Research Article

Phytochemical Analysis and Antimicrobial, Antinociceptive, and Anti-Inflammatory Activities of Two Chemotypes of *Pimenta pseudocaryophyllus* (Myrtaceae)

Joelma Abadia Marciano de Paula,1 Maria do Rosário Rodrigues Silva,2 Maysa P. Costa,2 Danielle Guimarães Almeida Diniz,3 Fabyola A. S. Sá,3 Suzana Ferreira Alves,3 Élson Alves Costa,4 Roberta Campos Lino,4 and José Realino de Paula3

1 Unit of Exact and Technologic Sciences, Goiás State University, Anápolis, Brazil  
2 Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Brazil  
3 Faculty of Pharmacy, Federal University of Goiás, Goiânia, Brazil  
4 Department of Physiological Sciences, Institute of Biological Sciences, Federal University of Goiás, Goiânia, Brazil

Correspondence should be addressed to Joelma Abadia Marciano de Paula, joelmapaula@uol.com.br

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Preparations from *Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum (Myrtaceae) have been widely used in Brazilian folk medicine. This study aims to evaluate the antimicrobial activity of the crude ethanol extracts, fractions, semipurified substances, and essential oils obtained from leaves of two chemotypes of *P. pseudocaryophyllus* and to perform the antinociceptive and anti-inflammatory screening. The ethanol extracts were purified by column chromatography and main compounds were spectrally characterised (1D and 2D 1H and 13C NMR). The essential oils constituents were identified by GC/MS. The broth microdilution method was used for testing the antimicrobial activity. The abdominal contortions induced by acetic acid and the ear oedema induced by croton oil were used for screening of antinociceptive and anti-inflammatory activities, respectively. The phytochemical analysis resulted in the isolation of pentacyclic triterpenes, flavonoids, and phenol acids. The oleanolic acid showed the best profile of antibacterial activity for Gram-positive bacteria (31.2–125 μg mL⁻¹), followed by the essential oil of the citral chemotype (62.5–250 μg mL⁻¹). Among the semipurified substances, *Ppm5*, which contained gallic acid, was the most active for *Candida* spp. (31.2 μg mL⁻¹) and *Cryptococcus* spp. (3.9–15.6 μg mL⁻¹). The crude ethanol extract and fractions from citral chemotype showed antinociceptive and anti-inflammatory effects.

1. Introduction

*Pimenta pseudocaryophyllus* (Gomes) L.R. Landrum (Myrtaceae) is a plant popularly known in Brazil as *pau-cravo*, *louro-cravo*, *louro*, *craveiro*, *craveiro-do-mato*, *chá-de-buque*, and *catuia* [1–6]. In folk medicine, the leaves have been used to produce a refreshing drink with calming, diuretic, and aphrodisiac properties, as well as to treat colds and their complications and digestive and menstrual problems [2, 4–6]. It is the only *Pimenta* species native to Brazil [1, 3], and recent studies have shown the occurrence of different chemotypes for this species; these are characterised, for example, by the predominance of citral or (E)-methyl isoeugenol in the essential oils [7].

Invasive infections caused by *Candida* spp. and *Cryptococcus neoformans* have increased significantly in recent years [8–11]. The cause of this rise is often related to immunodeficiency associated with transplantation [11] and acquired immunodeficiency syndrome (AIDS) [9], as well as the use of intravascular catheters [10], dialysis, and abusive use of glucocorticoids and broad-spectrum antibiotics [8]. The drugs available to treat these infections are often not selective, are toxic, or have narrow action spectra [12]; moreover, some species are resistant to antifungal agents [13].
In the pharmacotherapy of bacterial diseases, the use of antibiotics in recent decades has significantly reduced the incidence of many infectious diseases. On the other hand, the severe side effects from many of these substances and the emergence of multiresistant microorganisms have stimulated research on the development of new antibacterial agents that are more specific, effective, and safe [24, 25].

There is thus a consensus on the need for further research on new alternative treatments for bacterial and fungal infections. Efforts have been focused on investigating the antimicrobial properties of products from plants [12, 25]. In addition to extensive use in folk medicine in diseases related to the common cold, which often involve microbial and/or inflammatory processes, experimental data show that plants of the genus Pimenta (Myrtaceae) have antimicrobial potential [26, 27]. The essential oil of P. pseudocaryophyllus leaves collected in two geographical areas in the state of São Paulo was active against strains of C. albicans, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus [4]. In a previous study, we described the antimicrobial activity of the crude ethanol extract of leaves of this species collected in the Brazilian Cerrado against Gram-positive bacteria and Candida albicans [28], but we have not performed phytochemical studies for the isolation and identification of substances accountable for this activity.

Moreover, scientific studies have shown that Pimenta species, widely used in folk medicine, have analgesic and anti-inflammatory activities and are nontoxic in typical dosages [29–32]. Thus, the aims of this work were to carry out the phytochemical study, evaluate the antimicrobial activity of the crude ethanol extracts, fractions, semipurified substances, and essential oils obtained from leaves of two chemotypes of P. pseudocaryophyllus, and perform the screening of the antinociceptive and anti-inflammatory activities.

2. Material and Methods

2.1. General Experimental Procedures. The 1H and 13C one-dimensional and two-dimensional NMR spectra (Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC)) were obtained using a Bruker Avance III-500 spectrometer running at 500 MHz (1H) and 125 MHz (13C), using deuterated chloroform (CDCl₃) and deuterated dimethyl sulfoxide (DMSO-d₆) as solvents for nonpolar and polar samples, respectively. Tetramethylsilane (TMS) was used as an internal reference standard for chemical shifts (δ, ppm), and in some cases, the peak from the solvent was used for reference.

Essential oil samples were analysed by GC/MS using a QP5050A instrument (Shimadzu, Kyoto, Japan) with a capillary column of CBP-5 melted silica (30 m × 0.25 mm × 0.25 μm; 5% phenyl-methylpolysiloxane film) (Shimadzu, Kyoto, Japan). Additionally, helium was used with a flow rate of 1 mL min⁻¹ as a carrier gas, and a thermal profile of 60°C to 240°C with a gradient of 3°C min⁻¹ followed by a gradient of 10°C min⁻¹ up to 280°C, keeping a 5 min isotherm, was used. The ionisation energy of the detector was kept at 70 eV, and the sample injection volume following dilution in hexane (~10%) was 0.5 μL. The analysis was carried out in the scanning mode, with a 40–400 m/z mass interval and 1:5 injection ratio. The quantitative analysis was obtained by integrating the total ion chromatogram (TIC). The identification of the components was performed by comparing the mass and retention indices (RI) calculated using values for the mass and retention indices available in the literature [33]. The retention indices were calculated by coinjection with a mixture of hydrocarbons, C₆–C₃₂ (Sigma, MO, USA), applying the Van Den Dool and Kratz equation [34].

The mass spectra of the flavonoids were collected using a coupled LC/EM/EM: Varian 1200L (Walnut Creek, CA, USA) system with a quadrupole ion analyser and ionisation through electron impact, 70 eV in positive mode m/z [M + H]⁺. The m/z scanning spectrum was 100–900, and the ionisation chamber was kept at room temperature.

The HPLC was held on Waters equipment (MA, USA) equipped with quaternary pump, e2695 separation module, 2998 diode array detector (PDA), and Empower 2.0 data processing system. The Varian C-18 (250 × 4.6 mm) column was used at room temperature. The detection system was used to monitor at 360 nm for detection of flavonoids. The injection volume was of 30 μL and the run in isocratic mode was used as mobile phase 75% of A [acetonitrile/water (0.1% acetic acid)]—90:10 and 25% of B [MeOH (0.1% acetic acid)] at a constant flow of 1 mL min⁻¹. The maximal running time was 20 min. Quercitrin (Sigma) was used as reference standard. The samples were previously filtered through Millex (Millipore, MA, USA) membrane and the mobile phase in 0.45 μm PVDF membrane (Millipore).

For analytical and preparative thin-layer chromatography (ATLC, PTLC), chromatography plates prepared with G60 F₂₅₄ Vetec silica gel, or F₂₅₄ silica plates manufactured by Merck, RJ, Brazil, were used. Mixtures of organic solvents with the proper polarities for each analysed fraction were used as mobile phases. For detection of the components, the plates were observed under UV light at 254 nm and 365 nm and developed by the spraying of sulphuric vanillin followed by heating or exposure to a solution of 1% ethylamine diphenyl borate in methanol (NP) [35].

The column chromatography used silica gel G60 0.05–0.2 mm (Vetec, Brazil) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) as stationary phases. As mobile phases, mixtures of organic solvents in increasing order of polarity were used, according to the polarity profiles of the fractions undergoing the process.

2.2. Plant Material. The leaves of P. pseudocaryophyllus of the (E)-methyl isoeugenol and citral chemotypes were collected in São Gonçalo do Abaeté, MG, Brazil, in February 2008, at 18°20’ 58.4”S, 45° 55’23.4”W, and 864 m altitude. These two chemotypes are the most common found in the region of São Gonçalo do Abaeté and therefore were selected for this study.

The plant material was identified by Professor Carolyn Elinore Barnes Proença, Ph.D., of the Universidade de Brasília, and a voucher specimen (UFG-27159) was...
2.3. Preparation of Extracts and Fractions. Air-dried leaves were ground in a knife mill. The crude ethanol extract was obtained by maceration of the powdered material (1:5 w/v) of each chemotype in 95% EtOH (v/v) at room temperature, followed by filtration, and concentration on a rotary evaporator at a temperature below 40°C. The extracts were concentrated to a constant weight and named crude ethanol extract of (E)-methyl isoeugenol chemotype (EEC) and crude ethanol extract of citral chemotype (EEC).

Fifty grams of each extract (EEC and EEC) were dissolved in 250 mL MeOH/H2O (7:3) and subjected to liquid/liquid partitioning with solvents of increasing polarity (hexane, dichloromethane, and ethyl acetate). The final MeOH/H2O residual was concentrated in a rotary evaporator for elimination of the MeOH, and the resulting aqueous fraction was lyophilised. Therefore, four fractions were obtained from the crude extract of each chemotype: the hexane fraction (HFM and HFC), the dichloromethane fraction (DFM and DFC), the ethyl acetate fraction (EAFM and EAFC), and the aqueous fraction (AFM and AFC).

For obtaining of essential oils, leaves from each chemotype were subjected to hydrodistillation for 3 h in a Clevenger apparatus. The essential oils were dried with anhydrous Na2SO4, packaged in amber glass vials, and stored at −20°C until use. They were named essential oil of the citral chemotype (EOC) and essential oil of the (E)-methyl isoeugenol chemotype (EOC).

2.4. Isolation of the Chemical Constituents

HFC. The HFC (2.5 g) was fractionated on a silica gel column (1:40) eluted with a gradient of hexane-EtOAc (5–100%), EtOAc-MeOH (1:1), and MeOH (100%). Seventy-five 20 mL fractions were collected and evaluated by TLC [hexane-EtOAc mixtures (10–30%)], observed under 254/365 nm UV light, and detected with sulphuric vanillin reagent. Similar fractions were pooled, resulting in 14 new fractions (CH-1 to CH-14). Fractions CH-8 through CH-14 were rechromatographed on a silica gel column eluted isocratically with CH2Cl2-petroleum ether (7:3), resulting in Ppc-2 (22.6 mg).

HFM. The HFM (2.5 g) was fractionated on a silica gel column (1:40) eluted with 100% hexane-EtOAc (50–100%), EtOAc-MeOH (1:1), and MeOH (100%). One hundred 10 mL fractions were collected and evaluated by TLC [hexane-EtOAc (10–30%)], observed under UV light, and detected with sulphuric vanillin reagent, resulting in 14 new fractions (MH-1 to MH-14). Fractions MH-4 and MH-5 were rechromatographed on a silica gel column eluted isocratically with CH2Cl2-petroleum ether (7:3), resulting in Ppm-1 (106.2 mg) and Ppm-2 (75.8 mg). Fractions MH-9 and MH-10 were gathered and subjected to preparative TLC, and the semipurified fraction was rechromatographed on a silica gel column eluted isocratically with petroleum ether-ethyl acetone (40:10), resulting in Ppc-3 (59.7 mg). Fractions CEA-7, CEA-8, and CEA-9 showed typical spots of flavonoids with similar Rf values, so they were gathered and rechromatographed on a Sephadex LH-20 column eluted isocratically with MeOH-H2O (1:1). From this column, 175 fractions were collected and lyophilised after MeOH evaporation, evaluated by TLC, and grouped into 17 fractions (CEA-A to CEA-Q). The CEA-N fraction was subjected to preparative TLC [EtOAc-formic acid-acetic acid-H2O (100:11:11:26)], resulting in Ppc-4 (18.7 mg). CEA-K, CEA-L, and CEA-M were gathered and subjected to preparative TLC, and the semipurified fraction was rechromatographed on a Sephadex LH-20 column eluted with a gradient of acetone-H2O (1:1, 6:4, and 7:3). From this column, 93 1 mL fractions were collected and subjected to solvent evaporation, lyophilisation, and monitoring by TLC, which led to Ppc-5 (73.2 mg).

DFC. The DFC (1.0 g) was fractionated on a silica gel column (1:40) and eluted with a gradient of hexane-EtOAc (5–100%), EtOAc-MeOH (1:1), and MeOH (100%). Approximately 180 fractions of 1–10 mL were collected. The fractions were evaluated by TLC [isobutanol-acetic acid-water (14:1:5)] and exposed with UV light and 1% FeCl3 in 0.5 M hydrochloric acid. Fractions with the same chromatographic profiles were pooled, resulting in 11 new fractions (AC-1 to AC-11). AC-9 was reactive to FeCl3, resulting in Ppc-6 (83.1 mg).

DFM. The DFM (1.2 g) was fractionated on a silica gel column (1:40) and eluted with 100% hexane-EtOAc (50–100%), EtOAc-MeOH (1:1), and MeOH (100%). One hundred 10 mL fractions were collected and evaluated by TLC [hexane-EtOAc (10–30%)], observed under UV light, and detected with sulphuric vanillin reagent, resulting in 14 new fractions (MH-1 to MH-14). Fractions MH-4 and MH-5 were rechromatographed on a silica gel column eluted isocratically with CH2Cl2-petroleum ether (7:3), resulting in Ppm-1 (106.2 mg) and Ppm-2 (75.8 mg). Fractions MH-9 and MH-10 were gathered and subjected to isocratic silica gel column chromatography [hexane-CH2Cl2-MeOH (10:10:1)], which resulted in Ppm-3 (36.9 mg).

DFA. The low output from DFM hindered the use of chromatography columns in isolating possible compounds. Thus, TLC of the DFM was carried out with some of the semipurified fractions for Rf comparison to verify whether
some of the previously isolated compounds could also be found in this fraction. Runs were performed with DF_M, Ppc-1, Ppm-1, and Ppm-2 in CH_2Cl_2-petroleum ether (7:3), DF_M and Ppc-2 in petroleum ether-acetone (40:10), and DF_M and Ppm-3 in hexane-CH_2Cl_2-MeOH (10:10:1).

EAF_M. The EAF_M (1.2 g) was subjected to chromatography on a packed column with Sephadex LH-20 [MeOH (100%)]. Approximately 159 1-mL fractions were collected and analysed by TLC [EtOAc-formic acid-acetic acid-H_2O (100:11:11:26)]. Based on observation in UV light and detection with NP reagent, the fractions were pooled into four new fractions (MEA-1 through MEA-4). Fractions MEA-1 and MEA-2 showed typical spots of flavonoids with yellow-orange and greenish-yellow fluorescence after spraying with NP and observation under 365 nm UV light, with similar R_f values; thus, they were pooled and rechromatographed on a Sephadex LH-20 column eluted with a sequence of acetone-H_2O mixtures (1:1, 6:4, and 7:3). From this column, 99 fractions were collected and, after evaluation by TLC, resulted in Ppm-4 (273.4 mg).

AF_M. The AF_M (1.2 g) was subjected to chromatography in a packed column with Sephadex LH-20 [MeOH (100%)]. Approximately 117 1-mL fractions were collected. The fractions were monitored by TLC [isobutanol-acid acetic-water (14:1:5)] with use of the UV light and 1% FeCl_3 in 0.5 M HCl solutions were monitored by TLC [isobutanol-acid acetic-water (14:1:5)]. Approximately 117 1-mL fractions were collected. The extracts, fractions, essential oils, citral (Sigma), and semipurified substances were subjected to the microdilution test in broth for determining the minimum inhibitory concentration (MIC) in sterile 96-well microplates inoculated with bacteria, respectively, and itraconazole (Sigma) at an initial concentration of 16 μg mL\(^{-1}\) was used as a control for fungi. The microplates inoculated with bacteria were incubated at 35°C ± 2°C for 16–20 h and for 24 hours at this temperature for Staphylococcus. One hour before the end of the incubation period, each well received 20 μL of 0.5% triphenyl tetrazolium chloride (TTC), and the microplates were reincubated for approximately 30 min. The appearance of reddish colour was considered as proof of bacterial growth. The microplates inoculated with fungi were incubated at room temperature for 48 h (Candida spp.) and 72 h (Cryptococcus spp.). Fungal growth was checked visually, and the MIC was defined as the lowest concentration (μg mL\(^{-1}\)) of the sample fully capable of inhibiting bacterial and fungal growth.

2.5. Antimicrobial Activity. The extracts, fractions, essential oils, citral (Sigma), and semipurified substances were subjected to the microdilution test in broth for determining the minimum inhibitory concentration (MIC) in sterile 96-well microplates with “U”-shaped wells, as recommended by the Clinical and Laboratory Standards Institute (CLSI) [36, 37]. The experiments were performed in duplicate and repeated twice, independently, except for the semipurified substances. American Type Culture Collection (ATCC) standard strains and clinical isolates were used (Table 1); these are kept in the Laboratories for Bacteriology and Mycology at the Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, GO, Brazil.

Prior to testing, to reactivation the microbial cultures, the bacteria were transferred to Casoy broth and incubated at 37°C for 24 h, then transferred to inclined Casoy agar, and incubated at 37°C for an additional 24 h. The fungi were transferred to Sabouraud dextrose agar and incubated at room temperature for 24 to 48 h (Candida spp.) or 48 to 72 h (Cryptococcus spp.).

The culture medium used in the antibacterial activity test was 2x Müller Hinton broth (MH) and that used in the antifungal activity test was RPMI 1640. The samples were solubilised in 10% dimethyl sulfoxide (DMSO) and diluted in MH broth (antibacterial activity) to obtain a concentration of 2000 μg mL\(^{-1}\) or in RPMI (antifungal activity) to obtain a concentration of 1000 μg mL\(^{-1}\). The semipurified substances were used at concentrations according to the available amount of the substance. For the preparation of samples with essential oils, 0.02% Tween 80 was added. Vancomycin (Sigma; 32 μg mL\(^{-1}\)) and gentamicin (Sigma; 128 μg mL\(^{-1}\)) were used as controls for Gram-positive and Gram-negative bacteria, respectively, and itraconazole (Sigma) at an initial concentration of 16 μg mL\(^{-1}\) was used as a control for fungi. The microplates inoculated with bacteria were incubated at 35°C ± 2°C for 16–20 h and for 24 hours at this temperature for Staphylococcus. One hour before the end of the incubation period, each well received 20 μL of 0.5% triphenyl tetrazolium chloride (TTC), and the microplates were reincubated for approximately 30 min. The appearance of reddish colour was considered as proof of bacterial growth. The microplates inoculated with fungi were incubated at room temperature for 48 h (Candida spp.) and 72 h (Cryptococcus spp.). Fungal growth was checked visually, and the MIC was defined as the lowest concentration (μg mL\(^{-1}\)) of the sample fully capable of inhibiting bacterial and fungal growth.

2.6. Antinociceptive and Anti-Inflammatory Activity. These experiments were approved by the Animal Ethics Committee of the Federal University of Goiás, Protocol number

| Table 1: Microorganisms used in determining the minimum inhibitory concentration (MIC). |
|---------------------------------|----------------|----------------|
| Microorganisms                  | ATCC           | Clinical isolated |
| Gram (+) bacteria               |                |                  |
| Staphylococcus aureus           | 6538           |                  |
| Staphylococcus aureus           | 25923          |                  |
| Staphylococcus epidermidis      | 12229          |                  |
| Micrococcus luteus              | 9341           |                  |
| Micrococcus roseus              | 1740           |                  |
| Sporulated Gram (+)             |                |                  |
| Bacillus cereus                 | 14579          |                  |
| Bacillus atrophaeus             | 6633           |                  |
| Gram (−) bacteria               |                |                  |
| Escherichia coli                | 8739           |                  |
| Escherichia coli                | 11229          |                  |
| Enterobacter aerogenes          | 13048          |                  |
| Enterobacter cloacae            | HMA/FT502      |                  |
| Serratia marcescens             | 14756          |                  |
| Salmonella spp.                 | 19430          |                  |
| Pseudomonas aeruginosa          | 27483          |                  |
| Pseudomonas aeruginosa          | SPM1           |                  |
| Fungi                           |                |                  |
| Candida albicans                | 02             |                  |
| Candida parapsilosis            | 11-A           |                  |
| Candida parapsilosis            | 22019          |                  |
| Cryptococcus neoformans         | 28957          |                  |
| Cryptococcus neoformans         | L2             |                  |
| Cryptococcus gattii             | L1             |                  |

The experiments were performed in duplicate and repeated twice, independently, except for the semipurified substances.
146/2008. Male, young adult mice, weighing between 25 and 30 g, were transferred to the experimental room two days before the tests, kept in a light/dark cycle of 12 h at 22 ± 2°C in a noise-free facility, with water and food ad libitum. The food was taken away 12 h before the test, keeping the water available ad libitum at all times.

2.6.1. Evaluation of Antinociceptive Activity by Testing Abdominal Contortions Induced by Acetic Acid. The evaluation of abdominal contortions induced by acetic acid was carried out according to Koster et al. [38] and Lapa et al. [39]. Test groups consisting of 10 mice per dose of extract, fraction, standard drug or vehicle were used. The mice from the different experimental groups received intraperitoneal injections of 1.2% acetic acid solution (v/v, 10 mL kg⁻¹) 1 h after oral intake (gavage) of the extracts or fractions (in different doses) or indomethacin (standard drug) or vehicle (control). They were then placed under glass funnels and the contortions, contractions, and rotation of the abdomen followed by the extension of one or both back paws were counted over the subsequent 30 min.

The extracts, fractions, and essential oils were tested in the following doses: EE_M and EE_C—2000, 1000, and 500 mg kg⁻¹; FH_C—400 mg kg⁻¹; DF_C—240 mg kg⁻¹; EAFC—1160 mg kg⁻¹; AF_C—480 mg kg⁻¹; EC—60, 200, 600 mg kg⁻¹. Indomethacin was used at a dose of 10 mg kg⁻¹. The control groups received the vehicle used in the solubilization of each extract, fraction, or essential oil (10 mL kg⁻¹), so there were three control groups that received either 10% propylene glycol in CMC gel, 10% DMSO in water, or only water.

2.6.2. Anti-Inflammatory Activity by Testing Ear Oedema Induced by Croton Oil. Tests of ear oedema induced by croton oil were carried out according to Zanini et al. [40]. Groups of mice (n = 10) were treated (v.o.) with dexamethasone (2.0 mg kg⁻¹), vehicle (10% propylene glycol in CMC gel, 10 mL kg⁻¹), or EE_C (2000, 1000, and 500 mg kg⁻¹). One hour after treatment, each animal received 20 μL of a freshly prepared solution of croton oil (2.5% v/v) in acetone on the surface of the right ear. The left ear received the same volume of acetone. After 4 h, the animals were sacrificed and identical segments were taken from both ears. The formations and intensities of the oedema were expressed as the mean of differences in weight between the segments of the animals’ ears: the smaller the weight difference, the greater the potential for inhibition.

2.7. Statistical Analysis. The antinociceptive effects of the different extracts, fractions, and essential oils were expressed as the means (±SEM) and are shown as percentages relative to the control group (vehicle). The significant differences between treated and control groups (vehicle) were assessed by an ANOVA and a multiple comparison by Tukey-Kramer. P values <0.05 were considered statistically significant. The data analysis used the application GraphPad Prism 3.0.

3. Results and Discussion

3.1. Phytochemical Analysis. The phytochemical analysis of the fractions obtained from the crude ethanol extracts of P. pseudocaryophyllus leaves of the citral (E)-methyl isoeugenol chemotype enabled the identification of pentacyclic triterpenes (lupeol, α-amyrin, β-amyrin, oleanolic acid, betulinic acid, and ursolic acid), flavonoids (quercetin 3-O-α-L-rhamnopyranoside, quercetin 3-O-β-glucopyranoside, kaempferol 3-O-α-L-rhamnopyranoside, quercetin 3-O-α-arabinofuranoside, quercetin 3-O-α-arabinopyranoside, quercetin 3-O-β-arabino-pyranoside, and catechin), gallic acid, and ellagic acid (Table 2). These constituents, except ursolic acid, were found for the first time in this species. They were identified based on spectra of 1D and 2D [41] and 13C NMR (HSQC and HMQC) and with comparison with data in the literature (copies of the original spectra can be obtained from the corresponding author). The flavonoid quercitin was also identified based on the mass spectrum and analysis by HPLC in comparison with an authentic sample.

The results from the qualitative and quantitative analysis of volatile oils of P. pseudocaryophyllus, with the volatile constituents listed in order of elution, are found in Table 3. A total of 31 compounds were identified, accounting for 94–100% of the volatile components. There was a predominance of phenylpropanoid derivatives (97.5%) among the volatile components of the (E)-methyl isoeugenol chemotype, and almost all of the oil consisted of (E)-methyl isoeugenol (93.9%). The essential oil of the citral chemotype consisted mainly of oxygenated mono- and sesquiterpenes (69.6% and 13.7%, resp.), and the monoterpenic aldehydes neral and geranial, which are referred to as citral when their isomers are mixed, were the major components (27.59% and 36.49%, resp.).

3.2. Antimicrobial Activity. The MIC values of extracts, fractions, and essential oils, as well as citral (Sigma), are described in Table 4. Table 5 shows the MIC of the semipurified substances and of vancomycin, gentamicin, and itraconazole, which were used as controls. The results were discussed taking into account the classification by Holetz et al. [41], which has also been adopted by other authors [42–44], where a MIC lower than 100 μg mL⁻¹ represents good antimicrobial activity; a MIC from 100 to 500 μg mL⁻¹ represents moderate antimicrobial activity; a MIC from 500 to 1000 μg mL⁻¹ represents weak activity; a MIC above 1000 μg mL⁻¹ suggests that the substance is inactive. EE_C showed the lower MIC compared to EE_M for Gram-positive bacteria, and both were inactive for Gram-negative bacteria (Table 4). EE_C was the most active for fungal strains, with a MIC between 7.8 and 62.5 μg mL⁻¹. The lowest MIC of this extract was found for the strains of Cryptococcus (MIC = 7.8–15.6 μg mL⁻¹), while EE_M showed good-to-moderate activity (MIC = 15.6–125 μg mL⁻¹) (Table 4) for the yeasts studied.

Regarding the fractions and semipurified substances, the dichloromethanolic fractions of both chemotypes (DF_M and DF_C) were the most active for Gram-positive bacteria (MIC = 250–500 μg mL⁻¹) and were less active for the fungal...
| Substances semipurified in citral chemotype (mg) | Substances semipurified in (E)-methyl isoeugenol chemotype (mg) | Compounds identified (approximated amount—mg) | References |
|-----------------------------------------------|---------------------------------------------------------------|-----------------------------------------------|------------|
| **Ppc-1** (133.5)                             |                                                               | Lupeol (60.7), α-amyrin (20.2), and β-amyrin (52.6) | [14]       |
| **Ppc-2** (22.6)                              |                                                               | Oleanolic acid                                 | [14]       |
| **Ppc-3** (59.7)                              |                                                               | Quercetin                                      | [15, 16]   |
| **Ppc-4** (18.7)                              |                                                               | Quercetin 3-O-α-L-rhamnopyranoside—quercitrin  | [17]       |
| **Ppc-5** (73.2)                              |                                                               | Quercetin 3-O-α-L-rhamnopyranoside—quercitrin (46.2), quercetin | [17–19] |
| **Ppc-6** (83.1)                              |                                                               | 3-O-β-glycopyranoside—isoquercitrin (6.6), kaempferol 3-O-α-L-rhamnopyranoside—afzelin (6.6), and catechin (13.8) | [15, 19] |

| **Ppm-1** (106.2)                             | (E)-methyl isoeugenol                                         | Gallic acid, ellagic acid, and not identified derivatives | [15, 20] |
| **Ppm-2** (75.8)                              |                                                               | Lupeol (47.4), α-amyrin (9.5), and β-amyrin (18.9)    | [14]       |
| **Ppm-3** (36.9)                              |                                                               | Oleanolic acid (25.8), ursolic acid (7.4), and betulinic acid (3.7) | [14, 22] |
| **Ppm-4** (273.4)                             |                                                               | Quercetin 3-O-α-L-rhamnopyranoside—quercitrin (88.3), quercetin 3-O-β-glycopyranoside—isoquercitrin (8.8), kaempferol 3-O-α-L-rhamnopyranoside—afzelin (17.6), quercetin 3-O-α-arabinofuranoside—avicularin (8.8), quercetin 3-O-α-arabinopyranoside—guaijaverin (8.8), quercetin 3-O-β-arabinopyranoside (8.8), gallic acid (2.5), and catechin (105.8) | [15, 17–19, 23] |
| **Ppm-5** (203.6)                             |                                                               | Gallic acid and not identified derivatives          | [15]       |

* Acquired from 1H NMR data.
Table 3: Percentage chemical composition of essential oils from leaves of *P. pseudocaryophyllus* (E)-methyl isoeugenol and citral chemotypes.

| Component                            | RI   | MC  | CC  |
|--------------------------------------|------|-----|-----|
| (1) (2E)-Hexenol                     | 846  | 0.24|     |
| (2) UC                               | 918  | 0.05|     |
| (3) α-Tunene                          | 929  | 0.46|     |
| (4) Allyl isovalerate                 | 938  | 0.49|     |
| (5) UC                               | 947  | 0.47|     |
| (6) β-Pinene                         | 973  | 1.41|     |
| (7) 6-Methyl-5-hepten-2-one           | 980  | 0.71|     |
| (8) Dehydro-1,8-cineole              | 987  | 0.23|     |
| (9) Linalool                          | 1096 | 1.02|     |
| (10) exo-Isocitral                   | 1139 | 0.27|     |
| (11) (Z)-Isocitral                   | 1159 | 0.36|     |
| (12) UC                              | 1175 | 0.28|     |
| (13) (E)-Isocitral                   | 1177 | 0.51|     |
| (14) UC                              | 1203 | 0.19|     |
| (15) Nerol                           | 1224 | 1.05|     |
| (16) Neral                           | 1237 | 27.59|    |
| (17) Geraniol                        | 1250 | 2.13|     |
| (18) Geranial                        | 1266 | 36.49|    |
| (19) UC                              | 1332 | 1.61|     |
| (20) UC                              | 1358 | 0.49|     |
| (21) UC                              | 1368 | 2.1 |     |
| (22) α-Copaene                       | 1373 | 0.4 | 1.03|
| (23) β-Bourbonene                    | 1381 | 0.7 |     |
| (24) β-Elemene                       | 1389 | 0.31|     |
| (26) (E)-Caryophyllene               | 1416 | 1.7 | 1.55|
| (27) α-Copaene                       | 1425 | 0.27|     |
| (28) Aromadendrene                   | 1435 | 0.55|     |
| (29) α-Humulene                      | 1450 | 0.27|     |
| (30) (Z)-Methyl isoeugenol           | 1451 | 2.0 |     |
| (31) Allo-Aromadendrene              | 1457 | 0.57|     |
| (32) γ-Muurolene                     | 1472 | 0.36|     |
| (33) (E)-Methyl isoeugenol           | 1494 | 93.9|     |
| (34) γ-Cadinene                      | 1510 | 1.15|     |
| (35) δ-Cadinene                      | 1520 | 0.4 | 0.32|
| (36) UC                              | 1548 | 0.77|     |
| (37) Spathulenol                     | 1573 | 3.75|     |
| (38) Caryophyllene oxide             | 1578 | 8.88|     |
| (39) Humulene epoxide II             | 1604 | 1.07|     |

Monoterpene hydrocarbons 1.87
Oxygenated monoterpenes 69.65
Sesquiterpene hydrocarbons 2.5 7.08
Oxygenated sesquiterpenes 13.7
Other 1.44
Phenols 97.5

Yield (%, v/p) 0.8 0.8

RI: calculated retention index. MC: *P. pseudocaryophyllus* (E)-methyl isoeugenol chemotype. CC: *P. pseudocaryophyllus* citral chemotype. UC: unknown component.

The greatest inhibitory effect on Gram-positive bacteria of the compounds studied (MIC = 31.2 μg mL⁻¹ for *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12229, and *B. cereus* ATCC 14579; MIC = 62.5 μg mL⁻¹ for *S. aureus* ATCC 25923, *M. roseus* ATCC 1740, and *B. atrophaeus* ATCC 6633; MIC = 125 μg mL⁻¹ for *M. luteus* ATCC 9341) (Table 5). The phytochemical analysis showed that Ppc-2 is oleic acid, a pentacyclic triterpene with antibacterial activity [45–47], which was especially effective against Gram-positive bacteria. The TLC analysis of the DF₉ showed the presence of oleic acid, betulinic, and ursolic acids, which probably contributed to the observed antibacterial activity of this fraction [45–47]. **Ppc-4**, isolated from EAF₉ and composed of quercitrin, afzelin, isouercitrin, avicularin, guaijaverin, catechin, and gallic acid, showed activity against *C. neoformans* L2 and L1 (CIM = 62.5 μg mL⁻¹) but was not active against *Candida* (Table 5). Studies have shown that catechin and quercitrin, the main components of **Ppc-4**, inhibit the growth of some pathogenic fungi [48–50]. **Ppc-5**, obtained from the AF₉, showed an activity profile similar to this fraction, except for *C. parapsilosis* 11A (Table 5). The phytochemical analysis carried out on **Ppc-5** verified the presence of gallic acid and its derivatives, which are known for their antimicrobial activities [51–53], as well as the presence of sugars. The **Ppc-4** isolated from EAF₉, which was composed of quercitrin, showed moderate inhibition of *Candida* strains (MIC = 250 μg mL⁻¹) and good-to-moderate inhibition of the strains of *Cryptococcus* (MIC = 62.5 to 250 μg mL⁻¹) (Table 5), partly contributing to the antifungal activity of this fraction. **Ppc-5**, which was also isolated from fraction EAF₉, consisted of a mixture of flavonoids of which the major component is quercitrin and did not show activity under the conditions tested. **Ppc-6** contributed in part to the antifungal activity of AF₉, with MICs ranging from 62.5 to 125 μg mL⁻¹ for *Candida* strains (Table 5). The phytochemical analysis of **Ppc-6** showed ellagic acid to be the major component. Similar results for *Candida* spp., due to ellagic acid, were reported by Silva et al. [54]. These findings are promising in the search for new options against infections caused by *Candida* spp. and *Cryptococcus* spp., particularly with the continuous increase of opportunistic fungal resistance to available treatments, the emergence of rare species of *Candida* [55], and the increasing number of infections by *Cryptococcus neoformans* and *C. gattii* [56].

The essential oil of the citral chemotype (EO₉) showed good activity (MIC = 62.5 μg mL⁻¹) against *B. cereus* ATCC 14579 and moderate activity (MIC = 125–250 mL g μg mL⁻¹) against the remaining Gram-positive bacteria; in some cases, this essential oil showed better inhibition than citral (MIC = 125–250 μg mL⁻¹) (Table 4). Citral, the major component of the EO₉, is a monoterpene aldehyde mixture of the isomers neral and geranial. Aldehydes, such as formaldehyde and glutaraldehyde, are known to have strong antimicrobial activity, and several researchers have demonstrated the antimicrobial effect of citral [57–62]. It is suggested that the aldehyde group conjugated to the carbon-carbon double bond, which is present in neral and geranial, provides a highly electronegative chemical structure that may explain
| Microorganisms | EEₘ | HFₘ | DFₘ | EAFₘ | AFₘ | EOₘ | EEₖ | HFₖ | DFₖ | EAFₖ | AFₖ | EOₖ | Citral (Sigma) |
|----------------|-----|-----|-----|------|-----|-----|-----|-----|-----|------|-----|-----|----------------|
| Gram (+) bacteria |     |     |     |      |     |     |     |     |     |      |     |     |                |
| **S. aureus** ATCC 6538 | 1000 | 1000 | 500 | 1000 | >1000 | 500 | >1000 | 250 | 500 | 1000 | 250 | 125 |                |
| **S. aureus** ATCC 25923 | 1000 | 1000 | 500 | 1000 | >1000 | 500 | >1000 | 250 | 500 | 1000 | 125 | 125 |                |
| **S. epidermidis** ATCC 12229 | 1000 | 1000 | 250 | 1000 | >1000 | 1000 | 250 | 500 | 1000 | 125 | 250 |             |
| **M. luteus** ATCC 9341 | 1000 | 1000 | 250 | 1000 | >1000 | 500 | >1000 | 250 | 500 | 1000 | 125 | 125 |                |
| **M. roseus** ATCC 1740 | 1000 | 1000 | 500 | 500 | >1000 | 500 | >1000 | 250 | 500 | 1000 | 125 | 125 |                |
| **B. cereus** ATCC 14579 | 1000 | 1000 | 250 | 1000 | >1000 | 500 | >1000 | 250 | 500 | 1000 | 62.5 | 125 |                |
| **B. atrophaeus** ATCC 6633 | 1000 | 1000 | 500 | 1000 | >1000 | 500 | >1000 | 250 | 500 | 1000 | 125 | 125 |                |
| **Gram (−) bacteria** |     |     |     |      |     |     |     |     |     |      |     |     |                |
| **E. coli** ATCC 8739 | >1000 | >1000 | >1000 | 1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |                |
| **E. coli** ATCC 11229 | >1000 | >1000 | >1000 | 1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |                |
| **E. aerogenes** ATCC 13048 | >1000 | >1000 | >1000 | 1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |                |
| **E. cloacae** HMA/FTA502 | >1000 | >1000 | >1000 | 1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |                |
| **S. marcescens** ATCC 14756 | >1000 | >1000 | >1000 | 1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |                |
| **Salmonella spp.** ATCC 19430 | 1000 | 1000 | 1000 | 1000 | >1000 | 1000 | 1000 | 1000 | 1000 | >1000 | 1000 | 500 |                |
| **P. aeruginosa** ATCC 27483 | 1000 | 1000 | 1000 | 1000 | >1000 | 1000 | 1000 | 1000 | 1000 | >1000 | 1000 | 1000 |                |
| **P. aeruginosa** SPM1 | 1000 | 1000 | 1000 | 1000 | 500 | >1000 | 1000 | 1000 | 1000 | >1000 | 1000 | 1000 |                |
| **Fungi** |     |     |     |      |     |     |     |     |     |      |     |     |                |
| **C. albicans** 02 | 125 | 62.5 | >500 | 31.2 | 62.5 | >500 | 62.5 | 500 | 250 | 31.2 | 62.5 | 500 | 125 |                |
| **C. parapsilosis** 11A | 62.5 | 62.5 | 500 | 15.6 | 31.2 | >500 | 31.2 | 250 | 250 | 31.2 | 31.2 | 500 | 250 |                |
| **C. parapsilosis** ATCC 22019 | 62.5 | 62.5 | 500 | 31.2 | 62.5 | >500 | 31.2 | 250 | 250 | 31.2 | 31.2 | 500 | 125 |                |
| **C. neoformans** var. neoformans ATCC 28957 | 15.6 | 15.6 | >500 | >500 | 15.6 | 125 | 15.6 | 250 | 500 | 7.8 | 15.6 | 15.6 | 7.8 |                |
| **C. neoformans** var. neoformans L1 | 125 | 31.2 | 500 | >500 | 7.8 | 250 | 7.8 | 62.5 | 500 | 15.6 | >500 | 62.5 | 15.6 |                |
| **C. neoformans** var. gatti L1 | 125 | 31.2 | >500 | >500 | 31.2 | 250 | 15.6 | 500 | 500 | 15.6 | >500 | 62.5 | 31.2 |                |

EEₘ: ethanol extract of the (E)-methyl isoeugenol chemotype. HFₘ: hexane fraction of the (E)-methyl isoeugenol chemotype. DFₘ: dichloromethane fraction of the (E)-methyl isoeugenol chemotype. EAFₘ: ethyl acetate fraction of the (E)-methyl isoeugenol chemotype. AFₘ: aqueous fraction of the (E)-methyl isoeugenol chemotype. EOₘ: essential oil of the (E)-methyl isoeugenol chemotype. EEₖ: ethanol extract of the citral chemotype. HFₖ: hexane fraction of the citral chemotype. DFₖ: dichloromethane fraction of the citral chemotype. EAFₖ: ethyl acetate fraction of the citral chemotype. AFₖ: aqueous fraction of the citral chemotype. EOₖ: essential oil of the citral chemotype.
Table 5: Minimum inhibitory concentration (μg mL⁻¹) of semipurified substances of *P. pseudocaryophyllus*.

| Microorganisms         | Ppc-2 | Ppc-4 | Ppc-5 | Ppc-6 | Vanc. | Gent. | Ppm-1 | Ppm-3 | Ppm-4 | Ppm-5 | Itrac. |
|------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| **Gram (+) bacteria**  |       |       |       |       |       |       |       |       |       |       |        |
| *S. aureus* ATCC 6538  | 31.2  | >500  | 1000  | NC    | 2     | NC    | NC    | NC    | NC    | NC    | NC     |
| *S. aureus* ATCC 25923 | 62.5  | >500  | >1000 | NC    | 1     | NC    | NC    | NC    | NC    | NC    | NC     |
| *S. epidermidis* ATCC 12229 | 31.2 | 500   | >1000 | NC    | 1     | NC    | NC    | NC    | NC    | NC    | NC     |
| *M. luteus* ATCC 9341  | 125   | 500   | >1000 | NC    | 0.25  | NC    | NC    | NC    | NC    | NC    | NC     |
| *M. roseus* ATCC 1740  | 62.5  | >500  | 1000  | NC    | 0.5   | NC    | NC    | NC    | NC    | NC    | NC     |
| *B. cereus* ATCC 14579 | 31.2  | 500   | >1000 | NC    | 2     | NC    | NC    | NC    | NC    | NC    | NC     |
| *B. atrophaeus* ATCC 6633 | 62.5 | >500  | 1000  | NC    | 0.25  | NC    | NC    | NC    | NC    | NC    | NC     |
| **Gram (-) bacteria** |       |       |       |       |       |       |       |       |       |       |        |
| *E. coli* ATCC 8739    | >1000 | >500  | >1000 | NC    | NC    | 8     | NC    | NC    | NC    | NC    | NC     |
| *E. coli* ATCC 11229   | >1000 | >500  | >1000 | NC    | NC    | 2     | NC    | NC    | NC    | NC    | NC     |
| *E. aerogenes* ATCC 13048 | >1000 | >500  | >1000 | NC    | NC    | 0.125 | NC    | NC    | NC    | NC    | NC     |
| *E. cloacae* HMA/FTA502 | >1000 | >500  | >1000 | NC    | NC    | 4     | NC    | NC    | NC    | NC    | NC     |
| *S. marcescens* ATCC 14756 | >1000 | >500  | >1000 | NC    | NC    | 4     | NC    | NC    | NC    | NC    | NC     |
| *Salmonella* spp. ATCC 19430 | >1000 | >500  | >1000 | NC    | NC    | 2     | NC    | NC    | NC    | NC    | NC     |
| *P. aeruginosa* ATCC 9027 | >1000 | >500  | >1000 | NC    | NC    | 4     | NC    | NC    | NC    | NC    | NC     |
| *P. aeruginosa* SPM1   | >1000 | >500  | >1000 | NC    | NC    | 4     | NC    | NC    | NC    | NC    | NC     |
| **Fungi**              |       |       |       |       |       |       |       |       |       |       |        |
| *C. albicans* 02       | >600  | 250   | >500  | 62.5  | NC    | NC    | 500   | >500  | >500  | >500  | 31.2   | 1      |
| *C. parapsilosis* 11A  | >600  | 250   | >500  | 125   | NC    | NC    | 500   | >500  | >500  | >1000 | 1      |        |
| *C. parapsilosis* ATCC 22019 | >600 | 250   | >500  | 125   | NC    | NC    | 500   | >500  | >500  | >500  | 31.2   | 1      |
| *C. neoformans* var. neoformans ATCC 28957 | >600 | 62.5 | >500 | >500 | NC | NC | 250 | >500 | 500 | 15.6 | 2 |
| *C. neoformans* var. neoformans L2 | >600 | 62.5 | >500 | 500 | NC | NC | 250 | 500 | 62.5 | 3.9 | 2 |
| *C. neoformans* var. gatti L1 | >600 | 250 | >500 | 500 | NC | NC | 250 | 500 | 62.5 | 3.9 | 2 |

Ppc-2: oleanolic acid. Ppc-4: quercitrin. Ppc-5: quercitrin, isoquercitrin, afzelin, and catechin. Ppc-6: ellagic acid, gallic acid, and sugars. Ppm-1: (E)-methyl isoeugenol. Ppm-3: oleanolic acid, ursolic acid, and betulonic acid. Ppm-4: quercitrin, isoquercitrin, afzelin, catechin, avicularin, guaijaverin, and gallic acid. Ppm-5: gallic acid and sugars. Vanc.: Vancomycin (32 μg mL⁻¹). Gent.: gentamicin (128 μg mL⁻¹). Itrac.: itraconazole (16 μg mL⁻¹). NC: not carried out.
its activity [57]. The EO<sub>M</sub> was inactive against bacteria under the conditions tested. Its major component, the phenylpropanoid (E)-methyl isoeugenol, comprises almost all of the oil (93.9%). Structurally, (E)-methyl isoeugenol differs from eugenol because it has a methoxyl group at the C1 position of the ring instead of a hydroxyl group, and the double bond in its propenyl group is in a different position. The absence of the hydroxyl group in the phenolic structure may have contributed to the lack of activity of this compound. Griffin et al. [63] observed that the methylation of the hydroxyl group in eugenol, producing methyleugenol, resulted in a loss of activity for Gram-negative bacteria.

The EO<sub>C</sub> showed good activity against Cryptococcus (MIC = 15.6 μg mL<sup>−1</sup> to 62.5 μg mL<sup>−1</sup>), but the EO<sub>M</sub> showed moderate activity against strains of Cryptococcus (MIC = 125 μg mL<sup>−1</sup> to 250 μg mL<sup>−1</sup>) and showed no activity for species of Candida obtained from clinical isolates (MIC > 500 μg mL<sup>−1</sup>) (Table 4). The structure-activity relationship of the components of certain essential oils has been researched in phytopathogenic fungi, and there is little information on fungal pathogens in humans. For example, carbonyl α,β-unsaturated compounds, such as the monoterpene aldehydes neral and geranial, have strong antifungal activity [27, 64, 65], similar to their antibacterial activity. Little information regarding the antifungal mechanism of action of these aldehydes is available. The evaluation of the effects of citral on the membranes, organelles, and intracellular macromolecules of Aspergillus flavus spores has shown that citral damages the cell walls and membranes of spores, reducing their elasticity [66].

3.3. Antinociceptive and Anti-Inflammatory Screening. The EE<sub>M</sub> (v.o. 2000, 1000, and 500 mg kg<sup>−1</sup>) significantly reduced the number of abdominal contortions caused by the intraperitoneal injection of acetic acid in mice compared to animals that received only vehicle (control group) (Figure 1). There was no significant difference observed between the analgesic effects of the three doses used (data not shown).

The EE<sub>C</sub> (v.o. 2000 and 1000 mg kg<sup>−1</sup>) was able to inhibit significantly and in a dose-dependent manner, the abdominal contortions in mice induced by the intraperitoneal acetic acid compared with the control group and showed no significant difference compared to the group of animals treated with indomethacin (10 mg kg<sup>−1</sup>) (Figure 2).

Given the dose-dependent antinociceptive effect shown by EE<sub>C</sub> (Figure 2), this extract was selected to perform the screening of the antinociceptive activity of its fractions and essential oils, as well as to evaluate its anti-inflammatory activity by testing ear oedema induced by croton oil. HF<sub>C</sub> (400 mg kg<sup>−1</sup>), DF<sub>C</sub> (240 mg kg<sup>−1</sup>), and EA<sub>F</sub>C (1160 mg kg<sup>−1</sup>) significantly decreased the number of abdominal contortions induced by the intraperitoneal injection of acetic acid compared with the control group. However, there was no significant difference in the antinociceptive effect shown by the three fractions. The group of animals that received AF<sub>C</sub> (480 mg kg<sup>−1</sup>) showed a number of contortions similar to that of the group that received vehicle (Figure 3).

The EO<sub>C</sub> at doses of 60, 200, and 600 mg kg<sup>−1</sup> (v.o.) showed significant dose-dependent inhibitory effects on the abdominal contortions induced by intraperitoneal acetic acid in mice compared to the control group (Figure 4).

The ear oedema induction test showed that the EE<sub>C</sub> has antiedematogenic activity that is significantly greater than that of the control at all doses tested (Figure 5). Pretreatment with EE<sub>C</sub> (2000, 1000, and 500 mg kg<sup>−1</sup>) reduced the oedema from 14.3 ± 0.4 mg (control) to 10.9 ± 0.5, 11.3 ± 0.4, and 10.5 ± 0.5 mg, respectively.

The results obtained from this study showed that both EE<sub>M</sub> and EE<sub>C</sub> have antinociceptive activity. It was not possible to infer whether this action involves peripheral and/or central mechanisms, as the model of abdominal contortions induced by the intraperitoneal injection of acetic acid in mice is sensitive to analgesic substances that act centrally and/or peripherally and that show a wide range of mechanisms of action [39]. Similar results were observed in other species of the genus Pimenta [29, 30, 32].

The mixture of triterpenes lupeol and α- and β-amyrin isolated from HF<sub>C</sub> could be accountable for the observed activity. Several in vivo experiments have shown that, among the pharmacological activities attributed to these compounds, analgesic and anti-inflammatory effects are predominant [31, 67–72].

Oleanolic acid, which is widely distributed in the vegetable kingdom, has strong anti-inflammatory effects [73] that may have contributed to reducing the number of abdominal contortions induced by chemical stimulation in mice, as observed for the DF<sub>C</sub>.
Quercetin, quercitrin, and kaempferol have anti-inflammatory activities [74, 75], which may also have contributed for the analgesic activity of EAFc observed in this study.

Data from the literature report the antinociceptive activity of citral [76, 77], a major component of the EOc. Moreover, sedative, anxiolytic, and anticonvulsant effects were observed from the essential oils of Cymbopogon citratus in mice through the use of motor activity tests (“rotarod” and “open-field”), a hypnosedative activity test (sleep induced by barbiturate), an anxiolytic action test (“plus-maze” and “light-dark box”), and an anticonvulsant action test (seizures chemically induced by pentylenetetrazole) [78]. Therefore, the inhibition by the EOc of the contortions induced by chemical stimulation in mice in this study may be due to both peripheral and central mechanisms. However, the sedative and relaxing effects of citral, as well as the increase in sleep time induced by barbital, especially at high doses (200 mg kg\(^{-1}\)) [79], may have exerted a greater influence on reducing the number of contortions than the analgesic effects themselves.

4. Conclusion

Until now, no systematic phytochemical and biological study on citral and the (E)-methyl isoeugenol chemotypes of P. pseudocaryophyllus had been reported. Among the isolated substances, oleanolic acid obtained from the dichloromethane fraction of the citral chemotype showed the best profile of antibacterial activity against the Gram-positive microorganisms used in this research (MIC = 31.2 to 125 \(\mu g\) mL\(^{-1}\)), followed by the essential oil of the citral chemotype, which showed good activity (MIC = 62.5 \(\mu g\) mL\(^{-1}\)) against B. cereus ATCC 14579 and moderate activity (MIC = 125–250 \(\mu g\) mL\(^{-1}\)) against the other Gram-positive microbes.

The extracts, fractions, and essential oils from P. pseudocaryophyllus leaves showed several levels of antifungal activity against Candida spp. and Cryptococcus spp. The antifungal
activity shown by the ethyl acetate and aqueous fractions from both chemotypes, especially against *Candida albicans* (MIC = 31.2 to 62.5 μg mL⁻¹) and *C. parapsilosis* (MIC = 15.6 to 62.5 μg mL⁻¹), showed the potential of this species as a source of new antifungal alternatives.

In the models of abdominal contortions induced by acetic acid and ear oedema induced by croton oil in mice, the crude extract of the citral chemotype showed antinociceptive and anti-inflammatory effects, respectively. These effects may be related to the presence of the pentacyclic triterpenes lupeol, α-amyrin, and β-amyrin and the flavonoids quercetin, quercitrin, and afzelin.

**Conflicts of Interests**

The authors have no conflict of interests to declare.

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