ÁREA AGRÍCOLA

VEGETATIVE GROWTH OF *Hypericum perforatum* L. PLANTS TREATED WITH HIGH DYNAMIZED DILUTIONS OVER DIFFERENT GROWING SEASONS

CRECIMIENTO VEGETATIVO DE PLANTAS DE *Hypericum perforatum* L. TRATADAS CON DILUCIONES ALTAMENTE DINAMIZADAS DURANTE DIFERENTES TEMPORADAS DE CRECIMIENTO

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Abstract: Contextualization: The species *Hypericum perforatum* is widely used as a treatment for several diseases, especially depression. This plant is not native to Brazil and, therefore, it is not planted in the country. Brazil has high rates of occurrence of depression, same as the whole Latin America, being considered a public health problem, so its cultivation is considered as a potential treatment tool.
Knowledge gap: The cultivation of the species in the country is still inefficient, as the plant does not reach an adequate stage for flowering. Thus, further studies regarding the vegetative growth and the establishment of *H. perforatum* are needed, as it can provide great economic and health autonomy by addressing a public interest, using a treatment with practically no side effects, reducing drug costs for the country.
Purpose: Evaluate the effects of high dynamized dilutions and the influence of the seasons on vegetative growth and contents of bioactive compounds in *Hypericum perforatum* plants.
Methodology: Experiments were performed in two seasons: Spring/Summer and Summer/Autumn. The experimental plot consisted of 12 plants and 4 repetitions, totaling 48 plants per treatment. Five treatments were used consisting of homeopathic preparations of Kali carbonicum, *Natrum muriaticum*, *Phosphorus*, and *Silicea terra* at 12CH, and distilled water as control. The height of the longest branch, the total number of branches, shoot dry weight, and a number of dark glands were evaluated, as well as the amounts of phenolic compounds.
Results and conclusions: Homeopathic preparations affected *H. perforatum* plants differently over the cultivated seasons. In the Spring/Summer experiment, the Silicea terra treatment promoted higher plant growth than *Phosphorus*, but a similar rate to that of the other treatments. In the Summer/Autumn experiment, the homeopathies *Kali carbonicum*, *Natrum muriaticum*, and *Phosphorus* increased the vegetative growth in comparison to control. It was observed that in the experiment carried out in the Spring/Autumn, the plants of *H. perforatum* had difficulty in development. The formation

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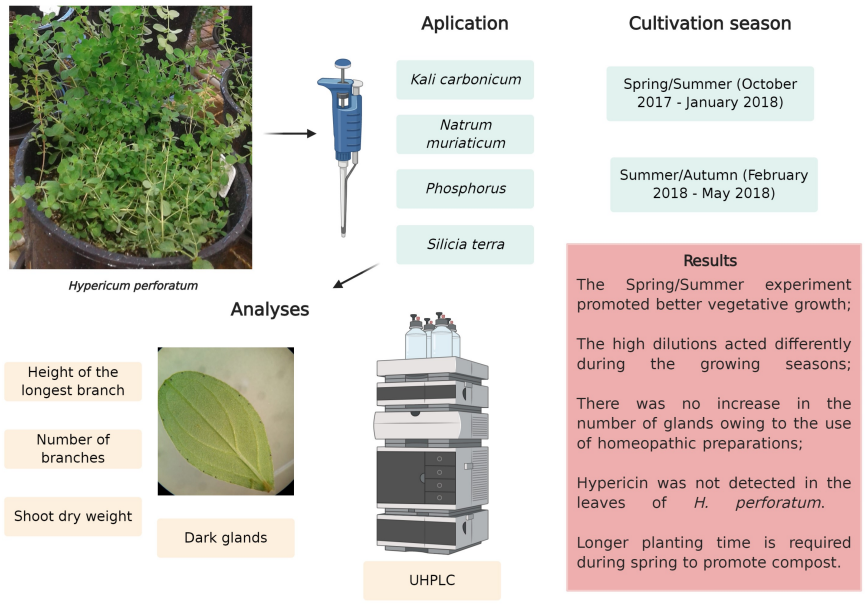
of dark glands was not stimulated by the use of homeopathic preparations. The hypericin compound was not detected in any sample of *H. perforatum* leaves. This suggests the need for an extended cultivation time for the naphthodianthrone compound to accumulate in the dark glands.

Keywords: Hypericin, Homeopathy, Medicinal plant, St John's wort .

Resumen: Contextualización: la especie *Hypericum perforatum* se usa ampliamente como tratamiento para varias enfermedades, especialmente la depresión. Esta planta no es originaria de Brasil y, por lo tanto, no se planta en el país. Brasil tiene altas tasas de ocurrencia de depresión, al igual que toda América Latina, siendo considerado un problema de salud pública, por lo que su cultivo se considera una posible herramienta de tratamiento.
Vacío de conocimiento: el cultivo de la especie en el país aún es ineficiente, ya que la planta no alcanza una etapa adecuada para la floración. Por lo tanto, es necesario llevar a cabo estudios adicionales sobre el crecimiento vegetativo y el establecimiento de *H. perforatum*, ya que puede proporcionar una gran autonomía económica y sanitaria, al atender un interés público usando un tratamiento que prácticamente no tenga efectos secundarios, lo que reduce los costos de los medicamentos para el país.
Propósito del estudio: evaluar los efectos de las diluciones altamente dinamizadas y la influencia de las estaciones sobre el crecimiento vegetativo y el contenido de compuestos bioactivos en plantas de *Hypericum perforatum*.
Metodología: los experimentos se realizaron en dos estaciones: Primavera/Verano y Verano/Otoño. La parcela experimental constó de 12 plantas y 4 repeticiones, para un total de 48 plantas por tratamiento. Se utilizaron cinco tratamientos que consistieron en preparaciones homeopáticas de *Kali carbonicum*, *Natrum muriaticum*, *Phosphorus* y *Silicea terra* a 12CH y agua destilada como control. Se evaluó la altura de la rama más larga, número de ramas, peso seco del brote y número de glándulas oscuras, así como las cantidades de compuestos fenólicos.
Resultados y conclusiones: las preparaciones homeopáticas afectaron a las plantas de *H. perforatum* de manera diferente durante las temporadas de cultivo. En el experimento de Primavera/Verano, el tratamiento con *Silicea terra* promovió un mayor crecimiento de las plantas que el *Phosphorus*, pero a una tasa similar a la de los otros tratamientos. En el experimento Verano/Otoño, las homeopatías *Kali carbonicum*, *Natrum Muriaticum* y *Phosphorus* aumentaron el crecimiento vegetativo en comparación con el control. Se observó que en el experimento realizado en la Primavera/Otoño, las plantas de *H. perforatum* tuvieron dificultad de desarrollo. La formación de glándulas oscuras no fue estimulada por el uso de preparaciones homeopáticas. El compuesto de hipericina no se detectó en ninguna muestra de hojas de *H. perforatum*. Esto sugiere la necesidad de un tiempo de cultivo prolongado para que el compuesto de naftodiantrona se acumule en las glándulas oscuras.

Palabras clave: Hipericina, Homeopatía, Planta medicinal, Hierba de San Juan.

GRAPHIC SUMMARY



Source: authors

1. INTRODUCTION

Hypericum perforatum, known as St. John's wort, has been used as a medicinal plant to treat different human diseases, mainly mild and moderate depression (Ng et al., 2017). The biological activity of *H. perforatum* is attributed to more than ten classes of secondary metabolites, including anthraquinones/naphthodianthrones, phloroglucinol, flavonoids, xanthones, volatile oils, vitamin C, tannins, proteins, carotenoids, and coumarins. However, hypericin and hyperforin have been the main compounds studied in this medicinal plant because of their well-known antidepressant effects (Mullaicharam and Halligudi, 2018).

Hypericin, an anthraquinone derivative, is naturally found in the yellow flowers of *H. perforatum*. It has antidepressant activity, resulting from an inhibitory effect on the neuronal uptake of norepinephrine, dopamine, γ-amino butyric acid and L-glutamate. It is accumulated in specialized morphological secretory structures know as dark glands (Gaid et al., 2016). Previous studies have reported that hypericin concentrations will depend on different factors, such as planting and harvesting time, the phenological stage of plants at harvest and use of appropriate treatments for phytosanitary maintenance (Southwell and Bourke, 2001).

Native from Europe, Asia, and North Africa, the species *H. perforatum* can abundantly grow up on pastures, roadsides, and environments modified by human activity (Crompton et al. 1998). However, the adaptation and cultivation of *H. perforatum* in Brazil is still ineffective, because the plant does not reach a proper size (~ 60 cm) and does not reach an adequate stage to flower. Considering the high therapeutic potential of *H. perforatum*, it needs to be grown in ecological systems, so that there are no negative changes

in the content of medicinal compounds that provide efficiency against diseases, such as depression (Faron et al., 2004).

Regulated by Normative Instruction No. 17/2014 by the Ministry of Agriculture, Cattle and Supplying for organic production (Ministério da Saúde, 2014b), homeopathic preparations have proven to be an effective and residue-free technology for use in agriculture (Teixeira and Carneiro, 2017; Sen et al., 2018). The application of homeopathic preparations can help the cultivation of medicinal plants on a more sustainable basis, eventually improving plant growth, and secondary metabolites biosynthesis and accumulation (Pereira et al., 2019).

In addition to the choice of residual-free treatments in medicinal plants (Ministério da Saúde, 2014a), the period of cultivation of the species needs to be determined, as this factor will also be determinant for the production of biomass and the biosynthesis of bioactive compounds. According to Soni et al. (2015), the growing season influences the availability and the amounts of bioactive compounds in medicinal plants, determining their phytotherapeutical potential. Planting and/or harvesting at the wrong time may impair the yield of secondary metabolites pharmacologically relevant, so it is very important to identify the best seasons for cultivation. In this sense, this study aimed to evaluate the use of high dynamized dilutions and the influence of seasons on the vegetative growth and content of phenolic compounds in *Hypericum perforatum* plants.

2. MATERIALS AND METHODS

Cultivation of *Hypericum perforatum*: The experiments were carried out in a culture room with controlled temperature and light at the Laboratory of Plant Health and Homeopathy and also in a greenhouse at the Epagri Experimental Station, located in the city of Lages (50º 19'46.93" W, 27º 48'28.746" S), Santa Catarina state, southern Brazil.

Two experiments were performed as follows: The Spring/Summer experiment, from October 2017 to January 2018 and the Summer/Autumn experiment, from February 2018 to May 2018. *H. perforatum* seeds were acquired from Feltrin Seeds®, showing a 64% germination rate, according to the manufacturer.

For production of the seedlings, the seeds were sown in a sowing tray filled with vermiculite and black earth, in a 2:1 ratio. The experiment used a randomized block design and the sowing trays were separated into blocks and transferred to a growth room at 25 ºC and 16h/8h photoperiod, under luminous intensity set up at 2.338 LUX provided by LED lamps. The sowing trays were placed on a plastic tray containing 200 mL until the seeds' emergence, 15 days. Five treatments were used, consisting of homeopathic preparations in the 12CH (twelfth order of the Hahnemannian centesimal dilution) of *Kali carbonicum*, *Natrum muriaticum*, *Phosphorus*, and *Silicea terra* and distilled water as a control. The matrices of the homeopathic preparations were acquired in a compounding pharmacy at 6CH. The preparations at 12CH were made according to the Brazilian Homeopathic Pharmacopeia (Ministério da Saúde, 2011).

The selection of homeopathic preparations was carried out using repertory language and in consultation with medical sources. The main characteristics of the species *H. perforatum* were analyzed, such as: sensitivity to cold, need for constant water and light, photosensitivity, and fragility. Using the materials listed, and according to the Homeopro® software, it was determined which homeopathic best approached the level of similarity.

All experiments were performed in a double-blind analysis, namely, the operator was unaware of the treatment to be used. *H. perforatum* seedlings were treated twice a week, dispensing 1 mL of homeopathic preparations per sowing cell directly in the soil, totaling eight applications in a 30-day experimental period.

Six weeks after sowing, the treated seedlings, with approximately 6 cm in height, were transplanted into pots (four plants per pot) and taken to a greenhouse. The experimental plot consisted of 12 plants and 4 repetitions, totaling 48 plants per treatment. 8.7liters pots containing vermiculite, black soil, and sheep

manure were used (1:1:1, v/v/v). 195 g natural phosphate per 360 liters of compost were used. In the greenhouse, homeopathic treatments were applied twice a week again and extended for one month until the end of the experiment, at 60 days after sowing.

After seed germination (15 days), two assessments per week were performed to measure the height of the main branch. The measurements were done from the base of the stem up to the highest leaf, and 17 evaluations of the main branch were carried out. At the end of the experiment (75 days), plant height, total number of branches and shoot dry weight were evaluated.

Sample collection and dark gland count: All the samples of the Spring/Summer experiment were collected in February 2019; whereas, for the Summer/Autumn experiment samples were harvested in June 2019. Dark glands were counted from a destructive sample of five leaves from each plant, totaling 240 leaves per treatment. The counting of dark glands was performed using a stereoscopic microscope (25x) on the adaxial face of the leaves. The remaining materials (leaves and roots) were placed in a force-air drying oven for 48 h at 50 °C.

Preparation of hydroalcoholic extracts: After comparing the data obtained in the general average of the dark gland count, the samples treated with the homeopathic preparation Silicea terra 12CH and the control ones were selected for further chromatographic analysis. The extracts were obtained by maceration using commercial ethyl alcohol 92% (v/v) and 36g and 29g dry shoot samples of the control plants and *Silicea terra*-treated plants, respectively, from the Spring/Summer experiment. Similarly, for the Summer/Autumn experiment, 1.05 g and 1.39g for plants treated with water and *Silicea terra*, respectively. Grinding was performed with 48 plants that were divided into four repetitions. The material was kept under maceration at room temperature and protected from light for seven days. After filtration, the solvent was removed by rotary evaporation at 45 °C to obtain the crude hydroalcoholic extract. The extracts obtained for each maceration step were combined, frozen, and lyophilized, yielding four crude extracts per treatment.

Ultra-Performance Liquid Chromatography (UPLC): The hydroalcoholic preparations of *H. perforatum* extracts were submitted to UPLC analysis for hypericin characterization. The samples were prepared by removing the nonpolar components by solid-liquid extraction using *n*-hexane as an extractor solvent. 0.5 g hydroalcoholic extract were added in 2 mL of n-hexane, and the procedure was repeated twice. All samples were analyzed in triplicate. The analytical standard of the hypericin compound (Sigma-Aldrich 95%) was used for identification of the target compound, according to its retention time and UV-vis spectroscopic profile.

The experiments were conducted using an Ultimate 3000 RS UPLC System (Thermo-Fisher Scientific, USA) as proposed by Brolis et al. (1998). Chromatographic separation was performed by using a reverse-phase C18 column (FR-Thermo Scientific 250×4.6 mm, 5 μm) coupled with a C18 guard column (Phenomenex®), thermostatized at 33°C, and a diode array detector (DAD). The mobile phase was eluted at flow rate at 1mL/min using the following linear gradient program (Brolis et al., 1998): A) water acidified with phosphoric acid 85% (99.7: 0.3 v/v); B) acetonitrile; C) methanol. Injection volume was 10 μl, and hypericin detection was achieved at wavelengths 240 nm, 270 nm, 320 nm, and 400 nm.

Statistical analysis: For the study variables (height of the largest branch, number of branches and shoot dry weight) one way analysis of variance (ANOVA - F test) was used for each season, and the assumptions of the model were checked using the Bartlett test (homoscedasticity) and the Shapiro-Wilk test (normality). In cases in which the assumptions of the model were not satisfied, the transformation proposed by Box-Cox was used, applying the optimal lambda for transformation. In cases where there was a significant effect of the treatments, the means were compared by Tukey's test.

To describe the behavior of the plant height variable over time, the logistic model was used, given by:

$$y_{ij} = \frac{\beta_{1i}}{\left(1 + e^{\frac{\beta_{2i} - t_j}{\beta_{3i}}}\right)} + \varepsilon_{ij} \quad (1)$$

[Ecuación 1]

where: β_{1i} represents the parameter associated with the asymptote for the ith treatment, β_{2i} is the parameter representing the numerical value associated with time at the curve's inflection point for the ith treatment. At this point the height value will be $\frac{\beta_{1i}}{2}$. β_{3i} is the scale parameter associated with the ith treatment, tj is the time in days associated with jth observation and is the error associated with the jth observation of the ith treatment.

The treatments were compared using the confidence intervals of the model parameters. All analyses were performed with the aid of scripts written in the R language, considering a 5% significance level.

Retention time (min) and mean of the peak area (%) are presented for the chromatographic analyses performed by UHPLC.

3. RESULTS AND DISCUSSION

Cultivation: The vegetative growth of *H. perforatum* in the Spring/Summer experiment was higher ($p < 0.05$) in plants treated with the homeopathic preparation with *Silicea terra* compared to the ones treated with *Phosphorus*, but it was similar to that of the other treatments. In the Summer/Autumn experiment, the treatments with *Natrum muriaticum* and *Kali carbonicum* provided the highest vegetative growth, and were statistically different from the control (Figure 1).

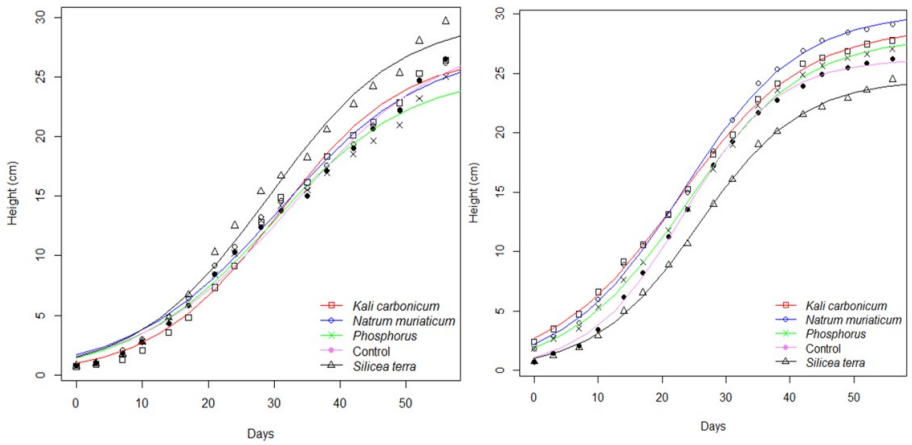


FIGURE 1.
Vegetative growth of the main branch of *Hypericum perforatum* plants with high dilution treatments. A) Spring/Summer experiment; B) Summer/Autumn experiment.
Source: authors.

The plants treated with *Silicea terra* were higher in comparison to those treated with Phosphorus, but their height was similar to that of plants treated with *Kali carbonicum*, *Natrum muriaticum*, and to the

height of the control treatment in the Spring/Summer experiment. In the Summer/Autumn experiment, Natrum muriaticum-treated plants differed from the Silicea terra-treated ones, but not from homeopathic preparations with *Kali carbonicum* and *Phosphorus*.

As far as growth period is concerned, the Spring/Summer plants treated with *Silicea terra* took 4 and a half days longer, on average, to reach 50% of the estimated height than plants treated with Phosphorus. For the Summer/Autumn experiment, *Silicea terra* was shown to act on plant height differently when compared to the Spring/Summer experiment, with a shorter time to reach 50% of the estimated height in comparison to other treatments.

TABLE 1.
Vegetative growth of *Hypericum perforatum* over time in two growing seasons and treated with homeopathic preparations.

Treatment	β_1 (plant height/cm)	β_2 (growing time/days) **	β_1 (plant height/cm)	β_2 (growing time/days) **
	Spring/Summer		Spring/Summer	
Kali-c	26.96 ab	30.53 a	28.91 a	22.57 b
Nat-m	27.56 ab	30.62 a	30.17 a	23.24 ab
Phos	25.34 b	29.49 a	28.04 ab	23.81 ab
Sil	29.99 a	29.19 a	24.53 c	25.71 a
Control	28.80 ab	32.91 a	26.22 bc	23.33 b

Source: Authors.
*Means followed by the same letter in the column within each variable do not differ significantly from one another (5% significance). Kali-c = Kali carbonicum; Nat-m = Natrum muriaticum; Phos = Phosphorus; Sil = Silicea terra.
**Estimated time for plant height to reach 50% of total growth. The parameter β_1 corresponds to the height (cm) of the plant over time and the parameter β_2 represents the number of days that the plant reaches 50% of the height estimated by the asymptote.

At the end of the experiment, the height of the longest branch in Spring/Summer plants was lower when the plants were treated with the homeopathic preparations *Kali carbonicum* and *Natrum muriaticum*. Phosphorus, Silicea terra, and the control treatments presented the highest averages. For the Summer/Autumn experiment, no statistical differences were detected for the treatments (Table 2).

TABLE 2.
Average height of the longest branch and number of branches (± standard error) of *Hypericum perforatum* plants treated with homeopathic preparations in two growing seasons.

Treatment	Height of longest branch (cm)	Number of branches	Shoot dry weight (g)
		Spring/Summer experiment	
Kali-c	37.88 ± 2.03 b	21.68 ± 3.05 ab	22.90 ± 3.26 ns
Nat-m	38.33 ± 2.02 b	18.92 ± 1.77 b	22.49 ± 3.24
Phos	41.92 ± 2.27 ab	22.02 ± 1.63 ab	28.20 ± 4.46
Sil	44.89 ± 2.58 ab	30.85 ± 3.54 a	29.20 ± 5.19
Control	48.53 ± 2.42 a	29.21 ± 3.64 ab	35.94 ± 4.86
Mean and error	42.32 ± 1.12	24.62 ± 1.39	27.74 ± 2.04
		Summer/Autumn experiment	
Kali-c	37.39 ± 1.20 ns	2.00 ± 0.39 ns	1.75 ± 0.19 ns
Nat-m	39.29 ± 1.24	2.44 ± 0.38	1.96 ± 0.20
Phos	37.88 ± 1.52	1.47 ± 0.28	1.38 ± 0.21
Sil	34.70 ± 1.59	3.17 ± 0.41	1.40 ± 0.15
Control	36.41 ± 1.19	2.13 ± 0.60	1.51 ± 0.17
Mean and error	37.19 ± 0.62	2.23 ± 0.20	1.59 ± 0.09

Source: Authors.
*Means followed by the same letter in the column do not differ significantly from one another (5% significance). ns= non-significant. Kali-c = Kali carbonicum; Nat-m = Natrum muriaticum; Phos = Phosphorus; Sil = Silicea terra.

The average number of branches of *H. perforatum* plants in the Spring/Summer experiment was higher with the *Silicea terra* treatment, unlike the *Natrum muriaticum*-treated plants, but similar to control and the other treatments. In the Summer/Autumn experiment, there were no statistical differences among the treatments for height and number of branches.

Regarding the influence of homeopathy on agriculture, Bonato and Silva (2003) state that homeopathic preparations behave like energy and, when dynamized, the wave frequency remains fixed, with variation only on its amplitude, thus being able to alter the responses in a negative or positive way. Homeopathic medical materials act in the form of vibration, acting on the amplitude of the wave and consequently reflecting on biological organisms (Kolisko and Kolisko, 1978; Silva et al., 2005). Andrade and Casali (2011) state that medical materials react on the electromagnetic field differently, depending on the vitality of the plant. Thus, the performance in the organisms will depend on the chosen homeopathic preparation, the Hahnemannian centesimal used, and even the application method.

Dark glands: The number of dark glands present in the leaves of *H. perforatum* did not differ according to the homeopathic preparations and the control (Table 3).

TABLE 3.
Number of dark glands in the leaves of *Hypericum perforatum* over time in two growing seasons and treated with homeopathic preparations.

Treatments	Dark glands (n°)	
	Spring/Summer experiment	Summer/Autumn experiment
Kali carbonicum 12CH	14.76 ± 0.44 ns	12.75 ± 0.54 ns
Natrum muriaticum 12CH	15.39 ± 0.41	13.37 ± 0.37
Phosphorus 12CH	14.64 ± 0.65	12.20 ± 0.43
Silicea terra 12CH	16.41 ± 0.56	13.28 ± 0.34
Control	16.24 ± 0.51	12.54 ± 0.38
Mean and error	15.51 ± 0.24	12.83 ± 0.19

Source: Authors.
*Means followed by the same latter in the column do not differ significantly from one another (5% significance). ns = non-significant.

The number of dark glands may vary according to the growth stage of *H. perforatum* plants and, consequently, it affects the production of hypericin. Kladar et al. (2015) reported that phenological stages, such as flowering (pre-bloom, full-bloom, and post-bloom), influence the secondary metabolite pathways of plants.

Chromatographic analysis: The hypericin substance was targeted, but there was no peak in the crude extract chromatograms that corresponded to its retention time. Other peaks with absorptions at wavelengths 240 nm, 270 nm, 320 nm were detected, but they could not be identified. In the Spring/Summer experiment, the hydroalcoholic extracts of the control and *Silicea terra*-treated plants presented 11 peaks in the chromatograms, while in the Summer/Autumn experiment, 12 peaks were detected in both treatments. The detected peaks mostly showed more nonpolar compounds that appeared in the final retention times.

Rizzo et al. (2019) reported that the biosynthesis of hypericin and even other metabolites are related to the development of dark glands. The authors explained that leaves are the parts used in therapeutic treatment, but flowers are the ones that have more dark glands and, consequently, more hypericin. At the time of cultivation performed in this study, there was no time to flowering, which presumably determined the non-detection of the compound. In this study, as *H. perforatum* plants were grown only for 75 days, one could speculate that plants did not reach the growth and developmental stages required for the biosynthesis and accumulation of hypericin, regardless of growing seasons.

4. CONCLUSIONS

Homeopathic remedies affected plants of *H. perforatum* differently during the growing seasons. Higher averages were observed in plant cultivation when planting started in Spring compared to Summer. The compound of interest, hypericin, was not detected in any sample of leaves of *H. perforatum*. This suggests the need for a longer cultivation time so that biosynthesis and accumulation of such secondary metabolite can occur in the dark glands.

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