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Evaluation of the allelopathic, genotoxic, and antiproliferative effect of the medicinal species *Psychotria brachypoda* and *Psychotria birotula* (Rubiaceae) on the germination and cell division of *Eruca sativa* (Brassicaceae)

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Biological assays are widely used to monitor toxic and allelopathic substances. The present study aimed to evaluate the allelopathic, genotoxic, and antiproliferative potential of aqueous extracts of *Psychotria brachypoda* (Müll. Arg.) Britton and *Psychotria birotula* Smith & Downs in two concentrations on the germination and cell division of *Eruca sativa* Hill. seeds. The biological assay was conducted in a controlled growth chamber. For monitoring the allelopathic effect, the following variables were evaluated: total number of germinated seeds, seedling root length, germination velocity index, and germination percentage. The means were compared using the Tukey test and orthogonal contrasts were undertaken to better compare the variables. To evaluate the antiproliferative and genotoxic effects, seedling roots were collected and the squashing technique was followed for preparation of slides. The results of the present study demonstrated that the medicinal species *Psychotria brachypoda* and *Psychotria birotula* inhibited root growth, germination velocity index, and germination percentage in seeds of arugula, in addition to inhibiting cell division and inducing chromosomal alterations in *Eruca sativa*. We conclude that the studied species have alellopathic, genotoxic, and antiproliferative effects on *Eruca sativa* in both concentrations studied.

Keywords: biological assays; alellopathy; *Psychotria brachypoda*; *Psychotria birotula*; *Eruca sativa*

Introduction

Brazil has a large diversity of plant species used in popular medicine due to the production of medicinal substances, which are most often the result of secondary metabolism in plants (Martins et al. 2000). These compounds participate in allelopathic activity and are present in all the plant's tissues, including leaves, flowers, fruits, roots, rhizomes, stems, and seeds (Gatti et al. 2004). It is considered that all plant organs have the potential to store allelochemicals, but the quantity and path by which they are emitted differs from species to species (Friedman 1995).

Currently, allelopathy has been recognized as an important ecological mechanism that influences the type of existing vegetation in an ecosystem, the dominance and succession of plants, the formation of communities, as well as crop management and productivity (Chou 1986, 1999).

For allelopathic studies, plants should be sensitive and have an effective response in a short time, even when low concentrations of allelochemicals are used. *Sorghum bicolor* L. (sorghum), *Cucumis sativus* L. (cucumber) (Belinelo et al. 2008), *Lactuca sativa* L. (lettuce), *Eruca sativa* Hill. (arugula) (Souza et al. 2005), and *Sesamum indicum* L. (sesame) (Oliveira 2009) have been cited in literature as good candidates.

Eruca sativa has a high photosynthetic capacity due to the large number of chloroplasts in the seed endosperm, allowing partial independence of the maternal tissues, in addition to reserve storages eventually accumulating in the embryo (Papini et al. 2010).

Biological assays for verifying allelopathy can be carried out on filter paper, inert substrates, hydroponics or soil (Weidenhamer et al. 1989), and the evaluated parameters are typically germination and root length (Belinelo et al. 2008). Besides germination, Souza et al. (2005) evaluated mitotic index (MI) and the germination velocity index (GVI).

Many medicinal species have been tested for their allelopathic effect, among them *Leucaena leucocephala* (Lam.) (Prates et al. 2000), *Cymbopogon citratus* (DC) Stapf., *Stevia rebbaudiana* (Bert) (Souza et al. 2005), *Arctium minus* (Hill) Bernh (Belinelo et al. 2008), *Solanum lycocarpum* A. St-Hil, *Solanum subumbellatum* Vell., *Solanum granuloso-leprosum* Dunal (Oliveira 2009), *Ruta graveolens* L. (De Feo et al. 2002), *Salvia officinalis* L. (Viecelli and Cruz-Silva 2009), and *Vernonia tweediana* Baker (Olguin et al. 2005).

Among the species of the genus *Psychotria* L., *P. brachypoda* (Müll. Arg.) Britton produces the alkaloid psycholatine, which contains a high pharmacological potential due to analgesic activity of the opioid,

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anxiolytic, and antipsychotic types, interacting with receptors of several neurotransmitter systems in the central nervous system (Fragoso 2007). Furthermore, *P. birotula* Smith & Downs produces the pyrrolidino indole alkaloids meso-chimonanthine and chimonanthine, also with pharmacological use (Brand et al. 2009).

Given the presence of alkaloids in *Psychotria brachypoda* and *P. birotula*, the aim of this study was to evaluate the allelopathic, genotoxic, and antiproliferative activity of different concentrations of extracts of *P. brachypoda* and *P. birotula* on the germination and cell division of *Eruca sativa* (arugula).

Materials and methods

Sampling of plant material

Plant material (leaves) of *Psychotria brachypoda* and *P. birotula* was collected in the municipality of Dom Pedro de Alcântara, Rio Grande do Sul State, Brazil, during the vegetative period of development, on 17 September 2010 and identified taxonomically (Mori et al. 1989). After sampling, the leaves were allowed to dry at room temperature for 90 days, for later preparation of extracts.

Extract preparation

Aqueous extracts were prepared by infusion in two concentrations (5 and 20 g l^{-1}) with dry leaves of *Psychotria brachypoda* and *P. birotula*. For the preparation of aqueous extracts, leaves were infused in boiling distilled water for 10 minutes, and then strained. The extracts were allowed to cool at room temperature before use in the biological assays.

Biological assay

In this study, for testing germination inhibition, rootlet growth, and analysis of cell division, seeds of *Eruca sativa* were used as test organisms. Seeds of *E. sativa* were distributed in 9 cm Petri dishes, lined with three sheets of filter soaked with 7 ml of each treatment and placed into plastic bags to prevent evaporation of the treatments. The treatments used were: T1 = distilled water, T2 = glyphosate 3%, T3 = *Psychotria brachypoda* extract at 5 g l^{-1}, T4 = *P. brachypoda* extract at 20 g l^{-1}, T5 = *P. birotula* extract at 5 g l^{-1}, and T6 = *P. birotula* extract at 20 g l^{-1}.

After soaking, the plates were kept in a growth chamber for 72 hours at 25°C and LD 16:8 photoperiod. Five replicates were used for each treatment, where each replicate contained 50 seeds in each Petri dish, in a completely randomized design.

Counts were carried out daily to calculate the GVI and compare the means of germinated seeds each 24 hours. For the calculation of germination inhibition, the count of the total number of seeds germinated at the end of 72 hrs was undertaken, followed by measuring their roots.

Mitotic index (MI)

After 72 hours of germination, the roots were collected and fixed in Carnoy's solution (3:1; ethanol: acetic acid) for 24 h at room temperature, then placed in 70% alcohol under refrigeration.

The squashing technique was used for slide preparation where the roots of *Eruca sativa* were placed in HCl 1N to hydrolyze for 5 min. Afterwards, the meristematic region was stained with 2% acetic orcein, and squashed with a glass rod over a coverslip (Guerra and Souza 2002). The slides were observed and analyzed using a LEICA microscope at 400×. A count was undertaken of all cells in division, and the occurrence of chromosomal alterations, and the mitotic index (MI) was calculated.

Two slides per replicate were analyzed, with 500 cells per slide and a total of 5000 cells per treatment. The MI was obtained by dividing the number of cells in division by the total number of cells and multiplying by 100.

Statistical analysis

The experimental design was completely randomized with six treatments and five replicates (each Petri dish was considered one replicate), and each replicate contained 50 seeds of *Eruca sativa*.

The means of the root lengths, the germination percentage, GVI, and the mean of the seeds germinated each 24 hrs were considered.

The data were tested for normality and transformed using the formula arcsine $\sqrt{x}/100$. The chi-square (χ^2) test was used for comparisons between the mitotic indices and the chromosomal alterations, using the program Bioestat 5.0 (Ayres et al. 2007), and the other data were submitted to ANOVA and compared with the Tukey test (5% probability) with the program Assistat version 7.6 Beta. The means of the germinated seeds in each treatment each 24 h were compared by orthogonal contrasts.

Results

In this study the effects of the aqueous extracts of the species *Psychotria brachypoda* and *P. birotula*, in two concentrations, on the seed germination and cell division of *Eruca sativa* were studied.

In Table 1 and Figure 1, the mean root length, GVI, and the percentage of germination in all treatments are presented. For the variable root length, all the treatments differed significantly from the negative control, including the positive control in glyphosate 3%, which was expected to decrease the root length of *Eruca sativa*.

The aqueous extracts of leaves of *Psychotria brachypoda* and of *P. birotula* interfered in the root development of *Eruca sativa*, with a higher inhibitory potential for the extracts of *Psychotria brachypoda*, and with an increasing concentration this effect was more damaging. For the species *P. birotula*, the effect did not vary

Table 1. Comparison of the mean root length, germination velocity index (GVI), and percentage of germination (%G) in each treatment.

Treatment	Mean of the root length	GVI	% G
Negative control	1.68 [a]	23.06 [a]	59.95 [a]
Positive control	0.78 [c]	6.84 [c]	16.66 [c]
Aqueous extract of *Psychotria brachypoda* 5 g l^{-1}	1.04 [bc]	21.93 [a]	54.94 [ab]
Aqueous extract of *Psychotria brachypoda* 20 g l^{-1}	0.85 [c]	18.98 [b]	50.59 [b]
Aqueous extract of *Psychotria birotula* 5g l^{-1}	1.17 [b]	21.34 [ab]	55.11 [ab]
Aqueous extract of *Psychotria birotula* 20 g l^{-1}	1.25 [b]	20.31 [ab]	54.36 [ab]
CV (%)	14.24975	7.63594	10.26165

Means followed by the same letter do not differ by the Tukey test at 5% probability of error.

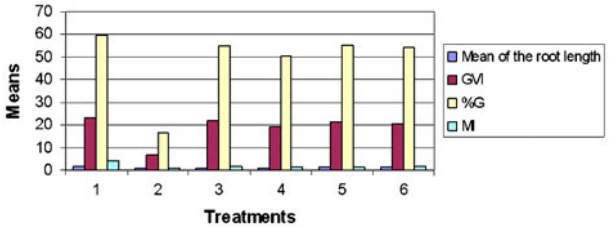

Figure 1. (Color online) Comparison of the mean root length, germination velocity index (GVI), percentage of germination (%G) and mitotic index (MI) in each treatment.

between the concentrations. Both *P. brachypoda* and *P. birotula* inhibited root growth in *Eruca sativa* (Table 1, Figure 1).

The GVI was not affected by applying the *Psychotria brachypoda* extract at 5 g l^{-1}; however, the concentration of 20 g l^{-1} had a negative effect on the GVI of seeds of *Eruca sativa*, while the extracts of *Psychotria birotula*, both at 5 g l^{-1} and 20 g l^{-1} inhibited the GVI of *Eruca sativa* without differences between the concentrations. Nevertheless, the extracts of the species in study can be considered allelopathic on the GVI of *Eruca sativa*; however, *Psychotria brachypoda* does not achieve the GVI in low concentrations and *P. birotula* presents an inhibitory effect in both the concentrations (Table 1, Figure 1).

The percentage of germination was affected by treatment with extracts of *Psychotria brachypoda* and *P. birotula*, where extracts of *P. brachypoda* demonstrated a higher potential in inhibiting germination when compared with the extracts of *P. birotula*. Still, for *P. brachypoda* the extract at the higher concentration was more efficient at inhibiting germination and for *P. birotula* there was no significant difference between the extracts at the different concentrations (Table 1, Figure 1).

Table 2 presents the values of the orthogonal contrasts, showing the differences between treatments, i.e. extracts of *Psychotria brachypoda* and *P. birotula* at the different concentrations and the negative and positive controls. All the treatments differed from one another, indicating that the extracts possess an allelopathic effect.

According to the contrasts there was an interaction between the six treatments and the three observation times, in other words, every 24 hours the behavior of each treatment changed (Table 2).

According to the orthogonal contrasts, there was no differences between the species under study; however, there was a difference between concentrations for both *Psychotria brachypoda* and *P. birotula*, where an increase in the concentration caused an increase in the allelopathic effect (Table 2, Figure 2).

In Table 3 the results for the phases of cell division of the analyzed cells are presented as well as the mitotic

Table 2. Comparison among the means of germination of the contrasts (c) between 24, 48, and 72 hours and the significance of the estimated contrast.

	Contrasts					
Treatment	C1	C2	C3	C4	C5	Means
Negative control	+5	0	0	0	0	30.73
Positive control	–1	+4	0	0	0	3.13
Aqueous extract of *Psychotria brachypoda* 5 g l^{-1}	–1	–1	+1	+1	0	28.00
Aqueous extract of *Psychotria brachypoda* 20 g l^{-1}	–1	–1	+1	–1	0	21.26
Aqueous extract of *Psychotria birotula* 5 g l^{-1}	–1	–1	–1	0	+1	26.53
Aqueous extract of *Psychotria birotula* 20 g l^{-1}	–1	–1	–1	0	–1	24.13
X 24 hours	7.9*	–10.8*	1.4 [ns]	9.8 [ns]	7.8*	—
X 48 hours	11.8*	–26.6*	–1.7 [ns]	6.8*	–1.4 [ns]	—
X 72 hours	10.5*	–28.1*	–1.8 [ns]	3.6 [ns]	0.8 [ns]	—

*Contrast between significant means by the F test at 5% of probability of error; ns between non-significant means by the F test at 5% of probability of error.

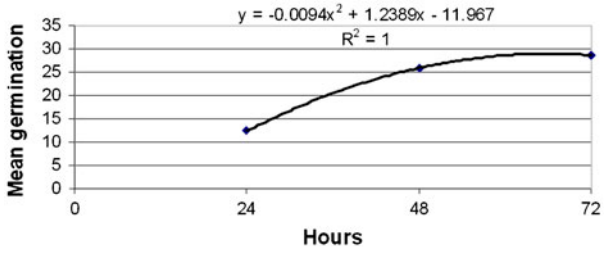

Figure 2. Regression analysis of seed germination in *Eruca sativa* over different time intervals (24, 48, and 72 hours).

index of each treatment. In this table it is possible to observe that all the treatments differ from the negative control (results for distilled water (T1) and the values for χ^2 are presented in Table 5). Glyphosate 3% was used as a positive control (T2) and was only significantly different from the negative control (T1), demonstrating that the extracts of *Psychotria brachypoda* and *P. birotula* possess antiproliferative effects (Table 3).

The treatments with the extract of *Psychotria brachypoda* at the concentrations of 5 and 20 g l^{-1} did not differ between each other and the same occurred for the extracts of *P. birotula* in both concentrations, where there was also not a difference between the extracts of the two species in study (Table 3). The comparison between the numbers of chromosomal alterations demonstrated that the only significant difference was between the positive and negative control (Table 4).

In the comparison between the treatments with the extracts of the species in study (T3XT4, T3XT5, T3XT6, T4XT5, T4XT6, and T5XT6), there was no difference and none of these treatments differed from the positive control. Like 3% glyphosate, the extracts from *Psychotria brachypoda* and *P. birotula* at 5 and 20 g l^{-1} showed genotoxic effects on the cell cycle of *Eruca sativa* (Table 4).

In the treatment with distilled water (T1 = negative control), no chromosomal alterations occurred, while the treatment with glyphosate 3% (T2 = positive control) had 25 chromosomal alterations. The other treatments presented the following number of chromosomal alterations: extract of *Psychotria brachypoda* at 5 g l^{-1} = 11 alterations, extract of *P. brachypoda* at 20 g l^{-1} = six alterations, extract of *P. birotula* at 5 g l^{-1} = 5 alterations, and extract of *P. birotula* at 5 g l^{-1} = six alterations (Table 4 and Figure 3).

Discussion

According to Souza Filho and Duarte (2007), allelopathy can have inhibitory or stimulatory effects, whereas in the present study the medicinal species *Psychotria brachypoda* and *P. birotula* demonstrated an inhibitory allelopathic effect on the seeds of *Eruca sativa.*

The extracts of *Psychotria brachypoda* and *P. birotula* interfered in the development of the roots of *Eruca sativa*, coinciding with the results observed by Stein et al. (2008) where the medicinal species *Plantago australis* Lam. and *Pl. brasiliensis* Sims. reduced the development of lettuce leaves. The essential oil of leaves of passion fruit also decreased the length of lettuce, tomato, and melissa roots (Rosado et al. 2005). The extracts of *Bidens pilosa* L. and *B. alba* (L.) DC inhibited the root

Table 3. Number of cells in interphase, mitosis, and mitotic (MI) index of root-tips of *Eruca sativa* for each treatment.

Treatment	Interphase	Prophase	Metaphase	Anaphase	Telophase	MI (%)
Negative control	4794	153	33	13	7	4.12 [a]
Positive control	4946	23	12	17	2	1.08 [b]
Aqueous extract of *Psychotria brachypoda* 5 g l^{-1}	4900	68	23	8	1	2 [b]
Aqueous extract of *Psychotria brachypoda* 20 g l^{-1}	4930	50	14	6	0	1.40 [b]
Aqueous extract of *Psychotria birotula* 5 g l^{-1}	4926	56	13	5	0	1.48 [b]
Aqueous extract of *Psychotria birotula* 20 g l^{-1}	4914	52	21	9	4	86 [b]

Means followed by the same letter do not differ in the chi-square test at 5% probability of error.

Table 4. Number of cells with chromosomal alterations in the root-tips of *Eruca sativa* for each treatment.

Treatment	Bridges in anaphase and telophase	Chromosomal breakage	Laggard chromosome	Total cells with alteration
Negative control	0	0	0	0 [a]
Positive control	0	12	13	25 [b]
Aqueous extract of *Psychotria brachypoda* 5g l^{-1}	5	4	1	11 [b]
Aqueous extract of *Psychotria brachypoda* 20g l^{-1}	3	2	1	6 [b]
Aqueous extract of *Psychotria birotula* 5g l^{-1}	2	0	3	5 [b]
Aqueous extract of *Psychotria birotula* 20g l^{-1}	3	0	3	6 [b]

Means followed by the same letter do not differ in the chi-square test at 5% probability of error.

Table 5. Chi-square (χ^2) values for the comparisons between the mitotic index (MI) of each treatment and between the amount of chromosomal alterations.

	χ^2 values	
Treatment	MI	Chromosomal alterations
T1×T2	91.234	24.938
T1×T3	37.878	10.988
T1×T4	68.917	5.996
T1×T5	64.021	4.998
T1×T6	50.798	5.996
T2×T3	13.955	5.425
T2×T4	2.090	11.609
T2×T5	3.166	13.294
T2×T6	7.418	11.609
T3×T4	5.386	1.468
T3×T5	3.954	2.246
T3×T6	1.074	1.468
T4×T5	0.113	0.091
T4×T6	1.667	0.000
T5×T6	0.915	0.091

growth of *Lactuca sativa* (Lima et al. 2011) and the leaf extracts of *Schinus terebinthifoIius* Raddi inhibited the root growth of *Brachiaria decumbens* (Forsski) Stapf. (Pessanha et al. 2010). *Bidens pilosa* and *Lactuca sativa* had their root and shoot growth affected by the extracts of *Plectranthus barbatus* Andrews (Azambuja et al. 2010), while the extracts of *Leucaena leucocephala* inhibited the root development of *Zea mays* L. (Prates et al. 2000; Pires et al. 2001).

Treatments with extracts of *Psychotria brachypoda* showed a lower germination percentage than treatments with the extracts of *P. birotula*, and the seeds of *Eruca sativa* suffered a more pronounced effect when treated with the extract of *Psychotria brachypoda* at 20 g l^{-1}. Still, the extracts of the species in study reduced the GVI of *Eruca sativa*, as *Psychotria brachypoda* does not achieve the GVI in low concentrations and *P. birotula* presents this effect at both concentrations.

Results of GVI inhibition and germination percentage were also observed by Mairesse et al. (2007) who observed a reduction in the percentage of germination of lettuce seeds with the use of extracts of *Amaranthus cruentes* L. (amaranth), *Blepharocalyx salicifolius* (Kunth) O. Berg (murta), *Casearia sylvestris* Sw. (crack-open), and *Grevillea banksii* R. Br. (kahiliflower). Borella and Pastorini (2009) reported that extracts of *Phytolacca dioica* L. also affect the percentage of germination, GVI, root length, shoot growth, fresh and dry weight, and this effect was enhanced with the increasing concentration of extracts.

The use of an ethanolic extract of *Eucalyptus citriodora* Hook. reduced the velocity of germination of *Bidens pilosa* (Ferreira et al. 2007). Also, an increase in the concentration of leaf extracts of *Schinus terebinthifoIius* inhibited the GVI of *Brachiaria decumbens* (Pessanha et al. 2010), and the extracts of *Plectranthus barbatus* reduced the GVI of seeds of *Bidens pilosa* and *Lactuca sativa* (Azambuja et al. 2010). The extracts of *Cymbopogon citratus* and *Stevia rebaudiana* reduced GVI in seeds of *Eruca sativa* and *Lactuca sativa* and the inhibitory effect was more pronounced with increasing concentration of extracts (Souza et al. 2005).

The different concentrations of *Psychotria brachypoda* and *P. birotula* have an antiproliferative effect on

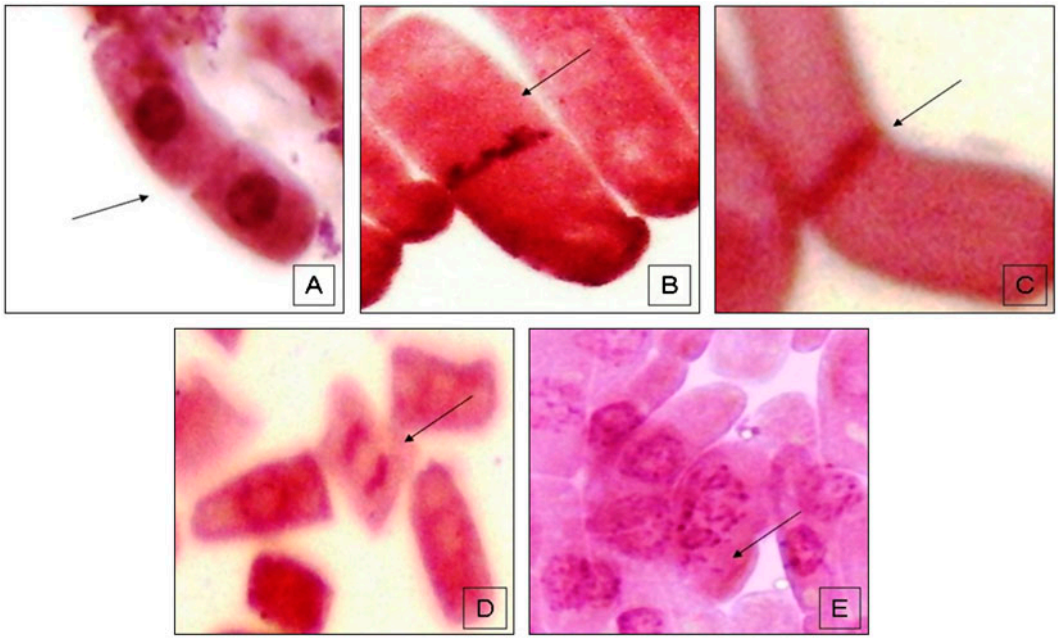

Figure 3. (Color online) Meristematic root cells of *Eruca sativa* submitted to treatments. (A) cells in interphase submitted to the treatment with the extract of *Psychotria brachypoda* at 5 g l^{-1}; (B, C) cells in metaphase submitted to the extract of *Psychotria brachypoda* at 20 g l^{-1}; (D) cell in telophase submitted to the extract of *Psychotria birotula* at 20 g l^{-1}; (E) cell in prophase irregular submitted to the extract of *Psychotria birotula* at 5 g l^{-1}.

the cell cycle of *Eruca sativa*, coinciding with the results observed by Souza et al. (2005) for the species *Cymbopogon citratus* on the cell cycles of *Eruca sativa* and *Lactuca sativa.* Furthermore, in a study carried out earlier by Prates et al. (2000), extracts of *Leucaena leucocephala* demonstrated an inhibitory effect both on root growth and MI of root cells in corn seedlings. *Psychotria brachypoda* and *P. birotula* showed antiproliferative and genotoxic effects on the cell cycle of *Eruca sativa.* Souza et al. (2005) reported that the medicinal species *Cymbopogon citratus* reduced MI in lettuce and arugula.

Other researchers have used plants for biomonitoring tests; among them Frescura et al. (2012) observed antiproliferative effects from the medicinal species *Luehea divaricata* Mart & Zucc. by the *Allium cepa* L. test. Fachinetto et al. (2007) found that *Achyrocline satureidoides* (Lam) DC has antiproliferative activity on the *Allium cepa* cell cycle, and Bakare et al. (2012) made observations indicating that an e-waste leachate contained substances capable of inducing cytotoxicity and somatic mutations in *Allium cepa*.

The elongation of the shoots and roots depends directly on cell division (mitosis), the formation of cambium and xylem vessels, and these structures are dependent on the partition of nutrients by the seedling (Hoffmann et al. 2007). Thus, it is understood that in the present study, the interference in the mitotic index also affected the development of roots, germination percentage, and the GVI of arugula seeds.

The allelopathic, genotoxic and antiproliferative effects can be associated with the facts that *Psychotria brachypoda* produces the indole alkaloid psycholatine (Fragoso 2007), and *P. birotula* produces the indole alkaloids meso-chimonanthine and chimonanthine (Brand et al. 2009). Since in the majority of the variables the inhibitory effect was more damaging at the higher concentrations, we believe it is due to the concentration of alkaloid present in the extract.

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