Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

The ethanolic extract of *Gomphrena celosioides* is not carcinogenic and has antigenotoxic effects and chemopreventive Properties

O extrato etanólico de *Gomphrena celosioides* não é carcinogênico e tem efeito antigenotóxico e propriedades quimiopreventivas

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Abstract

Gomphrena celosioides, popularly known as perpétua, perpétua brava, bachelor´s button and prostate globe amarahth, is used for the treatment of urinary tract disorders, kidney stones, for skin diseases, infectious diseases, gastrointestinal and respiratory conditions. Rich in phenolic acids and flavonoids, this plant has therefore a potential for use in cancer prevention. Given the above, the present research aimed to evaluate the carcinogenic effect of the ethanolic extract of *G. celosioides* (EEGc) in an alternative model of *Drosophila melanogaster* and the genotoxic and antigenotoxic effects in Swiss mice. The larval survival test and the detection of epithelial tumor clones were performed in *D. melanogaster*. The tested EEGc concentrations were 0.96, 1.92, 3.85 and 7.70 mg/mL. In Swiss mice, the genotoxicity and antigenotoxicity of doses of 100, 1,000 and 2,000 mg/Kg were evaluated. The results showed that EEGc at a concentration of 7.70 mg/mL reduced ($p<0.05$) larval survival. However, EEGc was not carcinogenic, and the lowest concentration (0.96 mg/mL) prevented ($p<0.05$) the basal occurrence of epithelial tumors. In mice, EEGc at the highest dose (2,000mg/Kg) increased the frequency of genomic lesions ($p<0.05$). Yet, none of the doses caused chromosomal lesions ($p>0.05$). When associated with cyclophosphamide, EEGc was antigenotoxic ($p<0.05$). The percentages of reduction of genomic damage ranged from 33.39 to 63.23% and of chromosomal damage from 20.00 to 77.19%. In view of the above, it is suggested that EEGc is not carcinogenic, has an antigenotoxic effect and chemopreventive properties.

Keywords: medicinal plant, Amaranthaceae, comet, micronucleus, splenic phagocytosis.

Resumo

Gomphrena celosioides, conhecida popularmente por perpétua, perpétua brava, bachelor´s button e prostate globe amarahth, é utilizada na medicina popular para o tratamento de distúrbios do trato urinário e pedra nos rins e para doenças de pele, infecciosas, gastrointestinais e respiratórias. Essa planta é rica em ácidos fenólicos e flavonóides e, portanto, tem potencial para uso na prevenção de câncer. Frente ao exposto, a presente pesquisa teve por objetivo avaliar o efeito carcinogênico do extrato etanólico de *G. celosioides* (EEGc) em modelo alternativo de *Drosophila melanogaster* e os efeitos genotóxicos e antigenotóxicos em camundongos Swiss. Em *D. melanogaster* foi realizado o teste de sobrevivência larval e para a detecção de clones de tumores epiteliais. As concentrações testadas do EEGc foram de 0,96, 1,92, 3,85 e 7,70 mg/mL. Em camundongos Swiss avaliou-se a genotoxicidade e antigenotoxicidade das doses de 100, 1.000 e 2.000 mg/Kg. Os resultados demonstraram que o EEGc na concentração de 7,70 mg/mL reduziu ($p<0,05$) a sobrevivência larval. No entanto, o EEGc não é carcinogênico e a menor concentração (0,96 mg/mL) preveniu ($p<0,05$) a ocorrência basal de tumores epiteliais. Nos camundongos o EEGc na maior dose (2.000mg/Kg) aumentou a frequência de lesões genômicas ($p<0,05$). Porém, nenhuma das doses causou lesões cromossômicas ($p>0,05$). Quando associado à ciclofosfamida o EEGc foi antigenotóxico ($p<0,05$). As porcentagens de redução de danos genômicos variaram de 33,39 a 63,23% e de danos cromossômicos de 20,00 a 77,19%. Frente ao exposto, sugere-se que o EEGc não é carcinogênico, tem efeito antigenotóxico e propriedade quimiopreventiva.

Palavras-chave: planta medicinal, Amaranthaceae, cometa, micronúcleo, fagocitose esplênica.

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Received: February 13, 2024 – Accepted: July 8, 2024

1. Introduction

Gomphrena celosioides, in Brazil popularly known as "perpétua" or "perpétua brava" and in other countries as "bachelor's button" and "prostate globe amarahth" (Myers et al., 2000; Fank-DeCarvalho and Graciano-Ribeiro, 2005; Vasconcelos et al., 2018), is a weed little consumed by wild animals (Fank-DeCarvalho and Graciano-Ribeiro, 2005). However, it is widely used in popular medicine for the treatment of urinary tract disorders and kidney stones (Prachi et al., 2009; Sharma and Vijayvergia, 2011; Vasconcelos et al., 2017a; Vasconcelos et al., 2018), in infectious skin diseases (Marcorini et al., 2020), gastrointestinal and respiratory illnesses (Vasconcelos et al., 2017a).

In spite of being little consumed by wild animals (Fank-DeCarvalho and Graciano-Ribeiro, 2005), its use is described as safe. According to Souleymane et al. (2014), the median lethal dose (LD50), in acute toxicity, for aqueous extract of *G. celosioides* is greater than 4000mg/Kg. For the ethanolic extract, it is higher than 2,000mg/kg (Macorini et al., 2022). In Addition, our group demonstrated that the ethanolic extract is also safe when administered to pregnant females and, therefore, did not cause genetic damage and did not alter reproductive performance and embryo-fetal development (Salustriano et al., 2022).

This use safety, which includes the absence of genotoxic damage, is a reliable indication to evaluate chemopreventive or antigenotoxic effects for *G. celosioides*. This fact is further corroborated by the constituents present in the ethanolic extract, which are phenolic acids and flavonoids (Vasconcelos et al., 2017a). Chemical compounds classes that can act as antioxidants, being antioxidants important as chemoprotectors (Kong and Lillehei, 1998; Santos; Cruz, 2001).

Given the above, the present research aimed to evaluate the carcinogenic effect of the ethanolic extract of *G. celosioides* (EEGc) in an alternative model of *Drosophila melanogaster* and the genotoxic and antigenotoxic effects in Swiss mice.

2. Materials and Methods

2.1. Botanical material and extract preparation

The ethanolic extract of *G. celosioides* was produced from aerial parts of the plant according to the method described in previous publications by our study group (Vasconcelos et al., 2017a; Salustriano et al., 2022). The EEGc evaluated in this study was also used by Vasconcelos et al. (2017a) and Salustriano et al. (2022) and the characteristics of the extract were, therefore, previously described in the literature.

Nevertheless, the extracts have been tested to confirm the same composition over the years (Vasconcelos et al., 2017a). Plant samples were collected in April 2014 in Paranaíba, Mato Grosso do Sul, Brazil [lat: −19.666667 long: −51.183333 WGS84], analyzed and cataloged by professor Dr. Josafá Carlos de Siqueira, at the Pontifícia Universidade Católica do Rio de Janeiro (PUC-RJ).

A voucher specimen was archived in the Herbarium of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo (FFCLRP/USP) under number SPFR-2962. The species was registered in the National Registry System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under the codes AF99A04 and ASEC80E.

2.2. Chemical agents

EEGc was solubilized in Tween 80 (1%) and subsequently diluted with distilled water. The concentrations used in the test for the detection of epithelial tumor clones by Epithelial Tumor Test (ETT) in *D. melanogaster* were 0.96, 1.92, 3.85 and 7.70 mg/mL. These concentrations were defined in a pilot experiment (data not shown).

The doses used in the study with mice were 100, 1,000 and 2,000mg/kg of body weight (b.w.) by gavage (orally – p.o.). According to Vasconcelos et al. (2017a), the dose of 100 mg/kg has a diuretic effect and that is the reason it was chosen as an effective and therapeutic dose. The other two doses were based on the study by Salustriano et al. (2022) and according to this study, the dose of 1,000mg/Kg is a safe dose (10x higher) than the effective/therapeutic dose (Brasil, 2020; OECD, 2015) and the dose of 2,000mg/Kg was established as the limiting dose according to the acute oral toxicity guidelines (OECD, 2008).

Doxorubicin (Rubidox, Bergamo, Brazil), diluted in distilled water, was used as a positive control, at a concentration of 2.17mg/mL (0.4mM) (Vasconcelos et al., 2017b), for the clone detection test of ETT in *D. melanogaster*.

Cyclophosphamide (Fosfaseron, Ítaca Laboratorios, REG. MS No. 1.2603.0056.002−1; Lot 063020, Brazil), diluted in distilled water, was used as a positive control, at a dose of 100 mg/kg b.w. intraperitoneally (i.p.), for tests with Swiss mice (Oliveira et al., 2023; Correa et al., 2024).

2.3. Experiment I

2.3.1. Survival test and detection of epithelial tumor clones in D. melanogaster

The epithelial tumors were evaluated in an alternative model of *D. melanogaster* (fly model).

The mutant lines of *D. melanogasters: Multiple Wing Hair* (mwh/mhw) and wts/TM3 were grown alone (Figure 1). Then, males of the mwh/mhw lineage and virgin females of the wts/TM3 lineage were selected and crossed (Figure 2; Figure 3).

Laying took place in a suitable laying medium (bottles containing a solid agar base (3% agar in water) and a layer of fresh biological yeast and sugar). After 72 ± 4 hours, the flasks were washed with reverse osmosis water and marked trans-heterozygous (wts+/+mwh) and balanced heterozygous (TM3, Sb1 +/+ mwh) larvae were obtained using a sieve.

To assess toxicity, the hatch test was performed (Rand, 2014). To this end, 50 larvae were transferred to glass bottles containing 1.5 g of instant mashed potato medium (brand Yoki Alimentos S. A., São Paulo, Brazil), as an alternative culture medium for *Drosophila* (Spano et al., 2001), moistened with 5 mL of different EEGc concentrations (0.96, 1.92, 3.85 and 7.70 mg/mL).

Tween 80 (1%) was used for the negative control and Doxorubicin (2.17mg/mL) for the positive control (Vasconcelos et al., 2017b). At the end of the collection,

the numbers of live and dead larvae were evaluated and described as a percentage. For the dose to be used in the next test, at least 70% of the larvae needed to be alive (Vasconcelos et al., 2017b).

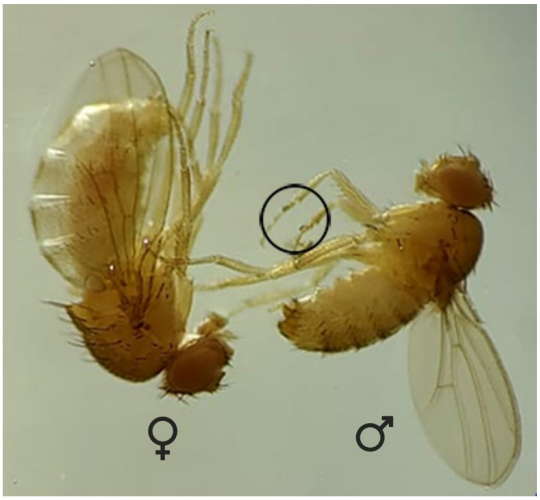

Figure 1. Females and Males of *D. melanogaster Multiple Wing Hair* (mwh/mhw) and wts/Sb[1] mutant lines.

A second group of bottles, with the same treatments, was prepared for the ETT test and the offspring were awaited to hatch. The descendants carrying the wts+/+mhw genotype were separated and identified by the presence of phenotypic wild hair (long and thin). The descendants, which have short and thick hair, belonging to the balanced heterozygous progeny (which have the presence of the balancing gene TM3, Sb1) were discarded since they do not have the gene under study.

After the metamorphosis process, adult flies were collected and preserved in bottles containing 70% ethanol. 200 individuals (males and females) of each concentration were analyzed, with genotype (wts+), which have normal (long) hair. Short-haired flies (Stubble) were not analyzed, as they do not have the wts gene. The individuals were analyzed in a hollow plate containing glycerin (Glycerol $C_3H_8O_3$) using brushes and a stereoscopic magnifying glass. The presence of tumors was counted in each of the segments (eyes, head, wings, body, legs, dumbbells). In the end, all tumors per individual were added together (Vasconcelos et al., 2017b).

2.4. Experiment 2

2.4.1. Animals and experimental design

Forty male mice (*Mus musculus*) of the Swiss lineage with an average weight of 40g were used. The animals

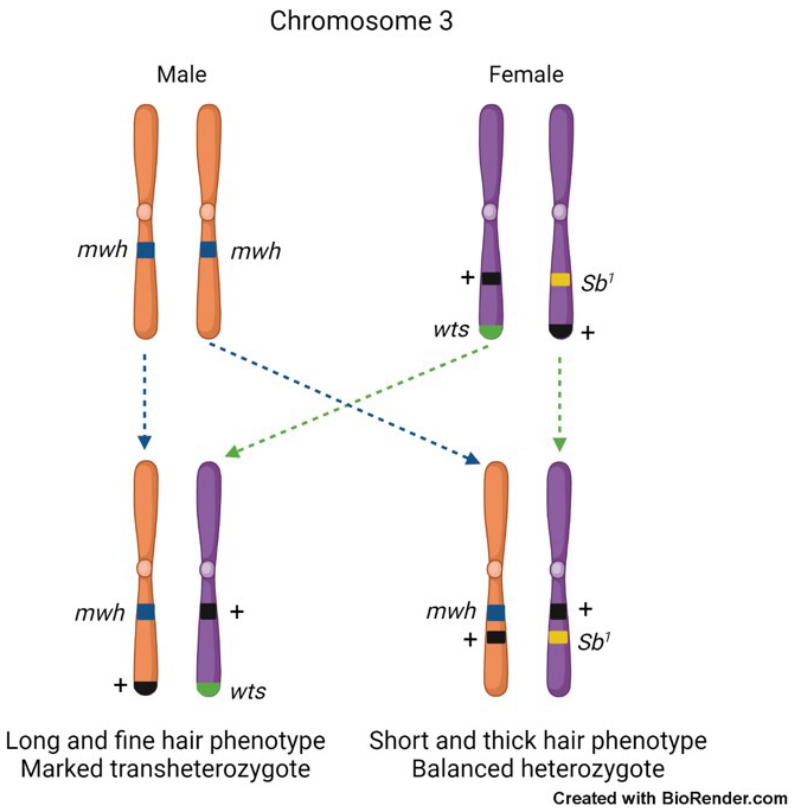

Figure 2. Didactic diagram of the lineage cross between males of the mwh/mhw and virgin females of the wts/Sb[1]. Chromosome 3 is represented containing the genotypes mwh/mhw and wts/Sb[1]. + indicates the wild-type allele.

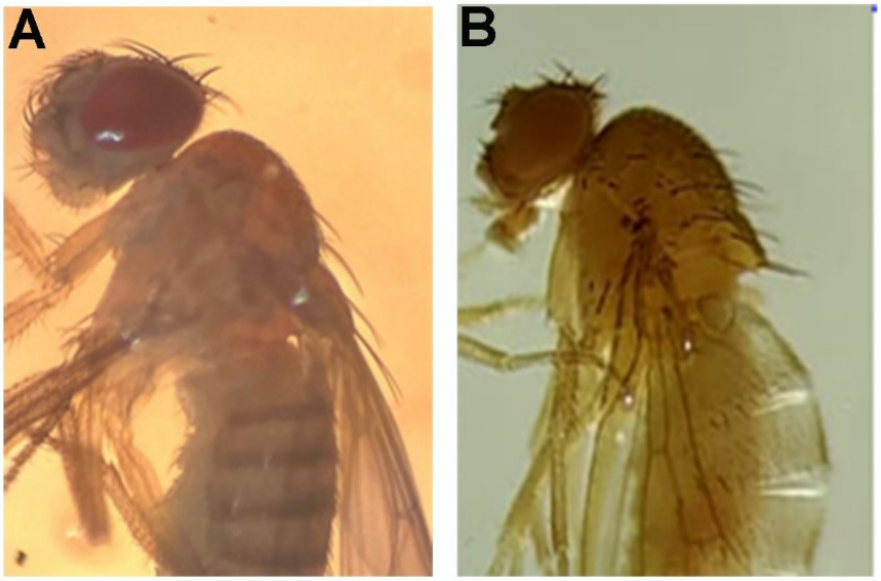

Figure 3. Phenotypic appearance of the descendants of males from the mwh/mhw lineage and virgin females from the wts/Sb[1] lineage. The offspring (males or females) with the long-hair phenotype (A) will present the mwh/wts genotype and those with the short-hair phenotype (B) will present the mwh/Sb[1] genotye.

underwent an adaptation period of 7 days, placed in mini-isolators (Alesco® ventilated rack) lined with shavings from Pinus sp. The animals were fed standard commercial food (Nuvital®) and filtered water under a free access system. Lighting was controlled by photoperiod (12h light/12h dark) and temperature maintained at 22 ± 2°C and humidity at 55 ± 10.

The research was carried out in accordance with the protocols of the Universal Declaration of Animal Rights and approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Mato Grosso do Sul (UFMS) under opinion number 965/2018.

Animals were distributed into 8 experimental groups (n = 5): Negative Control Group (CN) – the animals were treated with 0.1mL/10g of body weight (b.w.) of the EEGc vehicle (Tween 80 – 1%) via oral (v.o.) and 0.1mL/10g p.c. the cyclophosphamide vehicle (distilled water) intraperitoneally (i.p.); Positive Control Group (Cyclophosphamide) – the animals were treated with 0.1mL/10g p.c., p.o.) of the EEGc vehicle (Tween 80 – 1%) and 100mg/Kg of Cyclophosphamide (p.c., i.p.); Genotoxicity Groups EEGc100, EEGc1,000 and EEGc2,000 – the animals were treated with 100, 1,000 and 2,000 mg/kg (p.c.; v.o.) of EEGc, respectively + 0.1mL/10g (p.c.. i.p.) of the cyclophosphamide vehicle (distilled water); Antigenotoxicity Groups EEGc100+Cycle, EEGc1,000+Cycle and EEGc2,000+Cycle – the animals were treated with 100, 1,000 and 2,000 mg/Kg (p.c.; p.o.) of EEGc, respectively + 100mg/Kg of Cyclophosphamide (p.c., i.p.) (Figure 4).

2.4.2. Biological tests

Peripheral blood samples were collected through puncture of the tail vein, 24, 48 and 72 hours after administration of the test compounds. After 24 hours, peripheral blood was analyzed using the comet assay. At 24, 48 and 72 hours, peripheral blood was analyzed using the micronucleus technique. After these collections, the animals were euthanized by cervical dislocation followed by laparotomy for collection, weighing and macroscopic analysis of the organs (heart, lung, spleen, liver and kidneys). The spleen was used for evaluation of splenic phagocytosis after weighing. The biometric parameters were composed of the initial weight, final weight and absolute and relative weight of the organs.

2.4.2.1. Comet assay

The comet assay was performed according to Singh et al. (1988) and evaluated according to Kobayashi (1995). Twenty microliters of peripheral blood (20µL) were homogenized in 120µL of LPM agarose (0.5%). The mixture was then deposited on slides pre-covered with common agarose (5%). The slides were covered with a glass coverslip and cooled to 4°C for 20 minutes. Then, after removing the coverslips, the slides were immersed in a lysis solution (89.0ml of lysis stock – 2.5M NaCl, 100.0mM EDTA, 10.0mM Tris, pH 10.0 corrected with solid NaOH; 1 .0mL of Triton X-100 and 10.0mL of DMSO).

Lysis occurred for 1 hour at 4°C in a dark place. Then, the slides were placed in the electrophoresis vat and immersed in a buffer with pH >13.0 (300.0mM NaOH and 1.0mM EDTA) and DNA denaturation occurred for a period of 20 minutes at 4°C under the shelter from the light. Then, electrophoresis was performed at 25.0V and 300.0mA (1.25v/cm) for 20min. The slides were neutralized with buffer at pH 7.5 (0.4M Tris-HCl) for 3 cycles of 5 minutes each and left to air dry. Then, the slides were fixed with absolute ethyl alcohol for 10 minutes.

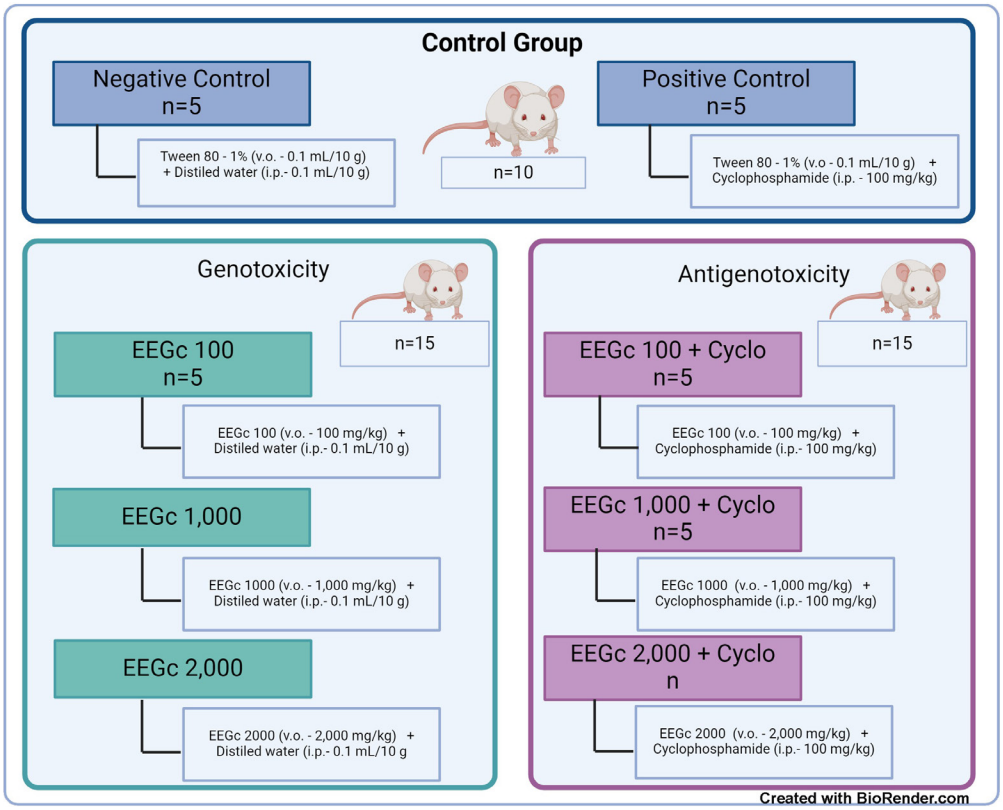

Figure 4. Mice experimental groups design: Negative Control Group – 0.1mL/10g body weight (b.w.) of the EEGc vehicle (Tween 80 – 1%) orally (p.o.) and 0.1mL/10g p.c. the cyclophosphamide vehicle (distilled water) intraperitoneally (i.p.); Positive Control Group - Cyclophosphamide (Cyclo) – 0.1mL/10g of Tween 80 (1%) (p.c., p.o.) and 100mg/Kg of Cyclophosphamide (p.c., i.p.); Genotoxicity Groups EEGc100, EEGc1,000 and EEGc2,000 – 100, 1,000 and 2,000 mg/Kg (p.c.; v.o.) of EEGc, respectively + 0.1mL/10g of distilled water (p.c., i.p.); Antigenotoxicity Groups EEGc100+Cycle, EEGc1,000+Cycle and EEGc2,000+Cycle – 100, 1,000 and 2,000 mg/Kg (p.c.; p.o.) of EEGc, respectively + 100mg/Kg of Cyclophosphamide (p.c., i.p.).

They were then stained with 100µL of ethidium bromide (20x10-3mg/mL). The slides were analyzed under an epifluorescence microscope (Bioval®) at 40x magnification, with a 420-490nm excitation filter and a 520nm barrier filter. 200 cells/animal were analyzed and classified into: class 0 – undamaged nucleoids that do not have a tail; class 1 – nucleoids with a tail smaller than the diameter of the nucleoid; class 2 – nucleoids with tails measuring between 1 and 2 times the diameter of the nucleoid; class 3 – nucleoids with a tail twice the diameter of the nucleoid (Kobayashi, 1995). Nucleoids from apoptotic cells, which are completely fragmented, are generally not counted (Speit et al., 1996).

2.4.2.2. Micronucleus assay in peripheral blood

Samples of 20µl of peripheral blood were deposited on a slide previously prepared with 20µl of Acridine Orange (1.0mg/ml) and covered with a coverslip. The slides were stored in a -20ºC freezer for at least 15 days. 2,000 cells/animal were analyzed under an epifluorescence microscope (Motic®; Model BA 410), with a 420-490nm excitation filter and 520nm barrier filter, at 400x magnification, as described by Hayashi et al. (1990), modified by Oliveira et al. (2009).

2.4.2.3. Splenic phagocytosis Assessment

The splenic phagocytosis test was performed by macerating 1/3 of the spleen in physiological solution using a Pasteur pipette. After successive aspirations, one hundred microliters of the cell suspension were placed on a slide previously stained with Acridine Orange (1 mg/mL). The slide was then covered with a coverslip and stored in a freezer for at least 15 days, until further analysis under a fluorescence microscope with a 420-490nm excitation filter and 520nm barrier filter, and 400x magnification. 200 cells/animal were analyzed. The assessment of the presence or absence of phagocytosis was performed as established by Hayashi et al. (1992) and modified by Carvalho et al. (2015).

2.5. Damage reduction percentage (RD%) calculation

Damage reduction percentages for the comet and micronucleus assays were scored according to Manoharan and Banerjee (1985) and Waters et al. (1990).

$$RD\% = \left[\frac{Positivecontrolmean - Associatedgroupmean}{Positivecontrolmean - Negativecontrolmean}\right] x100 \quad (1)$$

2.6. Statistical analysis

In order to evaluate the results of the epithelial tumor test in *D. melanogaster*, the non-parametric Mann–Whitney U test was used. In tests with mice, parametric data were analyzed by ANOVA/Tukey. Data were presented as mean ± standard deviation and the significance level established was p <0.05.

3. Results

3.1. Experiment I – Survival, estimation of somatic cell damage and tumor frequency in D. melanogaster

The survival test demonstrated that only the highest concentration of EEGc (7.70mg/mL) reduced (p<0.05) the survival rate of *D. melanogaster* larvae. However, all concentrations tested showed survival greater than 80% (Figure 5) and, therefore, were maintained for the subsequent test since the minimum acceptable survival is 70% (Vasconcelos et al., 2017b).

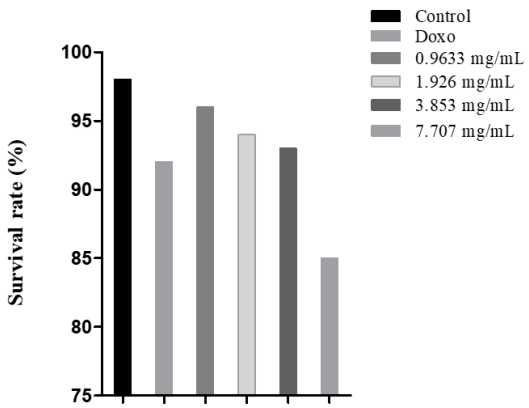

Figure 5. Survival rate of larvae treated with EEGc at concentrations of 0.96, 1.92, 3.85 and 7.70 mg/mL. Tween 80 (1%) was used for the negative control and doxorubicin for the positive control with the concentration of (2.17mg/mL).

ETT demonstrated that Doxorubicin increased (p<0.05) the frequency of tumor clones by 19.14x, proving its carcinogenic effect. The EEGc did not show a carcinogenic effect (p>0.05). Even the lowest concentration tested (0.96mg/mL) reduced (p<0.05) the basal frequency of tumors observed in the negative control (Table 1).

3.2. Experiment II – Genotoxic and antigenotoxic evaluation in mice

The evaluation of biometric parameters demonstrated that the initial weight, final weight and relative and absolute weight of the heart, lung, liver, kidneys and spleen were similar (p>0.05) between the different experimental groups (Figure 6).

Assessment of genomic damage, using the comet assay, demonstrated that cyclophosphamide was genotoxic and increased (p<0.05) the frequency of DNA damage by 12.04x. Isolated EEGc was not genotoxic (p>0.05) at the two lowest doses. However, the highest dose (EEGc2000) was genotoxic and increased (p<0.05) the frequency of genomic lesions by 5.37x. The EEGc associated with cyclophosphamide was antigenotoxic (p<0.05) for all doses tested and the percentages of damage reduction were 63.23, 49.91 and 33.39% for doses of 100, 1,000 and 2,000mg /Kg, respectively (Table 2).

The evaluation of chromosomal damage, using the micronucleus assay, demonstrated that cyclophosphamide is genotoxic and increased (p<0.05) the frequency of chromosomal damage by 3.59x, 3.13x and 2.87x at times 24, 48h and 72h. Isolated EEGc was not genotoxic (p>0.05) at any of the doses and at any of the times analyzed. However, the three doses were antigenotoxic (p<0.05) at 24 and 48h. The percentages of damage reduction were 64.42, 77.19 and 52.63% in 24h and 71.43, 63.26 and 40.82% for 48h in doses of 100, 1,000 and 2,000mg/Kg, respectively. Within 72 hours, EEGc was not antigenotoxic (p>0.05) and the percentages of damage reduction were 20.00, 36.67 and 63.33% for doses of 100, 1,000 and 2,000mg/Kg, respectively (Table 3).

The splenic phagocytosis assay demonstrated that none of the treatments (cyclophosphamide alone, EEGc alone and EEGc associated with cyclophosphamide) induced phagocytic activation (p>0.05) (Figure 7).

Table 1. Frequency of tumor clones observed in *D. melanogaster*, heterozygous for the wts tumor suppressor gene, treated with different concentrations of the EEGc.

Treatment EEGc	Total Flies	Number of tumors							Frequency
		Eye	Head	Wing	Body	Leg	Halter	Total	
NC	200	0	0	6	5	3	0	14	0,07
Doxo	200	0	3	186	21	58	1	269	1.34*
0.96	200	0	0	6	0	0	0	6	0.03*
1.92	200	0	0	7	2	4	0	13	0.06
3.85	200	0	3	5	5	3	0	16	0.08
7.70	200	0	3	8	6	5	0	22	0.11

Caption: EEGc – Ethanolic extract of *G. cellosioides*. NC – Negative control – Tween 80 (1%); Doxo - Positive Control – Doxorubicin (2.17mg/mL). Frequency – Amount of tumors / number of flies analyzed; *Indicates a statistically significant difference in relation to the negative control group. Statistical test: Mann Whitney (p<0.05).

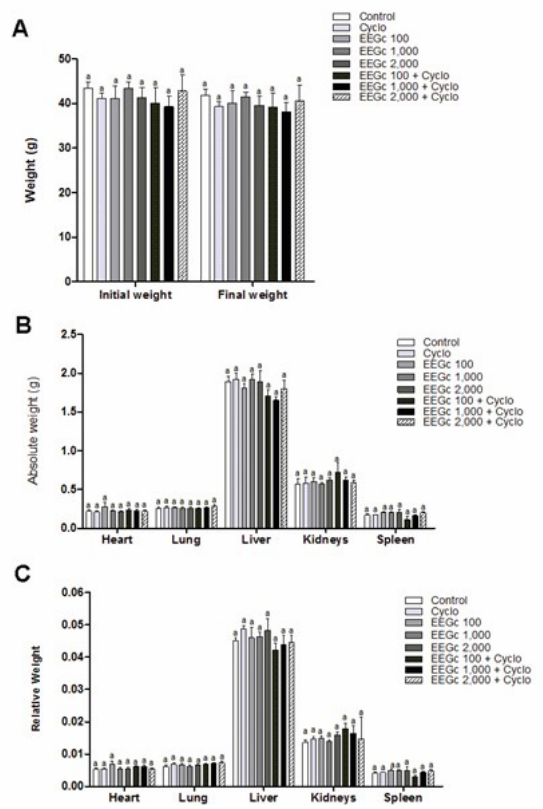

Figure 6. Mean values ± standard deviation of biometric parameters of animals treated with EEGc alone or in association with cyclophosphamide. A – Initial weight and final weight; B – Absolute weight of the organs; C – Relative weight of the organs. Negative Control Group (Control) – 0.1mL/10g of body weight (b.w.) of the EEGc vehicle (Tween 80 – 1%) orally (p.o.) and 0.1mL/10g p.c. the cyclophosphamide vehicle (distilled water) intraperitoneally (i.p.); Positive Control Group (Cyclo) – 0.1mL/10g of Tween 80 (1%) (p.c., p.o.) and 100mg/Kg of Cyclophosphamide (p.c., i.p.); Genotoxicity Groups EEGc100, EEGc1,000 and EEGc 2,000 – 100, 1,000 and 2,000 mg/Kg (p.c.; v.o.) of EEGc, respectively + 0.1mL/10g of distilled water (p.c., i.p.); Antigenotoxicity Groups EEGc100+Cyclo, EEGc1,000+Cyclo and EEGc2,000+Cyclo – 100, 1,000 and 2,000 mg/Kg (p.c.; p.o.) of EEGc, respectively + 100mg/Kg of Cyclophosphamide (p.c., i.p.). Statistical test: ANOVA/Tukey. Different letters above the bar indicate statistically significant differences ($p<0.05$).

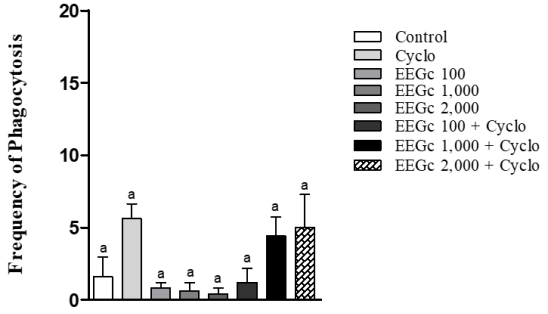

Figure 7. Mean values ± standard deviation of the frequency of cells with phagocytosis in the spleen of animals treated with EEGc alone or in association with cyclophosphamide. Negative Control Group (Control) – 0.1mL/10g of body weight (b.w.) of the EEGc vehicle (Tween 80 – 1%) orally (p.o.) and 0.1mL/10g p.c. the cyclophosphamide vehicle (distilled water) intraperitoneally (i.p.); Positive Control Group (Cyclo) – 0.1mL/10g of Tween 80 (1%) (p.c., p.o.) and 100mg/Kg of Cyclophosphamide (p.c., i.p.); Genotoxicity Groups EEGc100, EEGc1,000 and EEGc2,000 – 100, 1,000 and 2,000 mg/Kg (p.c.; v.o.) of EEGc, respectively + 0.1mL/10g of distilled water (p.c., i.p.); Antigenotoxicity Groups EEGc100+Cyclo, EEGc1,000+Cyclo and EEGc2000+Cyclo – 100, 1,000 and 2,000 mg/Kg (p.c.; p.o.) of EEGc, respectively + 100mg/Kg of Cyclophosphamide (p.c., i.p.). Statistical test: ANOVA/Tukey. Different letters indicate statistically significant differences ($p<0.05$). Statistical test: ANOVA/Tukey. Different letters above the bar indicate statistically significant differences ($p<0.05$).

4. Discussion

This study is pioneering in demonstrating that EEGc is not carcinogenic in *D. melanogaster* and has a chemopreventive effect on DNA damage induced by cyclophosphamide in Swiss mice. Furthermore, *G. celosioides* has already been described with low antitumor effect in vitro. The EEGc presented IC50 above 1,000µg/mL in cells of the KKU-100 (JCRB 1568) and KKU-213 (JCRB 1557) lineages (Promraksa et al., 2019) and above 250µg/mL for ethanolic and aqueous extracts in HepG2 cells (Chassagne et al., 2017). Additionally, the literature described that EEGc was not genotoxic in pregnant female Swiss mice (Salustriano et al., 2022).

The present study showed that EEGc has the potential to significantly reduce the survival of *D. melanogaster* larvae at concentrations equal to or greater than 7.70 mg/mL. However, all doses evaluated in the survival test were used for the ETT because larval survival was greater than 70% and this indicated that the concentrations tested did not affect larval development to the point of compromising the development of subsequent tests. The literature describes that survival of at least 70% of larvae is ideal for choosing doses that can be used in the ETT. Therefore, a mortality rate above 30% can compromise subsequent tests by not reaching the minimum number of adult individuals required for analyzes (Demir et al., 2013; Orsolin et al., 2015).

At ETT, it was observed that the lowest concentration of EEGc (0.96mg/mL) was able to significantly reduce the basal frequency of epithelial tumors in *D. melanogaster*. This effect suggests that the extract may have antitumor potential. However, its effect would possibly not be in the treatment of cancer since the chemotherapy effect, simulated in culture tests with cholangiocarcinoma cell lines (KKU-100 and KKU-213), has already refuted this hypothesis (Chassagne et al., 2017; Promraksa et al., 2019). Thus, it is inferred that its effect may be correlated with the prevention of DNA lesions that can predispose the individual to the development of cancer and, consequently, it would also have an effect on reducing the occurrence of this disease.

It is also noteworthy that the other EEGc concentrations (1.92 and 3.85mg/mL), including the concentration that significantly reduced larval survival (7.70mg/mL) do not have a carcinogenic effect in the alternative ETT model in *D. melanogaster*. This result allows us to infer safety of use since there is correspondence between the results obtained in the

Table 2. Mean values ± standard deviation of the frequency of cells with DNA damage, distribution of cells by damage classes, score and percentage of DNA damage reduction in animals treated with the EEGc alone or in combination with cyclophosphamide (Cyclo).

Experimental Groups	Cells with DNA damage	Classes				Score	%RD
		0	1	2	3		
Control	10.20±2.03[a]	189.80±2.03	10.20±2.03	0.00±0.00	0.00±0.00	10.20±2.03[a]	
Cyclo	122.80±8.31[d]	76.20±9.24	112.80±8.01	10±1.58	0.00±0.00	132.80±8.88[d]	
Genotoxicity							
EEGc 100	35.80±3.63[a,b]	164.20±3.62	35.80±3.62	0.00±0.00	0.00±0.00	35.80±3.62[a,b]	
EEGc 1,000	39.60±5.84[a,b]	160.40±5.84	39.60±5.84	0.00±0.00	0.00±0.00	39.60±5.84[a,b]	
EEGc 2,000	54.80±6.96[b,c]	145.20±6.96	54.80±6.96	0.00±0.00	0.00±0.00	54.80±6.96[b,c]	
Antigenotoxicity							
EEGc 100 + Cyclo	51.60±12.63[b,c]	148.80±12.41	50.80±12.18	0.40±0.24	0.00±0.00	51.60±12.63[b,c]	63.23%
EEGc 1,000 + Cyclo	66.60±9.82[b,c]	133.40±9.82	66.60±9.82	0.00±0.00	0.00±0.00	66.60±9.82[b,c]	49.91%
EEGc 2,000 + Cyclo	85.20±3.37[c]	112.80±4.49	84.60±2.98	0.60±0.40	0.00±0.00	85.80±3.76[c]	33.39%

Legend: Negative Control Group (Control) – 0.1mL/10g body weight (b.w.) of the EEGc vehicle (Tween 80 – 1%) orally (p.o.) and 0.1mL/10g p.c. the cyclophosphamide vehicle (distilled water) intraperitoneally (i.p.); Positive Control Group (Cyclo) – 0.1mL/10g of Tween 80 (1%) (p.c., p.o.) and 100mg/Kg of Cyclophosphamide (p.c., i.p.); Genotoxicity Groups EEGc100, EEGc1,000 and EEGc2,000 – 100, 1,000 and 2,000 mg/Kg (p.c.; v.o.) of EEGc, respectively + 0.1mL/10g of distilled water (p.c., i.p.); Antigenotoxicity Groups EEGc100+Cyclo, EEGc1,000+Cyclo and EEGc2,000+Cyclo – 100, 1,000 and 2,000 mg/Kg (p.c.; p.o.) of EEGc, respectively + 100mg/Kg of Cyclophosphamide (p.c., i.p.). Statistical test: ANOVA/Tukey. Different letters indicate statistically significant differences ($p<0.05$). %RD – Percentage of DNA Damage Reduction. Statistical test: ANOVA/Tukey. Different letters indicate statistically significant differences ($p<0.05$).

Table 3. Mean values ± standard deviation of the frequency of micronuclei and percentage of reduction in DNA damage in animals treated with the EEGc alone or associated with cyclophosphamide (Cyclo).

Experimental Groups	Micronuclei Frequency			%DR		
	24h	48h	72h	24h	48h	72h
Control	4.40±0.75[a,b]	4.60±1.03[a,b]	3.20±0.37[a,b]			
Cyclo	15.80±0.66[d]	14.40±0.93[d]	9.20±1.16[c]			
Genotoxicidade						
EEGc 100	2.80±0.66[a]	6.40±0.81[a,b,c]	2.80±0.37[a]			
EEGc 1,000	4.80±0.92[a,b]	4.00±1.30[a,b]	4.40±0.75[a,b,c]			
EEGc 2,000	4.40±1.03[a,b]	3.60±0.81[a]	7.20±1.32[a,b,c]			
Antigenotoxicidade						
EEGc 100 + Cyclo	8.00±0.89[b,c]	7.40±0.93[a,b,c]	8.00±2.07[b,c]	68.42%	71.43%	20.00%
EEGc 1,000 + Cyclo	7.00±1.67[a,b,c]	8.20±1.07[b,c]	7.00±0.84[a,b,c]	77.19%	63.26%	36.67%
EEGc 2,000 + Cyclo	9.80±0.86[c]	10.40±1.03[c,d]	5.40±0.51[a,b,c]	52.63%	40.82%	63.33%

Legend: Control Group (Control) – 0.1mL/10g body weight (b.w.) of the EEGc vehicle (Tween 80 – 1%) orally (p.o.) and 0.1mL/10g p.c. the cyclophosphamide vehicle (distilled water) intraperitoneally (i.p.); Positive Control Group (Cyclo) – 0.1mL/10g of Tween 80 (1%) (p.c., p.o.) and 100mg/Kg of Cyclophosphamide (p.c., i.p.); Genotoxicity Groups EEGc100, EEGc1,000 and EEGc2,000 – 100, 1,000 and 2,000 mg/Kg (p.c.; v.o.) of EEGc, respectively + 0.1mL/10g of distilled water (p.c., i.p.); Antigenotoxicity Groups EEGc100+Cyclo, EEGc1,000+Cyclo and EEGc2,000+Cyclo – 100, 1,000 and 2,000 mg/Kg (p.c.; p.o.) of EEGc, respectively + 100mg/Kg of Cyclophosphamide (p.c., i.p.). Statistical test: ANOVA/Tukey. Different letters indicate statistically significant differences ($p<0.05$). %RD – Percentage of DNA Damage Reduction. Statistical test: ANOVA/Tukey. Different letters indicate statistically significant differences ($p<0.05$).

models in *D. melanogaster* and those obtained in mammals (Eeken et al., 2002; Nepomuceno, 2015). The correspondence may be attributed due to the evolutionary conservation of tumor suppressor genes between *D. melanogaster* and mammals (Eeken et al., 2002). Furthermore, more than 50 genes have been mapped and characterized as tumor suppressor genes in *D. melanogaster*. Eight of them have a function in the embryonic development of the fly, 12 in brain development, 19 are expressed in cells of the imaginal discs, 25 in hematopoietic development and 10 in adult gonads (Justice et al., 1995) and among these genes, the most important wts (Nishiyama et al., 1999). It is also noteworthy that *D. melanogaster* has an enzyme system similar to that of mammals (Graf and Van Schaik, 1992).

These facts impelled the study to be continued. But no longer in the alternative model of *D. melanogaster* but in an animal model that more closely resembles humans and the model chosen was Swiss mice. The results with mice suggest that EEGc is not toxic since classic clinical signs of intoxication were not observed such as diarrhea, tremors, excessive salivation, convulsions, hypoactivity, ataxia, lethargy, tail curvature, hair standing on end, opacity of the eyes and mucous membranes, and/or reduction in food and water consumption, among others (OECD, 2008; Corso et al., 2019; Nunes et al., 2023; Rezende et al., 2023; Correa et al., 2024). This fact is confirmed by the absence of significant variations in weights at the beginning and end of the experiment. Since weight reduction is understood as an important indicator of toxicity (Upadhyay et al., 2019; Salustriano et al., 2022; Neves et al., 2023, 2024; Nunes et al., 2023; Oliveira et al., 2023; Ortiz et al., 2023; Rezende et al., 2023; Correa et al., 2024) as well as variations in the absolute and relative weights of organs (Oliveira et al., 2018a; Oliveira et al., 2018b).

Besides, it is noteworthy that the literature has already reported that acute toxicity for EEGc is greater than 2,000mg/Kg in Wistar rats for both sexes (males and females) (Macorini et al., 2022). This study even used the same extract used by us. It is also remarkable that this extract was classified in Category 5, according to the Globally Harmonized System, which is the least toxic or non-toxic (OECD, 2008; Macorini et al., 2022). In relation to the aqueous extract of *G. celosioides*, it was found that the acute toxicity is greater than 4000 mg/kg (Konan et al., 2014). Regarding subacute toxicity, the literature states that doses 75, 150 and 300mg/Kg, administered repeatedly over 28 days, have low toxicological potential in male and female Swiss mice (Macorini et al., 2022). These results support the findings of this study.

Complementary data to acute and subacute toxicity, which can corroborate the safety of use, are genotoxicity studies. Thus, this study suggests, in a pioneering way, that EEGc can cause DNA lesions, at the genomic level (assessed by the comet assay), at a dose equal to or greater than 2,000mg/Kg since this dose caused a 5.37x increase in frequency of damage observed in the control group. It is also noteworthy that the lowest doses showed a tendency to increase the frequency of injuries by 3.51x and 3.88x for doses of 100 and 1,000mg/Kg, respectively.

These results reveal a dose response curve, when an increase in dose generates an increase in the frequency of genomic lesions. At first, this data requires attention. However, when evaluating the frequency of chromosomal damage, it was found that EEGc was not genotoxic, that is, none of the doses tested increased the frequency of micronuclei. These data suggest that genomic damage (assessed by the comet test) was not fixed in the cellular genome in the form of chromosomal damage (assessed by the micronucleus test) indicating the absence of genotoxicity. This fact was corroborated by the absence of splenic phagocytosis observed for all experimental groups.

Previously, our research group had already reported that this EEGc did not cause chromosomal damage and did not activate splenic phagocytosis in treatments with these same doses, for up to 18 consecutive days, in pregnant female Swiss mice (Salustriano et al., 2022). These facts reinforce the safety of using EEGc as this extract did not present acute toxicity and has a low potential for subacute toxicity (Macorini et al., 2022), did not present genotoxicity in pregnant females and did not activate splenic phagocytosis (Salustriano et al., 2022) and the genomic damage observed in this study was not fixed in the cellular genome as indicated by our micronucleus assay results.

Genomic damage can be repaired (Fernández-Bertólez et al., 2022) and, therefore, may not be fixed in the cellular genome, causing less damage to the organism (Navarro et al., 2014; Oliveira et al., 2015; Correa et al., 2024). It is assumed that this fact, already reported in the literature, also occurred in this study. Furthermore, it is known that one of the main functions of the spleen is to promote blood filtration by removing damaged cells from it, including those with DNA damage, micronucleated cells, for example; senescent cells; and intraerythrocytic inclusions.

Thus, the study by Hayashi et al. (1990) served as the basis for more recent researches that use this methodology to measure the phagocytic activity of the spleen in response to genotoxic or antigenotoxic action (Navarro et al. 2014; Oliveira et al., 2015, 2018a, b, 2019, 2023; Berno et al., 2016; Schneider et al., 2016; Araujo et al., 2017; Ishikawa et al., 2017; Navarro et al., 2018; Ishikawa et al., 2018; Salustriano et al., 2022; Neves et al., 2023; Rezende et al., 2023; Correa et al., 2024; Neves et al., 2024). Therefore, as the spleen is involved in immunomodulation processes and phagocytosis is effective in sequestering anucleated or micronucleated cells, this action can mask the genotoxic effects of a substance or extract. Thus, the combination of genotoxicity and splenic phagocytosis methodologies strengthens the hypotheses tested by providing more robust data (Carvalho et al. 2015) and thus we can infer that EEGc is not genotoxic.

Another unprecedented finding from this study is the antigenotoxic effect of EEGc. Until now, the literature consulted did not indicate the chemopreventive effect of this extract. EEGc was able to reduce genomic damage (assessed by the comet) by up to 63.23%, with the lowest dose being the most effective (100mg/Kg). They were also efficient in preventing chromosomal damage (assessed by the micronucleus assay) by up to 77.19%, with the intermediate dose, in the 24-hour assessment, being the most efficient. For the antigenotoxicity data, the dose response pattern was not established.

The antigenotoxic and/or chemopreventive effect is the ability of a product to reduce the frequency of DNA damage. This effect can be due to desmutagenesis when, in short, one compound binds to another, preventing the genotoxic/mutagenic effect through an adsorption process, for example; or by bioantimutagenesis. In this other case, after damage occurs, the compound modulates the body's enzymes in an attempt to favor the correction of the genotoxic/mutagenic damage. Therefore, enzymes act as modulators of repair and replication (Kada and Shimoi, 1987; De Flora, 1998; Oliveira et al., 2006, 2007, 2009, 2013, 2014; Mantovani et al., 2008; Fedel-Miyasato et al., 2014a, b; Diab and Aboul-Ela, 2012; Rocha et al., 2016).

The daily use of antigenotoxic compounds could be a good cancer prevention strategy and the daily use of antigenotoxic compounds is called chemoprevention (Gomes et al., 1996; Surh et al., 1996; ALaqeel, 2024; Mughal et al., 2024; Dyshlyuk et al.,2024). For a long time, the literature has recorded an inversely proportional association between the consumption of fruits and vegetables and the development of cancer (Flagg et al., 1995; Weisburger, 1999, 2000; Zhang et al., 1999; Ferrari, 2001; Ferrari and Torres, 2002) and this fact encourages the need to eat adequately using natural and antigenotoxic products (Amaral et al., 2021).

Classical literature also records that the main natural inhibitors of carcinogenesis/genotoxicity are represented by antioxidants that are free radical blockers and also by cell death inducers, enzyme inhibitors, angiogenesis inhibitors, growth factor antagonists, hormones and injury repairers. of DNA (Kleiner, 1997; Kellof et al., 1999)

According to Salvador et al. (2012) in the genus *Gomphrena*, steroids, terpenoids, ecdysteroids, flavonoids, aurantiamide and protoalkaloids were isolated. According to Vasconcelos et al. (2017a), the aerial parts of *G. celosioides* are rich in malic acid, caffeic acid, ferulic acid, vanillic acid, catecin, Irisone B, Dimethoxy-flavone and caffeoyl-glucose. Thus, the presence of phenolic acids and flavonoids is verified, in the genus and species, and these chemical compounds can act as antioxidants.

Antioxidants, in general, act to protect against the formation of DNA-damaging substances and to intercept free radicals (which, once formed, begin their DNA-damaging activities) (Kong; Lillehei, 1998; Santos; Cruz, 2001; ALaqeel, 2024). Thus, the antigenotoxic and/or chemopreventive effect of EEGc can be attributed to its constituents.

In light of the above, it is considered that EEGc is safe to use and has a low genotoxic potential, as genomic damage is not fixed in the form of chromosomal damage in the cellular genome. Furthermore, EEGc showed a chemopreventive effect, which suggests therapeutic applicability in the prevention of DNA lesions that can increase the predisposition to cancer.

Acknowledgements

The authors are grateful to Prof. Dr. Josafá Carlos de Siqueira for plant material identification, to Prof. Dr. Alexandre Azenha Alves de Rezende for consulting on the Epithelial Tumor Test to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo - Grant process: 21/09693-5), to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico Process: 306603/2022-5, and 427859/2018-2), to FAEPEX-UNICAMP, to FUNDECT (Fundação de Apoio ao desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul) and to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support and scholarships.

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