UNIVERSIADE VILA VELHA – ES

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

COMPOSIÇÃO QUÍMICA E ATIVIDADE ANTI-INFLAMATÓRIA DO ÓLEO ESSENCIAL E DO EXTRATO ETANÓLICO DAS FOLHAS DE *Campomanesia phaea* (O. Berg.) Landrum

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Dissertação apresentada a Universidade Vila Velha, como pré-requisito do Programa de Pós-graduação em Ciências Farmacêuticas, para a obtenção grau de Mestra em Ciências Farmacêuticas

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Aprovada em 3 de outubro de 2019,

Banca Examinadora:

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Prof. (a). Dr. (a). Girlandia Alexandre Brasil (UVV)

Prof. Dr. Marcio Fronza (UVV)

Orientador

Dedico esse trabalho à presença de Deus na minha vida, na forma da minha família e meus amigos.

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LISTA DE ABREVIATURAS

ABTS - 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

ATCC - American Type Culture Collection

COX - Ciclooxigenase

DMSO - Dimethyl Sulfoxide

SD - Standard Deviation

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

ELISA - Enzyme Linked Immuno Sorbent Assay

EO - Essential oil of the leaves of *Campomanesia phaea*

EXT - Ethanolic extract of the leaves of *Campomanesia phaea*

FRAP - Ferric Reducing Antioxidant Power

GC-FID - Gas Chromatography – Flame Ionization Detector

GC-MS - Gas Chromatography – Mass Spectometry

IC50 - Half maximal inhibitory concentration

IL-6 - Interleukin 6

LPS - Lipopolysaccharide

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NBT - Nitroblue Tetrazolium Chloride

NF-κB - Nuclear Transcription Factor Kappa B

NO - Nitric Oxide

EO – Essential Oil

TNFα - Tumor Necrosis Factor Aplha

RESUMO

LORENÇONI, Mariane Fioroti, M. Sc, Universidade Vila Velha - ES, outubro de 2019. **Composição química e atividade anti-inflamatória do óleo essencial e do extrato etanólico das folhas de *Campomanesia phaea* (O. Berg.) Landrum.** Orientador: Marcio Fronza.

O uso terapêutico das plantas medicinais na saúde humana constitui-se como prática milenar, historicamente construída na sabedoria do senso comum que articula cultura e saúde. A utilização de tais práticas no Brasil, o qual possui uma das maiores diversidades vegetal do mundo, poderia trazer benefícios como: diminuição nos gastos com medicamentos, maior adesão aos tratamentos, valorização da cultura e, no que se refere ao uso de plantas medicinais e medicamentos fitoterápicos contribuições para a validação científica das espécies. Considerando tais aspectos, este estudo teve como objetivo investigar o perfil fitoquímico e o potencial farmacoterapêutico do óleo essencial (EO) e do extrato etanólico (EXT) das folhas de *Campomanesia phaea* em relação aos efeitos antioxidante e anti-inflamatório utilizando métodos químicos e bioensaios *in vitro* em cultura de células. Técnicas de Cromatografia Gasosa e Líquida acoplada a Espectrometria de Massas foram utilizadas para identificação dos metabólitos secundários principais. A atividade antioxidante foi determinada pelos métodos químicos do sequestro do radical 2,2- 2,2-difenil-1-picril-hidrazil (DPPH), ácido 2,2'-azinobis-3-etilbenzotiazolina-6-sulfonico (ABTS) e pelo Poder Antioxidante de Redução do Ferro (FRAP), além do efeito protetor ao dano oxidativo celular causado pelo peróxido de hidrogênio (H_2O_2) em cultura de macrófagos. A atividade anti-inflamatória e imunomoduladora foi avaliada quanto à influência sobre a produção de óxido nítrico e do ânion superóxido ($O_2^{\bullet-}$), e pela quantificação das citocinas pró-inflamatórias fator de necrose tumoral (TNF alfa) e interleucina 6 (IL-6) pela técnica de Ensaio de imunoabsorção enzimática (ELISA) e pela inibição do fator nuclear kappa B (NF-κB) por quimioluminescência. Foi obtido como resultado um total de 41 compostos identificados no óleo essencial (EO), sendo o (E)-cariofileno (14%) e o óxido de cariofileno (6,9%) os compostos majoritários. No extrato etanólico (EXT) foram encontrados 20 compostos, mas apenas três foram identificados. O EO e o EXT inibiram a produção de $O_2^{\bullet-}$ (99,0% e 52,9%) na concentração de 100 µg/mL; $NO^{\bullet-}$ intracelular (50,0% e 51,9%) e citocinas pró-inflamatórias IL-6 (41,0% e 82,9 %) e TNF-α (74,7% e 87,9%) na concentração de 50 µg/mL, respectivamente. Além disso, observou-se inibição da atividade do fator nuclear kappa B (EO 36,2% e EXT 40,9%) na concentração de 20 µg/mL. É possível concluir que em conjunto, os resultados indicaram que o EO e o EXT possuem potentes atividades anti-inflamatórias e podem ter uma promessa terapêutica no manejo de condições inflamatórias agudas e crônicas.

Palavras-chaves: Inflamação, óxido nítrico, citocinas, NF-κB, atividade anti-inflamatória

ABSTRACT

LORENÇONI, Mariane Fioroti, M. Sc, University Vila Velha - ES, october 2019.
Chemical composition and anti-inflammatory activity of essential oil and ethanolic extract of *Campomanesia phaea* (O. Berg.) Landrum leaves.
Advisor: Marcio Fronza.

The therapeutic use of medicinal plants in human health is an ancient practice, historically built on the wisdom of common sense that articulates culture and health. The use of such practices in Brazil, which has one of the largest vegetal diversities in the world, could bring benefits such as: decrease in medicine expenses, greater adherence to treatments, culture valorization and, as regards the use of medicinal plants and herbal medicines contributions to the scientific validation of the species. Considering these aspects, this study aimed to investigate the phytochemical profile and pharmacotherapeutic potential of the essential oil (EO) and ethanolic extract (EXT) of Campomanesia phaea leaves in relation to the antioxidant and anti-inflammatory effects using chemical methods and bioassays. *in vitro* in cell culture.
Gas and liquid chromatography techniques coupled to mass spectrometry were used to identify the main secondary metabolites. Antioxidant activity was determined by the chemical methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical sequestration (ABTS) and Ferric Reducing Antioxidant Power (FRAP), in addition to its protective effect against cellular oxidative damage caused by hydrogen peroxide (H2O2) in macrophage culture. Antiinflammatory and immunomodulatory activity was evaluated for the influence on nitric oxide and superoxide anion (O2 • -) production, and by the quantification of proinflammatory cytokines tumor necrosis factor (TNF alpha) and interleukin 6 (IL- 6) by Enzyme Linked Immuno Sorbent Assay (ELISA) technique and inhibition of nuclear factor kappa B (NF-κB) by chemiluminescence. A total of 41 compounds identified in the essential oil (EO) were obtained, being (E) -caryophyllene (14%) and caryophyllene oxide (6.9%) the major compounds. In the ethanolic extract (EXT) 20 compounds were found, but only three were identified. EO and EXT inhibited O2 • - production (99.0% and 52.9%) at a concentration of 100 µg / mL; • intracellular NO (50.0% and 51.9%) and pro-inflammatory cytokines IL-6 (41.0% and 82.9%) and TNF-α (74.7% and 87.9%) in the concentration of 50 µg / mL respectively. In addition, inhibition of nuclear factor kappa B activity (EO 36.2% and EXT 40.9%) was observed at a concentration of 20 µg / mL. It can be concluded that together, the results indicated that EO and EXT possess potent anti-inflammatory activities and it may hold therapeutic promise in the management of acute and chronic inflammatory conditions.

Keywords: Inflammation, nitric oxide, cytokines, NF-κB, antiinflammatory activity

Artigo cientifico

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Chemical composition and anti-inflammatory activity of essential oil and ethanolic extract of *Campomanesia phaea* (O. Berg.) Landrum leaves

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Abstract

Ethnopharmacological relevance: *Campomanesia* species are used in folk medicine for anti-inflammatory, -ulcerogenic, -diabetic, -obesity, and many other purposes.
Aim of the study: This study aimed to investigate the phytochemical profile and pharmacotherapeutic potential of the essential oil (EO) and ethanolic extract (EXT) of the leaves of *Campomanesia phaea* in relation to antioxidant and anti-inflammatory effects using chemical methods and *in vitro* bioassays in cell culture.
Materials and Methods: Gas and liquid chromatography techniques coupled to mass spectrometry were used to identify the main secondary metabolites. The antioxidant activity was determined by the chemical methods of radical sequestration of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and by ferric reducing antioxidant power (FRAP); in addition to the protective effect against cellular oxidative damage caused by hydrogen peroxide (H_2O_2) in macrophage culture. The anti-inflammatory and immunomodulatory activity was evaluated for the influence on the production of nitric oxide and superoxide anion ($O_2^{\bullet-}$), and by the quantification of proinflammatory cytokines tumor necrosis factor (TNF alpha) and interleukin 6 (IL- 6) through Enzyme Linked Immuno Sorbent Assay (ELISA) technique and inhibition of nuclear factor kappa B (NF-κB) through chemiluminescence.
Results: A total of 41 compounds were identified in the essential oil (EO), being (*E*)-caryophyllene (14%) and caryophyllene oxide (6.9%) the major compounds. In the ethanolic extract (EXT), three flavonoids from the flavanones group were identified: alpinetin *O*-dideoxy-hexoside, 5,7-dimethoxyflavanone and alpinetin. The EO and EXT inhibited the production of $O_2^{\bullet-}$ (99.0% and 52.9%) at a concentration of 100 μg /mL, intracellular $NO^{\bullet-}$ (50.0% and 51.9%) and proinflammatory cytokines IL-6 (41.0% and 82.9%) and TNF-α (74.7% and 87.9%) at a concentration of 50 μg/mL, respectively. In addition, inhibition of nuclear factor kappa B (EO 36.2% and EXT 40.9%) was observed at 20 μg/mL.
Conclusions: Taken together, the results indicated that EO and EXT possess potent anti-inflammatory activities and it may hold therapeutic promise in the management of acute and chronic inflammatory conditions.

Keywords: medicinal plants; inflammation; oxidative stress; nitric oxide; cytokines; NF-κB.

Abbreviations

ABTS - 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

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EO – Essential Oil

TNFα - Tumor Necrosis Factor Aplha

1. Introduction

Medicinal plants have been the source of virtually all medicines historically. The medicinal use of plants basically arises from approaches to popular knowledge that test its therapeutic use in the treatment and prevention of numerous diseases. In this way, popular medicine has become of great importance for science and has contributed significantly to the discovery of new substances and to the selection of species to be studied (de Albuquerque et al., 2007; Harvey et al., 2015; Newman and Cragg, 2016). In this sense, Brazil stands out as a potential source of these natural products for having the greatest plant biodiversity of the planet and also because most of its plants are little explored in relation to its pharmacological potential (Valli et al., 2018).

Among the many families of plants investigated, the Myrtaceae family deserves special interest. This family is composed of approximately 4,000 species and 133 genera distributed throughout the world, including *Campomanesia* (Wilson et al., 2001), genus which comprises approximately 42 species, of which 24 are found in Brazil (Landrum, 1986; Tokairin et al., 2018; Zappi et al., 2015). The species *Campomanesia phaea* (O. Berg) Landrum, popularly known in Brazil as cambuci or cambucizeiro, stands out mainly due to the importance of its fruits as a food source and due to the medicinal properties attributed to the plant (Kawasaki and Landrum, 1997; Santos et al., 2016).

Despite its popular use, comprehensive studies on this species are rare. Previous studies have reported a wide range of total phenolic, flavonoid, and anthocyanin contents in *C. phaea* extracts when compared to diverse native fruits, and this correlated to high antioxidant capacity (Genovese et al., 2008; Haminiuk et al., 2011). Phenolic compounds from *C. phaea* fruit such as ellagic acid and glycosylated quercetin derivatives were also found to be potent inhibitors of carbohydrate-hydrolyzing enzymes (α-amylase and α-glucosidase) in *in vitro* enzymatic assays (Gonçalves et al., 2010). Phenolic-rich extract from *C. phaea* was also effective in ameliorating tolerance to glucose as well as in reducing insulinemia and fasting glycemia and improving dyslipidemia by increasing HDL-cholesterol and decreasing the LDL-cholesterol (Donado-Pestana et al., 2015).

C. phaea leaves were identified as having essential oils with a predominance of sesquiterpenes, particularly caryophyllene oxide (11.7%) and β-selinene (6.9%). For the monoterpene fraction, the oil was characterized by linalool (11.1%) (Adati and De Oliveira Ferro, 2006). However, although there are some studies on the chemical characteristics of the *C. phaea* fruit and essential oil, and preliminary studies on their pharmacological properties of *C. phaea* fruits, detailed reports on the chemical composition of the essential oil and the extracts from *C. phaea* leaves and their biological activities are still lacking.

In this context, the objective of this study was to investigate the chemical composition and biological activities of essential oil and ethanolic extract from the leaves of *Campomanesia phaea*.

2. Materials and methods

2.1 Chemical and biochemical reagents

Reagents such as 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, nitroblue tetrazolium chloride (NBT), Np-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), gallic acid, and quercetin were obtained from Sigma Aldrich Chemical Co., St. Louis, MO, USA. L-N6-(1-Iminoethyl) lysine dihydrochloride (L-NIL) was obtained from Cayman Chemical Company. Immunoenzymatic assay (ELISA) kits for detection of cytokine TNF-α and IL-6 were obtained from eBioscience, San Diego, California, USA. Dual-Luciferase® Reporter Assay System 10-Pack was obtained from Promega Corporation, USA. All other solvents and reagents used were analytical grade and were obtained from various commercial sources.

2.2 Cell lines

The cell lines used were RAW 264.7 mouse macrophages (ATCC® TIB-71 ™); L929 fibroblast (ATCC® CCL-1 ™) obtained from subcutaneous, areolar, and adipose tissues of rats (*Mus musculus*); MV3 melanoma (0284 – BCRJ) obtained from human lymph nodes (*Homo sapiens*); and human embryonic kidney (HEK) cell transfected with a luciferase-expressing gene (Panomic, Fremont, CA) (retrovirus). Cells were obtained from the *Banco de Células do Rio de Janeiro (BCRJ)* and cultured in modified Eagle's medium (Dulbecco's Sigma Aldrich® Chemical Co. St. Louis, MO, USA) or high glucose modified culture medium (DMEM-Gibco-BRL Life Biotechnologies, Grand Island, NY, USA) and supplemented with 100 IU/ml penicillin and 100 μg/mL streptomycin and 10% fetal bovine serum (FBS-Sigma Aldrich® Chemical Co., St. Louis, MO, USA) at 37°C, in a humidified atmosphere containing 5% CO_2.

2.3 Collection and identification of plant material

The leaves of *Campomanesia phaea* (O.Berg) Landrum used in the study were collected in October/2017, in Santa Leopoldina town, (20º06'02"S 40º31'47"W), Serrana region of the State of Espírito Santo, Brazil, and were properly identified by botanist Ms. Solange Schneider.

An exsiccate of the plant (nº 2625) was prepared and stored in Vila Velha University's herbarium.

2.4 Collection of essential oil and ethanolic extract

The leaves of *C. phaea* were selected and dehydrated in a greenhouse with forced air ventilation at 40ºC until constant weight. Afterwards, they were pulverized in a knife mill and then subjected to extraction processes.

The essential oil (EO) of the leaves of *C. phaea* was extracted by hydrodistillation in a Clevenger apparatus, purified by freezing process, filtered with anhydrous magnesium sulfate, and stored in a freezer at -20ºC until chemical analysis.

The extract was obtained by ultrasonic assisted maceration in three cycles of 30 minutes each using 150 mL of ethanol (98 GL) as solvent. After extraction, the solvent was removed at 55°C with the aid of Rotavapor. For complete dehydration of the extract, it was subjected to lyophilization and then stored in a freezer at -20ºC until biological tests and chemical analyses were carried out.

2.5 Chemical composition of the essential oil

Quantitative chemical analysis of the volatile oil was performed in triplicate using Agilent® 7890A gas chromatography (GC) system operated with HP GC ChemStation data processing system version A.01.14 equipped with CombiPAL Autosampler System (CTC Analytics AG, Switzerland) and a Flame Ionization Detector (FID). Thymol was used as an internal standard. Samples were prepared by adding 10 µL stock solution of the internal standard to the known masses of the EO and diluting it with 1 mL of ethyl acetate. The injection volume was 1.0 µL, in split mode with 20:1 injection ratio. HP-5MS fused silica capillary column (30-m long x 250 µm internal diameter x 0.25 µm film thickness) (California, USA) was used. Helium was used as drag gas with flow of 1.0 mL/min; the injector and detector were maintained at 250°C. The condition of analysis was of ramp heating from 60 to 240°C with heating rate of 3°C/min. The content of the eluted constituents were expressed as percentages of relative area of the peaks without response factor.

Qualitative analyses were performed using Agilent® 7890A Chromatograph coupled to Agilent® MSD 5975C mass-selective detector (Agilent Technologies, California, USA) operated by electron impact ionization at 70 eV in sweep mode at a rate of 1.0 scan/s, with a mass acquisition interval of 40-400 m/z. The operating conditions were the same as those used in GC-DIC analyses. The chemical constituents were identified by comparing their retention indices relative to the co-injection of a standard solution of n-alkanes (C_8-C_{20}) and/or by

comparing mass spectra from the NIST/EPA/NHI database (The National Institute of Standards and Technology, 2008) and literature (Adams, 2007; Ткачёв, 2008). The retention indices were calculated using the Van de Dool & Kratz equation (1963) and for the assignments, retention indices described in literature were consulted (Adams, 2007; Ткачёв, 2008).

2.6 Ethanolic extract chemical composition

The identification analyses of the main secondary metabolites in the ethanolic extract of the leaves of *C. phaea* (EXT) were carried out through high-performance liquid chromatography using a Dionex Focused Ultimate 3000 chromatograph equipped with a diode array detector (DAD) coupled to a LCQ Fleet Ion Trap ThermoFisher Scientific mass spectrometer (Waltham, MA, USA). Samples were prepared at a concentration of 2 mg/mL using methanol as solvent, chromatographic grade from Tedia (Fairfield, OH, USA). Chromatographic conditions were Ascentis Express C_{18} column (100 mm x 4.6 mm; 2.7 µm particle size) Supelco (Bellefonte, PA, USA). The mobile phase was composed of 0.1% formic acid solution (A) and methanol (B) eluted at a flow rate of 0.5 mL/min and maintained at 35°C. Elution of the mobile phase was performed in gradient mode: 0 - 50 min (10-100% B); 50 - 55 min (100% B); and 55 - 60 min (10% B). The injection volume was 3 µL. A C_{18} guard column (5 mm × 4.6 mm ID, 2.7 µm particle size) Supelco (Bellefonte, PA, USA) was used to protect the analytical column. The parameters of the mass spectrometer were as follows: voltage capillary, 36 V; nebulization, 5.5 Bar; drying gas flow, 10 L/min; drying temperature, 250°C; and collision energy, 35 eV. Signal acquisition was carried out in the positive ionization mode in m/z range from 100 to 1000 and also for MS2 of the base peaks. The results in .raw files (manufacturer's extension) were converted to .mzML (open source) using MSConvert from ProteoWizard. The chromatograms in .mzML extension were processed using MzMine, and dereplication was executed after data loading on the Global Natural Products Social Molecular Networking (GNPS) spectra platform (Wang et al., 2016).

2.7 Free radical scavenging activity

The antioxidant activities of the EO and the ethanolic extract of *C. phaea* were determined by the colorimetric methods of ABTS free-radical scavenging (Re et al., 1999), DPPH free-radical scavenging (Scherer and Godoy, 2009), and reduction of iron (FRAP) (Benzie and Strain, 1996). Gallic acid, B-caryophyllene, and quercetin were used as positive controls. The experiments were executed in triplicate on two different days at least and the results were expressed as IC_{50} (µg/mL).

2.8 *In vitro* cytotoxicity evaluation

For the cytotoxicity analysis of the essential oil and ethanolic extract of *C. phaea*, the colorimetric MTT method proposed by Mosmann (1983) was used with modifications. For this analysis, three different cell lines were used: RAW264.7 (macrophage), L929 (fibroblast), and MV3 (melanoma). Cells were plated at a density of 7×10^4 cells/mL in 96-well plates and incubated (5% CO_2 at 37°C) overnight. The cells were exposed to different sample concentrations (7.8 - 500.0 μg/mL) and doxorubicin (10 μM), which was used as a positive control. After a 24h-incubation, 100 μL of 3-(4,5-dimethylthiazol-2yl)-diphenyl tetrazolium bromide (MTT) (1 mg/mL) was added to each well and the formed formazan crystals were dissolved in DMSO. The results were expressed in percent viability and the experiments were performed in triplicate on two different days at least.

2.9 *In vitro* indirect determination of nitric oxide

Indirect quantification of nitric oxide was carried out by determining the influence on the production of nitric oxide in LPS-activated RAW 264.7 macrophage culture (Marques et al., 2019). Macrophages were plated in 96-well plates at a density of 8×10^4 cells/mL and incubated (5% CO_2 at 37°C) for 24 h. Cells were exposed to different sample concentrations (essential oil and ethanolic extract) (1.0 - 100.0 μg/mL) and stimulated with LPS (1 μg/mL). L-NIL (50 μM) and gallic acid (10 μg/mL) were used as positive controls. After 24 h the cell supernatant was used for nitrite quantification using the Griess reagent (Green et al., 1982). The absorbance was determined on a microplate reader at 540 nm. The experiments were performed in triplicate on two different days at least and the results were expressed as mean ± SD of nitrite concentration (μM) of three independent experiments.

2.10 Reduction of superoxide anion production

The potential inhibitory effects of the essential oil and ethanolic extract on superoxide radical ($O_2^{\bullet-}$) production in LPS-activated RAW 264.7 macrophages were evaluated by superoxide assay (Marques et al., 2019; Pinho et al., 2011). Macrophages were plated in 96-well plates at a density of 8×10^4 cells/mL and incubated (5% CO_2 at 37ºC) for 24 h. Cells were exposed to different sample concentrations (1.0 - 100.0 μg/mL) and stimulated with LPS (1 μg/mL). Gallic acid (100 μg/mL) and tempol (12.5 mM) were used as positive controls. After 24 h the supernatant was removed and NBT (1 mg/mL) was added to the cells. After 2 h the cells were washed with methanol and the formed formazan crystals were dissolved in KOH (2 M) and DMSO, respectively. Absorbance was quantified on a microplate reader at 630 nm. The

experiments were performed in triplicate on two different days at least and the results were expressed as mean ± SD of superoxide anion production (%) of three independent experiments.

2.11 Preventive effect against oxidative damage in RAW 264. 7 caused by H_2O_2

The protective effect of the essential oil and ethanolic extract against the oxidative damage caused by hydrogen peroxide (H_2O_2) in macrophages was analyzed through the modified hydrogen peroxide method (Adetutu et al., 2011). RAW 264.7 macrophages were plated in 96-well plates at a density of 8 × 10 4 cells/mL. After 24 h the cells were exposed to different sample concentrations (1 - 100 μg/mL) and after 30 min, 0.75 mM H_2O_2 was added. Catalase (10 IU/mL) was used as a positive control. After 2 h of incubation, cell viability was evaluated by the MTT colorimetric method. The experiments were performed in triplicate on two different days at least and the results were expressed as mean ± SD of cell viability (%) of three independent experiments.

2.12 *In vitro* cytokine determination

For cytokine quantification, macrophage culture (RAW 264.7) was plated in 96-well plates at a density of 8 × 10 4 cells/mL and incubated (5% CO_2 at 37°C) for 24 h. Cells were exposed to different sample concentrations (essential oil and ethanolic extract) (1.0 - 100.0 μg/mL) and stimulated with LPS (1 μg/mL). Gallic acid (10 μg/mL) was used as positive control. After 24 h, the cell supernatant was removed and used for quantification of IL-6 and TNF-α cytokines employing an enzyme-linked immuno sorbent assay (ELISA) kit, with specific antibodies and standards for each cytokine tested according to the manufacturer's instructions (eBioscience® and Invitrogen®). The absorbance was quantified in microplate reader at 450 and 570 nm. The experiments were performed in triplicate and the results were expressed in pg/mL.

2.13 Nuclear factor kappa B activity determination

In order to determine the activity of nuclear factor kappa B (NF-kB), the capacity of the essential oil and ethanolic extract of *C.phaea* to inhibit NF-kB through luciferase expression was evaluated (Kondratyuk et al., 2012; Marques et al., 2019). HEK 293 human embryonic kidney cells transfected with the addition of NF-κB luciferase gene were plated (1 x 10^5 cells/mL) in 96-well plates and incubated for 48 h. Afterwards, the cells were exposed to a concentration of 20 μg/mL of each sample (oil and extract) followed by the addition of TNF-α (2 ng/mL - 0.5 ng/well) and incubated for another 6 h. The luciferase assay was then performed using the Promega Luc assay kit (Madison, WI) following the manufacturer's instructions. TPCK (4 μM) was used as NF-kB inhibition control. Luciferase activity was monitored using

a microplate luminescence reader (Mults-Mode, Filter Max F5, Molecular Devices Spectra, USA). The results were expressed as percentage of the NF-kB inhibitory activity. The experiments were performed in triplicate on two different days at least. Cell viability was examined in parallel using the SRB assay under the same experimental conditions.

2.14 Sulforhodamine B (SRB)

The SRB method was used to determine cell viability (Houghton et al., 2007; Vichai and Kirtikara, 2006). In summary, HEK 293 human embryonic kidney cells were plated in 96-well plates and cultured for 48 h. After that, the samples (oil and extract) were added at a concentration of 20 μg/mL and incubated for another 6 h. Afterwards, the cells were fixed through the addition of 20% trichloroacetic acid (TCA) at 4°C for 30 min followed by addition of SRB solution (0.4% SRB in 1% acetic acid) and incubation for 30 min. The plate was then washed with 1% acetic acid and dried, and the protein-bound dye was dissolved in 10 mM Tris base solution (pH 10.0). The absorbance was measured on a spectrophotometer at 515 nm. The experiments were performed in triplicate on two different days at least and the results were expressed as percent survival.

2.15 Statistical analysis of data

Statistical analyses were performed using GraphPad Prism 5 software (San Diego, CA, USA 176). Data were presented as mean ± standard deviation (SD). Statistical comparisons were carried out using univariate analysis of variance (ANOVA) followed by Turkey's post hoc. Values of $p<0.05$ were considered significant.

3. Results

3.1 Collection and chemical composition of the essential oil

Hydrodistillation of dehydrated *C. phaea* leaves produced a slightly greenish colored oil with characteristic aroma. The essential oil content was 0.3% (w/w). According to the chemical analyses, 40 compounds were identified in the essential oil of the leaves of this species, representing 95.75% of the total chemical composition of the oil (Table 1). Sesquiterpene hydrocarbon was the predominant chemical class (70.48%), with (*E*)-caryophyllene (14%) being the major constituent. As major constituents stand out, in order of elution: linalool (5.19%), α-copaene (5.87%), *allo*-aromadendrene (5.10%), γ-muurolene

(6.06%), β-selinene (5.50%), α-selinene (6.23%), δ-cadinene (6.04%), and caryophyllene oxide (6.87%). Relative areas lower than 5% were assigned to the other oil constituents.

Table 1. Chemical composition of the essential oil of *Campomanesia phaea* (O. Berg.) Landrum leaves.

Identified Compounds	RI	RT	Area %±DP
(*Z*)-3-hexen-1-ol	849	3.85	1.07±0.02
n-hexanol	862	4.06	0.13±0.04
α-Pinene	932	5.55	1.08±0.06
β-Pinene	976	6.72	0.62±0.03
1,8-Cineol	1027	8.34	0.53±0.01
(*Z*)-β-Ocimene	1030	8.44	0.91±0.02
Linalool	1100	10.97	5.19±0.27
α Terpineol	1190	14.77	0.81±0.04
α-Cubebene	1349	21.80	1.06±0.07
α-Ylangene	1371	22.75	0.89±0.00
α-Copaene	1376	22.96	5.87±0.21
β-Elemene	1392	23.67	0.45±0.02
α-Gurjunene	1409	24.42	0.50±0.02
(*E*)-Caryophyllene	1419	24.85	14.00±0.86
β-Copaene	1429	25.25	1.67±0.05
γ-Elemene	1434	25.46	0.41±0.08
Aromadendrene	1439	25.67	4.38±0.15
NI	1443	25.89	0.29±0.05
α-Humulene	1453	26.29	3.57±0.16
allo-Aromadendrene	1461	26.60	5.10±0.31
γ-Muurolene	1477	27.29	6.06±0.21
α-Amorfene	1480	27.44	1.20±0.00
β-Selinene	1486	27.69	5.50±0.31
α-Selinene	1495	28.06	6.23±0.14
NI	1500	28.29	1.75±0.02
Germacrene A	1506	28.52	0.47±0.01
γ-Cadinene	1514	28.86	3.04±0.15
δ-Cadinene	1524	29.25	6.04±0.04
Selina-4(15),7(11)-diene	1535	29.71	0.79±0.15
α-Cadinene	1538	29.82	0.46±0.20
Selina-3,7(11)-diene	1542	29.97	1.37±0.08
α-Calacorene	1553	30.43	0.50±0.01
Germacrene B	1557	30.60	0.88±0.07
E-Nerolidol	1561	30.89	0.81±1.27
Espathulenol	1569	31.45	2.55±0.04
Caryophyllene oxide	1584	31.68	6.87±0.08
Globulol	1594	32.10	0.60±0.52
NI	1603	32.45	0.65±0.07
NI	1610	32.72	0.60±0.03
1,10-di-epi-Cubenol	1623	33.25	0.33±0.05
1-epi-Cubenol	1629	33.48	0.99±0.17

T-Cadinol	1632	33.63	0.43±0.06
Cubenol	1643	34.01	1.21±0.01
α-Cadinol	1655	34.49	1.14±0.22
NI	1671	35.12	0.37±0.31
NI	1675	35.28	0.28±0.03
NI	1717	36.89	0.34±0.01
Total aliphatic alcohols			*1.20*
Total monoterpenes hydrocarbons			*2.61*
Total oxygenated monoterpenes			*6.53*
Total sesquiterpenes hydrocarbons			*70.48*
Total oxygenated sesquiterpenes			*14.93*
Total unidentified			*4.24*
Total			99.99

RI: Retention indices relative to series of n-alkanes (C8-C20) on HP-5MS column in elution order. NI: not identified. RT: retention time. SD: standard deviation (n=3)

3.2 Ethanolic extract chemical composition

Three x 30' ultrasonic assisted maceration of oven-dried leaves with forced air ventilation at 40°C of *C. phaea* using 98% ethanol as solvent yielded a strongly greenish colored extract with characteristic aroma. The ethanolic extract was analyzed in electrospray positive mode and monitored in the mass range of m/z 100-1000 m/z, and also with UV detection in the range from 200 to 400 nm. These analyses generated base peak (BPC) and UV chromatograms at 254 nm (Figure 1).

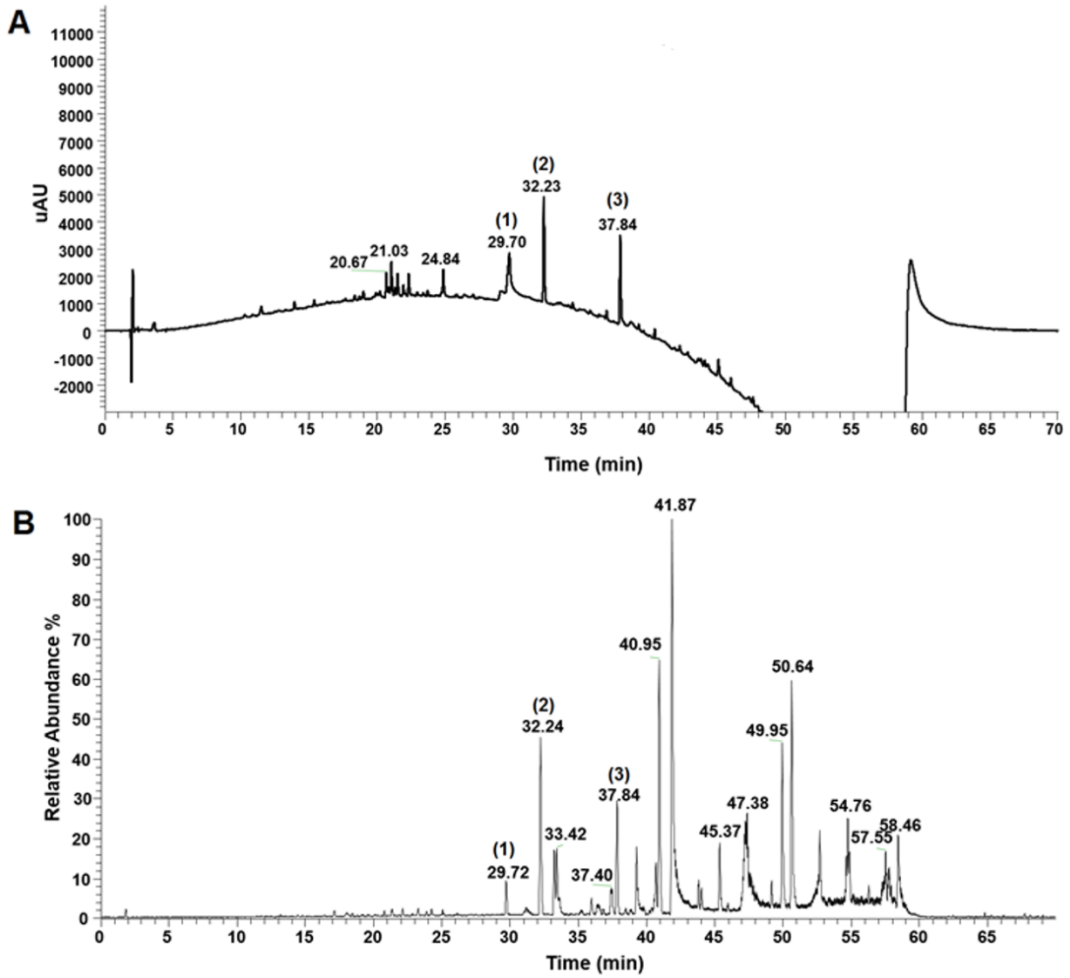

Figure 1. UV 254 nm (A) and base peak (BPC) (B) chromatograms of the ethanolic extract of *C. phaea*. Analytical conditions described in section 2.5. Peaks: (1) alpinetin O-dideoxyhexoside, (2) 5,7-dimethoxyflavanone, and (3) alpinetin.

The mass spectra obtained after LC-MS/MS analysis were loaded onto the Global Natural Products Social Molecular Network (GNPS) platform (Wang et al., 2016). Processing and comparing the spectral data of the ethanolic extract to the GNPS database led to the identification of three compounds: alpinetin O-dideoxyhexoside, 5,7-dimethoxyflavanone, and alpinetin (Table 2).

Table 2. Substances identified in *C. phaea* ethanolic extract.

Number	Compound *	t_R (min)	$[M + H]^+$	Fragment ions	λ_{MAX} (nm)
1	**Alpinetin O-dideoxyhexoside**	29.73	563	271, 167, 131, 103	287, 374
2	**5,7-Dimethoxy-flavanone**	32.23	285	181, 166, 138, 131	285, 374
3	**Alpinetin**	37.83	271	167, 131, 103	287, 374

t_R: retention time; $[M + H]^+$: protonated molecule; λ_{MAX}: ultraviolet absorption maxima. * (Wang et al., 2016).

The three identified compounds were flavonoids from the flavanone group. The compound that had a retention time of 29.73 min corresponds to the glycosylated derivative of the flavonoid alpinetin, whose protonated molecule of the aglycone moiety has an m/z ratio of 271. On the other hand, the difference in mass between the precursor ion (m/z 563) of the glycosylated molecule and the m/z ratio of aglycon (m/z 271) indicate the neutral loss of two deoxyhexoses (146 + 146 Da) (Kachlicki et al., 2016). The other identified flavanones were the aglycones 5,7-dimethoxy-flavanone (m/z 285) and alpinetin (m/z 271). These compounds presented 14Da as a difference of masses, which corresponds to the additional presence of a methyl in 5,7-dimethoxy-flavanone when compared to alpinetin.

3.3 Scavenging activity of the essential oil and ethanolic extract

The free radical scavenging activities of *C. phaea* essential oil (EO) and ethanolic extract (EXT) were determined using the DPPH, ABTS, and FRAP assays and the results are shown in Table 3. The EXT showed significant antioxidant activity with IC_{50} results comparable to quercetin and gallic acid. On the other hand, the EO showed no scavenging activity at the tested concentrations.

Table 3. Antoxidant activity of *C. phaea* essential oil and ethanolic extract estimated by the DPPH, FRAP, and ABTS assays.

	Antioxidant activity (IC_{50} µg/mL)		
Samples	**ABTS**	**DPPH**	**FRAP**
EO	>1000	>1000	>1000
EXT	8.25 ± 2.38 [a]	9.14 ± 1.37 [a]	1.04 ± 0.32 [a]
Gal-ac	4.76 ± 0.28 [a]	1.14 ± 0.03 [b]	3.31 ± 0.00 [a]
B-Caryophyllene	>1000	188.47 ± 1.05 [c]	>1000
Quercetin	4.85 ± 1.05 [a]	3.93 ± 1.05 [a]	2.75 ± 1.67 [a]

EO: *C. phaea* essential oil. EXT: *C. phaea* ethanolic extract
Different letters in the same column correspond to significant differences ($p<0.05$). Tests were performed in triplicate in three independent experiments at least and expressed as mean ± standard deviation.

3.4 *In vitro* citotoxicity of *C. phaea*

The cytotoxicities of the EO and EXT of *C. phaea* were evaluated by the MTT colorimetric method in the macrophage (RAW 264.7), fibroblast (L929), and melanoma (MV3) lines. The EO and EXT had no effect on cell viability at concentrations below 125 and 500 µg/mL, respectively. After defining the non-cytotoxic concentrations of the samples, it was set the maximum concentration of 100 µg/mL to be used in the subsequent cell culture assays.

3.5 Preventive effect against oxidative damage caused by H_2O_2 in RAW 264.7 macrophages

The preventive effects of the EO and EXT against hydrogen peroxide (H_2O_2)-induced cell damage were evaluated in RAW 264.7 macrophages. As observed in Figure 2, the EO did not show protective action against oxidative damage in any of the tested concentrations using this method; in contrast, the EXT showed a preventive effect of 32.6, 44.2, and 70.5% at concentrations of 10, 50, and 100 µg/mL, respectively.

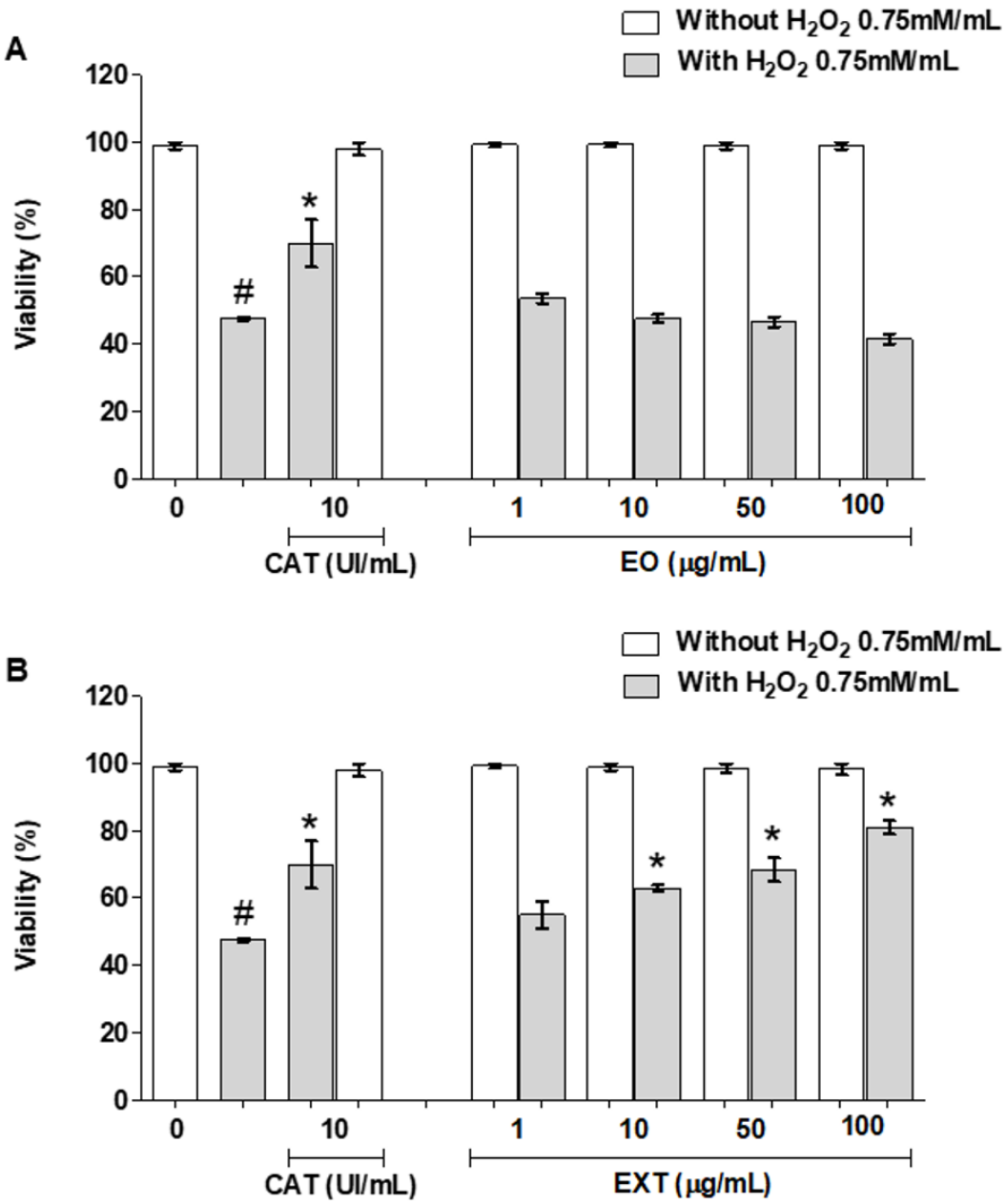

Figure 2. Protective effect of the essential oil (EO) (A) and ethanolic extract (EXT) (B) of *C. phaea* evaluated in RAW 264.7 macrophages against damage caused by hydrogen peroxide (H_2O_2). The macrophages were exposed to different sample concentrations in the presence or absence of H_2O_2. Catalase at 10 IU/mL (CAT) was used as positive control. The results were expressed as mean ± SD of three independent experiments. #Significant ($p<0.05$) compared to the negative control without H_2O_2. *Significant ($p<0.05$) compared to the H_2O_2 control.

3.6 *In vitro* indirect determination of nitric oxide

Indirect quantification of nitric oxide (NO) was carried out by determining the influence on nitrite production in LPS-activated RAW 264.7 macrophage culture. The EO and EXT dose-dependently inhibited NO production. Inhibitions of 50.0% and 51.9% in NO production were observed after exposure to EO and EXT at 50 µg/mL, respectively (Figure 3). Gallic acid (10

μg/mL) and the specific inhibition of i-NOS, L-NIL (50 μM) were used as positive controls, leading to inhibitions of 20.4 and 47.4% of NO production, respectively.

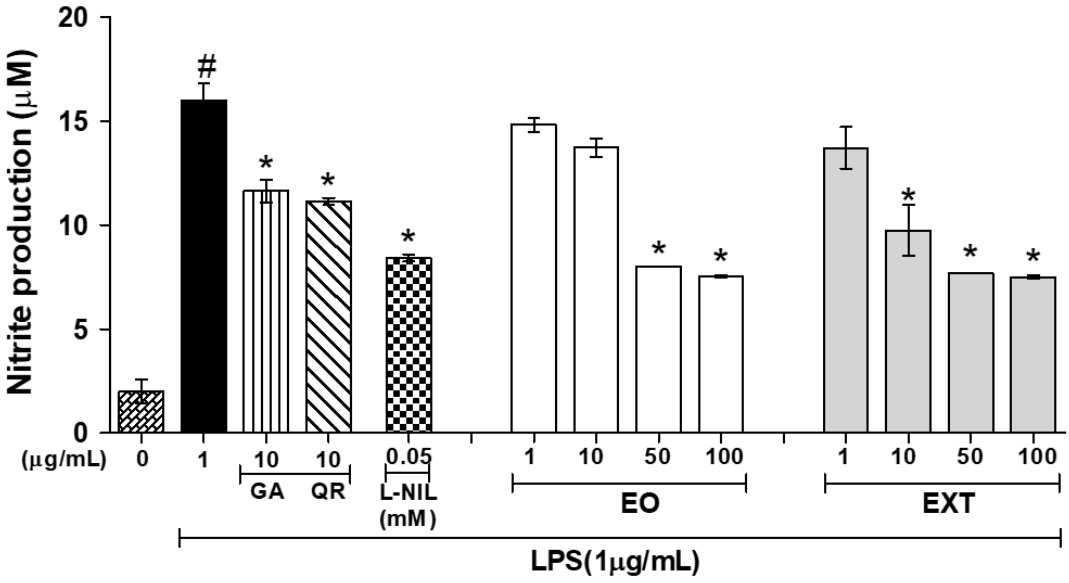

Figure 3. Effect of the essential oil (EO) and ethanolic extract (EXT) of *C. phaea* on the production of nitric oxide (NO). RAW 264.7 macrophages were exposed to different sample concentrations and stimulated with LPS (1 μg/mL) after 30 min. Gallic acid (GA) at 10 μg/mL, quercetin (QR) at 10 μg/mL, and L-NIL at 50 μM were used as positive controls. The results were expressed as mean ± SD of three independent experiments. #Significant ($p<0.05$) compared to the negative control without LPS. *Significant ($p<0.05$) compared to the control with LPS-induced cells.

3.7 *In vitro* superoxide determination

In a dose-dependent manner, at concentrations of 10, 50, and 100 μg/mL, the EO inhibited 31.6, 99.0, and 99.5%, respectively, while the EXT at 100 μg/mL inhibited 52.9% of superoxide radical ($O_2^{\bullet-}$) production in the LPS-activated RAW 264.7 macrophage culture. Gallic acid (100 μg/mL) and tempol (12.5 mM) were used as positive controls and inhibited 72.8 and 67.3% of $O_2^{\bullet-}$ production, respectively, as shown in Figure 4.

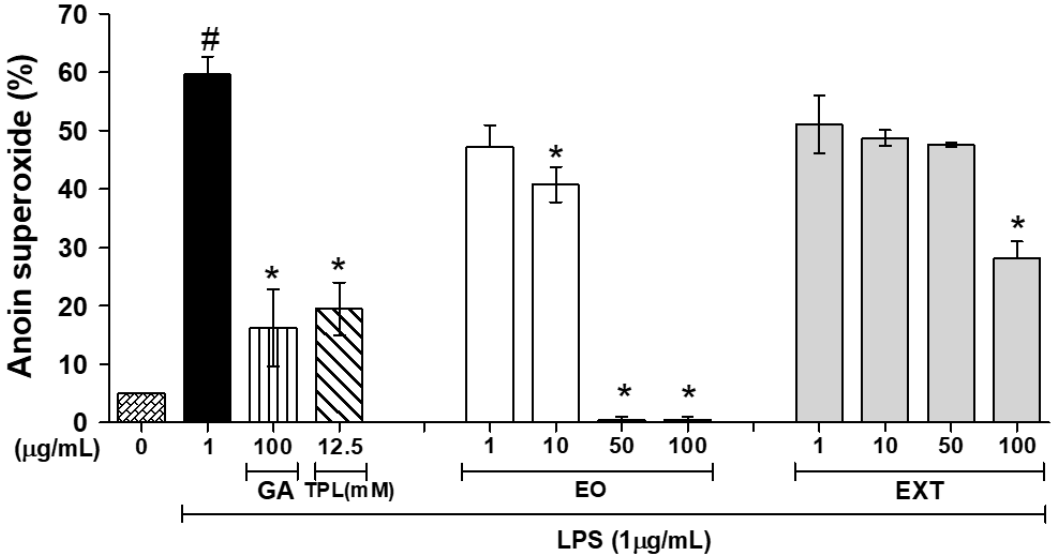

Figure 4. Effect of the essential oil (EO) and ethanolic extract (EXT) of *C. phaea* on the production of superoxide radical ($O_2^{\bullet-}$). The RAW 264.7 macrophages were exposed to different sample concentrations and stimulated with LPS after 30 min. Gallic acid 100 μg/mL (GA) and tempol 12.5 mM (TPL) were used as positive controls. The results were expressed as mean ± SD of three independent experiments. #Significant ($p<0.05$) compared to the negative control without LPS. *Significant ($p<0.05$) compared to the control with LPS-induced cells.

3.8 *In vitro* cytokine determination

The cell supernatant exposed to different EO and EXT concentrations was used for quantifications of IL-6 and TNF-α cytokines. Figure 5 shows that the EO inhibited, respectively, 41 and 46% of IL-6 production at concentrations of 50 and 100 μg/mL as well as 32.3, 49.5, 74.7, and 76.4% of TNF-α production at concentrations of 1, 10, 50, and 100 μg/mL. The EXT inhibited, respectively, 82 and 86.7% of IL-6 production at concentrations of 50 and 100 μg/mL as well as and 40, 73.8, 87.9, and 91.6% of TNF-α production at concentrations of 1, 10, 50, and 100 μg/mL.

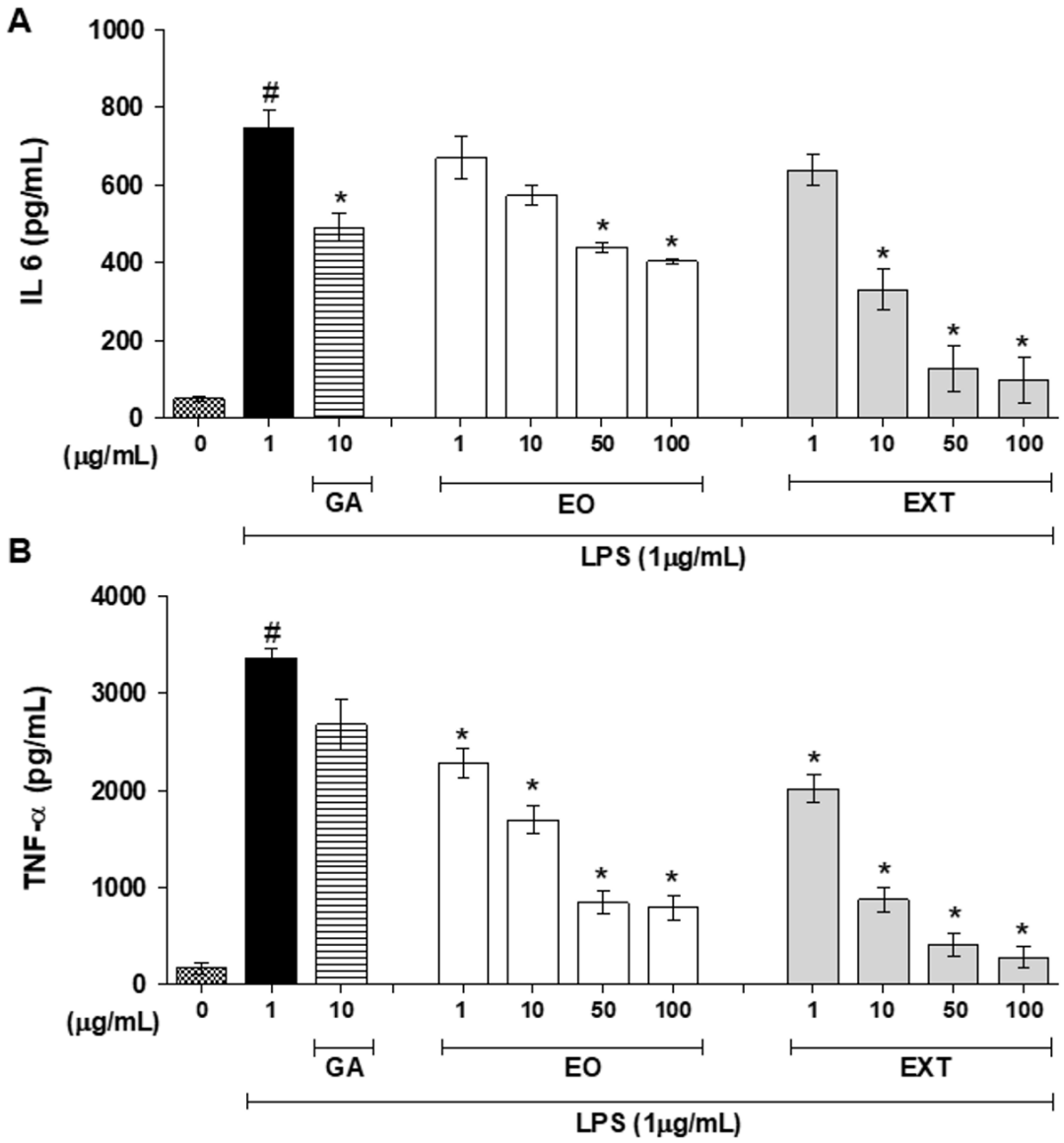

Figure 5. Effect of the essential oil (EO) and ethanolic extract (EXT) of *C. phaea* on the concentration of proinflammatory cytokines IL-6 (panel A) and TNF-α (panel B). RAW 264.7 macrophages were exposed to different sample concentrations in the presence or absence of LPS. Results were expressed as mean ± SD of three independent experiments. #Significant ($p<0.05$) compared to the negative control without LPS. *Significant ($p<0.05$) compared to the control with LPS-induced cells.

3.9 Nuclear factor kappa B activity determination

The luciferase reporter assay was used to detect the *in vitro* anti-inflammatory activity of the EO and EXT by quantifying the NF-κB inhibition. Cells from the HEK 293 lineage (human embryonic kidney) were used and the EO was tested at a 20 µg/mL. The EO and EXT had no cytotoxic effect on renal cells, exhibiting a survival rate of 93.8% ± 3.2 and 97.3% ± 4.0, respectively, as determined by the SRB assay. Also, they showed significant inhibition of 36.2 % ± 1.8 and 40.9% ± 12.6 of the NF-κB activity at the same concentration.

4. Discussion

In this study, we investigated the chemical composition of the essential oil and ethanolic extract of the leaves of *Campomanesia phaea* and, for the first time, its bioactive potential in relation to *in vitro* antioxidant and anti-inflammatory properties through LPS-stimulated RAW 264.7 macrophage culture. Based on the obtained results, it is suggested that the essential oil and mainly the ethanolic extract of *C. phaea* leaves have a promising anti-inflammatory effect that is mediated by the decrease in the production of pro-inflammatory mediators (IL-6 and TNF-α), NO, and O_2 induced by LPS in RAW 264.7 macrophages. Such anti-inflammatory effects can be explained, at least in part, by the NF-kB signaling pathway inhibition.

The main constituents of the essential oil of *C. phaea* leaves were the terpenoids (*E*)-caryophyllene (14%), caryophyllene oxide (6.9%), α-selinen (6.2%), γ-muurolene (6%), δ-cadinene (6%), α-copaene (5.9%), β-selinene (5.5%), linalool (5.2%), and *allo*-aromadendrene (5.1%). A previous study by Adati and Ferro (2006) identified as major compounds caryophyllene oxide (11.8%), linalool (11.1%), T-cadinol + T-muurolol (7.5%), β-selinene (6.9%), and β-caryophyllene (6.3%). The observed variations between the chemical compositions of the volatile oils of the *C. phaea* leaves reported in this study and those previously reported in the literature could be explained by genetic and envirnonmental factors such as season, rainfall, altitude, temperature, and vegetative cycle (Yang et al., 2018). To the best of our knowledge, the related literature does not report any scientific papers regarding the chemical composition of *C. phaea* leaves. Previous studies evaluated the ethanolic extract of the fruit and reported its high contents of phenolic compounds, including ellagitannins and proanthocyanidins (Donado-Pestana et al., 2015; Gonçalves et al., 2010; Tokairin et al., 2018).

The production of reactive oxygen species occurs in neutrophils and macrophages during inflammation and other processes of normal cellular metabolic activities (Lutz et al., 2019). Under conditions where the rate of generation of these free radicals is increased or the protective antioxidant mechanism is reduced, an imbalance occurs favouring free radical formation, leading to increased oxidative stress and subsequent tissue damage. ROS production increase has often been found in several pathophysiologies as well as other systemic complications (Barbosa et al., 2010; Lutz et al., 2019). In the present study, we observed that the ethanolic extract showed excellent scavenging capacity, comparable to quercetin and gallic acid. In the LPS-stimulated macrophage culture, both the extract and the essential oil had a negative effect on the production of nitric oxide and superoxide anions. These results indicate an important biological activity contributing to the control of oxidative stress and consequent suppression of the inflammatory process. In this context, several studies have demonstrated that

both NO and $O_2^{\bullet-}$ are involved in numerous pathophysiological processes and are therefore considered important therapeutic targets (Cinelli et al., 2019; Guzik et al., 2003).

In the last decade, the attraction towards medicinal and aromatic plants has been growing due to increasing consumer demand and interest in these plants for medicinal and many other applications. In addition to the capacity of plant extracts and essential oils in eliminating or reducing the formation of free radicals, there is also abundant evidence that they have important anti-inflammatory activities (Azab et al., 2016; Harvey et al., 2015; Miguel, 2010). For the first time in this context, this study demonstrated that the ethanolic extract and the essential oil of *C. phaea*, besides decreasing the production of NO and O_2, exhibited the capacity to suppress the production of pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and the signaling pathway of the transcription factor NF-κB.

The IL-6 and TNF-α are two important pro-inflammatory cytokines with relevant roles in inflammatory disorders (Sugimoto et al., 2016). Many essential oils and medicinal plants have demonstrated the correlation between the anti-inflammatory properties and their abilities for suppressing pro-inflammatory cytokines. *Rosmarinus officinalis* L. oil, mainly constituted by 1,8-cineole along with α-pinene, as well as camphor have demonstrated important anti-inflammatory effects that can be attributed due to the inhibition of prostaglandin synthesis or release, reduction of pro-inflammatory TNF-α, IL-1 and IL-6, and the potential to inhibit NF-κB transcription (Borges et al., 2019). *Cordia verbenacea* D. C. essential oil significantly decreased TNFα without affecting IL-1β production in carrageenan-injected rat paws (Passos et al., 2007). The ability to lowered endotoxin-induced in mice levels of peripheral nitrate/nitrite, interleukin (IL)-1β, IL-18, TNF-α, and interferon (IFN)-γ, production was attributed to cinnamaldehyde and linalool, major constituents of the essential oil of *Cinnamomum osmophloeum* leaves (Lee et al., 2018).

The transcription factor NF-κB is considered a pivotal mediator in the human immune system. The NF-κB signaling pathway is responsible for regulating the expression of a variety of genes involved in inflammatory responses, including proinflammatory cytokines, adhesion molecules, chemokines, and inducible enzymes such as iNOS (Sugimoto et al., 2016). Therefore, inhibition of the NF-κB signaling pathway has been widely explored as an important, effective therapeutic strategy for the treatment of various malignant inflammatory disorders (Liu et al., 2017). Our results indicate that the signaling pathway of NF-κB seems to be only partially involved in the possible molecular mechanism by which the essential oil and ethanolic extract of *C. phaea* inhibit oxidative stress and expression of proinflammatory mediators.

5. Conclusion

Our results suggest that both the oil and extract have bioactive compounds capable of blocking free radicals and/or inhibiting their intracellular actions; of inhibiting the production of important mediators of the inflammatory process, such as nitric oxide, superoxide anion, TNF-α, and IL-6; and of inhibiting the activation of the transcription factor NF-kB. These effects may explain, at least partially, the biochemical mechanisms that contribute to the antioxidant and anti-inflammatory effects demonstrated in the *in vitro* assays. In conclusion, this study demonstrated, particularly, that *C. phaea* extract could be explored as a valuable source of new and potent compounds with anti-inflammatory properties. Both oil and extract have therapeutic potentials for the modulation and regulation of macrophage activation and may provide safe and effective treatment options for a variety of inflammatory disorders.

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Conflict of interests

The authors have no conflicts of interest to report.

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