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Genotoxic evaluation of infusions of *Urera baccifera* leaves and roots in *Allium cepa* cells

[Evaluación genotóxica de infusiones de hojas y raíces de *Urera baccifera* en células de *Allium cepa*]

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Abstract

Context: The aqueous extracts of *Urera baccifera* Wedd. leaves and roots are used to inflammatory and infectious diseases in Brazilian folk medicine. Oxalic acid, a substance co-related with toxicity and stinging, was already quantified in this plant.

Aims: To evaluate the action of leaves and roots infusions (1, 30, 75 g/L) and the oxalic acid standard on mitosis as indicative of presumably antimitotic and genotoxic actions, using the *Allium cepa* test.

Methods: Oxalic acid was quantified in the roots and leaves infusions by High-performance liquid chromatography (HPLC-DAD), with the mobile phase of 25 mM phosphate buffer (pH 2.5): acetonitrile at 95:5 (v/v). To the genotoxicity test, onion bulbs were used. After the rootlets germination, each bulb was submitted for 24 h of the individual treatments. Were analyzed 1000 cells per bulb, in a total of 5000 cells per treatment.

Results: Results showed that all concentrations of roots infusions induced chromosomes abnormalities, except for the highest, that caused a substantial inhibition in the mitosis, precluding to be observed abnormalities. In the leaves infusions, only the two higher concentrations caused the highest values of damage in the cellular cycle. The oxalic acid also caused abnormalities in the mitosis, and may be considered responsible by part of the genotoxic action of *U. baccifera*.

Conclusions: Oxalic acid can be responsible by part of the chromosomal abnormalities caused by *U. baccifera*, although, there must have more metabolites that evoke the same effect promoting the genotoxic effect of this nettle.

Keywords: Chromosome abnormalities; oxalic acid; stinging nettle; toxicity; Urticaceae.

Resumen

Contexto: Los extractos acuosos de las hojas y raíces de *Urera baccifera* (L.) Ex.: Wedd. se utilizan en enfermedades inflamatorias e infecciosas en la medicina popular brasileña. El ácido oxálico, un compuesto co-relacionada con la toxicidad y el escozor, ha sido cuantificado en esta planta.

Objetivos: Evaluar la acción de las infusiones de las hojas y las raíces (1, 30, 75 g/L) y el estándar de ácido oxálico sobre la mitosis de raíces de *Allium cepa* como indicativo de presumibles acciones antimitóticas y genotóxicas.

Métodos: El ácido oxálico se cuantificó en las infusiones de raíces y las hojas por cromatografía líquida de alto rendimiento (HPLC-DAD), la fase móvil fue tampón de fosfato 25 mM (pH 2,5): acetonitrilo a 95:5 (v/v). Para la prueba de genotoxicidad se utilizaron bulbos de cebolla. Después de la germinación de las raicillas, cada bulbo se sometió a 24 h de tratamientos individuales. Se analizaron 1000 células por bulbo, un total de 5000 células por tratamiento.

Resultados: Los resultados mostraron que todas las concentraciones de infusiones de raíces indujeron anomalías cromosómicas, excepto la concentración más alta que causó una gran inhibición de la mitosis, lo que impide al estado de anomalías observadas. En las infusiones de hojas, sólo las concentraciones superiores indujeron los valores más altos de daños en el ciclo celular. El ácido oxálico también causó alteraciones en la mitosis.

Conclusiones: El ácido oxálico podría ser responsable de parte de las anomalías cromosómicas causadas por *U. baccifera*, aunque, hay que tener más metabolitos que evocan el mismo efecto, y actuar de forma sinérgica, favoreciendo el efecto genotóxico de esta ortiga.

Palabras Clave: Ácido oxálico; anomalías cromosómicas; ortiga brava; toxicidad; Urticaceae.

Abbreviations: AB: anaphasic bridges; AN%: chromosomes abnormalities perceptual; ANA: anaphase; BLC: breaks and lost chromosomes; BN: binucleate cells; DC: disorganised chromosomes; I: interphase; ME: metaphase; MI: mitotic index; MN: micronuclei; PRO: prophase; SD: standard deviation; Tel: Telophase

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INTRODUCTION

Urera baccifera (L.) Gaudich Ex. Wedd, popularly known as stinging nettle, is used to inflammatory diseases and rheumatic pains in Latin America folk medicine (Badilla et al., 1999a). Pharmacological activities of *U. baccifera* had already been confirmed by previous studies (Badilla et al, 1999a; Badilla et al, 1999b; Badilla et al, 2006; Martins et al, 2009; Onofre and Herkert, 2012; Gindri et al, 2014a), but few studies concerning their toxic activity were performed.

Crude extracts of *U. baccifera* roots and leaves present genotoxic effects in leukocytes, damage that was attributed to the presence of oxalic acid in the plant (Gindri et al., 2014b). The cytogenetic parameters induced by aqueous extracts of *U. baccifera* roots in *Allium cepa* show a non-significate reductions in the mitotic index, and a significate chromosome alterations during the division (Amat et al., 2002).

Allium cepa test is widely used to evaluate effects or damages that mutagenic agents might cause, especially by the capacity of this plant to be in a constant mitotic division, and also easily to identify the toxic effects and alterations that could occurring over a cell cycle of this plant (Tedesco and Laughinghouse IV, 2012).

Thus, in order to determine the potential anti-mitotic activity and genotoxicity actions of *Urera baccifera,* roots and leaves infusions were analyzed using *Allium cepa* test, with the standard oxalic acid as reference.

MATERIAL AND METHODS

Plant material

The studied materials were collected from their natural habitats, in São Francisco de Assis, Brazil (29°37.115' S, 054°53.970'W; height 150 m), in November, 2012. The botanical identification of the material was performed by Prof. Dr. Renato Záchia. A voucher specimen was deposited in Herbarium of the Federal University of Santa Maria, Biology Department, with the number 13.070.

Aqueous extracts were obtained according ethnotherapeutic procedures, by infusion, in 1, 30, and 75 g/L.

Oxalic acid quantification by high-pressure liquid chromatography

Oxalic acid was quantified in the infusions of roots and leaves following the Fu et al. (2006) methodology with minor modifications. High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto-Sampler (SIL-20A), equipped with Shimadzu LC-20 AT, reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV–VIS detector DAD SPD-M20A and Software LC Solution 1.22 SP1, C-18 column (4.6 mm x 150 mm) packed with 5 µm diameter particles. The mobile phase was 25 mM phosphate buffer (pH 2.5): acetonitrile at 95:5 (v/v), at a flow rate of 0.8 mL/min and 20 µL of each sample were injected. The peak was identified by comparison with the retention time of the standard (Fig. 1) solution, at a wavelength of 207 nm. The concentrations of the samples were 5.0 mg/mL in distilled water and were tested in triplicate. The calibration curve of oxalic acid in the concentration range of 1.0 - 5.0 mg/mL was made in triplicate, and the equation obtained was y = 9116848x + 5156373, R = 0.9967. The retention time of oxalic acid standard was 2.0 min.

Genotoxic evaluation

Onion bulbs cultivated without the application of herbicides or fungicides were obtained to this test. The bulbs were scraped at the root to promote the emergence of new roots. To set-up the experiment allowing rootlets to grow, all bulbs were placed initially in a small 50 mL plastic cup containing distilled water for three days to the rootlets can emerge. After this period, the bulbs were transferred to other clean and dry containers including the samples (treatments). They were used five groups of bulbs of *Allium cepa* for each treatment, one being a negative

control in distilled water and another for the positive control in glyphosate 2 and 15%.

The rootlets were submitted for 24 hours of the individual treatments. They were collected and immediately fixed in ethanol:acetic acid (3:1), also for 24 hours. Afterwards, the rootlets were removed from the fixing solution and transferred to ethanol 70%, where they were kept under refrigeration (4°C) until used. The rootlets were hydrolyzed in hydrochloric acid for five minutes and rinsed in distilled water just before the slides preparation.

They were analyzed 1000 cells per bulb, totaling 5000 cells per treatment (Tedesco and Laughinghouse IV, 2012). The slides were prepared for squashing technique (Guerra and Souza, 2002) and stained acetic orcein 2%, and then analyzed in a Leica microscope (Leica Microsystems, Germany).

Mitotic index (M), prophase (Pro), metaphase (Me), anaphase (Ana) and telophase (Tel) indexes were the cytological parameters studied, as well as chromosomes abnormalities (AN%), production of micronuclei (MN) and binucleated cells (BN) at interphase. The mitotic index (MI) were measured as the following equation:

MI = [total number of cells observed (cells in interphase + cells in division) / number of cells in interphase] x 100.

Statistical analysis

The obtained values were statistically analyzed by means of the chi-square (χ^2) test, with a level of probability of $p < 0.05$.

RESULTS AND DISCUSSION

Oxalic acid quantification in the infusions of *U. baccifera* leaves and roots are presented in the ensuing table (Table 1).

Table 1. Quantification of oxalic acid in the leaves and roots of *Urera baccifera*.

	Leaves	Roots
Oxalic acid (mg/g)	0.263 ± 0.098	0.154 ± 0.109

The values are mean ± standard deviation. The quantification was made in triplicate.

As observed in the oxalic acid quantification, the leaves presented a value almost two times bigger than the roots. In a previous study, *U. baccifera* crude extract presented oxalic acid in lea-ves (0.44 ± 0.05 mg/g) and roots (1.79 ± 0.22 mg/g) in a higher concentration (Gindri et al., 2014b), probably due the different extraction method performed – the prior study used maceration. Usually, the infusion is used to plant parts of the soft structure while decoction is used to rigid and woody plants (Tedesco and Laughinghouse IV, 2012). In this study, the infusion was used to leaves and roots to reproduce the popular use of *U. baccifera* in Brazil.

Oxalic acid and tartaric acid were already proposed as the possible persistent pain-inducing agents in the stinging hairs of *Urtica thunbergiana,* from Urticaceae (Fu et al., 2006). The presence of oxalic acid in the infusions of *U. baccifera* confirm the stinging and pain caused by this nettle.

The *Allium cepa* cell cycle can be taken into consideration after 24 hours, and is divided into interphase and dividing cells, comprehending the prophase, metaphase, anaphase and telophase phases (Tedesco and Laughinghouse IV, 2012). The cells observed in the different phases of cell division are exposed in the next table (Table 2) and can be observed in the ensuing figures (Figs. 1-3).

The observation of cells in interphase and division cells, as well as the mitotic index, are used as indicators of an adequate proliferation of the cells (Tedesco and Laughinghouse IV, 2012). In this study, the negative control distillated water presented a value of 3.38% of mitotic index (MI) and do not differ statistically from the negative control, glyphosate 2% (MI: 3.66%), leaves 75 g/L (MI: 4.00%) and roots 1 g/L (MI: 3.72%), all treatments allowing the regular mitosis in the *A. cepa* cells.

The major concentration of glyphosate 15% was capable of inhibit the mitosis in the test plant (MI: 0.16%), being not possible to observe division of the cells. The same occurs to the roots 75 g/L (MI: 0.34%) and leaves 1 g/L (MI: 1.40%) and 30 g/L (MI: 1.84%) with significant differences ($p < 0.05$) in those treatments. Studying the aqueous extracts of *U. baccifera* roots in 75 g/L at the same

method, Amat et al. (2002) observed no significant reductions in the mitotic index of *A. cepa*.

Table 2. Meristematic cells of *Allium cepa* in different steps of the cellular cycle, submitted to different treatments of *Urera baccifera* leaves and roots.

Treatment	Concentration	I	Pro	Me	Ana	Tel	MI
Negative Control - Water	-	4831	82	41	26	20	3.38[a]
Positive Control – Glyphosate (%)	2	4917	103	30	18	32	3.66[a,b]
	15	4992	5	3	-	-	0.16[c]
Oxalic acid (mg/mL)	1	4740	184	27	10	39	5.20[d]
Roots (g/L)	1	4814	117	26	24	19	3.72[b,f]
	30	4735	168	33	35	29	5.30[d]
	75	4983	14	-	3	-	0.34[e]
Leaves (g/L)	1	4930	53	3	-	14	1.40[g]
	30	2908	40	14	3	35	1.84[h]
	75	4800	114	34	21	31	4.00[f]

I: Interphase; Pro: Prophase; Me: Metaphase; Ana: Anaphase; Tel: Telophase; MI: Mitotic index.
Different letters represent statistically different results.
In this experiments were analyzed 1000 cells per bulb, totaling 5000 cells per treatment.

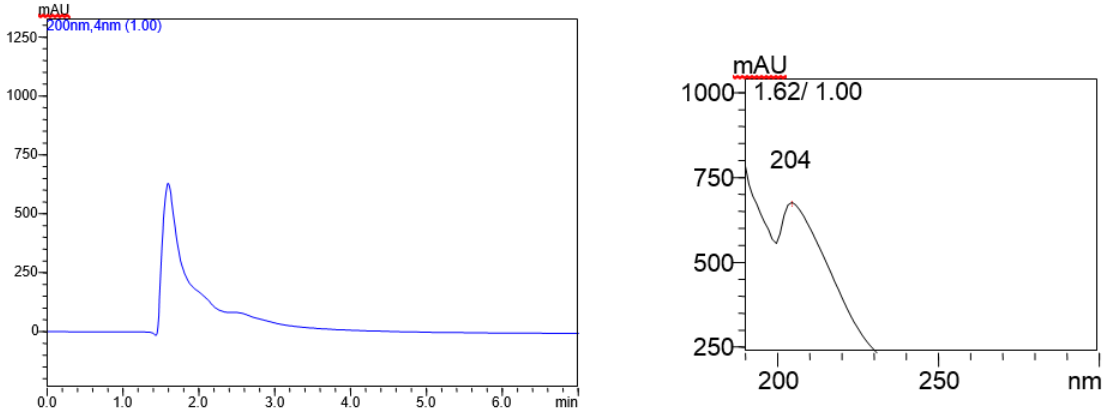

Figure 1: Chromatogram and DAD of the standard oxalic acid in 4 mg/mL.
The calibration curve with the standard and the samples were analyzed in triplicate.

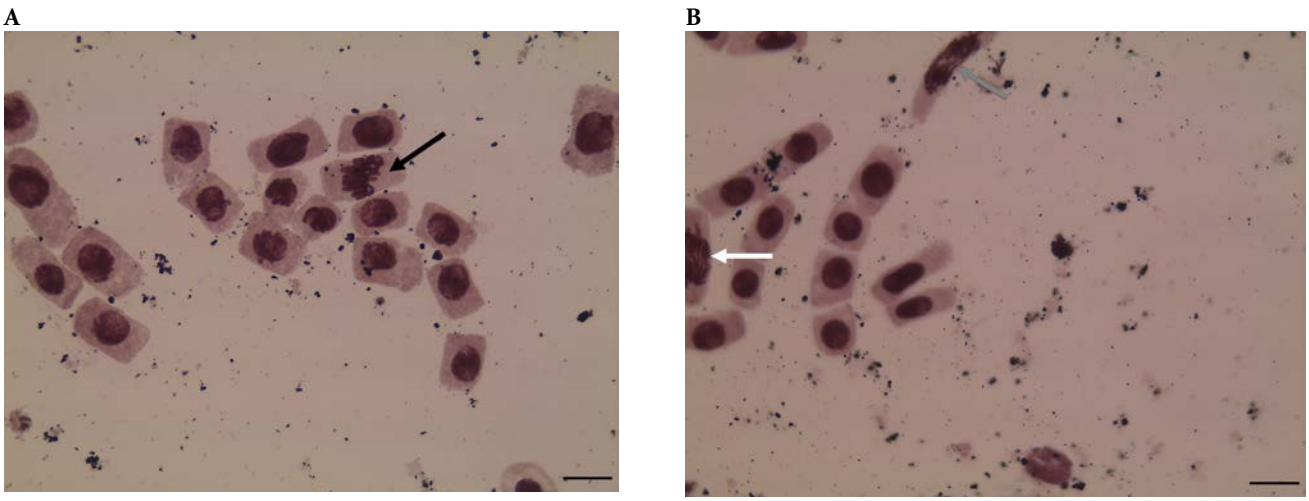

Figure 2. A and **B:** *Allium cepa* meristematic cells showing the alterations due to the action of oxalic acid. Black arrow: disorganization of the metaphase White arrow: lost chromosome; Gray arrow: anaphasic bridges. 40X. Scale: 10 µm.

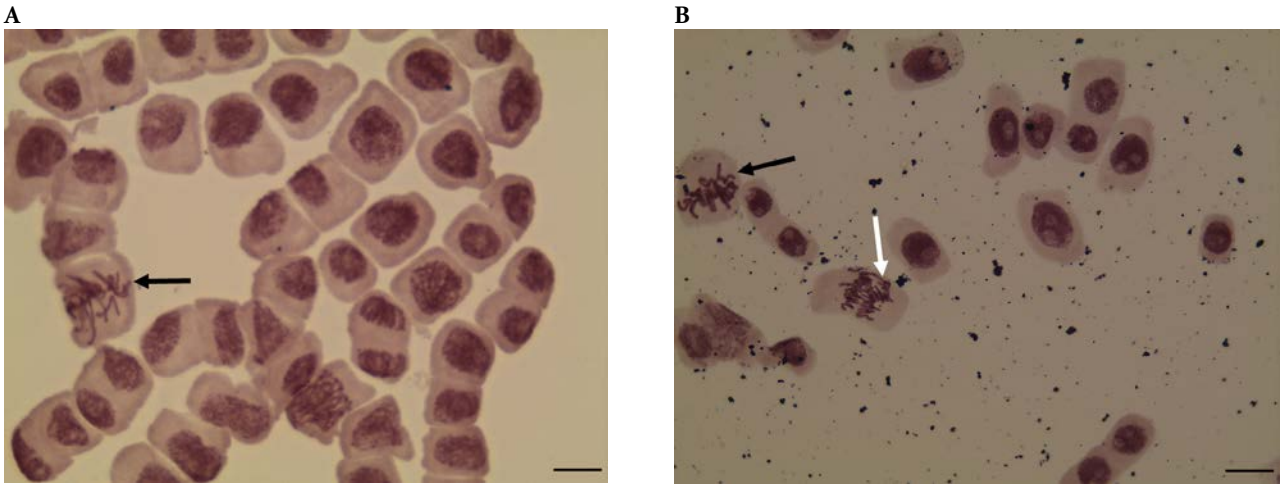

Figure 3. *Allium cepa* meristematic cells showing the alterations due to the action of *U. baccifera* infusions. **A**: roots infusion (1 g/L). **B**: leaves infusion (75 g/L). Black arrow: breaks and lost chromosomes during metaphase. White arrow: anaphasic bridges and lost chromosomes in anaphase. 40X. Scale: 10 µm.

The antiproliferative activity of plants was also observed to *Pluchea sagittalis* (Rossato et al., 2010), *Achirocline saturioides* (Fachinetto et al, 2007) and in high concentrations of *Bauhinia candicans* (Camparoto et al., 2002).

On the other hand, oxalic acid and roots 30 g/L induced mitosis in *A. cepa* cells, which was observed by the increase of division cells (PI, MeI, AI and TI) and the high value of MI (5.20 and 5.30%, respectively), being statistically different ($p < 0.05$) when compared to distillated water MI value (3.38%).

Aqueous extracts of "boldo" species' *Vernonia condensata, Plectrantus barbatus, Plectrantus amboinicus* also evoke an increase in the mitotic index when evaluated by the same method (Iganci et al., 2006).

Genotoxicity is the capacity of clastogenic agents to cause lesions in the genetic material. The evaluations of genotoxicity include, mainly, damage in the DNA, mutations and chromosomal alterations (Tedesco and Laughinghouse IV, 2012). The irregular cells observed (Figs. 1-3) among the

division cells are detailed in the ensuing table (Table 3).

During interphase, no irregularities were found. The major concentration of glyphosate 15% inhibit the mitosis in *Allium cepa* (MI: 0.16%), being not possible to observe division cells and cell abnormalities. The same occurs to the roots 75 g/L (MI: 0.34%), with significant differences. It is important to highlight that in the activity of the roots in 75 g/L was very similar to the glyphosate 15%, a non-selective systemic herbicide, what indicate that a tea of *U. baccifera* at this concentration can be dangerous to human health.

Taken into consideration the abnormalities observed (AN), the negative control (AN: 1.78%), with few cells presenting chromosomal abnormalities, presented no statistical differences from leaves 1 g/L (AN: 1.43%). In other words, the infusion of the leaves in low concentration caused no damage in *A. cepa* cells. Differently from oxalic acid (AN: 4.62%) and roots 30 g/L (AN: 5.56%), that have a perceptual of abnormalities almost three times higher, being observed breaks and lost chromosomes and disorganized chromosomes.

Table 3. Dividing cells, cellular aberration and chromosome abnormalities caused by *U. baccifer*a extracts in *A. cepa* cells.

Treatment	**Division cells**	**Cellular aberrations**					**Total of irregularities**	**AN%**
		AB	**BLC**	**BN**	**MN**	**DC**		
Negative control - Water	169	1	2	-	-	-	3	1.78
Positive control – Glyphosate (%)								
2	183	15	13	-	2	17	45	24.6
15	8	-	-	-	-	-	0	0
Oxalic acid (1 mg/mL)	260	1	1	-	-	10	12	4.62
Roots (g/L)								
1	186	3	3	-	-	11	17	9.14
30	265	2	6	-	1	6	15	5.66
75	17	-	-	-	-	-	0	0
Leaves (g/L)								
1	70	-	-	-	1	-	1	1.43
30	92	-	2	-	-	6	7	7.61
75	200	1	7	-	-	14	22	11.0

AB: Anaphasic bridges; BLC: Breaks and lost chromosomes; MN: micronuclei; BN: binucleated cells; DC: Disorganized chromosomes; AN%: Chromosomes abnormalities percentage.
In this experiment were analyzed 1000 cells per bulb, totaling 5000 cells per treatment.

The biggest percentage of chromosomes abnormalities was observed in the roots 1 g/L and the leaves 30 g/L and 75 g/L (9.14, 7.61 and 11.0%, respectively), as demonstrated in the Fig. 3. In this study, all concentrations of roots infusions induced damage in the cellular cycle, except for the highest (75 g/L), that caused a substantial inhibition of the mitosis. In the infusions of leaves, only the two higher concentrations caused the highest values of chromosomes abnormalities. Significant production of chromosome abnormalities were recorded in *U. baccifera* roots previously (Amat et al., 2002). It is important to highlight that Amat et al. (2002) use only the roots of *U. baccifera*, and in a concentration of 75 g/L. In this study was made a comparison between the roots and the leaves of the stinging nettle, since the leaves were tested and popularly used as an anti-inflammatory remedy (Badilla et al., 199a; 1999b; 2006). The *U. baccifera* aqueous extracts caused events that demonstrate the anti-proliferative ability (roots 75 g/L and leaves 1 and 30 g/L) and genotoxic potential (roots 30 g/L and leaves 30 and 75 g/L) on the *A. cepa* cell cycle. These conditions may result from various interactions of different chemical components present in the plant, which probably causes inhibitory effects on the cell cycle. Phenolics, flavonoids, alkaloids and condensed tannins were already quantified in this nettle, beyond the oxalic acid (Gindri et al., 2014b). To the flavonoid quercetin was attributed the anti-proliferative effect (De Souza et al., 2010). Rossato et al. (2010) reported that the anti-proliferative capacity obtained in their study by the same test can be attributed to the smallest concentration or type of bioactive substances. The low concentration of metabolites of *U. baccifera* were previously described (Gindri et al., 2014b).

Oxalic acid was responsible for 4.62% of chromosomes abnormalities (Fig. 2A-B), what could explain part of the cellular aberrations observed in the infusions. In plants, calcium oxalate deposition is common and their proposed functions include calcium regulation, ion balance (e.g. sodium and potassium), plant protection, tissue support (plant rigidity), detoxification (e.g. heavy metals or oxalic acid), and light gathering and reflection (Nakata, 2003). Oxalic acid in *U. baccifera* confirm the stinging and pain caused by this nettle, besides justified the genotoxicity caused by the infusions of *A. cepa* meristematic cells.

CONCLUSIONS

The results demonstrated that infusions at different concentrations of *U. baccifera* have genotoxic activity. Oxalic acid can be responsible in part of the chromosomal abnormalities caused by *U. baccifera*, although, an even broader question raised by this study concerns that there must have more metabolites that evoke the same effect, and act synergically, promoting the genotoxic effect of this nettle.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Amat AG, Yajia ME, Gonzalez CF, Lorca GL, Sanchez Gonzalez F, Riglos AG, Veron JR (2002) Evaluation of cytological parameters induced by aqueous extracts of seven plants used as antihypertensive agents in Argentine folk medicine. Lat Am J Pharm 21: 34-42.

Badilla B, Chaves F, Mora G, Poveda JL (2006) Edema induced by *Bothrops aspes* (Squamata: Viperidae) snake venom and its inhibition by Costa Rican plant extracts. Rev Biol Trop 54: 245-252.

Badilla B, Mora G, Lapa AJ, Emim JAS (1999a) Anti-inflammatory activity of *Urera baccifera* (Urticaceae) in Sprague-Dawley rats. Rev Biol Trop 47: 365-371.

Badilla B, Mora G, Poveda LJ (1999b) Anti-inflammatory activity of aqueous extracts of five Costa Rican medicinal plants in Sprague-Dawley rats. Rev Biol Trop 47: 723-727.

Camparoto ML, Teixeira RO, Mantovani MS, Vicentini VEP (2002) Effects of *Maytenus ilicifolia* Mart. and *Bauhinia candicans* Benth infusions on onion root-tip and rat bone-marrow cells. Genet Mol Biol 25: 85-89.

De Souza LFB, Laughinghouse IV HD, Pastori T, Tedesco M, Kuhn AW, Do Canto-Dorow TS, Tedesco SB (2010) Genotoxic potential of aqueous extracts of *Artemisia verlotorum* on the cell cycle of *Allium cepa*. Int J Environ Stud 67: 871-877.

Fachinetto JM, Bagatini MD, Durigon J, Silva ACF, Tedesco SB (2007) Efeito anti-proliferativo das infusões

de *Achyrocline satureioides* DC (Asteraceae) sobre o ciclo celular de *Allium cepa*. Rev Bras Farmacogn 17: 49-54.

Fu HY, Chen SJ, Chen RF, Ding WH, Kuo-Huang LL, Huang RN (2006) Identification of oxalic acid and tartaric acid as major persistent pain-inducing toxins in the stinging hairs of the nettle, *Urtica thunbergiana*. Ann Bot 98: 57-65.

Gindri AL, de Souza LB, Cruz RC, Boligon AA, Machado MM, Athayde ML (2014b) Genotoxic evaluation, secondary metabolites and antioxidant capacity of leaves and roots of *Urera baccifera* Gaudich (Urticaceae). Nat Prod Res 28: 37-41.

Gindri AL, Kubiça TF, Mario DN, Oliveira SM, Silva CR, Cabreira TN, Boligon AA, Ferreira J, Lovato LT, Weiblen R, Alves SH, Athayde ML (2014a) Antiviral, antimicrobial and anti-inflammatory activities of *Urera baccifera*. Afr J Pharm Pharmacol 8: 284-291.

Guerra M, Souza MJ (2002) Como observar cromossomos: um guia de técnica em citogenética vegetal, animal e humana. São Paulo: Funpec, 131 p.

Iganci JRV, Bobrowski VL, Heiden G, Stein VC, Rocha, BHG (2006) Efeito do extrato aquoso de diferentes espécies de boldo sobre a germinação e indice mitótico de *Allium cepa* L. Arq Inst Biol 73: 79-82.

Martins FO, Gomes MMR, Nogueira FLP, Martins GR, Romanos MTV, Kaplan MAC, Menezes FS (2009) *In vitro* inibitory effect of *Urera baccifera* (L.) Gaudich. extracts against herpes simplex. Afr J Pharm Pharmacol 3: 581-584.

Nakata PA (2003) Advances in our understanding of calcium oxalate crystal formation and function in plants. Plant Sci 164: 901-909.

Onofre SB, Herkert PF (2012) Antimicrobial activity of extracts obtained from *Urera baccifera* (L.) Gaudich. Adv Life Sci 2: 139-143.

Rossato LV, Tedesco SB, Laughinghouse IV HD, Farias JG, Nicoloso FT (2010) Alterations in the mitotic index of *Allium cepa* induced by infusions of *Pluchea sagittalis* submitted to three different cultivation systems. An Acad Bras Cienc 82: 857-860.

Tedesco SB, Laughinghouse IV HD (2012) Bioindicator of Genotoxicity: The *Allium cepa* Test, Chapter 8, In: Environmental Contamination, Srivastava JK (Ed.): InTech, Croatia.