



Growth Inhibition and Pro-apoptotic Action of *Eleusine indica* (L) Gaertn Extracts in Allium test

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Authors' contributions

This work was carried out in collaboration between both authors. Author AAO designed and conducted the experiment, data analysis, and wrote the manuscript. Author NFR supervised the execution and reviewed the writing. Both authors read and approved the final manuscript.

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ABSTRACT

Background: *E. indica* is a gramineae largely used in Brazilian traditional medicine for treatment of respiratory diseases. Previous research work has shown this plant to have glicoflavonoids constituents.

Place and Duration of Study: The study was carried out during four months in 2014 in the Laboratory of Molecular Biology, Department of Pharmacy, Lutheran University of Brazil (CEULJI/ULBRA), Ji-Paraná, Rondônia, Brazil.

Aims: This study aimed to determine possible mutagenicity and/or cytotoxicity activity of *E. indica*, using the Allium test to investigate root growth, mitotic index and micronuclei formation.

Methodology: We applied the Allium test to analyze the effects of *E. indica* compounds on genetic material. The plant aerial parts were dried, pulverized and prepared as aqueous extract. The experiment consisted in 5 assays: *E. indica* extracts in concentrations of 25%, 50%, and 100%; positive control (CuSO₄); and negative control (distilled water). Each assay was performed with 10 repetitions.

Results: Initial results showed inhibition of meristems growth and stable mitotic index. Differential analysis indicated influence in cell division process with a significant number of cells in metaphase

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that did not progress to the next mitotic cycle stage, and no significant presence of micronuclei.

Conclusion: Our results strongly suggest the plant having cytotoxic compounds with microtubule affinity interaction without mutagenicity activity.

Keywords: Medicinal plants; aqueous extract; cytotoxicity; *Allium cepa*; micronuclei. *Eleusine indica*.

1. INTRODUCTION

Medicinal plants have been used in diseases' treatment in the form of teas and infusions since the beginning of human history [1-3]. The Amazon region has a legacy of great genetic variability in plants and many of them are not yet cataloged species. A large array of plant species that have been used for a long time in traditional medicine have not yet been studied. One species that occurs in this region is the grass *E. indica*, pertaining to the Poaceae family, popularly known as Indian goosegrass; it is characterized as an upright plant, consisting of stem, hairless and striated, with heights ranging from 30 to 70cm, annual, herbal, with reproduction by seed, and with the ability to grow in any type of soil, preferably in environments with high temperatures and humidity [4].

In studies on medicinal purposes, this plant is cited for treatment of diseases related to the respiratory tract [5]. This fact was already confirmed in earlier work through the isolation of two flavones: the vitexin and the schaftoside, both with significant positive results in asthma treatment [6,7]. Studies have reported *E. indica* use in inflammatory processes, as nasal decongestant, as therapeutic for osteoporosis and plantar fasciitis, and in the form of infusion or decoction against pneumonia, influenza, and fever [8-11].

The compounds isolated from *E. indica* belong to the group of flavonoids which comprises more than 6000 substances known to have close relation with physiological processes in plants. Flavonoids regulate growth and development playing a role as a protective mechanism against pathogens and solar radiation. This substances' group has extensive ubiquity and diverse biological activities, which can be beneficial or harmful, and only a limited number of them have been studied with this regard [12,13,9].

The Allium test is an excellent biomarker for first screening of genotoxicity and mutagenicity of medicinal plants due to its low cost, reliability, and compliance with other tests. It is also appropriated to study cytotoxic effects of

medicinal plants, whereas the *Allium cepa* roots are in direct contact with the tested substance, allowing the evaluation in different concentrations. Chromosomal changes and division of meristematic cells in onion root are often used as an alert signal to advert people about the consumption of certain products [14,1]. Therefore, this study aimed to determine possible mutagenicity and/or cytotoxicity activity of *E. indica*, using the Allium test to investigate root growth, mitotic index and micronuclei formation.

2. MATERIALS AND METHODS

2.1 Collection of *E. indica*

E. indica samples were collected in the medicinal garden of Itapirema High School (EFA) in the city of Ji-Parana (Brazil) (Coordinates: 10°50'28.7"S, 62°01'47.3"W) during flowering stage (March, 2014). The plant was identified by the botanist Joseane B Barbosa, M.Sc, who was in charge of the university (CEULJI/ULBRA) Herbarium in which the voucher specimen was deposited under the number 007.

2.2 Phytochemical Compounds Extraction

The extracts were prepared following the folk medicine usage of *E. indica*. The fresh aerial parts were oven dried with air circulation for 24 hours at 44°C, and subsequently pulverized. The phytochemical compounds extraction was made by decoction. Distilled water (pH=7.2) was heated up to 90°C, then *E. indica* pulverized parts were added in a proportion of 100g per 1L during 15 minutes [15]. Thereafter, the solution was cooled to room temperature and filtered in filter paper (PRO Lab. Quantitative Filter Paper). The resultant solution consisted in the 100% concentration assay, here and after denominated Eleusine₁. For the 50% concentration assay, distilled water was added to the Eleusine₁ solution in 1:1 ratio, here and after denominated Eleusine₂. Finally for the 25% concentration assay, distilled water were used in 3:1 ratio, here and after denominated Eleusine₃.

2.3 Allium Test

The *A. cepa* (2n = 16) specimens were acquired in the local popular market, all of small size, uniform, same origin, not sprouted and healthy. The bulbs were germinated with the bottom dipped in a 30mL solution which were the 3 *E. indica* assays, the negative and positive control for a period of 72 hours at 24°C. The experiment had as negative control (NC), distilled water, and as positive control (PC), copper sulfate (CAS N°. 7758998; Sigma Chemical Co., St Louis, MO, USA) at a concentration of 0.006g/L⁻¹[16,17].

The experiment was conducted using 10 replicates for each assay. After the exposure period, the meristems were collected and placed in a solution of 3:1 (v/v) methanol/acetic acid at a temperature of 4°C during 12 hours for chemical fixation. Afterwards, the hydrolysis was performed in a 1 mol/L⁻¹ Hydrochloric acid (HCl) solution for 5 minutes in water bath at 60°C followed by distilled water wash.

Slides were made in duplicate and stained with Panotipo Rapido LB Kit (Record N°. 80002670065; Reny Lab Chemical and Pharmaceutical Co., Barbacena, MG, Brazil). The squash was performed after the coverslip addition [18]. Slide analysis was done by optical microscopy with 1000x magnification. The variables studied were: number of micronuclei formation per 1000 cells per slide, root number and root length measured after 5 days of exposure, and mitotic index (MI). The MI was calculated as shown in Equation 1, where CM is a number of cells in mitosis and TC is a total number of analyzed cells.

$$MI = CM / TC \times 100$$

2.4 Statistical Analysis

For statistical analysis, the collected data was treated in the statistical software R Core Team, version 3.0.2. The computed tests were ANOVA followed by Tukey's test and the nonparametric Pearson correlation test, with an adopted significance level of $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Physicochemical Characterization

The root number and root length levels are presented in Table 1. These results show that all

E. indica assays caused significant root growth inhibition in comparison with negative and positive control assays. The inhibitions in root number and root length were greater in increasing concentrations of *E. indica* extracts. Average root length was 3.99 with a Standard Deviation (SD) of 1.71 (3.99±1.71 cm) in negative control and 1.4±0.55 cm in positive control. However, average root length in Eleusine₃ assay decreased significantly in comparison to negative control (Table 1). Average root length in treatment assays also decreased significantly according to concentration. Statistical analysis shows that the values observed in Eleusine₃ and Eleusine₂ assays are significant ($P \leq 0.05$) and the values of Eleusine₁ are highly significant ($P < 0.001$)¹. When comparing data from treatment assays among themselves, no significant correlation was found ($r = 0.27$ and $P > 0.05$)².

Inhibition in germination process is not contingent upon extract concentration and dose escalation may not interfere in the inhibitory process [19]. Studies with *Cymbopogon citratus* popularly known as lemon grass and also belonging to the Poaceae family, found the plant aqueous extract having compounds with allelochemical effects on *Lactuca sativa* root growth [20]. The influence of some plants on root growth occurs by the production of secondary metabolic compounds that act inhibiting or promoting the germination process and the cell division process [21]. The allelochemicals are released into the atmosphere by plants in various ways: by volatilization, root exudation, leaching and decomposition of waste vegetable structures [21,22].

3.2 Mitotic Index

The number of cells in mitosis and respective mitotic index are presented in Table 2, the results are not considered statistically significant ($P > 0.05$). Considering that root growth was inhibited, we analyzed the mitotic cell phase by cell differential analysis and we found that *E. indica* assays had a large number of cells in metaphase, as shown in Fig. 1, with severe depression values for anaphase and telophase which was proven by comparison with negative and positive controls ($P \leq 0.001$).

¹ANOVA and Tukey's test.

²Pearson Correlation coefficient.

Table 1. Average root number and root length in treatments and control groups assays

Treatment groups	Concentrations	Average root number ± SD	Average root lengths (cm) ± SD
Negative Control	Distilled water	8.2±1.8	3.99±1.71
Positive Control (CuSO ₄)	0.006g/L ⁻¹	3.2±0.6	1.4±0.55
Eleusine ₃	25g/L ⁻¹	2.3±0.92	0.71±0.45*
Eleusine ₂	50g/L ⁻¹	2.45±1.08	0.75±0.47*
Eleusine ₁	100g/L ⁻¹	1.99±0.87	0.49±0.29***

* Significant value ($P \leq 0.05$); *** Highly significant value ($P < 0.001$).

Table 2. Number of cells in mitosis and mitotic index by treatment

Treatment	Interphase	Mitotic phase				Mitotic index
		Prophase	Metaphase	Anaphase	Telophase	
Negative control	16357	1265	1227	580	571	18.22
Positive control	16128	1500	1338	540	440	19.36
Eleusine ₃	16122	1778	1898	122***	80	19.39
Eleusine ₂	16288	1680	1897	99***	36	18.56
Eleusine ₁	16042	1922	1885	100***	51	19.79

*** Highly significant value regarding to the negative and positive control groups ($p \leq 0.001$)

MI measures the proportion of cells in the cell cycle M-phase and its inhibition can be interpreted as cellular death or a delay in the cell proliferation kinetics [23]. Reduction in the mitotic activity could be due to DNA synthesis inhibition or a blocking in the cell cycle G2 phase, preventing the cell from entering in mitosis [24]. Mitodepressive effects of some herbal extracts, including the ability to block DNA synthesis and nuclear proteins, were reported earlier [25,26]. Several other herbal extracts have been reported to inhibit mitosis [27-29].

The same effect was observed in earlier studies with chamomile inflorescence, with significant increase in the MI, and cell death concomitant with increased testing doses [30]. In a study conducted by [20], different results were reported for *C. citratus* extracts, in which root growth inhibition decreased MI. Flavonoids may interfere in the mitotic process, therefore induce apoptosis depending on its nature and concentration [13].

Cell differential analysis determined the absence of membrane cell boundaries in *E. indica* assays as shown in Fig. 1a. In cells with membrane absence the chromatin was in apoptotic process as in Fig. 1b. initially sparse and in transcription, beginning a rapid process of condensation and inactivation. Coarse lumps of heterochromatin were formed into the nucleus, with some changes in nuclear boundary. The inactivation of genetic material led to dismantling the

cytoskeleton and to its disorganization, as in Fig. 1c. causing cell deformation on its contours and undoing its joints [31].

Apoptosis is an essential process to maintain the development of living beings, as it is important to eliminate unnecessary or defective cells. During apoptosis, the cell undergoes morphological changes including cell shrinkage, loss of adhesion to the extracellular matrix and to neighboring cells, chromatin condensation, internucleosomal DNA fragmentation and formation of apoptotic bodies. This biological phenomenon, in addition to playing an important role in controlling many vital processes, is associated with numerous diseases such as cancer [31].

3.3 Micronuclei

The negative control assay showed 5.2±0.63 micronuclei formation, and the positive control had value of 28.4±1.77 micronuclei, both for every 2000 cells. In *E. indica* assays results for Eleusine₃, Eleusine₂ and Eleusine₁ were: 7.6±1.83, 7.2±3.76, and 9.1±1.66 micronuclei respectively, for every 2000 cells. The results reveal that aqueous extract of *E. indica* produces no mutagenic effect in meristems of *A. cepa* once the numbers indicate low significance ($P > 0.05$) in comparison with the negative control assay; additionally, no chromosomal aberrations were observed.

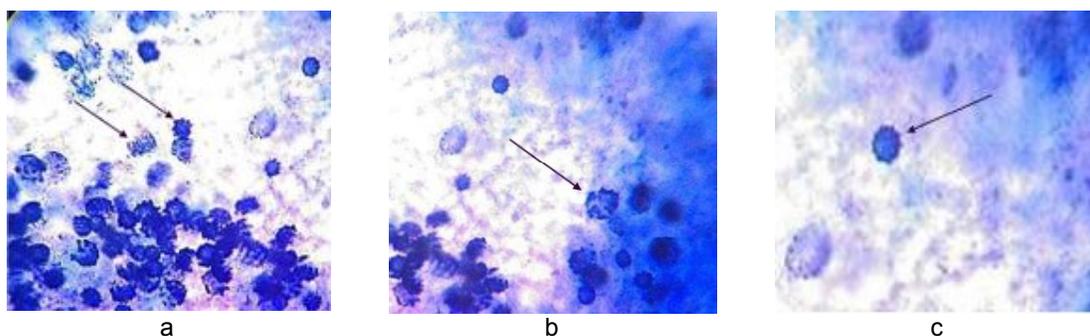


Fig. 1. Meristematic cells of *A. cepa* treated with *E. indica* observed under an optical microscope with a 1000x magnification. (a) Membrane damage in prophase and metaphase; (b) Apoptotic bodies; (c) Cell membrane deformation

While studying *Brachiaria brizanthiae*, also a Poaceae, [32] reported scarce micronucleus' formation, that occurs due to the meiotic behavior with slight formation of diploid accessions in the first division which is corrected in meiosis II leading to a normal cell division process. Bio-guided studies with *C. citratus* in *Allium* test did not lead to micronuclei formation [29]. In a study with *Aloe vera* extract, an average of 5.25 micronuclei was reported, which suggests that *E. indica*, although not belonging to the same family of *A. vera*, also leads to low significance values for micronucleus formation [1]. The low number of micronucleus formation induced by *E. indica* extract may be linked to its flavonol compounds: vitexin and schaftoside, which are antioxidants agents that act by sequestering free oxygen radicals [1,12].

4. CONCLUSION

Ultimately, our results indicate that when applied in higher concentrations *E. indica* extract has cytotoxic compounds with microtubule affinity interaction without mutagenicity activity. We used crude extracts of *E. indica* aerial parts. Experiments with crude extracts are appropriate because traditional medicinal herbs are generally used in the same way. Our findings in this study suggest that therapeutic use of *E. indica* requires additional investigation with isolated compounds regarding microtubules interaction in the cell cycle.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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