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Mutagenic Effect of Three Invasive Species through *Allium Cepa* Bioassay

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Abstract: Invasive alien species are a global threat to biodiversity that affects protected areas around the world. The occupation of new environments by these plants is a problem to be solved and it is essential to investigate all the aspects that allow this successful to find solutions to this question, such as its mutagenic effects. Thus, this study aimed to evaluate the mutagenic effect of leaves extracts of *Acacia mangium* Willd, *Artocarpus heterophyllus* Lam and *Eriobothrya japonica* (Thunb.) Lindl through *Allium cepa* bioassay. For this, *A. cepa* seeds were submitted to continuous and discontinuous (acute and chronic) treatments in medium with water (negative control) or four concentration of each extract (1, 5, 10 and 50 mg/mL). The mitotic index was affected at all concentrations of three extracts tested in all treatments, continuous and discontinuous. Aneugenic effects were not related to any treatment tested. *E. japonica* extract induced clastogenic effects at 1, 5 and 10 mg/mL in continuous treatment, 5 and 10 mg/mL in acute discontinuous treatment and at 10 mg/mL in chronic discontinuous treatment. Clastogenic effect was also observed at 10 mg/mL of *A. heterophyllus* extract in continuous and acute discontinuous treatments.

Key words: Mutagenicity, biological contamination, *acacia mangium* Willd, *artocarpus heterophyllus* Lam, *eriobothrya japonica* (Thunb.) Lindl.

1. Introduction

Biological contamination by invasive alien species is the second greatest global threat to biodiversity (first in islands and protected natural areas). In protected areas, its impact goes beyond the change in community structure. They interfere with nutrient cycling, hydrology and fire regime [1] and generate the fragmentation of habitats, which increase the degree of isolation of protected areas, their vulnerability and susceptibility to new invasions [2].

All protected areas in Brazil present some degree of biological invasion, such as the conservation units of Espírito Santo state (southeast of Brazil), and three of the most common invasive species are *Artocarpus heterophyllus* Lam, *Eriobotrya japonica* (Thunb.) Lindl, and *Acacia mangium* Willd.

The success of alien plants in the occupation of new environments is a global problem which is difficult to solve and it is essential that all aspects that allow this success be investigated in order to find solutions to this issue. There are few studies in this area and they are limited to ecological aspects, such as the study of the action of the allelochemicals produced by these species on growth parameters and development of the native species affected. Molecular aspects involved are neglected and represent an important research gap that can contribute significantly to the understanding of the invasion mechanisms, providing subsidies to assist in developing of effective strategies for the control and eradication of these species in the invaded ecosystems without affecting the native species.

Living beings in nature are constantly exposed to various substances that have the ability to interact with DNA (deoxyribonucleic acid), changing its structure quickly. In well-adapted populations, chromosomal alterations are often harmful to the cells [3] and affect vital processes, such as replication and gene transcription, which may lead cell death [4-7].

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Substances presented in the environment may cause damage to the genetic material by various mechanisms, interfering in the mitotic spindle, proteins repair activity and promoting chromosome damage. In addition, genotoxic agents have been divided into two classes, according to their mechanism of action, aneugenic, which affects the mitotic spindle apparatus, leading to aneuploidies; and clastogenic, whose action consists in causing injuries in the chromosomes [8].

The analysis of chromosomal alterations is one of the few direct methods for mutagenic evaluation and it is necessary to a test organism in constant mitotic division to observe the changes throughout the cell cycle, such as the inhibition of cell cycle, interruption in metaphases, emergence of bridges and micronuclei and the induction of numerical and structural chromosomal alterations, among others [9, 10].

Allium cepa test is one of the most used *in vivo* tests to evaluate the cytotoxic and genotoxic potential of secondary metabolites of plants in their different concentrations and different exposure times [9, 10]. This method allows the detection of damages in macroscopic parameters, such as alterations in color, size and morphology of roots and microscopic changes, such as alterations in mitosis [11-13]. The use of *A. cepa* test enables simultaneous and comparative evaluation of several parameters, such as germination, root size and morphology, dead and aberrant cells, and mitotic index [14, 15]. In addition, spontaneous chromosomal abnormalities are extremely rare in this species, which makes it possible to associate the damage occurred to the effect of the test substance [16].

The present work aimed to evaluate the cytotoxic, genotoxic and mutagenic action of leaves extracts of *Artocarpus heterophyllus* Lam, *Eriobotrya japonica* (Thunb.) Lindl and *Acacia mangium* Willd, alien species presented in protected area in Espírito Santo-Brazil, in order to verify if this was the invasion mechanism used by these species.

2. Method and Materials

2.1 Plant Material and Extract Preparation

The leaves of *Acacia mangium* Willd, *Artocarpus heterophyllus* Lam and *Eriobothrya japonica* (Thunb.) Lindl were collected, respectively, in Parque Estadual de Itaúnas, Instituto Estadual de Meio Ambiente e Recursos Hídricos and Parque Estadual de Pedra Azul. The plant material was collected between March and April, 2014 and one exsiccate of each species was deposited at Herbarium of the Universidade Federal do Espírito Santo, Brazil.

For preparing the extracts, 2 kg of the dry vegetable materials were added to 5 L of 99.3% ethyl alcohol (INPM) and subjected to exhaustion maceration for five days protected to the light. After the maceration period, the material was filtered and subjected to rotoevaporation in Tecnal Rotating Evaporator (TE 210 model) to obtain the crude ethanolic extracts.

2.2 Evaluation of Cytotoxic, Genotoxic and Mutagenic Activity in Allium Cepa Test

Thirty *Allium cepa* seeds, variety Baia Periform (Topseed-Agristar of Brazil LTDA) (2n = 16) − from a same lot, were germinated at room temperature in soaked filter paper in Petri plates with four distincts concentrations of ethanolic extracts of *Acacia mangium* Willd, *Artocarpus heterophyllus* Lam and *Eriobothrya japonica* (Thunb.) Lindl (1, 5, 10 and 50 mg/mL) and in deionized water (NC (negative control)). Each treatment was made in triplicate totalizing ninety seeds per treatment. The treatments were performed by:

Continuous treatment—the seeds were germinated directly in deionized water (CN) or one of the four concentrations of ethanolic extracts of *Acacia mangium Willd*, *Artocarpus heterophyllus* Lam or *Eriobothrya japonica* (Thunb.) Lindl (1, 5, 10 and 50 mg/mL).

Discontinuous treatment—the seeds were first germinated in deionized water until their radicles

reached 1 cm in length. Then, the radicles were transferred to other Petri plates and received their respective treatments (NC or one of four concentrations of the extracts). After 20 h (acute treatment), 45 radicles were collected randomly and the other 45 radicles were maintained in Petri's plate to receive the appropriate treatment until completion at 72 h (chronic treatment).

Subsequently, the radicles of continuous and discontinuous treatment were fixed in Carnoy 3:1, maintained at room temperature for 24 h and after stored at 10 ºC until the preparation of the slides for the cytological analyses. The fixed radicles were subjected to hydrolysis in 1 N hydrochloric acid at 60 ºC for 5 minutes, washed with deionized water and treated with Schiff Reagent for 2 h in the dark for staining. The maceration of the radicles was carried out with 1 drop of acetic orcein. Six slides of each treatment were analyzed with an optical microscope at 40 x, 1,000 cells for slide, 6,000 cells analyzed per treatment.

For analysis of the cytotoxic effect, the mitotic index was calculated by Eq. (1):

$$MI = \frac{\text{number of cells in division}}{\text{total of cells analyzed}} \times 100$$

To evaluate the AI (aneugenic index) was analyzed cells in division with irregular metaphase (c-metaphase), changes in anaphase (multipolar, delayed, etc.), changes in telophase (delayed), binucleated and multinucleated cells and chromosomal losses.

CI (clastogenic index) was evaluated by the frequencies of cells with micronuclei, grip, bridges and chromosomal breaks and cell death was determined. MI (mitotic index) and aberration index were analyzed for the acute discontinuous treatments and the germination index, mitotic indices and aberration were determined by the continuous treatment.

The data were statistically analyzed with the Chi-squared method, with significance levels fixed at $p < 0.05$ and $p < 0.01$.

3. Results and Discussions

In Tables 1-3, the results of the *A. cepa* test at continuous (CT) and acute (DT 20 h) and chronic (DT 72 h) discontinuous treatments with *Acacia mangium* Willd, *Artocarpus heterophyllus* Lam and *Eriobothrya japonica* (Thunb.) Lindl extracts are summarized. The Table 1 shows MI, AI, and CI observed in *A. cepa* seeds treated with 1, 5, 10 and 50mg/mL of *A. mangium* Willd ethanolic extract. The presence of cells in later stages of the division (metaphase, anaphase and telophase) decreases considerably when compared to the NC. This decrease interfered with MI and this reduction was statistically significant in all tested concentrations for the continuous and discontinuous treatments.

The concentrations of 10 and 50 mg/mL did not present cells in the advanced phases of the mitosis (metaphase, anaphase, telophase) in the continuous treatment (Table 1), suggesting that the phytochemicals presented in the medium may have compromised fundamental aspects of the replication of the genetic material. The scarcity of metaphase, anaphase and telophase indicate a delay or a block of mitosis [17], which suggests that the activation of the control point between G2 and mitosis, stagnating the cell cycle, occurs in its initial phase, avoiding chromosomal segregation.

MI is an indicator of adequate cell proliferation and is an important parameter to evaluate the negative influence of extracts in the cell cycle [10, 18]. The comparison between MI values of the treatment groups and the negative control group can be used to infer about the development of the exposed organism. Low MI values signalize the decrease in the growth and development [11, 19] and high MI values suggest uncontrolled cell division process, which may lead disordered cell proliferation and tumor development. MI decrease was observed in a study performed with

phytochemicals presented on fruit of *Z. limonella* Alston [20], similar to our study. On the other hand, Aguiar et al. [21] observed an increase in the MI in a study with phytochemicals produced by mycorrhizal fungus *Trichoderma sp.*, which suggests that theses metabolites may promote root tissue growth of the plants associated with fungus.

A. mangium Willd extract did not induce aneugenic and clastogenic abnormalities at continuous treatment by 50mg/mL (Table 1). The absence of aneugenic alterations, AI, can result in a cell cycle block in the first stages of mitosis. This suggests that there were no significant cell number in the stage of chromosome separation, the moment that these abnormalities can be observed. A high number of dead cells were observed in *A. mangium* Willd extract treatment groups (data not shown) and this early cell death can be another reason to the low frequency of aberrant cells observed (Table 1).

Cell division process of the radicles was affected at all concentrations and in all treatments with *Artocarpus heterophyllus* Lam extracts (Table 2). *A. heterophyllus* treatments showed highest number of cells in later phases of the cell division (Table 2) when compared with *A. mangium* (Table 1) and this amount of cell in later phases declined as the concentration of the *A. heterophyllus* extract increased. The seeds exposed to continuous treatment with 50 mg/mL of extract did not germinate and in the discontinuous treatment, germinated previously in water, the radicle cells did not advance beyond prophase, demonstrating some cytotoxic effect of *A. heterophyllus* Lam extract at 50 mg/mL, which may explain the absence of germination in continuous treatment.

MI is decreased by dose-dependent effect to continuous and chronic discontinuous treatments (Table 2), similar results to Ping et al. [22] in a study with *Euphorbia hirta* extract using *A. cepa* system. Dead cells were frequently observed after *A. heterophyllus* Lam treatment (data not shown), which may occur due to a disorder in the cellular antioxidative machinery, triggering the increase of oxidative stress and EROs production [14]. According to Patnaik et al. [14], this increase of EROs can lead genomic instability, mutagenic damages and apoptosis. Aneugenic index did not differ statistically to the negative control, which can be attributed to elevate number of dead cells, and clastogenic effect increased at 10 mg/mL dose in continuous treatment and at 1, 10 e 50 mg/mL in acute discontinuous treatment (Table 2).

Table 1 MI, AI and CI of *A. cepa* seeds at continuous (CT) and acute (DT 20h) and chronic (DT 72h) discontinuous treatment protocols with NC and four concentrations of *Acacia mangium* Willd extract.

Treatment	Concentration (mg/mL)	I	P	M	A	T	M.I (%)	A.I (%)	C.I (%)
NC	-	3145	2764	70	63	73	48.82 ± 3.68	0.29 ± 0.12	0.01 ± 0.01
CT	1	4522	1514	4	9	10	25.47** ± 3.43	0.09 ± 0.05	0.03 ± 0.03
	5	4128	1923	12	10	24	32.42* ± 2.34	0.24 ± 0.05	0.13 ± 0.11
	10	4498	1617	0	0	1	26.44** ± 2.62	0.00 ± 0.00	0.08 ± 0.08
	50	5135	898	0	0	0	14.91** ± 1.00	0.00 ± 0.00	0.00 ± 0.00
DT 20h	1	4619	1494	1	2	11	24.67** ± 1.96	0.09 ± 0.05	0.00 ± 0.00
	5	4593	1500	5	9	9	25.00** ± 2.50	0.11 ± 0.04	0.03 ± 0.02
	10	4949	1069	3	5	9	18.04** ± 1.35	0.06 ± 0.03	0.11 ± 0.11
	50	4721	1397	1	0	1	22.91** ± 2.70	0.00 ± 0.00	0.03 ± 0.02
DT 72h	1	4284	1691	19	19	58	29.57** ± 2.71	0.13 ± 0.04	0.13 ± 0.07
	5	4515	1695	17	9	40	28.21** ± 1.89	0.14 ± 0.03	0.06 ± 0.04
	10	4533	1499	23	18	46	26.05** ± 0.73	0.21 ± 0.08	0.06 ± 0.04
	50	5018	1015	2	1	2	16.92** ± 1.73	0.16 ± 0.07	0.01 ± 0.01

I−Interphase; P−Prophase; M−Metaphase; A−Anaphase; T−Telophase.

* $p < 0.05$; ** $p < 0.01$.

Table 2 MI, AI and CI of *A. cepa* seeds at continuous (CT) and acute (DT 20h) and chronic (DT 72h) discontinuous treatment protocols with NC and four concentrations of *Artocarpus heterophyllus* Lam extract.

Treatment	Concentration (mg/mL)	I	P	M	A	T	M.I (%)	A.I (%)	C.I (%)
NC	-	3145	2764	70	63	73	48.82 ± 3.68	0.29 ± 0.12	0.01 ± 0.01
CT	1	4180	1865	25	16	27	31.77* ± 3.33	0.13 ± 0.06	0.06 ± 0.04
	5	4555	1513	12	3	9	25.35** ± 4.42	0.19 ± 0.16	0.00 ± 0.00
	10	4404	1701	3	2	6	27.93** ± 3.09	0.00 ± 0.00	0.01* ± 0.01
	50	Did not germinate							
DT 20h	1	4715	1355	17	19	18	23.18** ± 2.95	0.14 ± 0.07	0.01* ± 0.01
	5	4243	1843	13	20	14	31.02* ± 3.89	0.25 ± 0.07	0.09 ± 0.09
	10	4579	1478	25	12	30	25.35** ± 2.13	0.25 ± 0.08	0.29* ± 0.25
	50	3952	2089	1	4	1	34.57* ± 1.83	0.01 ± 0.01	0.26* ± 0.26
DT 72h	1	4284	1691	19	19	58	29.57** ± 2.71	0.24 ± 0.07	0.00 ± 0.00
	5	4515	1695	17	9	40	28.21** ± 1.89	0.14 ± 0.03	0.01 ± 0.01
	10	4533	1499	23	18	46	26.05** ± 0.73	0.16 ± 0.07	0.01 ± 0.01
	50	5018	1015	2	1	2	16.92** ± 1.73	0.11 ± 0.05	0.21 ± 0.13

I−Interphase; P−Prophase; M−Metaphase; A−Anaphase; T−Telophase.

* $p < 0.05$; ** $p < 0.01$.

Table 3 MI, AI and CI Index of *A. cepa* seeds at continuous (CT) and acute (DT 20h) and chronic (DT 72h) discontinuous treatment protocols with NC and four concentrations of *Eriobothrya japonica* (Thunb.) Lindl extract.

Treatment	Concentration (mg/mL)	I	P	M	A	T	M.I (%)	A.I (%)	C.I (%)
NC	-	3145	2764	70	63	73	48.82 ± 3.68	0.29 ± 0.12	0.01 ± 0.01
CT	1	4549	1295	38	33	32	23.61** ± 4.42	0.49 ± 0.20	0.69** ± 0.36
	5	4523	1461	37	28	40	25.86** ± 1.59	0.52 ± 0.14	0.55** ± 0.36
	10	4318	1668	20	17	20	28.44** ± 4.37	0.53 ± 0.06	1.06** ± 0.37
	50	4416	1720	0	0	0	27.92** ± 3.41	0.06 ± 0.04	0.11 ± 0.08
DT 20h	1	4709	1250	18	15	25	22.02** ± 2.16	0.74 ± 0.07	0.19 ± 0.09
	5	5071	1106	23	8	14	18.51** ± 1.70	0.28 ± 0.09	0.55** ± 0.30
	10	4752	1368	57	15	42	23.95** ± 3.45	0.36 ± 0.13	0.39** ± 0.18
	50	4318	1798	15	11	9	30.02** ± 2.91	0.30 ± 0.07	0.06 ± 0.06
DT 72h	1	4522	1539	18	18	29	26.43** ± 3.69	0.37 ± 0.10	0.11 ± 0.05
	5	4541	1487	24	10	19	25.53** ± 2.66	0.21 ± 0.09	0.14 ± 0.09
	10	4686	1392	36	29	31	23.85** ± 4.10	0.20 ± 0.10	0.88** ± 0.04
	50	Cell did not react to pigmentation							

I−Interphase; P−Prophase; M−Metaphase; A−Anaphase; T−Telophase.

* $p < 0.05$; ** $p < 0.01$.

Eriobothrya japonica (Thunb.) Lindl. extract induced MI reduction at all concentrations tested in all treatments groups (Table 3). The interruption of cell division process was observed when radicles, in initial stages, were treated with 50 mg/mL in continuous treatment. In chronic discontinuous treatment, MI decreased in a dose-dependent relation (Table 3), indicating the interference of high phytochemicals concentration accumulation in radicles development.

Morphological changes were observed during *A. cepa* radicles development in all treatments groups at concentrations of 10 and 50 mg/mL. The radicles presented darkening, especially in the meristematic region, and this interfered in the permeability of Schiff Reactive in chronic discontinuous treatment at 50 mg/mL. Similar morphological alteration in radicles was observed in a toxic and genotoxic effects investigation with industrial effluents [17].

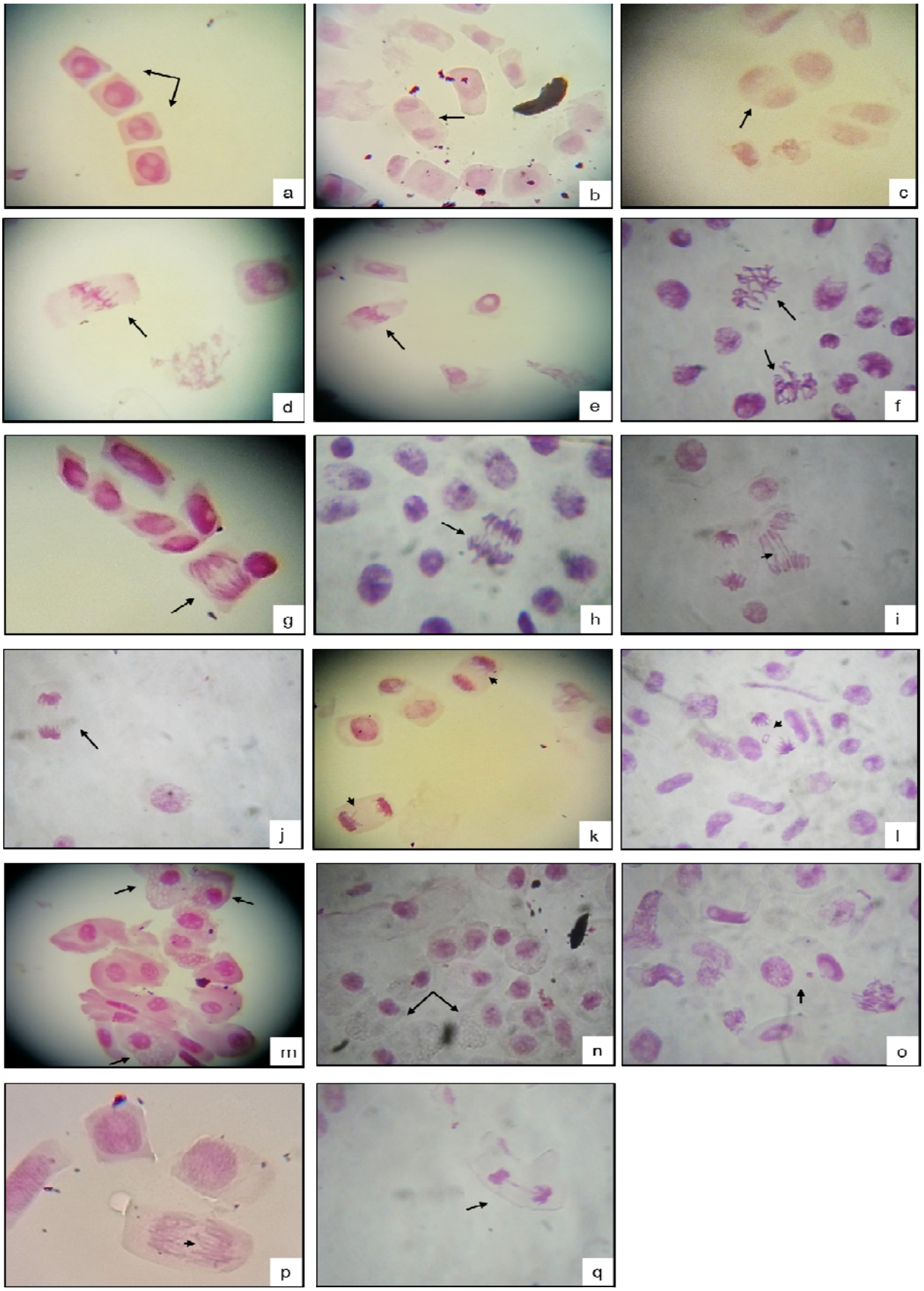
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Fig. 1 Anomalies observed in meristematic cells of *Allium cepa* radicles treated with ethanolic extracts of *A. mangium* Willd, *A. heterophyllus* Lam and *E. japonica* (Thunb.) Lindl.

Where: (a)−normal interphasic cells; (b-c)−binucleated cell; (d)−normal metaphase; (e)−adherence; (f)−c-metaphase (up) and adherence (down); (g)−normal anaphase; (h)−multipolar anaphase; (i)−delay anaphase (arrowhead); (j)−normal telophase; (k)−delay telophase (arrowhead); (l)−chromosome loss; (m-n)−cell death; (o)−micronucleated cell; (p)−anaphase with chromosome breakage (arrowhead); (q)−Chromosome bridge. Magnification: 400 X.

This morphological alteration made it impossible to differentiate the nuclear region and made cell analysis unviable. The frequency of dead cells increased considerably in the continuous and discontinuous acute treatments and at 10 mg/mL in chronic discontinuous treatment. Cell death can promote changes in cell permeability and this may difficult or block the staining of chronic discontinuous treatment at 50mg/mL.

Aneugenic index of the seeds treated with the extract of *E. japonica* (Thunb.) Lindl showed a tendency to increase, but this index was not statistically different to the negative control. Significant changes were observed in clastogenic index at de concentration of 1, 5 and 10 mg/ml of continuous treatment, in 5 and 10 mg/mL of acute discontinuous treatment and in 10 mg/mL of chronic discontinuous treatment, probably due to the high number of dead cells in these treatments.

Aneugenic and clastogenic index were not statistically different at the concentration of 50 mg/mL of *E. japonica* (Thunb.) Lindl. extract. These events may have occurred due to some protective effect or reversal of genotoxicity or may be attributed to changes in cellular permeability mechanisms. Phytochemical molecules presented in the concentration of 50 mg/mL are five times higher than the concentration of 10 mg/mL. This high concentration may promote quick saturation in membrane receptors, reducing considerably the input of phytochemical molecules into the cells, which may not be sufficient to cause changes in DNA and provide the cytotoxic effect observed in our study, MI reduction (Table 3).

The anomalies observed in the cells treated with the extracts of *A. mangium* Willd, *A. heterophyllus* Lam and *E. japonica* (Thunb.) Lindl. are shown in Fig. 1. Micronuclei were observed in the continuous treatments of all extracts (Tables 1-3) and in the chronic discontinuous treatment with *A. mangium* Willd extract (Table 1) and *E. japonica* (Thunb.) Lindl extract (Table 3), but the micronucleus frequency was not statistically significant for the extracts tested (Tables 1-3). The inhibition of the cell cycle may have contributed to the low number of micronuclei observed, since it is a result of a dysfunctional mitotic process in which chromosomal breaks occurred [9].

4. Conclusions

Understanding all the aspects that make it an invasive species is fundamental in search to efficient solutions to biological contamination issue. Therefore, the results showed at this paper reinforce the importance to investigate the intracellular aspects like modification in cell cycle and in the genetic material as a mechanism that facilitates the process of invasion by alien species.

In relation to *A. mangium* Willd, one of those mechanisms to involve cytotoxicity since mitotic index presented by *A. cepa* seeds treated with its extract showed statistical significant changes and this reduction can interfere negatively in the germination of native species. Thus, the cytotoxicity helps *A. mangium* Willd at occupying new environments. However, it is not due to genotoxic actions once were not aneugenic and clastogenic effect, probably due to the blocking of cell division process at prophase phase.

To *A. heterophyllus* Lam and *E. japonica* (Thunb) Lindl, their extracts induced cytotoxic and genotoxic/mutagenic effects beyond cytotoxicity. At this way, chromosomal anomalies and induction of

cell death are probably the main mechanism involved in genetic material damage induced by *E. japonica* (Thunb.) Lindl and *A. heterophyllus* Lam. Summarily the alien species *A. mangium* Willd, *A. heterophyllus* Lam and *E. japonica* (Thunb) Lindl are able to induce damages into the cells and to genetic material, and this may be one mechanism used by them to have advantages and successfully occupy new environments.

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Conflict of Interest

Authors declare no competing financial interests associated with the manuscript exist.

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