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## Cytotoxicity and Genotoxicity of *Tridax procumbens* L. in *Allium cepa*

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### ABSTRACT

With increased consumption of medicinal plants in alternative treatments and the search for new compounds with biological activity, the need arose to investigate the toxic, carcinogenic and teratogenic potential of these compounds. Among many species, *Tridax procumbens* stands out for being a plant commonly used in folk medicine, finding varying reports of its use. This study was conducted to investigate cytotoxic and genotoxic effects of ethanolic, aqueous and hydroethanolic extracts of this species and a phytochemical screening of different classes present in extract by HPLC-PAD. *Allium cepa* seedlings were exposed to extracts for 48 h. After this period, the seedlings were replaced in distilled water. Then the roots were hydrolyzed in hydrochloric acid (HCl) 1 N and stained with Schiff reactive. The roots were placed on slides, then was added a drop of 2% acetic carmine and covered with coverslips and were observed 5000 cells per treatment. It was found that different extracts led to a reduction in mitotic index when compared with negative control and still presented changes in death rate. In this context, results suggest that extracts of *T. procumbens* have inhibitory effect on mitosis and mutagens on cell division in root meristematic cells of *A. cepa*.

**Key words:** *Allium cepa*, cytotoxic, genotoxic, mitotic index, Schiff reactive, teratogenic potential

### INTRODUCTION

Biodiversity has been the source of biomolecules with great potential for biotechnological use being, among others, an important source for agronomic and pharmaceutical industries (Adams *et al.*, 2007; Elumalai *et al.*, 2011; Bussmann *et al.*, 2011). Alternative treatments, mainly with medicinal plants, are part of human culture and in many countries are of regular use especially because of its affordability and availability (WHO., 2002; Bussmann *et al.*, 2011). Among many species *T. procumbens* L. stands out for being a plant commonly used in folk medicine, with reports of its use as anticoagulant, antifungal, insect repellent, expectorant, antidiarrheal, antidysenteric, preventing hair loss and inducing cicatrization (Saraf *et al.*, 1991; Taddei and Rosas-Romero, 2000).

In the last decade the number of scientific papers on *T. procumbens* has increased significantly due to pharmacological use (Christudas *et al.*, 2012; Algariri *et al.*, 2013; Policegoudra *et al.*, 2014). Researches have shown some properties of *T. procumbens* as anti-inflammatory, hepatoprotective, healing, immunomodulatory, antimicrobial, antiseptic and hypotensive (Ravikumar *et al.*, 2005; Jachak *et al.*, 2011; Christudas *et al.*, 2012). Some of these works provide phytochemical characterization which already reported presence of flavonoids, luteolin, dexamethasone, glucotureoline,  $\beta$ -sitosterol, flavones, glycosides and quercetin (Subraman *et al.*, 1968; Saxena and Albert, 2005; Mundada and Shivhare, 2010). However, their toxic and mutagenic profile is still incipient and even absent in these studies.

Given this, studies of genotoxic potential have been conducted in order to assess the risks of ingesting different plants with medicinal purpose (Verschaeve and van Staden, 2008; Akintonwa *et al.*, 2009; Christudas *et al.*, 2012). Among assays concerning genotoxic potential, stands out the *A. cepa* because it can be used to determine both cytotoxic and genotoxic effects of many kinds of chemicals, including those of vegetable origin, being recognized for his sensitivity and capacity to interact with mutagens during the cell cycle (Fiskesjo, 1997; Carita and Marin-Morales, 2008; Olorunfemi *et al.*, 2011; Herrero *et al.*, 2012).

Considering that there are few reports about toxicological and mutagenic properties of *T. procumbens* in literature, this study was conducted to evaluate cytotoxic and genotoxic effects of ethanolic, hydroethanolic and aqueous extracts of this plant on *A. cepa* cells.

## MATERIALS AND METHODS

**Plant material and preparation of extracts:** The study was conducted in the years 2013 and 2014 over 18 months. The plant parts (branches, leaves and flowers) of *T. procumbens* were collected from specimens found in the Universidade Estadual Paulista-SP (22°39'42" S, 50°24'44" W, Altitude: 546 m). The species voucher is deposited in the scientific collection of the Plant Systematics laboratory, FCL, UNESP-Assis (HASSI) sobnumber 113. For preparation of the extracts, the plant parts were pooled, washed, dried in stove (40°C) and pulverized. The aqueous extract was obtained by mechanical agitation, in distilled water (proportion 1:10 (w:v) for 24 h at 24°C). Right after, it was vacuum filtered, frozen and lyophilized (model: L101, Liotop, Brazil) to obtain a dry extract. The hydroethanolic extract was obtained by mechanical agitation in a ethanol: water (70:30) solution in the proportion 1:10 (w:v) for 24 h. The process was repeated 3 times with the same plant material. Then, the obtained extract was filtered and taken to an rotary evaporator (model: MA120, Marconi, Brazil) at 60°C for the ethanol removal and later frozen and lyophilized to obtain a dry extract. The same was performed to the ethanolic extract, where only the ethanol: water (70:30) solution was substituted by absolute ethanol (IMPEX, Brazil), which had the dry extract obtained by rotary evaporator concentration followed by desiccation chamber at room temperature.

**Phytotoxicity in root meristematic cells of *Allium cepa*:** *Allium cepa* (onion) seeds were germinated in petri dishes, when roots of seedlings reached 1 cm length they were exposed to extracts at concentrations that presented highest activity in pre and post-emergence experiments for a period of 48 h. After this period, seedlings were replaced in Petri dishes containing distilled water until they reached 5 cm length average (recovery period). The entire experiment was conducted in a BOD (Biological Oxygen Demand) germination greenhouse conditions. Roots were fixed in Carnoy (absolute ethyl alcohol and glacial acetic acid, 3:1). Following for setting and analysis of roots, they were hydrolyzed 1 N in HCl at 60°C for 8 min and right after were stained with Schiff reactive for 2 h in the dark.

Roots were placed on slides, added a drop of 2% acetic carmine and covered with coverslips, after, they were crushed and fixed, analyses were performed using an optical microscope (100X) and 5000 cells per treatment were observed. Phytotoxic effects of the extracts were determined by mitotic index analysis (total number of dividing cells/total number of analyzed cells×100) and cell death index (total number of cells death/total number of analyzed cells×100). Chromosomal aberrations were also determined (aberrant anaphase and telophase) and their frequencies used to determine the rate of chromosomal changes (total number of cells changed/total number of analyzed cells×100). For *A. cepa* test statistical analysis, results were submitted to nonparametric Kruskal-Wallis and Mann-Whitney tests (analysis significance level of 5 and 1%) according to Leme and Marin-Morales (2009).

**High Performance Liquid Chromatography (HPLC):** Chromatographic separations were performed on high performance liquid chromatography (analytical, quaternary gradient) model PU-2089S Plus (Jasco®), coupled to a diode array detector with photo scan range 200-900 nm, MD-2015 model Plus (Jasco®), automatic injector model AS-2055 (Jasco®) with 50 mL loop and column oven model CO-2060 Plus. Jasco ChromPass (version 1.8.1.6) was used during the acquisition and processing of chromatographic data. Reverse phase column immobilized with octadecylsilane was used model Luna C<sub>18</sub> (2) 100A (Phenomenex®) of 250×4.6 mm i.d., with an average particle size of 5 µm with guard column (Phenomenex®) of 4×3 mm i.d.. An aliquot of 10 mg from ethanolic extract was dissolved in 1 mL of acetonitrile (ACN) 100% and filtered with syringe filter with pore size of 0.45 µm. Samples were monitored by PDA detector in a range of 200-600 nm. Chromatogram was obtained at 334 nm. Mobile phase: Acetonitrile+0.1% formic acid (A) and water+0.1% formic acid (B). Gradient: 10-35% of A in B during 60 min.

## RESULTS

**Phytotoxicity in root meristematic cells of *Allium cepa*:** Mitotic index of root meristematic cells of *A. cepa* exposed to different extracts (aqueous, hydroethanolic, ethanolic) of *T. procumbens* are presented in Table 1. For treatment at 20 mg mL<sup>-1</sup> concentration was observed mitotic 10.84, 08.90 and 11.30, respectively and the 3 extracts did not differ statistically between themselves and positive control (9.74). Only hydroethanolic shown significant difference when compared with negative control (14.52). For analysis of treatments chromosomal changes were observed the presence of binucleated cells in metaphase with bud in interphase, with lobulated nuclei and cells in metaphase, anaphase and telophase that showed up in an aberrant manner (Table 2). Chromosomal alterations index of treatments with ethanolic and hydroethanolic extracts showed no statistical difference when compared with negative control and each other. Only aqueous extract did not present significant difference when compared to positive control (Table 1). Similarly, death index was calculated based on total number of cells that presented above-mentioned changes. Treatments presented an increase in this index, however only hydroethanolic and aqueous differed from negative control but showed less than that observed for positive control (Table 1).

**Analysis by High Performance Liquid Chromatography (HPLC-PAD):** With aid of PAD detector performing scan in the spectral range of 200-600 nm, spectra in UV region for eluted peaks were obtained. Peaks with absorption bands typical of flavonoids (Fig. 1) which are recognized for presenting the band II, with maximum wavelength in spectral range of 240-290 nm, assigned to A-ring and band I, with maximum length in spectral range of 300-390 nm, assigned to B-ring, presenting higher incidence of molecules from group of flavones and flavonoids.

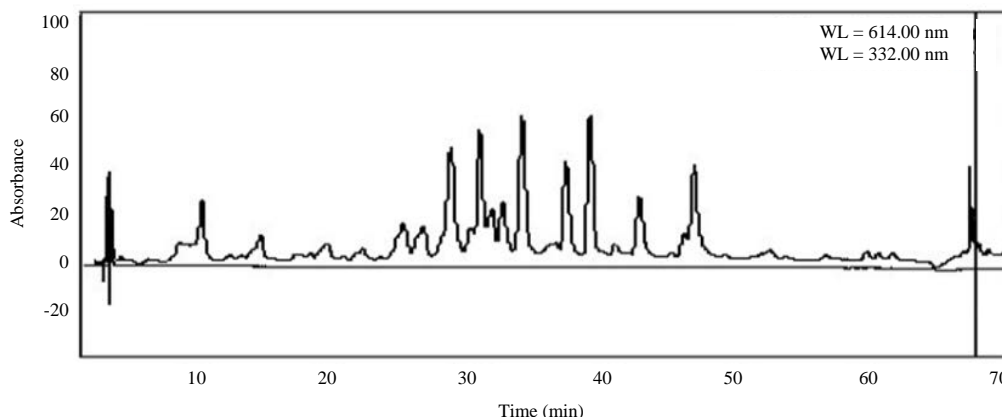


Fig. 1: Chromatographic profile of ethanol extract obtained by HPLC-PAD. Eluting system: A (Acetonitrile+0.1% formic acid) and B (Water+0.1% formic acid) Gradient: 10-35% A in B in 60 min. Phenomenex® Luna C18 column (250×4.6 mm id. 5 µm), HPLC (Jasco®), flow 1.0 mL min<sup>-1</sup>, λ = 334 nm, Injection volume: 20 µL, Column oven: 40°C

Table 1: Mitotic index, death index, index of chromosomal alterations of root meristem cells of *Allium cepa* treated with (ethanolic, hydroethanolic and aqueous) extracts of *Tridax procumbens* in 20 mg mL<sup>-1</sup> concentration negative control treated with water and positive control treated with 0.0077 µL mL<sup>-1</sup> of MMS

Different extracts (20 mg mL <sup>-1</sup> )	Mitotic index	Death index	Chromosomal alterations index
<b><i>Tridax procumbens</i></b>			
NC	14.52±0.17 <sup>a</sup>	0.80±0.58 <sup>a</sup>	0.67±0.45 <sup>a</sup>
Ethanolic	11.30±2.63 <sup>ab</sup>	0.82±0.23 <sup>a</sup>	0.48±0.31 <sup>a</sup>
Hydroethanolic	08.90±0.79 <sup>b</sup>	1.64±0.50 <sup>b</sup>	0.96±0.42 <sup>a</sup>
Aqueous	10.84±1.86 <sup>ab</sup>	1.50±0.62 <sup>b</sup>	1.02±0.35 <sup>b</sup>
MMS	09.74±2.50 <sup>b</sup>	3.20±1.17 <sup>c</sup>	1.98±0.59 <sup>b</sup>

NC: Native control, MMS: Methyl methanesulfonate, 5000 cells analyzed. Mean±Standard Deviation. Same letters in columns do not differ statistically averages evaluated with the Kruskal-Wallis test (p<0.05)

Table 2: Chromosomal aberrations and micronuclei of root meristem cells of *Allium cepa* treated with the (ethanolic, hydroethanolic and aqueous) extract of *Tridax procumbens* in 20 mg mL<sup>-1</sup> concentration. Negative control receiving only water and positive control treated with 0.0077 µL mL<sup>-1</sup> of MMS

Different extracts	Micronuclei	Metaphase aberrant	Anaphase aberrant	Telophase aberrant	Lobed nucleus	Chromosome breakage	Chromosomal loss
<b><i>Tridax procumbens</i></b>							
NC	0.7±0.05 <sup>a</sup>	2.0±0.06 <sup>a</sup>	1.2±40.03 <sup>a</sup>	0.02±0.01 <sup>a</sup>	0.02±0.00 <sup>a</sup>	0.5±0.07 <sup>a</sup>	0.0±0.00 <sup>a</sup>
Ethanolic	0.8±0.30 <sup>a</sup>	0.8±0.83 <sup>b</sup>	3.04±3.09 <sup>b</sup>	1.40±0.54 <sup>b</sup>	0.20±0.04 <sup>b</sup>	2.0±0.04 <sup>b</sup>	0.6±0.09 <sup>b</sup>
Hydroethanolic	3.0±0.08 <sup>c</sup>	4.6±0.50 <sup>c</sup>	3.20±1.09 <sup>b</sup>	1.04±0.07 <sup>b</sup>	0.40±0.09 <sup>b</sup>	3.0±0.04 <sup>b</sup>	0.8±0.09 <sup>b</sup>
Aqueous	2.2±0.03 <sup>b</sup>	3.2±0.37 <sup>d</sup>	5.80±2.28 <sup>c</sup>	1.20±0.04 <sup>b</sup>	0.00±0.00 <sup>b</sup>	2.6±0.05 <sup>b</sup>	0.9±0.07 <sup>b</sup>
MMS	5.4±0.07 <sup>c</sup>	7.4±0.34 <sup>e</sup>	6.60±0.54 <sup>c</sup>	1.00±0.24 <sup>b</sup>	4.20±0.63 <sup>c</sup>	6.6±0.31 <sup>b</sup>	2.6±0.09 <sup>c</sup>

NC: Negative control, MMS: Methyl methanesulfonate, 5000 cells analyzed. Mean±Standard Deviation. Same letters in columns do not differ statistically averages evaluated with the Kruskal-Wallis test (p<0.05)

## DISCUSSION

Research have shown that inhibition of root growth of *A. cepa* is probably related to reduction of mitotic index (Akinboro and Bakare, 2007; Da Silva *et al.*, 2012). In similarly way, reduction in number of dividing cells was observed in treatments performed with different extracts of *T. procumbens*, indicating an inhibitory effect in *A. cepa* root cells mitosis, with the highest incidence of aberrant cells (Table 1 and 2), as demonstrated in the work done by Kwankua *et al.* (2010) and Timothy *et al.* (2014). In accordance with reduction in mitotic index and increased frequency of aberrant cells, were found changes in treatments with *T. procumbens* in death index (Table 1).

Knoll *et al.* (2006) evidenced potential genotoxic activity in *Pterocaulon polystachyum* through *A. cepa* test, detecting an inhibitory capacity in cell division related to increasing concentrations of aqueous extracts. Same way as presented in study by De Pinho *et al.* (2010) for gorse (*Baccharis trimera*) evidencing mutagenic effects of infusion in plant cells of *A. cepa* and in cultured human cells.

Analysis of possible compounds involved with phytotoxic potential was performed by HPLC-PAD for ethanolic extract. Where it was possible to verify peaks with typical absorption bands of flavonoids (Fig. 1) and according to studies by Mabry *et al.* (1970) and Merken and Beecher (2000), these wavelengths correspond to the presence of flavones and flavonoid molecules groups, which are recognized to exhibit peaks for band II about 240-280 nm and peaks I band around 300-380 nm.

Genotoxic observed in biological assays with *A. cepa* may be related to the presence of phenolic compounds observed in chromatographic research, because it is known phytotoxic potential of many quinones and phenols may affect membrane permeability causing damage to DNA and proteins and causing lipid peroxidation, leading to cell's death (Appel, 1993; Yu *et al.*, 2003). These activities are related to the formation of radicals that donate electrons to molecular oxygen, forming radical superoxide ( $O_2^-$ ) that can still undergo a series of reactions becoming more reactive, hydroxyl ( $OH^-$ ) or until form the radical hydroperoxil ( $HO_2^-$ ) (Testa, 1995; Hammond-Kosack and Jones, 1996).

During calculation of mitotic index was observed that many cells were retained in the interphase stage leading to reduced rates of cell division. Salehzadeh *et al.* (2003) analyzing the sub-phases of interphase showed that DNA synthesis phase (S) and gap 2 (G2) are commonly altered by chemical substances capable of stimulating production of free radicals which can cause damage to DNA and protein synthesis. Further, according to Rahman *et al.* (2013), changes in these subphases may occur due to increased duration either inhibition the same caused by an accumulation of biological damage possibly caused by changes in specific proteins that control cell cycle transition (Roy *et al.*, 2006). Inderjit and Duke (2003) classified terpenoids and phenolic compounds as the main chemical groups associated with phytotoxic activity.

## CONCLUSION

These results suggest that extracts of *T. procumbens* have inhibitory effects on mitosis and genotoxic on cell division of *A. cepa*. However, it is necessary new genotoxic, mutagenic and other risk assessments *in vivo* seeking better understanding in benefit of human welfare.

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