Modification of Electrical Brain Wave by *Citrus sp.* Essential Oil Inhalation

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Abstract

The essential oil of orange (*Citrus sp*.) has been widely used in aromatherapy according to its anxiolytic effect. Electroencephalography (EEG) is one of the reliable neurological techniques used to study the brain and behavioral functions. This method has been used to record brain waves for frequency analysis. The present study examined the effect of citrus essential oil (EO) on EEG pattern in adult male Wistar rats. Animals were anesthetized with Zoletil® (60 mg/kg i.m.). Stereotaxic apparatus was used to fix rat skull for the implantation of 4 stainless steel screw electrodes over the frontal and parietal cortices using bregma as the landmark. Ampicillin (145 mg/kg i.m.) was daily injected for 4 days after the surgery and the animals were allowed to recover for 10 days. On the day of the experiment, individual rat EEG was recorded using PowerLab®/4s system. The EEG signals were displayed on LabChart® software and processed on a personal computer. The inhalation of EO (20 and 200 µl) and distilled water as control were performed in the EEG recording chamber. Frontal EEG analysis showed that EO (20 µl) increased the percent baseline powers in low frequency bands ranging from theta (4.7-6.6 Hz), alpha1 (7-9.4 Hz), alpha2 (9.8-12.5 Hz) to beta1 (12.9-18.4 Hz) waves. However, in the parietal cortex, only alpha2 and beta1 powers were significantly increased. In addition, EO 200 µl increased the percentage of the baseline powers only in the theta and the alpha2 bands in the frontal cortex. In conclusion, this study demonstrates EEG pattern in response to inhalation of citrus EO. The data may represent EEG biomarker of the EO in the central nervous system (CNS). However, it remains unknown in terms of its CNS mechanism.

J Physiol Biomed Sci. 2013; 26(1): 5-8

Keywords: *essential oil, citrus oil, electroencephalography, rat*

Plants in *Citrus* genus have been widely investigated for their anxiolytic effects both in human and animal models. In human, ambient odor of orange (*Citrus sinesis*) was applied in a dental office to reduce anxiety and improve mood in female patients.[1] Sweet orange aroma also produced an acute anxiolytic activity in healthy volunteers.[2] Animal models were often used for confirmation of essential oil effect on behavioral tests such as elevated plus maze task (EPM), light-dark box task and open field task (OFT). The effects of *C. sinensis* (sweet orange) essential oil on male Wistar rats and mice were evaluated in the elevated plus-maze and followed by the light/dark paradigm. The sweet orange essential oil was found to increase exploration on the open arms of the elevated plus-maze and time spent in the lit chamber of the light/dark paradigm, respectively.[3] Lemon oil had the strongest anti-stress effect in all three behavioral tasks which include EPM task, a forced swimming task (FST), and OFT in mice.[4] In terms of mechanism, the essential oils of plants in *Citrus* genus have been demonstrated to stimulate multiple receptor types. For example, lemon oil produced anti-stress via dopamine (DA) receptors and serotonin type 1A receptors ($5\text{-}HT_{1A}$).[4]

Previously, EEG technique has been used only for making inferences about overall states of sleep and wakefulness.[5] Up to date, the EEG technique has been used to record the brain activity following drug treatment of neurological diseases both in humans and rodents.[6,7] For example, diazepam is a well-known drug for the treatment of anxiety disorder. In rat model, it was found to increase beta and gamma oscillation.[6] However, long term use of diazepam has been found to develop many side effects such as sedation and memory impairments.[8,9] These aversive effects also lead to the development of tolerance, dependence, withdrawal symptoms and addiction.[10] Some other alternative treatments with safer effects have been searched. This study hypothesized that essential oil of orange has CNS action and produces specific pattern of electrical brain wave. The main purpose of this study was to investigate the effect of essential oil of orange (*Citrus sp.*) inhalation on EEG pattern in rats.

This work was presented at the 42nd Annual Scientific Meeting of the Physiological Society of Thailand, April 24-26, 2013, Phetchaburi, Thailand.

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Materials and Methods

Animals

Adult male Wistar rats, weighing approximately 300-350 g, were used. Animals were housed in a room maintained at 24 ± 1 °C with12 hr light/dark cycle (light on at 7:00 a.m.). The animals were housed in stainless steel home-cages with the dimension of 30 cm × 30 cm × 30 cm (length x width x high) and a single rat per cage with *ad libitum* access to food pellets and water. The experimental protocols for care and use of the experimental animals in the present study were approved and guided by the Animals Ethical Committee of Prince of Songkla University.

Citrus sp. essential oil (EO)

The EO was purchased from The Royal Project Foundation of Thailand (Kasetsart University, Bangkok, Thailand). The chemical analysis confirmed that the EO contains 95.88% Limonene. This ingredient was previously found to produce anxiolytic action in mice.[11] It was hypothesized to work through benzodiazepine-type receptors.[12]

Surgical procedure

For the surgical process, the animals were anesthetized with intramuscular injection of 60 mg/kg Zoletil® 100 (Virbac, Thailand). Stainless steel screw electrodes were stereotaxically implanted over the frontal cortex (AP; +3 mm, ML; 3 mm) and the parietal cortex (AP; -4 mm, ML; 4 mm) on the left side of the skull (Figure 1 A - C) by using bregma as the landmark. Electrode fixed at the midline over the cerebellum (AP, -12 mm; ML, 0 mm) was used as a reference and ground electrode. All electrodes were secured in place with acrylic resin (Unifasttrad, Japan) (Figure1 D).

After full recovery from surgery, the rats were acclimated to connection with cables for 2 hours in the inhalation apparatus consisting of a Plexiglas cylinder with 45 and 30 cm in height and diameter, respectively.

EEG recording

On the experimental day, baseline EEG was recorded for half an hour and 1-hour period following the inhalation of orange oil (20 and 200 μl) or distilled water. EEG signals were amplified with a low-pass 100 Hz, high-pass 0.1 Hz by a PowerLab/4SP system (AD Instruments) with 12-bit A/D converter, and stored in a personal computer through the Chart program software. The EEG signals were processed through 0.78 – 45 Hz band-pass filter: Delta band (0.8- 4.30 Hz), theta band (4.7-6.6 Hz), alpha1 band (7-9.4 Hz), alpha2 band (9.8-12.5 Hz), beta1 band (12.9-18.4 Hz), beta2 band (18.8-35.2 Hz) and gamma band (35.5-45 Hz). The digitized EEG data were segmented into 1024-point (50% overlap) and the signals were converted to power spectra by the fast Fourier transform algorithm (Hanning window cosine transform). Then the power spectra of 2.56-sec sweeps in each 60-min length of selected period of artifact-free signals were averaged to give the power spectra of the period.

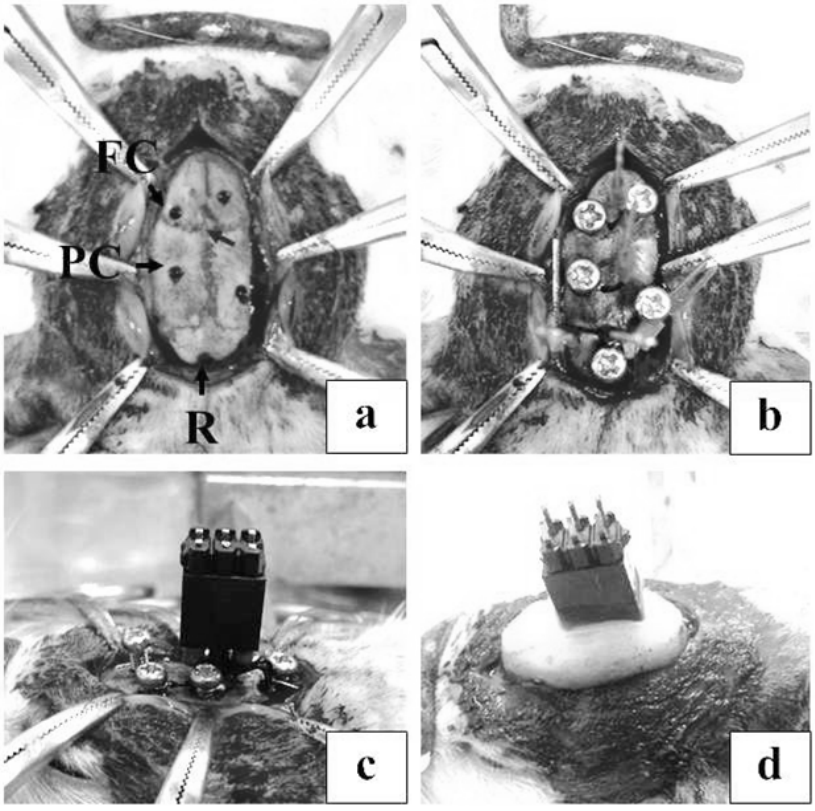

Figure 1 Electrodes implantation. (**a**) Locations of electrode implantation on the skull. FC = frontal cortex, PC = parietal cortex, R = reference at the cerebellum. Unlabeled arrow indicates bregma. (**b**) Stainless steel screw electrodes were implanted into the frontal and parietal cortices and the cerebellum as reference electrode. Two stainless steel screw anchors were implanted on the right side of the skull. (**c** and **d**) Female electrodes were inserted into the header socket and then acrylic dental cement was poured in to fix all electrodes on the rat skull

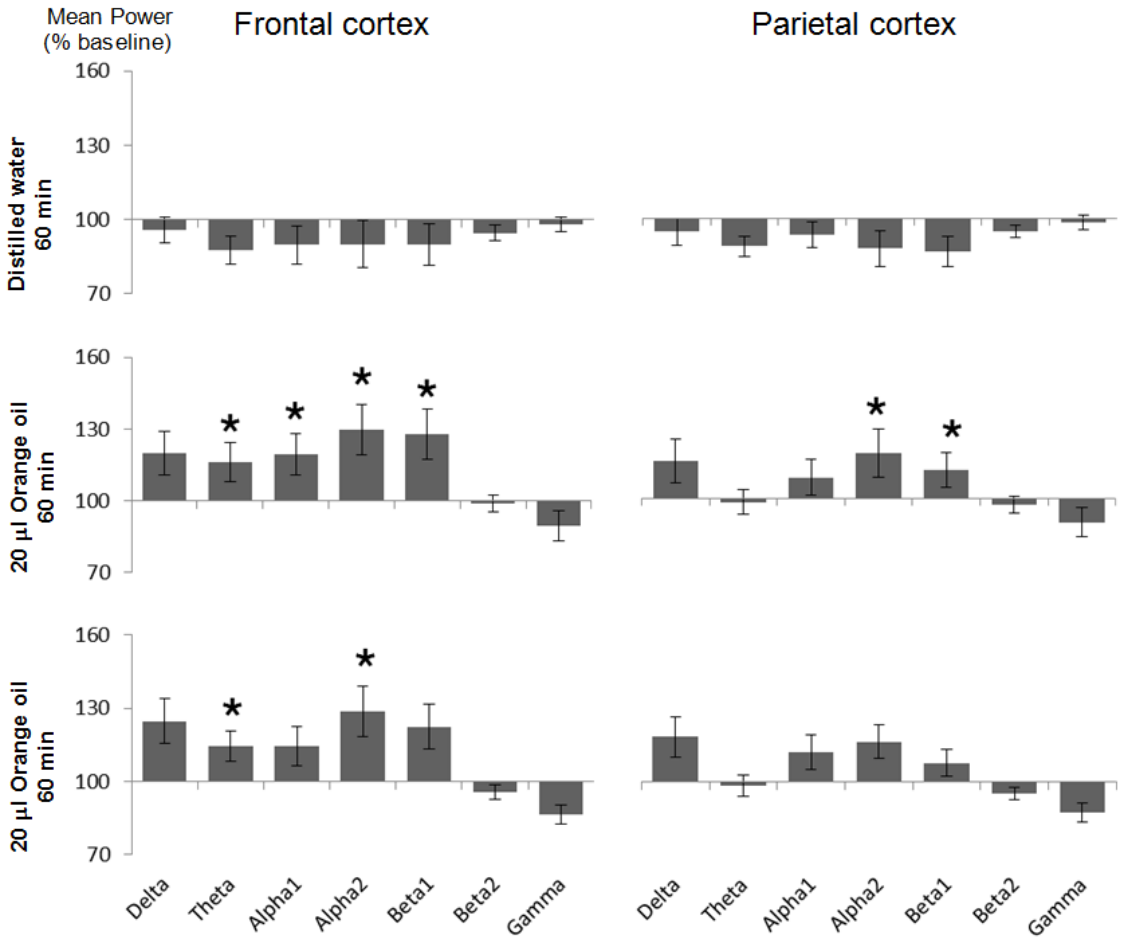

Figure 2 Effects of single inhalation with the 20 or 200 µl essential oil of orange, or distilled water (rats 8-10 per group) on EEG pattern. Mean percentage power from frontal cortex (left) and parietal cortex.[17] *P< 0.05 as compared with the distilled water group (one-way ANOVA followed by Tukey post hoc test).

Data analysis

Data from the frontal and parietal cortices were expressed in percentage of baseline power. Percentage baseline power of EEG was calculated from pre- and post-treatment. The pre-treatment was set to 100% and the post-treatment was computed to compare with 100% pre-treatment. EEG data was shown in mean ± SEM. Statistical difference between groups was tested using one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparison. The differences were considered statistically significant at $P < 0.05$.

Results

Results from inhaled EO or distilled water were presented in Figure 2. After a single treatment, EO at 20 µl (EO20) and 200 µl (EO200) were found to change percentage power in the frontal and parietal cortices. In one-way analysis of the frontal EEG, significant increases in power were seen in theta (F(2, 27) = 4.808; P = 0.017), alpha1 (F(2, 27) = 3.516; P = 0.045), alpha2 (F(2, 27) = 4.396; P = 0.023), and beta1 (F(2, 27) = 4.266; P = 0.025). No significant change was observed in the higher frequency ranges. Multiple comparisons also indicated significant effects of EO20 in the frequency range from theta to beta1 activities whereas EO200 had significant effects on theta and alpha2 only.

In the parietal EEG analysis, significant increases were specific only on alpha2 (F(2, 27) = 3.908; P = 0.033) and beta1 (F(2, 27) = 4.076; P = 0.029) bands. In particular, changes in EEG power were produced by EO20.

Discussion

This study aimed to screen the effect of essential oil on electrical brain activity. The electroencephalography (EEG) is the most popular technique used to detect the real-time activity of the brain. Basically, it is used to study the brain function. One of the principal advantages of EEG was that continuous brain function can be monitored with repetition of recording by using the same set of animals. It is also practical for longitudinal studies. The present studies examined electrical brain waves in rats inhaled with EO (20 and 200 µl). The results obtained in these studies demonstrated the quantitative effects of the EO inhalation on cortical neuronal activities

measured by EEG. The increase in percentage of baseline power produced by EO 20 µl were seen in theta, alpha1, alpha2 and beta1 activities in the frontal cortex. In addition, the EO also increased percentage of baseline power on the alpha2 and beta in the parietal cortex.

It was noted that all changes induced by EO were found mainly in the low frequency range. The increase in slow wave activities might suggest anxiolytic or sedative effects. Previously, diazepam effect on the electrical activity of the brain has been consistently examined in rats and found to increase the beta activity through benzodiazepine site on the $GABA_A$ receptors.[13,14] The present results also showed similar findings. However, whether or not the EO produced these EEG pattern via $GABA_A$ receptors remains to be clarified. Moreover, the CNS action of the citrus EO might be evaluated from brain states of the animals following the EO inhalation. In general, anxiolytic agents promote slow wave activity and sleep.[15] Thus, sleep-wake analysis might be needed in order to classify CNS effect of the citrus EO.

Conclusion

Inhalation of citrus EO in rats increased brain wave activity mainly in the slow frequency range. The pattern induced by the inhalation was found, in part, to resemble the effect of those standard anxiolytics. The frontal cortex exhibited relatively more sensitive to the inhalation than the parietal cortex.

Acknowledgement

This work was financially supported by grants from the Natural Products Research Center of Excellence (NPRCoE) and the Department of Physiology, Faculty of Science, Prince of Songkla University, Thailand.

Conflict of Interest

None to declare.

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