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ORIGINAL ARTICLE

Neuropharmacological activities of fruit essential oil from *Litsea cubeba* Persoon

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Abstract *Litsea cubeba* (Lauraceae) is woody plant endemic to Taiwan that is traditionally used as a spice. In the current study, several behavioral analyses were performed to evaluate the neuropharmacological activity of the essential fruit oil of *L. cubeba* in ICR mice. Oral administration of 100, 300 and 500 mg/kg of *L. cubeba* fruit oil significantly prolonged pentobarbitone-induced mouse sleeping time by 20.0, 110.8, and 159.6 %, respectively. In addition, after administration of *L. cubeba* oil, mice significantly increased the time spent in the open arms and number of entries into the open arms of an elevated plus maze compared to saline-treated mice suggesting that *L. cubeba* oil has anxiolytic activity. A tail-flick test conducted after treatment of mice with 500 mg/kg *L. cubeba* fruit oil also suggested that this oil has potent analgetic activity. According to GC/MS analyses, the essential fruit oil of *L. cubeba* oil consists of 23 compounds. The main components are geranial (37.16 %), neral (28.29 %), and *d*-limonene (22.90 %). We conclude that *L. cubeba* oil has a potent effect on the central nervous system of mice.

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Introduction

The World Health Organization (WHO) reported that in 2003 more than 450 million people suffered from mental or behavioral disorders [1]. Thus, discovery of new neuropharmacologically active phytocompounds as therapeutic alternatives for such disorders is of interest. The local name for *Litsea cubeba* (Lour.) Persoon (Lauraceae) is mountain pepper. Indigenous people in Taiwan traditionally use this plant to treat inflammation, headache, and intoxication [2]. Many studies have shown that essential oils of *L. cubeba* exhibit a range of bioactivities such as antitermite [3], antioxidant [4], larvicidal [5], and cytotoxic [6] activities. However, reports on the neuropharmacological activity of the essential oil from *L. cubeba* are rare. In this study, several animal behavioral analyses were performed to evaluate the neuropharmacological activities of the oil from the fruit of *L. cubeba* to understand its effect on the central nervous system. The composition of the essential oil was also characterized.

Materials and methods

Plant materials

Mature fruit of 15-year-old *L. cubeba* were collected from Huisun Experimental Forest, Nantou, Taiwan in June 2008. Prof. Yen-Hsueh Tseng from the Department of Forestry, National Chung-Hsing University, confirmed taxonomic identification, and voucher specimens were deposited at the Herbarium of the Department of Forestry, National Chung-Hsing University, Taiwan.

Fruit essential oil preparation

Fruits of *L. cubeba* (800 g) underwent water distillation for 6 h in a Clevenger-type apparatus, and oil content (ml/kg) was determined based on the dry weight of the fruit. The essential oil was stored in sample vials after deoxygenation with nitrogen prior to analysis by gas chromatography (GC) and GC–mass spectrometry (GC/MS).

GC/MS analyses of essential oil of *L. cubeba*

The compositions of the fruit essential oils were analyzed by GC/MS (HP G1800A; Hewlett Packard, USA), equipped with a DB-5 ms column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J & W Scientific). The temperature program was as follows: 40 °C for 1 min, then increased by 4 °C/min to 260 °C and held for 4 min. The other parameters were as follows: injection temperature, 270 °C; ion source temperature, 280 °C; EI, 70 eV; carrier gas, He at 1 ml/min; injection volume, 1 μl; spilt ratio, 1:50; and mass range, *m/z* 45–425. Quantification was obtained from percentage peak areas from the gas chromatogram. A Wiley/NBS Registry of Mass Spectral Data search and authentic reference compounds were used for substance identification. The Kovats retention index (KI), which is a parameter calculated in reference to *n*-alkanes that converts retention times into system-independent constants, was also confirmed [7]. Chromatography results expressed as area percentages were calculated with a response factor of 1.0.

Animals

Male ICR mice (4 weeks old, 25–28 g) were purchased from BioLasco (Taiwan). Mice were allowed 1 week to acclimatize before testing. They were housed under conditions of controlled temperature (25 ± 2 °C), relative humidity (55 ± 5 %), and lighting (light period 06:00–18:00), with food and water ad libitum. The animals were transferred to the laboratory at least 1 h before the start of each experiment. All animal experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals,* and Taiwan laws relating to the protection of animals, and were approved by the local ethics committee.

Behavioral analysis

During behavioral assays, all the activities of test mice were recorded for visual and automated quantitative analysis using a DSP CCD camera (Model: KMS-63F4) connected to a computer installed with Noldus software (Ethovision version 4.0, Noldus Information Technology, Wageningen, the Netherlands) for data acquisition.

Open-field test

The open-field test was used to evaluate the exploratory activity of the animals [8]. Open-field activity was measured in a wooden cage (76 cm × 76 cm × 40 cm) divided into 25 squares of equal area. The mice were divided into four groups ($n = 10$) and oral administered *L. cubeba* fruit oil, which were diluted with corn oil (100, 300, and 500 mg/kg/d) using a gastric feeding tube or corn oil orally for 8 days. On day 8, 1 h after *L. cubeba* fruit oil administration, each mouse was placed in the center of the open-field arena. The distance of movement, mean speed and the time spent immobile during a 5-min observation period were recorded to indicate the exploratory activity of mice.

Pentobarbital-induced sleeping time

The mice were divided into 5 groups ($n = 8$). The control group was injected with pentobarbitone sodium [45 mg/kg; intraperitoneally (i.p.)] only. The other groups were injected with pentobarbitone sodium (45 mg/kg; i.p.) sixty minutes after oral administration of *L. cubeba* fruit oil (100, 300 and 500 mg/kg/d), or zolpidem hemitartrate (0.3 mg/kg/d), respectively. The time elapsing between loss and recovery of the righting reflex was considered as sleeping time and recorded for control and treated animals [9].

Elevated plus-maze test

The elevated plus maze is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents [10]. The elevated plus maze for mice consists of two opposing open arms (32 cm × 6 cm) perpendicular to two opposing closed arms (32 cm × 6 cm) with walls (15 cm). The plus maze was elevated 50 cm above the floor. The control group was treated with saline only, and a positive control group was treated with trazodone hydrochloride (10 mg/kg), a well-known psychoactive compound with sedative and anti-depressant properties used clinically for relief of an anxiety disorder. The other groups were fed orally with *L. cubeba* fruit oil (100, 300 and 500 mg/kg/d) for 7 days, respectively. On day 7, mice were individually placed on the center of the maze. The number of entries and the time spent in the open arms of the apparatus were recorded during a 5-min observation period.

Tail-flick test

The tail-flick test measures pain response in animals. It is used in basic pain research and to measure the effectiveness of analgesics by observation of animal reaction to intense heat [11]. Mice that withdrew their tails from hot

water (52 °C) in 3.0 ± 1.0 s were pre-selected 24 h before the start of the experiment. The test mice were divided into five groups ($n = 10$). The control group was treated with saline only, the positive control group was treated with acetaminophen (60 mg/kg) and the experimental groups were fed *L. cubeba* fruit oil (100, 300, and 500 mg/kg/d) orally for 9 days. On day 9, the tail of each test mouse was placed in hot water (52 °C) 0, 30, 60, and 120 min after administration of *L. cubeba* fruit oil, acetaminophen or saline. Withdrawal of the tail from the hot water was taken as the end point. A cut-off time of 10 s was adopted to prevent damage to mouse tails [12].

Statistical analysis

Data are expressed mean ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($*P < 0.05$ and $**P < 0.01$) between the control (untreated) and treated cells were analyzed by Dunnett's test.

Results

Composition analysis of essential oil

Hydrodistillation of *L. cubeba* fruits yielded 18 ml/kg essential oil according to dry weight. Table 1 shows the results of GC/MS analyses of fruit essential oil from *L. cubeba*. A total of 23 compounds were identified from the fruit essential oil of *L. cubeba*. The main components were geranial (37.16 %), neral (28.29 %), and *d*-limonene (22.90 %). Geranial, neral and limonene made up around 90 % of the content of the fruit oil; the contents of other constituents are smaller than 1 %.

Effect of essential on open-field test

In the open-field test, the average total distance travelled by the vehicle control animals was 3802.3 ± 381.9 cm at a speed of 13.25 ± 1.22 cm/s; when mice received 100, 300, or 500 mg/kg of *L. cubeba* fruit oil, the distances travelled were reduced to 3002.4 ± 790.0, 2906.5 ± 325.1, and 2809.0 ± 452.9 cm, respectively; and the speed of movement decreased to 9.98–10.18 cm/s (Table 2). Oral administration of *L. cubeba* fruit oil did not result in any significant change in time spent immobile.

Effect of essential oil on pentobarbital-induced sleeping time in mice

The effect of oral administration of essential fruit oil of *L. cubeba* on pentobarbital-induced sleeping of mice is shown in Table 3. When treated with *L. cubeba* fruit oil (100, 300, and 500 mg/kg), pentobarbitone-induced mouse sleeping time was dose-dependently prolonged by 20.0 % (41.3 ± 12.8 min), 110.8 % (72.5 ± 19.8 min), and 159.6 % (89.3 ± 31.2 min), respectively (Table 3). In this study, zolpidem hemitartrate was used as a positive control; it prolonged the pentobarbitone-induced sleeping time from 34.4 ± 6.8 to 46.8 ± 3.39 min at the dosage of 0.3 mg/kg.

Table 1 Compositions of fruit essential oil from *Litsea cubeba*

Compound	Concentration (%)	KI	Identification[a]
α-Pinene	0.98	929	MS, KI, ST
Camphene	0.05	953	MS, KI, ST
Sabinene	0.16	967	MS, KI, ST
β-Pinene	0.87	969	MS, KI, ST
6-Methyl-5-hepten-2-one	0.31	983	MS, KI
β-Myrcene	2.06	987	MS, KI, ST
p-Cymene	0.03	1016	MS, KI, ST
d-Limonene	22.90	1023	MS, KI, ST
1,8-Cineol	0.78	1026	MS, KI, ST
Terpinolene	0.07	1082	MS, KI, ST
Linalool	0.87	1097	MS, KI, ST
Citronellal	1.00	1153	MS, KI, ST
Neral	28.29	1254	MS, KI, ST
Geranial	36.16	1277	MS, KI, ST
α-Terpinyl acetate	0.72	1349	MS, KI
Neryl acetate	0.17	1362	MS, KI
Geranyl acetate	0.76	1381	MS, KI
α-Copaene	0.05	1375	MS, KI, ST
β-Caryophyllene	0.49	1419	MS, KI, ST
β-Copaene	0.08	1429	MS, KI, ST
Elixene	0.24	1445	MS, KI
α-Caryophyllene	0.04	1450	MS, KI, ST
Caryophyllene oxide	0.02	1579	MS, KI, ST

KI Kovats index on a DB-5MS column in reference to *n*-alkanes, *ST* authentic standard compounds

[a] MS, NIST and Wiley libraries and the literature

Table 2 Effects of *L. cubeba* fruit oil on locomotor activity of mice in the open-field test

Treatment (mg/kg, p.o.)	Total distance travelled (cm)	Speed (cm/s)	Time spent immobile (s)
Control	3802.3 ± 381.9	13.25 ± 1.22	26.16 ± 5.9
Fruit oil (100)	3002.4 ± 790.0*	9.98 ± 3.13*	26.72 ± 11.59
Fruit oil (300)	2906.5 ± 325.1*	10.12 ± 1.02*	26.58 ± 4.94
Fruit oil (500)	2809.0 ± 452.9*	10.18 ± 1.99*	26.78 ± 3.07

Data are presented as mean ± SD from 10 animals. Observations were made 60 min after oral administration of vehicle (control) or fruit oil of *L. cubeba*

* $P < 0.05$ compared with vehicle-treated controls

Table 3 Effect of fruit oil of *L. cubeba* on pentobarbitone-induced sleeping time in mice

Treatment (mg/kg)	Mean sleeping time ± SD (min)
Control saline (20)	34.4 ± 6.8
Fruit oil (100)	41.3 ± 12.8*
Fruit oil (300)	72.5 ± 19.8***
Fruit oil (500)	89.3 ± 31.2**
Zolpidem hemitartrate (0.3)	46.8 ± 3.39*

All animals were treated with pentobarbitone sodium (45 mg/kg, i.p.) 60 min after oral administration of vehicle and fruit oil of *L. cubeba*

* $P < 0.05$, ** $P < 0.01$ compared with vehicle-treated controls

Effect of essential oil on mice in the elevated plus-maze

Next, the elevated plus-maze test was used to evaluate the anxiolytic activity of fruit essential oil of *L. cubeba*. Trazodone hydrochloride was used as a reference compound. Table 4 shows the effect of essential oil and trazodone hydrochloride on behavior in the elevated plus-maze test. The time of spent in the open arms was 9.72 ± 6.67 s, and number of entries into the open arms was 10.38 ± 8.70 for vehicle control (treated animals with saline). Independent *t* test revealed that administration of fruit oil (500 mg/kg) significantly increased the time spent in the open arms (36.55 ± 11.65 s) ($P < 0.01$) and number of entries into the open arms (25.75 ± 11.51 times) ($P < 0.05$), compared to the saline-treated group. The time spent in the open arms of mice that underwent trazodone hydrochloride treatment (10 mg/kg; p.o.) was 28.86 ± 10.31 s and the number of entries into the open arms was 21.63 ± 14.91 times.

Effect of essential oil on mice in the tail-flick test

In the tail-flick test, doses of 100 and 300 mg/kg of fruit essential oil of *L. cubeba* showed no significant effects. However, significant prolongation of reaction times could be seen at doses of 500 mg/kg at 30 min post-treatment (Fig. 1). Fruit oil treatment significantly prolonged ($P < 0.01$) the tail-flick time at up to 90 min post-treatment. With the 500 mg/kg dose, maximum analgesic activity was seen at 60 min post-treatment. The activity of positive control group, acetaminophen (90 mg/kg), was seen up to 90 min post-treatment.

Discussion

L. cubeba is a unique evergreen tree that grows in Asian countries. It is a well-known traditional Chinese medicine that has been used for "warming and dispersing coldness" as well as relieving pain for thousands of years [13].

Table 4 Effects of fruit essential oil of *L. cubeba* and trazodone hydrochloride on mouse behavior in the elevated plus-maze test

Treatment (mg/kg, p.o.)	Time spent in open arms (s)	Number of entries into open arms (times)
Control	9.72 ± 6.67	10.38 ± 8.70
Fruit oil (100)	18.73 ± 9.55	12.75 ± 9.98
Fruit oil (300)	28.07 ± 8.46**	18.43 ± 8.75
Fruit oil (500)	36.55 ± 11.65**	25.75 ± 11.51*
Trazodone hydrochloride (10)	28.86 ± 10.31**	21.63 ± 14.91*

Data are presented as mean ± SD from 10 animals. Observations were made 60 min following the oral administration of vehicle (control) or fruits oil of *L. cubeba*

* $P < 0.05$ compared with vehicle-treated controls. * $P < 0.05$, ** $P < 0.01$, compared with vehicle-treated controls

According to the results obtained by Luo et al., the oral, dermal, and inhalation 50 % lethal dose and concentration (LD_{50} and LC_{50}) of *L. cubeba* oil were determined. Results indicated that the oral 50 % lethal dose (LD_{50}), the dermal LD_{50}, and the inhalation 50 % lethal concentration (LC_{50}) are approximately 4000 mg/kg of body weight, in excess of 5000 mg/kg, and approximatively 12500 μg/ml, respectively. Luo and his coworkers [14], therefore, conclude that *L. cubeba* oil is slightly toxic. In addition, they also demonstrated that genetic toxicity testing of *L. cubeba* oil in vitro and in vivo was negative. *L. cubeba* is characterized by its pleasant citrus scent, particularly emitted from the fruits. The oil is distilled from the small, pepper-like fruits of the tree for commercial use. *L. cubeba* oil is rich in citral, which includes geranial (the *E*-isomer, also known as citral) and neral (the *Z*-isomer, also known as citral B), and has an intense lemon-like, fresh, sweet odor. Its fragrance is quite similar to lemongrass and lemon verbena, but smells even sweeter. According to a report by Ho and his coworkers [6], *L. cubeba* can be classified into three chemotypes: citral, limonene, and citronellal. In our study, the dominant component was citral composed of geranial (37.16 %) and neral (28.29 %); thus, our sample is classified as citral type *L. cubeba*.

Although considerable scientific evidence has proved that *L. cubeba* oil exhibits various bioactivities, studies on the neuropharmacological activity of this plant are rare. The present study demonstrated the neuropharmaological effect of *L. cubeba* fruit oil on mice. First of all, there is no statistical difference on weight change between the oil treatment groups and control group mice (as shown in Fig. 2, weight change in the mice elevated plus-maze test as an example). In standard animal behavioral evaluation tests, *L. cubeba* oil reduced the locomotor activity of mice. However, in the open-field test the time that the mice spent immobile did not increase after treatment with *L. cubeba*

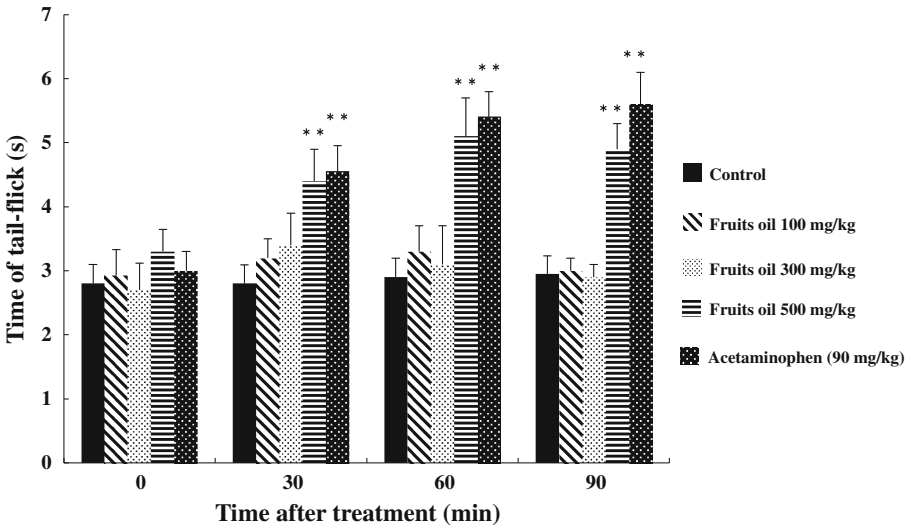

Fig. 1 Analgesic effect of *L. cubeba* fruit essential oil and acetaminophen (90 mg/kg) measured by the tail-flick test. Each bar represents values as mean ± SD ($n = 10$). $^{**}P < 0.01$ compared with vehicle-treated controls

oil (Table 2). Pretreatment with *L. cubeba* oil noticeably prolonged pentobarbital-induced sleeping time in a dose-dependent manner (Table 3). In the elevated plus-maze test for anxiety, *L. cubeba* oil obviously increased the time spent and number of entries into the open arms of the apparatus (Table 4) suggesting stress reduction. Moreover, the results of the tail-flick test of pain response suggested that high doses of *L. cubeba* oil have antinociceptive activity (Fig. 1). It is worthy to note that the analgesic only observed at the dosage of 500 mg/kg group. According to the review by de Sousa [15], limonene, neral, and geranial, which are also the major compounds in the *L. cubeba* essential oil, possess the analgesic effect. However, it needs to reach the action concentration. This neuropharmaological effect of *L. cubeba* oil is similar to that found with several other plants such as *Cymbopogon citratus* [16], *Lippia alba* [17], and *Melissa officinalis* [18] that have citral and limonene as main constituents. In a previous study [19], the essential oils from leaves of *C. japonica* were found to prolong the sleeping time in pentobarbital-treated of ICR mice. In addition, both the essential oil and one of its constituent monoterpenes, *d*-limonene, were found to possess potent anxiolytic and analgestic activities based on the results obtained from elevated plus-maze and writhing tests. Moreover, Fukumoto and colleagues [20] demonstrated that citrus essential oil containing components such as limonene and citral could decrease both physical and psychological stress. Dobetsberger and Buchbauer [21] also reported that citral and limonene possesses distinct stress-reducing activity. It is well adapted that $GABA_A$ (γ-amino butyric acid A) receptor mediates the majority of inhibitory neurotransmission in the mammalian central nervous system (CNS) and therefore it is a major focus in neuropharmacology research. $GABA_A$ receptor is ligand-gated chloride channels that mediate an inhibitory

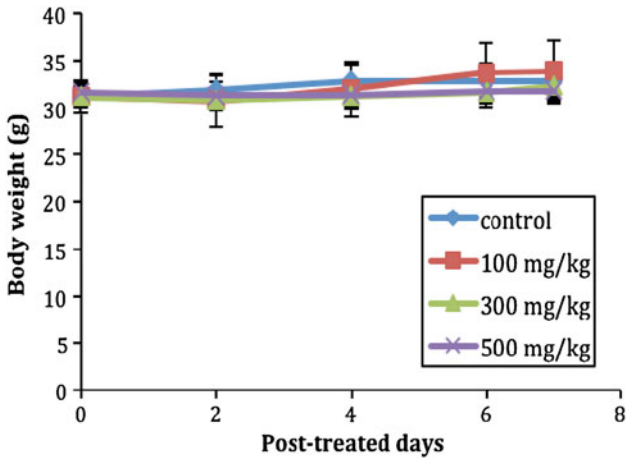

Fig. 2 Weight changes of mice in the elevated plus-maze test. The dosages of fruit essential oil for mice were 100 (*filled square*), 300 (*triangle*), and 500 (*open square*) mg/kg

effect by increasing the chloride influx into neurons, inducing membrane hyperpolarisation and neuronal inhibition. Recently, several studied have reported that monoterpenoids present within the essential oils of such herbal medicines have modulatory activity with GABA at several $GABA_A$ receptor subtypes. Zhou et al. [22] demonstrated the effect of limonene on brain neurotransmitter levels and behavior on rat. According to their results, limonene was applied for 1 week, significant changes concerning, for example GABA, 5-HIAA and 5-HT, were observed. Meanwhile, basal hypothalamic–pituitary–adrenal (HPA) activity was determined by corticosterone after administration of limonene for 1 week. Due to the increased concentration of GABA and the changes of other neurotransmitters, an anti-stress effect was assumed. A possible mechanism might involve stress-induced hypothalamic–pituitary–adrenal (HPA) inhibitory activity and a

stress-alleviating effect possibly mediated by the $GABA_A$ receptor. The neuropharmacology activities of *L. cubeba* oil might be regulated via the similar mechanism.

The results obtained in this study suggest possible application of *L. cubeba* oil as a functional food and/or pharmaceutical to aid central nervous system regulation. In conclusion, this unique indigenous aromatic plant showed strong neuropharmaological activity in animals. Applications of its compounds might be useful for regulation of the central nervous system. Further study of the compounds of *L. cubeba* oil and its mechanism of action are warranted.

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