

Phytochemical Approach and Evaluation of the Osmotic Fragility and Cytotoxic Activity of *Piptadenia stipulacea* (Benth) Ducke

Abordagem Fitoquímica e Avaliação da Fragilidade Osmótica e Atividade Citotóxica de *Piptadenia stipulacea* (Benth) Ducke

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ABSTRACT

Piptadenia stipulacea (Benth.) Ducke, Fabaceae, is a medicinal plant that can be found in the Caatinga is the only exclusively Brazilian biome. Various biological activities, such as antimicrobial, anti-inflammatory, antifungal and antioxidant activities are reported for this plant. This work investigated the chemical compounds present in the ethanolic extract of the leaves of *P. stipulacea* (EELPs), being used in the test of osmotic fragility in the blood of sheep and cytotoxic and genotoxic effect in the *Allium cepa* test with onion roots. Phytochemical analysis were performed by thin-layer chromatography. Results revealed the presence of tannin, alkaloids, saponins, coumarins, flavonoids and terpenes. 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* with different concentration EELPs (50 $\mu\text{g.mL}^{-1}$, 500 $\mu\text{g.mL}^{-1}$ e 1000 $\mu\text{g.mL}^{-1}$) no chromosomal abnormality were identified in the cell division process (Interphase, prophase, metaphase, anaphase, telophase) in relation to the control group. However, the EELPs at the concentration of 50 $\mu\text{g.mL}^{-1}$ presented a root development when compared to 500 $\mu\text{g.mL}^{-1}$ and 1000 $\mu\text{g.mL}^{-1}$. This demonstrates a pharmacological importance of this plant and low hemolysis index, cell membrane integrity and low toxicity absence of chromosomal abnormality. The results suggest that *P. stipulacea* may present antigenotoxic.

Keywords: *Piptadenia stipulacea*, phytochemical, haemolytic action, cytotoxicity, cell division

RESUMO

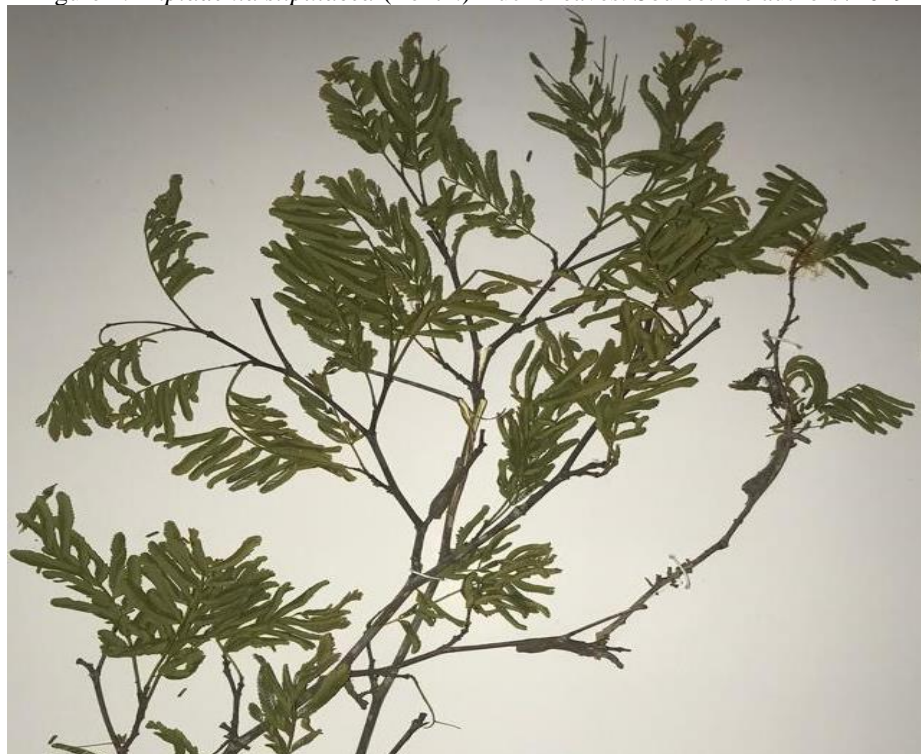
Piptadenia stipulacea (Benth.) Ducke, Fabaceae, é uma planta medicinal que pode ser encontrada na Caatinga, único bioma exclusivamente brasileiro. Diversas atividades biológicas, como antimicrobiana, antiinflamatória, antifúngica e antioxidante são relatadas para esta planta. Este trabalho investigou os compostos químicos presentes no extrato etanólico das folhas de *P. stipulacea* (EELPs), sendo utilizados no ensaio de fragilidade osmótica no sangue de carneiro e efeito citotóxico e genotóxico no ensaio *Allium cepa* com raízes de cebola. As análises fitoquímicas foram realizadas por cromatografia em camada delgada. Os resultados revelaram a presença de taninos, alcalóides, saponinas, cumarinas, flavonóides e terpenos. O ensaio de fragilidade osmótica eritrocitária mostrou baixos níveis de hemólise. Observações citológico com *Allium cepa* com diferentes concentrações de EELPs (50 $\mu\text{g.mL}^{-1}$, 500 $\mu\text{g.mL}^{-1}$ e 1000 $\mu\text{g.mL}^{-1}$) nenhuma anormalidade cromossômica foi identificada no processo de divisão celular (interfase, prófase, metáfase, anáfase, telófase) em relação ao grupo controle. Porém, os EELPs na concentração de 50 $\mu\text{g.mL}^{-1}$ apresentaram desenvolvimento radicular quando comparados a 500 $\mu\text{g.mL}^{-1}$ e 1000 $\mu\text{g.mL}^{-1}$. Isso demonstra a importância farmacológica desta planta e baixo índice de hemólise, integridade da membrana celular e baixa toxicidade ausência de anomalia cromossômica. Os resultados sugerem que *P. stipulacea* pode apresentar propriedades antigenotóxicas.

Palavras-chave: *Piptadenia stipulacea*, fitoquímica, ação hemolítica, citotoxicidade, divisão celular

1 INTRODUCTION

The study of medicinal plants is important not only for the confirmation of therapeutic uses, but also for the identification of potentially toxic, carcinogenic or teratogenic components (Silva et al., 2017; Silva et al., 2017). *Piptadenia stipulacea* (Benth.) Ducke (figure 1) is a typical tree of the caatinga, commonly known as white swear and easily found at roadsides, its wood is used in civil construction, fabrication of coal and also in forest restoration. It has been widely used for the treatment of relief of stomach pain, vomits, diarrhea and antithermic (Bezerra et al., 2011). Pharmacological studies showed properties such as anti-inflammatory, antifungal and antioxidant (Almeida et al., 2005). The microbiologic evaluation of the extract species showed that more one part of the plant had antimicrobial activity (Bezerra et al., 2011). 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% (Jain, C.N., 1986). 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* (Makinde et al., 1994). 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 (Jain, C.N., 1986; Perk, k., 1964). 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 (Arraes et al., 2012). 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 (Ribeiro et al., 1991). Prior knowledge of the chemical components found in vegetables is necessary, as it provides a list of their main metabolites. (Albuquerque; Andrade, 2002). Once the presence of certain chemical groups is detected, it is directed to future analyzes. However, little is known about is chemical constitution and therapeutic properties on the species *P. stipulaceae*, which suggests research on this aspect. Due to knowledge, that chemical analysis and the potential for human toxicity investigation are important tests for the medical use of plants, this work aimed evaluated the phytochemical screening of *Piptadenia stipulacea* the to investigate the sheep e osmotic fragility in blood of mutton cytotoxic and genotoxic on *Allium cepa*.

Figure 1. *Piptadenia stipulacea* (Benth.) Ducke leaves. Source: the authors /2019



2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

The species *Piptadenia stipulacea* (BENTH.) Ducke were collected in the city of Caruaru, state of Pernambuco, Brazil, in March 2018. The sample was certified by the biologist Marlene Barbosa from the Department of Botany at the Federal University of Pernambuco (UFPE) and it is deposited under the N°83426 in the Herbarium of the Center for Biological Sciences - (UFPE).

Figure 2. The Caatinga region in Caruaru, Pe, Brazil.



2.2 EXTRACTION AND PHYTOCHEMICAL SCREENING

The leaves of *P. stipulaceae* were weighed (50 g), were washed in running water, pulverized and extracted with ethanol (EOH) at room temperature for 72 hours. Then, the solvent was filtered and evaporated at 35°C under reduced pressure. A presence of alkaloids, triterpenoids, flavonoids, coumarins, and saponins was by tested by thin layer chromatography (TLC). The mobile phase used for flavonoids and was ethyl acetate-formic acid-acetic acid-water (AcOEt-HCOOH-AcOH-H₂O 100:11:11:27v/v), for coumarins ether-toluene-acetic 10% (50:50:50 v/v) was used. The visualization reagents used for flavonoids and alkaloids were respectively etilborilaminoester acid (Neu) and Dragendorff. For Coumarins, the visualization method used was UV light on 365nm (Wagner et al., 1996). The presence the saponins was test by mechanical shaking of the extract and visualization of foam (Simões et al., 2004). Formation of foam for 15 min was considered as positive for presence of saponins (Dewick, 2002). The presence of tannins was investigated by addition of iron chlorine 0,5M to the Fresh dilution of dried extract in saline solution (0.9 % NaCl) was prepared on the day of experiments.

2.3 OSMOTIC FRAGILITY ASSAY

The osmotic fragility assay followed the methodological procedures described by Dacie and Lewis (1975). 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 EELPs 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: $\% = \frac{Ab. \cdot 100\%}{1,49}$. 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 (Guerra, et al., 2002) 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 EELPs 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 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: $IM = \frac{NCM}{TNC} \times 10$. 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 through analysis of variance (ANOVA) followed by Student T and Tukey tests to compare the averages using the Graph Pad prism software (Version 5.0.). Considering $p < 0.05$ with a confidence level of 95%.

3 RESULTS AND DISCUSSION

3.1 PHYTOCHEMICAL APPROACH

The expressive use of medicinal plants promotes a growing need to understand the properties of vegetal compounds and their possible biological active behaviors. The yield of the crude extract was calculated in relation to the weight of the collected material and obtained after the evaporation route. The crude ethanolic extract of leaves the *P. stipulaceae* (EELPs) considering the respective masses, concentration and yields, resulted in the following data: leaf

mass 50g, concentration 18.43g mL, yields 36.86%. Results from the phytochemical study using ethanol extract of leaves of *P. stipulaceae* (EELPs) are shows in table 1.

TABLE 1. Phytochemical prospection of the ethanoic extract of leaves of the *P. stipulaceae* (EELPs)

Classes of Metabolites	Results
Tannin	+++
Alkaloids	+++
Saponins	+++
Terpenes	++
Coumarins	+++
Flavonoids	+++

Expressing result: less expressive; (++); more expressive (+++).

The EELPs showed the presence more expressive of tannin, alkaloids, saponins, coumarins and flavonoids, and less expressive of triterpenes. These results are consistent with the chemical prospecting carried out by Bezerra et al., (2011) which indicated the presence of tannins and other phenolic compounds, as well as the presence of saponins in the leaves extract of *P. stipulaceae*. Pharmacognostic studies conducted in Mexico point to tannins as one of the main compounds responsible for biological activities. It is identified that the high productivity of tannins may represent an industrial potential in the alternative generation of antimicrobials (Reviera-Arce et al., 2007). The presence of tannins was very expressive in study. The vegetation of the Caatinga has great botanical potential but little explored as to the knowledge of the chemical composition and the therapeutic potential of its vegetables. Among the species of this biome, the white jurema (*P. stipulaceae*) is abundant and appreciated as food for animals. The presence of alkaloids was confirmed in the EELPs. According to Bezerra et al., (2011) in the extracts of the stem bark, heartwood and leaf of *P. stipulaceae* (white jurema) the results were negative for alkaloids. Saponin compounds were found in the EELPs. They have several biological activities; the ones that should be highlighted are related to increases in immune response and rupture of erythrocytes such as immunologic adjuvant and hemolytic activities (Kaiser, Pavel, & Ortega, 2010). Flavonoids and coumarins compounds were qualitatively identified in our study are a class of polyphenols that are present in a relative abundance on vegetables secondary metabolites. Unexampled of a chemo preventive strategy is the use of group natural products known as flavonoids. They are polyphenolic compounds that occur naturally in foods of plant origin. Flavonoids are generally nontoxic and demonstrate a variety of biological activities such as anti-allergic, anti-inflammatory, anti-oxidative, free radical remove, , anti-mutagenic and modulator of enzyme activities (Heo et al., 2001; Agati et al., 2012).

3.2 OSMOTIC FRAGILITY ASSAY

According to Elias et al., (2014) 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 EELPs at different concentrations ($50 \mu\text{g.mL}^{-1}$, $100 \mu\text{g.mL}^{-1}$, $250 \mu\text{g.mL}^{-1}$, $500 \mu\text{g.mL}^{-1}$, $750 \mu\text{g.mL}^{-1}$, $1000 \mu\text{g.mL}^{-1}$) in the osmotic fragility test against sheep blood. The results of the effect of EELPs 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 (++) . A toxicidade de alguns metabólitos secundários presentes nos vegetais é bastante relatada (Silva et al, 2012). According to Dewick, (2002) alkaloids, even in small concentrations, are naturally toxic substances. Triterpene 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. (Dewick, 2002; Glauert et al., 1967; Karabaliev et al., 2003). The phytochemical investigation the ethanolic extract of leaves of *P. stipulaceae* (EELPs) revealed the presence of alkaloids and saponins, however it did not show signs of hemolysis in the osmotic fragility assay characterizing cell integrity. Flavonoids with a very expressive presence in our study, according to Costa-Lotufo et al., (2003) flavonoids have their cytotoxic activity established in different tumor cells. 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 (Barabino, et al., 2010). A number of toxicological tests are used to assess the concentrations and exposure time required for toxic agents to produce adverse effects on organisms (Braga, et al., 2015). The EELPs at different concentrations in the spectrophotometer reading, where the supernatant remained between 10-13%, presenting low hemolysis percentages and featuring no significant difference between the concentrations used (Table 2). According to Rangel et al. (1997), 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.

Table 2- Percentages of sheep blood hemolysis in the presence EELPs

EELPs	% hemolysis
50 µg/mL	10,73%
100 µg/mL	10,46%
250 µg/mL	10,77%
750 µg/mL	10,77%
1000 µg/mL	13,14%

3.3 ALLIUM CEPA ASSAY

Many natural compounds of plant origin are known to have chemopreventive properties. (Lima et al 2019). Mankind has always been exposed to many chemicals present in food and in pharmaceutical products, including those used in folk medicine. It has been documented that some natural compounds in foods and beverages for human consumption have an anti-mutagenic or anticarcinogenic effect. (Romero-Jiménez et al., 2005). 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 (Longhin, 2008). In addition to the common cytology parameters, such as mitotic index (MI) and chromosome abnormalities, the macroscopic root growth parameter of *Allium cepa* was also investigated. Table 2 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 (42 ± 1.91), metaphase (1.25 ± 0.75), anaphase (1.75 ± 0.62) and telophase (0.75 ± 0.47) in the concentrations $50 \mu\text{g.mL}^{-1}$, $500 \mu\text{g.mL}^{-1}$, $1000 \mu\text{g.mL}^{-1}$ EELPs during cell division: prophase (25.22 ± 1.73 ; 31.68 ± 3.45 ; 34.75 ± 0.91), metaphase (2.43 ± 0.0 ; 3.75 ± 1.26 ; 0.0 ± 0.0), anaphase (1.65 ± 1.52 ; 1.88 ± 0.40 ; 1 ± 0.57) and telophase (1.36 ± 0.66 ; 0.66 ± 0.47 ; 0.25 ± 0.25) 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 (Fernandes, et al., 2007). 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 decrease the number of dividing cells (Sharma, et al., 2012). Mitotic index was similar between treatments: control (45.75 ± 3.22) and EELPs at concentrations of $50 \mu\text{g.mL}^{-1}$ (39.66 ± 1.76), $500 \mu\text{g.mL}^{-1}$ (49.33 ± 5.20) and $1000 \mu\text{g.mL}^{-1}$ (35.25 ± 1.03).

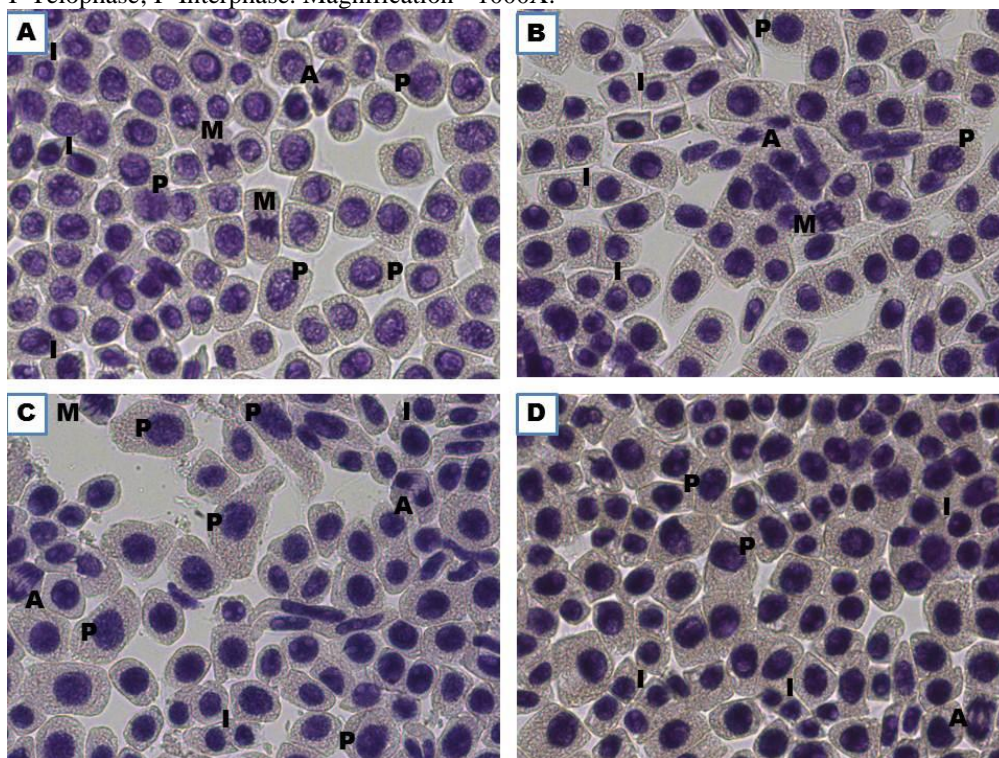
Table 2. EELPs cytological effects during the cell division process of the *Allium cepa* root (n=5).

Treatment	Interphase	Prophase	Metaphase	Anaphase	Telophae	MI %
Control	252.25 ± 29.62	42 ± 1.91	1.25 ± 0.75	1.75 ± 0.62	0.75±0.47	45.75 ± 3.22
50 µg/MI	253.65 ± 51.24	25.22 ± 1.73	2.43± 0.0	1.65 ± 1.52	1.36±0.66	39.66 ± 1.76
500 µg/MI	231.77 ± 22.69	31.68 ± 3.45	3.75 ± 1.26	1.88 ± 0.40	0.66±0.47	49.33 ± 5.20
1000µg/mL	234.25 ± 43.55	34.75 ± 0.91	0,0 ± 0.0	1 ± 0.57	0.25±0.25	35.25 ± 1.03

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.

It has been documented that some natural compounds in foods and beverages for human consumption have an anti-mutagenic or anticarcinogenic effect (Romero-Jiménez et al., 2005). [36]. 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. (Adeyemo, et al., 2013). 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 EELPs concentrations. No chromosomal abnormality or alteration in the phases of the cell divisions was found (Figure 3).

Figure 3. Photomicrographs of the cell division of the root *Allium cepa* in the presence of EELPs. Negative control (A), EELPs at concentrations 50 $\mu\text{g.mL}^{-1}$ (B), 500 $\mu\text{g.mL}^{-1}$ (C) e 1000 $\mu\text{g.mL}^{-1}$ (D). P- Prophase; M-Metaphase; A-Anaphase; T-Telophase; I- Interphase. Magnification - 1000X.



An alternative strategy currently in use is to consume anti-carcinogenic/anti-mutagenic substances that could prevent or reverse some of the effects produced by carcinogens (Romero-Jiménez et al., 2005). Table 3 shows the results of the EELPs effect on root growth of *Allium cepa*. Statistical analysis indicated difference between treated and control groups. Root growth of the negative control (5.47 ± 0.40), did not show significant difference when compared to the extract at a concentration of 50 $\mu\text{g.mL}^{-1}$ (4.39 ± 0.54) ($p > 0.05$). However, compared to concentrations of 500 $\mu\text{g.mL}^{-1}$ (0.69 ± 0.11) and 1000 $\mu\text{g.mL}^{-1}$ (0.35 ± 0.06), it caused growth inhibition ($p < 0.001$). Phytochemical analysis indicated the presence of relevant groups of secondary metabolites. The phytochemical study of the EELPs showed the presence of the following chemical groups: tannin, alkaloids, saponins, coumarins and flavonoids. This result indicates that the active compounds that show protective effect are present in greater amounts in these concentrations. As shown by the analysis of phytochemicals, EELPs presents

Table 3. Effects of EELPs 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	5.47 ± 0.40	0
50 µg.mL ⁻¹	4.39 ± 0.54	0
500 µg.mL ⁻¹	0.69 ± 0.11 ***	70.00
1000 µg.mL ⁻¹	0.35 ± 0.06 ***	82.92

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

4 CONCLUSION

Results of this study of *P. stipulaceae* leaves show that ethanol extract (EELPs) may present low level of toxicity. This demonstrates a pharmacological importance of this plant and low hemolysis index, cell membrane integrity and low toxicity absence of chromosomal abnormality. However, further experiments using different test-systems are required to establish adequate procedures for the medicinal use of this plant and to better characterize its properties.

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