sub>2</sub>, 100). High resolution MS, calculated for C<sub>15</sub>H<sub>16</sub>O<sub>3</sub> (M<sup>+</sup>) m/z: 244.1099; actual: 244.1093. GS/MS: RT 9.16.

**4-Hydroxy-3-methoxybenzaldehyde (vanillin) (VII)**

Colorless liquid from MeOH-H<sub>2</sub>O, mp 83-84.5°C, mol wt 152.14, and was bluish-green with FeCl<sub>3</sub>-K<sub>3</sub>[Fe(CN)<sub>6</sub>]. This compound was identified as 4-hydroxy-3-methoxybenzaldehyde by direct comparison with an authentic sample on TLC (R<sub>f</sub>, 0.82 in CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O=65:35:10, 0.59 in CHCl<sub>3</sub>:MeOH=9:1, 0.84 in BuOH:HAc:H<sub>2</sub>O=4:1:1), and mixed mp. IR cm<sup>-1</sup>: 3300 (OH), 1610, 1595, 1580, 1515 (aromatic).

**4-Hydroxy-3-methoxybenzoic acid (vanillic acid) (VIII)**

White odorless needle from MeOH-H<sub>2</sub>O, mp 210°C, mol wt 168.14, and with a positive Gibb's reaction. This compound was identified as 4-hydroxy-3-methoxybenzoic acid by direct comparison with an authentic sample on TLC (R<sub>f</sub>, 0.76 in CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O=65:35:10, 0.28 in CHCl<sub>3</sub>:MeOH=9:1, 0.88 in BuOH:HAc:H<sub>2</sub>O=4:1:1) and mixed mp.

IR cm<sup>-1</sup>: 3450 (OH), 2960, 2620, 1680, 1000. <sup>1</sup>H-NMR ( $\delta$  in acetone-d<sub>6</sub>): 3.30 (3H, s, OCH<sub>3</sub>), 6.81, 6.92, 7.25, 7.31 (each 2H, d, J=9.5 Hz, 8 $\times$ arom.-H), 8.38 (1H, s, OH, D<sub>2</sub>O-exchangeable). MS m/z (%): 244 (M<sup>+</sup>, 2.2), 137 (OC<sub>6</sub>H<sub>4</sub>CH<sub>2</sub>OCH<sub>3</sub>, 17.5), 107 (HO=C<sub>6</sub>H<sub>4</sub>=CH<sub>2</sub>, 100). High resolution MS, calculated for C<sub>15</sub>H<sub>16</sub>O<sub>3</sub> (M<sup>+</sup>) m/z: 244.1099; actual: 244.1093.

**Carrageenan-induced Paw edema**

The assay was performed as previously described by Winter (1962). GE fractions and phenolic compounds dissolved in 0.5% CMC-Na were administered orally 1 h prior to carrageenan injection. The control group received 0.5% CMC-Na only. 0.1 mL 1% carrageenan was injected intradermally into the subplantar of the right hind paw. The paw volume was measured initially and then 1, 2, 3, and 4 h after the carrageenan injection by plethysmometer (model 7140, UGO Basile, Italy).

**Arachidonic acid (AA)-induced edema**

AA-induced ear edema was performed by the method of Young *et al.* (1984). AA dissolved in acetone (2 mg/5  $\mu$ L) was applied to the inner and outer surfaces of the right ear. GE fractions and phenolic compounds dissolved in DMSO (5  $\mu$ L/ear) were painted on the right ear 5 min before the AA application. The left ear received the vehicle (acetone or DMSO). The ear thickness was measured 40 min after induction of inflammation using a micrometer (Mitutoyo, Mfg., Japan). The edema was measured as an increase in the ear thickness due to AA application.

**HAc-induced writhing response**

HAc-induced writhing test was carried out as described by Bentley *et al.* (1983). GE fractions and phenolic compounds dissolved in 0.5% CMC-Na were administered orally 1 h prior to HAc injection. HAc (0.7%, 0.1 mL/10 g) was administered intraperitoneally and 10 min after the injection the number of writhings were counted over 10 min.

**Cyclooxygenase activity**

Cyclooxygenase activity was measured following the procedure reported in the literature (Andreani *et al.*, 2004) using a cyclooxygenase activity kit. Briefly, COX-I (60 U/mL) or COX-II (30 U/mL), hematin (0.12  $\mu$ M) and Tris-phenol buffer (100 mM Tris, 0.5 mM phenol, pH 7.3) were added to the microtiter plate wells to obtain a reaction mixture. Then, phenolic compounds dissolved in DMSO were added at the concentration of 0.1, 0.3 and 1.0 mM, and incubated at room temperature for 1 h. The enzymatic reaction was started by adding cold COX chemiluminescent substrate and 100  $\mu$ M cold AA, and the chemiluminescent emission was measured immediately for 5 sec using a luminometer (FL600 Microplate Reader, Bio-Tek).

The cyclooxygenase activity was evaluated by integrated chemiluminescent signal for the read time in relative light units (RLU).

### Measurement of intracellular ROS generation

#### Cell culture

RBL-2H3 cells were cultured in Dulbecco's modified Eagle minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) and antibiotics (100 IU/mL penicillin G, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B) at 37°C with 5% CO<sub>2</sub>.

#### Cell viability

Cells ( $5 \times 10^5$ ) were diluted and cultured in 96 well plates, and treated with GE fractions and phenolic compounds for 24 h at 37°C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution was added to the medium at a final concentration of 0.5 mg/mL and further incubated for 4 h at 37°C. Culture medium was removed, and was replaced with the same volume of DMSO (Ferrari *et al.*, 1990). The absorbance of each well was measured at 570 nm using an ELISA reader (FL600 Microplate Reader, Bio-Tek).

### Measurement of ROS

Intracellular ROS were detected by employing the fluorescent probe dichlorofluorescein (DCF). The cells were incubated with 20 µM DCF diacetate in Krebs buffer for 1 h at 37°C. The cells were then washed and suspended in

Krebs buffer at a density of  $10^6$  cells/mL. GE fractions and phenolic compounds were added 10 min prior to induction, and intracellular ROS generation was induced by silica (2 mg/mL) for 30 min at 37°C. DCF fluorescence was measured using a fluorospectrophotometer (FL600 Microplate Reader, Bio-Tek) at the excitation 485 nm/emission 535 nm (Shen *et al.*, 1996).

### Measurement of DPPH radical scavenging

The 1,1-diphenyl-2-picrylhydroazyl (DPPH) assay is based on the scavenging activity of stable DPPH free radicals (Chen *et al.*, 1999). The reaction mixtures phenolic compounds at the concentration of 10, 30, and 100 µM dissolved in DMSO and 100 µM DPPH in an ethanol solution were incubated in the dark at room temperature for 30 min, and the absorbance values were measured at 517 nm using an ELISA reader.

### Data analysis

All the values were represented as mean±SEM. Significant differences between two values were calculated by a two-tailed Student's t-test, with p values < 0.05 considered statistically significant.

## RESULTS AND DISCUSSION

### Activity-guided separation of MeOH extract and fractions of GE based on anti-inflammatory activity

MeOH extracts of GE significantly inhibited carrageenan-

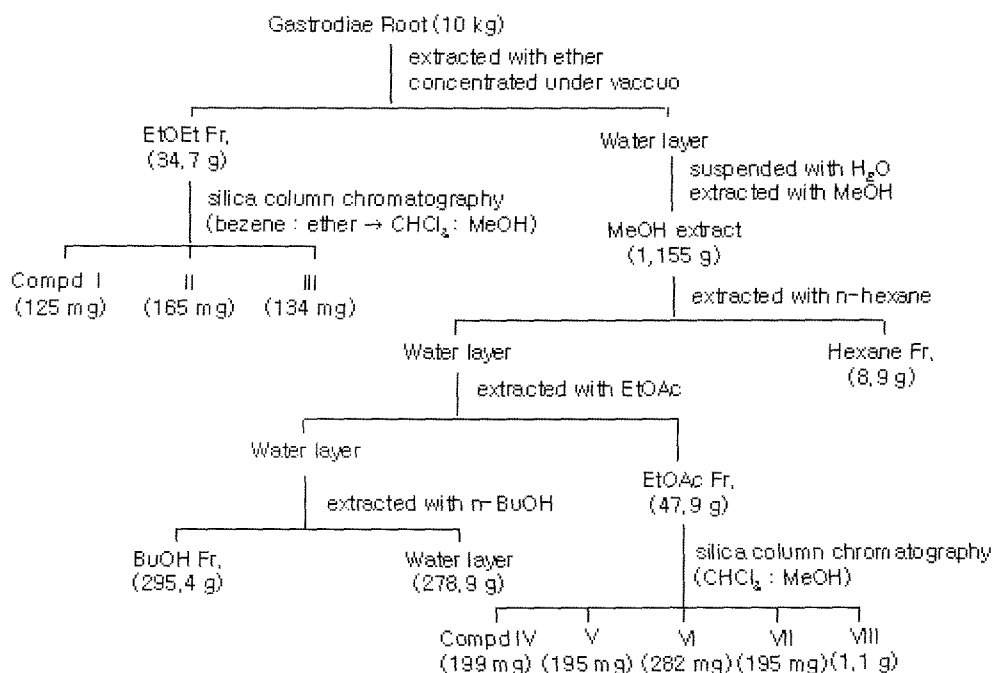
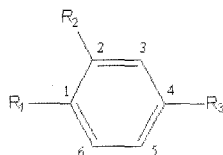


Fig. 1. Isolation of phenolic compounds from *Gastrodia elata*.

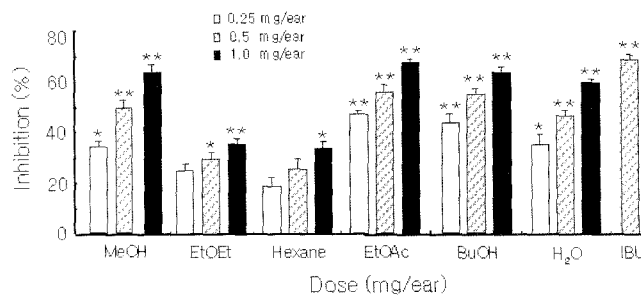


	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Compound I	-OH	-H	-CHO
Compound II	-OH	-H	-CH <sub>2</sub> OH
Compound III	-H	-H	-CH <sub>2</sub> OH
Compound IV	-OH	-H	-CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub> OH
Compound V	-OH	-H	-CH <sub>2</sub> OC <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> OCH <sub>3</sub>
Compound VI	-OH	-OCH <sub>3</sub>	-CH <sub>2</sub> OH
Compound VII	-OH	-OCH <sub>3</sub>	-CHO
Compound VIII	-OH	-OCH <sub>3</sub>	-COOH

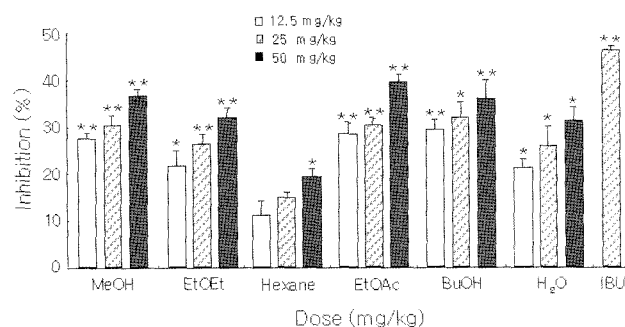
**Fig. 2.** Eight phenolic compounds isolated from *Gastrodia elata*. Compound I: 4-hydroxy benzaldehyde; Compound II: 4-hydroxybenzyl alcohol; Compound III: benzyl alcohol; Compound IV: bis-(4-hydroxyphenyl)methane; Compound V: 4-(4'-hydroxybenzyloxy) benzyl methyl ether; Compound VI: 4-hydroxy-3-methoxybenzyl alcohol; Compound VII: 4-hydroxy-3-methoxybenzaldehyde (vanillin); Compound VIII: 4-hydroxy-3-methoxy benzoic acid (vanillic acid).

induced paw edema at 25 mg/kg (Table I), HAC-induced writhing response at 12.5 mg/kg orally (Fig. 4), and AA-induced ear edema at 0.25 mg/ear cutaneously (Fig. 3). The EtOAc fraction at 12.5 mg/kg orally, and the BuOH, H<sub>2</sub>O and EtOEt fractions at 25 mg/kg orally significantly inhibited carrageenan-induced paw edema. In AA-induced ear edema, EtOAc, BuOH and H<sub>2</sub>O fractions at 0.25 mg/ear cutaneously significantly inhibited the edema. The anti-inflammatory activity of the EtOEt and H<sub>2</sub>O fractions at 0.25 mg/ear were less potent than that of ibuprofen at the same dose ( $P < 0.05$ ). Among the solvent fractions, the EtOAc fraction seems to be the most potent anti-inflammatory in the animal models of inflammation (Table I, Figs. 3 and 4).

The EtOEt and EtOAc fractions were chromatographed on a silica gel column with a CHCl<sub>3</sub>-MeOH system (Junko *et al.*, 2002; Taguchi *et al.* 1981; Lin *et al.* 1996) in order to isolate the biologically active agents. Eight phenolic compounds were isolated and identified as 4-hydroxy benzyl alcohol (I), 4-hydroxy benzaldehyde, benzyl alcohol (II), bis (4-hydroxy phenyl) methane (III), 4-(4'-hydroxybenzyloxy) hydroxybenzyl alcohol (IV), 4-(4'-hydroxybenzyloxy) benzyl methyl ether (V), 4-hydroxy-3-methoxybenzyl alcohol (VI), 4-hydroxy-3-methoxy-benzaldehyde (VII), and 4-hydroxy-3-methoxy benzoic acid (VIII) (Fig. 2).



**Fig. 3.** Inhibitory activities of fractions from *Gastrodia elata* MeOH extract on arachidonic acid-induced ear edema in mice. Ear edema (%) =  $(a-b)/b \times 100$ ; a: thickness of the right ear, b: thickness of the left ear. Inhibition (%) of ear edema =  $(a'-b')/a' \times 100$ ; a': ear edema of the control group, b': ear edema of the test group. Data represented as mean  $\pm$  SEM (n = 6). Significantly different from control (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ) as determined by the Student's t-test. MeOH: methanolic extract from *Gastrodia elata*; EtOEt: ether fraction of the methanolic extract; Hexane: n-hexane fraction of the methanolic extract; EtOAc: ethylacetate fraction of the methanolic extract; BuOH: n-butanol fraction of the methanolic extract; H<sub>2</sub>O: water fraction of the methanolic extract; IBU: ibuprofen.



**Fig. 4.** Analgesic activities of fractions from *Gastrodia elata* methanolic extract on HAC-induced writhing response in mice. Inhibition (%) =  $(a-b)/a \times 100$ ; a: number of writhings of the control group, b: number of writhings of the test group. Data represented as mean  $\pm$  SEM (n = 6). Significantly different from control (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ) as determined by the Student's t-test. All of fractions are same as Fig. 3.

### Anti-inflammatory and analgesic action of phenolic compounds

The phenolic compounds isolated from the EtOEt and EtOAc fractions were anti-inflammatory in a dose-dependent manner. In the carrageenan-induced paw edema, compound VII and VIII at 25 mg/kg orally and the other compounds at 50 mg/kg were significant anti-inflammatory for 4 h (Table II). Carrageenan-induced development of paw edema in rats is commonly correlated with the early exudative stage of inflammation, one of the important processes of inflammatory pathology (Ozaki, 1990). These results suggest that these compounds inhibit the early phase release of histamine, serotonin and kinins.

The later phases in the inflammatory response are suspected to be due to AA metabolites producing edema

**Table I.** Effects of fractions from *Gastrodia elata* of MeOH extract on carrageenan-induced paw edema in rats

Group	Dose (mg/kg p.o.)	Increase (%) of Paw Edema			
		1 h	2 h	3 h	4 h
Control	-	29.39±2.00	44.31±0.80	62.43±1.31	38.11±0.72
MeOH extract	12.5	15.64±1.43*	40.33±3.22	55.64±3.12	29.19±1.89*
	25	9.49±1.86**	31.12±1.34**	43.07±1.94**	25.95±3.86
	50	7.10±1.23**	28.42±1.63**	37.47±1.67**	23.20±1.35**
EtOEt extract	12.5	26.06±0.85	40.19±4.28	50.33±3.46	34.56±0.92
	25	13.78±0.53*	26.84±1.23**	46.07±3.06*	30.92±1.60*
	50	10.42±0.04*	17.12±0.73**	42.74±2.36**	27.05±0.38**
Hexane fraction	12.5	28.84±0.34	40.00±0.94	62.32±2.22	37.32±0.17
	25	22.43±1.12	36.73±4.01	51.90±1.39**	29.89±3.21*
	50	20.13±1.60*	31.94±2.78	45.75±1.91**	24.81±1.97**
EtOAc fraction	12.5	21.51±1.09	30.10±2.36*	45.03±0.40**	33.04±0.21*
	25	11.25±0.85*	27.77±1.70**	39.17±2.97*	29.32±1.87
	50	9.13±0.68**	16.94±1.41**	30.50±1.70**	24.42±1.91*
BuOH fraction	12.5	23.21±1.02	36.46±1.57*	48.83±3.50	33.39±3.33
	25	17.15±1.62*	32.76±2.58	44.78±3.43*	28.68±1.75*
	50	14.85±0.60*	26.26±2.40**	38.07±2.96**	22.37±0.56**
H <sub>2</sub> O fraction	12.5	22.25±1.90	40.90±3.80	55.77±2.40	35.74±1.59
	25	17.63±1.27*	29.03±2.21*	45.07±2.58*	31.24±1.50*
	50	13.28±0.56*	20.38±1.14**	38.82±2.50**	26.11±2.30*
Ibuprofen	25	10.79±0.70**	20.01±1.94**	30.05±2.33**	20.53±2.03*

Increase (%) of paw edema =  $(a-b)/b \times 100$ , where a is the increased hind-paw volume and b is the initial hind-paw volume. Data were represented as mean ± SEM. (n =6). Significant differences from control were determined by the Student's t-test, with \*represents  $p < 0.05$  and \*\*represents  $p < 0.01$ .

dependent on the mobilization of neutrophils (Just et al., 1998). In the AA-induced ear edema, compounds II, VII and VIII at 0.25 mg/ear cutaneously significantly inhibited edema induction compared to control ( $p < 0.01$ ) (Fig. 5). AA-induced inflammation, a common model of skin inflammation, involves the rapid formation of leukotrienes followed by vasoactive amine release and subsequent edema and neutrophil infiltration (Carlson et al., 1985; Crummey et al., 1987), and so these results indicate that these compounds can inhibit the formation of AA metabolites.

In the HAc-induced writhing response, compounds II, III, VI, VII, and VIII at 12.5 mg/kg orally significantly inhibited writhing compared to control ( $p < 0.05$ ) (Fig. 6). Acetic acid causes algia by liberating endogenous substances including serotonin, histamine, prostaglandin, bradykinin and substance P, which excite pain nerve endings (Collier et al., 1968; Raj, P.P., 1996). Therefore, our results indicate that the phenolic compounds might inhibit the synthesis and/or release of these endogenous substances.

The anti-inflammatory potency of the phenolic compounds at the lowest doses that had a significant effect

when compared to control was in the following order: Compound VII > Compound VIII > Compound II > Compound III > Compound I > Compound IV (Tables II, Figs. 5 and 6). These results indicate that the C-3 methoxy radical in phenolic compounds plays an important role in the anti-inflammatory action.

#### Inhibitory activity of phenolic compounds on COX-I and II activities

0.1 mM compound III significantly inhibited COX-I activity, though less potently than ibuprofen (Fig. 7A). 0.1 mM compound II significantly inhibited COX-II activity (Fig. 7B). COX is the key enzyme in AA metabolism, leading to the generation of prostaglandins (PGs) and thromboxanes (TXs) which mediate pain and edema associated with inflammation. These results indicate that compound II and III could inhibitor the generation of PGs and TXs in inflamed tissues.

#### Anti-oxidant activity of phenolic compounds

Compounds I, IV, and VII dose-dependently inhibited

**Table II.** Effects of phenolic compounds from *Gastrodia elata* on carrageenan-induced paw edema in rats

Group	Dose (mg/kg p.o.)	Increase (%) of Paw edema			
		1 h	2 h	3 h	4 h
Control	-	29.39±2.00	44.31±0.80	62.43±1.31	38.11±0.72
Compound I	12.5	24.69±0.79	40.86±2.09	61.79±1.38	37.94±0.55
	25	21.47±1.41	33.52±1.26**	49.65±1.42**	35.80±1.06
	50	20.11±1.57*	31.53±2.28*	42.82±2.56**	34.07±1.81
Compound II	12.5	21.56±0.45	34.96±1.78*	53.90±1.28*	37.64±0.57
	25	17.11±1.93*	30.13±1.78*	50.23±1.78*	34.46±0.72*
	50	11.19±0.98**	24.30±1.07**	39.30±0.15**	23.28±2.07*
Compound III	12.5	18.40±3.35	37.99±3.24	59.99±1.51	38.02±0.33
	25	15.71±0.93*	33.47±1.64*	46.94±1.72**	30.65±1.20*
	50	13.53±1.08**	31.27±1.10**	42.80±0.96**	28.56±1.14*
Compound IV	12.5	28.59±1.91	43.72±1.67	62.05±2.23	37.92±0.95
	25	24.11±2.44	35.32±1.00**	50.07±0.73**	35.87±0.88
	50	15.55±1.77*	30.53±1.57**	44.75±1.23**	31.84±0.89*
Compound VI	12.5	19.59±0.55*	36.40±1.43*	57.68±1.35	37.71±1.57
	25	15.16±1.23*	31.80±1.62*	48.92±2.38*	32.19±1.28*
	50	13.62±0.85*	22.21±1.03**	39.26±1.58**	26.47±0.83**
Compound VII	12.5	22.71±1.39	30.34±3.04*	40.59±2.42**	30.75±1.08*
	25	17.29±2.27*	24.90±2.19**	33.47±1.53**	25.98±0.71**
	50	14.04±0.70*	15.58±1.25**	30.63±1.28**	22.03±2.77*
Compound VIII	12.5	22.68±1.32	34.92±1.67*	49.47±2.54*	32.82±1.49
	25	16.30±0.25*	29.57±1.02**	39.71±1.07**	28.35±0.71**
	50	12.44±0.40*	27.49±1.40**	33.69±3.11**	21.26±2.02*
Ibuprofen	25	10.79±0.70**	20.01±1.94**	30.05±2.33**	20.53±2.03*

Increase (%) of paw edema =  $(a-b)/b \times 100$ , where a is the increased hind-paw volume and b is the initial hind-paw volume. Data were represented as mean  $\pm$  S.E.M. (n=6).

Significant differences from control were determined by the Student's t-test, with \* represents  $p < 0.05$  and \*\* represents  $p < 0.01$ .

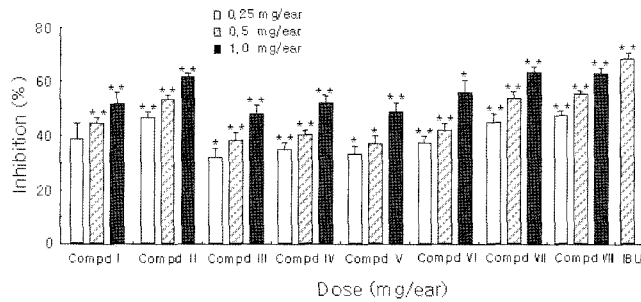
All compounds are same as Fig. 2.

silica-induced ROS generation (Fig. 8). Compounds IV, VII and I at 30 mM significantly inhibited silica-induced ROS generation by 43.4, 29.5 and 20.5%, respectively (Fig. 8). Compound IV was more potent than ascorbic acid at inhibiting ROS generation. In the DPPH free radical scavenging activity assay, compound VI at 30 mM significantly reduced free radical activity by 55.2% (Fig. 9). DPPH radical scavenging activity of compound VI was greater than that of ascorbic acid at 30 mM, but other phenolic compounds did not show any anti-oxidant activity. It has been reported that ROS such as superoxide anions, hydroxyl radicals and peroxynitrite participate in the process of inflammation in various tissues including the skin (Trenam *et al.*, 1992), and that hydroxyl radicals and peroxynitrite have strong oxidizing properties towards biomolecules, especially lipids of cell membranes (Halliwell and Chirico, 1993; Beckman and Crow, 1993). In addition

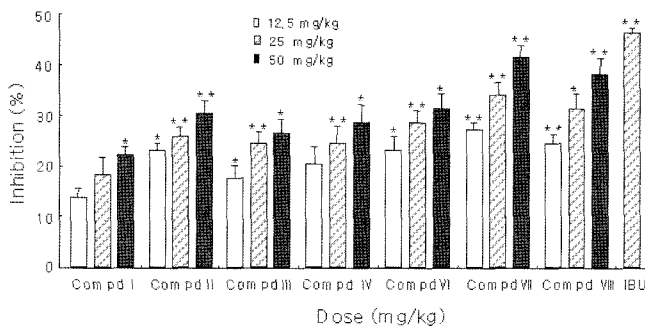
to being tissue damaging agents, ROS have another important role in inflammation. Free radicals activate AA metabolism (especially COX-2), pro-inflammatory cytokines (i.e. interleukins and tumor necrosis factor), and the apoptotic process. Therefore, phenolic compounds of GE that have scavenging activities toward these radicals and/or suppressive activities on lipid peroxidation may thus be expected to have therapeutic potential for several skin inflammatory diseases (Trenam *et al.*, 1992).

#### Structure-activity of phenolic compounds on anti-inflammatory action

Phenolic compounds isolated from the EtOEt and EtOAc fractions of GE have significant anti-inflammatory action in carrageenan-induced paw edema, AA-induced ear edema, and HAc-induced writhing response. Compound VII, 4-Hydroxy-3-methoxy benzaldehyde (vanillin), had the most



**Fig. 5.** Inhibitory activities of phenolic compounds from *Gastrodia elata* on arachidonic acid-induced ear edema in mice. Ear edema (%) =  $(a-b)/b \times 100$ ; a: thickness of the right ear, b: thickness of the left ear. Inhibition (%) of ear edema =  $(a'-b')/a' \times 100$ ; a': ear edema of the control group, b': ear edema of the test group. Data represented as mean  $\pm$  SEM (n = 6). Significantly different from control (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ) as determined by the Student's t-test. All compounds are same as Fig. 2.

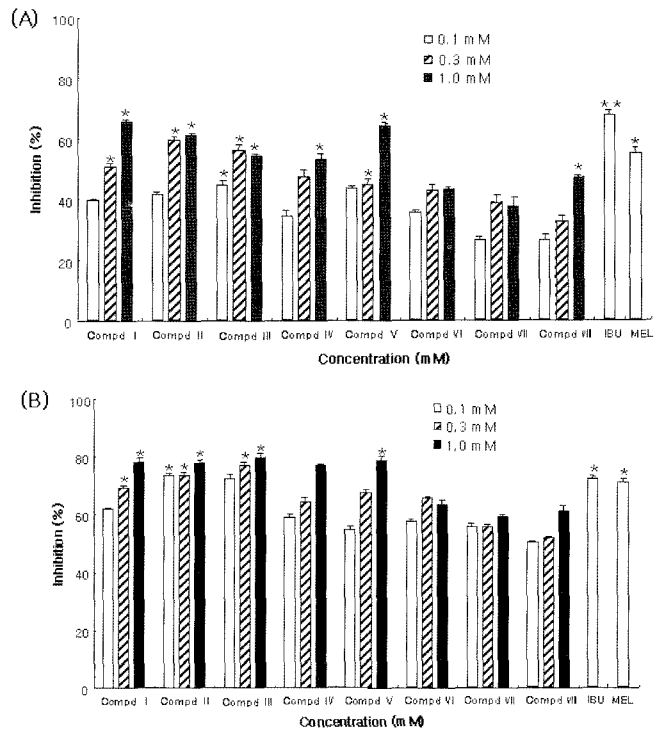


**Fig. 6.** Analgesic activities of phenolic compounds from *Gastrodia elata* on acetic acid-induced writhing response in mice. Inhibition (%) =  $(a-b)/a \times 100$ ; a: number of writhings of the control group; b: number of writhings of the test group. Data represented as mean  $\pm$  SEM (n = 6). Significantly different from control (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ) as determined by the Student's t-test. All compounds are same as Fig. 2.

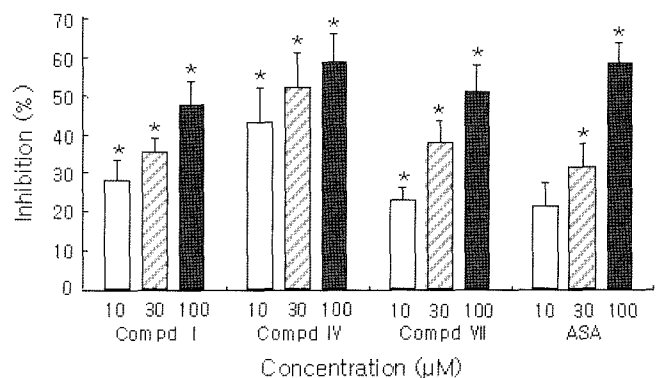
potent anti-inflammatory activity. The structure-activity relationship of phenolic compounds showed that C-4 hydroxy and C-3 methoxy radicals in benzyl alcohol or aldehyde have an important role in the mechanism of anti-inflammatory activity.

### Pharmacological mechanism of phenolic compounds of GE

The anti-inflammatory action of 4-hydroxy-3-methoxybenzaldehyde (compound VII), 4-hydroxy-3-methoxy benzoic acid (compound VIII) and 4-hydroxybenzaldehyde (compound I) was due to their anti-oxidant activity, whereas that of benzyl alcohol (compound III), 4-hydroxybenzyl alcohol (compound II) and 4-hydroxybenzaldehyde (compound I) was due to their inhibitory action of COX-I and II activity. Arachidonic acid is the precursor of a large family of compounds known as the eicosanoids, which includes cyclooxygenase-derived prostaglandins and lipoxygenase-derived leukotrienes. The eicosanoids possess a wide



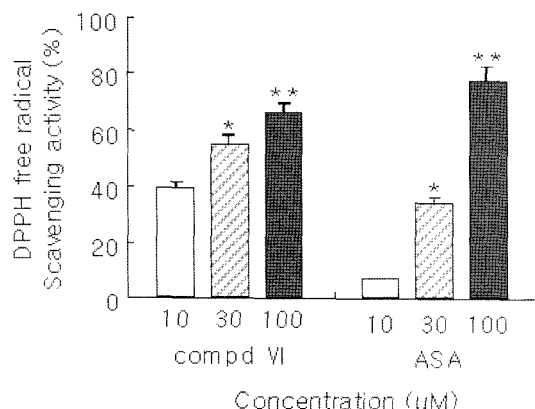
**Fig. 7.** Inhibitory activities of phenolic compounds from *Gastrodia elata* on cyclooxygenase (COX). (A) Inhibitory activities of phenolic compounds from *Gastrodia elata* on COX-I. (B) Inhibitory activities of phenolic compounds from *Gastrodia elata* on COX-II. Inhibition (%) =  $(a-b)/a \times 100$ ; a: cyclooxygenase units (Units/mL) of the control group, b: cyclooxygenase units (Units/mL) of the test group. Data represented as mean  $\pm$  SEM (n = 4). Significantly different from the control (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ) as determined by the Student's t-test. All compounds are same as Fig. 2.



**Fig. 8.** Anti-oxidant activity of phenolic compounds from *Gastrodia elata* on silica-induced intracellular ROS (reactive oxygen species) generation in RBL 2H3 cells. Data represented as mean  $\pm$  SEM (n = 4). Significantly different from control (\*:  $P < 0.05$ ) as determined by the Student's t-test. Compound I: 4-hydroxybenzaldehyde; Compound IV: bis-(4-hydroxyphenyl)methane; Compound VII: 4-hydroxy-3-methoxy benzoic acid (vanillin); ASA: ascorbic acid.

spectrum of biological activities, among which is their ability to mediate a number of the signs and symptoms





**Fig. 9.** Antioxidant activity of phenolic compounds of from *Gastrodia elata* in DPPH radical scavenging activity. DPPH radical scavenging activity (%) =  $(a-b)/a \times 100$ ; a: the absorbance of control at 517 nm, b: the absorbance of test group. Data represented as mean  $\pm$  SEM (n = 4). Significantly different from the control (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ) as determined by the Student's t-test. Compound VI: 4-hydroxy-3-methoxybenzyl alcohol; ASA: ascorbic acid.

associated with inflammatory reactions. In this experiment, some phenolic compounds had significant anti-inflammatory activity in AA-induced ear edema, which suggests that they were inhibiting the action or production of prostaglandins and leukotrienes.

### Biologically active agents of GE

The eight phenolic compounds that were isolated in this experiment seemed to be active agents, and compound VII from EtOAc fraction was the most active. Phytochemical studies of this plant have revealed the presence of several phenolic compounds including 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, gastrodin, parishin and 4,4'-dihydroxybenzyl sulfoxide (Zhou *et al.*, 1979; Taguchi *et al.*, 1981; Noda *et al.*, 1995; Lin *et al.*, 1996; Yun-Choi *et al.*, 1997, 1999). Among these, gastrodin, a phenolic glycoside, is a major constituent, accompanied by its aglycone, 4-hydroxybenzyl alcohol (Zhou *et al.*, 1979). Although gastrodin has been thought to be the major active component responsible for the clinical effects of GE, recent reports suggest that the pharmacological effects of GE cannot be explained by gastrodin alone (Junhua *et al.*, 1989). Consequently, it is likely that bioactive compounds other than gastrodin are contained in GE. Gastrodioside, a glycoside, has been isolated from the BuOH and EtOEt fractions of GE and its structure was elucidated as bis(4-hydroxybenzyl) ether mono- $\beta$ -D-glucopyranoside. Other compounds that have been isolated include 4-hydroxybenzyl alcohol, 4-hydroxybenzyl aldehyde, 4-hydroxybenzyl methyl ether, bis(4-hydroxybenzyl) methyl ether, bis(4-hydroxybenzyl) methyl ether, 4'-hydroxybenzyloxy-4-benzyl alcohol, 4( $\beta$ -D-glucopyranosyloxy) benzyl alcohol, tris(4- $\beta$ -D-glucopyranosyloxy) benzyl citrate (parishin), 1,2-bis(4- $\beta$ -D-

glucopyranosyloxy) benzyl citrate (parishin B), 1,3-bis(4- $\beta$ -D-glucopyranosyloxy) benzyl citrate (parishin C), 4,4'-hydroxybenzyl sulfoxide, 4,4'-hydroxybenzyl ether, bis(4-hydroxybenzyl)ether, 4'-hydroxybenzyl-4-hydroxy-3-(4"-hydroxybenzyl)benzyl ether (gastrol), 4,4'-dihydroxydiphenyl methane, 4,4',4"-trihydroxy-benzyl alcohol, bis [2,4(4'-hydroxybenzyl)]phenol, 4(2,5-di-hydroxybenzyl)phenol, 2,5-bis(4-hydroxy-benzyl)phenol, vanillyl alcohol,  $\alpha$ -acetylaminophenylpropyl-benzoylaminophenyl-propionate and 4-hydroxybenzyl b-sitosterol ether (Xiao *et al.*, 2002). Junko *et al.* (2002) isolated a new gastrol, 4'-hydroxybenzyl 4-hydroxy-3-(4"-hydroxybenzyl)benzyl ether, together with 10 known phenolic compounds from the EtOEt fraction of the MeOH extract of the rhizomes of GE, and the relaxant effects of these constituents on smooth muscle preparations isolated from guinea pig ileum were also studied in order to reveal their characteristic pharmacological activities. The MeOH extract of GE at 0.5 mg/mL reduced the contractions of electrically stimulated ileum, and also 4(4'-hydroxyphenyl)phenol, 4-4'-hydroxybenzyloxy(4"-benzyloxy)hydroxybenzyl alcohol, 2,4-bis(4-hydroxybenzyl)phenol, gastrol, bis(4,4'-dihydroxybenzyl) ether, 4-hydroxy-benzyl methyl ether and 4(4'-hydroxybenzyl) benzyl alcohol at 0.02 mg/mL significantly reduced the contraction of guinea pig ileum induced by acetylcholine and serotonin (Junko *et al.*, 2002). GE may have another anti-inflammatory compounds such as 4,4'-dihydroxybenzyl sulfoxide (Yun-Choi and Pyo, 1997), alpha-acetylphenylpropyl-alpha-benzyl-alpha-benzoylaminophenyl-propionate (Xiao *et al.*, 2002) and phenolic glycosides.

### Abbreviations

GE: *Gastrodia elata*  
 MeOH: methanol  
 EtOEt: ether  
 EtOAc: ethyl acetate  
 BuOH: butanol  
 HAc: acetic acid  
 DPPH: 1,1-diphenyl- 2-picrylhydroazyl  
 ROS: reactive oxygen species  
 COX: cyclooxygenase  
 CMC-Na: carboxymethyl cellulose, sodium salt  
 DMSO: dimethylsulfoxide  
 SEM: standard error of mean  
 IBU: ibuprofen  
 ASA: ascorbic acid

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