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Olive oil industry by-products. Effects of a polyphenol-rich extract on the metabolome and response to inflammation in cultured intestinal cell

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(Article begins on next page)

1 **OLIVE OIL INDUSTRY BY-PRODUCTS. EFFECTS OF A POLYPHENOL-RICH**  
2 **EXTRACT ON THE METABOLOME AND RESPONSE TO INFLAMMATION IN**  
3 **CULTURED INTESTINAL CELL**  
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39 *Abbreviations:* ABTS, 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonate; AFOA, aldehydic form of  
40  
41 oleuropein aglycone; d6-DSS, 2,2-dimethyl-2-silapentane-d6-5-sulfonic; DAFOA, dialdehydic  
42  
43 form of oleuropein aglycone; DMEM, Dulbecco's modified Eagle's medium; DOP, defatted olive  
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45 pomace; DPBS, Dulbecco's phosphate-buffered saline; GAE, gallic acid equivalent; GLUT2,  
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47 glucose transporter 2; IFN $\gamma$ , interferon gamma; IL-10, interleukin 10; IL-12p70, interleukin 12p70;  
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49 IL-1 $\alpha$ , interleukin 1 alpha; IL-1 $\beta$ , interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6,  
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51 interleukin 6; IL-8, interleukin 8; LDH, lactate dehydrogenase; MTT, methylthiazolyldiphenyl-  
52  
53 tetrazolium bromide; MUFA, mono unsaturated fatty acids; NADH, nicotinamide adenine  
54  
55 dinucleotide; PC, principal component; PCA, principal component analysis; PRE, polyphenol-rich  
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57 extract; PUFA, poly unsaturated fatty acids; SGLT1, sodium-glucose transporter 1; TAC, total  
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59 antioxidant capacity; TB, Trypan Blue; TE, trolox equivalent; TNF $\alpha$ , tumor necrosis factor alpha;  
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61 TPC, total phenol content  
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22 **Abstract**

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Over the past years, researchers and food manufacturers have become increasingly interested in olive polyphenols due to the recognition of their biological properties and probable role in the prevention of various diseases such as inflammatory bowel disease. Olive pomace, one of the main by-products of olive oil production, is a potential low-cost, phenol-rich ingredient for the formulation of functional food. In this study, the aqueous extract of olive pomace was characterized and used to supplement human intestinal cell in culture (Caco-2). The effect on the cell metabolome and the anti-inflammatory potential were then evaluated. Modification in the metabolome induced by supplementation clearly evidenced a metabolic shift toward a “glucose saving/accumulation” strategy that could have a role in maintaining anorexigenic hormone secretion and could explain the reported appetite-suppressing effect of the administration of polyphenol-rich food. In both basal and inflamed condition, supplementation significantly reduced the secretion of the main pro-inflammatory cytokine, IL-8. Thus, our data confirm the therapeutic potential of polyphenols, and specifically of olive pomace in intestinal bowel diseases. Although intervention studies are needed to confirm the clinical significance of our findings, the herein reported results pave the road for exploitation of olive pomace in the formulation of new, value-added foods. In addition, the application of a foodomics approach allowed observing a not hypothesized modulation of glucose metabolism.

35 **Keywords**

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Olive oil by-products, polyphenols, inflammation, glucose metabolism, NMR based metabolomics, foodomics

## 1. INTRODUCTION

Olive oil is one of the most representative food in the traditional Mediterranean diet and a key element associated with its health-protecting effects. Epidemiological studies indicate an inverse association between olive oil intake and the occurrence of different types of cancer, cardiovascular risk factors, age-related processes, chronic inflammatory disorders, and inflammatory bowel diseases (Buckland & Gonzalez, 2015; Cougnard-Grégoire et al., 2016; Guasch-Ferré et al., 2014; Psaltopoulou, Kostis, Haidopoulos, Dimopoulos, & Panagiotakos, 2011; Schwingshackl, Christoph, & Hoffmann, 2015). These health benefits are mainly attributed to the phenolic class, where oleuropein, tyrosol and their derivatives are the major constituents and make up around 90% of the total phenolic content of a virgin olive oil (Talhaoui et al., 2016). Olive oil production, an agro-industrial activity of vital economic significance for many Mediterranean countries, is associated with the generation up to 30 million tons of waste by-products per year (Chandra & Sathivelu, 2009), olive pomace being one of the main by-products (Mirabella, Castellani, & Sala, 2014).

Although olive mill wastes represent an important environmental problem in the Mediterranean area since their high organic acid concentration turns them into phytotoxic materials, they still contain valuable nutritional resources (Roig, Cayuela, & Sánchez-Monedero, 2006). After milling, only 2% of the phenolic compounds in olives is transferred to the oil and as much as 98% is retained in the cake. In particular, olive pomace has a high concentration of minerals, sugars and polyphenols (Dermeche, Nadour, Larroche, Moulouati, & Michaud, 2013), and its possible valorization as functional ingredient deserves attention and represents a challenge for the food industry (Aliakbarian, Casazza, & Perego, 2011).

In this work, the polyphenols retained in the defatted olive pomace (DOP) were water extracted and quantitative and qualitative characterized, and the polyphenol-rich extract (PRE) was then supplemented to a human intestinal cell line (Caco-2 cells). Caco-2 is a colonic tumor cell line which, when cultured, spontaneously exhibits enterocyte-like characteristics. Given the difficulties in maintaining long-lasting cultures of enterocytes, this cell line is a suitable *in vitro* model to carry out experiments trying to delineate the effect of exogenous compounds (Rodríguez-Juan et al., 2001). The effect of PRE supplementation on the cell metabolome was investigated. The metabolomic approach, based on high-resolution NMR spectroscopy, allows determining the cell perturbation induced on cells by different concentration of polyphenols from a holistic point of view (Nicholson, Lindon, & Holmes, 1999; Picone et al., 2013). Moreover, the anti-inflammatory effect of PRE was evaluated by measuring the secretion of different cytokines in basal condition and in cells exposed to a pro-inflammatory stimulus.

80 *In vitro* experiments represent the first step to evaluate the health effect of new functional  
81 ingredients. Although clinical studies are needed before drawing conclusions, results herein  
82 reported clearly evidenced the anti-inflammatory effect of PRE and pave the road to the exploitation  
83 of olive pomace waste as a functional ingredient.

## 85 2. MATERIAL & METHODS

### 87 2.1. Chemicals

88  
89 Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and Dulbecco's  
90 phosphate-buffered saline (DPBS) were purchased from Lonza (Basel, Switzerland). Trypan Blue  
91 (TB) was from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals and solvents were  
92 of the highest analytical grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

### 94 2.2. Polyphenol water extraction from defatted olive pomace (DOP) powder

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96 DOP powder was gently provided by ISANATUR SPAIN S.L. (Puente La Reina, Spain).  
97 Specifically, the by-product of olive oil extraction was dried and defatted based on the patent  
98 WO2013030426 with further developments to increase the process sustainability. DOP nutritional  
99 composition was analysed by producer (Table 1). Two g of DOP powder were suspended in 100 ml  
100 of distilled water, vigorously vortexed with a magnetic stirrer for 10 minutes at room temperature  
101 and sonicated at the maximum intensity for 44 minutes at 40 °C (Annegowda, Anwar, Mordi,  
102 Ramanathan, & Mansor, 2010). The solution was then centrifuged at 4,500g for 5 minutes and the  
103 supernatant sterilely filtered on 0.22 µm acetate cellulose filters. Two independent extractions were  
104 pooled to avoid technical inter-variability and kept at -20 °C until further analysis.

### 106 2.3. Total antioxidant capacity (TAC) and total phenol content (TPC)

107  
108 TAC was determined in PRE by the 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS)  
109 method as described by Di Nunzio, Toselli, Verardo, Caboni, and Bordoni (2013) and expressed as  
110 mM of trolox equivalent (TE). TPC content in PRE was determined using Folin-Ciocalteu's method  
111 adapted to a 96-well plate assay (Di Nunzio et al., 2013), and expressed as mg gallic acid equivalent  
112 (GAE)/mL.

114 2.4. *Polyphenol composition by RP-HPLC–DAD–MS*

115  
116 The polyphenol composition of the extract was determined using a liquid chromatography apparatus  
117 HP 1100 series from Agilent Technologies coupled to diode array and mass spectrometer detectors,  
118 as previously described by Gómez-Caravaca et al. (2013), with some modification. The sample was  
119 injected in a reverse phase column Poroshell 120 SB-C18 (3 x 100 mm, 2.7 µm) from Phenomenex,  
120 and the chromatogram was registered at 280 nm. The extracted compounds were identified by  
121 analysing UV and MS spectra and quantified by DAD detection. The quantification was performed  
122 by comparison to calibration curves of tyrosol, caffeic acid, oleuropein and rutin at 280 nm for the  
123 different class of phenols.

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125 2.5. *Caco-2 cell culture and supplementation*

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127 Caco-2 cells were seeded in 24-well and 96-well plates at  $1 \times 10^5$  cells/well concentration  
128 (cytotoxicity and inflammation assays) or in 100 mm Petri dishes at  $2.7 \times 10^6$  cells/well (NMR  
129 assay) and grown for 21 days. After complete differentiation, assessed by measuring the trans  
130 epithelial electric resistance of the cell monolayer using a commercial apparatus (Millicell ERS;  
131 Millipore Co., Bedford, MA), cells were supplemented for 24 h with 1 ml of serum-free DMEM  
132 containing different amount of PRE-polyphenols (10, 20, 50, 100, 200 and 500 µg/ml). To avoid  
133 interference due to the vehicle, some cells were supplemented with the same amount of water  
134 (control cells). To induce inflammation, in some experiments cells were exposed to interleukin 1  
135 beta (IL-1β) at 10 ng/mL for 24 h. The inflammatory stimulus was concomitant to PRE  
136 supplementation.

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138 2.6. *Cytotoxicity*

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140 PRE cytotoxicity was evaluated in Caco-2 cells by different assays:

- 141 - The number of total and viable cells was determined by staining cell populations with TB as  
142 reported by Di Nunzio et al. (2013) with slight modifications. Cell viability was expressed as a  
143 percentage of the total cell number.
- 144 - Cell viability was assessed using AlamarBlue® Cell Viability Reagent (Life Technologies Ltd.;  
145 Paisley, UK) and the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay with slight  
146 modifications (Di Nunzio et al., 2013; Valli, Taccari, Di Nunzio, Danesi, & Bordoni, 2018).  
147 Cell viability was expressed as a percentage of the viability in control cells, assigned as 100%.

148 - Lactate dehydrogenase (LDH) release was assessed determining its activity in the medium. The  
149 rate of nicotinamide adenine dinucleotide (NADH) oxidation was followed  
150 spectrophotometrically at 340 nm for 1 min (Di Nunzio, Valli, & Bordoni, 2016), and LDH  
151 activity was calculated using the extinction coefficient of NADH, normalized for cell cell count  
152 and expressed as mU/ml/10<sup>6</sup> cells.

## 154 2.7. HR <sup>1</sup>H-NMR

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156 Cells were scraped off and the pellet washed with ice-cold DPBS. Cells were then lysed by  
157 sonication and centrifuged at 21,000g for 10 min at 4 °C. Five hundred µl of supernatant were  
158 centrifuged at 14,000rpm for 5 min and then added to 10 µl of a D2O solution of 100 mM 2,2-  
159 dimethyl-2-silapentane-d6-5-sulfonic (d6-DSS) with a final concentration in the NMR tube of  
160 9.0909 mM. <sup>1</sup>H-NMR spectra were recorded at 298 K on a Bruker US+ Avance III spectrometer  
161 operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation  
162 (Bruker BioSpin, Karlsruhe, Germany). The HOD residual signal was suppressed by applying the  
163 CPMGPR1D sequence with presaturation. Each spectrum was acquired using 32 K data points over  
164 a 7183.908 Hz spectral width (12 ppm) and adding 256 transients. A recycle delay (D1) of 5 s and a  
165 90° pulse (P1) of 11.190 µs were set up. Acquisition time (2.28 s) and recycle delay were adjusted  
166 to be 5 times longer than the longitudinal relaxation time (T1) of the protons under investigation,  
167 which has been considered to be not longer than 1.4. The data were Fourier transformed and phase  
168 and baseline corrections were automatically performed using TopSpin version 3.0 (Bruker BioSpin,  
169 Karlsruhe, Germany). Signals were identified by comparing their chemical shift and multiplicity  
170 with Chenomx Profiler software data bank (ver. 8.1, Edmonton, Canada) and data in the literature  
171 (Ghini et al., 2017; Picone et al., 2013).

## 173 2.8. Cytokines secretion

174  
175 After 24 h supplementation, the level of the pro- and anti-inflammatory cytokines interferon gamma  
176 (IFNγ), interleukin 1 alpha (IL-1α), IL-1β, interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6  
177 (IL-6), interleukin (IL-8), interleukin 10 (IL-10), interleukin 12 p70 (IL-12p70), and tumor necrosis  
178 factor alpha (TNFα) was estimated in the cell media by the multiplex sandwich ELISA Ciraplex  
179 (Aushon; Billerica, USA) following the manufacturer's instructions. 96-well plates pre-spotted with  
180 protein-specific antibodies were used and luminescent signals were detected by Cirascan™ Imaging  
181 System. Results were expressed as pg/mL medium. The level of the pro-inflammatory IL-8 was

182 estimated in cell media also by AlphaLISA kits (IL-8 Immunoassay Research Kits; Perkin Elmer  
183 Inc., Waltham, MA, USA) following the manufacturer's instructions (Antognoni et al., 2017; Valli  
184 et al., 2016). 96-microwell plates (96 1/2 AreaPlate from Perkin Elmer) were used and read using  
185 an EnSpire™ plate reader (Perkin Elmer). Results were expressed as pg/mL medium.

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## 2.9. Statistical analysis

Compositional, cytotoxicity and inflammation data are mean  $\pm$  standard deviation (SD). Statistical differences were determined by the one-way analysis of variance (ANOVA) followed by Dunnett's test considering  $p < 0.05$  as significant. NMR spectra processing and statistical analyses were performed using R computational language (ver. 3.4.3) and MATLAB (ver R2008b, MathWorks Inc.). Each  $^1\text{H-NMR}$  spectrum was processed by means of scripts developed in-house. ANOVA followed by Tukey HSD multiple comparison was performed on the integral of each bin. The outcomes of ANOVA ( $p < 0.05$  as significant) pointed out metabolites affected in a positive or negative way (Picone et al., 2018).

## 3. RESULTS

### 3.1. PRE characterization

TAC and TPC of PRE are reported in Table 2. PRE phenolic profile and quantification are given in Fig. 1. As shown, 1- $\beta$ -D-glucopyranosyl acyclodihydroelenolic acid/Hydroxytyrosol and  $\beta$ -hydroxyverbascoside were the main phenols in PRE.

### 3.2. Cell count and viability

In Fig. 2, cell count and cell viability evaluated by TB (A), and by MTT assay, Alamar Blue, and LDH release (B) are reported. No modification in cell number, viability (TB and Alamar Blue) and LDH leakage was evidenced up to PRE concentration corresponding to 200  $\mu\text{g}$  of polyphenols/mL. At the highest PRE concentration, a significant increase of cell number and viability, and a reduction of LDH release were observed. The MTT assay evidenced an enhanced cell viability also at PRE- concentrations corresponding to 100 and 200  $\mu\text{g}$  of polyphenols/mL.

### 3.3. Effect on the metabolome

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To investigate the perturbation induced by supplementation on Caco-2 metabolome, PRE concentrations corresponding to 50, 200 and 500  $\mu\text{g}$  polyphenols/mL were used. These concentrations were selected on the basis of cytotoxicity results, 50  $\mu\text{g}/\text{mL}$  representing the highest concentration inducing no variation in cell viability, while 200 and 500  $\mu\text{g}/\text{mL}$  were the two highest concentrations causing an increase of cell viability. Before statistics, each  $^1\text{H}$ -NMR spectrum was processed by means of scripts developed in-house in R language. Chemical shift referencing was performed by setting the DSS signal to 0.00 ppm. Moreover, the alignment of the spectra was improved, where possible, using the *iCoshift* tool (Savorani, Tomasi, & Engelsen, 2010) available at [http:// www.models.life.ku.dk/algorithms/](http://www.models.life.ku.dk/algorithms/). The following spectral regions were removed prior to data analysis: the regions including only noise (the spectrum edges between 11.00 and 9.00 ppm and between 0.5 and -1.00 ppm), the NMR signal which is strongly affected by the residual solvent peak (water, between 4.00 and 5.00 ppm). After the Fourier transformation and prior to multivariate analysis, data underwent to a pre statistical improvement: spectra were first normalized to the unit area (Craig, Cloarec, Holmes, Nicholson, & Lindon, 2006) and then a points reduction by the “spectral binning” (Gartland, Beddell, Lindon, & Nicholson, 1991). The first operation is aimed at removing possible dilution effects. The second one avoids the effect of peaks misalignments among different spectra due to variations in chemical shift of signals belonging to some titratable acids. The binning operation is performed by subdividing the spectra into 720 bins, each integrating 60 data points (0.0109 ppm each). Principal component analysis (PCA) was performed on 720 bins. A representative  $^1\text{H}$  spectra of control and supplemented cell are shown in Fig. 3; to better appreciate small signals, spectra were split into two parts (A and B) and then magnified. To evaluate the effect of PRE on the cell metabolome and the variation within samples, a preliminary PCA was performed on the binned database (data not shown). The main function of PCA is to explain the highest portion of variance of the original data (Laghi, Picone, & Capozzi, 2014) and at the same time to reduce the number of variables of the dataset to two or three principal components. These new components correspond to a linear combination of the original ones and they are represented as a score plot where each principal component (PC) accounts for as much variance in the data as possible. PC1 vs PC2 account for 90% of the total variance and it is mainly located along PC1 (78%). The high variance explained by PC1 is because of the effect of PRE on cells’ metabolome which changes deeply from 50  $\mu\text{g}/\text{mL}$  to 500  $\mu\text{g}/\text{mL}$ . A second PCA was run considering only bins with a loading value greater than the standard deviation of the principal component loading for PC1 and PC2. Results are shown in the score plots of Fig. 4A. To gain insight into the observed spectral clustering, the PCA loadings were inspected and the result plotted in Fig. 4B. The increasing doses

250 of PRE exerted metabolic changes along PC1, resulting in positive values of this principal  
251 component at low doses, whilst at higher concentration of PRE the metabolic pathways switch to a  
252 different condition, giving rise to PC1 negative values, indicating a drastic change in the metabolic  
253 pathways. To confirm that bins in the loading barplot were significantly different, ANOVA  
254 followed by Tukey HSD multiple comparison test was performed on the integral of each compound  
255 (Table 3).

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### 257 3.4. *PRE anti-inflammatory activity*

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259 To evaluate PRE anti-inflammatory effect, cells were supplemented with the same concentrations  
260 used to assess the impact on metabolome. Cytokine secretion in the medium was evaluated by  
261 ELISA Ciraplex assay in basal condition and in cells exposed to an inflammatory stimulus (IL-1 $\beta$ ),  
262 and it is reported in Table 4.

263 In basal condition, only IL-8 was detectable in the medium of control cells. Supplementation with  
264 PRE caused a significant decrease of the pro-inflammatory IL-8, while IL-1 $\alpha$  slightly increased at  
265 the highest PRE concentration. In inflamed condition, an increased secretion of all cytokines was  
266 evident in control cells. Particularly, IL-8 concentration in the medium was 8 times higher than in  
267 basal condition. Co-administration of PRE phenols significantly reduced IL-8 secretion in a dose-  
268 related manner. Notably, in inflamed condition, IL-8 concentration in cells supplemented with 500  
269  $\mu\text{g/mL}$  phenols was lower than in control cells in basal condition. Supplementation with the highest  
270 PRE concentration also caused an increase in IL-1 $\alpha$  and IL-10 secretion compared to control cells.  
271 After 24 h, IL-1 $\beta$  concentration was lower than the supplemented concentration confirming its very  
272 short half-life in a biological system (Lopez-Castejon & Brough, 2011). IL-8 secretion was also  
273 determined using the AlphaLISA kit assay. As reported in Fig. 5, results obtained confirmed the  
274 previous ones.

275

## 276 4. **DISCUSSION**

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278 A relevant challenge for scientists and food industries is to turn food processing by-products and  
279 wastes into new ingredients exploiting the favorable properties that this low-price, yet very  
280 nutritious, commodities have (Moure et al., 2001). In this study, we focused on the possible  
281 exploitation of olive oil by-products evaluating the effect of PRE of olive pomace in cultured  
282 intestinal cells. The first step was to evaluate and characterize the PRE, and data obtained were  
283 consistent with previous results on olive pomace (De Marco, Savarese, Paduano, & Sacchi, 2007;

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284 Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012; Malapert, Reboul, Loonis, Dangles, & Tomao,  
285 2018).

286 Although extracts containing a high concentration of phenols can be considered high valued  
287 products, knowledge of their cellular effects is required to evaluate their potential as a value-added  
288 functional ingredient. To this aim, Caco-2 cells were supplemented with different concentrations of  
289 PRE providing a different amount of phenolic compounds. The possible cytotoxicity of PRE was  
290 assessed, and no cytotoxic effects were observed. On the contrary, the highest PRE concentration  
291 (500 µg polyphenols/mL) reduced LDH leakage and increased cell viability. The MTT assay  
292 revealed a significant increase of viability also in cells supplemented with lower PRE  
293 concentrations (100 and 200 µg polyphenols/mL). It could be due not only to the high sensitivity of  
294 the assay (Di Nunzio et al., 2017), but also to an increased mitochondrial activity. In fact, the  
295 measurement of cell viability using the MTT assay correlates with the mitochondrial metabolic  
296 capacity (Danesi et al., 2011). de Oliveira et al. (2016) already showed that resveratrol positively  
297 modulates mitochondrial activity. This effect could be ascribed to the incorporation of phenolic  
298 compounds that induces a deformation of the membrane surface toward a negative membrane  
299 curvature (Lopez et al., 2014) modulating pathways that define mitochondrial biogenesis,  
300 membrane potential, electron transport chain and adenosine triphosphate synthesis, and intra-  
301 mitochondrial oxidative status (Sandoval-Acuna, Ferreira, & Speisky, 2014). Although previous  
302 studies have identified specific metabolic properties of olive phenols (Rescifina, Chiacchio,  
303 Iannazzo, Piperno, & Romeo, 2010), their overall impact on the cell metabolome is still unknown.  
304 This issue was addressed in this study investigating the effects of PRE supplementation on the  
305 entire metabolome by <sup>1</sup>HNMR techniques combined with pattern recognition (Picone et al., 2011).  
306 Using an untargeted approach, we evidenced that olive polyphenols supplementation causes a huge  
307 dose-related perturbation of Caco-2 cells metabolome. In particular, PRE supplementation modified  
308 glucose homeostasis and metabolism. The increased intracellular concentration of glucose (both in  
309 phosphorylated and unphosphorylated form) was related to a reduced catabolization, as confirmed  
310 by the decreased concentration of pyruvate and lactate. *In vitro* and *in vivo* studies already reported  
311 that various polyphenols and their derivatives may impair the glycolytic pathway through the  
312 inhibition of phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase  
313 activity (Ishii et al., 2008; Li et al., 2016; Rodacka, Strumillo, Serafin, & Puchala, 2015). In  
314 addition, we could speculate that modification in glucose transport also occurred. Glucose is  
315 absorbed by intestinal cells mainly via the sodium-glucose transporter 1 (SGLT1), and it is then  
316 released in the blood stream via glucose transporter 2 (GLUT2) (Röder et al., 2014). Glucose  
317 transporters are inhibited by different polyphenols (Castro-Acosta, Lenihan-Geels, Corpe, & Hall,

318 2016; Schulze et al., 2014), and inhibition of GLUT2 is reported to be stronger than SGLT1  
319 (Farrell, Ellam, Forrelli, & Williamson, 2013; Manzano & Williamson, 2010). The increased  
320 glucose intracellular concentration could be also due to an increased gluconeogenesis from alanine,  
321 which significantly decreased in cells supplemented with the highest PRE concentration. A  
322 gluconeogenetic activity in Caco-2 cells has been already reported (Pan et al., 2003) and intestinal  
323 glucose *de-novo* synthesis has a role in the regulation of satiety and food intake (Mithieux et al.,  
324 2005). The modification of the concentration of the two ketogenetic aminoacids, leucine and  
325 isoleucine, and of myo-inositol and taurine, which are both linked to glucose metabolism, are also  
326 suggestive of a metabolic shift toward a “glucose saving/accumulation” strategy. The  
327 gastrointestinal tract releases hormones with important physiological roles in regulating  
328 plasma glucose levels, gut motility and satiety. Gut hormones cholecystokinin, glucagon-like  
329 peptide and peptide tyrosine-tyrosine have been shown to be the primary satiety signals, and their  
330 secretion is regulated by nutrient intake (Wren & Bloom, 2007) and by the concentration of  
331 intestinal glucose (Sun et al., 2017). Caco-2 cells secrete anorexigenic gut hormones, and secretion  
332 is induced by exposure to epigallocatechin-3-gallate, chlorogenic acid and ferulic acid (Song,  
333 Aihara, Hashimoto, Kanazawa, & Mizuno, 2015). The observed glucose accumulation after  
334 exposure to PRE could have a role in maintaining anorexigenic hormone secretion, and could  
335 explain the reported appetite-suppressing effect of the administration of polyphenol-rich food  
336 (Molan, Lila, & Mawson, 2008). Further studies are needed both *in vitro* and *in vivo* to verify this  
337 hypothesis and to understand its functional and clinical significance.

338 In basal condition, supplementation with the highest concentration of PRE-phenols significantly  
339 reduced IL-8 secretion; in inflammatory condition, the anti-inflammatory effect of PRE was dose-  
340 related. The major anti-inflammatory components in olives are tyrosol, hydroxytyrosol, oleuropein,  
341 verbascoside and their derivatives (Rigacci & Stefani, 2016). In particular, hydroxytyrosol is  
342 considered the major anti-inflammatory compound in aqueous olive extracts (Richard et al., 2011).  
343 In agreement, hydroxytyrosol and verbascoside were the main phenols in the PRE extract, and their  
344 rapid uptake by intestinal cells has been demonstrated in Caco-2 cells (Cardinali, Linsalata,  
345 Lattanzio, & Ferruzzi, 2011). Overall, our finding confirms the therapeutic potential of polyphenols  
346 in inflammatory bowel diseases (Rahman et al., 2018; Salaritabar et al., 2017).

347 The exploitation of by-products to formulate new food with added nutritional value is an innovative  
348 and sustainable strategy that meets current and future expectations of consumers about  
349 environmental impact, ethical issues, human health, and safety, maximizing the net benefit to  
350 society. To do it, by-products must be characterized and their effect in cells should be investigated  
351 in depth as a preparatory approach to human intervention trials. In this study, the anti-inflammatory

352 effect of an extract obtained from olive pomace has been clearly evidenced in cultured intestinal  
353 cells. A concomitant, not hypothesized modulation of glucose metabolism was observed,  
354 highlighting that NMR metabolomics is fundamental for studying the biological effect of bioactive  
355 compounds, particularly when they have a broad spectrum of mechanisms of action. Although  
356 *Caco-2* cells are often used to investigate the anti-inflammatory effects of food components  
357 (Antognoni et al., 2017; Romier-Crouzet et al., 2009), the Authors are aware that results obtained *in*  
358 *vitro* must be confirmed *in vivo*. Anyway, the present research confirm that the foodomics approach  
359 has a great potentiality in the assessment of the nutraceutical properties of bioactive compounds  
360 (Capozzi & Bordoni, 2013; Catalan, Barrubés, Valls, Sola, & Rubio, 2017; Ghini et al., 2017;  
361 Marcolini et al., 2015).

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369 **Author contributions**

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371 M.D.N. performed the experiments on cell culture and wrote the manuscript; G.P. carried out NMR  
372 and multivariate statistical analysis; F.P. performed HPLC analyses; A.B., M.F.C., F.C. and A.G.  
373 designed and supervised the study. All Authors critically contributed to the manuscript writing.

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375 **Conflicts of interest**

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377 The authors declare no conflict of interest. The funding sponsors had no role in the design of the  
378 study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, and in  
379 the decision to publish the results.

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585 **Table 1**

586 Nutritional composition of DOP.

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4	Humidity (g/100g)	3,53
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6	Protein (g/100g)	12,15
7		
8	Fat (g/100g)	3,7
9		
10	Total Fiber (g/100g)	30,8
11		
12	Insoluble Fiber (g/100g)	24,8
13		
14	Soluble Fiber (g/100g)	6,06
15		
16	Ash (g/100g)	10
17		
18	Carbohydrates (g/100g)	40
19		
20	Sodium (mg/kg)	182
21		
22	Saturated Fats (%)	19,74
23		
24	MUFA (%)	52,32
25		
26	PUFA (%)	27,92
27		
28	Total $\omega$ 3 (%)	2,7
29		
30	Total $\omega$ 6 (%)	25,17
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32	$\omega$ 3/ $\omega$ 6	0,11
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589 **Table 2**

590 TAC and TPC of PRE. TAC is expressed as mM trolox equivalent (TE). TPC is expressed as mg  
591 gallic acid equivalent (GAE)/mL. Data are mean  $\pm$  SD of two independent extractions analyzed in  
592 triplicate.

TAC (mM TE)	5.72 $\pm$ 0.05
TPC (mg GAE/mL)	0.69 $\pm$ 0.06

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595 **Table 3**

596 List of metabolites whose signals were assigned and integrated into the NMR spectra. **In each**  
 597 **condition, data are mean  $\pm$  SD of three samples coming from independent experiments.** Statistical  
 598 analysis was by the one-way ANOVA followed by Tukey HSD multiple comparison Test ( $p < 0.05$ )  
 599 for post hoc group comparisons. Similar letters indicate no significant difference.

Metabolites	Bins' Integrals ( $10^{-3}$ )			
	0 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$	200 $\mu\text{g/mL}$	500 $\mu\text{g/mL}$
UMP	0.62 $\pm$ 0.3 <sup>bc</sup>	1.42 $\pm$ 0.48 <sup>ab</sup>	1.99 $\pm$ 0.37 <sup>a</sup>	0.03 $\pm$ 0.40 <sup>c</sup>
Glucose-6-Phospate	1.39 $\pm$ 0.42 <sup>bc</sup>	1.23 $\pm$ 0.18 <sup>c</sup>	1.75 $\pm$ 0.18 <sup>ab</sup>	5.85 $\pm$ 0.87 <sup>a</sup>
o-Phosphocholine	5.28 $\pm$ 1.03 <sup>ab</sup>	6.27 $\pm$ 0.53 <sup>a</sup>	4.68 $\pm$ 0.72 <sup>b</sup>	2.81 $\pm$ 0.40 <sup>c</sup>
Lactate	3.72 $\pm$ 1.36 <sup>a</sup>	3.74 $\pm$ 1.08 <sup>a</sup>	3.70 $\pm$ 0.89 <sup>ab</sup>	1.37 $\pm$ 0.10 <sup>b</sup>
UDP-N-Acetylglucosamine	2.42 $\pm$ 0.18 <sup>c</sup>	2.29 $\pm$ 0.18 <sup>c</sup>	3.35 $\pm$ 0.64 <sup>b</sup>	8.48 $\pm$ 0.82 <sup>a</sup>
Glucose	5.27 $\pm$ 0.57 <sup>b</sup>	5.27 $\pm$ 0.12 <sup>b</sup>	5.93 $\pm$ 0.82 <sup>b</sup>	10.56 $\pm$ 1.48 <sup>a</sup>
Ethanol	5.21 $\pm$ 1.23 <sup>b</sup>	4.99 $\pm$ 1.83 <sup>b</sup>	6.73 $\pm$ 3.42 <sup>a</sup>	12.5 $\pm$ 2.48 <sup>a</sup>
myo-Inositol	4.16 $\pm$ 0.42 <sup>b</sup>	4 $\pm$ 0.87 <sup>b</sup>	4.94 $\pm$ 1.21 <sup>ab</sup>	8.33 $\pm$ 1.04 <sup>a</sup>
Glycine	20.03 $\pm$ 6.27 <sup>a</sup>	12.6 $\pm$ 2.73 <sup>a</sup>	12.5 $\pm$ 2.09 <sup>a</sup>	6.00 $\pm$ 1.35 <sup>b</sup>
Taurine	4.42 $\pm$ 1.34 <sup>a</sup>	4.67 $\pm$ 1.15 <sup>a</sup>	3.88 $\pm$ 0.38 <sup>ab</sup>	2.11 $\pm$ 0.02 <sup>b</sup>
Pyruvate	4.78 $\pm$ 1.17 <sup>a</sup>	4.02 $\pm$ 0.4 <sup>a</sup>	3.06 $\pm$ 0.41 <sup>b</sup>	1.18 $\pm$ 0.29 <sup>b</sup>
Alanine	8.37 $\pm$ 0.34 <sup>a</sup>	7.25 $\pm$ 0.24 <sup>a</sup>	5.04 $\pm$ 0.91 <sup>ab</sup>	1.33 $\pm$ 0.45 <sup>b</sup>
Leucine	4.66 $\pm$ 0.53 <sup>a</sup>	3.73 $\pm$ 0.62 <sup>b</sup>	3.48 $\pm$ 0.23 <sup>b</sup>	2.12 $\pm$ 0.67 <sup>c</sup>
Isoleucine	4.28 $\pm$ 0.6 <sup>a</sup>	4.23 $\pm$ 0.59 <sup>b</sup>	4.31 $\pm$ 0.34 <sup>b</sup>	1.72 $\pm$ 0.29 <sup>c</sup>

601 **Table 4**

602 Cytokine secretion in basal and inflamed condition. Data are expressed as pg/mL and are mean ±  
 603 SD of four samples in each condition from two independent experiments. Statistical analysis was by  
 604 the one way ANOVA (IL-1α in basal and stressed condition: p<0.05; IL-8 in basal and stressed  
 605 condition p<0.001; IL-10 in stressed condition p<0.01) using Dunnett's as post-test to compare  
 606 supplemented cells to corresponding controls (\* p<0.05; \*\* p<0.01; \*\*\* p<0.001). The limit of  
 607 detection were 4.75 fg/ml, 0.177 pg/ml, 3 fg/mL, 40.5 fg/ml, 9.72 fg/ml, 30.1 fg/ml, 59 fg/ml, 0.25  
 608 pg/ml, 0.74 pg/ml for IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, and TNFα  
 609 respectively.

Polyphenols (µg/mL)	0				50				200				500			
	0	50	200	500	0	50	200	500	0	50	200	500	0	50	200	500
	<b>IL-1β (10 ng/mL)</b>															
IFNγ	n.d.	0.02±0.03	0.03±0.04	0.02±0.03	0.05±0.01	0.04±0.03	0.08±0.03	0.09±0.03								
IL-1α	n.d.	0.75±0.72	0.87±0.58	1.48±0.66**	2.06±0.81	2.2±0.85	3.16±0.61	4.1±1.49*								
IL-1β	n.d.	0.03±0.06	0.06±0.1	0.01±0.02	1.21±0.03	0.3±0.0	0.12±0.08	1.13±2.14								
IL-2	n.d.	0.05±0.05	0.06±0.04	0.06±0.06	0.11±0.04	0.12±0.04	0.17±0.03	0.2±0.06								
IL-4	n.d.	0.03±0.04	0.01±0.02	0.01±0.01	0.09±0.04	0.07±0.04	0.12±0.05	0.17±0.05								
IL-6	n.d.	0.03±0.04	0.04±0.03	0.03±0.05	0.16±0.02	0.1±0.05	0.14±0.04	0.16±0.05								
IL-8	13.17±4.31	7.97±2.92*	7.84±1.87*	0.11±0.25***	105.2±28.02	21.96±5.25***	9.24±1.02***	0.88±0.4***								
IL-10	n.d.	0.05±0.09	0.04±0.06	0.07±0.13	0.17±0.06	0.17±0.11	0.25±0.09	0.37±0.04*								
IL-12p70	n.d.	0.3±0.68	0.3±0.66	0.49±1.1	1.64±0.74	1.19±0.99	2.71±1.08	3.29±0.86								
TNFα	n.d.	0.84±1.02	n.d.	n.d.	1.96±0.47	1.58±0.57	2.51±0.68	2.88±0.48								

611 **Figure Captions**

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3  
613 **Fig. 1.** Phenolic profile and concentration.

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614 Phenols concentration is expressed as  $\mu\text{g/mL}$ . Data are mean  $\pm$  SD of three determination. A  
6  
615 representative chromatogram is reported, and peak assignment is given in the table. DAFOA<sup>a</sup>,  
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616 DiAldehydic Form of Oleuropein Aglycone; AFOA<sup>b</sup>, Aldehydic Form of Oleuropein Aglycone  
10  
617 (5S,8R,9S).

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619 **Fig. 2.** Cell count and cell viability by TB and by MTT, Alamar Blue assay, and LDH release.

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620 Cell count and cell viability by TB (panel A) are expressed as number of cells per well. Cell  
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621 viability by MTT and Alamar Blue (panel B) are expressed as percentage of control cells (assigned  
19  
622 as 100%). LDH (panel B) is expressed as  $\text{mU/mL}/10^6$  cells. In each condition, data are mean  $\pm$  SD  
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623 of six samples obtained from two independent experiments. Statistical analysis was by the one way  
23  
624 ANOVA ( $p < 0.001$ ) using Dunnett's as post-test to compare supplemented cells to control ones (\*  
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625  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

26  
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28  
627 **Fig. 3.** 600.13 MHz <sup>1</sup>H NMR spectrum of 4 cell lysate at pH 7.33 and at different concentration of  
30  
628 PRE (0, 50, 200 and 500  $\mu\text{g/mL}$ ).

32  
629 Panel A: upfield and midfield region (0.5:4.60). Panel B: downfield region (4.60:9.00). **1**-Leucine  
34  
630 (d: 0.946), **2**-Valine (d: 0.978, d: 1.031), **3**-Isoleucine (t: 0.926, d: 0.978), **4**-Ethanol (t: 1.174, q:  
36  
631 3.651), **5**-Lactate (d: 1.318, q: 4.106), **6**-Alanine (d: 1.469), **7a**-Putrescine (m: 1.754, m: 3.048) only  
38  
632 in C200 and **7b**-2-Oxoglutarate (t: 2.429; t: 2.996) only in C500, **8**-Acetate (s: 1.909), **9**-Glutamate  
40  
633 (m: 2.0403, m: 2.118, m: 2.328), **10**-UDP-N-Acetylglucosamine (s: 2.0716, m: 5.505, m: 5.971, d:  
42  
634 7.9387), **11**-1,3-Dihydroxyacetone (s: 4.413), **12**-Choline (s: 3.192), **13**-Creatine (s: 3.028), **14**-  
44  
635 Creatine phosphate (s: 3.032), **15**-O-Phosphocoline (s: 3.209, m: 3.581, m: 4.155), **16**-Taurine (t:  
46  
636 3.249, t: 3.415), **17**-Myo-Inositol (dd: 3.528, t: 3.615, t: 4.056), **18**-Threonine (d: 1.378, d: 3.578,  
48  
637 m: 4.249), **19**-Glycine (s: 3.552), **20**-Pyruvate (s: 2.358), **21**-Glucose-1-phosphate (t: 3.391, q:  
50  
638 5.445), **22**-Uridine (m: 5.903, d: 7.860), **23**-Fumarate (s: 6.509), **24**-Tyrosine (d: 6.889, d: 7.184),  
52  
639 **25**-Phenylalanine (d: 7.321, t: 7.369, t: 7.421), **26**-Nicotinurate (q: 7.590, m: 8.244, dd: 8.703, d:  
54  
640 8.931), **27**-Hypoxanthine (s: 8.181, s: 8.201), **28**-Inosine (s: 8.224, s: 8.333), **29**-Formate (s: 8.444),  
56  
641 **30**-Glucose-6-phosphate (d: 5.229, m: 3.494), **31**-sn-Glycero-3-phosphocholine (s: 3.218, m:  
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642 4.313), **32**-UMP (d: 8.083, m: 5.938). Abbreviations for multiplicities are: s = singlet, dd = doublet  
60  
643 of doublets, d = doublet, t = triplet and m = multiplet (denotes complex pattern).

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645 **Fig. 4.** PCA scores plot of the <sup>1</sup>H-NMR spectrum for all samples performed on selected significant  
646 bins.

647 (A); PCA plot on the whole spectra dataset. (B); PCA plot on selected bins. The grey scaled arrow  
648 from high to low PC1's values stands for the increasing concentration of a pattern of metabolites  
649 due to the different PRE concentration. (C) Loadings barplot for the first principal component on  
650 the 93 selected bins. 1-2: "UMP"; 3-5: "Glu-6-Phospate"; 6-8: "sn-3-GlyPhoCholine"; 9-11: "o-  
651 PhospCholine"; 12-14: "Lactate"; 15-26: "Patterns of signals from different metabolites"; 27-29:  
652 "UDP-N-Acetylglucosamine"; 30-38: "Glucose"; 39-41: "Ethanol"; 42: "Myo-Inositol"; 43-45: "o-  
653 PhospCholine"; 46: "Glycine"; 47-48: "Myo-Inositol"; 49-60: "Glucose"; 61-63: "Taurine"; 64:  
654 "Glucose"; 65-67: "o-PhospCholine"; 68: "Choline"; 69: "Pyruvate"; 70-71: "Not Assigned"; 72-  
655 74: "Alanine"; 75:78: "Lactate"; 79-81: "Not Assigned", 82-84: "Ethanol"; 85-86: "Leucine"; 87-  
656 88: "Isoleucine"; 89-93: "Not Assigned".

657  
658 **Fig. 5.** IL-8 secretion in basal (A) and inflammatory condition (B).

659 IL-8 is expressed as pg/mL. In each condition data are mean ± SD of nine samples obtained from  
660 three independent experiments. Statistical analysis was by the one way ANOVA (p<0.001) using  
661 Dunnett's as post-test to compare supplemented cells to corresponding controls (\*\*\*) p<0.001).

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**Figure 1**

#	Compounds	Mass data ESI <sup>-</sup>	μg/ml
1	Quinic acid	191 [M-H] <sup>-</sup> , 111	73.7 ± 10.1
2	Hydroxytyrosol 4-β-D-glucoside	315 [M-H] <sup>-</sup> , 153	37.3 ± 9.3
3	1-β-D-glucopyranosyl acyclodihydroelenolic acid / Hydroxytyrosol	407 [M-H] <sup>-</sup>	111.5 ± 6.9
4	Tyrosol	137 [M-H] <sup>-</sup>	11.8 ± 0.1
5	<i>p</i> -coumaric acid	153 [M-H] <sup>-</sup> , 123	6.5 ± 0.2
6	Caffeic acid	179 [M-H] <sup>-</sup> , 135	7.4 ± 0.2
7	DAFOA <sup>a</sup> (Decardoxymethylated)	377 [M-H] <sup>-</sup> , 307	21.1 ± 2.3
8	Hydroxylariciresinolglucoside	537 [M-H] <sup>-</sup>	5.2 ± 0.1
9	β-Hydroxyverbascoside diastereoisomers	639 [M-H] <sup>-</sup>	114.9 ± 6.7
10	β-Hydroxyverbascoside diastereoisomers	639 [M-H] <sup>-</sup>	125.4 ± 7.2
11	Rutin	609 [M-H] <sup>-</sup>	12.0 ± 1.3
12	Verbascoside	623 [M-H] <sup>-</sup>	57.8 ± 4.4
13	Dihydro-oleuropein	543 [M-H] <sup>-</sup>	14.0 ± 1.7
14	Isoverbascoside	623 [M-H] <sup>-</sup>	19.4 ± 1.4
15	Deacetoxy oleuropein aglycone	319 [M-H] <sup>-</sup>	67.3 ± 4.0
16	Oxidized product of DAFOA <sup>a</sup>	335 [M-H] <sup>-</sup>	32.7 ± 0.6
17	Hydro-oleuropein	539 [M-H] <sup>-</sup>	11.7 ± 0.1
18	AFOA <sup>b</sup>	377 [M-H] <sup>-</sup>	10.3 ± 1.4
19	AFOA <sup>b</sup>	377 [M-H] <sup>-</sup>	9.8 ± 0.8
Total phenolic contents			749.2 ± 17.6

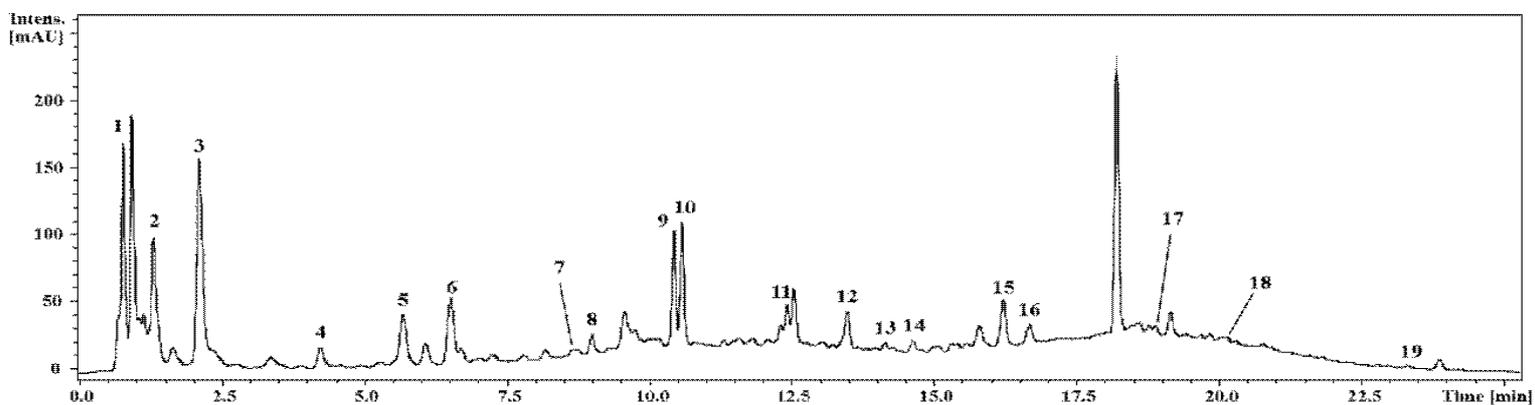


Figure 2

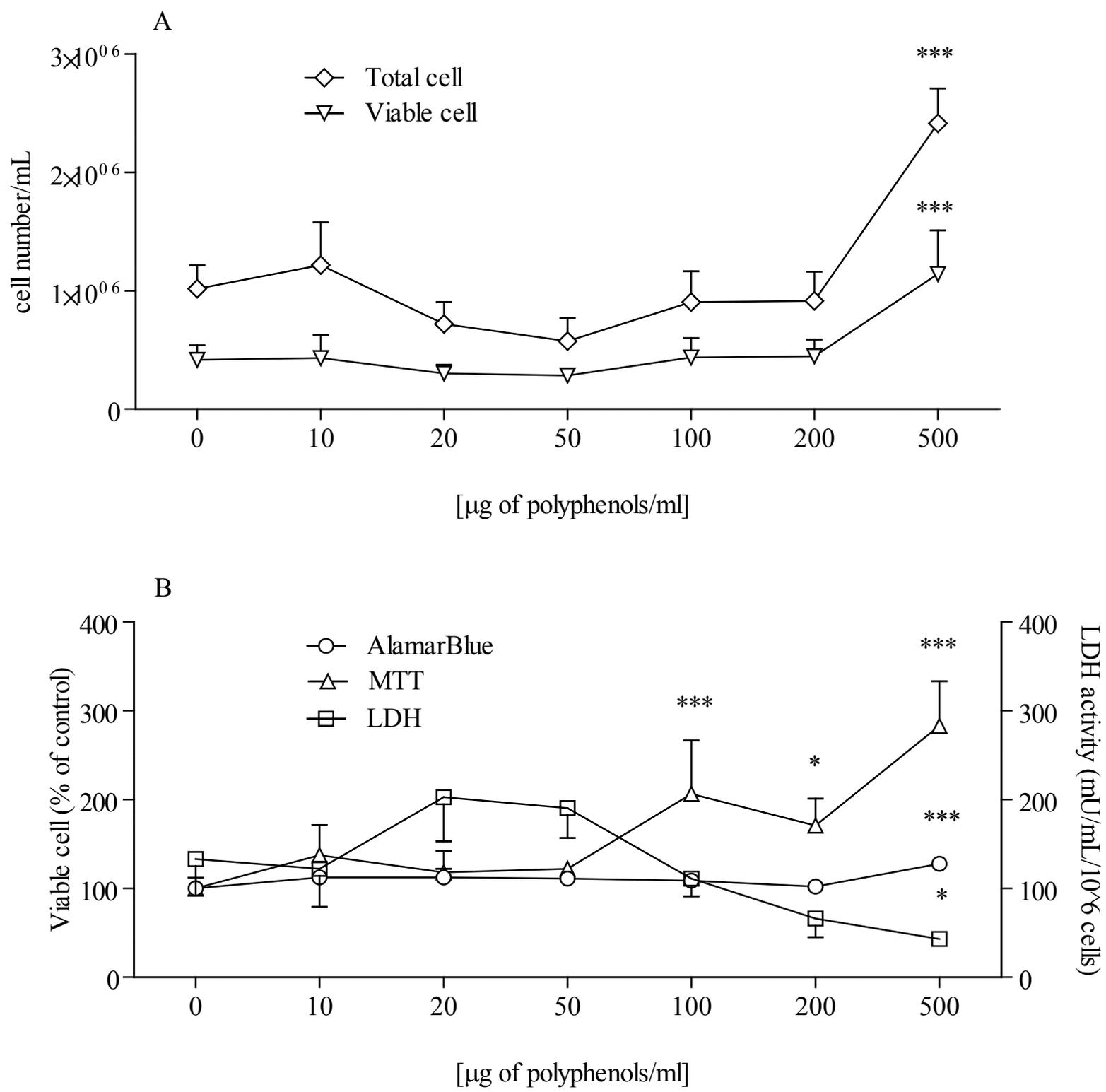


Figure 3

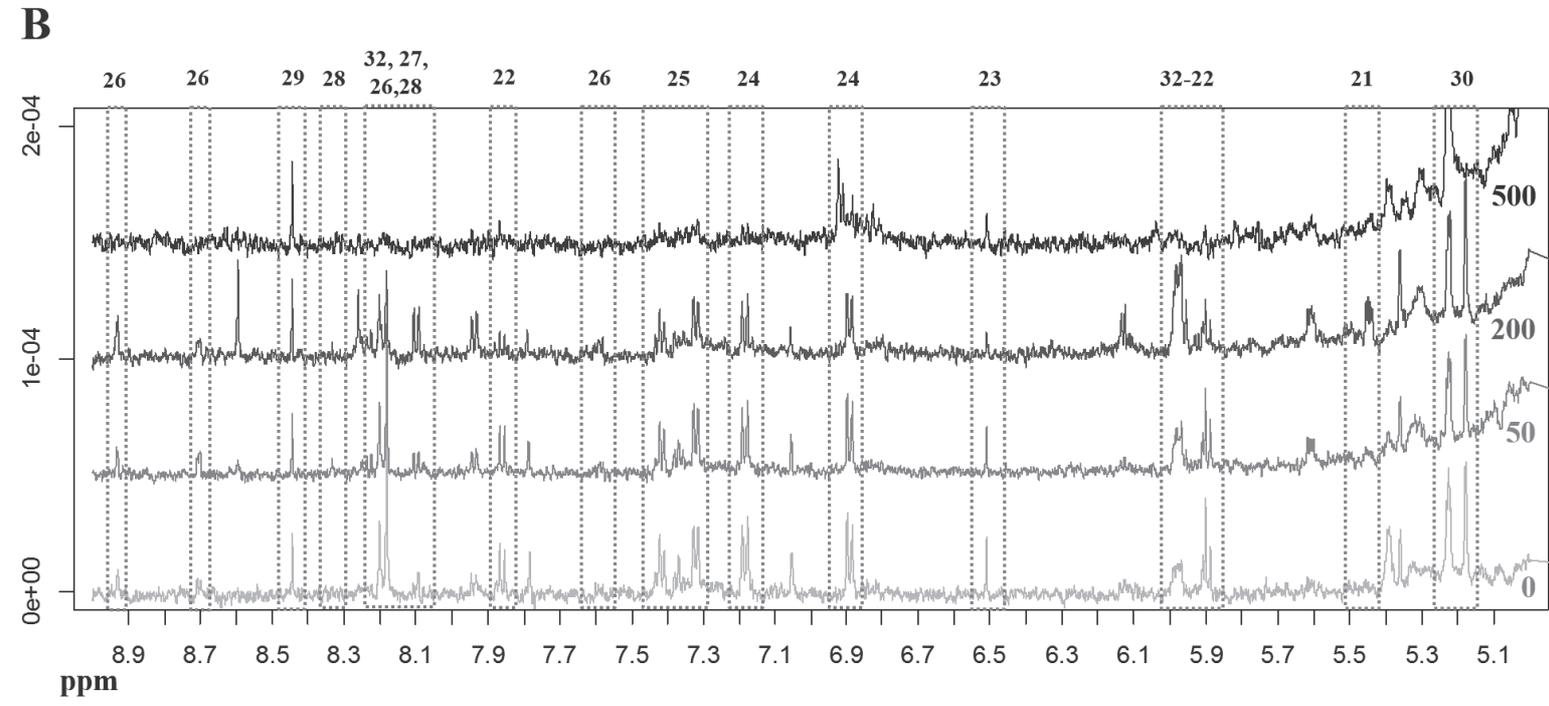
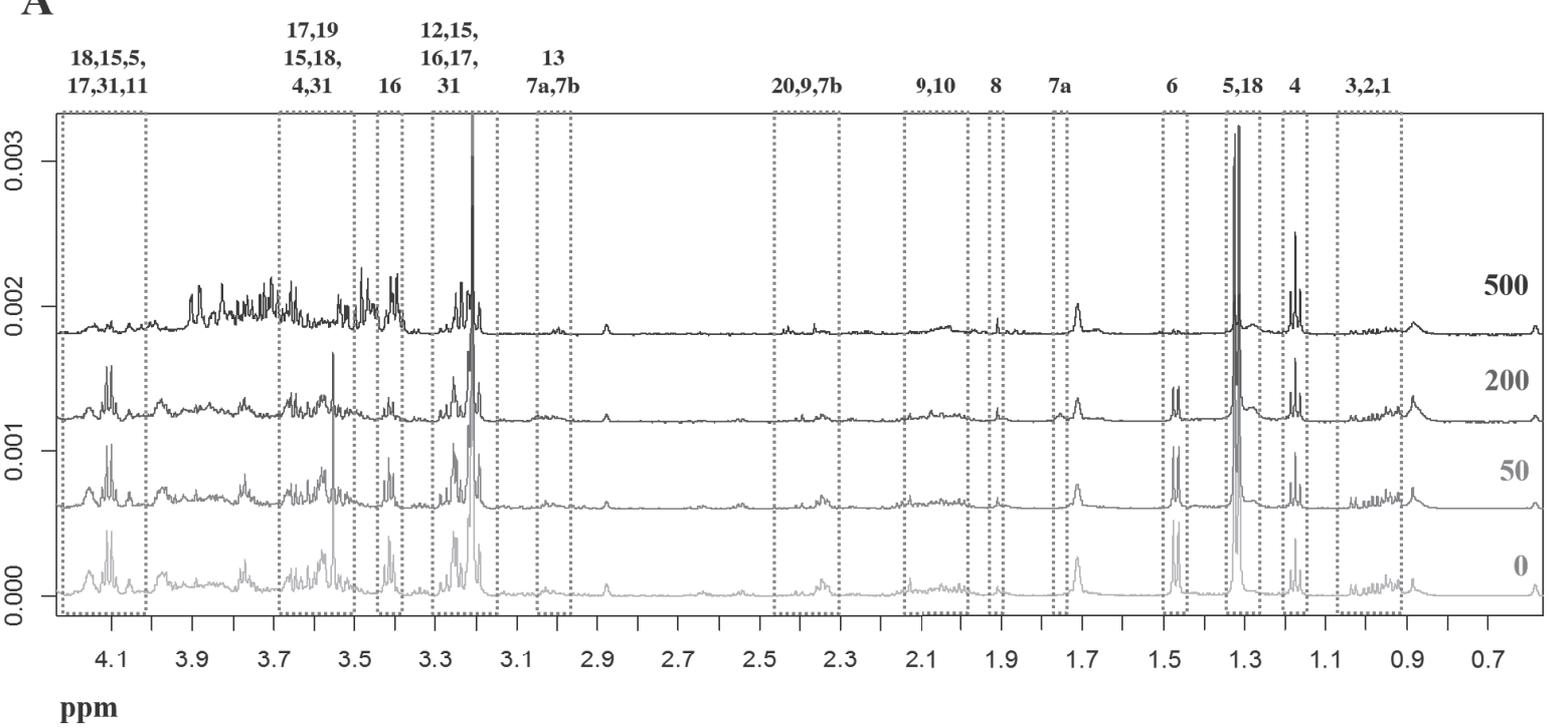
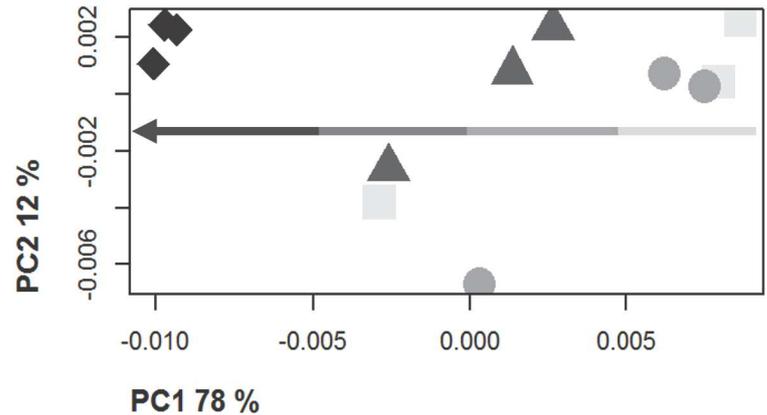
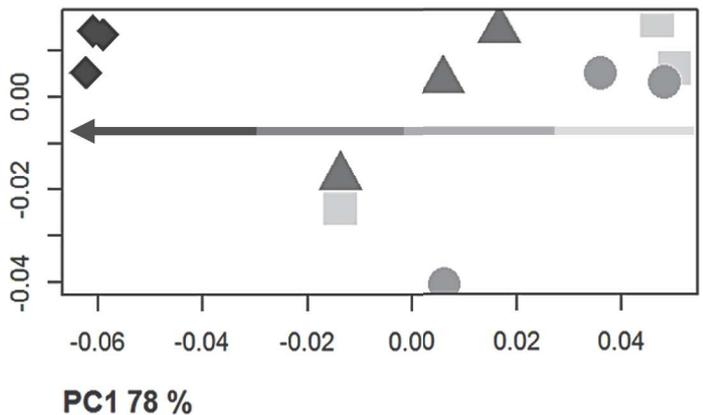


Figure 4

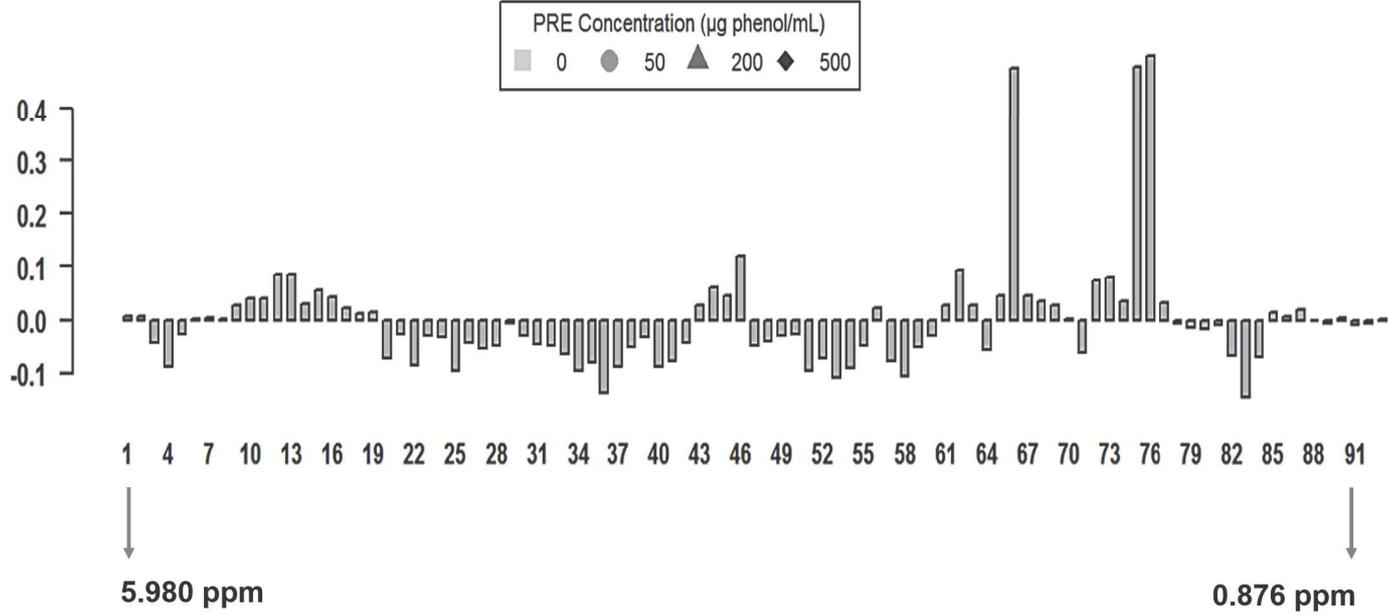
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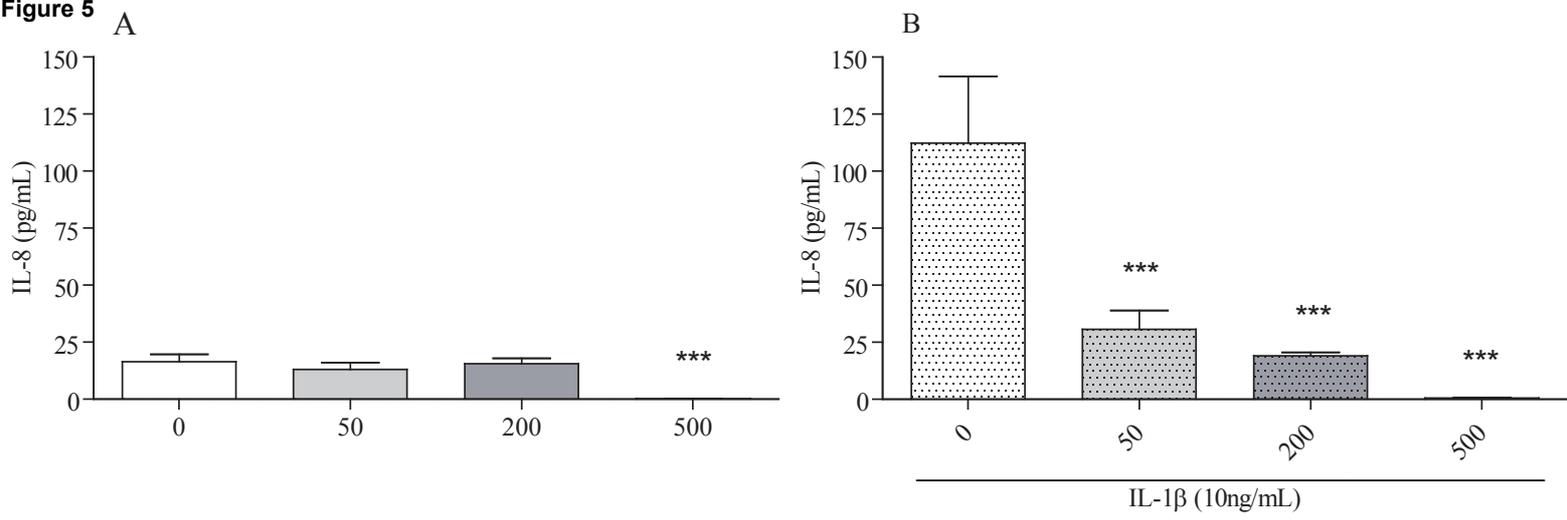
B



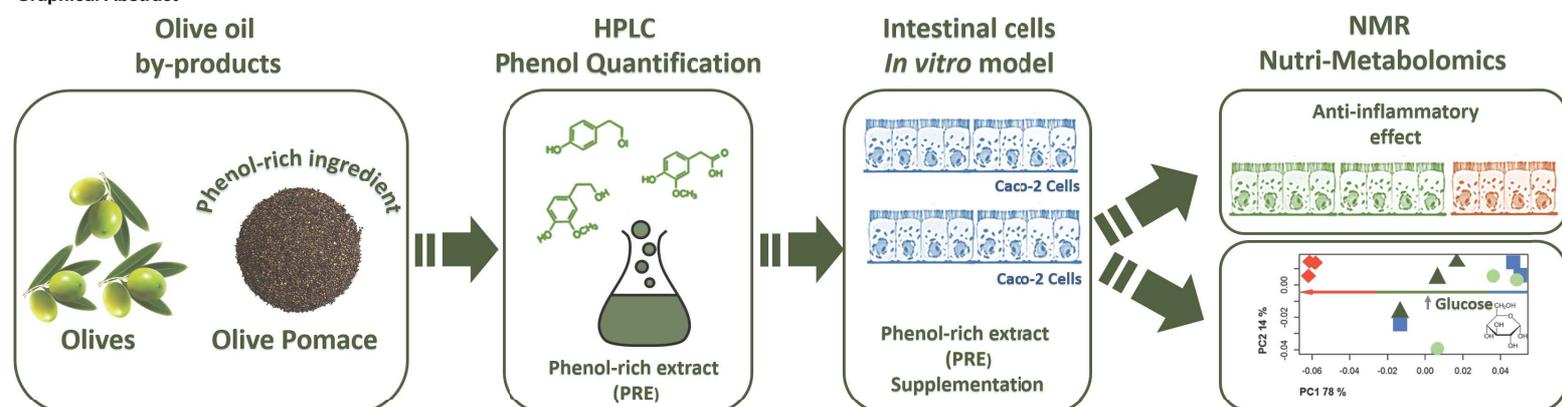
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**Figure 5**



\*Graphical Abstract



**OLIVE OIL INDUSTRY BY-PRODUCTS. EFFECTS OF A POLYPHENOL-RICH EXTRACT ON THE METABOLOME AND RESPONSE TO INFLAMMATION IN CULTURED INTESTINAL CELL**

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Mattia Di Nunzio, Gianfranco Picone, Federica Pasini, Maria Fiorenza Caboni, Andrea Gianotti, Alessandra Bordoni, Francesco Capozzi

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