Research Article

The Development of Sweet Basil Essential Oils Nanoemulsion for Scototaxis Behaviors in Zebrafish

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ABSTRACT

Sweet basil is an herb known for its essential oils, widely used in the food and fragrance industries. This essential oil contains a compound with calming properties; however, only a few preclinical studies show the anxiolytic effect of sweet basil essential oil. This study aimed to test the anxiety-related effect of sweet basil essential oil in zebrafish and develop nanoemulsion to enhance its therapeutic effects. Gas chromatography-mass spectrometry (GC-MS) identifies the anxiolytic compounds of sweet basil essential oil including estragole (100%) and linalool (25.52%). A nanoemulsion of sweet basil oil was developed and measured physical properties by zetasizer. We found that nanoemulsion had particles size of 52.96±14.69 nm and a dispersion value of 0.25±0.01. The scototaxis behavior test of zebrafish was performed, and the anxiety-related compounds (ethanol and fluoxetine) were compared with sweet basil essential oil and nanoemulsion. The anxiolytic effects of sweet basil essential oil and its nanoemulsion were found in zebrafish. These findings indicate that sweet basil essential oil, particularly as a nanoemulsion, has potential as an alternative treatment for anxiety-related behavior.

Keywords: Anxiety-related behaviors, *Ocimum basilicum* L., *Danio rerio* (Hamilton, 1822), Light/dark test

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Introduction

Medicinal plants have been valued for their therapeutic properties throughout history, offering treatments for various health conditions [1]. Across cultures, medicinal plants have been recognized for their healing potential, particularly in addressing ailments such as anxiety-related disorders [2]. Sweet basil (*Ocimum basilicum* L.; Lamiaceae) is an herb characterized by its small, square-shaped stem and purple branches [3]. This species is distributed in tropical and subtropical regions of Asia [4]. Sweet basil is a fragrant, local herb and an economical plant, valued for its essential oils used in the food and perfume industries [4]. Moreover, this essential oil has beneficial effects on the nervous system, decreasing depression and anxiety symptoms [5]. However, only one preclinical study shows the anxiolytic effect of sweet basil [6]. Therefore, this demonstrates the importance of these methods to evaluate this medicinal plant.

Anxiety disorders affect a significant portion of the global population, posing a considerable burden on mental health [7, 8]. Medicinal plants have gained attention for their anxiolytic properties, with traditional practices often supporting their therapeutic use [9]. Zebrafish (*Danio rerio* (Hamilton, 1822)) have emerged as a model organism for assessing the anxiolytic potential of medicinal compounds [10]. Zebrafish models such as the anxiety-related behavior test are explored to assess therapeutic efficacy [11]. The novel tank test is a behavioral assay used to evaluate anxiety-like responses in zebrafish. This test can be employed to assess the effects of medicinal plant extracts on anxiety behavior, offering valuable insights into their potential therapeutic applications [11].

Encapsulation techniques represent another approach to enhance stability, bioavailability, and targeted delivery of bioactive compounds from medicinal plants [12]. Emulsification or colloidal dispersion, one of encapsulation technique, is a two-phase system with a continuous phase that might be solid, liquid, or gaseous and a dispersed phase [13]. This technique protects sensitive bioactive constituents, improves their solubility, and controls their release [14]. Such techniques hold the potential to develop formulations that maximize the therapeutic effects of medicinal plants.

By combining methods like behavioral assays, and encapsulation techniques, in this study, we investigated the anxiolytic effect of sweet basil essential oil on zebrafish using scototaxis behavior test and developed the sweet basil nanoemulsion to enhance its therapeutic effects.

Materials and Methods

Essential oils and chemicals

Sweet basic essential oils, CAS number: 8015-73-4, were purchased from CHEMIPAN Company, Thailand. Tween 80 was obtained from Sigma-Aldrich while propylene glycol and ethanol were obtained from Ajax Finechem and Merck.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry analysis of sweet basil essential oils was carried out using the Agilent 7890B system and Agilent 7000C GC/MS Triple Quad (Perkin-Elmer, Inc. U.S.A) equipped with a fused silica column, packed with NP-5ms capillary column (30 m in length × 250 μm in diameter × 0.25 μm in thickness). Helium gas was used as carrier gas at a constant flow rate of 1 mL/min. For GC–MS

spectral detection, an electron ionization energy method was adopted with a high ionization energy of 70 eV (electron Volts) with fragments ranging from 33 to 500 m/z. The injection was used at a split ratio of 50:1, and the injector temperature was maintained at 250 °C (constant). The column oven temperature was set at 50 °C for 2 min, raised at 4 °C minute^{-1} up to 220 °C, and the final temperature was increased to 220 °C for 5 min.

The contents of phytochemicals presented in the test samples were identified based on a comparison of their retention time (min), peak area, peak height, and mass spectral patterns with the spectral database of authentic compounds stored in the NIST library [15].

Nanoencapsulation of Sweet basil essential oil

To enhance its therapeutic effects of sweet basil essential oil, emulsification was selected to encapsulate this essential oil. In this study, nanoemulsion was prepared by modified method from Zhang et al. [16]. Nanoencapsulation condition was optimized for developing nanoencapsulated sweet basil. Water phase was prepared with surfactant (propylene glycol): water (2:1), and oil phase was prepared with surfactant (tween 80): sweet basil essential oil (3:7). The oil phase was stirred in the magnetic stirrer at 1000 rpm speed for 1 hour. After oil phase preparation, the water phase was then dropped into oil phase with flow rate 1 mL minute^{-1} until total volume at 50 mL. The mixture phase was stirred in the magnetic stirrer at room temperature and speed 1000 rpm for 7 hours to develop sweet basil nanoemulsion. To prevent the degradation of the obtained nanoemulsion, it was stored at room temperature without light exposure.

Morphological analysis (zetasizer) and anxiolytic effect (light-dark test) of sweet basil essential oil nanoemulsion were performed after developing nanoencapsulation.

Zebrafish housing

Adult zebrafish (both male and female, age > 15 weeks) of the leopard phenotype (*leo*) were purchased from a commercial distributor, Chatuchak market. Not sex-specific 16 zebrafish (4 zebrafish per trial) were performed in each condition. Every condition was tested for 4 replicates.

These fish were experimentally naive and given at least two weeks to acclimate to the lab environment (e.g., the room and water temperature was kept at room temperature with illumination (a 14/10-h light/dark cycle (lights on 8.00 AM)) provided by fluorescent light tubes). Animals were kept at a ratio of 1 fish/100 mL in the 1.5 L acrylic box with tap water and fed once twice daily with SAKURA fish flakes.

The light/dark test of zebrafish

The anxiolytic effect of sweet basil compounds/formula was performed by light/dark test. Test was performed at daylight (08.00 AM – 5.00 PM). The expose time was modified from Cachat et al. [11] for acute treatment. Zebrafish was acutely exposed by immersion for 30 min to medicinal plant compounds/formula. Thirty minutes was chosen because several studies in zebrafish have shown that oral drug administration leads to peak serum concentrations within 40 minutes [17, 18]. The 10 μL L^{-1} sweet basil essential oils was selected based on previous study [19]. The 1%v/v ethanol was used for GABAergic drug reference [20], and the fluoxetine concentration 200 μg L^{-1} was used for serotonergic drug reference

[21]. DO (no less than 4 mg L^{-1}), pH (6.8-8.5), temperature (24-30°C), and fish weight were measured in every individual test [22].

The scototaxis behavior test (light/dark test) of zebrafish was modified from Maximino et al. [23]. After acute drug exposure, individuals were then placed in a 4-liter light/dark rectangular tank (20×15×30 (height×length×width) cm). This rectangular tank was divided into equal light/dark areas. The barrier in each light/dark sides placed at the 5-centimeter distance from center to restrain zebrafish. Before beginning scototaxis behavior test, the drug-treatment zebrafish were located and restrained in dark area for 5 minutes to habituate a light/dark tank. After dark-area restraining, the barriers were removed, and the zebrafish explored the light/dark area in a tank for 10 minutes. The videos of scototaxis behavior test were recorded by the smartphone for 10 minutes. Every test was performed between 8 AM and 5 PM. Behavioral endpoints were followed from Grossman et al. [24]. Time spent in light, number of entries to the light, latency to enter the light, and average entry duration were observed and analyzed by humans.

Statistical analysis

Data analysis was performed using R [25]. Graphs were made using ggplot2 [26]. Statistical analysis was done using one-way (treatment) ANOVA. Interactions from ANOVA were followed up with Dunnett's multiple comparison tests to examine the effects of sweet basil essential oil and sweet basil essential oil nanoemulsion [27] and pairwise t-test with False Discovery Rate (FDR) correction to compare the effects of two different formula (sweet basil essential oil and sweet basil essential oil nanoemulsion) [28]. For effect sizes, ANOVAs are reported as η^2 and t-tests as Cohen's d. The interpretation of effect sizes as small ($0.01<\eta^2<0.06$; $0.2<d<0.5$), medium ($0.06\leq\eta^2<0.14$; $0.5\leq d<0.8$) or large ($\eta^2\geq0.14$; $d\geq0.8$) based on Cohen [29].

Ethic permission

All experimental hosts were managed according to the guidelines approved by the Institute for Animals for Scientific Purpose Development (IAD) and the National Research Council of Thailand (NRCT) and the relevant document (No. U1-10673-2565) was approved by the committee. The guidelines and standards for animal care were used according to the Animal Ethics Committee of the Faculty of Science, Srinakharinwirot University. The animals were assigned to experimental groups randomly and belonged to the same batch. All animal tests were included in the analyses without exclusion and attrition. All experiments were performed as planned, and all endpoints assessed were included in the analysis.

Results and Discussion

Chemical analysis of sweet basil essential oil using gas chromatography-mass spectrometry (GC-MS) revealed 40 chemical compounds (Table 1), including active substances important to the nervous system in relieving anxiety in zebrafish, primarily Estragole (100%) and Linalool (25.52%). Estragole inhibits nerve cell excitation by blocking sodium ion channels in the Dorsal Root Ganglion (DRG), resulting in reduced neuronal excitation [30]. Linalool also affects the anxiety-related behaviors in animals. This compound inhibits the glutamatergic system in the central nervous system, specifically the N-methyl-D-aspartate receptor

(NMDAR), by binding to the glutamate recognition site as a competitive antagonist. This action closes the ion channel, preventing calcium ions from entering the cell and suppressing neurotransmission [31].

Table 1 The GC-MS profiles of sweet basil essential oil

Peak	RT (min)	Compound	CAS No.	Content %
1	6.945	α-Pinene	80-56-8	0.12
2	8.184	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	3387-41-5	0.02
3	8.259	β-Pinene	127-91-3	0.07
4	8.651	5-Hepten-2-one, 6-methyl-	110-93-0	0.13
5	8.79	β-Myrcene	123-35-3	0.04
6	9.856	Benzene, 1-methyl-3-(1-methylethyl)-	535-77-3	0.04
7	10.006	D-Limonene	5989-27-5	0.05
8	10.078	Eucalyptol	470-82-6	0.51
9	10.726	β-Ocimene	13877-91-3	0.12
10	11.546	2-Furanmethanol, 5-ethenyltetrahydro-α,α,5-trimethyl-, cis-	5989-33-3	0.07
11	12.092	trans-Linalool oxide (furanoid)	34995-77-2	0.09
12	12.544	Linalool	78-70-6	25.52
13	14.363	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, cis-	491-07-6	0.03
14	15.037	Cyclohexanone, 5-methyl-2-(1-methylethyl)-	10458-14-7	0.29
15	15.666	α-Terpineol	98-55-5	0.23
16	16.024	Estragole	140-67-0	100
17	17.41	Neral	106-26-3	0.37
18	18.419	2,6-Octadienal, 3,7-dimethyl-, (E)-	141-27-5	0.71
19	18.908	Anethole	4180-23-8	0.04
20	21.269	Eugenol	97-53-0	0.04
21	21.876	Copaene	3856-25-5	0.03
22	22.252	cis-3-Hexenyl cis-3-hexenoate	61444-38-0	0.04
23	22.407	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]-	515-13-9	0.07
24	22.817	Methyleugenol	93-15-2	0.05
25	23.246	Caryophyllene	87-44-5	0.42
26	23.781	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	17699-05-7	0.64
27	23.962	2,6-octadiene,1,1-diethoxy-3,7-dimethyl-	7492-66-2	0.08
28	23.984	cis-β-Farnesene	28973-97-9	0.06
29	24.301	Humulene	6753-98-6	0.26

Table 1 The GC-MS profiles of sweet basil essential oil (cont.)

Peak	RT (min)	Compound	CAS No.	Content %
30	24.444	(E)-β-Farnesene	18794-84-8	0.13
31	25.152	Germacrene D	23986-74-5	0.11
32	25.28	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-	13877-93-5	0.1
33	25.6	(Z)-1-Methyl-4-(6-methylhept-5-en-2-ylidene)cyclohex-1-ene	13062-00-5	0.05
34	26.007	β-Bisabolene	495-61-4	0.06
35	26.428	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1α,4aβ,8aα)]-	523-47-7	0.04
36	27.02	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-	15423-57-1	2.07
37	27.656	trans-4-Methoxycinnamaldehyde	24680-50-0	0.14
38	28.146			0.05
39	28.895			0.03
40	37.217	Hexadecanoic acid, methyl ester	112-39-0	0.03

Table 2 The zetasizer analysis of sweet basil essential oil nanoemulsion

Nanoemulsion properties	Sweet basil nanoemulsion (nm)	Nanoemulsion criteria (nm)	Reference
Particle size	52.96±14.69	1-100	[27-29]
Polydispersity index	0.25±0.01	0-0.03	[12]

The sweet basil nanoemulsion was then developed; and the particle size and polydispersity index were measured by zetasizer (Table 2). The produced nanoemulsion exhibited clear, homogeneous physical characteristics, classifying it as highly stable. In addition, we found that sweet basil nanoemulsion had particles with a size of 52.96±14.69 nanometers and a dispersion value of 0.25±0.01. This nanoemulsion is aligned with standard nanoemulsion properties (particle size 1-100 nanometers, dispersion value 0-0.3) [32-34]. The particle size falls within the 1-100 nanometer range for highly stable nanoemulsions, with smaller particles contributing to greater stability [12]. The distribution value of 0.25±0.01 indicates a narrow particle distribution, suggesting uniform and homogeneous particles, which further enhances the nanoemulsion's stability [12]. These properties collectively demonstrate that the sweet basil essential oil nanoemulsion exhibits the desired stability and potential for therapeutic use in anxiety treatment. However, there are some limitations to consider. For instance, while the zetasizer analysis provided insights into the nanoemulsion's properties and potential storage stability, further studies on long-term storage and large-scale applications are needed to better understand its physical properties.

A study on the anxiety-related effects of sweet basil essential oil (EOOB) and sweet basil essential oil nanoemulsion (EMEOOB) using the scototaxis behavior test in zebrafish is demonstrated in Figure 1. For time spent in light (Figure 1A), we found a large effect of treatment (P<0.001, η^2=0.56). Dunnett's multiple comparison tests indicated that EOOB (P<0.001) and EMEOOB (P<0.001) treatment groups swam in light area longer than control group. For number of entries to the light (Figure 1B), we found a large effect of treatment (P<0.001, η^2=0.39). Dunnett's multiple comparison tests indicated that EOOB (P<0.001) and EMEOOB (P<0.001) treatment groups swam to light zone more than control group. For average entries duration (Figure 1C), we found a medium effect of treatment (P=0.126, η^2=0.09). However, Dunnett's multiple comparison tests indicated that only EMEOOB (P=0.048) treatment groups spent time in light area per time longer than control group. For latency to enter the light (Figure 1D), we found a large effect of treatment (P=0.016, η^2=0.15). Dunnett's multiple comparison tests indicated that EOOB (P=0.009) and EMEOOB (P=0.015) treatment groups swam to light zone faster than control group. Ethanol inhibited startle responses and increased time spent in the bright area [35], while fluoxetine encouraged exploration of new areas by preventing serotonin reuptake and enhancing serotonergic signaling [36]. Sweet basil essential oil treatment similarly resulted in significant differences in all parameters compared to the control group. The anxiolytic effect of sweet basil essential oil is attributed to its main compound, linalool, which inhibits nerve impulses, leading to anesthetic effects [31]. Previous studies have identified linalool as an anxiolytic compound [37]. Milanos et al. [37] demonstrated that linalool enhances GABAergic currents in mice, and this compound has also been shown to inhibit glutamatergic activity [38, 39]. Estragole is also described as an anxiolytic compound [19, 30, 40]. Silva-Alves et al. [30] found that estragole blocks neuronal excitability by directly inhibiting Na^+ channels, which may contribute to its anxiolytic effects.

This behavioral study has limitations and should be mentioned. This study cannot fully explain anxiety-related behavior due to the absence of other biological methods. Future research should investigate cortisol levels to better understand potential differences in HPA axis function [41]. In addition, the various concentrations of sweet basil essential oil and nanoemulsion should be added to explore the different results of therapeutic efficiency. Meanwhile, we study acute responses of sweet basil essential oil on animal, humans usually take it chronically. Herz [42] noted that responses depend on human experience, environment, and genetics. While we cannot confirm anxiolytic or anxiogenic effects, this study highlights the potential for varied human responses.

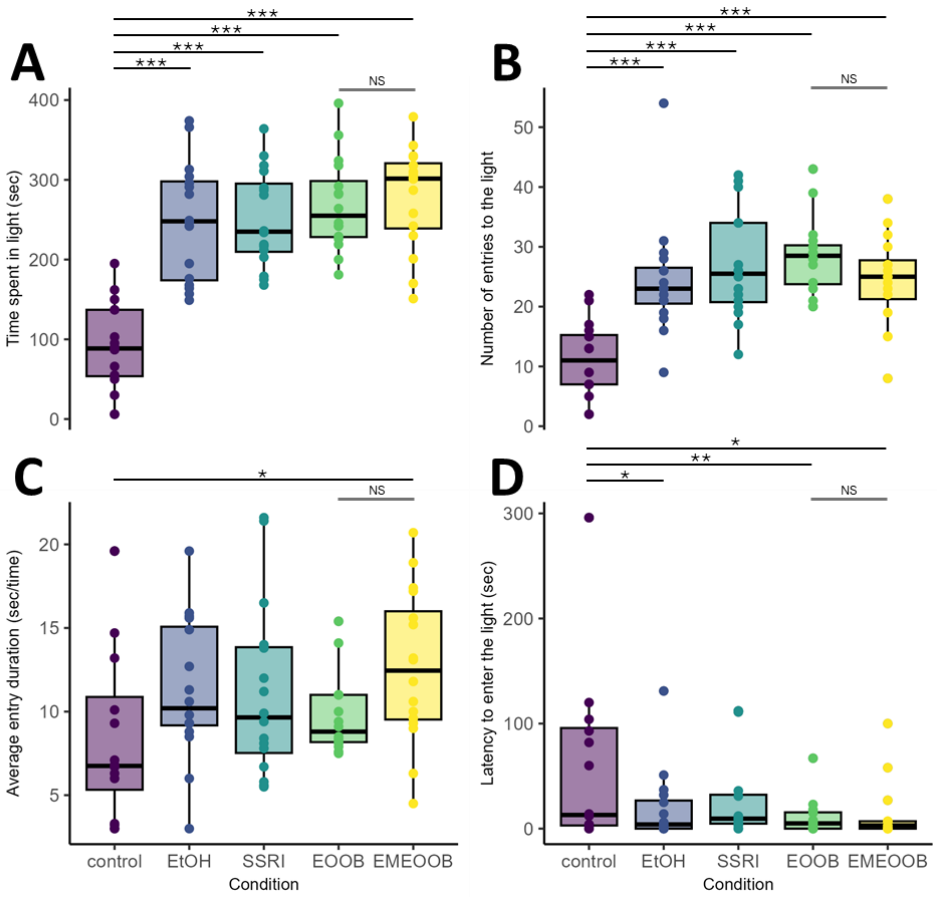

Figure 1 Influence of sweet basil essential oils and sweet basil nanoemulsion on scototaxis test. The effect of sweet basil essential oils and sweet basil nanoemulsion on (A) time spent in light, (B) number of entries to the light, (C) average entry duration, and (D) latency to enter the light. Boxplots indicate median (center line), interquartile range (box ends), and hinge±1.5 times the interquartile range (whiskers). ***P < 0.001, **P < 0.01, *P < 0.05 from Dunnett's multiple comparison tests within group (black line). (ns) P > 0.10 from pairwise t-test with FDR correction between sweet basil essential oils and sweet basil nanoemulsion (gray line). Abbreviations: EtOH = ethanol, SSRI = fluoxetine, EOOB = sweet basil essential oils, and EMEOOB = nanoemulsion of sweet basil.

Conclusions

This study indicates that basil essential oil, particularly with estragole and linalool as active compounds and shows promoting anxiolytic effects in animals. The sweet basil nanoemulsion, characterized by a particle size of 52.96±14.69 nanometers and a dispersion index of 0.25±0.01, meets standard criteria for nanoemulsions. The scototaxis behavior test explores that both sweet basil essential oil and sweet basil nanoemulsion affect the anxiety-related behavior in zebrafish. These results indicate that sweet basil nanoemulsion formulation preserves its anxiolytic potential. This makes it an option for anxiolytic applications and supports its potential in future research on alternative anxiety therapies.

Availability of data and material

The detailed methodology and numerical data are available from the corresponding author on reasonable request.

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