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Evaluation of the mutagenic and antimuutagenic activity of *Chlorella vulgaris* in a test of *Allium cepa*

Avaliação da atividade mutagênica e antimutagênica de *Chlorella vulgaris* em um teste de *Allium cepa*

Evaluación de la actividad mutagénica y antimutágena de *Chlorella vulgaris* en una prueba de *Allium cepa*

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Abstract

The microalgae *Chlorella vulgaris* is usually commercialized as nutraceutical although it has potential application in the pharmaceutical and cosmetic industries. Therefore, our objective in this research is to evaluate the mutagenic and antimutagenic action of the aqueous extract of *C. vulgaris* through the *Allium cepa* assay. Three concentrations of the aqueous extract of *C. vulgaris* were tested: 0.075, 0.15 and 0.30mg/mL. In the mutagenicity analysis, *A. cepa* meristematic cells were cultured in the presence of the aqueous extract of *C. vulgaris* with distilled water as negative control and copper sulfate as positive control. For antimutagenicity, pre-treatment, simple simultaneous and post-treatment protocols were used. 400 cells/treatment were analyzed under optical microscopy (40x). Data were analyzed by ANOVA (one-way) and Tukey tests, considering $p<0.05$. The aqueous extract of *C. vulgaris* did not show

mutagenicity in any of the three concentrations evaluated. About the antimutagenicity protocols, the harm reduction percentages were 94.7%, 94.1% and 96.2% (pre-treatment); 88.9%, 93.2% and 91.08% (simultaneous simple); and 85.2%, 84.5% and 94.7% (post-treatment) referring to concentrations of 0.075, 0.15 and 0.30 mg/mL, respectively. According to these results, the microalgae *C. vulgaris* did not show mutagenic action at the tested doses and it reduced genetic damage caused by copper sulfate.
Keywords: Microalgae; Copper sulfate; Mutagenicity test; DNA damage.

Resumo
A microalga *Chlorella vulgaris* é geralmente comercializada como nutracêutico, embora tenha potencial aplicação nas indústrias farmacêutica e cosmética. Portanto, nosso objetivo nesta pesquisa é avaliar a ação mutagênica e antimutagênica do extrato aquoso de *C. vulgaris* através do ensaio de *Allium cepa*. Foram testadas três concentrações do extrato aquoso de *C. vulgaris*: 0,075, 0,15 e 0,30mg/mL. Na análise de mutagenicidade, células meristemáticas de *A. cepa* foram cultivadas na presença do extrato aquoso de *C. vulgaris* com água destilada como controle negativo e sulfato de cobre como controle positivo. Para a antimutagenicidade, foram utilizados os protocolos de pré-tratamento, simultâneo simples e pós-tratamento. 400 células/tratamento foram analisadas em microscopia óptica (40x). Os dados foram analisados pelos testes ANOVA (one-way) e Tukey, considerando $p<0,05$. O extrato aquoso de *C. vulgaris* não apresentou mutagenicidade em nenhuma das três concentrações avaliadas. Sobre os protocolos de antimutagenicidade, os percentuais de redução de danos foram de 94,7%, 94,1% e 96,2% (pré-tratamento); 88,9%, 93,2% e 91,08% (simples simultâneos); e 85,2%, 84,5% e 94,7% (pós-tratamento) referentes às concentrações de 0,075, 0,15 e 0,30 mg/mL, respectivamente. De acordo com esses resultados, a microalga *C. vulgaris* não apresentou ação mutagênica nas doses testadas e reduziu os danos genéticos causados pelo sulfato de cobre.
Palavras-chave: Microalgas; Sulfato de cobre; Teste de mutagenicidade; Dano de DNA.

Resumen
La microalga *Chlorella vulgaris* se comercializa habitualmente como nutracéutico, aunque tiene una aplicación potencial en las industrias farmacéutica y cosmética. Por tanto, nuestro objetivo en esta investigación es evaluar la acción mutagénica y antimutagénica del extracto acuoso de *C. vulgaris* mediante el ensayo *Allium cepa*. Se probaron tres concentraciones del extracto acuoso de *C. vulgaris*: 0.075, 0.15 y 0.30 mg / mL. En el análisis de mutagenicidad, se cultivaron células meristemáticas de *A. cepa* en presencia del extracto acuoso de *C. vulgaris* con agua destilada como control negativo y sulfato de cobre como control positivo. Para la antimutagenicidad, se utilizaron protocolos de pretratamiento, simultáneos y postratamiento simples. Se analizaron 400 células / tratamiento bajo microscopía óptica (40x). Los datos fueron analizados por ANOVA (unidireccional) y pruebas de Tukey, considerando $p <0.05$. El extracto acuoso de Chlorella vulgaris no mostró mutagenicidad en ninguna de las tres concentraciones evaluadas. En cuanto a los protocolos de antimutagenicidad, los porcentajes de reducción de daños fueron 94,7%, 94,1% y 96,2% (pretratamiento); 88,9%, 93,2% y 91,08% (simultaneo simple); y 85,2%, 84,5% y 94,7% (postratamiento) referidos a concentraciones de 0,075, 0,15 y 0,30 mg / mL, respectivamente. Según estos resultados, la microalga Chlorella vulgaris no mostró acción mutagénica a las dosis probadas y redujo el daño genético causado por el sulfato de cobre.
Palabras clave: Microalgas; Sulfato de cobre; Prueba de mutagenicidad; Daño al ADN.

1. Introduction

The use of microalgae for medicinal and food purposes by eastern peoples is millenary, being part of their diet since antiquity, but in the west the consumption of this type of food still occurs on smaller scale. The pursue for a healthier life and, consequently, a better quality of life is a goal for many people, who see algae as an alternative to achieve this goal due to its high nutritional value and its anti-cancer, anti-diabetes, anti-inflammatory and antioxidants properties (Lauritano et al., 2016; Oliveira et al., 2021), which leads to demand and acceptance by consumers of this type of food (Koyande et al. 2019; Boukid & Castellari, 2021; Oliveira et al., 2021).

Among the most researched microalgae is *Chlorella vulgaris*, a unicellular green microalga of the Oocystacae family, rich in bioactives that reinforce the immunity of human beings, reducing the stress hormone cortisol (Aggarwal, 2020). Dantas et al. (2021), using extracts from the biomass of *C. vulgaris*, developed an alcoholic beverage whose in vivo tests showed that it played an important protective role in the physiology of brain cells. Another study developed by Saccomori et al. (2021) showed that microalgae produce biostimulants that act on the physiological processes of plants through phytohormones,

vitamins, oligosaccharides, among other molecules contained in the extracts, inducing growth, productivity, and aiding in the absorption of nutrients.

The phytochemical analysis of *Chlorella* performed by Soares (2021) found the presence of flavonoids, tannins, phenolic compounds, terpenes and saponins, mineral salts, vitamins, proteins, lipids and carbohydrates, in addition to bioactive compounds with great potential to be used against diseases such as hypertension, diabetes, among others (Cavalcanti et al., 2021). *C. vulgaris* is consumed in the form of powder, tablets, capsules or extracts, as a food supplement for humans and animals (Dantas et al., 2021). Its biological activities have been reported with the induction of cytokines IL-1 (interleukin-1), TNF-α (tumor necrosis factor alpha) and INF-γ (interferon gamma), antitumor activity, antioxidant activity, collagen synthesis in the skin and tissue regeneration, which contribute to the delay of aging (Bezerra et al., 2021).

According to Mesadri et al. (2021), compounds extracted from the biomass of *C. vulgaris* showed activity against Gram positive, Gram negative and antifungal bacteria. In an in vivo assay, the consumption of *C. vulgaris* regulated the immunohematopoietic system, increasing or restoring the host's own defenses, becoming able to inhibit malignant and infectious processes. The algaeal so showed to be effective in restoring myelosuppression through the normalization of the reduced number of hematopoietic precursors such as granulocytes and macrophages in the bone marrow (Morcelli, 2021).

The method of evaluating chromosomal alterations in *Allium cepa* L. roots is validated by the International Chemical Safety Program and the United Nations Environmental Program (Bispo et al., 2021), and this system allows an assessment of chromosomal damage, interference in the plant cell cycle in the DNA and the determination of toxicity through the observation of mutagenic, genotoxic and cytotoxic effects on the roots (Soares et al., 2021).

The *A. cepa* test system is frequently used due to its low cost, its ease to perform its recognized reliability (Dos Santos et al. 2021), and the fact that it is one of the ways to ensure the safety of food products and medicines, and it is able to demonstrate possible damage to the cell cycle due to the presence of harmful substances. The ability of a compound to affect the cell cycle provides useful information about its cytotoxic mechanisms of action (Ferreira et al., 2021).

The *A. cepa* test is capable of monitoring and identifying potential risks or benefits, as mutagenic effects can occur and be analyzed through chromosomal alterations (Agertt et al., 2021). Therefore, the objective of this work was to evaluate the mutagenic and antimutagenic activity of the aqueous extract of *C. vulgaris* at different concentrations using the *A. cepa* test.

2. Methodology

The study was carried out at the Laboratory of Histological and Embryological Techniques – LTHE of the Institute of Biological Sciences, University of Pernambuco – ICB/UPE.

The photosynthetic microorganism (MF) of *Chlorella vulgaris* (Utex, 1803) was obtained from the Laboratory of Bioactive Technology – LABTECBIO of the Federal Rural University of Pernambuco – UFRPE.

2.1 Cultivation of the photosynthetic microorganism and obtaining extracts

C. vulgaris was cultivated in a standardized culture medium, Bold's Basal Medium (Bischoff; Bold 1963). The cultivation began at a concentration of 50 mg/L, and when the microorganisms reached the exponential growth phase, which lasts 15 days, they were centrifuged at 10000 rpm, 4ºC for 10 minutes. The cells were lyophilized and then used in the preparation of extracts according to the methodology proposed by Chu et al. (2006), which consists of using 0.2M Tris-HCl buffer, pH 7.2 and shaking for 9 hours. After shaking, the extracts were centrifuged, this time at 8000 rpm, 4ºC and 10 minutes and then used in the following steps.

2.2 *Allium cepa* system

Onion seeds (*Allium cepa*) of the Baia Periforme variety, lot N. 0003801635037010 (Feltrin®) were used as vegetal biological material to evaluate the effects induced by copper sulfate (SC), the positive control, and by the aqueous extract of *C. vulgaris*. A total of 40 seeds per group were planted in Petri dishes, and to ensure that the concentration remained constant throughout the test, the dishes were wrapped with PVC film to prevent volatilization. Analyzes were performed in triplicate.

2.3 Assessment of mutagenic activity

A. cepa seeds were cultivated according to the protocols established by Anciã and Romão (2016), the negative control (NC) group's seeds were grown in 3 mL of distilled water for 120 hours and the Positive Control (CP) group's seeds were cultivated in 3 mL of distilled water for 72 hours, then the seeds were transferred to another Petri dish and soaked in a copper sulfate solution at the concentration of 0.006 mg/L for 48 hours.

The seeds were cultivated in 3 mL of distilled water for 72 hours and then transferred to another Petri dish and soaked in an aqueous extract of *C. vulgaris* at the concentrations of T1 (0.075 mg/mL), T2 (0.15 mg/mL) and T3 (0.30 mg/mL) for 48 hours.

2.4 Evaluation of the antimutagenic activity of *C. vulgaris*

The seeds of *A. cepa* were cultivated according to the protocols established by Anciã and Romão (2016), with adaptations:

Pre-treatment

The seeds were cultivated in 3 mL of distilled water for 24 hours and then submitted to the aqueous extract of *C. vulgaris* at the concentration of T4 (0.075 mg/mL), T5 (0.15 mg/mL) and T6 (0.30 mg/mL) for 48 hours. Subsequently, they were washed twice in distilled water, transferred to another plate, and soaked in an aqueous solution of SC at the concentration of 0.006 mg/mL for 48 hours.

Simple simultaneous

The seeds were cultivated in 3 mL of distilled water for 24 hours, then subjected to an aqueous extract of *C. vulgaris* (1.5 mL) at the concentrations of T7 (0.075 mg/mL), T8 (0.15 mg/mL) and T9 (0.30 mg/mL), associated with a copper sulfate solution (1.5 mL) at the concentration of 0.006 mg/mL, for 96 hours.

Post-treatment

The seeds were cultivated in 3 mL of distilled water for 24 hours, then subjected to an aqueous solution of SC at the concentration of 0.006 mg/mL for 48 hours, then they were washed twice in distilled water and transferred to another plate, soaked in 3 mL of aqueous extract of *C. vulgaris* at the concentrations of T10 (0.075 mg/mL), T11 (0.15 mg/mL) and T12 (0.30 mg/mL) for 48 hours.

After 5 days (120 h) of cultivation, the root tips (meristems) were removed and fixed in modified Carnoy (3:1, ethanol: acetic acid) for 24 hours, and after this period, they were transferred to tubes containing alcohol at the concentration of 70% and kept refrigerated until use. The roots were hydrolyzed in 1N HCl for 6 minutes at 60°C and then submitted to Giemsa staining (Merck®) for microscopic analysis. The slides were subjected to dry ice to aid the removal of the coverslip and then

the material was kept at room temperature to dry for 24h. Then, a new cover slip was glued onto the biological material with the aid of synthetic resin Entelan (Merck®) and again, there was a 24 hour wait to begin the analyses.

In each Petri dish, 40 seeds were placed to germinate, totalizing 14 dishes (1 dish/group). To ensure that the concentration remained constant throughout the test, the plates were wrapped with PVC film to prevent volatilization. To determine the number of cells in mitosis as well as the mitotic index, 20,000 cells/treatment were analyzed. In the analysis of chromosomal aberrations and damage reduction, 2,000 cells were evaluated. In all cases 400 cells/slide were analyzed under a CX31 Leica® optical microscope at the amplification rates of 40x and 100x.

According to Macedo et al. (2018), the mitotic index (MI) and the total frequency of aberrations (FTA) were both used to evaluate mutagenic and antimutagenic activity, through the relationships:

I.M (Mitotic Index)

$$\frac{\text{Number of dividing cells (prophase, metaphase, anaphase, telophase)}}{\text{Total cells observed}} \times 100$$

FTA (Frequency of Aberrations)

$$\frac{\text{Total aberrations}}{\text{Total cells}}$$

The harm reduction rate was used to assess antimutagenic activity, as follows:

%RD (Harm Reduction Percentage)

$$\frac{\text{Positive Control Mean - Associated Group Mean}}{\text{Positive Control Mean - Negative Control Mean}} \times 100$$

2.5 Statistical analysis

The values in the tables were expressed as mean ± standard error of the mean (e.p.m.). The differences between the groups were determined through Analysis of Variance (ANOVA – oneway), followed, when differences were detected, by the Tukey test, with a significance level of $p<0.05$. Statistical evaluations were performed using the Graphpad Prism® v5.01 program.

3. Results and Discussion

Cytotoxicity levels were assessed by the decrease in the mitotic index (MI) of cells subjected to exposure, evidenced in the positive control group, against copper sulfate (Messias et al, 2021; Meneguetti et al., 2014). Concentrations of 0.075, 0.15 and 0.30 mg/mL of the aqueous extract of *C. vulgaris* kept the DNA of the cells intact, similar to that observed in the negative control group, but diverged significantly in all mitotic phases when compared to the positive control group, indicating that the extract has no cytotoxic action.

According to Da Silva et al. (2015), the reduction in the mitotic index occurs due to the action of the chemical agent that can inhibit DNA synthesis, reducing the mitosis process, as observed in the positive control group (Table 1). The mitotic index is essential to assess cellular toxicity of certain chemical compounds, as it allows checking possible alterations through the increase or decrease of cell division rates.

According to Echeveste et al. (2017), stress caused by copper causes increases in cell volume, shifts in morphology, reduction in cell division rates, reduction of surface-volume ratio, and in the metal absorption rate, consequently there is also a reduction in its toxicity. Table 1 and 2 present the data related to the mutagenicity tests carried out with the aqueous extract of

C. vulgaris. Table 1 shows the number of cells in different stages of mitosis, according to the treatments performed. One may notice that the treatments performed with *C. vulgaris* did not cause significant chromosomal alterations when compared to the negative control group, but it is significantly different when compared to the positive control group. The mutagenicity observed in the positive control groups was confirmed by the increase in the number of chromosomal aberrations promoted by the deleterious action of copper sulfate.

Table 1. Number of cells in mitosis and mitotic index according to the applied treatment (*Chlorella vulgaris* e Copper sulphate) on the *Allium cepa* test system.

Treatment	**Mitotic Phase**					**IM±DP**
	Interphase	**Prophase**	**Metaphas**	**Anaphase**	**Telophase**	
CN	19554^{a}	206^{f}	89^{k}	83^{p}	68^{u}	2,23 ± 1,42
CP	$19794^{a,b,c,d,e}$	$96^{f,g,h,i,j}$	$42^{k,l,m,n,o}$	$43^{p,q,r,s,t}$	$25^{u,v,w,x,y}$	1,03± 0,45
T1	19566^{c}	205^{h}	96^{m}	79^{r}	62^{w}	2,21± 1,37
T2	19578^{d}	207^{i}	80^{n}	72^{s}	51^{x}	2,05 ± 0,91
T3	19564^{e}	215^{j}	88^{o}	78^{t}	55^{y}	2,18 ± 0,96

CN= Negative Control (Distilled H_2O). CP= Positive Control (Copper Sulfate - 0.006 mg/mL). T1= Aqueous extract of *C. vulgaris* (0.075 mg/ml). T2= Aqueous extract of *C. vulgaris* (0.15 mg/ml). T3= Aqueous extract of *C. vulgaris* (0.30 mg/ml). MI=Mitotic index+ Standard deviation of the mitotic index. Equal letters in the same column show a significant difference. *Significant value ($p<0.0005$). Source: Authors.

Chromosomal alterations evidenced in the *A. cepa* system are presented in Table 2. It is possible to observe the frequency of chromosomal aberrations produced in comparison to the damage caused by the different treatments performed and compared with the positive and negative control group. The micronucleus was the most frequent aberration, followed by sprout, bridge, loss and delay. It is possible to conclude that the number of aberrations in the different treatments performed did not differ significantly from the negative control group but was significantly different from the positive control group.

One way to assess the mutagenicity of substances is by observing the frequency of micronuclei (MN), chromosomal masses similar to the main nucleus and expressed in daughter cells as a consequence of damage to parental cells that were not repaired or that were repaired amiss (Costa et al. 2014; Figueira, 2017).

Regarding mitotic phase data, these are not significant in terms of cell division, with the largest number of cells in interphase. Fachinetto et al. (2007) claim that high concentrations of some compounds can stimulate or constrain the cell cycle. The mutagenicity verified in the positive control groups was confirmed by the increase in the number of chromosomal aberrations promoted by the deleterious action of copper sulfate. Marques et al. (2018) state that soils with high Cu content cause biochemical stress in plants, decreases in photosynthetic rate and respiration, thus causing the shortening of roots and upper part, in addition to decreasing root surface area and biomass.

Qin, Rong et al. (2015) tested copper-induced root growth by the *A. cepa* assay, showing that microtubules were the target sites for copper toxicity in root tip meristematic cells, and exposure to copper substantially impaired microtubule arrangements, increased DNA damage and suppressed cell cycle progression, as shown in Table 2.

Table 2. Chromosomal aberrations formed, every 2000 cells, analyzed as a function of the treatment (*Chlorella vulgaris* and copper sulfate) applied in the *Allium cepa* system.

Treatment	Chromosomal Aberrations (AC)							
	MNU	BRO	PON	QUE	PER	ATR	FTA	M±DP
C N	3^{a}	2^{f}	1^{k}	2	1^{o}	0	9	1,3± 1,05
C P	$71^{a,b,c,d,e}$	$21^{f,g,h,i,j}$	$11^{k,l,m,n}$	9	$8^{o,p,q}$	3	123	12,3± 3,65
T1	6^{c}	3^{h}	1^{m}	2	1^{p}	2	15	1,5 ± 1,27
T2	4^{d}	6^{i}	2^{n}	2	1^{q}	2	16	1,6 ± 0,51
T3	7^{e}	4^{j}	0	1	0	0	12	1,2 ± 1,03

Caption: Negative Control (Distilled H_2O). CP= Positive Control (Copper Sulfate - 0.006 mg/mL). T1= Aqueous extract of *C. vulgaris* (0.075 mg/ml). T2= Aqueous extract of *C. vulgaris* (0.15 mg/ml). T3= Aqueous extract of *C. vulgaris* (0.30 mg/ml). MNU - Micronucleus; BRO – Sprout; PON - Bridge; ATR – Delay; PER – Loss; THAT - Break; FTA - Total Frequency of Chromosome Changes; M±SD - Mean ± Standard deviation of the number of chromosomal alterations. Equal letters in the same column show a significant difference. *Significant value ($p<0.0005$). Source: Authors.

Table 3 and 4 present the data related to the antimutagenicity tests carried out with aqueous extracts obtained from the biomass of *C. vulgaris* on the deleterious action of copper sulphate. Antimutagenic agents presented on table 3are able to neutralize the harmful effects on DNA. These agents include natural and synthetic compounds. Based on their mechanism of action among antimutagens, several classes of compounds can be distinguished (Sloczyńska et al., 2014).

Table 3 shows that, regardless of the type of treatment used (pre-treatment, simple simultaneous or post-treatment), the chromosomal aberrations evidenced did not diverge from those found in the negative control group, nor did they diverge between the three groups tested, but it was significantly different from the positive control group. According to Chen et al. (2019) and Safaei et al. (2019), seaweed extracts have antioxidant and antimutagenic/anticarcinogenic activities due to β-carotene, lutein and chlorophyll-related derivatives isolated from this species, in addition to vanillic, fumaric, caffeic, protocatechuic and caftaric acid and phytol. The results of our study suggest that the compounds extracted from the biomass of *C. vulgaris* play a protective role for the DNA against the action of the aggressor agent. This is perceived due to the main damage occurred in the *A. cepa* system (micronuclei and sprouts). Islam et al. (2017) explain that phytol has antimicrobial, antiviral, antitumor, non-mutagenic, antiteratogenic, anti-inflammatory, antidiabetic properties, in addition to antispasmodic, anticonvulsant, antiallergic, anxiolytic, antidepressant, antinociceptive, antiallergic and immunoadjuvant activities.

Damages such as bridging were significantly different at the three concentrations tested for the pre-treatment group, whereasin the simultaneous single and post-treatment groups the results were different at the concentrations of 0.15 mg/mL and 0.30 mg/mL, respectively.

The seeds' cells submitted to pre-treatment suffered less damage to their DNA, demonstrating the protective action of *C. vulgaris*. According to Kolumbayeva et al. (2014), the microalgae extract is able to block the free radical production process caused by oxidative stress, reducing the probability of damage to the genome by the activation of the cell repair system.

According to Mesadri, Wagner and Fagundes (2021), microalgae have several enzymes and antioxidant substances that protect them against potentially harmful effects. These enzymes increase their activities when algae are exposed to stressful conditions. Rossato, Oliveira and Sagrillo (2021), in their study with decontamination of environments through bioremediation, the removal of heavy metals in 58%, including copper, demonstrating its potential for bioaccumulation of metals in its cell wall. Sayadi, Rashki and Shahri (2019) used modified *C. vulgaris* to function as a bioabsorbent in the heavy metals adsorption process, the process was endothermic, spontaneous and physicochemical and presented high adsorption efficiency.

According to Okuyama et al. (2018), the *Chlorella vulgaris* growth factor (Chlorella Growth Factor - CGF) is able to modulate the repair system, enhancing its role as chemoprotection through bioantimutagenesis. According to Zhao et al. (2014)

and Dextro (2019), when *C. vulgaris* is affected by stress caused by an aggressor agent, it releases secondary products such as chlorelina, a toxin with protective action and which inhibits the growth of some species of microorganisms.

Figure 3. Evaluation of chromosomal aberrations formed every 2000 cells analyzed according to the treatment adopted (Pre-treatment, simple simultaneous and post-treatment) applied in the *Allium cepa* system.

Treatment	Aberration						FTA	M±DP
	MNU	BRO	PON	QUE	PER	ATR		
C N	5^{a}	3^{l}	2^{w}	2	1	0	13	1,3 ± 1,05
C P	$71^{a,b,c,d,e,f,g,h,i,j,k}$	$21^{l,m,n,op,q,r,s,t,u,v}$	$11^{w,x,y,z}$	9	8	3	123	12,3 ± 3,65
T4	6^{c}	3^{n}	2^{y}	3	2	2	18	1,8 ±1,13
T5	6^{d}	4^{o}	3^{z}	4	2	0	19	2,0± 1,2
T6	5^{e}	3^{p}	3^{aa}	3	1	1	16	1,6 ± 0,84
T7	9^{f}	6^{q}	4	3	3	1	26	2,6 ± 1,26
T8	8^{g}	3^{r}	1^{bb}	4	3	1	20	2,0 ± 1,15
T9	7^{h}	6^{s}	4	3	1	1	22	2,2± 1,31
T10	12^{i}	7^{t}	4	4	3	1	31	3,1 ± 1,28
T11	13^{j}	9^{u}	5	3	2	0	32	3,2 ± 1,03
T12	9^{k}	6^{v}	2^{cc}	1	0	0	18	1,8 ± 0,63

Caption: Negative Control (Distilled H_2O). CP= Positive Control (Copper Sulfate - 0.006 mg/mL). T4= Pre-treatment (Aqueous extract of *C. vulgaris* - 0.075 mg/ml). T5= Pre-treatment (Aqueous extract of *C. vulgaris* - 0.15 mg/ml). T6= Pre-treatment (Aqueous extract of C. vulgaris - 0.30 mg/ml). T7= Simple simultaneous (Aqueous extract of *C. vulgaris* - 0.075 mg/ml). T8= Simple simultaneous (Aqueous extract of *C. vulgaris* - 0.15 mg/mL). T9= Simple simultaneous (Aqueous extract of *C. vulgaris* - 0.30 mg/mL). T10= Post-treatments (Aqueous extract of *C. vulgaris* - 0.075 mg/ml). T11= Post-treatments (Aqueous extract of *C. vulgaris* - 0.15 mg/ml). T12= Post-treatments (Aqueous extract of *C. vulgaris* - 0.30 mg/ml). MNU - Micronucleus; BRO – Sprout; PON - Bridge; ATR – Delay; PER – Loss; THAT - Break; FTA - Total Frequency of Chromosome Changes; M±SD= Mean ± Standard deviation of the mean of chromosomal alterations. *Significant value ($p<0.0001$). Equal letters in the same column show a significant difference. Source: Authors.

Table 4 highlights the reduction in the damage caused to the nuclei of *A. cepa* cells over the protective action of *C. vulgaris*. In this study, the concentration of copper sulfate removed by *C. vulgaris* was not determined, however, it is possible to verify that all tested protocols were able to reduce the damage caused by the aggressor agent by about 84%. Echeveste et al. (2017) report that high copper concentrations can cause changes in the physiology of some microalgal species. Copper is a compound commonly used by industries and is frequently utilized in the fields of information technology, civil construction and agricultural inputs, among others; however, it presents a risk to the environment as it is gradually capable of causing physiological changes, threatening the balance in aquatic ecosystems (Langston, 1990).

The pre-treatment, among all the protocols used, presented the best indexes, being the treatment with 0.30 mg/mL the best among all those used to prevent damage to the DNA, indicating that *C. vulgaris* has compounds that contribute to the protection against damage caused by copper sulphate. Osuna-Ruiz et al. (2016) explain that the antioxidant and antimutagenic activities are related by the content of flavonoids and chlorophylls and, to a lesser extent, by the phenolic compounds and the content of carotenoids evaluated in the green marine microalgae species.

Dextro et al. (2019) observed that copper is capable of affecting the morphology of chromosomes and the cell division cycle. Additionally Pinto et al. (2003) demonstrated that this metal causes oxidative damage in cells, increasing the production of reactive oxygen species. According to Magalhães (2014), among all microalgae species, the most sensitive to copper is *Chlorella vulgaris*.

The simple simultaneous treatment and the post-treatment presented values below those obtained by the pre-treatment, except for the T12 treatment (0.30 mg/mL), which presented a reduction rate similar to those of the pre-treatment with 0.075 mg/mL, suggesting that high doses of the aqueous extract of *C. vulgaris* can recover damage caused after exposure to copper sulfate in a similar way to those presented by pre-treatment. Okuyama et al. (2018) used the *C. vulgaris* growth factor to

reduce cell damage by methyl methanesulfonate in the post-treatment protocol, and found damage reduction ranging from 108.24 to 100.13% at the concentrations of 0.075, 0.15, 0.30 mg/ml.

Figure 4. Evaluation of the total damage frequency, average damage, cell damage reduction rate and mitotic index, evaluated every 2000 cells according to the treatment adopted (Pre-treatment, simple simultaneous and post-treatment) applied in the system *Allium* strain.

Treatment		**FTD**	**M±DP**	**%RD**	**IM±DP**
C Negative		9	0,93 ± 0,73	----	2,23 ± 1,42
C Positive		123	12,3 + 3,65	----	1,08 ± 0,45
Pre-treatment	T4	15	1,5 ± 1,26	94,74%	2,21 ± 1,37
	T5	16	1,6 ± 0,56	94,01%	2,05 ± 0,91
	T6	12	1,2 ± 1,03	96,20%	2,18 ± 0,96
Simple simultaneous	T7	18	1,8 ± 1,37	88,90%	2,53 ± 0,75
	T8	19	1,6 ± 1,15	93,27%	2,65 ± 0,57
	T9	16	1,6 ± 0,84	91,08%	2,55 ± 0,85
After treatment	T10	31	3,1 ± 1,28	85,23%	2,30 ± 0,69
	T11	32	1,3 ± 1,31	84,50%	2,48 ± 0,98
	T12	18	1,8 ± 0,63	94,74%	2,68 ± 1,20

Caption: Negative Control (H_2O). CP= Positive Control (Copper Sulfate – 0.006 mg/mL). T4= Pre-treatment (Aqueous extract of *C. vulgaris* – 0.075 mg/ml). T5= Pre-treatment (Aqueous extract of *C. vulgaris* – 0.15 mg/ml). T6= Pre-treatment (Aqueous extract of C. vulgaris – 0.30 mg/ml). T7= Simple simultaneous (Aqueous extract of C. vulgaris – 0.075 mg/ml). T8= Simple simultaneous (Aqueous extract of *C. vulgaris* – 0.15 mg/mL). T9= Simple simultaneous (Aqueous extract of *C. vulgaris* – 0.30 mg/mL). T10= Post-treatments (Aqueous extract of *C. vulgaris* – 0.075 mg/ml). T11= Post-treatments (Aqueous extract of C. vulgaris – 0.15 mg/ml). T12= Post-treatments (Aqueous extract of *C. vulgaris* – 0.30 mg/ml). FTD = Total damage frequency; M±SD – Mean ± Standard deviation of the mean number of damages; %RD= Damage Reduction Rate; MI= Mitotic index ± Standard deviation of the mitotic index. Source: Authors.

4. Conclusion

It was observed that the *Chlorella vulgaris* extract did not present mutagenicity action regardless of the treatment used and that the chromosomal aberrations evidenced did not differ from the negative control group and between the tested groups but were different from the positive control group.

When exposed to the aggressor agent, seeds subjected to pre-treatment with *C. vulgaris* suffered the least damage and when exposed to high doses of the extract in the post-treatment they were also able to reduce DNA damage in a similar way to the pre-treatment.

C. vulgaris plays a protective role on DNA against the action of the aggressor agent, and all tested protocols had high damage reduction rates, though the pre-treatment, among all the protocols, obtained the best indexes. Additional studies are required in order to elucidate the protection mechanisms used by microalgae.

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