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In vitro evaluation of the cytotoxic and genotoxic effects of ethanolic extracts of *Echinodorus macrophyllus*

Avaliação in vitro dos efeitos citotóxico e genotóxico do extrato etanólico de Echinodorus macrophyllus

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ABSTRACT

Echinodorus macrophyllus (Kunth.) Micheli (*E. macrophyllus*) is an aquatic plant belonging to the family Alismataceae, popularly known as chapéu-de-couro. Previous studies have shown that chapecoderins, echinophyllins, and echinolides are present in its chemical composition. Although there is evidence of *E. macrophyllus* having therapeutic properties (especially in the treatment of inflammatory diseases), it is necessary to carry out studies to investigate its safety for therapeutic use. Thus, the aim of this study was to evaluate the cytotoxic and genotoxic effects of ethanolic extracts from *E. macrophyllus* (EEEM) leaves on human peripheral blood mononuclear cells (PBMCs). PBMCs were collected from healthy volunteers for use in cytotoxicity (MTT) and genotoxicity (cytokinesis-block micronucleus cytome [CBMN cyt] and comet) assays and treated with concentrations of EEEM ranging from 1 to 500 µg/mL. The results of this study did not indicate cytotoxic or genotoxic effects of the EEEM for any of the concentrations evaluated. *E. macrophyllus* does not cause significant genetic damage to PBMCs in comet and CBMC assays, suggesting that the plant is safe and does not pose significant risks with regard to general consumption.

Keywords: chapéu-de-couro, CBMN-cyt, comet, DNA damage, mutagenesis

RESUMO

Echinodorus macrophyllus (Kunt.) Micheli (*E. macrophyllus*) é uma planta aquática da família Alismataceae, popularmente conhecida como chapéu-de-couro. Estudos prévios demonstraram que chapecoderinas, equinofilinas e equinólidos são os principais metabólitos presentes na planta, frequentemente associados às suas propriedades biológicas.

Embora haja evidências sobre as propriedades terapêuticas de *E. macrophyllus* (especialmente direcionada ao tratamento de doenças inflamatórias), é necessário a realização de estudos para a investigação do seu uso terapêutico. Assim, o objetivo deste estudo foi avaliar os efeitos citotóxicos e genotóxicos do extrato etanólico das folhas de *E. macrophyllus* (EEEM) em células mononucleadas do sangue periférico humano (PBMCs). PBMCs foram coletadas de voluntários saudáveis para a utilização no ensaio de citotoxicidade (MTT) e nos ensaios de genotoxicidade (citoma micronúcleo combloqueio da citocinese – CBMN cyt e cometa), e tratadas com concentrações do EEEM variando entre 1 a 500 µg/mL. Os resultados desse estudo não identificaram efeitos citotóxico ou genotóxico do EEEM em nenhuma das concentrações avaliadas. *E. macrophyllus* não induziu danos genéticos significativos em PBMC nos ensaios do cometa e CBMN cyt, sugerindo que a planta é segura e não apresenta riscos significativos para o consumo em geral.

Palavras-chave: chapéu-de-couro, CBMN-cyt, cometa, dano ao DNA, mutagênese

INTRODUCTION

Echinodorus macrophyllus (Kunth.) Micheli (*E. macrophyllus*) is an aquatic plant belonging to the family Alismataceae, popularly known as chapéu-de-couro (FERNANDES, 2012; NUNES, 2003). Morphologically, *E. macrophyllus* is characterized by petiolate, oval, cordiform, and acute base or acuminate leaves at the apex; it is dark green in color, 20-40 cm in length and 15-35 cm in width in the proximal region, with roughly 11-13 main nervures; it bears white hermaphrodite flowers, numerous and arranged in elongated clusters (LEITE, 2007; SANTOS, 2017).

E. macrophyllus has in its chemical composition chapecoderins A, B, and C echinophyllins and echinolides A and B (KOBAYASHI, 2000; SHIGEMORI, 2002). For other species of the *Echinodorus* genus, previous studies have identified the presence of caffeic acid, diterpenes, alkaloids, flavonoids, and other aromatic derivatives (MANNS, HARTMANN, 1993; SCHNITZLER, 2007; TANAKA, 2000).

The leaves of *E. macrophyllus* are widely used in folk medicine through the preparation of infusions (BARBOSA, 2013). The therapeutic use of the leaves of *E. macrophyllus* and other species of the *Echinodorus* genus is directed at the treatment of inflammatory diseases such as asthma and arthritis. In addition, studies have shown that *Echinodorus* extracts exhibit diuretic and antioxidant activities, making them potentially useful as auxiliaries in the control of blood pressure (LIMA PRANDO, 2015; PINTO, 2007; ROSA, 2017).

The concern with the safety of natural products has increased with the growing awareness that many plants can be effective for the treatment of diseases. Thus, information about their safety is needed because many plants contain diverse toxic substances for defense against viruses, bacteria, and fungi, which can cause negative effects in the human body (DA COSTA LOPES, 2000; VIDAL, 2010).

The assessment of safety is the main reason for conducting genotoxicity tests on plants. There are a number of reports that plants, their extracts, and the isolated substances derived from them exhibit mutagenic and genotoxic effects, including DNA damage, genetic mutations, and inheritable structural or numerical chromosomal damage, as well as promoting the development of carcinogenic processes (EDZIRI, 2011; GUNES-BAYIR, 2018; SPONCHIADO, 2016; YOUSSEF, ELAMAWI, 2020).

Chemical compounds that exhibit genotoxic effects can be identified in test batteries that include the

comet assay and the cytokinesis-block micronucleus cytome assay (CBMN cyt assay). Once exposed to a toxic substance, genetic material may undergo gene mutations, chromosomal damage, or DNA damage (BASU, 2018; FENECH, 2020; LU, 2017).

The assessment of genotoxicity indicators is important in determining the relationship between the damage caused and the substance to which the organism was exposed (LADEIRA, SMAJDOVA, 2017; MORO, 2013). Although there is evidence that *E. macrophyllus* exhibits therapeutic properties, it is necessary to carry out studies to investigate its safety for therapeutic use. Thus, the aim of this study was to evaluate the cytotoxic and genotoxic effects of ethanolic extracts from *E. macrophyllus* (EEEM) leaves on human peripheral blood mononuclear cells (PBMCs).

METHODOLOGY

Plant material and extraction procedure

E. macrophyllus leaves were collected at Sítio Pindorama in Governador Valadares – MG, November 2015. The specimen was identified by Dr. Vinícius Antônio de Oliveira Dittrich and deposited at the Herbarium Leopoldo Krieger (CESJ 70701) at Federal University of Juiz de Fora. The research was authorized by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN; no. A2D9094).

E. macrophyllus leaves were ground in a knife mill to obtain a fine powder. The powder (23.412g) was subjected to maceration at room temperature with ethyl alcohol 95° GL of analytical grade. Every two days the extract obtained was filtered and, after the last maceration, the filtered extract was concentrated using a rotary evaporator at 40° C to obtain the crude extract that was used to carry out the tests (LANG, 2011).

Isolation of human peripheral blood mononuclear cells (PBMCs)

The blood samples were collected from adult healthy volunteers, no tobacco users, and were not taking any type of medication. They were informed about the experimental procedures, signed consent form to participate in the study, and all the procedures were approved by the Ethics Committee of the Federal University of Juiz de Fora, protocol number 65620217.8.0000.5147. PBMCs isolation was made by the difference of gradient density Hypaque-1077 (Histopaque®, Sigma–Aldrich-USA) according to manufacturer instructions. After centrifugation (400 × g for 30 minutes at room temperature), PBMCs were found at the plasma/1077 interphase and collected carefully with a Pasteur pipette. After that, the cells were washed in PBS twice (240 × g for 10 minutes at room temperature) and resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, phytohemagglutinin (150 µg/mL), penicillin/streptomycin (50 IU/mL and 50 µg/mL, respectively) and 10% (v/v) fetal bovine serum (FBS). The cell was incubated at 37 ºC in a humidified atmosphere containing 5% CO2 and used for cytotoxicity and genotoxic assays.

Cytotoxicity assay

The cytotoxicity of the EEEM was evaluated by the 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) (MOSMANN, 1983), a method for determining cell viability by measuring the mitochondrial dehydrogenase action. This enzyme reduces MTT to water-insoluble blue formazan crystals. PBMCs were counted and plated (1×105 cells/well) in 96-well culture plates and allowed to stabilization at 37◦C in a 5% CO2 atmosphere for 24h. PBMCs were exposed to concentrations ranging from 1 – 500 µg/mL (1 µg/ml, 5 µg/ml, 10 µg/ml, 25

µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/mL, and 500 µg/mL) of EEEM for 24h. A negative control containing only cells in culture medium was also evaluated. After 24h incubation, the MTT solution (5 mg / mL) was added for 4 h at 37° C in a 5% CO2 atmosphere. The plate was centrifuged at 1500 rpm for 10 min. The medium was removed and replaced by 180 µL of dimethyl sulfoxide (DMSO), followed by mixing to dissolve the formazan crystals. Absorbance was measured at 570 nm after further 4 hours of incubation, when the formazan crystals produced in the MTT reaction were solubilized with DMSO. The result was calculated as a percentage as compared to the negative control (DMSO only).

The alkaline comet assay

The alkaline comet assay was performed to measure DNA damage according to the method described by SINGH (1988) and TICE (2000). PBMCs were exposed to EEEM at concentrations from 15-200µg/mL (15µg/mL, 30µg/mL, 100µg/mL, 150µg/mL, and 200 µg/mL) for 4h, and a negative control. After treatment time, 10 µL aliquot of the cell suspension was mixed with 120 µL of Low Melting Point (LMP) Agarose and added onto the slide previously prepared with standard agarose and kept at 4°C. The slides were exposed to the lysis solution (2, 5M NaCl, 100mM EDTA, 10M Tris, pH 10) at 4°C for 24h. After lysis, the slides were stabilized in an electrophoresis solution (300mM NaOH, 1mM EDTA, pH> 13) for 40 minutes. Finally, electrophoresis was performed for 20 minutes at a voltage of 25V (1V/cm). The slides were stained with ethidium bromide (20µg/mL) examined under fluorescence microscopy, using a 515-560 nm filter and a 590 nm filter barrier, in a 40X objective. One hundred nucleoids were analyzed in each slide and these were classified in 0, 1, 2, 3 and 4 according to the size and intensity of the tail. The damage index (DI) was calculated with the formula: DI = (1 x n1) + (2 x n2) + (3 x n3) + (4 x n4), were the n0–n4 represent the number of nucleoids with 0–4 damage level.

Cytokinesis-block micronucleus assay (CBMN-cyt assay)

DNA instability was evaluated using CBMN-cyt according to the method of FENECH (2007). PBMCs were stabilized in culture flasks at 37° C in a 5% CO2 atmosphere for 24h. Then, PBMCs were exposed to EEEM at concentrations from 25 – 250 µg/mL (25 µg/ml, 50 µg/ml, 100 µg/ml, and 250 µg/ml). Negative and positive control cultures were incubated simultaneously. As positive control, methyl methanesulfonate [MMS; 150 µg/mL - Sigma Aldrich, 129925] was used. After 20h of the administration of the treatment solutions, PBMCs were exposed to 3 mg/mL cytochalasin B for 28h to block cytokinesis. After 72h, cells cultures were harvested by standard procedures including incubation in hypotonic solution of sodium citrate, fixation with methanol/glacial acetic acid solution (3:1, v/v). After fixation, slides were prepared and stained with acridine orange (40µg/mL) for microscopic analysis. A total of 1000 binucleated (BN) cells was examined for each treatment to identification and scoring of micronuclei (MN), biomarker of chromosome breakage and/or whole chromosome loss, nucleoplasmic bridges (NPBs), biomarker of DNA misrepair and/or telomere end-fusions, and nuclear buds (NBuds), biomarker of elimination of amplified DNA and/or DNA repair complexes. The same slides were used to study the nuclear division index (NDI), by screening 500 cells for each treatment. NDI was calculated by the following formula: NDI = (M1 + 2*M2 + 3*M3 + 4*M4)/N, where M1–M4 represent the number of cells with 1–4 nuclei, respectively, and N is the number of cells scored.

Statistical analysis

The results presented represent the mean ± standard deviation of three independent experiments (n = 3). The data were submitted to analysis of variance

(ANOVA) with Tukey multiple comparison post-test, with the aid of the program GraphPad Prism 5.0. The value of p <0.05 was considered statistically significant for all parameters evaluated.

RESULTS

Cytotoxicity evaluated by MTT assay

In the MTT assay (Figure 1), it was observed that PBMCs exposed for 24 hours to EEEM concentrations below 250 µg/mL did not exhibit cytotoxicity characteristics. At a concentration of 500 µg/mL, a decrease in the cell viability of PBMCs was observed, but this decrease was not statistically significant (p > 0.05). ANOVA with a Tukey multiple comparison post-test was used to evaluate significance. Tests pertaining to all EEEM concentrations indicated cell viability greater than 70% and did not present a dose-dependent relationship.

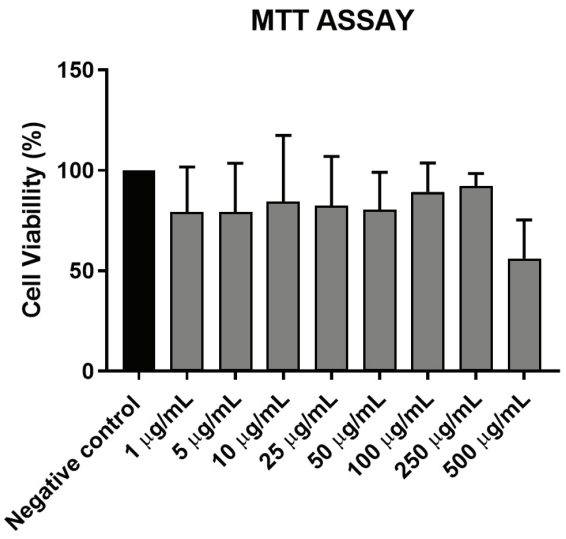

FIGURE 1. Cytotoxic effects of different concentrations of ethanolic extracts from *E. macrophyllus* (EEEM) leaves (1-500 µg/mL) on PBMCs after 24 hours of treatment in the MTT assay. Data represent the mean ± standard deviation (SD) of three independent tests. No significant difference was observed (p > 0.05). ANOVA followed by Tukey multiple comparison post-test.

The alkaline comet assay

We also investigated the occurrence of EEEM-induced genotoxicity using the comet assay. None of the concentrations evaluated caused significant DNA damage to the PBMCs. The results of the damage index are shown in Table 1; there was no significant induction on the damage index, nor was there any dose-dependent response (p > 0.05). ANOVA with a Tukey multiple comparison post-test was used to evaluate significance.

Table 1 also shows the distribution of the nucleoid classes, with no significant differences between the tested concentrations of EEEM and the negative control.

TABLE 1. Nucleoid damage classes and the damage index (DI) of cells exposed to different concentrations of ethanolic extract from *E. macrophyllus* (EEEM) leaves.

Treatments	Nucleoid Damage Classes					DI
	0	1	2	3	4	
Negative Control	24 ± 22	29 ± 10	26 ± 13	12 ± 13	9 ± 8	143 ± 83
15 µg/mL	28 ± 35	26 ± 14	24 ± 16	13 ± 6	9 ± 10	149 ± 86
30 µg/mL	16 ± 25	22 ± 13	29 ± 17	18 ± 12	15 ± 13	194 ± 98
100 µg/mL	16 ± 27	24 ± 4	31 ± 15	17 ± 9	12 ± 8	185 ± 76
150 µg/mL	14 ± 23	21 ± 13	30 ± 9	20 ± 16	15 ± 16	201 ± 99
200 µg/mL	12 ± 21	23 ± 11	31 ± 9	19 ± 12	16 ± 16	203 ± 90

Data from the alkaline comet assay after four hours of treatment. Data represent the mean ± standard deviation of three independent experiments. DI: damage index calculated with the formula DI = (1 x n1) + (2 x n2) + (3 x n3) + (4 x n4), where the n0-n4 represents the number of nucleoids with a 0-4 damage level. No significant difference was observed (p > 0.05). ANOVA followed by Tukey multiple comparison post-test.

Cytokinesis-block micronucleus cytome assay

We observed that in the CBMN cyt assay (Figure 2), EEEM did not significantly increase the frequency of MN, NPB, or Nbuds in PBMCs. Although the data might suggest an increased dose-dependency with regard to MN and NPB, no statistically significant difference was observed between the treated groups and the negative control groups ($p > 0.05$). Neither did we observe significant differences in the NDI, reinforcing the lack of cytotoxicity.

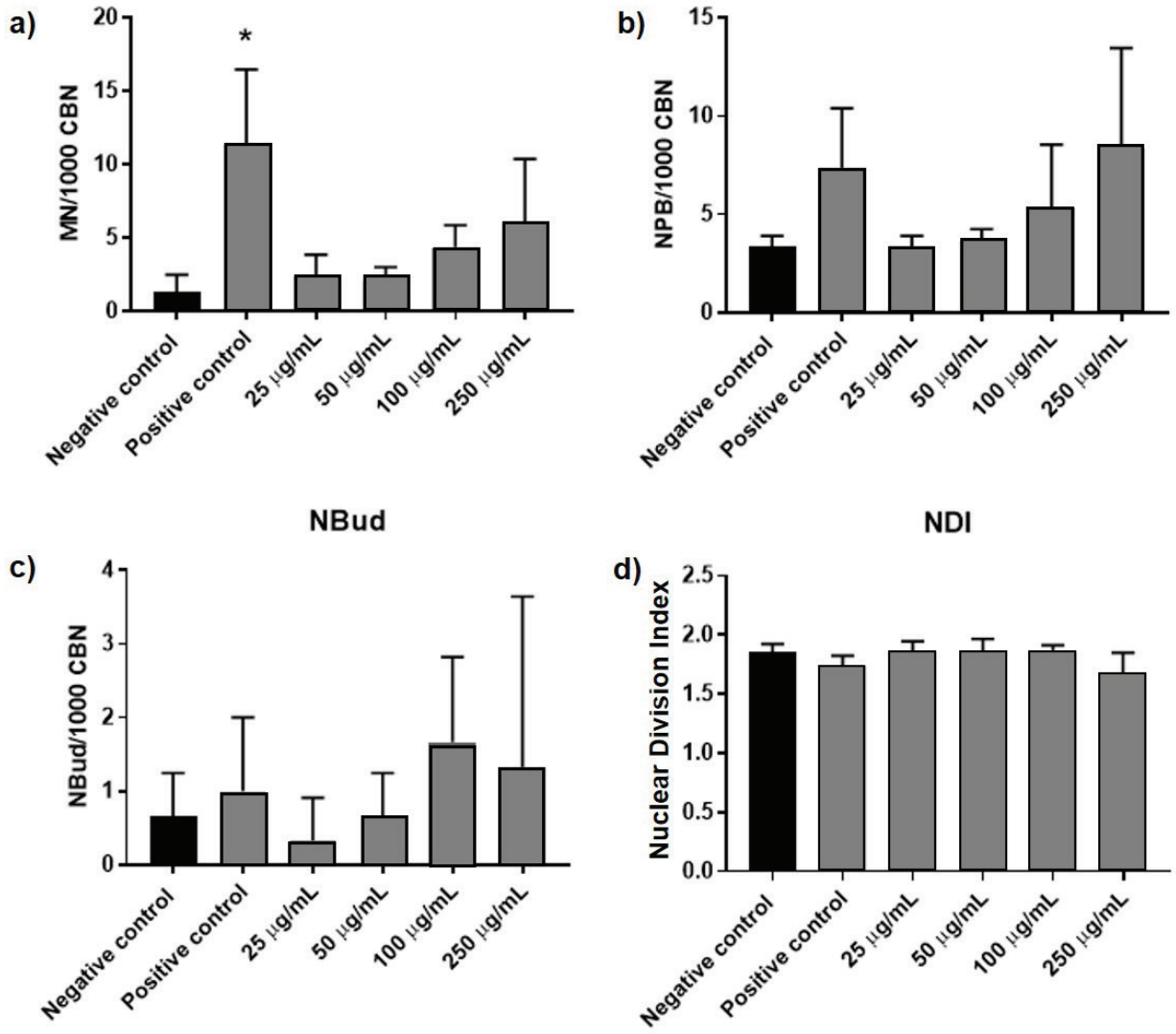

FIGURE 2. Genotoxic evaluation of ethanolic extracts from *w* (EEEM) leaves with cytokinesis-block micronucleus cytome assay. Frequency of (a) micronuclei (MN), (b) nucleoplasmatic bridges (NPB), (c) nuclear buds (NBUD), and (d) nuclear division indices (NDI) in PBMCs treated for 48 hours with different EEEM concentrations. Positive control groups were treated with methyl methanesulfonate (MMS) at a concentration of 150 µg/mL. MN, NPB, and NBud analyses of 3000 binucleated cells were conducted for each treatment group. 1500 cells were analyzed per treatment for NDI calculation. The data represent the mean ± standard deviation of three independent experiments. *These results were significantly different from the negative control ($p < 0.05$). followed by Tukey multiple comparison post-test.

DISCUSSION

E. macrophyllus presents medicinal properties effective against a variety of diseases; the evaluation of its potential cytotoxic and genotoxic effects is therefore important to ensure its safety for therapeutic uses. This study aimed to investigate the genotoxic potential of EEEM with regard to PBMCs. The results obtained in this study did not show any potential genotoxic effects.

According to VAZ (2016), the use of *E. macrophyllus* does not produce the clinical signs of toxicity in Wistar rats, and is therefore considered safe. Studies with other plant species of the genus *Echinodorus*, such as *Echinodorus grandiflorus*, have also shown no indication of significant toxicit (MARQUES, 2017). Despite this, the existing literature on *E. macrophyllus* offers very little information about its toxicity and genotoxicity. Ethnopharmacological information alone is insufficient to ensure the use of medicinal plants is safe (REYES-GARCIA, 2010). Thus, more studies are necessary to establish the safety of using and consuming *E. macrophyllus* (DA COSTA LOPES, 2000).

In this study, we found that EEEM at a concentration of 500 µg/mL caused a potential reduction in cell viability despite this, however, there was no statistical difference when compared with the negative control. Concentrations less than 250 µg/mL showed cell viability greater than 70%, even though no dose-dependent relationship was present. Similar results were observed in an *in vitro* cytotoxicity evaluation using hepatoma cell lines (HEC) and kidney cell lines (HEK); the higher doses of the *E. macrophyllus* aqueous extract were able to inhibit cell growth, although there was no sign of cell death (DA COSTA LOPES, 2000). In an *in vitro* study with J774 cells, no cytotoxic effects of the aqueous extract of *E. macrophyllus* were observed; however, there was an inhibition of cell growth that occurred when using concentrations of 600 µg/mL (PINTO, 2007). The data obtained in this study compared with that of other studies show that EEEM does not present cytotoxic activity.

The evaluation of the EEEM samples by comet assay also did not reveal significant damage to the DNA of the cells, and there was no dose-dependent relationship. Despite this, we observed that concentrations up 250 µg/mL caused a potential increase in DNA damage, but without statistical significance. Similar results were obtained in an in vivo study using an aqueous extract. The highest dose indicated a low to moderate frequency of DNA lesions in kidney cells, caused by both lyophilized and crude *E. macrophyllus* aqueous extract. In addition, genotoxicity with regard to blood and liver cells was not detectable at lower or highest doses (DA COSTA LOPES, 2000). EEEM was also used in an in vivo evaluation conducted by VAZ (2016), and no DNA damage was observed for any concentrations. The results of this study and previous results published in the literature suggest that *E. macrophyllus* does not cause significant DNA damage, especially when used at low concentrations.

HT-29 cells (a type of cancer cell) were exposed to a KPF5, a substance isolated from the Kakadu plum, a fruit native to Australia; exposure to a 0.5 mg/mL treatment resulted in an increase in MN frequency, and treatment with concentrations of 1.0 mg/mL caused a decrease in the nuclear division index (NDI), showing a cytostatic effect. The other biomarkers could not be quantified, since this treatment also decreased the number of binucleated cells (TAN, 2011). Our study showed a different result; EEEM presented a low frequency of micronulei with a dose-dependent relationship.

There was no significant influence on NDI, suggesting no cytostatic effect. However, the other markers (NPB and NBud) showed a dose-dependent relationship but no statistical difference. These data show that *E. macrophyllus* is not capable of inducing DNA damage but also suggest that high concentrations may interfere with DNA repair.

Other studies that also evaluate mutagenicity potential, such as the AMES test, also did not show a clear induction of mutagenicity when *E. macrophyllus* aqueous extracts were tested with Salmonella typhimurium strains TA97a, TA98, TA100, and TA102 (DA COSTA LOPES, 2000). However, VIDAL (2010) observed that a lyophilized extract of *E. macrophyllus* is mutagenic against Escherichia coli strains CC103 and CC104 and against the TA98 strain of Salmonella typhimurium.

CONCLUSION

The present study has indicated the absence of EEEM cytotoxicity, genotoxicity, and mutagenicity against PBMCs, under evaluated conditions. In conclusion, our study suggests the safety of, and absence of significant risks associated with using and consuming *E. macrophyllus*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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