Chemical composition and toxicity of the essential oil of *Aloysia* Paláu species (Verbenaceae) from South Brazil

Composição química e toxicidade do óleo essencial das espécies de *Aloysia* Paláu (Verbenaceae) do Sul do Brasil

Resumo

No Brasil existem nove espécies nativas de *Aloysia* que são pouco estudadas quanto às composições químicas e atividades biológicas. Assim, este estudo descreve a composição química determinada por CG-EM do óleo essencial de cinco espécies nativas e uma cultivada de *Aloysia* ocorrentes no Rio Grande do Sul e avalia a toxicidade dos óleos essenciais de *A. citrodora*, *A. lycioides* e *A. dusenii* através do bioensaio com *Artemia salina*. Um grupo de espécies apresentou 1,8-cineol como composto principal: *A. dusenii* (16,2%), *A. citrodora* (32,8%), *A. lycioides* coletada em...
Guaiña (49,5%) y A. lycioides recolectada en São Marcos (17,6%). En cuanto a las especies A. polygalifolia y A. virgata presentaron mayores proporciones de germacreno-D (11,2% y 12%, respectivamente) y A. chamaedryfolia presentó espatulenol (15,6%). La especie A. lycioides recolectada en Rosário del Sur tuvo β-Felandreno (23,7%) como compuesto mayoritario. Todos los aceites esenciales probados presentaron alta toxicidad contra Artemia salina con valores de CL50 entre 48,12 µg mL\(^{-1}\) y 55,96 µg mL\(^{-1}\).

**Palabras clave:** Artemia salina; Hidrodestilación; Rio Grande do Sul.

### 1. Introduction

Verbenaceae is a family of about 31 genera and approximately 918 species of herbs, shrubs, or small trees, mainly distributed in tropical and subtropical regions (Stevens, 2001). Among the most important genus are *Lippia* and *Aloysia* (Ricco et al, 2010). *Aloysia* comprises 30 species and is distributed from the South of the United States and Mexico until the north of Patagonia (Siedo, 2006), characterized by shrubby form, aromatic inflorescences, and known primarily for its essential oils (Hernandez et al, 2003). Nine species of *Aloysia* are native in Brazil, occurring all in the South region, and *A. citrodora* is found only under cultivation (Refloa, 2016).

Aromatic plants have been used since ancient times for their medicinal properties (Bakkali et al, 2008) and their odors are determined by the presence of essential oils (EOs), a mixture of volatile low-molecular-weight mono- and sesquiterpenes and other isoprenes (Singh et al, 2002). The EOs are involved in various ecological interactions including ones of medicinal properties such as bactericidal, fungicidal and antiviral properties (Chao et al, 2000), besides they may also exhibit cytotoxic activity (Sacchetti et al, 2005).

The brine shrimp lethality bioassay is rapid, simple, easily mastered, inexpensive, and requires small amounts of test material (Ghisalberti, 1993) to predict toxicity (McLaughlin, 1991). Since its introduction, this test has been successively employed to provide a frontline screen backed up by more specific and more sophisticated bioassays (Apu et al, 2010).

The present study reports the chemical composition by GC-MS of the essential oils of five native species of the genus *Aloysia* distributed in the Rio Grande do Sul - South Brazil and one cultivated species. Moreover, the cytotoxic activity of essential oil of the species *A. citrodora, A. lycioides* and *A. dusenii* were evaluated by brine shrimp bioassay.

### 2. Methodology

#### 2.1 Plant material

Aerial parts from six species of *Aloysia* (leaves, flowers, and stem) were collected at seven locations in the Rio Grande do Sul State, Brazil (Table 1). The samples were identified, and voucher specimens were deposited in the Herbarium of the Universidade de Caxias do Sul (HUCS) and Herbarium of the Universidade Federal do Rio Grande do Sul (ICN). The detailed data referent to each species can be found in Table 1.
Table 1. Location, Herbarium Number, and Collect Date of each specimens of Aloysia species used for chemical characterization.

| Plant Name                | Location                              | Herbarium Number | Collect Date |
|---------------------------|---------------------------------------|------------------|--------------|
| (I) Aloysia chamaedryfolia Cham. | Rosário do Sul – 106m                  | HUCS 40702       | April 2013   |
|                           | S 30°25'35.7" – W 55°16'30.8"         |                  |              |
| (II) Aloysia citrodora Paláu | Carlos Barbosa – 666m                  | HUCS 41907       | December 2013|
|                           | S 29°16'55.62" – W 51°29'34.58"       |                  |              |
| (III) Aloysia dasenii Moldenke | Garibaldi – 48 m                     | HUCS 39692       | February 2014|
|                           | S 29°13'58.51" – W 51°39'48.02"       |                  |              |
| (IV) Aloysia lycioides Cham. | Guaíba – 23 m                         | HUCS 40700       | December 2012|
|                           | S 30°10'47" – W 51°23'33"             |                  |              |
| (V) Aloysia lycioides Cham. | São Marcos – 746 m                    | HUCS 40699       | February 2013|
|                           | S 28°56'07.20" – W 51°07'23.81"       |                  |              |
| (VI) Aloysia lycioides Cham. | Rosário do Sul – 106 m                 | HUCS 40703       | April 2013   |
|                           | S 30°25'35.7" – W 55°16'30.8"         |                  |              |
| (VII) Aloysia polygalifolia Cham. | Guaíba – 23 m                   | HUCS 40701       | December 2012|
|                           | S 30°10'47" – W 51°23'33"             |                  |              |
| (VIII) Aloysia virgata (Ruiz & Pav.) Juss. | Três Passos – 451 m | ICN 161927       | April 2014   |
|                           | S 27°28'14" – W 23°59'52"             |                  |              |

Source: Authors (2021).

2.2 Essential oil extraction

After collection, aerial parts of Aloysia species were dried at room temperature, fragmented, and subjected to extraction. Essential oils were obtained by hydrodistillation in a Cleve apparatus for 1 hour (Agostini et al, 2009). Anhydrous sodium sulfate was employed to eliminate essential oil humidity. The essential oils were stored in airtight tubes, wrapped in aluminum foil, and stored in the freezer (-20 °C) prior to use.

2.3 Essential oil chemical characterization

Chromatographic analysis was performed using a gas chromatograph coupled to a mass spectrometer detector (GC-MS) and a gas chromatograph with a flame ionization detector (GC-FID) (Hewlett Packard 6890) (Tomazoni et al, 2016). The analyses used two capillary columns HP-Innowax (GC-FID: 30 m × 320 mm × 0.50 mm; GC-MS: 30 m × 250 mm × 0.50 mm), (Hewlett Packard, Palo Alto, USA). GC-MS analysis were carried out on the conditions: column temperature, 40 °C (8 min) to 180 °C at 3 °C/min, 180-230 °C at 20 °C/min, 230 °C (20 min); interface 280 °C; split ratio 1:100; carrier gas He (56 KPa); flow rate: 1.0 mL/min; ionization energy 70 eV; mass range 40-350; volume injected 0.4 µL diluted in hexane (1:10). GC-FID analyses were carried out on the conditions: column temperature, 40 °C (8 min) to 180 °C at 3 °C/min, 180-230 °C at 20 °C/min, 230 °C (20 min); injector temperature 250 °C; detector temperature 250 °C; split ratio 1:50; carrier gas H2 (34 KPa). The volume injected was 1 µL diluted in hexane (1:10). Identification of the individual components was based on comparing their GC retention times (R.T.) on polar columns and comparison with mass spectra of components by GC-MS. The components were identified by a combination of the mass spectrum of Wiley library and by comparison with data from literature (Adams, 2007). The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks was 100 %.

2.4 Brine-shrimp bioassay

The cytotoxicity bioassay was done according to Meyer’s procedure (Meyer et al, 1982) with modifications on the preparation of the samples. The essential oils tested were obtained from A. citrodora, A. lycioides (V) and A. dasenii.
Approximately 1 g brine shrimp eggs (*Artemia salina* - Flagner Soares de Souza Ind.) was hatched in a rectangular aquarium (10 × 20 cm) filled with artificial seawater, which was prepared with 1 L beaker of distilled water containing 30 g of commercial salt mixture (Azevedo Bento S.A. Comércio e Indústria). After 48 hours of incubation, the active shrimp (10-15) were collected by pipette. The nauplii were transferred to culture plates with diluted solutions of the essential oils in 1000 µg mL⁻¹, 500 µg mL⁻¹, 100 µg mL⁻¹, 50 µg mL⁻¹ e 20 µg mL⁻¹ with dimethyl sulfoxide (DMSO) 1%. The total volume was adjusted to 1 mL with artificial seawater. Three replications were done for each dose level and control with artificial seawater and DMSO 1%. After 24 hours, the survivors were counted. The absence of movement of nauplii for 5 minutes was regarded as dead. The bioassay was done three times independently, and the LC₅₀ (Lethal Concentration 50) and 95% CI (Confidence Intervals) were calculated using the Probit Analysis with the software IBM SPSS 21.0.

3. Results and Discussion

3.1 Chemical composition of the essential oils

Essential oils of six species of *Aloysia* (Table 1) were analyzed for chemical composition by GC-MS. The essential oils yields ranged from 0.2% (mL 100 g⁻¹ of dried leaves) for *A. chamaedryfolia* to 2.5% for *A. citrodora*, depending on the species (Table 2). But it also varied according to provenance from 0.8% for *A. lycioides* from Guaiba to 2.0% for *A. lycioides* from São Marcos (Table 2). A total of 25 (I), 36 (II), 23 (III), 16 (IV), 23 (V), 21 (VI), 13 (VII), and 26 (VIII) compounds were identified for each species or provenance, representing 56.1% to 88.4% of the composition of the essential oils (Table 2).
Table 2. Chemical composition, chemical groups, and yield of essential oils from aerial parts of *Aloysia* species: (I) *A. chamaedryfolia*; (II) *A. citrodora*; (III) *A. dusenii*; (IV) *A. lycioides* collected in Guaíba; (V) *A. lycioides* collected in São Marcos; (VI) *A. lycioides* collected in Rosário do Sul, (VII) *A. polygalifolia*, (VIII) *A. virgata*.

| Compound             | Group | I    | II   | III  | IV   | V    | VI   | VII  | VIII  |
|----------------------|-------|------|------|------|------|------|------|------|-------|
| α-Pinene             | HM    | 2.4  | 3.4  | 3.7  | 2.2  | 3.5  | 1.6  | -    | 1.1   |
| β-Thujene            | HM    | 0.2  | 0.2  | 0.8  | -    | 0.7  | -    | -    | -     |
| Camphene             | HM    | -    | 0.3  | -    | -    | -    | -    | -    | -     |
| β-Pinene             | HM    | 3.3  | 0.2  | 13.0 | -    | 11.6 | 1.2  | -    | -     |
| β-Phellandrene       | HM    | 0.8  | 9.3  | 1.7  | -    | 1.4  | 23.7 | -    | -     |
| Myrcene              | HM    | 0.5  | 1.0  | 1.8  | -    | 1.6  | -    | -    | -     |
| Limonene             | HM    | 1.2  | 6.0  | 9.9  | 2.9  | 8.6  | 0.4  | -    | -     |
| 1,8-Cineol           | OM    | 3.1  | 32.8 | 16.2 | 48.5 | 17.6 | 2.1  | -    | -     |
| γ-Terpinene          | HM    | -    | 1.8  | -    | 0.6  | -    | -    | -    | -     |
| p-Cymene             | HM    | 4.0  | 0.5  | 5.4  | 1.4  | 7.0  | 0.5  | -    | -     |
| 1-Octadien-3-ol      | OM    | 0.1  | 0.5  | 0.2  | -    | 0.1  | -    | -    | 0.4   |
| Δ-Elemene            | HS    | -    | -    | -    | -    | -    | -    | -    | 1.5   |
| Terpinol             | OM    | 0.2  | 2.2  | 0.3  | 2.4  | 0.5  | 1.5  | -    | -     |
| α-Terpinene          | OM    | -    | -    | -    | -    | -    | -    | -    | 0.4   |
| α-Cedrene            | HS    | -    | -    | -    | -    | -    | -    | -    | 0.6   |
| β-Bourbonene         | HS    | 0.6  | 0.3  | -    | -    | -    | -    | -    | 1.3   |
| Linalool             | OM    | 7.7  | 0.7  | 1.8  | 1.5  | 2.1  | 1.8  | 0.8  | 0.5   |
| Copaene              | HS    | -    | -    | -    | -    | -    | -    | -    | 0.4   |
| β-Cubebene           | HS    | -    | -    | -    | -    | -    | -    | -    | 1.5   |
| p-Caryophyllene      | HS    | -    | -    | -    | -    | -    | -    | -    | -     |
| β-Caryophyllene      | HS    | 1.8  | 1.0  | 6.5  | 0.3  | 4.2  | 1.8  | 10.8 | 7.2   |
| Terpine-4-ol         | OM    | 0.5  | 0.5  | 1.4  | 1.4  | 1.3  | 1.8  | -    | -     |
| Myrtenal             | OM    | 0.4  | -    | -    | -    | -    | -    | -    | -     |
| Alloaromadendrene    | HS    | -    | -    | -    | -    | -    | -    | -    | -     |
| α-Caryophyllene      | HS    | 1.4  | 0.4  | 0.9  | -    | 0.7  | 1.8  | 8.9  | 0.8   |
| γ-Murolene           | HS    | -    | -    | -    | -    | -    | -    | -    | 1.1   |
| cis-Verbenol         | OM    | 3.2  | -    | -    | -    | -    | -    | -    | -     |
| Myrtenyl Acetate     | HS    | 4.1  | 1.9  | 6.1  | -    | 2.7  | 1.3  | 11.2 | 12.0  |
| Germacrene-D         | HS    | 3.3  | 3.1  | 4.3  | -    | 2.6  | 0.8  | -    | -     |
| Bicyclogermacrene    | HS    | -    | -    | -    | -    | -    | -    | -    | -     |
| Carvone              | OM    | -    | -    | -    | -    | -    | -    | -    | 6.8   |
| 8-isopropenyl-1,1,5-dimethyl-5 iocadene | HS | - | - | - | - | 0.5 | - | - | - |
| Δ-3-Carene           | HM    | -    | 0.3  | -    | -    | -    | -    | -    | -     |
| β-Elemene            | HS    | -    | -    | -    | -    | -    | -    | -    | -     |
| α-Carene             | HM    | -    | 3.2  | -    | -    | -    | -    | -    | -     |
| Myrtenol             | OM    | 0.5  | -    | -    | -    | -    | -    | -    | -     |
| γ-Elemene            | HS    | -    | 0.2  | 0.6  | 0.2  | 0.4  | 1.4  | 7.1  | -     |
| p-Elemene            | OM    | 0.5  | -    | -    | -    | -    | -    | -    | -     |
| Menthaediol          | OM    | -    | -    | -    | -    | -    | -    | -    | 2.6   |
| α-Cubebene           | HS    | 0.7  | 0.8  | 0.2  | 0.7  | 0.4  | 0.8  | -    | -     |
| Δ-Cadinene           | OM    | 4.6  | 1.8  | 5.4  | 3.6  | 8.3  | 8.7  | 7.8  | 3.2   |
| Caryophyllene Oxide  | OM    | 4.6  | 1.8  | 5.4  | 3.6  | 8.3  | 8.7  | 7.8  | 3.2   |
| Epibiciclo-sesquiphendrene | OM | - | - | - | - | - | - | - | - |
| Nerolidol            | OS    | 0.8  | -    | -    | 0.6  | 4.6  | 1.1  | 1.6  | -     |
| Elemenol             | OS    | 0.9  | 4.4  | -    | 4.7  | 1.0  | 0.6  | -    | -     |
| α-Elemol             | OS    | -    | -    | -    | 15.3 | -    | -    | -    | -     |
| α-Selinene           | HS    | 0.3  | -    | -    | -    | -    | -    | -    | -     |
| Guaiol               | OS    | -    | -    | -    | -    | -    | -    | -    | 1.6   |
| Cedrol               | OS    | 10.3 | 0.1  | 2.3  | 0.2  | -    | 1.5  | 1.7  | -     |
| (-) Spathulenol      | OS    | 15.7 | 3.1  | 2.8  | 6.3  | 4.9  | 0.6  | -    | 4.9   |
| Calarene             | HS    | -    | -    | -    | -    | -    | -    | -    | 1.0   |
| β-Patchoulenol       | HS    | -    | -    | -    | -    | -    | -    | -    | 1.5   |
| Bulnesol             | OS    | -    | -    | -    | -    | -    | -    | -    | 0.9   |
| (+) Spathulenol      | OS    | -    | -    | -    | -    | -    | -    | -    | 10.6  |
| Caryophylladienol    | OS    | -    | -    | -    | -    | -    | -    | -    | 1.1   |
| Total identified     |       | 61.5 | 84.7 | 88.5 | 76.8 | 84.9 | 70.2 | 56.0 | 72.7  |

Oil yield v/v (%)

- 0.2 2.5 1.1 0.8 2.0 1.0 0.4 0.8

* (R.T.) Retention Time, (-) absence or no detected, (OM) oxygenated monoterpenes, (HM) hydrocarbons monoterpenes, (OS) oxygenated sesquiterpenes, (HS) hydrocarbons sesquiterpenes.

Source: Authors (2021).
The main compounds of each EO showed that each species presented a different composition. However, some constituents are conserved in several species, as linalool and β-caryophyllene, for example (Table 2). Possibly, the conservation of these constituents may have chemotaxonomic significance to maintain similar morphologic and biochemical characteristics, which will determine the biosynthesis of their secondary metabolism (Sousa et al, 2012). For A. lycioides, collections were performed in three different locations and resulted in different chemical compositions. Thereby, these species were separated into two groups: major compound 1,8-cineole (IV and V) and β-Phellandrene (VI). These differences in major compounds found among the A. lycioides from Guaiaba and São Marcos and from Rosário do Sul could be associated with the geographical origin of the material, but could also suggest different chemotypes. The species A. citrodora is known for the predominant presence of citral in their chemical composition (Zigadlo et al, 1994). However, our results did not show the presence of this component. These data may suggest a new chemotype for the species. These results are based on a local collection and do not analyze the intraspecific variation. The different chemistry can occur through the influence of environmental conditions and seasonal variations (Ricciardi et al, 2011). On the other hand, the composition of the essential oil of a plant is also genetically determined and usually specific to a particular organ and characteristic for their stage of development, giving rise to chemotypes in plants rich in essential oils. Tavares et al (2005) showed that differences in the composition of different chemotypes of Lippia alba are not only a product of the influence of environmental factors but mainly reflect the genotypic variation of these plants.

There were predominant monoterpenes (Table 3) in A. citrodora (59.6%), A. dusenii (63.1%), and A. lycioides (collected in Guaiaba 66.7%, collected in São Marcos 65.1%, and collected in Rosário do Sul 41.3%). The species A. virgata and A. polygalifolia showed higher levels of sesquiterpenes (45.4% and 66.1%, respectively), while A. chamaedryfolia presented a similar quantity of mono- and sesquiterpenes (29.4% and 28.9%, respectively). These classes of terpenes are related to different biological activities (Singh & Sharma 2015) what can explain the popular use of some Aloysia species for medicinal purposes (Santos et al, 2015).

### Table 3. Percentage of chemical groups (mono- and sesquiterpenes) in essential oils from Aloysia species: (I) A. chamaedryfolia; (II) A. citrodora; (III) A. dusenii; (IV) A. lycioides collected in Guaiaba; (V) A. lycioides collected in São Marcos; (VI) A. lycioides collected in Rosário do Sul, (VII) A. polygalifolia, (VIII) A. virgata.

| Chemical groups                  | I     | II    | III   | IV   | V    | VI   | VII  | VIII |
|---------------------------------|-------|-------|-------|------|------|------|------|------|
| Hydrocarbon monoterpenes        | 12.4  | 21.2  | 37.8  | 6.5  | 35.1 | 27.5 | 0.0  | 2.2  |
| Oxygenated monoterpenes         | 17.1  | 38.4  | 25.3  | 60.2 | 30.0 | 13.8 | 10.6 | 4.7  |
| **Total Monoterpenes**          | **29.4** | **59.6** | **63.1** | **66.7** | **65.1** | **41.3** | **10.6** | **6.9** |
| Hydrocarbon sesquiterpenes      | 13.3  | 10.7  | 22.6  | 1.5  | 15.0 | 8.5  | 42.8 | 43.5 |
| Oxygenated sesquiterpenes       | 15.6  | 14.4  | 2.7   | 8.6  | 5.1  | 20.5 | 2.6  | 22.6 |
| **Total sesquiterpenes**        | **28.9** | **25.1** | **25.3** | **10.1** | **20.1** | **29.0** | **45.4** | **66.1** |

Source: Authors (2021).

### 3.2 Cytotoxicity activities

As expected, the degree of lethality was directly proportional to the essential oil concentration (Table 4). The mortality rate of brine shrimp nauplii was drastically increased as the dose level was increased from 20 µg mL⁻¹ to 100 µg mL⁻¹. Moreover, a 100 % mortality was observed at 500 µg mL⁻¹ and 1000 µg mL⁻¹ dose levels for all essential oil evaluated. More than 80% of the nauplii remained active in control, with DMSO 1%.
Table 4. Toxicity of essential oils from Aloysia species on Artemia salina in different doses level with their LC50 and 95% confidence intervals determined by Probit Analysis.

| Species          | Percentage of death at 24 hours/dose (µg mL^-1) | LC50 (µg mL^-1) | 95% CI (µg mL^-1) |
|------------------|-----------------------------------------------|-----------------|-------------------|
|                  | 1000  | 500  | 100  | 50   | 20    | Control |
| A. citrodora     | 100.00| 100.00| 75.33| 32.41| 25.20| 15.26 | 55.96 | 45.97 – 67.49 |
| A. dusenii       | 100.00| 99.62| 74.89| 37.45| 29.88| 13.72| 48.98 | 43.74 – 54.58 |
| A. lycioides     | 100.00| 99.82| 73.73| 35.25| 33.60| 14.35| 48.12 | 40.10 – 57.14 |

The value of LC50, calculated from the 24-hour counts, was not different among the essential oils of the species tested. Therefore, the toxicity was considered high, and LC50 values were from 48.12 µg mL^-1 in A. lycioides (V) to 55.96 µg mL^-1 in A. citrodora. The similar toxicity of these essential oils can probably be explained by the main constituent, 1,8-cineol, present in all of them. According to Meyer et al (1982) the LC50 value under 1000 µg mL^-1 is pharmacologically active and toxic, classifying these as potentially of pharmacological interest as some authors have already related the brine shrimp lethality with the detection of antitumoral compounds in terrestrial plants (Carballo et al, 2002, Macken et al, 2000). However, there is no correlation between the degree of toxicity found for brine shrimp and the toxicity to mammalian cells and the brine shrimp test is used as a tool for approaching the real toxicity (Oliva et al, 2007). Oliva et al (2007) showed in their study the low toxicity of the essential oil from Aloysia tomentosa (LC50 968 µg mL^-1), and nontoxicity of essential oils from Aloysia polystachia (LC50 6459 µg mL^-1) and Aloysia triphylla (synonym for A. citrodora) (LC50 1279 µg mL^-1), differently of the high toxicity observed in this work for all species, especially A. citrodora. Nevertheless, the essential oil composition determined by them for A. triphylla (A. citrodora) was different and rich in limonene, citral, spathulenol, and thujone, showing the importance of chemical characterization to study biological activities.

4. Conclusion

This work is the first study evaluating the chemical composition of the essential oil of six species of Aloysia from the South of Brazil. The species showed different chemical compositions, but some constituents are conserved in several species. This chemical information can assist in the taxonomy of the genus. Also, the results demonstrated the cytotoxic activity of some Aloysia essential oils, showing that studies like this are essential in the screening for new substances with potential biological activities. Therefore, the following steps should be a more detailed evaluation of the toxicity presented by this essential oil to secure the safety of using these natural products.

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