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HEALTH SCIENCES

Antiulcer mechanisms of the hydroalcoholic extract from Aztec marigolds' medicinal and edible flowers (*Tagetes erecta L.*)

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Abstract: The antiulcer mechanisms of the dry extract of *T. erecta* flowers (DETe) were studied here. The acute ulcers induced by acidified ethanol or indomethacin were reproduced in mice pretreated with DETe (3 - 300 mg/kg). The antiulcer activity of DETe was also verified in mice pretreated with NEM, L-NAME, indomethacin, or yohimbine. The antisecretory effect of DETe was verified in rats, and its anti-*Helicobacter pylori* activity was determined in vitro. DETe (300 mg/kg, p.o) reduced the ethanol- or indomethacin-induced ulcer by 49 and 93%, respectively. The pre-treatment with L-NAME, NEM or yohimbine abolished the gastroprotective effect of DETe. However, DETe did not change the volume, acidity, or peptic activity in rats and did not affect *H. pylori*. This study expands knowledge about the antiulcerogenic potential of DETe, evidencing the role of nitric oxide, non-protein sulfhydryl groups, α2 adrenergic receptors, and prostaglandins, but not antisecretory or anti-*H. pylori* properties.

Key words: lutein, gastric ulcer, oxidative stress, phytotherapy, *H. pylori*.

INTRODUCTION

Gastric ulcer disease is a lesion that affects the gastric mucosa with multiple etiologies occurring because of an imbalance between the aggressive and protective factors in the gastric mucosa (Yandrapu & Sarosiek 2015). Among the protective factors are the mucus-bicarbonate layer, adequate blood flow, as well as the enzymatic, and non-enzymatic antioxidant defenses. These defenses are available in the gastric mucosa against the deleterious effect of aggressive endogenous factors such as increased gastric acid secretion, pepsin, and the production of reactive oxygen species (ROS) (Wallace 2008). The imbalance between these factors is exogenously favored through smoking, alcoholism, high caffeine consumption, prolonged use of certain types of drugs (particularly non-steroidal anti-inflammatory drugs, NSAIDs), and *Helicobacter pylori* infection (Nwose & Yee 2016, Burkitt et al. 2017). Among the symptoms of this disease, pain in the upper abdomen, indigestion, and a sensation of epigastric burning stand out.

The current pharmacological treatment of ulcers aims to attenuate gastric acid secretion, and histamine type 2 receptor antagonists (H2RA, such as ranitidine), and irreversible proton pump inhibitors (PPIs such as omeprazole) are

among the most prescribed drugs. In addition, some nonspecific antacids and antibiotics are also prescribed to treat gastric ulcers in the presence of *H. pylori* infection (Hernandes 2010). Although effective, anti-gastric acid-secreting drugs are associated with some inconveniences and adverse effects, including tolerance to H2RA, and a higher prevalence of osteoporosis, hypergastrinemia, and enterochromaffin-like cell hyperplasia due to the prolonged use of PPIs (Sheen & Triadafilopoulos 2011, Panday et al. 2014, Kuna et al. 2019).

Such factors drive the continuous search for alternative and/or adjuvant therapies to enrich the antiulcer therapeutic arsenal and the ethnobotanical knowledge can help us in this search because through the scientific validation of the gastroprotective potential of plants used to treat gastric disorders it is possible to open paths for new and unsuspected modes of action, as well as for effective and safe alternative therapies and/or adjuvants. As oxidative stress and the inflammatory process play a crucial role in the pathogenesis of gastric ulcers, medicinal plants with antioxidant effects with additional anti-inflammatory activity may represent promising candidates for gastroprotective purposes. In this sense, there has been considerable emphasis on health promotion activities performed by plants rich in carotenoids (Hosseini et al. 2017, Meurer et al. 2019, Mudumbi et al. 2019) due to their antioxidants (Pietta 2000) and anti-inflammatory (Maleki et al. 2019) activities.

Tagetes erecta L. (Asteraceae) is a native plant to Mexico and Guatemala, rich in carotenoids (Meurer et al. 2022, 2019, Burlec et al. 2021) and most likely naturalized in the rest of Central and South America, commonly known as Calendula flower or Aztec Marigold (PLANT USE 2021). Moreover, its flowers are used as an ingredient in salads and as natural food colorants since it is one of the most popular edible flowers worldwide (Setshogo 2005). Guided by its popular use, some studies have investigated the pharmacological properties of preparations from *T. erecta* flowers in gastrointestinal diseases and other systems.

Indeed, the spasmolytic effect of *T. erecta* was confirmed by Ventura-Martínez et al. (2018), which involves voltage-gated calcium channels, but not the nitric oxide pathway or the release of neurotransmitters from enteric neurons. Previously, our research group also showed that *T. erecta* extract can reduce the ulcerative colitis (UC) severity by attenuating inflammatory cytokine secretion and improving the endogenous antioxidant defense in dextran sodium sulfate (DSS)-induced UC in mice (Sindhu & Kuttan 2012, Meurer et al. 2019). Recently, the essential oil from *T. erecta* flowers presented a protective action on experimental gastric cancer and our research group described its healing gastric ulcer properties (Cui et al. 2021, Meurer et al. 2022). In the central nervous system, the effects of *T. erecta* flowers have also been studied and the serotonergic, nitrergic pathway, and sigma receptors are possibly involved in the antidepressant action of *T. erecta* in the mouse forced swim test (Khulbe et al. 2013). Whereas the findings from Pérez-Ortega et al. (2017) supported the anxiolytic and sedative-like properties of *T. erecta* by involving mainly serotonergic neurotransmission.

Carotenoids are compounds responsible for the yellow, orange, and red pigments of plants, fruits, and vegetables, and are well known for their potential antioxidant, anti-inflammatory and anti-apoptotic effects, playing an important role in reducing the risk of various diseases, including gastric ulcer (Stringueta et al. 2006, Sindhu & Kuttan 2012, Sivel et al. 2014). Concerning the carotenoids present in *T. erecta*, 80% is lutein and it is considered one of the

best sources of this compound (Sivel et al. 2014, Rayayuningsih et al. 2016).

Lutein extracted from *T. erecta* is effectively absorbed into the bloodstream, exerting several functions in the body, is mainly used to prevent damage to the ocular retina (Hadden et al. 1999, Sivel et al. 2014). Interestingly, the gastroprotective effect of lutein has already been described, and among the popular indications for medicinal use of *T. erecta* are digestive, diuretic, and sedative, in stomach diseases (Setshogo 2005, Mollik et al. 2010, Sindhu & Kuttan 2012, PLANT USE 2021).

This study was designed to add new advances in the knowledge about the antiulcer potential of this plant of the dry extract of *T. erecta* (DETe), expanding the data obtained earlier by Meurer et al. (2022). Therefore, this research extends our first study on the antiulcer effects of this extract, aiming to contribute to the continuity of validation of the gastroprotective efficacy of *T. erecta*, opening new ways to develop new pharmacological strategies for the management of gastric ulcers.

MATERIALS AND METHODS

Obtaining the extract and choice of doses

The dry extract of *T. erecta* (DETe), which was prepared using water plus ethanol (1:9) at an extraction temperature between 80°C and 85°C for 4 hours, was commercially obtained from the pharmaceutical ingredient supplier Pharmanostra (Campinas, Brazil). This commercial extract was produced by Natural Field (Shaanxi, China) under lot number #16E10-B019- 005810. The company declared that the extract contains xanthophyll content equal to 18%, of which 10% is represented by lutein. The phytochemical characterization of DETe was previously demonstrated by Meurer et al. (2019, 2022). The DETe doses used in this extract were based on data reported by Meurer et al. (2019, 2022) and allowed 1 Log between the doses to allow visualization of the dose-response curve if there is this effect profile.

Animals

Female mice Swiss, 3 months old (20-30 g) and Female rats Wistar, 3 months old (250-300 g) from the Central Animal Facility of UNIVALI (Santa Catarina, Brazil) and kept under controlled conditions of temperature (~25°C), and with a light and dark period of 12 hours each. All experiments were carried out following the ethical principles of animal experimentation recommended by the National Council for Animal Experimentation (CONCEA), approved by the Ethics Committee on Animal Use (CEUA) of the UNIVALI (008/20), according to the ARRIVE guidelines, and were performed following the International Standards and Ethical Guidelines on Animal Welfare.

Acidified ethanol-induced acute ulcer model in mice

To achieve the first data about the gastroprotective potential of DETe, the ethanol acidified- induced ulcers were employed as described by Morimoto et al. (1991) because this ulcerogenic agent promotes intense oxidative and hemorrhagic damage to the gastric mucosa. The mice were initially submitted to an 8-hour fast and divided into 7 groups (n=6). The positive control group was pretreated orally with carbenoxolone (CBX) at a dose of 200 mg/kg. The negative control group was treated with 10% dimethylsulfoxide solution (10 mL/kg), the vehicle used to solubilize the extract. Three groups were orally pretreated with extract at doses of 3, 30, and 300 mg/kg body weight, and one group received intraperitoneally (i.p) DETe at 30 mg/kg. The dose chosen for intraperitoneal administration was ten times lower than the effective dose by the

oral route as performed in Da Rosa et al. (2018), taking into account that in this route there are no impacts on the gastrointestinal tract for the absorption of the constituents.

The group that received DETe intraperitoneally was performed following Da Rosa et al. (2018) to evaluate if the gastroprotective potential of the extract is systemically obtained or needs topical contact to gastric mucosa or is due to their modifications after absorption from the oral route. The seventh group was composed of mice that were not exposed to acidified ethanol and therefore remained healthy and non-ulcerated, this group was named Naive. Then, the ulcerogenic agent acidified ethanol (0.3 M HCl plus 60% ethanol, 0.5 mL/20g) was administered to the animals orally one hour after the oral administration or 30 minutes after intraperitoneal administration. One hour after the ulcerogenic agent intake, the animals were euthanized in a CO_2/O_2 atmosphere. Then, the stomachs were removed and opened along the greater curvature, cleaned using a cotton swab and saline solution carefully, and scanned, to measure the hemorrhagic lesion area using appropriate software, the EARP®. The results were expressed as the total amount of injured area (mm^2).

Indomethacin-induced acute ulcer model in mice

Given the ulcerogenic potential of AINEs due to the reduction of prostaglandins bioavailability and in turn, mucus depletion in consequence of cyclooxygenase inhibition, the gastroprotective effect of DETe at the effective dose against ethanol acidified was measured like Somensi et al. (2020) to access the gastroprotective potential of this extract against different harmful agents and pathways. The mice were divided into three groups (n = 6) and pretreated orally with vehicle (Veh, 10% dimethylsulfoxide solution, 10 mL/kg), Carbenoxolone (CBX, 200 mg/kg) or DETe (300mg/kg, the gastroprotective dose against ethanol acidified). One hour later, animals received indomethacin [100 mg/kg, per os (p.o.)] to induce gastric injury. After 6 h, the animals were euthanized in a CO_2/O_2 atmosphere, and the stomachs were removed and opened along the greatest curvature. Lesions were quantified in each stomach, expressed in square millimeters using the EARP® program.

Antiulcer activity in mice pretreated with N-ethylmaleimide (NEM), N-ω-nitro-l-arginine methyl ester (L-NAME), indomethacin, and yohimbine

These experiments were carried out to evaluate the role of endogenous non-protein sulfhydryl compounds (NP-SH), nitric oxide (NO), prostaglandins (PGEs), and α2 adrenoreceptor in the gastroprotection exhibited by DETe (300 mg/kg, the gastroprotective dose against ethanol acidified) as described by Matsuda & Yoshikawa (1999), Arrieta et al. (2003) and Leite et al. (2009), with minor modifications. The mice were fasted for 8 h and pretreated with antagonists or inhibitors: NP-SH blocker (NEM - 10 mg/kg, i.p.), a NO synthase inhibitor (L-NAME - 70 mg/kg, i.p.), a non-selective cyclooxygenase inhibitor (indomethacin - 10 mg/kg, i.p) or a non-selective α2 adrenoreceptor antagonist (yohimbine - 2 mg/kg, i. p.). Further, 30 minutes after the pre-treatment with inhibitors or antagonists the mice were treated with vehicle (Veh, 10% dimethylsulfoxide solution, 10 mL/kg, p.o) or DETe (300 mg/kg, p.o). Then, 1 hour later, all animals received ethanol/HCl solution to induce gastric ulcer as described above. The animals were euthanized in a CO_2/O_2 atmosphere 1 hour after ulcer induction and their stomachs were removed, opened along the greater curvature, cleaned, and scanned, to measure the lesion area using appropriate software, EARP®.

Pylorus ligation

As described by Shay et al. (1945) with modifications, the rats were divided into 3 groups (n=6) and anesthetized with xylazine (10 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.). The positive control group received omeprazole (20 mg/kg, p.o) 30 minutes before the pylorus ligation. All rats were submitted to a laparotomy, where the pylorus was exposed and ligated. After this procedure, the animals received vehicle (Veh, 10% dimethylsulfoxide solution, 10 mL/kg) or DETe (300 mg/kg, the gastroprotective dose against ethanol acidified) intraduodenally (i.d). Subsequently, the abdominal wall was sutured, and the animals were euthanized after four hours in a CO_2/O_2 chamber, the stomach was removed, and the contents were collected. The volume of gastric juice (mL) was measured in a graduated cylinder after centrifugation (1,500 rpm, 15 min, 4°C), the pH was determined as a pH and total acidity (mEq/L/4h) was measured by titration with 10 mM sodium hydroxide.

Determination of peptic activity

As described by Anson (1938), 100 µL of gastric juice from pylorus ligated rats was incubated with 500 µL of bovine albumin (5 mg/mL prepared in 60 µM HCl) at 37°C for 10 min. Then, 1M Folin was added and incubated at 25°C for 30 min. The absorbance of each sample was inferred at 660 nm and the results were expressed in µM/mL/4h of tyrosine. The results were calculated by interpolation of obtained values on a standard tyrosine curve (30-1000 mmol/mL) because pepsin cleaves albumin releasing tyrosine residues.

Quantification of reduced glutathione (GSH)

As proposed by Sedlak & Lindsay (1968), the ulcerated tissues from mice pretreated with vehicle (10 mL/kg), CBX (200 mg/kg), or DETe (300 mg/kg, the gastroprotective dose against ethanol acidified) and submitted to acidified ethanol-induced ulcer model were weighed, and 200 mM potassium phosphate buffer (pH 6.5) was added to obtaining the homogenate. Afterward, 50 µL of homogenate was added to 40 µL of 12.5% trichloroacetic acid and centrifuged for 15 min at 3,000 rpm. Subsequently, triplicates of 10 µL aliquots of the supernatant, or distilled water, were added to 290 µL of 0.4 M TRIS buffer (pH 8.9). The reaction was started with the addition of 5 µL of 1 mM 5,5'-dithiobis 2-nitrobenzoic acid 5 min before the reading at 415 nm. Procedures were performed at 4ºC, and individual values were interpolated into a standard curve for GSH (1.25–10 µg/mL) with values expressed in µg GSH/g tissue.

Determination of lipid hydroperoxides (LOOH) levels

The total LOOH levels in the gastric ulcerated mucosa from mice pretreated with vehicle (10 mL/kg), CBX (200 mg/kg), or DETe (300 mg/kg, the gastroprotective dose against ethanol acidified) and submitted to acidified ethanol-induced ulcer model was measured using the iron II oxidation test in the presence of orange xylenol. For this, the ulcerated areas were homogenized in methanol (1:4) and centrifuged for 20 minutes at 8,900 rpm (4ºC). Supernatant and FOX-2 reagent [4 mM butylated hydroxytoluene (BHT), 250 mM $FeSO_4$, 25 mM H_2SO_4 and xylenol orange at 100 mM] were incubated for 30 minutes at 25°C. The reading was made at 560 nm. The results were expressed as mmol/mg of tissue using the extinction coefficient of 43.6/M/cm for H_2O_2, cumene hydroperoxide, or butyl hydroperoxide.

Quantification of the catalase (CAT) activity

To quantify the CAT activity, the method described by Aebi (1984) was determined. To perform this assay, the remaining homogenate from the ulcerated tissues of mice pretreated with vehicle

(10 mL/kg), CBX (200 mg/kg), or DETe (300 mg/kg, the gastroprotective dose against ethanol acidified) and submitted to acidified ethanol-induced ulcer model (obtained as described in quantification of reduced glutathione (GSH) was centrifuged at 9,000 rpm for 20 minutes at 4°C. The resulting supernatant for used to measure CAT activity as described by Aebi (1984). In this assay, triplicates of 5 µL of the supernatant from each sample or distilled water were added to 195 µL of a reaction solution (5 mM Tris/EDTA buffer, pH 8.0, 30% hydrogen peroxide, and distilled water) and immediately read at 240 nm. Results were expressed in µmoL H_2O_2/mg protein/min.

Quantification of superoxide dismutase (SOD) activity

The SOD activity was determined according to Marklund & Marklund (1974), and the supernatants used to measure SOD activity were obtained as described in this section, having been obtained from the ulcerated tissues of mice pretreated with vehicle (10 mL/kg), CBX (200 mg/kg) or DETe (300 mg/kg, the gastroprotective dose against ethanol acidified) and submitted to acidified ethanol-induced ulcer model. The reactions were performed in 200 mM Tris HCl buffer plus 2 mM EDTA at pH 8.5 at 25°C. In polypropylene tubes, triplicates of 20 µL of the supernatant aliquot were mixed with 442.5 µL of Tris buffer – EDTA. After, 25 µL of 1 mM pyrogallol was added and incubated for 20 min. The tubes were centrifuged at 4,000 rpm for 4 min at 4°C and the absorbance of the supernatant was read at 205 nm. The amount of protein that inhibited the reaction by 50% (IC_{50}) is equivalent to 1 unit (U) of SOD. Results were expressed in U of SOD/mg protein.

Determination of glutathione S-transferase (GST) activity

The total GST activity was determined as described by Habig et al. (1974) in the supernatants obtained as described in quantification of superoxide dismutase (SOD) activity, having been obtained from the ulcerated tissues of mice pretreated with vehicle (10 mL/kg), CBX (200 mg/kg) or DETe (300 mg/kg, the gastroprotective dose against ethanol acidified) and submitted to acidified ethanol-induced ulcer model. Briefly, the supernatant, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 Mm GSH, and 100 mM potassium phosphate buffer (pH 6.5) at 25°C were used in the reactions. The reaction of CDNB with GSH was monitored at 340 nm for 90 s. Specific activity was calculated using an extinction coefficient of 9.6/mM/cm for GSH, and the results were expressed in mmol GSH /min/mg protein.

Quantification of myeloperoxidase (MPO) activity

The method was performed as described by Bradley et al. (1982). The samples were homogenized with 200 mM potassium phosphate buffer (pH 6.5), and the obtained homogenate was centrifuged at 10,000 rpm for 20 min. Subsequently, the precipitate obtained was resuspended with 1 mL of 80 mM potassium phosphate buffer in the presence of 0.5% hexadecyltrimethylammonium. After the samples were again centrifuged (12,000 rpm, 20 min at 4°C), and triplicates of 30 µL aliquots of the supernatant or distilled water were added to 220 µL of a reaction solution (100 µL of 80 mM phosphate buffer, 85 µL of 22 mL phosphate buffer and 15 µL of 0.017% H_2O_2). The reaction was started with the addition of 20 µL of tetramethyl benzidine. The samples were then incubated for 3 min at 37°C, and the reaction was stopped by adding 30 µL of 1.46 M sodium acetate (pH = 3.0).

The MPO activity was determined at 620 nm, and the results were expressed as optical density unit (O.D.) /mg protein.

Antimicrobial assay

Bacterial strain and inoculum preparation

Helicobacter pylori (ATCC 43629) was provided by the Oswaldo Cruz Foundation, National Institute for Quality Control in Health, Collection of Reference Microorganisms in Health Surveillance (Rio de Janeiro). It was cultured on Mueller-Hinton agar (Kasvi, Spain) supplemented with aged (≥2 weeks old) sheep blood (5% v/v) and incubated at 37° C for 3-7 days under microaerobic conditions generated using a micro-aerobic GasPak EZ (Becton, Dickinson) in an anaerobic flask (Merk). To prepare the inoculum, the test bacteria were suspended in saline (0.89%) NaCl, adjusted to the 0.5 McFarland standard (1.5×10^8 CFU/mL), and diluted in Mueller Hinton broth (MHB) supplemented with 10% fetal bovine serum (FBS) to obtain the equivalent of 5×10^4 CFU/mL.

Minimum inhibitory concentration (MIC) against H. pylori

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the compound (Stenger Moura et al. 2021). The positive control Ampicillin was dissolved with DMSO and diluted with the culture medium to a concentration of 40 mg/mL. The negative control consisted of culture medium, solvent (DMSO), and inoculum of the bacteria. The proportion of DMSO did not exceed 1% in the medium. Serial double dilutions of the DETe samples were mixed with MHB supplemented with 10% FBS at 2.5 mg/mL. Final concentrations of 2500, 1250, 625, 312.5, 156.25, 78.12, 39.06, 19.53, 9.76, 4.88 µg/mL were tested. Each well was inoculated with *H. pylori* at a final concentration of 5×10^5 CFU/mL. Plates were incubated for 3 days in a micro-aerobic atmosphere at 37° C. After incubation, the plates were visually examined and the lowest concentration showing complete growth inhibition was recorded as the MIC for that compound. The test was performed in triplicate. The proportion of DMSO did not exceed 1% in the medium. The negative control consisted of culture medium, solvent (DMSO), and bacterial inoculum. Ampicillin was used as a positive control drug.

Statistical analysis

The results obtained were presented as means ± standard error of means (S.E.M., n=6). One-way or two-way analysis of variance (ANOVA) was used, followed by the Bonferroni or Dunnett post hoc tests, when applicable. Analyzes were performed using the Program for Windows, GraphPadPrism version 7.0 (GraphPad Software, San Diego, USA). A value of $p<0.05$ was considered significant.

RESULTS

Gastroprotective effect of DETe on ethanol/HCl-induced gastric ulcer in mice

As expected, oral administration of acidified ethanol ulcerated the gastric mucosa on average 113.7 ± 11.90 mm² in the vehicle group. As shown in figure 1a, the treatment with DETe at a dose of 300 mg/kg reduced the ulcer area by up to 48.8% when compared to the vehicle group. As expected, the group pretreated with CBX (200 mg/kg) also presented a reduction in the ulcer area compared to the vehicle-treated group ($p<0.05$). There were no differences between experimental groups treated with CBX and DETe ($p>0.05$). Representative images of these data are shown in figure 1b.

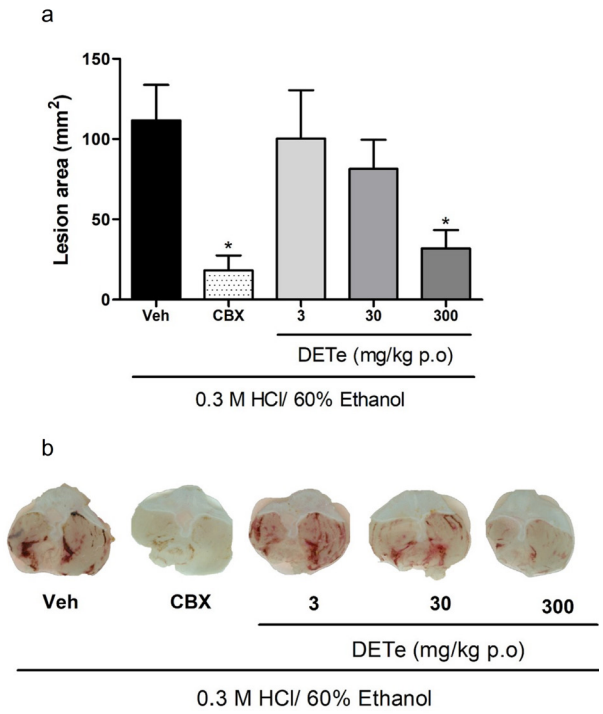

Figure 1. Gastroprotective effect of DETe on acute gastric ulcer induced by HCl/ethanol in mice: a - Effects of oral administration of DETe (at doses of 3, 30 and 300 mg/kg); b - Representative macroscopic images of the gastroprotective effects of DETe (at doses of 3, 30 and 300 mg/kg). Statistical analysis was performed using one-way ANOVA followed by Dunnet's test. *p<0.05 when compared to the vehicle. Veh: Vehicle; CBX: carbenoxolone (200 mg/kg). DETe: dry extract of *Tagetes erecta*.

Gastroprotective effect of intraperitoneal DETe on ethanol/HCl-induced acute gastric ulcer in mice

The gastroprotective effect of DETe against acute gastric ulcer induced by HCl/ethanol when the extract was administered intraperitoneally is abolished, and no significant difference between the group treated with DETe and the vehicle group was observed (Figure 2).

Gastroprotective effect of DETe on indomethacin-induced gastric ulcer in mice

The administration of indomethacin ulcerated the gastric mucosa of mice to an extent averaging 4.73 ± 0.93 mm^2 in the vehicle group.

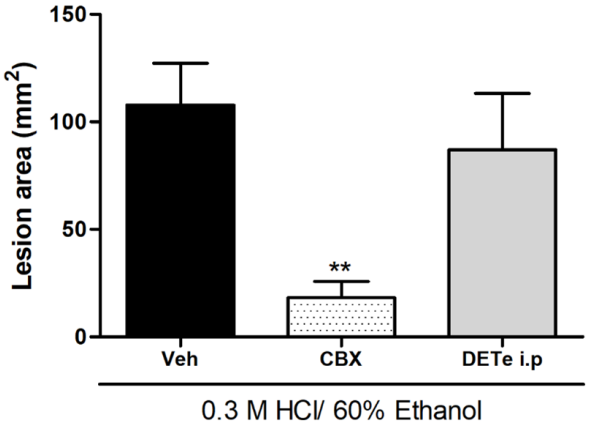

Figure 2. Gastroprotective effect of intraperitoneal DETe on acute gastric ulcer induced by HCl/ethanol in mice. One-way Anova followed by Dunnet's test. **p<0.01 when compared to the vehicle. Veh: Vehicle; CBX: carbenoxolone (200 mg/kg). DETe i.p: Intraperitoneal *Tagetes erecta* dry Extract (30 mg/kg).

Treatment with carbenoxolone reduced gastric ulcerations by 76.9% when compared to the vehicle group. As seen in figure 3, treatment with DETe at a dose of 300 mg/kg also reduced gastric ulcerations by 92.7%, compared to the vehicle group. In addition, the pretreated with CBX (200 mg/kg) also promoted a reduction in the ulcer area compared to the vehicle-treated group ($p<0.05$), but no differences were observed between experimental groups treated with CBX and DETe ($p>0.05$).

Effect of L-NAME, NEM, Indomethacin, and DETe on Ethanol/HCl-induced acute ulcer in mice

Pre-treatment with *N-ω-nitro-l-arginine methyl ester* (L-NAME) (Figure 4a), N-ethylmaleimide (NEM) (Figure 4b), yohimbine (Figure 4c), and indomethacin (Figure 4d) increased the ulcer area induced by acidified ethanol compared to the saline-pretreated group ($p<0.05$). Furthermore, pre-treatment with L-NAME, NEM, yohimbine, and indomethacin in animals treated with DETe abolished the extract's gastroprotective effect, indicating the participation of nitric oxide, sulfhydryl compounds, cyclooxygenases, and α2-adrenoceptors in its gastroprotective effect.

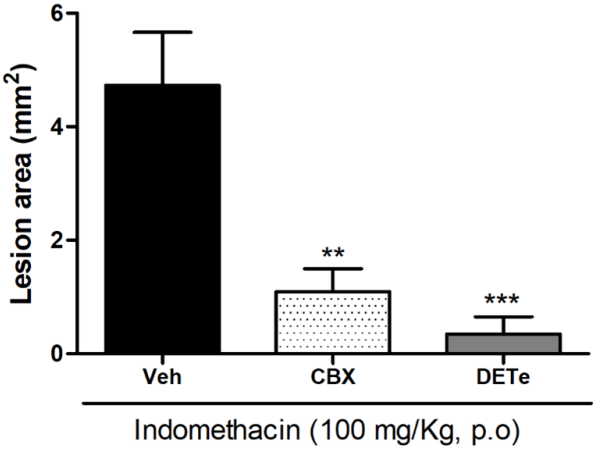

Figure 3. Gastroprotective effect of DETe on indomethacin-induced acute gastric ulcer in mice. One-way ANOVA followed by Dunnet's test. **p<0.001 and ***p<0.0001 when compared to the vehicle. Veh: Vehicle; CBX: carbenoxolone (200 mg/kg). DETe: Dry extract of *Tagetes erecta* (300 mg/kg).

Effect of DETe on the level of reduced glutathione (GSH) and lipid hydroperoxides (LOOH)

As shown in Table I, the GSH levels in the vehicle group were reduced by 18.7% when compared to the naive group (1306 ± 57.1 µg/mg of tissue). Carbenoxolone administration increased GSH activity by 13.9% when compared to vehicle. The group treated with DETe showed an increase of 36.0% in GSH when compared to the vehicle group (1062 ± 54.1 µg/g tissue). Also in Table I, in the LOOH levels, an increase in the vehicle group of 133.3% can be observed when compared to the naive group (1.5 ± 0.1 mmoL hydroperoxides/mg of tissue). Carbenoxolone administration reduced LOOH levels by 46.3% when compared to the vehicle group. Moreover, the group treated with DETe decreased by 48.6% when compared to the vehicle group (3.5 ± 0.5 mmoL hydroperoxides/mg tissue).

Effect of DETe on the SOD, CAT, and GST activities

Concerning the SOD activity, no statistical difference between the naïve, carbenoxolone, and vehicle-treated groups was found. However, in the group treated with DETe, this parameter was reduced in naive, carbenoxolone, and vehicle-treated groups ($p<0.05$). The analysis of CAT activity revealed a p-value equal to 0.06 between DETe and vehicle groups, and in the vehicle group, the CAT activity was increased by 44.82%, compared to the non-ulcerated group (Naive= 3.2 ± 0.4 µmoL H_2O_2/mg protein/min). The GST activity was increased by 322.6%, 461.3% and 338.7% in ulcerated groups pretreated with vehicle, carbenoxolone, and DETe, respectively, when compared to the naïve group.

Effect of DETe on the MPO activity

As shown in figure 5, the vehicle group increased the MPO activity by 79.6% when compared to the naive group (0.07 ± 0.01 mDO/mg of protein). The group treated with carbenoxolone or DETe reduced the MPO activity by 39.2% and 39, 1%, compared to the vehicle group (0.14 ± 0.01 mDO/mg protein). No differences were observed between groups treated with CBX and DETe ($p>0.5$).

Effect of DETe on gastric secretion and peptic activity

The volume of gastric juice in the vehicle group was 3.9 ± 0.24 mL, while in the same group the pH was 3.5 ± 0.18, reaching a total acidity of 29.3 ± 3.5 mEq[H^+]/mL, and peptic activity of 2092 ± 239.9 µM of tyrosine/mL/4 hours. The administration of DETe (300 mg/kg, i.d) did not change the volume, pH, acidity, or peptic activity when compared to the vehicle group. As expected, the administration of omeprazole (20 mg/kg) reduced acidity, and peptic activity. In addition, the pH of the gastric environment in the group treated with omeprazole was 6.6, as shown in Table II.

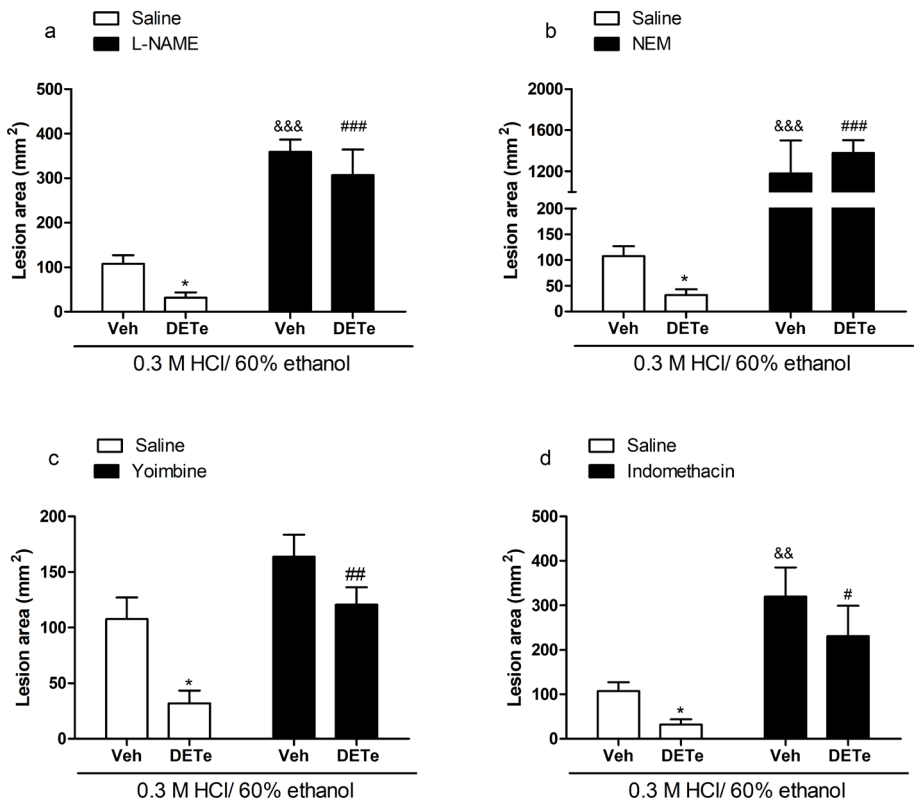

Figure 4. Effect of L-NAME (a), NEM (b), Yohimbine (c), Indomethacin (d), and DETe on Ethanol/ HCl-induced acute ulcer in mice. *p<0.05 compared to saline-pretreated vehicle by one-way ANOVA by Dunnet's test.; # p<0.05, ##p<0.01 and ###p<0.001 compared to DETe pretreated with saline and $^{\&\&}$p<0.01 and $^{\&\&\&}$p<0.001 compared to Veh group pretreated with saline by two-way ANOVA followed by Bonferroni's test. Veh: vehicle; DETe: Dry extract of *Tagetes erecta* (300 mg/kg, p.o); NEM: N-ethylmaleimide (10 mg/kg, i.p); L-NAME: N-ω-nitro-1-arginine methyl ester (70 mg/kg, i.p); yohimbine (2 mg/kg, i.p); Indomethacin (10 mg/kg, i.p).

Table I. Effects of DETe on oxidative parameters in the stomach of mice with the acidified ethanol-induced ulcer.

Parameters	Naive	Vehicle	Carbenoxolone	DETe
GSH	1306 ± 57.1	1062 ± 54.1[a]	1210 ± 53.5	1445 ± 70.7[e]
LOOH	1.5 ± 0.1	3.5 ± 0.5[c]	1.9 ± 0.1[d]	1.8 ± 0.2[d]
SOD	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.1 ± 0.1[c,d,h]
CAT	3.2 ± 0.4	5.8 ± 0.2[b]	3.7 ± 0.7[g]	4.5 ± 0.5[f]
GST	3.1 ± 0.7	13.1 ± 0.2[b]	17.4 ± 1.8[b]	13.6 ± 1.04[b]

GSH – Glutathione (µg/g tissue); LOOH - lipid hydroperoxides (mmoL hydroperoxides/mg tissue); SOD – Superoxide dismutase (U/mg of protein); CAT – Catalase (µmoL H_2O_2/mg protein/min); GST - Glutathione S-transferase (mmol GSH/mg protein/min); DETe - Dry extract of Tagetes erecta. One-way anova followed by Bonferroni test. [a]p<0.05, [b]p<0.005 and [c]p<0.0001 when compared to naive. and [d]p<0.001, [e]p<0.01, [f]p=0.06 and [g]p<0.05 when compared to vehicle. [h]p<0.05 when compared to CBX group.

Effects of DETe against H. pylori

Incubation of DETe at all concentrations tested did not show antimicrobial activity against *H. pylori* bacteria, not inhibiting bacterial growth and reaching a MIC value > 2000µg/mL (data not shown). The antibacterial agent ampicillin (SIGMA A-9518) was included in the assay as a positive control. A drug-free solution was also used as a negative control. The *H. pylori* strain was susceptible to ampicillin at MIC of 0.2 µg/mL.

DISCUSSION

Dry extracts of *T. erecta* flowers like the one investigated here are widely marketed and used

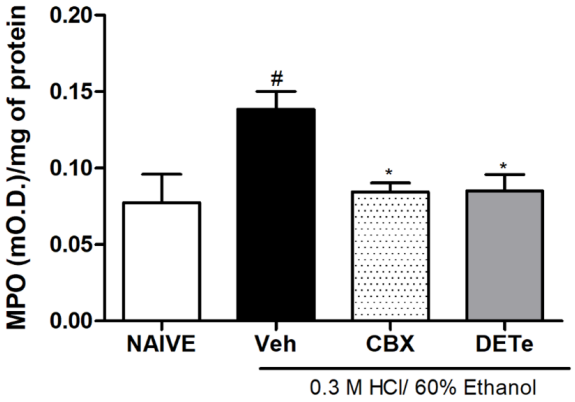

Figure 5. Effects of DETe on MPO levels in mice with ethanol-induced gastric ulcer. The animals were divided into non-ulcerated (Naive), ulcer treated with vehicle (Veh, 10 ml/kg, water + Tween 80 1%) and ulcer treated with DETe (300mg/kg). One-way ANOVA followed by Bonferroni's test. # p<0.05 compared to the naive and *p<0.05 compared to the vehicle. CBX: carbenoxolone (200 mg/kg). DETe: Dry extract *Tagetes erecta* (300 mg/kg).

worldwide due to the carotenoid lutein, which has vast biological potential. Furthermore, in traditional medicine *T. erecta* flowers are popularly used to treat gastrointestinal diseases and there is already experimental evidence of their beneficial potential in ulcerative colitis and gastric ulcers (Salehi et al. 2018, Meurer et al. 2019, 2022). In the face of this, the data obtained here bring new advances in the knowledge of the antiulcer potential of this extract, evidencing that its antiulcer actions occur in a way mediated by the strengthening of gastric protective factors with the participation of oxide nitric, non-proteic sulphydryl groups, prostaglandins, and adrenergic alpha-receptors, but not reducing gastric acid secretion and not having any effect against *H. pylori*.

Classically, the ethanol acidified-induced ulcer has been used to search for products or preparations with an antiulcer potential, including our research group (Alrashdi et al. 2012, Da Silva et al. 2016, Jan et al. 2019, Somensi et al. 2020, Boeing et al. 2021). This model was chosen because of its ulcerogenic and necrotizing potential, resulting in damage to the gastric mucosa, easily seen with the naked eye as hemorrhagic streaks in the gastric mucosa. Hemorrhagic lesions were evident in the gastric mucosa of animals exposed to acidified ethanol and pretreated with vehicle.

In contrast, was observed that the extract administered orally at 300 mg/kg (Figure 1a), but not by an intraperitoneal route at 30 mg/kg (Figure 2), reduced the ulcer area (hemorrhagic streaks) when compared to vehicle. These findings indicate that DETe has a topical effect on the gastric mucosa or that modifications dependent on gastric acidity may be necessary for the gastroprotective actions of the extract. Is important to emphasize that the dose of DETe tested intraperitoneally was ten times lower than that administered orally, this occurred because in the intraperitoneal route there is no pronounced first-pass effect as there is in the oral route and therefore its impact on the bioavailability after

Table II. Effect of DETe on gastric secretion and peptic activity.

Treatment	Dose (mg/Kg)	Volume (mL)	pH	mEq[H^+]/mL/4h	Peptic activity (μM tyrosine/mL/4 h)
Veh	-	3.8 ± 0.24	3.5 ± 0.18	29.3 ± 3.5	2092.0 ± 239.9
Ome	20	3.0 ± 0.08	6.6 ± 0.5[a]	17.1 ± 5.0 [a]	840.5 ± 322 [a]
DETe	300	3.5 ± 0.36	3.8 ± 0.18	31.4 ± 3.3	2670.0 ± 277.7

Veh: vehicle; Ome: omeprazole (20 mg/kg); DETe: Dry extract of *Tagetes erecta* (300 mg/kg). One-way ANOVA followed by Dunnet's test. [a]p<0.05 when compared to the vehicle.

abortion of the extract administered orally, this approach was already employed (Maria-Ferreira et al. 2014). Corroborating these findings, Sindhu & Kuttan (2012) found that oral administration of lutein reduced erosion and gastric bleeding in animals that received ethanol.

In indomethacin-induced ulcers, oral administration of DETe promoted a reduction in the lesion area when compared to the vehicle group (Figure 3). Similarly, Hobbenaghi et al. (2019) evaluated the effect of crocin, a carotenoid derived from *Crocus sativus*, on the indomethacin-induced intestinal ulcers, and concluded that crocin protects the intestinal mucosa through antioxidant, anti-inflammatory, and anti-apoptotic mechanisms. Additionally, Boyacioglu et al. (2016) evaluated the effects of lycopene on indomethacin-induced gastric ulcers and found that the treatment of 100 mg/kg of lycopene significantly reduced the area of gastric ulcers in the gastric mucosa. Thus, the gastroprotective effect of carotenoids in the indomethacin-induced ulcer model has been described in the literature and the lutein content present in DETe may contribute to the effect of the extract against NSAID-induced gastric mucosal aggression.

Concerning the lutein amount in DETe, the presence of this carotenoid was confirmed and estimated at 8.2% of DETe by Meurer et al. (2019, 2022). This finding agrees with Hadden et al. (1999), which evidenced that commercial extracts of *T. erecta* flowers contain trans-lutein as the major carotenoid component, up to 80% of the carotenoid fraction, with various cis-lutein isomers as minor components. In addition to carotenoids, such as lutein, the petals from the *Tagetes* genus contain several classes of secondary metabolites, including phenolic acids, flavonoids, triterpenes, alcohols, sterols, saponins, tannins, polysaccharides, and resins. Indeed, the Ultra-High-Performance Liquid Chromatography–electrospray ionization– mass spectrometry analysis of the DETe allowed the identification of five compounds: laricitrin, two laricitrin hexosides, ellagic acid, and myricetin (Meurer et al. 2019).

In addition to the gastroprotection promoted by carotenoid lutein, the gastroprotection promoted by ellagic acid, a compound present in DETe was already described (Sindhu & Kuttan 2012, Meurer et al. 2019). Beserra et al. (2011) evaluated the antiulcer effect of ellagic acid in models of acute ulcers induced by ethanol or indomethacin and chronic ulcer induced by acetic acid in rats. Therefore, the gastroprotection evoked by DETe would be mediated by carotenoid and non-carotenoid compounds present in the extract.

Given the promising results found against acidified ethanol and indomethacin ulcerogenic effects, it was then decided to investigate the mode of action involved in the gastroprotection promoted by DETe. It was decided to verify the contribution of NO, non-protein sulfhydryl compounds, prostaglandins, and α-adrenoreceptors in the gastroprotection exerted by the DETe. Together with prostanoids, NOS plays an important role as a vasodilator in the gastric mucosa promoting the removal or dilution of HCl or other harmful agents and in turn protects the gastric mucosa from injury (Tarnawski et al. 2012). In fact, the results showed in figure 4a that pre-treatment with L-NAME, a non-selective NO synthase inhibitor, increased the ulcerated area. Furthermore, the pre-treatment with L-NAME abolished the gastroprotective effect of the DETe, suggesting the involvement of NO in the effect promoted by the DETe (Figure 4a).

Non-protein sulfhydryl compounds also play important roles in the process of eliminating toxic substances from the gastric mucosa (Barros et al. 2016). Indeed, the administration

of a blocker of sulfhydryl compounds, such as NEM, promotes an increase in gastric damage in animals exposed to acidified ethanol, as shown in the results obtained in figure 4b. In addition, it was found that pre-treatment with NEM also abolished the effect of DETe (Figure 4b), also suggesting the participation of sulfhydryl compounds in the gastroprotective mechanism of the extract, which can be supported by the maintenance of DETe -induced GSH levels in the mucosa gastric (Table I).

Yohimbine is a non-selective antagonist of α_2 adrenergic receptors, which mediate the responses involved in the regulation of gastric secretion (Gyires et al. 2000). Yohimbine pre-treatment also inhibited the effects of DETe on ethanol/HCl-induced ulcers (Figure 4c). Like yohimbine, pre-treatment with indomethacin also reduced the effect of DETe, suggesting that α_2 adrenergic receptors and endogenous prostaglandins may be involved in the mechanism of action of DETe against the ulcerogenic capacity of acidified ethanol.

It is noteworthy that in the results shown in figure 4d, the ulcer was induced by acidified ethanol in animals pretreated with indomethacin at a dose ten times lower than that used when indomethacin was used as an ulcer inductor (figure 3). Thus, in animals ulcerated by acidified ethanol but pretreated with indomethacin (10 mg/kg) the extract does not exert a gastroprotective effect, probably due to the depletion of prostanoids necessary for this effect. On the other hand, as shown in figure 3, the extract prevented, that is, promoted gastroprotection in gastric lesions induced by indomethacin (100 mg/kg), indicating that in a prophylactic way the extract prevents ulcerogenic damage by NSAIDs.

Based on the results, it can be observed that the DETe uses several mechanisms of action to exert its effect. As described by Meurer et al. (2019, 2022), the extract is complex and has several phytoconstituents, which may be related to the effects obtained in the present study, since there is a diversity of molecules with different bioactive mechanisms.

The GSH was one of the antioxidant parameters evaluated in this study, which is a non-enzymatic antioxidant, composed of the amino acid glutamine, cysteine, and glycine and has a fundamental role in defending the body against oxidative stress (Patlevič et al. 2016).

As shown in Table I, the GSH levels increased in the group treated with DETe when compared to the vehicle group, indicating better preservation of this antioxidant resource, and showing that DETe promoted a positive effect in reducing oxidative stress since GSH depletion is associated with high oxidative damage in the gastric mucosa exposed to ethanol (Pérez et al. 2017). These findings corroborate the results of Meurer et al. (2019) where treatment with DETe (300 mg/kg) increased the availability of GSH in the colon of colitic mice when compared to vehicle. Similarly, Sindhu & Kuttan (2012) demonstrated, in their study with lutein at doses of 100mg/kg and 250mg/kg, increased gastric GSH in animals ulcerated with ethanol when compared to vehicle.

The oxidative process can also be characterized by lipid peroxidation of the membrane of cells in the ulcerated tissue (Lih-Brody et al. 1996, Leite 2014). Regarding the LOOH levels, an increase in the vehicle group compared to the naive group can be seen. However, in the group treated with DETe, this parameter was decreased (Table I), indicating that the extract can minimize damage to the lipid membranes of the gastric mucosa caused by the intense ROS generated resulting from exposure to ethanol. Corroborating the finding, Brito et al. (2018), found that pre-treatment with ethanol extract of *Spondias mombin* L., which has ellagic acid as

one of the bioactive compounds similar to DETe, reduced lipid peroxidation when compared to vehicle, in an ethanol-induced ulcer model.

Concerning the activity of SOD, an enzymatic antioxidant, no statistical difference between the naive and vehicle-treated groups was found. Conversely, Cabral et al. (2017) demonstrated that the administration of acidified ethanol promoted a reduction in SOD in the vehicle group when compared to the naive group. However, the group treated with DETe experienced a decrease in SOD activity when compared to the vehicle group. SOD is the first line of enzymatic defense, promoting the dismutation of the superoxide anion into hydrogen peroxide which is later catalyzed by other antioxidant enzymes (Patlevič et al. 2016). Despite the reduction in SOD activity, it is likely that the direct antioxidant effects, partly due to the presence of carotenoids and flavonoids, can exert a compensatory effect, which would therefore justify the reduction in lipid peroxide levels and maintenance of GSH levels even under reduced SOD activity.

In the analysis of the CAT activity a *p*-value in the borderline of statistical significance, equal to 0.06, was found between DETe group and the vehicle ulcerated group (Table I). Interestingly, as shown in Table I, the CAT activity was enhanced in-vehicle ulcerated group, which can reflect an antioxidant response against the elevated generation of hydroperoxides (substrates for CAT) after ulcer induction. Nevertheless, this response did not avoid the elevation in LOOH amount in the gastric mucosa of mice ulcerated by ethanol acidified and treated with vehicle. In contrast, the LOOH amount in the ulcerated group treated with DETe was reduced even with the CAT activity reduction (p=0.06 compared to vehicle), which can due to the reduction in hydroperoxides generation or due to direct antioxidant action of DETe compounds to stabilize the ROS produced.

The GST activity was increased in the ulcerated group treated with DETe when compared to the naive (Table I). The GST is a family of detoxification enzymes that catalyze the conjugation of GSH with a wide variety of endogenous and exogenous electrophilic compounds (Townsend & Tew 2003). The results obtained suggest that DETe promotes an increase in the action of this enzyme that participates in the prevention of ulcers by reducing the formation of free radicals and other xenobiotics. In addition, is possible to infer that the increase in GSH levels in the gastric mucosa of mice pretreated with DETe can be followed by the increase in GST activity because GSH is a cofactor to this detoxifying enzyme.

The MPO enzyme is considered an indirect marker of the presence of neutrophils in injured tissue. The ulcerated group treated with DETe showed reduced activity of this enzyme when compared to the ulcerated group treated with the vehicle (Figure 5), indicating a decrease in neutrophil recruitment. In the ulceration process, due to inflammation, there is the recruitment of leukocytes that promotes increased oxygen consumption to produce superoxide anion, leading to the production of numerous ROS, thus these results demonstrate a possible positive effect in reducing gastric inflammation and, consequently, reduction of gastric ulcers (Veen et al. 2009).

In clinical practice, gastric ulcers are treated with inhibitors of gastric acid secretion, especially proton pump inhibitors. The pylorus ligation methodology was used to verify the antisecretory potential of DETe. The results obtained demonstrate that the administration of DETe did not change the volume, pH, total acidity, and peptic activity (Table II). Therefore, the effects achieved by the administration of the extract are mediated by the strengthening of protective factors of the gastric mucosa as

demonstrated by Pereira et al. (2013) to *Tabebuia avellanedae*, but not by the reduction in the acidity of the content. gastric.

As reviewed by Wang (2014), numerous medicinal plants have been reported for their anti-*H. pylori* activity. Moreover, the anti-*H. pylori* effects of extracts or fractions rich in carotenoids have been revealed and for this reason, the antimicrobial activity of DETe was investigated in the present study (Molnár et al. 2010). Recently, Trinh et al. (2020) showed that the ethanolic extract of *Tagetes erecta* flowers showed inhibitory ability against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. However, in our experimental condition, the MIC of DETe against *H. pylori* was > 2000 µg/mL, suggesting that DETe does not possess antimicrobial effects against this ulcerogenic bacteria, at least directly. Despite its MIC value, the DETe administration could reduce inflammation in the gastric mucosa generated by *H. pylori* infection but experiments to test this hypothesis are awaited.

CONCLUSION

The dry extract of *T. erecta* showed an antiulcerogenic effect mediated by the reduction of oxidative stress and gastric inflammatory process, in addition to other complementary modes of action that include nitric oxide, the availability of sulfhydryl groups, alpha 2 adrenergic and prostanoid receptors. This effect may be due to the large number of carotenoids and flavonoids, especially lutein, present in this plant. Besides, the extract has no antisecretory activity in vivo and does not inhibit the growth of *H. pylori*. The articles, images and analysis tables used to support the findings of this study are available from the corresponding author upon request.

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