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Differences in the effects of essential oil from *Citrus junos* and (+)-limonene on emotional behavior in mice

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Citrus junos (Yuzu, CJ) is a traditional fruit in Japan and its essential oil (EO) has been used in food, cosmetics, and traditional medicine. The present study examined the influence of essential oil from *Citrus junos* (EOCJ) on emotional behavior in mice, and how its action differed in comparison to (+)-limonene (LI), its major component. The influence of inhaled administration (i.h.) of EOCJ for 90 minutes on mouse emotional behavior was examined using the light/dark box (LDB) test, open field (OF) test, and elevated plus-maze (EPM) test. In addition, gas chromatography (GC) was used in clarifying the amount of LI absorbed in the internal organs. Inhalation of EOCJ at 3.4 and 6.7 mg/L air indicated the tendency for an anxiolytic-like effect in LDB and EPM tests. In addition, an increase in locomotor activity was observed at 6.7 mg/L air EOCJ (i.h.) in the OF test. Inhalation of (+)-LI at 3.4 and 6.7 mg/L air indicated the same anxiolytic-like effect in EPM test as with EOCJ. In contrast, the anxiolytic-like effect of (+)-LI was smaller than that of EOCJ in the LDB test. Furthermore, an increase in locomotor activity was not observed at 6.7 mg/L air (+)-LI (i.h.).

Keywords: *Citrus junos*; essential oil; limonene; emotional behavior; anxiolytic-like effect

Introduction

Citrus junos (Yuzu, CJ) is a member of the *Citrus* genus in the family Rutaceae, and is produced chiefly on the southern mainland (Honshu) of Japan and on the southern coast of South Korea. CJ has been widely used in various areas as food and cosmetics and has garnered attention from Japan and the rest of the world because it has a peculiar smell compared to other citrus fruits. Moreover, it is a long-held belief in Japan that taking a bath with CJ fruits promotes blood circulation, cures chapped skin, and prevents colds. The essential oil from *C. junos* (EOCJ) has been used to improve metabolism and for relaxation. Sawamura and others have presented their work on EOCJ components in a number of papers (1–4). In addition, there are several papers that have specified the location within CJ that produce EOCJ components using isotope analysis (5–8). EOCJ was reported to inhibit the generation of the carcinogen *N*-nitrosodimethylamine (9, 10). Furthermore, anti-inflammatory (11) and anti-oxidative (12) effects of EOCJ have been reported. However, the anxiolytic-like effects of limonene (LI) and the EO from *C. aurantium* have been reported (13–15). Moreover, it has been reported that LI influenced monoamines, such as serotonins and dopamine (16–18). However, the influence of EOCJ on emotional behavior has not been reported. Here, we report on the influence of EOCJ on emotional behavior in mice, and how the effects of EOCJ differ from those of LI, its major component.

Experimental

Essential oil from Citrus junos (EOCJ)

Pericarps from CJ (Makino) Siebold ex Tanaka; Rutaceae) were collected in Kochi, Japan in January 2009. The pericarps were cut into small pieces and the EO was extracted by steam distillation for 2 hours. A yield of 0.2% (*v*/*w*) was obtained. This EO was purchased from Green Flask Co., Ltd, Tokyo, Japan.

Gas chromatography (GC) analysis

A Clarus 500 (Perkin–Elmer Inc., Waltham, MA, USA) was used for gas chromatography–mass spectroscopy (GC–MS) analysis, and a GC-2010 Plus (Shimadzu, Kyoto, Japan) was used for GC–flame ionization detection (FID) analysis. The details of GC analysis have been described elsewhere (19). Briefly, the EOCJ or authentic standards, diluted with hexane, were injected into a GC equipped with MS, which was operated under the electron impact ionization mode, and with FID. Two types of capillary columns were used: (1) an Equity-1 (30 m × 0.25 mm i.d., 0.25 μm, non-polar column; Sigma-Aldrich Japan K.K., Tokyo, Japan) and a CycloSil-B (30 m × 0.25 mm i.d., 0.25 μm, chiral

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column; Agilent Technologies, Inc., Santa Clara, CA, USA). The GC condition was as follows: carrier gas, helium (1.33 mL/minute); split rate, 50:1; inlet line temperature, 250°C; source temperature, 230°C; column temperature, 35°C for 5 minutes, 35°C to 215°C at 3°C/minute, then 215°C for 5 minutes; mass spectra, electron impact, 70 eV. Individual components were identified by comparison with authentic standards and/or from the GC-MS NIST library and/or retention index (RI) (20).

Identification and quantification of essential oil (EO) components

The composition of the EO obtained from a single plant species may exhibit seasonal and geographical variability. The Linear Retention Indices (LRIs) of the EO components on the Equity-1 column were determined in relation to a homologous series of *n*-alkanes containing 12 *n*-hydrocarbons (C_9–C_{16}). The LRIs were calculated according to the equation proposed by Kratz (21). Authentic standards were obtained from the following chemical supply companies. Sabinene was purchased from Extrasynthese (Lyon, France). β-Caryophyllene was acquired from Wako Pure Chemical Industries, Ltd (Osaka, Japan). (+)-α-Pinene, (–)-α-pinene, (+/–)-camphene, (–)-β-pinene, myrcene, α-phellandrene, *p*-cymene, (+)-LI, (–)-LI, γ-terpinene, terpinolene, linalool, and α-terpineol were obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Saturated alkanes, C_9–C_{16}, for calculating LRI were purchased from Tokyo Chemical Industry Co., Ltd. The individual peaks were identified by comparing their retention times and LRIs to those of authentic standards or data from the literature (20). Comparisons of fragmentation patterns in the mass spectra with those of authentic standards and/or those in the GC–MS NIST library (the NIST mass spectral search program for the NIST/EPA/NIH mass spectral library version 1.7, CA, USA) were also made. For quantitative evaluation, concentrations of all compounds were calculated by integrating their corresponding peak areas by FID. The quantitative analysis was done using the absolute calibration method of pentadecane and (+)-LI (Tokyo Chemical Industry Co., Ltd) (Table 1 and Figure 1).

Animals

Male ICR mice (Clea Japan, Tokyo, Japan), 5 weeks of age at the start of each experiment, were individually housed in cages for 1 week. The cages were placed in a room artificially illuminated by fluorescent lamps on a 12 hour light:12 hour dark schedule (light period: 08:00–20:00) and maintained at 24 ± 2°C. The mice were given free access to food and water. The mice had no prior exposure to either the EO or drug, and

Table 1. The concentrations (in g/L) of EOCJ (essential oil from *Citrus junos*) components.

Peak no.	Component	Reference LRI (DB-5)	Measurement LRI (Equity-1)	g/L	Chiral ratio (+/-)	Identifiaction
1	α-thujene	924	922	2.7		RI,MS
2	α-pinene	932	927	12.2	61/39	RI,MS,STD
3	sabinene	969	963	1.64		RI,MS,STD
4	β-pinene	974	965	7.2	100/0	RI,MS,STD
5	mycrene	988	986	14.0		RI,MS,STD
6	α-phellandrene	1002	994	trece		RI,MS,STD
7	unknown		1007	trace		RI,MS
8	p-cymene	1020	1011	66.6		RI,MS,STD
9	β-phellandrene	1025	1016	22.2		RI,MS
10	limonene	1024	1021	690.8	98/2	RI,MS,STD
11	trans-ocimene	1044	1042	0.6		RI,MS
12	γ-terpinene	1054	1049	20.3		RI,MS,STD
13	m-cymene	1082	1074	trace		RI,MS
14	terpinolene	1086	1078	1.0		RI,MS,STD
15	linalool	1095	1090	12.5		RI,MS,STD
16	coahuilensol	1166	1160	trace		RI,MS
17	p=methyl-acetophenone	1179	1173	trace		RI,MS
18	thymol	1289	1286	trace		RI,MS
19	tridecane	1300	1302	trace		RI,MS
20	β-caryophyllene	1417	1409	trace		RI,MS
21	β-farnesene	1454	1451	trace		RI,MS
22	β-vetivenene	1554	1558	trace		RI,MS
	monoterpene	19 compounds		851.8		
	sesquiterpene	3 compounds		trace		

Notes: The data are expressed as the mean of three replicates; trace, indicates below the quantitation limit, for example, trace < 0.03 g/L; the LRIs were derived from reference 20.

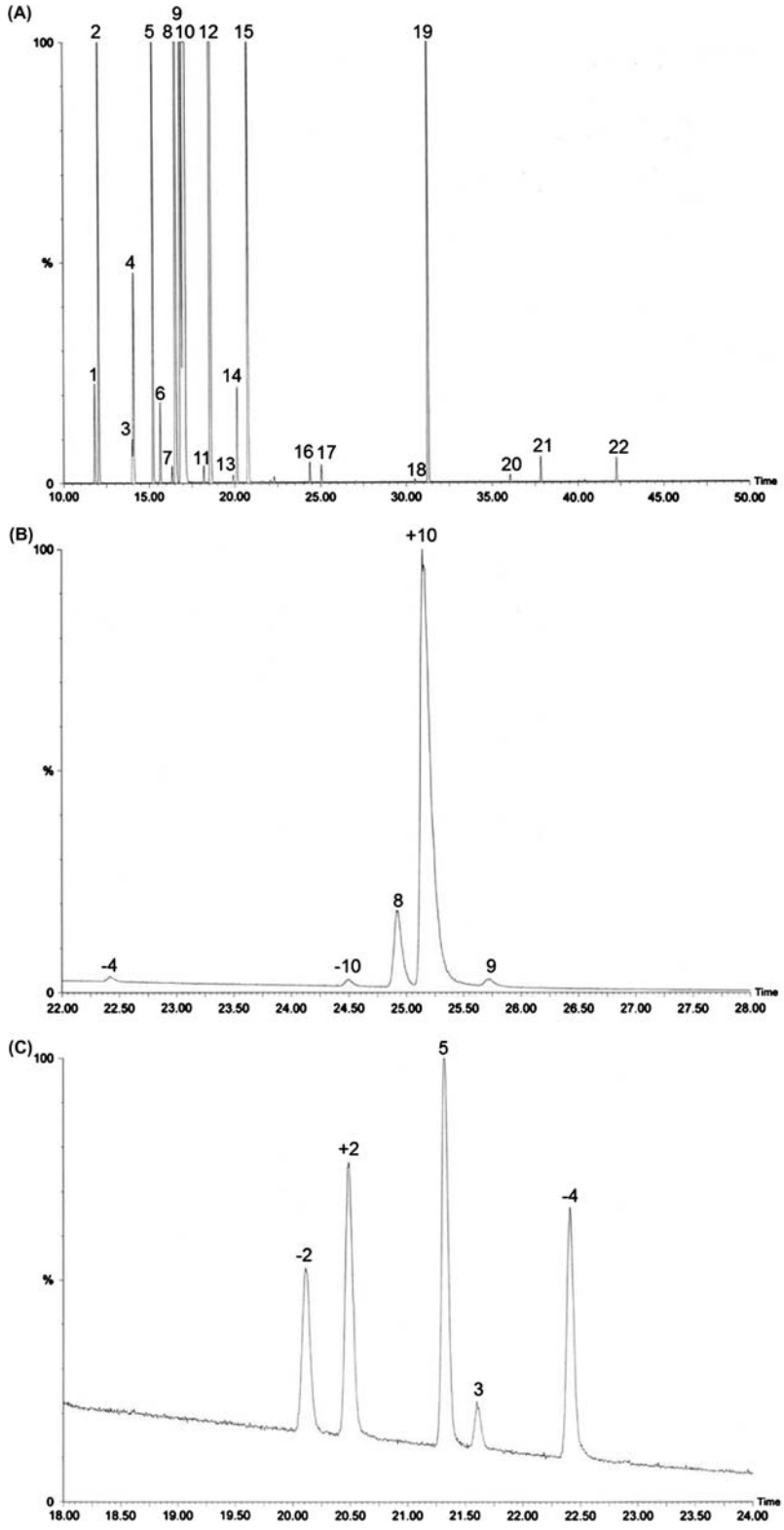

Figure 1. GC chromatogram of EOCJ (essential oil from *Citrus junos*) by GC–MS with Equity-1 (A) and with CycloSil-B (B, C). The compound number corresponds to Table 1.

each mouse was used once during the experiment. Five mice were used for each experiment by intraperitoneal injection (five mice × two kinds of concentrations = 10 mice) and by inhaled administration (i.h.) (five mice × five kinds of concentrations = 25 mice). Five mice were used for each experiment assessing drug distribu-

tion (five mice × four kinds of concentrations = 20 mice). In total, 55 mice were used. All experiments were conducted in accordance with the guidelines regarding the care of experimental animals, as approved by the Animal Research Committee at Toho University.

Drug administration

In a preliminary experiment, the benzodiazepine anxiolytic diazepam (Wako Pure Chemical Industries, Ltd) was dissolved in 0.1% Tween 80 (Kanto Chemical Co., Ltd, Tokyo, Japan)/saline and then injected intraperitoneally (i.p.) at a volume of 5 mL/kg body weight. Diazepam (3 mg/kg body weight) and 0.1% Tween 80/saline, as the control, were administered i.p. 30 min prior to the initiation of these tests as follows. These mice were placed in a glass container (5 L volume; 100 mm × 250 mm × 200 mm, length × width × height) at 200 lx for 30 minutes followed by 90 minutes.

In the main experiment, each mouse was placed in a glass container (5 L volume; 100 mm × 250 mm × 200 mm, length × width × height) at 200 lx, and then acclimatized for 30 minutes (22). A piece of 11-μm filter paper (GE Healthcare Japan, Tokyo, Japan) soaked in EOCJ was set on the upper side of the glass container. After the acclimatization period, mice were then exposed to water (control), EOCJ and (+)-LI at a concentration of 3.4 and 6.7 mg/L air (i.h.). Administration of EOCJ (i.h.) was initiated 90 minutes prior to these three tests as follows. The total time in the container was 30 + 90 minutes. Light/dark box (LDB) test in the first day was done, open field (OF) test was done on the second day, and elevated plus-maze (EPM) test was done on the third day. The details of this procedure have been described elsewhere (23). The EOCJ and (+)-LI on each filter paper were assumed to be completely volatized. The concentrations of EOCJ and (+)-LI were calculated based on this assumption.

LDB test: number of entries into the light compartment (times) and time in the light compartment (in seconds)

The LDB consisted of two compartments: a light compartment (300 mm × 300 mm × 300 mm, length × width × height) at 400 lx that was painted white and illuminated with a 100-W desk lamp, and a dark zone (150 mm × 300 mm × 300 mm, length × width × height) at 10 lx, which was painted black. The two compartments were separated by a partition with a tunnel (60 mm × 60 mm, width × height) to allow passage from one compartment to the other. Mice (n = 5 per group) were randomly assigned to experimental groups (including a water control). The experiments were performed between 08:00 and 20:00. Animals were placed in the center of the light compartment facing the wall opposite the tunnel. The number of entries into the light compartment and the total time spent in the light compartment were recorded over a 10-minute period (times and seconds, respectively). Each mouse was placed in the light compartment, and the behavior of each mouse was recorded for 10 minutes with a web camera (BWC-30L01/SV USB, Buffalo Inc., Aichi, Japan). Data were analyzed by the video tracking system ANY-maze (Stoelting Co., Wood Dale, IL, USA). The apparatus was cleaned thoroughly with water and organic solvent between trials (24). The details of this procedure have been described elsewhere (23).

OF test: total distance traveled (in meters)

To study the effect of the sample on locomotor activity, each animal was placed in the center of a black rectangular area (400 mm × 400 mm, length × width) surrounded by 450-mm-high walls. The apparatus was illuminated with a 200-lx light at floor level. Mice (n = 5 per group) were randomly assigned to experimental groups (including a control). The total distance (in meters) traveled during a 60-minute trial was recorded; the testing was conducted between 08:00 and 20:00. Each mouse was placed in the center of the rectangular area, and the behavior of each mouse was recorded for 60 minutes with a web camera (BWC-30L01/SV USB, Buffalo Inc., Aichi, Japan). Data were analyzed by the video tracking system ANY-maze (Stoelting Co., Wood Dale, IL, USA). The apparatus was cleaned thoroughly with water and organic solvent between trials (25). The details of this procedure have been described elsewhere (23).

EPM test: number of entries into the open arms (%), time in the open arm compartments (in seconds)

The elevated plus-maze (26) consists of two open arms (200 mm × 50 mm, length × width) and two closed arms (200 mm × 50 mm, length × width) with walls, crossing each other at right angles. The maze was located 600 mm above the floor. The apparatus was illuminated with a 200-lx light at floor level. Mice (n = 5 per group) were randomly assigned to experimental groups (including a control). Each mouse was placed at the crossing point of the maze, and the behavior of each mouse was subsequently recorded for 10 minutes by an attached camera. These data were then analyzed by ANY-maze (Stoelting Co., Wood Dale, IL, USA). The details of this test have been described elsewhere (23). Entry into the open arms (%) and time spent in the open arms (in seconds) in the EPM test were measured.

Collection of internal organs and sample preparation

The mice were euthanized by anesthetic and the internal organs (liver, kidney, and brain) were collected. To clarify the concentration and distribution of LI, internal

organs from an additional animal, treated with EOCJ and (+)-LI at 3.4 and 6.7 mg/L air, were collected soon after inhalation. The tissues were processed without venous drainage. Each internal organ was preserved at –80°C. The ice-cold internal organs were extracted with hexane by sonication and the extracts were analyzed by GC–MS and GC–FID, as described earlier. Quantitative analysis was done using the absolute calibration method of (+)-LI. Each amount of LI was corrected by the content ratio, and represents the mean ± standard error (n = 5).

Statistical analysis

Statistical differences between diazepam and control (0.1% Tween 80/saline group) in experiment one were determined using a two-sided Student's t test. Differences with $P < 0.05$ were considered significant. The statistical differences in the means of all results in experiment two were assessed using a one-way analysis of variance (ANOVA) with Dunnett's multiple comparison *post hoc* test using JMP software (SAS Institute Japan Ltd, Tokyo, Japan). Statistical significance was established at $P < 0.05$. A water group was analyzed as a control.

Results and discussion

Experiment one: Validation of method by diazepam. The evaluation method of anxiolytic-like behavior was assessed using diazepam (3 mg/kg mouse, i.p.) and showed significant increases (as positive control) in the number of entries into the light compartment of the LDB test, time spent in the light compartment of the LDB, the entry ratio to the open arms of the EPM, and time spent in the open arms of the EPM ($P < 0.05$ in all cases). Additionally, decreased locomotor activity was observed in the OF test. Therefore, the ability of these three tests to measure anxiolytic-like effects in mice was confirmed (Figure 2).

Experiment two: Anxiolytic-like behavior of (+)-LI and EOCJ. In the LDB test, mice administered 6.7 mg/L air EOCJ (i.h.) indicated a significant increase compared to the control in number of entries into the light compartment (Figure 3A). In the LDB test, mice administered 6.7 mg/L air (+)-LI (i.h.) and 3.4 and 6.7 mg/L air EOCJ (i.h.) indicated a significant increase compared to the control in the amount of time spent in the light compartment (Figure 3B).

In the EPM, the mice administered (+)-LI (i.h.) and EOCJ (i.h.) at 3.4 and 6.7 mg/L air showed an increasing tendency with respect to the entry ratio to the open arms and the time spent in open arms (Figure 3D and 3E). Furthermore, mice administered 3.4 mg/L air (+)-LI (i.h.) and 6.7 mg/L air EOCJ (i.h.) indicated a significant increase ($P < 0.05$) in the entry ratio to the open arms. Mice administered 6.7 mg/L air (+)-LI (i.h.) and 6.7 mg/L air EOCJ (i.h.) showed a significant increase ($P < 0.05$) in the time spent in open arms.

In the OF test, the mice administered (+)-LI (i.h.) did not indicate an increase in locomotor activity (Figure 3C). However, the mice administered 6.7 mg/L air EOCJ indicated an increase in locomotor activity.

However, the anxiolytic-like effect of (+)-LI was smaller than that of EOCJ in the LDB test. Indeed, in

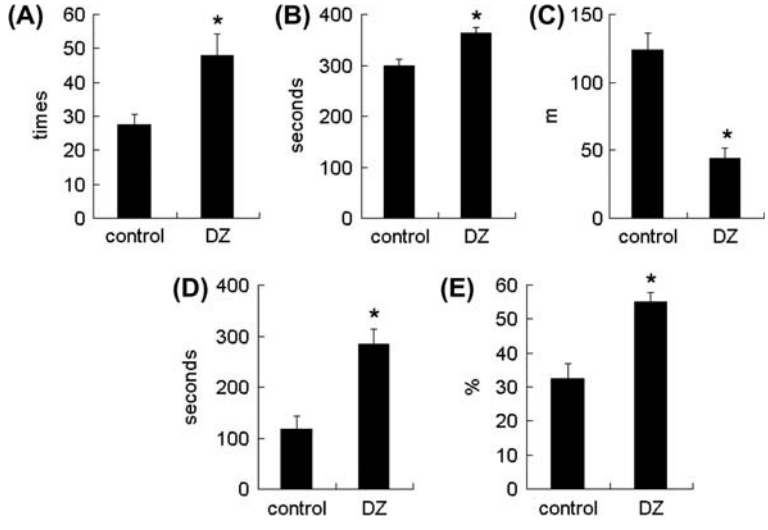

Figure 2. The anxiolytic-like effect of i.p. administration of 3 mg/kg diazepam, as assessed by the LDB test, OF test, and EPM test (mean ± standard error, n = 5). (A) Number of entries into the light compartment (times) in the LDB test, (B) time in the light compartment (in seconds) in the LDB test, (C) total distance traveled in the OF (in meters) in the OF test, (D) entry ratio in the open arms (%) in the EPM test, (E) time in the open arms (in seconds) in the EPM test. For the control 0.1% Tween 80/saline was used. DZ, diazepam. $^{*}P < 0.05$.

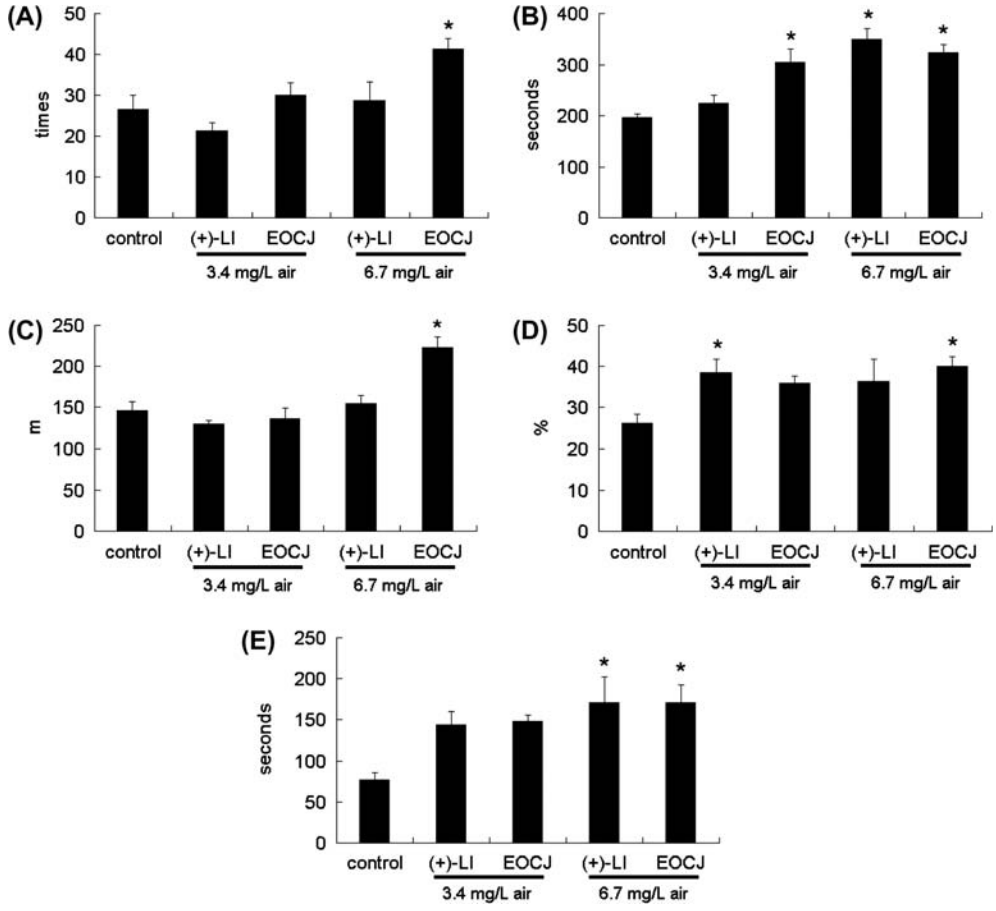

Figure 3. The anxiolytic-like effects of (+)-LI and EOCJ (3.4 and 6.7 mg/L air i.h.), as assessed using the LDB test, OF test, and EPM test (mean ± standard error, $n = 5$). (A) Number of entries into the light compartment (times) in the LDB test, (B) time in the light compartment (in seconds) in the LDB test, (C) total distance traveled in the OF (in meters) in the OF test, (D) entry ratio in the open arms (%) in the EPM test, (E) time in the open arms (in seconds) in the EPM test. Water was used as the control. (+)-LI, (+)-limonene. EOJC, essential oil from *Citrus junos*. $^{*}P < 0.05$.

the LDB test the anxiolytic-like effect of (+)-LI was smaller than that of EOCJ in terms of number of entries into the light compartment (Figure 3A), but not in relation to the time in the light compartment (Figure 3B). Additionally, the increase in locomotor activity was not observed with 6.7 mg/L air (+)-LI (i.h.).

Experiment three: Distribution of LI in mouse organs. To clarify the amount of the LI, the major component of EOCJ, absorbed after inhaled administration, the internal organs (liver, kidney, and brain) were analyzed. The amount of LI in the liver increased slightly with 6.7 mg/L air EOCJ compared to administration of 6.7 mg/L air (+)-LI (not significantly different) (Figure 4A). In the other internal organs (kidney and brain), the amount of LI was almost the same (Figure 4B and 4C).

It is thought that mixtures (multi-component substances), such as natural extracts, act according to the major component. However, the effects of multi-component mixtures cannot wholly be explained by the main component. For example, Japanese traditional (Kampo) medicine is composed of multiple components, and these were investigated with respect to psychological disease (27–34). We performed this research to clarify one aspect (anxiolytic-like effect) of the utility of a multi-component EO.

In this research, in investigating the differences between the influence of EOCJ and (+)-LI on emotional behavior in mice (EOCJ increased locomotor activity), it is thought that minor components other than (+)-LI play a role in the effects of EOCJ. (+)-LI comprises approximately 70% of EOCJ. The anxiolytic-like effect of (+)-LI is likely to predominate when administering low doses of EOCJ (3.4 mg/L air i.h.). However, the effect of the minor components on locomotor activity might have been more evident with administration of the high dose of EOCJ (6.7 mg/L air i.h.). That is, the higher dose might have produced both an anxiolytic-like effect and an increase in locomotor activity.

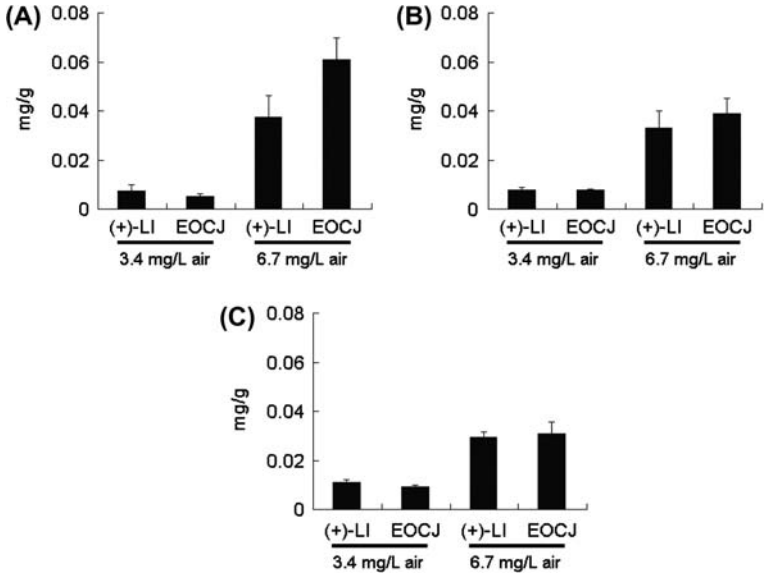

Figure 4. The distribution of LI (mg/g organ) on administration of (+)-LI and EOCJ (3.4 and 6.7 mg/L air i.h.). (A) In the liver, (B) kidney, (C) brain (mean ± standard error, $n = 5$). (+)-LI, (+)-limonene. EOJC, essential oil from *Citrus junos*.

It is thought that multi-component EOs will produce synergistic and diversified effects due to the interaction and cumulative effects of their various components. In this study, we were able to clarify an aspect of the mixture effect of EOCJ. The relationship between a mixture such as EO and the effects of its minor components is a question worthy of investigation. It is expected that future studies such as these will provide further clarification.

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