Synergistic Effect of Fragrant Herbs in Japanese Scent Sachets

Yumi Fujiwara, Michiho Ito

Department of Pharmacognosy, Graduate School of Pharmaceutical Science, Kyoto University, Kyoto, Japan

Key words
- sedative effect
- inhalation
- fragrant herbs
- synergistic effect

Abstract
The sedative activity of eight aromatic natural medicines that are traditionally used in Japanese scent sachets was examined using an open field test with mice. Galangal (*Kaempferia galanga*), patchouli (*Pogostemon cablin*), sandalwood (*Santalum album*), spikenard (*Nardostachys chinensis*), cinnamon (*Cinnamomum cassia*), clove (*Syzygium aromaticum*), star anise (*Illicium verum*), and borneol (*Dryobalanops aromatica*) distilled oils were used. These natural medicines have various pharmacological effects. For example, galangal has insecticidal activity and clove extracts possess strong total antioxidant activity. Aromatherapy, a well-known complementary medicine system that uses inhalation, has recently attracted much attention. The sedative activity of inhaled aromatic compounds or essential oils has been examined by measuring the spontaneous motor activity of mice in an open field test. The galangal, patchouli, sandalwood, spikenard, and borneol oils showed significant sedative effects. The effect was stronger for a mixture of the five oils than for any of the single oils. This suggests that the oil mixture may have synergistic activity. Sedative activity was not observed when inactive oils (cinnamon, clove, and star anise) were added to the mixture of the five active oils.

Abbreviations
- AUC: area under the curve
- FID: flame ionization detector
- GABA: γ-aminobutyric acid
- NREM: non-rapid eye movement
- PAH: perillaldehyde

Supporting information available online at http://www.thieme-connect.de/products

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In Japan, it is a traditional custom to enjoy the fragrance of natural medicines using scent bags or sachets known as koubukuro. Aromatic natural medicines that emit fragrances at room temperature, such as galangal (*Kaempferia galanga*), patchouli (*Pogostemon cablin*), sandalwood (*Santalum album*), spikenard (*Nardostachys chinensis*), cinnamon (*Cinnamomum cassia*), clove (*Syzygium aromaticum*), star anise (*Illicium verum*), and borneol (*Dryobalanops aromatica*) distilled oils were used. These natural medicines have various pharmacological effects. For example, galangal has insecticidal activity [1] and clove extracts possess strong total antioxidant activity [2]. However, the majority of studies use extracts, and there are very few that use essential oils. In animal experiments, the pharmacological effects of essential oils are usually evaluated after oral administration and abdominal or percutaneous injection, rather than after inhalation. Aromatherapy, a well-known complementary medicine system that uses inhalation, has recently attracted much attention, and some interesting effects have been reported. Lavender essential oil shows antianxiety and antidepressant effects [3] and olfactory stimulation with black pepper oil significantly improves the sensory and reflexive motor movement of swallowing [4]. Further studies and trials for the clinical use of essential oils are needed to improve their
effective use and increase the evidential background for therapies. The sedative activity of inhaled aromatic compounds or essential oils has been examined by measuring the spontaneous motor activity of mice in an open field test [5].

Sedative effects have also been reported for some of the aromatic natural medicines found in Japanese scent sachets administered by inhalation. For example, the sedative activity of spikenard extract was examined using a spontaneous vapor administration system [6], and the main component of sandalwood, santalol, significantly decreased the total waking time and increased the total non-rapid eye movement sleep time in rats [7]. In the present study, the chemical compositions of the distilled oil of natural medicines commonly used in Japanese sachets were analyzed, and their possible effects on spontaneous motor activity in mice were examined. Mixtures of oils were also investigated and we discuss the contribution of the results to the scientific background for traditional Japanese scent sachets.

Results

The analysis results for the eight essential oils are shown in Table 15S–85, Supporting Information. The structures of the two main components of each essential oil are shown in Fig. 1S, Supporting Information. The yield of the essential oils for each natural medicine was calculated as 0.26% (w/w) for galangal, 0.24% (w/w) for patchouli, 0.20% (w/w) for sandalwood, 0.23% (w/w) for spikenard, 0.47% (w/w) for cinnamon, 3.33% (w/w) for clove, and 1.88% (w/w) for star anise, per dry weight. The essential oils of galangal, sandalwood, clove, and star anise were colorless, and those of patchouli, spikenard, and cinnamon were pale yellow. Each oil had a characteristic odor.

Essential oils isolated by hydrodistillation were administered to mice by vapor inhalation, and their sedative activity was examined. The doses of $4 \times 10^{-4}$ to $4 \times 10^{-2}$ mg were calculated according to the range of doses that have been reported as effective for the same experimental system [8]. PAH is a major component in the essential oil of Perilla frutescens (Labiatae), which was previously reported to have sedative and antidepressant-like effects on mice [9, 10]. The results of the administration of the essential oils are shown in Fig. 1. The sedative effects of a fragrant compound administered by inhalation often draw a U curve. This is in common with other sedative volatile compounds [6]. Sedative activity was observed for galangal, patchouli, sandalwood, spikenard, and borneol crystals. The strongest activities were observed at doses of $4 \times 10^{-4}$ mg (galangal), $4 \times 10^{-3}$ mg (patchouli), $4 \times 10^{-4}$ mg (sandalwood), $4 \times 10^{-3}$ mg (spikenard), and $4 \times 10^{-3}$ mg (borneol crystals), and they were statistically significant (Fig. 1a–d, h, p < 0.05). During the first 10 min of administration, the locomotor activity of the mice decreased to approximately two-thirds that of the control (Fig. 2). In contrast, none of the cinnamon, clove, and star anise oils showed sedative activity. Administration of these non-sedative oils caused a continuous increase of locomotor activity from 30 min onward, or abnormal behavior such as jumping, rapid movement, and frequent excretion. The control mice became calm and their locomotor activity was reduced to nearly zero after 30 min.

There are a vast number of possibilities for generating combinations of eight essential oils and doses. We initially investigated a sedative effect for a mixture of the eight oils, although sedative activity was not observed at every concentration (data not shown). Therefore, mixtures of the five oils that showed a sedative effect were examined for synergistic effects. Furthermore, each of the inactive oils was added to the mixture of active oils, and the sedative activity of these mixtures was also examined. The doses of the oil mixtures were the most effective doses for single administration. The composition of the mixtures of five active oils is shown in Table 1. The results of the administration of oils are shown in Figs. 3 and 4. Mixtures of the five active oils were administered to mice, and the locomotor activity was decreased more than when the oils were administered individually. Although neither was significant, this result shows that the mixture of effective oils produced synergistic effects. When three inactive oils, namely, cinnamon, clove, and star anise, were added separately to the mixture of the five active oils, the activity of the mixtures was decreased.

Table 1S, Supporting Information, shows the boiling point temperatures under atmospheric pressure, vapor pressure at 25°C, and lipophilicity, indicated by logP of the main components of each oil. Generally, the volatility of compounds increases as the boiling point decreases, and the vapor pressure is higher. Higher lipophilicity increases the penetration rate through membranes and should increase the amount of compound absorbed. Three of the inactive oils have lower boiling points and higher vapor pressures compared with those of the five active oils. The activities of the oil mixtures containing an inactive oil were strongly affected by the main components of the inactive oils, probably because the inactive oils had a high volatility and were thus dominant in the vapor. Some of the active oils have a lower vapor pressure; however, patchouli alcohol (patchouli), α-santalol (sandalwood), and calarene (spikenard) have a high lipophilicity and showed a fast effect because they are absorbed faster. These results suggest that the potency, volatility, and lipophilicity of the oil components may affect sedative activity.

Discussion

The sedative effect of galangal hexane extract administered by inhalation has been examined. Huang [11] reported that inhaling the hexane extract at doses of 1.5 and 10 mg produced a significant reduction in locomotor activity. The two main aromatic compounds, ethyl p-methoxycinnamate and ethyl cinnamate, showed sedative effects at doses of 0.0014 and 0.0002 mg, respectively [11]. In the present report, the distilled galangal oil showed sedative activity at a dose of $4 \times 10^{-4}$. The distilled oil produced a sedative effect at a lower dose than the hexane extract. Organic solvents extract both the volatile and the nonvolatile compounds, whereas the distilled oil only contains the volatile compounds. For inhalation, where the volatile compounds are most important, the distilled oil is more effective than the hexane extract, and this produced a difference in the effective dose. The distilled oil produced sedative activity at one-third of the dose of a single compound, suggesting that the sedative activity observed in this report was caused by the whole oil.

The most abundant component in patchouli was patchouli alcohol (66.2%). Patchouli alcohol has been reported to be responsible for the sedative activity of Microtomena patchouli essential oil. The effective dose of patchouli alcohol is 75–750 μg [12]. This dose is about 25-fold greater than the effective dose of patchouli oil in this report, which was $4 \times 10^{-3}$ mg. As was the case with galangal, patchouli essential oil has a higher sedative activity compared with its individual components.

The most abundant compounds in sandalwood, α-santalol and trans-β-santalol, contribute to the characteristic odor of sandalwood oil and have many pharmacological effects [13, 14]. Santalol caused a significant increase in total NREM sleep time [7], suggesting that it has sedative activity.
Twenty-six components were identified in spikenard essential oil, and most of them were sesquiterpene compounds. Cedarwood or sandalwood oils, which have high sesquiterpene contents, are often used for inhalation to produce relaxing effects. Calarene, $\alpha$-gurjunene [6], and $\beta$-maaliene [8] have sedative activities at doses of 0.17%, 1.5%, and 0.014–0.14%, respectively. In contrast, the active dose observed in this paper was $4 \times 10^{-5}$ mg. These results indicate that the sedative activity is caused by multiple sesquiterpene components rather than a single compound. Fifteen compounds were identified in clove essential oil. It has been reported that eugenol may improve learning and memory, although the dose dependency was not investigated [15]. Tianpeng et al. reported that inhaling eugenol changed the amount of neurotransmitters in the hippocampus and cortex. Levels of glutamate and choline acetyltransferase, an excitatory neurotransmitter, were significantly increased, whereas that of GABA, an inhibitory neurotransmitter, was not. These results may indicate that the elevation of the levels of excitatory neurotransmitters was caused by inhaling eugenol. The excitatory motion of the mice in this study, such as rapid movement and jumping, was consistent with these results. Inhaling eugenol appeared to produce a central nervous stimulatory effect.

Aromatic natural medicines have characteristic scents. The most abundant components may contribute heavily to the scent; however, the sedative effects of the essential oils do not appear to arise from single compounds, because oils consist of small amounts of various different compounds. The pharmacological effects observed in this study could not be related to the individual main components of the essential oils, suggesting that the sedative effect arose from the whole oil.

The mixture of the five active oils decreased the locomotor activity of the mice more than the individual oils did. This showed that the mixture of active oils produces synergistic effects. The decrease in locomotor activity was small compared with the increase in the number of components, and it did not reach twofold that of the individual oils. This applied to mixtures of active oils that did not contain a large amount of a single component or components with an excitatory effect. Patchouli alcohol, borneol, and isoborneol constituted more than 50% of the mixture because they are lower effective compounds and bigger amounts were required for their highest activity. However, the combination of the species of the compounds in the mixture oil was different from what it was in each oil, and it might be dangerous to conclude that the activity can be attributed to those large amount constituents. However, it is certain that the mixture of active oils produces synergistic effects.

A possible biological mechanism behind the pharmacological activity may involve the cerebral limbic system [16]. When a volatile component molecule reaches the nose, it binds to an olfactory receptor on an olfactory cell. The fragrance information is transmitted to the cerebral limbic system and then to the hypothalamus, where it affects the autonomic nervous system and the endocrine system. Serizawa et al. reported that mice have more than 1000 olfactory receptor genes, and each olfactory cell expresses a single olfactory receptor gene for a receptor that detects a volatile component molecule with a specific structure. The axons of each olfactory cell in an olfactory bulb extend to one of 2000 glomeruli and form a neural circuit; thus, each glomerulus corresponds to one olfactory receptor [17]. Assuming that the sedative effect observed in this study only arises from this pathway, the results for the mixture of the five active oils can be explained as follows: The mixture of the five active oils showed a synergistic effect, although the spontaneous activity did not decrease in proportion to the increase in the number of compounds. If the proposed mechanism is accurate, when many volatile component molecules compete to bind to a limited number of olfactory receptors, not all of the molecules will bind, and consequently the sedative activity should correspond to the number of receptors.

When the inactive cinnamon, clove, and star anise oils were added individually to the mixture of the five active oils, there was a significant decrease in sedative activity. Interestingly, none of the resultant mixtures exhibited sedative activity, despite the small ratio of the inactive oil to the active oil mixture of 1:5. The most abundant components of the cinnamon, clove, and star anise inactive oils are cinnamaldehyde, eugenol, and anethole, respectively. Iwasaki et al. reported that an intravenous injection

### Table 1 - Composition of a mixture of the five active oils.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI</th>
<th>Peak area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-Patchouline</td>
<td>1681</td>
<td>0.4</td>
</tr>
<tr>
<td>$\alpha$-Copaene</td>
<td>1688</td>
<td>0.3</td>
</tr>
<tr>
<td>Aristolene</td>
<td>1784</td>
<td>0.7</td>
</tr>
<tr>
<td>Calarene</td>
<td>1811</td>
<td>4.2</td>
</tr>
<tr>
<td>$\alpha$-Gurjunene</td>
<td>1839</td>
<td>1.2</td>
</tr>
<tr>
<td>Seychellene</td>
<td>1865</td>
<td>2.0</td>
</tr>
<tr>
<td>Caladrene</td>
<td>1882</td>
<td>0.8</td>
</tr>
<tr>
<td>Isoborneol</td>
<td>1885</td>
<td>6.8</td>
</tr>
<tr>
<td>Borneol</td>
<td>1916</td>
<td>23.4</td>
</tr>
<tr>
<td>$\alpha$-Bulnesene</td>
<td>1929</td>
<td>0.3</td>
</tr>
<tr>
<td>Anethole</td>
<td>2024</td>
<td>0.5</td>
</tr>
<tr>
<td>$\beta$-Ionone</td>
<td>2100</td>
<td>1.1</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>2133</td>
<td>0.8</td>
</tr>
<tr>
<td>$\psi$-Globulol</td>
<td>2181</td>
<td>0.6</td>
</tr>
<tr>
<td>Spathulenol</td>
<td>2206</td>
<td>1.9</td>
</tr>
<tr>
<td>Ethyl cinnamate</td>
<td>2213</td>
<td>0.8</td>
</tr>
<tr>
<td>Patchouli alcohol</td>
<td>2242</td>
<td>18.8</td>
</tr>
<tr>
<td>$\alpha$-Santalol</td>
<td>2309</td>
<td>1.9</td>
</tr>
<tr>
<td>$\beta$-Santalol</td>
<td>2343</td>
<td>2.3</td>
</tr>
<tr>
<td>Ethyl $\rho$-methoxycinnamate</td>
<td>2431</td>
<td>2.5</td>
</tr>
<tr>
<td>Total identified</td>
<td></td>
<td>71.1</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>28.9</td>
</tr>
</tbody>
</table>

*Order of elution is on an InertCap-Wax column; *RI is the retention index, calculated against C10–C26 n-alkanes on an InertCap-Wax column; Peak area percentage was determined by calculating the peak area of the FID chromatogram.
of cinnamaldehyde increased adrenaline secretion in rats. The secretion of adrenaline occurs through the activation of the transient receptor potential ankyrin1 ion channel, which is involved with transmission of pain by volatile irritants, such as allyl isothiocyanate or cinnamaldehyde [18]. The administration of GABA elicited a bimodal response in locomotion that was not dose-dependent; a lower dose elicited a small increase in locomotion, whereas a higher dose elicited a reduction. In addition, GABA attenuated locomotion, although it did not abolish locomotion in dopamine-stimulated locomotor activity [19]. Therefore, our results suggest that the three main compounds of the inactive oil produced adrenaline secretion and showed an excitatory activity, whereas the inhibitory activity was mediated by GABA. The effect is not dose-dependent and does not completely suppress excitatory activity, meaning that it shows an overall excitatory effect. We investigated the sedative effect of inhaling vapors from eight aromatic natural medicines using the decrease in spontaneous motor activity of mice as a model for relaxation. We also examined the effect of mixtures of the five active essential oils, and observed a synergistic effect. Our results suggest that fragrance materials that have experiential benefits may also have a pharmacological sedative effect. This evidence can be used as the basis for possible medical applications and product development.

Materials and Methods

Eight fragrant herbs, galangal (K. galanga), patchouli (P. cablin), sandalwood (S. album), spikenard (N. chinensis), cinnamon (C. cassia), clove (S. aromaticum), star anise (I. verum), and borneol (D. aromatica), were purchased from Mitsuboshi Pharmaceutical Co., Ltd. The batch numbers of the purchased products are as follows: 567A (K. galanga), 591A (P. cablin), 2687D (S. album), 591C (N. chinensis), 7015(C. cassia), 517A (S. aromaticum), 585B (I. verum), 09 144206 (D. aromatica). Voucher specimens of eight fragrant herbs were deposited in the herbarium of the Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University [specimen numbers: EST-5009 (K. galanga), EST-5010 (P. cablin), 2687D (S. album), 591C (N. chinensis), 7015(C. cassia), 517A (S. aromaticum), 585B (I. verum), 09 144206 (D. aromatica)].
dodecane, tetradecane, hexadecane, octadecane (Wako Pure Chemical Industries Co., Ltd.), docosane, tetracosane (Nacalai Tesque Co., Ltd.), eicosane, pentacosane, and hexacosane (Tokyo Kasei Co., Ltd.) were prepared as the gas chromatography retention indices. All other chemicals and reagents used in this study were of the highest grade available.

Distillation of aromatic natural medicines
The aromatic natural medicines (100 g, each), except for borneol, were hydrodistilled for 2 h using the clevenger-type apparatus designated in the Japanese Pharmacopoeia 16th edition, and the distilled oil was captured in hexane. The essential oil was dried over anhydrous sodium sulfate and stored at −20 °C before analysis and animal experiments.

Gas chromatography and gas chromatography mass spectrometry analysis
Qualitative analysis of the volatile components was performed by GC/MS (6890GC/5975MSD, Agilent Technologies) under the following operating conditions: fused silica capillary column, DB-Wax (Agilent Technologies), 60 m × 0.25 mm, film thickness 0.25 μm; column temperature program for galangal: 60–240°C increasing at 3 °C/min, holding at 240°C for 30 min; column temperature program for other materials: 60–210°C increasing at 3 °C/min, holding at 210°C for 30 min; injector, 100 °C; carrier gas, helium, 26 cm/min; split ratio, 100:1; injection volume, 1 μL; ionization energy, 70 eV. Quantitative analysis of volatile components was performed by GC (G5000, Hitachi) with an FID under the following operating conditions: fused silica capillary column, InertCap-Wax (GL Sciences), 60 m × 0.25 mm, film thickness 0.25 μm; column temperature program, same as for GC/MS; injector, 100 °C; detector for galangal, 250 °C; detector for other materials, 220 °C; carrier gas, helium, 0.8 cm/min; split ratio, 100:1; injection volume, 1 μL. The retention indices of the components were calculated on the InertCap-Wax column using n-alkane standards. The compounds were identified by comparing the fragmentation pattern of the mass spectra with those available from the National Institute of Standards and Technology and flavors libraries. Quantitative analysis was achieved with an FID.

Animals
Animal experiments were designed following the recommendations of the Animal Research Committee of Kyoto University, Kyoto, Japan (approval number 2012–18). Experimental procedures involving the use and care of animals conformed to the institutional guidelines, which comply with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan (2006). Male 4-week-old ddY mice were purchased from Japan SLC. The mice were housed in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity of 50 ± 10% with a 12-h light-dark cycle before being used for experiments. They were fed standard pellet chow and water ad libitum. All behavioral observations were conducted between 10:00 and 17:00 at the same temperature and humidity.

Evaluation of spontaneous motor activity
The sedative activities of the fragrant components were evaluated in the mice by their spontaneous motor activity in an open field test described in a previous report [6]. The distilled oils and borneol crystals were dissolved in triethyl citrate (400 μL total) at concentrations ranging from 4 × 10−4 to 4 × 10−2 mg (v/v) in a glass cage (W 60 × L 30 × H 34 cm). The samples were dropped onto four filter paper disks (100 μL each), which were placed on the wall of the glass cage using adhesive tape. The solution vapor was allowed to fill the cage by natural diffusion for 60 min. A mouse was placed in the center of the cage and was monitored by a video camera for another 60 min. The frequency at which the mouse crossed the lines drawn on the bottom of the cage at 10 cm intervals was counted every 5 min for 60 min. AUC, indicating total locomotor activity over 60 min, was calculated by the trapezoidal rule [20]. All values are expressed as the mean ± SEM. Statistical analyses were carried out using Dunnett’s test using GraphPad Instat3 (GraphPad Software). A probability level of p < 0.05 was taken to be statistically significant in the analysis. The results were reproducible; therefore, five mice from each administration group were chosen at random for statistical analysis.

Supporting information
Analysis results for the eight essential oils, boiling point, vapor pressure, and lipophilicity of their main components, as well as structures of the two main components of each essential oil are available as Supporting Information.

Conflict of Interest
The authors declare no conflict of interest.

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