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Toxic or medicinal? New approach to the pharmacological and toxicological properties of *Echinodorus macrophyllus* leaves

Tóxico ou medicinal? Nova abordagem das propriedades farmacológicas e toxicológicas das folhas de *Echinodorus macrophyllus*

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Echinodorus macrophyllus contains some phytochemicals that can induce toxicity but are also promising as ingredients in products to promote and maintain health, improve the immune system, and prevent diseases. In this study, the aqueous and ethanolic extracts of leaves of this species were characterized by high-performance liquid chromatography, investigated in vitro regarding their modulating action on snake venom enzymes, and evaluated in digestive enzyme inhibition assays. The extracts inhibited the phospholipase activity induced by *Bothrops jararacussu* and *B. moojeni* venoms. The hemolytic activity induced *by Crotalus durissus terrificus* venom was potentiated by 30-73% by the ethanolic extract at the ratios analyzed. The thrombolytic activity induced by *B. moojeni* venom was inhibited by 51% and 34% by the aqueous and ethanolic extracts at a ratio of 1:5 (w:w), respectively. The clotting time induced by B. moojeni and *Lachesis muta muta* venoms was prolonged after incubation with either extract. The extracts also exerted antigenotoxic action on DNA fragmentation induced by *L. muta muta* venom. Both extracts lowered the enzymatic activity of α-glucosidases, and the ethanolic extract inhibited α-amylases. The inhibition of digestive enzymes suggests the extracts could decrease calorie availability from carbohydrate intake. Results confirm the potential therapeutic use of *E. macrophyllus* in the prevention and treatment of hemostatic changes, since the proteins of venoms inhibited by it exhibit high homology with human proteins involved in various pathophysiological processes, such as inflammatory responses and blood coagulation.
Keywords: phospholipase A_2 inhibitors, protease inhibitors, digestive enzyme inhibitors.

Echinodorus macrophyllus contém alguns fitoquímicos que podem induzir toxicidade, mas também são promissores como ingredientes em produtos para promover e manter a saúde, melhorar o sistema imunológico e prevenir doenças. Os extratos aquoso e etanólico das folhas dessa espécie foram caracterizados por cromatografia líquida de alta eficiência, investigados *in vitro* quanto à sua ação moduladora sobre enzimas de peçonhas de serpentes e avaliados em ensaios de inibição de enzimas digestivas. Os extratos inibiram a atividade da fosfolipase induzida pelas peçonhas de *Bothrops jararacussu* e *B. moojeni*. A atividade hemolítica induzida pela peçonha de *Crotalus durissus terrificus* foi potencializada em 30-73% pelo extrato etanólico nas proporções analisadas. A atividade trombolítica induzida pela peçonha de *B. moojeni* foi inibida em 51% e 34% pelos extratos aquoso e etanólico na proporção de 1:5 (p:p), respectivamente. O tempo de coagulação induzido pelas peçonhas de *B. moojeni* e *Lachesis muta muta* foi prolongado após a incubação com qualquer um dos extratos. Os extratos também exerceram ação antigenotóxica na fragmentação do DNA induzida pela peçonha de *L. muta muta*. Ambos os extratos diminuíram a atividade enzimática das α-glicosidases, e o extrato etanólico inibiu as α-amilases. A inibição das enzimas digestivas sugere que os extratos podem diminuir a disponibilidade calórica da ingestão de carboidratos. Resultados confirmam o potencial uso terapêutico de *E. macrophyllus* na prevenção e tratamento de alterações hemostáticas, uma vez que as proteínas das peçonhas inibidas por ela apresentam alta homologia com proteínas humanas envolvidas em diversos processos fisiopatológicos, como respostas inflamatórias e coagulação sanguínea.
Palavras-chave: inibidores de fosfolipases A_2, inibidores de proteases, inibidores de enzimas digestivas.

1. INTRODUCTION

Evidence of the use of plants for medicinal purposes dates back to the dawn of civilization and is considered one of the oldest practices employed by humans for the cure, prevention, and treatment of diseases. In many cases, medicinal plants have been used as the only resource in primary health care due to the ease of access, no required medical prescription, high cost of manufactured drugs, and belief in the absence of toxic effects.

In folk medicine, the leaves of the species *Echinodorus macrophyllus* (Kunth) Micheli of the family Alismataceae are widely used in the form of tea prepared by infusion or decoction for the treatment of infections, inflammatory and respiratory diseases, and renal dysfunction [1]. This species is found in southeastern Brazil, mainly in flooded areas of the states of Minas Gerais and São Paulo, and is popularly known in Brazil as chapéu de couro [2]. Studies have shown the presence of some phenolic compounds, such as flavonoids, triterpenes, alkaloids, and tannins, in *E. macrophyllus* [1, 3]. Although this plant is already listed in the Brazilian Pharmacopoeia (both in 1926 and in 1959), studies are still needed on its use as a phytotherapeutic agent, as it has great pharmacological importance [2].

Bioactive compounds have been investigated as enzyme modulators for therapeutic applications in the prevention and treatment of various diseases. In this sense, snake venoms are valuable tools for the characterization of enzyme modulators since they are rich sources of phospholipases A_2 (PLA_2) and proteases (metalloproteases and serine proteases) that participate in the maintenance of hemostasis and in inflammatory and immune responses. These enzymes may exhibit up to 96% functional and structural homology with enzymes present in the human body [4, 5], making it possible to investigate the effects of enzyme inhibitors on human enzymes by studying their effects on snake venoms.

Thus, in this study, the aqueous and ethanolic extracts of *E. macrophyllus* leaves were characterized in pharmacological and toxicity assays to evaluate their effects on the activity of PLA_2 and proteases (using snake venoms as the study material) and on the activities of digestive enzymes, in addition to assessing their antigenotoxic/genotoxic potential by the comet assay, aiming to increase the knowledge about the nutraceutical potential of these extracts.

2. MATERIALS AND METHODS

2.1 Plant material: *Echinodorus macrophyllus* leaves

The leaves of *E. macrophyllus* were collected in March 2019 at the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil (21° 14′ S, 45° 00′ W, 918 m altitude). The species was identified in the Herbarium of the Department of Biology of UFLA, and a voucher specimen was added to the ESAL Herbarium collection under registration number 30,251.

The leaves were washed in distilled water and dried in an oven for 72 hours at 35 °C. The dried leaves were ground in a Wiley mill, and the powder obtained was used for extraction. The aqueous extract was obtained by infusion for 30 minutes at a ratio of 1:25 (w:v) and then filtered, frozen, and lyophilized. The ethanolic extract (70% ethanol) was obtained at room temperature by static maceration, filtered, subjected to solvent removal in a rotary evaporator (at 45°C), frozen, and lyophilized. This study received the authorization to access the genetic patrimony (CGEN – Brazil) under the process numbers A5D79A6.

2.2. Phytochemical screening

The aqueous and ethanolic extracts of *E. macrophyllus* leaves were subjected to phytochemical screening for organic acids, alkaloids, azulenes, carotenoids, catechins, depsides and depsidones, coumarin derivatives, steroids and triterpenoids, flavonoids, cardiotonic glycosides, sesquiterpene lactones and other lactones, saponins, purines, and tannins. Specific reagents were used for each chemical group so that the reactions resulted in the development of the color and/or precipitate characteristic of each class of substances [6].

2.3. Determination of the phenolic composition of the extracts by high-performance liquid chromatography

Chromatographic analysis was performed using a Shimadzu high-performance liquid chromatograph equipped with two LC-20AT high-pressure pumps, an SPD-M20A UV–visible detector, a CTO-20AC oven, a CBM-20A interface, and a SIL-20A injection-type autosampler. The separations were performed using a Shim-pack VP-ODS-C18 column (250 mm × 4.6 mm) connected to a Shim-pack Column Holder precolumn (10 mm × 4.6 mm). The lyophilized extracts were dissolved in water (1:20, w:v), and the phenolic standards were filtered through a 0.45-µm membrane (Millipore®) and injected into the chromatograph. The phenolic compounds were identified by comparison with the retention times of the standards. The standards used were ferulic acid, salicylic acid, syringic acid, gallic acid, caffeic acid, p-coumaric acid, vanillic acid, chlorogenic acid, epicatechin, catechin, epigallocatechin gallate, resveratrol, and quercetin. Quantification was performed by constructing analytical curves obtained by linear regression, considering a coefficient of determination (R^2) of 0.99 [7].

2.4. Snake venom

Crystallized crude venoms were commercially purchased from Bioagents Serpentarium (Batatais, São Paulo). The venoms were weighed (10 mg) and dissolved in 1 mL of phosphate-buffered saline (PBS, pH 7.4) to perform the tests.

2.5. Collection of human blood

The protocols that required the use of human biological material were previously approved by the Human Research Ethics Committee (COEP) of the Federal University of Lavras under registration number CAAE: 56628316.0.0000.5148. The blood used was collected by venipuncture in tubes containing heparin (for the hemolytic activity and comet assays), citrate (for clotting activity), or no anticoagulant (for thrombolytic activity).

2.6. Activity of phospholipase A_2 and lysis of erythrocytes

Phospholipase and hemolytic activities were evaluated in solid medium as described by Gutiérrez et al. (1988) [8]. The gel for evaluating phospholipase activity was prepared with 0.01 mol L^{-1} $CaCl_2$, egg yolk lecithins (phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine) 1:3 v:v in PBS (pH 7.4), 1% bacteriological agar, and 0.005% sodium azide. The medium was poured at 45-50°C into Petri dishes. For hemolytic activity, the gel was prepared by replacing the egg yolk phospholipids with a concentrate of human erythrocytes (1% hematocrit). After solidification of the gels, the treatments were applied into holes, and the Petri dishes were kept in a cell culture chamber for 12 hours at 37°C.

The inhibition of phospholipase A_2 was evaluated by preincubation of the aqueous and ethanolic extracts of *E. macrophyllus* leaves for 30 minutes at 37°C with *Bothrops atrox*, *B. jararacussu*, and B. moojeni venoms (30 µg) at different ratios (venom:extract, w:w). The inhibition of hemolytic activity was evaluated using *B. jararacussu*, *B. moojeni*, and *Crotalus durissus terrificus* venoms (50 µg). Controls containing only venom or plant extract were also evaluated.

The translucent halos formed around the holes in the gel were measured (millimeters). The activities are expressed as percentages, considering the controls containing only venoms as having 100% activity.

2.7. Effects on thrombus dissolution, citrated plasma coagulation, and fibrinogenolysis

Thrombolytic activity was evaluated in human blood clots formed in vitro as described by Cintra et al. (2012) [9]. The clots were incubated for 24 hours at 37°C with samples containing *B. moojeni* and *Lachesis muta muta* (40 μg), PBS, or venom preincubated (30 minutes at 37°C) with *E. macrophyllus* extracts. The activities were quantified by measuring the volume of liquid released by each thrombus. Controls containing only venom were defined as having 100% activity, and the mean of the volumes obtained in the negative control (PBS) was subtracted from the means of the other treatments.

The effects of *E. macrophyllus* extracts on plasma coagulation were evaluated using the method described by Rodrigues et al. (2000) [10]. The extracts were previously incubated with *B. moojeni* and *Lachesis muta muta* venoms, the mixtures were then added to tubes containing citrated plasma (200 μL), and the time until clot formation was measured. The minimum coagulant dose was defined as the smallest amount of venom capable of inducing coagulation in 50-180 seconds [11].

For the evaluation of fibrinogenolytic activity, 15% (w:v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used. The *E. macrophyllus* extracts were preincubated with *B. moojeni* venom (50 μg) for 30 minutes at 37°C, followed by the addition of fibrinogen and another 90 minutes of incubation. The samples were subjected to electrophoresis to observe the α, β, and γ chains of fibrinogen, which was used as a control. Controls containing fibrinogen, fibrinogen and extracts, and fibrinogen and venom were performed.

2.8. Evaluation of genotoxicity/antigenotoxicity

The comet assay was used to detect DNA damage in human leukocytes. The treatments containing *Lachesis muta muta* venom (50 μg in 300 μL of PBS) with the *E. macrophyllus* extracts were incubated for 30 minutes at 37°C. Then, 300 μL of blood was added to each treatment, and it was kept in a cell culture chamber for 4 hours at 37°C. Aliquots of 75 μL of the incubated samples were transferred to tubes containing 225 μL of low-melting-point agarose, and three slides were prepared for each treatment [12]. The cells included in the slides were subjected to osmotic lysis and then electrophoresis (30 V, 30 mA for 30 minutes). Next, the nucleoids were incubated in neutralization solution (0.4 M Tris-HCl, pH 7.4) for 25 minutes, fixed with ethanol, stained with propidium iodide, and observed under an epifluorescence microscope. The nucleotide fragmentation levels were determined as described by Collins et al. (1997) [13] with adaptations [14].

2.9. Kinetic assays with digestive enzymes: with and without gastric fluid simulation

The enzymes used in these assays were porcine pancreatic lipase (EC 3.1.1.3) type II (Sigma), porcine pancreatic α-amylase (EC 3.2.1.1) type VI B (Sigma), and porcine pancreatic trypsin (EC 3.4.21.4) (Merck). A-glucosidase (EC 3.2.1.20) was obtained from the fresh porcine duodenum as per Pereira et al. (2010) [15].

α-Amylases: The activity of α-amylases was determined according to the method proposed by Noelting and Bernfeld (2004) [16]. Thus, 50 μL of the sample and 50 μL of α-amylase were preincubated for 20 minutes in a water bath at 37°C. The substrate used was 1% starch (0.05 mol L^{-1} Tris, 38 mmol L^{-1} NaCl, and 0.1 mmol L^{-1} $CaCl_2$, pH 7.0). Samples and substrate (100 μL) were incubated for 10, 20, 30, or 40 minutes. The reactions were stopped by adding 3.5 dinitrosalicylic acid (200 μL), and the product was quantified in a spectrophotometer at 540 nm.

α-Glucosidases: The activity of α-glucosidases was determined according to Kwon et al. (2008) [17] using 5 mmol L^{-1} p-nitrophenyl-α-D-glucopyranoside as the substrate, prepared in 0.1 mol L^{-1} citrate-phosphate buffer, pH 7.0. In the assay, 50 μL of *E. macrophyllus* extracts and 100 μL of enzyme were incubated for 10, 20, 30, or 40 minutes in a 37°C bath after adding 50 μL

of substrate. The reactions were stopped by adding 1,000 μL of 0.05 mol L^{-1} NaOH, and the product was quantified in a spectrophotometer at 410 nm.

Lipases: Lipase activity was measured according to Souza et al. (2011) [18] using 8 mmol L^{-1} p-nitrophenyl palmitate as the substrate, prepared in 0.05 mmol L^{-1} Tris-HCl and 0.5% Triton X-100 buffer, pH 8.0. In the assay, 50 μL of the extracts and 100 μL of the enzyme were incubated for 10, 20, 30, or 40 minutes in a 37°C bath after adding 50 μL of the substrate. The reactions were stopped by being put in an ice bath and by adding 1,000 μL of 0.05 mmol L^{-1} Tris-HCl buffer, pH 8.0. The product was quantified in a spectrophotometer at 410 nm.

Trypsins: Trypsin activity was measured as described by Erlanger et al. (1961) [19]. Thus, 200 μL of the extracts and 200 μL of enzyme were incubated in a bath at 37°C for 10, 20, 30, and 40 minutes after adding 800 μL of the substrate p-benzoyl-DL arginine p-nitroanilide (BApNA), prepared in 0.05 mmol L^{-1} Tris buffer, pH 8.2. The reactions were stopped by adding 200 μL of 30% acetic acid, and the product was quantified in a spectrophotometer at 410 nm.

2.9.1. Gastric fluid simulation

The enzymatic activities were also evaluated after incubation of the extracts with simulated gastric fluid (prepared according to The United States and Pharmacopeia, 2005) [20] for 1 hour in a bath at 37°C. Before the activity assays, the incubated samples were neutralized with sodium bicarbonate to pH 7.2.

2.9.2. Data analysis

To meet the safety range of 40 to 80% enzyme inhibition, different concentrations of the aqueous and ethanolic extracts were evaluated.

The enzyme inhibitions were obtained by determining the slopes of the lines (absorbance × time) corresponding to the values obtained for the control enzyme (without extracts) and enzymes + sample (aqueous or ethanolic extracts) in the activity assays. The slope of the line corresponds to the product formation rate per minute of reaction. The presence of inhibitory agents results in a flatter slope. The absorbance values were converted into product micromoles based on data obtained from standard curves prepared with glucose (for α-amylase) and p-nitrophenol (for glucosidase and lipase). For trypsin, the inhibition units were calculated from the molar extinction coefficient of BApNA, determined by Erlanger et al. (1961) [19].

2.10. Statistical analysis

The results are presented as the mean of triplicates ± standard deviation obtained in three independent assays. The data were statistically evaluated by analysis of variance, and the means were compared by the Scott-Knott test in R statistical software [21]. Statistical significance was accepted at $p < 0.05$.

3. RESULTS AND DISCUSSION

The results of the phytochemical screening indicated the presence of catechins, flavonoids, steroids, triterpenes, coumarins, saponins, and alkaloids in the ethanolic extract of *Echinodorus macrophyllus*. In the aqueous extract, only flavonoids, depsides, and depsidones were detected.

The levels of phenolic compounds in both *E. macrophyllus* extracts are shown in Table 1 and Figure 1. The aqueous extract had 2.4 times as much phenolic compounds as the ethanolic extract. The extracts had different compositions, given that epicatechin and ferulic acid were detected only in the aqueous extract.

Table 1. Concentration of phenolic compounds in the aqueous and ethanolic extracts of Echinodorus macrophyllus leaves.

Phenolic compounds (mg100 g^{-1})	Aqueous extract	Ethanolic extract
Gallic acid	22.40 ± 0.40	15.13 ± 0.67
Catechin	125.29 ± 2.77	145.93 ± 1.00
Chlorogenic acid	30.34 ± 0.49	18.00 ± 0.46
Epicatechin	155.46 ± 0.89	nd
p -coumaric acid	290.71 ± 4.71	89.44 ± 1.17
Ferulic acid	31.43 ± 0.34	nd
Resveratrol	21.13 ± 0.30	18.00 ± 0.48
∑ Phenolic compounds	676.76	286.5

The results correspond to the means of triplicates and the calculated standard deviations. nd = not detected.

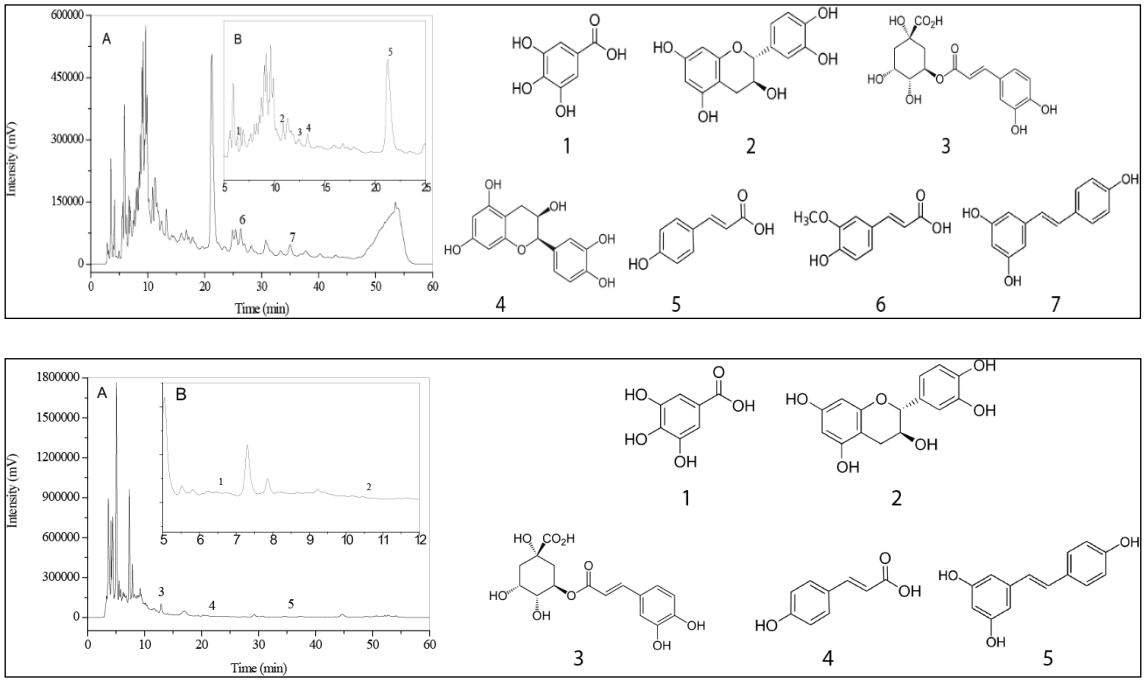

Figure 1. Upper image: (A) Chromatogram of the aqueous extract of Echinodorus macrophyllus leaves, with identification of the peaks: 1. gallic acid (time = 6.301 min); 2. catechin (time = 10.864 min); 3. chlorogenic acid (time = 12.429 min); 4. epicatechin (time = 13.272 min); 5. p-coumaric acid (time = 21.225 min); 6. ferulic acid (time = 26.222 min); and 7. resveratrol (time = 35.000). (B) Expansion of minutes 5-25 of the chromatogram. Lower image: Chromatogram of the ethanolic extract of Echinodorus macrophyllus leaves, with identification of the peaks: 1. gallic acid (time = 6.560 min); 2. catechin (time = 10.867 min); 3. chlorogenic acid (time = 12.592 min); 4. p-coumaric acid (time = 21.468 min); and 5. resveratrol (time = 35.017 min). (B) Expansion of minutes 5-12 of the chromatogram.

Phenolic compounds have aroused great interest in the medical field because they have pharmacological properties, such as cytotoxic and chemoprotective activities [22]. Long-term intake of these compounds may reduce the incidence of numerous diseases that affect the world population, such as different types of cancer, type 2 diabetes, cardiovascular diseases, and nervous system diseases [23].

Some bioactive compounds, such as saponins, flavonoids, alkaloids, triterpenes, and steroids, have already been identified in the species *E. macrophyllus* [1], as observed in the present study. Flavonoids have been related to the anti-inflammatory effect of this species [1, 24]. The flavonoid-enriched fraction of the aqueous extract of *E. macrophyllus* can inhibit carrageenan-induced inflammation in mice, in addition to reducing the levels of nitric oxide and leukotriene

B4 and the migration of neutrophils [24]. Flavonoids can also protect platelets against peroxidative stress, modulating the activity of enzymes involved in arachidonic acid metabolism, reducing the release of reactive oxygen intermediates, and inhibiting platelet activation and clot formation [25, 26].

Thus, the phenolic compounds present in plants represent promising molecules for use as therapeutic agents. However, the efficacy of these compounds depends on their metabolism and bioavailability [23].

3.1 Activity of phospholipase A_2 and lysis of erythrocytes

The aqueous and ethanolic extracts of *E. macrophyllus* significantly potentiated the phospholipase activity induced by *Bothrops atrox* venom, by 6.2% and 12.5% at the ratios evaluated (Figure 2A). For *B. jararacussu* venom, 7.1% inhibition was observed after incubation with the aqueous extract at ratios of 1:0.1 to 1:2.5 (w:w) and with the ethanolic extract at all ratios (Figure 2B). Only the aqueous extract, at the highest ratios, inhibited (7%) the phospholipase activity induced by *B. moojeni* venom (Figure 2C).

Phospholipase A_2 of snake venoms catalyzes the hydrolysis of phospholipids in the ester bond of carbon 2, causing damage to cell membranes and releasing lysophospholipids and fatty acids. The hydrolysis of phospholipids results in activation of the inflammatory response and coagulation cascade due to the production of eicosanoids through the action of cyclooxygenases and lipoxygenases on the structure of arachidonic acid [27]. Several research groups have investigated natural compounds with inhibitory action on phospholipases from snake venoms, since they have high structural and functional homology with human phospholipases, acting in several physiological processes, allowing us to simulate the effects of these compounds in animals [5]. The inhibitory activity on phospholipase A_2 associated with the presence of phenolic compounds, such as gallic acid, caffeic acid, and epigallocatechin gallate, has been described for different plant extracts with inhibitory and/or preventive action on inflammatory processes [28]. Thus, the inhibition of phospholipases observed in the present study can be partially attributed to the presence of phenolic acids and flavonoids in the extracts of *E. macrophyllus*, since these are associated with a lower production of inflammatory mediators with action on phospholipases and cyclooxygenases [29].

The hemolytic activity is shown in Figure 2. The erythrocyte lysis assay has been used as an *in vitro* experimental model to investigate the toxic and anti-cytotoxic effects of a wide variety of natural, chemical, and synthetic compounds. In the present study, the plant extracts did not induce hemolysis under the concentrations and incubation times evaluated. However, the extracts altered the hemolytic activity exerted by the venoms. The aqueous extract of *E. macrophyllus* at a ratio of 1:5 (venom:extract, w:w) inhibited 100% of the hemolytic activity induced by *B. jararacussu* venom (Figure 2D). Regarding the hemolysis induced by *B. moojeni* venom, the aqueous and ethanolic extracts exerted statistically significant inhibition, ranging from 11 to 100% and 30 to 100%, respectively (Figure 2E). Both aqueous and ethanolic extracts potentiated the hemolysis induced by the venom of *C. durissus terrificus*, with greater intensity at the ratios 1:0.5 (68%) and 1:1 (73%), respectively (Figure 2F).

Studies conducted by Assafim et al. (2011) [30] demonstrated that the ethanolic extract of *Hypericum brasiliense* inhibited 100% of the hemolytic activity induced by *B. jararaca* venom at a ratio of 1:50 (w:w). In contrast, Simão et al. (2015) [31] reported the induction of hemolysis by the aqueous extract of *Pereskia grandifolia*, possibly resulting from interactions of the extract constituents with structures that make up the erythrocyte membranes. These researchers partially attributed the hemolytic action to the presence of saponins in the *P. grandifolia* extract. Saponins can interact with sterols present in the plasma membrane of erythrocytes, increasing membrane permeability and allowing the excessive entry of ions and water into cells, resulting in their rupture [32]. Considering the results obtained in the present study, the saponins present in the *E. macrophyllus* extracts may have potentiated the hemolytic action exerted by the venoms in some analyzed ratios.

Thus, the synergistic and additive effects of phenolic compounds present in the extracts of *E. macrophyllus* can be observed in the inhibition and/or potentiation of the activities exerted by phospholipase A_2 and proteases (metalloproteases and serine proteases), with the effects varying according to the concentration evaluated. Phenolic compounds can also bind to the active site or to a coordination site of ionic cofactors, promoting conformational changes in enzymes and consequently altering their catalytic activity [5].

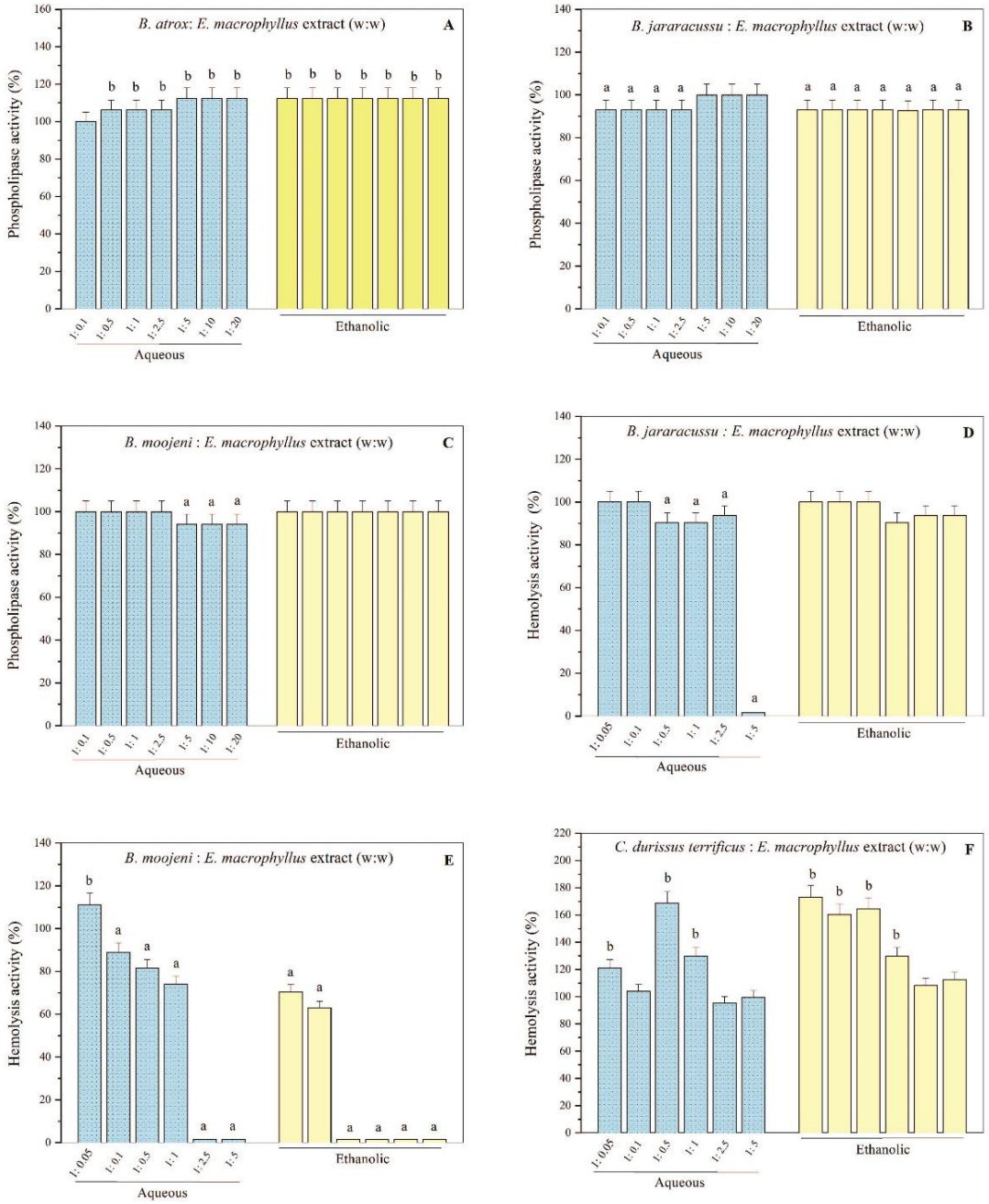

Figure 2. Phospholipase activity (%) induced by venoms of Bothrops atrox (A), Bothrops jararacussu (B), and Bothrops moojeni (C) preincubated with the aqueous or ethanolic extract of Echinodorus macrophyllus leaves. Hemolytic activity (%) induced by venoms of Bothrops jararacussu (D), Bothrops moojeni (E), and Crotalus durissus terrificus (F) preincubated with the aqueous or ethanolic extract of Echinodorus macrophyllus leaves. Controls (+) containing only venoms (30 µg for phospholipase and 50 µg for hemolysis) were considered to have 100% activity. The results correspond to the means of triplicates of data obtained AT each ratio (venom:extract, w:w) and the calculated standard deviations. a: differs significantly from the respective positive control by the Scott-Knott test ($p < 0.05$) in inhibition. b: differs in potentiation.

3.2 Effects on thrombus dissolution, citrated plasma coagulation, and fibrinogenolysis

The thrombolytic activity induced by *B. moojeni* and *Lachesis muta muta* venoms previously incubated with aqueous and ethanolic extracts of *E. macrophyllus* is shown in Figure 3. The aqueous extract potentiated thrombolysis induced by *B. moojeni* venom at ratios of 1:0.5 and 1:1 (w:w) 35 and 25%, respectively. At the 1:5 ratio, 51% inhibition was observed. The ethanolic extract inhibited thrombolytic activity at the ratios 1:1, 1:2.5, and 1:5 (w:w). At the highest ratio, the inhibition was 34% (Figure 3A). For *L. muta muta* venom, the thrombolytic activity was potentiated 43% by the aqueous extract (1:0.5, w:w) and 47% by the ethanolic extract (1:2.5 and 1:5, w:w) (Figure 3B).

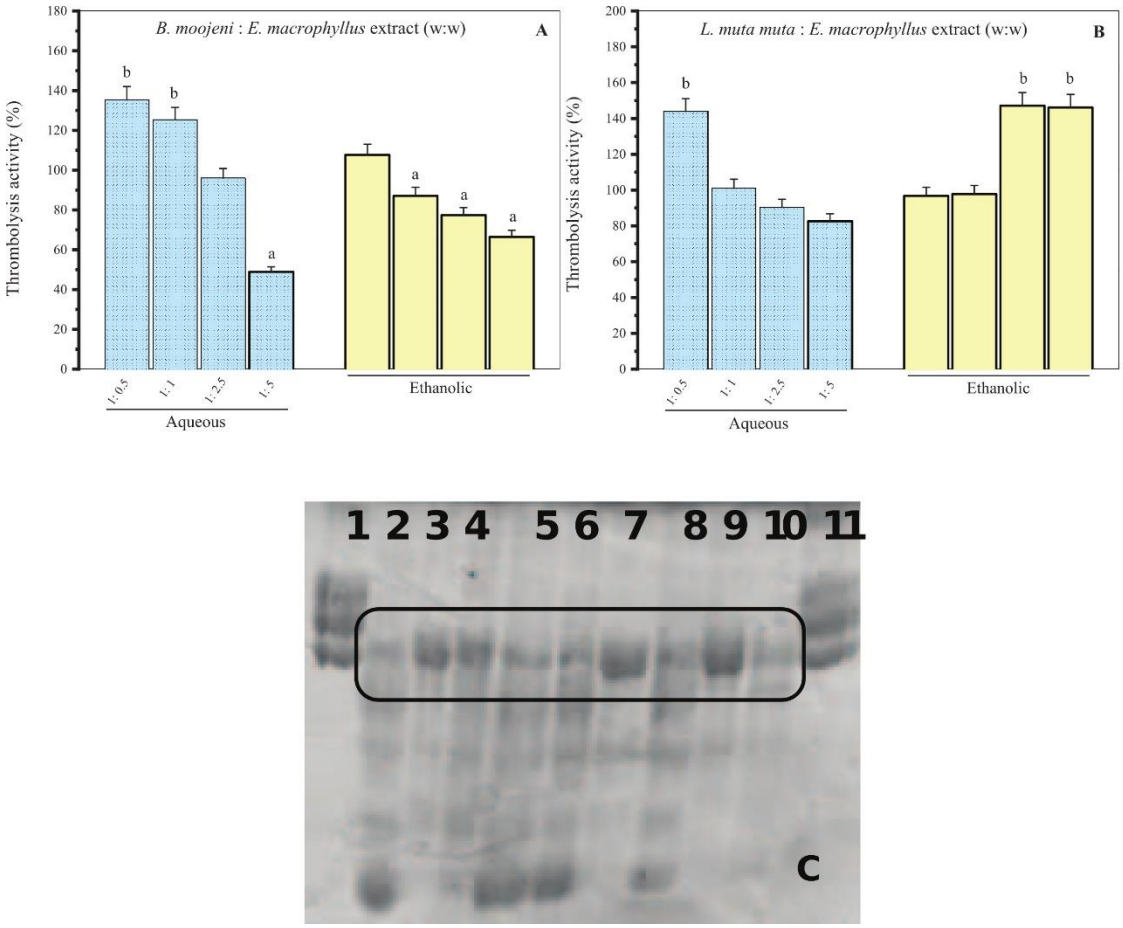

Figure 3. Thrombolytic activity (%) induced by Bothrops moojeni (A) and Lachesis muta muta (B) snake venom preincubated with aqueous and ethanolic extracts of Echinodorus macrophyllus leaves. Controls (+) containing only venoms (40 µg) were considered to have 100% activity. The results correspond to the means of triplicates and the calculated standard deviations. a: differs significantly from the respective positive control by the Scott-Knott test ($p < 0.05$) in inhibition. b: differs in potentiation. Fibrinogenolysis activity: (C) Electrophoresis profile (SDS-PAGE) to evaluate the effect of extracts from Echinodorus macrophyllus on the fibrinogenolysis induced by Bothrops moojeni venom. 1: fibrinogen (60µg); 2: B. moojeni (60 µg) + fibrinogen; 3: B. moojeni + fibrinogen + aqueous extract from E. macrophyllus (AEEM) (1: 0.5; w:w); 4: B. moojeni + fibrinogen + AEEM (1:1; w:w); 5: B. moojeni + fibrinogen + AEEM (1:2.5; w:w); 6: B. moojeni + fibrinogen + AEEM (1:5; w:w); 7: B. moojeni + fibrinogen + ethanolic extract from E. macrophyllus (EEEM) (1:0.5; w:w); 8: B. moojeni + fibrinogen + EEEM (1:1; w:w); 9: B. moojeni + fibrinogen + EEEM (1:2.5; w:w); 10: B. moojeni + fibrinogen + EEEM (1:5; w:w); 11: fibrinogen (60 µg).

Thrombin is a serine protease that plays a key role in blood coagulation and wound healing processes and catalyzes the conversion of fibrinogen into fibrin [33]. However, the accumulation

of fibrin within the blood vessels, induced, for example, by excessive activation of thrombin and factor X, results in thrombosis, which is the most frequent cardiovascular disease and the leading cause of mortality worldwide [34]. Therefore, one of the strategies for the treatment of thrombotic diseases is to reduce the activity of enzymes of the blood coagulation cascade, such as thrombin, with the use of plant compounds, given that synthetic inhibitors for these enzymes are associated with serious side effects such as bleeding [34].

Choi et al. (2015) [35] reported the inhibitory activity of the flavonoid kaempferol (30 µg) on thrombin and factor Xa as well as a 71% reduction in the formation of fibrin polymers. In the same context, the ethanolic extract of the leaves of *Brownea rosa-de-monte* was described as having an inhibitory effect on the thrombin-like proteases present in the venom of *B. asper* [36]. In turn, the action of the methanolic extract of *Leucas lavandulifolia* on the lysis of clots was associated with the presence of tannins and alkaloids in its composition [37].

In the present study, different ratios of the extracts incubated with *B. moojeni* or *L. muta muta* venom increased the dissolution of blood thrombi (Figure 3), suggesting that the different compounds present in the extracts, especially flavonoids and alkaloids, could act as thrombin inhibitors, with potential application in the treatment of thrombotic diseases [35, 38] or as enhancers of hemorrhagic proteases.

Fibrinogen is a plasma glycoprotein that plays an important role in platelet coagulation and aggregation, consisting of three pairs of polypeptide chains (α, β, and γ chains), linked together by disulfide bonds [39]. The breakage of its chains can lead to changes in the coagulation process with consequent induction of bleeding and/or formation of blood clots. We evaluated the fibrinogenolytic potential of venom toxins since the use of fibrinogen as a substrate allowed us to determine the action of proteases and their inhibitors on the blood coagulation cascade and other processes dependent on it, in addition to allowing the characterization of natural compounds with anti-venom properties.

The aqueous extract of *E. macrophyllus* partially inhibited the fragmentation of fibrinogen molecules induced by proteases present in *B. moojeni* venom at ratios of 1:0.5 and 1:1 (w:w) (Figure 3, Lines 3 and 4). For the ethanolic extract, similar activity was observed at the ratios 1:0.5 and 1:2.5 (Figure 3, Lines 7 and 9). Fibrinogenases induce the formation of blood clots through the formation of fibrin networks resulting from the breakdown of fibrinogen chains, so the extracts can be considered protease inhibitors that act in the reduction of clot formation. The results of the clotting activity corroborate the inhibition exerted by the extracts on fibrinogenolysis, since the preincubation of snake venoms with the extracts resulted in an increase in the clotting time of the citrated plasma (Table 2).

For the coagulation induction tests in citrated plasma, predetermined minimum coagulant doses were used, which were 5 µg and 10 µg of *B. moojeni* and *Lachesis muta muta* venoms, respectively. In the analysis of the results, standard values obtained from coagulograms performed in the routine practice of clinical analysis laboratories were considered. Thus, times obtained in the treatments that were ≥ 10 seconds more than the times obtained in the positive control were considered significantly different, since some coagulation cascade processes can occur at between 10 and 24 seconds, such as prothrombin activation (usually between 10 and 14 seconds) and partial activation of thromboplastin (usually between 24 and 40 seconds).

Table 2. Effect of the aqueous and ethanolic extracts of Echinodorus macrophyllus leaves on the clotting activity induced by Bothrops moojeni and Lachesis muta muta snake venom on citrated human plasma.

		Clotting time (s)	
	Venom: extract (w: w)	***Bothrops moojeni***	***Lachesis muta muta***
Aqueous extract	1:0.5	75.33 ± 3.79	90.00 ± 6.08
	1:1	94.00 ± 4.36[a]	123.67 ± 8.74[a]
	1:2.5	98.00 ± 10.56[a]	146.33 ± 7.23[a]
	1:5	122.67 ± 5.13[a]	162.00 ± 4.36[a]
	1:10	136.67 ± 1.15[a]	163.33 ± 9.45[a]
Ethanolic extract	1:0.5	90.33 ± 6.66[a]	118.33 ± 9.07[a]
	1:1	96.00 ± 4.51[a]	133.33 ± 2.52[a]
	1:2.5	110.67 ± 7.51[a]	140.00 ± 6.24[a]
	1:5	115.67 ± 3.79[a]	157.00 ± 5.13[a]
	1:10	120.67 ± 2.08[a]	181.67 ± 8.33[a]
	Control	**69.67 ± 3.21***	**96.00 ± 7.37****

* Control was performed with 5 µg of the evaluated venom. ** Control was performed with 10 µg of the evaluated venom. The results are presented as the mean of triplicates ± standard deviation. a differs from the respective positive controls by at least 10 seconds (significant by the Scott-Knott test at $p < 0.05$).

The aqueous and ethanolic extracts of *E. macrophyllus* preincubated with *B. moojeni* venom at the analyzed ratios promoted clotting times of citrated plasma that were 24 to 67 seconds longer and 21 to 51 seconds longer than the control time, respectively. The aqueous extract, when preincubated with *L. muta muta* venom, promoted an increase in clotting time at the ratios analyzed: 1:1 (w:w) (28 sec.), 1:2.5 (50 sec), 1:5 (66 sec.), and 1:10 (67 sec.). The ethanolic extract, acting on the same venom at all ratios analyzed, induced plasma clotting times of 22 to 85 seconds, which were also longer than those of the control.

To prevent blood loss, the hemostatic system requires a balance between fibrin formation (coagulation) and its dissolution (fibrinolysis); therefore, any change in this system can cause severe problems such as thrombosis or hemorrhage [40]. The development of coagulopathies is related to excessive local or systemic activation of coagulation or to excessive local or systemic fibrinolytic activity [41]. Enzymes involved in the coagulation cascade include hemorrhagic metalloproteases that act as anticoagulants; procoagulant metalloproteases that induce the formation of fibrin networks, such as fibrinogenolytic networks; serine proteases, which can also act as anticoagulants but with less efficiency because they are responsible for the formation of unstable clots; and some phospholipase A_2 proteins that can also act as procoagulants [42].

Thus, the search for inhibitors of phospholipases and proteases in plant extracts is highly important in the context of pathophysiological maintenance and/or in the control of hemostasis disorders, since the action of plant compounds in the formation of complexes with calcium ions can result not only in the inhibition of phospholipase A_2 enzymes but also in anticoagulant action, as calcium is an essential cofactor for the activity of human proteases that act in the coagulation cascade [43].

Both *E. macrophyllus* extracts, when preincubated with different venoms, increased the clotting time, probably because they acted as inhibitors of some coagulant enzymes. These extracts may be exploited in the future to treat cardiovascular diseases. To understand these actions, further studies are still needed to determine their effective and safe doses and formulations, allowing the exploration of the pharmacological potential of the extracts.

3.3 Comet assay

In the fragmentation results of DNA molecules, there was no significant difference between the nucleoids treated with the *E. macrophyllus* extracts when incubated with the *L. muta muta* venom and the nucleoids without treatment (negative control) in terms of the distribution of

damage classes 1 to 4 (Table 3). These findings highlight the antigenotoxic potential of the extracts.

Table 3. Mean number of nucleoids per comet class after treatment with aqueous and ethanolic extracts of Echinodorus macrophyllus leaves previously incubated with Lachesis muta muta venom.

Treatments			Comet classes (Damage%)				
			0 (≤5)	**1**(5–20)	**2** (20-40)	**3** (40-85)	**4** (≥85)
C (-)			49.9±5.4^{a}	48.0±7.1^{a}	2.1±0.8^{b}	0.0±0.0^{c}	0.0±0.0^{b}
C (+) *			2.1 ±0.8^{d}	12.8±6.2^{c}	44.3±5.4^{a}	31.9±7.3^{a}	8.9±4.7^{a}
C (+) **			10.1±4.4^{c}	31.1±0.7^{b}	39.6±6.1^{a}	9.7±3.2^{b}	6.4±0.7^{a}
Venom: *E. macrophyllus* extract (w:w)	Aqueous	(1:0.5)	35.7±7.0^{b}	57.2±4.5^{a}	10.3±1.7^{b}	0.0±0.0^{c}	0.0±0.0^{b}
		(1:1)	36.2±4.8^{b}	56.0±8.3^{a}	7.7±3.0^{b}	0.0±0.0^{c}	0.0±0.0^{b}
	Ethanolic	(1:0.5)	33.8±2.8^{b}	59.2±2.5^{a}	6.0±0.3^{b}	0.0±0.0^{c}	0.0±0.0^{b}
		(1:1)	34.5±3.8^{b}	55.2±0.8^{a}	11.5±2.2^{b}	0.0±0.0^{c}	0.0±0.0^{b}

C (-): only blood solution. C (+) *: *Lachesis muta muta* (50 µg mL^{-1}). C (+) **: Doxorubicin (100 µg mL^{-1}), a genotoxic antitumor drug. The values represent the average of three tests performed with blood from different volunteers, with 300 nucleoids per treatment/volunteer, totaling 900 nucleoids/treatment. Data followed by the same letters in a column do not differ by the Scott-Knott test ($p > 0.05$).

The mean frequency of damage (Figure 4A) and the values of arbitrary units (AUs) (Figure 4B) showed that both extracts of *E. macrophyllus* reduced the fragmentation of DNA molecules induced by the *L. muta muta* venom, so they had antigenotoxic action. The AU values were 74.3 and 78.2 for the aqueous and ethanolic extracts, respectively, which were approximately one-third the value obtained for the positive control (venom), at 232.7.

Controls of the pure extracts at concentrations of 250 µg mL^{-1} and 50 µg mL^{-1} were also performed, and the aqueous and ethanolic extracts at the highest concentration induced DNA fragmentation, possibly due to abrupt changes in the cell incubation environment. The damage levels were 24% and 13% lower, respectively, than those observed for *L. muta muta* venom (data not shown).

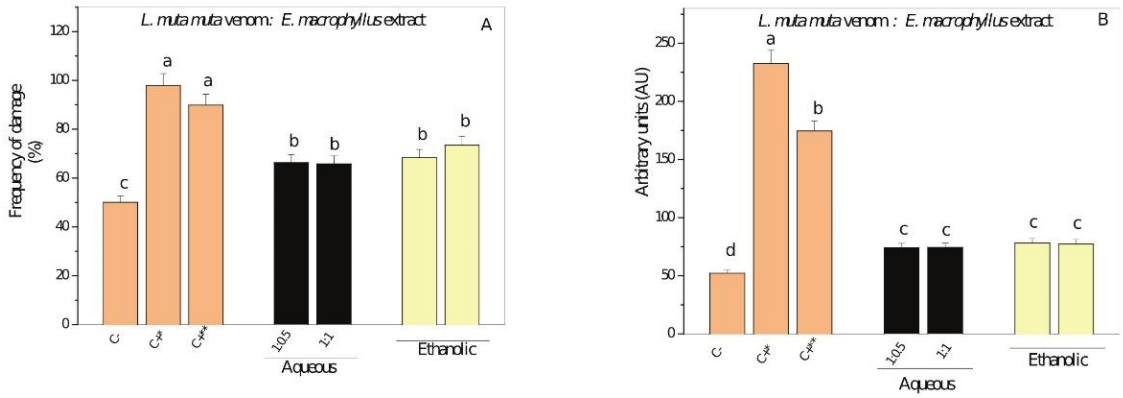

Figure 4. Comet assay. (A) Frequency of damage (%). (B) Arbitrary units, calculated according to Collins (2004). Data marked with the same letters do not differ by the Scott-Knott test ($p > 0.05$).

DNA damage assessed by the comet assay is widely used in genotoxicity assays and studies on DNA damage and repair mechanisms since it is a highly sensitive, safe, and reliable technique for assessing damage in individual cells [44]. The aqueous extract of *E. macrophyllus* was previously evaluated by Lopes et al. (2000) [45], and these authors found no *in vitro* cytotoxicity in liver or kidney epithelial cells of mice. Vaz et al. (2016) [44] described the absence of genotoxic action of the ethanolic extract of the same plant species at the doses evaluated. The effect of crude extracts of the species *Echinodorus grandiflorus* on leukocyte DNA molecules of Swiss mice has also been analyzed [46], and nongenotoxic damage scores were observed, similar to those obtained in the negative control. According to the same researchers, the absence of genotoxicity was attributed to the presence of compounds present in the extracts such as saponins, steroids, and flavonoids that are considered protective of the stability of protein molecules and DNA. In the present study, flavonoids, steroids, and saponins were also identified in the *E. macrophyllus* extracts, suggesting the participation of these compounds in the protection of DNA molecules, explaining the antigenotoxic action against the toxins present in *L. muta muta* venom.

3.4 Inhibition of digestive enzymes

The results of the modulatory effects of the aqueous and ethanolic extracts of *E. macrophyllus* on the enzymes α-amylase, α-glucosidase, lipase, and trypsin, evaluated before and after exposure to the simulated gastric fluid, are shown in Table 4.

The ethanolic extract showed 53.62% inhibition of the enzyme α-amylase, and after exposure to the simulated gastric fluid, the inhibition increased 27.5%. For α-glucosidase activity, the aqueous and ethanolic extracts of *E. macrophyllus* showed inhibition of 1.00 and 0.70 μmol min^{-1} g^{-1}, respectively, which are equivalent to 60.0% and 42.84%. However, the aqueous and ethanolic extracts, after passing through the gastric fluid simulation, showed inhibitory actions of 70.46% and 49.54%, respectively. The increase in enzyme inhibition exerted by the extracts after exposure to the simulated gastric fluid can be explained by the ionization of active groups resulting from the change in pH. The inhibition of α-amylase and α-glucosidase can be used as a strategy to control postprandial hyperglycemia, which is a risk factor for the development of micro- and macrovascular complications resulting from diabetes mellitus [47]. Some phenolic compounds, due to their ability to bind to proteins, have already been reported as inhibitors of glycosidic hydrolases.

Table 4. Effect of the aqueous and ethanolic extracts of Echinodorus macrophyllus leaves on the activity of digestive enzymes, evaluated before and after gastric fluid simulation.

Enzyme	***Echinodorus macrophyllus***							
	Aqueous extract				**Ethanolic extract**			
	Inhibition before exposure		**Inhibition after exposure**		**Inhibition before exposure**		**Inhibition after exposure**	
	(UIE)[a]	**(%)**	**(UIE)[a]**	**(%)**	**(UIE)[a]**	**(%)**	**(UEI)[a]**	(%)
α-Amylase	ns	26.29	nd	nd	7.85±1.21	53.62	11.59±0.39	68.36
α-Glycosidase	1.00±0.09	60.00	1.03±0.04	70.46	0.70±0.05	42.84	0.72±0.04	49.54
Lipase	ns	37.10	nd	nd	nd	27.24	nd	nd
Trypsin	ns	11.95	nd	nd	nd	26.73	nd	nd

Data from five replicates, with mean ± standard deviation.

[a] The *Echinodorus macrophyllus* extract measured for each of the enzymes was diluted to provide an inhibition between 40% and 80%, to ensure the reliability of the results.

UIE = Unit of inhibit enzyme at μmol min^{-1} g^{-1} sample (extract)

nd = inhibition not determined

ns = inhibition not significant

According to Justino et al. (2018) [47], the presence of compounds such as chlorogenic acid, caffeic acid, catechin, quercetin, and rutin in *Annona muricata* extracts is related to their inhibitory action on α-amylase and α-glucosidase. These researchers suggest a mechanism of enzymatic inhibition by the action of many hydroxyl groups present in phenolic compounds. Inhibition of α-glucosidase activity was also reported for the hydroalcoholic extract of *Moringa stenopetala*, which is rich in phenolic compounds and flavonoids [48]. The presence of flavonoids in the extracts of *E. macrophyllus* may also have contributed to the inhibitory action on the enzymes α-amylase and α-glucosidase, since these compounds have been described as causing antiatherogenic and antihyperglycemic effects, oxidation of lipoproteins, platelet aggregation, and vascular reactivity [49]. Franco et al. (2018) [50] reported lower than 40% inhibition of α-glucosidase activity in the ethanolic extract of the species *Echinodorus grandiflorus*, although they observed high inhibitory activity on lipase.

However, in the present study, both extracts of *E. macrophyllus* showed a low percentage of inhibition of lipase and trypsin activity. The inhibition of lipase would add to the beneficial effects of the extracts because it represents a promising method to treat obesity and diabetes mellitus, considering that in addition to hyperglycemia, these metabolic diseases are associated with high levels of triglycerides in the blood, resulting from high-fat diets and sedentary lifestyles [48]. Thus, by inhibiting lipase, the absorption of free fatty acids would be reduced, which would decrease the hyperlipidemia associated with these diseases [51]. The low inhibition rates exerted by the extracts of *E. macrophyllus* on the activity of trypsin can favor the therapeutic use of this plant because trypsin inhibitors present in the diet may interfere with the normal and systemic metabolism of some organs, such as the pancreas, liver, and muscle, thus altering the growth rate of young animals [52].

4. CONCLUSION

Echinodorus macrophyllus extracts have bioactive substances that give them potential therapeutic uses in the treatment of various diseases with inflammatory origins or progression related to the imbalance of the hemostatic system. These extracts could modulate the enzymatic activity of phospholipase A_2 (involved in the generation of eicosanoids with action mainly in the inflammatory response and blood coagulation) and proteases (fibrinogenolytic and thrombin-like), mainly by the action of phenolic compounds that interact with catalytic sites of enzymes, cofactor binding sites or even hydrophobic regions present in these molecules. The extracts also stood out for their antigenotoxic activity, giving them a possible protective effect on cellular components. Future studies may define safe and effective formulations, doses, and administration forms that enable the therapeutic use of this plant species.

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