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Antioxidant Activity of Chia Flour as a Food Supplement in a Cellular Model: Repercussions of Processing and *In Vitro* Digestion

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32 **Abstract**

33   Food processing and digestion can alter bioactive compound composition of food, affecting

34 their potential biological activity. In this study, we evaluated the direct and protective antioxidant

35 effects of polyphenols extracted from defatted chia flour (DCF) (salviaflaside, rosmarinic and

36 fertaric acid as major compounds), sweet cookies supplemented with DCF (CFC) (same major

37 compounds), and their digested fractions (rosmarinic acid, salviaflaside, fertaric and salvianolic

38 E/B/L acid as major compounds) in HepG2 cells in basal and in oxidative stress conditions. DCF

39 showed protective antioxidant effects by decreasing reactive oxygen species (ROS) and protein

40 oxidation products (POP) while increasing reduced glutathione (GSH). Additionally, CFC revealed

41 similar protective effects and even showed enhanced modulation of the antioxidant system due to

42 the activation of antioxidant enzymes. However, the digested fractions only decreased ROS,

43 indicating continued antioxidant effects. This study underscores the importance of evaluating

44 manufacturing and digestion effects to confirm a food's antioxidant properties.

45

46 **Highlights**

47   • Defatted chia flour (DCF) and chia flour cookies (CFC) showed antioxidant effects on

48   HepG2 cells.

49   • DCF and CFC decreased ROS and protein damage by increasing antioxidant defenses.

50   • *In vitro* gastrointestinal digestion decrease antioxidant activity of polyphenol supplemented

51   cookies.

52   • Digested CFC reduced ROS but did not modulate GSH levels nor antioxidant enzyme

53   activity.

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58

Graphical Abstract

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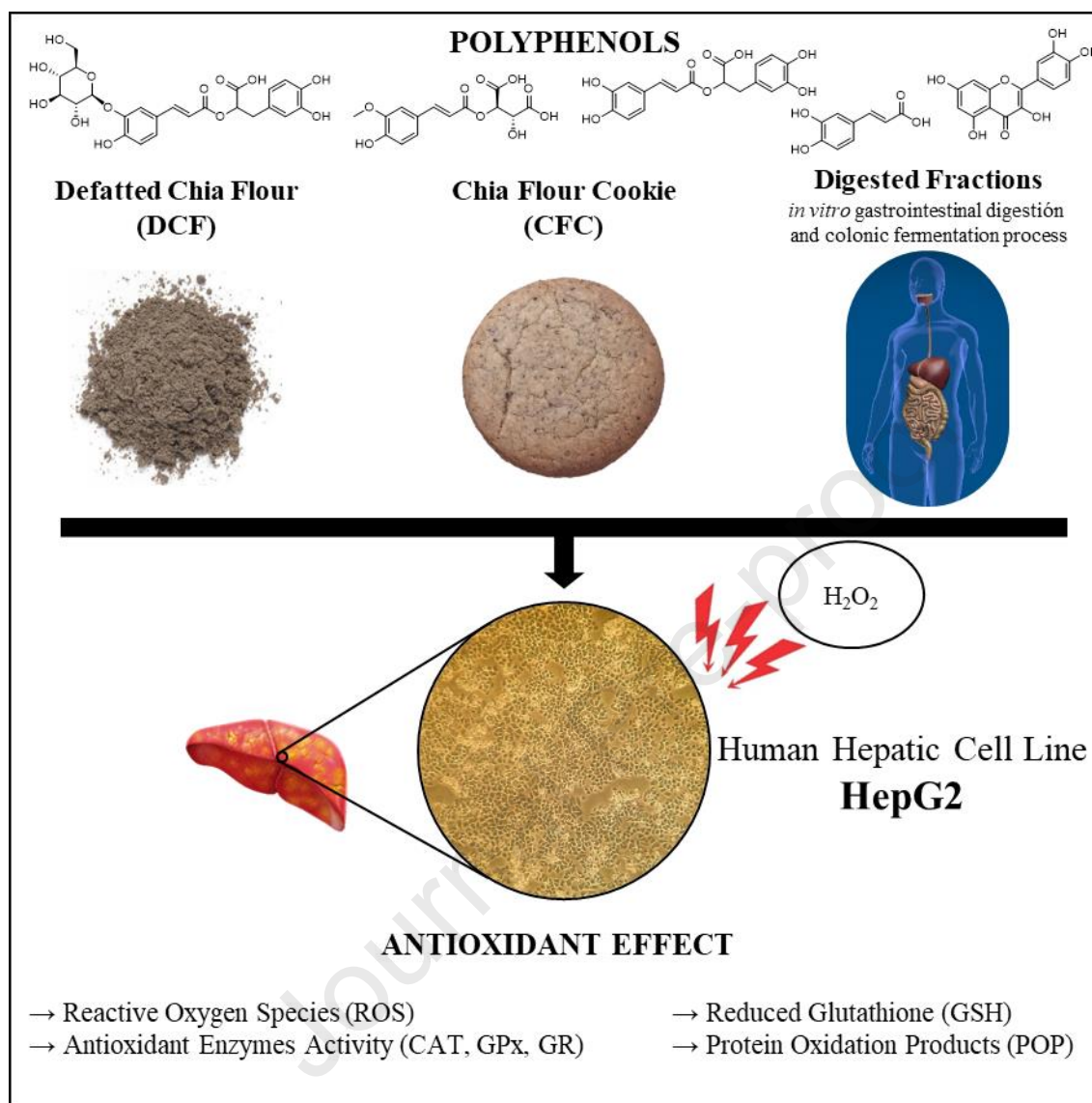
1. Introduction

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].

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

87 HepG2 cells are a validated model of the human liver that has been well characterized and widely

88 used in biochemical and nutritional studies [2].

89   A vegetal ingredient that has gained popularity in recent years is chia (*Salvia hispanica* L.)

90 seeds. These seeds are well-known for their high content of ω-3 fatty acids and antioxidant

91 compounds [8]. They are used in different food matrices, with bakery products being the most

92 common [9]. Several published articles have reported on how the processing and the gastrointestinal

93 digestion affect the antioxidant capacity of these seeds using *in vitro* chemical methods [10-14].

94 However, most of these *in vitro* colorimetric methods are based on the ability of the compounds to

95 scavenge free radicals. Different studies have demonstrated that it is highly unlikely that

96 polyphenols could act by this direct scavenging mechanism in biological environments, due to the

97 low physiological concentration and reaction rates compared to endogenous antioxidants [15-17].

98 Therefore, sometimes results obtained by *in vitro* methods are not extrapolated to the real biological

99 activity. Thus, more complex models like cell culture or *in vivo* experiments are necessary. Few

100 research studies have been conducted in this field using cell culture models. Ref. [18] and [19],

101 have studied the antioxidant activity of different plants of the *Salvia* genus (the same as chia) in cell

102 culture, showing promising results. Ref. [20] examined the effect of chia flour extract on

103 carbohydrate metabolism in HepG2 cells. Also, several articles have shown the antioxidant effect of

104 rosmarinic acid, one of the major compounds present in chia flour [21-23]. In previous studies by

105 our research group, we determined the polyphenol profile and content of defatted chia flour made

106 from an industrial by-product after oil extraction. Additionally, we evaluated how processing and

107 gastrointestinal digestion affect polyphenol profile and antioxidant activity (using *in vitro* chemical

108 methods) when this flour was added to sweet biscuits [12]. However, to our knowledge, there are no

109 previous studies focused on the antioxidant activity of chia flour using cellular models, nor in the

110 modification of this biological activity due to food-making processes and gastrointestinal digestion.

111 These type of studies should be considered as the initial steps for assessing the potential

112 bioactivity of a new extract/compound before they are evaluated in *in vivo* assays, wheter in

5

113   animals or humans [17, 24, 25]. Thus, in this work, we use cell culture experiments with HepG2 to

114   study how processing and gastrointestinal digestion affect the antioxidant effect of defatted chia

115   flour, evaluating the enzymatic and non-enzymatic endogenous antioxidant system, as well as

116   different oxidative damage markers.

117

118   **2. Material and Methods**

119   **2.1. Chemical and Reagent**

120   Ultra-pure water (<18 MΩ·cm<5 μgL−1 TOC) was obtained from a purification system

121   Arium 61316-RO plus Arium 611 UV (Sartorius, Germany). Methanol (HPLC grade) and formic

122   acid (puriss. p.a. for mass spectroscopy) were provided by J. T. Baker (State of Mexico, Mexico)

123   and Merck (California, USA), respectively. Commercial standards of polyphenolic compounds

124   were obtained from Extrasynthese (Genay, France), Sigma-Aldrich (Steinheim, Germany), and

125   Fluka (Dorset, U.K.). Filters (0.45 μm, HVLP04700) were obtained from Millipore (São Paulo,

126   Brazil). Porcine enzymes used in *in vitro* gastrointestinal digestion and all reagents used for redox

127   markers in cell culture were purchased from Sigma-Aldrich (Buenos Aires, Argentina). SnakeSkin

128   dialysis bags with a molecular weight cut-off of 10 kDa and a width of 22 mm, and Hypersep SPE

129   500 mg/2.8 mL C18 cartridges were obtained from ThermoFisher SCIENTIFIC. Anaerobic

130   atmosphere generation bags were purchased from Mitsubishi Gas Chemical (Tokyo, Japan).

131   Dulbecco's modified Eagle medium was obtained from Sigma-Aldrich and fetal bovine serum from

132   Natocor (Córdoba, Argentina). All other reagents were of analytical grade.

133

134   **2.2. Sample Preparation**

135   **2.2.1. Defatted Chia Flour and Chia Flour Cookie**

136   First, defatted chia flour (DCF) was obtained by milling the cold-pressed deoiled fraction of

137   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 watts; 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

7

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 aminoacids, and 2 mM L-glutamine. HepG2 cells grew in a cell incubator (thermo) 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

8

189   allowed to observe an effect of polyphenol extracts at concentrations similar to physiological ones

190   (data not shown) [26,27].

191

192   **2.5. Cell Viability**

193   Cytotoxicity of the stress model and the polyphenol treatments were measured using a flow

194   cytometer to determine the fluorescence intensity of the vital dye trypan blue [28].

195   Briefly, cells were seeded in a 96-well plate (2.5 x 10^4 cells/well) and incubated for 24 h.

196   Then, they were exposed to polyphenol samples and the stressor as described in **section 2.3.** After

197   this, cells were rinsed with PBS and suspended in 0.002% trypan blue in PBS. Fluorescence

198   intensity was measured by flow cytometry (Attune, ThermoFischer) with emission at 660 nm.

199   Results were analyzed using Flow Jo Software (Tree Star), and collected as the mean fluorescence

200   intensity of each sample.

201

202   **2.6. Reactive Oxygen Species Measurement**

203   Reactive oxygen species (ROS) levels were quantified using dichloro-dihydro-fluorescein

204   diacetate (DCFH-DA) [29]. Cells were seeded in a 96-well plate (2.5 x 10^4 cells/well) and

205   incubated for 24 h. Then, they were exposed to polyphenol samples and the stressor as described in

206   **section 2.3.** After this, cells were rinsed with PBS and incubated with DCFH-DA 10 μM in DMEM

207   without FBS for 15 min at 37 °C. Next, the supernatant was discarded, cells were rinsed and then

208   re-suspended in PBS. Fluorescence intensity was measured by flow cytometry with emission at 530

209   nm. Results were analyzed using Flow Jo Software (Tree Star), and collected as the mean

210   fluorescence intensity of each sample.

211

212   **2.7. Antioxidant Enzyme Activities**

213   To assess the activity of the endogenous enzymatic antioxidant system, the activities of

214   catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were measured using

215   different methods based on enzymatic kinetics and the disappearance of their substrates. UV-Vis

9

216 spectrophotometry was performed using a microplate reader, SynergyHT (Biotek). The activity of

217 each enzyme was expressed as nkat/mg protein, where 1 kat represents the conversion of 1 mol of

218 substrate per second. Protein content was measured using the Bradford method [30].

219   Cells were seeded in a 24-well plate (2.5×10^5 cells/well), incubated for 24 h, and then

220 exposed to polyphenol samples and the stressor as described in **section 2.3.** After this, cells were

221 rinsed with PBS and harvested in a phosphate buffer 0.1 M pH= 6.5 with 20% glycerol, EDTA

222 (ethylenedinitrilotetraacetic acid) 1 mM, and DTE (dithioerythritol) 1.4 mM. The cell homogenate

223 was centrifuged at 13000 g and 4 °C for 10 minutes, and the supernatant was used to measure the

224 activity of the three enzymes.

225   CAT was measured following the decrease in absorbance of H_2O_2 at 240 nm, and GPx and

226 GR were determined measuring the decrease in NADPH absorbance at 340 nm using the methods

227 described in Ref. [31] and [32].

228

229   **2.8. Reduced Glutathione**

230   The most important endogenous non-enzymatic antioxidant, reduced glutathione (GSH),

231 was measured following the method described in Ref. [33]. This method is based on the

232 derivatization of GSH with o-phthalaldehyde (OPA) to form an indole, which can be quantified by

233 fluorescence.

234   To carry out this, cells were seeded in 6-well plates (5×10^5 cells/well), incubated for 24 h,

235 and then exposed to polyphenol samples and the stressor as described in **section 2.3**. Then, cells

236 were rinsed with PBS and harvested in a phosphate buffer 0.1 M pH= 8 with EDTA 5 mM. The cell

237 homogenate was centrifuged at 13,000 g and 4°C for 10 minutes, and the supernatant was mixed

238 with OPA 0.25 mM (final concentration) in a 96-well plate. The mixture was incubated for 20

239 minutes in the dark at room temperature, and then its fluorescence was measured at an excitation

240 wavelength of 360 nm and an emission wavelength of 460 nm. GSH concentration was expressed as

241 µg GSH/mg protein and calculated using a calibration curve prepared with commercial GSH (0.02-

242 20 µg/mL) and treated in the same way as samples.

243

2.9. Protein Oxidation

245   Protein oxidation products (POP) were quantified using the protein carbonyl group method

246 [34]. Reactive oxygen species can react with the side chain of certain amino acids and generate

247 carbonyl groups, which can be derivatized with the reagent 2,4-dinitrophenylhydrazine (DNPH) to

248 form a colored hydrazone. Thus, POP can be quantified by measuring this product through

249 spectrophotometry after the described derivatization.

250   To perform this method, cells were seeded in 100 mm plates (1 x 10^6 cells/well), incubated

251 for 24 h, and then exposed to polyphenol samples and the stressor as described in **section 2.3**. Then,

252 cells were rinsed with PBS and harvested in a phosphate buffer 50 mM pH= 7.5. The cell

253 homogenate was centrifuged at 13,000 g at 4°C for 10 minutes, and the supernatant was divided

254 into two aliquots (sample and blank). DNPH 5 mM in HCl 2 M was added to the sample and

255 incubated in the dark for 10 minutes. After this, 5% TCA was added, and the mixture was

256 centrifuged for 3 minutes at 11,000 g. The pellet was rinsed twice with ethanol:ethyl acetate (1:1)

257 and then dissolved in guanidine 6 M in a phosphate buffer 20 mM pH= 2.3. The blank was treated

258 in the same manner as the samples, but no DNPH was added. The absorbance of samples and

259 blanks was measured at 366 nm, and the difference between them was used to calculate the POP

260 concentration (ε= 6.22 mM^{-1}cm^{-1}), expressed as µM/mg protein.

261

2.10. Statistical Analysis

263   For statistical analysis, the software INFOSTAT was used [35]. Three independent

264 experiments were carried out in triplicate for each parameter measured (n= 9). All results were

265 expressed as a percentage of the negative control samples to improve visualization. Normality and

266 homoscedasticity were evaluated graphically and numerically (using tools from the R library

267 Multisplus) through Shapiro-Wilk's tests. General and Linear Mixed Models were used to evaluate

268 significant differences between treatments. The treatment was included as a fixed effect, and the

269 independent experiment number was included as a random effect. In the case of significance (p<

270 0.05), a LSD Fisher comparison test was performed to reveal differences between the means.

271 Statistical analyses were performed separately for the direct and protective effects. Besides, paired

272 differences were analyzed between the negative and positive controls of stress. Data are expressed

273 as mean ± SE.

274

275 **3. Results**

276 **3.1. Polyphenol Profile and Content**

277 Twenty-five compounds were tentatively identified in DCF (**Table 1** of *Supplementary*

278 *Information*) belonging to the hydroxycinnamic acid, flavonoids, organic acid, and amino acid

279 families. The major compound was salviaflaside, followed by rosmarinic acid and fertaric acid

280 (**Table 2** of *Supplementary Information;* adapted from Ref. [12]).

281 After the cookie-making process, only 11 compounds were detected and quantified in CFC.

282 Some compounds increased their relative concentration, such as quinic acid, danshensu, tryptophan,

283 quercetin dihexoside, kaempferol dihexoside, and salviaflaside. On the other hand, caftaric acid,

284 fertaric acid, and rosmarinic acid decreased their relative concentrations. However, salviaflaside and

285 rosmarinic acid were still the major compounds.

286 After *in vitro* gastrointestinal digestion, only 8 compounds were found in the small intestine

287 dialyzable fraction of CFC. This sample showed increased relative concentrations of caftaric acid,

288 salviaflaside, and rosmarinic acid. Besides, caffeic acid, a compound from DCF but not detected in

289 CFC, was detected in this digested fraction. On the other hand, lower relative concentrations of

290 quinic acid and tryptophan were observed compared to undigested CFC. In addition, quercetin

291 dihexoside, kamepferol dihexoside, and quercetin hexoside were not detected in this fraction.

292 Finally, 9 compounds were found in the large intestine dialyzable fraction of CFC, with rosmarinic

293 acid exhibiting the highest relative concentration, even surpassing salviaflaside. Besides, salvianolic

294 acid E/B/L and quercetin hexoside were detected in this fraction but not in the previous one. Lower

295 relative concentrations of caftaric acid, tryptophan, fertaric acid, caffeic acid, and salviaflaside were

296 observed. No quinic acid was detected in this fraction [12].

297

298 **3.2. Antioxidant Activity of Defatted Chia Flour (DCF) and Chia Flour Cookie (CFC)**

299 **3.2.1. Cell Viability**

300 To evaluate the effect of the different samples on cell bioavailability, the trypan blue assay

301 was performed. Since the trypan blue assay is a dye-exclusion test, an increase in fluorescence was

302 associated with an increase in cell death. Results are summarized in **Fig. 1A**.

303 No differences were observed between the negative control and DCF, CFC, and CC (Control

304 Cookie). On the other hand, the oxidative stress stimulus caused an increase in cell death by

305 approximately 16% compared to the negative control, which could not be prevented by the different

306 polyphenol treatments.

307

308 **3.2.2. Reactive Oxygen Species**

309 Regarding the direct effect of polyphenols, no differences between the negative control and

310 DCF were observed. However, both CC and CFC decreased ROS levels, showing that the

311 supplemented cookie had a greater effect (**Fig. 1B**). Under oxidative stress conditions, different

312 results were observed. The positive control showed a significant increase with respect to the

313 negative control. Under this condition, DCF showed an antioxidant effect, decreasing ROS levels,

314 similar to CFC. CC also showed a protective effect, but it was lower than in the other two samples.

315

316 **3.2.3. Antioxidant Enzymes**

317 Concerning the direct effect of the polyphenol extracts, only CFC showed a significant

318 increase in the CAT and GR activities, while no modifications in the other enzymes were observed

13

319 (**Fig. 2A, 2B and 2C**). In addition, exposure to H_2O_2 did not show effects on the activity of any of

320 the three enzymes measured. However, under this stress condition, CC and CFC extracts raised GPx

321 activity.

322

323 ### 3.2.4. Glutathione Content

324 Regarding the direct effect of polyphenol extracts, only CFC showed a significant increase

325 in GSH levels compared to the negative control. Under stress conditions, the positive control

326 showed a decrease in the level of GSH compared to the negative control, which could be partially

327 prevented by DCF and CFC pretreatment (**Fig. 2D**).

328

329 ### 3.2.5. Protein Oxidation

330 Regarding protein damage, DFC did not present any effect compared to the negative control

331 under basal conditions, while both cookies showed a significant decrease in protein oxidation

332 products (**Fig. 3**). Exposure to H_2O_2 did not produce any changes in protein oxidation. However,

333 under these oxidative stress conditions, both DCF and CFC helped to decrease POP levels

334 compared to the positive control.

335

336 ### 3.3. Antioxidant Activity of Digested Chia Flour Cookies

337 ### 3.3.1. Cell Viability

338 The results of the effect of the gastrointestinal digestion samples on cell viability are

339 summarized in **Fig. 4A.** No modifications in cell viability were observed following treatment with

340 small and large intestine dialyzable fractions of CFC and CC, similar to the undigested samples.

341

342 ### 3.3.2. Reactive Oxygen Species

343 First, small intestine dialyzable fraction of CC showed antioxidant activity under basal

344 conditions, reducing ROS levels compared to the blank negative control. However, the same

345   fraction of CFC exhibited a greater effect (**Fig. 4B**). Regarding the protective effect of these small

346   intestine dialyzable fractions, CC did not present significant variations with respect to the positive

347   control of stress, but CFC continued to demonstrate antioxidant activity. Concerning large intestine

348   dialyzable fractions, on the other hand, both CC and CFC exhibited reduced ROS levels for direct

349   and protective effects with respect to the negative and positive controls, with no differences

350   between them (**Fig. 4B**).

351

352   **3.3.3. Antioxidant Enzymes**

353   In contrast to the results obtained for undigested CFC, the small intestine dialyzable fraction

354   decreased CAT activity under basal conditions (**Fig. 5A**). However, for the large intestine

355   dialyzable fraction, both CFC and CC increased CAT activity under oxidative stress conditions

356   (**Fig. 5B**). GPx and GR activity showed no differences among treatments (**Fig. 5C to 5F**).

357

358   **3.3.4. Reduced Glutathione**

359   Results obtained for GSH levels in digested samples are summarized in **Fig. 5G and 5H.**

360   Under basal conditions, only small intestine dialyzable fraction of CC increased GSH values

361   compared to the negative control; however, no differences were observed under oxidative stress

362   conditions for any treatment. Regarding large intestine dialyzable fractions, different results were

363   observed. Under basal conditions, only CFC fraction increased GSH levels with respect to control,

364   but under oxidative stress conditions, it was the CC fraction treatment that showed this effect.

365

366   **3.3.5. Protein Oxidation**

367   **Fig. 6A and 6B** summarizes the results obtained for POP in the case of fractions obtained

368   after the *in vitro* gastrointestinal digestion process. Small intestine dialyzable fractions of CC and

369   CFC exhibited no effects, either direct or protective. In the case of large intestine dialyzable

370   fractions, POP levels showed an increase under basal conditions due to CFC. However, this fraction

371 did not exhibit any effect under oxidative stress conditions. On the other hand, the CC fraction did

372 not reveal any impact under basal conditions, but it increased POP levels under oxidative stress

373 conditions.

374

375 **4. Discussion**

376 Polyphenols are bioactive compounds well-known for their antioxidant activity. Their mode

377 of action primarily depends on their chemical structure and the surrounding environment (normal or

378 basal conditions or in the presence of oxidative stress). Therefore, it is importante to assess the

379 effect of processing and gastrointestinal digestion on the polyphenol profile and, consequently, on

380 their potential bioactivity. In this regard, this study aimed to evaluate the biological activity of

381 polyphenolic extract from defatted chia flour, the polyphenolic extract from sweet cookies

382 supplemented with chia flour and the samples obtained after simulating the gastrointestinal

383 digestion of this cookie.

384

385 **4.1. Direct effect of samples in HepG2 cells in basal conditions**

386 **4.1.1. Impact of Deffated Chia Flour (DCF) processing during cookie making on the**

387 **antioxidant activity**

388 DCF did not show any direct effects on human liver HepG2 cells under basal conditions

389 (**Fig. 1**, **2,** and **3**). Similarly, Ref. [19] observed no effects on cell viability when HepG2 cells were

390 directly exposed to rosemary extracts (including *salvia* genus and chia) at concentrations of 5-20

391 μg/mL. Additionally, Ref. [21] did not observe any effects on ROS, GPx, and CAT when the same

392 cell line was treated with rosmarinic acid (one of the major compounds in DCF) at a concentration

393 of 1 μM.

394 However, when this flour was added into sweet cookies, at the same polyphenol

395 concentration, different results were obtained. Cell exposure to this supplemented cookie improved

396 the function of the endogenous antioxidant system, increasing CAT and GR activity as well as GSH

16

397 concentration compared to the control levels (**Fig. 2**). Consequently, there was a decrease in ROS

398 levels (**Fig. 1B**) and protein oxidative damage (POP) (**Fig. 3**). Therefore, this modification in

399 antioxidant effect could be attributed to the changes in the polyphenol profile caused by processing,

400 such as the decrease in relative concentrations of rosmarinic, caftaric, caffeic, or salvianolic acids

401 and the increase in salviaflaside or quinic acid. Furthermore, the food matrix (a cookie made of

402 wheat flour without DCF supplementation, named Control Cookie (CC)) also showed a decrease in

403 ROS and POP with respect to the negative control (**Fig. 1B** and **3**). However, the antioxidant effect

404 of CFC was greater, which could be attributed to the polyphenol content coming from the added

405 chia flour. To our knowledge, there are currently no reports on how processing affects direct

406 antioxidant activity in cell models under basal conditions, including the food matrix without

407 supplementation. However, similar to this study, Ref. [18] found higher values of GSH when

408 HepG2 cells were treated with a 90% methanol extract of *Salvia officinalis* with respect to cells

409 under basal conditions. However, no effects were observed when cells were treated with an aqueous

410 extract of the same plant, showing that the differences in the polyphenol profile of the extracts

411 could have different biological effects. In addition, Ref. [36] observed improvements in cell

412 viability only when Caco-2 cells were treated with bread supplemented with defatted olive pomace

413 but not when exposed to non-supplemented bread. In summary, in this study, changes in the

414 polyphenol profile and relative concentrations of each compound, caused by physical and chemical

415 processes during the cookies manufacturing, improved the antioxidant activity of DCF under basal

416 conditions.

417

418 **4.1.2. Impact of *in vitro* gastrointestinal digestion of cookies on the antioxidant activity**

419 Regarding changes in the antioxidant effect caused by *in vitro* gastrointestinal digestion, the

420 small intestine dialyzable fraction of CFC decreased ROS levels compared to the blank negative

421 control, even to lower levels than the same fraction of CC (**Fig. 4B**). However, it did not show

422 significant differences in GSH and POP (**Fig. 5G** and **6A**). In the case of the large intestine

17

423   dialyzable fraction, CFC also showed lower ROS levels than the blank negative control but without

424   significant differences with respect to the same fraction of CC (**Fig. 4B**), suggesting a decrease in

425   the antioxidant activity of this fraction compared to the previous one. Furthermore, even though

426   large intestine dialyzable fraction of CFC showed a significant increase in a non-enzymatic

427   antioxidant like GSH (**Fig. 5H**), this fraction also increased POP (**Fig. 6B**). In some cases,

428   polyphenol treatments could increase protein oxidation under basal conditions. Ref. [37] found that

429   tannic, ellagic, and gallic acids slightly increased POP levels in CHO cells. However, they proved

430   that these compounds act as antioxidants and decrease DNA damage under the same conditions. In

431   short, changes occurring during *in vitro* gastrointestinal digestion, such as an increase in rosmarinic,

432   caftaric, and salvianolic acids and a decrease in salviaflaside and quinic acid relative concentrations,

433   mainly decreased the antioxidant effects of CFC under basal conditions.

434

435   **4.2. Protective effect of samples in HepG2 cells in oxidative stress conditions**

436   **4.2.1. Impact of Deffated Chia Flour (DCF) processing during cookie making on the**

437   **antioxidant activity**

438   On the other hand, the effects of pretreatment with polyphenols could have different effects

439   when cells are exposed to oxidative stress conditions. In this study, exposure to H_2O_2 10 mM for 1

440   h increased cell death and ROS by 16% and 88%, respectively (**Fig. 1B**). Besides, GSH levels were

441   significantly lower compared to the negative control (**Fig. 2D**). However, no effect of the stressful

442   stimulus was observed in the activity of antioxidant enzymes or POP levels (**Fig. 2A-C** and **3**). Ref.

443   [38] also observed a decrease in the viability of SK-N-MC cells when treated with 300 µM of H_2O_2

444   for 24 h. Additionally, Ref. [39] showed that H_2O_2 exposure did not modify GR and superoxide

445   dismutase (SOD) activities, even when ROS levels were increased. Finally, even though we

446   expected an increase in POP levels caused by H_2O_2 exposure, it has been shown that carbonyl

447   formation due to protein oxidation depends on the time of exposure to H_2O_2, reaching a maximum

448   at 0.5 h of exposure and decreasing thereafter until no differences are observed with basal cells at 2

18

449 it [40]. This could explain why no differences were observed between basal and oxidative stress

450 conditions for control samples.

451   Unlike the effects of DFC observed under basal conditions, this extract showed important

452 protective antioxidant activity under oxidative stress cell conditions, as it increased GSH (**Fig. 2D**)

453 and decreased ROS and POP levels (**Fig 1B** and **3**). Furthermore, the changes in the polyphenol

454 profile mentioned above during processing almost did not modify the antioxidant effect observed

455 under stressed conditions. CFC did not exhibit significant differences with respect to DCF in ROS,

456 GSH, and POP levels but showed greater effects than CC (**Fig. 1B**, **2D,** and **3**), again suggesting

457 that the antioxidant activity could be attributed to the polyphenol content of the supplemented

458 cookie. The only difference observed between DCF and CFC was the increase observed in GPx (the

459 only antioxidant enzyme that was not modified under basal conditions). However, CC also

460 increased GPx, so this effect might be caused by the food matrix and not the polyphenols. In line

461 with this, Ref. [41] also observed equivalent antioxidant activities in cell viability for grape and

462 wine at the same concentration in Caco-2 cells exposed to H_2O_2, even when the wine polyphenol

463 profile had been modified during processing. Besides, Ref. [42] also described lower POP levels in

464 HepG2 cells treated with green coffee bean extract or its purified major compounds, with analogous

465 results. Additionally, Ref. [43] found lower levels of POP in 3T3 cells treated with quercetin,

466 catechin, or a mixture of both, with no differences among treatments.

467

468   **4.2.2. Impact of *in vitro* gastrointestinal digestion of cookies on the antioxidant activity**

469   Regarding digested samples, small intestine dialyzable fraction of CFC demonstrated

470 antioxidant activity by decreasing ROS levels, contrary to the CC fraction, which did not show

471 significant differences compared to the positive control (**Fig. 4B**). However, none of the small

472 intestine dialyzable fractions of both cookie formulations exhibited effects on any of the other

473 parameters measured. Similarly, large intestine dialyzable CFC also decreased ROS compared to

474 the blank positive control, but with no differences with respect to the CC fraction (**Fig. 4B**).

19

475   Likewise, the large intestine dialyzable fraction of both cookies equally increased CAT (**Fig. 5B**).

476   Ref. [44] also found a protective effect on the generation of ROS in HepG2 cells treated with

477   digested cookies prepared with wholegrain flour from different wheat species. Nevertheless, in that

478   study, authors did not carry out colonic fermentation or include a cookie formulation made with

479   common wheat flour. Besides, Ref. [45] described lower ROS and POP levels, and a partial

480   recovery of GSH compared to the positive control in cells incubated with Yerba Mate extracts.

481   However, these outcomes were not observed in the case of treatments with the main circulating

482   metabolites after digestion. To sum up, the protective antioxidant activity of CFC strongly

483   decreased after *in vitro* gastrointestinal digestion, but still demonstrated effects in reducing ROS

484   levels.

485

486   **4. Conclusions**

487   In conclusion, this study sheds light on the intricate interplay between polyphenols, the food

488   matrix, and their effects on cellular antioxidant responses. Under basal conditions, Defatted Chia

489   Flour (DCF), a rich polyphenol industrial by-product, did not exhibit direct effects on human liver

490   HepG2 cells. However, when incorporated into Chia Flour Cookies (CFC), the cell exposure to this

491   supplemented cookie showed significant improvements in the endogenous antioxidant system,

492   resulting in decreased ROS levels and protein oxidative damage. These modifications in antioxidant

493   effects could be attributed to changes in the polyphenol profile induced by processing, like a

494   decrease in relative concentration of rosmarinic and caftaric acids and an increase in salviaflaside.

495   Furthermore, the study examined the impact of in vitro gastrointestinal digestion on the

496   antioxidant activity of CFC. The results indicated that the small intestine dialyzable fraction of CFC

497   reduced ROS levels, but the large intestine dialyzable fraction showed a detriment in this

498   antioxidant effect. These changes were associated with polyphenols potentially available after

499   complete digestion that, interestingly, some of them were opposite to those observed during the

500   production of CFC, which contributed to the improved antioxidant activity of DCF.

20

501   Additionally, the study explored the effects of polyphenol pretreatment under oxidative

502   stress conditions. While DCF did not exhibit significant effects under basal conditions, it displayed

503   strong protective antioxidant activity when cells were exposed to oxidative stress. This protective

504   effect was largely retained in the CFC, emphasizing the potential of polyphenols to mitigate

505   oxidative stress-induced damage. On the other hand, digested samples lost the ability to improve the

506   functioning of the measured endogenous antioxidant system markers and protect against protein

507   damage, however were able to decrease ROS levels.

508   In summary, this research underscores the complex and context-dependent nature of

509   polyphenol-mediated antioxidant effects, which are influenced by factors such as the food matrix,

510   processing, and the cellular environment. These findings contribute to a better understanding of

511   how polyphenols can be harnessed for their potential health benefits and emphasize the need for

512   further investigation into their multifaceted actions in different physiological and pathological

513   contexts.

514

522

523   **Credit Authorship Contribution Statement**

524   **Agustín Lucini Mas:** investigation, formal analysis, writing original draft. **María Eugenia**

525   **Sabatino:** investigation, methodology, formal analysis. **Martin Gustavo Theumer:** Methodology,

526   review & editing. **Daniel Alberto Wunderlin:** Conceptualization, resources, project

21

527 administration. **Maria Veronica Baroni:** Conceptualization, resources, visualization, supervision,

528 project administration

529

530 **Declaration of Competing Interest**

531 The authors declare that they have not known competing financial interests or personal relationships

532 that could have influenced the work reported in this paper.

533

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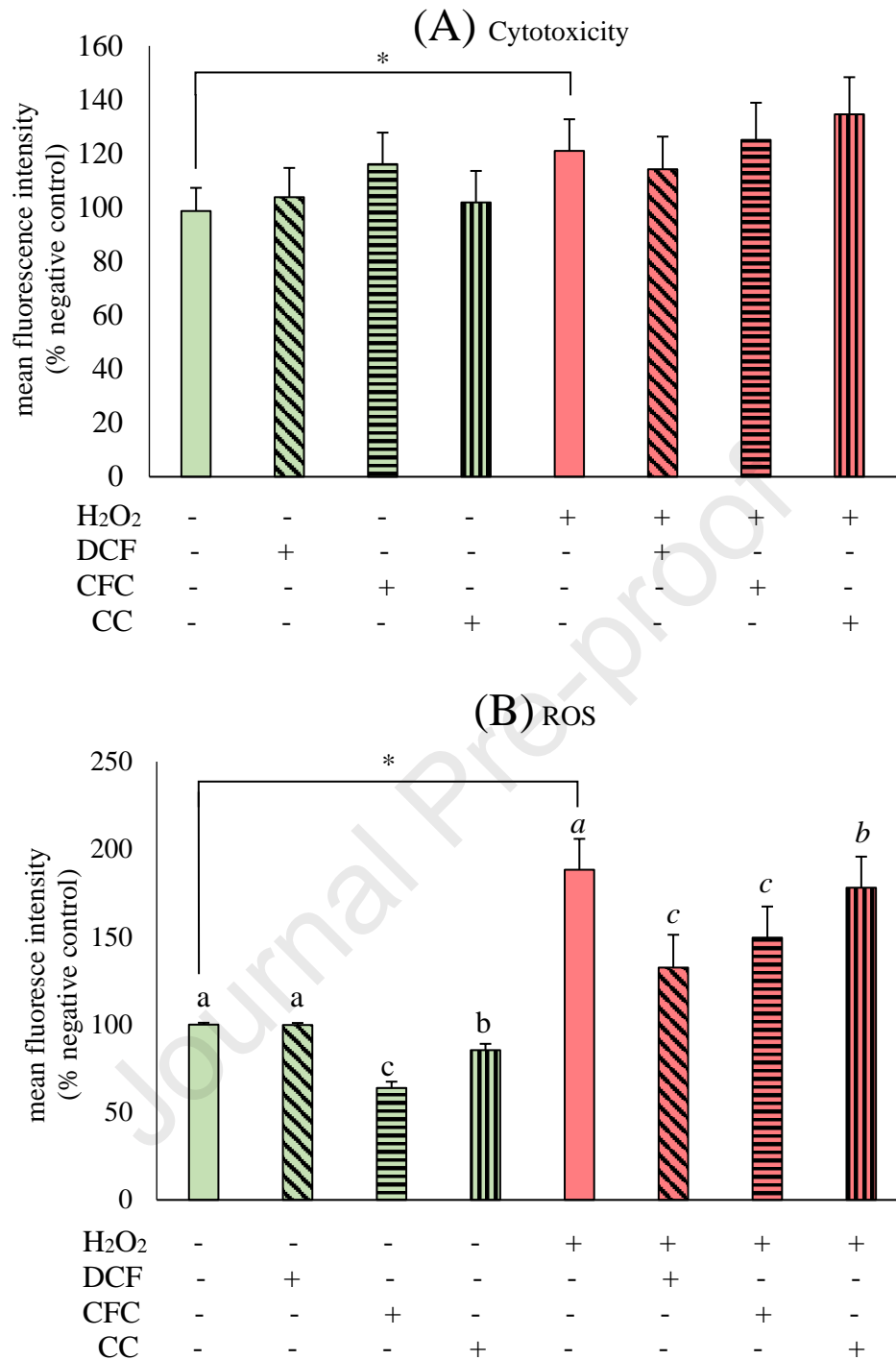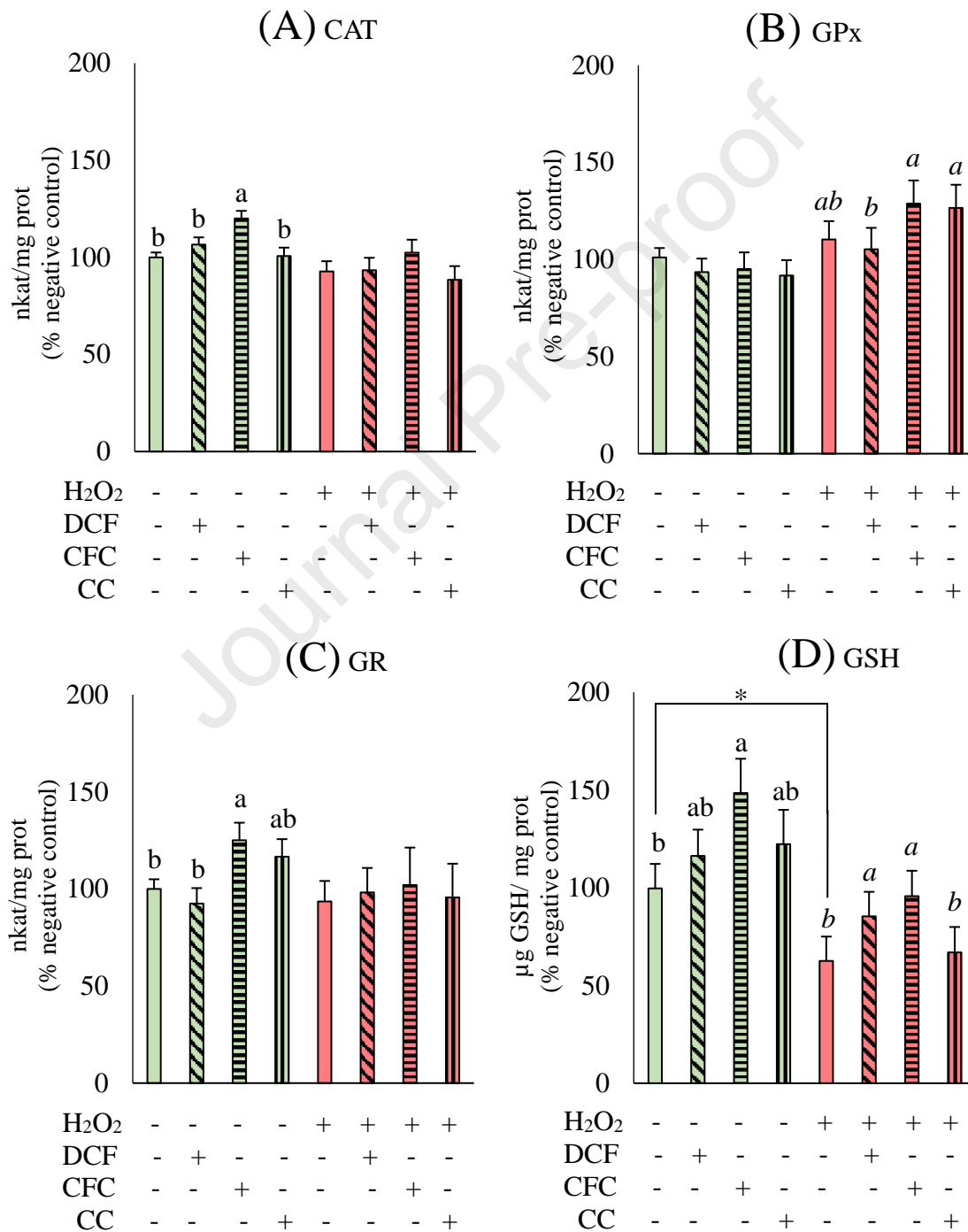
Fig. 1: Effect of Defatted Chia Flour (DCF), Chia Flour Cookie (CFC), and Control Cookie (CC) exposure on **(A):** cytotoxicity and **(B):** reactive oxygen species (ROS) levels in HepG2 cells under basal and oxidative stress conditions. DCF and CFC exposures were performed at 1 µg/mL, and CC with the 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).
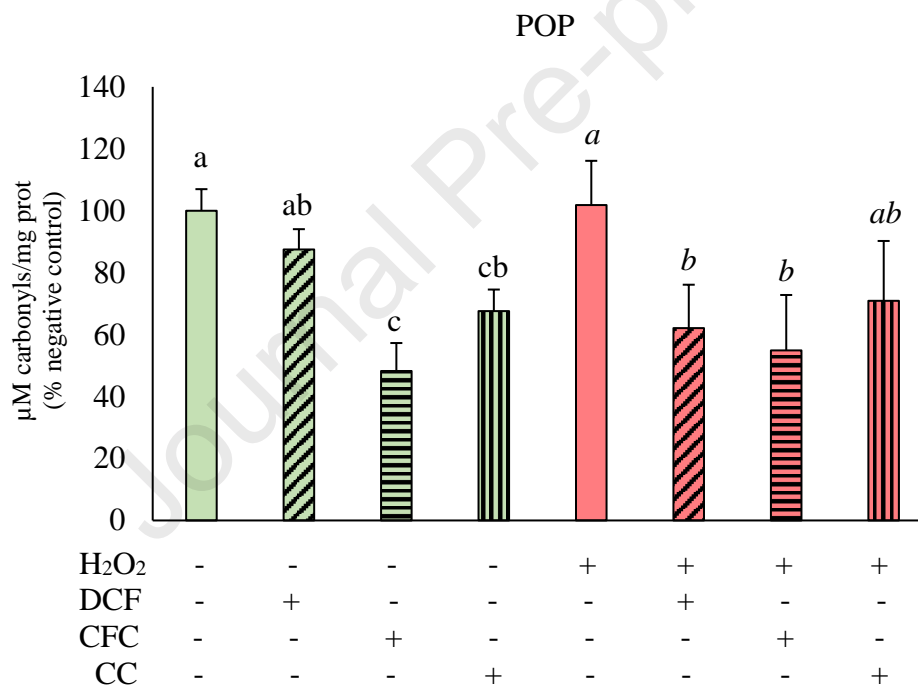
Fig. 2: Effect of Defatted Chia Flour (DCF), Chia Flour Cookie (CFC), and Control Cookie (CC) exposure on antioxidant enzymes **(A):** catalase (CAT), **(B):** glutathione peroxidase (GPx), **(C):** glutathione reductase (GR), and **(D):** reduced glutathione (GSH) in HepG2 cells under basal and oxidative 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).

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).
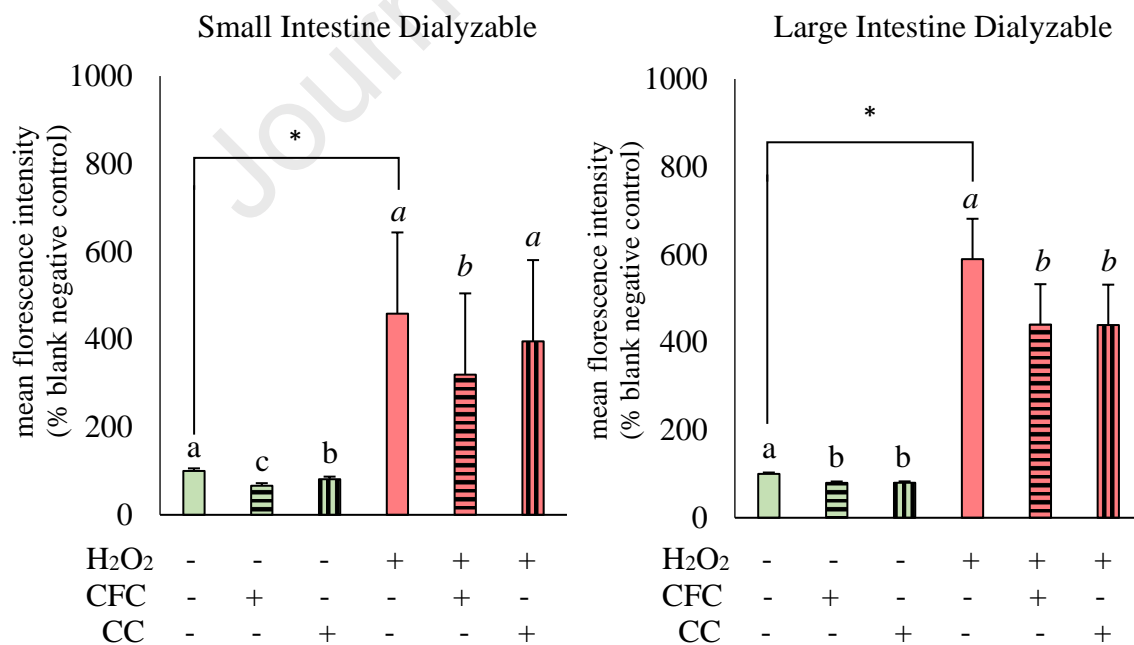
(A) Cytotoxicity
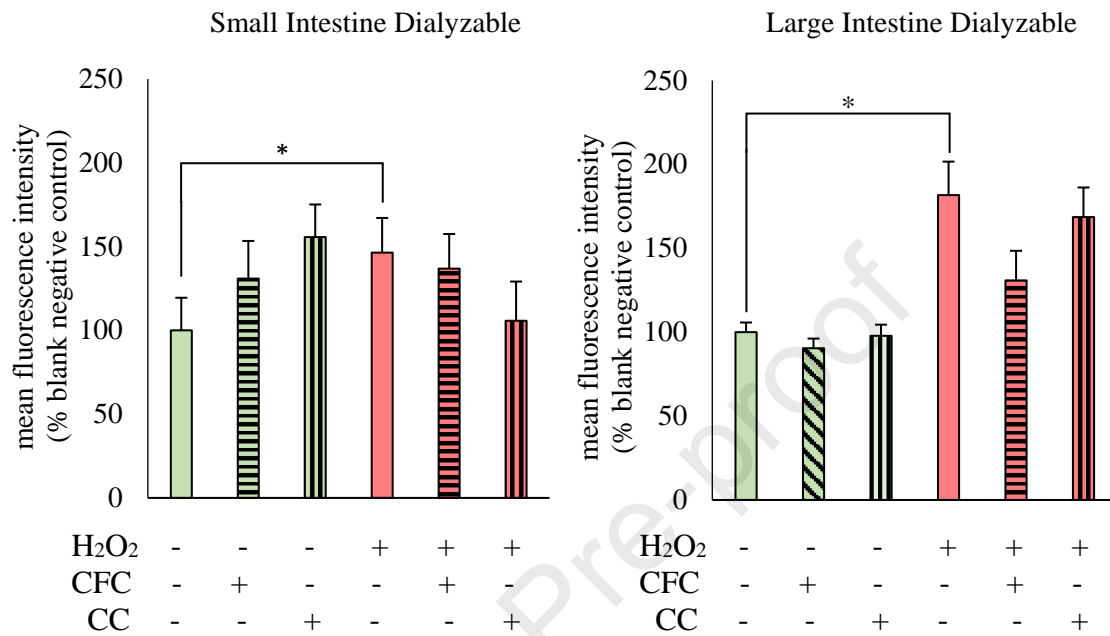
(B) ROS

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).
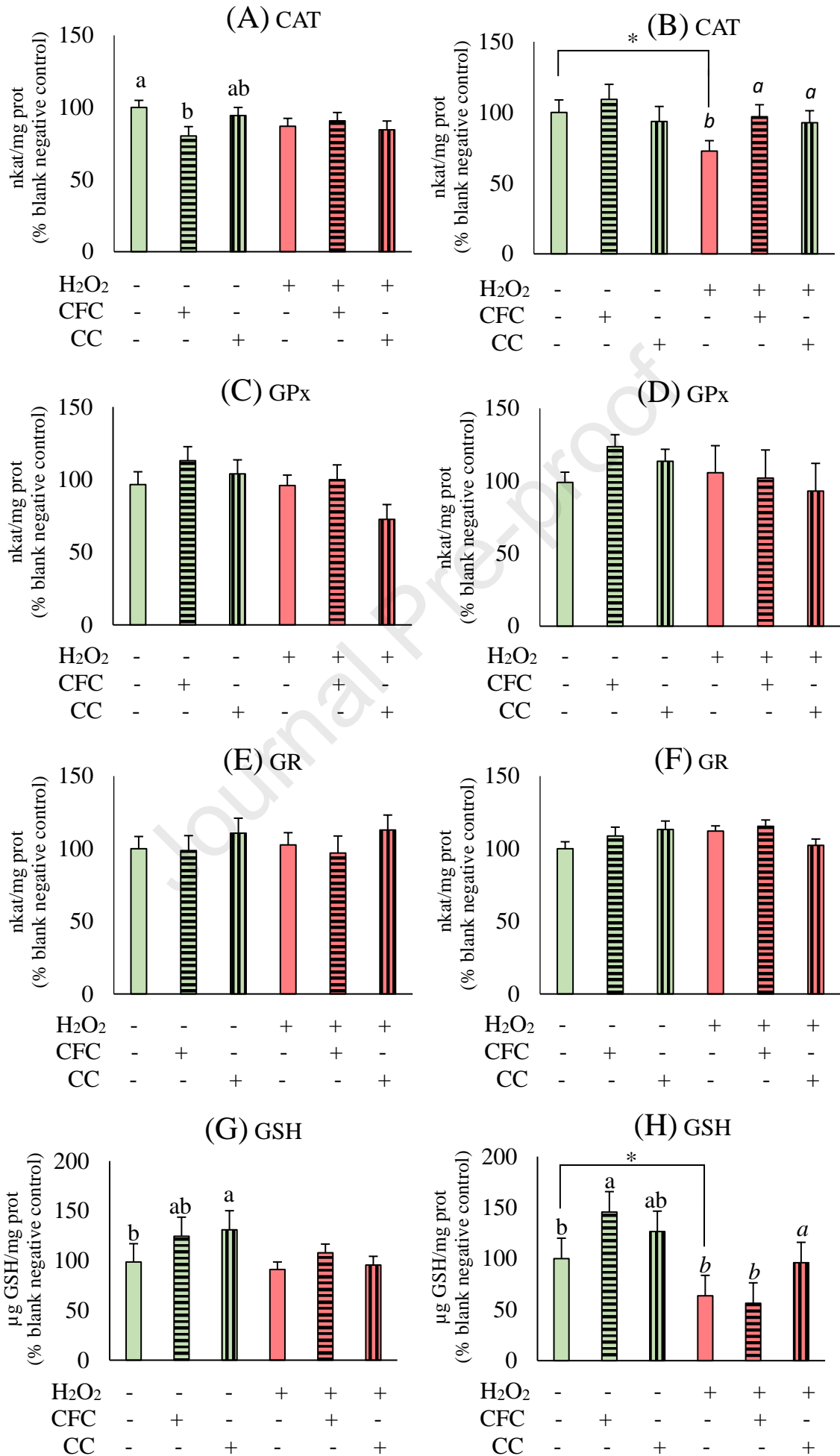
(A) CAT

(B) CAT

(C) GPx

(D) GPx

(E) GR

(F) GR

(G) GSH

(H) GSH

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).
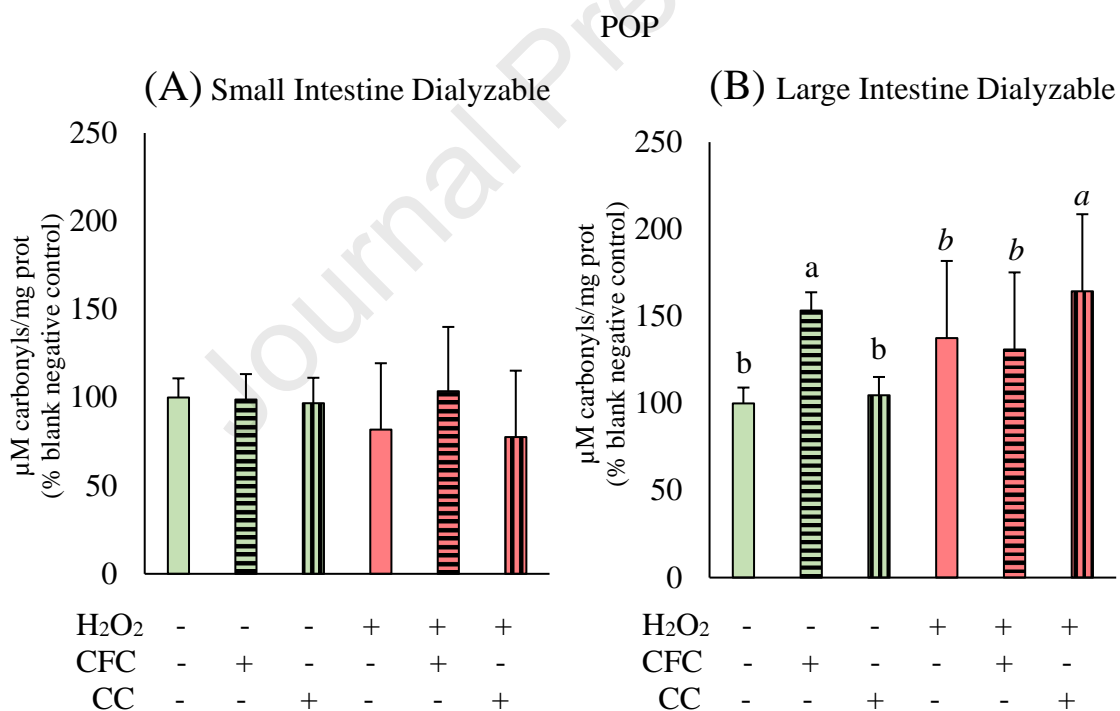
POP

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).

Declaration of interests

Χ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: