Pharmaceutical Potentials of Selected Aromatic Spices:
Pharmacological and Phytochemical Evaluation of Cinnamon (*Cinnamomum cassia*), West African Black Pepper (*Piper guineense*) and Tree Basil (*Ocimum gratissimum*)

（香辛料の薬物様作用：3 種の香辛料、桂皮、西アフリカ黒胡椒
および木性バジルの薬理学的、植物化学的評価）

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Preface

Phytomedicine is one of the most reliable means for health benefits in different systems of traditional medicine, including Kampo, Ayurveda, Chinese and African Traditional medicines. According to the World Health Organization (WHO), 80% of the world population relies on traditional medicine, mainly herbs. Compounds and extracts derived from plants are the basis of ≥30% of modern drugs, and herbal medicine remains a promising alternative treatment for many diseases. Over the last decade, there has been a revival of interest in the use of phytomedicines all over the world, particularly for preventive means. Nutraceuticals, defined as "foodstuffs that provide health or medical benefits in addition to their basic nutritional values" would be a sort of phytomedicine that is quite familiar in daily life. Spices or culinary herbs are often found useful for treating some common diseases and wounds in their traditional usage, and they offer potential as nutraceuticals. Cinnamon, West African black pepper and tree basil which are common Cameroonian kitchen spices were investigated for their potency as nutraceuticals and discussed for pharmaceutical development.

緒言

健康増進や疾病予防を目的としたセルフメディケーション、補完代替医療また、漢方をはじめとする伝統医学による治療では、植物性素材が多く用いられる。日本では生薬は医薬品として取り扱われるが、生薬の中には、桂皮（シナモン）や薄荷（ミント）など、効果効能をうたわなければ香辛料（食品）やいわゆるハーブとして取り扱うことができるものも多くある。近代医薬品の入手が容易ではない地域などでは、身近な入手し易い素材で疾病予防やプライマリーケアができることは望ましいことであり、香辛料やハーブにその可能性が期待される。本研究では、カメルーンの台所でよく見かける香辛料およびハーブのうち 3 種をとりあげ、それぞれ薬用作用を評価した。すなわち、（1）桂皮入り食餌の継続投与による胃潰瘍予防活性、（2）西アフリカ黒胡椒精油の吸入投与によるマウスの鎮静活性および抗不安活性、（3）木性バジル精油の吸入投与によるマウスの鎮静活性、抗不安活性および抗鬱活性, について検討した。その結果、これらの香辛料は胃潰瘍の予防や軽い不安、鬱を軽減する効果が期待できることが明らかとなった。

Chapter 1

Regular ingestion of cinnamomi cortex pulveratus offers gastroprotective activity in mice

Abstract The present study investigated the gastroprotective effects of a cinnamon diet using different gastric ulcer mouse models. Dose dependency and the effective dose period of administration of a cinnamon powder diet were established using the water immersion stress gastric ulcer model. A cinnamon powder diet significantly protected mice against ulceration by stress, ethanol, HCl and oral administration of aspirin, but not against ulceration by oral administration of indomethacin or subcutaneous administration of indomethacin or aspirin. Such a diet conferred protection against gastric ulcers at an effective concentration of 100 mg cinnamon powder per gram of food after administration for four weeks and the active compound of the cinnamon powder for gastroprotective activity was identified as cinnamaldehyde. These findings indicate that regular ingestion of cinnamon powder offers gastroprotection presumably through a cytoprotective mechanism but the efficacy against NSAIDs-induced gastric ulcers may be limited.

Keywords *Cinnamomum cassia* powder · Gastric ulcer · Gastroprotection · Regular ingestion · Synergism

Introduction

Gastric ulcer disease is prevalent in many parts of the world. Although chronic disease is primarily caused by *Helicobacter pylori* infection, gastric ulcers are aggravated by the use of nonsteroidal anti-inflammatory drugs (NSAIDs), excessive alcohol intake and stress [1, 2]. Several naturally occurring agents, including spices, are known to augment the mucosal defense so that the gastric mucosa can resist strong irritants such as concentrated ethanol, acid, and NSAIDs [3].

Cinnamomum cassia Blume (Lauraceae), known as cinnamon in its stem bark form, is listed in the Japanese Pharmacopoeia. It is a common spice that is often included in Kampo formulae for stomach problems such as gastric ulcers [4], and aqueous extracts of C. cassia have been shown to have anti-ulcerogenic potential [5, 6]. Cinnamon is commonly used in powder form, although scientific evidence of the gastroprotective benefits of whole cinnamon powder is lacking, and the effects of long term ingestion have not been established. This study therefore aimed to investigate the gastroprotective benefits of regular ingestion of C. cassia powder and attempted to clarify the active compounds and mechanisms of action involved in its gastroprotective activity.

Materials and methods

Animal care

Three-week-old male ddY mice (12 g) purchased from Japan SLC, Shizuoka, Japan were used for this study. They were kept under an ambient temperature of 25 ± 2 ºC and a relative

humidity of 50–60 % with a light–dark cycle of 12 h. Animals were fed laboratory-made pellet chow and water ad libitum. Animal experiments were designed following recommendations by the Animal Research Committee of Kyoto University, Kyoto, Japan (Approval number 2010–22). Experimental procedures involving animals and their care were conducted in conformity with institutional guidelines that complied with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan (2006).

Drugs and Reagents

Cinnamomi cortex used in this study was purchased from Vinh Phuc Co. Ltd (Tam Ky, Vietnam) and was pulverized by Mitsuboshi Co. (Nara, Japan). Indomethacin and sucralfate were purchased from Nacalai Tesque (Kyoto, Japan). Sucralfate was dissolved in 1 % carboxymethylcellulose (CMC) and administered orally (500 mg/kg) as a positive control 45 min before ulcerogenesis. All other drugs and reagents used in this study were of the highest grade commercially available.

Administration of cinnamon powder

Cinnamon powder was administered to animals via their diet for 4 weeks prior to ulcerogenesis. The cinnamon diet was prepared as follows: C. cassia powder was mixed with powdered animal feed at a concentration of 100 mg/g of feed. Distilled water was added to make pellets approximately 1.5 cm in diameter. The pellets were frozen at -20 ºC

for 24 h and freeze-dried for 24 h. Control groups were administered similarly prepared pellets without cinnamon.

Determination of dose and dose period of cinnamon powder

To determine the most effective dose of cinnamon powder, four groups of mice (n = 10/group) were administered cinnamon diets containing varying concentrations of cinnamon powder for 4 weeks. The diet of group 1 contained 2 mg of cinnamon powder per g of feed, group 2 contained 10 mg/g, group 3 contained 100 mg/g and the control group was fed pellet chow without cinnamon powder. Ulcers were evoked in all groups by water immersion stress at 7 weeks and mucosal injury measurements were made as described below. To determine the optimal dose period, mice were divided into three groups. Mice in group 1 were administered the cinnamon diet 1 week prior to ulcerogenesis, mice in group 2 were placed on the cinnamon diet for 2 weeks preceding gastric ulcer induction while those in group 3 were placed on the cinnamon diet for 4 weeks preceding gastric ulcer induction. All animals were fed simple pellet chow when they were not on the cinnamon diet. Control groups were fed pellet chow without cinnamon powder for 4 weeks.

Gastric ulcer induction

Mice were divided into four groups of 10, and each group was subjected to a different method of ulcerogenesis. All animals were fasted for 24 h on water before ulceration and were sacrificed by i.p. injection of sodium pentobarbital anaesthesia (500 mg/kg of body weight). The methods of ulcerogenesis were as follows: (1) Water immersion stress induced ulcerogenesis: Mice were restrained in stress cages and immersed up to their xiphoid in a

water bath maintained at 23 ºC for 8 h prior to sacrifice according to the method of Kuwayama and Eastwood (1985) [7]. (2) Absolute ethanol-induced ulcerogenesis: Animals were orally administered 99.5 % absolute ethanol at a dose of 5 ml/kg body weight 1 h before sacrifice. (3) HCl-induced ulcerogenesis: 0.6 M HCl was administered orally to animals at a dose of 5 ml/kg body weight 1 h before sacrifice. (4) NSAID-induced ulcerogenesis: Each group received one of the following treatments 4 h before sacrifice; Group 1 were orally administered indomethacin (35 mg/kg body weight), group 2 were orally administered aspirin (200 mg/kg body weight), group 3 received a subcutaneous injection of indomethacin (35 mg/kg body weight), and group 4 received a subcutaneous injection of aspirin (200 mg/kg body weight). Indomethacin was dissolved in 5 % $NaHCO_3$, while aspirin was dissolved in 5 % arabia gum before administration. All administered doses were based on previously reported data [8–10].

Gastric mucosal lesion measurement

After sacrifice, mouse stomachs were removed and incised along the greater curvature. Gastric contents were emptied, and the stomachs were rinsed with saline and fixed in 1 % formalin. Gastric mucosal lesions were observed under a microscope at 10× magnification, and the lengths of all ulcerogenic lesions per stomach were measured. The sum of the lengths of all ulcerogenic lesions per stomach was taken as the ulcer index, which represented the severity of gastric injury.

Measurement of gastric mucosa thickness

Mice were divided into two groups of 20. One group was placed on the cinnamon diet for 4 weeks while the control group was fed ordinary pellet chow for 4 weeks. After sacrifice, the stomachs were removed, fixed in 1 % formalin then in 10 % formalin. Fixed stomachs were embedded in paraffin, and cut at the pyloric antrum into 5-µm thick sections. The stomachs of control group mice were cut at the same location as those of the cinnamon treated group. The cut sections were immediately dipped in distilled water and mounted on slides. These were deparaffinized by soaking in xylene twice for 10 min each, rehydrated through graded ethanol solutions (100 and 95 %) twice for 3 min each, then rinsed in distilled water. Rehydrated slides were stained using the Periodic Acid- Schiff staining method. Histological examination was performed using a light microscope equipped with an ocular micrometer at 400× magnification and the thickness of the gastric mucosa was measured.

Extraction of cinnamon powder and isolation of active components

Fifty grams of cinnamon powder was extracted using a Sohxlet apparatus with ethyl acetate to afford approximately 5 g of extract and 36 g of debris. The ethyl acetate extract of *C. cassia* (EACC) was evaporated under reduced pressure using a rotary evaporator. The headspace of the extract was analyzed for complete solvent eradication by solid phase micro extraction with gas chromatography (SPME-GC). The analysis was performed on a G7000-M9000/3DQMS system (Hitachi, Tokyo, Japan) under the following operating conditions: column, fused silica capillary column TC-WAX (Hewlett Packard, Palo Alto, CA), 60 m × 0.25 mm, 0.25 µm film thickness; column temperature, 40–120 ºC increasing at a rate of

16 ºC/min, 5 min at 120 ºC, 120–130 ºC increasing at 1 ºC/min, 15 min at 130 ºC, 130–200 ºC increasing at 20 ºC/min, 20 min at 200 ºC; carrier gas, He, 147.1 kPa; ionization energy, 15 eV. To investigate the active components, EACC was fractionated using silica gel open column chromatography (Wakogel C-200, Wako, Osaka, Japan) and eluted with chloroform:AcOEt (2:1) to afford three fractions (Fr. 1–3).

The biological activity of EACC and that of the fractions were investigated by administering them to mice using the same technique for cinnamon powder described earlier. EACC and fraction doses were calculated according to their proportional yields from cinnamon powder to allow for assessment of the effect of each component on the activity of whole cinnamon powder. Prevention of gastric ulcer by EACC and its fractions was examined using the ethanol-induced ulcer model described earlier because it afforded more visual and distinct ulcerogenic lesions in control groups than the other ulcerogens. Moreover, the percentage protection against gastric ulcers that cinnamon powder afforded when investigated by using the ethanol model was higher than that of the other ulcerogenic agents.

EACC fractions were further purified by gel permeation chromatography (GPC) (LC-918 recycling high performance liquid chromatography (HPLC) system, JAIGEL-1H and -2H columns, 20 mm × 600 mm (Japan Analytical Industry, Tokyo, Japan), eluted with CHCl3 (flow rate 3.0 ml/min), and preparative thin layer chromatography (PTLC) followed by HPLC and gas chromatography-mass spectrometry (GC/MS) analysis was performed. GC/MS analysis was performed on an Automass (JEOL, Tokyo, Japan) under the following operating conditions: column, fused silica capillary column TC-WAX (Hewlett Packard), 60 m × 0.25 mm, film thickness, 0.25 μm; column temperature, 40–130 ºC increasing at a rate of 2 ºC/min, 25 min at 130 ºC, 130–140 ºC increasing at 2 ºC/min, 15 min at 140 ºC, 140–200 ºC increasing at 15 ºC/min, 30 min at 200 ºC; injector, 180 ºC, carrier gas, He, 45

cm/min; column head pressure, 100 kPa; injection volume,1 µl; ionization energy, 70 eV. Compounds were identified by comparing their retention times, mass and ion spectra from an MS data library (NIST 02), and authentic standards. Purified compounds were analyzed by nuclear magnetic resonance (NMR) and Fab-MS to elucidate structures.

Administration of cinnamaldehyde (CA)

CA was administered to mice using the same method of administration for cinnamon powder described earlier to confirm its gastroprotective activity. Mice were divided into three groups of 10. Group 1 was administered a diet consisting of CA at a concentration of 0.98 mg per/g of feed for 4 weeks. The diet of group 2 contained 9.8 mg CA/g of feed and control group 3 was administered ordinary mouse feed for 4 weeks. At 7 weeks, ulcers were evoked by water immersion stress and gastric mucosa injury was measured previously described.

Statistical analyses

Data were expressed as means for all animals in treatment groups (n = 10). The Student's t test, and the analysis of variance (ANOVA) test followed by Tukey–Kramer's, Bonferroni's or Dunnett's multiple comparison tests were used to analyze data.

Results

Effect of different doses and dosing periods of cinnamon on the prevention of gastric ulcer

Figure 1a shows the dose-dependent preventative effect of cinnamon powder on gastric ulcers caused by water immersion stress. A 2 and 10 mg/g had no significant effects on preventing gastric ulcers compared with the control group, while the 100 mg/g dose was significantly effective for preventing gastric ulcers compared with the control group ($p < 0.01$).

The optimal dose period was investigated over 1–4 weeks. As shown in Fig. 1b, the longer the period of administration, the better the gastroprotective benefit of a cinnamon powder diet. Gastric ulcers were significantly inhibited in mice that were placed on a cinnamon diet for 4 weeks compared with the control group ($p < 0.01$). Consequently, a cinnamon powder dose of 100 mg/g over a 4-week period was used for subsequent experiments.

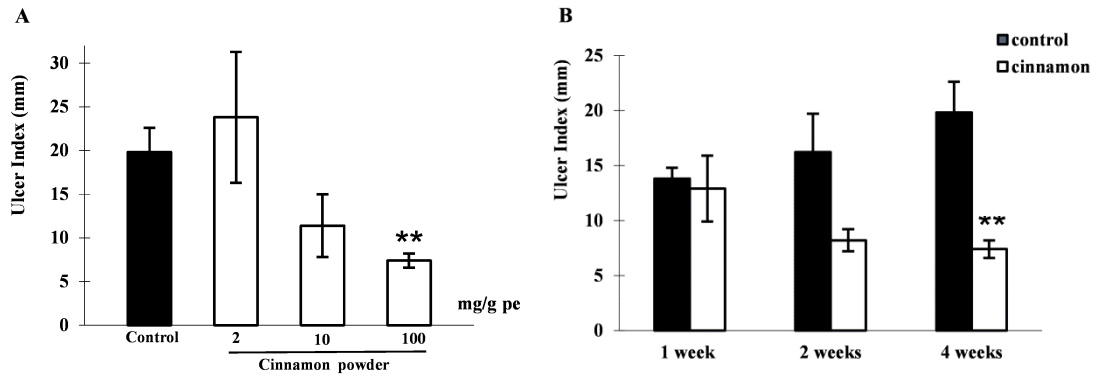

Fig. 1 Optimization of cinnamon powder diet dose and dosing period for preventing gastric ulcers induced by water immersion stress. **a**, Graph showing dose dependency of cinnamon powder after 4-week administration. Data are presented as mean ± SEM values of 10 mice. Statistical differences between cinnamon (*light bars*) versus the control group (*dark bars*) were calculated by ANOVA, followed by Tukey-Kramer's multiple comparison test. **b**, Graph showing effective dose period of cinnamon powder diet (100 mg/g per feed) over 1-, 2- and 4-week periods. Statistical differences versus the control group were calculated by the Student's *t*-test. * $P < 0.05$ ** $P < 0.01$ versus Control

Effect of 4-week cinnamon powder diet on ulceration by water immersion stress in mice

Humans are continually being exposed to different stressful conditions, and stress is known to aggravate gastric ulcer disease. Hence we investigated the effects of regular ingestion of cinnamon powder on stress-induced gastric ulcer. Permission to carry out this experiment was obtained from the committee regulating the ethical use of animals for scientific research in Kyoto University. The effect of a 4-week administration of cinnamon powder on water immersion stress-induced ulceration in mice is shown in Fig. 2. The mean ulcer index of mice in the cinnamon treated group was significantly lower (8 mm) compared with the control group (14 mm; $p < 0.01$), indicating that regular ingestion of cinnamon powder may

inhibit stress induced ulcers. Physiological stress is thought to increase surface cell loss from fundic mucosa that is accompanied by a depression in epithelial proliferation [7].

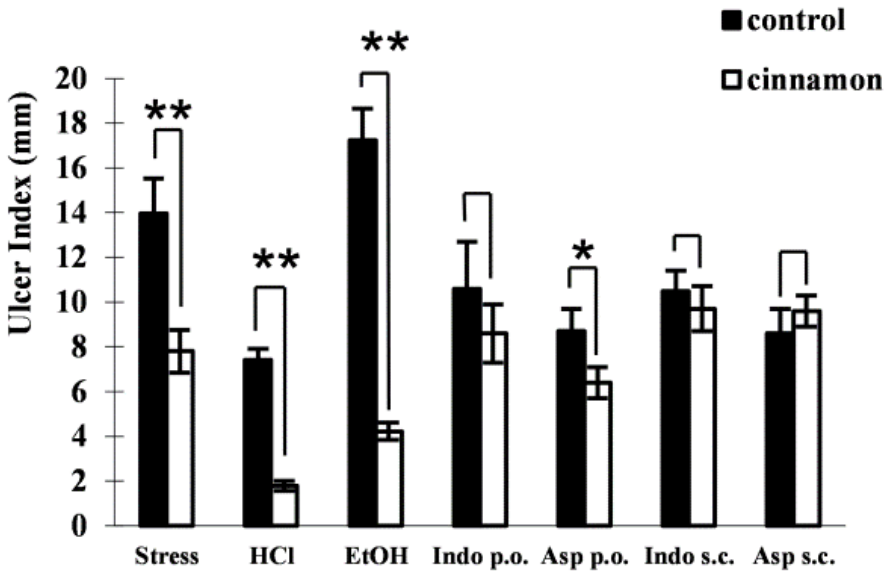

Fig. 2 Graph showing effect of a 4-week cinnamon powder diet on gastric ulcers caused by water immersion stress (Stress), administration of HCl or ethanol (EtOH), oral administration of indomethacin (Indo p.o.) or aspirin (Asp p.o.), or subcutaneous administration of indomethacin (Indo s.c.) or aspirin (Asp s.c.). Data are presented as mean ± SEM values of 10 mice. Statistical differences between cinnamon (*light bars*) versus the control group (*dark bars*) were calculated by the Student's *t* test. $^{*}P < 0.05$ $^{**}P < 0.01$ versus Control

Effect of 4-week cinnamon powder diet on ulceration by oral administration of ethanol and HCl in mice

It is well established that intragastric administration of noxious agents such as ethanol causes acute hemorrhagic erosion of the gastric mucosa in humans and other animals [11]. In the present study, mice ulcerated by oral administration of absolute ethanol or 0.6 M HCl had significantly lower ulcer index values (4.2 mm) than control groups if they were treated with cinnamon powder (17.3 mm; $p < 0.01$; Fig. 2). These values compare with the mean ulcer index value in the HCl-induced ulcer group of mice fed a cinnamon diet of 1.8 mm versus the control 7.4 mm ($p < 0.01$). HCl and ethanol are known to cause gastric ulcers by imparting direct physical damage to the gastric mucosa and eroding the mucosal layer [11]. The ethanol-induced ulcer model afforded a more visual and distinct ulcerogenesis than control groups, and a greater percentage protection against gastric ulcers following the cinnamon diet than the HCl-induced ulcer model.

Effect of 4-week cinnamon powder diet on ulceration by NSAIDs in mice

As shown in Fig. 2, when ulcerogenesis was induced by oral administration of aspirin, a 4 week administration of cinnamon powder significantly protected mice against gastric ulcers ($p < 0.05$). Mice that were fed the cinnamon diet and underwent oral aspirin ulceration had a mean ulcer index value of 6.4 mm (26.45 % protection) while the control group had a mean ulcer index value of 8.7 mm. However, the 4-week administration of cinnamon powder did not protect mice against gastric ulcers induced by oral administration of indomethacin. The mean ulcer index value of mice that were fed the cinnamon diet and underwent oral indomethacin ulceration was 8.6 mm (18.87 % protection) which was not statistically

different from the control group with a mean ulcer index value of 10.6 mm. No significant differences in ulcer index values were observed with ulceration induced by the subcutaneous administration of either indomethacin or aspirin in the cinnamon-treatment groups compared with the control groups (Fig. 2).

Effect of 4-week cinnamon diet on gastric mucosa thickness

The effect of the 4-week cinnamon powder diet on the thickness of mice gastric mucosa was next investigated (Supplementary Figure 1). The cinnamon treated group of mice tended to have a thicker gastric mucosa compared with the control group. Statistical analysis was carried out using the student's *t* test to compare the mean thickness of the mucus layer of the cinnamon treated group with that of the control group. It was found that, the mean gastric mucosa thickness of the 4-week cinnamon-treated group (287.76 ± 9.162 µm) was significantly greater than that of the control group, which had an average thickness of 235.265 ± 7.717 µm.

Effect of EACC and fractions on ulceration by ethanol in mice

The 4-week administration of EACC (10 mg/g and 20 mg/g) to mice caused a significant dose-dependent decrease in ulcer index values compared to control groups (Fig. 3). Fractionation of EACC yielded 7.65 g of fraction 1 (a thick oily greenish yellow liquid), 0.58 g of fraction 2 (green amorphous powder), and 2.86 g of fraction 3 (a fibrous brownish semi-solid mass). The 4-week administration of fraction 1 (84 mg/g) and fraction 2 (6 mg/g) resulted in significant gastroprotective activity compared with the control group, suggesting that active gastroprotective constituents are contained in ethylacetate fractions 1 and 2. The

mean ulcer index values of mice in the different treatment groups were 8.6 mm for the EACC 10 mg/g group, 6.3 mm for the EACC 20 mg/g group, 10.5 mm for fraction 1, 11.7 mm for fraction 2, 17.3 mm for the sucralfate-treated group, and 8.5 mm for the control group. However, the percentage of gastroprotection afforded by whole cinnamon powder (75.7 %) was higher than that of either EACC 10 mg/g (50.3 %), EACC 20 mg/g (63.5 %), fraction 1 (39.3 %), or fraction 2 (32.4 %). The mean ulcer index of fraction 1, 2 and 3 treated groups were all significantly greater than that of the cinnamon powder treated group (Fig. 3).

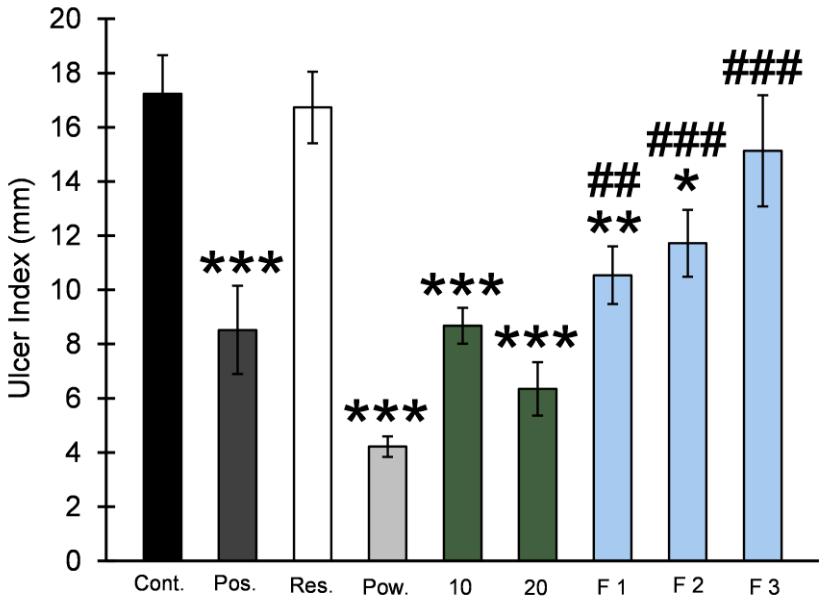

Fig. 3 Graph showing the effects of EACC and fractions on ethanol-induced ulceration in mice. Represented treatment groups were control (Cont.), positive control (Pos.), cinnamon residue (Res.), cinnamon powder (Pow.), 10 mg/g cinnamon extract (10), 20 mg/g cinnamon extract (20), fraction 1 (F1), fraction 2 (F2), and Fraction 3 (F3). Data are presented as mean ± SEM values of 10 mice. Statistical differences versus the control group were calculated by ANOVA, followed by Bonferroni's multiple comparison test. *** $P < 0.001$, ** $P < 0.01$, * $P > 0.05$ versus Control. ### $P < 0.001$, ## $P < 0.01$ versus Cinnamon powder

Chemical composition of EACC and fractions

Phytochemical analysis of EACC by HPLC revealed that it consisted of CA, cinnamyl alcohol, cinnamyl acetate, coumarin and other unidentified compounds. GC/MS analysis of fraction 1 revealed that it contained mainly CA with some copaene. HPLC analysis of fraction 2 showed that it consisted mainly of CA and its alcohol, which was confirmed by H-

NMR and C-NMR analyses after purification (Supplementary Figure 2). These results suggest that CA might play a role in the gastroprotective benefits of a cinnamon diet

Effect of CA on ulceration by water immersion stress

CA was administered to mice at doses of 0.98 mg and 9.8 mg/g of feed. CA was found to be significantly effective in preventing gastric ulcers at both doses of 0.98 mg and 9.8 mg/g of feed compared to the control group at $p < 0.05$ (Fig. 4), which provided further evidence for the beneficial role of CA in the gastroprotective activity of a cinnamon powder diet.

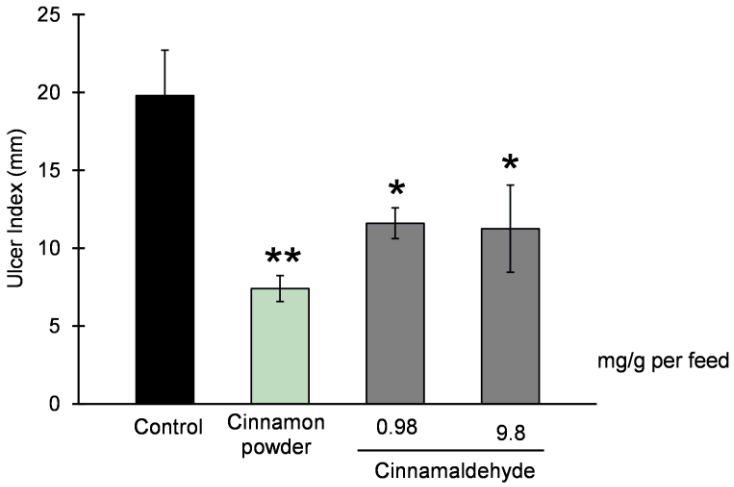

Fig. 4 Graph showing effect of cinnamaldehyde on ulceration induced by water immersion stress in mice. Represented treatment groups were control, cinnamon powder, 0.98 mg/g and 9.8 mg/g cinnamaldehyde. Data are presented as mean ± SEM values of 10 mice. Statistical differences versus the control group were calculated by ANOVA, followed by Dunnett's and Tukey-Kramer's multiple comparison tests. ** $P < 0.01$, $^{*}P < 0.05$ versus control

Discussion

The present study revealed that cinnamon powder exhibits potent gastroprotective activity in mice when consumed over a long period. The anti-ulcerogenic potential of a single dose oral administration of the aqueous extract of cinnamon powder has previously been reported [5, 6, 12]. However, the gastroprotective benefits of cinnamon powder administered in the form in which it is used in herbal medicine and as a spice is demonstrated here for the first time.

In the present study, ulcerogenesis was carried out using different models. A cinnamon diet clearly conferred effective protection against gastric ulcers induced by stress, ethanol or HCl. These models of ulceration induce gastric ulcer by a mechanism related to direct physical damage on the gastric mucosa thus suggesting a cytoprotective benefit of the cinnamon diet. The effects of the cinnamon diet on gastric ulcers caused by NSAIDs were also investigated. NSAIDs are thought to induce gastric damage by the nonspecific inhibition of cyclooxygenase (COX) and by markedly decreasing mucosal prostaglandin levels [13–18]. Cinnamon diet did not attenuate ulceration induced by subcutaneous injection of indomethacin or aspirin, but reduced the severity of gastric ulcers caused by oral administration of indomethacin although this effect was not significant. On the other hand, cinnamon diet significantly attenuated gastric ulcer caused by oral administration of aspirin. This effect was however weaker than that achieved against necrotizing agents. These results indicate that the efficacy of cinnamon diet against NSAIDs-induced gastric ulcers seems to be limited. It is thought that oral administration of aspirin might function as a mucosal barrier breaker rather than just as a COX inhibitor. This might partly explain why the gastroprotection against ulceration by oral administration of aspirin was somewhat effective. Results of histological examination revealed that the 4-week cinnamon diet appeared to increase the thickness of the gastric mucosa. However, it is unclear whether the thickening

of the gastric mucosa is as a result of increase in mucus secretion or whether this effect is directly associated with the gastroprotective benefit of cinnamon diet. We speculate that prostaglandin mediation might be involved in the gastroprotective activity of cinnamon diet but likely plays only a minor role. Other mechanisms different from prostaglandin synthesis might also be pertinent to the gastroprotective activity of cinnamon diet. These might include inhibition of gastric acid secretion, enhancement of gastric mucus secretion, mucosal blood flow increase, antioxidant activity, afferent sensory nerve stimulation or HSP gene expression [19–26]. Further studies are required to clarify this.

We identified cinnamaldehyde as an active component of the gastroprotective benefits of a cinnamon diet. This finding is consistent with that of Harada *et al*. [27] who reported that cinnamaldehyde inhibited ulcerogenesis induced by water immersion stress. Shaik and colleagues also found that cinnamaldehyde had antiulcerogenic potential [28]. However, our present work showed that cinnamon powder exhibited greater gastroprotection than either EACC or the active fractions, leading us to surmise that cinnamaldehyde may not be the sole active compound responsible for the gastroprotection conferred by a cinnamon diet. Tanaka *et al*. [6] reported that 3-(2-hydroxyphenyl)-propanoic acid and its O-glucoside isolated from the aqueous extract of cinnamon had an anti-ulcerogenic effect on serotonin-induced gastric ulcers in rats. However, they suggested that, unlike the active compounds, the aqueous extract showed multiple anti-ulcerogenic effects that were caused by more than two active components with different pharmacological effects. Eugenol, another compound present in cinnamon has also been shown to possess anti-ulcerogenic potential [29]. Taken together, these results suggest that a synergistic activity between active compounds is likely to account for the gastroprotective activity of cinnamon powder. Indeed, this is in agreement with the definition of ‘active ingredients’ as described in the guidelines of natural medicine issued by World Health Organization (Fact Sheet number 134).

We conclude that regular ingestion of cinnamon powder may offer gastroprotective benefits via cytoprotection, which validates its use in Kampo medicine prescriptions for stomach problems. Our work and that of others suggests that cinnamon powder may be useful as an alternative therapy in the management of recurrent or non-*H. pylori* gastric ulcers. However, the efficacy against NSAIDs induced gastric ulcers appears to be limited.

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Chapter II

Behavioral effects of inhaled essential oils from two spices from Cameroon

Section I

Inhalation of the Essential Oil of *Piper guineense* from Cameroon Shows Sedative and Anxiolytic-Like Effects in Mice

Inhalation of the Essential Oil of *Piper guineense* from Cameroon Shows Sedative and Anxiolytic-Like Effects in Mice

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The aromatherapeutical potential of *Piper guineense* essential oil was investigated in mice *via* inhalation administration, and the active compounds were identified. An open field test and light/dark transition test were used to evaluate the sedative and anxiolytic activities of this essential oil, respectively. *P. guineense* essential oil showed significant sedative activity at an effective dose of 4.0×10^{-5} mg per cage compared to the control group. It also showed potent anxiolytic effect at a dose of 4.0×10^{-6} mg per cage. The main compounds of *P. guineense* essential oil were linalool (41.8%) and 3,5-dimethoxytoluene (10.9%). These two main compounds were shown to play a major role in the sedative activity of *P. guineense* essential oil. These results suggest that inhalation of *P. guineense* essential oil might induce a mild tranquilizing effect.

Key words *Piper guineense*; inhalation; sedative; linalool; 3,5-dimethoxytoluene

Essential oils (EOs) are gaining considerable recognition in complementary therapies for the treatment of several mental illnesses such as bipolar disorder, attention deficit hyperactivity disorder, anxiety, and depression. EOs have been used for massage, inhalation, and skin application and are considered a holistic complementary therapy to increase comfort and reduce stress.[1,2] Fragrance inhalation reportedly induces sedative or stimulative effects on brain function in humans.[1,3–6] The first drugs used to treat diseases of the central nervous system (CNS) were based on natural sources, specifically plants.[7] Due to the adverse effects encountered with the use of many conventional anxiolytic drugs,[8] plants with molecules that produce CNS effects are attractive targets for the development of new drugs.[7]

In Africa, phytotherapy still plays an important role in the management of diseases, especially among populations with very low incomes.[9] *Piper guineense* Schum. & Thonn. (Piperaceae), a forest liana with gnarled branchlets spiraling up to shrubs of approximately 10 m, is native to Africa and indigenous to Cameroon.[10] The small spherical fruit, which is known as "bush pepper" in Cameroon, is a popular spice sold in local markets. It is used mainly as a condiment; however, the fruits, leaves, and roots of *P. guineense* have also found diverse medicinal uses in African traditional medicine. They are used to treat convulsion, rheumatism, respiratory diseases, gastrointestinal diseases, and venereal diseases and for uterine muscle stimulation.[10–14] An aqueous extract of *P. guineense* fruits reportedly exhibits an anticonvulsant effect[15–17]; however, it is not known whether the essential oil of *P. guineense* (PGEO) shows any behavioral effect. Anticonvulsant agents are known to have a suppressing effect on the CNS.[8] Based on the reported anticonvulsant activity of *P. guineense* fruit, and on our results of preliminary screening of the essential oils of selected aromatic plants from Cameroon for their sedative effects, PGEO was selected as a potential sedative agent. In this study, the aromatherapeutical potential of PGEO from Cameroon was investigated *via* inhalation, and the chemical constituents and active compounds responsible for its activity were identified.

The authors declare no conflict of interest.

MATERIALS AND METHODS

Animal Care Four-week-old male ddY mice (20–30 g) purchased from Japan SLC (Shizuoka, Japan) were used for this study. They were kept under an ambient temperature of 25±2°C and a relative humidity of 50–60% with a light–dark cycle of 12 h. The animals were fed pellet chow and water *ad libitum*. The animal experiments were designed following the recommendations of the Animal Research Committee of Kyoto University, Kyoto, Japan (Approval number 2010–22). Experimental procedures involving animals and their care were conducted in conformity with institutional guidelines that complied with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan (2006). All experiments were conducted between 10:00–17:00 h under the same conditions.

Plant Materials Dried fruits of *P. guineense* were purchased from the Mfoundi market in Yaounde (central region, Cameroon) in May 2011. The vendor (Ngah Brigitte, shed number 11) obtained fruits of *P. guineense* (collected from the wild) from suppliers in the east and south regions of Cameroon (personal communication). A specimen of dried fruits of *P. guineense* (specimen number: EST-4977) was deposited in the herbarium of Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University.

Drugs and Reagents Diazepam (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and lavender oil (Nacalai Tesque Inc., Kyoto, Japan) were used as positive controls. Triethyl citrate (TEC; Merck, Darmstadt, Germany), a non-sedating odorless solvent, was used to dissolve the fragrant components. *R*-(−)-Linalool and 3,5-dimethoxytoluene were purchased from Nacalai Tesque and Wako, respectively. All chemicals used in this study were of the highest grade available.

Isolation of PGEO and Fractionation PGEO was prepared by hydrodistillation of dried fruits for 2 h using a Clevenger apparatus, as designated in the Japanese Pharmacopeia (JP XV). The oil was captured in hexane, dried with

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anhydrous sodium sulfate, concentrated and stored in sealed vials at 4°C until analysis. Fractionation of PGEO was carried out using silica gel column chromatography. The column was eluted with hexane–acetone (6:1) to give Fractions 1–4, and then washed with absolute acetone to give Fraction 5.

Behavioral Testing Apparatus. Open Field Test The sedative effect of PGEO was evaluated based on mouse spontaneous locomotor activity in an open field test. The open field test apparatus used has been described previously.[18–20] The open field consisted of a closed glass cage (W 60 cm×L 30 cm×H 34 cm). The samples were administered to the mice by inhalation. The doses administered are expressed as milligrams of PGEO in 400 μL TEC per cage. The administration procedure was as follows: 4 filter-paper discs were adhered to 4 corners of the inner walls of the glass cage. PGEO was charged on the filter paper discs and the cage was closed, so that the vapor pervaded the cage by natural diffusion. Sixty minutes after charging the sample, a mouse was placed in the center of the cage and monitored by a video camera for 60 min. The frequency that the mouse crossed the lines drawn on the floor of the cage (at 10 cm intervals) was counted every 5 min for 60 min. The area under the curve (*AUC*), which represented total locomotor activity, was then calculated.

Light/Dark Transition Test The light/dark box test is a widely used behavioral test for anxiolysis.[21] The light/dark transition apparatus consisted of 2 equally sized compartments; a light area (30 cm×L 30 cm×H 34 cm) illuminated by a 6.5 W desk LED lamp, and a dark area (30 cm×L 30 cm×H 34 cm) blackened with black plastic sheets. The two compartments were separated by a black wall with an aperture (small doorway) in its center (5 cm×5 cm) to allow passage from one compartment to the other. PGEO was charged in both compartments for 60 min in accordance with the open field test. Thereafter, a mouse was placed in the center of the lit area facing the tunnel and the following parameters were recorded using a video camera during a 15 min test period: (1) latency time for the first crossing to the dark compartment; (2) the number of crossings between the light and dark compartments; and (3) the total time spent in the illuminated area. A mouse was considered to have entered the new area when all 4 legs crossed the threshold of the compartment. Diazepam was used as a positive control. The dose of diazepam administered was determined based on literature reports[21] and on observations made in a preliminary experiment.

Qualitative and Quantitative Analyses of PGEO Qualitative analysis of PGEO was carried out on an Agilent 6850 series gas chromatograph connected to an MSD 5975 with the following operation conditions: column: fused silica capillary column, DB-wax (HP), 60 m×0.25 mm×0.25 μm; column temperature program: 90–190°C, increasing at a rate of 2°C/min, holding at 90°C for 2 min and at 190°C for 10 min; injector temperature: 110°C; carrier gas: helium, 25 cm/s; column head pressure: 100 kPa; ionization energy: 70 eV; injection volume: 1.0 μL. Quantitative analysis was carried out on a Hitachi G-5000 equipped with a flame ionization detector (FID) with the following conditions: column: fused silica capillary column, TC-wax (HP), 60 m×0.25 mm×0.25 μm, (CP-Chirasil-Dex CB, 25 m×0.25 mm×0.25 μm for chiral analysis); column temperature: same as GC/MS; injector: 180°C, detector: 200°C, FID; carrier gas: helium, 0.8 mL/min; split ratio: 29:1; column head pressure: 200 kPa; injection volume: 1 μL. The linear retention indices of the constituents were determined using a series of *n*-alkanes as standards. The chemical compounds were identified by use of NIST 2 and flavors libraries and the identities of most compounds were confirmed by comparison of their retention indices and mass spectra with those of reference standards or published data.[22]

Statistical Analysis Data are expressed as the mean± standard error of the mean. Statistical analyses were performed using Student's *t*-test or one-way analysis of the variance (ANOVA) followed by Dunnett's test using GraphPad Instat (GraphPad Software, San Diego, CA, U.S.A.). A probability level of $p<0.05$ was considered to be statistically significant.

RESULTS

Phytochemical Analysis of PGEO The dried fruits of *P. guineense* afforded 0.2% (w/w) EO with a greenish color and sharp characteristic odor. Table 1 shows the 21 identified constituents listed in their order of elution from the DB-wax column. Linalool (41.8%) and 3,5-dimethoxytoluene (10.9%) were found to be the principal constituents of PGEO. Stereochemical characterization of linalool in PGEO using GC equipped with a Chirasil-Dex column revealed the *R*-(−) enantiomer was predominant (abundance ratio of *R*-(−):*S*-(+) linalool was 4.37:1). Phytochemical analyses of the fractions of PGEO revealed that linalool was the main compound of Fractions 3 and 4 (66.3 and 39.9%, respectively) whereas 3,5-dimethoxytoluene was not detected in these fractions. Fraction 2 contained 3,5-dimethoxytoluene (45.3%) and linalool (30.4%) as the main compounds. Neither linalool nor 3,5-dimethoxytoluene was detected in Fraction 1.

Sedative Activity of PGEO Figure 1 shows the locomotor activity following the administration of PGEO *via* inhala-

Table 1. Chemical Composition of *Piper guineense* Essential Oil

Compound	RI[a]	Peak area (%)
α-Pinene	1041	1.8
Camphene	1094	4.8
β-Pinene	1134	9.2
β-Phellandrene	1139	2.3
3-Carene	1168	2.2
D-Limonene	1218	0.9
p-Cymene	1288	1.2
α-Copaene	1508	1.5
Camphor	1537	2
Linalool	1551	41.8
β-Elemene	1601	2.1
Caryophyllene	1611	3.6
Aromadendrene	1664	1.5
Isoborneol	1679	2.4
α-Humulene	1681	1.4
α-Terpineol	1704	4.1
γ-Elemene	1838	1.2
3,5-Dimethoxytoluene	1853	10.9
Safrole	1880	1.6
Caryophyllene oxide	1996	1.6
Elemol	2106	0.9
Guaiol	2107	1

a) Retention indices. Compounds listed in their order of elution from the DB-wax column.

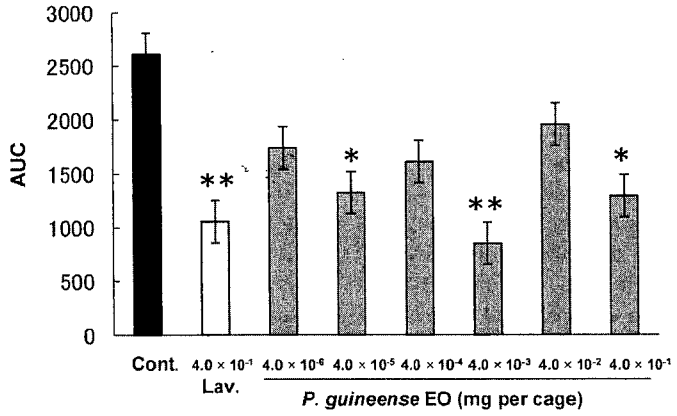

Fig. 1. Sedative Activity of *Piper guineense* Essential Oil

Total spontaneous locomotor activity of mice that received vehicle (triethyl citrate, 400 μL) and *Piper guineense* essential oil (4.0×10^{-6}–4.0×10^{-1} mg per cage; EO). Data are shown as the mean±standard error of the mean of 6 mice. Statistical differences *vs.* the control group (Cont.) were calculated using analysis of the variance followed by Dunnett's test. *$p<0.05$, **$p<0.01$. Lav.; lavender oil (4.0×10^{-1} mg per cage).

tion at doses ranging from 4.0×10^{-6} to 4.0×10^{-1} mg per cage. A hormetic biphasic dose response pattern, revealed as a u-shaped curve, was observed, in which efficacy was optimal at a concentration of 4.0×10^{-3} mg. PGEO administered at 4.0×10^{-5} and 4.0×10^{-1} mg also showed a significant decrease in locomotor activity. However, the most effective concentration was 4.0×10^{-3} mg, which showed a reduction in locomotor activity that was comparable to that of lavender oil.[23] With respect to additional behavioral observations made on excretion, grooming, and rearing (data not shown), the inhibition of locomotor activity induced by 4.0×10^{-1} mg PGEO was considered to be as a result of drug intoxication and not sedation. The *AUC* values of the 4.0×10^{-5} and 4.0×10^{-3} mg PGEO-treated groups were significantly smaller than the control values, and the decrease in locomotor activity produced by these concentrations was statistically significant ($p<0.05$ and $p<0.01$, respectively), suggesting a potential sedative effect of PGEO. The non-linear hormetic dose response pattern is known to be quite predominant in anxiolytic drug screening tests. It is partially explained by the phenomenon that an agonist may bind to two subtypes of receptors, with one activating a stimulatory pathway while the other activates an inhibitory one.[24] Nevertheless, the doses administered in this study are only given as the concentration of samples administered per cage. Due to the low concentration of the compounds and the simplicity of our apparatus, it was not feasible to measure the true concentration of compounds in the vapor phase that saturated the cage. However, in a previous study,[18] headspace measurement of compounds in the vapor phase (using an SPME/GCMS technique) revealed that the dose administered correlates positively with the amount of compound in the vapor state.

Anxiolytic-Like Activity of PGEO In the light/dark transition test, anxiolytic-like activity is represented by an increased duration in the light area and increased movement between the two compartments.[21] Diazepam, dissolved in corn oil (0.5 mg/kg) and administered intraperitoneally at 30 min prior to testing, significantly increased the total time spent in the light area and the number of transitions between both compartments compared to the vehicle (corn oil). This confirmed that the experimental apparatus was valid. The administration of PGEO at a concentration of 4.0×10^{-6} mg per cage significantly increased the total time spent in the light area as well as the number of transitions between the light and dark compartments (Figs. 2A, B). This suggested that PGEO might induce an anxiolytic-like effect, thus confirming its tranquilizing property. The administration of PGEO did not cause a significant change in the latency to enter the dark compartment.

Effects of PGEO Fractions on Mouse Locomotor Activity Fractions 1–4 of PGEO were administered individually to mice by inhalation at doses ranging from 4.0×10^{-6} to 4.0×10^{-2} mg per cage. Fractions 2, 3, and 4 induced a significant decrease in locomotor activity and the strongest effect was observed at a concentration of 4.0×10^{-5} mg (Figs. 3A–C). Fraction 2 was more potent than Fractions 3 and 4. Fraction 1 did not induce a significant decrease in locomotor activity compared to the control group. This suggested that Fractions 2, 3, and 4 contained the active ingredients of PGEO; thus, these fractions were analyzed for their chemical composition by GC/MS. As described earlier, the main compound of Fraction 2 was 3,5-dimethoxytoluene, while that of Fractions 3 and 4 was *R*-(−)-linalool. The effects of *R*-(−)-linalool and 3,5-dimethoxytoluene on motor activity were further examined to confirm their role in the sedative effect of PGEO.

Effects of *R*-(−)-Linalool and 3,5-Dimethoxytoluene on Mouse Locomotor Activity Figures 4A and 4B show the effect of the main compounds identified from the PGEO active fractions on locomotor activity. *R*-(−)-Linalool was administered at concentrations ranging from 4.0×10^{-6} to 4.0×10^{-1} mg per cage. It significantly decreased the locomotor activity of mice at concentrations of 4.0×10^{-5} and 4.0×10^{-3} mg, with the 4.0×10^{-5} mg dose being the most potent. 3,5-Dimethoxytoluene also significantly decreased the locomotor activity of mice at concentrations of 4.0×10^{-5} and 4.0×10^{-2} mg. The 4.0×10^{-1} mg dose caused abnormal actions such as excess excretion. A mixture of *R*-(−)-linalool and 3,5-dimethoxytoluene at a ratio of 3 : 2 (*i.e.*, the ratio of both compounds in Fraction 2) was evaluated to elucidate their interaction (Fig. 4C). This mixture was found to induce a significant decrease in locomotor activity at doses of 4.0×10^{-5} and 4.0×10^{-3} mg. Taken together, these results confirmed that *R*-(−)-linalool and 3,5-dimethoxytoluene might play a major role in the inhibition of locomotor activity of PGEO alongside the other minor constituents. The biphasic dose response pattern observed for the mixture of *R*-(−)-linalool and 3,5-dimethoxytoluene was

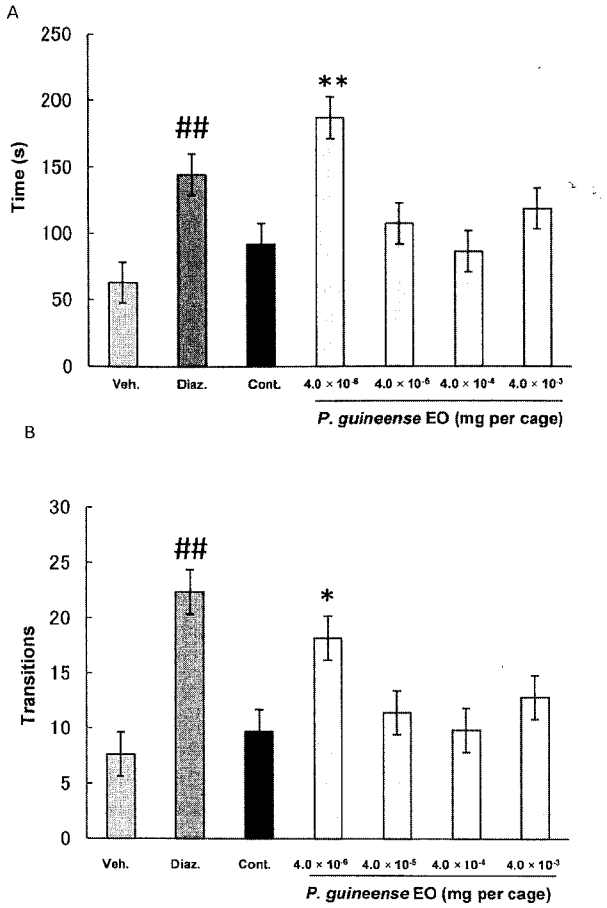

Fig. 2. Anxiolytic-Like Activity of *Piper guineense* Essential Oil

Anxiolytic-like activity of mice that received vehicle (corn oil; Veh.), diazepam (0.5 mg/kg; Diaz.), control (triethyl citrate 400 μL; Cont.), and *Piper guineense* essential oil (EO); A: time spent in the light area, B: number of transitions between the compartments. Data are shown as the mean±standard error of the mean of 10 mice. Statistical differences were calculated using analysis of the variance followed by Dunnett's test; $*p<0.05$, $**p<0.01$ compared to the control group (triethyl citrate), and Student's *t*-test, $^{\#\#}p<0.01$ compared to vehicle (corn oil).

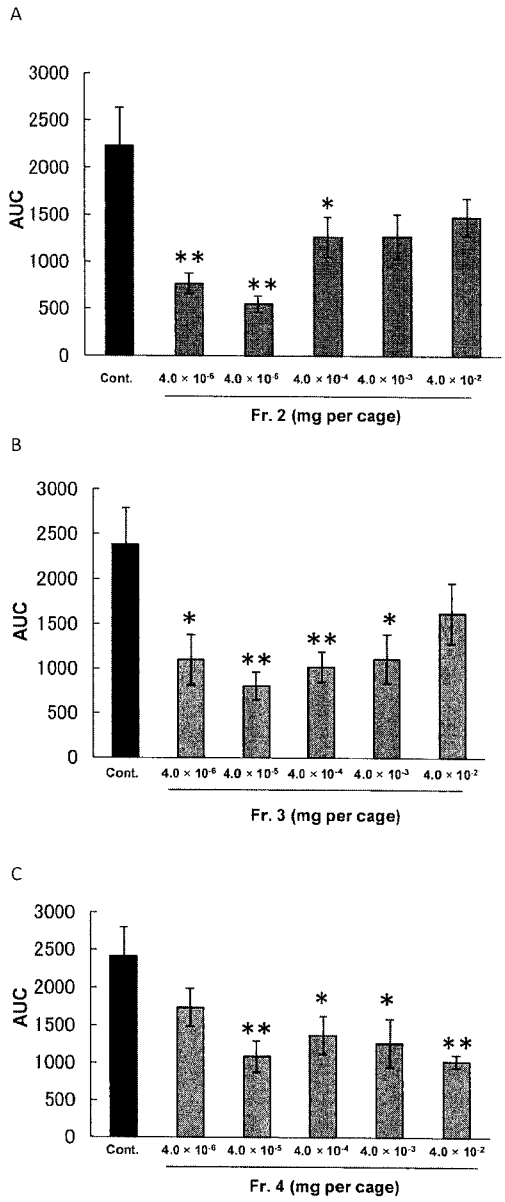

Fig. 3. Effects of PGEO Fractions on Mouse Locomotor Activity

Total spontaneous locomotor activity of mice treated with *Piper guineense* essential oil fractions; A: Fraction (Fr.) 2, B: Fr. 3, and C: Fr. 4. Data are shown as the mean±standard error of the mean of 6 mice. Statistical differences *vs.* the control group (Cont.) were calculated using analysis of the variance followed by Dunnett's test. $*p<0.05$, $**p<0.01$.

not observed with Fraction 2. This might have resulted from the influence of the other compounds present in Fraction 2 or from the difference in the purity of the *R*-(−)-linalool enantiomer.

DISCUSSION

This study revealed that PGEO from Cameroon exerts inhalative, sedative, and anxiolytic effects in mice. This tranquilizing effect upon inhalation of PGEO is being reported herein for the first time. The potency of PGEO was comparable to that of the EO of *Lavandula angustifolia*.[25]

The phytochemical composition of PGEO is known to be influenced by geographical and climatic conditions.[10] Several chemotypes have been reported, including the dillapiole, β-caryophyllene, β-pinene, and linalool types.[26] In Cameroon, the chemical composition of PGEO obtained from different regions revealed some differences in their main compounds. For example, Jirovetz *et al.*[26] reported β-caryophyllene as the main compound of PGEO obtained from the littoral region of Cameroon. Amvam Zollo *et al.*[27] reported a β-pinene type of PGEO obtained from the central region of Cameroon. Menut *et al.*[28] and Tchoumbounang *et al.*[10] also reported the β-pinene type of PGEO obtained from the west region of Cameroon. However, in this study, linalool was identified as the main compound of PGEO obtained from the east and south regions of Cameroon, with a yield of 41.8% w/w. This indicated that *P. guineense* originating from the east and south regions of Cameroon could offer a potential source of naturally occurring linalool. This variety of *P. guineense* might be a valuable resource for conservation, just like "poivre de penja" (an exotic variety of *P. nigrum* cultivated in a volcanic valley in the Moungo region of Cameroon, which is currently under intellectual property protection).[29]

Recent studies have revealed that stereochemistry influences the physiological effects of odorants.[30–32] Hoferl and colleagues[32] demonstrated that *R*-(−)-linalool, but not *S*-(+)-linalool, showed stress relieving effects on human subjects.

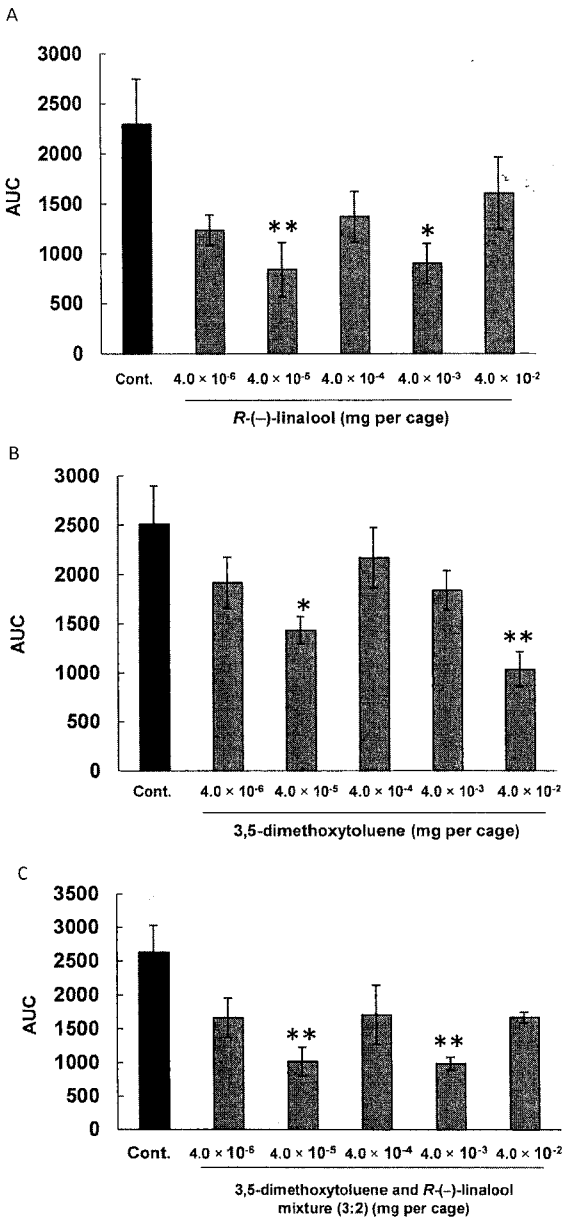

Fig. 4. Effects of *R*-(−)-Linalool and 3,5-Dimethoxytoluene on Mouse Locomotor Activity

Total spontaneous locomotor activity of mice treated with vehicle (triethyl citrate 400 μL; Cont.), *R*-(−)-linalool (4A), 3,5-dimethoxytoluene (4B), and a mixture of *R*-(−)-linalool and 3,5-dimethoxytoluene (4C). Data are shown as the mean±standard error of the mean of 6 mice. Statistical differences *vs.* the control group were calculated using analysis of the variance followed by Dunnett's test. $*p<0.05$, $**p<0.01$.

In this light, the stereochemical characterization of linalool in PGEO was performed and it was found to contain mainly the *R*-(−)-linalool enantiomer. Additionally, chemical composition analysis revealed that the phenolic methyl ester 3,5-dimethoxytoluene was the second main compound of PGEO, with a yield of 10.9%. This compound is abundant in family Rosaceae and is known to be the principal compound of the Chinese Rose, which is famous for its musky smell.[33] Nevertheless, in family Piperaceae, 3,5-dimethoxytoluene has only been reported to be present in *P. lenticellosum*.[34] Thus, our work presents the first report of the presence of 3,5-dimethoxytoluene in PGEO.

Wisanine, a piperidine-type alkaloid isolated from the roots of *P. guineense*, has been reported for its anti-aggressive, sedative, tranquilizing, and anticonvulsant activities.[35–37] In the present study, two active compounds that might play a major role in the sedative activity of PGEO were identified, namely, *R*-(−)-linalool and 3,5-dimethoxytoluene. Linalool is well known to exhibit CNS depressant activity.[38,39] However, 3,5-dimethoxytoluene has received very little scientific exploration, with the exception of a report by Nakamura *et al.*[40] on the alleged sedative effect of the fragrance. To the best of our knowledge, our work presents the first scientific evidence on the neuropharmacological effects of 3,5-dimethoxytoluene *via* inhalation in an animal behavioral experimental model. Furthermore, we investigated the pharmacological interaction of linalool and 3,5-dimethoxytoluene. We found that, although linalool and 3,5-dimethoxytoluene are quite potent sedative compounds, the potency of a mixture of these two compounds was not greater than when the compounds were administered singly or of the whole EO. This finding corroborates that of Nakamura *et al.*[40] who stated that "the sedative effect of 3,5-dimethoxytoluene is not preferable when its balance with other aromatic components is taken into consideration." β-Pinene, a monoterpene reported to possess potent sedative activity[41] was detected in substantial amount in PGEO (9.2%). It is possible that β-pinene might have contributed to the sedative effect of PGEO. Taken together, it is suggested that a synergistic interaction between the numerous compounds identified in PGEO might account for its sedative activity.

A sedative drug decreases activity, moderates excitement, and calms the recipient, whereas a hypnotic drug produces drowsiness and facilitates the onset and maintenance of a state of sleep that resembles the electroencephalographic characteristics of natural sleep.[8] Pentobarbital is a hypnotic agent that potentiates γ-aminobutyric acid (GABA)-mediated postsynaptic inhibition through the allosteric modification of $GABA_A$ receptors. Drugs that possess CNS depressant activity decrease the time for the onset of sleep, prolong the duration of sleep, or both.[42] PGEO was found to promote the onset of sleep, and it significantly reduced sleep latency compared to the control group. However, unlike chlorpromazine, it showed no effect on the elongation of pentobarbital-induced sleep, even at doses higher than the sedative dose (data not shown). These results indicate that PGEO might act as a mild tranquilizer, but not as a hypnotic agent. On the other hand, the results could also imply that PGEO might exert its sedative effects only partially *via* the GABAergic receptor system. Ashorobi and Akintoye[16] also made a similar observation. They found that an aqueous extract of *P. guineense* fruits showed anticonvulsant activity without prolonging ketamine-induced sleep, and they suggested that a non GABA-mediated pathway might be involved in the anticonvulsant effect of *P. guineense*. In addition, linalool reportedly exhibits its CNS depressant activity *via* a variety of mechanisms, among which are the glutamatergic and nicotinic receptors.[43,44] Further studies are required to elucidate the tranquilizing effects and mechanism of action of PGEO.

In conclusion, our study reveals that PGEO from the east and south regions of Cameroon possesses a potent inhalative tranquilizing effect. Two active compounds, namely, *R*-(−)-linalool and 3,5-dimethoxytoluene, were shown to play a major role in the inhalative sedative effect of PGEO. These results could be useful in the development of alternative therapies for the management of CNS-related conditions. In

addition, *P. guineense* can be easily obtained and exploited in a sustainable manner.

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Section II

Sedative, Anxiolytic and Antidepressant-like Effects of Inhalation of the Essential Oil of *Ocimum gratissimum* L. from Cameroon in Mice

ABSTRACT

In this study, the behavioral effects of inhalation of the essential oil of *Ocimum gratissimum* from Cameroon were investigated. The open field test, light/dark box test, tail suspension test and Rota-rod test were used to assess the sedative, anxiolytic-like, antidepressant-like and motor coordination effects respectively, in mice. GC and GC/MS analyses were performed to investigate the chemical composition of *O. gratissimum* essential oil. Phytochemical analysis revealed thymol (68%) as main compound of *O. gratissimum* essential oil. Inhalation of *O. gratissimum* essential oil showed potent sedative, anxiolytic and antidepressant-like effects in mice, and did not cause any deleterious effects on motor coordination. It is suggested that a synergistic effect of the constituents of *O. gratissimum* essential oil might account for its sedative activity. In conclusion, inhalation of *O. gratissimum* essential oil might have aromatherapeutic potential.

Keywords: Sedative, Anxiolytic, Antidepressant, Inhalation, *Ocimum gratissimum***,** Thymol

1. Introduction

According to the World Health Organization (WHO) [1] "mental health is as important as physical health to the overall well-being of individuals, societies and countries. Yet only a small minority of the 450 million people suffering from a mental or behavioural disorder is receiving treatment". WHO [2] also reported that 80% of the world's population used natural remedies and traditional medicines for the treatment of their ailments. As such, it is very important to establish scientific validation of the effectiveness of these natural remedies. Aromatherapy is continuously gaining recognition as an alternative/conventional therapy for the management of several mental disorders especially in the US, UK, France and Germany [3]. Essential oils and fragrance compounds have been reported to elicit pharmacological effects on the CNS in experimental animals and humans [4-9]. Despite the widespread use of inhaled essential oils in aromatherapy for the treatment of anxiety, depression, insomnia, mental exhaustion etc, experimental data on psychopharmacological properties of inhaled essential oils are surprisingly scarce [4, 10].

In the search for new therapeutic products for the treatment of neurological or psychiatric disorders, medicinal plant research worldwide has demonstrated the pharmacological effectiveness of different plant species [11, 12]. The African continent and Cameroon in particular holds an enormous resource in terms of floral biodiversity and its medicinal plants have remained a main reservoir of phytochemicals for pharmaceutical drug development [13-16]. However, documentation and scientific validation of the medicinal potentials of plants commonly used in traditional medicine is lacking [13, 15].

O. gratissimum (lamiaceae) is an aromatic medicinal plant found in the wild or cultivated throughout the tropics and subtropics. In West Africa, it is commonly found around village huts and gardens and cultivated for medicinal and culinary purposes [17]. It is commonly known as "masepo" in Cameroon and is used for flavouring a local well known dark fish

sauce called “Bongo Tjobi” [18]. *O. gratissimum* is used in African Traditional Medicine for the treatment of several diseases including epilepsy, fever, diarrhea, mental illness, fungal infections, cold and convulsion [19]. Leaf extracts of *O. gratissimum* [17] and the essential oil administered orally [20-22] have been reported to show anticonvulsant, anxiolytic, CNS depressant, and antinociceptive activities respectively. However, there are no reports on the aromatherapeutical effects of *O. gratissimum* essential oil. In this study, psychopharmacological effects of inhalation of the essential oil of *O. gratissimum* from Cameroon were investigated, and the chemical constituents and active compounds responsible for its activity were identified. The anxiolytic activity and antidepressant-like activities were also evaluated.

2. Materials and methods

2.1 Animal care

Four-week-old male ddY mice (20-30 g) purchased from Japan SLC (Shizuoka, Japan) were used for this study. They were kept under an ambient temperature of 25 ± 2°C and a relative humidity of 50–60% with a light-dark cycle of 12 h. The animals were fed pellet chow and water *ad libitum*. All animal experiments were designed following the recommendations of the Animal Research Committee of Kyoto University, Kyoto, Japan (Approval numbers 2011-19, 2012-18, 2013-17). Experimental procedures involving animals and their care were conducted in conformity with institutional guidelines that complied with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan (2006). All experiments were conducted between 10:00–17:00 h under the same conditions.

2.2 Plant materials

Dried aerial parts of *O. gratissimum* were used for this study. The plant material originated from the wild in Batibo, Northwest region Cameroon. A voucher specimen of *O. gratissimum* (specimen number: EST-4975) was deposited in the herbarium of Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University.

2.3 Drugs and reagents

Benzylacetone (Tokyo Kasei), Diazepam (Wako Pure Chemical Industries Ltd., Osaka, Japan), and Fluoxetine (Nacalai Tesque Inc., Kyoto, Japan) were used as positive controls. Thymol was purchased from Nacalai Tesque. Triethyl citrate (TEC; Merck, Darmstadt, Germany), a non-sedating odorless solvent, was used to dissolve the essential oil and fragrant compounds. All chemicals used in this study were of the highest grade available.

2.4 Isolation of OGEO and fractionation

The essential oil of *O. gratissimum* (OGEO) was prepared by hydrodistillation of dried aerial parts for 2 h using a Clevenger apparatus, as designated in the Japanese Pharmacopeia (JP XVI). The oil was captured in hexane, dried with anhydrous sodium sulfate and concentrated. Headspace of the oil was analysed by solid phase micro extraction (SPME) and gas chromatography/mass spectrometry (GC/MS) to confirm that the oil was void of substantial amount of hexane. The pure essential oil obtained was stored in sealed vials at 4 °C until analysis. Fractionation of OGEO was carried out using preparative thin layer chromatography (TLC). The TLC plates were developed with petroleum ether:acetone (5:1) and partitioned to obtain fractions 1–3. Fractions 1 and 3 were evaluated for their biological activity.

2.5 Behavioral testing apparatus

2.5.1 Open field test

The sedative effect of OGEO was evaluated based on mouse spontaneous locomotor activity in an open field test. The open field test apparatus used was described previously [23]. The doses administered are expressed as milligrams of OGEO in 400 μL TEC per cage. The administration procedure was as follows: 4 filter-paper discs were adhered to 4 corners of the inner walls of the glass cage. OGEO was charged on the filter paper discs and the cage was closed, so that the vapor pervaded the cage by natural diffusion. Sixty minutes after charging the sample, a mouse was placed in the center of the cage and monitored by a video camera for 60 min. The frequency that the mouse crossed the lines drawn on the floor of the cage (at 10 cm intervals) was counted every 5 min for 60 min. The area under the curve (AUC) of locomotor activity counts per 5 min (X-axis) and time (Y-axis) which represented total spontaneous locomotor activity was then calculated. Benzylacetone, a fragrant compound which had been previously reported to show a sedative effect upon inhalation was used as positive control [23, 24]. Benzylacetone was administered by inhalation at a dose of 4.0×10^{-3} mg per cage.

2.5.2 Light/dark transition test

The light/dark box test is a widely used behavioral test for evaluating the effect of anxiolytic agents [25]. The light/dark transition apparatus consisted of 2 equally sized compartments; a light area (30 cm × L 30 cm × H 34 cm) illuminated by a 6.5 W desk LED lamp, and a dark area (30 cm × L 30 cm × H 34 cm) blackened with black plastic sheets. The two compartments were separated by a black wall with an aperture (small doorway) in its center (5 cm × 5 cm) to allow passage from one compartment to the other. OGEO was charged in both compartments for 60 min in accordance with the open field test. Thereafter, a mouse was

placed in the center of the lit area facing the tunnel and the following parameters were recorded using a video camera during a 15 min test period: (1) the number of crossings between the light and dark compartments; and (2) the total time spent in the illuminated area. A mouse was considered to have entered the new area when all 4 legs crossed the threshold of the compartment. Diazepam was used as a positive control [26].

2.5.3 Tail suspension Test

The tail suspension test is a widely used behavioral model for testing the effect of antidepressant agents [27]. Tail suspension test was carried out by a method described by Steru and colleagues (1985) [28]. Mice were suspended from the edge of a table (63 cm high) by an adhesive tape placed approximately 1 cm from the tip of the tail. The mice were considered immobile when they stopped to make any struggling movements and hung passively. Immobility time was recorded for a period of 6 min. A reference compound fluoxetine was administered at a dose of 20 mg/kg p.o 1 h prior to testing, to confirm the validity of the apparatus. Fluoxetine was dissolved in physiological saline (vehicle) and administered at an injection volume of 10 ml/kg. The dose of fluoxetine administered was determined based on literature report [29] and on observations made in a preliminary experiment. OGEO was administered to mice by inhalation in the open field arena for 30 min before the tail suspension test.

2.5.4 Rota-rod Test

The effect of active doses of OGEO on motor coordination was assessed using a Rota-rod Treadmill 660C (Muromachi Kikai CO; LTD), 5 cm diameter, 20 cm high, at accelerating speed (40 rpm; 5 mins). The latency to fall from the rotating treadmill was recorded for 5 min.

OGEO was administered to mice by inhalation in the open field arena for 30 min before the Rota-rod test.

2.5.5 Qualitative and quantitative analyses of OGEO

Qualitative analysis of OGEO was carried out on an Agilent 6850 series gas chromatograph connected to an MSD 5975 with the following operation conditions: column: fused silica capillary column, DB-wax (HP), 60 m × 0.25 mm × 0.25 µm; column temperature program: 40–200 °C, increasing at a rate of 4 °C/min, holding at 40 °C for 2 min and at 190 °C for 20 min; injector temperature: 110 °C; carrier gas: helium, 25 cm/s; column head pressure: 100 kPa; ionization energy: 70 eV; injection volume: 1.0 µL. Quantitative analysis was carried out on a Hitachi G-5000 equipped with a flame ionization detector with the following conditions: column: fused silica capillary column, TC-wax (HP), 60 m × 0.25 mm × 0.25 µm; column temperature program: same as GC/MS; injector: 180 °C, detector: 200 °C, FID; carrier gas: helium, 0.8 mL/min; split ratio: 100:1; column head pressure: 200 kPa; injection volume: 1 µL. The linear retention indices of the constituents were determined using a series of *n*-alkanes as standards. The chemical compounds were identified by use of NIST 2 and flavors libraries and the identities of most compounds were confirmed by comparison of their retention indices and mass spectra with those of reference standards or published data [30].

2.5.6 Statistical analysis

Data are expressed as the mean ± standard error of the mean. Statistical analyses were performed using Student's *t*-test or one way analysis of the variance (ANOVA) followed by Dunnett's test using GraphPad Instat (GraphPad Software, San Diego, CA, USA). A probability level of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Phytochemical analysis of OGEO

The dried aerial parts of *O. gratissimum* afforded 0.7% (w/w) EO with an amber color and sharp thyme-like odor. Table 1 shows the 22 identified constituents listed in their order of elution from the DB-wax column. Thymol was found to be the principal constituent of OGEO (68.0%). Phytochemical analyses of the fractions of OGEO revealed that thymol was the main component of Fraction 3 (95.2%). The main components of Fraction 1 were β-selinene, γ-terpinene, *trans*-caryophyllene and *p*-cymene (31.0, 28.4, 14.8 and 9.7%, respectively), whereas thymol was not detected in this fraction.

Table 1: Phytochemical constituents of *Ocimum gratissimum* essential oil

Compound	RI	Peak area %
α-pinene	1032	trace
myrcene	1168	0.5
α-terpinene	1188	0.5
γ-terpinene	1222	3.0
p-cymene	1316	5.9
dihydro-*p*-cymene	1449	0.5
1-octen-3-ol	1457	0.8
α-copaene	1506	0.5
linalool	1552	0.6
terpinene-4-ol	1619	4.3
trans-caryophyllene	1619	2.6
o-cresol	1663	0.6
α-humulene	1690	0.4
α-terpineol	1711	0.6
borneol	1719	0.6
β-selinene	1744	4.0
α-selinene	1748	1.2
α-panasinen	1788	0.6
p-cymen-8-ol	1864	0.4
caryophyllene oxide	2010	1.9
thymol	2146	68.0
carvacrol	2177	2.5

RI: Retention indices. Compounds are listed in order of their elution from DB-Wax column.

3.2 Effect of OGEO on mouse spontaneous locomotor activity

Figure 1 shows the locomotor activity following the administration of OGEO via inhalation at doses ranging from 4.0×10^{-10} to 4.0×10^{-1} mg per cage. A biphasic dose response pattern was observed. Mouse spontaneous locomotor activity was enhanced at low doses ranging from 4.0×10^{-10} to 4.0×10^{-6} mg per cage, whereas higher doses (4.0×10^{-5} to 4.0×10^{-1} mg per cage) suppressed mouse spontaneous locomotor activity. A significant decrease in locomotor activity was observed at doses 4.0×10^{-4} to 4.0×10^{-1} mg per cage in a dose dependent manner. The AUC values of the 4.0×10^{-4} to 4.0×10^{-1} mg OGEO-treated groups were significantly smaller than the control values, and the decrease in locomotor activity produced by these concentrations was statistically significant ($P < 0.05$ and $P < 0.01$),

suggesting a potential sedative effect of OGEO. The most effective concentrations were 4.0×10^{-3} mg and 4.0×10^{-2} mg which showed a reduction in locomotor activity that was comparable to that of benzylacetone. Transitions of total locomotor activity per 5 min were compared among administered doses. Mice administered OGEO at the dose of 4.0×10^{-3} to 4.0×10^{-2} mg became calm from the 20th minute. By the 30th minute, mouse locomotor activity dropped to nearly zero (data not shown). This indicated that an administration period of about 30 minutes was necessary for OGEO to be effective at the active doses.

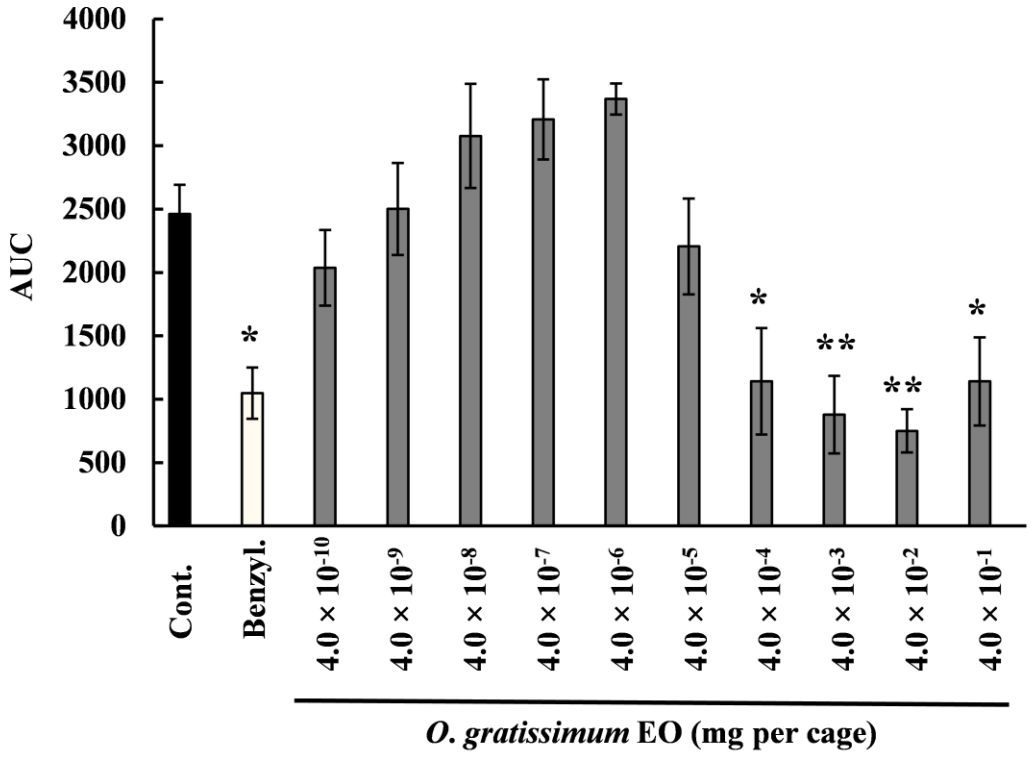

Fig 1: Sedative activity of *Ocimum gratissimum* essential oil. Total spontaneous locomotor activity of mice that received vehicle (triethyl citrate, 400 µL) and *Ocimum gratissimum* essential oil (4.0×10^{-10} – 4.0×10^{-1} mg per cage; EO). Data are shown as the mean ± standard error of the mean of 6 mice. Statistical differences vs. the control group (Cont.) were calculated using analysis of the variance followed by Dunnett's test.*$p < 0.05$, **$p < 0.01$. Benzyl.; Benzylacetone (4.0×10^{-3} mg per cage).

3.3 Effects of OGEO fractions on mouse locomotor activity

Fractions 1 and 3 of OGEO were administered individually to mice by inhalation at doses ranging from 4.0×10^{-8} to 4.0×10^{-1} mg per cage. Fraction 2 was not tested because the composition of the fraction was a mixture of fractions 1 and 3, and the amount of the fraction was insufficient for the tests using mice. Both Fraction 1 and 3 induced a significant decrease in locomotor activity compared to the control groups. This suggested that these two fractions contained active ingredients of OGEO. Fraction 3 appeared to be more potent than Fraction 1. Fraction 1 showed a significant decrease in locomotor activity at a dose of 4.0×10^{-1} mg while Fraction 3 showed a significant decrease in locomotor activity at doses of 4.0×10^{-5} and 4.0×10^{-4} mg per cage (Fig. 2A and 2B). The active doses of fraction 3 (4.0×10^{-5} and 4.0×10^{-4} mg per cage) were lower than those of OGEO (4.0×10^{-4} and 4.0×10^{-1} mg per cage). Thus, the effect of the main compound of Fraction 3 (thymol) was further examined to confirm its role in the sedative effect of OGEO.

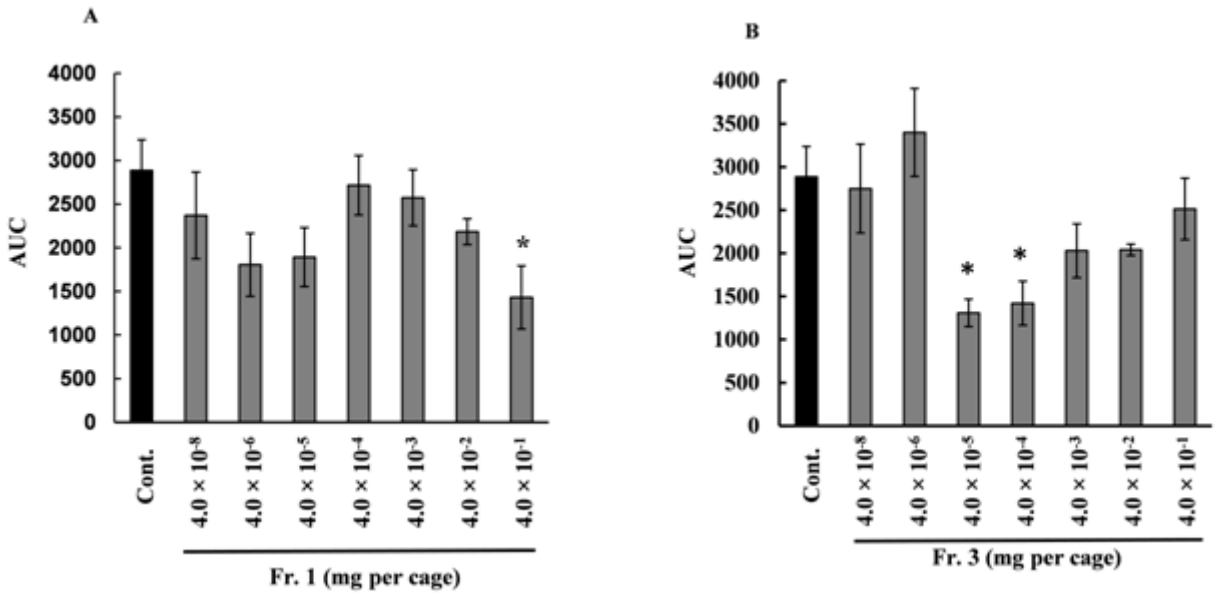

Fig 2: Effects of OGEO fractions on mouse locomotor activity. Total spontaneous locomotor activity of mice treated with *Ocimum gratissimum* essential oil fractions; A: Fraction (Fr.) 1 and B: Fraction 3. Data are shown as the mean ± standard error of the mean of 6 mice. Statistical differences vs. the control group (Cont.) were calculated using analysis of the variance followed by Dunnett's test.*$p < 0.05$.

3.4 Effects of thymol on mouse locomotor activity

Figure 3 shows the effect of thymol on mouse spontaneous locomotor activity. Thymol was administered at concentrations ranging from 4.0×10^{-10} to 4.0×10^{-1} mg per cage. It significantly decreased the locomotor activity of mice at concentrations of 4.0×10^{-5} and 4.0×10^{-4} mg. This indicated that thymol might possess a sedative activity. Thymol also significantly decreased mouse spontaneous locomotor activity at a dose of 4.0×10^{-2} mg. However, the inhibition of locomotor activity at the 4.0×10^{-2} mg dose was considered to be as a result of muscle relaxation because the performance of mice that were administered this

dose of thymol showed shorter latency to fall off the Rota-rod treadmill compared to the control group (Fig. 6). A comparison of the effect of thymol and OGEO on mouse spontaneous locomotor activity was made to elucidate their relative potencies. Both thymol and OGEO showed a biphasic dose response pattern. At low doses (phase 1), the dose patterns of thymol and OGEO were quite similar with a linear increase in locomotor activity from the dose of 4.0×10^{-10} to 4.0×10^{-6} mg per cage. However, at higher doses (4.0×10^{-5} to 4.0×10^{-1} mg per cage), thymol and OGEO showed different dose patterns. The therapeutic window of OGEO was wider and the potency of OGEO was greater than that of thymol. The dose dependent decrease in locomotor activity observed with OGEO at higher doses was not observed in thymol.

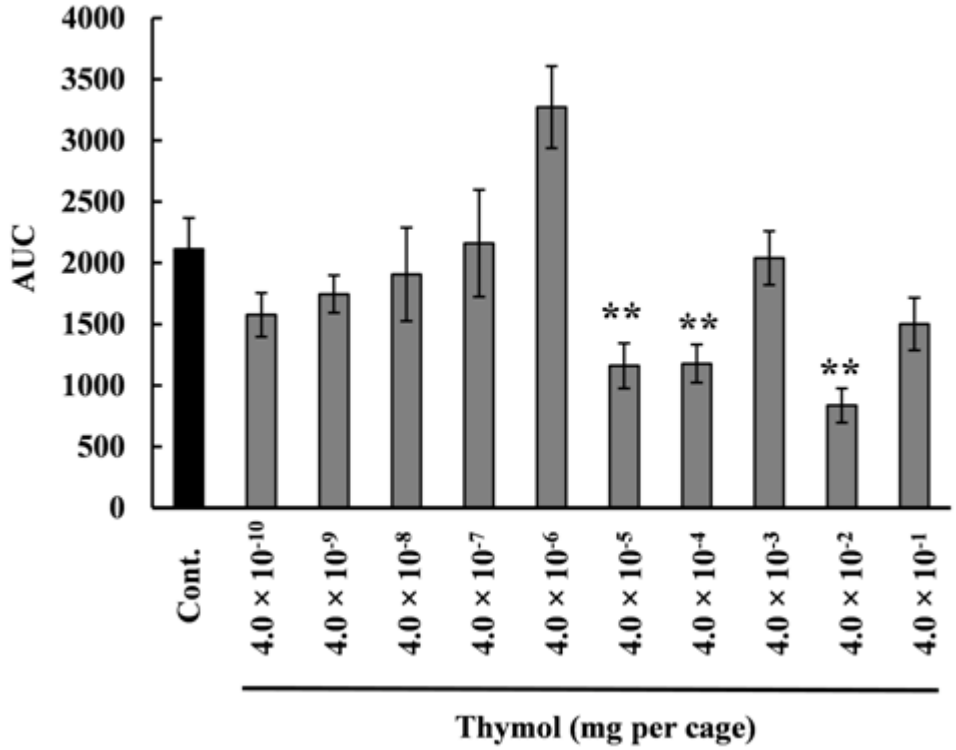

Fig 3: Effects of thymol on mouse locomotor activity. Total spontaneous locomotor activity of mice treated with vehicle (triethyl citrate 400 µL; Cont.) and thymol. Data are shown as the mean ± standard error of the mean of 6 mice. Statistical differences vs. the control group were calculated using analysis of the variance followed by Dunnett's test.*$p < 0.05$, **$p < 0.01$.

3.5 Anxiolytic-like activity of OGEO

In the light/dark transition test, anxiolytic-like agents typically increase the time spent in the light area and the movements between the two compartments [25]. The validity of the experimental system was confirmed using diazepam as positive control. Diazepam, dissolved in corn oil (0.5 mg/kg) and administered intraperitoneally at 30 min prior to testing, significantly increased the total time spent in the light area and the number of transitions between the two compartments compared to the vehicle (corn oil) [26]. The administration of OGEO at a concentration of 4.0×10^{-4} mg per cage significantly increased the total time spent

in the light area as well as the number of transitions between the light and dark compartments (Fig. 4A and 4B). This suggested that OGEO might induce an anxiolytic-like effect.

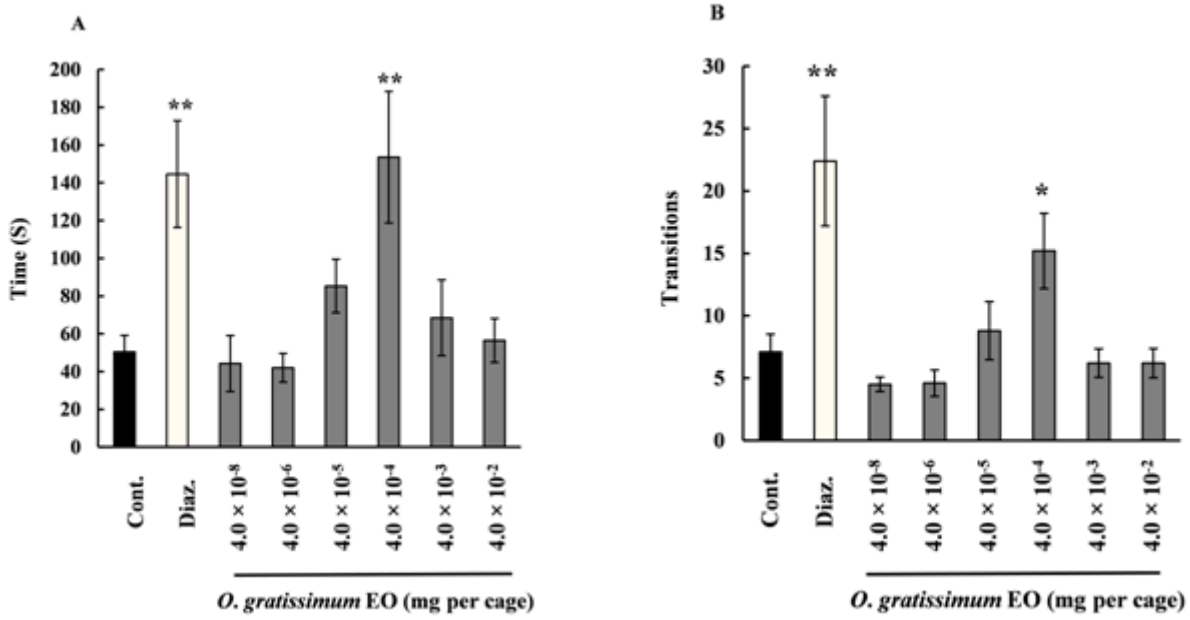

Fig 4: Anxiolytic-like activity of mice that received control (triethyl citrate 400 µL; Cont.), diazepam (0.5 mg/kg; Diaz.), and *Ocimum gratissimum* essential oil (EO); A: time spent in the light area, B: number of transitions between the compartments. Data are shown as the mean ± standard error of the mean of 10 mice. Statistical differences were calculated using analysis of the variance followed by Dunnett's test. *$p < 0.05$, **$p < 0.01$.

3.6 Antidepressant-like activity of OGEO

In the tail suspension test, antidepressant-like activity is represented by a decrease in immobility time [27]. Fluoxetine, dissolved in 0.9% physiological saline (20 mg/kg) and administered orally 1 h prior to testing, significantly shortened the immobility time compared to the vehicle (saline). This confirmed that the experimental system was valid. OGEO was

administered at doses of 4.0×10^{-10} to 4.0×10^{-1} mg per cage. At doses of 4.0×10^{-3} to 4.0×10^{-1} mg per cage, OGEO significantly decreased the immobility time compared to the control group (Fig. 5). This suggested that OGEO might induce an antidepressant-like effect. OGEO at a dose of 4.0×10^{-6} mg per cage caused an increase in immobility time compared to control group.

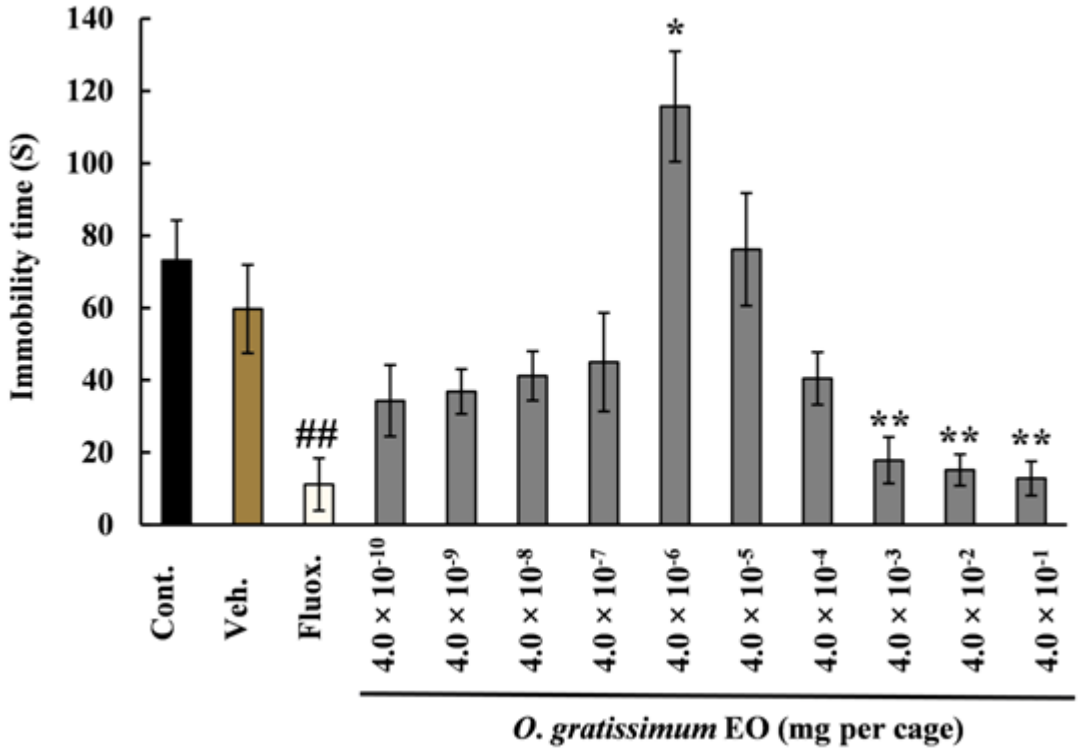

Fig 5: Effect of inhalation of OGEO on immobility time in Tail Suspension Test. Data are shown as the mean ± standard error of the mean of 6 mice. Statistical differences vs. the control group were calculated using analysis of the variance followed by Dunnett's test.*p < 0.05, **p < 0.01 compared to the control group (triethyl citrate), ##p < 0.01 compared to vehicle. Veh.; Vehicle (Saline), Fluox.; Fluoxetine (20 mg/kg p.o., 1 h before test).

3.7 Effect of OGEO on motor coordination

OGEO was administered to mice by inhalation at doses of 4.0×10^{-3} to 4.0×10^{-1} mg per cage and 30 minutes later, the Rota-rod test was performed. There were no significant differences in the latency to fall off the treadmill at the tested doses of OGEO treated group compared to control group (Fig. 6). This indicated that OGEO had no deleterious effects on motor coordination at the tested doses. Fraction 1, at the dose of 4.0×10^{-1} mg per cage and thymol at the dose of 4.0×10^{-2} mg per cage were also tested in the Rota-rod test. Fraction 1 showed no deleterious effects on motor coordination at the tested dose. However, thymol at a dose of 4.0×10^{-2} mg per cage showed a relatively short latency to fall off the Rota-rod treadmill, although this effect was not significant (Fig. 6). This indicated that the decrease in locomotor activity exhibited by thymol at a concentration of 4.0×10^{-2} mg might not be as a result of sedation.

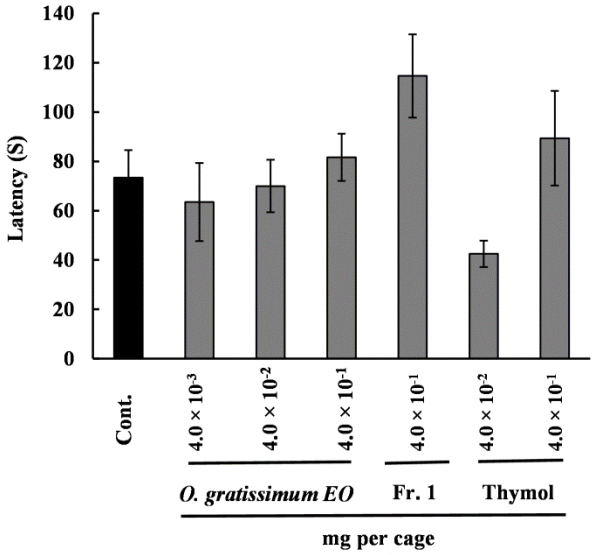

Fig 6: Effects of OGEO, fraction 1 and thymol on motor coordination in Rota-rod test. Data are shown as the mean ± standard error of the mean of 6 mice. Statistical differences vs. the control group were calculated using analysis of the variance followed by Dunnett's test.

4. Discussion

4.1 Behavioral effects of OGEO

In the current study, the psychopharmacological effects upon inhalation of the essential oil of *Ocimum gratissimum* were demonstrated. *O. gratissimum* appeared to have potent sedative and anxiolytic-like activities in mice without producing motor impairments. These results suggest that inhaled OGEO might elicit a tranquilizing effect. *O. gratissimum* also appeared to show potent antidepressant-like activity at doses of 4.0×10^{-3} to 4.0×10^{-1} mg per cage. These doses did not cause increase in locomotor activity in the open field test, indicating that OGEO indeed possessed antidepressant properties. This suggests that inhalation of OGEO might exert a thymoleptic effect. Taken together, these results indicate that inhalation of the essential oil of *O. gratissimum* might be effective in the management of affective disorders.

4.2 Active principles of OGEO

Several chemotypes of OGEO such as the eugenol, geraniol and thymol types have been reported [31]. Although the eugenol type has been extensively studied, very few studies have focused on the geraniol or thymol types. OGEO used in this study was the thymol type (68%). Assays with fractions of OGEO and single compound revealed that thymol is an active compound in the inhalative sedative effect of OGEO. However, other monoterpene compounds present in OGEO such as linalool, *p*-cymene, 1-octen-3-ol, have been previously reported to show sedative effect upon inhalation in mice [26, 32-34]. It is suggested that other active compounds, though present only in small amounts in OGEO might have worked in synergy with thymol to contribute to the inhalative sedative effects of OGEO. Furthermore, comparison of the sedative activities of OGEO and thymol indicated that OGEO was more potent than thymol, suggesting that the whole essential oil of *O. gratissimum* might be more

beneficial than its active compounds. This finding is consistent with that of Galindo *et al*. 2010 [35] who reported that the anticonvulsant and sedative effects of oral administration of the eugenol type essential oil of *O. gratissimum* might be due to a synergistic interaction of its components.

4.3 Selectivity and dose response patterns of psychoactive agents

According to Brunton *et al*. 2011 [36] drugs that selectively modify the CNS function (eg anti-depressants, tranquilizers, sedatives, hypnotics, certain stimulants, anti-psychotics etc) may cause either depression or excitation. In some instances, a drug may produce both effects simultaneously on different systems. Although selectivity of action may be remarkable, it is usually over-estimated as a drug usually affects several CNS functions to varying degrees [36]. For example, Brunton *et al*. [36] also stated that although the public often considers alcoholic beverages as a stimulant, ethanol is a CNS depressant. However, mild doses of alcohol appear to disinhibit the CNS function. Although this view is controversial, Harvey 1962 [37] asserted that "the disinhibition hypothesis is more than plausible and has actually proved most useful in ordering the effects of a wide range of doses of depressant drugs. To think of depressant drugs as acting simple along the vertical axis of the CNS, and not to consider the possible actions of these drugs along non-vertical dimensions of the CNS system, is to disregard the empirical findings." According to Calabrese 2005, [38] the non-linear hormetic-like biphasic dose response pattern (inverted U-shape or low dose stimulation, high dose inhibition) is known to be quite predominant in anxiolytic drug screening tests. It is assumed to result from a mixed agonist/antagonist effect [38]. Published data indicate that while several essential oils and fragrance compounds have been reported to have CNS depressant effects, others such as chamomile essential oil, peppermint essential oil and its constituents have been shown to have psychomotor stimulant effects on the CNS [4, 39, 40]. These psychomotor stimulating essential

oils are suggested to be potentially useful in the treatment of mental fatigue or Attention Deficit Hyperactivity Disorder (ADHD) [40, 41]. Interestingly, some pure compounds such as eugenol and 1,8-cineole have been simultaneously reported to show stimulatory and sedative effects on the CNS [35]. It is therefore important that the pharmacological effects of essential oils and fragrance compounds be tested over a wide dose range in order to elucidate their effects.

4.4 Biphasic effect of OGEO on spontaneous locomotor activity

In the present study, OGEO evaluated at doses ranging from 4.0×10^{-10} to 4.0×10^{-1} mg per cage showed a biphasic effect on mouse spontaneous locomotor activity. At low doses, OGEO enhanced mouse locomotor activity while at higher doses, a dose dependent decrease in locomotor activity was observed. The enhancement of locomotor activity showed by OGEO might have been brought about by thymol (main compound of OGEO) since thymol showed a similar increase in locomotor activity at low doses. Elhabazi et al, (1996) [42] reported that the aqueous extract of *Thymus broussonetii* had anxiolytic effects in the light/dark box test, meanwhile the ethylacetate extract enhanced locomotor and exploratory activities. Thymol and thyme essential oil on the one hand, have been reported to show stimulating effects upon inhalation in mice [4, 39]. On the other hand, *in vitro* reports have revealed that thymol is a potent GABA agonist, implying that it might have sedative properties [43, 44]. We might therefore conclude that OGEO and thymol might be CNS depressants but might exert stimulant-like effects at mild doses. To the best of our knowledge this is the first report elucidating the dual effect (dose-related) of OGEO or thymol on locomotor activity *in vivo*. These results suggest that the doses at which OGEO or other thymol-rich essential oils would be administered need to be studied carefully with respect to the desired effect targeted.

4.5 Dose-effect relationship in the antidepressant-like effect of OGEO

The antidepressant-like effect of OGEO was also investigated at doses ranging from 4.0×10^{-10} to 4.0×10^{-1} mg per cage in the tail suspension test. While a significant anti-depressant-like effect was observed at doses of 4.0×10^{-3} to 4.0×10^{-1} mg per cage, mice showed a depressed-like behavior with a significant increase in immobility time at the dose of 4.0×10^{-6} mg. In the open field test, OGEO showed a biphasic dose pattern with locomotor activity increase from 4.0×10^{-10} mg dose up to 4.0×10^{-6} mg dose and then a decrease in locomotor activity from this dose downward. This implied that the 4.0×10^{-6} mg dose represented the peak for locomotor activity enhancement. In addition to marked increase in ambulation, mice administered OGEO at this dose (4.0×10^{-6} mg) showed excited-like behavior in the open field test such as jumping, increased rearing and grooming (data not shown). This further confirmed the stimulant-like effect of this dose. Stimulation of the CNS by stimulant drugs is known to be followed by a period of mental depression [45]. Although adequate scientific data about the relationship between depression and the use of recreational drug is lacking, many recreational drugs can cause depression or anxiety [46]. According to Mulholland, [47] in depression, the level of neurotransmitters which control our emotions are altered, and recreational drugs affect these neurochemicals. For example; dopamine is affected by cocaine, amphetamine and ecstasy, serotonin is affected by ecstasy and LSD and noradrenaline is affected by amphetamines and opiates, and it is largely these three chemicals on which antidepressant medicines work [47]. This might explain why the 4.0×10^{-6} mg OGEO dose induced a depressed-like behavior in the tail suspension test. Furthermore, the inverted U-shape dose response has been reported for several psychoactive drugs such as benzodiazepines, amphetamine, cocaine, nicotine and morphine [38, 48]. Although further studies are required to elucidate the underlying mechanisms for the activities of OGEO at different doses, our results highlight the importance of testing the effects of psychoactive

essential oils over a wide dose range. It is worth mentioning that the doses administered in this study are only given as the concentration of samples administered per cage. Due to the low concentration of the compounds and the simplicity of our apparatus, it was not feasible to measure the true concentration of compounds in the vapor phase that saturated the cage. However, in a previous study, [32] headspace measurement of compounds in the vapor phase (using an SPME/GCMS technique) revealed that the dose administered correlates positively with the amount of compound in the vapor state.

5. Conclusion

The use of essential oils for the treatment of CNS related disorders seems to be a promising aspect in the field of complementary and alternative medicine. We previously reported that inhalation of the essential oil of *Piper guineense* from Cameroon shows sedative and anxiolytic effects in mice [26]. It is hoped that the aromatherapeutic potentials of indigenous African aromatic plants would be explored further as they could be valuable resources and beneficial in complementary and/or alternative therapy for the management of mental disorders. In conclusion, this study demonstrated that inhalation of the essential oil of *O. gratissimum* shows potent sedative, anxiolytic-like and antidepressant-like effects in mice. These results could be useful in the development of complementary and/or alternative therapies for the management of CNS related disorders with less invasiveness. Furthermore, *O. gratissimum* has a good potential for exploitation because it is a commonly used spice, cheap, accessible and can be easily cultivated.

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Conclusion and future perspectives

Three aromatic spices, namely, cinnamon, West African black pepper and tree basil, were administered to mice by oral ingestion or inhalation of their fragrances, and their pharmacological and phytochemical evaluations were carried out. The findings demonstrated that spices and herbs have pharmaceutical potentials and their exploitation could be beneficial in preventive, alternative and complementary therapies for some common diseases or disorders, such as gastric ulcer, anxiety and depression.

The development of nutraceuticals is a recent trend in the food and pharmaceutical industries, as focus on the use of nutraceuticals for healthcare among consumers grows stronger. Nutraceuticals are thought of in a broad sense as foods, food derivatives or food products that reportedly provide health or medical benefits, including the prevention and treatment of disease. Such products range from health and functional foods, dietary supplements, herbal products to isolated nutrients. Cinnamon, West African black pepper and tree basil are edible and hence can be considered as foods. In addition to their value as foods, they also offer medicinal benefits as demonstrated in this study. It is suggested that these spices and herb could serve as useful nutraceutical resources, or potential resources for development of pharmaceuticals.

Natural materials (herbs, animals, minerals etc) have a long history of usage for health benefits. Despite the accumulation of knowledge and experience of their usage in traditional medicines, the integration of conventional and traditional medicines is still in progress. It takes time partly because of the lack of scientific evidence for their acclaimed effects. Establishment of scientific evidence, as well as safe, mild or non-invasive modes of administration (such as incorporation into the diet or inhalation of fragrances as shown in this study or other nutraceutical modes of usage) would facilitate the optimal exploitation of these natural materials for health benefits. This type of research is of importance and it is hoped that

many more potential nutraceutical natural materials would eventually be fully exploited.

List of Publications

1. **Regular ingestion of Cinnamomi cortex pulveratus offers gastroprotective activity in mice.**

   Joan Manjuh Tankam, Yuki Sawada and Michiho Ito

   Journal of Natural Medicines (2013) 67 (2): 289-295

2. **Inhalation of the essential oil of *Piper guineense* from Cameroon shows sedative and anxiolytic-like effects in mice**

   Joan Manjuh Tankam and Michiho Ito

   Biological and Pharmaceutical Bulletin (2013) 36 (10): 1608-1614

3. **Sedative, anxiolytic and antidepressant-like effects of inhalation of the essential oil of *Ocimum gratissimum* L. from Cameroon in mice**

   Joan Manjuh Tankam and Michiho Ito

   Journal of Pharmacognosy and Phytochemistry (2014) 2(5): 1-9

出版目録

1. Regular ingestion of Cinnamomi cortex pulveratus offers gastroprotective activity in mice.
（桂皮入り食餌の継続投与による胃潰瘍予防活性）
Joan Manjuh Tankam, Yuki Sawada and Michiho Ito
2013 年 4 月発行　Journal of Natural Medicines
第 67 巻 第 2 号 289 頁～295 頁に掲載

2. Inhalation of the essential oil of *Piper guineense* from Cameroon shows sedative and anxiolytic-like effects in mice
（西アフリカ黒胡椒精油の吸入投与によるマウスの鎮静活性および抗不安活性）
Joan Manjuh Tankam and Michiho Ito
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3. Sedative, anxiolytic and antidepressant-like effects of inhalation of the essential oil of *Ocimum gratissimum* L. from Cameroon in mice
（木性バジル精油の吸入投与によるマウスの鎮静活性、抗不安活性および抗鬱活性）
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