



# Protective role of jaboticaba *Plinia peruviana* peel extract in copper-induced cytotoxicity in *Allium cepa*

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## Abstract

Jaboticaba *Plinia peruviana* (Poir.) Govaerts is a Brazilian berry that presents high levels of polyphenols, which may play a key role in preventing cytotoxic and genotoxic effects of harmful agents. Although copper is an essential micronutrient that plays an important role in organisms, high copper concentrations may trigger toxicity to animals and plants. Here, we investigated whether *Plinia peruviana* hydroalcoholic extract prevents copper-induced cytotoxicity in *Allium cepa* root cells. Five different anthocyanins and phenolic compounds were identified in *Plinia peruviana* extract. Importantly, the exposure to 1.53 mg/L copper for 24 h impaired mitotic index, as well as increased mitosis disturbances and triggered DNA damage. Pre-incubation with *Plinia peruviana* extract (0.25 g/L and 0.75 g/L) for 3 h prevented copper-induced changes in the mitotic index and reduced the number of abnormal cells. In conclusion, we suggest that *Plinia peruviana* peel extract has protective effects against cellular and genetic disturbances induced by copper.

**Keywords** DNA damage · Mitotic index · Protection · Polyphenols · Anthocyanins · Copper toxicity

## Introduction

*Plinia peruviana* (Poir.) Govaerts is a native Brazilian berry known as “Jaboticaba.” It belongs to the Myrtaceae family and is widely distributed in tropical and

subtropical regions in Central and South America and Australia (Mabberley 1997). Jaboticaba is an edible fruit, considered a good dietary source of ellagic acid and other antioxidants (Abe et al. 2012; Neves et al. 2018). Jaboticaba is widely used in the food and pharmaceutical

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industries due to its medical, antioxidant, and antibacterial properties (Mazzarino et al. 2018; Oliveira et al. 2018). In folk medicine, *Plinia* sp. has been used to treat stomach disorders, throat afflictions, and diabetes (Stasi and Hiruma-Lima 2002). Epidemiological studies show that rich dark-colored fruits reduce the incidence of cardiovascular diseases, diabetes, stroke, and cancer (Crozier et al. 2009; Boeing et al. 2012). The presence of anthocyanins (ATH) and depsides in these fruit, which has strong antioxidant, anti-inflammatory, and antigenotoxic properties, may help explain these effects (Borges et al. 2014).

Mounting evidence suggests that consumption of fruit and vegetables is correlated with a low incidence of oxidative stress-related diseases (e.g., cancer and metabolic disorders) (Ramirez-Tortosa et al. 2001; Leite et al. 2011; Wang et al. 2013; Guo and Ling 2015; Belwal et al. 2017). Moreover, ATH-enriched extracts prevent tumor cell proliferation and DNA damage induced by heavy metal exposure in plants (Glińska et al. 2007; Wang et al. 2011; Leite-Legatti et al. 2012; Fernandes et al. 2013; Wang et al. 2014; Khan et al. 2016). *Plinia trunciflora* (O. Berg.) Kausel [syn. *Plinia peruviana* (Poir.) Govaerts] extracts present high polyphenol levels and reduce amiodarone-induced cell death in human lung fibroblasts (Calloni et al. 2016). Although their antioxidant effects are commonly associated with the presence of ATH and phenolic compounds (Sacchet et al. 2015), there are no studies regarding their potential protective role against metal-induced cytotoxicity.

Copper is an essential micronutrient that plays a key role in biological processes, as a cofactor of various enzymes. However, accidents, occupational hazards, and industrial and agricultural practices may increase copper levels in the environment. Thereby, copper may become an environmental contaminant (Gaetke and Chow 2003; Wang and Björn 2014). Studies have shown that high copper concentrations cause toxicity to plants and animals (Feigl et al. 2013; Pal et al. 2013). Copper toxicity may involve oxidative stress (Gaetke and Chow 2003), growth inhibition, disturbances in cell division, and DNA damage (Qin et al. 2015), thus culminating in cytotoxicity and genotoxicity in various organisms, including plants (Yildiz et al. 2009; Avery 2011; Sies 2015; Wang et al. 2016).

In order to investigate the potential antigenotoxicity of *Plinia peruviana* extract (PPE) against copper-induced cytotoxicity, we used *Allium cepa* as a model organism due to its high sensibility to harmful agents, providing reliable data when compared to other existing approaches (Leme and Marin-Morales 2009). Importantly, meristematic *Allium cepa* cells have been largely used to detect DNA damage, such as chromosomal aberrations (CA), nuclear abnormalities (NA), and cell cycle arrest (Leme and Marin-Morales 2009).

## Materials and methods

### Materials

Copper sulfate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) was obtained from Neon Commercial LTDA (São Paulo, SP, Brazil). All other chemicals were analytical grade. In this study, we used *Allium cepa* bulbs of a commercial variety (Baia Periforme) in order to ensure homogeneity in the biological responses measured.

### Plant material

All pieces of fruit of *Plinia peruviana* were collected in the city of Alpestre (in the state of Rio Grande do Sul, Brazil) ( $27^\circ 10' 56.82''$  S and  $53^\circ 7' 19.55''$  O) and taxonomically identified. The specimens were deposited at the university herbarium, as described elsewhere (Sacchet et al. 2015).

### Preparation of PPE

The PPE of fruit peel was prepared as reported previously (Veggi et al. 2011). Briefly, fruit peels (1 kg) were dried at  $40^\circ\text{C}$  for 48 h. Later, 1000 mL of absolute ethyl alcohol and distilled water (70%:30%) were added, and the mixture was protected from light for 72 h. Then, the solvent was evaporated, and the extract was freeze-dried for the conduction of further assays. For cytotoxicity and genotoxicity assays, PPE was dissolved in distilled water at two concentrations (0.25 g/L and 0.75 g/L), and the pH of the aqueous solution was adjusted to 6.5 using NaOH 1 N.

### Total phenolic compounds

Total phenolic compounds were determined as described elsewhere (Singleton and Rossi 1965). The analysis is based on the reduction of the phosphomolybdic-phosphotungstic acid complex by phenolic compounds to a blue product that is measured at 750 nm. Results were expressed as gallic acid equivalents (mg gallic acid equivalents/100 g extract), and the values were expressed as means of triplicate analysis.

### Quantification of compounds by HPLC-DAD

All chemicals were analytical grade. Acetonitrile and acetic acid were purchased from Merck (Darmstadt, Germany). Cyanidin chloride, cyanidin 3-O-glucoside chloride, malvidin chloride, malvidin 3-O-glucoside chloride, and delphinidin 3-O-glucoside chloride were acquired from ChromaDex. High-performance liquid chromatography-diode array detection (HPLC-DAD) was performed as described previously (Bitencourt et al. 2016). A Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan)

was equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, a SPD-M20A diode array detector, and the software LC solution 1.22 SP1. Reverse phase chromatography was performed under gradient conditions using C18 column (4.6 mm × 150 mm) packed with 5- $\mu$ m diameter particles; the mobile phase was water containing 1% acetic acid (A) and acetonitrile (B), and the composition gradient was 13% of B until 10 min and changed to obtain 20%, 30%, 50%, 60%, 70%, 20%, and 10% B at 20, 30, 40, 50, 60, 70, and 80 min, respectively, following the method described by Bitencourt et al. (2016) with slight modifications. PPE was analyzed at a concentration of 15 mg/mL. The flow rate was 0.6 mL/min, injection volume was 40  $\mu$ L, and the wavelength was set at 520 nm. All samples and the mobile phase were filtered through a 0.45- $\mu$ m membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standard references were prepared in the HPLC mobile phase at a concentration range of 0.030–0.500 mg/mL. Chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (300 to 700 nm). Calibration curve for each substance was cyanidin chloride,  $Y = 13,054x + 1245.8$  ( $r = 0.9997$ ); cyanidin 3-O-glucoside chloride,  $Y = 12,695x + 1184.3$  ( $r = 0.9999$ ); malvidin chloride,  $Y = 12,749x + 1267.9$  ( $r = 0.9996$ ); malvidin 3-O-glucoside chloride,  $Y = 13,158x + 1305.2$  ( $r = 0.9995$ ); and delphinidin 3-O-glucoside chloride,  $Y = 12,539x + 1183.1$  ( $r = 0.9997$ ). All chromatography operations were carried out at room temperature and run in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10  $\sigma/S$ , respectively, where  $\sigma$  is the standard deviation of the response and  $S$  is the slope of the calibration curve (Bitencourt et al. 2016).

## Experimental design

Treatments were performed based on a previous report (Glińska et al. 2007). Five healthy *Allium cepa* bulbs ( $n = 5$ ) were used for each experimental group. Bulbs were maintained in the dark under specific temperature conditions (26 °C) and exposed to a saline solution containing  $\text{KNO}_3$  (0.51 g/L),  $\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$  (1.18 g/L),  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  (1.23 g/L),  $\text{KH}_2\text{PO}_4$  (0.14 g/L), and EDTA (0.005 g/L), for 72 h at pH 6.5, for growth of primordial roots. Later, roots were initially exposed to an aqueous PPE solution (0.25 g/L and 0.75 g/L), pH 6.5, for 3 h. Then, the roots were incubated with an aqueous solution of copper 1.53 mg/L ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) for 24 h. Roots allocated in the PPE group were kept in the PPE for 3 h and later kept in the saline solution until the end of the incubation period. The control group was

maintained in the saline solution throughout the experimental period. A group of bulbs were exposed to copper and were not exposed to the extracts. PPE concentrations were chosen based on pilot studies, which revealed that 0.25 and 0.75 g/L did not show toxicity in *Allium cepa* meristematic cells (Fig. S1). Copper concentration (1.53 mg/L) was selected based on its potential toxicity, as reported elsewhere (Hu et al. 2015).

## *Allium cepa* analysis

The protective role of PPE against copper-induced genotoxicity was assessed using the *Allium cepa* test (Fiskesjö 1985). After the exposure period, the roots of each bulb were fixed in methanol:acetic acid (3:1). The roots were submitted to acid hydrolysis in acetic acid 45%:HCl 1 N (9:1) at 50 °C for 5 min. To prepare the slides, meristematic regions (approximately 2 mm) were removed, covered with a coverslip, and squashed into a drop of 2% acetic orcein solution. To measure the cytotoxicity of treatments, we assessed the mitotic index (MI), which was obtained by counting the total number of mitosis within 1000 cells. CA such as fragments, breaks, losses, bridges, delays, adherence, multipolarity, and disturbed mitosis within different cell division stages (metaphase, anaphase, and telophase) were used to quantify genotoxicity (Leme and Marin-Morales 2009). We analyzed 500 mitoses for each experimental group to quantify CA. NA characterized by morphological changes in the structure of interphasic nuclei, such as lobulated nuclei, nuclear buds, and polynuclear cells, were also assessed. We analyzed 1000 interphase cells for each experimental group to quantify NA. Furthermore, the total number of abnormal cells was obtained by adding up the total amount of CA and NA obtained for each experimental group. All analyses were performed under a light microscope (model Olympus CX31BRASFA) at  $\times$  1000 magnification.

## Statistical analysis

Because all data was normally distributed (Kolmogorov-Smirnov test) and homoscedastic (Bartlett's test), results were expressed as the mean  $\pm$  S.E.M and analyzed by two-way analysis of variance (ANOVA), followed by Student-Newman-Keuls multiple comparison test. Statistical significance was set at  $p \leq 0.05$ .

## Results

### Total phenolic compounds and HPLC analysis

Total phenolic compounds in PPE were 1276 mg GAE/100 g extract. HPLC fingerprinting of PPE revealed the presence of cyanidin (retention time,  $R_t = 10.27$  min; peak 1;  $51.69 \pm$

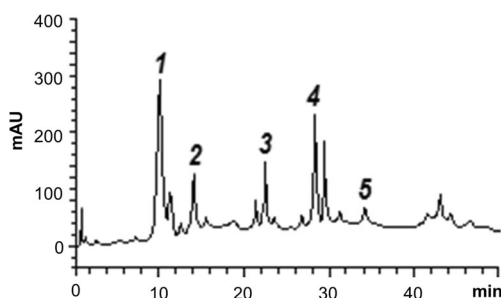
0.17 mg/g), malvidin ( $R_t = 12.03$  min; peak 2;  $23.08 \pm 0.06$  mg/g), delphinidin 3-O-glucoside ( $R_t = 22.19$  min; peak 3;  $30.17 \pm 0.23$  mg/g), cyanidin 3-O-glucoside ( $R_t = 27.85$ ; peak 4;  $38.11 \pm 0.40$  mg/g), and malvidin 3-O-glucoside ( $R_t = 31.96$  min; peak 5;  $4.71 \pm 0.01$  mg/g) (Fig. 1).

### Allium cepa analysis

Cells in various phases of mitosis after the experimental period were assessed by light microscopy. For MI, two-way ANOVA yielded significant effects of copper ( $F_{(1, 20)} = 42.64$ ;  $p = 0.0001$ ) and of a PPE  $\times$  copper interaction ( $F_{(2, 20)} = 6.936$ ;  $p = 0.0052$ ). Post hoc analyses revealed that copper decreased the MI (1.03%) when compared to the control group (10.8%). Nonetheless, pre-incubation with 0.25 g/L and 0.75 g/L PPE for 3 h attenuated these effects (6.8% for both groups) (Fig. 2a).

Regarding the NA, we verified significant effects of PPE ( $F_{(2, 19)} = 11.07$ ;  $p = 0.0006$ ), of copper ( $F_{(1, 19)} = 15.97$ ;  $p = 0.0008$ ), and of a PPE  $\times$  copper interaction ( $F_{(2, 19)} = 5.961$ ;  $p = 0.0098$ ), where PPE prevented the copper-induced NA at both concentrations (Fig. 2b). Conversely, pretreatment with PPE did not prevent the occurrence of CA following copper exposure (Fig. 2c). CA and NA found in *Allium cepa* root cells after the exposure period include adherences, bridges, vagrant forms, multipolar mitosis, disturbed mitosis, fragments, sticky chromosomes, irregular nucleus, nuclear buds, mini cells, and binucleated cells (Fig. 3). CA were classified as shown in Table 1. A higher frequency of total CA was observed in the copper-exposed group. Furthermore, sticky chromosomes were the majority of aberrations caused by copper.

Concerning the total number of abnormal cells, two-way ANOVA revealed significant effects of PPE ( $F_{(2, 20)} = 10.55$ ;  $p = 0.0007$ ), of copper ( $F_{(1, 20)} = 28.06$ ;  $p = 0.0001$ ), and of a PPE  $\times$  copper interaction ( $F_{(2, 20)} = 7.488$ ,  $p = 0.0037$ ). Post hoc analyses showed that pre-incubation with 0.25 g/L and 0.75 g/L PPE prevented the increase in the total number of abnormal cells in copper-exposed roots (Fig. 2d).



**Fig. 1** Chromatogram obtained from jaboticaba *Plinia peruviana* extract (PPE). Cyanidin (peak 1), malvidin (peak 2), delphinidin 3-O-glucoside (peak 3), cyanidin 3-O-glucoside (peak 4), and malvidin 3-O-glucoside (peak 5)

### Discussion

In this study, we report a preventive effect of PPE against copper-induced cytotoxicity for the first time. Our data showed that PPE prevented the increase of NA, reduced the number of abnormal cells, and attenuated the decrease in cell cycle progression caused by copper. Overall, we suggest a protective role of PPE against copper-induced cytotoxicity in the *Allium cepa* root model.

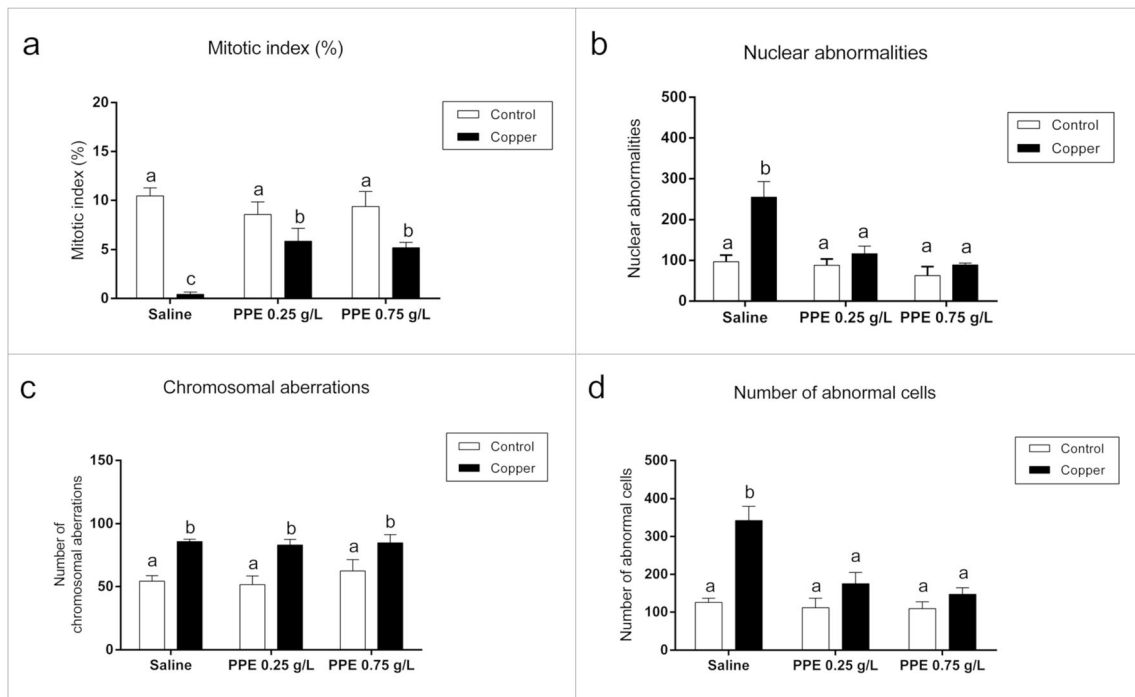
Although copper is an essential element, its levels in the environment must be tightly controlled since elevated copper concentrations may become toxic (Flemming and Trevors 1989). Copper exposure in *Allium cepa* increases DNA damage (CA) and suppresses cell cycle progression (Hemachandra and Pathiratne 2015; Qin et al. 2015). In addition to the potential toxicity to plants, chronic copper toxicity has been related to liver and kidney damage, as well as to the enhancement of inflammation and oxidative stress, promoting neurotoxicity (Gaetke and Chow 2003; Becaria et al. 2006; Pal 2014). Importantly, the copper concentration used here (1.53 mg/L) can be found in drinking water (Harvey et al. 2016). Nonetheless, we observed that the respective concentration increases DNA damage and decreases cell cycle progression, suggesting a potential cytotoxicity.

Recently, studies have identified the phytochemical composition of jaboticaba *Plinia* sp., which constitutes a good source of nutrients, such as minerals, soluble and insoluble fibers, and phenolic compounds, as well as ATH in these fruit peels (Lago et al. 2011; Mazzarino et al. 2018; Neves et al. 2018). ATH belongs to a group of flavonoids that is subdivided into different subclasses (flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanidins) (Howes 2018). Although most ATH found in jaboticaba peels are cyanidin-3-O-glucoside and delphinidin-3-O-glucoside, the presence of cyanidin, malvidin, and malvidin 3-O-glucoside was also reported (Sacchet et al. 2015). This data is in accordance with our study, because these ATH were identified in PPE. Moreover, the presence of these substances accounts for the fruit's antioxidant activity (Leite et al. 2011; Leite-Legatti et al. 2012; Sacchet et al. 2015).

Our analysis revealed that the values of total phenolic compounds in *Plinia peruviana* were similar to blueberries ( $12.00 \pm 0.77$  to  $14.81 \pm 1.58$  g GAE  $\text{kg}^{-1}$ ) (Dai et al. 2009), and lower than *Myrciaria jaboticaba* ( $32.15$  g GAE  $\text{kg}^{-1}$ ) and *Myrciaria cauliflora* ( $31.63 \pm 0.1$  g GAE  $\text{kg}^{-1}$ ) (Reynertson 2007; Leite-Legatti et al. 2012). Other phenolic compounds were detected in *Plinia* sp., including gallic acid, ellagic acid, isoquercitrin, quercimeritrin, quercitrin, myricitrin, and quercetin (Neves et al. 2018).

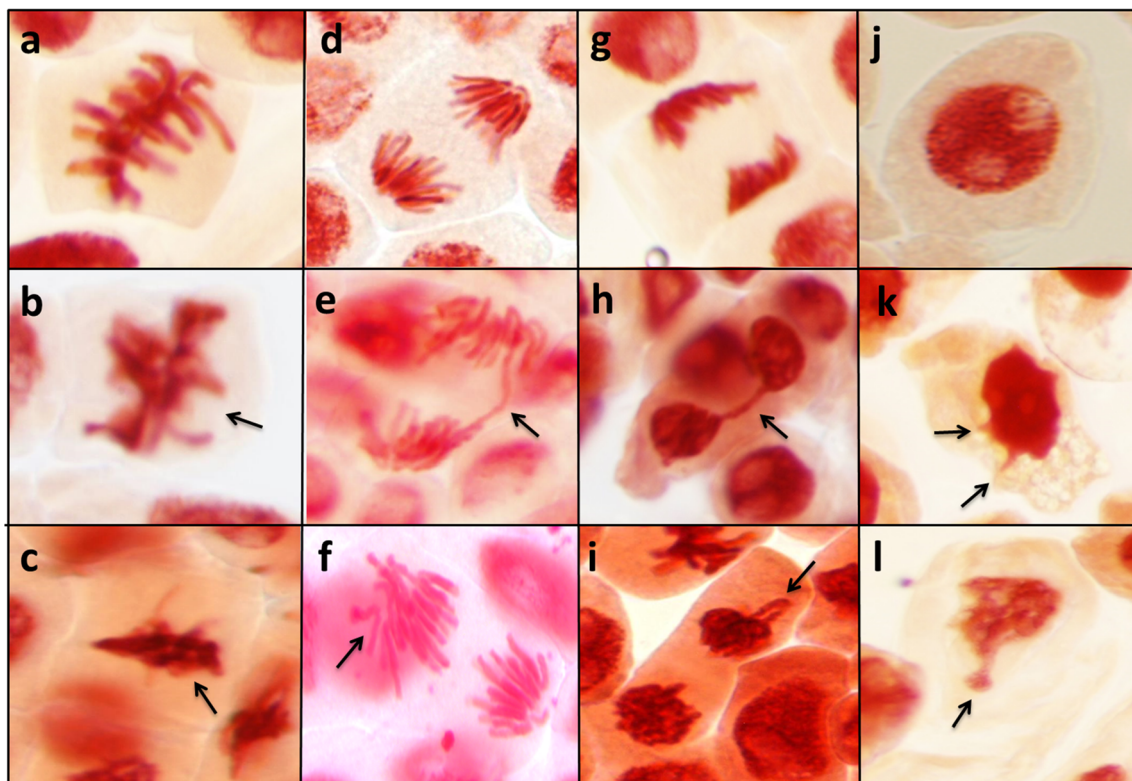
Plants are indeed rich sources of secondary metabolites (terpenes, phenolic compounds, alkaloids), and these compounds probably act synergistically to provide a more effective response than a single molecule (De Marino et al. 2014).





**Fig. 2** Effects of *Plinia peruviana* extract (PPE) on copper-induced cytotoxicity in *Allium cepa* root cells. **a** Mitotic index (MI). **b** Nuclear abnormalities (NA). **c** Chromosomal aberrations (CA). **d** Number of abnormal cells. Results are expressed as means  $\pm$  S.E.M. and analyzed by

two-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Distinct letters denote statistical differences ( $n = 4\text{--}5$  replicates,  $p \leq 0.05$ )



**Fig. 3** Representative figure showing chromosomal aberrations (CA) and nuclear abnormalities (NA) measured in this study. **a** Normal metaphase. **b** Sticky chromosomes. **c** Adherence. **d** Normal anaphase. **e** Bridge in

anaphase. **f** Anaphase with chromosome loss. **g** Normal telophase. **h** Bridge in telophase. **i** Laggard chromosome. **j** Normal cell in interphase. **k** Irregular nuclei. **l** Nuclear bud

**Table 1** Effects of *Plinia peruviana* extract (PPE) on copper-induced chromosomal aberrations in root cells of *Allium cepa*. Data are expressed as means ± S.E.M. and analyzed by one-way ANOVA followed by

Student-Newman-Keuls multiple comparison test. Distinct letters denote statistical differences ( $n = 4\text{--}5$  replicates,  $p \leq 0.05$ )

Metal	Pretreatment		Chromosomal aberrations (CA)							
	0.25 g/L	0.75 g/L	Bridges	Sticky	Delays	Disturbed mitosis	Multipolar anaphase	Losses	Breaks	Total number of CA
–	–	–	1.6±0.6	0.0±0.0 <sup>a</sup>	19.9±7.5	5.8±2.3	0.5±0.5	0.2±0.2	0.2±0.2	65.2±11 <sup>a</sup>
+	–	–	1.6±0.8	73.00±5.3 <sup>b</sup>	11.4±2.8	6.0±2.4	0.4±0.4	1.0±0.5	0.0±0.0	85.8±1.9 <sup>b</sup>
–	+	–	1.6±0.5	7.3±3.8 <sup>a</sup>	18.6±2.0	18±2.0	0.5±0.5	0.7±0.2	0.0±0.0	51.9±6.8 <sup>a</sup>
–	–	+	0.8±0.4	2.7±1.0 <sup>a</sup>	25.7±8.7	25±8.7	0.0±0.0	1.0±0.3	0.0±0.0	62.4±9.0 <sup>a</sup>
+	+	–	0.4±0.4	53.2±12.9 <sup>b</sup>	12.2±4.48	12.2±4.4	0.2±0.2	1.7±0.7	0.0±0.0	83.0±4.5 <sup>b</sup>
+	–	+	1.0±0.36	57.2±15.9 <sup>b</sup>	8.7±4.8	8.7±4.8	0.0±0.0	0.8±0.4	0.1±0.1	84.8±6.4 <sup>b</sup>

Here, pretreatment with PPE mitigates copper-induced cell cycle suppression in *Allium cepa*. Evidence suggests that ATH-enriched extracts protect cells against metal-induced toxicity (Glińska et al. 2007; Posmyk et al. 2009b), and that flavonoids may form strong complexes with copper ions (Ríha et al. 2014). The chelating properties of ATH can lead to metal sequestration, thereby reducing the toxic effects triggered by metals (Glińska and Gapińska 2013). Inside cells, metals can be chelated by compounds with SH-groups, as well as phytochemicals, glutathione, organic acids, and other ligands (Glińska and Gapińska 2013).

Despite copper-induced DNA damage in *Allium cepa*, we observed a trend for the PPE pretreatment to decrease some types of CA (e.g., sticky chromosomes and bridges). Previous data has shown that jaboticaba peel extract did not reduce chromosomal damage induced by cyclophosphamide (Leite-Legatti et al. 2012). Conversely, antigenotoxic effects of jaboticaba seed extracts and *Myrciaria dubia* juice were reported (Da Silva et al. 2012; Silva et al. 2016), showing that such effects are relatively controversial and that more studies are needed to clarify this point.

Additionally, polyphenol-rich jaboticaba extract prevents reduced cell viability and changes in complex-I activity and ATP biosynthesis caused by amiodarone in human lung fibroblast cells (Calloni et al. 2016). Moreover, Caprioli et al. (2016) have shown the presence of cyanidin-3-glucoside as the major constituent among phenolics, revealing potent radical scavenger activity in *Lonicera caerulea* extracts. Experimental evidence suggests that polyphenols may exert their roles by distinct mechanisms: modulating the metabolic activation of mutagens (Zhang et al. 1997), stimulating detoxification enzymes (Posmyk et al. 2009a), scavenging free radicals (Sacchet et al. 2015), and forming strong ligand complexes with ions of metals, such as Fe and Cu (Ferguson 2001; Havsteen 2002). The chelating properties can lead to metal isolation, such as molybdenum (Mo) sequestration in the epidermis of *Brassica sp.* (Hale et al. 2001). Basically, we showed that the protective properties of PPE against copper-

induced cytotoxicity in *Allium cepa* root meristems could be associated with the presence and interaction of ATH and phenolic compounds identified in this fruit. However, further studies are needed to elucidate the mechanisms underlying the beneficial effects of PPE against copper exposure in *Allium cepa* roots.

### Conclusion

In summary, our data shows a protective role of PPE against copper-induced cellular and genetic disturbances in *Allium cepa* meristematic tissue. This study supports the first evidence that ATH and phenolic compounds present in *Plinia peruviana* peels may help protect organisms against copper-induced toxicity. Considering these findings, jaboticaba extract can be considered a promising candidate as a protector against toxic agents and its consumption through diet could be stimulated.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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