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Pinetonina™, an Intranasally Administered Essential Oil Preparation, Is Effective in Decrease of Cortisol Levels and on the Glutamate Release Modulation

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Abstract

Background: Most anxiety complaints are treated with pharmacological measures involving barbiturates and benzodiazepines, in which they may end up causing tolerance and pharmacological dependence. Integrative approaches such as aromatherapy are used in addition to medications to improve sleep quality and reduce anxiety. Thus, Pinetonin™, a phytocomplex obtained from a blend of essential oils aims to aid in the symptoms of stress and anxiety. **Methods:** The cytotoxicity of Pinetonin™ was evaluated MTT assay using fibroblasts and astrocytes showed reduction in the cell viability only at high concentrations. Evaluation of intracellular calcium and determination of residual glutamate in the supernatant of astrocyte cultures showed agonist action of dihydroxyphenylglycine (DHPG) increasing linearly the concentration of intracellular calcium and the glutamate levels in the supernatants of the cultures. On the other hand, cultures of astrocytes treated with Pinetonin™ showed residual glutamate levels in the supernatants reducing proportionally, as well as, intracellular calcium reduction. The determination of salivary cortisol showed a significant reduction in salivary cortisol levels in the group that received Pinetonina™. The evaluation of the electroencephalo-

gram in patients treated with Pinetonina™ had a significant increase ($P < 0.05$) in the frequency (Hz) of the alpha and beta waves. **Results:** A reduction in dose-dependent cell viability was observed when compared to cultures of Pinetonin™ treated fibroblasts with control culture. When Pinetonin™ and linole are administered in astrocytic cells, there was a reduction of the intracellular concentration of Ca^{2+} against a control group treated with DHPG agonist. The evaluation of salivary cortisol demonstrated a reduction when the patient group was treated with Pinetonin™ by purchasing the results against the group of patients treated with saline. Reinforcing the relaxed state of that group, alpha waves have been increased and reductions in beta waves. **Conclusion:** The results obtained after intranasal administration of Pinetonina™ suggest that this phytocomplex can help reduce the symptoms of stress and the better quality of life.

Keywords

Pinetonin, Anxiety, Cortisol, Astrocites, Glutamate

1. Background

Affecting almost 13% of the world's population, anxiety disorders are among the most common psycho-socio-behavioral problems that justify the importance and interest in the areas applied to psychopharmacology. Psychoanalytic, behavioral, genetic, biological, cognitive and psychodynamic theories are used as tools to explain the etiology of anxiety disorders since it is a disease of heterogeneous disorders [1]. Most complaints related to these problems are treated with pharmacological measures involving benzodiazepines and barbiturates, in addition to other anxiolytic agents, which in the majority of cases can generate tolerance and consequently pharmacological dependence [2].

Thus, to reduce the costs of pharmacotherapy and, in synergy, to complement the medicinal treatments, the essential oils appear as new therapeutic modalities. This integrative practice has the actions as anti-inflammatory, anti-oxidants helping to reduce anxiety and stress [3].

To guarantee the emotional balance and the physical and mental estares several essential oils are obtained [4] [5]. Currently, with the goals of improving sleep quality and also reducing the effects of anxiety, integrative approaches like aromatherapy are used in synergy with medications [6].

In addition, studies have demonstrated the effect of olfactory stimulation on various physiological systems, among which, anxiety-related systems. In this sense, the practice of using essential oils via the olfactory system had a positive effect on the reduction of anxiety, increasing sleep quality and stabilization of blood pressure in patients with cardiovascular disease in the intensive care unit [7]. In other studies, it was possible to observe that the inhalation of essential oils reduced blood pressure in rats after stressful responses [8], and that the olfactory

stimulus with essential oils, aided in the stabilization of the mood of stroke patients and who showed symptoms of anxiety [9].

Lavender (*Lavandula angustifolia*) belongs to the family Lamiaceae (*Labiatae*), produces an essential oil with composition rich in linalyl acetate, pinene, limonene, linalool and lavandulol. [10] The essencial oil from *Lavandula* species (lavender) exhibit various biological activities, namely anti-microbial, antimutagenic, anti-inflammatory and analgesic properties [11].

However, the most explored activity of lavender essential oil is its use as an anxiolytic and as an adjuvant in the treatment of insomnia, even though there is no evidence that Lavender does not have a sedative effect, olfactory use can improve the quality of sleep reducing insomnia [12].

Foeniculum vulgare Mill, commonly known as fennel, is an aromatic plant widely cultivated in temperate and tropical regions [13]. The chemical constituents of *F. vulgare* include essential oil, fatty acid, phenylpropanoids, monoterpenes, sesquiterpenes and coumarins. It also contains triterpenoids, tannins, flavonoids, cardiac glycosides, saponins, and other types of compounds [14]. In traditional medicine, *F. vulgare* is used to encourage menstruation and lactation, stimulate gastrointestinal motility, relieve intestinal gas accumulation, improve eyesight, alleviate productive coughs, ease spasm, promote courage and mental strength, reduce stress and nervousness and produce calming [15].

Therefore, this work aims to evaluate a blend of essential oils in order to verify if their inhalation may contribute to the reduction of anxiety and the quality of life of the volunteers, using as indicative of these activities, the analysis of alpha (α) and beta (β) obtained by electroencephalogram.

2. Materials and Methods

2.1. Cell Culture

Both cell lines were kindly provided by Prof. Dr. Eduardo Finger, Coordinator of the Laboratory of Molecular Biology, Anhembi Morumbi University. The Fibroblast (CCD-1072Sk) and astrocyte (GIBCO N7805100) lines, were cultured in ISCOVE™ and DMEM/F12 medium, respectively, both with 10% fetal bovine serum, 0.292 g/L L-glutamine, 1.0 g/L D-glucose, 2.2 g/L $NaHCO_3$, 10,000 IU Penicillin and 0.060 g/L Streptomycin. Cells were maintained in 25 cm^2 flasks (1×10^5 cells/mL) in a humid oven with 5% CO_2 atmosphere at 37˚C. In all experiments the cultures of fibroblasts and astrocytes were submitted to the cell viability test using the trypan blue dye and the reading was performed in haemocytometric chamber by light microscopy. All experiments described were performed when cell viability was equal to or greater than 95%. In all assays, the incubation time was 12 h and the concentrations of Pinetonina™ ranged from 1% - 10%.

2.2. MTT Reduction to Formazan Assays

The reduction test [3-(4,5-Dimethylthiazol-2-yl) 2,5-Diphenyl Tetrazolium Bro-

mide] (MTT) is used with great success to estimate the number of viable cells in an initial screening of drugs. Its interpretation is indicative of cellular metabolic activity, and the site of occurrence of redox reactions includes both mitochondria and the cytosol. The reduction of the salt of MTT to formazan is carried out mainly by the enzyme succinate dehydrogenase, and results in insoluble formazan crystals in violet color. The intensity of staining is used to measure mitochondrial activity and consequently cell viability (MOSMANN *et al.*, 1983). Therefore, 5×10^4 cells/well were treated with different concentrations of Pinetonina™ (0.5%, 1.0%, 1.5% and 2.0%). After that, 10 µL of the 5 mg/mL MTT solution (Sigma-Aldrich) was added to each well. After 4 hours, the culture was collected in 15 ml tubes and centrifuge at 1500 rpm for 5 minutes, the supernatant from each well discarded and the pellet with the crystals formed on the bottom of the plate dissolved with 100 µL of pure ethanol and then homogenized on a plate shaker for 15 minutes. Optical density was measured by the microplate reader (FlexStation® 3 multimode benchtop reader) at 540 nm.

2.3. Evaluation of Intracellular Calcium Concentration

Measurements of the cytosolic calcium concentration ($[Ca^{2+}]_i$) were performed by the spectrofluorometric method using the fura-2 acetoxymethyl ester (Fura 2-AM) method [16]. Neuronal cells (4×10^5 cells/ml) were resuspended in Tyrode solution (Inmilimolar (mM): Sodium Chloride (NaCl) 137.0; Potassium Chloride (KCl) 2.7; Clacium Chloride Dihydratade ($CaCl_2 \cdot 2H_2O$) 1.4; Magnesium Chloride Hexahydrate ($MgCl_2 \cdot 6H_2O$) 0.5; Sodium Dihydrogen Phosphate Monohydrate ($NaH_2PO_4 \cdot H_2O$) 0.4; 16.0 Carbonic acid (H_2CO_3), Glucose supplemented with serum bovine albumin (BSA, 0.2% w/v) and incubated with Fura 2-AM (5 micromolar (µM), 23˚C) for 1 hour under stirring to be continued. Measurements of $[Ca^{2+}]_i$ were performed on a spectrofluorimeter. Maximum and minimum rates of 340/380 were achieved by administration of the cellular permeabilizer digitonin (50 µM) and Egtazic Acid (EGTA) (4 mM) + Sodium Hydroxid (NaOH) (0.04 N) added at the end of the experiments. The functional response of the cells was assessed by the $[Ca^{2+}]_i$ alteration in response to treatment with dihydroxyphenylglycine (DHPG), Linalool, Pinetonina™ and the combination Linalol and DHPG, in the following concentrations: 1.0%, 2.5% and 5.0% compared with basal levels.

2.4. Glutamate Supernatant

Astrocytes maintained in culture and treated according to item 2.4 were cultured in 96-well plates containing complete culture medium. After treatment with DHPG, Linalol, Pinetonina™ and the combination Linalol and DHPG, at the following concentrations: 1%, 2.5% and 5%, the culture supernatant was collected and analyzed by the glutamate assay kit (Biovision) according to the manufacturer's instructions. A standard curve generated by different concentrations of glutamate in DMEM—Dulbecco's Modified Eagle Medium—was used to calcu-

late the concentrations of glutamate by the ordinary least squares equation $y = 0.073X + 0.122$ was used to quantify the results.

2.5. Evaluation of the Electroencephalogram (Powerlab)

2.5.1. Volunteers

The protocols were approved by the local Ethics Committee and written consent was obtained from each subject. To obtain waves recorded in the electroencephalogram, 30 volunteers, with no evidence of nasal obstruction, aged between 18 and 35 years, all male, university students, with an average of 4.5 hours of sleep per night and without pre-disease existing, were evaliueted after signing the free and informed consent form. Three (3) groups were formed, namely: Control group (without intranasal application); Saline group and Pinetonina™ group. Each volunteer applied 2 (two) "puffs" to each nostril, either Pinetonina™ or saline. One of the groups, however, did not receive any kind of intranasal application, being this group the one used to take basal electroencephalogram waves. After the application of the intranasal doses (puffs), either Pinetonina™ or saline, each of the volunteers waited in a segregated place, without intense noise and without excessive light for 30 minutes, and then underwent electroencephalogram evaluation. The group that received no intranasal application performed the same procedure as the groups receiving either Pinetonina™ or saline.

2.5.2. Electroencephalogram (EEG)

Three disposable electrodes were implanted for epidural electroencephalogram recording. The electrodes were positioned bilaterally on the frontal bone and the occipital cortex (posterior 7.4 milimeters (mm) bregma, lateral ± 5.0 mm). The reference electrode was placed posteriorly in a known anatomical region with external occipital protuberance. The cables were connected to a socket, which was secured together with the electrodes. The electroencephalogram was measured continuously before and 20 minutes after inhalation of Pinetonina™ or saline in experiment 1 and in the case of the control experiment the recording was performed without inhalation of any agent. The electroencephalographic signal was digitized online at a sampling rate of 200 Hertz (Hz) and subjected to off-line spectral analysis. The power spectra were calculated for consecutive 2.56 s intervals and 0.39 Hz frequency compartments using the labchart analysis system. In this study, the global electroencephalographic (0 - 30 Hz) frequency band was divided into two frequency bands: α (8 - 13 Hz) and β (15 - 30 Hz).

2.6. Statistical Analysis

The results were presented as mean + SEM (standard error of the mean). The results were submitted to statistical analysis by analysis of variance (ANOVA) of one way, followed by Tukey test. Values of $P < 0.05$ were considered significantly different. The analysises were performed in the program GraphPad Prism version 5.0. The statistical analyzes used in the trials involving the powerlab were expressed as percentage control of the baseline values and represented by means

± SEM. The significance of the differences between the mean values of the EEG experiment was determined by repeated measures of variance analysis (ANOVA) followed by the Student-Newman-Kewls (SNK) test for multiple comparisons. All statistical analyzes were performed using the Statistical Package for the Socail Science (SPSS) 22.0 program (SPSS, Chicago, IL), and a 5% probability of type I errors was used to determine statistical significance. In all cases, $P < 0.05$ was taken as the level of significance.

3. Results

3.1. MTT Reduction Test for Formazan

The results obtained with the MTT test are shown in Figure 1. It is possible to observe a reduction in the viability of fibroblasts and astrocytes in a dose-dependent manner. In addition, only the 10% concentration promoted a significant reduction in cellular viability when compared to the control group (untreated group), considering a value of $P < 0.05$.

3.2. Evaluation of Intracellular Calcium and Determination of Residual Glutamate in the Supernatant of Astrocyte Cultures

The results presented in Figure 2(a) show the agonist action of DHPG increasing linearly the concentration of intracellular calcium. The glutamate levels released in the culture medium of these astrocytes were observed, as well as the linear increase of this excitatory amino acid. In Figure 2(b), we observed that linalool, one of the main components of the Pinetonina™ blend, promoted reduction in intracellular calcium levels. When looking at the astrocytes treated with Pinetonina™ (Figure 2(c)), it is possible to note that intracellular calcium levels are reduced starting from the 2.5% concentration. Finally, the 2D figure indicates that previous treatment with linalol reversed the increase of intracellular calcium and residual glutamate in the culture medium of the astrocytes.

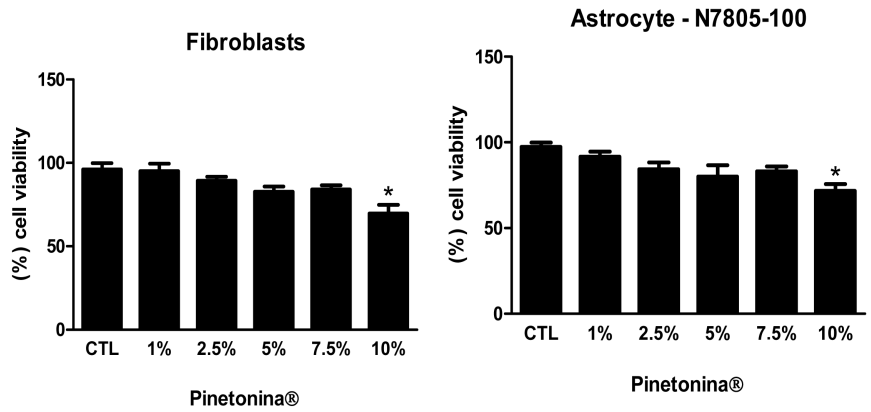

Figure 1. Results of cell proliferation through the MTT reduction assay after 24 hours exposure with different concentrations of Pinetonin™. Prior to the start of the tests, the cells were depleted of fetal bovine serum. (*) $P < 0.05$—significant in relation to the control, ANOVA, Tukey. Trials performed in triplicate. GraphPad Prism V.5.0.

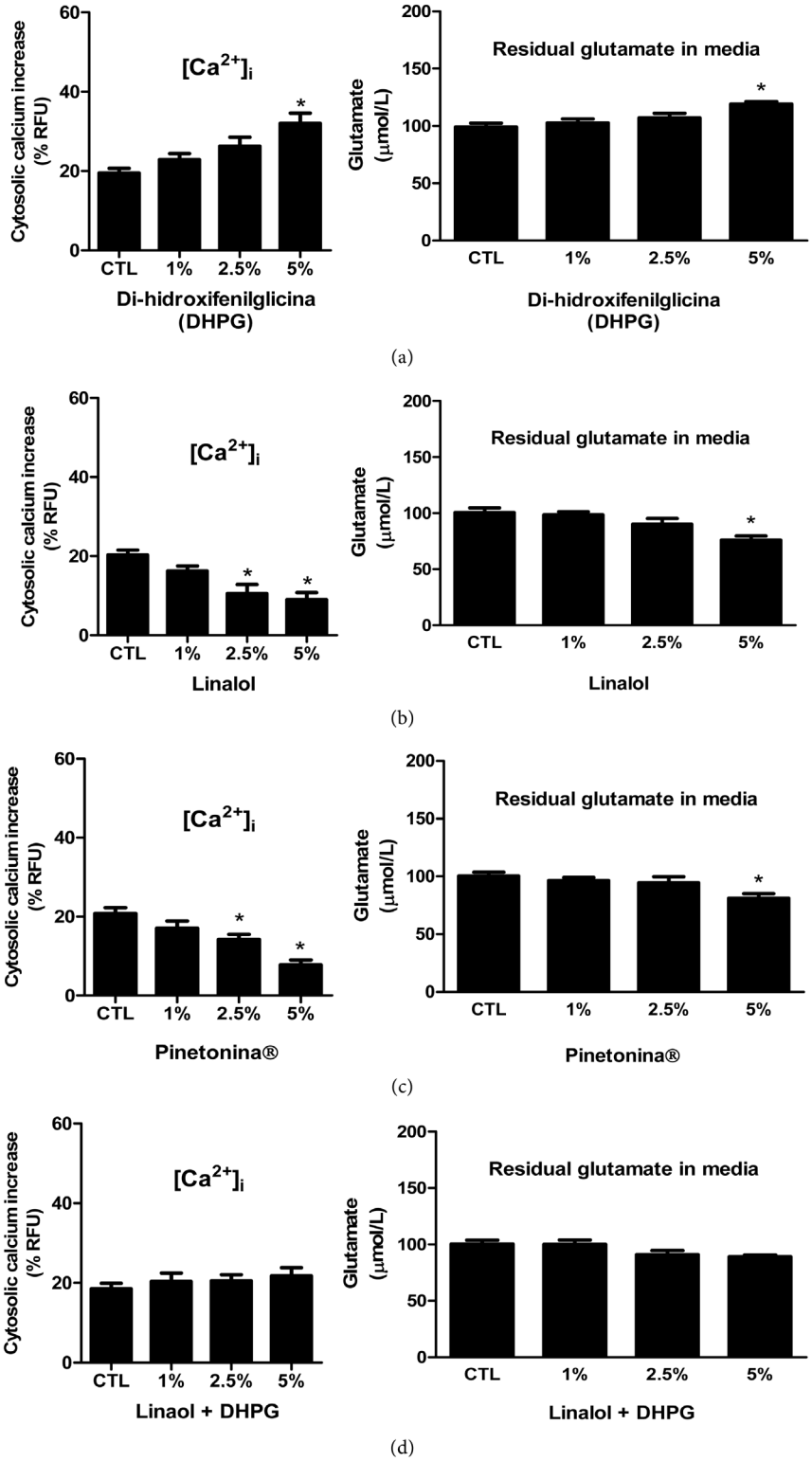

Figure 2. Levels of intracellular calcium and glutamate residue in the supernatants of astrocyte cultures. (a) cultures treated with the glutamatergic receptor agonist, DHPG; (b) cultures treated with linalool; (c) cultures treated with Pinetonin™; (d) cultures previously treated with linalool and then with DHPG. (*) $P < 0.05$—significant in relation to the control, ANOVA, Tukey. Trials performed in triplicate. GraphPad Prism V.5.0.

3.3. Determination of Salivary Cortisol

After using Pinetonin™ for 15 consecutive days, twice daily, volunteers collected saliva for determination of salivary cortisol. Figure 3 shows a significant reduction ($P < 0.05$) in salivary cortisol levels in the Pinetonin™ group. The groups treated with saline in the same scheme did not present a significant reduction.

3.4. Evaluation of the Electroencephalogram (Powerlab)

The evaluation of the electroencephalogram of the volunteers who received Pinetonina™ and saline, in addition to those who did not receive any type of product, showed that Pinetonina™ influenced the alpha (α) and beta (β) waves. Volunteers treated with Pinetonina™ had a significant increase ($P < 0.05$) in the frequency (Hz) of the alpha waves, which are waves related to relaxation, visualization and creativity (Figure 4(a)). In Figure 4(b), it was possible to observe that the volunteers who received Pinetonina™ presented reduction in the frequency of beta waves (β), waves these related to the alert, concentration and cognition.

4. Discussion

Recent studies have shown that essential oils have several biological activities, including anti-inflammatory activity [17] [18] [19] [20], antimicrobial [20] [21] and sedative properties [22] [23]. Essential oils such as those obtained from the *Lavandula spp.* genus have been extensively studied with the objective of evaluating their effects and describing possible biological interactions in order to prove their mechanism of action [20] [24] [25] [26] [27]. In this context, a phytocomplex was developed, called Pinetonina™ with the objective of assisting in stress situations and improving sleep quality. Our results, *in vitro*, demonstrated that Pinetonina™ did not show cytotoxicity in the evaluated strains (fibroblasts

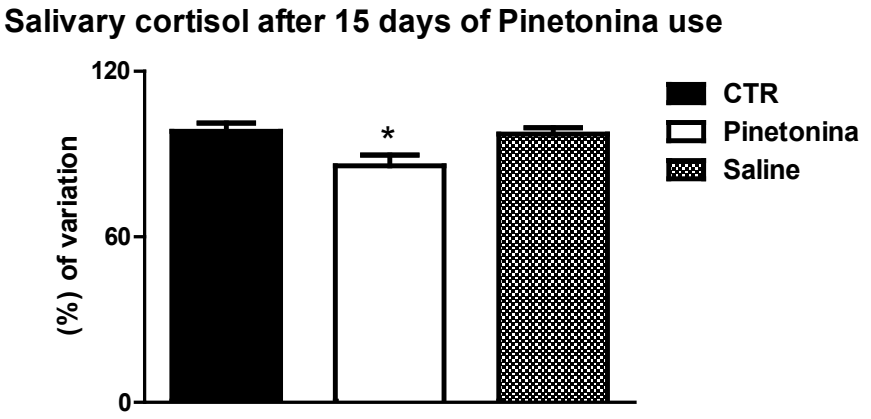

Figure 3. Variation of the level of salivary cortisol between groups of volunteers who received Pinetonina™, saline and without treatment. The group treated with Pinetonina™ obtained lower mean (%) when compared to the control group (without treatment) and when compared to the group treated with saline. (*) $P < 0.05$—significant in relation to the control, ANOVA, Tukey. Trials performed in triplicate. GraphPad Prism V.5.0.

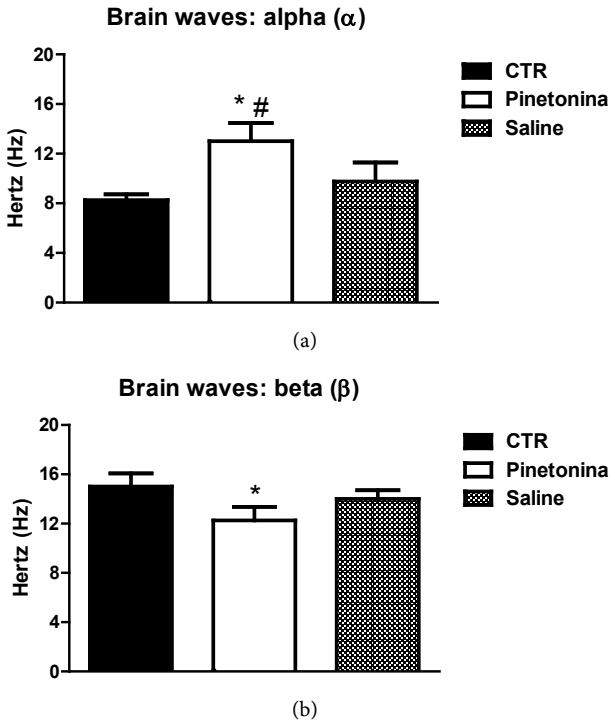

Figure 4. Frequency pattern of alpha and beta brain waves. (a) significant increase in the alpha wave frequency of the Pinetonin™ group when compared to the saline and control groups (without intranasal application); (b) reduction in beta-wave frequency of the Pinetonin™ group when compared to saline and control groups. (*) $P < 0.05$—significant in relation to the control, ANOVA, Tukey. Trials performed in triplicate. GraphPad Prism V.5.0.

and astrocytes), and the only tested concentration that significantly reduced cell viability in both strains was 10%, a concentration very high when compared to what is observed in clinical practice [18]. These results corroborate data found in the literature on the toxicity of some essential oils [25] [28] [29]. In order to propose a possible mechanism of action of Pinetonia™, astrocyte lineage was used to assess its ability to modulate the release of glutamate, an amino acid that participates in the Central Nervous System (CNS) as an excitatory neurotransmitter [30] [31] and the mobilization capacity of intracellular calcium, a phenomenon related to the release of glutamate and several other neurotransmitters stored in vesicles [30]. The results indicated that the action of the DHPG agonist on the astrocyte line increased the concentration of intracellular calcium and a proportional increase in glutamate levels was observed in the supernatant obtained from the supernatant of these cultures. On the other hand, when the astrocyte culture was previously treated with linalool, one of the main components of the essential oil of the species *Lavandula spp*. [25] [27] [32], which are part of the composition of Pinetonina™, we observed reduction in intracellular calcium levels, with consequent reduction of residual glutamate in the supernatant of astrocyte cultures. Thus, when analyzing the results obtained with Pinetonina™ it is possible to observe the same behavior regarding calcium mobilization in the as-

trocytes that were obtained with linalool, besides reducing the residual glutamate in the supernatant of the cultures. Studies showing the intracellular calcium modulation capacity with consequent release of glutamate by astrocytes allow us to infer the hypothesis that components present in the composition Pinetonina™ could reduce, albeit partially, the release of glutamate allowing a greater action of the inhibitory neurotransmitter Gamma-Amino Butyric Acid (GABA) responsible for part of the sedative action in the CNS [33] [34]. In order to evaluate *in vivo* the effects of stress-related and relaxation-related Pinetonin™, we performed salivary cortisol dosing of the volunteers after 15 consecutive days of use of the product. When compared to control volunteers (who received no treatment) and placebo volunteers (saline), we observed a significant reduction in salivary cortisol levels. This study shows the probably indirect influence of calcium and glutamate on the release of cortisol through the modulation of release of Corticotropin Releasing Hormone (CRH) [26] [33] [35] [36], which would ultimately signal the release of adrenocorticotrophic hormone (ACTH) for the production of cortisol in the adrenal cortex. The idea of a lower production of cortisol would be related to the greater ease of melatonin release, since the latter hormone is elevated when cortisol is reduced [37] [38], thus aiding in sleep quality [39] [40] [41]. Finally, the electroencephalogram evaluation of the volunteers showed that Pinetonina™ influenced the alpha (α) and beta (β) waves. The results showed that volunteers treated with Pinetonina™ had a significant increase ($P < 0.05$) in the frequency (Hz) of alpha waves, which are waves related to relaxation, visualization and creativity [41] [42], whereas volunteers who received Pinetonina™ presented a reduction in the frequency of beta waves (β), these waves related to the alert [42] [43] [44].

5. Conclusion

Based on the *in vitro* tests, it can be suggested that the compound Pinetonina™ is not considered cytotoxic, since only from the concentration of 10% showed to significantly reduce cell viability in cell lines evaluated. After determination of intracellular calcium and determination of glutamate in the supernatant of the astrocyte cultures, it can be deduced that Pinetonin™ was able to reduce the calcium concentration and the release of glutamate (excitatory neurotransmitter) into the supernatant of the astrocyte cultures, suggesting amodulating activity between excitatory and inhibitory systems in the CNS. Regarding the *in vivo* tests, the salivary cortisol dosage of the volunteers that made intranasal application of Pinetonina™, allows us to conclude that the levels of this hormone, which in situations of stress is high, and has their values reduced. As melatonin levels begin to rise as cortisol levels begin to decline, we may suggest that Pinetonin™, in daily stress situations where cortisol levels begin to decay much later than normal, would reduce cortisol circulating, facilitating the increase of melatonin, making the patients who use Pinetonina™ have better quality of sleep and less stress. Associated with the reduction of salivary cortisol, patients who underwent

intranasal application showed an increase in the CNS alpha wave pattern on the electroencephalogram, indicating that Pinetonin™ may promote relaxation in individuals using the compound, while reducing beta brain waves, responsible for the alert, and other aspects of the CNS. Thus, it was possible to prove that the inhalation of Pinetonina™ reduces the anxiety, providing an increase in the quality of life of the volunteers.

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Availability of Data and Materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions MJ, FA, VMSA and CRO conceived and designed the experiments; MSAS, FRS, LMS, RAB performed the experiments; MJ, LMS, RAB, VC and CRO analyzed the data; MJ, VMSA, MSAS, VC and CRO wrote the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Research Ethics Committee of Anhembi Morumbi University (registration number 2.624.363).

Competing Interests

The authors declare that they have no competing interests.

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Abbreviations

ACTH: Adrenocorticotrophic hormone, ANOVA: Analyses of variance, BSA: Serum bovine albumin, Ca^{2+}: Calcium ions, $CaCl_2 \cdot 2H_2O$: Calcium chloride dihydrate, CNS: Central nervous system, CRH: Corticotropin release hormone, DHPG: Dihydroxyphenylglycine, DMEM: Dulbecco's modified eagle medium, EEG: Electroencephalogram, EGTA: Egtazic acid, Fura-2-AM: Fura-2-AcetoxyMethylester, GABA: Gamma amino butyric acid, H_2CO_3: Carbonic acid, Hz: Hertz, KCl: Potassium chloride, $MgCl_2 \cdot 6H_2O$: Magnesium chloride hexahydratade, mm: Millimeter, mM: Milimolar, μM: Micromolar, NaCl: Sodium chloride, $NaH_2PO_4 \cdot H_2O$: Sodium dihydrogen phosphate monohydrate, SEM: Standard error of the mean, SNK: Student-Newman-Kewls, SPSS: Statistical package for the social science.