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Bioprospection of *Eugenia brasiliensis*, a Brazilian native fruit, as a source of anti-inflammatory and antibiofilm compounds

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ABSTRACT

The anti-inflammatory and antibiofilm activities as well as toxicity and chemical profile of *Eugenia brasiliensis* pulp extract (EBE), were evaluated. EBE chemical profile and phenolic content were determined by LC–MS/MS. EBE was tested for its *in vitro* and *in vivo* anti-inflammatory activity, including TNF-α release, NF-κB activation, neutrophil migration and paw edema. The MIC/MBC and antibiofilm activities were tested against methicillin sensitive and resistant *Staphylococcus aureus*, *Escherichia coli, Pseudomona aeruginosa, Streptococcus mutans,* and *Lactobacillus acidophilus*. EBE acute toxicity was evaluated in *Galleria mellonella* and RAW 264.7 macrophage. EBE total phenolic content was 389.88 ± 3.48 mg GAE/g with identified polyphenols. EBE decreased TNF-α release *in vivo* and *in vitro*, NF-κB activation, neutrophil influx into peritoneal cavity, and it showed maximal inhibition of paw edema after 2 h. MIC of EBE ranged from 62.5–500 µg/mL while MBC values were > 500 µg/mL, with a decrease in *L. acidophilus* biofilm formation. EBE showed negligible toxicity in larvae and macrophage cells. Our findings open new perspectives concerning EBE application as source of anti-inflammatory and antibiofilm molecules as a functional food, pharmaceutical lead or agribusiness commodity.

1. Introduction

Brazil is widely known by harboring a huge biodiversity, sheltering approximately 22% of worldwide species in its territory (about 1.8 million species) [1]. Therefore, the Brazilian Atlantic rainforest has been considered as a hotspot for discovery of new bioactive molecules and functional foods [1–4]. However, most of the Brazilian native species remain scientifically unknown in terms of chemical composition and biological activity, for instance, the genus *Eugenia*.

Nowadays, there are studies which have proven several species to possess biological properties including antibacterial (*E. umbelliflora*, *E. jambolana*), antifungal (*E. leitonii, E. brasiliensis*) antioxidant (*E. leitonii, E. brasiliensis, E. involucrata, E. myrcianthes*), anti-inflammatory (*E. leitonii, E. aurata, E. punicifolia*) and others [5–10]. While some *Eugenia* species have their chemical profile identified, most studies fail to relate their chemical composition with biological activity. Based on empirical knowledge, the fruit and leaves of *Eugenia* species are consumed in folk medicine.

Eugenia brasiliensis Lam. *(Myrtaceae)* commonly known as "grumixama", "grumixameira" and "Brazilian cherry" is a tree whose fruit is approximately 2.0 cm in diameter, and whose peel is smooth, shiny and possesses different colors. There are some varieties of *E. brasiliensis* such as yellow or white in γ-variety, red in β-variety and dark purple in α-variety (used in this study), with the former being the most common one [11,12] (a picture of the fruit is available from http://www.colecionandofrutas.org/eugeniabrasili.htm). Several parts of *E. brasiliensis* such as leaves, fruits, and bark wood have been used in folk medicine as diuretic, astringent and for the treatment of rheumatism [13]. In addition, the pulp of this species is commonly consumed in the form of juice and frozen pulp, or as a fresh fruit. *E. brasiliensis* represents an economic potential due to the commercial cultivation and the attractive sensorial attributes in its composition [14].

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A study with the essential oil from the leaves of *E. brasiliensis* showed antimicrobial activity against several pathogens such as *S. aureus, P. aeruginosa,* and *E. coli.* [15,16]. Another study showed that crude extracts from leaves (*E. brasiliensis*) and seeds (*E. brasiliensis* and *E. leitonii*) had antifungal activity against *Candida albicans* biofilms, with better results than the gold standard nystatin [6]. Nevertheless, the antimicrobial and antibiofilm activities of *E. brasiliensis* crude extract on pathogenic bacteria remain to be investigated.

E. brasiliensis seeds, leaves and pulp have also shown promising anti-inflammatory activity by reducing almost 50% of the neutrophil influx into the inflammatory site [8]. Likewise, another study showed that *E. brasiliensis* leaves and its ethyl acetate fraction act by reducing the neutrophil migration into the peritoneal cavity, thereby exerting anti-inflammatory activity [17]. Nevertheless, there is a need to elucidate the mechanism of action of its anti-inflammatory activity. Considering the ethnopharmacology, we evaluated the anti-inflammatory and antibiofilm activities of EBE, as well as its *in vivo* toxicity and polyphenolic profile. Our findings reveal new perspectives concerning the EBE application as a promising source of anti-inflammatory and antibiofilm compounds.

2. Material and methods

2.1. Plant material

The fruit of *Eugenia brasiliensis* Lam. (grumixama) was collected in Atlantic rainforest area, a local farm (Rare fruit ranch) (S 23° 23′, W 45° 39′) in the city of Paraibuna, São Paulo, Southeastern Brazil, between the months of February and March 2012. The collection was carried out under permission of the Brazilian Ministry of Environment (Council for the Administration and Management of Genetic Heritage–CGEN/ CNPq, # 010907/2014-9). It was deposited in the herbarium of the "Luiz de Queiroz" College of Agriculture at the University of São Paulo (ESALQ/ USP, Piracicaba, São Paulo) the botanical specimen (voucher number ESA056895).

2.2. Preparation of extract

To prepare the extract, 5 g of lyophilized Eugenia *brasiliensis* purple pulp were extracted using 50 mL of a mixture of ethanol (EtOH) and water (H_2O) (4:1 v/v, respectively), and submitted to ultrasound for three times (30 minutes each time). Next, the extract was filtered, evaporated and lyophilized. The dry EBE was stored at −20 °C. In all assays, the extract was diluted in saline (NaCl, 0.9%, w/v) [8].

2.3. Phytochemical profile

The phytochemical profile of the EBE (the same extract used in this study) was recently evaluated by Gas Chromatography Coupled to Mass Spectrometry (GC/MS), as published elsewhere [8]. To validate and expand the EBE chemical profile, we carried out a chemical analysis by liquid chromatography coupled with mass spectrometry (LC–MS/MS) and total phenolic content analysis.

2.3.1. LC–MS/MS analysis

A total of 10 mg of EBE was added to 750 µL of methanol plus 750 µl of an aqueous solution of 0.1% formic acid. The tandem mass spectrometry (MS/MS) system employed for analyte identification was a Quattro triple quadrupole (Micromass, Manchester, UK) equipment fitted with a Z-electrospray (ESI) interface operating on positive and negative ion modes. The temperatures of source block and desolvation gas were set at 100 °C and 350 °C, respectively. Nitrogen was used as both drying (nearly 380 L/hr) and nebulizing (nearly 38 L/hr) gas, while argon was used as collision gas. The cone voltages employed during the analyses ranged from 20 to 50 V and the collision energy ranged from 15 to 25 eV among the analytes analyses. For identities confirmation, the analyses were carried out in the multiple-reaction monitoring (MRM) mode. Table 1 presents the ion transitions under MRM mode utilized to monitor analytes. The prepared samples were introduced into the MS/MS system by direct infusion under a flow rate at 10 µL/min [9].

Table 1
Polyphenols identified in EBE by LC–MS/MS analysis.

Compound	Molecular ion $[M+H]^+$ or $[M-H]^-$ (m/z)	MS/MS ion
1 - Catechin or Epicatechin	$[M-H]^-$: 289	245, 205, 137
2 - Ellagic acid pentoside	$[M-H]^-$: 433	301
3 - Quercetin hexoside	$[M-H]^-$: 463	301
4 - Cyanidin-3-arabinoside or Cyanidin-3-xyloside	$[M+H]^+$: 419	287
5 - Delphinidin-3-pentoside	$[M+H]^+$: 435	303
6 - Cyanidin-3-galactoside or cyanidin-3-glucoside	$[M+H]^+$: 449	287
7 - Delphinidin-3glucoside	$[M+H]^+$: 465	303
8 - Malvidin-3-glucoside	$[M+H]^+$: 493	331

2.3.2. Total phenolic content

Aliquots of 20 µL of EBE and gallic acid standard were added to the wells of microplates containing a solution of 10% Folin–Ciocalteu. After 5 min, 75 µL of a solution of 4% sodium carbonate was added to the wells. The absorbance was measured at 740 nm by means of UV-mini 1240 spectrophotometer after 2 h (Shimadzu Corp., Kyoto, Japan). The total phenolic content was expressed as gallic acid per gram of EBE (GAE/g) [18].

2.4. Anti-inflammatory activity

2.4.1. Cell culture and viability assays

RAW264.7 macrophage (BCRJ code: 0212; Rio de Janeiro, Brazil) was cultured in endotoxin-free Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS) plus 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and L-glutamine (37 °C, 5% CO_2). RAW 264.7 cells (5×10^5 cells/mL) were cultured in 96-well plates and incubated for 24 h prior to treatment with EBE at 0.02, 0.2, 2, 20 and 200 µg /mL or vehicle (0.9% saline, negative control) for 24 h. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.3 mg/mL) was added to each well and the plates were incubated for 3 h (37 °C, 5% CO_2). The supernatant was removed and 100 µL of isopropanol were added. The absorbance was measured at 470 nm using an ELISA microplate reader and the results were expressed as cell viability units [19].

2.4.2. NF-kappa B activation assay

RAW 264.7 cells (3×10^5 cell/well) stably transfected with the NF-κBpLUC gene to express luciferase by the transcription factor NF-kB were cultured in 24-well plates and incubated overnight (37 °C, 5% CO2). The cells were pretreated with EBE at 2, 20 and 200 µg/mL or vehicle (0.9% saline) for 1 h prior to lipopolysaccharide (LPS) stimuli (1 µg/mL) for 4 h. After cell lysis (lysing buffer), 25 µL of the suspension was mixed to reaction reagent (luciferase containing luciferin at 0.5 mg/mL). The quantification of luminescence was performed using a microplate reader (SpectraMax M3, Molecular Devices). The results were expressed as luminescent relative units [20].

2.4.3. Cytokine assay in vitro (RAW 264.7)

RAW 264.7 cells (2×10^5 cells/well) were cultured in 96-well plates and incubated overnight (37 °C, 5% CO_2). The cells received EBE at 2, 20 and 200 µg/mL or vehicle (0.9% saline) for 30 min prior to LPS stimuli (1 µg/mL) for 4 h under the same conditions. The supernatant was collected and the level of TNF-α was determined using an ELISA