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Pectin-rich extracts from olives inhibit proliferation of Caco-2 and THP-1 cells.

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Abstract.

Three olive modified pectin extracts have been produced by heat and acid treatment of alperujo, the major by-product of olive oil production. Their effect on proliferation of the colon carcinoma Caco-2 and the leukemia monocytic THP-1 cell lines has been studied. All pectin preparations inhibited the growth of Caco-2 and THP-1 cells at concentrations ranging from 1 to 10 mg/ml. Interestingly, none of the pectins inhibited the growth of confluent Caco-2 cells, showing the specificity of the antiproliferative effect for the transformed Caco-2 phenotype. In addition, all the pectins inhibited agglutination of red blood cells by galectin-3, a lectin involved in tumor growth, metastasis, and immune cell regulation that has been proposed as a mediator of the anti-tumor effects of modified pectins. In addition, determination of caspase-3 activation in THP-1 cells indicates that treatment with olive pectins triggers apoptosis. These results supporting their possible use of as health-promoting food ingredients or supplements.

Keywords: colon Caco-2 cancer cells; leukemia monocytic THP-1; alperujo; proliferation; galectin-3; caspase.

1. Introduction.

Pectins are very complex heteropolysaccharides that are found in higher plants as structural elements of the cell wall. They are a component of soluble dietary fiber, which is poorly digested in the small intestine but ferments in the colon and plays a significant role in many physiological processes. Thus, pectin has prebiotic properties,

anti-inflammatory activity, and regulates intestinal passage. In addition it aids in controlling diabetes, and in the prevention of obesity and cancer (Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011; Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006). Some possible applications of pectins in tissue engineering, cancer treatment, and gene delivery have been proposed (Morris, Belshaw, Waldron, & Maxwell, 2013; Munarin, Tanzi, & Petrini, 2012).

Modification of pectins by heat and/or chemical treatments yields lower molecular weight fragments that can be more biologically active (Naqash, Masoodi, Rather, Wani, & Gani, 2017). Thus, modified citrus pectins are lower molecular weight pectins that are produced by enzymatic, chemical or heat treatment of citrus pectin, yielding pectins of molecular weight up to 10KDa. It has been found that these pectins inhibit or block aggregation of cancer cells, cellular adhesion, and metastasis (Nangia-Makker, et al 2002).

Although citrus fruits and apple pomace are the major sources of commercial pectin, other sources include residues from agricultural and food industries, which could lead to a revalorization of materials that are other way discarded. This is the case of alperujo, the by-product resulting from the continuous biphasic extraction of olive oil. Alperujo is a combination of the olive vegetative water and solids (skin, seeds, pulp, and pieces of stones) from the olive-pomace mill waste (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, Fernández-Bolaños, 2013) (Babbar, Dejonghe, Gatti, Sforza, & Elst, 2016). Pectic polysaccharides rich in arabinose represent one third of the olive cell walls (Coimbra, Cardoso, & Lopes-da-Silva, 2010). Several million tons of alperujo are produced every year in Spain alone, representing a disposal challenge because of its high content in organic matter and phytotoxic compounds.

We have described two procedures for thermal processing of alperujo at 50-80^{o}C (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Munoz, García, & Fernández-Bolaños, 2015) or 160^{o}C (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Munoz, & Fernández-Bolaños, 2015) that facilitates extraction of residual olive oil as well as solubilization of pectic polysaccharides and other components of interest. The resulting modified pectins extracts, which we named Pectoliv, have good physicochemical properties and health-promoting properties in vitro, in some cases superior to commercial pectin supplements. More recently, Pectoliv samples produced as described (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolaños, 2015), and also subjected to hydrolysis using trifluoroacetic acid, have shown to inhibit proliferation of human bladder cancer cell lines (Bermudez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Fernández-Bolaños, & Sánchez-Carbayo, et al, 2019). Now, we have used chemical treatment using citric acid or sulfuric acid to the treatment at 160^{o}C in order to increase the chances that the resulting modified pectins have positive biological effects. The aim of this study was to determine the effect of these Pectoliv extracts on the proliferation of two different cell lines, Caco-2 and THP-1, in order to establish whether these extracts may inhibit the growth of tumors. The effect of a commercial modified citrus pectin (MCP), Pectasol-C, was also determined for comparison. In addition, the effect of the modified pectins on agglutination of red blood cells by galectin-3 has been determined in order to assess the possible interaction of these pectins with galectin-3. It has been claimed that the beneficial health effects of modified pectins are related to binding to galectin-3, a lectin present at multiple cellular locations that has been implicated in tumorigenesis and cancer progression (Newlaczyl & Yu, 2011). Activation of caspase-3 and release of lactate dehydrogenase have also been determined in order to assess whether apoptosis or plain cytotoxicity are involved in any possible effect on cell proliferation.

2. Material and methods.

2.1 Materials

Human recombinant galectin-3, neutral red, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma. Alperujo, the by-product resulting from the biphasic extraction of olive oil, was provided by the oil extraction factory "Oleicola el Teja" (Córdoba, España). 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 Isolation and purification of olive pectin extracts.

Alperujo, the main by-product of olive oil production, was steam-treated at 160 ºC for 30 min according to the procedure previously described (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Munoz, & Fernández-Bolaños, 2015). The fraction released by heat treatment was ultra-filtered using a 3 kDa membrane. The resulting low molecular weight pectins were precipitated in 80 % ethanol and allowed to dry (Pectoliv-1). The same procedure was also carried out in the presence of 0.5% (w/w) citric acid or hydrochloric acid during steam treatment for production of Pectoliv-2 and Pectoliv-3, respectively.

2.3 Characterization of pectin extracts.

Galacturonan (anhydrogalacturonic acid) was determined according to the m-hydroxydiphenyl method described by Blumenkrantz & Asboe-Hansen (1973) for uronic acids. Glycosyl compositions were determined by gas chromatography (GC) after conversion to alditol acetates. Individual neutral sugars were analyzed from samples with initial trifluoroacetic acid (TFA) hydrolysis (2 N TFA at 121 ºC for 1 h)

prior to reduction, acetylation and analysis by GC (Englyst & Cummings, 1984) using inositol as internal standard. Calibration was performed using a series of standard solutions of L-rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-mannose (Man) and D-xylose (Xyl). The chromatographic conditions were described by Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent & Fernández-Bolaños (2012).

Total phenolic content was determined using the Folin−Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents (Singleton & Rossi, 1965). Protein was determined using the micro Kjeldahl method by applying the conversion factor 6.25 (Banks-Gibson, 1904). Ash was determined according to the AOAC procedure (AOAC, 1990).

The molecular weight distribution of the purified polysaccharide-enriched extracts was estimated by high performance size exclusion chromatography (HPSEC) using two different columns (300 X 7.8 mm i.d., Tosoh Bioscience LLC, King of Prussia, PA) in sequence: TSKgel GMPWXL (dextran MW<50000KDa) and TSKgel G3000PWXL (dextran MW<60KDa), as described previously by Dos-Santos, Jiménez-Araujo, Rodríguez-Arcos, & Fernández-Trujillo (2011). The system was calibrated by regression analysis using 252, 110, 70, 40, 6 kDa dextrans and glucose standards.

2.4 Cell culture and treatment.

Caco-2 and THP-1 cells were kept at 5% CO_2 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, 1% non-essential amino acids, 100 u/ml penicillin, and 100 g/ml streptomycin. 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 renewed once in 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 pectin extracts were dissolved in Hank´s Balanced Salt Solution (HBSS) at 100 mg/ml, heated at 100^{o}C for 30 minutes, and diluted with culture medium as required. Cells were seeded in 96 well microplates (4 x 10^4 or 14 x 10^4 cells/well, 50 µl/well). Extracts were added in the same volume (50 µl/well) to achieve a final concentration of 0.37 to 10 mg extract/ml. Caco-2 and THP-1 cells were incubated for up to 4 and 9 days, respectively. In addition to the cell viability assay as shown below, cells were inspected under the phase contrast microscope.

2.5 Cell proliferation assays

Proliferation of adherent (Caco-2) and suspended (THP-1) cells was determined by measuring viability at different times using the neutral red and 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 reduction of MTT were dissolved by addition of 100 µL HCl (0.1 N) in isopropanol, and absorbance at 570 nm with subtraction of background at 630 nm was measured using a plate reader (Girón-Calle et al., 2010; Kops, West, Leach, & Miller, 1997). Data on cell proliferation was analyzed by one way analysis of variance followed by Tukey's test for pairwise multiple comparisons (n=6).

2.6 Assays for LDH release and caspase activation.

A commercial lactate dehydrogenase (LDH) cytotoxicity assay kit (Pierce-Thermo Scientific, Rockford IL) was used to determine LDH release into the culture medium. The assay is based on the conversion of lactate to pyruvate catalyzed by LDH resulting in reduction of NAD^+ to NADH, which is then used by diaphorase to reduce a tetrazolium salt into a red formazan that absorbs at 490 nm. Reagents were prepared and reactions carried out as instructed in the kit. Incubations for the LDH assay were carried out in 96 well plates using the same routine culture medium as described above, but containing only 5 % FBS and no phenol red to reduce background absorbance. Absorbance was measured at 490 nm with subtraction of background at 680 nm using a plate reader. Absorption of a no reagent blank was subtracted from the samples. Two sets of replicate incubations were carried out for each set of treatments, one for determination of LDH in the extracellular medium, and one for determination of total LDH by treatment with lysis buffer. LDH release was given as the percentage of total LDH that was released into the extracellular medium.

Caspase-3 activity was measured using the EnzChek caspase-3 assay kit (Molecular Probes, Eugene OR). This kit is based on a bis amide derivative of rhodamine, rhodamine 110 bis-(N-CBZ-L-aspartyl-Lglutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD–R110), which is non-fluorescent and has two peptides blocking amino groups in the fluorophore rhodamine 110. Caspase-3 targets these peptides and releases the fluorophore, which has excitation and emission peaks at 496 and 520 nm, respectively. Cells were seeded in 12 well plates, collected by trypsinization, washed, and cell lysates were prepared and assayed for caspase-3 activity as instructed in the kit.

2.7 Agglutination assay

Glutaraldehyde-fixed, trypsinized rat erythrocytes were prepared as follows for agglutination assays. The pellet resulting from centrifugation of rat blood at 500 g for

10 min was washed three times by resuspension in PBS and centrifugation at 500 g for 10 minutes. The pellet was then resuspended in PBS (10% v/v) containing 0.5% (v/v) glutaraldehyde and incubated in a shaker for 1 hour at room temperature. Erythrocytes were recovered by centrifugation and washed with PBS three times as previously described, and were resuspended in PBS (20 % v/v) containing sodium azide (0.1% w/v). The fixed erythrocytes were trypsinized by incubation with trypsin (1 mg/ml erythrocytes) for 30 minutes at 37^{o} C, and washed with PBS three times as described above (Marquardt, & Gordon, 1975).

Agglutination assays were carried out by incubating the trypsinized, glutaraldehyde fixed rat erythrocytes (40 μL, 1.6 mg, 4 % w / v in PBS buffer) in the presence of galectin 3 (0.5 μg/well) in 96 U shape well microplates for 1 hour at room temperature. Because the commercial galectin-3 preparation included lactose as a stabilizer, it was previously dialyzed against phosphate saline buffer in order to remove the sugar. . Galectin-3 was added to erythrocytes as a negative control and incubations of erythrocytes with no sample or no galectin-3 added were used as a positive control.

2.8 Statistic analysis

The SigmaPlot program (Systat Software Inc., San Jose, California) was used for ANOVA and logistic four parameter non-linear regression analyses.

3. Results

3.1 Pectin extraction and characterization.

The chemical composition of the three Pectoliv preparations and MCP are shown in table 1, upper panel. The most important difference between Pectoliv and MCP is the

content in phenolics, which was between 6 and 8 % in the former, but only 0.15 % in the latter. Content in neutral sugars and proteins was also higher in Pectoliv, while content in uronic acid was higher in MCP. The three Pectoliv preparations cannot be considered pectin preparations according to the US Pharmacopeia because they do not meet the criteria of having a minimum content of galacturonic acid of at least 74%. The most important differences in the composition among the three olive extracts were the contents in uronic acid and neutral sugars, which decreased from Pectoliv 1 to Pectoliv 3.

Analysis of the sugar composition by gas chromatography revealed that the three Pectoliv preparations have similar compositions as compared to MCP (table 1, lower panel). About half of the sugar residues were uronic acid, and the percentage of xylose and galactose were between 13 and 20 %. Rhamnose, arabinose, mannose, and glucose were between 2 and 5 %, and fucose was hardly detectable. The sugar composition of MCP was clearly different, having a lot more glucuronic acid, 91 %, and lower concentrations of all other sugars.

As shown in figure 1, estimation of molecular weight by size exclusion chromatography indicated that the Pectoliv preparations included molecules ranging from a few kDa up to several hundred kDa, with a peak and / or shoulder with an average size between 40 and 6 KDa. This is consistent with profiles previously described for other Pectoliv preparations that also suffered acid treatment, and with the modified pectin nature of these extracts (Bermudez-Oria et al 2019). MCP had a similar profile, plus an additional peak corresponding approximately to the molecular weight of single sugars.

3.2 Effect of the modified pectins on cell proliferation.

Caco-2 and THP-1 cells were used as in vitro models in order to determine the effect of the modified pectins in proliferation of cancerous cells. Caco-2 are adherent cells that were originally isolated from a human colon carcinoma (Engle, Goetz, & Alpers, 1998), and THP-1 cells grow in suspension and were isolated from a monocytic leukaemia patient (Tsuchiya et al., 1980). Caco-2 cells were exposed to increasing concentrations of the modified pectins for up to 7 days, and cell proliferation was determined by measuring uptake of the vital stain neutral red at the end of the different incubation periods. These treatments were carried out in complete culture medium by addition of the modified pectins at the same time the cells were seeded. A concentration-dependent inhibition of cell proliferation was caused by MCP and Pectolivs as shown in figure 2. While the lowest concentration, 0.37 mg/ml, did not inhibit proliferation at all, the highest concentration, 10 mg/ml, completely inhibited proliferation. Pectolivs at 3.33 mg/ml inhibited proliferation completely, but the same concentration of MCP still allowed some proliferation. Interestingly, treatment for 7 days with 1.11 mg/ml of any of the modified pectins actually enhanced cell proliferation.

THP-1 cells were exposed to the same concentrations of MCP and Pectolivs than Caco-2 cells, but because these cells grow faster, exposure was carried out for up to four days instead of seven (figure 3). Proliferation was inhibited by MCP and Pectolivs even at the lowest concentration. A comparison of the doses that caused inhibition of proliferation by half (ED50) after incubation for three days is shown in table 2. These data highlights the fact that Caco-2 cells were more susceptible to inhibition of

proliferation than THP-1 cells, and that the effect of Pectoliv was higher than that of MCP.

As mentioned before, incubation of Caco-2 cells with MCP and Pectolivs at low concentration for seven days reversed the inhibition seen after shorter incubation times, and actually enhanced proliferation (figure 2). This might be related with the fact that Caco-2 cells differentiate into an enterocyte-like phenotype when they are allowed to grow as confluent monolayers (Engle et al., 1998). In order to test this hypothesis, Caco-2 cells were seeded at a higher density and allowed to grow for two days before exposure to the models pectins. This time frame allows for the cells to be exposed while they are differentiating but still growing, although at a lower rate than the same cells in non-confluent conditions. This experiment revealed that the differentiating Caco-2 cells were mostly immune to the inhibitory effect of the modified pectins (figure 4). Although there were some statistically significant effects on cell proliferation, these were lower than 10 % at the best and do not follow any concentration dependent pattern.

3.3 Activation of caspase-3 and LDH release.

Inhibition of cell proliferation can be due to an actual slow-down of cell growth, or to induction of cell death via apoptosis and/or necrosis. Caspase-3 is one of the effector caspases that are activated by initiator proteases of the same family, which mediate both the intrinsic and extrinsic pathways for apoptosis. Release of the cytosolic enzyme lactate dehydrogenase (LDH) to the extracellular medium is a hallmark of cell membrane rupture and is used as a cytotoxicity assay. While apoptosis is a programmed

chain of events that results in cell death, cellular death by necrosis is characterized by random cell disruptions that cause release of cellular contents without mediation of the caspase pathway. Activation of caspase-3 and LDH release were determined after incubation of THP-1 cells with MCP and Pectoliv 1 to 3 in order to gain insight on whether these treatments caused programmed cell death and/or necrosis.

As shown in figure 5, treatment with any of the Pectoliv at a concentration of 10 mg/ml for 48 hours caused activation of caspase-3, while treatment with MCP did not. The activation of caspase-3 by Pectolivs somehow resembles the proliferation curves shown in figure 3, indicating that apoptosis might be involved in the antiproliferative effect of these samples. LDH release into the media after incubation with Pectolivs or MCP was lower than 10 % in all cases and no significant difference was found as compared to control (not shown).

3.4 Effect of modified pectins on agglutination of red blood cells by galectin-3

The possible disruption of the agglutination of red blood cells by galectin-3 was used to assess the possible interaction of this protein with MCP and Pectolivs. Concentrations between 4 and 60 mg/ml were tested to determine inhibition of the agglutination caused by 0.5 mg/well galectin-3. Both MCP and Pectolivs were able to inhibit the agglutination of red blood cells by galectin-3, although Pectolivs proved to be more effective than MCP, as shown in table 2.

4. Discussion

Our group has developed procedures for thermal treatment of the alperujo by-product at low (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, Garcia, et al.,

2015a) and high (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolaños, 2015b) temperature so that both extraction of the residual oil and release of bioactive components are facilitated. Fractions resulting from the high-temperature treatment have been called Pectoliv in general because of their relatively high content in pectin, although they do not meet the USP criteria for pectins of having a content in galacturonic acid of at least 74%. The Pectoliv-1 preparation that has been used in the present work has been produced by high temperature treatment of alperujo as described (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolaños, 2015b), while Pectoliv-2 and Pectoliv-3 represent modifications of the original procedure to include treatment with citric and sulphuric acid, respectively. Steam treatment promoted the cleavage of the galacturonic backbone with 47.4-52.5% of uronic acid, together arabinogalactan, as a branched region of pectin, and other polysaccharides differing in their sugar composition, and probably originating from arabinoxylan, glucomannan or xyloglucan, which were previously described as constituents of olive pulp (Jiménez, Guillén, Fernández-Bolaños, & Heredia, 1994). The acid treatment together the high temperature did affect the uronic acid content or reduce the molecular weight, as might be expected by acid hydrolysis (Diaz, Anthon, & Barrett, 2007).

All Pectoliv preparations in general have a complex chemical composition characterized by the formation of complexes of proteins, polyphenols and pectins at high temperatures. The composition of Pectoliv reflects the fact that olive oil extraction and subsequent thermal treatment of the alperujo by-product leads to formation of complexes that link pectins, proteins and phenols together (Capasso, De Martino, & Arienzo, 2002)(Bermudez-Oria et al 2019). Differences in the composition of the olives and the resulting by-product of olive oil extraction, as well as modifications in the thermal treatment, often result in variability in the composition of different Pectoliv

preparations. This is especially true for the content in phenols that can vary from 4 up to 59% (w/w) (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolaños, 2015)(Bermudez-Oria et al 2019). Pectoliv 1 to 3 in this paper are characterized by a quite homogeneous composition with a relatively low content in phenols, 6-8 %, as compared to other Pectolivs.

Caco-2 cells have been used as a model of exposure to components in the diet and drugs (Giron-Calle et al., 2004; Sánchez-Vioque et al., 2016), and as a model of absorption in the gut when they are allowed to differentiate in vitro to an enterocyte-like phenotype (Artursson, Palm, & Luthman, 2001; Girón-Calle et al., 2010). When Caco-2 cells are allowed to grow in confluent cultures they differentiate into polarized cells that resembles the healthy enterocyte in morphology and physiology (Engle et al., 1998). Thus, the same cell line can be used to model both cancerous, transformed cells, and healthy epithelial cells (Giron-Calle et al., 2004). Results of exposure of Caco-2 cells to MCP and Pectolivs reveal that inhibition of proliferation is dependent on the transformed Caco-2 phenotype, supporting the view that this phenomenon is not due to non-specific toxicity to cells. Because we are dealing with an in vitro model of tumors in the colon, the antiproliferative activity of Pectolivs would not depend on the absorption, or lack of, from the gut into the bloodstream.

THP-1 cells complement very well Caco-2 cells as an in vitro model of exposure of tumors to bioactive components. This is so because they are cells of monocytic nature, thus belonging to the immune system, and grow in suspension. Exposure of THP-1 cells to MCP and Pectolivs also revealed a marked inhibition of proliferation. The antiproliferative activity of Pectolivs on both Caco-2 and THP-1 cells was higher than the activity of MCP. Previous report show that pectins, including MCP, apple pectins, and pectin oligosaccharides inhibit proliferation by inducing apoptosis (Jackson

et al., 2007; Leclere et al., 2015; Li et al., 2012), reviewed in (Leclere, Van Cutsem, & Michiels, 2013). Activation of caspase-3 as shown here clearly shows that induction of apoptosis may as well be involved in the antiproliferative effects of Pectolivs. Surprisingly, we did not find activation of caspase-3 by MCP as compared to the control, but this might be due to the fact that MCP has a lower inhibitory activity than Pectolivs, which is just starting to show after two days of treatment. The lack of release of LDH upon treatment with Pectolivs further support the view that apoptosis, and not non-specific cellular toxicity, is involved in the inhibition of cells proliferation.

Several reports found that interaction with galectin-3 appears to be involved in the antitumor effects of pectins, and MCP has been characterized as a galectin-3 antagonist. Our own group recently found a decreased expression of galectin-3 in bladder cancer cells lines treated with Pectoliv, and inhibition of galectin-3 agglutination of red blood cells by the same Pectoliv preparations (Bermudez-Oria et al 2018). Our data is consistent with these results, showing that antiproliferative activity is paralleled by inhibition of agglutination by galectin-3. Interestingly, expression of galectin-3 correlates with transformation and invasiveness in colon cancer as determined in a large number of primary and metastatic tumors (Schoeppner, Raz, Ho, & Bresalier, 1995) and (Nakamura et al., 1999). Other report described an initial decrease in expression of galectin-3, followed by an increase in expression, during colon cancer progression (Sanjuan et al., 1997). Considering these reports, it is tempting to propose that expression of galectin-3 decreases upon differentiation of Caco-2 cells, and that this might explain why proliferation of confluent Caco-2 cells is no longer inhibited by the modified pectins. Nevertheless, galectin-3 expression in the plasma membrane of Caco-2 cells increased between four and eight times with differentiation, as determined using three different proteomic approaches to study changes in protein expression during

differentiation of these cells (Pshezhetsky et al., 2007), and it has actually been reported to be necessary for differentiation of an epithelial cell line (Hikita et al., 2000). Galectin-3 expression in Caco-2 cells may also be increased by exposure to xenobiotics (Isoda, Talorete, Han, & Nakamura, 2006). It should be kept in mind that galectin-3 can be found in different intracellular locations and carries out different functions, so that simply considering expression levels may fail to indicate whether this protein is involved or not in a phenomenon.

Galectin-3 is also involved in regulation of the immune system, and it is especially expressed in monocytes and macrophages, including the THP-1 cell line. In these cells, it is upregulated upon stimulation with lipopolysaccharide (Dabelic, Novak, Goreta, & Dumic, 2012). Galecting-3 is involved in cancers of the blood cells, especially leukemia (Ruvolo, 2016). The THP-1 cell line is one example of how cells of the immune system can express galectin-3 in different locations, and can at the same time bind and respond to this protein when present in the extracellular medium, modulating immune response and progression and metastasis of blood cell cancers (Dabelic et al., 2012).

In conclusion, our data clearly shows that the three Pectoliv preparations used for this study inhibit proliferation of two in vitro models of cancerous cells. One is a model of tumors in the colon, and the other is a model of leukemia. The inhibitory effect was higher than the effect of MCP that was used as reference material. In parallel with this activity, the three Pectoliv preparations induced apoptosis as determined by activation of caspase-3, and inhibited agglutination of red blood cells by galectin-3.

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Figure captions

Figure 1. Molecular weight distribution of Pectoliv 1 to 3 and MCP by size exclusion chromatography. Number above the peaks represent molar masses of dextran standards.

Figure 2. Effect of Pectoliv 1, 2 and 3, and MCP on proliferation of Caco-2 cells. Cells ($4x10^3$ cells/well) were seeded in the presence of increasing concentrations of the modified pectin preparations and allowed to proliferate for one to seven days. Cell number was then estimated by determination of neutral red uptake. Data represent average of six replicates, error bars are not shown for clarity. Asterisks indicate statistically significant difference between treatment and vehicle control for each incubation period (one way ANOVA-Tukey test, $p<0.05$).

Figure 3. Effect of Pectoliv 1, 2 and 3, and MCP on proliferation of THP-1 cells. Cells ($4x10^3$ cells/well) were seeded in the presence of increasing concentrations of the modified pectin preparations and allowed to proliferate for up to four days. Cell number was then estimated by measuring reduction of MTT. Data represent average of six replicates, error bars are not shown for clarity. Data represent average of six replicates, error bars are not shown for clarity. Asterisks indicate statistical significant difference between treatment and vehicle control for each incubation period (one way ANOVA-Tukey test, $p<0.05$).

Figure 4. Effect of Pectoliv1 to 3 and MCP on proliferation of confluent Caco-2 cells. Cells ($14x10^3$ cells/well) were seeded and incubated for two days. Increasing concentration of CMP and Pectolivs were then added and cells were incubated for up to seven more days. Cell number was estimated by determination of neutral red uptake. Data represent average of six replicates, error bars are not shown for clarity. Data represent average of six replicates, error bars are not shown for clarity. Asterisks indicate statistically significant difference between treatment and vehicle control for each incubation period (one way ANOVA-Tukey test, $p<0.05$).

Figure 5. Caspase-3 activation in cells treated with the modified pectins. THP-1 cells ($4x10^3$ cells/well) were seeded in the presence of the modified pectins (10 mg/ml) and incubated for 24 and 48 hours. As a positive control, cells were treated with camptothecin for 4 hours. Data represent average of five replicates, error bars represent standard deviation. Asterisks indicate statistically significant differences between treatments and vehicle control (one way ANOVA-Tukey test, $p<0.05$).

Table1. Chemical composition (% weight, upper panel) and sugar composition (% molar composition, lower panel) of MCP and Pectoliv.

	MCP	Pectoliv-1	Pectoliv-2	Pectoliv-3
		% (w/w)		
Uronic acid	51.52 ± 7.78	40.94 ± 4.27	30.74 ± 1.66	24.57 ± 0.97
Neutral sugar	5.24 ± 0.07	37.03 ± 0.84	34.11 ± 1.95	23.8 ± 1.20
Phenol	0.15 ± 0.01	6.70 ± 0.38	6.33 ± 0.31	7.92 ± 0.51
Protein	3.69 ± 0.06	9.63 ± 0.13	9.31 ± 0.25	7.50 ± 0.38
Ash	20.33 ± 0.19	3.61 ± 0.5	13.81 ± 0.56	26.00 ± 1.64
Moisture	11.43 ± 0.01	6.20 ± 0.01	6.08 ± 0.01	7.7 ± 0.01
Total %	92.36	104.11	100.38	97.49
		% molar		
Rhamnose	1.691 ± 0.11	3.95 ± 0.38	5.43 ± 0.25	5.02 ± 0.4
Fucose	0.12 ± 0.03	0.05 ± 0.01	0.03 ± 0.01	nd
Arabinose	1.08 ± 0.14	6.78 ± 0.22	4.43 ± 0.14	3.21 ± 0.09
Xylose	0.49 ± 0.04	17.70 ± 0.36	19.45 ± 1.8	17.17 ± 1.19
Mannose	0.56 ± 0.07	2.40 ± 0.79	3.95 ± 0.3	3.95 ± 0.01
Galactose	4.67 ± 0.08	13.78 ± 0.79	14.69 ± 0.34	15.91 ± 1.32
Glucose	0.63 ± 0.01	2.84 ± 0.26	4.60 ± 0.2	3.95 ± 0.28
Uronic acid	90.77 ± 0.35	52.50 ± 5.51	47.42 ± 2.54	50.79 ± 2.01

Table 2. Inhibition of cell proliferation and inhibition of erythrocyte agglutination by galectin-3 in the presence of the modified pectins. ED50 (mg/ml) for inhibition of cell proliferation after incubation for three days was calculated using logistic four parameter non-linear regression of some of the data plotted in figures 2 (Caco-2 cells) and 4 (THP-1 cells). Inhibition of agglutination is expressed as the minimum concentration of modified pectins (mg/ml) inhibiting agglutination of red blood cells by galectin-3.

	MCP	**Pectoliv-1**	**Pectoliv-2**	**Pectoliv-3**	**camptothecin**
ED50 Caco-2	2.56	1.31	1.96	2.01	0.04
ED50 confluent Caco-2	-	-	-	-	0.23
ED50 THP-1	> 10	3.89	4.67	3.75	0.01
lowest concentration inhibiting agglutination	32	8	8	4	

Figure 1

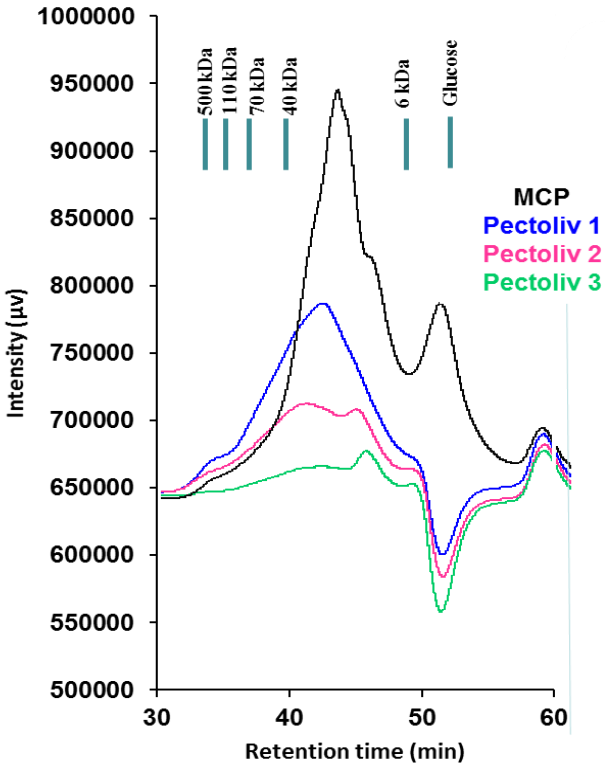
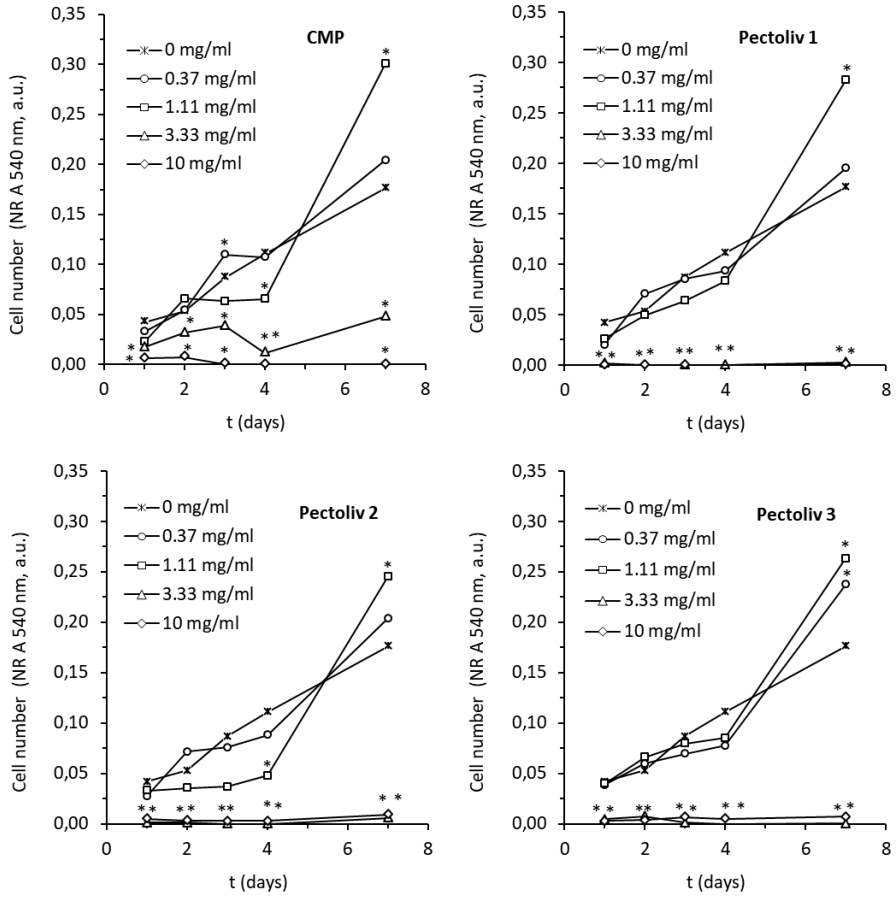

500 kDa
110 kDa
70 kDa
40 kDa
6 kDa
Glucose
MCP
Pectoliv 1
Pectoliv 2
Pectoliv 3
Intensity (µv)
1000000
950000
900000
850000
800000
750000
700000
650000
600000
550000
500000
30
40
50
60
Retention time (min)

Figure 2


CMP
Pectoliv 1
Pectoliv 2
Pectoliv 3
0 mg/ml
0.37 mg/ml
1.11 mg/ml
3.33 mg/ml
10 mg/ml
Cell number (NR A 540 nm, a.u.)
t (days)
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0,05
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0,15
0,20
0,25
0,30
0,35
0
2
4
6
8

Figure 3

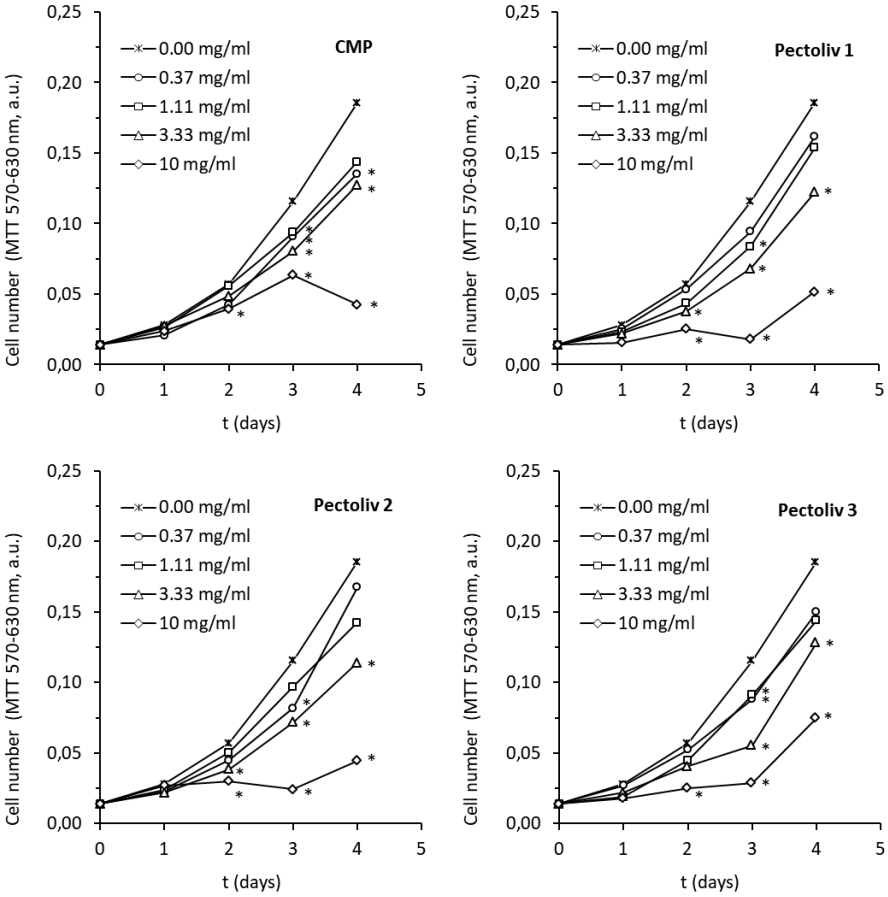

CMP
Pectoliv 1
Pectoliv 2
Pectoliv 3
0.00 mg/ml
0.37 mg/ml
1.11 mg/ml
3.33 mg/ml
10 mg/ml
Cell number (MTT 570-630 nm, a.u.)
t (days)
0,00
0,05
0,10
0,15
0,20
0,25
0
1
2
3
4
5

Figure 4

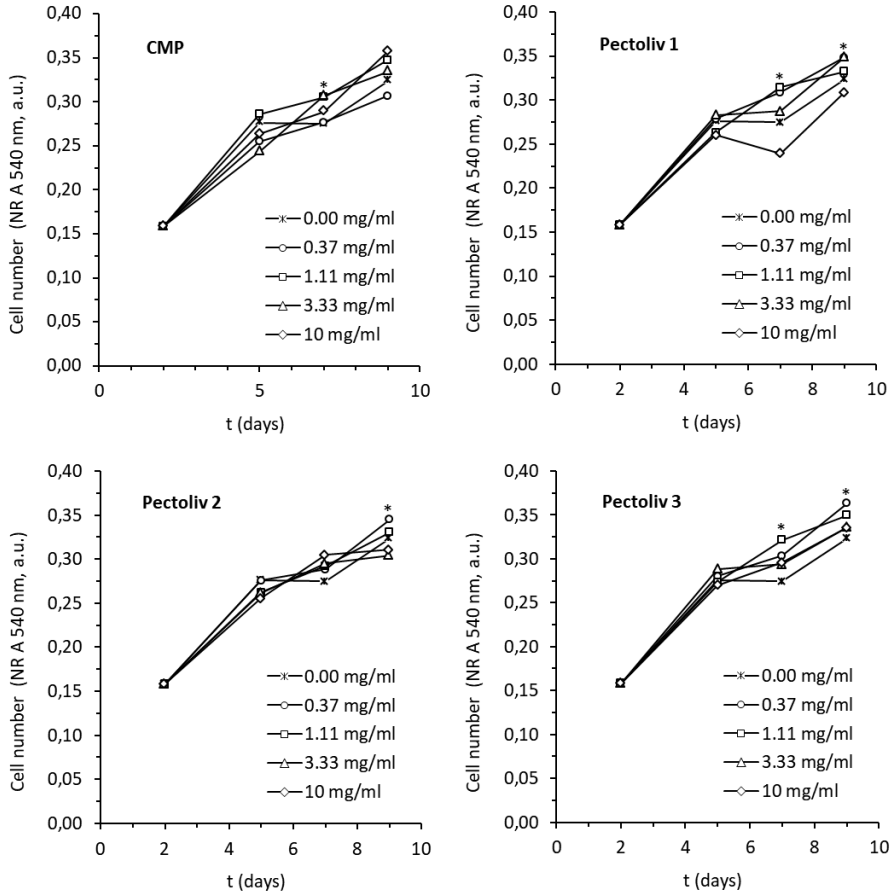
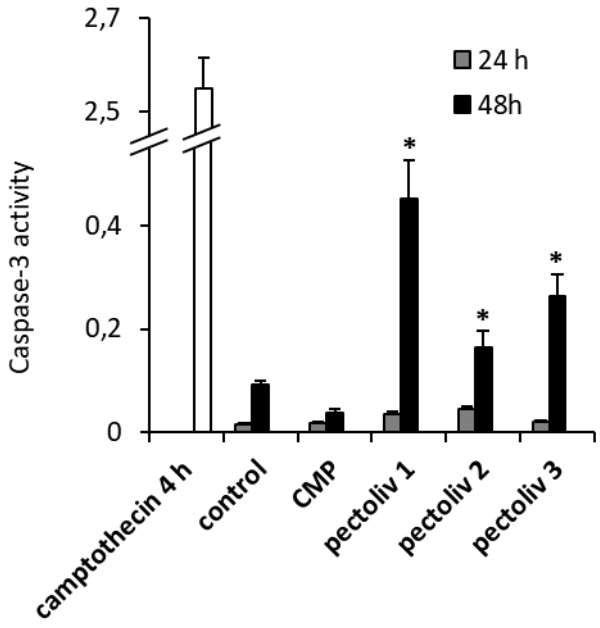

CMP
Pectoliv 1
Pectoliv 2
Pectoliv 3
Cell number (NR A 540 nm, a.u.)
t (days)
0.00 mg/ml
0.37 mg/ml
1.11 mg/ml
3.33 mg/ml
10 mg/ml

Figure 5


2,7
2,5
0,4
0,2
0
Caspase-3 activity
24 h
48h
*
*
*
camptothecin 4 h
control
CMP
pectoliv 1
pectoliv 2
pectoliv 3