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BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
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Articulo Original / Original Article

Anti-inflammatory and antioxidant properties of *Bertholletia excelsa* (H.B.K) bark extract

[Propiedades antiinflamatorias y antioxidantes del extracto de corteza de *Bertholletia excelsa* (H.B.K)]

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Section Pharmacology

Received: 30 September 2021
Accepted: 9 March 2022
Accepted corrected: 30 April 2022
Published: 30 July 2023

Citation:
Silva MJA, Boleti APA, Acho LDR, Campos JF, Neto JPM, Guimaraes A, Silva FMA, Koolen HHF, Santos EL, Lima ES
Anti-inflammatory and antioxidant properties of *Bertholletia excelsa* (H.B.K) bark extract
Bol Latinoam Caribe Plant Med Aromat
22 (4): 472 - 487 (2023).
https://doi.org/10.37360/blacpma.23.22.4.35

Abstract: *Bertholletia excelsa* is native to the Amazon Rainforest and is popularly known as the Brazil nut. It has socioeconomic importance due its nuts being a great export product. There are few studies in the literature regarding the biotechnological potential of its bark, although it is used in folk medicine. The aim of this study was to determine the chemical constituents, anti-inflammatory and antioxidant properties of *B. excelsa* bark extract (BEB). Twelve substances were identified by LC/MS/MS, and cytotoxicity tests were carried out, as well as analyses of nitric oxide production and elimination of free radicals. BEB caused cytoprotection against oxidative stress in macrophages, increased HMOX-1 expression, overcame the antioxidant effects of GPx-1 and reduced its expression and was able to inhibit leukocyte migration in use peritonitis. BEB efficiently attenuated oxidative stress due to its antioxidant and anti-inflammatory properties and, as such, can be used as a safe and effective source of a natural herbal medicine.

Keywords: *Bertholletia excels*; Antioxidant; Anti-inflammatory; Nitric oxide; Peritonitis.

Resumen: *Bertholletia excelsa* es originaria de la selva amazónica y se la conoce popularmente como nuez de Brasil. Tiene importancia socioeconómica debido a que sus frutos secos son un gran producto de exportación. Existen pocos estudios en la literatura sobre el potencial biotecnológico de su corteza, aunque se utiliza en medicina popular. El objetivo de este estudio fue determinar los componentes químicos, las propiedades antiinflamatorias y antioxidantes del extracto de corteza de *B. excelsa* (BEB). Se identificaron 12 sustancias mediante LC/MS/MS y se realizaron pruebas de citotoxicidad, así como análisis de producción de óxido nítrico y eliminación de radicales libres. BEB causó citoprotección contra el estrés oxidativo en macrófagos, aumentó la expresión de HMOX-1, superó los efectos antioxidantes de GPx-1 y redujo su expresión y fue capaz de inhibir la migración de leucocitos en la peritonitis de uso. BEB atenuó eficazmente el estrés oxidativo debido a sus propiedades antioxidantes y antiinflamatorias y, como tal, puede utilizarse como una fuente segura y eficaz de un medicamento a base de hierbas naturales.

Palabras clave: *Bertholletia excelsa*; Antioxidante; Antiinflamatorio; Óxido nítrico; Peritonitis.

INTRODUCTION

Inflammation is associated with many common diseases and is a complex process that involves a network of cytokines that are responsible for the expression of many pro-inflammatory genes. Phagocytic cells and the oxidative stress generated by these mediators play an important role in the inflammatory response (Wang *et al*., 2011; Kang *et al*., 2015). In this sense, plants have been a source of new products for the development of medicines for thousands of years, and an impressive number of phytomedicines have been isolated from natural sources, which have, among their activities, relevant anti-inflammatory and antioxidative properties (Gardner *et al*., 1997).

Bertholletia excelsa is one of the treasures of the Amazon Rainforest and its fruits are an important export product from several regions. The Brazil nut trees are part of the botanical family Lecythidaceae, of the genus *Bertholletia* and, due to their large size, received the name *excelsa* (Melo *et al*., 2015). Many studies have been carried out on this plant, mainly in regards to the use of its nuts as a dietary supplement; however, research is necessary regarding the use of other parts of the plant such as its bark.

The biological properties of plants can be understood by the presence of phytochemicals, such as tannins, flavonoids, ascorbic acid, tocopherol, and secondary metabolites including polyphenols, identified in this species (Kwon *et al*., 2015). Several studies have shown that these bioactive compounds can act as inhibitors of molecular targets/pro-inflammatory mediators in inflammatory responses and eliminators of free radicals in the prevention of diseases related to oxidation. An ethnopharmacological investigation and bioassay-guided isolation of these compounds may lead to the discovery of more effective and safer therapeutic agents or plant extracts for treating diseases linked to oxidative stress and inflammation (Cuzzocrea *et al*., 2001).

It is a huge tree and its bark is used as a medicine by the local populations of the Amazon, but few studies have explored the chemical or pharmacological potential of its bark. Therefore, the present study investigated the phytochemical profile, and the antioxidant and anti-inflammatory properties of *B. excelsa* bark extract against oxidative stress in *in vitro* and *in vivo* tests.

MATERIAL AND METHODS

Plant Material Collection

The bark from branches of *Bertholletia excelsa* 1000 g was obtained from trees in Muiracupuzinho, highway AM-010, Km 56, Amazonas state, Brazil. A voucher was deposited at the UFAM Herbarium under N° 10348.

Preparation of the B. excelsa bark extract

The air-dried bark 1,000g was extracted by maceration at 60°C over three days, using a 7:3 mixture of ethanol: water 3 x 2 L. The liquid extracts were combined and dried using spray drier equipment (MD1.0, Labmaq, Ribeirao Preto, SP, Brazil).

Mass Spectrometry Analysis

A UHPLC-MS system, composed of an Accela 600 liquid chromatography system, was coupled to an LTQ mass spectrometer with a linear ion trap mass analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Electrospray ionization in negative mode (ESI-) was used to assess the composition of *B. excelsa*. Mass spectra (MS) were acquired in the range of *m/z* 50 to 1000, and tandem mass spectra (MS/MS) was acquired by collisional induced dissociation (CID) of trapped precursor ions. Helium was used as collisional gas. Tentative identifications were made by manual interpretation of MS/MS spectral data that were compared with those previously reported. Chromatographic separations were achieved with a Kinetex C_{18} column (2.6 µm, 30 x 4.6 mm, 100 Å pore size) (Phenomenex) column using a binary mobile phase. Solvent A was ultrapure water and solvent B was methanol. A gradient elution at 28°C was performed as follows: 0 - 15 min 20–100% at a flow rate of 0.4 mL/min. The autosampler temperature was held at 20°C and the injection volume was 5 µL. Ionization parameters were according to a previous method (Koolen *et al*., 2012) as follows: capillary voltage - 4.5 kV, cone voltage 5 V, sheet gas 25 arb, auxiliary gas 5 arb. Collision energies were applied as an increasing ramp from 2 to 50 eV.

2,2-Diphenyl-1-Picrylhydrazyl Radical-Scavenging Activity (DPPH)

The elimination activity of $DPPH^{\bullet}$ radical was measured according to the method of (Buritis & Bucar, 2000). Different BEBs (0-100 µg/mL) were tested in $DPPH^{\bullet}$ (100 mM) for 15 min and then the reading was performed. Gallic acid and ascorbic acid

were used as a standard and DMSO as a negative control. The antioxidant activity was obtained using the following equation: % inhibition = 100 – (ABS/ABS control sample) × 100, where abs is the absorbance.

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) Assay ($ABTS^{\bullet+}$)

The $ABTS^{\bullet}$+ radical assay was based on the method of (Re *et al*., 1999) with modifications. Initially, a solution of $ABTS^{\bullet}$ + (7 mM) and other components was prepared, then added BEB (0-100 µg/mL), incubation and reading. Gallic acid and ascorbic acid were the positive controls and negative DMSO. The antioxidant activity was obtained using the following equation: % inhibition = 100 - (ABS sample/ABS control) × 100, where ABS is the absorbance.

Anion Superoxide Radical-Scavenging Assay ($O_2^{\bullet-}$)

The elimination activity of the superoxide anion radical $O_2^{\bullet -}$ was measured using the NBT method of (Öztürk *et al*., 2007). The reagents NADH 390 µM, NBT 250 µM, PMS 10 µM and the BEB extract (0-100 µg/mL) were used for later reading. Gallic acid was used as a positive control. The percentage of inhibition of the generation of superoxide anion radical from three parallel measurements was calculated using the following formula: % inhibition = 100 - (final ABS of the sample - initial ABS sample) × 100 ABS control, where ABS is the absorbance.

Xanthine Oxidase Inhibitory Activity In Vitro (XO)

Xanthine oxidase activity was determined by measuring the formation of uric acid from xanthine according to (Shukor *et al*., 2018). Reagent 1, the enzyme xanthine oxidase and BEB (0-100 µg/mL), DMSO, and allopurinol were used. Two incubations were carried out and uric acid was added. Calculation of the uric acid concentration was performed to determine the percentage of enzyme inhibition.

Cell culture

The murine macrophage cell line J774 was acquired from the Cell Bank of Rio de Janeiro, RJ and was cultured at 37°C in a humidified incubator with 5% CO_2 in RPMI-1640 medium that contained 10% fetal bovine serum FBS, 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen).

Cell viability assay

J774 macrophages 1x10^6 cells/well were grown in 96-well tissue culture plates and were exposed to various concentrations of BEB (0-100 µg/mL) for 24 h (Mosmann, 1983). After incubation, the culture medium containing BEB was removed and the cells were washed with 0.1 mL of 0.1 M phosphate buffered saline PBS, pH 7.4, at 37ºC, followed by the addition of 300 µL of MTT solution 1 mg/mL in RPMI-1640 medium and incubation for 3 h at 37ºC. The medium was discarded and the dark-blue formazan crystals formed in intact cells were dissolved in DMSO and the absorbance at 540 nm was measured with a microplate reader (DTX 800, Beckman, CA, USA).

J774 macrophages 1×10^6 cells/well in 96-well plates were incubated at 37°C with 250 µM of H_2O_2 for 24 h with or without BEB and then treated with the MTT solution 1 mg/mL for 3 h. The dark-blue formazan crystals formed in intact cells were dissolved in DMSO, and the absorbance at 540 nm was measured with a microplate reader (DTX 800, Beckman). The results are expressed as the percentage of MTT reduction relative to the absorbance of control cells.

Cellular Antioxidant Activity

DCFH-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals mainly derived from H_2O_2 and be converted into its fluorescent product, DCF, which is retained within the cells (Wolfe & Liu, 2007). J774 cells 6 × 10^4 cells/well in 96-well plates were incubated at 37°C with 10 µM DCFH-DA for 1 h. Afterwards, the cells were washed with Hank's buffer treated with BEB (0.005, 0.05, 0.5, 5 and 50 µg/mL) and 250 µM of H_2O_2. The fluorescence was immediately measured with an excitation wavelength of 485 nm and emission wavelength of 520 nm using a microplate reader (DTX 800, Beckman, CA, USA). Controls with/without DCFH-DA were made. Quercetin was used as the positive control. Intracellular antioxidant activity was expressed as the percentage of inhibition of intracellular ROS produced by H_2O_2-exposure.

$NO^{\bullet}$ production assay

Nitric oxide $NO^{\bullet}$ production by J774 cells was assayed by measuring the accumulation of nitrite in the culture medium using a Griess test (Green *et al*., 1982). After incubation of the cells 1×10^6 cells/mL with BEB in different concentrations of 0-100 µg/mL, these were incubated for 24 h with lipopolysaccharide LPS 1µg/mL at 37°C in a 5% CO_2 incubator. In this test, nitric oxide was measured in

cell supernatant by means of the Griess reaction at 560 nm and sodium nitrite was used as a standard.

RNA extraction and cDNA Synthesis

Total RNA was extracted from J774A.1 cell lines. First-strand cDNA synthesis was performed with random hexamer-primed Superscript III™ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

Real-time quantitative PCR (RT-qPCR)

TaqMan® gene expression assays for nuclear factor erythroid derived 2, (Nfe2l2), heme oxygenase 1 (Hmox1), peroxiredoxin 2 (Prdx2), glutathione peroxidase 1 (Gpx1), mitogen-activated protein kinase 1 (Mapk1), nitric oxide synthase 2 (Nos2), interleukin 1 beta (Il1b) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encoding-gene as internal reference were performed. Real-time PCR (qPCR) was performed using a thermal cycler (StepOne Plus, Applied Biosystems, Waltham, MA, USA). qPCR reactions were performed in triplicate with the following thermocycling conditions: 96°C for 10 min, 35 cycles, each for 15 sec at 96°C and at 60°C for 2.5 min. The 10.0 µL reaction mixture was composed of 5.0 µL TaqMan™ Universal PCR Master Mix (Applied Biosystems™), 1.0 µL (~50 ng cDNA), 0.5 µL TaqMan® and 3.5 µL nuclease-free water.

Rearing and Maintenance of Caenorhabditis elegans

To perform the *in vivo* toxicity assay, we used the wildtype N2 strain of the nematode *Caenorhabditis elegans*. The specimens were incubated at 20°C in Petri dishes containing nematode growth medium (NGM) agar and fed with *Escherichia coli* strain OP50-1. The nematode culture was synchronized through treatment of pregnant hermaphrodites with 2% sodium hypochlorite and 5M sodium hydroxide.

Assessment of Toxicity in C. elegans

A toxicity assay for the BEB was performed using *C. elegans* in 96-well plates. Each well contained 10 nematodes at the L4 stage, which were incubated for 24 h at 20°C with BEB at different concentrations (0.5 - 500 *µ*g/mL) in M9 medium. After this period, nematode viability was evaluated by repeatedly touching the worms with a microspatula. A stereomicroscope (Motic SMZ-140 & W10X/23 model, BC, Canada) was used to manipulate and examine the nematodes. The data were calculated from two independent experiments in duplicate and according to Hunt (2017).

In vivo assay of LPS-induced peritonitis in mice

Acute inflammation in mice was performed by intraperitoneal injection of bacterial LPS into the peritoneal cavity of the mice (LPS 500 ng/cavity/0.2 mL), according to the methodology of (Ajayi *et al*., 2017) with modifications. The study was approved by the Animal Research Ethics Committee of the Federal University of Amazonas (Cert. No. 048/2019). During the experiments, all precepts were followed to ensure the greatest comfort and the least possible suffering to the animals. Initially, the mice were divided into 5 groups (n=4 animals/group) for oral pretreatment with a saline vehicle (0.2 mL/10g), dexamethasone (1 mg/kg) and BEB extract (50 and 100 mg/kg). After one hour of treatment, the LPS inflammation inducer (500 ng/0.2 mL) was administered in the intraperitoneal cavity in the test groups and only saline in the negative control group. After four hours of treatment, the animals were euthanized with ketamine (180 mg/kg) and xylazine (30 mg/kg) given intraperitoneally. After this procedure, the peritoneal cavity was washed with 10 mL of cold PBS buffer 10 mM, pH 7.4 containing EDTA 3 mM. The abdominal cavity was gently massaged three times and the cell suspension was aspirated with a syringe. The aliquots of the abdominal fluid were placed in 15 ml Falcon® tubes, centrifuged and resuspended with 200 µL of PBS buffer, then an aliquot 1:1 Turk's solution and cell concentrate was removed and total leukocyte counts were performed in a Newbauer chamber. At the end, the aliquots of the peritoneal fluid were frozen in a -80°C freezer for further analysis of inflammatory cytokines.

Statistical analysis

All data were obtained as mean ± standard error of the mean SEM and as statistically significant differences between groups, and an analysis of variance ANOVA and Dunnett's post-test were performed to compare the treatment with the control with the use of the software GraphPad Prism 6. The results were considered significant when the value of p was <0.05.

RESULTS

Phytochemical analysis

A total of twelve compounds were identified in the extract analysis, but it was not possible to identify of

the three compounds Figure No. 1. The deprotonated ions $[M–H]^−$ and their corresponding fragment ions observed from CID experiments were compared to fragmentation routes previously described by a manual interpretation. For the analysis of BEB Table No. 1, the peak eluted at 1.92 min displayed the deprotonated molecule at *m/z* 191, which fragments to the ion at *m/z* 173 and m/z 111, and allowed us to identify it as quinic acid (Bataglion *et al*., 2015). Main peak 2 (Rt 2.04 min) was identified as gallic acid based on its deprotonated molecule at *m/z* 169 and the characteristic carbon dioxide loss (−44 Da), and gave the fragment $[M–CO_2–H]−$ at *m/z* 125 (Bataglion *et al*., 2015). Peaks 3 and 4 eluted at 2.41 and 2.54 min, and at *m/z* 353 and 355, respectively, and displayed unusual fragmentation, which was not positively identified; thus both were described as unknown compounds.

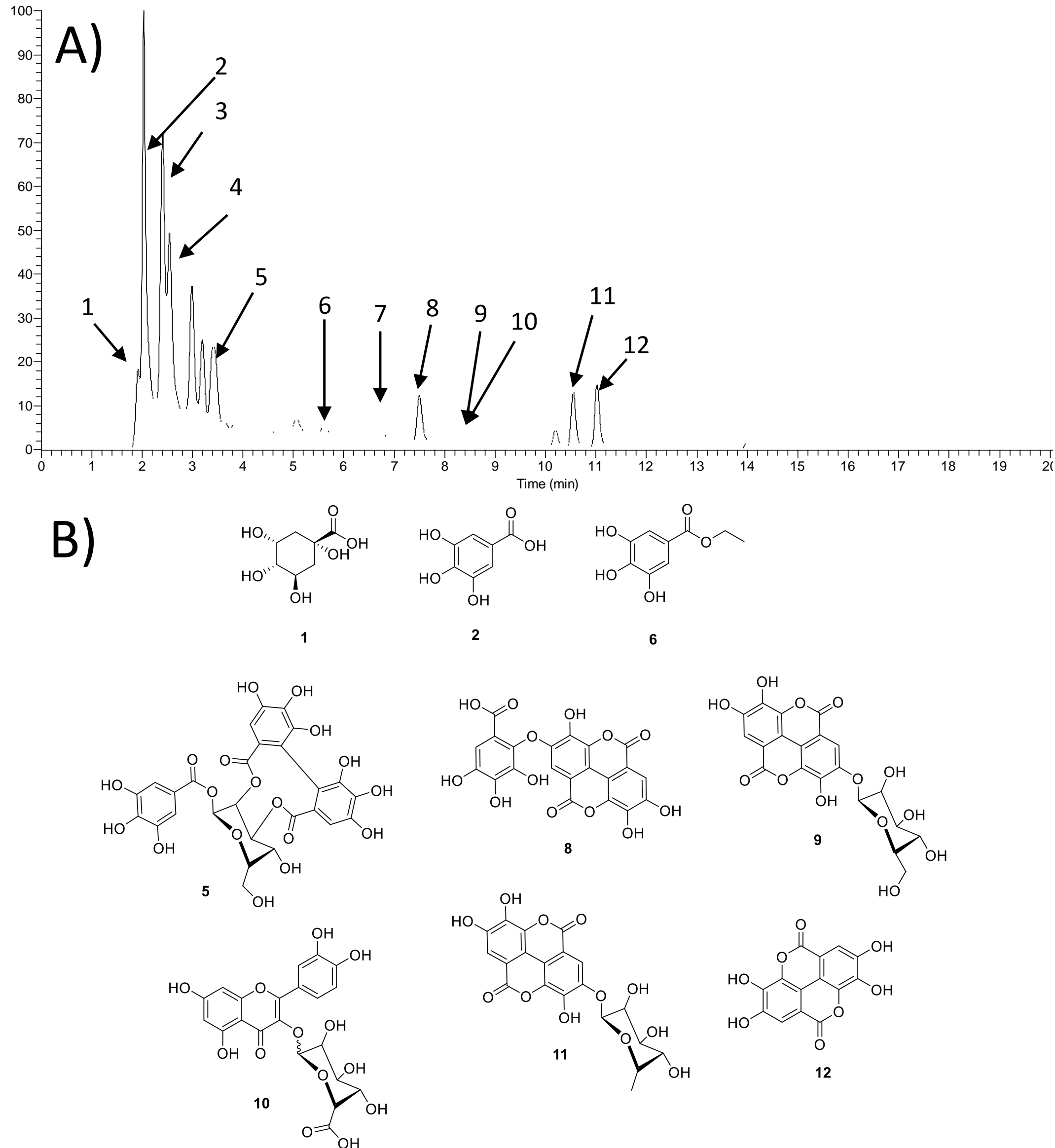

Figure No. 1
(A) Total ion chromatogram of phenolic compounds identified in the extract of *B. excelsa* by HPLC-MS/MS.
(B) Chemical structures of the identified compounds

Table No. 1
UHPLC–ESI-MS/MS analysis of phenolic compounds in *B. excelsea* extract

Peak number	Compound	Retention time (min)	$[M - H]^-$	MS Fragments	Class
1	Quinic acid	1.92	191	173, 111	Organic acid
2	Gallic acid	2.04	169	125	Benzenoid acid
3	Unknown	2.41	353	335, 309, 265, 247, 221	-
4	Unknown	2.54	355	337, 311	-
5	HHDP-galloyl-glucose	3.01	633	301	Ellagitannin
6	Ethyl gallate	3.18	197	169, 125	Benzenoid ester
7	Unknown	3.44	291	247	-
8	Valoneic acid dilactone	7.50	469	425	Ellagic acid derivative
9	Ellagic acid hexoside	10.19	463	301	Ellagic acid derivative
10	Quercetin-3-*O*-glucuronide	10.37	477	301	Flavonoid derivative
11	Ellagic acid desoxyhexoside	11.06	447	300	Ellagic acid derivative
12	Ellagic acid	11.93	301	257, 229	Ellagic acid derivative

The ellagitanin HHDP-galloyl-glucose was identified eluting at 3.01 min (peak 5) based on the parent *m/z* 633 and its main fragment ion at *m/z* 301 (Fracassetti *et al*., 2013). Ethyl gallate eluted at 3.18 min (peak 6) based on its typical fragmentation ions (*m/z* 197 → 169 and *m/z* 169 → 125), and was related to those observed for the gallic acid (Verdam *et al*., 2017). Peak 7 (3.44 min) was also designated as unknown since it was not possible to interpret its fragmentation. The compound eluting at 7.50 min (peak 8) was identified as the valoneic acid dilactone. This was based on its precursor *m/z* 469 and main fragment at *m/z* 425, as previously described (Wyrepkowsk *et al*., 2014). Peaks at 10.19, 10.37 and 11.06 min compounds 9, 10 and 11 displayed equal fragmentation patterns with *m/z* 301 as the main ion product (Fracassetti *et al*., 2013). The parent *m/z* at 463, 477 and 447 enabled the identification of these derivatives as being ellagic acid glucoside (Otterbein & Choi, 2000), quercetin-3-*O*-glucuronide (Surh *et al*., 2008) and ellagic acid deoxyglucoside (Pearce *et al*., 2007; Melo *et al*., 2015). Ellagic acid (*m/z* 301) was identified eluting at 11.93 min (peak 12) based on the main fragments *m/z* 257 and 229, as previously described for this compound (Pedrosa *et al*., 2016).

Antioxidant properties of B. excelsa bark extract in vitro

To evaluate the antioxidant activity, we began by investigating its $DPPH^\bullet$, $ABTS^{\bullet+}$ and anion superoxide radical scavenging activity. Thus, as presented in Table No. 2, BEB can be considered a free radical scavenger and antioxidant. It showed % inhibition of free radicals at 72.37 ± 0.5, 88.61 ± 0.4 and 76.52 ± 0.58 for $DPPH^\bullet$, $ABTS^{\bullet+}$, and $O2^{\bullet-}$, respectively. Antioxidant activity of BEB was similar to that of the gallic acid standard, which showed a % inhibition at 98.89 ± 1.0, 92.15 ± 0.7, 81.91 ± 2.36, respectively.

Furthermore, BEB inhibited 85.03 ± 1.54% of the activity of the xanthine oxidase at 100 µg/mL, with IC_{50} at 18.0 ± 1.15 µg/mL, and these results contribute to $O_2^{\bullet-}$inhibition since this radical is generated during the assay Table No. 2. Our results indicate that the considerable antioxidant activity of BEB can be associated with the phenolic and flavonoid compounds present. The total phenolic and flavonoid content was 277.21 ± 2.76 GAE mg/g and 52.0 ± 2.27 QE mg/g, respectively, which demonstrates a large amount of phenolic compounds, and these are greater than the flavonoid content.

Table No. 2
Free radical scavenging activity (IC_{50} in microgram per milliliter) of BEB and antioxidant standard

Sample	DPPH	ABTS	$O_2^{-\bullet}$	XO
BEB	6.36±0.8	41.4±0.5	38.6±1.35	18±1.15
Gallic acid	3.25±0.2	3.53±0.05	24.23±1.25	-
Allopurinol	-	-	-	2.24±0.03

Values are means ± SEM. DPPH (*n*=3), ABTS (*n*=3), anion radical superoxide ($O_2^{-\bullet}$, n=3), xanthine oxidase (XO, n=3)

Effect of B. excelsa bark extract on cell viability

In order to investigate the cytotoxicity of BEB on the J774 macrophage cell line, the cells were seeded in wells of 96-well culture plates at a density of 1 x 10^6 cells/well. The influence of BEB and quercetin at various concentrations on cellular toxicity was first analyzed using the MTT assay, without the influence of hydrogen peroxide. During a 24 h period of incubation, BEB and quercetin did not affect J774A1 cell viability Figure No. 2.

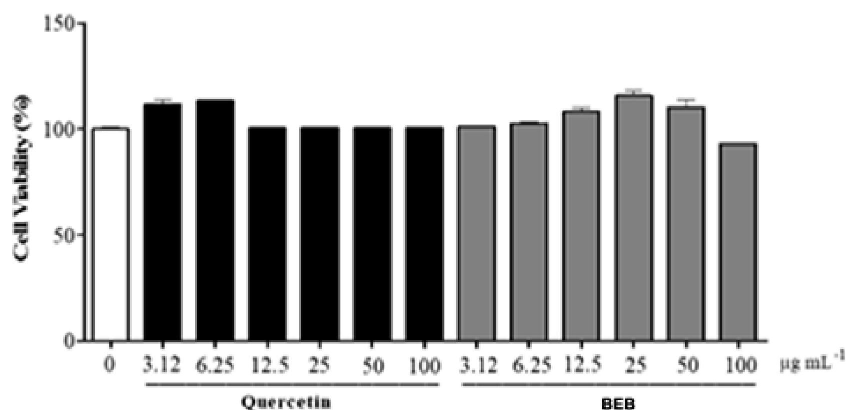

Figure No. 2
Effect of BEB and quercetin on cellular viability in J774 macrophage. J774 macrophages (1x10^6 cells/well) were cultivated in 96-well tissue culture plates and exposed to various concentrations of BEB and quercetin (3.12-100 µg/mL) for 24 h. The values are means ± SD of three replicates. **p*<0.05 compared to control vehicle treated cells

Inflammatory activity of B. excelsa bark extract

We next investigated whether BEB inhibits $NO^{\bullet}$ production in LPS-induced J774 macrophage cells Figure No. 3. Nitric oxide is a well-known inflammatory mediator and vasodilator and may induce oxidative stress in pathological conditions (Abramson, 2008). The effect of BEB in inhibiting nitrite levels induced by LPS in macrophage can be determined by Griess tests. BEB decreases nitrite levels in a concentration-dependent manner. The maximum inhibition by BEB was achieved at a concentration of 100 µg/mL, with 40 ± 0.18% of reduction.

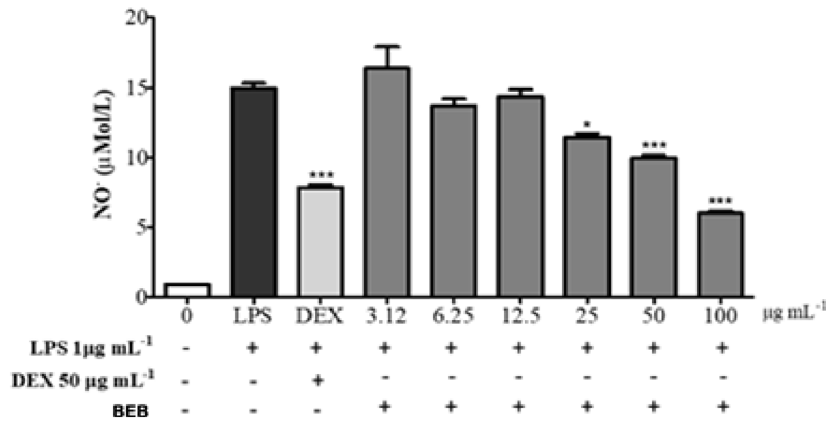

Figure No. 3
Effect of the BEB on NO• production in LPS-stimulated J774 cells. Production of NO• was assayed in culture supernatants of macrophages stimulated with LPS (1 *µ*g/mL) for 24 h, in the presence of the BEB (3.12–100 *µ*g/mL). The nitrite values are the mean ± SD from three independent experiments. The values are means ± SD of three replicates. **p*<0.05 and ****p*<0.001 compared to control vehicle treated cells

Cellular Antioxidant Activity of B. excelsa bark extract

The present study showed that BEB could protect murine macrophages from oxidative damage. This antioxidant activity in cells indicated that at *5* µg/mL the BEB reduced the levels of intracellular ROS promoted by the presence of H_2O_2, with IC_{50}=4.57 µg/mL (with confidence interval between 1.659 to 12.62) by 60 ± 1.49%. Dichlorofluorescein is a probe that is trapped within cells and is easily oxidized to fluorescent dichlorofluorescein DCF. The decrease in cellular fluorescence compared to the control cells indicates the antioxidant capacity of the extract (Wolfe & Liu, 2007). The oxidation inhibition values of the quercetin 50µg/mL were not significantly different when compared with BEB Figure No. 4.

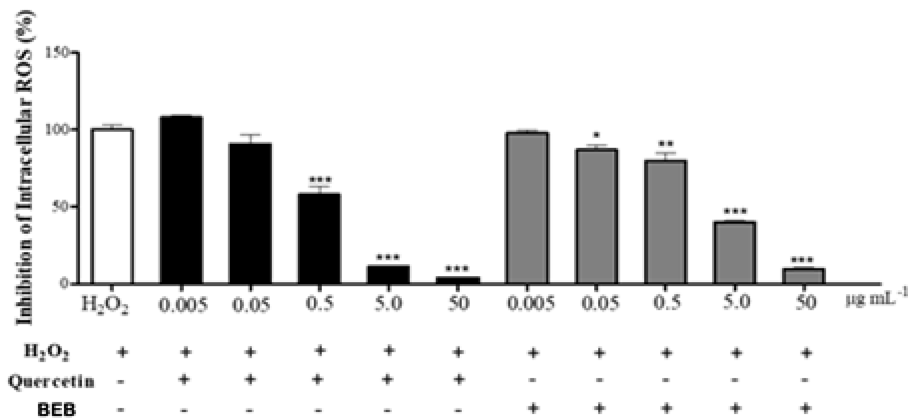

Figure No. 4
Antioxidant activity measured by DCF assay of BEB and quercetin (Q). J774 macrophages (1x 10^6 cell/well) were preloaded for 30 min with 10 µM of DCFH-DA. Afterwards, cells were treated with 0.005-50 µg/mL of BEB and/or quercetin, and 250 µM of H_2O_2. The fluorescence levels were measured with an excitation wavelength of 485 nm and emission wavelength of 520 nm. Results were expressed in oxidation inhibition (%). The values are means ± SD of three replicates. **p*<0.05, ***p*<0.01 and ****p*<0.001 compared to oxidation promoted by H_2O_2

The cytotoxic effects of BEB were examined using a MTT assay in order to determine non-toxic concentrations for inflammatory and antioxidant activity test. BEB did not show any significant toxic effect on the growth of cells. Therefore, in order to determine whether BEB prevents oxidative stress by inhibiting ROS generation, we measured intracellular ROS concentrations with DCFH-DA, and found that treatment of J774 cells with H_2O_2 induced a marked rise in oxidative stress, which was characterized by excessive ROS production. This was followed by a reduction in the levels of intracellular ROS by BEB. Quercetin, at the level of concentration used in this study, has been shown to exhibit strong suppression of intracellular ROS generation, and BEB was as efficient as the antioxidant standard in scavenging ROS (Sittisart & Chitsomboon, 2014).

Macrophages are vital for the recognition and elimination of microbial pathogens, and its survival has critical importance to the host defense system (Grom & Mellins, 2010; Bhaskaran *et al*., 2012). Previous studies have demonstrated that the virulence of some bacteria is due to their ability to trigger the death of activated macrophages via the stimulation of ROS production (Bhaskaran *et al*., 2012). Our results showed anti-inflammatory activity of BEB in the same concentrations of its antioxidant activity.

By using the MTT assay, the present study also showed that the exposure of macrophages to H_2O_2 resulted in a reduction of cell viability. However, pretreatment with different concentrations of BEB, with or without H_2O_2, greatly diminished loss of cell viability when compared with quercetin, the positive control.

***B. excelsa* bark extract induces expression of antioxidant genes**

To investigate the effect of BEB on the expression of antioxidant-related genes, real time PCR analysis was performed Figure No. 5 A-D and we verified the intensity of expression using different concentrations of BEB and quercetin standard (Q) and 250 μM of H_2O_2. First cell viability was analyzed using an MTT assay Figure No. 6 A-B and a mortality rate of 40% at 250 μM H_2O_2 was observed, and was high enough to be comparable to antioxidant agents. The analyses with BEB were carried out in concentrations of 10, 50 and 100 μg/mL, and a concentration range with higher cell protection. We chose a concentration of 10 μg/mL of quercetin since this showed the best results for cell protection.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a key transcription factor that is sensitive to redox and that translocates to the nucleus and binds to the antioxidant response. The sequences located in the promoter regions of genes that encode various antioxidant proteins, such as heme oxygenase 1 (HO-1), increase as a result of oxidative stress. Induction of these antioxidant proteins is essential for the protection of cells from oxidative stress caused by ROS such as H_2O_2. Exposure of cells at a 100 μg/mL of BEB for 24 h resulted in an increase 294% in the levels of expression of HMOX-1 and showed that BEB has the ability to significantly upregulate these antioxidant cellular events Figure No. 6B.

Concomitantly, we observed elevated levels of GPx1 expression in the positive control H_2O_2 and a reduction of 53% in GPx1 expression of macrophages treated with BEB Figure No. 6C. This shows that its function is specific to H_2O_2 degradation, since GPxs are a family of enzymes homologous to the selenocysteine (Sec)-containing mammalian GPx-1 that uses GSH as an obligate co-substrate in the reduction of hydrogen peroxide to water (Lubos *et al*., 2011). When we compared the levels of GPx1 expression in the standard quercetin, the standard was significantly higher compared with BEB. The largest reduction in expression of BEB at 50 and 100 μg demonstrates its effectiveness in capturing free radicals.

BEB was also effective in reducing the expression of pro-oxidant genes. Gene expression analysis Figure No. 6D also demonstrated that, in the presence of H_2O_2, there was a significant increase in the expression of the NOS2 gene, a nitric oxide synthase responsible for the synthesis of nitric oxide NO, and which plays an important physiological role as a potent vasodilator. However, its expression can be increased in pathological conditions (Bayir *et al*., 2007). In the presence of BEB, a significant reduction 64% in NOS2 expression was observed. The antioxidant effect of BEB proved to be superior to the effects of quercetin at 100 μM. Considering that the samples and H_2O_2 were co-incubated, this may possibly have caused the inhibition of ROS by antioxidant compounds before the oxidative action of H_2O_2

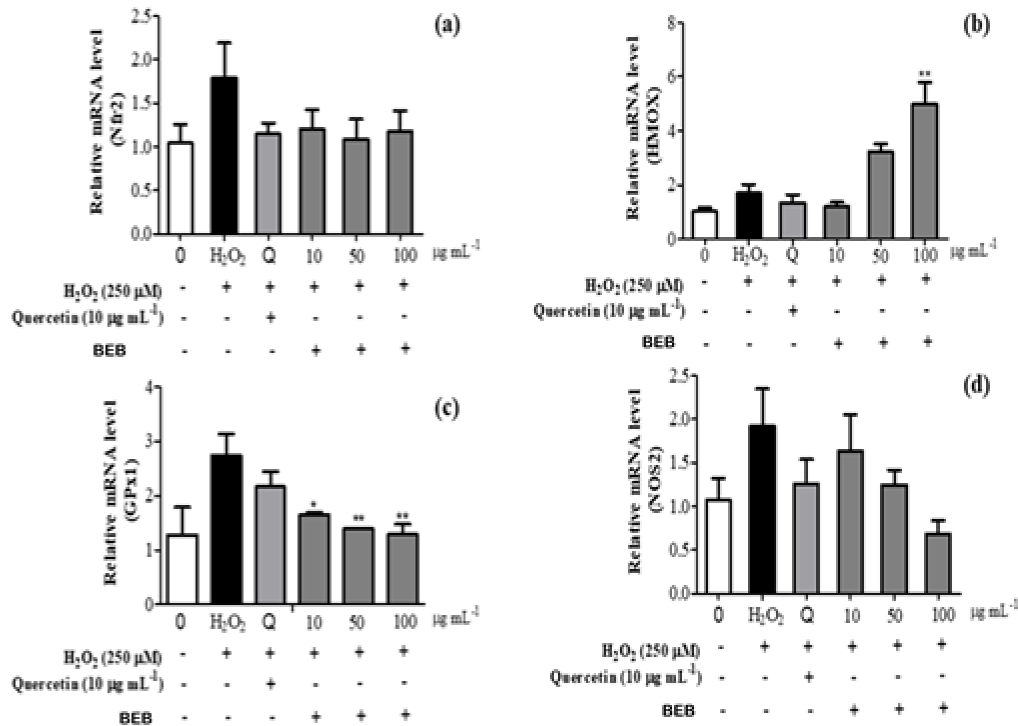

Figure No. 5
Antioxidant action of BEB on pathway of pro-oxidant and antioxidant genes. J774 macrophages (1x10^6 cells/well) were cultivated in 96-well tissue culture plates and exposed to 250 µM of H_2O_2 and concentrations of BEB (10, 50 and 100 µg/mL) and quercetin (10 µg/mL) for 24 h. Relative mRNA levels were determined for the following genes: (A) NFR2, (B) HMOX-1, (C) GPX-1 and (D) NOS2. The values are means ± SD of three replicates. *p<0.05 compared to control vehicle treated cells

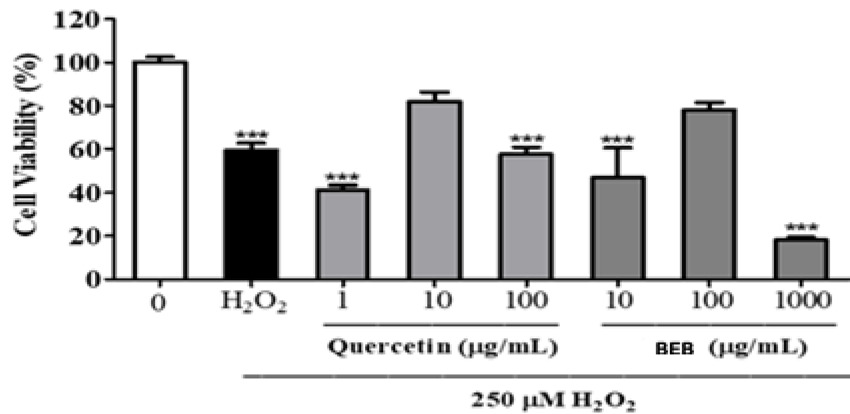

Figure No. 6
Cytotoxic action of BEB and quercetin in cellular viability in H_2O_2-stimulated J774 macrophages. J774 macrophages (1x10^6 cells/well) were cultivated in 96-well tissue culture plates and exposed to 250 µM of H_2O_2 and concentrations of BEB (10, 100 and 1000 µg/mL) and quercetin (1, 10 and 100 µg/mL) for 24 h. The values are the means ± SD of three replicates. *p<0.05 compared to control vehicle treated cells

The response of an inducible heme-degrading enzyme, heme oxygenase-1 (HO-1), which is an antioxidant enzyme, for a wide range of cellular stresses, exhibits adaptive responses to oxidative stress including skeletal muscle cells (Kang *et al*., 2015). BEB induces HO-1 expression to afford protection of macrophages against cytotoxicity and protects cells from H_2O_2-induced cell death by inducing antioxidant enzymes, including HO-1. The most significant finding in our present study is the demonstration of the involvement of the Nrf2 pathway in BEB-mediated HO-1 gene induction.

Our research demonstrated that the GPx1 expression levels were elevated in cells stimulated with H_2O_2. This demonstrates GPx1's specific function for H_2O_2 degradation. The GPx expression levels in the sample containing quercetin showed themselves to be significantly elevated compared to the other samples. This can be attributed to quercetin having a lower efficiency when compared to BEB. This occurs even before a natural increase in gene expression caused by the phenolic compounds by moving the Nrf2 factor to enhance the production of these enzymes (Kaspar *et al*., 2009). We observed the greatest reduction in the expression at 100 µg/mL of BEB, which demonstrates its effectiveness in capturing free radicals. These data corroborate the graphical analysis, and the levels at which the presence of phenolic compounds such as ellagitannin derivatives, such as ellagic acid, its glycosides and valoneic acid dilactone present in quercetin and BEB, may be responsible for the potential observed.

Our findings were similar to the negative control, which is in accordance with previous observations regarding this class of compounds in which they were classified being potent antioxidants. This has been previously determined for punicalagin isolated from pomegranate *Punica granatum*, and different ellagitannins from different heartnut varieties *Juglans ailanthifolia* var. *cordiformis* (Cerdá *et al*., 2004; Li *et al*., 2006).

Toxicity in C. elegans

BEB was not toxic to the nematodes after 24 h of incubation in concentrations up to 100 µg/mL when compared with the control group Figure No. 7. Only at high concentrations did it reduce the viability of the nematode.

Previous studies have shown that *C. elegans* can be used as an alternative *in vivo* model for obtaining rapid results in toxicity studies in order to assess the pharmacological and toxic effects of drugs (Leung *et al*., 2008; Bonamigo *et al*., 2017). *C. elegans* is a multicellular organism with a high reproduction rate and short life cycle, which makes it an excellent *in vivo* model for complementing cell culture-based systems (Leung *et al*., 2008). Furthermore, BEB shows no significant toxic effects in an *in vivo C. elegans* experimental model.

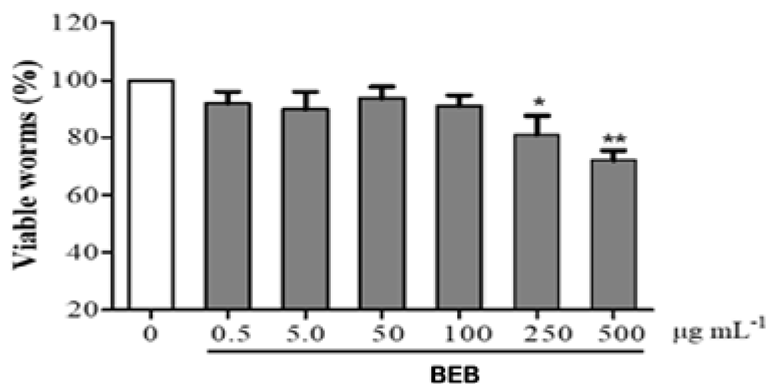

Figure No. 7
Toxicity of BEB against *C. elegans*. *C. elegans* (L4) were incubated for 24 h at 20°C with BEB (0.5-500 µg/mL). Data are expressed as mean ± SEM. *p<0.05 compared to control vehicle treated cells

In vivo assay of LPS-induced peritonitis in mice

BEB was assessed for its ability to inhibit leukocyte migration to the peritoneal cavity of previously inflamed mice. Thus, the animals were pre-treated orally with doses of LPS 500 ng/cavity, saline (0.2 mL/10g), dexamethasone (1 mg/kg) and BEB extract (50 and 100 mg/kg). After one hour of treatment, LPS was administered intraperitoneally in the groups,

though not in the saline control group and the duration of the experiment was 4 hours. The collection of the peritoneal lavage was performed and the count of the total leukocytes marked in the inflammatory response was performed.

As seen in Figure No. 8, BEB was able to reduce total leukocytes in a dose-dependent manner 50 and 100 mg/kg, and significantly decreased the inflammatory process induced by LPS. However, when compared to the reference drug, BEB at a dose of 100 mg/kg, it showed a response similar to the 1 mg/kg dexamethasone standard. BEB at a dose of 50 mg/kg did not show a statistically significant difference.

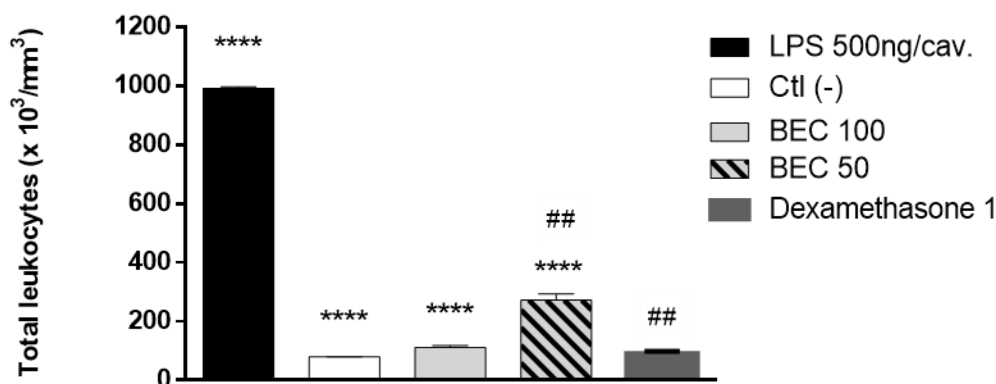

Figure No. 8
Total leukocytes from the peritoneal cavity of mice in an assay of lipopolysaccharide (LPS) stimulated peritonitis after four hours of treatment. Data are expressed as mean ± SEM.
$^{****}p<0.0001$ compared to control LPS 500 ng/cavity and
$^{\#\#}p<0.001$ when compared to the positive control group dexamethasone 1 mg/kg

DISCUSSION

Inflammation is a physiological process that is initiated in response to infection or tissue damage. It is a mechanism aimed at tissue repair after injury and consists of a cascade of cellular and microvascular events that aim to remove damaged tissue and generate new tissue. It can be induced by oxidative stress that occurs when cellular homeostasis, normally involving a fine balance between free radical generation and its detoxification by cellular antioxidants, is disturbed (Dhuna *et al*., 2013). Antioxidant activities are known to increase proportionally in relation to the polyphenol content, mainly due to their redox properties, and products derived from medicinal plants can be used as new therapeutic agents due to the presence of secondary metabolites, which include groups such as polyphenols (Galeno *et al*., 2014).

The use of medicinal plants for the treatment and control of diseases that affect man comes from ancient medicinal practices. Their effect in the treatment of inflammatory diseases mostly comes from the use of plants that have active constituents present in their extracts. Among the main active compounds present in these plants are chemical groups such as polyphenolic compounds, flavonoids, terpenoids and tannins, which are secondary metabolites that act on different targets involved in inflammatory processes (Komakech *et al*., 2019). In BEB, it was possible to identify the presence of chemical groups such as tannins (elagitanin derivatives) in its composition, which are responsible for the vasoconstrictor effect that favors tissue regeneration (healing), and flavonoids that have anti-inflammatory, antioxidant and anti-radical effects. BEB demonstrated itself to be a prolific source of these compounds, especially ellagitannin derivatives; mainly ellagic acid and its glycosides. In this sense, the bark of *B. excelsa* constitutes a reliable alternative source for obtaining active polyphenolic compounds. This is also observed for *Punica granatum* and *Quercus petraea* (Konig *et al*., 1994; Tanaka & Nishioka, 1986). Furthermore, the radical-scavenging activities of BEB increased in a concentration-dependent manner for $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $O_2^{\cdot-}$, and these results are confirmed by the inhibition of xanthine oxidase.

The cytotoxic effects of BEB were examined

using a MTT assay in order to determine non-toxic concentrations for inflammatory and antioxidant activity test. BEB did not show any significant toxic effect on the growth of cells. Therefore, in order to determine whether BEB prevents oxidative stress by inhibiting ROS generation, we measured intracellular ROS concentrations with DCFH-DA, and found that treatment of J774 cells with H_2O_2 induced a marked rise in oxidative stress, which was characterized by excessive ROS production. This was followed by a reduction in the levels of intracellular ROS by BEB. Quercetin, at the level of concentration used in this study, has been shown to exhibit strong suppression of intracellular ROS generation, and BEB was as efficient as the antioxidant standard in scavenging ROS (Sittisart & Chitsomboon, 2014).

Macrophages are normal cells that are vital for the recognition and elimination of microbial pathogens, and the survival of macrophages is of critical importance to the host defense system (Grom, 2012). Several previous studies have demonstrated that the virulence of some bacteria is due to their ability to trigger the death of activated macrophages via stimulating ROS production. Our results showed anti-inflammatory activity of BEB in the same concentrations of its antioxidant activity.

By using the MTT assay, the present study also showed that the exposure of macrophages to H_2O_2 resulted in a reduction of cell viability. However, pretreatment with different concentrations of BEB, with or without H_2O_2, greatly diminished loss of cell viability when compared with quercetin, the positive control.

As has been well documented, the response of an inducible heme-degrading enzyme, heme oxygenase-1 (HO-1), which is an antioxidant enzyme, for a wide range of cellular stresses, exhibits adaptive responses to oxidative stress including skeletal muscle cells (Kang *et al*., 2015). Therefore, targeted induction of this enzyme may be considered an important therapeutic strategy for protection against oxidative damage. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a major transcription factor of HO-1, and BEB induces HO-1 expression to afford the protection of macrophages against cytotoxicity and protects cells from H_2O_2-induced cell death by inducing antioxidant enzymes, including HO-1. The most significant finding in our study is the demonstration of the involvement of the Nrf2 pathway in BEB-mediated HO-1 gene induction.

Nitric oxide ($NO^{\bullet}$) is an important chemical mediator generated by endothelial cells, macrophages, and neurons involved in several biological processes. An elevated production of $NO^{\bullet}$ could promote several diseases. Oxygen reacts with nitric oxide to generate nitrite and peroxynitrite anions that act as free radicals (Bayir *et al*., 2007; Moukette *et al*., 2015). Our results showed that BEB could combine with oxygen and inhibit the generation of peroxynitrite anions. Similar studies have shown prevention of DNA damage, lipid peroxidation, and increased protein expression and genomic expression of NOS isoforms (NOS 1, 2, 3), if the organism is supplemented by extracts containing large amounts of antioxidant compounds (Colombo *et al*., 2015).

Our research demonstrated that the GPx1 expression levels were elevated in cells stimulated with H_2O_2. This demonstrates GPx1's specific function for H_2O_2 degradation. The GPx expression levels in the sample containing quercetin showed themselves to be significantly elevated compared to the other samples. This can be attributed to quercetin having a lower efficiency when compared to BEB. This occurs even before a natural increase in gene expression caused by the phenolic compounds by moving the Nrf2 factor to enhance the production of these enzymes (Kaspar *et al*., 2009).

We observed the greatest reduction in Nrf2 expression when the cells were treated with 100 μg/mL of BEB, which demonstrates its effectiveness in capturing free radicals. These data can be explained by presence of phenolic compounds such as ellagitannin derivatives, ellagic acid, its glycosides, and valoneic acid dilactone in BEB. Our findings were similar to the negative control, which is in accordance with previous observations regarding this class of compounds in which they were classified being potent antioxidants. This has been previously determined for punicalagin isolated from pomegranate (*Punica granatum*), and different ellagitannins from different heartnut varieties (*Juglans ailanthifolia* var. *cordiformis*) (Li *et al*., 2006; Cerdá *et al*., 2004).

Previous studies have shown that *C. elegans* can be used as an alternative *in vivo* model for obtaining rapid results in toxicity studies in order to assess the pharmacological and toxic effects of drugs (Leung *et al*., 2008; Bonamigo *et al*., 2017), since it is a multicellular organism with a high reproduction rate and short life cycle, which makes it an excellent *in vivo* model for complementing cell culture-based systems (Leung *et al*., 2008). It is possible to classify this nematode as a relatively simple organism, which

can possibly be used in a wide range of studies, and, as such it enjoys the advantage of not having its use regulated by ethics committees, since it is an invertebrate (Hirotsu *et al*., 2015).

In the *in vivo* assay of LPS-induced peritonitis in mice, BEB was able to inhibit the migration of leukocytes from the peritoneal cavity (inflammatory site) of the animals when compared to the dexamethasone control. This response may have been promoted by the inhibition of NFkβ, ERK1/2, MAPK and STAT signaling pathways that are related to the inflammatory process and which promote the transcription of different genes that modulate inflammation. However, additional assays are needed to assess gene expression *in vivo*.

The interaction of neutrophils recruited at the site of inflammation with resident cells and local inflammatory mediators can lead to the production of several mediators, including cytokines/chemokines, degradation enzymes, oxygen and nitrogen species, and metalloproteases that can further amplify the inflammatory response and trigger damage to neighboring tissues.

CONCLUSIONS

Bark extract from *B. excelsa* could protect macrophages from cytotoxicity caused by H_2O_2 by decreasing expression of ROS production. This extract also presents *in vitro* and *in vivo* anti-inflammatory activities. In addition, the upregulation of HO-1 expression affords protection of macrophages against cytotoxicity and protects cells from H_2O_2-induced cell death by inducing antioxidant enzymes, including GPX1. The mechanisms of action involved in the findings described in this study may be related to the presence of certain compounds present in the extract, which in previous research have already demonstrated the activities shown here. However, the specific mechanisms involved in their crosstalk with upstream and downstream signaling molecules still need to be better understood.

ACKNOWLEDGMENTS

For this study, the authors acknowledge CNPQ, CAPES and FAPEAM for their financial support. We also thank the Analytical Center at UFAM for the chemical analyses.

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