

Inhibitory Effects of the Fragrance Inhalation of Essential Oil from *Acorus gramineus* on Central Nervous System

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The present study was designed to evaluate central inhibitory effects of the essential oil from *Acori graminei* Rhizoma (AGR), the dry rhizomes of *Acorus gramineus* SOLANDER (Araceae) upon fragrance inhalation (aroma therapy). Preinhalation of the oil markedly delayed the appearance of pentylenetetrazole-induced convulsion. Furthermore, inhalation impressively inhibited the activity of γ -aminobutyric acid (GABA) transaminase, a degrading enzyme for GABA as the inhalation period was lengthened. The GABA level was significantly increased and glutamate content was significantly decreased in mouse brain by preinhalation of the essential oil. The above results suggest that the anticonvulsive effect of this AGR oil is originated by the enhancement of GABA level in the mouse brain, because convulsion depends partially on GABA concentration which can be properly preserved by inhibiting GABA transaminase. Moreover, fragrance inhalation progressively prolonged the pentobarbital-induced sleeping time as inhalation time was lengthened. Ten hour inhalation corresponded almost to the effect (145% increase) of oral administration (60 mg/kg). This sedative effect after inhalation or oral administration of AGR essential oil suggests that this oil may act on the CNS *via* the GABAergic system. The inhibitory activity of preinhalation of the essential oil on lipid peroxidation, to which the anticonvulsive action is attributed, also supported the above results, confirming and amplifying our previous reports on the CNS inhibitory effects of AGR.

Key words *Acorus gramineus*; essential oil; anticonvulsive effect; sedative effect; aroma therapy

Acori graminei Rhizoma (AGR), the dry rhizomes of *Acorus gramineus* SOLANDER (Araceae), is officially listed in the Korean Pharmacopoeia which records its sedative, digestive, analgesic, diuretic, and antifungal effects.

This herbal drug has been reported to be responsible for various pharmacological actions on the central nervous system (CNS): The water extract or methanol extract of AGR antagonized pentylenetetrazole-induced convulsion, potentiated pentobarbital-induced sleeping time, induced sedation, and decreased spontaneous activity in mice.^{1,2} Receptor-ligand binding studies suggested that the interactions with the central dopamine receptors and the GABA_A-benzodiazepine receptor mediate these central inhibitory actions.² Besides that, the extracts of composite drugs containing AGR as a main component are clinically used for improvement of learning and memory and for stroke.^{3,4} However, the essential oil of AGR has not been thoroughly evaluated. Recently we reported that this oil inhibited glutamate-induced excitotoxicity in a concentration-dependent manner, leading to neuroprotective effects on cultured cortical neurons through the blockade of NMDA receptor activity,⁵ and exhibited antioxidative effects in *in vivo* and *in vitro* assay.⁶

The rhizomes and leaves of *Acorus gramineus* are known to contain 0.11–0.42% of essential oil consisting of 30 kinds of compounds⁷ which may affect the CNS after direct inhalation of its fragrance. Pure fragrance compounds and essential oils with sedative properties influenced the motility of mice in inhalation studies under standardized conditions.⁸ To date, only a few papers have reported on sedative or activating properties of some essential oils and their constituents on animals under standardized experimental procedures.^{9–12}

We now report the effects of an essential oil of AGR on the sedative and anticonvulsant properties and also antioxi-

dative activity after fragrance inhalation.

MATERIALS AND METHODS

Materials Dried roots of *A. gramineus* were purchased from a traditional herb market located in Yeongchon, Korea and identified by Prof. Byung-Soo Kang, College of Oriental Medicine, Dongguk University, Gyeongju, Korea. A voucher specimen of this plant material is on deposit at the Herbarium (DG-AGR9801) of this college. GABA, glutamate, α -ketoglutaric acid, β -NADP, pentylenetetrazole, *o*-phthalaldehyde, 2-aminoethylisothiuronium bromide, pyridoxal-5-phosphate, chlorpromazine hydrochloride, and sodium pentobarbital were obtained from Sigma Co. (St. Louis, MO, U.S.A.). All other chemicals and reagents were of the highest grade available.

Animal Studies Male 8-week-old outbred ICR mice with mean weights of 28.5 g were housed in groups of seven under standardized conditions (room temperature: 21 \pm 2 °C, relative humidity: 50–60%, light–dark rhythm: 12 h cycle). A special cage (Three-Shine Co., Seoul, Korea) was used for inhalation of the fragrance: 2 g of fragrance oils on a petri dish (8.5 cm diameter) were put in each cage (W 26 \times L 22 \times H 20 cm) for evaporation. The cage cap was equipped with a special filter which passed minimum breathing air. Concentration of the fragrance in the cage was not determined. Essential oil was inhaled two times per day (for 3 h every morning and afternoon) for 7, 14 and 30 d, respectively.

Preparation of Essential Oil The dried and pulverized rhizomes of the plant (600 g) were extracted three times with 21 of *n*-hexane at room temperature for 48 h, then filtered. The filtrate was evaporated under 80 °C to remove hexane,

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which was further eliminated *in vacuo* for 5 min. at room temperature to give 6.6 g of greenish-brown essential oils.

In Vivo GABA Transaminase Assay Animals were anesthetized with diethyl ether, and perfused with normal saline to exclude any disturbances from intravascular substances. The whole brain was isolated, and then homogenized with a glass Teflon homogenizer in 4 volumes of 0.1 M K.P. buffer (pH=7.4). Homogenates were centrifuged in 600×g for 10 min at 4 °C, supernatant was collected and recentrifuged in 10000×g for 20 min at 4 °C. Postmitochondrial fractions were ultracentrifuged (Kontron T-2080, Switzerland) in 105000×g for 1 h, and the supernatant was used as an enzymatic source in the GABA transaminase assay.¹³ GABA, α -ketoglutaric acid, 0.15 M potassium phosphate buffer (pH=8.0) and tissue homogenates were incubated in 37 °C for 30 min, followed by the addition of NADP⁺. The amount of NADPH generated in the brain tissue for 20 min was measured by spectrophotometer (Ultrospec 2000, Pharmacia, U.S.A.) at 340 nm as an activity of GABA transaminase.

Determination of GABA/Glutamate Levels in Brain Concentrations of GABA and glutamate in the brains were measured using a modified method of Allen and Griffiths.¹⁴ Tissues were homogenized in 0.3 M triethanolamine buffer, pH 6.8, containing 1 mM of aminoethylisothiuronium bromide and 2 mM pyridoxal-5'-phosphate, then centrifuged at 15000×g for 20 min. Postmitochondrial fraction from each extract was resuspended in 200 mM potassium phosphate buffer, deproteinized, and then centrifuged. Supernatants were filtered by membrane filter (0.2 μ m: 13 mm), *o*-phthalaldehyde-derivatized GABA and glutamate were used for the detection of fluorescence in the HPLC measurement (LC-10AD, Shimadzu, Japan). An Inertsil ODS-3 (150×4.6 mm I.D., 5 μ m) column was used. HPLC was performed using gradients of methanol (20% to 0% in 40 min) in 10 mM potassium acetate buffer (pH 6.5), at a flow rate of 0.6 ml/min. The amounts of GABA and glutamate in brains were represented as nmole per milligram protein.

In Vivo Anticonvulsant Activity Assay In the group of convulsive dose of pentylenetetrazole (PTZ)-treated mice,¹⁵ PTZ (70 mg/kg) was injected subcutaneously 1 h after the last inhalation. Onset time of convulsion, recovery time and lethality were recorded. We classified the signs shown in each degree of convulsions: 0 (no sign); 1 (paralysis and fibrillations); 2 (raising forelimbs and convulsions); 3 (weak jerking); 4 (strong jerking and jumping, squeaking); 5 (lengthy of typical tonic-clonic seizure, expiration). We also calculated duration (Σ [the time each convulsion concluded—the time each convulsion began]/total frequency of convulsion), recovery time (time to complete recovery after first convulsion), and severity (Σ [(degree of convulsion)×(frequency of convulsion at each degree)]/total frequency of convulsion).

Lipid Peroxidation Assay According to the method of Ohkawa *et al.*,¹⁶ tissue homogenates, 8.1% sodium dodecyl sulfate, 20% acetate buffer (pH=3.5) and 0.8% 2-thiobarbituric acid were incubated for 1 h at 95 °C, and then cooled to room temperature. The thiobarbituric acid reactive substance (pink color) in the reactant was transferred to a mixture of *n*-butanol : pyridine (15 : 1) and its absorbance was measured at 532 nm as the degree of lipid peroxidation. The level of lipid

peroxides was expressed in terms of MDA equivalents (nmoles MDA/g of tissue).

Pentobarbital Induced-Sleeping Time Assay Mice were administered with sodium pentobarbital (50 mg/kg in saline) intraperitoneally, 2, 5 and 10 h after fragrance inhalation and 30 min after oral administration (60 mg/kg in Tween 80). Sleeping time was recorded from the disappearance of the righting reflex until its recovery and compared with those of the control group and the chlorpromazine-treated group (positive control).

GC/MS Analysis For qualitative and quantitative analysis of the components of an essential oil, gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett Packard 5890 series II instrument connected to an Automass 50 (JEOL). The operating conditions were as follows: column fused silica capillary column, TC-wax (Hewlett Packard), 60 m×0.25 mm, film thickness=0.25 μ m; column temperature: 40—300 °C increasing at 5 °C/min to 150 °C, then 15 °C/min to 300 °C, ending at 300 °C for 10 min; injector: 180 °C; carrier gas: nitrogen at a flow rate of 30 cm/s; column head pressure: 180 kPa; injection volume: 0.5 ml; ionization energy: 70 eV; ion source temperature: 200 °C. Chemical components were identified by comparing their retention times and mass spectra with those of authentic samples or by comparing their mass spectra with those in the MS data library (NBS library). The relative amount of each component was determined by calculating the peak area of the TIC chromatogram.

Statistical Analysis Data are expressed as the mean±S.E. of the number (*n*) of experiments. Statistical analysis of difference was determined by Student's *t*-test or ANOVA, followed by post hoc multiple comparison analysis package (Systat Inc., Evanston, IL, U.S.A.).

RESULTS

GABA Transaminase Inhibition The inhalation of an essential oil of AGR led to inhibition of the activity of GABA transaminase. In all groups that inhaled the fragrance, production of NADPH (nmoles/mg protein/h) by GABA transaminase was significantly ($p<0.05$) lower than that of the PTZ-treated group. The level of NADPH at 7 d inhalation (total 6 h per day) was decreased to 65.4% of the PTZ-treated group (Fig. 1). The longest inhalation (30 d) showed no large difference from 7 or 14 d treatment.

GABA and Glutamate Levels Preinhalation of an essential oil increased the brain GABA level in a convulsive dose of PTZ-treated mice as shown in Table 1. Brain GABA contents (nmole/mg protein) were significantly ($p<0.05$) decreased in the PTZ (70 mg/kg)-treated mice compared with the control group. However, GABA levels were significantly ($p<0.05$) increased by preinhalation, almost to the control level by 30 d inhalation of the fragrance. In the same experiment, brain glutamate content was strongly increased in the PTZ-treated group and decreased almost to the control level ($p<0.05$) after fragrance inhalation for 30 d.

Anticonvulsant Activity The effect of the preinhalation of an essential oil on PTZ-induced convulsion in mice was observed. As the inhalation period lengthened, all inhaling groups had a delayed appearance of convulsion compared with the period induced by PTZ alone (Table 2). Recovery

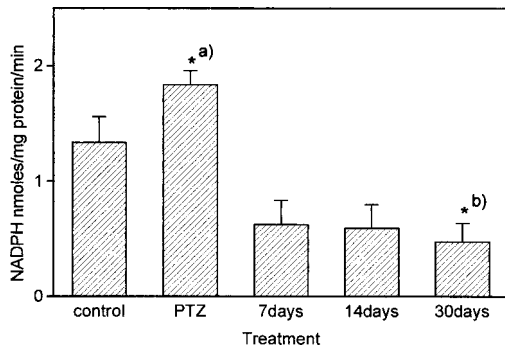


Fig. 1. Effect of the Preinhalation of Fragrance on the Brain GABA Transaminase Activity *in Vivo*

The assay procedure was described in experimental methods. Values are the mean±S.E. (n=7). a) p<0.05, significantly different from control, b) p<0.05, significantly different from PTZ-treated group.

Table 1. Effect of the Preinhalation of Fragrance from *Acori graminei* Rhizoma on the Brain GABA and Glutamate Levels in a Convulsive Dose of PTZ-Treated Mice

Treatment	Content (nmol/mg protein)	
	GABA	Glutamate
Control	2.76±0.14	9.45±0.42
PTZ	1.64±0.22 ^a	18.24±0.56 ^a
7 d	2.15±0.18 ^b	14.56±0.32 ^b
30 d	2.58±0.28 ^b	10.06±0.48 ^b

Mice inhaled an essential oil two times per day (3 h every morning and afternoon) for 7 and 30 d. Values represent the mean±S.E. (n=7). a) p<0.05, significantly different from control, b) p<0.05, significantly different from PTZ-treated group.

Table 2. Effect of the Preinhalation of Fragrance from *Acori graminei* Rhizoma on PTZ-Induced Convulsion in Mice

Treatment	Onset time (min)	Duration (min)	Recovery time (min)	Severity	Lethality (%)
Control	2.2±1.6	1.5±0.5	8.5±2.3	5.8±0.8	71.4
7 d	6.3±2.1	1.2±0.5	7.2±1.8	5.0±0.9	14.3
14 d	7.7±2.9	0.9±0.2	5.4±2.5*	3.9±0.6*	14.3
30 d	13.5±4.2*	0.5±0.1*	2.8±0.8*	1.8±0.4*	0

Mice inhaled the essential oil two times per day (3 h every morning and afternoon) for 7, 14 and 30 d. Values represent the mean±S.E. (n=7). * p<0.05, significantly different from the control group.

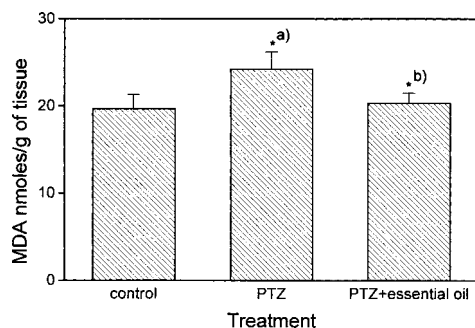


Fig. 2. Effect of Fragrance Inhalation on Brain Lipid Peroxidation in Pentylene-tetrazole (PTZ)-Treated Mice

Mice were administered PTZ (70 mg/kg, subcutaneous injection) one hour after the final inhalation and decapitated 30 min after injection. Values are the mean±S.E. (n=7). a) p<0.05, significantly different from control, b) p<0.05, significantly different from PTZ-treated group.

time and severity were significantly ($p<0.05$) decreased in the PTZ-treated mice compared with control. For the longest period (one month preinhalation) the inhibition was most effective, exhibiting 67.1% reduction of recovery time and 69.0% decrease of severity compared with control. Lethality was also strongly decreased from 71.4% for control to no death with 30 d of inhalation.

Anti-lipid Peroxidation Preinhalation of fragrance for 7 d had an inhibitory effect on lipid peroxidation as shown in Fig. 2. Brain lipid peroxidation (MDA nmoles/g of tissues) was significantly ($p<0.05$) increased in the PTZ-treated mice compared with control (23.6% increase). However, lipid peroxides were significantly ($p<0.05$) diminished almost to the level of control by fragrance inhalation.

Sleeping Time Prolongation Preinhalation of fragrance also time-dependently prolonged the pentobarbital-induced sleep (Fig. 3). At the longest period (10 h inhalation), the sleeping time was increased to 139% of the control, which was comparable to the effect of 60 mg/kg oral administration (145% increase). Chlorpromazine hydrochloride (10 mg/kg, *p.o.*), a positive control, showed 222% prolongation as compared with the control group.

GC/MS Analysis The results of GC-MS analysis of the essential oil are shown in Table 3. (+)-Calarene (retention time=35.86 min) has not yet been reported to be contained in this plant.

DISCUSSION

Liao *et al.*²⁾ reported the central inhibitory effects of a water extract of AGR, which decreased the locomotor activ-

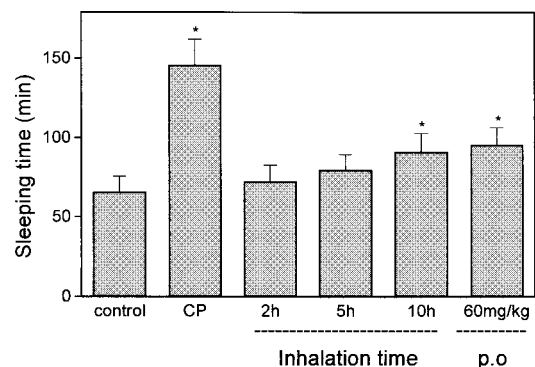


Fig. 3. Effect of Preinhalation and Oral Administration of Fragrance on Pentobarbital-Induced Sleeping Time in Mice

The data are shown as the mean±S.E. (n=7). CP (chlorpromazine, 10 mg/kg) was administered *per os*. * p<0.05, significantly different from control.

Table 3. Composition of the Essential Oil from the Rhizomes of *Acorus gramineus*

Retention time (min)	Compound ^{a)}	Content (%)	
		Before inhalation	After inhalation ^{b)}
28.18	Camphor	0.31	0.00
29.11	Borneol	0.19	0.03
34.72	Methyl eugenol	0.64	0.16
35.58	<i>cis</i> -Methylisoeugenol	1.71	0.40
35.64	β -Caryophyllene	0.33	0.09
35.74	γ -Cadinene	0.17	0.05
35.86	(+)-Calarene	0.10	0.03
36.55	β -Guaiane	0.11	0.03
36.88	Elemicin	0.18	0.05
37.16	Euasarone	17.05	9.28
37.62	β -Asarone	40.35	73.37
38.28	α -Uasarone	12.34	6.90

a) Compounds having over 0.1% content before inhalation are listed. b) After inhalation for 30 d, the essential oil was directly analyzed by GC/MS under the same conditions as the conditions before inhalation.

ity, increased the pentobarbital-induced sleep, and exhibited a weak anticonvulsant effect and a GABA agonist-like action. The essential oil of AGR, however, has not yet been thoroughly evaluated for its actions on the CNS. In our previous reports,^{5,6)} we demonstrated the neuroprotective action of AGR essential oil against excitotoxicity through the blockade of NMDA receptor activity and antioxidative activities in *in vivo* and *in vitro* assays. Consistent with those results, the present study introduced the effects of essential oil of AGR on sedation and convulsion by way of fragrance inhalation. This is the important difference between the present work (essential oil test after inhalation) and our former report (total extract test after oral administration).

Preinhalation the essential oil of AGR markedly delayed the appearance of PTZ-induced convulsion. PTZ is known to block the action of GABA in the CNS, inducing convulsion.¹⁷⁾ Furthermore, we found that inhalation of the essential oil of AGR inhibited GABA transaminase, a degrading enzyme for GABA which is a major inhibitory neurotransmitter in the mammalian brain, *in vivo* as the inhalation period was lengthened from 7 to 30 d. This result of enzyme inhibition supported the above anticonvulsant effect of fragrance inhalation on PTZ-induced convulsion, because the proper level of GABA in brain plays an important role in anticonvulsion. Finally, we have determined the level of GABA as well as the content of glutamate, an excitatory neurotransmitter, in mouse brain. The GABA level was significantly ($p < 0.05$) increased and glutamate content was significantly ($p < 0.05$) decreased by preinhalation of the essential oil compared with each control group. The above results suggest that the anticonvulsive effect of the essential oil of AGR is partially supported by the enhancement of GABA level in the mouse brain, because convulsion depends partially on GABA concentration which can be properly preserved by inhibiting GABA transaminase.

To investigate whether the preinhalation of this oil also affects sedation in the CNS, the prolongation of sleep duration was estimated. Fragrance inhalation progressively prolonged the pentobarbital-induced sleeping time as inhalation time was lengthened. The longest inhalation time (10 h) nearly

corresponded to the effect of oral administration (60 mg/kg), which is, however, much weaker than that of chlorpromazine, a positive control.

Finally, we examined the antioxidative activity of the essential oil of AGR after fragrance inhalation in mice. It has been stated that the excitatory amino acid (*e.g.*, glutamate) is released by oxidative stress conditions to afford neuronal excitation.¹⁸⁾ Liu and Mori¹⁹⁾ also suggested that the anticonvulsive effect of Gastrodiae Rhizoma may be attributable to the antioxidant activity of its active components. We assayed the antioxidative effect of the essential oil of AGR *in vivo*. The content of brain peroxy-lipids (MDA mol/g of tissue) was significantly ($p < 0.05$) increased in PTZ-treated mice compared with control. However, this lipid peroxidation was significantly ($p < 0.05$) diminished almost to the level of control by preinhalation of the essential oil fragrance. This antioxidant activity may be related to the anticonvulsant effect of the essential oil. The antioxidant activity of this oil has been determined *in vitro* by us,⁶⁾ and showed significant inhibition of lipid peroxidation, aldehyde oxidase and xanthine oxidase activities, but weak DPPH radical scavenging activity.

AGR essential oil was analyzed by a GC-MS system to exhibit twelve compounds (over 0.1% content) including β -asarone as a main component (40.35% of total hexane fraction) (Table 3). β -Asarone itself has been reported to cause generalized convulsions and potentiate the Metrazol seizures, while α -asarone (12.34% content) has a definite tendency to protect against convulsions and modified electro-shocks, however, the hypnotic potentiating property of β -asarone is more than that of α -isomer.²⁰⁾ Moreover, neither compound exerts any effect on the spontaneous motor activity. So, our results suggest that the anticonvulsant or sedative effect of the essential oil after inhalation may not originate from β -asarone, but from other components including α -asarone. To confirm this, the content change of individual components was analyzed by GC-MS before and after inhalation. As shown in Table 3, the contents of all components except β -asarone were lower after inhalation. The increase of β -asarone content after inhalation may result from the decrease of other components. Because β -asarone is a convulsant and a cancer suspect agent but has very low volatility,²¹⁾ the inhalation without β -asarone would be more useful than oral administration for therapeutic application.

In conclusion, the present study confirmed and amplified our previous reports on the CNS inhibitory effects of AGR, showing its anticonvulsive and sedative actions by direct inhalation of the essential oil fragrance.

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