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Analysis of cytotoxicity and genotoxicity of sweeteners using *Allium cepa* bioassays

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ABSTRACT. Sweeteners are food additives consumed worldwide, especially by individuals with restrictive diets. This study evaluated the cytotoxic and genotoxic effects of sweeteners widely sold in Brazil through a bioassay with *Allium cepa*. Onion bulbs were exposed to different concentrations of sweeteners, and their root cells were stained with acetic carmine and analyzed under an optical microscope. Distilled water was used as a negative control, and glyphosate as a positive control. The results demonstrated that the mitotic index (MI) and the chromosome aberration index (CAI) of the three sweeteners tested did not differ significantly from the negative control, indicating that they are not toxic or mutagenic additives. This result is positive for consumers of these products, especially those who need to replace sugar due to dietary restrictions. However, they should be consumed with caution.

Keywords: Aspartame; stevioside; sodium saccharinand; sodium cyclamate and food.

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Introduction

Over the decades, food industries have developed new techniques to win over their consumers, whether through internal or external modification of their products. In this context, several natural and synthetic molecules serve as tools to enable such changes and are called food additives (Carocho, Morales, & Ferreira, 2017).

Food additives are substances added to foods to enhance their physical, chemical, and sensory characteristics while not altering the nutritional values of products (Mooradian, Smith, & Tokuda, 2017; Trasande, Shaffer, & Sathyanarayana, 2018; Valluzzi et al., 2019). In Brazil, the regulation of the use of food additives is the responsibility of the National Health Surveillance Agency (ANVISA), which, in turn, is based on international health bodies such as the Food and Drug Administration (FDA); the European Food Safety Authority (EFSA); the Food Chemical Codex (FCC); the Brazilian Pharmacopoeia; Officially recognized pharmacopoeias, according to Collegiate Board Resolution - RDC nº 511, dated 27 May 2021; the European Commission; and the Joint FAO/WHO Expert Committee on Food Additives - JECFA); (Agência Nacional de Vigilância Sanitária [ANVISA], 2023); Oliveira et al., 2017a).

Sweeteners are food additives used to give a sweet taste to food due to its low caloric value. In this way, they are valuable substances for individuals with diabetes or who follow a low-calorie diet (Fernstrom, 2015; Pearlman, Obert, & Casey, 2017). Among the types of sweeteners currently allowed by ANVISA and highly prevalent in the Brazilian market, sodium saccharin, sodium cyclamate, aspartame, and steviol glycosides stand out (ANVISA, 2023; Zanini, Araújo, & Martínez-Mesa, 2011).

Although they have diverse origins and chemical structures, all these compounds share the characteristic of having an extremely high sweetening power compared to sucrose (table sugar). However, the maximum level of sweetness to be achieved with a sweetener is often lower in relation to sucrose since, in high doses, these additives have a "bitter" or "metallic" taste (Antenucci & Hayes, 2014; Magnuson, Carakostas, Moore, Poulos, & Renwick, 2016; Whitehouse, Boullata, & McCauley, 2008). In recent years, it has been possible to notice an exponential growth in the consumption of sweeteners, mainly caused by the increase in cases of diabetes and the widespread commercialization of diet products such as juices and soft drinks (Dunford et al., 2020; Guo, Li, Liu, Shi, & Gao, 2021; Martyn et al., 2018). According to estimates, the world consumption of these products accounted for around 1.3 billion dollars in 2020 (Alves, Rodrigues-Silva, Ribeiro, & Rath, 2021). In Brazil, a 2019 study showed that the use of sweeteners reaches 13.4% in a portion of 41,433 individuals, and research suggests that there is a rapid increase in this value within the Brazilian market (Arrais et al., 2019; Barraj, Scrafford, Bi, & Tran, 2021).

However, allied to this growth, research has emerged that has put the safety of such compounds to the test, stating that the ingestion of these molecules – even in safe doses – can lead mainly to erythrocyte damage, blood hypercoagulation, overexpression of inflammatory cytokines, neural cell dysfunction, exacerbated formations of free radicals and genotoxicity (Bandyopadhyay, Ghoshal, & Mukherjee, 2008; Choudhary & Pretorius, 2017; Magnuson et al., 2016; Pearlman et al., 2017; Whitehouse et al., 2008).

Despite being contested, few studies on the toxicity of sweeteners have received notoriety in Brazil (Alves et al., 2021; Oliveira et al., 2017b). The use of plant bioassays to assess the genotoxicity of isolated compounds proves to be useful in these cases, as they have a high sensitivity and susceptibility to DNA mutations (Iganci, Bobrowski, Heiden, Stein, & Rocha, 2022; Patnaik, Achary, & Panda, 2013).

The superior plant *Allium cepa* stands out for providing good practical conditions in bioassays and has been validated by the World Health Organization, the United Nations Environmental Program, and the United States Environmental Protection Agency (Roberto, Jamal, Malaspina, & Marin-Morales, 2016). It is the recommended plant because it has rapid cell proliferation in its radicles, with large chromosomes and in small numbers (2n = 16), thus facilitating the induction, detection, and subsequent visualization of chromosomal damage (FISKESJÖ, 1985; Leme & Marin-Morales, 2009; Oliveira et al., 2017a).

This study evaluated the cytotoxic and genotoxic effects of artificial and natural sweeteners on *Allium cepa* bulbs exposed to different concentrations typically used daily.

Material and methods

The study was carried out at the Microbiology Laboratory of the *Universidade do Contestado*, Concórdia campus. *Allium cepa* bulbs were purchased commercially in Concórdia – Santa Catarina, Brazil, being medium-sized, uniform, from the same origin, non-germinated and healthy. The central parenchyma of the budding crowns was removed, and then the bulbs were placed in flasks with distilled water for 72 hours in the dark and at a temperature of 23 ± 3°C to stimulate the growth of new roots. When the emerged rootlets reached 2–2.5 cm in length, the bulbs were submitted in duplicate to each sweetener sample for 24, 48, and 72 hours, with the negative control (NC) being performed with distilled water and the positive control (CP) with glyphosate at a concentration of 15 mg mL^{-1} (Mercado & Caleño, 2020). The sweetener concentrations were based on RDC nº18 of March 24, 2008 (Agência Nacional de Vigilância Sanitária [ANVISA], 2008) and Normative Instruction - Nº. 211, of March 1, 2023 (ANVISA, 2023) and on the manufacturer's guidelines, where allowed and duplicated values were selected, expressed through drops of sweeteners 50 mL^{-1} of water (Table 1).

Table 1. Concentrations of sweeteners used in *Allium cepa* bulbs.

Treatment	Concentration (in drops 50 mL)
Aspartame (*Brand A*)	10
	20
Stevioside (*Brand B)*	10
	20
Sodium Saccharinand	10
Sodium Cyclamate (*Brand C)*	20

After the exposure time, three specimens of roots were selected from each bulb, washed with distilled water, and fixed in Carnoy's solution (ethanol: glacial acetic acid [3:1 v/v]) for 24 hours. Next, the collected roots were washed with distilled water and subjected to hydrolysis in HCl (hydrochloric acid) 1N at 60ºC for 11 minutes. Finally, the apical region of the rootlets was removed, macerated, and stained with acetic carmine for visualization under the microscope, where 1,000 cells per sample were counted. The mitotic index (MI) was calculated through the ratio of the number of mitotic cells (MC)/total number of cells X 100. The chromosomal aberration index (CAI) was calculated through the ratio of the number of aberrant cells (CA)/total number of cells X 100 (Islam et al., 2017).

Statistical analysis

The data obtained in this study were expressed as mean values with ± standard deviation (SD) for each concentration. Statistical analyses were performed using the R 4.0.2 software by one-sided analysis of variance (ANOVA) with a significance level of $p < 0.05$, considering a 5 x 3 x 2 factorial scheme (treatments, times, and concentrations).

Results

Tables 2 and 3 show the effects of different types and sweetener concentrations on *Allium cepa* root cells over exposure periods of 24, 48, and 72 hours. The mitotic index (MI) was used to evaluate cytotoxicity, while the chromosomal aberration index (CAI) was considered to assess genotoxicity.

Table 2. Cytotoxic and genotoxic analysis of *Allium cepa* root cells exposed to distilled water (negative control), glyphosate (positive control) and different sweeteners, at a concentration of ten drops, over 24, 48 and 72 hours.

Treatment	Time (hours)	MC	AC	MI (%)	CAI (%)
Glyphosate	24	146 ± 4.0 ^{cA}	30.5 ± 1.5 ^{aC}	14.6 ± 0.4 ^{cA}	3.1 ± 0.2^{aC}
	48	114 ± 4.0 ^{dB}	38.5 ± 1.5 ^{aB}	11.4 ± 0.4 ^{dB}	3.9 ± 0.2^{aB}
	72	90.5 ± 1.5 ^{eC}	51.5 ± 2.5 ^{aA}	9.1 ± 0.1 ^{daC}	5.2 ± 0.3^{aA}
Water	24	275 ± 2.0 ^{aB}	0.5 ± 0.5 ^{cNS}	27.5 ± 0.2 ^{aB}	0.1 ± 0.1 ^{cNS}
	48	282 ± 3.0 ^{bAB}	0.0 ± 0.0 ^{dNS}	28.2 ± 0.3 ^{bAB}	0.0 ± 0.0^{dNS}
	72	286 ± 5.5 ^{bA}	1.0 ± 1.0 ^{dNS}	28.7 ± 0.5 ^{bA}	0.1 ± 0.1^{dNS}
Aspartame	24	276.5 ± 4.5 ^{bA}	12 ± 1.0 ^{bC}	27.7 ± 0.5 ^{bA}	1.2 ± 0.1^{bC}
	48	266.5 ± 1.5 ^{cB}	14 ± 2.0 ^{bB}	26.7 ± 0.2 ^{cB}	1.4 ± 0.2^{bB}
	72	244.5 ± 4.5 ^{dC}	20 ± 1.0 ^{bA}	24.5 ± 0.4 ^{dC}	2.0 ± 1.0^{bA}
Stevioside	24	279 ± 3.0 ^{aA}	0.5 ± 0.5 ^{cB}	27.9 ± 0.3 ^{aC}	0.1 ± 0.1^{cB}
	48	290 ± 1.0 ^{aB}	2.5 ± 0.5 ^{cdB}	29 ± 0.1 ^{aB}	0.3 ± 0.1^{cdB}
	72	302.5 ± 4.5 ^{aC}	5.5 ± 0.5 ^{cA}	30.3 ± 0.4 ^{aA}	0.6 ± 0.1^{cA}
Sodium Saccharin and Sodium Cyclamate	24	276.5 ± 3.5 ^{abA}	2.0 ± 1.0 ^{cC}	27.7 ± 0.4 ^{abA}	0.2 ± 0.1^{cC}
	48	267 ± 1.5 ^{cB}	4.5 ± 0.5 B^{c}	26.8 ± 0.1 ^{cB}	0.5 ± 0.1^{cB}
	72	255 ± 1.5 ^{cC}	8 ± 1.0 ^{cA}	25.6 ± 0.2 ^{cC}	0.8 ± 0.1^{cA}

Data were expressed as mean ± SD. Lowercase superscript letters between each treatment x time group (ten drops) and uppercase letters within each treatment x time group indicate significant differences from each other (ANOVA, Tukey's test, $p < 0.05$). ns = not significant. Mitotic Cells (MC); Aberrant cells (AC); Mitotic index (MI); Chromosomal aberration index (CAI).

Table 3. Cytotoxic and genotoxic analysis of *Allium cepa* root cells exposed to distilled water (negative control), glyphosate (positive control) and different sweeteners, at a concentration of twenty drops, during the exposure period of 24, 48, and 72 hours.

Treatment	Time (hours)	MC	AC	MI (%)	CAI (%)
Glyphosate	24	146 ± 5.7 $^{d A}$	30.5 ± 2.1 $^{a C}$	14.6 ± 0.6 $^{d A}$	3.05 ± 0.2 $^{a A}$
	48	114 ± 5.7 $^{c B}$	38.5 ± 2.1 $^{a B}$	11.4 ± 0.6 $^{c B}$	3.85 ± 0.2 $^{a B}$
	72	90.5 ± 2.1 $^{d C}$	51.5 ± 3.5 $^{a A}$	9.05 ± 0.2 $^{d C}$	5.15 ± 0.4 $^{a C}$
Water	24	275 ± 2.8 $^{a ns}$	0.5 ± 0.7 $^{c ns}$	27.5 ±0.3 $^{a ns}$	0.05 ± 0.1 $^{c ns}$
	48	282 ± 4.2 $^{a ns}$	0 ± 0.0 $^{d ns}$	28.2 ± 0.4 $^{a ns}$	0 ± 0.0 $^{d ns}$
	72	286.5 ± 7.8 $^{b ns}$	1 ± 1.4 $^{d ns}$	28.65 ± 0.8 $^{b ns}$	0.1 ± 0.1 $^{d ns}$
Aspartame	24	245.5 ± 6.4 $^{c A}$	15.5 ± 2.1 $^{b C}$	24.55 ± 0.6 $^{c A}$	1.55 ± 0.2 $^{b C}$
	48	235.5 ± 4.9 $^{b A}$	24.5 ± 2.1 $^{b B}$	23.55 ± 0.5 $^{b A}$	2.45 ± 0.2 $^{b B}$
	72	213.5 ± 7.8 $^{c B}$	31 ± 2.8 $^{b A}$	21.35 ± 0.8 $^{c B}$	3.1 ± 0.3 $^{b A}$
Stevioside	24	267 ± 5.7 $^{ab C}$	1.5 ±0.7 $^{c B}$	26.7 ±0.6 $^{ab C}$	0.15 ± 0.6 $^{c B}$
	48	299.5 ± 9.2 $^{a B}$	4 ± 1.4 $^{cd B}$	29.95 ± 0.9 $^{a B}$	0.4 ± 0.1 $^{cd B}$
	72	322 ± 4.2 $^{a A}$	8.5 ± 2.1 $^{c A}$	32.2 ± 0.4 $^{a A}$	0.85 ± 0.2 $^{c A}$
Sodium Saccharin and Sodium Cyclamate	24	255.5 ± 7.8 $^{bc A}$	2.5 ± 0.7 $^{c B}$	25.55 ± 0.8 $^{bc A}$	0.25 ± 0.1 $^{c B}$
	48	242.5 ± 9.2 $^{b AB}$	6.5 ± 2.1 $^{c B}$	24.25 ± 0.9 $^{b AB}$	0.65 ± 0.2 $^{c B}$
	72	226.5 ± 3.5 $^{c B}$	11 ± 2.8 $^{c A}$	22.65 ± 0.4 $^{c B}$	1.1 ± 0.3 $^{c A}$

Data were expressed as mean ± SD. Lowercase superscript letters between each treatment x time group (twenty drops) and uppercase letters within each treatment x time group indicate significant differences from each other (ANOVA, Tukey's test, $p < 0.05$). ns = not significant. Mitotic Cells (MC); Aberrant cells (AC); Mitotic index (MI); Chromosomal aberration index (CAI).

Tables 2 and 3 show an increase in the AC rate in relation to the degree of toxicity of the analyzed substance. The three sweeteners tested did not show enough chromosomal aberrations to be equalized to the positive control (glyphosate), remaining close to the negative control (distilled water).

For the evaluation of aberrant cells, several types of mutations were accounted for: formation of micronuclei, dysmorphic or retarded chromosomes, chromosomal bridges, chromosomal disorganization, and chromosomal fragments (Figure 1). Such aberrations were present at all exposure times and in the three sweeteners tested but in more significant amounts at a concentration of 20 drops (Table 3).

As for the MI, at a concentration of 10 drops, the effects found for the three sweeteners ranged from 24.5 to 30.3% in the three exposure times (Table 2). The sweeteners aspartame and sodium saccharin + sodium cyclamate also showed similar results ($p < 0.05$) at this concentration. With 20 drops, the variation was from 21.35 to 32.2% (Table 3); the positive control, on the other hand, presented a variation of 9 to 14%, indicating a low inhibition of cell multiplication caused by sweeteners and, consequently, a low cytotoxic effect of these additives.

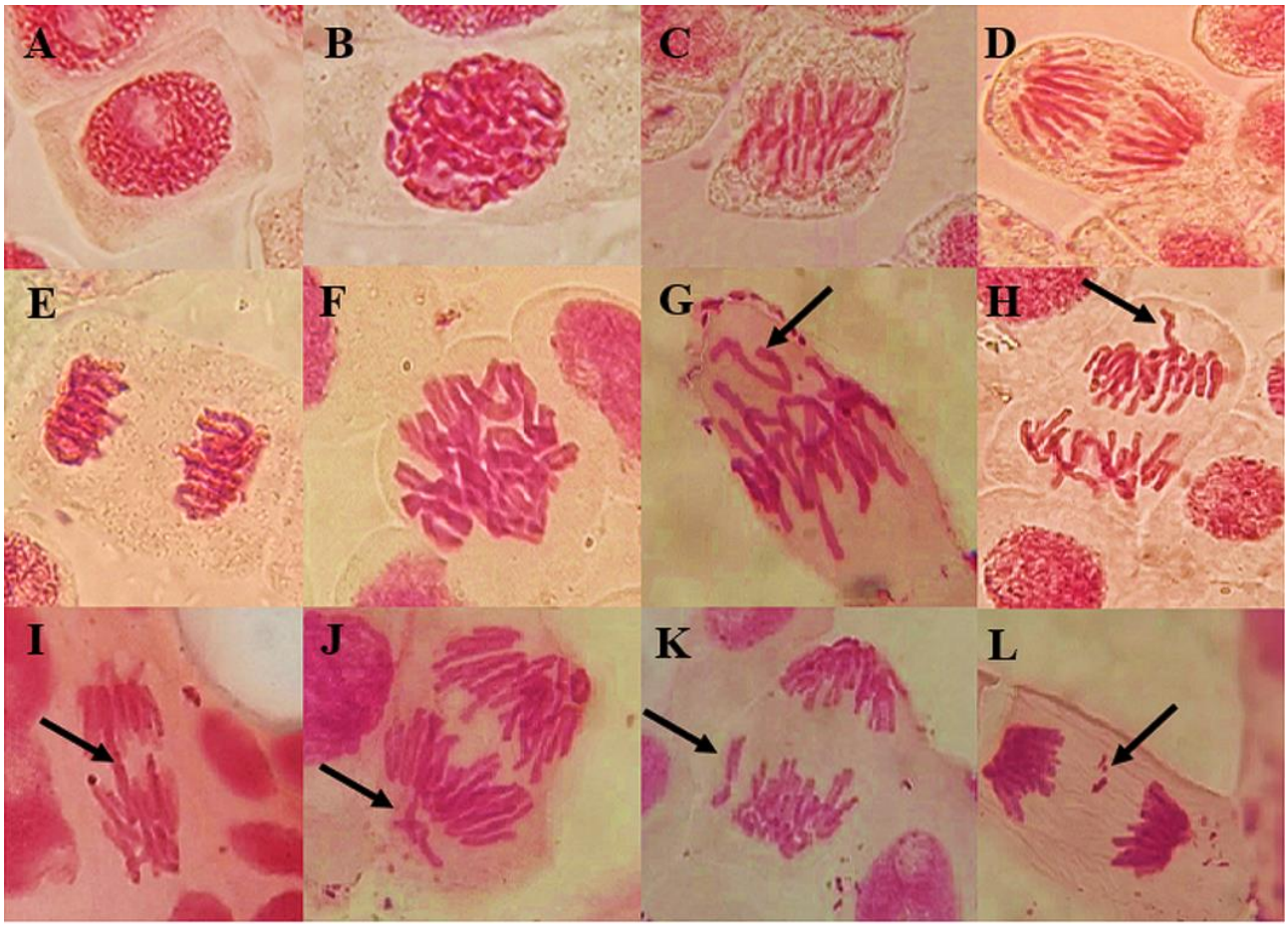

Figure 1. Cellular variations found in *Allium cepa* roots exposed to sweeteners. Stages of mitotic division in *Allium cepa* cells, at 1000x magnification under an optical microscope. From **a** to **e**, normal stages of cell division. **a** – Interphase; **b** – Prophase; **c** – Metaphase; **d** – Anaphase; **e** – Telophase; **f** – Chromosomal disorganization during prophase; **g** – Chromosomal disorganization during metaphase and lagging chromosome (arrow); **h** – Chromosomal disorganization during anaphase and lagging chromosome (arrow); **i** – Chromosomal bridge (arrow) during anaphase; **j**, **k**, and **l** – Chromosome fragments (arrows). Source: Prepared by the author.

Although relatively high when compared to the negative control, the CAI of the sweeteners differed mainly from the positive control, both in the concentration of 10 or 20 drops. According to Tukey's test ($p < 0.05$), the sweeteners and the negative control were far from the positive control (Tables 2 and 3), thus suggesting that the additives studied also do not have a significant genotoxic effect. Regarding the exposure time within each treatment group presented in Tables 3 and 4, only the negative control did not show a significant effect ($p < 0.05$).

To better observe the effect of the concentration of the aspartame, stevioside, and sodium saccharin + sodium cyclamate sweeteners, Table 4 presents the mean values of the treatments in the concentrations of 10 and 20 drops and the Tukey test ($p < 0.05$).

Table 4. Mean values of treatments for cytotoxic and genotoxic analysis of *Allium cepa* root cells exposed to distilled water (negative control), glyphosate (positive control) and different sweeteners in concentrations of ten and twenty drops.

Treatment	Concentration (drops)	MC	AC	MI (%)	CAI (%)
Glyphosate	10	116.8 ± 27.9 ^{nsD}	40.2 ± 10.6 ^{nsA}	11.7 ± 2.8 ^{nsD}	4.0 ± 1.1 ^{nsA}
	20	116.8 ± 27.9 ^{nsE}	40.2 ± 10.6 ^{nsA}	11.7 ± 2.8 ^{nsE}	4.0 ± 1.1 ^{nsA}
Water	10	281.2 ± 5.8 ^{nsB}	0.5 ± 0.5 ^{nsD}	28.1 ± 0.6 ^{nsB}	0.0 ± 0.0 ^{nsD}
	20	281.2 ± 5.8 ^{nsB}	0.5 ± 0.5 ^{nsD}	28.1 ± 0.6 ^{nsB}	0.0 ± 0.0 ^{nsD}
Aspartame	10	262.5 ± 16.4 ^{aC}	15.3 ± 4.2 ^{aB}	26.3 ± 1.6 ^{aC}	1.5 ± 0.4 ^{aB}
	20	231.5 ± 16.4 ^{bD}	23.7 ± 7.8 ^{bB}	23.2 ± 1.6 ^{bD}	2.4 ± 0.8 ^{bB}
Stevioside	10	290.5 ± 11.8 ^{nsA}	2.8 ± 2.5 ^{nsCD}	29.1 ± 1.2 ^{nsA}	0.3 ± 0.3 ^{nsCD}
	20	296.2 ± 27.7 ^{nsA}	2.8 ± 2.0 ^{nsC}	29.6 ± 2.8 ^{nsA}	0.5 ± 0.4 ^{nsC}
Sodium Saccharin and Sodium Cyclamate	10	266.5 ± 10.5 ^{aC}	4.8 ± 3.0 ^{nsC}	26.7 ± 1.1 ^{aC}	0.5 ± 0.3 ^{nsC}
	20	241.5 ± 14.5 ^{bC}	6.7 ± 4.3 ^{nsC}	24.2 ± 1.5^{bC}	0.7 ± 0.4 ^{nsC}

Data were expressed as mean ± SD. Lowercase superscript letters within treatments x concentration (ten and twenty drops) and uppercase letters between each treatment x concentration group indicate significant differences from each other (ANOVA, Tukey's test, $p < 0.05$). ns is not significant. Mitotic Cells (MC); Aberrant cells (AC); Mitotic index (MI); Chromosomal aberration index (CAI).

Discussion

Although consumed worldwide and considerably useful for individuals with restrictive diets, sweeteners have long-term effects that are still unknown. In the case of saccharin and aspartame, recent studies with rats have shown that these substances may be involved in negative physiological changes, such as changes in liver enzymes, superoxide formation, increased rate of apoptosis in various tissues, and deposition of amyloid plaques in the brain (Alkafafy, Ibrahim, Ahmed, & El-Shazly, 2015; Anbara, Sheibani, Razi, & Kian, 2021; Ashok, Poornima, Wankhar, Ravindran, & Sheeladevi, 2017; Whitehouse et al., 2008). On the other hand, studies with stevioside have not yet shown any evidence of toxicity (Rotimi et al., 2018; Uçar, Yılmaz, Yılmaz, & Kılıç, 2017; Urban, Carakostas, & Brusick, 2013).

Research with *Allium cepa* proved useful in this case, given the practicality, replicability, and reliability of this test. Because they have a stable karyotype, root cells of *Allium cepa* show a visible and expressive response – through nucellar mutations – when exposed to a substance with a potential cytotoxic effect (Aydın & Liman, 2020; Fiskesjö, 1985; Koç & Pandir, 2018; Leme & Marin-Morales, 2009). In the present work, the cytotoxic and genotoxic potential of different sweeteners was tested in *Allium cepa* cells, using glyphosate as a positive control and distilled water as a negative control.

The mitotic index (MI) is represented by the total number of cells present in the cell cycle, and its increase or decrease is directly related to a substance's cytotoxicity level. Therefore, if the MI is below the negative control, it is possible to assume that the substance in question caused an unfavorable change in the cell multiplication mechanisms; on the other hand, MI values above the negative control may indicate a reduction in the time of the mitotic cycle and an increase in the cell division rate, which is often harmful to the cell by inducing chromosomal mutations (Al-Ahmadi, 2013; Leme & Marin-Morales, 2009). Chromosomal aberrations mainly originate from problems during the mitotic spindle, consequently generating disturbances in the separation and organization of chromosomes (Ribeiro et al., 2016).

In the results obtained, no significant difference in MI or CAI was observed between the treatment values with the sweeteners and the negative control. However, a slight increase in aberrant cells is observed with the administration of aspartame in 10 and 20 drops compared to the other sweeteners (Table 4). This finding is complemented by research by Anbara et al., (2021) in which rats subjected to high doses of this sweetener (40, 80, and 160 mg Kg^{-1}) showed hormonal dysregulation and damage to mRNA molecules; therefore, additional research is needed to determine the specific cytotoxic and genotoxic dose of this additive in eukaryotic media.

In their analysis, Das, Hazra, Sengupta, Hazra, and Chattopadhyay, (2021) evaluated - through different concentrations - the cytotoxicity and genotoxicity of saccharin in *Allium cepa* bioassays. The author obtained a considerable decrease in MI, especially in the treatment with 1% of saccharin in 12 hours of exposure, in addition to an increase in chromosomal aberrations and a visible reduction in the length of the roots of onions; however, the concentrations used were higher when compared to those in Table1, and it should be noted that the saccharin used was in its pure form; these factors may justify the results obtained by the author.

Inversely to other sweeteners, the stevioside treatment showed a subtle increase in MI, whereas few chromosomal aberrations occurred. In their study, Scaria, Kamath, and Chakraborty, (2017) analyzed the administration of stevioside (250 mg Kg^{-1}) in rats with induced diabetes, evaluating the following parameters: glucose, urea, creatinine, albumin, total protein, total cholesterol, triglycerides, high-density lipoproteins (HDL) and antioxidant enzymes such as superoxide dismutase, catalase, and lipid peroxidase. Their findings showed an improvement in the hyperglycemic condition, a decrease in triglycerides, urea, and total cholesterol, and stabilization of the oxidative status compared to the positive control group. Additionally, (Khare & Chandra, 2019) found that using purified stevioside (10µM) in breast tumor cells decreased the replication of these cells, which may explain the cytoprotective effect and the low induction of chromosomal mutations found in the current study.

Conclusion

The present study showed that the sweeteners Aspartame, Sodium Saccharin, Sodium Cyclamate, and Stevioside did not have relevant cytotoxic and genotoxic effects on *Allium cepa* cells at the concentrations and times tested. This result is positive for consumers of these products, given the practicality of these additives and the need to replace common sugar in individuals with dietary restrictions. However, further research is needed to clarify possible cellular changes, especially using bioassays closer to human cells.

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