Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

# Bioactive Compounds in Persea americana var. Quintal Byproducts: Exploring Their Antioxidant Potential and Inhibitory Effects on LDL Oxidation, Lipid Peroxidation, and Nitric Oxide Production

**Maria Sol Peña Carrillo**
Universidade Federal de Uberlândia
**Romário Divino Vilarinho Galvão**
Universidade Federal de Uberlândia
**Ana Luiza Silva Borges**
Universidade Federal de Uberlândia
**Vinícius Prado Bittar**
Universidade Federal de Uberlândia
**Iasmin Aparecida Cunha Araújo**
Universidade Federal de Uberlândia
**Mário Machado Martins**
Universidade Federal de Uberlândia
**Diego Godina Prado**
Universidade Federal de Uberlândia
**Françoise Vasconcelos Botelho**
Universidade Federal de Uberlândia
**Alberto de Oliveira**
Universidade Federal de Uberlândia
**Neide Maria Silva**
Universidade Federal de Uberlândia
**Allisson Benatti Justino**
allissonbjustino@ufu.br
Universidade Federal de Uberlândia
**Foued Salmen Espindola**
Universidade Federal de Uberlândia

**Research Article**

**Keywords:** Persea americana, Quintal by-products, Antioxidant activity, Oxidative stress, LDL oxidation, Phenolic compounds

**Posted Date:** September 9th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-10549533/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

**Additional Declarations:** No competing interests reported.

## Abstract

Oxidative stress results from an imbalance between reactive oxygen species and the body's antioxidant defenses, leading to cellular, lipid, protein, and neuronal damage, and is associated with numerous diseases. Medicinal plants represent valuable sources of natural antioxidant compounds capable of mitigating such damage. Persea americana (Lauraceae), whose fruit — the avocado — is widely consumed globally and cultivated in warm climates, generates by-products (peel and seed) comprising 25–33% of fruit weight. These are typically discarded, resulting in economic loss, environmental issues, and unexplored bioactive potential. This study evaluated the antioxidant potential of ethanolic extracts and organic fractions from Quintal avocado by-products, focusing on inhibitory effects on LDL oxidation, lipid peroxidation, and nitric oxide (NO) production. Extracts were obtained by maceration and fractions by liquid-liquid partitioning. HPLC-ESI-MS/MS identified procyanidins, catechins, and quercetin, particularly in the ethyl acetate and n-butanol fractions from peel (FAPC, FBPC) and seed (FAPS, FBPS). These fractions exhibited high activity in FRAP and DPPH assays and protected over 50% of erythrocytes from AAPH-induced oxidation at 1 mg/mL. Intrinsic hemolytic activity remained below 25% for FAPS and FAPC, and 0.04% for FBPS and FBPC. At 2 µg/mL, all four fractions inhibited LDL oxidation and peroxidation in vitro, and at 25 µg/mL suppressed lipid peroxidation in cardiac tissue. No cytotoxic activity was observed. FAPC and FBPC inhibited NO production at 100 and 10 µg/mL, respectively. These findings suggest that P. americana var. Quintal by-products are a promising source of bioactive compounds for treating oxidative stress-related diseases.

Clinical trial number: not applicable.


## 1. Introduction

Oxidative stress is a condition characterized by the excessive production of reactive oxidant species (ROS) and the inadequate efficiency of antioxidant systems to neutralize them (1, 2). This state can cause a variety of metabolic disturbances that collectively contribute to the development of several chronic and degenerative diseases affecting neuronal, muscular, digestive, endocrine, and cardiac systems (1, 3, 4).

In the context of cardiovascular diseases such as atherosclerosis, several studies have shown that key pathogenic mechanisms—including the oxidation of low-density lipoproteins (LDL), inflammatory processes, and endothelial dysfunction—are closely associated with oxidative stress (5, 6). In this sense, the search for natural antioxidant sources has gained increasing importance. Historically, medicinal plants have been a vital source of newbioactive compounds capable of neutralizing ROS and to treat various diseases related to oxidative stress (6, 7).

*Persea americana* is a tree species belonging to the Lauraceae family, whose fruit, the avocado, is highly consumed in the world and is cultivated in warm climate regions (8). Typically, only the avocado pulp is utilized, generating waste (seed and peel) that constitutes between ~ 25% of the fruit's total weight(9). Unfortunately, these by-products are often discarded without additional applications, posing potential environmental concerns (10).

Currently, several studies on phytochemical compounds have demonstrated the pharmacological potential of avocado by-products, including anti-inflammatory, antihypertensive, antidiabetic, hypoglycemic, antimicrobial, and antifungal activities, as well as applications in dermatology and insect control (9, 11, 12, 13, 14, 15, 16, 17, 18). These properties are associated with the presence of saponins, acetogenins, caffeic acid, hydroxybenzoic acid, and predominantly polyphenols such as procyanidins (including catechin and epicatechin) (19, 14, 20). However, it is important to note that both the bioactivity and chemical composition can vary depending on the avocado variety being studied (18).

Avocado Quintal is a Brazilian variety cultivated from March to July, especially in regions with a warm climate (21). This variety is the least investigated and is mainly characterized by its large size and weight, ranging from 600 to 1000 g per fruit, unlike other smaller varieties that weigh approximately 150 to 400 g (22).

Previous studies have reported the medicinal properties of by-products from various avocado varieties; however, research on the Quintal variety is scarce. Consequently, this research aims to evaluate the antioxidant potential of the ethanolic extract and organic fractions derived from the by-products of *Persea americana* var. Quintal, as well as their inhibitory potential on LDL oxidation, lipid peroxidation, and NO production.

## 2. Methodology

# 2.1 Reagents

Ethanol, methanol, and organic solvents hexane, dichloromethane, ethyl acetate, and n-butanol were purchased from Vetec Quimica Fina Ltda (Duque de Caxias, Rio de Janeiro, Brazil). Dimethyl sulfoxide was purchased from Merck (Darmstadt, HE, Germany).

Folin-Ciocalteu, gallic acid, vanillin, catechin, quercetin, Sodium nitrate, aluminum chloride, sodium carbonate, Folin-Ciocalteu, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, 2,4,6-Tris(2-pyridyl)-s-triazine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), fluorescein, n-acetilcistenía, 2'-7'-dichlorodihydrofluorescein (DCFH-DA ), potassium chloride, potassium bromide, 2-Thiobarbituric acid, aprotinin from bovine lung, were purchased from Sigma Aldrich Sigma Aldrich (St. Louis, MO, USA).

# 2.2 Plant material

Avocado fruits (Persea americana Mill. cv. Quintal) were obtained in March 2022 from “O Rei do Abacate,” a licensed vendor operating at CEASA Uberlândia (Centro de Abastecimento de Produtos Agrícolas), Minas Gerais, Brazil. This period corresponds to the peak seasonal availability of the Quintal cultivar in the region. The fruits originated from a commercial orchard located in the agricultural corridor between the municipalities of Uberlândia and Uberaba, Minas Gerais (approximate coordinates: 18°55'08" S, 48°16'37" W), an area recognized for its favorable conditions for avocado cultivation.

Only undamaged fruits of uniform size (400–450 g), with intact pedicels and no visible signs of mechanical injury or disease, were selected. The fruits were immediately transported to the laboratory in ventilated plastic crates under ambient temperature conditions, with a transit time of less than 30 min. To standardize ripening, samples were stored in a dark room for 48–72 h until reaching optimal maturity, determined by slight yield to finger pressure at the apical end.

After ripening, peels and seeds were manually separated, yielding a total of 616.98 g of peel and 706.79 g of seeds from thirteen Quintal avocados. Fruit authenticity was confirmed based on macroscopic and sensory characteristics, including shape, texture, and size.

# 2.3 Extraction and fractionation

The collected material was pulverized and subjected to a static maceration process with 98% ethanol (1:5 w/v) for seven days at room temperature and in the absence of light. Subsequently, the solutions were filtered and rotary-evaporated (Buchi Rotovapor R-215) at 40°C. The ethanolic extracts obtained from the seed (EEPS) and peel (EEPC) were weighed and frozen for subsequent lyophilization (L 101, Liotop). To obtain organic fractions, 20 g of EEPS and 20 g of EEPC were diluted in hydro-methanol (9:1 w/v). Then, each sample solution was filtered and partitioned by order of polarity: hexane (FHPS and FHPC), dichloromethane (FDPS and FDPC), ethyl acetate (FAPS and FAPC), and n-butanol (FBPS and FBPC). The residue from the partitioning process constitutes the hydro-methanolic fraction (FMPS and FMPC). The obtained extracts were collected at -20°C, lyophilized, and stored until the date of analysis.

# 2.4 Phytochemical Prospection

# 2.4.1 Total phenolic content

Total phenolic content in EEPC, EESP, and the organic fractions (1 mg/mL in ethanol) was assessed using the Folin–Ciocalteu method (23). The samples were incubated with a Folin-Ciocalteau solution for 6 minutes at 25°C. Then, the mixture was incubated with 7% of sodium carbonate for 2 hours at 25 ºC in the absence of light. Gallic acid was used as a standard to quantify phenols, and readings were taken at 760 nm (Versamax, Molecular Devices, EUA). Results were expressed in milligrams of gallic acid equivalents per gram (mg GAE/g).

## 2.4.2 Total flavonoid content

The determination of the total flavonoid content of EEPC, EESP, and organic fractions (1 mg/mL in ethanol) was conducted using the method described by Popova et al. (24). The samples were incubated with 5% sodium nitrite for 6 minutes at 25°C. Then, the mixture was incubated with 10% de of aluminum chloride for more six minutes at 25 ºC in the dark. Quercetin was used as standard to construct an analytical curve to determinate the flavonoid content, and readings were taken at 425 nm (Versamax, Molecular Devices, EUA). Results were expressed in milligrams of quercetin equivalents per gram (mg QE/g).

## 2.4.3 Condensed tannin content

The determination of the condensed tannin content of EEPC, EESP, and organic fractions (1 mg/mL in ethanol) was conducted using the method described by Schofield et al. (25). The samples were incubated with 4% de vanillin and HCL for 6 minutes at 25°C. Catechin was used as standard to construct an analytical curve to determinate the condensed tannin content, and readings were taken at 500 nm (Versamax, Molecular Devices, EUA). Results were expressed in milligrams of catechin equivalents per gram (mg CE/g).

## 2.5 *In-vitro* antioxidant activity

## 2.5.1 FRAP

For this assay, EEPS and EEPC and organic fractions (1 mg/mL in ethanol) were mixed with FRAP reagent (300 mM sodium acetate buffer at pH 3.6, 10 mM TPTZ, and 20 mM ferric chloride in a ratio of 10:1:1) for 6 minutes at 37°C in the absence of light in a 96-well microplate. Ascorbic acid (ACAS) and gallic acid (GAAC) were used as positive controls. The analytical standard curve was prepared using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and readings were taken at 593 nm (EnSpire spectrophotometer PerkinElmer). The results were expressed as µmol of Trolox equivalents (µmol TE/g) (26).

## 2.5.2 DPPH

This assay evaluated antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical method (27).Serial dilutions of each extract and fraction were prepared in ethanol. These dilutions were incubated with DPPH (60 mM) in a microplate for 30 minutes at 37°C in the dark. Absorbance was measured at 517 nm using an EnSpire spectrophotometer (PerkinElmer). The inherent absorbance of DPPH served as a control, and absorbances of samples diluted in ethanol without DPPH were considered as blanks. ACAS and GAAC were used as positive controls. The DPPH scavenging capacity of the samples were determined using the following formula: DPPH (%) = [(Abs DPPH - Abs sample) / (Abs DPPH - A blank)] x 100

## 2.5.3 ORAC

The ethanolic extracts EEPS and EEPC and their fractions were diluted in ethanol (0.1 mg/mL) and then incubated with fluorescein (0.085 nmol/L in 75 mM phosphate buffer, pH 7) for 15 minutes at room temperature. Subsequently, AAPH (153 mmol/L) was added to initiate the reaction, and the fluorescence intensity (485 nmex/528 nmem) was measured at 37°C for 90 minutes with readings every 2 minutes (EnSpire, PerkinElmer). The antioxidant capacity was determined using an analytical curve constructed with trolox as a standard (µmol Trolox equivalents/g). Quercetin was used as a positive control

due to its compatibility with the fluorescein-based ORAC system and its reproducible fluorescence protection kinetics (28, 29).

## 2.6 Erythrocyte hemolysis assay mediated by AAPH

### 2.6.1 Preparation of erythrocyte suspension

To evaluate the inhibition of AAPH-mediated oxidation in red blood cells, a modified version of the method proposed by Zhao et al. (30) was employed. Blood samples were obtained from healthy, non-smoking volunteers using heparinized vacutainer tubes, in accordance with the ethical principles outlined in the Declaration of Helsinki, and the study was approved by the local Ethics Committee (protocol no. 61082522.4.0000.5704; approval no. 5.671.038). The collected samples were centrifuged at 1500 × g for 10 min to isolate erythrocytes. The resulting cell pellet was washed three times with 1× PBS buffer (pH 7.4), with centrifugation at 1500 × g for 10 min between each wash, in order to remove plasma components and obtain purified erythrocytes. Subsequently, a 20% (v/v) erythrocyte suspension was prepared in 1× PBS (pH 7.4) and used for further analyses.

### 2.6.2 Oxidative hemolysis assay

The organic fractions FDPC, FDPS, FAPC, FAPS, FBPC, and FBPS (2,5 mg/ml) were incubated with ES solution for 20 min at 37°C with low agitation. Then, the mixtures were incubated with AAPH (200 mM) with moderate agitation at 37°C for 2 h. After incubation, 100 µL of each sample was collected, diluted in 1X PBS (pH 7.4), and centrifuged at 1500 g for 10 minutes. The absorbance of the supernatant was recorded at 540 nm by a spectrophotometer (EnSpire, PerkinElmer). AA (1.5 mM) and N-acetylcysteine (NAC) (2 mM) were chosen as negative controls, and distilled water as positive control. ES solution and PBS were mixed as a blank control. The percent oxidative hemolysis was calculated as follows: oxHemolysis (%) = ((Abs sample – Abs. blank)/(Abs. Controle (+) – Abs. blank)). To evaluate the intrinsic hemolytic activity of each extract, 200 µL of 1X PBS was added instead of 200 µL of AAPH (30).

### 2.6.3 Generation of reactive oxygen species (ROS)

The relative intracellular level of ROS was measured using the assay with 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA). In each sample, distilled water was added to induce hemolysis. Subsequently, the mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. Afterward, the supernatants were collected and incubated with a buffer (sodium phosphate 20 mM with KCl 140 mM, pH 7.4) and DCFH-DA for 3 minutes at room temperature and in the absence of light. The generation of ROS was recorded by detecting the fluorescence intensity on a fluorescence reader (EnSpire, PerkinElmer) (Ex = 488 nm, Em = 525 nm) (31).

## 2.7 Inhibition of Oxidation and Peroxidation of low-density lipoprotein (LDL)

### 2.7.1 Isolation and induction of LDL Oxidation

The LDL isolation was performed following the method already described by Chung et al. (32). Blood samples were collected from healthy individuals in vacutainer tubes with heparin, following ethical principles approved by the local Ethics Committee (Uberlandia-Minas Gerais, Brazil) based on protocol 5,671,038. The samples were centrifuged at 3000 rpm for 10 minutes at 4°C. Subsequently, potassium bromide (0.327g/mL of plasma), aprotinin (5 µL/mL), benzamidine (5 µL/mL), 0.5 M PMSF (0.5 µL/mL), chloramphenicol (20 µL/mL), and sodium azide (10 µL/mL) were added to the collected plasma. Then, the mixture was ultracentrifuged at 40,000 rpm for 2 hours and 30 minutes at 4°C. The LDL band was collected and dialyzed using 1X PBS (pH 7.4) for 36 hours to obtain the isolated LDL. The final protein concentration was accessed by Bradford methodology (33). The organic fractions FDPC, FDPS, FAPC, FAPS, FBPC, FBPS and quercetin (0,5 µg/mL, 1 µg/mL and 2 µg/mL) were incubated with LDL isolate (50 µg/mL) and $CuSO_4$ (5 µM) to induce oxidation. LDL in solution with PBS was used as a negative control, while LDL in solution with PBS plus copper served as the positive control. The process was

monitored over 3 hours at 37°C in a microplate, with readings taken every 2 minutes at 234 nm (EnSpire, PerkinElmer). The level of oxidation was quantified by calculating the area under the curve (AUC) of absorbance over time.

## 2.7.2 Induction of LDL Peroxidation

Lipid peroxidation was determined according to thiobarbituric acid reactive species (TBARs) assay (34). After the LDL oxidation induction assay, the samples were taken directly from the plate and incubated with 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) for 2 hours at 100 ºC. Subsequently, butanol was added to each mixture, and they were centrifuged at 5000 xg for 3 minutes. The obtained supernatant was collected to measure fluorescence intensity at 515 nm excitation / 553 nm emission (EnSpire, PerkinElmer). Lipid peroxidation was quantified using a malondialdehyde (MDA) standard curve, and results were expressed as µmol MDA equivalents per milliliter.

## 2.8 *Ex-vivo* analysis in cardiac tissue

The hearts were extracted from healthy C57BL/6 mice (6–8 weeks old), sourced from the Center for Bioterism and Experimentation of the Federal University of Uberlandia (UFU) with official approval from the animal ethics committee (Craft No.7/2022/CEUA/PROPP/REITO-UFU of July 14, 2022). The animals were euthanized, and the hearts were extracted, transported in liquid nitrogen, and subsequently stored at -80°C. The hearts were weighed, homogenized in a sodium phosphate buffer (20 mM) with potassium chloride (140 mM) (pH 7.4) (1:10 m/v), and then centrifuged at 3,000 g for 10 minutes at 4°C. The resulting supernatant was exposed to the oxidizing agent AAPH (100 mM) and treated with six different organic fractions of *P. americana* (FDPS/C, FAPS/C, and FBPS/C) at concentrations of 50, 25 and 12.5 µg/mL. Incubation took place for 1 hour at 37°C with agitation every 10 minutes (35). For positive control, only AAPH was used without treatment, and buffer served as the negative control. Subsequently, these samples were submitted to Bradford essay and TBARS assay.

## 2.9 Anti-inflammatory activity

## 2.9.1 Cell Culture

RAW 264.7 macrophages (*ATCC TIB-71*) were cultured in 75 cm² flasks using complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. After reaching appropriate confluency, cells were detached using a cell scraper and seeded into 96-well plates at a density of $5 \times 10^4$ cells/well in 200 µL of RPMI medium. Cells were allowed to adhere for 24 hours prior to subsequent experimental procedures.

## 2.9.2 Cell Viability assay

Cell viability was assessed in a separate assay under non-stimulated conditions in order to determine the intrinsic cytotoxicity of the tested fractions. RAW 264.7 macrophages were treated with the extracts at concentrations of 1000, 500, 100, and 10 µg/mL for 24 h. After treatment, the culture medium was removed and MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, USA) was added at a concentration of 50 µg/well. Plates were incubated for 4 h at 37°C in a humidified atmosphere containing 5% $CO_2$ to allow formazan crystal formation. Subsequently, the supernatant was removed, and dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells maintained in RPMI medium (36).

## 2.9.3 LPS-induced inflammatory post-treatment model

The anti-inflammatory potential of the fractions was evaluated using a post-treatment model in LPS-stimulated RAW 264.7 macrophages (37). After a 24 h adhesion period, cells were stimulated with lipopolysaccharide (LPS, 1 µg/mL) for 24 h to induce an inflammatory response. Cells maintained in RPMI medium without LPS were used as non-stimulated controls. Following LPS stimulation, the medium was replaced, and cells were treated with the extracts at concentrations of 1000,

500, 100, and 10 µg/mL for an additional 24 hours. This experimental design allows the evaluation of the ability of the fractions to modulate nitrite production after the inflammatory stimulus has been established, mimicking a post-treatment or therapeutic-like condition rather than a preventive approach.

### 2.9.4 Nitric Oxide (NO) Concentration Assay

The production of NO was estimated based on the accumulation of nitrite ($NO_2^-$) using the Griess reagent, as previously described (38). After the post-treatment incubation period, culture supernatants were collected and mixed with an equal volume (50 µL) of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine (v/v) in 2.5% phosphoric acid). The reaction mixture was incubated at room temperature for 10 min. Absorbance was measured at 570 nm using a microplate reader, and nitrite concentrations were determined using a sodium nitrite standard curve. Results were expressed as percentage of inhibition of NO production relative to LPS-stimulated cells.

## 2.10 HPLC-ES-MS/MS Analysis

The FAPC, FBPC, FAPS, and FBPS fractions were analyzed by high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI/MS). These fractions were selected based on their performance in previous biological assays. The analysis was carried out using an Agilent Infinity 1260 liquid chromatograph coupled to a high-resolution mass spectrometer (Agilent® model 6520B) equipped with an ESI source. Each fraction was prepared at a concentration of 5 mg/mL in HPLC-grade methanol and filtered through 0.22 µm syringe filters. The mobile phase consisted of 0.1% formic acid in water (v/v) (solvent A) and methanol (solvent B). The gradient program was as follows: 10% B at 0 min, increasing to 98% B from 0 to 10 min, and held at 98% B from 11 to 17 min. Ionization parameters were set at 220°C, 20 psi nebulizer pressure, 8 L/min drying gas flow, and a capillary voltage of 4.5 kV. Tandem mass spectra (MS/MS) were acquired using collision energies ranging from 5 to 30 eV to achieve optimal fragmentation. MS data were analyzed using Agilent MassHunter® software to obtain exact mass values, and the resulting spectra were compared to previously published data and reference databases, including PubChem and MassBank.

## 2.11 Statistical Analysis

The results were expressed as mean ± standard deviation, except for the IC50 calculation, where the results were expressed as mean ± standard error of the mean. Analysis of variance between groups was conducted using the One-Way ANOVA test with Tukey's post-test for multiple comparisons. Values of $p < 0.05$ were considered significant. Both statistical analyses and graph plotting were performed using GraphPad Prism 8.0 software.

# 3. Results

## 3.1 Phytochemical prospection

In the phytochemical screening analysis of *P. americana* var. Quintal seeds, FAPS exhibited a notable concentration of polyphenols, tannins, and flavonoids (365.90 ± 29.47 mg GAE/g, 1099.34 ± 45.02 mg EGC/g, and 1307.01 ± 18.98 mg QCT/g, respectively). Following closely are FBPS and FDPS, both displaying similar quantities (Table 1). The FAPC fraction showed the highest values for phenols, tannins, and flavonoids, with values of 297.00 ± 13.28 mg GAE/g, 1639.00 ± 53.43 mg EGC/g, and 537.70 ± 5.17 mg QCT/g, respectively. EBPC quantified a value of 941.70 ± 36.43 for tannins and 348.83 ± 13.29 for flavonoids. Compared to FAPC and FBPC, FAPS and FBPS exhibit higher concentrations of phenols, tannins, and flavonoids. FHPS, FHPC, FMPS and FMPC showed lowest quantities compared to the other organic fractions (Table 1). Although not the lowest overall, the ethanolic extracts from seed and peel (EEPS and EEPC) presented lower levels of total phenols (84.86 ± 7.17 and 111.30 ± 0.65 mg GAE / g), flavonoids (354.61 ± 13.73 and 285.11 ± 11.37 mg QCT / g), and condensed tannins (189.70 ± 55.98 and 599.50 ± 21 mg EGC/ g) compared specifically to the ethyl acetate and n-butanol fractions, indicating that the fractionation process effectively concentrated polyphenols in the more polar fractions.

Table 1
Total phenols, flavonoids and condensed tannis content of the ethanolic extract of *P. americana* var. Quintal by-products and its organic fractions. Values are expressed as mean ± standard deviation, and different letters indicate statistical significance ($p < 0.05$).

| EEPS | Total phenols (mg GAE/g) | Flavonoids (mg QE/g) | Condensed tannins (mg CE/g) |
|---|---|---|---|
| | **84.86 ± 7.17** □□□□ | **189.70 ± 55.98** □□ | **354.61 ± 13.73** □□ |
| FHPS | 7.88 ± 4.98 □□ | 22.86 ± 19.80 □ | 393.92 ± 9.99 □□ |
| FDPS | 14.80 ± 11.16 □□ | 226.90 ± 20.57 □□ | 474.12 ± 14.9 □ |
| FAPS | 365.90 ± 29.47 □ | 1099.34 ± 45.02 □ | 1307.01 ± 18.98 □ |
| FBPS | 148.71 ± 14.54 □ | 389.81 ± 4.43 □ | 446.20 ± 12.08 □□ |
| FMPS | 33.02 ± 7.09 □ | 53.09 ± 19.44 □□ | 38.27 ± 2.48 □ |
| EEPC | 111.30 ± 0.65 □ | 599.50 ± 21.86 □ | 285.11 ± 11.37 □ |
| FHPC | 1.63 ± 0.81 □ | 31.51 ± 4.88 □ | 259.54 ± 10.19 □□ |
| FDPC | 21.26 ± 2.75 □ | 13.28 ± 0.93 □ | 233.20 ± 3.25 □ |
| FAPC | 297.00 ± 13.28 □ | 1639.00 ± 53.43 □ | 537.70 ± 5.17 □ |
| EBPC | 171.60 ± 5.35 □ | 941.70 ± 36.43 □ | 348.83 ± 13.29 □ |
| EMPC | 21.82 ± 1.46 □ | 97.43 ± 4.07 □ | 65.46 5.02 □ |

## 3.2 Antioxidant activity

The antioxidant capacity of EEPS and its fractions was determined using the FRAP, ORAC, and DPPH methods. In the FRAP analysis, the FAPS fraction exhibited the highest antioxidant capacity (787.7 ± 24.35 µmol Trolox eq/g), however, show significant difference compared to the GAAC and ACAS controls (915 ± 11,81 e 916 ± 6,64 µmol Trolox eq/g respectively). The FDPS and FBPS fractions also demonstrated substantial antioxidant activities, with values of 442 ± 29.77 and 409.6 ± 10.96 µmol Trolox eq/g, respectively (Fig. 1a). In the ORAC assay, EEPS, FAPS, and FBPS (4.049 ± 55.20 µmol Trolox eq/g, 2.966 ± 69.60 e 2.966 ± 122.28 µmol Trolox eq/g respectively) showed high antioxidant capacities, similar to the quercetin (4147 ± 27,2 µmol Trolox eq/g) (Fig. 1b). In the DPPH method, FAPS demonstrated the highest antioxidant potential with an $IC_{50}$ of 3.55 ± 13.46 µg/mL, followed by FDPS and FBPS (Fig. 1c). Collectively, the FAPS fraction demonstrated the most promising antioxidant performance across all assays, with FDPS and FBPS also showing noteworthy activities.

Similarly, the antioxidant capacity of the ethanolic peel extract (EEPC) and its organic fractions was evaluated using the FRAP, ORAC, and DPPH assays. In the FRAP assay (Fig. 2a), the FAPC fraction exhibited the highest antioxidant value (872.6 ± 22.58 µmol Trolox equivalents/g), with no significant difference compared to the controls, GAAC and ACAS, which showed values of 910.2 ± 9.21 and 913.5 ± 4.93 µmol Trolox eq/g, respectively. Conversely, the FBPC fraction also demonstrated antioxidant capacity (562.5 ± 54.53 µmol Trolox eq/g), though this value was significantly different from the controls. In the ORAC assay (Fig. 2b), EEPC and its organic fractions were able to inhibit AAPH-induced oxidation, although their antioxidant capacities were significantly lower than that of the quercetin standard (3147 ± 25.4 µmol Trolox eq/g). Among them, EEPC (3049 ± 39.03 µmol Trolox eq/g) and FAPC (2882 ± 12.36 µmol Trolox eq/g) were the closest in value to quercetin. In the DPPH assay, FAPC stood out with an $IC_{50}$ of 0.56 ± 7.97 µg/mL, which was even lower than that of the control ASAC (0.80 ± 7.48 µ/mL), with no significant difference between them. FBPC also showed notable activity with $IC_{50}$ values of 1.17 ± 11.12 µg/mL (Fig. 2c). Overall, FAPC and FBPC were the fractions that presented the most promising results across all three assays.

## 3.3 Inhibition of AAPH-Induced Erythrocyte Hemolysis

Based on previous results and the observed phytochemical profile, the ability to protect against AAPH-induced hemolysis was evaluated in the dichloromethane, ethyl acetate, and n-butanol fractions of seed and peel (Fig. 3a). It was observed that the FBPS (35.25 ± 2.35%), FDPC (30.10 ± 1.00%), FAPC (46.87 ± 2.72%), and FBPC (20.47 ± 0.026%) fractions remarkably protected against oxidation in blood cells and showed significant differences compared to + C (70.51 ± 1.69%). However, they did not achieve the same level of protection as NAC (0.60 ± 0.44%) and ASAC (0.72 ± 0.38%). The presence of reactive oxygen species (ROS) was evaluated (Fig. 3b), and the results indicate that FBPS (931 ± 15 RFU), FAPC (1366 ± 123 RFU), and FBPC (869 ± 4 RFU) exhibited low ROS values compared to C+ (2468 ± 28 RFU) with $p < 0.0001$.

In the hemolytic assay without AAPH, it was observed that the NAC (0.88 ± 0.50%) and ASAC (1.41 ± 0.66%) controls were non-hemolytic and showed no significant difference compared to the negative control (PBS 1X, 0.03 ± 0.03%), similar to the FBPS (0.03 ± 1.07%) and FBPC (2.52 ± 1.17%). On the other hand, FAPS (19.06 ± 0,92%) and FAPC (24.22 ± 0.95) exhibited moderate hemolytic activity (> 25%) with significant differences compared to both the positive ($H_2O$, 96.44 ± 3.56%) and negative controls. FDPS (54.32 ± 1.95%) and FDPC (97.42 ± 3.28%) exhibited a pronounced hemolytic activity exceeding 50%. Notably, FDPC showed no significant difference compared to the positive control.

## 3.4 Inhibition of LDL Oxidation and Peroxidation

The inhibition of $Cu^{2+}$-induced LDL peroxidation was evaluated for the organic fractions FDPS/PC, FAPS/PC, and FBPS/PC (Fig. 4). The quercetin control was able to inhibit LDL oxidation at concentrations of 0.5 and 1 µg/mL, and lipid peroxidation at 0.5, 1.0, and 2.0 µg/mL, without showing significant differences compared to the C- (LDL without $Cu^{2+}$). For the seed-derived fractions, FAPS was able to inhibit oxidation at concentrations of 0.5 (17.28 ± 10.96 AUC) and 1.0 µg/mL (7.17 ± 2.54 AUC), also reduced MDA levels at 1.0 and 2.0 µg/mL (0.33 ± 0.49 and 0.45 ± 0.75 nmol MDA/mg protein, respectively). Similarly, FBPS inhibited LDL oxidation at 1.0 µg/mL (20.03 ± 2.28 AUC) and 2.0 µg/mL (4.76 ± 0.89 AUC), and lipid peroxidation at a concentration of 2.0 µg/mL (0.36 ± 0.38 nmol MDA/mg prot).

For the peel-derived fractions, both FAPC and FBPC inhibited LDL oxidative damage at concentrations of 1.0 µg/mL (5.72 ± 1.43 and 11.07 ± 4.07, respectively) and 2.0 µg/mL (16.61 ± 3.58 and 5.08 ± 0.57, respectively). Regarding TBARS, FAPC was able to fully reduce lipid peroxidation at a concentration of 2.0 µg/mL (0.02 ± 0.37), even outperforming quercetin at all three concentrations. Similarly, FBPC reduced MDA levels at 2.0 µg/mL (0.23 ± 0.19 38 nmol MDA/mg prot). No significant differences were observed in any of the results compared to the negative control (native LDL).

## 3.5 Inhibition of Lipid Peroxidation in Cardiac Tissue in an Ex Vivo Model

The ability to inhibit AAPH-induced lipid peroxidation in cardiac tissue was assessed (Fig. 5). The fractions FAPS, FBPS, FAPC, and FBPC (50 µg/mL) were able to reduce MDA levels to 0.104 ± 0.018; 0.117 ± 0.007; 0.098 ± 0.013, and 0.098 ± 0.023 mmol MDA/mg prot. respectively, showing significant differences compared to C+ (0.394 ± 0.002 mmol MDA/mg prot.) with $p < 0.0001$. Even at lower concentrations (12.5 µg/mL), these same fractions exhibited significant differences compared to C+ with $p < 0.0001$.

## 3.6 Cytotoxicity in macrophages RAW 264.7

The cytotoxic effects of the organic fractions (FDPS, FAPS, FBPS, FDPC, FAPC, and FBPC) were evaluated using the MTT assay (Fig. 6). FAPS, FBPS, FAPC, and FBPC did not exhibit cytotoxic effects at any of the tested concentrations (1000–10 µg/mL), showing no significant differences compared to the control group (cells maintained in RPMI medium without LPS or treatment). In contrast, In contrast, FDPS and FDPC showed marked cytotoxicity, with cell viability below 25% at the highest concentrations (1000, 500, and 100 µg/mL).

## 3.7 Determination of NO levels

The ability of the organic fractions to modulate nitric oxide (NO) production was evaluated in LPS-stimulated RAW 264.7 macrophages using a post-treatment model (Fig. 7). The results reveal that the fractions FDPS and FDPC show a marked inhibition of NO production, especially at low concentrations (10 µg/mL), with highly significant differences compared to the LPS group. However, it is essential to consider that these fractions showed cytotoxic activity. In contrast, FAPC and FBPC significantly decreased NO production across the tested concentrations while maintaining cell viability, indicating a potential anti-inflammatory effect under post-stimulation conditions. FAPS displayed a non-linear, concentration-dependent behavior, with increased NO levels at the highest concentration and reduced levels at lower concentrations, suggesting a biphasic modulatory effect, and FBPS showed limited or inconsistent inhibition of NO production under the evaluated conditions.

## 3.8 HPLC-MS/ESI

As observed in the previous assays, FAPC, FBPC, FAPS and FBPS demonstrated remarkable antioxidant activity, preventing LDL and cardiac tissue oxidation and a potential to inhibit the production of NO. Furthermore, they did not exhibit hemolytic or cytotoxic activities. Therefore, these fractions were submitted to HPLC-MS/ESI analysis to identify their main bioactive compounds (Table 2). Subsequently, the dichloromethane fractions (FDPS and FDPC) were also included in the analysis to provide a more comprehensive characterization of the chemical profile of the organic extracts (Table 2).

Table 2
Compounds identified in FDPC, FAPC, FBPC, FDPS, FAPS and FBPS extracts of *P. americana* var. Quintal by HPLC-ESI-MS/MS in negative ionization mode.

| RT (min) | *m/z* [M-H]$^-$ | Exact Mass | Error (ppm) | Fragments (*m/z*) MS/MS | Molecular formula | Tentative identity | Sample | Reference |
|---|---|---|---|---|---|---|---|---|
| 0.77 | 209.0668 | 209.0667 | 0.48 | 15 eV: 101, 89, 71, 59 | C7H14O7 | Mannoheptulose | FAPC, FDPC, FBPC | (39) |
| 0.83 | 133.0144 | 133.0142 | 1.5 | 15 eV: 115, 72, 71 | C4H6O5 | Malic acid | FAPC, FAPS, FBPC, FBPS, FDPC, FDPS | (39) |
| — | 191.0565 | 191.0561 | -1.57 | 20 eV: 127, 111, 93, 87, 85 | C7H12O6 | Quinic acid | FAPC, FAPS, FBPC, FBPS, FDPC, FDPS | (40) |
| 1.02 | 117.0194 | 117.0193 | 0.85 | 15 eV: 99, 96, 73, 55 | C4H6O4 | Succinic acid | FAPC, FAPS, FBPC, FBPS | (39) |
| 2.01 | 315.0718 | 315.0722 | -1.27 | 20 eV: 153, 152, 109, 108 | C13H16O9 | Dihydroxybenzoic acid glucoside | FBPC, FBPS | (41) |
| 2.37 | 315.1085 | 315.1085 | 3.81 | 20 eV: 153, 135, 119, 113, 101, 89, 59 | C14H20O8 | Hydroxytyrosol glucoside | FAPS, FBPS, FDPS | (22, 42) |
| 2.69 | 353.0881 | 353.0878 | 0.85 | 20 eV: 191, 179, 135 | C16H18O9 | Chlorogenic acid isomer I | FAPS, FBPS, FDPS | (39) |
| 2.97 | 341.0884 | 341.0878 | 1.76 | 25 eV: 179, 161, 135, 89, 59 | C15H18O9 | Caffeic acid glucoside | FAPC, FBPC | (39) |
| — | 577.1355 | 577.1351 | 0.69 | 20 eV: 451, 425, 407, 289, 287, 245, 125 | C30H26O12 | Procyanidin B-type ((epi)catechin dimer) isomer I | FAPC, FAPS, FBPS, FBPC | (43, 44) |

Note: a: The flavan-3-ol epimer was distinguished by elution order on a C18 column according to Dou et al. (47); b: [M – 2H]$^{-}$; c: The compound 593.1300 was proposed based on the fragments 447, 301 and 300 that indicates the presence of quercetin-*O*-rhamnoside and quercetin in structure, also the lost of 146 u to form fragment 447 can be explain for lost of one unit of *p*-coumaric acid – $H_2O$; d: The compounds m/z 755.1626 and 739.1678 was proposed based on the pattern of fragments, the ion fragment m/z 593 indicates the presence of Quercetin-O-p-coumaroyl)-rhamnoside, the ion fragment 447 indicates the presence os quercetin-rhamnoside, ions fragments 300 and 301 indicate the presence of flavonoid quercetin and fragments 163 indicate the presence of p-coumaric acid and 179 of ferulic acid, also the proposal was based on similarity with isomer kaempferol-glucoside (m/z 447) reported by (56).

| RT (min) | *m/z* [M-H]$^-$ | Exact Mass | Error (ppm) | Fragments (*m/z*) MS/MS | Molecular formula | Tentative identity | Sample | Reference |
|---|---|---|---|---|---|---|---|---|
| 3.02 | 1153.2611 | 1153.2619 | 0.69 | 30 eV: 1027, 983, 865, 863, 701, 577, 575, 287 | C60H50O24 | Procyanidin B-type ((epi)catechin quatramer) isomer I | FAPC, FAPS, FBPC | (44) |
| 3.07 | 865.1976 | 865.1985 | -1.04 | 30 eV: 739, 713, 695, 577, 407, 289, 287, 245, 125 | C45H38O18 | Procyanidin trimer B-type ((epi)catechin trimer) isomer I | FAPC, FBPC | (45, 40) |
| 3.37 | 337.0931 | 337.0929 | 0.59 | 20 eV: 191, 173, 163, 119 | C16H18O8 | Coumaroylquinic acid | FBPS | (40, 46) |
| 3.39 | 289.0721 | 289.0718 | 1.04 | 20 eV: 271, 245, 221, 203, 179, 151, 139, 125, 123, 109 | C15H14O6 | Catechin | FAPS, FBPS, FAPC, FDPS | (47, 44, 46) |
| 3.64 | 577.1360 | 577.1351 | 1.56 | 20 eV: 451, 425, 407, 289, 287, 151, 125 | C30H26O12 | Procyanidin B-type ((epi)catechin dimer) isomer II | FAPC, FAPS, FBPC | (43, 44) |
| 3.74 | 863.1829 | 863.1829 | 0 | 30 eV: 711, 573, 451, 411, 289 | C45H36O18 | Procyanidin trimer A-type ((epi)catechin trimer) | FAPS, FBPS, FDPS | (40) |
| 3.76 | 353.0884 | 353.0878 | 1.70 | 20 eV: 191 | C16H18O9 | Chlorogenic acid isomer II | FBPC, FBPS | (39) |
| 4.04 | 865.1977 | 865.1985 | -0.92 | 30 eV: 739, 713, 695, 577, 407, 289, 287, 245, 125 | C45H38O18 | Procyanidin trimer B-type ((epi)catechin trimer) isomer II | FAPC, FAPS, FBPC, FBPS | (45, 40) |
| 4.21 | 720.1609 | 720.1590 | 2.64 | 20 eV: 1315, 1152, 863, 720, 635, 575, 449, 289, 125 | C75H62O30 | Procyanidin B-type ((epi)catechin pentamer) | FBPC, FBPS | (48, 49) |
| — | 1008.7231 | 1008.7241 | -0.99 | 20 eV: 1727, 1439, 1151, | C105H86O42 | Procyanidin B-type ((epi)catechin heptamers) | FBPC, FBPS | (49) |

Note: a: The flavan-3-ol epimer was distinguished by elution order on a C18 column according to Dou et al. (47); b: [M – 2H]$^{-}$; c: The compound 593.1300 was proposed based on the fragments 447, 301 and 300 that indicates the presence of quercetin-*O*-rhamnoside and quercetin in structure, also the lost of 146 u to form fragment 447 can be explain for lost of one unit of *p*-coumaric acid – $H_2O$; d: The compounds m/z 755.1626 and 739.1678 was proposed based on the pattern of fragments, the ion fragment m/z 593 indicates the presence of Quercetin-O-p-coumaroyl)-rhamnoside, the ion fragment 447 indicates the presence os quercetin-rhamnoside, ions fragments 300 and 301 indicate the presence of flavonoid quercetin and fragments 163 indicate the presence of p-coumaric acid and 179 of ferulic acid, also the proposal was based on similarity with isomer kaempferol-glucoside (m/z 447) reported by (56).

| RT (min) | *m/z* [M-H]$^-$ | Exact Mass | Error (ppm) | Fragments (*m/z*) MS/MS | Molecular formula | Tentative identity | Sample | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | 1008, 863, 577, 289, 125 | | | | |
| — | 1153.2582 | 1153.2619 | -3.20 | – | C60H50O24 | Procyanidin B-type ((epi)catechin quatramer) isomer II | FBPC, FBPS | (44) |
| 4.25 | 864.1904 | 864.1907 | -0.34 | – | C90H74O36 | Procyanidin B-type ((epi)catechin hexamers) | FBPC, FBPS | (49) |
| 4.30 | 289.0719 | 289.0718 | 0.35 | 20 eV: 245, 221, 203, 179, 161, 151, 125, 123, 109 | C15H14O6 | Epicatechin | FAPS, FAPC, FBPC, FBPS, FDPC, FDPS | (47, 44, 46) |
| 4.87 | 163.0403 | 163.0401 | 1.23 | 15 eV: 119, 93 | C9H8O3 | Coumaric acid | FDPC | (39, 41) |
| 4.97 | 575.1187 | 575.1195 | -1.39 | 20 eV: 449, 423, 407, 289, 287, 125 | C30H23O12 | Procyanidin A-type ((epi)catechin dimer) | FAPS, FAPC | (50, 40) |
| 5.15 | 577.1354 | 577.1351 | 0.52 | 15 eV: 451, 425, 407, 289, 161, 125 | C30H26O12 | Procyanidin B-type ((epi)catechin dimer) isomer III | FAPC, FAPS | (43, 44) |
| 5.18 | 193.0508 | 193.0506 | 1.04 | 20 eV: 178, 134 | C10H10O4 | Ferulic acid | FDPC | (39) |
| 5.72 | 579.2095 | 579.2083 | 2.07 | 20 eV: 417, 181 | C28H36O13 | Syringaresinol-glucoside | FDPC | (39) |
| 5.97 | 463.0878 | 463.0882 | -0.86 | 20 eV: 301, 300, 271, 255, 151 | C21H20O12 | Quercetin-O-hexoside | FAPC, FAPS, FBPC | (40) |
| 5.82 | 435.1306 | 435.1297 | 2.07 | 20 eV: 345, 315, 273, 197 | C21H24O10 | Phloretin-C-hexoside (Nothofagin) | FAPC, FBPC | (51) |
| 6.02 | 477.0689 | 477.0679 | 8.17 | 20 eV: 301, 255, 151 | C21H18O13 | Quercetin-O-glucuronic acid | FBPC | (52) |
| 6.43 | 447.0934 | 447.0933 | 0.22 | 20 eV: 301 | C21H20O11 | Quercetin-O-rhamnoside | FAPC, FDPC | (50) |

Note: a: The flavan-3-ol epimer was distinguished by elution order on a C18 column according to Dou et al. (47); b: [M – 2H]$^{-}$; c: The compound 593.1300 was proposed based on the fragments 447, 301 and 300 that indicates the presence of quercetin-*O*-rhamnoside and quercetin in structure, also the lost of 146 u to form fragment 447 can be explain for lost of one unit of *p*-coumaric acid – $H_2O$; d: The compounds m/z 755.1626 and 739.1678 was proposed based on the pattern of fragments, the ion fragment m/z 593 indicates the presence of Quercetin-O-p-coumaroyl)-rhamnoside, the ion fragment 447 indicates the presence os quercetin-rhamnoside, ions fragments 300 and 301 indicate the presence of flavonoid quercetin and fragments 163 indicate the presence of p-coumaric acid and 179 of ferulic acid, also the proposal was based on similarity with isomer kaempferol-glucoside (m/z 447) reported by (56).

| RT (min) | *m/z* [M-H]$^-$ | Exact Mass | Error (ppm) | Fragments (*m/z*) MS/MS | Molecular formula | Tentative identity | Sample | Reference |
|---|---|---|---|---|---|---|---|---|
| 6.58 | 263.1293 | 263.1289 | 1.52 | 15 eV: 219, 204, 163, 153, 138 | C15H20O4 | Abscisic acid | FDPC | (39) |
| 6.86 | 207.0662 | 207.0663 | -0.48 | 20 eV: 179, 161, 135, 134, 133 | C11H12O4 | Ferulic acid derivative | FDPC | (53) |
| 7.05 | 431.0975 | 431.0984 | -2.09 | 20 eV: 285, 284, 255, 227, 151 | C21H20O10 | Kaempferol-O-rhamnoside | FAPC | (54) |
| 7.05 | 461.1087 | 461.1089 | -0.43 | 30 eV: 315, 314, 300, 299, 285, 284, 271, 257, 243 | C22H22O11 | Isorhamnetin-rhamnoside | FAPC | (55) |
| 7.26 | 301.0361 | 301.0354 | 2.33 | 20 eV: 273, 255, 179, 151, 121, 107 | C15H10O7 | Quercetin | FAPC, FDPC | (44) |
| 8.15 | 593.1300 | 593.1301 | -0.17 | 25 eV: 447, 301, 300 | C30H26O13 | Quercetin-O-(O-p-coumaroyl)-rhamnoside | FAPC | — (c) |
| 8.95 | 755.1626 | 755.1618 | 1.06 | 30 eV: 609, 593, 447, 301, 300, 179 | C39H32O16 | Quercetin-O-(O-caffeoyl-O-p-coumaroyl)-rhamnoside | FAPC, FDPC | — (d) |
| 9.28 | 739.1678 | 739.1668 | 1.35 | 30 eV: 593, 447, 301, 300, 163 | C39H32O15 | Quercetin-O-(di-O-p-coumaroyl)-rhamnoside | FAPC, FDPC | — (d) |

Note: a: The flavan-3-ol epimer was distinguished by elution order on a C18 column according to Dou et al. (47); b: [M – 2H]$^-$; c: The compound 593.1300 was proposed based on the fragments 447, 301 and 300 that indicates the presence of quercetin-*O*-rhamnoside and quercetin in structure, also the lost of 146 u to form fragment 447 can be explain for lost of one unit of *p*-coumaric acid – $H_2O$; d: The compounds m/z 755.1626 and 739.1678 was proposed based on the pattern of fragments, the ion fragment m/z 593 indicates the presence of Quercetin-O-p-coumaroyl)-rhamnoside, the ion fragment 447 indicates the presence os quercetin-rhamnoside, ions fragments 300 and 301 indicate the presence of flavonoid quercetin and fragments 163 indicate the presence of p-coumaric acid and 179 of ferulic acid, also the proposal was based on similarity with isomer kaempferol-glucoside (m/z 447) reported by (56).

## 4. Discussion

There are currently few studies evaluating the phytochemical composition and therapeutic potential of the by-products (seeds and peel) of different avocado varieties. However, most of these studies have concentrated on the Hass and Fuerte varieties, leaving limited research on the Quintal variety and its organic fractions. Therefore, this work evaluated the potential of the seed and peel extracts and their organic fractions in terms of their antioxidant activity and inhibition of oxidation and peroxidation of LDL and cardiac tissue, and the production of NO.

In the phytochemical prospecting analysis conducted in this study, the ethanolic extract of the peel (EEPC) exhibited a higher concentration of phenolic compounds compared to the ethanolic seed extract (EEPS), although the difference

between them was not statistically significant (111.30 ± 0.65 and 84.86 ± 7.17 mg GAE/g respectively). Previous studies which showed that peel extracts across different variants had higher polyphenols (9, 57, 58) suggesting that the accumulation of this compounds in the epidermis is a form of protection against ultraviolet radiation, as well as serving as a means of defense against certain pathogens and predators. In general terms, studies have shown that the highest phenolic contents are common in the peel and seeds of the fruit (59, 20).

Amado and collaborators (9) have reported the phytochemical composition of different avocado varieties, highlighting that Quintal peel extracts exhibit higher levels of phenolic compounds and flavonoids compared to other cultivars. In comparison, the phenolic and flavonoid contents obtained in the present study for both extracts and organic fractions were markedly higher, exceeding 100 mg GAE/g and 200 mg QCT/g, respectively (Table 1). Although variations in phytochemical content have been widely reported across avocado varieties (13, 16, 20), these differences are often attributed to environmental conditions, agronomic practices, extraction methods, and cultivar characteristics. Overall, the Quintal variety has been consistently described as a promising source of bioactive compounds, with its peel and seed exhibiting strong antioxidant potential and high nutritional value (9, 60)

Following liquid–liquid partitioning, a differential redistribution of phenolic compounds, flavonoids, and condensed tannins was observed, indicating that fractionation enables the selective enrichment of metabolites according to their polarity. In this context Ethyl acetate and n-butanol fractions, particularly FAPC and FBPC, showed the highest concentrations of phenolic compounds, flavonoids, and condensed tannins (Table 1), which was consistent with their superior biological activity across antioxidant and anti-inflammatory assays. These findings demonstrated that solvent fractionation is an effective strategy for concentrating polymeric phenolic compounds in plant extracts.

To further characterize the phytochemical profile of the selected fractions, HPLC-ESI-MS/MS analysis was performed. This analysis revealed a complex phytochemical profile dominated by flavan-3-ols (catechin and epicatechin), procyanidins (B- and A-type oligomers with varying degrees of polymerization), phenolic acids (chlorogenic acid isomers, caffeic and ferulic acid derivatives), and flavonols such as quercetin and its glycosylated and acylated forms (Table 2). These compounds are widely recognized for their antioxidant activity through hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms, which is consistent with the antioxidant responses observed in the different assays (61, 20, 58).

Among the evaluated samples, the FAPC fraction showed the highest antioxidant capacity. In the DPPH radical scavenging assay, this fraction presented an $IC_{50}$ value of 0.56 ± 7.97 µg/mL, which was lower than that of ascorbic acid (0.80 ± 7.48 µg/mL), indicating a higher efficiency in neutralizing free radicals. This behavior was further supported by the FRAP assay, in which FAPC reached a reducing capacity of 872.6 ± 22.58 µmol Trolox eq/g, with no statistically significant difference when compared to the reference antioxidant (913.5 ± 4.93 µmol Trolox eq/g). The strong antioxidant performance of this fraction can be attributed to the combined presence of catechin, procyanidins A and B (dimers and trimers), quercetin, and chlorogenic acid, whose structural features favor both HAT and SET reactions.

In addition, the EEPC, FBPC, EEPS, and FBPS fractions also demonstrated relevant antioxidant activity. Regarding DPPH radical scavenging capacity, these fractions required lower concentrations to reach 50% antioxidant effect when compared to avocado byproducts from the Hass variety. Velderrain et al. (58) reported $EC_{50}$ values of 0.072 ± 0.012 mg/mL for EEPC and 0.091 ± 0.003 mg/mL for EEPS, whereas lower values were observed in the present study, indicating enhanced radical scavenging efficiency. These findings highlight the strong relationship between phenolic composition and antioxidant activity in *Persea americana* var. Quintal byproducts, reinforcing their potential as a valuable source of natural antioxidants.

The FRAP, DPPH, and ORAC methods showed that FDPS/PC, FAPS/PC, and FBPS/PC have a high antioxidant potential. However, it was necessary to evaluate the capacity to inhibit oxidative hemolysis, because this method is a good experimental model that can measure the percentage of inhibition of damage to erythrocyte membranes induced by free radicals, emulating processes that occur in human cells (62, 63). The FBPS, FAPC, and FBPC fractions were able to protect erythrocytes from AAPH-induced oxidation (Fig. 3a), a result that was supported by intracellular ROS detection. The same

fractions showed low intensities of intracellular ROS compared to the positive control (erythrocytes treated only with AAPH) (Fig. 3b). Also, the hemolytic activity of FBPC and FBPS was practically nil, while FAPC and FAPS were less than 25%.

Oxidative stress is considered a cardiovascular risk factor, as it stimulates the oxidation of low-density lipoproteins (ox-LDL), resulting in the development of atherosclerosis (64, 65). Therefore, it is necessary to study antioxidant compounds of exogenous origin as a therapeutic strategy targeting oxidative stress. The organic fractions FAPC, FBPC, FAPS, and FBPS were able to inhibit LDL oxidation and lipid peroxidation at 2.00 µg/mL (Fig. 4), making these fractions promising for the development of antioxidant therapies. Their bioactivity is associated with the presence of flavonoids, particularly quercetin and its glycosylated and acylated derivatives, detected in the analyzed fractions (Table 2). These quercetin-derived flavonols have been widely reported to exert antioxidant and anti-inflammatory effects, influencing lipid metabolism and contributing to the modulation of atherosclerosis-related processes. According to Poznyak et al. (66), flavonoids such as quercetin are associated with the regulation of LOX-1–mediated pathways, which play a central role in ox-LDL uptake and endothelial dysfunction. LOX-1 is one of the main endothelial receptors for oxidized LDL and is directly involved in the promotion of endothelial cell apoptosis (65, 67).

Cardiovascular diseases have mainly been associated with the oxidation of circulating lipoproteins and the role of oxidized LDL in atherosclerosis (5). However, the heart is a highly oxidative organ in which cardiomyocyte renewal is virtually absent, making it particularly vulnerable to the accumulation of lipid peroxidation products formed as a result of oxidative damage (68, 69). Due to the immense need for nutrients and oxygen, the heart contains a large number of mitochondria compared to other tissues, being the main source of intracellular ROS, presumably the largest endogenous source of lipid peroxidation (LP) in the heart (70). Although mitochondria have antioxidant systems that keep aldehyde levels (such as MDA) low, these are compromised by various pathological states and aging (70) which, in turn, leads to more cardiovascular diseases. The organic fractions FAPC, FBPC, FAPS, and FBPS demonstrated promising results in inhibiting lipid peroxidation induced by AAPH in cardiac tissue (Fig. 5). At a concentration of 50 µg/mL, they effectively reduced MDA levels to 0.104 ± 0.018; 0.117 ± 0.007; 0.098 ± 0.013, and 0.098 ± 0.023 mmol MDA/mg prot, respectively, exhibiting significant differences compared to the control (C+) with 0.394 ± 0.002 mmol MDA/mg prot.

Specifically, there are no previous studies on the lipid peroxidation (LP) inhibitory potential of avocado by-products in cardiac tissue; however, there are studies that have managed to inhibit MDA levels in other tissues using avocado peel and seed extracts. (71) indicated that avocado seed extract was able to protect the pancreas from Fe2+-induced lipid peroxidation *in vitro*, showing an $IC_{50}$ of 60.61 µg/mL. The authors consider that this protective effect can be attributed to phenolic compounds due to their ability to chelate $Fe^{2+}$, eliminate free radicals, and act as reducing agents. Gianazza et al. (68) also argue that natural flavonoids successfully inhibit the end products of advanced lipoxidation. This inhibitory effect is mainly associated with the capacity of flavonoids to interrupt lipid peroxidation chain reactions through hydrogen atom transfer and electron donation mechanisms, as well as to reduce the formation and reactivity of lipid-derived aldehydes such as malondialdehyde(68, 69). This justifies the inhibition of peroxidation by FAPC, FBPC, FAPS, and FBPS, as they have a high content of phenolic compounds and flavonoids.

In addition to oxidative damage and lipid peroxidation, the oxidation of low-density lipoproteins is known to trigger inflammatory responses by promoting the release of pro-inflammatory cytokines and nitric oxide (NO), particularly in macrophages involved in atherogenesis. The inflammatory process is an organism's defense mechanism against various pathogens and involves the participation of various mediators, such as TNF-α (proinflammatory cytokine) and NO, which are secreted mainly by macrophages (20). Excessive release of these mediators (TNF-α and/or NO) is related to atherosclerosis, diabetes, cancer, immune diseases, and other inflammatory diseases (19).

In these circumstances, it is necessary to use drugs capable of reducing or inhibiting this event. Hence, the evaluation was conducted to assess the capability of ethanolic extracts and organic fractions from Quintal avocado by-products to modulate NO generation in LPS-stimulated RAW 264.7 macrophages. In this context, a post-treatment model was applied, in which cells were first exposed to LPS and subsequently treated with the extracts, enabling the evaluation of their capacity

to regulate NO production after the inflammatory response had been established. This approach better reflects a therapeutic scenario, where intervention occurs after the onset of oxidative and inflammatory processes. The FAPC and FBPC fractions were able to suppress NO production at concentrations of 100 and 10 µg/mL, respectively (Fig. 7), and these fractions did not show cytotoxic activity at any of the concentrations analyzed (Fig. 6).

Tremocoldi in their research (20) reported that avocado peel extract from the Forte variety was able to reduce NO production (8.5 µM) and suppress TNF-α (495.3 pg/mL). The same authors suggest that the anti-inflammatory activity of the peel extract may be related to the high content of phenolic compounds such as procyanidin B1 and epicatechin, which are capable of acting on different pathways of the inflammatory process, such as the production of TNF-α and NO, as well as inhibiting the action of free radicals. The extracts FAPC, FBPC, FAPS, and FBPS contained high levels of procyanidins (Table 2).

According to Terra et al. (72), these compounds exert their anti-inflammatory effects by inhibiting the activity of ERK1/2 (a protein kinase), which, when activated, phosphorylates proteins that stimulate the expression of pro-inflammatory genes. Procyanidins are also capable of inhibiting IKKβ activity, an enzyme responsible for phosphorylating IκB—a protein that keeps NF-κB inactive. Once IκB is phosphorylated, NF-κB is released and translocates into the nucleus, where it activates genes involved in inflammation, immune responses, and the production of reactive oxygen species.

## 5. Conclusions

In summary, this study explored the bioactive potential of by-products from the *of P. americana* var. Quintal avocado variety. Fractions from these by-products, particularly FAPC and FBPC, are rich in phenolic compounds and exhibit strong antioxidant properties, suggesting potential applications in treating cardiovascular diseases related to oxidative stress. Additionally, FAPC and FBPC show anti-inflammatory effects without any cytotoxic or hemolytic activity, indicating their safety for potential use. Overall, these findings highlight the potential of *P. americana* var. Quintal by-products as a valuable source of bioactive compounds for therapeutic development, contributing to both health benefits and environmental sustainability in the avocado industry.

## Declarations

# Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

# Author Contributions

Author contributions are detailed on the title page.

# Ethics Approval

This study was performed in line with the principles of the Declaration of Helsinki. Procedures involving human blood samples were approved by the local Research Ethics Committee (protocol no. 61082522.4.0000.5704; approval no. 5.671.038). Procedures involving animals (C57BL/6 mice) were approved by the Animal Ethics Committee (CEUA/PROPP/REITO) of the Federal University of Uberlândia (protocol no. 7/2022, approved July 14, 2022), and all animal handling followed institutional and national guidelines for the care and use of laboratory animals.

**Consent to Participate**

Informed consent was obtained from all individual human volunteers included in the study prior to blood collection.

**Consent to Publish**

Not applicable — this manuscript does not contain any individual person's data in any form (images, videos, or identifying information).

# Funding

This work was supported by the Foundation for Research Support of the State of Minas Gerais – FAPEMIG (grant APQ-02185-24) and the National Council for Scientific and Technological Development – CNPq (442716/2024-9). A.B.J. received a fellowship from the Coordination for the Improvement of Higher Education Personnel (CAPES). F.S.E. received a research productivity fellowship from CNPq (303853/2025-5).

# Author Contribution

María Sol Peña Carrillo: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Romário Divino Vilarinho Galvãona: Methodology, Validation, Investigation, Data curation. Ana Luiza Borges: Methodology, Validation, Investigation, Data curation. Vinícius Prado Bittar: Methodology, Validation, Investigation, Data curation. Allisson Benatti Justino: Methodology, Validation, Investigation, Data curation. Iasmin Aparecida Cunha Araújo: Methodology, Validation, Investigation, Data curation. Mário Machado Martins: Methodology. Diego Godina Prado: Validation, Investigation, Data curation. Françoise Vasconcelos Botelho: Methodology, Formal Analysis. Alberto de Oliveira: Validation, Investigation, Data curation. Neide Maria Silva: Formal analysis, Investigation. Foued Salmen Espíndola: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & and editing, Visualization, Supervision, Project administration, Funding acquisition.

# Acknowledgments

The authors thank the Institute of Biotechnology of the Federal University of Uberlândia (UFU) for the infrastructure and support provided for this study. M.S.P.C. also thanks FAPEMIG for financial support.

# Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## References

1. Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity*, *2017*. https://doi.org/10.1155/2017/8416763
2. Rebuglio, C., Biavatti, M., Oliveira, P., De Mello, B., Almeida, A., & Bueno, G. (2021). View of Estresse oxidativo: uma introdução ao estado da arte / Oxidative stress: an introduction to the state of art. *Brazilian Journal of Development*, *7*(1), 10152–10168. https://ojs.brazilianjournals.com.br/ojs/index.php/BRJD/article/view/23823/19133
3. Sánchez, V., & Méndez, N. (2013). Estrés oxidativo, antioxidantes y enfermedad ARTÍCULO DE REVISIÓN. *Rev Invest Med Sur Mex Julio-Septiembre*, *20*(3), 161–168.

4. Zorrilla, A. (2002). El envejecimiento y el estrés oxidativo. *Rev Cubana Invest Biomed*, *3*(21), 178–185.
5. Chen, K., & Keaney, J. F. (2012). Evolving concepts of oxidative stress and reactive oxygen species in cardiovascular disease. *Current Atherosclerosis Reports*, *14*(5), 476–483. https://doi.org/10.1007/s11883-012-0266-8
6. Xu, D. P., Li, Y., Meng, X., Zhou, T., Zhou, Y., Zheng, J., Zhang, J. J., Li, H., & Bin (2017). Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources. *International Journal of Molecular Sciences 2017*, *18*(1), 96. https://doi.org/10.3390/ijms18010096
7. Zafar, F., Asif, H. M., Shaheen, G., Ghauri, A. O., Rajpoot, S. R., Tasleem, M. W., Shamim, T., Hadi, F., Noor, R., Ali, T., Gulzar, M. N., & Nazar, H. (2023). A comprehensive review on medicinal plants possessing antioxidant potential. *Clinical and Experimental Pharmacology and Physiology*, *50*(3), 205–217. https://doi.org/10.1111/1440-1681.13743
8. Hurtado-Fernández, E., Fernández-Gutiérrez, A., & Carrasco-Pancorbo, A. (2018). Avocado fruit— Persea americana. *Exotic Fruits*, 37–48. https://doi.org/10.1016/B978-0-12-803138-4.00001-0
9. Amado, D. A. V., Helmann, G. A. B., Detoni, A. M., de Carvalho, S. L. C., de Aguiar, C. M., Martin, C. A., Tiuman, T. S., & Cottica, S. M. (2019). Antioxidant and antibacterial activity and preliminary toxicity analysis of four varieties of avocado *(Persea americana* Mill). *Brazilian Journal of Food Technology*, *22*, e2018044. https://doi.org/10.1590/1981-6723.04418
10. Figueroa, J. G., Borrás-Linares, I., Lozano-Sánchez, J., & Segura-Carretero, A. (2018). Comprehensive identification of bioactive compounds of avocado peel by liquid chromatography coupled to ultra-high-definition accurate-mass Q-TOF. *Food Chemistry*, *245*, 707–716. https://doi.org/10.1016/J.FOODCHEM.2017.12.011
11. Borja, D., & Goetschel, L. (n.d.). Phytochemical and proximal study of Avocado (Persea americana) Hass and Fuerte By-products.
12. Coman, V., Teleky, B., Mitrea, L., Martau, G., Szaco, K., Calinoiu, F., & Vodnar, C. (2019). Bioactive potential of fruit and vegetable wastes. *Advances in Food and Nutrition Research*, 1–22. 10.1016/bs.afnr.2019.07.001. https://sci-hub.se/
13. da Silva, G. G., Pimenta, L. P. S., Melo, J. O. F., Mendonça, H., de Augusti, O. P., R., & Takahashi, J. A. (2022). Phytochemicals of Avocado Residues as Potential Acetylcholinesterase Inhibitors, Antioxidants, and Neuroprotective Agents. *Molecules (Basel Switzerland)*, *27*(6). https://doi.org/10.3390/MOLECULES27061892
14. Dabas, D., Shegog, R. M., Ziegler, G. R., & Lambert, J. D. (2013). Avocado (Persea americana) Seed as a Source of Bioactive Phytochemicals. *Current Pharmaceutical Design*, *19*(34), 6133–6140. https://doi.org/10.2174/1381612811319340007
15. Ekom, S. E., Tamokou, J. D. D., & Kuete, V. (2022). Methanol extract from the seeds of Persea americana displays antibacterial and wound healing activities in rat model. *Journal of Ethnopharmacology*, *282*, 114573. https://doi.org/10.1016/J.JEP.2021.114573
16. Lyu, X., Agar, O. T., Barrow, C. J., Dunshea, F. R., & Suleria, H. A. R. (2023). Phenolic Compounds Profiling and Their Antioxidant Capacity in the Peel, Pulp, and Seed of Australian Grown Avocado. *Antioxidants (Basel Switzerland)*, *12*(1). https://doi.org/10.3390/ANTIOX12010185
17. Melgar, B., Dias, M. I., Ciric, A., Sokovic, M., Garcia-Castello, E. M., Rodriguez-Lopez, A. D., Barros, L., & Ferreira, I. C. R. F. (2018). Bioactive characterization of Persea americana Mill. by-products: A rich source of inherent antioxidants. *Industrial Crops and Products*, *111*, 212–218. https://doi.org/10.1016/J.INDCROP.2017.10.024
18. Soledad, C. P. T., Paola, H. C., Enrique, C., Israel, O. V., GuadalupeVirginia, R. L. I., N. M., & Raúl, Á. S. (2021). Avocado seeds (Persea americana cv. Criollo sp.): Lipophilic compounds profile and biological activities. *Saudi Journal of Biological Sciences*, *28*(6), 3384–3390. https://doi.org/10.1016/J.SJBS.2021.02.087
19. Bangar, S. P., Dunno, K., Dhull, S. B., Kumar Siroha, A., Changan, S., Maqsood, S., & Rusu, A. V. (2022). Avocado seed discoveries: Chemical composition, biological properties, and industrial food applications. *Food Chemistry*: *X*, 16. https://doi.org/10.1016/J.FOCHX.2022.100507
20. Tremocoldi, M. A., Rosalen, P. L., Franchin, M., Massarioli, A. P., Denny, C., Daiuto, É. R., Paschoal, J. A. R., Melo, P. S., & De Alencar, S. M. (2018). Exploration of avocado by-products as natural sources of bioactive compounds. *Plos One*, *13*(2). https://doi.org/10.1371/JOURNAL.PONE.0192577

21. Dias, C., Sampaio, A. C., Midori, I., & Yamanishi, O. K. (2011). Avocado production chain in the state of São Paulo (Brazil) / La cadena de producción de aguacate en el estado de São Paulo (Brazil). *Proceedings VII World Avocado Congress 2011 (Actas VII Congreso Mundial Del Aguacate 2011)*, 5–9.
22. Salazar-López, N. J., Domínguez-Avila, J. A., Yahia, E. M., Belmonte-Herrera, B. H., Wall-Medrano, A., Montalvo-González, E., & González-Aguilar, G. A. (2020a). Avocado fruit and by-products as potential sources of bioactive compounds. *Food Research International*, *138*, 109774. https://doi.org/10.1016/J.FOODRES.2020.109774
23. Kupina, S., Fields, C., Roman, M. C., & Brunelle, S. L. (2018). Determination of Total Phenolic Content Using the Folin-C Assay: Single-Laboratory Validation, First Action 2017.13. *Journal of AOAC International*, *101*(5), 1466–1472. https://doi.org/10.5740/JAOACINT.18-0031
24. Popova, M., Bankova, V., Butovska, D., Petkov, V., Nikolova-Damyanova, B., Sabatini, A. G., Marcazzan, G. L., & Bogdanov, S. (2004). Validated methods for the quantification of biologically active constituents of poplar-type propolis. *Phytochemical Analysis: PCA*, *15*(4), 235–240. https://doi.org/10.1002/PCA.777
25. Schofield, P., Mbugua, D. M., & Pell, A. N. (2001). Analysis of condensed tannins: a review. *Animal Feed Science and Technology*, *91*(1–2), 21–40. https://doi.org/10.1016/S0377-8401(01)00228-0
26. Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Analytical Biochemistry*, *239*(1), 70–76. https://doi.org/10.1006/ABIO.1996.0292
27. Blois, M. S. (1958). Antioxidant Determinations by the Use of a Stable Free Radical. *Nature 1958*, *181*(4617), 4617. 1199-1200. https://doi.org/10.1038/1811199a0
28. Carrillo-Martinez, E. J., Flores-Hernández, F. Y., Salazar-Montes, A. M., Nario-Chaidez, H. F., & Hernández-Ortega, L. D. (2024). Quercetin, a Flavonoid with Great Pharmacological Capacity. *Molecules*, *29*(5), 1000. https://doi.org/10.3390/molecules29051000
29. Zhang, Q., Yang, W., Liu, J., Liu, H., Lv, Z., Zhang, C., Chen, D., & Jiao, Z. (2020). Identification of Six Flavonoids as Novel Cellular Antioxidants and Their Structure-Activity Relationship. Oxidative Medicine and Cellular Longevity, 2020, 4150897. https://doi.org/10.1155/2020/4150897
30. Zhao, L., Chen, J., Su, J., Li, L., Hu, S., Li, B., Zhang, X., Xu, Z., & Chen, T. (2013). In vitro antioxidant and antiproliferative activities of 5-hydroxymethylfurfural. *Journal of Agricultural and Food Chemistry*, *61*(44), 10604–10611. https://doi.org/10.1021/jf403098y
31. Sommerfeld, S., Mundim, A. V., Silva, R. R., Queiroz, J. S., Rios, M. P., Notário, F. O., Ronchi, A. A. M., Beletti, M. E., Franco, R. R., Espindola, F. S., Goulart, L. R., & Fonseca, B. B. (2022). Physiological Changes in Chicken Embryos Inoculated with Drugs and Viruses Highlight the Need for More Standardization of this Animal Model. *Animals*, *12*(9). https://doi.org/10.3390/ani12091156
32. Chung, B. H., Wilkinson, T., Geer, J. C., & Segrest, J. P. (1980). Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *Journal Lipid Research*, *21*, 284–291. https://doi.org/10.1016/S0022-2275(20)39807-2
33. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3
34. Yagi, K. Simple procedure for specific assay of lipid hydroperoxides in serum or plasma. Methods in Molecular Biology (, & Clifton, N. J. (1998). 108, 107–110. https://doi.org/10.1385/0-89603-472-0:107
35. Komino, E. A., Ramos, A., Souza, A. V., De, Caixeta, D. C., Bittar, V. P., Borges, A. L., Botelho, F. V., & Espindola, F. S. (2023). *Annona crassiflora Mart. Fruit Peel Polyphenols Preserve Cardiac Antioxidant Defense and Reduce Oxidative Damage in Hyperlipidemic Mice*.
36. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

37. Suriyaprom, S., Srisai, P., Intachaisri, V., Kaewkod, T., Pekkoh, J., Desvaux, M., & Tragoolpua, Y. (2023). Antioxidant and Anti-Inflammatory Activity on LPS-Stimulated RAW 264.7 Macrophage Cells of White Mulberry (Morus alba L.) Leaf Extracts. *Molecules*, *28*(11), 4395. https://doi.org/10.3390/MOLECULES28114395
38. Bryan, N. S., & Grisham, M. B. (2007). Methods to Detect Nitric Oxide and its Metabolites in Biological Samples. *Free Radical Biology & Medicine*, *43*(5), 645. https://doi.org/10.1016/J.FREERADBIOMED.2007.04.026
39. Serrano-García, I., Olmo-García, L., Pedreschi, R., Vílchez-Quero, J. L., González-Fernández, J. J., Hormaza, J. I., & Carrasco-Pancorbo, A. (2025). Characterisation of avocado fruits from different Iberian regions: Integrating ion mobility in non-targeted LC-MS metabolomics. *Food Chemistry*, *481*, 143937. https://doi.org/10.1016/j.foodchem.2025.143937
40. Llorent-Martínez, E. J., Spínola, V., & Castilho, P. C. (2017). Phenolic profiles of Lauraceae plant species endemic to Laurisilva forest: A chemotaxonomic survey. *Industrial Crops and Products*, *107*(January), 1–12. https://doi.org/10.1016/j.indcrop.2017.05.023
41. Zhu, T., Li, N., Zhao, C., Liu, J., Liu, Y., Chen, J., Dong, Y., Tian, Y., Hong, X., & Xiong, H. (2025). Comprehensive characterization and identification of chemical constituents of Pingwei Powder by ultra high performance liquid chromatography tandem quadrupole-time-of-flight tandem mass spectrometry. *Journal of Chromatography Open*, *7(January)*. https://doi.org/10.1016/j.jcoa.2025.100203
42. Zhang, Y., Wen, M., Zhou, P., Tian, M., Zhou, J., Zhang, L. Analysis of chemical composition in Chinese olive leaf tea by, & UHPLC-DAD-Q-TOF (2020). -MS/MS and GC–MS and its lipid-lowering effects on the obese mice induced by high-fat diet. Food Research International, 128(September 2019), 108785. https://doi.org/10.1016/j.foodres.2019.108785
43. Loyola, A. C. F. E., Silva, V. D. M., Silva, M. R., Rodrigues, C. G., Dos Santos, A. N., Melo, J. O. F., Augusti, R., & Fante, C. A. (2021). Use of paper spray mass spectrometry for determining the chemical profile of green cavendish banana (Musa AAA) peel and pulp flours and evaluation of its functional potential. *Journal of the Brazilian Chemical Society*, *32*(5), 953–963. https://doi.org/10.21577/0103-5053.20200243
44. Said, R., Ben, Hamed, A. I., Mahalel, U. A., Al-Ayed, A. S., Kowalczyk, M., Moldoch, J., Oleszek, W., & Stochmal, A. (2017). Tentative characterization of polyphenolic compounds in the male flowers of Phoenix dactylifera by liquid chromatography coupled with mass spectrometry and DFT. *International Journal of Molecular Sciences*, *18*(3), 1–18. https://doi.org/10.3390/ijms18030512
45. KajdŽanoska, M., Gjamovski, V., & Stefova, M. (2010). HPLC-DAD-ESI-MSn identification of phenolic compounds in cultivated strawberries from Macedonia. *Macedonian Journal of Chemistry and Chemical Engineering*, *29*(2), 181–194. https://doi.org/10.20450/mjcce.2010.165
46. Younis, I. Y., Khattab, A. R., Selim, N. M., Sobeh, M., Elhawary, S. S., & Bishbishy, M. H. E. (2022). Metabolomics-based profiling of 4 avocado varieties using HPLC–MS/MS and GC/MS and evaluation of their antidiabetic activity. *Scientific Reports*, *12*(1), 1–15. https://doi.org/10.1038/s41598-022-08479-4
47. Dou, J., Lee, V. S. Y., Tzen, J. T. C., & Lee, M. R. (2007). Identification and Comparison of Phenolic Compounds in the Preparation of Oolong Tea Manufactured by Semifermentation and Drying Processes. *Journal of Agricultural and Food Chemistry*, *55*(18), 7462–7468. https://doi.org/10.1021/jf0718603
48. Sannomiya, M., Montoro, P., Piacente, S., Pizza, C., Brito, A. R. M. S., & Vilegas, W. (2005). Application of liquid chromatography/electrospray ionization tandem mass spectrometry to the analysis of polyphenolic compounds from an infusion of Byrsonima crassa Niedenzu. *Rapid Communications in Mass Spectrometry*, *19*(16), 2244–2250. https://doi.org/10.1002/rcm.2053
49. Wollgast, J., Pallaroni, L., Agazzi, M. E., & Anklam, E. (2001). Analysis of procyanidins in chocolate by reversed-phase high-performance liquid chromatography with electrospray ionisation mass spectrometric and tandem mass spectrometric detection. *Journal of Chromatography A*, *926*(1), 211–220. https://doi.org/10.1016/S0021-9673(01)00994-3
50. Bystrom, L. M., Lewis, B. A., Brown, D. L., Rodriguez, E., & Obendorf, R. L. (2008). Characterisation of phenolics by LC–UV/Vis, LC–MS/MS and sugars by GC in Melicoccus bijugatus Jacq. 'Montgomery' fruits. *Food Chemistry*, *111*(4),

1017–1024. https://doi.org/https://doi.org/10.1016/j.foodchem.2008.04.058

51. Llorent-Martínez, E. J., Spínola, V., Gouveia, S., & Castilho, P. C. (2015). HPLC-ESI-MSn characterization of phenolic compounds, terpenoid saponins, and other minor compounds in Bituminaria bituminosa. *Industrial Crops and Products*, *69*, 80–90. https://doi.org/https://doi.org/10.1016/j.indcrop.2015.02.014
52. Li, Z. H., Guo, H., Xu, W., Bin, Ge, J., Li, X., Alimu, M., & He, D. J. (2016). Rapid Identification of Flavonoid Constituents Directly from PTP1B Inhibitive Extract of Raspberry (Rubus idaeus L.) Leaves by HPLC-ESI-QTOF-MS-MS. *Journal of Chromatographic Science*, *54*(5), 805–810. https://doi.org/10.1093/chromsci/bmw016
53. Baleiras-Couto, M. M., Guedes, R., Duarte, F. L., Fortes, A. M., & Serralheiro, M. L. (2023). Untargeted Metabolomics Discriminates Grapes and Wines from Two Syrah Vineyards Located in the Same Wine Region. *Fermentation*, *9*(2). https://doi.org/10.3390/fermentation9020145
54. Araujo, N. M. P., Arruda, H. S., dos Santos, F. N., de Morais, D. R., Pereira, G. A., & Pastore, G. M. (2020). LC-MS/MS screening and identification of bioactive compounds in leaves, pulp and seed from Eugenia calycina Cambess. *Food Research International*, *137*(March), 109556. https://doi.org/10.1016/j.foodres.2020.109556
55. Simirgiotis, M. J., & Schmeda-Hirschmann, G. (2010). Direct identification of phenolic constituents in Boldo Folium (Peumus boldus Mol.) infusions by high-performance liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry. *Journal of Chromatography A*, *1217*(4), 443–449. https://doi.org/10.1016/j.chroma.2009.11.014
56. Cádiz-Gurrea, M., de la Fernández-Arroyo, L., Joven, S., J., & Segura-Carretero, A. (2013). Comprehensive characterization by UHPLC-ESI-Q-TOF-MS from an Eryngium bourgatii extract and their antioxidant and anti-inflammatory activities. *Food Research International*, *50*(1), 197–204. https://doi.org/10.1016/j.foodres.2012.09.038
57. Dixon, R. A., & Paiva, N. L. (1995). Stress-Induced Phenylpropanoid Metabolism. *The Plant Cell*, *7*(7), 1085–1097. https://doi.org/10.1105/TPC.7.7.1085
58. Velderrain, G. R., Quero, J., Osada, J., Martín-Belloso, O., & Rodríguez-Yoldi, M. J. (2021). Phenolic-Rich Extracts from Avocado Fruit Residues as Functional Food Ingredients with Antioxidant and Antiproliferative Properties. *Biomolecules 2021*, *11*(7), 977. https://doi.org/10.3390/BIOM11070977. *11*.
59. Rodríguez-Carpena, J. G., Morcuende, D., Andrade, M. J., Kylli, P., & Estevez, M. (2011). Avocado (Persea americana Mill.) phenolics, in vitro antioxidant and antimicrobial activities, and inhibition of lipid and protein oxidation in porcine patties. *Journal of Agricultural and Food Chemistry*, *59*(10), 5625–5635. https://doi.org/10.1021/JF1048832
60. Mardigan, L. P., Dos Santos, V. J., da SILVA, P. T., Visentainer, J. V., Gomes, S. T. M., & Matsushita, M. (2018). Investigation of bioactive compounds from various avocado varieties (Persea americana Miller). *Food Science and Technology*, *39*, 15–21. https://doi.org/10.1590/FST.34817
61. Rojas, A., Fuentes, E., Cádiz-Gurrea, M., de la Rodriguez, L., Villegas-Aguilar, L., Palomo, M. D. C., Arráez-Román, I., D., & Segura-Carretero, A. (2022). Biological Evaluation of Avocado Residues as a Potential Source of Bioactive Compounds. *Antioxidants (Basel Switzerland)*, *11*(6). https://doi.org/10.3390/ANTIOX11061049
62. Aguilera-Aguirre, S., Meza-Espinoza, L., Hernández-Mendoza, A., Vallejo-Córdoba, B., González-Córdova, A. F., Montalvo-González, E., Aguilera-Aguirre, S., Meza-Espinoza, L., Hernández-Mendoza, A., Vallejo-Córdoba, B., González-Córdova, A. F., & Montalvo-González, E. (2018). Bromelia pinguin y Bromelia karatas. *TIP Revista Especializada En Ciencias Químico-Biológicas*, *21*, 13–21. https://doi.org/10.22201/FESZ.23958723E.2019.1.142. Evaluación de la capacidad de inhibición de hemólisis oxidativa y actividad antimicrobiana de fracciones peptídicas obtenidas de la hidrólisis de proteínas de huevo, leche y soya usando proteasas extraídas de.
63. Ibrahim, I. H., Sallam, S. M., Omar, H., Rizk, M., & Ibrahim, I. H. (2006). Oxidative Hemolysis of Erythrocytes Induced by Various Vitamins. *International Journal of Biomedical Science: IJBS*, *2*(3), 295. https://doi.org/10.59566/ijbs.2006.2295
64. Batty, M., Bennett, M. R., & Yu, E. (2022). The Role of Oxidative Stress in Atherosclerosis. Cells 2022, Vol. 11, Page 3843, 11(23), 3843. https://doi.org/10.3390/CELLS11233843

65. Kattoor, A. J., Pothineni, N. V. K., Palagiri, D., & Mehta, J. L. (2017). Oxidative Stress in Atherosclerosis. *Current Atherosclerosis Reports*, *19*(11), 1–11. https://doi.org/10.1007/S11883-017-0678-6/METRICS
66. Poznyak, A. V., Grechko, A. V., Orekhova, V. A., Chegodaev, Y. S., Wu, W. K., & Orekhov, A. N. (2020). Oxidative Stress and Antioxidants in Atherosclerosis Development and Treatment. *Biology*, *9*(3). https://doi.org/10.3390/BIOLOGY9030060
67. Kattoor, A. J., Goel, A., & Mehta, J. L. (2019). LOX-1: Regulation, Signaling and Its Role in Atherosclerosis. *Antioxidants*, *8*(7). https://doi.org/10.3390/ANTIOX8070218
68. Gianazza, E., Brioschi, M., Fernandez, A. M., & Banfi, C. (2019). Lipoxidation in cardiovascular diseases. *Redox Biology*, *23*. https://doi.org/10.1016/J.REDOX.2019.101119
69. Halliwell, B. (2000). Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? *Cardiovascular Research*, *47*(3), 410–418. https://doi.org/10.1016/S0008-6363(00)00097-3
70. Martín-Fernández, B., & Gredilla, R. (2018). Estrés oxidativo mitocondrial y envejecimiento cardíaco. *Clínica e Investigación En Arteriosclerosis*, *30*(2), 74–83. https://doi.org/10.1016/J.ARTERI.2017.12.002
71. Oboh, G., Odubanjo, V. O., Bello, F., Ademosun, A. O., Oyeleye, S. I., Nwanna, E. E., & Ademiluyi, A. O. (2016). Aqueous extracts of avocado pear (Persea americana Mill.) leaves and seeds exhibit anti-cholinesterases and antioxidant activities in vitro. *Journal of Basic and Clinical Physiology and Pharmacology*, *27*(2), 131–140. https://doi.org/10.1515/JBCPP-2015-0049
72. Terra, X., Palozza, P., Fernandez-Larrea, J., Ardevol, A., Blade, C., Pujadas, G., Salvado, J., Arola, L., & Blay, M. T. (2011). Procyanidin dimer B1 and trimer C1 impair inflammatory response signalling in human monocytes. *Free Radical Research*, *45*(5), 611–619. https://doi.org/10.3109/10715762.2011.564165

## Figures

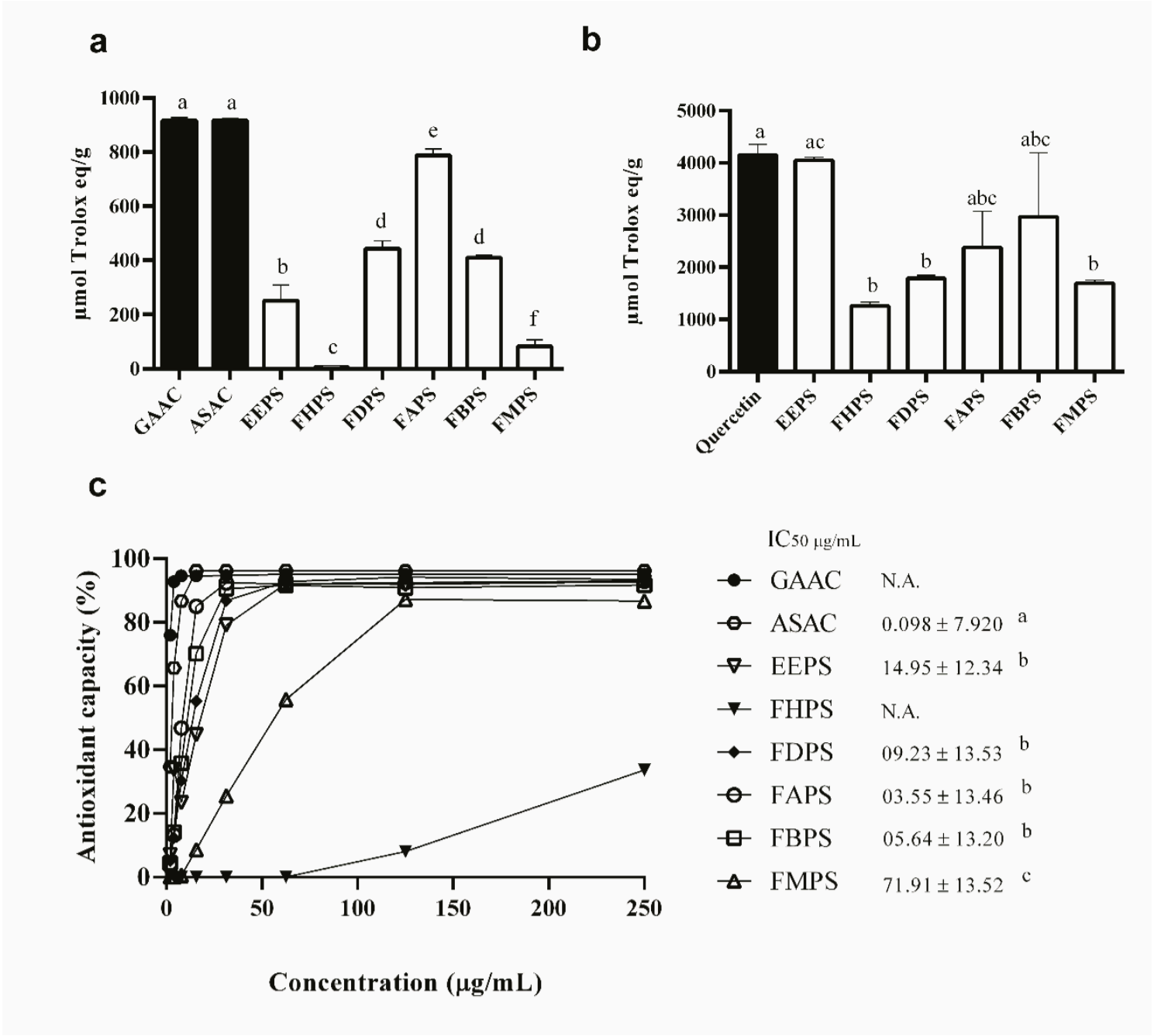



**Figure 1**

Antioxidant activity of EEPS and its organic fractions determined by the FRAP (A), ORAC (B), and DPPH (C) methods. Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$). note: Gallic acid (GAAC) and ascorbic acid (ASAC) were used as controls for A and C, while quercetin was used for B. EEPS: ethanolic extract of of *P. americana* var. Quintal seed; FHPS: hexane fraction; FDPS: dichloromethane fraction; FAPS: ethyl acetate fraction; FBPS: n-butanol fraction; FMPS: hydromethanolic fraction. N.A.: means no activity.

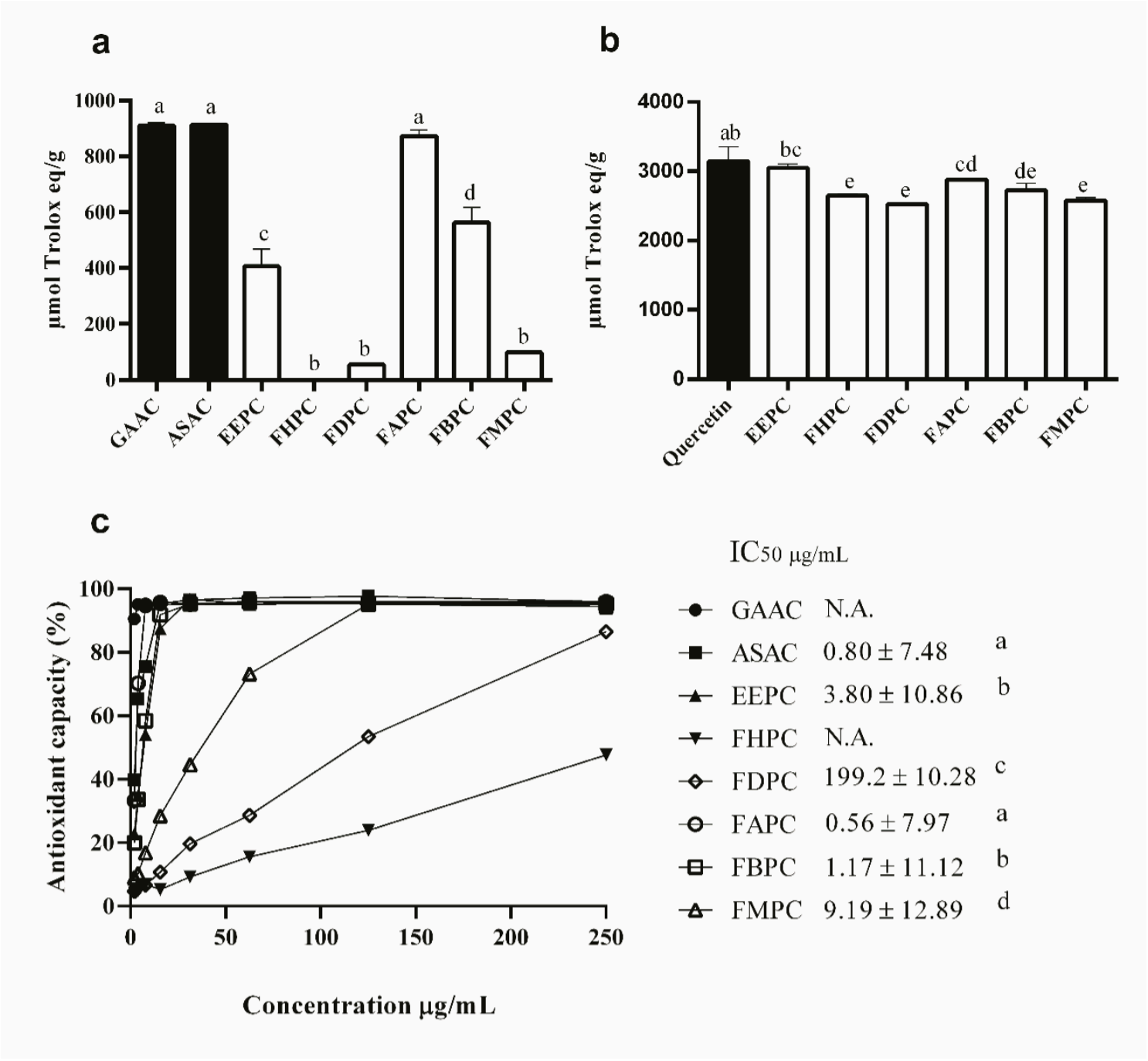



**Figure 2**

Antioxidant activity of EEPC and its organic fractions determined by the FRAP (A), ORAC (B), and DPPH (C) methods. Gallic acid (GAAC) and ascorbic acid (ASAC) were used as controls for A and C, while quercetin was used for B. Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$). Note: EEPC: ethanolic extract of of *P. americana* var. Quintal peel; FHPC: hexane fraction; FDPC: dichloromethane fraction; FAPC: ethyl acetate fraction; FBPC: n-butanol fraction; FMPC: hydromethanolic fraction. N.A.: means no activity.

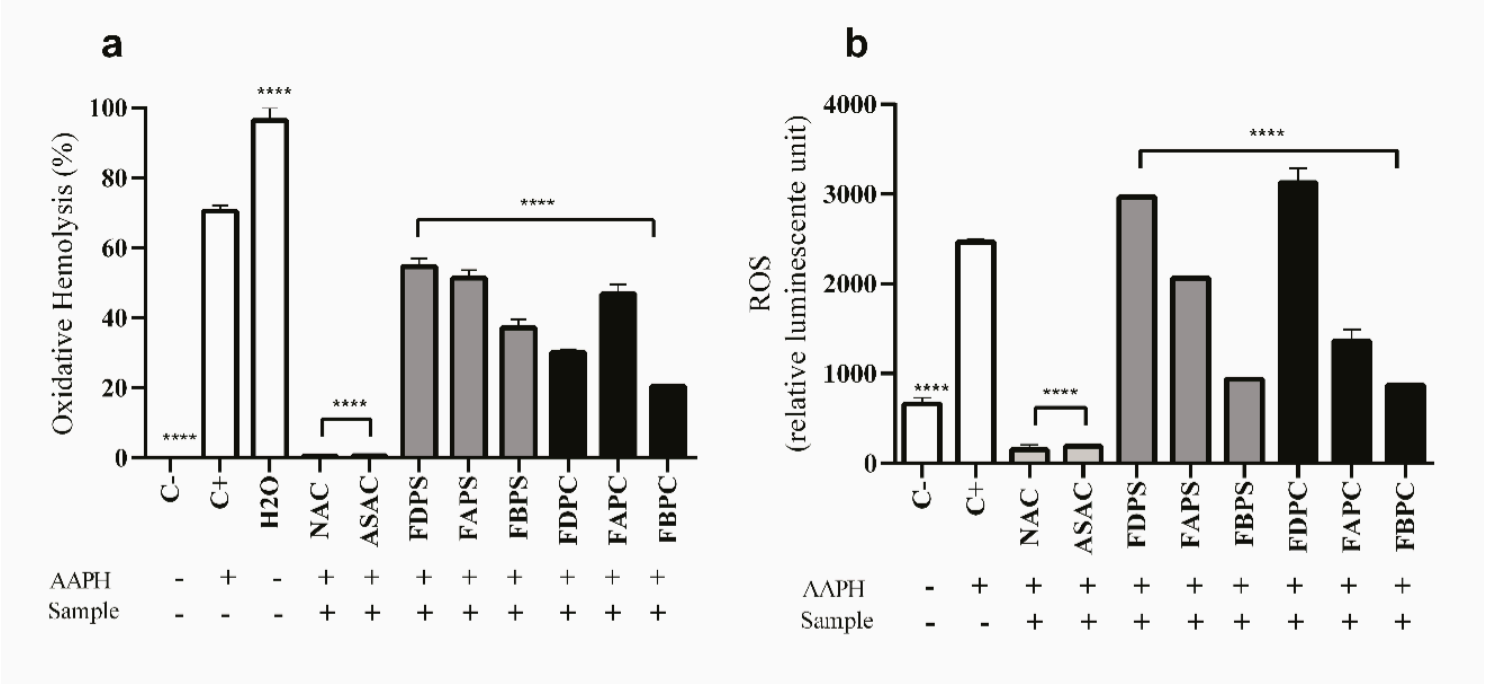



**Figure 3**

Induction of Erythrocyte Hemolysis by AAPH (A) and Reactive Oxygen Species (ROS) Production. Values are expressed as mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to C+. Note: C-: 1X PBS buffer; C+: AAPH; NAC: N-acetylcysteine; ASAC: ascorbic acid FDPS: seed dichloromethane fraction; FAPS: seed ethyl acetate fraction; FBPS: seed n-butanol fraction; FDPC: peel dichloromethane fraction; FAPC: peel ethyl acetate fraction; FBPC: peel n-butanol fraction.

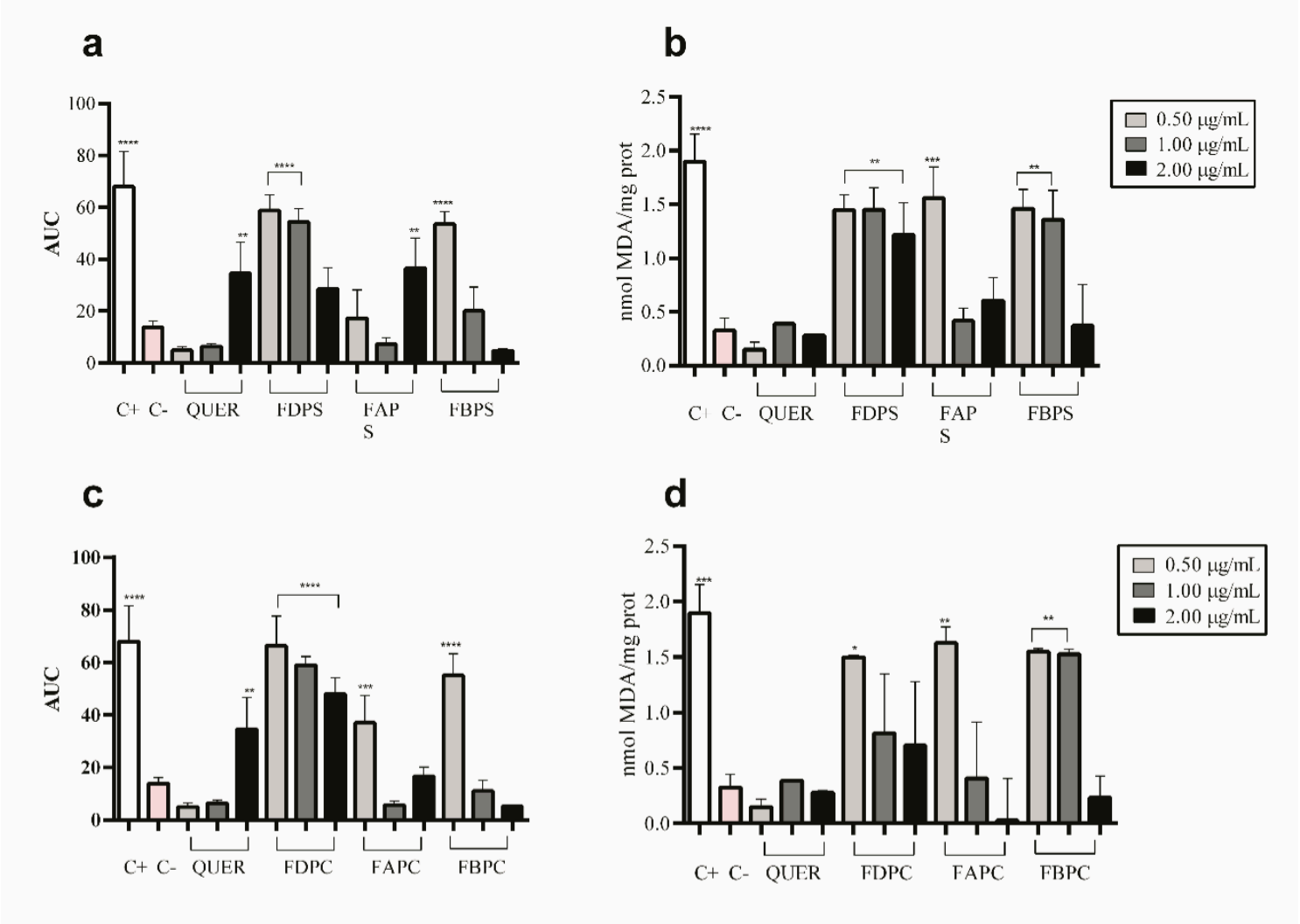



**Figure 4**

Evaluation of inhibition of LDL oxidation (A and C) and peroxidation (B and D) by fractions FDPS/PC, FAPS/PC, FBPS/PC, and quercetin. Values are expressed as mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to native LDL. Note: C+: LDL with Cu2+; C-: LDL; FDPS: seed dichloromethane fraction; FAPS: seed ethyl acetate fraction; FBPS: seed n-butanol fraction; FDPC: peel dichloromethane fraction; FAPC: peel ethyl acetate fraction; FBPC: peel n-butanol fraction; AUC: Area Under the Curve.

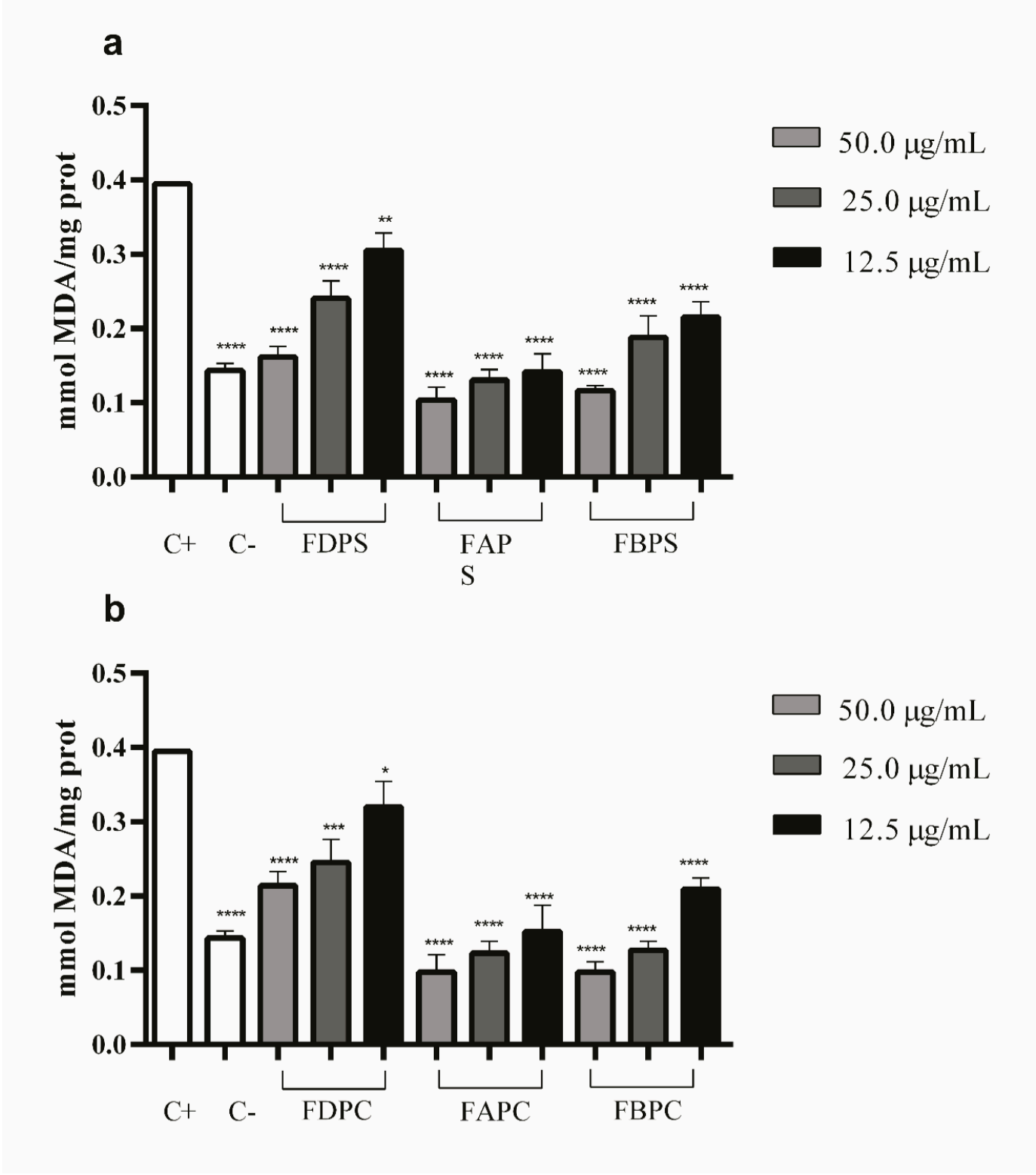



**Figure 5**

Analysis of the inhibition capacity of lipid peroxidation induced by AAPH in cardiac tissue by fractions derived from the seed (A) and peel (B). Values are expressed as mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to C+. Note: C-: cardiac tissue with buffer only; C+: oxidized cardiac tissue with AAPH and without treatment; FDPS: seed dichloromethane fraction; FAPS: seed ethyl acetate fraction; FBPS: seed n-butanol fraction; FDPC: peel dichloromethane fraction; FAPC: peel ethyl acetate fraction; FBPC: peel n-butanol fraction.

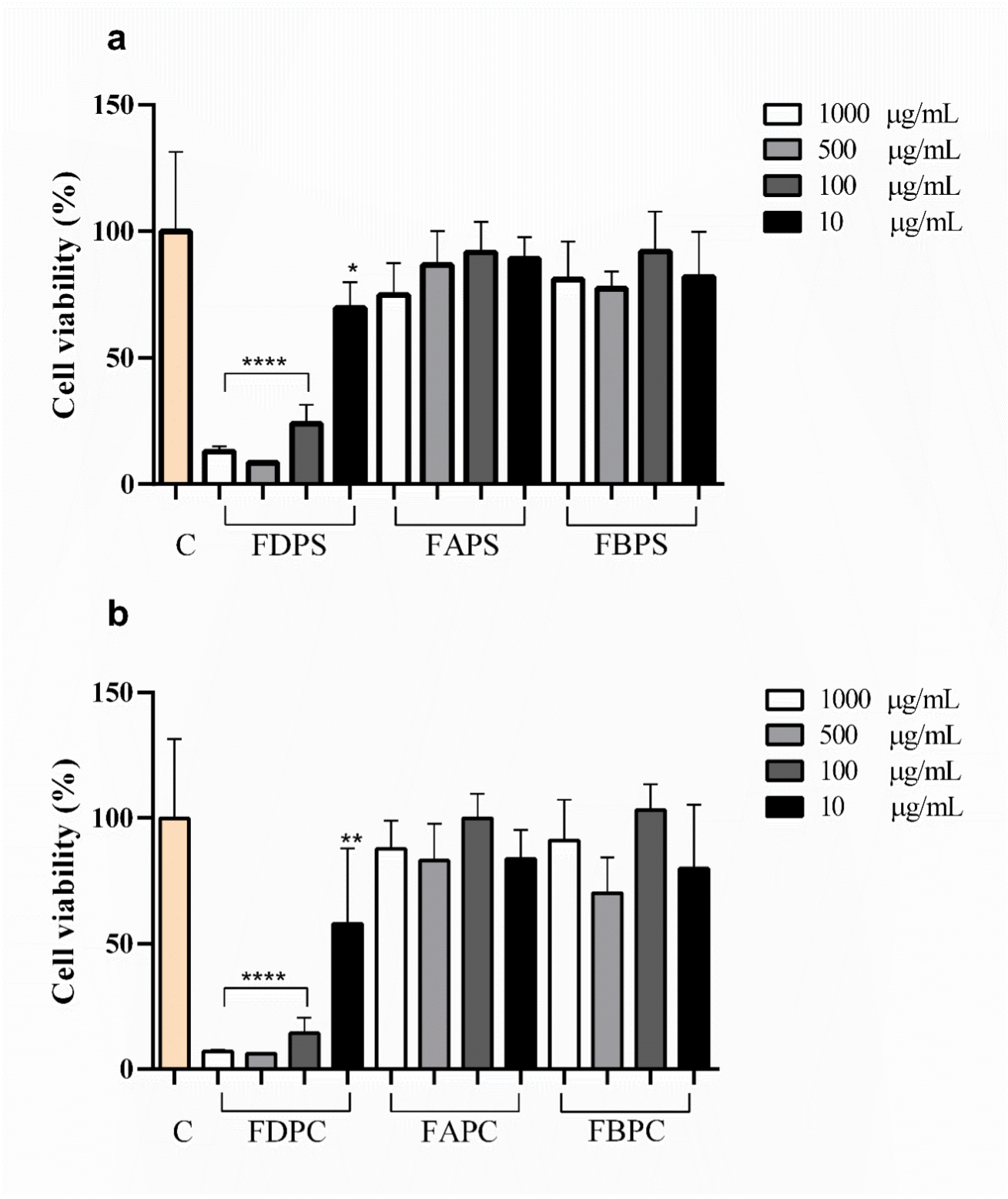



**Figure 6**

Cytotoxic Evaluation of Organic Fractions from Seed (A) and Peel (B) in RAW 264.7 Macrophages. Values are expressed as mean ± standard deviation * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to Control (RPMI). Note: FDPS: seed dichloromethane fraction; FAPS: seed ethyl acetate fraction; FBPS: seed n-butanol fraction; FDPC: peel dichloromethane fraction; FAPC: peel ethyl acetate fraction; FBPC: peel n-butanol fraction.

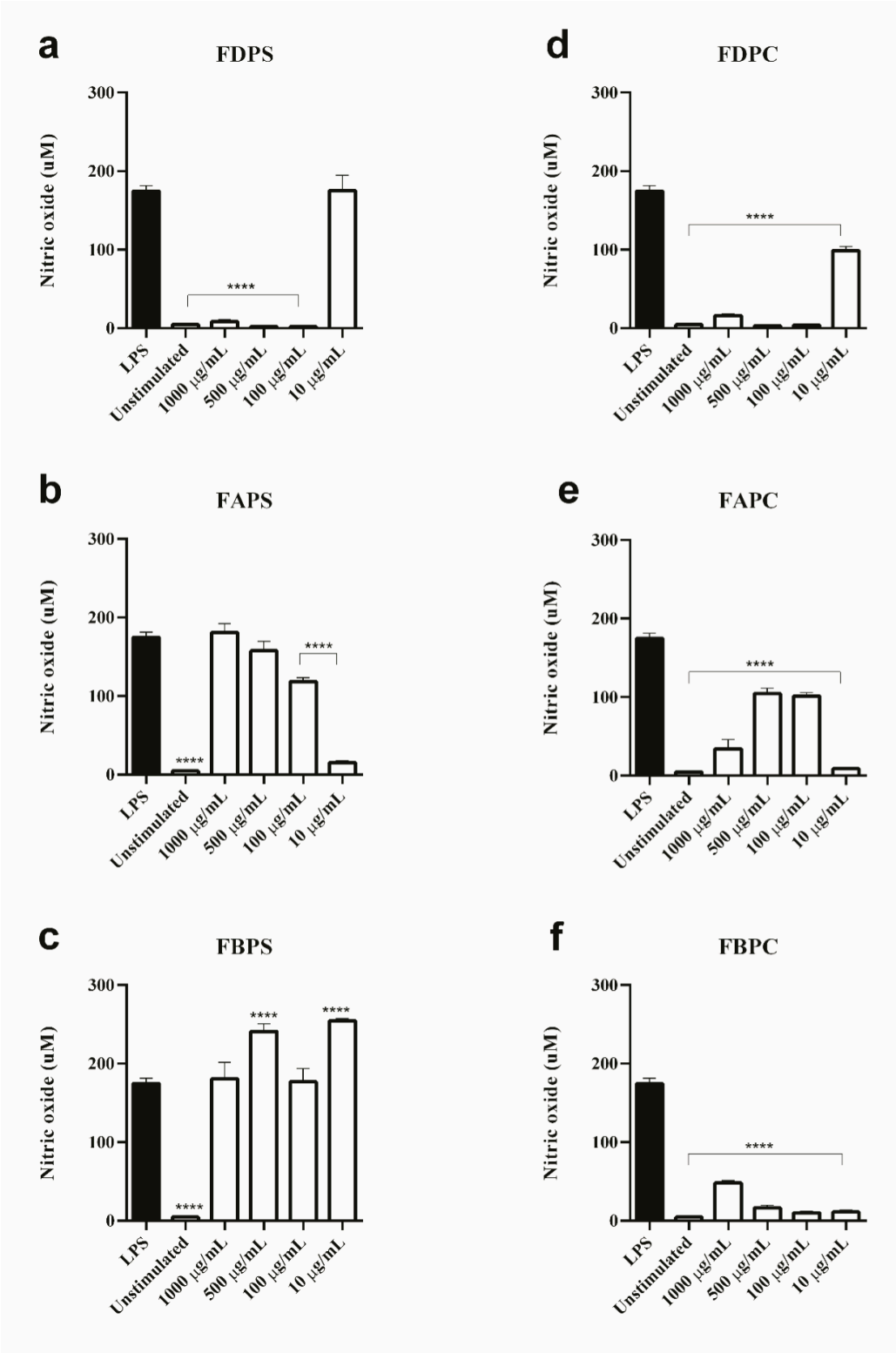



**Figure 7**

Inhibitory capacity of organic fractions derived from seed (A–C) and peel (D–F) on nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages. Values are presented as mean ± standard deviation (SD). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to the LPS group. Note: FDPS: seed dichloromethane fraction; FAPS: seed ethyl acetate fraction; FBPS: seed n-butanol fraction; FDPC: peel dichloromethane fraction; FAPC: peel ethyl acetate fraction; FBPC: peel n-butanol fraction.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx