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Chemistry

Doxorubicin induces DNA damage in peripheral blood mononuclear cells: *Althernantera Brasiliana (L.) Kuntze* extract is a cytoprotector?

Doxorubicina induz dano ao DNA em células mononucleares de sangue periférico: extrato de *Althernantera Brasiliana (L.) Kuntze* é um citoprotetor?

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ABSTRACT

Doxorubicin (DOXO) is a chemotherapeutic agent that exerts cytotoxic effects through oxidative stress. Given the need to neutralize the cellular damage caused by chemotherapy drugs in healthy cells, this study aims to investigate the activity of *Althernantera brasiliana (L.) Kuntze* against DNA damage induced by DOXO in peripheral blood mononuclear cells (PBMC). For this purpose, a hydroethanolic extract from flowers and leaves of Althernantera brasiliana (HEAB) containing phenolic compounds, flavonoids, and condensed tannins was prepared. The phytochemicals of EHAB showed a lower IC50 value than ascorbic acid, which was associated with its ability to chelate iron ions. Despite its antioxidant properties, EHAB did not show a protective effect on DOXO-induced DNA damage in PBMCs. In addition, the treatment with DOXO and varying concentrations of EHAB (50 µg/mL and 100 µg/mL) decreased cell viability and increased late apoptosis PBMCs, suggesting a synergistic cytotoxic effect of EHAB when used in combination with DOXO. Therefore, even though EHAB shows antioxidant properties in vitro, it does not appear to be an alternative to protect blood cells from genotoxic effects induced by DOXO.

Keywords: Phytotherapy; Penicillin; DNA damage; Cytotoxicity; Oxidative stress

RESUMO

A doxorrubicina (DOXO) é um agente quimioterápico que exerce efeitos citotóxicos por meio do estresse oxidativo. Diante da necessidade de neutralizar o dano celular causado por drogas quimioterápicas em células saudáveis, este estudo teve como objetivo investigar a atividade de *Althernantera brasiliana*

(L.) Kuntze contra danos ao DNA induzidos por DOXO em células mononucleares de sangue periférico (PBMC). Para tanto, foi preparado um extrato hidroetanólico de flores e folhas de Althernantera brasiliana (HEAB) contendo compostos fenólicos, flavonoides e taninos condensados. Os fitoquímicos do EHAB apresentaram um valor de IC50 menor do que o ácido ascórbico, o que foi associado à sua capacidade de quelar íons de ferro. Apesar de suas propriedades antioxidantes, o EHAB não mostrou um efeito protetor no dano ao DNA induzido por DOXO em PBMCs. Além disso, o tratamento com DOXO e concentrações variadas de EHAB (50 µg/mL e 100 µg/mL) diminuiu a viabilidade celular e aumentou PBMCs de apoptose tardia, sugerindo um efeito citotóxico sinérgico de EHAB quando usado em combinação com DOXO. Portanto, embora o EHAB apresente propriedades antioxidantes in vitro, não parece ser uma alternativa para proteger as células sanguíneas dos efeitos genotóxicos induzidos pelo DOXO.

Palavras-chave: Fitoterapia; Penicilina; Dano ao DNA; Citotoxicidade; Estresse oxidativo

1 INTRODUCTION

Cancer is one of the most important causes of death and a barrier to increasing life expectancy worldwide. Chemotherapy is the most used therapeutic method for the treatment of cancer, which may involve the use of antimetabolite drugs (e.g., methotrexate), DNA-interactive agents (e.g., cisplatin and doxorubicin), mitotic inhibitors (e.g., taxanes), hormones and targeting agents. Despite the beneficial effect of chemotherapeutic agents in inducing the death of cancer cells, they can also produce cytotoxic effects on non-tumor cells, thus leading to a dichotomy between cancer and normal cells. Hence, it is essential to develop therapeutic strategies for mitigating the side effects of chemotherapy (Bray, 2018; Sung, 2021).

Doxorubicin (DOXO) is a chemotherapeutic agent from the anthracycline family used in the treatment of different types of cancer (Cuomo et al., 2019). Although its mechanism of action is complex, DOXO is known to interfere with nucleic acid synthesis and induce the production of free radicals that can damage cell membranes and DNA (Rivankar, 2014). In this regard, DOXO has proven to be highly successful in regressing several neoplasms, including, among others, breast carcinoma, lung, bladder, thyroid, and ovarian cancer, and bone sarcomas. However, the clinical use of DOXO is limited due to its different side effects, e.g., cardiotoxicity, myelosuppression, and liver damage (Cappetta et al., 2017; Moslehi, 2016).

The phytochemicals with known antioxidant and cytoprotective effects are a promising alternative to improve the efficiency of cancer treatments and reduce adverse reactions (Choudhari et al., 2020). *Alternanthera brasiliana (L.) Kuntze*, also known as penicillin, is a plant used for folk medicine in different countries to treat viral infections, gastric, hepatic, renal, and respiratory disorders. This plant also shows promising properties as an emollient, antidiarrheal, anti-inflammatory, vermifuge, antimicrobial, and analgesic agent. Although almost all organs of A. brasiliana are used in traditional medicine, leaves are the most widely used in therapeutics (Samudrala, 2015; Souza et al., 1998; Salvador et al., 2004). Some species of the *Alternanthera* family contain alkaloids, steroids, flavonoids, saponins, and terpenoids as secondary metabolites (Tomei, 2018). In this regard, Brochado et al. (2003) identified six flavonoids in leaves of A. Brasiliana, which may exhibit antioxidant activity. Therefore, it is interesting to examine the potential protective effects of A. brasiliana against cellular oxidative damage.

Chemotherapy with DOXO displays several side effects on non-target cells, including oxidative stress and DNA damage. On the other hand, antioxidant phytochemicals from A. Brasiliana have already been identified. This study aims to assess the potential cytoprotective effects of a hydroethanolic extract of *A. brasiliana* (HEAB) against DNA damage and cytotoxic activity induced by DOXO in peripheral blood mononuclear cells (PBMC).

2 METHODS

2.1 Preparation of the hydroethanolic extract from *Althernantera Brasiliana (L.) Kuntze*

A specimen of Althernantera brasiliana was identified by a biologist from the University of Cruz Alta (Brazil) and deposited at the University Herbarium (Registration Number: 1125). Leaves and flowers of Althernantera brasiliana collected at the municipality of São Luiz Gonzaga-RS were dried in an oven with air circulation (± 40°C) and then

crushed in a knife mill. Next, hydroethanolic maceration (EtOH:H2O 3:2, v/v) was carried out for seven days (first maceration), and the material was filtered and concentrated in a rotary evaporator. The experimental procedure was repeated to carry out a second maceration of the plant material, obtaining a hydroethanolic extract of Althernantera brasiliana (HEAB), which was finally lyophilized for further use (Simões et al., 2010).

2.2 Quantification of phytochemical compounds in HEAB

2.2.1 Total phenolic content

The determination of total polyphenols was carried out according to the Folin-Ciocalteau method described by Swain and Hillis (1959). For this purpose, one (1) mg sample was diluted in 1 mL water, and then 0.25 N Folin-Ciocalteu solution and 0.5M sodium carbonate were added. The mixture was incubated for 2 h, and absorbance was measured using a spectrophotometer at a wavelength of 725 nm. All measurements were carried out in triplicate. The total polyphenol content was expressed in equivalent milligrams of gallic acid per gram of dry extract based on the standard gallic acid calibration curve.

2.2.2 Total flavonoid content

The dosage of total flavonoids was determined according to the method described by Zhishen et al. (1999). One (1) mg sample was diluted in 1 mL water, and then 5% sodium nitrite, 2% aluminum chloride, and 1M sodium hydroxide were added. The absorbance was read with a spectrophotometer at 510 nm. The measurements were performed in triplicate, and dosage was calculated based on the standard calibration curve of quercetin. The total flavonoid content was expressed in milligrams of quercetin per gram of extract.

2.2.3 Condensed tannin content

The determination of condensed tannins was carried out as described by Morrison et al. (1995). The sample was diluted in methanol at a concentration of 100 mg/mL. Subsequently, 0.1 mL sample was mixed with 0.9 mL methanol, and then 2.5 mL vanillin solution (1 g vanillin diluted in 100 mL methanol) and 2.5 mL hydrochloric acid solution (8 mL hydrochloric acid diluted in 100 mL methanol) were added. After incubation for 10 min at 60°C, absorbances were determined with a spectrophotometer at 500 nm. Measurements were carried out in triplicate. The condensed tannin content was expressed in milligrams of catechin per gram of extract based on the standard catechin calibration curve.

2.3 Identification of antioxidant mechanisms

The ability to remove reactive nitric oxide (NO) species was assessed according to the protocol described by Sreejayan and Rao (1997). Different concentrations of extract or ascorbic acid (positive control) were mixed with 10 mM sodium nitroprusside. After 150 min, 0.5 mL Griess reagent was added, and absorbances were measured at 546 nm. The negative control consisted of replacing the extract with phosphate-buffered saline (PBS). The NO removal rate was calculated using the equation: % NO removal = (1 - sample absorbance/control absorbance) x 100. The IC50 value, i.e., the concentration estimated to inhibit 50% of the biochemical function, was also calculated.

To evaluate the scavenging activity of DPPH (1,1-diphenyl-2-picryl-hydrazyl) radicals, EHAB samples were mixed with an ethanolic solution of DPPH (0.3 mM). After 30 min, absorbances were read at 518 nm. Measurements were carried out in triplicate. The DPPH removal rate was determined using the equation as follows: % removal = 100 – [(Sample absorbance – blank absorbance)] x 100] / control absorbance. A solution of DPPH in ethanol and vitamin C were used as negative and positive controls, respectively. The IC50 value was also calculated (Mensor et al., 2001).

The chelating activity of iron ions was determined according to the protocol described by Deker (1990) with minor modifications. After mixing EHAB solution with Fe2+, 2,4,6-tripyridyl-S-triazine (TPTZ) (5 mM) was added to generate a complex with free Fe2+ ions, forming a blue color precipitate that can be measured spectrophotometrically at 593 nm. The chelating activity was calculated using the following equation: Chelating activity (%) = (Control absorbance – Sample absorbance/Control absorbance) x 100. The IC50 value was also calculated.

2.4 Evaluation of the cytoprotective activity of HEAB

2.4.1 Sample collection

This study was approved by the Research Ethics Committee of the University of Cruz Alta (project approval number: 5.340.591). Individuals who agreed to participate in the study signed a free and informed consent form. Ten (10) healthy individuals i.e., six women and four men, with a mean age of 35.2 ± 12.5 years, who studied or worked at the University of Cruz Alta, were randomly selected to participate voluntarily. From each individual, 20 mL of peripheral venous blood was collected for PBMC isolation.

PBMC isolation was performed using a density gradient with Hystopaque reagent (Sigma, USA) (d=1,077 g/mL). For this purpose, blood samples were added into 15 mL Falcon® Type tubes containing Hystopaque at a 1:2 ratio, and then the tubes were centrifuged at 500g for 25 min at room temperature. After centrifugation, PBMC that remained at the interface between the reagent and the plasma were aspirated using Pauster pipettes. PBMCs were resuspended in a new tube and washed twice with phosphate-buffered saline. After washing, cell concentration and viability was determined with a hemocytometer using Trypan Blue staining. The cell samples showing viability higher than 95% were used in further experiments.

2.4.2 In vitro exposure

PBMC were divided into eight treatment groups at a concentration of 3x106 cells per plate/group and incubated in RPMI 10% fetal bovine serum. The in vitro exposure protocol was initiated with 30 min pre-treatment with EHAB, followed by 2 h treatment with DOXO and EHAB at 37ºC. The experimental groups were as follows: control group, three HEAB treatment groups (10 µg/mL, 50 µg/mL, and 100 µg/mL), and four DOXO treatment groups (5 µM, 5 µM + HEAB 10 µg/mL, 5 µM + HEAB 50 µg/mL, and 5 µM + HEAB 100 µg/mL). The concentrations for each treatment were based on the concentration curve, using the highest DOXO concentration capable of causing at least 50% DNA damage induction compared to non-exposed cells and the highest HEAB concentration that did not induce cytotoxicity (data not shown).

2.5 Tests to assess cytotoxicity

2.5.1 DNA damage-sensitive γH2Ax detection

PBMC at a density of $0.5x10^6$ cells were used to assess DNA damage. First, cells were fixed with Perm/Fix buffer (BD Biosciences, USA) for 40 min at 4°C, washed twice, and resuspended in 100 µl Perm/Wash buffer (BD Biosciences). Next, 5 µL anti-γH2Ax PE antibody were added to 100 µL Perm/Wash buffer and incubated for 60 min at 4°C. Finally, cells were washed and resuspended in 300 µL Perm/Wash Buffer. The fluorescence intensity was measured using a BD Accuri C6 Plus flow cytometer (BD Biosciences, USA). Data were expressed as median fluorescence intensity (MFI).

2.5.2 Cell death detection

PBMC at a density of $0.5x10^6$ cells were used to assess cell death induced by apoptosis or necrosis. Cells from each group were incubated with 4 µL annexin and 4 µL propidium iodide (PI) (BD Biosciences) in binding buffer (10 mM HEPES / NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) for 15 min at room temperature in the dark. After

incubation, fluorescence intensity was measured in twenty thousand events using the BD Accuri C6 Plus cytometer. Apoptosis and necrosis were determined by the emission of green fluorescence (annexin) and red fluorescence (propidium iodide), respectively. The combined green and red fluorescence was also examined to determine late apoptosis. Data were expressed as percentage of cells undergoing apoptosis, necrosis, and late apoptosis.

2.6 Statistical analysis

Data distribution analyses were performed using the Shapiro–Wilk test. Afterward, data showing normal distribution were submitted to the one-way Analysis of Variance followed by the Tukey's test. The p values $p < 0.05$ were considered statistically significant.

3 RESULTS

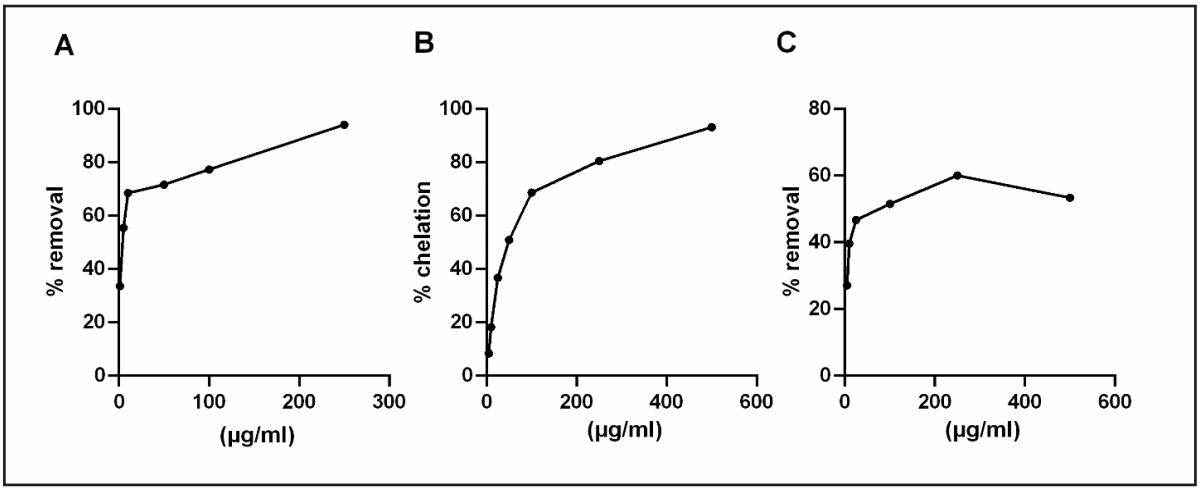
As shown in Table 1, HEAB shows a considerable content of phenolic compounds, flavonoids, and condensed tannins. The antioxidant effects of HEAB are summarized in Figure 1 and Table 2. EHAB exhibited scavenging activity toward DPPH and NO radicals and chelating abilities on iron ions at a proportional rate to the concentration. Moreover, EHAB had a higher mean inhibitory concentration than vitamin C (positive control), thus showing higher scavenging DPPH activity and chelating effects than the positive control.

Table 1 – Quantification of EHAB phytochemical compounds

Extract content	
Total phenolic compounds (mg AG/g)	100,1 ± 40,3
Total Flavonoids (mg QE/g)	20,3 ± 23,8
Condensed Tannins (mg CA/g)	3.688,8 ± 234,1

Data presented as mean ± standard error of mean (n = 3). AG: gallic acid; QE: quercetin; CA: catechin

Figure 1 – HEAB antioxidant mechanisms



All experiments were performed in triplicate and data are presented as mean. DPPH radical (A) Scavenging ability. (B) Iron ion chelating activity. (C) Radical scavenging activity of nitric oxide (NO).

Table 2 – HEAB antioxidant mechanisms

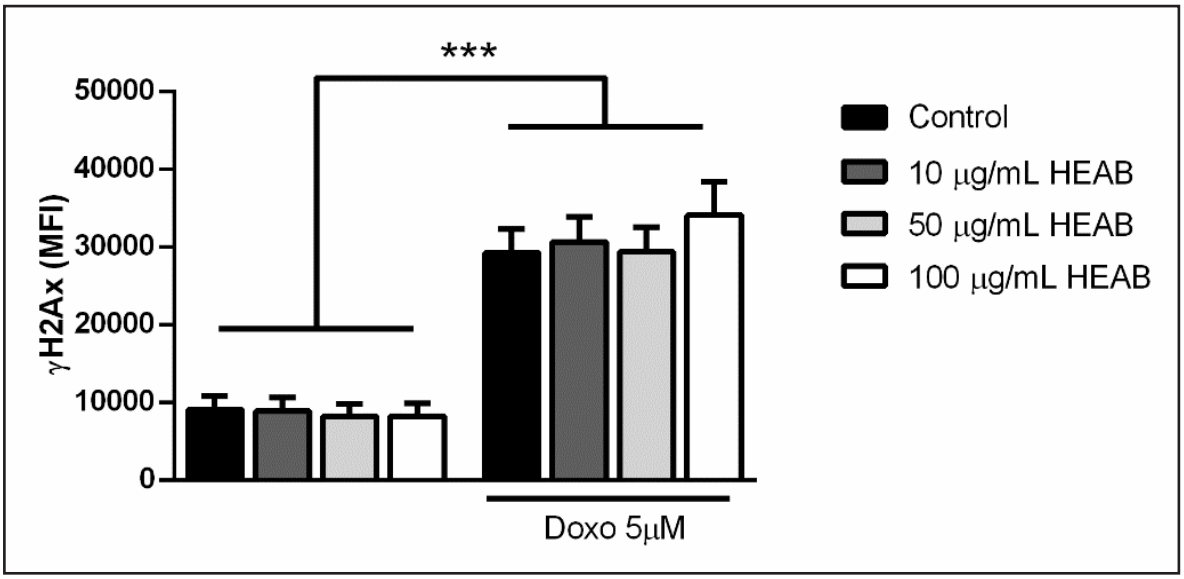
	HEAB (µg/mL)	Ascorbic acid (µg/mL)
DPPH Removal	IC50 = 1,91	IC50 < 5
Chelating activity	IC50 = 152,56	IC50 > 500
NO removal	IC50 = 127,89	IC50 = 95,86

IC50 = mean inhibitory concentration; NO = nitric oxide. Ascorbic Acid used as a positive control

We also assessed the potential cytoprotective effects of EHAB on PBMC. As shown in Figure 2, 5 µM DOXO-induced phosphorylation of the DNA damage-sensitive marker γH2Ax in PBMCs. On the other hand, EHAB did not prevent DOXO-induced DNA damage at any tested concentration.

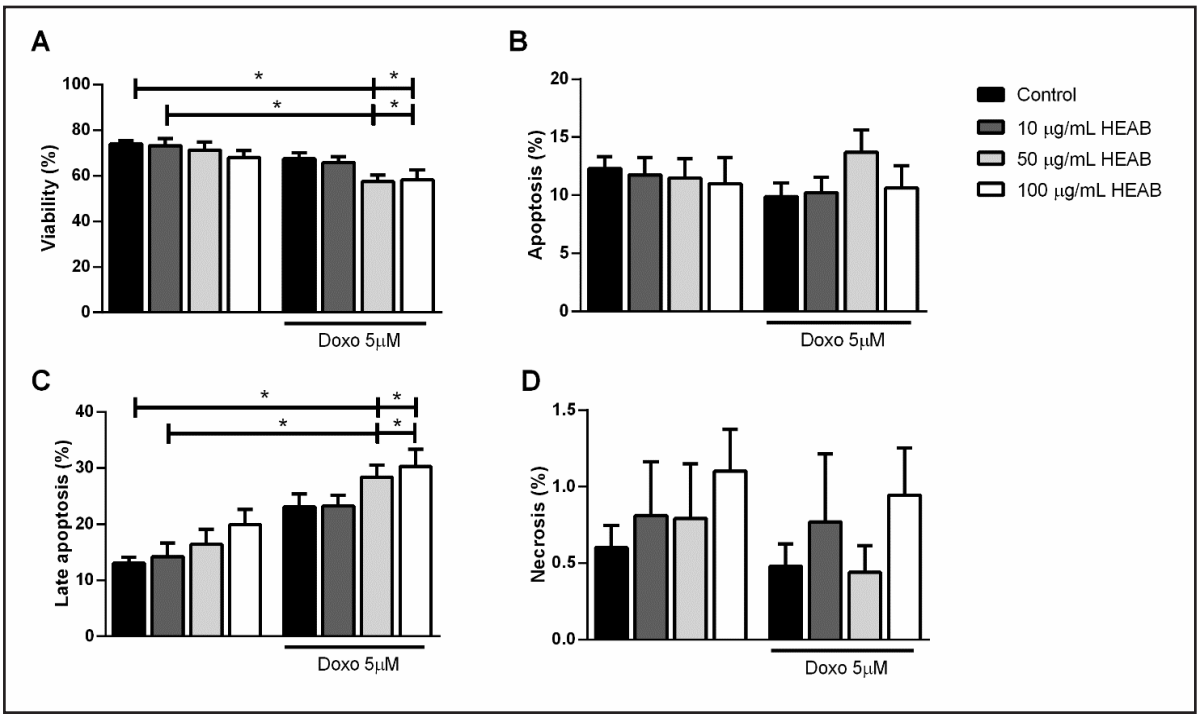
Furthermore, we found that PBMC treated with DOXO and HEAB at different concentrations either did not significantly decrease cell viability (Figure 3A) or increase apoptosis (Figure 3B), late apoptosis (Figure 3C), and necrosis (Figure 3D). However, the combined treatment of DOXO with 50 µg/mL or 100 µg/mL EHAB significantly decreased viability (Figure 3A) and increased late apoptosis (Figure 3C), suggesting that EHAB and DOXO show a negative synergistic effect.

Figure 2 – Effect of HEAB and DOXO on DNA damage in PBMC



***p <0.001, data expressed as mean ± standard error, statistically significant differences assessed by One-Way Anova test followed by Tukey post-test, n=10 in each group. γH2Ax: Phosphorylated Histone 2Ax, DNA damage marker, MFI: Median Fluorescence Intensity, Doxo: Doxorubicin, HEAB: *Hydroethanolic Extract of Althernanthera brasiliensis*

Figure 3 – Effect of EHAB and DOXO on cytotoxicity in PBMC



*p<0.05, data expressed as mean ± standard error, statistically significant differences assessed by One-Way Anova test followed by Tukey post-test, n=10 in each group. Doxo: Doxorubicin, HEAB: Hydroethanolic Extract of *Althernanthera brasiliensis*. (A) % of viable cells – negative annexin and propidium iodide; (B) % of cells undergoing apoptosis – positive annexin; (C) % of cells in late apoptosis – positive annexin and propidium iodide; (D) % of cells in necrosis - positive propidium iodide

4 DISCUSSION

The use of medicinal plants has increasingly gained interest due to their beneficial effects in the treatment and prevention of different pathophysiological conditions (Choudhari et al., 2020). To our knowledge, this is the first study assessing the potential protective effects of a hydroethanolic extract from leaves and flowers of Alternanthera brasiliana against DOXO-induced cell damage.

We detected a considerable concentration of phenolic compounds, flavonoids, and condensed tannins in EHAB. Therefore, our study demonstrates that aerial parts of Alternanthera brasiliana are excellent sources of phytochemicals with potential biological and pharmacological activities. A previous study showed that the genus *Alternanthera (Amaranthaceae)* is characterized by a high diversity of phytochemicals, including flavonoids, steroids, saponins, alkaloids, triterpenoids, glycosides, and phenolic compounds (Singla et al., 2022). In line with these findings, the characterization of an alcoholic extract obtained from aerial organs of A. brasiliana using chromatography methods confirmed the presence of several phytochemicals, including n-hexadecanoic acid, linoleic acid, clionasterol, α-tocopherol, and α-amyrin (Alencar et al.; 2020). In addition, phytochemicals such as kaempferol, stigmasterol, quercetin, vitexin, ferulic acid, and caffeic acid have also been identified in several *Alternanthera* species (Singla et al., 2022).

The compounds identified in the present study have already been identified in other plant species, and they are well-known to show diverse pharmacological activities, especially an antioxidant effect. Here, we observed that HEAB exhibits antioxidant properties, including neutralizing activity toward NO and free radicals and chelating abilities on ferrous ions. We also demonstrated that HEAB has a higher capacity to neutralize NO radicals than vitamin C, a potent antioxidant. It has been proposed that phytochemicals with antioxidant properties in HEAB may decrease the risk of suffering several health disorders (Ponte, 2021), likely by preventing genotoxic and cytotoxic damage.

The adverse effects of DOXO on cardiomyocytes have mainly been associated with mitochondrial dysfunction and the generation of reactive oxygen species (ROS). Cardiomyocytes have relatively low levels of enzymes with antioxidant activity (e.g., superoxide dismutase, catalase), and therefore they are more susceptible to DOXO-induced cellular damage. Moreover, cardiotoxicity can be prevented by exogenous administration of compounds capable of neutralizing oxidative stress, including DNA damage. In this regard, it has been shown that PBMCs exhibit a similar response as cardiomyocytes under exposure to DOXO (Todorova, 2012). Hence, we used PBMC as a model to test such effects.

We observed that the treatment with DOXO induces in vitro DNA damage in PBMCs. Although we did not find a protective effect of EHAB on DNA damage, a previous study showed that quercetin, a phytochemical present in Alternanthera species, reduces DOXO-induced oxidative stress and DNA damage in cardiomyocytes while simultaneously maintaining cell viability (Dong et al, 2014).

The incubation time and dose of the treatment with DOXO tested in this study were insufficient to induce cell death by apoptosis or necrosis. Furthermore, HEAB showed no cytotoxic effects per se against PBMCs. Surprisingly, we found that the combined treatment of DOXO with 50ug/mL or 100ug/mL HEAB decreased cell viability and increased late apoptosis. These results suggest that HEAB potentiates DOXO-mediated cytotoxicity and thus may be a promising agent against tumor cells.

We did not detect a protective effect of HEAB after exposure to DOXO; however, phytochemicals from *Alternanthera* species with inhibitory effects against ROS and apoptosis have already been identified (Wu et al., 2013). Further characterization of these phytochemicals, including their mechanisms of action and dose safety, is needed before their pharmacological use.

5 CONCLUSION

HEAB contains different classes of phytochemical compounds and exhibits in vitro antioxidant effects. Despite its antioxidant activity, HEAB did not show a cytoprotective effect against DOXO-induced DNA damage in PBMC, indicating that this extract is not a promising alternative to minimize the occurrence of side effects in chemotherapy. Further analyses using HEAB at different concentrations to those tested in our experiments and employing cancer cell lines as cellular models are required to assess in detail its synergistic cytotoxic effects when combined with DOXO.

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