Sedative activities of essential oils from Beninese medicinal plants via inhalation administration and structure-activity relationships of their active compounds (ベナン産薬用植物精油の吸入投与による鎮静活性と活性化合物の構造活性相関研究)

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Preface

In evolutionary terms, humans have greatly benefited from plants and their secondary metabolites. Since the dawn of humanity, plants have served as a source of food for human and animals, fibers for clothes and medication for human ailments. In the great diversity of the plant kingdom, essential oils have particularly attracted much attention recently. Essential oils are concentrated hydrophobic liquid produced by glandular trichomes and other secretory structures, and they contain volatile compounds with complex mixtures of hydrocarbons and oxygenated hydrocarbons which are extracted from herbs, flowers, and other plant parts. They have served for a long time in many traditional healing systems and they have demonstrated efficacy for treating people with anxiety, pain, bipolar disorder, attention deficit hyperactivity disorder (ADHD), stress and depression. They have multiple usages and address every component of the human or animal body such as their use by oral administration, inhalation, diffusers, baths, or massages.

Anxiety, insomnia, depression, ADHD and post-traumatic stress disorder (PTSD) are the most frequent mental illnesses in the world and they impose high economic, personal and society burden, not mentioning their multiple side effects and controversial efficacy. Currently, the pathogenesis of these diseases still remains ambiguous, and many factors, including stress, lead to their occurrence. Stress is defined as an alteration in an organism's physiological homeostasis, resulting from the exposure to certain internal or external events or situations. There is a strong component with these ailments as they sometimes occur in tandem. Studies have demonstrated that essential oils are effective in reducing depression, anxiety, and stress symptoms in both animal models and humans. However, there is still a lack of scientific evidence of the pharmacological activities of essential oils regarding stress-related disorders and their action mechanisms remains unclear. Their use could prove to be hazardous as their chemical composition is diverse and using these therapies with no scientific investigation could do more harm than good.

Africa is rich in many plant species, and especially in towns and rural villages of the Republic of Benin, traditional healers and market herbalists believe that fragrant species can be of interest through their sedative or sleep promoting activity. Briefly, first in this thesis, I collected 3 plant species from the Republic of Benin (West Africa), namely *Lantana camara* L., *Dysphania ambrosioides* (L.) Mosyakin & Clemants and

Chromolaena odorata (L.) R.M.King & H.Rob, and they were investigated for their sedative or sleep-promoting effects via inhalation administration to mice. Further, the active compounds were identified and investigated for their structure-activity relationships. Second, essential oils constituents belong principally to two different chemical groups: terpenoids (monoterpenes and sesquiterpenes) and phenylpropanoid derivatives. It has been suggested that their constituents could exert their biological activities through modulation of the GABAergic receptor system as GABA is the major inhibitory neurotransmitter in the central nervous system. Therefore, I have explored the implication of the GABAergic system in the sedative activities of essential oils constituents extracted from the plant species, and explained their action mechanisms. Finally, sesquiterpene compounds which are less studied that monoterpenes were investigated for their molecular descriptors and I performed a QSAR and SAR study in order to better understand the structures in cause for activity. The data presented in this thesis can be helpful for the management of complex stress-related CNS disorders such as ADHD or PTSD. This thesis is divided in 3 essential chapters.

Abstract

In **Chapter 1**, I present the sleep promoting effects of 3 plant species from the Republic of Benin. First, I investigated the chemical composition of essential oil from the leaves of *L. camara* (LCEO), evaluated its sedative effects in mice via inhalation administration, and identified the compounds responsible for activity. The results indicated that via inhalation administration, sabinene (38.81%) and 1,8-cineole (28.90%) were the main compounds in cause for the sedative activity of LCEO. Thus, they could be considered as promising candidates for the management CNS-associated diseases. Second, I analyzed the chemical composition of the essential oil from the leaves of *D. ambrosioides* (DAEO), and its sleep-promoting effects via inhalation administration in ddY mice. Ascaridole (35.52%) and *p*-cymene (47.16%) were the major components, and both compounds were evaluated for sedative activity by using the caffeine-treated excitatory mouse model. They were highly effective in decreasing locomotor activity of excited mice by more than 50%. Further investigations indicated that the GABAergic system mediates the sedative effect. The results further extend the knowledge on their use as potential, promising natural products for the management of sleep disorders and CNS-related ailments. Last, essential oil from the leaves of *C. odorata* (COEO) was investigated for its sedative activity in mice. The results showed that COEO significantly reduced mice locomotor activity and analysis of chemical composition of the oil indicated that caryophyllene oxide (43.75%) was the major compound. In order to understand the action mechanisms, caryophyllene oxide was tested for its effects on the CNS by using a caffeine pre-excited mice test and a pentobarbital sleeping-induced test in mice. The results demonstrated that caryophyllene oxide is a CNS-depressant; however, it does not operate via the GABAergic receptor system. Its precursor, *β*-caryophyllene, was also investigated and it demonstrated a similar pattern of sedative activity. We believe these results further extend actual knowledge on these naturally occurring sesquiterpenes.

In **Chapter 2**, I wanted to further understand the activities of monoterpene 1,8-cineole identified in *L. camara*, which demonstrated a novel double U-shaped curve pattern of sedative activity, as described in Chapter 1. Therefore, I present here the anxiolytic and antidepressant activities of naturally occurring monoterpene 1,8-cineole and its structural isomer 1,4-cineole. Anxiety-like behaviors were evaluated by using the light–dark box test (LDB) and marble-burying test (MBT) and depression-like symptoms were tested by

using the forced swimming test (FST) and tail suspension test (TST). The results demonstrated that 1,8-cineole and 1,4-cineole both possessed an anxiolytic and antidepressant effect. Further, the role of the $GABA_A$/benzodiazepine receptor system in the anxiolytic effects of 1,8- and 1,4-cineole was investigated through co-administration of flumazenil, a GABAergic system antagonist. The results suggested that only 1,8-cineole affects the $GABA_A$/benzodiazepine receptors. In this chapter, I demonstrated that inhaled 1,8- and 1,4-cineole prevented anxiety and depressive-like symptoms in classic mice models.

Sesquiterpene compounds such as caryophyllene oxide, were previously demonstrated in Chapter 1, to be sedative even at low doses. A previous work has investigated QSAR study of monoterpene compounds. However, sesquiterpenes which are more complex and possess stronger odor in plants are not yet studied for possible QSAR or SAR analysis. Therefore, in **Chapter 3**, as a part of my endeavor to shed light on the effects of sesquiterpenes on the CNS, a detailed study on the properties of selected sesquiterpenes was performed by using their ADMET properties. From an initial list of one hundred and fourteen sesquiterpenes, a rigorous selection of eighteen sesquiterpenes was conducted, and these compounds were further divided into two groups: training set and external validation set of nine sesquiterpenes each. The training set was evaluated for sedative activity in mice via inhalation and the results demonstrated that all compounds were sedative, except for one compound, curzerene. QSAR study was performed using simple regression and multiple regression models, and a multiple regression model was the best fit to predict sedative activity of the sesquiterpenes. Further, molecular determinants were analyzed to find the key parameters needed for sedative activity. Molar refractivity and the number of hydrogen bonds acceptors were suggested to be statistically important in predicting more than 82% of the sedative activity. Therefore, in this chapter, I suggest novel QSAR models that could be useful in predicting sedative activity of sesquiterpenes, thus accelerating the process of drug development.

Publication notes

Chapter 1 is based on the investigation of sedative activities of 3 plants materials from the Republic of Benin and their action mechanisms, which appeared in 3 different journals: **Journal of Natural Medicines [1]**, **Journal of Natural Products [2]** and **Pharmaceuticals [3]**. Chapter 2 is about the anxiolytic and antidepressant activities of monoterpenes cineoles, which appeared in the journal **Molecules [4]**. Chapter 3 is based on a paper describing molecular determinants and QSAR models on sedative activity of sesquiterpene compounds, which appeared in the journal **Planta Medica [5]**. In total, the content of this thesis is based on 5 scientific publications as presented in the following list.

List of publications by the author

1- **Dougnon, G**., Ito, M. (2020). Sedative effects of the essential oil from the leaves of *Lantana camara* occurring in the Republic of Benin via inhalation in mice. Journal of natural medicines, 74(1), 159–169. https://doi.org/10.1007/s11418-019-01358-9

2- **Dougnon, G**., Ito, M. (2021). Role of Ascaridole and *p*-Cymene in the Sleep-Promoting Effects of *Dysphania ambrosioides* Essential Oil via the GABAergic System in a ddY Mouse Inhalation Model. Journal of natural products, 84(1), 91–100. https://doi.org/10.1021/acs.jnatprod.0c01137

3- **Dougnon, G**., Ito, M. (2021) Essential Oil from the Leaves of *Chromolaena odorata*, and Sesquiterpene Caryophyllene Oxide Induce Sedative Activity in Mice. Pharmaceuticals, 14, 651. https://doi.org/10.3390/ph14070651

4- **Dougnon, G**., Ito, M. (2020). Inhalation Administration of the Bicyclic Ethers 1,8- and 1,4-cineole Prevent Anxiety and Depressive-Like Behaviours in Mice.

Molecules (Basel, Switzerland), 25(8), 1884. https://doi.org/10.3390/molecules25081884

5- **Dougnon, G**., Ito, M. (2022). Molecular descriptors and QSAR models for sedative activity of sesquiterpenes administered to mice via inhalation. Planta Medica. DOI: 10.1055/a-1770-7581

Chapter I. Sleep promoting effects of 3 aromatic plant species collected from the Republic of Benin (West Africa): *Lantana camara* L., *Dysphania ambrosioides* (L.) Mosyakin & Clemants and *Chromolaena odorata* (L.) R.M.King & H.Rob.

I. General Experimental Procedures

1. GC and GC/MS analysis

Qualitative analysis of *L. camara* essential oil (LCEO) was carried out using an Agilent 6850 series gas chromatograph connected to an MSD 5975 system (Agilent Technologies). The following operating conditions were employed: column, fused silica capillary column, DB-wax (HP), 60 m × 0.25 mm × 0.25 μm; column temperature, 60–200 °C, increasing at a rate of 2 °C/min, holding at 65 °C for 5 min, increasing at 0.5 °C/min until 77 °C then at 20 °C/min, and holding at 200 °C for 5 min. Injector temperature, 160 °C; carrier gas, helium, 25 cm/s; column head pressure, 100 kPa; ionization energy, 70 eV; injection volume, 1.0 μL; MS interface temperature, 150 °C/min; MS mode, electron impact (EI); detector voltage, 0.4 kV; mass range, 35-300 u; scan speed, 300 u/s. We performed qualitative analysis of *D. ambrosioides* essential oil (DAEO) and *C. odorata* essential oil (COEO) using a Shimadzu GCMS-QP2020NX gas chromatograph mass spectrometer connected to a Nexis GC-2030 system (Shimadzu Corporation, Kyoto, Japan). For DAEO, I used the following operating conditions: fused silica capillary column, DB-wax (HP), 60 m × 0.25 mm × 0.25 μm; column temperature, 60°C–220°C, holding at 60°C for 2 min, increasing at a rate of 3°C/min until 220°C; injector temperature, 210°C; carrier gas, helium, 25 cm/s; column head pressure, 100 kPa; ionization energy, 70 eV; injection volume, 1.0 μL; MS interface temperature, 150°C/min; MS mode, electron impact (EI); detector voltage, 0.4 kV; mass range, 35–300 u; and scan speed, 300 u/s. For COEO, I used the following operating conditions: fused silica capillary column, DB-wax (HP), 60 m × 0.25 mm × 0.25 μm; column temperature starting at 60°C and increasing at a rate of 3°C/min until 220°C; injector temperature, 210°C; carrier gas, helium, 25 cm/s; column head pressure, 100 kPa; ionization energy, 70 eV; injection volume, 1.0 μL; MS interface temperature, 150°C/min; MS mode, electron impact (EI); detector voltage, 0.4 kV; mass range, 35–300 u; and scan speed, 300 u/s.

Quantitative analysis were performed using a Hitachi G-5000 GC system (Hitachi, Ltd., Ibaraki, Japan) equipped with a flame ionization detector (FID) as follows: fused silica capillary column, TC-wax (HP), 60 m × 0.25 mm × 0.25 μm; column temperature, same as for GC-MS; injector temperature, 200°C; detector, FID, 220°C; carrier gas, helium, 0.8 mL/min; split ratio, 100:1; column head pressure, 200 kPa; and injection volume, 1 μL. We determined the linear retention indices of each constituent using *n*-alkanes as standards and identified chemical compounds by comparing their retention indices (RIs) and mass spectra (MS) using NIST Special Database 2 (https://www.nist.gov/srd/nist-special-database-2) or by comparison with authentic samples.

2. Experimental animals

Four-week-old male ddY mice (20–30 g) were purchased from Japan SLC (Shizuoka, Japan). Animals were housed in colony cages under a 12 h/12 h light/dark cycle at 25 ± 2 °C and a relative humidity of 50–60%. They were fed pellet chow and water ad libitum and allowed to accommodate to these conditions for 1 week before experiments. Animal

experiments were conducted following the recommendations of the Animal Research Committee of Kyoto University, Kyoto, Japan (approval number, 2014-14-3).

Experimental procedures involving animals and their care were conducted in accordance with the institutional guidelines and in compliance 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). All experiments were conducted between 10:00 and 17:00 under identical conditions.

3. Drugs and Reagents

Lavender oil and benzylacetone (all of purity: > 95%, Tokyo Kasei, Japan), well-known sedative agents [1–3], were used as a positive control. Triethyl citrate (TEC; Merck, Darmstadt, Germany), a non-sedating odorless solvent, was used to dissolve the fragrant components. Sabinene (purity: ≥ 97%) was purchased from Chem Cruz Chemicals, Santa Cruz Biotechnologies (Dallas, USA). 1,8-Cineole (purity: > 85%) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). *p*-Cymene (purity ≥ 98%) and α-terpinene (purity > 80%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Caryophyllene oxide (purity ≥ 95%) was purchased from Wako Pure Chemical Industries (Osaka, Japan) and *β*-caryophyllene (purity > 90%) was purchased from Tokyo Chemical Industry Co. LTD (Tokyo, Japan).

All chemicals used were of the highest grade available.

4. Behavioural experiments

4.1. Open-field test procedure

The sedative effects in mice were evaluated using an open-field test (Figure 1), as previously described [1]. Administered doses were expressed as milligrams of sample per 400 μL of TEC, following previous experiments [3–6]. Four pieces of filter paper were placed in the four corners of the inner walls of the glass cage (60 cm wide, 30 cm long, 34 cm high) using adhesive tape. A sample was deposited on each piece of filter paper and the cage was closed so that the vapor pervaded by natural diffusion. Sixty minutes after charging the sample, a mouse was placed in the center of the cage and subjected to video surveillance for another 60 min. During monitoring, the frequency each mouse crossed lines drawn at 10 cm intervals on the floor of the cage was counted every 5 min. The area under the curve (AUC) of locomotor activity counts per 5 min (*y*-axis) and time (*x*-axis), representing total locomotor activity, was calculated by trapezoidal rule.

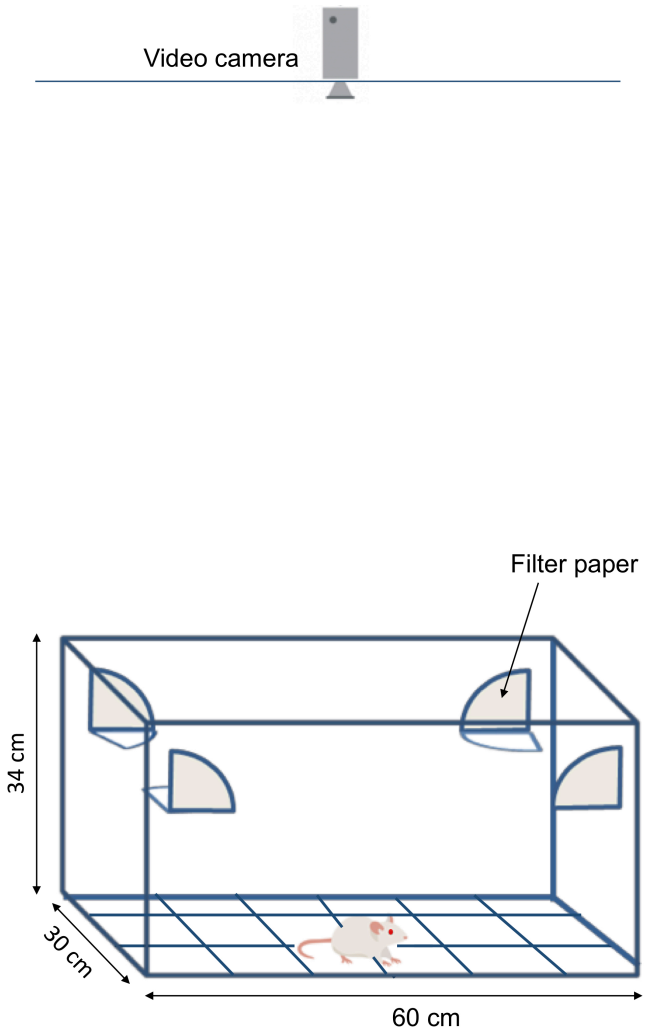


Figure 1: The open-field arena

4.2. Caffeine-Induced Stimulation of Locomotor Activity in Mice

We intraperitoneally administered caffeine to mice at a dose of 25 mg/kg. Next, I placed each mouse in a cage filled with vapor from the sample solution 30 min after administration and measured locomotor activity for another 1 h, as described in the open-field test. Diazepam (5 mg/kg) used as a positive control was administered orally 30 min before caffeine administration. The test was performed as described by Takemoto et al. [7] with modifications.

4.3. Pentobarbital-Induced Sleeping Test

We administered sodium pentobarbital (42 mg/kg) intraperitoneally to mice (n = 6/group) and immediately placed them in a cage filled with vapor of the sample solution (400 µL/cage). Sleeping time was defined as the time difference between loss and

recovery of the righting reflex. Diazepam (3 mg/kg) used as a positive control was administered orally 30 min before sodium pentobarbital injection [7]. To evaluate the action mechanism of molecules in the CNS, 3 mg/kg of flumazenil, a specific $GABA_A$-benzodiazepine receptor antagonist, was administered intraperitoneally 15 min before diazepam administration.

4.4. Rota-Rod Test

A Rota-rod treadmill (MK-600; Muromachi Kikai Co., Ltd., Japan) was used to perform the experiment. To adapt the mice to the Rota-rod, the mice (n = 6/group) underwent a 2-day training period during which they were placed on the rotating rod first at 5 rpm for 3 min (mode 1) and then at 40 rpm for a total of 3 trials/day. Mice that failed to remain on the rotating rod after two trials were excluded. On day 3, the Rota-rod test was performed. After 60 min of sample inhalation or after 30 min of 5 mg/kg diazepam intraperitoneal administration, the mice were placed on the rotating rod, and the duration (up to 3 min) that the mice remained on the rotating rod was recorded.

5. Evaluation of the amount of compound evaporated in the cage before inserting the mice

The amount of compound evaporated in the glass cage was measured using methodology previously described [8,9]. Each compound was dissolved in TEC and dropped onto a filter paper. The filter paper was weighed and placed in a closed glass cage for 60 min, after what it was removed and weighed again. Weight difference from immediately after dropping the sample and after removal of the filter paper was regarded as the amount of compound evaporated per hour in the glass cage.

6. Statistical analysis

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

II. Sedative Effects of *Lantana camara* L. Essential Oil

1. Introduction

Lantana camara Linn (Verbenaceae) is the most widespread species of the *Lantana* genus. It is an evergreen climbing aromatic shrub that can grow up to 2–4 m in height. Its flowers are small, tubular and are various colors, from red to pink, white, yellow, and violet. The stems are quadrangular, and generally armed with hooked prickles. The fruits are green and turn black when ripe [10]. *L. camara* is native to tropical and sub-tropical regions of America and has now been introduced as an ornamental plant in various parts of the world. The main chemotypes reported in the literature are sesquiterpene hydrocarbons, namely *ß*-caryophyllene, germacrene D, and bicyclogermacrene; monoterpenes are represented by limonene, sabinene, and *ß*-phellandrene; and the oxygenated compounds are mainly davanone, 1,8-cineole, caryophyllene oxide, and (*E*)-nerolidol [11,12].

L. camara is used in many parts of the world to treat a number of disorders. In Tanzania, it is used to treat malaria [13], whereas in India, essential oil from the leaves is used for its antiseptic and antifungal properties [14]. In the Republic of Benin, leaves are used in traditional medicine to treat skin diseases [15], and essential oil from the leaves of *L. camara* (LCEO) is claimed to demonstrate sedative effects via inhalation administration. Several aromatic natural medicines have been used as incense and perfume for their relaxing or energizing effects [9,16], and excellent results were obtained using essential oils in young patients suffering from attention deficit hyperactivity disorder (ADHD) [17]. Also, inhalation administration of the vapor of herbal drug oil has caused significant decrease in mice locomotor activity and this supports the argument for their traditional usage as relaxing and possible anxiolytic or antidepressant agents via inhalation administration [1–3,6,18]. To the best of my knowledge, only a few data are available concerning the chemical composition of LCEO occurring in Benin and its sedative effects remain not yet studied.

Herein, the chemical composition of LCEO occurring in Benin was investigated as well as its sedative effects in mice via inhalation administration, and the components responsible for the sedative activity were identified.

2. Material and methods

2.1. Plant material

Fresh leaves of *L. camara* (Figure 2) were collected in October 2016 from Houedo in the Republic of Benin (West Africa) and air dried. GPS coordinates of the collection site were latitude 6°33′37.133″ and longitude 2°22′26.567″. Identification was confirmed by Gaudence Julien Djego of the Laboratory of Botany and Applied Ecology, University of Abomey-Calavi, and vouchers were deposited in the Herbarium of Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University,

Japan (specimen number EST-5024) and the National Herbarium of Benin (specimen number AA 6677/HNB).

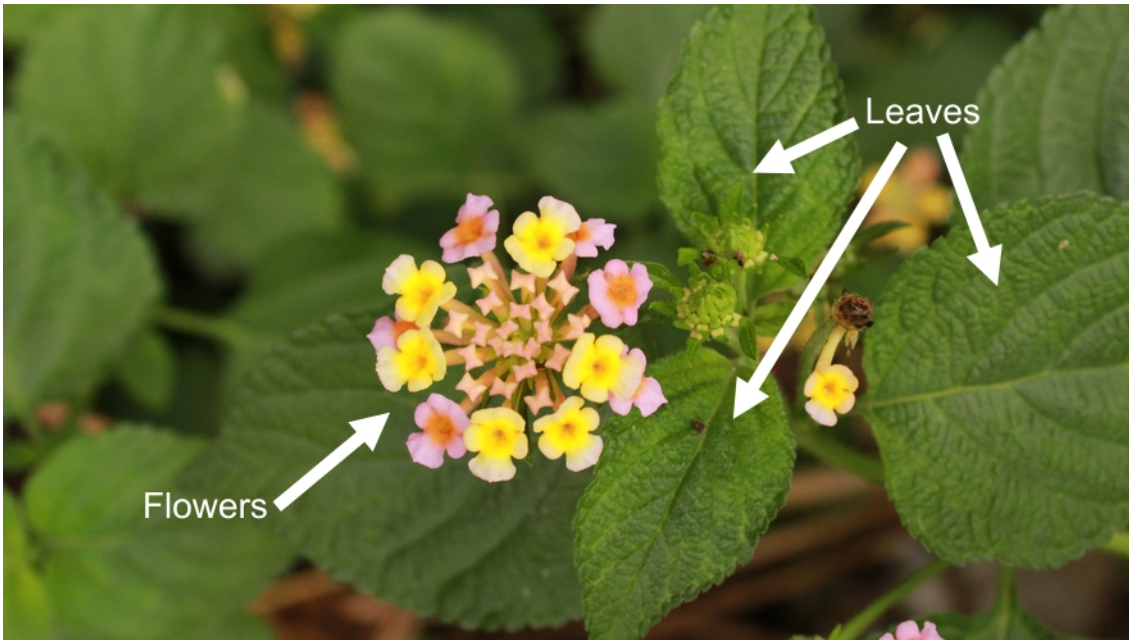


Figure 2: Leaves of *Lantana camara* in their natural environment

2.2. Extraction, fractionation of LCEO and chemical composition analysis

LCEO was extracted by hydrodistillation of *L. camara* leaves (56.60 g) for 2 h using a Clevenger apparatus as recommended in the Japanese Pharmacopoeia (JP17; http://jpdb.nihs.go.jp/jp17e/). The oil was captured in hexane, dried over anhydrous sodium sulfate, and concentrated. The headspace of the oil was then analyzed by Solid Phase Micro Extraction (SPME)-GC/MS to confirm that the oil was free of hexane. The obtained oil was stored in sealed vials at 4 °C until analysis. LCEO was subjected to silica gel column chromatography for fractionation (column diameter: 16 mm; column height: 170 mm), and the column was eluted with hexane/acetone (4:1, about 250 mL) to give fractions 1 and 2. Both fractions were evaporated and subjected to GC/MS analysis to confirm that no remaining solvent was present before analysis of biological activity.

3. Results and Discussion

3.1. Chemical composition analysis of LCEOs from diverse origins

The results of the GC and GC/MS analyses of LCEO are presented in Table 1 according to their retention indices on a DB-wax column and are compared in their ratio with LCEOs from other regions.

Table 1: Phytochemical constituents of Beninese LCEO and comparison of their ratio in LCEOs of different origins

Compound[a]	Retention index		Peak area (%)							
	Calculated[b]	Literature[c]	Present study[d]	Ref. 1	Ref. 2	Ref. 3	Ref. 4	Ref. 5	Ref. 6	Ref. 7
α-Pinene	1025	1025	5.38	-	1.90	0.31	-	3.68	-	-
β-Pinene	1105	1104	3.74	3.40	2.10	0.56	-	2.59	-	-
Sabinene	1117	1119	**38.81**	**21.50**	**14.70**	0.15	-	**9.02**	**-**	**-**
3-Carene	1146	1147	6.06	-	1.70	-	-	2.26	-	-
Myrcene	1159	1160	3.21	2.10	-	0.02	-	0.63	-	-
Limonene	1199	1198	3.38	-	-	0.11	-	1.62	-	-
1,8-Cineole	1215	1212	**28.90**	**23.40**	**15.80**	0.12	**10.75**	2.83	-	0.78
(+)-2-Bornanone	1532	1529	2.59	-	-	-	-	-	-	-
(*Z*)-*p*-Menth-2-en-1-ol	1566	1565	1.57	0.10	-	-	-	-	-	-
***β*-Caryophyllene**	1609	1608	4.69	**10.90**	**8.90**	**13.26**	**16.37**	**11.98**	4.80	**9.90**
Terpinen-4-ol	1612	1612	0.33	2.50	1.70	0.27	-	tr	0.10	0.90
α-Humulene	1696	1689	0.13	4.40	-	1.38	**8.22**	6.17	4.90	-
Bicyclogermacrene	1736	1734	0.21	-	2.80	-	3.65	2.59	-	-
ar-Curcumene		1777	-	-	-	**24.69**	-	0.78	0.60	-
Caryophyllene oxide		1987	-	-	-	-	2.98	-	-	**21.75**
(*E*)-Nerolidol		2031	-	1.30	5.90	0.25	-	2.28	**43.40**	**10.39**
Davanone		2040	-	-	-	-	2.88	**15.94**	-	-
Spathulenol		2133	-	-	3.40	1.48	-	0.68	-	**14.95**

[a]Order of elution determined using a DB-wax column.
[b]Retention index, calculated against C10–C26 *n*-alkanes on a DB-wax column.
[c]Retention index, taken from the NIST library.
[d]Peak area percentage was determined by calculating the peak area of the FID chromatogram in GC analyses.
Ref. 1: Seme (Rep. of Benin) [19]; Ref. 2: Nigeria [12]; Ref. 3: Cameroon [20]; Ref. 4: India [21]; Ref. 5: Madagascar [20]; Ref. 6: Cuba [22]; Ref. 7: Nigeria [23]; tr: trace; -: not detected.

In the present study, a total of 13 compounds were identified, with the main components being represented by monoterpene hydrocarbons (60.58%) and oxygenated monoterpenes (33.39%); sabinene and 1,8-cineole (38.81% and 28.90% respectively) were the main ones (Figure 3).

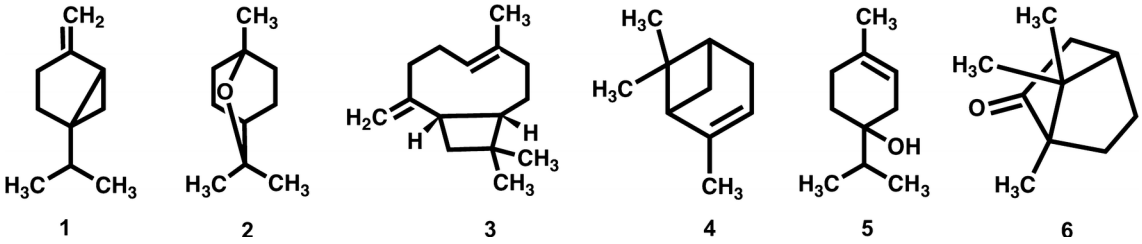


Figure 3: Chemical structures of the major constituents of LCEO and fractions

Sabinene (**1**), 1,8-Cineole (**2**), *ß*-Caryophyllene (**3**), *α*-Pinene (**4**), Terpinen-4-ol (**5**), and (+)-2-Bornanone **(6)**

Similar to my study, a previous work conducted in another region of the Republic of Benin (Seme) showed that the main compounds of LCEO were sabinene (21.5%) and 1,8-cineole (23.4%) [19]. LCEO is known to demonstrate an important quantitative and qualitative variability in the chemical contents of its oil. Therefore, I compared the results in the present study with previous studies on LCEOs from different regions.

Our results showed similar qualitative composition with LCEOs from Nigeria (wild), Cameroon, and Madagascar (cultivated), which all contained sabinene, 1,8-cineole, α-pinene, and *ß*-caryophyllene at various concentrations [20,24], whereas LCEO from India (wild) showed notable difference with my study and contained no trace of sabinene [21]. The presence or absence of sabinene and 1,8-cineole in LCEO from northern Brazil (wild) depended on the collection site and time of collection [25,26], whereas neither were found in LCEO from Cuba (cultivated) [22]. Citral was the major compound identified in five varieties of *L. camara* from Egypt (wild) [27]; however, it was not detected in samples from Nigeria or Benin. More, a high content of sesquiterpene hydrocarbon compounds with mainly *ß*-caryophyllene (13.26%) and ar-curcumene (24.69%) was reported in LCEO from Cameroon [20], while LCEO from China (wild) was represented by *α*-humulene (9.3%) and germacrene-D (15.8%) [28]. Monoterpene hydrocarbons are mainly represented by *α*-phellandrene (16.4%) and limonene (16.5%) in LCEO from northern Brazil [25], and sabinene (11.4%), *α*-pinene (4.1%), and *ß*-pinene (2.8%) in LCEO from Madagascar [29]. Among the oxygenated compounds, caryophyllene oxide (21.75%) in LCEO from Nigeria [23], 1,8-cineole (10.75%) in LCEO from India [21], and davanone (15.9%) in LCEO from Madagascar [20] have mainly been reported.

The chemical composition of parts of plants is reported to be affected by the developmental stage, genetic, geographical, and seasonal factors [12,30,31]. Thus, the differences in chemical composition of LCEO in the present study with LCEOs from other regions could be explained by variability in climate, altitude, time of collection and soil, or the method of extraction of the oil.

3.2. Evaluation of LCEO and fractions sedative effects when administered to mice via inhalation

To investigate the effects of LCEO on mice locomotor activity, mice were administered via inhalation doses of 0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg LCEO dissolved in 400 µL of TEC. Benzylacetone, a well-known sedative agent [1–3], was used as positive control and it significantly decreased mice locomotor activity by 61% ($p < 0.05$) as compared to the control group. This indicated that the experimental model for sedative activity in this study was validated. A decrease in locomotor activity was observed at all doses of LCEO in a dose-dependent manner and AUC values were significantly reduced at doses of 0.0004, 0.004 and 0.04 mg by 68%, 48% and 67% respectively ($p < 0.05$), when compared to the control (Figure 4a). The sedative effects produced by the doses of 0.0004 mg and 0.04 mg LCEO were the most effective and locomotor activity dropped nearly to zero after 20-30 min of inhalation administration (Figure 4b). Previous studies on essential oils from *Ocimum gratissimum* L., *Piper guineense* Schumach. & Thonn. and *Zingiber zerumbet* (L.) Roscoe ex Sm. have also reported sedative effects in a dose-dependent manner, supporting the argument that, similar to LCEO, there is a great variety of plant essential oil showing sedative activity, and possibly useful for the treatment of central nervous system-related diseases [2,3,6].

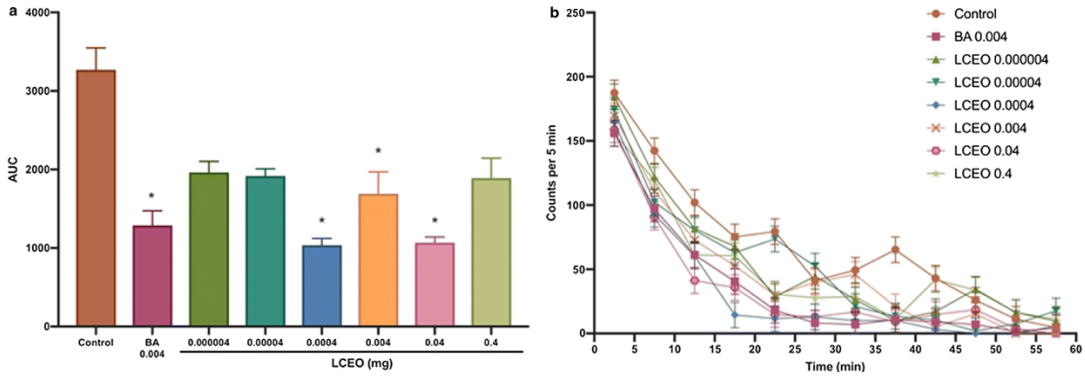


Figure 4: Total spontaneous motor activity (a) and locomotor activity transition (b) of mice treated with LCEO (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg)

Data represent the mean ± SEM of 6 mice. Statistical differences vs. the control group were calculated using ANOVA followed by Dunnett's test. *$p < 0.05$.

The LD_{50} value for 1,8-cineole, one of the main components of LCEO, was 3849 mg/kg [32]. Regarding sabinene, the other main component of LCEO, there were no adverse effects on nidation, reproduction, fetal development, or maternal survival when 224 mg/kg/day sabinene was administered orally for 6-15days to pregnant mice and rats [33]. In my study, the highest administered dose, 0.4 mg LCEO, contains 0.15 mg sabinene and 0.11 mg 1,8-cineole, which are much lower than the concentrations required to induce toxicity. More, during all the experiments, abnormalities, such as an increase in urination or defecation, were not noticed.

To identify the components responsible for activity, LCEO was fractionated to give two fractions which were further investigated for sedative activity. The main components

of fraction 1 (367 mg, yellowish) were sabinene (33.95%), 1,8-cineole (32.95%), *β*-caryophyllene (15.65%), and *α*-pinene (8.75%), while fraction 2 (129 mg, pale yellow) consisted of terpinen-4-ol (61.63%) and (+)-2-bornanone (38.37%). The structures of the different components are represented in Figure 3. Both fractions were administered individually to the mice by inhalation at doses of 0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg per 400 µL of TEC. As shown in Figure 5a, fraction 2 induced a significant decrease in locomotor activity at 0.004 mg ($p < 0.05$). Terpinen-4-ol, one of the major compounds of fraction 2, causes a decrease in spontaneous locomotor activity in mice [34], and its sedative effects are greater than or equal to that of eugenol when administered at the same concentration in silver catfish [35]. The other major component of fraction 2, (+)-2-bornanone, has sedative, anesthetic, and analgesic properties [36,37]. These two compounds therefore seem to explain the sedative activity of fraction 2.

Fraction 1 caused a significant decrease in mouse locomotor activity at doses of 0.0004, 0.004 and 0.04 mg, with the strongest effect observed at 0.004 mg (Figure 5b, $p < 0.05$). Fraction 1 appeared more effective than fraction 2, suggesting that it contained the most active components of LCEO. Of the main compounds of fraction 1, *α*-pinene is an antidepressant previously found to have sedative activity upon inhalation in mice [38,39], whereas *β*-caryophyllene has been reported to possesses sedative, local anesthetic, antidepressant, and anxiolytic activity via a CB2 receptor agonist [3,40]. *β*-Caryophyllene and *α*-pinene are present in relatively high amounts in fraction 1 and could therefore be responsible for the sedative effects. However, sabinene and 1,8-cineole comprised a larger portion of fraction 1 and LCEO, and therefore their sedative effects on mice were examined further.

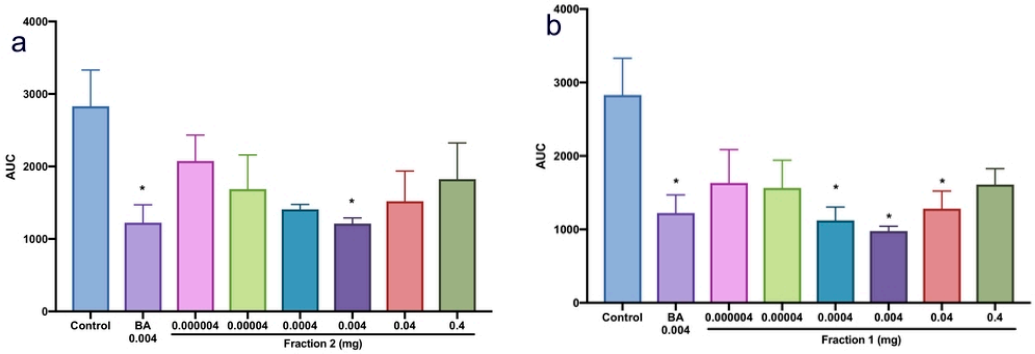


Figure 5: Total spontaneous motor activity of mice treated with fraction 2 and fraction 1 (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg) (a and b respectively)

Data represent the mean ± SEM of 6 mice. Statistical differences vs. the control group were calculated using ANOVA followed by Dunnett's test. *$p < 0.05$.

3.3. Effects of the main compounds sabinene and 1,8-cineole on locomotor activity in mice

The weight of the total administered compounds on filter paper was measured before and after the experiment for sabinene and 1,8-cineole. The weight of each compound was found to be reduced by 80-90% after administration for 1h, indicating that a large portion

of the administered sample had effectively evaporated in the air before the mice were placed in the glass cage.

Sabinene and 1,8-cineole were administered individually to the mice by inhalation at doses of 0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg per 400 µL of TEC. Sabinene decreased locomotor activity at all doses, with a significant effect at the doses of 0.0004 and 0.004 mg (Figure 6a, $p < 0.05$). 1,8-Cineole showed a sedative effect similar to that of LCEO (Figure 6b); however, only 0.0004 mg ($p < 0.05$ when compared to control) demonstrated true sedative activity, because 0.04 mg did not induce a significant effect and the mice displayed excitation behavior such as jumping and rearing. Similar effects have been observed for hexahydroxyzerumbone derivatives [3] and basil essential oil [41].

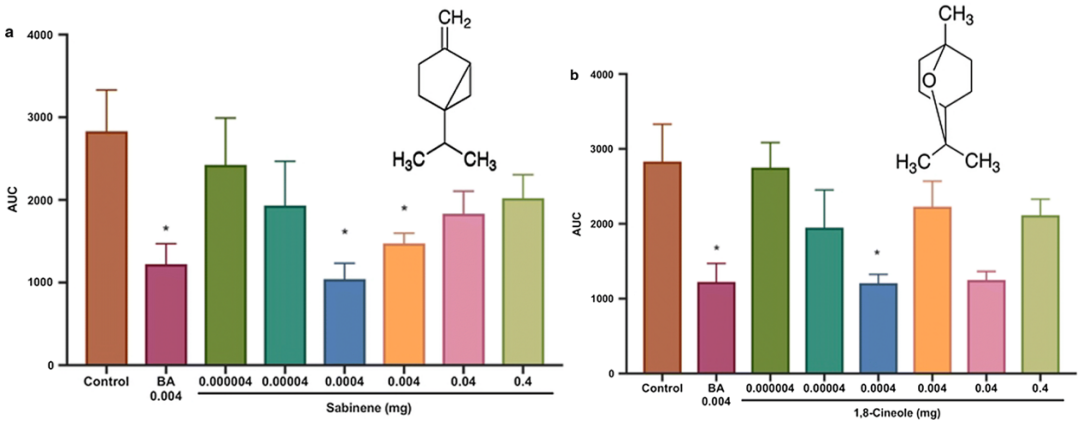


Figure 6: Total spontaneous motor activity of mice treated with sabinene and 1,8-cineole (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg) (a and b respectively)

Data represent the mean ± SEM of 6 mice. Statistical differences vs. the control group were calculated using ANOVA followed by Dunnett's test. *$p < 0.05$.

Sabinene, a natural bicyclic monoterpene, is one of the chemicals that contribute to the flavor of black pepper and is a major constituent of carrot and nutmeg seed oils [42]. It has also been reported to act as an anti-inflammatory agent [43]. Sabinene is a double-bond isomer of thujene, a major constituent of *Hedyosmum brasiliense* Mart., which is also used as a sedative [44]; this suggested a possible sedative activity of sabinene. 1,8-Cineole is a naturally occurring saturated monoterpene found mainly in the essential oils of *Eucalyptus* and has been reported to have smooth muscle relaxant, anti-inflammatory, and antinociceptive effects [45,46]. Also, the sedative effect of two *Eucalyptus* species was suggested to be due to their high 1,8-cineole content [47]. In the present study, I found evidence that sabinene and 1,8-cineole could be used as a potent sedative agent via inhalation. A future study should examine the relationship between the structure and activity of each sabinene, 1,8-cineole and their derivative compounds in order to elucidate the structural features with significant sedative activity.

3.4. Effects of a mixture of sabinene and 1,8-cineole administered to mice via inhalation

Both of the main compounds of LCEO (sabinene and 1,8-cineole) showed sedative activity, and a mixture (ratio 1:1) of these compounds was tested to understand their role in this activity. A mixture of sabinene and 1,8-cineole was administered to mice via inhalation at doses of 0.00008, 0.0008, 0.008, 0.08 or 0.8 mg per 400 µL of TEC, which resulted in a decrease in locomotor activity in a dose-dependent manner. Analysis of AUC values showed a significant decrease at doses of 0.0008, 0.008 and 0.08 mg by 65%, 52% and 63% respectively ($p < 0.05$) as compared to the control group (Figure 7a). Mice in the 0.0008 and 0.08 mg administered groups calmed within 20-30 min (Figure 7b), as it was observed in LCEO.

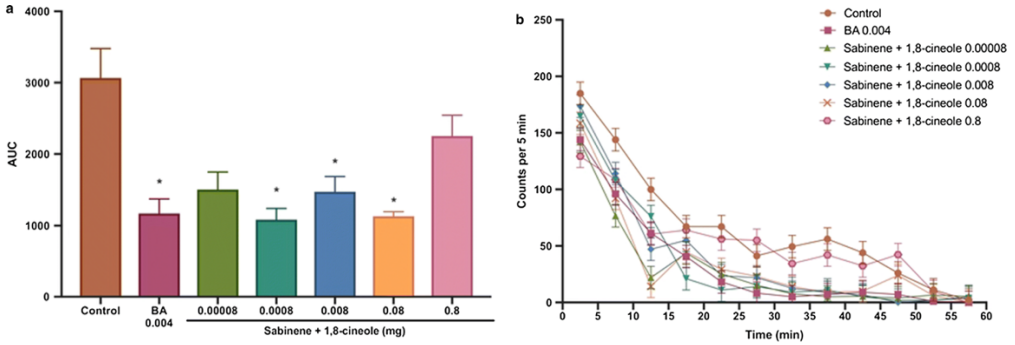


Figure 7: Total spontaneous motor activity (a) and locomotor activity transition (b) of mice treated with a mixture of sabinene and 1,8-cineole (0.00008, 0.0008, 0.008, 0.08 or 0.8 mg)

Data represent the mean ± SEM of 6 mice. Statistical differences vs. the control group were calculated using ANOVA followed by Dunnett's test. *$p < 0.05$.

More, comparison of locomotor activity transition of mice treated with the mixture of sabinene and 1,8-cineole with that of mice treated with LCEO showed that their sedative activity described a similar decrease in the time course. This result suggested that both sabinene and 1,8-cineole were the principal components responsible for sedative activity of LCEO. However, it is important to observe that LCEO contained other compounds than sabinene and 1,8-cineole, and these compounds though present only in a small amount might have combined to cause a sedative activity in LCEO. Similarly, some authors have previously reported a synergistic action of various constituents present in essential oils from plant material [2,48].

The identification of active components is an important step to investigate the effects of plant material. The doses applied to rodents are known to be very different from the effective doses in humans. However, the findings of the present study indicate that LCEO and its related compounds might be useful for the sedation of individuals with dementia, attention deficit hyperactivity disorder, insomnia, or other psychological disorders [8,9]. Previous studies on *O. gratissimum*, *P. guineense*, *Microtoena patchoulii* (C.B.Clarke ex Hook.f.) C.Y.Wu & S.J.Hsuan, *Z. zerumbet*, and *Heracleum afghanicum* Kitam. [2,4,6,9], as well as the present paper, have shown that a number of volatile components from the essential oils of various plants potently reduce the locomotor activity in mice following inhalation administration. Studies are currently underway to investigate the relationship between the structure and activity of LCEO components, and their possible beneficial

actions for the treatment of anxiety and depression. The results of these studies could lead to the use of LCEO as a natural medicine in the management of diseases related to the central nervous system.

4. Conclusion

A great variability in the chemical composition of LCEOs from different regions was demonstrated in the present paper. We also showed that LCEO collected during the rainy season in Houedo (Republic of Benin), possesses strong sedative activity when administered via inhalation, and the major components of the oil, namely sabinene and 1,8-cineole, were identified as principal active compounds. The reduction of locomotor activity may indicate relaxing, anxiolytic or antidepressant effects. Therefore, further investigations using animal models of anxiety and depression are now required to better understand the activities of LCEO and its related compounds.

III. Sleep-promoting effects of *Dysphania ambrosioides* (L.) Mosyakin & Clemants Essential Oil

1. Introduction

Insomnia, or sleeplessness, is a major sleep disorder caused by various factors, such as psychological or chronic stress (e.g., anxiety or depression), physical disturbance (e.g., chronic pain from arthritis or headache), or drugs (e.g., caffeine or nicotine) [49]. It is a major public health issue in both developed and developing countries and affects more than 30% of adults population, with one in seven adults having chronic or long-term insomnia [50,51]. Insomnia is also associated with depression and anxiety, leading to decreased mental activity, memory loss, autonomic dysfunction, or decreased immune function [52]. Current treatments include antidepressants, anxiolytics, sedatives, or hypnotics, such as benzodiazepines (e.g., diazepam, clonazepam) or other drugs (e.g., zolpidem, doxepin) [53]. Unfortunately, these drugs can lead to psychomotor disorders, drug dependence, addiction, tolerance, and memory impairment, and their efficacy is controversial. Therefore, it is necessary to search for new sedative or sleep-promoting molecules with fewer side effects and potent efficacy.

Essential oils commonly contain diverse compounds naturally synthesized by many plants, with acyclic, monocyclic, or bicyclic structures and unsaturated, saturated, or aromatic structural elements [54]. They often have pharmacological effects, such as antifungal, antibacterial, antioxidant, sedative, anxiolytic, and antidepressant activities, due to their diversity and abundance in nature [55–59]. Recently, they have attracted great interest because when administered via the inhalation route (e.g., inhaled essential oils from *Lavandula angustifolia* Mill., *Rosmarinus officinalis* L., *Ferulago angulata* (Schltdl.) Boiss., and agarwood), they have the potential to treat central nervous system (CNS) disorders, such as stress, insomnia, anxiety, and depression [1,60–62].

Dysphania ambrosioides (L.) Mosyakin & Clemants (epazote, Mexican tea, or wormseed), previously known as *Chenopodium ambrosioides* L., is an herbaceous shrub of the Amaranthaceae family native to tropical America. *D. ambrosioides* leaves have pharmacological effects, such as anthelmintic and vermicide, antifungal, antioxidant, antimicrobial, anti-inflammatory, and analgesic activities [55,63–67]. The essential oil from the leaves of *D. ambrosioides* (DAEO) shows great variability in mono- and sesquiterpenes, with ascaridole, carvacrol, *p*-cymene, α-terpinene, and limonene being the major compounds [64,68,69]. In the Republic of Benin (West Africa), with a rich variety of medicinal plants with multiple beneficial properties [70], *D. ambrosioides* is locally known as "*azogbidiwa*". DAEO is used as a traditional medicine mainly for its antibacterial properties, and traditional healers and local populations believe it has sedative activity upon inhalation. To date, however, there are no comprehensive data on DAEO's ability to induce sedation, and the molecular mechanisms underlying its sedative activity have not been reported.

In this work, I investigated for the first time, the sedative effects of DAEO using the open-field test in mice. We also investigated DAEO's chemical composition using phytochemical techniques, such as gas chromatography (GC) and gas chromatography–

mass spectrometry (GC-MS), and compared my data with existing literature in order to identify DAEO's main active compounds and determine their effects on the CNS. In addition, I confirmed DAEO's sleep-promoting effect and action mechanism through the gamma-aminobutyric acid ($GABA_A$)-benzodiazepine receptor system by performing different behavioral investigations, such as caffeine excitation and pentobarbital sleep-induced tests. Finally, I conducted the Rota-rod test to eliminate any potential neuromuscular dysfunction that could cause bias. The data herein reported constitute a solid database of DAEO's chemical composition from different parts of the world and demonstrates pharmacological evidence of DAEO's benefits in sleep. In addition, the data are a promising lead for the management of CNS and stress-related disorders with simple inhalation of natural products.

2. Material and Methods

2.1. Sample collection

We collected fresh *D. ambrosioides* leaves (

Figure 8) in October 2016 from Cotonou in the Republic of Benin (West Africa) and air-dried them. Identification was confirmed by Gaudence Julien Djego of the Laboratory of Botany and Applied Ecology, University of Abomey-Calavi (Benin), and vouchers were deposited in the Herbarium of Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan (specimen number EST-5037), and the National Herbarium of Benin (specimen number AA 6676/HNB).

Figure 8: Leaves of *Dysphania ambrosioides*

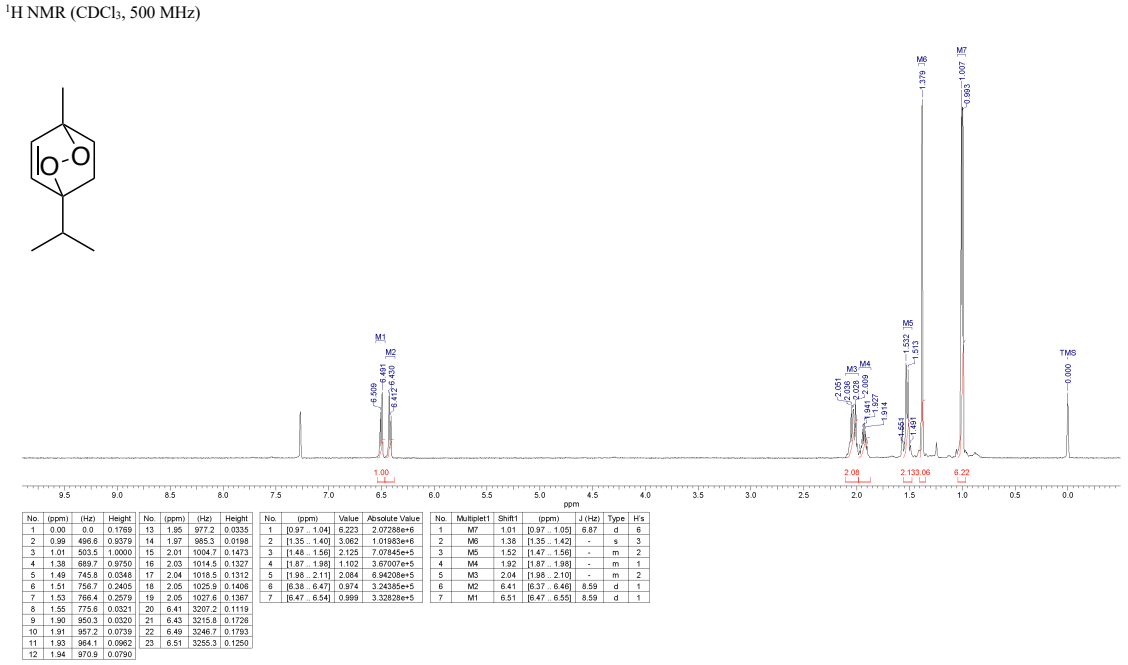
2.2. Extraction and Fractionation of DAEO

We extracted DAEO by hydrodistillation of 32.04 g of *D. ambrosioides* leaves for 3 h using a Clevenger apparatus, as recommended in the Japanese Pharmacopoeia (JP17; http://jpdb.nihs.go.jp/jp17e/). DAEO (780 mg; yield 2.43%) was captured in hexane, dried over anhydrous sodium sulfate, and then concentrated. The headspace of the oil was analyzed by solid phase microextraction (SPME)-GC-MS to confirm that it was hexane free. The obtained oil was stored in sealed vials at 4°C until analysis.

For fractionation, DAEO was subjected to silica gel column chromatography (column diameter 16 mm; column height 170 mm), and the column was eluted with ~250 mL of hexane/acetone (4:1) to obtain fractions 1 and 2. Before future behavioral investigation, each fraction was evaporated and subjected to GC-MS analysis to confirm that no solvent was remaining.

2.3. Synthesis of ascaridole

Ascaridole (1.91 g; 58% yield) was synthetized from *α*-terpinene, following previous methodology, and spectroscopic data (Figure 9) were in good agreement with those reported [71]. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-LA 500 (500 MHz for ^{1}H). Chemical shifts are presented in ppm relative to tetramethylsilane (1H, 0.00). Ascaridole: 1H NMR (500 MHz, $CDCl_3$): δ 1.01 (d, J = 6.9 Hz, 6H), 1.38 (s, 3H), 1.47-1.56 (m, 2H), 1.87-1.98 (m, 1H), 1.98-2.10 (m, 2H), 6.41 (d, J = 8.6 Hz, 1H), 6.41 (d, J = 8.6 Hz, 1H). We are thankful to Assistant Professor Yosuke Yamaoka (Department of Synthetic Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University) for his great help with the synthesis of ascaridole.



No.	(ppm)	(Hz)	Height
1	0.00	0.0	0.1769
2	0.99	498.6	0.9579
3	1.01	503.5	1.0000
4	1.38	689.7	0.9750
5	1.49	745.8	0.0348
6	1.51	756.7	0.2405
7	1.53	766.4	0.2579
8	1.55	775.6	0.0321
9	1.90	950.3	0.0320
10	1.91	957.2	0.0739
11	1.93	964.1	0.0962
12	1.94	970.9	0.0790

No.	(ppm)	(Hz)	Height
13	1.95	977.2	0.0335
14	1.97	985.3	0.0198
15	2.01	1004.7	0.1473
16	2.03	1014.5	0.1327
17	2.04	1018.5	0.1312
18	2.05	1025.9	0.1408
19	2.05	1027.6	0.1367
20	6.41	3207.2	0.1119
21	6.43	3215.8	0.1726
22	6.49	3246.7	0.1793
23	6.51	3255.3	0.1250

No.	(ppm)	Value	Absolute Value
1	[0.97 .. 1.04]	6.223	2.07288e+6
2	[1.35 .. 1.40]	3.062	1.01983e+6
3	[1.48 .. 1.56]	2.125	7.07845e+5
4	[1.87 .. 1.98]	1.102	3.67007e+5
5	[1.98 .. 2.11]	2.084	6.94208e+5
6	[6.38 .. 6.47]	0.974	3.24385e+5
7	[6.47 .. 6.54]	0.999	3.32826e+5

No.	Multiplet1	Shift1	(ppm)	J (Hz)	Type	H's
1	M7	1.01	[0.97 .. 1.05]	6.87	d	6
2	M6	1.38	[1.35 .. 1.42]	-	s	3
3	M5	1.52	[1.47 .. 1.56]	-	m	2
4	M4	1.92	[1.87 .. 1.98]	-	m	1
5	M3	2.04	[1.98 .. 2.10]	-	m	2
6	M2	6.41	[6.37 .. 6.46]	8.59	d	1
7	M1	6.51	[6.47 .. 6.55]	8.59	d	1

Figure 9: ^{1}H NMR spectrum of ascaridole ($CDCl_3$, 500 Mhz)

3. Results and Discussion

3.1. Chemical composition of DAEOs from various regions

Table 2 summarizes the results of GC and GC-MS analyses of DAEO. We identified 11 compounds, which represented 90% of DAEO, with 35.5% 1-methyl-4-propan-2-yl-2,3-dioxabicyclo[2.2.2]oct-5-ene (ascaridole) and 47.2% 1-methyl-4-isopropylbenzene (*p*-cymene) (Figure 10). We next compared the composition of DAEO from this study with samples from different parts the world.

Table 2: Chemical composition of DAEO

N°	Compound[a]	Calculated RI[b]	Literature RI[c]	Peak area (%)[d]
1	(+)-4-Carene	1185	1149	5.1
2	*D*-Limonene	1204	1198	0.87
3	*γ*-Terpinene	1247	1245	0.4
4	***p*-Cymene**	1278	1270	**47.2**
5	1,3,8-*p*-Menthatriene	1394	1411	0.1
6	*p*-Mentha-1,5,8-triene	1426	1453	0.3
7	1-Hexanol, 2-ethyl-	1482	1487	1.3
8	Benzaldehyde	1518	1518	0.6
9	**Ascaridole**	1801	1810	**35.5**
10	Isoascaridole	1914	1851	0.6
11	*trans*-Ascaridole glycol	2043	2073	0.7
	Unidentified			7.3
	Total			100

[a]Order of elution determined using a DB-wax column.
[b]Retention index, calculated against C10–C26 *n*-alkanes on a DB-wax column.
[c]Retention index, taken from the NIST library.
[d]Peak area percentage determined by calculating the peak area of the FID chromatogram in GC analyses.
DAEO, essential oil obtained from *Dysphania ambrosioides* (L.) Mosyakin & Clemants leaves; RI, retention index; GC, gas chromatography.

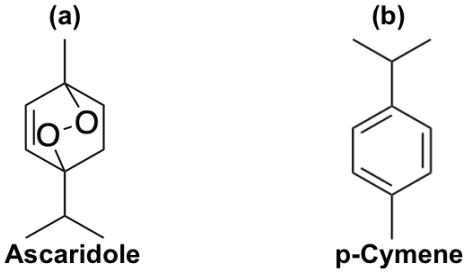


Figure 10: Chemical structures of ascaridole (a) and *p*-cymene (b)

We found a great quantitative and qualitative variation in the chemical composition of DAEO (Figure 11). Similar to my results, ascaridole (14.75%–61.92%) and *p*-cymene (20.2%–36.3%) were the major components of DAEO in samples from India, Cuba, Togo, Cameroon, China, and Argentina [69,72–77]. However, in samples from East Mediterranean, Yemen and Brazil, while ascaridole (46.9%–61.3%) was the main component [68,78,79], *p*-cymene was relatively low (2%–8.1%). In contrast, in some samples from Cameroon, Brazil, Nigeria, Mexico, and India, *p*-cymene (14.5%–50%) was the main component [55,80–85], while ascaridole was low or non-existent (0%–6.2%). Isoascaridole, the isomer of ascaridole, which was detected in trace amounts in this study (0.58%), was one of the main components in some samples from Yemen (54.2%) and China (13%) [79,86], while some samples from Cameroon, India, Morocco, and Nigeria mainly contained α-terpinene (47.37%–63.6%) [55,69,81,85,87], which was not detected in my sample. Overall, most of the chemical profiling of DAEO from different parts of the world showed between 10 and 15 components, with ascaridole and *p*-cymene being the predominant ones.

Until recently, ascaridole was known as a compound characteristic to DAEO; however, my results showed that both ascaridole and *p*-cymene are exclusive compounds present in almost all DAEO samples in relatively important doses. Therefore, these two compounds should be considered in DAEO-related studies. The great variability in the chemical composition of DAEO from different parts of the world can be explained by differences in climate, altitude, time of collection and soil, or the method of extracting DAEO, and the chemical composition is sensitive to the developmental stage and genetic, geographical, and seasonal factors.

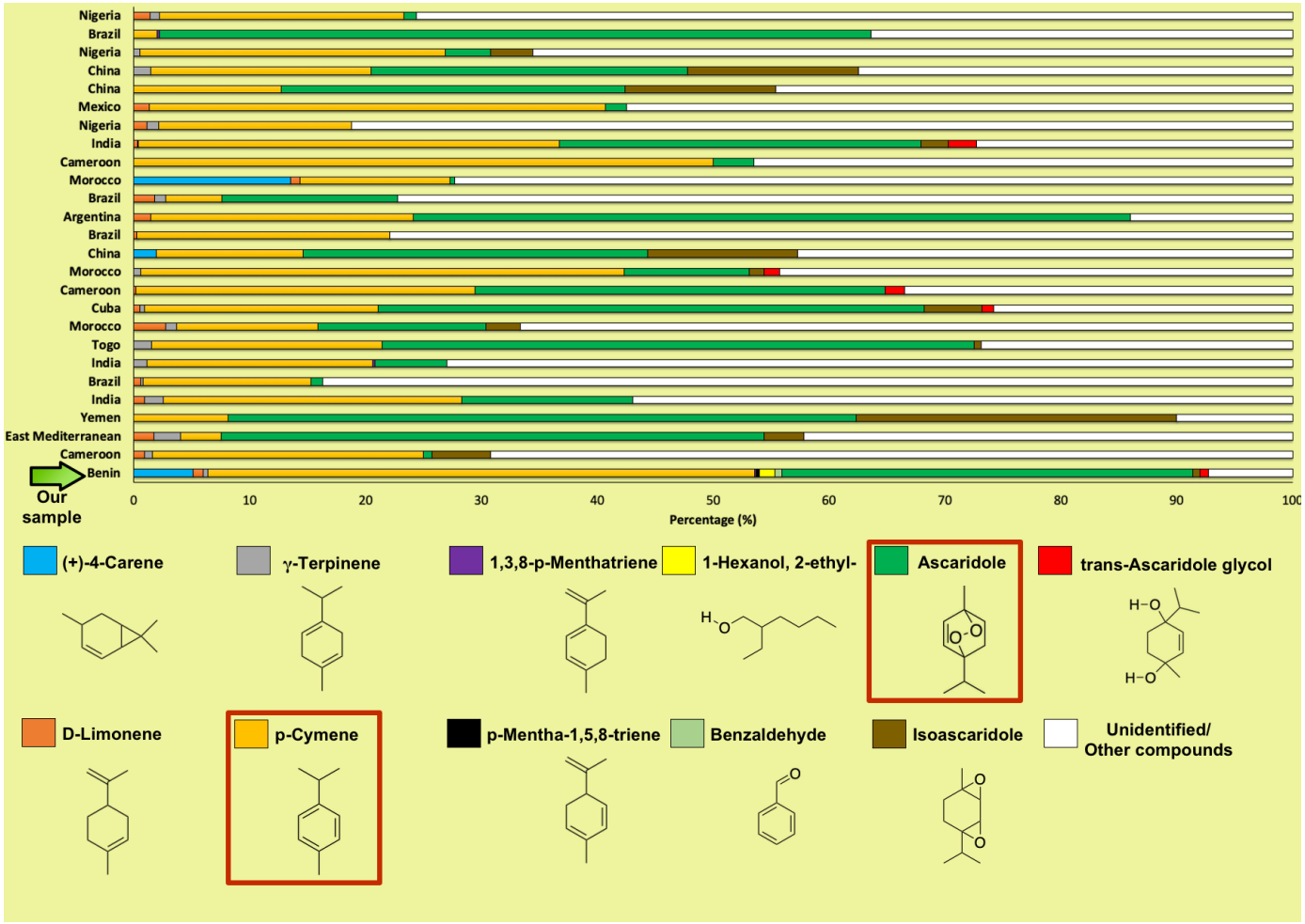


Figure 11: Chemical composition of DAEO in my study compared to DAEO from different parts of the world.

Ascaridole and *p*-cymene (red frame) were the predominantly reported compounds in most of DAEO from different parts of the world. DAEO, essential oil obtained from *Dysphania ambrosioides* (L.) Mosyakin & Clemants leaves.

3.2. Sedative effects of DAEO and its fractions

Following previous experiments [1,88,89], I administered different inhalation doses of DAEO (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg) dissolved in 400 μL of triethyl citrate (TEC); the control group was given only TEC. Lavender oil (positive control) significantly decreased locomotor activity by 62% ($P < 0.001$) compared to the control group. DAEO decreased locomotor activity at all doses in a dose-dependent manner ($F[7, 40] = 5.691$; $P < 0.001$), with an inverted U-shaped dose–response curve. The area under the curve (AUC) values significantly decreased at 0.000004, 0.0004, 0.004, 0.04, and 0.4 mg per 400 µL of TEC by 42%, 40%, 45%, 62%, and 66%, respectively, compared to the control group (Figure 12a). With regard to locomotor activity transition (Figure 12b), mice started sleeping from 15 min after inhalation of DAEO until their locomotor activity completely stopped. Given the strong sedative effect of DAEO via inhalation on mice, I was curious about both the action mechanisms and the compounds responsible for the decrease in locomotor activity as well as the unconventional dose–response curve.

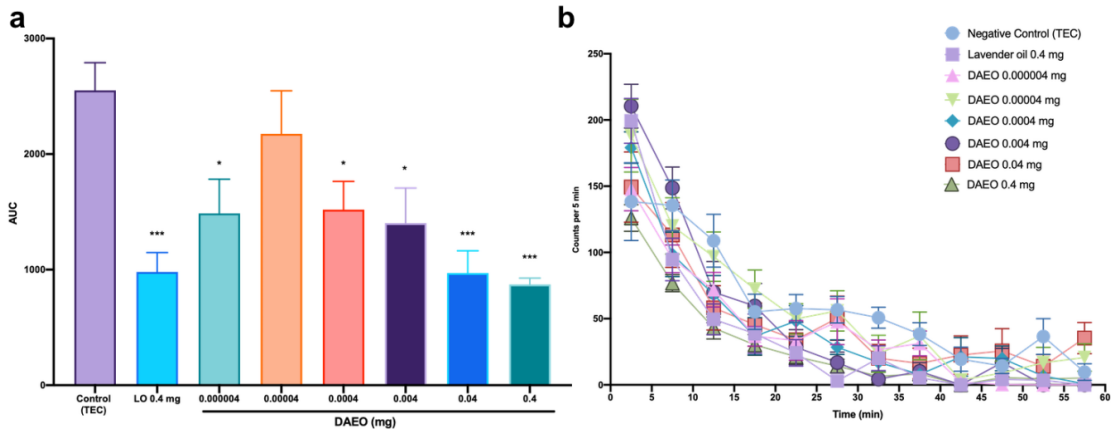


Figure 12: Total spontaneous locomotor activity (a) and locomotor activity transition (b) of mice treated with different doses of DAEO (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg per 400 µL of TEC).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using one-way ANOVA, followed by Dunnett's test. *$P < 0.05$; ***$P < 0.001$. DAEO, essential oil obtained from *Dysphania ambrosioides* (L.) Mosyakin & Clemants leaves; TEC, triethyl citrate; LO, lavender oil; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

To identify the compounds responsible for the decrease in locomotor activity, I fractionated DAEO and obtained two fractions, which I analyzed for pharmacological activity. To investigate their possible sedative activity, fraction 1 (221 mg), mainly containing ascaridole, and fraction 2 (473 mg), mainly containing *p*-cymene, were administered separately via inhalation to mice at 0.0004, 0.004, and 0.04 mg per 400 µL of TEC. Bioactivity-guided DAEO fractionation showed that both fractions are active. Fraction 1 significantly decreased locomotor activity at all three doses ($F[4, 25] = 15.22$; $P < 0.001$) by 65%, 63%, and 85%, respectively, compared to the control group (Figure 13). Fraction 2 decreased locomotor activity, mainly at 0.004 and 0.04 mg per 400 µL of TEC ($F[4, 25] = 15.29$; $P < 0.001$) by 72%–73% compared to the control group (Figure

14). These results indicated that both fractions contain active ingredients, which demand further investigations of ascaridole and *p*-cymene.

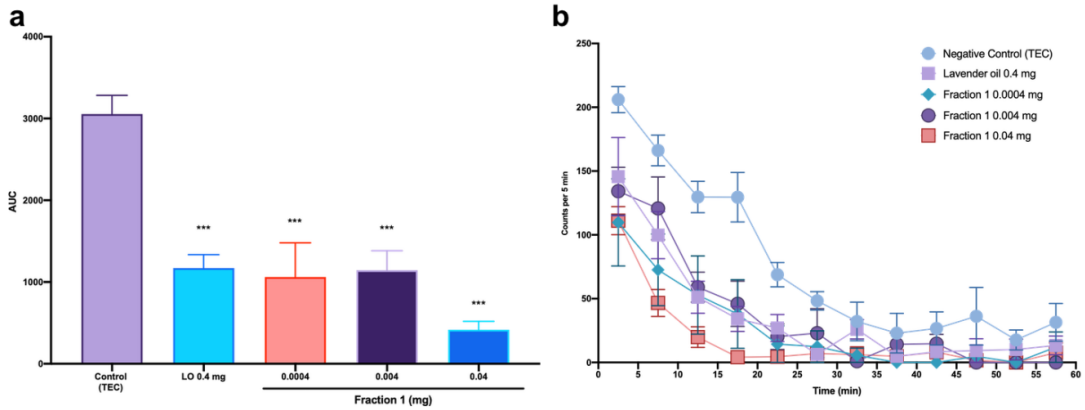


Figure 13: Total spontaneous locomotor activity (a) and locomotor activity transition (b) of mice treated with different doses of fraction 1 (0.0004, 0.004, or 0.04 mg per 400 µL of TEC).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using one-way ANOVA, followed by Dunnett's test. ***$P < 0.001$. TEC, triethyl citrate; LO, lavender oil; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

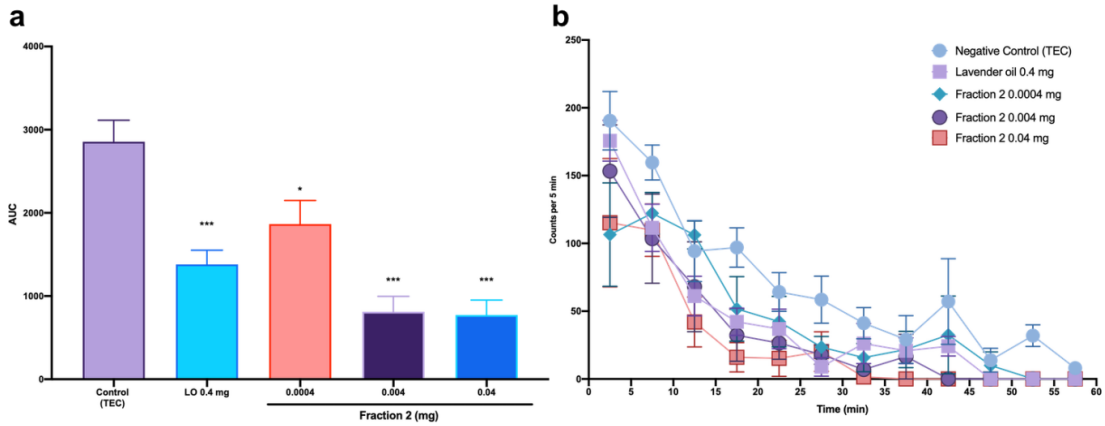


Figure 14: Total spontaneous locomotor activity (a) and locomotor activity transition (b) of mice treated with different doses of fraction 2 (0.0004, 0.004, or 0.04 mg per 400 µL of TEC).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using one-way ANOVA, followed by Dunnett's test. *$P < 0.05$; ***$P < 0.001$. TEC, triethyl citrate; LO, lavender oil; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

3.3. Sedative activities of ascaridole and *p*-cymene

Mice were administered ascaridole and *p*-cymene individually at the same doses as DAEO. Benzylacetone (positive control), a compound with reported sedative activity [1,88], significantly decreased locomotor activity in the tests for ascaridole and *p*-cymene by 56% and 63%, respectively ($P = 0.004$ and $P < 0.001$, respectively). The weights of ascaridole and *p*-cymene were measured on filter paper before and after experiments. The weight of each compound decreased by 80%–90% after administration for 1 h, indicating

that a large portion of the administered sample effectively evaporates in air before the mice are placed in the open-field arena, which is consistent with previous studies [88].

Ascaridole is an organic heterobicyclic peroxide that is mainly found in essential oils of *Chenopodium* sp. and *Peumus boldus* Molina [90], and has antinociceptive and anti-inflammatory, antimalarial, antitrypanosomial, larvicidal, and nematocidal activities [67,91,92]. In this study, ascaridole significantly decreased locomotor activity at doses of 0.00004–0.4 mg per 400 µL of TEC by 64%, 52%, 67%, 64%, and 56% respectively, compared to the control group (F[7, 40] = 4.206; P = 0.001) (Figure 15). In a previous study, 100 mg/kg ascaridole increased sleeping time and decreased locomotor activity when administered intraperitoneally to mice [93]. In addition, plant species mainly containing ascaridole, such as *Achillea arabica* Kotschy, have a sedative effect, as shown by a decrease in locomotor activity in an open-field test, and anxiolytic activity, as shown by an increase in the time spent in the open arm of the elevated plus maze test [94].

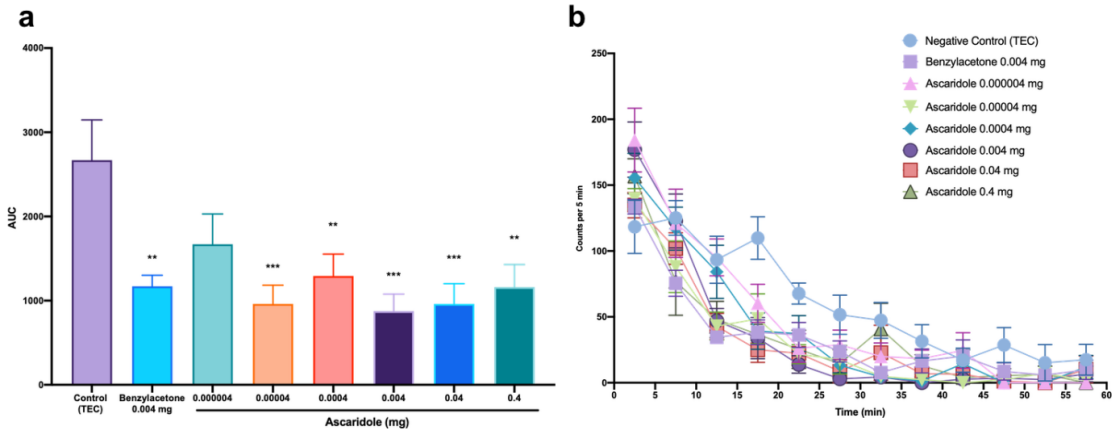


Figure 15: Total spontaneous locomotor activity (a) and locomotor activity transition (b) of mice treated with different doses of ascaridole (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg per 400 µL of TEC).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using one-way ANOVA, followed by Dunnett's test. **P < 0.01; ***P < 0.001. TEC, triethyl citrate; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

In this study, I demonstrated for the first time the sedative activity of ascaridole upon inhalation in mice. In recent years, administration by inhalation has become a simple and non-invasive way of giving drugs and is being used to investigate sedative, analgesic, anxiolytic, and antidepressant activities in both humans and animals [1,88,95–98]. The most effective dose of ascaridole in my study was 0.004 mg per 400 µL of TEC, with a U-shaped dose–response curve [1] from 0.0004 to 0.4 mg per 400 µL of TEC. However, on the left side, the pattern of the curve changed at 0.00004 mg per 400 µL of TEC, and a lower dose of 0.000004 mg per 400 µL of TEC formed a double U-shaped dose–response curve, as previously reported [88]. However, I found no statistical significance, and the effect was considered irrelevant. Regarding the curve pattern and the mechanisms I will discuss later, I believe that lower doses, such as 0.0000004 mg per 400 µL of TEC, or higher doses, such as 4 or 40 mg per 400 µL of TEC, on both sides of the curve would form a more sophisticated curve pattern with multiple biphasic effects. Nevertheless, it is first essential to understand the results reported from 0.000004 to 0.4 mg per 400 µL of TEC with regard to my previous experiments. In this sense, why a low dose of 0.00004

mg per 400 µL of TEC is effective is unclear and, in addition to the unconventional dose–response pattern observed, demands further investigation.

p-Cymene is a naturally occurring monoterpene with two geometric isomers, *m*-cymene and *o*-cymene, the latter not occurring naturally [99]. *p*-Cymene has a characteristic woody and spicy odor and has been recognized as safe by the US Food and Drug Administration [100]. *p*-Cymene is found in the essential oils of many plant species, such as *D. ambrosioides* (16.2%) [64], *Origanum vulgare* L. (11.5%–35.7%) [101], and *Thymus* sp. (9.80%–15.2%) [102]. Studies have reported its analgesic, antinociceptive, and anti-inflammatory properties [103,104], in addition to its use as an antioxidant, antibacterial, antifungal, and antiparasitic agent [105–108]. Interestingly, among monoterpenes, *p*-cymene is the biological precursor of carvacrol, which has sedative, anxiolytic, and antidepressant activities and interacts with different receptors sites in the CNS [109,110]. This study showed for the first time the sedative activity of inhaled *p*-cymene in a mouse model. Inhaled *p*-cymene showed a pattern similar to DAEO, with all doses being significantly effective (Figure 16). The most effective doses were 0.000004, 0.00004 mg per 400 µL of TEC, and 0.004–0.4 mg per 400 µL of TEC with a decrease of 69%, 57%, 61%, 67%, and 68%, respectively, compared to the control group (F[7, 40] = 8.554; $P < 0.001$). Similar to my results, *p*-cymene showed depressant activity on the CNS, such as a decrease in spontaneous locomotor activity, analgesia, and sedation, at intraperitoneal doses of 50 and 100 mg/kg [104]. The sole exception when comparing the results of *p*-cymene and DAEO is that the top edge of the inverted U-shaped dose–response curve is 0.00004 mg per 400 µL of TEC for DAEO and 0.0004 mg per 400 µL of TEC for *p*-cymene. One possible reason is that DAEO contains multiple compounds, while *p*-cymene is a single compound. A mixture of different compounds in diverse ratios can change the configuration in DAEO. More, ascaridole and *p*-cymene are individually effective at 0.00004 mg per 400 µL of TEC, while, interestingly, DAEO is not. This supports my hypothesis that other active or inactive compounds in DAEO, such as benzaldehyde, γ-terpinene, and (+)-4-carene, can have antagonistic or synergistic effects at these doses. Fujiwara et al. [16] obtained the same results, showing that a mixture of five different active oils produces a synergistic or antagonistic effect, leading to diminution or increase of pharmacological activity, compared to individual oils.

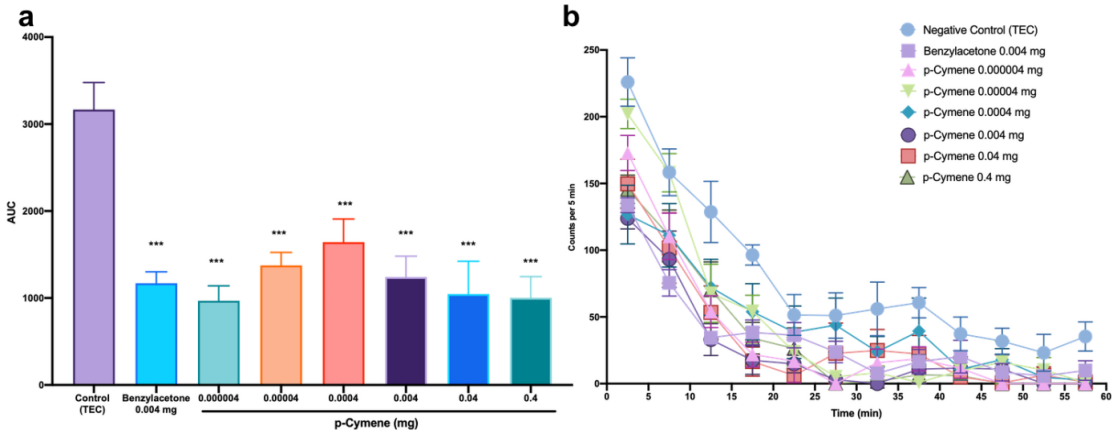


Figure 16: Total spontaneous locomotor activity (a) and locomotor activity transition (b) of mice treated with different doses of *p*-cymene (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg per 400 µL of TEC).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using one-way ANOVA, followed by Dunnett's test. ***$P < 0.001$. TEC, triethyl citrate; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

3.4. Suggested mechanisms for the activity of ascaridole and *p*-cymene

We showed evidence of how ascaridole and *p*-cymene can be used as potent sedative agents via inhalation. *p*-Cymene is more potent than ascaridole, and both compounds play a major role in DAEO's sedative activity. Of particular interest are the inverted U-shaped dose–response curves formed by *p*-cymene and DAEO in decreasing locomotor activity in mice, even at low doses. Similar dose–response curves have been previously reported in several compounds targeting the CNS [111–114], and these dose–response curves have also been reported as nonmonotonic dose–response curves (NMDRCs). NMDRCs are often represented by a U shape (maximal responses at both extremities of the curve and the nadir at intermediate levels) or an inverted U shape (nadirs at both extremities of the curve and maximal responses at intermediate levels), as shown in this study. However, more complicated dose–response curves exist, with maximal responses at multiple intermediate levels and both extremities [88,115,116]. According to Kohn and Melnick [116], NMDRCs require the existence of excessive unoccupied receptors that are available to bind with a compound and in case of an inverted U-shaped curve, recruitment of the compound is weaker compared to an endogenous ligand, acting as an activator or a repressor. At low doses, both the endogenous ligand and the compound bind to receptors and activate a response; however, at high doses, the receptors become fully occupied, leading to an opposed pharmacological response. Relevantly, the effects of high doses can be due to the action via the binding of multiple receptors, while the effects of low doses can be due to the action via only a single receptor. However, although these arguments sound scientifically valid and can be relied upon, additional studies should be conducted.

The median lethal dose (LD_{50}) of ascaridole is 400 mg/kg in mice [117], which is 1000-fold the highest dose administered in this study. The LD_{50} of *p*-cymene is 4750 mg/kg [118], and 0.4 mg DAEO contains 0.14 mg of ascaridole and 0.19 mg of *p*-cymene. These are insignificant doses to induce toxicity. In addition, in all my experiments, abnormalities, such as jumping, rearing, or an increase in urination or defecation, were not reported. At 25°C, the vapor pressure of ascaridole and *p*-cymene is 1.78 and 1.46 mmHg, respectively, and their boiling point is 112°C–115°C and 177.1°C, respectively, which could explain their easy absorption due to high volatility. Hu et al. investigated the mean plasma levels of ascaridole and *p*-cymene following administration to rats, and results showed that they are rapidly absorbed into the circulatory system [119]. In addition, ascaridole and *p*-cymene are both derived from the conjugated symmetric α-terpinene, and possess the same alkylbenzene skeletal structure. The similarity in structure, physical properties, and biosynthesis pathway can explain the potential activity of ascaridole and *p*-cymene. To further investigate the action mechanisms of ascaridole and *p*-cymene, I performed the open-field test using an excitant agent, caffeine, on the CNS.

3.5. Effects of caffeine-induced stimulation of locomotor activity in mice

A sleep-promoting effect is better observed in a test using caffeine to induce locomotor hyperactivity in mice. Following intraperitoneal administration of caffeine, I measured the total spontaneous locomotor activity for 1 h every 5 min (Figure 17). Caffeine, a strong excitant particularly acting on the cerebral cortex, significantly increased spontaneous locomotor activity by 68% ($P < 0.001$) compared to the control group, which received no drug. Diazepam (positive control) decreased the excitation provoked by caffeine by more than half ($P < 0.001$), which validated the experimental model. Interestingly, when caffeine-treated mice were exposed to the most efficient dose of ascaridole (0.004 mg per 400 µL of TEC), spontaneous locomotor activity decreased by 47% ($P = 0.003$), which was similar to the decrease in the diazepam group, indicating inhibition of the caffeine-induced increase in spontaneous locomotor activity. Similarly, the most efficient dose of *p*-cymene (0.000004 mg per 400 µL of TEC) decreased spontaneous locomotor activity of mice previously excited with caffeine by 50% ($P = 0.001$). These results showed that ascaridole and *p*-cymene are both potential depressants of the CNS. Similarly, inhalation of cedrol, a major component of cedarwood oil, reverses the activity of caffeine [120].

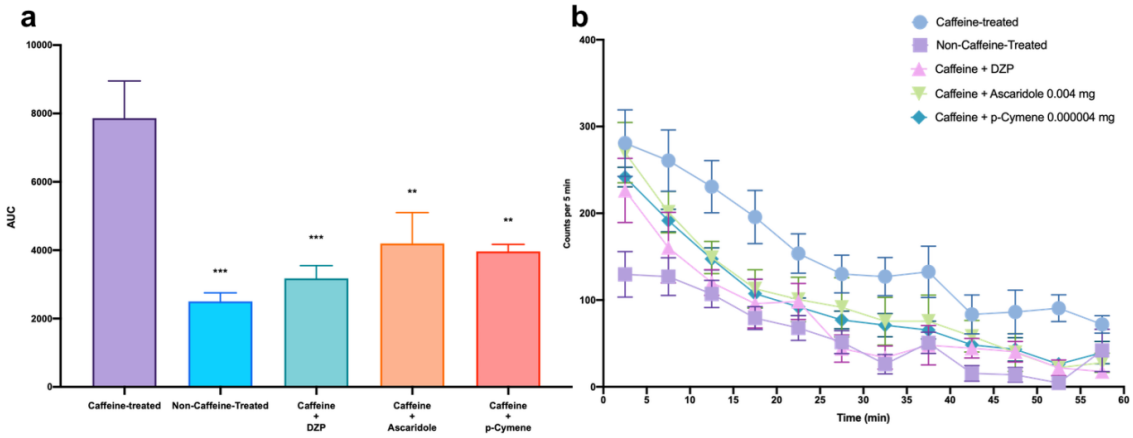


Figure 17: Total spontaneous locomotor activity (a) and locomotor activity transition (b) of mice excited with caffeine (25 mg/kp intraperitoneally) and treated with ascaridole (0.004 mg per 400 µL of TEC) or *p*-cymene (0.000004 mg per 400 µL of TEC).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's *t*-test or ANOVA, followed by Dunnett's test. $^{**}P < 0.01$; $^{***}P < 0.001$. DZP, diazepam; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

3.6. Evaluation of the role of the GABAergic receptor system in ascaridole and *p*-cymene activities

Pentobarbital has sleeping or sedative activity by enhancing the activity of GABA receptors. Therefore, it is possible to evaluate the possible action of pharmacological agents on the GABAergic system by using the pentobarbital-induced sleeping test. In regard to my previous results, I hypothesized that ascaridole and *p*-cymene prolong the

pentobarbital-induced sleeping time upon inhalation by depressing the CNS. To verify the experimental model, I first administered diazepam to pentobarbital-treated mice. Diazepam prolonged the pentobarbital-induced sleeping time by 62% ($P < 0.001$) (Figure 18). Similarly, ascaridole and *p*-cymene prolonged the sleeping time of mice by 42% and 77%, respectively ($P = 0.002$). These results validated my hypothesis and confirmed that the mechanisms underlying the sedative activity of ascaridole and *p*-cymene are through the action via the GABA receptor. To better understand the action mechanisms, I administered flumazenil, a $GABA_A$ receptor antagonist, together with ascaridole and *p*-cymene each. Interestingly, flumazenil decreased the duration of sleep by 31%, confirming that after being absorbed into the circulatory system, ascaridole and *p*-cymene reach the CNS and act on the receptors of neurotransmitters specifics to $GABA_A$ receptors to induce sedation. Essential oil components such as 1,8-cineole, (+)-borneol, camphor, and α-thujone also show potentiation of $GABA_A$ receptors [98,121].

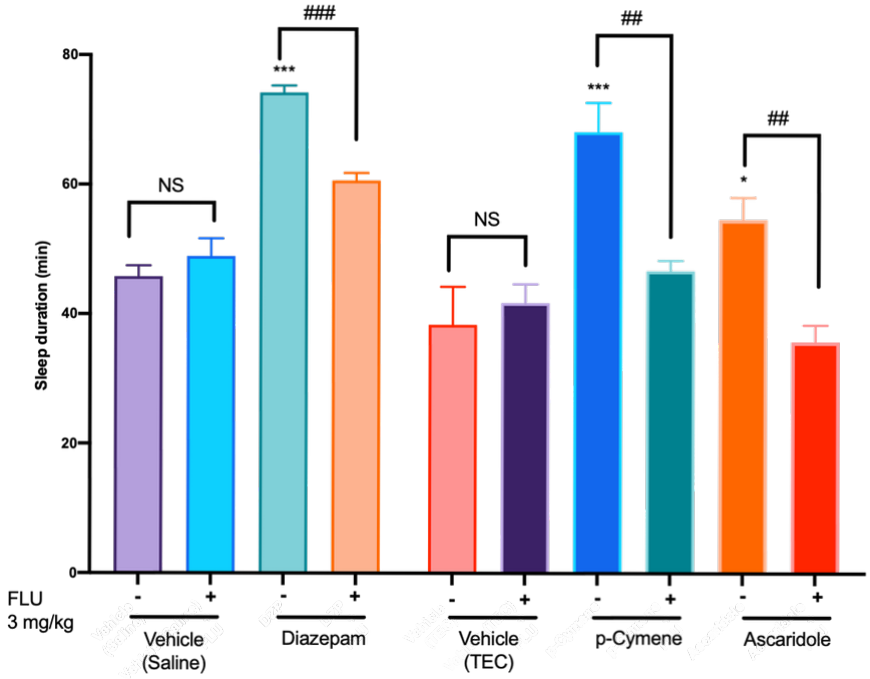


Figure 18: Sleep duration in the pentobarbital-induced sleeping test of mice treated with ascaridole (0.004 mg per 400 µL of TEC) or *p*-cymene (0.000004 mg per 400 µL of TEC), diazepam (3 mg/kg), or flumazenil (3 mg/kg).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's *t*-test or ANOVA, followed by Dunnett's test. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. the control group; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ for flumazenil treatment vs. treatment without flumazenil. TEC, triethyl citrate; FLU, flumazenil; NS, not significant; SEM, standard error of the mean; ANOVA, analysis of variance.

Further, a decrease in locomotor activity can be due to muscular dysfunction, and to ensure my results were due to sedation and not motor dysfunction, I conducted the Rota-rod test in mice.

3.7. The rota-rod test shows no motor function impairment in mice

The Rota-rod test is a simple and efficient way of assessing motor coordination and balance in rodents. It has been used to investigate the effects of essential oils or volatile compounds on the CNS [7,122]. Diazepam (positive control) significantly decreased the latency of mice to fall from the bar ($P = 0.03$), indicating a myorelaxant effect (Figure 19). However, neither ascaridole nor *p*-cymene had a motor coordination defect and there was no significant change in the latency of mice to fall compared to the control group ($P = 0.84$). Therefore, the decrease in locomotor activity in this study is not associated with motor incoordination or imbalance. The same results were previously demonstrated for other volatile compounds, such as aristolen-1(10)-en-9-ol or *Matricaria chamomilla* L. essential oil, administered via inhalation [7,123]. However, it is important to note that some monoterpenes, such as myrcene and citral, have sedative activity associated with motor system incoordination at high doses when administered intraperitoneally [59], indicating the beneficial effects of administration by inhalation.

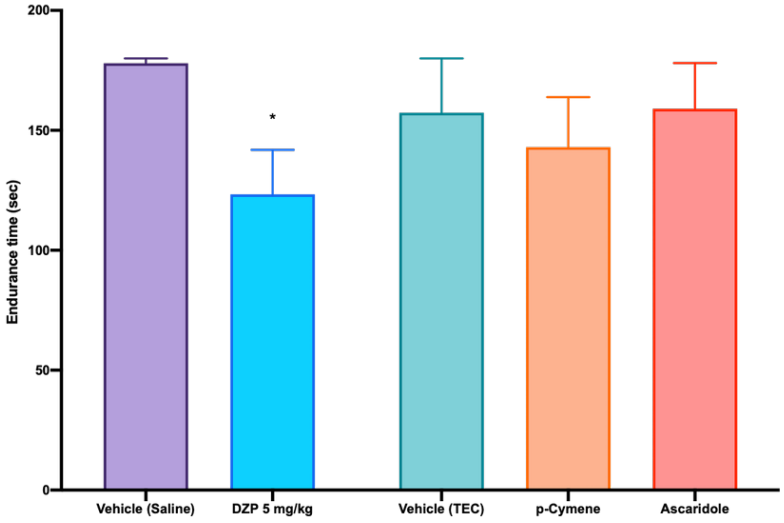


Figure 19: Endurance time in the Rota-rod test of mice treated with ascaridole (0.004 mg per 400 µL of TEC) or *p*-cymene (0.000004 mg per 400 µL of TEC).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's *t*-test or ANOVA, followed by Dunnett's test. *$P < 0.05$. DZP, diazepam; TEC, triethyl citrate; SEM, standard error of the mean; ANOVA, analysis of variance.

4. Conclusion

DAEO from different parts of the world show an interesting variability in their chemical composition. DAEO has a sleep-promoting effect via administration by inhalation, as confirmed by a decrease in locomotor activity in mice to nearly zero. Ascaridole and *p*-cymene, major components of DAEO, individually decrease spontaneous locomotor activity and prolong the pentobarbital-induced sleeping time in mice, indicating that they might be majorly responsible for DAEO's sedative effect. A decrease in locomotor activity is often associated with relaxing, anxiolytic, or

antidepressant activity. Therefore, DAEO and its related compounds might be useful for sedating individuals with insomnia, attention deficit hyperactivity disorder, dementia, or other psychological disorders. However, further investigations using animal models of anxiety and depression are required to better understand the activities of DAEO and its related compounds.

IV. Sleep-promoting effects of *Chromolaena odorata* (L.) R.M.King & H.Rob essential oil

1. Introduction

Essential oils have been used for their beneficial properties in human medicine, due to their abundance and diversity in nature. Recently, they have demonstrated antifungal, antibacterial, sedative, anxiolytic, antidepressant, antioxidant, and analgesic activities [56,62,94,98,124,125]. However, there are still multiple aspects of essential oils that have not been explored and need unravelling. Inhalation administration is a safe and recognized way of administering essential oils, and it has proven to be effective especially when addressing the central nervous system (CNS) [5,60,96].

Chromolaena odorata (L.) R.M.King & H.Rob (Compositae), previously known as *Eupatorium odoratum* L., is a scrambling perennial shrub native to the Americas, with straight stems which bear three-veined, opposite, ovate-triangular leaves and with a shallow, fibrous root system [126]. It grows up to 2–3 m in height, and can reach up to 5–10 m. Within its native range, *C. odorata* shows marked morphological variability in terms of flower color, leaf shape and smell of the crushed leaves. In some parts of the world, several forms and their intermediates co-occur, while in others, the population appears homogeneous [127]. *C. odorata* has been the subject of numerous ethnobotanical and ethnopharmacological investigations in the world for its various pharmacological properties such as wound healing activity, antioxidant, stomachic, hemostatic, anti-malarial and antimicrobial activities [128–131].

The essential oil from the leaves of *C. odorata* (COEO) has frequently revealed a diversity of chemotypes. For example, in a report from Cameroon and Congo [132], *α*-pinene and *p*-cymene were the main compounds whereas, in a sample from India [133], geijerene, *α*-copaene, 3-carene and *β*-caryophyllene have been identified as the major constituents. Another report from Ivory Coast [134] identified pregeijerene and germacrene-D as major compounds. Therefore, the chemical composition of COEO is quite diverse and interesting, which demands further exploration.

In the Republic of Benin (West Africa) which is rich in multiple plant species [70], *C. odorata* is praised for its numerous therapeutic effects such as antiseptic and antibacterial activities. The leaves are applied on wounds which accelerate their healing, and are also used to treat skin irritations. In addition, the inhaled leaves are used to treat respiratory diseases. The plant exhibits a strong fragrance and existing data on its chemical composition suggests the presence of some compounds reported to have sedative activity upon inhalation, such as *α*-pinene or *p*-cymene [97,125]. From the above, I hypothesized that COEO could possess a sedative activity when inhaled. To the best of my knowledge, no study has evaluated the sedative activity of COEO, nor investigated its molecular mechanisms. Therefore, the present study was undertaken to shed light on the sedative activity of COEO. Together with the evaluation of the action mechanisms via the gamma-aminobutyric acid ($GABA_A$)-benzodiazepine receptor system, it also investigated the chemical composition of the oil in order to identify the key components responsible for activity. The data reported in the present study adds a plus to the scientific knowledge of the plant and introduces a novel literature for the treatment of CNS and stress-related disorders by using essential oils from plant material.

2. Materials and Methods

2.1. Plant Material

Fresh leaves of *C. odorata* (

Figure 20) were collected in October 2016 from Abomey-Calavi in the Republic of Benin (West Africa) and air-dried. Identification was confirmed by Gaudence Julien Djego of the Laboratory of Botany and Applied Ecology, University of Abomey-Calavi (Benin), and vouchers were deposited in the Herbarium of Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan (specimen number EST-5038), and the National Herbarium of Benin (specimen number AA 6675/HNB).

Figure 20: Leaves of *Chromolaena odorata*

2.2. Preparation and Fractionation of COEO

We extracted COEO by hydrodistillation of 66.98 g of *C. odorata* leaves for 3 h using a Clevenger apparatus, as recommended in the Japanese Pharmacopoeia (JP17; http://jpdb.nihs.go.jp/jp17e/). COEO (690 mg; yield 1.03%) was captured in hexane, dried over anhydrous sodium sulfate, and then concentrated. The headspace of the oil was analyzed by solid phase microextraction (SPME)-GC-MS to confirm that it was hexane free. The obtained oil was stored in sealed vials at 4°C until analysis.

For fractionation, COEO was subjected to silica gel column chromatography (column diameter 16 mm; column height 170 mm), and the column was eluted with about 250 mL

of hexane/acetone (4:1) to obtain fractions 1 and 2. Before the behavioral investigations, each fraction was evaporated until there was no solvent remaining.

3. Results and Discussion

3.1. Chemical composition of COEOs from different regions

The results of the chemical analyses of COEO performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) are presented in Table 3. In total, about 15 compounds were identified, representing more than 95% of the oil. A comparison of the composition of COEO in the present study (Republic of Benin, West Africa) with COEOs from different regions of the world was performed, to better understand the various chemotypes existing in *C. odorata*.

Table 3: Chemical composition of COEO.

N°	Compounds[a]	Peak area (%)[b]	Calculated RI[c]	Literature RI[d]
1	Geijerene	5.68	1298	1338
2	*α*-Copaene	2.84	1461	1491
3	Benzene, 1,2,4-triethyl-	1.14	1501	1501
4	Linalool	3.98	1504	1543
5	Pinocarvone	1.70	1535	1575
6	*β*-Caryophyllene	1.70	1559	1588
7	*β*-Copaen-4-*α*--ol	2.27	1571	1594
8	*trans*-Pinocarveol	0.57	1611	1661
9	Anisole	7.39	1627	
10	*trans*-Verbenol	4.55	1633	1680
11	Germacrene D	7.39	1661	1708
12	Verbenone	2.84	1664	1720
13	*p*-Mentha-1,5-dien-8-ol	0.57	1673	1738
14	Naphthalene	9.09	1690	1755
15	Caryophyllene oxide	43.75	1916	1986
	Unidentified	4.55		
	Total	100		

[a]Order of elution determined using a DB-wax column.
[b]Peak area percentage determined by calculating the peak area of the FID chromatogram in GC analyses.
[c]Retention index, calculated against C_{10}–C_{26} *n*-alkanes on a DB-wax column.
[d]Retention index, taken from the NIST library.
COEO, essential oil obtained from *Chromolaena odorata* leaves; RI, retention index; GC, gas chromatography.

As demonstrated in Figure 21, I found and report here the existence of various chemotypes in COEOs. A study conducted in Nigeria demonstrated that the oil was dominated by *α*-pinene (42.2%), *β*-pinene (10.6%) and germacrene D (9.7%). Another work performed in the Republic of Benin (Godomey) revealed similar composition in addition to pregeijerene (14.6%) as major constituent. Other chemotypes of COEO were represented by geijerene (26.34%), *α*-copaene (17.87%) and *β*-caryophyllene (11.14%) in a sample from India. Phytol (11%) was a major constituent in a sample from Ivory Coast, whereas it was rarely present in other studies and was not detected in my sample. In Thailand, only pregeijerene (40.6%) and dauca-5,8-diene (16.75%) were mainly represented. Similarly, camphor (15.46%) was detected in a sample from Nigeria, and 9,12,15-octadecatrienoic acid, (*Z*,*Z*,*Z*) (25.38%), *n*-hexadecanoic acid (13.34%) and phytol (11.02%) were reported in a study from Malaysia, which emphasizes my argument that *C. odorata* demonstrates a huge variation in the chemical composition of their essential oils. In total, geijerene, pregeijerene, germacrene D, *α*-pinene, *β*-pinene, *β*-caryophyllene and caryophyllene oxide were the compounds frequently reported in all COEO analyzed. However, my study conducted in Abomey-Calavi (Republic of Benin), revealed caryophyllene oxide as the major constituent (43.75%), followed by naphthalene (9.09%). Reasons explaining the chemical variability in COEOs are probably geographical conditions such as location of the plant, difference in altitude, soil, time of collection or method of extraction of the oil. It is of note to mention that caryophyllene oxide is reported as the major constituent in other compositae such as *Artemisia campestris* L. (8.5-38.8%), and other plant species such as *Melaleuca styphelioides* Sm. (43.78%) or *Ocotea acutifolia* (Nees) Mez (56%) [135–137].

In addition to the comparison between COEOs from different regions, I analyzed the frequency of occurrence of COEOs components. Results were remarkable and demonstrated that all reported COEOs can be categorized in 2 equal phenotypes: monoterpene hydrocarbon-oil type (frequency = 44.04%) and sesquiterpene hydrocarbon-oil type (frequency = 41.58%). Of which, monoterpene hydrocarbons are represented majoritarily by pregeijerene and *α*-pinene, and sesquiterpene hydrocarbons are represented by germacrene D and *β*-caryophyllene. Oxygenated sesquiterpenes-oil type is less occurring (frequency = 6.90%) and the key compound in this class is caryophyllene oxide. This strengthens my argument to the huge diversity in COEOs and explains the importance of investigating the chemical composition of essential oils prior to their pharmacological evaluation.

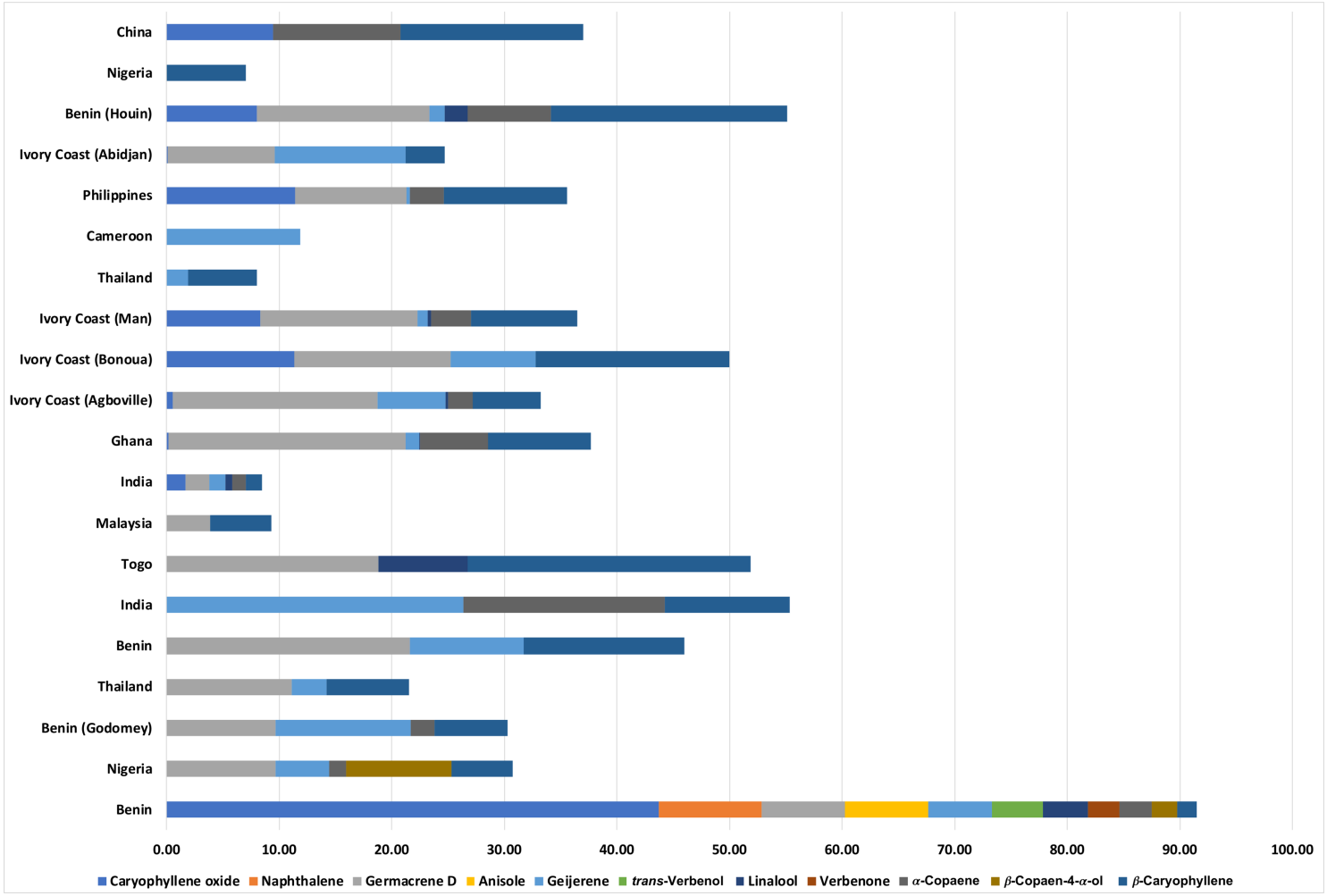


Figure 21: Chemical composition of the main compounds in COEO from my study compared to COEO from other studies in the world.

Only components reported in this study are presented in comparison of their ratio with COEOs from other regions in the world. COEO, essential oil obtained from *C. odorata* leaves.

3.2. Sedative activity and bioactivity-guided fractionation of COEO

In regard to my previous experiments [88,89,138], mice were administered different inhalation concentrations of COEO (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg/cage (volume of the cage 61.2L). Lavender oil was used as positive control and triethyl citrate (TEC), as negative control. As showed in Figure 22A, lavender oil significantly decreased locomotor activity by more than 66% (P = 0.001) in comparison to the control group. COEO decreased locomotor activity at concentrations from 0.00004 to 0.04 mg/cage ($F[7, 40] = 4.497$; $P < 0.001$), describing a double U-shaped curve. The most effective concentrations were 0.04 and 0.00004 mg/cage, which decreased locomotor activity by 71% and 64% respectively, in comparison to the control group. In all groups, mice started to sleep from about 25 min after inhalation sample administration, until their activity was reduced to zero (Figure 22B).

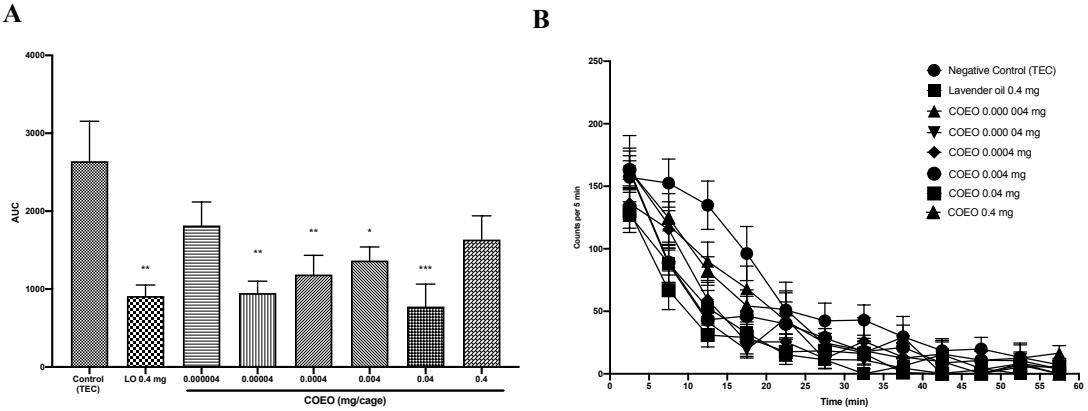


Figure 22: Total spontaneous locomotor activity (A) and locomotor activity transition (B) of mice treated with COEO (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg/cage).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's t-test or ANOVA, followed by Dunnett's test. *P < 0.05; **P < 0.01; ***P < 0.001. COEO, essential oil obtained from *C. odorata* leaves; TEC, triethyl citrate; LO, lavender oil; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

To identify the compounds responsible for the decrease in locomotor activity, COEO was fractionated, to give two fractions. Each fraction was analyzed and fraction 1 only induced sedative activity in mice. As showed in Figure 23, it significantly decreased locomotor activity at 0.0004 mg/cage by 56% (P = 0.03), compared to the control group. Because fraction 1 comprise caryophyllene oxide as major compound, I hypothesized that caryophyllene oxide could be the compound in cause for COEO sedative activity.

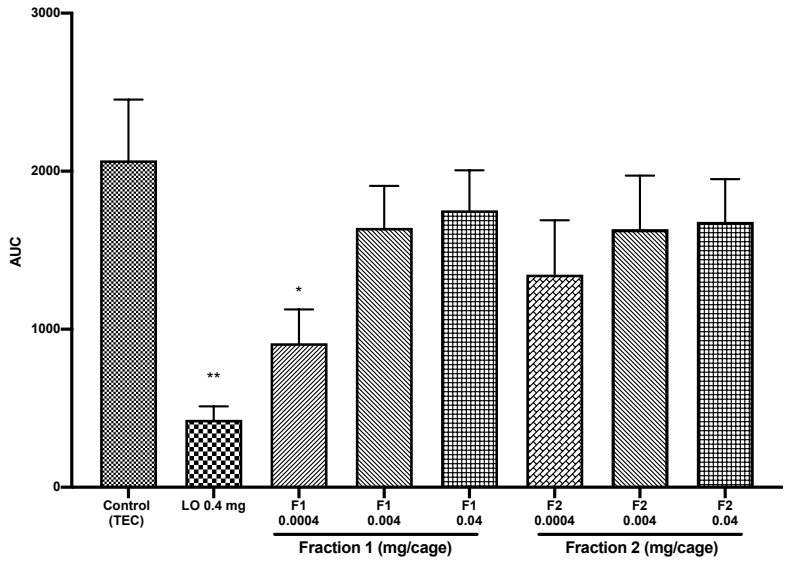


Figure 23: Total spontaneous locomotor activity of mice treated with fraction 1 and 2 (0.0004, 0.004, or 0.04 mg/cage).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's t-test or ANOVA, followed by Dunnett's test. *P < 0.05; **P < 0.01. TEC, triethyl citrate; LO, lavender oil; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

3.3. Caryophyllene oxide induced sedative activity in mice

Caryophyllene oxide was administered to the mice at the same concentrations as COEO. Benzylacetone was used as positive control [1,88], and as showed in Figure 24, it significantly decreased locomotor activity (P = 0.04), thus validating the experimental model. In the present study, caryophyllene oxide significantly decreased locomotor activity at 0.0004 and 0.04 mg/cage by 56% and 57% respectively, compared to the control group (F[7, 36] = 2.436; P = 0.04) (Figure 24). Likewise, caryophyllene oxide previously induced sedative activity in mice when administered via inhalation at 0.45 mg/cage [3]. In addition, caryophyllene oxide was a major compound of *Baccharis uncinella* DC. essential oil which demonstrated sedative activity in male albinos mice [139]. In the same way, it also promoted sedation in silver catfish [140,141].

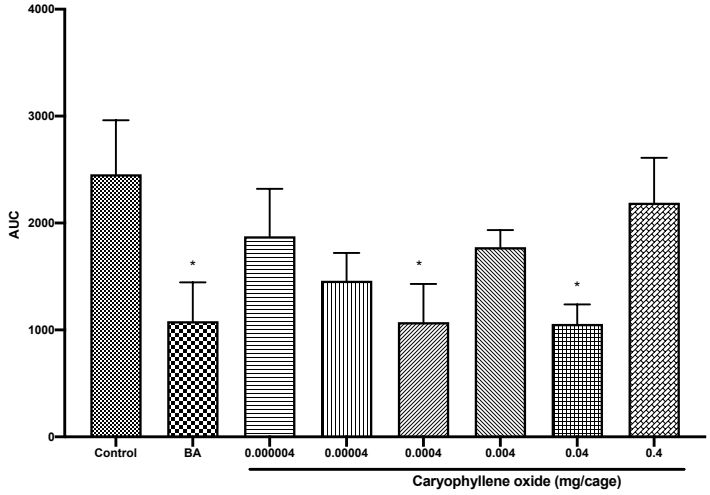


Figure 24: Total spontaneous locomotor activity of mice treated caryophyllene oxide (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg/cage).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's t-test or ANOVA, followed by Dunnett's test. *P < 0.05. TEC, triethyl citrate; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

To confirm that caryophyllene oxide truly evaporated in the cage before the mice are inserted, a similar experiment to the open-field arena was performed [88]. The results demonstrated that more than 90% of caryophyllene oxide already evaporated in the air before the mice are inserted, thus confirming that the sedative activity observed is due to the action of caryophyllene oxide. In addition, caryophyllene oxide is the oxidation product of *β*-caryophyllene. Its pharmacological activities include anticarcinogenic, anti-inflammatory, antioxidant, antiviral and analgesic properties [142–144]. In previous years, analysis of the essential oil of *Cannabis sativa* L. revealed the presence of mainly sesquiterpene compounds among which caryophyllene oxide was surprisingly identified as the compound sensed by hashish detection dogs [145].

A concentration of 0.04 mg/cage caryophyllene oxide seemed to be more sedative and the results described a double U-shaped dose-response curve. We have previously reported such dose-response curve [88,98,125], which appears to be the effect of the presence of multiple receptors with different affinities [116]. Comparison of the results of caryophyllene oxide with those of COEO showed that COEO was more sedative at lower concentrations. As a matter of fact, COEO 0.00004 mg/cage was statistically significant, although the described double U curve was similar to that of caryophyllene oxide. Previous studies have agreed that administration of the whole essential oil from plant material is more beneficial than sole administration of their separate compounds [1,88,125]. In the present study, COEO was demonstrated to be more beneficial than the individual administration of caryophyllene oxide. One possible explanation is that in COEO, different compounds are mixed in different ratios, and they can act in synergy to improve the effects of COEO. Accordingly, Fujiwara et al.[16] showed that a mixture of different active oils was beneficial to individual oils, because of a possible synergistic action.

3.4. Effect of Caryophyllene oxide on pre-excited mice with caffeine

To better understand the mechanisms underlying the sedative activity of caryophyllene oxide, I first investigated its action on the CNS. Caffeine was administered to mice to induce excitation and thereafter, caryophyllene oxide (0.04 mg/cage) was given to the mice. As demonstrated in Figure 25, caffeine increased the mice locomotor activity by more than 68% (P = 0.003). Similar to diazepam (positive control), upon administration of caryophyllene oxide, mice locomotor activity was decreased by 61% (P = 0.001), indicating that caryophyllene oxide is a powerful CNS depressant. In previous studies monoterpenes and sesquiterpenes were also effective in decreasing the excitation provoked by caffeine [120,125].

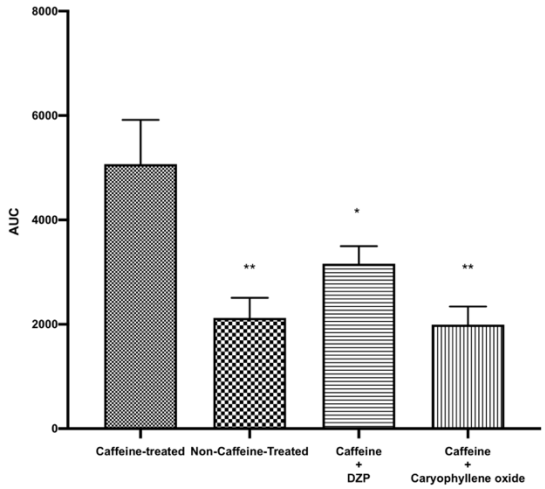


Figure 25: Total spontaneous locomotor activity of mice excited with caffeine (25 mg/kp intraperitoneally) and treated with caryophyllene oxide (0.04 mg/cage).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's t-test or ANOVA, followed by Dunnett's test. *P < 0.05; **P < 0.01. DZP, diazepam; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

3.5. Effects of Caryophyllene oxide on the Pentobarbital-Induced Sleeping Test in mice

In an attempt to verify the action of caryophyllene oxide on the GABAergic receptor system, mice were administered inhaled caryophyllene oxide after pentobarbital injection. Diazepam, positive control, prolonged the pentobarbital-induced sleeping time by 102% (P < 0.001) and the effect was reversed by 33% following flumazenil administration (Figure 26).

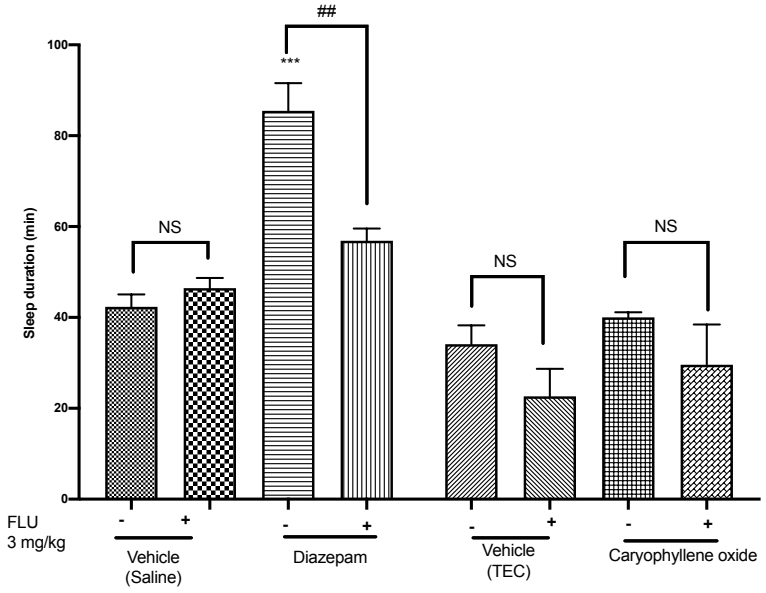


Figure 26: Sleep duration in the pentobarbital-induced sleeping test of mice treated with caryophyllene oxide (0.04 mg/cage), diazepam (3 mg/kg), or flumazenil (3 mg/kg).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's t-test or ANOVA, followed by Dunnett's test. ***P < 0.001 vs. the control group; $^{\#\#}$P < 0.01 for

flumazenil treatment vs. treatment without flumazenil. TEC, triethyl citrate; FLU, flumazenil; NS, not significant; SEM, standard error of the mean; ANOVA, analysis of variance.

However, caryophyllene oxide only prolonged the sleeping time of mice by 17% and this effect was not statistically significant. More, administration of flumazenil did not change the sleeping time, which suggested that caryophyllene oxide does not operate via the GABAergic receptor system. Conversely, a work in which caryophyllene oxide was extracted from *Psidium guajava* L. hexanolic leaves, demonstrated that it increased the sleeping time induced by pentobarbital [146]. In this study, authors utilize different dosage and methodology and caryophyllene oxide obtained only in traces was administered intraperitoneally whereas my study was performed on pure caryophyllene oxide administered via inhalation. These differences could explain the divergences in the studies. Although I suggest that the action mechanism of caryophyllene oxide is not facilitated by the GABAergic receptors system, other monoterpene and sesquiterpene compounds such as ascaridole or aristolen-1(10)-en-9-ol have previously demonstrated good sedative activity via the $GABA_A$ receptors system [7,98,121]. For instance, numerous studies have suggested the action mechanism of caryophyllene oxide to be mediated via the cannabinoid (CB) receptor system, especially the CB2 receptor [147].

Further, mice were subjected to the Rota-rod test to confirm that the sedative activity of caryophyllene oxide was not the effect of a deficit in motor function. The results demonstrated that there was no significant change in the latency of mice to fall compared to the control group ($P = 0.16$). Therefore, it follows that the decrease in locomotor activity in my study is not associated with motor incoordination (Figure 27).

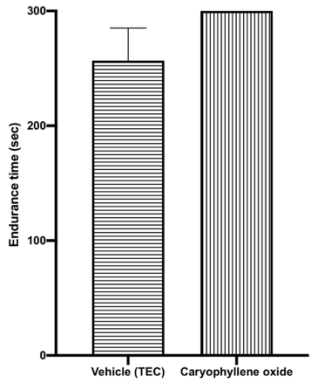


Figure 27: Endurance time in the Rota-rod test of mice treated with caryophyllene oxide (0.04 mg/cage).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's t-test or ANOVA, followed by Dunnett's test. TEC, triethyl citrate; SEM, standard error of the mean; ANOVA, analysis of variance.

3.6. Structure-activity relationships of caryophyllene oxide and its precursor β-caryophyllene

Caryophyllene oxide is the oxidation product of *β*-caryophyllene, and both compounds are frequently reported in COEOs (frequency of occurrence = 6.15% and 12.01%, respectively). More, *β*-caryophyllene and caryophyllene oxide are natural substances approved as flavoring agents by the Food and Drug Administration (FDA) and by the European Food Safety Authority (EFSA). Therefore, I implied that it could be of interest to compare their physical properties and activities. Analysis of their physicochemical properties (Table 4) [148,149] indicated that both compounds are low volatiles with high affinity to the non-aqueous layer of membranes.

Table 4: Physicochemical properties of *β*-caryophyllene and caryophyllene oxide.

	***β*-Caryophyllene**	**Caryophyllene oxide**
Appearance	Colorless to pale yellow clear oily liquid	Pale yellow white crystalline solid
Molecular Weight	204.35 g/mol	220.35 g/mol
Formula	C_{15} H_{24}	C_{15} H_{24} O
Odor	Spicy	Woody
Optical Rotation	-5.00 to -10.00	-65.00 to -75.00
Melting Point	129.00 to 130.00 °C at 14.00 mm Hg	60.00 to 62.00 °C at 760.00 mm Hg
Boiling Point	254.00 to 257.00 °C at 760.00 mm Hg	279.68 °C at 760.00 mm Hg
Vapor Pressure	0.013000 mmHg at 25 °C	0.007000 mmHg at 25 °C
Flash Point	205.00 °F	> 212.00 °F
logP (o/w)	6.777	4.429

For this reason, when absorbed by the olfactory cells, they can stick to the nasal mucosa and exert their activity. More, they are considered safe with no reported toxicity in studies. As a matter of fact, the median lethal dose (LD_{50}) of *β*-caryophyllene and its oxide are both >5000 mg/kg in mice, which is times-fold the highest dose administered in my study, indicating that the sedative activity in my study is exempt of any sign of toxicity.

β-caryophyllene is a sesquiterpene compound commonly reported in essential oils from plants such as *Cinnamomum verum* J.Presl and *Syzygium aromaticum* (L.) Merr. & L.M. Perry [150]. In plants, *β*-caryophyllene is sometimes found with isocaryophyllene or its oxidation product, caryophyllene oxide. Studies have elucidated its action mechanism as a selective agonist of the CB2 cannabinoid receptors with no interaction with the CB1 receptors. More, it is considered as a dietary cannabinoid of pharmaceutical promise with multiple pharmacological activities [151]. Its biological effects include anti-inflammatory, anticarcinogenic, antimicrobial, antioxidant, and analgesic activities [152–154].

We investigated herein the sedative activity of *β*-caryophyllene. In my open field test, *β*-caryophyllene in general demonstrated a reduction in locomotor activity of mice. However, these effects were only statistically significant at a lower concentration of 0.00004 mg/cage, which is lower than that of caryophyllene oxide. As presented in Figure 28, mice locomotor activity was reduced by more than 61% in regard to the control group ($P = 0.009$).

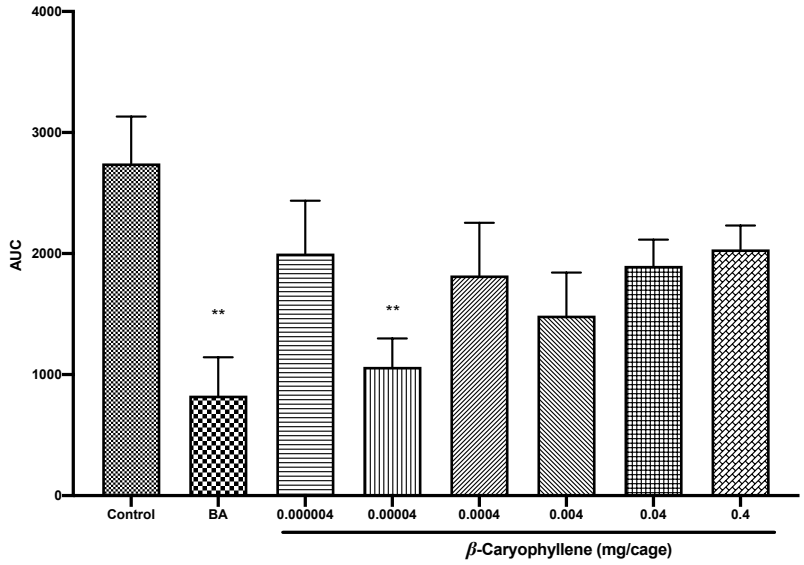


Figure 28: Total spontaneous locomotor activity of mice treated with β-caryophyllene (0.000004, 0.00004, 0.0004, 0.004, 0.04, or 0.4 mg/cage).

Data are shown as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated using Student's t-test or ANOVA, followed by Dunnett's test. **P < 0.01. TEC, triethyl citrate; AUC, area under the curve; SEM, standard error of the mean; ANOVA, analysis of variance.

In the same way, *β*-caryophyllene was also reported sedative in a previous study [3] and more, it demonstrated sedative and anxiolytic activities following oral administration to mice [155]. Comparison with the results of caryophyllene oxide revealed the same double U-shaped curve pattern; however, as *β*-caryophyllene seems to be more effective at lower doses, this indicated that the existence of an epoxy functional group (Figure 29A) could render the activity less effective in caryophyllene oxide. Ogawa et al. [3] also previously made similar assertions in their study on *Z. zerumbet* related compounds. More, the tridimensional structure of both compounds (Figure 29B and Figure 29C) indicated that the nine-membered ring cycle in caryophyllene oxide makes it a more complex structure, which I suggest, could render difficult interactions with the biological layers. *β*-Caryophyllene on the other side, which appears freer to bind to receptors, could possibly interact easily with receptors, leading to better reactivity. Recent papers have indicated that the action mechanism of *β*-caryophyllene does not involve $GABA_A$/benzodiazepine nor 5-HT 1A receptors, but could be mediated via the cannabinoid CB2 receptors system, as it was also suggested for caryophyllene oxide [155,156]. Further, new studies suggest that CB2 receptors could be involved in the treatment of emotional behaviors such as anxiety [156], which could explain the sedative potential of *β*-caryophyllene and caryophyllene oxide. Overall, *β*-caryophyllene is quite similar to its oxide, and more investigations on these particular sesquiterpenes could broaden actual knowledge on their cellular targets.

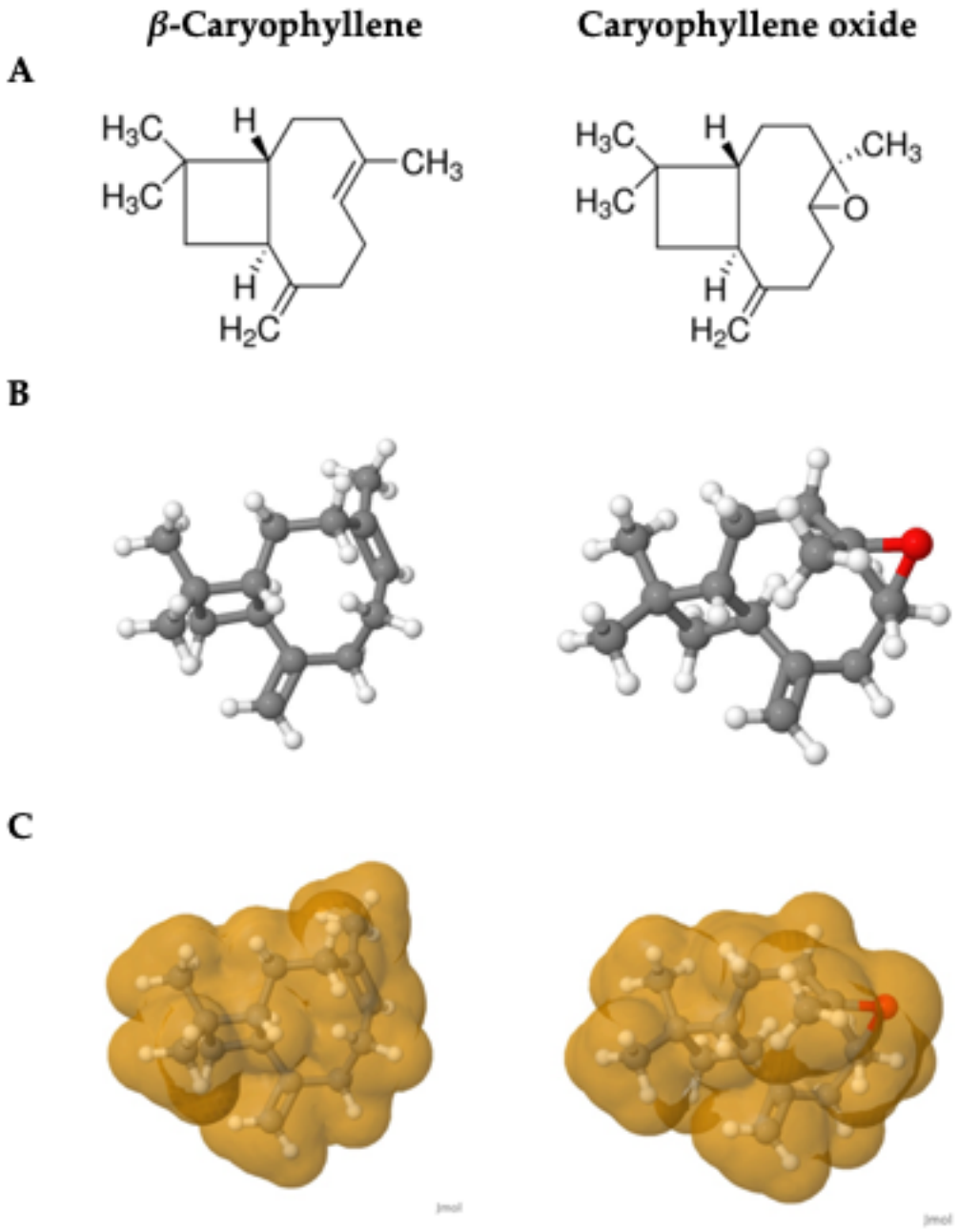


Figure 29: Comparison of the structures of β-caryophyllene and caryophyllene oxide.

Bidimensional structures (A); Tridimensional structures (B); Tridimensional structures with Van der Vaals forces (C). The sole difference between the structures is the epoxy group in caryophyllene oxide. Structures were drawn by using Jmol [157].

4. Conclusions

In the present study, I have demonstrated an important variation in the essential oil composition of COEOs from different regions in the world. COEO from the Republic of Benin was investigated for sedative activity and its major constituent, sesquiterpene caryophyllene oxide, revealed to be a key component in the sedative activity. In an attempt to shed light on the mechanisms of action, I demonstrated that the GABAergic receptors system were not involved in the sedative activity, and suggested that binding to other systems such as the

cannabinoid receptor system could explain the activity. Therefore, further investigations on the action mechanism of caryophyllene oxide and similar compounds is needed.

In total, COEO and its sesquiterpene components could be potentially used in the treatment of CNS-related disorders, and in a different chapter, I will investigate the structure-activity relationship of selected sesquiterpenes compounds, to better understand their pharmacological activities and action mechanisms in the CNS.

Chapter II. The cineole monoterpenes: evaluation of their anxiolytic and antidepressant activities following inhalation administration in mice.

1. Introduction

Anxiety is one of the most common mental illnesses and affects more than one-eighth of the adult population worldwide [158]. A pharmacotherapeutic approach is typically used to treat anxiety, but these therapies are associated with several side effects, such as memory disturbance, interactions with other drugs and dependence. Another important psychiatric condition is depression, with a substantial morbidity and high suicide rate. The medicines currently used to treat depression often demonstrate shortcomings, such as slow onset of action, low response rates and drug resistance development, which limits their utilisation. For many years, anxiety and depression were considered to be separate pathologies, but presently, the use of benzodiazepines for treatment of anxiety only is being slowly replaced with antidepressants that can treat depression and major anxiety disorders [159]. Aside from their side effects and limited utilisation, these medicines contribute to a high economic burden of disease and are long-term or life-long treatments. Considering these limitations, there is a need for novel psychopharmacological approaches that are efficient with fewer side effects. Recently, natural products have been considered to be promising for treatment of anxiety and depression, and several studies have investigated essential oils from plant materials because they have demonstrated excellent results in the central nervous system (CNS), such as an anxiolytic, relaxant, sedative, antinociceptive or antidepressant effects [2,160,161].

Eucalyptol, which is 1,8-cineole or 1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane (Figure 30a), is a monoterpene ether present in essential oils derived from many plants, such as eucalyptus and rosemary. Various pharmacological activities, such as sedative, antihypertensive, anti-inflammatory and antinociceptive effects, of 1,8-cineole have been demonstrated [46,88,162]. A minor component of plant extracts containing 1,8-cineole that is found in *Elettaria cardamomum* (L.) Maton (cardamom) and *Piper cubeba* Bojer is 1,4-cineole or 1-methyl-4-(1-methylethyl)-7-oxabicyclo [2.2.1] heptane (Figure 30b), a natural monoterpene stereoisomer of 1,8-cineole, which has also exhibited interesting bioactivity, such as an anxiolytic-like action in mice via oral administration, and has been shown to be a precursor for microbial hydroxylation [163,164].

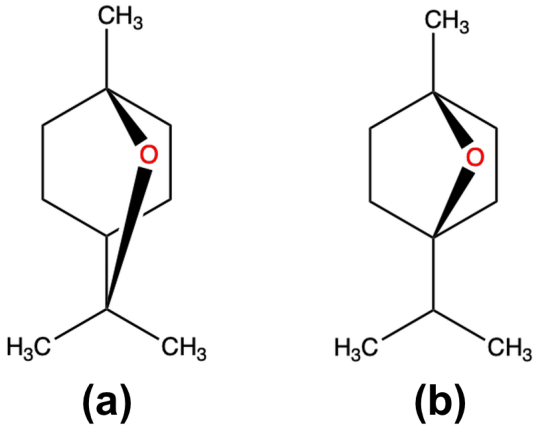


Figure 30: Structural formula of (a) 1,8-cineole and (b) 1,4-cineole.

Emerging research on the biological effects of 1,8- and 1,4-cineole has shown promising results in humans and animals [165,166]. Studies have shown the anxiolytic or antidepressant-like activity of monoterpenes [6,159,163,167–169], but to the best of my knowledge, no studies have been published about the activities of 1,8- and 1,4-cineole in ameliorating anxiety and depressive-like symptoms in mice via inhalation administration.

Therefore, in the present study, I evaluated the anxiolytic-like effects of 1,8- and 1,4-cineole by using two classic mice behavioural models of anxiolytic screening known as the light–dark box test (LDB) and marble-burying test (MBT), studied the possible involvement of the GABAergic transmission system in the anxiolytic activities of 1,8- and 1,4-cineole by using flumazenil (FLU; an antagonist of the GABAergic transmission system), evaluated the antidepressant activities of 1,8- and 1,4-cineole by using the forced swimming test (FST) and tail suspension test (TST), which are mice models of depression, and assessed any possible deficit in neuromuscular function that could bias the behavioural activities by performing the horizontal wire test (HWT) in mice.

2. Materials and Methods

2.1. Chemical and reagents

Triethyl citrate (purity >98%, Merck), a non-sedating odourless solvent, was used to dissolve the fragrant components. Benzylacetone (purity >95%, Tokyo Chemical Industries, Ltd.), flumazenil (purity > 98%, Wako Pure Chemical Industries, Ltd.), diazepam (purity >98%, Wako Pure Chemical Industries, Ltd.) and fluoxetine (purity >98%, Tokyo Chemical Industries, Ltd.) were used as positive controls. 1,4-Cineole (purity >85%) was obtained from Acros Organics. 1,8-Cineole (purity >85%) was purchased from Wako Pure Chemical Industries, Ltd. All chemicals used were of the highest grade available.

2.2. Experimental animals

Four-week-old male ddY mice (20–30 g) were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in colony cages under a 12 h/12 h light/dark cycle at 25°C ± 2°C and a relative humidity of 50%–60%. They were fed pellet chow and water *ad libitum* and allowed to accommodate to these conditions for 1 week before the experiments. Animal experiments were conducted following the recommendations of the Animal Research Committee of Kyoto University, Kyoto, Japan (approval number, 2014-14-3; first approved 27 December 2014 and renewed annually until 14 March 2018). Experimental procedures involving animals and their care were conducted in accordance with the institutional guidelines and in compliance 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 and 17:00 under identical conditions.

2.3. Behavioural experiments

Anxiety symptoms were evaluated by using the LDB and MBT while depressive-like behaviours were investigated by using the FST and TST, as previously demonstrated [2,170–172] with minor modifications. Inhalation administration was performed as described in the OFT. The sedative effects and motor coordination were evaluated by using the OFT and HWT, respectively [2,173]. To avoid any potential bias in the experiments, each treatment group was recorded by using a video camera and data was analysed blinded to the group by a trained observer. In all my experiments, each animal was used only once.

2.3.1. Light–dark box test (LDB)

The LDB is a widely used behavioural test for evaluating the anxiolytic effects of drugs. The test is based on the innate aversion of rodents to brightly lit areas and on their spontaneous exploratory behaviour in response to a novel environment and to light [170]. The apparatus consisted of two equally sized compartments (30 × 30 × 34 cm each); a light area illuminated by a 60-watt desk LED lamp, and a dark area blackened with black plastic sheets. The two compartments were separated by a black wall with an aperture (small doorway) in its centre (5 × 5 cm) to allow passage from one compartment to the other. In both compartments,1,8- or 1,4-cineole was charged for 60 min in accordance with the open-field test. Thereafter, mice (n = 10/group) were individually placed in the centre of the lit area facing the tunnel, and activity was recorded by using a video camera during a 15-min test period, after which the number of entries in the light box (NELB) and time spent in the light box (TSLB) were counted. Diazepam 0.5 mg/kg *i.p.* was used as a positive control, as previously described [2].

2.3.2. Marble-burying test (MBT)

The MBT is an effective method for testing the anxiolytic-like properties of a particular substance. The test is based on the defensive burying behaviour observed in rodents in response to aversive stimuli, such as shock; noxious food; or unanimated objects, such as glass marbles [174]. Mice (n = 10/group) were individually introduced into the apparatus that consisted of a Plexiglas cage (42 × 34 × 15 cm) with a floor filled with a 5-cm deep layer of sawdust and containing 20 distributed glass marbles (15-mm diameter). Each test lasted 30 min, and a marble was considered as hidden when it was at least two-thirds covered by sawdust. The sawdust and marbles were washed with soap and water, cleaned with ethanol 70% and dried with a paper towel, and then the next mouse was tested.

2.3.3. Open-field test (OFT)

The sedative effects of 1,4-cineole on mice were evaluated using an OFT, as previously described [2,3,5,6,18,41]. Administered doses were expressed as milligrams of sample per 400 µL of TEC following previous experiments [4–6]. Four pieces of filter paper were placed in the four corners of the inner walls of the glass cage (60-cm wide, 30-cm long, 34-cm high) by using adhesive tape. On each piece of filter paper, 1,4-cineole was deposited, and the cage was closed so that the vapour pervaded by natural diffusion. Sixty minutes after charging the sample, mice (n = 6/group) were individually placed in the centre of the cage and subjected to video surveillance for another 60 min. During monitoring, the number of times a mouse crossed lines drawn at 10-cm intervals on the floor of the cage was counted every 5 min. The area under the curve (AUC) of locomotor activity counts per 5 min (Y-axis) and time (X-axis), representing total locomotor activity, was calculated according to the trapezoidal rule.

2.3.4. Horizontal wire test (HWT)

The HWT estimates motor coordination and muscle relaxation [173]. The test was performed by treating the mice according to a slight modification of the method described by Bonetti et al. [175]. The mice were lifted by their tails and allowed to grasp a horizontally strung wire (2-mm diameter, 30-cm long) placed 25 cm above a table with their forepaws, after which they were released. The number of mice from each treatment group (n = 10/group) that did not grasp the wire with their forepaws or actively grasp the wire with at least one hind paw

within a 10-sec period was recorded. Diazepam (5 mg/kg, *i.p.*) was used as a positive control and administered 30 min before the test.

2.3.5. Forced swimming test (FST)

The FST is the most widely used test for assessment of antidepressant activity. The procedure used is the same as previously described by Porsolt et al. [176] with some minor modifications. After treatment with saline, fluoxetine (20 mg/kg, *p.o.*), TEC, or 1,8- or 1,4-cineole, the mice (n = 10/group) were individually forced to swim in a transparent Plexiglas cylinder (40-cm high and 20-cm in diameter) containing a 15-cm depth of water at 25°C ± 2°C. The water was renewed after each mouse was tested. During the session, immobility time was recorded by using a video camera. The total duration of immobility was measured during the last 4 min of a single 6-min test session. The mice were considered to be immobile when they remained floating motionless or when they only made movements necessary to keep their heads above water.

2.3.6. Tail suspension test (TST)

The TST is a widely used behavioural model for testing the effect of antidepressant agents. The test was carried out by using a method described by Steru et al. [172]. Mice (n = 10/group) were individually suspended from the edge of a suspension box (63-cm high) by an adhesive tape placed approximately 1 cm from the tip of the tail. The mice were considered to be immobile when they stopped making any struggling movements and hung passively. The immobility time was recorded for a period of 6 min. Fluoxetine (20 mg/kg *p.o.*) was administered as positive control 1 h prior to testing, to confirm the validity of the apparatus.

2.4. Statistical analysis

All values are expressed as the mean ± standard error of mean (SEM). Statistical analyses were performed by using Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. GraphPad InStat software version 7 (GraphPad Software, Inc.) was used to perform all statistical analyses. P values < 0.05 were considered to be indicative of statistical significance.

3. Results and Discussion

In Chapter I, I demonstrated that 1,8-cineole, one of the major compounds of the essential oil extracted from *L. camara* leaves, exhibited good sedative activity via inhalation in mice [88]. Because sedative activity is often associated with anxiolytic or antidepressant-like activity [2,6,177,178], further investigations of the effects of 1,8-cineole and its related compounds on the CNS were required. This investigation was based on a structure–activity study showing that 1,8-cineole and its stereoisomer 1,4-cineole prevented anxiety and depressive-like symptoms in mice. To test this hypothesis, classic mice models of anxiety and depression, including the LDB, MBT, FST, TST, open-field test (OFT) and HWT, were used. These tests are based on exposure of animals to a stressful condition and a specific test for measuring behavioural and physiological responses. In all experiments, the mice were administered inhalation doses of 0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg 1,8- or 1,4-cineole

dissolved in 400 µL of triethyl citrate (TEC) individually on the basis of previous experiments [2,6,41].

3.1. Anxiolytic-like activities of 1,8- and 1,4-cineole by using the LDB

The LDB is considered to be one of the most widely validated tests for assaying the activities of anxiolytic agents. Reportedly, in the LDB, anxiolytic drugs tend to increase the number of entries in the light box (NELB) and the time spent in the light box (TSLB) [170]. Our results showed that diazepam 0.5 mg/kg *i.p.*, used as a positive control, significantly increased TSLB and NELB by 84% and 57%, respectively, relative to the levels in the vehicle (saline) group. A dose of 1,8-cineole 0.0004 mg significantly increased TSLB by 75% relative to the level in the control group [$F(11,108) = 14.79$, $p < 0.001$] (Figure 31a); however, it was decreased by 20% relative to that after dosing with diazepam. Similarly, 0.0004 mg 1,8-cineole significantly increased NELB by 40% relative to the level in the control group [$F(11,108) = 13.37$, $p < 0.001$] (Figure 31b), but decreased it by 17% relative to the level when using diazepam. These results validated the experiment and suggested an anxiolytic-like activity of 1,8-cineole slightly inferior to that of diazepam.

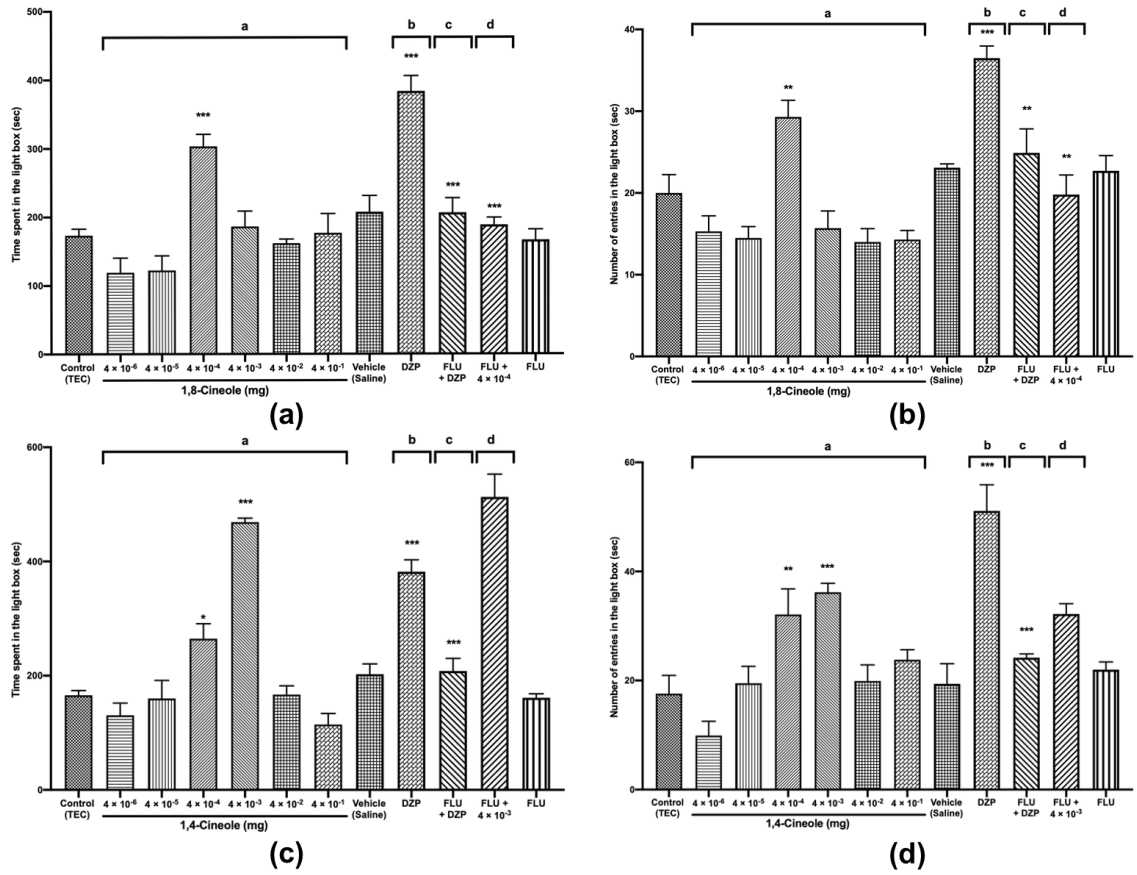


Figure 31: The Light–dark box test (LDB) of mice treated with 1,8- (a, b) or 1,4-cineole (c, d) (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg), diazepam (0.5 mg/kg) or flumazenil (2.5 mg/kg).

The parameters analysed were time spent in the light box (TSLB) (a, c) and number of entries in the light box (NELB) (b, d). The values are given as the mean ± SEM of 10 mice. The letter **a** indicates a significant difference when compared with the control (TEC); the letter **b** indicates a significant difference when compared with the vehicle (saline); the letter **c** indicates a significant difference when compared with diazepam; the letter

d indicates a significant difference when compared with 1,8-cineole 0.0004 mg or 1,4-cineole 0.004 mg. Statistical differences vs. the control group were calculated by using Student's t test or ANOVA followed by Dunnett's test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Our findings are supported by the study of Kim et al. [179], who showed that inhaled 1,8-cineole, significantly reduced preoperative anxiety in humans. Similarly, administration of the essential oil from *Achillea wilhelmsii* C. Koch, which contains 1,8-cineole as a major compound (20.8%), showed anxiolytic activity [160].

We found that 1,4-cineole at doses of 0.0004 and 0.004 mg significantly increased TSLB by 60% and 182%, respectively [$F (11,108) = 38.20$, $p < 0.001$] (Figure 31c) and significantly increased NELB by 83% and 110%, respectively, relative to the levels in the control [$F (11,108) = 13.11$, $p < 0.001$] (Figure 31d). Additionally, the effect of 1,4-cineole 0.004 mg was better than that of 0.0004 mg in both TSLB and NELB and 23% greater than that of diazepam. These results indicated a potential anxiolytic effect of 1,4-cineole. Our inhalation administration of 1,4-cineole showed results similar to those of Gomes et al. [163] who demonstrated that oral administration of 1,4-cineole 400 mg/kg had anxiolytic activity in mice. These results suggested that 1,8- and 1,4-cineole could be used as anxiolytic agents depending on the dose and route of administration.

3.2. The role of the $GABA_A$/benzodiazepine receptor system in the anxiolytic activity of 1,8- and 1,4-cineole

Several mechanisms have been proposed to explain anxiety and depression symptoms, and studies have frequently reported mediation via the GABAergic transmission system [41,161,180]. GABA is the primary inhibitory neurotransmitter in the CNS, and it has been reported that one third of all CNS neurons are thought to be mediated via the GABAergic transmission system [181]. Therefore, I investigated the implication of the GABAergic system transmission in the anxiolytic activity of 1,8- and 1,4-cineole. FLU 2.5 mg/kg *i.p.*, an antagonist of benzodiazepine drugs, was administered together with 1,8-cineole 0.0004 mg and 1,4-cineole 0.004 mg in the LDB. During the tests with 1,8-cineole, FLU alone did not alter TSLB and NELB (Figure 31a and Figure 31b, respectively). However, pre-treatment of FLU with diazepam significantly reduced TSLB by 45% [$F (11,108) = 16.79$, $p < 0.001$] (Figure 31a) and NELB by 34% [$F (11,108) = 13.37$, $p < 0.001$] (Figure 31b) relative to the tests with diazepam alone. These results confirmed the antagonistic effect of FLU on diazepam. Similarly, treatment of 1,8-cineole 0.0004 mg with FLU significantly reduced TSLB and NELB by 38% and 34%, respectively, relative to the tests with 1,8-cineole 0.0004 mg (Figure 31a and Figure 31b), suggesting an antagonistic effect of FLU on 1,8-cineole. Some authors have previously suggested that 1,8-cineole may exert its activity through the GABAergic transmission system [168,182].

During the tests with 1,4-cineole, the FLU, diazepam, and diazepam + FLU groups showed similar results to those of the tests with 1,8-cineole, which validated the experiments. However, in both TSLB and NELB, the anxiolytic effect of 1,4-cineole 0.004 mg was not reversed by co-administration with FLU (Figure 31c and Figure 31d), indicating that the anxiolytic effect of 1,4-cineole was not mediated via $GABA_A$/benzodiazepine receptors. These results are consistent with those of Gomes et al. [163] who found that 1,4-cineole administered orally to mice did not involve benzodiazepine receptors. Further investigations on 1,4-cineole need to be conducted in future experiments to elucidate the possible role of $5\text{-}HT_{1A}$, D1 or noradrenergic receptors in the anxiolytic activity.

3.3. Anxiolytic-like activities of 1,8- and 1,4-cineole by using the MBT

To further corroborate the anxiolytic activity observed in the LDB, I also performed the MBT in which rodents tend to bury glass marbles. This behaviour is interpreted as related to anxiety; consequently, anxiolytic drugs tend to reduce the number of marbles buried (NMB) [167]. Results similar to those of the LDB were observed for both 1,8- and 1,4-cineole in the MBT. When compared with the NMB of the control, that of the mice treated with 1,8-cineole 0.0004 mg was reduced by 26% [$F(8,81) = 5.297$, $p < 0.001$], which indicated an anxiolytic-like effect of 1,8-cineole (Figure 32a). The NMBs of the mice treated with 1,4-cineole at doses of 0.0004 and 0.004 mg were reduced by 29% and 38%, respectively [$F(8,81) = 6.431$, $p < 0.001$], relative to those of the control (Figure 32b), indicating an anxiolytic-like activity of 1,4-cineole.

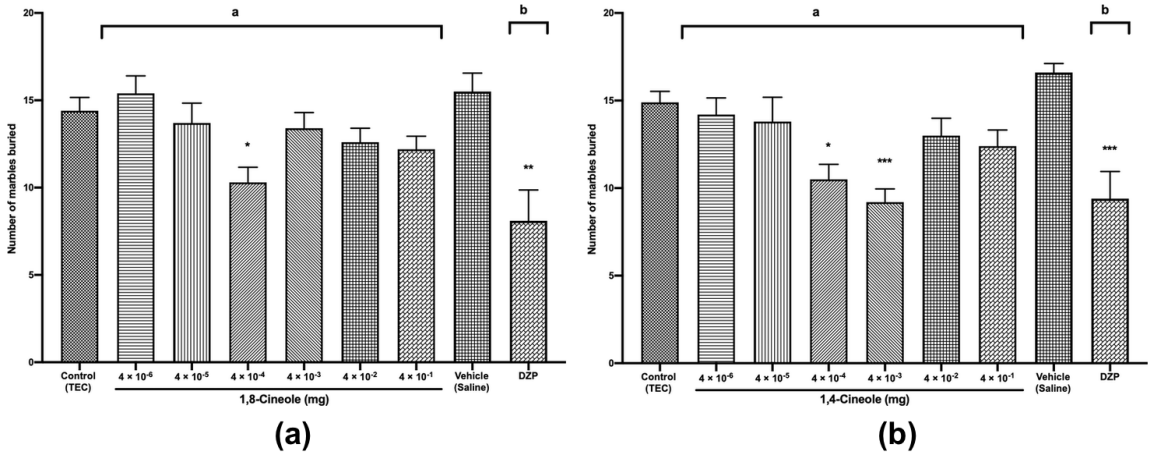


Figure 32: The marble-burying test (MBT) of mice treated with 1,8- (a) or 1,4-cineole (b) (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg) or diazepam (0.5 mg/kg).

The parameter analysed was number of marbles buried (NMB). The values are given as the mean ± SEM of 10 mice. The letter **a** indicates a significant difference when compared with the control (TEC); the letter **b** indicates a significant difference when compared with the vehicle (saline). Statistical differences vs. the control group were calculated by using Student's t test or ANOVA followed by Dunnett's test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Moreover, 1,4-cineole 0.004 mg had a superior anxiolytic-like effect to that of 1,4-cineole 0.0004 mg and was greater than that of diazepam, as observed in the LDB. Similar to 1,8- and 1,4-cineole, other monoterpenes and derivatives of monoterpenes have been demonstrated to possess anxiolytic-like activity; such as that shown by epoxy–limonene administered at 25, 50 and 75 mg/kg *i.p.* in Swiss mice [167] and of carvacryl acetate administered at 50 and 75 mg/kg *i.p.* in mice [169], which demonstrated good anxiolytic activities relative to those of the vehicle.

3.4. Sedative effects of 1,8- and 1,4-cineole by using the OFT

To further investigate the effects of 1,8- and 1,4-cineole on the CNS, I explored the sedative activity of 1,4-cineole in the OFT. The OFT utilises behavioural changes in rodents exposed to a novel environment and has been used to detect sedative activity in mice [2,5,6,18]. Mice administered 1,4-cineole at doses of 0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg per 400 µL of TEC showed a decrease in locomotor activity at all doses. As shown in Figure 33a, there

was a significant decrease in mice locomotor activity at doses of 1,4-cineole 0.0004 and 0.04 mg by 70% and 59%, respectively [F (7,40) = 5.236, p < 0.001], relative to those of the control. Moreover, analysis of locomotor activity transition (Figure 33b) showed that the sedative effects produced by the doses of 0.0004 mg and 0.04 mg 1,4-cineole were the most effective and locomotor activity dropped nearly to zero 15–20 min after inhalation administration.

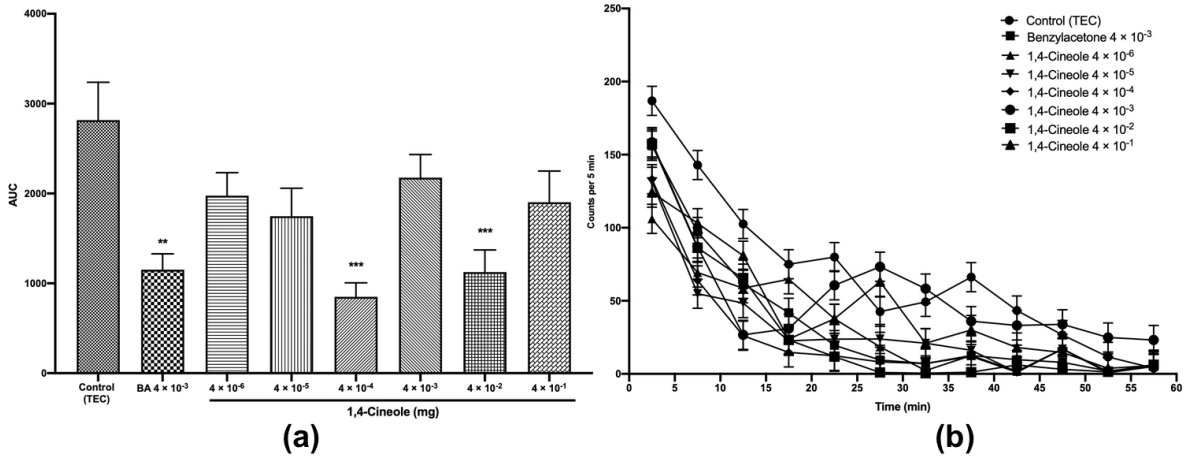


Figure 33: Total spontaneous motor activity (a) and locomotor activity transition (b) of mice treated with 1,4-cineole (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg).

The values are given as the mean ± SEM of six mice. Statistical differences vs. the control group were calculated by using Student's t test or ANOVA followed by Dunnett's test. (*p < 0.05, **p < 0.01, ***p < 0.001).

Controversially, Gomes et al. [163] showed that 1,4-cineole 100 and 200 mg/kg *p.o* did not induce any change in mice locomotor activity. Differences in the results could be due to the differences in the doses administered and routes of administration. Additionally, Gomes et al. [163] performed the OFT for 5 min, whereas I performed it for 60 min. It has been reported that the sedative activity is observed generally 20–30 min after drug administration [2,3,5,6,18,41]. Moreover, Gomes et al. [163] concluded that a higher dose of 1,4-cineole could demonstrate sedative activity because their pentobarbital test indicated a possible sedative effect for 1,4-cineole. The sedative effect of 1,8-cineole was studied in my previous study [88] and it showed a significant decrease in locomotor activity at 0.0004 mg. Taken together, my results observed in the LDB, MBT and OFT suggest that 1,8- and 1,4-cineole exhibit anxiolytic effects associated with sedative action in mice. Similar to the activity in 1,8- and 1,4-cineole, anxiolytic activity has been often associated with sedative effects in other terpenoids, such as linalool, nerol, limonene epoxide and thymol and in essential oils from *O. gratissimum*, *Telfairia occidentalis* Hook.f., *P. guineense* and *Citrus aurantium* L. [2,6,167,177,178,183,184].

3.5. Evaluation of a possible peripheral neuromuscular blockage by using the HWT

A deficit in motor coordination would likely affect performance of the mice in the OFT. Therefore, I investigated the effects of 1,8- and 1,4-cineole in the HWT, a classic animal model used to evaluate peripheral neuromuscular blockage. Our findings showed that diazepam 5 mg/kg *i.p.* as a positive control significantly decreased the percentage of mice grasping the wire by 60% and 50% in the tests with 1,8- and 1,4-cineole, respectively (p < 0.001), relative

to the levels in the control (Figure 34a and Figure 34b), indicating a myorelaxant effect of diazepam.

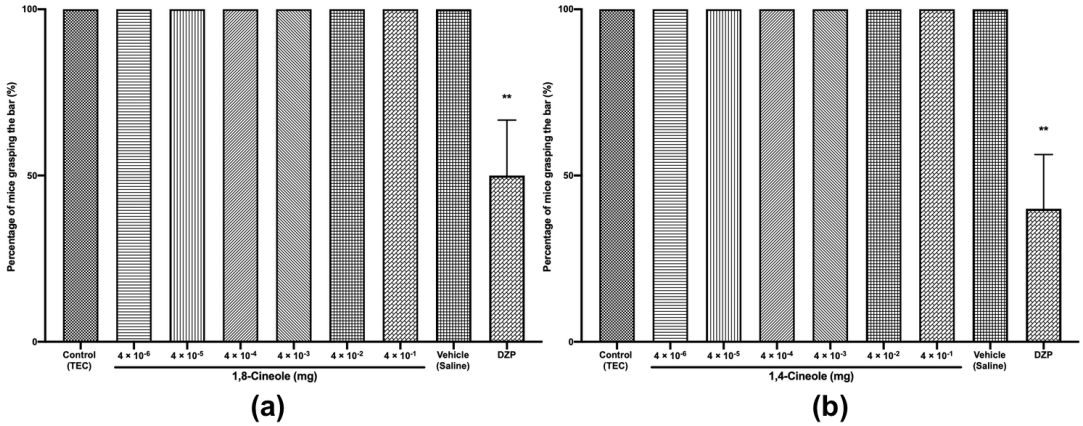


Figure 34: Horizontal wire test (HWT) of mice treated with 1,8- (a) or 1,4-cineole (b) (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg) or diazepam (5 mg/kg).

The values are given as the mean ± SEM of 10 mice. Statistical differences vs. the control group were calculated by using Student's t test or ANOVA followed by Dunnett's test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

In contrast, no change was observed after treatment with 1,8- or 1,4-cineole at doses of 0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg per 400 µL of TEC relative to the levels in the control, indicating a lack of myorelaxation effect at these doses. Consequently, the observed decrease in locomotor activity is probably not related to peripheral neuromuscular blockage but may involve neurons that control CNS activity. Similar results were obtained for the methyl and isopropyl N-methylanthranilates from *Choisya ternata* Kunth and for quercetin [173,181]. The LD_{50} value for oral administration of 1,8-cineole in mice is 3849 mg/kg [32], whereas the LD_{50} value for 1,4-cineole is 3100 mg/kg in rats [185]. The maximum administered dose in my study was 0.4 mg, which is much lower than the toxic doses. Moreover, abnormalities, such as an increase in urination or defecation, twisting, tremors, seizures, catalepsy and stereotypical behaviours, were not noticed, suggesting that the effect is probably not toxic.

3.6. Antidepressant-like activities of 1,8- and 1,4-cineole by using the FST and TST

In addition to the anxiolytic-like activities, I investigated 1,8- and 1,4-cineole, for their activity in the most widely used animal models for antidepressant drug screening, the FST [171] and TST [172]. In these tests, mice are forced to swim or are hung by their tails in an inescapable situation; the mice first demonstrate vigorous activity trying to escape the threatening environment and finally become immobile as a symptom of behavioural despair. Substances that decrease immobility time are known to demonstrate antidepressant properties in humans. In the FST, 1,8-cineole at doses of 0.0004 and 0.04 mg induced a significant decrease in the immobility time of mice by 44% and 39%, respectively, relative to the times in the control group [$F(8,81) = 5.400$, $p < 0.001$] (Figure 35a).

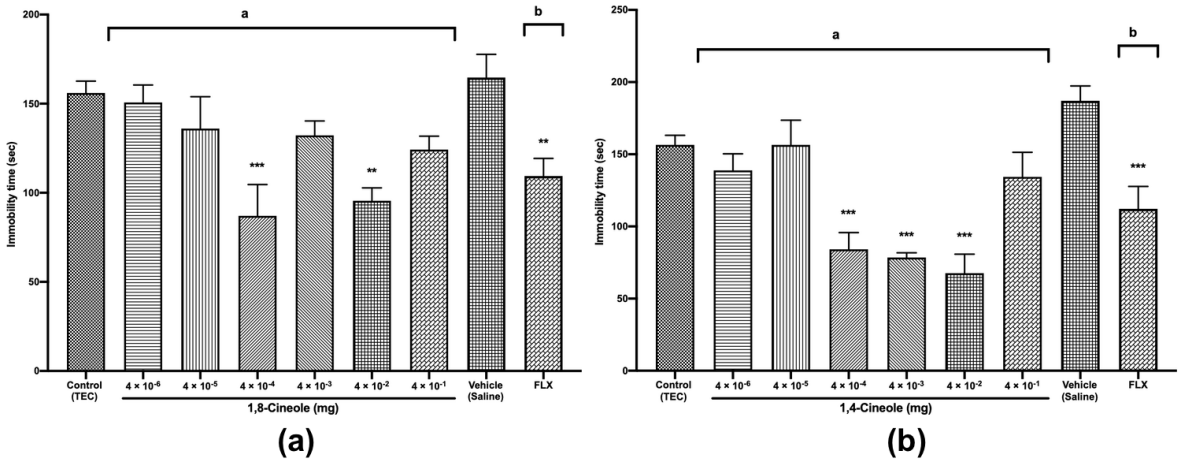


Figure 35: The Forced swimming test (FST) of mice treated with 1,8- (a) or 1,4-cineole (b) (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg) or fluoxetine (20 mg/kg).

The parameter analysed was immobility time in each test. The values are given as the mean ± SEM of 10 mice. The letter **a** indicates a significant difference when compared with the control (TEC); the letter **b** indicates a significant difference when compared with the vehicle (saline). Statistical differences vs. the control group were calculated by using Student's t test or ANOVA followed by Dunnett's test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Treatment with the antidepressant drug FLX at 20 mg/kg *p.o.*, used as a positive control, also significantly reduced the immobility time by 40% relative to the time in the vehicle (saline) group. Additionally, 1,8-cineole at doses of 0.0004 and 0.04 mg had greater antidepressant effects by 21% and 13%, respectively, relative to those for FLX. Antidepressant effects were previously observed in mice after oral administration of *R. officinalis* containing 45% 1,8-cineole [186]. On the other hand, no antidepressant activity was detected for 1,8-cineole 1 mg/kg administered intraperitoneally [168]; however, the authors suggested that the single-dose regimen of 1,8-cineole 1 mg/kg *i.p.* could be insufficient to potentially produce an antidepressant effect. As shown in Figure 35b, 1,4-cineole at doses of 0.0004, 0.004 and 0.04 mg induced dose-dependent significant decreases in the immobility times of mice by 46%, 49% and 56%, respectively, relative to those in the control group [$F(8,81) = 10.60$, $p < 0.001$]. Compared with the effect of FLX, the effects of 1,4-cineole at doses of 0.0004, 0.004 and 0.04 mg were 24%, 29% and 39% greater, indicating potent antidepressant activity of 1,4-cineole. In contrast to my study, Gomes et al. [163] found possible depressive activity of 1,4-cineole 400 mg/kg *p.o*. The differences in the results could also be explained by differences in the administered doses and routes of administration. Sousa et al. [187] made the same observations, suggesting that the administration route or difference in experimental models could explain differences in the pharmacological effects.

In the TST, as shown in Figure 36a, similar to the results observed in the FST, 1,8-cineole at doses of 0.0004 and 0.04 mg significantly reduced the immobility times in mice by 62% and 55%, respectively, relative to those in the control group [$F(8,81) = 7.563$, $p < 0.001$].

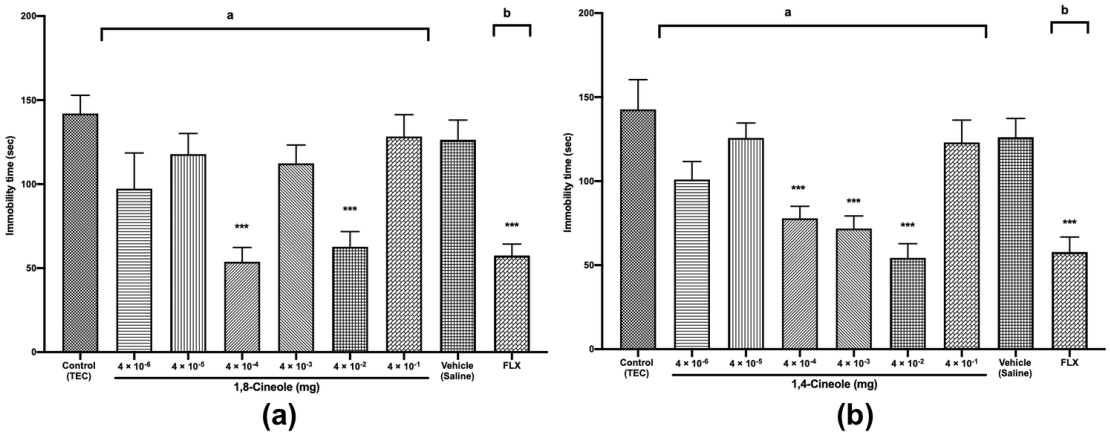


Figure 36: The Tail suspension test (TST) of mice treated with 1,8- (a) or 1,4-cineole (b) (0.000004, 0.00004, 0.0004, 0.004, 0.04 or 0.4 mg) or fluoxetine (20 mg/kg).

The parameter analysed was immobility time in each test. The values are given as the mean ± SEM of 10 mice. The letter **a** indicates a significant difference when compared with the control (TEC); the letter **b** indicates a significant difference when compared with the vehicle (saline). Statistical differences vs. the control group were calculated by using Student's t test or ANOVA followed by Dunnett's test. (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 36b shows that 1,4-cineole at doses of 0.0004, 0.004 and 0.04 mg significantly decreased the immobility times in mice by 45%, 49% and 62%, respectively, relative to those of the control group [$F(8,81) = 9.290$, $p < 0.001$]. The TST for 1,8- and 1,4-cineoles showed results similar to those of the FST, confirming the validity of my experiments. Similar to the activities of 1,8- and 1,4-cineoles, antidepressant activity was previously demonstrated for terpinen-4-ol contained in *Origanum majorana* L. [188], *β*-pinene and linalool (principal constituents of *Litsea glaucescens* Kunth) [189] and for the essential oil of *R. officinalis* [186].

3.7. Relationship structure-activity of 1,8- and 1,4-cineole

The two cineole isomers investigated in the present study have identical molecular weights (MW = 154.25 g/mol) and molecular formulas ($C_{10}H_{18}O$) but they differ in the position of the oxygen bridge connecting atoms 1 and 4 in 1,4-cineole or atoms 1 and 8 in 1,8-cineole. However, despite the structural similarity, their biological actions and mechanisms were different, which makes it interesting to compare the possible relationships between their physical and biochemical characteristics and their activities. The position of a functional group in a compound is reported to likely have activation or inhibitory effects on their activity. In this way, the study of Miyoshi et al. [5] on benzylacetone and derivatives demonstrated that a series of benzylacetone isomers differing only in the position of the ketone group showed different sedative activity levels, with the most sedative isomers being compounds with a ketone group on the carbon adjacent to C3. Additionally, aromatic compounds with isomeric structures have previously demonstrated various effects via activation of the olfactory bulb [190]. The boiling point of 1,8-cineole is 176°C–177°C, which is higher than 172°C–174°C for 1,4-cineole. Compounds with higher boiling points often have reduced vaporisation that may contribute to weakening of behavioural activity, which could explain the greater activity of 1,4-cineole than that of 1,8-cineole. Differences in conformation can also affect the activity of compounds. Miyoshi et al. [5] demonstrated that different steric configurations of the oxygen atom affected the activity of 4-phenyl-2-butanol. Another study also demonstrated that *cis*-isomers were 10-fold more potent than *trans*-isomers of compounds [3]. Regarding 1,8- and 1,4-cineole, the

spatial orientation of the dimethyl side chain differed between the two molecules, as shown in Figure 37.

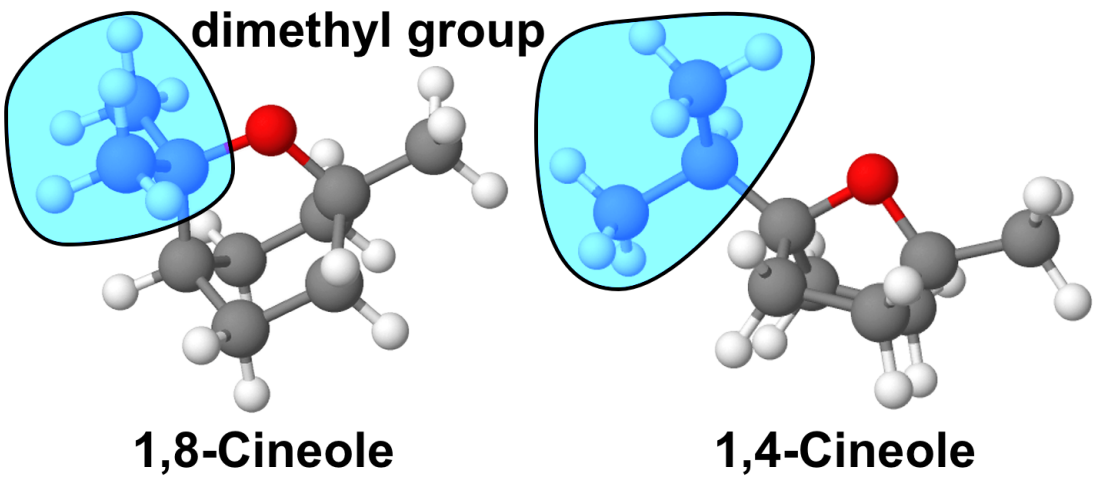


Figure 37: Tri-dimensional conformation showing the position of the free dimethyl group (circled in blue) of 1,8-cineole compared with that of 1,4-cineole.

In 1,4-cineole, the dimethyl group is free and located far from the hexyl ring, whereas in 1,8-cineole, the dimethyl group is involved in the formation of a heterocyclic ring [191]. This difference made 1,4-cineole a more flexible structure with a freely rotating dimethyl group that can interact with several types of olfactory receptors. Therefore, the differences in structural, biochemical and physical properties could explain the differences in the behavioural activities of 1,8- and 1,4-cineole.

4. Conclusions

Inhalation administration is a non-harmful method of administration that can be applied to any patient without distinction of age or mental or physical condition. This study demonstrated that 1,8- and 1,4-cineole possessed good sedative, anxiolytic and antidepressant activities when administered to mice via inhalation. The study findings suggest that 1,8- and 1,4-cineole could be considered for the treatment of CNS-related pathologies, such as post-traumatic stress disorder, attention deficit hyperactivity disorder, insomnia, anxiety or depression.

Chapter III. Molecular descriptors analysis and QSAR models for sedative activity of sesquiterpenes administered via inhalation in mice.

1. Introduction

Stress is defined as a state of threatened homeodynamic balance provoked by several intrinsic and extrinsic, real or perceived, challenges or stimuli defined as stressors [192]. Several drugs that act on the central nervous system (CNS), such as antiepileptics, benzodiazepines, and opioids, have been used to address stress-related disorders [193,194]. However, these treatments vary in efficacy and tolerability and are often accompanied by severe effects and drug–drug interactions [195]. Therefore, alternative agents with minor side effects and high efficacy are urgently required. Naturally occurring products are increasingly being considered, and essential oils have received much attention owing to their therapeutic effects on the CNS, particularly related to sedative activity [5,7,88,125,139]. The beneficial effects of monoterpenes were previously shown during the search for volatile compounds that are responsible for the sedative activity via inhalation. 1,8-cineole, for instance, displays good sedative, anxiolytic, and antidepressant activities when administered via inhalation to mice [98]. Other monoterpenes, such as ascaridole, *p*-cymene, *l*-menthol and geraniol also present sleep-promoting effects [125,138].

Sesquiterpenes (C15-isoprene unit) are less volatile natural products found in plants that possess greater stereochemical diversity and stronger odors compared with monoterpenes (C10-isoprene unit) [196]. Sesquiterpenes can be acyclic, monocyclic, bicyclic, or tricyclic, and have shown various properties *in vivo* or *in vitro*, such as sedative, anti-inflammatory, antimicrobial, or antitumor activities. For example, aristolen-1(10)-en-9-ol has a sedative activity in mice via the GABAergic system [7]; tagitinins C, F and A exhibit anti-inflammatory activity on human neutrophils by decreasing lipopolysaccharide-induced interleukin-6, interleukin-8 and tumor necrosis factor alpha production [197]; confertdiolide is active against the paw mouse edema induced by carrageenan [198]; *epi*-cubenol inhibits the *Staphylococcus aureus* NorA multidrug efflux pump [199]; cynaropicrin has cytotoxic and pro-apoptotic activities on leucocyte cancer cells [200]; parthenolide inhibits nuclear factor κB involved in inflammatory and immune responses [201]; and zerumbone and centratherin show respectively antitumor and genotoxic activities, *in vitro* and *in vivo* [202,203]. Caryophyllene oxide was recently identified as the sesquiterpene compound responsible for the sedative activity of *Chromolaena odorata* (L.) R.M.King & H.Rob (devil weed) essential oil in mice [204]. Its isomer, *β*-caryophyllene, has also shown sedative activity [3,204], suggesting a causal relationship between structure and activity, and between stereochemical diversity and sedative activity of sesquiterpene compounds. Computational methods are used to predict the pharmacokinetic properties of molecules to avoid wasting time and resources. Indeed, various useful *in silico* absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties can be assessed even with a large data set [205]. Although few exceptions are reported [206,207], *in silico* tools are generally faster and cheaper than any conventional laboratory methods for drug discovery [208], and scientists can easily control pharmacokinetic and toxicity properties of molecules through structural modification, as it was observed for antidiabetic or anti-hyperlipidemic drugs [209,210]. Consequently, in this study, physiochemical and *in silico* ADMET attributes of commonly reported sesquiterpenes were investigated to select the best candidates for drug evaluation. Selected sesquiterpenes were investigated for their sedative activity in mice, and regression analyses were performed to construct quantitative structure–activity relationship (QSAR) models for predicting the sedative activity of sesquiterpenes. For the first time, QSAR models that could successfully explain and predict the sedative activity of sesquiterpene compounds were established. These QSAR models may help in optimizing the development of novel sesquiterpenes with sedative activity.

2. Material and Methods

2.1. General experimental procedures

Eighteen sesquiterpene compounds with good predicted ADMET properties were selected from an initial list of 114. This list was obtained from literature data [211–213] and divided into training and test sets. Thus, nine compounds in the training set were investigated for the sedative activity in mice at three different doses between 4×10^{-4} and 4×10^{-2} mg/cage (cage volume = 0.065 m^3). Quantitative analyses of chemical structures can help in explaining and predicting physiological activities [214]. However, various chemical structures and physicochemical properties of sesquiterpenes makes it difficult to predict their sedative properties accurately. Nevertheless, QSAR studies are useful for analyzing and predicting pharmacological effects, pharmacokinetic parameters, and several metabolic modifications [214]. To the best of my knowledge, no report on a QSAR study conducted on sedative sesquiterpenes is available. Therefore, the compounds in the test set (good hits whose activities are unknown) were completed with six external sesquiterpenes with sedative activity obtained from previous studies using the same experimental protocol as in our study [3,215,216]. These sesquiterpenes were used to build QSAR models with simple and multiple regression analyses to explain and predict their sedative properties based on data from the experimental set of compounds. It is important to mention that although the number of compounds in the training set is small, it allows the construction of a valid predictive model which can be used to predict activity of novel sesquiterpenes. Several models of QSAR studies have been successfully developed based on small sets of compounds [217–224].

2.2. Selection of compounds and screening method

The original set of 114 sesquiterpenes was selected among naturally occurring and frequently reported sesquiterpenes in the literature [211–213]. ADME physicochemical properties of selected compounds were predicted using the Swiss ADME online web server [225]. This platform is a web-based tool that provides free access to a pool of fast and robust predictive models for physicochemical properties, pharmacokinetics, drug-likeness, and medicinal chemistry friendliness [225].

Briefly, chemical structures were collected in simplified molecular-input line-entry system (SMILES) format from the NCBI PubChem compound database (https://pubchem.ncbi.nlm.nih.gov/) and processed using the Swiss ADME software. Physicochemical properties, lipophilicity, and solubility were considered in the analysis [225,226]. Cutoff values were set using well-known rules, such as Lipinski's RO5, Ghose's rule, and Veber's rule [227–229]. These rules were selected because they respond well to the estimated solubility and permeability of compounds in biological layers. Indeed, the RO5 predicts that poor absorption or permeation is more likely to occur when there are more than 5 hydrogen bond donors, 10 hydrogen bond acceptors, the MW is >500, and the calculated log P is >5 (or MLogP > 4.15) [227]. Ghose estimates that compounds can be good hits when the calculated log P is between −0.4 and 5.6, MW is between 160 and 480, MR is between 40 and 130, and the total number of atoms is between 20 and 70 [229]. According to Veber's rule, compounds that meet only 2 criteria of 10 or fewer rotatable bonds and polar surface area equal to or less than 140 or 12 or fewer hydrogen bond donors and acceptors will have a high probability of good oral bioavailability. However, using restrictive criteria with different filters could discard some interesting chemicals [230]. Nevertheless, although other interesting rules

such as the beyond rule of five (bRo5) exist [231–234], sesquiterpenes in this study do not have a MW >500 Da. Therefore, the bRo5 was not considered in the analysis. Molecular parameters for drug likeliness were MW, number of hydrogen bond donors (nHBDon), nHBAcc, lipophilicity (log P), aqueous solubility (log S), topological polar surface area (TPSA), number of rotatable bonds (nRotB), and MR [214,225]. The Swiss vector machine algorithm was used for predicting properties [214,225]. Furthermore, toxicity was predicted using DataWarrior software. This approach uses a two-dimensional scaling algorithm and employs vector-based or non-vector-based descriptors to visualize chemical or pharmacophore spaces of large data sets [235]. After ADMET evaluation, 18 molecules (Table 5 and Figure 38) were selected for acceptable pharmacokinetic properties with no toxicity, which met cutoff values proposed for Lipinski's RO5, Ghose's, and Veber's rules.

Table 5: Names and physiochemical properties of selected 18 sesquiterpenes and additional six sesquiterpenes for the test set.

	Sesquiterpenes	Formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	Lipinski's violations	Ghose's violations	Veber's violations
Training set													
1	α-bisabolol	C15H26O	222.37	4	1	1	72.36	20.23	3.76	−3.34	0	0	0
2	Cedrol	C15H26O	222.37	0	1	1	68.56	20.23	3.54	−3.66	0	0	0
3	Curcumol	C15H24O2	236.35	1	2	1	69.73	29.46	2.9	−3.02	0	0	0
4	Curzerene	C15H20O	216.32	2	1	0	68.74	13.14	3.98	−4.21	0	0	0
5	Farnesol	C15H26O	222.37	7	1	1	73.96	20.23	4.32	−4.17	0	0	0
6	Germacrone	C15H22O	218.33	0	1	0	70.88	17.07	3.62	−3.37	0	0	0
7	Nerolidol	C15H26O	222.37	7	1	1	74	20.23	4.19	−3.8	0	0	0
8	Nootkatone	C15H22O	218.33	1	1	0	68.98	17.07	3.57	−3.56	0	0	0
9	Zerumbone	C15H22O	218.33	0	1	0	70.62	17.07	3.57	−3.68	0	0	0
Test set (experimental activity unknown)													
1	α-cadinol	C15H26O	222.37	1	1	1	70.72	20.23	3.43	−3.26	0	0	0
2	α-santalol	C15H24O	220.35	4	1	1	68.04	20.23	3.57	−3.44	0	0	0
3	Cubebol	C15H26O	222.37	1	1	1	68.82	20.23	3.5	−3.62	0	0	0
4	Cubenol	C15H26O	222.37	1	1	1	70.72	20.23	3.52	−3.48	0	0	0
5	δ-cadinol	C15H26O	222.37	1	1	1	70.72	20.23	3.44	−3.26	0	0	0
6	Humulene epoxide	C15H24O	220.35	0	1	0	69.91	12.53	3.72	−3.67	0	0	0
7	Rotundone	C15H22O	218.33	1	1	0	69.24	17.07	3.56	−3.43	0	0	0
8	Santonin	C15H18O3	246.3	0	3	0	68.15	43.37	2.4	−2.81	0	0	0
9	t-Muurolol	C15H26O	222.37	1	1	1	70.72	20.23	3.42	−3.26	0	0	0

Table 5: Names and physiochemical properties of selected 18 sesquiterpenes and additional six sesquiterpenes for the test set. (continued)

	Sesquiterpenes	Formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	Lipinski's violations	Ghose's violations	Veber's violations
Test set (activity collected from literature [3,215,216])													
1	α-Gurjunene	C15H24	204.35	0	0	0	67.14	0	3.26	−3.69	1	0	0
2	Calarene	C15H24	204.35	0	0	0	66.88	0	3.2	−4.04	1	0	0
3	Caryophyllene Oxide	C15H24O	220.35	0	1	0	68.27	12.53	3.15	−3.45	0	0	0
4	Hexahydroxyzerumbol	C15H30O	226.4	0	1	1	73.01	20.23	3.25	−4.65	0	0	0
5	Hexahydroxyzerumbone	C15H28O	224.38	0	1	0	72.05	17.07	3.05	−4.45	0	0	0
6	Valerena-4,7(11)-diene	C15H24	204.35	1	0	0	69.04	0	3.43	−3.91	1	0	0

Properties were analyzed by the Swiss ADME software

MW: molecular weight, nHBDon: number of hydrogen bonds donors, nHBAcc: number of hydrogen bonds acceptors, log P: lipophilicity, log S: aqueous solubility, TPSA: topological polar surface area, nRotB: number of rotatable bonds, MR: molar refractivity.

2.3. Construction of QSAR models

2.3.1. Training and external validation sets

The 18 selected sesquiterpenes were divided into two equal groups. Nine commercially available sesquiterpenes, for which the sedative activity was investigated in this study, were assessed as a training set (Figure 38A). The sedative activity was evaluated using an open field test, for each dose between 4×10^{-4} and 4×10^{-2} mg/cage. The activity of the remaining nine sesquiterpenes (Figure 38B) was predicted using equations built with the training set. In addition, because the actual sedative activity of these compounds is unknown, existing data on the sedative activity of six sesquiterpenes were used as an external test set of compounds [3,215,216]. These compounds are presented in Figure 38C.

The molecular properties of compounds are important determinants of their pharmacological activities. Investigating the relevance of molecular properties to the sedative activity was conducted by analyzing differences among 10 important molecular properties of compound [236]. These included MW, TPSA, log P, MR, nHBAcc, nHBDon, nRotB, number of rings, number of fused rings, and hydrophilicity. All descriptors above were calculated using the PaDEL-Descriptor package [237].

The values obtained from the calculation of molecular descriptors were used to construct simple and multiple regression models for compounds in the training set. Each model was evaluated using standard least squares as a selection pressure, and regression diagnosis and other statistical analyses were performed using JMP statistical discovery and Microsoft Excel software. The AUC values obtained from biological data were used as the dependent variable in QSAR analyses (Table 7).

2.3.2. Statistical analysis

The performance of the developed QSAR model was evaluated using r^2 (the squared correlation coefficient), q^2_{LOO} (cross-validated correlation coefficient), F-test (Fischer's value) for statistical significance, and pred_r^2 (r^2 for the external test set). The main utility of a QSAR model is its capability to be replicated by the model. However, only when the following conditions are satisfied, a QSAR model will be robust and predictive: $r^2 > 0.6$, $q^2_{LOO} > 0.5$, $r^2 - q^2_{LOO} < 0.3$, and pred_$r^2 > 0.6$ [238–240].

2.3.3. Internal validation

The model's predictive power was evaluated using the Leave-One-Out Cross-Validation (LOO-CV) method following the methodology described previously [221,222,238,239,241–244]. The q^2 cross-validation correlation coefficient for the LOO method was calculated according to Eq. 3. Each data points were removed once, and the model was built using the remaining data points (compounds) to calculate the q^2_{LOO} statistics for the developed QSAR models. The activity of the removed compound was thus predicted using the generated equation. This was repeated until all compounds were omitted at least once, and their predicted activities were used to calculate the validation parameters, i.e., q^2_{LOO}, absolute percentage error (APE), mean absolute percentage error (MAPE), and standard deviation of error of prediction (SDEP) [220,221]. APEs of predictions were calculated for all data points, and the average

(i.e., MAPE) were calculated using Eqs. 4 and 5, respectively. MAPE is commonly used because it allows for easy interpretation of the average difference between the predicted and actual value of compounds in the training set. SDEP for the LOO method was calculated according to Eq. 6.

$$q^2_{Loo} = 1 - \frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{\sum_{i=1}^{n}(y_i - \bar{y}_i)^2}$$ **(Eq. 3)**

$$APE = \frac{|y_i - \hat{y}_i|}{y_i} \times 100$$ **(Eq. 4)**

$$MAPE = \frac{\sum_{i=1}^{n} APE}{n}$$ **(Eq. 5)**

$$SDEP = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n}}$$ **(Eq. 6)**

where $\hat{y}_i$ is the estimated value, y_i the experimental value, $\bar{y}_i$ the average value.

Furthermore, the r^2 values are proportional to the number of descriptors in the model, which is unreliable in determining the predictive response of the model. As such, r^2 is adjusted, which is defined as in Eq. 7.

$$r^2 adj = \frac{r^2 - p\,(n-1)}{n-p+1}$$ **(Eq. 7)**

where p is the number of descriptors in the equation and n is the number of compounds in the training set.

The numerical changes between the r^2 and r^2adj should be <0.3, showing that the number of descriptors used in the QSAR model is acceptable and vice versa [238,241,242,245,246].

2.3.4. External validation

It is noteworthy that good r^2 values are insufficient in validating the model. Therefore, more parameters must be established to highlight the predictive capability of the models. The optimal combination of the training and test sets was subjected to MLRplusValidation 1.3 program (http://dtclab.webs.com/software-tools) [243] to internally and externally validate the model generated based on acceptable model criteria of the test set [222,238,242,243,245] for a robust QSAR model with good predictive potential, such as:

pred_$r^2 > 0.6$

$\frac{r^2 - r_0^2}{r^2} < 0.1$ or $\frac{r^2 - r'^2_0}{r^2} < 0.1$

$0.85 < k < 1.15$ or $0.85 < k' < 1.15$

r^2_m (overall) > 0.5

r^2_m (overall) is calculated according to Eq.8:

$$r^2_m = r^2 - (1 - \sqrt{r^2 - r_0^2})$$ **(Eq. 8)**

where r^2 is the squared correlation coefficient between the observed and predicted activities, r^2_0 is the squared correlation coefficient between the predicted and observed activities, and k and k′ are the regression slopes passing through the origin.

The predicted value of r^2 in the external test set (pred_r^2) is calculated according to Eq. 9:

$$pred\_r^2 = 1 - \frac{\sum(y_{pred(test)} - y_{(test)})^2}{\sum(y_{(test)} - \bar{y}_{(training)})^2}$$ **(Eq. 9)**

where $y_{pred(test)}$ and $y_{(test)}$ are predicted, and observed activity for the test set and $\bar{y}_{(training)}$ is the mean activity value of the training test.

Both summations are over all molecules in the test set. The pred_r^2 value indicates the current model's predictive power for the external test set [238,242,245]. The statistical significance of the model is determined by the F-test, whose high value is of interest.

2.3.5. Y-randomization test

The Y-randomization test ensured the robustness of the QSAR model. In this technique, the dependent variable vector is randomly shuffled, and a new QSAR model is developed using the original independent variable matrix. The random number simulation has shown that the probability of chance correlation and, with it, the degree of inflation of internal figures of merit is considerable in small data sets and in case of low object-to-variable ratios. Therefore, assessing the probability of chance correlation with the help of a permutation test (scrambling) when the variable selection is applied became common practice in QSAR studies [222,242]. The new QSAR models (after several repetitions) are expected to have low r^2 and q^2 values. If the opposite happens, an acceptable QSAR model cannot be obtained for the specific modeling method and data.

2.4. Drugs and reagents

Benzylacetone (purity > 95%) was obtained from Tokyo Kasei and used as a positive control. Triethylcitrate, a nonsedating odorless solvent, was used to dissolve fragrant components. Nerolidol (purity > 97%) and nootkatone (purity > 97%) were purchased from Tokyo Chemical Industries. Farnesol (purity > 90%) was purchased from Wako Pure Chemical Industries. Curzerene (purity > 97%) was purchased from MedChemExpress. Curcumol (purity > 99%) and cedrol (purity > 98%) were purchased from BLD Pharm. α-Bisabolol (purity ≥ 93%) was purchased from Sigma Aldrich. Zerumbone (purity ≥ 98%) was purchased from Funakoshi Adipogen Life Sciences. Germacrone (purity ≥ 98%) was purchased from Cayman Chemicals. All chemicals used were of the highest grade available.

2.5. Animals

Four-week-old male ddY mice, weighing 20–30 g, were purchased from Japan SLC. Mice were housed in colony cages under a 12/12-h light/dark cycle at 25°C ± 2°C and 50%–60% relative humidity. Animals were fed pellet chow, given water *ad libitum,* and allowed to acclimatize for 1 week before experiments. Animal experiments were conducted in compliance with the Animal Research Committee guidelines of Kyoto University (approval no. 2014-14-3; first approved on December 27, 2014 and renewed annually until March 14, 2021). Experimental procedures involving animals and their care were conducted in compliance with institutional guidelines and the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the authority of the Ministry of Education, Culture, Sports, Science and Technology, Japan (2006). All experiments were conducted between 10:00 a.m. and 5:00 p.m. under identical conditions.

2.6. Open-field test

Compound sedative activity was evaluated using mice and counting spontaneous motor movement in an open-field test as previously described [88,125,204]. Briefly, each compound was completely dissolved in 400-μL triethylcitrate and dropped onto four filter paper disks. The vapor from the solution pervaded glass cages (W: 60 cm, L: 30 cm, H: 34 cm; volume = 0.065 m^3) by natural diffusion. The total compound amount present was 4×10^{-4}, 4×10^{-3}, or 4×10^{-2} mg/cage.

Sixty minutes after charging the sample, a mouse was placed in the center of the cage and monitored with a video camera for 1 h. During monitoring, the frequency of each mouse crossing lines drawn at 10-cm intervals on the cage floor was counted every 5 min. The AUC

of locomotor activity counts per 5 min (y-axis) and time (x-axis), representing the total locomotor activity, was calculated using the trapezoidal rule. Statistical analyses were performed by employing Dunnett's test using GraphPad Software. A probability level of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Structural and physicochemical properties

The first part of this study gathered information on physicochemical properties to select the best compounds for further analysis. A total of 114 naturally occurring and frequently reported sesquiterpenes were evaluated against key physicochemical parameters by using the Swiss ADME web server [214,225]. A comprehensive list of all investigated sesquiterpenes with reported pharmacokinetic properties is shown in Table 6. For instance, the molecular weight (MW) of sesquiterpenes varies from 128.17 to 262.26, and log P values vary from 1.47 to 4.97. The prediction was thus built by considering a broad range of parameters, thereby reducing the risk of compound selection at random. Furthermore, the Swiss ADME web server could predict values of some sesquiterpenes that were higher than the cutoff value set using Lipinski's rule of five (RO5). This was observed in case of naphthalene, bicyclogermacrene, and aromadendrene, justifying categorizing these sesquiterpenes as bad "hits." Some previous studies using the Swiss tool confirmed its utility in predicting molecular properties [225]. Finally, 18 compounds (Figure 38) were selected that did not violate Lipinski's RO5, Ghose's rule, and Veber's rule. For further investigations, all 18 were predicted to lack significant toxicity and were divided into two equal groups: training (Figure 38A) and test (Figure 38B) sets. In addition, an external validation test set (Figure 38C) was evaluated as explained in the Material and Methods section.

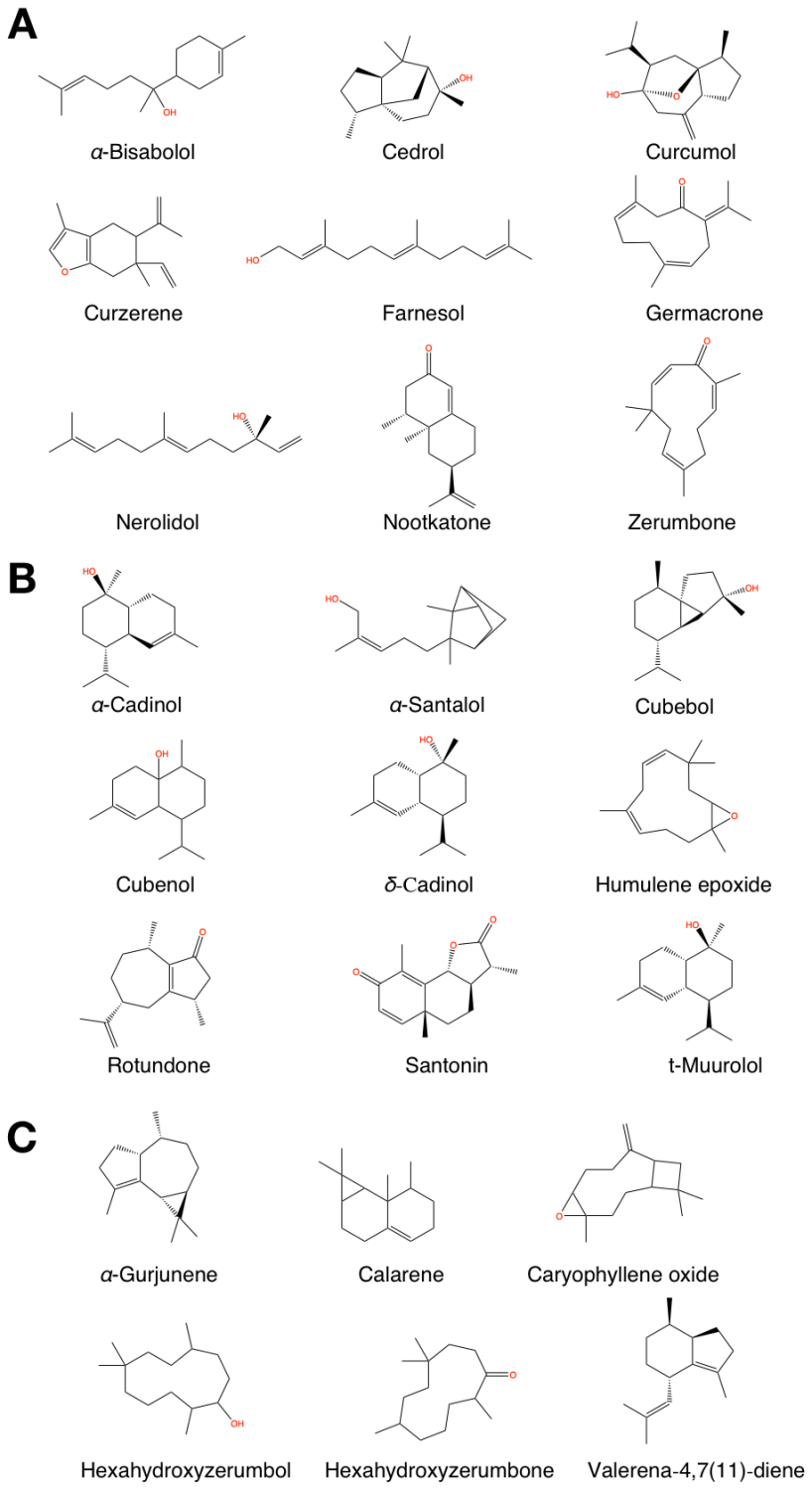
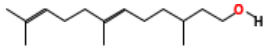
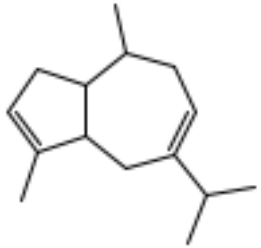
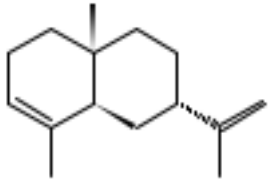
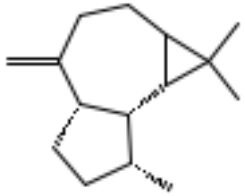
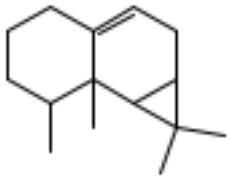
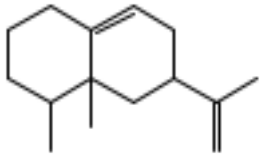
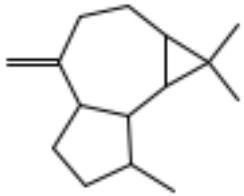
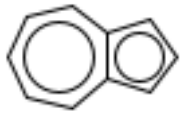
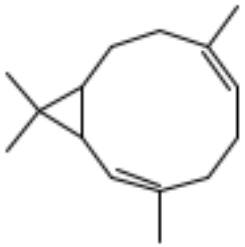


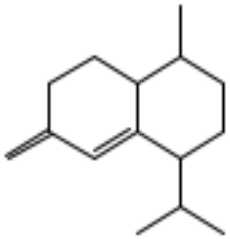
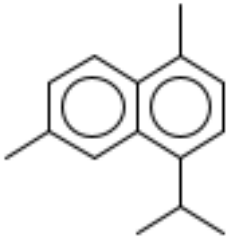
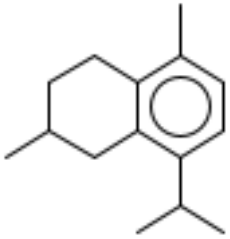
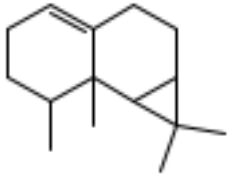
Figure 38: Chemical structures of sesquiterpenes in the training set (A), test set (B), and external test set (C).

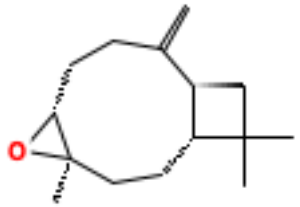
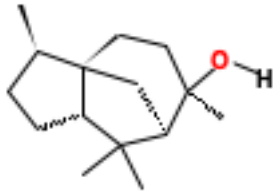
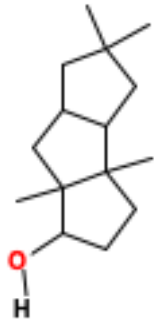
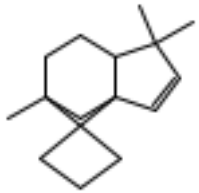
The training set included α-bisabolol, cedrol, curcumol, curzerene, farnesol, germacrone, nerolidol, nootkatone, and zerumbone. The test set included α-cadinol, α-santalol, cubebol, cubenol, δ-cadinol, humulene epoxide, rotundone, santonin, and t-muurolol. The external test set included hexahydroxyzerumbone, hexahydroxyzerumbol, caryophyllene oxide, calarene, α-gurjunene, and valerena-4,7(11)-diene.

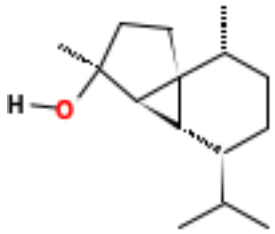
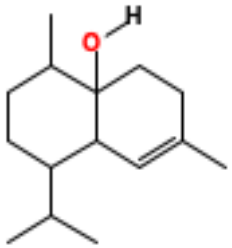
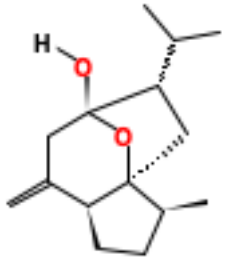
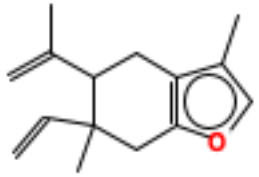
Table 6: Names, structures and physiochemical properties of the 114 analyzed sesquiterpenes.

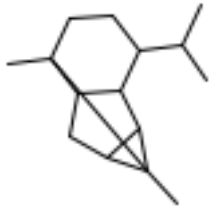
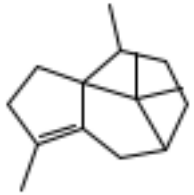
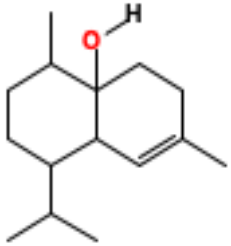
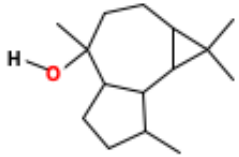
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
2,3-dihydrofarnesol		C15H28O	224.38	8	1	1	74.43	20.23	4.47	-4.34	0	0	0
3,7-guaiadiene		C15H24	204.35	1	0	0	69.04	0	4.06	-3.54	1	0	0
7-epi-*α*-selinene		C15H24	204.35	1	0	0	68.78	0	4.39	-4.32	1	0	0
Allo-aromadendrene		C15H24	204.35	0	0	0	67.14	0	4.34	-4.07	1	0	0
Aristolene		C15H24	204.35	0	0	0	66.88	0	4.37	-4.04	1	0	0

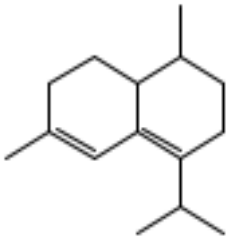
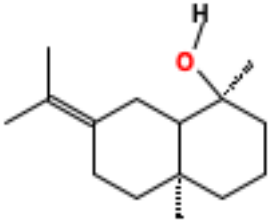
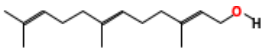
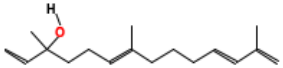
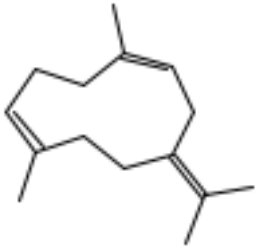
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Aristolochene		C15H24	204.35	1	0	0	68.78	0	4.42	-4.34	1	0	0
Aromadendrene		C15H24	204.35	0	0	0	67.14	0	4.34	-4.07	1	0	0
Azulene		C10H8	128.17	0	0	0	43.06	0	3.02	-3.39	0	2	0
Bicyclogermacrene		C15H24	204.35	0	0	0	68.78	0	4.15	-3.72	1	0	0

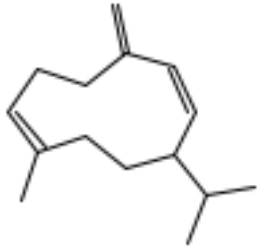
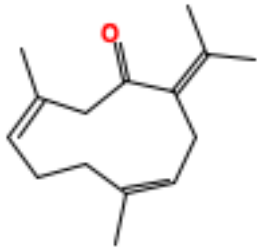
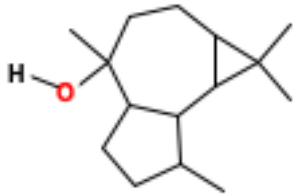
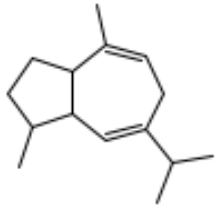
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Bicyclosesqui-phellandrene		C15H24	204.35	1	0	0	69.04	0	4.25	-4	1	0	0
Cadalene		C15H18	198.3	1	0	0	68.46	0	4.76	-5.01	1	0	0
Calamene		C15H22	202.34	1	0	0	68.23	0	4.57	-4.5	1	0	0
Calarene		C15H24	204.35	0	0	0	66.88	0	4.37	-4.04	1	0	0

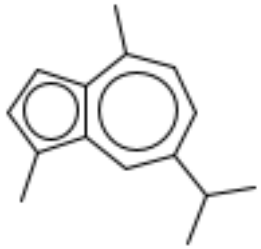
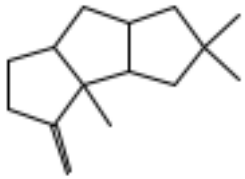
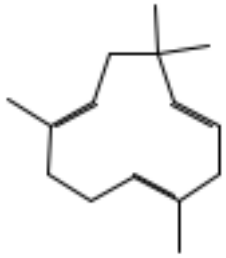
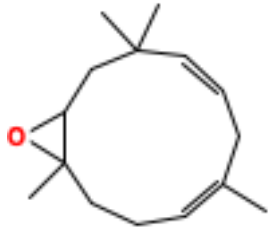
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Caryophyllene oxide		C15H24O	220.35	0	1	0	68.27	12.53	3.68	-3.45	0	0	0
Cedrol		C15H26O	222.37	0	1	1	68.56	20.23	3.54	-3.66	0	0	0
Ceratopicanol		C15H26O	222.37	0	1	1	68.26	20.23	3.59	-3.8	0	0	0
Clovene		C15H24	204.35	0	0	0	66.62	0	4.65	-4.74	1	0	0

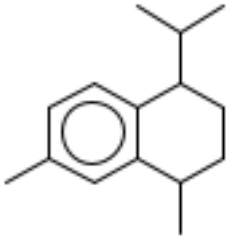
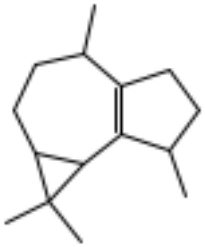
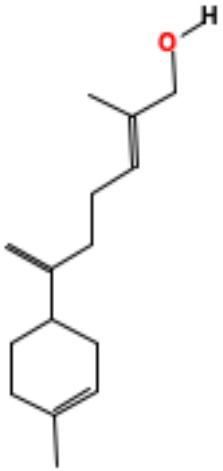
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Cubebol		C15H26O	222.37	1	1	1	68.82	20.23	3.5	-3.62	0	0	0
Cubenol		C15H26O	222.37	1	1	1	70.72	20.23	3.52	-3.48	0	0	0
Curcumol		C15H24O2	236.35	1	2	1	69.73	29.46	2.9	-3.02	0	0	0
Curzerene		C15H20O	216.32	2	1	0	68.74	13.14	3.98	-4.21	0	0	0

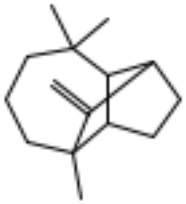
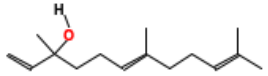
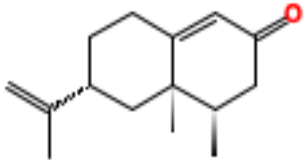
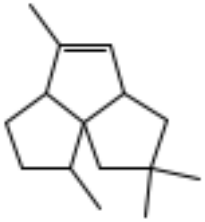
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Cyclosativene		C15H24	204.35	1	0	0	65.24	0	4.32	-4.11	1	0	0
Cyperene		C15H24	204.35	0	0	0	66.88	0	4.4	-3.83	1	0	0
Epi-cubenol		C15H26O	222.37	1	1	1	70.72	20.23	3.52	-3.48	0	0	0
Epiglobulol		C15H26O	222.37	0	1	1	68.82	20.23	3.42	-3.57	0	0	0

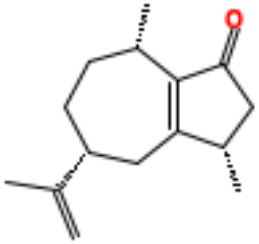
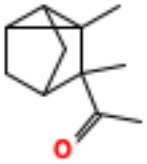
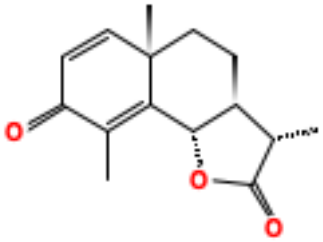
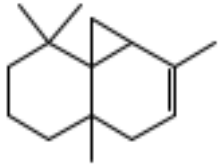
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Epizonarene		C15H24	204.35	1	0	0	69.04	0	4.18	-3.57	1	0	0
Eudesm-7(11)-en-4-ol		C15H26O	222.37	0	1	1	70.46	20.23	3.69	-3.67	0	0	0
Farnesol		C15H26O	222.37	7	1	1	73.96	20.23	4.32	-4.17	0	0	0
Fokienol		C17H28O	248.4	9	1	1	83.14	20.23	4.72	-4.28	1	0	0
Germacrene B		C15H24	204.35	0	0	0	70.68	0	4.6	-4.74	1	0	0

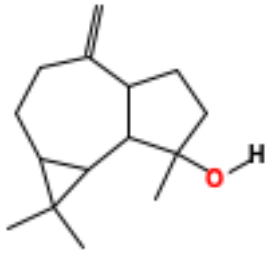
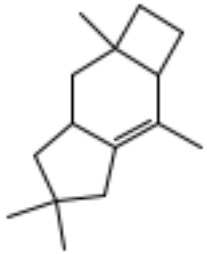
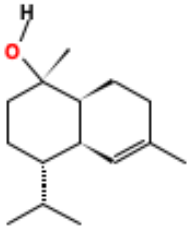
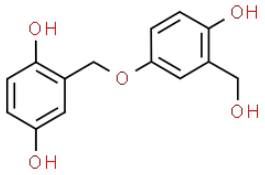
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Germacrene D		C15H24	204.35	1	0	0	70.68	0	4.3	-4.03	1	0	0
Germacrone		C15H22O	218.33	0	1	0	70.88	17.07	3.62	-3.37	0	0	0
Globulol		C15H26O	222.37	0	1	1	68.82	20.23	3.42	-3.57	0	0	0
Guaia-6,9-diene		C15H24	204.35	1	0	0	69.04	0	4.14	-3.74	1	0	0

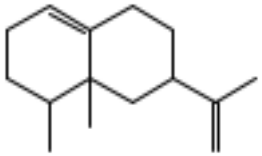
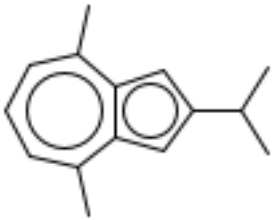
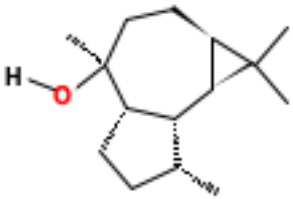
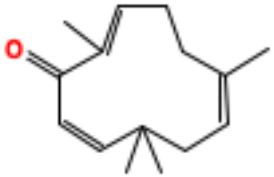
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Guaiazulene		C15H18	198.3	1	0	0	67.58	0	4.53	-4.5	1	0	0
Hirsutene		C15H24	204.35	0	0	0	66.88	0	4.51	-4.26	1	0	0
Humulene		C15H24	204.35	0	0	0	70.42	0	4.26	-3.97	1	0	0
Humulene epoxide		C15H24O	220.35	0	1	0	69.91	12.53	3.72	-3.67	0	0	0

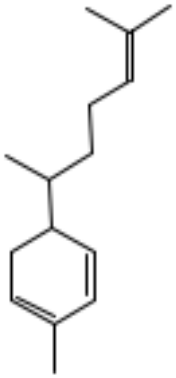
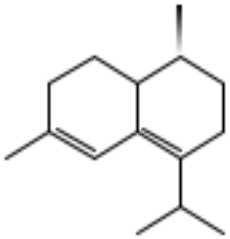
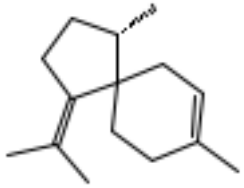
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Isocalamenene		C15H22	202.34	1	0	0	68.07	0	4.58	-4.55	1	0	0
Isoledene		C15H24	204.35	0	0	0	67.14	0	4.26	-3.67	1	0	0
Lanceol		C15H24O	220.35	5	1	1	71.84	20.23	3.71	-3.37	0	0	0

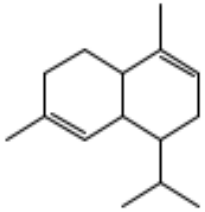
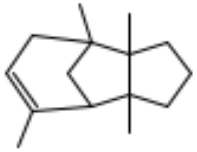
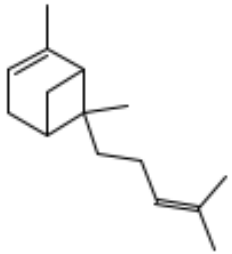
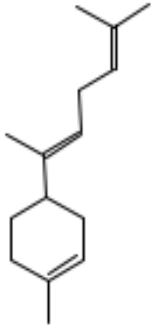
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Longicyclene		C15H24	204.35	0	0	0	64.98	0	4.39	-4.26	1	0	0
Longifolene		C15H24	204.35	0	0	0	66.88	0	4.5	-4.31	1	0	0
Naphtalene		C10H8	128.17	0	0	0	43.95	0	3.1	-3.45	1	2	0
Nerolidol		C15H26O	222.37	7	1	1	74	20.23	4.19	-3.8	0	0	0
Nootkatone		C15H22O	218.33	1	1	0	68.98	17.07	3.57	-3.56	0	0	0
Pentalenene		C15H24	204.35	0	0	0	66.88	0	4.39	-4.06	1	0	0

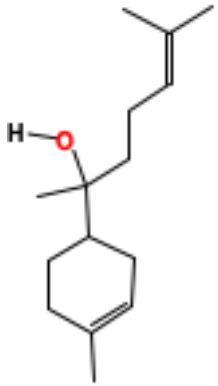
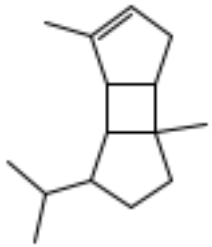
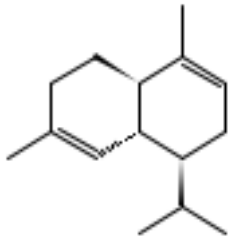
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Rotundone		C15H22O	218.33	1	1	0	69.24	17.07	3.56	-3.43	0	0	0
Santalone		C11H16O	164.24	1	1	0	48.33	17.07	2.36	-2.12	0	0	0
Santonin		C15H18O3	246.3	0	3	0	68.15	43.37	2.4	-2.81	0	0	0
Sesquichamene		C15H24	204.35	0	0	0	66.62	0	4.48	-4.11	1	0	0

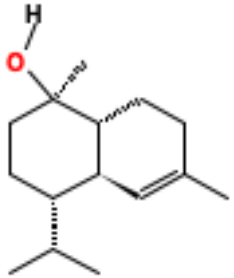
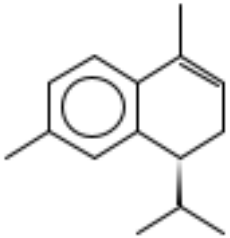
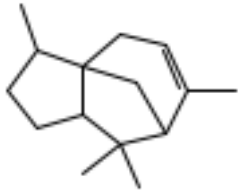
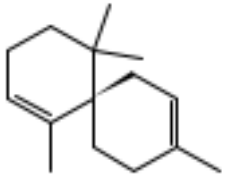
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Spathulenol		C15H24O	220.35	0	1	1	68.34	20.23	3.26	-3.17	0	0	0
Sterpurene		C15H24	204.35	0	0	0	66.88	0	4.42	-3.78	1	0	0
t-muurolol		C15H26O	222.37	1	1	1	70.72	20.23	3.42	-3.26	0	0	0
Terrestrol E		C14H14O5	262.26	4	5	4	69.62	90.15	1.47	-2.92	0	0	0

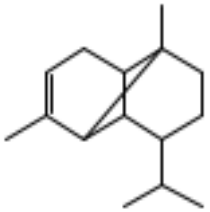
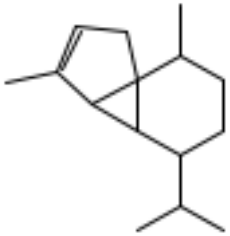
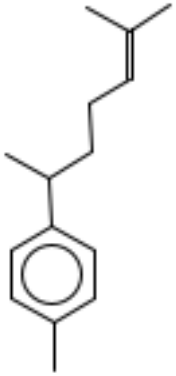
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Valencene		C15H24	204.35	1	0	0	68.78	0	4.41	-4.34	1	0	0
Vetivazulene		C15H18	198.3	1	0	0	67.58	0	4.55	-4.5	1	0	0
Viridiflorol		C15H26O	222.37	0	1	1	68.82	20.23	3.43	-3.57	0	0	0
Zerumbone		C15H22O	218.33	0	1	0	70.62	17.07	3.57	-3.68	0	0	0

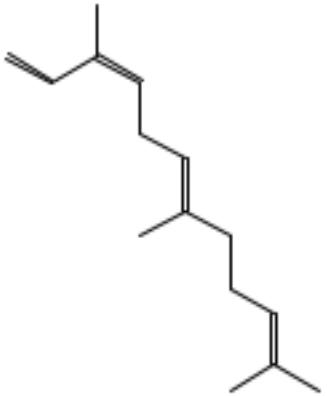
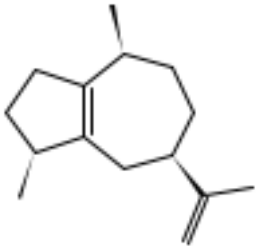
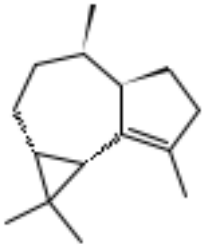
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
Zingiberene		C15H24	204.35	4	0	0	70.68	0	4.47	-4.1	1	0	0
Zonarene		C15H24	204.35	1	0	0	69.04	0	4.17	-3.57	1	0	0
α-alaskene		C15H24	204.35	0	0	0	68.78	0	4.33	-4	1	0	0

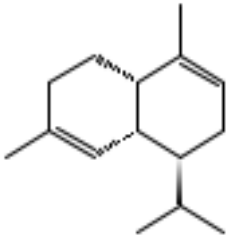
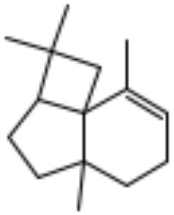
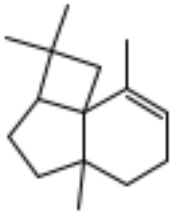
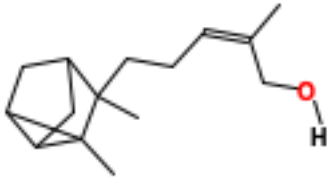
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
α-amorphene		C15H24	204.35	1	0	0	69.04	0	4.08	-3.61	1	0	0
α-Barbatene		C15H24	204.35	0	0	0	66.62	0	4.48	-4.11	1	0	0
α-bergamotene		C15H24	204.35	3	0	0	68.78	0	4.7	-4.97	1	0	0
α-bisabolene		C15H24	204.35	3	0	0	70.68	0	4.77	-4.92	1	0	0

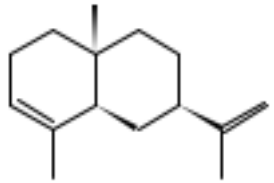
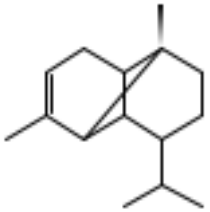
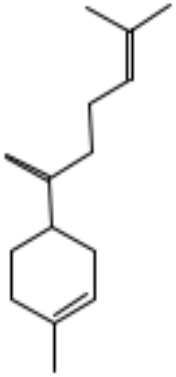
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
α-bisabolol		C15H26O	222.37	4	1	1	72.36	20.23	3.76	-3.34	0	0	0
α-bourbonene		C15H24	204.35	1	0	0	67.14	0	4.29	-3.86	1	0	0
α-cadinene		C15H24	204.35	1	0	0	69.04	0	4.08	-3.61	1	0	0

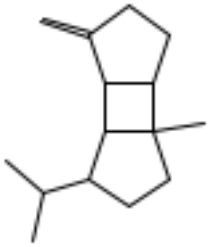
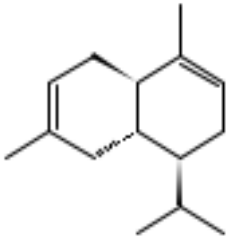
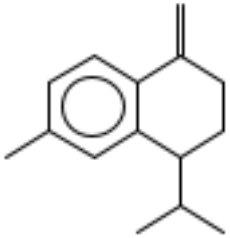
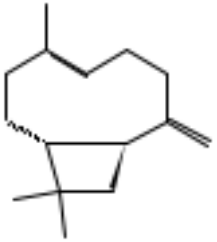
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
α-cadinol		C15H26O	222.37	1	1	1	70.72	20.23	3.43	-3.26	0	0	0
α-calacorene		C15H20	200.32	1	0	0	68.39	0	4.38	-4.09	1	0	0
α-cedrene		C15H24	204.35	0	0	0	66.88	0	4.36	-4.02	1	0	0
α-chamigrene		C15H24	204.35	0	0	0	68.52	0	4.29	-3.9	1	0	0

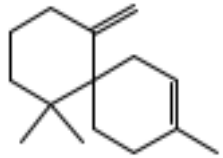
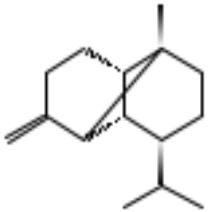
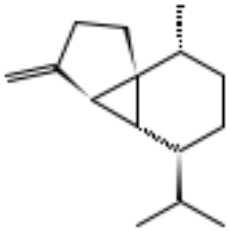
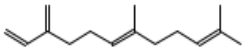
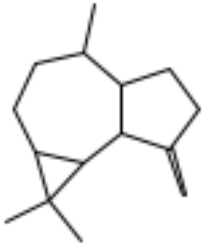
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
α-copaene		C15H24	204.35	1	0	0	67.14	0	4.3	-3.86	1	0	0
α-cubebene		C15H24	204.35	1	0	0	67.14	0	4.31	-3.86	1	0	0
α-curcumene		C15H22	202.34	4	0	0	69.55	0	4.86	-4.52	1	0	0

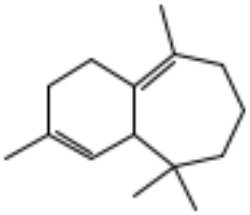
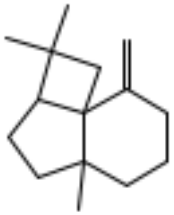
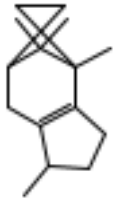
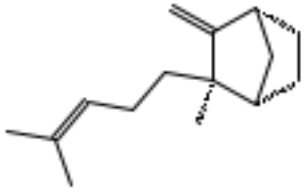
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
α-farnesene		C15H24	204.35	6	0	0	72.32	0	4.96	-4.57	1	0	0
α-guaiene		C15H24	204.35	1	0	0	69.04	0	4.3	-3.93	1	0	0
α-gurjunene		C15H24	204.35	0	0	0	67.14	0	4.27	-3.69	1	0	0

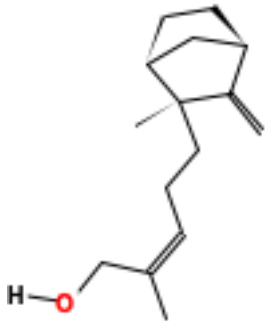
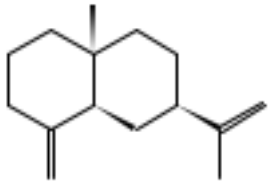
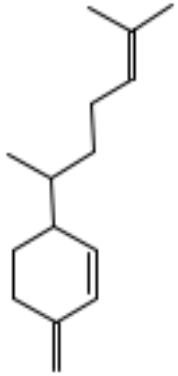
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
α-muurolene		C15H24	204.35	1	0	0	69.04	0	4.07	-3.61	1	0	0
α-panasinsene		C15H24	204.35	0	0	0	66.62	0	4.52	-4.2	1	0	0
α-Panasinsene		C15H24	204.35	0	0	0	66.62	0	4.52	-4.2	1	0	0
α-santalol		C15H24O	220.35	4	1	1	68.04	20.23	3.57	-3.44	0	0	0

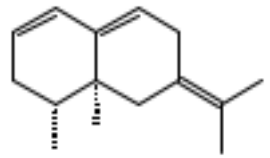
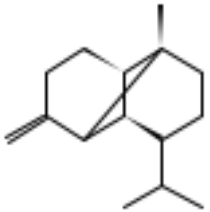
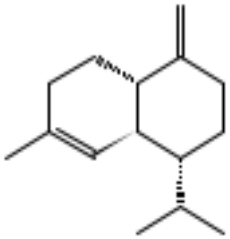
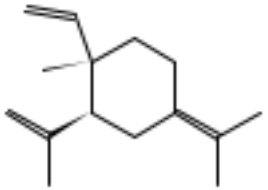
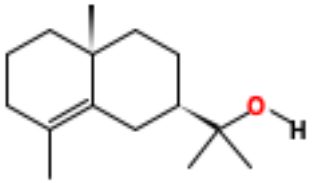
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
α-selinene		C15H24	204.35	1	0	0	68.78	0	4.4	-4.32	1	0	0
α-ylangene		C15H24	204.35	1	0	0	67.14	0	4.3	-3.86	1	0	0
β-bisabolene		C15H24	204.35	4	0	0	70.68	0	4.83	-4.89	1	0	0

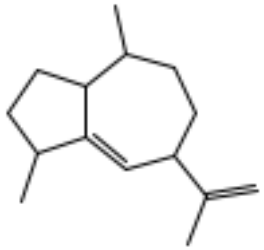
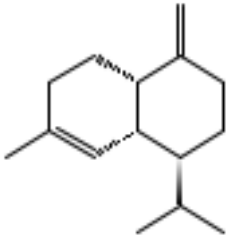
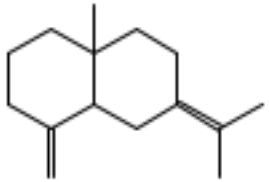
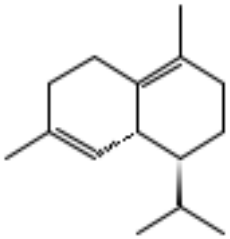
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
β-bourbonene		C15H24	204.35	1	0	0	67.14	0	4.4	-4.01	1	0	0
β-cadinene		C15H24	204.35	1	0	0	69.04	0	4.06	-3.56	1	0	0
β-calacorene		C15H20	200.32	1	0	0	68.39	0	4.48	-4.24	1	0	0
β-caryophyllene		C15H24	204.35	0	0	0	68.78	0	4.24	-3.87	1	0	0

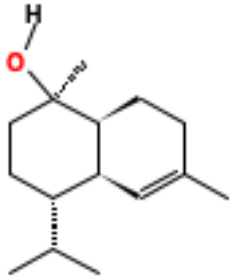
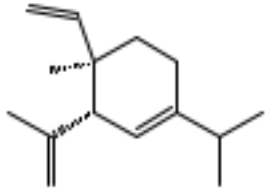
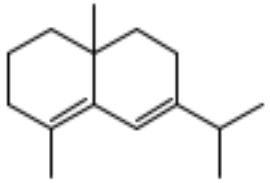
Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
β-chamigrene		C15H24	204.35	0	0	0	68.52	0	4.39	-4.05	1	0	0
β-copaene		C15H24	204.35	1	0	0	67.14	0	4.4	-4.01	1	0	0
β-cubebene		C15H24	204.35	1	0	0	67.14	0	4.4	-4.01	1	0	0
β-farnesene		C15H24	204.35	7	0	0	72.32	0	4.97	-4.44	1	0	0
β-gurjunene		C15H24	204.35	0	0	0	67.14	0	4.33	-4.07	1	0	0

Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
β-Himachalene		C15H24	204.35	0	0	0	68.78	0	4.2	-3.59	1	0	0
β-Panasinsene		C15H24	204.35	0	0	0	66.62	0	4.62	-4.35	1	0	0
β-patchoulene		C15H24	204.35	0	0	0	66.88	0	4.4	-3.81	1	0	0
β-santalene		C15H24	204.35	3	0	0	68.78	0	4.69	-4.81	1	0	0

Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
β-santalol		C15H24O	220.35	4	1	1	69.94	20.23	3.87	-4.05	0	0	0
β-selinene		C15H24	204.35	1	0	0	68.78	0	4.5	-4.47	1	0	0
β-sesquiphellandrene		C15H24	204.35	4	0	0	70.68	0	4.56	-4.25	1	0	0

Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
β-vetivenene		C15H22	202.34	0	0	0	68.31	0	4.13	-3.89	1	0	0
β-ylangene		C15H24	204.35	1	0	0	67.14	0	4.4	-4.01	1	0	0
γ-cadinene		C15H24	204.35	1	0	0	69.04	0	4.18	-3.76	1	0	0
γ-elemene		C15H24	204.35	2	0	0	70.42	0	4.56	-4.35	1	0	0
γ-eudesmol		C15H26O	222.37	1	1	1	70.46	20.23	3.6	-3.29	0	0	0

Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
γ-gurjunene		C15H24	204.35	1	0	0	69.04	0	4.33	-4.36	1	0	0
γ-muurolene		C15H24	204.35	1	0	0	69.04	0	4.17	-3.76	1	0	0
γ-selinene		C15H24	204.35	0	0	0	68.78	0	4.39	-4.06	1	0	0
δ-cadinene		C15H24	204.35	1	0	0	69.04	0	4.14	-3.43	1	0	0

Sesquiterpenes	Structure	Molecular formula	MW	nRotB	nHBAcc	nHBDon	MR	TPSA	Log P	Log S	RO5 violations	Ghose's violations	Veber's violations
δ-cadinol		C15H26O	222.37	1	1	1	70.72	20.23	3.44	-3.26	0	0	0
δ-elemene		C15H24	204.35	3	0	0	70.42	0	4.49	-4.23	1	0	0
δ-selinene		C15H24	204.35	1	0	0	68.78	0	4.34	-3.76	1	0	0

Properties were analyzed by the Swiss ADME software.
MW: molecular weight, nHBDon: number of hydrogen bonds donors, nHBAcc: number of hydrogen bonds acceptors, log P: lipophilicity, log S: aqueous solubility, TPSA: topological polar surface area, nRotB: number of rotatable bonds, MR: molar refractivity.

3.2. Sedative activity

Sesquiterpenes in the training set, such as α-bisabolol, cedrol, curcumol, curzerene, farnesol, germacrone, nerolidol, nootkatone, and zerumbone, were investigated for their sedative activity at doses in the range of 4×10^{-4}–4×10^{-2} mg/cage (cage volume: 0.065 m^3). Of note, all compounds demonstrated considerable sedative activity, except curzerene, which did not possess any sedative activity within the range of tested doses (**Error! Reference source not found.**).

3.2.1. Presence of oxygen-containing groups and sedative activity

The sedative activity was analyzed by considering several structural features. Various compounds in the training set included an oxygen in their structure. However, a close evaluation of compound structures revealed that only curzerene and curcumol have an oxygen-containing group within the ring. Curzerene was observed to not possess any sedative activity at any tested dose, whereas curcumol, with a conventional U-shape, exhibited its best activity at 4×10^{-3} mg/cage. Curzerene's oxygen is fused with an aromatic ring, which could render the structure more complex and increase the difficulty of interacting with olfactory receptors (Figure 40). The oxygen occurring in curcumol is not in an aromatic ring and can freely bind to receptors. Similarly, a flexible oxygen-containing group is present in other compounds in this study, such as cedrol, α-bisabolol, nerolidol, and farnesol, all of which showed sedative activity. Therefore, an oxygen-containing group in the molecule may be essential for the sedative activity, but the sedative activity may require an oxygen group that is freely movable to interact with molecular receptors. Butylbenzene, which lacks a ketone group, was indeed not sedative compared with benzylacetone in a previous study [5]. Similarly, α-humulene, which has no oxygen in its structure, did not show any sedative activity compared with zerumbone, which has a ketone group in its chemical structure [18]. Moreover, a ketone group was previously shown to be important for neural activation of the dorsal olfactory bulb in rabbits [247], and a SAR study using HEK293 cells with forced expression of olfactory receptor identified hydroxyl (-OH) and aldehyde (-CHO) groups as determinants of olfactory receptor activation [248]. Similar to the observation in this study, these previous studies showed the importance of having a reactive oxygen-containing group in the molecule for sedative activity.

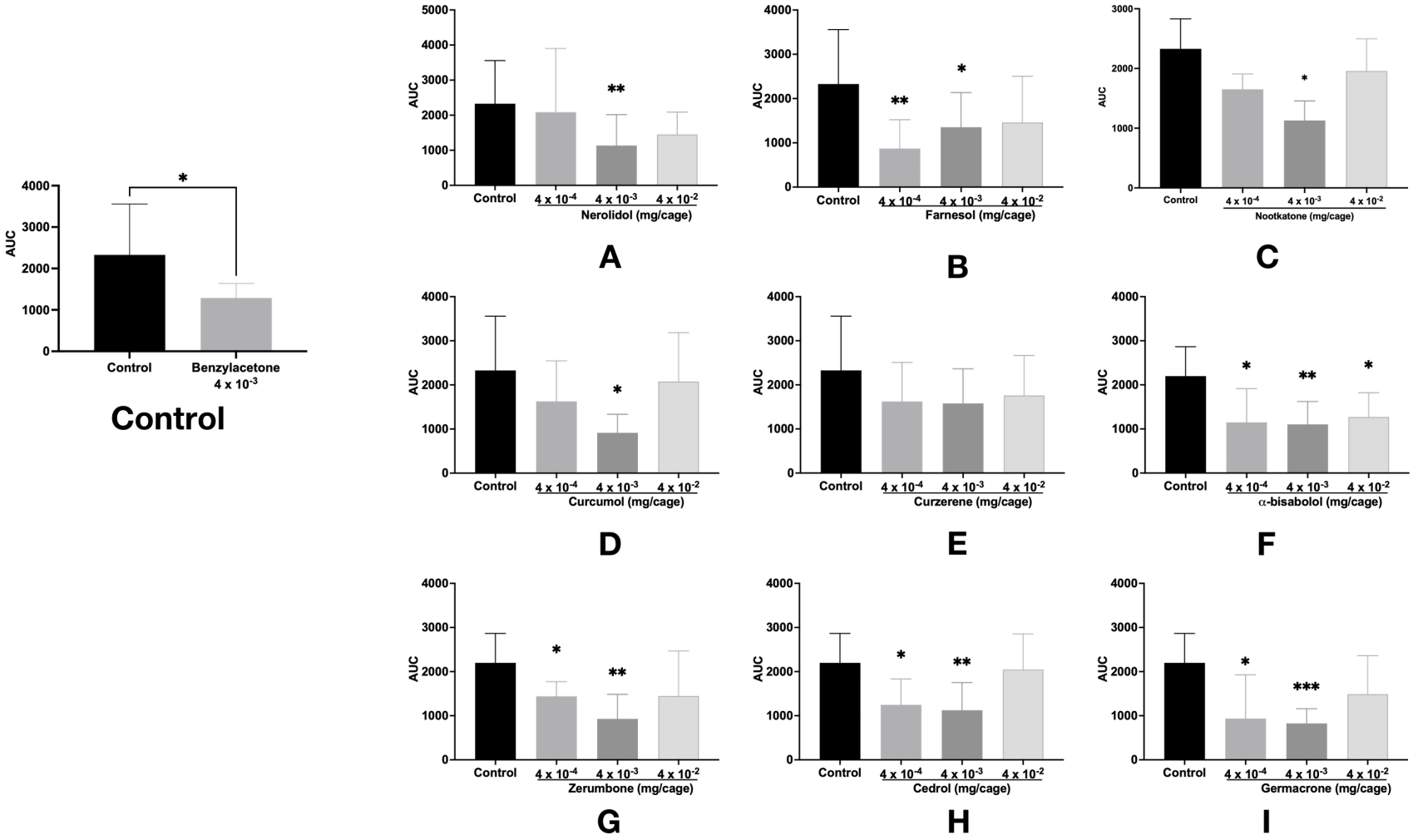


Figure 39: Total spontaneous locomotor activity of mice treated with α-bisabolol, cedrol, curcumol, curzerene, farnesol, germacrone, nerolidol, nootkatone, or zerumbone (4 × 10−4, 4 × 10−3, or 4 × 10−2 mg/cage).

Data are mean ± SD of six mice. Statistical differences vs. control mice were calculated using Student's t-test or ANOVA followed by Dunnett's test. * $p < 0.05$; ** $p < 0.01$. TEC, triethyl citrate; AUC, area under the curve representing total locomotor activity; SD, standard deviation; ANOVA, analysis of variance.

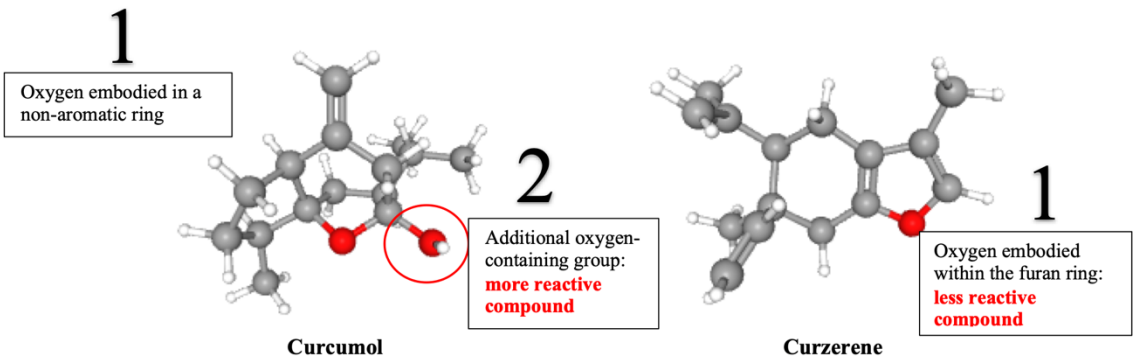


Figure 40: Comparison of 3D structures of curcumol and curzerene.

3.2.2. Alcohol group position and sedative activity

An oxygen atom was shown to be important for sedative activity; however, the effect of the specific position of the oxygen molecule in sesquiterpenes was not investigated. Nerolidol and farnesol, both acyclic sesquiterpenes, have similar molecular formulas ($C_{15}H_{26}0$). The difference between the two compounds lies in the position of the alcohol group on C1 for farnesol and C3 for nerolidol. This difference radically alters the sedative activity. Nerolidol presents a U-shaped curve for the sedative activity with the lowest sedative dose of 4×10^{-3} mg/cage. By contrast, farnesol shows dose-dependent sedation with the lowest effective dose of 4×10^{-4} mg/cage. The position of the alcohol group in farnesol thus allows for its better interaction with molecular receptors. Therefore, it was suggested that the oxygen-containing group's position is also important for the sedative activity. Miyoshi *et al.* [5] previously demonstrated comparable results for benzylacetone and its derivatives, suggesting that a specific ketone group position was required for the activation of sensory receptors.

3.2.3. Parental (original) carbonyl cation and sedative activity

The parental carbonyl cation in the biosynthetic pathway of plants was further investigated to understand its effects on molecule reactivity. Notably, a similarity between the origin of the parental carbonyl cation and sedative activity was observed in the 18 selected compounds. Nootkatone, germacrone, and curcumol originated from an (*E,E*)-germacradienyl cation (10,1 closure of farnesyl cation), whereas α-bisabolol and cedrol originated from the bisabolyl cation (7,1 closure of nerolidyl cation) (Figure 41). Sesquiterpenes originating from the (*E,E*)-germacradienyl cation displayed a higher sedative activity than molecules originating from the bisabolyl cation. Thus, although substituting compounds with a hydroxyl group is important, the original pathway of compound synthesis is also a determining factor for sedative activity. Furthermore, ~ six sesquiterpenes in the study with good ADMET properties originated from the (*E,E*)-germacradienyl cation. It might be intriguing to consider products derived from this pathway for future investigation on pharmacological properties of sesquiterpenes.

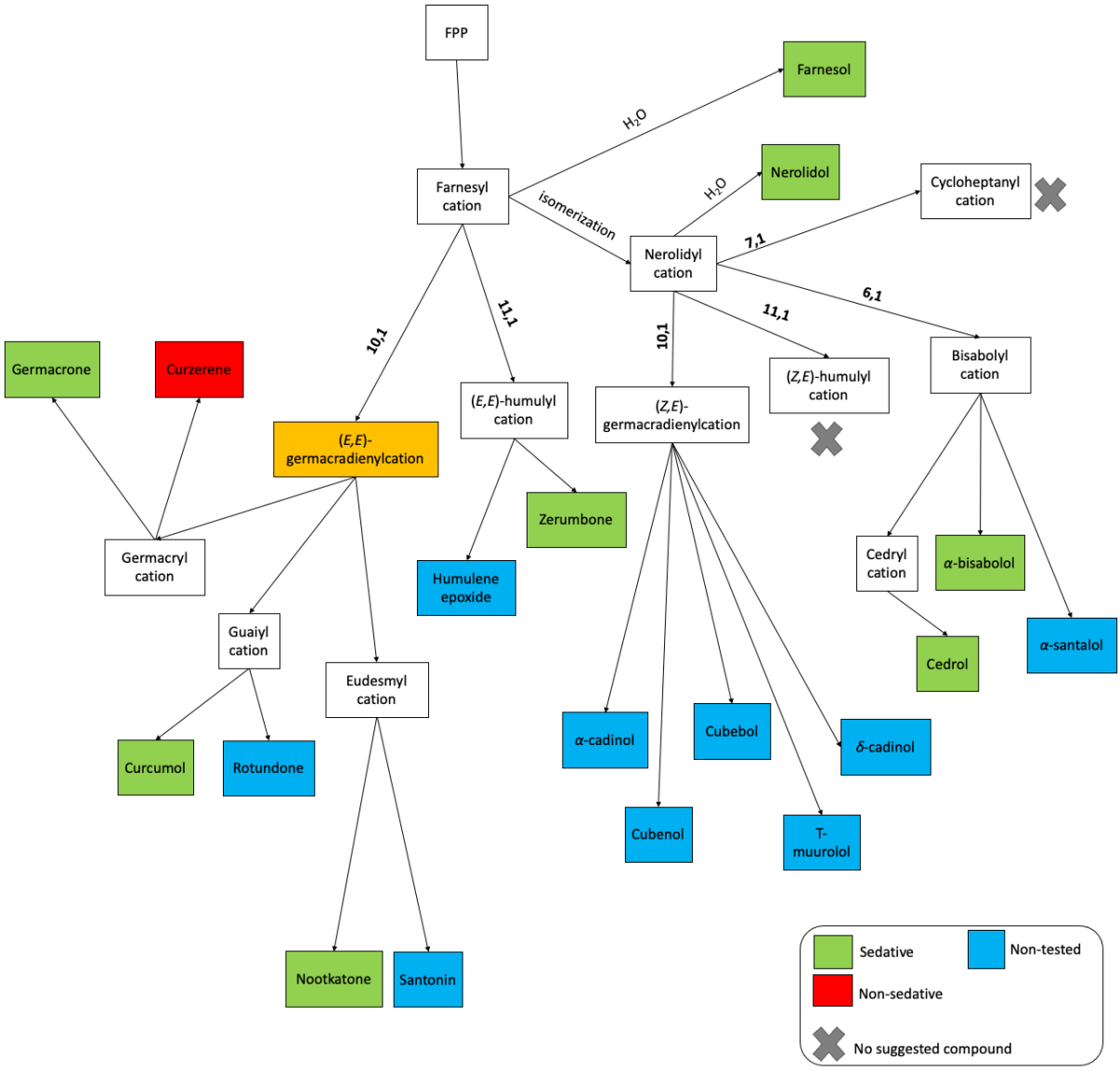


Figure 41: Original pathway of synthesis of sesquiterpenes and their sedative activity.

3.2.4. Ring size and sedative activity

Analysis of the 18 sesquiterpenes indicated that among nine sesquiterpenes in the training set, three possessed a monocyclic ring: one C6-ring with a long linear chain (α-bisabolol) and two with two large C15 rings (germacrone and zerumbone). These compounds showed a sedative activity at a lower dose of 4×10^{-4} mg/cage and demonstrated a U-shaped curve, suggesting that monocyclic sesquiterpenes, even with large rings, such as germacrone or zerumbone, can be expected to show sedative activities at low doses. Nevertheless, cedrol and curcumol, tricyclic compounds with complex structures, also show sedative activities at low doses. Therefore, although the ring size affects the structural shape and molecular interactions, it is not considered as a determining parameter for the sedative activity. All molecules with large rings have an oxygen-containing group in common, suggesting that regardless of the ring size, an additional factor important for the sedative activity is the presence of freely movable oxygen in the molecule, as previously mentioned.

3.3. QSAR study of molecular descriptors and sedative activity

QSAR studies were used for determining the important structural features that dictate the sedative activity of sesquiterpene compounds used in this study. Molecular descriptors were calculated for the structures as described in the Materials and Methods section. Standard least square methodology was used for QSAR studies with single and multiple regression models analysis. Sesquiterpenes were considered at their most sedative dose in the QSAR models, and area under the curve (AUC) values were used as objective variables for assessing sedative and non-sedative properties (Figure 42).

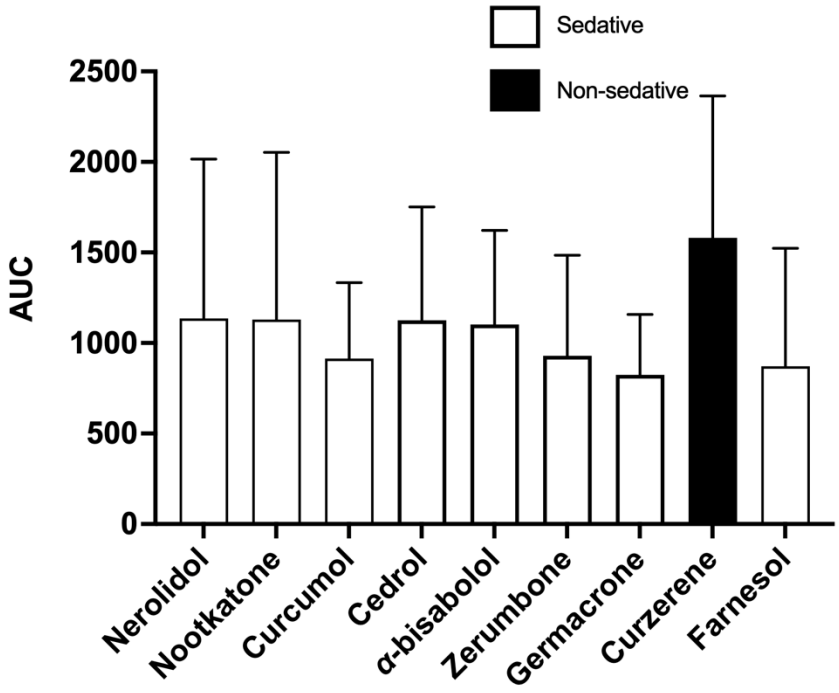


Figure 42: Sedative and non-sedative sesquiterpenes in the training set using experimentally obtained AUC values.

Data are mean ± SD of six mice. AUC, area under the curve representing total locomotor activity; SD, standard deviation.

3.3.1. Simple regression analysis

Simple regression analyses (SRA) indicated that molar refractivity (MR) showed the most significant correlation with AUC values for analyzed sesquiterpene compounds (Figure 43), as shown in Eq. 1:

$Predicted\ activity\ AUC\ (SRA) = 2946.51 - 27.45 \times MR$ **(Eq. 1)**

N = 9, $r^2 = 0.66$, F = 13.45, SDEP = 142.72

N is the number of compounds used to build the QSAR model, r^2 denotes the squared correlation coefficient, and F represents the Fisher's test statistics.

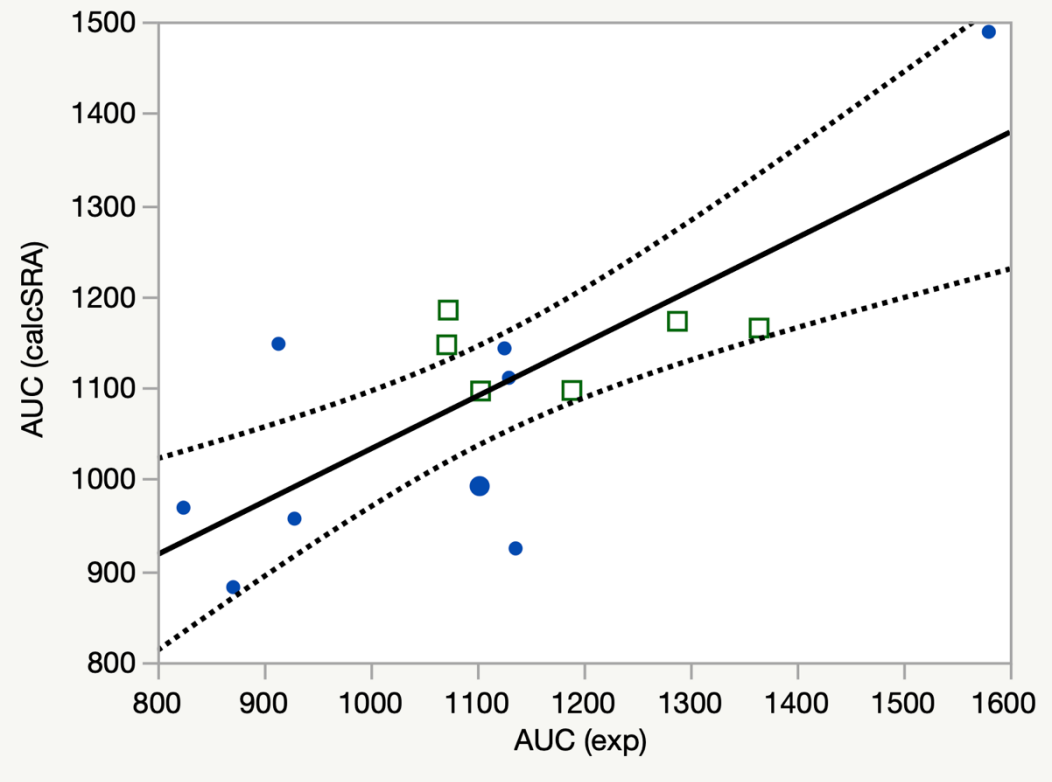


Figure 43: Fitness plot showing calculated versus experimental values of sedative activity using Eq. 1 obtained by simple regression analysis (SRA).

$r^2 = 0.59$, F = 18.95 (p = 0.0008). Blue points represent training set compounds, whereas the green square represents test set compounds. The curved lines represent the 95% confidence interval for the regression line.

MR is a measure of the overall polarity of a molecule. It is related to the molecular size and London dispersion forces, which act during the drug–receptor interaction. Relative solubility, the partition coefficient, P, of a substance between octan-1-ol and water, was a determining factor for the sedative activity in monoterpenes [5]. However, sesquiterpenes are larger compounds with higher MWs, and the discovery of MR as a molecular factor implicated in the sedative activity of sesquiterpenes is notable. Most compounds gain entry into cell membranes via passive diffusion, and the diffusion rate depends on the molecule size [249]. Accordingly, MR values show the best statistical fit with AUC among molecular descriptors. The negative coefficient of this parameter in the model indicates that an increase in the MR of sesquiterpenes decreases the AUC values, resulting in better sedative activity. For instance, when comparing curzerene and curcumol, curcumol possesses a higher sedative activity. Previous studies also identified MR as a crucial factor for predicting antimalarial activity [244] and anti-human immunodeficiency virus activity of some arylsulfonamides [222].

The model obtained using Eq. 1 had a regression coefficient $r^2 = 0.66 > 0.6$, and F-test value (F = 13.45, p = 0.008), suggesting that MR is important; however, r^2 is not adequately strong and multiple regression models with plural descriptors are thus required to enhance prediction performance.

3.3.2. Multiple regression analysis

Combinatorial optimization with 10 descriptors was used to explain the variance of AUC using a genetic algorithm. However, too many attributes would possibly increase the chance of obtaining a statistically significant correlation. Therefore, the number of attributes in any analysis should be as low as possible to avoid any “chance” factor. Furthermore, the number

of descriptors in a multiple regression equation should be 2 when the data set comprises fewer than 20 compounds [250]. Therefore, the number of descriptors was limited to two, and variable selection was performed using the standard least square method as a validation system. A model comprising MR and number of hydrogen bond acceptors (nHBAcc) descriptors (Eq. 2) provides a good correlation of the structural properties of sesquiterpenes with observed sedative activity.

$$Predicted\ activity\ AUC\ (MRA) = 2670.31 - 20.39 \times MR - 206.65 \times nHBAcc \quad \textbf{(Eq. 2)}$$

N = 9, $r^2 = 0.82$, F = 13.60, $r^2adj = 0.76$, SDEP = 116.45, $q^2_{LOO} = 0.71$, pred_$r^2 = 0.83$

N is the number of compounds used to construct the QSAR model, r^2 denotes the squared correlation coefficient, and F represents the Fisher's test statistics.

Eq. 2. could successfully discriminate sedative and non-sedative sesquiterpenes with more than 82% accuracy ($r^2 = 0.82$, $p = 0.0059$). MRA was superior to SRA in predicting sedative activity, and an external set of six compounds (Figure 38C) was collected from the literature [3,215,216] to confirm the prediction ability of Eq. 2 in a real test set data. Additionally, the ability of the model to predict the sedative activity of sesquiterpenes was validated by performing leave-one-out internal cross-validation (LOO-CV), calculating pred_r^2 for the test set, and estimating the mean absolute percentage error (MAPE) and standard deviation of error of prediction (SDEP) parameters. Furthermore, the robustness of the model was examined by performing a Y-randomization test [222,241,242,245] as described in the Materials and Methods section.

The internal cross-validated $q^2_{LOO} = 0.71$, indicates good internal predictive power of the model. In addition, external validation of the model (pred_$r^2 = 0.83 > 0.6$) showed that the model properly predicts the activity of new sesquiterpenes. Sum all, the r^2 and q^2_{LOO} statistics of the QSAR analysis and the value of $r^2 - q^2_{LOO} = 0.11 < 0.3$, indicate a reasonably good predictive power of the generated Eq. 2. Furthermore, a small SDEP value of 116.45 for a mean value for $AUC_{(exp)}$ of 1068.14 indicated that a significant proportion of the sedative activities of sesquiterpenes can be explained by the QSAR developed in Eq. 2. Additionally, a small value for the corresponding MAPE (9.46), which indicates good prediction power of the developed equation, suggested that the model was adequately strong to predict the sedative activity of sesquiterpenes (Table 7).

The nHBAcc, which is the other descriptor found to be important based on the QSAR model shown in Eq. 2, is the atom, ion, or molecule component of a hydrogen bond that does not supply the bridging (shared) hydrogen atom. Ketones are particularly active in forming hydrogen bonds by accepting hydrogens, which could explain why nHBAcc was identified as a determinant of the sedative activity in my multiple regression model. The negative coefficient of the nHBAcc descriptor in Eq. 2 suggests that compounds with higher values for this descriptor would have a higher sedative activity. For instance, santonin, which had the highest nHBAcc in my set of compounds, was predicted to have the best sedative activity according to my model.

The statistical modeling parameters of the selected model passed the acceptability criteria [222,238–242,244] as shown in Table 8, demonstrating that the predictive power of the developed model is considerably good and is reliable in predicting the sedative activity of other structurally related compounds. Furthermore, a fitness plot (Figure 44A and Figure 44B) was obtained, representing and comparing the actual and predicted activities of the molecules of the training set and the test sets, showing the existence of a good correlation with most data points in the 95% confidence interval.

Table 7: Experimental, calculated, and predicted (AUC) sedative activity according to MRA.

Sesquiterpenes	AUC $_{(exp)}$	AUC $_{(calc)}$	AUC $_{(predLOO)}$	APE
Training set				
α-Bisabolol	**1102.50**	1012.05	997.24	**9.55**
Cedrol	**1125.83**	1123.86	1123.19	**0.24**
Curcumol	**913.75**	920.90	982.98	**7.58**
Curzerene	**1580.31**	1587.41	1649.53	**4.38**
Farnesol	**871.25**	930.02	951.73	**9.24**
Germacrone	**824.58**	994.54	1025.78	**24.40**
Nerolidol	**1136.25**	961.50	915.83	**19.40**
Nootkatone	**1130.00**	1099.97	1095.47	**3.06**
Zerumbone	**928.75**	985.64	996.81	**7.33**
MAPE				**9.46**
Test set (experimental activity unknown)				
α-cadinol	-	1100.23	-	-
α-santalol	-	1095.10	-	-
Cubebol	-	1146.33	-	-
Cubenol	-	1100.23	-	-
δ-cadinol	-	1100.23	-	-
Humulene epoxide	-	1033.91	-	-
Rotundone	-	1123.34	-	-
Santonin	-	658.61	-	-
t-Muurolol	-	1100.23	-	-
External test set (activity collected from literature)				
α-Gurjunene	**1288.12**	1352.48	-	-
Calarene	**1364.73**	1347.02	-	-
Caryophyllene Oxide	**1073.01**	1154.92	-	-
Hexahydroxyzerumbol	**1071.67**	1126.74	-	-
Hexahydroxyzerumbone	**1103.39**	1089.23	-	-
Valerena-4,7(11)-diene	**1189.09**	1296.33	-	-

The AUC $_{(exp)}$ values are the biological activity of sesquiterpene compounds in the training set (from pharmacological activity evaluation of the compounds as described in the Material and Methods section in this study) and test set (from literature data [3,215,216]). The AUC $_{(calc)}$ values correspond to the predicted activity of the compounds obtained using Eq. 2. The AUC $_{(predLOO)}$ values denote the predicted sedative activity based on the LOO prediction method. MAPE is the mean of absolute percentage error (APE).

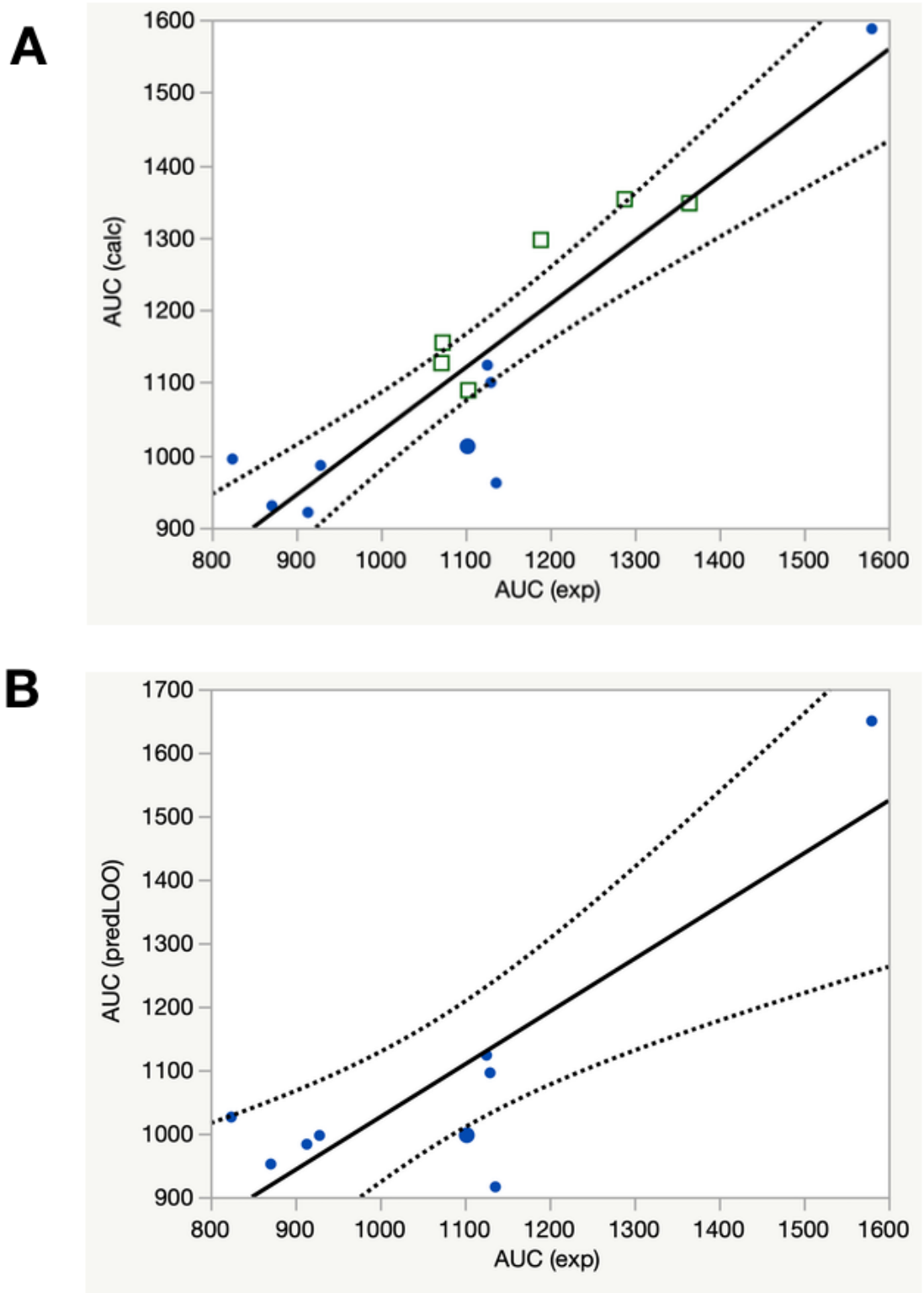


Figure 44: Fitness plot showing calculated versus experimental values of sedative activity, $r^2 = 0.82$, F = 61.23 ($p < 0.0001$) (A) and predicted LOO versus experimental values of sedative activity, $r^2 = 0.73$, F = 18.58 ($p = 0.0035$) (B) according to Eq. 2.

Blue points represent training set compounds, whereas the green square represents test set compounds. The curved lines represent the 95% confidence interval for the regression line.

Table 8: Summary of important statistical parameters obtained with MRA analyses (at 95% significance level).

	Value from the present study	**Threshold value** [238–240,242]	**Software response**
Internal Validation			
r	0.9	>0.8 (in vivo data)	PASSED
r^2	0.82	>0.6	PASSED
$r^2 - r^2adj$	0.06	<0.3	PASSED
Leave-One-Out Cross-Validation (LOO-CV)			
q^2_{LOO}	0.71	>0.5	PASSED
r^2_m	0.64	>0.5	PASSED
External Validation			
pred_r^2	0.83	>0.6	PASSED
$r^2_0 - r'^2_0$	0.03	<0.3	PASSED
k	0.96	0.85 < k < 1.15	PASSED
$\frac{r^2 - r_0^2}{r^2}$	0.00029	<0.1	PASSED
k'	1.04	0.85 < k' < 1.15	PASSED
$\frac{r^2 - r'^2_0}{r^2}$	0.03518	<0.1	PASSED
Test set size	N = 6	N > 5	PASSED

Furthermore, the difference between calculated and experimental values (residual) of the biological activities is shown in Figure 45.

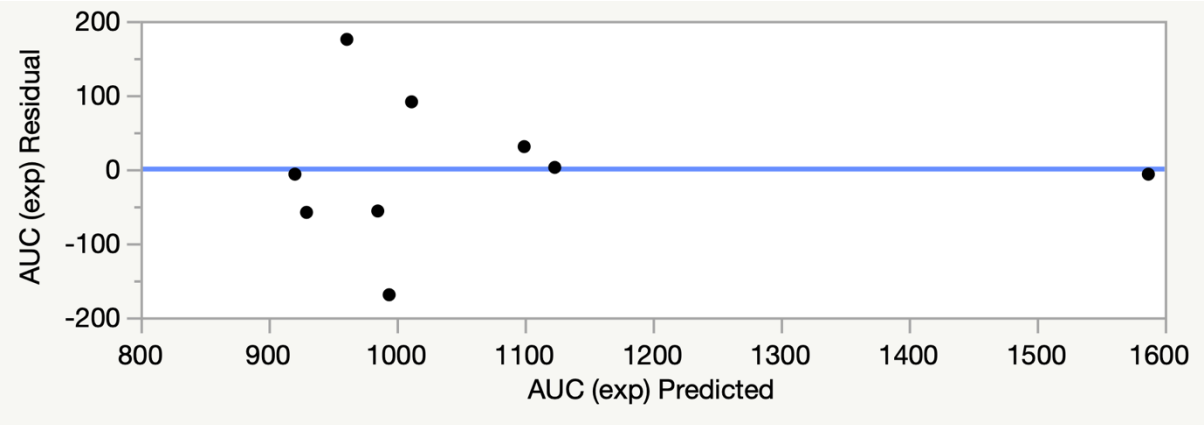


Figure 45: Residual plot between calculated and experimental values.

Judging from Figure 45, the residual values obtained are small enough, thus implying that the obtained model can predict the activity of new sesquiterpenes with sedative activity. Finally, the low r^2 and q^2 values from the Y-randomization test (Table 9) indicated that the results of Eq. 2 are not because of chance correlation or structural dependency of the training

set [222,241–243]. Furthermore, the correlation matrix shows poor correlation between the descriptors MR and nHBAcc, as shown in Table 10.

Table 9: Results of the Y-randomization test.

Model	r	r^2	q^2
Original	0.91	0.82	0.71
Random 1	0.23	0.05	−2.30
Random 2	0.14	0.02	−4.83
Random 3	0.21	0.05	−0.65
Random 4	0.28	0.08	−2.12
Random 5	0.38	0.15	−3.32
Random 6	0.67	0.45	−4.93

Table 10: Intercorrelation between selected molecular descriptors.

	MR	nHBAcc
MR	1	
nHBAcc	−0.4605	1

4. Conclusion

The evaluation of pharmacokinetic and physicochemical properties during drug discovery is a critical task. One hundred fourteen sesquiterpenes were evaluated *in silico* using a screening procedure based on ADMET properties. Eighteen sesquiterpenes that did not violate Lipinski's, Ghose's, and Vebers's rules and with no suggested toxicity were selected for additional investigation. Half of the compounds were evaluated for the sedative activity in mice, and the results showed that this activity differed with the presence and position of an oxygen-containing group, such as alcohol or ketone, and relied on the parental carbonyl cation. Furthermore, QSAR analyses having high accuracy revealed that molecular descriptors could be identified to discriminate between sedative and non-sedative sesquiterpene compounds. Six additional sesquiterpenes were used externally to validate the QSAR model with MRA. MR and nHBAcc were suggested to permit accurate prediction of sedative activity, and the QSAR model was validated by several statistical tests such as $r^2 = 0.82 > 0.6$, $q^2_{LOO} = 0.71 > 0.5$, and low values of r^2 and $q^{2,}$ for Y-randomization tests. Therefore, the constructed QSAR model may contribute to developing novel sesquiterpenes for treating CNS-related diseases such as insomnia, anxiety, depression, post-traumatic stress disorder and attention deficit hyperactivity disorder.

General Conclusion and Perspectives

In this thesis, 3 aromatic plant species were collected in the Republic of Benin (West Africa) and investigated for their chemical composition and sedative or sleep-promoting effects in mice. The plant species were Lantana camara L., Dysphania ambrosioides (L.) Mosyakin & Clemants and Chromolaena odorata (L.) R.M.King & H.Rob. They demonstrated good sedative activity in mice, which confirms their usage in traditional medicines, and I have conducted a structure-activity relationship study on their chemical constituents. The main constituents of the oils were investigated and chemically elucidated. In addition, I explored the action mechanisms which suggested that the GABAergic system might be in cause for activity, or binding to multiple receptors with different affinities might explain the activities. Futhermore, a QSAR study and evaluation of molecular determinants of sedative activity of sesquiterpenes compounds was conducted to predict sedative activity of complex molecules such as sesquiterpenes, until now less investigated for their activities on the CNS in comparison to monoterpenes. Sum all, I present scientific evidence of the use of traditional medicines to treat stress and possibly complex CNS-related diseases, and the outcome of this work might be useful in accelerating the drug discovery process.

We suggest in future studies to explore more herbal medicines, as they contain surprisingly interesting molecules with various activities. In order to do so, it would be interesting to collaborate with traditional healers and present them with the results of these studies because they are the ones who use the plants and transmit knowledge from generation to generation, although they have no scientific evidence of their treatments. Most of these people are illiterate and with scientists coming to get their recipies without collaborating with them, they are getting more reticent to provide useful informations. Essential oils can be beneficial to the CNS-ailments, and the molecules of essential oils are known to reach the brain and exert their effect through the olfactory system or the respiratory system. As these pathways would suggest different action mechanisms and interactions of molecules with multiple receptors, there is still great work to be done in order to better understand the whole picture. Exploration of cellular and molecular mechanisms may provide additional insight into the future therapeutic use of essential oils. QSAR studies can help accelerate these investigations, as in QSAR, once the structure of a hit is known, its activities can be predicted using other compounds with similar structures. Computational chemistry and molecular modeling softwares can therefore be used as effective tools in identifying binding site interactions and structure-activity relationships. Nowadays, SAR provides valuable information in drug discovery and development. It is principally used for discovering and developing new compounds, and as demonstrated in this thesis, it allows the determination of important chemical groups that interact with molecular receptors to exhibit activity. More SAR and QSAR studies with 3D structures of sesquiterpenes and molecular docking should be conducted in order to allow researchers to go further than simply identifying active or inactive molecules, but to actually predict the activity of unknown potential hits. Therefore, rational structural modifications or insertion of new chemical groups to improve sedative activity of essential oils in general, and sesquiterpenes in particular are expected as a result.

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