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4 **Polyphenols associated to pectic polysaccharides account for most of the**
5 **antiproliferative and antioxidant activities in olive extracts.**

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ABSTRACT

Extracts rich in pectic polysaccharides associated to polyphenols were obtained from alperujo, the by-product of olive oil extraction, by different thermal treatment and subsequent chemical treatments. The resulting “Pectoliv” extracts exhibited strong antioxidant activities and a high antiproliferative capacities *in vitro* against colon carcinoma Caco-2 and leukemia monocytic THP-1 cell lines. Bleaching of the extracts with sodium chlorite removed the phenolic compounds and significantly reduced antiproliferative and antioxidant activities, confirming that these were due mainly to the associated polyphenols present in Pectoliv. However, even after bleaching, these activities remained very similar to that of a commercial citrus modified pectin obtained by chemical treatment from citrus pectin, which has been implicated in cancer treatment and prevention. Thus Pectolivs have a high potential as antiproliferative or antioxidant agents.

49 **Keywords:** cancer; alperujo; modified citrus pectin; antiproliferative activity; antioxidant
50 activity.

1. Introduction

Alperujo is one of the most important by-products generated by the olive oil manufacturing process using the continuous two-phase extraction system, with over 4 million tonnes generated annually in Spain. Alperujo is a combination of the olive vegetative water and solids (skin, seeds, pulp, and stones) from olive-pomace mill waste (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2013). Alperujo is a source of substances of high added value, including phenols, carbohydrates and proteins (Navarro, Fiore, Fogliano, & Morales, 2015)(Troise, Fiore, Colantuono, Kokkinidou, Peterson, & Fogliano, 2014). The thermal treatment of alperujo (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz & Fernández-Bolaños, 2012), which allows an easy separation of the solid and liquid phases, also results in the solubilization of compounds of high added value in the liquid phase. For example, considerable amounts of pectic polysaccharides can be obtained from the aqueous fraction, because one third of the olive-pulp cell wall is made up of arabinose-rich pectin polysaccharides (Cardoso, Coimbra, & Lopes da Silva, 2003).

Pectins are a complex polysaccharide found in the cell wall of higher plants. They contribute to soluble dietary fiber, which is incompletely processed by human digestive enzymes and not absorbable in the small intestine, but is fermented by colon bacteria (Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006). Moreover, it is associated with gastrointestinal health and recently there is evidence of the prevention and treatment of cancer (Wollowski, Rechkemmer, & Pool-Zobel, 2001; Mudgil & Barak, 2013).

Pectin treated with pH adjustment – by alkali or acid, heat or enzyme, and known as “modified pectin” – has been broken into small fragments that, in theory, with a molecular weight around 10 kDa, can be absorbed and pass into the blood circulation (Zhang, Gao,

Shi & Zhang, 2007). Several studies proved that modified pectin has an anticancer effect, causing the reduction of cell proliferation, migration and adhesion, as well as triggering the induction of apoptosis and anti-metastasis (Nangia-Makker et al., 2002; Liu, Huang, Yang, Lu, & Yu, 2008; Maxwell, Belshaw, Waldron, & Morris, 2012; Zhang, Xu, & Zhang, 2015; Maxwell et al., 2016). Generally, it is thought that modified pectin is biologically active due to its galactan side chains that can bind to the pro-metastatic protein Galectin-3 (Maxwell et al., 2012). Galectin-3 is a target protein that when overexpressed promotes metastasis and protects cancer cells from apoptosis (Nangia-Makker et al., 2002), and when its activity is blocked, inhibits cell adhesion (Maxwell et al., 2012) and enhances the induction of apoptosis (Liu et al., 2008).

Citrus fruits and apple pomace are the major sources for commercial pectin, although other sources are being introduced. Alternative sources include residues from agricultural and food industries, which could lead to a revalorization of waste materials that are otherwise discarded, as is the case with alperujo. Previous studies carried out by our group indicate that the pectic material obtained from alperujo by hydrothermal treatment (160° C/30 min) (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolaños, 2015a) or by gentle heat treatment (50–80° C) (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, Garcia, & Fernandez-Bolaños, 2015b) showed different physical characteristics and biological properties. In both cases, it had a high oil capacity and emulsifying activity, and a higher capacity for binding bile acids and glucose than commercial citrus pectin. A high antiproliferative activity was observed in different bladder cancer lines from alperujo-derived pectin extracts rich in polyphenols compared with a modified citrus pectin (MCP) (Pectasol-C) or cisplatin (CDDP), a known anticancer agent (Bermúdez-Oria et al., 2019a). In addition, other alperujo-derived pectin extracts containing phenols inhibited proliferation of Caco-2 and THP-1 cancer cells, and also

inhibited hemagglutination by galectin-3. These inhibitory effects were even higher than those of MCP (Bermúdez-Oria et al., 2019b).

The first objective of the present work was to investigate the effect of different chemical or thermal treatments on the composition, antioxidant effect, and antiproliferative activity on Caco-2 cells of pectic extracts obtained from alperujo. The second objective was to determine whether polyphenols are related with these activities. For this, polyphenols were removed from some of the extracts by bleaching using chlorite before assessing antioxidant capacity and the effect on proliferation of Caco-2 and THP-1 cells.

2. Material and Methods

2.1 Materials

Neutral red and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma. Pectasol-C is a commercial modified citrus pectin supplement from Econugenics (Santa Rosa, California). Caco-2 and THP-1 cells were provided by the European Collection of Authenticated Cell Cultures, Public Health England. Cell culture media and serum were from Gibco, Thermo Fisher Scientific.

2.2 Raw material

Olive pomace, or “alperujo” (a semi-solid residue composed of olive peel, pulp, seeds, and ground stones), was collected directly after the two-phase centrifugal system used in a local pomace processing mill (Marchena, Sevilla, Spain) for the extraction of olive oil.

2.3 Isolation and purification of extracts from alperujo

The hydrothermal treatment of alperujo (Fernandez-Bolaños et al., 2010, Patent no.

ES2374675) was performed using a steam treatment reactor prototype, designed by our research group at the Instituto de la Grasa (Seville, Spain). Fresh alperujo samples (8 Kg) were treated with saturated steam for 60 min at a temperature of 80° C or 120° C, or for 30 min at a temperature of 160° C. Heating of the alperujo was performed by direct steam injection, enhancing the contact between the steam and the alperujo. After closing the steam inlet valve, the pressure was reduced to atmospheric pressure at a controlled rate. The wet material was centrifuged at 4700 g (Comteifa, S.L., Barcelona, Spain) to separate the solid and liquid phases, with subsequent ultrafiltration of the liquid phase at 3000 Da. The liquid phase higher than 3000 Da was concentrated to 2 L in a rotary evaporator, and precipitated with 70% EtOH. The alcohol-insoluble residue was freeze-dried and named according to the temperature at which it was obtained: Pectoliv-80, Pectoliv-120 and Pectoliv-160. Part of the Pectoliv-80 and -120 extracts were divided and a basic hydrolysis carried out with 1 M NaOH for 1 hour, with subsequent neutralization with 1 M HCl. The liquid was lyophilized, and the extract were named Pectolive-80A and -120A (Fig. 1).

2.4 Bleaching of Pectoliv

Pectoliv extracts were treated with acid followed by bleaching according to Renard & Thibault (1996) with some modifications. A bleaching solution was prepared by mixing acetic acid (CH₃COOH 0.2% w/v) and sodium chlorite (NaClO₂ 0.2% w/v). Briefly, MCP and Pectoliv-80, -80A and -160 (7.5 % w/v) were added to the bleaching solution. The mixture was heated at 70° C for 1 hour. These steps were repeated twice. The samples were precipitated with 70% EtOH, filtered and rinsed with EtOH. The bleached phenolic residues were removed by washing and ultrafiltration at 1000 Da, and then lyophilized. Bleached extracts were named Pectoliv-80B, -80AB, -160B and MCP-B (Fig. 1).

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152 Figure 1. Scheme of the extraction procedure employed to obtain the five different extracts,
153 named Pectoliv, from olive by-product by different thermal and chemical treatments and
154 yield (g/dry kg alperujo). Three of the extracts were bleached with sodium chlorite in acidic
155 conditions.

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157 *2.5 Characterization of pectin extracts*

158 Galacturonan (anhydrogalacturonic acid) was determined according to the m-
159 hydroxydiphenyl method described by Blumenkrantz & Asboe-Hansen (1973) for uronic
160 acids.

161 Glycosyl compositions were determined by gas chromatography (GC) after
162 conversion to alditol acetates. Individual neutral sugars were analyzed from duplicate
163 samples with initial TFA hydrolysis (2 N TFA at 121° C for 1 h) prior to reduction,
164 acetylation and analysis by GC (Englyst, Wiggins, & Cummings, 1984) using inositol as an
165 internal standard. Calibration was performed using a series of standard solutions of L-
166 rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-mannose (Man)
167 and D-xylose (Xyl). The chromatographic conditions were described by Lama-Muñoz,
168 Rodríguez-Gutiérrez, Rubio-Senent & Fernández-Bolaños (2012).

169 Total phenolic content was determined using the Folin–Ciocalteu spectrophotometric
170 method and expressed as grams of gallic acid equivalents (Singleton & Rossi, 1965).

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172 *2.6 Relative molecular weight determination*

173 The homogeneity and molecular weight distribution of the purified Pectoliv extracts
174 were estimated by high performance size exclusion chromatography (HPSEC) using two
175 different columns (300 X 7.8 mm i.d., Tosoh Bioscience LLC, King of Prussia, PA) in

sequence TSK gel GMPWXL (dextran MW<50000 kDa) and TSKgel G3000PWXL (dextran MW<60 kDa) as described previously (Dos-Santos, Jiménez-Araujo, Rodríguez-Arcos, & Fernández-Trujillo, 2011). The system was calibrated with standard dextrans of 500, 110, 70, 40, and 6 kDa using a regression curve.

2.7 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay is based upon the inhibition of peroxy radical-induced oxidation initiated by the thermal decomposition of 2, 2'-azobis(2-amidino-propane) dihydrochloride (AAPH). The reactive oxygen species (ROS) generated from this thermal decomposition quench the signal from the fluorescent probe fluorescein. The antioxidant capacity of the samples was assayed according to Ou, Hampsch-Woodill, & Prior (2001) with minor modifications. Samples were diluted with sodium phosphate buffer (10 mM, pH 7.4) and 25 µL of each were transferred to a microplate. The blank well received 25 µL phosphate buffer while standards received 25 µL Trolox solutions (10-140 µM). Then 150 µL of 1 µM fluorescein was added to all wells. After incubation (37° C, 15 min), 25 µL AAPH (250 mM) was added to each well to initiate the reaction and readings were taken every 5 min for 90 min (Ex. 485 nm, Em. 538 nm) in a microplate reader (Fluoroskan Ascent™, Thermo Scientific™). Results were calculated using the difference of areas under the fluorescein decay curve between the blank and the sample and expressed as µM Trolox equivalents.

2.8 Cell culture and treatment

Caco-2 and THP-1 cells were cultured and treated as described (Bermúdez-Oria et al, 2019b). Cells were cultured in Dulbecco's Modified Eagle Medium (1000 mg/ml glucose, 110 mg/ml pyruvate, and 580 mg/ml glutamine) supplemented with 10% fetal

bovine serum (FBS), 1% non-essential amino acids, 100 U/ml penicillin, and 100 g/ml streptomycin and incubated at 37° C with 5% CO₂. Fetal bovine serum was heat-inactivated at 56° C for 30 minutes. Caco-2 cells were subcultured once a week using trypsin-ethylenediaminetetraacetic acid, and medium was replenished once between passages. THP-1 cells were subcultured every 2–3 days by resuspension in fresh medium. Treatments were carried out under the same standard culture conditions. The lyophilized Pectoliv extracts were dissolved in Hank's Balanced Salt Solution (HBSS) at 100 mg/ml, heated at 100° C for 30 minutes, and diluted with culture medium as required. Cells were seeded in 96-well microplates (4 x 10⁴ or 14 x 10⁴ cells/well, 50 µl/well). Extracts were added to the same volume (50 µl/well) to achieve a final concentration of 10% v/v HBSS. Caco-2 and THP-1 cells were incubated for up to 9 and 4 days, respectively. In addition to the cell proliferation assay (described in 2.9), cells were periodically inspected under the phase contrast microscope.

2.9 Cell proliferation assays

Proliferation of adherent (Caco-2) and suspended (THP-1) cells was determined by measuring proliferation at different times using the neutral red and thiazolyl blue tetrazolium bromide (MTT) assays, respectively. For the neutral red assay, cells in 96-well plates were incubated in fresh culture medium containing the vital stain neutral red (50 µg/ml) for 30 minutes. Cells were then washed using HBSS, and the stain was extracted using acetic acid (75 µl, 1% (v/v) in ethanol 50% (v/v)). Absorbance was measured at 550 nm using a plate reader (Borenfreund & Puerner, 1985; Girón-Calle, Alaiz, & Vioque, 2010). For the MTT assay, cells in 96-well plates were incubated in culture medium containing MTT (0.5 mg/ml) for 60 minutes. The blue formazan crystals formed by the reduction of MTT were dissolved by the addition of 100 µL HCl (0.1 N) in isopropanol,

and absorbance at 570 nm (with subtraction at 630 nm) was measured using a plate reader (Kops, West, Leach, & Miller, 1997; Girón-Calle et al., 2010).

3. Results

3.1. Preparation of the extracts from alperujo

Five extracts were obtained from olive oil by-product by different thermal and chemical treatments. Pectoliv-80 and Pectoliv-120 were obtained from mild thermal treatments, using the conventional temperatures for the recovery of pectin from fruits, 80° C or 120° C for 60 min, respectively. Extracts Pectoliv-160 was obtained from thermal treatments at 160° C for 30 min, with the objective of reducing the molecular weight to gain a product similar to a heat-modified water-soluble pectin preparation, which presents a known antiproliferative effect (Lefsih et al., 2018).

In order to obtain other extracts of low molecular weight by chemical treatment, Pectoliv-80 and Pectoliv-120 were hydrolyzed with alkali (NaOH). The resulting products were named Pectoliv-80A and Pectoliv-120A, respectively. The same chemical treatment is used for production of PectaSol-C, a commercial modified citrus pectin (MCP) extracted from citrus pectin (Jackson et al., 2007), although some authors indicate that MCP may be enzymatically modified (Maxwell et al., 2012). The latter extracts were modified in a variety of ways to provide an array of extracts with varying chemical composition and molecular weight.

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3.2 Effect of the olive extracts (Pectoliv) and MCP on cell proliferation of Caco-2 cells

The effect of each of the five different extracts – differing in their method of preparation, thermal or chemical treatment – on the proliferation of Caco-2 colon cancer

cells was investigated to understand whether the different extraction methods affected the extracts' biological effect. Cells were treated with 0.35, 1.10, 3.50 and 10 mg/mL of Pectoliv extracts or commercial MCP for up to 7 days. At 0.35 and 1.10 mg/mL, neither the five Pectoliv extracts nor MCP inhibited cell proliferation (Figure 2). Incubation with 3.5 mg/mL Pectoliv-80 inhibited cell proliferation completely, while the inhibition caused by Pectoliv-80A was not as high. Pectoliv-80, -80A, -120 and -160 completely inhibited cell proliferation at 10 mg/mL from the first day of treatment. Pectoliv-120A and MCP reduced proliferation to a minor extent, reaching a maximum inhibition of 75% and 56% at day 7, respectively. Several studies indicate that pectins with a lower molecular weight, including Pectasol-C, exhibit greater bioactivity than pectins with a higher molecular weight (Hayashi, Gillen, & Lott, 2000; Maxwell et al., 2012). However, our results show that Pectoliv-80 has a higher inhibitory effect than Pectoliv-80A, which has suffered hydrolysis by treatment with alkali. This result suggests that molecular weight alone is not indicative of bioactivity.

3.3. Antioxidant activities of Pectoliv and MCP

The antioxidant activity of the five Pectoliv extracts and MCP were evaluated according to their oxygen radical absorbance capacity (ORAC). The five Pectoliv extracts showed higher antioxidant capacity (with values between 120 and 387 $\mu\text{mol Trolox equivalent / g extract}$) than the commercial MCP (68 $\mu\text{mol Trolox equivalent / g extract}$) (Figure 3). These results were similar to those obtained in previous studies with other Pectoliv extracts (Bermúdez-Oria et al., 2019). Pectoliv-80 showed the highest antioxidant activity, followed by Pectoliv-80A, which can be explained because these extracts contained two-fold more phenolic compounds than the other extracts, as seen in the

chemical characterization (Table 1). The ORAC assay confirmed that these phenols partially maintained their antioxidant activity after their theoretical polymerization and binding to the polysaccharide fraction, which indicates certain availability of the catechol groups, responsible for antioxidant activity (Spizzirri et al., 2009). These results coincide with those obtained from the cell proliferation assay in Caco-2 cells, where a direct relationship between a major antiproliferative activity for Pectoliv-80 and Pectoliv-80A and their higher antioxidant activity was observed. These findings are also in agreement with those of other authors who published similar observations with different degrees of potency depending on the type of cancerous cell line and antioxidant extract studied (Seeram et al., 2006; Tow, Premier, Jing, & Ajlouni, 2011; Baby, Antony, & Vijayan, 2017; Losada-Echeberria, Herranz-Lopez, Micol, & Barrajon-Catalan, 2017). In addition, although MCP was recently shown to be an effective antioxidant (Hawach, Boujaoude, & Abdel-Massih, 2016; Ramachandran, Wilk, Malnick, & Eliaz, 2017), our data, using the ORAC assay, showed that MCP had a lower antioxidant activity than the Pectoliv extracts, which also coincided with the lowest antiproliferative activity. Effectively, the Pectoliv extracts have important antioxidant properties, including free-radical scavenging, acting as hydrogen donors to the peroxy radical generated in the ORAC assay. Peroxy radicals are reactive species generated during lipid oxidation in food and living tissues (Lobo, Patil, Phatak, & Chandra, 2010; Phaniendra, Jestadi, & Periyasamy, 2015). Thus, these ORAC values can be considered to be of biological relevance as a reference of antioxidant effectiveness. Therefore, there appears to be a direct relationship between the antioxidant activity of each extract and its antiproliferative activity.

3.4. Chemical composition and molecular weight of the Pectoliv extracts

The uronic acid content of the extracts increased with temperature up to 120° C/ 60

min, with a slight decrease at 160° C/ 30 min (Table 1). However, alkali treatment did not affect the uronic acid content or reduce the molecular weight, as might be expected by chemical β elimination (Renard & Thibault, 1996). Indeed, certain depolymeration with the reduction of molecular weight was only observed when the treatment temperature was increased (Figure 4). The ratio of uronic acids to neutral sugars was between 1 and 1.5 for different Pectoliv extracts, which is consistent with previous results reported by Rubio-Senent et al., (2015a), compared to a ratio of 9 for MCP. The US Pharmacopeia defines pectin as a polysaccharide with not less than 74% of galacturonic acid (Rolin, 2002), yet all characterized Pectoliv extracts had a content of 49%–61% uronic acids and so cannot be considered as pectins. However, Pectoliv extracts had a high proportion of uronic acids so they could be considered as “pectin-like polysaccharides” with a high proportion of associated neutral sugars and phenols.

The composition of neutral sugars showed that the extracts were rich in arabinose and galactose, with rhamnose, xylose and glucose present in minor proportions, which is indicative of arabinogalactan being an integral, branched region of pectin. The phenol content was higher in Pectoliv-80 and -80A, with 8% and 11% respectively, more than double the amount of the other extracts, while the phenol content for MCP was very low, at only 0.15%. The presence of these phenolic compounds could be due to the possible interaction between the polysaccharides of the olive cell wall and the hydrophilic compounds, such as phenols, which occurs during the rupture of fruit tissues by olive crushing and further malaxation of the olive paste before oil extraction. In addition, oxidation, condensation and/or polymerization reactions (via enzymatic or non-enzymatic action) provide a significant quantity of non-carbohydrate polymeric material associated with the cell wall polysaccharides (Obied, Allen, Bedgood, Prenzler, & Robards, 2005). These findings in Pectoliv-80 and -80A were probably due to their high polyphenol

concentration (since polyphenols bind to pectin polysaccharides), and correlate with their high antioxidant and antiproliferative activity.

3.5. Bleaching of Pectoliv extracts: chemical composition and antioxidant activity

Due to the presence of chemical linkages between polyphenols and carbohydrates, pectin-bound polyphenols are difficult to eliminate. In order to confirm the components of the extracts responsible for their antiproliferative activity, polyphenols in Pectoliv-80, -80A and -160, and MCP, were eliminated by bleaching using sodium chlorite in acid medium. The resulting low-molecular weight oxidized products were eliminated by dialysis. The bleached extracts were characterized and their antioxidant activities measured. Although the phenolic compounds were degraded or solubilized, in accordance with Gellerstedt (2009), the bleached extracts did not show a substantial modification in uronic acid or sugar composition (Table 2). The ratio of uronic acids to neutral sugars (0.7–2) was very similar to unbleached Pectoliv for MCP-B. The uronic acid content in the bleached samples varied between 24% and 56%, with a decrease in the MCP-B (37%) with respect to MCP (52%) or an increase from 39% to 56% for Pectoliv-160-B.

Bleaching caused a drastic decrease in the antioxidant capacity of Pectoliv extracts (Figure 3), reducing the ORAC activity from 120–387 $\mu\text{mol/g}$ to 34–94 $\mu\text{mol/g}$ extract, and with ORAC disappearing completely in MCP-B. These results suggest a clear correlation between antioxidant activity and the presence or absence of the phenolic compounds in the Pectoliv extracts.

3.6. Effect of bleached Pectoliv extracts on the proliferation of Caco-2 and THP-1 cell lines

In order to assess the correlation between the presence of phenolic compounds in the extracts and their antiproliferative effect on cells, the Caco-2 colon cancer and THP-1 leukemia cancer cell lines were treated with bleached extracts. Following the exposure of Caco-2 cells to increasing concentrations (2.5, 5 and 10 mg/mL) of bleached and unbleached Pectoliv extracts (-80-B, -80AB, -160-B and -80, -80A, -160, respectively), we observed that at 2.5 mg/mL, and more clearly at 5 mg/mL (Figure 5A), all the unbleached Pectoliv extracts inhibited cell proliferation compared to the bleached samples and the control, with a clear time dependent effect. It is remarkable that the inhibitory activities of bleached Pectoliv were similar to bleached and unbleached MCP for the same concentration and the same treatment time. This effect was even more evident at higher concentrations. For example, at 10 mg/mL, Pectoliv-80, -80A, and -160 completely inhibited proliferation after 1 or 2 days of treatment (with a continued inhibition of proliferation from there on), while the corresponding bleached Pectoliv extracts allowed proliferation to a similar extent as MCP and MCP-B.

Next, THP-1 cells were treated with the same range of concentration of bleached and unbleached extracts for 4 days. Treatment with 2.5 mg/ mL unbleached Pectoliv-80 and -160 resulted in reduced cell proliferation with respect to the control, and almost completely inhibited proliferation at 5 mg/ mL (Figure 5B). Similar to the results with Caco-2 cells, bleaching of these Pectolivs greatly reduced the antiproliferative activity. At 10 mg/ mL, proliferation was almost completely inhibited by the three unbleached Pectoliv extracts. At this concentration, bleaching of these three Pectolivs allowed for a proliferation rate similar, although still lower, than the proliferation of THP-1 cells exposed to either bleached or unbleached MCP.

Therefore, elimination of phenols by bleaching greatly reduced the antiproliferative

activity of Pectolivs on both Caco-2 and THP-1 cells, resulting in an effect similar or superior to the antiproliferative effect of MCP. Consequently, the phenols linked to polysaccharides seem to be responsible for the majority of the antiproliferative effect observed for the Pectoliv extracts isolated from the by-product of olive oil extraction. Our findings are consistent with reports showing an antiproliferative effect of polyphenols or polyphenol-rich extracts on Caco-2, THP-1 and other cancer cell lines (Tsan, White, Maheshwari, Bremner, & Sacco, 2000; Girón-Calle et al., 2004; Megías et al. 2009; Gul, Ahmad, Kondapi, Qureshi, & Ghazi, 2013; Sánchez-Vioque et al., 2016). In addition, a polyphenol mixture from olive oil processing waste was described to decrease the production of superoxide anion in THP-1 cells (Léger, Kadiri-Hassani, & Descomps, 2000). Since high levels of ROS are present in cancer cells and may help tumor survival by inactivation of the caspases involved in apoptosis induction (Halliwell, 2007), the Pectoliv extracts, via their free-radical scavenging capacity, could help to reduce the concentrations of ROS and induce apoptosis.

4. Conclusions

This study revealed that the Pectoliv extracts had a high antioxidant and antiproliferative activity on Caco-2 and THP-1 cell lines, as compared to MCP. Interestingly, the extracts obtained at the lowest temperature, Pectoliv-80 and Pectoliv-80A, had the highest antiproliferative and antioxidant activity, consistent with them having the highest content of phenolic compounds. Bleaching caused an important decrease in the antiproliferative activity of all Pectoliv extracts, reaching levels similar to those found for MCP. Moreover, bleaching produced a drastic decrease in the antioxidant activity of the Pectoliv extracts. Therefore, we showed that the high antioxidant and antiproliferative activity observed in the extracts from alperujo was mostly due to their content of phenolic compounds.

However, other components in these extracts, possibly pectic polysaccharides, may also be involved in the antiproliferative activity, as indicated by the fact that the antiproliferative activity of bleached Pectolivs was still similar or even superior to that of commercial MCP. These Pectolivs are products with a high potential for application in the pharmaceutical industry.

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Declarations of interest

None.

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Figure captions

Figure 1. Scheme of the extraction procedure employed to obtain the five different extracts, named Pectoliv, from olive by-product by different thermal and chemical treatments and yield (g/dry kg alperujo). Three of the extracts were bleached with sodium chlorite in acidic conditions.

Figure 2. Anti-proliferative activity of the extracts obtained by thermal (Pectoliv-80, -120 and -160) or thermal and chemical (Pectoliv-80A and -120A) treatment at four different concentration, as compared to MCP. Neutral red uptake was determined after incubation of Caco-2 cells for 1 to 7 days. Data are the mean of three replicates $\pm$ SD. * indicate statistical significant difference between sample and vehicle control for each incubation period and # indicate statistical difference of the all samples for the day of incubation (one way ANOVA-LSD test, $p < 0.05$).

Figure 3. Antioxidant capacity of Pectoliv extracts (unbleached and bleached extracts) compared to those for commercial modified citrus pectin (MCP). Oxygen radical capacity (ORAC) is expressed as $\mu\text{mol Trolox equivalent /g extract}$. Average, and standard deviations are shown ($n = 3$).

Figure 4. Refractive index elution profiles of the Pectoliv extracts obtained by high performance size exclusion chromatography using two TSK gel columns (GMPWXL and G3000PWXL) placed in a series. Numbers above the peaks represent molar masses of dextran standards.

Figure 5. Effect of unbleached and bleached Pectolive extracts on proliferation of (A) Caco-2 and (B) THP-1 cells. Cells were cultured in 96-well plates (5×10^3 cells/well) in culture medium containing 2.5, 5 and 10 mg/mL of extract (Pectoliv-80, -80A, -160, and MCP unbleached and bleached) and a control (without extract). For the Caco-2 cells, Uptake of neutral red by Caco-2 cells was determined after incubation for 1, 2, 3, 4 or 7 days, and reduction of MTT by THP-1 cells was determined after incubation for 1, 2, 3 or 4 days. Data represent the mean of three incubations $\pm$ SD. * indicate statistical significant

difference between sample and vehicle control for each incubation period, # indicate statistical difference of the all samples for the day of incubation (one way ANOVA-LSD test, $p < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

627 **Figure 5**
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632 **Table 1.** Chemical composition (g/100 g) and glycosyl residue composition (% molar ratio)
 633 of Pectoliv extracts obtained by thermal (Pectoliv-80, -120 and -160) or thermal and
 634 chemical (Pectoliv-80A and -120A) treatment as compared to modified citrus pectin
 635 (MCP). The data shown are mean $\pm$ standard deviation. Rha: rhamnose; Xyl: xylose; Man:
 636 mannose; Gal: galactose; Glc: glucose; UrA: uronic acids. N.D. not detected.

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	Pectoliv					
	MCP	80	80 A	120	120 A	160
Uronic acid	51.52 $\pm$ 7.78	26.98 $\pm$ 2.6	29.93 $\pm$ 0.92	42.29 $\pm$ 1.87	48.51 $\pm$ 1.27	38.67 $\pm$ 0.59
Neutral sugar	5.24 $\pm$ 0.07	23.33 $\pm$ 3.00	30.22 $\pm$ 0.92	28.78 $\pm$ 0.90	29.92 $\pm$ 0.60	36.28 $\pm$ 1.93
Phenol	0.15 $\pm$ 0.01	8.08 $\pm$ 0.1	10.93 $\pm$ 0.32	4.54 $\pm$ 0.13	4.71 $\pm$ 0.06	4.93 $\pm$ 0.08
Total	56.91	58.39	71.08	75.61	83.14	79.88
UrA	90.77 $\pm$ 0.35	51.46 $\pm$ 0.00	49.22 $\pm$ 0.00	58.97 $\pm$ 0.00	61.51 $\pm$ 0.00	50.67 $\pm$ 0.00
Rha	1.69 $\pm$ 0.11	4.47 $\pm$ 0.11	5.82 $\pm$ 0.36	5.49 $\pm$ 1.97	6.03 $\pm$ 0.37	6.28 $\pm$ 0.16
Fuc	N.D	0.22 $\pm$ 0.01	0.31 $\pm$ 0.02	0.21 $\pm$ 0.04	0.22 $\pm$ 0.00	0.32 $\pm$ 0.01
Ara	1.08 $\pm$ 0.14	22.62 $\pm$ 2.30	23.11 $\pm$ 0.28	12.50 $\pm$ 0.98	11.00 $\pm$ 0.31	14.44 $\pm$ 0.52
Xyl	0.49 $\pm$ 0.04	1.93 $\pm$ 0.13	2.12 $\pm$ 0.05	4.21 $\pm$ 0.24	3.75 $\pm$ 0.09	5.84 $\pm$ 0.21
Man	0.56 $\pm$ 0.07	1.70 $\pm$ 0.08	1.82 $\pm$ 0.03	2.49 $\pm$ 0.29	2.44 $\pm$ 0.04	3.64 $\pm$ 0.18
Gal	4.67 $\pm$ 0.08	11.97 $\pm$ 0.82	13.36 $\pm$ 0.07	12.15 $\pm$ 0.51	11.29 $\pm$ 0.35	14.60 $\pm$ 0.56
Glc	0.63 $\pm$ 0.01	3.37 $\pm$ 0.26	3.70 $\pm$ 0.05	3.45 $\pm$ 0.18	3.41 $\pm$ 0.06	3.28 $\pm$ 0.01

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641 **Tabla 2.** Chemical composition (g/100 g) and glycosyl residue composition (% molar ratio)
 642 of bleached Pectoliv extracts (Pectoliv-B) and commercial modified citrus pectin (MCP-B).
 643 The data shown are mean $\pm$ standard deviation. Rha: rhamnose; Xyl: xylose; Man:
 644 mannose; Gal: galactose; Glc: glucose; UrA: uronic acids. N.D. not detected.

	Pectoliv-B			
	MCP-B	80	80 A	160
Uronic acid	37.03 $\pm$ 4.94	29.41 $\pm$ 2.83	23.79 $\pm$ 2.45	56.45 $\pm$ 1.51
Neutral sugar	4.52 $\pm$ 0.51	29.54 $\pm$ 3.20	33.71 $\pm$ 5.10	24.82 $\pm$ 1.78
Phenol	N.D			
Total	41.55	58.95	57.50	81.27
UrA	87.33 $\pm$ 2.64	49.45 $\pm$ 6.02	41.53 $\pm$ 4.96	69.49 $\pm$ 1.40
Rha	3.56 $\pm$ 1.68	5.29 $\pm$ 0.97	5.18 $\pm$ 1.52	5.47 $\pm$ 0.29
Fuc	0.29 $\pm$ 0.05	0.36 $\pm$ 0.01	0.36 $\pm$ 0.08	0.32 $\pm$ 0.01
Ara	1.61 $\pm$ 0.09	23.42 $\pm$ 2.71	24.83 $\pm$ 1.16	7.88 $\pm$ 0.22
Xyl	0.94 $\pm$ 0.09	1.71 $\pm$ 0.13	6.96 $\pm$ 8.75	2.97 $\pm$ 0.13
Man	0.38 $\pm$ 0.13	2.46 $\pm$ 0.03	2.09 $\pm$ 0.32	2.21 $\pm$ 0.82
Gal	3.59 $\pm$ 0.48	14.21 $\pm$ 1.95	15.84 $\pm$ 0.90	10.04 $\pm$ 0.23
Glc	2.31 $\pm$ 0.31	3.09 $\pm$ 0.28	3.22 $\pm$ 0.32	1.63 $\pm$ 0.02

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