Are salty liquid food flavorings *in vitro* antitumor substances?

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ABSTRACT

The objective of this study was to evaluate the antiproliferative, cytotoxic and genotoxic potential of salty liquid synthetic flavorings of Butter, Cheddar Cheese and Onion. The antiproliferative potential (2.9-1500 µg/mL) was assessed by MTT assay after 72h using the human tumor lines SF-295 (glioblastoma), OVCAR-8 (ovarian), HCT-116 (colon) and HL-60 (promyelocytic leukemia) and primary cultures of murine Sarcoma 180 (S180) and peripheral blood mononuclear cells (PBMC). *Allium cepa* bulbs were exposed to growing respective doses (1 mL and 2 mL). Only Butter and Cheddar flavorings revealed cytotoxic activity on cancer cells, with IC_{50} values ranging from 125.4 µg/mL (Cheddar - HCT-116) to 402.6 µg/mL (Butter - OVCAR-8). Butter flavoring was the most cytotoxic on PBMC (136.3 µg/mL) and increased cell division rate in relation to the mitotic index but did not cause cellular aberrations. Onion and Cheddar flavorings reduced the mitotic index after 24h and 48h exposure, but only Onion flavoring resulted in cellular aberrations and mitotic spindle abnormalities, such as anaphase and telophase bridges, micronucleated cells, conchicine-metaphases and amplifications. So, Butter, Onion and/or Cheddar flavorings caused significant changes in the division of meristematic cells of *A. cepa* and presented cytotoxic action even on decontrolled proliferating human tumor cells.

Key words: food additives, *Allium cepa*, antiproliferative action, neoplasic cells, toxicity.

INTRODUCTION

Food additives are compounds intentionally added, with no nutritional benefit, aiming to modify the physical, chemical, biological or sensory characteristics of food products. Among these, flavoring substances are compounds with sensory properties that provide flavor and aroma to various types of foods (Constant et al. 2007). They have complex chemical formulation including diluents, antioxidants, defoamers, preservatives, emulsifiers, stabilizers, acidity regulators, flavor enhancers, antiwetting agents, anti-caking agents, dyes, solvents for

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extraction and processing, approved by the European Food Safety Authority (EFSA) and, nationally, by the Brazilian National Health Surveillance Agency (*Agência Nacional de Vigilância Sanitária - ANVISA*).

Flavorings are considered a controversial progress of the food industry, once many health experts suggest that these additives, as well as food dyes, contribute significantly to impaired diet and for the triggering of pathologies (Cheeseman 2012). In this way, researchers report that the use of flavoring substances, mainly the synthetic ones, raises a number of questions regarding their toxicity in systemic and cellular level, and point out the urgent need for studies assessing the toxic potential of food flavoring (Wrolstad and Culver 2012). ANVISA (2007) states as constant and priority the improvement of safety on the use of additives in food formulation.

In this context, cytotoxic methods revolutionized cell-based drug screening by offering a high-throughput screening colorimetric assay, whereas they simplified sample processing which requires no radioisotope but they are sensitive enough for miniaturization into 96-well plate formats, as seen in MTT, ATP and Alamar Blue[TM] assays. These methods have been very used in the search for new substances with antiproliferative activity and toxic potencial (Skehan et al. 1990, Al-Nasiry et al. 2007, Leite et al. 2014, Ferreira et al. 2015, Monção et al. 2015).

Plant bioassays have been considered highly sensitive and simple in monitoring the cytotoxic and genotoxic effects of chemical compounds (USEPA) (Iganci et al. 2006). *Allium cepa* L. (onion), through the meristematic regions of roots, has been indicated as an effective test organism for evaluating the toxicity at the cellular level (Caritá and Marin-Morales 2008). This test system has excellent kinetic properties of proliferation and large and few chromosomes (2n = 16), which facilitates detection of cellular aberrations (Herrero et al. 2012). It also allows verifying changes in cell division (mitotic index), such as increased or decreased cell proliferation rates in tissues exposed to chemicals of interest (Tabrez et al. 2011).

In the context, this study evaluated the cytotoxicity and genotoxicity of synthetic food flavorings of butter, onion and cheddar on vegetal normal and human and murine tumor cells. These flavorings were chosen because of their wide use as additives by the food industry, especially in the production of frozen foods, and due to their elevated consumption by the population.

MATERIALS AND METHODS

CHEMICALS

Salty liquid synthetic flavorings of butter, cheddar cheese and onion, identical to natural, were purchased from a specialized retailer in the national and international marketing of synthetic food additives located in northeastern Brazil. Flavorings, with oily appearance, were packed in amber bottle with a capacity of 100 mL. Doxorubicin and Alamar Blue[TM] (Resazurin) were purchased from Sigma Aldrich Co. (St Louis, MO, USA). All flavorings were diluted in dimethylsulfoxide (DMSO) to a final concentration of 50 mg/mL (stock solution).

In vitro ANTIPROLIFERATIVE ASSAYS

The antiproliferative potential of the salty liquid synthetic flavorings was assessed after 72h exposure using the human lines SF-295 (glioblastoma), OVCAR-8 (ovarian carcinoma), HCT-116 (colon carcinoma) and HL-60 (promyelocytic leukemia) and primary cultures of murine Sarcoma 180 (S180) cells and of human peripheral blood mononuclear cells (PBMC).

Cell culture was performed in plastic culture flasks (Corning, 25 cm^2, 50 mL volume for adherent cells and 75 cm^2, 250 mL volume for cell suspension) using the RPMI 1640 culture medium supplemented with 10 % fetal bovine serum, 2 mM glutamine, 100 U/mL penincillin and 100 μg/mL streptomycin, at 37 °C with 5 % CO_2.

Quantification of cell proliferation of the flavorings (2.9-1500 µg/mL) was spectrophotometrically determined using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter) at a wavelength of 595 nm for MTT assay and at wavelengths of 570 and 595 nm in Alamar Blue assays. Control groups (negative and positive) received the same amount of DMSO (0.1 %). Doxorubicin was used as positive control (0.005-5.0 µg/mL).

Cytotoxic activity evaluation against human tumor cell lines

The cytotoxicity of the flavorings against different histological types of human cancer was assessed using the MTT method (Mosmann 1983), which analyzes the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a purple formazan product. Briefly, cells were plated in 96-well plates (0.3-0.7 x 10^5 cells/well) and incubated to allow cell adhesion. After 24h of incubation, the substances previously dissolved in pure, sterile DMSO were added to the plates (2.9-1500 µg/mL).

After 69h of incubation, plates were removed and centrifuged at 1500 rpm for 15 min. The supernatant was aspirated and 200 µL of 10 % MTT solution in RPMI 1640 were added; the plate was then incubated at 5 % CO_2 for 3h. Next, plates were again centrifuged, the supernatant was aspirated and the precipitate was resuspended in 150 µL pure DMSO and stirred for about 10 min until complete dissolution of the formazan crystals. The reading was performed using a plate spectrophotometer (DTX 880 Multimode Detector, Beckman Coulter) at a wavelength of 595 nm.

In vitro evaluation on the murine Sarcoma 180 tumor

Adult female Swiss mice (*Mus musculus* Linnaeus, 1758) were obtained from the animal facilities of the Universidade Federal do Piauí (UFPI), Teresina, Brazil. They were kept in well-ventilated cages under standard conditions of light (12h with alternate day and night cycles) and temperature (27 ± 2 °C) and were housed with free access to commercial rodent stock diet (Nutrilabor, Campinas, Brazil).

S180 tumor cells are kept in the peritoneal cavities of Swiss mice in the Laboratory of Experimental Oncology of the Federal University of Ceará since the mid 1980s and a tumor sample was recently donated for our laboratory at UFPI. All procedures were approved by the Committee on Animal Research at the UFPI (Process n° 008/2015) and followed the Brazilian (*Colégio Brasileiro de Experimentação Animal* - COBEA) and International Standards on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council).

Ascite-bearing mice between 7 and 9 days post-inoculation were euthanized by cervical dislocation and a suspension of Sarcoma 180 cells was harvested from the intraperitoneal cavity under aseptic conditions. The suspension was centrifuged at 500 X g for 5 min to obtain a cell pellet and washed three times with RPMI medium. Cell concentration was adjusted to 0.5 x 10^6 cells/mL in supplemented RPMI 1640 medium, plated in a 96-well plate and incubated with increasing concentrations of the flavorings [(2.9-1500 µg/mL). Cell proliferation was determined by the Alamar Blue assay after 72 h of cell culture at 37 °C with 5 % CO_2. Twenty-four hours before the end of incubation, 20 µL of stock solution (0.156 mg/mL) of Alamar Blue™ were added to each well. The absorbance was measured at 570 nm and 595 nm and the drug effect was quantified as the control percentage as described by Ferreira et al. (2011) with one modification, since the measurement was performed after 24h following the Alamar Blue addition.

Antiproliferative study on peripheral blood mononuclear cells by Alamar Blue assay

Heparinized human blood (from healthy, non-smoker donors who had not taken any drug for

at least 15 days prior to sampling, aged between 18-35 years-old) was collected and PBMC were isolated by a standard method of density-gradient centrifugation over Ficoll-Hypaque. PBMC were washed and resuspended (3 x 10^5 cells/mL) in supplemented RPMI 1640 medium and phytohemagglutinin (4 %). Then, PBMC were plated in 96-well plates (3 x 10^5 cells/well). After 24h, the flavorings were added to each well (2.9-1500 μg/mL), and the cells were incubated during 72h. Twenty-four hours before the late incubation, 10 μL of stock solution (0.312 mg/mL) of Alamar Blue™ were added to each well (Ferreira et al. 2015). The absorbance was measured as described above. All studies were performed in accordance with Brazilian research guidelines (Law 466/2012, National Council of Health, Brazil) and with the Declaration of Helsinki.

EVALUATION OF THE CYTOTOXIC AND GENOTOXIC POTENTIAL IN ROOT MERISTEM CELLS OF *Allium cepa*

The manufacturing recommendations indicate the use of 1 mL of flavoring in 300 g of mass. Thus, test with *A. cepa* were carried out using onion bulbs with an average weight of 300 g and flavoring doses of 1 and 2 mL.

Root meristem cells for cytogenetic analysis

Bulbs of *A. cepa* (onion variety baia) were allowed to root in bottles with distilled water at room temperature (± 25 °C), aerated, until obtaining roots of about 2 cm in length. For analysis of each dose (treatment), an experimental group with five bulbs was established. Before placing the roots in contact with their respective doses, some roots were collected and fixed to work as control of the bulb itself.

The remaining roots of the five bulbs of each dose evaluation were placed in their respective solutions for 24h, comprising the exposure time of 24h (24h TE). Afterwards, some roots were removed and fixed. The remaining roots of each bulb were returned to their respective solutions and remained for additional 24h, comprising the exposure time of 48h (48h TE). After this period, roots were collected and fixed again. Exposure times of 24 and 48h were chosen to evaluate the effects of these doses on more than one cell cycle. All roots were in direct contact with the test solution. Roots were fixed in Carnoy 3: 1 (ethanol: acetic acid) at room temperature for 24h. For each collection, we took, on average, three roots per bulb.

Preparation and reading of slides

On average three slides were mounted per bulb, following the method of Guerra and Souza (2002). Each slide was stained with two drops of 2 % acetic orcein and examined under a light microscope with magnification of 400X. A sum of 1,000 cells were analyzed for each bulb, totaling 5,000 cells for each control and treatments.

We analyzed cells in interphase, prophase, metaphase, anaphase and telophase. The number of cells in interphase and in division was calculated for each control and exposure time to determine the mitotic index (MI). The effect of the flavoring doses was also evaluated by the quantification of micronucleated cells, C-metaphases, anaphase and telophase bridges, gene amplification, cells with adhesions, nuclear buds and multipolar anaphase.

STATISTICAL ANALYSIS

For cytotoxicity assays, the IC_{50} values and their 95 % confidence intervals were obtained by nonlinear regression using the Graphpad program (Prisma 5.0, Intuitive Software for Science, San Diego, CA). All *in vitro* studies were carried out in duplicate represented by independent biological evaluations. Outcomes with *Allium cepa* technique were analyzed by Chi-square test (χ^2) and differences were considered significant statistically when $p < 0.05$.

RESULTS AND DISCUSSION

New perspectives have hardly indicated the need for toxicological and cellular evaluations of flavorings in order to rethink, develop and reorganize, based on the new discoveries, novel technical documents by the regulatory agencies that regulate the use of food additives (Konishi et al. 2011). Firslty, we evaluated the cytotoxic activity of flavoring substances on four tumor cell lines using the MTT test. MTT is a rapid, sensitive, and inexpensive method to analyze the viability and the metabolic state of the cell based on the conversion of the yellow salt MTT into a purple crystal blue by mitochondrial enzymes in metabolically active cells (Skehan et al. 1990) sensitive, and inexpensive method for measuring the cellular protein content of adherent and suspension cultures in 96-well microtiter plates. The method is suitable for ordinary laboratory purposes and for very large-scale applications, such as the National Cancer Institute's disease-oriented in vitro anticancer-drug discovery screen, which requires the use of several million culture wells per year. Cultures fixed with trichloroacetic acid were stained for 30 minutes with 0.4 % (wt/vol. As described in table I, only the flavorings of Cheddar and Butter showed cytotoxic activity with IC_{50} values ranging from 125.4 µg/mL (Cheddar - HCT-116) to 402.6 µg/mL (Butter - OVCAR-8), although none have revealed cytotoxicity against leukemic cells. It was also verified the antiproliferative action on primary culture of PBMC after 72h of incubation. Butter flavoring was the most cytotoxic on human normal proliferating leukocytes [136.3 (111.3-167.0) µg/mL] (Table I).

In an attempt to envisage an antitumor action upon *in vivo* assessments, it was determined the activity on Sarcoma 180 cells using the Alamar Blue assay. This metodology incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. This technique has been extensively used to assess different cell types in toxicological, environmental, antimicrobial and cytotoxic susceptibility tests. It presents higher sensibility when compared to other cytotoxicity assays, since smaller amount of cells and steps are required converting it into a suitable method to evaluate proliferation in primary cultures of normal and tumor cells from mice, rats and humans (Gonzales and Tarloff 2001, Al-Nasiry et al. 2007, Rampersad et al. 2012, Schoonen et al. 2012, Bezerra et al. 2015,

TABLE I

In vitro cytotoxic evaluation of the Onion, Cheddar and Butter flavorings on human cancer lines by MTT assay and on primary culture of Sarcoma 180 (S180) cells and peripheral blood mononuclear cells (PBMC) quantified by Alamar Blue assay after 72h of incubation.

Flavoring	IC_{50} (µg/mL)*					
	SF-295	OVCAR-8	HL-60	HCT-116	S180	PBMC
Onion	> 1500	> 1500	> 1500	> 1500	> 1500	> 1500
Cheddar	301.8 248.3 − 366.8	385.8 332.3 − 448.2	> 1500	125.4 102.1 − 154.0	> 1500	> 1500
Butter	402.6 305.1 − 531.3	383.2 313.4 − 468.7	> 1500	250.7 194.1 − 323.9	> 1500	136.3 (111.3 − 167.0)
Doxorubicin	0.2 0.13 − 0.23	0.3 0.17 − 0.31	0.02 0.01 − 0.02	0.1 0.09 − 0.17	1.9 1.4 − 2.4	0.97 0.52 − 1.80

*Data are presented as IC_{50} values and 95 % confidence intervals for leukemia (HL-60), colon (HCT-116), glioblastoma (SF-295) and leukemia (HL-60) human cancer lines and for murine S180 and peripheral blood mononuclear cells. Doxorubicin (Dox) was used as positive control. Experiments were performed in duplicate.

Ferreira et al. 2015). Herein, we did not found antiproliferative action of the flavorings against these malignant S180 cells, exhibiting negative results as those found with leukemia cells (Table I). In fact, cell type antiproliferative specificity is observed with substances with a pool of chemicals, and this is probably due to the presence of different classes of compounds (Cragg et al. 1994). Hence, the use of more than one cell line is considered necessary for detection of cytotoxic compounds.

Since S180 cells are maintained in peritoneal cavity of mice, it is likely that their behavior are similar to those presented by HL-60 suspended cells. Maybe, flavorings are interfering on tumor adhesion to the extracellular matrix (ECM) (in this case is the plastic flask), which causes cytotoxic action. Adhesion to the ECM is essential for survival and propagation of adherent cells. Loss of adhesion activates apoptosis known as anoikis. Previously, some works with Brefeldin A (BFA), a mycotoxin that causes endoplasmic reticulum stress in eukaryotic cells, preferentially induces cell death in MDA-MB-231 (breast carcinoma) suspension cultures in comparison with adherent cultures (Tseng et al. 2014).

Although *in vitro* cytotoxicity studies by MTT assay and similar tecniques are effective in finding new substances with antiproliferative potential, they do not determine the mechanism of action (Berridge et al. 1996). To evaluate the action of these three flavorings, we also used root meristem cells of *A. cepa* to achieve more detailed data about their toxicity at the cell level. This test system is widely used in the screening of cytotoxicity and genotoxicity of chemicals (Herrero et al. 2012) and has satisfactory similarity to results with other test organisms (Arung et al. 2011, Gomes et al. 2013, Oliveira et al. 2013).

Table II describes the number of cells in interphase and in different stages of cell division and values of the mitotic index from root meristem *A. cepa* cells treated with Butter flavoring. There was a significant increase in the rate of cell division in relation to the MI in all doses and times tested in comparison with the control. None of the doses caused cell aberrations or interfered in the MI between 24h and 48h exposure since mitotic spindle alterations or micronucleus are not observed ($p > 0.05$).

TABLE II

Total number of cells analyzed on the cell cycle of root tips of *Allium cepa* treated with 1 and 2 mL of Butter flavoring at exposure times of 24 and 48h.

Dose	ET	Undifferentiated cells/Interphase	P	M	A	T	Cells in division	MI (%)
	C	4464	202	187	82	65	536	10.7
1 mL	24h	3711	438	397	301	153	1289	25.8*
	48h	3830	413	389	219	149	1170	23.4*
	C	4381	190	177	152	100	619	12.4
2 mL	24h	3892	345	289	271	203	1108	22.2*
	48h	3830	392	279	298	201	1170	23.4*

ET – Exposure Time; C – Negative control; MI – Mitotic Index. *Mean values of MI were analyzed by Chi-square test (χ^2) and were significantly different when $p < 0.05$.

According to Cavalcantti et al. (2012), diacetyl (2,3-butanedione), a diluent at large quantities in the chemical composition of the Butter flavoring, cause obliterative bronchiolitis, a very common disease in workers at microwave popcorn factories,

which blocks the bronchioles and compromises lung function. Whittaker et al. (2008) analyzed the mutagenic potential of this chemical compound in a gene mutation assay with rat lymphoma cells (L5178Y line) and observed that diacetyl caused

significant damage to loci on chromosome 11 of these cells and caused functional loss of the locus for thymidine kinase enzyme, causing uncontrolled proliferation of cells of the cell line used.

High doses of diacetyl are cytotoxic because of its potential to replace thymine with guanine. This change may affect the control of cell division (More et al. 2012), which can explain, at least in part, the increasing in proliferating of the root meristem cells. In fact, some studies state that diacetyl might be considered a tumor activator. Likewise, Potera (2012), in a study with mice, found that diacetyl led to fibroblast proliferation in the lungs of these animals due to increased expression of genes responsible for the production of cytokines. Thus, the results obtained by More et al. (2012) and Potera (2012) confirm the results obtained in this study, when the doses used stimulated a significant increase in cell division.

Table III presents the number of cells in interphase and in different stages of cell division

and values of the mitotic index from root meristem *A. cepa* cells treated with Cheddar flavoring. It was observed that 1 mL and 2 mL of Cheddar flavoring caused dose dependent reduction in the mitotic index after 24h (15.0 and 6.5 %) and 48h (6.0 and 6.3 %) exposure, respectively ($p < 0.05$).

Table IV shows the number of cells in interphase and in different stages of cell division and values of the mitotic index from root meristem cells of *A. cepa* treated with Onion flavoring (ET 24 and 48h). This flavoring reduced cell division at 24 or 48h exposure for both doses of 1 mL (3.5 and 2.1 %) and 2 mL (2.5 and 2.6 %) ($p < 0.05$). However, no statistical differences were found between doses ($p > 0.05$). In both ET and doses, it was noted increase of cells in prophase, especially at ET 48h, suggesting that Onion flavoring in such conditions interfered on formation or configuration of mitotic spindles, but additional studies will be performed to confirm it.

TABLE III

Number of cells analyzed on the cell cycle of root tips *Allium cepa* treated with 1 and 2 mL of Cheddar flavoring at exposure times of 24 and 48h.

Dose	ET	Undifferentiated cells/Interphase	P	M	A	T	Cells in division	MI (%)
	C	3541	1205	116	85	53	1459	29.2
1 mL	24h	4249	544	107	44	56	751	15.0*
	48h	4702	114	106	43	35	298	6.0*
	C	3426	465	55	32	22	1574	31.5
2 mL	24h	4777	213	57	31	22	323	6.5*
	48h	4685	282	18	07	08	315	6.3*

ET – Exposure Time; C – Negative control; MI – Mitotic Index. *Mean values of MI were analyzed by Chi-square test (χ^2) and were significantly different when $p < 0.05$.

TABLE IV

Total number of cells analyzed on the cell cycle of root tips of *Allium cepa* treated with 1 and 2 mL of Onion flavoring at exposure times of 24 and 48h.

Dose	ET	Undifferentiated cells /Interphase	P	M	A	T	Cells in division	MI (%)
	C	4561	161	105	82	91	439	8.8
1 mL	24h	4826	129	14	22	09	174	3.5*
	48h	4896	101	00	01	02	104	2.1*
	C	4454	270	86	58	132	546	10.9
2 mL	24h	4874	101	16	09	00	126	2.5*
	48h	4871	124	05	0	00	129	2.6*

ET – Exposure Time; C – Negative control; MI – Mitotic Index. *Mean values of MI were analyzed by Chi-square test (χ^2) and were significantly different when $p < 0.05$.

In table V, it is also demonstrated that both treatments with Onion flavoring at doses of 1 mL and 2 mL reduced the mitotic index (24h: 3.5 %, 48h: 2.1 % / 24h: 2.5 %, 48h: 2.6 %) and increased anaphase and telophase bridges (48h: 13 and 24h: 27), micronucleated cells (24h: 113, 48h: 125 / 24h: 175, 48h: 118), C-metaphases (48h: 22 and 33) and amplifications (24h: 67), respectively. So, cellular aberrations [1 mL (24h: 182, 48h: 160); 2 mL (24h: 202, 48h: 153)] were higher when compared to the negative control ($p<0.05$) (Fig. 1).

Thus, under these conditions, Onion flavoring revealed cytotoxic activity, as demonstrated by high antiproliferative rates and genotoxicity activity, promoting formation of micronuclei and mitotic spindle abnormalities in the vegetal cells. These results differ from those obtained with *in vitro* assays with human and murine cells, on which this food additive was not cytotoxic. There were no studies assessing the toxicity at the cellular level of this food flavoring.

Unfortunately, there are no data about chemical constituents of these flavorings. Some few reports described toxicity studies with compounds also found in additives. Among the flavorings, some of them inhibit microbial proliferation, we can cite potassium benzoate, sodium benzoate and potassium nitrate. They are clastogenic, mutagenic and cytotoxic compounds on normal peripheral human blood cells (Brasil 1999, Mpountoukas et

al. 2010, Zequin et al. 2011). Moreover, boric acid, citric acid, potassium citrate and sodium citrate are cytotoxic on meristematic cells of *A. cepa* (Brasil 1999, Türkoğlu 2007). Benzyl alcohol is frequently used as diluent in foods to maintain uniformity and facilitate incorporation and dispersion of flavors. It was showed that high concentrations of benzyl alcohol promoted significant damage to the mitotic spindle and, therefore, interfered in cell division of human blood cells (Demir et al. 2010). However, these results can not be extrapolated for the present results obtained with flavorings of butter, cheddar cheese, since their ingredients are unclear yet. It is important to emphasize that for each class of aroma and flavor additives, about thirty chemical compounds are represented (ANVISA 2007). Among them, the class with usage restriction of some of its constituents is solvent for extractions, such as agaric acid, aloin, berberine, coumarin, hydrocyanic acid, hypericin, pulegone, safrole and isosafrole, santonin and alpha and beta tuyona that have maximum tolerable limits in food flavorings (Brasil 1999). Moreover, the food flavoring sparteine, allyl hexanoate and quinine were prohibited by these agencies in the early 1980 due to outcomes from evaluation studies of acute and long-term tests carried out in different systems (Konishi et al. 2011).

Recent studies have shown that food flavoring can be highly toxic when used for prolonged peri-

TABLE V

Number of anaphase and telophase bridges, micronucleated cells, colchicine metaphases, amplifications and total number of cellular aberrations in each control and at doses of 1 and 2 mL of Onion flavoring.

Dose	ET	Anaphase and telophase bridges	Micronucleated cells	C-metaphase	Amplifications	Total number of cellular aberrations
1 mL	C	1	0	0	0	01
	24h	0	113	2	67	182*
	48h	13	125	22	0	160*
2 mL	C	1	0	0	0	01
	24h	27	175	0	0	202*
	48h	2	118	33	0	153*

ET – Exposure Time; C – Negative control. *Mean values of MI were analyzed by Chi-square test (χ^2) and were significantly different when $p<0.05$.
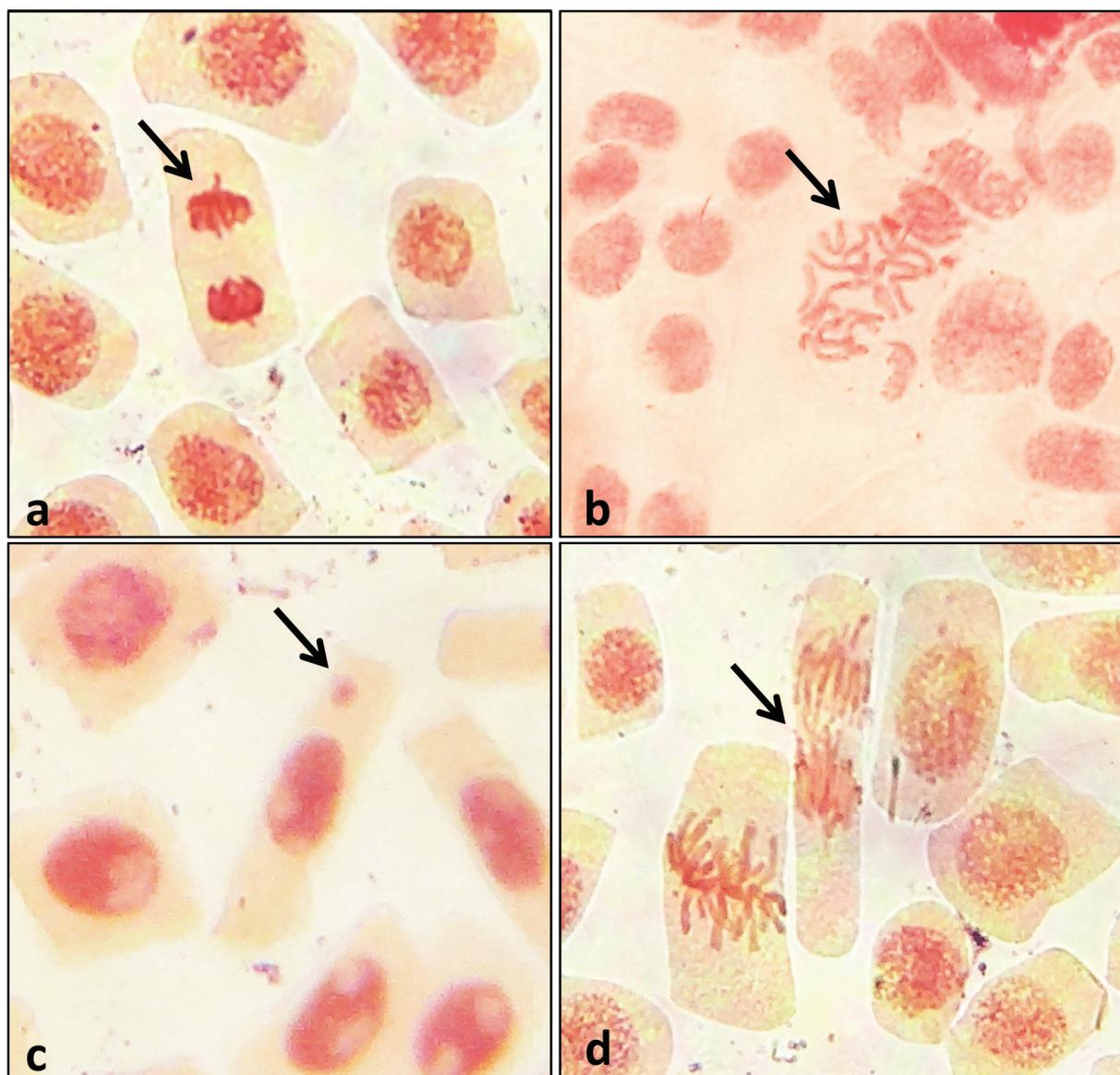
Figure 1 - Cellular aberrations and mitotic spindle abnormalities caused by Onion flavoring on root meristem cells of *Allium cepa* after 24h and/or 48h of treatment. **a**: late anaphase in chromosome; **b**: metaphase in chromosome not aligned in the equatorial plate; **c**: micronucleus; **d**: anaphase bridges. Staining with acetic orcein and examination by light microscope. Magnification, 400X.

ods, promoting hyperactivity in children with and without attention deficit (Stevens et al. 2015), causes blood hemoglobin concentration decreasing, drastic changes in liver function, reduces body weight gain of mice, triggers allergies, skin hypersensitivity and has poor digestion in humans (Anderson et al. 2013, Voltolini et al. 2014). Nevertheless, flavoring additives are poorly studied in the toxicological point

of view and a few studies have assessed the toxicity of food flavoring at the cellular level, differently from most of the dyes used in food industry, which have been widely studied in diverse bioassays and have well-defined values of Acceptable Daily Intake (ADI) (Honorato et al. 2013).

In Brazil, ANVISA (2007) states that high doses of flavorings can cause irritant and narcotic

actions and produce chronic toxicity in digestive tract in the long-term when indiscriminately used. Nevertheless, this regulatory agency as well as EFSA fails to inform the limits of daily intake for these additives and does not report the doses which are considered high nor which flavoring causes cellular or specific organ damage.

Salinas (2002) also believes that food additives have cellular toxicity in the long term. However, similarly to ANVISA (2007), he does not determine which are considered high and low doses, nor define the flavoring causing this type of action. So, although the use of flavoring is permitted by the Ministry of Health and ANVISA, it is necessary and urgent to develop studies to determine the toxic potential and safety doses of these food flavors (Honorato et al. 2013).

CONCLUSIONS

Butter, Onion and/or Cheddar flavorings caused significant changes in the cell division (and cellular aberrations) of root meristem cells of *A. cepa* and presented cytotoxic action even on decontrolled proliferating human tumor cells. So, it is important to conduct supplementary bioassays to check on the cellular level how these additives promote cell death in cells from different types of organisms [plant and mammals (mice and humans)]. *In vivo* toxicological investigations with mammals and further *in vitro* studies are in progress to detail this mechanism, taking into consideration that balance of matrix metalloproteinases (MMP) production by tumor cells may be damaged and to activate cell death (Kessenbrock et al. 2010).

ACKNOWLEDGMENTS

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