

## Allelopathy, toxicity and phytochemical profile of aqueous extracts from *Aspidosperma pyriforme* and *Combretum leprosum*

Alelopatia, toxicidade e perfil fitoquímico de extratos aquosos de *Aspidosperma pyriforme* e *Combretum leprosum*

Alelopatía, toxicidad y perfil fitoquímico de extractos acuosos de *Aspidosperma pyriforme* y *Combretum leprosum*

Received: 04/01/2021 | Reviewed: 04/08/2021 | Accept: 04/11/2021 | Published: 04/23/2021

**José Carlos da Silveira Pereira**

ORCID: <https://orcid.org/0000-0002-1813-0247>  
Faculdade de Enfermagem Nova Esperança de Mossoró, Brazil  
E-mail: [carlosbiotech@gmail.com](mailto:carlosbiotech@gmail.com)

**Karina Maia Paiva**

ORCID: <https://orcid.org/0000-0001-8492-9375>  
Universidade do Estado do Rio Grande do Norte, Brazil  
E-mail: [karinampaiva@hotmail.com](mailto:karinampaiva@hotmail.com)

**Naama Jessica de Assis Melo**

ORCID: <https://orcid.org/0000-0002-1437-3436>  
Universidade Federal Rural do Semi-Árido, Brazil  
E-mail: [naama.melo@ufersa.edu.br](mailto:naama.melo@ufersa.edu.br)

**Tallyson Nogueira Barbosa**

ORCID: <https://orcid.org/0000-0002-4502-4220>  
Universidade Federal de Pelotas, Brazil  
E-mail: [tallyson\\_n\\_b@hotmail.com](mailto:tallyson_n_b@hotmail.com)

**Patrícia Lígia Dantas de Morais**

ORCID: <https://orcid.org/0000-0001-9317-1164>  
Universidade Federal Rural do Semi-Árido, Brazil  
E-mail: [plmorais@hotmail.com](mailto:plmorais@hotmail.com)

**Juliana Rocha Vaez**

ORCID: <https://orcid.org/0000-0003-1298-0395>  
Universidade Federal Rural do Semi-Árido, Brazil  
E-mail: [juaeaz@ufersa.edu.br](mailto:juaeaz@ufersa.edu.br)

**Salvador Barros Torres**

ORCID: <https://orcid.org/0000-0003-0668-3327>  
Universidade Federal Rural do Semi-Árido, Brazil  
E-mail: [sbtorres@ufersa.edu.br](mailto:sbtorres@ufersa.edu.br)

**Marcos Antonio Nobrega de Sousa**

ORCID: <https://orcid.org/0000-0001-6550-6609>  
Universidade Federal de Campina Grande, Brazil  
E-mail: [marcosandesousa@gmail.com](mailto:marcosandesousa@gmail.com)

### Abstract

This study characterizes the allelopathic effect in the germination of *Allium cepa* seeds, and toxic on the species *Artemia salina*, of aqueous extracts of leaves of *Aspidosperma pyriforme* and *Combretum leprosum* and the constituent phytochemical compositions. For this, *Allium cepa* seeds were germinated in systems containing aqueous extracts (200, 400 and 800 mg.L<sup>-1</sup>) and distilled water, to evaluate aspects of germination, mitotic phases, mitotic index and limit value of cytotoxicity. The toxicity of the aqueous extracts was evaluated in *Artemia salina*. The extracts were evaluated qualitatively and quantitatively when the substances present to define the phytochemical profile. The aqueous extract of *A. pyriforme* negatively affects the germination process in the hypocotyl and seedling growth at 800 mg.L<sup>-1</sup>. The LC<sub>50</sub> found for the aqueous extract of *A. pyriforme* was 4986 mg.L<sup>-1</sup>. The effect of *C. leprosum* extract on germination resulted in an increase in the dry matter of the root at 400 mg.L<sup>-1</sup> and in the density of the dry matter of the root at 800 mg.L<sup>-1</sup>. In addition, it reduces the seedling matter at 200 mg.L<sup>-1</sup>, corresponding to the trend observed in the mitotic index, in which this concentration presented a sublethal score for the limit value of cytotoxicity. The maximum concentration evaluated was not sufficient to determine an LC<sub>50</sub> in *A. salina*. The phytochemical profiles of both species demonstrated classes of substances with potential pharmacological application. This information is important because these species are commonly used as food for farm animals and for purposes in folk medicine.

**Keywords:** Mofumbo; Pereiro; Caatinga; Metabolites.

### Resumo

Este estudo caracteriza o efeito alelopático, na germinação de sementes de *Allium cepa*, e tóxico, sobre a espécie *Artemia salina*, de extratos aquosos de folhas de *Aspidosperma pyriforme* e *Combretum leprosum* e as composições fitoquímicas constituintes. Para isso, sementes de *Allium cepa* foram germinadas em sistemas contendo extratos aquosos (200, 400 e 800 mg.L<sup>-1</sup>) e água destilada, para avaliar aspectos de germinação, fases mitóticas, índice mitótico e valor limite de citotoxicidade. A toxicidade dos extratos aquosos foi avaliada em *Artemia salina*. Os extratos foram avaliados qualitativa e quantitativamente quando as substâncias presentes para definir o perfil fitoquímico. O extrato aquoso de *A. pyriforme* afeta negativamente o processo de germinação no hipocótilo e no crescimento de plântulas a 800 mg.L<sup>-1</sup>. A CL<sub>50</sub> encontrado para o extrato aquoso de *A. pyriforme* foi de 4986 mg.L<sup>-1</sup>. O efeito do extrato de *C. leprosum* na germinação resultou no aumento da matéria seca da raiz a 400 mg.L<sup>-1</sup> e na densidade da matéria seca da raiz a 800 mg.L<sup>-1</sup>. Além disso, reduz a matéria de plântula a 200 mg.L<sup>-1</sup>, correspondendo à tendência observada no índice mitótico, em que essa concentração apresentou escore subletal para o valor limite de citotoxicidade. A concentração máxima avaliada não foi suficiente para determinar um CL<sub>50</sub> em *A. salina*. Os perfis fitoquímicos de ambas espécies demonstraram classes de substâncias com potencial aplicação farmacológica. Esta informação é importante porque essas espécies são comumente usadas como alimento para animais de produção e para fins na medicina popular.

**Palavras-chave:** Mofumbo; Pereiro; Caatinga; Metabólitos.

### Resumen

Este estudio caracteriza el efecto alelopático, en la germinación de semillas de *Allium cepa*, y tóxico, sobre la especie *Artemia salina*, de extractos acuosos de hojas de *Aspidosperma pyriforme* y *Combretum leprosum* y las composiciones fitoquímicas constituyentes. Para ello se germinaron semillas de *Allium cepa* en sistemas que contienen extractos acuosos (200, 400 y 800 mg.L<sup>-1</sup>) y agua destilada, para evaluar aspectos de germinación, fases mitóticas, índice mitótico y valor límite de citotoxicidad. Se evaluó la toxicidad de los extractos acuosos en *Artemia salina*. Los extractos fueron evaluados cualitativa y cuantitativamente cuando las sustancias presentes para definir el perfil fitoquímico. El extracto acuoso de *A. pyriforme* afecta negativamente el proceso de germinación en el hipocótilo y crecimiento de plántula a 800 mg.L<sup>-1</sup>. La CL<sub>50</sub> encontrada para el extracto acuoso de *A. pyriforme* fue de 4986 mg.L<sup>-1</sup>. El efecto del extracto de *C. leprosum* sobre la germinación resultó en un aumento de la materia seca de la raíz a 400 mg.L<sup>-1</sup> y en la densidad de la materia seca de la raíz a 800 mg.L<sup>-1</sup>. Además, reduce la materia de plántula a 200 mg.L<sup>-1</sup>, correspondiente a la tendencia observada en el índice mitótico, en el cual esta concentración presentó un puntaje subletal para el valor límite de citotoxicidad. La concentración máxima evaluada no fue suficiente para determinar una CL<sub>50</sub> en *A. salina*. Los perfiles fitoquímicos de ambas especies demostraron clases de sustancias con potencial aplicación farmacológica. Esta información es importante porque estas especies se utilizan comúnmente como alimento para animales de granja y con fines de medicina popular.

**Palabras clave:** Mofumbo; Pereiro; Caatinga; Metabolitos.

## 1. Introduction

The dissemination of the therapeutic characteristics of plants is mainly due to popular observations of the use and effectiveness of these plants, even without the knowledge of their chemical constituents. The interest of researchers in various areas, such as botany, pharmacology and phytochemistry, is therefore aroused, which, together, increase the information on natural medicine, such as flora in the Brazilian semi-arid region (Maciel et al., 2002; Melo et al., 2017). Caatinga, an exclusively Brazilian biome, has a semi-arid, hot and dry climate, basically resulting in xerophytic vegetation (Almeida et al., 2005), with many endemic plant species in the biome (Melo & Andrade, 2007).

Many of the plant species may have potential phytochemical and pharmacological, but require scientific studies to prove these biological activities. Therefore, plant bioassays can be used for monitoring of the bioactivity of extracts, fractions and isolated compounds of plants (Noldin et al., 2003). The study of these effects on other plants is called allelopathy, which analyzes the easily visible effects of allelochemicals on the growth and development of plants (Bhadoria, 2011). For this, the germination test with vegetables is a model widely used to assess the potential allelochemical of extract or isolated substances, so that when a compound interferes with the cellular metabolism, for example, include reduced germination index; seeds darkened and swollen; reduced root or radicle and shoot or coleoptile extension; swelling or necrosis of root tips; curling of the

root axis; discoloration, lack of root hairs; increased number of seminal roots; reduced dry weight accumulation; and lowered reproductive capacity. They can reveal its toxic and/or cytotoxic action (Bhadoria, 2011; Luz et al., 2012).

*Aspidosperma pyrifolium* Mart. (Apocynaceae), known as pereiro, have your extracts popularly used by humans in cases of heart problems, diarrhea and as a sedative (Almeida et al., 2005), anti-inflammatory urinary tract disease (Agra et al., 2007), visceral antinociceptics (stomach pain, cramps), for alleviating itching and dermatitis and as calmative (Albuquerque et al., 2007), and with neuroprotective, antioxidant and anti-inflammatory effects in a Parkinson's disease model in rats (Araújo et al., 2018). Are also applied in the control of phytopathogens and with antimalarial effect moderate (Muñoz et al., 2000), which effect is attributed to the presence of indole alkaloids (Mitaine-Offer et al., 2002), whom are associated to the insecticides effects (Trindade et al., 2008). *A. pyrifolium* is one of the most important toxic plants of Caatinga. For this species have been reported natural abortion cases involving goats, sheep and cattle, but confirmed experimentally only in goats (Medeiros et al., 2004). Lima and Soto-Blanco (2010) have previously shown that this species has caused death in pregnant rats, hemolytic activity and side effects in *A. salina*, considered a good indicator of toxicity.

The species *Combretum leprosum* Mart. (Combretaceae), popularly known as mofumbo (Lira et al., 2002), is used in folk medicine as a healing agent and prevention of skin rashes and wounds clean (Horinouchi et al., 2013), expectorant and antitussive (Agra et al., 2007), sedative, antidiarrheal, bronchitis, influenza, whooping cough, diphtheria, heartburn, hemostatic (Albuquerque et al., 2007; Paulino et al., 2012) and also as antiophidic (Mors et al., 2000). Pharmacological studies with extracts and compounds isolated from different parts of this species, suggest different biological activities, such as: anticholinesterase effects (Facundo et al., 2005), antiulcerogenic (Nunes et al., 2009), antinociceptive (Longhi-Balbinot et al., 2012), anti-inflammatory and antiproliferative (Horinouchi et al., 2013), natural antioxidant (Evaristo et al., 2017) and against the promastigote form of *Leishmania amazonenses* (Teles et al., 2011).

The objective of this study was evaluate the allelopathic effect on *Allium cepa* L. (indicator organism), toxicity on *Artemia salina* and characterize the associated phytochemical profile of aqueous extracts from *Aspidosperma pyrifolium* Mart. and *Combretum leprosum* Mart.

## 2. Methodology

### Plant material

Leaves of *A. pyrifolium* Mart. & Zucc., Apocynaceae, and *C. leprosum* Mart., Combretaceae, were collected in the municipal of Russas, CE (4°50'59.9"S 37°53'29.9"W and 4°50'28.6 "S 37°54'05.5"W, respectively), in April 2015. Botanical classification was checked with The Plant List (<http://www.theplantlist.org>; accessed May 2015). The taxonomic identification was obtained in the Herbário Dárdano de Andrade-Lima (UFERSA) under codes 14525 and 10195, respectively. The samples were placed in kraft paper bags and transported to the Laboratório de Genética e Evolução (LAGENE - UFERSA) for drying and obtaining the extract.

### Extraction

The aqueous extracts of dried plant material were obtained by the methodology described per Matsumoto et al. (2010), with modifications. The leaves were selected, dried at room temperature and ground in a blender to obtain a fine powder. The aqueous extracts 10% (w/v) were prepared by diluting 100 g of each material in distilled water by magnetic stirring at 4 °C for 24 h. The material was filtered mesh of fine cloth, followed by vacuum filtration with filter paper (14 µm).

### Germination test

Bioassays were performed on a germination chamber of type Biochemical Oxygen Demand (B.O.D.) with controlled temperature of 20 °C and photoperiod of 12 h. *Allium cepa* seed, of variety NUN 1205 F1, were placed in gerbox-type boxes (11 x 11 cm) lined with a double layer of paper blotter moistened with 10 mL of solution of different treatments: *A. pyrifolium* extract (200; 400 and 800 mg.L<sup>-1</sup>) and *C. leprosum* extract (200; 400 and 800 mg.L<sup>-1</sup>) and distilled water (negative control). Four replications statistics with 50 seeds were used in each replicate in a completely randomized experimental design.

The seed physiological quality was assessed following the recommendations of the Regras para Análise de Sementes (Brasil, 2009) with counts at 24 h intervals until the twelfth day, thus obtaining the first germination count, germination and germination speed index (GSI). Were considered germinated the seeds that presented radicle with at least 50% of seed size (Ferreira & Aquila, 2000).

The germination data and the radicle length were calculated relative growth index (RGI) and germination index (GI) in accordance with Varnero et al. (2007), to verify the influence of the treatments.

The calculation of these values was carried out following equations:

$RGI = RLS / RLC$ , where RLS is the radicle length of the sample and RLC is the radicle length of the negative control;

$GI = RGI \times (GSS / GSC) \times 100$ , where, RGI is the relative growth rate, GSS is the number of germinated seeds in the sample and GSC is the number of germinated seeds in the negative control.

The RGI values obtained were classified into three categories, according to the toxic effects (Young et al., 2012): Inhibition of root elongation (I) when the value obtained for RGI is between 0 and 0.8; no significant effect (NSE) when the value obtained for RGI is equal to or between 0.8 and 1.2; and stimulation of root elongation (S) when the value obtained for RGI is greater than 1.2. The GI values were classified into three categories, according to the presence of phytotoxic substances (Zucconi, 1981): absence or low concentration of phytotoxic substances when the value obtained for GI is greater than or equal to 80; moderate presence of phytotoxic substances when the value obtained for GI is between 50 and 80; and high concentration of phytotoxic substances, when the value obtained for GI is less than or equal to 50.

Following the methodology of Pereira et al. (2009) with modifications, to obtain the lengths of the seedlings and their parts (hypocotyl and root), the cotyledons were removed and the hypocotyls separated from the roots. The hypocotyls and roots were then placed in separate paper bags, which were kept in a greenhouse at  $60 \pm 1$  °C for 72 h. At the end of this period, the root, hypocotyl and seedling dry matter mass (RDM, HDM and SDM, respectively) were obtained in milligrams. Results were expressed as average weights, ie the weight of dry mass divided by the number of seedlings placed in the paper bag to dry. The weight of dry mass per seedling was obtained from the sum of the average dry mass weights of the hypocotyl and root.

Additionally, the weight of dry biomass per centimeter, ie the root, hypocotyl and seedling dry biomass density (RDBD, HDBD and SDBD, respectively) was evaluated. To obtain the values of this variable, expressed in milligrams per centimeter of seedling, mg.cm<sup>-1</sup>, the following formula was used (Pereira et al., 2009):

$$DB = \text{Weight} / \text{Length}$$

This value was obtained from the weight and length measurements of each seedling of the plot.

### Cytogenetic analysis

The mitotic index (MI) test was performed using the crush technique (Guerra & Souza, 2002). When the roots reached 2.0 cm in length (approximately five days after the start of the assay) (Leme et al., 2008), the roots (two roots from each replica) were placed in Carnoy's fixative solution (ethanol:acetic acid - 3:1) for 24 h. To prepare the slides, the roots were

removed from the fixative, washed in distilled water (three baths of 5 min each), hydrolyzed in 1 mol.L<sup>-1</sup> HCl at 60 °C for 11 minutes and washed once more in distilled water. Then, using tweezers and a scalpel blade, the hood (apical portion of the root) of approximately 1 mm to 2 mm in length was removed and placed under a blade. One drop of 2% acetic carmine was added and stained for 5 minutes. The coverslip was then placed on the slide and squashed with the thumb with reasonable pressure (Guerra & Souza, 2002).

The slides of each bioindicator were analyzed by scanning method under optical microscope for observation at 400X magnification, 4 replicates of 1000 cells/treatment, with a total of 4000 cells per treatment. The mitotic index was obtained by dividing the number of cells in mitosis by the total number of cells observed and multiplying by 100, and the presence of prophase, metaphase, anaphase and telophase were analyzed. The cytotoxicity limit value was also calculated according to the equation: Cytotoxicity limit value = MIS / MIN x 100, where, MIS is the mitotic index of the sample, and MIN is the mitotic index of the negative control.

### **Toxicity assay**

Toxicity of the extracts leaves was performed according to Rodriguez et al. (2010) protocol. Eggs hatching from *Artemia salina* was performed in a cultivation solution containing 18 g NaCl and 5 g NaHCO<sub>3</sub> in a final volume of 1 liter of distilled water, under constant light and aeration for 48 h. Ten nauplii hatched were separated and transferred to 24-well plates containing 100 µL cultivation solution for *A. salina* and 400 µL extract at maximum concentration of 10000 mg.L<sup>-1</sup> per well. Toxicity assay was performed in triplicate. The nauplii dead number were determined and the LC50 was calculated by non-linear regression.

### **Phytochemical analysis**

The methodology according to Matos (2009) and Barbosa et al. (2004) was used to determine the presence of organic acids, sugars, alkaloids, anthraquinones, azulenes, quaternary bases, compounds phenolics, steroids, lactones, foaming saponines and terpenes. Total extractable polyphenols were quantified according to Larrauri et al. (1997) and anthocyanins and yellow flavonoids according to Francis (1982).

### **Statistical analysis**

The statistical evaluations were performed by Shapiro-Wilk normality test and performed analysis of variance (ANOVA) with Tukey post-test ( $p < 0.05$ ) to relative growth index, germination index, plant growth, dry matter and dry biomass density of *Allium cepa* roots, hypocotyl and seedlings and mitotic index. All analyzes were performed using the Grahpad 8.01 Prism software.

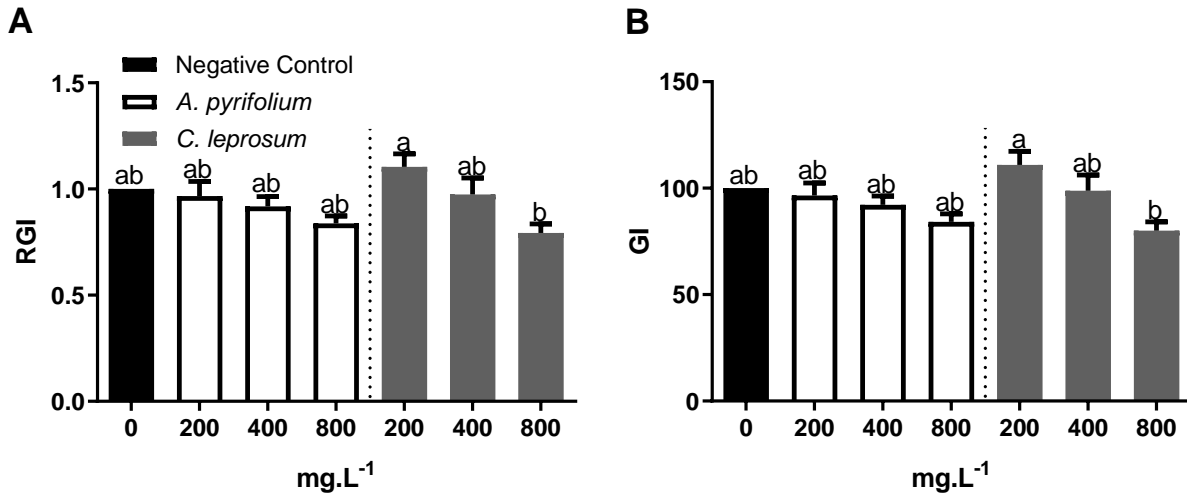
## **3. Results and Discussion**

The results of the germination test, first count and germination speed index (GSI) did not differ significantly ( $p > 0.05$ ). Therefore, it can be stated that the extracts did not have allelopathic effects on germination, as all treatments had similar results and indiscriminately showed high germination percentage at the beginning of the test, about 70% of seeds germinated on the third day, reaching the summit between the fifth and sixth day.

Similar results of this work were described by Borges et al. (2011), which also found no allelopathic effect of aqueous extracts of castor seeds on the germination of seeds of *A. cepa*. Ferreira and Aquila (2000) argue that the allelopathic effect often does not occur on germination or germination rate, but can affect other process parameter, such as the length of roots.

The influence of the extracts on the length of roots and germination were assessed by relative growth index (RGI) and germination index (GI) (Figure 1).

**Figure 1.** Relative growth index (RGI) and germination index (GI) of *Allium cepa* under the effect of aqueous extract of *A. pyriformium* and *C. leprosum*.



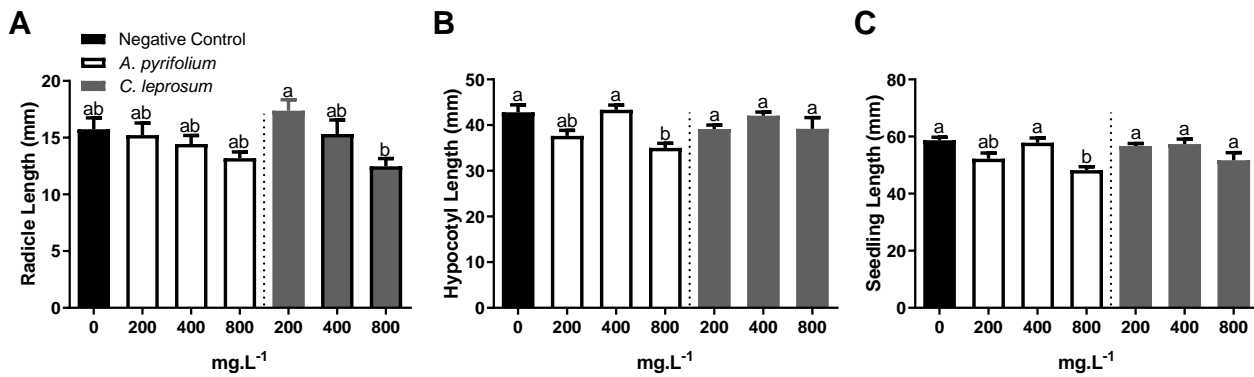
Mean±SEM followed by the same letter in the same species and control do not differ statistically by Tukey test ( $p > 0.05$ ). Source: Authors.

The obtained RGI (Figure 1A) had no significant effect on radicle growth (RG, as according Young et al. (2012), RGI values comprised between 0.8 and 1.2 range exhibit this behavior, as observed in Figure 2A). However, significant differences were observed between extracts of *C. leprosum* with 200 and 800 mg.L<sup>-1</sup>, apparently revealing inversely proportional effect between the RGI and concentration, which was confirmed by the equation of ( $y = -0.1556x + 1.2683$ ,  $R^2 = 0.991$ ). The reduction of root growth under treatment with *C. leprosum* extract with 800 mg.L<sup>-1</sup> can be related to the presence of mildly phytotoxic metabolites (Varnero M et al., 2007).

Varnero M et al. (2007) proposed that the germination index (GI) is a better indicator than the relative growth index (RGI) to characterize the phytotoxic potential of an organic material. This fact was not observed for the studied samples with GI greater than 80% (Figure 1B). According Zucconi (1981), GI values  $\geq 80\%$  indicates the absence of phytotoxic substances or they are in low concentration.

Root growth (RG) was not significantly affected by extracts in relation to the negative control (Figure 2A). Regarding hypocotyl length, an 18.2% reduction was identified in relation to the negative control in roots treated with aqueous extract of *A. pyriformium* 800 mg.L<sup>-1</sup> (Figure 2B). Hypocotyls treated with aqueous extract of *Combretum leprosum* did not differ from controls (Figure 2B). As for seedling length, obtained by summing the length of the roots and hypocotyls, only *A. pyriformium* 800 mg.L<sup>-1</sup> extracts reduced the length significantly compared to the control, 17.9% (Figure 2C). Thus, the RGI, GI and radicle length values obtained demonstrate that the extracts of *A. leprosum* and *C. pyriformium* not affect the germination and root development *Allium cepa* at the concentrations tested. Nevertheless, the seedling, especially its hypocotyl, has its growth compromised when exposed to *A. pyriformium* 800 mg.L<sup>-1</sup> extract.

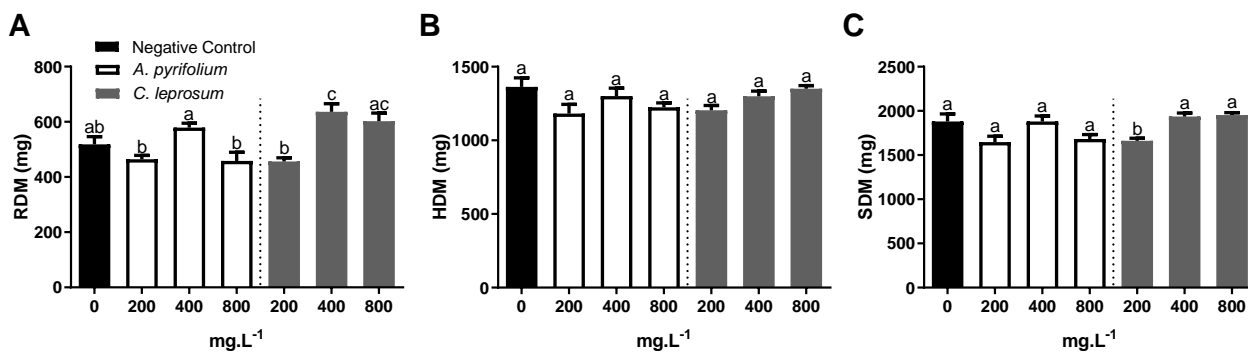
**Figure 2.** Plant growth of *Allium cepa* roots, hypocotyl and seedlings under the effect of the aqueous extract of *A. pyrifolium* and *C. leprosum*.



A: Radicle length of *A. cepa*; B: Hypocotyl length of *A. cepa*; C: Seedling length of *A. cepa*. Mean±SEM followed by the same letter in the same species and control do not differ statistically by Tukey test ( $p > 0.05$ ). Source: Authors.

Although there was no effect on root length with both extracts, *C. leprosum* 400 mg.L<sup>-1</sup> extract affected root dry matter mass, increasing by 22.9% compared to the control (Figure 3A), suggesting thickening of the root, which may be associated with the mitotic index, explored later. Hypocotyl dry matter analysis showed no change in the treated groups (Figure 3B). In the meantime, when seedlings were evaluated, only *C. leprosum* 200 mg.L<sup>-1</sup> extract negatively affected the dry matter mass (Figure 3C), reducing 11.7% in relation to the control. This effect corroborates the tendency to reduce root dry matter (Figure 3A), although this group does not differ statistically from the control.

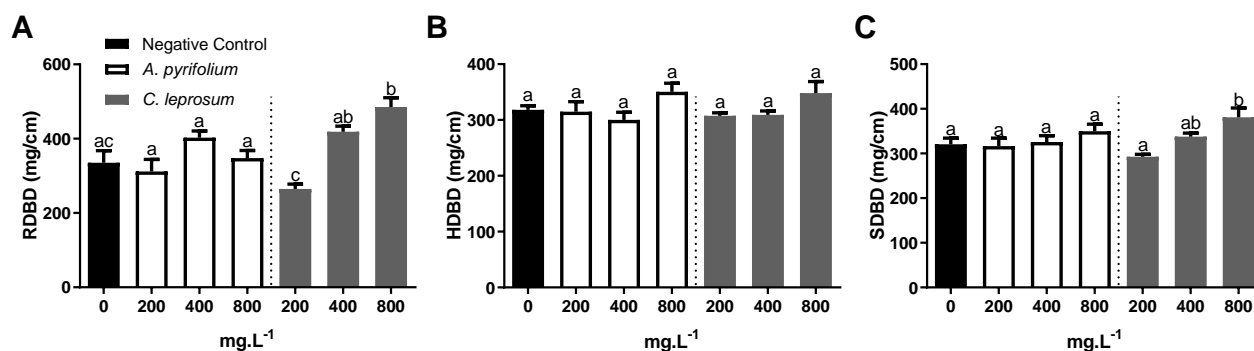
**Figure 3.** Dry matter of *Allium cepa* roots, hypocotyl and seedlings under the effect of the aqueous extract of *A. pyrifolium* and *C. leprosum*.



A: Root dry matter (RDM) of *A. cepa*; B: Hypocotyl dry matter (HDM) of *A. cepa*; C: Seedling dry matter (SDM) of *A. cepa*. Mean±SEM followed by the same letter in the same species and control do not differ statistically by Tukey test ( $p > 0.05$ ). Source: Authors.

Biomass density, a parameter that correlates dry matter with seedling length, did not identify any significant influence of *A. pyrifolium* extract on the negative control (Figure 4, B and C). However, the effect of *C. leprosum* 800 mg.L<sup>-1</sup> extract on the increase (44.7%) of the root dry biomass density (Figure 4A) is remarkable, corresponding to the reduction of its growth (Figure 2A), characterizing the cell growth by root thickening. This condition led to the same response in the evaluation of seedling dry biomass density, with an 18.8% increase in seedlings treated with the extract (Figure 4C). Hypocotyls did not change their biomass density (Figure 4B).

**Figure 4.** Dry biomass density of *Allium cepa* roots, hypocotyl and seedlings under the effect of the aqueous extract of *A. pyriformium* and *C. leprosum*.



A: Root dry biomass density (RDBD) of *A. cepa*; B: Hypocotyl dry biomass density (HDBD) of *A. cepa*; C: Seedling dry biomass density (SDBD) of *A. cepa*. Mean $\pm$ SEM followed by the same letter in the same species and control do not differ statistically by Tukey test ( $p > 0.05$ ). Source: Authors.

These growth inhibitions (Figure 2A, B and C) observed in *Allium cepa* may occur due to the presence of sesquiterpenolactones, which potentially may inhibit DNA synthesis, impairing normal cell division, as observed with reduced mitotic index in *A. cepa* cells treated with aqueous extract of *Distephanus angulifolius* (DC.) H. Rob & B. Kahn., which contains substances belonging to the class (Chukwujekwu & Van Staden, 2014). Similarly, alkaloids present in the *Erythrina* family (Amaryllidaceae) inhibit DNA and protein synthesis (Parsons & Williams, 2000), this group being quite common in *A. pyriformium* (Nogueira et al., 2014).

Congruent to these effects, some heavy metals in the extracts may infer reduction of cell division, as well as those obtained from *Azadirachta indica* A. Juss., *Mangifera indica* L., *Cymbopogon citratus* (DC.) Stapf and *Morinda lucida* Benth., which present in their constitution zinc, copper, manganese, iron, cadmium and lead in different concentrations (Ajasa et al., 2004; Akinboro & Bakare, 2007; Haider et al., 2004).

In contrast, coumarins and flavonoids have been attributed to antiproliferative and antioxidant processes (Ammar et al., 2008; Kostova, 2006). Antioxidants are known to be universal anti-mutagenic agents (Giri et al., 1998; Odin, 1997; Sarkar et al., 1997) and their common representatives are ascorbic acid (vitamin C) and  $\beta$ -carotene, but also tannins (Kaur et al., 2000), aurones (Kaur et al., 2009; Zampini et al., 2008), flavonoids (Perez-Carreón et al., 2002; Zhai et al., 1998), saponins (Lee et al., 1999), phenols and terpenoids (Ananthi et al., 2010) that have anti-mutagenic effects and all but the last two were found in the aqueous extract of *Erythrina velutina* Willd., which has antigenotoxic effect (Silva et al., 2013).

The hydroxyl group present in phenolic compounds has redox properties, allowing to act as a reducing agent (Pietta, 2000; Shahidi et al., 1992). Nevertheless, Ivanova et al. (2005) suggest that not all polyphenols have antioxidant activity. Many of these substances can be identified in both extracts (data presented in tables 1 and 2), which may be correlated to the variability of effects (reduction or increase) in the different concentrations of the tested extracts.

The evaluation of mitosis cells under the treatment of *A. pyriformium* aqueous extract showed that they did not differ statistically. The mitotic index (MI) of this treatment presented mean values equal to or higher than those observed in the negative control, reflecting in cytotoxicity limit values (CLV)  $\geq 100\%$  (Table 1). An MI decrease below 22% of the control causes lethal effects on test organisms (Antosiewicz, 1990), while a decrease below 50% usually has sublethal effects (Panda & Sahu, 1985) and it's called cytotoxic limit value (Sharma, 1983).



**Table 1.** Mitotic index and cytotoxicity limit value of *Allium cepa* under the effect of aqueous extract of *A. pyrifolium* and *C. leprosum*.

Treatment	Prophase	Metaphase	Anaphase	Telophase	MI (%)	CLV (%)	
Negative Control	6.3 ± 0.6 <sup>ab</sup>	7.5 ± 1.6 <sup>a</sup>	14.5 ± 2.3 <sup>a</sup>	3 ± 0.4 <sup>a</sup>	3.1 ± 0.3 <sup>ab</sup>	-	
<i>A. pyrifolium</i>	200 mg.L <sup>-1</sup>	13.3 ± 1.1 <sup>a</sup>	11.8 ± 1.2 <sup>a</sup>	12.3 ± 2.6 <sup>a</sup>	5.5 ± 0.9 <sup>a</sup>	4.3 ± 0.3 <sup>a</sup>	137
	400 mg.L <sup>-1</sup>	9.8 ± 3.5 <sup>a</sup>	8 ± 3.1 <sup>a</sup>	8.5 ± 2.9 <sup>a</sup>	7.3 ± 2.5 <sup>a</sup>	3.4 ± 1.1 <sup>a</sup>	107
	800 mg.L <sup>-1</sup>	7.5 ± 1.5 <sup>a</sup>	7 ± 1.5 <sup>a</sup>	12.5 ± 1.7 <sup>a</sup>	4.3 ± 1.8 <sup>a</sup>	3.1 ± 0.6 <sup>a</sup>	100
<i>C. leprosum</i>	200 mg.L <sup>-1</sup>	2.8 ± 0.75 <sup>b</sup>	3.8 ± 1.3 <sup>a</sup>	2.8 ± 1.2 <sup>b</sup>	2.5 ± 1 <sup>a</sup>	1.2 ± 0.2 <sup>b</sup>	38 <sup>Sb</sup>
	400 mg.L <sup>-1</sup>	10 ± 0.58 <sup>a</sup>	10.8 ± 2.4 <sup>a</sup>	17 ± 3.5 <sup>a</sup>	3.5 ± 0.5 <sup>a</sup>	4.1 ± 0.6 <sup>a</sup>	132
	800 mg.L <sup>-1</sup>	7.5 ± 1.6 <sup>a</sup>	6.8 ± 3.9 <sup>a</sup>	9.5 ± 2.2 <sup>ab</sup>	3.3 ± 0.8 <sup>a</sup>	2.7 ± 0.7 <sup>ab</sup>	86

Mean±SEM followed by the same letter in the same species and control do not differ statistically by Tukey test (p > 0.05). Source: Authors.

In the analysis of MI in the *C. leprosum* extract bioassay (Table 1), it was observed that the values did not differ statistically from the control. Nevertheless, 38% CLV was observed for cells treated with *C. leprosum* 200 mg.L<sup>-1</sup>, a value categorized as a sublethal effect. This effect is due to the reduction in the total number of dividing cells, characterized by a 1.2% MI, which is 61.3% lower than the control, and a significant reduction in the number of prophase and anaphase cells. Higher concentrations did not have the same effect, this may have been due to the dissociation of some allelochemicals due to the larger water volume of 200 mg.L<sup>-1</sup> treatment compared to 400 and 800 mg.L<sup>-1</sup>, leading to a reduction in the number of dividing cells, which reflected in the cytotoxicity limit value (CLV).

The cell cycle control system performs regulatory processes based on checkpoints, the three main ones being: beginning or G1/S; G2/M; metaphase-anaphase transition (Morgan, 2006). Thus, despite the reduction of cells treated with *C. leprosum* 200 mg.L<sup>-1</sup> (Table 1), the analysis of this last checkpoint (data not shown), as well as mitotic index, does not suggest cell cycle blockade for both extracts.

There is a linear correlation between macroscopic and microscopic parameters, so that in *Allium cepa*, with the reduction of root growth, there is also a reduction in the number of dividing cells, ie, the mitotic index (Akinboro & Bakare, 2007; Borges et al., 2011; FISKESJÖ, 1985), which occurs in the apical meristem and can be observed in association with the appearance of stunted roots, indicating growth retardation and cytotoxicity (Yıldız et al., 2009), this correlation may also be putatively associated with dry matter, as it is the product of multidimensional cell growth and stretching. However, this correlation only occurred with the reduction of dry matter of *C. leprosum* 200 mg.L<sup>-1</sup> treated seedlings corresponding to the sublethal effect of cytotoxicity.

Although CLV is related to cell multiplication, values higher than 100% did not lead to increase of macroscopic parameters, except for *C. leprosum* 400 mg.L<sup>-1</sup> which induced 132% CLV (Table 1) and increase of dry matter mass of the roots (Figure 3A). The correlation may still be questioned when considering treatment with *C. leprosum* 800 mg/mL which presented safe cytotoxicity limit value (86%, Table 1), but promoted the increase of root and seedling dry biomass density (Figure 4A and C). Theoretical and practical studies should be performed to better characterize CLV higher than 100%, uncommon in the literature.

The aqueous extract of *C. leprosum* showed no toxicity to *Artemia salina*, with an no calculable LC<sub>50</sub> value, since the maximum concentration of extract obtained did not present toxicity higher than 30% and dose-independent effect, resulting in the low suitability of the model for this material at the evaluated concentrations (R<sup>2</sup> = 0.12). However, aqueous extract of *A. pyrifolium* showed high toxicity to *A. salina*, with LC<sub>50</sub> of 4986 mg.L<sup>-1</sup> (R<sup>2</sup> = 0.93), while negative control (cultivation solution) showed no toxicity (Table 2).

**Table 2.** Toxicity of aqueous extracts of *A. pyriformium* and *C. leprosum* on *Artemia salina*.

	LC <sub>50</sub> (mg.L <sup>-1</sup> )
Aqueous extract of <i>C. leprosum</i>	-
Aqueous extract of <i>A. pyriformium</i>	4986
Cultivation solution	No toxicity

Source: Authors.

*Aspidosperma. pyriformium* is recognized as one of the most toxic plants in the Caatinga, with a record of teratogenic effect, hemolytic activity and adverse effects in *A. salina*, a model organism in toxicity assessment (Lima & Soto-Blanco, 2010; Medeiros et al., 2004). An aqueous solution of the ethanolic extract with lethal effect on 46.2% of the nauplii of *A. salina* at 30 mg/mL (corresponding to 30000 mg.L<sup>-1</sup>) was evaluated. The discrepancy between the effect observed in our study and that of Lima and Soto-Blanco (2010) is probably associated with a distinct chemical composition from the extracts used, perhaps favored by a synergistic effect in our case.

There is a positive correlation between lethality and cytotoxicity (Carballo et al., 2002; Meyer et al., 1982) for compounds that do not require metabolic activation (Solis et al., 1993). In this context, our study performed a biochemical approach to better characterize these extracts.

Qualitative phytochemical tests described in Table 4 revealed the presence of primary metabolism compounds, such as organic acids and carbohydrates (reducing sugars). However, they have not identified the presence of polysaccharides in aqueous base extracts.

**Table 3.** Phytochemical profile presents in aqueous extract of *A. pyriformium* and *C. leprosum*.

Metabolite	<i>A. pyriformium</i>	<i>C. leprosum</i>
Organic Acids	+	+
Sugars		
Reducing Sugars	+	+
Polysaccharides	-	-
Alkaloids	+	-
Anthraquinones	-	-
Azulenes	+	+
Quaternary Bases	-	-
Phenolic Compounds		
Deposides and Depsidones	-	+
Phenols and Tannins	+	+
Flavonoids	+	+
Flavonols, Flavanones, Flavanonols and Xanthones	+	-
Catechins	-	+
Steroids	+	+
Lactones		
Coumarin Derivatives	-	-
Sesquiterpenelactones and other lactones	-	-
Foaming saponins	-	+
Terpenes		
Carotenoids	+	-
Triterpenoids	-	-

+ = presente; - = absent. Source: Authors.

*Aspidosperma pyriformis* presented different alkaloids widely studied for the species (Mitaine-Offer et al., 2002; Nogueira et al., 2014; Trindade et al., 2008) and carotenoids, commonly associated with photoreception, photoprotection, antioxidant protection and cancer cells cytotoxicity (Esteban et al., 2015). Azulenes, which possess anti-inflammatory properties (Guarrera et al., 2001), were found in both species. Phenols, tannins and flavonoids were also observed in both species. Flavonoids have antiproliferative effects (Ammar et al., 2008) and are antioxidants, along with tannins, saponins, terpenes and phenols (Ananthi et al., 2010). Anthraquinones, phenols, tannins, flavonoids and triterpenoids have previously been reported in *A. pyriformis* (Almeida et al., 2005; Nunes et al., 2018).

The fact that the extracts studied did not affect plant health is important for situations in which it aims to control pests associated with plants, be they bacteria, fungi, or insects. Some studies have shown the effect of insecticide *A. pyriformis*. The aqueous extract of the bark are repellent larval of the first instar and ovicidal on diamondback moth, *Plutella xylostella* (Linnaeus 1758) (Lepidoptera: Plutellidae) (Torres et al., 2006), while the rich ethanol fraction alkaloid has excellent insecticidal properties against larvae of diamondback moth (Trindade et al., 2008).

The depsides, depsidones and foaming saponins were observed in *C. leprosum*. Extracts and substances of *C. leprosum* have also been studied for its pharmacology effects of human interest, such as anti-inflammatory (Horinouchi et al., 2013), anti-nociceptive (Longhi-Balbinot et al., 2012), gastroprotective and antiulcerogenic (Nunes et al., 2009) and antibacterial (Evaristo et al., 2014). Organic acids, reducing sugars, phenolic compounds, foaming saponins and triterpenoids have previously been reported in *C. leprosum* (Evaristo et al., 2014; Facundo et al., 1993; Facundo et al., 2005; Lopes et al., 2010; Nunes et al., 2009).

Quantitative biochemical analysis revealed the presence of phenolic substances (Table 4), which are commonly associated with antioxidant processes, and are known to be anti-mutagenic (Giri et al., 1998; Odin, 1997; Sarkar et al., 1997). In our study, anthocyanins and flavonoids (Table 4) were quantified in extracts of *A. pyriformis* and *C. leprosum*, the latter were reported in *C. leprosum* (Facundo et al., 1993).

**Table 4.** Metabolite dosage in aqueous extract of *A. pyriformis* and *C. leprosum*.

	Species / Concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )*	
	<i>A. pyriformis</i>	<i>C. leprosum</i>
Total Extractable Polyphenols	1036,11	3057,68
Yellow Flavonoids	8,18	8,45
Anthocyanins	0,62	0,27

\*Dosage in micrograms of metabolites per gram of liquid extract. Source: Authors.

The high content of polyphenols in the extracts, the composition of which possibly contains phenols, tannins, depsids and depsidones, probably acts in antagonism to toxic allelochemicals present in the extracts, so that no linear dose-response effect can be identified under macroscopic and microscopic parameters of the exposed tissues of *Allium cepa*. Many constituent compounds of the extracts can act synergistically or disguise their effects on germinal, cellular and toxicological aspects; more research is needed to better cytotoxic and phytochemical characterization of *Aspidosperma pyriformis* and *Combretum leprosum*.

#### 4. Conclusion

The aqueous extract of *Aspidosperma pyriformis* leaves not have allelopathic effect at the concentrations tested. *Combretum leprosum* extract showed toxic effects at lower concentrations in some analysis, and may be

associated with the dissociation factor of each component of the extract, causing them to have different effects at each concentration, not following a dose response model. The phytochemical profile of aqueous extracts of *C. leprosum* and *A. pyrifolium* demonstrated the presence of compounds with potential for pharmacological application. Therefore, future pharmaceutical analysis may be accompanied by analyzes of cytotoxicity or toxicity in cell and animal models.

## Acknowledgments

The authors thank the Universidade Federal Rural do Semi-Árido (UFERSA) for funding and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for a scholarship.

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