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Artigo

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Fragilidade Osmótica Eritrocitária e Atividade Citotóxica do Extrato Aquoso de *Indigofera Suffruticosa* Mill (Fabaceae)

Resumo: *Indigofera suffruticosa* Mill (Fabaceae) ocorre em abundância no nordeste do Brasil e tem intenso uso popular no tratamento de processos infecciosos e inflamatórios. Diversas atividades biológicas, como anti-inflamatória, anticâncer, antitumoral, protetor do fígado e baixa toxicidade são relatados para esta planta. O objetivo deste estudo foi investigar a fragilidade osmótica e propriedade citotóxica do extrato aquoso de folhas de *I. suffruticosa*. O extrato aquoso de folhas de *I. suffruticosa* (EAFIs) foi obtido por maceração, filtração e posteriormente liofilizado, sendo utilizados no ensaio de fragilidade osmótica com sangue de carneiro e efeito citotóxico no ensaio *Allium cepa*. A fragilidade osmótica eritrocitária apresentou baixo nível de hemólise, tanto pela avaliação qualitativa do sobrenadante quanto pelo resultado do percentual hemolítico. Para a avaliação citotóxica com *Allium cepa*s não foram identificadas anormalidades cromossômicas como também diferenças no processo de divisão celular (interfase, prófase, metáfase anáfase e telófase) entre o grupo controle e os grupos submetidos às diferentes concentrações do EAFIs (50 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$ e 1000 $\mu g.mL^{-1}$). Contudo, o extrato na concentração de 50 $\mu g.mL^{-1}$ em relação às concentrações de 500 $\mu g.mL^{-1}$e 1000 $\mu g.mL^{-1}$ apresentou maior desenvolvimento radicular. Concluindo-se que o AEFIs demonstrou baixo índice de hemólise e ausência de anormalidade cromossômica ou alteração nas fases das divisões celulares, sugerindo integridade da membrana celular e baixo nível de toxicidade.

Palavras-chave: *Indigofera suffruticosa;* ação hemolítica; citotoxicidade; divisão celular.

Abstract

Indigofera suffruticosa Mill (Fabaceae) occurs in abundance in northeastern Brazil and has intense popular use in the treatment of infectious and inflammatory processes. Various biological activities, such as anti-inflammatory, anticancer, antitumor, liver protector and low toxicity are reported for this plant. The aim of this study was to investigate the osmotic fragility and cytotoxic properties of aqueous leaf extracts from *I. suffruticosa*. The aqueous extract of *I. suffruticosa* leaves (AELIs) was obtained by maceration, filtration and later lyophilized, being used in the osmotic fragility test with sheep blood and cytotoxic effect in the *Allium cepa* assay. The erythrocyte osmotic fragility test showed low hemolysis levels, in the qualitative evaluation of the supernatant and in the result of the hemolytic percentage. Cytological examination with *Allium cepa,* no chromosomal abnormality were identifie, neither in the process of cell division (Interphase, prophase, metaphase, anaphase, telophase) between the control group and the groups of the experiment with different concentration of AELIs (50 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$ e 1000 $\mu g.mL^{-1}$). No chromosomal abnormalities or alterations in the cell division phases were found. However, the extract at the concentration of 50 $\mu g.mL^{-1}$ presented a root development when compared to 500 $\mu g.mL^{-1}$ and 1000 $\mu g.mL^{-1}$. It is concluded that the AELIs shows low hemolysis index and absence of chromosomal abnormality or alteration in the phases of the cell divisions, suggesting cell membrane integrity and low toxicity level.

Keywords: *Indigofera suffruticosa;* hemolytic action; cytotoxicity; cell division.

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Erythrocyte Osmotic Fragility and Cytotoxic Activity of the Aqueous Extract of Leaves of *Indigofera Suffruticosa* Mill (Fabaceae)

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1. Introduction

Erythrocyte osmotic fragility (EOS) can be defined as the erythrocyte resistance to hemolysis, evaluated by the use of buffered solutions of NaCl in distilled water in concentrations decreasing from 0.85 % to 0 %.[1] Cell volume control through the active elimination of solutes is one of the mechanisms by which lysis of the erythrocyte membrane is prevented *in vivo.*[2] Several intrinsic and extrinsic factors influence osmotic fragility of erythrocytes, such as erythrocyte shape, volume and size, type and amount of hemoglobin, as well as differences in membrane viscoelasticity and chemical and structural composition of erythrocytes or increase thereof under different conditions.[1,3] Toxicological studies are an important tool to evaluate the adverse effects of chemical compounds on living organisms. Among these, toxicology seeks to investigate the diversity of chemical or physical agents and vegetal nature, during any periods of life.[4]

The *Allium cepa* test is commonly used in toxicological assays, in studies with plant extracts, to evaluate macroscopic aspects such as alteration of color, shape and root size, in addition to microscopic characteristics such as alterations in the division of onion meristematic cells.[5]

Indigofera suffruticosa Mill (Fabaceae) occurs abundantly in northeastern Brazil and has intense popular use in the treatment of infections, inflammations and other processes, showing no reports of side effects that are harmful to humans. The phytochemical investigation of leaf extracts of *I. suffruticosa* revealed the presence of alkaloids, flavonoids, steroids, proteins, carbohydrate triterpenes and indigo.[6] Pharmacological studies have shown that aqueous extract of leaves of *I. suffruticosa* present the following activities: anti-inflammatory,[6] embryotoxic,[7] antimicrobial,[8] anticancer and antitumor alternative therapy.[9] The aqueous extract of *I. suffruticosa* leaves has been shown to inhibit the development of egg hatching, larval ecdysis and the deterrent effect on oviposition of *Aedes aegypti* mosquitoes.[10] Mice with sarcoma 180 showed liver response after sub-chronic treatment with *I. suffruticosa* extract.[11] The phytochemical study of three (3) bis-indolic alkaloid fractions; Indigo and Indirubin from *I. suffruticosa* leaves were identified, isolated and purified.[10] However, few reports are available on the toxicological properties of *I. suffruticosa*, especially its genotoxic effects. Due to the anti-inflammatory, anticancer and antitumor properties, liver protective agent and low toxicity level, previously reported, our hypothesis is that the plant has low hemolytic effects and cytotoxicity. The aim of this study was to evaluate the effect of aqueous extract of *I. suffruticosa* leaves on the osmotic fragility of sheep blood, cytotoxic and genotoxic properties on *Allium cepa*.

2. Materials and Methods

2.1. Plant material

The *Indigofera suffruticosa* species was collected in the neighborhood of Várzea in the city of Recife, in March 2018. It was cataloged at the UFP - Geraldo Mariz Herbarium of the Federal University of Pernambuco (UFPE) under the number 83424 at the UFPE Bioscience Center.

2.2. Obtaining the aqueous extract of leaves of *Indigofera suffruticosa* (AELIs)

The extracts were obtained through the maceration method described by Leite *et al*.[6] The newly collected dried leaves (300 g) were reduced to small fragments and the extraction procedure occurred at room temperature for 72 hours with distilled water in a 1:100 ratio. After that the extract was filtered and subjected to lyophilization at -18 °C to 13.3 °C in a yield of 2.38 g.

2.3. Osmotic fragility assay

The osmotic fragility assay followed the methodological procedures described by Dacie and Lewis.[12] Samples of commercial lamb blood were purchased from Laborclin® (25 µL). Then, 5mL of 0.9 % NaCl saline was distributed in 7 tubes. In tube 0, 25 µL of lamb blood was added and incubated for 30 minutes. The following tubes from 1 to 6

received AELIs at the concentrations: 1000 µg.mL^{-1}; 750 µg.mL^{-1}; 500 µg.mL^{-1}; 250 µg.mL^{-1}; 100 µg.mL^{-1}; 50 µg.mL^{-1} respectively. Then each tube received 25 µL of lamb blood and was incubated for 30 minutes, subsequently centrifuged for 3500 rotations per minute for 15 minutes. Afterwards, the supernatant was placed in the Bioplus spectrophotometer with wavelength 540nm. The hemolysis percentage was obtained based on the formula:

$$\% = \frac{Ab. \; . \; 100\%}{1{,}49}$$

For negative control, 0.9 % isotonic sodium chloride solution and the positive control with distilled water were used, which were submitted to the same procedures used in the test samples. The assay was performed in triplicate and the hemolytic percentage was set with positive control being designated as 100 %. The hemolysis degree was qualitatively evaluated by reddish tone (hemolysis) in the supernatant obtained after centrifugation. They were attributed to the intensity of hemolysis, where a (+) cross indicates slight hemolysis, two crosses (++) significant hemolysis and three crosses (+++) indicates intense hemolysis.

2.4. *Allium cepa* assay

The experiment was carried out following previously described methodological procedures,[13] with adaptations. Onion bulbs were obtained at a local market and chosen according to their size (approximately 3.5 cm diameter) and appearance. The external cataphylls and old roots were removed with care, and the bulbs were washed, dried and kept at 4 °C until the beginning of the experiment. The treatment was simultaneous for each concentration, and including the positive control, five bulbs were used. They were placed in vials filled with AELIs solution at concentrations of 50 µg.mL^{-1}, 500 µg.mL^{-1}, 1000 µg.mL^{-1}, with 5 replicates each; distilled water was used as negative control. Root length evaluation was performed for 96 hours. Three major roots were measured by bulb with the aid of a pachymeter, collected, washed in distilled water, hydrolyzed with 1 mol.L^{-1} HCl for 10 minutes. They were then crushed and placed in glass microscope slides with a drop of 45 % acetic acid for 5 minutes. The roots were stained with 15 % hematoxylin for 15 minutes for each concentration of the extract and control, ten preparations were analyzed (1000 cells per concentration and control) a submitted to a "blind" test at a magnification of x1000. The mitotic index (MI) calculation, which was established as the ratio between the number of cells in division and the total number of cells analyzed. For MI the following equation was performed:

$$IM = \frac{NCM}{TNC} X \; 100$$

NCM: number of cells in mitosis. TNC: total number of counted cells. From the values obtained in the above equation, it was possible to evaluate the cytotoxic potential of the samples in inhibiting or increasing cell proliferation.

2.5. Statistical analysis

Results are presented as mean ± standard deviation (SD). The data were analyzed by means of analysis of variance (ANOVA) followed by Tukey and Bonferroni's tests by using GraphPad Prism (version 5.0), considering $p<0.05$ with a confidence level of 95 %.

3. Results and Discussion

3.1. Osmotic fragility assay

According to Elias *et al*.,[14] osmotic fragility can be influenced by several factors such as shape, volume and size of the erythrocyte, as well as the type and amount of hemoglobin, differences in its membrane viscoelasticity and chemical and structural composition, it has also been emphasized that the cell shape is affected by changes in cell membrane composition. Study investigated the effect of AELIs at different concentrations (50 µg.mL^{-1}, 100 µg.mL^{-1}, 250 µg.mL^{-1}, 500 µg.mL^{-1}, 750 µg.mL^{-1}, 1000 µg.mL^{-1}) in the osmotic fragility test against sheep blood. The results of the

effect of AELIs on the repeated concentrations on sheep blood were evaluated qualitatively, the supernatant and the precipitate remained transparent, with no signs of hemolysis, characterizing red blood cell integrity, similar to the negative control (-). Whereas in the positive control, supernatant presented a reddish hue, characterizing hemolytic action (++). According to Leite[6] extracts of leaves, stems and fruits of *I. suffuticosa* has mainly lectinic activity, with rabbit erythrocytes being inhibited by carbohydrates and glycoproteins. Lectin refers to a class of proteins of non-immunological origin, which can agglutinate erythrocytes thanks to their property of reversibly binding to carbohydrates. They can act as cell reconnaissance sites.The phytochemical investigation of leaf extracts of *I. suffruticosa* revealed the presence of alkaloids, flavonoids, steroids, proteins, carbohydrate, triterpenes and indigo coumarin.[8] In the case of the species *I. suffruticosa* our results are compatible with some data already recorded in these studies. The toxicity of some secondary metabolites present in the vegetables is quite relatada.[15] According to Dewick,[16] alkaloids, even in small concentrations, are naturally toxic substances. Triterpenic saponins are natural compounds also associated with toxicity, due to their ability to cause haemolysis. Its hemolytic effect is the result of the ability to interact with the cell membrane elements of red blood cells, especially with cholesterol molecules, causing a deformation in the membrane and as a consequence, causing extravasation of the content intracellular.[16,17,18]

Diseases associated with metabolic changes can modify the proportion of phospholipids and cholesterol in the erythrocyte membrane and thus affect osmotic fragility. Mature erythrocytes are unable to perform lipid synthesis due to absence of the acetyl CoA carboxylase enzyme, and thus the cell membrane undergoes changes in its lipid composition according to the changes of the lipids present in circulation.[19] A number of toxicological tests are used to assess the concentrations and exposure time required for toxic agents to produce adverse effects on organisms.[20]

The AELIs at different concentrations in the spectrophotometer reading, where the supernatant remained between 9-10 %, presenting low hemolysis percentages and featuring no significant difference between the concentrations used. According to Rangel *et al.*,[21] a hemolysis percentage between 0-40 % is characterized as low, between 40-80 % considered moderate and above 80 % considered as high. In the present study, the hemolysis percentage was considered low, since it did not exceed 10 %, suggesting cellular membrane integrity.

3.2. *Allium cepa* assay

The *Allium cepa* test is commonly used in toxicological tests, in studies using plant extracts, to evaluate macroscopic parameters such as color change, shape, root size, as well as microscopic parameters such as changes during the meristematic division of onion.[22]

In addition to the common cytology parameters, such as mitotic index and chromosome abnormalities, the macroscopic root growth parameter of *Allium cepa* was also investigated. Table 1 shows results of the mitotic index, mitotic activity and chromosomal abnormalities that were not found in the *Allium cepa* meristem with the different treatments. Statistical analysis did not indicate any change in mitotic activity. The cytological effect of the control group during the cell division process presented the following percentages: prophase (22 ± 0.70), metaphase (4.50 ± 0.95), anaphase (4.7 ± 1.10), telphase (2 ± 0.40) in the concentrations 50 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$, 1000 $\mu g.mL^{-1}$AELIs during cell division: prophase (33.75 ± 4.32; 49.5 ± 8.09; 34.75 ± 6.45); metaphase (6 ± 1.08; 3.50 ± 0 .50; 1.75 ± 1.03) anaphase (2.25 ± 0.75; 2.25 ± 0.25; 0.50 ± 0.50) and telophase (1.75 ± 0.25; 1.50 ± 0.50; 0.50 ± 0.28) respectively. Generally, the MI is a reliable parameter that allows estimating the frequency of cell division, which is generally used for the screening of cytotoxic agents.[23] A significant reduction of MI may be due to the mitodepressive action of substances, so agents used to interfere in the normal cell cycle may can decrease the number of dividing

cells.[24] Mitotic index was similar between treatments: control (13.3 ± 2.09) and AELIs at concentrations of 50 µg.mL^{-1} (17.5 ± 3 .57), 500 µg.mL^{-1} (22.7 ± 5.57) and 1000 µg.mL^{-1} (17.8 ± 3.38).

Table 1. AELIs cytological effects during the cell division process of the *Allium cepa* root (n=5)

Treatment	Interphase	Prophase	Metaphase	Anaphase	Telophase	MI %
Control	219.25 ± 18.80	22 ± 0.70	4.50 ± 0.95	4.75 ± 1.10	2 ±0 .40	13.3 ± 2.09
50 µg/mL	219.50 ± 5.26	33.75 ± 4.32	6 ± 1.08	2.25 ± 0.75	1.75 ± 0.25	17.5 ± 3.57
500 µg/mL	201.50 ± 1.32	49.5 ± 8.09	3.50 ± 0.50	2.25 ± 0.25	1.50 ± 0.50	22.7 ± 5.57
1000µg/mL	165.25 ± 6.34	34.75 ± 6.45	1.75 ± 1.03	0.50 ± 0.50	0.50 ± 0.28	17.8 ± 3.38

Values are mean ± standard deviation (SD) (n= 1000 cells/group); $p<0.05$ significantly different (at same cell division phase); (ANOVA followed by *one way* Tukey *post test*); MI: mitotic index

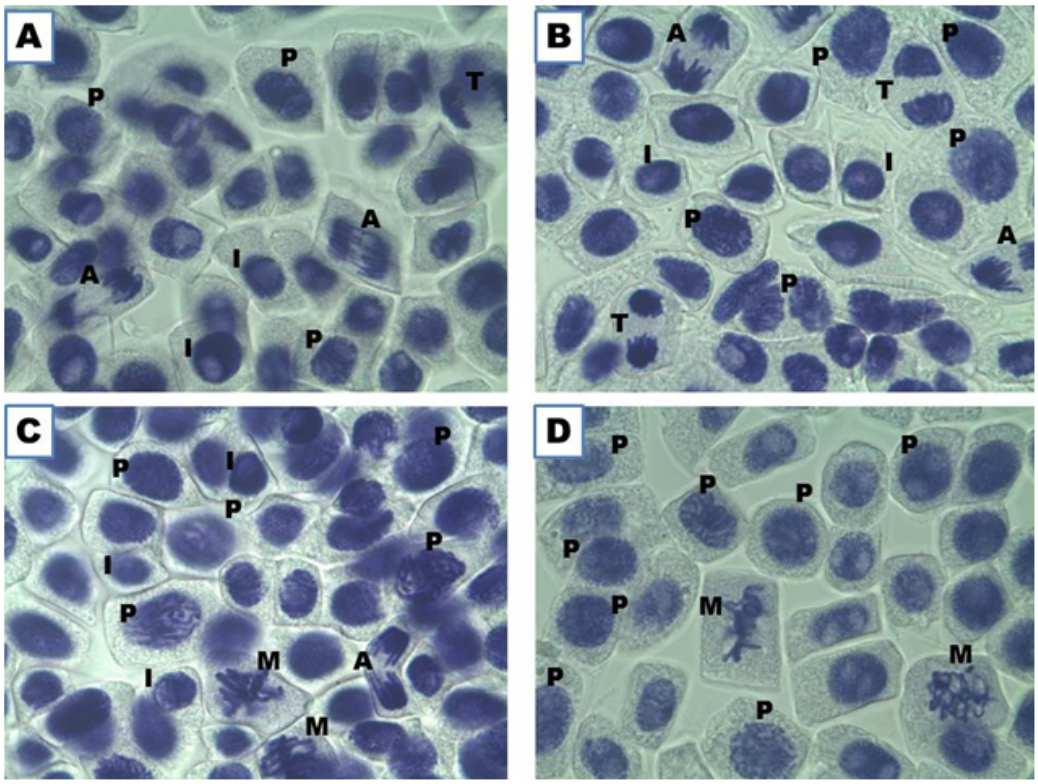

Figure 1. Photomicrographs of *Allium cepa* root cells in the presence of AELIs. Negative control **(A)**, AELIs at concentrations 50 µg.mL^{-1} **(B)**, 500 µg.mL^{-1} **(C)** e 1000 µg.mL^{-1} **(D)**. P-Prophase; M-Metáfase; A-Anaphase; T-Telophase; I- Interphase. Magnification - 1000X

Toxicity studies in genetic material or genotoxicity are designed to determine chemicals that can perturb and modify the genetic material causing gene or chromosomal mutations. Several of assay systems, especially in vitro systems, have been devised to detect the genotoxic effect of different substances, results are usually used as indicators for mutagenic effects.[25]

Cytological observations during the cell division phases (interphase, prophase,

metaphase, anaphase and telophase) of the *Allium cepa* roots of the control were similar to the different AELIs concentrations. No chromosomal abnormality or alteration in the phases of the cell divisions was found (Figure 1). Table 2 shows the results of the AELIs effect on root growth of *Allium cepa*. Statistical analysis indicated difference between treated and control groups. Root growth of the negative control (4.10 ± 0.21), did not show significant difference when compared to the extract at a concentration of 50 $\mu g.mL^{-1}$ (4.68 ± 0.25) ($p>0.05$). However, compared to concentrations of 500 $\mu g.mL^{-1}$ (1.23 ± 0.09) and 1000 $\mu g.mL^{-1}$ (0.70 ± 0.08), it caused growth inhibition ($p<0.001$).

In a study reported in the literature on acute toxicity with Swiss albino mice using aqueous extract of *I. suffruticosa* leaves, low order of toxicity was found.[6] It has also been demonstrated that the aqueous extract of leaves of *I. suffruticosa* inhibits the development of egg hatching, larval ecdysis and has a deterrent effect on oviposition of *Aedes aegypti* mosquitoes.[10] Mice with sarcoma 180 presented tumor inhibition with low order of toxicity when treated with the extract at the dose of 50 $mg.kg^{-1}$ (ip) by infusion (64.53 %) and maceration (62.62 %)

Table 2. Effects of AELIs on Allium root growth of *Allium cepa* (n = 5 onions/concentration or group)

Treatment	Average root length in time: 96hs (cm)	Reduction of root growth (%)
Control	4.10 ± 0.21	0
50 µg.mL-1	4.68 ± 0.25	0
500 µg.mL-1	1.23 ± 0.09 ***	70.00
1000 µg.mL-1	0.70 ± 0.08 ***	82.92

Values are mean ± standard deviation (SD); $p<0.05$ significantly different; (ANOVA followed by two way Bonferroni's test); ***Significantly different from the control group

4. Conclusion

In our study the erythrocyte osmotic fragility and meristematic onion cells in the presence of AELIs demonstrated low

hemolysis index and absence of chromosomal abnormality or alterations in the cell divisions phases, suggesting cell membrane integrity and low toxicity, corroborating with the previously reported activities on the *I. suffruticosa* species.

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