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Stress amelioration and anti-inflammatory potential of Shiikuwasha (Citrus depressa Hayata) essential oil, limonene, and γ-terpinene

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Abstract

Shiikuwasha (Citrus depressa Hayata) essential oil (SEO) extracted from Shiikuwasha fruit pulp contains two major volatile components, limonene and γ-terpinene (56.56–57.31 and 24.41–24.98%, respectively). In this study, we showed that the aroma streams of SEO, limonene, and γ-terpinene exhibited significant physiological stress amelioration effects in nine healthy female panelists tested in a 30-min visual display task. While SEO administration significantly reduced work errors by 15 min compared to the odorless control condition, the limonene stream reduced work errors only at the first and fourth 5-min intervals, and the γ-terpinene effect lasted only for the initial 5-min. However, γ-terpinene inhalation reduced stress by significantly increasing post-work electrocardiogram R–R interval variability and lowering electroencephalogram beta wave powers at the midline occipital Oz position. Additionally, SEO, limonene, and γ-terpinene displayed anti-inflammatory activity in vitro by suppressing the pro-inflammatory markers NO and IL-1β in BV-2 microglial cells in a dose-dependent manner.

Keywords: Anti-inflammatory, Aroma inhalation, Citrus essential oil, Shiikuwasha pulp, Stress amelioration

1. Introduction

Shiikuwasha (Citrus depressa Hayata), also known as Hirami lemon or thin-skinned flat lemon, is a citrus cultivar that grows naturally in the southernmost prefecture of Japan, Okinawa. This small citrus fruit is commonly harvested from its unripe to ripe stages from September to December and is rich in beneficial phytochemical substances, such as phenolics, flavonones, and polymethoxylated flavones [1,2]. Citrus pulp is one of the main byproducts produced when fruits, such as Shiikuwasha, are squashed to release its juice [3–5]. Shiikuwasha essential oil (SEO), which is originally stored in the oil glands of fruit peels, can be extracted from fruit pulp through various extraction methods such as steam distillation and cold centrifugation. The resulting essential oils contain a large number of volatile components, which are mostly used as flavoring substances in the food and beverage industries [2,3].

Essential oils from citrus species possess various bioactive compounds such as limonene, linalool, and 1,8-cineole, which confer many health benefits such as anti-inflammatory, antioxidant, anticancer, and antimicrobial activities [3,6–8]. Moreover, essential oils and their volatile aroma components exhibit various anti-neuroinflammatory properties in microglial cells, implying their dietary benefits in reducing neurodegenerative disorders [9,10]. These
volatile substances may also be used as scent ingredients in direct inhalation therapeutic treatments, also known as aromatherapy [11,12]. Aromatherapy has been considered a complementary and alternative medicine for stress reduction, relaxation improvement, attention performance stimulation, and so on. However, several studies merely presented suggestive evidence of aromatherapeutic benefits, suggesting that more definitive studies are needed to understand the nature of its biological potency [11,13,14].

Physiological stress is considered a neurological disorder; thus, treating it as a serious mental health condition is recommended [15,16]. Plant-based food materials, including essential oils, contain complex formulations of aroma components that can influence sniff-induced neurological activity in the olfactory system [7,8,17]. Pleasant odors of inhaled volatile components are intended to stimulate sedative effects via specific nasal olfactory stimuli in the amygdala and hippocampus of the human brain, which are storehouses of emotions and memories [13,17]. Thus, they are considered to have neuroprotective effects against stress-induced depression-like behaviors, including anxiety, dementia, Alzheimer’s disease, and brain inflammation [11,14]. Basic clinical diagnostic observations such as electrocardiography (ECG) and electroencephalography (EEG) might provide elementary signals of changes in cardiac and brain activities, respectively, which could appropriately display the effects of aroma inhalation on human subjects [13–15]. In addition, the neuroprotective effects of aroma substances can be assessed by their ability to suppress the expression of pro-inflammatory signaling molecules and cytokines [6,10,18].

Our previous study has detailed the odor-active substances in SEO as key aroma components using an extended aroma extract dilution analysis [4]. Moreover, there is currently an upsurge of interest in exploring the potent biofunctions of citrus essential oils and their aroma compounds, in which studies on SOE are still lacking [6–8]. To the best of our knowledge, no prior study has explored the use of essential oils from Shiiikuwasha pulp to ameliorate neurological stress or inflammation. This study aimed to investigate the stress amelioration effects of SEO and its two primary aromatic components, limonene and γ-terpinene, via inhalation in human subjects and to evaluate their anti-inflammatory activities against stress markers in vitro using microglial cell culture. Therefore, the effects of SEO potential as well as its key aroma components on neuroprotective benefits and possible associations or trends in both studies could be assessed. The volatile aroma constituents of SEO were initially characterized using gas chromatography-flame ionization detection/mass spectrometry (GC-FID/MS). The aroma stream of these scent materials was then flow-controlled and administered to subjects over visual display work in front of an electronic monitor, while their physiological, respiratory, and brain conditions were observed using ECG and EEG monitoring. Nitric oxide (NO) and interleukin (IL)-1β were used as pro-inflammatory markers in lipopolysaccharide (LPS)-stimulated microglial cells.

2. Materials and methods

2.1. Reagents

Anhydrous sodium sulfate was purchased from Merck KGaA (Darmstadt, Germany). Limonene (≥99.0%), γ-terpinene (≥98.5%), and LPSs from Escherichia coli O55:B5 were purchased from Sigma–Aldrich (St Louis, MO, USA). Authentic standards for the identification of volatile aroma components were obtained from Sigma–Aldrich and Tokyo Chemical Industry (Tokyo, Japan). RPMI-1640, sodium nitrite, and squalane were obtained from Wako Pure Chemical Industries (Osaka, Japan). Dimethyl sulfoxide was purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and fetal bovine serum (FBS) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Griess reagent comprised of 2.5% sulfanilamide (Sigma–Aldrich), 0.05% N-(1-naphthyl) ethylenediamine (Sigma–Aldrich), and 2.5% phosphoric acid (Wako Pure Chemical Industries). CellTiter 96™ Aqueous One Solution Cell Proliferation Assay reagent containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was obtained from Promega Corporation (Madison, WI, USA).

2.2. Shiiikuwasha essential oil (SEO) extraction

The essential oil was extracted from the pulp by-product of Shiiikuwasha fruit obtained in three batches from October to December 2015. Briefly, 50 g of pulp was extracted with 400 mL of distilled water using a Clevenger-type hydrodistillation apparatus for 3 h. Subsequently, the collected crude oil was centrifuged at 2900×g for 5 min at 4 °C (Himac CF-5RX, Hitachi Koki Co. Ltd., Tokyo, Japan), and the purified oil was then dehydrated using anhydrous sodium sulfate for 12 h at 4 °C. All extractions were performed in triplicates. SEO was stored in sealed vials at −30 °C prior to analysis.
2.3. Volatile aroma components analysis

The volatile aroma components of SEO were measured based on a previous study [4]. Briefly, 1 μL of essential oil was injected at a split ratio of 1:50 into an Agilent 7890A GC-FID system (Agilent J&W, Santa Clara, CA, USA). The volatile aroma components were separated using an Agilent J&W DB-Wax column (60 m × 0.25 mm i.d., 0.25-μm film thickness) under a helium stream at a flow rate of 32 cm/s. The initial oven temperature was 40 °C, which was maintained for 2 min, then increased to 200 °C at a rate of 2 °C/min, and finally held for 38 min. The GC injector and FID were set at 250 °C. Aroma components were identified based on linear retention indices (RIs) upon measurement of the homologous series of C7–C30 alkanes, followed by comparison of the MS patterns of the peak with MS data from the National Institute of Standards and Technology (NIST) MS Library, Version 2008, and MS patterns of authentic standards. The MS patterns of the peaks were acquired using an Agilent 5975C MS system under the following conditions: both ion source and interface temperatures were set at 230 °C, electron impact ionization at 70 eV, and mass acquisition range of 29–450 amu. The composition was expressed as the relative concentration of the FID peak area response. All analyses were performed in triplicate.

2.4. Aroma inhalation administration and visual display terminal (VDT) test

The VDT test that might cause stress fatigue was displayed through a 21.3-inch LCD monitor (RDT214S, 1600 × 1200 pixels, 300 cd/m², Mitsubishi Electric Corp., Tokyo, Japan) in a 1.3 × 1.8 × 2.0 m sealed acoustics sound room during February–March 2016, as previously described (Fig. 1a) [13]. The participants were asked to assess the correct quick response of VDT work by clicking a mouse button on a straight odd-ball picture as the true target that was randomly presented on the monitor at 20 ms/s and appeared for 20% of the total presentation during VDT (Fig. 1b). Additionally, non-straight (slopped) odd-ball pictures, as false targets, also randomly appeared to distract visual performance. They were 5° and 10° left-slopped odd balls (10 and 30% presentation frequencies, respectively) and 5° and 10° right-slopped odd balls (10 and 30% presentations, respectively). The experimental design of the study was approved by the Research Ethics Committee of Kyushu University (approval number and date: No. 42 and January 29, 2016, respectively), which was obtained through a review of the proposed study protocol and participants’ data protection. This study was conducted in accordance with the principles of the Declaration of Helsinki. Verbal informed consent was obtained from all participants prior to the experiment.

Stress amelioration effects of SEO of December produce, limonene, and γ-terpinene were measured through inhalation administration in nine female adult healthy subjects (age 20.4 ± 0.7 years, range:19–21 years) against a VDT test for 30 min. The female subject group was chosen to avoid psychological and physiological differences between genders, and individuals without smelling problems or respiratory disorders were selected as panelists. The participants were asked not to drink alcohol or smoke for at least 1 d prior to the experiment. Room temperature was maintained at 25 °C. Each participant was asked to perform the VDT test four times at an interval of 1 week, first in the absence of aroma administration (control condition), and then aroma administration with SEO, limonene, and γ-terpinene in a randomized schedule. Random arrangements for SEO, limonene, and γ-terpinene aroma stream administrations were generated using an online randomization tool, Research Randomizer (https://www.randomizer.org/). The aroma substances were administered at a final concentration of 0.2% (v/v) in squalene as a carrier oil by forced air stream at a flow rate of 1 L/min to the VDT test room. Odorless squalene was streamed at the same velocity as that in the control condition. The participants were aware of the existence of the odor compared to the control condition, but they were not informed which odor was applied or when they were in the odor or odorless condition throughout the experimental period.

2.5. Stress amelioration monitoring

The relative amelioration potential of SEO of December produce, limonene, and γ-terpinene against neurological stress were determined through ECG and EEG analyses using a biosignal amplifier system equipped with Ag/AgCl electrodes (Polymate Mini, Miyuki Giken Co., Ltd., Tokyo, Japan). Complex demodulation amplitudes (low frequency, LF:0.04–0.15 Hz; high frequency, HF:0.15–0.4 Hz) and R–R intervals of heart rate variabilities, and band powers of electrical brain activity values during 30 min VDT work were measured, subsequently the neurological conditions were compared to that of 5 min period before (baseline) and after (recovery) the VDT work [13]. The ECG rhythm signals of the successive interval against the immediately preceding R–R interval
were monitored from the left and right wrist positions as a two-dimensional Lorenz scatter plot at both baseline and recovery periods. The EEG band signals were amplified, and the band frequencies were filtered between 1 and 100 Hz, and recorded at four midline head positions (z, zero) at a rate of 512 Hz for alpha, beta, delta, and theta brainwaves (7.0–12.0, 15.0–28.0, 0.5–3.0, and 3.0–7.0 Hz, respectively).

2.6. NO and IL-1β anti-inflammatory assays

The anti-inflammatory activities of SEOs of different fruit ripening stages, limonene, and γ-terpinene were evaluated using NO- and IL-1β-production inhibitory assays [9]. Briefly, SEO, limonene, and γ-terpinene were dissolved in dimethyl sulfoxide at 100 mg/mL, filtered, and diluted with RPMI-1640 (without phenol red indicator) to a concentration range of 3.91–500 μg/mL. BV2 microglial cells (Banca Biologica e Cell Factory, Genoa, Italy) were seeded in a 24-well plate (5.0 × 10^5 cells/500 μL/well) in RPMI-1640 medium with phenol red containing 10% FBS and maintained under 5% CO₂ at 37 °C ambience for 24 h. Afterwards, the spent medium was replaced with 400 μL of fresh RPMI-1640 containing SEO, limonene, or γ-terpinene, and incubated for 30 min. Next, 5 μL of LPS solution (100 μg/mL) was added to induce inflammation and the cells were incubated for 24 h. The resulting culture supernatant was collected and used for NO and IL-1β measurements. For NO measurement, 100 μL of supernatant was added to 100 μL Griess reagent, and the absorbance of the solution was measured at 540 nm using a PowerWave XS2 microplate reader (BioTek Instruments, Winooski, VT, USA). The amount of NO produced was calculated using a standard calibration curve for sodium nitrite (0–100 μM). The IL-1β assay was performed using the Mouse IL-1β ELISA Ready-SET-Go™ kit (e-Bioscience, Inc., San Diego, CA, USA) according to the manufacturer’s instructions. The absorbance was measured at 450 nm using a PowerWave XS2 microplate reader (BioTek Instruments, Winooski, VT, USA).
Instruments) and IL-1β levels were determined using a standard curve of the respective recombinant cytokines (0–500 pg/mL). The effects of SEO, limonene, and γ-terpinene on cell viability were assessed using the MTS assay [19]. All analyses were performed in triplicates.

2.7. Statistical analysis

Each result was expressed as the mean value and standard deviation or standard error, and statistical differences between parameters such as extraction yield, volatile aroma composition, and anti-inflammatorv activity were examined using the Tukey–Kramer honestly significant difference (HSD), while Student’s t-test was applied for the stress amelioration effect (JMP, SAS Institute, Cary, NC, USA).

3. Results

3.1. Volatile aroma components of Shiikuwasha essential oil

The yield of SEO extracted from Shiikuwasha pulp markedly declined with fruit maturation (108.2–66.3 g oil/kg pulp) (Fig. 2a). SEOs from different maturation stages comprised of 56.56–57.31% limonene and 24.41–24.98% γ-terpinene, followed by moderate concentrations of p-cymene, α-terpineol, α-pinene, myrcene, terpinolene, β-pinene, and α-thujene (Fig. 2b). Specifically, the relative concentration of limonene significantly increased from 56.82% in SOE of October produce to 57.31% in November produce (p < 0.05) and then the concentration declined to 56.56% at the ripening stage. Conversely, the lowest concentration of γ-terpinene was recorded in the SOE of November produce (24.41%), whereas there was approximately 0.41% increase in the December batch. Furthermore, p-cymene, α-terpineol, and terpinolene concentrations significantly increased, while the levels of myrcene, α-pinene, β-pinene, and α-thujene in SEO decreased over the 3 months of fruit ripening.

3.2. Stress amelioration effects of Shiikuwasha essential oil, limonene, and γ-terpinene

The stress amelioration effects of the SEO of December produce and its two predominant aroma components, limonene and γ-terpinene were evaluated over a 30 min-VDT work on a display monitor. There was no significant difference in performance on hitting true odd-ball targets in every 5 min work interval between SEO, limonene, and γ-terpinene aroma stream administrations compared to the control (or without aroma), which ranged from 74.12 to 84.57% and 61.99–73.65% at the first and last intervals, respectively (Fig. 1c). However, SEO aroma administration significantly reduced rates of error performance due to lesser false hits up to 15 min of VDT course compared to control, i.e., first interval: 8.82 vs. 19.67%; second interval: 7.47 vs. 18.21%; third interval: 5.57 vs. 12.53% (p < 0.05) (Fig. 1d). Similarly, aroma streams of limonene also scored fewer mistakes in the first and fourth intervals, whereas the impact of γ-terpinene was observed only in the first 5-min.

EEG monitoring showed no significant difference in the complex demodulation high-frequency (HF) amplitude of participants’ heart rate variability when panelists received SEO, limonene, and γ-terpinene aroma inhalations compared to those without aroma stream at 5-min before and after VDT work (Fig. 3a). The ratio of low frequency (LF)/HF due to aroma administrations was also recorded statistically unchanged at both baseline and recovery periods that ranged from 2.0–2.1 (vs. 1.9 at control condition) and 2.0–2.2 (vs. 2.0 at control), respectively (Fig. 3b). On the other hand, there were remarkable changes in the R–R intervals of ECG heart rate variabilities in the recovery period compared to before VDT work when panelists received limonene and γ-terpinene streams, while SEO aroma administration failed to alter the Lorentz plots as in the odorless control condition (Fig. 4). A slight increase in participants’ R–R intervals was recorded upon limonene administration (Fig. 4c), while the mean R–R intervals were significantly increased from 653.2 ms at baseline period to 733.0 ms at the recovery period due to γ-terpinene influence (p < 0.05) (Fig. 4d).

EEG monitoring at midline occipital Oz before, during, and after VDT tasks generally showed similar alteration patterns of bioelectrical brainwave activity of alpha, beta, delta, and theta on aroma stream administrations from SEO, limonene, and γ-terpinene compared to the control condition (Fig. 5). The relative power of the alpha band sharply declined in the first 5-min of VDT compared to the baseline period due to SEO administration from 14.7% to 10.8%, but there were no significant differences between SEO inhalation and control over all stages, as well as for the two aroma compounds (Fig. 5a). Interestingly, the γ-terpinene aroma stream consistently lowered brain bioelectrical activity at beta spectral wave over 30-min VDT compared to other aroma administrations and odorless conditions (Fig. 5b). Moreover, significant reductions of relative power of beta band of γ-
terpinene stream were recorded at before and after the course compared to control (12.4 vs. 10.8% and 11.8 vs. 9.9%, respectively) \((p < 0.05)\).

3.3. Anti-inflammatory activities of Shiikuwasha essential oil, limonene, and \(\gamma\)-terpinene

In vitro anti-inflammatory effects of SEO of different fruit-ripening stages and those of its two predominant aromatic components, limonene and \(\gamma\)-terpinene, were evaluated in BV2 murine microglial cells. None of the tested concentrations \((3.91 \text{ to } 500 \mu g/mL)\) had a significant effect on cell viability \((p < 0.05)\) (Fig. 6). The anti-inflammatory effects of these aroma compounds were tested using an LPS-induced inflammation model in which NO and IL-1\(\beta\) cytokine are well-known pro-inflammatory mediators (Fig. 7). Notably, SEO of December
batch, which was produced from mature Shiikuwasha fruits, even at the lowest concentration of 7.8 μg/mL inhibited LPS-induced NO production in microglial cells compared to cells treated with LPS alone (46.9 versus 50.6 μM; p < 0.05). A dose dependent inhibition of LPS-induced NO production was observed from 15.6 to 500 μg/mL (44.3–4.5 μM; p < 0.01) (Fig. 7a). A similar inhibitory effect on NO production was also observed with essential oils from October and November batches (which were extracted from immature fruits), and with authentic limonene and γ-terpinene molecules (Fig. 7b).

Unlike in the NO inhibitory assay, higher concentrations of SEO were necessary to inhibit IL-1β production (Fig. 7c and d). Significant suppression of this LPS-induced IL-1β was observed from 31.3 μg/mL of SEO of December batch (p < 0.05).

Fig. 3. (a) Complex demodulation-high frequency (HF) amplitude and (b) low frequency (LF)/HF ratio of heart rate variabilities measured via electrocardiogram recorded before and after visual display terminal (VDT) tasks with administration of odorless squalane (control), Shiikuwasha essential oil (SEO), limonene, and γ-terpinene. Each value is expressed as the mean ± standard error (N = 9).

Fig. 4. RR interval Lorenz plots of ECG heart rate variabilities recorded before and after visual display terminal (VDT) tasks with administration of (a) odorless squalane (control), (b) Shiikuwasha essential oil (SEO), (c) limonene, and (d) γ-terpinene. Each value is expressed as the mean ± standard error (N = 9); significant difference of mean values is indicated as * at p < 0.05.
Better inhibitory activity was observed at higher essential oil concentrations, wherein the IL-1β signal was significantly reduced to 2.1 pg/mL at 500 μg/mL (p < 0.01). Similar results were observed for SEO from the November batch, while limonene displayed a lower anti-inflammatory potential against IL-1β (Fig. 7d). In contrast, γ-terpinene showed enhanced anti-inflammatory effects at higher concentrations (500 μg/mL), as in the SEO of the October or December batch. The anti-inflammatory potential of SEOs at 250–500 μg/mL was comparable to that of the reference control, dexamethasone, at 4–20 μM (data not shown).

4. Discussion

Shiikuwasha pulp is also one of the major by-products of processes that include the use of the fruit, such as the production of vinegar from unripe fruit (October and November) and juice production from ripe fruit (December). The decline in SEO extraction can be attributed to the decrease in oil glands in the fruit peels during ripening, which is the primary source of essential oil in Shiikuwasha pulp [2]. Nevertheless, SEOs from different maturation stages possessed comparable aroma constituents that differed significantly from those of other citrus cultivars, including predominant limonene (56.56–57.31%) and γ-terpinene (24.41–24.98%). These two major constituents possess high aroma activities (flavor dilution factor = 243) and are responsible for emitting unique aroma properties to SEO, in which limonene can produce citrus, green, and minty odors, while γ-terpinene has waxy and woody scents [4]. In contrast, most of the essential oils derived from common citrus cultivars are predominantly composed of limonene e.g. 96.2% in grapefruit (Citrus paradisi), 95.1% in tangerine (Citrus reticulata var tangerine), 94.9% in orange (Citrus sinensis), 93.9% pummelo (Citrus grandis), and 71.0% in lime (Citrus limonia), and with lower γ-terpinene proportions that ranged from 0 to 12% (11.9% in lime) [20,21]. The compositional ratio of these two monoterpene hydrocarbons could be used as an important index for monitoring the aroma quality of Shiikuwasha fruit.

Fig. 5. EEG bioelectrical brainwave activity of (a) alpha, (b) beta, (c) delta, and (d) theta band power densities at midline occipital Oz recorded before, during, and after VDT tasks with administration of odorless squalane (control), Shiikuwasha essential oil (SEO), limonene, and γ-terpinene. Each value is expressed as the mean ± standard error (N = 9); significant differences of aroma inhalation administration compared to control are indicated as * at p < 0.05.
and its derived food and beverage products, including essential oils. On the other hand, relative concentration fluctuations in the SOE were observed in other important volatile terpene substances, such as α-pinene, α-terpineol, and α-pinene, which is consistent with a previously reported study, indicating that terpene biosynthesis has continuously occurred during the ripening of Shiikuwasha fruits [2].
The SEO of December produce was used in the stress amelioration effects experiment for its practical application approach because the essential oil obtained from by-products of Shiikuwasha juice manufacturing has more consistent agro-industrial availability as ripe fruit harvest than unripe fruits from the October or November harvest season. Pleasant citrus aromas from SEO administration could improve work performance in the VDT test by reducing mistakes in the first 15-min period, which is two and three times longer than that of limonene and \( \gamma \)-terpinene streams, respectively. The complexity of the aroma composition of the essential oil, which provides a balanced enjoyable scent, could be responsible for this augmentation phenomenon. Therefore, inhalation of this essential oil and its aroma compounds could improve the attention and concentration of panelists, and thus work quality by reducing errors that could lead to typing mistakes in electronic visual tasks such as VDT. However, these aroma administrations did not have particular effects on the panelists’ respiratory states, as indicated by unchanged ECG heart rate signal amplitudes, that is, HF variability and LF/HF ratio. HF amplitude ordinarily signifies cardiac parasympathetic nerve activity, while LF/HF value data can indicate the sympathovagal balance index. Thus, alteration of these parameters compared to the control condition might provide primary indications of physiological changes in the respiratory system [22]. Interestingly, a similar occurrence was previously recorded for aroma inhalation of fir tree (\textit{Abies sibirica}) essential oil, which might indicate that inhalation of flow-controlled essential oil streams does not affect breathing issues, even when used during electronic visual work for a certain period of time (up to 30 min) [13].

Moreover, to evaluate the relaxation effect of aroma administration over VDT work, R–R intervals of heart rate variabilities, which could directly indicate vagal activity, were measured. The increase in panelists’ R–R intervals due to limonene and \( \gamma \)-terpinene administration could indicate relaxed deep breathing outcomes with elevated parasympathetic nervous system activity after completing the VDT task [13,23]. However, a similar effect was not
observed for SOE administration, indicating that participants’ parasympathetic nerves could not benefit from the complex combination of aroma sensations from the volatile aroma components of the essential oil. This phenomenon suggests that the relieving effect of inhaling less complex odorants, that is, minty citrus scent from limonene or woody odor γ-terpinene, could stimulate beneficial physiological senses, such as relaxation and stress relief over electronic visual tasks, and thus has a potent contribution to physiological stress reduction.

The aroma stream from γ-terpinene also significantly reduced the bioelectrical activity of beta spectral power at midline occipital Oz, indicating that participants might experience reduced active concentration that could promote less anxious thinking for 5 min of aroma inhalation at and after finishing the visual task. The reduction of this beta band, particularly in the occipital lobe, has also been considered an indication of physiological stress reduction by reducing tension, inducing relaxation, and improving rest quality [24,25]. The high odor strength and aroma activity of γ-terpinene in Shiikuwasha [4] could promote its potent aroma stream application in neurological stress amelioration during electronic visual tasks. However, the study outcomes were limited to healthy young women.

To measure the potent in vitro anti-inflammatory effects of SEO of different fruit ripening stages, in which the oil could be obtained by local artisans regardless of the harvest season, SEOS of October–December batches were evaluated in BV2 murine microglial cells via NO and IL-1β inhibition assays. Differential activities against the productions of these pro-inflammatory substances exerted by SEO from different harvesting months could be due to intrinsic differences in the chemical composition of SEO extracted from fruit pulps at different maturation stages [2]. Moreover, SEO and γ-terpinene showed better anti-inflammatory activities at higher concentrations against IL-1β, while limonene displayed a lower effect, indicating that various possibilities of interaction and mechanism of action may occur between the chemical components in the essential oil, including these two predominant monoterpenes. Nevertheless, our findings show that the essential oil from Shiikuwasha, as well as its aroma-active components, has potent anti-inflammatory activities in suppressing stress marker production, such as NO and IL-1β. These bioactive substances are known to alter the gene expression responsible for the production of these pro-inflammatory cytokines and also block or stimulate key upstream signaling pathways, thus affecting their production [10,26–28]. This result confirmed the anti-inflammatory potential of not only essential oil derived from Shiikuwasha pulp but also its aroma-active components, such as limonene and γ-terpinene, in microglial cells, which differs from previously reported citrus essential oils’ in vitro anti-inflammatory activity studies [6,28].

The observed anti-inflammatory activity in glial cells highlights the potential use of SEO and its aromatic constituents for neural cell stress reduction, and thus could act as an anti-neuroinflammatory agent. However, since SEO stream administration did not significantly alter participants’ physiological, respiratory, and brain power states, there was no direct association between stress amelioration and the anti-inflammatory activity of SEO. Nevertheless, both studies revealed promising neuroprotective biological activities of γ-terpinene. Such neuroprotective benefits have also been observed for other plant essential oils and their chemical components [9,11]. The outcome of our study would encourage further complex biomolecular research studies, such as active component interactions, receptor binding, and signaling mechanisms [29,30]. Taken together, the findings of this study may lead to the endorsement of practical applications of citrus essential oils for possible complementary and alternative treatments, such as aromatherapy, and should also promote the beneficial use of agro-industrial waste materials, including citrus pulp.

5. Conclusion

The essential oil extracted from Shiikuwasha pulp was mainly composed of monoterpenes, of which limonene, γ-terpinene, p-cymene, α-terpineol, α-pinene, myrcene, terpinolene, β-pinene, and α-thujene were the main aroma compounds. The relative proportions of limonene and γ-terpinene in SEO were found to be distinct from those in other common citrus cultivars, which imparts superior and unique aroma characteristics to SEO. We found that the administration of the SEO aroma stream in human subjects greatly improved mental focus, as determined by the reduction in error hits by 15 min in the electronic visual task. While limonene reduced error hits in the visual task only at the first and fourth 5-min intervals, γ-terpinene effects lasted for the initial 5-min only. However, γ-terpinene provided a physiological relieving effect by significantly increasing the R–R interval spectral variability of ECG signals after completing the 30 min-task. It also alters EEG beta wave activity, which signifies less tension at the midline occipital Oz, and thus provides evidence for γ-terpinene stress reduction activity. Furthermore, we show that SEO
extracted from Shiikuwasha fruit and its two predominant compounds, limonene and γ-terpinene, have significant anti-inflammatory potential by suppressing pro-inflammatory markers such as NO and IL-1β in microglial cells.

Conflict of interest

None.

Acknowledgements

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