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Chemical composition of the essential oil of *Aloysia triphylla* (L'Hér) Britton due to water deficit and seasonality

[Composición química de aceite esencial de *Aloysia triphylla* (L'Hérit) Britton en función de la deficiencia hídrica y estacionalidad]

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Abstract: The essential oil has change in relation to environmental variations with changes according to exposure to stress and seasonal period. This study aimed to evaluate the chemical composition of essential oil of the Aloysia triphylla subjected to different periods of drought in the four seasons. The experiment was conducted in greenhouse, in randomized block design in a bifactorial 5x4. The treatments consisted of five periods of water deficit (3, 6, 9, 12 days without irrigation and daily irrigation) and four seasons (spring, summer, autumn and winter), with five repetitions. The evaluations were conducted in the middle of each season. The essential oil components showed changes only in relation to the seasons, did not differ between periods of water deficit. Citral decreased concentration in winter and increased in the fall and summer. The winter season favored minority components.

Keywords: Cidró, Citral, Geranial, Secundary metabolites.

Resumen: El aceite esencial tiene alteraciones en relación a las variaciones ambientales con modificaciones de acuerdo con la exposición al estrés y periodo estacional. El presente trabajo tuvo como objetivo evaluar la composición química de aceite esencial de Aloysia triphylla sometida a diferentes periodos de déficit hídrico, en las cuatro estaciones del año. El experimento fue conducido en invernadero plástico, con un delineamiento experimental de bloques casualizados en esquema bifactorial 5x4. Los tratamientos fueron constituidos de cinco periodos de déficit hídrico (3, 6, 9, 12 días sin irrigación e irrigación diaria) y cuatro estaciones (primavera, verano, otoño e invierno), con cinco repeticiones. Las evaluaciones fueron realizadas en la mitad de cada estación del año. Los componentes de aceite esencial presentaron variación solamente en relación a las estaciones del año, no difiriendo entre los periodos de deficiencia hídrica. El citral disminuyó su concentración en el invierno y aumento en el otoño y verano. La estación de invierno favoreció los componentes minoritarios.

Palabras clave: Cidró, Citral, Geranial, Metabolitos secundarios.

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INTRODUCTION

Aloysia triphylla (L'Herit) Britton (cidró) is a large shrub (2 to 3 meters), erect and branched belonging to Verbenaceae family. It is an aromatic and astringent plant probably originated in Chile. It is rich in volatile oil and may act as a mild sedative (Lorenzi & Matos, 2008). In aromatherapy it is used against digestive, nervous and acne problems. In cooking it is used in the preparation of liqueurs, juices, breads and adding flavor to meat.

The plants produce a wide variety of organic compounds which often not appear to have a direct function in its growth and development. Such substances are known as secondary metabolites, secondary products or natural products (Taiz & Zeiger, 2013). Among the physiological functions of secondary metabolites in plants is cited protective action in relation to environmental stresses, such as those associated with changes in temperature, water content, light intensity, UV exposure and nutritional deficiency (Castro *et al.*, 2005).

We highlight among the secondary metabolites the essential oils, which are complex mixtures and have the characteristics of volatility and low molecular weight. Usually are liquid oily appearance, odoriferous, soluble in organic solvents and in water has limited solubility (Simoes et al., 2010). Present in its chemical composition phenylpropanoids, monoterpenes and sesquiterpenes, with predominance of monoterpenes (85%) and sesquiterpenes (10 to 15%) (Cardoso *et al.*, 2001).

The biosynthesis of essential oils is influenced by climatic factors, soil conditions and harvest time (Taveira *et al.*, 2003). The plant has different behavior in different seasons. It goes through changes which affect the concentration of the active ingredients according to seasonality. Environmental factors such as rainfall, temperature, wind, soil fertility, latitude, altitude and seasonality, intervene, significantly, in the production of these compounds (Pinto & Bertolucci, 2002). This causes that there is no standardization in regard to the decision of the best time to place the collection of medicinal and aromatic plants rich in essential oils (Chagas *et al.*, 2011).

Studies in order to evaluate the effect of water stress on essential oil composition of *Ocimum basilicum* demonstrated that under conditions of stress, there was a yield of essential oil twice as great, still observed a significant change in oil constitution with reduction in the percentage of sesquiterpenes and increased percentage of linalool and metilchavicol (Simon *et al.*, 1992).

The low water availability may influence several physiological and metabolic processes in the plant, such as stomatal closure, decline in growth rate, solute accumulation and antioxidants and the expression of specific stress genes (Silva & Casali, 2000). In aromatic species the water deficit may affect besides development, the essential oil content, making the production of active ingredients is greater in environments without water deficit (Marques *et al.*, 2009). Water deficit may also influence the chemical composition of essential oil. The monoterpene composition in some species of *Cymbopogon* spp. may vary according to the intensity and duration of the water stress. With 45 days of moderate stress, there is an increase of geraniol and the reduction of citral *C. nardus* L., yet in *C. pendulus* Stapf is an increase in citral. With 90 days of moderate stress, Geraniol no reduction in *C. nardus* and increased citral *C. pendulus* (Singh-Sangwan *et al.*, 1994).

The constituents of essential oils may vary, and may be terpene terpene hydrocarbons, simple and terpene alcohols, aldehydes, ketones, phenols, oxides, peroxides, ethers, furans, organic acids, lactones, coumarins and even sulfur compounds. Such compounds are presented in different concentrations, having usually one of the components as the majority, existing in other lower levels and some in very low quantities, called trace (Simões *et al.*, 2010).

Chemical analysis of the essential oil from *A. triphylla*, revealed that the predominant constituent is citral also limonene, citronellol, geraniol, alpha and beta pinene, cineol, ethyl eugenol, linalol, valerianic acid, beta-caryophyllene, among others (Lorenzi & Matos, 2008). Were identified in the essential oil of Cidró the geranial (26.1 to 37.3%), neral (18.0 to 29.3%), limonene (9.21 to 15.8%) and nerolidol (2.7 – 24.3%), besides other components in lower amount (Paulus *et al.*, 2013). Citral term originates from Latin citrus, meaning lemon, lemongrass. It is a monoterpênico aldehyde of formula $C_{10}H_{16}O$, is known in forms geranial (trans citral or E-entgen) and neral (citral cis or Z-zusammen) (Leal *et al.*, 2003).

Facing the scarcity of information about the behavior of *A. triphylla* front to water stress, it has as aim to identify changes in production and essential oil composition of that species subject to water deficit associated with seasonality.

MATERIAL AND METHODS

The sutdy local characterization

The study was conducted in protected environment at the Federal University of Santa Maria/Frederico Westphalen - RS Campus, with geographical location of 27º 23 'S, 53 25' O and altitude of 490 m in the period from April 2013 to May 2014. According to the Köppen climate classification, the climate is Cfa, with annual average temperature of around 18º C, with highs in the summer can reach 41° C and minimum in winter reaching values below 0° C. The average annual rainfall is high, usually between 1.800 and 2.100 mm, well distributed throughout the year (Alvares *et al.*, 2013).

Implantation and conduct of the experiment

The experimental design was a randomized complete block in a bifactorial 5x4, with five repetitions. The treatments consisted of five periods of water deficit (0, 3, 6, 9 or 12 days) and four seasons (winter, spring, summer and fall). The experimental units were composed of two plants of *A. triphylla*, spaced 0.8 m from each other and 1.0 m between the lines, totaling 10 plants of evaluation.

The seedlings were produced in tubes with commercial substrate, from a matrix of *A. triphylla* located in the garden of medicinal species of UFSM/Frederico Westphalen Campus, using the stem cuttings method, with stakes of 25 cm in length. Applied indole-butyric acid in stakes at a concentration of 1000 ppm, aiming to fast rooting them. The stakes received two daily waterings.

After rooting seedlings were transplanted to the greenhouse on April 12th 2013 with prior soil tillage without the application of fertilizer. Irrigation was performed with drip tapes daily during the growing season, the water deficit periods being applied only in times of evaluation, as described above.

The cultivation environment in which it was carried out this work is characterized by presenting coverage arc-shaped with plastic film 150 microns thick, with a ceiling height of 3.5 meters high and dimensions of 10 meters wide and 20 meters of lenght. The curtain management was performed daily in order to facilitate ventilation, reducing the temperature amplitude, to prevent movement of strong winds and the water inlet arising from rainfall. Inside the same installed a compact weather station model ISIS S1220 Squiter of Brazil with the aim to characterize the environment within which gave conduction type.

Periods of exposure to water deficit were three days without receiving irrigation, six days without irrigation, nine days without irrigation, 12 days without irrigation and treatment with irrigation maintained over the whole period, being carried harvest soon after. The effect of seasonality was measured from the collection of plants, which were performed in the periods that marked the half of each season, being on winter July 29th to August 8th, in the spring of November 6th - 15th, in summer February 7th - 16th and in the autumn May 15th - 26th.

Obtention of essencial oil

The essential oil was obtained from fresh leaves in all seasons and was extracted by hydrodistillation in Clevenger apparatus modified for 3 hours, with three replicates per treatment. Composite samples were used, the mass of which varied according to the season due to leaves of availability: in winter we used 60 g fresh weight per repetition in the spring, summer and autumn were used 350 grams of fresh weight per repetition. Each repetition consisted of two plants, harvested at 8 am.

The chromatographic analysis was conducted to knowledge the oil composition. The material for analysis was placed in vials and sent to the Extractive Vegetables Laboratory belonging to the Federal University of Santa Maria in Santa Maria, where the chromatographic analysis were realized.

Evaluations and statistical analysis

The analyse by GC-MS TIC was performed using an Agilent 6890 gas chromatograph coupled with a selective mass detector Agilent 5973, under the following conditions: HP5-MS column (5% - phenyl - 95% - metylsiloxano, 30 mx 0,25 mm x 0,25 millimeters); EIMS: 70 eV. Operating conditions: flow divider 1: 100; temperature program 40-260° C; 40º C for 4 min; ramp, 4° C/min; He charger gas; 1ml/min flow; injector and detector temperature, 220° C; temperature 250º C interface.

The constituents of essential oils were identified based on retention index (RI) and mass spectral fragmentation models, in comparison with the literature data (Adams, 2009). The concentration of the constituents was calculated using the total area of its peaks, related to the total area of all the constituents of the sample obtained by analysis using gas chromatography with ionization detector flame (IDF), the result being expressed in percentage.

Table 1
Chemical constituents of the essential oil (%) *Aloysia triphylla* according to the seasons of the year. Frederico Westphalen, RS. 2015.

Componente	Média das Estações (%)													
	Wint	**SD**	**M**	**Sp**	**SD**	**M**	**Sum**	**SD**	**M**	**Aut**	**SD**	**M**	**Ikcal.**	**Ikref.**
6-metil-5-hepten-2-ona	1.7 b	0.7	1.6	3.7 a	1.4	3.6	1.7 b	0.4	1.7	2.9 a	0.5	3.1	983	986
β-pinene	_	_	_	0.3 a	0.1	0.4	0.3 a	0.1	0.4	0.2 b	0.0	0.3	991	983
limonene	3.1 d	1.5	3.3	16.2 b	3.0	17.1	18.4 a	2.3	18.5	11.2 c	1.4	11.0	1027	1027
Z-ocimene	_	_	_	0.6 b	0.3	0.5	0.4 b	0.2	0.5	1.1 a	0.1	1.1	1049	1050
Linalool	0.6 b	0.1	0.6	0.7 a	0.1	0.8	0.7 a	0.1	0.8	0.6 b	0.0	0.6	1102	1100
Z-limonene oxide	0.5 a	0.1	0.4	0.4 a	0.1	0.4	0.3 a	0.2	0.3	_	_	_	1134	1136
E-verbenol	0.6 a	0.3	0.8	0.4 b	0.1	0.5	0.4 b	0.1	0.4	0.3 b	0.1	0.3	1139	1144
Isocitral <exo->	_	_	_	0.3 a	0.0	0.4	0.2 b	0.0	0.3	0.3 a	0.0	0.3	1147	1144
2-pineno-4-ol	_	_	_	0.2 b	0.0	0.3	0.3 a	0.1	0.3	0.2 c	0.0	0.3	1150	1143
Z-Isocitral	_	_	_	0.5 b	0.3	0.3	0.5 b	0.1	0.5	0.8 a	0.1	0.9	1166	1164
Rosefuran epoxide	_	_	_	0.3 ab	0.1	0.4	0.4 a	0.1	0.3	0.2 b	0.0	0.2	1177	1177
E-Isocitral	_	_	_	0.4 d	0.1	0.5	0.8 c	0.1	0.8	1.3 b	0.1	1.3	1184	1180
Z-geraniol	3.2 a	1.0	1.1	2.2 a	0.9	2.1	2.7 a	0.5	1.2	1.9 a	0.9	1.5	1232	1229
β-Citral	18.8 b	3.0	20.0	17.6 b	5.6	19.3	20.6 ab	1.4	22.1	24.5 a	1.1	24.6	1242	1240
E-geraniol	2.3 a	0.9	0.6	2.6 a	0.9	2.5	3.1 a	0.5	1.1	2.4 a	1.3	1.7	1258	1258
α-Citral	25.6 b	4.7	26.7	25.0 b	4.0	25.6	26.7 ab	1.8	28.5	31.4 a	1.5	31.7	1272	1273
Geranic acid	6.6 a	1.9	7.1	1.3 b	0.6	1.4	1.0 b	0.5	1.2	_	_	_	1361	1359
Neryl acetate	1.0 a	0.2	0.7	0.5 b	0.1	0.5	0.4 b	0.0	0.5	0.3 b	0.1	0.4	1367	1366
α-Cubebene	2.8 a	1.4	2.3	0.4 b	0.2	0.5	0.6 b	0.2	0.5	1.6 b	1.6	2.7	1377	1372
Geraniol acetate	6.8 a	2.6	6.4	3.9 b	0.6	3.8	2.6 c	0.2	2.6	3.0 c	0.2	3.0	1386	1384
β-Caryophyllene	4.3 ab	0.3	4.3	3.6 bc	1.5	3.4	3.4 c	1.1	3.5	4.6 a	0.2	4.6	1419	1418
α- Caryophyllene	0.5 a	0.3	0.4	0.4 a	0.1	0.5	0.4 a	0.1	0.4	0.4 a	0.1	0.4	1451	1453
Aromadendrene	1.1 a	0.4	0.4	0.3 a	0.0	0.3	0.2 a	0.1	0.3	0.2 a	0.0	0.3	1460	1460
Geranyl propanoate	2.2 a	0.3	2.4	0.3 b	0.0	0.4	0.9 b	0.8	0.3	2.3 a	0.1	2.3	1476	1477
α-Curcumene	2.2 a	1.0	2.0	2.9 a	0.3	3.0	2.4 a	0.7	2.7	0.3 b	0.1	0.3	1483	1483
ϒ-Cadinene	0.5 a	0.2	0.5	0.4 a	0.0	0.4	0.6 a	0.6	0.4	0.5 a	0.2	0.6	1515	1513
E-Nerolidol	1.4 a	0.1	0.6	0.8 ab	0.2	0.8	0.4 b	0.1	0.4	0.6 b	0.0	0.6	1565	1563
Espathulenol	5.1 a	0.9	4.9	3.3 b	0.5	3.2	2.0 c	0.4	1.8	2.1 c	0.2	2.2	1578	1580
Caryophyllene oxide	7.2 a	1.3	8.0	5.1 b	0.9	5.2	5.8 b	0.7	6.1	2.3 c	0.3	2.3	1583	1584
δ- Cadinol	1.1 b	0.2	1.2	1.5 a	0.3	1.4	1.3 a	0.3	1.4	1.0 b	0.1	1.0	1642	1640
α-Cadinol	0.5 a	0.2	0.5	_	_	_	0.6 a	0.6	0.2	_	_	_	1654	1654
Total médio identificado	95.93			97.48			98.16			98.67				

Notes: Wint = Winter; Sp = Spring; Sum = Summer; Aut = Autumn; IKcal = Kovats Index calculated; IKref = reference Kovats Index; _ = component not detected; SD = standard deviation; M = Median. Means followed by the same letter on the line do not differ by Tukey test at 5% probability.

The results were submitted to variance analysis using the software Genes and the means were compared by Tukey test (Cruz, 2013).

RESULTS AND DISCUSSION

There was no significant interaction between water deficit period and the seasons for essential oil components. Chromatographic analysis of extracted essential oils showed great variability of the components in the different seasons of the year, with no significant difference between the periods of water deficit.

These results corroborate with those obtained with the essential oil chemical analysis Malaleuca alternifólia plant, under water stress, there was no change in the concentration of the main constituents of oil, even when the plants were exposed to severe water deficit, which caused decrease oil content, but it maintained the concentration of the components (Silva *et al.*, 2002).

In relation to the seasons, the components had different standard deviations, and noted lower values in summer and autumn (months with a lower temperature range) and higher standard deviation values in winter and spring (months when major changes in climatological elements). The components of greatest interest (limonene, β-citral, α-citral, Espathulenol and Caryophyllene oxide) showed higher standard deviations averaged area, reaching 5.6 for β-citral spring (Table 1). For all oscillations, the median components did not differ much from the average, with values both above and below the averages observed for the compounds.

The identified major components in the essential oil samples were limonene (3.2 – 18.5%), β-Citral (17.6 – 24.6%), α-Citral (25.1 – 31.5%), caryophyllene oxide (2.3 – 7.2%), and 6-methyl-5-hepten-2-ona, β-pinene, Z-ocimene, linalool, limonene oxide-Z, E-verbenol, isocitral <exo->, 2-pinene-4-ol isocitral <(Z)->, epoxide rosefurano, E-Isocitral, Z-geraniol, E-geraniol, geranic acid, ethyl nerila, α-cubebeno, geraniol acetate, β-caryophyllene, α-caryophyllene, aromadendrene, geraniale propionate, α-curcumene, Y-cadinene, nerolidol, spathulenol, δ- cadinol and α-cadinol (Table 1).

The mixture of isomers (β-Citral and α-Citral) generically called citral, are the main constituents of commercial interest the essential oil of Cidró (Lorenzi & Matos, 2008). These were the components found in greater quantity in the four seasons of the year, and in autumn and summer, their concentrations were higher when the α-Citral has come to represent 31.5% oil components of *A. triphylla* and β-Citral 24.6%, to total concentration of citral of 56.0% (Table 1). These results are in agreement with other studies to evaluate the essential oil composition of *A. triphylla* in the 12 months of the year, which verified that the higher levels of α-citral (37.2 and 37.4%) and β-Citral (29.3 and 29.0%), mainly found in January and March (summer) (Paulus *et al.*, 2013). Studies evaluating the effect of seasonality in the constituents of *Lippia alba*, found that citral had higher concentration in autumn season, followed by the summer, and in this work the production at these seasons did not differ statistically (Castro *et al.*, 2002).

Such variations are probably related to the physiological cycle of the plant, because in the winter there is a period of low biosynthetic activity. Studies oil composition of *A. triphylla* showed that in winter, the total sesquiterpenes (synthesis of more complex compounds) showed 22% level, while total amount of monoterpenes showed 54% (Botrel *et al.*, 2010). The Figure 1 shows that in winter there was the highest concentration of sesquiterpenes (36.3%), and this was decreasing in other evaluation stations, coming to 19.45% in the autumn.

The highest concentration of monoterpenes was found in summer and autumn (78.1% and 77.6% respectively). Studies show that the difference between total monoterpenes and sesquiterpenes decrease during other seasons, and in spring, this ratio was almost 1:1 (Botrel *et al.*, 2010), different than shown in Figure 1, where the difference between these components only increased in the spring, summer and fall.

In plants, terpenes usually act as phytoalexins, insect repellents, pheromones, plant hormones, signaling molecules, allelochemicals, attraction agents for pollination and defense against herbivores (Burt, 2004). The monoterpenes are smaller and low-density molecules, which facilitates its volatilization, causing act as attractive to pollinators (Amaral *et al.*, 2015), a fact that may explain the higher concentration of citral in the summer and autumn, period in which the flowering of *A. triphylla*, at which time the plant needs to attract pollinators. This factor may be associated with high concentration of monoterpenes found in summer and autumn (Figure 1), the time that *A. triphylla* blooms in southern Brazil.

The percentage of these components affects the commercial importance of essential oil from *A. triphylla*, as well as some low percentage of undesirable components, such as geraniol and nerol which are reduced forms of neral and geranial, which may affect the desired properties essential oil (Tabatabaie & Nazari, 2007). In the analysis performed in this study were found Z-geraniol or nerol (1.9 to 3.3%) and E-geraniol (from 2.3 to 3.2%), both of which have constant behavior in all seasons of the year, not changing its concentration in essential oil.

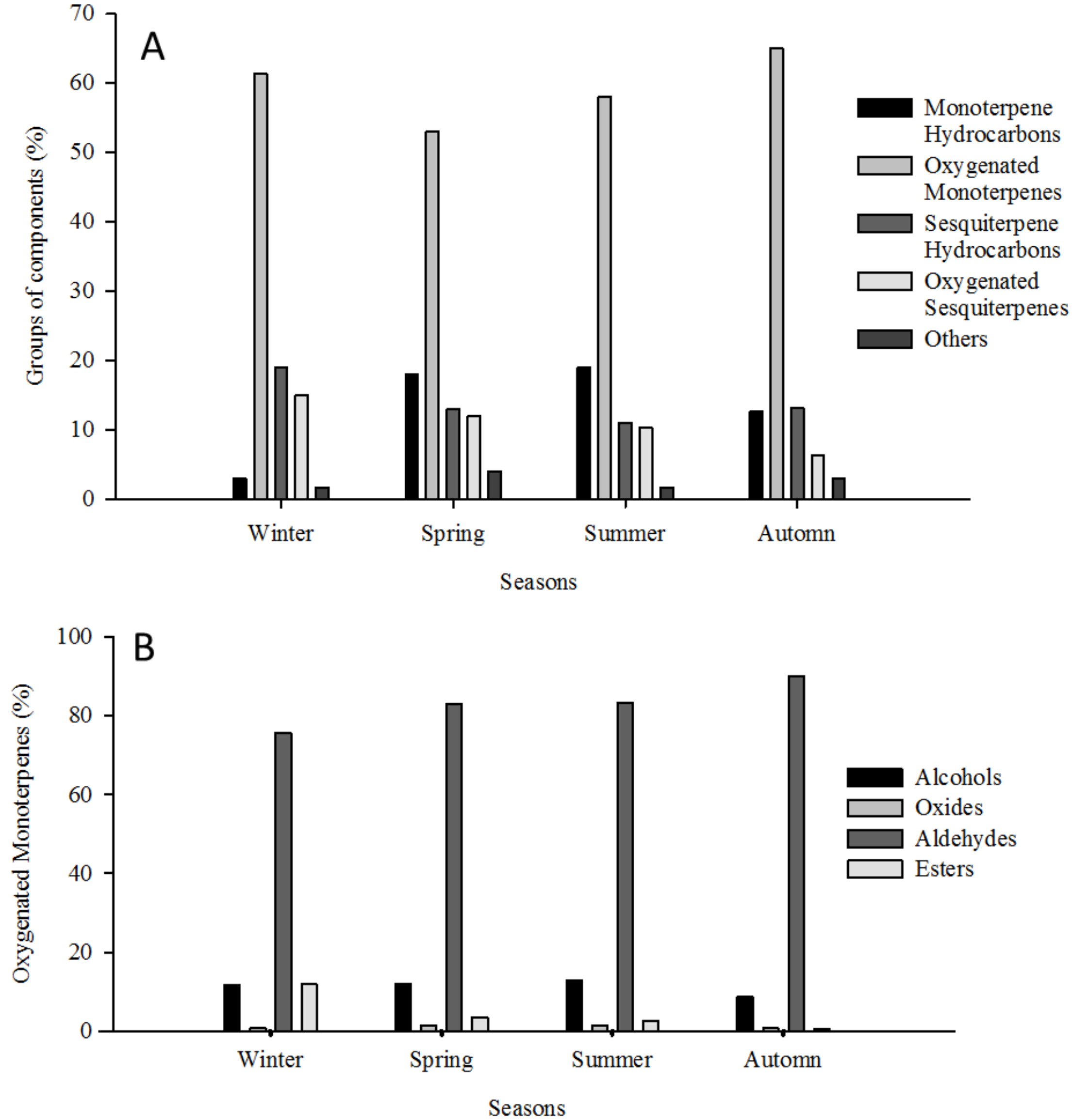

Figure 1
Percentage of classification of the different components of *Aloysia triphylla* oil.
A= Groups of components; B = Oxygenated Monoterpenes. Frederico Westphalen, RS. 2015.

The plant developmental stages associated with the conditions of cultivation environment are likely to influence the essential oil composition (Paulus *et al.,* 2013). The intensity of solar radiation can modify the content and composition of the essential oil through activation of sensitive enzymes to light, forming part of the route of mevalonic acid, the precursor of terpenes, the main constituents of essential oils (Souza *et al.,* 2011). The light intensity and temperature have varied over the day, acting directly on the primary processes, such as respiration and photosynthesis may influence indirectly the production of secondary metabolites and the essential oil composition, which has its dependent synthesis products of primary metabolism (Santos & Inneco, 2003).

Another essential oil important component of *A. triphylla* is limonene, which had its highest concentration in the summer season (18.5%), keeping up with intermediate values in spring and autumn, reducing drastically in winter (3,2%) (Table 1). Studies of the essential oil composition of *A. triphylla* in Dois Vizinhos, Paraná, found a limonene concentration in essential oil between 9 and 14%, with the highest concentrations from September to April, reducing the months from May to August (Paulus *et al.,* 2013).

There were found two sesquiterpenes components in the essential oil with relatively large content in the samples, they caryophyllene oxide (2.3 to 7.2%) and spathulenol (2.1 to 5.1%), with the highest concentrations of both the the winter season. The sesquiterpenes usually are larger, heavier and less volatile, and often protection functions such as antimicrobial activity and fungitoxic action (Amaral et al., 2015), and winter is a more rainy season and conducive to the development of pathogens. These two components were identified in the oil of *A. triphylla* in any month of evaluation Dois Vizinhos, Paraná (Paulus *et al.,* 2013). The elevated content of spathulenol and caryophyllene oxide found in the Winter season demonstrates that it is possible to obtain higher concentrations of these compounds in adverse or less-ideal conditions. These results are pertinent to the pharmaceutical industry which works with these components, and are relevant to the goals of several studies related to the application of said components.

It was also found β-caryophyllene in the essential oil samples (3.4 to 4.6%). The β-caryophyllene and sesquiterpenes caryophyllene oxide have anticarcinogenic activity, and β-caryophyllene also has activity related to gastric cytoprotection (Souza *et al.,* 2006). The component acetate geranila was also found in higher concentration in the winter season, reaching 6.9%, while in summer its concentration reached 2.6% (Table 1).

Overall, almost all of the essential oil components of *A. triphylla* had interference from seasonality, some standing out in the winter season and the other in the summer or autumn. Few components that increased concentration in the spring, especially linalool, which increased to 0.8%. This factor may be related to vegetative growth that has greatly increased in this season, with the need for increased production of primary metabolites, for the growth of the plant. In southern Brazil, where the four seasons are well defined, these variations become more evident, resulting in productive change and quality of the evaluated material.

CONCLUSIONS

Thus, we can conclude that the components of the essential oil of *A. triphylla* were not influenced by water deficit applied. The seasonality has great control on essential oil composition, and the component of greatest interest, citral, is present in greater amounts in the summer and autumn. The winter season favors the concentration of minor components, especially caryophyllene oxide and spathulenol.

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