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Antiproliferative effect of the tree and medicinal species *Luehea divaricata* on the *Allium Cepa* cell cycle

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The medicinal and tree species *Luehea divaricata* is known as 'açoita-cavalo' and widely used for wood, reclamation, and in popular medicine. The aim of this study was to evaluate the antiproliferative and genotoxic effects of infusions of two populations of this species on the *Allium cepa* cell cycle. Cells of root tips of *Allium cepa* were used as an *in vivo* test system for monitoring the genotoxicity of this medicinal plant. Leaves and bark of two populations of *Luehea divaricata* were collected during the vegetative stage and used to prepare infusions in two concentrations: for the leaves (6 g/L and 30 g/L) and two concentrations for the bark (32 g/L and 160 g/L), using distilled water as negative control and glyphosate 3% as positive control. For this study, 10 groups of four bulbs were utilized, with one group of bulbs for each treatment. The slides were prepared by the squashing technique, scoring 4000 cells for each group of bulbs. The mitotic index (MI) was calculated and then a statistical analysis was performed using chi-square (χ^2). The results showed that *Luehea divaricata* infusions in both populations caused a reduction of MI compared to control, and in both analyzed concentrations there was no significant genotoxic effect in comparison to the negative control, however there was a significant difference in relation to the positive control for both populations. The antiproliferative effect of leaf extracts increased with a greater concentration and among bark extracts no significant difference occurred between the two concentrations. The studied populations did not show genetic variability regarding the antiproliferative effect.

Keywords: *Allium cepa*; Genotoxicity; *Luehea divaricata*

Introduction

The economic potential of native Brazilian medicinal species is huge, such that these species are considered a resource to be preserved and utilized. Studies characterizing the germplasm of species are essential to optimize the use of native medicinal resources of a country and conserve the available plant genetic diversity (Pereira *et al*. 2006).

we have *Luehea divaricata* Martius (Tiliaceae), commonly known as 'açoita-cavalo' (Pio Corrêa 1984; Alice et al 1995) is one of the numerous plant species found in Brazil. *Luehea divaricata* is native to the Brazilian states: Bahia, Rio de Janeiro, São Paulo, Minas Gerais, Goiás, Mato Grosso do Sul, Paraná, Santa Catarina (SC), and Rio Grande do Sul (RS) (Alice et al. 1995). In a study by Vaccaro and Longhi (1995), a floristic description of some of the remaining forest areas of the Upper Uruguay-RS were undertaken and one of the most important species was *L. divaricata*. Furthermore, Ruschel *et al*. (2003) carried out a study on the diversity of species in forests used commonly for timber in the High Uruguay-SC region and *L. divaricata* was one of the most dominant species.

The 'açoita-cavalo' is a pioneering tree in various forest formations. A heliophytic and selective hygrophilic species, it is characteristic of alluvial forests where it can be very frequent or even abundant. In the dense and high forests, especially in terrains with gentle slopes and deep soils, the species can be rare or absent altogether. In rocky soils and steep slopes at the top, it reappears with high frequency, demonstrating that the species has two distinct ecological environments for its development in the primary forest. Also, it is very common in semi-devastated woods and brushwoods (Reitz *et al*. 1988).

According to Reitz *et al*., 1988 and Lorenzi (1992) the 'açoita-cavalo' is considered one of the 55 most important trees for reforestation of degraded areas as well as *Blepharocalyx salicifolius* (HB & K) Berg., *Cedrela fissilis* L, *Diatenopteryx sorbifolia* Radlk., *Enterolobium contortisiliquum* (Vell.) Morong, *Myrocarpus frondosus*, *Paretecoma peroba* (Record & Mell) Kuhlm., *Scutia buxifolia* Reiss., 'salveiro', and *Tabebuia avel-*

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lanedae Lorentz ex Griseb. Furthermore, this species has a great medicinal value, with anti-inflammatory, anti-anemic, diuretic, and mouth aseptic effects by the infusion of the leaves and decoction of the stem (Pio Corrêa 1984; Alice *et al*. 1995).

The phytochemical studies of the species indicate that this plant has a large amount of tannins and monoterpenes, both in the bark and in the stem (Lorenzi and Matos 2002). Bortoluzzi *et al*. (2002 apud Müller 2006), in phytochemical analysis of *Luehea divaricata* leaves observed the presence of flavonoids, saponins, tannins and mucilage catechisms, and according to Souza *et al*. (2005), the extracts of this species are potentially allelopathic.

The consumption of tea by the population can suppress the effects of mutagens that are acting on the human organism (Silva *et al*. 2004). However, Vicentini *et al*. (2001) reported that teas and herbal infusions might contain toxic substances with mutagenic effects. Due to the heavy use of medicinal plants, toxicity and mutagenicity studies are needed to contribute to a safe and effective use.

Mitotic and replication indexes are used as indicators of adequate cell proliferation (Gadano *et al*. 2002), which can be measured through the plant test system of *Allium cepa* L. (Fachinetto and Tedesco 2009), and the method of chromosome aberration in roots of *A. cepa* is validated by the International Program on Chemical Safety (IPCS, WHO) and the United Nations Environmental Programme (UNEP) as an efficient test for the analysis and monitoring *in situ* of the genotoxicity of environmental substances (Cabrera and Rodriguez 1999; Silva *et al*. 2004).

Several factors influence the production of secondary metabolites of medicinal plants, whose synthesis is frequently affected by environmental conditions. Thus, variations in the overall index and/or relative proportions of secondary metabolites in plants may occur and Gobbo-Neto and Lopes (2007) cite the main factors that can alter the production or concentration of secondary metabolites in plants, such as seasonality, circadian rhythms, development stage, temperature, water availability, UV radiation, soil nutrients, altitude, and atmospheric composition. Besides these, we emphasize that genetic differences can be found among the different populations studied.

The aim of this study was to evaluate the anti-proliferative and genotoxic effects of the infusions of two populations of the tree and medicinal species *Luehea divaricata*, using the plant test system *Allium cepa.*

Materials and methods

Leaves and bark of two populations of *Luehea divaricata* were collected in the municipality of São Francisco de Assis, Rio Grande do Sul, Brazil. Being population 1 (Pop 1) in São José do Inhandijú, brushwood region (29”29’29.49”S and 54”55’13.67”O), and population 2 (Pop 2) in Passo do Leão, on a riverbank (29”32’18.65”S and 55”01’16.85”O). The main difference between the sampling sites was due to greater availability of water at the site where population 2 was collected. The material of each population was collected during the vegetative period of development.

The leaves and bark of each population remained in room temperature for a 90-day period, and then aqueous extracts (teas) were prepared. Aqueous extracts were prepared from the leaves through infusion for 10 minutes in two concentrations for each population at 6 and 30 g/L. The use of these concentrations was based on general human consumption and 5 times more concentrated, to simulate the highest consumption, because people tend to prepare teas in more concentrated doses for it to have a stronger effect, without knowing the true effect based on scientific data, only on popular beliefs.

Using the bark of *Luehea divaricata,* aqueous extracts were prepared by decoction for 10 minutes at concentrations of 32 and 160 g/L for each population.

The meristematic cells of *Allium cepa* were used to assess the morphological and structural cell alterations and to determine the mitotic index (MI). Ten groups of 4 bulbs were placed to root in water, which constituted 10 treatments, each with 4 replicates. The following treatments were used: T1- distilled water (negative control); T2- glyphosate 3% (positive control); T3- infusions of leaves of *Luehea divaricata* (6 g/L) of Pop 1; T4- infusions of leaves of *Luehea divaricata* (30 g/L) of Pop 1; T5- infusion of leaves of *Luehea divaricata* (6 g/L) of Pop 2; T6- infusion of leaves of *Luehea divaricata* (30 g/L) of Pop 2; T7- decoction of bark of *Luehea divaricata* (32 g/L) of Pop 1; T8- decoction of bark of *Luehea divaricata* (160 g/L) of Pop 1; T9- decoction of bark of *Luehea divaricata* (32 g/L) of Pop 2; and T10- decoction of bark of *Luehea divaricata* (160 g/L) of Pop 2.

The protocol used followed the methodology by Fiskesjö (1993) with modifications used by Knoll *et al*. (2006). The bulbs were grown 4 days before exposure to treatments and not 3; since there is variation in rootlet growth in onions, we allowed all bulbs to achieve a minimum of 0.5cm before being placed in the treatments.

After 4 days, the negative control group (T1) remained in water and the other groups of onion bulbs were subject to different treatments for 24 hours more and then, the roots were collected and fixed in 3:1 fixative (ethanol: acetic acid) for a 24-hour period and stored in 70% ethanol under refrigeration.

The positive control used was glyphosate, as it is shown to induce chromosomal alterations in meristematic cells of *Allium cepa* (Souza *et al*. 2010).

For analysis of cell division of the onion bulb roots, rooted in water, and subjected to the different treatments, rootlets were collected with approximately 2 cm, which were hydrolyzed in HCl 1N for 5 minutes, using the meristematic region only, and were stained with 2% acetic orcein (adapted from Guerra and Souza, 2002). The meristematic region was squashed with the aid of a glass rod and the material was placed on a slide. The slides

were observed under the microscope at 400X magnification and analyzed. The total count of cells was made in division, occurrence of chromosomal alterations, and the MI was calculated.

The MI was obtained by dividing the number of cells in division by the total number of cells observed and multiplied by 100. 1000 cells were counted per bulb, preparing 2 slides for each bulb, counting 500 cells per slide totaling in 4000 cells observed per group of bulbs for each of the treatments.

The statistical analysis was performed by the Chi-square test (x^2), with the aid of the program BIOESTAT 5.O (Ayres *et al*. 2007).

Results

Table 1 shows the number of cells observed in interphase and in different phases of division during the cell cycle of *Allium cepa*, as well as the values of MI, for 4000 cells analyzed. It was also observed that MI values did not differ between the two populations of *Luehea divaricata*, and when compared to the negative control, there was inhibition of cell division.

When comparing the leaf extract of Pop 1 and Pop 2 in lower concentrations, T3 and T5 ($x^2 = 0.389$) and the highest concentration, T4 and T6 ($x^2 = 0.729$) it was observed that these did not differ significantly, as also seen in the bark extract of Pop 1 and Pop 2 in the lower concentration, T7 and T9 ($x^2 = 1.002$) and in the higher concentration, T8 and T10 ($x^2 = 1.000$) which also did not differ significantly.

The leaf extracts of Pop 1 in the concentration of 6 g/L differed significantly from the extract at 30 g/L of the same population ($x^2 = 57.278$), and in Pop 2, the leaf extracts in the concentration of 6 g/L also differed significantly from the extract at 30 g/L ($x^2 = 56.405$).

For the bark extract of *Luehea divaricata* of Pop 1, no significant difference occurred between concentrations of 32 g/L and 160 g/L ($x^2 = 6.005$). The same occurred for the bark extracts of Pop 2 in concentrations of 32 g/L and 160 g/L ($x^2 = 7.374$).

When comparing the extracts of leaf and bark of Pop 1 (T3 and T7), in concentrations of 6 g/L and 32 g/L respectively, a significant difference between these extracts was observed ($x^2 = 64.366$), being that a difference was also observed for T5 and T9, of Pop 2 ($x^2 = 62.852$). However, when comparing the results obtained for the extracts of leaves (30 g/L) and of bark (160 g/L) of Pop 1 (T4 and T8: $x^2 = 9.010$) and of Pop 2 (T6 and T10: $x^2 = 10.304$) no significant difference between the extracts was observed.

The positive control (T2) and all the other treatments (T3 to T10) differed significantly from the negative control (T2: $x^2 = 26.781$; T3: $x^2 = 63.405$; T4: $x^2 = 193.857$; T5: $x^2 = 54.632$; T6: $x^2 = 183.176$; T7: $x^2 = 202.215$; T8: $x^2 = 219.882$; T9: $x^2 = 191.138$; T10: $x^2 = 216.846$). Furthermore, T3 and T5 did not differ from the positive control regarding MI, being $x^2 = 0.0132$ and $x^2 = 0.0668$ respectively.

The positive control presented 53 cells with aberrations, being 22 bridges during anaphase/telophase (Figure 1A and 1B), 8 cells with chromosomal breakage (Figure 1C), and 23 with laggard chromosomes (Figure 1D).

The leaf extracts at the concentration of 6 g/L of Pop 1 showed only 1 cell with a laggard chromosome and at 30 g/L showed 1 cell with a micronucleus (Table 2).

In Table 2, the leaf extracts of Pop 2 at the concentration of 6 g/L presented 5 cells with chromosomal aberrations, 3 having anaphasic bridges, 1 a laggard chromosome, and 1 a micronucleus. At 30 g/L, there was only 1 cell with a laggard chromosome.

There were no aberrations for the bark extract of Pop 1 at both 32 g/L and 160 g/L. However, for Pop 2, at the concentration of 32 g/L there was 1 laggard chromosome, and at 160 g/L, there were no aberrations.

In Table 2, the number of cells that presented chromosomal aberrations and the types of aberrations that occurred in each of the treatments for 4000 analyzed cells are presented.

When comparing the aberrations of treatments T2 to T10 with the negative control (T1), the following values were obtained for x^2: T2 = 52.653; T3 = 1.000; T4 = 1.000; T5 = 4.997; T6 = 1.000; T7 = 0; T8 = 0;

Table 1. Number of cells in interphase and mitosis, as well as the mitotic index of root-tips of *Allium cepa* for each treatment.

Treatment		Interphase	Prophase	Metaphase	Anaphase	Telophase	MI (%)
Distilled Water*	T1	3786	115	40	25	34	5.35[a]
Glyphosate **	T2	3872	25	56	27	13	3.02[b]
Leaf extracts at 6 g/L (Pop 1)	T3	3920	43	17	08	12	2.00[b]
Leaf extracts at 30 g/L (Pop 1)	T4	3991	04	02	0	03	0.22[c]
Leaf extracts at 6 g/L (Pop 2)	T5	3912	23	27	20	18	2.20[b]
Leaf extracts at 30 g/L (Pop 2)	T6	3987	07	03	02	01	0.32[c]
Bark extracts at 32 g/L (Pop 1)	T7	3994	06	0	0	0	0.15[c]
Bark extracts at 160 g/L (Pop 1)	T8	4000	0	0	0	0	0.00[c]
Bark extracts at 32 g/L (Pop 2)	T9	3990	03	04	02	01	0.25[c]
Bark extracts at 160 g/L (Pop 2)	T10	3999	0	01	0	0	0.2[c]

* Negative control
** Positive control

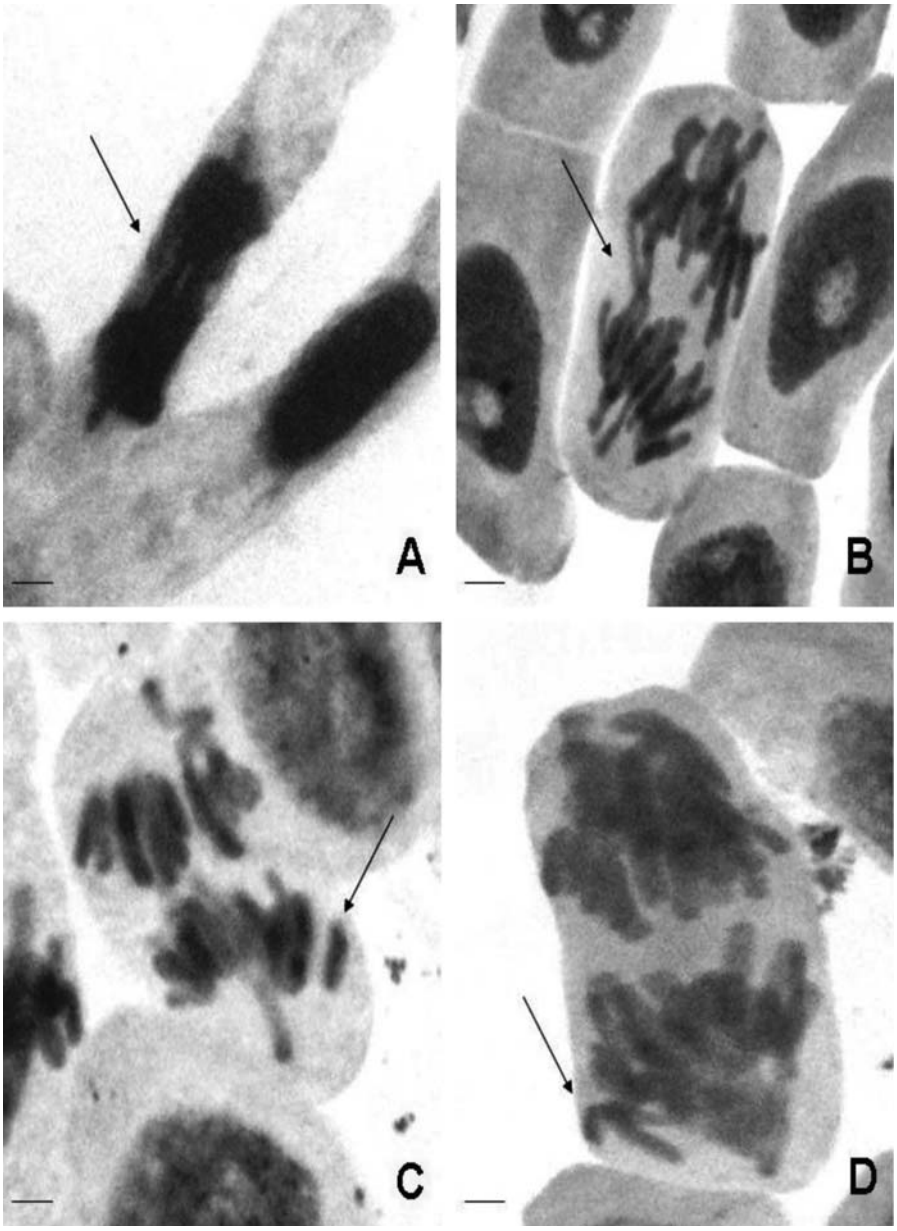

Figure 1. Cells with arrows indicating observed aberration in the treatment with glyphosate 3% (positive control). A) Cell in telophase with a bridge; B) Cell in anaphase with a bridge; C) Cell in anaphase with chromosomal breakage; D) Cell in anaphase with laggard chromosome. Scale = 10 μm.

T9 = 1.000; and T10 = 0. Where the negative control differed only from the positive control and none of the treatments from the extracts differed from the negative control. All the treatments differed from the positive control, where the χ^2 values were as follows: T3 = 49.740; T4 = 49.740; T5 = 39.440; T6 = 49.740; T7 = 52.653; T8 = 52.653; T9 = 49.740, and T10 = 52.653.

Comparing the number of aberrations in each treatment, these do not differ among each other: T3 and T4: $\chi^2 = 0$; T3 and T5: $\chi^2 = 2.665$; T3 and T6: $\chi^2 = 0$; T3 and T7: $\chi^2 = 1.000$; T3 and T8: $\chi^2 = 1.000$; T3 and T9: $\chi^2 = 0$; T3 and T10: $\chi^2 = 1.000$; T4 and T5: $\chi^2 = 2.665$; T4 and T6: $\chi^2 = 0$; T4 and T7: $\chi^2 = 1.000$; T4 and T8: $\chi^2 = 1.000$; T4 and T9: $\chi^2 = 0$; T4 and T10: $\chi^2 = 1.000$; T5 and T6: $\chi^2 = 2.665$; T5 and T7: $\chi^2 = 4.997$; T5 and T8: $\chi^2 = 4.997$; T5 and T9: $\chi^2 = 2.665$; T5 and T10: $\chi^2 = 4.997$; T6 and T7: $\chi^2 = 1.000$; T6 and T8: $\chi^2 = 1.000$; T6 and T9: $\chi^2 = 0$; T6 and T10: $\chi^2 = 1.000$; T7 and T8: $\chi^2 = 0$; T7 and T9: $\chi^2 = 1.000$; T7 and T10: $\chi^2 = 0$; T8 and T9: $\chi^2 = 1.000$; T8 and T10: $\chi^2 = 0$; and T9 and T10: $\chi^2 = 1.000$.

Discussion

In this study, to evaluate the antiproliferative and genotoxic effects of the extracts of two populations of *Luehea divaricata*, commonly known as 'açoita-cavalo', we

Table 2. Number of cells with chromosomal aberrations for each treatment.

Treatment		Anaphasic and telophasic bridges	Chromosomal breakage	Laggard chromosome	Cells with micronucleus	Total cells with aberrations
Distilled Water*	T1	0	0	0	0	0 [a]
Glyphosate **	T2	22	08	23	0	53 [b]
Leaf extracts at 6 g/L (Pop 1)	T3	0	0	01	0	01 [a]
Leaf extracts at 30 g/L (Pop 1)	T4	0	0	0	01	01 [a]
Leaf extracts at 6 g/L (Pop 2)	T5	03	0	01	01	05 [a]
Leaf extracts at 30 g/L (Pop 2)	T6	0	0	01	0	01 [a]
Bark extracts at 32 g/L (Pop 1)	T7	0	0	0	0	0 [a]
Bark extracts at 160 g/L (Pop 1)	T8	0	0	0	0	0 [a]
Bark extracts at 32 g/L (Pop 2)	T9	0	0	01	0	01 [a]
Bark extracts at 160 g/L (Pop 2)	T10	0	0	0	0	0 [a]

* Negative control
** Positive control

applied the plant test system *Allium cepa*. The *A. cepa* test has been used by various researchers that carry out this test together with tests in animals and the results obtained are similar (Teixeira *et al*. 2003; Vicentini *et al*. 2001), demonstrating its effectiveness. In addition, the *A. cepa* test is considered an excellent bioindicator for the first screening of genotoxicity of medical plants, being a low cost test, reliable, and agreeing with other genotoxicity tests (Bagatini *et al*. 2007).

The results of this study showed that leaf and bark extracts of the studied *Luehea divaricata* populations exhibit antiproliferative effects, that is, they possess activity capable of inhibiting cell division.

The leaf extracts of the populations demonstrate a dose dependent inhibitory effect on the MI with the increase of extracts from 6 g/L to 30 g/L. However, for the bark extracts of both Pop 1 and 2, even though there was an increase in the concentration, there was not a significant increase in inhibition, where both the concentrations 32 g/L and 160 g/L showed high reduction in the MI of the meristematic root-tips of *Allium cepa.*

In these concentrations of tea, the presumed compounds responsible for inhibiting the cell cycle do not present mutagenic effects, and in other species such as *Solidago microglossa* DC and *Pterocaulon polystachyum* DC studied by Bagatini *et al.* (2009) and Knoll *et al*. (2006), flavonoids did not demonstrate mutagenecity, but antiproliferative capacity, showing that they could assist in the anti-aging process, inhibiting cell division.

The leaf and bark extracts of both populations 1 and 2 presented few chromosomal abnormalities, not differing significantly from the negative control, which preliminary demonstrates the safety in using these teas at the concentrations of 6 g/L and 30 g/L for the leaves and 32 g/L and 160 g/L for the bark, though in higher concentrations there can be different genotoxic effects.

There were also chromosomal abnormalities observed in the positive control, glyphosate (Table 2). A study of another herbicide, Avenoxan, whose active ingredient is 2,4-D was undertaken by Gul *et al*. (2006) on its cytogenetic effects on *Allium cepa* and *Allium sativum* L. Two concentrations of the herbicide were used and induced chromosomal abnormalities, such as laggard chromosomes, multipolar anaphase, multinuclei, stickyness, anaphase and telophase bridges, and chromosomal fragments.

There was not a significant difference between the two studied populations, indicating that the production of medicinal substances, which according to Martins *et al*. (2000) are most often resultant of plant secondary metabolism, did not differ between Pop 1 and Pop 2, even with these populations being from different locations and habitats. The same result was reported by Fachinetto and Tedesco (2009) when evaluating two different populations of *Baccharis trimera* (Less) DC and *Baccharis articulata* (Lam) Pers., finding that the results of cell inhibition and genotoxic effects did not differ between the populations, where only one population had a difference in MI among the concentrations.

Luehea divaricata has tannins and flavonoids, among other chemicals in its chemical composition. Therefore, inhibition of cell division may be due to the presence of tannins and flavonoids, according to Teixeira *et al*. (2003), which comment that enzymatic activity associated with tannins may be responsible for inhibiting the cell division in *Allium cepa*. Furthermore, Fachinetto *et al*. (2007) found that *Achyrocline satureidoides* (Lam) DC has antiproliferative activity on the *Allium cepa* cell

cycle, and this species has tannins and flavonoids in its chemical composition, as well as *L. divaricata*.

Another example is the medicinal species *Pterocaulon polystachyum* studied by Knoll *et al*. (2006), containing tannins and flavonoids in its chemical composition, and presenting antiproliferative activity on the *Allium cepa* cell cycle.

Sultan and Çelik (2009) investigated the genotoxic and antimutagenic extracts of the species *Capparis spinosa* L., because the aerial parts of this plant are widely used. The authors comment that in evaluating the effect of the complex mixture of the extracts of *C. spinosa* on *Allium cepa* cells, they found the aqueous extract not being genotoxic, though potentially antimutagenic.

In this study, we can only indirectly assign the action of the chemical compounds present in both extracts of *Luehea divaricata* used and tested with the *Allium cepa* test. However, we suggest that in future work the extraction of each of the products be carried out and analyzed. Although, in the case of aqueous extracts (teas), we hypothesize that the predominant substances are responsible for the inhibitory action on cell division.

Cragg and Newman (2005) reported that many of the agents used in cancer therapy are derived from natural sources and were discovered from cytoxicity tests, by inhibiting cancer cell proliferation in models *in vitro* or *ex vitro*. Therefore, it is of great importance to perform further studies on medicinal species that inhibit cell cycles, which is the case of *Luehea divaricata*, which demonstrates this capability especially with the high rate of MI inhibition on the *Allium cepa* cell cycle using the bark extracts.

Conclusions

It is concluded through the results of this study that the leaf and bark extracts of two populations of *Luehea divaricata* in concentrations of 6 g/L and 30 g/L for the leaves, and 32 g/L and 160 g/L for the bark, have antiproliferative action and do not show genotoxic effects on the cell cycle of *Allium cepa*. The extracts of the leaves have a dose dependent antiproliferative activity with increasing concentration of the extract, indicating possibilities of its use in the preparation of antitumor medicaitons. This species has to be preserved due to its high value in the recovery of degraded areas and their medicinal potential.

The extracts of the leaves have a dose dependent antiproliferative activity with increasing concentration of the extract, indicating possibilities of its use in the preparation of antitumor medications. Due to its therapeutic value, this species should be protected.

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