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Triplaris gardneriana seeds extract exhibits *in vitro* anti-inflammatory properties in human neutrophils after oxidative treatment

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ABSTRACT

Ethnopharmacological relevance: *Triplaris gardneriana* Wedd. (Polygonaceae family) is a plant species from Brazilian semiarid region which is used in local traditional medicine for the treatment of inflammatory conditions such as hemorrhoids.

Aim of the study: In this study, the *in vitro* anti-inflammatory activity of different concentrations of ethanolic extract from *T. gardneriana* seeds (EETg) was performed in order to contribute to the knowledge about etnomedicinal use of this plant species.

Materials and methods: The anti-inflammatory properties were evaluated through different approaches, such as *in vitro* protein anti-denaturation test, scavenging of reactive oxygen species (ROS) and myeloperoxidase (MPO) inhibition in human neutrophils activated by phorbol-12-myristate-13-acetate (PMA). Besides that, molecular docking was performed to provide new insights about the interaction between the major phenolic components in the plant extract and MPO.

Results: EETg was characterized showing a total phenol content of 153.5 ± 6.3 μg gallic acid equivalent/mg extract, ability to remove hydrogen peroxide (H_2O_2) in a concentration-dependent manner and had a spectroscopic profile which suggests the presence of hydroxyl groups. EETg was able to prevent protein denaturation ranging from 40.17 to 75.09%. The extract, at 10 and 20 μg/mL, was able to modulate neutrophils pro-inflammatory functions, such as degranulation and burst respiratory. In both assays, the EETg had anti-inflammatory effect comparable to nonsteroidal anti-inflammatory drugs. Among the main phenolic compounds of EETg, quercitrin, quercetin and catechin showed the highest binding affinity *in silico* to MPO.

Conclusion: This study demonstrated, for the first time, that the anti-inflammatory effect of *T. gardneriana* seeds occurs due to its modulatory effect on human neutrophil degranulation and free-radical scavenging activity.

1. Introduction

Inflammation is a biological defense mechanism caused by the interruption of tissue homeostasis due to the presence of biological, chemical or physical agents in the body (Ambriz-Pérez et al., 2016). However, in many inflammatory situations, it occurs an excessive activation of phagocytes and free radicals production which leads to an increase of vascular permeability, protein denaturation, alterations in cell membrane and proinflammatory cytokines secretion (e.g. tumor necrosis factor alpha - TNF-α). Clinical evidences suggest that these events are important components for the development of several diseases including inflammation-associated chronic illnesses, such as insulin resistance, type 2 diabetes mellitus, memory loss, neurodegenerative and cardiovascular diseases, besides that imbalanced immune response that generates susceptibility to infections (Hussain et al., 2016). This reinforces the need for antioxidant and anti-inflammatory agents, which can prevent oxidative stress and inflammation (Murugan and Parimelazhagan, 2014).

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The treatment of inflammatory conditions is usually done with the non-steroidal anti-inflammatory drugs (NSAID) administration, which frequently induces adverse effects like gastric irritation and kidney damage (Sharma et al., 2018). In recent years, natural products have received great prominence due to the anti-inflammatory effects of plant extracts and/or their isolated compounds (Azab et al., 2016). Studies have shown that phenolic compounds can exert a modulatory effect on cellular biomarkers related to oxidative stress and inflammation, which would be capable of reducing the risk of many chronic diseases. The ability of these molecules to reduce inflammation is based on the following events: firsty, direct action as antioxidants; secondly, interference in oxidative stress signaling and finally, suppression of pro-inflammatory signaling transductions (Zhang and Tsao, 2016).

In traditional Brazilian medicine, plants from Polygonaceae family are used as alternative pharmacotherapies in the treatment of inflammatory conditions and associated illnesses. Several plants of *Polygonum* and *Triplaris* genera exert hemostatic action, required in the treatment of internal hemorrhages, such as uterus and hemorrhoids (Silva-Brambilla and Moscheta, 2001). *Triplaris gardneriana* Wedd., commonly known as “pajeú”, is a native plant species of the Northeast semiarid region which popular therapeutic uses include toothache, sore throat, bronchitis, rheumatism, enteritis and gastritis. Its bark or root are used to treat inflammation of internal organs and leaves for hemorrhoids relief (Macedo et al., 2015; Magalhães et al., 2019). The dosage forms are varied, the most frequent as infusion and decoction are followed by sitz bath, water maceration, cataplasm and, exceptionally, syrups (Macedo et al., 2018).

According to some reports, many seeds from Northeastern Brazilian plants are exploited in order to discover pharmacological properties that support their use in folk medicine. This plant organ is not merely a site to accumulate organic materials for nutritional purposes, but also features low molecular weight compounds (secondary metabolites) with possible biomedical application (Ferreira et al., 2011). Some scientific evidences showed the antibacterial, anticholinesterase and antioxidant benefits of *T. gardneriana* seeds (Farias et al., 2013). However, unlike its barks and leaves, there are no reports in the literature proving the anti-inflammatory effects of seeds.

Phytochemical investigations revealed terpenes, saponins and phenolic compounds in *T. gardneriana* seeds. Previous study from our research group was able to establish its phenolic profile using HPLC-DAD technique, revealing the presence of caffeic, ellagic and chlorogenic acids (phenolic acids) along with catechin, epicatechin, kaempferol, quercetin and quercitrin (flavonoids) as major phenolic entities (Almeida et al., 2017).

In this perspective, the present study aimed to amplify the data about the anti-inflammatory activity of *T. gardneriana* by evaluating the effect of its seed extract on protein denaturation, pro-inflammatory mechanisms of human neutrophils measured by reactive oxygen species (ROS) production and myeloperoxidase (MPO) activity. In addition, we aimed to determine the binding free energy and interaction with MPO for the main phenolic compounds of EETg, previously detected by our research group.

2. Materials and methods

2.1. Chemical and drugs

Gallic acid, quercetin, Folin-Ciocalteu reagent, phorbol-12-myristate-13-acetate (PMA), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 3,3′,3,5′-tetramethylbenzidine (TMB), Hank's balanced salt solution (HBSS), 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium was acquired from Medley (Brazil). All other reagents were of analytical grade.

2.2. Plant extract preparation

For the plant samples collection, the study presented the following registrations: 47978-1 (Authorization and Information System on Biodiversity – SISBIO) and AF21C1 (National System for Management of Genetic Heritage and Associated Traditional Knowledge – SISGen). Briefly, *T. gardneriana* mature seeds were collected in Tamboril, Ceará, Brazil (04° 49 ′56 ″S, 40° 19′ 14″ W) in March 2013. The voucher specimen (EAC39600) was deposited in the Herbarium Prisco Bezerra at the Federal University of Ceará (Fortaleza, Brazil). Then, 500 g of seeds were pulverized and extracted with 99% ethanol (1:2 w/v) at room temperature (25 °C) for 3 days (Farias et al., 2013). The extractive solution was concentrated under reduced pressure in a rotary evaporator (TE-210 Tecnal, Piracicaba, Brazil) until complete solvent elimination. The ethanolic extract from *T. gardneriana* seeds (EETg) was placed in an hermetically sealed flask and stored in a freezer at - 20 °C. The yield was calculated as follows: Yield (%) = (extract mass/pulverized seeds mass) x 100.

2.3. Chemical characterization

EETg was characterized in relation to its phenolic composition by Folin-Ciocalteu colorimetric method (Pessoa et al., 2016) as well as infrared spectroscopy (Gonzaga et al., 2013). The total phenol content was determined by reaction with Folin-Ciocalteu reagent followed by absorbance reading at 700 nm in a spectrophotometer (Epoch, Take 3 module, BioTek, Winooski, USA). The analysis was performed in triplicate and results expressed in micrograms of gallic acid equivalents per milligram of extract. Afterwards, the extract was prepared in KBr pellets. The spectrum profile in infrared region was obtained using a Shimadzu FT-IR spectrophotometer model 8300 (Shimadzu Corporation, Kyoto, Japan). The maximum absorption was reported in wavenumbers (cm^{-1}) which was then used to determine organic groups indicative of secondary metabolites present in EETg.

2.4. Hydrogen peroxide (H_2O_2) scavenging assay

Different concentrations of EETg (10–200 μg/mL) were added to H_2O_2 solution (4 mM) prepared in phosphate buffer (pH 7.4). After 10 min of reaction at room temperature, absorbance value was read at 230 nm against a blank solution containing extract in buffer without H_2O_2 (Yen and Chen, 1995). Gallic acid was used as standard phenolic for comparison. The scavenging potential was estimated as EC_{50} (concentration capable of eliminating 50% of the oxidizing species). The assay was performed in triplicate.

2.5. Protein denaturation method

The ability of EETg in preventing *in vitro* protein denaturation was verified by egg albumin denaturation inhibition assay (Singh and Patra, 2018), with modifications. The test solution consisted of 150 μL of albumin from fresh hen's egg [in 5% phosphate-buffered saline - PBS] and 50 μL of different concentrations of EETg (0.05–20.0 μg/mL in PBS buffer), incubated at 37 °C in a water bath for 30 min. PBS buffer was used as blank. Denaturation was induced by maintaining the reaction mixture at 70 °C for 10 min. After cooling, turbidity was measured spectrophotometrically at 660 nm. The protein denaturation inhibition was calculated according to the following expression: Inhibition (%) = ($Abs_{control}$ - $Abs_{treated\ sample}$/$Abs_{control}$) x 100. The control consisted of albumin without treatment with samples. The assay was performed in triplicate and used diclofenac sodium (25.0 μg/mL in PBS buffer) as standard drug.

2.6. Human neutrophils isolation

Human leukocyte-rich blood from healthy adults was collected at

Pharmaceutical and Cosmetics Studies Center (CEFAC) - Fortaleza, Brazil, after approval by the Research Ethics Committee (CEP) from Federal University of Ceará (register number 3.227.799). Neutrophils, with viability of 90 ± 2.0% established by the exclusion with Trypan Blue, were isolated according to Lucisano and Mantovani (1984) and later modified by Kabeya et al. (2002).

2.7. Neutrophils viability assay

Neutrophils (5.0×10^6 cells/mL) were incubated at 37 °C with EETg (1–200 μg/mL), quercetin (10 μg/mL, antioxidant standard), dimethyl sulfoxide - DMSO (1% v/v, control/quercetin vehicle), HBSS (untreated cells) or Triton X-100 (0.2% v/v, cytotoxic standard) for 30 min, afterwards MTT was added to the medium. After 3 h under a 5% CO_2 atmosphere, the cells were washed with PBS buffer and the formazan product was dissolved in 100 μL of pure DMSO. The absorbance values were recorded at 550 nm and cell viability was expressed by percentage. The test was performed in triplicate and repeated three times (Mosmann, 1983).

2.8. ROS production by human neutrophils

Human neutrophils suspension (5.0×10^6 cells/mL) was incubated with different concentrations of EETg (0.05–20 μg/mL) or quercetin (50 μg/mL) at 37 °C for 15 min. Then, the chemiluminescent probe luminol (280 μM) was added to the medium and the mixture was incubated at 37 °C for further 5 min. PMA (0.1 μM) was added to the cell culture and chemiluminescence responses (CL-lum) were measured in a luminometer (Synergy HT, BioTek Instruments, Winooski, USA). The light emission was recorded in c.p.m. (counted photons per minute), for 20 min at 37 °C. CL-lum spontaneous production by unstimulated cells was also measured. The readings run in triplicate and the assay was repeated three times. The results were expressed as percentage of chemiluminescence emission corresponding to the ROS production (Lopes et al., 2018).

2.9. Neutrophil degranulation: myeloperoxidase (MPO) activity

Human neutrophil suspension (5.0×10^6 cells/mL) was pretreated at 37 °C for 15 min with EETg (0.05–20.0 μg/mL), indomethacin (36 μg/mL) or HBSS (untreated cells). Cells were stimulated by the addition of PMA (0.1 μM) for 15 min at 37 °C. The material was centrifuged for 10 min at 4 °C. To aliquots (50 μL) of supernatants PBS (100 μL) was added, followed by phosphate buffer (50 μL, pH 7.0) and H_2O_2 (0.017%). After 5 min at 37 °C, TMB (1.84 mM, 20 μL) was added and the reaction was stopped after 3 min with H_2SO_4 (4 M, 30 μL). The absorbance values were measured at 450 nm, the readings were done in replicate (three to six) and repeated three times. The results were expressed as percent MPO inhibition by stimulated human neutrophils (Úbeda et al., 2002).

2.10. Myeloperoxidase docking with phenolic acids and flavonoids

The myeloperoxidase structure was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/) with the ID: 5FIW (Bonnefond and Cavarelli, 2015). The Chimera 1.8 software (Pettersen et al., 2004) was used to remove the B and D chains, waters and other molecules, and add hydrogen to the residues (the Asp98 was considered protonated (Gau et al., 2016)). The ligands (major phenolic acids and flavonoids previously identified in EETg) were built in the software Avogadro 1.1.1 considering the pH = 7.4 (Hanwell et al., 2012), following the semi-empirical PM6 (Stewart, 2007) geometry optimization using the program MOPAC2012 (Stewart, 2012). Ligands and protein in the *pdbqt* format were generated by AutoDockTools, where the phenolic compounds were considered flexible (with PM6 charges), and the enzyme rigid (with Gasteiger charges) (Morris et al., 2009). The Fe(II) partial charge (1.263) were obtained from PM6 optimization, considering the Fe(II) bounded with a water molecule, the lateral chain of histidine, and the heme group (with the water dielectric constant). AutoDock Vina 1.1.1 program was used for the blind docking (Trott and Olson, 2010), using a grid box of 66 × 66 x 76, and the coordinates x = -51.05, y = −-14.50, z = −-22.33, with an exhaustiveness of 100. As a model of the binding pose, it was selected the compound's conformer with lowest binding energy (ΔG). The results from docking were analyzed using the Discovery Studio Visualizer 17.2 software (Dassault Systèmes BIOVIA, 2017).

2.11. Statistical analysis

Results were expressed as mean ± standard deviation (SD) and the comparison of means was performed using analysis of variance (ANOVA) followed by Dunnett test. Differences were considered statistically significant when $p < 0\ 05$.

3. Results and discussion

The extract (EETg) showed a yield of 22.25%. Among the soluble and extractable constituents in the preparation, it is important to highlight the occurrence of phenolic compounds. These correspond to a group of phytochemicals with recognized antioxidant properties (Abdullah et al., 2017) including phenolic acids and flavonoids. The total phenol content in EETg was 153.53 ± 6.29 μg gallic acid equivalent/mg of extract, lower but close to the value of total phenol (181.8 ± 0.07 μg gallic acid equivalent/mg of extract) of *Calligonum azel* extract, a species from Polygonaceae family located in the arid region of Tunisia (Bannour et al., 2016). In general, these results refer to low or intermediate molecular weight polyphenols, which are usually extracted with ethanol (Sant'anna et al., 2012).

In order to contribute to its chemical characterization, the spectroscopic profile of EETg was determined (Fig. 1). The plant extract showed strong absorptions in the region between 3000 and 3500 cm^{-1}, which suggests the presence of hydroxyl group (O–H), a primordial characteristic of phenolic compounds. Similarly, it was observed a somewhat strong absorption in the region of 1700 cm^{-1}, indicating the occurrence of carbonyl functional group (C═O), usually found in organic acids (Silva Júnior et al., 2006). In addition, bands between 800 and 1800 cm^{-1} indicated an absorption pattern peculiar of aromatic compounds such as tannins and flavonoids (Farias et al., 2013). Previous work described chromatographic profile of EETg showing the presence of different polyphenols (Lopes Neto et al., 2017), such as

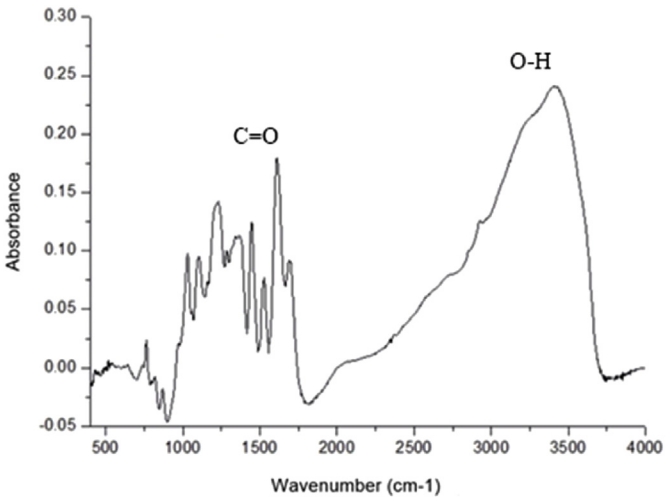

Fig. 1. Infrared absorption spectrum of *Triplaris gardneriana* seeds extract (EETg).

catechin and epicatechin-3-*O*-gallate, molecules rich in organic groups mentioned above, thus corroborating with the findings in the present infrared spectrum.

Although identification of individual phenolic compounds present in *T. gardneriana* seeds has been the focus of a previous publication by our group, we used the Fourier transform infrared (FT-IR) spectroscopy in order to generate a spectrum that could be regarded as a metabolic "fingerprint" of the sample and even evaluate the qualitiy of this botanical material. It is a common knowledge for researchers dealing with natural products that the analytical technique aforementioned that does not resolve the concentrations of secondary metabolites, but provides an overview of the composition of a plant tissue and it is a method requested by Pharmacopoeias of many countries (Liu et al., 2006).Since ROS are commonly associated with the onset of inflammatory processes and their production is considered a potential target to anti-inflammatory drugs (Rivera et al., 2018), the present study evaluated the *in vitro* antioxidant activity of EETg through its direct interaction with H_2O_2, a non-radical species with physiological relevance. Among the hydroperoxides, H_2O_2 is the most used as a model of oxidative stress. In biological systems, when it is not rapidly eliminated, it is converted to hydroxyl radical, which in turn, causes cellular damages (Meng et al., 2017). The extract was able to remove H_2O_2 in a concentration-dependent manner (EC_{50} = 28.23 ± 1.52 μg/mL). Its neutralizing effect was higher than that of gallic acid (EC_{50} = 46.60 ± 1.50 μg/mL) and even more so than that of flavonoids isolated from *Rumex hastatus* (EC_{50} = 275 μg/mL), member of the same botanical family that *T. gardneriana* (Ahmad et al., 2015). The capacity of plant extracts, more precisely of their phenolic constituents, to act as *in vitro* antioxidants has been the subject of several studies. This activity are due to the hydroxyl groups linked to their chemical structures (Hussain et al., 2016). Therefore, EETg could be used as possible therapeutic agents against ROS-mediated diseases, scavenging diffusible species across cellular membranes, like H_2O_2. Based on that, the promising activity of EETg against this oxidant species can serve as the basis for more elaborate antioxidant studies. Previous work showed that EETg has protected the MCF-7 (human breast adenocarcinoma) cell line against oxidative stress, as well as restored its redox balance after treatment with *tert*-butyl hydroperoxide (TBHP) solution or menadione (Almeida et al., 2017).

Protein denaturation is one of the main problems documented in inflammatory conditions, such as in rheumatoid arthritis (Padmanabhan and Jangle, 2012). EETg was evaluated for the ability to inhibit heat-induced protein denaturation (Fig. 2). Inhibition percentages were 75.09 and 62.84% at the two highest concentrations of EETg, 10 and 20 μg/mL, respectively. These values were higher than presented by the treatment with diclofenac sodium 25 μg/mL (49.44%), standard nonsteroidal anti-inflammatory. It is noteworthy that the anti-denaturation action of biologically relevant pure compounds and plant extracts is desirable when used in low concentrations (Williams et al., 2008).

The molecular mechanism of tissue proteins denaturation involves alteration of their three-dimensional structures, more precisely, disturbances in their hydrophobic interactions, hydrogen and disulfide bonds (Murugan and Parimelazhagan, 2014). Subsequently, proteins lose their biological functions and there is an enhancement of the inflammatory process. In this context, phenolic compounds are capable, for example, of binding to cations and other biomolecules, thereby stabilizing protein entities (Alhakmani et al., 2014). We hypothesize that polyphenols-albumin interactions lead to the protein stabilization via multiple binding sites, creating a series of hydrophobic interaction, which might subsequently be complemented by hydrogen bonds. This could partly explain the fact that several studies suggest that phenolic preparations have the ability to protect globular proteins against physical or chemical stressors (Prigent et al., 2003; Paun et al., 2018). EETg, as reported previously, contains a variety of phenolic compounds, which corroborate this hypothesis. Likewise, the ability of

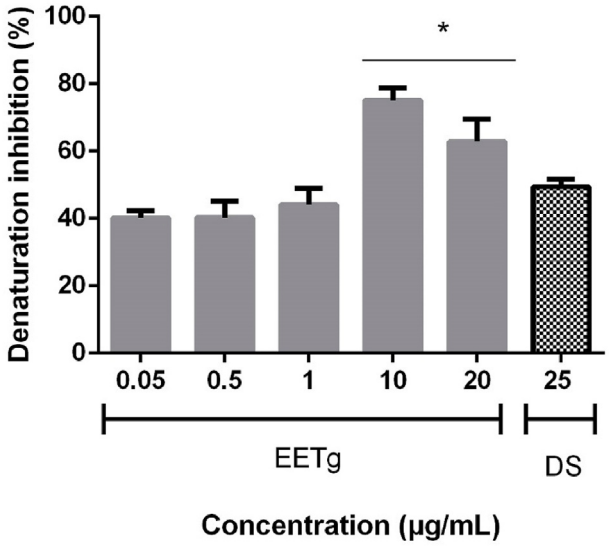

Fig. 2. Protective effect of *Triplaris gardneriana* seeds extract (EETg) on heat-induced protein denaturation. DS: diclofenac sodium. The bars represent mean ± standard deviation. * *vs* DS, significantly different when $p < 0.05$ (ANOVA and Dunnet as the *post hoc* test).

aqueous leaf extracts of both green tea and black tea (*Camellia sinensis*) to avoid *in vitro* protein denaturation is attributed to its expressive flavonoid content (Chatterjee et al., 2012).

The acute toxicity assessment of plant extracts is essential to ensure its safe use as an anti-inflammatory agent. Therefore, MTT assay is used in toxicological screening of extracts as well as of their isolated compounds. The experiment is based on the MTT reduction by mitochondrial dehydrogenase into the purple formazan (Ghagane et al., 2017). In this purpose, the effect of EETg on the viability of human neutrophils was determined using MTT assay. The exposure of neutrophils to EETg (1–200 μg/mL) did not cause significant reduction in the cellular viability (98.26–110.36% viable cells), when compared with the response of the untreated group (HBSS) (100% viable cells) (Fig. 3). The same pattern was observed for quercetin (10 μg/mL) whereas the cytotoxic agent Triton-X (0.2% v/v) promoted the mortality of approximately 90% of neutrophils tested. This result is reinforced by that described to *T. americana* barks hydroalcoholic extract (2 mg/kg body weight) which showed no mortality or symptoms indicative of toxicity in experimental rats (Camones et al., 2010).

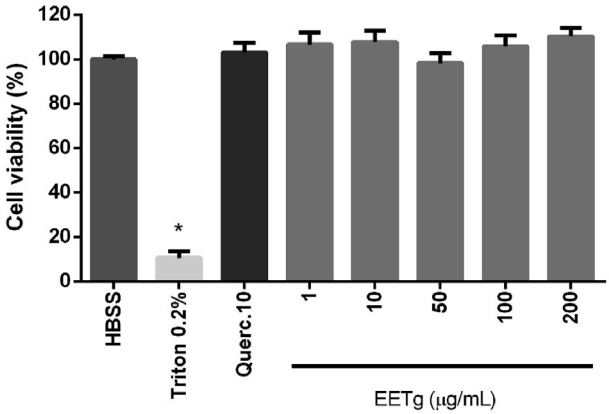

Fig. 3. Effect of *Triplaris gardneriana* seeds extract (EETg) on the human neutrophils viability determined by MTT assay. HBSS: Hank's balanced salt solution; Querc. 10: quercetin (10 μg/mL). Analyzes were performed in triplicate in three different days. The bars represent mean ± standard deviation. **vs* HBSS, significantly different when $p < 0.05$ (ANOVA and Dunnet as the *post hoc* test).

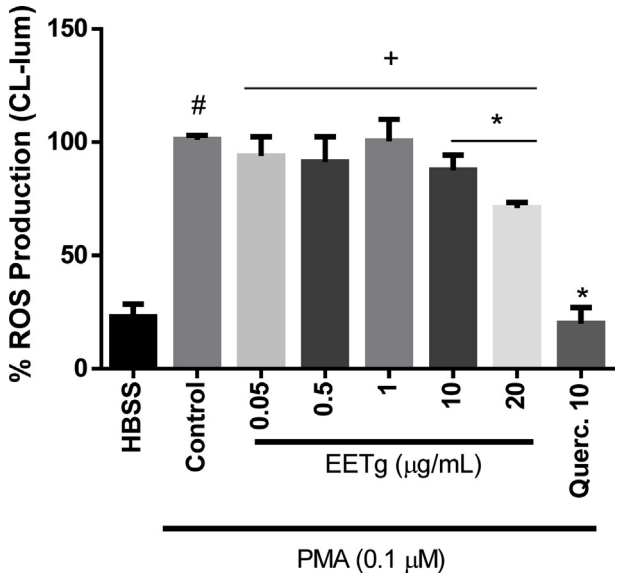

Fig. 4. *In vitro* inhibitory effect of *Triplaris gardneriana* seeds extract (EETg) on human neutrophil reactive oxygen species (ROS) generation elicited by phorbol-12-myristate-13-acetate (PMA). HBSS: Hank's balanced salt solution; Control: cells stimulated with PMA; Querc. 10: quercetin (10 µg/mL). Analyzes were performed in triplicate in three different days. The bars represent mean ± standard deviation. #*vs* HBSS, **vs* Control and +*vs* Querc, significantly different when $p < 0.05$ (ANOVA and Dunnet as the *post hoc* test).

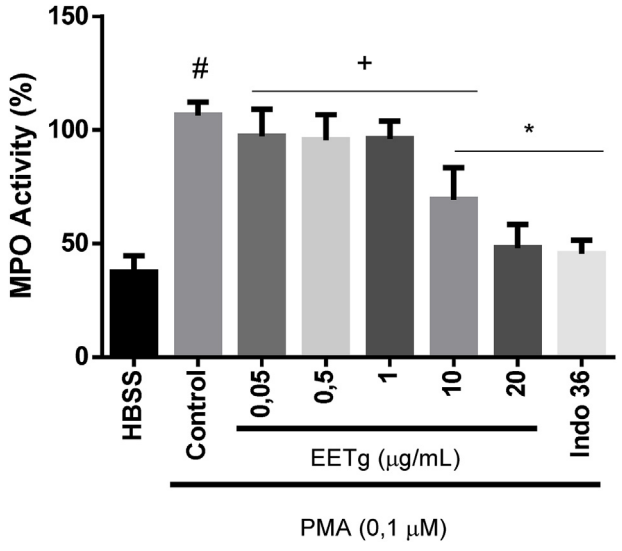

Fig. 5. Effect of *Triplaris gardneriana* seeds extract (EETg) on myeloperoxidase (MPO) enzymatic activity released from human neutrophil stimulated by phorbol-12-myristate-13-acetate (PMA). HBSS: Hank's balanced salt solution; Control: cells stimulated with PMA; Indo 36: indomethacin (36 µg/mL). Analyzes were performed in triplicate in three different days. The bars represent mean ± standard deviation. #*vs* HBSS, **vs* Control and +*vs* Indo, significantly different when $p < 0.05$ (ANOVA and Dunnet as the *post hoc* test).

Assuming that EETg presents antioxidant action on human neutrophils metabolism, these cells were stimulated with PMA and had their total ROS production measured by the chemiluminescence probe luminol (CL-lum) (Fig. 4). PMA, a synthetic phorbol ester analogue, is capable of activating the signal transduction in the neutrophil, inducing strong respiratory burst and, consequently, resulting in the release of superoxide anion, hydrogen peroxide and others MPO-derived species (Vorobjeva and Pinegin, 2016). Thus, addition of PMA provided about 4-fold increase in ROS production (control) when related to

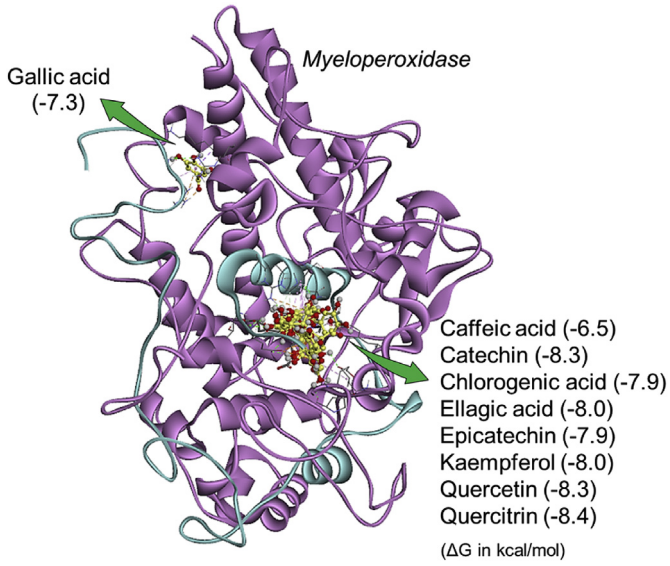

Fig. 6. Docking simulation between the myeloperoxidase (MPO) and the major phenolic acids and flavonoids present in *Triplaris gardneriana* seeds extract (EETg). MPO chains A and C are shown in blue and pink color, and the phenolic compounds are represented by carbon atoms in yellow color. Predicted binding free energy, ΔG (kcal/mol), values are indicated in parentheses. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

unstimulated cells (HBSS). Treatment with EETg suppressed the neutrophil response in 13.42 and 30.07% at concentrations of 10 and 20 µg/mL, respectively. This effect was much less significant when compared to that of quercetin (81.12%), used as standard in this and subsequent tests (Fig. 4). Another study described results of ROS production inhibition with intermediate values between those of our extract and that of quercetin, that is, the highest concentration of *Abelmoschus esculentus* leaves aqueous extract (10 µg/mL) was able to inhibit up to 40% ROS production in PMA-stimulated neutrophils (Tsumbu et al., 2012). Similarly, but not using neutrophils, *T. americana* bark methanolic extract showed *in vitro* antioxidant activity measured by CL-lum probe (Desmarchelier et al., 1997).

Reduction of CL-lum results from the action of EETg possibly eliminating H_2O_2 and hypochlorous acid - HOCl (substrate and product of the peroxidase, respectively) and hindering the catalytic activity of MPO itself (Figueiredo-Rinhel et al., 2017). As previously discussed in this work, the extract neutralizes H_2O_2 in a concentration-dependent manner and this reinforces the idea that part of luminol signal block is due to the antioxidant action of EETg.

Recently, neutrophil inhibition has been introduced as a part of a strategy for pharmacological modulation of inflammatory and oxidative stress processes. Bioactive extracts and their constituents, such as phenolic compounds, may inhibit the free radicals release by neutrophils and activation of these cells. Some flavonoids, phenolic acids and tannins inhibit the formation of pro-inflammatory signaling molecules. For instance, ROS act as secondary messengers, closely associated to both acute and chronic inflammations (Denev et al., 2014; Ondua et al., 2019). Therefore, the inhibitory effect of EETg on PMA-activated neutrophils seems be related to a direct ROS scavenging activity of the plant extract, specially its polyphenol content. Furthermore, it is important to point out the extract did not induce neutrophil oxidative response per se (data not shown). It is known that quercetin, one of the phenolic compounds in *T. gardneriana*, prevents phagocytes activation, affecting the expression of proinflammatory cytokines, free radical formation, lysosomal enzyme release, degranulation and phospholipid metabolism (Guazelli et al., 2018). This fact could explain, in part, how sometimes a purified phenolic compound would perform better in

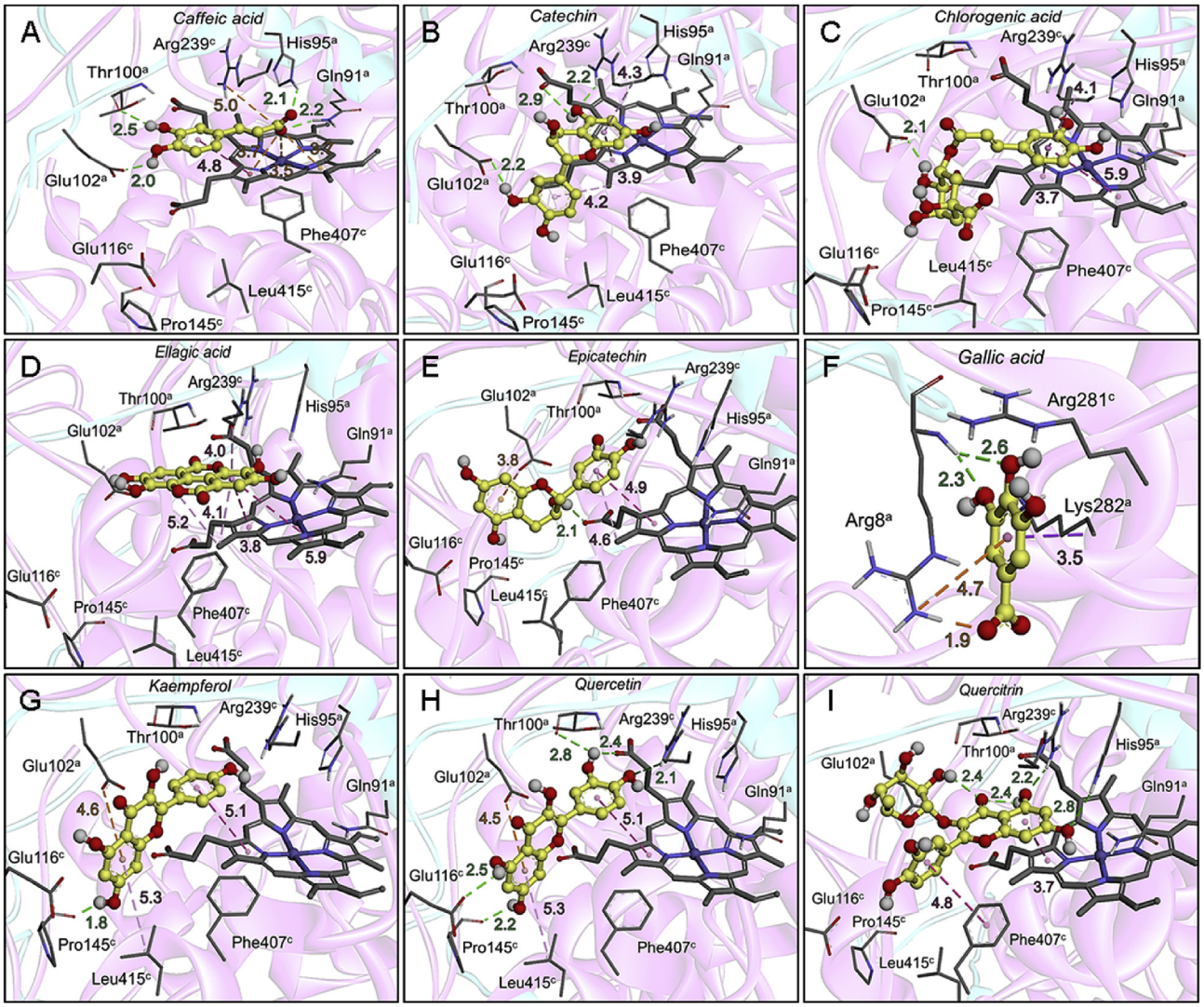

Fig. 7. Interactions between the phenolic compounds and myeloperoxidase (MPO) (A: caffeic acid, B: catechin, C: chlorogenic acid, D: ellagic acid, E: epicatechin, F: gallic acid, G: kaempferol, H: quercetin, and I: quercitrin). Hydrogen bonds, electrostatic (salt-bridge, π-anion and π-cation), and hydrophobic (π-alkyl, π-π) interactions are shown in green, orange, and purple dot lines, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inhibiting neutrophil oxidative metabolism than the plant extract. However, when an extract is used, there is a good chance that synergism between its active compounds acting on other inflammation-related processes would be lost when each of these constituents is isolated. In addition, plant extracts continue to be used for therapeutic interventions since the purification of anti-inflammatory compounds still seems a complicated task (Azab et al., 2016). The moderate effect of EETg may be associated with its low permeability due to the insufficient quantity of lipophilic antioxidant compounds that could interact with the cell membrane and reach the cytosol of the tested cells (Tsumbu et al., 2012). Previous work identified the presence of quercetin as a constituent of EETg, as well as other flavonoids with intermediate lipophilic character such as catechin, quercitrin and kaempferol (Almeida et al., 2017). Thus, it is possible to infer that, perhaps, the mode of action of the extract is similar to that already described for quercetin purified or that is partly justified by the presence of quercetin in EETg composition.

MPO is a peroxidase released during the neutrophils and monocytes degranulation. It produces HOCl from H_2O_2 and chloride anions to kill bacteria and other pathogens. Its clinical significance has been the focus of many researchers, because it may contribute to the control of several non-microbial chronic inflammatory processes (Yuan et al., 2017). Thus, the ability of EETg in inhibit MPO activity, one of the major neutrophilic enzymes, was verified after PMA induction (Fig. 5). *T. gardneriana* extract significantly inhibited the catalytic activity of MPO, reaching percentages between 30.53 and 51.89% (10 and 20 μg/mL, respectively) in relation to the control group (100% release). It is important to point out these concentrations were the same which prevented *in vitro* protein denaturation. In a similar way, the ethanolic extract of *Gentiana lutea* roots (10 μg/mL), a perennial herb used for anti-inflammatory purposes in Europe, inhibited 31% of MPO activity (Nastasijević et al., 2012). The highest concentration of EETg presented inhibition profile similar to that of indomethacin 36 μg/mL (54.42%), standard drug.

In acute inflammation, neutrophils cause tissue damage via the excessive release of their granule enzymes, such as MPO. Numerous nonsteroidal anti-inflammatory drugs, anilines and phenols reversibly inhibit MPO (Nastasijević et al., 2012). Phenolic compounds found in *T. gardneriana* such as gallic acid, catechin and epigallocatechin gallate show anti-inflammatory potential demonstrated by inhibition of MPO-dependent HOCl formation in activated neutrophils or by damages to MPO/HOCl-induced biomolecules (Nayeem et al., 2016; Tian et al.,

2017). Accordingly, it would be desirable for ETTg to exhibit a similar effect. This data about EETg phenolic profile could be interesting for further analysis since the MPO inhibition might be structure dependent. Specific phenolic compounds present in *T. gardneriana* seeds were therefore investigated.

Molecular docking was used to understand the binding modes of the major phenolic compounds from EETg (caffeic acid, catechin, chlorogenic acid, ellagic acid, epicatechin, gallic acid, kaempferol, quercetin, and quercitrin), previously identified and quantified by our research group (Almeida et al., 2017), in MPO protein. According to the docking, except for gallic acid, all the compounds interact in the active site of MPO (Fig. 6), contributing to the inhibition. The predicted binding free energy (ΔG) indicates that the molecules interact in a favorable thermodynamical process (ΔG < 0.0 kcal/mol) with the protein. Catechin, quercetin, and quercitrin presented the lowest ΔG value (Fig. 6), indicating a better affinity for MPO when compared with the other phenolic compounds studied. This probably occurred due to the higher lipophilicity of these three flavonoids in relation to gallic acid might favor their access to the MPO active site.

The binding analysis of the phenolic acids and flavonoids shown which hydrogen bonds, electrostatic, and hydrophobic interactions present an essential role in the ligand-receptor complexes stabilization (Fig. 7). The presence of quinic acid and rhamnose in chlorogenic acid and quercitrin, respectively, alter the binding pose of these molecules when compared with caffeic acid and quercetin (Fig. 7A, C, 7H and 7I). Epicatechin, kaempferol, and quercetin demonstrated a similar binding mode, with their flavan B ring interacting with the pyrrole ring from heme, while catechin and quercitrin interact with the A ring (Fig. 7C, E, 7G, 7H, and 7I). Only the caffeic acid shown O···Fe coordination (Fig. 7A), while the other molecules (except gallic acid) interacts with the heme group by π-π interaction (mainly ellagic acid - Fig. 7D). The binding pose obtained from quercetin and epicatechin has practically the same pattern when compared with the previous studies of Shiba et al. (2008) and Gau et al. (2016). As related by Gau et al. (2016), Gln91, His95, and Arg239 are key amino acids residues involved in the interactions. Moreover, it was observed that Glu102 is also important due the hydrogen bonds and π-anion interactions, besides of the heme group. In fact, the residues His95, Glu102, Arg239, Phe366, and Phe407 are essential to form hydrogen bond and hydrophobic interactions with the inhibitor (Tian et al., 2017). In this way, the docking data obtained are in accordance with the literature and collaborates for the explanation of MPO inhibition by EETg.

Overall, the results show that EETg since at low concentrations modulates the human neutrophils oxidative metabolism and degranulation, also suggesting that it might be active at other inflammatory pathways (proteins denaturation, e.g.). According to literature, these effects may be associated at least in part with the synergism of phenolic compounds in plant extract (Figueiredo-Rinhel et al., 2017; Lopes et al., 2018). Corroborating this hypothesis, recently we showed that the anti-neuroinflammatory effect of *Camelia sinensis* extract was related to the presence of polyphenols, epicatechin and epigallocatechin gallate in plant extract (Pinto et al., 2015).

4. Conclusion

The present study contributed to ethnomedicinal validation of *T. gardneriana* for the treatment of inflammatory disorders. *In vitro* activities showed that EETg has protective effect against protein denaturation, in addition to modulating some neutrophil effector functions, such as degranulation and burst respiratory without cytotoxic signs in these cells. A computer docking simulation also proposed the binding of EETg phenolic compounds to the catalytic center of MPO. These findings suggest that EETg could exert *in vivo* protective effects against inflammatory mechanisms that occur in different pathological conditions. More efforts are needed to find out more information about the mechanisms behind its anti-inflammatory action of EETg.

Authors' contribuition

This article was generated with data of a doctoral thesis by the student J.J.L.N, supervised by A.F.U.C. The contributions were as follows: J.J.L.N., T.S.A., L.K.A.M.L, T.R.M and A.F.U.C. conceived and designed the experiments; J.J.L.N., T.S.A., T.R.M., L.C.P.A.F., P.A.N. and K.F.N. performed the experiments; J.J.L.N., T.S.A., A.F.U.C., L.K.A.M.L. and J.B.T.R. analyzed the data; J.J.L.N., T.S.A. and A.F.U.C. wrote the paper.

Declaration of competing interest

The authors declare no competing interests.

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