Genetics and Molecular Biology, 38, 1, 93-100 (2015)

DOI: http://dx.doi.org/10.1590/S1415-475738120140162

Research Article

Genotoxic potential of the latex from cotton-leaf physicnut (*Jatropha gossypiifolia* L.)

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Abstract

Jatropha gossypiifolia L. (Euphorbiaceae), popularly known as cotton-leaf physicnut, is a milky shrub notable for its medicinal properties. The present study aimed to evaluate the toxic, cytotoxic and genotoxic effects of the latex of *J. gossypiifolia*, using *Allium cepa* L. as test system. Seeds of *A. cepa* were exposed to five concentrations of the latex (1.25; 2.5; 5; 10 and 20 mL/L) in order to evaluate parameters of toxicity (evaluation of root growth), cytotoxicity (mitotic index frequency) and genotoxicity (frequency of chromosome alterations). The latex showed a significant decrease in root mean growth value as well as mitotic index for the tested concentrations, except for 1.25 mL/L, when compared to results from the negative control. The 1.25, 2.5 and 5 mL/L concentrations induced significant chromosome adherences, C-metaphases and/or chromosome bridges, as genotoxic effects. The significant frequency of chromosome bridges also indicated mutagenic potential for chromosomes of *J. gossypiifolia* as discussed in the paper. Considering that the latex is used in popular therapies, and that the test system *A. cepa* presents good correlation with tests carried out in mammals, it can be pointed out that its use for medicinal purposes may be harmful to human health especially if ingested.

Keywords: *Allium cepa* test, medicinal plant, plant latex, genotoxicity evaluation.

Received: May 21, 2014; Accepted: August 16, 2014.

Introduction

For thousands of years, several plants have been used in popular medicine. Despite being considered the main source of antimutagenics and antioxidants (Çelik and Aslantürk, 2010) some of their phytochemicals may cause adverse reactions or have potential of interacting with other medications, generating toxic, cytotoxic, mutagenic and genotoxic effects (Pawlowski *et al.*, 2012; Ping *et al.*, 2012; Ray *et al.*, 2013). However, for many of them there is little information available regarding the potential health risks at short, mid- or long term (Nunes *et al.*, 2012).

Jatropha gossypiifolia L. (Euphorbiaceae), commonly known as bellyache bush, black physicnut or cotton-leaf physicnut, is a shrub that contains a characteristic latex largely used for medicinal purposes, though in an empirical way (Cordeiro and Secco, 2014). The leaves are used *in natura* or in compresses, and are considered to have anti-malarian (Jansen *et al.*, 2010), insecticidal (Valencia *et al.*, 2006), anti-inflammatory (Oliveira *et al.*, 2010) and antimicrobial (Dhale and Birari, 2010; Gaikwad *et al.*, 2012) properties. The root and stem have cytotoxic (Nazeema and Girija, 2013), anti-malarian, leishmanicidal, antimicrobial, insecticidal, molluscicidal (reviewed by Sabandar *et al.*, 2013) and anti-inflammatory (Bhagat *et al.*, 2013) properties. The seeds and fruits are used against influenza, and also as laxative (Sabandar *et al.*, 2013), sedative, analgesic or anti-diarrheal agents (Apu *et al.*, 2013). The latex, in turn, is bactericidal (Gaikwad *et al.*, 2012) and molluscicidal (Matos, 2004). In Brazil, the topical application of latex *in natura* is employed against wounds and bites of venomous animals (Stasi and Hiruma-Lima, 2002), and its ingestion in diluted form is used for treatment of diarrhea by indigenous peoples (Curto, 1993). In India, the solution of latex with mustard oil (*Brassica campestris*) and clove oil (*Syzygium aromaticum*) is applied onto painful gum or teeth (Punjaji, 2012), whereas in the south of Ni-

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geria it is used to reduce nosebleed and wounded skin (Oduola *et al.*, 2007).

Despite its medicinal properties, the latex of *J. gossypiifolia in natura*, in direct contact with the skin, may produce caustic and irritating effects (Parente and Rosa, 2001). Moreover, the aqueous extract of latex was shown to be toxic for fish as well as upon intraperitoneal administration to house mice, presenting significant reduction of acetylcholinesterase (AChE) inhibition in the first (Singh and Singh, 2012; Pratap and Singh, 2013) and seizures in the latter (Singh and Singh, 2012). The inhibition of AChE prevents the degradation of choline neurotransmitters, such as acetylcholine, and prolongs the signal transmission through synapses. Consequently, it may lead to lethal paralysis by hyperstimulation of the nervous system (Stansley, 1993). The toxic effect may be mainly associated to cyclic peptides of the latex (Horsten *et al.*, 1996; Auvin-Guette *et al.*, 1997; Pratap and Singh, 2013), which show cytotoxic activity, as related for other *Jatropha* species (reviewed by Sabandar *et al.*, 2013). Therefore, complementary studies are still necessary for the latex of *J. gossypiifolia* to be safely employed for medicinal purposes, starting with the evaluation of its toxic potential.

Whereas the toxicity tests in animals lead to their death, alternative analysis should be considered. In this sense, genotoxicity tests using the *Allium cepa* test system showed a good correlation with the test system of mammals (Rank and Nielsen, 1994), indicating its use as an alternative for monitoring the genotoxic potential of chemical compounds (Fachinetto *et al.*, 2007). Additionally, *A. cepa* stands out among other plants due to presenting large chromosomes and in few number ($2n = 16$) in its cells (Fiskesjo, 1985). Moreover, the *A. cepa* test system has high sensitivity in detecting chemical and environmental agents (Leme and Marin-Morales, 2009). This system is easy to use and presents itself as a suitable bioindicator for the first screening of genotoxicity, thanks to its low cost, reliability and concordance with other genotoxicity tests. This way it contributes in the preliminary genotoxicity assessment of compounds for medicinal purposes (Bagatini *et al.*, 2007). Thus, the present work aimed at analyzing the toxic, cytotoxic and genotoxic effects of the latex from *J. gossypiifolia* by means of the *A. cepa* test system.

Material and Methods

Biological material

Latex of *J. gossypiifolia* was collected from an adult plant in Teresina (PI, Brazil) in January 2013. Herbarium specimens containing leaves, flowers and fruits were stored in the Afrânio Fernandes at the State University of Piauí Herbarium (UESPI – Teresina, Brazil); voucher specimen number: HAF 03111. The seeds of *A. cepa* (cv. Vale Ouro IPA – 11) used in the bioassays were kindly provided by the Agronomic Institute of Pernambuco (IPA, Recife, Brazil).

The latex of *J. gossypiifolia* was extracted following removal of the leaf petioles using pruning shears, at 8 to 9 am. The latex was immediately stored in Falcon tubes wrapped in aluminum foil in order to reduce the oxidation process. The latex was then transported, in cooling box containing ice, to the Laboratory of Plant Genetics and Biotechnology (Genetics Department, UFPE) where it was diluted in distilled water to yield five different latex concentrations (1.25; 2.5; 5; 10 and 20 mL/L) to be used at *A. cepa* assay.

Allium cepa assay

One hundred seeds of *A. cepa* (cv. Vale Ouro IPA - 11) were germinated in Petri dishes containing filter paper moistened with distilled water, at room temperature. When the rootlets reached about 1 cm in length, they were transferred to the five cited latex concentrations (one dish for each concentration) for 24 h. Distilled water was used as negative control (NC); MMS (methyl methanesulfonate 4 x 10^{-4} M), a drug with clastogenic activity, and the herbicide Trifluralin (0.84 ppm of the active agent), a substance with aneugenic activity (Fernandes *et al.*, 2007), were used as positive controls. When rootlets reached about 1.5 cm length, the material was fixed in Carnoy (ethanol:acetic acid 3:1, v/v) for 6-8 h, at room temperature, and stored at -20 °C until slide preparation.

For slide preparation, the root tips were washed three times in distilled water, for 5 min each time, and hydrolyzed at 60 °C for 10 min in HCl 1 N. After hydrolysis, the root tips were again washed in distilled water and transferred to amber glass bottles containing Schiffs reagent, in which they remained for 2 h in the dark. After this time, the root tips were washed until complete removal of the reagent, transferred onto slides, squashed with one drop of 2% acetic carmine, and mounted with Entellan®.

Latex toxicity was evaluated according to the mean root length variation (in centimeters) of 30 roots per treatment. The experimental unit consisted of one individual root (one root per specimen). Cytotoxicity and genotoxicity were evaluated by scoring 5,000 meristematic cells (experimental unit: slide with 500 cells, with a total of 10 analyzed slides per treatment) under light microscope (400 x). The assessed aspects were: (1) mitotic index (cytotoxicity) and (2) chromosome alteration index (genotoxicity). The last one includes alterations resulting from aneugenic effects (*e.g.* C-metaphases, metaphase with chromosome adherences, loss chromosomes, multipolar anaphases, binucleate cells, polyploid metaphases, and other alterations) or clastogenic effects (*e.g.* chromosome fragments in metaphase or anaphase, chromosome bridges and other alterations). Micronuclei can be result from either aneugenic or clastogenic effects.

Photographs of the best cells were taken in a Leica conventional microscope (DM 500) (1000 x), using a digital camera (Nikon 14.0 Megapixels). Images were adjusted

for brightness and contrast, in gray tones using Adobe Photoshop CS6.

Statistical Analysis

The toxicity values were expressed as averages, whereas the values of cytotoxicity and genotoxicity were expressed as frequencies. Statistical analysis to evaluate data distribution with regard to normality was carried out by Lilliefors test (D) in the program Assistat 7.7 (Silva and Azevedo, 2002), while homogeneity of variance was evaluated by the Cochran test in the program BioEstat 5.3 (Ayres *et al.*, 2007). Data which did neither present normal distribution nor homogeneity were analyzed by the non-parametric test of Kruskal-Wallis, followed by the *a posteriori* Student-Newman-Keuls test ($p < 0.05$) in the program BioEstat 5.3 (Ayres *et al.*, 2007). The data on cytotoxicity were the only presenting normal distribution and homogeneity of variance, and were analyzed by the parametric test of Scott-Knott ($p < 0.05$) in the program Assistat 7.7 (Silva and Azevedo, 2002).

Results

The tests of toxicity and cytotoxicity carried out with seeds of *A. cepa* exposed to the different concentrations of latex from *J. gossypiifolia* showed a significant reduction in root mean growth and in mitotic index (MI) values of all tested latex concentrations, when compared to NC, except for 1.25 mL/L. In these analyses, the reduction was partially or entirely dose-dependent for root mean growth and in mitotic index, respectively (Table 1).

With regards to chromosome alterations (CA), the increase in the total indexes (Table 2; Figure 1) was highly significant when compared with NC results, except for the 10 and 20 mL/L concentrations. From these results, we may infer that *J. gossypiifolia* latex has a genotoxic action. On the other hand, the nemployed empirically in the popular medicine of maon-significant CA frequencies found for the higher latex concentrations (10 and 20 mL/L) may result from MI reduction with these treatments.

When the chromosome alterations were analyzed separately, metaphases with chromosome adherences (Figure 1A) have been found in all the treatments carried out with the latex; nevertheless significant NC related results were only recorded for the three lower concentrations (1.25; 2.5 and 5 mL/L). The presence of C-metaphases (Figure 1B) was significant only for 2.5 and 5 mL/L concentrations (Table 2). Both chromosome adherences and C-metaphases are result from aneugenic mechanisms.

Other non-significant alterations in chromosome segregation during anaphase and telophase were registered, such as chromosome losses (Figure 1C) and multipolarities (Figure 1D-E; Table 2). Additionally, polyploid (Figure 1F) and binucleated cells (Figure 1G), nuclear buds (Figure 1H) and lobulated nuclei (Figure 1I) have been found in all analyzed treatments, although no significant alterations have been observed for any concentration (Table 2).

Chromosome fragments (Figure 1J) and chromosome bridges (Figure 1K; Table 2), arising from clastogenic effects, were also registered, but only the presence of chromosome bridges was significant for concentrations 1.25, 5 and 10 mL/L. Non-significant micronucleus (Figure 1L) frequencies were also observed.

Discussion

The latex of *J. gossypiifolia* is broadly used in popular medicine in many countries. However, this plant has phytochemicals in its composition such as alkaloids and cyclic peptides, which may be toxic to users (Singh and Singh, 2012; Pratap and Singh, 2013). In the present study, the test system *A. cepa* was used to evaluate the toxic, cytotoxic and genotoxic effects of different concentrations (1.25; 2.5; 5; 10 and 20 mL/L) of latex from *J. gossypiifolia*. The concentrations tested were selected based on a usage concentration recommended in popular medicine (approximately 10 mL/L, corresponding to one tablespoon in one liter of water). Apart from the recommended concentration, others were also tested based on results of latex toxicity in fish, which varied from 10 to 21.5 mL/L (Singh and Singh, 2012; Pratap and Singh, 2013), but mainly on antimicrobial activity data, which varied from 1.9 to 4.4 mL/L (Patil *et al.*, 2012) for the latex of *J. gossypiifolia* and from 0.5 to 10 mL/L for the latex of *J. curcas* (Arekemase *et al.*, 2011).

The bioassays carried out here revealed dose-dependent toxicity (mean root growth) and cytotoxicity (mitotic index) for all analyzed treatments except for the lowest concentration (1.25 mL/L). These effects may be attributed to various chemical substances present in the latex of *J. gossypiifolia*, mainly cyclic peptides (CPi), such as cyclogossines A (Horsten *et al.*, 1996) and B (Auvin-Guette *et al.*, 1997), terpenes (Patil *et al.*, 2012) and alkaloids that have cytotoxic activity (Sabandar *et al.*, 2013). Peptides present in other species of the genus *Jatropha* have also been reported as cytotoxic, such as integerrimides A and B of *J. integerrima* (Mongkolvisut *et al.*, 2006) and the curcacyclins A and B of *J. curcas* (Insanu *et al.*, 2012).

Cyclic peptides have a cyclical conformation (Sakai *et al.*, 1996), which facilitates their passing through cell membranes, as well as absent exposure of the C- and N-terminal groups to exopeptidases (Wu *et al.*, 2007). These characteristics may be related to the ease with which these peptides enter and remain inside cells of *A. cepa*, causing toxic effects, as observed here and in previous studies with latex of *J. curcas* in *Artemia salina* as well as in cell culture of human ovarian cancer (Insanu *et al.*, 2012). On the other hand, terpenes, such as diterpenes, are some of the most toxic compounds in *Jatropha* (Devappa *et al.*, 2011), whereas lipophilic nature (Wink, 2012) facilitates their entry and remaining inside cells of *A. cepa*, causing toxic effects. Additionally, terpenes in latex may be associated

with a reduction in calcium concentration, thus inhibiting protein kinase C (PKC), as observed in leaf ethanol extract of *J. gossypiifolia* (Silva *et al.*, 1995; Paes *et al.*, 2012), decreasing cell proliferation (Alberts *et al.*, 2010). Similar results were observed with inhibitors of PKC in *A. cepa* (Blume *et al.*, 2008), *Arabidopsis thaliana* (Sheremet *et al.*, 2010) and *Nicotiana tabacum* (Sheremet *et al.*, 2012).

In the current study, most chromosome alterations (CA), such as chromosome adherences and C-metaphases, were considered a result of genotoxic effects, since they represent damage to the genetic material which is not necessarily fixed in the organism (Leme and Marin-Morales, 2009; Mazzeo *et al.*, 2011). When these alterations can be repaired, they are not transmitted to descendant cells (Ventura-Camargo *et al.*, 2011). The presence of chromosome adherences and C-metaphases confirms the interference of phytochemicals of *J. gossypiifolia* latex in the assembly, stabilization and/or inactivation of spindle fibers, characterizing an aneugenic activity of the latex.

Aneugenic activities in metaphase may generate other types of cell abnormalities such as multipolar anaphases, nuclear buds, lobulated nuclei, and polyploid cells (Fernandes *et al.*, 2009). However, these alterations were not significant in the present study, what indicates that chromosome adherences and C-metaphases did not contribute to further alterations, and suggests a reversible mechanism for them (Odeigah *et al.*, 1997).

Chromosome bridges, chromosome fragments and part of micronucleus formation are related to clastogenic activity (Fenech *et al.*, 2011). Chromosome bridges and fragments, for instance, could be the result of chromosome breakage-fusion-bridge cycles, elucidated for the first time by Barbara McClintock in the 1930s in maize chromosomes (reviewed by Jones, 2005). However, chromosome

Table 1 - Values of mean length for the root tips of *Allium cepa* and mitotic indexes in meristematic cells, observed 24 h after *Jatropha gossypiifolia* latex treatment at different concentrations.

Latex treatment (mL/L)	Mean root length (cm)	Mitotic index (%)
Distilled water (NC)[1]	3.32 ± 0.90	51.57 ± 2.69a[5]
1.25	3.21 ± 0.89	51.77 ± 6.63a
2.5	2.43 ± 0.64**[4]	44.33 ± 5.53b
5	2.11 ± 0.64**	36.70 ± 5.27c
10	2.28 ± 0.85**	36.32 ± 5.43c
20	1.87 ± 0.62**	22.02 ± 8.00d
MMS (4 x 10^{-4} M)[2]	2.78 ± 0.63	36.67 ± 7.11c
Trifluralin (0.84 ppm)[3]	2.38 ± 0.69**	23.00 ± 2.76d

[1]NC: negative control. [2]MMS (Methyl methanesulfonate): positive control. [3]Trifluralin: positive control. [4]Significant in the Kruskal-Wallis test with *a posteriori* Student-Newman-Keuls test (*p < 0.05; ** p < 0.01). [5]Scott-Knott test (p < 0.05; averages followed by the same lowercase letter are not significantly different). The results refer to analysis of 5,000 cells per treatment.

Table 2 - Frequency of chromosome alterations in meristematic cells of *Allium cepa* root tips, observed 24 h after *Jatropha gossypiifolia* latex treatment at different concentrations.

Chromosome alteration	Negative control	Latex treatment (mL/L)					Positive control	
	Distilled water	1.25	2.5	5	10	20	MMS[1] (4 x 10 -4 M)	Trifluralin (0.84 ppm)
Metaphases with chromosome adherences	0.66 ± 0.32	1.46 ± 0.47**[2]	1.29 ± 0.68*	1.28 ± 0.24**	0.86 ± 0.35	0.76 ± 0.34	0.11 ± 0.18	3.25 ± 1.86**
C-metaphases	0.02 ± 0.06	0.11 ± 0.15	0.19 ± 0.18*	0.37 ± 0.25**	0.11 ± 0.15	0.09 ± 0.16	0.07 ± 0.10	0.39 ± 0.55*
Chromosome losses	0.02 ± 0.06	0.15 ± 0.17	0.15 ± 0.19	0.06 ± 0.12	0.14 ± 0.11	0.13 ± 0.17	0.00 ± 0.00	0.08 ± 0.10
Binucleated cells	0.07 ± 0.10	0.04 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.94 ± 1.29
Polyploid cells	0.04 ± 0.12	0.04 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.10	0.02 ± 0.06	0.00 ± 0.00	0.30 ± 0.39
Multipolar anaphases	0.00 ± 0.00	0.02 ± 0.06	0.00 ± 0.00	0.02 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.30 ± 0.44
Lobulated nucleus	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.18	0.02 ± 0.06	0.02 ± 0.06	0.00 ± 0.00	0.02 ± 0.06	5.28 ± 3.85**
Nuclear buds	0.07 ± 0.13	0.22 ± 0.14	0.11 ± 0.24	0.31 ± 0.27	0.14 ± 0.19	0.17 ± 0.24	0.13 ± 0.15	1.24 ± 0.64**
Micronucleus	0.35 ± 0.31	0.66 ± 0.53	0.77 ± 0.56	0.93 ± 0.93	0.57 ± 0.32	0.46 ± 0.58	4.55 ± 2.55**	2.18 ± 1.27**
Chromosome fragments	0.02 ± 0.00	0.02 ± 0.06	0.02 ± 0.06	0.07 ± 0.17	0.02 ± 0.06	0.02 ± 0.06	0.17 ± 0.06**	0.04 ± 0.08
Chromosome bridges	0.00 ± 0.00	0.27 ± 0.20**	0.09 ± 0.13	0.22 ± 0.24*	0.27 ± 0.26**	0.04 ± 0.08	0.00 ± 0.00	0.19 ± 0.22**
Total	1.25 ± 0.62	2.99 ± 1.03**	2.68 ± 1.15*	3.28 ± 1.35**	2.26 ± 0.58	1.69 ± 1.10	5.05 ± 2.26**	14.19 ± 7.10**

[1]MMS (Methyl methanesulfonate). [2]*Significant in the Kruskal-Wallis test with *a posteriori* Student-Newman-Keuls test (* p < 0.05; ** p < 0.01). The results refer to analysis of 5,000 cells per treatment.

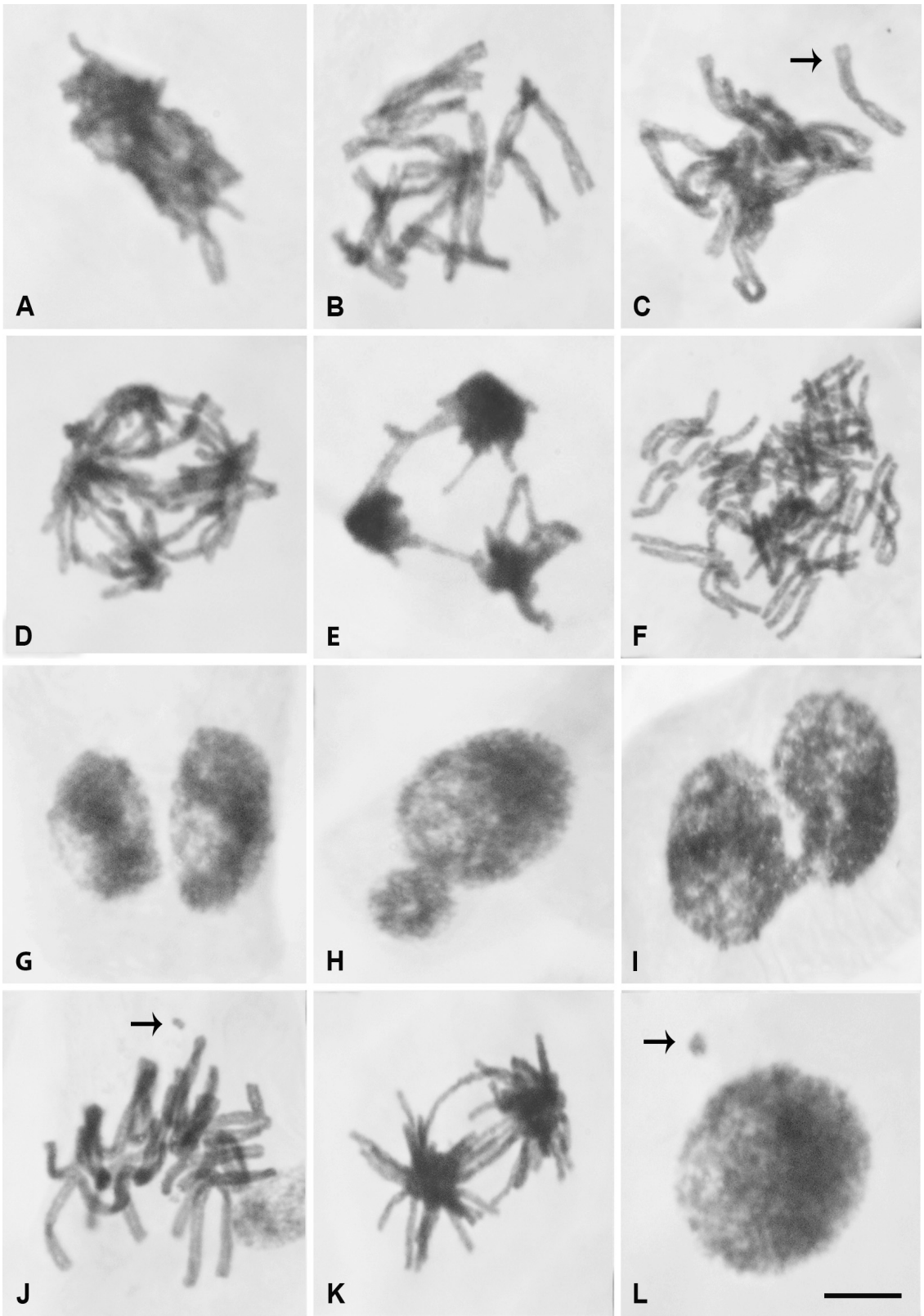

Figure 1 - Chromosome alterations after *Jatropha gossypiifolia* latex treatment of *Allium cepa* meristematic cells. (A) Chromosomal adherence (1.25 mL/L). (B) C-metaphase (5 mL/L). (C) Metaphase with chromosome loss (2.5 mL/L). (D) Multipolar anaphase with chromosome bridges (5 mL/L). (E) Multipolar telophase with chromosome bridges (5 mL/L). (F) Polyploid metaphase (10 mL/L). (G) Binucleated cell (10 mL/L). (H) Nucleus with nuclear bud (5 mL/L). (I) Lobulated nucleus (2.5 mL/L). (J) Metaphase with chromosome fragment (5 mL/L). (K) Anaphase with chromosome bridge (10 mL/L). (L) Micronucleated cell (1.25 mL/L). Arrows in (C), (J) and (L) indicate chromosome loss, chromosome fragment and micronucleus, respectively. Bar: 10 μm (for all pictures).

fragments could also be induced by several factors involved in DNA breaks (Fenech, 2000). On the other hand, micronuclei formation could be induced by both aneugenic and clastogenic activities, related to entire chromosomes or chromosome fragments (respectively) not incorporated into the main nucleus during the cell cycle (Fenech *et al.*, 2011). In the present results, from these three clastogenic alterations, only chromosome bridges had a significant increase.

The clastogenic alterations triggered by the latex indicate a chromosome mutagenic potential. This type of alteration represents damage to the genetic material at chromosome level that could not be repaired by the cell, being possibly transmitted to descendant cells (Grant, 1978). Furthermore, J. gossypiifolia latex caused toxicity and cytotoxicity at the tested concentrations, except for 1.25 mL/L. It also generated significant alterations in the assembly and stabilization of the mitotic spindle fibers, resulting in disturbance of the cell cycle and chromosome alterations for all concentrations. Based on these last results, it can be inferred that the mechanism of action of the latex also has aneugenic nature.

Although *J. gossypiifolia* is considered an important potential plant for the generation of pharmacological and/or biotechnological products (Félix-Silva *et al.*, 2014), overall, the tests performed indicated toxicity, cytotoxicity and genotoxicity of *J. gossypiifolia* latex, including the concentrations acknowledged as antimicrobial (1.9 and 4.4 mL/L) (Patil *et al.*, 2012). Considering that the latex of *J. gossypiifolia* is employed empirically in the popular medicine of many countries, and that the *A. cepa* test system presents good correlation with the tests carried out in mammals (Rank and Nielsen, 1994), it should be pointed out that it may potentially harm the human health especially if ingested. In addition, other studies with this plant material are necessary in order to elucidate the mechanisms of action of its bioactive compounds, and to reduce its toxicity while keeping its therapeutic action, for further use of isolated latex compounds in the pharmaceutical industry.

Acknowledgments

We are grateful to the Federal University of Pernambuco (UFPE) and the State University of Piauí (UESPI) for providing the installations and infrastructure for realization of the present study; to Dr. Francisco Soares Filho, for taxonomic identification of the botanic material (*J. gossypiifolia*); and to Dr. Fátima de Oliveira Pires for technical help with microscopy. This work was supported by fellowship within the Post-Graduation Promotion Program (PROF) from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil), and research grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and FACEPE (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco).

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Associate Editor: Carlos F.M. Menck