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Phytotoxicity of *Solanum aculeatissimum* Jacq. leaves extract

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The objective of this study was to evaluate the phytotoxicity of *Solanum aculeatissimum* Jacq. leaves ethanolic extract in seeds germination, development and fixation of *Lactuca sativa* seedlings. The same study also aimed to assess the mitotic index of lettuce roots meristematic cells, quantification of phenols and total flavonoids and triage by mean of phytochemical testing of the main secondary metabolites classes. Bioassays of germination, development of root and hypocotyl were carried out in Petri dishes using achenes of *Lactuca sativa* L. cv. 'Grand Rapids' (lettuce). Concomitantly, were evaluated the physico-chemical characteristics (pH, osmotic potential and electrical conductivity), mitotic index, quantification of total phenols and flavonoids and determination of phytochemical profile of the treatments extract. The results obtained in the bioassays demonstrate that the ethanol extract of *S. aculeatissimuma* presents phytotoxic potential in the development of lettuce seedlings, given that the concentration of 20 mg/ml showed greater inhibition (41% of germination). The extract contains significant amounts of antioxidants, total flavonoid and phenols, where the concentration 1000µg/mL showed higher values (86.50%). Furthermore, it was possible to observe the presence of compounds with allelopathic activity in the phytochemical screening test as coumarins, tannins, terpenes, flavonoids and alkaloids. Given the above it is clear that the ethanolic extract of *S. aculeatissimum* presents allelopathic substances with phytotoxic activity that can affect the germination and development of other plant species in their natural environment.

Key words: Allelopathy, antioxidants, 1,1-diphenyl-2-picrylhydrazyl (DPPH) test, *Lactuca sativa*.

INTRODUCTION

The term allelopathy can be described as the interference of a plant in the growth and establishment of another (including microorganisms) by mean of the liberation of chemical compounds in the environment (Rice, 1984). These interactions can proportionate positive or negative responses in the target organism, and these substances

are denominated allelochemicals, which in their vast majority are originated from the secondary metabolism of plants (Blanco, 2007). Generally, allelochemicals act by promoting cellular and metabolic changes, including modifications in membranes functionality, the absorbance of nutrients and water, the photosynthetic and respiratory activities, protein synthesis and enzyme activity, and in the genetic material promoting RNA and DNA alterations (Inderjit, 2006).

Many studies aimed to better understand and identify plants with allelophatic potential in the environment, researchers believe that in the future allelochemicals can become herbicides, insecticides and even alternative nematicides, aiming to lower the presence of chemical defensives in the environment (Dias and Dias, 2007). In this context, many agronomic studies have been applied to the discovery of new substances with phytotoxic action originated from plants with allelophatic potential, aiming the control of weeds and plant pathogens (Ferreira and Aquila, 2000; Souza filho et al., 2006; Santana et al., 2006; Moreira et al., 2008; Pelegrini and Cruz-silva, 2012). Among angiosperms, the Solanaceae family is considered one of the most important because it presents a vast diversity of active secondary metabolites, which many of these metabolites present elevated antioxidant capacity (Ribeiro et al., 2007). Having as main identified component the espirostano and solasodine glycoalkaloids, very common in the *Solanun* genus, considered the most important in the family (Kohara et al., 2005), also by the allelopathic activity discovered in glycolyse alkaloids of the green fruits of *Solanum crinitum* (Alves, 2003; Lu et al., 2011; Ohyama et al., 2013; Muruhan et al., 2013).

Inside the *Solanum* genus, the *Solanum aculeatissimum* species, popularly known in Brazil as “Joá do mato” stands out for being a plant with invasive characteristics for many crops cultivated in Brazil, such as pastures, citrus orchards, gardens and grass, coffee plantations, also found in natural open fields, cerrado and Atlantic rainforest (Lorenzi, 2006). It is a semi woody weed that can reach 50 cm in height, easily characterized by the excessive number of prickles all over the plant’s end (Mentz and Oliveira, 2004). Its fruit is used directly in edema and skin conditions such as boils (Rodrigues and Carvalho, 2001). Studies point out that the methanolic extract of the leaves present a potential source of substances with antibacterial properties, among them the rutin flavonoid (Pereira, 2006).

Given the above information, this study aimed to evaluate the phytotoxic potential of the ethanolic extract of *S. aculeatissimum*, in the germination of seeds and in the growth and development of seedlings of *Lactuca sativa*. The same project also aimed to evaluate the mitotic index of lettuce roots meristematic cells, quantification of phenols and total flavonoids and triage by mean of phytochemical testing of the main secondary metabolites classes.

MATERIALS AND METHODS

Experimental procedures

The bioassays were carried out in the Plant Physiology and Phytotherapics Laboratory of the Biological Sciences Department, College of Science and Letters of Assis, Universidade Estadual Paulista (UNESP), Assis-SP, Brazil.

Sampling and preparation of the plant material

The leaves of *S. aculeatissimum* were sampled from specimens present in the College of Science and Letters, UNESP-Assis/SP (22°32’20’’S and 50°22’60’’W). The taxonomic identity was carried out with the collaboration of Dr. Renata Giassi Udulutsch, professor of State University of São Paulo “Júlio de MesquitaFilho”, Assis campus. After the samples collection, the leaves were selected and dried in forced-air oven at a temperature of 40°C for 24 h, right after, they were grinded and the resulting powder was stored in dark plastic flasks.

Preparation of the ethanolic extract

The ethanolic extract was prepared by mechanic maceration of the plant’s powder, with ethanol in PA (IMPEX, Brazil) (at a concentration of 1:10 [p/v]) for 24 h at room temperature. The extract was then filtered at low pressure under vacuum, a methodology similar to the one performed by Rutherford and Powrie (1993), Hajhashemi et al. (2003) and Boligon et al. (2009). The extraction was repeated three times with the same plant material. The resulting extracts were combined and concentrated in rotary evaporator (model: MA120, Marconi, Brazil) at a mean temperature of 60°C, then the dried residue was used in the biological assays, according to the work of Áquila (2000) and Sadraeietai (2003).

Germination bioassay (pre-emergent)

The bioassay was carried out in Petri dishes (8x8 mm) lined with germination paper, which was dampened with 1 ml of the extract diluted with distilled water for the concentrations of 5, 10 and 20 mg/ml. Fifty achenes of lettuce cv. Grand rapids were sowed by dish, separated in experimental groups and control (distilled water), incubated for 48 h in growth chamber (model: 411/FPD, Nova Ética, Brazil) in relative humidity 80 to 90% and temperature 23±2°C (Alves et al., 2004). The experimental design was fully randomized, with six repetitions of each treatment concentration or control. The germination was monitored every 6 h, with the projection and geotropic curve of the root being the germination evaluation criteria, as described by Ferreira and Áquila (2000), Ferreira et al. (2008) and Maraschin-Silva and Áquila (2006a). With the results obtained, the following indexes were calculated.

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Germination: G% = [∑ni/A].100

Mean time of germination: $\bar{t} = \frac{\sum_{i=1}^{k} niti}{\sum_{i=1}^{k} ni}$

Mean speed of germination: $\bar{v} = \frac{CV}{100} = \frac{1}{\bar{t}}$

Where, A: Total number of achenes sowed; ni: Number of achenes germinated in each instant (ti); CV: coefficient of variation; ti: the time gap between the beginning of the experiment and the observation time and k: last day of observation (Labouriau, 1983; Santana and Ranal, 2004; Pereira et al., 2009).

Physical-chemical characteristics (pH, osmotic potential and electric conductivity)

The pH of the *S. aculeatissimum* diluted extract was determined directly in the treatment solutions, using a pH meter (Tecnopon ®, model MPA210, Brazil). The osmotic potential measurement of the extracts was carried out using dilutions of polyethylene glycol (PEG-6000) to produce the osmotic potentials of -1.0 to -0.02 MPa, such as described my Villela et al. (1991) and Mazzafera (2003). The Brix refraction measurement, for each concentration of PEG-6000 and the extract was determined by a refractometer ABBÉ and the values were used to calculate the water potential, as described by Bakke et al. (2006). The electric conductivity was determined in the treatment solutions, using a conductivity meter (Instrutherm® CD-860).

Length of the radicle and hypocotyl (post-emergent)

In the growth bioassay, thirty seedlings of lettuce (with approximately 2 mm of main root length) were put in petri dishes (8x8 mm) with 1 ml of extract (5, 10 and 20 mg mL^{-1}), and distilled water, as described above. The dishes were put in growth chamber (model: 411/FPD, Nova Ética, Brazil), under the same conditions of the germination assay, as described by Ferreira et al. (2008) and Maraschin-Silva and Áquila (2006b). The experimental design was fully randomized, with six repetitions of each treatment concentration or control. The experiment was monitored at 24 and 48 h, the length of the primary root and hypocotyl were measured with digital caliper rule (modelo: IP65, DIGIMESS ®, Brasil).

Mitotic index

For the mitotic index analysis, the primary roots of *L. sativa* seedlings (approximately 2 mm length of the primary root) were collected and prepared by the squash technique (Guerra and Souza, 2002; Mahajan and Sharma, 2008). First, the roots were fixated in Carnoy solution (ethanol : glacial acetic acid 3:1) for 2 h, hydrolyzed in HCl 5N for 15 min at room temperature, washed with distilled water and colored with 5% of carmin acetic acid. The cells were observed under optic microscope, with magnification of 100x and 5000 cells were analyzed for each treatment. The mitotic index (MI) was obtained from the equation, MI=(m/T)x100, where m= the number of cells in mitosis and T= total number of cells (Pires et al., 2001; Tabur and Oney, 2009).

Quantification of total phenols and flavonoids

The quantification of phenols and total flavonoids was performed in the ethanol diluted extract with the concentrations of 25, 50, 75, 100, 250, 500 and 1000 µg/ml. For the determination of phenols content, the Folin and Ciocalteu (1927) method was carried out. For each 0.5 ml of extract in the different concentrations were added 5 ml of distilled water and 0.25 ml of Folin-Ciocalteu reagent. After 3 min, 1 ml of saturated Na_2CO_3 at 10% was added to the mixture and stored for 1 h. The absorbance was read at 725 nm using UV-Vis spectrophotometer (Model: SP220, BIOSPECTRO, Brazil). All tests were performed in triplicate and the results were expressed in mg of galic acid by gram of extract.

For the quantification of the extracts total flavonoids was carried out determination by mean of UV-Vis spectrophotometer (model: SP220, BIOSPECTRO, Brazil) reading and the samples were prepared as described by Zhishen et al. (1999), based in the complexation of flavonoids and $AlCl_3$. An aliquot of 250 µl of different concentration extracts were mixed with 1.25 ml of distilled water and 75 ml of $NaNO_2$ 5%. After 6 min, 150 ml of a 10% $AlCl_3/H_2O$ solution was added. Passed 5 more min, 0.5 ml of a 1 M NaOH solution was added and following the total volume was completed by the addition of 2.5 ml of distilled water. The samples were agitated in a vortex agitator and the absorbance measure at 510 nm. All the tests were performed in triplicate and the results expressed in mg of rutin by gram of extract.

Determination of DPPH radical scavenging activity

The DPPH radical (1,1-diphenyl-2-picrylhydrazyl, Sigma, EUA) scavenging activity was determined according to the methodology proposed by Bilos (Manian et al., 2008). The dried ethanolic extract of each sample was dissolved in ethanol (75%) in different concentrations (25, 50, 75, 100, 250, 500, 1000 µg/ml), following they were mixed with 5 ml of DPPH solution ($1.5x10^{-4}$M). The extracts reacted with the radical for 30 min at low luminosity, then the readings were performed in an UV-Vis spectrophotometer at 517 nm wavelength. The antioxidant activity calculation was carried out according to the formula: I%=[(control - sample)/control]x100. The galic acid (Vetec-QuímicaFina, Brazil) was used as reference. Triplicates were performed for the analyses. EC50 was calculated using linear regression.

Ferric-ion reducing antioxidant power (FRAP) assay

The FRAP assay was performed as previously described by Pulido et al. (2000) with some modifications. 2.7 ml of FRAP reagent, freshly prepared was mixed with 270 µl distilled water and 90 µl of each sample. Then this mixture was maintained in water bath at 37°C for 30 min. The FRAP reagent contained 2.5 ml of 10 mM TPZ solution in 40 mM HCl, plus 2.5 of 20 mM $FeCl_2 6H_2O$, plus 25 ml of 0.3 M acetate buffer (pH 3.6). Readings was done at the absortion maximum (595 nm). Solutions of known Trolox concentration was used for calibration. The final results were expressed as micromole Troloxequivalents (TE) per grams of extract (µmol TE/g of E.).

Evaluation pro-oxidant activity by methods relative electrophoresis mobility (REM)

REM was adopted from Hsieh et al. (2005) and Toda (2005). Bovine Serum Albumin - BSA (2 mg/ml) was diluted in PBS (10 mM, pH 7.4) and incubated with Cu^{2+} (2 mM) and H_2O_2 (0.25 mM) at 37°C for 24 h in the presence or absence of the herbal ethanolicextract (1000 and 500 µg/ml). Electrophoresis of BSA was performed using polyacrylamide gels (SDS-PAGE), it was prepared according to the standard technique (Encor biotechnology inc.). Running gel solution was utilized in 12% of Acrylamide, and the

Table 1. Effect of ethanolic extract of *S. aculeatissimum*on the germination and growth of *L. sativa*.

Extract (mg/ml)	Germination (%)	Mean time (h)	Mean speed (h)	Radicle length (mm)	Hypocotyl length (mm)	Mitotic index
0	98.66±1.00[a]	17.08±5.34[a]	0.07±0.04[a]	13.77±4.74[a]	3.49±0.66[a]	11.23±1.23[a]
5	92.00±3.10[a]	21.38±0.86[a]	0.05±0.00[ab]	04.81±0.62[b]	2.38±0.39[b]	10.97±1.83[a]
10	92.00±5.21[a]	26.58±1.19[b]	0.04±0.00[ab]	05.06±0.98[b]	2.52±0.56[b]	09.98±2.09[a]
20	41.33±19.0[b]	38.88±2.82[c]	0.02±0.00[b]	02.86±0.75[c]	2.01±0.55[c]	03.45±0.29[b]

The data was presented in averages±standard deviation. [a]Averages with at least one equal letter, in the column, indicate absence of significant difference (p>0.05), by the Tukey test. [b]Mitotic index = (total number of cells in division / total number of cells analyzed x 100), with at least one equal letter, in the column, indicate absence of significant difference, by the Qui-squared test (χ^2< 0.05).

Table 2. pH, osmotic potential and electric conductivity of the ethanolic extract of *S. aculeatissimum*nas in different concentrations (5, 10 and 20 mg/ml).

Extract (mg/ml)	pH	Osmotic potential (MPa)	Electric conductivity (mS/cm)
0	4.87	0.00	0.50
5	4.78	0.10	0.86
10	4.74	0.46	1.60
20	4.81	1.26	2.91

stacking gel at 5%. Proteins were stained with 0.25% Coomassie Blue R-250. Results were expressed in the REM in mm using that of native BSA as the base.

Extracts phytochemical profile determination

The phytochemical tests were carried out according to the procedures described by Sivasankari et al. (2010) to identify components, such as flavonoids, alkaloids, terpenes, triterpenoids, condensed tannins, hydrolysable tannins, coumarins, saponins, glycosides and phenols.

Statistical analysis

The data was analyzed by variance analysis and Tukey test (α =0.5). These tests were performed using the BioEstat software (version: 5.0), according to Santana and Ranal (2004) and Pereira et al. (2009). For the mitotic index analysis, the qui-squared test was performed to identify a positive answer between the experimental groups and control, according to the analyses proposed by Ribeiro et al. (2003).

RESULTS

In Table 1, the germination indexes were presented. The germinability of seeds treated with the concentrations 5 and 10 mg/ml did not present significant difference (the percentage of germination to the concentrations of 5 and 10 mg/ml was 92%), but presented values with significant difference to the 20 mg/ml treatment (percentage of germination of 41.33%) being that only in the last concentration there was significant difference to the control. Considering the mean germination time, the 5 mg/ml concentration did not present significant difference compared to the control, however it differed to the 10 and 20 mg/ml treatments, while these two treatments differed among their selves, where the 20 mg/ml reached the highest value (38.88 h). For the germination speed all concentrations (5, 10 and 20 mg/ml) did not present significant difference among each other, and the only treatment that differed from the control was the 20 mg/ml.

In the analyses of the radicle and hypocotyl it was observed that all treatments (5, 10 and 20 mg/ml) differed statistically from the control, where the concentrations of 5 and 10 mg/ml did not present significant difference. The concentration of 20 mg/ml expressed the lowest radicle length (2.86 mm) and hypocotyl (2.01 mm), being significantly different from the other concentrations. For the mitotic index, it was observed that the concentrations of 5 and 10 mg/ml did not present significant difference in comparison with the control group, only the 20 mg/ml presented significant difference from the other treatments, obtaining the lowest mitotic index of all (3.45).

Table 2 presents the physical-chemical characteristics of the ethanolic extract of *S. aculeatissimum* leaves. pH, osmotic potential and electric conductivity were measured for each concentration (5, 10 and 20 mg/ml) and the control group (distilled water). In the pH analysis, control presented the highest value (pH=4.87) compared to the treatment, while the 10 mg/ml concentration presented the lowest value (pH=4.74) among the concentrations. For the osmotic potential and electric conductivity, the 20 mg/ml concentration presented the highest values (1.26 MPa for osmotic potential and 2.91

Table 3. Antioxidant activity, total phenols and total flavonoids of the *Solanum aculeatissimum* ethanolic extract in different concentrations (mg/ml).

Extract (mg/ml)	Total phenols[a]	Total flavonoids[a]	% Antioxidant activity[b]	FRAP[c]
25	-	1.64	04.49	-
50	-	1.70	03.78	-
75	-	1.67	05.75	-
100	-	1.70	08.09	-
250	0.81	1.96	23.30	-
500	3.12	2.46	51.25	40.67
1000	3.95	3.42	86.50	55.11

[a]Average values ± standard deviation of triplicates for total phenols (equivalent mg/g of galic acid extract), total flavonoids amount (equivalent quercetin mg/g of extract); [b]Average values ± standard deviation of triplicates for test cleaning of DPPH radical scavenging activity; [c]Micromole Trolox equivalents (TE) per mg of dried extract (µmol TE/g of E.).

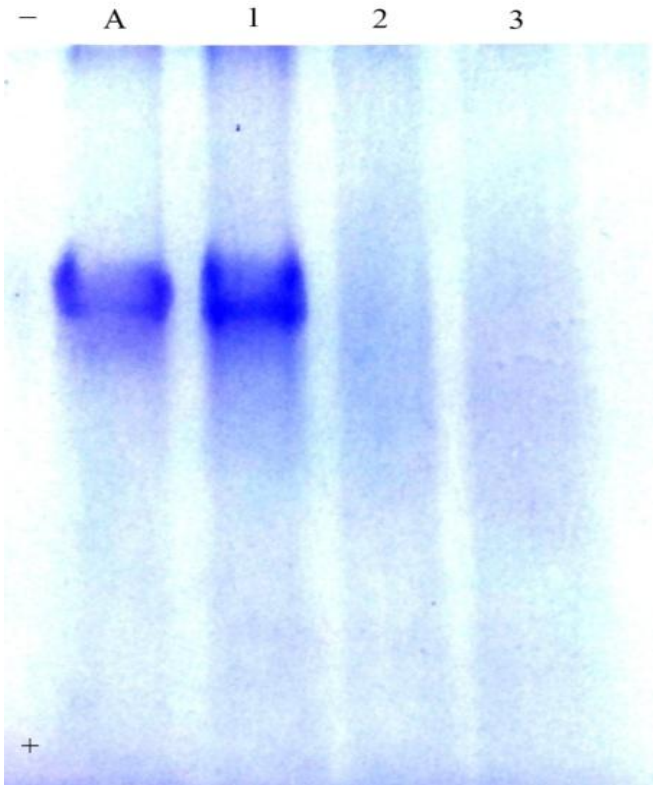

Figure 1. Effect of ethanolic extract from *S. aculeatissimum* and Cu_2+/H_2O_2 on migration of BSA with PAGE (Incubation period was 10 days). A: Native BSA, 1: BSA with Cu_2+/H_2O_2, 2: BSA with Cu2+/H_2O_2 and ethanolic extract (500 µg/ml), 3: BSA with Cu_2+/H_2O_2 and ethanolic extract (1000 µg/ml).

mS/cm for electric conductivity) compared to the other concentrations and control group.

In Table 3 are found the amounts of antioxidant, phenols and total flavonoids of the *S. aculeatissimum* ethanolicextractin different concentration (25, 50, 75, 100, 250, 500 and 1000 µg/ml), for these it was observed a dose dependence for the antioxidant activity and, phenols and total flavonoids determination, the EC50 was calculated at the concentration 556.38 µg for the antioxidant activity in DPPH assay. In the FRAP assay was found 55.11 (µmol TE/g of E.) at the concentration of 1000 µg/mL of extract.

It is possible to view electrophoresis (Figure 1) gel which was extensive fragmentation of the protein resulting from the action of BSA in conjunction extract used in two concentrations of 500 and 1000 µg/ml (lane 2 and 3), fragmentation was much higher compared to positive control who owned copper and hydrogen peroxide only in acting protein (lane 1).

The phytochemical triage of the *S. aculeatissimum* ethanolic extract is presented in Table 4. It is possible to observe the presence of coumarins, hydrolysable tannins and triterpenes, but in low visualization. For alkaloids the visualization was moderate and for flavonoids it was observed the highest visualization and test sensibility.

DISCUSSION

According to Lorenzi (2006), *S. aculeatissimum* presents high competitive capacity due to its invasive characteristics, being characterized as a weed. This peculiar characteristic of the species shows susceptibility in presenting substances of ecological importance, such as allelochemicals, as confirmed in the bioassays of this study. With the exposed, the obtained results of this study indicate that the *S. aculeatissimum* ethanolic extract possesses compounds capable of interfering in the germination index, radicle growth of lettuce and mitotic index of meristematic cells at the same laboratory conditions. Such results corrobarate to studies done by Silva et al. (2012) which showed that the concentrations of 10 and 20 $mg.mL^{-1}$ of the ethanol extract of leaves of *Zanthoxylum rhoifolium* Lam interfered significantly in the germination index and radicle growth of lettuce. According to Ferreira and Aquila (2000) and Inderjit

Table 4. Phytochemical triage of the *S. aculeatissimum* ethanolic extract.

Phytochemical components	Intensity
Alkaloids	++
Coumarins	+
Steroids	-
Flavonoids	+++
Condensed tannins	-
Hydrolysable tannins	+
Triterpens	+

+ Low visualization; ++ moderate visualization; +++ high visualization; - not visualized.

(2001), such changes may be directly linked with cellular and metabolic alterations, including modification in the membranes functioning, absorption of nutrients and water, photosynthetic and respiratory activities, cellular growth, expression and synthesis of nucleic acids and several other alterations.

pH and osmotic potential are factors that can interfere in the germination process, however the results obtained in the present study demonstrate that they stayed inside the acceptable standards of what is considered appropriate for germination and initial growth (Aquila et al., 2012) (Table 2). These evaluations are necessary, because plant extracts can present specific solutes that can change water properties, thereby being able to proportionate a false positive result for the experiment. Studies carried out by Ferreira and Áquila (2000) and Borella (2009) demonstrated that sugars, amino acids and organic acids are solutes that can mask the allelopathic effect of the extracts by pH interference or for being osmotic active.

Whereas the extract activity on the radicle length, Aquila et al. (1999) and Omezzine and Haouala (2013) showed that allelochemicals can act in different manners, depending in the environment that the target plant inhabits, once both reflect different physiological states. The results of the present study show the effects on seeds germination and lettuce seedlings development (Table 1). The results presented in the seedling development (post-emergent test), at the end of the 48 h of experimentation, present a significant reduction in the seedlings radicle and hypocotyl growth treated with the three extracts concentration, when compared to the control group (Table 1). This data showed that the extract, beyond presenting cytotoxic characteristics in the germination process as evaluated by the mitotic index determination, also presented phytotoxic action for the development of seedlings, corroborating study performed by Candido (2013) in different target plants.

Study performed by Inderjit (2011) demonstrated that plant extracts tested in bioassays for the investigation of possible allelopathic potential, are constituted by a blend of substances with primary and secondary origins of plant metabolism. According to Ahmad et al. (2011) the allelochemicals that act in pre-emergent, post-emergent and phytotoxicityare the benzoquinones, coumarins, flavonoids, terpenoids, lactones, mucilage and alkaloids that can be associated with effects on germination, plant development and possibly cell division, as demonstrated in this study.

Considering that allelochemicals are related to oxidative stress, became opportune the antioxidant evaluation of the *S. aculeatissimum* ethanolic extract, present in this study. Mori and Schroeder (2004) demonstrate that oxidative stress is a process that occurs in the plant tissues, where by action of some enzymes the superoxide radical is transformed in water. One of the many effects of allelochemical in plants is the production control and accumulation of oxygen reactive specimens (ORSs), which accumulate in cells as answer to the allelochemicals and are responsible by the cellular death (Gallice, 2011). Another mechanism related to the ORSs formation is the action of allelochemicals over the NADPH oxidase, enzyme responsible for the donation of NADPH electrons to an acceptor (O_2) forming superoxide (Foreman et al., 2003).

Thus, it was possible to analyze the antioxidant activity of the ethanolic extract, presenting activity of 86.50% in the concentration of 1000 µg/ml (Table 3). This activity is related to the presence of phenolic compounds electron donators, characterizing a reduction action, descendent of the vast presence of flavonoids in the *S. aculeatissimum* extract. It is known that flavonoids possess ideal structures for the scavenging of free radicals, being antioxidants with great reduction capacity (Ozçelik et al., 2011). With the exposed, this study also evaluated the presence of phenols and total flavonoids, for the concentration 1000 µg/ml was observed 3.95 mg equivalent grams of galic acid extract of total phenols. According to these results and according to studies mentioned above, is possible sugest that the free radicals elimination activity, just like the elevated level of poliphenols present in the extract, can be correlated with the action mechanism, characterized by the allelopathic effect of *S. aculeatissimum*.

Extracts in the presence of Cu^{2+} / H_2O_2 fragmentation of the protein was more severe, there was an enhancement by the extracts, this is a suggestion the one way how the phytotoxicityis expressed in the extract studied in this work. Considering the invasive characteristic of *S. aculeatissimum* and the results obtained in this study, it is possible to conclude that this species presents allelopathic substances with phytotoxic activity capable of interfering directly in stabilization and development of other species in their natural environment.

Conflict of Interest

The authors have not declared any conflict of interest.

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