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Research report

Lemon oil vapor causes an anti-stress effect via modulating the 5-HT and DA activities in mice

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Abstract

We examined the anti-stress action of the essential oils of lavender, rose, and lemon using an elevated plus-maze task (EPM), a forced swimming task (FST), and an open field task (OFT) in mice. Lemon oil had the strongest anti-stress effect in all three behavioral tasks. We further investigated a regulatory mechanism of the lemon oil by pre-treatments with agonists or antagonists to benzodiazepine, 5-HT, DA, and adrenaline receptors by the EPM and the FST. The anti-stress effect of lemon oil was significantly blocked by pre-treatment with frumazenil, benzodiazepine receptor antagonist, or apomorphine, a nonselective DA receptor agonist. In contrast, agonists or antagonists to the 5-HT receptor and the alpha-2 adrenaline receptor did not affect the anti-stress effect of lemon oil. Buspirone, DOI, and mianserine blocked the antidepressant-like effect of lemon oil in the FST, but WAY100,635 did not. These findings suggest that the antidepressant-like effect of lemon oil is closely related with the 5-HTnergic pathway, especially via 5-HT_{1A} receptor. Moreover, the lemon oil significantly accelerated the metabolic turnover of DA in the hippocampus and of 5-HT in the prefrontal cortex and striatum. These results suggest that lemon oil possesses anxiolytic, antidepressant-like effects via the suppression of DA activity related to enhanced 5-HTnergic neurons.
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Keywords: Lemon oil; Distress; Anxiolytic; Antidepressant; 5-HT; DA

1. Introduction

Pure fragrance compounds and essential oils have various effects on human and other mammalian species when inhaled or ingested, and many studies of these compounds and oils have been conducted. Some of those effects, such as the control of emotion and mood, (e.g. sedative [4,5,29], anxiolytic [6,9,11,12,29], antidepressant [26,27], hypnotic [11,36,38], alert [11,38]), antispasmodic [6], control of the autonomic nervous system activity [20,35] and endocrine system [7,30], strengthening of the immune system by stimulating the production of white blood cells [16], pain mitigation [7], anti-tumor [17], increase in lipolysis [31] etc., has been documented. Among the properties of pure fragrance compounds and essential oils, the emotional and behavioral modulations, although there are many anecdotal or empirically speculated efficacious effects, is often difficult to examine and demonstrate in a scientifically controlled conditions. Furthermore, the mechanisms of the effects of each fragrance compound or essential oil have not yet been made clear and seem to differ for each fragrance.

Some studies have suggested that essential oils affect the modulation of the central neurotransmitter system. Linalool, a major component of lavender oil, is reported to have an effect on glutamate receptors in vitro [15]. Pinene, which is one of the components of lemon oil, and lavender, hinokitiol, eugenol, citronellol, and citronellal are reported to potentiate the responses in the presence of GABA at low concentrations and inhibit the responses in the presence of GABA at high concentrations in vitro [1]. *Hypericum perforatum* L (St. John's wort) is supposed to inhibit the synaptosomal uptake of 5-HT in rats [19], and therefore the 5-HT concentration of basal nuclei may increase. In addition, *H. perforatum* L is also reported to increase extracellular dopamine levels in the rat prefrontal cortex [40].

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Among the many effects of essential oil, anxiolytic and antidepressant effects are very helpful for psychiatry and psychopharmacology, since combining the medicine and essential oil can reduce the dose of those medicines and essential oil may help prevent the side effects of the anxiolytic and antidepressant medicines. All clinically available anxiolytics and antidepressants have limited clinical efficacy because of their adverse side effects, such as the amnestic effect of benzodiazepine.

In the present study, we examined the effects of inhaling lavender, rose and lemon oil vapor on mice in behavioral analyses, using the elevated plus-maze task (EPM), the open field task (OFT) and the forced swimming task (FST). Furthermore, we investigated which neurotransmitter activity was related to the anti-stress effect, by using agonists or antagonists to the benzodiazepine (BZP), 5-HT, DA and adrenaline receptors in behavioral tasks, and measured their metabolic turnovers in the prefrontal cortex, the hippocampus, and the striatum by HPLC.

2. Materials and methods

2.1. Animals

ICR strain mice were obtained from Tokyo Laboratory Animal Co., Ltd. (Tokyo, Japan) at 5 weeks old and used in all experiments after an adaptation period of 1 week. Male mice were used, because female rats showed a reduced aversion to the open arms compared to male rats in the EPM [22] and we observed similar results in mice. The mice were maintained at a controlled temperature ($22 \pm 2\,^{\circ}C$) and on a regular light/dark cycle (7:00–19:00 h, light), and all animals had free access to food and water. The animals were naive to the essential oil and drugs, and each mouse was used once only. The number of animals per group was five or 10. All experiments were conducted in accordance with the guidelines regarding the care of experimental animals, as approved by the Animal Research Committee at Tottori University.

2.2. Inhalation of essential oil

The essential oils of lavender, rose, and lemon were supplied by Soda Aromatic Co., Ltd. (Tokyo, Japan). To be efficiently vaporized, the essential oil was mixed with the same volume of ethanol and was then soaked up by cotton set on the upper side of an inhalation box. In all experiments, 1 ml of the essential oil was vaporized in the container (30 cm × 23 cm × 15 cm). Ethanol was applied as the control treatment. The inhalation of essential oil was started 90 min before the behavioral tasks.

2.3. Drug application

Diazepam (0.5 mg/kg, 1.0 mg/kg) (Takeda Pharmaceutical Co. Ltd, Osaka, Japan), a BZP receptor agonist, was diluted with 0.9% saline. Flumazenil (1.0 mg/kg, 5.0 mg/kg), a BZP receptor competitive antagonist, buspirone (2.0 mg/kg, 6.0 mg/kg), a 5-HT_{1A} receptor agonist, WAY100,635 (0.1 mg/kg, 0.3 mg/kg, 1.0 mg/kg), a 5-HT_{1A} receptor antagonist, (±)-2-5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) (0.3 mg/kg, 1.0 mg/kg), a 5-HT_{2A} receptor agonist, mianserin (1.0 mg/kg, 3.0 mg/kg), a 5-$HT_{2A/C}$ receptor agonist, fluoxetine (1.8 mg/kg, 3.0 mg/kg, 3.5 mg/kg, 10.0 mg/kg), a selective serotonin reuptake inhibitor (SSRI), imipramine (30 mg/kg, 50 mg/kg), a tricyclic antidepressant and a 5-HT and NA uptake inhibitor, apomorphine (0.5 mg/kg, 1.5 mg/kg, 3.0 mg/kg), a nonselective DA receptor agonist, clonidine (0.05 mg/kg, 0.1 mg/kg), a selective α2 adrenergic receptor agonist, and yohimbine (0.5 mg/kg, 1.5 mg/kg, 2.5 mg/kg, 4.0 mg/kg), a selective α2 adrenergic receptor antagonist (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in 0.9% saline. Haloperidol (0.5 mg/kg, 1.0 mg/kg), a D_2, D_3, D_4 receptor antagonist, was dissolved in acidic 0.9% saline under heated condition. All drugs and vehicle were injected intraperitoneally and an injection volume was 0.2 ml/30 g.

2.4. Behavioral analyses

2.4.1. Elevated plus-maze task

The EPM was performed according to the protocol used in the previous report with slight modifications. In brief, the mice were placed in the experimental room to facilitate adaptation at least 1 h before the task. All tasks were carried out during the light period (11:00–15:00), in a counterbalanced random order. Each mouse was placed at the center of crossed arms, and then its behavior was recorded for 5 min using a video camera (CCD-TR 313, Sony, Tokyo, Japan). Between subjects, the maze floor was thoroughly cleaned with cotton and ethanol. The mice received an injection of saline or vehicle or drugs 30 min before the task, except fluoxetine and imipramine. Fluoxetine was injected 120 min before the task, and imipramine was injected 60 min before the task.

Parameters consisted of the percentage of entry into the open arms and the amount of time spent on the open arms, which were determined by replaying the videotape.

2.4.2. Forced swimming task

The FST was carried out according to the method of Porsolt et al. [32]. Mice were dropped individually into glass cylinders (height 25 cm, diameter 10 cm) containing 10 cm of water, maintained at 23–25 °C. Mice were left in the cylinder for 6 min. After the first 2 min, the total duration of immobility in mice was measured during a 4 min task. The mouse was judged to be immobile when it remained floating passively in the water. The mice received an injection in a way similar to that used in the EPM.

2.4.3. Open field task

The OFT apparatus consisted of a clear acrylic box (30 cm × 30 cm × 35 cm) with a lid. The floor was divided by drawn lines into 36 area of about 25 sq. cm. The task was performed in a light- and sound-attenuated shield box with a dim light. The mice received an injection of saline (i.p.) 30 min before the task.

Each mouse's behavior was then recorded by video camera for 45 min. We evaluated the following behavior at 5 min intervals: the locomotor activity counts (the frequency across the squares) and the rearing counts (the frequency of rearing around the wall). Between subjects, the box was thoroughly cleaned with cotton and ethanol.

2.5. Quantification of monoamines and their metabolites by HPLC

Ninety and 180 min after mice were treated with the inhalation of lemon oil, they were decapitated and the whole brain of each animal was removed and immediately stored at −80 °C until monoaminergic determination.

The samples were homogenized in 1 ml of 0.1 M potassium perchlorate containing 0.2 mM sodium bisulfite and 0.2 mM EDTA2Na, and 10 ng isoproterenol was added to each sample as an internal standard to control for procedural losses. The homogenates were centrifuged (20,000 × *g*, 15 min at 0 °C), and supernatants were used for monoamine determination using high performance liquid chromatography with electrochemical detection (HPLC-ECD, Eicom, Kyoto, Japan).

Separation of monoamines was performed according to the procedure described previously [37]. Monoamines dopamine (DA) and serotonin (5-HT) as well as their main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-3-indoleacetic acid (5-HIAA), were measured. Standards were run concurrently, and concentrations of unknowns were determined by comparison to peak areas of standards after correction for recovery of the internal standard.

2.6. Statistical analysis

All results are expressed as means ± S.E. Differences between treatment groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A probability level of $P < 0.05$ was taken to be statistically significant in the analyses.

3. Results

3.1. Behavioral changes by inhalation of essential oils

Total numbers of entries into the open and closed arms in the EPM were not significantly changed by inhalation of ethanol, lavender, rose, or lemon oil. On the other hand, the percentage of entries into the open arms was significantly increased in the lemon oil-treated mice ($F(2, 30) = 18.29$; $P < 0.0001$) (Fig. 1A-b). The time spent on the open arms was also significantly increased in the lemon oil-treated mice ($F(2, 30) = 8.024$; $P = 0.0018$) (Fig. 1A-c).

An antidepressant effect of lemon oil was more apparent in the FST ($F(2, 15) = 5.841$; $P = 0.0169$) (Fig. 1B). Lavender and rose oil had no effect on the immobility time in the FST.

In the OFT, both the locomotor activity and the rearing counts for 45 min were lower in the mice treated with lavender or lemon oil inhalation than in mice in other groups (Fig. 1C), and a significant effect was determined only in the lemon oil-treated group ($F(2, 15) = 6.936$; $P = 0.0113$ and $F(2, 15) = 11.53$; $P = 0.0020$). Lavender oil had tendencies to decrease in the locomotion and rearing counts, however those were not significant from ethanol-treated mice.

These results show that lemon oil vapor has anxiolytic, antidepressant-like, and sedative effects and suggest that lemon oil vapor has a suppressive effect on distress in mice.

3.2. Role of GABA receptor in the anti-stress effect of lemon oil

We used diazepam, a BZP receptor agonist, and flumazenil, a BZP receptor competitive antagonist, to estimate the role of GABA receptor in the lemon oil-induced anti-stress reaction. Diazepam and flumazenil were injected 30 min before the

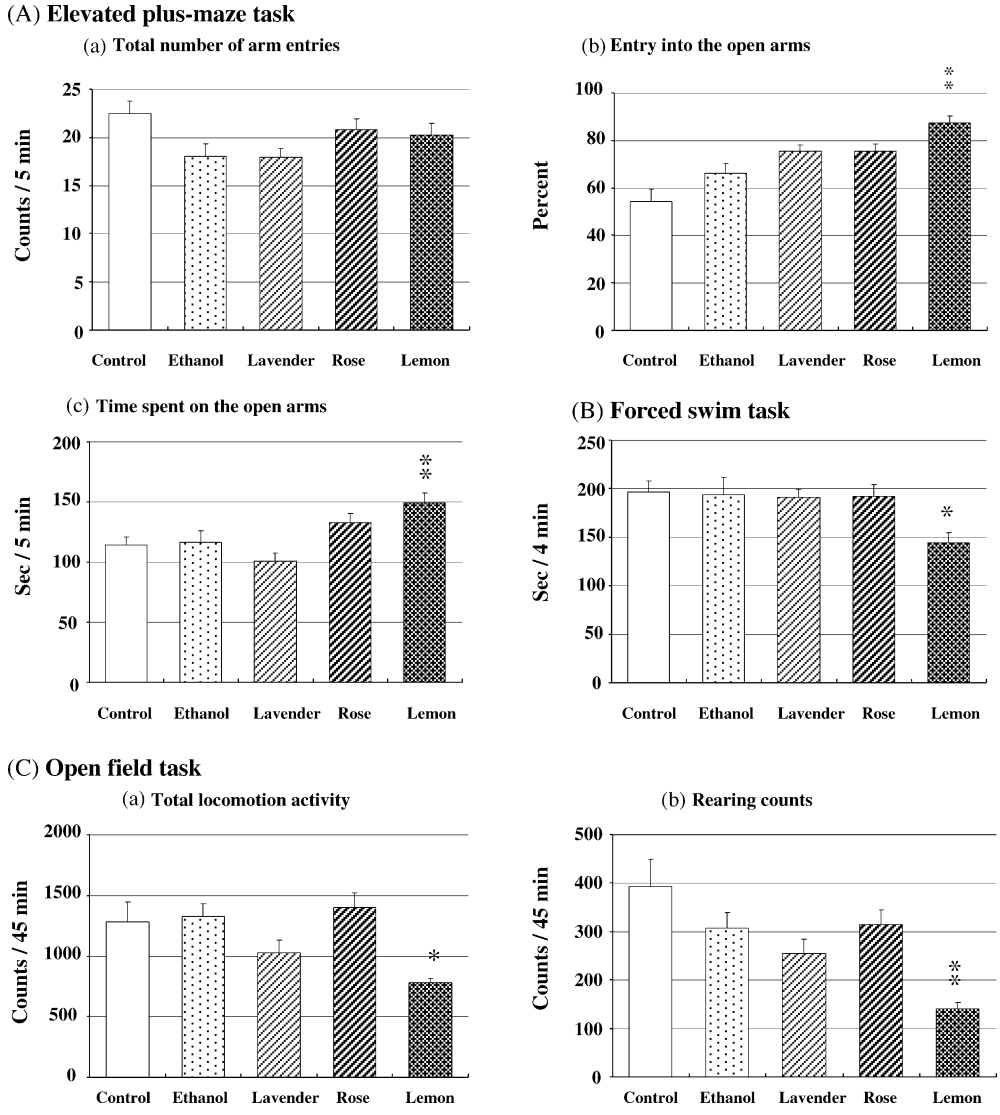

Fig. 1. Effects of the inhalation of essential oil vapor on the elevated plus-maze task (A, $n = 10$), forced swim task (B, $n = 5$), and open field task (C, $n = 5$) in mice. The graphs (A) represent the total number of open arm entries (a), the percentage of entries into the open arms (b), and the time spent on the open arms (c) during a 5 min period. The graph (B) represents the immobility duration time during a 4 min period. The graphs (C) represent the total locomotor activity counts (a) and rearing counts (b) for 45 min. Each value represents the mean ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the ethanol group by one-way ANOVA with Tukey's multiple comparison test.

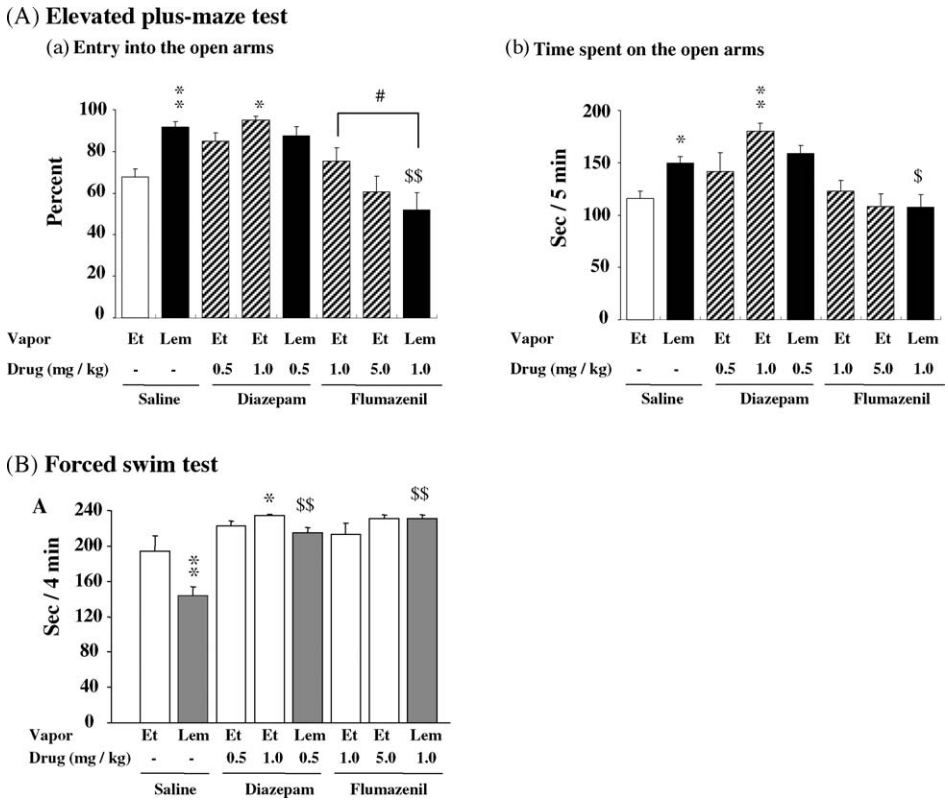

Fig. 2. Effects of BZP receptor agonist and antagonist on the elevated plus-maze task (A) and forced swim task (B) in the mice that inhaled lemon oil vapor. The graph (A-a) represents the percentage of entries into open arms, and the graph (A-b) represents the time spent on the open arms during a 5 min period. The graph (B) represents the immobility duration time during a 4 min period. Each value represents the mean ± S.E. The number of mice was 10 in ethanol alone and lemon oil alone in EPM task, and five in the other group, respectively. $^{*}P<0.05$, $^{**}P<0.01$ compared to the ethanol + saline group, \$ $P<0.05$, \$\$ $P<0.01$ compared to the lemon oil + saline group, $^{\#}P<0.05$ compared between flumazenil (1.0 mg/kg) alone and the combination of lemon oil inhalation and flumazenil injection. All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Lem: lemon oil vapor inhalation.

tasks. The percentage of entries ($F(2, 20)=18.56$; $P<0.0001$) and the time spent on the open arms ($F(2, 20)=12.93$; $P=0.0003$) in the EPM showed an increase in mice treated with diazepam (Fig. 2A). However, the combination of lemon oil and diazepam did not show any effect on these two parameters ($F(2, 20)=1.438$; $P=0.2620$ and $F(2, 20)=0.7271$; $P=0.4970$). In contrast, flumazenil significantly blocked the anti-stress effect of lemon oil in both percent of entry ($F(2, 20)=10.90$; $P=0.0009$) and time spent on the open arms ($F(2, 20)=4.916$; $P=0.0145$) (Fig. 2A-a).

The immobility duration in the FST significantly increased following a treatment with diazepam (1.0 mg/kg) ($F(2, 20)=4.817$; $P=0.0272$). The antidepressant-like effect of lemon oil was significantly reversed by treatments with diazepam ($F(2, 20)=39.72$; $P<0.0001$) or flumazenil ($F(2, 20)=28.81$; $P<0.0001$) (Fig. 2B).

3.3. Role of 5-HT receptor activity under lemon oil vapor inhalation

We used buspirone, a 5-HT_{1A} receptor agonist, WAY 100,635, a 5-HT_{1A} receptor antagonist, DOI, a 5-HT_{2A} receptor agonist, mianserin, a 5-$HT_{2A/C}$ receptor agonist, fluoxetine, a SSRI, and imipramine, a tricyclic antidepressant and a 5-HT and NA uptake inhibitor, to estimate the role of 5-HT receptor in the lemon oil-induced anti-stress reaction.

The percentage of entries into open arms showed a significant increase in the EPM in mice treated with buspirone ($F(2, 20)=5.729$; $P=0.0113$), though the time spent on the open arms did not show any effects. The combination of lemon oil and buspirone had a tendency to enhance an increase in the percentage of entries into open arms, however it was not potentiative ($F(2, 20)=3.151$; $P=0.0658$) (Fig. 3A).

WAY 100,635 showed a tendency to decrease the percentage of entries and the time spent on the open arms with all doses, however there were no significant differences ($F(3, 25)=0.7462$; $P=0.5352$ and $F(3, 25)=1.853$; $P=0.1718$) (Fig. 3A). The combination of lemon oil and WAY 100,635 did not show any potentiative effects.

DOI ($F(2, 20)=0.720$; $P=0.5010$) and mianserin ($F(2, 20)=2.710$; $P=0.0951$) did not have apparent effects in the percentage of entries into open arms in the EPM. DOI and mianserin showed a tendency to increase time spent on the open arm; however, there were no significant differences from the control group ($F(2, 20)=0.1446$; $P=0.8668$ and $F(2, 20)=0.0706$; $P=0.9321$) (Fig. 3A). The immobility duration in the FST did not reduce following a treatment with not DOI nor mianserin (Fig. 3B). The combination of lemon oil and DOI (0.3 mg/kg)

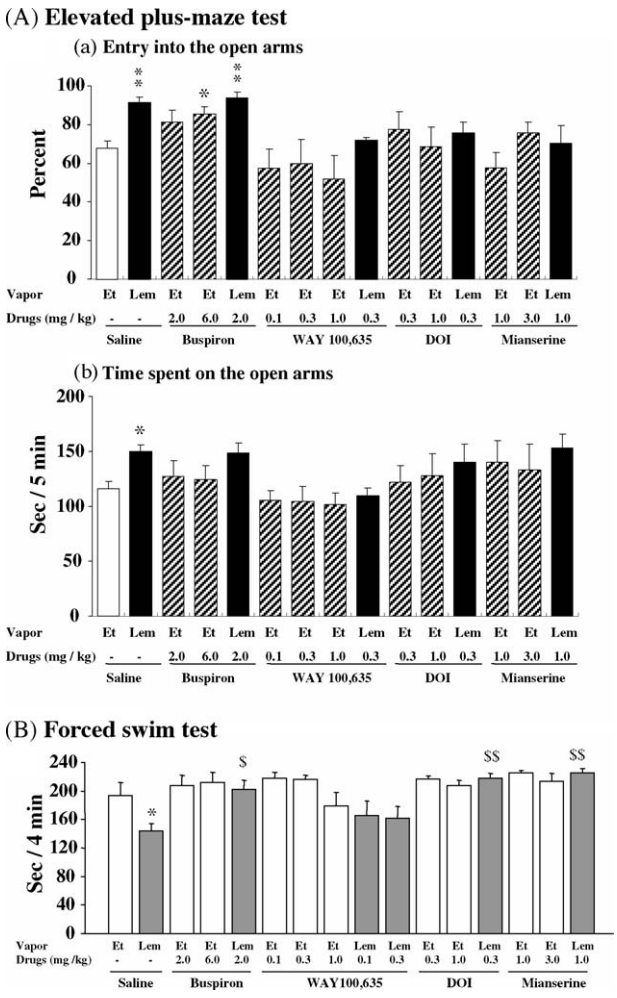

Fig. 3. Effects of 5-HT agonist and antagonist on the elevated plus-maze task (A) and forced swim task (B) in the mice that inhaled lemon oil vapor. The graph (A-a) represents the percentage of entries into open arms, and the graph (A-b) represents the time spent on the open arms during a 5 min period. The graph (B) represents the immobility duration time during a 4 min period. Each value represents the mean ± S.E. The number of mice was 10 in ethanol alone and lemon oil alone in EPM task, and five in the other group, respectively. $^{*}P<0.05$, $^{**}P<0.01$ compared to mice that ethanol + saline group, \$ $P<0.05$, \$\$ $P<0.01$ compared to lemon oil + saline group. All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Lem: lemon oil vapor inhalation.

or mianserin (1.0 mg/kg) significantly erased the lemon effect.

Fluoxetine, a SSRI, caused a significant increase in both percentage of entries ($F(2, 20)=9.257$; $P=0.0016$) and time spent on the open arms ($F(2, 20)=12.10$; $P=0.0004$). However, the lemon oil did not enhance the anti-stress effect of fluoxetine (Fig. 4A). In contrast, imipramine, a tricyclic antidepressant and a 5-HT and NA uptake inhibitor, showed a significant increase in percentage of entries into open arms in the EPM ($F(2, 20)=5.282$; $P=0.0164$). Moreover, there were no significant differences between lemon oil alone and the combination of imipramine and lemon oil.

Interestingly, buspirone ($F(2, 20)=9.209$; $P=0.0028$), DOI ($F(2, 20)=36.46$; $P<0.0001$), and mianserine ($F(2, 20)=49.16$; $P<0.0001$) blocked the antidepressant-like effect of lemon oil in the FST, but WAY100,635 did not ($F(2, 20)=0.610$; $P=0.5563$) (Fig. 3B). In addition, imipramine had an apparent antidepressant effect at a dose of 50 mg/kg ($F(2, 20)=6.908$; $P=0.0082$), and fluoxetine did not show any antidepressant effect at the dosages we used in this study (3 or 10 mg/kg) ($F(2, 20)=0.8449$; $P=0.4504$) (Fig. 4B), even though fluoxetine blocked the antidepressant-like effect of lemon oil ($F(2, 20)=9.636$; $P=0.0020$).

3.4. Role of DA receptor under lemon oil vapor inhalation

We used apomorphine, a nonselective DA receptor agonist, and haloperidol, a D_2, D_3, D_4 receptor antagonist, to estimate the role of DA receptor in the lemon oil-induced anti-stress reaction.

Apomorphine showed an anti-stress effect in both the percentage of entries into open arms ($F(2, 20)=4.393$; $P=0.0302$) and the time spent on the open arms ($F(2, 20)=0.5170$; $P=0.6090$) in the EPM (Fig. 5A). These effects of apomorphine were erased by treatment with lemon oil ($F(2, 20)=6.665$; $P=0.0073$). In contrast, lemon oil did not affect the haroperidol-treated mice ($F(2, 20)=2.063$; $P=0.1595$).

The highest dose of apomorphine (3.0 mg/kg) significantly reduced the immobility duration in the FST ($F(2, 20)=3.811$; $P=0.0443$) (Fig. 5B). Moreover, the combination of lemon oil and apomorphine (0.5 mg/kg) or haloperidol (0.5 mg/kg) did not show any significant effect in the FST.

3.5. Role of α2 adrenergic receptor with lemon oil vapor inhalation

We used clonidine, a selective α2 adrenergic receptor agonist, and yohimbine, a selective α2 adrenergic receptor antagonist, to estimate the role of α2 adrenergic receptor in the lemon oil-induced anti-stress reaction.

Yohimbine showed a significant increase in the percentage of entries into open arms ($F(2, 20)=5.363$; $P=0.0175$) and the time spent on the open arms in the EPM ($F(2, 20)=5.120$; $P=0.0202$), although clonidine had no effect (Fig. 6A). The combination of lemon oil and clonidine or yohimbine did not show any alteration on the effect of lemon oil alone.

A low dose of clonidine (0.05 mg/kg) or yohimbine (1.5 mg/kg) blocked the antidepressant-like effect of lemon oil in the FST ($F(2, 20)=7.096$; $P=0.0083$ and $F(2, 20)=11.29$; $P=0.0014$) (Fig. 6B).

3.6. Changes in the contents of monoamines and their metabolites in the brain following lemon oil inhalation

Ethanol inhalation did not have any significant effect on the DA or DOPAC contents in the brain. The inhalation of lemon oil vapor for 90–180 min significantly increased the DA content in the hippocampus (Table 1). The contents of DOPAC in the prefrontal cortex and hippocampus also significantly increased following inhalation of lemon oil vapor for 180 min. The DA/DOPAC ratio was not particularly changed in any of the three brain areas.

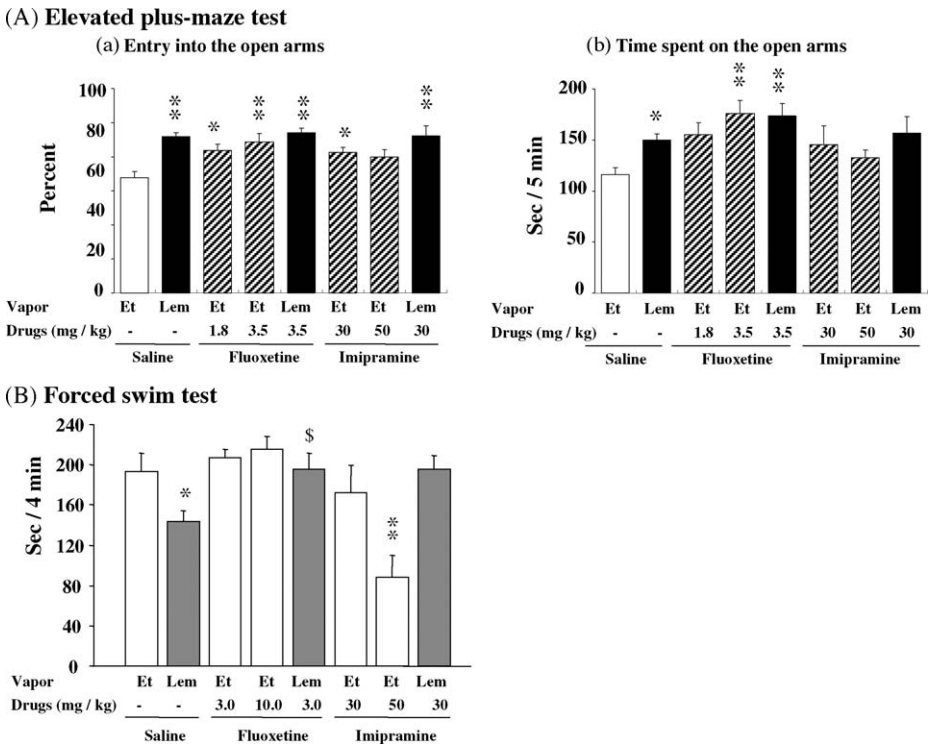

Fig. 4. Effects of a SSRI and a tricyclic antidepressant on the elevated plus-maze task (A) and forced swim task (B) in the mice that inhaled lemon oil vapor. The graph (A-a) represents the percentage of entries into open arms, and the graph (A-b) represents the time spent on the open arms during a 5 min period. The graph (B) represent the immobility duration time during a 4 min period. Each value represents the mean ± S.E. The number of mice was 10 in ethanol alone and lemon oil alone in EPM task, and five in the other group, respectively. $^{*}P<0.05$, $^{**}P<0.01$ compared to the ethanol + saline group, \$ $P<0.05$ compared to the lemon oil + saline group. All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Lem: lemon oil vapor inhalation.

Ethanol inhalation caused a significant increase of 5-HT content in the prefrontal cortex. Furthermore, the inhalation of lemon oil vapor for 180 min resulted in a significant increase of 5-HT in the prefrontal cortex compared with ethanol inhalation. 5-HIAA contents were more apparently affected by treatment with lemon oil vapor in all three brain regions (Table 1). Moreover, 5-HIAA/5-HT ratios in the hippocampus and striatum were also enhanced by lemon oil inhalation for 90 or 180 min.

4. Discussion

The present study shows that the inhalation of lemon oil vapor induced anxiolytic and antidepressant-like effects in the EPM and FST. Furthermore, significantly reduced locomotor activity and rearing behavior in the OFT were observed after 90 min of lemon oil vapor inhalation in male mice. There are many reports showing the anxiolytic [6], sedative [14], antispasmodic [39], and antidepressant [26] effects of lemon, lemon odor, or

Table 1
Dopamine, serotonin, and their metabolites in the brain regions in lemon oil inhaled mice

Brain region	Group	DA	DOPAC	DOPAC/DA	5-HT	5-HIAA	5-HIAA/5-HT
Prefrontal cortex	Control	0.30 ± 0.05	0.18 ± 0.04	0.61 ± 0.11	0.99 ± 0.17	1.35 ± 0.20	1.40 ± 0.13
	Ethanol	0.18 ± 0.04	0.17 ± 0.02	1.13 ± 0.23	1.66 ± 0.22 a	1.63 ± 0.22	1.06 ± 0.21
	Lemon 90	0.31 ± 0.09	0.35 ± 0.09	1.15 ± 0.08 b	2.04 ± 0.17 b	3.22 ± 0.23 b,d	1.60 ± 0.13
	Lemon 180	0.24 ± 0.04	0.34 ± 0.03 a,d	1.60 ± 0.30 a	2.37 ± 0.21 b,c	3.22 ± 0.39 b,d	1.46 ± 0.32
Hippocampus	Control	6.38 ± 2.22	2.06 ± 0.67	0.40 ± 0.08	1.53 ± 0.38	0.94 ± 0.12	0.76 ± 0.16
	Ethanol	5.90 ± 1.69	2.40 ± 0.66	0.50 ± 0.12	1.71 ± 0.35	1.18 ± 0.23	0.75 ± 0.13
	Lemon 90	12.52 ± 1.78 c	4.56 ± 1.16	0.36 ± 0.06	2.48 ± 0.39	2.54 ± 0.39 b,c	1.05 ± 0.14
	Lemon 180	20.57 ± 5.84 c	5.54 ± 1.02 a,c	0.31 ± 0.05	2.59 ± 0.23 a	3.10 ± 0.34 b,d	1.19 ± 0.07 a,c
Striatum	Control	40.17 ± 12.68	8.71 ± 2.15	0.27 ± 0.05	3.98 ± 1.08	1.43 ± 0.27	0.46 ± 0.12
	Ethanol	47.72 ± 5.66	13.69 ± 1.84	0.31 ± 0.06	4.56 ± 0.71	1.92 ± 0.25	0.47 ± 0.09
	Lemon 90	45.45 ± 3.22	17.42 ± 1.99 a	0.40 ± 0.06	4.21 ± 0.25	3.37 ± 0.43 b,c	0.79 ± 0.06
	Lemon 180	66.76 ± 8.10	14.86 ± 1.01 a	0.25 ± 0.06	4.35 ± 0.32	5.06 ± 0.43 b,d	1.16 ± 0.06

HPLC determination of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in the prefrontal cortex, hippocampus and striatum in lemon oil inhaled mice. Data are expressed in pmol/mg tissue (mean ± S.E., $n=5$). (a) $P<0.05$, (b) $P<0.01$ compared with control group, (c) $P<0.05$, (d) $P<0.01$ compared with ethanol group, respectively.

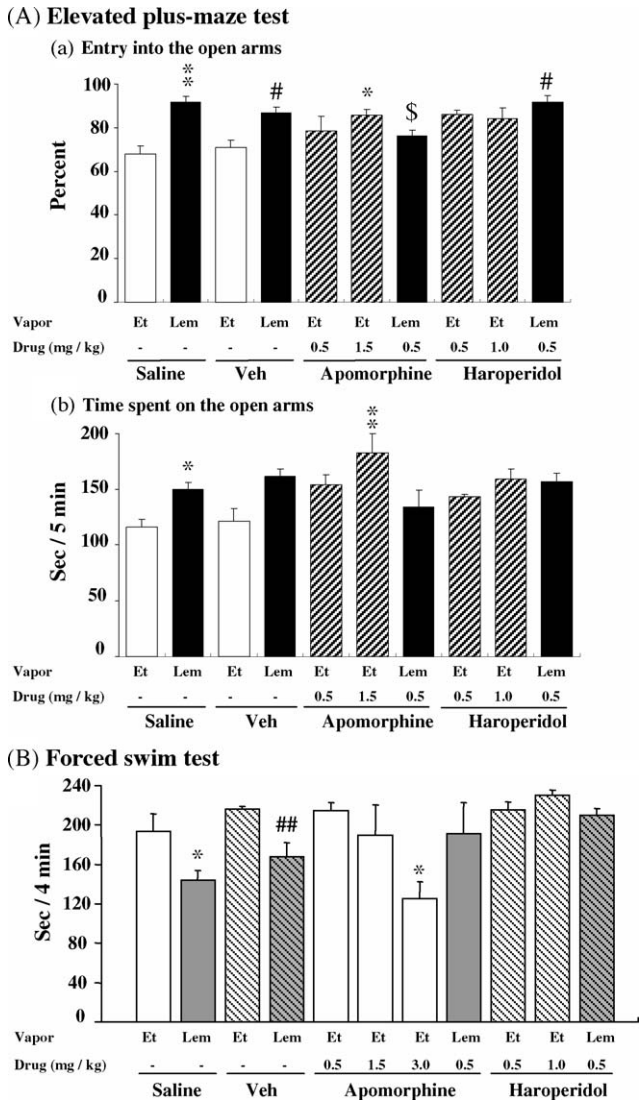

Fig. 5. Effects of DA agonist and antagonist on the elevated plus-maze task (A) and forced swim task (B) in the mice that inhaled lemon oil vapor. The graph (A-a) represents the percentage of entries into open arms, and the graph (A-b) represents the time spent on the open arms during a 5 min period. The graph (B) represents the immobility duration time during a 4 min period. Each value represents the mean ± S.E. The number of mice was 10 in ethanol alone and lemon oil alone in EPM task, and five in the other group, respectively. $^{*}P<0.05$, $^{**}P<0.01$ compared to the ethanol + saline group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared to the ethanol + vehicle group, $\$ P<0.05$ compared to the lemon oil + saline group. All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Lem: lemon oil vapor inhalation, Veh: injection of saline with a few drops of hydrochloric acid 1N.

limonene in mice or rats. Limonene is one of the major components of lemon oil. In humans, it has been shown that ambient lemon odor can improve creativity, mood, and perceived health [25]. Heart rate changes have also been reported after exposure to lemon essential odor [24]. However, the precise mechanisms of these psychological or pharmaceutical effects induced by lemon oil vapor are still unknown.

In the present study, we showed that lemon oil vapor enhanced entries into the open arm in the EPM under pretreatment with buspirone, a 5-HT_{1A} receptor agonist. Lemon oil had a similar effect under the pretreatment with haloperidol, a D_2, D_3, D_4 receptor antagonist. In addition, flumazenil, a BZP receptor antagonist, blocked the anti-stress effect of lemon oil. It is well known that the 5-HT neurons originating from the midbrain raphe nuclei innervate both the substantia nigra (SN) and the ventral tegmental area (VTA). In addition, terminal areas of the substantia nigra pars compacta (SNc) and VTA, such as the striatum or the nucleus accumbens, receive an input from 5-HT neurons originating in the raphe nuclei [13]. These findings suggest that lemon oil vapor possibly affects on the response to the DAnergic activity via modulation of 5-HTnergic and/or GABA-BZP receptor complex.

In the FST, the progesterone $GABA_A$-modulatory metabolite 5α-pregnan-3α-ol-20-one (allopregnanolone) is released and rapidly metabolized after an acute stress session [33]. Briones-Aranda et al. [3] reported that either forced swimming or a high dose of allopregnanolone can block the anxiolytic effect of diazepam. In our present study, diazepam had the anti-stress effect, and flumazenil caused a stressful reaction in the EPM. On the other hand, neither diazepam nor flumazenil showed an antidepressant effect in the FST, and in fact these two drugs blocked the antidepressant-like effect of lemon oil in the FST. Briones-Aranda et al. [3] suggested that forced swimming produces conformational changes in the GABA-BZP complex that alter the pharmacological profile of BZP. The results of these previous reports together with our present results suggest that the lemon oil-induced antidepressant-like effect does not occur via a direct GABA-BZP pathway, because lemon oil caused the antidepressant-like effect even in the forced swimming condition that was not suited to the agonist to BZP receptor.

On the other hand, lemon oil vapor did not enhance the pharmaceutical effects of antidepressant drugs including fluoxetine and imipramine in the FST, although these drugs blocked the antidepressant effect of lemon oil vapor. Imipramine is widely used as a positive control in the FST. Interestingly, imipramine completely blocked the antidepressant-like effect of lemon oil, although imipramine showed a strong effect when used alone. Exploring this reversible effect of imipramine on the function of lemon oil may give us useful information for undersanding the mechanism of the antidepressant-like effect of lemon oil.

A prominent participation of 5-HT in depression and anxiety is generally recognized [18,23], although the complex of emotional states cannot be reduced to imbalances of a single neurotransmitter. In the present study, buspirone, DOI, and mianserine blocked the antidepressant-like effect of lemon oil in the FST, but WAY100,635 did not (Fig. 3B). These findings suggest that the antidepressant-like effect of lemon oil is closely related with the 5-HTnergic pathway, especially via 5-HT_{1A} receptor. However, further investigations are necessary to clarify the precise signal transduction induced by lemon oil. It is also well known that the modulating and control of emotion are related to changes in the contents of monoamines and their metabolites in the limbic system.

In the present study, the determination of monoamines and their metabolites in the brain of mice showed that the inhala-

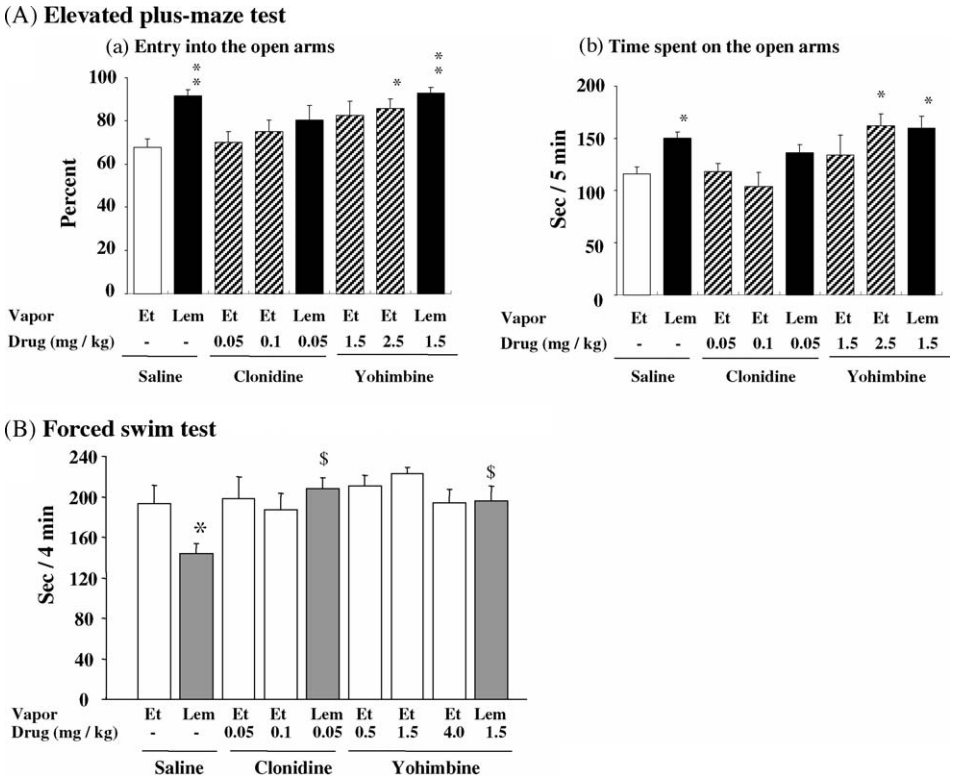

Fig. 6. Effects of adrenaline α2 agonist and antagonist on the elevated plus-maze task in the mice that inhaled lemon oil vapor. The graph (a) represents the percentage of entries into open arms, and the graph (b) represents the time spent on the open arms during a 5 min period. The graph (B) represents the immobility duration time during a 4 min period. Each value represents the mean ± S.E. The number of mice was 10 in ethanol alone and lemon oil alone in EPM task, and five in the other group, respectively. $^{*}P<0.05$, $^{**}P<0.01$ compared to the ethanol + saline group, $ $P<0.05$ compared to the lemon oil + saline group. All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Lem: lemon oil vapor inhalation.

tion of lemon oil vapor enhanced the synthesis of 5-HT in the prefrontal cortex and the hippocampus significantly. The turnover ratio of 5-HT was more enhanced in the hippocampus and the striatum than in the prefrontal cortex. The results of the DOPAC/DA ratio and DA content in the brain regions suggest that the DAnergic activity in the prefrontal cortex was significantly enhanced by inhaling lemon oil vapor. Furthermore, the inhalation of lemon oil vapor accelerated the DA synthesis in the hippocampus and the striatum.

The enhanced 5-HIAA/5-HT ratio that we observed in the hippocampus and striatum in the mice that inhaled lemon oil vapor was consistent with the higher 5-HT turnover in the hippocampus and striatal 5-HTergic terminals, whereas the DA content in the hippocampus was higher in the mice that inhaled lemon oil vapor than that in mice that inhaled ethanol. It has been reported that DA and 5-HT fibers normally compete for striatal target sites during postnatal development, and DA might tonically suppress the release of neurotrophic factors that promote 5-HT axon growth [28]. Although the precise modulating mechanisms of lemon oil for the 5-HT and DA neurons are still unknown, we think that activated 5-HTergic neurons suppress the DAergic neurons under the lemon oil inhalation condition.

Renard et al. [34] have reported that DA concentration in the whole brain of mice increased from the 5th min of the FST and returned to the basal level after 20 min. DOPAC concentration increased after a 20 min task period. On the other hand, 5-HT concentration increased after an 8 min task period. Noradrenaline was not modified during the FST. This report suggests that DA activity is more rapidly induced by a higher stressor like the FST than by a mild stressor. Interestingly, lemon oil vapor induced anti-stress effects under both mild and higher stressors in our study. Considering that, we conclude that lemon oil may enhance 5-HT activity at first, and then 5-HTnergic neurons modulate the DAnergic system. On the other hand, some reports have shown that 5-HT has a stimulatory effect in hyperlocomotion. For example, systemic administration of 5-HT_2 receptor antagonists prevented hyperactivity, whereas intrastriatal injection of 5-$HT_{2A/2C}$ agonists increased locomotion [2]. In contrast, nonselective 5-HT receptor agonists or 5-HT transporter inhibitors possibly reduced hyperactivity in neonatally DA-depleted rats [10,21]. These reports suggest that 5-HT acts as an inhibitory locomotor in the DA-depleted model.

In the present study, lemon oil inhalation increased 5-HT and 5-HIAA content in the prefrontal cortex. This result may be related to the idea that locomotion is normally under DAergic control and to a previous observation showing that partial 5-HT depletion did not affect spontaneous locomotion [8]. Haloperidol pretreatment, however, enhanced the anxiolytic effect of lemon oil vapor. It is, therefore, possible that the enhanced 5-HTnergic system inhibits stressful behavior under the lemon oil

inhalation without DA activity. It is also well known that 5-HT can exert both stimulatory and inhibitory effects on psychological behaviors, probably depending on complex interactions with other neurotransmitters systems.

Thus, we conclude that lemon oil possesses anxiolytic, antidepressant-like effects. Lemon oil possibly reduces distress by modulating GABAnergic, serotonergic, and dopaminergic systems in the brain. This effect might be caused by a suppression of DA activity via enhanced 5-HTnergic neurons under the lemon oil inhalation condition.

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