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Article

Allelopathic interference of *Hovenia dulcis* extracts on the germination and development of *Parapiptadenia rigida*

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Abstract - This paper reports the influence of ethanolic extracts of fresh and dried leaves, pseudofruits, seeds, bark and roots of the invasive plant *Hovenia dulcis* Thunb. on seed germination and seedling development of the native tree *Parapiptadenia rigida* (Benth.) Brenan. Fresh adult and young organs of *H. dulcis* were extracted by maceration, while the extracts of the corresponding dried and pulverized plant material were obtained by percolation using soxhlet. The parameters germination, emergence velocity index (EVI), length and dry weight of radicles and hypocotyls of *P. rigida* were evaluated under influence of *H. dulcis* extracts in concentrations of 0, 25, 50, 75 and 100 mg mL^{-1}. Extracts of fresh plant materials provided yields varying between 5.7 and 9.7%, while extracts of dried organs showed values ranging from 14.0 to 35.5%. The allelopathic activity of extracts on *P. rigida* differed according to the plant organ of the donor species and showed to be concentration dependent, in addition to being influenced by the state of the extracted plant material. The allelopathic effects of *H. dulcis* extracts on *P. rigida* affected different stages of its development and were caused by allelochemicals present in distinct plant organs.

Interferência alelopática de extratos de *Hovenia dulcis* na germinação e desenvolvimento de *Parapiptadenia rigida*

Resumo - Este trabalho relata a influência de extratos etanólicos de folhas frescas e secas, pseudofrutos, sementes, cascas e raízes da planta invasora *Hovenia dulcis* Thunb. Na germinação de sementes e no desenvolvimento de plântulas da árvore nativa *Parapiptadenia rigida* (Benth.) Brenan. Órgãos adultos e jovens frescos de *H. dulcis* foram extraídos por maceração, enquanto os extratos do material vegetal seco e pulverizado correspondentes foram obtidos por percolação com soxhlet. Os parâmetros germinação, índice de velocidade de emergência (IVE), comprimento e massa seca de radículas e hipocótilos de *P. rigida* foram avaliados sob a influência de extratos de *H. dulcis* nas concentrações de 0, 25, 50, 75 e 100 mg mL^{-1}. Os extratos de materiais vegetais frescos proporcionaram rendimentos que variaram entre 5,7 e 9,7%, enquanto os extratos de órgãos secos apresentaram valores que variaram de 14,0 a 35,5%. A atividade alelopática dos extratos sobre *P. rigida* diferiu de acordo com o órgão vegetal da espécie doadora e mostrou-se dependente da concentração, além de ser influenciada pelo estado do material vegetal extraído. Os efeitos alelopáticos dos extratos de *H. dulcis* sobre *P. rigida* afetaram diferentes estágios de seu desenvolvimento e foram causados por aleloquímicos presentes em órgãos vegetais distintos.

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Introduction

Allelopathy may be considered an interference of one plant over another, providing positive or negative effects on individuals through the production of chemical constituents released into the environment (Zhang et al., 2020). Such a phenomenon includes processes involving complex mixtures of secondary metabolites produced by plants, which may present phytotoxic activities when in high concentrations, affecting not only seed germination, but also seedling development (Shan et al., 2023; Xu et al., 2023). These mixtures, also known as allelochemicals, are produced by different plant organs and vary in concentration and composition. They can regulate the relation between organisms, which consequently influences the behavior of the population of a species (Hierro & Callaway, 2021; Shan et al., 2023).

One of the most used procedures in the initial phase of prospection of an allelopathic activity is the evaluation of crude extracts. Once the production of secondary metabolites by a plant may be influenced by factors such as plant material state (fresh or dried), age and organ (Pant et al., 2021; Lou et al., 2023), these conditions should be considered during extract production. Plant extracts can be obtained through numerous methods of which maceration and percolation are considered standard options. While maceration is considered the method of choice for fresh plant material, percolation can only be performed with dried and pulverized plant organs (Kokilananthan et al., 2022).

Hovenia dulcis, which is also known as the Japanese grape, is a species native to Asia. The large and rapidly growing tree, is recognized as one of the most important invasive in Brazil, and therefore, this species has been considered a problem for the conservation of national ecosystems (Ribeiro et al., 2019; Schmidt et al., 2020; Reis et al., 2021; Silva et al., 2023). It has been introduced into different biomes in Brazil, including the Pampa, Atlantic Forest of *Alto Paraná*, Araucaria forest, Cerrado, interior forests of Bahia State and Hill Forests of the *Serra do Mar* (Silva et al., 2023). Extracts obtained from the leaves and pseudofruits of *H. dulcis* presented allelopathic activity on *Lactuca sativa* L. seeds (Wandscheer et al., 2011), which are used as a bioindicator species for allelopathic evaluation.

Considering the invasive potential of *H. dulcis* in southern Brazilian native forest fragments (Ribeiro et al., 2019; Schmidt et al., 2020; Reis et al., 2021), its effects over the development of species which naturally occurs in these areas are important to evaluate. The authors' observations at the collection site corroborate the literature reports about the invasiveness of *Hovenia dulcis*, since there is an orchard of this species, where there are practically no native trees around its individuals. One of the native species that proved to be most sensitive, avoiding proximity to *H. dulcis*, is *Parapiptadenia rigida*. In this context, the aim of this study was to investigate the possible effects of different concentrations of ethanolic extracts obtained from fresh and dried leaves, pseudofruits, seeds, bark and roots of young and adult *H. dulcis* plants on germination and seedling development of *Parapiptadenia rigida*, a south Brazilian native tree.

Material and methods

The plant organs of *Hovenia dulcis* Thunberg were harvested at the State Foundation for Agricultural-Forestry Research (Fundação Estadual de Pesquisa Agropecuária Florestas, FEPAGRO Florestas), which is located in Santa Maria, State of Rio Grande do Sul (RS), Brazil (S 29°41' 08"; W 53°48'42"). Plant material was obtained from adult plants in a 25-year-old plantation, and from young plants from a 3-year-old natural regeneration. Harvests were carried out on 09/30/2007 (young and adult leaves and roots), 10/28/2007 (young and adult bark) and 06/01/2008 (pseudofruits with seeds).

After harvesting, distinct plant organs from young and adult trees were separated, and part of the fresh material was submitted to maceration in ethanol (three times, always with solvent renewal, for at least seven days each) as presented by Okselni et al. (2023). The extractive liquids from the first, second and third macerations from each plant organ were gathered. A portion of fresh plant material was submitted to drying in an oven with forced air circulation at 40 °C until constant weight was obtained, followed by grinding in Willey mill type with meshes of 6 µ. Pulverized material was extracted by percolation using a Soxhlet apparatus (Ezhilarasan et al., 2021). Extracts were concentrated under reduced pressure and transferred to desiccators for two weeks followed by removal of residual moisture by phosphorus pentoxide. Extraction yields obtained were calculated considering the plant material mass used and expressed in % (w/w).

Before biological assay, stock solutions (100 mg mL^{-1}) were prepared from the crude dry extracts using ethanol 95%, which were further diluted in order to obtain three other concentrations (75, 50 and 25 mg mL^{-1}). A control treatment containing only distillated water was also evaluated

and the pH of the final solutions was determined (Santos et al., 2015).

Seeds of the target species *Parapiptadenia rigida* (Benth.) Brenan were acquired from FEPAGRO Florestas (batch 57/09). The design of the allelopathic bioassay was randomized with four repetitions of 24 seeds in each treatment. For the experiments, cell culture plates containing 12 cells with 22 mm diameter lined with blotter paper were used. The amount of extract/water used to moisten the substrates was 0.302 mL/cell, which corresponds to the liquid volume of the substrate (Brasil, 2022). After transferring the solutions to the cells, the boxes were placed in an oven at 35 ºC for 24 h in order to achieve total solvent evaporation. The seeds were disinfected with 2% sodium hypochlorite (Santos et al., 2020). Cells containing one *P. rigida* seed each were irrigated with distillated water (0.302 mL/cell) and transferred to a biochemical oxygen demand (BOD) chamber set at 12/12 h photoperiod and temperature of 25 ± 2 °C. Germination was monitored daily for five days. The experiment was concluded when germination stabilized in all repetitions of the treatment in two consecutive counts. The parameters evaluated initially were germination (G) and emergence velocity index (EVI). On the last day, the results of lengths and dry weights of radicles and hypocotyls were also obtained.

The results obtained for germination were transformed into arc sin (√%/100) (Pivetta et al., 2014) and expressed in percent according to the Equation 1.

$$G\% = \left(\Sigma_{nj} \;\; N^{-1}\right).100 \qquad (1)$$

where: Σ_{nj} = number of germinated seeds in relation to the number of seeds available to germinate (N).

Daily counts were performed in order to obtain the EVI, while computing normal and abnormal seedlings, which showed necrosis, until the last day of the experiment. This index was expressed according the equation of Mcguire (Soleymani, 2019), as presented in Equation 2.

$$EVI = G_1/N_1 + G_2/N_2 + ...G_n/N_n,\ G_1\ G_2 ...G_n \qquad (2)$$

where: G = number of germinated seeds in the day; N = number of days after the start of the bioassay.

Biometric parameters of length were obtained by measurements of radicles and hypocotyls, and results were expressed in cm (Carvalho et al., 2022). Thereafter, plant material was weighed on a precision scale for moisture calculation and afterwards placed in an oven at 60 ºC until weight stabilization. Then, the material was transferred to desiccators with silica for posterior weight determination. Results are expressed by means and the data of germination, EVI, radicle and hypocotyl lengths, radicle and hypocotyl dry weight were submitted to analysis of variance (ANOVA) followed by Tukey test at significance levels of 1 or 5%.

Results

Results of pH analysis of *Hovenia dulcis* ethanolic extracts showed values ranging between 5.38 and 6.65 (data not shown), while the pH from control (distilled water) was 5.30. The yields of the extracts obtained by maceration ranged between 5.72 and 9.66%, while the extracts obtained by percolation varied from 14.00 to 35.45% (Table 1).

Regarding the extracts obtained by maceration of fresh plant material, the ones obtained from adult leaves, adult and young roots impaired the germination of *P. rigida* seeds when compared to control (Figure 1A). The extract obtained from adult roots was the most effective, showing activity at 75 mg mL^{-1}. For the extracts obtained by percolation, adult leaves and pseudofruits were the only ones that affected the germination of *P. rigida* at the highest concentration (100 mg mL^{-1}) when compared to control (Figure 1B).

The emergency velocity index (EVI) of *P. rigida* seeds proved to be more sensitive to the extracts actions, being significantly affected by young barks, adult leaves, adult roots, young roots, pseudofruits and seeds when compared to control (Figure 1C). In these cases, young barks, adult leaves, adult roots and young roots influenced EVI at 75 mg mL^{-1}, while pseudofruits and seeds interfered with this parameter at 50 and 100 mg mL^{-1}, respectively. All the extracts obtained by percolation influenced the EVI of seeds, except those obtained from adult roots (Figure 1D). The young barks extract significantly affected EVI at 75 mg mL^{-1}, while adult barks, adult leaves, young roots, pseudofruits and seeds impaired it at 50 mg mL^{-1} compared to control. On the other hand, the young leaves extract demonstrated to be the most injurious, thus influencing this parameter at the lowest concentration.

All *H. dulcis* extracts obtained from fresh plant material impaired *P. rigida* radicle length at 25 mg mL^{-1} with exception of seeds, which significantly affected it at 50 mg mL^{-1}, when compared to control (Figure 2A). Considering the highest

concentration tested, young barks, adult leaves, adult roots, young roots and pseudofruits extracts stood out as the most toxic, differing statistically from the others (Figure 2B). Additionally, all extracts influenced radicle dry weights at 25% concentration, except adult barks, which was effective at 50 mg mL^{-1} (Figure 2C). Regarding the action of all extracts at 50 mg mL^{-1}, those obtained from the adult roots were the most efficient at affecting radicle dry weight, hence differing from the other extracts (Figure 2D).

As already observed for the radicles, hypocotyl development was impaired by all extracts obtained from fresh plant material at 25 mg mL^{-1}, with the exception of seeds, which interfered significantly in lengths at 50 mg mL^{-1} compared to control (Figure 3A). For the 50 mg mL^{-1} concentration, the adult roots extract impaired the hypocotyl length more intensely, while for 100 mg mL^{-1} significantly higher lengths were observed for the seeds submitted to adult barks and seeds extracts (Figure 3B). The dry seedling weights were also affected by all maceration extracts by at least 50 mg mL^{-1} with exception of seeds (Figure 3C). It is important to highlight that the extracts obtained from adult leaves, adult roots, young roots and pseudofruits demonstrated activity at the lowest concentration tested. For the concentrations 50 and 100 mg mL^{-1}, the seeds extract stood out by induced the highest dry weight (Figure 3D). At the highest concentration, it did not differ from the adult barks extract.

All extracts obtained from dried plant material significantly impaired the radicle length of *P. rigida* at 25 mg mL^{-1} (Figure 4A). The extract of young barks at 50 mg mL^{-1} provided an increase in the growth of the species radicle, compared to the result provided by the extract in the previous concentration. When the performance of the extracts was compared at 100 mg mL^{-1}, young barks, adult leaves, young leaves, adult roots, young roots and pseudofruits were the most toxic, since seedlings presented no radicles (Figure 4B). The 25 mg mL^{-1} concentration of all extracts influenced the dry weights of this seedling part, with exception of young leaves and young roots, which showed significant effect only at the highest concentration when compared to control (Figure 4C). Although the seeds extract at the lowest concentration provided a lower dry weight result, when compared to the control, the extract at 50 mg mL^{-1} provided a result that did not differ from the control. Except for the seeds, all the other extracts extensively impaired root development at 100 mg mL^{-1} (Figure 4D).

The highest concentration of all extracts obtained from dried plant material impaired hypocotyl development (Figure 5A). However, those obtained from adult barks, young barks, adult leaves, adult roots, young roots and pseudofruits stood out, since they already had allelopathic effect at the lowest concentration. At 100 mg mL^{-1}, adult barks and seeds extracts presented the longest hypocotyl lengths, thus differing from the others (Figure 5B). Therefore, the hypocotyl dry weight was just not influenced by the adult barks extract (Figure 5C). All other extracts significantly interfered with this variable at least at the highest concentration, thus differing from control. At the highest concentration, adult barks extract presented the highest hypocotyl dry weight, differing from the other extracts (Figure 5D).

Table 1. Average yields (%) of different *Hovenia dulcis* extracts obtained by maceration and percolation methods.

Method	Adult barks	Young barks	Adult leaves	Young leaves	Adult roots	Young roots	Pseudo fruits	Seeds
Fresh plant material / Maceration	6.4	7.4	8.2	-	6.1	5.7	9.7	6.5
Dried plant material / Percolation	28.4	14.0	17.0	31.3	20.5	35.5	22.7	15.87

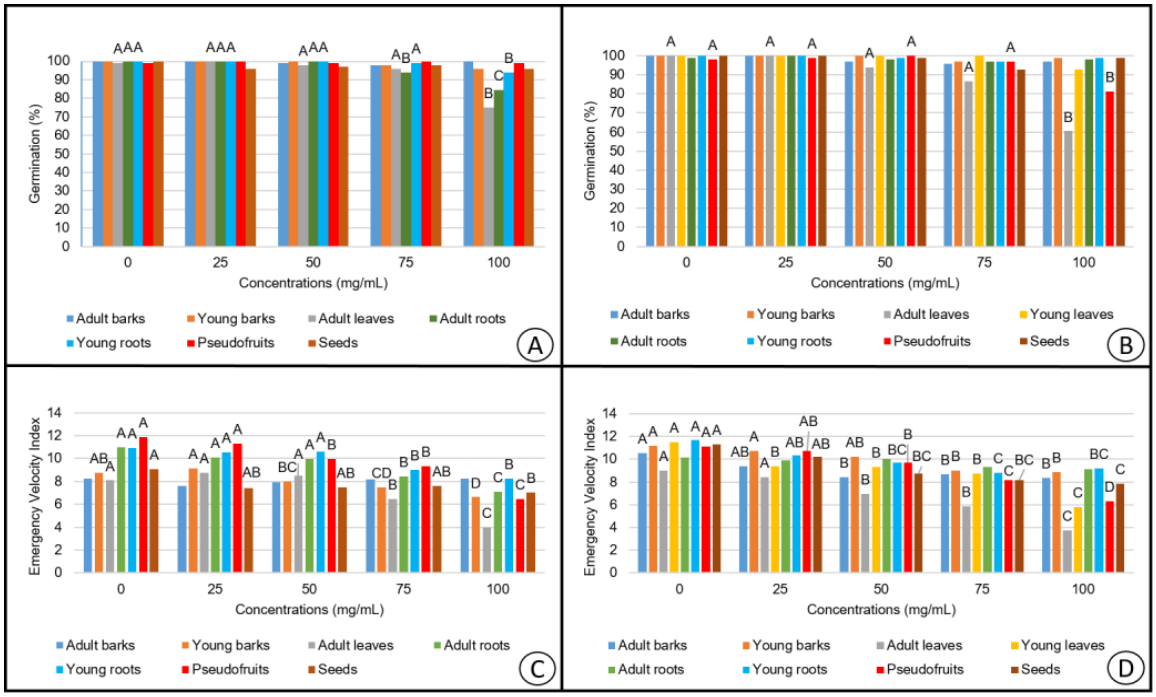

Figure 1. Average germination (A and B) and emergency velocity index values (C and D) of *Parapiptadenia rigida* seeds submitted to the action of different *Hovenia dulcis* extracts (mg mL^{-1}) obtained from maceration (A and C) and percolation (B and D) methods (n = 24). Different uppercase letters in columns indicate statistical differences (P < 0.01) between concentrations of the extracts.

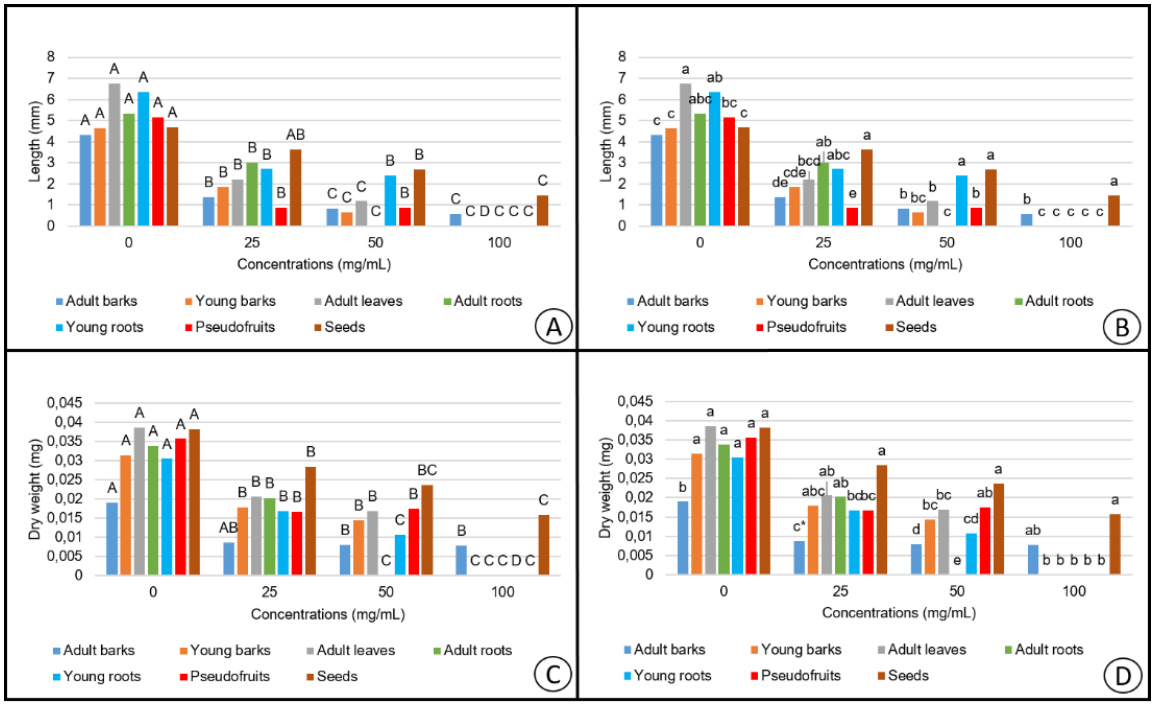

Figure 2. Average values of lengths (A and B) and dry weights (C and D) of *Parapiptadenia rigida* radicle submitted to the action of different *Hovenia dulcis* extracts (mg mL^{-1}) obtained from maceration method (n = 24). Different uppercase letters in columns indicate statistical differences (P < 0.01) between concentrations of the extracts (A and C). Different lowercase letters in columns indicate statistical differences (P < 0.01) between extracts in the same concentrations (B and D). Means followed by different lowercase letters in columns indicate statistical differences (P < 0.05) between extracts in the same concentrations.

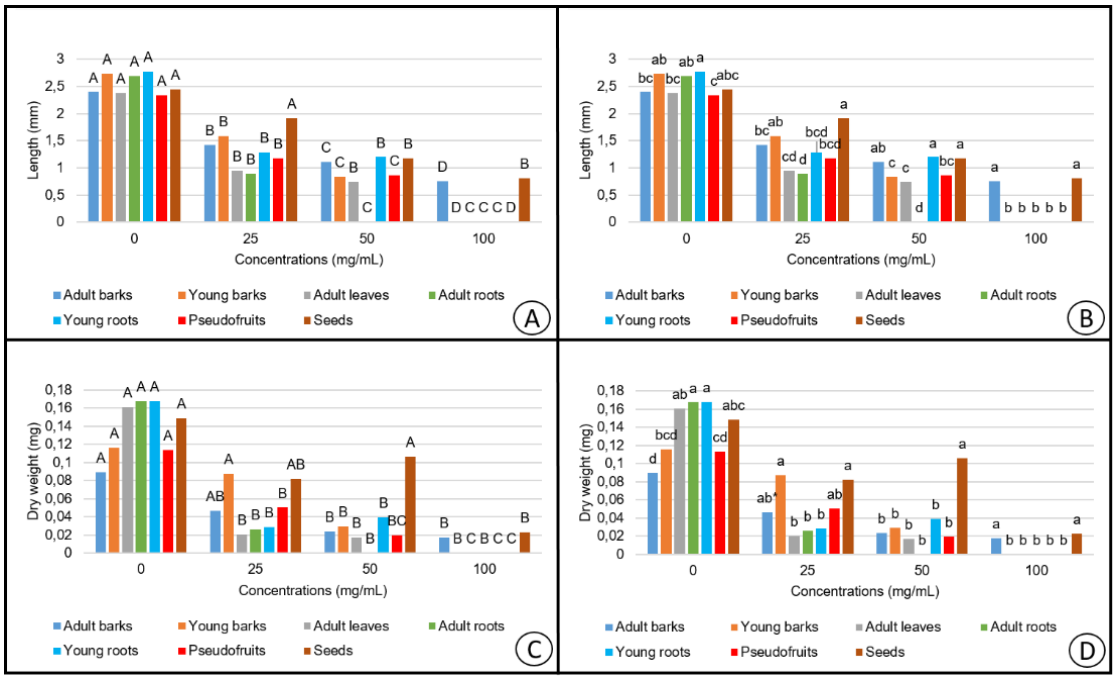

Figure 3. Average values of lengths (A and B) and dry weights (C and D) of *Parapiptadenia rigida* hypocotyl submitted to the action of different *Hovenia dulcis* extracts obtained from maceration method (n = 24). Different uppercase letters in columns indicate statistical differences (P < 0.01) between concentrations of the extracts (A and C). Different lowercase letters in columns indicate statistical differences (P< 0.01) between extracts in the same concentrations (B and D). Means followed by different lowercase letters in columns indicate statistical differences (P < 0.05) between extracts in the same concentrations.

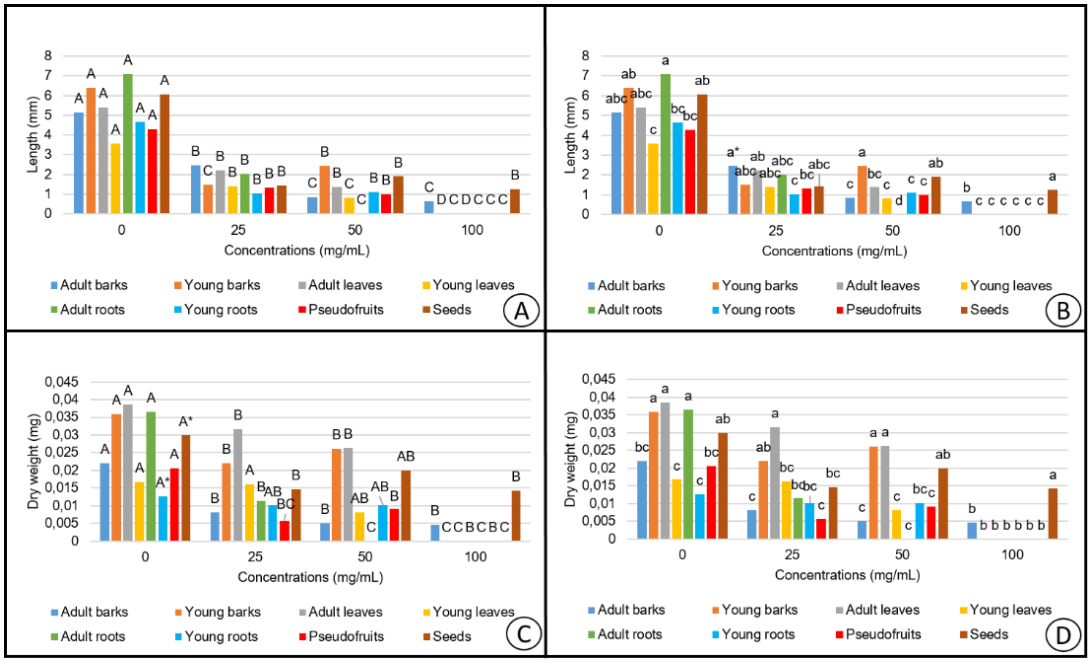

Figure 4. Average values of lengths (A and B) and dry weights (C and D) of *Parapiptadenia rigida* radicle submitted to the action of different *Hovenia dulcis* extracts (mg mL^{-1}) obtained from percolation method (n= 24). Different uppercase letters in columns indicate statistical differences (P<0.01) between concentrations of the extracts (A and C). Different lowercase letters in columns indicate statistical differences (P<0.01) between extracts in the same concentra-tions (B and D). * = Means followed by different uppercase or lowercase letters in columns indicate statistical differences (P<0.05) between concentrations of the extracts or extracts in the same concentrations. .

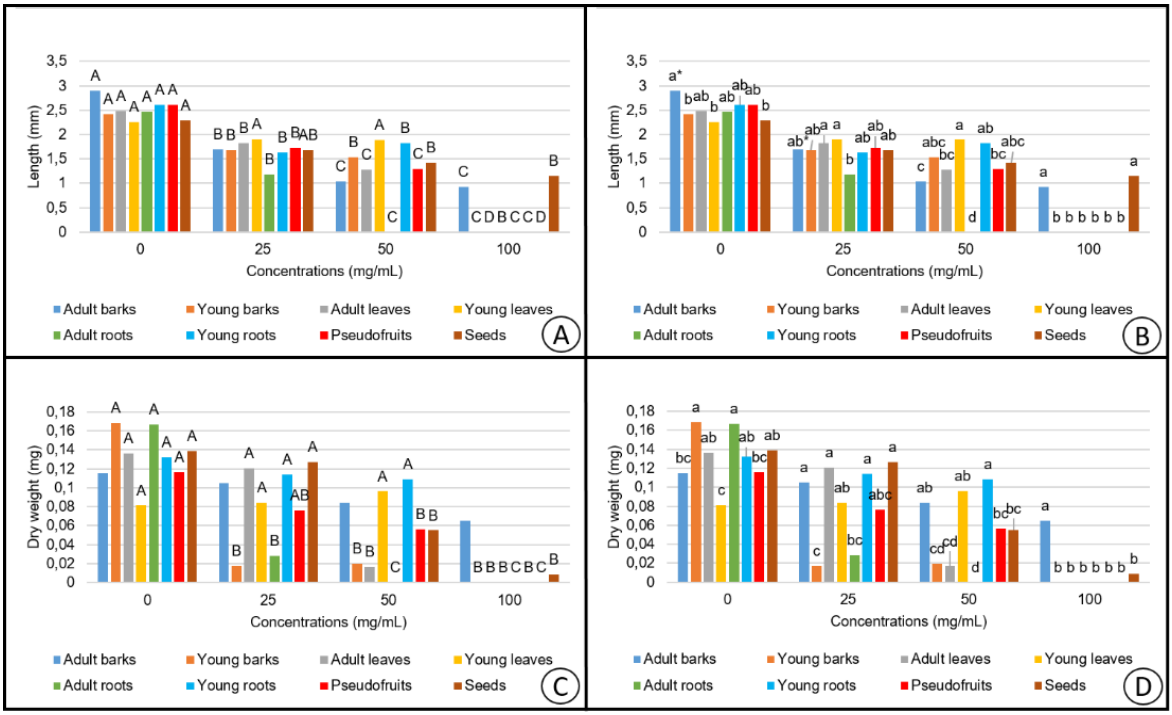

Figure 5. Average values of lengths (A and B) and dry weights (C and D) of *Parapiptadenia rigida* hypocotyl submitted to the action of different *Hovenia dulcis* extracts (mg mL^{-1}) obtained from percolation method (n = 24). Different uppercase letters in columns indicate statistical differences ($P < 0.01$) between concentrations of the extracts (A and C). Different lowercase letters in columns indicate statistical differences ($P < 0.01$) between extracts in the same concentrations (B and D). Means followed by different lowercase letters in columns indicate statistical differences ($P < 0.05$) between extracts in the same concentrations.

Discussion

Allelopathy is often promoted by mixtures of multi-target secondary metabolites that have been selected during evolution for chemical defense (Hisashi & Midori, 2023). For the donator species, the literature presents phytochemical studies with leaves, seeds, fruits and root bark. These studies have revealed that *Hovenia dulcis* leaves present triterpenoid saponins (Cai et al., 2021) and the odoriferous monoterpenoid glycoside kenposide A and B, besides the sesquiterpenoid glycoside icariside C (Li et al., 2022). In addition, saponins (Cai et al., 2021) and flavonoids (Ngo, et al., 2021; Janilkarn-Urena et al., 2023) were identified in the seeds and fruits of *H. dulcis*. On the other hand, alkaloids (Godoi et al., 2023) and saponins (Cai et al., 2021; Sferrazza et al., 2021) were isolated from the root bark.

Secondary metabolites are not constant in plants and their production can be influenced by numerous factors (Pant et al., 2021). In addition to soil composition, allelochemicals and their concentrations may vary according to environmental conditions, plant age and organ, among others (Carraro-Lemes et al., 2019; Bazargani et al., 2021). Therefore, the present study separated distinct plant organs of the donor species before extraction, in order to separately analyze allelopathic activity in an attempt to understand the strategies used by *H. dulcis* to invade areas of native Brazilian forests.

Regarding the pH results, all values found were within the expected range of between 3.94 and 6.65, already described by Wandscheer et al. (2011) when analyzing extracts of *H. dulcis* pseudofruits and leaves. According to Santos et al. (2015), extreme acid (below 3.0) or alkaline (above 11.0) values may interfere in the germination process. Crude extracts can be osmotically active and contain substances such as sugars, amino acids and organic acids that may interfere in pH (Ferreira & Aquila, 2000). However, considering the experimental data our results corroborate the former reports, since no influences of pH were observed. In addition, it was evidenced that the responses of the seeds/seedlings were dependent on the plant organ, the state of the extracted plant material (fresh or dried) and the concentration of the solution.

The extract yields obtained from fresh and dry plant material by two different extraction methods were compared, and percolation showed higher yields. These results were expected, since maceration uses room temperature and fresh, coarsely divided plant

material. On the other hand, dried and pulverized plant material was used in order to obtain the percolate samples in a soxhlet apparatus. The fragment size of plant material to be extracted is one of the determining factors for extractive yield, since the processes of diffusion of the low molecular weight metabolites to the solvent is facilitated in the case of particles of smaller size (Loum et al., 2021). However, soxhlet extraction presents an important disadvantage. During extraction process the extractive solution is heated, which may result in the degradation of thermo unstable bioactive components (Mahanta et al., 2021; Drăghici-Popa et al., 2023). A chemical alteration of the extractives may also occur during the drying process of the plant material. Consequently, substances resulting from the plant withering are obtained, which can lead to bioactivity loss (Pandino et al., 2020). Comparing the results of the allelopathic activity, while considering both extraction methods, *Parapiptadenia rigida* germination and emergence velocity index were more affected by adult root extracts obtained from fresh plant material compared to the extract from the corresponding dried organ. Fresh adult root macerate affects the target species at 75% concentration, while the extract obtained from the same dried plant organ by percolation did not affect these two parameters in any of the tested concentrations. These findings indicate that drying plant material and/or extraction at high temperatures caused the destruction of bioactive substances, as already described by Belwal et al. (2022).

The effects of *H. dulcis* extracts of different plant organs on *P. rigida* seeds are concentration dependent and indicate distinct behavior regarding germination and emergence velocity index (EVI) parameters. Differences in allelopathic responses provided by distinct plant organs of the same species were already reported in studies with *Chenopodium ambrosioides* L. (Jiménez-Osornio et al., 1996) and *Artemisia absinthium* L. (Delachiave et al., 1999). According to Hickman et al. (2021), allelochemical effects are concentration dependent and the substances can act in many different processes simultaneously. Additionally, a determined target species may have distinct responses to a particular concentration of a specific substance. According to Yuan et al. (2021), the allelochemicals were possibly developed by *H. dulcis* while aiming to provide a competitive advantage over the invaded community, which was named as *novel weapon hypothesis*. However, in addition to the observed phytotoxic activity of the *H. dulcis* extracts, a hormesis effect was observed when the allelochemicals provided by young barks and seeds were applied at 50 mg mL^{-1} on *P. rigida* seeds. Hormesis is considered an adaptive response characterized by stimulation at a low dose, which causes inhibition at a high dose (Hickmann et al., 2021; Agathokleous & Calabrese, 2022).

Parapiptadenia rigida germination was impaired by the extracts obtained by both extraction methods, especially by those products obtained from fresh leaves and roots, using the maceration method, as well as by dried leaves and pseudofruits extracted by percolation. According to Shan et al. (2023), the effects caused by allelochemicals on germination and seedling growth can be considered secondary manifestations of reactions that initially occurred at molecular and cellular levels. Additionally, the changes in germination pattern may be caused by several effects on DNA transcription and transduction, functioning of secondary messengers, respiration, membranes permeability, and conformation of enzymes and receptors. Germination and germination speed are commonly analyzed parameters in the same experiment since they may show different susceptibilities in allelopathic evaluations (Begum et al., 2024). This is consistent with the results found in the present study, in which not all the extracts that impaired EVI affected germination.

Exotic invasive species are predominantly responsible for the loss of biological diversity. When introduced to other environments that have no enemies, they tend to adapt themselves and reproduce. Additionally, the exotic invasive species occupy the area of the native ones by creating changes in natural ecological processes, and may become dominant after the adaptation period, which is also related to the production of allelochemicals (Xu et al., 2023). In addition, Kong et al. (2024) reported that species which co-occur invest more in secondary metabolites with allelopathic properties, indicating that the invader reacts evolutionarily to the selection imposed by interspecific competition.

According to Schmidt et al. (2020), abiotic factors facilitate *H. dulcis* invasion, whereas the biotic ones present some resistance that hinders the naturalization process. Furthermore, the occurrence of semi-open vegetative type or intermediate forest succession stage gather favorable conditions for the establishment of *H. dulcis*, thus increasing invasive potential. In addition, our results suggest that the highly invasive potential of *H. dulcis* may be derived from allelopathic effects that occur sequentially caused by bioactive compounds present in distinct plant organs that affect different stages of development of native plants, as detected for *P. rigida*.

Conclusions

The allelopathic activity of *Hovenia dulcis* extracts on *Paraptadenia rigida* seeds differed according to the plant organ of the donor species and showed to be concentration dependent and influenced by the state of the starting plant material, whether dried or fresh. In general, germination and emergence velocity index are more affected by extracts of adult leaves, whereas seedling development underwent significant impairment under the influence of adult roots extracts. The allelopathic effects of *H. dulcis* extracts affected different stages of *P. rigida* development and were caused by bioactive compounds present in distinct plant organs, explaining the low occurrence of *P. rigida* in the natural environment close to *H. dulcis*.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' Contributions

Dane Block Araldi: conceptualization, formal analysis, investigation, methodology. **Carlos Garrido Pinheiro:** writing - original draft. **Ervandil Corrêa Costa**: conceptualization, supervision. **Danilo Boanerges Souza**: investigation methodology. **Berta Maria Heinzmann:** conceptualization, writing, review & editing.

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