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## Isolation of Sedative Principles from *Perilla frutescens*

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The MeOH extract of *Perilla frutescens* leaves, which are used as a sedative in Chinese traditional medicine, was fractionated to isolate its active constituents. The progress of the fractionation was followed by measuring the prolongation of hexobarbital-induced sleep in mice. The activity was mainly due to the presence of both perillaldehyde and stigmasterol.

**Keywords**—*Perilla frutescens*; Labiatae; sedative; perillaldehyde; stigmasterol; phytosterol

### Introduction

*Perilla frutescens* (Labiatae), an annual aromatic herb used as a spice and medicine, consists of several chemotypes with respect to the chemical composition of essential oils.<sup>1-3)</sup> In China's traditional medicine, a chemotype containing perillaldehyde (PA) as a major component of the essential oil has been considered to be most effective as a sedative or an antidote. Recently, Sugaya *et al.*<sup>4)</sup> reported that the oral administration of an aqueous extract (4.0 g/kg body wt.) of perilla leaf as well as that of PA (100 mg/kg) prolonged sleep induced by hexobarbital-Na in mice. Woo and Shin<sup>5)</sup> detected sedative activity in MeOH (70%) extract (125 mg/kg *i.p.*) of perilla leaf, although the active principle was not identified.

This paper reports that sedative activity found in the leaf extract of PA chemotype is largely due to the combined effect of two constituents, PA and stigmasterol.

### Materials and Methods

**Plant Material**—Leaves of *Perilla frutescens* BRITTON var. *acuta* KUDO cultivated in fields were harvested in early August. Leaves (3 kg) which had been air-dried in the shade for 2 weeks were extracted with MeOH (36 l) at room temperature for another 2 weeks. For the preparation of test samples for bioassay, the aqueous fraction of the extract was freeze-dried, while the fractions in organic solvents were concentrated under reduced pressure at 40 °C.

**Pharmacological Test**—Test samples suspended in 1% carboxymethylcellulose (CMC) or dissolved in olive oil were administered to male mice of ddY strain (5 to 6 weeks old, 18–22 g) at a dose of 0.1 ml/10 g body wt. All experiments were carried out at 25 ± 1 °C, between 1 and 5 p.m. Bioassays of sedative activity were carried out according to the methods of Takagi *et al.*<sup>6,7)</sup> and Sugaya *et al.*<sup>4)</sup> Animals were fasted for 24 h prior to the experiment. One hour after the oral administration (*p.o.*) of test samples, hexobarbital suspended in 1% CMC in physiological saline was administered intraperitoneally (*i.p.*) at a dose of 50 mg/kg body wt. to mice placed in a box. Those animals that stopped moving in the box within 15 min after hexobarbital injection were immediately transferred to another box, where those individuals that stayed immobile for more than 3 min were judged to be asleep. The sleeping time was defined as the time required for mice to regain spontaneous movements after having been transferred to the second box. Mice that failed to fall asleep within 15 min after hexobarbital administration were excluded from the experiments.

Data obtained from control experiments (total of 198 mice) were as follows: the percentage of mice that fell asleep on hexobarbital administration, 74%; the time required for inducing sleep, 5.98 ± 0.16 min; the average sleeping time, 26.5 ± 0.97 min. Student's *t*-test was employed for the statistic evaluation of experimental data.

**Chemical Analysis**—Phytosterols isolated from perilla leaves were identified by gas liquid chromatography (GLC) and mass spectrum (MS) comparisons with authentic samples. GLC: stainless steel column (3 mm × 1 m) packed with 1% SE 30 in Chromosorb W; column temperature, 240 °C; carrier gas, N<sub>2</sub> at a flow rate of 40 ml/min; detector, FID.<sup>8)</sup> Retention times: campesterol (CA) 9.0, stigmasterol (ST) 9.8,  $\beta$ -sitosterol (SI) 11.6 min. MS (JEOL JMS-01SG-2): found (M<sup>+</sup>) CA 401, ST 413, and SI 415. The identification of PA was done by GLC, in comparison with an authentic sample. GLC: stainless steel column (3 mm × 2 m) packed with 17% PEG-6000 on Chromosorb W (30–60 mesh); column temperature, 170 °C; carrier gas, N<sub>2</sub> at a flow rate of 30 ml/min; detector, FID.<sup>3)</sup> Retention time of PA: 5.6 min.

## Results and Discussion

### Isolation of Active Substances

Oral administration of the MeOH extract (2 g/kg body wt.) of perilla leaves resulted in a remarkable prolongation (84%,  $p < 0.01$ ) of sleeping time in hexobarbital-treated mice. In order to isolate the active constituents, the extract was fractionated by the procedure shown in Fig. 1 and each fraction was tested for sedative effect on mice at a dose which was calculated from the proportion of the weight of each fraction to the weight of its mother fraction.

When the MeOH extract was distributed between CHCl<sub>3</sub> (2.5 l) and water (0.5 l), the sedative activity was recovered in the former. The CHCl<sub>3</sub> layer was separated by chromatography on a silica gel column (Wakogel C-100, 200 g), using *n*-hexane (2 l), CHCl<sub>3</sub> (2 l), and MeOH (1 l) as developing solvents successively. The CHCl<sub>3</sub> eluate, which showed the greatest activity, was then subjected to Wakogel C-200 (100 g) column chromatography, and the CHCl<sub>3</sub> eluate was grouped into fr. 1 (0.5 l) containing PA and fr. 2 (1.0 l) without PA on the basis of thin layer chromatography (TLC) examination of every 50-ml fraction. Although fr. 2 prolonged hexobarbital-induced sleep significantly, none of its 3 subfractions eluted from a Wakogel C-200 column (100 g) with a mixture of CHCl<sub>3</sub> and MeOH (3:1) was found to be effective on the prolongation of sleep at the dose used for the pharmacological test. Therefore, fr. 1, which showed greater sedative activity, was chromatographed in the same system to yield an active fraction 1-2. However, a further attempt to purify this fraction by re-chromatography using the same gel (80 g) and solvent gave only fractions with lower activity. Thus, the first 2 fractions, 1-2-1 and 1-2-2, were combined and chromatographed on Wakogel C-200 (70 g) using a mixture of *n*-hexane and CHCl<sub>3</sub> (2:1). As a result, 2 active fractions 2-1 and 2-3 were obtained. The latter fraction, which contained no PA, lost its activity when fractionated by chromatography using CHCl<sub>3</sub>-acetone (5:1) as a solvent, so that no further investigation of this fraction was carried out. On the other hand, fraction 2-1 showed the presence of PA and 3 colored spots on a TLC plate sprayed with anisaldehyde-H<sub>2</sub>SO<sub>4</sub> reagent. Accordingly, this fraction was separated by preparative TLC into 3 fractions, 2-1-1, 2-1-2, and 2-1-3, which contained PA and phytol, phytosterols, and linoleic acid, respectively. However, none of these fractions tested at the given dose was effective in the prolongation of sleeping time. These results suggested that the sedative activity might be due to an interaction of PA with another substance which had been separated by TLC into a different fraction. To check this possibility, artificial mixtures of PA and each one of the main components, I, II, and III, isolated from fractions 2-1-1, -2, and -3, respectively, were tested for sedative activity.

As shown in Table I, only a mixture of PA and II from fr. 2-1-2 significantly increased the hexobarbital-induced sleeping time of mice. Chlorpromazine, which was used as the reference drug, showed remarkable prolongation of the sleep at doses of 1.0 and 5.0 mg/kg (74 and 122%, respectively). Component II yielded white, mica-like crystals when recrystallized from a mixture of *n*-hexane and ethyl acetate, and was found to consist of three phytosterols; SI, ST, and CA in a proportion of 4.5:1.1:1.0 by GLC and MS analyses.<sup>8)</sup>

### Interaction between PA and Phytosterols

GLC analysis showed that the contents of PA and phytosterols in fr. 2-1 with a high

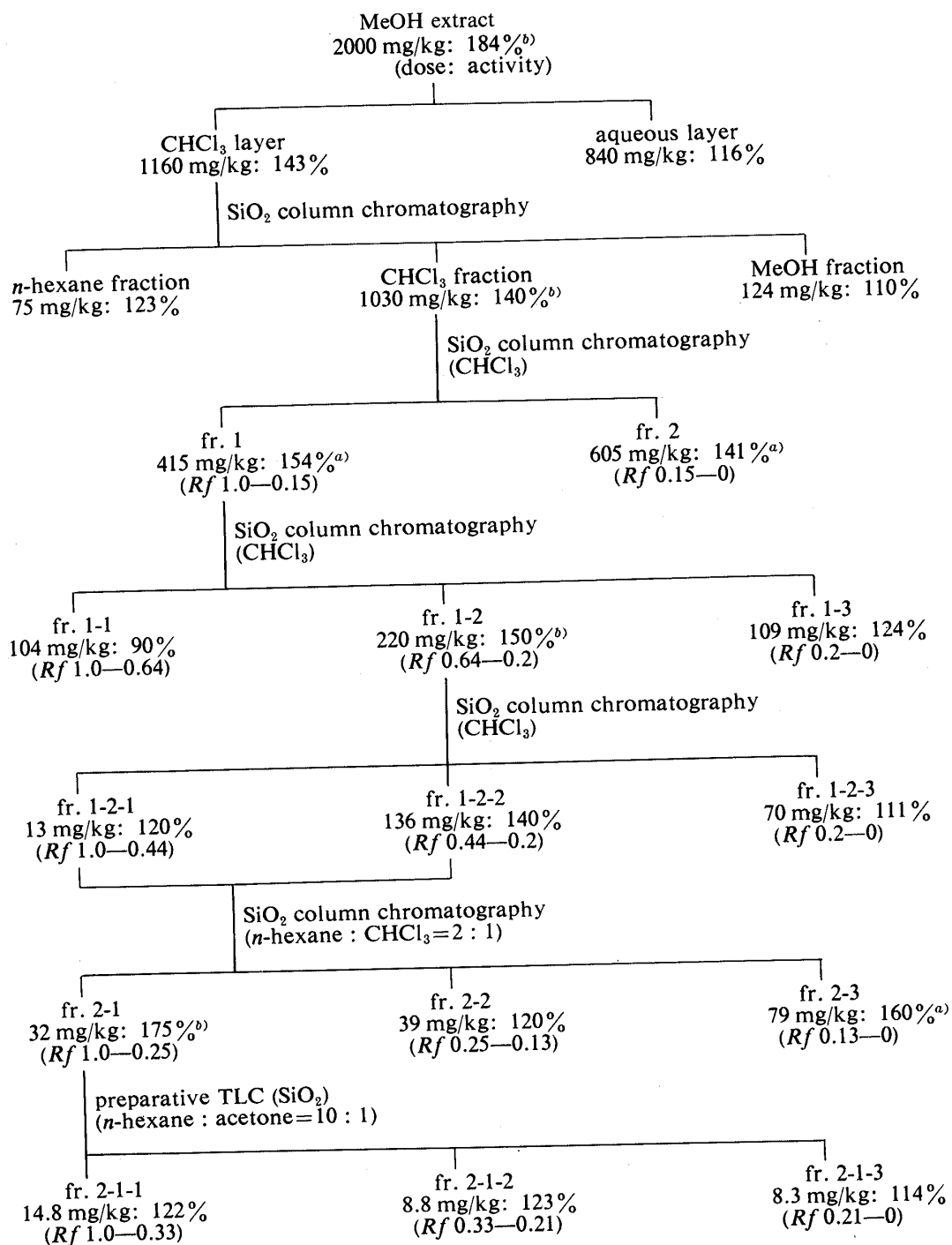


Fig. 1. Procedure for Fractionation of the Perilla Leaf Extract, and the Sedative Activity of Each Fraction

Every fraction was orally administered to a group of 8 mice. *Rf* values were examined by TLC on silica gel plates with  $\text{CHCl}_3$  as the developing solvent. Control = 100%. a)  $p < 0.05$ . b)  $p < 0.01$ .

sedative activity (175%) were 8.3 and 17.6%, respectively. It is therefore calculated that the doses of PA and phytosterols in fr. 2-1 administered to mice were approximately 2.7 and 5.6 mg/kg body wt., respectively. Taking these estimates into consideration, various combinations of PA (0–10 mg/kg) and the mixture of phytosterols mentioned above (0–10 mg/kg) were tested for sedative activity. Figure 2 shows that such simultaneous administration of PA and phytosterols remarkably prolonged the sleeping time in a dose-dependent

TABLE I. Effect of Simultaneous Oral Administration of Perillaldehyde (PA) and Three Isolated Components (I, II, and III) on Hexobarbital-Induced Sleep in Mice

Drug combination (mg/kg) (mg/kg)	No. of mice	Sleeping time (min)	
		(Mean $\pm$ S.E.)	Relative activity
Control (olive oil)	20	28.2 $\pm$ 2.3	100
PA (2.5)+I (4.0)	6	31.3 $\pm$ 3.8	111
PA (2.5)+II (4.0)	7	40.0 $\pm$ 2.2 <sup>a)</sup>	142
PA (2.5)+II (12.5)	5	40.6 $\pm$ 2.0 <sup>a)</sup>	144
PA (10.0)+II (31.0)	7	40.9 $\pm$ 4.3 <sup>a)</sup>	145
PA (2.5)+III (4.0)	6	26.2 $\pm$ 1.9	93
Control (olive oil)	7	23.4 $\pm$ 3.0	100
Chlorpromazine (1.0)	7	40.7 $\pm$ 3.2 <sup>b)</sup>	174
Chlorpromazine (5.0)	8	52.0 $\pm$ 8.0 <sup>b)</sup>	222

a)  $p < 0.05$ . b)  $p < 0.01$ .

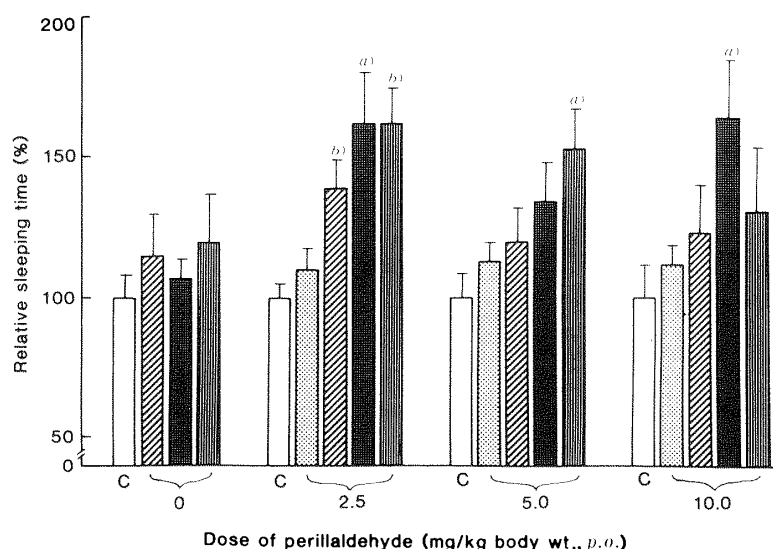


Fig. 2. Combined Effect of Perillaldehyde (PA) and Phytosterols on Hexobarbital-Induced Sleep in Mice

In addition to PA (diagonal lines), a mixture of phytosterols ( $\beta$ -sitosterol-stigmasterol-campesterol, 4.5:1.1:1.0) was simultaneously administered to mice at oral doses of 2.5 (dotted), 5.0 (cross-hatched), and 10.0 (vertical lines) mg/kg body wt. C indicates the control for each experimental block. a)  $p < 0.05$ . b)  $p < 0.01$ .

manner, although no dose-dependent response was observed when PA (0–10 mg/kg) alone was administered to the mice. However, Sugaya *et al.*<sup>4)</sup> have reported that dose-dependency was observed at a higher dose range of PA (0–100 mg/kg). As might have been expected, the combination of 2.5 mg/kg PA and 5 mg/kg phytosterols showed an activity nearly equivalent to that of fr. 2-1. The sleeping time, which was increased by 20% at the most over the control by a single administration of either PA or phytosterols, could be increased by 60% by simultaneous administration of the two components. When the doses of both PA and phytosterols were as high as 10 mg/kg body wt., however, no significant prolongation of sleep was observed.

In an attempt to determine the effective ingredient of phytosterols, bioassays were performed with a nearly pure (over 95%) sample of ST and with a mixture of SI and CA (55:45) (Table II). A significant prolongation of sleep was observed when ST (0.75,

TABLE II. Combined Effect of Perillaldehyde (PA) and Stigmasterol (ST) or a Mixture of  $\beta$ -Sitosterol and Campesterol (SI-CA<sup>a</sup>) on Prolongation of Hexobarbital-Induced Sleep in Mice

Drug combination (mg/kg) (mg/kg)	No. of mice	Sleeping time (min)	
		(Mean $\pm$ S.E.)	Relative activity
Control (olive oil)	40	25.8 $\pm$ 1.3	100
ST (0.75)	6	28.9 $\pm$ 3.1	112
ST (5.0)	6	27.6 $\pm$ 4.0	107
ST (0.75) + PA (2.5)	13	36.9 $\pm$ 2.7 <sup>b</sup>	143
ST (5.0) + PA (2.5)	13	37.9 $\pm$ 2.9 <sup>b</sup>	147
ST-CA(3.0) + PA (2.5)	8	32.3 $\pm$ 3.6	125
SI-CA (5.0) + PA (2.5)	4	27.1 $\pm$ 2.0	105

Each drug was administered orally. a) A mixture of  $\beta$ -sitosterol (SI) and campesterol (CA) at a ratio of 11:9. b)  $p < 0.01$ .

5.0 mg/kg) and PA (2.5 mg/kg) were combined, although no significant effect was observed with ST alone. Since 0.75 mg of ST is approximately equivalent to the amount of ST contained in 5 mg of the isolated phytosterols, it is reasonable that the activities of these two samples were found to be quite similar. In contrast, the combination of PA (2.5 mg/kg) and the SI-CA mixture (3 or 5 mg/kg) showed no noticeable sedative activity. These results seem to indicate that a considerable portion of the sedative activity of the leaf extract is caused by the combination of PA and ST. Furthermore, a decrease of spontaneous movement was observed in most of the mice from about 30 min after the administration of a PA-ST mixture until the time of hexobarbital injection. It is of interest that ST, which is widely distributed in higher plants, exhibits a specific interaction with another compound (PA). It should be noted that neither PA nor ST showed any visible toxic effect on mice and that the LD<sub>50</sub> values of these compounds are greater than 1 g/kg body wt. (*p.o.*). Furthermore, no mouse died within 72 h after the simultaneous oral administration of PA (1 g/kg) and ST (1 g/kg).

It has been reported that phytosterols are present in such mammalian tissues as adrenal gland<sup>9)</sup> and brain,<sup>10)</sup> and that phytosterols contained in the diet might accumulate in the cell membrane.<sup>11)</sup> According to Nes and Heftmann,<sup>12)</sup> ST increases the fluidity of the cell membrane. On the other hand, PA is known to inhibit the laryngeal reflex of cat and the excitation of the membranes of snail's neurons as well as frog's sciatic nerve fibers.<sup>4)</sup> It is likely therefore that the inhibitory action of PA on the central nervous system might be strengthened through an unknown effect of ST on the cell membrane.

The synergistic interaction observed between chemically unrelated constituents of the perilla leaf seem to be a good example of the general idea that various pharmacological activities of crude drugs are often generated by interactions between different constituents.

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