Revista Brasileira de Farmacognosia 29 (2019) 656–664

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Petiveria alliacea, a plant used in Afro-Brazilian smoke rituals, triggers pulmonary inflammation in rats

Thiago Cagliumi Alves [a], Eliana Rodrigues [a,*], João H.G. Lago [b], Carla M. Prado [c], Carlos Eduardo N. Girardi [d], Debora C. Hipólide [d]

[a] Centro de Estudos Etnobotânicos e Etnofarmacológicos, Universidade Federal de São Paulo, Diadema, SP, Brazil
[b] Centro de Ciências Naturais e Humanidades, Universidade Federal do ABC, Santo André, SP, Brazil
[c] Departamento de Biociências, Universidade Federal de São Paulo, Santos, SP, Brazil
[d] Departamento de Psicobiologia, Universidade Federal de São Paulo, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 12 February 2019
Accepted 18 June 2019

Keywords:
Ethnonobotany
Incense
Airway toxicity
Inhalation
Smoker

ABSTRACT

Petiveria alliacea L., Phytolaccaceae, a plant used in Afro-Brazilian religious smoke rituals is reported to have "harmonic properties" (anxiolytic effect) by ethnobotanical survey. In the present work, we analyzed the chemical composition of volatiles produced by leaves of *P. alliacea*, using headspace gas chromatography/mass spectrometry and its potential anxiolytic and toxic effects in smoke-exposed rats. Locomotor activity and anxiety-like behavior were allocated into groups, according to substance administration: acute (locomotor activity) or chronic (anxiety-like behavior) burning charcoal or to smoke from *P. alliacea*. Inflammatory cell counts in the bronchoalveolar lavage and morphometric analysis in airway were assessed. Animals exposed to *P. alliacea* smoke had no locomotor activity or elevated plus maze open arm exploration impairment, while lungs had lower number of macrophages in bronchoalveolar fluid and an increased number of mononuclear and polymorphonuclear cells in the peribronchovascular region. Chemical analysis of plant material allowed the identification of dimethylsulfide (18.7%), diethylsulfide (33.4%) and nerolidol (25.8%) as main volatile compounds. Taken together, prolonged exposure to *P. alliacea* smoke does not induce anxiolytic effects, but histological analyses indicate a possible pulmonary inflammatory response.

Introduction

Pharmacological studies based on ethnobotany and/or ethnopharmacology usually consider oral administration, but only rarely report on the use of "cigarettes", and smoke rituals, and inhalation by both methods concurrently. This study can therefore hopefully make a contribution to this less commonly examined route of administration.

Smoke rituals involve the burning of plants, usually using charcoal as a base for combustion, and are used in a number of different spiritual rituals. The smoke released in the burning is considered to serve as a means of communication with the supernatural, being a way to please, honor, or even to make requests or reach Gods faster. The use of incense originated in Ancient Egypt, having been used by the Romans, Greeks and in various civilizations throughout history (Lucas, 1930). The word perfume derives from *per* (to) and *fumum* (smoke), indicating that this use began with the burning of plants (Lewington, 2003). In the contemporary world, accounts of the use of incense are recorded in the following religions and beliefs: Buddhism (Yü, 2013), Christianity (Chapple, 2008), Islam (Tunçel, 2011), Judaism (Amar, 2007), Shamanism (Antonio et al., 2010) and Hinduism (Mazumdar and Mazumdar, 2015), involving a variety of plants.

Antonio et al. (2010) portrayed the role of smoke in Brazilian religions, describing the various rituals involved in this practice. One example of the use of smoke is in Afro-Brazilian religions in which the incense is known as *defumador*. The function of the incense is of such importance that religious ceremonies can only begin after the smoke ritual — where both the environment and the people taking part are exposed to the smoke of the burning herbs, in order to *̈balance the energies"*, drive away bad spirits and attract the good ones (Lima, 1979).

According to an ethnonobotanical study conducted by our research group of an Afro-Brazilian religion in Brazil, called 'Umbanda' (Garcia et al., 2016), the *'seven herbal'* incense is used in almost all ceremonies. According to the priests (*mediums*) it is used

* Corresponding author.
E-mail: e.rodrigues@unifesp.br (E. Rodrigues).

https://doi.org/10.1016/j.bjp.2019.06.005

for *“balance and harmony in the environment*,*̈cleansing, harmony, diseases of the spiritual plane*änd “to calm”. It is also said to have a possible anxiolytic effect. Among the seven plants that comprise the incense, *Petiveria alliacea* L., Phytolaccaceae, was described by the *mediums* as the one that was the most important in relation to this effect, with the leaves being the part used. Other studies have reported the importance of this plant in smoke rituals. According to Ruanda (1954), 20 types of incense were used in ‘Umbanda’, of which eighteen had as one of the constituents this plant. Known popularly as *amansa-senhor* (tame the master), it was used by slaves in Brazil in the preparation of a potion. This was mixed with the food of the masters to slowly intoxicate them and prevent the slave women from being harassed by them (Gomes et al., 2008). This plant is still one of the most important ingredients in the *tira-capeta* (removing the devil) “cigarette”, which has traditionally been used for its narcotic effects, and whose biological effect on the Central Nervous System (CNS) has already been previously studied by our group (Rodrigues et al., 2008).

Petiveria alliacea is an herbaceous plant widely distributed in the tropical regions of Central and South America, the Caribbean and Africa, and is popularly used in folk medicine as a potential source for the treatment of different CNS disorders including anxiety, depression, pain, epilepsy and memory impairments (for a review see Luz et al., 2016). Different fractions of *P. alliacea* obtained from the fresh whole plant, aerial parts, or roots and administered intraperitoneally and/or orally have indicated distinct pharmacological properties in classical behavioral models (Gomes et al., 2005; Blainski et al., 2010). A recent study by Blainski et al. (2010) noted that the hydro-alcoholic extract of the aerial parts had an anxiogenic effect, and the same extract made from the root parts showed an anxiolytic effect. Gomes et al. (2008) reported a possible depressor effect of nonpolar fractions obtained from the hydroalcoholic extract of *P. alliacea* root.

Considering the large number of *P. alliacea* preparations, their known pharmacological effects and the possible presence of active compounds, studies investigating the therapeutic effects of its smoke are still quite scarce. Some studies report that the use of incense in rituals can result in poor indoor air quality and is associated with respiratory diseases (Bérubé et al., 2007), allergies (Lin et al., 2008) and increases carcinogenic potential (Chiang and Liao, 2006; Liao and Chiang, 2006; Navasumrit et al., 2008; Chiang et al., 2009; Hu et al., 2009; Chuang et al., 2012). Some contemporary studies report similar effects in incense users outside the religious context (Hussain et al., 2014; He et al., 2015; Seow and Qing, 2016). This subject has been discussed by academic science for more than 50 years; Schoental and Gibbard (1967) studied the chemical composition of Chinese incense and its possible carcinogenic potential because of the high incidence of naso-pharyngeal cancer among the Chinese. Some of these authors attribute these effects to particulate matter (PM), generated during the combustion processes (Chiang et al., 2009; Chuang et al., 2012). In Buddhist temples in Asia, where incense burning occurs during religious rituals, the presence of PM and polycyclic aromatic hydrocarbons (PAH), a carcinogenic substance responsible for many cases of throat cancer, have been reported (Chiang and Liao, 2006; Tang et al., 2010). Furthermore, some studies have shown that exposure to PM can also induce systemic inflammation and other effects (Rivero et al., 2005).

To reproduce the effects of plants and other inhaled substances in animal models, there are already some standardized methodologies. Two types of methodologies in particular have been used according to the needs of the study. The first, nose-only exposure (NOE), is a technique in which only the mouse nostril is exposed to the smoke, thus simulating a smoker; the second is called whole-body exposure (WBE), which aims to mimic the conditions of passive smoking (Harris et al., 2010). As an example, the use of an enclosure to expose the experimental animal to essential oil inhalation used by Almeida et al. (2004) to verify the anxiolytic effects obtained from rose oil (*Rosa* spp. - Rosaceae).

Against this background, the present study analyzed the chemical composition of the volatile compounds produced by the leaves of *P. alliacea* and investigated its potential anxiolytic and toxic effects in laboratory animals, simulating the route of administration of the site where the ritual is performed.

Materials and methods

Animals

Male Wistar rats from the Center of Animal Models Development for Medicine and Biology, Universidade Federal de São Paulo (Unifesp), weighing between 350–450 g were housed in Plexiglas cages (five animals per cage), kept at a controlled temperature (21 ± 2 °C) on a 12/12 light cycle Food and water were available *ad libitum*, except during the time of the experiment. All procedures were performed with the approval of the Ethics Committee of Unifesp (CEP 1185/10) and the approval of the CNPq for access the genetic patrimony (CNPq 010040/2011-0).

Plant material

Petiveria alliacea L., Phytolaccaceae, was collected by the agronomist Ílio Montanari Jr. Msc, in March 2010. A voucher specimen (no. 886) has been deposited at the herbarium of the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agricolas (CPQBA), Universidade Estadual de Campinas, Campinas, Brazil.

Inhalation apparatus

To expose the rats to the smoke, we used a rectangular acrylic inhalation chamber with dimensions of 40 cm × 30 cm × 30 cm. There was a 12-mm diameter hole in the center of the floor to allow the connection of a compressed air system, with a removable lid to allow the rats to be placed inside the chamber. The compressed air system comprised a Laboport N 811 K pneumatic pump (KNF NeuerbergerdaTM, USA) with a flow rate of 10 l/min connected to a L̈connection embedded in the hole in the chamber to transfer air to the inside. The charcoal was ignited with a blowtorch type lighter (Honest^{TM}, China) on a small metal mesh (5 cm diameter × 1 cm height) and placed inside the chamber.

The amount of dried plant was 25 mg or 50 mg which were chosen by an estimation of amount plant smoke inhaled by humans, based on the volume of a known ritual site (3 × 108 cm^3), the amount of plant smoke released during the ritual (300 g) and total human body mass (3250 kg) present in an actual ritual. We, thus, have calculated an approximate proportion to the experimental condition, based on cage dimensions and rat’s body mass. The plant was placed on the charcoal for 60 s. The burnt material was then removed, and the animal was placed inside of the chamber and exposed to the smoke for 60 s. The animal was then transferred to an individual plexiglas cage and remained there for 15 min before being subjected to the behavioral tests. The charcoal group was submitted to the exact same procedure, except that no dried plant was added onto the burning charcoal.

A portable oxygen meter (DG 400 – Instrutherm^{TM}, Brazil) was used to check the amount of O_2 in the chamber and recorded 21% — the normal atmospheric level.

Treatments

Diazepam 2 mg/kg, *i.p.* (Germed Pharma®, Sa˜o Paulo, Brazil), saline (NaCl 0.9%, Sigma-Aldrich, Brazil, *i.p.*), charcoal 2.3 g (Zoghal

Ghaleb®, Iran) and dried, milled aerial parts of *P. alliacea* (25 or 50 mg).

Behavioral assessment

Behavioral tests were carried out from 9:00 to 12:00 am, and recorded using a digital video camera. The subsequent analysis was made and rechecked by a qualified, blinded observer. Fig. 1 illustrates the experiment design diagram for behavioral tests.

Open field test

Locomotor activity was evaluated in an open field (Britton and Britton, 1981) apparatus consisting of a circular arena (80 cm diameter), surrounded by a wall 50 cm in height. The apparatus floor was divided into three concentric circles, subdivided in nineteen equal sectors for observation of locomotion. Each rat was placed individually in the center of the apparatus, and the following behaviors were scored for 5 m in. (1) locomotion, measured as the number of sectors crossed with all four paws; (2) number of rearings, measured as the number of times the animal stood on its hind legs anywhere in the apparatus. The open field floor was cleaned with diluted ethanol after each trial. Fifteen min before being tested, each animal had been individually exposed in the inhalation chamber (PTV25, PTV50 and charcoal groups).

Elevated plus maze (EPM) test

The protocol used in the present study was based on Pellow et al. (1985), with some modifications. To evaluate anxiety-like behavior, animals were subjected to *P. alliacea* smoke exposure for 5 days per week over 28 days (PTV25, PTV50 and Charcoal groups). Additional standard groups: Diazepam, 2 mg/kg (Based on Pellow et al., 1985) and saline group were tested in EPM, 30 min after injections. The EPM test consisted of two open (50 × 10 × 1 cm) and two closed (50 × 10 × 30 cm) arms, arranged perpendicularly, elevated 50 cm above the floor. Each rat was placed on the center of the apparatus (10 × 10 cm) and the following behaviors were scored for 5 m in. (1) number of open arms entries; (2) number of closed arms entries; (3) time spent in open arms; (4) time spent in closed arms. After each trial, the apparatus was cleaned with diluted ethanol to eliminate any possible bias due to odors left by the previous rat. An anxiety index was calculated according to Mazor et al. (2009) considering the frequency and time spent in the open arms in relation to the total exploration of the apparatus.

The anxiety index was calculated as follows:

$$\text{Anxiety index} = \left[\frac{\left(\left(\frac{\text{open arms time}}{\text{total maze time}}\right) \text{x } 100\right) + \left(\left(\frac{\text{open arms entries}}{\text{total arms entries}}\right) \text{x } 100\right)}{2}\right]$$

Anxiety index values range from 0 to 100, with an increase in the index expressing increased anxiety-like behavior.

Bronchoalveolar lavage fluid (BALF)

At the end of the evaluation in the EPM, animals were euthanized by using chloral hydrate and the anterior chest wall was opened, animals were exsanguinated via the abdominal aorta, and the BALF was collected as previously described (Toledo et al., 2013) The trachea was cannulated and the BALF was obtained by washing the airway lumina with 2 × 0.5 ml of sterile saline. The recovery volume was over 95% of the instilled fluid and was put into a test tube on ice. To perform total and differential cell counting, the BALF was centrifuged at 800×*g* at 5 °C for 10 min and the cell pellet was resuspended in 0.2 ml of sterile saline. The total number of viable cells was determined in a Neubauer hemocytometer counting chamber.

Differential cell counts were performed in cytocentrifuge preparations of BALF (450 rpm for 6 min) (Cytospin, Cheshire, UK) stained with Diff-Quick (Biochemical Sciences Inc., Swedesboro, NJ). At least 300 cells were counted according to standard morphologic criteria.

Lung morphometry

After BALF collection, the lungs were infused with 2 ml of formaldehyde 4% and were removed en bloc. The lungs were fixed with 4% formaldehyde for 24 h and then transferred to 70% ethanol prior to paraffin embedding. This technique is constantly used in our group to evaluate inflammatory cells infiltrated around airways (Prado et al., 2006; Pinheiro et al., 2015; Bittencourt-Mernak et al., 2017; Camargo et al., 2018). Five-micrometer thick sections from embedded paraffin lungs were stained with hematoxylin and eosin (H and E) (Prado et al., 2006). To determine the number of polymorphonuclear (PMN) and mononuclear cells (MN) in the bronchovascular area (between the bronchial epithelium or endothelium and the airway or vessel adventitia) using light microscopy, we counted the number of PMN and non-PNM cells in areas of peribronchovascular inflammation in the field of view of the eyepiece in three to four areas of three to five airway walls/vessels (20 fields per animal). The results are expressed by cells per unit of area (10^4 μm^2). All analyses were performed in randomly selected transversely sectioned airways at a magnification of 1000× by a blinded researcher.

Analysis of plant material by headspace-GC/MS

To perform the headspace-GC/MS, approximately 500 mg of *P. alliacea* fresh leaves were placed in a glass headspace vial. After be sealed with PTEE/silicone septum and aluminum crimp cap, the vial was heated at 150 °C in order to evaporate the volatile compounds which were analyzed by GC-MS. This analysis was performed in a Shimadzu QP5050-A gas chromatograph/mass spectrometer, using a DB-5 capillary column (30 m × 0.35 mm × 0.2 μm). The injector temperature was set at 150 °C. The column temperature program started at 40 °C (10 min) and was increased 50 °C to 250 °C at 5 °C/min and keep at 250 °C (10 min). The mass spectrometer was operated in the electron ionization (EI) mode with ion source temperature at 200 °C, interface temperature at 250 °C and mass range at *m/z* 100–400. Recorded mass spectra were compared with those available in the NIST library.

Statistical analysis

Statistica 12.0 (StatSoft, Inc.) was used for statistical analyses. Behavioral scores for the elevated plus-maze and open field tests, and histopathological data were analyzed by one-way analysis of variance (ANOVA). The Newman–Keuls test was used *a posteriori* to check the differences between groups. When data was not normally distributed, as evidenced by Shapiro–Wilk test, non-parametric Kruskal–Wallis tests were performed. In these cases, the *post-hoc* Mann–Whitney U-test was used to identify significant pairwise differences. For all statistical procedures, significance was set at $p < 0.05$.

Results

Animals body weight variation

One-way ANOVA indicated no significant difference among the four experimental groups in terms of animals body weight variation after the experiment period of 28 days ([$F(4, 45) = 0{,}55$, $p = 0{,}12$]).

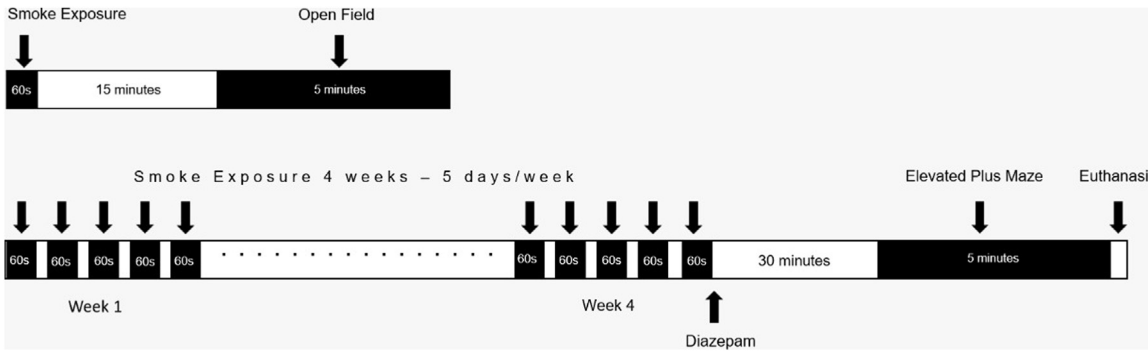

Fig. 1. Experiment design diagram for behavioral tests.

Open field test

ANOVA detected no group difference for ambulation, measured as the number of sectors crossed in the open field ([F(3,36) = 0.69; p = 0.56]) and for number of rearings [F(3,36) = 0.27; p = 0.85].

Elevated plus maze test (EPM)

One-way ANOVA indicated no significant group effect in the percentage of time spent in the open arms [$F_{(4,45)} = 0.81$; $p = 0.53$] but a significant group effect on the percentage of open arm entries [$F_{(4,45)} = 7.17$; $p < 0.01$]. Newman–Keuls *post-hoc* analyses revealed that animals that received diazepam injection entered the open arms more than all the other groups ($p < 0.01$). One-way ANOVA detected a significant group effect on the anxiety index [$F_{(4,45)} = 3.71$; $p = 0.01$], Newman–Keuls test *post-hoc* analyses revealed that anxiety index was lower in the DZP group than in the control ($p < 0.05$), charcoal ($p < 0.04$), PTV25 and PTV50 ($p < 0.05$). The results are depicted in Fig. 2.

Inflammatory cells in the bronchoalveolar lavage

The macrophage, neutrophil, eosinophil and lymphocyte counts in the BALF samples from all the experimental groups are presented in Fig. 3. Kruskal–Wallis test revealed a statistically significant group difference for macrophage cells percentage [$H_{(3)} = 7.82$; $p = 0.05$]. Mann–Whitney test indicated that rats submitted to repeated exposure to *P. alliacea* smoke (PTV25 and PTV50 groups) had a reduced percentage of macrophage cells than rats in the saline group (U = 4.0; $p = 0.05$ and U = 2.0; $p = 0.03$, respectively). Kruskal-Wallis test revealed no significant group effect for neutrophil [$H_{(3)} = 6.52$; $p = 0.08$], eosinophil [$H_{(3)} = 5.30$; $p = 0.15$] and lymphocyte [$H_{(3)} = 3.45$; $p = 0.32$] cell percentages.

Lung morphometry analyses

For lung morphometry analyses, one-way ANOVA showed that polymorphonuclear cells around the airway were increased in rats exposed to smoke in the chamber [$F_{(3,24)} = 5.03$; $p < 0.01$]. Post-hoc analysis revealed an increase in cells around the airways in the charcoal, PTV25 and PTV50 groups compared to the saline group ($p < 0.01$). One-way ANOVA [$F_{(3,22)} = 4.41$; $p = 0.01$] revealed an increase in the number of mononuclear cells. Newman-Keuls test post hoc analyses revealed an increase in mononuclear cells around airways in rats exposed to smoke in the chamber compared to saline (charcoal vs saline, $p = 0.01$; PTV25 vs saline, $p = 0.02$; PTV50 vs saline, $p = 0.03$) (Fig. 4). Photomicrographs of airways can be observed in Fig. 5 from charcoal, PTV25 and PTV50 groups showed increased infiltrate of inflammatory cells around airways,

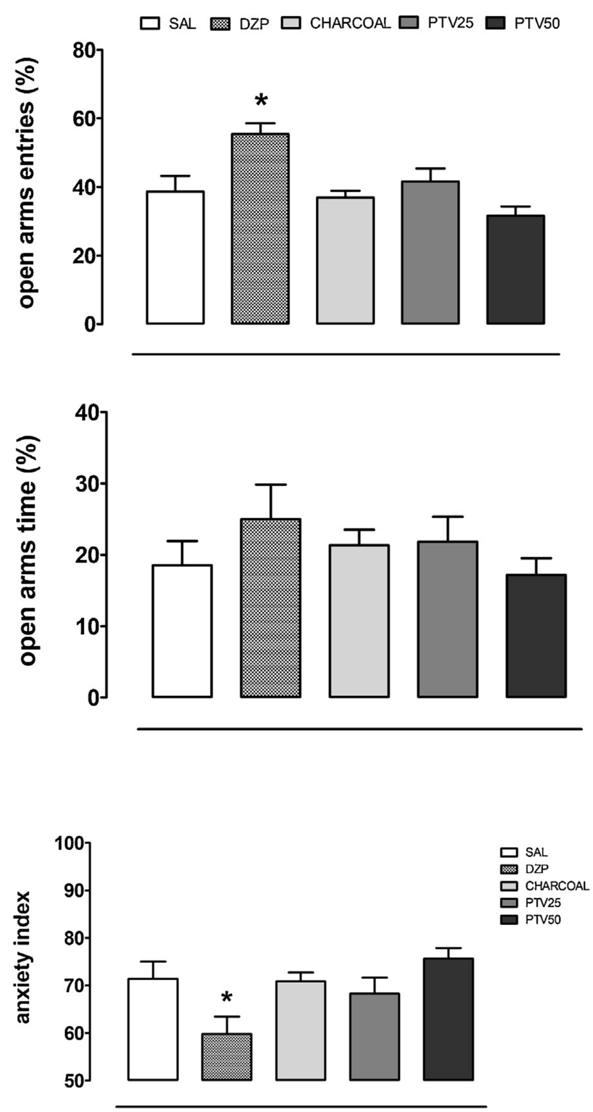

Fig. 2. Effects of *Petiveria alliacea* smoke exposure and diazepam injection on anxiety-related parameters in the EPM. Rats were exposed to *Petiveria alliacea* smoke for 28 days (PTV25, PTV50 and Charcoal groups). Diazepam and saline groups (DZP, 2 mg/kg, i.p.) were tested in the EPM 30 minutes after intraperitoneal injections. Each bar represents mean ± SEM of percentage of time in open arm, the percentage of open arm entries or anxiety index. Significant differences: *p < 0.05 compared with all the other groups (ANOVA followed by Newman–Keuls test).

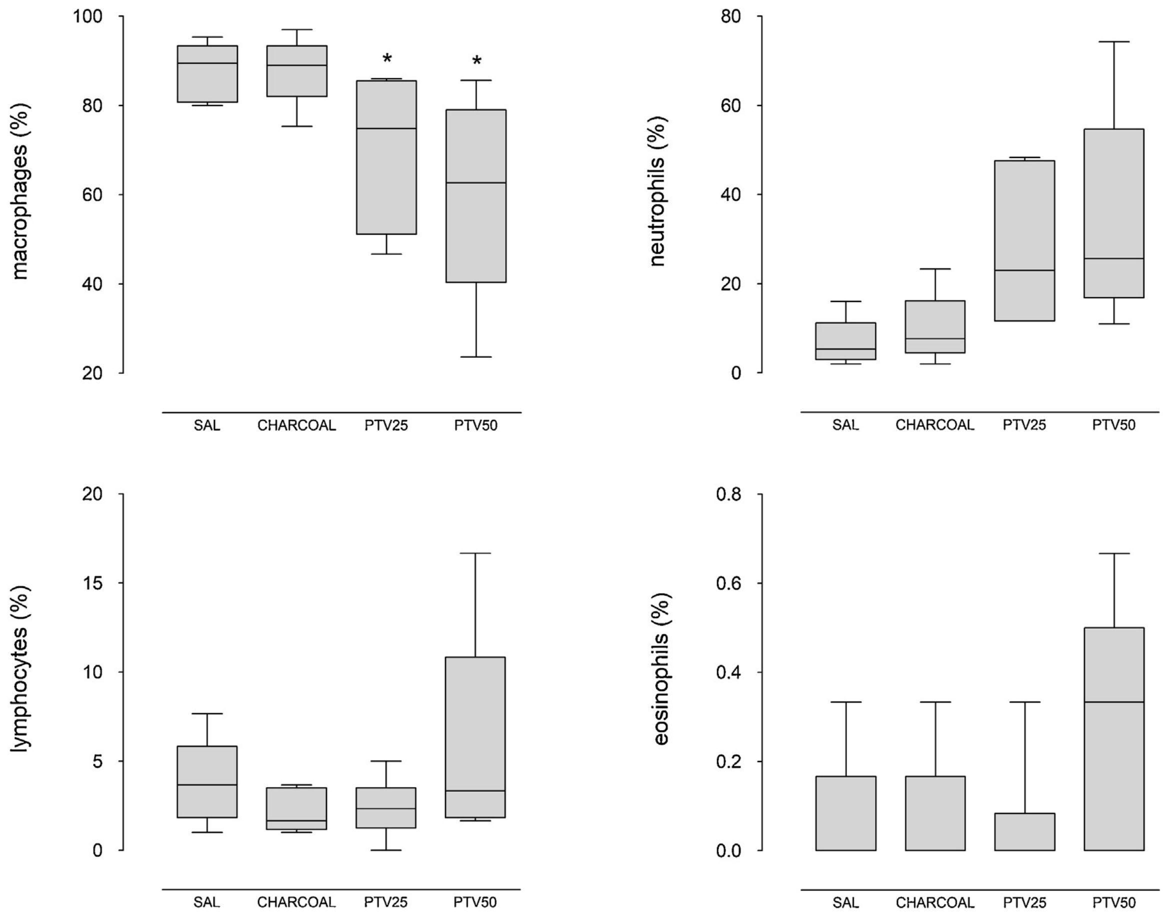

Fig. 3. The graphs show the median with a 95% confidence interval of macrophages, lymphocytes, eosinophils and neutrophils percentage cell counts in bronchial alveolar lavage fluid (BALF) samples from rats exposed to *Petiveria alliacea* smoke. (n = 5–6 per group). Animals submitted to subchronic expose *Petiveria alliacea* smoke (PTV25 and PTV50 groups) reduced percentage cell counts than saline, $^{*}p < 0.05$, Mann Whitney test.

Table 1
Identified volatile compounds from leaves of *Petiveria alliacea* by headspace GC/MS analysis.

Peak	R_t/min	Relative amount/%	Identified compound
1	2.3	18.7	Dimethylsufide
2	3.1	33.4	Diethylsufide
3	7.9	6.1	α-pinene
4	9.3	9.9	β-pinene
5	20.7	4.6	Di-n-propyldisulfide
6	43.2	25.8	Nerolidol
Total		98.5	

epithelial thickness, and peribronchiolar edema around airways compared to the saline group.

Chemical analysis from leaves of Petiveria alliacea *by head-space GC/MS*

The chemical analysis of volatiles from leaves of *P. alliacea* (Fig. 6 and Table 1) showed a predominance of nerolidol (25.8%), dimethyl and diethylsulfides (18.7 and 33.4%, respectively). Other minor compounds such as α−(6.1%) and β− (9.9%) pinenes as well as di-*n*-propyldisulfide (4.6%) were also identified. The occurrence of sulfides and polisufildes as volatiles found in the leaves of *P. alliacea* are in concordance with previous reports in which several sulfur derivatives were detected (Luz et al., 2016).

Discussion

Recently, we have conducted an ethnobotanical survey indicating that the ritual use of *P. alliacea* smoke has a potential anxiolytic effect in humans (Garcia et al., 2016). Herein, we present the first study to investigate the effects of *P. alliacea* smoke on animal behavior.

Petiveria alliacea is suggested to have a possible anxiolytic effect and is used in Afro-Brazilian religion to create "balance and harmony in the environment", and "to calm" (Garcia et al., 2016). Using an elevated plus maze (EPM), the present study investigated the potential anxiolytic effect of the smoke produced by the leaves of *P. alliacea* in laboratory animals after inhaling the smoke, simulating site conditions where the ritual is performed. The EPM is a widely used apparatus to test anxiety-like behavior in rodents and is sensitive to anxiolytic drugs that act on $GABA_A$ receptors, such as benzodiazepines, increasing the number of visits and the time spent in the open arms (Pellow and File, 1986). Our results confirmed previous findings that administration of diazepam produced anxiolytic-like effects, characterized by an increase in the percentage of exploration of the open arms of the EPM (Pellow et al., 1985) and a decrease in the anxiety index, which expresses decreased anxiety-like behavior. In contrast, no effect on anxiety-like behavior was observed in rats chronically exposed to the smoke produced by the *P. alliacea.*

Locomotor activity assessed by total ambulation and vertical exploration in the open field was not affected by exposure to smoke produced by the *P. alliacea* leaves. Measuring oxygen levels during

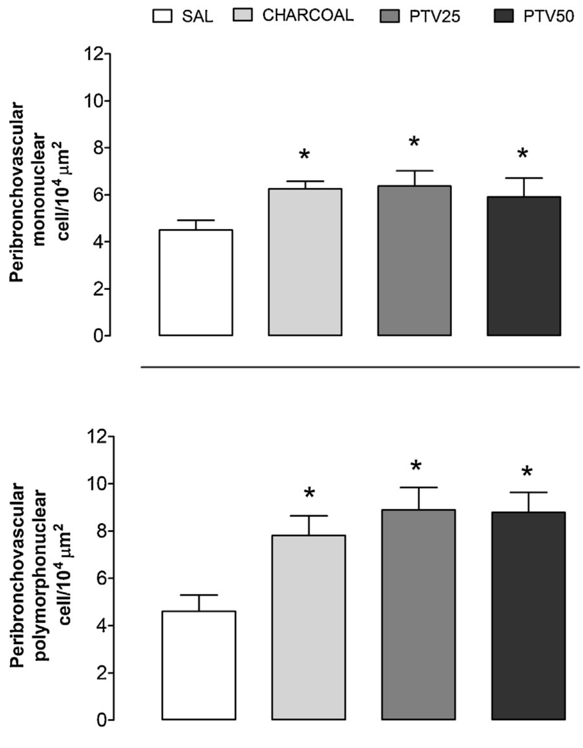

Fig. 4. Lung morphometry analyses indicated that polymorphonuclear and mononuclear number cells around the airway are increased in rats exposed to smoke Charcoal, PTV25 and PTV50 groups compared to saline ($p < 0.03$, Newman–Keuls test).

the experiment, which were found to be normal, eliminated the possibility of any hypoxic effect.

Some important considerations should be taken into account, including the fact that extracts obtained from different parts of *P. alliacea* produce diverse effects on animal behavior. Other studies using non-polar extracts of the plant found that root extracts produced a slight decrease in spontaneous locomotor activity, and non-polar extracts of leaves caused hyperexcitability (Cifuentes et al., 2001). A previous study by Blainski et al. (2010) reported that the hydro-alcoholic extract of the aerial parts had an anxiogenic effect, but extracts from the roots showed an anxiolytic effect. Gomes et al. (2008) reported a possible depressor effect of non-polar fractions obtained from hydro-alcoholic extract of *P. alliacea* root. These studies showed that the main constituents were benzyl polysulfides (Benavides et al., 2001), triterpenes, coumarins, and flavonoids (Suarez and Monache, 1992). As reported by Wolfman et al. (1994) and Viola et al. (1995), flavonoids act as GABAergic modulators based on their structural similarity with benzodiazepines. However, in the present study the volatile compounds found in the leaves of *P. aliaceae* were also sulfur derivatives, but predominately those containing methyl, ethyl and *n*-propyl groups. This chemical difference could be, at least in part, associated to distinct pharmacological aspects (Asmadi et al., 2011; Ross and Mazza, 2010; Tymchyshyn and Xu, 2010).

The *mediums* interviewed during the ethnobotanical survey conducted by Garcia et al. (2016) pointed to *P. alliacea* as one of the *šstrongest"* substances with regard to its *"harmonizing effects"*. However, the cult participants were not exposed to smoke from this single plant, but a mixture of this with six other plants in the *'seven herb'* incense), namely rosemary (*Rosmarinus officinalis* L., Lamiaceae), myrrh (*Commiphora* spp., Burseraceae), incense (*Pittosporum* spp., Pittosporaceae), benzoin (*Styrax* spp., Styracaceae), lavender (*Lavandula dentata* L., Lamiaceae) and rue (*Ruta graveolens*

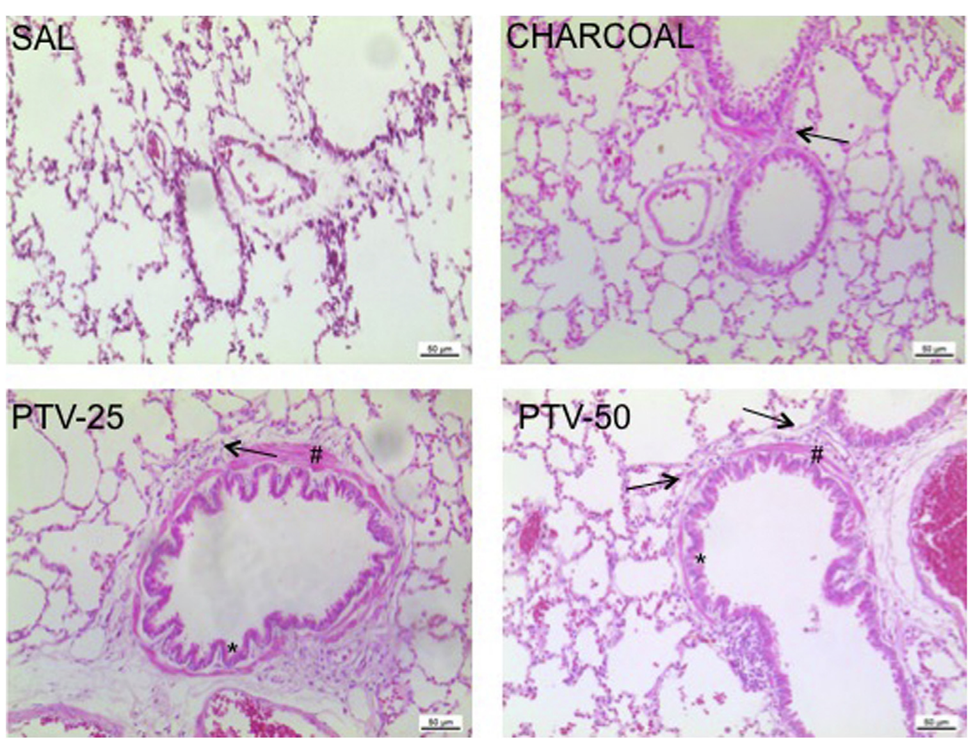

Fig. 5. Photomicrographs of airways from one animal from the four experimental groups studied stained with H&E: saline (Control), charcoal, PTV-25 and PTV-50. It is clearly demonstrated that charcoal and PTVs adminitration induce inflammatory cells infitrate around airways associated to peribronchiolar edema (arrows). We also found an airway epithelial (*) and airway smooth muscle (#) thickeness in PTV groups compared to saline.

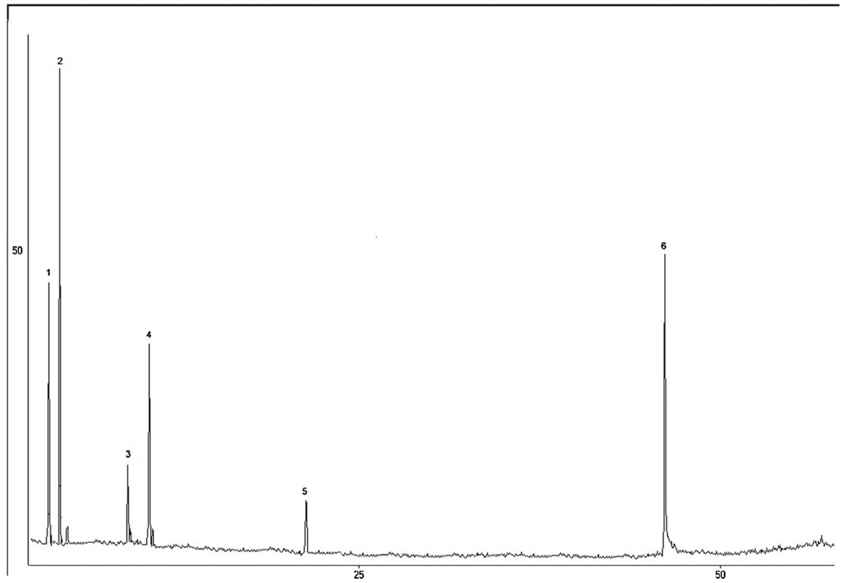

Fig. 6. Headspace GC/MS chromatogram of the fresh leaves of *Petiveria alliacea.*

L., Rutaceae). The possible effects mentioned by the *mediums* could be due to another of these plants or the combined effect of the mixture of compounds emitted by the smoke of these plants.

It was initially hypothesized that any possible anxiolytic effect of *P. alliacea* would only happen after repeated exposure, as the congregation of the temple are exposed to the smoke from the burning plants once a week, for an extended period. However, this hypothesis was not supported by this research as the animals exposed chronically did not show evidence of changes in their levels of anxiety in relation to the control groups.

Therefore, the possible calming effect, reported by the priests in our previous study (Garcia et al., 2016), which was attributed to smoke from the burning plants, may be associated with other factors such as longer exposure to the smoke; the species used, combination of burnt plants and the synergistic interaction between theme; and changes in the chemical composition of the plants depending on the part used, and the place and time of harvest. It could also be attributed to the ritual, the environment and the beliefs that surround and psychologically direct the medium's trance. Thus, a more detailed study is required, looking at all the plants individually as well as any synergistic interaction between them. The study of all the seven herbs used could reveal an anxiolytic effect similar to that reported in the smoking of the *tira-capeta* "cigarette", in which this plant is also present, along with eight other plants. In previous studies, we found that the *tira-capeta* induced a biphasic response, with intense initial stimulation of the CNS, followed by a general depressor state; it also decreased sleep latency and increased total sleep time (Rodrigues et al., 2008). In addition, further study of the plant could reveal an anxiolytic effect in parts other than the leaves, as reported by Blainski et al. (2010) and Gomes et al. (2008), who observed an anxiolytic effect of the root, although the method of administration and extract investigated were different from those used here.

The ritual use of smoke can involve the collection of plants in urban or rural areas near the place of worship or the products may be purchased ready for use; in both cases a large arsenal of Brazilian and African flora is used (Antonio et al., 2010). In the case of commercial material, the identity of the plants used is often unknown, raising questions about the authenticity of the product (in terms of its traditional composition) and the safety of its use (Soares et al., 2013).

Regarding the evaluation of the toxicity of exposure to *P. alliacea* smoke, we observed that the PTV25 and PTV50 groups had a significantly reduced number of macrophages. According to Cho et al. (1986) macrophages are phagocytic cells and thus this reduction reflects a mechanism that prevents the pulmonary defense inflammatory process. In a survey conducted by Tao et al. (2003), it was shown that reactive oxygen species (ROS) establish an oxidant-dependent relationship with macrophages causing them to suffer intracellular oxidation and thus induce them to apoptosis. Such a mechanism suppresses the phagocytic process, and promotes the release of pro-inflammatory cytokines. The polymorphonuclear cells (PMN) are potential signs of the airway inflammatory process as they are commonly found in patients with chronic bronchitis and/or lung infections (Strassburg et al., 2004). The particulate materials present in the smoke could have an effect on the PMN, either directly or indirectly increasing the ongoing pulmonary inflammation. However, its mechanism of action remains not fully understood. In the present study, the PMN were directly analyzed around the airways and bronchial vessels of the animal after exposure to *P. alliacea* smoke. In the groups exposed to some kind of smoke: charcoal (charcoal group) or the *P. alliacea* smoke (PTV25 and PTV50 groups) there was a significantly greater number of these cells, evidence of the toxicity of smoke entering the airways. Mononuclear cells, also identified as signaling inflammation (Szilasi et al., 2006), were also found in greater numbers in all groups exposed to some kind of smoke, either charcoal or *P. alliacea*.

All these indicators lead us to the hypothesis that the particulate materials and/or gas products generated during burning are primarily responsible for the inflammatory process in the groups in question.

Exposure to smoke, regardless of its source, is, as this and many other studies have shown (Barregard et al., 2008), an important factor in airway inflammation and can lead to serious diseases such as Chronic Obstructive Pulmonary Disease (COPD) and asthma. It is also important to note that many of individuals who attend these rituals, which use smoke, may already have some previous respiratory disease such as asthma, rhinitis or chronic bronchitis, and that exposure to this type of environment could aggravate their existing disease. Obviously, although we have clearly shown that exposure to smoke, whether from the plant or charcoal, induced pulmonary inflammation, other studies need to be undertaken to investigate the functional consequences of this histopathologic response, the mechanisms involved and the relationship between the size of the smoke particles and the diseases present.

Studies evaluating the toxicity of the use of incense in indoor or outdoor environments focus on particulate matter, gas products and many organic compounds (Lin et al., 2008), but few mention that incense is made of plants and so, the studies do not report on composition of the plants in the incense sticks.

Conclusion

Our results demonstrate that exposure to *P. alliacea* smoke has no effect on locomotor activity or anxiety-like behavior evaluated by open field and elevated plus maze, respectively. Chemical analysis results by headspace GC/MS from leaves of *P. alliacea* showed the presence of terpenoids (α−/β−pinenes and nerolidol) and sulfur derivatives including dimethyl and diethylsulfides as well as di-*n*-propyldisulfide. Lung morphometry results suggest possible toxicity in all groups of animals exposed to smoke, indicating that the use of smokers in ritual contexts can cause harmful effects regardless of the plant. However, bronchoalveolar lavage analysis point that the toxicity effect is potentiated in the groups of animals, which inhaled smoke from the *P. alliacea*. Thus, our results suggest that prolonged exposure to smoke from *P. alliacea* produced by the burning method using charcoal is indicative of a possible lung and airway inflammation. The results of this study therefore open new perspectives into research on the inhalation of smoke in these ritual contexts, and show they pose a risk to the health of practitioners of different religions around the world.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article

Conflict of interest

The authors declare no competing financial interest.

Author contributions

Conceptualization: DCH, ER and TCA; data curation: DCH and TCA; formal analysis: DCH, ER, TCA, JHGL, CMP and CENG; funding acquisition: DCH and ER; investigation: DCH and TCA; methodology: DCH, ER, TCA, JHGL, CENG and CMP; project administration: DCH; statistical analysis: CENG; supervision: DCH and TCA; writing original draft: DCH, ER and TCA; writing review and editing: DCH, ER, TCA, JHGL, CENG and CMP.

Acknowledgments

This work was supported by CNPq; CAPES and Associação Fundo de Incentivo à Pesquisa. The authors would like to thank Ílio Montanari Júnior from the Chemical, Biological and Agricultural Pluridisciplinary Research Center, University of Campinas, Brazil for providing the *P. alliacea* plant. The authors wish to express their gratitude to José Roberto Jardim (Respiratory Division and Pulmonary Rehabilitation Center, Universidade Federal de São Paulo) and Nathalia Montouro Pinheiro for their expert help and contributions to the study. Finally we would like to thank Cássio Ribeiro who made this study possible at his Umbanda Center.

References

Almeida, R.N., Motta, S.C., de Brito Faturi, C., Catallani, B., Leite, J.R., 2004. Anxiolytic-like effects of rose oil inhalation on the elevated plus-maze test in rats. Pharmacol. Biochem. Behav. 77, 361–364.

Amar, J.P., 2007. Patterns of prayer: The Hcombining dot belowûssõyô incense rite of forgiveness. Orient. Chr. Periodica. 73, 379–416.

Antonio, R.L., Scalco, N., Medeiros, T.A., Soares, J.A.R., Rodrigues, E., 2010. Smoke of ethnobotanical plants used in healing ceremonies in Brazilian culture. In: Rai, M, Acharya, D, Ríos, J.J. (Eds.), Ethnomedicinal Plants: Revitalizing of Traditional Knowledge of Herbs. Science Publisher, Enfield, pp. 166–191.

Asmadi, M., Kawamoto, H., Saka, S., 2011. Thermal reactivities of catechols/pyrogallols and cresols/xylenols as lignin pyrolysis intermediates. J. Anal. Appl. Pyrolysis 92, 76–87.

Barregard, L., Sällsten, G., Andersson, L., Almstrand, C., Gustafson, P., Andersson, M., Olin, A., 2008. Experimental exposure to wood smoke: Effects on airway inflammation and oxidative stress. Occup. Environ. Med. 65, 319–324.

Benavides, P.J.C., Young, M.C.M., Giesbrecht, A.M., 2001. Antifungal polysulphides from *Petiveria alliacea* L. Phytochem. Rev. 57, 743–747.

BéruBé, K., Balharry, D., Sexton, K., Koshy, L., Jones, T., 2007. Combustion-derived nanoparticles: Mechanisms of pulmonar toxicity. Clin. Exp. Pharmacol. Physiol. 34, 1044–1050.

Bittencourt-Mernak, M.I., Pinheiro, N.M., Santana, F.P., Guerreiro, M.P., Saraiva-Romanholo, B.M., Grecco, S.S., Caperuto, L.C., Felizardo, R.J., Câmara, N.O., Tibério, I.F., Martins, M.A., Lago, J.H., Prado, C.M., 2017. Prophylactic and therapeutic treatment with the flavonone sakuranetin ameliorates LPS-induced acute lung injury. Am. J. Physiol. Lung. Cell. Mol. Physiol. 1, L217–L230.

Blainski, A., Piccolo, V.K., Mello, J.C.P., de Oliveira, R.M., 2010. Dual effects of crude extracts obtained from *Petiveria alliacea* L. (Phytolaccaceae) on experimental anxiety in mice. J. Ethnopharmacol. 128, 541–544.

Britton, D.R., Britton, K.T., 1981. A sensitive open field measure of anxiolytic drug activity. Pharmacol. Biochem. Behav. 15, 577–582.

Camargo, L.D.N., Righetti, R.F., Aristóteles, L.R.C.R.B., Dos Santos, T.M., de Souza, F.C.R., Fukuzaki, S., Cruz, M.M., Alonso-Vale, M.I.C., Saraiva-Romanholo, B.M., Prado, C.M., Martins, M.A., Leick, E.A., Tibério, I.F.L.C., 2018. Effects of anti-il-17 on inflammation, remodeling, and oxidative stress in an experimental model of asthma exacerbated by LPS. Front. Immunol. 5, http://dx.doi.org/10.3389/fimmu.2017.01835.

Chapple, C.K., 2008. Sacrifice and sustainability. World Views Environ. Cult. Relig. 12, 221–236.

Chiang, K.C., Liao, C.M., 2006. Heavy incense burning in temples promotes exposure risk from airborne PMs and carcinogenic PAHs. Sci. Total. Environ. 372, 64–75.

Chiang, K.C., Chio, C.P., Chiang, Y.H., Liao, C.M., 2009. Assessing hazardous risks of human exposure to temple airborne polycyclic aromatic hydrocarbons. J. Hazard. Mater. 166, 676–685.

Cho, D.Y., Pace, L.W., Beadle, R.E., 1986. Cerebral cryptococcosis in a horse. Vet. Pathol. 23, 207–209.

Chuang, H.C., Jones, T., BéruBé, K., 2012. Combustion particles emitted during church services: Implications for human respiratory health. Environ. Int. 40, 137–142.

Cifuentes, C.M., Gómez-Serranillos, M.P., Iglesias, I., Villar del Fresno, A.M., Morales, C., Paredes, M.E., Cáceres, A., 2001. Neuropharmacological profile of ethnomedicinal plants of Guatemala. J. Ethnopharmacol. 76, 223–228.

Garcia, D., Ribeiro, C., Santos, J.F.L., Soares, J.A.R., Antonio, R.L., Santos, T.S.D. dos, Rodrigues, E., 2016. Defumadores com possível efeito ansiolítico utilizados no centro de Umbanda Caboclo Ubirajara e Exú Ventania, Diadema, SP: Um Estudo Etnofarmacológico. Ethnoscientia 1, 1–12.

Gomes, P.B., Oliveira, M.M.D.S., Nogueira, C.R.A., Noronha, E.C., Carneiro, L.M.V., Bezerra, J.N.S., Neto, M.A., Vasconcelos, S.M.M., Fonteles, M.M.F., Viana, G.S.B., Sousa, F.C.F.D., 2005. Study of antinociceptive effect of isolated fractions from *Petiveria alliacea* L.(tipi) in mice. Biol. Pharm. Bull. 28, 42–46.

Gomes, P.B., Noronha, E.C., Melo, C.T.V., Bezerra, J.N.S., Neto, N.A., Lino, C.S., Vasconcelos, S.M.M., Viana, G.S.B., Sousa, F.C.F., 2008. Central effects of isolated fractions from the root of *Petiveria alliacea* L. (tipi) in mice. J. Ethnopharmacol. 120, 209–214.

Harris, A.C., Mattson, C., LeSage, M.G., Keyler, D.E., Pentel, P.R., 2010. Comparison of the behavioral effects of cigarette smoke and pure nicotine in rats. Pharmacol. Biochem. Behav. 96, 217–227.

He, Y.Q., Xue, W.Q., Shen, G.P., Tang, L.L., Zeng, Y.X., Jia, W.H., 2015. Household inhalants exposure and nasopharyngeal carcinoma risk: A large-scale case-control study in Guangdong, China. BMC Cancer 15, http://dx.doi.org/10.1186/s12885-015-2035-x.

Hu, M.T., Chen, S.J., Huang, K.L., Lin, Y.C., Lee, W.J., Chang-Chien, G.P., Tsai, J.H., Lee, J.T., Chiu, C.H., 2009. Characteritization of, and health risks from, polychlorinated dibenzo-*p*-dioxins/dibenzofurans from incense burned in a temple. Sci. Total Environ. 407, 4870–4875.

Hussain, T., Al-Attas, O.S., Al-Daghri, N.M., Mohammed, A.A., De Rosas, E., Ibrahim, S., Vinodson, B., Ansari, M.G., El-Din, K.I.A., 2014. Induction of CYP1A1, CYP1A2, CYP1B1, increased oxidative stress and inflammation in the lung and liver tissues of rats exposed to incense smoke. Mol. Cell. Biochem. 391, 127–136.

Lewington, A., 2003. Plants for People. Eden Project Books, London.

Liao, C.M., Chiang, K.C., 2006. Probabilistic risk assessment for personal exposure to carcinogenic polycyclic aromatic hydrocarbons in Taiwanese temples. Chemosphere 63, 1610–1619.

Lima, D.B.F., 1979. Malungo, Decodificação do Umbanda: Contribuição à História Das Religiões. Civilização Brasileira, Rio de Janeiro.

Lin, T.C., Krishnaswamy, G., Chi, D.S., 2008. Incense smoke: Clinical, structural and molecular effects on airway disease. Clin. Mol. Allergy 6, 1–9.

Lucas, A., 1930. Cosmetics, perfumes and incense in ancient Egypt. J. Egypt. Archaeol. 16, 41–56.

Luz, D.A., Pinheiro, A.M., Silva, M.L., Monteiro, M.C., Prediger, R.D., Maia, C.S.F., Fontes-Júnior, E.A., 2016. Ethnobotany, phytochemistry and neuropharmacological effects of *Petiveria alliacea* L.(Phytolaccaceae): A review. J. Ethnopharmacol. 185, 182–201.

Mazor, A., Matar, M.A., Kaplan, Z., Kozlovsky, N., Zohar, J., Cohen, H., 2009. Gender-related qualitative differences in baseline and post-stress anxiety responses are not reflected in the incidence of criterion-based PTSD-like behaviour patterns. World J. Biol. Psychiatry 10, 856–869.

Mazumdar, S., Mazumdar, S., 2015. Lived Experience of Religion: Hindu Americans in Southern California. In: Brunn, S.D., Gilbreath, D.A. (Eds.), The Changing World Religion Map: Sacred Places, Identities, Practices and Politics. Springer, Netherlands, pp. 1405–1419.

Navasumrit, P., Arayasiri, M., Hiang, O.M.T., Leechawengwongs, M., Promvijit, J., Choonvisase, S., Ruchirawat, M., 2008. Potential health effects of exposure to carcinogenic compounds in incense smoke in temple workers. Chem. Biol. Interact. 173, 19–31.

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open: Closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Method. 14, 149–167.

Pellow, S., File, S.E., 1986. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. Pharmacol. Biochem. Behav. 24, 525–529.

Pinheiro, N.M., Miranda, C.J., Perini, A., Câmara, N.O., Costa, S.K., Alonso-Vale, M.I., Caperuto, L.C., Tibério, I.F., Prado, M.A., Martins, M.A., Prado, V.F., Prado, C.M., 2015. Pulmonary inflammation is regulated by the levels of the vesicular acetylcholine transporter. PLoS One 27, e0120441.

Prado, C.M., Leick-Maldonado, E.A., Yano, L., Leme, A.S., Capelozzi, V.L., Martins, M.A., 2006. Effects of nitric oxide synthases in chronic allergic airway inflammation and remodeling. Am. J. Respir. Cell. Mol. Biol. 35, 457–465.

Rivero, D.H.R.F., Soares, S.R.C., Lorenzi-Filho, G., Saiki, M., Godleski, J.J., Antonangelo, L., Dolhnikoff, M., Saldiva, P.H.N., 2005. Acute cardiopulmonary alterations induced by fine particulate matter of Sao Paulo, Brazil. Toxicol. Sci. 85, 898–905.

Rodrigues, E., Gianfratti, B., Tabach, R., Negri, G., Mendes, F.R., 2008. Preliminary investigation of the central nervous system effects of' Tira-capeta' (Removing the Devil), a cigarette used by some Quilombolas living in Pantanal Wetlands of Brazil. Phytother. Res. 20, 1248–1255.

Ross, K., Mazza, G., 2010. Characteristics of lignin from flax shives as affected by extraction conditions. Int. J. Mol. Sci. 20, 4035–4050.

Ruanda, A.D., 1954. Banhos, Defumações, e Amacis da Umbanda, Fifth ed. Editora espiritualista, Rio de Janeiro.

Schoental, R., Gibbard, S., 1967. Carcinogens in Chinese Incense Smoke. Nature 216, 612.

Seow, W.J., Qing, L., 2016. Domestic incense use and lung cancer in Asia: A review. Rev. Environ. Health 31, 155–158.

Soares, J.A.R., Kato, E.T.M., Bugno, A., Galduróz, J.C.F., Marques, L.C., Macrini, T., Rodrigues, E., 2013. Informal trade of psychoactive herbal products in the city of Diadema, SP, Brazil: Quality and potential risks. J. Evid. Based. Integr. Med., http://dx.doi.org/10.1155/2013/894834.

Strassburg, A., Droemann, D., van Zandbergen, G., Kothe, H., Dalhoff, K., 2004. Enhanced PMN response in chronic bronchitis and community-acquired pneumonia. Eur. Respir. J. 24, 772–778.

Suarez, L.E.C., Monache, F.D., 1992. 6-*C*-Formyl and 6-*C*-hydroxymethyl flavanones from Petiveria alliacea. Phytochemistry 31, 2481–2482.

Szilasi, M., Dolinay, T., Nemes, Z., Strausz, J., 2006. Pathology of chronic obstructive pulmonary disease, Pathol. Oncol. Res. 12, 52–60.

Tang, K., Li, Y., Zhang, Z., Gu, Y., Xiong, Y., Feng, G., He, L., Qin, S., 2010. The PstI/RsaI and DraI polymorphisms of CYP2E1 and head and neck cancer risk: A meta-analysis based on 21 case-control studies. BMC Cancer, http://dx.doi.org/10.1186/1471-2407-10-575.

Tao, F., Gonzalez-Flecha, B., Kobzik, L., 2003. Reactive oxygen species in pulmonary inflammation by ambient particulates. Free. Radic. Biol. Med. 15, 327–340.

Toledo, A.C., Sakoda, C.P., Perini, A., Pinheiro, N.M., Magalhães, R.M., Grecco, S., 2013. Flavonone treatment reverses airway inflammation and remodelling in an asthma murine model. Br. J. Pharmacol. 168, 1736–1749.

Tunçel, G., 2011. Two brass censers in the Museum of Turkish and Islamic art, Istanbul, in the chronology of Turkish metal Works. Milli. Folklor. 89, 257–264.

Tymchyshyn, M., Xu, C., 2010. Liquefaction of bio-mass in hot-compressed water for the production of phenolic compounds. Bioresour. Technol. 101, 2483–2490.

Viola, H., Wasowski, C., Levi de Stein, M., Silveira, R., Dajas, F., Medina, J.H., Paladini, A.C., 1995. Apigenin, a component of *Matricaria recutita* flowers, is a central benzodiazepine receptors-ligand with anxiolytic effects. Planta Med. 61, 213–216.

Yü, C.F., 2013. Passing the Light: The Incense Light Community and Buddhist Nuns in Contemporary Taiwan. University of Hawai'i Press, Hawai.

Wolfman, C., Viola, H., Paladini, A., Dajas, F., Median, J.H., 1994. Possible anxiolytic effects of chrysin, a central benzodiazepine receptor ligand isolated from Passiflora coerulea. Pharmacol. Biochem. Behav. 47, 1–4.