



The Effects of Inhaled *Limnophila aromatica* Essential Oil on Brain Wave Activities and Emotional States in Healthy Volunteers: a Randomized Crossover Study

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Abstract

Background and objectives: *Limnophila aromatica* (Lam.) Merr., a characteristic odorous vegetable, is commonly used in Thai dishes and folk medicine. *Limnophila aromatica* essential oil has never been investigated on brain activities before. This study aimed to evaluate the effects of *L. aromatica* essential oil inhalation on brain wave activities through EEG recordings and emotional states among healthy volunteers using a randomized crossover design. **Methods:** *Limnophila aromatica* essential oil was extracted from the aerial parts by hydro-distillation method and its chemical components were analyzed by GC/MS. The essential oil was diluted to 8% v/v with sweet almond oil. Twenty-four healthy participants aged between 18 and 25 years, recruited from general public, inhaled both *L. aromatica* essential oil and sweet almond oil with seven-day washout period. Nicolet EEG v32 was used to record brain activities and the self-evaluated questionnaire on emotional states in Thai version of The Geneva Emotion and Odor Scale was used to measure the subjective feelings of the participants. Data were analyzed using paired t-test with a significance level of 0.05. **Results:** *Limnophila aromatica* essential oil increases theta and alpha wave and induced more relaxed feelings compared to the vehicle. **Conclusion:** The inhalation of *L. aromatica* essential oil could cause the relaxing effects on positive mood states and the relaxation of brain state.

Keywords: brain wave activities; emotional states; *Limnophila aromatica*; rice paddy herb

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Introduction

Limnophila aromatica (Lam.) Merr. (Scrophulariaceae), rice paddy herb or “Phak Kha Yang” (in Thai) is a characteristic odorous vegetable, a tropical flowering plant widely used as a medicinal plant and a spice in local dishes or a raw salad in Southeast Asia including Vietnam,

Malaysia and Thailand. It can be grown in flooded paddy fields. In Vietnam, *L. aromatica* is also called “Vietnamese coriander” because of its aromatic flavors of lemon oil and cumin. In Malaysia, it can grow well in lowland paddy fields. Local people consume it raw or cooked as

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an ingredient of a dish. In Thailand, *L. aromatica* is commonly used in Thai dishes due to its characteristic odor [1]. The plant is consumed raw or steamed. Local people use it to add flavor and give umami taste to local dishes such as soups and curries [2-3]. The plant is extensively used in Asian indigenous system of medicine for menstrual problems, wounds, dysentery, fever, elephantiasis and as a galactagogue, aperient, appetizer, digestive, carminative agent [4]. It possesses diuretic and muscle relaxant properties [2]. Previous studies reported that *L. aromatica* could possess pharmacological properties because of its anti-cancer, vascular protective, anti-proliferative and antioxidant activities [1,5,6]. The main compounds of volatile oil from *L. aromatica* analyzed by GC/MS varied from Z-ocimene, terpinolene, camphor, limonene, +-trans-isolimonene, a-pinene to a-caryophyllene [7]. The odors of essential oils are recognized to be therapeutically effective due to both the physiological and psychological effects through brain wave activities and emotional states. Electroencephalogram (EEG) has been widely used for studying brain activities associated with higher mental functions in humans affected by the essential oil inhalation [8-9]. The odor molecules stimulate olfactory receptor cells in the nose, open channels leading to the olfactory sensory neurons send the electrical signals to the brain via olfactory bulb and higher olfactory cortex [10]. EEG recording can measure neural excitations due to the changes in electric potentials via electrodes placed on the scalp. The major signals reflecting brain electrical activity consist of 4 frequency characteristic waves i.e. delta (0-3.99 Hz), theta (4-7.99 Hz), alpha (8-13 Hz) and beta (>13 Hz) waves. Various regions of the brain emit these characteristic brain waves simultaneously [11]. EEG showed that alpha waves, which are associated with relaxation, increased in the presence of relaxing odors such as lavender oil and citronella oil [12-13]. On the other hand, jasmine oil and rosemary oil have been reported that both essential oils increased beta waves for the presence of stimulating effect [14-15]. The essential oil inhalation has several effects on psychological well-being, emotions and behaviors by emotional states questionnaire. In Thailand, a number of studies have been conducted on the effects of essential oil inhalation in these parameters i.e. EEG, and emotional states. [12-15]. Most essential oils are

rather diluted in fixed oils or carrier oils, which are considered to be inert and used for dilution purposes such as canola, sunflower, olive, jojoba, and sweet almond oils [16]. The sweet almond oil is a natural fixed oil obtained from the kernel of *Prunus dulcis* (Mill.) D.A. Webb that has been widely used as a non-olfactory stimulating carrier oil [17]. *Linnophila aromatica* essential oil has never been evaluated in clinical research especially on brain wave activities. This plant can be consumed safely as it has been traditionally used as a medicinal plant and a spice in Thai medicine and Thai cuisine due to its characteristic odor [1-3]. So, this study aimed to evaluate the effects of *L. aromatica* aerial part essential oil inhalation on brain wave activities through EEG recordings and emotional states among healthy volunteers using a randomized crossover design. The chemical constituents of *L. aromatica* essential oil as the intervention were investigated as well.

Material and Methods

Ethical considerations

The research proposal was approved by the University's Ethical Review Committee for the Research Involving Human Research Subjects (Health Science Group) (COA No. 154/2018). The protocol of the study was in accordance with Helsinki declaration (Thai Clinical Trials Registration No. TCTR20181119005). The participants signed the informed consents prior to entrance to the study.

Linnophila aromatica essential oil

Linnophila aromatica was collected from the northeast of Thailand (Maha Sarakham Province) and authenticated by one of the authors (Nijsiri Ruangrunsi). The voucher specimen (LA530161) was deposited at the Herbarium of College of Public Health Sciences, Chulalongkorn University. The essential oil was extracted from the fresh aerial parts by hydro-distillation method using a Clevenger apparatus [18].

Gas chromatography mass spectrometry

The essential oil constituents were analyzed by gas chromatography/mass spectrometry using Thermo Finnigan Trace GC Ultra equipped with the Finnigan DSQ MS detector. BPX5 fuse silica column (30 m × 0.25 mm, 0.25 µm film thicknesses) was used as stationary phase. The oven temperature started from 60 °C to 240 °C

with a constant rate of 3 °C/min. The carrier gas was helium with the flow rate of 1 mL/min. Mass spectra was performed by EI positive mode. The chemical constituents were identified by comparing mass spectra and retention time indices with NIST 05 Mass Spectral library and Kovat retention indices reported in the literature [19]. Quantification of each component was performed by peak area ratio and demonstrated in percentage.

Randomized crossover study

Within-participant design was used by assigning each participant to receive both *L. aromatica* oil and sweet almond oil. A randomized two-group crossover design was performed. Each participant was randomly assigned to receive firstly *L. aromatica* essential oil or sweet almond oil by choosing the drawing lots. The washout period was seven days. The experimental study was conducted during July 2018 to April 2019.

Participants

Twenty-four healthy volunteers were recruited from general public. The sample size was calculated by computer G*Power 3.1 regarding to the previous study [14] (type II error at 0.05, power of test at 80%, effect size at 1.2 and dropout rate at 20%).

Inclusion and exclusion criteria

Healthy participants aged 18-25 years with normal body mass index (BMI) [20] were recruited. Inclusion criteria were right-handed participants evaluated by the Edinburgh Handedness Inventory scale [21], normal sense of smell screened using the n-butyl alcohol test [22], nonsmokers, no symptoms of upper respiratory infection, hypertension or cardiovascular disease, no current or past history related to neurological illness, epilepsy and loss of consciousness longer than 30 minutes and not taking neither central nervous system medication nor sedative drugs. Preliminary study found that 8% v/v of *L. aromatica* essential oil was most acceptable among volunteers, so this concentration was used in this study. The pleasantness of *L. aromatica* essential oil (8 % v/v in sweet almond oil) was tested using Odor familiarity five-point Likert scale. The one whose pleasantness between 2-4 was included. The participants who were allergic to essential oil or had a headache were excluded from the experiment.

Intervention administration

Limnophila aromatica essential oil was diluted to 8 % v/v with sweet almond oil (TCFF, Thailand). One mL was administered via an oxygen pump connected to a respiratory mask through a plastic tube with the air flow set of 2 L/min. One mL of the sweet almond oil was used as control.

Electroencephalography

Nicolet EEG v32 from Natus Neurology Company, USA was used. The set of 21 electrodes with one additional ground were placed in accordance with the international 10-20 system at Fp1, Fp2, F3, F4, F7, F8, Fz, C3, C4, Cz, P3, P4, Pz, T3, T4, T5, T6, O1, O2. LOC and ROC were placed for eye movements. EEG jelly was inserted into each electrode to keep the impedance below ten kilo-ohm. Reference electrodes were applied to measure for electrical activity at the ear lobes (A1 and A2), behind auricles and for detection of eye movements. The areas of interest were grouped into the left anterior area (Fp1, F3, F7), the right anterior area (Fp2, F4, F8), left posterior area (P3, T5, O1), right posterior area (P4, T6, O2) and the central (Fz, Cz, Pz) brain regions.

The power spectra of the respective frequency bands were recorded, interpreted by fast fourier transformation and expressed as delta (0-3.99 Hz), theta (4-7.99 Hz), alpha (8-12.99 Hz) and beta (13-30 Hz).

Emotional states

Thai version of The Geneva Emotion and Odor Scale [23] was used. The questionnaire comprised of 12 items of the emotional states i.e. good, bad, active, drowsy, fresh, relaxed, stressed, frustrated, romantic, annoyed, calm and disgusted. The questionnaire was verified by the advisor, the co-advisors, specialists in Thai interpretation and physiology. The ten-millimeter visual analog scale was chosen in this study to assess the emotional condition. The reliability was done by 20 participants in preliminary study and the Cronbach's α value was at 0.752.

Procedures

The participants were asked not to use hair spray, antiperspirants and any perfumes, nor drink alcoholic and caffeinated beverages. They did not feel fatigued or drowsy on the day of the experiment. Each participant had four appointment dates for EEG and emotional state

experiments using *L. aromatica* oil and the sweet almond oil with seven-day washout period.

Each participant was asked to sit in a quiet room with the temperature at $24 \pm 1^\circ\text{C}$, relative humidity at 50-65% in a comfortable chair. Concerning the EEG, the first and second appointment dates consisted of two processes. Firstly, EEG activity was recorded during eyes opened and eyes closed condition for ten min as rest when the participant felt comfortable. Secondly, the intervention (*L. aromatica* oil or the sweet almond oil) was applied for eight min while the EEG activity was recorded while eyes were closed simultaneously. Regarding emotional states, each participant was asked to sit in the same condition. Firstly, each participant felt comfortable for ten min then the participant was requested to evaluate emotional states by questionnaire. Secondly, the intervention (*L. aromatica* oil or the sweet almond oil) was applied for ten min and the participant was asked to evaluate the emotional states again.

Statistical analysis

SPSS version 22 was used for data analysis. Shapiro-Wilk test was performed to verify normal distribution of the data. The comparison was done by paired t-test using $p < 0.05$ for statistical significance.

Results and Discussion

The chemical composition of *L. aromatica* essential oil and GC chromatogram is shown in table 1 and figure 1.

Limonene was the major compound (48.95%) followed by 18.04% cis-4-caranone and 14.84% perilla aldehyde. The chemical composition was similar to that of Tucker et al. study (53.12% limonene and 12.19% cis-4-caranone) [24] but it was different from that of Bhuiyan et al. report (39.21% Z-ocimene and 17.24% terpinolene) [25] as well as that of Vairappan and Nagappan (30.06% sabinene and 14.06% terpinen-4-ol). Therefore, these odor effects were based on the limonene major type of *L. aromatica* essential oil [26]. Twenty-four healthy volunteers participated: five males and 19 females aging 19.67 ± 0.76 years with normal BMI of $20.03 \pm 2.32 \text{ kg/m}^2$ (height $1.61 \pm 0.08 \text{ m}$ and weight $52.25 \pm 10.41 \text{ kg}$). *Limnophila aromatica* essential oil was diluted with sweet almond oil (8% v/v). Hence, the effects from sweet almond oil were studied as the control. The repeated measurement was designed that each participant was one's own control. To minimize the selection bias from the inhalation sequence, the crossover study was performed with a seven-day washout period to eliminate carry-over effects.

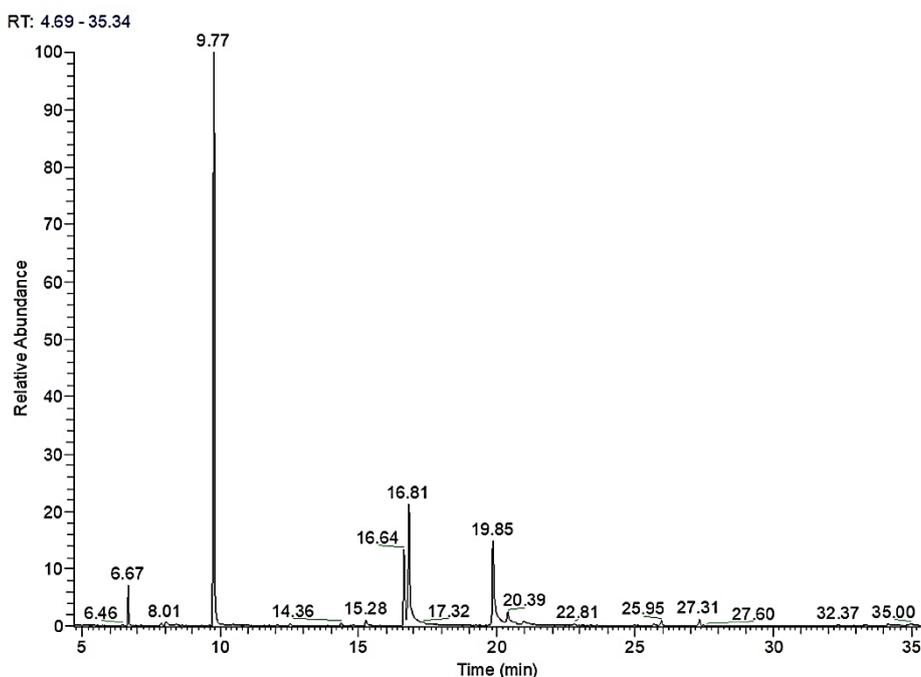


Figure 1. GC chromatogram of *Limnophila aromatica* essential oil

The effects of aromas on the nervous system may be divided into two different forms of arousal, the cortical arousal such as brain wave activity and the autonomic arousal such as heart rate, blood pressure, skin conductance, etc. that can be used as indicators for the measurement of the essential oil effects [7]. For sweet almond oil inhalation, the feelings of stress decreased significantly ($p=0.046$). For *L. aromatica* inhalation, the feelings of relaxation and calmness increased significantly ($p=0.002$ and $p=0.009$ respectively). The comparison of emotional state responses between *L. aromatica* and sweet almond oil showed that the changes of the relaxed feelings in *L. aromatica* were statistically significantly higher than those in sweet almond oil ($p=0.049$) (table 2).

The effects on EEG were displayed in table 3. For sweet almond oil, there were no significant changes in the band powers of delta, theta, alpha and beta of all brain regions. For *L. aromatica*, the alpha power of right anterior, center and right posterior brain regions increased significantly ($p=0.040$, $p=0.015$, $p=0.017$ respectively). The comparison of EEG between the two inhalations showed that there were significant changes in the power of theta and alpha waves. The power of theta over right anterior brain regions decreased significantly ($p=0.031$). There were statistically

significant increases in the alpha power of most regions namely left anterior, right anterior, center and right posterior brain regions ($p=0.037$, $p=0.039$, $p=0.015$, $p=0.046$, respectively).

The emotional state and EEG responses from *L. aromatica* essential oil were in accordance.

The alpha wave is dominant in mentally relaxed state, whilst beta wave is dominant in concentrated thought or tense mental state representing stimulating effect [7]. The results in this study indicated the relaxing effects of *L. aromatica* essential oil inhalation. Alpha, theta and delta increasing power have been found in relaxation state [27]. The effects on increasing theta and alpha were also found by the inhalation of lavender oil, a well-known relaxing odor [28]. The emotional state also indicated the relaxing effects of *L. aromatica* essential oil. However, *L. aromatica* increased only relaxation whilst lavender oil increased relaxation, freshness, active feelings but decreased drowsiness [12].

Kwaingjai et al. studied brain wave modification by *Citrus* sp. essential oil that comprised 96% of limonene in rat model and found that citrus oil increased the powers in low frequency bands ranging from theta (4.7-6.6 Hz), alpha 1 (7-9.4 Hz), alpha 2 (9.8-12.5 Hz) to beta 1 (12.9-18.4 Hz) waves in the frontal cortex and only alpha 2 and beta 1 in the parietal cortex.

Table 1. The chemical compositions of *Limnophila aromatica* essential oil*

Chemical component	Retention time (min)	Kovat's Index	Percentage
alpha-Pinene	6.67	939	2.79
Sabinene	7.87	975	0.25
Amyl vinyl carbinol	8.01	979	0.75
Myrcene	8.38	990	0.22
Limonene	9.77	1030	48.95
<i>trans</i> -beta-Ocimene	10.45	1050	0.21
Linalool	12.52	1096	0.20
Camphor	14.36	1146	0.35
Borneol	15.28	1169	0.69
<i>trans</i> -4-Caranone	16.64	1196	8.42
<i>cis</i> -4-Caranone	16.81	1200	18.04
Perilla aldehyde	19.85	1271	14.84
<i>para</i> -Mentha-1-en-9-ol	20.39	1295	1.75
Limonene aldehyde	20.99	1328	0.32
Neoisodihydrocarveol acetate	25.69	1359	0.22
beta-Caryophyllene	25.95	1419	0.52
alpha-Humulene	27.31	1454	0.74
alpha-Cadinene	32.37	1538	0.21
10-epi-gamma-Eudesmol	34.18	1623	0.27
gamma-Eudesmol	35.00	1632	0.26

*The chemical components were identified by comparing mass spectra and retention time indices with NIST 05 Mass Spectral library and Kovat retention indices reported in the literature [19]. Quantification of each component was performed by peak area ratio and demonstrated in percentage.

Table 2. The emotional states between sweet almond oil and *Linnophila aromatica* essential oil

Emotion	Sweet almond oil (SO) n = 24			<i>L. aromatica</i> essential oil (LO) n = 24			p-value between SO and LO change
	Mean score (SD)		p-value	Mean score (SD)		p-value	
	Rest	Intervention		Rest	Intervention		
1) good	4.78(2.31)	5.51(1.92)	0.114	4.86(2.52)	5.55(2.73)	0.151	0.957
2) bad	1.34(2.28)	0.92(1.29)	0.312	1.14(1.08)	0.86(1.11)	0.391	0.730
3) active	3.44(2.44)	3.21(2.19)	0.612	3.31(1.98)	3.79(2.23)	0.180	0.167
4) drowsy	1.94(1.78)	2.88(2.53)	0.096	2.43(2.35)	2.14(2.05)	0.639	0.116
5) fresh	3.92(2.16)	3.90(1.93)	0.984	3.81(1.84)	4.53(2.38)	0.258	0.172
6) relaxed	4.33(1.76)	4.61(2.03)	0.554	3.85(1.76)	5.78(2.86)	0.002*	0.049*
7) stressed	1.17(1.27)	0.94(1.12)	0.046*	1.37(1.58)	1.09(1.54)	0.365	0.872
8) frustrated	0.96(0.87)	0.88(1.32)	0.714	1.18(1.40)	1.41(2.00)	0.595	0.523
9) romantic	1.55(1.58)	2.16(2.18)	0.130	1.97(2.06)	2.58(2.16)	0.116	0.986
10) annoyed	0.85(1.14)	0.52(0.62)	0.110	0.77(1.27)	0.95(1.57)	0.639	0.141
11) calm	3.48(1.78)	3.68(2.26)	0.713	3.82(2.10)	5.28(2.50)	0.009*	0.171
12) disgusted	0.39(0.48)	0.40(0.59)	1.000	0.50(0.54)	0.51(0.60)	0.923	0.978

*Significant difference, p value < 0.05

Table 3. Brain wave power between sweet almond oil and *Linnophila aromatica* essential oil

Area	Sweet almond oil (SO) n = 24			<i>L. aromatica</i> essential oil (LO) n = 24			p-value between SO and LO change
	Mean power (SD)		p-value	Mean power (SD)		p-value	
	Rest	Intervention		Rest	Intervention		
Delta power (μV^2)							
Left anterior	6.10(1.94)	6.22(1.57)	0.674	6.11(1.52)	5.94(2.16)	0.688	0.540
Right anterior	6.54(2.06)	6.42(1.69)	0.672	6.98(1.49)	6.79(2.08)	0.638	0.886
Center	8.70(2.34)	8.60(1.66)	0.837	7.80(3.13)	8.37(3.98)	0.422	0.413
Left posterior	3.72(1.24)	3.89(1.29)	0.385	3.78(1.02)	3.72(1.38)	0.729	0.393
Right posterior	3.49(1.81)	3.46(1.37)	0.931	3.23(1.35)	3.33(1.39)	0.659	0.760
Theta power (μV^2)							
Left anterior	3.88(1.94)	3.69(1.70)	0.118	3.84(2.40)	4.93(3.55)	0.130	0.066
Right anterior	4.31(2.16)	4.09(1.81)	0.134	4.26(2.35)	5.30(3.36)	0.093	0.031*
Center	7.91(3.74)	7.57(3.27)	0.478	6.92(2.42)	7.54(5.45)	0.620	0.433
Left posterior	2.90(1.43)	2.89(1.30)	0.954	3.05(1.86)	3.12(1.85)	0.839	0.820
Right posterior	2.80(2.11)	2.67(1.52)	0.723	3.05(1.86)	2.60(1.28)	0.240	0.564
Alpha power (μV^2)							
Left anterior	3.69(1.91)	3.44(1.69)	0.411	4.44(2.86)	6.02(4.09)	0.068	0.037*
Right anterior	4.07(2.18)	3.83(1.94)	0.521	4.74(2.58)	6.15(4.29)	0.040*	0.039*
Center	4.07(2.18)	3.83(1.94)	0.885	8.40(4.25)	11.14(5.94)	0.015*	0.015*
Left posterior	5.28(3.00)	5.34(4.50)	0.942	6.58(3.82)	7.76(5.36)	0.157	0.369
Right posterior	5.48(3.07)	5.41(4.29)	0.925	6.41(4.07)	8.81(5.26)	0.017*	0.046*
Beta power (μV^2)							
Left anterior	3.37(1.40)	3.21(1.54)	0.191	3.38(1.02)	3.20(0.97)	0.175	0.860
Right anterior	3.42(1.39)	3.33(1.89)	0.667	3.34(1.14)	3.29(1.08)	0.748	0.894
Center	4.84(1.35)	4.46(1.10)	0.101	4.50(1.44)	4.60(1.35)	0.683	0.166
Left posterior	3.37(0.99)	3.45(1.35)	0.714	3.92(1.71)	3.54(1.32)	0.178	0.209
Right posterior	3.44(1.94)	3.26(1.73)	0.558	3.45(1.60)	3.83(2.11)	0.427	0.326

* Significant difference, p value < 0.05

The increase in slow wave activities might suggest anxiolytic or sedative effects [29]. Sowndhararajan et al. studied the olfactory stimulation of limonene on human EEG and found increase in high beta waves that represented mental stress, tension and anxiety increasing [30]. According to the fact that each essential oil is composed of a variety of volatile organic compounds, its odor and also property is characteristic which is different from the pure compound. Lorig *et al.* demonstrated that the mixture of vanillin and phenylethyl alcohol

produced a pattern of brain electrical activity different from each single compound.

Furthermore, the undetectable low concentrations of lavender oil and spice apple could affect brain activities and human moods [31,32]. The olfactory system plays an important role to the physiological and psychological of the essential oils. The olfactory information, from the odor molecules attach to cilia of the olfactory receptor cells and is transmitted directly to the higher olfactory cortex in the corticomедial amygdala portion of the brain through

olfactory tract where the signaling process is decoded and olfactory interpretation and response occurs [9]. EEG recording is a non-invasive and painless technique for investigation of the brain electrical activities induced by the essential oils.

The essential oil from the aerial parts of *Limnophila aromatica* comprised of 48.95% limonene, 18.04% cis-4-caranone and 14.84% perilla aldehyde. The randomized crossover study in 24 healthy participants revealed that *L. aromatica* essential oil significantly increased the relaxed and calm feelings as well as the alpha and theta wave powers that represent the relaxing effect of this essential oil.

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Author contributions

Pakamon Thanatuskitti conducted the experimental studies; Vorasith Siripornpanich supervised the part of electroencephalography; Winai Sayorwan supervised the parts of participant recruitment and emotional state questionnaire; Chanida Palanuej and Nijisiri Ruangrunsi supervised the part of *Limnophila aromatica* essential oil extraction and chemical analysis, the study concept and the manuscript revision. All authors were involved in the study design, data analysis and manuscript preparation.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

EEG: electroencephalogram; GC: gas chromatography; MS: mass spectrometry; EI: electron ionization; NIST: National Institute of Standards and Technology, United States

Department of Commerce; TCFF: Thai-China Flavours and Fragrances Industry Co., Ltd.; SO: sweet almond oil; LO: *Limnophila aromatica* essential oil