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Allelochemical Potential of Alecrim-de-Campinas (*Holocalyx balansae* Micheli) on Germination and Growth of Species of Agro-Economic Interest

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HIGHLIGHTS

- *Holocalyx balansae* shows allelopathic potential, inhibiting weed growth and germination.
- Bioassays show morning glory and sourgrass are highly sensitive, with reduced growth.
- Kaempferitrin (HB-1 compound) affects growth, reducing elongation, notably in lettuce seedlings.
- *H. balansae* metabolite show strong potential as natural herbicides, especially for sourgrass.

Abstract: Herbicides selects resistant weed populations, such as morning glory (*Ipomoea triloba* L.) and sourgrass (*Digitaria insularis* (L.) Fedde). Natural compounds represent an alternative control method for weeds. Released into the environment, those metabolites can influence other organisms by allelochemical interactions. Alecrim-de-campinas *(Holocalyx balansae* Micheli) produces cyanogenic glycosides, which are toxic metabolites. Therefore, can crude extract, aqueous extracts, and *H. balansae* metabolites affect the germination and growth of morning glory, sourgrass, and lettuce? Crude extract (CE) and the ethyl acetate fraction (EAF) were obtained and their allelochemical potential evaluated by lettuce germination. Aqueous extracts (EAq) were tested on the germination and growth of lettuce, morning glory, and sourgrass. The HB-1 compound was isolated from EAF and its effect was tested on the growth of the weeds and on the germination and growth of lettuce. CE, EAF, and EAq reduced germination and inhibited

growth, especially affecting root elongation. This study found no difference in lettuce germination under HB-1 solutions. The compound reduced seedling elongation, the bioassays caused the emergence of abnormal seedlings with root necrosis, yellowish and poorly expanded cotyledons and eophylls. The evaluated seedlings showed reduced growth and weight and morphological changes.

Keywords: Weeds; bioassays; germination; seedling growth; kaempferitrin; flavonoid; aqueous extracts.

INTRODUCTION

The Fabaceae family is considered, according to [1], one of the three largest families of native plants in Brazil, of which half are endemic species, distributed across all national biomes. This family has immense ecological and economic importance. The genus *Holocalyx* belongs to the subfamily Papilionoideae, standing out for having only one species in this taxon, *Holocalyx balansae* Micheli. (sin. *H. glaziovii*), popularly known as alecrim-de-campinas. This arboreal species is not endemic to the national flora and occurs in seasonal semideciduous forest, a common formation in the state of Paraná [2].

H. balansae is a very common species in the afforestation of urban environments and is highly appreciated for its timber potential [3]. However, it is considered a toxic tree as it has cyanogenic glycosides [4-7], a nitrogenous secondary metabolite. Secondary metabolites are bioactive compounds in plants that can exert numerous functions, especially protect individuals against biotic and abiotic factors. One of the biotic characteristics that can influence plant growth is the phenomenon known as allelopathy, a biochemical interaction between plants and microorganisms in natural environments by the production and release of secondary metabolites, also called allelochemicals. Allelochemicals, in turn, stimulate or inhibit the development of neighboring plants [8-10]. As the phenomenon occurs under natural conditions, researchers have replicated allelopathy in laboratory tests. Due to its toxic nature, *H. balansae* may represent an unexplored source of bioactive compounds with biotechnological applications. In this context, allelopathy experiments seek to evaluate the phytotoxicity of plant extracts [11], enabling the identification of natural compounds with allelochemical activity in target species, such as weeds.

Weeds are a problem for agriculture since the continuous and excessive use of chemicals selects plant populations that can resist current conventional herbicides [12,13]. However, with scientific and biotechnological advancement, research offers alternative methods to manage weeds, such as applying secondary metabolites with allelochemical properties as natural herbicides (or bioherbicides). Brazil and several other countries have two very common weed species: morning glory (*Ipomoea triloba* L.) and sourgrass (*Digitaria insularis* (L.) Fedde). Reports suggest that populations of both species have shown biotypes resistant to common commercial herbicides [14,15]. These plants feature among the weeds that cause most inconvenience to farmers in cultivated areas [16]. Thus, finding bioactive natural compounds that are less harmful to the environment to control these species may represent an alternative tool to the use of synthetic chemicals due to the existence of biotypes resistant to the most commonly used conventional herbicides [11,17].

Research in Brazil has largely sought potential bioactive compounds such as bioherbicides [18]. This stems from the biodiversity of its native flora, an aspect which attracts the attention of biotechnological researchers in view of the increase in deforestation in the country [4,19]. Phenolic and nitrogenous compounds constitute the most common chemical compounds in the Fabaceae family. According to [20], few species of the Papilionoideae subfamily had their chemical potential studied but flavonoids configure major compounds in the individuals in this taxon.

In view of the toxicity potential of *H. balansae* and the importance of prospecting for new compounds with biotechnological applications, such as allelopathy, this study aimed to investigate the phytotoxic effects of *H. balansae* leaflet extracts and its isolated metabolite on the germination and initial growth of lettuce, morning glory, and sourgrass.

MATERIAL AND METHODS

Plant material study and crude extract preparation

Branches of *H. balansae* were collected from a tree on the university campus in Maringá-PR. The leaflets were separated and dried at room temperature and ground in a knife mill (yield 500 g), with a subsequent extraction with hexane and methanol that resulted in 7.3 g of hexane extract (EHex) and 43.92 g of crude extract (CE), respectively. The extracts were concentrated in a rotary evaporator (±36 °C).

Crude extract fractionation and phytochemical characterization of EAF

An aliquot of CE (30 g) was solubilized with distilled water and methanol (H_2O:MeOH 7:3). Then, the solution was partitioned with solvents of increasing polarity (liquid:liquid) in chloroform (1.7 g of chloroform fraction $FCHCl_3$) and ethyl acetate (4.9 g of ethyl acetate fraction EAF). The residual volume was evaporated and concentrated in 21.2 g of hydromethanolic fraction (FH_2O:MeOH). A light-yellow amorphous EAF precipitate (80 mg) was subjected to RMN ^{1}H (300 MHz) (Table 1), ^{13}C (75 MHz), gCOSY, gHSQC, and gHMBC analysis for identification (Figure S1-S4 online supplementary material).

Solubilization of crude extract, ethyl acetate fraction, and aqueous extracts from leaflets of *H. balansae*

Aliquots of CE and EAF were solubilized and diluted with distilled water to 50 mL (m/v) at four concentrations: CE 0.25, 0.25, 0.5, and 1.0 $g.L^{-1}$ and EAF 0.32, 0.63, 1.26, and 2.52 $g.L^{-1}$. Aqueous extracts (EAq) were prepared after milling 0.50, 1.0, 2.0, 4.0, 8.0, and 10.0 g of the dried leaflets in a blender with 100 ml of distilled water, followed by filtration under a cotton gauze and centrifuged for 5 min at 4,000 rpm. The supernatants were collected and concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, and 10.0% were obtained (m/v).

Preparation of the HB-1 compound solution for lettuce germination and the initial growth bioassays

An aliquot of the HB-1 compound (kaempferitrin) was weighed and diluted in distilled water to obtain concentrations of 0.125, 0.25, 0.5 and 1.0 $g.L^{-1}$ to evaluate lettuce germination. A stock solution of HB-1 compound was prepared and 2.7 mg of it were weighed and solubilized in 40 µl of methanol with further dilution to 135 ml of distilled water (m/v). With the stock solution, dilutions were made to obtain concentrations of 15.0, 10.0, 5.0, 2.5, 1.0, 0.5, and 0.1 $mg.L^{-1}$,which were used in the growth bioassays with lettuce, morning glory, and sourgrass seedlings.

Germination bioassays with aqueous extracts of *H. balansae* leaflets

The weed species morning glory (*Ipomoea triloba*) and sourgrass (*Digitaria insularis*) were acquired from the company Agro Cosmos and the lettuce cypsela (*Lactuca sativa* L. cv. greatlakes 659), at a local market. The morning glory seeds were submerged in pure sulfuric acid (H_2SO_4) for 40 min to overcome dormancy and then washed in running water for 5 min [21,22]. Bioassays were carried out in 9 cm diameter Petri dishes with two filter paper discs and 6 ml of EAq from the leaflets of *H. balansae*. In total, 25 lettuce cypselae and 25 morning glory seeds were placed on each plate. For each concentration, five plates were used (n=5), totaling 100 seeds in each test. Plates with only distilled water were used as a control. The plates were kept in a germination chamber illuminated by four cold white Super-LED 8-W lamps and 14.4-W LED strips for 48 h at 25 °C for lettuce and 30 °C for morning glory, distributed entirely at random in a 12 h light/dark photoperiod. In the germination bioassays with sourgrass, 6 cm diameter Petri dishes were used with two filter paper discs and 3 ml of aqueous extracts. In total, 10 sourgrass caryopsis were placed in each Petri dish and each concentration consisted of 10 repetitions (n=10), totaling 100 caryopses. Plates were used only with distilled water as a control. Randomly distributed plates were kept in a germination chamber at 30 °C under a photoperiod of 12 h light/dark regime for 72 h. Germination was checked at 24 h intervals in any bioassay and those seeds that showed radicle protrusion (± 2 mm) were considered germinated. Then, germination percentage (%G), mean germination time ($MGT.days^{-1}$), and germination speed index (GSI) were calculated according to [23].

Analysis of initial growth with aqueous extracts of *H. balansae* leaflets and solutions of the HB-1 compound

Seeds were previously germinated for 48 (lettuce and morning glory) and 72 h (sourgrass) in distilled water. After radicle protrusion, seedlings were transferred to Petri dishes in the same way as for germination bioassays. After incubation, root and shoot length (cm) were obtained on a graph paper from five weed and 10 lettuce seedlings. The seedlings grown under the solutions of the HB-1 compound were dried in an oven at 60 °C for 48 h and weighed on an analytical scale to obtain their dry mass (g). Results are expressed in column charts with the standard error of the mean (SEM).

Statistical analyses

The experimental design was completely randomized and results were subjected to analysis of variance (ANOVA) followed by Dunnet's multiple comparison test ($p<0.05$). When the assumptions of the parametric analysis were not met, data were analyzed using the Kruskal-Wallis test followed by Dunn's test of multiple comparisons ($p<0.05$). Statistical calculations were performed on GraphPad Prism® 8 and all results were expressed using SEM in tables and column graphs to compare them to the control of the bioassays with the evaluated conditions that showed a 5% significance statistical difference.

RESULTS

This study identified the light-yellow amorphous precipitate obtained from EAF as the glycosilated flavonoid 3.7-di-O-α-L-aminopyrosylcampherol (kaempferitrin) (Figure 1). Table 1 shows the main signs of HB-1 that enabled the identification of the compound. Using the gHMBC contour map (Figure S4, online supplementary material), it was possible to confirm the positions of the rhaminoses (Rha) on the C-3 and C-7 carbons.

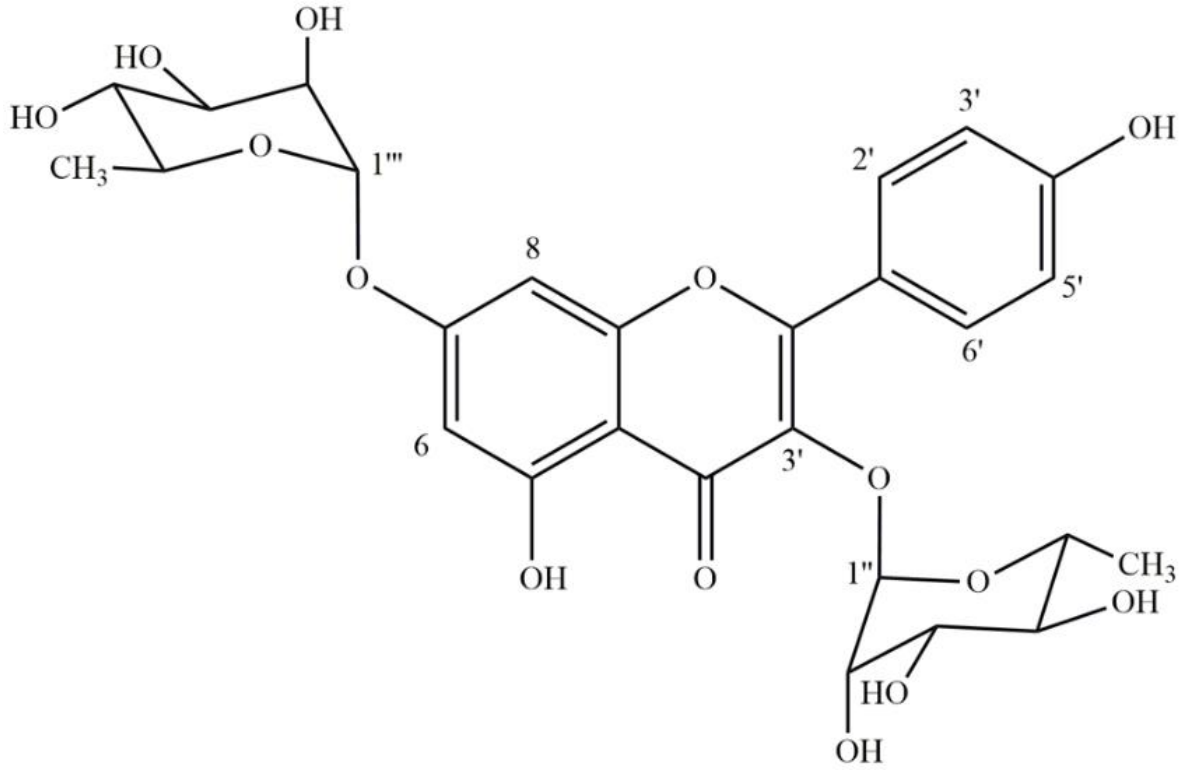


Figure 1. Molecular structure of the compound HB-1 (3,7-di-O-α-L-raminopyranosyl kaempferol) (kaempferitrin).

Table 1. NMR ^{1}H e ^{13}C of HB-1 compound (kaempferitrin)

H	δ_H (^{1}H)	C	δ_c (^{13}C)*
3' e 5'	6,93 (*J* = 8,7 Hz)	3' e 5'	116,6
2' e 6'	7,79 (*J* = 8,7 Hz)	2' e 6'	132,0
6	6,46 (*J* = 1,8 Hz)	6	100,6
8	6,72 (*J* = 1,8 Hz)	8	95,6
1''	(d) 5,39 (*J* = 1,8 Hz)	1''	103,5
1'''	(s) 5,56	1'''	99,9
6''	(d) 0,92 (*J* = 5,7 Hz)	6''	17,7
6'''	(d) 1,25 (*J* = 6,0 Hz)	6'''	18,1

*(δ-ppm; 300 e 75 MHz; CD_3OD)

Lettuce germination bioassays with CE and *H. balansae* leaflet fraction

Germination analysis showed that CE and FAE of *H. balansae* leaflets significantly altered the germination performance of the lettuce cypselae with the increase of the evaluated concentrations. The germination percentage (%G) significantly decreased with increasing concentrations, reducing it by an average of 80% of the %G in the most concentrated solution of CE and FAE compared to the control. Average germination time (AGT) was higher with increasing concentrations, when compared to the control, and was significantly higher at the 0.5 CE and in 1.85 and 20 $g.L^{-1}$ FAE concentrations. Both CE and FAE caused an average of one-day delay in cypselae germination, thus decreasing the germination speed index (GSI) so that with increasing concentration the number of cypselae germinated in the period proportionally decreased, which significantly altered the GSI value in the more concentrated solution, decreasing about 6% the GSI (Table 2 and Table 3).

Table 2. Germination performance of lettuce (*L. sativa*) under crude extract of alecrim-de-campinas leaflets (*H. balansae*).

Parameters	**Control**	**0,125 $g.L^{-1}$**	**0,25 $g.L^{-1}$**	**0,5 $g.L^{-1}$**	**1,0 $g.L^{-1}$**
%G	100 ± 0,0	44 ± 4,3	17 ± 6,4*	22 ± 6,2	8 ± 3,7*
MGT ($days^{-1}$)	1,11 ± 0,02	1,66 ± 0,09	1,60 ± 0,12	1,96 ± 0,04*	1,50 ± 0,50
GSI (ø)	23,6 ± 0,2	7,4 ± 0,9	2,8 ± 0,8*	2,9 ± 0,8*	1,0 ± 0,5*

*Indicates statistical difference compared to the control, using Dunn's test ($p<0.05$) means±SEM. %G germination percentage, MGT mean germination time, GSI germination speed index.

Table 3. Germination performance of lettuce (*L. sativa*) under ethyl acetate fraction of alecrim-de-campinas leaflets (*H. balansae*).

Parameters	**Control**	**0,32 $g.L^{-1}$**	**0,63 $g.L^{-1}$**	**1,26 $g.L^{-1}$**	**2,52 $g.L^{-1}$**
%G	100 ± 0,0	97 ± 1,9	91 ± 5,7	70 ± 14,7	15 ± 12,5*
MGT ($days^{-1}$)	1,11 ± 0,02	1,75 ± 0,07	1,79 ± 0,07	1,85 ± 0,12*	2,0 ± 0,0*
GSI (ø)	23,6 ± 0,2	15,1 ± 1,0	13,9 ± 1,6	10,6 ± 3,2	1,9 ± 1,6*

*Indicates statistical difference compared to the control, using Dunn's test ($p<0.05$) means±SEM. %G germination percentage, MGT mean germination time, GSI germination speed index.

Lettuce germination bioassays with the EAq of *H. balansae* leaflets

The EAq of *H. balansae* leaflets affected lettuce cypsela germination as its %G gradually decreased with increasing concentrations of EAq from 1% on ward, with statistically significant differences between the control and the 8% concentration. MGT increased with the concentrations, showing statistically significant differences between the control and the 4 and 8% concentrations, which delayed the germination of lettuce cypselae by about one day. As a consequence of the germination delay, the number of cypselae germinated every 24 h decreased proportionally and they are statistically different from the control at 4 and 8% concentrations. Due to experimental problems, it was not possible to evaluate the effect of EAq at a 0.25% concentration on lettuce germination. Regarding weed germination, morning glory %G suffered significant inhibition from 0.25% concentrations onward. MGT increased from the 0.5% concentration, with significant differences compared to the control at 4 and 8%. GSI decreased proportionally with increasing concentrations, with statistical differences in relation to the control from 0.5% onwards. Sourgrass caryopsis was also susceptible to allelochemicals from *H. balansae*, failing to germinate at concentrations greater than 0.5%. Sourgrass MGT significantly increased by 26 and 101% at the evaluated concentrations, which delayed germination by one day at 0.5% concentration. GSI proportionally decreased with statistical differences at the 0.5% concentration (Table 4).

Table 4. Germination performance of lettuce cypselas (*L. sativa*), morning glory (*I. triloba*) and sourgrass (*D. insularis*) under aqueous extracts of alecrim-de-campinas leaflets (*H. balansae*).

Species	Parameters	Control	0.25 %	0.5 %	1.0 %	2.0 %	4.0 %	8.0 %
Lettuce (*L. sativa*)	%G	97±1,0	np	98±1,2	93±1,9	92±3,7	88±2,8	76±4,6*
	MGT (days^{-1})	1,04±0,03	np	1,03±0,02	1,08±0,05	1,3±0,09	2,0±0,0*	1,98±0,01*
	GSI	23,8±0,5	np	24,1±0,3	22,4±0,9	20±1,7	11±0,4*	9,8±0,7*
Morning glory (*I. triloba*)	%G	93±1,92	80±2,83**	77±4,44**	72±4,32**	67±3,0**	33±2,52**	x
	MGT (days^{-1})	1,02±0,01	1,01±0,01	1,13±0,04	1,14±0,02	1,23±0,04*	1,20±0,09*	x
	GSI	23±0,54	19,9±0,66	18±1,28**	16,8±1,13**	14,9±0,85**	7,5±0,84**	x
Sourgrass (*D. insularis*)	%G	61±5,47	58 ± 6,11	36±2,67**	x	x	x	x
	MGT (days^{-1})	1,28±0,08	1,62±0,09**	2,58±0,10**	x	x	x	x
	GSI	5,23±0,37	4±0,31	1,45±0,12*	x	x	x	x

**Indicates statistical difference compared to the control, using Dunnet's test ($p<0.05$) means±SEM. *Indicates statistical difference compared to the control, using Dunn's test ($p<0.05$) means±SEM. %G germination percentage, MGT mean germination time, GSI germination speed index, np non performed; x no germination registered

Bioassays of initial growth of lettuce and weeds with the EAq of leaflets of *H. balansae*

The EAq from the leaflets of *H. balansae* inhibited the growth of lettuce, sourgrass, and morning glory seedlings. Lettuce seedlings were quite susceptible to the phytotoxic effects of EAq as there was no growth at concentrations above 2%. From 0.5% onward, root length was proportionally inhibited to increasing concentrations (28–60% on average). Shoot elongation decreased, and at the 2% concentration, it showed significant differences from the control (Figure 2a). Lettuce seedlings affected by EAq showed many symptoms, including necrotic seedlings, delayed expansion, and cotyledon opening with blackening of the root apex (Figure 2b).

The evaluated weed species were also susceptible to the phytotoxic effects of the allelochemicals in the EAq. Morning glory seedlings decreased as concentrations increased and showed statistical differences in root growth compared to the control from 0.5% onward, with an average reduction of 28–60% in elongation. The same occurred with shoot growth, with statistical differences from the control starting at 1%. No morning glory seedling growth was recorded at concentrations above 4% (Figure 2c). The bioassays showed seedlings with abnormal growth, stunted roots, absent ramifications, and non-expanded cotyledons in the seminal tegument (Figure 2d). Sourgrass seedling root length showed a significant reduction around 19–57%, proportional to EAq concentrations, as did shoot growth, with statistical differences from 0.5% onward. This research found no sourgrass growth at concentrations above 1% (Figure 2e). Several assays showed the emergence of seedlings with smaller and thinned eophylls blades and roots with severe tissue necrosis (Figure 2f).

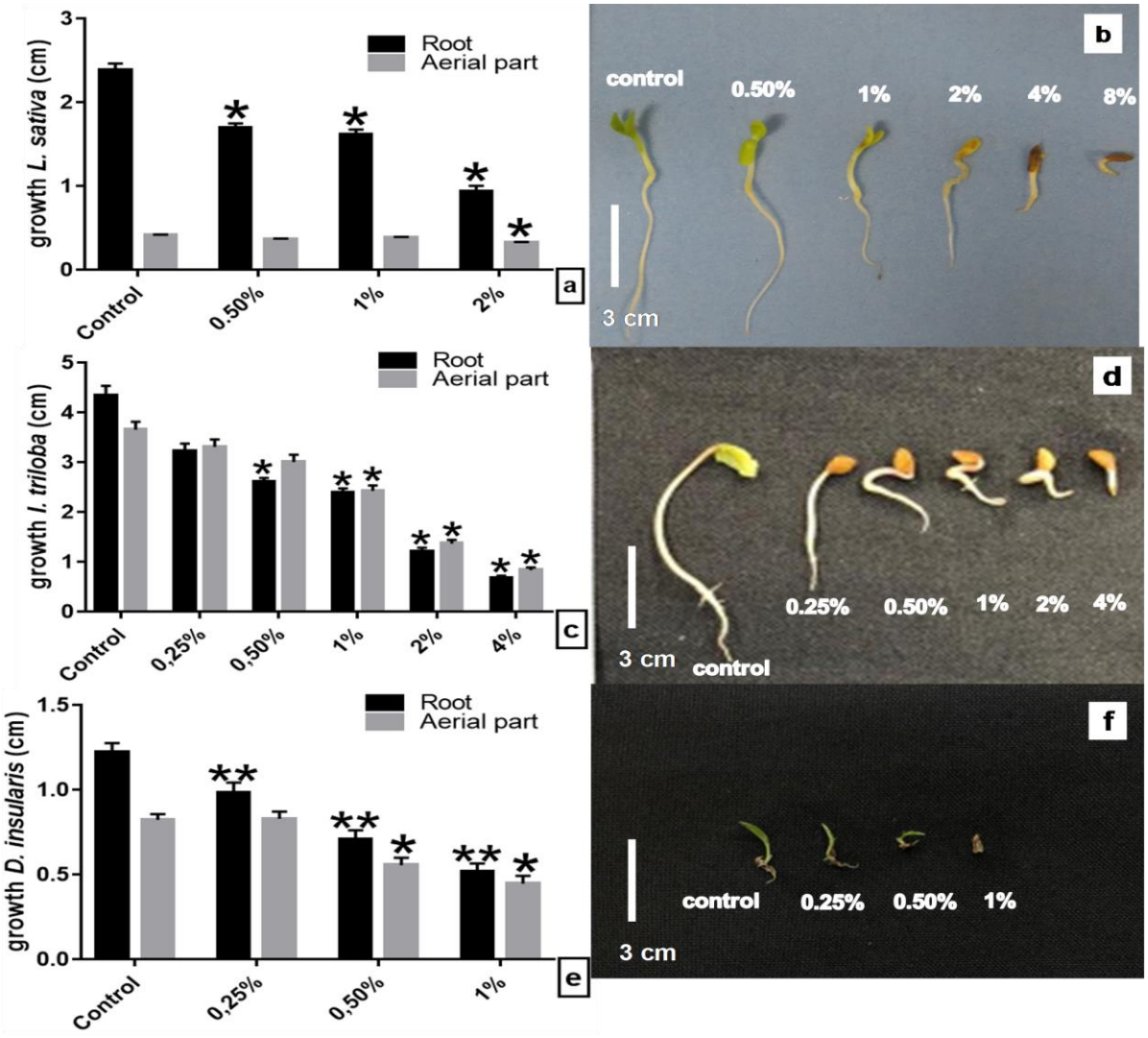


Figure 2. Lettuce seedling growth (*L. sativa*) (a), appearance of lettuce seedlings (*L. sativa*) (b), growth of morning glory seedlings (*I. triloba*) (c) appearance of morning glory seedlings (*I. triloba*) (d), growth of sourgrass seedlings (*D. insularis*) (e), appearance of sourgrass seedlings (*D. insularis*) (f) under aqueous extracts of alecrim-de-campinas leaflets (*H. balansae*). **Indicates statistical difference compared to the control using Dunnet's test ($p<0.05$) means±SEM. *Indicates statistical difference compared to the control, using Dunn's test ($p<0.05$) means±SEM (3 cm scale).

Bioassays with HB-1 compound isolated from EAF from *H. balansae* leaflets

The solutions of the HB-1 compound showed no phytotoxicity on the germination of lettuce cypselae, as evinced by no statistical differences for the evaluated germination parameters (Table 5). However, the growth of lettuce seedlings changed in contact with the solutions of the HB-1 compound. The 10 $mg.L^{-1}$ concentration stimulated root growth. However, lower concentrations reduced growth, in which 1 and 5 $mg.L^{-1}$ of the solution inhibited root growth by 78 and 44%, respectively, concentrations in which the seedlings showed the smallest roots in the bioassays. Concentrations from 0.1 $mg.L^{-1}$ on ward inhibited shoot development (except under 10 and 15 $mg.L^{-1}$). This research found the smallest hypocotyls under 0.1 and 1 $mg.L^{-1}$ (Figure 3a). The evaluated concentrations of the HB-1 compound reduced the dry mass of lettuce seedlings in all bioassays, with statistical differences for 0.1, 0.5, and 5 $mg.L^{-1}$ (Figure 3b). The emergence of abnormal seedlings in the most effective concentrations was common. Under 1 $mg.L^{-1}$, lettuce seedlings showed browning and root atrophy, at 5 $mg.L^{-1}$, cotyledons were yellowish and roots short and thicker. However, at higher concentrations of kaempferitrin, the roots of the seedlings were longer but tapered and translucent (Figure 3c).

The HB-1 compound affected morning glory seedlings, root elongation decreased under all concentrations compared to control, with significant differences at 0.1, 1, 2.5, 5, and 15 $mg.L^{-1}$. This study found the smallest roots in seedlings maintained at 1, 2.5, and 5 $mg.L^{-1}$ concentrations, with an average growth inhibition of 28%. This research observed similar results for shoot growth, as all concentrations inhibited air elongation. The 1, 2.5, and 5 $mg.L^{-1}$ concentrations significantly inhibited hypocotyl length (about 30%) (Figure 4a). The dry weight of seedlings decreased under all concentrations, with differences in relation to the control at 0.1, 0.5, 2.5, 10, and 15 $mg.L^{-1}$ (Figure 4b). Morning glory seedlings in contact with solutions of the HB-1 compound had cotyledons contained in the seminal tegument and pale roots with no ramifications compared to the control at the highest concentrations (5–15 $mg.L^{-1}$) (Figure 4c).

The growth of sourgrass seedlings was susceptible to the effect of the HB-1 compound because all concentrations reduced root length, with statistically significant differences from 0.5 $mg.L^{-1}$ on ward, except under 10 $mg.L^{-1}$. This study found the smallest roots in seedlings treated with 0.5–2.5 $mg.L^{-1}$ concentrations, which inhibited root growth by an average of 23%. Shoot growth was also affected, with statistical differences compared to the control in most concentrations. This study found the smallest hypocotyls under the lowest concentration (0.1 $mg.L^{-1}$), with an average of 13% of inhibition on aerial growth (Figure 5a). Regarding dry weight, the solution of the HB-1 compound in the 15 $mg.L^{-1}$ concentration reduced the dry mass of seedlings by 19% (Figure 5b). In the other concentrations, the compound did not affect seedling dry weight. The highest concentrations of the compound solution affected the expansion of leaf blades when compared to the control, whereas the lowest concentrations slightly thickened the roots (Figure 5c).

Table 5. Germination performance of lettuce (*L. sativa*) under solutions of the HB-1 compound (kaempferitrin) isolated from the ethyl acetate fraction of leaflets of alecrim-de-campinas (*H. balansae*)

Parameters	**Control**	**0,03 $g.L^{-1}$**	**0,06 $g.L^{-1}$**	**x0,12 $g.L^{-1}$**	**0,24 $g.L^{-1}$**
%G	40 ± 4,3	38 ± 3,8	46 ± 8,4	35 ± 9,0	39 ± 3,0
MGT ($days^{-1}$)	1,6 ± 0,1	1,7 ± 0,0	1,6 ± 0,0	1,5 ± 0,1	1,7 ± 0,0
GSI	6,9 ± 0,7	6,1 ± 0,6	8,0 ± 1,7	6,4 ± 1,5	$6,4 \pm 0,5^{ns}$

NS: No statistical differences were registered. %G germination percentage, MGT mean germination time, GSI germination speed index.

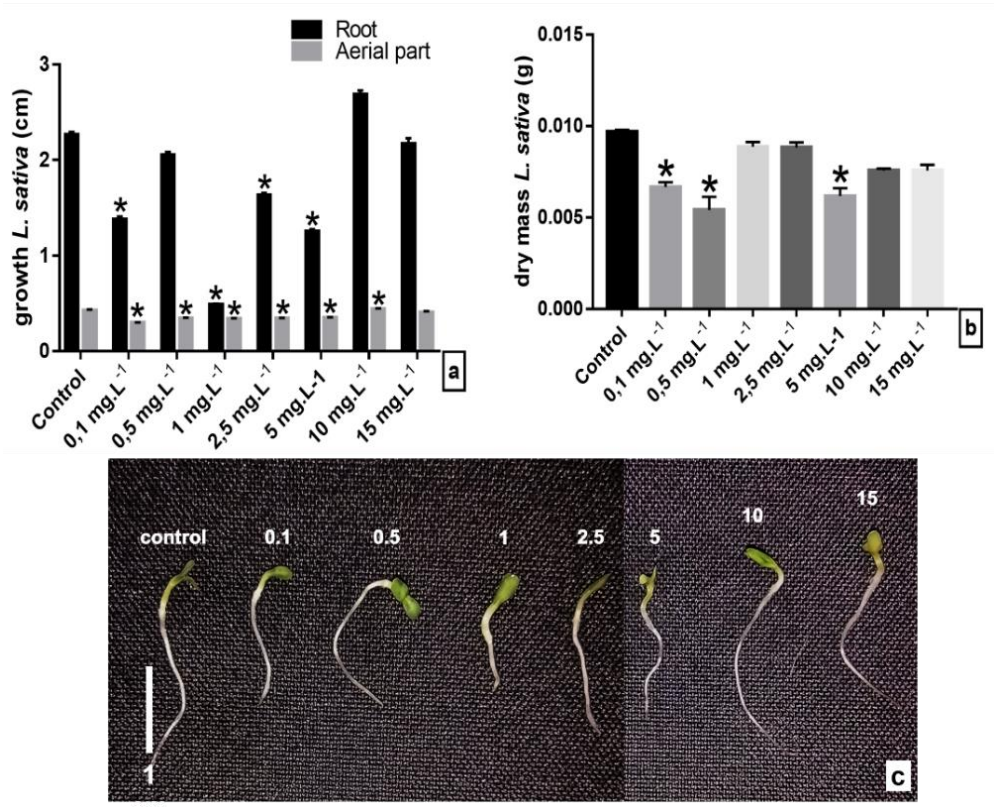


Figure 3. Lettuce (*L. sativa*) seedling growth (a), dry mass of lettuce (b), appearance of lettuce seedlings (c) under solutions of the HB-1 compound (kaempferitrin) isolated from the ethyl acetate fraction of alecrim-de-campinas (*H. balansae*) leaflets. *Indicates statistical difference compared to the control using Dunn's test ($p<0.05$) means±SEM (1 cm scale).

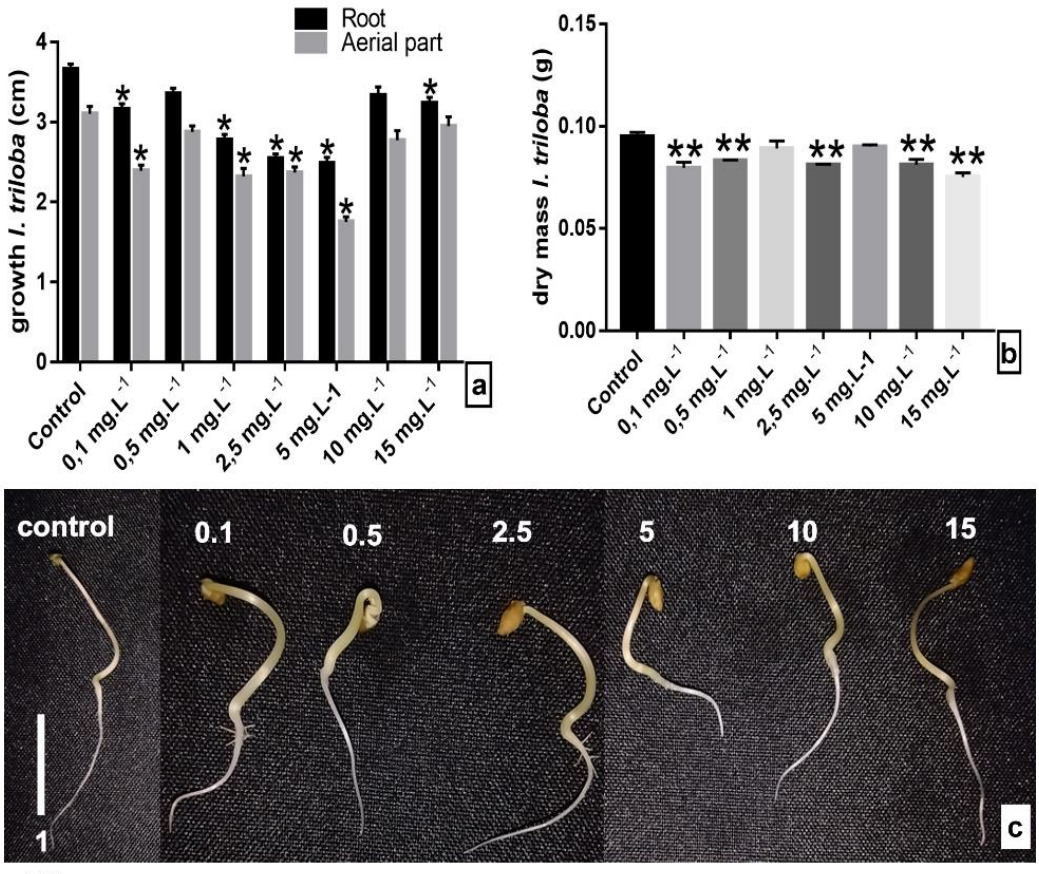


Figure 4. Morning glory (*I. triloba*) seedling growth (a), dry mass of morning glory (b), appearance of mourning glory seedlings (c) under solutions of the HB-1 compound (kaempferitrin) isolated from the ethyl acetate fraction of alecrim-de-campinas (*H. balansae*) leaflets. **Indicates statistical difference compared to the control using Dunnet's test ($p<0.05$) means±SEM. *Indicates statistical difference compared to the control using Dunn's test ($p<0.05$) means±SEM (1 cm scale).

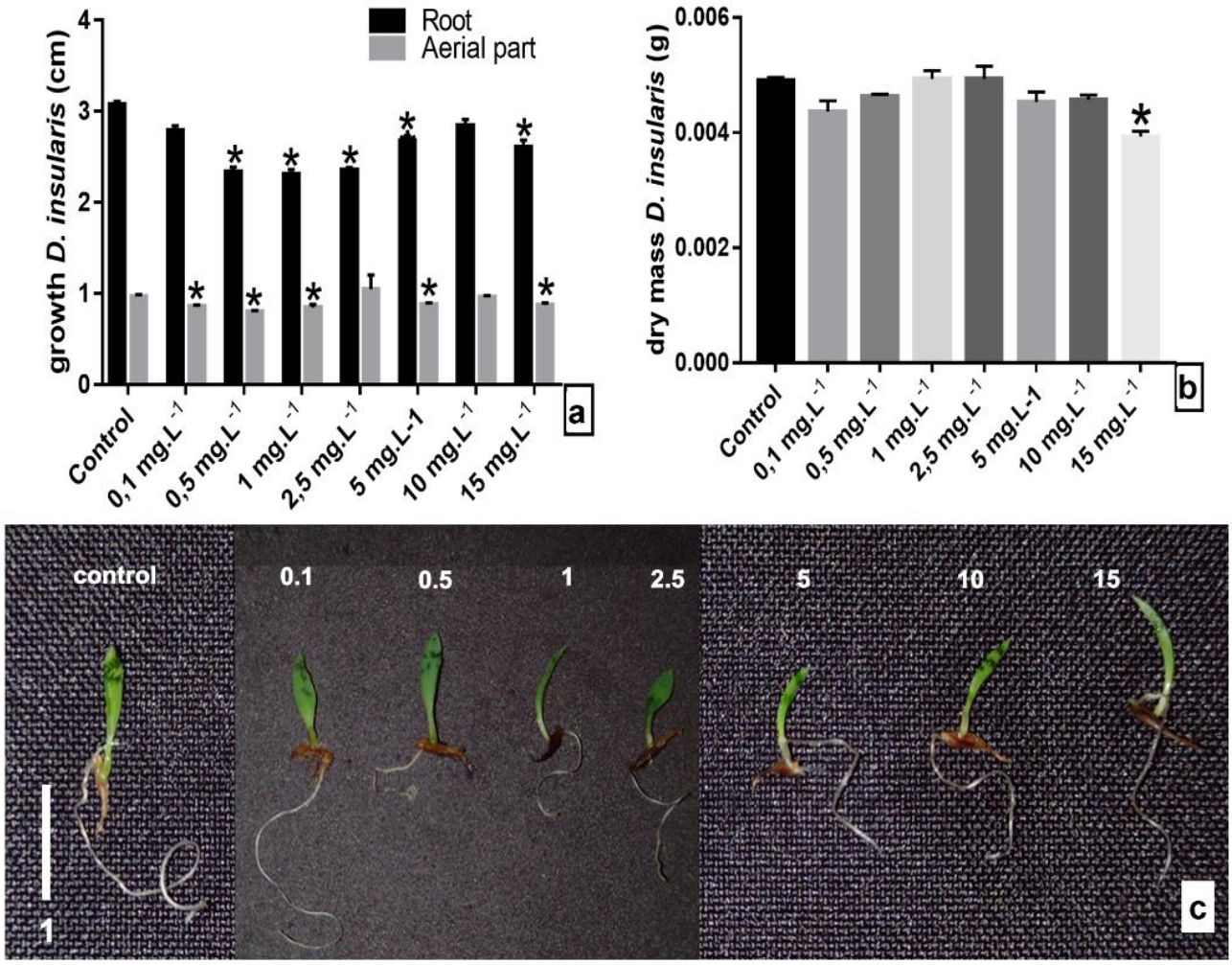


Figure 5. Sourgrass (*D. insularis*) (a), dry mass of sourgrass (b) and appearance of sourgrass seedlings (c) under solutions of the HB-1 compound (kaempferitrin) isolated from the ethyl acetate fraction of alecrim-de-campinas (*H. balansae*) leaflets. *Indicates statistical difference compared to the control using Dunn's test ($p<0.05$) means±SEM (1 cm scale).

DISCUSSION

The phytochemical prospection of the EAF characterized the glycosylated flavonoid kaempferitrin (HB-1 compound). Its structure was determined by 1D and 2D NMR spectra (300 MHz and 75 MHz; CD3OD) and by comparison with the literature [24]. Kaempferitrin constitutes a major component in *Holocalyx balansae* leaflets and was previously reported by [25] in this plant. This is the first report on the allelopathic potential of the major compound extracted from *H. balansae*, a native Fabaceae species with limited ecological and phytochemical characterization.

Bioassay results show the phytotoxic effects on germination and growth of the evaluated species. Lettuce germination under CE and EAF showed a dose-dependent effect in the results of germination parameters since the increase in solution concentrations proportionally reduced these parameters. Moreover, the highest concentration evaluated most effectively inhibited germination, time, and number of germinated seeds (Table 2 and Table 3).

Unlike most reports in the literature, in the EAq bioassays, weed germination was the most susceptible, rather than that of lettuce, as neither morning glory seeds showed germination in concentrations above 4% nor sourgrass in those above 0.5%, whereas lettuce cypselae germinated at all concentrations. Thus, sourgrass germination was the most sensitive to the phytotoxic effects of the allelochemicals of the EAq of the leaflets of *H. balansae*. However, under CE and EAF, lettuce was quite sensitive (Table 4), resembling [26], in which the EAq of lilac tasselflower (*Emilia sonchifolia*) was more phytotoxic to the germination of the weed beggar-ticks (*Bidens pilosa*) than to lettuce. The organic fractions of sorghum (*Sorghum bicolor*) in

[27] and signal grass (*Urochloa ruziziensis*) in [28] also affected the germination parameters of morning glory and sourgrass under increasing concentrations of bioassay solutions.

Lettuce germination is usually more sensitive, as, according to [9,29], it is a widely used species as it has high sensitivity to numerous allelochemicals. However, the germination of lettuce cypselae under solutions of the HB-1 compound showed no significant change in parameters (Table 5). This result may be related to the concentrations of the compound used in the bioassay since the method adopted may affect experimental results [8,30]. In the germination process, allelochemicals can especially affect the mobilization of seed reserves and the reactivation of energy metabolism, essential steps for seed germination. Thus, the deleterious effects of allelochemicals can delay or inhibit seed germination [31]. Allelopathic effects are often associated with changes in germination speed and time due to metabolic disorders such as inactivation of enzymatic activity [9,23]. According to [32,33] allelochemicals inhibit hormonal and enzymatic activity (gibberellins and α-amylase) during germination, modifying embryo cell division and expanding and affecting root protrusion. These reports agree with our results, as the decrease in germination parameters (especially MGT and GSI) can be attributed to the allelochemicals in the solutions. Thus, we propose that the metabolites in the tested materials have targeted one or more of these previously reported processes in the seeds, by increasing the time required for germination and by decreasing the number of seeds germinated in the evaluation periods of the bioassays (every 24 h).

Germination is less sensitive to the action of allelochemicals than plant development [9]. Thus, when evaluating growth, it is possible to obtain a wider range of results than simply germination of the tested seeds [34]. Results show that the initial growth bioassays with the EAq and solutions of the HB-1 compound, effectively modified the growth and caused morphological alterations in the seedlings of the evaluated species. EAq inhibited the growth of the evaluated seedlings (root and shoot) in a dose-dependent manner, i.e., as concentrations increase, structure elongation significantly decreased in such a way that the higher concentrations resulted in lower seedling growth. The roots of lettuce seedlings showed greater sensitivity to allelochemicals than its aerial part since they decreased in greater proportions (Figure 2a). On the other hand, for weed seedlings, shoot length decreased as the roots under the allelochemicals in the EAq. Of the analyzed species, sourgrass proved to be more sensitive to the metabolites in EAq than lettuce and morning glory since it showed no seedling growth at concentrations above 1% (Figure 2e, and 2f). These results resemble those of [31], who, when analyzing aqueous extracts of tickberry (*Lantana camara*) at concentrations ranging from 2.5 to 10%, observed a gradual inhibition of the growth of oat seedlings, which was related to the phytotoxic effects of allelochemicals in cell division, which makes it impossible for the organ to grow.

In the bioassays with the solutions of the HB-1 compound, results for the growth of lettuce seedlings show that the HB-1 compound affected the development of its organs (especially its root) since the decrease in lettuce root elongation was greater than that of the shoot (Figure 3a). The opposite was observed for morning glory seedlings, as both organs were sensitive and showed greater reduction (Figure 4a). Among the analyzed species, sourgrass seedlings were the least sensitive to the HB-1 compound (Figure 5a). However, EAq significantly affected them, as mentioned earlier (Figure 2e). The dry mass of the evaluated seedlings generally decreased under the action of the HB-1 compound (Figure 3b, 4b, and 5b).

Kaempferitrin was found in the n-butanolic fraction of *Aeschynomene fluminensis,* according to [35], this fraction inhibited the growth of lettuce, morning glory, and sourgrass seedlings and caused structural changes in them. This indicates that the results in this study can be attributed to the phytotoxic effects of the HB-1 compound, since it is a major component in *H. balansae* leaflets.

The appearance of abnormal seedlings, as previously reported, is related to the allelochemical effect of metabolites on plant development, of which root necrosis and atrophy are the most frequent symptoms [9]. This observation agrees with our experiments since atrophies and root necrosis, branching absence, cotyledon yellowing and expansion delay, and eophyll opening (Figure 2b, 2d, 2f, 3c, 4c, and 5c) were common symptoms of morphological alterations to the seedlings. According to [36], these structural changes stem from the direct action of allelochemicals in the root system because the elongation of the structure depends on cell divisions that, if inhibited, compromise their development and affect plant establishment. According to [37], roots are generally more sensitive to allelochemicals than other structures. This may explain our results as shoot growth was inhibited but in smaller proportions compared to root growth. According to [38], roots are possibly the main target of allelochemicals as they are in contact with the metabolites and directly exposed. Thus, the changes in growth in this study and the appearance of abnormal seedlings evince the allelochemical influence of the constituent metabolites of the EAq of *H. balansae* leaflets, which reduced structure elongation and thus reduced seedling mass.

According to the literature, in addition to the kaempferitrin (HB-1 compound), reports show other compounds as *H. balansae* constituents, such as glycosides-cyanogen, holocalin [4-7], prunasin [6-39], the phytosteroid stigmasterol, and flavonoids [40] were found. Kaempferitrin is a glycosylated flavonoid and, according to [41,42], has several bioactive properties. According to [20], phenolic compounds are a characteristic class of Fabaceae species and many phenolic compounds have allelochemical properties [43]. Flavonoids are allelochemicals often associated with the hormone auxin, which can act in the activation of hormone oxidation pathways and in the inhibition of mitochondrial metabolism and the cell division of meristematic tissues [44,45]. Since kaempferitrin is a flavonoid and a major constituent in *H. balansae*, the inhibitory effects reported here may be attributed to this allelochemical, as flavonoids are known to affect hormonal signaling and interfere with processes such as auxin transport, cell division, and overall plant development, including germination and growth.

As previously stated, other than kaempferitrin, cyanogenic glycosides are also present in *H. balansae*, and are highly toxic secondary metabolites because they release hydrogen cyanide (HCN) when degrading their molecules. Plants use this physiological strategy to avoid predators. Thus, these compounds are extremely toxic [5,46-49]. Therefore, *H. balansae* is considered a toxic plant and can represent an unexplored source of natural products with many applications. Additionally, the steroid stigmasterol, also found in this species, has reported phytotoxic activity [50].

The phytotoxic effects observed in germination and seedling growth of the target species are likely related to the bioactive properties of kaempferitrin and the other secondary metabolites identified in *H. balansae*. Therefore, these natural compounds may have acted reducing growth, mass gain, and modifying seedling morphogenesis. It is possible to propose that these compounds in *H. balansae* caused the results found in this study, indicating its phytotoxic potential. Allelochemicals influence seed germination and seedling development through multiple physiological and biochemical pathways. As previously stated, they can disrupt the mobilization of seed reserves and energy metabolism, inhibit enzymatic activities, and interfere with hormonal signaling including all of which are vital for cellular development. These effects germination and seedling development, which compromises their vigor and establishment [9,23,32].

The impact of these processes highlights the biotechnological applications of allelochemicals, such as kaempferitrin present in *H. balansae*, especially in the development of bioherbicides to control weeds like sourgrass, this species showed the greatest sensitivity to the tested materials in this study. In this context, the application of bioherbicides in cultivated areas can delay or even inhibit the germination of unwanted plants, helping reduce competition with crops. As bioproducts, these compounds remain in the soil for less time and are easily leached than chemical pesticides, which reduces the likelihood of them interfering with cultivated plants, allowing them to germinate and develop properly [11,17]. Notably, the differential sensitivity between the tested species indicates a degree of selectivity in the allelopathic potential of *H. balansae* metabolites. Since, sourgrass showed the highest susceptibility, whereas lettuce was less affected by the kaempferitrin. This selectivity is advantageous for potential agricultural applications, where minimizing damage to non-target crops is essential. Considering that wild poinsettia and sourgrass have resistant biotypes to common pesticides [14,15], plants natural compounds provide an alternative to reduce environmental impacts associated with synthetic pesticides. Thus, incorporating allelopathic species or their metabolites into agroecological farming aligns with sustainable practices and offers different solutions for weed control. This highlights the potential of allelochemicals as biotechnological resources to help and even enhance production in agroecological systems.

CONCLUSION

The evaluated species were quite susceptible to the phytotoxic effects of *H. balansae* in both their germination and seedling growth. Sourgrass showed a high rate of germination inhibition due to the absence of germinated caryopsis at EAq concentrations above 0.5%, characterizing it as the most sensitive target species to the allelochemicals in *H. balansae*. Lettuce germination remained unaffected by the HB-1 compound but the growth of seedlings in contact with the HB-1 compound showed significantly reduced root and shoot elongation. The EAq also drastically reduced the seedling growth of the three species evaluated, of which lettuce was the most sensitive; an expected result since lettuce is highly sensitive to many substances. All species underwent morphological changes in contact with the metabolites in the tested material, showing atrophies and root necrosis, delay shoot expansion, and tissue discoloration as the most common symptoms of the allelochemical influence of the metabolites. Thus, the study provided new insights into the bioactivity of the native Brazilian species, *H. balansae*, highlighting its potential for producing bioherbicides and its biotechnological applications in weed control.

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