



## Preventive supplementation with fresh and preserved peach attenuates CCl<sub>4</sub>-induced oxidative stress, inflammation and tissue damage<sup>☆</sup>

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### Abstract

The present study was elaborated to comparatively evaluate the preventive effect of different peach-derived products obtained from preserved fruits (Syrup and Preserve Pulp Peach [PPP]) and from fresh peels and pulps (Peel and Fresh Pulp Peach [FPP]) in a model of liver/renal toxicity and inflammation induced by carbon tetrachloride (CCl<sub>4</sub>) in rats. Tissue damage (carbonyl, thiobarbituric acid reactive species and sulfhydryl), antioxidant enzymes activity (catalase and superoxide dismutase) and inflammatory parameters [tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  levels, and receptor for advanced glycation end-products (RAGE) and nuclear factor (NF) $\kappa$ B-p65 immuncontent] were investigated. Our findings demonstrated that Peel, PPP and FPP (200 or 400 mg/kg) daily administration by oral gavage for 30 days conferred a significant protection against CCl<sub>4</sub>-induced antioxidant enzymes activation and, most importantly, oxidative damage to lipids and proteins as well as blocked induction of inflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$ , RAGE and NF $\kappa$ B. This antioxidant/anti-inflammatory effect seems to be associated with the abundance of carotenoids and polyphenols present in peach-derived products, which are enriched in fresh-fruit-derived preparations (Peel and FPP) but are also present in PPP. The Syrup – which was the least enriched in antioxidants – displayed no protective effect in our experiments. These effects cumulated in decreased levels of transaminases and lactate dehydrogenase leakage into serum and maintenance of organ architecture. Therefore, the herein presented results show evidence that supplementation with peach products may be protective against organ damage caused by oxidative stress, being interesting candidates for production of antioxidant-enriched functional foods.

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**Keywords:** Peach; NF $\kappa$ B-p65; RAGE; Antioxidant; Anti-inflammatory; Carbon tetrachloride

### 1. Introduction

The liver and kidney play pivotal roles in the systemic control of energetic metabolism, blood homeostasis as well as phase I/II detoxification and excretion of a myriad of xenobiotics present in food, medicines, alcohol and other toxicants humans are exposed to daily in the contemporary lifestyle [1]. Xenobiotics became extremely important in the last decades with the advances in food technology, which make use of several preservatives, sweeteners and dyes to improve taste, color and shelf time of products. Nonetheless, several therapeutic drugs may exert hepato- and renal toxicity, which range

from simple painkiller drugs such as acetaminophen and other nonsteroidal anti-inflammatory drugs, to others like allopurinol, interferon-beta1a, duloxetine (antidepressant) and some classical chemotherapeutic drugs such as cisplatin and cyclophosphamide [2]. In addition, high blood sugar levels in uncontrolled diabetes may cause liver and renal inflammation and damage, which severely impair organ functioning [3,4]. Imbalance in the detoxification systems may be caused by a series of acute and chronic exposure to xenobiotics, which associated with genetic factors can lead to hepatocellular/renal apoptosis and inflammation. Chronic liver injury/disease is frequently featured by development of local or disseminated fibrosis and impairment in energetic and xenobiotic metabolism, whereas renal disease affects excretion of waste products and toxins produced in liver from blood circulation and electrolytes homeostasis, regulation of blood pressure and hormone secretions [5].

Free radicals and nonradicals reactive oxygen species (ROS) are widely believed to contribute to development of several age-/

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xenobiotic-related diseases by causing oxidative stress and oxidative damage [6]. When an imbalance between oxidants and antioxidants occurs, an excess of ROS forms, causing oxidative damage in vulnerable targets such as membrane unsaturated fatty acids, protein thiols and DNA bases [7]. Excessive ROS may contribute to the aging process as well as to chronic diseases such hepatic fibrosis, cancer, atherosclerosis, neurodegenerative diseases and diabetes [6,8]. Taking into account that it has been established that oxidative stress and inflammation play a critical role in chronic liver damage and renal injuries [5,9], the search for druggable signaling mechanisms to counteract liver/renal damage is an area of interest among scientists. On the other hand, the variety of antioxidants present in vegetable sources – associated with the genetic improvement and food technology – makes the discovery of functional foods a good and safe strategy to prevent the chronic development of organ injuries.

It has been proved that fruits and vegetables have plenty of antioxidant compounds including flavonoids and carotenoids, which have been associated with lower risk of stroke, coronary heart disease, and markers of inflammation and oxidative stress in adults [10]. Of relevance for this study, carotenoids are divided into two major structural groups: (a) oxygen-containing molecules as xanthophylls such as lutein and  $\beta$ -cryptoxanthin, and (b) unoxxygenated carotenes which include hydrocarbon carotenoids that are either cyclized, such as  $\alpha$ -carotene and  $\beta$ -carotene, or linear, like lycopene [5,11]. High carotenoid intake leads to significantly reduced risk of several chronic and degenerative diseases such as coronary diseases, some types of cancer and other ROS-related conditions [4,5,12,13].

Fruit phenolic compounds influence the visual appearance (pigmentation and browning), taste (astringency), and potential health-promoting properties of different fruit products [14]. The health benefits related to fruit consumption seem to be related, at least in part, to the content of antioxidant phenolic metabolites [14]. The main classes of polyphenols in peaches are flavonoids, including chlorogenic acid, quercetin, (–)-epicatechin and (+)-catechin [15,16]. Polyphenol consumption is associated with the reduction of pathologies associated to inflammation and oxidative stress, including some types of cancer, cardiovascular diseases and diabetes [15,17,18].

The present study was performed to investigate the protective effect of a standard diet supplemented with peach in an animal model of liver/renal toxicity and inflammation induced by carbon tetrachloride ( $\text{CCl}_4$ ) *in vivo*. Different parts of fresh peaches (peels and pulps) were administered separately to animals, and their effects were compared with the effects of canned peach pulps preserved in sucrose-based syrup, one of the most widespread manufactured versions for commercialization of this fruit. We monitored markers of (a) tissue damage [serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin and lactate dehydrogenase (LDH) levels, and histochemistry]; (b) oxidative stress and oxidative damage to biomolecules [superoxide dismutase (SOD) and catalase (CAT) activities, and nitrotyrosine, protein carbonylation, sulphydryl and lipoperoxidation (thiobarbituric acid reactive species {TBARS}) levels]; (c) inflammatory parameters [tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  levels, and receptor for advanced glycation end-products (RAGE) and nuclear factor (NF) $\kappa$ B-p65 immunocontent]. High-performance liquid chromatography (HPLC) was carried out to evaluate five carotenoids (all-*trans*-lutein, zeaxanthin,  $\beta$ -cryptoxanthin,  $\alpha$ -carotene and  $\beta$ -carotene) and six polyphenols (neochlorogenic acid, chlorogenic acid, rutin, quercetin, catechin and epicatechin) in different peach samples. Total phenolic content of each peach-derived product was determined using the Folin–Ciocalteu reaction. Blood glucose and weight were also monitored. The effect of treatments on liver and kidneys protection was determined by performing assays in serum and tissues.

## 2. Materials and methods

### 2.1. Chemicals

The following standards for carotenoids were purchased from Sigma-Aldrich (St. Louis, MO, USA):  $\beta$ -cryptoxanthin (purity >97%),  $\beta$ -carotene (purity >93%),  $\alpha$ -carotene (purity >95%) and zeaxanthin (purity >95%). Lutein standard (purity >95%) was purchased from Indofine (Hillsborough, NJ, USA). The HPLC-grade solvent methyl-*tert*-butyl was purchased from Scientific Hexis (São Paulo, Brazil). Methanol, formic acid (Sigma-Aldrich, St. Louis, MO, USA) and purified water (Milli-Q system, Millipore, MA, USA) were used for mobile phase preparation. Chlorogenic acid (95%), rutin hydrate (94%), quercetin dihydrate (98%), (+)-catechin hydrate (99%) and (–)-epicatechin (90%) were purchased from Sigma-Aldrich. CAT (EC 1.11.1.6), SOD (EC 1.15.1.1), thiobarbituric acid (TBA), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and  $\text{CCl}_4$  were from Sigma-Aldrich. Enzyme-linked immunosorbent assay (ELISA) microplates were from Greiner Bio-One (Monroe, NC, USA), and ELISA TMB spectrophotometric detection kit was from BD Biosciences (San Diego, CA, USA). TNF- $\alpha$ , IL-1 $\beta$ , nitrotyrosine, NF $\kappa$ B-p65, RAGE and  $\beta$ -actin primary antibodies and secondary horseradish-peroxidase-linked antibodies were from Cell Signaling Technology (Beverly, MA, USA). Purified recombinant TNF- $\alpha$  protein was from Abcam (Cambridge, UK), and IL1- $\beta$  was from BD. Bilirubin, LDH, AST and ALT activity kits were from Labtest (Minas Gerais, Brazil). The peach samples were provided by Embrapa Clima Temperado (Rio Grande do Sul, Brazil). Blood glucose meter OneTouch Ultra was from Johnson & Johnson (New Brunswick, NJ, USA).

### 2.2. Preparation of peach samples

The Maciel peach variety was developed by the unit of temperate climate of the Brazilian Agricultural Research Corporation (Embrapa Clima Temperado) by controlled hybridization. The seeds were laminated in chamber at  $4^\circ\text{C} \pm 1^\circ\text{C}$ , and then seedlings were cultivated in a greenhouse and subsequently transplanted to the seedlings experimental field. Fruits were obtained from this field (Pelotas, Rio Grande do Sul, Brazil; location coordinates:  $-31^\circ 30' 57.44''$ ,  $-52^\circ 33' 11.52''$ ). Immediately after harvesting the fruits, the pulps and peels were separated (the pits were removed and discharged) and frozen at  $-20^\circ\text{C}$  prior to lyophilization. These pulps and peels obtained from the fresh fruits were named Fresh Peach Pulps (FPP) and Peels, respectively, in this study (Fig. 1). Fruits were also used to prepare a sucrose-syrup-based preserve by the standard manufacturing procedure used for industrial-scale production of commercial canned peach. Briefly, the fruits were cut in half and the pits separated by twist. The fruits were subjected to a soda (NaOH 50%) shower-based peeling procedure in a treadmill and immediately washed to remove the soda. The remaining peel-free pulps were placed into cans, and hot sucrose-based syrup (water:sucrose 80:20) was added. Cans were sealed, subjected to sterilization and then cooled. The cans contained 820 g of total weight (pulps in syrup) and 485 g of drained weight. After 4 months, the cans were opened, and the pulps and the syrup were separated and subjected to lyophilization. The peach pulps obtained by this process were named Preserve Peach Pulps (PPP) in this study. The separated Syrup was designated as such and was also lyophilized and administered to animals in order to evaluate its nutritional effects, as it may extract active compounds from the pulps during the 4 months of preservation into cans. The frozen samples of FPP, Peels, PPP and Syrup were subjected to lyophilization at the same time. Lyophilization was carried out in L108 Liotop equipment (Liobras, São Paulo, Brazil) at the Embrapa Clima Temperado experimental unit. The lyophilized samples were preserved at  $-20^\circ\text{C}$ , dissolved in ultrapure water at the moment of the experiment and then centrifuged ( $4000g \times 3$  min) to precipitate rough particles (always protected from light and temperature). Supernatant suspension was collected and used for experiments. For each assay, serial dilutions were obtained from the stock solution of the different lyophilized samples and were used for oral administration. Aliquots of these diluted samples were also subjected to HPLC and other analytical analyses described below.

### 2.3. Bromatological analysis of lyophilized peach-derived samples

The total protein content was determined by the Kjeldahl method using the correction factor of 6.25 [19]. The lipid content was determined by the Soxhlet method [20] (Foss 2055 model Soxtec, Hillerød, Denmark). The carbohydrate content was determined according to the method of Lane–Eynon that is based on reducing a known amount of alkaline copper reagent (Fehling) to cuprous oxide [21]. The methodology used for the determination of fiber was proposed by and performed according to Association of Official Analytical Chemists [22]. Analyses were performed in triplicate. Results were expressed as grams per 100 grams (g/100 g) of dry matter.

### 2.4. Animals and experimental design

Male Wistar rats (21 days old) were obtained from our breeding colony. They were caged in groups of four animals with free access to standard commercial food (Chow Nuvilab CR-1 type; Curitiba, PR, Brazil). Chow nutritional composition consisted of total protein (22%), vegetal fiber (8%), minerals (10%), calcium (1.4%) and phosphorous (0.8%). Enrichment by kilograms is as follows: vitamin A (12,000 IU), vitamin D3 (1800 IU), vitamin E (30 IU), vitamin K3 (3 mg), vitamin B1 (5 mg), vitamin B2 (6 mg), vitamin B6 (7 mg), vitamin B12 (20  $\mu$ g), niacin (60 mg), folic acid (1 mg), biotin (0.05 mg), choline (600 mg), iron (50 mg), copper (10 mg), zinc (60 mg), manganese

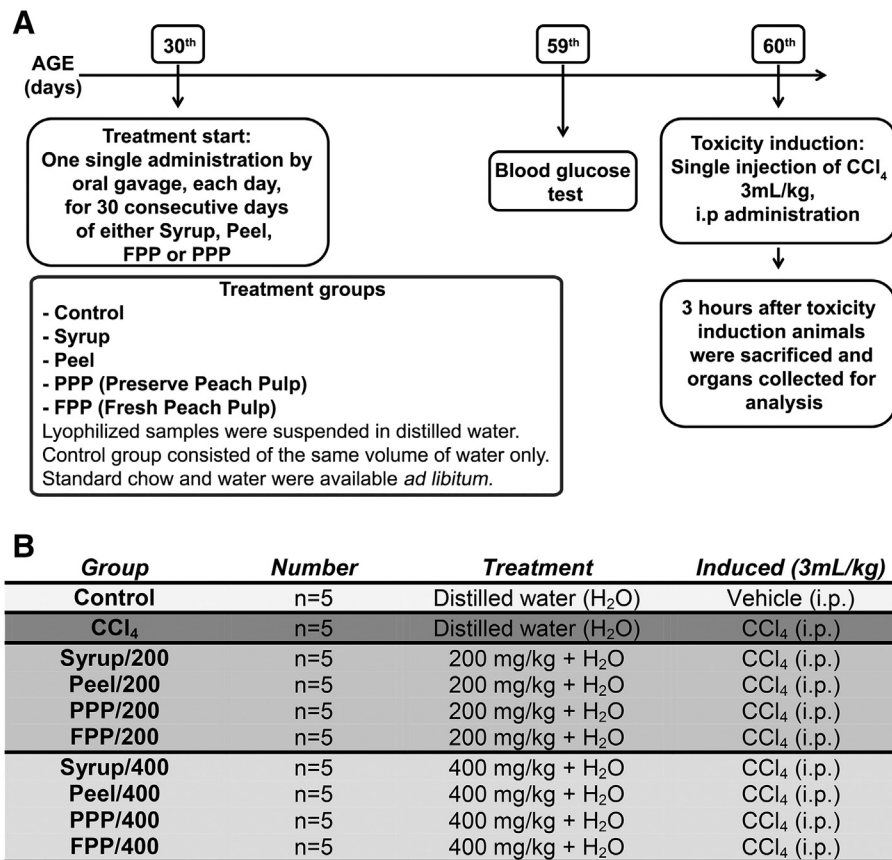


Fig. 1. Experimental design of administration of peach-derived products by oral gavage and acute toxicity induction to animals. Timeline (A); detailed scheme of experimental design (B).

(60 mg), cobalt (1.5 mg), iodine (2 mg), selenium (0.05 mg), lysine (100 mg) and methionine (300 mg). Rats were maintained in a 12-h light–dark cycle in a temperature-controlled colony room (21°C). All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health [23]. Our research protocol (no. 23944) was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal do Rio Grande do Sul. Sixty-five healthy animals were utilized for this study. A pilot test was performed with five animals to determine optimal dose of CCl<sub>4</sub>.

A process of adaptation to gavage manipulation was started at the 21st day of life of animals. The timeline design is shown at Fig. 1. At the 30th day of life, the weight of the animals was evaluated (animals weighed 90 to 110 g), and the treatments with the different peach-derived products were started. Treatments consisted of one single oral administration by gavage, each day, for 30 consecutive days, of either Syrup, Peel, PPP or FPP lyophilized samples previously resuspended in distilled water to constitute doses of 200 or 400 mg/kg. Standard rodent chow and water were available *ad libitum* during all treatment. At the 29th day of treatment (59th day of life of animals), a blood sample was withdrawn to check blood glucose levels. At the 30th day of treatment, a single ip injection of CCl<sub>4</sub> (3 ml/kg dissolved in olive oil) was administered for the acute toxicity induction. Three hours after injection, animals were euthanized, and the organs and serum were collected for analyses.

#### 2.5. Weight gain and blood glucose levels

One day before euthanasia, animals were fasted for 8 h [24], and a blood glucose test was performed. The blood sample was taken by a small tail puncture immediately before and 20, 40, 60 and 120 min after treatment administration. At each time, glucose was measured with a glucose meter (OneTouch Ultra).

#### 2.6. Cytokines levels in serum (IL-1 $\beta$ and TNF- $\alpha$ )

TNF- $\alpha$  and IL-1 $\beta$  were quantified by indirect ELISA. Serum was incubated in an ELISA plate. After 24 h, plates were washed three times with Tween–Tris-buffered saline (TTBS, 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20). Subsequently, 200  $\mu$ l of anti-TNF- $\alpha$  or anti IL-1 $\beta$  (1:1000) was added, and incubation was carried out for 24 h at 4°C. The plates were washed three times with TTBS and

incubated with rabbit or mouse IgG peroxidase-linked secondary antibody (1:1000) for 2 h according to product datasheets. After washing the plate three times with TTBS, 200  $\mu$ l of substrate solution (TMB spectrophotometric ELISA detection kit) was added to each well and incubated for 15 min. The reaction was stopped with 50  $\mu$ l/well of 12 M sulfuric acid, and the plate read at 450 nm in a microplate reader.

#### 2.7. Measurement of ALT, AST, bilirubin and LDH in serum

ALT, AST and LDH activities and bilirubin concentrations in serum were determined using Labtest kits (Minas Gerais, Brazil) according to manufacturer instructions.

#### 2.8. Antioxidant enzymes (SOD and CAT)

CAT and SOD activities were quantified in tissue homogenates (liver and kidney). Tissues were homogenized with 50-mM phosphate buffer (KH<sub>2</sub>PO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub>, pH 7.4). CAT activity was evaluated by following the rate of H<sub>2</sub>O<sub>2</sub> absorbance decrease at 240 nm [25]. Results are expressed as units of CAT/mg of protein. The activity of SOD was measured by quantifying the inhibition of superoxide-dependent adrenaline autooxidation to adrenochrome, which was monitored at 480 nm for 10 min (32°C) in a spectrophotometer [26]. Results are expressed as units of SOD/mg of protein.

#### 2.9. Oxidative damage markers (carbonyl, TBARS, sulfhydryl and nitrotyrosine)

As an index of protein oxidative damage, the carbonyl groups were determined based on its reaction with 2,4-dinitrophenylhydrazine as previously described [27]. Lipoperoxidation was determined from the quantification of TBARS originated from reaction of TBA with lipoperoxides in an acid-heating medium [28]. After precipitation with trichloroacetic acid 10%, supernatant was mixed with 0.67% and heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Protein nitrotyrosine was quantified by indirect ELISA in serum. Oxidative status of thiol groups was assessed by quantification of total reduced sulfhydryl (SH) groups in samples [29]. Briefly, for total SH content measurement, 60- $\mu$ g sample aliquot was diluted in phosphate-buffered saline (NaCl, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>) and 5,5'-dithionitros 2-nitrobenzoic acid (10 mM) and read in a spectrophotometer at 412 nm after 60 min of incubation in room temperature.

## 2.10. Protein assay

Total protein was quantified by Bradford assay and used to normalize all data [30].

## 2.11. Western blotting to liver and kidney proteins

To perform immunoblot experiments, the tissue was prepared using radioimmunoprecipitation assay buffer protocol. The proteins (30 µg/well) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes with Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Protein loading and electroblotting efficiency were verified through Ponceau S staining, and the membrane was washed with TTBS (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20). Membranes were incubated for 20 min at room temperature in SNAP i.d. 2.0 Protein Detection System (Merck Millipore, Billerica, MA, USA) with each primary antibody (anti-RAGE, anti-NFκB-p65 or anti β-actin; 1:500 dilution range) and subsequently washed with TTBS. Anti-rabbit or -mouse IgG peroxidase-linked secondary antibody was incubated for an additional 20 min in SNAP (1:5000 dilution) and washed again, and the immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit (Thermo Scientific; Luminol/Enhancer and Stable Peroxide Buffer, Rockford, IL USA). Densitometric analysis of the films was performed using Image J. software. Blots were developed to be linear in the range used for densitometry. All results were expressed as a relative ratio to β-actin.

## 2.12. Histological analysis

Formalin-fixed samples of liver were dehydrated, diaphonized and embedded in paraffin according to protocols for routine histological procedures. Five-micrometer-thick sections of the paraffin-embedded tissues were obtained and stained by means of hematoxylin–eosin histochemical (H&E) method. The sections were examined microscopically for evaluation of histological changes [31].

## 2.13. Quantification of carotenoids in peach-derived products by HPLC

Samples for HPLC analysis (500 mg) were dispersed in 20 ml of purified water, shaken for 15 min, filtered through a 0.45-mm membrane (Millipore, HVLP) and analyzed by LC. The content of neochlorogenic acid was calculated by the chlorogenic acid linear equation. The content of rutin, quercetin, catechin and epicatechin was calculated by the linear equations of the corresponding reference substances. The carotenoids were separated on a polymeric reversed phase column (YMC C<sub>30</sub> 250 µm×4.6 µm; particle size of 3 µm) with a mobile phase gradient elution starting with water/methanol/methyl-*tert*-butyl ether (MTBE) at 5:90:5 and reaching 0:95:5 after 12 min, 0:89:11 after 25 min, 0:75:25 after 40 min and 00:50:50 after 60 min with a flow rate of 1 ml/min at 33°C [32]. The spectra were analyzed between 250 and 600 nm, and the chromatograms were processed at a fixed wavelength of 450 nm for carotenoids. Identification was performed by comparison of peak retention times obtained in each sample with the retention times of standards analyzed under the same conditions.

Quantifications were performed constructing standard curves for the carotenoids in the following concentration ranges: 5–4000 µg/ml for β-carotene, 2–200 µg/ml for α-carotene, 1–1000 µg/ml for all-*trans*-lutein, 4–4000 µg/ml for cryptoxanthin and 1–500 µg/ml for zeaxanthin. The standards were dissolved in MTBE and analyzed under the same conditions.

The limits of detection (LODs) and limits of quantification (LOQs) were determined as previously described [33]. The following LOD and LOQ scores were, respectively obtained:  $6.5 \times 10^{-2}$  and  $10.9 \times 10^{-2}$  mg/kg for β-carotene,  $6.9 \times 10^{-3}$  and  $1.2 \times 10^{-2}$  mg/kg for lutein,  $2.1 \times 10^{-2}$  and  $3.5 \times 10^{-2}$  mg/kg for cryptoxanthin,  $9.6 \times 10^{-2}$  and  $1.6 \times 10^{-2}$  mg/kg for zeaxanthin, and  $2.0 \times 10^{-2}$  and  $3.3 \times 10^{-2}$  mg/kg for α-carotene.

## 2.14. Quantification of polyphenols by HPLC *in vitro*

The LC analysis of peach samples was performed [14] using a Shimadzu Prominence equipment (Kyoto, Japan) coupled to an SPD-20A UV/VIS detector. The stationary phase was a Phenomenex RP-18 column (Synergi Fusion 150×4.6-mm i.d.; 4-µm particle size) guarded by a Phenomenex precolumn (2×4-mm i.d., 10-µm particle size). The mobile phase consisted of (A) formic acid 0.5% (v/v) and (B) methano:formic acid (100:0.1, v/v). The gradient elution was 5% B (0–5 min), 5%–12% B (5–10 min), 12% B (10–13 min), 12%–29% B (13–35 min), 29%–46% B (35–50 min), 46%–80% B (50–52 min), 80% B (52–57 min) and 80%–100% B (57–60 min). The column was washed with methanol for 3 min and reequilibrated with 5% B for 5 min before the next analysis. The flow rate was 1 ml min<sup>-1</sup>, and the injection volume was 10 µl. The chromatograms were recorded at 280 nm and 340 nm, and the analysis was carried out at room temperature. Chlorogenic acid, rutin hydrate, quercetin dihydrate, (+)-catechin hydrate and (–)-epicatechin were used as external standards. They were dissolved in methanol and diluted to obtain the concentrations between 1 and 20 µg/ml. These solutions were filtered through a 0.45-mm membrane filter (Millipore, HVLP). The linear equations were  $y=23,548x-10,618$  ( $r^2=0.9977$ ),  $y=14,166x-7037$  ( $r^2=0.9971$ ),  $y=23,096x-10,331$  ( $r^2=0.9962$ ),  $y=5868x+751$  ( $r^2=0.9993$ ) and  $y=54261x-409$  ( $r^2=0.9997$ ) for chlorogenic acid, rutin hydrate, quercetin dihydrate, (+)-catechin hydrate and (–)-epicatechin, respectively.

## 2.15. Determination of total phenolic content

Total phenolic content of peaches and derivatives was determined using the Folin–Ciocalteu method [34]. One hundred microliters of Folin–Ciocalteu reagent was mixed with 100 µl of sample, and then 200 µl of Na<sub>2</sub>CO<sub>3</sub> 35% was added. The volume was completed to 1900 µl with ultrapure H<sub>2</sub>O and then homogenized. After 10 min, the absorbance was measured at 725 nm and compared to a gallic acid calibration curve. Total polyphenols in samples were expressed as gallic acid equivalents [35].

## 2.16. Statistical analysis

Statistical analysis was performed with GraphPad 5.0 software. The results were expressed as mean±standard error (S.E.M.). Data were evaluated by one-way analysis of variance followed by Tukey's *post hoc* test. Differences were considered significant when  $P<.05$ .

## 3. Results

### 3.1. Bromatological analysis

Table 1 shows the results obtained for bromatological analysis of the different lyophilized products of peach – Syrup, Peel, PPP and FPP. Lipid, protein, dietary fiber, reducing sugar, total sugar and calories contents were measured. In general, fruits present moderate quantities of lipids [36]. We observed here lipid values below 1.5 g/100 g in all lyophilized samples, as expected. The highest protein content was found in Peel ( $4.4 \pm 0.03$ ), followed by FPP ( $3.0 \pm 0.05$ ). PPP showed lower amounts of protein ( $1.3 \pm 0.1$ ) compared to FPP, possibly as consequence of a cooking process triggered by the addition of hot syrup into the cans during the manufacturing process. The Syrup samples had the lowest values for lipids, proteins and dietary fiber but presented the highest values for total sugar. The FPP presented higher amounts of fiber ( $5.8 \pm 0.08$ ). The carbohydrate content found in FPP ( $63.9 \pm 0.8$ ) and Peel ( $46.5 \pm 0.01$ ) was lower than that in PPP ( $78.6 \pm 1.5$ ) and Syrup ( $98.2 \pm 1.5$ ). Calories of each sample were calculated based on these data. FPP (277.7 kcal/100 g) and Peel (205.1 kcal/100 g) presented lower caloric index followed by the PPP (328.7 kcal/100 g). Syrup presented the highest caloric content due to its high index of carbohydrates (403.8 kcal/100 g). Considering the elevated content of sugars present in the Syrup, we decided to monitor blood glucose levels and body weight variations during the period of administration of the different peach-derived products by oral gavage to the animals.

### 3.2. Quantification of carotenoids, polyphenols and total phenolic content in peach-derived products

The health benefits related to fruit consumption seem to be related to the content of secondary metabolites presenting antioxidant activities. Therefore, we analyzed the content of carotenoids and polyphenols in the peach-derived products. The chemical composition analysis by HPLC of Peel, PPP and FPP samples showed presence of (a) all-*trans*-lutein, (b) zeaxanthin and (c) β-cryptoxanthin as the major

Table 1  
Nutritional composition of peach-derived products – Syrup, Peel, PPP and FPP.

Compounds	Peach-derived products			
	Syrup	Peel	PPP	FPP
Lipid	0.3±0.1	1.4±0.2	1.0±0.09	1.1±0.3
Protein	0.1±0.05	4.4±0.03	1.3±0.1	3.0±0.05
Dietary fiber	0.1±0.07	1.0±0.004	4.6±0.04	5.8±0.08
Reducing sugar	34.1±0.4	17.1±0.5	35.3±0.01	19.3±0.3
Total sugar	98.2±1.5	46.5±0.01	78.6±1.5	63.9±0.8
Total kcal/100 g	403.8	205.1	328.7	277.7

Values expressed as the mean±standard deviation as g/100 g of dry matter.

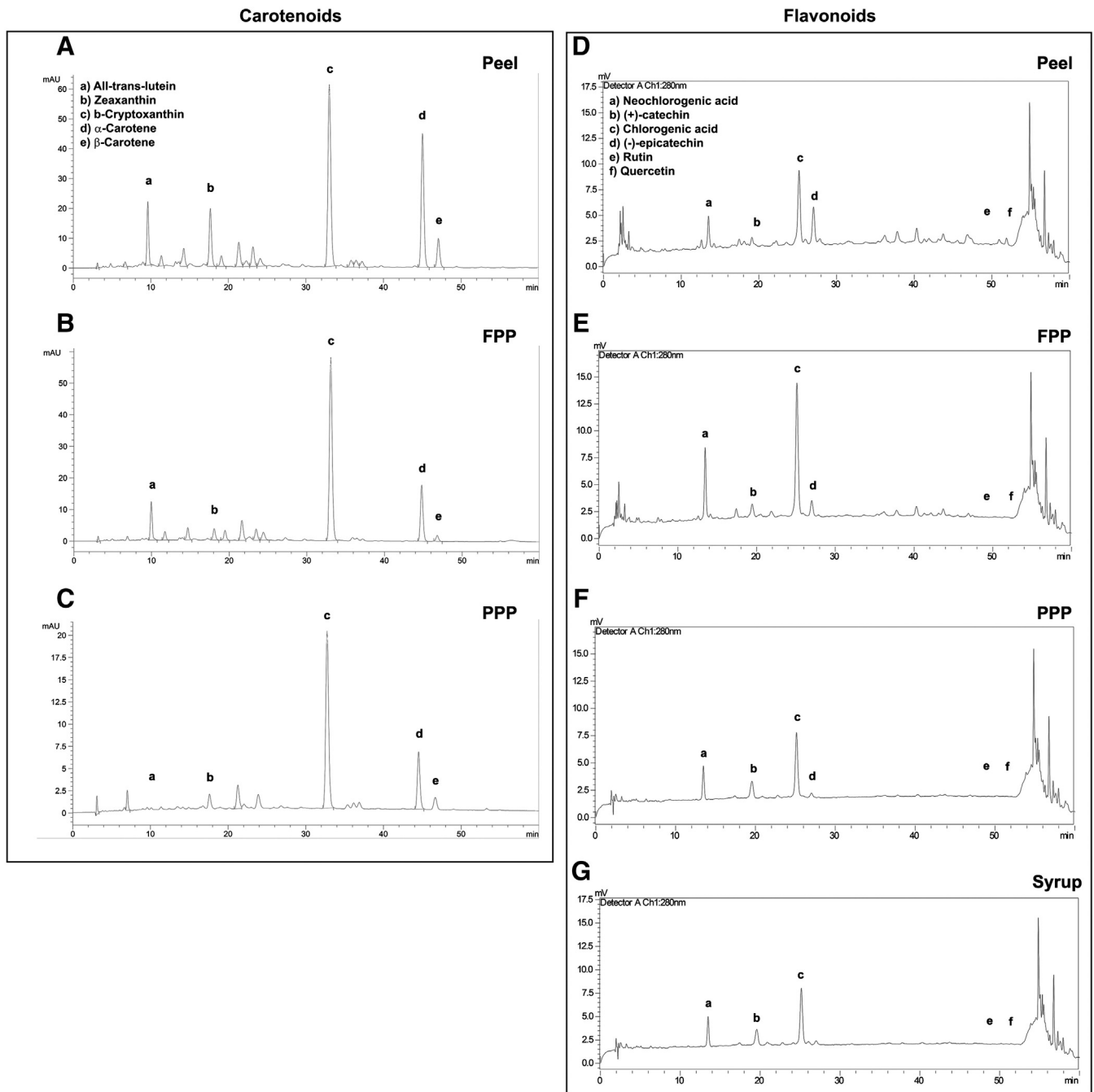


Fig. 2. Representative HPLC chromatograms of the different peach-derived products. Left side: Peel (A), FPP (B) and PPP (C). Each peak represents one carotenoid: (a) all-*trans*-lutein; (b) zeaxanthin; (c)  $\beta$ -cryptoxanthin; (d)  $\alpha$ -carotene; (e)  $\beta$ -carotene. Right side: Peel (D), FPP (E), PPP (F) and Syrup (G). Each peak represents one polyphenol: (a) neochlorogenic acid; (b) (+)-catechin; (c) chlorogenic acid; (d) (-)-epicatechin; (e) rutin and (f) quercetin.

compounds; furthermore, (d)  $\alpha$ -carotene and (e)  $\beta$ -carotene were also identified (Fig. 2). Interestingly, Peel and FPP, which also presented a better performance in protecting against oxidative damage and inflammation, were enriched in antioxidant carotenoids such as all-*trans*-lutein, zeaxanthin,  $\alpha$ -carotene and  $\beta$ -carotene compared to PPP (Table 2). Our chemical composition analysis of polyphenols was conducted using six different standards: (a) neochlorogenic acid, (b) (+)-catechin, (c) chlorogenic acid, (d) (-)-epicatechin, (e) rutin and (f) quercetin. Only four of these compounds were detected in our samples of peach-derived products: neochlorogenic acid, (+)-catechin, chlorogenic acid and (-)-epicatechin (Fig. 2, Table 2). Chlorogenic acid was the compound that presented the highest concentrations, which

are observed mainly in FPP samples. The presence of (-)-epicatechin was detected only in samples derived from fresh fruits (i.e., FPP and Peel). Rutin and quercetin were not detected in our samples. Quantification of total phenolics showed that FPP (2656.25  $\mu\text{g/g}$ ) and Peel (3350.196  $\mu\text{g/g}$ ) presented higher concentrations of polyphenols, while PPP (1456.446  $\mu\text{g/g}$ ) and Syrup (1281.836  $\mu\text{g/g}$ ) were less enriched in such compounds (Table 2).

### 3.3. Effect of treatments on weight gain and blood glucose levels

The animals were divided into groups receiving different treatments in addition to the standard diet; treatments consisted of one single oral

Table 2  
Quantification of carotenoids, polyphenols and total phenolic content in peach-derived products – Syrup, Peel, PPP and FPP.

Peak	Rt range (min)	Compounds	Carotenoids (µg/g)			
			Peach-derived products			
			Syrup	Peel	PPP	FPP
A	17.9–19.8	All-trans-lutein	ND	6.78±0.3	0.93±0.8	1.528±0.3
B	20.0–21.7	Zeaxanthin	ND	3.287±0.2	0.725±0.1	2.371±0.2
C	32.6–43.43	β-Cryptoxanthin	ND	39.149±0.1	12.718±0.2	35.755±0.1
D	38.4–38.53	α-Carotene	ND	1.441	ND	ND
E	42.95–43.43	β-Carotene	ND	37.657±0.4	6.397±0.1	15.22±0.4
Total			ND	88.284	20.77	54.874
Polyphenols (µg/g)						
A	13.4	Neochlorogenic acid	150±0.01	130±0.01	140±0.01	270±0.01
B	19.4–19.7	(+)-Catechin	320±0.01	ND	300±0.01	210±0.01
C	24.8–25	Chlorogenic acid	390±0.01	430±0.04	400±0.01	790±0.02
D	27	(-)-Epicatechin	ND	610±0.01	ND	250±0.01
E	49	Rutin	ND	ND	ND	ND
F	52	Quercetin	ND	ND	ND	ND
Total			860	1.170	840	1.520
Total phenolic content						
Total		Gallic acid equivalents	1281.836±076	3350.196±153	1456.446±124	2656.25±173

ND, not detected. The values are expressed as mean±standard deviation (µg/g).

administration by gavage, each day, for 30 consecutive days of either Syrup, Peel, PPP or FPP lyophilized samples previously resuspended in distilled water at the doses of 200 or 400 mg/kg. Experimental design is described at Section 2.4 and shown in Fig. 1. We observed that both doses (200 and 400 mg/kg) did not affect the weight of animals as all groups maintained the same proportions of weight gain along the period of treatment (Fig. 3A and C). The Syrup and PPP daily treatment at doses of 200 mg/kg (Fig. 3B) promoted a transient increase of glycemia, which peaked by 20 and 40 min (102±1.6 mg/dl) and returned to basal levels after 120 min (89±1.0 mg/dl). In animals receiving 400 mg/kg, glycemic curves displayed the same profile, although glycemia peaked at higher levels (112±0.5 mg/dl) compared to the groups receiving 200 mg/kg. However, it is important to note that glycemic levels in all groups increased and returned to normal levels 120 min after the oral administration at the 29th day of treatment, indicating no differences in glycemic variations between the treatments.

### 3.4. Effects of Syrup, Peel, PPP and FPP on tissue damage serum biomarkers

Table 3 shows the effect of the different peach products on CCl<sub>4</sub>-induced tissue damage. Treatment with a single dose of CCl<sub>4</sub> caused a threefold increase in the levels of AST and ALT transaminases, confirming the induction of tissue toxicity. Bilirubin, which is frequently more affected in severe hepatotoxicity, only increased 1.5-fold. At dose of 200 mg/kg, only FPP was able to reduce AST activity; all other markers did not change with this dose or other products. On the other hand, pretreatment with 400-mg/kg/day PPP and FPP for 30 days significantly prevented leakage of AST and ALT. Bilirubin was decreased only with FPP group. LDH test evidenced that one single administration of CCl<sub>4</sub> to Wistar rats resulted in a 10-fold increase of LDH activities in serum, and FPP,

PPP and Peel showed capacity to protect tissues against cytotoxic damage.

### 3.5. Cytokine (TNF-α and IL-1β) levels in serum

Taking into account that organ inflammation is an important component of tissue damage signaling leading to systemic inflammation, we assessed serum levels of the inflammatory cytokines IL-1β and TNF-α after CCl<sub>4</sub> administration. The levels of these markers were significantly increased by CCl<sub>4</sub> treatment compared with controls, indicating that damage from CCl<sub>4</sub> causes inflammatory responses as expected. Again, treatments with 200 mg/kg of peach-derived products prior to toxicity insult did not prevent CCl<sub>4</sub>-induced cytokine production (Fig. 4A and B), whereas 400-mg/kg Peel blocked IL-1β (Fig. 4A) and FPP blocked TNF-α induction by CCl<sub>4</sub> (Fig. 4B).

### 3.6. Status of oxidative stress parameters (nitrotyrosine in serum and SOD and CAT in liver and kidney)

As observed for the aforementioned parameters, treatments with 200 mg/kg of peach-derived products did not prevent CCl<sub>4</sub> effects on nitrotyrosine levels. In agreement with the inhibitory effect upon IL-1β and TNF-α, FPP and Peel daily administration at 400 mg/kg decreased protein nitration (nitrotyrosine), indicating that these peach-derived products can successfully block the effect of reactive nitrogen species such as peroxynitrite on protein damage (Fig. 5A). We also observed that CCl<sub>4</sub> also promoted a significant increase in the activity of superoxide detoxification enzymes (SOD) in renal and hepatic tissues (~twofold increase), and the treatment with 400 mg/kg of Peel and FPP blocked induction of this enzyme by CCl<sub>4</sub> in liver but not in kidney (Fig. 5B and D). The hydrogen peroxide detoxification enzyme CAT was 2-fold and 30-fold increased in liver and renal tissues after CCl<sub>4</sub>-induced damage (Fig. 5C and E). At 400 mg/kg, treatment with PPP and FPP significantly inhibited CAT activity induction by CCl<sub>4</sub> in both tissues; Peel blocked CAT induction in liver, and a nonsignificant trend towards decrease was also observed in kidney.

### 3.7. Oxidative damage assays in liver and kidney

Hepatic and renal oxidative damage was determined through carbonyl (protein damage by hydroxyl radical), sulfhydryl (cysteine oxidation) and TBARS assays (lipoperoxidation). In both liver and kidney, exposure to CCl<sub>4</sub> modulated all parameters of oxidative damage toward a pro-oxidative status/damage. FPP treatment seems to be the most effective in inhibiting oxidative damage as it inhibited protein carbonylation, formation of lipoperoxides/TBARS and cysteine oxidation in liver (*P*<.05) as well as kidney (*P*<.001) (Fig. 6 A-F). Treatment with Peel also exerted significant antioxidant activity by preventing carbonylation (liver, Fig. 6A), lipoperoxidation (liver and kidney, Fig. 6C and F) and sulfhydryl oxidation in liver (Fig. 6B). Syrup treatment was ineffective to counteract oxidative damage, and PPP only blocked lipoperoxidation in both tissues (Fig. 6C and F), suggesting modest effects on these parameters at the tested doses.

### 3.8. Western blot for NFκB and RAGE in liver and kidney tissues

Fig. 4A and B data showed that CCl<sub>4</sub> increases the levels of IL-1β and TNF-α, which are important proinflammatory gene products. Western blot analysis revealed that the amount of hepatic and renal NFκB-p65 protein and RAGE – which are important upstream mediators of inflammatory genes such as TNF-α and IL-1β – increased markedly after CCl<sub>4</sub> administration. CCl<sub>4</sub>-induced NFκB-p65 protein level was significantly attenuated by Peel and FPP in liver (Fig. 7A). Treatments with Peel, PPP and FPP, but not

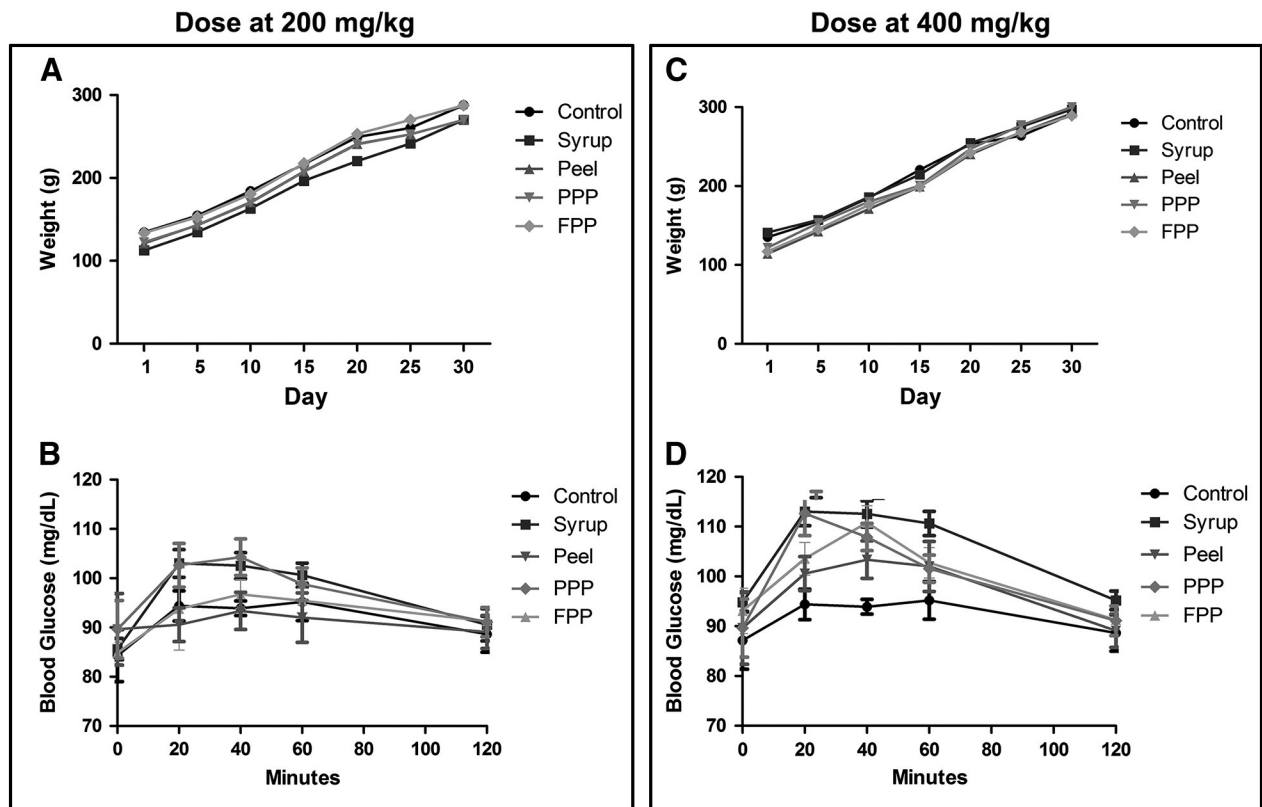


Fig. 3. Animals' weight during treatments with peach-derived products at 200 mg/kg (A) and 400 mg/kg (C). Blood glucose of animals during treatments with peach-derived products at 200 mg/kg (B) and 400 mg/kg (D). Values represent mean  $\pm$  S.E.M. No significant differences were observed. Two-way analysis of variance and Tukey's multiple comparison *post hoc* test were applied.

Syrup, were able to prevent NF $\kappa$ B-p65 induction by CCl<sub>4</sub> in kidneys (Fig. 7C). The effects of CCl<sub>4</sub> on RAGE protein content were broadly inhibited by Peel, PPP and FPP, but not Syrup, in liver (Fig. 7B) and kidney (Fig. 7D) tissues.

### 3.9. Liver histology

Histological analysis of control animals liver sections showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, and visible central veins (Fig. 8A). The liver sections from CCl<sub>4</sub> treatments revealed liver injuries, such as hydropic degeneration and nuclear polymorphism, which were characterized by presence of hepatocytes cloudy swelling with pale cytoplasm and poorly delineated and displaced nuclei (Fig. 8B). Analysis of hepatic histopathological lesions indicated that treatment with Peel (Fig. 8D), PPP (Fig. 8E) and FPP (Fig. 8F) markedly ameliorated the morphology of liver after CCl<sub>4</sub> insult. Syrup group (Fig. 8C) did not display improvements compared to CCl<sub>4</sub> group.

## 4. Discussion

In the last decade, there has been a growing interest in understanding how the intake of different fruits and vegetables as well as its respective processed foods can lead to health benefits in the short and long term. The presence of a variety of carotenoids, ascorbic acid, citric acid and polyphenols amongst other compounds makes fruits and vegetables able to interfere with antioxidant, inflammatory and coagulative systems [10,13,37]. Understanding how industrial processing affects biological properties of vegetables is also subject of interest in the field. Here, we focused on the study of different parts of

fresh (Peel and FPP) and canned peaches (Syrup and PPP) and evaluated their preventive role in liver and renal damage caused by CCl<sub>4</sub> exposure in rats in order to address its possible application as a functional food.

CCl<sub>4</sub> is widely utilized to induce liver fibrosis, cirrhosis and general toxicity in rats [38–40]. In our model, a single CCl<sub>4</sub> dose was injected at the last day of a 30-day period in which rats received a daily single-dose administration of Syrup, Peel, PPP or FPP by oral gavage, characterizing it as a preventive strategy.

Treatments consisted of a daily administration of a single dose of each of the peach-derived products. Since animals were exposed to a standard diet (rodent chow and water *ad libitum*), we might conclude that addition of regular peach consumption to a regular, healthy diet might exert some beneficial effects against toxic insults. The peach-derived products

Table 3

ALT, AST and LDH activities and bilirubin concentrations in serum of animals treated with peach-derived products (Syrup, Peel, PPP and FPP at 200 and 400 mg/kg/day for 30 days) and subjected to injection of CCl<sub>4</sub> (3 ml/kg).

Group	ALT (U/L)	AST (U/L)	Bilirubin (mg/ml)	LDH (U/L)
Control	31.53 $\pm$ 2.5	119.13 $\pm$ 3.4	10.50 $\pm$ 0.2	10.5 $\pm$ 1.5
CCl <sub>4</sub>	83.80 $\pm$ 9.8	315.9 $\pm$ 11.7	15.27 $\pm$ 0.8	104.4 $\pm$ 1.1
Syrup/200	70.30 $\pm$ 10.51	229.5 $\pm$ 39.3	15.97 $\pm$ 0.3	101.8 $\pm$ 6.4
Peel/200	63.1 $\pm$ 4.4	234.1 $\pm$ 32.3	15.31 $\pm$ 0.2	102.5 $\pm$ 8.1
PPP/200	65.9 $\pm$ 5.9	240.4 $\pm$ 40.5	15.05 $\pm$ 0.3	101.5 $\pm$ 12.1
FPP/200	62.15 $\pm$ 4.8	205.2 $\pm$ 16.5 *	14.28 $\pm$ 0.3	82.9 $\pm$ 4.2
Syrup/400	71.49 $\pm$ 9.7	289.2 $\pm$ 18.1	14.35 $\pm$ 0.6	107.3 $\pm$ 1.7
Peel/400	62.00 $\pm$ 8.7	248.0 $\pm$ 17.22	13.91 $\pm$ 0.4	78.47 $\pm$ 2.4 ***
PPP/400	42.11 $\pm$ 8.1 **	202.3 $\pm$ 12.57 **	13.43 $\pm$ 0.8	56.99 $\pm$ 1.7 ***
FPP/400	40.94 $\pm$ 4.8 **	176.9 $\pm$ 21.47***	11.01 $\pm$ 1.1 *	59.79 $\pm$ 2.4 ***

Asterisks represent differences in relation to CCl<sub>4</sub>-treated group (\**P*<.05, \*\**P*<.01 and \*\*\**P*<.001). Values represent mean  $\pm$  S.E.M. One-way analysis of variance and Tukey's multiple comparison *post hoc* test were applied for all data.

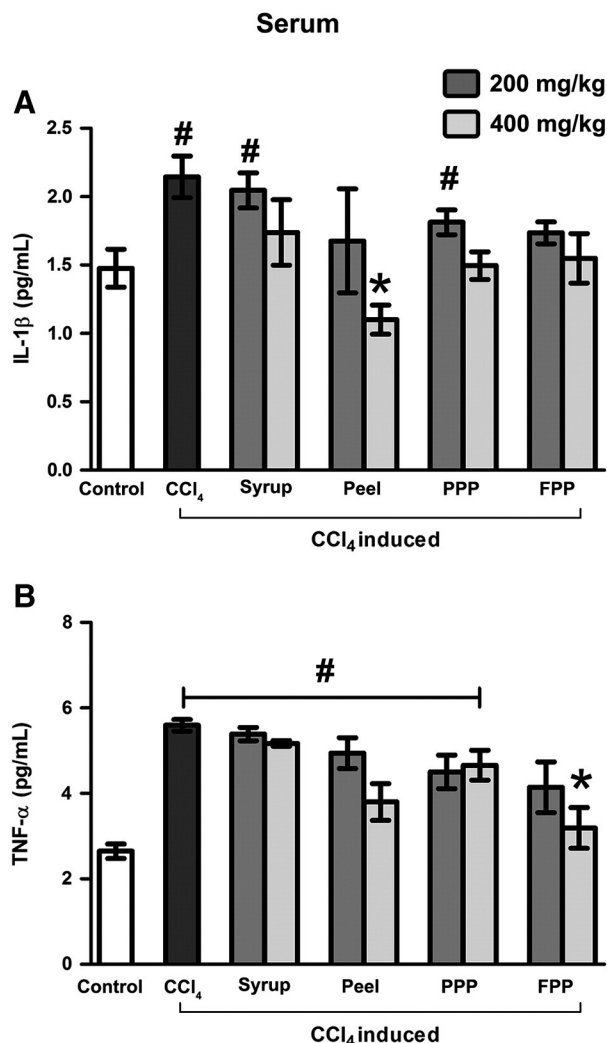


Fig. 4. Effects of treatment with peach-derived products (Syrup, Peel, PPP and FPP at 200 and 400 mg/kg a day for 30 days) on serum levels of IL-1 $\beta$  and TNF- $\alpha$  in CCl<sub>4</sub>-treated rats. IL-1 $\beta$  (A) and TNF- $\alpha$  (B) levels were quantified by ELISA as described in "Materials and methods". #Denotes difference compared to control group; \*denotes difference compared to CCl<sub>4</sub>-treated group. Values represent mean  $\pm$  S.E.M. One-way analysis of variance and Tukey's multiple comparison *post hoc* test were applied for all data.

analyzed here contained a small fraction of lipids. Lipids obtained from plant foods are thought to exert an important role in increasing the bioavailability of carotenoids, unsaturated fatty acids and liposoluble vitamins (such as vitamins A and E) [36,41]. Also, the increased fiber intake elicited by the treatments with Peel, FPP and PPP may also confer some health benefits, as diets rich in fiber are associated to lower levels of serum LDL, decreased risk of coronary heart disease, reduced blood pressure, enhanced weight control, better glycemic control, reduced risk of certain forms of cancer and improved gastrointestinal function [42]. Based on literature comparison, FPP can be considered an appropriate source of dietary fiber (5.8 g fiber/100 g) [43].

Along the treatments, all groups presented similar weight gain (Fig. 3A and C). At the 29th day, blood glucose was measured, and a glycemic curve after the administration of the dose at this day was assessed. The results showed that the different peach-derived products did not induce significant variations in blood glycemic levels. We had concern that significant differences in the variation of blood glucose levels after the administration of peach-derived products could be associated to physiological changes that would affect some of the effects studied here. It is important to note, however, that comparing the effects of each peach-derived product

with other compounds with established glycemic index and the determination of glycated albumin levels are necessary for a complete screening regarding blood glucose variations.

In the course of hepatic/tissue damage, plasmatic membrane dysfunction and necrotic cell death release a series of transaminases, LDH amidst other enzymes into blood circulation, which are considered indicators of liver/tissue injury [44]. In our model, different peach-derived products exerted varying degrees of protection when based on detection of serum transaminases, LDH and bilirubin caused by CCl<sub>4</sub>. FPP, Peel and PPP were the most efficient to prevent leakage of these markers, mostly LDH. As LDH is not totally specific for liver and renal damage, and AST also can be used as marker of cardiac damage, we confirmed the preventive effect of Peel, FPP and PPP treatments by direct histological analyses in liver tissues, and the protective effect was clear. However, the group treated with Syrup presented no detectable effect in these parameters.

CCl<sub>4</sub> is a nonpolar compound which tends to interact more efficiently with lipid structures as cell membranes, thus propagating free radical reactions toward intracellular compartments [45]. Then, we would expect to find out a significant damage to lipids following CCl<sub>4</sub> exposure. In biological systems, lipid peroxidation creates a series of stable toxic aldehydes products, and TBARS have been frequently used as an indicator of lipid peroxidation. Increased lipoperoxidation, protein carbonylation levels, and decreased total thiol content facilitate intra- and intermolecular cross-links of proteins, which in turn induce conformational changes leading to increased hydrophobicity, formation of protein aggregates and oxidative damage to proteins, inducing generalized cellular dysfunction and favoring the maintenance of the pro-oxidative state [9,46]. Indeed, lipid peroxidation seems to be an important mechanism whereby CCl<sub>4</sub> affects cell integrity once the levels of TBARS in CCl<sub>4</sub>-treated animals were massively increased mainly in kidney tissues, in agreement with Refs. [7,47]. Treatments with Peel, PPP and FPP (400 mg/kg), but not Syrup, effectively protected against lipid peroxidation in liver and renal tissues possibly due to its antioxidant and free radical scavenging activities. Although kidney has showed higher TBARS, the peach-derived products were able to protect this tissue even at the lowest dose, suggesting a greater capacity of peaches in preventing renal injury. Protein carbonylation increased in both tissues, and there was a decrease of protein thiol content, indicating that CCl<sub>4</sub> injury also affects protein redox state, which may affect protein structure and function, and consequently pathways and organelles functioning [48]. In our model, treatment with Peel protected liver against sulphydryl oxidation and protein carbonylation, whereas only FPP was efficient in renal tissue (at 400 mg/kg) for the same markers.

The effect of Peel and FPP against protein nitration (3-nitrotyrosine marker) suggests that these fractions affect nitric oxide (NO) and its derivative nitration-active compound peroxynitrite metabolism. NO is frequently increased by inflammatory signals which activate nitric oxide synthase enzymes through NF $\kappa$ B pathway, playing a role in carcinogenesis, chronic infection, inflammation and neurodegeneration [49,50]. High levels of superoxide can interact with NO in tissues and form the highly diffusible nitrogen species peroxynitrite, a highly reactive intermediate, which can increase DNA damage and initiate lipid peroxidation [51]. Increases in peroxynitrite lead to protein tyrosine residues modification to form nitrotyrosine adduct, affecting protein structure and function. For example, tyrosine nitration of mitochondrial manganese-dependent SOD results in loss of enzymatic activity [52], and in serum, peroxynitrite targets mainly albumin, thus forming 3-nitrotyrosine groups [53]. We believe that increases in the activation of inflammatory pathways such as NF $\kappa$ B – as observed in Fig. 7 – as well as induction of RAGE (which signals downstream by inducing NF $\kappa$ B) in the presence of CCl<sub>4</sub>-induced free radicals are promoting formation of peroxynitrite.



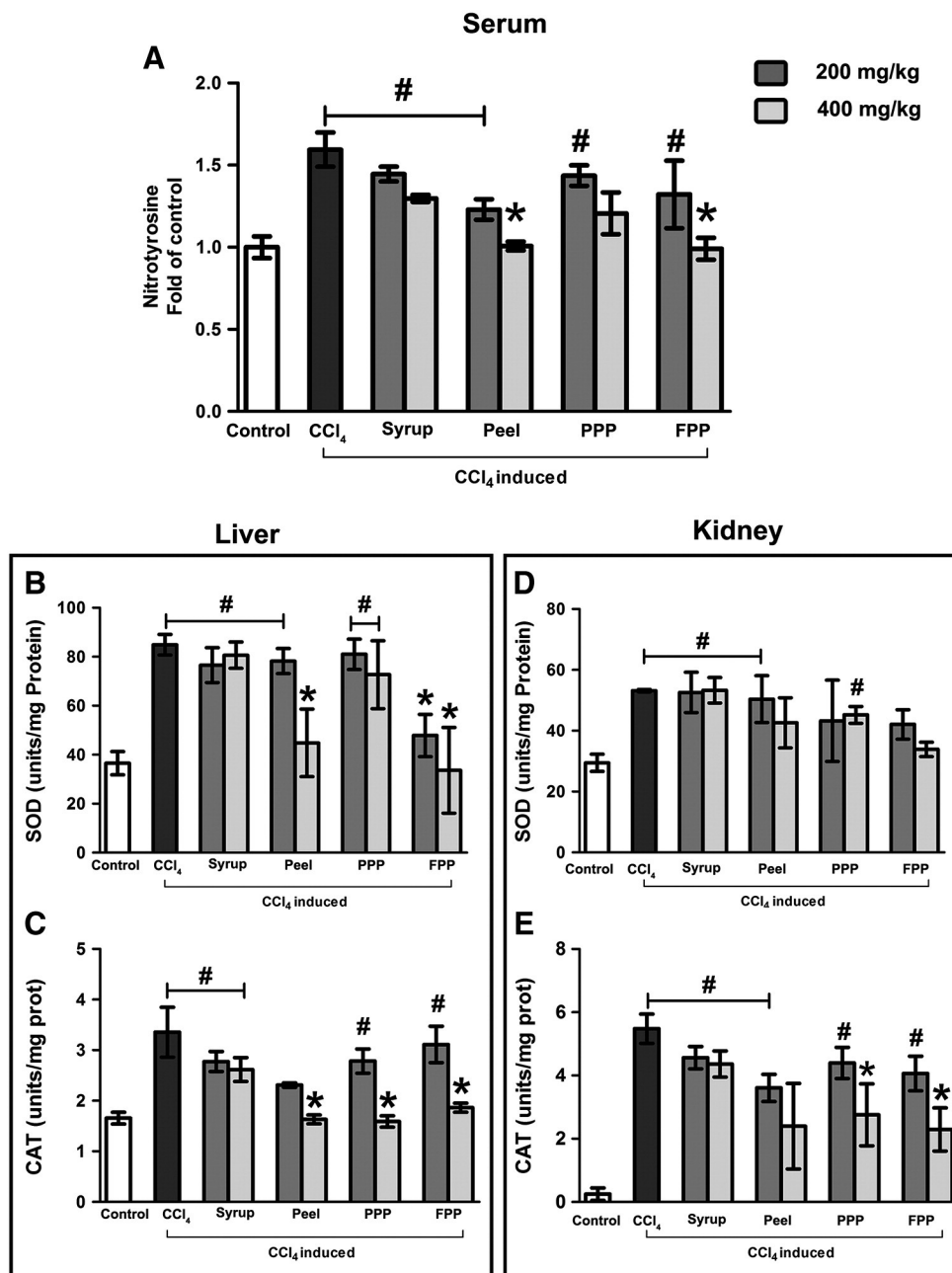


Fig. 5. Effects of treatment with peach-derived products (Syrup, Peel, PPP and FPP at 200 and 400 mg/kg a day for 30 days) against CCl<sub>4</sub>-induced oxidative stress. Nitrotyrosine levels (A) in serum were evaluated by ELISA. SOD (B) and CAT activity (C) in liver homogenates. SOD (D) and CAT activity (E) in kidney. #Denotes difference from control group; \*represents difference from CCl<sub>4</sub>-treated group. Values represent mean±S.E.M. One-way analysis of variance and Tukey's multiple comparison *post hoc* test were applied for all data.

Then, treatment with peach-derived products might be inhibiting nitrotyrosine formation by blocking either NFκB or superoxide radical formation. The antioxidant effect of Peel and FPP in preventing ROS accumulation is evident from their inhibitory effect on SOD and CAT activities induction by CCl<sub>4</sub> in liver. SOD and CAT are in the first line of antioxidant defense mechanisms by protecting cells against superoxide and hydrogen peroxide through sequential detoxification reactions. These reactions, in turn, decrease hydrogen peroxide availability to react with transition metals to form the most dangerous free radical, hydroxyl [53]. CCl<sub>4</sub> toxicity might result in significantly increases in SOD and CAT activities possibly as an attempt to counteract free radicals in liver and kidney. One could conclude that if Peel and FPP treatments are decreasing antioxidant enzymes

activity, this would enhance oxidative stress. However, taking into account that animals received a daily treatment with peach-derived products for 30 days prior to CCl<sub>4</sub> injection, we interpret that Peel and FPP treatments provided an antioxidant capacity to block CCl<sub>4</sub>-induced oxidative damage (as observed from the decreased level of oxidative damage markers and liver histology, Fig. 6A–C); thus, the substrates for SOD and CAT were not available to cause detectable increases in their activities. The understanding on how a diet with fruits enriched with antioxidant compounds can prevent tissue damage involves an interesting and complicated cross-talk among (a) level and type of oxidants generated by the stressor agent; (b) organ intrinsic antioxidant enzymatic and nonenzymatic defenses; and (c) type, bioavailability and specific free-radical/oxidant

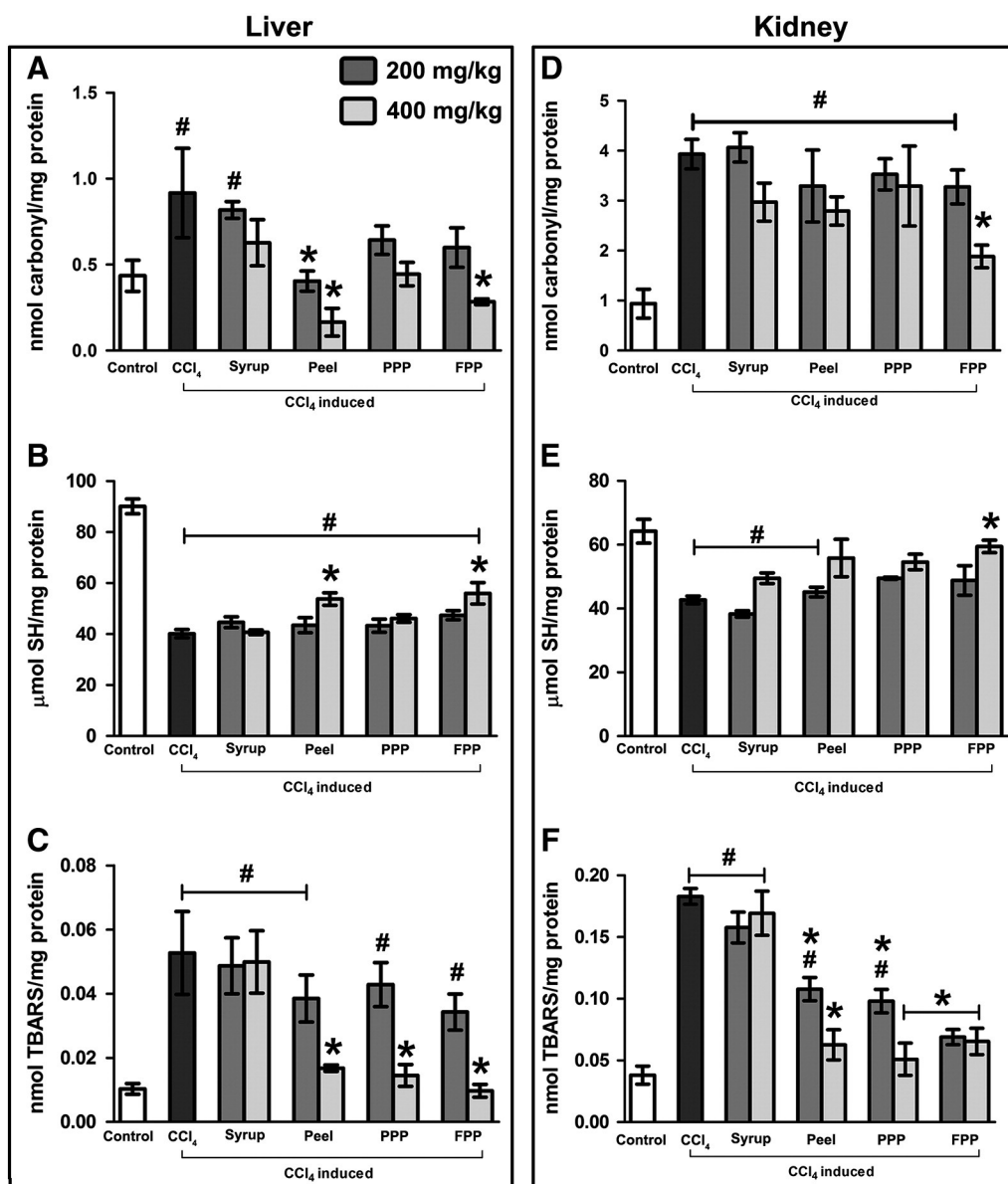


Fig. 6. Effects of treatment with peach-derived products (Syrup, Peel, PPP and FPP at 200 and 400 mg/kg a day for 30 days) against  $\text{CCl}_4$ -induced oxidative damage. Protein carbonyl levels (A), free sulfhydryl groups (B) and TBARS levels (C) in liver homogenates. Carbonyl (D), free sulfhydryl groups (E) and TBARS levels (F) in kidney. #Denotes difference to control group; \*represents difference to  $\text{CCl}_4$ -treated group. Values represent mean  $\pm$  S.E.M. One-way analysis of variance and Tukey's multiple comparison *post hoc* test were applied for all data.

quenching activity of the antioxidants present in the respective fruit. For example, we did not detect preventive effects of FPP on SOD induction in kidney, but FPP still blocked sulphhydryl oxidation, carbonylation and lipoperoxidation as well as prevented transaminases and LDH leakage in  $\text{CCl}_4$  intoxication model, showing that the understanding of the antioxidant systems as a whole is important to make a conclusion on extracts' usefulness as antioxidants.

Inflammatory processes are frequently accompanied by alterations in the tissue structure. Such alterations may result from tissue damage due to active proteases or toxic moieties released by inflammatory cells [53]. NF $\kappa$ B is a transcription factor that has been recognized as one of the factors involved in a series of pathological conditions, principally inflammation and cancers. NF $\kappa$ B consists of a heterodimer of p65/p50 retained in the cytoplasm as an inactive tertiary complex associated with inhibitory proteins known as nuclear factor of kappa light

polypeptide gene enhancer in B-cells inhibitor, alpha (I $\kappa$ Bs) [54]. After stimuli such as, for example, TNF- $\alpha$ , I $\kappa$ B phosphorylation by IKKs leads to proteasome degradation of I $\kappa$ B, releasing NF $\kappa$ B to the nucleus [54,55]. RAGE ligand-dependent activation was shown to downstream activate NF $\kappa$ B, members of the MAPK family and the PI3K pathway, leading to induction of proinflammatory cytokines such as TNF and interleukins and enhancing reactive species production and oxidative-stress-related cell damage [56]. RAGE is capable of inducing *de novo* synthesis of NF $\kappa$ B and NF $\kappa$ B targets RAGE promoter elements, which results in cycles of increasing states of proinflammatory cytokine production upon RAGE activation [47,57]. In our model,  $\text{CCl}_4$  caused hepatic and renal inflammation as observed from the increased levels of NF $\kappa$ B and RAGE as well as its downstream targets TNF- $\alpha$  and IL-1 $\beta$ ; necrosis and inflammatory infiltrates were also confirmed by liver histology [58], evidencing an inflammatory landscape accompanying liver/renal

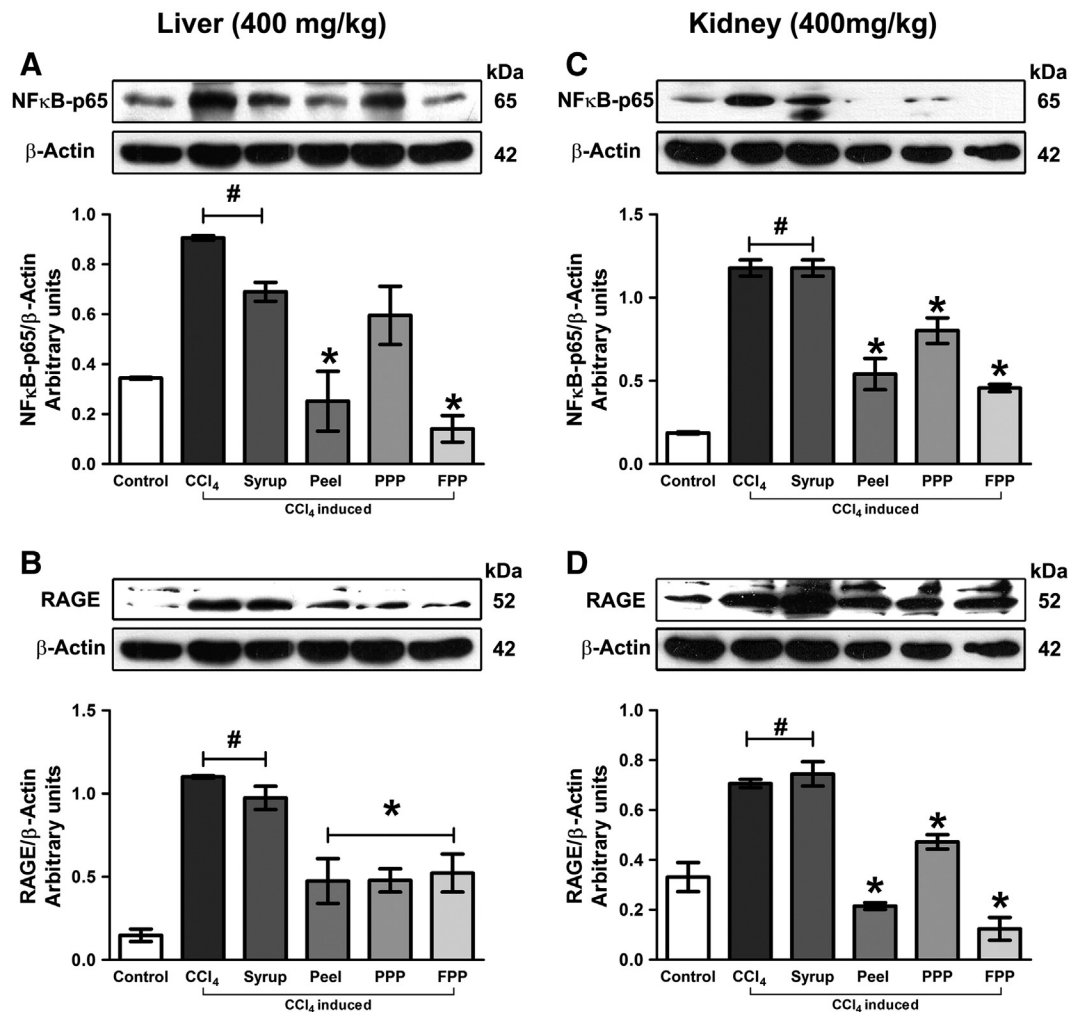


Fig. 7. Effects of treatment with peach-derived products (Syrup, Peel, PPP and FPP at 400 mg/kg a day for 30 days) on NFκB-p65 and RAGE protein content in rat liver and kidney. After treatments, animals were subjected to CCl<sub>4</sub> injection, and Western blot analysis was performed. The immunocontent of NFκB-p65 (A) and RAGE in liver (B). Immunocontent of NFκB-p65 (C) and RAGE in kidney (D). #Denotes difference to control group; \*represents difference to CCl<sub>4</sub>-treated group. Each figure depicts representative Western blots gels plus mean ± S.E.M. quantification values. One-way analysis of variance and Tukey's multiple comparison *post hoc* test were applied for all data.

damage. Treatment with Peel, FPP and PPP blocked the increases in NFκB and RAGE in liver and kidney tissues caused by CCl<sub>4</sub>, and some effect on serum TNF-α and IL-1β was observed with 400 mg/kg of FPP and Peel, respectively. TNF-α and IL-1β are proinflammatory cytokines and play key roles in the induction and perpetuation of inflammation in macrophages [59]. Prolonged excessive production of TNF-α has been implicated to contribute to the pathology of liver damage and systematic toxicity [39,51], and it might lead to marked cellular death. IL-1β is rapidly expressed in response to tissue damage [60]. Previous studies have hypothesized that IL-1β may directly activate hepatic stellate cells (HSCs) through autocrine signaling and stimulate the matrix metalloproteinases produced by HSCs within the space of Disse, resulting in liver fibrogenesis [59,60]. The inhibition of TNF-α and IL-1β release by FPP and Peel, respectively, can be attributed not only to its antioxidant effect but also to a possible direct effect on inflammatory pathways such as NFκB.

Studies carried out over the past few years have shown that dietary carotenoids and polyphenols are associated with reduced oxidative stress [61,62]. In addition, carotenoids and polyphenols are antioxidants frequently present in fruits, especially in peaches, and play a role in the prevention of damage caused by harmful ROS, which are continuously produced in the body during normal cellular functioning or are introduced from exogenous sources [63]. Here,

we added the information to contribute with the concept that carotenoids and polyphenols also collaborate with the antioxidant potential of peaches. The ability of plants to scavenge ROS seems to be related to the chemical structure of phenolic compounds [64,65]. Our findings suggest that the Peel, FPP and PPP may present some important antioxidant properties, probably related to their carotenoids and phenolic content. In our tests to detect carotenoids in the peach-derived products administered to animals, all-*trans*-lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene were identified in Peel, PPP and FPP samples, but Syrup did not present any detectable amount of these compounds. The abundance of carotenoids was higher in Peel followed by FPP and PPP.

Previous studies demonstrated that chlorogenic acid is one of the most abundant polyphenol in fruits (including peaches) and may provide health-promoting advantages to consumers [7,15]. Our HPLC analyses detected significant amounts of chlorogenic acid in all peach-derived products, including Syrup. The polyphenol content of FPP was around twofold higher than in other samples, indicating that (a) the pulp of peaches is the site where these compounds are more elevated and (b) a significant amount of pulp polyphenols is degraded or lost to the Syrup during the preservation time. (+)-Catechin and (–)-epicatechin are flavonoids that are both found in

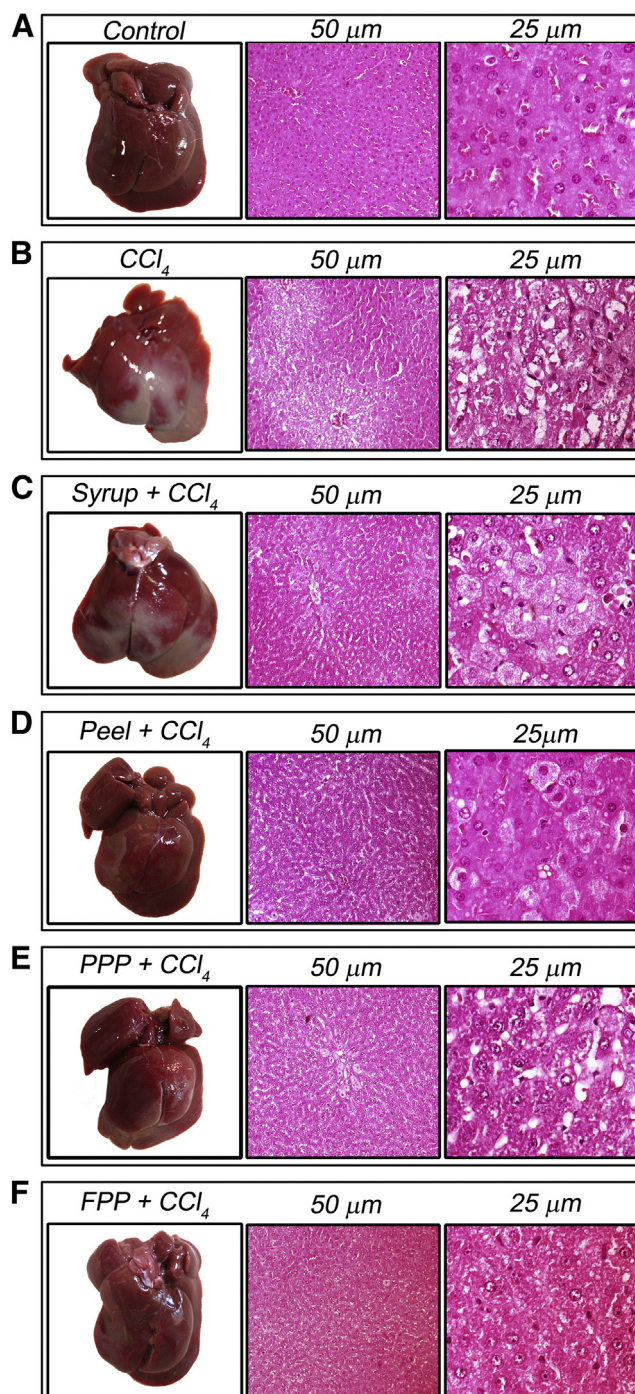


Fig. 8. Effects of treatment with peach-derived products (Syrup, Peel, PPP, and FPP 400 mg/kg a day for 30 days) on liver morphological and histological characteristics (H&E staining, original magnification of 50  $\mu\text{m}$ , and approximation of 25  $\mu\text{m}$ ). Liver organ and liver tissue of normal rats (A).  $\text{CCl}_4$  group (B). Syrup+ $\text{CCl}_4$  group (C). Peel+ $\text{CCl}_4$  (D). PPP+ $\text{CCl}_4$  (E). FPP+ $\text{CCl}_4$  (F).

green tea, black tea, wine and other plant foods, such as fruits [66]. (–)-Epicatechin was detected only in samples obtained from fresh fruits, i.e., Peel and FPP. It has been previously observed that administration of (–)-epicatechin enhanced antioxidant defense in rats [67] and also that (+)-catechin and (–)-epicatechin are able to delay lipid oxidation and  $\beta$ -carotene depletion in human plasma incubated with a free radical generator *in vitro*[68].

This different carotenoid and polyphenols profiling is in even consonance with the elevated antioxidant activity exerted by treatments with Peel and FPP (compared to PPP and Syrup) observed here. The current knowledge allows some interpretations of these

results. During the manufacturing process, peaches receive a large amount of sucrose aiming preservation into cans. Sugars are directly related to increases in advanced glycation end-product (AGE) formation in biological systems, and in turn, AGEs enhance the expression and activation of RAGE. In animals treated with Syrup, RAGE content was similar to that observed in the  $\text{CCl}_4$  group. However, in animals treated with PPP, where peaches also were exposed to high amounts of sugar but kept significant levels of carotenoids and polyphenols,  $\text{CCl}_4$ -induced RAGE was reduced in both tissues. PPP also was able to prevent the increase in RAGE, transaminases, LDH and some oxidative stress markers – such effect

was not observed in Syrup treatments. These observations, altogether, suggest that the processing of peaches for the production of canned-syrup-based preserves of peach pulps affects some of the antioxidant properties that might be conferred by the fresh fruits. Nonetheless, it must be pointed that, although PPP antioxidant effects were decreased compared to FPP in our study, we still observed some significant protective effects conferred by PPP treatment.

Based on the present data, we suggest that the consumption of peaches as part of a healthy, equilibrate diet may confer significant protective properties against insults to liver and kidneys to humans. Extrapolating our results to a human context, the highest dose of lyophilized peach sample tested here (400 mg/kg a day) could be easily obtained in the diet based in the application of the human equivalent dose (HED) calculation that considers the body surface area normalization method [69]. This calculation results in an HED for FPP of 64.86 mg/kg/day, which equates to a dose of 4.540 mg of FPP for a 70-kg person per day. A single fresh fruit that is available for consumers in the market may vary in size, but the average weight is about 100 g. On the other hand, the lyophilization process used here caused an average decrease of 80% in the weight of each peach sample. Therefore, considering a dry weight of about 20 g for each fruit, an individual weighing 70 kg would require the equivalent of 3 to 5 g of our sample of FPP to equate with the highest dose for rats evaluated in this study (400 mg/kg). This amount could be obtained by ingesting two to three peaches a day. Another recently published study also indicated a similar consumption (two to three peaches per day) to inhibit tumor growth and metastasis of MDA-MB-435 breast cancer cells *in vivo* [15].

## 5. Conclusion

In conclusion, Peel, PPP and FPP treatments appear to bring a significant inhibition of CCl<sub>4</sub>-induced damage either in the level of organ morphology and serum markers or in oxidative damage to macromolecules (TBARS, carbonyl, SH content levels). Our investigation shows a direct comparison between antioxidant effects of fresh and industry-processed fractions of peaches (Syrup, Peel, PPP and FPP) in an *in vivo* model of liver/renal toxicity, comparing their potentials and associating these to their content of carotenoid, polyphenols and phenolic antioxidants. Peel, FPP and PPP treatments also attenuated inflammation markers in liver and kidney as they blocked the stimulatory effects of CCl<sub>4</sub> on NFκB, RAGE, nitrotyrosine, TNF-α and IL-1β. Further experiments using low dose/long term or high dose/short term and other damaging agents may provide additional mechanism whereby peach consumption may be associated to prevention of tissue damage by pro-oxidant agents.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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