



## CELLULAR AND MOLECULAR BIOLOGY

### Cytogenotoxicity and protective effect of piperine and capsaicin on meristematic cells of *Allium cepa* L.

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**Abstract:** Piperine and capsaicin are important molecules with biological and pharmacological activities. This study aimed to evaluate the cytogenotoxic and protective effect of piperine and capsaicin on *Allium cepa* cells. *A. cepa* roots were exposed to negative (2% Dimethylsulfoxide) and positive (Methylmethanesulfonate, MMS, 10 µg/mL) controls, and four concentrations (25-200 µM) of piperine or capsaicin (alone) or associated before, simultaneously or after with the MMS. Only the lowest concentration of piperine (25 µM) showed a protective effect because it was not genotoxic. Piperine and capsaicin were cytotoxic (50, 100 and 200 µM). Piperine (50 to 200 µM) caused a significant increase in the total average of chromosomal alterations of in *A. cepa* cells. For capsaicin, the genotoxic effect was dose-dependent with a significant increase for all concentrations, highlighting the significant presence of micronuclei and nuclear buds for the two isolates. In general, bioactive compounds reduced the total average of chromosomal alterations against damage caused by MMS, mainly micronuclei and/or nuclear buds. Therefore, the two molecules were cytotoxic and genotoxic at the highest concentrations, and did not have cytoprotective action, and the lowest concentration of piperine demonstrated important chemopreventive activity.

**Key words:** Bioactive compounds, mitotic index, chromosomal alterations, protection against cell damage.

## INTRODUCTION

Peppers comprise groups of plants of the genera *Piper* and *Capsicum* (Salazar et al. 2016) and are among the main spices appreciated worldwide. Peppers are functional foods, as they have a potential medicinal application with protective and therapeutic action (Antonio et al. 2018, Rather & Bhagat 2018). The chemoprotective potential of piperamides has already been observed in *P. nigrum* and *P. longum* (Lai et al. 2012, Grinevicius et al. 2017) and in some species of *Capsicum* there are many biologically active metabolites, such as capsaicinoids, capsinoids and carotenoids (Luo et al. 2011,

Bertão et al. 2016). Protection can result from the interaction of molecules with mutagenic agents in the intracellular and/or extracellular (desmutagenic) media and in the damage repair process (bioantimutagenic) (Santos et al. 2012, Asita et al. 2015, Andrade et al. 2016).

The detection of phytochemicals capable of modulating genotoxic, mutagenic and carcinogenic effects show satisfactory results for polyphenols, carotenoids, alkaloids, tannins (Li et al. 2018, Ribeiro et al. 2018, Sá et al. 2019). There are several studies using natural extracts of *Piper* and *Capsicum* for analysis of genotoxicity and antigenotoxicity, however,

studies evaluating isolated molecules are more frequent, given the improvement of the methods for extraction of these compounds and access to these substances commercially (Bley et al. 2012, Rather & Bhagat 2018).

Piperine (1-piperilpiperidine) is an alkaloid isolated mainly from *P. nigrum*, known as black pepper, but can also be found in other representatives of the family Piperaceae (Meghwal & Goswami 2013). The structural characteristics of piperine are divided into three parts: (1) an aromatic ring with a methylenedioxy bridge, associated with the antioxidant and anti-tumor potential; (2) a conjugated dienone system, which performs lipophilic interactions with various molecular residues; and (3) a piperidine ring that creates an amide bond, responsible for insecticidal and anti-tumor actions, in addition to interacting with enzymatic systems, leading to biotransformation effects (Qu et al. 2015, Singh & Choudhary 2015).

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is an alkaloid/amide compound found in *Capsicum* fruit and can account for 1% of the mass of peppers (Fattori et al. 2016). Regarding the structure and activity of capsaicin, stand out the regions: (1) aromatic, responsible for most of the antioxidant activity; (2) amide bond, responsible for the effect of analgesia and antinociceptive activity, and (3) the aliphatic chain, related to analgesic activity and with an important role in the total polarity of the molecule, giving it a hydrophobic character (Huang et al. 2013).

Piperine and capsaicin also have an antiparasitic and immunomodulatory effect (Soutar et al. 2017, Vurmaz et al. 2019). In addition, studies on the antiproliferative, mutagenic/genotoxic/protective and anti-apoptotic/pro-apoptotic effects have been carried out on different types of tumor cells, but these studies have contradictory results for both piperine

and capsaicin (Singh & Duggal 2009, Fattori et al. 2016). Toxicogenic and/or protective effects depend on the dose and the group of cells treated. Understanding the modulatory activity, finding effective concentrations that are safe for the body is especially important. Although the evaluation of genotoxicity/antigenotoxicity of piperine and capsaicin has been carried out *in vitro* and *in vivo* tests (Thiel et al. 2014, Fernandez-Bedmar & Alonso-Moraga 2016), studies that show their effects using chromosomal changes such as cytogenetic biomarkers are incipient and there are no reports on the biological effects of piperine and capsaicin in a plant test system. Thus, the present study can show whether the isolates interact with spindle fibers and / or with the DNA and whether they have a modulating effect against the mutagenic agent.

The test system for chromosomal changes in *Allium cepa* L. is widely cited in the literature as a bioindicator for the evaluation of cytotoxicity, genotoxicity and protective effect of chemical compounds, as it has rapid cell multiplication, large and few chromosomes, which allows better analysis of structural and numerical changes (Leme & Marin-Morales 2009, Bonciu et al. 2018). Its low cost and agreement with other similar tests, which involve the manipulation and sacrifice of animals, justifies its extensive use in toxicogenetic bioassays (Eren & Özata 2014). Besides that, it has a good correlation with cytotoxicity and genotoxicity tests *in vitro* or *in vivo* (Eren & Özata 2014, Sá et al. 2019).

Considering the importance of piperine and capsaicin as spices used in gastronomy and popular medicine (Bley et al. 2012, Muhammad et al. 2018), the present study aimed to investigate the cytotoxic, genotoxic, protective or modulating effects against the damages caused by methylmethanesulfonate, using the chromosomal alterations test in meristematic cells of *A. cepa*.

## MATERIALS AND METHODS

### Tested compounds

Piperine  $\geq$  97% CAS (94-62-2), capsaicin  $\geq$  95% CAS (404-86-4) and Methylmethanesulfonate (MMS, CAS 66-27-3) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### DNA-damaging agent

MMS was used to induce DNA damage in meristematic cells of *A. cepa*. MMS (10  $\mu$ g/mL) is an alkylating agent with direct activity, inducing disturbances such as DNA breaks, bridges and chromosome loss, which are also expressed as micronuclei (Bianchi et al. 2016, Couto et al. 2019). MMS was used in *A. cepa* test because it has high genotoxicity at low concentrations. In addition, MMS shows less cytotoxic effect, allowing cells to continue the cell cycle and those chromosomal changes can be visualized in different phases of cell division (Bianchi et al. 2015).

### *Allium cepa* bioassay

One hundred *A. cepa* (cv. Vale Ouro IPA-11) seeds per Petri dish were germinated with distilled water in an incubator (BOD SL - 224<sup>®</sup>) under a 12 h photoperiod and temperature of 24°C for three days at the Genetics Laboratory (LABGENE) of the Center for Natural Sciences of UESPI, Teresina-PI. After germination, seeds with roots of approximately 1 cm were exposed to different treatments to assess the cytogenotoxicity and antigenotoxicity of the bioactive piperine and capsaicin, according to Nantes et al. (2014) and Couto et al. (2019).

In the genotoxicity test, seeds were transferred to the negative controls (NC) (Dimethylsulfoxide - 2% DMSO in distilled water), solvent (SC) (distilled water), positive MMS I (Methylmethanesulfonate, 10  $\mu$ g/mL dissolved in DMSO 2%) and MMS II (dissolved only in distilled

water) and for treatments with piperine or capsaicin in concentrations of 25, 50, 100 and 200  $\mu$ M for 48 h. The protective effect was performed by exposing the germinated seeds to piperine or capsaicin before, simultaneously or after the MMS, representing the pre, simultaneous and post treatments, respectively (Rocha et al. 2016). The pretreatment assesses demutagenic action, the simultaneous treatment assesses both demutagenic and bioantimutagenic activity and the posttreatment indicates bioantimutagenic action (Fedel-Miyasato et al. 2014, Felicidade et al. 2014).

The concentrations were previously determined from studies with the isolates and this range of concentrations is commonly tested on molecules with pharmaceutical potential. In addition, equal or similar concentrations are tested in chemotherapeutic trials dissolved in 2% DMSO (Greenshields et al. 2015, Siddiqui et al. 2017).

After the treatments carried out, roots were fixed in Carnoy (3 ethanol: 1 acetic acid) for 6-8 h and stored at -20°C until the slide was prepared. To mount the slides, roots were washed three times in distilled water for 5 min each and hydrolyzed at 60°C for 10 min in 1N HCl. After hydrolysis, roots were again washed in distilled water and transferred to amber glass flasks, containing the Schiff Reactive, where they remained in the dark for 2 hours. Roots were then washed, until the reagent was completely removed, transferred to slides, where they were crushed in a drop of 2% acetic carmine and mounted with Entellan<sup>®</sup> (107960; Merck Millipore) (Almeida et al. 2015).

Cytotoxicity, genotoxicity and antigenotoxicity were evaluated by counting 5,000 meristematic cells per treatment (500 cells/slides, with a total of 10 slides analyzed per treatment) under a light microscope (Zeiss Primo Star with AxioCam 105 color camera) at 400x magnification in the

Soil Analysis Laboratory (LASO) of the Center for Agricultural Sciences (CCA) UFPI, Teresina-PI.

For each treatment, the mitotic index (MI, cytotoxicity) and mean chromosomal alteration (genotoxicity) resulting from aneugenic action (metaphase with chromosomal adhesion, C-metaphase, chromosomal loss, multipolar anaphase, binucleated cells) were evaluated for each treatment among others and/or clastogenic action (chromosomal fragments in metaphase or anaphase, chromosomal bridges and other alterations) (Leme & Marin-Morales 2009). Cytotoxicity was also analyzed by the mean value of cells and cells in death process (CDP), which have characteristics such as: heteropicnotic nucleus and displaced to the periphery of the cell, vacuolization and swelling of the cytoplasm (Bianchi et al. 2010). To determine the MI, the number of cells in different phases of mitosis was divided by the total number of cells. For chromosome alterations, the number of alterations was divided by the total number of cells.

Protective effect was assessed by analyzing the percentage of damage reduction (% DR) for each treatment with piperine or capsaicin, according to the following formula: % DR =  $[(a - b)/(a - c)] \times 100$  Where: a = average of MMS chromosomal alterations; b = average of chromosomal alterations in each treatment and c = average of chromosomal alterations in the NC) (Waters 1990).

### Data analysis

Data were analyzed using the Kruskal-Wallis non-parametric test, followed by the Student-Newman-Keuls a posteriori test ( $p < 0.05$ ), in the BioEstat 5.3 software (Ayres & Ayres 2007), for comparison between the means of the controls and groups treated.

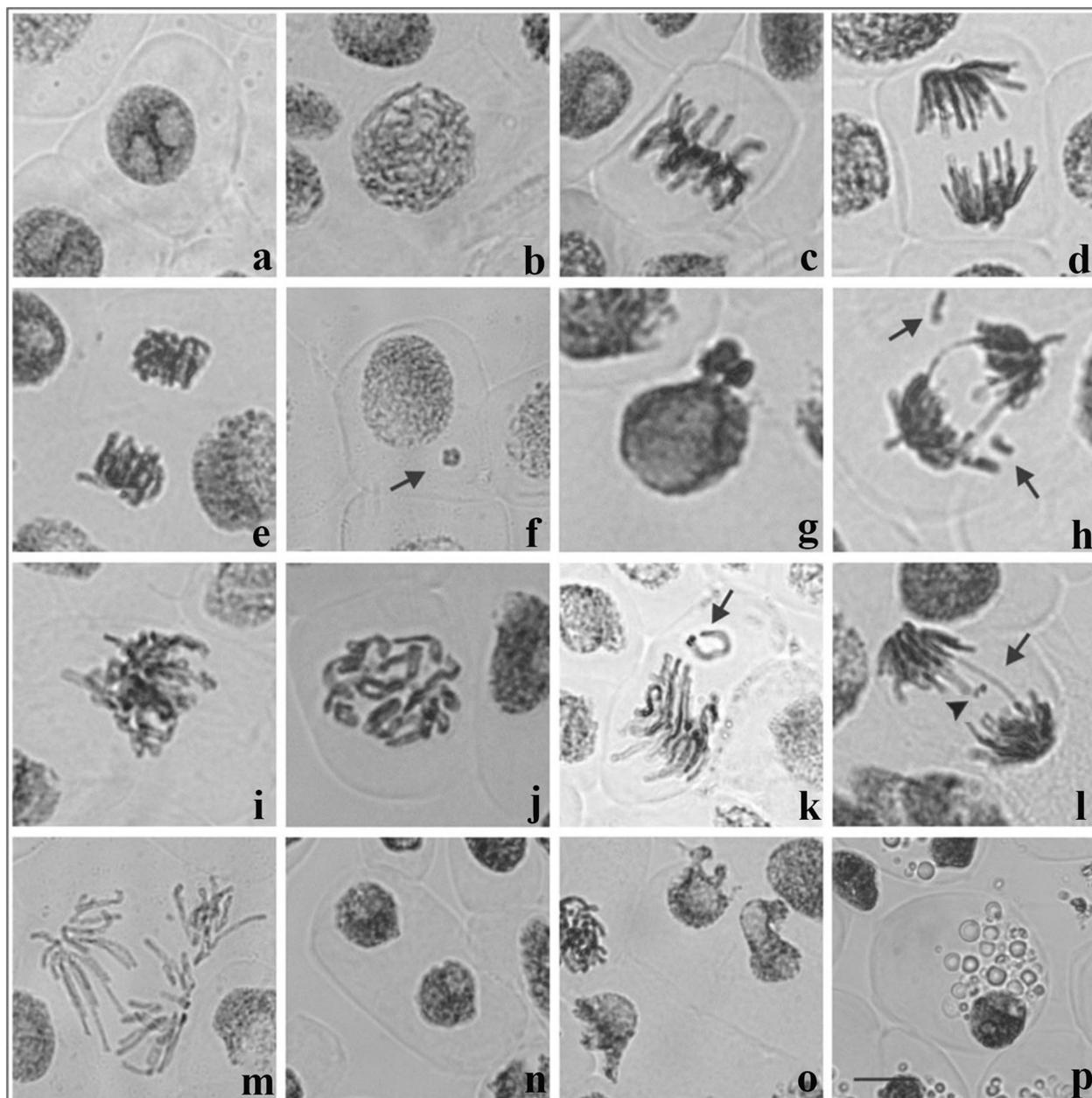
## RESULTS

### Cytogenotoxicity of piperine and capsaicin

Piperine and capsaicin were cytotoxic at concentrations from 50 to 200  $\mu\text{M}$ , since there was a significant reduction in the mitotic index (MI) of the meristematic cells of *A. cepa* in relation to the NC, being dose dependent when exposed to capsaicin. Piperine and capsaicin significantly reduced the prophases (50 to 200  $\mu\text{M}$ ). Additionally, the lower percentage of prophases in capsaicin resulted in a significant reduction in the other phases of the *A. cepa* cell cycle, mainly in 200  $\mu\text{M}$  (Table I).

*A. cepa* cells in death process (CDP) were not significant when compared to the NC, however there was an increase in CDP in all concentrations of piperine and at the lowest (25  $\mu\text{M}$ ) and higher (200  $\mu\text{M}$ ) of capsaicin, which may have contributed to the cytotoxic effect (Table I, Figure 1p).

Piperine caused a significant increase in the total average of chromosomal alterations (genotoxic effect) at concentrations of 50 to 200  $\mu\text{M}$  in *A. cepa* cells when compared to NC. For capsaicin, the genotoxic effect was dose-dependent with a significant increase for all concentrations (Table I). Piperine and capsaicin caused different types of chromosomal alterations and normal cells: a) normal interphase; b) normal prophase; c) normal metaphase; d) normal anaphase; e) normal telophase; f) micronucleus (arrow); g) interphase with nuclear bud; h) chromosomal breaks (arrow); i) chromosomal adherence; j) C-metaphase; k) chromosomal loss (arrow); l) chromosomal bridge (arrow) and chromosomal break (arrow head); m) multipolar anaphase; n) binucleated cell; o) nuclear alteration; p) cells under death process (heteropicnotic nucleus displaced for cell periphery; vacuolization and cytoplasm swelling) (Table II, Figure 1a-p), but



**Figure 1.** Chromosomal and nuclear alterations observed by the analysis of meristematic cells from *Allium cepa* roots. a) normal interphase. b) normal prophase. c) normal metaphase. d) normal anaphase. e) normal telophase. f) micronucleus (arrow). g) interphase with nuclear bud. h) chromosomal breaks (arrow). i) chromosomal adherence. j) C-metaphase. k) chromosomal loss (arrow). l) chromosomal bridge (arrow) and chromosomal break (arrow head). m) multipolar anaphase. n) binucleated cell. o) nuclear alteration. p) cells under death process (heteropicnotic nucleus displaced for cell periphery; vacuolization and cytoplasm swelling). Bar: 10  $\mu\text{m}$  (for all images). All chromosomal changes were observed for piperine and capsaicin, except multipolar anaphase for capsaicin.

**Table I. Phases of mitosis, mitotic index, cells in death process (CDP) and total chromosomal alterations in meristematic cells of *A. cepa* exposed to different concentrations of piperine or capsaicin.**

Treatment ( $\mu\text{M}$ )	Phases of mitosis (%)				Mitotic Index (%)	CDP (%)	Chromosomal alteration
	Prophase	Metaphase	Anaphase	Telophase			
NC	15.93 $\pm$ 0.87	1.33 $\pm$ 0.75	1.62 $\pm$ 0.60	0.98 $\pm$ 0.34	19.86 $\pm$ 1.16	0.04 $\pm$ 0.12	4.13 $\pm$ 2.28
MMS I	10.57 $\pm$ 1.50**	1.16 $\pm$ 0.51	1.09 $\pm$ 0.46	0.67 $\pm$ 0.50	14.09 $\pm$ 1.96**	0.77 $\pm$ 1.80	59.52 $\pm$ 13.34**
Piperine							
25	15.38 $\pm$ 0.84	1.36 $\pm$ 0.46	0.97 $\pm$ 0.39	1.21 $\pm$ 0.45	18.93 $\pm$ 1.21	0.08 $\pm$ 0.24	6.63 $\pm$ 3.23
50	14.13 $\pm$ 1.05*	1.46 $\pm$ 0.49	1.44 $\pm$ 0.33	1.08 $\pm$ 0.46	18.11 $\pm$ 1.06*	0.51 $\pm$ 1.29	11.93 $\pm$ 2.69**
100	12.90 $\pm$ 1.42**	1.31 $\pm$ 0.79	1.01 $\pm$ 0.52	1.01 $\pm$ 0.52	16.22 $\pm$ 1.94**	0.27 $\pm$ 0.60	11.63 $\pm$ 6.44*
200	14.27 $\pm$ 0.84*	1.23 $\pm$ 0.51	1.26 $\pm$ 0.59	1.10 $\pm$ 0.67	17.85 $\pm$ 0.81*	0.47 $\pm$ 1.03	15.05 $\pm$ 3.79**
Capsaicin							
25	14.39 $\pm$ 0.61	1.24 $\pm$ 0.38	1.02 $\pm$ 0.42*	1.43 $\pm$ 0.64	18.08 $\pm$ 0.97	0.13 $\pm$ 0.40	13.31 $\pm$ 9.71*
50	13.00 $\pm$ 0.91*	1.05 $\pm$ 0.39	0.99 $\pm$ 0.37*	1.39 $\pm$ 0.37	16.42 $\pm$ 1.38*	0.02 $\pm$ 0.06	14.55 $\pm$ 3.46**
100	9.37 $\pm$ 1.85**	0.93 $\pm$ 0.41	0.81 $\pm$ 0.47**	0.79 $\pm$ 0.49	11.90 $\pm$ 2.12**	0.00 $\pm$ 0.00	15.36 $\pm$ 4.94**
200	4.64 $\pm$ 2.55**	0.15 $\pm$ 0.22**	0.17 $\pm$ 0.25**	0.24 $\pm$ 0.33**	5.21 $\pm$ 3.05**	1.74 $\pm$ 2.30	16.78 $\pm$ 6.73**

Data are means  $\pm$  SD (Standard Deviation). NC: Negative Control (Dimethylsulfoxide - 2% DMSO in distilled water). MMS I: 10  $\mu\text{g}/\text{mL}$  of Methylmethanesulfonate dissolved in DMSO 2%. \*Significant by Kruskal-Wallis test with a posteriori Student-Newman-Keuls test (\*  $p < 0.05$ ; \*\* $p < 0.01$ ) when compared to NC. Data are for 5,000 cells/treatment. The DMSO 2% was used as a negative control, but how the results were statistical identical to solvent (distilled water), the data using water were omitted. The MMS I and MMS II (dissolved only in distilled water) also were statistical identical, the data using MMS II were omitted.

only micronuclei (MN) and nuclear buds (NB) were significant (50 to 200  $\mu\text{M}$ ) in the piperine treatment. In capsaicin, the same changes were significant at all concentrations and chromosomal adherence was significant at concentrations of 50 and 100  $\mu\text{M}$ .

### Modulation of cell damage by piperine and capsaicin

As for the modulatory effect, there was no significant difference in MI and CDP in the three protocols (pre, simultaneous and posttreatment) exposed to piperine or capsaicin in relation to MMS I. Further, in capsaicin there were significant reductions in MI in the pre (100 and 200  $\mu\text{M}$ ), simultaneous (25 and 100  $\mu\text{M}$ ) and posttreatment (200  $\mu\text{M}$ ) (Table III).

As for the different phases of mitosis, cells treated with piperine or capsaicin showed no significant difference in relation to MMS I in the three protocols. Further, in capsaicin, there

was a reduction in prophases, metaphases and anaphases in the three protocols, and telophases only in the posttreatment (Table III). The observed results showed that piperine or capsaicin was not able to neutralize the cytotoxic action of MMS I.

A significant reduction in the total average of chromosomal alterations in *A. cepa* when exposed to piperine was verified in the pre (57.93 to 85.66%), simultaneous (58.71 to 74.07%) and posttreatment (54.38 to 64.16%) for all concentrations in relation to MMS I. In capsaicin, the reduction of chromosomal alterations was also observed in the pre (70.39 to 86.21%) and simultaneous (36.36 to 51.89%) for all concentrations, while in the posttreatment, only the highest concentration (200  $\mu\text{M}$ ) had the reduction (35.84%). These results reinforce the interaction of piperine or capsaicin with MMS, modulating genotoxic action of MMS, however there was no protective effect (except for the

lowest concentration of piperine), since these molecules alone were genotoxic (Table III).

The significant reduction in MN and/or NB was verified in all concentrations of the pre and simultaneous treatments in relation to MMS I when exposed to piperine or capsaicin. In the posttreatment, there was also a reduction in the same changes in all concentrations with piperine and for capsaicin occurred only in the highest concentration (Table IV).

**DISCUSSION**

The cytotoxic effect of piperine and capsaicin may be related to a significant reduction in prophases (50 to 200 µM). The lower percentage of prophases in capsaicin resulted in a significant decrease in the other phases of the *A. cepa* cell cycle, mainly in 200 µM (Table I). The observed results are reinforced by previous studies showing the cytotoxicity of piperine or capsaicin in concentrations equal to and/or similar to the present study, which promoted the arrest of tumor cells in G1 (Ouyang et al. 2013, Fofaria et al. 2014) and/or G2/M (Yaffe et al. 2013, Greenshields et al. 2015, Zhang et al. 2015, Siddiqui et al. 2017).

An increase in CDP in all concentrations of piperine and at the lowest and highest concentrations of capsaicin may also have contributed to the cytotoxic effect. Similar results were observed in tumor cells treated with piperine or capsaicin, which caused oxidative stress by reactive oxygen species (ROS) (Yaffe et al. 2013), reactive nitrogen species, inhibition of NADH-oxidoreductase (an enzyme that stimulates cell activity and proliferation) and rupture of the mitochondrial membrane permeability (Pramanik et al. 2011, Qian et al. 2016, Cho et al. 2017).

**Table II. Chromosomal alterations in meristematic cells of *A. cepa* exposed to different concentrations of piperine or capsaicin.**

Treatment (µM)	Chromosomal alteration										
	MN	NB	CB	BC	CA	Cm	CL	CBr	MA	MA	NA
NC	0.84 ± 1.08	2.82 ± 1.62	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.31	0.10 ± 0.30	0.19 ± 0.39	0.00 ± 0.00	0.10 ± 0.31	0.10 ± 0.31	0.00 ± 0.00
MMS I	33.56 ± 7.66**	23.43 ± 5.80**	0.46 ± 0.64*	0.28 ± 0.64	0.38 ± 0.50	0.00 ± 0.00	0.84 ± 1.01	0.28 ± 0.45	0.10 ± 0.31	0.10 ± 0.31	0.19 ± 0.39
Piperine											
25	3.04 ± 1.74	2.54 ± 2.39	0.00 ± 0.00	0.10 ± 0.31	0.38 ± 0.50	0.09 ± 0.29	0.29 ± 0.65	0.10 ± 0.30	0.10 ± 0.31	0.10 ± 0.31	0.00 ± 0.00
50	3.81 ± 3.22*	7.55 ± 4.78*	0.18 ± 0.37	0.00 ± 0.00	0.19 ± 0.40	0.00 ± 0.00	0.10 ± 0.30	0.10 ± 0.31	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
100	3.48 ± 4.48*	6.77 ± 5.21*	0.09 ± 0.30	0.00 ± 0.00	0.36 ± 0.47	0.09 ± 0.30	0.37 ± 0.47	0.09 ± 0.30	0.09 ± 0.30	0.09 ± 0.30	0.19 ± 0.40
200	6.98 ± 2.64**	6.65 ± 3.72*	0.10 ± 0.31	0.00 ± 0.00	0.48 ± 0.68	0.09 ± 0.29	0.28 ± 0.45	0.19 ± 0.40	0.29 ± 0.46	0.29 ± 0.46	0.00 ± 0.00
Capsaicin											
25	6.80 ± 6.53*	4.88 ± 3.82*	0.00 ± 0.00	0.00 ± 0.00	1.07 ± 1.63	0.00 ± 0.00	0.19 ± 0.40	0.19 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.39
50	5.53 ± 2.57*	6.74 ± 6.53*	0.09 ± 0.30	0.20 ± 0.63	1.60 ± 1.41*	0.10 ± 0.31	0.10 ± 0.31	0.19 ± 0.39	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.31
100	6.58 ± 2.74**	6.74 ± 2.88*	0.00 ± 0.00	0.00 ± 0.00	1.55 ± 1.43*	0.00 ± 0.00	0.00 ± 0.00	0.38 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.30
200	7.69 ± 3.86**	7.69 ± 3.86*	0.00 ± 0.00	0.36 ± 0.75	1.27 ± 1.58	0.00 ± 0.00	0.09 ± 0.30	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.28

Data are means ± SD (Standard Deviation). NC: Negative Control (Dimethylsulfoxide - 2% DMSO in distilled water). MMS I: 10 µg/mL of Methylmethanesulfonate dissolved in DMSO 2%. MN: Micronucleus. NB: Nuclear Bud. CB: Chromosomal Breaking. BC: Binucleated Cell. CA: Chromosomal Adherence. Cm: C-metaphase. CL: Chromosomal Loss. CBr: Chromosomal Bridge. MA: Multipolar Anaphase. NA: Nuclear Alteration. \*Significant by Kruskal-Wallis test with a posteriori Student-Newman-Keuls test (\* p < 0.05; \*\*p < 0.01) when compared to NC. Data are for 5,000 cells/treatment. The DMSO 2% was used as a negative control, but how the results were statistical identical to solvent (distilled water), the data using water were omitted. The MMS I and MMS II (dissolved only in distilled water) also were statistical identical, the data using MMS II were omitted.

**Table III. Phases of mitosis, mitotic index, cells in death process (CDP) and total chromosomal alterations and percentage of damage reduction (%DR) in meristematic cells of *A. cepa* exposed to different concentrations of piperine or capsaicin.**

Treatment ( $\mu\text{M}$ )	Phases of mitosis (%)				Mitotic index (%)	CDP (%)	Total chromosomal alterations	%DR
	Prophase	Metaphase	Anaphase	Telophase				
NC	15.93 $\pm$ 0.87**	1.33 $\pm$ 0.75	1.62 $\pm$ 0.60	0.98 $\pm$ 0.34	19.86 $\pm$ 1.16**	0.04 $\pm$ 0.12	4.13 $\pm$ 2.28**	-
MMS I	10.57 $\pm$ 1.50	1.16 $\pm$ 0.51	1.09 $\pm$ 0.46	0.67 $\pm$ 0.50	14.09 $\pm$ 1.96	0.77 $\pm$ 1.80	59.52 $\pm$ 13.34	-
<i>Pre-treatment</i>								
<i>Piperine + MMS I</i>								
25	11.60 $\pm$ 1.08	1.10 $\pm$ 0.47	1.00 $\pm$ 0.28	1.00 $\pm$ 0.44	14.70 $\pm$ 1.66	1.03 $\pm$ 1.42	27.43 $\pm$ 5.29*	57.93
50	11.24 $\pm$ 1.06	0.75 $\pm$ 0.31*	0.77 $\pm$ 0.40	1.01 $\pm$ 0.45	13.78 $\pm$ 1.33	0.02 $\pm$ 0.06	15.06 $\pm$ 5.44**	80.27
100	10.80 $\pm$ 0.49	1.18 $\pm$ 0.48	0.77 $\pm$ 0.28	0.85 $\pm$ 0.43	13.60 $\pm$ 0.88	0.24 $\pm$ 0.43	13.39 $\pm$ 4.33**	83.28
200	10.42 $\pm$ 0.85	0.90 $\pm$ 0.39	0.66 $\pm$ 0.36*	0.56 $\pm$ 0.32	12.53 $\pm$ 1.06	0.45 $\pm$ 0.84	12.07 $\pm$ 2.83**	85.66
<i>Simultaneous treatment</i>								
<i>Piperine + MMS I</i>								
25	9.12 $\pm$ 2.36	0.13 $\pm$ 0.40	1.03 $\pm$ 0.38	0.66 $\pm$ 0.32	11.93 $\pm$ 2.27	0.22 $\pm$ 0.70	18.49 $\pm$ 5.68**	74.07
50	8.14 $\pm$ 2.63	1.15 $\pm$ 0.47	0.56 $\pm$ 0.39*	0.91 $\pm$ 0.38	10.77 $\pm$ 3.21	0.43 $\pm$ 0.70	19.64 $\pm$ 22.09**	72.00
100	9.23 $\pm$ 1.40	1.24 $\pm$ 0.65	0.62 $\pm$ 0.33*	0.90 $\pm$ 0.37	11.99 $\pm$ 1.60	0.00 $\pm$ 0.00	22.09 $\pm$ 6.65**	67.57
200	8.13 $\pm$ 2.12	0.78 $\pm$ 0.38	0.69 $\pm$ 0.38	0.85 $\pm$ 0.63	10.62 $\pm$ 2.28	0.00 $\pm$ 0.00	27.00 $\pm$ 8.19*	58.71
<i>Post-treatment</i>								
<i>Piperine + MMS I</i>								
25	8.79 $\pm$ 1.29	1.27 $\pm$ 0.55	1.22 $\pm$ 0.39	0.97 $\pm$ 0.47	12.25 $\pm$ 2.04	0.24 $\pm$ 0.77	23.98 $\pm$ 7.96**	64.16
50	10.40 $\pm$ 0.97	1.47 $\pm$ 0.55	1.20 $\pm$ 0.50	1.21 $\pm$ 0.61	14.26 $\pm$ 1.32	0.52 $\pm$ 1.10	25.04 $\pm$ 6.39**	62.25
100	9.55 $\pm$ 1.04	1.13 $\pm$ 0.34	1.12 $\pm$ 0.45	1.24 $\pm$ 0.51	13.03 $\pm$ 1.55	0.00 $\pm$ 0.00	28.49 $\pm$ 8.27**	56.02
200	9.35 $\pm$ 1.02	1.36 $\pm$ 0.59	0.96 $\pm$ 0.50	0.93 $\pm$ 0.36	12.59 $\pm$ 1.41	0.26 $\pm$ 0.82	29.40 $\pm$ 7.05**	54.38
<i>Pre-treatment</i>								
<i>Capsaicin + MMS I</i>								
25	12.21 $\pm$ 1.36	0.82 $\pm$ 0.43	0.54 $\pm$ 0.33*	0.41 $\pm$ 0.29	13.97 $\pm$ 1.84	0.00 $\pm$ 0.00	11.77 $\pm$ 4.95**	86.21
50	9.58 $\pm$ 1.60	0.46 $\pm$ 0.28**	0.39 $\pm$ 0.25**	0.39 $\pm$ 0.25	10.83 $\pm$ 1.91	0.25 $\pm$ 0.55	18.66 $\pm$ 4.05**	73.77
100	6.15 $\pm$ 1.49*	0.37 $\pm$ 0.39**	0.24 $\pm$ 0.29**	0.40 $\pm$ 0.28	7.71 $\pm$ 2.10**	0.08 $\pm$ 0.19	20.53 $\pm$ 8.21**	70.39
200	5.48 $\pm$ 1.72**	0.44 $\pm$ 0.28**	0.27 $\pm$ 0.24**	0.34 $\pm$ 0.29	6.54 $\pm$ 2.10**	1.01 $\pm$ 1.58	18.64 $\pm$ 6.71**	73.80
<i>Simultaneous treatment</i>								
<i>Capsaicin + MMS I</i>								
25	8.16 $\pm$ 1.91*	0.82 $\pm$ 0.61	0.34 $\pm$ 0.19**	0.37 $\pm$ 0.29	9.69 $\pm$ 1.96*	0.00 $\pm$ 0.00	39.38 $\pm$ 6.93*	36.36
50	9.87 $\pm$ 1.61	0.90 $\pm$ 0.38	0.42 $\pm$ 0.25*	0.82 $\pm$ 0.36	12.00 $\pm$ 1.23	0.00 $\pm$ 0.00	35.01 $\pm$ 15.16**	44.24
100	8.71 $\pm$ 1.99	0.71 $\pm$ 0.28*	0.24 $\pm$ 0.15**	0.47 $\pm$ 0.12	10.19 $\pm$ 2.02*	0.00 $\pm$ 0.00	30.78 $\pm$ 7.63**	51.89
200	8.95 $\pm$ 1.91	0.79 $\pm$ 0.28	0.39 $\pm$ 0.31**	0.40 $\pm$ 0.21	10.53 $\pm$ 1.95	0.52 $\pm$ 1.63	37.07 $\pm$ 11.46*	40.53

**Table III. Continuation.**

Treatment ( $\mu\text{M}$ )	Phases of mitosis (%)				Mitotic index (%)	CDP (%)	Total chromosomal alterations	%DR
	Prophase	Metaphase	Anaphase	Telophase				
Post-treatment Capsaicin + MMS I								
25	9.09 $\pm$ 1.68	1.50 $\pm$ 0.49	0.68 $\pm$ 0.24	0.81 $\pm$ 0.44	12.09 $\pm$ 2.10	0.40 $\pm$ 0.86	50.18 $\pm$ 11.64	16.86
50	8.76 $\pm$ 1.54	1.20 $\pm$ 0.48	0.84 $\pm$ 0.39	0.81 $\pm$ 0.36	11.61 $\pm$ 1.80	0.00 $\pm$ 0.00	61.60 $\pm$ 13.31	-3.75
100	8.19 $\pm$ 1.03	1.27 $\pm$ 0.37	0.85 $\pm$ 0.43	0.16 $\pm$ 0.55*	11.49 $\pm$ 1.76	0.46 $\pm$ 0.82	57.46 $\pm$ 9.74	3.72
200	4.45 $\pm$ 1.54**	0.46 $\pm$ 0.54*	0.21 $\pm$ 0.28**	0.27 $\pm$ 0.28	5.38 $\pm$ 2.39**	1.64 $\pm$ 3.02	39.67 $\pm$ 14.23*	35.84

Data are means  $\pm$  SD (Standard Deviation). NC: Negative Control (Dimethylsulfoxide - DMSO 2% in distilled water). MMS I: 10  $\mu\text{g}/\text{mL}$  of Methylmethanesulfonate dissolved in 2% DMSO (positive control). Pre-treatment (piperine or capsaicin + MMS). Simultaneous treatment (piperine or capsaicin added simultaneously with MMS). Post-treatment (MMS + piperine or capsaicin). \* Significant in the Kruskal-Wallis test with a posteriori Student-Newman-Keuls test (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ) when compared to MMS I. The results refer to the analysis of 5,000 cells per treatment. The DMSO 2% was used as a negative control, but how the results were statistical identical to solvent (distilled water), the data using water were omitted. The MMS I and MMS II (dissolved only in distilled water) also were statistical identical, the data using MMS II were omitted.

Pro-oxidant action of piperine or capsaicin may have resulted in the genotoxic effect, as ROSs increase the risk of DNA damage, including the division of cells with unrepaired or poorly repaired damage, leading to mutations (Kehrer & Klotz 2015). The genotoxic effect observed in piperine and capsaicin are, mainly, the result of significant MN and NB. The MN observed are due to clastogenic and/or aneugenic damage not repaired or erroneously repaired in parental cells, is easily observed in daughter cells as a structure similar to the main nucleus, but in a reduced size (Fernandes et al. 2007, Leme & Marin-Morales 2009). NB may be related to the formation of MN, through the elimination of extra genetic material in the main nucleus of the cell, or it may be due to the aggregation of a delayed chromosome by the nuclear envelope, before being fully reincorporated into the main nucleus (Bianchi et al. 2015). While chromosomal adherences are a type of abnormality that involves the protein in the chromatin matrix and not necessarily the DNA itself; it can also be irreversible and lead to cell death (Fernandes et al. 2009).

Piperine and capsaicin are potential antimutagenic and anticarcinogenic compounds (Abo-Zeid & Farghaly 2009, Fernandez-Bedmar & Alonso-Moraga 2016). In this way, we sought to evaluate the protective or modulatory effect of both molecules on damage induced by MMS I in the pre, simultaneous and posttreatment protocols.

The two molecules are alkaloids, which have both antioxidant and pro-oxidant action (capable of generating free radicals) depending on the dose and the group of treated cells (Rather & Bhagat 2018, Macáková et al. 2019). Probably, the molecules acted as pro-oxidants in the cells of *A. cepa*, potentiating the cytotoxic action of MMS I, mainly for capsaicin, which promoted significant reductions in the MI. According to Bianchi et al. (2016), ROS may be associated with decreased MI in *A. cepa* cells, as they cause lipid peroxidation, changes in membrane fluidity and DNA damage. In response to these damages, there is usually a delay in the mitotic cycle, mainly in the G1 and/or G2 phases, to allow the cells to repair the damage induced before replicating their DNA and starting mitosis (Feng et al. 2010).

**Table IV. Chromosomal alterations in meristematic cells of *A. cepa* exposed to different concentrations of piperine or capsaicin.**

Treatment (µM)	Chromosomal alteration									
	MN	NB	CB	BC	CA	Cm	CL	CBr	MA	NA
NC	0.84 ± 1.08**	2.82 ± 1.62**	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.31	0.10 ± 0.30	0.19 ± 0.39	0.00 ± 0.00	0.10 ± 0.31	0.00 ± 0.00
MMS I	33.56 ± 7.66	23.43 ± 5.80	0.46 ± 0.64	0.28 ± 0.64	0.38 ± 0.50	0.00 ± 0.00	0.84 ± 1.01	0.28 ± 0.45	0.10 ± 0.31	0.19 ± 0.39
<i>Pre-treatment</i>										
<i>Piperine + MMS I</i>										
25	13.31 ± 4.31**	12.23 ± 2.88**	0.38 ± 0.67	0.00 ± 0.00	0.10 ± 0.31	0.00 ± 0.00	0.85 ± 0.54	0.29 ± 0.46	0.18 ± 0.39	0.10 ± 0.31
50	6.09 ± 3.58**	7.41 ± 4.83**	0.74 ± 0.74	0.09 ± 0.29	0.18 ± 0.38	0.00 ± 0.00	0.09 ± 0.27	0.46 ± 0.66	0.00 ± 0.00	0.00 ± 0.00
100	4.69 ± 3.05**	7.56 ± 2.94**	0.39 ± 0.50	0.00 ± 0.00	0.37 ± 0.65	0.00 ± 0.00	0.19 ± 0.40	0.19 ± 0.40	0.00 ± 0.00	0.00 ± 0.00
200	4.48 ± 2.39**	6.47 ± 2.92**	0.48 ± 0.81	0.00 ± 0.00	0.18 ± 0.57	0.00 ± 0.00	0.09 ± 0.30	0.38 ± 0.67	0.00 ± 0.00	0.00 ± 0.00
<i>Simultaneous treatment</i>										
<i>Piperine + MMS I</i>										
25	11.09 ± 4.40**	6.65 ± 4.38**	0.19 ± 0.39	0.00 ± 0.00	0.19 ± 0.38	0.10 ± 0.30	0.00 ± 0.00	0.18 ± 0.39	0.10 ± 0.30	0.00 ± 0.00
50	8.95 ± 4.71**	9.87 ± 3.57**	0.28 ± 0.48	0.00 ± 0.00	0.36 ± 0.63	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.57	0.00 ± 0.00	0.00 ± 0.00
100	10.55 ± 4.75**	10.03 ± 2.76**	0.19 ± 0.41	0.00 ± 0.00	0.65 ± 0.77	0.00 ± 0.00	0.19 ± 0.39	0.48 ± 0.82	0.00 ± 0.00	0.00 ± 0.00
200	12.61 ± 7.05**	13.54 ± 3.76	0.28 ± 0.63	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.40	0.28 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.29
<i>Post-treatment</i>										
<i>Piperine + MMS I</i>										
25	13.86 ± 6.05**	8.91 ± 3.56*	0.09 ± 0.29	0.00 ± 0.00	0.66 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.47 ± 0.65	0.00 ± 0.00	0.00 ± 0.00
50	13.32 ± 2.82**	11.05 ± 4.62*	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.70	0.08 ± 0.26	0.00 ± 0.00	0.19 ± 0.40	0.00 ± 0.00	0.00 ± 0.00
100	15.88 ± 6.56**	11.67 ± 3.96**	0.00 ± 0.00	0.00 ± 0.00	0.57 ± 0.67	0.00 ± 0.00	0.10 ± 0.31	0.28 ± 0.44	0.00 ± 0.00	0.00 ± 0.00
200	16.24 ± 6.20**	12.63 ± 6.20**	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.83	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.39	0.00 ± 0.00	0.00 ± 0.00
<i>Pretreatment</i>										
<i>Capsaicin + MMS I</i>										
25	5.67 ± 2.22**	5.45 ± 3.63**	0.10 ± 0.31	0.00 ± 0.00	0.17 ± 0.37	0.00 ± 0.00	0.09 ± 0.29	0.19 ± 0.40	0.00 ± 0.00	0.10 ± 0.31
50	9.76 ± 3.16*	7.79 ± 2.61**	0.19 ± 0.40	0.00 ± 0.00	0.73 ± 0.37	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.39	0.00 ± 0.00	0.00 ± 0.00
100	8.13 ± 5.64**	10.22 ± 5.78*	0.59 ± 1.88	0.00 ± 0.00	0.75 ± 1.08	0.00 ± 0.00	0.28 ± 0.62	0.37 ± 0.48	0.10 ± 0.31	0.09 ± 0.29
200	8.01 ± 3.30**	9.60 ± 4.30*	0.00 ± 0.00	0.09 ± 0.28	0.46 ± 0.68	0.00 ± 0.00	0.10 ± 0.31	0.10 ± 0.31	0.09 ± 0.29	0.10 ± 0.31
<i>Simultaneous treatment</i>										
<i>Capsaicin + MMS I</i>										
25	25.47 ± 4.44	12.31 ± 3.84*	0.19 ± 0.40	0.00 ± 0.00	0.66 ± 0.65	0.10 ± 0.31	0.37 ± 0.90	0.09 ± 0.29	0.00 ± 0.00	0.19 ± 0.40
50	20.64 ± 8.54*	11.89 ± 6.63*	0.68 ± 0.65	0.09 ± 0.29	0.76 ± 0.89	0.27 ± 0.86	0.39 ± 0.81	0.29 ± 0.46	0.00 ± 0.00	0.00 ± 0.00
100	19.07 ± 5.80**	10.50 ± 4.24**	0.45 ± 0.65	0.00 ± 0.00	0.46 ± 0.65	0.00 ± 0.00	0.19 ± 0.60	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.30

**Table IV. Continuation.**

Treatment ( $\mu\text{M}$ )	Chromosomal alteration									
	MN	NB	CB	BC	CA	Cm	CL	CBr	MA	NA
200	20.59 $\pm$ 8.21*	14.84 $\pm$ 4.86	0.39 $\pm$ 0.69	0.10 $\pm$ 0.32	0.86 $\pm$ 1.05	0.10 $\pm$ 0.31	0.10 $\pm$ 0.31	0.10 $\pm$ 0.31	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
<i>Post-treatment</i>										
<i>Capsaicin + MMS I</i>										
25	31.53 $\pm$ 8.44	17.16 $\pm$ 4.47	0.10 $\pm$ 0.58	0.09 $\pm$ 0.30	0.74 $\pm$ 0.84	0.20 $\pm$ 0.63	0.00 $\pm$ 0.00	0.18 $\pm$ 0.38	0.09 $\pm$ 0.30	0.00 $\pm$ 0.00
50	42.56 $\pm$ 11.74	16.50 $\pm$ 5.00	0.10 $\pm$ 0.31	0.00 $\pm$ 0.00	0.81 $\pm$ 0.67	0.09 $\pm$ 0.28	0.91 $\pm$ 1.03	0.37 $\pm$ 0.48	0.09 $\pm$ 0.28	0.17 $\pm$ 0.36
100	37.85 $\pm$ 7.86	17.35 $\pm$ 2.11	0.10 $\pm$ 0.31	0.10 $\pm$ 0.32	0.86 $\pm$ 0.83	0.18 $\pm$ 0.38	0.48 $\pm$ 0.69	0.55 $\pm$ 0.63	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
200	26.70 $\pm$ 10.25	11.82 $\pm$ 6.16**	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.28 $\pm$ 0.46	0.29 $\pm$ 0.66	0.28 $\pm$ 0.64	0.19 $\pm$ 0.41	0.10 $\pm$ 0.31	0.00 $\pm$ 0.00

Data are means  $\pm$  SD (Standard deviation). NC: Negative Control (Dimethylsulfoxide - DMSO 2% in distilled water). MMS I: 10  $\mu\text{g}/\text{mL}$  of Methylmethanesulfonate dissolved in DMSO 2%. MN: Micronucleus. NB: Nuclear Bud. CB: Chromosomal Breaking. BC: Binucleated Cell. CA: Chromosomal Adherence. Cm: C-metaphase. CL: Chromosomal Loss. CBr: Chromosomal Bridge. MA: Multipolar Anaphase. NA: Nuclear Alteration. Pre-treatment (piperine or capsaicin + MMS). Simultaneous treatment (piperine or capsaicin added simultaneously with MMS). Post-treatment (MMS + piperine or capsaicin). \* Significant in the Kruskal-Wallis test with a posteriori Student-Newman-Keuls test (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ) when compared to MMS I. The results refer to the analysis of 5,000 cells per treatment. The DMSO 2% was used as a negative control, but how the results were statistical identical to solvent (distilled water), the data using water were omitted. The MMS I and MMS II (dissolved only in distilled water) also were statistical identical, the data using MMS II were omitted.

In general, the tested compounds modulated the genotoxic effect of MMS I. In the pre, the isolated compound (piperine or capsaicin) may have interacted directly with MMS I in the intracellular environment in *A. cepa* cells. For the simultaneous, the reduction in damage to the cell can be a result of both the demutagenic and bioantimutagenic action (Nantes et al. 2014) by the tested bioactive agents. In posttreatment, piperine also promoted a reduction in damage induced by MMS I by the bioantimutagenic action, which acts in DNA repair mechanisms, inducing the reversion of the mutagenic effect and/or preventing the fixation of mutations (Dametto et al. 2017). In capsaicin, a similar result for the posttreatment was observed only in the highest concentration. These results reinforce the interaction of piperine or capsaicin, modulating the genotoxic action of MMS, however there was no protective effect, since these molecules alone were genotoxic with a significant production of MN and NB. Moreover, the %RD was also a result of a significant reduction of the same

alterations, reinforcing the possible interaction between isolates with MMS I.

Only the lowest concentration of piperine (25  $\mu\text{M}$ ) was not genotoxic and showed a protective effect in all protocols (pre, simultaneous, and post), showing demutagenic and bioantimutagenic action, which increases the interest in further studies on this concentration as a chemoprotective. Other studies have also shown satisfactory results regarding the protective effect of piperine in the test for chromosomal changes in mouse bone marrow cells induced by cyclophosphamide and mitomycin C (Wongpa et al. 2007, Abo-Zeid & Farghaly 2009). Piperine also decreased the genotoxic (comet assay) and mutagenic (test micronucleus) effect induced by aflatoxins in chickens (Cardoso et al. 2016). The cytochrome P450 enzyme is responsible for 50% of the metabolism of therapeutic agents, and the comparison of the presence of this enzyme complex leads to the conclusion that plants have a lower concentration of antioxidant enzymes compared to mammals and insects (Leme & Marin-Morales 2009, Rocha et al. 2016).

MMS was used in the present study as a DNA damage inducer in the *A. cepa* assay. There are two main mechanisms by which this compound can act. The first is its known capacity for alkylation and methylation, which can cause breaks in the double strand of DNA and inhibition of the replication fork (Chatterjee & Walker 2017). The second is its induction of high levels of oxidative stress, which can lead to apoptosis, cell death and DNA damage (Lackinger et al. 2001, Jiang et al. 2016). Studies demonstrate the ability to deplete Glutathione-S-transferase (Liu et al. 1996) and Glutathione (Siddique et al. 2019) levels by MMS, which impairs cellular antioxidant defenses and leads to the accumulation of ROS generated as byproducts of normal cellular metabolism (Raza 2011).

Piperine or capsaicin probably neutralized and/or modulated the action of MMS I by the two mechanisms mentioned, since the direct genotoxic action of MMS I was reduced in the protocols used. Moreover, piperine or capsaicin may also have acted by neutralizing the ROS resulting from the action of MMS, since the isolates are alkaloids and have antioxidant activities, neutralizing the action of free radicals (Kaur & Arora 2015, Tsoi et al. 2015).

Based on the results obtained in the present study, piperine and capsaicin showed a cytotoxic effect, except for the lowest concentration, associated mainly with the reduction of prophases in *A. cepa*, and genotoxic effect with emphasis on MN and NB, except for the lowest concentration of piperine. Even with no cytoprotective effect, the analyzed compounds reduced chromosomal alterations (MN and NB) in most protocols and concentrations, which reinforces the possible interaction with MMS. However, only the lowest concentration of piperine (25  $\mu$ M) was not genotoxic and showed a protective effect in all protocols,

while the other concentrations of the tested molecules alone were genotoxic. Thus, the lowest concentration of piperine demonstrated important chemopreventive activity, which is indirectly correlated with the prevention and/or treatment of genetic diseases, such as cancer. Nevertheless, further studies are required to elucidate possible mechanisms of interaction between biocompounds and MMS.

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#### Author contributions

Marcondes Soares Dias conducted the entire study, analysis and interpretation of data and writing of the manuscript. Erasmo Pereira do Vale Junior and Bianca Cristina dos Santos participated in the analysis and interpretation of the data. Francielle Alline Martins contributed to the discussion and text review and Ana Paula Peron, co-supervised the research, was responsible for the study design and review. Pedro Marcos de Almeida participated in all stages from the idealization and design of the study, as well as analysis, review and interpretation of data.

