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Toxicity of food flavorings to ex-vivo, in vitro and in vivo bioassays

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Abstract:

This study evaluated the toxicity of food flavorings of mint, cinnamon and lemon in meristem root cells of *Allium cepa*, in pure form (as marketed) and in the concentrations of 12.5, 25, and 50%, after 24 and 48 hours of exposure; in *Vero* cell culture evaluated by MTT test and in nauplii of *Artemia salina*, both tests used flavorings in pure form and in the concentrations of 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50%, after 24 hours of exposure. The three flavorings, in all treatments and times of analysis considered, caused significant inhibition of cell division. However, the flavorings did not cause cellular alterations to the evaluated meristems. All evaluated treatments significantly reduced the viability of the evaluated cell line and promoted 100% lethality of *A. salina* nauplii. The evaluated flavorings, under the established study conditions, promoted wide and significant toxicity.

Keywords: flavoring additive, toxic potential, *Allium cepa* , MTT, *Artemia salina* .

Introduction

Food additives or micro-ingredients have become mandatory in modern food, mainly because of their ability to maintain a long-time quality of food marketed in supermarkets (Xu et al., 2013; Adami & Condi, 2016). Among these substances, aroma and flavor additives are of particular relevance because they give or enhance aroma and flavor to the most varied types of processed foods (Konishi, Hayashi, & Fukushima, 2011; Sales et al., 2017).

Classified as natural, nature-identical synthetic and artificial synthetic, food flavorings have complex formulation, consisting of a variety of chemical compounds, such as diluents, antioxidants, antifoams, preservatives, emulsifiers, stabilizers, acid regulators, flavor enhancers, anti-wetting agents, anti-clogging agents, dyes, and extraction and processing solvents (Brasil, 2007). These flavoring additives are authorized worldwide by the Food and Agriculture Organization (FAO) (Xu et al., 2011), and in Brazil by the National Health Surveillance Agency (ANVISA) by means of Resolution RDC 2 of January 15th, 2007 (Brasil, 2007;

Sales et al., 2017). It is important to note that, according to the Codex Alimentarius (2009), the flavoring formula in general is standardized worldwide.

Although conferring essential organoleptic properties on processed foods, and authorized by food safety agencies, most of the flavorings used in the industry do not have established Acceptable Daily Intake (ADI), since to date there is still very little research on toxicological evaluations considering these additives (Konishi et al., 2012; Koca, Erbay, & Kaymark-Ertekin, 2015). Thus, it is relevant to carry out studies that determine the toxic, cytotoxic, genotoxic and mutagenic potential of flavor additives (Koca et al., 2015; Marques, Silva, Ferreira, & Peron, 2015; Sales et al., 2017). In addition, there is an urgent need to establish the toxicity of these substances due to the knowledge that some types of cancer result from the interaction between endogenous and environmental factors, and the most notable of the agents is the diet, particularly when it is made up of excess processed foods (Xu et al., 2011; Koca et al., 2015).

Root meristems of *Allium cepa* L. are considered in the scientific environment an efficient test for the preliminary screening of the cytotoxicity and genotoxicity of chemical compounds (Herrero et al., 2012; Ventura-Camargo, Angelis, & Marin-Morales, 2016). The efficiency of this bioassay is mainly because A. cepa has a reduced chromosome number (2n = 16), which allows the detection of disturbances in the cell proliferation index, and cellular alterations (Silva, Sales, Santos, & Peron, 2017). Furthermore, the cytotoxicity of substances of interest can be observed through viability via reduction of the MTT salt (tetrazolium salt), through the pyruvate dehydrogenase enzymatic complex. This in vitro assay is based specifically on mitochondrial functionality and allows the determination of cytotoxicity through cell viability in cell lines against chemical compounds (Ma et al., 2016).

Another relevant test organism for the initial assessment of the toxicity of chemical compounds is Artemia salina (Anostraca) (Rosa et al., 2016). Nauplii of this species are used as biological test to evaluate the toxic potential of useful natural and/or synthetic substances (Silva et al., 2017). The lethality of this organism has been used to identify biological responses, in the which variables such as death or life are the only ones involved (Meyer et al., 1982; Paredes et al., 2016; Silva et al., 2017).

Thus, the present study aimed to evaluate, through different treatments or concentrations, the toxic, cytotoxic and genotoxic potentials of synthetic flavoring additives similar to the natural ones of mint, cinnamon and lemon, to the root meristem cells of *A. cepa*, in *Vero* cells evaluated via MTT test and A. salina nauplii. The mentioned flavorings were selected for study because they are widely found in industrialized sweets, such as candies, gums, ice cream, mousses, cookies and cakes, and also because there are no studies in the literature evaluating the toxicity of these additives.

Material and methods

Obtaining flavorings and determination of treatments for toxicity assessment

Aroma and flavor additives, nature-identical synthetic, commercially available in non-greasy liquid form of mint, cinnamon and lemon were obtained from a food additive manufacturing industry located in the city of São Paulo, State of São Paulo, Brazil, specialized in the domestic and international marketing of food additives.

In the *A. cepa* test, the roots of bulbs were exposed to each flavoring from the following treatments: pure flavoring (without dilution) and flavoring dissolved in distilled water in the concentrations of 12.5, 25, and 50%. In the MTT and A. salina tests, the following treatments were established: pure flavoring (without dilution) and flavoring diluted in aqueous solution of synthetic sea salt (30 g L^{-1}) at concentrations of 0.78,0 1.56, 3.12, 6.25, 12.5, 25, and 50%.

Cytotoxicity and genotoxicity test in root meristem cells of Allium cepa L.

For the evaluation of the flavorings in root meristems, initially, onion bulbs were placed in aerated flasks with distilled water to obtain 2.0 cm long roots. For the analysis of the all treatment, an experimental group with five onion bulbs was established. Before placing the roots in contact with their respective treatments, some roots were collected and fixed to serve as control of the bulb itself, which was identified as analysis time 0 hour (0h).

Then, the remaining roots were placed in their respective treatments for 24 and 48 hours, procedures called exposure times 24 and 48h, where root collection was performed every 24 hours. A negative control was prepared only with distilled water, in which roots were also collected at 0, 24, and 48h. All roots collected during the experiment were fixed in Carnoy 3:1 (ethanol:acetic acid) for up to 24 hours.

Slides were mounted according to the protocol proposed by Guerra and Souza (2002) and analyzed under an optical microscope with a 40x objective. For each bulb, 1,000 cells were analyzed, totaling 5,000 cells for each control group (0h), 24h exposure time group and 48h exposure time group. Cells were counted in interphase and during cell division, and the mitotic index was calculated, thus determining the cytotoxic potential. Genotoxicity was evaluated through micronuclei frequency, and aneugenic or mitotic spindle alterations were evaluated through the frequency of colchicine metaphases, anaphase and telophase bridges, gene amplifications, cells with adhesion, nuclear buds and multipolar anaphases. The results were analysed using software R, with significance level 5%, using the non-parametric Kruskal-Wallis test with Dunn`s posterior test.

Cytotoxic activity evaluation against Vero cell line

The cytotoxicity of food flavoring against Vero cells was evaluated by the MTT (4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (Sigma-Aldrich, Germany) method, through the quantization of viable cells. Cells were cultured in 96-well plates (TPP, Trasadingen, Switzerland), at a density of 2×10^5 cells $well^{-1}$. After a 24h incubation period, at 37°C in an atmosphere of CO_2, the culture medium was removed and the cells were washed three times with serum-free L-15; the following treatments were established: pure flavoring (without dilution) and flavoring diluted in MM (L-15 medium with 2% serum) at concentrations of 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50%. Untreated controls were performed by the addition of 200 #L of MM.

Cells were then incubated for 24 hours. The medium was then removed and 50 #L of MTT solution (5 mg mL^{-1}) was added. Plates were reincubated for 4h. After that, the MTT solution was removed, 100 #L of DMSO was added to dissolve formazan crystals, and the plates were gently shaken, until crystals were completely dissolved. The solubilized product was quantified by spectrophotometry at 492 nm (reference at 620 nm). Results were expressed as % viability cell considering absorbance control cells as 100% viable.

Toxicity test in nauplii of Artemia salina Leach. (Artemiidae)

The toxicity test of aroma and flavor micro-ingredients against *A. salina* was carried out according to the protocol proposed by Meyer et al. (1982) and Paredes et al. (2016) with minor modifications. *A. salina* eggs were incubated in solution of synthetic sea salt (30 g L^{-1}) in a glass vessel equipped with a dark compartment and another with artificial light. Water was maintained at room temperature under constant stirring and aeration for 48h until hatching of the larvae. With the aid of a Pasteur pipette, the larvae or microcrustacean nauplii (n = 10) were transferred to test tubes containing 3 mL of each treatment. The control was prepared

only with solution of synthetic sea salt (30 g L^{-1}). All treatments were analyzed in triplicate, and the number of dead larvae were counted after 24h of exposure.

Results and discussion

Based on the results in Table 1, the mitotic indices obtained for the root meristem cells of *A. cepa* exposed to cinnamon, lemon and mint flavorings, in pure form and in the three concentrations analyzed, at 24 and 48h exposure, were significantly lower than the observed cell division indices for their respective 0h exposure time. Furthermore, the cell division indices for the 24 and 48h exposure times of all treatments also demonstrated significant inhibition of cell division to the meristematic tissue when compared to the mitotic index obtained for the negative control (distilled water) related to the same exposure times. No significant cellular changes were found in the meristematic cells exposed to the treatments with the flavorings evaluated. Thus, under the conditions of analysis established for this bioassay, the microingredients tested were cytotoxic, but not genotoxic.

TABLE 1.
Mitotic indices observed in root meristem tissue of Allium cepa at 0, 24, and 48 hours exposure times to cinnamon, lemon and mint food flavorings, evaluated pure and at three concentrations.

	ET/MI (%)			
Co		0h	24h	48h
		41.9^{a}	41.7^{a}	41.3^{a}
	TR	0h	24h	48h
Flavoring	Pure	42.1^{a}	7.1^{b*}	4.8^{b*}
	50%	40.9^{a}	12.1^{b*}	10.3^{b*}
Mint	25%	39.9^{a}	14.1^{b*}	10.1^{b*}
	12.5%	40.1^{a}	16.9^{b*}	15.3^{b*}
	Pure	39.9^{a}	5.1^{b*}	2.9^{b*}
	50%	41.3^{a}	10.9^{b*}	8.9^{b*}
Cinammon	25%	37.9^{a}	10.6^{b*}	9.4^{b*}
	12.5%	40.0^{a}	13.1^{b*}	9.2^{b*}
	Pure	39.1^{a}	3.1^{b*}	1.7^{b*}
	50%	39.0^{a}	9.4^{b*}	7.2^{b*}
Lemon	25%	41.1^{a}	14.1^{b*}	12.9^{b*}
	12.5%	40.1^{a}	13.8^{b*}	11.4^{b*}

Co: Distilled water control; ET: exposure time; TCI: Total number of cells in interphase; TCD: Total number of dividing cells. Values followed by different letters, in the same treatment, are significantly different from each other by the software R, with significance level 5%, using the non-parametric Kruskal-Wallis test with Dunn`s posterior test, the 5% level. *Significant MI/ET of aromas to the specific MI/ET of the negative control.

As mentioned by Herrero et al. (2012), mitotic indices significantly lower than the control indices - such as those observed in the present study for cinnamon, lemon and mint flavors - indicate the presence of agents whose toxic action impairs the growth and development of exposed organisms. In addition, these authors state that the inhibition of cell proliferation triggered by cytotoxic compounds in tissues of intense cellular proliferation and normal performance, as used in this research, is very harmful to the organism by inhibiting or limiting the replacement of cells, altering the production of proteins and result in dysfunction of the organ where it is located. Such losses, according to Valavanidis, Vlachogranni, Fiotakis, and Loridar (2013) and

Zilifdar et al. (2014) can significantly compromise the cellular division of the affected tissue or organ and trigger and/or potentiate cancerous processes.

In relation to the condition that flavorings have shown cytotoxic but not genotoxic potential to *A. cepa*, Sales et al. (2017) point out that the drastic inhibition of division in normal tissues may occur by the action of agents that affect the integrity of the nuclear spindle during mitosis, promoting significant chromosomal derangement. Considering that the principle of the cell cycle is the formation of identical cells, the production of new cells with significant changes in structure and/or chromosome number make cell functioning unfeasible and tend to be eliminated from tissues with normal performance, which may lead to a significant antiproliferative effect.

For the tetrazolium reduction test, the nine treatments analyzed for the three flavorings reduced the cell viability of the Vero cell culture by 100%, demonstrating significant cytotoxicity of the food additives under study. These results corroborate the significant cytotoxicity results presented in Table 1. Results of the toxicity obtained in A. salina showed that nine treatments analyzed for cinnamon, mint and lemon flavorings caused 100% lethality of nauplii exposed to these substances. In addition, there was a significant relationship between the results of toxicity observed for bioassays, in vivo and in vitro.

According to the Technical Regulation on Aroma and Flavor Additives, approved by ANVISA in 2007, and still in force, the formulation of any synthetic food flavoring is standardized, being the responsibility of the food safety agencies the inspection of its composition (Brasil, 2007). However, the specific composition of cinnamon, lemon and mint flavorings has not been found in the technical documents of regulatory agencies, labels, manufacturers websites and scientific literature.

Nevertheless, there are studies showing toxicity at the cellular level of chemical constituents with diluent and preservative actions, according to Resolution RDC 2 as of January 15, 2007, in the basic formulation of the aroma and flavor additives, and that validate the data obtained for the three flavorings evaluated in the present study. Among these compounds is benzoic alcohol, responsible for maintaining uniformity and facilitating the incorporation and dispersion of aromas in food products. In the analysis of the cellular action of this diluent, Demir, Kogaglu, and Kaya (2010) found that this alcohol promoted significant damage to the mitotic spindle, and, consequently, the cell division of human peripheral blood cells.

Another diluent found in the flavoring formulation is diacetyl (2,3-butadione). Whittaker, Clarke, San, Begley, and Dunkel (2008) reported that this compound in gene mutation assay in rat lymphoma caused significant damage to the loci of chromosome 11 of these cells, causing loss of expression of genes for thymidine kinase enzyme. Still, More, Raza, and Vince (2012) verified that the diacetyl diluent had the potential to replace thymine with guanine in regions of euchromatin and to cause disruption of hydrogen and disulfide bonds in the tertiary structure of enzymes involved in the process of cell division. In the composition of food flavorings, preservative compounds are also found: boric acid, citric acid, potassium citrate and sodium citrate (Brasil, 1999), which, according to Tükoğlu (2007), resulted in a significant reduction in the cell division index of root meristem cells of *A. cepa*, proving to be cytotoxic.

Conclusion

From the results obtained, it was verified that the cinnamon, lemon and mint flavorings induced, under the conditions established for the present study, broad toxicity to the root meristems of *A. cepa*, in Vero cells evaluated by MTT test, and *A. salina* nauplii. Further studies are necessary to define the toxic potential of additives, for example in rodent bioassays, to ensure the safety of the population in consuming such additives.

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