Anais da Academia Brasileira de Ciências (2018) 90(3): 3165-3174
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201820180144
www.scielo.br/aabc | www.fb.com/aabcjournal

Hibiscus acetosella extract protects against alkylating agent-induced DNA damage in mice

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Manuscript received on February 15, 2018; accepted for publication on May 11, 2018

ABSTRACT

Hibiscus acetosella was shown to exert beneficial effects in humans and animal models however, the effects of this plant on DNA are unknown. The aim of this study was to determine the antigenotoxic and antimutagenic effects of *H. acetosella* extracts on alkylating agent methyl methanesulfonate (MMS) *in vivo* in mice. Initially, we performed analysis of phenolic compounds in extracts of *H. acetosella* by high-performance liquid chromatography (HPLC). Next, mice were divided into 8 groups and treated with distilled water or plant extract (0.1 ml/10 g) by gavage for 15 days, followed by intraperitoneal (ip) administration of saline solution or MMS (40 mg/Kg b.w) on day 16. Caffeic acid, following by gallic acid, gallocatechin, coumaric acid, and 3,4-dihydroxybenzoic acid were found to be present in extracts of *H. acetosella* leaves. In peripheral blood analysis of groups receiving pretreatment with *H. acetosella* at doses of 50 or 100 mg/kg plus MMS decreased DNA damage as evidenced by comet assay and Micronucleus assays relative to MMS alone. These results suggested that *H. acetosella* extracts exerted protective effects dose dependent against genotoxicity and mutagenicity induced by alkylating agents.

Key words: *Hibiscus acetosella*, antigenotoxicity, comet assay, antimutagenicity, micronucleus test.

INTRODUCTION

The low availability of currently available drugs have driven research in herbal medicine, with the goal of identifying new pharmacotherapeutic agents (Ouédraogo et al. 2012, Venancio et al. 2016, Ferreira et al. 2016, Trindade et al. 2016). In this context, medicinal plants are often used for either prevention or treatment of several human diseases (Gemelli et al. 2015, dos Santos et al. 2016, Niwa et al. 2016). The efficacy of these medicinal plants is often attributed to their antioxidant activities (Gülçin et al. 2006, Trindade et al. 2016, Ferreira et al. 2016). Thus, plants used in traditional medicine may constitute an important source of new biologically active compounds (Da-Costa-Rocha et al. 2014).

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In Latin America, biodiversity is extensive; 40% of the countries in Latin America, including Brazil, have more plant species than most other nations worldwide (Michán and Llorente-Bousquets 2009). In this context, natural plant extracts of the Malvaceae family were examined as potential sources of biologically active compounds (Kiessoun et al. 2012). The Malvaceae family consists of 75 genera and 1500 species, which are widely distributed and found primarily in tropical and subtropical regions (Wang et al. 2012).

The Hibiscus genus is the largest of the Malvaceae family, consisting of approximately 300 species of annual or perennial herbs, shrubs, and trees (Wang et al. 2012). Some investigators suggested that plants of this genus possess antioxidant properties (Farombi and Fakoya 2005, Rosa et al. 2006, Olalye and Rocha 2007, Mohamed et al. 2013, Ehsan et al. 2015, Kapelula et al. 2017) owing to their potent scavenging effects on reactive oxygen species (ROS) and free radicals such as hydrogen peroxide (H_2O_2). Components of this genus including flavonoids and polyphenolic compounds are often active dietary constituents that contribute to these protective effects by exerting antioxidant actions. Indeed, Kapepula et al. (2017) observed that although the species present different compositions, *H. acetosella, H. cannabinus and H. sabdariffa* displayed high cellular antioxidant activity with IC50 values ranging from 0.5 to 3 μg mL−1.

Pharmacological investigations of the Hibiscus genus reported that species of this genus displayed biological activities, including anti-inflammatory, antimicrobial, antidiabetic, hepatoprotective, and anticancer activities (Adetutu et al. 2004, Farombi and Fakoya 2005, Olalye and Rocha 2007, Rosa et al. 2006). Therefore, the aim of this study was to determine whether *Hibiscus acetosella* extracts exerted antigenotoxic and antimutagenic capacities *in vivo* in a mouse model by using alkylating agents to induce DNA damage.

MATERIALS AND METHODS

PLANT COLLECTION AND EXTRACT PREPARATION

H. acetosella WeLw ex Hiern leaves were obtained from the Pe. Dr. Raulino Reitz Herbarium of University of Southern Santa Catarina (UNESC), City of Criciúma, Santa Catarina State, Brazil. The plant material used in the experiment was collected in a single time of the year (September), in order to avoid the possible change of chemical compounds in different seasons. An exsicta of the plant was previously identified by the botanic Dra. Vanilde Citadini Zanette and kept in the herbarium (register no: CRI:8551). Preparation of the extract was carried out as previously described (Carvajal-Zarrabal et al. 2009). The specimens were dried in an oven at 40°C for 40 hr. Subsequently dried leaves were crushed, to yield a powder, and 196.7 g of the material was subjected to maceration in 1 L ethanol at 70% (proportion of 1:5) for 15 days. Then the mixture was filtered, and solvent was eliminated using a rotary evaporator, yielding 57 g sample. The extract was diluted with distilled water for administration to animals by gavage.

ANALYSIS OF PHENOLIC COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Aliquots (10 μl) of ethanolic extract (10 mg/ml dry weight) were analyzed using a liquid chromatograph (Shimzadu LC-10A) equipped with a C18 reverse-phase column (Vydac 218TP54; 250 mm × 4.6 mm Ø, 5 μm, 30 °C) and a UV-Vis spectrophotometric detector operating at 280 nm. Elution was carried out using water: acetic acid: n-butanol (350:1:10, v/v/v) as the mobile phase, with a flow rate of 0.8 ml/min. Detection of the compounds of interest was performed using co-chromatography and comparisons of retention times with standard compounds (gallocatechin, gallic acid, protocatechuic acid, coumaric acid, and

caffeic acid; Sigma, St. Louis, MO, USA), under the same experimental conditions. Quantification of the phenolic compounds was carried out using an external standard curve for gallic acid (5–300 μg/ml, $r^2 = 0.9958$; y = 35158x), considering the peak areas of interest to calculate concentration. The presented values correspond to means of three injections per sample. The concentrations of phenolic compounds are expressed as μg/g dry mass.

ANIMALS

In the present study, 48 male Swiss albino mice (weighing approximately: 25 g; age: 7–8 weeks) were obtained from the Animal Center of the University of Southern Santa Catarina (UNESC, Brazil). All procedures involving animals and their care were performed in accordance with national and international laws and guidelines for the use of animals in biomedical research. The experimental procedures were approved by the local ethics committee for animal use (CEUA – UNESC; approval no.: 130/2011). Mice were randomized by weight and housed in polycarbonate cages with steel wire tops (6 animals per cage). Mice were housed under conditions of controlled room temperature (22 ± 2°C) and humidity (55% ± 10%) and exposed to alternating 12 hr light/dark cycle. On the last day of the experiment, the animals were acclimated in the laboratory for 30 minutes with controlled temperature of 22 ± 1ºC. Care was taken to minimize suffering and reduce external sources of stress, pain, and discomfort for the animals. Only the minimum number of animals necessary to produce reliable scientific data was used.

EXPERIMENTAL DESIGN

Animals were divided into 8 groups of 6 mice per group. The animals were treated as described in Table I. Mice in different groups were treated with distilled water or plant extracts (50, 100, or 200 mg/kg; 0.1 ml /10 g body weight) by daily gavage for 15 days, followed by intraperitoneal (ip) administration of saline solution, MMS (40 mg/Kg b.w).

The doses of *H. acetosella* extracts used in this study were selected based on previous studies using

TABLE I
Experimental design to evaluate antigenotoxicity and antimutagenicity of *Hibiscus acetosella* extracts.

Group	Dose	Period	Day 16	Day 16 (4 h)	Day 17 (24 h)
1 (Negative control)	Distillated water	15 days	Saline solution	Peripheral blood collection	Death and collection of bone marrow
2 (Positive control)	Distilled water	15 days	MMS		
3	Extract (50 mg/kg)	15 days	Saline solution		
4	Extract (100 mg/kg)	15 days	Saline solution		
5	Extract (200 mg/kg)	15 days	Saline solution		
6	Extract (50 mg/kg)	15 days	MMS		
7	Extract (100 mg/kg)	15 days	MMS		
8	Extract (200 mg/kg)	15 days	MMS		

MMS: methyl methanesulfonate.

other species of Hibiscus with biological activities in animal models (Adetutu et al. 2004, Liu et al. 2010, Olalye and Rocha 2007).

All treatments were made at the same time each day. Four hr after administration, peripheral blood was collected by tail vein without anesthesia. Twenty-four hr later, animals were killed by cervical dislocation, and bone marrow removed immediately. The samples were then processed for comet assays and micronucleus (MN) tests.

COMET ASSAY

Comet assays were carried out under alkaline conditions, as previously described by Tice et al. DC, March 25-26, 1999, an expert panel met to develop guidelines for the use of the single-cell gel (SCG(2000), and visual scores were classified according to the method of Collins et al. (1997). Peripheral blood samples were collected in heparinized tubes and kept on ice. Blood cell aliquots (5 µl) were embedded in low melting agarose (0.75%, w/v; 95 or 80 µl, respectively). These mixtures were then placed onto microscope slides, which were precoated with normal melting point agarose (1.5%, w/v) and furnished with cover slips (two slides per sample). The slides were briefly placed on ice, and cover slips removed. The base slides were immersed in freshly prepared lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid [EDTA], 10 mM Tris, pH 10.0–10.5) and then incubated for 20 min in freshly prepared alkaline buffer (300 mM NaOH, 1 mM EDTA, pH > 13). Electrophoresis (15 min/300 mA; 25 V; 0.7 V/cm) was then performed using the same buffer. All of these steps were carried out in minimal indirect light. Following electrophoresis, slides were neutralized with 400 mM Tris (pH 7.5) and stained with ethidium bromide solution (10 mg/ml). The images of 100 randomly selected cells (50 cells from each of the two replicate slides) were analyzed for each individual mice. DNA damage index (DI) were calculated by visually separating cells into 5 classes according to tail size (0 = no tails to 4 = maximum-length tails). An individual DI was thus obtained for each sample and consequently for each group studied. The group DI ranged between 0 (completely undamaged = 100 cells × 0) and 400 (maximum damage = 100 cells × 4). Visual scores of comet assays are considered a reliable evaluation method and are typically consistent with computer-based image analysis methods. All slides were coded for blind analysis.

PERCENTAGE OF DAMAGE REDUCTION (DR%)

The MMS DR% upon *Hibiscus acetosella* extracts administration was calculated as follows: [MMS mean-the mean of a plant extracts]/ (MMS mean-control group mean). Results were multiplied by 100 to obtain DR%. This procedure was performed to evaluate DR% in the comet assay according to Mauro et al. (2013).

MICRONUCLEUS (MN) TESTS

MN tests were performed and analyzed according to the guidelines of the Gene-Tox program described by Mavournin et al. (1990). Bone marrow was extracted from mice femurs, and smears were placed directly onto microscope slide using a drop of fetal calf serum (FCS). Slides were stained with Giemsa (5%), air-dried, and coded for blind analysis. To avoid false-negative results and as a toxicity measure, the ratio between polychromatic and normochromatic erythrocytes (PCE: NCE) was determined for 200 erythrocytes per animal. The presence of micronuclei (MN) was measured for 2000 erythrocytes for each animal (1000 from each of the two slides), using bright-field microscopy (1000× magnification). For individual animals, mean values of micronucleated polychromatic erythrocytes (MnPCE) and micronucleated normochromatic erythrocytes (MnNCE) were used

as the experimental unit. The variation was based upon differences between mice of different groups.

STATISTICAL ANALYSIS

Statistical analyses were carried out using the Bioestat 5.8.4 software package. Results are expressed as mean values ± standard deviations. The normality of variables was evaluated using the Kolmogorov-Smirnov test. Statistical analyses for all groups were carried out using one-way analysis of variance (ANOVA). When ANOVA showed significant differences ($p < 0.05$), post-hoc analysis was performed with Tukey's test.

RESULTS

QUANTITATIVE AND QUALITATIVE ANALYSIS OF PHENOLIC COMPOUNDS BY HPLC

Chromatographic analyses showed higher levels of caffeic acid, following by gallic acid, gallocatechin, coumaric acid, and 3,4-dihydroxybenzoic acid, in extracts of *H. acetosella* (Figure 1 and Table II).

COMET ASSAYS: GENOTOXICITY AND ANTIGENOTOXICITY

Data demonstrated that MMS alone (group 2) significantly increased DNA damage using comet assays (DI) in peripheral blood ($P < 0.05$) (Figures 2a and 2b). Also, the figure 2a show the genotoxic effects of different doses of *H. acetosella,* constituting groups 3–5 (50 mg/kg,100 mg/kg and 200 mg/kg, respectively). In this way, we can see that peripheral blood of groups treated with *H. acetosella* extracts at doses of 100 and 200 mg/kg showed increased DNA damage in relation to control group ($P < 0.05$).

Next, we evaluated the antigenotoxic effects of different doses of *H. acetosella*, constituting groups 6–8 (50 mg/kg plus MMS, 100 mg/kg plus MMS, and 200 mg/kg plus MMS, respectively) in relation to the DNA damage induced in groups 2 (positive control MMS) (Figure 2b). Peripheral blood of groups pretreated with *H. acetosella*

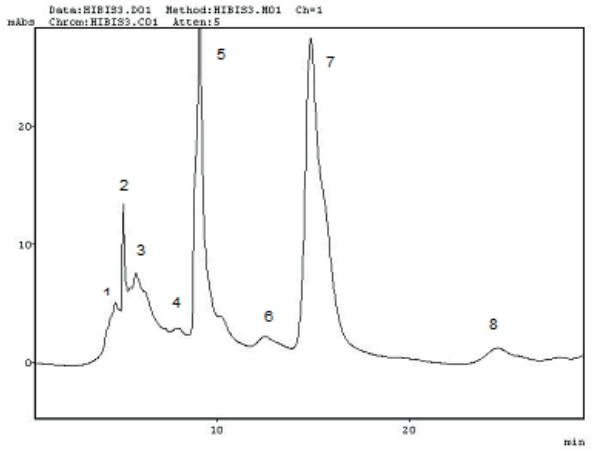

Figure 1 - Chromatographic profiles of phenolic compounds of *H. acetosella* extracts (HPLC, 280 nm). Peak 1: gallocatechin; 2: unidentified compound; 3: gallic acid; 4: 3,4-dihydroxybenzoic acid; 5: unidentified compound; 6: unidentified compound; 7: caffeic acid; 8: coumaric acid.

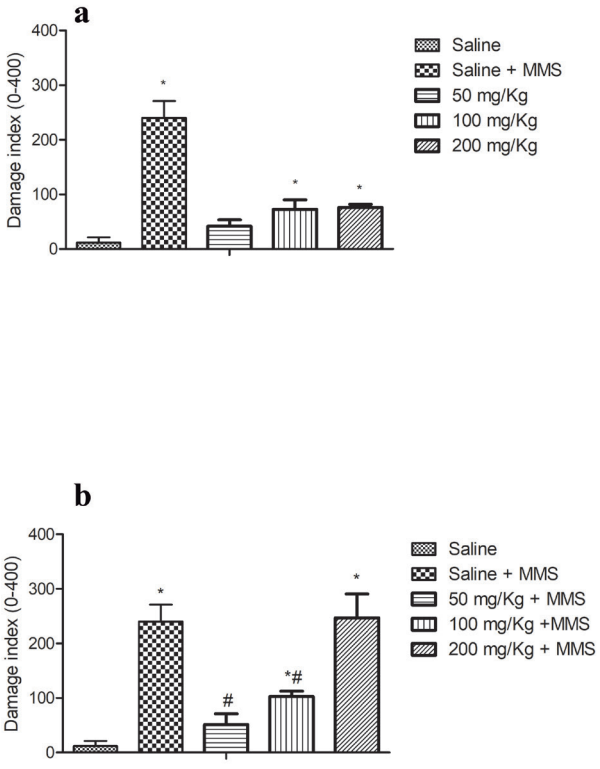

Figure 2 - Genotoxic (**a**) and Antigenotoxic (**b**) effects of different doses of *H. acetosella* in the blood cells of mice. *$p < 0.05$ compared with saline; $^{\#}p < 0.05$, compared with saline plus MMS, using ANOVA and Tukey test.

TABLE II
HPLC analysis of phenolic compounds (µg/mL, dry weight) in ethanolic extracts of *Hibiscus acetosella*. Values are expressed as means ± standard deviations.

Compound	Concentration (µg/mL)
Caffeic acid	51.13 ± 2.60
Coumaric acid	3.72 ± 0.43
3,4 protocatechuic dihydroxybenzoic acid	3.46 ± 0.67
Gallocatechin	4.13 ± 1.06
Gallic acid	14.22 ± 3.71

TABLE III
Values obtained using MN tests in bone marrow at different doses of *Hibiscus acetosella*.

Treatment	Number of animals	MnPCEs	PCE/NCE
Negative control	6	2.50 ± 1.05	0.53±0.03
Positive control MMS	6	8.20 ± 3.83*	0.54 ± 0.04
Extract 50 mg/kg	6	2.17 ± 1.17#	0.55±0.03
Extract 100 mg/kg	6	1.80± 1.30#	0.55±0.04
Extract 200 mg/kg	6	2.00 ± 1.67#	0.54±0.03

MMS: methyl methanesulfonate.
Data are means ± standard deviations (n = 6 animals). A total of 2000 polychromatic erythrocytes (PCEs) were analyzed per animal.
*Significant difference compared with the negative control, $p < 0.05$ (ANOVA, Tukey test).
#Significant difference compared with the positive control MMS, $p < 0.05$ (ANOVA, Tukey test).

extracts at doses of 50 or 100 mg/kg plus MMS showed decreased DNA damage in relation to those treated with MMS alone ($P < 0.05$) (Figure 2b). For the percentages of peripheral cells, mice that received extracts at doses of 50 and 100 mg/kg showed reductions of 78.8% and 57.2% for DI, respectively, compared with those of the positive control group (MMS). In animals treated with the higher dose of 200 mg/kg, damage caused by MMS could not be prevented ($P > 0.05$) (Figure 2b).

MN tests: mutagenicity and antimutagenicity

Animals treated only with the MMS showed high levels of MnPCEs in the bone marrow compared with that in control animals (Table III). Next, we evaluated the mutagenic effects of different doses of *H. acetosella*, constituting groups 3–5 (50 mg/kg, 100 mg/kg, and 200 mg/kg, respectively). No significant differences in MnPCE levels were observed between the negative control and the groups treated with *H. acetosella* extracts (Table III).

Table IV shows antimutagenic effects of different doses of *H. acetosella*, constituting groups 6–8 (50 mg/kg plus MMS, 100 mg/kg plus MMS, and 200 mg/kg plus MMS, respectively). Oral administration of extracts at low and intermediate doses (50 and 100 mg/kg) significantly decreased MnPCE levels compared with that in the MMS group (Table IV). The inhibition percentages were 88.2% and 85.4%, respectively. No significant differences in MnPCE levels were observed between the MMS and 200 mg/kg plus MMS groups (Table IV).

DISCUSSION

Medicinal plants are widely used and may have critical applications as nutraceuticals and phytomedicine. A literature review revealed that plants of genus Hibiscus are rich in phenolics, flavonoids, and tannins (Ramirez-Rodrigues et al. 2011). In our experiments, we show that administration of H. acetosella extracts at low dose (50 mg/kg) was not genotoxic or mutagenic and still protected DNA from damage caused by the alkylating agent (MMS). Besides, our results suggest that the protective effect of H. acetosella extracts appears to be dose dependent. Collectively, our results offer new insight into the H. acetosella extracts impacts in the in the DNA damage index.

In the present study, caffeic acid, following by gallic acid, gallocatechin, coumaric acid, and 3,4-dihydroxybenzoic acid were found to be present in extracts of *H. acetosella* leaves, as previously reported (Tsumbu et al. 2012, Kapelula

TABLE IV
Values obtained using MN tests in bone marrow at different doses of *Hibiscus acetosella* after administration of MMS.

Treatment	Number of animals	MnPCEs	Reduction
Negative control	6	2.50 ± 1.05	-
Positive control MMS	6	8.20 ± 3.83*	-
Extract 50 mg/kg + MMS	6	3.17 ± 1.33#	88.2%
Extract 100 mg/kg + MMS	6	3.33 ± 0.82#	85.4%
Extract 200 mg/kg + MMS	6	5.50 ± 3.45	-

MMS: methyl methanesulfonate.
Data are means ± standard deviations (n = 6 animals). A total of 2000 polychromatic erythrocytes (PCEs) were analyzed per animal.
*Significant difference compared with the negative control, $p < 0.05$ (ANOVA, Tukey test).
#Significant difference compared with the positive control MMS, $p < 0.05$ (ANOVA, Tukey test).

et al. 2017). These compounds have been of particular interest for their potential bioactive properties and promising role as alternative treatment in several illnesses. In this sense, Li et al. (2015) in study *in vitro* showed that caffeic acid-treated cells generated lower levels of ROS that induced signal-regulated kinase (ERK) signaling pathway. Inhibition of ERK signaling blocked the caffeic acid -induced improvement of cell viability and protection against DNA damage caused by H_2O_2 treatment. Similarly, studies in human and animal models suggest that gallic acid reduces oxidatively damaged DNA in lymphocytes, liver, colon and lungs and protects these organs against γ-irradiation-induced strand breaks and formation of oxidatively damaged DNA-bases (Chakraborty et al. 2009, Nair and Nair 2013). Thus, these findings of phytochemicals reflected the important potential applications of these extracts.

The genotoxicity of *H. acetosella* extracts were evaluated and the dose of 100 and 200 mg/kg utilized presented effect genotoxic, suggesting that the protective effect of *H. acetosella* extracts appears to be dose dependent. However, mice that received 50 and 100 mg/kg of the extract plus MMS showed reduced DNA damage compared with MMS group, attenuating the genotoxic activity of the alkylating agent. The similar findings were previously reported by Rosa et al. as test media, it has been shown that HME strongly inhibited the mutagenic action of H(2(2006, 2007) that observe both antigenotoxic and antimutagenic effects against oxidative DNA damage of other species of Hibiscus (*Hibiscus tiliaceus*). Furthermore, the treatment with a dose of 200 mg/kg was genotoxic and not able to prevent DNA damage caused by MMS. However, the comet assay is a sensitive test that can detect small changes in the number of strand breaks within a nucleus, which can be repair (Collins et al. 1997). Thinking about that, we also performed MN test and observe that no dose of *H. acetosella* extracts was mutagenic. Also, the half-maximal lethal dose (LD_{50}) of Hibiscus extracts in rodents was found to be more than 5000 mg/kg, suggesting that the extract was virtually nontoxic (Ali et al. 2005).

Our MN test of mutagenicity in bone marrow were different of the findings of comet assays. We show that no dose of *H. acetosella* extracts was mutagenic. Also, pretreatment with *H. acetosella* decreased the damage caused by alkylating agent (MMS), primarily when used at a dose of 50 and 100 mg/kg of the extract. Notably, comet assays detect strand breaks, whereas MN tests provide

a measure of both chromosome breakage and chromosome loss and have been shown to be a sensitive indicator of chromosome damage (Mavournin et al. 1990). In accordance with our results, Olvera-García et al. (2008) and Farombi and Fakoya (2005) showed strong *in vitro* and *in vivo* antimutagenic activity of phenolic compounds in H. sabdariffa extracts. The antioxidant activity of phenolic compounds has been shown to inhibit mutations because they can scavenge free radicals or induce antioxidant enzymes (Hochstein and Atallah 1988). Thus, the present results suggest that the genoprotective assignments of *H. acetosella* may be similar as others antioxidants components, such vitamin C, that have the ability to compete with DNA alkylation targets, reducing the activity of alkylating agents, also having an important role in the regulation of DNA repair enzymes (Cooke et al. 1998).

On the other hand, has been proposed that other mechanisms than the antioxidant activities could be involved in the protective effect of these phenolic compounds. In this way, evidences suggest an ability of phenolic compounds to interact with signaling pathways that modulate apoptosis (Spencer 2007), including the nuclear factor-κB or mitogen-activated protein kinase (MAPK) pathways (Ramassamy 2006). Furthermore, phenolic acids have exhibited anti-inflammatory and anti-cancer properties (Ambriz-Pérez et al. 2016). Therefore, others biological mechanisms associated to antigenotoxic and antimutagenic action of *H. acetosella* extracts on alkylating agent can be involved.

In conclusion, under the described experimental conditions, administration of *H. acetosella* extracts at dose of 50 and 100 mg/kg was shown to have protective effects against the genotoxicity and mutagenicity induced by alkylating agents, probably due to the antioxidant activities of phenolic compounds. Besides, this protective effect appears to be dose dependent. Additional biochemical investigations are necessary to elucidate the mechanisms of action of *H. acetosella* extracts, which were found to have a role in protection against DNA damage. Thus, the results supported the utilization of this plant may have potential therapeutic interest and could justify their use in traditional medicine and local nutraceutical resources.

ACKNOWLEDGMENTS

We thank UNESC for financial support. The authors declare that they have no conflicts of interest.

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