

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

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The aromatherapeutic 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 aromatherapeutic potential of PGEO from Cameroon was investigated via inhalation, and the chemical constituents and active compounds responsible for its activity were identified.

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 $25 \pm 2^\circ\text{C}$ 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 reports²¹ 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 (%)
<i>α</i> -Pinene	1041	1.8
Camphene	1094	4.8
<i>β</i> -Pinene	1134	9.2
<i>β</i> -Phellandrene	1139	2.3
3-Carene	1168	2.2
<i>ν</i> -Limonene	1218	0.9
<i>p</i> -Cymene	1288	1.2
<i>α</i> -Copaene	1508	1.5
Camphor	1537	2
Linalool	1551	41.8
<i>β</i> -Elemene	1601	2.1
Caryophyllene	1611	3.6
Aromadendrene	1664	1.5
Isoborneol	1679	2.4
<i>α</i> -Humulene	1681	1.4
<i>α</i> -Terpineol	1704	4.1
<i>γ</i> -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.

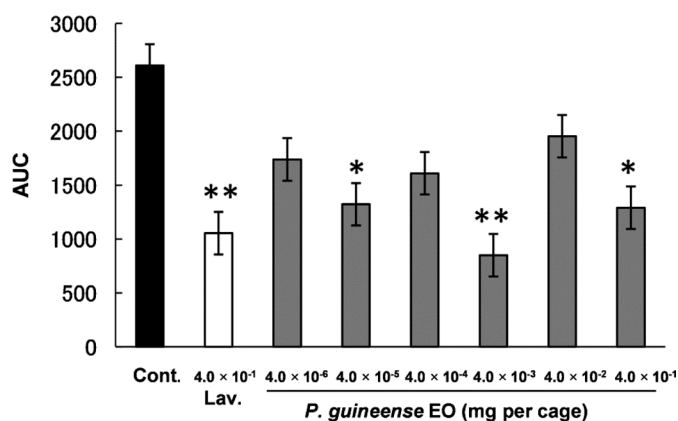


Fig. 1. Sedative Activity of *Piper guineense* Essential Oil

Total spontaneous locomotor activity of mice that received vehicle (triethyl citrate, 400 μ L) and *Piper guineense* essential oil (4.0×10^{-6} – 4.0×10^{-1} mg per cage; EO). Data are shown as the mean \pm 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

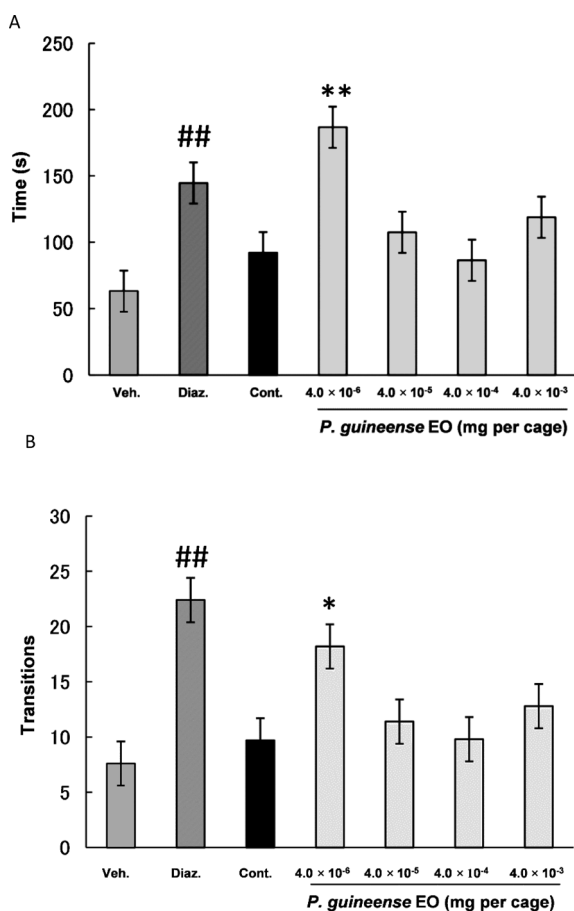


Fig. 2. Anxiolytic-Like Activity of *Piper guineense* Essential Oil

Anxiolytic-like activity of mice that received vehicle (corn oil; Veh.), diazepam (0.5 mg/kg; Diaz.), control (triethyl citrate 400 μ L; 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 \pm 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).

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 Tchoumboung *et al.*¹⁰⁾ also reported

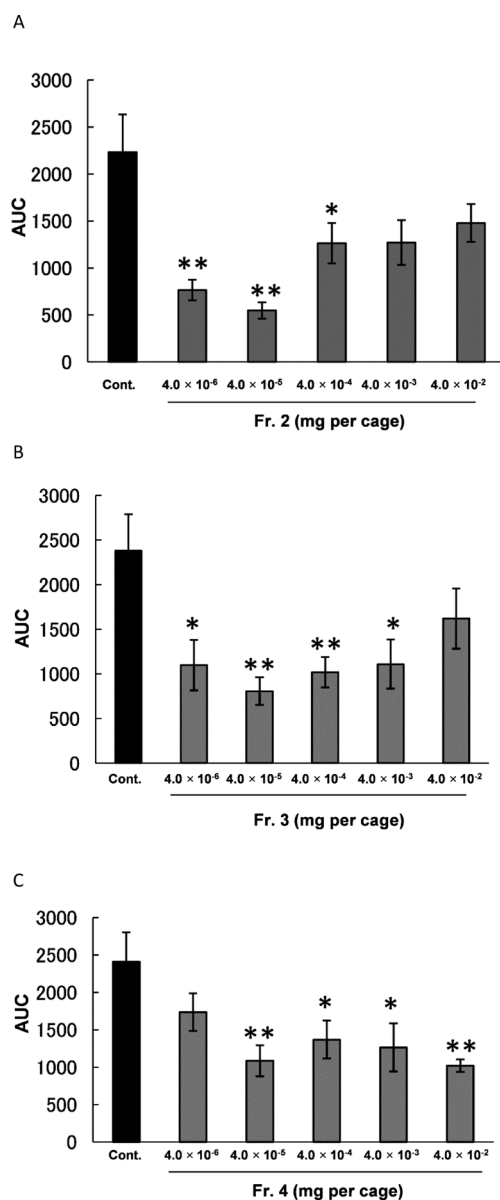


Fig. 3. Effects of PGEO Fractions on Mouse Locomotor Activity

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 \pm 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.

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 Mounjo 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 colleagues³²⁾ demonstrated that *R*(-)-linalool, but not *S*(+)-linalool, showed stress relieving effects on human subjects.

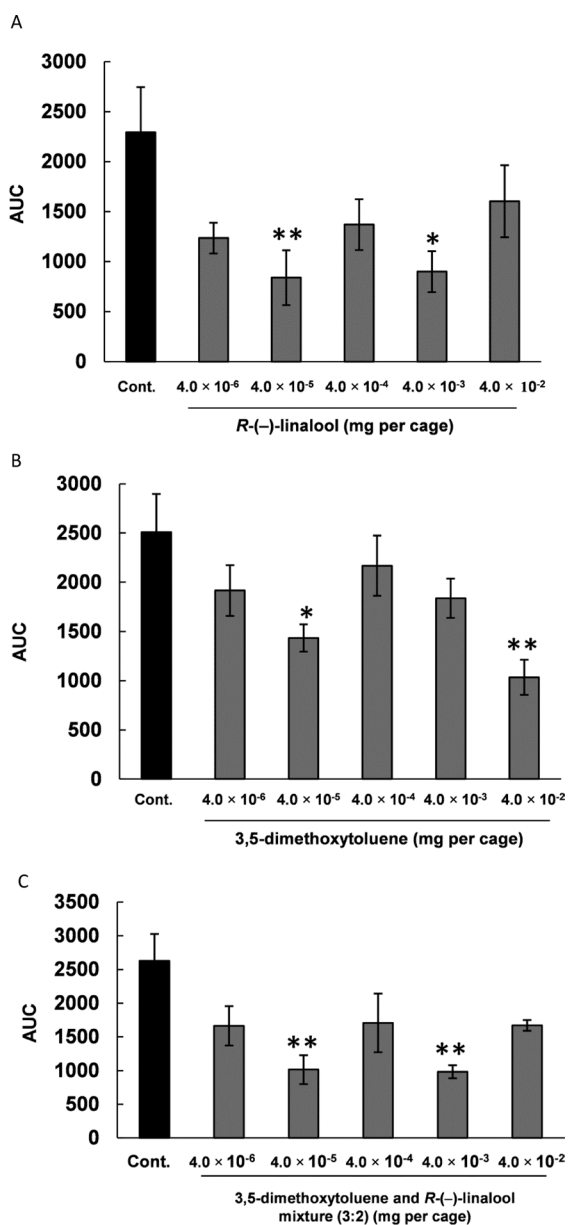


Fig. 4. Effects of *R*(-)-Linalool and 3,5-Dimethoxytoluene on Mouse Locomotor Activity

Total spontaneous locomotor activity of mice treated with vehicle (triethyl citrate 400 μ L; 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 \pm 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 activity⁴¹ 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 Akintoye¹⁶ 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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