UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

JOSY GOLDONI LAZARINI

Phytochemical, toxicity and evaluation of anti-inflammatory and antioxidant activities of *Eugenia neonitida* Sobral (pitangatuba), a Brazilian native fruit

Fitoquímica, toxicidade e avaliação das atividades anti-inflamatória e antioxidante de *Eugenia neonitida* Sobral (pitangatuba), uma fruta nativa do Brasil

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Tese apresentada *à* Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Odontologia, na área de Farmacologia, Anestesiologia e Terapêutica.

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Orientador: Prof. Dr. Pedro Luiz Rosalen
Coorientador: Dr. Marcelo Franchin

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RESUMO

As frutas nativas brasileiras (FNB) podem ser classificadas como “superfrutas”, devido à sua rica composição polifenólica que pode modular o processo inflamatório, bem como reduzir a produção de espécies reativas de oxigênio e nitrogênio (ERO/ERN). Este estudo determinou a composição polifenólica, avaliou o mecanismo de ação anti-inflamatório, a atividade antioxidante e perfil de toxicidade sistêmica do extrato, fração (F3) e subfração (S8) de *Eugenia neonitida* (*En*e). Os compostos presentes em *En*e, F3 e S8 foram quantificados e identificados por LC-ESI-QTOF-MS. Para os estudos de atividade anti-inflamatória *in vitro,* utilizou-se cultura de macrófagos RAW 264.7 transfectados (NF-kB-pLUC gene) e quantificação de TNF-α e CXCL2/MIP-2, por meio de ELISA. Para a avaliação do mecanismo de ação anti-inflamatório *in vivo*, foram conduzidos os ensaios de migração de neutrófilos induzida por carragenina, quantificação de TNF-α e CXCL2/MIP-2, microscopia intravital e expressão de molécula de adesão por Western Blotting. Para a atividade antioxidante, foram utilizados os ensaios contra ERO/ERN (ROO•, O2• -, HOCl e NO •). Por fim, o perfil de toxicidade sistêmica foi testado em larvas de *Galleria mellonella*. A análise química revelou a presença de ácido hidroxibenzoico, flavanoides e elagitaninos para *En*e e F3 e derivados de quercetrina, ácido vanílico e ácido cumárico para S8. *En*e reduziu, *in vitro,* a ativação de NF-κB e níveis de TNF-α, entretanto, não reduziu CXCL2/MIP-2; *in vivo,* diminuiu a migração de neutrófilos bem como os níveis de TNF-α e CXCL2/MIP-2. Com relação à fração, a F3 reduziu a ativação de NF-κB, os níveis de TNF-α e CXCL2/MIP-2 (*in vitro* e *in vivo*) e migração de neutrófilos. Finalmente, S8 reduziu ativação de NF-κB, níveis de TNF-α e CXCL2/MIP-2 (*in vitro e in vivo*) e migração neutrofílica, comprovada por microscopia intravital e redução da expressão de ICAM-1 (molécula de adesão) em endotélio. Em geral, *En*e, F3 e S8 exibiram atividades contra ERO/ERN e nenhuma das amostras induziu a toxicidade sistêmica em *G. mellonella*. Assim, *En*e, F3 e S8 não apresentaram toxicidade e atuaram como anti-inflamatório e antioxidante, devido à sua composição fenólica. A *E. neonitida* é uma promissora fonte de compostos bioativos pouco explorada, que pode favorecer setores como o agronegócio, indústrias farmacêutica e alimentícia proporcionando o desenvolvimento de novos produtos ou insumos para a saúde humana.

Palavras-chave: *Eugenia neonitida*, fruta nativa, anti-inflamatória, antioxidante.

ABSTRACT

Brazilian native fruits (FNB) can be classified as “superfruits” due to their rich polyphenolic composition that can modulate the inflammatory process as well as reduce the production of reactive oxygen and nitrogen species (ROS/RNS). This study determined the polyphenolic composition, evaluated the mechanism of anti-inflammatory action, antioxidant activity and systemic toxicity profile of the extract, fraction (F3) and subfraction (S8) of *Eugenia neonitida* (*En*e). The compounds present in *En*e, F3, and S8 were quantified and identified by LC-ESI-QTOF-MS. For the studies of anti-inflammatory activity *in vitro*, RAW 264.7 macrophages (NF-kB-pLUC gene) transfected were used stimulated with LPS and quantification of inflammatory cytokines by ELISA. For the evaluation of the anti-inflammatory mechanism of action *in vivo*, neutrophil migration tests were performed in the peritoneal cavity of mice challenged with carrageenan, quantification of inflammatory cytokines *in vivo* using ELISA, intravital microscopy (rolling and leukocyte adhesion) and expression of adhesion molecule (ICAM-1) by westernblott. For antioxidant activity, tests against ROS/RNS (ROO •, O2 • -, HOCl and NO •) were used. Finally, the systemic toxicity profile was tested in *Galleria mellonella* larvae. Chemical analysis revealed the presence of hydroxybenzoic acid, flavanoids and ellagitannins for *En*e and F3 and derivatives of quercetrin, vanillic acid and cumaric acid for S8. *En*e reduced the activation of NF-κB and TNF-α levels *in vitro*, however, it did not reduce CXCL2/MIP-2; *En*e treated *in vivo* decreased neutrophil migration as well as TNF-α and CXCL2/MIP-2 levels. Regarding the fraction, F3 reduced the activation of NF-κB, the levels of TNF-α and CXCL2 / MIP-2 (*in vitro* and *in vivo*) and migration of neutrophils. Finally, S8 reduced activation of NF-κB, levels of TNF-α and CXCL2 / MIP-2 (*in vitro* and *in vivo*) and neutrophil migration confirmed by intravital microscopy and reduced expression of ICAM-1 (adhesion molecule) in endothelium. In general, *En*e, F3 and S8 exhibited activities against ROS/RNS and none of the samples induced systemic toxicity in *G. mellonella*. Thus, *En*e, F3 and S8 showed no toxicity and exibited anti-inflammatory and antioxidant activities due to their phenolic composition. *E. neonitida* is a promising source of bioactive compounds little explored, which can favor sectors such as food industries, pharmaceutical and agribusiness, providing the development of new products for human health.

Keywords: *Eugenia neonitida*, native fruit, anti-inflammatory, antioxidant

SUMÁRIO

1 INTRODUÇÃO

A mata Atlântica é um rico ecossistema que abrange 15% do território brasileiro. Presente em 17 estados, esse bioma apresenta características geográficas como amplitude altitudinal que favorece a grande diversidade e endemismo, incluindo mais de 20 mil espécies da flora (de Araújo et al., 2019). Inseridas na floresta atlântica, as espécies de frutas nativas brasileiras (FNB) constituem um valor inestimável tanto ao seu patrimônio genético, quanto na riqueza de nutrientes e compostos bioativos. Entretanto, esse bioma com toda sua biodiversidade subutilizada, atualmente encontra-se ameaçado pelo desmatamento (11,399 hectares em 2018), restando apenas 12,4% de vegetação original (de Araújo et al., 2019; SOS Mata Atlântica).

Desde o descobrimento do Brasil (1500) até o presente século (XXI), a maioria das FNB permanecem inexploradas devido à falta de conhecimento e, consequentemente, são desvalorizadas na comercialização e no consumo. Este desinteresse brasileiro não se restringe apenas à produção e agregação de valor econômico, mas sobretudo na exploração das qualidades bioativas e funcionais das frutas (de Araújo et al., 2019).

Observa-se, do ponto de vista agroindustrial, um crescimento no consumo e produção de frutas nativas brasileiras consolidadas, como, por exemplo, o açaí. Nos últimos anos, o estado do Pará vem se destacando por ser o maior produtor nacional de açaí e em 2018, aumentou a produção para 1,43 milhões de toneladas de frutos, plantados em 190 mil hectares. Essa produção é de extrema relevância no contexto socioambiental e econômico, gerando cerca de 100 mil empregos diretos e indiretos para o sustento de famílias, bem como iniciativas de sustentabilidade e preservação de biomas nativos (Carneiro et al., 2013; FUNDAÇÃO INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2018).

Atualmente, associa-se o açaí à imagem de alimento saudável, no entanto, outras frutas nativas brasileiras podem possuir propriedades equivalentes ou superiores às do açaí, e que devido à baixa popularidade, falta de pesquisa e geração de conhecimento correm o risco de serem extintas (Carneiro et al., 2013).

Myrtaceae é uma das famílias de plantas mais comuns encontradas no Brasil, chegando a mais de 1000 espécies espalhadas de norte a sul. Cerca de 50% são nativas do Bioma Mata Atlântica (de Araújo et al., 2019). Nesse contexto, as espécies frutíferas do gênero *Eugenia*, pertencente à família Myrtaceae, representam

aproximadamente 400 espécies, as quais seus frutos apresentam um grande potencial econômico, farmacêutico e nutricional, uma vez que são fontes de moléculas com atividades biológicas (bioativas) (Sardi et al., 2017; de Araújo et al., 2019). Estudos recentes com diversas espécies frutíferas do gênero *Eugenia* confirmam seu potencial bioativo e que consequentemente tem ocasionado um aumento do interesse pelo consumo *in natura,* produtos, entre outros (Infante et al., 2016; Sardi et al., 2017).

No contexto de bioatividade, estudos conduzidos por Infante et al (2016) utilizando extratos de diversas partes das espécies de *Eugenia leitonii* (araçá-piranga), *Eugenia involucrata (*cereja do rio grande*), Eugenia brasiliensis* (grumixama) e *Eugenia myrcianthes* (ubajaí) revelaram atividades antioxidante e anti-inflamatória. Neste estudo, os autores identificaram compostos majoritariamente fenólicos como a epicatequina e ácido gálico, sendo possivelmente atribuída a eles as atividades biológicas evidenciadas.

Em outro estudo anti-inflamatório, o extrato de semente da *E. leitonii* reduziu a ativação do fator nuclear–κB (NF-κB), níveis de citocinas, expressão de moléculas de adesão e rolamento, e consequentemente, alterando o influxo de neutrófilos no processo inflamatório. Os compostos majoritários, como elagitaninos e antocianinas foram identificados e atribuídos a eles a atividade anti-inflamatória observada (Lazarini et al., 2016).

Um estudo bioprospectivo conduzido por Lazarini e colaboradores (2018) revelou que o extrato da polpa de *E. brasiliensis* apresentou atividade anti-inflamatória por meio da redução da ativação do NF-κB, nível da citocina do tumor de necrose tumoral-α (TNF-α), migração de neutrófilos e diminuição do edema de pata induzido por carragenina em camundongos. O extrato também apresentou atividade antimicrobiana contra *Lactobacillus acidophilus* na forma de biofilme*.* Quimicamente, foram identificados moléculas como quercetina, epicatequina e antocianinas e as atividades *in vivo* e *in vitro* encontradas foram atribuídas à presença deste mesmos compostos químicos (Sardi et al., 2017).

Recentemente, um estudo de revisão compreensiva publicada por Chang, Alasalvar e Shahidi (2019) classificaram de forma pioneira algumas espécies de frutas nativas como acerola, goji berry, pitanga*,* açaí, entre outras, como “superfrutas”. As principais características para a classificação são os altos níveis de compostos bioativos, como flavonoides, bem como a atividade antioxidante. As frutas nativas brasileiras em geral, também poderiam ser classificadas como “superfrutas” uma vez

que apresentam os elevados teores de compostos bioativos e atividade antioxidante, requisitos apontados na revisão.

Estudos epidemiológicos sugerem que a ingestão regular de polifenois, como flavonoides identificados nas frutas nativas do gênero *Eugenia*, tem sido associada na prevenção de doenças inflamatórias crônicas geradas pelo estresse oxidativo, como aterosclerose, diabetes, obesidade, distúrbios cardiovasculares, câncer, artrite, entre outros (Domeneghini e Lemes 2011; Slavin e Lloyd 2012; Lavecchia et al., 2013). Essas doenças inflamatórias crônicas, geralmente iniciadas sem sintomas clínicos, estão relacionadas ao estresse oxidativo gerado por espécies reativas de oxigênio/nitrogênio (ERO/ERN) em células, resultando em danos celulares, e consequentemente, destruição de tecidos (Lavecchia et al., 2013).

As espécies reativas podem desencadear a ativação de fatores nucleares importantes, como, por exemplo, o NF-κB, aumentando, por exemplo, a produção de citocinas (TNF-α, IL-1β, entre outras) e quimiocinas (CXCL2/MIP-2, IL-8, entre outras), expressão de moléculas de adesão (P, E-selectinas) e rolamento (I-CAM-1, V-CAM) e influxo de neutrófilos (Lavecchia et al., 2013; Mittal et al., 2014; Soares et al., 2019). Nesse contexto, um estudo publicado em uma das melhores revistas de imunologia destaca novos alvos moleculares para tratamento de doenças inflamatórias, dentre eles os inibidores de selectinas, integrinas, citocinas, quimiocinas entre outros (Mackay C., 2008).

O nosso grupo de pesquisa vem buscando, ao longo dos anos, novas fontes de moléculas e novos alvos moleculares com atividade anti-inflamatória. Recentemente, realizamos um estudo de bioprospecção de atividade anti-inflamatória com frutas nativas brasileiras inexploradas. Nesse *screening,* utilizamos onze extratos de polpa de frutas nativas brasileiras a saber: *Byrsonima lancifolia* (murici guassú); *Eugenia stipitata* (araçá-boi); *Jacaratia dodecaphylla* (jaracatiá-mamão); *Spondias mombin* (cajá); *Byrsonima arthropoda* (murici vermelho); *Acnistus arborescens* (fruta-do-sabiá); *Campomanesia phaea* (cambuci); *Sagerectia elegans* (cambuiti-cipó); *Eugenia neonitida* (pitangatuba); *Rubus rosaefolius* (morango silvestre) e *Solanum alternopinatum* (juquirioba).

A atividade anti-inflamatória das espécies foram avaliadas por meio do ensaio de migração de neutrófilos induzido por carragenina em camundongos e observamos que do total de 11 extratos, 6 extratos apresentaram redução do influxo de neutrófilos no peritônio dos camundongos e 1 extrato (*Eugenia neonitida*) se

destacou dos demais, devido à sua excelente atividade anti-inflamatória, o qual selecionamos para os ensaios posteriores (Soares et al., 2019). O extrato de *E. neonitida* (pitangatuba) apresentou a redução do influxo de neutrófilos em 65%, bem como maior rendimento de extração (45%).

Portanto, o objetivo desde trabalho foi determinar a composição química, avaliar o mecanismo de ação anti-inflamatório, atividade antioxidante por meio de espécies reativas de oxigênio/nitrogênio e o perfil de toxicidade sistêmica *in vivo* da fração purificada (F3), subfração (S8) e do extrato bruto de *Eugenia neonitida* (*En*e) por meio de um estudo bioguiado.

2 ARTIGOS

2.1 Anti-inflammatory and antioxidant potential, *in vivo* toxicity, and polyphenolic composition of *Eugenia neonitida* (pitangatuba), an unexplored Brazilian native fruit.

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Abstract

Unexplored Brazilian native fruits are a rich source of polyphenolic compounds that can act as anti-inflammatory and antioxidant agents. This study determined the polyphenolic composition, anti-inflammatory mechanism of action, antioxidant activity and systemic toxicity of *Eugenia neonitida* extract (*Ene*) and its purified fraction (F3) obtained through bioassay-guided fractionation. Phenolic compounds present in *Ene* and F3 were tentatively identified by LC-ESI-QTOF-MS. The anti-inflammatory activity of *Ene* and F3 was tested *in vitro* and *in vivo* through NF-κB activation, cytokine release and neutrophil migration assays. The samples were tested for their effects against reactive species (ROO•, O2•-, HOCl and NO•) and for their toxicity in *Galleria mellonella* larvae model. It was identified the presence of hydroxybenzoic acid, flavanols, ellagitannins, flavone and flavonols. *Ene* and F3 reduced NF-κB activation, cytokine release and neutrophil migration, with F3 being three-fold more potent. Overall, F3 exhibited strong antioxidant effects against biologically relevant radicals, and neither *Ene* nor F3 were toxic to larvae. In conclusion, *Ene* and F3 revealed the presence of phenolic compounds that decreased the inflammatory parameters evaluated and inhibited reactive oxygen/nitrogen species. *E. neonitida* is a source of bioactive compounds that may provide benefits for human health.

Keywords: *Eugenia neonitida;* anti-inflammatory; antioxidant; polyphenols.

Introduction

The Brazilian Atlantic rainforest is a rich ecosystem which has been extensively threatened by deforestation. With only 12% of the original area left, it shelters numerous fauna and flora species, including approximately 50% endemic edible fruit trees. Occurring in the Atlantic rainforest, Brazilian native fruit (BNF) species are part of a yet unknown, neglected and underutilized biodiversity, especially for bioprospecting novel molecules with biological properties [1-2].

The genus Eugenia (Myrtaceae family) has approximately 400 native species occurring in the Atlantic rainforest. Brazilian native species have great economical potential for fresh fruit consumption; production of juice, jam and ice cream; as well as for pharmaceutical and nutritional use [2]. Recent studies reported that Eugenia spp. have anti-cancer (*Eugenia uniflora* and *Eugenia brasiliensis*), anti-inflammatory and antioxidant effects (*Eugenia leitonii*, *E. brasiliensis, Eugenia stipitata, Eugenia myrcianthes* and *Eugenia involucrata*), among others [2-5]. Currently, some traditional fruits and BNF species, such as *E. uniflora* and *Euterpe oleracea,* have been termed “superfruits” due to their high levels of phytonutrients (bioactive molecules) that can improve regular consumers’ health [6].

Eugenia fruit species contain a rich nutritional value (minerals and vitamins) and are a promising source of phytonutrients, such as carotenoids, flavanols (catechin, epicatechin), anthocyanins (cyanidin, delphinidin, malvidin), flavonols (kaempferol, quercetin), phenolic acids (gallic acid), and others [2-6]. Scientific evidence suggests that regular intake of bioactive molecules contained in native fruits has been associated with a reduced risk of developing oxidative stress-related diseases, such as diabetes, obesity, cardiovascular disorders, cancer, arthritis, chronic inflammatory diseases etc [2-9]. The latter commonly go unnoticed and are triggered by oxidative stress generated by oxygen/nitrogen reactive species (ROS/RNS), resulting in cell damage and tissue injury [9]. ROS and RNS can overly activate an important signaling pathway associated with inflammation, the nuclear factor κB (NF-κB), and thereby they render the inflammatory process more destructive than resolutive [4,9,10]. Thus, the search for, and application of, novel bioactive compounds able to control the inflammatory process while modulating ROS/RNS generation is required.

Recently, our research group reported the anti-inflammatory and antioxidant activity of five BNF species [4]. *Eugenia neonitida* showed the strongest *in vivo* anti-

inflammatory activity and thus was selected for further analysis in the present study. *E. neonitida*, popularly known as *“Pitangatuba”, “pitangola”, “pitangão”* or *“pitanga-amarela”,* is a shrub tree approximately two meters high, with dark green leaves. Its fruit is 4 cm long and 3 cm wide, has pleasant bittersweet taste with juicy pulp to consume *in natura* or in beverages, juices and jams [11]. While *E. neonitida* has been used in folk medicine to treat skin depigmentation and vitiligo – because it contains a photosensitizing substance [11], its anti-inflammatory activity, ROS/RNS scavenging activity, and phytochemical composition, remain to be determined.

Thus, our study hypothesis was that the phytochemical compounds present in *E. neonitida* have ROS/RNS scavenging effects and, consequently, inhibit important pro-inflammatory biomarkers. To test our hypothesis, we determined the phytochemical composition of *E. neonitida* extract (*Ene*) and its purified fraction (F3) and tested them for their anti-inflammatory mechanism of action, antioxidant activity and systemic toxicity *in vivo*.

Material and methods

Reagents

The reagents were purchased from Tedia (Fairfield, OH, USA): formic acid. Millipore Milli-Q System (Millipore SAS, Molsheim, France): purified water. J.T. Baker (Phillipsburg, NJ, USA): acetonitrile, methanol and ethanol. Sigma-Aldrich (St. Louis, MO, USA): Roswell Park Memorial Institute (RPMI), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, DMSO (dimethylsulfoxide), carrageenan, dexamethasone, diaminofluorescein-2 (DAF-2), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium nitroprusside, sodium hypochlorite solution (NaOCl), nitrotetrazolium blue chloride (NBT), dibasic potassium phosphate, 2,2-azobis(2-methylpropionamidine) β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), rhodamine 123, dihydrochloride (AAPH) and fluorescein sodium salt. Gibco (Grand Island, NY, USA): fetal bovine serum. Applied Biological Materials Inc. (Richmond, BC,Canada): RAW 264.7 macrophages transfected with the NF-kB-pLUC gene. MERCK KGaA (Frankfurt, Germany): Silicagel 60 (0,063-0,200 mm). Promega Corporation (Madison, WI, USA): luciferin. Amresco, Inc. (West Chester, Pensilvania, EUA): Lysis buffer TNT, mixture of TRIS BASE and Tween 20. R&D Systems, Inc:

TNF-α and CXCL2/MIP-2 kits. Supelco, Bellefonte, PA, USA): LC-18 SPE cartridges 2 g.

Plant material and extract preparation

Previous access to BNF material was granted by the Council for Genetic Heritage Management (CGEN #AD4B64F; Brazilian Ministry of Environment). *E. neonitida* Sobral samples were collected once in a local farm ("Rare Fruit", "*Frutas Raras*" in Portuguese), located in the city of Campina do Monte Alegre, São Paulo, Southeastern Brazil. The farm region is in the Atlantic rainforest subtropical region (Cfb - Köppen climate classification), with an altitude of 612 meters (S 23º 53' 57.06"; W 48º 51' 24.68"). Fruit samples were collected from November to February 2016, and the specimens were deposited in the herbarium of the "Luiz de Queiroz" College of Agriculture at the University of São Paulo/ ESALQ/USP (Piracicaba, São Paulo), under voucher number HPL 5279. For extract preparation, lyophilized *E. neonitida* pulp (50 g) was mixed with 500 mL of ethanol and water (80:20, v/v; respectively). The mixture was submitted to three ultrasound cycles (30 min each), filtered to separate the liquid content, evaporated and lyophilized. *E. neonitida* hydroalcoholic extract (*Ene*) was stored at -20°C until further use.

E. neonitida extract fractionation

Freeze-dried *Ene* was submitted to fractionation using an open dry column chromatography on normal phase silica gel. It was used for elution a mixture of ethyl acetate:methanol:water (77:13:10, v/v), and six fractions with different polarities were obtained. The fractions were monitored using Thin Layer Chromatography (TLC). Fluorescent compounds were visualized under ultraviolet (UV) light at 366 nm wavelength. *Ene* and its most active fraction (F3) were submitted to LC-ESI-QTOF-MS analysis.

Phytochemical analysis

Sugar removal

To remove the sugar content of Ene, 500 mg of the extract was diluted with 5 mL of water and centrifuged (5000 x g for 15 min). The precipitate was dissolved in HCL solution (pH = 2.0) and filtered. In parallel, LC-18 SPE cartridges were conditioned with acidic water and methanol (pH = 2.0). Then, 5 mL of Ene were added to each cartridge until the total liquid content passed through the column. This wash process was repeated with acidic water to remove the sugars. Finally, the compounds of interest were eluted with methanol, and sugar-free Ene was recovered and stored at -20°C until further use.

High-Resolution Mass Spectrometry analysis (LC-ESI-QTOF-MS)

LC-ESI-QTOF-MS analysis was carried out using a chromatograph (Shimadzu Co., Tokyo) with a quaternary pump LC-30AD, photodiode array detector (PDA) SPD-20A. Reversed phase chromatography was performed using Phenomenex Luna C18 column (4.6 x 250 mm x 5 µm). High-resolution mass spectrometry MAXIS 3G – Bruker Daltonics (Bruker Daltonics, Bremen, Germany) was fitted with a Z-electrospray (ESI) interface operating in negative ion mode with a nominal resolution of 60,000 m/z. Twenty microliters of Ene and F3 were injected into a liquid chromatography system. The conditions for the analysis of Ene were as follows: nebulizer at 2 Bar; dry gas at 8 L/min; temperature at 200 ºC and HV at 4500 V. The mobile phase consisted of two solvents: (A) water/formic acid (99.75/0.25, v/v) and (B) acetonitrile/formic acid/water (80/0.25/19.75, v/v). The flow rate was 1 mL/min, and the gradient was initiated with 10 % B, increasing to 20 % B (10 min), 30 % B (20 min), 50 % B (30 min), 90 % B (38 min), and decreasing to 10 % B (45 min), completing after 55 min. The conditions for the analysis of F3 was similar to those of Ene, except for the mobile phase, which consisted of two solvents: water/formic acid (99.75/0.25, v/v) (A) and acetonitrile (100) (B). The flow rate was 1 mL/min, and the gradient was initiated with 10 % solvent B, increasing to 30 % B (20 min), 50 % B (32 min), 95 % B (38 min), 95 % B (60 min), and decreasing to 10 % B (75 min), completing after 80 min. External calibration was carried out using the software MAXIS 3G – Bruker Daltonics 4.3 to check for mass precision and data analysis. The identification of compounds was performed tentatively by comparison of exact mass, MS/MS mass spectra and molecular formulae available from the scientific literature.

Evaluation of anti-inflammatory activity

Cell culture and viability *in vitro* assay

RAW 264.7 macrophages transfected with the NF-κBpLUC gene to express luciferase was obtained from American Type Culture Collection (ATCC) and were cultured in endotoxin-free RPMI 1640 medium supplemented with fetal bovine serum (10 %, v/v), penicillin (100 U/mL), streptomycin sulfate (100 µg/mL) and L-glutamine (37 °C, 5 % CO_2). Macrophages were cultured in 96-well plates (5 × 10^5 cells/mL) and incubated overnight. *En*e at 10, 30, 100, 300 and 1000 µg /mL and F3 at 0.1, 10, 30, 100 and 300 µg /mL, or culture media (negative control), were added to each well and incubated for 24 h. All groups were stimulated with 10 ng/mL lipopolysaccharide (LPS), except the negative control. After this period, the supernatant was removed and MTT solution (0.3 mg/mL) was added to the wells. The plates remained incubated for 3 h (37 °C, 5% CO_2). It was removed the supernatant 100 µL of DMSO were added to all wells. Plate absorbance was measured at 470 nm using an ELISA microplate reader.

NF-κB activation, and TNF-α and CXCL2/MIP-2 levels

Macrophage cells transfected were cultured in 24-well plates (3 × 10^5 cells/mL). The cells were treated with *En*e or F3 at 30, 100 and 300 µg/mL for 30 min before LPS stimulation (10 ng/mL) for 4 h, except the culture media control (negative control). After 4 h, the cell lysis buffer and 25 µL of luciferase reagent (luciferin at 0.5 mg/mL) were added to each well. Luminescence was measured using a white microplate reader (SpectraMax M3, Molecular Devices). In addition, according to the protocols by the manufacturers, it was determined TNF-α and CXCL2/MIP-2 levels using an ELISA microplate reader. The results were expressed in pg/mL.

In vivo anti-inflammatory assays

Animals

It was purchased from CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil) male C57BL/6JUnib mice, SPF (specific-pathogen free), weighing between 22 and 25 g. All animals were housed *in vivarium* under humidity (40 – 60 %) and temperature (22 ± 2 °C) control in 12 h light-dark cycle, with

access to food and water *ad libitum*. The animals were deprived of food for 8 h before oral administration of *En*e or F3. This study was carried out in strict accordance with the guidelines for the care and use of animals. The protocol was approved by the Institutional Ethics Committee on Animal Research of the University of Campinas (CEUA/UNICAMP, Protocol Number 4371-1, approved on 09/23/2016).

Inhibition of neutrophil migration

Mice received orally (via gavage) single doses of *En*e (3 or 10 mg/kg) or F3 (3 or 10 mg/kg). The negative control group received oral administration of 0.9% saline (vehicle) and 2 mg/kg dexamethasone. After 1 h, all animals received an inflammatory challenge by intraperitoneal (i.p.) injection of the flogistic agent carrageenan (500 µg/cavity) for 4 h, except the vehicle group. After 4 h, the animals were sacrificed, it were washed their peritoneal cavity and recovered in order to count for the total number of leukocytes and neutrophils. The results were expressed as number of neutrophils *per* cavity. In addition, according to the protocols by the manufacturers, it was determined TNF-α and CXCL2/MIP-2 levels using an ELISA microplate reader. The results were expressed in pg/mL.

Evaluation of reactive oxygen/nitrogen species (antioxidant activity)

Peroxyl radical ($ROO^{\bullet}$)

The $ROO^{\bullet}$ scavenging capacity of *En*e and F3 was determined as previously described [14]. Briefly, 30 µL of *En*e or F3 plus 60 µL of fluorescein, and 110 µL of a AAPH solution, were transferred to a microplate. The reaction was performed at 37 ºC and absorbance was measured every minute for 2 h at 485 nm (excitation) and 528 nm (emission) using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Trolox standard was used at concentrations ranging from 12.5 to 400 µM. The results were expressed as µmol/Trolox equivalents per g of extract/fraction (*En*e and F3) [4].

Superoxide anion ($O_2^{\bullet-}$)

The capacity of *En*e and F3 to scavenge $O_2^{\bullet-}$ generated by the NADH/PMS system was determined. It was mixed in the microplate 100 µL of NADH, 50 µL of NBT, 100 µL of *En*e or F3 (at different concentrations) and 50 µL of PMS. The assay was performed at 25 ºC and absorbance was measured after 5 min at 560 nm. A control

was prepared replacing the sample with the buffer, and a blank was prepared for each sample dilution replacing PMS and NADH with the buffer. Absorbance was measured in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) and the results were expressed as IC_{50}, the mean quantity (µg/mL) of *En*e or F3 required to quench 50 % of the superoxide radicals [12].

Hypochlorous acid (HOCl)

The HOCl scavenging activity of *En*e and F3 was measured by monitoring their effects on HOCl-induced oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123, with modifications. HOCl was prepared using a 1% NaOCl solution, adjusting the pH to 6.2. The reaction mixture contained *En*e or F3 at different concentrations, phosphate buffer (pH 7.4), DHR, and HOCl, in a final volume of 300 µL. The assay was carried out at 37 ºC in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) and fluorescence was measured immediately at 528 ± 20 nm (emission) and 485 ± 20 nm (excitation). The results were expressed as IC_{50} (µg/mL) of *En*e or F3 [4].

Nitric oxide ($NO^{\bullet}$)

The nitric oxide ($NO^{\bullet}$) activity was determined using diaminofluorescein-2 (DAF-2) as a $NO^{\bullet}$ probe. Briefly, 50 µL of *En*e or F3 plus 50 µL of SNP solution, 50 µL of buffer and 50 µL of DAF solution were added to the wells (96-well plate). Changes in fluorescence (excitation = 495 nm, emission = 515 nm) were measured in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) over a 120-min period at 5-min intervals. The results were expressed as IC_{50} (µg/mL) of *En*e or F3 [4].

Systemic toxicity

Galleria mellonella model

In order to provide preliminary evidence on the potential toxic effects of *En*e and F3, their acute systemic toxicity was determined in *G. mellonella* larvae model. of *En*e and F3 at 0.01, 0.1, 0.3, 1, 3 and 10 g/kg were tested. Larvae with no signs of melanization, weighing 200 to 300 mg, were randomly selected for each group (n = 15). An aliquot of 10 µL of *En*e, F3 or control (0.9% NaCl, w/v) were injected into their hemocoel via the last left proleg using a Hamilton syringe (Hamilton, Reno, NV). The

larvae were incubated at 30 °C and their survival was monitored at selected intervals for up to 72 h. Larvae with no movements upon touch were counted as dead [13].

Statistical analysis

The data were checked for normality and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The results were expressed as mean ± standard deviation (SD). For studies of survival, curves for larvae groups treated and untreated were compared using the Log-rank (Mantel-Cox) test. The results were considered significant at $P \leq 0.05$.

Results

Phytochemical analysis

As seen in Table 1, the chemical analysis revealed 16 compounds in *Ene*, 13 compounds in F3, and 8 compounds present in both samples. Overall, hydroxybenzoic acid, flavanols, ellagitannins, flavone and flavonols were detected in the samples. The chemical fractionation process is shown in supporting information S1 Fig: Visual aspect of *Ene*, chemical fractionation, yield and TLC.

Compound	Rt (min)	Molecular formula	$[M-H]^-$	MS fragments (m/z)	*Ene*	F3
Hydroxybenzoic acid						
Gallic acid	4.6	$C_7H_6O_5$	169.0105	**125.0209**, 117.9917, 140.5531, 150.3022	-	+
Syringic acid hexoside	14.7	$C_{15}H_{20}O_{10}$	359.1259	153.0888, **197.0771**, 212,6754, 119.0322	+	+
Sinapic acid-*O*-hexoside I	19.9	$C_{17}H_{22}O_{10}$	385.1409	**179.1035**, 119.0319, 135,1143, 223.0879	+	+
Sinapic acid-*O*-hexoside II	20.1	$C_{17}H_{22}O_{10}$	385.1409	**179.1044**, 135.1152, 113.0207, 223.0874	-	+
Flavanols						
(Epi)catechin	11.4	$C_{15}H_{14}O_6$	289.0726	**289.0726**, 245.0814, 179.0340, 205.0473	+	-
(Epi)catechin derivative	12.8	$C_{15}H_{14}O_6$	401.0886	**289.0725**, 245.0825, 205.0508, 179.0342	+	-
Ellagitannins						
Ellagic acid	20.2	$C_{14}C_6O_8$	300.9990	**300.9999**, 229.5014, 255.2311, 257.0124	+	-
Galloyl-HHDP-hexoside	8.3	$C_{27}H_{22}O_{18}$	633.0559	300.9922, **301.9946**, 169.0092, 463.0512, 481.0551	-	+
Di-HHDP-galloyl-glucose derivate I	10.9	$C_{41}H_{27}O_{26}$	467.0369 $[M-2H]^{2-}$	**275.0200**, 343.0106, 300.9988,169.0503	+	+
Di-HHDP-galloyl-glucose I	10.9	$C_{41}H_{27}O_{26}$	935.0797	**299.0203**, 275.0199, 300.9994, 633.0743	+	-
Di-HHDP-galloyl-glucose derivate II	11.8	$C_{41}H_{27}O_{26}$	467.0372 $[M-2H]^{2-}$	**275.0200**, 169.0146, 301.000, 343.0100	+	-
Di-HHDP-galloyl-glucose II	11.8	$C_{41}H_{27}O_{26}$	935.0806	**275.0200**, 299.0206, 633.0737, 300.99898	+	-
Di-HHDP-galloyl-glucose derivate III	16.6	$C_{41}H_{27}O_{26}$	467.0365 $[M-2H]^{2-}$	**300.9996**, 423.1872, 169.0141, 275.0247	+	-
Flavone						
Apigenin-7-*O*-glucoside	14.0	$C_{21}H_{20}O_{11}$	431.1810	205.1177, 121.0290, 119.0320, **269.2089,** 153.0176, 241.9622, 311.4209	-	+
Flavonols						
Quercetin-*O*-hexoside I	20.2	$C_{21}H_{20}O_{12}$	463.0887	**301.0328**,271.02445,463.0884, 243.0800	+	+
Quercetin-*O*-hexoside II	20.5	$C_{21}H_{20}O_{12}$	463.0890	**301.0352**, 271.0248, 243.0802, 463.0889	+	+
Quercetin-*O*-(*O*-galloyl)-hexoside	21.2	$C_{28}H_{24}O_{16}$	615.0996	**301.0357**, 271.0248, 179.0982, 151.0039	+	+
Quercetin-*O*-acethylhexoside	22.4	$C_{23}H_{22}O_{13}$	505.0994	221.0238, **301.0335**, 506.1011, 463.0024	+	-
Quercetin-3-malonylglucoside	22.4	$C_{24}H_{22}O_{15}$	549.0889	300.0279, **301.0328**, 271.0249, 243.0807	+	+
Kaempferol-3-*O*-glucoside	23.2	$C_{21}H_{20}O_{11}$	447.0940	**285.0335**, 284.0335, 255.0299, 227.0851	+	+

Bold values indicate the main fragments; Rt = retention time; [M-H]- (negative ionization mode) experimental mass of compound; + detected/ - not detected.

In vitro anti-inflammatory assays

Viability assay, NF-κB activation and TNF-α and CXCL2/MIP-2 levels

As seen in Fig 1A, treatment with *Ene* at concentrations up to 300 µg/mL did not significantly affect cell viability of LPS-stimulated macrophages as compared to the culture media control ($P > 0.05$). However, cells treated with *Ene* at 1000 µg/mL showed reduced cell viability as compared to the control ($P < 0.05$). As expected, LPS treatment (10 ng/mL) did not affect cell viability ($P > 0.05$).

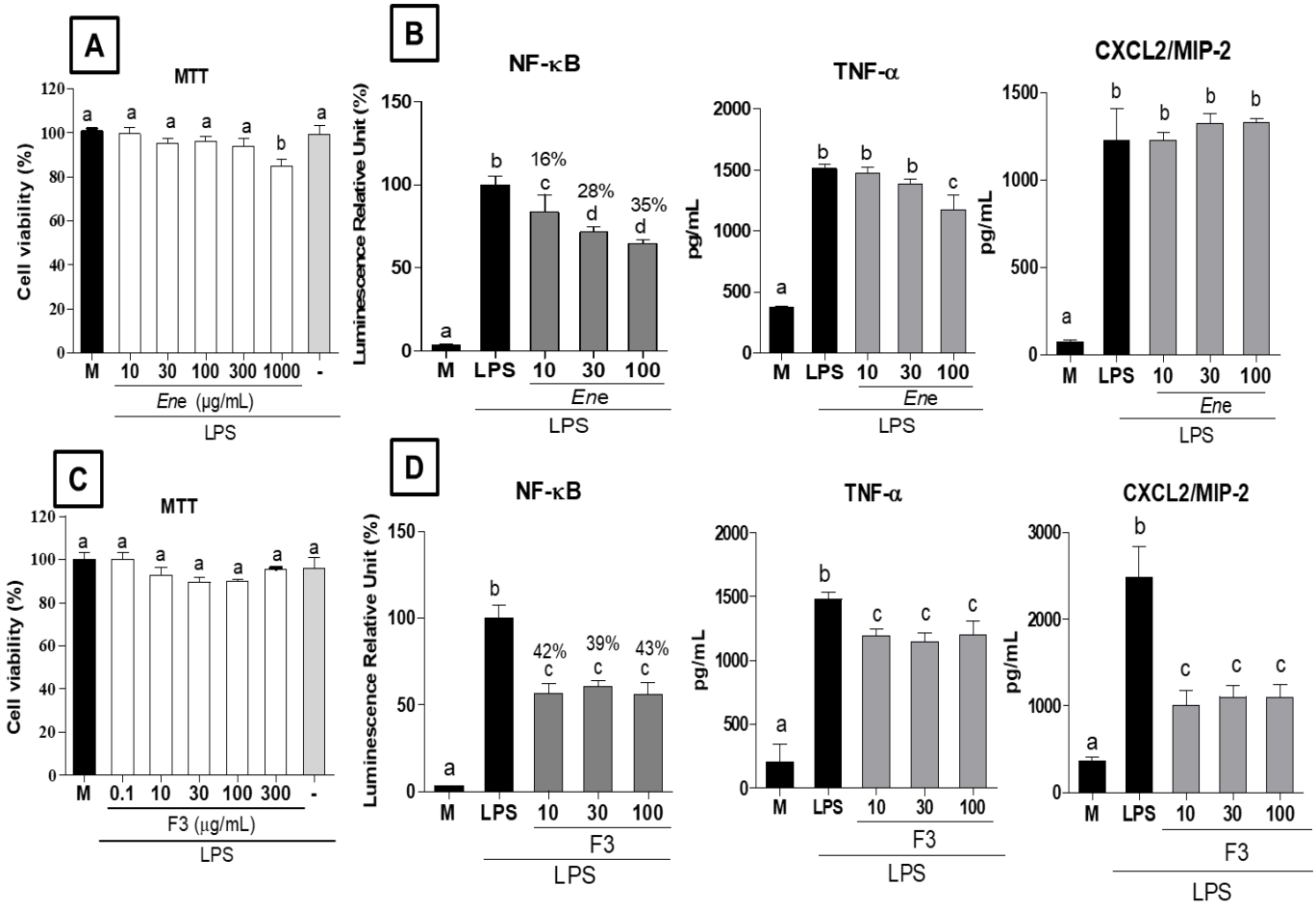

Fig 1. The effects of *Ene* and F3 on cell viability and inflammation markers in RAW 264.7 macrophages. Effects of culture medium (M), LPS (10 ng/mL) (-),*Ene* (10, 30, 100, 300 and 1000 µg/mL) and F3 0.1, 10, 30, 100 and 300 µg/mL on RAW 264.7 macrophages. **(A and C)** Evaluation of RAW 264.7 macrophages viability after 24h. **(B and D)** Evaluation of NF- kB activation and and release of TNF-α and CXCL2/MIP-2 on RAW 264.7 macrophages. The results were expressed as mean ± SD, $n = 4$. Different letters indicate statistical difference and the symbol % indicate decrease in NF- kB activation. ANOVA One-way followed by Tukey's post-hoc test, $P < 0.05$.

The Fig 1B shows that treatment with *Ene* (10, 30 and 100 µg/mL) significantly reduced NF-κB activation by 16%, 28% and 35%, respectively, as

compared to LPS-treated cells. *En*e reduced TNF-α levels only when tested at 100 µg/mL ($P < 0.05$) and 10 and 30 µg/mL did not affect TNF-α release. Likewise, *En*e treatment did not affect the release of CXCL2/MIP-2 by macrophages at any tested concentration as compared to LPS-treated cells ($P > 0.05$, Fig 1B). The purified fraction (F3) from *En*e did not affect macrophage viability at any tested concentration as compared to culture media control and LPS-treated cells ($P > 0.05$; Fig 1C). Lastly, macrophage cells treated with F3 (10, 30 and 100 µg/mL) had NF-κB activation significantly reduced (42%, 39% and 43%, respectively) as compared to LPS-treated cells ($P < 0.05$; Fig 1D).). Lastly, F3 decreased the release of TNF-α and CXCL2/MIP-2 in at all tested concentrations when compared to their respectively LPS-treated cells control ($P < 0.05$; Fig 1D). The MTT and NF-κB activation screening for all fractions is shown in supporting information S2 Fig: cytotoxicity and NF-κB activation of fraction derived from *En*e on RAW 264.7 cells.

In vivo anti-inflammatory assays

The inhibitory effects of *En*e and F3 on neutrophil influx were determined. Mice pre-treated with *En*e at 3 and 10 mg/kg showed a significant decrease in neutrophil influx (58% and 70%, respectively) as compared to the control carrageenan (Fig 2A). As expected, mice that received dexamethasone showed a significant decrease in neutrophil migration (74%). Interestingly, there was no statistical difference in terms of neutrophil influx between mice treated with *En*e (at both doses) and those treated with the positive control dexamethasone, a gold-standard corticosteroid widely used in medicine and dentistry ($P > 0.05$).

Treatment with *En*e at 3 and 10 mg/kg also reduced the release of TNF-α and CXCL2/MIP-2 into the peritoneal cavity of mice as compared to each control group (Fig 2B and Fig 2C). Mice treated with F3 at 3 and 10 mg/kg showed reduced neutrophil migration (53% and 41%, respectively) in relation to the control group ($P < 0.05$; Fig 2D).

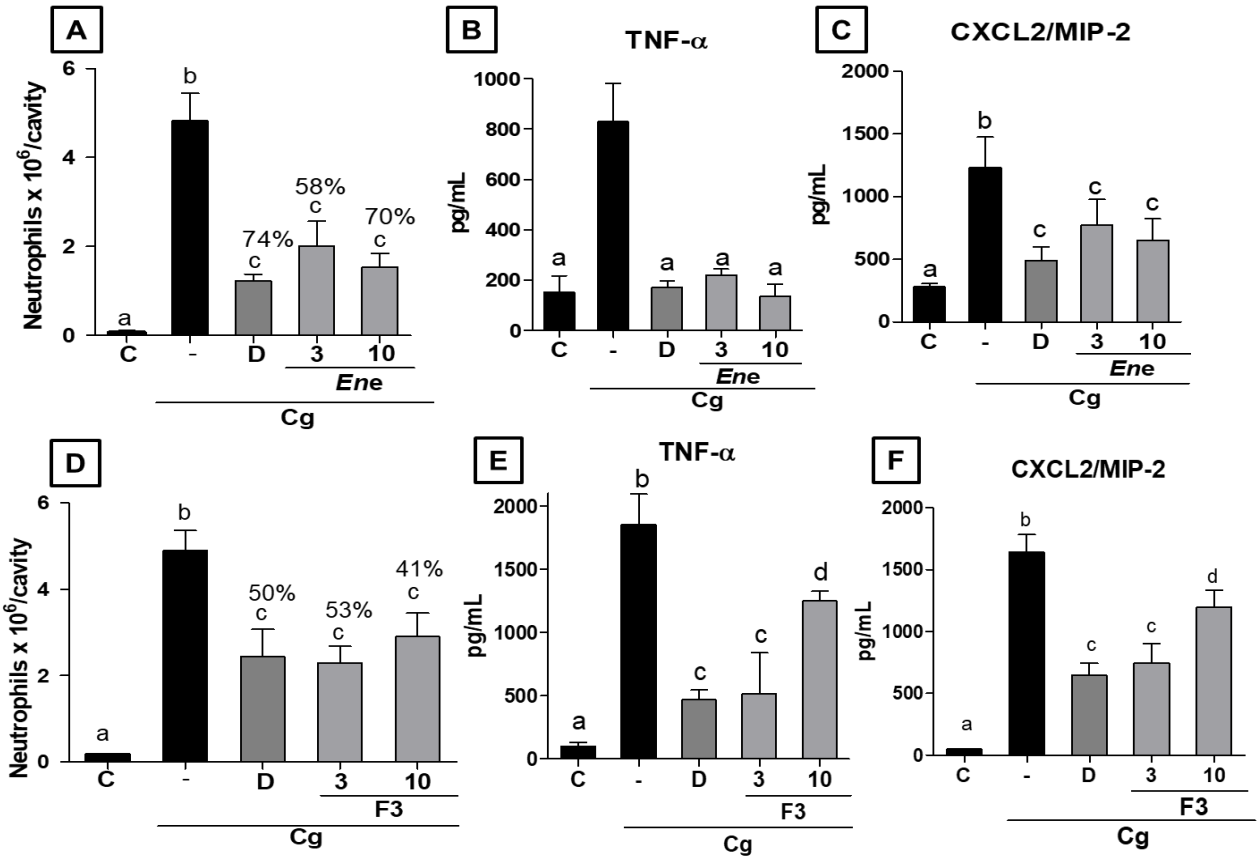

Fig 2. The effects of *Ene* and F3 on neutrophil migration and cytokine release *in vivo.* Effects of vehicle (C), carrageenan (-), dexamethasone (D; 2 mg/kg), *Ene* or F3 (3 and 10 mg/kg) on neutrophil migration into the peritoneal cavity of mice induced by i.p. administration of carrageenan (−) (500 µg/cavity). **(A and D)** Effects of the treatments on neutrophil migration into the peritoneal cavity of mice induced by i.p. administration of carrageenan. **(B and E)** Effects of the treatments on the release of TNF-α (1.5 h) in mice. **(C and F)** Effects of the treatments on the release of CXCL2/MIP-2 (3 h) in mice. The results were expressed as mean ± SD, *n* = 5-6. Different letters indicate statistical difference and all groups were compared to each other. ANOVA one-way followed by Tukey's post-test, $P < 0.05$.

In our study, the positive control dexamethasone reduced neutrophil migration by 50% ($P < 0.05$). Mice treated with F3 (3 and 10 mg/kg) showed reduced levels of TNF-α and CXCL2/MIP-2 when compared to the control group ($P < 0.05$; Fig 2E and Fig 2F). Given the ant-inflammatory potential of *Ene* and F3, both samples were further tested for their antioxidant activity (ROS/RNS scavenging capacity).

Reactive oxygen/nitrogen species

Antioxidant activity of *Ene* and F3 against peroxyl radical (ROO•), superoxide anion ($O_2^{\bullet-}$), hypochlorous acid (HOCl) and nitric oxide (NO•). We investigated the antioxidant activity of *Ene* and F3 against oxygen/nitrogen reactive species, which are biologically equivalent to human metabolism.

As seen in Table 2, while *Ene* was the most active sample to deactivate the superoxide anion $O_2^{\bullet-}$ (172 µg/mL), F3 showed the highest capacity to quench peroxyl radical (680 µmol TE/g extract), hypochlorous acid HOCl (1.13 µg/mL) and nitric oxide NO• (5.05 µg/mL) as compared to *Ene*. The results for the radicals $O_2^{\bullet-}$, HOCl and NO• are expressed as IC_{50} - which means the amount of substance required to deactivate 50 % of the radicals. Overall, F3 seems to have a greater concentration of compounds with both antioxidant and anti-inflammatory properties.

Table 2. Antioxidant activity of *Ene* and F3 against peroxyl radical (ROO•), superoxide anion ($O_2^{\bullet-}$), hypochlorous acid (HOCl) and nitric oxide (NO•).

Sample	ROO•	$O_2^{\bullet-}$	HOCl	NO•
	µmol TE/g extract	µg/mL	µg/mL	µg/mL
Ene	250 ± 0.008[a]	172.00 ± 14.90[a]	16.68 ± 1.17[a]	11.48 ± 1.84[a]
F3	680 ± 0.02[b]	849.00 ± 4.04[b]	1.13 ± 0.15[b]	5.05 ± 1.07[b]

ROO• is expressed as µm TE/mg of extract (TE = Trolox equivalent), $O_2^{\bullet-}$, HOCl and NO• are expressed as IC_{50} (µg/mL). The results were expressed as mean ± SD (standard deviation), $n = 3$. Different letters in the same column indicate statistical difference ($P < 0.05$), according to Student's t test.

Toxicity of Ene and F3 in G. mellonella model

G. mellonella larvae were injected diferent doses of *Ene* and F3 and their survival monitored for 72 h. As seen in Fig 3, larvae treated systemically with *Ene* and F3 did not demonstrate toxic effects at any tested dose ($P > 0.05$), indicating negligible toxicity in this *in vivo* model.

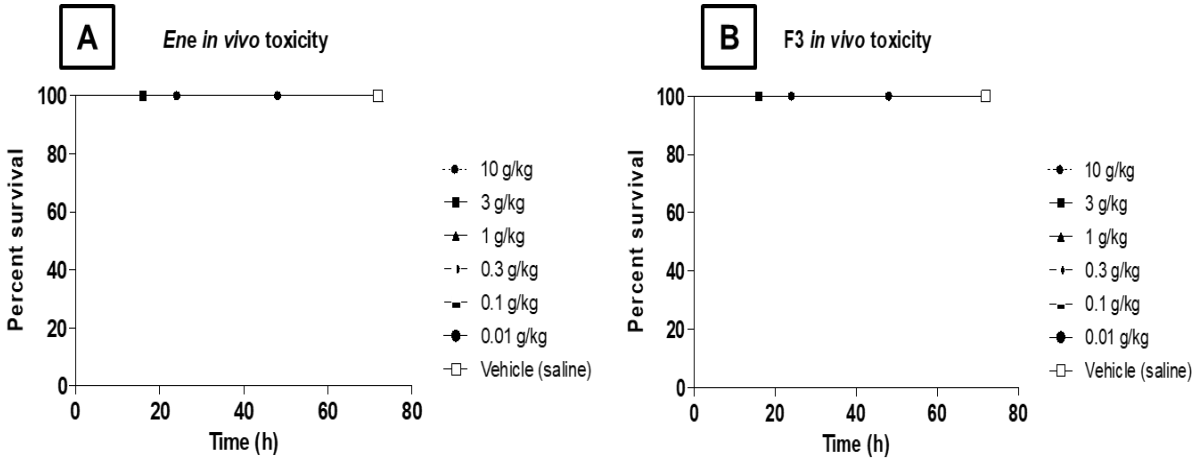

Fig 3. Systemic toxicity of *Ene* and F3 in *G. mellonella* larvae model. Larvae were treated with *Ene* and F3 at 0.01, 0.1, 0.3, 1, 3 and 10 g/kg or vehicle (saline), and had their survival monitored up to 72 h ($P > 0.05$, Log-rank test).

Discussion

It is estimated that approximately 1.7 million deaths per year are related to the low intake of fruits and vegetables, despite fruits are widely cultivated worldwide. A minimum daily intake of 400 g is required for prevention of chronic diseases and several micronutrient deficiencies [14]. BNF species can be an important ally to one's health as functional foods while fostering agribusiness and protecting deforestation in threatened rainforests and biomes.

In this bioguided study, we demonstrated that *E. neonitida* extract and its fraction (F3) have (i) anti-inflammatory and (ii) antioxidant activity and (iii) low toxicity *in vivo. Ene* and F3 samples were submitted to chemical analysis for tentative identification of compounds based on exact masses, MS/MS spectra and molecular formulas. Gallic acid (*m/z* 169.0105), identified in F3, is an ion produced at *m/z* 125 due to loss of a CO_2 [15]. Another compound presented a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 359.1259, yielding MS2 fragments at *m/z* 197 (loss of a hexosyl moiety; [syringic acid-H]$^-$) and 153 (base peak; [syringic acid-H-CO_2]$^-$), suggesting that it could be a syringic acid hexoside in *Ene* and F3 [16]. *m/z* 385.1409 were tentatively identified as isomers of sinapic acid-*O*-hexoside [17].

(Epi)catechin (*m/z* 289.0726) (flavanol class) and (epi)catechin derivative (m/z 401.0886) were also found in *Ene*, but not in F3. These compounds are stereoisomers characterized by the molecular ion [M-H- 289]-. The fragments of MS2 corresponding

to *m/z* 245, *m/z* 205 and *m/z* 179 indicate that the compound is (epi)catechin [12,18]. The ion *m/z* 245 may result from a loss of a CO_2 group [M-H-44]- by decarboxylation or by CH_2CHOH- group loss [15].

Ellagic acid (*m/z* 300.9990), which was detected in *En*e, belongs to the class of organic compounds known as ellagitannin. This compound is well known for its antioxidant activity with free radical scavenging properties [19]. Ellagitannin compounds were found in both samples, although galloyl-HHDP-hexoside (*m/z* 633.0559) was detected only in F3. Two di-HHDP-galloyl-glucose (*m/z* 935) isomers and three di-HHDP-galloyl-glucose derivate (*m/z* 467 [M-2H]-2) isomers were identified in *En*e and F3, which presented the fragments *m/z* 633 (M-HHHDP) and *m/z* 301 (ellagic acid) [18,20,21]. Apigenin-7-*O*-glucoside (*m/z* 431.1810) was detected only in F3 based on the *m/z* 269 fragment, which is typical of apigenin [22].

The following flavonols were identified in *En*e and F3: quercetin-*O*-hexoside (*m/z* 463); quercetin-*O*-(*O*-galloyl)-hexoside (*m/z* 615.0996), which produced *m/z* 179 and 151 corresponding a quercetin fragmentation pattern; quercetin-3-malonylglucoside (*m/z* 549.0889), which presented the *m/z* 301 fragment, a typical ion following the loss of a hexose molecule [M-H-162]- [4,23,24]. Finally, the kaempferol-3-*O*-glucoside (*m/z* 447.0940) was identified in *En*e and F3 composition [25].

The compound quercetin-*O*-acethylhexoside, present only in *En*e, was tentatively identified as quercetin-acylated-hexoside based on literature data. This compound showed *m/z* 505.0994 with fragmentation at *m/z* 463 due to loss of an acetyl group (42 Da) and *m/z* at 301 due to loss of a hexoside residue (162 Da) [26].

It is worth noting that some compounds were present in F3, but not in *En*e. This can be explained by the fact that F3 was obtained through a fractionation process, that is, chemical purification, in a way that compounds previously undetectable become more concentrated and therefore detectable by LC-ESI-QTOF-MS.

Once *En*e and F3 were chemically characterized, we next tested both samples for their anti-inflammatory activity in different models. Inflammation is an orchestrated host response involving different cell types, such as macrophages. The inflammatory response leads to an intracellular increase of ROS and RNS by macrophages, which can activate the NF-κB intracellular pathway resulting in gene transcription and release of inflammatory mediators, such as TNF-α and CXCL2/MIP-2 [27,28]. Subsequently, inflammatory mediators increase protein expression on the surface of endothelial cells (selectins and integrins) that control the neutrophil flow into the inflammatory focus [4].

In our study, *Ene* and F3 reduced NF-κB activation in macrophages, decreased the release of TNF-α and CXCL2/MIP-2 and, consequently, mitigated neutrophil migration in mice. Some chemical compounds as ellagitannin identified in *Ene* and F3, have been shown to inhibit NF-κB activation and to modulate neutrophil influx during inflammation [13,21,29]. Quercetin and kaempferol are known to reduce NF-κB activation, TNF-α and IL-1β release, as well as to decrease leukocyte recruitment and oxidative stress [5,13,30-32].

Our findings show that *Ene* and F3 modulated neutrophil migration into the inflammatory focus. Although *Ene* is a chemically crude extract, a complex mixture of bioactive compounds at low concentration, its inhibitory activity on neutrophil influx did not differ from that of the gold standard dexamethasone, which is a pure monodrug. These findings illustrate the potential of *E. neonitida* as a functional food to reduce or prevent inflammatory diseases and promote health.

In two similar studies, the extracts of *E. leitonii* seeds and *E. brasiliensis* pulp (from the same Eugenia genus) reduced neutrophil influx into the inflammatory focus, with no difference from dexamethasone, and also decreased NF-κB activation [13,35]. Interestingly, these three species (*E. leitonii, E. brasiliensis* and *E. selloi)* share two compounds in their chemical composition, ellagic acid and quercetin, which may be related for the anti-inflammatory activity observed [13,33].

Despite the benefits of the inflammatory response, an exacerbated presence of neutrophils and other cells generating excessive ROS/RNS production may create an imbalance in pro- and antioxidants, ultimately damaging tissues, DNA, proteins, and other host components [34].

Here, *Ene* and F3 were further tested for their ROS/RNS scavenging activity. Our findings indicate that F3 was more effective than *Ene* in this regard, despite the fact that the fractionation process was guided by the anti-inflammatory data. *Ene* was more effective in quenching the superoxide anion (O_2•) compared to F3, which can be explained by the synergism among the phenolic compounds present in the crude extract. Four Eugenia spp. were previously tested for their O_2• radical scavenging activity and the authors reported IC_{50} ranging from 215 to 402 µg/mL, indicating that *E. neonitida* is more potent than those native species. [5]. The superoxide anion contributes to metabolic oxidative stress, resulting in cell damage and genomic instability. Hence, the inhibition of this radical can be an important strategy to prevent diseases related to oxidative stress [34].

The ORAC (oxygen radical absorbance capacity) assay is a direct method to measure hydrophilic and lipophilic chain-breaking antioxidant capacity against the peroxyl radical, generated by AAPH via hydrogen atom transfer reactions [35]. In the context of inflammation, ROS present in the lipid tissue are converted into peroxyl radical and may cause cell membrane damage, neoplasia and, most likely, several inflammatory chronic and degenerative diseases [34,36]. Our findings showed that the peroxyl radical scavenging capacity of F3 was three-fold greater than that of *Ene*, which might be due to the chemical composition of the fraction.

We next determined the ROS (hypochlorous acid - HOCl) scavenging activity of the samples. *Ene* and F3 exhibited different IC_{50} values, which indicated that F3 was approximately 16-fold more potent than *Ene* to scavenge HOCl. Although *Ene* is a crude extract, its HOCl radical scavenging activity was comparable to that of Trolox (IC_{50} of 134 µg/mL), a standard drug [37]. As compared to other native fruits, *Ene* and F3 were more active than *E. brasiliensis* and *E. leitonii* pulp (IC_{50} of 42 and 109 µg/mL, respectively) [5].

The samples were further tested for their nitric oxide (NO•) scavenging capacity. In this regard, F3 was approximately 2-fold more effective than *Ene*. A previous study investigated the NO• scavenging capacity of *E. stipitata*, a BNF belonging to Eugenia genus, and observed an equivalent activity (IC_{50} of 6.95 µg/mL) [4]. We detected the presence of quercetin derivatives in both samples. This flavonoid is known to be more potent than other antioxidant nutrients, such as vitamin C and vitamin E [38]. The different potency observed in the samples can be due to the synergism and chemical composition present in the crude extract and its fraction.

This is the first report on the ROS/RNS scavenging activity *E. neonitida* extract and its purified fraction (F3). It is known that ROS and RNS are important components in the inflammation process for their capacity to promote cell / tissue damage in the organism. The question that remains is how and how much ROS and RNS contribute to the inflammatory response. Thus, controlling the balance between oxidation and anti-oxidation through exogenous antioxidants obtained from the diet (*e.g.*, daily fruit intake) is a promising strategy to prevent the onset of inflammatory diseases [10,38].

This is also the first report on the *in vivo* systemic toxicity of *Ene* and F3 in *G. mellonella* larvae. This is a simple, a low-cost, and validated model widely used, because the results obtained in this model correlate with those observed in mammals [39]. In our study, it was not possible to find the lethal dose (LD_{50}) of the samples even

when testing a dose 1000 times higher (10 g/kg) than that used in mice (10 mg/kg). In a previous study with other BNF *(E. leitonii* seed and *E. brasiliensis* leaf extracts), the authors showed LD_{50} of 1.5 g/kg for both extracts using *G. mellonella* model. However, we evaluated the pulp extract in our study, which seems to have negligible toxicity in this model as compared to other parts of Eugenia spp. fruits [13].

Conclusions

In summary, *En*e and F3 inhibited neutrophil migration by reducing NF-κB activation and exhibited free radical scavenging activity due to the presence of flavonols and hydroxybenzoic acid class compounds. *E. neonitida,* an unexplored Brazilian native fruit, which is yet not popular and not broadly commercialized.

E. neonitida has proven to be a good source of bioactive compounds, even better than the best fruits traditionally consumed, and was shown to modulate the inflammatory process. This study adds value to the unexplored Brazilian native fruit that have emerged as a source of bioactive compounds providing benefits for human health (functional foods), to contribute to the development of agribusiness for many families, and also contributing to biodiversity conservation, particularly in the Brazilian Atlantic Rainforest.

Supporting Information:

S1 Fig. Visual aspect of *En*e, chemical fractionation, yield and TLC.

S2 Fig. Cytotoxicity and NF-κB activation of fraction derived from *En*e on RAW 264.7 cells.

Author Contributions

Conceived and designed the experiments: SMA, JGL, PLR. Performed the experiments: JGL, JCS, BDN. Analyzed the data: MF, APM, JGL, JCS. Contributed reagents/materials/analysis tools: SMA, PLR. Wrote the paper: SMA, PLR, JGL, JCS, APM**.**

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Conflicts of Interest

The authors declare no conflicts of interest.

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Artigo 1

Supplementary Material

S1 Fig. Visual aspect of *Ene*, chemical fractionation, yield and TLC.

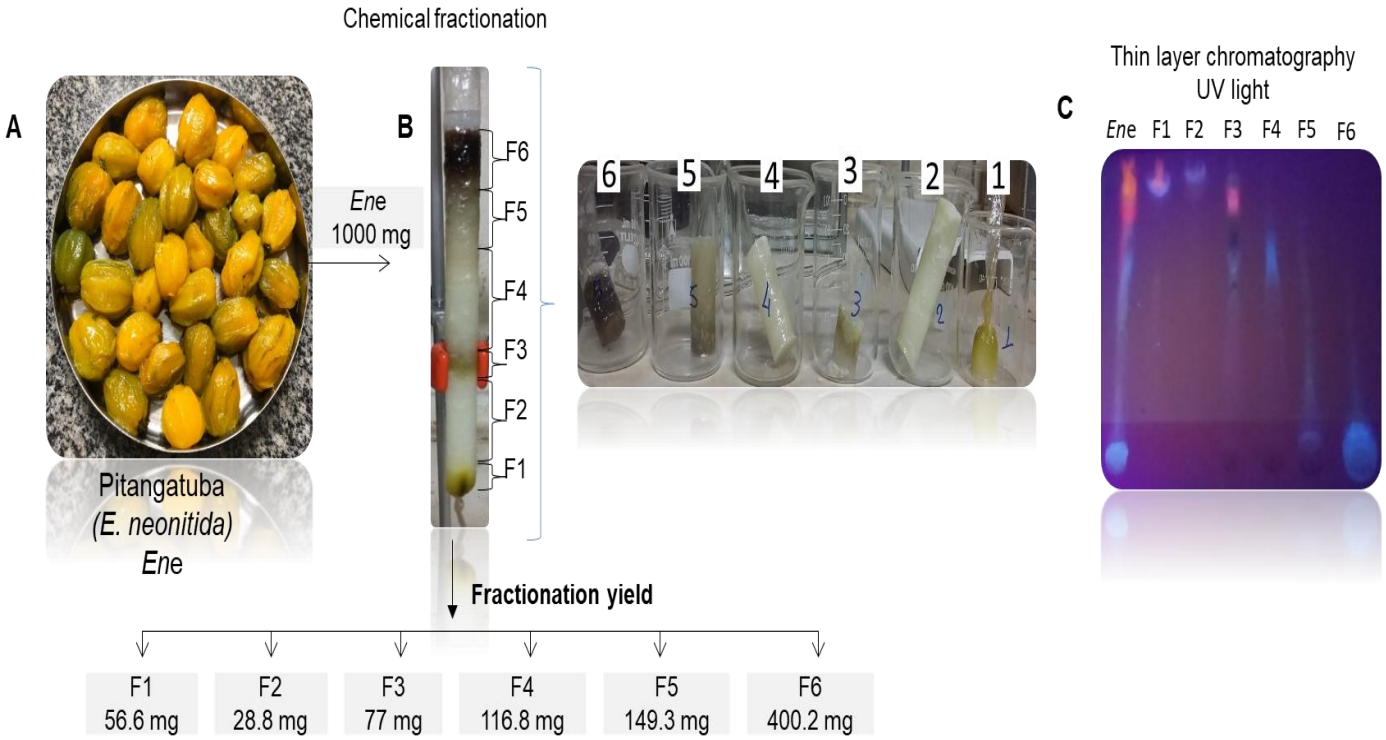

Figure S1. Visual aspect of *Ene*, chemical fractionation, yield and TLC. Visual aspect of *E. neonitida* extract – *Ene* (common name: pitangatuba) (**A**). Chemical fractionation of *Ene* resulting in 6 fractions and their respective yield (**B**). Thin layer chromatography visualized in UV light (366 nm) **(C)**.

Supplementary Material

S2 Fig. Cytotoxicity and NF-κB activation of fraction derived from *Ene* on RAW 264.7 cells.

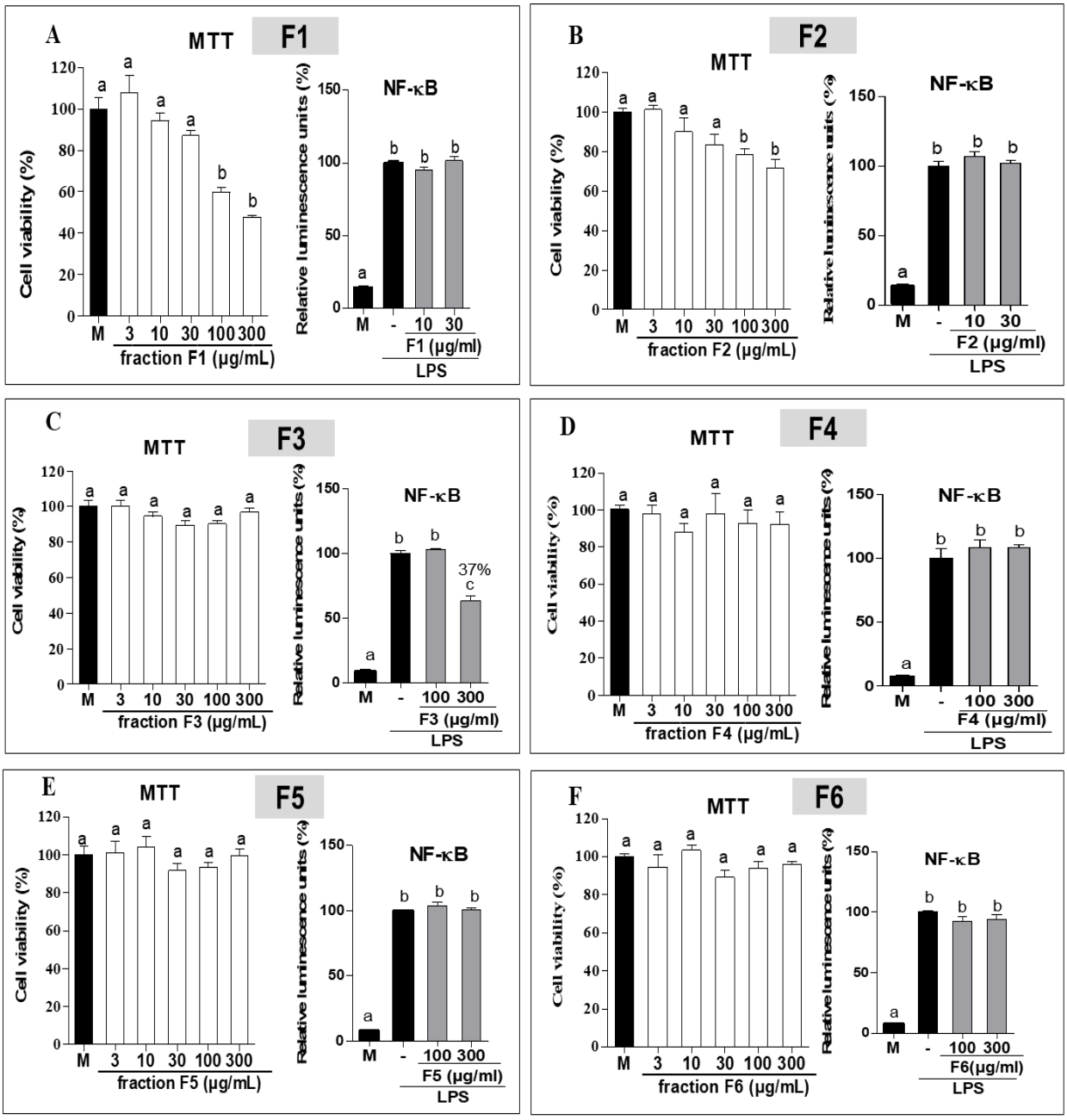

Figure S2. Cytotoxicity and NF-κB activation of fraction derived from *Ene* on RAW 264.7 cells. To assess cytotoxicity (MTT), macrophages were treated with medium RPMI (M) and fractions F1-F6 (**A-F**, respectively) at 3, 10, 30, 100 and 300 µg/mL for 24h. To assess the NF-κB activation, macrophages were treated with medium RPMI (M), fractions F1-F6 (**A-F**, respectively) at 10, 30, 100 or 300 µg/mL and stimulated with LPS (10 ng/ml) for 4h. The results were expressed as mean ± SD, n = 4-6. All groups were compared to each other and different letters indicate statistical difference. One-way ANOVA followed by Tukey's post-test, $p < 0.05$.

Artigo 2. Phytoactives from *Eugenia neonitida* (pitangatuba), a Brazilian native superfruit, decreases neutrophil migration: involvement of NF-κB, ICAM-1 expression, cytokine production and ROS and RNS scavenging.

Artigo a ser submetido no periódico Food Chemistry (IF: 5.399).

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Abstract

A large number of Brazilian native fruits, classified as “superfruits” due to their rich phytochemical composition and bioactivity, remain poorly explored for the development of food and pharmaceutical products. We determined the phytochemical composition, anti-inflammatory mechanism of action, ROS/RNS scavenging capacity and systemic toxicity of purified subfraction (S8) of *E. neonitida*. The phytochemical composition was assessed of S8 by LC–ESI-QTOF-MS. The anti-inflammatory mechanism of action of S8 was tested *in vitro* and *in vivo* through NF-κB activation, cytokine levels, neutrophil migration, intravital microscopy and expression of ICAM-1, S8 also was tested against oxygen/nitrogen reactive species and for its toxicity in G*alleria mellonella* larvae model. At S8 composition were identified quercetrin, vanillic acid and coumaric acid. The S8 decreased NF-κB activation, TNF-α and CXCL2/MIP-2 levels, neutrophil migration and expression of ICAM-1. S8 exhibited high antioxidant effects against ROS/RNS and did not induced toxic effects on *G. mellonella* larvae. *E. neonitida* is a promising source of bioactive compounds unexplored, which can promote sectors such as agribusiness, pharmaceutical and food industries generating development of new products or supplies for human health.

Keywords: *Eugenia neonitida,* anti-inflammatory, ROS and RNS, native fruit.

1. Introduction

The natural products are one of the most important source of bioactive compounds with biological activities. At this context, the Brazilian Atlantic rainforest may include 1 – 8% of the world's total species possessing a high diversity and endemism of species of plants reaching more than 20.000 species, however, this ecosystem is threat of extinction. (Ribeiro, Metzger, Martensen, Ponzoni, & Hirota, 2009).

Regarding to Atlantic rainforest plants diversity, the *Eugenia* genera (Myrtacea family) have highlighted for its biological activities and to represents great potential for fresh fruit consumption, areas such as agro-industrial food (production of juice, jam and ice cream), cosmetic, nutritional and pharmaceutical (Lazarini et al., 2018; Soares et al., 2019; Araujo, Neri-Numa, de Paulo Farias, da Cunha, & Pastore, 2019).

In the context of bioactivity, plants of the *Eugenia* genera showed activities such as antimicrobial, anticancer, antioxidant, and anti-inflammatory (Infante, Rosalen, Lazarini, Franchin & Alencar, 2016; Sardi, Freires, Lazarini, Infante, Alencar & Rosalen 2017; Araujo et al., 2019). These activities revealed in *Eugenia* genera are intrinsically related to its chemical composition such as Kaempferol (*Eugenia brasiliensis, Eugenia myrcianthes, Eugenia leitonii*), catechin/epicatechin (*E. brasiliensis, E. leitonii*), quercetin (*E. brasiliensis, E. myrcianthes, E.leitonii*), ellagic acid and apigenin (*Eugenia stipitata*) (Infante et al., 2016, Lazarini et al., 2018; Soares et al., 2019).

Some Brazilian native fruit have been considered "superfruits" due to possess high levels of antioxidant and bioactive compounds that may contribute to the prevention and treatment of inflammatory diseases (Chang, Alasalvar & Shahidi, 2018).

In the context of inflammation, this process is orchestrated by several types of cells that can be triggered by a trauma, tissue injury or pathogens. The tissue resident macrophage cell since activated by LPS (*lipopolissacharide),* for instance, increase the nuclear factor kappa B (NF-κB) transcription leading to release of pro-inflammatory cytokine and chemokines. These proteins stimulate endothelial cells to express proteins of rolling (selectins as P and E) and adhesion (integrins as I-CAM1) in order to attract neutrophils and lymphocytes into the tissue (Taniguchi, & Karin, 2018; Maleki, Crespo, & Cabanillas, 2019).

Since internalized, these cells produces reactive oxygen species (ROS), reactive nitrogen species (RNS) and different proteases leading to fibrosis formation, cell proliferation, and tissue damage. However, this process not contained can became a chronic inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, asthma, cancer, atherosclerosis, and diabetes usually initiated without an important clinic symptoms are generated by oxidative stress by ROS/RNS (Lavecchia, Rea, Antonacci, & Giardi, 2013; Ashley, Weil, & Nelson, 2012; Peres, Menezes, Teixeira, & Cunha, 2016).

Currently, due to food production and the life style, the inflammatory diseases and its chronicity have been associated with consumption of ultra-processed food containing saturated or trans-fat that increase ROS/RNS generation (Maleki et al., 2019; Soares et al., 2019). Therefore, it is of paramount relevance to the search of novel sources of bioactive molecules that simultaneously interfere in ROS/RNS generation and decrease the inflammatory process modulating novel pathways and targets (Lazarini et al., 2016; Maleki et al., 2019).

Previously, our group submitted five unexplored Brazilian native fruit species in an anti-inflammatory in vivo screening. We selected the *Eugenia neonitida* Sobral due to its promising anti-inflammatory activity by inhibits neutrophil migration. *E. neonitida* (synonyms *Eugenia neonitida* Cambess and *Eugenia selloi* O. Berg) is commonly known as “pitangatuba”, “pitangão” and “pitanga-amarela” occurs primarily at the restinga region Rio de Janeiro and Espirito Santo states. This Eugenia specie is a bush measuring up to 2.5m of high and its fruit has an oblong shape, bright yellow color exhaling an intense bittersweet scent (Vilar, Silva, Coelho, Silva & Srur 2006; Araujo et al., 2019). Unfortunately, most studies published about E. neonitida have characterized the physicochemical composition only.

There is a continuing need of scientific studies to describe the bioactivities such as anti-inflammatory, antioxidant and phytochemical identification of *E. neonitida* subfractions (purified chemically). Our hypothesis is that the chemical compounds present in *E. neonitida* and its subfraction purified may interfere in ROS/RNS scavenging capacity and consequently in inflammation process.

Thus, we determined the phytochemical composition, anti-inflammatory mechanism of action, ROS/RNS scavenging capacity and systemic toxicity of purified subfraction of *E. neonitida*.

2. Materials and methods

2.1 Reagents

The reagents were purchased from Tedia (Fairfield, OH, USA): formic acid. Millipore Milli-Q System (Millipore SAS, Molsheim, France): purified water. J.T. Baker (Phillipsburg, NJ, USA): acetonitrile, methanol and ethanol. Sigma-Aldrich (St. Louis, MO, USA): Roswell Park Memorial Institute (RPMI), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, DMSO (dimethylsulfoxide), carrageenan, dexamethasone, diaminofluorescein-2 (DAF-2), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium nitroprusside, sodium hypochlorite solution (NaOCl), nitrotetrazolium blue chloride (NBT), dibasic potassium phosphate, 2,2-azobis(2-methylpropionamidine) β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), rhodamine 123, dihydrochloride (AAPH) and fluorescein sodium salt. Gibco (Grand Island, NY, USA): fetal bovine serum. Applied Biological Materials Inc. (Richmond, BC,Canada): RAW 264.7 macrophages transfected with the NF-kB-pLUC gene. MERCK KGaA (Frankfurt, Germany): Silicagel 60 (0,063-0,200 mm). Promega Corporation (Madison, WI, USA): luciferin. Amresco, Inc. (West Chester, Pensilvania, EUA): Lysis buffer TNT, mixture of TRIS BASE and Tween 20. R&D Systems, Inc: TNF-α and CXCL2/MIP-2 kits. Supelco, Bellefonte, PA, USA): LC-18 SPE cartridges 2 g.

2.2 Plant Material and chemical purification.

Firstly, we registered the access of the Brazilian native fruit at the Council for Genetic Heritage Management (CGEN #AD4B64F; Brazilian Ministry of Environment). Shortly, the *Eugenia neonitida* Sobral was collected between November and February 2016, in Atlantic rainforest region, municipality of ‘Campina do Monte Alegre’ (S 23º 53' 57.06"; W 48º 51' 24.68"), state of São Paulo, Southeastern of Brazil. The specimen was deposited under voucher number HPL 5279 in the herbarium of the “Luiz de Queiroz” College of Agriculture at the University of São Paulo (ESALQ/USP, Piracicaba, São Paulo. *E. neonitida* was extracted by ultra-sound plus a mixture of ethanol and water (80:20, v/v; respectively). The *E. neonitida* extract was further fractionated using an open dry column chromatography on normal phase silica gel and

we selected the more bioactive fraction (F3) based on a bioguided-study for anti-inflammatory activity using the NF-κB activation on macropage culture.

2.3 Chemical subfractionation

In order to confer specificity and refinement, we carried out a subfractionation with the F3 initiating to 100 mg in a column chromatography on reverse phase C18. The elution initiated with a mixture of water:methanol (50:50 v/v) in an increased concentration gradient in scale to 10% until the final 100% of methanol. We obtained twelve subfractions named S1 to S12 that were monitored during the elution by UV light (366nm). In addition, all subfractions obtained were monitored by a thin layer chromatography (TLC) visualizing the fluorescent substances under UV light at the wavelengths of 366 nm. Ten subfractions were submitted to anti-inflammatory bioguided assay using NF-κB activation to select the most bioactive one subfraction to follow to the biological refinement assays. The most bioactive one was the subfraction S8 and was submitted to liquid chromatography coupled with high-resolution mass spectrometry analysis (LC-ESI-QTOF-MS). The subfractionation yield and process are shown on Supplementary material Figures S1 (subfracionation phase) and S2 (subfractionation yield).

2.4 Chemical composition analysis.

2.4.1 High-Resolution Mass Spectrometry analysis (LC-ESI-QTOF-MS).

The LC-ESI-QTOF-MS analysis was carried out using a chromatograph (Shimadzu Co., Tokyo) with a quaternary pump LC-20AD, photodiode array detector (PDA) SPD-20A. Reversed phase chromatography was performed using Phenomenex Luna C18 column (4.6 x 250 mm x 5 µm). High-resolution mass spectrometry MAXIS 3G – Bruker Daltonics (Bruker Daltonics, Bremen, Germany) was fitted with a Z-electrospray (ESI) interface operating in negative ion mode with a nominal resolution of 60,000 m/z. Twenty microliters of S8 was injected into a liquid chromatography system. The conditions follows: nebulizer at 2 Bar; dry gas at 8 L/min; temperature at 200 ºC and HV at 4500 V. The mobile phase consisted of two solvents: water/formic acid (99.75/0.25, v/v) (A) and acetonitrile/formic acid/water (80/0.25/19.75, v/v) (B). The flow rate was 1 mL/min, and the gradient was initiated with 10 % solvent B, increasing to 30 % B (20 min), 50 % B (32 min), 95 % B (38 min), 95 % B (60 min), and decreasing to 10 % B (75 min). An external calibration was carried out using the

software MAXIS 3G – Bruker Daltonics 4.3 to check for mass precision and data analysis. The identification of compounds was performed by comparison of exact mass, mass spectrum MS/MS and molecular formula available in the scientific literature (Soares et al., 2019).

2.5 Anti-inflammatory assays

2.5.1 Cell culture and MTT assay.

Macrophages (RAW 264.7) was cultured in endotoxin-free RPMI 1640 medium supplemented with fetal bovine serum (FBS; 10 % v/v), penicillin (100 U/mL), streptomycin sulfate (100 µg/mL) and L-glutamine (37 °C, 5 % CO_2). For the experiment, RAW 264.7 cells was cultured in 96-well plates (5 × 10^5 cells/mL); after 24h, the cells received S8 at 3, 10, 30, 100 and 300 µg /mL or medium (negative control) was added to each well and incubated for 24 h. All groups were stimulated with 10 ng/mL lipopolysaccharide (LPS), except the negative control. After this period, the supernatant was removed and MTT solution (0.3 mg/mL) was added to the wells. The plates remained incubated for 3 h (37 °C, 5% CO2). It was removed the supernatant 100 µL of DMSO were added to all wells. Plate absorbance was measured at 470 nm using an ELISA microplate reader (Lazarini et al., 2018). The effect of subfractions on cytotoxicity in RAW 264.7 cells is available in Supplementary material Figure S3.

2.5.2 Nuclear factor-κB activation and cytokines levels.

Macrophage cells transfected were cultured in 24-well plates (3 × 10^5 cells/mL). The cells were treated with S8 at 10, 30 and 100 µg/mL for 30 min before LPS stimulation (10 ng/mL) for 4 h, except the culture media control (negative control). After 4 h, the cell lysis buffer and 25 µL of luciferase reagent (luciferin at 0.5 mg/mL) were added to each well. Luminescence was measured using a white microplate reader (SpectraMax M3, Molecular Devices). In addition, according to the protocols by the manufacturers, it was determined TNF-α and CXCL2/MIP-2 levels using an ELISA microplate reader. The results were expressed in pg/mL (Soares et al., 2019). Effect of subfractions on NF-κB activation in RAW 264.7 cells is available in Supplementary material Figure S4.

*2.6 In vivo a*nti-inflammatory assays.

2.6.1 Animals.

It was purchased from CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil) male C57BL/6JUnib mice, SPF (specific-pathogen free), weighing between 22 and 25 g. All animals were housed *in vivarium* under humidity (40 – 60 %) and temperature (22 ± 2 °C) control in 12 h light-dark cycle, with access to food and water *ad libitum*. The animals were deprived of food for 8 h before oral administration of *En*e or F3. This study was carried out in strict accordance with the guidelines for the care and use of animals. The protocol was approved by the Institutional Ethics Committee on Animal Research of the University of Campinas (CEUA/UNICAMP, Protocol Number 4371-1, approved on 09/23/2016).

2.6.2 Inhibition of neutrophil migration.

Mice received orally (via gavage) a single dose of S8 at 3 and 10 mg/kg and the bioactive fraction (originated the S8) at 3 mg/kg was used as an internal control. The positive control received orally dexamethasone (2 mg/kg) and negative control 0.9% saline (vehicle). All animals, except the vehicle group, received carrageenan i.p. (500 µg/cavity) 1 h after the oral treatment. After 4 h, the mice were sacrificed, and their peritoneal cavity was washed and recovered in order to count the total number of leukocytes and neutrophils. The results were expressed as number of neutrophils *per* cavity. In addition, according to the protocols by the manufacturers, it was determined TNF-α and CXCL2/MIP-2 levels using an ELISA microplate reader. The results were expressed in pg/mL (Lazarini et al., 2016).

2.6.3 Intravital microscopy.

Based on results of neutrophil migration we select S8 at 3 mg/kg to test the anti-inflammatory activity by intravital microscopy. Mice were pretreated orally with S8 at 3 mg/kg 60 min prior to the injection i.p. of carrageenan (500 µg/cavity). Leukocytes rolling and adhesion were rated by intravital microscopy after 2 or 4 h of the inflammatory stimulus, as previously described (Bueno-Silva et al., 2016).

2.6.4 Adhesion (ICAM-1) protein expression by western blotting.

Mice were pretreated orally of S8 at 3 mg/kg in a single dose 1 h before i.p. administration of carrageenan (500 µg/cavity). After 4 h, the animals were sacrificed, their mesenteric tissues exposed, isolated and quantified by Bradford method. 50 µg of protein were transferred to a membrane (nitrocellulose) and the α-tubulin was ran in parallel as a molecular weight standard. It was used 5% of nonfat milk to block the membrane for 1 h at 4 °C in TBS-T. After this period, the membrane was incubated with anti-ICAM-1 (1:500) and α-tubulin (1:500) overnight at 4 ºC. The membrane were incubated with anti-mouse or anti-goat conjugated to peroxidase (1:5000) diluted in TBS-T containing 5% of nonfat milk for 1 h at room temperature. The bands of the specific antibody were visualized with chemiluminescence ECL for 60 s and exposed to documentation system (UVITEC Imaging Systems). Finally, a computer-based imaging system (ImageJ; National Institutes of Health, Bethesda, MD, USA) was utilized to measure the intensity of the optical density of the bands (Lazarini et al., 2016).

2.7 Antioxidant activity

2.7.1 Peroxil radical ($ROO^{\bullet}$).

$ROO^{\bullet}$ scavenging capacity of S8 was determined as previously described Melo et al., 2015. Briefly, 30 µL of S8 plus 60 µL of fluorescein, and 110 µL of a AAPH solution, were transferred to a microplate. The reaction was performed at 37 ºC and absorbance was measured every minute for 2 h at 485 nm (excitation) and 528 nm (emission) using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Trolox standard was used at concentrations ranging from 12.5 to 400 µM. The results were expressed as µmol/Trolox equivalents per g of extract/subfraction S8.

2.7.2 Superoxide anion ($O_2^{\bullet-}$).

The capacity of S8 to scavenge $O_2^{\bullet-}$ generated by the NADH/PMS system was determined. It was mixed in the microplate 100 µL of NADH, 50 µL of NBT, 100 µL of S8 and 50 µL of PMS. The assay was performed at 25 ºC and absorbance was measured after 5 min at 560 nm. A control was prepared replacing the sample with the buffer, and a blank was prepared for each sample dilution replacing PMS and NADH with the buffer. Absorbance was measured in a microplate reader (Molecular Devices,

LLC, Sunnyvale, CA, USA) and the results were expressed as IC_{50}, the mean quantity (µg/mL) of S8 required to quench 50 % of the superoxide radicals (Melo et al., 2015).

2.7.3 Hypochlorous acid (HOCl).

The HOCl scavenging capacity was measured by monitoring the effects of S8 on HOCl-induced oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123, with modifications. HOCl was prepared using a 1% NaOCl solution, adjusting the pH to 6.2 by adding 10% H_2SO_4 solution. The concentration of this solution, prepared in 100 mM phosphate buffer (pH 7.4), was measured at 235 nm using the molar absorption coefficient 100 M/cm. The reaction mixture contained the S8 (7.5 µg/ml), 100 mM phosphate buffer (pH 7.4), 1.25 µM DHR, and 5 µM HOCl, in a final volume of 300 µL. The assay was carried out at 37 ºC in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) and the fluorescence was measured immediately at 528 ± 20 nm (emission) and 485 ± 20 nm (excitation). The results were expressed as IC_{50} (µg/mL) of S8 (Melo et al., 2015).

2.7.4 Nitric oxide ($NO^{\bullet}$).

The nitric oxide ($NO^{\bullet}$) activity was determined using diaminofluorescein-2 (DAF-2) as a $NO^{\bullet}$ probe. Briefly, 50 µL of S8 plus 50 µL of SNP solution, 50 µL of buffer and 50 µL of DAF solution were added to the wells (96-well plate). Changes in fluorescence (excitation = 495 nm, emission = 515 nm) were measured in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) over a 120-min period at 5-min intervals. The results were expressed as IC_{50} (µg/mL) of S8 (Soares et al., 2019).

2.8 Systemic toxicity

2.8.1 Galleria mellonella model.

To test the potential toxic effects of S8, we determined the systemic acute toxicity in *Galleria mellonella* larvae model. The Larvae (200 to 300 mg) with no signs of melanization were randomly selected for each group (n = 15) received an aliquot of 10 µL of S8 (0.03, 0.1, 0.3, 1, 3 and 10 mg/kg) or control (0.9% NaCl, w/v). All treatments were injected into the hemocoel of each larva via the last left proleg using a Hamilton syringe (Hamilton, Reno, NV). The larvae were incubated at 30 °C and their survival was monitored at selected intervals for up to 72 h. Larvae with no movements upon touch were counted as dead (Soares et al., 2019).

2.9 Statistical analysis.

The results were expressed as mean ± standard deviation (SD). The data were checked for normality and the differences between groups were analyzed using one way or two-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's post-hoc tests. Survival curves of treated and untreated larvae were compared using Log-rank (Mantel-Cox) test. The results were considered significant at $p < 0.05$.

3. Results and discussion

3.1 Chemical composition analysis.

Total phenolic content and High-Resolution Mass Spectrometry analysis (LC-ESI-QTOF-MS). The chemical composition were tentatively identified by exact masses, MS/MS spectra and molecular formula. As seen in Table 1, the chemical analysis revealed the presence of quercetin-3-*O*-rhamnoside (quercitrin), vanillic acid-*O*-hexoside and coumaric acid-*O*-hexoside.

Table 1. Chemical Identification of S8 using LC-ESI-QTOF-MS technique.

Compound	Rt (min)	Molecular formula	$[M-H]^-$	MS fragments (m/z)
Quercetin-3-*O*-rhamnoside (quercitrin) I	22.2	$C_{21}H_{20}O_{11}$	447.0548	**299.9895**
Quercetin-3-*O*-rhamnoside (quercitrin) II	22.2	$C_{21}H_{20}O_{11}$	895.1163 [2M−H] -	**447.0542**, 299.9896
Quercetin-3-*O*-rhamnoside (quercitrin) III	22.4	$C_{21}H_{20}O_{11}$	447.0549	**299.9899**
Quercetin-3-*O*-rhamnoside (quercitrin) IV	22.4	$C_{21}H_{20}O_{11}$	895.1164 [2M−H] -	**447.0504**, 299.9904, 300.9976
Quercetin-3-*O*-rhamnoside (quercitrin) V	22.9	$C_{21}H_{20}O_{11}$	447.0546	**299.9897**, 300.9958
Quercetin-3-*O*-rhamnoside (quercitrin) VI	23.1	$C_{21}H_{20}O_{11}$	447.0549	**299.9899**, 300.9964, 447.0553
Quercetin-3-*O*-rhamnoside (quercitrin) VII	23.5	$C_{21}H_{20}O_{11}$	447.0549	299.9899, 300.9964, 447.0553
Quercetin-3-*O*-rhamnoside (quercitrin) VIII	23.6	$C_{21}H_{20}O_{11}$	447.0544	299,9099 (100); 447,0530 (38)
Vanillic acid-*O*-hexoside I	31.7	$C_{14}H_{18}O_9$	329.2319	**329.2305, 171.1010,** 167.1294
Vanillic acid-*O*-hexoside II	32.1	$C_{14}H_{18}O_9$	329.2316	**329.2305, 171.1010,** 167.1078
Vanillic acid-*O*-hexoside III	32.3	$C_{14}H_{18}O_9$	329.2305	**329.2305, 171.1010,** 166.8398, 108.5162
Coumaric acid-*O*-hexoside I	45.7	$C_{15}H_{18}O_8$	325.1835	**183.0107,** 163.6136, 145.2988**,** 119.0504
Coumaric acid-*O*-hexoside II	46.5	$C_{15}H_{18}O_8$	325.1831	**183.0107,** 163.6418, 145.2988, 119.0518

Rt = retention time; bold values are the main fragments; negative ionization mode.

HPLC-DAD-ESI-MS analysis revealed the presence of eight isomers of quercetin-3-*O*-rhamnoside with precursor ion at m/z 447. The second-generation product ion spectra of the precursor ion at m/z 447.0549 [M – H]− fragmented further to the aglycone (m/z 300.9964/301) by losing a rhamnosyl unit (–146 unified atomic mass unit) (Hashim, Abas, Shaari, & Lajis et al., 2012).

The hydroxybenzoic acids identified were three isomers of vanillic acid-*O*-hexoside (m/z 329) which showed the characteristic fragmentation patterns by the loss of 162 Da (glycosidic moiety). This was originated by the deprotonated aglycones vanillic acid (m/z 167) and fragmentation gave rise to fragment ions at m/z 108 ([M−H–162–CH_3–CO_2]−) (Fischer, Carle & Kammerer et al., 2011).

As for hydroxycinnamic acids, was observed two isomers of coumaric acid-*O*-hexoside (RT = 45.7 and 46.5) which showed a loss of the sugar moiety

[M − H − 162]− to yield coumaric acid (characteristic 163 → 119 fragmentation) (Vallverdú-Queralt, Jauregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010).

This is the first time that all phenolic compounds are identified in the subfraction (S8) of *Eugenia neonitida*. Thus, after identified the compounds present in S8, we initiate our proposal evaluating the anti-inflammatory mechanism of action by NF-κB activation and levels of TNF-α and CXCL2/MIP-2.

3.2 Anti-inflammatory activities in macrophages. As seen in Figure 1A, the S8 at 3, 10, 30 and 100 µg/mL did not affect cell viability when compared to the culture medium control ($p > 0.05$). The Figure 1B shows macrophages pretreated with S8 at 10, 30 and 100 µg/mL had significantly reduced NF-κB activation (34%, 76% and 93%, respectively) as compared to LPS-treated cells ($p < 0.05$). In addition, as seen in Figure 1 C, the S8 treatment at 30 and 100 µg/mL affect the TNF-α level compared to control LPS and also affect the release of CXCL2/MIP-2 at 100 µg/mL compared to control LPS ($p < 0.05$).

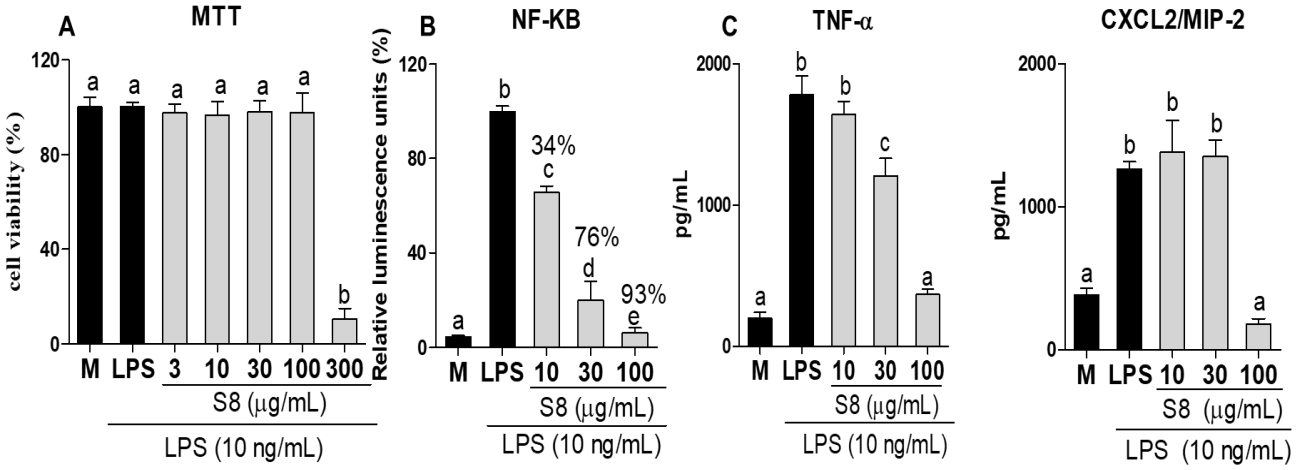

Figure 1. Macrophage viability, NF-κB activation and release of TNF-α and CXCL2/MIP-2. RAW 264.7 macrophages pretreated with culture medium (M), LPS (-; 10 ng/mL) and S8 at 3, 10, 30, 100 and 300 µg/mL. **(A)** evaluation of RAW 264.7 macrophages viability after 24h. (**B**) evaluation of NF-kB activation for 4 h. **(C)** release of TNF-α and CXCL2/MIP-2 on RAW 264.7 macrophages 4 h. The results were expressed as mean ± SD, *n* = 4. Different letters indicate statistical difference and the symbol % indicate decrease in NF- kB activation. ANOVA one-way followed by Tukey's post-hoc test, *P* < 0.05.

Macrophages are innate immune cells that produce cytokines and chemokine which in turn recruit inflammatory cells and initiate the adaptive immune responses (Gong et al., 2019). Different pattern recognition receptors (PRRs) as lipopolysaccharide (LPS) can activate the macrophages by binding in Toll-like receptors, specially 4 (TLR4) triggering intracellular signaling pathways that lead to activation of transcription nuclear factor as the NF-κB and others (Gong et al., 2019. Since activated, this pleiotropic factor promote genes encoding pro-inflammatory increasing the release of cytokines and chemokines (TNF-α and CXCL2/MIP-2 respectively), matrix metalloproteinases (MMPs), expression of selectins and integrins which are responsible for the leukocyte rolling and adhesion towards the inflammatory focus (Taniguchi, & Karin, 2018).

Our data showed that S8 inhibited the NF-κB activation and consequently the levels of TNF-α and CXCL2/MIP-2 *in vitro* that may be related to the chemical composition of S8. Since verified the S8 activity on inflammatory mediators, we next tested the anti-inflammatory activities of S8 on *in vivo* assays.

3.3 Anti-inflammatory activities in mice. The capacity of S8 inhibit neutrophil influx was evaluated *in vivo*. At the Figure 2A, mice treated with S8 at 3 and 10 mg/kg showed a significant ($p < 0.05$) decrease in neutrophil influx (48% and 52%, respectively) compared control group. In addition, mice pretreated with dexamethasone, a positive control, also showed a significant ($p < 0.05$) decrease (55%) in neutrophil migration. Interestingly, there was no statistical difference in the neutrophil influx of mice treated with S8 (both doses) and dexamethasone, a gold-standard corticosteroid widely used in medicine and dentistry ($p > 0.05$).

As seen in Figure 2B and C, S8 reduced the release of TNF-α and CXCL2/MIP-2 levels in the peritoneal cavity on mice at 3 and 10 mg/kg as compared to each control group ($p < 0.05$).

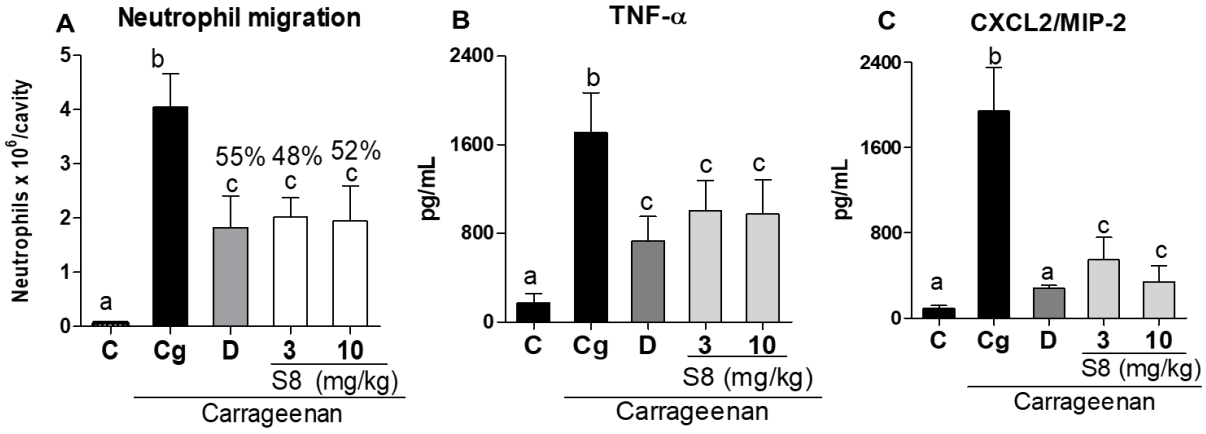

Figure 2. S8 inhibit neutrophil migration and cytokines release *in vivo.* Effects of vehicle (C), carrageenan (Cg), dexamethasone (D; 2 mg/kg), S8 (3 and 10 mg/kg) on neutrophil migration into the peritoneal cavity of mice induced by i.p. administration of carrageenan (500 µg/cavity). **(A)** Effects of the treatments on neutrophil migration into the peritoneal cavity of mice induced by i.p. administration of carrageenan. **(B)** Effects of the treatments on the release of TNF-α (1.5 h) in mice. **(C)** Effects of the treatments on the release of CXCL2/MIP-2 (3 h) in mice. The results were expressed as mean ± SD, *n* = 5-6. Different letters indicate statistical difference and all groups were compared to each other. ANOVA one-way followed by Tukey's post-test, $P < 0.05$.

Cytokines and chemokine are predominantly released by macrophages. TNF-α is an important cytokine transmit information from one cell to another becoming a key player in important biological process such as immunity, cell growth, cell migration, and others (Peres et al., 2016; Udalova, Monaco, Nanchahal, & Feldmann, 2016). Chemokines, as CXCL2/MIP-2, represent a large family of chemotactic factors important to promote inflammation since that chemokines attract neutrophils to inflammatory focus and stimulate the expression of selectins (P and E-selectin) and integrins (V and I-CAM1) on endothelial cells leading to increase of leukocyte rolling and adhesion (Peres et al., 2016). In our study, we demonstrated *in vivo* that S8 decreased significantly the TNF-α and CXCL2/MIP-2 release. Donder et al (2018) founded that rats chronically treated with quercetrin, a flavonoid also identified in S8 composition, decreased TNF-α levels and exerted a protective effect in colitis-induced.

In another study using colitis *in vivo* model, the authors founded that quercetrin decreased the macrophages and neutrophils infiltrated, iNOS and NF-κB expression (Camuesco et a., 2004). Also, Comalada et al (2005) showed reduction on

colitis progression in rats due to decreased of MPO (myeloperoxidase) and iNOS levels.

It is important to notice that flavonois and other molecules as vanillic acid can suffer extensively metabolization and their metabolites once cross the circulation exerting, may produce biological effects more bioactive than precursor structures. Di gesso et al (2015) showed that a metabolite of vanillic acid (vanillic acid glucuronide) reduced significantly the release of TNF- α on THP-1 human cells.

Differently of macrophages, the neutrophils are the most abundant cells on innate immune system representing a crucial role in acute and chronic inflammation. Neutrophils also has the capacity to phagocyte and be internalized by diapedese to the inflammatory focus (Peres et al., 2016). During the phagocytosis, neutrophil release ROS/RNS such as superoxide radical $O2^{-}$, hydrogen peroxide (H_2O_2), hypochlorous (HOCl), nitric oxide (NO) and others.

The increase of oxidative stress can modulate numerous redox-sensitive transcription factors including NF-κB and activator protein 1 (AP-1) which in an uncontrolled way increase the inflammatory mediators leading to DNA and tissue damage. Studies have shown that the exacerbated presence of neutrophils contribute to tissue damage due to the presence of ROS/RNS and inflammation resulting in the onset of several conditions such as diabetes, alzheimer, cardiovascular diseases, cancers and others (Luster, Alon & Andrian., 2005; Muñoz, & Costa 2013; Glennon-Alty, Hackett, Chapman, & Wright, 2018).

The discovery of novel drugs and strategies that control the exacerbated leukocytes influx to the inflammatory focus has been a strong effort. Thus, the modulation of S8 at the transcription factors as NF-κB, cytokines and chemokines levels, neutrophil migration, it can be a promising strategy to control the inflammatory process. In order to prove in real-time whether S8 decrease the leukocyte rolling and adhesion at the endothelial cells, we use the intravital microscopy assay and analyzed the ICAM-1 expression.

3.4 Intravital microscopy and ICAM-1 expression. The S8 at 3 mg/kg decreased significantly the leukocyte rolling (Figure 3A) and adhesion (Figure 3B) molecules in mesenteric microcirculation of mice after carrageenan injection. We also prove mechanistically that S8 reduced significantly the I-CAM1 expression in endothelial cells.

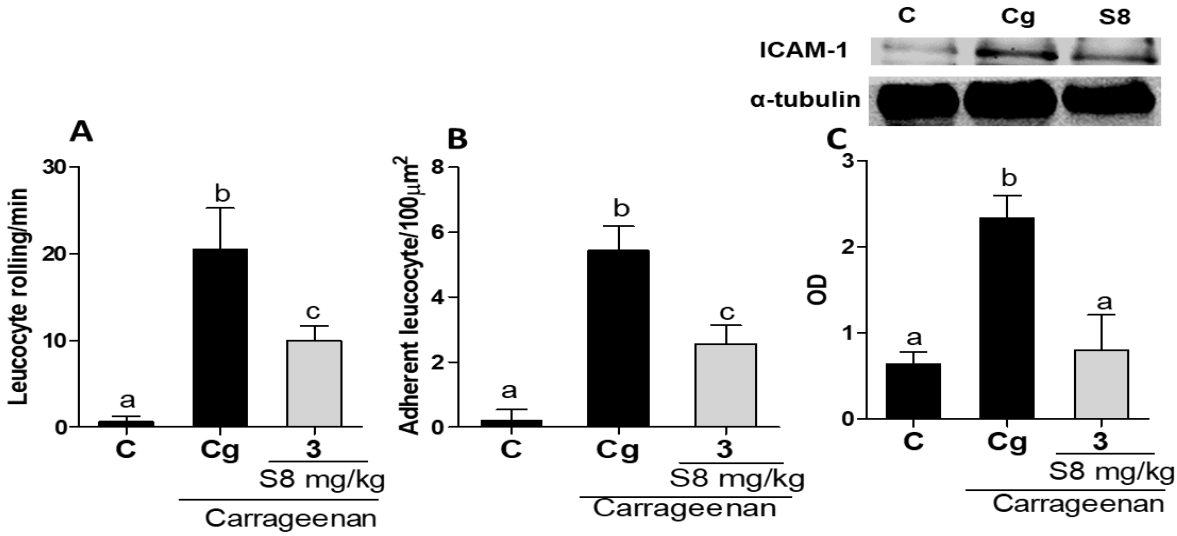

Figure 3. S8 reduced rolling and adhesion of leukocytes by intravital microscopy and ICAM-1 expression. **(A and B)** Mice were pretreated with vehicle (C) or S8 at 3 mg/kg 60 min before carrageenan (Cg; 500 µg/cavity) injection and leucocytes were counted by rolling and adhesion at the vessel. **(C)** Expression of I-CAM1 on endothelial cell of the mice 3.5 h after i.p. injection of carrageenan.The optical densities of the ICAM-1 bands were normalized to those of the α-tubulin bands and are represented as arbitrary units of an optical density ratio. The results were expressed as mean ± SD, n = 6. Different letters indicate statistical difference. One-way ANOVA followed by Tukey's post-test, $p < 0.05$.

Integrin as adhesion molecule ICAM-1 is expressed constitutively in low levels on vascular endothelial cells, some lymphocytes and monocytes. Cytokines released by macrophages can stimulate endothelial cells to express the ICAM-1 in its surface increasing the firm adhesion of leukocytes on vascular endothelium and subsequent the leucocyte recruitment (Peres et al., 2016).

Taken together, the S8 reduced the NF-κB activation leading reduction of TNF-α and CXCL2/MIP-2 levels, neutrophil rolling and adhesion, and expression of ICAM-1.

Finally, we evaluated the of S8 to deactivate the ROS/RNS formation.

3.6 *Antioxidant activity of S8 on peroxil radical (ROO•), superoxide anion ($O_2^{\bullet-}$), hypochlorous acid (HOCl)* and *nitric oxide (NO•).* The radicals HOCL and NO• are expressed as IC_{50} that is the amount required to deactivate 50 % of these radicals.

As seen in Table 2, S8 showed activity against the reactive specie, as follows: 180 ± 0.01 µmol TE/g S8 (ROO•), 2.43 ± 0.15 µg/mL (HOCl) and 3.9 ± 0.15

µg/mL (NO•). However, it was no possible to determine the IC_{50} for superoxide anion (O2•-).

Table 2. Antioxidant activity of S8 on peroxil radical (ROO•), superoxide anion (O2•-), hypochlorous acid (HOCl) and nitric oxide (NO•).

Sample	ROO•	$O_2^{\bullet-}$	HOCl	NO•
	µmol TE/g S8	µg/mL	µg/mL	µg/mL
S8	180 ± 0.01	n.a.	2.43 ± 0.15	3.9 ± 0.15

ROO• is expressed as µmol TROLOX/mg extract, n.a. = no activity was found within the tested concentration range (14 pg/mL to 500 µg/mL); HOCL and NO• are expressed as IC_{50} (µg/mL); mean ± SD; n=3.

The antioxidant scavenging of ROS/RNS is a defense mechanism primarily due to several systems as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. However, when this balance system fails there are an increase of ROS/RNS production that beyond the detoxification capacity of the cells becoming in lipid peroxidation, damage in DNA, protein, and tissue (Kim et al., 2013; Hegazi et al., 2019). In inflammatory context, ROS stimulate the activation of neutrophils and macrophages cells increasing the release of inflammatory mediators (cytokines and nuclear factors) that consequently leads to tissue injury and this process can became in a feedback loops (Hegazi et al., 2019).

Coupled to anti-inflammatory activity, S8 showed ROS/RNS scavenging capacity against radicals ROO•, HOCl and NO• that can be related to the chemical composition. Comparing our results to another Brazilian native fruit, S8 showed higher activity for HOCl and NO• radicals when compared to the *E. stipitata* crude extract (14.64 and 6.95 µg/mL respectively) (Soares et al., 2019). In summary, S8 showed interesting anti-inflammatory activity reducing inflammatory precursors leading to reduction of neutrophil influx and additionally, showed higher activity against radicals ROO•, HOCl and NO•. For this purpose, it has increasing an interest for therapeutic use of compounds, for instance in S8 that act against ROS/RNS (antioxidants), to treat diseases associated with oxidative stress.

Finally, to verify whether S8 induce systemic toxicity effect, we carried out an assay using *G. mellonella* larvae. As seen in Figure 6, the treatment systemically with S8 did not exert toxic effects on the larvae up to 10 mg/kg.

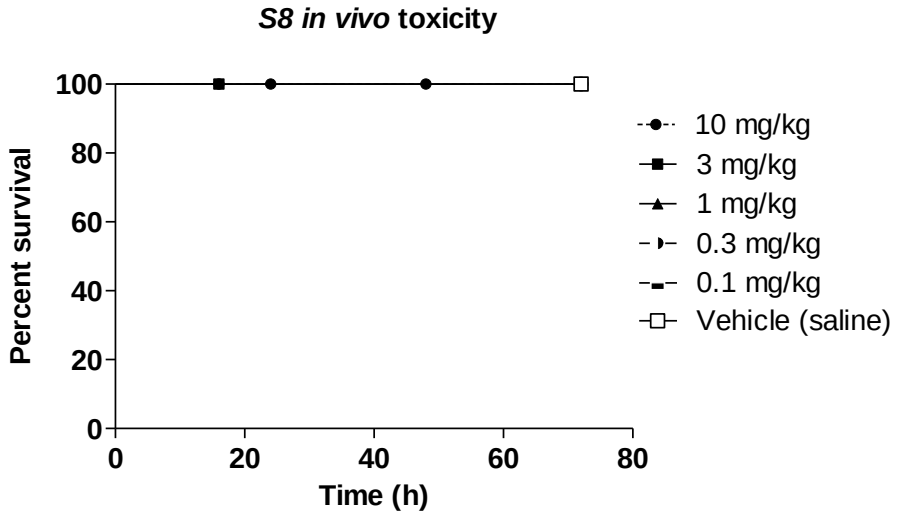

Figure 6. Effect of S8 on systemic toxicity in *Galleria mellonella* larvae. The larvae were treated with S8 at 10, 3, 1, 0.3 and 0.1 mg/kg or vehicle (saline) and their survival was recorded over 72 h ($p > 0.05$, Log-rank test).

This widely model used to the preliminary assessment of toxicity is advantageous for its low-cost, rapid results and most importantly reducing the numbers of animals due to results correlation of mammals (vertebrates) including ROS production, phagocytic hemocytes and others (Rochelle et al., 2016; Malmquist, Rogan, & McGillivray., 2019).

In our study, the maximum dose used in larvae, 10 mg/kg, which also correspond a highest dose used in mice assays, did not induce a toxic effect. In a study with the fruit extract of *Eugenia stipitata (*Brazilian native fruit belonging to the same genus of *E. neonitida),* the authors evaluated the toxic profile in G. *mellonella* model. Similarly, the *E. stipitata* did not induce toxic effects in any dose tested and this fact can be related to the presence of quercetin (without the Orhamnoside molecule portion) in the extract. Thus, this important data may indicate a potentially safe use of this purified subfraction of *E. neonitida,* however, other clinically relevant toxicity models must be considered to validate these findings.

In summary, the non-toxic S8 inhibited neutrophil migration by reducing NF-κB activation leading to decrease of cytokines levels, expression of ICAM-1 and ROS/RNS scavenging capacity due to the presence of polyphenols compounds. Based on the comprehensive review published by Chang, Alasalvar & Shahidi (2018), *E. neonitida* can be classified as superfruit since to meet the requirements acting as antioxidant and anti-inflammatory. *E. neonitida* has gained attention as a promising

source of bioactive compounds suggesting that this native superfruit can be consumed providing benefits for human health (functional foods).

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Artigo 2

2.2.1 Supplementary material

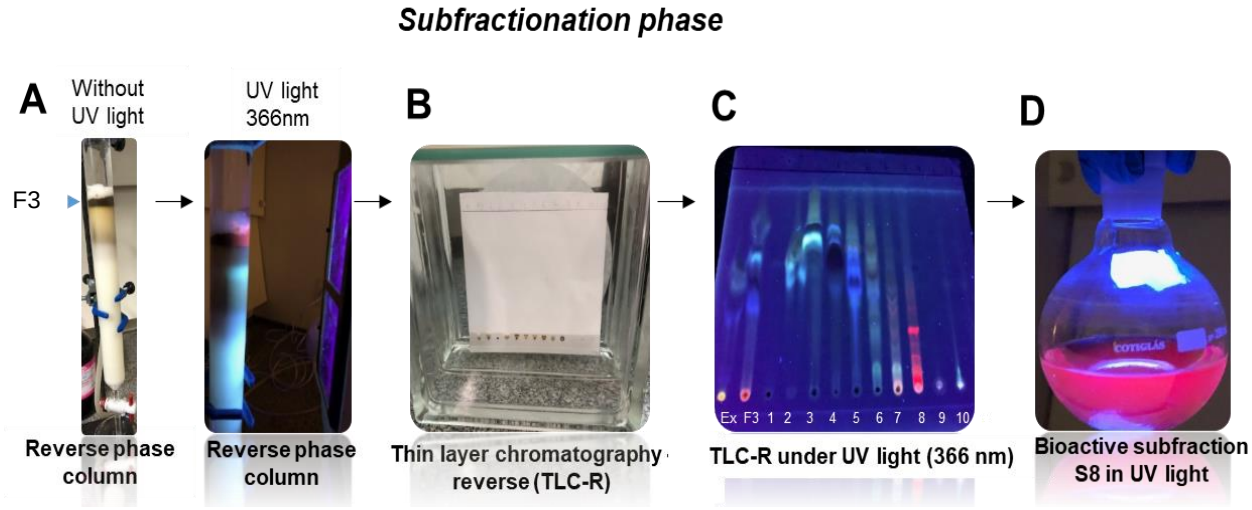
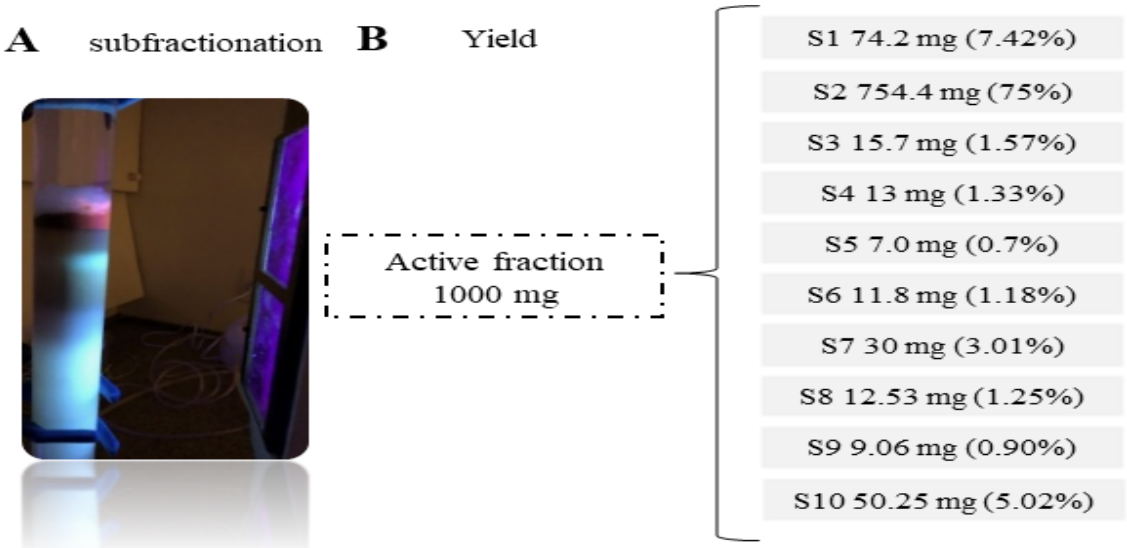


Figure S1: Subfracionation process. Eluition of *E. neonitida* fraction (derivated F3) in reverse silica column without and with UV light (366 nm) **(A).** Thin layer cromatography reverse (TLC-R) with total subfractions **(B)**. TLC-R with fractions S1 to S10 under UV light **(C).** Visual aspect f S8 in a large flat bottom under UV light **(D)**.

2.2.2 Supplementary material

Subfractionation yield



FigureS2: Subfractionation yield. Eluition of *E. neonitida* fraction (F3) in reverse silica column under UV light (366 nm) **(A).** Exact mass value (mg) of each subfraction obtained (originated from 1000 mg of bioactive fraction) **(B)**.

2.2.3 Supplementary material

Effect of subfractions on cytotoxicity in RAW 264.7 cells

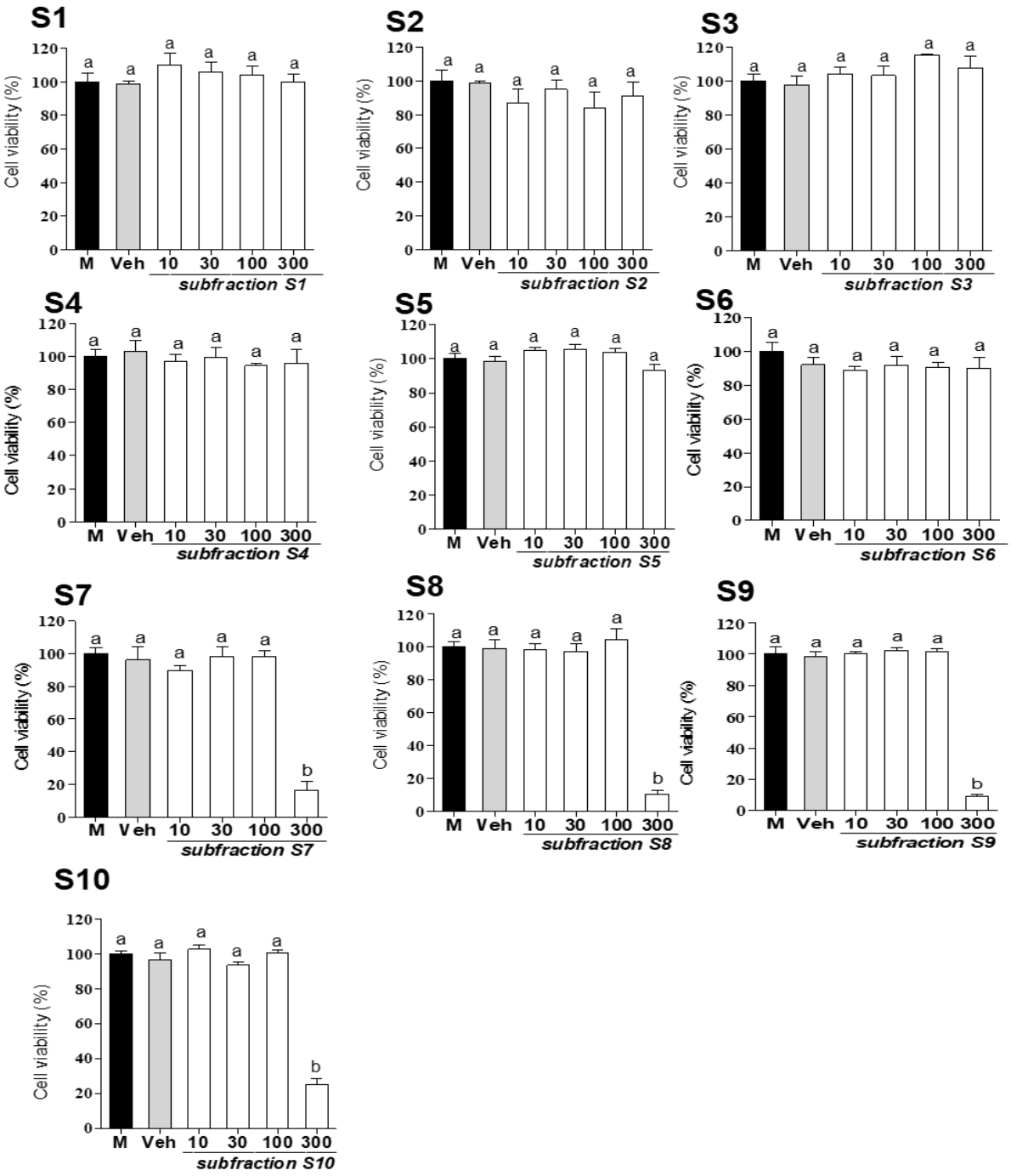


Figure S3. Effect of subfractions on cytotoxicity in RAW 264.7 cells. To assess cytotoxicity (MTT), macrophages were treated with medium RPMI (M) and subfractions S1-A10 at 3, 10, 30, 100 and 300 µg/mL for 24h. The results were expressed as mean ± SD, n = 4-6. All groups were compared to each other and different letters indicate statistical difference. One-way ANOVA followed by Tukey's post-test, $p < 0.05$.

2.2.4 Supplementary material

Effect of subfractions on NF-κB activation in RAW 264.7 cells.

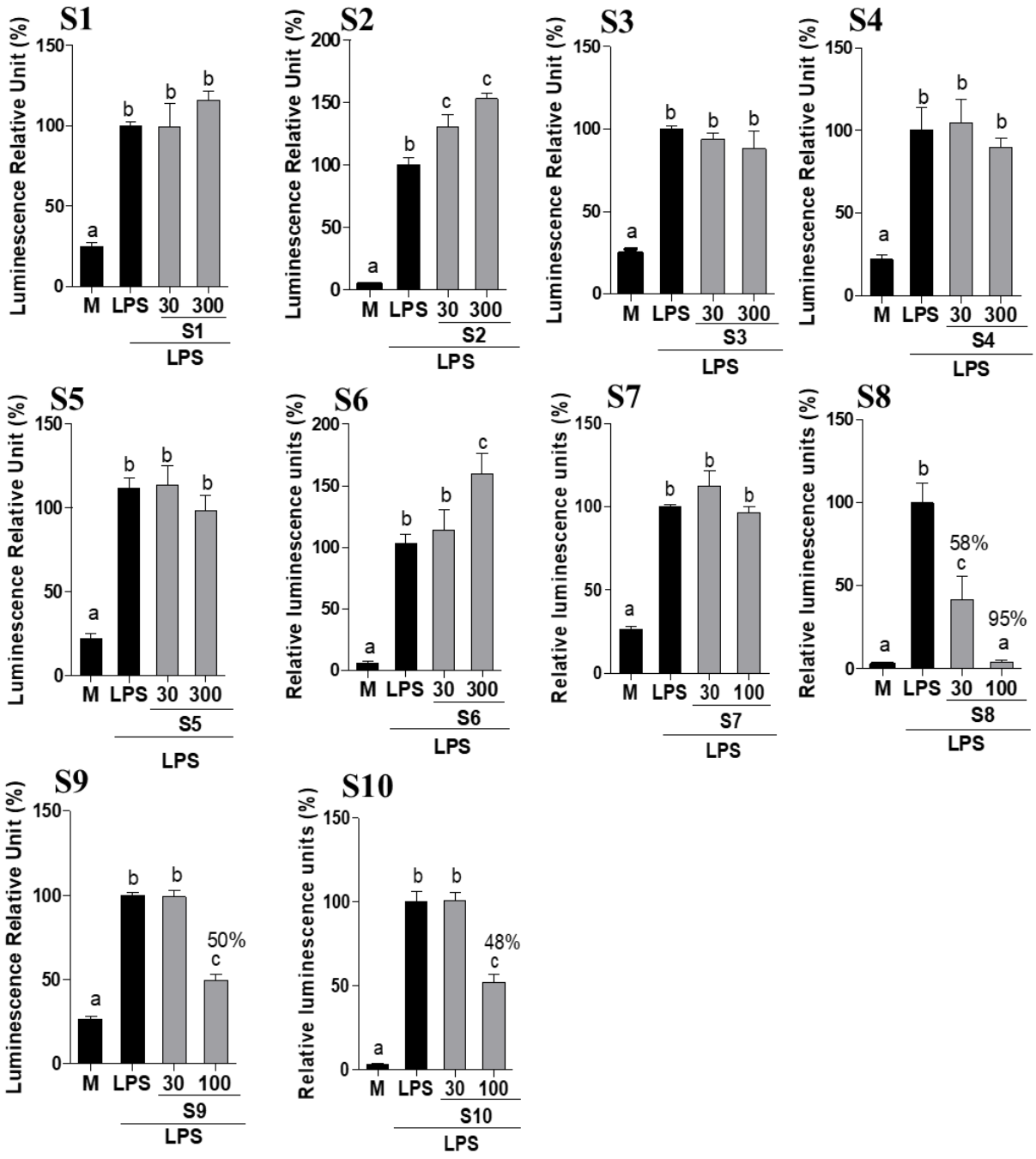


Figure S4. Effect of subfractions on NF-κB activation in RAW 264.7 cells. Macrophages were treated with medium RPMI (M), subfractions S1-S10 at 10, 30, 100 or 300 µg/mL and stimulated with LPS (10 ng/ml) for 4h. The results were expressed as mean ± SD, n = 4-6. All groups were compared to each other and different letters indicate statistical difference. One-way ANOVA followed by Tukey's post-test, $p < 0.05$.

3. DISCUSSÃO

A Organização Mundial de Saúde (OMS) relaciona o baixo consumo de frutas e legumes como um dos maiores riscos de mortalidade e, segundo o relatório da OMS/FAO, estima-se que cerca de 1,7 milhão de pessoas morram por ano em todo o mundo (2,8%) devido ao baixo consumo de frutas e vegetais (World Health Organization, 2017).

Segundo o mesmo relatório, é necessário ingerir 400 g diários de frutas e legumes, excluindo tubérculo, para prevenir o surgimento de doenças crônicas como cardiovasculares, câncer, diabetes e obesidade. Além disso, a ingestão insuficiente de frutas e legumes pode ocasionar globalmente cerca de 14% de mortes por câncer gastrointestinal, 11% por doenças isquêmicas do coração e cerca de 9% por derrame (World Health Organization, 2017, Soares et al., 2019).

Nesse contexto, apesar do consumo pela população de frutos bem conhecidos como, por exemplo, manga, abacaxi, melão, maracujá, entre outros, as frutas nativas brasileiras (FNB), representam uma importante estratégia alimentar, pois além de suas riquezas nutricionais, os compostos bioativos presentes podem prevenir o surgimento de doenças crônicas. Além disso, as FNB, até o momento inexploradas, possuem um enorme potencial de utilização como alimento funcional, expandindo a agricultura familiar, protegendo o desmatamento de muitas áreas de florestas tropicais e biomas únicos, além de gerar riquezas para o país (Chang, Alasalvar, Shahidi, 2019; De Araújo et al., 2019).

Com o objetivo de agregar valor científico às FNB inexploradas, nosso grupo de pesquisa conduziu um estudo de bioprospecção bioguiado pela atividade anti-inflamatória *in vitro* (ativação de NF-κB) e *in vivo* (migração de neutrófilo induzido por carragenina) com 11 extratos de polpas. Assim, o extrato da polpa de *Eugenia neonitida* (*En*e) foi selecionado e utilizando um delineamento experimental bioguiado, prosseguimos com o fracionamento e subfracionamento, gerando a fração ativa (F3) e subfração ativa (S8), que conferiu refinamento e especificidade ao estudo.

Primeiramente, identificamos de forma tentativa a composição química dos compostos presentes no *En*e, F3 e S8. A identificação por tentativa se baseia em comparar os parâmetros químicos obtidos pelo equipamento LC-ESI-QTOF-MS (espectrômetro de massas de alta resolução) com os dados disponíveis da literatura, como, por exemplo, a relação massa/carga (*m/z*), padrão de fragmentação e massa

molecular. Assim, a análise química por tentativa revelou 16 compostos e seus derivados presentes no *En*e, 13 na F3 e 3 na S8.

Resumidamente, foram identificados na composição de *En*e ácido siringíco hexosideo, ácido sinapico O-hexosideo I, (epi)catequina, derivados de elagitaninos, ácido elágico, derivados de quercetina e Kaempferol 3-*O*-glucoside.

Na composição da F3 identificou-se o ácido gálico, ácido siríngico, ácido sinapico-*O*-hexosideo I e II, derivados de elagitaninos, apigenina-7-*O*-glucosideo, derivados de quercetina e kaempferol 3-*O*-glucoside.

Finalmente na S8 foi possível identificar três compostos sendo esses a quercetina-3-Orhamnosideo (quercitrina), ácido vanillic-*O*-hexosideo e ácido coumarico-*O*-hexosideo.

Devido ao fracionamento do *En*e, verificou-se que, a F3 concentrou mais compostos em sua composição como o ácido gálico, ácido sinapico-*O*-hexosideo II e apigenina-7-*O*-glucosideo quando comparado com o *En*e (extrato que originou a F3). Esse fato pode ser explicado devido ao processo de fracionamento que concentrou mais os compostos na F3 sendo possível identificar esses novos compostos de forma mais precisa pela técnica de LC-ESI-QTOF-MS. A mesma racional segue para a S8 na qual identificamos três classes de compostos e seus derivados (isômeros).

Após identificado a composição química das amostras, iniciamos a avaliação da atividade anti-inflamatória.

A resposta inflamatória ocorre de forma orquestrada e que envolve diferentes tipos de células como macrófagos, neutrófilos entre outros. Essa resposta ocasiona um aumento intracelular de ERO e ERN por macrófagos, que podem ativar vias intracelulares como a do NF-κB (Thulasingam et al., 2011; Taniguchi & Karin, 2018).

Uma vez ativado, o NF-κB libera dímeros denominados de p65 e p50 para o núcleo celular dos macrófagos que induzem a transcrição gênica e liberação de mediadores inflamatórios, como citocinas (IL-1β, TNF-α entre outras) e quimiocinas (CCL2, CXCL1/KC, CXCL2/MIP-2, entre outras). Esses mediadores uma vez liberados, aumentarão a expressão de proteínas na superfície das células endoteliais (selectinas e integrinas) que controlam o fluxo de neutrófilos a ser internalizado no foco inflamatório (Soares et al., 2019).

Em nosso estudo, constatamos que o *En*e, F3 e S8 reduziram *in vitro* a ativação de NF-κB e níveis da citocina TNF-α. Nos ensaios *in vivo,* as amostras

diminuiram os níveis de TNF-α e CXCL2/MIP-2 e por consequência podem diminuir a expressão de moléculas de adesão e rolamento de neutrófilos acarretando na diminuição da migração de neutrófilos. A redução da ativação do NF-κB por *En*e, F3 e S8 pode ocorrer devido a interferência de proteínas intracelulares como a fosforilação de IκBα, completo IKK quinase, subunidades p65 e p50, entre outros.

Em relação aos resultados obtidos de migração de neutrófilo em camundongos, o *En*e e F3 apresentaram atividade anti-inflamatória reduzindo o influxo de neutrófilos na cavidade peritoneal dos animais e, além disso, não diferiram estatisticamente do controle positivo dexametasona, uma monodroga padrão-ouro amplamente utilizada na medicina o tratamento de doenças crônicas. Esse fato é importante uma vez que, embora o *En*e e F3 sejam quimicamente uma mistura complexa de compostos bioativos em baixa concentração, a atividade evidenciada na redução do influxo de neutrófilos não diferiu da dexametasona, indicando a potência anti-inflamatória das amostras.

De forma similar, não houve diferença da migração de neutrófilo em camundongos tratados com S8 (3 mg/kg) e dexametasona, indicando que a S8 se manteve equipotente ao controle gold standard.

Para a comprovação por qual(is) mecanismo(s) a S8 reduziria o influxo de neutrófilos, verificamos a expressão de moléculas de adesão e rolamento por meio da microscopia intravital. Como resultado, houve redução no rolamento bem como na adesão de leucócitos e por meio da técnica de western blotting pudemos comprovar a diminuição da expressão da integrina I-CAM1 no endotélio.

A atividade anti-inflamatória verificada no *En*e, F3 e S8, pode ser relacionada com os compostos químicos identificados. Na composição do *En*e e F3, estão presentes derivados de Di-HHDP-galloyl-glucose e ácido elágico que podem inibir a ativação do NF-κB e modular o fluxo de neutrófilos durante a inflamação (Lazarini et al., 2016).

A quercetina e o kaempferol, ambos identificados no *En*e e F3, também são conhecidos por reduzir a ativação do NF-κB, liberação dos níveis de TNF-α, IL-1β, diminuir o recrutamento de leucócitos e o estresse oxidativo (Infante et al., 2016; Lazarini et al., 2016; Chekalina et al., 2018; Sharma et al., 2019).

O composto quercitrina, identificado na composição da S8, foi testado no modelo de colite *in vivo* e foi constatada redução do número de células como os macrófagos e neutrófilos no lavado tecidual dos animais, bem como modulação da via

iNOS e NF-κB (Camuesco et a., 2004). Em outro estudo utilizando o modelo de colite em ratos tratados cronicamente, a quercitrina mostrou redução na progressão da colite, diminuição dos níveis de MPO e iNOS (Comalada et al., 2005).

Apesar da resposta inflamatória ser benéfica inicialmente, a presença exacerbada de neutrófilos e outras células que produzem ERO/ERN podem contribuir para o desequilíbrio de moléculas pró e antioxidantes, gerando danos aos tecidos, DNA, proteínas e outros (Kadioglu et al., 2015).

A eliminação de ERO/ERN foi avaliada com *Ene*, F3 e S8. Nossos achados indicam que, em geral, a F3 mostrou melhor desempenho na desativação de ERO quando comparado ao *Ene*, apesar do fracionamento ter sido bioguiado pelo potencial anti-inflamatório. O *Ene* apresentou melhor atividade na redução do ânion superóxido (O2 •) comparado ao F3. Esse fato pode ser explicado devido ao sinergismo entre os compostos fenólicos presentes no extrato bruto. Infelizmente, não foi possível mensurar a capacidade de desativação do ânion superóxido quando tratado com S8.

Infante e colaboradores (2016) encontraram para o extrato da semente de *E. leitonii* IC_{50} 260 µg/ml para o radical O2• sendo o melhor resultado encontrado quando comparado com outras espécies de *Eugenia* do mesmo estudo. O *Ene* demonstrou maior potencial (IC_{50} 172 µg/ml) na desativação desse radical comparado com o *E. leitonii.* O ânion superóxido contribui para o estresse oxidativo metabólico, resultando em dano celular e instabilidade genômica, e sua inibição pode ser um alvo importante para prevenir doenças relacionadas ao estresse oxidativo (Pinho-Ribeiro et al., 2016).

Para avaliar a capacidade de desativação do radical peroxil (ROO•) utilizamos o método ORAC (capacidade de absorção de radicais de oxigênio). Esse método mede diretamente a capacidade antioxidante de quebra de cadeia hidrofílica e lipofílica contra o radical peroxil, gerado pelo AAPH, envolvendo reações de transferência de átomos de hidrogênio (Huang, Ou & Prior, 2005).

No contexto da inflamação, as ERO presentes no tecido lipídico são convertidas em radical peroxil e podem causar danos à membrana celular, neoplasia e provavelmente várias doenças inflamatórias crônicas e degenerativas (Vinholes & Vizzotto, 2017; Pinho-Ribeiro et al., 2019). Comparando os resultados, a F3 apresentou capacidade aproximadamente 3 vezes maior contra o radical peroxil do que o *Ene* seguido da S8.

A potência em absorver radicais de oxigênio (ORAC) se concentrou na F3, seguido do *En*e e por último a S8, esse fato está relacionado à composição química da F3 que pode atuar de forma sinérgica uma vez que concentrou compostos majoritariamente das classes dos flavonoides e ácidos hidroxibenzoicos. A atividade anti-inflamatória observada pela S8 não é diretamente relacionada com desativação do radical peroxil (ROO•) uma vez que o estudo foi bioguiado pela atividade anti-inflamatória e não antioxidante.

Avaliamos também a capacidade de desativar outra ERO, o ácido hipocloroso (HOCl). *En*e, F3 e S8 exibiram diferentes valores de IC_{50}. A F3 apresentou aproximadamente 16 vezes mais potencia de desativação desse radical comparado ao *En*e e 2 vezes mais comparado ao S8. Classificando a atividade de forma decrescente teremos F3 (1,13 µg/ml), S8 (2,43 µg/ml) e *En*e (16,68 µg/ml). Comparado com outros frutos nativos, *En*e, F3 e S8 foram mais ativos que os extratos da polpa de *Eugenia brasiliensis* e *Eugenia leitonii* (42 e 109 µg/mL, respectivamente) (Infante et al., 2016). De forma similar a inativação do radical peroxil, a atividade anti-inflamatória observada pela S8 não é diretamente relacionada com a desativação do ácido hipocloroso.

A última espécie reativa avaliada foi o óxido nítrico (NO•). A S8 (3,9 µg/mL) foi a mais potente na desativação da espécie reativa de nitrogênio quando comparada de forma decrescente com a F3 (5,05 µg/mL) e *En*e (11,48 µg/mL). A S8 demostrou aproximadamente 1,3 vezes mais atividade em comparação com a F3 e 3 vezes mais ativa que *En*e.

Em um estudo conduzido por Soares e colaboradores (2019), os autores investigaram a capacidade de eliminação de NO• de *Eugenia stipitata*, uma FNB pertencente ao mesmo gênero *Eugenia*, e observaram uma atividade equivalente (6,95 µg/mL) a F3 e inferior a S8. Essa diferença pode ser explicada pelo processo de fracionamento e subfracionamento que concentraram compostos, como os flavonoides, conhecidos por serem antioxidantes mais potentes que as vitaminas C e E (Aguirre et a., 2011).

Detectamos a presença de derivados da quercetina nas três amostras estudadas, entretanto, a maior atividade para a espécie NO• foi observada na S8 seguido da F3 e por último na *En*e e esse fato pode ser devido a concentração dos derivados de quercetina após o subfracionamento. O aumento da atividade anti-

inflamatória observada pela S8 pode estar relacionada com a desativação da espécie reativa de nitrogênio.

É a primeira vez que a capacidade de eliminação de ERO/ERN foi determinada para *E. neonitida,* sua fração F3 e subfração S8. No entanto, a questão que permanece é como e quanto os ERO/ERN contribuem para a resposta inflamatória.

Assim, o controle do equilíbrio entre oxidação e anti-oxidação por meio de antioxidantes exógenos obtidos da dieta (por exemplo, ingestão diária de frutas) é uma estratégia promissora para prevenir o aparecimento de doenças inflamatórias (Mittal & Siddiqui, 2014; Chang, Alasalvar & Shahidi, 2018). Esses dados ilustram o potencial da *E. neonitida* como alimento funcional para reduzir ou prevenir doenças inflamatórias e promover a saúde.

Finalmente, este é o primeiro estudo que avaliou a toxicidade sistêmica *in vivo* do *En*e sua F3 e S8 em larvas de *G. mellonella*. Este é um modelo de baixo custo, simples e validado, amplamente utilizado na literatura, principalmente porque os resultados obtidos se correlacionam com os observados em mamíferos (Freires et al., 2017). Em nosso estudo, não foi possível encontrar a dose letal (LD_{50}), mesmo utilizando mil vezes mais (10 g/kg) a dose máxima administrada em camundongos (10 mg/kg). Para a S8, verificamos que a dose máxima administrada em camundongos (10 mg/kg) também não foi tóxica para as larvas na mesma dose.

Em um estudo anterior com outros FNB utilizando extratos de sementes de *E. leitonii* e folhas de *E. brasiliensis*, os autores demostraram a LD_{50} de 1,5 g/kg para ambos os extratos usando o modelo *G. mellonella* (Sardi et al., 2017). Em outro estudo utilizando polpa de *E. brasiliensis*, os autores encontraram que a dose de 10 g/kg foi capaz de matar 10% da população das larvas comparado ao controle (Lazarini et al., 2018). Em nosso estudo, avaliamos o extrato da polpa de *E. neonitida*, pertencente ao mesmo gênero *Eugenia*, e não encontramos até 10 g/kg toxicidade, indicando maior segurança no consumo de polpa de *E. neonitida* do que em outras partes dos frutos de *Eugenia*.

Em resumo, o *En*e, F3 e S8 reduziram a ativação de NF-κB, níveis de citocinas e consequentemente o rolamento, adesão e migração de neutrófilos desativaram espécies reativas de oxigênio e nitrogênio e não induziram a toxicidade *in vivo*, e apresentaram compostos fenólicos em sua composição. *E. neonitida*, uma fruta nativa do Brasil, pouco explorada comercialmente poderia ser utilizada para

geração de emprego e renda, aumentando a qualidade de vida dos consumidores e contribuindo assim para o desenvolvimento do agronegócio e do emprego para muitas famílias.

4 CONCLUSÃO

Conclui-se que:

- Para o *Ene* e F3 identificou-se a presença de ácido hidroxibenzoico, derivados de flavanoides e elagitaninos e para S8 derivados de quercetrina, ácido vanílico e ácido cumárico;
- O *Ene*, F3 e S8 apresentaram atividade anti-inflamatória por meio da redução da migração de neutrófilos, diminuição da ativação do NF-κB e níveis de citocinas e quimiocinas.
- A S8 também reduziu o rolamento e adesão de neutrófilo confirmado por microscopia intravital e expressão de molécula de adesão por Western Blotting;
- O *Ene*, F3 e S8 exibiram atividade sequestrante de ROS/RNS (ROO •, O2•, HOCl e NO •);
- O *Ene*, F3 e S8 não induziram toxiciddae em larvas de *G. Mellonella* nas doses testadas.

A *E. neonitida* (pitangatuba), uma superfruta nativa do Brasil, é uma promissora fonte de molécula(s) bioativas(s) com potencial anti-inflamatório e antioxidante que pode favorecer setores como o agronegócio, indústrias farmacêutica e alimentícia proporcionando benefícios à saúde humana (alimentos funcionais) e também contribuindo para a conservação da biodiversidade, principalmente da Mata Atlântica brasileira.

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[1] * De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed

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ANEXO 1 – Informativo CCPG/0001/2015

UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
Pró-Reitoria de Pós-Graduação
Fone: (019)3521-4149
Fax: (019)3521-4885

PROC. Nº 01P-3736/2002

INTERESSADA : **COMISSÃO CENTRAL DE PÓS-GRADUAÇÃO (CCPG)**

ASSUNTO : NORMAS SOBRE O FORMATO E A IMPRESSÃO DE DISSERTAÇÃO E/OU TESES (INFORMAÇÃO CCPG/001/2015)

DELIBERAÇÃO CCPG-Nº 284/2015

A COMISSÃO CENTRAL DE PÓS-GRADUAÇÃO DA UNIVERSIDADE ESTADUAL DE CAMPINAS, em sessão realizada em 09/09/2015, tomou ciência e aprovou, por unanimidade, à alteração da redação da Informação CCPG/001/2015, que trata da regulamentação das normas sobre o formato das dissertações de mestrado e teses de doutorado.

Encaminhe-se às CPG's, Diretoria Acadêmica (DAC), Biblioteca Central (BC) e à Gráfica.

CCPG, 09 de setembro de 2015.

Profª. Drª. **RACHEL MENEGUELLO**
Presidente
Comissão Central de Pós-Graduação

[12] Universidade Estadual de Campinas. Pró-Reitoria de Pós-graduação. Comissão Central de Pós-Graduação. Informação CCPG 001/2015. Campinas: Unicamp; 2015 [acesso 2015 Nov 30]. Disponível em: http://www.prpg.unicamp.br/arquivosnormas/infoccpg001_2015.pdf.

ANEXO 2 – Deliberação da Congregação Nº 306/2010

UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

PROCESSO: 00-P-00000/0000

INTERESSADO: COORD. DE PÓS-GRADUAÇÃO

DELIBERAÇÃO DA CONGREGAÇÃO Nº 306/2010

A Congregação da Faculdade de Odontologia de Piracicaba, em sua 146ª Reunião Ordinária realizada em 06/10/2010, aprovou o(a) exigências mínimas para defesas de dissertação e tese em formato alternativo a vigorar aos discentes ingressantes a partir do ano de 2011, sendo de um artigo submetido para publicação em revista científica indexada na base Lilacs ou superior para defesa de dissertação de mestrado e um artigo submetido para publicação em revista científica indexada na base Medline ou superior para defesa de tese de doutorado.

Piracicaba, 06 de outubro de 2010.

Jacks Jorge Junior
Diretor

ANEXO 3: Certificado de aprovação do Comitê de Ética no Uso de Animais

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que a proposta intitulada **Bioprospecção da atividade anti-inflamatória de frutas nativas brasileiras inexploradas em camundongos**, registrada com o nº **4371-1**, sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen e Josy Goldoni Lazarini**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA)**, **tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP**, em **23 de setembro de 2016**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**01/10/2016-01/10/2019**
Vigência da autorização para manipulação animal:	**01/10/2016-01/10/2019**
Espécie / linhagem/ raça:	**Camundongo isogênico / C57BL/6J**
No. de animais:	**252**
Peso / Idade:	**04 semanas / 20g**
Sexo:	**machos**
Origem:	**CEMIB/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

Campinas, 23 de setembro de 2016.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

ANEXO 4 – Comprovante autorização do Conselho de Gestão do Patrimônio Genético (CGEN)

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AD4B64F

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	AD4B64F
Usuário:	UNICAMP
CPF/CNPJ:	46.068.425/0001-33
Objeto do Acesso:	Patrimônio Genético
Finalidade do Acesso:	Pesquisa

Espécie
Eugenia stipitata
Byrsonima lancifolia
Jacaratia spinosa
Spondias mombin
Byrsonima arthropoda
Acnistus arborescens
Campomanesia phaea
Sageretia elegans
Eugenia selloi
Rubus rosaefolius
Solanum alternatopinnatum
Eugenia leitonii
Eugenia myrcianthes

ANEXO 4 – Comprovante autorização do Conselho de Gestão do Patrimônio Genético (CGEN) - continuação

Eugenia brasiliensis

Garcinia brasiliensis

Eugenia involucrata

Título da Atividade:	**Fitoquímica e avaliação de atividades biológicas (antioxidante, anti-inflamatória, antimicrobiana e outras) de frutas nativas brasileiras inexploradas**

Equipe

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Parceiras Nacionais

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Resultados Obtidos

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ANEXO 5 – Comprovante de submissão do artigo à revista científica internacional

PLOS ONE: Notification of co-authorship on manuscript - [EMID:520eaa28e41f589b]
PLOS ONE <em@editorialmanager.com>
Ter, 04/02/2020 18:10
Para:
• Josy Goldoni Lazarini <josy662@hotmail.com>
PONE-D-20-03291
Anti-inflammatory and antioxidant potential, toxicity, and polyphenolic composition of (pitangatuba), an unexplored Brazilian native fruit.
Dr. Pedro Rosalen

Dear Josy Lazarini,

You are receiving this email because you have been listed as an author on a manuscript recently submitted to PLOS ONE, which is entitled "Anti-inflammatory and antioxidant potential, toxicity, and polyphenolic composition of (pitangatuba), an unexplored Brazilian native fruit.".

The corresponding author for the submission process is: Dr. Pedro Rosalen
The full author list for the submission is: Josy Goldoni Lazarini; Marcelo Franchin; Jackeline Cintra Soares; Bruno Dias Nani; Adna Prado Massarioli; Severino Matias de Alencar; Pedro Rosalen

You are not required to confirm your co-authorship of this submission, but if you would like to add an ORCID iD please click the link below to confirm co-authorship and link your ORCID iD.
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Kind regards,
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ANEXO 6 – Comprovante de divulgação da pesquisa em mídias sociais

A pesquisa com as frutas nativas brasileiras repercutiram na mídia social com entrevistas em programas de rádios, sites e televisão. Abaixo disponibilizamos links para acesso.

Reportagens em Televisão

a) Mata Atlântica possui *berries* antioxidantes. **TV BANDEIRANTES,** 10/10/2017. Disponível em: http://noticias.band.uol.com.br/jornaldaband/videos/ultimos-videos/16329629/mata-atlantica-possui-berries-antioxidantes.html. Acessado em 23/11/2019.

b) Frutas brasileiras pouco conhecidas têm poder anti-inflamatório e antioxidante. **TV RECORD,** 09/10/2017. Disponível em: https://noticias.r7.com/fala-brasil/videos/frutas-brasileiras-pouco-conhecidas-tem-poder-anti-inflamatorio-e-antioxidante-09102017. Acessado em23/11/2019.

c) Pesquisadores da Unicamp e Esalq descobrem 'superfrutas' nativas da mata atlântica. **TV GLOBO EPTV Campinas e região**, 29/11/2017. Disponível em: http://g1.globo.com/sp/campinas-regiao/jornal-da-eptv/videos/t/edicoes/v/pesquisadores-da-unicamp-e-esalq-descobrem-superfrutas-nativas-da-mata-atlantica/6322222/. Acessado em 23/11/2019

d) Fernando Gabeira: as frutas nativas da Mata Atlântica. **GLOBONEWS: PROGRAMA FERNANDO GABEIRA,** 29/10/2017**.** Disponível em: https://globosatplay.globo.com/globonews/v/6252197/ ou http://g1.globo.com/globo-news/fernando-gabeira/videos/v/fernando-gabeira-as-frutas-nativas-da-mata-atlantica/6252006/. Acessado em23/11/2019.

e) Frutas menos conhecidas mostram-se poderosas e benéficas para a saúde. **TV GLOBO: PROGRAMA BEM ESTAR.** 21/12/2017. Disponível em: https://globoplay.globo.com/v/6371806/). Acessado em 23/11/2019.

ANEXO 6 – Comprovantes de divulgação da pesquisa em mídias sociais - Continuação

Reportagens em sites

a) Frutas pouco conhecidas têm alto poder anti-inflamatório e antioxidante. **Agência FAPESP,** 02/10/2017. Disponível em: http://agencia.fapesp.br/frutas_pouco_conhecidas_tem_alto_poder_antiinflamatorio_e_antioxidante/26295/. Acessado em 23/11/2019.

b) Frutas pouco conhecidas têm alto poder anti-inflamatório e antioxidante. **CNPq,** 02/10/2017. Disponível em: http://cnpq.br/web/guest/noticiasviews/-/journal_content/56_INSTANCE_a6MO/10157/5851642. Acessado em 23/11/2019.

c) Estudos atestam potencial bioativo de frutas nativas. **Faculdade de Odontologia de Piracicaba,** 16/11/2015. Disponível em: https://www.fop.unicamp.br/index.php/pt-br/portal-departamentos/morfologia-main/fop/1270-potencial-bioativo-de-frutas-nativas). Acessado em 23/11/2019.

Reportagens em rádios

a) Conheça as 'superfrutas' encontradas na Mata Atlântica que pesquisadores tentam salvar da extinção. **BBC Brasil,** 08/11/2017. Camilla Costa. Disponível em: http://www.bbc.com/portuguese/brasil-41904036#. Acessado em 23/11/2019.

b) Pesquisa mostra que cinco frutas da Mata Atlântica têm elevadas propriedades antioxidantes e anti-inflamatórias. **CBN Nacional,** 07/10/2017. Júlia Faria. Disponível em: http://cbn.globoradio.globo.com/media/audio/127173/pesquisa-mostra-que-cinco-frutas-da-mata-atlantica.htm. Acessado em 23/11/2019.

ANEXO 7 – Comprovantes de premiações em congressos científicos

CERTIFICADO

Certificamos que o trabalho intitulado "**Antioxidant and anti-inflammatory activities from super brazilian native fruit**", de autoria de *Josy Goldoni Lazarini*; Jackeline Cintra Soares; Bruno Dias Nani; Marcelo Franchin; Adna Prado Massarioli; Severino Matias de Alencar; Pedro Luiz Rosalen*, foi **Vencedor na categoria PAINEL, área BÁSICA,** no XIII Seminário de Pós-Graduação, realizado nos dias 26 e 27 de Abril de 2018, na Faculdade de Odontologia de Piracicaba - UNICAMP.

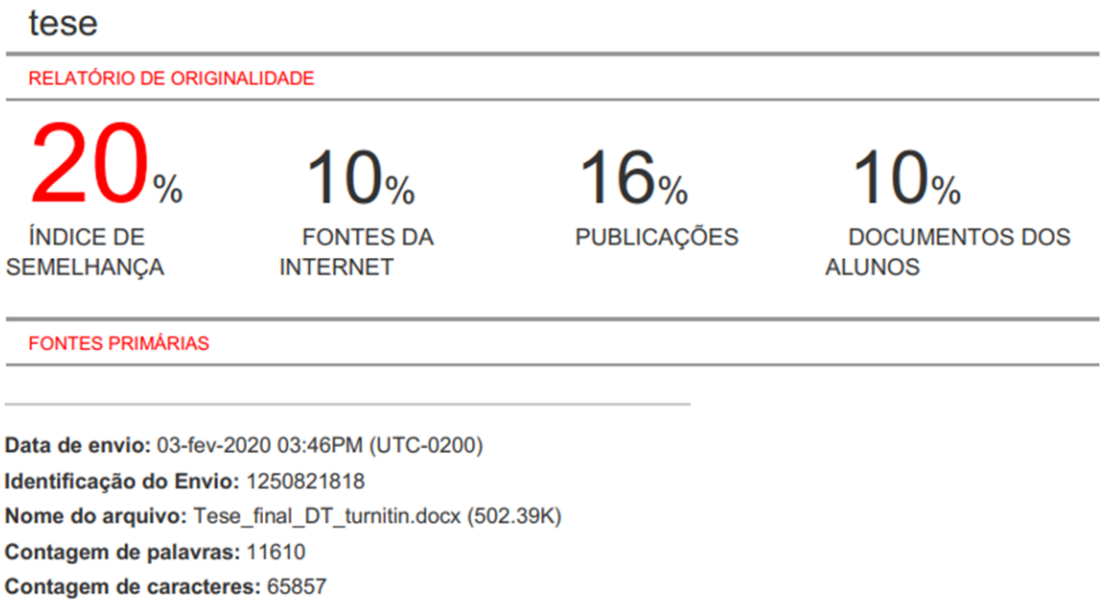
Piracicaba, 27 de Abril de 2018

Cínthia Machado Tabchoury

Profa. Dra. Cínthia Pereira Machado Tabchoury
Coordenadora do Seminário de Pós-Graduação FOP/Unicamp

ANEXO 7 – Comprovantes de premiação em congressos científicos - continuação

ANEXO 8 – Relatório de originalidade do sistema Turnitin



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