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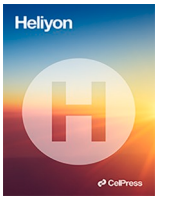

Research article

Antioxidant activity of chia flour as a food supplement in a cellular model: Repercussions of processing and *in vitro* digestion

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ABSTRACT

Food processing and digestion can alter bioactive compound composition of food, affecting their potential biological activity. In this study, we evaluated the direct and protective antioxidant effects of polyphenols extracted from defatted chia flour (DCF) (salviaflaside, rosmarinic and fertaric acid as major compounds), sweet cookies supplemented with DCF (CFC) (same major compounds), and their digested fractions (rosmarinic acid, salviaflaside, fertaric and salvianolic E/B/L acid as major compounds) in HepG2 cells in basal and in oxidative stress conditions. DCF showed protective antioxidant effects by decreasing reactive oxygen species (ROS) and protein oxidation products (POP) while increasing reduced glutathione (GSH). Additionally, CFC revealed similar protective effects and even showed enhanced modulation of the antioxidant system due to the activation of antioxidant enzymes. However, the digested fractions only decreased ROS, indicating continued antioxidant effects. This study underscores the importance of evaluating manufacturing and digestion effects to confirm a food's antioxidant properties.

1. Introduction

Polyphenols are a wide family of plant-based organic compounds with a special chemical structure characterized by having one or more aromatic rings with hydroxyl groups and different substituents. These compounds have been subject to extensive research due to their different biological activities, such as anti-inflammatory, antimicrobial, antidiabetic, and anticancer activities, among others. However, they are primarily known for their antioxidant effects [1]. It has been proven that these compounds could prevent or reverse the effects of oxidative stress that the organism could suffer from various factors, including but not limited to radiation, exposure to toxic chemicals, unhealthy habits like smoking, and poor nutritional diets. Polyphenols present diverse biological mechanisms of action [2]. While some compounds could interact with the endogenous enzymatic antioxidant system, improving its activity, other compounds could increase the level of non-enzymatic antioxidant components, or they may also modify the expression of genes or proteins related to the antioxidant response [3]. Therefore, in recent years, the consumption of polyphenol-rich diets has been promoted to prevent the development of oxidative stress and related diseases [4].

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However, the processing of vegetal ingredients during food manufacturing, as well as the subsequent gastrointestinal digestion, could modify the composition and concentration of the different bioactive compounds [5,6]. Since the biological activity and mechanism of polyphenol compounds depend on their chemical structure and concentration, any changes that occur during these processes could modify the resulting effect. Besides, vegetal ingredients usually have a mixture of polyphenol compounds, so they might exhibit synergistic or antagonistic actions [7]. Consequently, the study of the antioxidant activity of polyphenol compounds present in food has become an intriguing and relevant matter that needs to be accurately examined. Different antioxidant activity assays have been used in this research area; however, cultured cells have gained popularity in recent years. The use of cultured cells allows for the evaluation of the underlying mechanisms of oxidative stress and the effects of antioxidant agents. In particular, human hepatoma HepG2 cells are a validated model of the human liver that has been well characterized and widely used in biochemical and nutritional studies [2].

A vegetal ingredient that has gained popularity in recent years is chia (*Salvia hispanica* L.*)* seeds. These seeds are well-known for their high content of ω-3 fatty acids and antioxidant compounds [8]. They are used in different food matrices, with bakery products being the most common [9]. Several published articles have reported on how the processing and the gastrointestinal digestion affect the antioxidant capacity of these seeds using *in vitro* chemical methods [10–14]. However, most of these *in vitro* colorimetric methods are based on the ability of the compounds to scavenge free radicals. Different studies have demonstrated that it is highly unlikely that polyphenols could act by this direct scavenging mechanism in biological environments, due to the low physiological concentration and reaction rates compared to endogenous antioxidants [15–17]. Therefore, sometimes results obtained by *in vitro* methods are not extrapolated to the real biological activity. Thus, more complex models like cell culture or *in vivo* experiments are necessary. Few research studies have been conducted in this field using cell culture models. Ref. [18,19], have studied the antioxidant activity of different plants of the *Salvia* genus (the same as chia) in cell culture, showing promising results. Ref. [20] examined the effect of chia flour extract on carbohydrate metabolism in HepG2 cells. Also, several articles have shown the antioxidant effect of rosmarinic acid, one of the major compounds present in chia flour [21–23]. In previous studies by our research group, we determined the polyphenol profile and content of defatted chia flour made from an industrial by-product after oil extraction. Additionally, we evaluated how processing and gastrointestinal digestion affect polyphenol profile and antioxidant activity (using *in vitro* chemical methods) when this flour was added to sweet biscuits [12]. However, to our knowledge, there are no previous studies focused on the antioxidant activity of chia flour using cellular models, nor in the modification of this biological activity due to food-making processes and gastrointestinal digestion. These type of studies should be considered as the initial steps for assessing the potential bioactivivity of a new extract/compound before they are evaluated in *in vivo* assays, wheter in animals or humans [17,24,25]. Thus, in this work, we use cell culture experiments with HepG2 to study how processing and gastrointestinal digestion affect the antioxidant effect of defatted chia flour, evaluating the enzymatic and non-enzymatic endogenous antioxidant system, as well as different oxidative damage markers.

2. Materials and methods

2.1. Chemical and reagent

Ultra-pure water (<18 MΩ cm < 5 μgL^{-1} TOC) was obtained from a purification system Arium 61316-RO plus Arium 611 UV (Sartorius, Germany). Methanol (HPLC grade) and formic acid (puriss. p. a. for mass spectroscopy) were provided by J. T. Baker (State of Mexico, Mexico) and Merck (California, USA), respectively. Commercial standards of polyphenolic compounds were obtained from Extrasynthese (Genay, France), Sigma-Aldrich (Steinheim, Germany), and Fluka (Dorset, U.K.). Filters (0.45 μm, HVLP04700) were obtained from Millipore (São Paulo, Brazil). Porcine enzymes used in *in vitro* gastrointestinal digestion and all reagents used for redox markers in cell culture were purchased from Sigma-Aldrich (Buenos Aires, Argentina). SnakeSkin dialysis bags with a molecular weight cut-off of 10 kDa and a width of 22 mm, and Hypersep SPE 500 mg/2.8 mL C18 cartridges were obtained from ThermoFisher SCIENTIFIC. Anaerobic atmosphere generation bags were purchased from Mitsubishi Gas Chemical (Tokyo, Japan). Dulbecco's modified Eagle medium was obtained from Sigma-Aldrich and fetal bovine serum from Natocor (Córdoba, Argentina). All other reagents were of analytical grade.

2.2. Sample preparation

2.2.1. Defatted chia flour and chia flour cookie

First, defatted chia flour (DCF) was obtained by milling the cold-pressed deoiled fraction of chia seeds from commercial plantations in the province of Salta (Argentina).

Then, sweet cookies were prepared following the recipe in Ref. [12]. A control cookie (CC) formulation was prepared using wheat flour, caster sugar, vegetable shortening, powdered skimmed milk, $NaHCO_3$, NaCl, and water. The other formulation used was prepared with DCF as a replacement for 10 % wheat flour and named as chia flour cookie, or CFC.

Polyphenols were obtained by extraction 4 times with methanol:water (1:1) assisted by ultrasound (400 W; 40 Khz), in a solid: solvent proportion of 1:5 during 15 min at room temperature.

2.2.2. Digested fractions of sweet cookies

CFC and CC were subjected to *in vitro* gastrointestinal digestion simulated in four stages: the digestive process in the mouth, stomach, small intestine, and large intestine (including colonic fermentation). In this process, the corresponding enzymes and pH were used for each step. Besides, during the small intestine step, a dialysis bag (10 KDa molecular weight cut-off) was used to replicate the

passive absorption of polyphenolic compounds through the intestinal membrane. This sample was named as small intestine dialyzable. The same dialysis process was used during colonic fermentation. The fraction that went through the dialysis membrane represented the fraction that might be absorbed into the circulatory system through passive diffusion in the large intestine and was identified as large intestine dialyzable. In addition, a blank sample without a cookie was equally processed to discard the effect of the digestive reagents on the cell culture assays.

In summary, defatted chia flour (DCF), control cookie (CC), chia flour cookie (CFC), the small intestine dialyzable fraction, and the large intestine dialyzable fractions of CC and CFC were assayed in cell culture (Fig. S1 of *supplementary information*), previously determining the profile and content of polyphenolic compounds.

2.3. Determination of polyphenol profile and content by HPLC- DAD- QTOF

The determination of the polyphenol profile and quantification of each sample were performed following the methods described in Ref. [12]. Briefly, all samples were purified using a solid-phase extraction C18 cartridge and then analyzed using an HPLC-DAD-QTOF. Finally, the samples were evaporated and re-suspended in DMSO at the required concentration.

2.4. Cell cultivation

Human Hepatoma Cells (HepG2) gifted from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultivated with DMEM containing 10 % of fetal bovine serum, 100 units/mL penicilin, 100 μg/mL streptomycin, 100 μM non-essential amino acids, and 2 mM L-glutamine. HepG2 cells grew in a cell incubator (ThermoFischer) maintaining 5 % CO_2 at 37 °C.

When cells reached an appropriate density in the plates, four different treatments were assayed:

- *Negative control*: cells were cultivated with DMEM containing 10 % of fetal bovine serum, the above-described amount of antibiotics and supplements, and the vehicle of phenolic compounds in samples (0.1 % v/v, final concentration of DMSO).
- *Positive control of stress*: cells were exposed to DMEM containing 2.5 % of fetal bovine serum and 10 mM H_2O_2 for 1 h.
- *Direct effect of polyphenols*: cells were cultivated with 1 μg/mL of the different phenolic compounds samples (DFC, CFC, CC, and small and large intestine dialyzable fractions) in DMEM containing 10 % of fetal bovine serum, the above-described amount of antibiotics and supplements for 24 h. In the case of the control cookie and reaction blank, cells were exposed to a dilution equal to the CFC fractions.
- *Protective effect of polyphenols*: cells were cultivated as in the direct effect treatment but exposed to 10 mM H_2O_2 as in the positive control of stress.

The H_2O_2 concentration used in this study was determined in previous assays using (0.01–20 mM). The selected condition was chosen as it showed an increase in reactive oxygen species but allowed to observe an effect of polyphenol extracts at concentrations similar to physiological ones (data not shown) [26,27].

2.5. Cell viability

Cytotoxicity of the stress model and the polyphenol treatments were measured using a flow cytometer to determine the fluorescence intensity of the vital dye trypan blue [28].

Briefly, cells were seeded in a 96-well plate (2.5×10^4 cells/well) and incubated for 24 h. Then, they were exposed to polyphenol samples and the stressor as described in section 2.3. After this, cells were rinsed with PBS and suspended in 0.002 % trypan blue in PBS. Fluorescence intensity was measured by flow cytometry (Attune, ThermoFischer) with emission at 660 nm. Results were analyzed using Flow Jo Software (Tree Star), and collected as the mean fluorescence intensity of each sample.

2.6. Reactive oxygen species measurement

Reactive oxygen species (ROS) levels were quantified using dichloro-dihydro-fluorescein diacetate (DCFH-DA) [29]. Cells were seeded in a 96-well plate (2.5×10^4 cells/well) and incubated for 24 h. Then, they were exposed to polyphenol samples and the stressor as described in section 2.3. After this, cells were rinsed with PBS and incubated with DCFH-DA 10 μM in DMEM without FBS for 15 min at 37 °C. Next, the supernatant was discarded, cells were rinsed and then re-suspended in PBS. Fluorescence intensity was measured by flow cytometry with emission at 530 nm. Results were analyzed using Flow Jo Software (Tree Star), and collected as the mean fluorescence intensity of each sample.

2.7. Antioxidant enzyme activities

To assess the activity of the endogenous enzymatic antioxidant system, the activities of catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were measured using different methods based on enzymatic kinetics and the disappearance of their substrates. UV–Vis spectrophotometry was performed using a microplate reader, SynergyHT (Biotek). The activity of each enzyme was expressed as nkat/mg protein, where 1 kat represents the conversion of 1 mol of substrate per second. Protein content was measured using the Bradford method [30].

Cells were seeded in a 24-well plate (2.5×10^5 cells/well), incubated for 24 h, and then exposed to polyphenol samples and the stressor as described in section 2.3. After this, cells were rinsed with PBS and harvested in a phosphate buffer 0.1 M pH = 6.5 with 20 % glycerol, EDTA (ethylenedinitrilotetraacetic acid) 1 mM, and DTE (dithioerythritol) 1.4 mM. The cell homogenate was centrifuged at 13000 g and 4 °C for 10 min, and the supernatant was used to measure the activity of the three enzymes.

CAT was measured following the decrease in absorbance of H_2O_2 at 240 nm, and GPx and GR were determined measuring the decrease in NADPH absorbance at 340 nm using the methods described in Refs. [31,32].

2.8. Reduced glutathione

The most important endogenous non-enzymatic antioxidant, reduced glutathione (GSH), was measured following the method described in Ref. [33]. This method is based on the derivatization of GSH with o-phthalaldehyde (OPA) to form an indole, which can be quantified by fluorescence.

To carry out this, cells were seeded in 6-well plates (5×10^5 cells/well), incubated for 24 h, and then exposed to polyphenol samples and the stressor as described in section 2.3. Then, cells were rinsed with PBS and harvested in a phosphate buffer 0.1 M pH = 8 with EDTA 5 mM. The cell homogenate was centrifuged at 13,000 g and 4 °C for 10 min, and the supernatant was mixed with OPA 0.25 mM (final concentration) in a 96-well plate. The mixture was incubated for 20 min in the dark at room temperature, and then its fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. GSH concentration was expressed as μg GSH/mg protein and calculated using a calibration curve prepared with commercial GSH (0.02–20 μg/mL) and treated in the same way as samples.

2.9. Protein oxidation

Protein oxidation products (POP) were quantified using the protein carbonyl group method [34]. Reactive oxygen species can react with the side chain of certain amino acids and generate carbonyl groups, which can be derivatized with the reagent 2,4-dinitrophenylhydrazine (DNPH) to form a colored hydrazone. Thus, POP can be quantified by measuring this product through spectrophotometry after the described derivatization.

To perform this method, cells were seeded in 100 mm plates (1×10^6 cells/well), incubated for 24 h, and then exposed to polyphenol samples and the stressor as described in section 2.3. Then, cells were rinsed with PBS and harvested in a phosphate buffer 50 mM pH = 7.5. The cell homogenate was centrifuged at 13,000 g at 4 °C for 10 min, and the supernatant was divided into two aliquots (sample and blank). DNPH 5 mM in HCl 2 M was added to the sample and incubated in the dark for 10 min. After this, 5 % TCA was added, and the mixture was centrifuged for 3 min at 11,000 g. The pellet was rinsed twice with ethanol:ethyl acetate (1:1) and then dissolved in guanidine 6 M in a phosphate buffer 20 mM pH = 2.3. The blank was treated in the same manner as the samples, but no DNPH was added. The absorbance of samples and blanks was measured at 366 nm, and the difference between them was used to calculate the POP concentration ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$), expressed as μM/mg protein.

2.10. Statistical analysis

For statistical analysis, the software INFOSTAT was used [35]. Three independent experiments were carried out in triplicate for each parameter measured (n = 9). All results were expressed as a percentage of the negative control samples to improve visualization. Normality and homoscedasticity were evaluated graphically and numerically (using tools from the R library fitdistrplus) through Shapiro-Wilk's tests. General and Linear Mixed Models were used to evaluate significant differences between treatments. The treatment was included as a fixed effect, and the independent experiment number was included as a random effect. In the case of significance ($p < 0.05$), a LSD Fisher comparison test was performed to reveal differences between the means. Statistical analyses were performed separately for the direct and protective effects. Besides, paired differences were analyzed between the negative and positive controls of stress. Data are expressed as mean ± SE.

3. Results

3.1. Polyphenol profile and content

Twenty-five compounds were tentatively identified in DCF (Table 1 of *Supplementary Information*) belonging to the hydroxycinnamic acid, flavonoids, organic acid, and amino acid families. The major compound was salviaflaside, followed by rosmarinic acid and fertaric acid (Table 2 of *Supplementary Information;* adapted from Ref. [12]).

After the cookie-making process, only 11 compounds were detected and quantified in CFC. Some compounds increased their relative concentration, such as quinic acid, danshensu, tryptophan, quercetin dihexoside, kaempferol dihexoside, and salviaflaside. On the other hand, caftaric acid, fertaric acid, and rosmarinic acid decreased their relative concentrations. However, salviaflaside and rosmarinic acid were still the major compounds.

After *in vitro* gastrointestinal digestion, only 8 compounds were found in the small intestine dialyzable fraction of CFC. This sample showed increased relative concentrations of caftaric acid, salviaflaside, and rosmarinic acid. Besides, caffeic acid, a compound from DCF but not detected in CFC, was detected in this digested fraction. On the other hand, lower relative concentrations of quinic acid and tryptophan were observed compared to undigested CFC. In addition, quercetin dihexoside, kamepferol dihexoside, and quercetin

hexoside were not detected in this fraction. Finally, 9 compounds were found in the large intestine dialyzable fraction of CFC, with rosmarinic acid exhibiting the highest relative concentration, even surpassing salviaflaside. Besides, salvianolic acid E/B/L and quercetin hexoside were detected in this fraction but not in the previous one. Lower relative concentrations of caftaric acid, tryptophan, fertaric acid, caffeic acid, and salviaflaside were observed. No quinic acid was detected in this fraction [12].

3.2. Antioxidant activity of defatted chia flour (DCF) and chia flour cookie (CFC)

3.2.1. Cell viability

To evaluate the effect of the different samples on cell bioavailability, the trypan blue assay was performed. Since the trypan blue assay is a dye-exclusion test, an increase in fluorescence was associated with an increase in cell death. Results are summarized in Fig. 1A.

No differences were observed between the negative control and DCF, CFC, and CC (Control Cookie). On the other hand, the oxidative stress stimulus caused an increase in cell death by approximately 16 % compared to the negative control, which could not be prevented by the different polyphenol treatments.

3.2.2. Reactive oxygen species

Regarding the direct effect of polyphenols, no differences between the negative control and DCF were observed. However, both CC and CFC decreased ROS levels, showing that the supplemented cookie had a greater effect (Fig. 1B). Under oxidative stress conditions, different results were observed. The positive control showed a significant increase with respect to the negative control. Under this condition, DCF showed an antioxidant effect, decreasing ROS levels, similar to CFC. CC also showed a protective effect, but it was lower

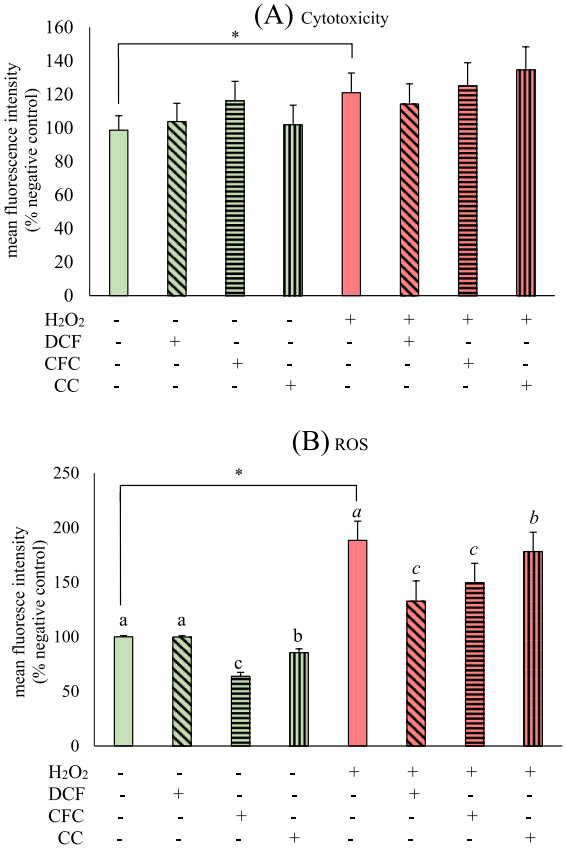

Fig. 1. Effect of Defatted Chia Flour (DCF), Chia Flour Cookie (CFC), and Control Cookie (CC) exposure on **(A):** cytotoxicity and **(B):** reactive oxygen species (ROS) levels in HepG2 cells under basal and oxidative stress conditions. DCF and CFC exposures were performed at 1 μg/mL, and CC with the same dilution as CFC. Asterisks (*) indicate paired significant differences ($p < 0.05$) between the negative and positive controls. Different letters indicate significant differences between treatments. Statistical analysis was performed separately for the direct (regular font) and protective effects (italics).

than in the other two samples.

3.2.3. Antioxidant enzymes

Concerning the direct effect of the polyphenol extracts, only CFC showed a significant increase in the CAT and GR activities, while no modifications in the other enzymes were observed (Fig. 2A, B and 2C). In addition, exposure to H_2O_2 did not show effects on the activity of any of the three enzymes measured. However, under this stress condition, CC and CFC extracts raised GPx activity.

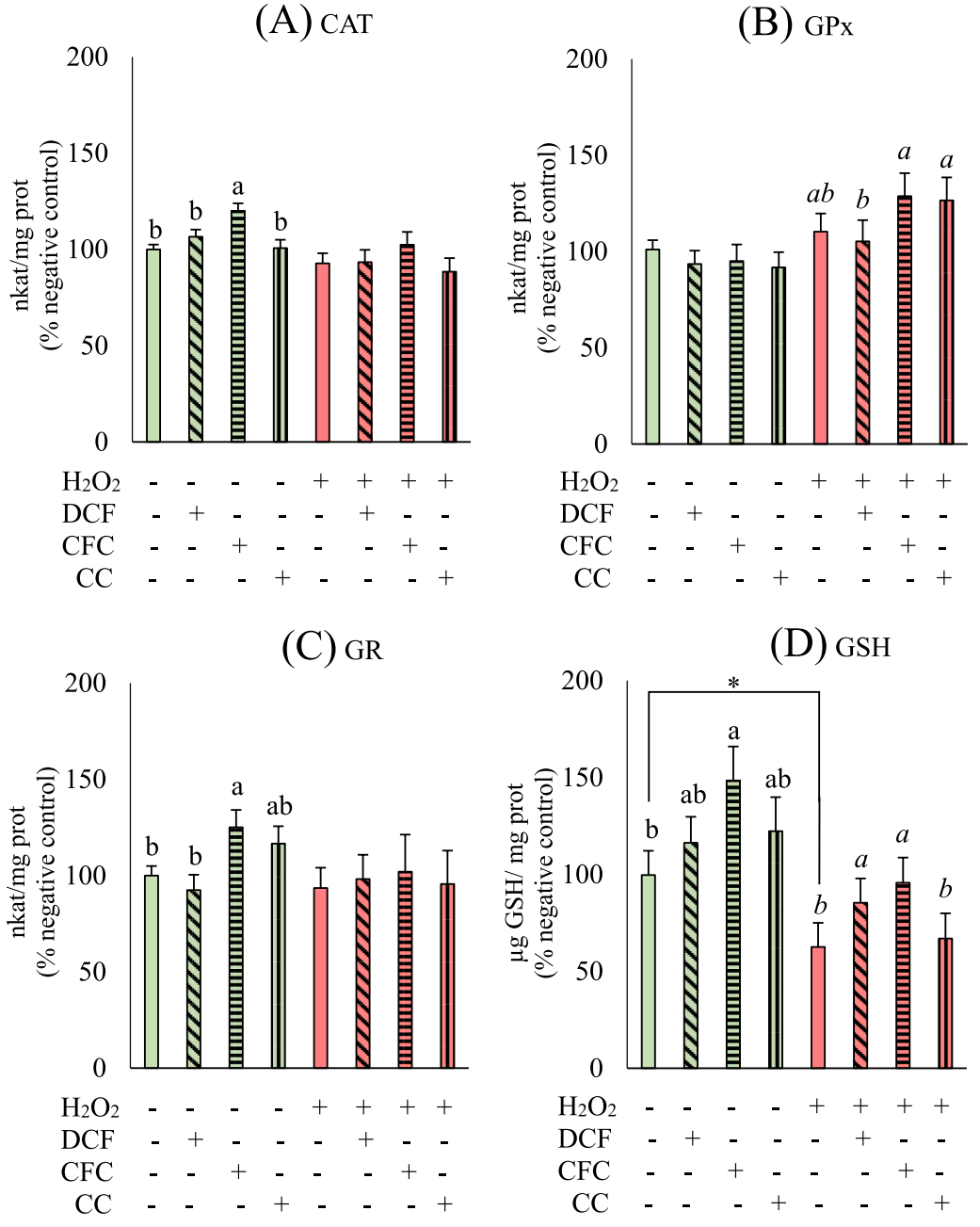

Fig. 2. Effect of Defatted Chia Flour (DCF), Chia Flour Cookie (CFC), and Control Cookie (CC) exposure on antioxidant enzymes **(A):** catalase (CAT), **(B):** glutathione peroxidase (GPx), **(C):** glutathione reductase (GR), and **(D):** reduced glutathione (GSH) in HepG2 cells under basal and oxidative stress conditions. DCF and CFC exposures were performed at 1 µg/mL, and CC with the same dilution as CFC. Asterisks (*) indicate paired significant differences ($p < 0.05$) between the negative and positive controls. Different letters indicate significant differences between treatments. Statistical analysis was performed separately for the direct (regular font) and protective effects (italics).

3.2.4. Glutathione content

Regarding the direct effect of polyphenol extracts, only CFC showed a significant increase in GSH levels compared to the negative control. Under stress conditions, the positive control showed a decrease in the level of GSH compared to the negative control, which could be partially prevented by DCF and CFC pretreatment (Fig. 2D).

3.2.5. Protein oxidation

Regarding protein damage, DFC did not present any effect compared to the negative control under basal conditions, while both cookies showed a significant decrease in protein oxidation products (Fig. 3). Exposure to H_2O_2 did not produce any changes in protein oxidation. However, under these oxidative stress conditions, both DCF and CFC helped to decrease POP levels compared to the positive control.

3.3. Antioxidant activity of digested chia flour cookies

3.3.1. Cell viability

The results of the effect of the gastrointestinal digestion samples on cell viability are summarized in Fig. 4A. No modifications in cell viability were observed following treatment with small and large intestine dialyzable fractions of CFC and CC, similar to the undigested samples.

3.3.2. Reactive oxygen species

First, small intestine dialyzable fraction of CC showed antioxidant activity under basal conditions, reducing ROS levels compared to the blank negative control. However, the same fraction of CFC exhibited a greater effect (Fig. 4B). Regarding the protective effect of these small intestine dialyzable fractions, CC did not present significant variations with respect to the positive control of stress, but CFC continued to demonstrate antioxidant activity. Concerning large intestine dialyzable fractions, on the other hand, both CC and CFC exhibited reduced ROS levels for direct and protective effects with respect to the negative and positive controls, with no differences between them (Fig. 4B).

3.3.3. Antioxidant enzymes

In contrast to the results obtained for undigested CFC, the small intestine dialyzable fraction decreased CAT activity under basal conditions (Fig. 5A). However, for the large intestine dialyzable fraction, both CFC and CC increased CAT activity under oxidative stress conditions (Fig. 5B). GPx and GR activity showed no differences among treatments (Fig. 5C to F).

3.3.4. Reduced glutathione

Results obtained for GSH levels in digested samples are summarized in Fig. 5G and H. Under basal conditions, only small intestine dialyzable fraction of CC increased GSH values compared to the negative control; however, no differences were observed under

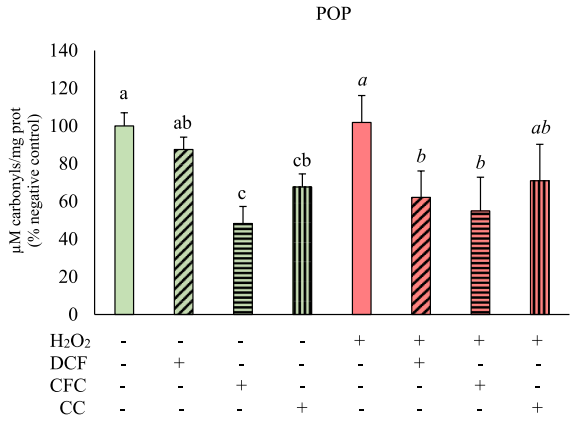

Fig. 3. Effect of Defatted Chia Flour (DCF), Chia Flour Cookie (CFC), and Control Cookie (CC) exposure on protein oxidation products (POP) in HepG2 cells under basal and oxidative stress conditions. DCF and CFC exposures were performed at 1 μg/mL, and CC with the same dilution as CFC. Different letters indicate significant differences ($p < 0.05$) between treatments. Statistical analysis was performed separately for the direct (regular font) and protective effects (italics).

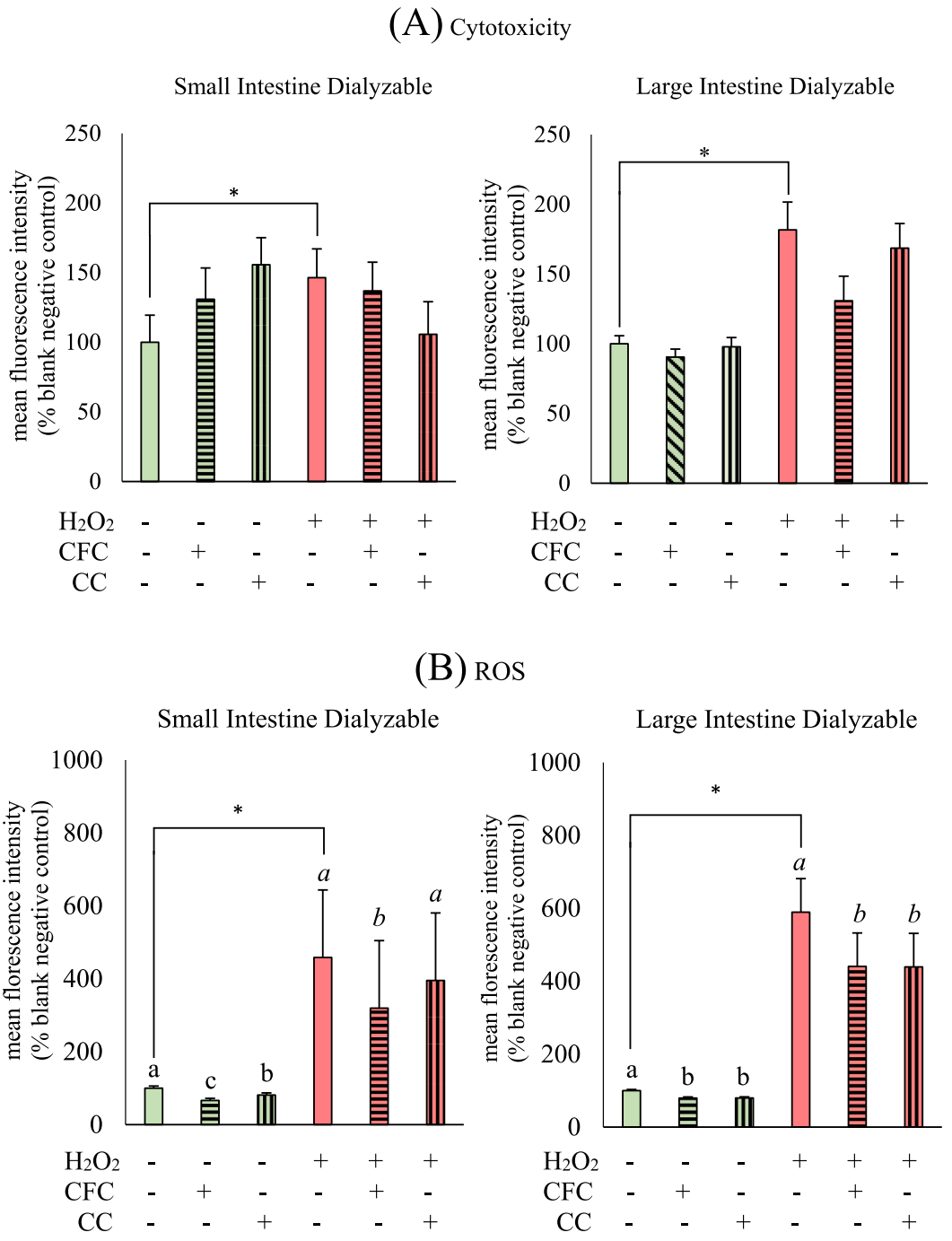

Fig. 4. Effect of Small and Large Intestine Dialyzable fractions of Chia Flour Cookie (CFC) and Control Cookie (CC) exposures on **(A):** cytotoxicity and **(B):** reactive oxygen species (ROS) levels in HepG2 cells under basal and oxidative stress conditions. The exposure with CFC fractions was performed at 1 μg/mL, and CC with the same dilution. Asterisks (*) indicate paired significant differences ($p < 0.05$) between the negative and positive controls. Different letters indicate significant differences between treatments. Statistical analysis was performed separately for the direct (regular font) and protective effects (italics).

oxidative stress conditions for any treatment. Regarding large intestine dialyzable fractions, different results were observed. Under basal conditions, only CFC fraction increased GSH levels with respect to control, but under oxidative stress conditions, it was the CC fraction treatment that showed this effect.

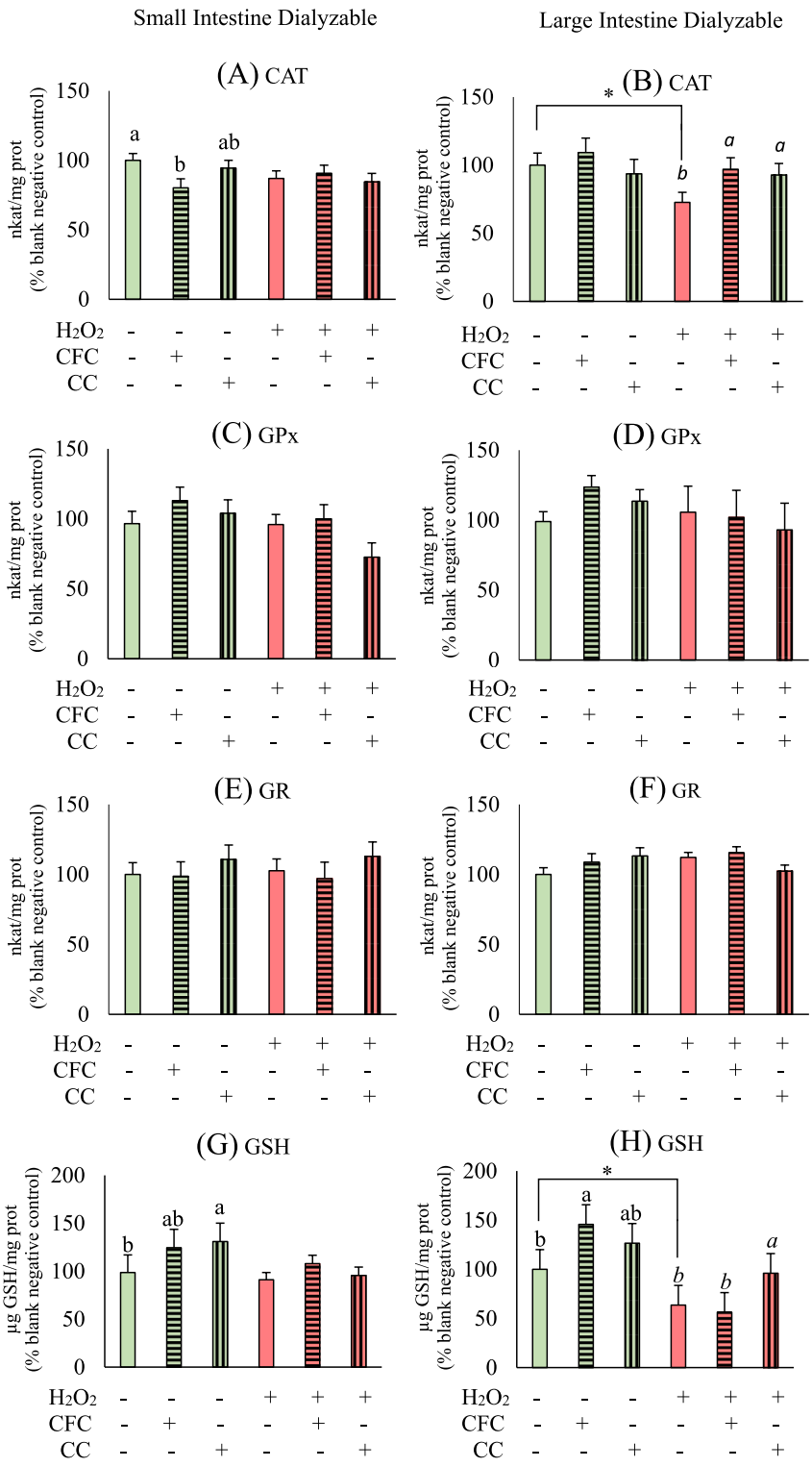

Fig. 5. Effect of Small and Large Intestine Dialyzable fractions of Chia Flour Cookie (CFC) and Control Cookie (CC) exposures on antioxidant enzymes **(A–B):** catalase (CAT), **(C–D):** glutathione peroxidase (GPx), **(E–F):** glutathione reductase (GR), **(G–H):** and reduced glutathione (GSH) in HepG2 cells under basal and oxidative stress conditions. The exposure with CFC fractions was performed at 1 µg/mL, and CC with the same dilution. Asterisks (*) indicate paired significant differences ($p < 0.05$) between the negative and positive controls. Different letters indicate significant differences between treatments. Statistical analysis was performed separately for the direct (regular font) and protective effects (italics).

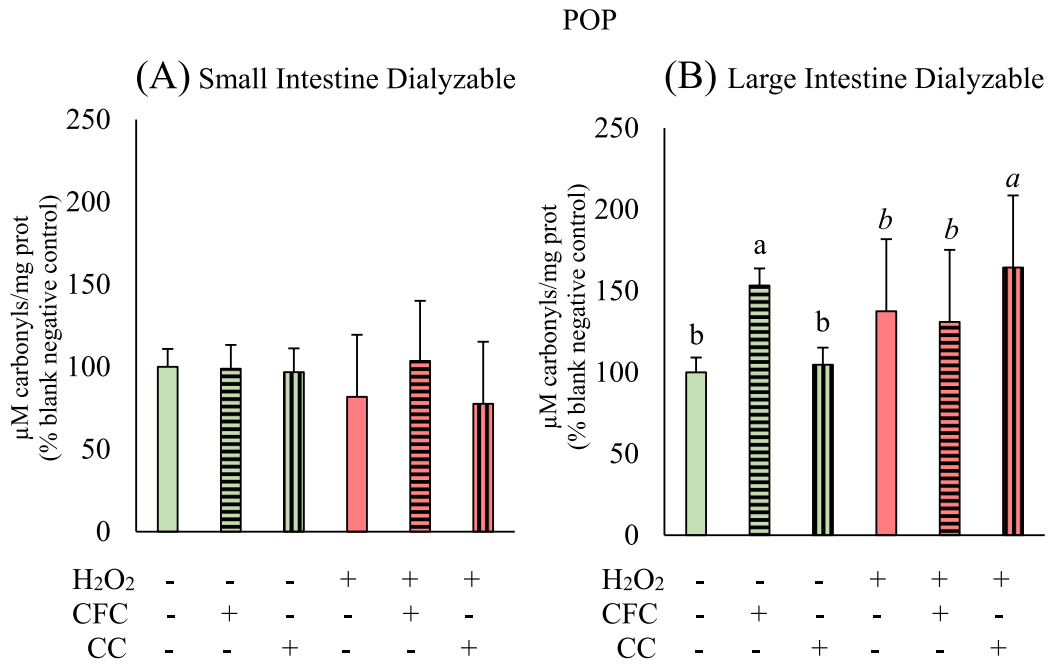

Fig. 6. Effect of **(A):** Small Intestine Dialyzable fraction and **(B):** Large Intestine Dialyzable fraction of Chia Flour Cookie (CFC) and Control Cookie (CC) exposures on protein oxidation products (POP) in HepG2 cells under basal and oxidative stress conditions. The exposure with CFC fractions was performed at 1 µg/mL, and CC with the same dilution. Different letters indicate significant differences ($p < 0.05$) between treatments. Statistical analysis was performed separately for the direct (regular font) and protective effects (italics).

3.3.5. Protein oxidation

Fig. 6A and B summarizes the results obtained for POP in the case of fractions obtained after the *in vitro* gastrointestinal digestion process. Small intestine dialyzable fractions of CC and CFC exhibited no effects, either direct or protective. In the case of large intestine dialyzable fractions, POP levels showed an increase under basal conditions due to CFC. However, this fraction did not exhibit any effect under oxidative stress conditions. On the other hand, the CC fraction did not reveal any impact under basal conditions, but it increased POP levels under oxidative stress conditions.

4. Discussion

Polyphenols are bioactive compounds well-known for their antioxidant activity. Their mode of action primarily depends on their chemical structure and the surrounding environment (normal or basal conditions or in the presence of oxidative stress). Therefore, it is importante to assess the effect of processing and gastrointestinal digestion on the polyphenol profile and, consequently, on their potential bioactivity. In this regard, this study aimed to evaluate the biological activity of polyphenolic extract from defatted chia flour, the polyphenolic extract from sweet cookies supplemented with chia flour and the samples obtained after simulating the gastrointestinal digestion of this cookie.

4.1. Direct effect of samples in HepG2 cells in basal conditions

4.1.1. Impact of Deffated chia flour (DCF) processing during cookie making on the antioxidant activity

DCF did not show any direct effects on human liver HepG2 cells under basal conditions (Figs. 1, **2,** and **3**). Similarly, Ref. [19] observed no effects on cell viability when HepG2 cells were directly exposed to rosemary extracts (including *salvia* genus and chia) at concentrations of 5–20 µg/mL. Additionally, Ref. [21] did not observe any effects on ROS, GPx, and CAT when the same cell line was treated with rosmarinic acid (one of the major compounds in DCF) at a concentration of 1 µM.

However, when this flour was added into sweet cookies, at the same polyphenol concentration, different results were obtained. Cell exposure to this supplemented cookie improved the function of the endogenous antioxidant system, increasing CAT and GR activity as well as GSH concentration compared to the control levels (Fig. 2). Consequently, there was a decrease in ROS levels (Fig. 1B) and protein oxidative damage (POP) (Fig. 3). Therefore, this modification in antioxidant effect could be attributed to the changes in the polyphenol profile caused by processing, such as the decrease in relative concentrations of rosmarinic, caftaric, caffeic, or salvianolic acids and the increase in salviaflaside or quinic acid. Furthermore, the food matrix (a cookie made of wheat flour without DCF supplementation, named Control Cookie (CC)) also showed a decrease in ROS and POP with respect to the negative control (Figs. 1B and

3). However, the antioxidant effect of CFC was greater, which could be attributed to the polyphenol content coming from the added chia flour. To our knowledge, there are currently no reports on how processing affects direct antioxidant activity in cell models under basal conditions, including the food matrix without supplementation. However, similar to this study, Ref. [18] found higher values of GSH when HepG2 cells were treated with a 90 % methanol extract of *Salvia officinalis* with respect to cells under basal conditions. However, no effects were observed when cells were treated with an aqueous extract of the same plant, showing that the differences in the polyphenol profile of the extracts could have different biological effects. In addition, Ref. [36] observed improvements in cell viability only when Caco-2 cells were treated with bread supplemented with defatted olive pomace but not when exposed to non-supplemented bread. In summary, in this study, changes in the polyphenol profile and relative concentrations of each compound, caused by physical and chemical processes during the cookies manufacturing, improved the antioxidant activity of DCF under basal conditions.

4.1.2. Impact of in vitro gastrointestinal digestion of cookies on the antioxidant activity

Regarding changes in the antioxidant effect caused by *in vitro* gastrointestinal digestion, the small intestine dialyzable fraction of CFC decreased ROS levels compared to the blank negative control, even to lower levels than the same fraction of CC (Fig. 4B). However, it did not show significant differences in GSH and POP (Figs. 5G and 6A). In the case of the large intestine dialyzable fraction, CFC also showed lower ROS levels than the blank negative control but without significant differences with respect to the same fraction of CC (Fig. 4B), suggesting a decrease in the antioxidant activity of this fraction compared to the previous one. Furthermore, even though large intestine dialyzable fraction of CFC showed a significant increase in a non-enzymatic antioxidant like GSH (Fig. 5H), this fraction also increased POP (Fig. 6B). In some cases, polyphenol treatments could increase protein oxidation under basal conditions. Ref. [37] found that tannic, ellagic, and gallic acids slightly increased POP levels in CHO cells. However, they proved that these compounds act as antioxidants and decrease DNA damage under the same conditions. In short, changes occurring during *in vitro* gastrointestinal digestion, such as an increase in rosmarinic, caftaric, and salvianolic acids and a decrease in salviaflaside and quinic acid relative concentrations, mainly decreased the antioxidant effects of CFC under basal conditions.

4.2. Protective effect of samples in HepG2 cells in oxidative stress conditions

4.2.1. Impact of Deffated chia flour (DCF) processing during cookie making on the antioxidant activity

On the other hand, the effects of pretreatment with polyphenols could have different effects when cells are exposed to oxidative stress conditions. In this study, exposure to H_2O_2 10 mM for 1 h increased cell death and ROS by 16 % and 88 %, respectively (Fig. 1B). Besides, GSH levels were significantly lower compared to the negative control (Fig. 2D). However, no effect of the stressful stimulus was observed in the activity of antioxidant enzymes or POP levels (Fig. 2A–C and **3**). Ref. [38] also observed a decrease in the viability of SK-N-MC cells when treated with 300 μM of H_2O_2 for 24 h. Additionally, Ref. [39] showed that H_2O_2 exposure did not modify GR and superoxide dismutase (SOD) activities, even when ROS levels were increased. Finally, even though we expected an increase in POP levels caused by H_2O_2 exposure, it has been shown that carbonyl formation due to protein oxidation depends on the time of exposure to H_2O_2, reaching a maximum at 0.5 h of exposure and decreasing thereafter until no differences are observed with basal cells at 2 h [40]. This could explain why no differences were observed between basal and oxidative stress conditions for control samples.

Unlike the effects of DFC observed under basal conditions, this extract showed important protective antioxidant activity under oxidative stress cell conditions, as it increased GSH (Fig. 2D) and decreased ROS and POP levels (Figs. 1B and 3). Furthermore, the changes in the polyphenol profile mentioned above during processing almost did not modify the antioxidant effect observed under stressed conditions. CFC did not exhibit significant differences with respect to DCF in ROS, GSH, and POP levels but showed greater effects than CC (Figs. 1B, 2D, and 3), again suggesting that the antioxidant activity could be attributed to the polyphenol content of the supplemented cookie. The only difference observed between DCF and CFC was the increase observed in GPx (the only antioxidant enzyme that was not modified under basal conditions). However, CC also increased GPx, so this effect might be caused by the food matrix and not the polyphenols. In line with this, Ref. [41] also observed equivalent antioxidant activities in cell viability for grape and wine at the same concentration in Caco-2 cells exposed to H_2O_2, even when the wine polyphenol profile had been modified during processing. Besides, Ref. [42] also described lower POP levels in HepG2 cells treated with green coffee bean extract or its purified major compounds, with analogous results. Additionally, Ref. [43] found lower levels of POP in 3T3 cells treated with quercetin, catechin, or a mixture of both, with no differences among treatments.

4.2.2. Impact of in vitro gastrointestinal digestion of cookies on the antioxidant activity

Regarding digested samples, small intestine dialyzable fraction of CFC demonstrated antioxidant activity by decreasing ROS levels, contrary to the CC fraction, which did not show significant differences compared to the positive control (Fig. 4B). However, none of the small intestine dialyzable fractions of both cookie formulations exhibited effects on any of the other parameters measured. Similarly, large intestine dialyzable CFC also decreased ROS compared to the blank positive control, but with no differences with respect to the CC fraction (Fig. 4B). Likewise, the large intestine dialyzable fraction of both cookies equally increased CAT (Fig. 5B). Ref. [44] also found a protective effect on the generation of ROS in HepG2 cells treated with digested cookies prepared with wholegrain flour from different wheat species. Nevertheless, in that study, authors did not carry out colonic fermentation or include a cookie formulation made with common wheat flour. Besides, Ref. [45] described lower ROS and POP levels, and a partial recovery of GSH compared to the positive control in cells incubated with Yerba Mate extracts. However, these outcomes were not observed in the case of treatments with the main circulating metabolites after digestion. To sum up, the protective antioxidant activity of CFC strongly decreased after *in vitro* gastrointestinal digestion, but still demonstrated effects in reducing ROS levels.

5. Conclusions

In conclusion, this study sheds light on the intricate interplay between polyphenols, the food matrix, and their effects on cellular antioxidant responses. Under basal conditions, Defatted Chia Flour (DCF), a rich polyphenol industrial by-product, did not exhibit direct effects on human liver HepG2 cells. However, when incorporated into Chia Flour Cookies (CFC), the cell exposure to this supplemented cookie showed significant improvements in the endogenous antioxidant system, resulting in decreased ROS levels and protein oxidative damage. These modifications in antioxidant effects could be attributed to changes in the polyphenol profile induced by processing, like a decrease in relative concentration of rosmarinic and caftaric acids and an increase in salviaflaside.

Furthermore, the study examined the impact of *in vitro* gastrointestinal digestion on the antioxidant activity of CFC. The results indicated that the small intestine dialyzable fraction of CFC reduced ROS levels, but the large intestine dialyzable fraction showed a detriment in this antioxidant effect. These changes were associated with polyphenols potentially available after complete digestion that, interestingly, some of them were opposite to those observed during the production of CFC, which contributed to the improved antioxidant activity of DCF.

Additionally, the study explored the effects of polyphenol pretreatment under oxidative stress conditions. While DCF did not exhibit significant effects under basal conditions, it displayed strong protective antioxidant activity when cells were exposed to oxidative stress. This protective effect was largely retained in the CFC, emphasizing the potential of polyphenols to mitigate oxidative stress-induced damage. On the other hand, digested samples lost the ability to improve the functioning of the measured endogenous antioxidant system markers and protect against protein damage, however were able to decrease ROS levels.

In summary, this research underscores the complex and context-dependent nature of polyphenol-mediated antioxidant effects, which are influenced by factors such as the food matrix, processing, and the cellular environment. These findings contribute to a better understanding of how polyphenols can be harnessed for their potential health benefits and emphasize the need for further investigation into their multifaceted actions in different physiological and pathological contexts.

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CRediT authorship contribution statement

Agustin Lucini Mas: Writing - original draft, Investigation, Formal analysis. **María Eugenia Sabatino:** Methodology, Investigation, Formal analysis. **Martin Gustavo Theumer:** Writing - review & editing, Methodology. **Daniel Alberto Wunderlin:** Resources, Project administration, Conceptualization. **María Verónica Baroni:** Visualization, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24125.

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