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Research Article

Evaluation of the Anti-inflammatory Activity of *Equisetum arvense* and *Baccharis trimera* Fractions

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Abstract

Inflammation is a natural response of the body to defend itself against potential threats and can be reduced through physical activity, proper nutrition, and the use of herbal medicines, which are medicinal plants. In the study, we aim to examine the anti-inflammatory effects of the volatile and ethanolic fractions of two commonly used medicinal plants, *Equisetum arvense,* and *Baccharis trimera*. The essential oils were obtained by hydrodistillation of the fresh leaves of the plants, while the ethanolic extracts were obtained using classical methodologies. All fractions were tested for anti-inflammatory activity, evaluating their ability to stabilize the red blood cell membrane and inhibit the spreading, and phagocytosis by macrophages, at concentrations varying from 200 to 600 μg mL^{-1}. The results of the experiments suggest that the ethanolic fraction of *B. trimera* shows promising results compared to the positive controls. Our investigations thus contribute to the specialized literature on the use of herbal medicines around nutrition, providing guidance for future studies on these fractions.

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Introduction

Inflammation is a response of the human body against various physiological and pathological processes, aimed at protecting the body against infections [1,2]. There are two types of inflammation: chronic and acute [3]. The use of commercial medications has been common in the treatment of inflammatory processes [4]. Another way to treat inflammation is through physical activity, as it promotes greater release of T regulatory cells, less secretion of immunoglobulins, and interferes with the th1/th2 balance, resulting in the production of th1 cells [5]. However, the use of herbal medicines and the ethnopharmacological knowledge of substances present in medicinal plants have gained prominence for their role as antagonists in the treatment of recurrent inflammatory diseases individuals' daily lives [6,7].

Natural products, such as plant extracts, contain metabolites that can have beneficial effects on the body, such as inhibiting the formation of free radicals and modulating enzymes, known as special metabolites [8]. In folk medicine, many medicinal plants are already being used for various purposes, including supplementary, pharmacological, dietary applications, among others [9]. These fractions can be consumed in their natural form, as extracts, infusions, and other preparations. The special metabolites present in plants are produced by them for purposes such as pollination, controlling microbiological activity, defense, and attraction. These metabolites can be applied to humans for various purposes, such as treating or preventing inflammation [10]. Recent estimates from the literature suggest that over the last 40 years, approximately 23.5% of approved drugs or pharmaceuticals have been derived from medicinal plants [11]. Therefore, it is justifiable to propose more detailed studies on the biological potential of substances from Brazilian flora, as well as plants used in its traditional medicine.

Among the various cultivable plants in this region,

medicinal plants with phytotherapeutic effects known in popular medicine as *Equisetum arvense* L. and *Baccharis trimera* have great biological potential that is still little explored for various applications [12]. *E. arvense* L is popularly known as "cavalinha" and is listed in the 1st edition of the Brazilian Pharmacopoeia, as well as on the National List of Medicinal Plants of Interest to the Unified Health System of Brazil (RENISUS). It is highly rich in minerals and is indicated by folk medicine for mild diuresis, swelling, remineralization, and inflammation [12]. "Carqueja" (*B. trimera*) is used to treat gastric and liver disorders, in addition to having antimicrobial properties and providing protection for the liver and stomach [13]. However, there are few studies on the anti-inflammatory activities of these plants and their fractions. Therefore, additional research is needed to explore the potential of these plants in inflammation, as the infusions or extracts of *B. trimera* and *E. arvense* have been used extensively by ethnopharmacology to increase the performance of physical practices. The purpose of this study is to extract polar and nonpolar fractions from *E. arvense* and *B. trimera*, and to test their anti-inflammatory potential.

Materials and methods

Collection and identification of species

Intact leaves of *E. arvense* L. *e B. trimera* were collected from a native plant greenhouse, in the city of Orizona, GO, Brazil, with geographic coordinates 17°02'13.6"S 48°18'22.1"W. During the collection of plant samples, various parts of the species were obtained, including leaves and branches free from herbivory. All parts collected were sent as exsiccates for botanical classification of the plant species. The taxonomic classification was carried out by professor Dr. Marcus Vinícius Vieitas Ramos, and the exsiccates were stored in the herbarium of the Instituto Federal Goiano – Campus Urutaí- GO.

Obtaining natural product fractions

Leaves of *B. trimera* and *E. arvense* were used to extract volatile fractions, with 90 g and 174 g of fresh plant material samples used, respectively. This extraction was done through hydrodistillation in a Clevenger-type apparatus, adapted to a round-bottom flask, described in the literature and in subsequent works by the research group [14,15]. The fresh leaves were manually miniaturized, and a volume of 400 mL of distilled water was added to the flask, and the hydrodistillation process was carried out for 180 minutes. The organic fraction obtained was chemically dried with anhydrous magnesium sulfate and then filtered to remove the solid. To obtain the ethanolic extracts, freshly collected leaves (120 g in both cases) were dried in an oven, miniaturized, and placed in a 2 L flask [16]. A volume of 1200 mL of ethanol (P.A., 95%, Neon, São Paulo-SP, Brazil) was added to this mixture. It was manually stirred for 7 days and then filtered. The extract was evaporated at 40 °C under reduced pressure to dryness in a rotary evaporator (Fisatom 801 model, Fisatom, São Paulo-SP, Brazil). The remaining fraction was dried at room temperature in a desiccator. The fractions obtained were stored in a hermetically sealed amber flask and kept under refrigeration until used for biological analysis.

Evaluation of anti-inflammatory activities

To assess the anti-inflammatory effects of *B. trimera* and *E. arvense*, three experiments were conducted. Initially, for the evaluation of the human red blood cell (HRBC) membrane stabilization, the method proposed by Singh, et al. 2020 and Fracasso, et al. 2023 was employed, with some modifications [17,18]. The evaluation of the stabilization of the HRBC membrane was performed by creating a suitable biological medium to test the stability of the red blood cell membrane. In this test the evaluation of the *in vitro* anti-inflammatory activity was conducted with red cell homogenates (10%, w/v). Peripheral blood samples were obtained by the Laboratory of Clinical Analysis Bioanalysis, in the city of Cândido Mota (SP). The samples that were donated corresponded to the leftovers of whole human blood that would be discarded after carrying out the analyses by the laboratory. Materials were from 12 healthy volunteers of both genders, aged between 25 and 35 years, who were not taking any medication or toxic substance, chosen according to the research criteria. The Informed Consent Form (ICF) was prepared, and the experimental protocol was approved by the Ethics in Research Committee (ERC) (Process 14382719.4.0000.5401). This involved adding 2 mL of hyposaline solution (0.18%), 1 mL of sodium phosphate buffer (0.1 M, pH 7.4), 1 mL of analyzed samples of the fractions tested at three different concentrations (200, 400, and 600 μg mL^{-1}), and 0.5 mL of HRBC solution. The hemoglobin content in the suspension was measured using a spectrophotometer at a wavelength of 560 nm. 1 mL of dexamethasone 40 μg mL^{-1} served as a positive control, while 1 mL of 0.9% saline solution was the negative control [19]. The anti-inflammatory activity was calculated using Equation 1.

$$\text{Anti-inflammatory Activity (\%)} = (E0 - ET)/E0 \times 100 \quad (1)$$

Where:

E0 - Absorbance of the negative control group

ET - Absorbance of treatment groups

Next, the tests spreading and the phagocytosis were performed with murine macrophages of the 264.7 RAW (ATCC TIB-71). The macrophage strain was thawed and cultivated in a cell culture flask with Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 culture medium at 37 °C, 5% CO_2. The anti-inflammatory efficacy was performed when the culture reached about 70% - 80% confluence. At this moment cells were harvested using a cell scraper, counted in a Neubauer chamber, and centrifuged at 1500 rpm for 5 min. Then, the supernatant was discarded, and the cells were resuspended in

a culture medium to reach the desired concentration for each experiment.

The spreading by macrophages was determined using the method described by Bastos, et al. (2012). Samples of *B. trimera* and *E. arvense* fractions were analyzed at three different concentrations (200, 400, and 600 µg mL^{-1}) [19]. Dexamethasone 40 µg mL^{-1} was used as a positive control and 0.9% saline solution was used as a negative control. Slides were prepared and examined under an optical microscope at 400x magnification, with a total count of 100 cells. This test was conducted in triplicate [20]. The inhibition of spreading was calculated using the following expression (Equation 2).

$$\text{Spreading Inhibition (\%)} = (E0 - ET)/E0 \times 100 \quad (2)$$

Where:

E0 - The average number of spread cells in the negative control group

ET - The average number of spreading cells in the treated groups.

The phagocytosis assay performed by macrophages followed the methodology described by Azedo, et al. (2012). Samples of *B. trimera* and *E. arvense* fractions were analyzed at three different concentrations (200, 400 and 600 µg mL^{-1}). As a positive control, dexamethasone was used (concentration of 40 µg mL^{-1}), and saline solution (0.9%) was the negative control. The prepared slides were examined under an optical microscope at 400x magnification, with a total count of 100 cells. The experiment was conducted in triplicate, with the Phagocytosis Inhibition (PI) calculated according to the following expression (Equation 3) [21].

$$\text{Phagocytosis Inhibition, PI (\%)} = (E0 - ET)/E0 \times 100 \quad (3)$$

Where:

E0 - Average number of cells in the negative control group that phagocytosed Zymosan particles

ET - The average number of cells in the treated groups that phagocytosed Zymosan particles.

Statistical analysis

The data of the *in vitro* experiments were expressed in terms of mean ± standard deviation. Statistical analysis was performed using BioEstat® (version 5.0) software (Brazil). To verify the statistical differences between the groups a one-way analysis of variance (ANOVA) was performed according to the experimental protocol, followed by Tukey's multiple comparison test. For all analyzes a p - value of < 0.05 was considered significant.

Results and discussion

In the chemistry of natural products, the diverse potential of special metabolites is utilized to promote the bioprospecting of plant molecules or fractions with the goal of expanding the pharmacological applicability of herbal medicines [22,23]. The Brazilian flora is an invaluable source of compounds, constituting approximately 25% of the world's total. Therefore, it is believed that the Brazilian flora holds great potential in providing compounds for various uses, especially in traditional medicine. However, some plants lack in-depth studies on their pharmacological applications. As a result, the use of extracts, infusions, fresh consumption, or essential oils from various plants, such as *E. arvense* and *B. trimera*, requires further detailed studies to scientifically demonstrate the biological role of certain compounds or mixtures of compounds [24].

Based on the vast variety of medicinal plants in Brazil, the extensive genus called *Equisetum* stands out, comprised of around 30 different species, all of which have uses in folk medicine. Furthermore, this genus can be considered one of the oldest in the world [24]. Among the plants of the *Equisetum* genus, *Equisetum arvense* L. stands out, a plant popularly known as "cavalinha", which is generally used to combat fluid retention. According to a study by Boeing (2021), *E. arvense* L. showed results on its diuretic effect, treatment of genitourinary diseases, inflammation, wound healing, and other benefits [24]. Although its diuretic effect has been proven in animal models and clinical trials, more studies are needed to prove its effectiveness in humans in terms of anti-inflammatory activities. In another study conducted by Monte (2004), the antinociceptive and anti-inflammatory effects of *E. arvense* in extract form were evaluated in mice at dosages of 10, 25, 50, and 100 $mg.kg^{-1}$. Studies indicate that the use of the extract contains antinociceptive effects and anti-inflammatory properties [25].

The species *Baccharis trimera*, also known as "carqueja", is a medicinal plant widely found in South America and used in ethnopharmacology to treat diseases associated with liver and gastric problems [11]. *B. trimera* also exhibits cardioprotective effects, possibly due to its lipid-lowering action and inhibition of free radicals' production [26]. Gene and collaborators (1996) describe a potential anti-inflammatory action in aqueous extracts of *B. trimera*, comparing it with the action of non-steroidal anti-inflammatory drugs [27]. Other biological activities include gastric protection, hepatoprotection, weight loss effects, and anthelmintic, antifungal, antiparasitic, and antiviral properties, among others [15]. In this sense, *E. arvense* and *B. trimera* are excellent sources of metabolites, and the need for research is evident, especially in exploring the anti-inflammatory activities of their fractions, which have been minimally studied in the literature.

The essential oils obtained from *E. arvense* (EA-EO) and *B. trimera* (BT-EO) are colorless and green, respectively, with a relative percentage of 1.7% and 0.5% (w/w). We also chose to obtain classes of compounds generally of high polarity and/or

high molecular weight, such as alkaloids, triterpenes, organic acids, and flavonoids, among others. The extracts of *E. arvense* and *B. trimera* were both presented in the form of brown oils, with extraction yields of 2.7% and 6.2% (w/w). According to Oliveira and collaborators (2012), the essential oil of *B. trimera* was obtained at a percentage of 0.5% (w/w), which corroborates the mass obtained in our extraction. In another study by Oliveira and collaborators (2012), the aqueous extract of *B. trimera* was obtained with a yield of 14.1% (w/w), demonstrating the low effectiveness in obtaining compounds from the ethanolic extract of the plant specimen [28-30]. Comparing the percentages of volatile fractions obtained with the literature, reports reveal a percentage of 0.2% for *E. arvense* [31]; and for the extract, Monte (2004) and collaborators describe a percentage of 14.0% (w/w) yield [25]. These data indicate that the extractions were quite efficient for the organic phases obtained, however, it is necessary to study whether obtaining an ethanolic extract is appropriate, or even whether the plant itself can develop different amounts/natures of metabolites depending on its region, climate, treatment and other aspects [32].

The inflammation process involves the release of several hydrolytic enzymes from the lysosome, which can cause damage to surrounding tissues and organelles, generating a wide range of disorders [33]. One of the viable alternatives to access data regarding potential inflammatory processes is the assay stabilization of the red blood cell membrane. We chose to use four fractions obtained in different concentrations to understand the dose and biological response. These fractions were applied at concentrations of 200, 400, and 600 µL mL^{-1} for the volatile fractions obtained in the form of Essential Oil (EO) and non-volatiles obtained in the form of ethanolic extract (EE) of *E. arvense* (EA) and *B. trimera* (BT). Data analysis suggests moderate stabilization of the red blood cell membrane by the BT-EE fraction, which is the most active fraction evaluated. Initially, a slight correlation of increased stabilization of red blood cell membranes at higher concentrations is noticeable, as seen at a concentration of 600 µg mL^{-1} (Absorbance of 65.50 ± 0.13 %, $p < 0.05$). These data align with Oliveira and collaborators, who used Carrageenan-induced Pleurisy to identify the anti-inflammatory potential in *B. trimera* fractions [28-30].

Figure 1 depicts spreading inhibition carried out by macrophages compared to the positive control (Dexamethasone 40 µg mL^{-1}). The functional analysis of the spread of mononuclear phagocytes is crucial in the study of the inflammatory process. The functional capacity of these cells, which participate in processes such as lysis and engulfment of particles or microorganisms, generation of hydrogen peroxide, adhesion, and emission of microvilli, is closely influenced by inflammation [20,31]. Although they differ significantly from the positive control, the spread analysis does not show drastic differences between the fractions. It is evident that the only fraction showing a spread greater than 50% is BT-EE, supporting the red blood cell stabilization data (Table 1). Data from this experiment for *E. arvense* and *B. trimera* are being reported for the first time.

The action of the phagocytic cell is an important factor in determining the immune processes. The ability of phagocytes to engulf Zymosan particles compared to a positive control is an indicator of anti-inflammatory capacity [20]. Table 2 demonstrates a relationship between the treatment of essential oils and ethanolic extract from *B. trimera* and *E. arvense* at concentrations of 200 to 600 µg mL^{-1} and the phagocytosis inhibition carried out by macrophages (PI) compared to a positive control (Dexamethasone 40 µg mL^{-1}) and a negative control. The data corroborates the other two experiments and can be considered promising for BT-EE 600 µg mL^{1} ($p < 0.05$).

It was observed that all treatments with the tested fractions showed significant differences ($p < 0.05$) compared to the negative control. Upon comparing the experiments, BT-EE is a promising fraction when compared to the positive control, as outlined in Tables 1,2. This corroborates with the literature,

Table 1: Evaluation of the stabilization of the red blood cell membrane in EO and EE fractions of *E. arvense* and *B. trimera*.

Treatments	% Absorbance	Standard deviation
Negative Control*	0	0
Positive Control**	98.97	0.34
EA-EO - 200 µg mL^{-1}	37.22	0.06
EA-EO - 400 µg mL^{-1}	40.53	0.42
EA-EO - 600 µg mL^{-1}	44.50	0.12
EA-EE - 200 µg mL^{-1}	39.23	0.05
EA-EE - 400 µg mL^{-1}	46.82	0.01
EA-EE - 600 µg mL^{-1}	58.60	0.23
BT-EO - 200 µg mL^{-1}	45.87	0.02
BT-EO - 400 µg mL^{-1}	47.80	0.02
BT-EO - 600 µg mL^{-1}	65.50	0.13
BT-EE - 200 µg mL^{-1}	40.11	0.01
BT-EE - 400 µg mL^{-1}	44.15	0.00
BT-EE - 600 µg mL^{-1}	47.52	0.03

* Negative Control = Physiological solution 0.9%; ** Positive control = Dexamethasone 40 µg mL^{-1}.

Table 2: Assessment of phagocytosis inhibition (PI) performed by macrophages on EO and EE fractions of *E. arvense* and *B. trimera*.

Treatments	% PI	Standard Deviation
Negative Control*	0	0
Positive Control**	73.64*	1.61
EA-EO - 200 µg mL^{-1}	31.16*	0.56
EA-EO - 400 µg mL^{-1}	34.58*	1.45
EA-EO - 600 µg mL^{-1}	46.37*	1.12
EA-EE - 200 µg mL^{-1}	51.60*	2.00
EA-EE - 400 µg mL^{-1}	54.57*	1.93
EA-EE - 600 µg mL^{-1}	58.73*	1.15
BT-EO - 200 µg mL^{-1}	25.04*	3.59
BT-EO - 400 µg mL^{-1}	29.87*	1.69
BT-EO - 600 µg mL^{-1}	38.12*	1.87
BT-EE - 200 µg mL^{-1}	46.43*	0.44
BT-EE - 400 µg mL^{-1}	67.60*	2.52
BT-EE - 600 µg mL^{-1}	73.44*	1.39

* Negative Control = Physiological solution 0.9%; ** Positive control = Dexamethasone 40 µg mL^{-1}.

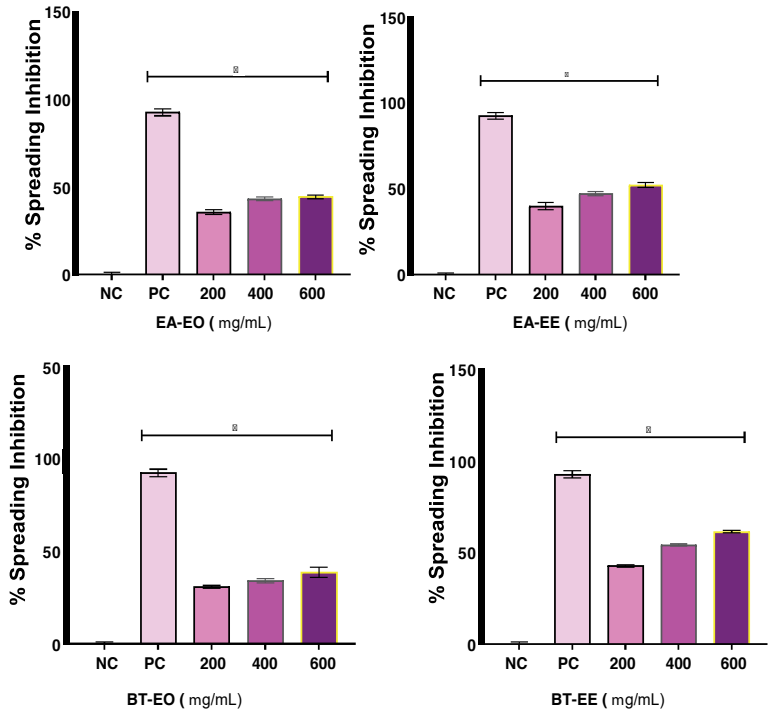

Figure 1: Evaluation of the spreading inhibition carried out by macrophages in EO and EE fractions of *E. arvense* and *B. trimera*. NC (Negative Control) = Physiological solution 0.9%; PC (Positive control) = Dexamethasone 40 μg mL^{-1}.

specifically a study conducted by Gene and collaborators (1996) which describes the anti-inflammatory activity of *B. trimera* [27]. This activity is attributed, in principle, to the prevention of prostaglandin biosynthesis through the inhibition of cyclooxygenase, as well as the fraction's ability to reduce dextran-induced swelling [33,34]. This study is a preliminary basis, but further access to information is needed regarding the toxicity of the fractions, their chemical identification, quantification of other inflammation modulators, mainly on inflammation-related genes in isolated cell populations, and the pharmacodynamic response of isolated cell populations with protein-based assays. Up to now, we infer the use of therapeutic plants, such as *B. trimera*, can influence immune reactions and lead to anti-inflammatory effects. The promising results of *B. trimera* make it a valuable therapeutic plant for further future studies.

Conclusion

In the present study, essential oils and ethanolic extracts of *E. arvense* and *B. trimera* were obtained. The stabilization of the red blood cell membrane, inhibition of spreading, and phagocytosis process by macrophages were evaluated. In all experiments, we observed that the most polar fraction of *B. trimera* (BT-EE) exhibited significant anti-inflammatory activity compared to positive controls. Additionally, a moderate anti-inflammatory activity *in vitro* was found for the essential oils of both species, as well as for the ethanolic extract of *E. arvense*. These results suggest the need for further study on the mode of action and biochemical properties of the investigated fractions to identify potential molecules that provide anti-inflammatory activity in the natural product. New studies are being conducted to evaluate the cytotoxicity of the promising fraction, and the antioxidant activity of BT-EE, and determine the chemical constitution through LC-MS^n and fraction isolation processes are underway.

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AFM and CFV worked in obtaining the natural products and acted in anti-inflammatory data analysis. JARF and LS acted in an anti-inflammatory analytical process. MVVR classified the botanical species. HJD worked on the conception, discussion of results, writing of the manuscript, revision, and approval of the final version of the manuscript. All authors agree with the submission of the manuscript and declare that they have not submitted it to another journal during the review process.

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