Original Article

Effects of different products of peach (*Prunus persica* L. Batsch) from a variety developed in southern Brazil on oxidative stress and inflammatory parameters *in vitro* and *ex vivo*

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Antioxidant, anti-glycation and anti-inflammatory activities of fresh and conserved peach fruits (*Prunus persica* L. Batsch) were compared. Fresh peach pulps, peels, preserve peach pulps and the preserve syrup were prepared at equal concentrations. Rat liver, kidney and brain cortex tissue slices were pre-incubated with peach samples, subjected to oxidative stress with $FeSO_4$ and hydrogen peroxide. Fresh peach pulps and peel conferred higher protection against cytotoxicity and oxidative stress than preserve peach pulps in most tissues. Release of tumor necrosis factor-α and interleukin-1β was also significantly decreased by Fresh peach pulps and peel, followed by preserve peach pulps. Total phenolic determination and HPLC analysis of carotenoids showed that the content of secondary metabolites in Fresh peach pulps and peel is significantly higher than in preserve peach pulps, while the syrup had only small or trace amounts of these compounds. Fresh peach pulps and Peel demonstrated high antioxidant and anti-inflammatory effects preventing against induced damage.

***Key Words*: antioxidant, anti-inflammatory, peach, protective effect**

Poor dietary intake of fruits and vegetables constitute a risk factor for several diseases such as cancer, coronary heart disease, stroke and insulin resistance.[1,2] The regular consumption of fruits and vegetables is associated to prevention of esophageal, stomach, pancreatic, bladder and cervical cancers; fruits and vegetables-enriched diets may prevent 20% of most types of cancers.[3] A meta-analysis of cohort studies observed that the risk of developing coronary heart disease and stroke decreased significantly for each additional portion of fruit consumed per day, indicating a protective effect.[4] It was also reported that fruit dietary intake may be associated with a reduced risk of Alzheimer's disease and lower cognitive decline with age.[5] Some fruit and vegetable also may play an important role in delaying the onset of Alzheimer's disease, particularly among those who are at high risk for the disease.[6]

Free radicals and related species (collectively known as reactive species) are constantly produced by cells as result of aerobic metabolism. Excessive production of reactive species may lead to oxidative stress, which results in oxidative damage to lipids, proteins and DNA. Consequently, increased risk for developing diseases associated oxidative stress, such as cancer, cardiovascular diseases and neurodegenerative conditions, may arise.[7] To cope with reactive species, cells must maintain an adequate pool of enzymatic and nonenzymatic antioxidants to properly clean/detoxify these species. Among the nonenzymatic antioxidants, exogenous compounds obtained from the diet exert an important role in the detoxification of free radicals and, in turn, in disease prevention. Phenolic compounds and carotenoids obtained from dietary vegetables and fruits exert prominent roles in the protection against oxidative damage.[8] A reduced risk of developing diseases commonly associated to oxidative stress has been associated to diets enriched in these compounds.[3]

Different varieties of peaches (*Prunus persica* L. Batsch) are highly consumed worldwide. Peach is the most important stone fruit crop in many western countries, being grown in Europe, North and South America at a fair range of different climate conditions and types of soils. Peaches are appreciated in different cultures mainly due to their flavor and nutritional value; however, studies on potential benefits of peaches consumption to human health are still incipient. Peaches present many secondary metabolites, such as penolic compounds, carotenoids and tocopherols that present important biological actions and are associated to disease prevention, as mentioned above. Nonetheless, most pharmacological studies focused on the biological activities enriched fractions and/or isolated forms of these compounds and only few studies evaluated the potential of peaches and its derivate products as functional foods. Recently, consumers over the world have been increasingly searching for foods that have a clear role in heath-promotion or disease prevention, so producers have been considering such preferences when developing new varieties of agricultural products. In the case of fruits, the present trend is the reinforcement of the content or availability of plant endogenous compounds with potential antioxidant, anti-glycemic, anti-inflammatory and anti-tumoral activities, without affecting other nutritional and flavor-associated properties.

In Brazil, peaches of the Maciel variety have been developed at temperate climate for consumption of the fresh fruit as well as its derivate products, such as juice and syrup-preserved pulp. However, little is known about potential health benefits of this commercial variety of peach and, especially, about the biological activity of the main products commercially available from peaches, such as the fresh fruit and the syrup-preserved pulp. In

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this regard, this study has been conducted to determine the cytoprotective, antioxidant and anti-inflammatory properties of peaches of the Maciel variety, developed by Embrapa (Brazilian Agricultural Research Corporation), using *in vitro* and *ex vivo* assays. Our results indicate that fresh peach pulps (FPP) and peels exhibit antioxidant, anti-glycation and anti-inflammatory properties, and that some of these properties are also present in syrup-based peach pulp preserves (PPP).

Material and Methods

Chemicals. Catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), thiobarbituric acid (TBA), ferrous sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2) were from Sigma-Aldrich (St. Louis, MO). ELISA microplates were from Greiner Bio-One (Monroe, LA) and ELISA TMB spectrophotometric detection kit was from BD Biosciences (San Diego, CA). Tumor necrosis factor alpha (TNF-α) rabbit polyclonal antibody, Interleukin-1 beta (IL-1β) rabbit polyclonal antibody and anti-rabbit immunoglobulin linked to peroxidase were from Cell Signaling (Danvers, MA). Purified recombinant TNF-α protein was from Abcam (Cambridge, UK) and IL-1β was from BD. MilliQ-purified H_2O was used for preparing solutions. Lactate dehydrogenase (LDH) activity kit was from Labtest (Lagoa Santa, Minas Gerais, Brazil). The following HPLC standards were purchased from Sigma (St. Louis, MO): β-cryptoxanthin (purity>97%), β-carotene (purity>93%), α-carotene (purity>95%), zeaxanthin (purity>95%). Lutein (purity>95%) was purchased from Indofine (Hillsborough, NJ). HPLC-grade solvents, including methyl-*tert*-butyl and methanol, were purchased from Scientific Hexis (Jundiaí, São Paulo, Brazil). The peach samples were provided by Embrapa Clima Temperado.

Animals. Adult male Wistar rats (60 days-old; weighing 280–300 g) were obtained from our breeding colony. They were caged in groups of four animals with free access to standard commercial food (CR1 lab chow, Nuvilab, Curitiba, Paraná, Brazil) and water and were maintained in a 12-h light–dark cycle (7:00–19:00) in a temperature-controlled colony room (21°C). All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health.[9] Our research protocol was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal do Rio Grande do Sul. Ten healthy animals were utilized for this study. A pilot test was performed with three animals to determine optimal induction of hydroxyl-mediated damage by Fenton reaction ($FeSO_4$ and H_2O_2).

Preparation of peach samples. The Maciel variety was developed by Embrapa Clima Temperado by controlled hybridization. The seeds were laminated in chamber at 4 ± 1°C and then seedlings were cultivated in greenhouse for later being transplanted to the seedlings experimental field. Fruits were obtained from this field (Pelotas, Rio Grande do Sul, Brazil, location coordinates: –31°30'57.44", –52°33'11.52"). Immediately after harvesting the fruits, the peel and pulps were separated and frozen at –20°C (the pits were removed and discharged). Fruits were also used to prepare syrup-based preserves by an industrial processing, which is the same procedure used for production of large-scale commercial canned peach preserves. Briefly, the fruits are cut in half and the pits are separated by twist. The fruits are subjected to a soda (NaOH 50%) shower-based peeling procedure in a treadmill and immediately washed to remove the soda. Pulps are placed into cans and hot sucrose-based syrup (water:sucrose = 80:20) is added. Cans are 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 four months, the cans were opened and the pulps and the syrup were separated and subjected to lyophilization. The samples of fresh pulp and peel were also 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°C, dissolved in ultrapure water at the moment of the experiment and then centrifuged (4,000 × *g* for 3 min) to precipitate rough particles (always protected from light and temperature). Supernatant suspension was collected and used for experiments. For each assay, different serial dilutions were obtained from the stock solution of the different peach derived samples and were used in all experiments. Aliquots of these diluted samples were subjected to HPLC analysis.

Tissue slice preparation. The animals were killed by decapitation. Kidney, liver and brain cortex were quickly removed and were chopped in slices weighing 40 ± 5 mg (Fig. 1). Tissue slices were prepared with a tissue chopper device (Redding, California) and immediately placed on ice. Tissue was maintained in pre-warmed Kreb's Ringer Hepes (KRH) oxygen-equilibrated solution (2.5 mM Hepes, 118 mM NaCl, 2.85 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM KH_2PO_4, 1.18 mM $MgSO_4$, 5 mM β-hydroxybutyrate, and 4.0 mM glucose, pH 7.4) over a period of 1 h. The samples were washed for 30 min in test tubes containing 2 ml KRH in a shaking water bath (60 oscillations/min) at 37°C under 95% O_2/5% CO_2. The KRH was replaced with incubation medium containing the treatments and incubated for 1 h. After this pre-incubation, the $FeSO_4$ (1 mM) and H_2O_2 (100 mM) were added and incubated for 30 min to generate the Fenton reaction.[10] After incubation the medium was collected, centrifuged at 3,000 × *g* for 5 min and used for cytotoxicity and inflammatory parameters estimation (see below). The tissue slices were homogenized in phosphate buffer 50 mM (PB, KH_2PO_4 and K_2HPO_4 pH of 7.4) and centrifuged at 6,000 × *g* for 5 min at 4°C. Protein content of the incubation medium and homogenates were determined by Bradford method for data normalization.[11]

Total reactive antioxidant potential (TRAP assay). The total reactive antioxidant potential (TRAP) was used as an index of non-enzymatic antioxidant capacity. This assay is based on the quenching of peroxyl radicals generated by AAPH [2,2azobis(2-amidinopropane)] by antioxidants present in a given sample.[12] Briefly, a chemical system that generates peroxyl radicals at a constant rate (an AAPH-containing buffer) is coupled to a luminescent reactant (luminol) which emits photons proportionally to its oxidation. The reaction was initiated by injecting luminol to the 0.1 M glycine buffer (pH 8.6) containing AAPH that resulted in steady luminescence emission. Equal amounts of samples are then added to this reaction system, and the luminescence emission at the moment following this addition (t = 0) is recorded. This initial emission reflects the production of free radicals by AAPH at the first moment right after sample addition and is related to the endogenous oxidant state of the sample. Following incubation, the thermal decomposition of AAPH produces luminescence at a constant rate ("system"), and the presence of free radical scavengers in the added sample will decrease this rate according to its content of non-enzymatic antioxidants. Sample addition decreases the peroxyl-derived luminescence proportionately to its antioxidant potential. We followed TRAP luminescence emission for 60 min and calculated the area under the curve (AUC) relative to the system without samples (which was considered as 100% of luminescence emission at all time points), using Trolox as a standard antioxidant for comparison.[13] The luminescence emission was recorded in a Micro Beta luminescence counter (Perkin Elmer, Waltham, MA).

Protein glycation assay. Bovine serum albumin (BSA, 10 mg/ml) in phosphate buffer (50 mM, pH 7.4) containing 0.02% (w/v) sodium azide was pre-incubated with the extracts at final concentrations of 1, 10 and 100 μg/ml. Glucose (25 mM) and fructose (25 mM) solutions were added to the reaction mixture. All the reagents and samples were sterilized by filtration through 0.25 μm membrane filters. Each solution was incubated at 37°C for 21 days in the dark in a capped tube. BSA glycation during this period

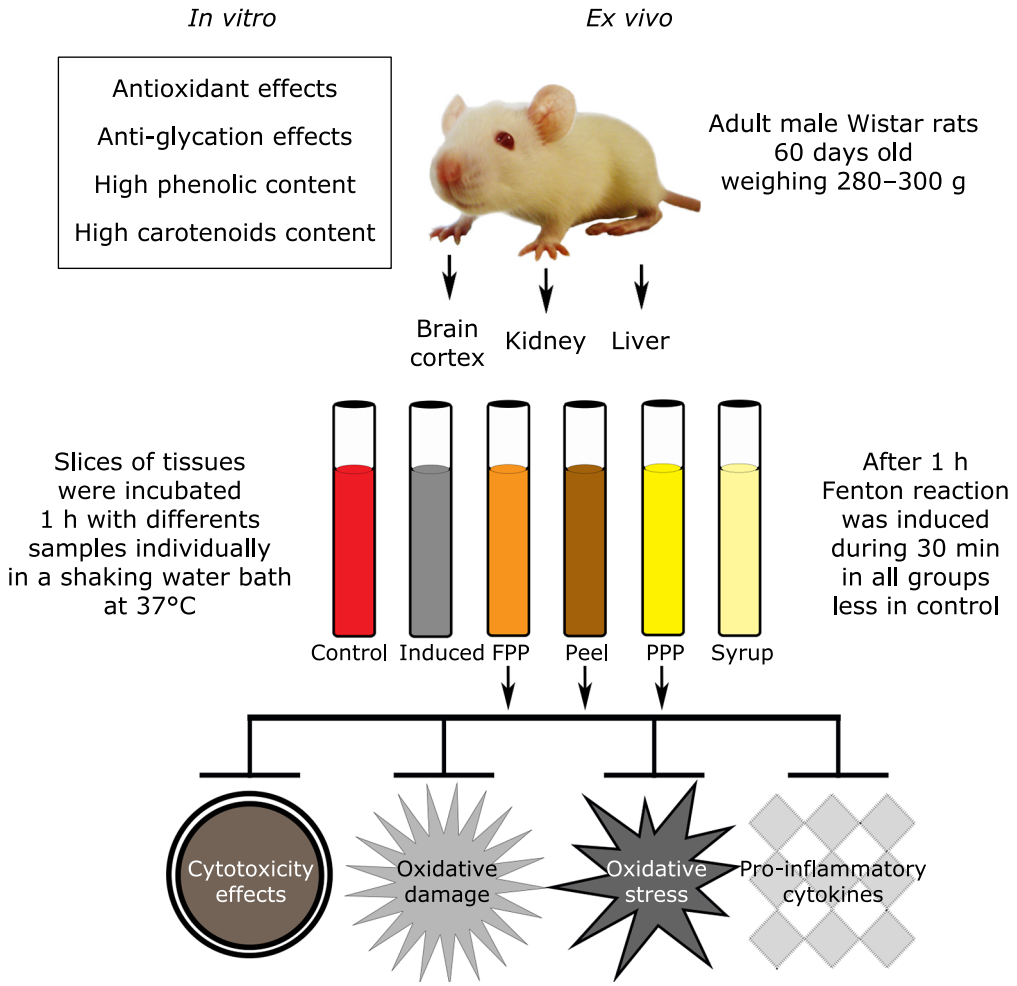

Fig. 1. Graphical abstract

resulted in fluorescent product formation, which was quantified in a fluorimeter (F2000, Hitachi Ltd., Tokyo, Japan) with an excitation wavelength of 350 nm and an emission wavelength of 450 nm.[14,15] Glycation inhibition was calculated as follows: Inhibition % = 1 – (As – Ab)/(Ac – Ab) × 100, where As = fluorescence of the incubated mixture with sample, Ac = the fluorescence of the incubated mixture without sample (positive control for induced glycation) and Ab = the fluorescence of the sample as a blank control.[16]

Determination of total phenolic content. Total phenolic content of peaches and derivatives was determined using the Folin–Ciocalteu method.[17] One hundred µl of Folin–Ciocalteu reagent were mixed to 100 µl of sample and then 200 µl of Na_2CO_3 35% were added. The volume was completed to 1,900 µl with ultra-pure H_2O and then homogenized. After 10 min, the absorbance was measured at 725 nm and compared to a gallic acid calibration curve. Total phenols in samples were determined as gallic acid equivalents.[18]

Quantification of carotenoids by High-Performance Liquid Chromatography (HPLC). Carotenoid analysis was performed using an HPLC system (Agilent series 1100, Santa Clara, CA) equipped with an online degasser, a quaternary pump, and an automatic injector. The carotenoids were separated on a polymeric reversed phase column (YMC C_{30} 250 µm × 4.6 µm; particle size of 3 µm) with a mobile phase gradient elution starting with water/methanol/MTBE (*Methyl tert-butyl ether*) 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.[19] The spectra were conducted between 250 and 600 µm, 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 and co-injection of standards. Quantifications were performed constructing standard curves for the carotenoids in the following concentration ranges: 5–4,000 µg/ml for β-carotene, 2–200 µg/ml for α-carotene, 1–1,000 µg/ml for all-trans-lutein, 4–4,000 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 (LOD) and limits of quantification (LOQ) were determined as previously described by Long and Winefordner.[20] The following LOD and LOQ scores were, respectively obtained: 6.5×10^{-2} and 10.9×10^{-2} mg/kg for β-carotene; 6.9×10^{-3} and 1.2×10^{-2} mg/kg for lutein; 2.1×10^{-2} and 3.5×10^{-2} mg/kg for cryptoxanthin; 9.6×10^{-2} and 1.6×10^{-2} mg/kg for zeaxanthin; and 2.0×10^{-2} and 3.3×10^{-2} mg/kg for α-carotene.

Cytotoxicity: measurement of LDH activity. The cell viability of the tissue slices was assessed by LDH activity into the incubation medium. This assay was performed by using a commercial kit for LDH (Code: 86-2/30) from Labtest (Lagoa Santa, Minas Gerais, Brazil) according to the manufacturer's instructions. The change in absorbance at 500 nm was followed in a

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SpectraMAX 190 plate reader (Molecular Devices, Sunnyvale, CA).

Oxidative stress parameters. Catalase (CAT, EC 1.11.1.6) activity was evaluated by following the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm.[21] The activity of SOD was measured by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a sample buffer; adrenochrome formation was monitored at 480 nm for 10 min (32°C) in a spectrophotometer.[22]

Sulfhydryl groups quantification. Oxidative status of thiol groups were assessed by quantification of total reduced sulfhydryl (SH) groups in samples.[23] Briefly, for total SH content measurement, a 60 μg sample aliquot was diluted in phosphate buffer saline (PBS) and 10 mM 5,5'-dithionitrobis 2-nitrobenzoic acid, and read in a spectrophotometer at 412 nm after a 60 min incubation.

Lipid peroxidation. The formation of thiobarbituric acid reactive species (TBARS) was quantified by an acid-heating reaction with thiobarbituric acid. TBARS formation is a widely adopted parameter for oxidative damage on lipids.[24] After precipitation with trichloroacetic acid 10% (TCA), supernatant was mixed with 2-thiobarbituric acid (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 carbonylation. The formation of carbonyl groups was used as a parameter for oxidative damage to proteins, based on the reaction with dinitrophenylhidrazine (DNPH).[25] Proteins were precipitated by the addition of 10% TCA and re-solubilized in DNPH. Then, the absorbance was read in a spectrophotometer at 370 nm.

Quantification of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). To determine TNF-α and IL1-β concentration in the incubation medium, we used an indirect enzyme-linked imunosorbent assay (ELISA) procedure. Samples were normalized according protein content and added to ELISA plates. The antigen was incubated for 24 h at room temperature, washed 3 times with Tween-Tris buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) and then primary antibody (1:1000 dilution range) was added and incubated for 24 h at 4°C followed by secondary antibody incubation (rabbit anti-IgG, 1:1000 dilution range) for 3 h at room temperature. The immunoreactivity (1:1) was detected using a spetrophotometric detection kit from BD Biosciences. The reaction was stopped with sulfuric acid, and samples read at 450 nm. Purified recombinant TNF-α (Abcam) and IL-1β (BD) were used for standard curve calculation.[26]

Statistical analysis. The results of measurements were expressed as mean ± SEM. Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All peach samples (fresh peach pulp, peel, preserve of peach pulp and preserve peach syrup), when incubated alone (i.e., in absence of the $FeSO_4/H_2O_2$ hydroxyl generating system), did not exert significant statistical effects to all parameters analyzed here (data not shown). All results were calculated in GraphPad Prism 5.01 software.

Results and Discussion

It is generally accepted that functional foods and nutraceuticals confer health benefits by improving parameters related to resistance against toxicity, oxidative stress and inflammation in different systems. Thus, we chosen to use kidney, liver and brain cortex as these organs are representative of different systems in the body and are major regulators of intermediary metabolism in mammals (including humans). Experiments were conducted with an incubation system using slices of rat liver, kidney and brain cortex, as this is a rapid method to evaluate biochemical effects of compounds in structured organs. This *ex vivo* model retains most of the original histological structure of the organ and hence preserves its physiological characteristics.[10,27]

We first evaluated the total antioxidant capacity of the different samples obtained from peach and derivate products. We suspended the lyophilized samples of FPP, peel, PPP and preserve peach syrup in water at the same concentration each (20 μg/ml) and subjected them to the TRAP assay. This assay is widely used to determine the non-enzymatic antioxidant capacity in plant extracts, which is mostly dependent on the content of secondary metabolites with redox activity.[13] The results showed that the peel has the highest antioxidant activity compared with other samples; the FPP also had a significant antioxidant capacity (Fig. 2A and B). PPP and syrup had no significant effects. Trolox (200 nM), hydrophilic analogue of α-tocopherol, was used as a standard antioxidant.

The total antioxidant reactivity (TAR) index indicates the instantaneous decrease in luminescence associated with the sample addition into the peroxyl-generating system. While TRAP indicates the quantity of antioxidants presents in the plant extracts, the TAR indicates their antioxidant effectiveness. Peel and FPP had the highest TAR indexes, compared to PPP and syrup (Fig. 2C). This result indicates that the both the FPP and the peel have a high content of molecules with significant antioxidant activity, which is probably associated to the composition of secondary metabolites, as seen in previous works.[28] When comparing the peel and FPP with other samples it is evident that the samples from fresh fruits (i.e., peels and FPP) had a higher antioxidant activity than samples from preserves (PPP and syrup). These findings suggest that some properties of the peaches are lost by the preserves over time or during the processing procedure, which agrees with previous observations showing that biological properties of industrialized/canned fruits are lower than in fresh fruits.[29] It is also possible that the high antioxidant potential of the fresh peaches is associated to its preservation capacity over time, as it is known that antioxidants help to preserve flavor and nutritional value of foods. Natural and synthetic antioxidants are widely used in the food preservation industry for this reason, and it might be possible that in syrup-based peach preserves they are oxidized over time, preserving other components of nutritional value of oxidation and consequent degradation.

Glycation is a spontaneous non-enzymatic amino-carbonyl reaction between reducing sugars and long-lived proteins and lipids. Glycation is one major form of chemical modifications to biomolecules that compromise their function and have been recently implicated in the molecular basis of several diseases, such as diabetes, cardiovascular pathologies and neurodegenerative diseases.[30] These chemical modifications frequently result in the formation of the so-called advanced glycation endproducts (AGE). Glycation is a source of reactive oxygen species (ROS), causing oxidative stress, which in turn may trigger the production and release of inflammatory mediators.[31] Besides, both AGE and oxidative stress enhance the expression of the receptor for advanced glycation endproducts (RAGE) in cells, which further activates pro-inflammatory pathways and NADPH oxidase-derived ROS production.[32] Antioxidants are reported to prevent the oxidative reaction of sugars with proteins and thus inhibit the formation of Amadori products, which is an early step in AGE formation.[15] Several reports indicate that production of radicals and highly reactive oxidants is increased by glycated proteins under physiological conditions.[15] We subjected isolated albumin to a glycation protocol through incubation with glucose and fructose during 21 days. At the end of the incubation period, albumin glycation was significantly inhibited by peel and FPP by 40% at different doses (Fig. 2D). PPP also inhibited albumin glycation, but at a lower extent (around 10%), while the syrup alone, probably due to its high sucrose content (more than 20%), enhanced glycation by 30%.

We next evaluated the effects of FPP, peel, PPP and syrup on parameters of cytotoxicity, oxidative stress and inflammation by using an *ex vivo* approach. Rat kidney, liver and brain cortex tissue

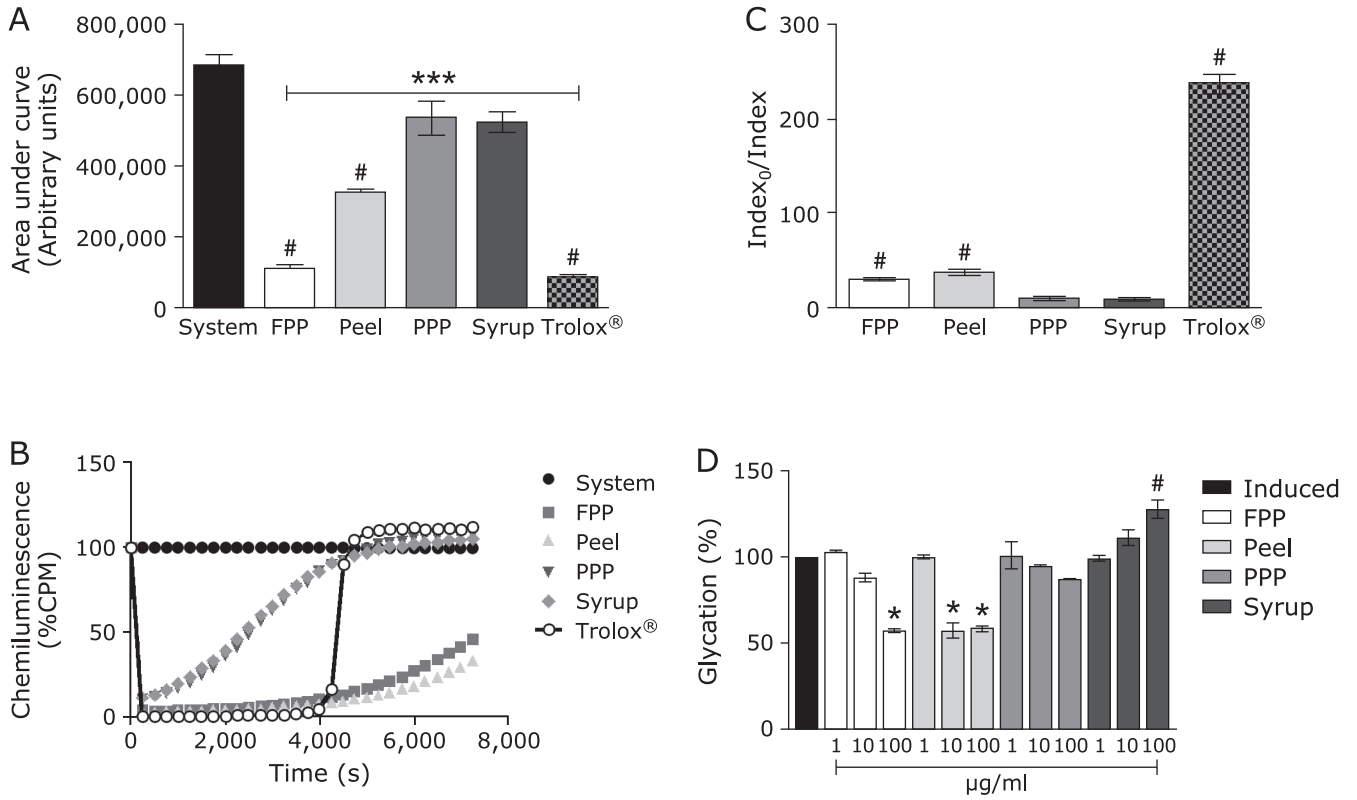

Fig. 2. Antioxidant and anti-glycation profile. Equal concentrations (20 μg/ml) of lyophilized samples of fresh peach pulp (FPP), peel, preserve peach pulp (PPP) and syrup were compared. (A) The total reactive antioxidant potential (TRAP) was performed as described in "material and methods" section and the area under curve values were calculated. "System" denotes the peroxyl-radical production system. Trolox was used as standard antioxidant for comparison. (B) Kinetics of chemiluminescence intensity (% counts per min [CPM]) are also depicted. (C) The TAR index, estimated by the instantaneous decrease in chemiluminescence when samples are added to the system, was calculated from the same assay. ***Different from system (p<0.0001), #different from syrup and PPP groups (p<0.0001). (D) Percentage of *in vitro* albumin glycation by glucose and fructose for 21 days in the presence of FPP, peel, PPP or syrup at 1,10 and 100 μg/ml. *Different from glycation-induced group (p<0.0001), #different from glycation-induced group and from asterisk-marked groups (p<0.05) using one-way ANOVA (Tukey's post hoc). Values in graphic bars represent mean ± SEM (triplicate experiments).

slices were isolated and pre-incubated with the different samples obtained from peaches and its products (80 μg/ml) for 60 min. Then we subjected the tissue slices to an oxidative insult by incubation in a hydroxyl radical production system with $FeSO_4$ 1 mM and H_2O_2 100 mM for 30 min. LDH activity in the incubation medium was assessed as a parameter of cytosolic leakage (cytotoxicity). The oxidative insult by the $FeSO_4/H_2O_2$ system (hydroxyl generating system) increased LDH activity in the incubation medium of all tissues analyzed (Fig. 3A, D and G). In kidneys (Fig. 3A), FPP, peel and PPP prevented the increase in LDH caused by the hydroxyl generating system, indicating a protective effect. In liver (Fig. 3D), FPP and peel had a significant protective effect. In brain cortex (Fig. 3G), only FPP had a significant effect on LDH activity.

The $FeSO_4/H_2O_2$ system induces cytotoxicity by oxidative stress, as consequence of Fenton reaction. Antioxidant enzymes are known to be induced in response to reactive species.[33] CAT and SOD have their activities increased when H_2O_2 and superoxide radicals are overproduced during cellular oxidative stress. Thus, enhanced CAT and SOD activities are common parameters indicative of a increased state of reactive species production.[34] As expected, incubation with the $FeSO_4/H_2O_2$ system increased CAT and SOD activities in tissue slices. In kidney (Fig. 3B) and brain cortex (Fig. 3H) slices, pre-incubation with FPP and peel significantly inhibited the activation of CAT by incubation with the $FeSO_4/H_2O_2$ system. In liver (Fig. 3E) only FPP was able to inhibit the CAT activities. SOD activation induced by $FeSO_4/H_2O_2$ system was prevented in kidney (Fig. 3C) by FPP, peel and PPP. In brain cortex (Fig. 3I), a inhibition of SOD activities was observed with FPP and peel as in liver (Fig. 3F). Since SOD and CAT activities are generally enhanced in conditions of increased substrate production, these results altogether suggest that the pre-treatments carried out here conferred antioxidant protection to kidney and brain cortex.

We also measured parameters of oxidative damage in biomolecules to assess the antioxidant properties of peaches to tissue slices. The oxidative damage to the proteins in tissue slices was measured by determining levels of the carbonyl groups based on the reaction of the groups with dinitrophenylhydrazine (DNPH). Formation of protein carbonyl groups is a well-known parameter of protein oxidation.[25] Protein carbonylation was greatly enhanced by the $FeSO_4/H_2O_2$ system in all tissues, but pre-incubation with FPP protected all tissues against this effect (Fig. 4A, D and G). PPP was able to prevent carbonyl formation in kidney (Fig. 4A). We also measured the total content of thiol groups, which indicates the level of protein SH groups oxidation, as SH groups are oxidized in response to pro-oxidant stimuli.[23] Protein SH oxidation was not prevented statically by any pre-treatment (Fig. 4B, E and H), however in liver (Fig. 4E) FPP group had no difference to control group indicating a possible protection.

Lipid peroxidation is considered one of the basic mechanisms involved in reversible and irreversible cell and tissue damage. Lipid peroxidation has been implicated in the pathogenesis of many diseases. In liver, it is an early marker of cell membrane

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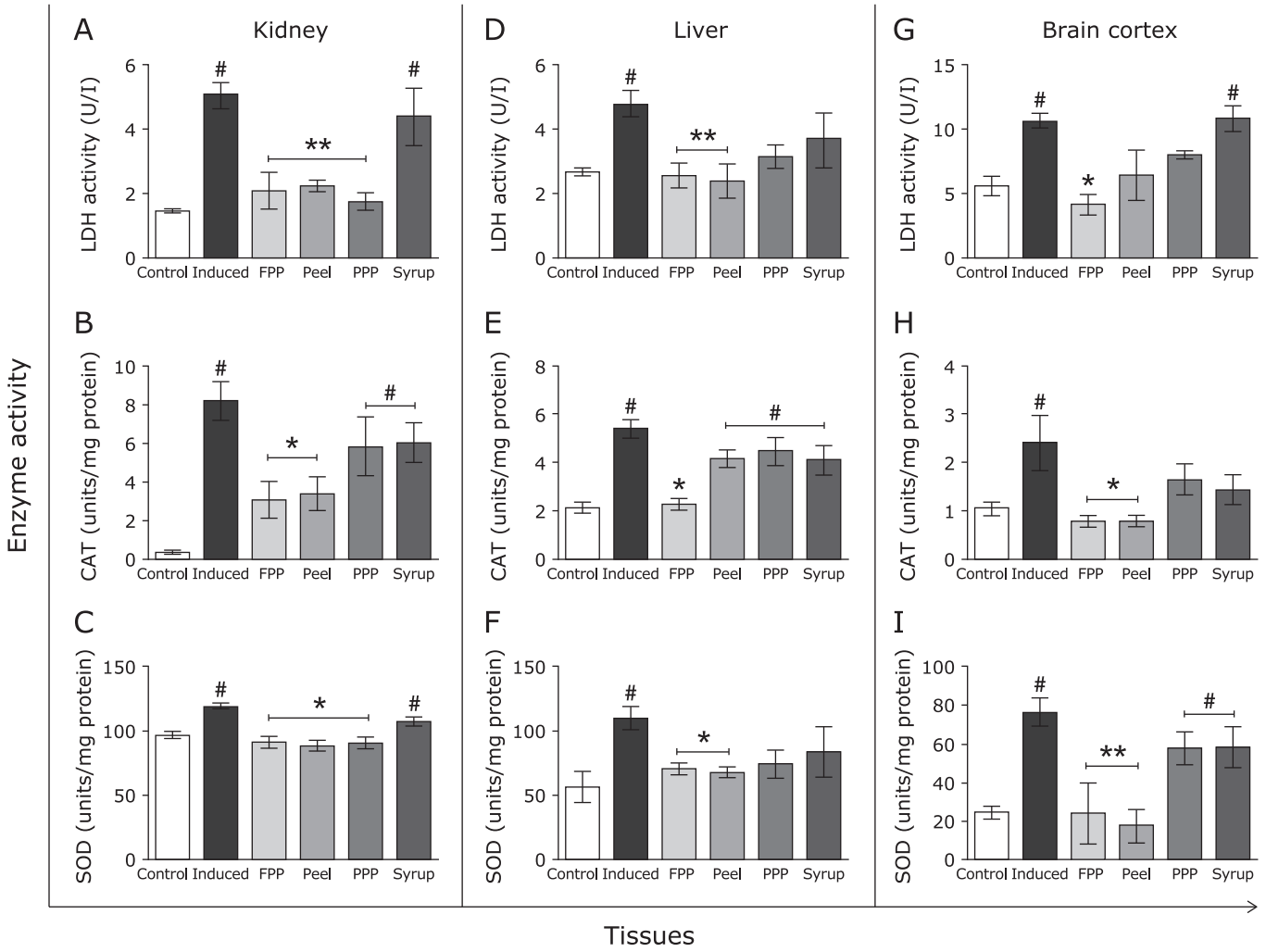

Fig. 3. Effects of FPP, peel, PPP and syrup on cytotoxicity and antioxidant enzyme activities in tissue slices subjected to oxidative stress. Kidney, liver and brain cortex slices were pre-incubated with FPP, peel, PPP or syrup (80 μg/ml each) for 60 min and then subjected to oxidative damage by incubation with $FeSO_4$ 1 mM and H_2O_2 100 mM for 30 min (stress-induced group). (A) LDH activity in the incubation medium was analyzed as a parameter for cytotoxicity (cell rupture). (B) CAT and (C) SOD activities were assessed in homogenized tissue slices of Kidney. Letters (D), (E), (F) represent respectively the same protocols to liver homogenate, and (G), (H), and (I) to brain cortex. #Different from control group (p<0.0001) **different from stress-induced group (p<0.001) and *(p<0.05). One-way ANOVA (Tukey's post hoc) was applied. Values in graphic bars represent mean ± SEM (triplicate experiments, n = 6 per group).

damage associated with the subsequent leakage of hepatotoxicity markers to the bloodstream.[35] Lipid peroxidation (expressed as TBARS) was significantly increased in samples treated with the $FeSO_4/H_2O_2$. Pretreatment with peel significantly reduced increase in TBARS formation in all tissue slice samples (Fig. 4C, F and I). In brain cortex slices (Fig. 4I), FPP also had a protective effect. The observation that peach peels presented antioxidant activity mainly in the lipid fraction (Fig. 4C, F and I), while FPP had a major antioxidant effect to soluble protein fractions (Fig. 4A, D and G) suggest that different secondary compounds present in distinct parts of the fruit (i.e., pulp and peel) are responsible for these effects.

In response to acute or chronic infection, the production and release of TNF-α and IL-1β is increased. These cytokines trigger pro-inflammatory signal cascades in tissues, enhancing reactive species production and further cytokine expression and release. In order to analyze the potential anti-inflammatory effects of peaches on tissues, TNF-α and IL-1β levels in the incubation medium were quantified by ELISA as previously described.[26] The incubation with the $FeSO_4/H_2O_2$ system led to a significant increase in the levels of TNF-α and IL-1β in the incubation medium of all tissues, indicating an acute inflammatory response (Fig. 5). In kidney tissue (Fig. 5A and B), the FPP, peel and PPP prevented the release of TNF-α and IL-1β. In the liver Fig. 5C and D), only the peel caused a similar effect, preventing the increase of TNF-α and IL-1β release caused by the pro-oxidant insult. FPP also inhibited the release of TNF-α in brain cortex (Fig. 5E).

As mentioned earlier, plant secondary metabolism is responsible for the synthesis of many compounds that exert important biological activities in animal cells when ingested as part of animal diet. Phenolic compounds are found in many different foods, especially fruits and vegetables.[35] Dietary phenolic compounds have been considered essential for prevention of oxidative stress-mediated diseases.[36] Polyphenols obtained from the diet are known to inhibit the free radical production derived from xenobiotic toxic agents, thus reducing the risk of liver disease.[37] Carotenoids are photosynthetic pigments that provide much of the different colors seen in plants and constitute an important part of the diet of many animals. In humans, carotenoids-enriched diets

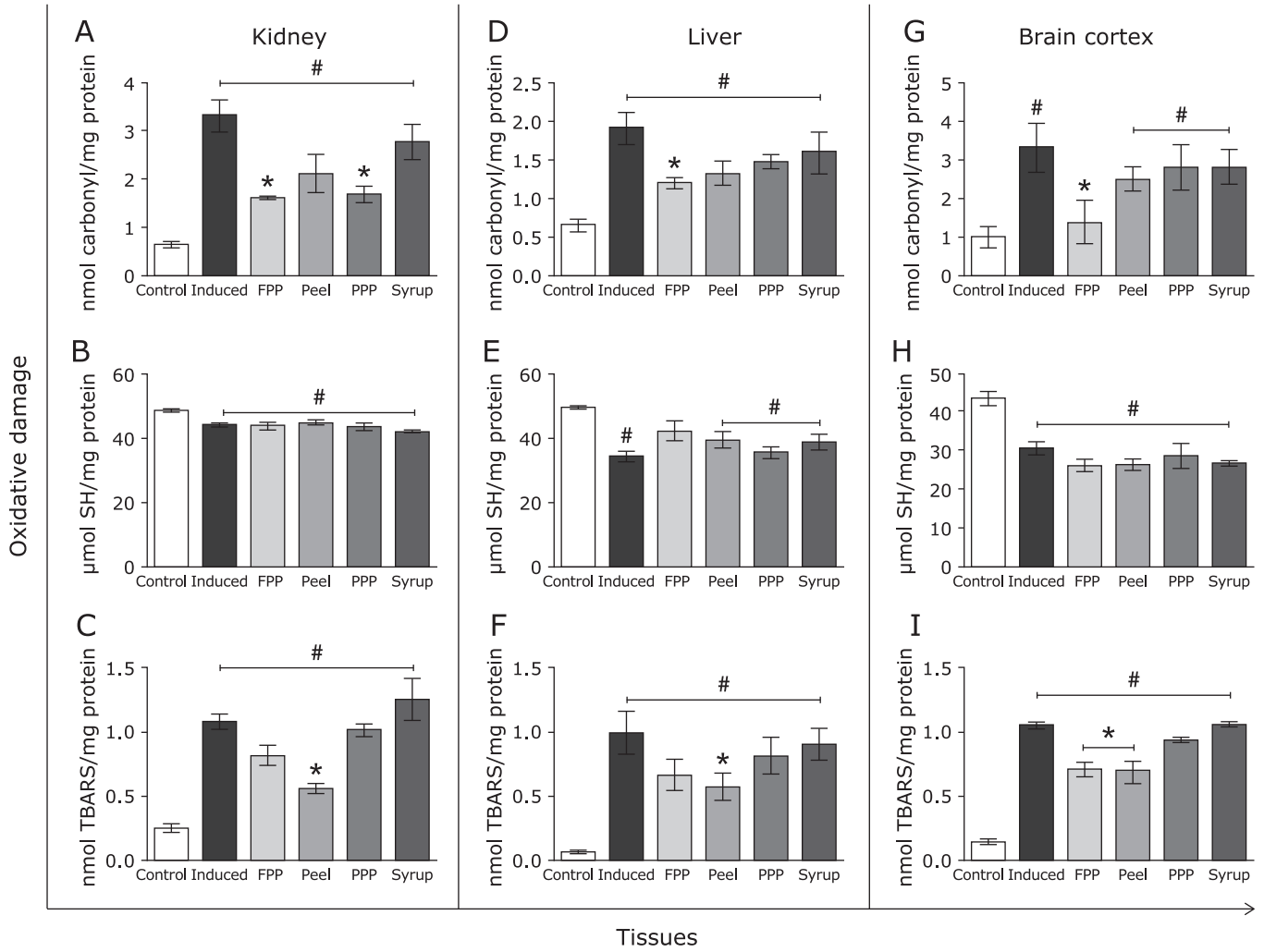

Fig. 4. Effects of FPP, peel, PPP and syrup on biomolecule oxidative damage. Kidney, liver and brain cortex slices were pre-incubated with FPP, peel, PPP or syrup (80 μg/ml each) for 60 min and then subjected to oxidative damage by incubation with $FeSO_4$ 1 mM and H_2O_2 100 mM for 30 min (stress-induced group). Tissues were homogenized and analyzed for (A), (B), (C) protein carbonylation, reduced sulphydryl content and TBARS content in kidney. The same assays were conducted to liver (D), (E), (F) and brain cortex (G), (H), (I). #Different from control group (p<0.05), *different from stress-induced group (p<0.05) using one-way ANOVA (Tukey's post hoc). Values in graphic bars represent mean ± SEM (triplicate experiments, n = 6 per group).

have been linked to prevention of certain cancers and eye diseases.[38] As FPP, peels, PPP and syrup presented different effects in our *in vitro* and *ex vivo* assays, we evaluated the differences between the content of phenolic compounds and carotenoids in these products.

We performed a determination of the total phenolic content of the peach-dervived samples by the Folin–Ciocalteau method and observed a higher content of total phenolics in peels and FPP compared to PPP and syrup, we used the gallic acid as standard (Fig. 6A). In a previous study with this same variety (Maciel) of peach, chlorogenic acid was found to be present in high amounts in lyophilized samples from the whole fruit.[28] Chlorogenic acid is one of the most abundant polyphenols in fruits and it may be one of the main phenolic compounds exerting the biological activities observed here. We also performed a quantification of five common carotenoid compounds (all-trans-lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene) in these samples by HPLC (Fig. 6B). Both FPP and peel presented higher concentrations of all carotenoids evaluated, while the PPP samples presented lower levels of these compounds with exception of α-carotene, which was not detected. On the other hand, there were no detectable amounts of any of the carotenoids analyzed in syrup samples. In previous studies, it was observed that peach peels exhibited a 2 to 27-fold higher antioxidant activity than the fruit pulps.[39] In general, the main differences between these fruit parts are the richest protein content of peels and the higher carbohydrate content in the pulp.[40] However, as we have seen here, the amount of carotenoids and phenolic compounds between these fruit parts may differ.

High concentration of phenolic compounds has been correlated with higher antioxidant activity in dietary fruits such as strawberry, raspberry, blueberry, peach, apricot and pear.[41] However, isolated phenolic compounds, carotenoids and vitamins with known antioxidant properties (such as vitamin A) are not able to exert antioxidant and anti-inflammatory actions at the same level as when obtained from fruit extracts such as nectarine, peach and plum, which suggests an important role for the synergism among the antioxidants in the mixture.[42] It is fairly possible that other molecules present in the samples studied here can also account for the biological effects observed in the present work. These include tocopherols (vitamin E), ascorbate, vitamin D, flavonoids other secondary metabolites. We observed here a rough correlation

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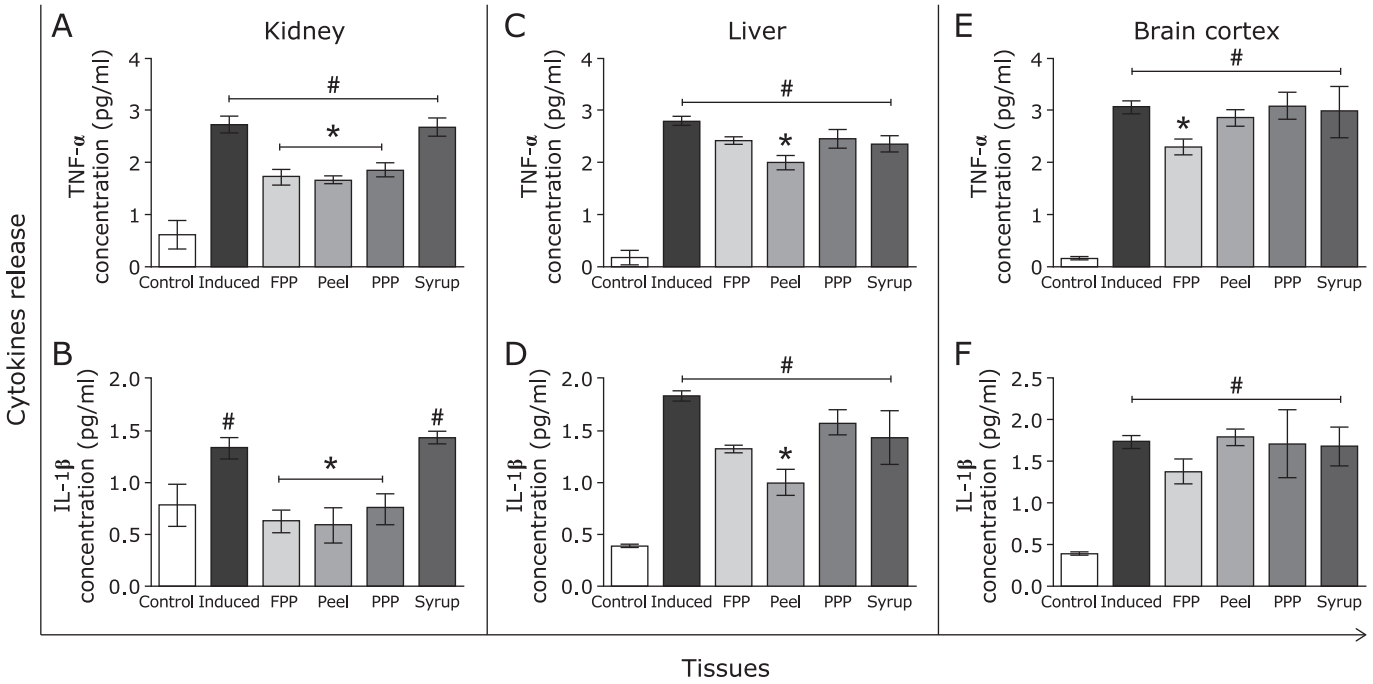

Fig. 5. Effects of FPP, peel, PPP and syrup on interleukin release. Kidney, liver and brain cortex slices were pre-incubated with FPP, peel, PPP or syrup (80 μg/ml each) for 60 min and then subjected to oxidative damage by incubation with $FeSO_4$ 1 mM and H_2O_2 100 mM for 30 min (stress-induced group). The incubation medium was collected and analyzed by ELISA. (A) TNF-α of kidney, (C) liver and (E) brain cortex was quantified. IL1-β levels in (B) kidney, (D) liver and (F) brain cortex were evaluated too. #Different from control group (p<0.0001), *different from stress-induced group (p<0.05) using one-way ANOVA (Tukey's post hoc). Values in graphic bars represent mean ± SEM (triplicate experiments, n = 6 per group).

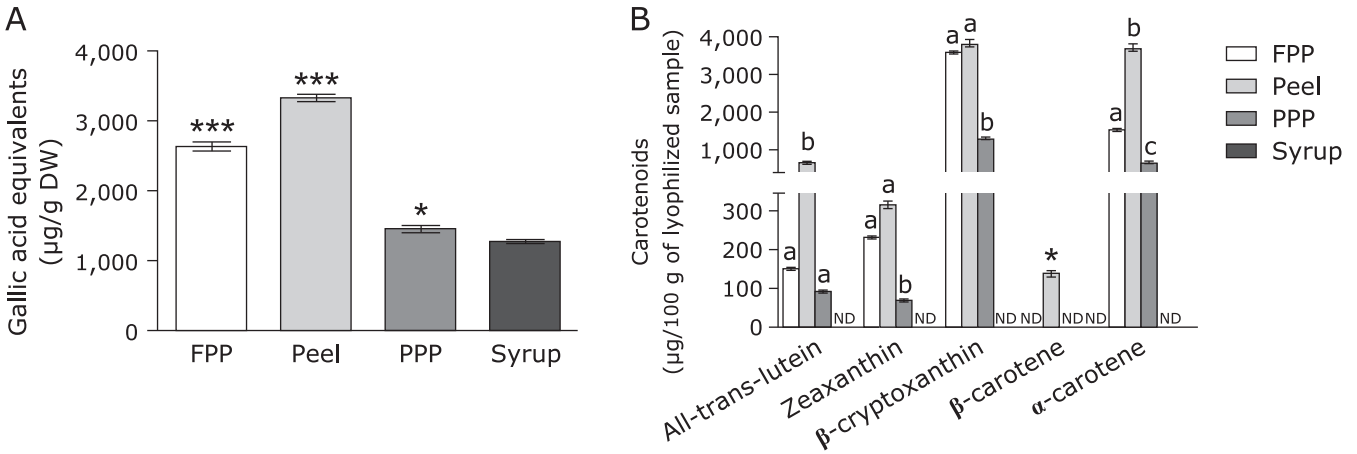

Fig. 6. Total phenol content and HPLC quantification. (A) Suspensions of FPP, peel, PPP and syrup (100 μg/ml) were analyzed for total phenolic content. Values are expressed in μg of gallic acid equivalents per gram of dry weight of samples (DW). ***Different from syrup and PPP groups (p<0.0001), *different from syrup group (p<0.05). B) HPLC quantification of major carotenoids in FPP, peel, PPP and syrup samples. Values are expressed in μg of each compound per 100 g of lyophilized sample. Letters denote same degree of significance between groups for each carotenoid (p<0.05), *different from all other groups. Values in graphic bars represent mean ± SEM (triplicate experiments, n = 3 per group).

between some of the biological effects exerted by different peach-derived samples and their content of phenolic compounds and carotenoids in samples. However, we cannot rule out the role of other compounds in such effects, particularly as we used an aqueous system to ressuspend lyophilized samples, thus enhancing the bioavailability of compounds other than carotenoids.

Carotenoids are hydrophobic compounds and their bioavailability is highly dependent on the solubility properties of their environment. It is very likely that the effects observed here would vary if peach samples had been subjected to extraction methods using apolar solvents, which would increase the availability of carotenoids to tissue slices. However, in this work we had as major aim not to perform a search for active compounds in peaches, but to explore the potential biological activities of peaches and its derivate products in the form they are consumed by humans. Thus, the use of extraction methods specific for hydrophobic compounds would therefore alter their bioavailability in relation to their form of consumption. With this in mind, we chosen the lyophilization of samples followed by water ressupension as in pilot experiments this has proven the most suitable form of administrating equal

amounts of sample (considering their dry weight) in a system (aqueous) with physiological significance. Thus, it is not surprising that we have detected the presence of hydrophobic compounds such as carotenoids in these water-based suspensions, as the same happens in all fruit juices where hydrophobic-derived compounds are readily detectable and present a significant bioavailability after ingestion.

Conclusions

FPP, peels, PPP and syrup present different antioxidant, anti-glycation and anti-inflammatory properties, as assessed by *in vitro* and *ex vivo* assays. The assessment of antioxidant and anti-inflammatory effects in liver, kidney and brain cortex slices showed significant differences between the peach-derived products; FPP and peel presented the highest antioxidant and anti-inflammatory properties, followed by PPP. Syrup had no significant effect in all assays. We observed that the content of phenolic compounds and carotenoids is significantly higher in FPP and peels, followed by PPP, and a low levels of phenolic compounds plus undetectable levels of carotenoids in syrup. Further studies will address the effects of the consumption of these products derived from peaches *in vivo* models, as well as the role of the micronutrients and their effects.

Authors' Contributions

J.G. conducted all the animal studies and drafted the manuscript. N.S., K.K., performed oxidative stress assays. R.C.B., performed assays of total phenolic content, protein glycation and total reactive antioxidant potential. K.S.M., conducted High-Performance Liquid Chromatography (HPLC) assays. C.S.G. and T.K.R., performed oxidative damage assays. M.S.M. was responsible by ELISA assays. M.V. and M.C.B.R. performed peach production, collection and lyophilization. J.C.F.M. and D.P.G. supervised and coordinate this work. All authors have read and approved the final manuscript.

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Abbreviations

CAT	catalase
$FeSO_4$	ferrous sulfate
FPP	fresh poulp peach
H_2O_2	hydrogen peroxide
IL-1β	interleukin-1 beta
LDH	lactate dehydrogenase
PBS	phosphate buffer saline
PPP	preserve poulp peach
RAGE	receptor for advanced glycation endproducts
SH	sulfhydryl
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive species
TNF-α	tumor necrosis factor alpha

Conflict of Interest

No potential conflicts of interest were disclosed.

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