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Identification of a cytotoxic molecule in heat-modified citrus pectin

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ABSTRACT

Modified forms of citrus pectin possess anticancer properties. However, their mechanism of action and the structural features involved remain unclear. Here, we showed that citrus pectin modified by heat treatment displayed cytotoxic effects in cancer cells. A fractionation approach was used aiming to identify active molecules. Dialysis and ethanol precipitation followed by HPLC analysis evidenced that most of the activity was related to molecules with molecular weight corresponding to low degree of polymerization oligogalacturonic acid. Heat-treatment of galacturonic acid also generated cytotoxic molecules. Furthermore, heat-modified galacturonic acid and heat-fragmented pectin contained the same molecule that induced cell death when isolated by HPLC separation. Mass spectrometry analyses revealed that 4,5-dihydroxy-2-cyclopenten-1-one was one cytotoxic molecule present in heat-treated pectin. Finally, we synthesized the enantiopure (*4R,5R*)-4,5-dihydroxy-2-cyclopenten-1-one and demonstrated that this molecule was cytotoxic and induced a similar pattern of apoptotic-like features than heat-modified pectin.

1. Introduction

Despite the development of "smart" or targeted drugs as new therapeutic approach for cancer patients, refractory disease still represents a high hurdle hampering successful chemotherapy. More than 60% of anti-cancer drugs in use nowadays are derived from natural products (Cragg & Newman, 2005, 2013). Vinca-alkaloids, camptothecin, etoposide or taxanes are such examples. Successful remission can be achieved with chemotherapy in most cases but refractory diseases and relapses remain a major obstacle. Indeed, resistance to chemotherapy develops and is still a high hurdle to get over for efficient cancer patient treatment. New molecules are thus needed that could be used alone or in combination with currently used drugs to enhance therapeutic success. Among promising avenues, pectin and pH- or heat-modified pectin have demonstrated chemopreventive and antitumoral activities against some aggressive and recurrent cancers (Leclere, Van Cutsem, & Michiels, 2013).

Pectins are abundant and complex polysaccharides present in the primary cell wall of plants. It is composed of homogalacturonan (HG), substituted galacturonans, rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). HG is a polymer of α-1,4-linked-galacturonic acid. HG residues can be methyl-esterified at the C-6 carboxyl or acetylated at the O-2 or O-3 depending from the pectin source. Side chains of RG-I and RG-II, whose structure can be very complex, are attached to a backbone of HG (Harholt, Suttangkakul, & Vibe Scheller, 2010; Mohnen, 2008). Citrus pectin consists mainly of HG, lower amount of RG-I and very few RG-II structural domains (Yapo, Lerouge, Thibault, & Ralet, 2007). Acidic pH has been used to modify pectin and generate lower molecular weight fragments able to compete with galectin-3 (Gao et al., 2012). Galectin-3 is a lectin that possesses a conserved carbohydrate-binding domain that specifically recognizes ß-galactoside moieties. It has been described to be overexpressed in metastatic cancers (de Oliveira et al., 2010) and promotes cell migration and survival (Fortuna-Costa, Gomes, Kozlowski, Stelling, & Pavao, 2014). Several works have demonstrated its anti-cancer activities both in vitro and in vivo (Chauhan et al., 2005; Inohara & Raz, 1994; Nangia-Makker et al., 2002).

In another study, pectin can also be modified by heat treatment. Different polysaccharide fractions isolated from ginseng rich

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in HG stopped cell cycle in G2/M phase on colon cancer HT-29 cells. On the other hand, fractions rich in HG and modified by heat treatment exerted a much higher anti-proliferative activity, which was accompanied by caspase-3 activation and apoptosis induction (Cheng et al., 2011). Similarly, rhamnogalacturonan I domain-rich pectin isolated from potato inhibited in vitro HT-29 cell proliferation and provoked a cell cycle arrest in G2/M phase. This inhibition was due to a decrease in cyclin B1 expression and in CDK-1 activity (Cheng et al., 2013). Kang et al. (2006) also produced a citrus pectin-derived oligosaccharide, which was biologically active, but they used irradiation instead of heat treatment. Pectin irradiated with 20 kGy and then dialyzed (molecular weight <10,000) inhibited cancer cell growth.

Jackson et al. (2007) showed that different treatment protocols of pectin can lead to differences in pectin apoptosis-inducing activity and that heat-fragmented pectin has a cytotoxic effect in galectin-3 expressing but also in non-expressing prostate cancer cells. This indicates that the active molecules in pH-modified and heat-treated pectin are not the same. Treatment of heat-modified pectin with pectinmethylesterase to cleave galacturonosyl carboxymethylesters and/or with endopolygalacturonase to remove non-methylesterified HG did not change the pro-apoptotic activity of the modified pectin. On the other hand, mild base treatment that eliminated the ester bounds destroyed the pro-apoptotic activity. Cytotoxic activity thus requires a base-sensitive linkage in oligosaccharides other than a carboxymethylester bound. Size analyses of the active fragments suggested low mass (10–20 kDa) oligosaccharides (Jackson et al., 2007). Recently, we showed that citrus pectin modified by heat treatment induced cell death in HepG2 and A549 cells. The induced cell death differs from classical apoptosis since a different pattern of caspase 3 cleavage was detected. Autophagy was also induced (Leclere et al., 2015).

However, besides these different studies, the nature of the active molecules in both products is not known. In this work, we used a fractionation approach of heat-modified citrus pectin aiming to identify molecules exerting a cytotoxic activity toward cancer cells. We report herein the isolation, synthesis and structural analysis of one such molecule, 4,5-dihydroxy-2-cyclopenten-1-one.

2. Material and methods

2.1. Fractionation of citrus pectin by heat treatment

Heat fragmented citrus pectin (HFCP) was obtained according to the method described by Jackson et al. (2007). A solution of 0.1% citrus pectin (Sigma P9135) composed (on a dry weight basis) of 74% homopolygalacturonic acid, 8.7% neutral sugars, 6.7% methoxy groups and 2.5% Na, was heated at pH 4.2 for 60 min at 121 °C under a pressure of 1 bar. The solution was then frozen at −80 °C and lyophilized. The dry material was stored at 4°C. Galactose, galacturonic acid, glucose and glucuronic acid monomers (1 g/l, Sigma) were heat-treated at pH 2.9 for 7.5 h.

2.2. Cell culture and pectin incubation

HepG2, A549, A431, HeLa, MDA-MB-231 and MCF10A cells were obtained from the American Type Culture Collection HepG2 cells were cultured in DMEM medium (Gibco 31825-023), A549 cells in MEM medium (Gibco 41090-028), A431 cells in DMEM high glucose and pyruvate (Gibco 41966-029), Hela cells in MEM, HEPES, GlutaMAX supplement (Gibco 42360-024) supplemented with sodium pyruvate (100 mM, Gibco 11360-039) and MEM non-essential amino acids solution (100x, Gibco 11140-035) and MDA-MB-231 cells in RPMI 1640 medium (Gibco 21875-034). For routine culture, media were supplemented with 10% fetal bovine serum (Gibco 10270), and the cells were kept at 37 °C in an atmosphere of 95% air and 5% CO_2. MCF10A cells were cultured in DMEM/F12 medium (Gibco 11320-074) containing 5% horse serum (Gibco 16050-122), 20 μg/mL EGF, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin and 100 ng/mL cholera toxin. They were maintained in culture in 75 cm^2 polystyrene flasks (Corning) with Basal Medium Eagle (BME, Invitrogen), supplemented with L-glutamine (2 mM, supplied by Sigma-Aldrich). For routine culture, both media were supplemented with 10% fetal bovine serum (Gibco 10270) and cells were kept at 37 °C in an atmosphere of 95% air and 5% CO_2. For treatment, cells were allowed to adhere for 24 h after seeding. Media were discarded, cells washed twice with PBS (Lonza BE17-516F) and placed in their corresponding medium, without serum, containing the following: 3 mg/mL of filter-sterilized HFCP, 3 mg/mL of filter-sterilized citrus pectin or 50 μM etoposide, used as a positive control. Negative controls were cells incubated in media alone.

2.3. Cell viability assay

HepG2 cells were seeded at 50 000 cells/well in 24-well plates before treatments for 24 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma M2128) solution was prepared at a concentration of 2.5 mg/mL in phosphate-buffered saline and 500 μL were added per well. After 2 h at 37°C and 5% CO_2 atmosphere, media and MTT solution were removed before adding lysis buffer. Optical density was measured 1 h later at 570 nm using a microplate spectrophotometer (Biorad × Mark Microplate spectrophotometer) and *Microplate manager 6* software. The MTT test measures the number of metabolically active (viable) cells.

2.4. Cell cytotoxicity assay

HepG2 cells were seeded at 50 000 cells/well in 24-well plates before incubation for 24 or 48 h. For each well, lactate dehydrogenase activity was measured in the supernatant, in the detached cells and in adherent cells after lysis in PBS containing 10% Triton X100 (Merck 9036-19-5). Lactate dehydrogenase activity was detected by colorimetric assay using a cytotoxicity kit (Roche 11644 793 001) and a microplate spectrophotometer. Cytotoxicity percentages were calculated by the ratio of the quantity of LDH present in the supernatant and in detached cells on the total quantity of LDH as in the formula: $100 \times (a+b)/(a+b+c)$ where a = supernatant LDH; b = detached cells LDH; c = adherent cells LDH. 0% LDH release means that all cells are viable while 100% LDH release indicates that all the cells are dead.

2.5. Western blot analysis

Cell lysates were prepared in lysis buffer (40 mM Tris; pH 7.5, 150 mM KCl, 1 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail (Complete from Roche Molecular Biochemicals; 1 tablet in 2 mL of H_2O, added at a 1:25 dilution) and phosphatase inhibitor buffer (25 mM $NaVO_3$, 250 mM PNPP, 250 mM α-glycerophosphate and 125 mM NaF, at a 1:25 dilution). The medium was centrifuged, and pelleted cells were added to cell lysates. The lysates were then centrifuged at 12,000 × g for 5 min, and the supernatants were collected. The proteins (15 μg) were denatured with the addition of LDS sample buffer (Invitrogen NP0007) and heated to 70 °C for 10 min. The proteins were resolved on a 4–12% NuPAGE (Invitrogen) gel and transferred to a low-fluorescence membrane (Millipore IPFL00010). The membranes were kept for 1 h in LiCor blocking solution and probed overnight with either an anti-caspase-3 rabbit antibody (Cell Signaling #9662S) that recognizes the full-length and cleaved forms of caspase-3 at a dilution of 1/500, an anti-PARP mouse antibody (BD Biosciences #551025) at a dilution of 1/1000 or a mouse anti-ubiquitin antibody

(LifeSensor VU101) at a dilution of 1/1000. All antibodies were diluted in LiCor solution containing 0.1% Tween 20 (Sigma P1379). The membranes were rinsed with PBS + 0.1% Tween 20 and incubated with fluorochrome-conjugated anti-mouse or anti-rabbit antibodies at a dilution of 1/10,000 for 1 h in the dark at room temperature. The membranes were rinsed with PBS + 0.1% Tween 20 and dried before being scanned and analyzed using Odyssey software. For anti-ubiquitin labeling, the membranes were washed in PBS after transfer, fixed in PBS containing 0.5% glutaraldehyde (pH 7) and rinsed three times in PBS before blocking. The membranes were rinsed once more with PBS + 0.1% Tween 20 and dried before being scanned and analyzed using Odyssey software.

2.6. Dialysis

Regenerated cellulose dialysis membranes Spectra/Por (SpectrumLab, USA) with cut off of 3500 or 6–8000 Da were used. Before being used, membranes were bathed in distilled H_2O at 100 °C for 10 min then rinsed with cold distilled H_2O. Membranes were then filled with HFCP, clipped and bathed in distilled H_2O (volume 10 fold larger than the sample volume) at 4 °C, under light agitation. The dialysis solution was changed every 2 h for 6 h. Both in and out solutions were then frozen at −80 °C and lyophilized. The dry material was stored at 4 °C.

2.7. Precipitation with ethanol

500 mL aqueous pectin solution at 1 mg/mL were heat treated, then concentrated by evaporation at 4 °C up to a volume of about 200 mL. Ethanol was then added to reach a final concentration of 70% or 85%. Precipitation took place overnight at 4 °C. The samples were then centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was recovered and evaporated up to half the volume. Then distilled H_2O was added to eliminate the remaining ethanol and the solution was lyophilized.

2.8. HPLC analysis

2.8.1. Ion exchange chromatography

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC–PAD) was performed using a Dionex ICS 3000 (Sunnyvale, USA) in order to identify and compare the degree of polymerization of polygalacturonic acid and HFCP. Chromatographic separation of 50 μL polysaccharide samples was performed on a Dionex CarboPac PA-100 column (4 × 250 mm). NaOAc gradient and constant 0.1 M NaOH at a flow rate of 1 mL/min were used under constant helium pressure as the mobile phase.

2.8.2. Reverse phase chromatography

Separation of heat-modified galacturonic acid and HFCP and collection of peak at 3.9–4.4 min was performed with High Performance Reverse Phase Chromatography Dionex ICS 3000 (Sunnyvale, USA) equipped with UV detection at 210 nm. Chromatographic separation of 50 μL samples was performed on Alltima HP C18 column (4.6 mm × 250 mm) (Grace Davison Discovery Sciences). Constant elution of aqueous 0.1% formic acid at a flow rate of 1 mL/min was used under constant helium pressure as the mobile phase.

Data analysis was performed with Chroméléon (Dionex-Thermo Fisher, USA) version 6.70 SP3.

2.9. Mass spectrometry analysis

Samples were analyzed by mass spectrometry, using an ESI-QTOF maXis 4G UHR-TOF (Bruker). Direct injection was performed by a syringe pump at a flow rate of 3 μL/min. MS spectra were acquired for 1 s in the *m*/*z* range between 50 and 400 *m*/*z*. Raw data were processed using DataAnalysis 4.0 and smart formula 3D (Bruker). Smart Formula 3D, a formula generation software based on the combination of MS and MSMS information, was used to generate the best possible chemical structure from one *m*/*z* ratio and its MS/MS spectrum.

2.10. 4,5-Dihydroxy-2-cyclopenten-1-one synthesis

The synthesis of (4*R*,5*R*)-4,5-dihydroxy-2-cyclopentenone **8** has been achieved through a 7 step synthetic sequence starting from the commercially available D-ribose **1** (Fig. 1). The protection of **1** by an *iso*propylidene afforded the furanoside **2** which was transformed into **3** via iodination of the primary alcohol in **2**. The reductive elimination of **3**, using Zinc under acidic conditions generated the acyclic intermediate **4**. The addition of vinyl Grignard reagent on the volatile aldehyde **4** provided a second olefin necessary for the formation of the cycle. Ring closing metathesis of the dienes afforded the cyclopentenol **6** which was oxidized by the Dess–Martin reagent to form the cyclopentenone **7**. The acetonide in **7** was then cleaved under acidic conditions affording target molecule **8** in 20% yield over 7 steps.

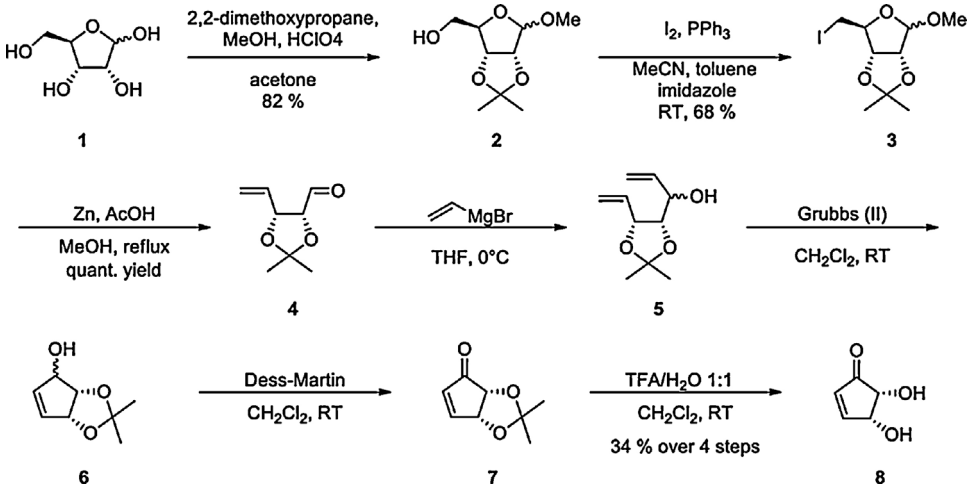
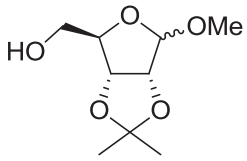


Fig. 1. Synthesis of molecule **8**.

2.10.1. Materials and methods

All reactions were carried out under an argon atmosphere. Yields refer to chromatographically and spectroscopically homogeneous materials. Reagents and chemicals were purchased from Sigma-Aldrich or Acros at ACS grade and were used without purification. All reactions were performed using purified and dried solvents: tetrahydrofuran (THF) was refluxed over sodium-benzophenone, dichloromethane (CH_2Cl_2) was refluxed over calcium hydride (CaH_2). All reactions were monitored by thin-layer chromatography (TLC) carried out on Merck aluminum roll silica gel 60-F254 using UV light and a molybdate-sufuric acid solution as revelator. Merck silica gel (60, particle size 0.040–0.063 mm) was employed for flash column chromatography and preparative thin layer chromatography using technically solvent distilled prior to use as eluting solvents. NMR spectra were recorded on a JEOL ECX 400 with solvent peaks as reference. All compounds were characterized by ^1H and ^{13}C NMR as well as by ^1H–^1H and ^1H–^{13}C correlation experiment when necessary. The following abbreviations are used to describe the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Chemical shifts (δ) are reported in ppm and referenced indirectly to TMS *via* the solvent (or residual solvent) signals.

2.10.2. 1-Methoxy-2,3-O-isopropylidene-α/β-D-ribofuranoside (2α/2β)

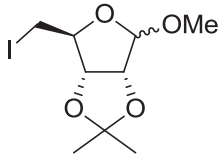


2

The anomeric mixture **2α/2β** (1.09 g, 82%) was prepared from D-ribose **1** (1 g, 6.66 mmol) according to the procedure of Klepper, Jahn, Hickmann, and Carell (2007).

^1H NMR (400 MHz, $CDCl_3$): δ = 4.98 (s, 1H_β), 4.85 (d, J = 5.9 Hz, 1H_β), 4.63 (d, J = 5.5 Hz, 1H_α), 4.58 (d, J = 5.9 Hz, 1H_β), 4.49–4.47 (m, 1H_α), 4.43 (m, 1H_β), 4.40–4.37 (m, 1H_α), 3.84–3.6 (m, 5H, $H_{\beta\alpha}$), 3.46 (s, 3H_α), 3.44 (s, 3H_β), 3.25–3.22 (dd, J = 10.7, J = 2.6 Hz, 1H_β), 1.55 (s, 3H_α), 1.48 (s, 3H_β), 1.37 (s, 3H_α), 1.32 (s, 3H_β).

2.10.3. Methyl 5-Deoxy-5-iodo-2,3-O-isopropylidene-β-D-ribofuranoside (Chen, Ye, lLiu, & Schnelle, 2012; Palmer & Jager, 2001)



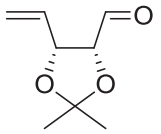
3

To a solution of **2** (1 g, 4.9 mmol, 1.0 equiv.) in a mixture of MeCN/toluene 1:1 (16 mL) was added imidazole (500 mg, 7.35 mmol, 1.5 equiv.) and triphenylphosphine (1.4 g, 5.4 mmol, 1.1 equiv.). After the addition of I_2 (1.4 g, 5.4 mmol, 1.1 equiv.) in portions, the reaction was stirred at RT for 2 h. TLC (Cyclohexane/EtOAc 8:2) indicated the formation of the major product and the presence of starting material. An additional addition of imidazole (500 mg, 7.35 mmol, 1.5 equiv.), triphenylphosphine (1.4 g, 5.4 mmol, 1.1 equiv.) and I_2 (1.4 g, 5.4 mmol, 1.1 equiv.) was performed and the reaction was stirred for 1 h at RT. Water (12 mL) and sodium thiosulfate (728 mg) were then added until the solution became clear. The organic layer was separated, dried over $MgSO_4$ and concentrated. The residue was purified through silica gel using Cyclohexane: EtOAc 9:1 as eluent to afford the product **3** (1 g, 68%) as an oil. The analytical data were identical to literature data.

^1H NMR (400 MHz, $CDCl_3$): δ = 5.05 (s, 1H), 4.77 (d, J = 5.8 Hz, 1H), 4.63 (d, J = 5.8 Hz, 1H), 4.44 (dd, J = 10.1, J = 5.9 Hz, 1H), 3.37 (s, 3H), 3.28 (dd, J = 5.9, J = 9.9 Hz, 1H), 3.16 (t, J = 10.1 Hz, 1H), 1.48 (s, 3H), 1.33 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 112.8, 109.8, 87.6, 85.5, 83.1, 55.4, 26.5, 25.1, 6.8.

*2.10.4. 4,5-Dideoxy-2,3-O-isopropylidene-D-erythro-4-pentenose **4** (Chen et al., 2012; Palmer & Jager, 2001)*



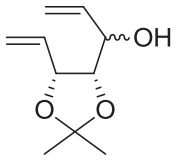
4

To a stirred solution of **3** (500 mg, 1.6 mmol, 1.0 equiv.) in MeOH (5 mL) was added Zn powder (115 mg, 1.76 mmol, 1.1 equiv., Aldrich, dust, <10 μm) in one batch. After addition of AcOH (11 μL, 0.19 mmol, 0.12 equiv.), the resulting mixture was heated to reflux for 4 h. Then, the reaction was cooled down to RT and filtered through a plug of celite followed by washing the cake with a mixture of THF/hexane (1:1, 3 mL) and MeOH (100 mL). The filtrate was then concentrated *in vacuo*. The aldehyde (liquid oil) was volatile, so the pressure was kept above 20 mbar. The residue was used in the next step without further purification.

^1H NMR (400 MHz, $CDCl_3$): δ = 9.56 (d, J = 3.2 Hz, 1H), 5.76 (ddd, J = 6.8, J = 10.3, J = 17.2 Hz, 1H), 5.47 (d, J = 17.2 Hz, 1H), 5.33 (d, J = 10.5 Hz, 1H), 4.86 (t, J = 6.8, J = 7.5 Hz, 1H), 4.42 (dd, J = 3.2, J = 7.5 Hz, 1H), 1.63 (s, 3H), 1.45 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 200.7, 131.3, 119.7, 111.3, 82.2, 79.1, 27.4, 25.3.

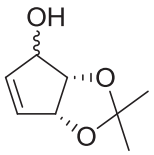
*2.10.5. (4S,5R)-1-(2,2-Dimethyl-5-vinyl-[1,3]dioxolan-4-yl)-prop-2-en-1-ol **5** (Smith, Han, Breslin, & Beauchamp, 2005; Yang, Ye, & Schneller, 2004)*



5

To a stirred solution of **4** (100 mg, 0.64 mmol) in dry THF (0.7 mL) was added vinylmagnesium bromide (0.75 mL, 0.75 mmol, 1.2 equiv., 1 M in THF) at −78 °C. After addition, the reaction was stirred for 10 min at the same temperature then for 1 h at 0 °C, before saturated solution of ammonium chloride (10 mL) and brine (10 mL) were added. The aqueous layer was separated and extracted with ethyl acetate (3 × 20 mL). The combined organic solutions were dried over $MgSO_4$ and concentrated under reduced pressure to give a mixture of diastereoisomers **5a/5b** (d.r. = 65:35), which was used in the next step without further purification. The analytical data were identical to literature data (Yang et al., 2004).

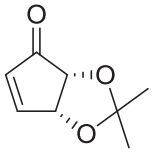
*2.10.6. (4R,5S)-4,5-O-Isopropylidenecyclopenten-1-ol **6** (Chen et al., 2012; Smith et al., 2005; Yang et al., 2004)*



6

A solution of compound **5** (120 mg, 0.65 mmol) in fresh distilled dichloromethane (1.56 mL) was bubbled with N_2 for 15 min to remove O_2. To this solution was added under argon Grubbs's second generation catalyst (5.5 mg, 0.0065 mmol, 0.01 equiv.). After overnight at RT, the solvent was concentrated under reduced pressure to give acetonide **6**, which was used in the next step without further purification. The analytical data were identical to literature data (Yang et al., 2004).

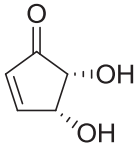
2.10.7. (4R,5R)-4,5-O-Isopropylidene-2-cyclopentenone ***7*** *(Chen et al., 2012; Smith et al., 2005; Yang et al., 2004)*



7

To a solution of **6** (101 mg, 0.65 mmol) in dichloromethane (5.4 mL) at 0 °C was added Dess–Martin periodinane (413 mg, 0.975 mmol, 1.5 equiv.). After addition, the reaction was warmed to room temperature and stirred for 2 h, before saturated $NaHCO_3$ solution (5 mL) and $Na_2S_2O_3$ solution (2 mL) were added. The aqueous layer was separated and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic solutions were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give the compound **7**, which was used in the next step without further purification. The analytical data were identical to literature data (Yang et al., 2004).

2.10.8. (4R,5R)-4,5-dihydroxy-2-cyclopentenone ***8*** *(Sugahara, Fukada, & Iwabuchi, 2004)*



8

To a solution of **7** (100 mg, 0.65 mmol) in dichloromethane (60 mL) was added TFA/H_2O 1:1 (2 mL). After addition, the reaction was stirred 16 h at RT. Then, the solvent was concentrated in vacuo and the residue was purified through preparative TLC by using $CHCl_3$/Acetone 6/4 as eluant to give the final compound (25 mg, 34% over 4 steps) as a liquid oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.38 (dd, J = 6.2, J = 2.9 Hz, 1*H*), 5.91–5.77 (m, 2*H*), 5.00 (d, J = 6.2 Hz, 1*H*), 2.81 (d, J = 5.5 Hz, 1*H*).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.8, 161.5, 132.9, 71.22, 68.99.

HRMS (ESI^+): meas. 137.014 calc. 137.021 for $C_5H_6NaO_3$.

3. Results

3.1. Low molecular fraction of HFCP induces HepG2 cell death

In order to study the cell death-inducing effects of HFCP, HepG2 cells were exposed to different concentration of HFCP. HFCP induced cytotoxic effect in a concentration-dependent manner after 24 h of incubation, as measured by a LDH release assay (Fig. 2A). On the other hand, unmodified pectin did not affect cell viability. At 3 mg/mL, HFCP induced cell death to a higher extent than etoposide at 50 μM, which was used as a positive control. It must be noted that etoposide is still much more potent in eliciting apoptosis in comparison with HFCP when analyzed mass-wise (etoposide 50 μM = approximately 30 μg/mL in comparison to the 3.0 mg/mL of HFCP in Fig. 2A). Its effect was also time-dependent and observed in two other cancer cell lines (Leclere et al., 2015). The concentration of 3 mg/mL was chosen as the reference for the following experiments. These effects were confirmed in a MTT assay assessing the number of viable cells (Fig. 2B). A kinetic analysis has also been performed (Fig. 2C). The results showed that a strong cytotoxic effect was observed for HFCP, but not for untreated pectin already after 24 h of incubation, similarly to what was observed for etoposide.

In order to identify the fraction and/or the molecules in HFCP that are responsible for its cytotoxic activity, we used a stepwise approach with different separation techniques and the subsequent "active" fractions were tested for their cytotoxic activity on HepG2 cells. When pectin is heat-treated, two degradation mechanisms can occur: ß-elimination and acidic hydrolysis (Kral & McFeeters, 1998). Both lead to pectin fragmentation at the level of the osidic bounds, generating fragments of different degrees of polymerization (DP). We thus attempted to separate the active fragments according to their size. HFCP was first dialyzed using a membrane with a cut off of 6–8000 Da. As shown in Fig. 2D, dialyzed HFCP completely lost its cytotoxic activity. Anion exchange HPLC analysis of dialyzed HFCP in comparison to HFCP and to a standard composed of oligogalacturonic acid with DPs lower than 6 (Fig. 2E) evidenced that dialyzed HFCP contained a mixture of fragments with a large variation of degree of polymerization (from monomer to DP ≥ 15). The analysis was performed in the presence of NaOH, which demethylated the fragments: a preliminary HPLC analysis had shown that all fragments were methylesterified (data not shown). Comparison between HFCP and dialyzed HFCP indicated that it was mainly molecules with a DP of 1, i.e. monomers, or neutral sugars that were lost upon dialysis. In order to confirm this observation and to investigate whether these low molecular weight fragments would display the cytotoxic activity, dialysis of HFCP with a cut off of 3500 Da was performed and the permeat (out fraction) was recovered and tested for potential activity. Fig. 3A shows that the permeat contained active molecules that exerted a concentration-dependent cytotoxic activity in HepG2 cells.

A second approach using ethanol precipitation was developed to confirm that the active fragments were of low molecular weight. Increasing the concentration of ethanol added to a solution induces the precipitation of molecules with lower and lower molecular weight, leaving the very low molecular weight molecules dissolved in the supernatant. We showed that the molecules from HFCP that were still soluble in 70 and 85% ethanol were the active ones (Fig. 3A), confirming that they were of very low molecular weight. Indeed, less than 20% of galacturonic acid monomers and less than 5% galactose precipitate in the presence of 85% ethanol (data not shown).

Since pectin is mainly composed of polygalacturonic acid, we hypothesized that using oligogalacturonic acid with a DP lower than 6 would reproduce the effect observed with the supernatant obtained after ethanol precipitation and of the permeat obtained after dialysis. However, this was not the case (Fig. 3B): polygalacturonic acid with a DP lower than 6 did not induce HepG2 cell death. Of note, polygalacturonic acid with a DP higher than 7 had no effect either. Two hypotheses can be proposed to explain this result: either, the cytotoxic activity did not originate from (poly)galacturonic acid whatever its DP but from neutral sugars derived from the rhamnogalacuronans of pectin; or

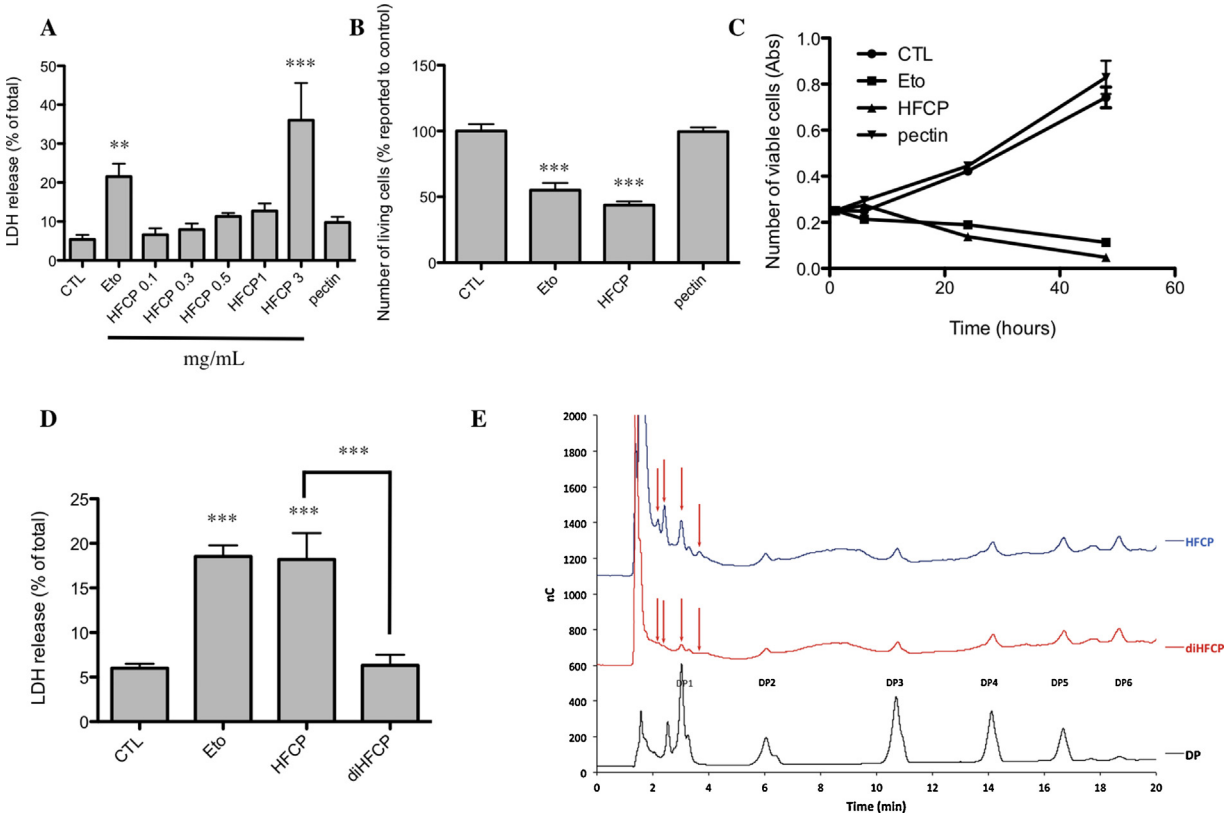


Fig. 2. *Low molecular fraction of HFCP induces HepG2 cell death.* (A) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), different concentrations of heat fragmented citrus pectin (HFCP) or 3 mg/mL citrus pectin (Pectin) for 24 h. Cytotoxicity was assayed using LDH cytotoxicity detection kit and results are expressed in percentage of total LDH activity. (B) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL heat fragmented citrus pectin (HFCP) or 3 mg/mL citrus pectin (Pectin) for 24 h. Cell viability was assayed with MTT assay and results are expressed in number of living cells (% reported to control). (C) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL heat fragmented citrus pectin (HFCP) or 3 mg/mL citrus pectin (Pectin) for different incubation time. Cell viability was assayed with MTT assay. Data are means ± 1 SD ($n = 3$). (D) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL heat fragmented citrus pectin (HFCP) or 3 mg/mL dialyzed HFCP (diHFCP, cut off 6–8000 Da) for 24 h. Cytotoxicity was assayed using LDH cytotoxicity detection kit. Data are means ± 1 SD ($n = 3$). Statistical analyses performed were ANOVA I with Bonferroni post-hoc tests. p Values in comparison to the control are **: $p < 0.01$; ***: $p < 0.001$. (E) Elution profile from HPLC separation (column PA100 with 0.2 to 0.5 M NaAc gradient and constant 0.1 M NaOH) of HFCP, dialyzed HFCP (diHFCP, cut off 6–8000 Da) or of oligogalacturonic acid with a degree of polymerization lower than 6 (DP).

(poly)galacturonic acid was chemically modified by heat treatment to generate the active molecule(s). The second hypothesis seems to be the correct one since heat-treatment of polygalacturonic acid with a DP lower than 6 generated molecules with cytotoxic activity toward HepG2 cells (Fig. 3C).

Since heat treatment seemed to be necessary to generate the cytotoxic activity and since HPLC analyses revealed that molecules with a molecular weight corresponding to a DP of 1 were contained in the active fraction of HFCP, we investigated whether heat treatment of galacturonic acid monomer would also generate cytotoxic molecule(s). Fig. 3D shows that, while galacturonic acid monomer at 3 mg/mL did not induce HepG2 cell death, heat-modified galacturonic acid monomer was toxic for HepG2 cells at 1 mg/mL. This was confirmed by measuring the number of viable cells using a MTT assay: heat-modified galacturonic acid monomer decreased the number of viable cells in a concentration-dependent manner after 24 h of incubation (Fig. 4A). It is important to note that the cytotoxic activity was not generated by heat treatment of neutral sugars like galactose or glucose. On the other hand, heat-treated glucuronic acid induced a similar cytotoxic activity than heat-treated galacturonic acid (Fig. 4B).

In order to investigate whether HFCP contained the same molecule(s) than heat-modified galacturonic acid monomer, both products were analyzed by HPLC using a C18 column and formic acid 0.1% in water as the eluent, which allowed a separation according to the hydrophobic properties of the molecules. The results showed that there was a peak at 3.9–4.4 min of elution that was detected in HFCP and heat-treated galacturonic acid monomer (see arrow in Fig. 5A), which was not observed in untreated pectin or in untreated galacturonic acid monomer. Moreover, the height of this peak was increased when pectin was heat-treated for 7.5 h in comparison to the usual 60 min treatment, suggesting that increasing the time of treatment generated more of the active molecule(s). This peak was collected, lyophilized and tested for its cytotoxic activity in HepG2 cells. The molecule(s) contained in this sample was(were) more active that heat-modified galacturonic acid monomer: at 0.25 mg/mL, these molecules induced a decrease of 93% in cell viability while heat-modified galacturonic acid monomer diminished cell viability by 46% (Fig. 5B).

3.2. 4,5-Dihydroxy-2-cyclopenten-1-one is one of the active molecules of HFCP

In order to investigate the structural features of the molecule(s) contained in heat-treated galacturonic acid monomer, mass spectrometry analyses were performed. Mass spectrometry spectra of heat-treated galacturonic acid monomer in comparison to unmodified galacturonic acid showed the presence of new peaks which might have derived from the active molecule(s): $M/Z = 115$, 137, 191, 200 and 281 (see circles in Fig. 5C). The peak with $m/z = 137$ (Fig. 5D) seemed to be the most interesting one because this peak was also present in the mass spectrometry analysis of

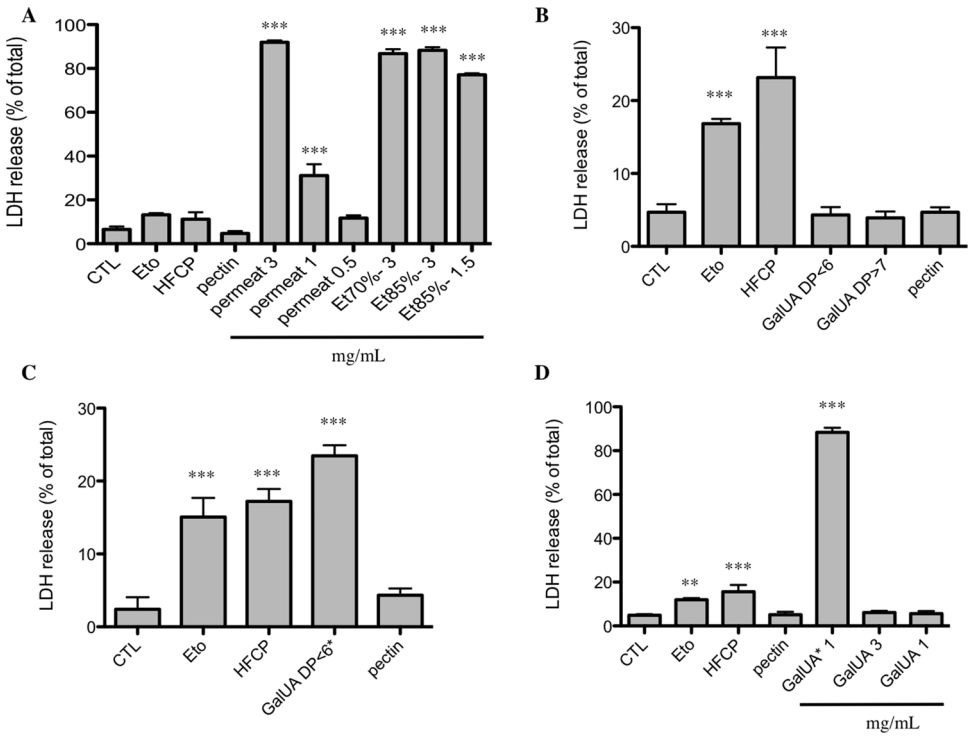


Fig. 3. *Heat-modified galacturonic acid induces HepG2 cell death.* (A) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL heat fragmented citrus pectin (HFCP), 3 mg/mL citrus pectin (Pectin), different concentrations of the permeat obtained after HFCP dialysis (cut off 3500 Da) or different concentration of the supernatant of HFCP from ethanol precipitation (70 or 85% ethanol) for 24 h. Cytotoxicity was assayed using LDH cytotoxicity detection kit and results are expressed in percentage of total LDH activity. (B) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL HFCP, 3 mg/mL polygalacturonic acid with a degree of polymerization lower than 6 (GalUA DP < 6), 3 mg/mL polygalacturonic acid with a degree of polymerization higher than 7 (GalUA DP > 7) or 3 mg/mL citrus pectin (Pectin) for 24 h. Cytotoxicity was assayed using LDH cytotoxicity detection kit. (C) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL heat-modified polygalacturonic acid with a degree of polymerization lower than 6 (GalUA DP < 6*) or 3 mg/mL citrus pectin (Pectin) for 24 h. Cytotoxicity was assayed using LDH cytotoxicity detection kit. (D) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL HFCP, 3 mg/mL pectin, 1 mg/mL heat-modified galacturonic acid (GalUA*) or 1 and 3 mg/mL galacturonic acid (GalUA) for 24 h. Cytotoxicity was assayed using LDH cytotoxicity detection kit. Data are means ± 1 SD (n = 3 or 4). Statistical analyses performed were ANOVA I with Bonferroni post-hoc tests. P values in comparison to the control are **: $p < 0.01$; ***: $p < 0.001$.

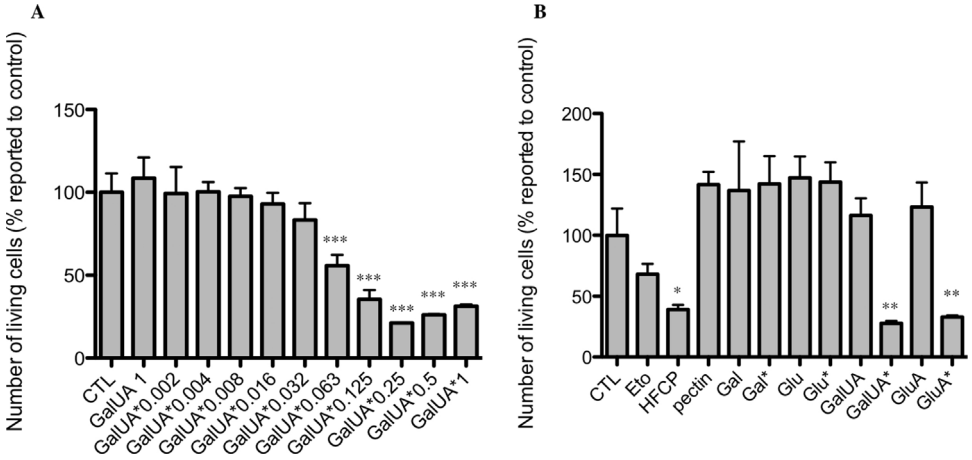


Fig. 4. Heat-modified galacturonic acid heat-modified glucuronic acid induced HepG2 cell death in a concentration-dependent manner. (A) HepG2 cells were incubated with medium alone (CTL), 1 mg/mL galacturonic acid (GalUA) or different concentration of heat-modified galacturonic acid (GalUA*) for 24 h. Cell viability was assayed with MTT assay and results are expressed in number of living cells (% reported to control). Data are means ± 1 SD (n = 3 or 4). Statistical analyses performed were ANOVA I with Bonferroni post-hoc tests. p Values in comparison to the control are ***: $p < 0.001$. (B) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL citrus pectin (Pectin) or with galactose (Gal), heat-modified galactose (Gal*), glucose (Glu), heat-modified glucose (Glu), galacturonic acid (GalUA), heat-modified galacturonic acid (GalUA*), glucuronic acid (GluA) or heat-modified glucuronic acid (GluA*), all at 3 mg/mL for 24 h. Cell viability was assayed with MTT assay. Data are means ± 1 SD (n = 3 or 4). Statistical analyses performed were ANOVA I with Bonferroni post-hoc tests. p Values in comparison to the control are *: $p < 0.05$; **: $p < 0.01$.

4,5-dihydroxy-2-cyclopenten-1-one (Fig. 6A), a molecule derived from heat treatment of uronic acid, uronic acid derivatives or a substance containing a saccharide compound which contains uronic acid, such as pectin. This molecule was claimed to possess anti-cancer activity (Koyama et al., 2000). According to these authors, 4,5-dihydroxy-2-cyclopenten-1-one presents a m/z value of 105 when ionized with one H^+ and of 137 when ionized with one Na^+. MS/MS analysis of this peak from heat-treated galacturonic

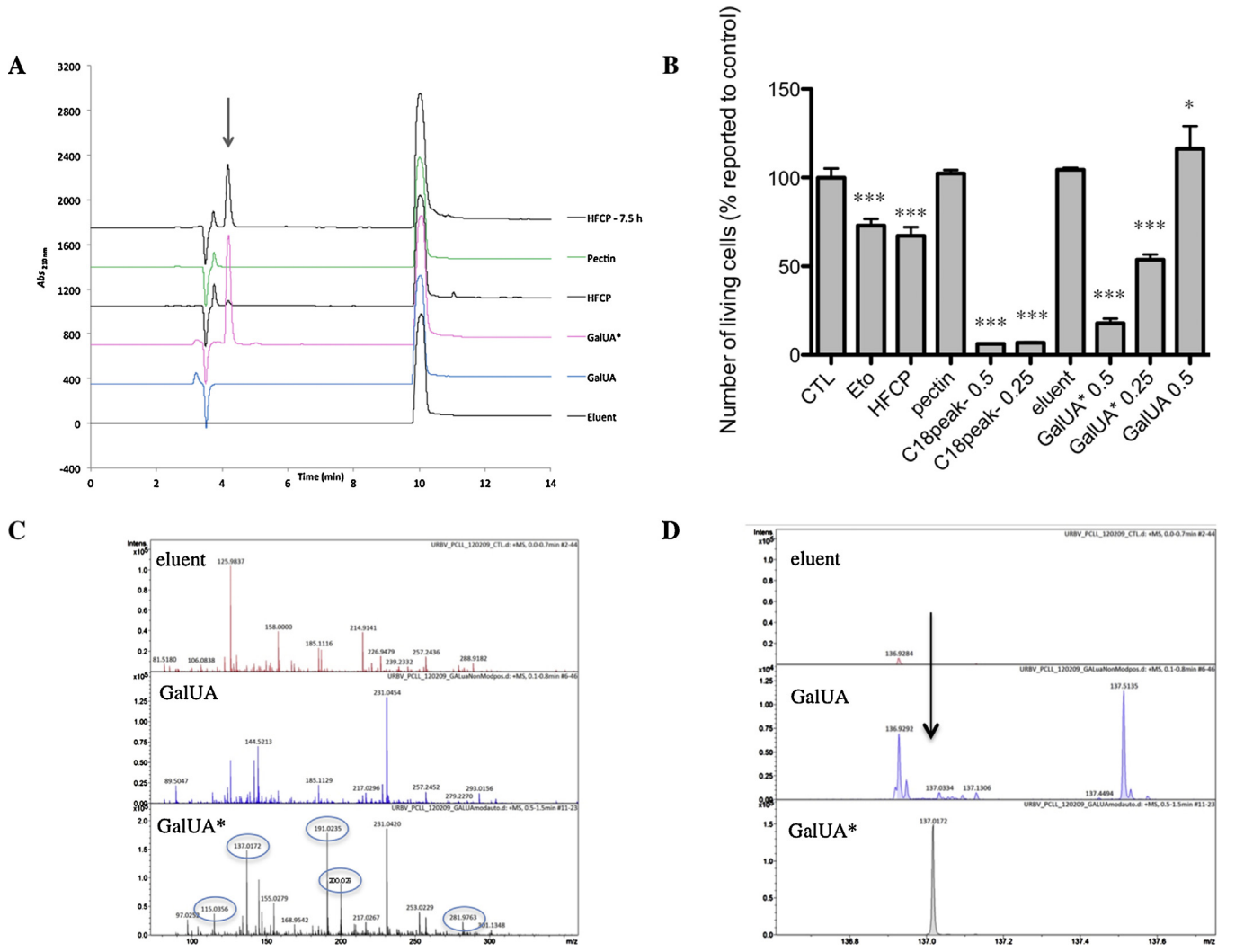


Fig. 5. *Heat-modified galacturonic acid and HFCP contain the same molecule that induces HepG2 cell death.* (A) Elution profile from HPLC separation (C18 reverse phase with 0.1% formic acid in water as the eluent) of pectin modified during 7.5 h by heat treatment, pectin, HFCP, heat-modified galacturonic acid (GalUA*), galacturonic acid (GalUA) and of the eluent. The arrow points at the 3.9–4.4 min peak. (B) HepG2 cells were incubated with medium alone (CTL), 50 μM etoposide (Eto), 3 mg/mL heat fragmented citrus pectin (HFCP), 3 mg/mL citrus pectin (Pectin), different concentrations of the molecules contained in the 3.9–4.4 min peak from C18 separation of heat-modified galacturonic acid, the corresponding volume of the HPLC eluent (formic acid) or different concentration of heat-modified galacturonic acid (GalUA*) or 0.5 mg/mL galacturonic acid (GalUA) for 24 h. Cell viability was assayed with MTT assay and results are expressed in number of living cells (% reported to control). Statistical analyses performed were ANOVA I with Bonferroni post-hoc tests. Data are means ± 1 SD ($n = 3$ or 4). p Values in comparison to the control are ***: $p < 0.001$. (C) MS spectra of the 3.9–4.4 min peak from C18 separation of galacturonic acid (GalUA) or heat-modified galacturonic acid (GalUA*) in comparison to the eluent. (D) Zoom of the spectra from (C) on the peak with $m/z = 137$.

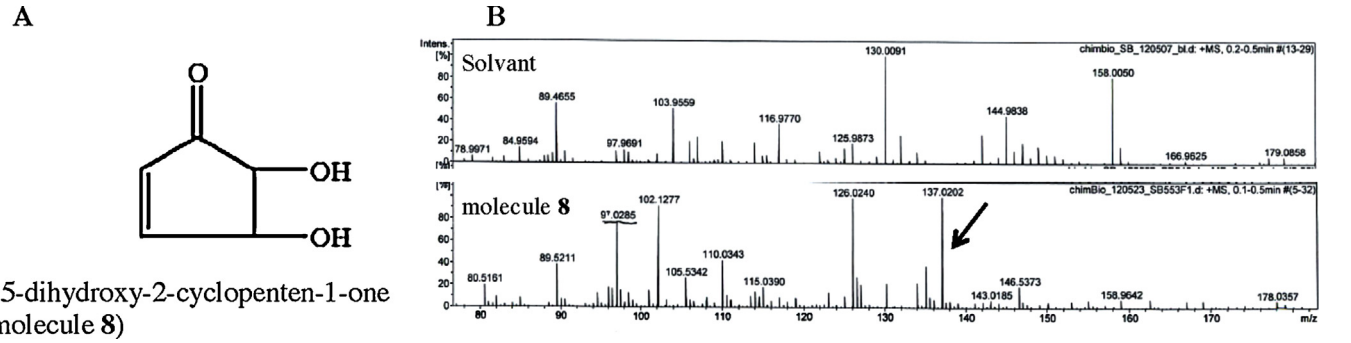


Fig. 6. *4,5-Dihydroxy-2-cyclopenten-1-one is one cytotoxic molecule present in HFCP.* (A) Chemical structure of 4,5-dihydroxy-2-cyclopenten-1-one (molecule **8**). (B) MS/MS spectra of molecule **8** in comparison to the solvent (0.1% formic acid).

acid (Fig. 7A) and smart Formula 3D analysis suggested the formula $C_5H_6NaO_3$, which corresponds to 4,5-dihydroxy-2-cyclopenten-1-one ionized with one Na^+. Theoretical fragmentation of 4,5-dihydroxy-2-cyclopenten-1-one gives the exact same peaks as the one observed in the MS/MS spectrum (data not shown). Spectra of MS analysis of HFCP in comparison to untreated pectin are very difficult to interpret because numerous peaks were detected (Fig. 7B). However, peaks with m/z values of 115 and 191, as

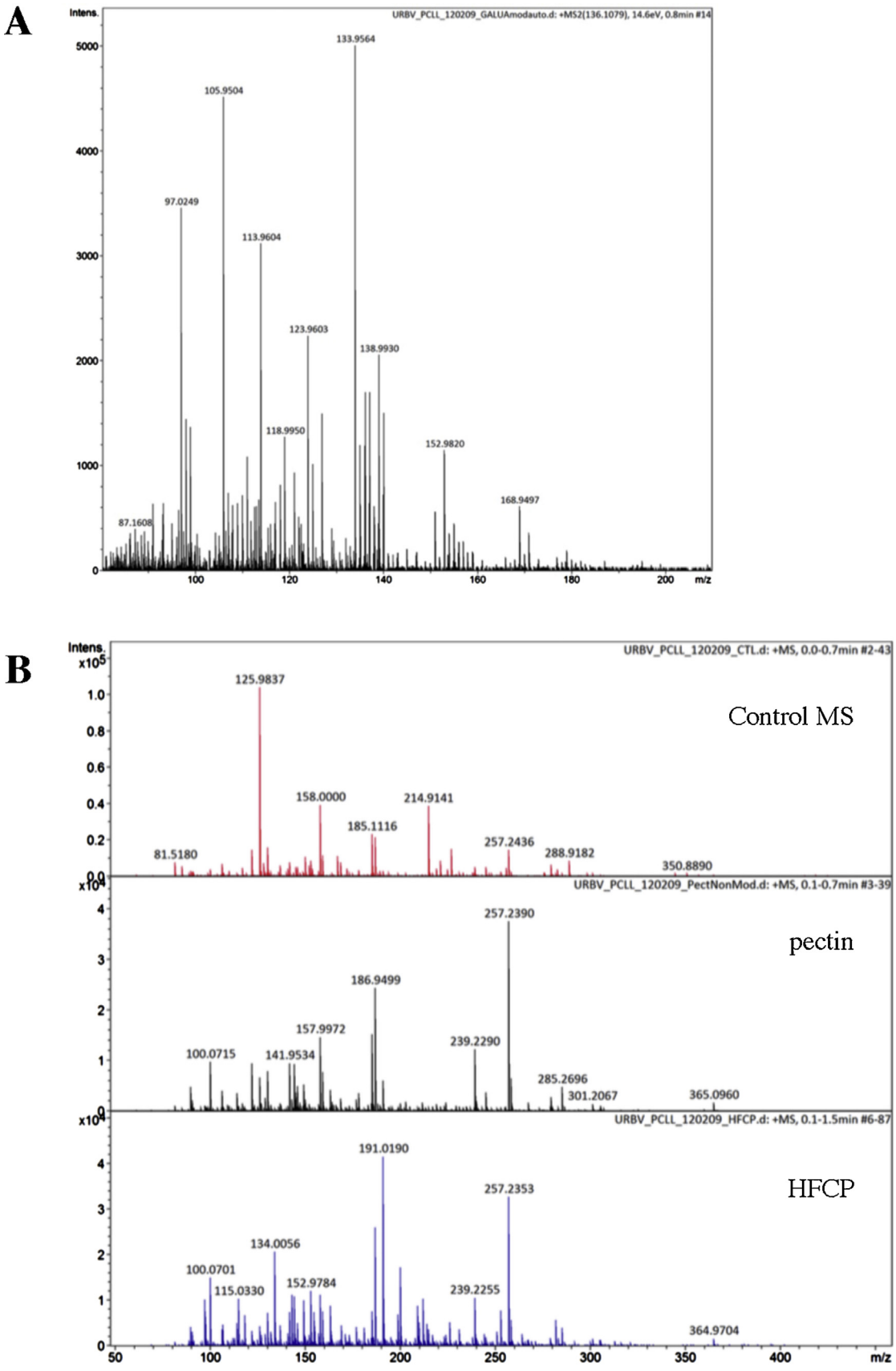


Fig. 7. *Structural analysis of HFCP and HFCP-derived molecules*. (A) MS/MS analysis of heat-modified galacturonic acid, peak with a $m/z = 137$. (B) MS/MS analysis of pectin and of heat-fragmented citrus pectin (HFCP).

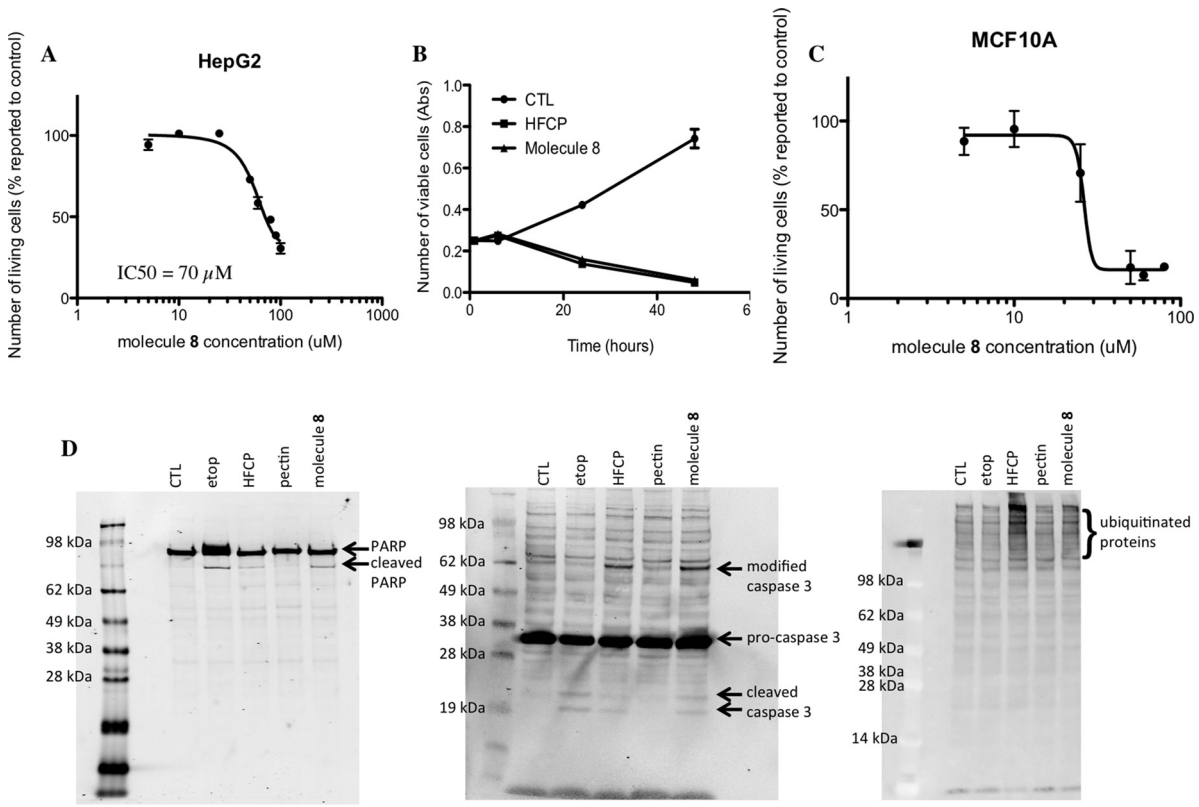


Fig. 8. *4,5-Dihydroxy-2-cyclopenten-1-one is cytotoxic.* (A) HepG2 cells were incubated with medium alone (CTL) or with different concentrations of molecule **8** for 24 h. Cell viability was assayed with MTT assay and results are expressed in number of living cells (% reported to control). Data are means ± 1 SD ($n = 3$). (B) HepG2 cells were incubated with medium alone (CTL), with 3 mg/mL heat-fragmented citrus pectin (HFCP) or with 80 μM of molecule **8** for different incubation time. Cell viability was assayed with MTT assay. Data are means ± 1 SD ($n = 3$). **(C)** MCF10A cells were incubated with medium alone (CTL) or with different concentrations of molecule **8** for 24 h. Cell viability was assayed with MTT assay. Data are means ± 1 SD ($n = 6$). The IC50 has been determined using Prism software. (D) HepG2 were incubated with medium alone (Ctl-), 50 μM etoposide (Etop), 3 mg/mL heat-fragmented citrus pectin (HFCP), 3 mg/mL citrus pectin (Pectin) or with 80 μM of molecule **8** for 24 h. Cleaved caspase-3, cleaved PARP and protein ubiquitination were detected by western blot analysis.

observed in the profile of heat-treated galacturonic acid, were revealed in HFCP, which were not present in the profile of untreated pectin. A peak with m/z of 137 was also detected but with a very low abundance.

It is to mention that MS analysis of heat-modified galacturonic acid monomer also revealed a peak with m/z values of 191 in addition to the 137 one. MS/MS analysis of these two peaks generated fragments with a m/z value of 134 and 97, which suggested that heat treatment of galacturonic acid may lead to the formation of dimers or trimers of 4,5-dihydroxy-2-cyclopenten-1-one. However, this still needs to be confirmed.

In order to confirm that 4,5-dihydroxy-2-cyclopenten-1-one could lead to such a fragmentation pattern in MS/MS analysis and to study its possible cytotoxic activity, this molecule was synthetized in our laboratory. ^{1}H and ^{13}C NMR analyses confirmed the structure of the synthetized molecule **8** while MS analysis showed that the fragmentation of 4,5-dihydroxy-2-cyclopenten-1-one indeed led to a peak with a m/z value of 137 (Fig. 6B). These results show that 4,5-dihydroxy-2-cyclopenten-1-one was one of the molecule generated by heat treatment of pectin and of galacturonic acid.

We then evaluated the cytotoxic activity of 4,5-dihydroxy-2-cyclopenten-1-one on HepG2 cells. Fig. 8A shows that this molecule induced a strong decrease in the number of living cells, in a concentration-dependent manner: a decrease in cell viability of about 50% was observed at 80 μM, indicating a very potent molecule. Indeed, in comparison, etoposide provoked a decrease in cell viability of 27% at 50 μM and heat-treated galacturonic acid, a decrease of 47% at 0.25 mg/mL (Fig. 5A). 0.25 mg/mL of galacturonic acid corresponds to 1.3 mM.

Then, we wanted to investigate whether these molecules would display a specific cytotoxic activity toward cancer cells. The cytotoxic activity of 4,5-dihydroxy-2-cyclopenten-1-one was assessed in MCF10A cells in comparison to HepG2 cells. MCF10A cells are an immortalized, non-transformed epithelial cell line derived from human fibrocystic mammary tissue. They are often considered as a normal control for cancer cells. As shown in Fig. 8B, HFCP induced a toxic effect in MCF10A similar to the one observed for HepG2 cells (compared with Fig. 2B). A kinetic analysis was performed (Fig. 2C). The results showed that a strong cytotoxic effect was observed for molecule **8**, similarly to what was observed for HFCP. (*4R*,*5R*)-4,5-dihydroxy-2-cyclopenten-1-one also led to MCF10A cell death to a similar extent than the one observed in HepG2 cells: a decrease in cell viability of 80% was observed at 80 μM (Fig. 8C).

A more quantitative approach to assess HFCP activity was performed by determining its IC50 using 5 different cancer cell lines as well as MCF10A. Results showed that the IC50 varied from 0.8 to 3 mg/mL, with no difference between the cancer cell lines and MCF10A cells (Fig. 9). These results indicate that HFCP is probably not specific to cancer cells. It has to be noted that etoposide was also as toxic for MCF10A cells as for HepG2 cells (data not shown) while etoposide is presently used in clinics to treat several types of cancer. Similar effects were obtained for another non-cancerous cell type, BJ-1 dermal fibroblasts (data not shown).

Finally, since HFCP was shown to induce specific features of apoptotic-cell death, i.e. classical PARP cleavage but atypical cleavage pattern of caspase 3 (Leclere et al., 2015), we wanted to investigated whether would induce cell death by a similar pathway. For that, PARP and caspase 3 cleavage was studied by western

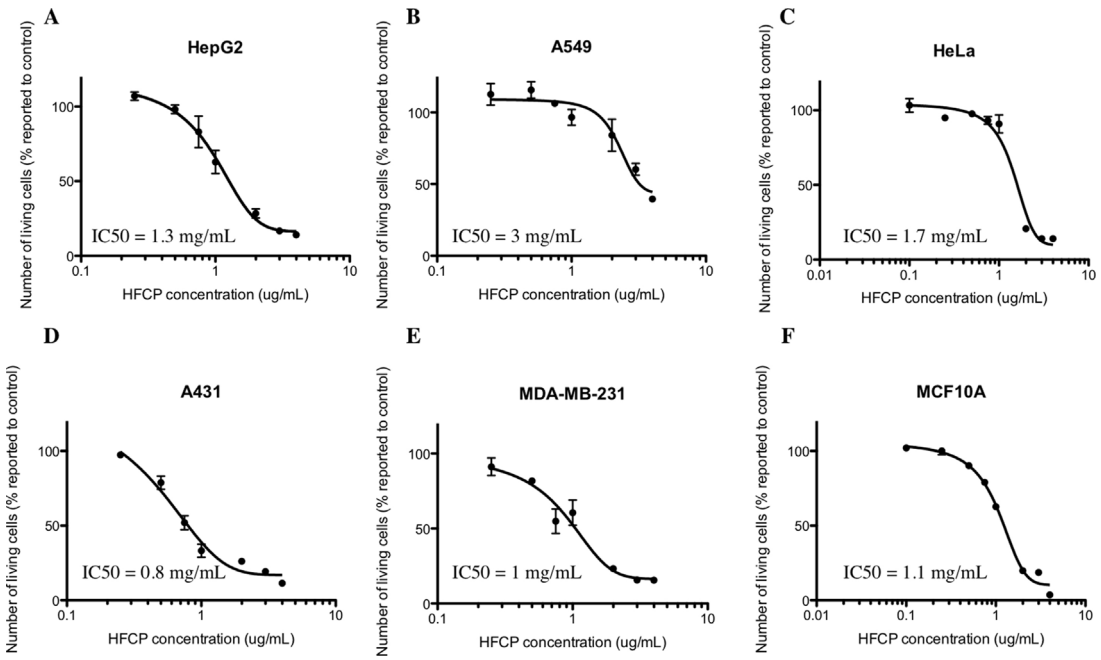


Fig. 9. *HFPC IC50 on different cell lines*. HepG2 (A), A549 (B), HeLa (C), A431 (D), MDA-MB-231 (E) and MCF10A (F) cells were incubated with medium alone or different concentrations of heat fragmented citrus pectin (HFCP) for 24h. Cell viability was assayed with MTT assay and results are expressed in number of living cells (% reported to control). Data are means ± 1 SD ($n = 3$). The IC50 has been determined using Prism software.

blot analysis in HepG2 cells incubated in the presence of (*4R*,*5R*)-4,5-dihydroxy-2-cyclopenten-1-one at 80 μM in comparison to 3 mg/mL HFCP. (*4R*,*5R*)-4,5-dihydroxy-2-cyclopenten-1-one, similarly to etoposide and to HFCP induced the cleavage of PARP. On the other hand, HFCP induced an atypical caspase 3 modification since a band of about 60 kDa was observed, which was not observed in the extract of cells incubated in the presence of etoposide. (*4R*,*5R*)-4,5-dihydroxy-2-cyclopenten-1-one led to the same caspase 3 migration pattern than the one induced by HFCP (Fig. 8D). Because HFCP was shown to trigger caspase 3 activation via protein ubiquitination and caspase 8 activation (Leclere et al., 2015), protein ubiquitination was assessed by a western blot analysis. An early increase in ubiquitination was observed when the cells were incubated in the presence of HFCP or (*4R*,*5R*)-4,5-dihydroxy-2-cyclopenten-1-one for 6 h but not in the presence of medium alone, etoposide or unmodified pectin (Fig. 8D).

4. Discussion

Pectin fragmented and modified by pH- or heat-treatment was shown to have potent anti-cancer effects (Leclere et al., 2013). A few hints have been unraveled: in pH-modified pectin, a functional motif of the β-1 → 4 galactan fragment, lying in the terminal residues, is required for its inhibitory activity on galectin-3 (Gao et al., 2012). On the other hand, low molecular weight pectin fragments bearing a base-sensitive linkage other than a carboxymethylester-bound seems to be responsible for their cytotoxic activity toward prostate cancer cells (Jackson et al., 2007). However, the exact structure of the compounds generated in modified pectin that are responsible for these activities is currently unknown.

To address this issue, we used several separation methods and tested the different fractions for their cytotoxic activity on HepG2 cells. It was determined that acidic sugars with low degree of polymerization (down to 1, i.e. monomers), mainly methyl esters, cleaved from homopolygalacturonans and chemically modified by the heat treatment displayed the cytotoxic activity. Mass spectrometry analysis of these molecules identified 4,5-dihydroxy-2-cyclopenten-1-one as one molecule present in the active fraction and chemipure (*4R*,*5R*)-4,5-dihydroxy-2-cyclopenten-1-one synthetized in our laboratory recapitulated the activity. It has to be noted that this molecule was probably not the only active molecule present in HFCP and that synergistic effects might have resulted from their association in this mixture.

The known mechanisms of pectin degradation upon heat treatment are ß-elimination and acidic hydrolysis (Diez-Dacal & Perez-Sala, 2012; Kral & McFeeters, 1998). However, these two types of reactions cannot explain the generation of 4,5-dihydroxy-2-cyclopenten-1-one **8**. Since we noted that HFCP and heat-treated galacturonic acid momoners were brown and smelling like caramel, it reminded us of the non-enzymatic browning of fruits upon storage or heating (Bharate & Bharate, 2012). The presence of galacturonic acid favors this process (Ibartz, Garza, & Pagan, 2008). This phenomenon is due to the Maillard reaction initiated by the condensation of a carbonyl group from a reductive sugar by an amine group from an amino acid or a protein. This reaction involves several complex and interconnected processes leading to the formation of glucosamines, ketosamines via Amadori rearrangements, of diketosamines and to the degradation of these compounds, resulting in the production of hydroxymethylfurfural. Some of these hydroxymethylfurfurals have toxic effects (Bauer-Marinovic, Taugner, Florian, & Glatt, 2012). Similarly, Products from the Maillard reaction are able to induce DNA single and double strand breaks (Hiramoto, Ishihara, Sakui, Daishima, & Kikugawa, 1998), to inhibit cell proliferation, probably by interfering with microtubules (Marko et al., 2002) and to display anti-tumoral activities (Marko et al., 2003).

How do these molecules exert their cytotoxic effects? 4,5-Dihydroxy-2-cyclopenten-1-one possesses an α, β unsaturated carbonyl group. This kind of chemical bound may form covalent adducts with thiols through a Michael conjugate addition (Nakayachi et al., 2004), which leads to the denaturation of proteins. This has been described for another molecule possessing an α, β unsaturated carbonyl group, 3-hydroxy-4-[(*E*)-(2-fyryl) methylidene]methyl-3-cyclopentene-1,2-dione, which reacts with cysteines in tubulin, thus impeding microtubule formation and

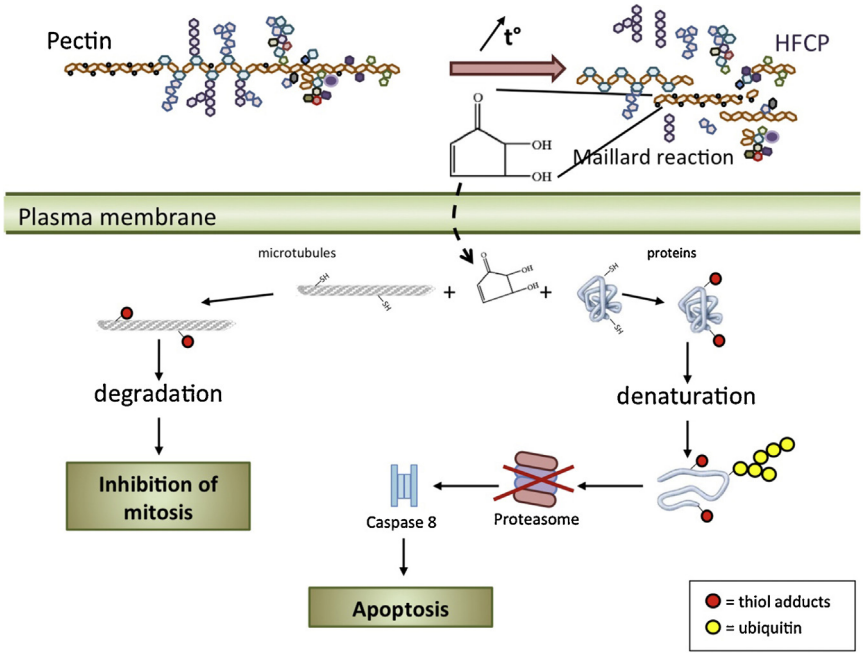


Fig. 10. *Schematic representation of HFCP-induced cell death.* Heat treatment triggers Maillard reactions that generate 4,5-dihydroxy-2-cyclopenten-1-one. This compound is able to form covalent adducts on cysteines, which leads to protein denaturation and ubiquitination. Tubulin adducts induce the degradation of this protein, hence inhibiting mitosis. On the other hand, ubiquitinylated protein aggregates inhibit proteasome, leading to caspase 8 activation and apoptosis.

cell cycle (Marko et al., 2002). It is important to note that HepG2 cells incubated in the presence of HFCP displayed a decrease in the abundance of tubulin (data not shown). Another family of molecules displaying an α, β unsaturated carbonyl group are cyclopentenone-containing prostaglandins. Some of them have anti-cancer properties (Diez-Dacal & Perez-Sala, 2012; Sanchez-Gomez, Cernuda-Morollon, Stamatakis, & Perez-Sala, 2004). Moreover, prostaglandins J inhibit the activity of ubiquitin isopeptidase, leading to polyubiquitinylated misfolded protein accumulation (Mullally, Moos, Edes, & Fitzpatrick, 2001). Similarly, cyclopentenone-containing prostaglandins provoke the denaturation and aggregation of the UCH-L1 protein (ubiquitin C-terminal hydrolase-L1), which is a deubiquitination enzyme, via a reaction with the thiol group of cysteines (Koharudin et al., 2010). Finally, an increase in cyclopentenone-containing prostaglandin level was detected in ischemic brain, that led to ubiquitination and aggregation of proteins in neurons (Liu et al., 2013). We actually observed an accumulation of polyubiquitinylated proteins in HepG2 cells exposed to HFCP (Leclere et al., 2015) as well as to (*4R,5R*)-4,5-dihydroxy-2-cyclopenten-1-one (Fig. 8C). We further demonstrated that this accumulation of polyubiquitinylated proteins initiated autophagy, as a protective mechanism, but that this process was overwhelmed, leading to caspase 8 activation and atypical apoptosis cell death (Leclere et al., 2015). It must be noted that cells incubated in the presence of 4,5-dihydroxy-2-cyclopenten-1-one displayed similar apoptotic-like features, suggesting that it is one of the active molecules in HFCP. The whole process is summarized in Fig. 10.

To the best of our knowledge, it is the first time that results regarding the effects of modified pectin on normal cells are reported. A similar cytotoxic effect was observed in several cancer cell lines and two normal cell types both for HFCP and 4,5-dihydroxy-2-cyclopenten-1-one. This observation urges everyone to look for the possible toxicity of new putative anti-cancer agent toward normal cells. However, future chemical modification of 4,5-dihydroxy-2-cyclopenten-1-one may decrease its toxicity toward normal cells, while keeping it for cancer cells, hence enhancing its specificity, as was performed for podophyllotoxin that became etoposide, one of the most used anti-cancer agents nowadays. In conclusion, this work has unraveled a new avenue to develop new drugs with a new mechanism of action that could overcome resistance of cancer cells to conventional chemotherapies.

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