**Antiproliferative activity of the olive extract rich in polyphenols and modified pectin
on bladder cancer cells**

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Running title: Olive extracts and bladder cancer

ABSTRACT

Bladder cancer (BC) is one of the most common human cancers. There is an interest in controlling and treating BC and other types of cancer via the use of natural substances and/or combination chemotherapy. Modified forms of pectin have been reported to possess anticancer bioactivity related to the interaction of galactosyl, a main component of pectin, with galectin-3, a carbohydrate binding protein that is overexpressed on many types of cancer cells. In this study, the antiproliferative effect on BC of novel modified pectins extracted from olives was evaluated. Pectoliv extracts, with high polyphenol content associated to polysaccharides rich in pectin, exhibited an important anti-proliferative capacity in vitro against four human bladder cancer cells lines, RT112, T24, J82, and SCaBER. Pectoliv treatment reduced the expression of galectin-1 and galectin-3 and significantly inhibited the agglutination of erythrocytes. Thus Pectoliv may have the potential for development as a novel galectin-3 inhibitor.

Keywords: bladder cancer; modified pectin; alperujo; proliferation; galectin-3; galectin-1

1. Introduction

According to the American Cancer Society, BC is one of the most common cancers among the human population, with 80470 new cases estimated for 2019 in the US.¹ It is associated with high morbidity and mortality rates if not treated optimally. Advanced disease is best treated with sytemic cisplatin-based chemotherapy. There is intense interest in finding other approaches to controlling and treating BC, such as the use of natural substances.

The olive oil manufacturing process, by the continuous two-phase extraction system, generates oil and a by-product that is a combination of liquid (olive vegetative water) and solid olive-pomace waste (skin, peel, pulp and piece of stones), called “alperujo”. An important component of olive pomace is the cell wall material that is mainly composed of cellulose, xylan, glucurunoxytan, xyloglucan and pectic material rich in arabinose.³ The crushing of olives forms the olive paste, and its slow mixing at moderate temperature (milling and malaxation) before oil extraction provokes cell rupture and interactions between cell wall polysaccharides and hydrophilic compounds (phenols, proteins, etc). Oxidation, condensation and/or polymerization reactions by enzymatic or non-enzymatic action provide an important non-carbohydrate polymeric material associated with cell wall polysaccharides.⁴

Previous studies by our group analyzed the pectin material isolated from hydrothermal-treated alperujo (160° C/ 30–60 min)⁵ and from a gentle heat-treated alperujo (50–80° C/1–2 h).⁶ In both cases, the pectins presented different and characteristic properties. Following “gentle” heating, the pectin-like polysaccharides with high polyphenol content showed a high antioxidant activity. The harsh hydrothermal treatment favored the recovery of pectin with a low molecular weight that resembled modified pectin, extracted and modified from numerous sources, mainly citrus^{7,8} or apple.⁹

Modified pectin, consisting of small and short chain pectin molecules, can be

absorbed by the intestinal tract and leads to beneficial biologic activities. For example, evidence suggests that modified pectin can act as an inhibitor of the pro-metastatic protein galectin-3,¹⁰ induce apoptosis, reduce proliferation and inhibit cell adhesion in numerous cancer cell lines.¹¹⁻¹³ Although the mechanisms of pectins' anticancer bioactivity are unknown, interaction with pectin galactan side-chains was postulated to inhibit the function of the pro-metastatic protein galectin-3, inhibiting its capacity to promote cell adhesion and migration, hence preventing tumor growth.¹⁴ Another protein with a carbohydrate recognition domain, galectin-1, has been described as a molecule capable of influencing metastasis and shown to play a role in apoptosis and cell growth regulation.¹⁵

In this study, the anti-proliferative activity of several polysaccharide- and polyphenol-rich extracts obtained from steam-treated alperujo at 150° C for 30 min were investigated in four distinct bladder cancer cell lines and compared with the effect of a commercial modified citrus pectin (MCP) and with two doses of cisplatin commonly used in chemotherapy. Also, the isolated material was characterized and the antioxidant and the hemagglutination activity were evaluated using in vitro assays. In addition, the secretion of galectin-1 and galectin-3 proteins were quantified upon exposure of BC cells to olive extract and modified citrus pectin (MCP).

2. Materials and Methods

2.1. Isolation and purification of olive extracts, named Pectoliv, from alperujo

Five olive extracts (Pectoliv) were isolated from steam-treated alperujo at 150° C for 30 min according to the procedure previously described.⁵ Briefly, samples 1, 3 and 4 were prepared from the fraction released from heat treatment, which was ultra-filtered using a 3 kDa membrane. The fraction with a size greater than 3 kDa was precipitated with 85% ethanol and then purified by ethylenediaminetetracetic acid (EDTA) complexation and

dialysis through a 1 kDa membrane, followed by percolation through a strong ionic exchanger resin. The purified extracts are referred to as Pectoliv 1, Pectoliv 3, and Pectoliv 4.

Samples 2 and 5 were prepared from samples 1 and 4, respectively, which were subsequently treated with a chemical hydrolysis at 95° C with 2 N trifluoroacetic acid during 3 h in order to remove the neutral sugars and increase the proportion of galacturonan backbone with respect to neutral polysaccharides. Precipitation with 85% ethanol facilitated the precipitation of an insoluble material containing the extracts. The resulting precipitates were named Pectoliv 2 and Pectoliv 5. (Pectoliv 1→Pectoliv 2)(Pectoliv 4→Pectoliv 5).

Alperujo (olive pomace) was collected directly after the two-phase centrifugal system used in local pomace processing (Oleícola el Tejar, Córdoba, Spain) for the extraction of olive oil at three different times throughout the olive season.

2.2. Characterization of the isolated olive extracts

Galacturonan (anhydrogalacturonic acid) was estimated according to the m-hydroxydiphenyl method described by Blumenkrantz & Asboe-Hansen¹⁶ for uronic acids.

Glycosyl compositions were determined by gas chromatography (GC) after their conversion to alditol acetates, and quantified as alditol acetates. Individual neutral sugars were analyzed from duplicate samples with initial trifluoroacetic acid (TFA) hydrolysis (2 N TFA at 121° C for 1 h) prior to reduction, acetylation, and analysis by GC.¹⁷ Inositol was used as an internal standard. Calibration was performed with a series of standard sugar solutions of L-rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-mannose (Man) and D-xylose (Xyl). The chromatographic conditions utilized were previously described.¹⁸

Total phenolic content was determined by the Folin–Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents.¹⁹ Protein was determined by the micro Kjeldahl method.²⁰ The analysis of ash was performed according to the Association of Official Agricultural Chemists procedure (AOAC).²¹

2.3. *Relative molecular weight determination*

The homogeneity and molecular weight distribution of the purified polysaccharide-enriched extracts were 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<50000 kDa) and TSKgel G3000PWXL (dextran MW<60 kDa) as described previously.²² The system was calibrated with standard dextrans of 252, 110, 70, 40, and 6 kDa, and glucose, using a regression curve.

2.4. *Culture of bladder cancer cell lines*

Four bladder tumor cell lines were obtained from the American Type Culture Collection and grown under standard tissue culture protocols, one transitional superficial or non-muscle invasive (RT112), two muscular invasive (J82, T24), and one squamous (ScaBER). Cells were grown for no longer than four to six passages and harvested at 75–90% confluence. After harvesting, cell pellets were washed three times in cold PBS and frozen at -20° C for protein extraction. To test inter-assay reproducibility, all experiments were evaluated in triplicate in independent runs.

2.5. *Cisplatin and pectin exposure*

Cells at a minimum of 70% confluence were exposed to Pectoliv extracts 1-5 (3.1, 6.3, and

12.5 mg/mL), commercial modified citrus pectin (MCP) (PectaSol-C, Econugenics, Santa Rosa, California) (3.1, 6.3, and 12.5 mg/mL) and cisplatin (CDDP) (25 and 50 μ M) and observed at different time points for each functional assay.

2.6. Proliferation assay - MTT

Ninety-six-well plates were seeded in triplicate, each with 5×10^5 cells/well in DMEM containing 10% FBS and incubated for 24 h. Proliferation of bladder cancer cells was determined by measurement of proliferation after 24 h of treatment with Pectoliv 1-5, MCP and cisplatin using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT cell proliferation kit (Roche Diagnostics) was used as recommended by the manufacturer. The number of viable cells is determined based on the formation of purple formazan crystals by the enzymes in viable cells. After 24 h incubation with the treatment samples, media was removed from the well plates, and 100 μ L MTT reagent (50 μ g/mL) added per well at 37° C in 5% CO₂. After 4 h incubation, the supernatant was removed and 100 μ L DMSO added per well to dissolve blue formazan crystals, and the absorbance read at 595 nm. All experiments were evaluated in triplicate in independent runs.

2.7. Protein analysis of galectins in supernatants of bladder cancer cell lines (ELISA assay)

Human bladder cancer cell lines were treated with different Pectoliv extracts or MCP before or after cisplatin treatment for 24 h. Cell supernatants were analyzed by enzyme-immunoanalyses using galectin-1 and galectin-3 ELISA kits (Calbiochem) and absorbance measured at 450 nm. Protein solutions were analyzed in duplicate in independent runs using Bradford Reagent (Sigma-Aldrich) and absorbance measured at 595 nm. All samples

were prepared in triplicate and absorbance measurements taken for each sample in duplicate.

2.8. Hemagglutination assay

A semi-quantitative agglutination assay was carried out by incubating the trypsinized, glutaraldehyde-fixed rat erythrocytes (40 μ L, 1.6 mg, 4% w/v in PBS buffer) in the presence of increasing concentrations of Pectoliv and MCP in 96-well U-bottom microplates for 1 h at room temperature. Agglutinating activity was categorized as negative or positive for each well. Galectin-3 (0.5 μ g/well) 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.

2.9. Statistical analysis

Results were expressed as mean values $\pm$ standard deviations. STATGRAPHICS® plus software was used for statistical analysis. Comparisons amongst samples were made using one-way analysis of variance (ANOVA) and the LSD method. A p-value <0.05 was considered significant.

3. Results

3.1. Obtention and chemical characterization of olive extracts

The isolation and purification of olive extracts Pectoliv 1, Pectoliv 2 and Pectoliv 3 was carried out using three different samples of alperujo obtained from hydrothermal treatments at 150° C for 30 min, following a procedure similar to that used for the isolation and purification of extracts rich in pectic polysaccharides as previously reported.⁵ Only one repetition of the thermal treatment and purification process was made. However, these three

195 samples of Pectoliv were obtained from three different samples of “alperujo” treated by
196 hydrothermal treatment at three different times. The composition of this isolated material
197 (Table 1A) showed a total sugar content (the sum of the percentage of uronic acid and
198 neutral sugar) was 65–75%, with a very high ratio of neutral and acidic polysaccharides
199 (2.5 to 10) in comparison with the previously reported extract (1.1 to 2.6); whereas the
200 phenolic compounds presented values of 17–24%, more than double that reported for the
201 material isolated in previous work.⁵ This difference was probably due to the wide
202 variability of alperujo samples (variety of olive, state of ripening, influence of season) and
203 the different treatment conditions. The phenolic compounds seem to be responsible for the
204 brown color of the extracts. These pigments from olive mill waste were identified as
205 catechol-melanin macromolecules derived from the polymerization action of the phenolic
206 compounds linked to polysaccharides, protein and fatty acids.⁴

207 Analysis of the neutral sugar composition of the isolated extracts (Table 1A)
208 showed the presence of Ara, Gal, and Xyl in high proportions. Minor amounts of Rha, Man
209 and Glc were also present in the three samples. Steam treatment promoted the cleavage of
210 the galacturonic backbone and the arabinan and galactan side chains, together with other
211 polysaccharides differing in their sugar composition, and probably originating from
212 arabinoxylan, glucomannan or xyloglucan, which were previously described as constituents
213 of olive pulp.³

214 The samples Pectoliv 1 and 4 were transformed by acid hydrolysis in order to
215 reduce the ratio of neutral and acidic polysaccharides and to obtain a more similar
216 composition to commercial MCP, a known inhibitor of galectin-3. Chemical analysis of
217 Pectoliv 2 and 5 compared with Pectoliv 1 and 4 showed a substantial loss of neutral sugars
218 and an increase of uronic acid, of 1.92 or 2.46 times, respectively (Table 1B). However, the
219 phenolic compounds in Pectoliv 2 increased from 23.9 to 72.3 g/ 100 g extract, while in

Pectoliv 5 there was practically no change, although the values were very high in comparison with MCP, in which phenolic compounds were practically absent. In this case, and following the indications of the US Pharmacopeia, that define pectin as having not less than 74% of galacturonic acid, Pectoliv 5 (with 84% of uronic acid) was similar to MCP (91%) and was the only sample that could be considered as modified pectin from alperujo. Nevertheless, the other samples could be considered pectin-like polysaccharides and all have a high proportion of associated phenols (Table 1B).

3.2. *Determination of molecular size distribution*

Pectoliv 1 had an approximate molecular size distributed in the range of 110 KDa and 2 KDa, while Pectoliv 3 and 4 ranged approximately between 500 KDa and 2 KDa (Figure 1). In the three samples, the highest proportion of compounds eluted in the zone with an average size between 70 KDa and 2 KDa. Reduction of the neutral polysaccharides after acid hydrolysis was reflected in the relative molecular size of Pectoliv 2 and 5, with a decrease in the intensity of zones corresponding to high molecular weight eluates, indicating a breakage of the polysaccharide chains into smaller ones. Both extracts eluted between 40 and 2 KDa, with a maximum peak close to 16 KDa, similar to the molecular size of MCP (3–13 kDa according to specifications), thus they theoretically can be considered as systemically bioactive.

3.3. *Antiproliferative effect on bladder cancer cells*

We evaluated the functional effect of the five Pectoliv extracts at 12.5 mg/mL on four different human bladder cancer cell lines and compared this with the effect on proliferation of a commercial MCP at the same concentration or cisplatin at 25 and 50 μ M. Pectoliv exposure produced an important inhibition of proliferation after 24 h in all the bladder

cancer cell lines studied, as observed by the MTT assay (Figure 2). One only repetition was realized by each of the five Pectoliv samples on the four bladder cancer cell lines due to the results were very similar for each five samples. Moreover, the inhibitory effect was higher with respect to commercial MCP for all cancer cell lines. MCP was recently reported to suppress T24 and J82 bladder cancer cell proliferation after 72 h of treatment at 5–20 mg/mL by sulforhodamine B (SRB) assay.⁸

The squamous SCaBER cells were the most sensitive to Pectoliv extracts, with an 80% reduction in cell proliferation, whereas RT112 cells were the least sensitive with a reduction of 20–40%. The olive-derived extracts had a similar or even more effective antiproliferative effect on SCaBER cells than cells treated with cisplatin, a common chemotherapeutic agent. It is interesting to note that none of the Pectoliv extracts inhibited the growth of confluent cultures of human colon carcinoma Caco-2 cells lines (unpublished results). When Caco-2 cells are allowed to grow in confluent cultures they differentiate and transform into healthy epithelial cells.^{23,24}

Further proliferation analyses were performed in these two cell lines comparing each Pectoliv extract versus MCP at increasing concentrations. The results showed that the olive-derived extracts affected cell proliferation more considerably than MCP in both the more sensitive (SCaBER, Figure 3A) and less sensitive (RT112, Figure 3B) cell lines. This higher efficacy could be due to the presence of phenols, which are practically absent in the MCP. However, despite the different amounts of phenols in the extracts, the decreases of cell proliferation in SCaBER cells were very similar; whereas in RT112 cells, the Pectoliv 2 sample, with a higher value of phenolic compounds (72%), showed a somewhat lower inhibition of proliferation. Furthermore, no relationship was observed between the different ratios of neutral/acid polysaccharides and the comportment of Pectoliv 1, 3, and 4 with respect to extracts 2 and 5. Thus, structure-function studies on the bioactivity of the

Pectoliv extracts are required for a better understanding of their anticancer effects. However, the high antioxidant activity (293–1062 $\mu\text{mol Trolox/g}$ extract) (Figure 4) and the presence of an important amount of phenolic compounds found in the extracts could be related to their antiproliferative activity. These findings are in agreement with previously published observations of different degrees of potency depending on the type of cancerous cell line and antioxidant extract studied.^{25,26}

3.4. *Galectin-1 and galectin-3 secretion upon exposure to Pectoliv extracts*

An elevated expression of galectin-1 and galectin-3 has been reported in bladder cancer cells.^{27,28} Galectin-1 and -3 are β -galactose-binding proteins involved in different steps of tumor progression (regulation of proliferation, apoptosis, cell cycle, metastasis and angiogenesis) and are potential targets for anti-cancer therapy.^{28,29} In the case of galectin-1, its secretion by tumor cells suppresses the tumor immune response through the induction of apoptosis of activated T cells.³⁰ Cytoplasmic galectin-3 is a well-known anti-apoptotic agent.³¹ In addition, the targeting of galectin-3 by MCP was been demonstrated in T24 and J82 bladder cancer cells.⁸ Thus, we explored the secretion of galectin-1 and galectin-3 proteins in RT112 and SCaBER cells when exposed to Pectoliv extracts and MCP by ELISA in cell supernatants. Our results show that Pectoliv treatment slightly suppressed galectin-1 and -3 protein expression, whereas MCP significantly reduced the expression of galectin-1 (Figure 5A, B). The levels of galectin-1 and -3 only increased when SCaBER cells were treated with Pectoliv-1 or, in the case of RT112 cells, with Pectoliv-2, the extract with the highest phenolic concentration. The inhibition of galectin-1 expression by MCP, and to a lesser extent by Pectoliv, is interesting since galectin-1 constitutes an important mechanism of tumor-immune escape due to the apoptosis of T cells. Its inhibition could potentially be targeted as an effective immune response against tumor cells, as proposed by the group of Rabinovich.³⁰

3.5. Inhibition of galectin-3 mediated hemagglutination

The galectin inhibitory activity of the five Pectoliv extracts isolated from olive by-products and commercial MCP were evaluated using the hemagglutination assay. The minimum inhibitory concentration (MIC) of pectin required to inhibit the galectin-3-mediated agglutination of red blood cells, was determined.

In the assays, MCP inhibited agglutination at an MIC of 32 mg/mL, whereas Pectoliv extracts showed a much more potent agglutination inhibition, with MICs of 4–8 mg/mL (Figure 6). The potential inhibition mechanism for Pectoliv and MCP could be different. In the case of MCP, the binding of galectin-3 to β -D-galactoside residues present on metastatic cancer cells may be blocked.³¹ In contrast, the hemagglutination inhibitory activity of Pectoliv could be attributed to the interactions of polyphenols present in the hydrocolloid extracts, which include in their molecular structure both aromatic rings and hydroxyl groups, with the galectin-3 protein preventing the agglutination of lectin.

4. Conclusions

Our study showed that Pectoliv extracts, obtained from thermally-treated alperujo, are constituted of pectin-like polysaccharides with different side chains made up of neutral sugars that are strongly bound to the phenol polymeric component. In this paper, we demonstrated that Pectoliv extracts show a high antioxidant capacity and an important suppression of bladder cancer cell proliferation *in vitro*. We also found that treatment of Pectoliv and commercial MCP was associated with decreased expression of galectin-1 (a protein that induces the apoptosis of T-cells, conferring immune privilege to tumor cells) and galectin-3 (an anti-apoptotic protein). Our findings confirm that Pectoliv significantly inhibits the agglutination of erythrocytes and could potentially be developed as a novel galectin-3 inhibitor. These results clearly indicate that the five Pectoliv preparations

obtained from the by-product of olive oil extraction and used for this study inhibit proliferation of four in vitro models of bladder cancerous cells of the similar form and confirm its antiproliferative efficacy. Future research based on the anticancer properties of the Pectoliv extracts that include *in vivo* tests and clinical studies, are required to attest their functionality in humans.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5.

		Sample	Concentration tested (mg/ml)				
			4	8	16	32	64
Pectoliv		MCP	NI	NI	NI		I
	1			I	I	I	I
	2			I	I	I	I
	3			I	I	I	I
	4		NI		I	I	I
	5		NI		I	I	I
		C+ C-

Figure 7

Table 1. Chemical composition of Pectoliv extracts of steam-treated olive by-product (alperujo) and commercial modified citrus pectin (MCP) expressed as g/100 g sample. Glycosyl residue composition analysis (% molar ratio). A) Pectoliv samples 1, 3, and 4; B) Pectoliv samples 2 and 5 and MCP. The data shown are mean $\pm$ standard deviation. Rha: rhamnose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; UrA: uronic acids.

A)	Pectoliv		
	1	3	4
Uronic acid	5.80 $\pm$ 1.05	16.48 $\pm$ 1.72	18.46 $\pm$ 1.96
Neutral sugar	59.91 $\pm$ 3.24	59.20 $\pm$ 15.10	46.91 $\pm$ 1.93
Phenol	23.92 $\pm$ 3.33	16.80 $\pm$ 0.35	22.75 $\pm$ 1.67
Protein	0.99 $\pm$ 0.26	3.34 $\pm$ 0.13	4.16 $\pm$ 0.20
Ash	2.30 $\pm$ 0.22	1.73 $\pm$ 0.14	3.22 $\pm$ 0.19
	92.92	97.56	95.50
% molar			
Rha	5.24 $\pm$ 0.15	3.68 $\pm$ 1.01	5.03 $\pm$ 0.17
Ara	20.66 $\pm$ 1.37	23.75 $\pm$ 6.30	29.19 $\pm$ 1.33
Xyl	26.37 $\pm$ 1.92	26.20 $\pm$ 6.46	7.05 $\pm$ 0.31
Man	7.34 $\pm$ 0.06	3.56 $\pm$ 1.11	4.37 $\pm$ 0.13
Gal	22.21 $\pm$ 0.83	15.79 $\pm$ 3.69	19.04 $\pm$ 0.69
Glc	8.88 $\pm$ 0.26	6.12 $\pm$ 1.63	7.96 $\pm$ 0.31
UrA	8.80 $\pm$ 1.67	25.63 $\pm$ 2.67	28.71 $\pm$ 3.05

B)	MCP	Pectoliv	
		2	5
Uronic acid	51.52 ± 7.78	11.14 ± 0.59	45.43 ± 6.53
Neutral sugar	5.24 ± 0.07	15.30 ± 1.59	8.64 ± 1.09
Phenol	0.15 ± 0.01	72.30 ± 1.73	19.16 ± 1.02
Protein	3.69 ± 0.06	0.52 ± 0.01	8.13 ± 1.73
Ash	20.33 ± 0.19	1.95 ± 0.20	7.02 ± 0.18
	80.93	101.21	88.39
% molar			
Rha	1.69 ± 0.11	15.42 ± 2.62	2.94 ± 0.39
Ara	1.08 ± 0.14	1.14 ± 0.07	1.08 ± 0.51
Xyl	0.49 ± 0.04	1.97 ± 0.13	1.23 ± 0.26
Man	0.56 ± 0.07	31.25 ± 2.51	7.57 ± 0.54
Gal	4.67 ± 0.08	1.29 ± 0.00	0.72 ± 0.10
Glc	0.63 ± 0.01	6.55 ± 0.44	2.37 ± 0.12
UrA	90.77 ± 0.35	42.07 ± 0.27	83.96 ± 13.33

Figure captions

Figure 1: Scheme of isolation and purification procedure of Pectoliv extracts.

Figure 2: Refractive index elution profiles from the Pectoliv 1-5 extracts obtained by high performance size exclusion chromatography with two TSK gel columns (GMPWXL and G3000PWXL) placed in a series. Number above the peaks represent molar masses of dextran.

Figure 3: Effect of Pectoliv extracts and MCP at 12.5 mg/mL or cisplatin (CDDP) at 25 and 50 μ M on the proliferation of human bladder cancer cell lines (RT112, J82, T24 and SCaBER) by the MTT assay. Cells were treated with Pectoliv and MCP at the indicated concentration for 24 h at 37° C. Proliferation (%) is expressed as relative percentages to cell control. Data represent the mean of three incubations $\pm$ SD. * indicates statistical significance with respect to MCP. # indicates statistical significance with respect to CDDP 25 μ M ($p < 0.05$). Bars represent mean $\pm$ SD.

Figure 4: Anti-proliferative activity of Pectoliv or MCP at three concentrations (3.1, 6.3, and 12.5 mg/mL) for 24 h on A) SCaBER and B) RT112 cells. Cell proliferation was analyzed by MTT assay. Proliferation (%) is expressed as relative percentages to cell control. Data represent the mean of three incubations $\pm$ SD. Asterisks indicate statistical significance with respect to MCP ($p < 0.05$). Bars represent mean $\pm$ SD.

Figure 5: Antioxidant capacity of Pectoliv 1-5 extracts compared to those for commercial citrus pectin and commercial modified citrus pectin (MCP). Oxygen radical capacity (ORAC) is expressed as μ Mol Trolox/g extract. Each bar is the average value of three replicates. The error bars represent standard deviations ($n = 3$).

Figure 6: Effect of Pectoliv, MCP and cisplatin on the expression of galectin-1 and galectin-3 in RT112 (less sensitive) and SCaBER (more sensitive) bladder cancer cells. (A, B) The cells were treated for 24 h with Pectoliv and MCP (12.5 mg/mL). At the protein level, the expression was assessed by ELISA assay. The data are shown as mean $\pm$ SD of triplicate experiments. Asterisks indicate not statistical significance respect cell control ($p < 0.05$).

Figure 7: The inhibitory activity on galectin-3 mediated erythrocyte agglutination (inhibition, I and non-inhibition, NI) of Pectoliv extracts and MCP at different

concentrations. Positive control (C⁺): incubations of erythrocytes with no sample or galectin-3; negative control (C⁻): galectin-3 added to erythrocytes.