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Phenolic compounds from *Gastrodia* rhizome and relaxant effects of related compounds on isolated smooth muscle preparation

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Abstract

Gastrol (**1**), together with 10 known phenolic compounds, has been isolated from the MeOH extract of the rhizomes of *Gastrodia elata* Blume (Orchidaceae), and their structures were elucidated by detailed spectral analyses including by 2D NMR spectroscopic analyses. 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.

Keywords: *Gastrodia* rhizome; *Gastrodia elata*; Orchidaceae; Gastrol; Phenolic compound; Smooth muscle relaxant effect; Postsynaptic nerve

1. Introduction

Gastrodia rhizome, the steamed and dried rhizomes of *Gastrodia elata* Blume (Orchidaceae) (Chinese name: Tienma), are very important Chinese herbal medicines used for the medical treatment of headaches, migraine, dizziness, epilepsy, rheumatism, neuralgia, paralysis and other neuralgic and nervous disorders (Tang and Eisenbrand, 1992). So far, we have investigated vasorelaxant effects of the *Uncaria* hook for which its clinical use is similar to that of the *Gastrodia* rhizome. As a result, the Ca^{2+} channel blocking and α-adrenoceptor-blocking activities of *Uncaria* alkaloids were found (Yano et al., 1991; Horie et al., 1992; Matsumiya et al., 1999). Therefore, we are also very interested in pharmacologically active constituents from *Gastrodia elata*.

Phytochemical studies of this plant have revealed the presence of several phenolic compounds such as 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 and Pyo, 1997; Yun-Choi et al., 1999). Among them, a phenolic glucoside, gastrodin, 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 *Gastrodia* rhizome, recent reports suggest that the pharmacological effect cannot be explained by gastrodin alone (Junhua and Guilian, 1989). Consequently, it is likely that bioactive compounds other than gastrodin are contained in the *Gastrodia* rhizome.

In this paper we describe the isolation and structural elucidation of several phenolic compounds including a new compound, named gastrol (**1**). In addition, the effect of these constituents on contraction provoked by neuronal spasmogens on isolated guinea-pig ileum was investigated.

2. Results and discussion

The MeOH extract from *Gastrodia* rhizome was separated into *n*-hexane, EtOAc, *n*-butanol and H_2O-soluble fractions. After evaluating the activity of the EtOAc solubles, this was fractionated by a series of column chromatographic and prep. HPLC separations to yield 11 phenolic compounds including a new compound (**1**) as one of the major active components. The remaining known compounds (**2–11**) were characterized as **2–6** (Taguchi et al., 1981), **7** (Noda et al., 1995), **8** (Yun-Choi and Pyo, 1997), **9**, **10** (Taguchi et al., 1981) and **11** (Noda et al., 1995), by comparison of their

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spectral data with those in the literature (Fig. 1). Gastrodin was not found in the EtOAc-soluble fraction.

The UV (λ_{max} 263 nm) and IR (ν_{max} 3448, 1100, 1029 cm^{-1}) absorptions of **1** strongly indicated a phenolic structure. The molecular formula of **1** was determined as $C_{21}H_{20}O_4$ from HR–FABMS (*m/z* 375.0964 $[M+K]^+$ and DEPT NMR spectra analyses, indicating 12 equivalents of unsaturation. The ^{13}C NMR spectrum of **1** showed 17 carbon signals including three signals [δ 34.8 (C-7″), 71.1 (C-7′), 71.3 (C-7)] due to methylene carbons, and four intense signals (δ 115.1, 115.2, 129.7, 129.9), suggesting the presence of two symmetric benzene rings. Three signals [δ 153.9 (C-4), 154.5 (C-4″), 156.1 (C-4′)] implied three phenolic carbon atoms in the molecule. The ^1H NMR spectrum of **1** revealed signals for 17 unique hydrogens. Three 2H singlets [δ 3.81 (H-7″), 4.28 (H-7), 4.29 (H-7′)] are considered as signals due to benzylic hydrogens. The cross-peaks between the signal at δ 6.67 (2H, *d*, $J=8.0$ Hz, H-3″, H-5″) and the signal at δ 6.99 (2H, *d*, $J=8.0$ Hz, H-2″, H-6″), and also between the signal at δ 6.69 (2H, *d*, $J=8.3$ Hz, H-3′, H-5′) and the signal at δ 7.02 (2H, *d*, $J=8.3$ Hz, H-2′, H-6′) in ^1H–^1H COSY of **1** indicated two sets of 4-benzylphenol unit, coupled with the consideration of DEPT and HMQC NMR spectral analyses. An additional 1, 2, 4-trisubstituted benzene ring was deduced from two cross-peaks between the signal at δ 6.68 (2H, *d*, $J=8.1$ Hz, H-5) and the signal at δ 6.95 (2H, *dd*, $J=8.1$, 2.0 Hz, H-6), and between the signal at δ 6.88 (2H, *d*, $J=2.0$ Hz, H-2) and the signal at δ 6.95 (2H, *dd*, $J=8.1$, 2.0 Hz, H-6). These phenolic partial structures were connected as shown in Fig. 2 by careful examination of the HMBC spectrum (8 Hz) and by comparison of the NMR spectroscopic data of **1** with those of structurally related compounds (**5**, **7** and **11**). Differential NOE experiments of **1** also supported this structure. For example, irradiation of the benzylic hydrogen at δ 3.81 (H-7″) caused the enhancement of the signals at δ 6.99 (H-2″) (13.3%) and δ 6.88 (H-2) (7.8%), as shown in Fig. 2. On the other hand, one of the benzylic hydrogen signals at δ 4.28 (H-7) displayed NOE enhancements with two signals at δ 6.88 (H-2) and at δ 6.95 (H-6). Thus, the above data lead us to propose the structure of the new compound **1** to be 4′-hydroxybenzyl 4-hydroxy-3-(4″-hydroxybenzyl)benzyl ether.

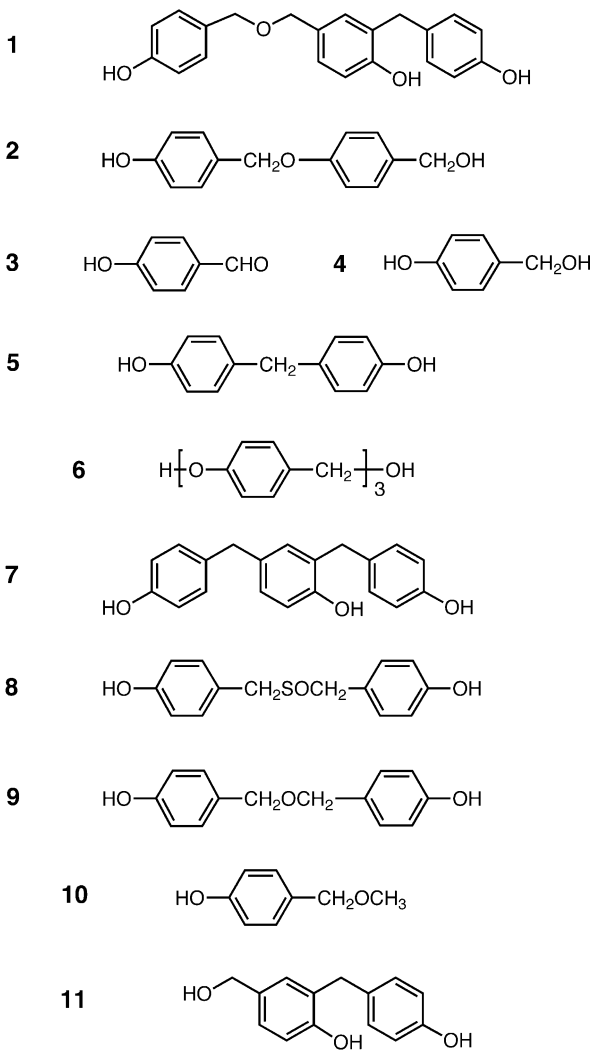

Fig. 1. Structures of **1** and related phenolic compounds isolated from *Gastrodia elata*.

We tried to elucidate pharmacological activities of *Gastrodia* rhizome using isolated guinea-pig ileum preparations (Watanabe et al., 1997; Yamamoto et al., 1999). In guinea-pig ileum, the MeOH extract (3 mg/ml) of the *Gastrodia* rhizome alone did not induce either contraction or relaxation under resting conditions (data not shown), but inhibited the electrically-induced twitch contraction in a concentration-dependent manner as shown in Fig. 3. Because the extract has a spasmolytic effect on the small intestine, we next investigated its effects on contractions induced by serotonin (10 μM) and acetylcholine (10 μM) in isolated guinea-pig ileum. Acetylcholine acts on muscarinic M_3 receptors on smooth muscle cells, leading to the induction of ileal contraction. On the other hand, serotonin acts on serotonin $5HT_3$ and $5HT_4$ receptors on the parasympathetic postsynaptic nerve to elicit acetylcholine release from the nerve ending (Holzer, 1998). The released acetylcholine induces ileal contraction. The MeOH extract (3 mg/ml) inhibited serotonin-induced contraction ($49.2\pm14.7\%$ inhibition, $n=4$, $P<0.01$ vs. control). On the other hand, the extract failed to inhibit acetylcholine-induced contraction ($-10.4\pm3.1\%$ inhibition, $n=4$, not significant in comparison with control). The EtOAc fraction (0.3 mg/ml) from the MeOH extract exhibited a marked inhibition ($92.6\pm4.4\%$ inhibition, $n=4$, $P<0.001$ vs. control) on serotonin-induced contraction. A weak inhibitory effect on acetylcholine-induced contraction was also observed ($32.1\pm5.3\%$ inhibition, $n=4$, not significant in comparison with control). In contrast,

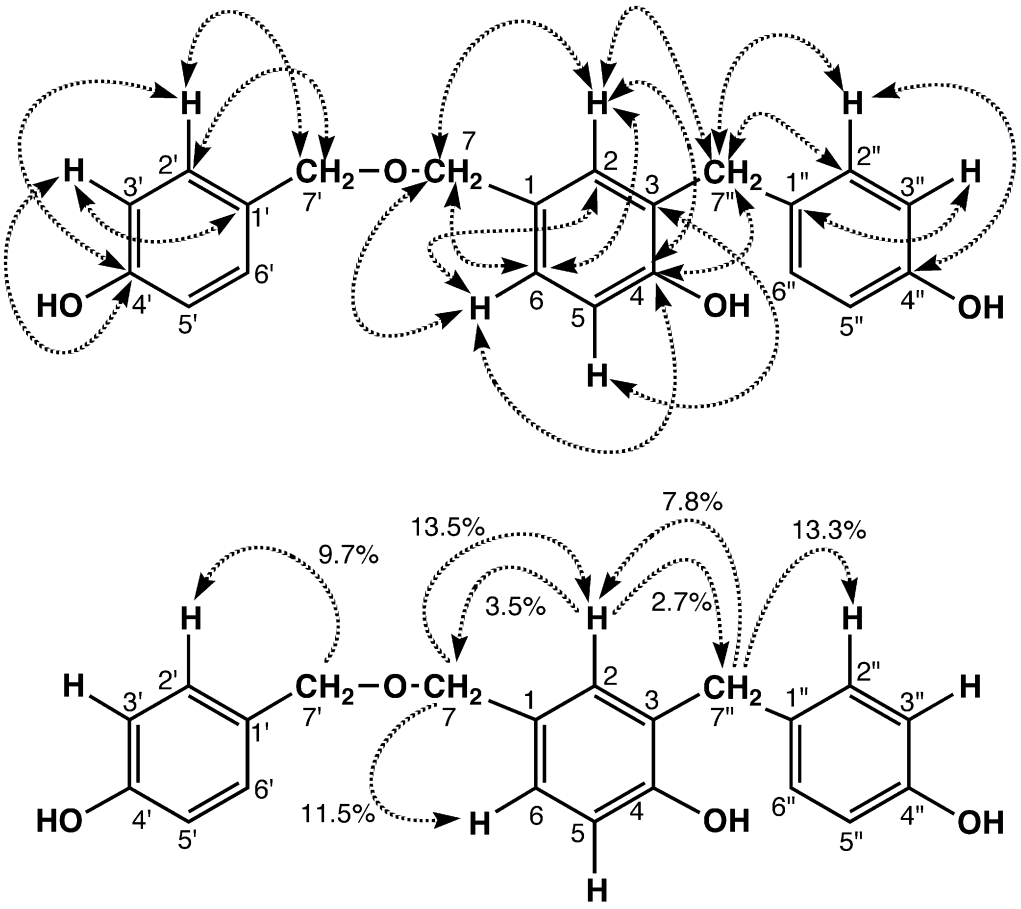

Fig. 2. HMBC correlations and NOEs data for compound **1**.

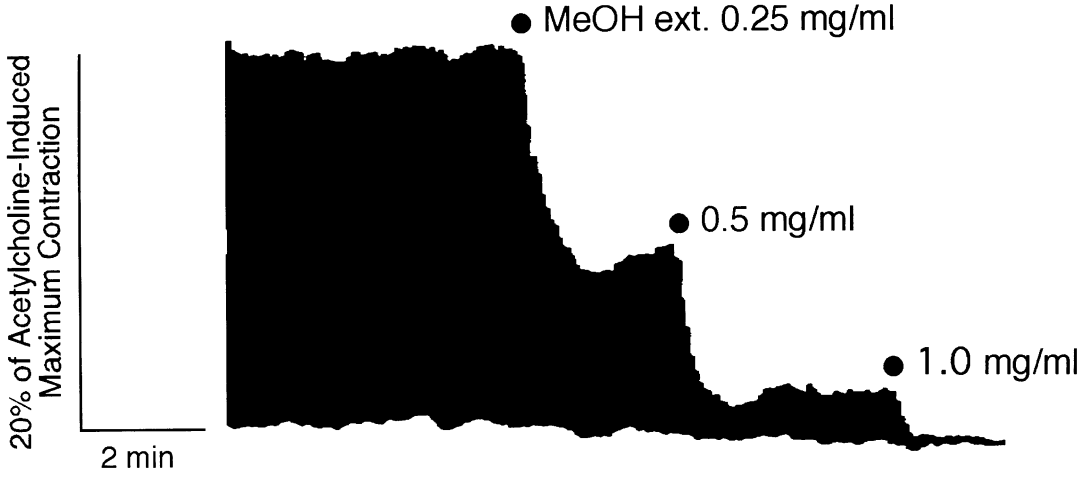

Fig. 3. Typical recording showing effect of *Gastrodia elata* extract on twitch contraction induced by electrical stimulation in isolated guinea-pig ileum. The addition of the extract was achieved in a cumulative manner. Ordinate scale represents 20% of maximum contraction induced by acetylcholine (10 μM). Abscissa represents the time. The trace shown is one of four experiments.

H_2O-soluble fraction extract (0.3 mg/ml) did not have any inhibitory effects on ileal contraction induced by acetylcholine ($-3.6\pm5.2\%$ inhibition, $n=4$, not significant in comparison with control) or by serotonin ($-5.7\pm7.0\%$ inhibition, $n=4$, not significant in comparison with control). Further, the *n*-hexane and *n*-butanol fraction (each 0.3 mg/ml) did not have any inhibitory effects on ileum contractions induced by either acetylcholine or by serotonin (data not shown). Consequently, active constituents that have an inhibitory effect on serotonin-induced contraction are concluded to be present in the EtOAc fraction.

The effects of several phenolic compounds isolated from the EtOAc fraction on acetylcholine- and serotonin-induced contractions were investigated at 0.02 mg/ml. The MeOH extract or the EtOAc fraction at 0.02 mg/ml did not show any effects on spasmogens-induced contractions (data not shown). As shown in Fig. 4, it was found that the activities of the constituents are more potent than those of the extract and the fraction. Compounds **5** and **6** (0.02 mg/ml) markedly inhibited serotonin-induced contraction, but only moderately inhibited acetylcholine-induced contraction in the ileum preparation (Fig. 4). Compound **7** and the new compound **1** inhibited acetylcholine- and serotonin-induced contractions to the same extent. Accordingly, the mechanisms of the spasmolytic effects of **1** and **7** may be different from those of either the EtOAc fraction or compounds **5** and **6**. The EtOAc fraction as well as **5** and **6** are more effective on serotonin-induced contraction than on acetylcholine-induced contraction. It

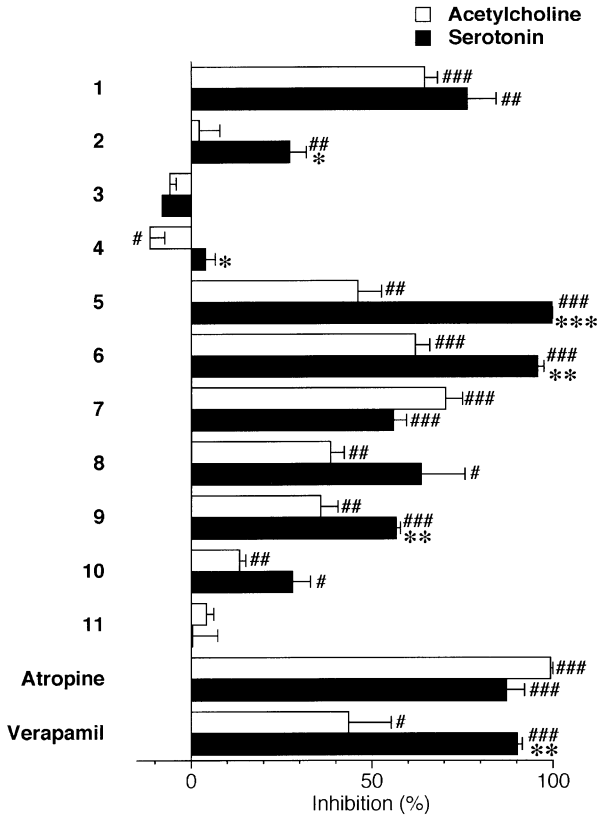

Fig. 4. Effects of constituents of *Gastrodia elata* on contractions induced by acetylcholine (white column) and serotonin (black column) in isolated guinea-pig ileum. The constituents (0.02 mg/ml), atropine (1 μM) or verapamil (3 μM) were added into the organ bath 7 min before addition of acetylcholine (10 μM) or serotonin (10 μM). Contraction (%) is expressed as a percentage against control contraction induced by acetylcholine or serotonin in the absence of samples. Each value shows the mean±S.E.M. of four to five animals. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, significantly different when compared with values immediately before the addition of each sample (paired *t*-test). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significantly different from the acetylcholine-stimulated group (Student's *t*-test).

was considered that the crude EtOAc fraction, and compounds **5** and **6** preferentially inhibit neurogenic contraction rather than inhibit contraction elicited by direct activation of smooth muscle cells. On the other hand, our preliminary experiments with isolated rat aorta suggested that the EtOAc fraction and compound **5** have a Ca^{2+} channel-blocking effect. Therefore we chose verapamil as a positive control.

In order to establish whether the EtOAc fraction and **5** have an inhibitory effect on the parasympathetic nerve rather than on the smooth muscle, the effects of these samples on neurogenic contractions in guinea-pig ileum were next examined. We employed serotonin, nicotine and capsaicin to induce neurogenic contraction of isolated ileal smooth muscle. These contractile agents act on the corresponding receptors on the parasympathetic postsynaptic nerves to elicit acetylcholine release from nerve endings. The MeOH extract inhibited neurogenic contraction induced by serotonin, nicotine and capsaicin in a concentration-dependent manner (Fig. 5A), but did not inhibit the contractions induced by acetylcholine and histamine, which act on the corresponding receptors on smooth muscle (MeOH extract at 2.4 mg/ml: acetylcholine contraction, 13.8±9.5% inhibition, $n=4$, not significant in comparison with control; histamine contraction, 15.5±9.1% inhibition, not significant in comparison with control). The EtOAc fraction and **5** markedly inhibited the neurogenic contraction at lower concentrations compared with the MeOH extract (Figs. 5B–D). On the other hand, their inhibitory effects on acetylcholine- and histamine-induced contractions were less potent than those on the neurogenic contraction. Verapamil inhibited some spasmogen-induced contractions with similar potencies. These effects result from the blockade of Ca^{2+} entry through Ca^{2+} channels in smooth muscle cells. The hallmarks of the inhibitory effect of verapamil are different from those of the MeOH extract, the EtOAc fraction and **5**. Taken together, it is suggested that the MeOH extract, the EtOAc fraction and **5** elicit a preferential inhibition on parasympathetic nerve rather than on smooth muscle.

In summary, the present results showed that several phenolic constituents from *Gastrodia* rhizome inhibited neurogenic contraction in isolated guinea-pig ileum, and lead us to speculate that the pharmacological effect of *Gastrodia* rhizome cannot be explained only by the presence of gastrodin as previously described (Junhua and Guilian, 1989). Several of the isolated constituents (e.g. **5** and **6**) are thought to have an inhibitory effect on neurotransmitter release induced by the stimulation of nicotine, serotonin and vanilloid receptors, whereas the new compound gastrol (**1**) appears to affect acetylcholine-induced contraction more directly. These pharmacological activities may be related to the clinical use of Gastrodia rhizome, such as treatment of headaches, migraine, dizziness and other neuralgic and nervous affections.

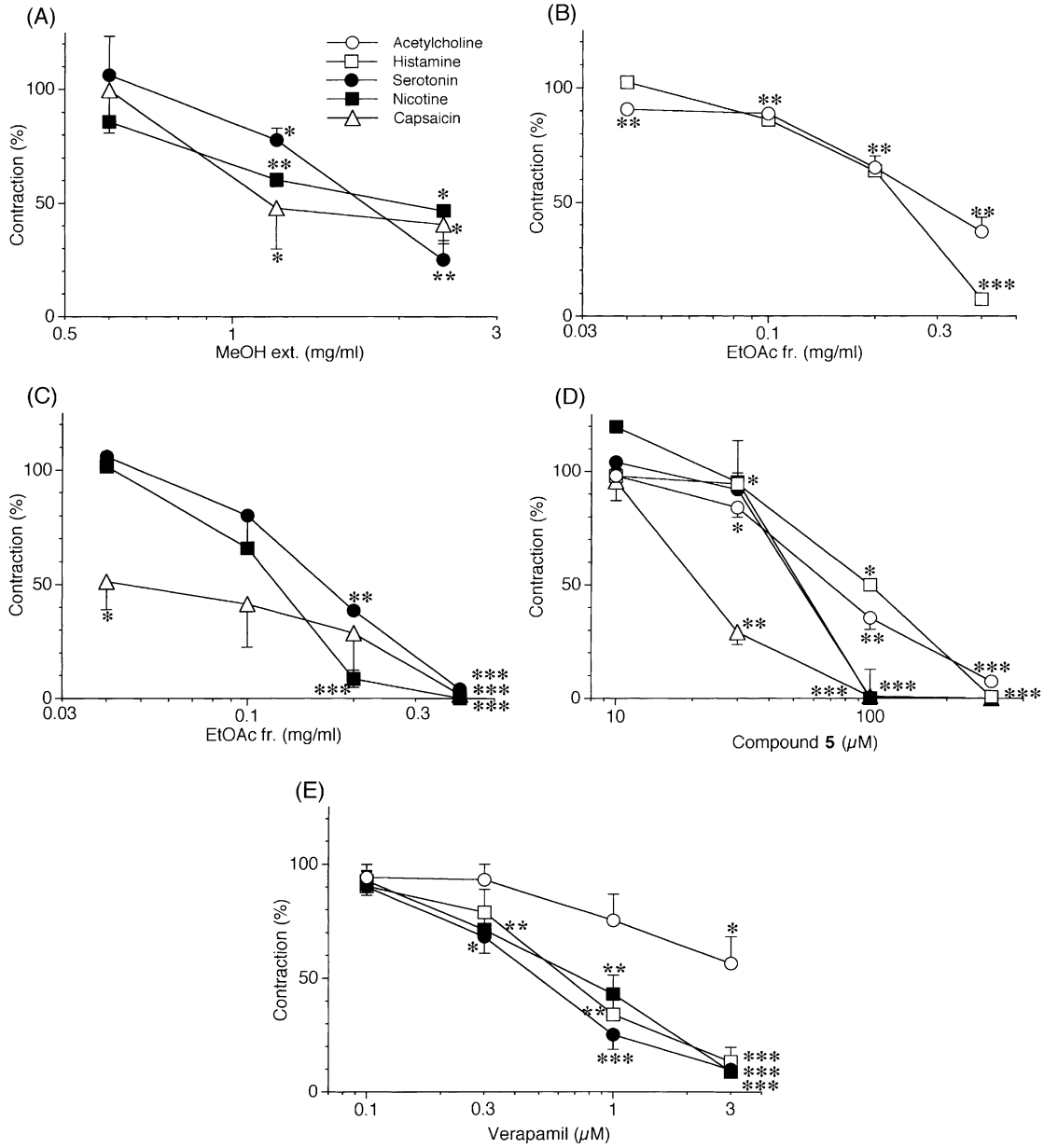

Fig. 5. Effects of MeOH extract (A), EtOAc fraction (B, C), compound **5** (D) and verapamil (E) on contraction induced by acetylcholine, serotonin, histamine, nicotine and capsaicin in isolated guinea-pig ileum. Each sample was added into the organ bath 7 min before the addition of acetylcholine (10 μM), histamine (3 μM), serotonin (10 μM), nicotine (30 μM) or capsaicin (3 μM). Contraction (%) is expressed as a percentage against control contraction induced by acetylcholine or serotonin in the absence of samples. Each value shows mean±S.E.M. of four to five animals. $*P<0.05$, $**P<0.01$, $***P<0.001$, significantly different when compared with values immediately before the addition of each sample (paired t-test). The abscissa is presented in logarithmic scale.

3. Experimental

3.1. General

IR spectra were recorded on a JASCO FT/IR-300E spectrometer by the diffuse reflection measurement method. UV spectra were taken on a Hitachi U-3400 spectrophotometer in MeOH. NMR spectra were recorded on a Jeol JNM-ALPHA 500 spectrometer (500 M Hz for ^{1}H and 125 M Hz for ^{13}C) with tetramethylsilane as an internal standard. FABMS were taken on a Jeol JMS-HX-110A mass spectrometer in *m*-nitrobenzylalcohol (NBA) matrix in the positive ion mode. Column chromatography was performed with Kieselgel 60 (230–400 mesh) (Merck), and the following solvent systems were used; $CHCl_3$–MeOH, benzene–EtOAc. TLC was carried out on precoated silica gel $60F_{254}$ (0.25 mm) (Merck). For prep. HPLC (LiChrosorb Si 60 column, 6 mm i.d. × 250 mm) (Kanto Chemical Co., Japan), $CHCl_3$–MeOH (9:1) was used.

3.2. Plant material

Rhizomes of *G. elata* Blume were purchased from either Tochimoto Tenkaido Co., Ltd. (Osaka, Japan) or were bought in Yunnan (China) by Dr. Qing Lin, and verified by Dr. Qing Lin, Yunnan Institute of Traditional Chinese Medicine and Materia Medica, Kunming, Yunnan, China. A voucher specimen (JH015393) is deposited in the herbarium, Graduate School of Pharm. Sci., Chiba University, Japan.

3.3. Extraction and isolation

Dried and ground rhizomes of *G. elata* (3.0 kg) were extracted with MeOH (5 l × 3) at 45 °C. The MeOH extract, after removal of the solvent by evaporation, was suspended in H_2O and partitioned with *n*-hexane, EtOAc and *n*-butanol, successively. After evaluating the activity of the resulting fractions, a portion of the EtOAc-soluble fraction (5.9 g) was applied to a silica gel column (36 mm × 46 cm, 250 g) eluted with solvent 1 ($CHCl_3$–MeOH) of increasing polarities (10:0 to 50:50, each of fractions collected 400 ml) to give 13 fractions. Fr. 3 (528 mg) was again subjected to silica gel (22 mm × 27 cm, 50 g) chromatogaphy with solvent 2 (benzene–EtOAc) of increasing polarities (10:0 to 80:20) to yield **10** (250 mg). Fr. 4 (555 mg) was applied to a silica gel column (50 g) with solvent 2 (10:0 to 80:20) to yield **2** (2.8 mg), **3** (45 mg) and **10** (212 mg). Fr. 7 (171 mg) was subjected to silica gel column chromatography (14.5 mm × 20 cm, 15 g) with solvent 2 (10:0 to 80:20) to yield **5** (3.5 mg), **6** (5.7 mg) and **11** (10 mg). Fr. 8 (204 mg) and 9 (123 mg) were combined and repeatedly subjected to silica gel chromatography with solvents 1 and 2 in a similar manner to yield **4** (0.8 mg) and **9** (154 mg). Frs. 10–12 (total 349 mg) were combined and applied to a silica gel column (22 mm × 21 cm, 40 g) with solvent 2 (10:0 to 0:10) to yield **7** (3.5 mg), **8** (7.6 mg) and a fraction (42 mg) rich in **1**. Compound **1** (15 mg) was finally purified from the **1**-rich fraction by prep. HPLC.

3.3.1. Compound *1* (gastrol)

Pale yellow oil, UV λ_{max} MeOH nm [log ε] : 263 [4.4], IR ν_{max} $CHCl_3$ cm^{-1} : 3448, 1509, 1261, 1100, 1029, 802, positive HR–FABMS *m/z*: 375.0964 $[M+K]^+$; calc. for $C_{21}H_{20}O_4K$: 375.0998, FABMS *m/z*: 337 $[M+H]^+$, 375 $[M+K]^+$, ^1H NMR ($CDCl_3+CD_3OD$*, 500 MHz; ppm) : 3.81 (2H, *s*, H-7″), 4.28 (2H, *s*, H-7), 4.29 (2H, *s*, H-7), 6.67 (2H, *d*, *J* = 8.0 Hz, H-3″, H-5″), 6.68 (1H, *d*, *J* = 8.1 Hz, H-5), 6.69 (2H, *d*, *J* = 8.3 Hz, H-3′, H-5′), 6.88 (1H, *d*, *J* = 2.0 Hz, H-2), 6.95 (1H, *dd*, *J* = 8.1, 2.0 Hz, H-6), 6.99 (2H, *d*, *J* = 8.0 Hz, H-2″, H-6″), 7.02 (2H, *d*, *J* = 8.3 Hz, H-2′, H-6′), ^{13}C NMR ($CDCl_3$ + CD_3OD*, 125 M Hz; ppm): 34.8 (C-7″), 71.1 (C-7′), 71.3 (C-7), 114.9 (C-5), 115.1 (C-3′, C-5′), 115.2 (C-3″, C-5″), 127.3 (C-6), 128.0 (C-3), 129.1 (C-1), 129.2 (C-1′), 129.7 (C-2′, C-6′), 129.9 (C-2″, C-6″), 130.6 (C-2), 131.6 (C-1″), 153.9 (C-4), 154.5 (C-4″), 156.1 (C-4′), * a few drops.

3.4. Guinea-pig ileum preparation

Male albino guinea-pigs (Dunkin-Hartley) weighing 300–400 g purchased from Takasugi Lab. Animals Co. Ltd. (Saitama, Japan) were stunned by a blow on the head and exsanguinated. Contraction of smooth muscle was measured in ileal preparation as described previously (Watanabe et al., 1997; Yamamoto et al., 1999). Briefly, the ileum was isolated and placed in Tyrode solution (mM): NaCl, 136.9; KCl, 2.6; $CaCl_2$, 1.8; $MgCl_2$, 1.1; NaH_2PO_4, 0.4; $NaHCO_3$, 11.9 and glucose, 5.6. The ileum was set up under 1 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 32 °C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2. At the start of each experiment a maximum response to acetylcholine (3 μM) was obtained in each tissue to check its suitability. Contractions were isotonically recorded by using a displacement transducer (NEC, San-ei Instruments Ltd., Type 45347, Tokyo, Japan), DC strain amplifier (San-ei 6M92, Tokyo, Japan) and a DC recorder (Hitachi, Mod 056, Tokyo, Japan).

In the experiments of electrical stimulation, tissues were stimulated through platinum needle-ring (a ring was placed 20 mm above the base of the needle 5 mm in length) electrodes using square wave pulses of supra maximal voltage. The ileum was transmurally stimulated with monophasic pulses (0.2 Hz) and 0.1 msec duration by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan).

The control contraction was induced by the first addition of a spasmogen. After washout, samples were added into the organ bath 7 min before the second addition of a spasmogen. The second spasmogen-induced contraction was measured in samples-treated ileum preparation. The height of contraction induced by spasmogens was measured in the absence or the presence of samples. Contraction (%) was determined by dividing the contractile height in sample-treated condition by that in non-treated condition. To obtain inhibition (%), this value was subtracted from 100.

The extract, the fraction and isolated pure compounds were dissolved in dimethylsulfoxide. Stock solutions were stored at −80 °C, and fresh dilution was made daily in the nutrient solution. The final concentration of dimethylsulfoxide is less than 1%.

3.5. Statistical analysis

All data are shown as the mean ± S.E.M. of results obtained from four to five animals. Statistical analyses were performed with two-tailed paired t-test for paired

observations of two groups, and two-tailed Student′s t-test for unpaired observations of two groups. P value <0.05 was considered statistically significant.

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