



Supercritical CO₂ extraction of *Aloysia gratissima* leaves and evaluation of anti-inflammatory activity

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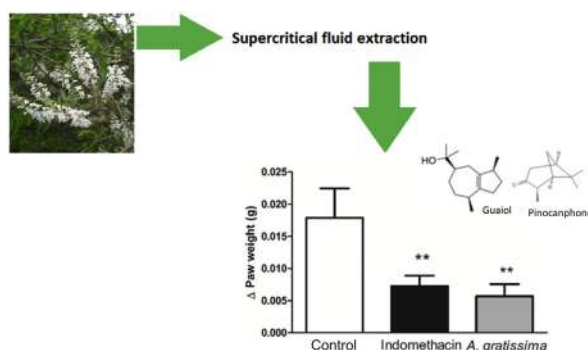
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HIGHLIGHTS

- The temperature and the pressure influenced in the extraction process.
- There was an increase of yield when high temperature and pressure were combined.
- The major compounds were guaiol and pinocampone.
- Spathulenol, β -elemene, caryophyllene and caryophyllene oxide contribute to the anti-inflammatory activity.

GRAPHICAL ABSTRACT



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ABSTRACT

This work aims to evaluate the effects of temperature and pressure on the extraction yield and chemical composition of the supercritical CO₂ (SCCO₂) extracts of *A. gratissima* leaves (EAG) and then to investigate *in vivo* anti-inflammatory activity of the extracts. The leaves of *A. gratissima* were extracted with supercritical CO₂ at 40, 50 and 60 °C and 15, 17.5 and 20 MPa for 120 min. The anti-inflammatory activity was performed with the EAG obtained at 60 °C and 20 MPa. The anti-inflammatory activity was investigated by the carrageenan-induced paw edema and myeloperoxidase (MPO) activity in paw tissue. The chemical analysis showed the presence of guaiol, pinocampone, caryophyllene oxide and spathulenol as the major chemical components of the EAG. These compounds may have contributed to the significant reduction of carrageenan-induced paw edema and MPO activity in the first 3 h after the injury induction.

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1. Introduction

The species belonging to the Verbanaceae family have hydrocarbon compounds, of which more than half are sesquiterpenes [1]. Among the plants belonging to this family, it can be highlighted *Aloysia gratissima*, a native aromatic plant from southern Brazil [2]. *Aloysia gratissima* is known in folk medicine, being studied for

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viricidal [3], nematocide [4], antioxidant [5], antidepressant, neuroprotective [2], antibacterial and antiedematogenic [6] activities. There are no reports of anti-inflammatory activity for this plant in the scientific literature.

Inflammation is a reaction of the body to infection or tissue damage. According to Trevisan et al., [7], inflammation is an important defense mechanism of the host, being characterized by redness, swelling, pain, heat and dysfunction of tissues and organs. Non-steroidal anti-inflammatory drugs (NSAIDs) are first-line drugs used to reduce the harmful events associated with inflammation. The disadvantages of the use of NSAIDs, such as the high risk of gastrointestinal mucosal damage, ulcers and erosions, kidney damage, increased blood pressure and heart diseases [8,9] can be minimized when they are replaced with efficient medicinal plants derived compounds [10]. The use of plants with anti-inflammatory properties received most interest today. In Brazil, medicinal plants play an important role in public health, and the Ministry of Health has released a list of medicinal plants with anti-inflammatory activity [11]. Regarding the therapeutic potential against inflammatory diseases, several species of aromatic plants rich in mono and diterpenes have shown important results, such as *Origanum vulgare* [12], and *thymus vulgaris* [13], among others [14,15]. Previous studies have shown that the *Aloysia gratissima* plant is rich in mono and diterpenes [16] which demonstrates possible anti-inflammatory properties.

In the study of medicinal plants, the elaboration of the extracts is one of the most important phases. Supercritical fluid extraction appears as an alternative method of extraction compared to conventional techniques such as steam distillation or Soxhlet extraction, because it avoids solute contamination with solvent residues and the degradation of thermo sensitive compounds [17]. Carbon dioxide is the most commonly used solvent for supercritical extraction due to its favorable properties [18]. Supercritical CO₂ produces high-quality extracts rich in terpenes from plant material with anti-inflammatory activity of properties [12]. In this sense, the objective of this work was to investigate the anti-inflammatory activity of *A. gratissima* leaves extract obtained by supercritical CO₂ (SCCO₂) under conditions that presented the highest extraction yield and the identification of the chemical components present in the extract.

2. Materials and methods

2.1. Materials

Carbon dioxide (99.9 % purity) was purchased from Air Liquide Brazil. Indomethacin and carrageenan were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant material

Leaves of *A. gratissima* were collected in December 2017 (summer), in the municipality of Erval Grande, RS, in the south of Brazil (27°23'14.3S, 52°33'49'W). This region presents a humid subtropical mesothermic climate. The plant was identified by a botanist (Dr. Adriano Dias de Oliveira). The plant voucher specimen is in the herbarium of Unochapecó (voucher UNO 3700). After collection, the leaves were manually separated and dried at room temperature (25 °C) to constant weight (about five days), then packed in plastic bags, identified and stored at 4 °C until extraction process.

2.3. Extraction of the *Aloysia gratissima* leaves by SCCO₂

The extraction setup and procedure are detailed elsewhere [19,20]. The *A. gratissima* leaves were extracted by SCCO₂ at temperatures of 40, 50 and 60 °C and pressure of 15, 17.5 and 20 MPa.

Table 1

Results of yields according to the temperatures, pressures and densities studied in the SCCO₂ extraction from EAG.

Run	Temperature (°C)	Pressure (MPa)	Density (kg/m ³) ^a	Yield (wt%)
1	40.0 (-1)	15.0 (-1)	748.26	1.13
2	60.0 (+1)	15.0 (+1)	561.86	1.22
3	40.0 (-1)	20.0 (-1)	830.31	1.72
4	60.0 (+1)	20.0 (+1)	694.68	4.42
5	50.0 (0)	17.5 (0)	717.99	1.82
6	50.0 (0)	17.5 (0)	717.99	1.18
7	50.0 (0)	17.5 (0)	717.99	1.49
8	35.8 (-1.41)	17.5 (0)	824.64	0.79
9	65.0 (+1.41)	17.5 (0)	599.82	2.10
10	50.0 (0)	13.9 (-1.41)	621.33	1.25
11	50.0 (0)	21.0 (+1.41)	779.04	2.82

The ∞ was calculated as a function of the number of independent variables ($n=2$), in equation: $\alpha = (2^n)^{1/4} = 1.41$.

^a Estimated from [47].

Approximately 11.03 ± 0.09 g of *A. gratissima* leaves were loaded into the extraction vessel. Carbon dioxide was loaded into the extraction vessel and was kept in contact with the sample matrix during 30 min for system stabilization. After stabilization, the extract from *A. gratissima* was collected by using a mass CO₂ flow rate of 2 g min⁻¹ over 2 h, following Scapinello et al. [20]. The amount of extract was determined gravimetrically (w/w) with respect to dry mass of leaves. The experimental design used in this study was the CCRD (2² central composite rotational design), with 3 central points and 4 axial points, as shown in Table 1.

The output variable was the yield of the extract obtained by SCCO₂ and the experimental results were analyzed according to the software Statistica® 7.0, as analysis of variance (ANOVA) to estimate the statistical parameters, with $p < 0.05$ adopted as significant.

2.4. Gas chromatography coupled to mass spectrometry – GC/MS analysis

Extracts of *A. gratissima* (EAG) were analyzed by Agilent GC/MS (7890B) and connected to a FID (Agilent Technologies, Palo Alto, CA, USA). The analysis conditions are described by Scapinello et al. [21], as follows: Agilent19091S capillary column, dimension: 30 m × 250 μm × 0.25 μm. He was carrier gas, adjusted to 1.0 mL·min⁻¹, oven temperature program was 40 °C at 4.0 min to 240 °C at a rate of 10 °C·min⁻¹ and up to 300 °C at a rate of 40 °C·min⁻¹ (maintained for 5 min), injector temperature was 280.0 °C, FID at 300 °C, sample injection volume 1 μL, split ratio 1:20. MS transferline temperature was set to 150 °C and the source of ions temperature was set at 230 °C. The ionization energy was set at 70 eV, and mass range m/z 40–400. For quantification purposes, area percent of each individual component in the chromatogram obtained by FID were used. Chemical compounds in the extracts were identified with the help of equipment library (Agilent P/N G1033A) with peak areas employed to calculate the content of each compound. To analyze, the extracts were solubilized in dichloromethane.

2.5. Animals

Male Swiss mice (25–35 g) were used in the *in vivo* experiments. The animals were bred at Unochapecó Animal Colony. The animals were held at 22 ± 2 °C, with a light/dark cycle of 12 h (lights on at 6 a.m. to 6 p.m.), fed standard laboratory feed and water *ad libitum*, following ethical principles of animal research (Ethics Committee of the Chapecó Regional Community University, number 004-18, Brazilian law No. 11794 - Brazil, 2016, 2008 and Council of the European Communities - Directive of 24 November 1986, 86/609/EEC).

The animals were fasted for a period of 2 h (no water restriction) prior to oral administration.

Mice were treated with volumes of $10 \text{ mL} \cdot \text{kg}^{-1}$, according to their weight, by the oral route (p.o.) (by gavage), intraperitoneal (i.p.) and intraplantar (i.pl.) depending on the specific protocol of each experiment. Mice were treated with the extract obtained under the experimental conditions that resulted in the highest yield (RUN 4), because no significant difference of the chemical composition between the extracts was observed. Solubilization of the extract and substances was performed in saline (NaCl 0.9 %) with the aid of 1 % Tween 80 (v/v) and ultrasound. The tested doses of the extract were chosen based on the study of Zeni et al. [5].

2.6. Carrageenan-induced paw edema

The animals ($n = 18/\text{group}$) were injected subcutaneously under the plantar surface of the right hind paw with $20 \mu\text{L}$ carrageenan ($300 \mu\text{g} / \text{paw}$, in 0.9 % NaCl), 1 h after EAG ($10 \text{ mg} \cdot \text{kg}^{-1}$), indomethacin ($10 \text{ mg} \cdot \text{kg}^{-1}$) or vehicle - control group - (0.9 % NaCl, 1 % Tween 80, $10 \text{ mL} \cdot \text{kg}^{-1}$) oral administration. The test was performed in accordance to Batista et al. [22]. The left hind paw was injected with saline. The formation of the hind paw edema was described as $\Delta \text{paw thickness} = \text{test paw thickness} - \text{basal paw thickness}$; also, the $\Delta \text{paw weight}$ (assessed using a laboratory analytical balance) was calculated as $\text{inflamed paw weight} - \text{non inflamed paw weight}$. Paw thickness was measured using a caliper [23].

2.7. Myeloperoxidase activity

The quantification of myeloperoxidase (MPO) activity was performed in the paws of animals submitted to the carrageenan-induced paw edema test. The inflamed hind paw tissue was collected 3 h after the induction of inflammation. Animals from the same group ($n = 18$ mice/group) were used to make a sample (the tissues from 4 to 5 animals were pooled in order to make a sample). The paw tissue was homogenized in a 50 mM Tris-HCl (pH 7.4) buffer and centrifuged at 4°C . The pellet was resuspended in 500 μL of potassium phosphate buffer (80 mM, pH 5.4) containing 0.5 % hexadecyltrimethylammonium bromide (HTAB). Subsequently, it was centrifuged at 11,000 g (20 min, 4°C). To the supernatant, it was added 7.5 % H_2O_2 and 18.4 nM 3,3',5,5'-tetramethylbenzidine (TMB). The MPO levels were determined at 620 nm and expressed as units of milli optic density (O.D.) / mg of protein as described by Bradley et al. [24].

2.8. Statistical analysis

The results are expressed as the mean $\pm$ S.E.M. of n animals per group. Data were analyzed by means of one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. Statistical analyses were performed using Graph Pad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA). Values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Extraction and chemical profile

The solvating power of the fluid is influenced by pressure and temperature thus favoring the extraction of the target dissolved in the supercritical fluid [25]. Table 1 shows the results of the extraction yield of *A. gratissima* leaves obtained by SCCO_2 for different temperature and pressure values according to the experimental design. The highest extraction yield was found at 60°C and 200 bar, 4.42 wt%. However, at 35.8°C and 17.5 MPa, the lowest extraction yield was obtained, 0.79 wt%. This behavior was also reported by

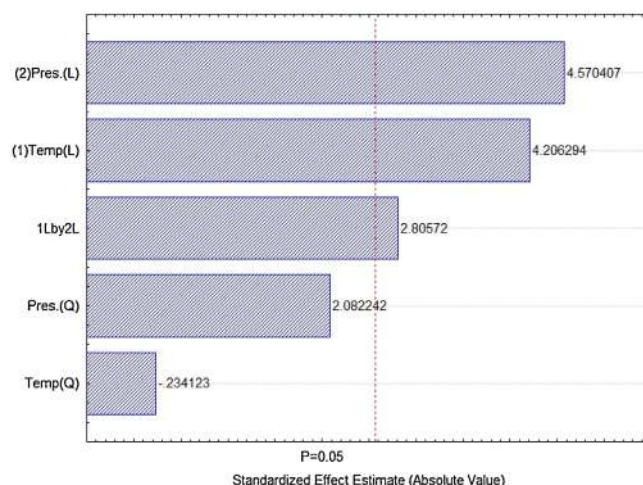


Fig. 1. Pareto chart for the experimental design showing the effect of temperature and pressure on the extraction yield.

Haloui and Meniai [26], but with pressure values different from those used in the present work. The pressure was also determined by other authors as being the more influential variable on the extraction yield [27–29].

From the Pareto chart (Fig. 1) it can be seen the influence of temperature and pressure on the extraction process. The interaction effect of such variables acts positively on the process yield whereas a negative effect is observed at the upper and lower levels, which is a result of the combination of solute vapor pressure and solvent density, hence affecting the migration/solubility of the extract into the solvent phase [30]. It is well-known from the vast literature available that, a rise in temperature at constant pressure, though decreases the solvent density, may lead to an increase of the extraction yield due to the enhancement of vapor pressure of the more volatile components being extracted.

Scapinello et al. [18] obtained extracts with supercritical CO_2 of the fruits of *M. azedarach* in a wide range of temperature and pressure, with the best condition in terms of extraction yield found at 60°C and 25 MPa, 5.40 wt%. Machmudah et al. [31], using seeds of *Rosa canina* L. (Rosaceae), observed increased productivity with increasing pressure at all temperatures, with higher gains observed for pressures above 300 bar and at higher temperatures.

The chemical composition of the extracts analyzed by GC / MS is shown in Table 2. The major compounds of EAG were guaiol, pinocanphone and (-)- trans-pinocarvilla acetate, besides the presence of other minor compounds like pinocarvil, (-)- trans-pinocarvil acetate, γ -elemene, caryophyllene oxide, (-)- spathulenol, myrtenol, isopinocampnone, humulene. Also, bunesol and caryophyllene present a similar content and intermediate amount compared to the whole compound characterization shown in Table 2.

Our findings support the results obtained by Trovati [32], in Clevenger extraction of leaves of *A. gratissima* where the presence of isopinocampnone (25.4 %), guaiol (12.7 %), and pinocampnone (7.2 %) was observed.

It is worth noticing that there were no significant variations of the extract's compositions in the temperature and pressure ranges investigated in this work, most probably due to changes in CO_2 density.

The chemical compound guaiol is a sesquiterpene alcohol, being found in medicinal plants, mainly cypress and guaiacum. It has an aroma similar to pine aroma, being characteristic of terpene compounds, besides having antibacterial and antitumor activities [33]. Additionally, guaiol was identified in a hexane extract from another

Table 2Identification by GC / MS and content by GC/FID of the chemical compounds of the SCCO₂ extracts of the leaves of *A. gratissima*.

Chemical compound	Area of each compound (%) by GC/FID										
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10	Run 11
Pinocarvil	3.43	3.11	2.60	3.61	4.17	4.37	3.75	4.55	4.44	4.05	4.04
Pinocanphone	9.36	10.47	8.70	8.89	12.15	12.68	7.34	12.20	12.49	12.23	11.08
Isopinocampnone	3.06	3.30	2.73	2.94	3.82	3.97	2.39	4.32	3.80	3.80	3.54
Myrtenol	3.31	3.13	2.57	2.85	3.95	3.64	4.03	4.25	4.22	3.49	3.89
(-)- trans-pinocarvila acetate	9.57	9.84	9.01	10.47	10.51	9.84	9.21	12.07	10.77	11.25	10.21
Caryophyllene	7.64	6.95	7.01	8.10	7.37	7.76	6.57	8.37	6.83	8.22	7.40
γ - Elemene	1.73	1.33	2.0	2.02	1.80	2.16	1.28	1.22	1.94	1.35	1.69
Humelene	2.83	2.23	2.79	2.61	3.0	2.37	2.98	2.48	2.42	3.20	3.03
(-)- Spathulenol	4.71	4.63	5.22	4.40	6.60	6.68	8.16	6.0	6.27	6.65	6.09
Caryophyllene oxide	3.9	3.82	5.77	6.40	6.67	7.08	8.46	5.69	7.19	7.28	6.78
Guaiol	23.5	21.44	21.16	22.04	29.39	19.99	24.06	20.36	19.63	20.08	20.42
Bunesol	7.77	7.54	8.43	7.91	7.41	6.26	10.46	3.75	8.41	5.03	8.29
Total	80.81	77.79	77.99	82.24	96.84	86.8	88.69	85.26	88.41	86.63	86.46

sample of the species that grows in Brazil, along with spathulenol [34]. Other authors have identified similar compounds, as well as different proportions of guaiol in *A. gratissima* oils, such as 2.6 % [35], 11.5 % [36], 12.5 % [32], 6.7 % [37] and 6.27 % [1]. These differences can be attributed to the environmental conditions and the extraction method to obtain the extract.

The second compound quantified in relative high amount was pinocampnone, and due to the high content of pinocampnone and isopinocampnone, the EAG can be compared to the extract of *Hysosopus officinalis*, which is widely marketed in the perfumery area [38].

Duschatzky et al. (2004) reported in their study with *A. gratissima*, caryophyllene oxide (15.8 %) as the major compound obtained by the Clevenger extraction method. Caryophyllene oxide is linked to plant extracts, being of ecological importance to the plant, and a having great value for the perfume industry due to its characteristic aroma, mainly the isomers of caryophyllene and α -humulene [39]. In addition, caryophyllene oxide has anticarcinogenic activities [40].

The chemical identification of EAG also showed the presence of spatulenol, an oxygenated sesquiterpene, which has immunomodulatory and antibacterial activity [41]. The terpenes are well known for their pharmaceutical properties, such as antimicrobial, anti-inflammatory and antitumor properties [42]. Extracts derived from medicinal and / or aromatic plants are important because they possess many biological effects, mainly anti-inflammatory, antioxidant, antifungal and antibacterial.

3.2. Carrageenan test

The induction of inflammation by carrageenan is used for screening new drugs with anti-inflammatory potential [43]. Carrageenan is a sulphated polysaccharide, considered as a phlogistic inducer. It evokes an acute inflammatory process, which develops rapidly due to the action of several proinflammatory mediators. Carrageenan elicits hyperalgesia and edema, as well as exacerbated sensitivity to thermal and mechanical stimuli in the inflamed tissue [22,43].

Herein, the EAG (10 mg kg⁻¹) significantly reduced mice paw edema from the 1st hour up to the 3rd hour after carrageenan i.p. administration. After 3 h of inflammation induction, the EAG lost its effects, unlike the positive control, indomethacin. The indomethacin-treated group showed a significant reduction of paw edema during all observation times (Fig. 2).

According to Carvalho [44], the paw edema induced by carrageenan is characterized by three steps, which are related to the involved mediators. The first step (the first 90 min of the test) is related to the release of histamine and serotonin, which

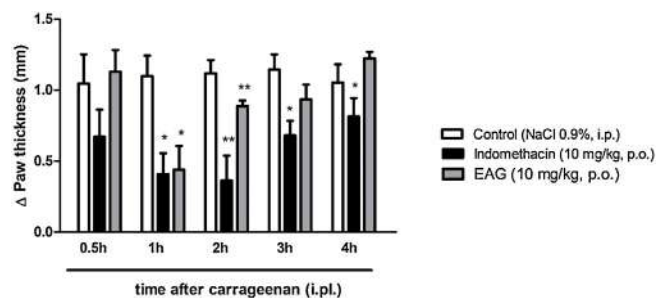


Fig. 2. Effect of *A. gratissima* leaves supercritical extract (EAG) on the carrageenan – induced paw edema. Mice were treated with vehicle (saline + 1 % tween 80, 10 ml / kg p.o., n = 18, control group), or EAG (10 mg kg⁻¹ p.o., n = 18) or indomethacin (10 mg kg⁻¹ p.o., n = 18) 1 h before intraplantar (i.p.) carrageenan administration (300 μ l / paw). One-way ANOVA followed by Student-Newman-Keuls: *p < 0.05 and **p < 0.01, compared to the vehicle treated (control) group. Results expressed as mean $\pm$ S.E.M.

are responsible for vasodilation and increase in vascular permeability, thus triggering the onset of the inflammatory process. In the second step (90–150 min), the activation of kinins begins, which induce increased blood vessel permeability and prostacyclin biosynthesis. The third step (after 150 min), involves the increase of prostaglandins synthesis in the inflamed tissue, at this stage there is also poly morphonuclear leukocytes infiltration.

Considering the EAG effects on the carrageenan-induced paw edema test, it may be inferred that its anti-inflammatory activity is not related to a decrease in prostaglandin synthesis, but to an inhibition in the release of histamine, serotonin and kinins. This hypothesis deserves further studies.

At the peak of EAG's effect (3 h after the injection) in the carrageenan-induced paw edema test, mice were euthanized and the weight of the inflamed (carrageenan) and non-inflamed (saline) paws was assessed. There was no significant difference between the Δ paw weight of indomethacin-treated and EAG-treated animals. However, the Δ paw weight from these groups were significantly (p < 0.01) lower than that of vehicle-treated animals (control group) (Fig. 3). These results corroborate the Δ paw thickness (mm) of the EAG and indomethacin-treated animals, which were significantly (p < 0.05) lower than the Δ paw thickness (mm) of the control group.

Vandresen et al. [6] demonstrated that the crude aqueous extract of leaves of *A. gratissima* caused a reduction of 23.6 % in carrageenan-induced ear edema in mice when compared to the animals treated with vehicle. Even though the compositions of water and supercritical CO₂ extracts are different, our results with the EAG are somehow consonant to those obtained by Vandresen et al. [6].



Fig. 3. Effect of *A. gratissima* leaves supercritical extract (EAG) on the Δ paw weight (g) (= inflamed paw weight (g) – non inflamed paw weight (g)), after euthanasia of mice receiving carrageenan i.p. (300 μ g / paw) injection. The mice were treated with vehicle (saline + 1 % tween 80, p.o., 10 ml / kg n = 18, control group), or EAG (10 mg kg⁻¹, p.o., n = 18) or indomethacin (10 mg kg⁻¹ p.o., n = 18) 1 h before carrageenan injection. One-way ANOVA, post hoc Student-Newman-Keuls: **p < 0.01 compared to the vehicle-treated (control) group. Results expressed as mean $\pm$ S.E.M.

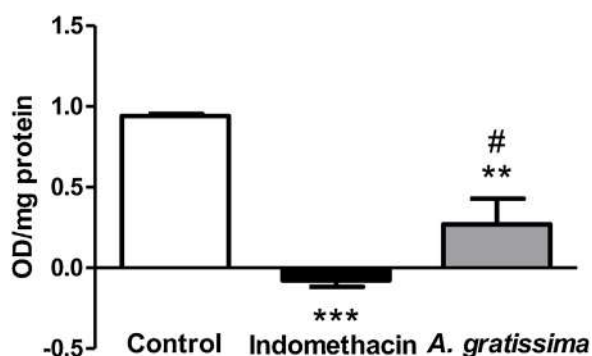


Fig. 4. Effect of *A. gratissima* leaves supercritical extract on the myeloperoxidase levels (optical density (OD)/mg protein) after euthanasia of mice receiving carrageenan i.p. (300 μ l / paw) injection. The mice were treated with vehicle (saline + 1 % tween 80, p.o., 10 ml / kg n = 18, control group), or EAG (10 mg kg⁻¹, p.o., n = 18) or indomethacin (10 mg kg⁻¹, p.o., n = 18) 1 h before carrageenan injection. One-way ANOVA, post hoc Student-Newman-Keuls: **p < 0.01, *** p < 0.01 compared to the vehicle-treated (control) group. #p < 0.05 compared to indomethacin-treated mice. Results expressed as mean $\pm$ S.E.M.

3.3. Myeloperoxidase activity

To assess the protective effects of EAG on carrageenan-induced inflammatory response, it was evaluated the myeloperoxidase (MPO) activity (shown in Fig. 4), which is an indirect measure of neutrophil migration to paw tissue. The paw tissue samples were collected 3 h after the algogenic stimulus, considering the peak of EAG effect in the carrageenan-induced paw edema test.

The MPO activity in paw tissue was significantly reduced by mice pre-treatment with indomethacin ($p < 0.001$) and EAG ($p < 0.01$) (Fig. 4). These results are in accordance to the carrageenan-induced edema, which was prevented by the EAG (Figs. 2 and 3). Considering that neutrophils and macrophages are related to MPO activity in inflamed tissues [24], we infer that the EAG reduces carrageenan-induced inflammation, probably by inhibiting neutrophils and macrophages recruitment.

Spathulenol, β -elemene and caryophyllene oxide are the major compounds found in the essential oil of *C. argyrophyllus*, which reduced carrageenan-induced paw edema at 30 mg kg⁻¹, besides inhibiting the myeloperoxidase activity [45]. The essential oil of *C. sellowianus*, enriched in guaiol, demonstrated *in vitro* anti-inflammatory activity, since it was able to reduce neutrophils chemotaxis [14]. Additionally, the anti-inflammatory activity of caryophyllene oxide is well established [43,46]; however, studies about the anti-inflammatory effects of pinocamphone are scarce. Therefore, we may suggest that the presence of spathulenol, β -elemene, caryophyllene, caryophyllene oxide and guaiol contribute

to the anti-inflammatory activity of the EAG demonstrated in the present study. Also, our study is the first one to demonstrate the *in vivo* anti-inflammatory activity of an extract rich in pinocamphone.

4. Conclusions

The supercritical CO₂ extracts of *A. gratissima* leaves (EAG) showed anti-inflammatory properties. The MPO activity in paw tissue was significantly reduced by mice pre-treatment with indomethacin in accordance to the carrageenan-induced edema, which was prevented by the EAG. The temperature and the pressure influenced the extraction process, there was an increase of yield when high temperature and pressure were combined. Among the EAG chemical constituents, guaiol, pinocamphone, spathulenol and caryophyllene oxide were the major ones, which could be associated to the anti-inflammatory activity of EAG in mice.

Declaration of Competing Interest

The authors have no conflict of interest that could affect the publication of this manuscript.

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Anti-inflammatory and antinociceptive effects of *Aloysia gratissima* leaves essential oil: An *in vivo* study

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ABSTRACT

Background and aim: *Aloysia gratissima* is used in popular medicine as an analgesic and sedative. However, studies on its biological activities are still scarce. Therefore, this work aimed to evaluate the antinociceptive and anti-inflammatory effect of *A. gratissima* leaves essential oil (OAG) in mice.

Experimental procedure: The OAG was obtained by hydrodistillation and its composition was determined by gas chromatography-mass spectrometry. The sesquiterpenes pinocamphone, β -pinene, and guaiol were the major constituents of OAG. The effect of OAG (1, 10, and 30 mg/kg, p.o.) was assessed by pre-treating male mice 1h before the formalin assay, carrageenan-induced peritonitis test, or the paw edema induced by arachidonic acid (AA). The acute toxicity of OAG (2000 mg/kg, p.o.) was also investigated.

Results and conclusion: The minimum effective dose of OAG in the formalin test was 1 mg/kg. This dose did not affect the locomotor activity of mice. The OAG decreased the number of leukocytes/mm³ in the peritoneal exudate of mice and reduced paw edema 45 min after the arachidonic acid injection. OAG treatment elicited a reduction in NPSH levels in the paw tissue and increased NPSH levels in mice blood plasma and did not cause mice mortality. The OAG is devoid of acute toxicity and shows anti-inflammatory activity, which can be related to the presence of pinocamphone, guaiol, and β -pinene and is mediated, at least in part, by mechanisms that involve the participation of NPSH.

1. Introduction

Inflammation is a protective response to tissue damage and/or microbial invasion, regulated by a cascade of many molecular interactions and biochemical reactions. It plays a central role in the pathogenesis of various disorders such as rheumatoid arthritis, cancer, and cardiovascular complications.^{1,2} The therapeutic approach commonly used against the inflammatory process involves non-steroidal anti-inflammatory drugs (NSAIDs), which are associated with several adverse effects such as nausea, vomiting, kidney and liver failure,^{3–5} as well as gastrointestinal lesions, manifested mainly as gastric ulcers.^{5,6} Therefore, the identification of new organic molecules and bioactive

compounds is essential to develop effective alternative drugs for the treatment of inflammatory diseases. Despite the desirable therapeutic effects, the potentially toxic and mutagenic effects of anti-inflammatory drugs should not be underestimated.⁷

The use of traditional medicinal plants is significantly related to the survival of the human species. The World Health Organization (WHO) acknowledged that ~80 % of the world's population is still dependent on traditional medicine.⁸ Indeed, several lifesaving drugs were originally isolated from plants or are semisynthetic analogs from phytochemicals.^{9,10} In this context, Brazil has the greatest total biodiversity in the world, with more than 45,000 species of superior plants.^{2,11} Consequently, the Brazilian population uses medicinal plants to treat several acute and chronic diseases.¹¹ Among the species popularly

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Abbreviations

AA –	arachidonic acid
GC-MS –	gas chromatography coupled to mass spectrometry
i.p. –	intraperitoneal
i.pl. –	intraplantar
MPO –	myeloperoxidase
NPSH –	non-protein thiols
NSAIDs –	non-steroidal anti-inflammatory drugs
O.D. –	optical density
OAG –	essential oil of <i>Aloysia gratissima</i> leaves
p.o. –	per os

known for their pharmacological actions are those of the genus *Aloysia*, which belongs to the Verbenaceae family.^{12,13} This genus contains 34 cataloged species, 12 of which can be found in Brazil.^{14,15}

Aloysia gratissima (Gillies & Hook.) Tronc. is a shrub that can reach a maximum height of 3 m.¹⁶ It is popularly known in Brazil as “Lavanda-do-Brasil”, “Erva-de-Nossa-Senhora”, and “Herva Sagrada” and is widely distributed in America, occurring from the United States to Argentina. Studies conducted in Brazil revealed that this species is popularly used for headaches, “nervous problems”, disorders of the digestive and respiratory systems, and inflammatory pain in Rio Grande do Sul state.^{17–19}

The infusion of *A. gratissima* leaves, which is enriched in polyphenols, is widely used in traditional medicine.^{14,20} The essential oil of *A. gratissima*, in turn, presents more than 70 % of hydrocarbons, of which more than half are sesquiterpenes.²¹ This is particularly interesting since the pharmacological action of plants is related to the presence of metabolites such as terpenes, flavonoids, and phytosterols.^{22,23} Considering that the main advantage of essential oils is to obtain mixtures characterized by two or three major compounds at high concentrations compared to other components present in minor quantities,²⁴ we employed here the essential oil of *A. gratissima* leaves.

Previous studies conducted by our research group showed that the extract obtained by Supercritical Fluid with carbon dioxide (SCCO₂) from *A. gratissima* leaves is rich in terpenes such as guaiaol, pinocampnone, caryophyllene, caryophyllene oxide, spathulenol.^{13,19} Additionally, we have shown its antinociceptive effect in mice, which is mediated by ATP-sensitive K⁺ channels, without causing changes in locomotor activity or motor coordination.¹³ Noteworthy, we have also demonstrated the anti-inflammatory action of *A. gratissima* extract, which prevented carrageenan-induced paw edema and reduced myeloperoxidase (MPO) activity, without toxicological effects at the dose of 2000 mg/kg.¹⁹ Despite these promising results, to the best of our knowledge, there are no studies on the antinociceptive and anti-inflammatory action of *A. gratissima* essential oil.

Considering the above-described scenario, it is important to move forward on the investigation of the effects of *A. gratissima*, with a special focus on its essential oil, using animal models of inflammation/nociception, as well as to elucidate its mechanisms of action. Therefore, the present work aimed at evaluating the antinociceptive and anti-inflammatory activities of the essential oil of *A. gratissima* leaves in models that use different stimuli in mice and investigate the mechanisms of action involved in the anti-inflammatory activity, that have not been explored yet.

2. Materials and methods

2.1. Chemicals

Formalin was purchased from Merck® (Darmstadt, Germany), indomethacin was obtained from Aspen Pharma (Durban, South Africa),

and dexamethasone from Aché Laboratórios Farmacêuticos (Guarulhos, SP). Carrageenan was obtained from Sigma Chemical Co. (St. Louis, MO, USA), and arachidonic acid was purchased from Dehon Farmácia de Manipulação (Santa Catarina, Brazil). The ELISA Kit for PGE₂ was obtained from Elabscience® (USA). Indomethacin and dexamethasone were the NSAID and steroidal anti-inflammatory drugs used as positive controls, respectively.^{25–27}

2.2. Plant material

A. gratissima leaves were collected in April 2021 (fall), in the municipality of Erval Grande, RS, Southern Brazil (27° 23 '14.3S, 52°33'49'W). This region has a humid subtropical mesothermal climate. The plant specimen was deposited in the Herbarium of the Community University of Chapecó (Herbário Unochapecó, SC, Brazil) under the accession number UNO 3700. After collection, the leaves were manually separated from the branches and dried without exposure to sunlight at room temperature. After being packed in plastic bags, samples were identified and stored at 4 °C until the extraction process. All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SisGen (Register #A22F841).

2.3. Extraction of *Aloysia gratissima* leaves by hydrodistillation

The essential oil was obtained from the leaves of *A. gratissima* by hydrodistillation, using the Clevenger apparatus. The experimental extraction procedure was based on the study by Ricciardi et al.,²⁸ adapted by our research group. We used deionized water and the Clevenger-type extractor set as drag liquid and the leaves were extracted for 120 min. The dried leaves of *A. gratissima* were ground in an industrial blender and 50 g of dry plant material were weighed for each 300 mL of deionized water, loaded into a 1000 mL distillation flask, and coupled to the ball condenser and heating mantle for 120 min.

After the extraction, the oil was collected and dried over anhydrous sodium sulfate (Na₂SO₄) and kept refrigerated at 4 °C in an amber bottle. The oil from the leaves of *A. gratissima* (OAG) was kept under these conditions until the following analyses were carried out.^{29,30}

2.4. GC/MS analysis

OAG's chemical composition was analyzed by Agilent GC/MS (7890B) gas chromatography coupled to a quadrupole mass spectrometer (5977A) (Agilent Technologies, Palo Alto, CA, USA). The GC/MS system's experimental conditions were described by Souza et al. (2020)¹³ and Scapinello et al. (2018),³¹ with minor modifications. Briefly, the system conditions were Agilent 19091S capillary column, dimension: 30 m × 250 µm × 0.25 µm. The mobile phase flow (carrier gas: He) was adjusted to 1.0 mL/min. The GC temperature program was 40.0 °C at 4 min to 240.0 °C at a rate of 10 °C/min and up to 300.0 °C at a rate of 40.0 °C/min (maintained for 5 min). The injector temperature was 280.0 °C, sample injection volume 1 µL, and split ratio 1:20. The MS transferline temperature was set to 150.0 °C and the source of ions temperature was set at 230.0 °C. For GC–MS detection, an electron ionization system was used with ionization energy set at 70 eV, and mass range atm/z 40–400. The chemical components present in the extract were identified by comparison with the equipment library (Agilent P/N G1033A) and comparing retention times; the experimental Kovats retention indices (dK_{Iexp}) were calculated using a non-isothermal method according to Van Den Dool and Dec. Kratz.³² The equation used was:

$$I_x = 100n + 100 \frac{(t_x - t_n)}{(t_{n+1} - t_n)}$$

Where t_x is the retention time of the compound of interest, t_n is the

retention time of the n-alkane that elutes immediately before t_x , and t_{n+1} is the retention time of the n-alkane that elutes immediately after t_x , this calculation was made relative to n-alkanes C7–C30.

The relative amounts of each component were calculated using their respective peak areas in the chromatogram. The extract was solubilized in 10 mg/mL of dichloromethane to be analyzed.

2.5. *In vivo* experiments

2.5.1. Animals

Male Swiss mice (or nulliparous females, in the case of toxicity experiments) aged 8 weeks (weight between 25 and 35 g) were obtained from the animal facility of the Community University of the Chapecó Region (Unochapecó). The animals were kept in a controlled environment under standard conditions ($22 \pm 2^\circ\text{C}$) with a 12 h light/dark cycle (lights on at 6 a.m.) and air humidity (40–60 %). They were housed in groups of 3–5 animals per cage (length: 28 cm, width: 19 cm, and height: 12.5 cm) and had free access to standard laboratory chow (Biobase®) and water. On experimental days, the animals were placed in the experimental room for at least 1 h before the experiments. In the case of oral administrations (gavage), they were deprived of food for 2 h before the administrations. The experimental groups consisted of 6 mice each and the sample size was calculated using the online calculator from the University of São Paulo (FOB-USP) (<http://estatistica.bauru.usp.br/calculoamostral/calculos.php>) considering $\alpha = 5\%$ and $\beta = 80\%$. The protocol for this study was approved by the institutional ethics committee (Approval number #022/2020) and all experimental practices followed the ethical precepts of animal research described in the Brazilian Law no. 11.794 – Brazil,^{31,33} Council of the European Union Communities – Decree of September 22, 2010, 2010/63/EU³³ and the ARRIVE Guidelines.³⁴ Following the experiments, mice were euthanized by isoflurane inhalation, and cervical dislocation was used to confirm the death.

Mice were treated with volumes of 10 mL/kg, respectively according to their weight, through oral gavage (p.o.) or intraperitoneal (i.p.) routes and with 20 μL by intraplantar (i.pl.) injection, according to each experiment's specific protocol. Solubilization of the oil and substances was performed in saline (NaCl 0.9 %) with the aid of 1 % Tween 80 (v/v) and ultrasonic bath (30 min at 20°C and 100 kHz). The oil's tested doses were chosen based on the study of Zeni et al. (2013) and Souza et al. (2020a; 2020b).^{13,19,35}

The experiments chosen by the authors cannot be fully replaced by *in vitro* assays because, to date, there is no biomarker with acceptably high sensitivity and sufficient specificity to replace the use of animals.^{26,36} Furthermore, it is important to assess inflammation through *in vivo* methods because the pharmacokinetics and pharmacodynamic parameters are generally non-reproducible from *in vitro* assays to *in vivo* assays.^{26,37}

2.5.2. Acute toxicity

The acute toxicity study was based on Guideline 423³⁹ of the Organization for Economic Cooperation and Development (OECD). Female mice ($n = 6$) were orally treated with OAG at 2000 mg/kg. The control group ($n = 3$) received the administration of the vehicle (0.9 % NaCl + 5 % Tween 80, 10 mL/kg, p.o.). Thereafter, the animals were observed with special attention during the first 4 h after treatment and daily for 15 days. The occurrence of mice death, and signals such as piloerection, hypothermia, palpebral ptosis, abdominal writhing, muscle tone alterations, shacking, hind paw paralysis, salivation, bronchial secretion, and seizures were registered. Additionally, body weight and food consumption were recorded for every 2 days during the experimental period (15 days). At the end of the experiment, the macroscopic aspect of the organs (liver, kidneys, adrenal glands, spleen, lungs, heart, and brain), as well as their relative weight (%), were registered.

2.5.3. Formalin-induced paw licking test

The experimental procedure was similar to the one described by Santos and Calixto (1997).³⁸ Briefly, animals ($n = 6/\text{group}$) were orally treated with vehicle (control group, 0.9 % NaCl + 1 % Tween 80), OAG (1, 10, and 30 mg/kg), and indomethacin (10 mg/kg), the positive control, 1 h before^{3,19} the injection of 2 % formalin (20 $\mu\text{L}/\text{paw}$) into the right hind paw (i.pl.). Immediately after the formalin injection, the time spent licking, biting, or lifting the injected hind paw (nociceptive behavior) was recorded during the first phase (0–5 min, neurogenic phase) and the second phase (15–30 min, inflammatory phase) of the test. The experiment was video-recorded and performed in a double-blind and randomized manner. The dose that presented the best result in this experiment was chosen to be used in the open-field test and other experiments of nociception.

2.5.4. Open field test

The open-field test was performed to evaluate the possible OAG effects on the locomotor and exploratory activities of mice. The experimental protocol was based on the method described by Müller et al. (2012).³⁹ The animals ($n = 6/\text{group}$) received OAG's minimal effective dose (1 mg/kg, p.o.) in the formalin writhing test, 1 h before being exposed to the open-field arena. The control group was treated with vehicle (10 mL/kg, 0.9 % NaCl + 1 % Tween 80, p.o.). The arena consisted of a water-proofed MDF black box ($40 \times 30 \times 30\text{ cm}$). After the habituation (5 min), the animals were video recorded by 10 min in the arena. The total distance traveled by the animals (m), average speed (m/s), the number of rearings, groomings, and fecal bolus expelled during the test were analyzed using the ANY-maze® software (Stoelting CO., Wood Dale, IL, USA).

2.5.5. Carrageenan-induced peritonitis

The experimental procedure followed Lapa (2003)⁴⁰ and Oliveira-Tintino et al. (2018),⁴¹ with minor modifications previously performed by our research group.²⁶ Mice were treated orally with vehicle (control group, 10 mL/kg, 0.9 % NaCl + 1 % Tween 80), dexamethasone (10 mg/kg)⁴² or OAG (1 mg/kg) 1 h before the injection of 1 % carrageenan (0.25 mL, i.p.). After 4 h, the animals were euthanized and 3 mL of heparinized PBS (5 UI/mL) were used to rinse the peritoneal cavity. The peritoneal exudates were collected, diluted in Türk's solution (1:10), and used for the subsequent measurement of MPO, PGE2, and total leukocyte counting in a Neubauer chamber.

2.5.6. Arachidonic acid-induced paw edema

This assay was performed as described by Oliveira-Tintino et al. (2018),⁴¹ with minor modifications.¹⁹ Animals were orally treated with vehicle (control group, 10 mL/kg, 0.9 % NaCl + 1 % Tween 80), OAG (1 mg/kg) or dexamethasone (10 mg/kg)³⁹, 1 h before the injection (20 μL) of 1 % arachidonic acid (AA) in the right hind paw (i.pl.), while 0.9 % NaCl was administered to the left hind paw. Paw thickness (mm) was measured with a Mitutoyo digital caliper (500-196-20 B Abs, 150 mm) at 15, 30, 45, 60, 75 and 90, 105, 115, and 120 min after the intraplantar administration of AA and expressed as $\Delta\text{paw thickness}$ ($\Delta\text{paw thickness} = \text{paw thickness at the specific time point} - \text{thickness foot base}$). The basal thickness of the mice's hind paws was measured before the i.pl. injection. After the experimental protocol, mice were euthanized and the blood and plantar tissue were collected to evaluate the NPSH content.

2.6. *Ex vivo* assays

2.6.1. NPSH and TBARS levels

This assay was performed in the blood and paw tissue of the animals submitted to the AA-induced paw edema test. Samples of paw tissue ($n = 6$) consisted of a random pool of 5 animals of the same group.¹⁹ Nevertheless, it was not necessary to pool the blood samples of the animals. Paw tissue was homogenized (1:10 m/v) in a 50 mM Tris-HCl

solution (pH 7.4) at 4 °C. Blood was collected in heparinized syringes and centrifuged for 10 min at 3000 rpm. Afterward, 4 % trichloroacetic acid was added to the supernatant to determine NPSH levels. In the other fraction of the supernatant, trichloroacetic acid 40 % was added to determine TBARS levels, then the tubes were centrifuged again for 10 min at 3000 rpm and the final supernatant was used for the assays. The NPSH content (nmol NPSH/mg of tissue or mL) was determined according to Ellman (1959),⁴³ with minor modifications,²⁵ and lipid peroxides were estimated using thiobarbituric acid reactive substances (TBARS, expressed as nmol MDA/mg tissue or mL) according to Ohkawa et al. (1979).⁴⁴

2.6.2. Myeloperoxidase activity

Myeloperoxidase (MPO) activity was investigated in the peritoneal exudate of animals submitted to the carrageenan-induced peritonitis test (described in 2.5.5). Samples were stored at –80 °C until the analysis of MPO activity according to Bradley et al. (1982)⁴⁵ and Camponogara et al. (2019).⁴⁶ MPO levels were determined at 620 nm and expressed as O.D./mg of protein. Total proteins were quantified by the method of Lowry et al. (1951).⁴⁷

2.6.3. Prostaglandin E2 levels

The concentration of PGE2 in the peritoneal exudates from the animals submitted to carrageenan-induced peritonitis (described in 2.5.5) was measured using a commercial ELISA kit following the manufacturer's instructions (Elabscience, Catalog number E-EL-0034). Absorbance was spectrophotometrically determined at 450 nm and the concentration of PGE2 is expressed as pg/mL.

2.7. Statistical analysis

Data normality was assessed using Bartlett's test. The exclusion criteria were defined as outliers (mean $\pm$ 2 S.D.) data points. Data from the acute toxicity study (food intake and relative body weight) were analyzed by two-way repeated measures analysis of variance (ANOVA) (Factor 1: Treatment, Factor 2: Time). The relative weight of the organs was analyzed by the Unpaired *t*-test. The results of the formalin test, open-field, carrageenan-induced peritonitis, and AA-induced paw edema, as well as data from *ex vivo* experiments, were analyzed by One-way ANOVA followed by Bonferroni's test. Statistical analyses were performed using Graph Pad Prism 8.0 for Windows (GraphPad Software, San Diego, California, USA). Results are expressed as the mean $\pm$ S.E.M. of *n* animals per group. *p* values less than 0.05 (*p* < 0.05) were considered significant.

3. Results

The chemical characterization of OAG by GC/MS identified 97.4 % of the total chemical compounds, indicating the presence of 44 different chemical constituents, including the volatile terpenes pinocamphone (13.57 %), guaiol (10.54 %) and β -pinene (8.51 %) as major compounds among others in smaller amounts (Table 1).

Results from the OAG's acute oral toxicity are demonstrated in Fig. 1. The relative body weight of mice treated with OAG (2000 mg/kg) was not significantly different from the weight of the control group during the experimental period (Fig., 1a). Nevertheless, the relative weight of the control group significantly (*p* < 0.05) increased from the 9th to the 15th day of observation. Also, the group treated with OAG presented a significant increase in the relative body weight from the 12th to the 15th of the observation (*p* < 0.0001). The food intake of mice treated with OAG (2000 mg/kg) is depicted in Fig. 1b. The food intake of animals treated with OAG was significantly higher than the food intake of the vehicle-treated animals (control group) 24 h after the oral administration (*p* < 0.05). However, there was a significant decrease (*p* < 0.0001) in the food intake of animals treated with vehicle (control group) on the last day of observation when compared to all the other days of

Table 1

Chemical composition of essential oil from leaves of *A. gratissima*.

Compound	RT	CAS no.	eKlit	dKlexp	RC (%)
α -Thujene	4.456	2867-05-2	931	931	0.14
α -Pinene	4.55	80-56-8	933	939	1.31
Sabinene	5.021	3387-41-5	975	979	0.35
β -Pinene	5.076	127-91-3	980	984	8.51
β -Myrcene	5.186	123-35-3	992	993	0.94
<i>o</i> -Cymene	5.629	527-84-4	1027	1030	0.17
D-limonene	5.678	5989-27-5	1018	1034	1.46
Eucalyptol	5.739	470-82-6	1044	1040	0.29
β -Ocimene	5.87	13877-91-3	1050	1051	0.22
β -Linalool	6.509	78-70-6	1105	1105	0.6
Terpineol	6.549	7299-41-4	1125	1108	1.14
cis-Verbenol	6.662	13040-03-4	1128	1118	0.47
α -Campholenal	6.857	4501-58-0	1130	1135	0.37
L-trans-Pinocarveol	7.05	547-61-5	1155	1152	4.01
cis-Verbenol	7.108	1845-30-3	1144	1157	1.85
Pinocamphone	7.303	547-60-4	1163	1174	13.57
Trans-3-pinanone	7.462	15358-88-0	1176	1188	4.43
α -Terpineol	7.621	98-55-5	1200	1201	0.16
(–)-Myrtenol	7.697	515-00-4	1212	1208	2.72
Verbenone	7.872	80-57-9	1207	1224	0.14
cis-Carveol	7.927	1197-06-4	1231	1229	0.4
Carvone	8.22	99-49-0	1249	1256	0.19
(–)-cis-Myrtanol	8.324	514-99-8	1258	1266	0.19
Thymol	8.69	89-83-8	1297	1299	0.25
(–)-trans-Pinocaryyll acetate	8.806	1686-15-3	1300	1310	8.26
α -Copaene	9.634	3856-25-5	1394	1391	0.22
(–)- β -Bourbonene	9.744	5208-59-3	1391	1401	0.5
β -Elemen	9.778	515-13-9	1394	1405	0.39
Caryophyllene	10.114	87-44-5	1444	1439	5.05
Humulene	10.45	6753-98-6	1477	1473	1.64
Alloaromadendrene	10.524	25246-27-9	1377	1481	0.26
D-Germacrene	10.713	23986-74-5	1503	1500	4.87
γ -Elemene	10.86	29873-99-2	1492	1516	2.95
β -Guaiene	11.208	88-84-6	1533	1554	0.32
Elemol	11.33	639-99-6	1562	1567	0.45
Elixene	11.455	3242-08-8	1488	1581	4.36
Germacrene D-4-ol	11.611	198991-79-6	1595	1597	0.43
(–)-Spathulenol	11.651	77171-55-2	1682	1602	2.7
Caryophyllene oxide	11.718	1139-30-6	1613	1609	3.34
Guaiol	11.801	489-86-1	1614	1619	10.54
β -Eudesmol	11.935	473-15-4	1632	1634	2.31
Cubenol	12.225	21284-22-0	1651	1667	0.51
Bulnesol	12.427	22451-73-6	1675	1690	4.03
Total					97.01 %

Note: RT – Retention time; eKlit: Retention indices NIST – National Institute of Standards and Technology Library; dKlexp = Kovats indices, retention indices relative to index C7–C30 n-alkanes; CAS no. = Chemical Abstracts Service (CAS) registry number; RC (%) = Relative concentration.

observation. Concerning the group treated with OAG, the same reduction in food intake was observed: there was a significant (*p* < 0.01) decrease in the food intake on day 15 compared to days 3, 6, 9, and 12 and also between days 9 and 12 compared to days 3 and 6 (*p* < 0.0001). After 15 days of evaluation, the animals were euthanized, and the relative weight of the organs was evaluated. There were no significant differences between the relative weight of organs (brain, heart, thymus, adrenal glands, kidneys, spleen, and liver, Fig. 2a–g) of the animals treated with OAG (2000 mg/kg, p.o.) and vehicle (control group). Furthermore, no deaths were recorded and therefore the oral toxicity of OAG can be classified in category 5 (acute lethal toxicity is greater than

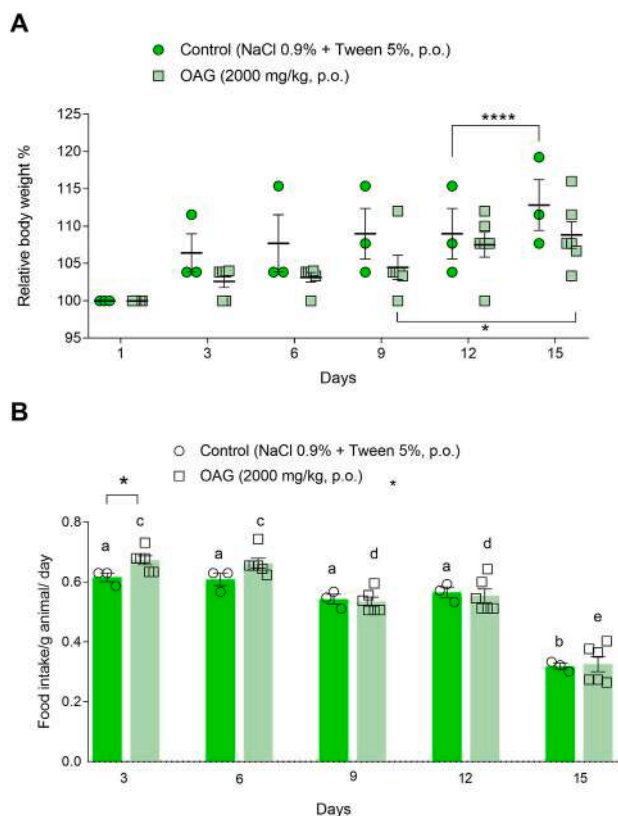


Fig. 1. Effect of acute treatment of *A. gratissima* leaves essential oil (OAG) (2000 mg/kg, p.o.) on relative body weight (%) in female Swiss mice ($n = 3-6$ mice/group) (A) and (%) food intake (g food intake/g mice/day) (B). Two-way repeated measures ANOVA followed by Bonferroni. Results expressed as mean $\pm$ S.E.M. (A) **** $p < 0.0001$. (B) * $p < 0.05$. ^a significant ($p < 0.05$) difference from ^b significant ($p < 0.05$) difference from ^a ^c significant ($p < 0.05$) difference from ^d and ^e significant ($p < 0.05$) difference from ^c and ^d.

2000 mg/kg) according to the OECD Globally Harmonized Classification System.⁴⁸

Fig. 3 illustrates the results from the formalin test. The nociception time (s) of animals treated with indomethacin (10 mg/kg, p.o.) was significantly reduced when compared to the vehicle-treated mice (control group) at the first ($p < 0.01$, Fig. 4a) and second ($p < 0.0001$, Fig. 4b) phases of the test. Also, animals treated with the OAG at all tested doses (1, 10, and 30 mg/kg, p.o.) presented a significant reduction in the nociceptive behavior (s) in both phases [first phase, $p < 0.001$, Fig. 4a; second phase, $p < 0.0001$, Fig. 4b] of the formalin test in comparison to the control group. Considering these results, we implemented the minimum effective dose of 1 mg/kg of OAG for the subsequent behavioral assessments.

The open field test was performed to rule out alterations due to the effect of OAG on the locomotor activity of mice, as well as a false positive or negative in the nociceptive results.^{13,49} We evaluated the distance traveled (m) (Fig. 4a), the average speed (m/s) (Fig. 4b), the number of rearings (Fig. 4c), groomings (Fig. 4d), and the number of fecal bolus (Fig. 4e) expelled during the session. There were no significant differences in any parameter analyzed between the groups treated with vehicle (control group), indomethacin (10 mg/kg), and OAG (1 mg/kg).

The results from the peritonitis test are depicted in Fig. 5. The number of leukocytes/mm³ in the peritoneal exudate from the animals orally treated with dexamethasone (10 mg/kg) ($p < 0.001$) and OAG (1 mg/kg) ($p < 0.01$) was significantly decreased when compared to the vehicle-treated mice (Figs. 3–5a). The MPO activity (Fig. 5b) was significantly ($p < 0.05$) reduced in the peritoneal exudate from the

group treated with dexamethasone (10 mg/kg). The group treated with OAG (1 mg/kg) presented a tendency ($p = 0.058$) to decrease the MPO activity in the peritoneal exudate. Nevertheless, no significant differences in the concentration of PGE2 (Fig. 5c) were observed in the peritoneal exudate between the experimental groups.

Fig. 6 illustrates the results from the AA-induced paw edema test. The animals treated with OAG (1 mg/kg) showed a significant reduction in the thickness of the AA-injected paw (similar to the group treated with dexamethasone (10 mg/kg)) from 60 min (after the intraplantar injection of AA) up to the last measurement at 120 min ($p < 0.0001$) when compared to the vehicle-treated (control) group (Fig. 6a). The animals submitted to the AA-induced paw edema test that received dexamethasone (10 mg/kg) and OAG (1 mg/kg) presented a significant ($p < 0.0001$) decrease in NPSH levels in the paw tissue (nmol NPSH/mg of tissue) in comparison to the control group (Fig. 6b). However, the NPSH levels in the blood plasma (nmol NPSH/mL) of groups that were treated with dexamethasone (10 mg/kg) or OAG (1 mg/kg) were significantly ($p < 0.001$) higher than the NPSH levels of vehicle-treated animals (Fig. 6c). The TBARS levels (nmol MDA/mg of tissue) in the paw tissue were not different between groups (Fig. 6d). Nevertheless, the TBARS levels in the blood plasma (Fig. 6e) of the groups that were treated with dexamethasone (10 mg/kg) or OAG (1 mg/kg) were significantly ($p < 0.001$ and $p < 0.05$, respectively) lower than the TBARS levels (nmol MDA/mL) of the treated animals.

4. Discussion

The use of medicinal plants is very important as it represents the primary healthcare needs of a significant part of the world population.⁸ In this context, ethnopharmacological research has advanced in recent years for the discovery of bioactive natural products.¹⁰ In this scenario, the popular use of vegetal species, such as *A. gratissima*, represents an alternative to a significant part of the population in South America to heal pain and inflammatory processes.²⁰ In this study, we show that the essential oil obtained from the leaves of *A. gratissima* by hydro-distillation (OAG) presents antinociceptive and anti-inflammatory activities in mice models using different stimuli, which corroborates the traditional use of *A. gratissima* as analgesic and anti-inflammatory. Additionally, we provide some insights on the mechanisms involved in the anti-inflammatory activity of OAG and demonstrate that the acute OAG administration (at 2000 mg/kg, p.o.) did not elicit mice mortality during 14 days of observation and, therefore, is devoid of acute toxicity.

The chemical characterization of OAG revealed the major compounds pinocampone (13.57 %), a monoterpene hydrocarbon, guaial (10.54 %), an oxygenated sesquiterpenoid, and β -pinene (8.51 %), monoterpene hydrocarbon. Chemical compounds such as monoterpenes and/or sesquiterpenoids are known to exhibit a wide range of pharmacological activities, including antinociceptive, anti-inflammatory, antimicrobial, and antitumor activities,⁵⁰ which turn the OAG's chemical composition very promising in terms of biological activity potential. Recently, we demonstrated that the extract from the leaves of *A. gratissima* obtained by supercritical fluid (with carbon dioxide - SCCO₂)^{13,19} is enriched in volatile terpenes, being guaial (18.5 %), pinocampone (11.4 %), caryophyllene oxide (6.76 %) and spathulenol (6.17 %), the main constituents. The chemical composition of SCCO₂ extract^{13,19} and OAG are very similar. In line with these observations, these compounds have been found in species of the genus *Aloysia*.⁵¹

Initially, we evaluated the OAG's acute toxicity according to the OECD Guideline 423.³⁹ Our results demonstrate that the OAG did not interfere with the gain weight of female mice treated during the 15 days of observation. These results are particularly interesting since changes in body weight might be related to the toxic effects of substances.⁵² Likewise, the food intake of the OAG and vehicle-treated groups was not different, except for the first day of observation, when OAG-treated animals consumed significantly more food than the control mice. The essential oil of *A. gratissima* presents sedative effects^{6,7} and we observed

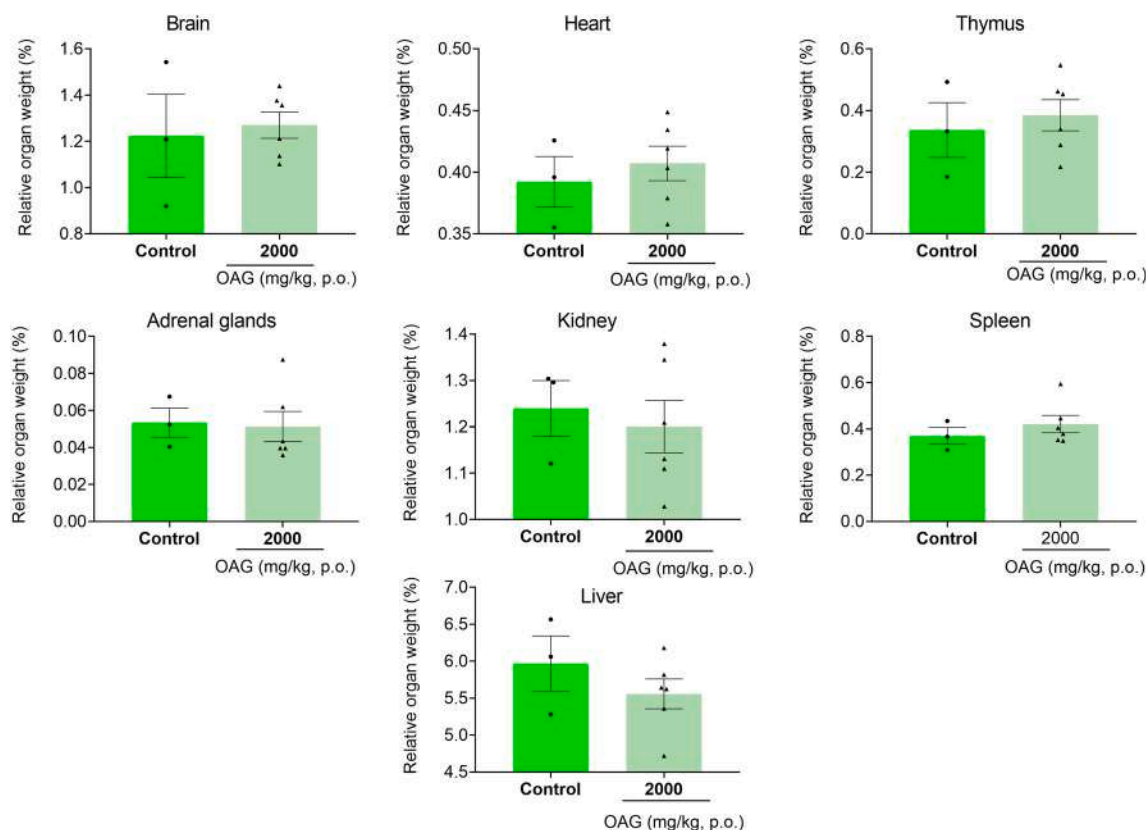


Fig. 2. Effect of *A. gratissima* leaves essential oil (OAG) on the relative (%) organ weight of female Swiss mice in the acute oral toxicity test. The brain, heart, thymus, adrenal glands, kidneys, spleen, and liver were evaluated. Mice ($n = 3-6$ mice/group) were treated orally with vehicle (0.9 % NaCl + 1 % tween, 10 ml/kg) or OAG (2000 mg/kg). Unpaired *t*-test.

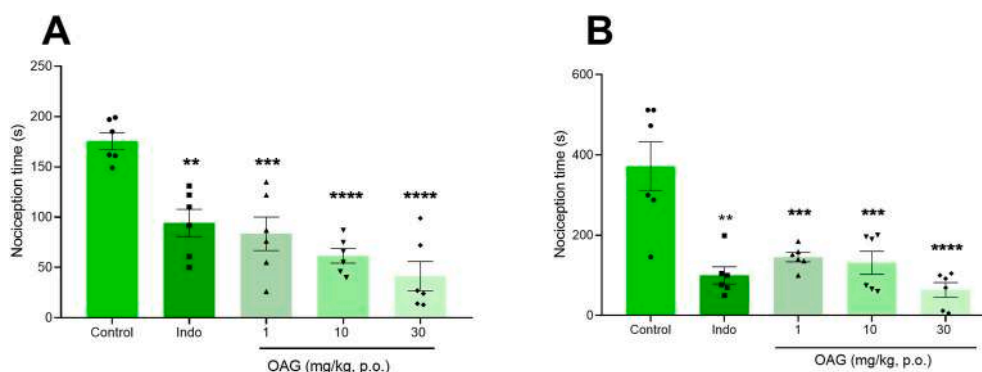


Fig. 3. Effect of *A. gratissima* leaves essential oil (OAG) on the formalin test in mice. Nociceptive behavior was considered as the time (s) of lifting, biting, or licking the paw in the first phase (A: 0–5 min) and the second phase (B: 15–30 min) of the test. Mice ($n = 6$ mice/group) were treated with vehicle (V: 0.9 % NaCl + 1 % Tween 80, 10 ml/kg), indomethacin (10 mg/kg), or OAG (1, 10 and 30 mg/kg) 1 h before administration of 2 % formalin i.p.l. One-way ANOVA followed by Bonferroni: ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$ are different from the vehicle group. Results expressed as mean $\pm$ S.E.M.

that there was intense sedation after the administration of OAG at 2000 mg/kg. Therefore, after a period without ingesting food, the increase in food intake might represent a post-fasting compensation. Additionally, a decrease in food intake over the days was observed for both experimental groups. This finding may be related to the estrous cycle of mice since it is known that on the days of pro-estrus, food consumption is markedly reduced.⁵³ In this sense, the reduction in food intake observed in both experimental groups might be related to this physiological condition. Nevertheless, we did not monitor the estrous phase of mice, which is a limitation of our study.

The OAG (2000 mg/kg, p.o.) did not induce any alteration in the relative weight (%) of the brain, heart, thymus, spleen, adrenal glands,

kidneys, and liver of mice. These findings demonstrate that the *A. gratissima* essential oil is less harmful to the mice organism than the SCCO₂ extract since Souza et al. (2020)¹³ found a significant decrease in the relative weight of the spleen and adrenal glands in mice treated with SCCO₂ extract of *A. gratissima* leaves. Zeni et al. (2013)¹² studied the toxicological effects of *A. gratissima* aerial parts aqueous extract and demonstrated that it induces liver toxicity in male mice at 2000 mg/kg. These differences between the findings may be related to mice gender and the extraction type used in the experiments. In any case, extracts and/or oil from *A. gratissima* proved to be harmless, as there were no deaths after oral administration at a dose of 2000 mg/kg. In this sense, the OAG could be included in Category 5 of the Harmonized System of

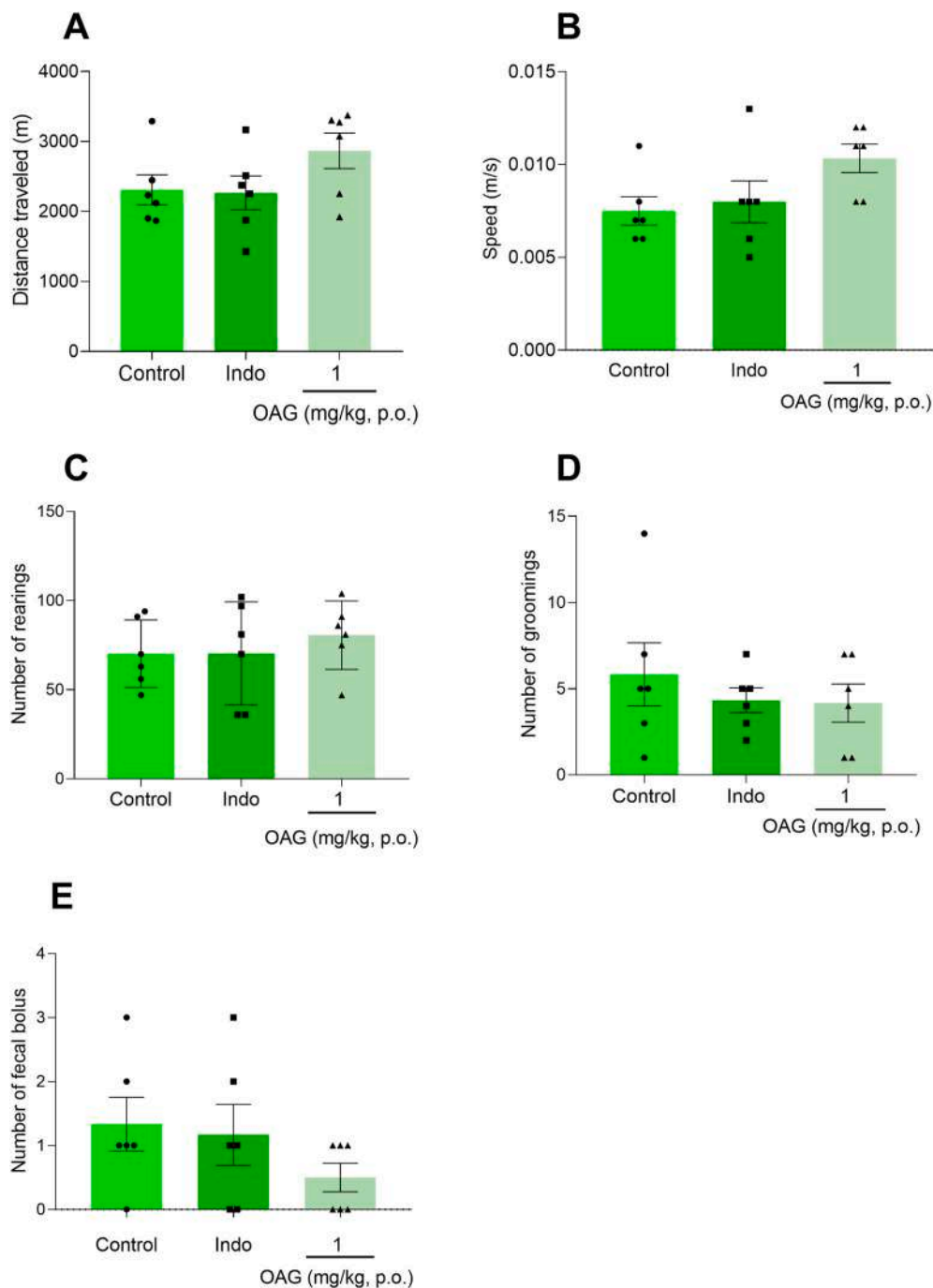


Fig. 4. Effect of *A. gratissima* leaves essential oil (OAG) on locomotor activity in mice (open field test). A: distance traveled (m); B: speed (m/s); C: number of rearings; D: number of groomings; E: number of fecal bolus expelled during the session. Mice ($n = 6$ mice/group) were orally treated with vehicle (0.9 % NaCl + 1 % Tween 80, 10 ml/kg), indomethacin (10 mg/kg) or OAG (1 mg/kg). One-way ANOVA. Each column represents the mean $\pm$ S.E.M.

Global Classification,⁴⁸ since its LD₅₀ is above 2000 mg/kg. Nevertheless, we did not investigate the toxicological effects of OAG using alternative models instead of mammals; nor perform a subacute or chronic oral administration of OAG to investigate its toxicological effects, which is a limitation of our study and deserves to be further studied.

It is known that NSAIDs, drugs commonly used to treat inflammatory processes, present several adverse effects, that are related to toxicity.³⁻⁶ Noteworthy, the acute oral LD₅₀ of indomethacin, the NSAID we employed as the positive control, is 13 mg/kg⁵⁴ in mice. Herein, we demonstrated that the acute oral LD₅₀ of OAG is > 2000 mg/kg, which acknowledges the security of the OAG and may represent an advantage

of the OAG compared to conventional NSAIDs, such as indomethacin.

The formalin test is an animal model that is widely used for the screening of analgesic/anti-inflammatory compounds.^{55,56} It aims to explore the analgesic effect of substances through the central and peripheral mechanisms of pain.^{13,41} Two distinct phases of pain are included in this test: phase I consists of neurogenic nociception, where there is direct stimulation of nociceptors via C fibers to the dorsal horn of the spinal cord after substance P secretion. Phase II (late phase) consists of inflammation-induced nociception due to the release of serotonin, histamine, bradykinin, and prostaglandin from the inflamed tissue by formalin.⁵⁷ We demonstrate that the OAG presents antinociceptive activity in both phases of the formalin test in mice, which may suggest that

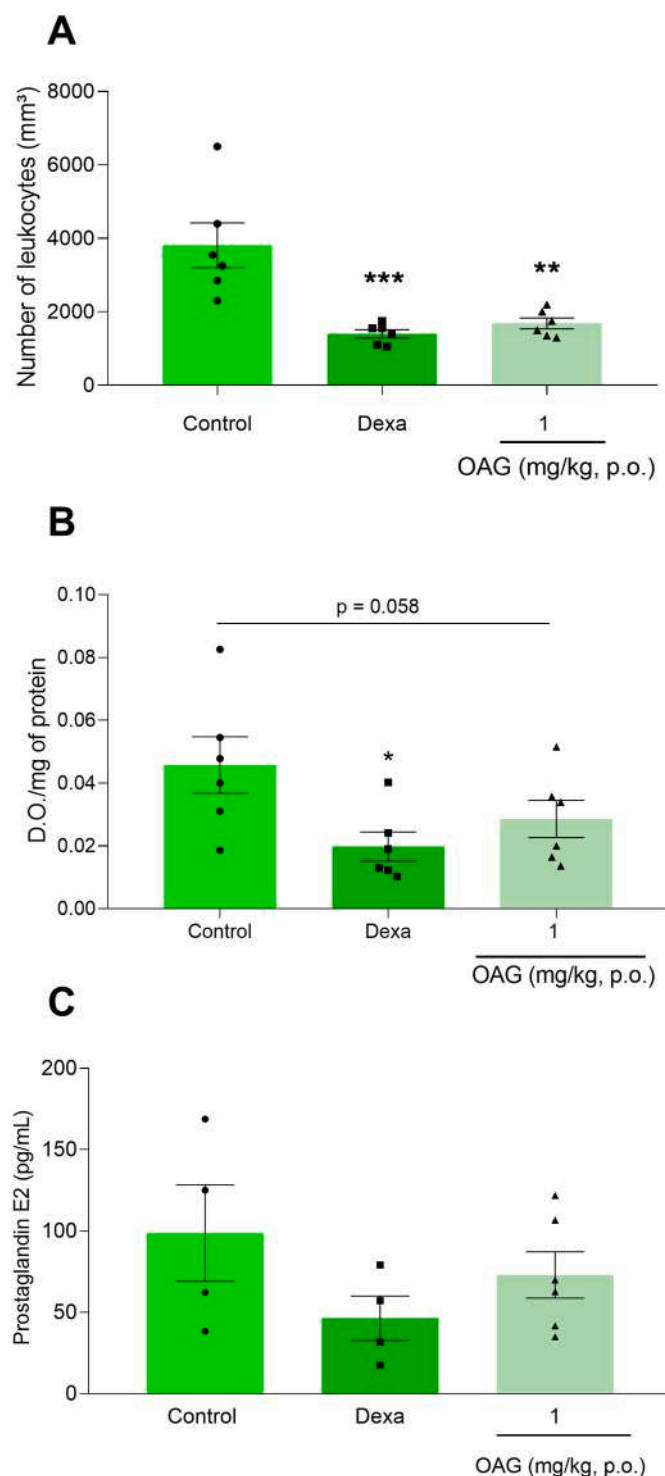


Fig. 5. Effect of *A. gratissima* leaves essential oil (OAG) on carrageenan-induced peritonitis inflammation assay. A: total leukocyte count/mm³; B: myeloperoxidase activity (optical density/mg of protein) and C: PGE2 levels (pg/mL) in the peritoneal exudate. Mice (n = 6 animals/group) were orally treated with vehicle (V: 0.9 % NaCl + 1 % Tween 80, 10 ml/kg), dexamethasone (10 mg/kg), or OAG (1 mg/kg) 1 h before of administration of 1 % carrageenan i.p. (0.25 mL). One-way ANOVA followed by Bonferroni: *p < 0.05; **p < 0.01 and ***p < 0.001 are different from the vehicle group. Results expressed as mean ± S.E.M.

OAG could be effective against neurogenic and inflammatory pain. Similar results were related by Souza et al. (2020),¹³ with *A. gratissima* SCCO₂ extract. Considering that the chemical composition of both OAG and SCCO₂ extract are similar, we may infer that the main constituents, mono, and sesquiterpenes, may be associated with their pharmacological activity.

The action of secondary metabolites such as β -pinene, pinocampone, guaiol, and spathulenol, which may be responsible for the anti-inflammatory effect of OAG, is suggested by Souza et al. (2020)¹³ that investigated the biological potential of *A. gratissima* leaves extracts obtained by CO₂ supercritical fluid. In line with these observations, Apel et al. (2010)⁵⁸ demonstrated that an extract from *Myrciaria tenella* leaves, enriched in these compounds, present anti-inflammatory activity. Furthermore, Oliveira Júnior et al. (2017)^{40,59} showed that a *Croton* sp. extract obtained by hydrodistillation, enriched in caryophyllene and caryophyllene oxide, presents antinociceptive and anti-inflammatory properties. Phytochemical and pharmacological studies also provide evidence that guaiol is an important secondary metabolic with pharmacological activities.^{60,61} In this sense, we suggest that the antinociceptive and anti-inflammatory activities of OAG may be due to the presence of the above-cited secondary metabolites.

Herein, we used the formalin test to determine the minimum effective dose of OAG, which was 1 mg/kg. Before the performance of other behavioral tests related to the anti-inflammatory activity of OAG, we carried out the open field test, aiming to evaluate the OAG effects on the locomotor activity of the animals. The open field test demonstrated that OAG at 1 mg/kg did not induce undesirable locomotor effects and, therefore, cannot be considered a stimulant or sedative.⁶² Therefore, we can discard false positive/negative results of OAG in the subsequent behavioral tests that were carried out in this study.

Carrageenan-induced peritonitis is related to cell recruitment during inflammation and depends on the orchestrated release of local mediators that are responsible for vascular changes and cell migration to the peritoneal cavity provoked by the phlogistic stimulus.^{63,64} In addition, there is the production and release of inflammatory mediators, which recruit leukocytes, such as neutrophils, that participate in the inflammatory process.^{65,66} The peritonitis test is divided into two phases: the early one occurs around 1 h after the phlogistic agent administration, and the late phase, occurs between 3 and 6 h after administration and results in a polymorphonuclear leukocytes infiltration.^{26,41,67} Herein, we evaluated the effect of OAG in this experimental approach for the first time. The OAG reduced leukocyte infiltration 4 h after carrageenan application. This evidence demonstrates that its anti-inflammatory mechanism of action involves the inhibition of leukocyte migration in the peritoneal cavity (as well as the generation of AA metabolites and vascular permeability) similar to the steroidal anti-inflammatory dexamethasone.⁶⁸

Afoulous et al. (2013)⁶⁹ demonstrated that β -pinene is present in the essential oil of *Cedrelopsis* sp. leaves, which has anti-inflammatory activities, and this finding is consonant to ours since we found β -pinene (8.51 %) in the chemical composition of OAG. Furthermore, Apel et al. (2010)⁵⁸ and Tung et al. (2008)⁷⁰ reported the anti-inflammatory activity of isolated terpenes, specifically caryophyllene oxide, inhibiting the production of nitric oxide (NO) and prostaglandins E (PGE2) elicited by bacterial lipopolysaccharide (LPS). Thus, the presence of caryophyllene oxide (3.34 %) in OAG may contribute to its anti-inflammatory effect.

MPO enzyme activity is considered a direct marker of neutrophil and macrophage infiltration and its inhibition is an indicator of anti-inflammatory action.^{46,71,72} As expected, there was a reduction in the total leukocyte count in the peritoneal exudate from the animals treated with dexamethasone. However, there was no significant inhibition of MPO enzyme activity in the peritoneal exudate of OAG-treated animals. Noteworthy, there was a tendency to reduce MPO activity in the peritoneal exudate of that group (p = 0.058). This result is somehow paradoxical since the total leukocyte count of the OAG-treated group was

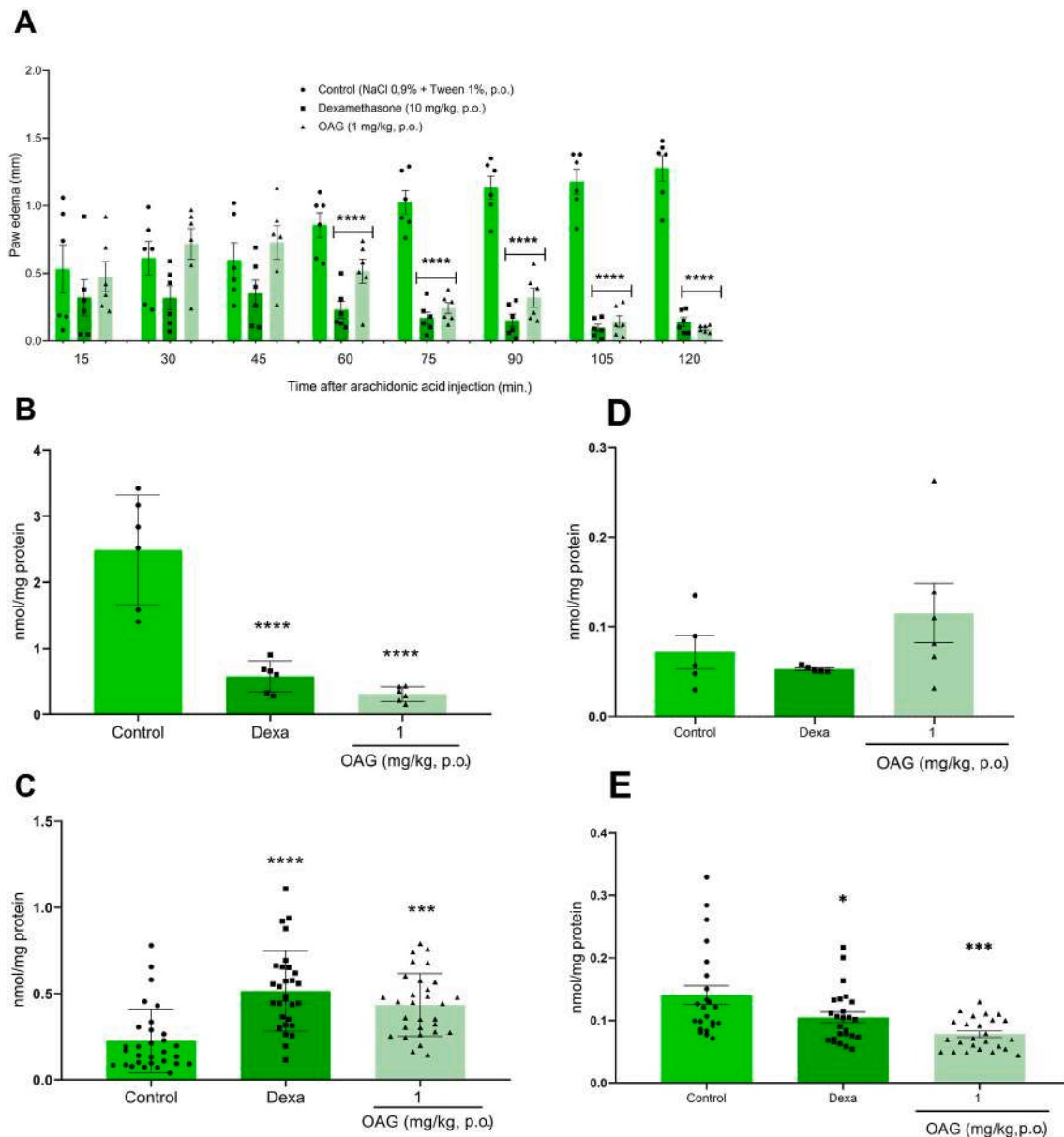


Fig. 6. Effect of *A. gratissima* leaf essential oil (OAG) on paw inflammation induced by the intraplantar injection of arachidonic acid. Mice ($n = 6$ animals/group) were orally treated with vehicle (V: 0.9 % NaCl + 1 % Tween 80, 10 ml/kg), dexamethasone (10 mg/kg) or OAG (1 mg/kg) 1 h after administration of 20 μ L of 1 % arachidonic acid i.pl. The paw edema was measured at 15, 30, 45, 60, 75, 90, 105, and 120 min after the intraplantar injection of arachidonic acid (A). The NPSH and TBARS levels were assessed in the plantar tissue (nmol NPSH/mg of tissue and nmol MDA/mg of tissue, respectively) (B, D) and the blood plasma (C, E) (nmol NPSH/mL and nmol MDA/mL, respectively) of the animals 120 min after the arachidonic acid injection. Results from (A) were analyzed by two-way repeated measures ANOVA followed by Bonferroni. Data from (B–D) were analyzed by one-way ANOVA followed by Bonferroni: * $p < 0.05$; *** $p < 0.001$ and **** $p < 0.0001$ different from the vehicle group. Results expressed as mean $\pm$ S.E.M.

reduced when compared to the control group. Nonetheless, we did not perform the differential leukocyte count in the peritoneal exudate of mice, which is a limitation of our study. Considering that MPO is highly expressed in neutrophils and macrophages, the leukocytes present in the peritoneal exudate of OAG-treated animals may be composed of cells that differ from the vehicle and dexamethasone-treated groups, but this hypothesis deserves further investigation.

We also investigated the effect of OAG on the production of PGE₂, the main inflammatory prostaglandin produced by COX-2, in peritoneal exudates from mice intraperitoneally injected with carrageenan. Interestingly, there were no differences in the PGE₂ levels between the experimental groups. Considering that activated macrophages release PGE₂,^{73,74} we suggest that this result is consonant with the lack of effect on the MPO activity of OAG-treated animals.

According to Barth (2016),⁷⁵ carrageenan is a classic inducer of inflammation, but the mechanisms by which it acts are still poorly understood, so, as in this study we performed the total leukocyte count and not just neutrophils or macrophages, this might suggest that carrageenan did not stimulate macrophage recruitment.

The AA metabolism pathway is one of the main mechanisms of induction of inflammation.⁷⁶ According to Sunita et al. (2012)⁶⁸ and Morgan (2021),²⁶ the model of paw AA injection is useful to detect lipoxygenase inhibitors. The measures of paw edema of mice treated with OAG reduced after 60 min of injection with AA and the reduction was maintained until the end of the analysis, in a similar way to the group treated with dexamethasone. Souza et al. (2020),¹⁹ report that the supercritical extract of *A. gratissima* leaves (10 mg/kg) reduced paw edema in mice induced with carrageenan up to 3 h after injection. The

anti-inflammatory effect of OAG in this model is possibly attributed to its chemical compounds, such as α -pinene (1.31 %), caryophyllene (5.05 %), and germacrene D (4.87 %). Li et al. (2016)⁷⁷ report that monoterpenes such as α -pinene have anti-inflammatory and analgesic effects in murine models and Oliveira-Tintino et al. (2018)⁴¹ describes that caryophyllene is an anti-inflammatory compound of a non-steroidal nature, that, in addition to the anti-inflammatory potential, has analgesic, antipyretic and platelet-inhibiting action.⁴¹

The antioxidant properties of the terpenes present in OAG cannot be attributed to just one compound, but to the whole, in a significant way.^{71,72} We evaluated whether the anti-inflammatory effect of OAG is linked to mechanisms of antioxidant action since reactive oxygen species are molecules that play an important role in the progression of inflammatory disorders. The production of oxygen free radicals triggered by inflammation leads to a decrease in NPSH levels, as a consequence of its consumption during oxidative stress and cell lysis.^{20,72} Animals treated with OAG and dexamethasone showed increased levels of NPSH in blood as well as a reduction in TBARS levels, which is widely used to assess the relative degree of lipid oxidation during inflammation.⁷³ These findings suggest that the OAG may exert its anti-inflammatory effect by activating the organism's antioxidant defense system. In the paw tissue, we found a reduction in NPSH levels of OAG and dexamethasone-treated animals and no alterations in the TBARS levels. The arachidonic acid-induced paw edema is related to an acute inflammatory process, which results in the formation of reactive oxygen species that can damage cells and tissues. In this context, the decrease in NPSH levels may indicate a consumption of this antioxidant, to avoid oxidative stress, at least, at the inflammation site. Briefly, these results demonstrate that oral administration of OAG can exert an anti-inflammatory effect and modulate the cellular antioxidant defense system.²⁰ Considering that inhibition of PGE2 is not involved in the mechanism of OAG anti-inflammatory action, we infer that this mechanism of action may represent an advantage in comparison to several vegetal species that exhibit anti-inflammatory properties through the inhibition of cyclooxygenases (COX), such as *Camellia sinensis*⁷⁸, *Zingiber officinale*⁷⁹, *Salix alba*⁸⁰. The non-selective inhibition of COX is related to several adverse events, including gastrointestinal damage.^{5,6}

5. Conclusion

The oil from the leaves of *A. gratissima* (OAG) presents different chemical components, being the terpenes pinocamphone, guaiol, and β -pinene the major ones. The OAG is devoid of acute toxicity and showed significant anti-inflammatory properties, which are, at least in part, mediated by reduced leukocyte recruitment and NPSH consumption. Considering that major terpenes present in OAG are known to be antinociceptive and anti-inflammatory, the biological activity of OAG found here may be attributed to the presence of these compounds. Our findings, therefore, support the traditional use of *A. gratissima* as analgesic and anti-inflammatory in popular medicine.

CRediT authorship contribution statement

L.G.M., Conceptualization, Resources, Supervision, Funding acquisition; M.A.S., K.K., O.S., A.C.P., M.B., M.S.Z.S., S.C.M., I.B., Investigation, Data curation, Formal analysis; J.V.O., J.S., J.D.M., Conceptualization, Resources, Supervision; M.A.S., J.S., J.D.M., L.G.M., Writing – original draft, Writing - review & editing.

Declarations of interest

None.

Ethics statement

The animal experiments were approved by the institutional ethics

committee (Approval number #022/2020). All methods were carried out in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>) for the reporting of animal experiments.

Data statement

All data generated and/or analyzed during this study are included in this publication article. Notwithstanding, all datasets are made available by the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Original Article

Antinociceptive effect and mechanism of supercritical carbon dioxide extract of *Aloysia gratissima* leaves in mice

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ABSTRACT

Background: *A. gratissima* is a shrub used in folk medicine as analgesic and sedative. However, studies on its antinociceptive activity are scarce. This research aimed to evaluate the antinociceptive effect of a supercritical carbon dioxide (SCCO₂) extract of *A. gratissima* leaves (EAG) in mice.

Methods: *A. gratissima* leaves were subjected to extraction with supercritical CO₂ (60 °C, 200 bar). The chemical composition of EAG was determined by gas chromatography–mass spectrometry (GC–MS). The antinociceptive profile of the extract (1, 10 and 30 mg/kg, p.o.) was established using acetic acid-induced abdominal contraction tests and formalin-induced paw-licking tests. The open field and rota-rod tests were used to evaluate a possible interference of EAG on mice motor performance. The contribution of the opioid system and adenosine triphosphate (ATP) sensitive K⁺ channels in the mechanism(s) of EAG action was evaluated by specific receptor blockers. EAG's acute toxicity was investigated using OECD 423 guideline.

Results: The GC–MS revealed the presence of sesquiterpenes (guaiaol and pinocamphone) in the EAG. Doses of 10 mg/kg and 30 mg/kg significantly reduced the number of abdominal writhes and paw licking time in mice in the formalin test. The EAG did not affect the locomotor activity and motor coordination of the mice. The antinociceptive effect of the EAG was prevented by glibenclamide in the mice formalin test, unlike naloxone pre-treatment. The acute administration of EAG caused no mortality.

Conclusion: *A. gratissima* leaves possess antinociceptive effect, mediated by K⁺ channels sensitive to ATP.

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At a glance of commentary

Scientific background on the subject

Aloysia gratissima aerial parts are traditionally used as sedative and for the treatment of headache. The essential oil from *A. gratissima* presents sesquiterpenes, which are related to the pharmacological action of plants.

What this study adds to the field

Our study demonstrated the presence of sesquiterpenes (guaialol and pinocamphone) in the supercritical CO₂ extract of *Aloysia gratissima* leaves and confirmed its antinociceptive activity in mice. This effect is mediated by K⁺ channels sensitive to ATP.

The scientific interest in researching compounds derived from plant extracts is associated with the knowledge of folk medicine [1]. The secondary metabolites present in these natural compounds can have their biological activity evaluated through in vivo experimental tests, so that their functionalities as drugs, food and cosmetics can be proven [2,3]. Among the species popularly known for their pharmacological actions, are the ones from the *Aloysia* genus, belonging to the Verbenaceae family, native to South America [4].

In Brazil, the species *Aloysia gratissima* (Gillies & Hook) Tronc. is commonly grown as an ornamental plant, being known as "Alfazema do Brasil" [5]. Aerial parts of this plant are used for the treatment of headache, bronchitis, as well as anxiety, depression and digestive disorders [6,7]. The essential oil from *A. gratissima* presents more than 70% of hydrocarbons, from which more than half are sesquiterpenes. This is particularly interesting, since the pharmacological action of plants is related to the presence of metabolites such as alkaloids, terpenes, flavonoids and phytosterols [5].

The use of natural products in the food and pharmaceutical industry, provides alternative results to current needs [8]. Additionally, obtaining compounds through pressurized fluids, such as carbon dioxide and propane, have been presented as alternatives, due to the use of non-toxic, non-flammable and low-cost solvents [8]. In this context, the extraction and study of the compounds present in these plant matrices through supercritical fluids is an interesting approach [8].

The presence of terpenes in plants, such as caryophyllene, caryophyllene oxide, guaialol, pinocamphone and spathulenol is described by several authors as the possible reason for the antinociceptive action of different extracts that present these compounds [9–12]. Furthermore, it has been demonstrated that terpenes are able to exert antinociceptive effects by opening K⁺ channels [13], which leads to K⁺ efflux and cell membrane hyperpolarization [14]. This effect, therefore, reduces the excitability of peripheral neurons and the release of neurotransmitters (e.g. substance P) in the spinal cord [15–17]. Different types of K⁺ channels are involved in antinociception: the adenosine triphosphate (ATP)-sensitive K⁺ channels, activated via several different drugs (including terpenes such as

caryophyllene oxide and guaialol [11]) in both the central and the peripheral nervous system; and the G-protein-regulated inwardly rectifying K⁺ channels, that open through the G-protein activation in neurons [18]. These kinds of K⁺ channel activations are both involved in opioid induced antinociception [14]. Interestingly, some terpenes exert their antinociceptive activity by activating opioid receptors [19–22], thus reducing neurons' excitability and the release of neurotransmitters.

Considering the presence of terpenes in *A. gratissima*, it becomes important to investigate, for the first time, the effect of this plant species on animal models of nociception and its mechanisms of action. The present work evaluated the antinociceptive activity of a supercritical CO₂ extract of *A. gratissima* leaves in models that use different stimuli in mice, and also investigated the mechanisms of action involved in its antinociceptive activity.

Materials and methods

Chemicals

Carbon dioxide (99.9% non-liquid purity phase) was purchased from Air Liquide®. Acetic acid and formaldehyde were purchased from Merck®. Ketorolac and glibenclamide were obtained from EMS Sigma Pharma®. Naloxone and morphine were purchased from Cristália® and indomethacin was obtained from Sigma®.

Plant material

A. gratissima leaves were collected in December 2017 (summer), in the municipality of Erval Grande, RS, South Brazil (27°23'14.3S, 52°33'49 'W). This region presents a humid subtropical mesothermic climate. The plant specimen was deposited in the Herbarium of the Community University of Chapecó (Herbário Unochapecó, SC, Brazil) under the accession number UNO 3700. After collection, the leaves were manually separated and dried at room temperature for five days. Afterwards, they were packed in plastic bags, identified and stored at 7 °C until extraction process.

Extraction of the *A. gratissima* leaves by supercritical CO₂ (SCCO₂)

The experimental extraction apparatus and procedure have been described in detail in other studies of the research group [23]. *A. gratissima* leaves were extracted with SCCO₂ at 60 °C and 200 bar (density 724 kg/m³). Approximately 11.03 ± 0.09 g of *A. gratissima* leaves were placed into the extraction vessel. Then, CO₂ was pumped into the bed, which had two 300 mesh wire disks at both ends and was held in contact with the sample array to allow the system to stabilize at the same condition as the experiment by 30 min. After stabilization, the extract from *A. gratissima* was then collected by opening the micrometric valve with 2 h of extraction time.

GC/MS analysis

Extract of *Aloysia gratissima* leaves (EAG's) chemical composition was analyzed by Agilent GC/MS (7890B) gas chromatography coupled to a quadrupole mass spectrometer (5977A) (Agilent Technologies, Palo Alto, CA, USA). GC/MS system's experimental condition were described by Scapinello et al. (2018) [24] with some modifications. Briefly, the system conditions were: Agilent 19091S capillary column, dimension: 30 m $\times$ 250 μ m $\times$ 0.25 μ m. The mobile phase flow (carrier gas: He) was adjusted to 1.0 mL min⁻¹. The GC temperature program was 40.0 °C at 4 min to 240.0 °C at a rate of 10 °C min⁻¹ and up to 300.0 °C at a rate of 40.0 °C min⁻¹ (maintained for 5 min). The injector temperature was 280.0 °C, sample injection volume 1 μ L, split ratio 1:20. The MS transferline temperature was set to 150.0 °C and the source of ions temperature was set at 230.0 °C. For GC–MS detection, an electron ionization system was used with ionization energy set at 70 eV, and mass range m/z 40–400. The chemical components presented in the extract were identified by comparison with the equipment library (Agilent P/N G1033A). The relative amounts of each individual component were calculated using their respective peak areas in the chromatogram. The extract was solubilized in dichloromethane to be analyzed.

In vivo experiments

Animals

Male Swiss mice (25–35 g) bred at the Community University of Chapecó Region (Unochapecó) bioterium were used in all pharmacological experiments. The animals were kept in a controlled environment under standard conditions (22 $\pm$ 2 °C) with a light/dark cycle of 12 h (lights on at 6:00 a.m. to 6:00 p.m.), fed standard laboratory feed and water *ad libitum*. Animal care and experiments were conducted in accordance with the animal research ethical principles, approved by the Ethics Committee of the university (Approval number 004–18), in accordance with Brazilian law No. 11794 and Council of the European Communities; Directive of 24 November 1986 (86/609/EEC). The animals were fasted for a period of 2 h (no water restriction) prior to administration of any test substance.

Mice were treated with volumes of 10 mL/kg, according to their weight, through oral gavage (p.o.) or intraperitoneal (i.p.) routes and with 20 μ L by intraplantar (i.pl.) injection, according to each experiment's specific protocol. Solubilization of the extract and substances was performed in saline (NaCl 0.9%) with the aid of 1% Tween 80 (v/v) and ultrasound. The extract's tested doses were chosen based on the study of Zeni et al. (2013) [4].

Acute toxicity

The acute toxicity study was based on Guideline 423 (2001) of the Organization for Economic Cooperation and Development (OECD). According to OECD 423, the study in female mice is recommended for the possible sensitivity to the acute toxic effects of the chemicals used in the research [25]. Female mice ($n = 6$) were orally treated with EAG at 2000 mg/kg. The control group ($n = 3$) received the administration of vehicle (0.9% NaCl,

1% Tween 80, 10 mL/kg). Thereafter, the animals were observed with special attention during the first 4 h after treatment and daily for 14 days. The occurrence of mice death, and signals such as piloerection, hypothermia, palpebral ptosis, abdominal writhing, muscle tone, shacking, hind paw paralysis, salivation, bronchial secretion and seizures were registered. Additionally, body weight and food consumption were recorded for 13 days. At the end of the experiment, the macroscopic aspect of the organs (liver, kidneys, adrenal glands, spleen, lungs, heart and brain) as well as their relative weight (%) were registered.

Acetic acid-induced writhing response

The abdominal writhing test induced by acetic acid has been used as a screening tool to evaluate analgesic or anti-inflammatory agents, as acetic acid induces an inflammatory response in the abdominal cavity, with subsequent activation of nociceptors [26]. In this test, mice writhing is a response to the pain characterized by episodes of arching of the back, extension of hind limbs and contraction of abdominal musculature [27]. Mice ($n = 6$ –8/group) were treated with EAG (1, 10 and 30 mg/kg, p.o.) or vehicle (0.9% NaCl + 1% Tween 80, p.o., control group) 1 h before the injection (i.p.) of acetic acid (0.6%, 10 mL/kg). Immediately after the injection, the animals were gently placed in a transparent apparatus (20 cm diameter glass box) and the number of abdominal contortions was counted cumulatively over a period of 20 min [28] by a human observer blind to the treatment. The positive control was the non-steroid anti-inflammatory indomethacin (10 mg/kg, p.o.). The dose that presented the best result in this experiment was chosen to be used in the other tests of nociception.

Open-field test

The open-field test was performed in order to evaluate the possible EAG effects on locomotor and exploratory activities of mice. The experimental protocol was based on the method described by Müller et al. (2012) [29]. The animals ($n = 6$ /group) received orally the minimal effective dose (10 mg/kg, p.o.) of EAG that was able to reduce the nociceptive behavior in the acetic-acid writhing test, 1 h before being exposed to the open-field arena. The control group was treated with vehicle (0.9% NaCl + 1% Tween 80, p.o.). The arena consisted in an acrylic box (40 $\times$ 30 $\times$ 30 cm), with the floor divided into 24 equal squares. After the habituation (5 min), the number of squares crossed with the four paws (crossing), rearings and groomings was recorded for 10 min. The number of fecal bolus was counted after the test.

Rota-rod test

The rota-rod test was used to evaluate the EAG effects on mice motor coordination. This test was performed as described by Neves et al. (2010) [30] with minor modifications. The apparatus was a cylinder (4 cm of diameter), rotating at 3 rpm. Animals were individually habituated to the apparatus for 5 min. Twenty-four hours later, they were trained for 5 min and only the ones that were able to stay 90 s on the rotating rod were selected for testing. Immediately after, mice ($n = 7$ /group) were orally treated with vehicle (NaCl 0.9% + 1% tween 80), indomethacin (10 mg/kg) or EAG (10 mg/kg). One hour

Table 1 Chemical composition of the *A. gratissima* leaves supercritical CO₂ extract.

Chemical compound	Area of each compound (%)
Pinocarvil	5.31
Pinocamphone	11.4
Isopinocamphone	3.52
Myrtenol	3.24
(-)- trans-pinocarvila acetate	10.5
Caryophyllene	7.63
γ – Elemene	7.28
Humelene	2.97
(-)- Spathulenol	6.17
Caryophyllene oxide	6.76
β - Cubebene	8.21
Guaiol	18.5
Bunesol	4.67
Total	96.1

later, mice performance in the rota-rod was evaluated considering the longest time of permanence on the apparatus and the number of falls, in a 5 min period.

Formalin test

The experimental procedure was similar to the one described by Santos and Calixto (1997) [31]. Briefly, the animals ($n = 6$ /group) were orally treated with vehicle (0.9% NaCl + 1% Tween 80) or EAG (10 mg/kg) 1 h before the injection of 2% formalin (20 μ L/paw, i.p.) in the right hind paw. Indomethacin (administered orally 1 h before the behavioral test, 10 mg/kg) was the positive control. Immediately after the formalin injection, the time spent licking, biting or lifting the injected hind paw (nociceptive behavior) was registered during the first (0–5 min, neurogenic phase) and the second phases (15–30 min, inflammatory phase) of the test.

Involvement of the opioid system and K⁺ channels sensitive to ATP

In order to assess the opioid system's involvement in the antinociceptive action mechanism of the EAG, mice were pre-treated with naloxone (a non-selective opioid receptor

antagonist; 2 mg/kg, i.p.) or vehicle (0.9% NaCl, i.p.) [32,33]. After 15 min, the animals ($n = 4$ –7/group) were treated with EAG (10 mg/kg, p.o.), morphine (opioid receptor agonist, positive control, 5 mg/kg, s.c.) or vehicle (0.9% NaCl, 1% Tween 80, p.o.).

The involvement of the ATP-sensitive K⁺ channels in EAG's antinociceptive effect was evaluated according to Zapata-Morales et al. (2017) [34]. Briefly, the animals ($n = 4$ –7/group) were pre-treated with glibenclamide (K⁺ channels sensitive to ATP blocker; 20 mg/kg, i.p.) or vehicle (0.9% NaCl, i.p.). The EAG (10 mg/kg, p.o.), ketorolac (non-steroid anti-inflammatory that presents antinociceptive effects mediated by ATP-sensitive K⁺ channels [35], positive control, 20 mg/kg, i.p.) or vehicle (0.9% NaCl + 1% Tween 80, p.o.) were administered to the animals 15 min after the pretreatment.

One hour after the treatments, the nociceptive behavior was evaluated in the formalin test, immediately after the i.p. injection of formalin 2%.

Statistical analysis

Statistical analyses were carried out using Graph Pad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Results from the investigation of the mechanism of action were analyzed by two-way ANOVA followed by Student-Newman-Keuls test. Rota-rod test results and toxicity data were analyzed by two-way repeated measures ANOVA. The relative weight of the organs in the toxicity test was analyzed by Unpaired t test. The results are expressed as the mean $\pm$ S.E.M. of n animals per group. Values of p less than 0.05 ($p < 0.05$) were considered significant.

Results

Table 1 shows the extract's chemical composition. The GC/MS analysis revealed that several terpene compounds are present in the EAG. The major compounds found in EAG were guaiol (18.50%) and pinocamphone (11.40%), in addition to the

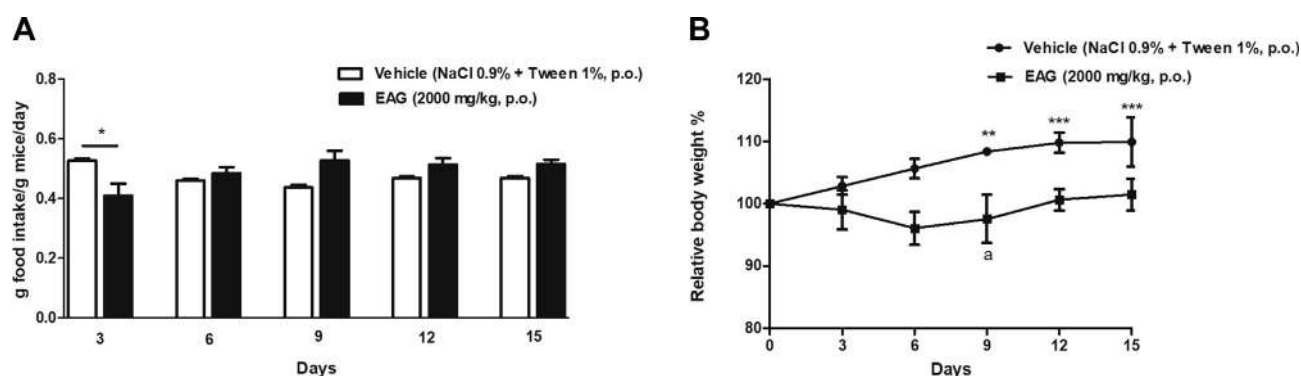


Fig. 1 Effect of the *A. gratissima* leaves supercritical extract (EAG) acute treatment (2000 mg/kg, p.o.) on food intake (A) (g food intake/g mice/day) and relative body weight (%) (B) of female Swiss mice. Data are expressed as mean $\pm$ S.E.M. ($n = 3$ –6 mice/group). Two-way repeated measures ANOVA followed by Student-Newman-Keuls. ** $p < 0.01$ and *** $p < 0.001$ represent differences in relation to the first measure (Day 0) in the same group treatment. * $p < 0.05$ different from the vehicle-treated group at the same day of treatment.

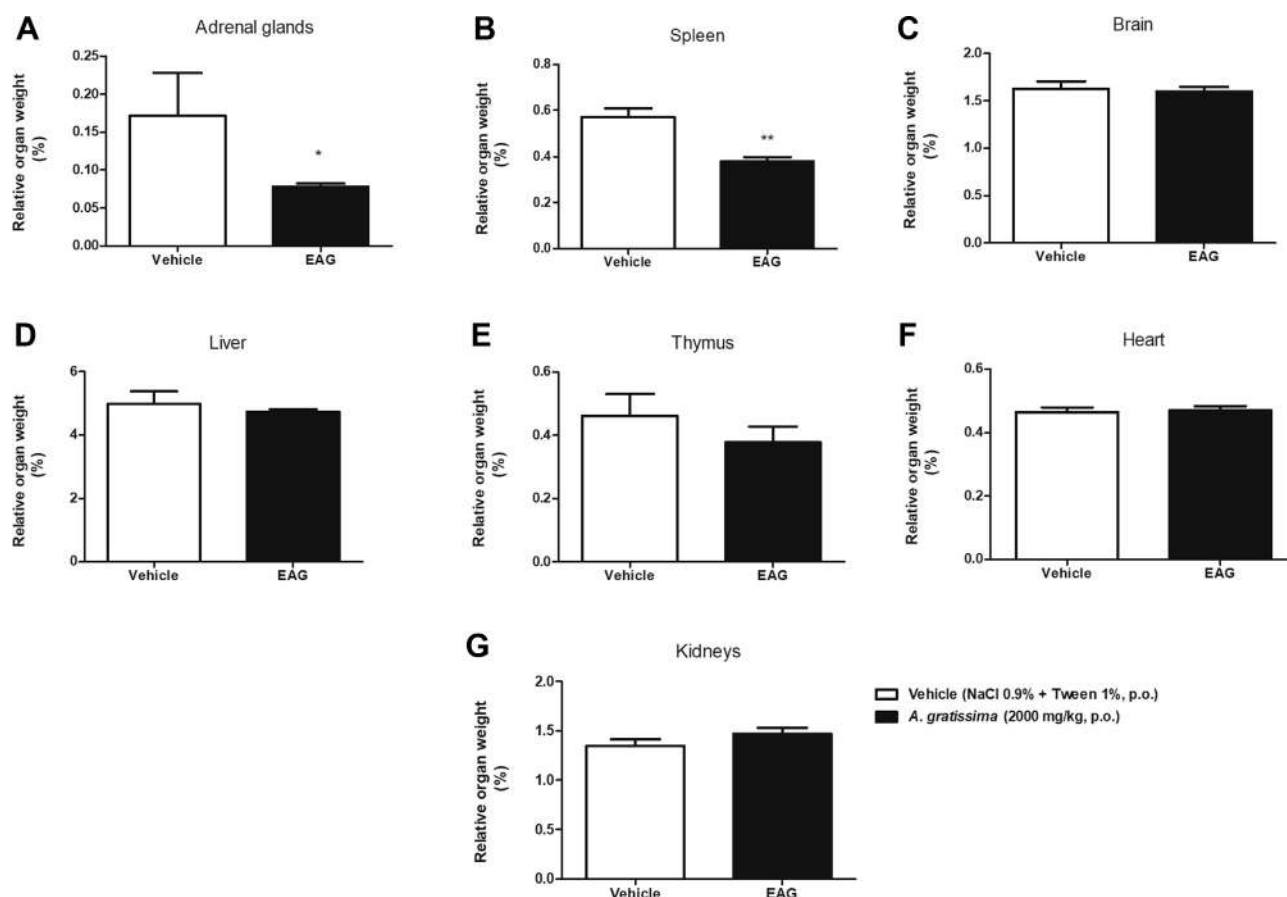


Fig. 2 Effect of *A. gratissima* leaves supercritical extract (EAG) on the relative weight of female Swiss mice organs (%) in the acute oral toxicity test. (A) adrenal glands; (B) spleen (C) brain; (D) liver; (E) thymus; (F) heart and (G) kidneys. The mice ($n = 3-6$) were orally treated with vehicle (NaCl 0.9% + tween 1%, 10 ml/kg) or EAG (2000 mg/kg). Unpaired t test, * $p < 0.05$, ** $p < 0.01$.

presence of other compounds such as pinocarvil, (–)-trans-pinocarvilacetate, γ -elemene, bunesol, caryophyllene, caryophyllene oxide, (–)-spathulenol, myrtenol, isopinocampheol, β -cubebene, in smaller quantities.

The administration of EAG (2000 mg/kg, p.o.) induced an intense sedation in the mice, which lasted 45 min. After recovery, mice fed normally, with no locomotor activity changes. Food intake [Fig. 1A] of EAG-treated mice was significantly ($p < 0.05$) lower than of vehicle-treated mice at the 3rd day after treatment. Mice treated with vehicle presented a significant increase in the body weight [Fig. 1B] at the 9th ($p < 0.01$), 12th ($p < 0.001$) and 15th ($p < 0.001$) days of observation in comparison to initial weight. There were no changes in the body weight of EAG-treated animals during the period of observation. Also, no death was recorded.

Mice treatment with EAG (2000 mg/kg, p.o.) did not cause changes in the relative weight (%) of the brain, liver, kidneys, lung, heart and thymus [Fig. 2]. However, there was a significant decrease in the relative weight of the adrenal glands ($p < 0.01$) and spleen ($p < 0.05$) of EAG-treated mice in comparison to vehicle-treated mice.

The acetic acid injection evokes abdominal contractions in rodents, since it induces the peripheral production of several pro-inflammatory mediators. The EAG at 10 and 30 mg/kg ($p < 0.05$) and indomethacin at 10 mg/kg ($p < 0.01$) reduced the

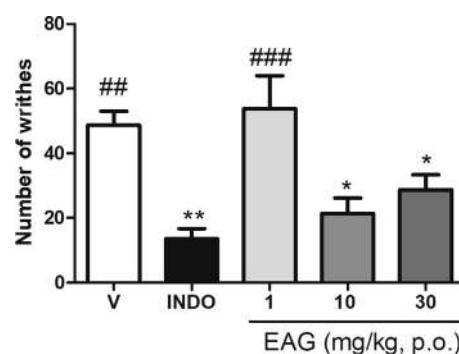


Fig. 3 Effect of a supercritical CO₂ extract of *Aloysia gratissima* leaves (EAG) in the acetic acid-induced abdominal writhing test in mice. V: vehicle treated group (0.9% NaCl + 1% Tween, 10 mL/kg p.o., $n = 8$). INDO: indomethacin (10 mg/kg p.o., $n = 6$) or EAG (1, 10 and 30 mg/kg p.o., $n = 6-8$), 1 h prior to acetic acid administration. Each column represents the mean $\pm$ S.E.M. One-way ANOVA followed by the Student-Newman-Keuls test, *** $p < 0.01$ and * $p < 0.05$ different from the vehicle group; ### $p < 0.001$ and ## $p < 0.01$ different from the INDO group.

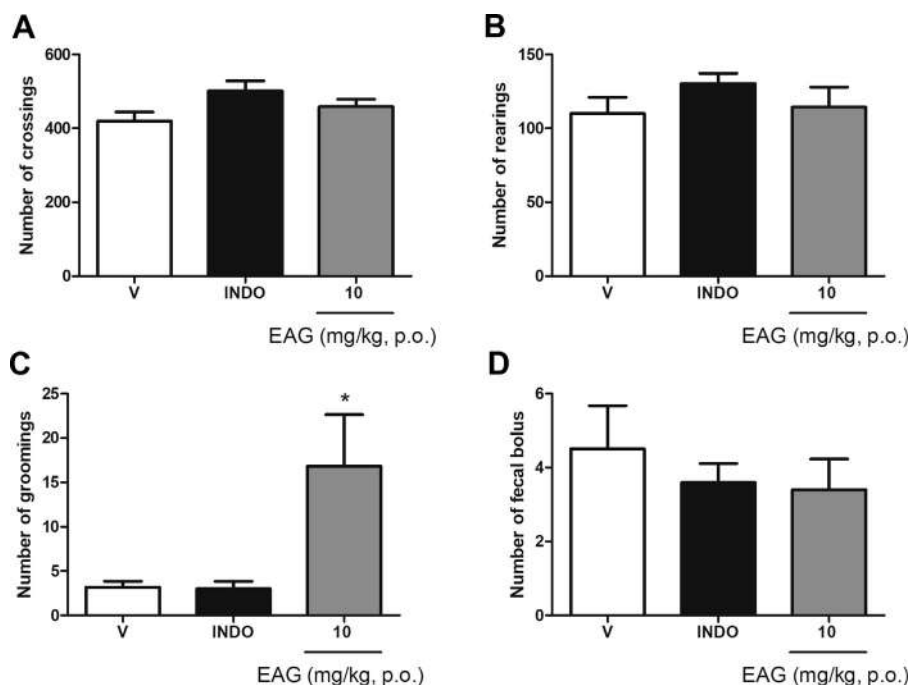


Fig. 4 Effect of a supercritical CO₂ extract of *Aloysia gratissima* leaves (EAG) on mice locomotor activity (open field test). A: number of crossings. B: number of rearings. C: number of groomings; D: number of fecal bolus. V: vehicle treated group (0.9% NaCl + 1% Tween, p.o., n = 6). INDO: indomethacin (10 mg/kg, n = 6). EAG (10 mg/kg, p.o., n = 6). Each column represents the mean $\pm$ S.E.M. One-way ANOVA followed by the Student-Newman-Keuls test, * $p < 0.05$ different from the vehicle group.

number of abdominal writhes provoked by the injection of acetic acid when compared to the vehicle group. On the other hand, the EAG at the lowest dose (1 mg/kg) was not effective in reducing acetic acid-induced writhes [Fig. 3]. The abdominal contortion test was applied to investigate the lowest effective dose, in order to continue the other tests.

Considering that a non-specific effect of the EAG on animals' locomotion could influence the results of the antinociceptive tests and, therefore cause false positive or negative results, we investigated the effects of the minimal effective dose of EAG (10 mg/kg, p.o.) in the open field test. Treatment with EAG and indomethacin (10 mg/kg, p.o.) did not alter the numbers of crossings [Fig. 4A], rearings [Fig. 4B] and the number of fecal bolus [Fig. 4D]. The grooming number [Fig. 4C] for EAG-treated animals was significantly ($p < 0.05$) higher when compared to the groups treated with indomethacin or vehicle.

Additionally, the treatment with EAG (10 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.) did not change mice motor coordination, compared to the vehicle group (NaCl 0.9%, p.o.) [Table 2].

The EAG at 10 mg/kg as well as indomethacin produced antinociception in the first phase ($p < 0.01$ and $p < 0.05$, respectively) – the neurogenic phase [Fig. 5A] – and in the second phase ($p < 0.001$) – inflammatory pain phase [Fig. 5B] of the formalin test. The investigation of the EAG action mechanism revealed that mice underwent pre-treatment with naloxone did not reverse the EAG antinociceptive activity in the two phases of formalin test [Fig. 6A and B], showing that the opioid system is unlikely to be involved in antinociceptive action. However, EAG's effect was prevented by mice who had pre-treatment with glibenclamide in the two formalin phases [Fig. 7A and B], thus suggesting that the K⁺ channels sensitive to ATP are involved in the mechanism of antinociceptive effect of EAG.

Table 2 Effect of a supercritical CO₂ *A. gratissima* leaves extract (EAG) on mice motor coordination (assessed by the Rota–Rod test). The animals (n = 7/group) were orally treated with vehicle (NaCl 0.9% + 1% tween 80), indomethacin (10 mg/kg) or EAG (10 mg/kg) 1 h before the test. Data are expressed as Mean $\pm$ S.E.M. Results were analyzed by Two-Way repeated measures ANOVA.

Group	Length of stay (s)	Number of falls
Vehicle	300.0	0.0
Indomethacin	300.0	0.0
EAG	299.6 $\pm$ 0.3	0.11 $\pm$ 0.03

Discussion

The solubility of chemical components obtained in the supercritical fluid is related to the fluid density. When CO₂ is used in the extraction of oils, it demonstrates good capacity for solubilization due to low polarity, without altering the chemical composition and the extract produced is free of solvent contamination [36]. The chemical composition of the *A. gratissima* leaves supercritical extract analyzed by GC/MS revealed the presence of terpene compounds, being guaiaol and

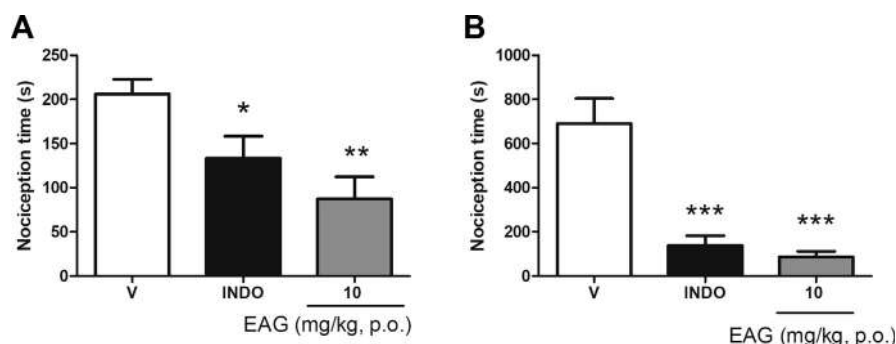


Fig. 5 Effect of a supercritical CO₂ extract of *Aloysia gratissima* leaves (EAG) on the formalin test. Nociceptive behavior was considered as the time (s) of elevation, biting or licking of the paw in the first phase (A: 0–5 min) and second phase (B: 15–30 min) of the test. Mice (n = 6 per group) were treated with vehicle (V: NaCl 0.9% + 1% tween 80, 10 ml/kg), Indomethacin (INDO: 10 mg/kg) or EAG (10 mg/kg) 1 h prior to administration of formalin 2% i.p. One-way ANOVA followed by Student-Newman-Keuls: **p* < 0.05; ***p* < 0.01 and ****p* < 0.001 are different from the vehicle group. Results expressed as mean ± S.E.M.

pinocamphone the major ones. These compounds are commonly found in species from the *Aloysia* genus, and their antibacterial and antitumor activities have already been demonstrated [37,38]. Terpenes have been known to show a wide range of pharmacological activities, including effects on the central nervous system, antinociceptive, anti-inflammatory, antimicrobial and antitumor properties [39].

Mice treatment with the EAG (2000 mg/kg, p.o.) did not change the weight of the brain, liver, kidneys, lung, heart and thymus. However, there was a significant decrease in the relative weight of the spleen and adrenal glands in EAG-treated mice. Moreover, EAG-treated animals did not gain weight as vehicle-treated ones. This may suggest a sign of toxicity, which should be further investigated in a repeated dose toxicity test. The decrease in food intake of EAG-treated animals at the 3rd day after administration may be due to the sedation caused by the extract [6,7]. Zeni et al. (2013) [13] studied the toxicological effects of a hydroalcoholic extract of *A. gratissima* aerial parts and demonstrated that it induces hepatic toxicity in male mice at 2000 mg/kg. However, in the present study, we demonstrated the acute toxicity of supercritical *A. gratissima* leaves extract against mice adrenal

glands and spleen. The differences between both findings may be due to the gender of mice and the type of extraction used in the experiments. Nonetheless, both extracts from the same vegetal species have proven to be harmless, since no deaths occurred after the oral administration at 2000 mg/kg. Our results show that the EAG could be included in Category 5 of the Harmonized Global Classification System (OECD Guideline 423, 2001) [25], since its LD₅₀ is above 2000 mg/kg.

It is known that the intraperitoneal acetic acid injection in rodents evokes abdominal writhing, since it induces the peripheral production of several pro-inflammatory mediators, such as prostaglandins, bradykinin, substance P, prostacyclin and other cytokines, which, therefore, excite the nociceptors in the nerve endings [40]. The protective effect of substances against the noxious chemical stimulus may be an indication for a decreased production of these mediators, thus causing a reduction in the number of writhes [41]. The EAG at 30 mg/kg reduced abdominal writhing (on average 41%) compared to the vehicle group, while the dose of 10 mg/kg resulted in an average reduction of 56%, both doses being similar to the group that received indomethacin, the positive control. These results may be related to the presence of pinocamphone and

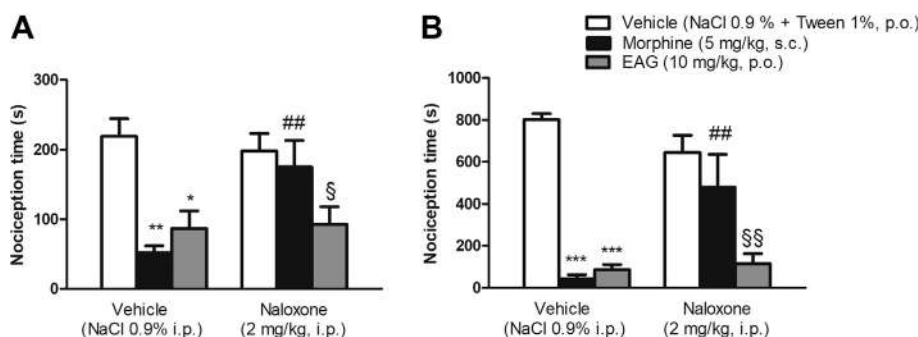


Fig. 6 Effect of mice pre-treatment with naloxone (2 mg/kg, i.p.) on the antinociceptive effect of a supercritical CO₂ extract of *Aloysia gratissima* leaves (EAG) (10 mg/kg) on the formalin test. Nociceptive behavior in the first phase (A, 0–5 min) or second phase (B, 15–30 min) of the test. Morphine (5 mg/kg, s.c.) was used as positive control. Results expressed as mean ± S.E.M. (n = 4–7 mice/group). Two-Way ANOVA followed by Student-Newman-Keuls test, **p* < 0.05; ***p* < 0.01; ****p* < 0.001 compared to the vehicle plus vehicle-treated group. ##*p* < 0.01 compared to the vehicle plus morphine-treated group; §*p* < 0.05; §§*p* < 0.01 compared to the vehicle plus naloxone-treated group and morphine plus naloxone-treated group.

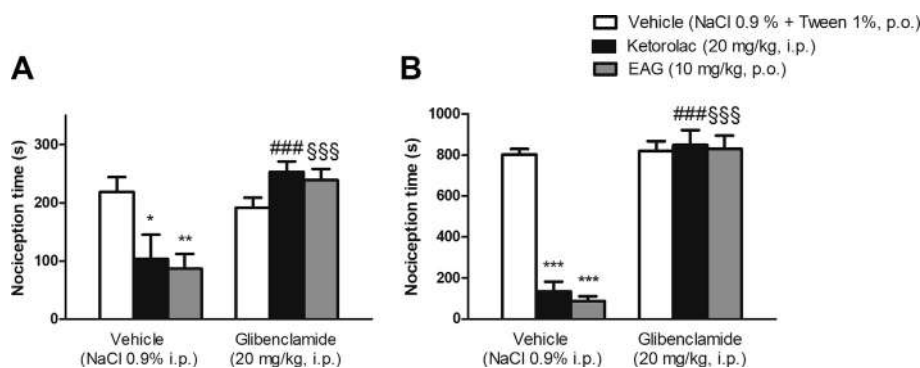


Fig. 7 Effect of mice pre-treatment with glibenclamide (20 mg/kg, i.p.) on the antinociceptive effect of a supercritical CO₂ extract of *Aloysia gratissima* leaves (EAG) (10 mg/kg, p.o.) on the formalin test. Nociceptive behavior in the first phase (A, 0–5 min) or second phase (B, 15–30 min) of the test. Ketorolac (20 mg/kg, i.p.) was used as positive control. Each column represents the mean $\pm$ S.E.M. (n = 4–7 mice/group). Two-Way ANOVA followed by the Student-Newman-Keuls test, * p < 0.05; ** p < 0.01; *** p < 0.001 compared to vehicle plus vehicle-treated group. ### p < 0.001 compared to the vehicle plus ketorolac-treated group; \$\$\$ p < 0.01 compared to vehicle plus EAG-treated group.

guaïol associated to caryophyllene oxide and spathulenol in EAG's chemical composition [42,43].

The acetic acid model is a preliminary test used to screen new analgesic drugs and was applied in our study to investigate the minimal antinociceptive dose of EAG, in order to continue the other tests. The lowest effective dose was used because of lower adverse effect's possibility. In order to investigate EAG's false-positive results in the nociception tests, mice behavior in the open field and rota-rod tests were evaluated [44].

The results obtained in the open field tests demonstrate that EAG (10 mg/kg) did not induce undesirable effects on the animals' locomotor activity and is not sedative or hyper-stimulant. The number of groomings was the only behavior changed in comparison to the vehicle group. Considering that the grooming behavior has been used to measure pharmacologically induced anxiolytic-like effects in rodents [44,45], we may infer that the increase in mice grooming elicited by the EAG might be related to its anxiolytic property [6,7].

In order to investigate EAG's effect on mice motor coordination, we used the rota-rod test. The results of indomethacin, vehicle and EAG treated animals were not different between them in the rota-rod test, suggesting that EAG does not impair the motor coordination and, therefore does not induce false-positive results in other behavioral tests.

The formalin test aims to explore the analgesic effect of substances through central and peripheral pain mechanism [46]. The antinociceptive activity may occur in two distinct phases. In the first phase, neurological pain is induced by the chemical stimulation of afferent sensory fibers, particularly C fibers, whereas in the late phase, pain is caused by inflammatory mediators' production such as: prostaglandins, histamine, bradykinin, and serotonin [47].

The EAG elicited antinociception both in the first and second phases of the formalin test in mice and therefore, we can suggest that the EAG is effective in the treatment of neurogenic as well as inflammatory pain. Indeed, EAG's antinociceptive and anti-inflammatory activities are probably related to its main constituents. The presence of guaïol and spathulenol may be responsible for the EAG anti-

inflammatory effect, as suggested by the study of Apel et al. (2010) [10], with *Myrciaria tenella* leaves extract, enriched in these compounds.

A number of authors evaluated the antinociceptive and anti-inflammatory activities of plant extracts that present the caryophyllene oxide as one of the main constituents. De Oliveira Júnior et al. (2017) [11] reported that *Croton conduplicatus*' extract obtained by hydrodistillation showed antinociceptive and anti-inflammatory activities, being the caryophyllene and caryophyllene oxide the main constituents of the extract. Caryophyllene oxide is also the main component of *Myrcia pubiflora* DC leaves essential oil, obtained by hydrodistillation, and its antinociceptive and anti-inflammatory activities have been proven in *in vivo* experimental models [12].

Opioids act at the cellular level by binding to the opioid receptors present throughout the central nervous system, so the ultimate effect is the reduction of neuronal excitability, resulting in reduced neurotransmission of nociceptive impulses [48]. Pure opioid agonists (such as morphine) have high affinity for opioid receptors. To verify whether opioid receptors mediate EAG's antinociceptive effect, mice were pre-treated with naloxone, an opioid antagonist. Naloxone did not prevent the EAG's antinociceptive activity in the two phases of the formalin test, suggesting that the opioid system is unlikely to be involved in its antinociceptive action.

Herein, we also investigated the involvement of ATP-sensitive K⁺ channels in the mechanism of EAG's effect, by pretreating mice with glibenclamide (K⁺ channel blocker). Several studies support the hypothesis that the opening of the K⁺ channels mediate antinociception by inducing neurons hyperpolarization [49]. Therefore, K⁺ channels, especially those sensitive to ATP, are particularly involved in the nociceptive responses [49]. Several G protein coupled receptors (including opioid receptors) that stimulate the opening of K⁺ channels are involved in antinociception [49]. Our results demonstrate that the combined administration of glibenclamide (K⁺ channel blocker) and EAG blocked the antinociceptive action of EAG in the two phases of the formalin test. In this context, our findings strongly suggest that the peripheral antinociceptive action of EAG might be related to the activation of K⁺ channels sensitive

to ATP. Nevertheless, this activation appears to occur independently from the stimulation of opioid receptors, since naloxone did not prevent the EAG's antinociceptive activity. Indeed, the effect of terpenes as activators of K^+ channels sensitive to ATP is well known [13,50]. Corroborating our results, De Oliveira Júnior et al. (2017) [11] demonstrated that the antinociceptive effect of *Croton conduplicatus* essential oil - which contains caryophyllene oxide and guaiaol - is mediated by K^+ channels sensitive to ATP.

Conclusion

Taken together, our results demonstrated the antinociceptive action of supercritical extract of *A. gratissima* leaves in mice, which is mediated by ATP-sensitive K^+ channels.

Fundings

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Conflicts of interest

The authors declare no conflicts of interest.

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Review Article

Biological Properties of *Aloysia gratissima* (Gillies & Hook.) Tronc. (Verbenaceae)

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Aloysia gratissima is a plant native to America, with applications in folk medicine for a wide range of diseases, such as bronchial infections, lung disorders, nervous system disorders (depression, anxiety), and inflammatory processes, among others. However, investigations about this species and its biological actions are still scarce. This literature review was carried out using articles published in the past 30 years on the PubMed, SciELO, and Web of Science platforms, with the focus on the method of extraction, chemical composition, and clinical and preclinical studies on the pharmacological properties of *A. gratissima*. We noticed that the main constituents of *A. gratissima* are guaiol, pinocampnone, β -pinene, and 1,8-cineole. Additionally, preclinical studies reveal that *A. gratissima* extracts present antidepressant, anti-inflammatory, antinociceptive, antibacterial, antifungal, and virucidal effects. The results also demonstrate that there is a greater interest on the part of researchers from 2012 onwards in studying *A. gratissima* extracts with potential for possible new drugs.

1. Introduction

The scientific interest in compounds derived from plant extracts is associated with the knowledge of popular medicine [1]. Oils and/or extracts derived from plants can be obtained from different parts of vegetal species, including leaves, stems, roots, seeds, flowers, and fruits. [2]. It is estimated that approximately 25% of all drugs currently prescribed are derived from plants [3]. Thus, there is a notable interest from the academic community and the pharmaceutical industry in developing safer and more effective drugs to treat health disorders [4]. Additionally, natural products are active against microorganisms, such as fungi, viruses, and bacteria, responsible for several infectious diseases. These pathologies are becoming more difficult to treat due to the increasing resistance of microorganisms to antibiotics, and in some cases, there is no treatment option [5].

Among the plants found in Brazil, we highlight the genus *Aloysia* (Verbenaceae), originally from South America, containing 34 cataloged species, 12 of which can be found in Brazil [6, 7]. *Aloysia gratissima* (Gillies & Hook.) Tronc. is a shrub that can reach a maximum height of 3 m, has an irregular growth pattern, and may present thorns on the branches. Its leaves are simple and small (1–2 × 0.5–0.8 cm) [8]. The buds are strong and herbaceous, and the flowers are white, fragrant, in bunches, with intense flowering. The foliage is persistent and blooms between spring and summer. This plant is ornamental, due to the intensity of flowering and the pleasant aroma of the flowers [9, 10].

A. gratissima, popularly known in Brazil as “lavanda-do-brasil,” “erva-de-nossa-senhora,” “erva sagrada,” and “garupá” is widely distributed in America, occurring from the United States to Argentina. It was recently found in the central areas of the continent, northern Argentina, Brazil, Paraguay, Uruguay, and Mexico [9, 11].

In Argentina, according to Ricciardi et al. [9], *A. gratissima* is known as “Angel,” “oreganillo,” “azahar del campo,” and “niño rupá” (name recognized by the Guaraní Indians). In Uruguay, it is called “cedron del monte” and in the United States, “whitebrush.” *A. gratissima* was previously classified by other botanical names, such as *Verbena gratissima* Gill. et Hook, *Aloysia lycioides* Cham., and *Lippia lycioides* (Cham.) Steudel. [7].

In Rio Grande do Sul, Brazil, *A. gratissima* is popularly used mainly for headaches, “nerve problems, disorders of the digestive and respiratory systems such as colds and bronchitis,” and inflammatory pain [11–13]. In Santa Catarina, Brazil, its use was observed in two locations—Blumenau and Guabiruba—as sedative and antidepressant [14].

The infusion of the aerial parts of this plant is widely used in Argentina as a tonic, for digestive disorders, as diaphoretic and aphrodisiac. In addition, the infusion prepared with flowers and leaves is used in Bolivia as carminative and sedative, and as antispasmodic in Paraguay and Uruguay [15].

Considering the importance of the popular use of native plants in southern Brazil and their different biological activities, this study aims to systematically examine scientific studies on the biological effects and chemical composition of *A. gratissima*.

2. Materials and Methods

This study used a qualitative method (literature review) and included articles published in the past 30 years. The articles were obtained using the keyword *A. gratissima* in the PubMed, SciELO, and Web of Science databases. Afterwards, the articles were selected according to the following inclusion criteria: articles with keywords in the title and/or abstract, articles about extracts of plant products or isolated compounds, and clinical or preclinical studies on pharmacological properties. In this sense, we excluded articles related to environmental toxicology and agroecology in this study.

3. Results and Discussion

3.1. Selection of Articles. In the initial search, 50 articles were identified: 14 from PubMed, 16 from SciELO, and 23 from Web of Science. In total, 15 articles were indexed in two or more databases and considered only once. After a detailed analysis regarding the titles, abstracts, and full texts, 15 articles that did not meet the search criteria were excluded. Thus, 23 articles were finally selected. The flowchart presented in Figure 1 details the progressive selection of the study.

The findings of the literature review carried out in the present study are compiled in Table 1. It was noted that there was an increase in the number of articles about *A. gratissima* after the year 2012. In some cases, there is more than one publication by the same author. This fact may be correlated to the occurrence of the plant in South America [11] (Table 1).

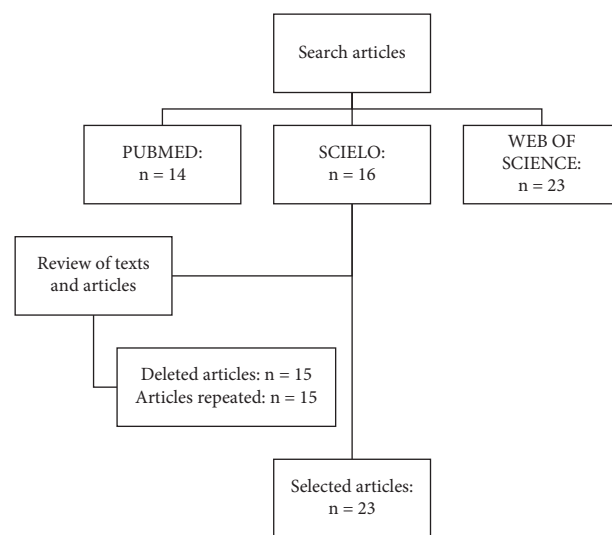


FIGURE 1: Flowchart of articles selection for the review.

The methodologies adopted for extraction and the parts of the plant that were used (leaves, flowers, branches, and stems) are different between the studies. The identification of the compounds present in the oils and/or extracts was similar in all articles, with gas chromatography (GC) being the method employed by the majority of studies. It was noticed that articles about *A. gratissima* are associated with *in vitro* and *in vivo* assays. Biological properties of this species, such as virucidal [22], nematocidal [33], antioxidant [29], sedative/anesthetic [6], antidepressant-like neuroprotective [14], antibacterial [28], antinociceptive, anti-inflammatory [13, 26], and antispasmodic [18], have been reported. There are no clinical studies on *A. gratissima* in the literature.

3.2. Chemical Composition of *Aloysia gratissima*. The chemical composition of the plant may vary according to the method of extracts and oils obtention, as well as the period of the harvest. Ricciardi et al. [9] performed the steam distillation of the aerial parts of the plant in three different harvest periods: spring (reproductive), autumn (vegetative), and summer (postreproductive) and in three different regions, such as São Lorenzo, Rio Empedrado, and Perichón. The authors identified elemene, viridiflorol, β -caryophyllene, and α -thujone as the main constituents found in the extracts, with high levels of sesquiterpenes (34.3–51.1%). During spring, the monoterpenes content was 7.6%, while sesquiterpenes had higher results in autumn (47.2%) and summer (51.1%). The thujone isomers appeared exclusively in samples from the São Lorenzo region, and the contents varied according to the considered ontogenic stage (6.8–17.5%). The authors confirmed the presence of these compounds through IR spectral performances, with a sample/oil fraction of 1/1, showing the deformation of the carbonyl group (5.72 μ m). This variation of carbonyl groups was also evaluated by gas chromatography according to the time of year, resulting in higher values in spring (19.3%), autumn (13.7%), and summer (8.2%) [9].

TABLE 1: Description of plant compounds and research aspects of the studies included in the systematic review.

Reference/year	Part of the plant	Extraction method	Chemical composition		Biological activities
Benovit et al. (2015) [6]	Leaves	Hydrodistillation/Clevenger	1,8-Cineole (18.54%), sabinene (9.5%), guaiol (6.79%), and bicyclogermacrene (5.12%)	<i>In vivo</i>	Sedative/anesthetic
Arze et al., (2013) [16]	Leaves	Hydrodistillation/Clevenger	Sabinene (30–35%), β -pinene (8–10%), and β -caryophyllene (~8%)	—	—
Bersan et al., (2014) [17]	Leaves	Hydrodistillation/Clevenger	E-Pinocamphone (16.07%), β -pinene (12.01%), guaiol (8.53%), E-pinocarveol acetate (8.19%), and α -caryophyllene (7.19%)	<i>In vitro</i>	Antimicrobial
Consolini et al. (2011) [18]	Leaves	Decoction	—	<i>In vivo</i>	Antispasmodic
Freires et al. (2015) [19]	Leaves	Hydrodistillation/Clevenger	Guaiol (29.63%), trans-pinocarveol (13.16%), bulnesol (11.79%), and myrtenol (5.3 1%)	<i>In vitro</i>	Antimicrobial
Galvez et al. (2020) [20]	Leaves	Hydrodistillation/Clevenger	β -Thujone ($36.1 \pm 0.1\%$), α -thujone ($32.2 \pm 0.2\%$), 1,8-cineol ($10.7 \pm 0.1\%$), and sabinene ($6.2 \pm 0.2\%$)	<i>In vitro</i>	Antifungal
Garcia et al. (2003) [21]	Leaves	Hydrodistillation/Clevenger	Caryophyllene oxide (15.84%), cadinol (17.37%), chrysanthenyl acetate (5.61%), limonene oxide (5.29%), and β -caryophyllene (4.77%)	<i>In vivo</i>	Virucidal—JUNV e HSV-1
Garcia et al. (2018) [22]	Leaves and fruits	Hydrodistillation/Clevenger	1,8-Cineole (17.64%) and guaiol (10.33%)	<i>In vivo</i>	Antileishmanial activity
Hister et al. (2009) [23]	Leaves	Hydrodistillation/Clevenger	—	<i>In vitro</i>	Inhibition of tumor cells growth
Ricciardi et al. (2006) [9]	Leaves	Hydrodistillation/Clevenger	β -Caryophyllene (25.1%), germacrene D (10.1%), bicyclogermacrene (12.6%), and α -thujone (10.7%)	—	—
Santos et al. (2015) [24]	Leaves	Hydrodistillation/Clevenger	1,8-Cineole (13.7%), germacrene D (13.4%), β -caryophyllene (12.7%), and β -pinene (11.7%)	<i>In vitro</i>	Antibacterial and antifungal
Soler (1986) [25]	Leaves	—	β -Pinene (8%)	—	—
Souza and Wiest (2007) [11]	—	Decoction	—	<i>In vitro</i>	Bacteriostatic
Souza et al. (2020) [13]	Leaves	Supercritical fluid with carbon dioxide	Guaiol (18.50%) and pinocamphone (11.40%)	<i>In vivo</i>	Anti-inflammatory
Souza et al. (2020) [26]	Leaves	Supercritical fluid with carbon dioxide	Guaiol (18.50%) and pinocamphone (11.40%)	<i>In vivo</i>	Antinociceptive
Trovati et al. (2009) [27]	Leaves	Hydrodistillation/Clevenger	Isopinocampone (<i>trans</i> -3-pinanone; 25.4%), limonene (15.1%), and guaiol (12.7%)	—	—
Vandresen et al. (2010) [28]	Leaves	Cold maceration/ethanol	α -bisabolol	<i>In vitro</i>	Antibacterial and Antiedematogenic effect
Zeni et al. (2011) [14]	Leaves	Hydrodistillation/Clevenger	—	<i>In vivo</i>	Antidepressant-like effect
Zeni et al. (2013) [29]	Leaves	Decoction	Ferulic acid (11.57–86.40 mg/100 g), trans-cinnamic acid (7.97–56.92 mg/100 g), and p-coumaric acid (0.00–16.06 mg/100)	<i>In vivo</i> and <i>in vitro</i>	Toxicity and antioxidant
Zeni et al. (2013) [30]	Stems and leaves	Decoction	—	<i>In vivo</i>	Antidepressant-like effect
Da Silva et al. (2006) [31]	Leaves	Cold maceration/ethanol	Guaiol, bisabolol, and spathulenol	—	—
Santos et al. (2013) [32]	Leaves	Hydrodistillation/Clevenger	Transpinocarveyl acetate (17.6%), trans-pinocamphone (16.3%), and guaiol (11.5%)	<i>In vitro</i>	Antibacterial and antifungal

Benovit et al. [6] used the Clevenger extraction and identified the presence of 1,8-cineole, sabinene, guaiol, and bicyclogermacrene in the pure oil extraction of *A. gratissima*. However, after the oil fractionation, the compounds E-(-)-pinocamphone, (-)-caryophyllene oxide, (-)-guaiol, and

(+)-spathulenol were identified. The two above-cited studies were carried out in the Santa Maria city, Rio Grande do Sul (Brazil). Arze et al. [16] identified the constituents sabinene, β -pinene, and β -caryophyllene in the essential oil of *A. gratissima* collected in the Cochabamba Province of

Mizque (Bolivia). The essential oil of *A. gratissima* collected in the São Carlos city, São Paulo (Brazil), studied by Trovati et al. [27], presents isopinocampone (25.4%), limonene (15.1%), and guaiol (12.7%) as its major constituents. In this study, oxygenated monoterpenes were the main groups, representing 32.6% of the total oil; oxygenated sesquiterpenes were 24.6% of the oil, followed by monoterpene hydrocarbons (18.3%) and hydrocarbon sesquiterpenes (10.9%), among others (8.3%).

In Uruguay, Soler et al. [25, 34] report the chemical compounds present in the oil obtained from the leaves of *A. gratissima* as sabinene (30–35%), β -pinene (8–10%), and β -caryophyllene (~8%), and in the flowers of the species, the main components are sabinene (19–10%) and β -pinene (11–5%).

The majority of studies about the species *A. gratissima* are based on extractions by the hydrodistillation method (Clevenger). However, there are a few articles that carried out the supercritical fluid extraction of the plant. Souza et al. [13, 26] found volatile terpenic compounds in the extract of *A. gratissima* leaves obtained by supercritical fluid with carbon dioxide (SCCO₂). The main compounds found in the supercritical extract were guaiol (18.50%) and pinocampone (11.40%), in addition to the presence of other compounds such as (-)-trans-pinocarvyl acetate (10.50%), b-cubeben (8.21%), caryophyllene (7.63%), γ -elemene (7.28%), caryophyllene oxide (6.76%), (-)-spatulenol (6.17%), pinocarvyl acetate (5.31%), bunsol (4.67%), isopinocampone (3.52%), myrtenol (3.24%), and humelene (2.97%) in smaller amounts. These findings agree with the chemical composition of the plant reported by other studies that used different methods of extraction. There were no significant variations in the composition of the extracts according to the pressures and temperatures operated during the extraction process. The chemical structures of the main compounds found in *A. gratissima* can be seen in Figure 2.

Santos et al. [32] evaluated the leaves and parts of the inflorescence of *A. gratissima* through hydrodistillation by Clevenger. According to the authors, the inflorescence of *A. gratissima* presented a higher content of oil (0.56%) than its leaves (0.35%). The gas chromatography-mass spectrometry (GC/MS) analysis revealed the presence of monoterpenes in the oil derived from the leaves (18.7% nonoxygenated and 22.6% oxygenated), while the essential oil of the inflorescence presented sesquiterpenes (30.4% nonoxygenated and 44.5% oxygenated), which corresponds to 74.9% of the total sesquiterpenes. Additionally, the authors [32] describe that the main compounds found in the essential oil of the leaves of *A. gratissima* were trans-pinocarvyl acetate (17.6%), trans-pinocampone (16.3%), and guaiol (11.5%). Nevertheless, there is a higher content of guaiol (19.5%), germacrene B (10.5%), bulnesol (10.0%), e-caryophyllene (8.9%), and caryophyllene oxide (8.24%) in the inflorescences.

Furthermore, guaiol was identified in a hexane extract from another sample of *A. gratissima* species in Brazil, together with spatulenol [31]. Other authors have identified different proportions of guaiol in *A. gratissima* oils, such as

29.63% [19], 22.04% [13], 12.7% [27], 18.5% [13], 11.5% [24], 10.33% [13], 8.53% [35], 6.7% [6], and 2.6% [36]. These differences can be attributed to the environmental conditions (e.g., period of harvest) and the extraction method [13].

Zeni et al. [29] evaluated *A. gratissima* oil obtained by hydrodistillation, through HPLC and identified ferulic acid (11.57–86.40 mg/100 g), trans-cinnamic acid (7.97–56.92 mg/100 g), and p-coumaric acid (0.00–16.06 mg/100 g) as the most abundant phenolic acids in the studied samples. The authors also analyzed the seasonality of the oil's compounds and found that it presented higher contents of caffeic, ferulic, and t-cinnamic acids in winter when compared to the summer. However, chlorogenic acid contents were more abundant in summer.

Da Silva et al. [31] evaluated the leaves of *A. gratissima* obtained from the extraction with ethanol at room temperature, in south-central Brazil. The extract was fractionated with hexane, dichloromethane, ethyl acetate, and methanol. The chromatographic analysis was carried out on silica gel eluted with hexane solvent and the main compounds found in the fractions were guaiol, bisabolol, and spathulenes. The dichloromethane fraction was chromatographed with chloroform/methanol, demonstrating the presence of triterpenoids. The ethyl acetate extract was chromatographed with hexane/ethyl acetate and resulted in the compounds betulinic acid, oleanolic acid, and ursolic acid. Finally, the methanol fraction was chromatographed on Sephadex LH20, using water/methanol, and the compounds found in the fraction were the phenylethanoid verbascoside and arenarioside.

The essential oil of *A. gratissima* contains more than 70% hydrocarbons, of which more than half are sesquiterpenes [36]. Freires et al. [19] indicated the presence of volatile compounds, mainly oxygenated mono- and sesquiterpenes, in addition to sesquiterpenic hydrocarbons. This is particularly interesting since the pharmacological effects of plants are related to the presence of metabolites such as alkaloids, terpenes, flavonoids, and phytosterols [26].

3.3. Anti-Inflammatory and Antinociceptive Activities.

Terpenic compounds are highly known for their pharmaceutical properties, such as antimicrobial, anti-inflammatory, and antitumor [37]. It is possible to assess the anti-inflammatory activity through the carrageenan-induced ear or paw edema assays. The assay is divided into three phases: the first phase (the first 90 minutes of the test) is related to the release of histamine and serotonin, the second phase (90–150 minutes) involves the activation of kinins, and the third phase (after 150 minutes) is related to the increased synthesis of prostaglandins in the inflamed tissue. In the last stage, there is also polyinfiltration of morphonuclear leukocytes.

Regarding the anti-inflammatory activity of *A. gratissima*, Vandresen et al. [28] demonstrated that the aqueous extract of its leaves caused a reduction of 23.6% in ear edema induced by carrageenan in mice when compared to animals treated with vehicle. This study corroborates the results found by Souza et al. [13], who demonstrated that an

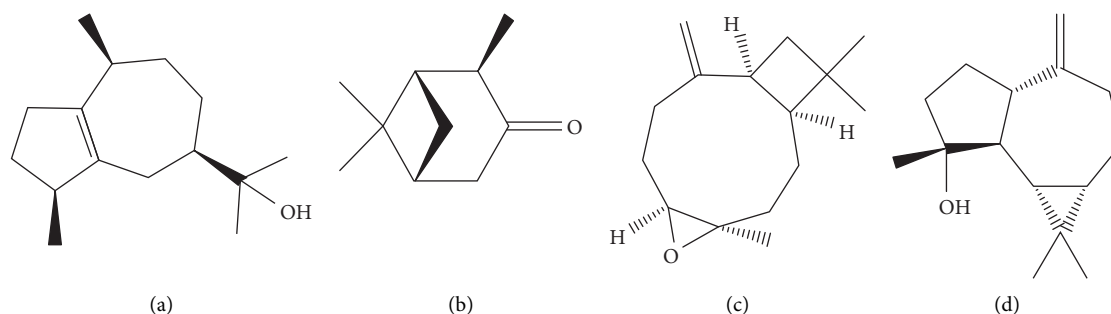


FIGURE 2: Chemical structure of the main compounds found in *Aloysia gratissima*: (a) guaiaol, (b) pinocamphone, (c) caryophyllene oxide, and (d) spathulenol.

extract of *A. gratissima* leaves obtained by SCCO_2 is enriched in guaiaol and elicited a reduction in paw edema during the first 3 hours after the carrageenan intraplantar administration in mice. The authors suggest that the anti-inflammatory activity of the *A. gratissima* supercritical extract is not related to a decrease in the synthesis of prostaglandins but to an inhibition of the release of histamine, serotonin, and kinins. Nevertheless, further studies on the anti-inflammatory activity of this vegetal species are still necessary.

Souza et al. [26] obtained promising results with the extract of *A. gratissima* obtained by supercritical extraction with carbon dioxide. Their research demonstrated that in the acetic acid-induced abdominal writhing test in mice, the extract at 30 mg/kg (p.o.) caused a reduction of 41% in the abdominal writhes, while the dose of 10 mg/kg caused a reduction of 56%, with the number of writhes from the groups treated with both doses being statistically similar to the positive control group (indomethacin). In the formalin assay, *A. gratissima* induced antinociceptive activity on both phases of the test; hence, it is effective on both neurogenic and inflammatory pain [26]. According to Apel et al. [38], these results are probably related to its main constituents, guaiaol, and spathulenol.

Souza et al. [13] also investigated the involvement of opioid receptors in the mechanism of antinociceptive action of the *A. gratissima* extract obtained by SCCO_2 through pharmacological antagonism with naloxone in the formalin test in mice. It was observed that naloxone did not prevent the antinociceptive activity of *A. gratissima* in both phases of the formalin test, suggesting that the opioid system is unlikely to be involved in the mechanism of antinociceptive action. This result shows promising pharmacological potential for the species, since the use of opioid agents is related to the development of dependence and tolerance, in addition to side effects including sedation, constipation, and urinary retention, among others [39]. Aiming to elucidate the mechanism of action of *A. gratissima* extract, the authors demonstrated the involvement of ATP-sensitive K^+ channels by pretreating mice with glibenclamide (K^+ -channel blocker). This mechanism of antinociceptive action may be related to the terpenes present in this species [25].

3.4. Antimicrobial Effects. The study performed by Freires et al. [19] analyzed the bioactive fractions (BF) of *A. gratissima* against *Streptococcus mutans* in order to assess the thickness, biovolume, and morphology of biofilms treated with the BF. Briefly, the author points out that BF from *A. gratissima* had a significant effect on bacterial viability—acting as bactericide [24]—and also affected a key characteristic of the pathogenicity of *S. mutans*—the production of extracellular polysaccharides (EPS) [24]. The authors report that there was a disruption of the integrity of the biofilm; therefore, the BF may have created porosity. There was no change in the thickness, but the biomass of EPS was significantly reduced ($15.63 \pm 2.56 \mu\text{m}^3/\mu\text{m}^2$; $p < 0.05$). One of the putative mechanisms of *A. gratissima* BF action may be related to inhibition of glycosyltransferase activity.

Santos et al. [24] evaluated the antimicrobial and antifungal activities of *A. gratissima* oil. The antimicrobial activity was evaluated against the microorganisms *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Acinetobacter baumannii* (ATCC 17978), *Escherichia coli* (ATCC25922), and *Pseudomonas aeruginosa* (ATCC 27853). The antibacterial effect was evaluated by the microdilution method to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The antifungal activity was evaluated using the yeasts *Cryptococcus neoformans* (ATCC 32264) and *Candida albicans* (ATCC 10231) and the filamentous fungi *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (ATCC 26934), *Rhizopus* sp. (CL 35), *Microsporium canis* (C112), *Microsporium gypseum* (C115), *Trichophyton mentagrophytes* (ATCC 9972), *Trichophyton rubrum* (C137), and *Epidermophyton floccosum* (C114).

Regarding antibacterial activity, the essential oil of *A. gratissima* was active against all tested microorganisms, and *B. cereus* was the most sensitive species (MIC = 1,000 $\mu\text{g}/\text{mL}$; MBC = 2,000 $\mu\text{g}/\text{mL}$). Regarding the antifungal activity, the essential oil showed moderate activity against *M. gypseum* (MIC = 1,000 $\mu\text{g}/\text{mL}$), *Epidermophyton flake-sum* (MIC = 1,000 $\mu\text{g}/\text{mL}$), *T. rubrum* (MIC = 1,000 $\mu\text{g}/\text{mL}$), *C. neoformans* (MIC = 1,000 $\mu\text{g}/\text{mL}$), and *C. albicans* (CIM = 1,000 $\mu\text{g}/\text{mL}$) and strong activity against *T. mentagrophytes* (MIC = 500 $\mu\text{g}/\text{mL}$) [24].

Bersan et al. [17] also evaluated the anti-microbial activity of *A. gratissima* oil by microdilution against the following microorganisms: *C. albicans* (CBS 562), *Streptococcus sanguis* (ATCC 10556), *Streptococcus mitis* (ATCC 903), *Porphyromonas gingivalis* (ATCC 33277), and *Fusobacterium nucleatum* (ATCC 25586). The *Aloysia* oil significantly inhibited the biofilm growth of *P. gingivalis*, *S. sanguis*, and *S. mitis* (9.0%; $p \leq 0.05$).

Galvez et al. [20] reported that the essential oil from the aerial parts of *A. gratissima* shows moderate antifungal activity against toxigenic *Fusarium* (MIC = 0.6–1.2 mg/mL) and was mainly inactive against *Aspergillus* species.

In addition to the chemical investigation of the *A. gratissima* oil, Santos et al. [32] studied the variable activity against Gram-positive bacteria (*Bacillus subtilis* CCT 2576, *S. aureus* CCT 2740, and *Streptococcus pneumoniae* ATCC 11733), Gram-negative bacteria (*Salmonella choleraesuis* CCT 4296 and *P. aeruginosa* ATCC 13388), and fungus (*C. albicans* ATCC 10231). The antimicrobial activity varied according to the microorganisms evaluated: the essential oil demonstrated an MIC 0.8 mg/mL against *P. aeruginosa* and MIC 0.6 mg/mL for *S. pneumoniae*. The essential oil of the inflorescence showed activity against *P. aeruginosa* (MIC 0.15 mg/mL), *S. pneumoniae* (MIC 0.025 mg/mL), and *C. albicans* (0.02 mg/mL). According to the authors, the essential oil of the inflorescence was more effective against the microorganisms than the essential oil of the leaf, and it was especially pronounced against the Gram-negative bacteria *P. aeruginosa*, the Gram-positive bacteria *S. pneumoniae*, and the yeast *C. albicans*.

Regarding the antiprotozoan effect of *A. gratissima*, the study by Garcia et al. [22] reports promising results of the *A. gratissima* oil, acting directly on *Leishmania amazonensis* (WHOM/BR/75/Josefa) parasite by affecting the kinetoplast, plasma membrane, and mitochondrial matrix. The authors correlate these results with the chemical composition of the oil, being guaiol the main compound. They also report that the oil affected the promastigote growth regardless of the dose and obtained inhibitions between 31% and 85% of the parasite growth, resulting in IC₅₀ = 25 µg/mL, after 48 h and IC₅₀ = 14 µg/mL, after 72 h. Garcia et al. [22] also investigated the effect of *A. gratissima* oil on intramacrophage amastigotes load. It was found a reduction in 85% of the amastigote intracellular load (at 2 µg/mL of *A. gratissima* oil, similar to the reduction promoted by 1 µg/mL of amphotericin B).

The authors state that guaiol can be directly metabolized by parasites, generating toxic products, or by host cells, generating a leishmanicidal metabolite. Garcia et al. [22] consider that *A. gratissima* can contribute to the development of new antiprotozoal drugs.

The chemical compound guaiol, widely found in species of the genus *Aloysia*, is sesquiterpenoid alcohol found in medicinal plants, mainly in Cypress and Guaiacum woods. This compound shows antibacterial and antitumor activities [40]. In this sense, Souza and Wiest [11] reported antimicrobial activity of *A. gratissima* against the *Rhodococcus equi* and *Pasteurella* sp. (isolated from the animal clinic) bacteria.

3.5. Virucidal Effect. Garcia et al. [21] studied the virucidal activity of *A. gratissima* oil obtained by hydrodistillation against herpes simplex virus type 1 (HSV-1), Junin virus (JUNV), and dengue virus type 2 (DEN-2). Satisfactory results were found for the JUNV, with VC₅₀ values ranging from 52 to 90 ppm. The VC₅₀ against DNA/HSV-1 was 65 ppm; therefore, it is considered less susceptible than the JUNV virus. However, *A. gratissima* oil was not effective against the DEN-2.

3.6. Antioxidant Effect. Zeni et al. [29] found promising results related to the antioxidant activity of *A. gratissima* oil. The authors analyzed the lyophilized oil of *A. gratissima* for total polyphenols (TP), total carotenoids (TC), total flavonoids (TF), and sequestering activity of free radicals.

The main compounds found in the oil were lutein (0.30–1.30 mg/g) and trans- β -carotene (0.24–1.86 mg/g). Other compounds were revealed by HPLC, such as ferulic acid (11.57–86.40 mg/100 g), trans-cinnamic acid (7.97–56.92 mg/100 g), and p-coumaric acid (0.00–16.06 mg/100 g), which were the most abundant phenolic acids in the studied samples. Caffeic, chlorogenic, gallic, vanillic, and protocatechuic acids were also found in the samples. As mentioned by the authors, climatic factors can significantly influence the accumulation of phenolic acid in the tissues of *A. gratissima*, indicating that studies on biological effects of the extracts/oils should take into account the influence of seasonality on the metabolic profile of this species.

Regarding the inhibition of free radicals, the author describes that the extracts depend on the concentration and synergistic effects of phytonutrients and may even be affected by other components involved in the cell complex, such as enzymatic components and glutathione. Furthermore, Zeni et al. [29] observed that the polyphenolic profiles revealed that some phenolic acids are present in a higher concentration than others, according to the month of the plant harvest, presenting an equilibrium throughout the seasons. Thus, it is indicated that the phenolic compounds significantly contributed to the antioxidant capacity of *A. gratissima*, with $R^2 = 0.9489$ ($p < 0.05$), revealing the EC₅₀ value of 1 mg/mL. Zeni et al. [29] also report that the antioxidant activity of *A. gratissima* is directed towards cellular lipid environments, such as organelle membranes.

3.7. Effects on the Central Nervous System. Zeni et al. [14] studied the *in vivo* antidepressant-like activity of *A. gratissima* oil obtained by steam dragging the leaves. The authors used the forced swimming test (FST) and the tail suspension test (TST) in mice. Their results show a reduction in the immobility time of mice treated with *A. gratissima* oil in both tests. Also, they demonstrated that the antidepressant-like activity occurs through the inhibition of N-methyl-D-aspartate (NMDA) receptors. Therefore, the authors suggest that this species has the potential to develop new drugs with antidepressant effects.

Benovit et al. [6] demonstrated the anesthetic and sedative properties of *A. gratissima* using silver catfish as an animal model. The authors performed a bio-guided

fractionation of the essential oil and demonstrated the importance of (+)-spathulenol in its sedative and anesthetic properties.

3.8. Toxicological Evaluation of *A. gratissima*. Garcia et al. [22] evaluated the toxicity of *A. gratissima* oil against host macrophages by the XTT method. The phagocytosis capacity of macrophages was not inhibited by the oil. Nevertheless, the authors report that there was an increase of around 50% of the phagocytosis of macrophages after treatment with *A. gratissima* oil.

In the *in vivo* toxicological evaluation of *A. gratissima*, Souza et al. [26] and Zeni et al. [29] reported the use of Swiss mice treated with 2,000 mg/kg (p.o.). During the 14 days of observation, there was no mice death in both studies.

Souza et al. [26] reported that there was intense sedation 45 minutes after the oral administration, but after this period, the animals fed normally and did not present locomotor changes. During the 14 day observation period, the authors report that there was no difference in the food intake and body weight between the group treated with the supercritical extract of *A. gratissima* leaves and the control group. Also, the oral administration of supercritical extract of *A. gratissima* leaves did not cause changes in the relative weight of the brain, liver, kidneys, lung, heart, and thymus. However, there was a significant decrease in the relative weight of the spleen and adrenal glands in the supercritical-extract-treated mice. These findings may indicate a sign of toxicity that should be further investigated in a repeated dose toxicity test.

It is important to note that there is a difference between the extraction methods performed in the studies by Zeni et al. [29] (hydroalcoholic extract) and by Souza et al. (extraction by SCCO₂). Nevertheless, the results of both studies agree with each other regarding the findings of the plant material toxicity.

4. Conclusions

South America stands out in scientific studies with the species *A. gratissima*. Terpenes are the main chemical compounds present in *A. gratissima*, being Guaiol, Pino-canfene, β -elemene, β -caryophyllene, spathulenol, trans-pinocamphone, and trans-pinocarveol acetate the main ones. It is observed that the extraction method used in the studies does not impact significantly in the chemical composition of the extracts; however, the period of harvest does. Taken together, the data found in the 21 reviewed studies suggest that *A. gratissima* has promising potential to be a source of anti-inflammatory, antinociceptive, antimicrobial, and/or antidepressant compounds.

Data Availability

The data supporting this systematic review are from previously reported studies and data sets, which have been cited.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Seasonal variation in the chemical composition, antimicrobial and antioxidant potential of *Aloysia gratissima* leaves essential oil

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ABSTRACT

This study aimed to evaluate the effect of seasonality on the chemical composition and the antimicrobial and antioxidant activities of the essential oil from *Aloysia gratissima* leaves (AGO). The essential oils were extracted from dried and crushed *A. gratissima* leaves using hydrodistillation. The chemical composition of AGO was analyzed by gas chromatography-mass spectrometry (GC-MS). Antioxidant activity was assessed using the DPPH assay, while antimicrobial activity was determined by the minimum microbicidal concentration method. Across the four seasons, 33 to 45 chemical compounds were identified, accounting for 93.76 %–97.92 % of the oils. Among these, β-pinene, pinocamphone and *trans*-pinocarvyl acetate were predominant in three seasons, whereas guaiaol was the major compound exclusively in autumn. The essential oils exhibited promising antimicrobial activity, particularly against *Staphylococcus aureus* and *Bacillus cereus*. This study represents the first seasonal characterization of the chemical composition and biological activities of AGO in Brazil.

1. Introduction

Essential oils are natural volatile compounds produced by plants and can be extracted from various plant parts, including flowers, buds, stems, leaves, seeds, twigs, roots, fruits, bark, and wood (Bakkali et al., 2008; Tariq et al., 2019). Essential oils are classified as herbal medicines and are used as integrative treatments for various conditions, including anxiety, depression, and infectious diseases. In addition, they exhibit antifungal, antibacterial, anticancer, and wound-healing properties (Sharifi-Rad et al., 2017). Due to these properties and their strong aromas, several essential oils are already utilized in the cosmetics and perfume industries (Katekar et al., 2023), as well as in the food industry (Scapinello et al., 2023).

One prominent group of compounds found in plant essential oils is terpenes, hydrocarbons formed by isoprene units, which have several proven pharmacological activities (Correia et al., 2008; Leite et al., 2021). The literature reports the main properties of these compounds, including antidepressant effects, anticonvulsant, anti-inflammatory,

antinociceptive, antimicrobial, antioxidant, and anticancer properties (Souza et al., 2007; Perazzo et al., 2008).

Aromatic plants with ethnopharmacological applications are among the most extensively studied in scientific research (Appiah et al., 2017). In this context, *Aloysia gratissima* (Gillies & Hook.) Tronc., commonly known as *Alfazema do Brasil*, *Erva Santa* or *Garupá*, holds a prominent place in South American folk medicine (Ricciardi et al., 2006). This aromatic species, belonging to the Verbenaceae family and native to South America, is highly valued in academic research due to its rich essential oil content and well-documented medicinal properties.

Studies on *A. gratissima* primarily focus on leaf extracts, utilizing methods such as hydrodistillation, decoction, cold extraction, and supercritical extraction. Reported biological activities of *A. gratissima* include sedative/anesthetic, anti-inflammatory, antinociceptive, antidepressant-like, and antileishmanial effects in *in vivo* studies. Additionally, *in vitro* studies have demonstrated antimicrobial and antifungal activities, antiedematogenic effects, and inhibition of tumor cell growth (Souza et al., 2022).

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The essential oil extracted from *A. gratissima* contains more than 70 % hydrocarbons, with sesquiterpenes accounting for over half of its composition. This is particularly noteworthy, as these secondary metabolites are closely associated with the pharmacological activities of essential oils (Bersan et al., 2014; Benovit et al., 2015; Garcia et al., 2018).

It is important to consider that the chemical composition of plants can fluctuate significantly with the seasons, affecting the quality and consistency of essential oils. These variations in essential oil composition can influence their therapeutic properties, such as antioxidant activity (Gasparetto et al., 2016; Castro et al., 2019; Silva et al., 2019; Kabdal et al., 2022). Therefore, this study aimed to evaluate the influence of seasonality on the chemical composition, as well as the

antimicrobial and antioxidant potential, of *Aloysia gratissima* leaf essential oil.

2. Results and discussion

2.1. Extraction yield

The chromatographic profile of AGO revealed the presence of 33–45 chemical compounds, accounting for 97.4 % in autumn, 96.46 % in winter, 94.35 % in spring, and 97.92 % in summer (Table 1). During the autumn season, the prominent constituents of AGO were pinocamphone (13.57 %), guaïol (10.54 %), and β-pinene (8.51 %). In winter and spring, the dominant compounds were pinocamphone (21.44 % and

Table 1
The chromatographic profile of the *Aloysia gratissima* essential oils.

Compound	Peak	RT (min)	RI NIST	RI EXP	Autumn	Winter	Spring	Summer
Relative Concentration (%)								
α-thujene	1	4.456	902	931	0.14	–	–	0.26
α-pinene	2	4.55	948	939	1.31	1.70	1.77	2.23
Sabinene	3	5.021	964	979	0.35	0.50	–	0.82
β-pinene	4	5.076	948	984	8.51	12.16	10.32	15.05
β-myrcene	5	5.186	997	993	0.94	–	0.21	2.55
o-cymene	6	5.629	1034	1030	0.17	–	1.79	0.16
d-limonene	7	5.678	1018	1034	1.46	2.35	–	3.06
Eucalyptol	8	5.739	1059	1040	0.29	0.21	0.25	0.31
β-ocimene	9	5.87	1032	1051	0.22	0.29	0.27	1.09
β-linalool	10	6.509	1105	1105	0.60	0.80	–	0.73
Terpineol	11	6.549	1158	1108	1.14	1.30	2.10	0.93
cis-verbenol	12	6.662	1128	1118	0.47	0.55	0.33	0.40
α-campholenal	13	6.857	1155	1135	0.37	0.52	0.33	0.46
L-trans-pinocarveol	14	7.05	1135	1152	4.01	4.50	4.66	2.97
cis-verbenol	15	7.108	1128	1157	1.85	2.36	2.15	1.43
Pinocamphone	16	7.303	1109	1174	13.57	21.44	14.09	13.74
Isocamphopinone	17	7.462	1160	1188	4.43	–	4.73	5.22
α-terpineol	18	7.621	1158	1201	0.16	–	0.17	0.17
(–)-myrtenol	19	7.697	1212	1208	2.72	2.95	3.16	1.60
l-verbenone	20	7.872	1191	1224	0.14	–	–	–
cis-carveol	21	7.927	1225	1229	0.40	0.29	0.35	0.23
Carvone	22	8.22	1239	1256	0.19	–	0.19	–
(–)-cis-myrtanol	23	8.324	1245	1266	0.19	–	0.17	–
Thymol	24	8.69	1294	1299	0.25	–	–	–
(–)-trans-pinocarvyl acetate	25	8.806	1307	1310	8.26	9.59	8.75	7.55
α-copaene	26	9.634	1390	1391	0.22	0.33	0.27	0.30
(–)-β-bourbonene	27	9.744	1374	1401	0.50	0.63	0.54	0.47
β-elemene	28	9.778	1387	1405	0.39	1.30	1.20	0.4
Caryophyllene	29	10.114	1494	1439	5.05	6.51	5.49	6.27
Humulene	30	10.45	1479	1473	1.63	2.18	1.68	1.86
Alloaromadendrene	31	10.524	1386	1481	0.26	0.41	0.32	0.31
d-germacrene	32	10.713	1501	1500	4.87	–	–	6.49
B-cubebene	33	–	–	–	–	6.46	4.74	–
γ-elemene	34	10.86	1492	1516	2.95	1.92	2.46	3.57
B-guaiene	35	11.208	1533	1554	0.30	–	–	0.24
Elemol	36	11.33	1540	1567	0.45	0.39	0.78	0.24
Elixene	37	11.455	1492	1581	4.36	3.91	3.33	4.21
Germacrene D-4-ol	38	11.611	1597	1597	0.42	–	–	0.30
(–)-spathulenol	39	11.651	1603	1602	2.70	0.42	2.13	0.64
Caryophyllene oxide	40	11.718	1614	1609	3.34	2.29	2.74	1.86
Guaïol	41	11.801	1614	1619	10.54	5.02	7.31	6.42
B-eudesmol	42	11.935	1644	1634	2.31	0.81	1.83	1.03
Cubedol	43	12.021	1647	1644	0.44	–	0.45	–
Cubenol	44	12.225	1651	1667	0.50	0.48	0.36	0.20
Bulnesol	45	12.427	1683	1690	4.03	1.89	2.93	2.15
Total					97.4	96.46	94.35	97.92
Compound group		Relative Concentration (%)						
		Autumn	Winter		Spring		Summer	
Monoterpene hydrocarbons		46.7	39.4		41.7		46.2	
Oxygenated monoterpenes		11.1	12.1		13.9		10.3	
Sesquiterpene hydrocarbons		26.7	30.3		27.8		28.2	
Oxygenated sesquiterpenes		15.6	18.2		16.7		15.4	
Total		100.0	100.0		100.0		100.0	

Note: Caption: RT – Retention time; RI NIST – National Institute of Standards and Technology Library Linear Retention Index; RI EXP – Experimental retention index.

14.09 %, respectively), β -pinene (12.16 % and 10.32 %, respectively), and *trans*-pinocarvyl acetate (9.59 % and 8.75 % respectively). In summer, the main constituents were β -pinene (15.05 %), pinocamphone (13.47 %), and *trans*-pinocarvyl acetate (7.55 %). Notably, despite the variations observed among seasons, these differences were not statistically significant ($p < 0.05$), according to the biplot test. Oxygenated monoterpenes have been identified as the predominant group in most characterized profiles of *Aloysia* species essential oils (Mohammadhosseini et al., 2022). A similar pattern was observed in AGO, with oxygenated monoterpenes, such as pinocamphone and *trans*-pinocarvyl acetate, being the dominant compounds. Additionally, oxygenated sesquiterpenes, such as guaialol, and hydrocarbon monoterpenes, such as β -pinene, were also detected, consistent with previous reports on *A. gratissima* essential oils (Trovati et al., 2009; Benovit et al., 2015; Avila et al., 2023).

In the summer, when the AGO yield was 1.14 % (w/w), the essential oil exhibited the highest content of β -pinene (15.05 %) and the lowest content of *trans*-pinocarvyl acetate (7.55 %). Notably, pinocamphone (13.74 %) remained one of the dominant compounds. These results closely align with the research conducted by Souza et al. (2020), who reported that the *A. gratissima* leaves extract, collected in summer, obtained using supercritical carbon dioxide extraction, yielded 4.42 % (w/w), with guaialol (18.55 %), pinocamphone (11.40 %) and *trans*-pinocarvyl acetate (10.55 %) identified as the major components.

The concentrations of chemical constituents in AGO varied depending on the season of collection, leading to the presence or absence of certain compounds. For instance, α -thujene was detected in AGO from autumn (0.14 %) and summer (0.26 %) but was absent in winter and spring. Similarly, thymol was detected only in autumn (0.25 %), which may be associated with its biosynthesis under low sunlight conditions (Kabdal et al., 2022).

Furthermore, the relative proportions of the constituents exhibited notable fluctuations across seasons. For instance, β -pinene accounted for 8.51 % of the total composition in autumn, increasing to 15.05 % in summer, while the sesquiterpene guaialol reached its peak in autumn (10.54 %). According to Ramakrishna and Ravishankar (2011), certain secondary metabolites may occur at lower levels during spring and summer due to the active growth of shoots and leaves. As plant growth slows down, the accumulation of specific secondary metabolites tends to increase during autumn and winter.

Pinocamphone and *trans*-pinocarvyl acetate exhibited their highest concentrations in winter (21.44 % and 9.59 %, respectively). It is well established that certain plant species upregulate the synthesis of secondary metabolites, such as pinocamphone and *trans*-pinocarvyl acetate, in response to cold and water stress (Olennikov et al., 2017; Muñoz-Acevedo et al., 2019). Consequently, the increased levels of these metabolites may serve as a protective mechanism against low temperatures and other environmental stressors prevalent in winter, including rainautumn. The chemical profile of AGO fluctuates seasonally, reflecting the dynamic nature of plant metabolism and the significant influence of climatic conditions (Silva et al., 2019; Zanusso et al., 2023).

The extraction yields of *A. gratissima* essential oils using hydrodistillation were 1.07 % in autumn, 0.80 % in winter, 1.09 % in spring, and 1.14 % in summer (w/w) (Fig. 1). Notably, no significant differences were observed among the seasons when comparing the percentage of AGO extracted from the dry plant matrix. Interestingly, Azevedo et al. (2020), suggest that volatile oil production in plants tends to increase at higher temperatures, such as in summer and spring, despite the potential risk of significant evaporation losses. Conversely, in winter, volatile oil production may decrease, which can be explained by the natural source-sink mechanism, where secondary metabolites are degraded and their chemical components redirected toward maintaining primary metabolism (Erb and Kliebenstein, 2020).

The yield of essential oil is a critical factor in determining the viability of studying a plant species for its bioactive properties. Different plant species exhibit variations in essential oil production, with yields

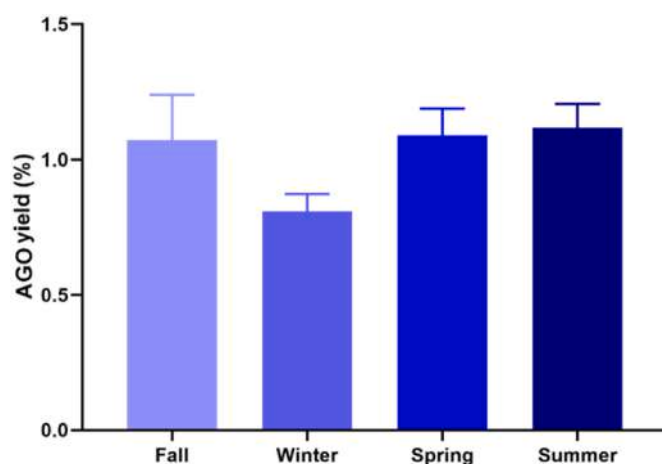


Fig. 1. Extraction yields of essential oils from *A. gratissima* leaves using hydrodistillation (% w/w).

ranging from 0.05 % to 10.0 % of the plant mass. Although the yields obtained may be relatively low compared to those of classic aromatic plants (Zheljazzkov et al., 2013), they still autumn within the typical range observed in leaf-based studies, with yields of up to 2 %. Thus, *A. gratissima* is a viable option for essential oil extraction, particularly considering the absence of significant yield variations between seasons.

However, factors beyond just the yield must be considered, such as the chemical composition of essential oils, their bioactive properties, and potential applications. These aspects provide valuable data into the true potential of *A. gratissima* as a source of bioactive substances. In Fig. 2, the cluster analysis reveals three main categories of AGO samples. Fig. 2A depicts three distinct clusters, showcasing both heterogeneity between groups and compatibility within each group. Winter stands out as the season with the most distinct chemical composition compared to the other seasons, whereas the transitional seasons of spring and autumn exhibit notable similarity in terms of chemical characteristics. Albeit different from the other seasons, summer shows a closer resemblance to spring and autumn.

Upon analyzing the chemical composition of the plant, we observed four distinct groups, as depicted in Fig. 2B. The most prominent cluster, labeled group 4 in Fig. 2B, consisted of compounds such as pinene (β and α), guaialol, *trans*-pinocarvyl acetate, and pinocamphone. Another notable group, labeled group 1 in Fig. 2B, included germacrene-D, isocamphopinone, caryophyllene, elixene, and pinocarveol. The remaining two clusters, group 2 and group 3 in Fig. 2B, encompassed the majority of the compounds and demonstrated a higher degree of similarity among themselves.

This information holds great importance in uncovering the rich chemical diversity within the essential oils of *A. gratissima* produced in different seasons. The identification of distinct clusters sheds light on the presence of diverse chemical compounds, each potentially harboring unique bioactive properties. These distinct chemical characteristics and compound groupings have the potential to greatly impact the range of applications for *A. gratissima* essential oil, opening promising avenues for further exploration into its therapeutic and cosmetic properties, among other potential uses.

2.2. Antimicrobial activity

Table 2 presents the results of the MIC and MMC assays conducted with AGO, revealing its potential inhibitory effect against the Gram-positive bacteria *S. aureus* and *B. cereus* in all seasons. Interestingly, AGO exhibited a lower effect against Gram-negative bacteria (*E. coli*, *S. enterica*, and *P. mirabilis*). The fungus *C. albicans* displayed susceptibility to oils obtained from the winter and summer seasons.

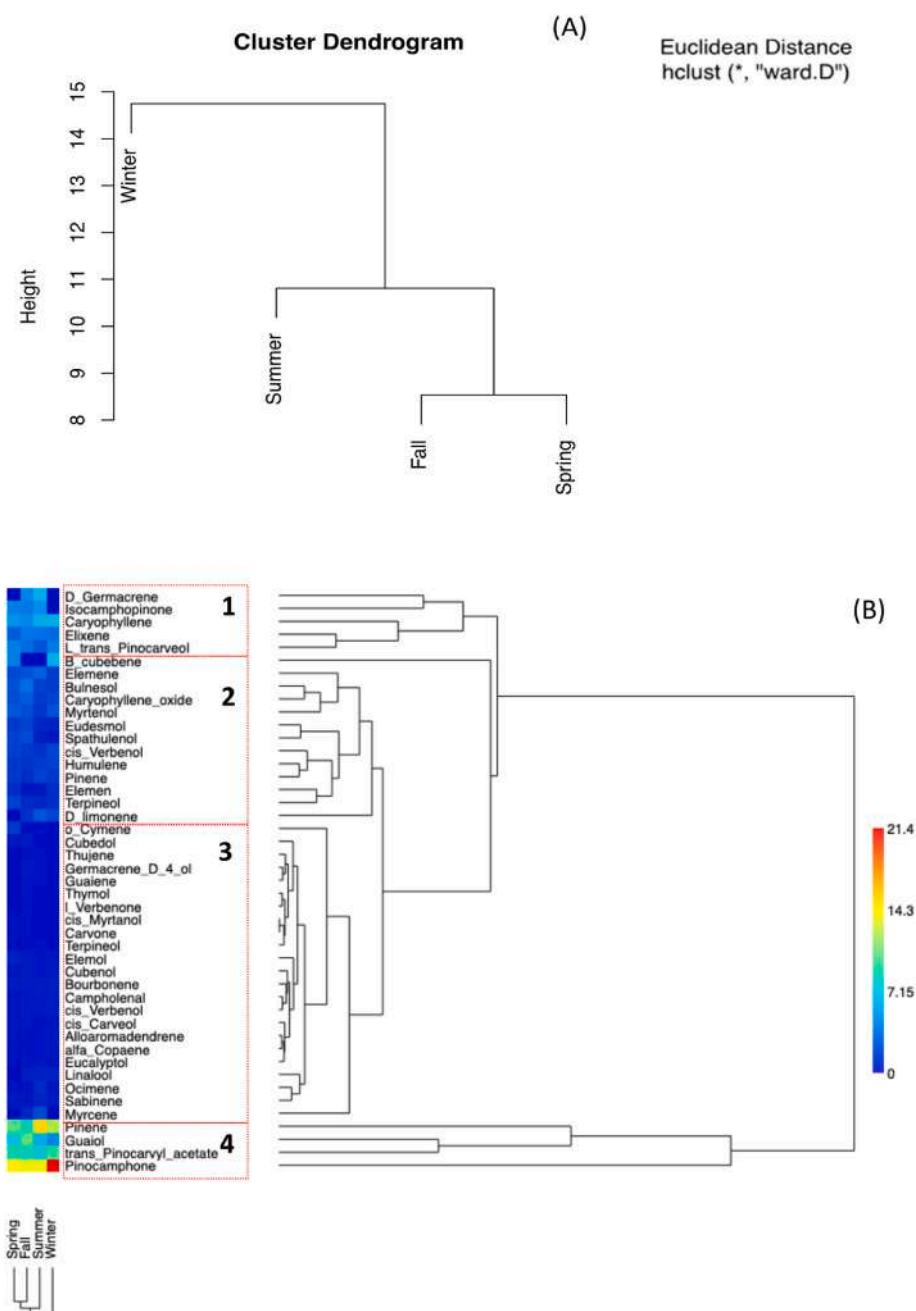


Fig. 2. Biplot of cluster analysis of essential oils constituents from *A. gratissima* leaves collected in four seasons. A) Chemical compounds between seasonal periods; B) Similarity between the chemical compounds of oils.

The resistance observed in Gram-negative bacteria compared to Gram-positive bacteria can be attributed to their unique structural characteristics, including a double phospholipid membrane that provides protection against lipophilic solutes. Additionally, the presence of porins acts as a selective barrier to hydrophilic solutes, preventing the penetration of compounds such as antibiotics or specific plant-derived secondary metabolites (González-Alamilla et al., 2019).

The majority of AGO constituents, such as pinocamphone, guaiol, β -pinene, and *trans*-pinocarvyl acetate, along with minor compounds such as caryophyllene oxide, α -cadinol, γ -elemene, spathulenol, caryophyllene and humulene, belong to the sesquiterpenoid group. Many of these sesquiterpenoids have been reported to possess antibacterial and antifungal properties (Ahmadi et al., 2010; Ma et al., 2016; Yin et al., 2020). Notably, guaiol, a sesquiterpenoid alcohol commonly found in *Aloysia* genus species, including *A. gratissima*, has been reported to

exhibit antibacterial and antitumor activities (Yang et al., 2016).

The presence of β -pinene (8.51–15.05 %), one of the major compounds, significantly contributes to antimicrobial activity. Research by Leite et al. (2007), highlights the inhibitory effects of isolated β -pinene and α -pinene on the growth and viability of Gram-positive bacteria. Similarly, studies by Burt (2004) and Gasparetto et al. (2016), confirm the potent antibacterial activity of α -pinene. β -pinene is known to disrupt cell integrity and impede crucial processes such as respiration and ion transport, ultimately leading to cell death (Carson et al., 2002). These results underscore the significance of these compounds as natural antimicrobial agents with potential for combating bacterial infections.

The proportions of chemical compounds and their interactions with other components present in the plant material, such as proteins and carbohydrates, can impact the extraction yield and the properties of the extracted oil, including its antimicrobial activity (Arruda et al., 2021).

Table 2
Antibacterial and antifungal activity of *A. gratissima* essential oil in different seasons.

Season	Gram-positive bacteria MIC/MMC (ppm)		Gram-negative bacteria MIC/MMC (ppm)			Fungus MIC/MMC (ppm)
	<i>S. aureus</i>	<i>B. cereus</i>	<i>E. coli</i>	<i>S. enterica</i>	<i>P. mirabilis</i>	<i>C. albicans</i>
Autumn	1560/6250	1560/6250	12500/25000	>25000/ NP	12500/25000	12500/25000
Winter	3120/3120	3120/3120	12500/12500	12500/12500	25000/25000	3120/12500
Spring	3120/12500	6250/6250	25000/25000	25000/25000	25000/25000	12500/12500
Summer	6250/6250	6250/12500	12500/25000	25000/ >25000	25000/25000	6250/12500

Note: Not Performed (NP); Minimal Microbicidal Concentration (MMC); Minimal Inhibitory Concentration (MIC).

Oliveira et al. (2016), explain that the antibacterial activity of essential oils can be attributed to the lipophilic nature of monoterpenes, which directly damage the cell membrane and disrupt the balance of cellular pH and inorganic ions.

To address multicollinearity, a variance inflation index analysis was conducted, resulting in the selection of 35 out of the 45 chemical compounds of AGO, which played a significant role in the context of multicollinearity. In contrast, only ten chemical compounds, α -thujene, β -pinene, *o*-cymene, *D*-limonene, β -ocimene, *cis*-verbenol, α -campholenal, pinocamphone, *l*-verbenone and α -copaene, were found to be free from multicollinearity. These chemical compounds played a significant role in explaining the variability (0.016 and 0.028) observed in MMC and MIC. When examining the RDAs, Axis 1 accounted for 58 % and 70 % of the variability (0.009 and 0.02), respectively, while axis 2 explained 20 % and 17 % (0.003 and 0.004), respectively, for MMC and MIC. These results are visually represented in Fig. 3, which illustrates the MMC (Fig. 3A and B) and MIC (Fig. 3C and D).

Regarding antimicrobial activity (Fig. 3A), we observed a negative correlation ($p < 0.05$) on Axis 1 for the compounds *o*-cymene ($r = -0.60$), α -thujene ($r = -0.44$), β -ocimene ($r = 0.55$), and β -pinene ($r =$

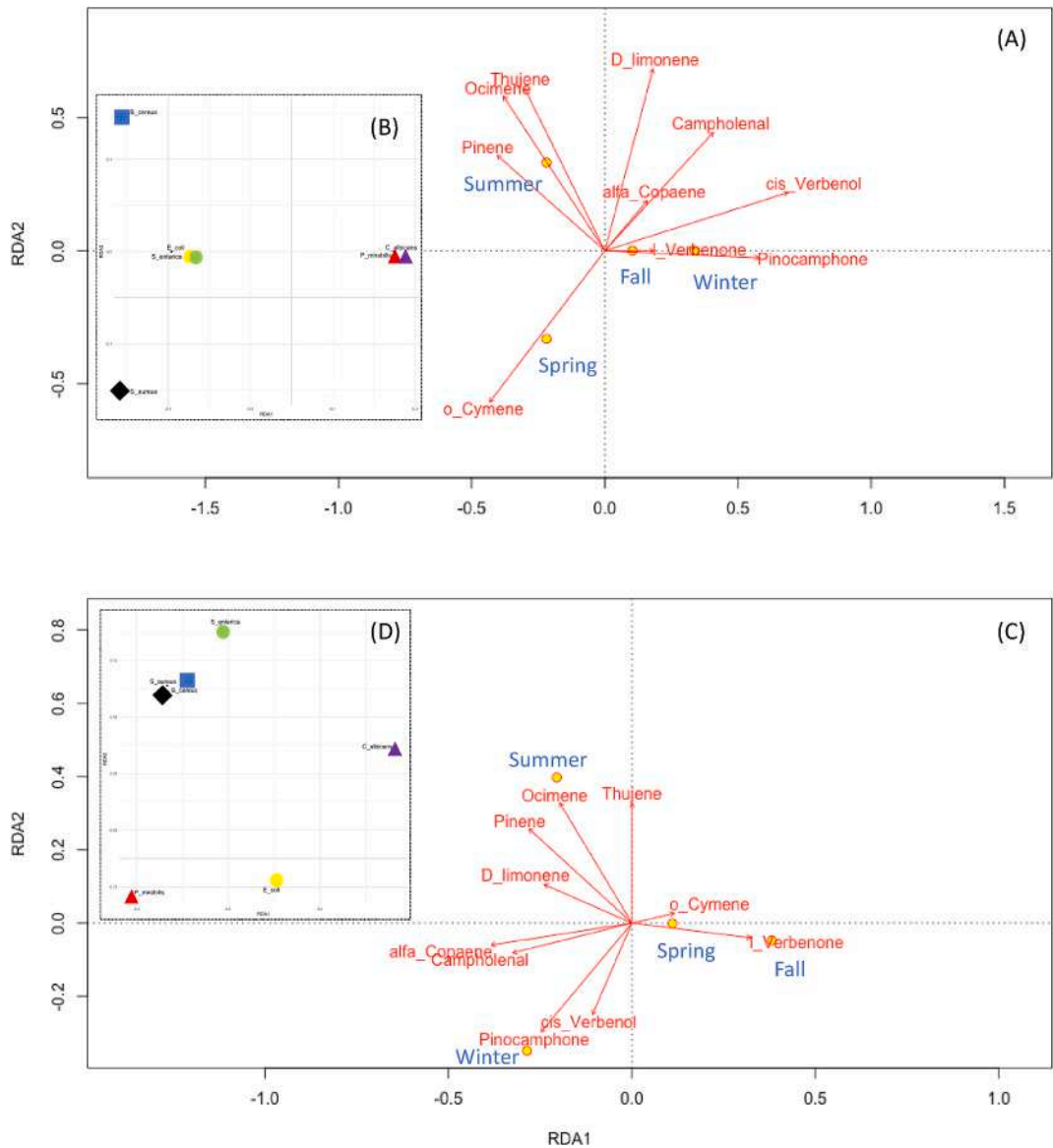


Fig. 3. Redundancy analysis (RDA) of AGO chemical compounds in seasonal periods and the relationship between types of bacteria. Note: *S. aureus* – black diamond; *B. cereus* – blue square; *E. coli* – yellow circle; *S. enterica* – green circle; *P. mirabilis* – red triangle; *C. albicans* – purple triangle; A and B) MMC; C and D) MIC.

–0.75) in relation to the MMC of *B. cereus*, *S. aureus*, *E. coli*, and *S. enterica*, particularly in the AGO samples from spring and summer. The negative values indicate that lower oil concentration is linked to higher efficiency, as described by Duarte et al. (2023). However, we found a positive correlation for the compounds α -limonene ($r = 0.26$), α -campholenal ($r = 0.67$), α -copaene ($r = 0.65$), l-verbenone ($r = 1.00$), *cis*-verbenol ($r = 0.95$), and pinocamphone ($r = 1.00$), which were associated with *P. mirabilis* and *C. albicans* MMC, mainly in the autumn and winter AGO samples.

Analyzing MIC values, as depicted in Fig. 3B, we discovered a noteworthy pattern. There was a negative correlation with Axis 1 for various chemical constituents found in winter and summer AGO. Notably, pinocamphone ($r = -0.64$), α -copaene ($r = -0.99$), α -campholenal ($r = -0.97$), *cis*-verbenol ($r = -0.40$), α -thujene ($r = 0.00$), β -pinene ($r = -0.74$), β -ocimene ($r = -0.52$), and α -limonene ($r = -0.92$) exhibited inhibitory effects against *P. mirabilis*, *S. aureus*, *B. cereus*, and *S. enterica*. Conversely, we observed a positive correlation with Axis 1 for l-verbenone ($r = 0.99$) and *o*-cymene ($r = 0.97$), which were associated with the inhibition of *C. albicans* and *E. coli*, particularly in the autumn and spring AGO samples.

Furthermore, according to the Monte Carlo statistical test outlined in Table 3, two chemical compounds stood out as highly significant. *o*-cymene ($r = 0.043$) and *cis*-verbenol ($r = 0.043$) demonstrated strong correlations with MMC, while pinocamphone ($r = 0.042$) emerged as the primary chemical compound responsible for the MIC of the AGO. These findings suggest the potential role of mono and sesquiterpene hydrocarbons in the observed antimicrobial activity. Thus, this study reveals the antimicrobial potential of *A. gratissima* and underscores the significant impact of its composition on the antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as yeast.

2.3. Antioxidant activity

Antioxidant activity is a complex process involving multiple mechanisms, such as preventing hydrogen abstraction, scavenging free radicals, inhibiting chain initiation, decomposing peroxides, enhancing reducing capacity, and binding transition metal ion catalysts (Fernandes et al., 2017). Table 4 illustrates the DPPH radical scavenging potential of AGO from different seasons. The IC₅₀ values, representing the 50 % scavenging capacity, varied significantly among the seasons. Winter AGO exhibited the highest antioxidant potential (IC₅₀ = 4.58 ± 0.38 µg/mL), and showed a notable difference compared to autumn AGO (IC₅₀ = 8.07 ± 0.08 µg/mL), spring (IC₅₀ = 8.97 ± 1.13 µg/mL), and summer (IC₅₀ = 7.98 ± 0.24 µg/mL). A lower IC₅₀ value indicates higher antioxidant activity. To ensure the reliability of the results, ascorbic acid (AA) was used as a positive control, this being a organic compound belonging to the group of unsaturated polyhydroxy alcohols and is considered one of the primary antioxidants of low molecular weight (Gegotek and Skrzydlewska, 2023).

Table 3
Statistical result of the redundancy analysis (RDA) of the correlation of the chemical composition of *A. gratissima* leaves essential oil in relation to Minimal Bactericidal Concentration (MMC) and Minimal Inhibitory Concentration (MIC).

Compound	MMC				MIC			
	RDA1	RDA2	r2	Pr(>r)	RDA1	RDA2	r2	Pr(>r)
α -thujene	–0.44	0.90	0.89	0.417	0.00	1.00	0.70	0.583
β -pinene	–0.75	0.66	0.56	0.583	–0.74	0.68	0.96	0.375
<i>o</i> -cymene	–0.60	–0.80	0.98	0.043	0.97	0.23	0.09	0.792
α -limonene	0.26	0.97	0.96	0.250	–0.92	0.40	0.45	0.750
β -ocimene	–0.55	0.84	0.93	0.500	–0.52	0.86	0.96	0.292
<i>cis</i> -verbenol	0.95	0.30	1.00	0.043	–0.40	–0.92	0.49	0.708
α -campholenal	0.67	0.74	0.70	0.583	–0.97	–0.24	0.74	0.458
Pinocamphone	1.00	–0.05	0.64	0.750	–0.64	–0.77	0.98	0.042
l-verbenone	1.00	0.00	0.06	1.000	0.99	–0.12	0.70	0.750
α -copaene	0.65	0.76	0.12	1.000	–0.99	–0.15	0.99	0.167

Note: Taken from the RDA1 and RDA2 vectors of the Monte Carlo test. Significant results are in bold.

Table 4
Antioxidant activity of *A. gratissima* essential oil at different seasons of the year.

Season	IC ₅₀ of DPPH radical scavenging (µg/mL)
Autumn	8.07 ± 0.38**** ^a
Winter	4.58 ± 0.08***
Spring	8.97 ± 1.13**** ^b
Summer	7.98 ± 0.24**** ^b

Note: Values are means ± standard deviation (n = 4). One-way ANOVA followed by Bonferroni test: ***p < 0.01 and ****p < 0.0001 different from AA, ^a p < 0.01 different from winter and ^b p < 0.001 different from winter.

The antioxidant activity of essential oils is closely related to the concentration of key compounds. AGO predominantly consists of monoterpene and sesquiterpene compounds, exhibiting remarkable structural variability due to isoprene unit formation. This structural diversity may correlate with the observed biological effects, including antioxidant activity (Masyita et al., 2022). Among the monoterpenes in AGO, β -pinene stands out as a naturally occurring structural isomer with various pharmacological activities, including antioxidant, antimicrobial, and antifungal properties (Zielińska-Blajet and Feder-Kubis, 2020).

3. Experimental

3.1. Plant material and extraction

In this study, leaves of *A. gratissima* were collected from a well-characterized population in Erval Grande municipality, Rio Grande do Sul, Brazil (27°23'14.3S, 52°33'49W), across four distinct seasons: autumn (March 29, 2022), winter (July 25, 2022), spring (December 21, 2022) and summer (January 29, 2023). For each season, multiple individuals (20) were sampled on a single collection day. This approach ensured that the collected material was representative of the seasonal variation within the population and minimized potential confounding factors such as extended temporal variability within a season. To further enhance the representativeness, all collected leaves were pooled, dried, and homogenized prior to essential oil extraction. This method effectively reduced individual-level variability and provided a composite sample that reflects the overall chemical composition of the population for each season. By adopting this strategy, we ensured that the observed seasonal variations in chemical composition and biological activities are robust and indicative of population-level patterns, rather than being artifacts of individual variability or sampling limitations.

The plant specimen is in the Herbarium of the Community University of the Chapecó Region under number UNO 3700. This species was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under registration number A22F841. After collection, the leaves were manually separated from the branches and dried without exposure to sunlight at room

temperature for approximately eight days. After being packed in plastic bags, samples were stored at 4.0 °C until the extraction process.

Leaves of *A. gratissima* (crushed) were submitted extraction by hydrodistillation was carried out in a Clevenger-type apparatus, with 300 g of sample for 1.8 L of distilled water were added, during 120 min. *A. gratissima* essential oil (AGO) obtained were dried with anhydrous sodium sulfate and stored at 4.0 °C until subsequent analyses. The yield of AGO was calculated according to equation:

$$\text{Extraction yield (\%)} = \frac{\text{AGO mass (g)}}{\text{Initial mass (g)}} \times 100$$

3.2. GC-MS analysis

The AGO chemical composition was analyzed by Agilent GC-MS (7890B) gas chromatography coupled to a quadrupole mass spectrometer (5977A) (Agilent Technologies, Palo Alto, CA, USA). The GC-MS system experimental conditions were described by Scapinello et al. (2018) and Souza et al. (2020), with some modifications. The Agilent 19091S capillary column (30 m × 250 µm × 0.25 µm) was used and the mobile phase flow (carrier gas: He) was adjusted to 1.0 mL/min. The GC temperature program was 40.0 °C for 4 min to 240.0 °C at a rate of 10 °C/min and up to 300.0 °C at a rate of 40.0 °C/min (maintained for 5 min). The injector temperature was 280.0 °C and the sample injection volume 1 µL (split ratio 1:20). The MS transfer line temperature was set to 150.0 °C and the source of ions temperature at 230.0 °C. For GC-MS detection, an ionization energy set at 70 eV and mass range at *m/z* 40–400. The chemical components present in the extract were identified by comparison with the equipment library (Agilent P/N G1033A) and comparing retention times; homologous C8–C30 n-alkane series by determining the linear retention index (LRI) according to van Den Dool and Dec. Kratz (1963). The relative amounts of each individual component were calculated using their respective peak areas in the chromatogram.

3.3. Antimicrobial assays

The antibacterial activity of AGO was evaluated against two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (NCTC 10320) and three Gram-negative bacteria, *Escherichia coli* (ATCC 8739), *Salmonella enterica* (ATCC 13076) and *Proteus mirabilis* (ATCC 35659), and still the *Candida albicans* (ATCC 10231) fungus. These strains were a standard reference of the American Type Culture Collection (ATCC) (Rockville, MD, USA) and Ceremic (C) – Mycological Reference Center (Facultad de Ciencias y Bioquímicas Farmacéuticas, Rosario, Argentina) and were obtained from the Food Analysis Laboratory - LANAL, Chapecó, SC, Brazil. The culture medium used for the bacteria was Mueller-Hinton agar, while Sabouraud Dextrose 4.0 % agar was used for the cultivation of fungi.

The minimum inhibitory concentration (MIC) of AGO was determined by the agar dilution assay according to CLSI (2009), with some modifications from Gasparetto et al. (2016). A series of dilutions was prepared at concentrations ranging from 25000 to 48 ppm, total volume of 1 mL. Afterwards, a volume of 1 µL of inoculum suspension was added to each tube, with the exception of sterile control. The oil-free solution was also used as a blank control. Each test was repeated until similar results were obtained. Tubes were incubated at 36.0 °C for 24 h for bacteria and 30.0 °C for 48 h for fungi. MIC was visually recorded after 24 h for bacteria and 48 h for fungi. Then, the minimum microbicidal concentration (MMC) was performed. The MIC was defined as the lowest concentration in ppm capable of inhibiting microbial growth.

3.4. Antioxidant assays

Ethanol solutions of AGO were prepared at concentrations from 0.8 to 10 µg/mL. From a 0.3 mM DPPH solution, 0.87 µL were added to 213

µL of an AGO ethanolic solution and allowed to react at room temperature and away from light for 30 min. After, the absorbance was read in a UV-Vis spectrophotometer at 517 nm and converted into the percentage of antioxidant activity (AA) using the following equation:

$$\text{AA (\%)} = 100 - \left[\frac{(\text{Abs.sample} - \text{Abs.blank}) \times 100}{\text{Abs.control}} \right]$$

As a blank, 213 µL of the solution and 87 µL of ethanol were used. As a control, 213 µL of ethanol and 87 µL of DPPH solution were used and all the analyses were performed in quadruplicate (Mensor et al., 2001). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.5. Statistical analysis

AGO yield and antioxidant activity are presented as mean ± S.E.M. Data normality was assessed using the Barlett test. Statistical analyses were performed using Graph Pad Prism 8.0 for Windows (GraphPad Software, San Diego, California, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni test, comparing the seasons; p values of less than 0.05 were considered significant. Multivariate analyses to detect similarity by Euclidean distance in AGO constituents in the seasonal periods was evaluated by a biplot analysis using the Past Program, using the grouping parameters indicated by Dormann et al. (2013) and Legendre (1998). The Redundancy Analysis (RDA) was conducted to investigate the relationship between antimicrobial activity (MIC and MMC), considering the different chemical compounds in seasonal AGO. The statistical significance of the correlation between them was determined using the Monte Carlo test with 5000 permutations (p < 0.05, performed in R program, version 4.1.3–2022-03-10).

4. Conclusions

The essential oil of *A. gratissima* exhibited similar yields across all seasons, albeit with varying chemical compositions. Terpenes were identified as the primary chemical constituents, including pinocamphe, β-pinene, *trans*-pinocarvyl acetate, and guaiaol, all of which are widely recognized for their antimicrobial properties. The essential oil demonstrated effectiveness against a range of Gram-positive and Gram-negative bacteria, as well as the fungus *C. albicans*. Notably, seasonality was found to influence the oil's antimicrobial and antioxidant activity. However, no direct correlation was observed between the concentrations of individual compounds and antimicrobial effects. These results contribute significantly to our understanding of the unique properties of *A. gratissima* essential oil, highlighting its potential as a natural product for applications in pharmacology, cosmetics and agriculture.

CRediT authorship contribution statement

Maryelen A. Souza: Writing – original draft, Investigation, Formal analysis, Data curation. **Ketelin Kunh:** Methodology, Formal analysis, Data curation. **Otávio Sanaioito:** Methodology, Formal analysis. **Monica S.Z. Schindler:** Validation, Methodology, Investigation, Formal analysis. **Adriana Gasparetto:** Methodology, Investigation, Data curation. **Kanandra T. Bertoncello:** Validation. **Renan de Souza Rezende:** Validation, Supervision, Investigation, Data curation. **J. Vladimir Oliveira:** Writing – review & editing, Supervision, Project administration. **Jaqueline Scapinello:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Data curation, Conceptualization. **Jacir Dal Magro:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Liz G. Müller:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Data curation, Conceptualization.

Data statement

All data generated and/or analyzed during this study are included in this publication article. All datasets are made available by the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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APLICAÇÃO DE ÓLEOS ESSENCIAIS EM ALIMENTOS: UMA REVISÃO SOBRE DESAFIOS E PERSPECTIVAS

APPLICATION OF ESSENTIAL OILS IN FOOD: A REVIEW ON CHALLENGES AND PERSPECTIVES

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Resumo

Os óleos essenciais são uma importante fonte de compostos bioativos com propriedades antioxidantes e antimicrobianas que demonstram potencial para atuarem como aditivos naturais em alimentos. São metabólitos secundários de plantas constituídos principalmente de terpenos e fenilpropanoides. Esses óleos são insolúveis em água, voláteis e possuem odor forte e característico. Porém, essas características podem afetar a incorporação desses óleos nos alimentos. Atualmente os óleos essenciais vêm sendo estudados com diversas alternativas de utilização em nanoemulsões, nanocápsulas, nano e micropartículas poliméricas. Neste contexto, o objetivo deste estudo foi realizar uma revisão de literatura sobre os óleos essenciais e suas funcionalidades na área alimentícia. Para realizar a pesquisa foram utilizadas as seguintes palavras-chaves: óleo essencial em alimentos, atividades antimicrobiana e antioxidante e métodos de encapsulamento disponíveis nos bancos digitais. Resultando na importância da utilização destes óleos nas indústrias de carnes, lácteos e outras, considerando que o mesmo pode ser empregado como alternativa aos aditivos tradicionais. O uso de tecnologias de encapsulação tende a auxiliar na estabilização dos óleos, reduzindo os impactos organolépticos nos alimentos, assim como, a liberação de seus constituintes (metabólitos secundários), favorecendo as suas potencialidades e aumentando a vida útil dos produtos alimentícios.

Palavras-chaves: Antimicrobiano. Encapsulação. Metabólitos secundários.

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Abstract

Essential oils are compounds that demonstrate the potential to act as natural additives in food products due to the antimicrobial and antioxidant. They are secondary plant metabolites consisting of terpenes and phenylpropanoids. These oils are insoluble in water, volatile and have a strong and characteristic flavor. However, these characteristics can affect the incorporation of these oils in foods. Currently, essential oils have been studied with several alternatives for use in nanoemulsions, nanocapsules, nano and polymeric microparticles. In this context, the objective of this study was to carry out a literature review on essential oils and their functionalities in the food area. To carry out the research, the following keywords were used: essential oil in food, antimicrobial activity and encapsulation methods available in digital banks. Resulting in the importance of using these oils in the meat, dairy and other industries, considering that it can be used as an alternative to traditional synthetic additives. The use of improved technologies tends to help in the stabilization of oils, thus reducing the impacts, reduces organoleptics in foods, as well as the release of their constituents (secondary metabolites), favoring their potential and increasing the self-life of food products.

Keywords: Antimicrobial. Encapsulation. Secondary metabolites.

1. INTRODUÇÃO

Os óleos essenciais (OEs) são compostos líquidos, voláteis, com odor e cor característicos, formados a partir de metabólitos secundários de plantas, presentes em todos os órgãos desta, como brotos, flores, folhas, caules, galhos, sementes, frutas e cascas. De acordo com a Organização Internacional de Padronização (ISO - *International Organization for Standardization*), óleo essencial é um produto obtido a partir de uma matéria-prima natural de origem vegetal, por destilação a vapor, hidrodestilação, ou prensagem à frio, esta última principalmente do epicarpo de frutas cítricas (Barros; Assis; Mendes, 2014; Sharma *et al.*, 2021).

São constituídos de uma mistura complexa de compostos poucos solúveis em água, principalmente metabólitos secundários da classe dos terpenos e fenilpropenos. Os terpenos são hidrocarbonetos compostos por várias unidades de isopreno, e podem ser classificados pelo número de unidades de isopreno na molécula (mono, sesqui e diterpenos). Os fenilpropenos são compostos fenólicos ou polifenóis, podem variar de moléculas simples, tais como ácidos fenólicos, para compostos altamente polimerizados, como taninos. Dentre os fenilpropenos presentes nos OEs, o mais conhecido é o eugenol (Hassoun; Çoban, 2017; Dima; Dima, 2015; Ribeiro-Santos *et al.*, 2018; Stevanović *et al.*, 2018).

Na natureza, os compostos presentes nos OEs têm a função de proteger a planta contra-ataques de predadores, como insetos e microrganismos, além de atrair polinizadores para dispersão de pólenes e sementes. Seus constituintes lhes conferem características de aroma, cor e funções antissépticas e são armazenados em células secretoras, epidérmicas, cavidades, canais ou tricomas glandulares presentes em todos os órgãos das plantas. Além de serem utilizados na fabricação de perfumes, devido às suas propriedades aromáticas, os OEs também são aplicados na produção de fármacos e cosméticos funcionais, como sedativos, analgésicos, anestésicos locais, antimicrobianos e anti-inflamatórios (Silveira *et al.*, 2012; Lavabre, 2019). Juntamente com os extratos, os OEs se enquadram como aromatizantes naturais permitidos para aplicação em alimentos (Brasil, 2007; Bakalli *et al.*, 2008).

Com o aumento da produção de alimentos em escala mundial e da preocupação com a segurança, é indispensável o uso de aditivos alimentares que conservem a qualidade e as características desejáveis. Durante os últimos anos observa-se uma mudança no comportamento dos consumidores de alimentos industrializados que exigem, cada vez mais, alimentos naturais e seguros para a saúde. O consumo de aditivos sintéticos, como corantes, aromatizantes e conservantes estão sendo associados a vários problemas de saúde, com efeitos adversos agudos ou crônicos, como alergias, hipersensibilidade alimentar, hiperatividade e câncer. Este fato vem impulsionando uma transformação no desenvolvimento de novos produtos pelo setor industrial, através principalmente da utilização de matérias-primas naturais nas formulações dos alimentos (Ibarz; Augusto, 2017).

O interesse em produtos naturais, como os OEs, está associado principalmente as suas diferentes propriedades biológicas, como antioxidante, antimicrobiana, analgésica, antidiabética e anti-inflamatória (Kumar; Naraian, 2019; Batiha *et al.*, 2020). Devido a essas características, a indústria de alimentos, cada vez mais, está empregando-os na formulação de produtos ou incorporando-os em embalagens. Sendo que um óleo essencial ideal para indústria de alimentos seria aquele que está disponível em grandes volumes, como coproduto e que é geralmente reconhecido como seguro (GRAS – *Generally Reconized as Safe*). Há diversas variáveis que interferem no rendimento da extração dos OEs, sendo alguns deles a escolha da planta, época de colheita, parte da planta utilizada, o solvente e o método de extração os mais significativos (Calo *et al.*, 2015).

A utilização dos OEs de forma industrial pode apresentar algumas limitações como a grande variabilidade da composição química, interações com a matriz alimentar, toxicidade, características sensoriais peculiares, necessidade de padronização prévia da forma de aplicação no produto, natureza volátil e susceptibilidade à oxidação, sendo necessário o desenvolvimento de tecnologias e de pesquisas para encontrar alternativas que superem essas barreiras (Ribeiro-Santos *et al.*, 2018; Stevanović *et al.*, 2018).

Como uma das alternativas para a indústria alimentícia, processos de encapsulação ou produção de nanoemulsões, são tecnologias que contribuem para melhorar a estabilidade, direcionar a ação dos compostos bioativos, prolongar a vida útil, melhorar a biodisponibilidade, bem como facilitar a incorporação dos óleos essenciais no alimento. Outra opção é a incorporação do óleo essencial em embalagens, com o desenvolvimento de embalagens ativas, que permitem a interação entre o alimento e a embalagem, promovendo atividade antimicrobiana e antioxidante, sem a necessidade de o óleo ser incorporado diretamente ao alimento (Prakash *et al.*, 2018; Hadidi *et al.*, 2020).

Estudos com aplicação de óleos essenciais crescem no âmbito científico, a fim de comprovar as atividades terapêuticas disseminadas pelo conhecimento popular, sendo necessária realizar uma abordagem mais ampla sobre os efeitos dos óleos essenciais aplicados em alimentos na qualidade, segurança e aceitação sensorial, almejando fornecer uma visão abrangente das suas propriedades antimicrobianas, antioxidantes e aromáticas, bem como identificar possíveis desafios e oportunidades para sua aplicação na indústria alimentícia.

Nesse contexto, o objetivo deste estudo foi realizar uma revisão da literatura sobre a aplicação de óleos essenciais em alimentos, principalmente produtos cárneos, de forma direta ou através de técnicas de nanoemulsões e encapsulação.

2. MATERIAL E MÉTODOS

Este estudo utilizou um método qualitativo (revisão de literatura) e incluiu artigos publicados nos últimos 15 anos. Foram utilizadas as seguintes bases de dados: *Web of Science*,

Scopus, *Pubmed*, *Springer Nature* e *Scientific Eletronic Library*. Como critério de busca foram utilizadas as seguintes palavras-chaves: *essential oils*, *bioactive compounds*, *antimicrobial*, *antibacterial*, *antioxidant*, *food*, *food packaging*, *meat*, *nanoemulsion* e *encapsulation*, bem como diferentes combinações dessas palavras. A pesquisa ocorreu entre os meses de fevereiro e julho de 2023.

3. RESULTADOS E DISCUSSÃO

3.1 Seleção dos artigos

Um número expressivo de artigos científicos sobre OEs foi encontrado, e após refinar a busca de acordo com as palavras-chaves, a partir das áreas química e de alimentos, com análise detalhada dos títulos e resumos, os textos completos que apresentavam a avaliação de atividades antimicrobiana e antioxidante de OEs puros, bem como na forma de nanoemulsões ou encapsulados, e a avaliação destas atividades *in situ*, principalmente em produtos cárneos, foram os selecionados, resultando em um total de 66 artigos científicos.

3.2 Aplicação de óleos essenciais como conservantes naturais em alimentos

A contaminação bacteriana causa grandes perdas à indústria de alimentos, além de resultar em sérios problemas de saúde pública devido a ocasionar doenças de origem alimentar. A partir disso, houve a adesão de antimicrobianos, antioxidantes e outros conservantes neste ramo, para que houvesse redução de danos (Becerril; Nerín; Silva, 2020). Contudo, nos últimos anos tem havido uma queda na busca por alimentos processados em função do conhecimento da população em relação ao potencial carcinogênico dos conservantes alimentícios, fato que é alertado pela Organização Mundial da Saúde (OMS, 2015). Em função disso, a indústria de alimentos têm estudado alternativas naturais a esses conservantes sintéticos, como os óleos essenciais extraídos de diferentes partes de plantas (Radünz *et al.*, 2019).

Os óleos essenciais costumam ter atividades antioxidante e antimicrobiana satisfatórias. A sua eficácia como antimicrobianos é relacionada principalmente à hidrofobicidade, que permite que entrem nas células dos microrganismos e causem alterações na permeabilidade das membranas, gerando alterações na estrutura e destruição da célula (Mesic *et al.*, 2021). Devido a estas propriedades, os óleos essenciais estão sendo amplamente estudados e explorados pelas indústrias alimentícias, seja pela adição direta no produto, bem como no desenvolvimento de embalagens ativas para extensão da vida útil dos alimentos (Pelissari *et al.*, 2009; Negi, 2012; Tavares *et al.*, 2014; Ouedrhiri *et al.*, 2016).

A agência regulatória de saúde dos Estados Unidos (*Food and Drug Administration* - FDA) reconhece a segurança dos óleos essenciais de acordo com o Código de Regulamentos Federais (FDA, 2019). Destacando-se os OEs reconhecidos como seguros pela FDA (2019): Alecrim (*Rosmarinus officinalis*); Baga de zimbro (*Juniperus communis*); Bergamota (*Citrus bergamia*); Cal (*Citrus aurantifolia*); Camomila Romana (*Anthemis nobilis*); Canela (*Cinnamomum zeylanicum*); Cassia (*Cinnamomum cassia*); Coentro (*Coriandrum sativum*); Cominho (*Cuminum cyminum*); Cravo-da-Índia (*Eugenia caryophyllata*); Erva-cidreira (*Cymbopogon flexuosus*); Erva-doce (*Foeniculum vulgare*); Gengibre (*Zingiber officinale*); Gerânio (*Pelargonium graveolens*); Hortelã (*Mentha spicata*); Hortelã-pimenta (*Mentha piperita*); Laranja azeda (*Citrus aurantium*); Laranja Selvagem (*Citrus sinensis*); Limão (*Citrus limon*); Manjeriço (*Ocimum basilicum*); Manjerona (*Origanum majorana*); Melissa (*Melissa officinalis*); Orégano (*Origanum vulgare*); Pimenta preta (*Piper nigrum*); Sálvia (*Salvia sclarea*); Tangerina

(*Citrus reticulado*); Tomilho (*Thymus vulgaris*); Toranja (*Citrus paradisi*); Ylang Ylang (*Cananga odorata*). Além disso, a União Europeia aprovou o uso destes também em embalagens de alimentos, estratégia que diminui a ingestão, considerando que níveis elevados geram toxicidade (Becerril; Nerín; Silva, 2020).

Fernández-López e Viuda-Martos (2018), realizaram uma revisão bibliográfica e descobriram centenas de artigos publicados relacionados às atividades antioxidante e antimicrobiana de óleos essenciais. Quando filtraram a busca por tipos de alimentos, encontraram 657 artigos relacionados à conservação de frutas, 403 relacionados à conservação de vegetais, 415 relacionados à conservação de peixes, 410 relacionados à conservação de carnes, 216 relacionados à conservação de leite e derivados e 97 relacionados à conservação de panificados.

De fato, numerosos estudos comprovam a eficácia dos óleos essenciais na conservação de alimentos, principalmente de origem animal. Em queijos, por exemplo, o uso dos OEs é amplamente avaliado através de estudos *in vitro* e em alguns casos com avaliações sensoriais que determinam a eficiência da aplicação no produto. Os principais OEs como orégano, capim-limão, tomilho e cravo da Índia demonstram eficácia como antimicrobianos naturais, principalmente sobre agentes patogênicos de importância na indústria láctea como *Staphylococcus aureus*, *Escherichia coli* e *Listeria monocytogenes* (Durço *et al.*, 2020). Outras matrizes como bebida láctea, iogurte e até o leite fluido também foram avaliadas em estudos com aplicação de OEs. Até mesmo em doce de leite a aplicação dos OEs de cravo e de canela demonstraram ação antioxidante notável quando comparado com amostras controle, preservando ainda as características sensoriais do produto, com aceitação pelos consumidores equivalente ao produto controle (Antigo *et al.*, 2017).

Já em produtos cárneos a aplicação é ainda mais frequentemente estudada, principalmente em derivados cárneos como salames, hambúrgueres e linguiças. Nestes casos, a combinação do aroma dos OEs é mais facilmente equilibrada sensorialmente, principalmente os OEs extraídos de especiarias como orégano, alecrim, cravo e manjerição, associando as características aromáticas às propriedades funcionais (Boskovic *et al.*, 2019).

Tofiño-Rivera *et al.* (2017), constataram que as espécies de *Salmonella* spp., *Staphylococcus aureus* e *Escherichia coli* são os microrganismos mais comumente encontrados na contaminação de embutidos cárneos. Estes autores avaliaram a substituição de conservantes químicos na produção de linguiças artesanais por OEs de tomilho (*Thymus vulgaris*) e cravo-da-Índia (*Syzygium aromaticum*). Apesar de os conservantes químicos serem responsáveis por menor desenvolvimento microbiano nos produtos finais em comparação com os produtos preparados com os óleos essenciais, os embutidos produzidos com os OEs se destacaram nos atributos cor e sabor nas análises sensoriais conduzidas, além de também demonstrarem satisfatória conservação, com inibição de crescimento para *Salmonella* spp., *S. aureus* e *E. coli* (Tofiño-Rivera *et al.*, 2017). Resultados similares foram obtidos por Šojić *et al.* (2019), os quais relataram significativa atividade antioxidante e antimicrobiana do óleo essencial de coentro (*Coriandrum sativum*) quando aplicado como aditivo alimentar em linguiças de porco cozidas.

As atividades antioxidante e antimicrobiana do óleo essencial de limão (*Citrus limon*) foram avaliadas por Hsouna *et al.* (2017), frente a *Listeria monocytogenes* inoculada em carne de gado picada. O óleo demonstrou excelente atividade antioxidante medida através da eliminação de radicais 2,2-difenil-1-picrilhidrazil (DPPH). A Concentração Inibitória Mínima (CIM) variou de 0,039 a 1,25 mg/mL para bactérias Gram-positivas e de 0,25 a 2,5 mg/mL para bactérias Gram-negativas, e o óleo inibiu o crescimento de *L. monocytogenes* nos pedaços de carne. Os autores concluem que essas concentrações do óleo são promissoras na prevenção da contaminação

e do crescimento de bactérias patogênicas, em especial *L. monocytogenes*, no armazenamento de carne bovina picada a 4,0 °C.

Alanazi *et al.* (2018), investigaram os efeitos inibitórios de constituintes de óleos essenciais (cinamaldeído, eugenol, isotiocianato de alila e carvacrol) contra a germinação e o crescimento vegetativo de esporos de *Clostridium perfringens* tipo A *in vitro* e *in situ* (carne de frango). Cinamaldeído, eugenol e carvacrol a 0,05 – 1,0% inibiram a germinação dos esporos de todos os *C. perfringens* isolados em meio TGY (Trypticase Glucose Levedura), e todos os constituintes (0,05 - 1,0 %) impediram o desenvolvimento e o crescimento vegetativo dos esporos de *C. perfringens* em meio TGY. Contudo, apenas o isotiocianato de alila (0,5 - 2,0%) impediu o crescimento de esporos de *C. perfringens in situ*, o que o torna efetivo no controle de esporos e células vegetativas de *C. perfringens* isolados em meio laboratorial.

Um novo método de aplicação de óleo essencial de tomilho (*Thymus vulgaris*) foi aplicado por Hernández *et al.* (2018), durante a secagem de carne, na forma de vapor diretamente em uma câmara de secagem. A carga microbiana foi avaliada nas amostras tratadas e comparada com amostras controle. Para amostras de carne inoculada com *Escherichia coli*, os autores relatam que a Concentração Inibitória Mínima do óleo contra este microrganismo após 6 h de secagem a 55 °C foi de 0,028 mL/L de ar. O estudo conclui que este método é uma opção viável e alternativa aos conservantes usuais para reduzir a carga microbiana na carne seca.

Através da demanda por produtos alimentícios minimamente processados, seguros e de qualidade, surgem as necessidades de mais estudos para verificação da eficácia dos filmes e revestimentos comestíveis na extensão de vida útil de alimentos, sendo uma inovação do conceito de embalagem ativa biodegradável, vista como desenvolvimento sustentável (Durango; Soares; Arteaga, 2011).

Nesse sentido, Tavares *et al.* (2014), desenvolveram uma cobertura comestível à base de alginato acrescida dos óleos essenciais de alecrim (*Rosmarinus officinalis*) e orégano (*Origanum vulgare*) para uso no produto lácteo ricota. Os autores verificaram ausência de coliformes a 45 °C, estafilococos e *Salmonella ssp.* nas amostras contendo óleo essencial (alecrim e orégano), analisadas durante 21 dias, e não houve diferença significativa entre as amostras em relação à análise sensorial, concluindo que a adição da cobertura comestível em ricota é uma alternativa viável para a conservação do produto.

O estudo da aplicação de uma embalagem ativa, que consistia em uma matriz de biopolímero (celulose/proteína de soro de leite) nanoestruturada reforçada com partículas de dióxido de titânio (1%) e gotículas de óleo essencial de alecrim (2%) foi realizado por Alizadeh-Sani, Mohammadian, McClemets (2020), na preservação de carne de cordeiro refrigerada a 4,0 °C por 15 dias, a fim de substituir as embalagens de plástico à base de petróleo. As amostras de carne foram analisadas periodicamente quanto à contagem de colônias e estabilidade química (pH, oxidação lipídica, lipólise). O estudo concluiu que a embalagem diminuiu significativamente o crescimento microbiano, a oxidação lipídica e a lipólise das amostras no período, o que resultou em um aumento na vida útil para aproximadamente 15 dias.

Neste estudo, um material de embalagem à base de biopolímero foi preparado usando um método de fundição, que consistia em uma matriz de nanofibra de celulose/proteína de soro de leite contendo partículas de dióxido de titânio (1% TiO₂) e gotas de óleo essencial (2% óleo de alecrim) como componentes funcionais.

Xavier *et al.* (2021), propuseram uma embalagem de quitosana funcionalizada com uma nanocápsula de zeína carregada com óleo essencial de pimenteira (*Cinnamodendron dinisii*) para a conservação de carne moída. O óleo essencial apresentou alta atividade antioxidante, que foi mantida após o nanoencapsulamento e incorporação à matriz de quitosana. Sua atividade

antimicrobiana foi significativa contra *Salmonella typhimurium* e *Shigella flexneri*. Na análise *in situ*, o óleo resultou na menor degradação oxidativa em comparação aos grupos controles. Ademais, o processo de nanoencapsulamento protegeu o óleo essencial da degradação térmica, o que garantiu sua efetividade na conservação, e, segundo os autores, essa formulação pode ser utilizada para aumentar o tempo de prateleira de carnes.

Najjaa *et al.* (2020), desenvolveram extrato de *Allium sativum* seco por congelamento, seco em forno e seu óleo essencial microencapsulado pela técnica de *spray dryer* como potenciais agentes conservantes de carne picada. No estudo, os autores compararam a atividade antibacteriana de cada formulação, e identificaram que apenas o óleo essencial microencapsulado e o alho seco por congelamento, ambos na concentração de 20% (m/m), bem como o óleo essencial microencapsulado a 15% (m/m), aumentaram em 4 dias a vida útil da carne picada.

Assim, é expressivo o potencial biológico que os óleos essenciais apresentam frente a maioria dos microrganismos de importância na indústria alimentícia, bem como processos de encapsulação demonstram melhora nas atividades, conforme relatam diversos estudos científicos, garantindo que estes possuem aplicabilidade como agentes conservantes em alimentos, principalmente de origem animal, como produtos cárneos e lácteos.

3.3 Desenvolvimentos recentes em nanoemulsões e em técnicas de encapsulamento de óleos essenciais

Os compostos bioativos presentes nos óleos essenciais são, em geral, sensíveis a fatores externos, como luz, calor e oxigênio, ou seja, são facilmente acometidos por oxidação, volatilização e degradação. Em função disso, para tornar viável a sua utilização pela indústria, pode-se fazer uso de técnicas de proteção, como o encapsulamento. Esse processo possibilita minimizar as interações dos óleos essenciais com o ambiente externo, serve como forma de liberação controlada, aumento do tempo de ação, e ainda ajuda a mascarar características sensoriais indesejáveis, sem diminuir sua ação (Asbahani *et al.*, 2015; Kaur *et al.*, 2020; Radünz *et al.*, 2020).

O encapsulamento pode ser realizado por meio de diversas técnicas, protegendo os óleos essenciais com uma ou mais camadas de um agente de revestimento. Este pode ser composto, por exemplo, por partículas poliméricas, como a quitosana, que é um polímero natural que apresenta propriedade antimicrobiana, podendo resultar em sinergismo com o óleo essencial. Polímeros naturais como a caseína, maltodextrina, alginato de sódio e poli(L-ácido láctico) são comumente empregados como agentes encapsulantes para óleos essenciais. Ademais, a formação de emulsões de óleos essenciais, um dos sistemas de encapsulamento mais utilizados, permite a incorporação de componentes hidrofóbicos em uma matriz hidrofílica (Pateiro *et al.*, 2021).

Indústrias como a alimentícia, farmacêutica, cosmética e agroquímica têm demonstrado grande interesse na utilização de dispersões coloidais para encapsular, proteger e liberar componentes bioativos lipofílicos visando aumentar sua biodisponibilidade. Uma das tecnologias aplicadas é o desenvolvimento de nanoemulsões, que consistem em dispersões coloidais termodinamicamente instáveis compostas por dois líquidos imiscíveis, um disperso no outro em partículas menores (raio entre 100 e 500 nm) que as de emulsões convencionais (McClements, 2012).

Nanoemulsões podem ser dispersões de óleo em água (O/A), água em óleo (A/O) ou água em óleo em água (A/O/A), e sua estabilidade é provida por um surfactante adequado. Surfactantes comumente utilizados nesse tipo de formulação são lecitina, proveniente da gema do ovo ou da soja, além do desoxicolato de sódio (sal biliar), polioxil-35 óleo de rícino (Cremophor® EL), polissorbato 20, 40, 60 e 80, Span 20, 40, 60 e 80 (monolaurato de sorbitan), entre outros. Além

disso, conservantes, antioxidantes e quimioprotetores também são empregados na sua composição (Ansarian *et al.*, 2022).

Em função de serem sistemas instáveis, para formar nanoemulsões é necessária a entrada de energia externa para que os componentes separados possam ser convertidos em uma dispersão coloidal, e esta deve, no mínimo, exceder a energia livre positiva associada ao aumento da área de contato entre as fases oleosa e aquosa. Os métodos possíveis de serem empregados na formação desse sistema são os de alta energia, que depende de ações mecânicas para reduzir o tamanho, a exemplo da emulsificação e homogeneização a alta pressão (por meio de microfluidizador, homogeneizador a pistão e ultrassom) e de baixa energia de emulsificação (emulsificação espontânea, método de inversão de fase) (Singh *et al.*, 2017).

Apesar da instabilidade, esse tipo de formulação não costuma apresentar problemas como cremação, floculação, coalescência e sedimentação, que são comuns em emulsões convencionais (Arianto; Cindy, 2019). Outras vantagens apresentadas pelas nanoemulsões são a menor evaporação de óleos, melhora na solubilidade de óleos essenciais e menor toxicidade, aumento da biodisponibilidade de moléculas lipofílicas, entre diversas outras, que tornam interessante a sua utilização em óleos essenciais (Das *et al.*, 2019; Kaur *et al.*, 2020).

Diversos estudos confirmam a qualidade das nanoemulsões de óleos essenciais, demonstrando que estas são adequadas para incorporação em formulações alimentares, para prevenir e controlar o crescimento microbiano e prolongar a vida útil. Das *et al.* (2019), relatam que a nanoemulsão do óleo essencial de camomila (*Matricaria chamomilla*) aumentou significativamente as atividades antibacteriana e antifúngica em comparação ao óleo essencial em emulsão convencional, frente aos microrganismos *E. coli*, *S. aureus*, *B. subtilis*, *P. aeruginosa*, *S. pyogenes*, *C. albicans* e *C. tropicalis*. Resultados semelhantes foram encontrados por Maccelli *et al.* (2020), que avaliaram a atividade antimicrobiana da nanoemulsão do óleo essencial das folhas de *Satureja montana* L., frente a microrganismos *E. coli*, *S. aureus*, *L. monocytogenes*, *P. aeruginosa* e *K. pneumoniae*, com valores de Concentração Inibitória Mínima variando de 0,39 a 6,25 mg/mL.

O óleo essencial de orégano (*Origanum vulgare*) também foi estudado por Moraes-Lovison *et al.* (2017), através da incorporação de nanoemulsões do óleo essencial em patê de frango, as quais exibiram boa ação antimicrobiana frente a *S. aureus* e *E. coli*, com Concentração Bactericida Mínima de 0,90 e 3,32 mg/mL, respectivamente. Os autores ainda relatam que a incorporação das nanoemulsões em patê de frango não alterou as características físico-químicas do produto cárneo, o que o torna adequado para ser incorporado em formulações de alimentos para controlar o crescimento bacteriano e prolongar a vida útil.

Balasubramani *et al.* (2017), encapsularam o óleo essencial das folhas de *Vitex negundo* L. e prepararam a nanoemulsão por meio do método de baixa energia de emulsificação. Esta demonstrou melhor atividade antimicrobiana que o óleo essencial não encapsulado frente a *E. coli*, *Enterobacter aerogenes* e *Enterococcus faecalis*, bem como aumento da atividade antioxidante de 28,87 para 23,26 µg/mL (IC₅₀) através do teste da eliminação de radicais 2,2-difenil-1-picrilhidrazil (DPPH). Os autores atribuíram esses resultados ao aumento da área superficial e da estabilidade causados pela nanoemulsão.

Ademais, Yazgan, Ozogul e Kuley (2019), investigaram a ação da nanoemulsão do óleo essencial de limão (*Citrus limonum*) sobre microrganismos de origem alimentar (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterococcus faecalis* e *Salmonella*) em comparação ao óleo essencial não encapsulado. Os autores relatam que a nanoemulsão teve maior atividade antimicrobiana sobre todos os patógenos, exceto para *K. pneumoniae*, e que a conversão do óleo

em nanoemulsão resultou em melhora dessa propriedade. Eles ainda sugerem que o uso dessa nanoemulsão pode ter potencial como antimicrobiano natural na indústria alimentícia.

Referente aos outros métodos de encapsulamento, diversos estudos foram realizados, com destaque para o emprego na conservação de alimentos. Radünz et al. (2019), encapsularam o óleo essencial de alho (*Syzygium aromaticum* L.) com alginato de sódio e emulsificantes a fim de utilizá-lo como antimicrobiano sem interferência do seu aroma no alimento. O encapsulamento teve alta eficiência e as partículas resultantes apresentaram menor atividade antioxidante e maior atividade antimicrobiana (*in vitro*, microrganismos *S. aureus*, *E. coli*, *L. monocytogenes* e *S. Typhimurium*) em comparação ao óleo não encapsulado. A atividade antimicrobiana, *in situ*, em hambúrguer, do óleo essencial a 3,04 mg/mL teve resultados superiores aos do conservante nitrito, com valores de 3,84 e 4,11 log UFC/g para *S. aureus*, para o óleo e conservante, respectivamente, considerado a análise realizada sete dias após a preparação dos hambúrgueres.

Silva et al. (2020), utilizaram a técnica de ultrassonificação para encapsular componentes de óleos essenciais (cuminaldeído e isoeugenol) com metil- β -ciclodextrina a fim de aumentar sua solubilidade em água e seus efeitos antimicrobianos. Os resultados dos testes realizados confirmaram o aumento na estabilidade e na solubilidade dos componentes do óleo, na atividade antioxidante e na atividade antimicrobiana frente a *S. aureus* e *E. coli* em comparação aos componentes não encapsulados.

O óleo essencial de canela (*Cinnamomum verum*) foi encapsulado em uma permutita ($\text{Ag}^+/\text{Zn}^{2+}$) nanoporosa a fim de promover liberação prolongada do óleo e efeito antimicrobiano sinérgico como conservante em embalagem de alimentos. As partículas encapsuladas demonstraram atividade antimicrobiana frente a *Aspergillus niger* e *Penicillium* sp. em comparação à permutita/óleo não encapsulada, que não apresentou atividade antimicrobiana para estes microrganismos. Pads de etilcelulose modificados pelas partículas encapsuladas foram testados quanto à atividade antimicrobiana *in situ* na conservação de amora chinesa e demonstraram incidência de decomposição significativamente menor que o grupo controle (NIU et al., 2018).

Biduski et al. (2019), encapsularam óleo essencial de alecrim (*Rosmarinus officinalis*) com o emulsificante anidrido octenil succínico (OSA-starch) por meio da técnica de *eletrospray*, e a eficiência de encapsulação foi avaliada, resultando em valores de 82 a 98%. Em resumo, anidrido octenil succínico (OSA-starch) produziu emulsões estáveis e adequadas para a encapsulação pela técnica de *eletrospray* - especialmente a emulsão com 20% de etanol (v/v), que resultou em menores gotículas e maior estabilidade que as emulsões com maior concentração de etanol.

A técnica de *spray dryer* é uma das mais utilizadas para transformar compostos líquidos em pó desidratado, e o encapsulamento de OEs por *spray dryer* é muito promissora, considerando que este equipamento é bastante difundido em nível industrial, facilitando sua aplicação em diversos setores, bem como resultar em boa retenção de compostos voláteis, sem alterar significativamente a composição química dos OEs, mantendo a atividade biológica (MINOZZO et al., 2021).

Locali-Pereira et al. (2020), microencapsularam o óleo essencial de pimenta rosa (*Schinus terebinthifolia*) em matrizes de camadas simples ou duplas pela técnica de *spray dryer* a fim de modular sua volatilidade e propriedades antimicrobianas. As partículas com camada simples foram estabilizadas com proteína de soja isolada, enquanto as de camada dupla, com proteína de soja isolada/alta metoxil pectina. As microcápsulas de camada dupla demonstraram maior conservação dos componentes voláteis do óleo, menor adsorção de água em armazenamento em umidade relativa maior ou igual a 75 % e maior atividade antimicrobiana em comparação às de camada simples. Ainda, ambas inibiram o crescimento de *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria*

monocytogenes e *Listeria innocua* *in vitro*. *In situ* (leite), ambas reduziram o crescimento bacteriano, enquanto o óleo não encapsulado não demonstrou inibição satisfatória. Os autores concluíram que a microencapsulação melhorou as propriedades do óleo essencial estudado e destacaram seu potencial como um conservante natural em produtos lácteos.

Uma pesquisa foi realizada sobre encapsulação do óleo essencial de tomilho (*Thymus vulgaris*) e avaliação do potencial antimicrobiano *in vitro* e *in situ* em produtos cárneos do tipo hambúrguer. Microcápsulas de caseína-maltodextrina foram produzidas por secagem por pulverização (*spray dryer*) e avaliadas quanto à eficiência de encapsulação, estabilidade térmica, compostos químicos e morfologia, além da atividade antimicrobiana nos hambúrgueres após cocção. Como resultado, os autores observaram que as cápsulas contendo o óleo essencial foram responsáveis pela inibição *in vitro* e *in situ* dos microrganismos *S. aureus*, *E. coli*, *L. monocytogenes* e *S. Typhimurium*. Além disto, as cápsulas apresentaram alta eficiência de encapsulação (88,9 %) e estabilidade térmica, o que permitiu maior proteção do óleo essencial. O efeito antimicrobiano do óleo essencial encapsulado frente a *S. aureus*, no produto, foi verificado após 14 dias de ensaio, quando o óleo essencial não encapsulado não apresentou essa atividade nesse tempo. Os autores do estudo relatam que esse resultado é devido à liberação lenta de compostos voláteis que ficam protegidos pelo agente encapsulante (Radünz *et al.*, 2020).

Considerando que a produção de alimentos exige diferentes processos, em especial térmicos, a aplicação de óleos essenciais como agentes conservantes é relatada como mais bem sucedida quando processos de proteção são empregados, uma vez que OEs apresentam compostos voláteis, que podem evaporar ou até mesmo se decompor sob as condições de processamento. Processos de encapsulação (micro e nano) são relatados na literatura como eficazes na manutenção das atividades antioxidantes e antimicrobianas, bem como na preservação dos compostos bioativos dos OEs.

3.4 Principais limitações de aplicações de óleos essenciais em alimentos

É notório que os óleos essenciais apresentam promissoras propriedades para a conservação de alimentos. No entanto, é importante reconhecer suas principais limitações. A volatilidade dos compostos aromáticos presentes nos óleos essenciais pode resultar em degradação destes ao longo do tempo, reduzindo sua eficácia como aditivo alimentar. Nesse sentido, a encapsulação torna-se uma técnica atraente na preservação de compostos instáveis, facilitando sua aplicação na indústria alimentícia. Cabe ressaltar que, por vezes, técnicas de encapsulação podem apresentar alguns inconvenientes como: gerar microcápsulas não uniformes; produção de pós muito finos que podem necessitar de processamento adicional; causar alterações em materiais sensíveis ao calor; e limitação na escolha de materiais de parede, os quais devem possuir baixa viscosidade em concentrações relativamente altas (Mahdavi *et al.*, 2014).

A estabilidade dos óleos essenciais em diferentes condições de processamento e armazenamento também pode ser um desafio, pois fatores como temperatura, luz e oxigênio podem afetar sua composição e qualidade. Portanto, é necessário realizar estudos detalhados para determinar as concentrações ideais, métodos de aplicação e formas de proteção dos óleos essenciais, a fim de maximizar seus benefícios e minimizar suas limitações no uso em alimentos.

Pesquisas envolvendo OEs são realizadas principalmente na intenção de promover uma alimentação segura, com um melhor controle microbiológico, evitando a transmissão de doenças por alimentos (conhecidas como DTAs) (Bellik, 2014; Noshirvani *et al.*, 2017). Os principais constituintes dos óleos essenciais, os metabólitos secundários, podem ter sua produção pelas plantas alterada devido às variações do meio ambiente, tais como: sazonalidade, temperatura, disponibilidade hídrica, altitude, nutrientes, ritmo circadiano e desenvolvimento, o que irá

influenciar na atividade biológica do óleo essencial. A alteração do perfil químico faz com que estudos similares, com os mesmos OEs e microrganismos possam apresentar resultados diferentes (Zhao; Rhee, 2022).

Estudos também destacam a maior sensibilidade de bactérias Gram-positivas ao uso de OEs, uma vez que as espécies Gram-negativas vêm demonstrando maior resistência, aumentando a necessidade de promover estudos mais focados no mecanismo de ação dos OEs na estrutura celular dos microrganismos (Santurio *et al.*, 2011; Beraldo *et al.*, 2013; Majolo *et al.*, 2014). A sensibilidade de um microrganismo a um determinado óleo essencial geralmente é avaliada pela determinação da Concentração Inibitória Mínima (CIM), que representa a menor quantidade do agente capaz de inibir o crescimento microbiano, via técnicas como a diluição em placas ou microdiluição em tubos (Oliveira *et al.*, 2009; CLSI, 2012). Os óleos essenciais apresentam boa atividade antimicrobiana quando a CIM é, em geral, inferior a 25 mg/mL, sendo resultados acima disso pouco promissores para a continuidade do estudo e aplicação do óleo *in situ*.

Produtos naturais como extratos e óleos essenciais são misturas complexas de compostos, assim, ressalta-se a importância de avaliar se o efeito investigado é de algum composto majoritário ou um efeito sinérgico. A avaliação do perfil fitoquímico do óleo utilizado por métodos cromatográficos e/ou espectroscópicos é primordial para identificar os principais constituintes, e estes testes demandam instrumentação de valor consideravelmente elevado, bem como técnicas de fracionamento e purificação, que são demasiadamente demoradas e complexas, para aplicação em novos testes, almejando detectar o composto (ou compostos) responsáveis pela ação antimicrobiana (Nazzaro *et al.*, 2013; Stashenko; Martinez, 2018).

A aplicação de óleos essenciais em alimentos também deve ser realizada com cautela pois, alguns óleos essenciais podem apresentar potencial toxicidade quando utilizados em concentrações elevadas. Testes *in vivo* avaliando doses seguras para administração oral devem ser realizados para garantir a segurança (Stevanović *et al.*, 2018).

Os óleos essenciais são opções mais naturais e benéficas para conservação de alimentos, mas seu alto custo em comparação aos conservantes usuais pode ser um desafio para sua adoção generalizada na indústria alimentícia. Uma das principais razões para o elevado custo dos óleos essenciais é o processo de extração, que exige equipamentos e mão de obra qualificada. Em geral, o rendimento de extração de óleo essencial é relativamente baixo, com valores médios de 0,1% a 5%, com base no peso seco da planta utilizada, o que contribui para o seu preço mais elevado (Başer; Buchbauer 2016; Bazana; Codevilla; Menezes, 2019). No entanto, à medida que a demanda por alimentos mais naturais e saudáveis aumenta, através da crescente preocupação com uma melhor qualidade de vida, é possível que o uso de óleos essenciais se torne cada vez mais viável e acessível.

4. CONSIDERAÇÕES FINAIS

Os óleos essenciais demonstram ser uma alternativa atraente e potencial para aplicação em alimentos e suas embalagens e, novas possibilidades estão sendo buscadas pelos pesquisadores, devido principalmente às suas propriedades antioxidantes e antimicrobianas. No entanto, sua incorporação em alimentos é um desafio devido à volatilidade, susceptibilidade à degradação química e seu aroma e sabor marcantes, bem como o elevado custo. O encapsulamento se mostra como uma abordagem eficaz para superar algumas dessas desvantagens, pois essa estratégia protege o óleo, proporciona uma liberação controlada de compostos ativos e ameniza os odores intensos associados aos óleos essenciais. Mais estudos sobre sistemas alimentares reais são essenciais e necessários para fornecer resultados confiáveis, através de pesquisas sobre as interações desses com o produto alimentício e/ou sua embalagem.

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