福岡大医紀（*Med. Bull. Fukuoka Univ.*）：48(1), 31-38, 2021

Effects of Inhaled Linalool on Anxiety-Related Behaviors and Frontal Cortical Serotonin Levels in Mice

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Abstract

The scent of plants is known to affect the mental and physical condition of animals. Linalool is one of the main components of lavender scent, and its inhalation has been suggested to have an anxiolytic effect in animals ; however, its mechanism has been unclear.

In this study, we conducted behavioral analysis experiments using an elevated plus-maze test and measured monoamine levels in prelimbic prefrontal cortex（PL-PFC）, a brain region thought to be related to anxiety, using microdialysis to investigate the mechanism underlying the anxiolytic effects of linalool inhalation in mice, and the mechanism underlying the effects of linalool's inhalation on brain transmitters. In an elevated plus maze test, the behavior of the mice in the maze was recorded for 5 minutes and their spontaneous locomotor activity and anxiety-related behaviors were assessed. While preforming microdialysis samples were 4 continuously collected and measured every 10 minutes for a total of 180 minutes from 120 minutes before treatment to 60 minutes after treatment.

The results showed that the inhalation of linalool prolonged the rate of time spent in the open arm of the elevated plus-maze test（Saline：3.12±1.72%, Linalool：33.17±11.49, p=0.037）. Microdialysis measurement of the frontal cortical serotonin concentration showed that serotonin the concentration was significantly increased in the diazepam injection group in comparison to the control group, whereas no significant change was observed in the linalool inhalation group（Saline：95.36±13.64%, Linalool：62.2±11.49, p=0.037, Diazepam：191.99±47.28, p=0.0082）. This suggests that the mechanism of the anxiolytic effect of linalool may not be involved in the serotonergic transmission of 5-HT1A receptors in the PL-PFC.

Key words : Aromatherapy, Anxiety, drug therapy, Behavior, Animal, drug effects, Monoterpenes, therapeutic use, Plant Extracts, pharmacology

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Introduction

Aromatherapy using essential oils is an ancient form of traditional medicine and folk medicine that has been practiced in Egypt, The Arabian peninsula, and Europe since ancient times[10)11)]. In modern times, aromatherapy is widely used around the world for personal physical and mental health, relaxation, stress relief, and the relief of chronic pain, depression, cognitive disorders, anxiety, insomnia, and stress-related disorders[12)]. In Japan, led by essential oil companies and the beauty and cosmetic industries, it is used as a hobby, beauty treatment, and relaxation tool. In the medical and nursing care fields, it is used as an alternative therapy to alleviate pain, reduce anxiety. It is also used to improve the environment of hospital rooms.

The main physiological effects of aromatherapy, which are sedative[1)-4)], anxiolytic[5)-7)], and antidepressant effects[8)], have been extensively reported. Many studies have also been conducted to elucidate the effects of plant-derived essential oils and aromatic species used in aromatherapy on the central nervous system (CNS) in animals and humans[2)3)13)14)7)15)16)]. Among them, the dose-dependent sedative effect of linalool[17)] is a common component of major essential oils (e.g., lavender, citrus, lemon balm, lemongrass, rosemary, and bergamot) traditionally used in sedatives, analgesics, sleeping pills, and anxiolytics. In addition, anticonvulsant effects related to the regulation of glutamate transmission[17)19)], which affects autonomic neurotransmission and antihypertensive effects have been reported[20)]. Furthermore, the inhalation of linalool has been reported to have sedative and anxiolytic effects, increasing social interaction and reducing aggressive behavior[25)].

However, research on the mechanisms and psychopharmacological properties of essential oil inhalation in the brain remain scarce. In the present study, we measured the serotonin concentration in extracellular fluid in the prelimbic prefrontal cortex (PL-PFC) using microdialysis and performed a behavioral analysis to investigate the mechanism of the anxiolytic effects of inhaled linalool in mice.

Materials and Methods

Animals

This experiment was conducted using 36 six-week-old male C57BL/6N mice purchased from CLEA Japan, Inc. Mice were randomly assigned to the linalool group (n=12), the diazepam group (n=12), and the control group (n=12). During the two weeks leading up to the experiment, the mice were housed in a control room at 22±1℃ in three cages on a 12-hour light/dark cycle (lit at 8:00 AM) with ad libitum access to food and water. In addition, the mice were handled twice for two minutes per time during the period leading up to the experiment. Mice were moved from the rearing room to the laboratory on the day of the experiment and were divided into the different treatment groups. The protocol for this experiment was in accordance with the guidelines for the welfare and use of laboratory animals of the National Institute of Health and was approved by the Ethical Review Committee for Animal Experiments of Fukuoka University (approval number 1805012).

Materials

In this study, linalool and diazepam were acquired from Sigma-Aldrich Japan. Saline was administered to the control groups.

Inhalation device

The inhalation device was constructed from a double-bottomed plastic container capacity: 4.4L). Perforated aluminum board and a piece of filter paper (50×50 mm square filter paper) were placed underneath it for linalool transpiration. In the linalool exposure group, 5μl of linalool was dripped into the container to make the concentration of linalool in the container 1 mg/l when it was completely volatilized, and the mice in the container inhaled it. The linalool concentration was determined based on previous studies[21)]. In the control group, saline was dripped and transpired using the same apparatus. A hole (diameter: 3.5 cm) was drilled in the center of the container lid for breathing and the insertion of microdialysis tubes. The containers were washed and dried after each experiment to ensure that there were no residual aromatic components.

Administration of anti-anxiety drugs

Diazepam was intraperitoneally injected at a dose of 10 mg/kg.

Microdialysis

Microdialysis was performed with reference to the methods of previous studies[22)]. After acclimatization mice

were anesthetized by the intraperitoneal administration（10 μl/g）of three mixed anesthetics（medetomidine 0.4 mg/kg, midazolam 2.0 mg/kg, and butorphanol 2.5 mg/kg）and the fixation of a stereotaxic device（Kopf 900；David Kopf Instruments, Tujunga CA）. After fixation, a microdialysis guide cannula was embedded in the PL-PFC（AP ＋1.9 mm, L ＋0.3 mm, and V −1.8 mm from bregma）The guide cannula was fixed to the skull using bone anchor screws and dental resin（acrylic cement）. Postoperatively, the animals were housed in a single individual cage for at least 24 hours until the microdialysis experiments were performed. The microdialysis probe（CX-01；EICOM, Kyoto, Japan）was perfused continuously at a rate of 2.0 μl/min. At 120 min after the start of probe perfusion, both the linalool and control groups were exposed and diazepam was administered by intraperitoneal injection to the diazepam group.

Dialysate samples were collected every 10 minutes for 120 minutes before exposure or administration and 60 minutes after exposure or administration, and high-performance liquid chromatography（HPLC）of the resultant samples was preformed to measure monoamines in the brains of the mice at the time of exposure. We also assessed anxiety-like behavior with an elevated plus maze（EPM）test as a behavioral experiment after perfusion was completed.（At the end of each experiment, the mice were decapitated and the position of the dialysis probe was checked）.

Elevated plus maze test

The anxiolytic-like effects of linalool were evaluated using an elevated plus-maze test[23]. Serotonin levels were measured by microdialysis, then the EPM test was performed for five minutes.

The EPM apparatus consisted of a plastic crossed open arm（50×10 cm）, a closed arm（50×10×40 cm）and a central square（10×10 cm）. The device was 60 cm in height（Fig. 1）. The maze was placed in a room that was artificially illuminated by three 60 W white fluorescent lights. All evaluated parameters were videotaped during the 5-min session and analyzed by Panlab Smartver 3.0.06.

Statistical analysis

An ANOVA and Dunnett's tests were performed to analyze the results of each procedure. The JMP software profram（version 12.2）was used to perform all of the analyses. The results were expressed as the mean±standard error of the mean. P values of ＜0.05 were considered to indicate statistical significance.

Fig. 1：Elevated plus maze test（EPM）
The elevated plus maze consisted of a plastic crossed open arm（50×10 cm）, a closed arm（50×10×40 cm）, and a central square（10×10 cm）with a 60 cm high device.

Results

Elevated plus-maze test

As shown in Figure 2, in the EPM test, no significant differences were observed among the linalool-inhalation, diazepam-injection, and control groups in terms of the total travel distance. A comparison by analysis of variance showed significant results, so a comparison of the groups by Tukey-kramer's SD test showed no significant difference. Therefore, we concluded that it cannot be said to show a sedative effect. (ANOVA : $p=0.048$; Tukey-Kramer : Saline vs. Linalool, $p=0.060$; Saline vs. Diazepam, $p=0.113$; Diazepam vs. Linalool, $p=0.949$)

The time spent in the open arm by the linalool-inhalation group was significantly increased in comparison to the control group ($P<0.05$). This trend was also observed in the diazepam-injection group a ; however, the difference did not reach statistical significance (Figure 3-A). The distance traveled within the open arm was also significantly increased in the linalool-inhalation and diazepam-injection groups in comparison to the control group, as shown in Figure 3-B ($P<0.05$).

Microdialysis experiment

Based on the results of the measurements, the serotonin concentrations in the material before and after administration were calculated and the change in the serotonin concentration after administration was compared to the pre-dose levels. As a result, the serotonin peak in the diazepam-injection group was significantly increased in comparison to the control group. On the other hand, the group that inhaled linalool showed a decrease in the serotonin peak ; however, this was not superior to that in the control group (Figure 4).

Discussion

Anxiety-related disorders are among the most common psychiatric disorders in modern society. Approximately 5.3% of Japanese adults and 18.2% of U.S. adults have met the diagnostic criteria for at least one anxiety disorder within the past 12 months[24], and the development of effective treatments and medications for anxiety disorders seems very important. The first-line medications for anxiety disorders include serotonin selective reuptake inhibitors (SSRIs), benzodiazepines that act on gamma-aminobutyric acid (GABA) receptors, and serotonin receptor agonists, which are associated with numerous side-effects (headache, somnolence, dizziness, sexual dysfunction, and serotonin syndrome) ; in particular, benzodiazepine anxiolytics are associated with harmful effects such as dependence, abuse, retrograde amnesia, and hyper-sedation. Thus there is a need for new solutions.

Various alternative medicines are used to supplement the weaknesses of Western medical approaches. Among them, aromatherapy is considered a useful tool for its sedative and anxiolytic effects due to its long history, simple method of using essential oils extracted from plants by

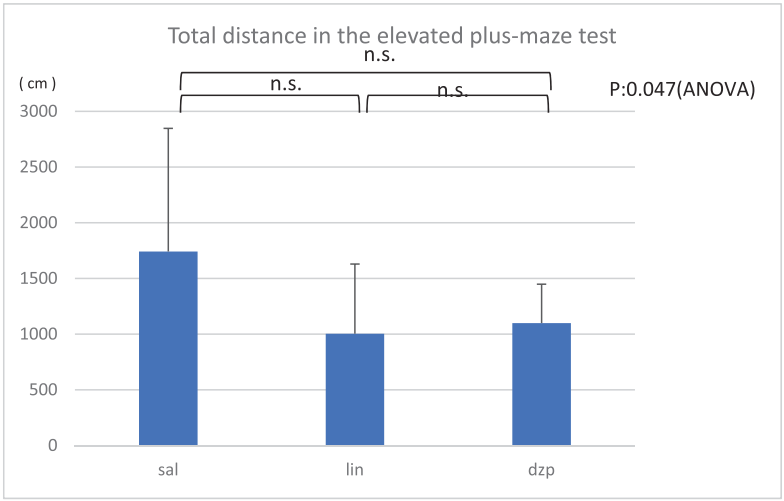

Fig. 2 : Total distance in the elevated plus-maze test.
Mice on an elevated plus maze were recorded for 5 minutes and assessed for spontaneous locomotor activity and anxiety states.
Data are presented as means±SEM from12 mice.
Abbreviations ; sal for saline, lin for linalool, dzp for diazepam, n.s. for not specific, and SEM for standard error of the mean.

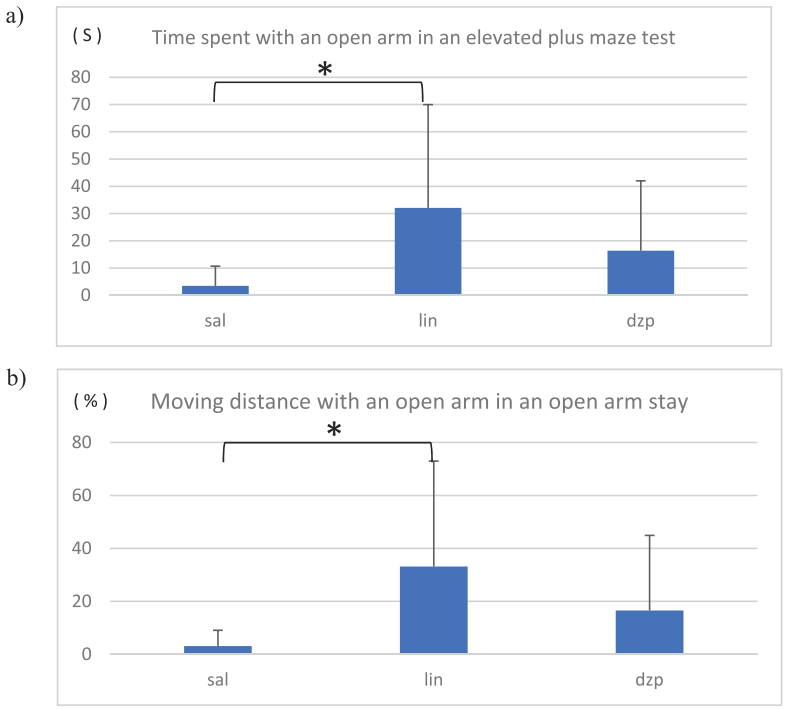

Fig. 3：a）Time spent with in the an open arm in an elevated pluse maze test.
b）Moving distance within the open arm in an open arm stay
Mice on an elevated plus were recorded for 5 minutes and assessed for spontaneous locomotor activity and anxiety states.
Data are presented as means±SEM from12 mice.
*：$p<0.05$, vs. saline
Abbreviations；sal for saline, lin for linalool, dzp for diazepam, and SEM for standard error of the mean.

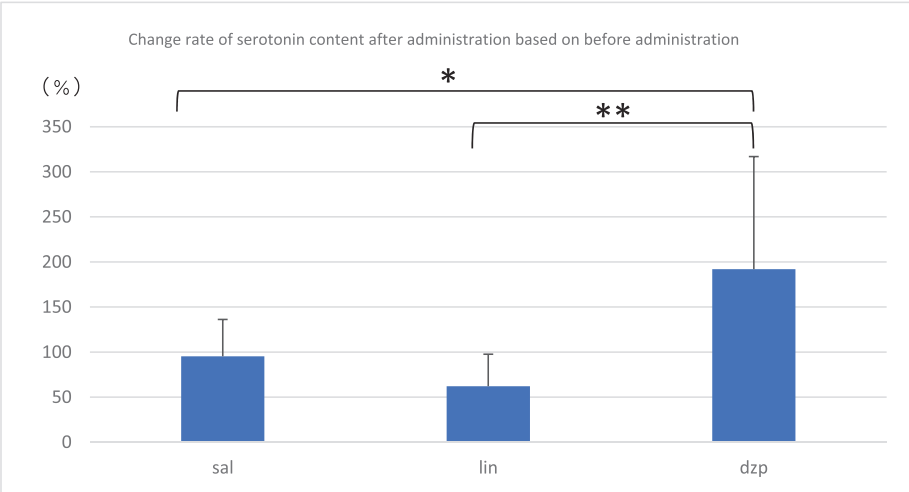

Fig. 4：Mean percentage change in serotonin concentration after treatment versus before treatment.
Samples were collected continuously for a total of 180 minutes from 120 minutes before to 60 minutes after treatment and measured collectively every 10 minutes.
Data are presented as means±SEM from12 mice.
*：$P<0.05$ vs. saline **：$P<0.05$ vs. linalool
Abbreviations；sal for saline, lin for linalool, dzp for diazepam, and SEM for standard error of the mean.

inhalation or application, and the fact that it has still left the realm of use as a relaxation method in Japan[1)–7)].

In aromatherapy, a wide variety of plant essential oils can be used depending on the symptoms and the purpose for which aromatherapy is beins preformed. In the present study, using an EPM test we confirmed that exposure to linalool— a common component of essential oils traditionally used as sedatives, analgesics, sleep medications, and anxiolytics—induced anxiolytic effects in mice. This result was consistent with previous studies[25)–27)].

First, in the EPM test, total distance traveled by the control, diazepam, and linalool groups did not differ to a statistically significant extent indicating that neither the inhalation of linalool nor the injection of diazepam had a sedative effect during the behavioral analysis. Benzodiazepines usually have anxiolytic and sedative effects, however, the benzodiazepine group in this study showed an anxiolytic effect without sedative effects. In the linalool–inhalation group, although the effect of linalool was attenuated by the diffusion of the aroma, the route of administration（i.e., inhalation）enabled continuous administration for a relatively long period of time and its effect was not accompanied by sedation.

Second, the inhalation of linalool significantly increased both the time spent in the open arm and the distance traveled in the open arm and had a stronger anxiolytic effects in comparison to those observed in either the control group or the diazepam–injection group.

Although the anxiolytic effects of linalool inhalation have been widely reported[1)5)–7)], the mechanism of this effect is often unknown. In the present study, we conducted real–time microdialysis experiments in the control, diazepam–injection, and linalool–inhalation groups.

The brain serotonin concentration after the intraperitoneal administration of diazepam was significantly increased in comparison to the control group, as shown in Figure 4. This result may reflect the anxiolytic effect of diazepam, as reported previous study[28)].

On the other hand, in the linalool group, brain serotonin levels in the PL–PFC during the inhalation period were lower than those in the control group, although the difference was not statistically significant.

Some previous studies have reported that the administration of flumazenil completely abolished the anxiolytic effects of linalool exposure, while the administration of WAY100635, a 5–HT1A receptor antagonist, was not associated with a significant changes in anxiolytic effects[29)–31)]. Taken together with the results of the present experiments, the anxiolytic effects elicited by linalool inhalation are based on a mechanism that does not involve serotonergic transmission associated with the PL–PFC 5–HT1A receptor（e.g., a GABA receptor–related anxiolytic mechanism）or a brain region that is not included in the PL–PFC（e.g., the hippocampus）. Thus, further research on these targets would improve our understanding of the mechanisms of aromatherapy–induced anxiolysis. Since aromatherapy is widely used as a traditional alternative therapy, it is presumed that patients with anxiety disorders often use aromatherapy in conjunction with pharmacotherapy for self–care or in mental clinic waiting rooms. However, these results suggest that linalool and benzodiazepines have different serotonergic properties in the PL–PFC, a brain region related to anxiety, which may cause unintended effects such as counteraction, attenuation, or even potentiation when used together. The risks of the concomitant use of multiple medications —not just benzodiazepines— in clinical practice for anxiety disorders and depression have been discussed in recent years；thus, alternative therapies such as aromatherapy are gaining attention as a treatment with fewer side–effects.

Further research should be conducted to investigate on the pharmacological mechanism of action of aromatherapy and its interaction with anxiolytic drugs. Specifically, studies should be performed to measure the effects in brain regions outside the PL–PFC and to analyze the effects of aromatherapy in a more continuous manner. Furthermore, validation experiments should be conducted using the combination of benzodiazepines and linalool, and to compare the effects of aromatherapy with those of anxiolytic drugs other than benzodiazepines.

Funding Sources

This research received no specific grant from any funding agency in the public, commercial, or not–for profit sectors.

Acknowledgements

We thank Sydney Kevin Heintz, from WORDVICE Group（https://wordvice.jp/）and Brian Quinn, from Japan Medical Communication for editing a draft of this manuscript.

References

1) Lehrner J, Eckersberger C, Walla P, Postsch G, Deecke L：Ambient odor of Orange in a dental office reduces anxiety and improves mood in female patients. Physiology & Behavior 71：83-86, 2000.
2) Buchbauer G, Jirovetz L, Jager W, Dietrich H, Plank C：Aromatherapy evidence for sedative effects of the sential oil of lavender after inhalation. Z. Naturforsch. [C]：1067-107246, 1067-1072, 1991.
3) Buchbauer, G., Jirovetz, L., Jager, W., Plank, C., Dietrich, H：Fragrance compounds and essential oils with sedative effects upon inhalation. Journal of Pharmaceutical Sciences. 82：660-664, 1993.
4) Elisabetsky, E., Souza, G. P. C., Santos, M. A. C., Siqueira, I. R., Amador, T.：Sedative properties of Linalool. Fitoterapia 15(5)：407-414, 1995.
5) Carvalho-Freitas, M. I. R., Costa, M.：Anxiolytic and sedative effects of extracts and essential oils from Citrus aurantium L. Biological and Pharmaceutical Bulletin 25(12)：1629-1633, 2002.
6) Cooke, B., Ernst, E.：Aromatherapy a system at icreview. British Journal of General Practice 50：493-496, 2000.
7) Diego, M. A., Jones, N. A., Field, T., etal., Aromatherapy positively affects mood, EEG pattern sofalertness and math computations. International Journal of Neuroscience 96(3-4)：217-224, 1998.
8) Komori, T., Fujiwara, R., Tanida, M., Nomura, J：Potential antidepressant effects of lemon odor in rats. Eur. Neuropsychopharmacology. 5(4)：477-480, 1995.
9) Linck, V. M., daSilva, A. L., Figueiro, M., Piato, A. L., Herrmann, A. P., Birck, F. D., Caramao, E. B., Nunes, D. S., Moreno, P. R. H., Elisabetsky, E：Inhaled linalool induced sedation in mice. Phytomedicine 16(4)：303-307, 2009.
10) Buchbauer, G：Uber biologische Wirkungen von Duftstoffenund atherischen Olen. Wiener Medizinische Wochenschrift 154：539-547, 2009.
11) Toda, M., Morimoto, K.：Effect of lavender aroma on Salivary endocrinological stress markers. Archives of Oral Biology 53(10)：964-968, 2008.
12) Perry, N., Perry, E.：Aromatherapy in the management of psychiatric disorders clinical and neuropharmacological perspectives. CNS Drugs 20(4)：257-280, 2006.
13) Hongratanaworakit, T., Buchbauer, G：Relaxing effects of ylangylang oil on humans after transdermal absorption. Phytotherapy Research 20(9)：758-763, 2006.
14) Gedney, J. J., Glover, T. L., Fillingim, R. B.：Sensory and affective pain discrimination after inhalation of essential oils. Psychosomatic Medicine 66：599-606, 2004.
15) Buckle, J：Aromatherapy. Nursing Times 89：32-35, 1993.
16) Moss, Cook, J., Wesner, K., Duckett, P.：Aromas of rosemary and lavender Essential oils differentially affect cognition and mood in healthy adults. International Journal of Neuroscience. 113：15-38, 2003.
17) Elisabetsky, E., Brum, L. F., Souza, D. O.：Anticonvulsant properties of linalool in glutamate-related seizure models. Phytomedicine 6(2)：107-113, 1999.
18) Brum, L. F., Elisabetsky, E., Souza, D. O：Effects of linalool on [(3)H] MK801 and [(3)H] muscimol binding in mouse cortical membranes. Phytotherapy Research. 15：422-425, 2001.
19) SilvaBrum, L. F., Emanuelli, T., Souza, D. O., Elisabetsky, E.：Effects of linalool on glutamate release and uptake in mouse cortical synaptosomes. Neurochemical Research. 26(3)：191-194, 2001.
20) Tanida et al：Olfactory stimulation with scent of lavender oil affects autonomic neurotransmission and blood pressure in rats, Neuroscience Letters 398：155-160, 2006.
21) Flávia Negromonte Souto-Maior, Fabíola Lélis de Carvalho：Anxiolytic-like effects of inhaled linalool oxide in experimental mouse anxiety models. Pharmacol Biochem Behavior 100(2)：259-63, 2011.
22) Saitoh, A., Suzuki, S., Soda, A., Ohashi, M., Yamada, M., Oka, J. I., Yamada, M：The delta opioid receptor agonist KNT-127 in the prelimbic medial prefrontal cortex attenuates veratrine-induced anxiety-like behaviors in mice. Behavioural Brain Research, 336：77-84, 2017.
23) Pellow S, Chopin P, File SE, Briley M：Validation of open：closed arm enries in an elevated plus-maze as a measure of anxiety in the rat. Journal of Neuroscience Methods. 14：149-167, 1985.
24) Demyttenaere, K., Bruffaerts, R., Posada-Villa, J., Gasquet, I., Kovess, V., Lepine, J. P., et al：Prevalence, severity, and unmet need for treatment of mental disorders in the World Health Organization world mental health surveys. Journal of the American

Medical Association. Vol. 291, issue 21：2581 90：1538−3598, 2004.
25）Linck V. M., Da Silva A. L., Figueiro M., Caramao E. B., Moreno P. R., Elisabetsky E：Effects of inhaled Linalool in anxiety, social interaction and aggressive behavior in mice. Phytomedicine 17：679−683, 2010.
26）Takahashi M., Satou T., Ohashi M., Hayashi S., Sadamoto K., Koike K：Interspecies comparison of chemical composition and anxiolytic−like effects of lavender oils upon inhalation. Natural Product Communications. 6：1769−1774, 2011.
27）Zhang N., Zhang L., Feng L., Yao L.：The anxiolytic effect of essential oil of Cananga odorata exposure on mice and determination of its major active constituents. Phytomedicine 23：1727−1734, 2016.
28）Stancheva S, Petkov VV：Effects of chronic diazepam and medazepam treatment on the level of biogenic monoamines in different rat brain areas. Acta Physiol Pharmacol Bulg. 14(3)：26−31, 1988.
29）Rudolph U, Crestani F, Benke D, Brunig I, Benson J A, Fritschy J M, et al.：Benzodiazepine actions mediated by specific g−aminobutyric acidA receptor subtypes. Nature 401：796−800, 1999.
30）Low K, Crestani F, Keist R, Benke D, Brunig I, Benson J A, et al.：Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290：131−134, 2000.
31）Harada H, Kashiwadani H, Kanmura Y, Kuwaki T：Linalool odor−induced anxiolytic effects in mice. Frontiers in Behavioral Neuroscience 12. 241, 2018.

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「The authors declare no conflict of interest.」