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Exceptional antioxidant, non-cytotoxic activity of integral lemon pectin from hydrodynamic cavitation

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Abstract: Lemon pectin extracted along with water-soluble flavonoids and other phytochemicals from citrus industry's waste lemon peel via hydrodynamic cavitation in water, directly at pre-industrial scale and further isolated via freeze drying, shows exceptionally high antioxidant and non-cytotoxic activity. Preliminary investigation indicates also significant antimicrobial activity. These findings open the route to the development of new nutraceutical and healthcare application of a versatile biopolymer endowed with new functionality, rapidly and conveniently obtained from an abundant by-product of the agrofood industry.

Keywords: pectin; antioxidant; lemon; flavonoids; hydrodynamic cavitation; IntegroPectin

Introduction

Pectin is the natural hydrocolloid most valued in the food industry where it is widely used as a stabilizing additive as well as to enhance the food textural properties [1]. Chiefly derived from lemon peel and to a minor extent from apple pomace commercial pectin is industrially obtained through an hydrolytic process carried out with the aid of diluted mineral acids at relatively high temperatures [2].

Besides generating large amounts of effluents in the form of diluted mineral acid solution, the process invariably degrades the structure of pectin found in the fruit peel, lowering the molecular weight and partly also the degree of esterification (DE). For example, compared to sulphuric acid more expensive citric acid recovers pectin of higher molecular weight (improving the viscosity and the flow properties of pectin gel) from citrus fruit peels [3].

Perhaps not surprisingly, given its ubiquitous presence in fruits and vegetables, pectin is a prebiotic dietary fiber exerting multiple physiological and biological functions including significant anticancer, antiobesity and heavy metal-binding capacity, which make it increasingly used as an active ingredient by the pharmaceutical, nutraceutical and food industries [4].

From microwave-assisted extraction at high temperature (110 °C) [5], through subcritical water extraction [6] and microwave hydrodistillation and gravity [7], several new acid-free extraction methods have shown to afford pectin of improved structural and functional properties on both laboratory and pre-industrial scale.

Amid them, perhaps the most promising method from the industrial viewpoint is the extraction based on controlled hydrodynamic cavitation (HC) [8]. Originally demonstrated in the citrus fruit field with waste orange peel on semi-industrial scale (42 kg of raw material in 142 L water) [9], the process quickly affords extraction and separation of water soluble and insoluble bioproducts with practically no degradation neither of pectin nor of the valued phytochemicals contained in the orange peel.

The DE of orange pectin obtained by HC followed by freeze-drying (17%) [9] was found to be low, as in the case of pectin derived from microwave hydrodistillation, hydrodiffusion and gravity (MHG) followed by freeze-drying (29%) [7]. We remind that pectin with DE <50% does not require sugar or acidic conditions to gel, making it particularly well suited for food, pharmaceutical, and nutraceutical applications [10].

Extending the hydrodynamic cavitation process to waste lemon peel (WLP) we show in the following that said new form of pectin isolated via freeze drying shows exceptionally high antioxidant and non-cytotoxic activity. Preliminary investigation indicates also significant antimicrobial activity.

Results and Discussion

Figure 1 shows a lemon pectin sample obtained after lyophilization. Dubbed IntegroPectin, such pectin is colored in yellow, and has a delicate fragrance pointing to the presence of lemon terpenes. Figure 1 also shows the industrial waste lemon peel undergoing grinding in ice with a blender prior to the hydrocavitation-assisted extraction process. Necessary for circulating the water-WLP mixture through the pump and the reactor, such pretreatment will easily be performed automatically in an industrial-grade system.

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Figure 1. Lemon pectin obtained after freeze drying (*top*), and waste lemon peel undergoing grinding with a blender prior to the hydrocavitation-assisted extraction process (*bottom*).

The HC extraction device, including a closed hydraulic loop (total volume capacity around 230 L), a centrifugal pump (Lowara, Vicenza, Italy, ESHE 50-160/ 75) with 7.5 kW nominal mechanical power and rotation speed of 2900 rpm, and a Venturi-shaped HC reactor, has been described in detail elsewhere [9]. The main structural difference with the extraction process applied to waste orange peel is that now the HC-assisted extraction was carried out in a sealed reactor in order to minimize the loss of the lemon peel's volatile components. This was one of the recommendations arisen from the previous study describing the waste orange peel treatment [9].

In detail, 34 kg of fresh waste lemon peel obtained from organically grown Siracusa lemons (*Citrus limon*, cultivar 'femminello') by an in-line extractor at a juice factory kindly donated by a citrus company based in Sicily were first ground in ice with an electric blender and then added along with 120 L of tap water to the HC device. The reactor was sealed and cavitation started.

The whole process lasted 60 min and consumed 6.70 kWh of electric energy, thus the specific consumed energy was 0.22 kWh per kg of fresh WLP. No forced heat dissipation was applied and the temperature rose from 10°C up to 42°C after 60 min. After completion, the liquid phase was collected and sent for lyophilization. The lyophilization process, which lasted a few days, was carried out in parallel using several 250 mL balloons connected to the pump of a Labconco FreeZone 4.5 Liter benchtop freeze dry system (less-consuming drying operations that could be implemented at industrial scale might use freeze drying after concentration, a process common in the citrus juice industry). The yellow, perfumed pectin thereby obtained (Figure 1) was stable and retained its yellow color during storage at room temperature and in direct contact with air.

The HC-based citrus peel extraction is actually so effective [9], that virtually all water-soluble compounds are brought in solution. Furthermore, to explain the exquisite smell of lemon IntegroPectin we make the hypotheisis that, as it happens with waste orange peel extraction carried out under similar HC conditions, the citrus essential oil contained in the peel is emulsified in a ultrastable nanoemulsion dispersed in the aqueous phase [9]. Accordingly, we call IntegroPectin (In-Pec) the pectin obtained with this method.

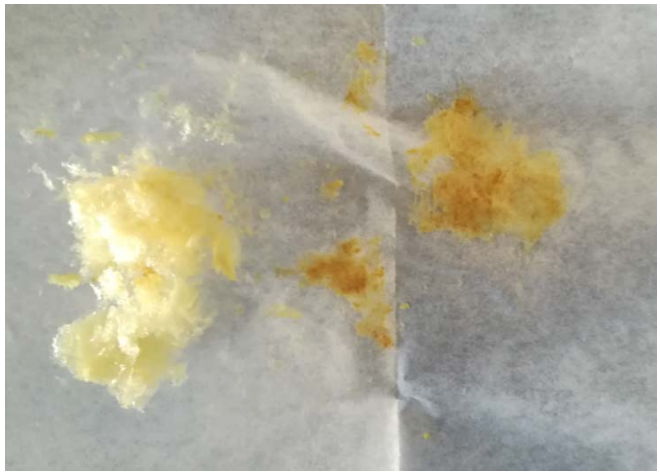

Figure 2. Integral lemon pectin obtained via hydrodynamic cavitation of waste lemon peel (Int-Pec, *left*), and after thermal treatment at 200°C for 5 min (Int-Pec-Hs, *right*).

After dehydration, the product was pulverized and 100 mg of the powder dissolved in 5 mL of phosphate-buffered saline (PBS) solution (pH = 7.4; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_3PO_4). For the heat-stressed sample preparation, the In-Pec powder was exposed at 200°C for 5 min. Figure 2 shows sample of both pectins.

An aliquot (100 mg) of the resulting heat-stressed IntegroPectin (In-Pec-Hs) powder was dissolved in 5 mL of PBS (pH = 7.4;

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137 mM NaCl, 2.7 mM KCl, 8 mM Na_3PO_4). The solutions were magnetically stirred for an hour and filtered by using a 0.45 µm sartorius filter, aliquoted (1 mL/vial), and stored at -20°C.

The total phenolic content was calculated according to an adapted Folin-Ciocalteu (F-C) colorimetric assay [11]. Aliquots (0.2 mL) of the In-Pec and In-Pec-Hs were made up to 5 mL with distilled water, followed by addition of 0.5 mL of Folin-Ciocalteu reagent. After 3 min, 1 mL of aqueous Na_2CO_3 (20% w/v) was added to each mixture subsequently made up to 10 mL with triple distilled water. The samples were then stored for 2 h at room temperature after which the absorbance of the solutions was measured at 765 nm by using a spectrophotometer (Shimadzu UV-2401 Dual-Beam UV-Vis). Quantification employed a gallic acid standard curve.

Reporting polyphenols in terms of gallic acid equivalents (GAE) per dry gram of pectin, results in Figure 2B point to high total phenolic content for both the IntegroPectin (0.88 mg GAE/g) and, though slight lower, for the heat-stressed derivative (0.81 mg GAE/g). For comparison, the amount of polyphenols in the lemon peel vary, depending on the cultivar, between 5.12 x 10^{-3} and 8.30 x 10^{-3} mg GAE/g [12], pointing to adsorption and concentration of the waste lemon peel (peel and residual pulp) polyphenols, solubilized in water by HC, at the surface of the freeze-dried pectin.

The Oxygen Radical Absorbance Capacity ORAC assay was performed according to slightly modified published procedures [13]. The reaction was carried out by using a 96-well plate: 160 µL of 0.04 µM fluorescein in 0.075 M Na-K phosphate buffer pH 7.0, 20 µL of diluted phenolic extract, or 20 µL of 100 µM Trolox. The mixture was incubated for 10 min at 37°C in the dark. After incubation, 20 µL of 40 mM 2,2'-Azobis-(2-methylpropionamidine) dihydrochloride (AAPH) solution was added. The microplate was immediately placed in a microplate reader (Thermo Scientific Fluoroskan Ascent F2 Microplate), and the fluorescence recorded (excitation and emission wavelengths at 485 and 527 nm, respectively) every 5 min for 60 min.

The ORAC value refers to the net area under the curve of fluorescein decay in the presence of the Klamin® phenolic extract or Trolox, minus the blank area. The activity of the sample expressed in µmol of Trolox equivalents (TE) per g of In-Pec or In-Pec-Hs was calculated by using Equation 1:

$$ORAC = k \times a \times h \times [(S_{sample} - S_{blank})/(S_{Trolox} - S_{blank})] \quad (1)$$

where k is the final dilution of the water-soluble extract; a is the ratio between the volume (in L) of the water-soluble extract and the grams of In-Pec or In-Pec-Hs; h is the final concentration of Trolox expressed as µmol/L; and S is the area under the curve of fluorescein in the presence of sample, Trolox, or buffer (blank) solution.

All reaction mixtures were prepared in triplicates, and at least three independent assays were performed for each sample.

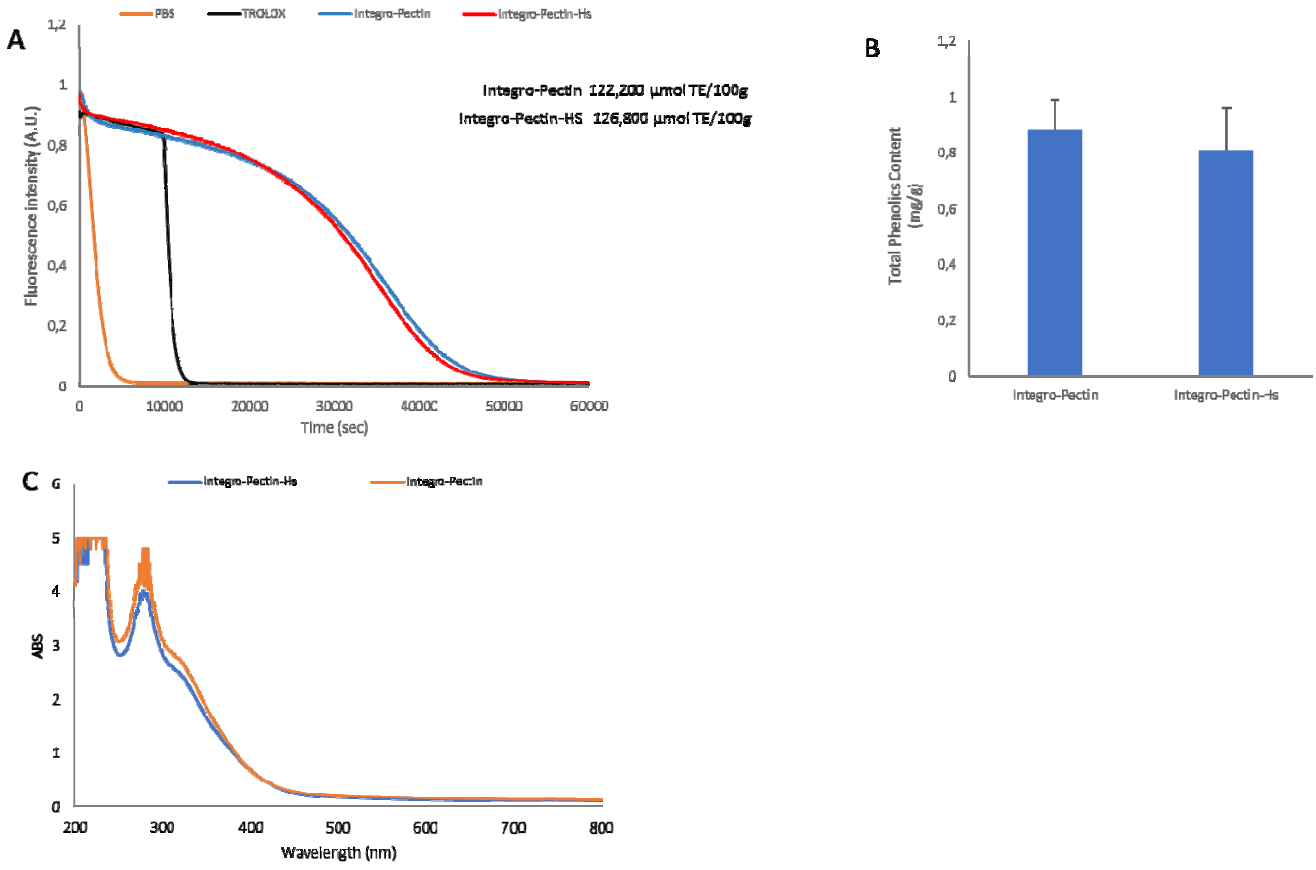

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Figure 3. Antioxidant activity of In-Pec and In-Pec-Hs pectin samples, expressed as µmol of Trolox equivalents (TE) per 100 g of pectin (ORAC assay, A); total phenolics content expressed mg of gallic acid equivalents per g of extract from the F-C assay (B); U V-vis absorption spectra in the 200 to 800 nm range (C).

To test the antioxidant properties of IntegroPectin as such or thermally stressed, powders of In-Pec and In-Pec-Hs were dissolved in PBS and submitted to ORAC and Folin-Ciocalteu assays. The antioxidant activity of the pectin samples, expressed as *µ*mol of Trolox equivalents (TE) per g of pectin for ORAC assays and as mg of gallic acid equivalents per g of extract for the F-C assay is displayed in Figures 3A and 3B, respectively.

The ORAC values are remarkably high for both IntegroPectin (122,200 *µ*mol TE/100g) and its heat-stressed derivative (126,800 *µ*mol TE/100g). For comparison, freeze-dried olive mill wastewater rich in olive polyphenols has an ORAC of 201,100 *µ*mol TE/100g [14], and black raspberry fruit has 16,210 *µ*mol TE/100g (on a dry matter basis) [15].

For the toxicity assay, we used human epithelial cells of the A549 cell line widely employed as epithelial cell model for drug metabolism [16]. The cells were cultured with RPMI 1640 medium (Celbio, Milano, Italy) supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen, Milano, Italy), 2 mM *l*-glutamine (Sigma-Aldrich, Italy), and 1% antibiotics (50 mg mL^{-1} penicillin and 50 mg mL^{-1} streptomycin, Sigma-Aldrich, Italy). The cells were maintained in a humidified 5% CO_2 atmosphere at 37 ± 0.1 °C.

The A549 cells were then treated with 0.25, 0.5, and 1 mg/mL of In-Pec or In-Pec-Hs for 24 h or with *tert*-butyl hydroperoxide (TBH, a model for organic hydroperoxides formed in pathological conditions widely employed to study intact biological systems [17]), pure (75 µM) or combined with In-Pec and In-Pec-Hs at different concentrations (0.25, 0.5, and 1 mg/mL) for 24 h. Untreated A549 cells were used as control. An optical microscope (Zeiss Axio Scope) equipped with a camera (Axiocam) and 20 × objectives, was utilized to analyze the morphology of the cells.

The cell viability was measured by the MTS assay (Promega Italia, Milano, Italy). In detail, 1 × 10^4/ml A549 cells were plated in 96 well plate. After 24 h, these cells were untreated (control) or treated with In-Pec or In-Pec-Hs. The cell proliferation assay reagent MTS 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was used according to the manufacturer's instructions. After the cell treatment, a 20 µL aliquot of the MTS solution was added to each well and incubated with the cells for 1 h at 37 °C, 5% CO_2. The absorbance at 490 nm was measured with the GloMax Discover Microplate Reader (Promega Italia, Milano, Italy). Results were expressed as the percentage of the MTS reduction with control samples as reference and are presented as mean value ± standard deviation (SD).

To assess the generation of Reactive Oxygen Species (ROS), the treated A549 cells were placed in a 96-well microplate. Some of A549 cells were treated with TBH (75 µM) alone or in combination with either In-Pec or In-Pec-Hs for 24 h. After that, a small aliquot of dichlorofluorescein diacetate (DCFH-DA) (1 mM) was added to each sample, placing the dyed cell samples in the dark for 10 min at room temperature. After washing with PBS, the cells were analyzed by an Axio Scope 2 a fluorescence microscope (Zeiss, Oberkochen, Germany). The UV-vis absorption spectrum in the 200-800 nm range points to the presence of phytocomplexes as shown by peaks at 250 nm and 370 nm (Figure 2C).

The complete absence of cytotoxicity for both IntegroPectin and heat-stressed IntegroPectin is shown by full retention of cell viability after 24 incubation of different pectin concentrations added to A549 cells. The MTS assay (Figure 4A) shows that no toxicity was detected at *all* the utilized concentrations compared with the control. The result was confirmed by microscopic observation of cellular morphology in which correct cell shape was observed at any pectin concentration (Figure 4B) confirming the absence of any cell damage.

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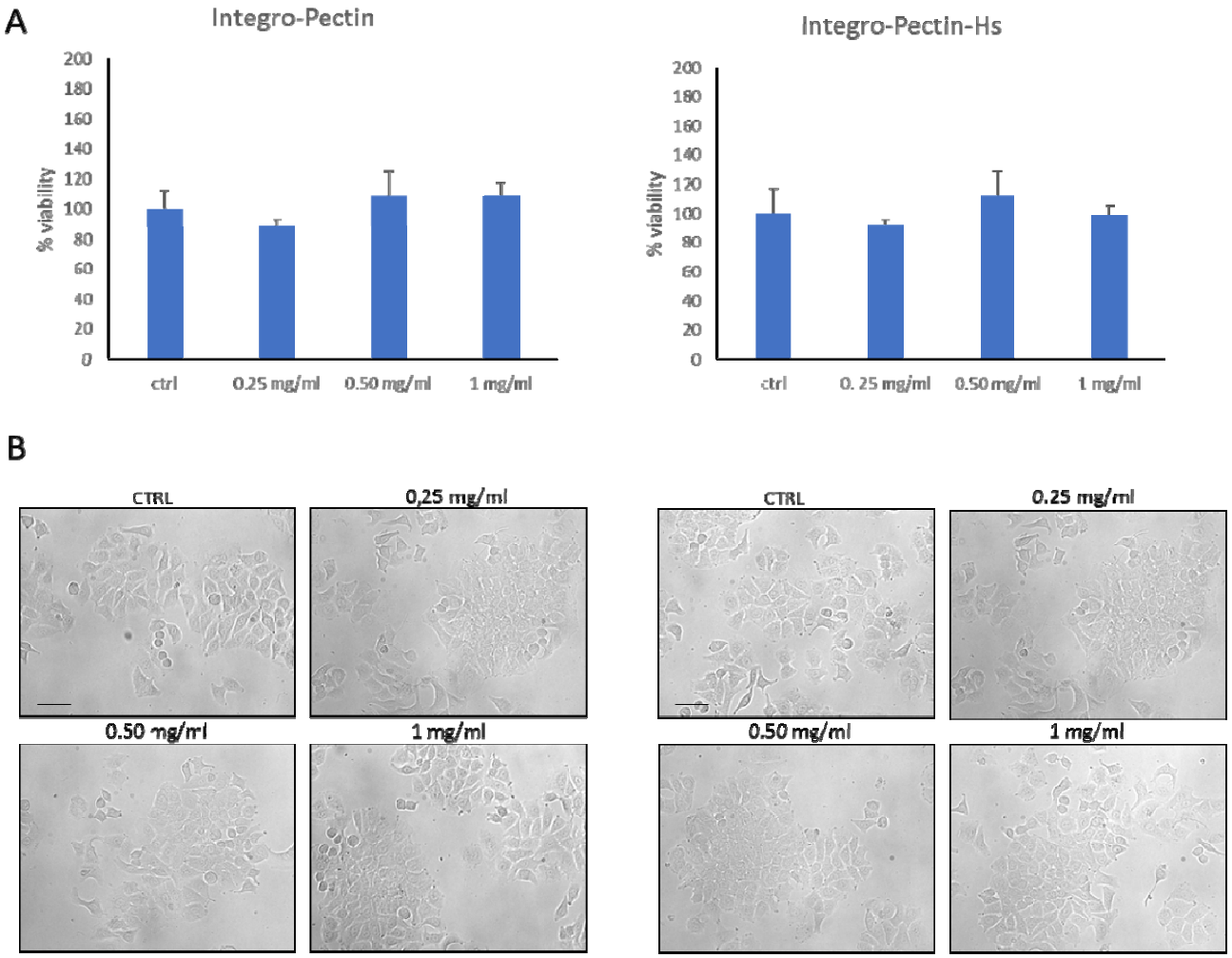

Figure 4. Cell viability at different integral pectin and heat-stressed pectin concentrations compared with control (A), and cellular morphology at different pectin concentrations compared with control (B). Bar = 100 µm, Control = CTRL.

Finally, as shown by the results of the dichloro-dihydro-fluorescein diacetate (DCFH-DA) quantitative assay for oxidative stress assessment of treated cells, IntegroPectin prevents oxidative stress (Figure 5).

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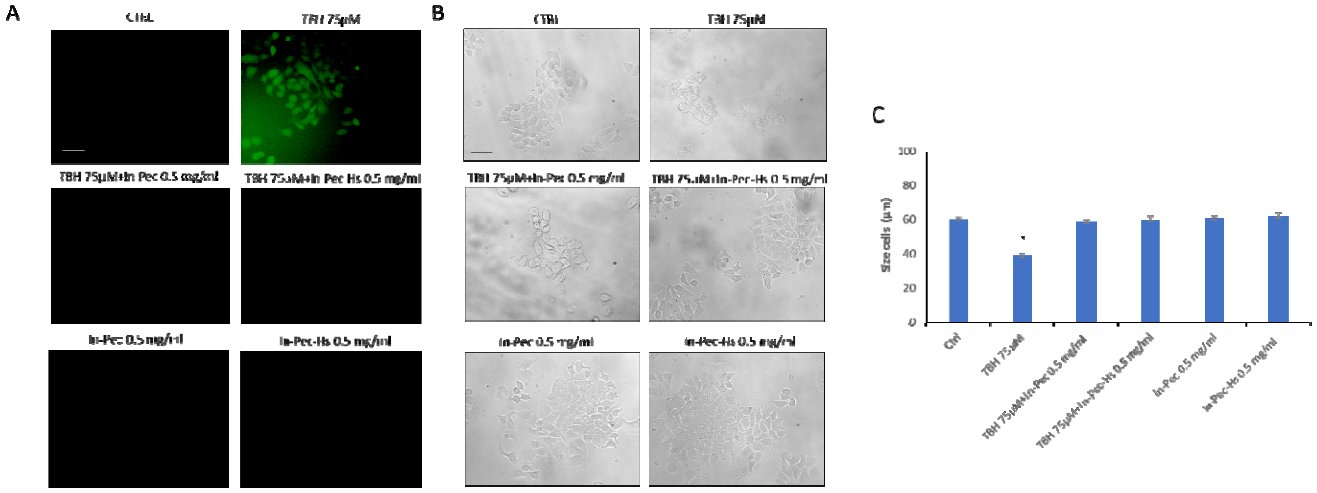

Figure 5. TBH-induced stress on A549 human epithelial cells (A, *top*), and stress inhibition due to both integral lemon pectin Int-Pec (A, *middle*) and heat-stressed lemon pectin Int-Pec-Hs (A, *bottom*) in 0.5 mg/mL concentration; microscopic observations (B, Bar = 100 µm) and cell size of the TBH-induced altered cells following addition of each pectin in 0.5 mg/mL concentration (C).

After 24 h of incubating the A549 cells with TBH alone or in combination with Int-Pec or Int-Pec-Hs, both the Int-Pec and Int-Pec-Hs were found to be able to inhibit TBH-induced stress (Figure 5A). The results were also confirmed by microscopic observation (Figures 5B and 5C) in which a significant recovery of the TBH-induced altered cell morphology and size was observed.

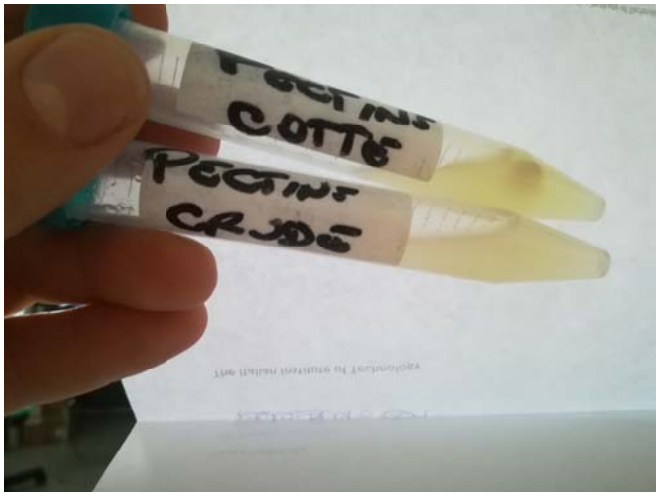

Figure 6. Integral lemon pectin (*crude*), and heat-stressed integral pectin (*cotte*) after two-week incubation in phosphate-buffered saline solution.

These results suggest that the coexisting bioactive components in the integral pectin obtained via hydrodynamic cavitation of waste lemon peel from organically grown lemon fruits in Sicily remain viable even after the application of a significant heat stress.

Only the strong antimicrobial activity of limonene [18] is diminished by the thermal treatment. Indeed, as shown in Figure 6, after incubating for 2 weeks both lemon IntegroPectin (Int-Pec) and heat-stressed IntegroPectin (Int-Pec-Hs) with the PBS buffered solution, mold formation is observed only for the heat-stressed pectin. This finding suggests that the amount of terpenes in the newly HC-extracted pectin diminishes after the pectin thermal treatment at 200 °C.

Outlook and Conclusions

In conclusion, lemon pectin obtained for the first time via hydrodynamic cavitation of waste lemon peel in water (directly at pre-industrial scale) exerts a significant antioxidant activity and shows no toxic effects on human epithelial cells even at the remarkably high concentration of 1 mg/mL.

These findings open the route to the development of new nutraceutical and healthcare application of a versatile biopolymer endowed with new functionality, rapidly and conveniently obtained from an abundant by-product of the agrofood industry using the simple, low cost, scalable and efficient hydrodynamic cavitation extraction process applied to waste lemon peel suspended in water, while meeting all the principles of natural product green extraction [8,9].

We ascribe these remarkable findings to the concomitante presence of bioflavoinds and nanoemulsified lemon oil at the surface of the freeze-dried IntegroPectin.

Lemon flavonoids are stable even under heat treatment conditions (121°C, 15 min) in acidic solution [19]. Acordingly, we find a minor decrease of the exceptionally high phenolic content of IntegroPectin for heat-stressed IntegroPectin exposed at 200°C for 5 min.

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Well known for their powerful antioxidant activity, the flavonoid glycosides in the peel of lemon fruit include six flavanon glycosides (eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, and neohesperidin), and three flavone glycosides (diosmin, 6,8-di-*C*-β-glucosyldiosmin (DGD), and 6-*C*-β-glucosyldiosmin (GD). Eriocitrin, neoeriocitrin and DGD show higher antioxidant activity, in comparison to the other biophenols [19].

Furthermore, eriocitrin especially abundant in lemons (and scarce in other citrus fruits), has stronger antioxidative activity than α-tocopherol in the low-density lipoprotein oxidation system [20]. Lemon flavonoids are also well known to exert anti-inflammatory activity through several mechanisms, from antioxidant and radical scavenging activities through modulation of the production of other proinflammatory molecules and of proinflammatory gene expression [21]. Hence, the results of experiments aimed to evaluate the anti-inflammatory of the newly obtained lemon pectin will be reported soon.

Preliminary observation of the antimicrobial activity of IntegroPectin (Figure 6) was lately confirmed identifying its remarkable activity against *Staphylococcus aureus* [22]. Hydrodynamic cavitation, in conclusion, is one of the enabling technologies of the emerging lemon bioeconomy [23]. Most likely, IntegroPectin will be widely produced and used for multiple applications soon.

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