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ORIGINAL ARTICLE

Wei-Wen Cheng · Chien-Tsong Lin · Fang-Hua Chu
Shang-Tzen Chang · Sheng-Yang Wang

Neuropharmacological activities of phytoncide released from *Cryptomeria japonica*

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Abstract Forest-air bathing and walking (*shinrin-yoku*) is beneficial to human heath. In this study the phytoncide (volatile compounds) released from *Cryptomeria japonica* plantation forest was characterized by using solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). The main volatile compounds were α-pinene (19.35%), β-myrcene (16.98%), D-limonene (15.21%), and γ-muurolene (7.42%). Furthermore, the neuropharmacological activity of the essential oils from leaves of *C. japonica* (ECJ) was evaluated by several animal behavior tests. ECJ could prolong the sleeping phase of ICR (imprinting control region) mice in the pentobarbital-induced sleeping time model. Furthermore, both EJC and one of its monoterpenes, D-limonene, possessed potent anxiolytic and analgesic activities based on the results obtained from elevated plus maze and writhing tests. The volatile compounds released from *C. japonica* provide relaxing and stress-relieving effects on mice, and further study on the effect of phytoncide on humans is worthwhile.

Key words *Cryptomeria japonica* · Essential oil · Neuropharmacological activities · Phytoncide · SPME

Introduction

It is well known that plant-derived essential oils exhibit multiple biological properties, such as antifungal activity,[1,2] insecticidal activity,[3] and anti-inflammation activity.[4] The World Health Organization[5] reported that around 450 million people suffer from mental or behavioral disorders. Thus, finding new neuropharmacologically active compounds from plants as therapeutic treatments for such disorders has progressed constantly. Phytoncide is defined as the antimicrobial volatile organic compounds emitted from plants. In chemical terms, the main components of phytoncide are closely related to essential oils produced from plants. Peoples in Asian countries, such as Japan, Taiwan, China, and Korea, believe that forest-air bathing and walking (*shinrin-yoku*) have various benefits to human health.[6,7] Walking in forest areas and breathing phytoncide emitted from the trees is not only pleasant and refreshing but also is beneficial for stress management and relaxation.[8]

Cryptomeria japonica (Japanese cedar or sugi) is native to Japan, and in Taiwan is one of the most important plantation conifers. Many researchers have shown that essential oils from *C. japonica* (ECJ) exhibit several bioactivities, including antisilverfish,[9] antitermite,[10] antimosquito,[11] and antifungal activity.[12] However, to the best of our knowledge, there is no report of neuropharmacological activity of the essential oil from *C. japonica*. Although forest-air bathing and walking are strongly promoted by the Forestry Bureau of Taiwan, there is a lack of information concerning the composition of phytoncide emitted from natural forests as well as scientific evidence for improving human health. In the present study, the phytoncide (volatile compounds) emitted from the leaves of *C. japonica* was characterized by using the solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS). In addition, several animal behavior tests were used to evaluate the neuropharmacological activities of ECJ. The results showed that ECJ and one of its representative constituents, D-limonene, have significant neuropharmacological activities.

Materials and methods

Plant materials

Phytoncide of *Cryptomeria japonica* was collected from the 35-year-old sugi plantation in the Experimental Forest of

W.-W. Cheng · C.-T. Lin · S.-Y. Wang (✉)
Department of Forestry/Core Lab of Plant Metabolomics in Biotechnology Development Center, National Chung-Hsing University, No. 250 Kuo-Kuang Road, Taichung 402, Taiwan
Tel. +886-4-22840345; Fax +886-4-22873628
e-mail: taiwanfir@dragon.nchu.edu.tw

F.-H. Chu · S.-T. Chang
School of Forestry and Resource Conservation, National Taiwan University, Taipei 106, Taiwan

National Taiwan University. Fresh leaves of *C. japonica* were obtained from the same stand in July 2007. The samples were identified by Dr. Yen-Hsueh Tseng of National Chung-Hsing University (NCHU). The voucher specimen [SYW 001 (TCF)] was deposited in the herbarium of NCHU.

Phytoncide collection by SPME

To obtain and analyze phytoncide from leaves, the SPME technique was used to collect the volatile compounds from the natural stand. A SPME holder and carboxen-polydimethylsiloxane (75 µm) were purchased from Supelco (Bellefonte, USA). The SPME fibers were conditioned by heating in a hot injection port of a gas chromatograph (GC) at 250°C for 20 min to remove contaminants before use. The leaves of *C. japonica* were put into a zip-lock bag, and a SPME fiber was introduced into the bag and exposed to the headspace of the bag for 30 min. The conditions selected in this study were based on a previous experiment where complete optimization of the extraction conditions was carried out.[13] After 30 min of adsorption, the SPME fiber was removed from the zip-lock bag and immediately frozen at –20°C until analysis. Finally, the SPME fiber was inserted into the injection port of the GC with SPME-liner where the terminal description occurs at 250°C for 30 s.

Essential oil preparation

Leaves of *C. japonica* (500 g) were cut into small pieces (2–3 cm), and subjected to water distillation for 6 h in a Clevenger type apparatus, followed by determination of oil content based on leaf dry weight. Essential oil was stored in sample vials after deoxygenation with N_2 prior to analysis by GC and GC-MS.

GC-MS analysis of essential oil

The compositions of essential oils were analyzed by GC-MS (HP G1800A; Hewlett Packard, USA), equipped with a DB-5ms 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^{-1} to 260°C, and held for 4 min. The other parameters were as follows: injection temperature, 270°C; ion source temperature, 280°C; ionization energy, 70 eV; carrier gas, He at 1 ml min^{-1}; injection volume, 1 µl; split ratio, 1:50; mass range, *m/z* 45–425. Quantification was obtained from percentage peak areas from the gas chromatogram. Wiley (V. 7.0)/NBS (V. 2.0) Registry of Mass Spectral Database libraries search and authentic reference compounds were used for substance identification. Chromatographic results expressed as area percentages were calculated with a response factor of 1.0.

Animals

Male ICR (imprinted control region) mice (4 weeks old, 25–28 g) were purchased from BioLasco (Taiwan). Mice were allowed 1 week to acclimatize before testing. They were housed with conditions controlled to 25° ± 2°C and 55% ± 5% relative humidity, with lighting from 06:00 to 18:00 h, and food and water ad libitum. The animals were transferred to the laboratory at least 1 h before the start of the experiment.

Behavioral analysis

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

Pentobarbital-induced sleeping time

The mice were divided into seven groups (n = 7). The control group received pentobarbitone sodium [45 mg kg^{-1}; intraperitoneal (i.p.)] only. The other groups were injected with pentobarbitone sodium (45 mg kg^{-1}; i.p.) 60 min after oral administration (p.o.) of EJC (100, 300, or 500 mg kg^{-1}), D-limonene (200 mg kg^{-1}), α-pinene (200 mg kg^{-1}) or zolpidem hemitartrate (0.3 mg kg^{-1}). The time elapsed between loss and recovery of the righting reflex was recorded as sleeping time and recorded for control and pretreated animals.[14]

Elevated plus maze test

The elevated plus maze is a widely used behavioral assay for rodents and it has been validated to assess the antianxiety effects of pharmacological agents.[15] The elevated plus maze for mice consisted of two opposing open arms (32 × 6 cm) perpendicular to two opposing closed arms (32 × 6 cm) with walls (15 cm). The plus maze was elevated 50 cm above the floor. The control group was treated with saline only, the positive control group was treated with trazodone hydrochloride (10 mg kg^{-1}), and the others groups were fed orally with the EJC (100, 300, or 500 mg kg^{-1} day^{-1}; p.o.) or D-limonene (100 mg kg^{-1} day^{-1}; p.o.) for 7 days. At day 7, mice were individually placed on the center of the maze. The number of entries and the time spent in opened arms were recorded during a 5-min observation period.

Writhing test

Writhing test was assessed by the acetic acid abdominal constriction test, which is a chemical visceral pain model.[15] Briefly, mice were divided into six groups (n = 8): treated with EJC (100, 300, or 500 mg kg^{-1} day^{-1}; p.o.), acetamino-

phen (60 mg kg^{-1} day^{-1}; p.o.), D-limonene (100 mg kg^{-1} day^{-1}; p.o.), or saline only for 10 days. At day 10, mice were injected intraperitoneally (i.p.) with 10 ml kg^{-1} of 0.9% acetic acid solution after 60 min of oral administration of EJC. Starting 10 min after acetic acid administration, the number of writhing episodes was counted in a 10-min period.

Statistical analysis

Data were expressed means ± standard deviation (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 phytoncide

Leaves of 35-year-old *Cryptomeria japonica* were subjected to water distillation. The yield of oil obtained from fresh leaves was 24.6 ml kg^{-1}. Table 1 shows the results of GC-MS analyses of leaf essential oil from *C. japonica*. The main components were elemol (18.22%), 16-kaurene (11.63%), 3-carene (9.66%), sabinene (9.37%), terpinene-4-ol (9.06%), α-eudesmol (5.70%), α-pinene (5.62%), and D-limonene (5.26%). In addition to determining the composition of EJC, we also used SPME to collect the phytoncide, which was emitted from *C. japonica* under natural conditions. As shown in Table 1, the composition and dominant constituents are different between essential oil and natural emitted phytoncide. α-Pinene (19.35%) was the dominant compound, which was emitted from the leaves of living *C. japonica*, followed by β-myrcene (16.98%), D-limonene (15.21%), and γ-muurolene (7.42%). Several volatiles were only found in natural released phytoncide, including β-ocimene, copaene, α-cedrene, β-elemene, longifolene, γ-muurolene, α-caryophyllene, and cedrol.

Effect of phytoncide on pentobarbital-induced sleeping time in mice

The effect of oral administration of ECJ on pentobarbital-induced sleeping of mice is shown in Table 2. When treated

Table 1. Composition analysis of phytoncide from leaves of *Cryptomeria japonica*

Compound	KI[a]	Content in essential oil (%)	Content in phytoncide (%)	Identification[b]
3-Hexen-1-ol	868	–[c]	2.55	MS, KI
α-Thujen	921	0.79	0.93	MS, KI
α-Pinene	929	5.62	19.35	MS, KI, ST
Camphene	941	0.96	1.37	MS, KI, ST
Sabinene	967	9.37	4.65	MS, KI
β-Pinene	969	0.19	–	MS, KI
β-Myrcene	983	2.59	16.98	MS, KI, ST
3-Carene	1006	9.66	2.51	MS, KI, ST
α-Terpinene	1010	1.90	1.48	MS, KI, ST
p-Cymene	1016	0.21	1.54	MS, KI, ST
D-Limonene	1023	5.26	15.21	MS, KI, ST
β-Ocimene	1038	–	0.79	MS, KI
γ-Terpinene	1052	3.08	2.14	MS, KI, ST
α-Terpinolene	1082	1.58	1.86	MS, KI, ST
Unknown	1109	–	2.44	MS, KI
Linalool	1116	0.47	–	MS, KI, ST
Terpinene-4-ol	1174	9.06	0.56	MS, KI
α-Terpinol	1183	0.51	–	MS, KI, ST
Linalyl acetate	1259	–	0.61	MS, KI, ST
Bornyl acetate	1278	0.76	2.59	MS, KI
Unknown	1325		0.32	MS, KI
α-Longipinene	1355	–	0.92	MS, KI
Copaene	1380	–	0.97	MS, KI
β-Elemene	1397	–	2.05	MS, KI
Longifolene	1405	–	3.48	MS, KI
α-Cedrene	1416	–	1.05	MS, KI
α-Caryophyllene	1450	–	0.74	MS, KI
γ-Muurolene	1469	–	7.42	MS, KI
δ-Cadinene	1525	0.53	–	MS, KI
Elemol	1548	18.22	–	MS, KI
Cedrol	1596	–	5.48	MS, KI, ST
δ-Seliene	1609	4.36	–	MS, KI
α-Eudesmol	1655	5.70	–	MS, KI
16-Kaurene	2040	11.63	–	MS, KI

[a] Kovats index on a DB-5ms column in reference to *n*-alkanes
[b] MS, NIST and Wiley libraries and literature; KI, Kovats index; ST, authentic standard compounds
[c] not detected

Table 2. Effect of essential oils from leaves of *C. japonica* (EJC) on pentobarbitone sleeping time in mice

Treatment	Mean sleeping time ± SD (min)
Control saline (20 ml kg^{-1})	37.2 ± 2.87
EJC (100 mg kg^{-1})	45.7 ± 4.71*
EJC (300 mg kg^{-1})	54.4 ± 4.74**
EJC (500 mg kg^{-1})	62.3 ± 11.15***
D-Limonene (200 mg kg^{-1})	43.0 ± 4.79*
α-Pinene (200 mg kg^{-1})	37.5 ± 3.48
Zolpidem hemitartrate (0.3 mg kg^{-1})	46.8 ± 3.39*

All animals were treated with pentobarbitone sodium (45 mg kg^{-1}, i.p.) 60 min after oral administration of vehicle and essential oils from leaves of *C. japonica*
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with vehicle-treated controls

Table 3. Effects of ECJ, D-limonene, and trazodone hydrochloride on the behavior of mice in the elevated plus maze test

Treatment		Time spent in open arms (s)	Number of entries into open arms
Vehicle	Concentration (mg kg^{-1})		
Control	–	12.68 ± 8.67	18.88 ± 11.7
Essential oil[a]	100	19.14 ± 9.01	39.67 ± 18.4
Essential oil[a]	300	25.27 ± 8.87*	61.67 ± 25.9**
Essential oil[a]	500	32.96 ± 6.27**	63.17 ± 8.9**
D-Limonene[a]	100	34.38 ± 12.61**	69.5 ± 35.1**
Trazodone hydrochloride[a]	10	28.86 ± 10.31*	25.63 ± 14.9

Data are presented as mean ± SE ($n = 10$). Observations were made 60 min after oral administration of vehicle (control) or essential oils from leaves of *C. japonica*
* $P < 0.05$; ** $P < 0.01$, compared with vehicle-treated controls
[a] Oral administration

with ECJ (100, 300, or 500 mg kg^{-1}), the pentobarbitone sleeping time for mice was dose-dependently prolonged from 37.2 min to 45.7, 54.4, and 62.3 min, respectively. When the mice treated with 200 mg kg^{-1} of D-limonene and α-pinene, which are the representative volatiles of EJC, the pentobarbitone sleeping times were 43.0 ± 4.79 min and 37.5 ± 3.48 min. D-Limonene caused a slightly prolonged sleeping time in mice. The hypnotic drug zolpidem hemitartrate (dosage 0.3 mg kg^{-1}) was used as a positive control, and the sleeping time was increased from 37.2 min to 46.8 min.

Effect of phytoncide on mice in the elevated plus maze

In this study, the elevated plus maze test was performed to evaluate the anxiolytic activity of EJC. Trazodone hydrochloride was used as a reference compound. Table 3 demonstrates the effects of EJC, D-limonene, and trazodone hydrochloride on the behavior of mice in the elevated plus maze test. The time spent in open arms was 12.68 ± 8.67 s, and the number of entries into open arms was 18.88 ± 11.7 times for vehicle control (animals treated only with saline). The independent Dunnett's test revealed that administration of EJC (500 mg kg^{-1}) significantly increased the time spent in open arms (32.96 ± 6.27 s) and the number of entries into open arms (63.17 ± 8.9 times) ($P < 0.01$) compared with the saline-treated group. The time spent in open arms for D-limonene treatment (100 mg kg^{-1}; p.o.) was 34.38 ± 12.61 s and the number of entries into open arms was 69.5 ± 35.1 times ($P < 0.01$).

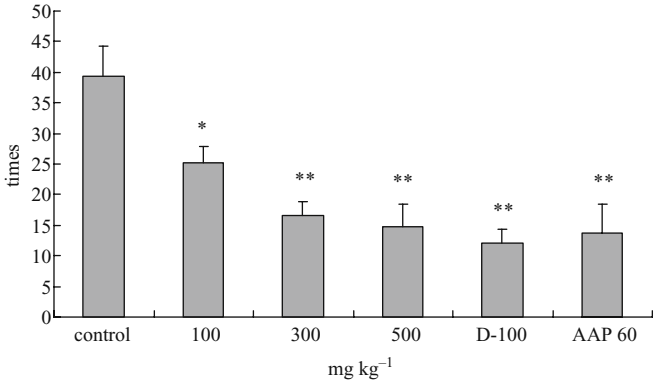

Fig. 1. Analgesic effect of essential oil and D-limonene (*D-100*) from *Cryptomeria japonica* leaf (Writhing test) in mice. Each bar represents mean ± standard error ($n = 8$). *Asterisk*, $P < 0.05$; *double asterisk*, $P < 0.01$ compared with vehicle-treated controls. *AAP 60*, 60 mg kg^{-1} acetaminophen

Effect of phytoncide on mice in writhing test

The acetic acid abdominal test is a popular method to evaluate the antinociceptive activity of natural products on mice. As shown in Fig. 1, the writhing times in mice without treatment were 39.25 ± 5.06. However, the number of writhing times in mice was reduced to 25.17 ± 2.79, 16.67 ± 2.25, and 14.71 ± 3.68 times, after administration of 100, 300, and 500 mg kg^{-1} of EJC, respectively. On the other hand, mice administered with D-limonene (100 mg kg^{-1}) had the number

of writhing episodes reduced by 69.0%, which was equal to the effect of administration of 90 mg kg^{-1} acetaminophen.

Discussion

Many studies have demonstrated that plant-derived essential oils exhibit a variety of biological properties, such as antianxiety,[16,17] sedative,[18] analgesic,[19,20] and anticonvulsant effects.[21] The present results suggest that phytoncide from *Cryptomeria japonica* exhibits a significant neuropharmacological effect on mice. Pretreatment with ECJ noticeably prolonged the pentobarbital-induced sleeping time in a dose-dependent manner. In the antianxiety effect assay (elevated plus maze test), ECJ obviously increased the time spent in open arms and the number of entries into open arms. Moreover, ECJ demonstrated antinociceptive activity in the writhing test.

The representative fragrance compound, D-limonene, emitted from *C. japonica* forests also exhibited antianxiety and antinociceptive effects in the mice. Silva et al.[22] reported that isopulegol, a monoterpenoid, could decrease depression and anxiety. Furthermore, citral, myrcene, and limonene can reduce locomotor activity and increase muscle relaxation.[23] Thus, it is reasonable to conclude that phytoncide possesses neuropharmacological activity. The results of this study support the concept that phytoncide from *C. japonica* exerts depressor activity on the central nervous system, and as such further study of the effects of phytoncide on humans is worthwhile.

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