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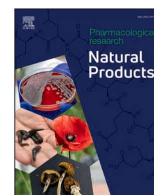

In vitro activity of *Citrus* IntegroPectin against lung cancer cells☆

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ABSTRACT

Citrus IntegroPectin bioconjugates obtained through acoustic cavitation in water of different *Citrus* fruit (lemon, red orange, and sweet orange) processing waste show substantial anticancer activity *in vitro* against human non-small cell lung cancer cells. Dissolved in aqueous phase at different concentrations, all bioconjugates tested affected long-term proliferation and cell migration of adenocarcinoma cells of line A549. Compared to the bioconjugate sourced from sweet orange, IntegroPectin phytocomplexes from lemon and red orange were more effective in reducing colony formation activity. Pointing to significant reduction in cancer cell progression, these first results concerning lung cancer cells support further investigation of these new low methoxyl pectins rich in citrus flavonoids and RG-I regions for the treatment of cancer.

1. Introduction

Being one of the most commonly diagnosed and harmful types of cancer, lung cancer is the main cause of cancer-related mortality [2]. With about 85 % share, non-small-cell lung cancer (NSCLC) is the most common form of lung tumor, with adenocarcinoma (40 %) and squamous cell carcinoma (30 %) being the main subtypes [3]. Despite progress in detection and advancements in standard treatments, NSCLC is frequently diagnosed at an advanced stage and is associated with a poor prognosis and high mortality rate. For instance, even though immune checkpoint blockade lung cancer treatments have extended the survival of patients with NSCLC [4], the 26 % 5-year survival rate remains low [5].

From adoptive cell transfer and its forms including tumor-infiltrating lymphocytes therapy [6], through new drugs enhancing the immune system's ability to fight cancer by blocking the proteins cancer cells use to evade immune cells [7], plentiful biomedical research is aimed to develop new therapies for NSCLC.

Amid said new treatments, numerous natural products sourced from plants or from algae capable to modulate the immune system, apoptosis, arrest cancer cell proliferation, and combat carcinogenic agents are being widely investigated in both *in vivo* and *in vitro* studies [8].

Driving mitochondrial-mediated apoptosis in A549 NSCLC cells of the A549 cell line and binding aurora B kinase involved in lung cancer growth, quercetin has shown promising results in the treatment of lung cancer [9].

Present in most fruits where it acts also as "molecular glue", pectin is a heteropolysaccharide consisting of a galacturonic acid polymer comprising homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), arabinogalacturonan (AG), and

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xylogalacturonan (XGA) regions. Approximately, it consists of repeating units of (1 → 4)-α-D-GalA (galactopyranosyluronic acid) residues, partly methyl-esterified at O-6 position (and at lower extent also acetyl-esterified at O-2 or O-3), interrupted by branched regions composed of (1 → 2)-α-L-rhamnose units (RG-I regions) further binding neutral sugars including galactose, arabinose, xylose, and fructose [10].

Commercially extracted as high methoxyl (HM) pectin (degree of esterification (DE)> 50 %) from citrus peels or apple pomace via prolonged hydrolysis promoted by dilute mineral acid at 70–80 °C, pectin is isolated via precipitation with isopropyl alcohol. Low methoxyl (LM) pectin having DE < 50 %, commercially produced by controlled hydrolysis of HM pectin, gels without requiring sugar in a broad pH range in the presence of small amounts of Ca^{2+} ions. In general, pectin is a highly bioactive and health-beneficial substance [11]. Its "modified" version rich in galactose neutral sugar residues abundant in RG-I galactan and arabinogalactan side chains obtained by hydrolysis at high pH and high temperature of commercial citrus pectin binds to lung cancer cells galactoside-binding galactin-3 protein limiting cancer cell proliferation [12]. Subsequent research found that said modified citrus pectin (MCP, the heat-treatment of citrus pectin results in the production of lower molecular weight pectin by β-elimination and reduction in degree of esterification) is an anti-metastatic agent capable of inhibiting proliferation and metastasis for numerous cancers [13].

Following its extraction via hydrodynamic cavitation (HC) in water only of the fresh residue of the industrial manufacturing of different *Citrus* fruit (lemon, orange and grapefruit) juices sourced from organically grown fruits, IntegroPectin is a phytocomplex of LM pectin showing broad-scope bioactivity [14]. Its remarkable antioxidant, anti-inflammatory, cardioprotective, neuroprotective, mitoprotective, antimicrobial and anticancer properties have been ascribed to its unique molecular structure (ultralow degree of methylation and abundant RG-I regions), coupled to the abundance of terpenes and flavonoids at its surface [14].

Showing the general applicability of cavitation in water only (no added acid, base or organic solvent) to extract the IntegroPectin (and CytroCell micronized cellulose) from fresh citrus processing waste (CPW), we recently demonstrated that acoustic cavitation (AC) can be employed affording IntegroPectin and CytroCell structurally analogous to those sourced via HC [15].

In this study we report the outcomes of *in vitro* investigation of the anti-cancer properties of three *Citrus* fruit IntegroPectin bioconjugates sourced via AC of fresh lemon, red orange and sweet orange industrial processing waste. Using adenocarcinoma lung cancer cells A549, we evaluated cell viability, colony formation ability, and motility. Long-term proliferation and cell migration indeed are crucial processes in cancer progression. Metastasis arises from the ability of cancer cells to move from their original site to other parts of the body, forming secondary tumors, leading to uncontrolled growth. The biology of metastatic SCLC involves both molecular and cellular mechanisms [16]. As put it by Sage and co-workers, the development of effective therapies requires to prevent metastatic spread [16].

2. Materials and methods

2.1. IntegroPectin isolation

Three *Citrus* fruit IntegroPectin bioconjugates were obtained via acoustic cavitation of fresh citrus processing waste as previously described [15]. In detail, lemon, sweet orange, and red orange CPW obtained from industrial citrus fruit squeezing in Sicily was kindly provided by OPAC Campisi (Siracusa, Italy). The CPW was packed in cardboard boxes and stored in a cold chamber at 4 °C during the transportation. All raw CPW samples were stored in a freezer at –20 °C and brought to room temperature prior to the AC-assisted extraction.

In brief, an aliquot (300 g) of CPW at room temperature was added with 3 L of ultrapure water obtained using a Barnstead Smart2Pure Water Purification System (Thermo Fisher Scientific, Waltham, MA, USA) and homogenized with a domestic electric blender by grinding twice for 30 s at high speed each time. The resulting mixture was extracted using the UIP2000hdT (20 kHz, 2000 W) industrial sonicator (Hielscher Ultrasonics, Teltow, Germany) equipped with a hydraulic pump operating at 1.43 L/min. The extraction process was carried out in continuous flow-mode for 30 min at 50 % of amplitude, in pulse condition (50 s on - 50 s off), setting the maximum work temperature at 50 °C. The power supplied to the digital probe-type sonicator was set at 800 W. After extraction was complete, the mixture was filtered through a cotton cloth in order to separate the insoluble fraction from the aqueous phase. The aqueous phase containing the IntegroPectin in solution was further filtered through a Büchner funnel by passing the mixture through a filter paper (Whatman, grade 589/3, retention < 2 μm) placed in the funnel. Eventually, IntegroPectin was isolated by freeze-drying using a FreeZone 4.5 Liter Benchtop Freeze Dry System (Labconco, Kansas City, MO, USA).

2.2. IntegroPectin solutions in PBS

A sample of each IntegroPectin was dispersed in PBS (phosphate-buffered saline, pH 7.4, purchased from Gibco Invitrogen, New York USA) at a concentration of 20 mg/mL. The resulting mixture was sonicated for 3 min to achieve a homogeneous solution. For each IntegroPectin, the resulting solution was stored at 4 °C prior to the biological tests

2.3. Quantification of flavonoids and phenolic acids

An amount of 70 mg of IntegroPectin derived from lemon, sweet orange, and red orange was suspended in a 5 mL solution of ethanol and water at a 4:1 (v/v) ratio. The suspension was homogenized using a vortex mixer and subsequently treated in a sonication bath for 3 min to ensure efficient dispersion of the material and complete extraction of flavonoid compounds. The resulting suspensions were filtered through a 0.22 μm nylon membrane filter before analysis by high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD).

Chromatographic separation was performed on a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and a diode array detector. A Synergy Hydro-RP C18 column (150 mm × 4.6 mm; 80 Å pore size; 4 μm particle size) purchased from Phenomenex (Torrance, CA, USA) served as the stationary phase. The mobile phase consisted of a 0.1 % (v/v) trifluoroacetic acid aqueous solution (solvent A) and methanol (solvent B) at a flow rate of 1.5 mL/min, applied under gradient conditions as follows: 0–2 min isocratic (A:B = 90:10), 2–17 min gradient (A:B = 90:10 to A:B = 50:50), 17–19 min isocratic (A:B = 50:50), and 19–20 min gradient (A:B = 50:50 to A:B = 90:10). Chromatograms were recorded at 280 nm. Under these conditions, the retention times for gallic acid (GA), *p*-coumaric acid (CA), hesperidin (HESP), naringenin (NAR), and kaempferol (KA) were 1.74, 7.82, 9.27, 9.50 and 14.40 min, respectively. Quantification of flavonoids and phenolic acids was achieved using calibration curves constructed from five standard solutions for each analyte. The calibration linear equations obtained were as follows:

GA (10–1 μg/mL concentration range):
$y = 1.016 + 14.252x$ ($R^2 = 0.996$).
CA (50–3.12 μg/mL concentration range):
$y = 32.05 + 69.682x$ ($R^2 = 0.999$).
NAR (43–2.7 μg/mL concentration range)
$y = 11.202 + 10.727x$ ($R^2 = 0.998$).
KAE (10–0.62 μg/mL concentration range)
$y = 1.1958 + 32.177x$ ($R^2 = 0.996$)

The calibration curve for hesperidin too was obtained from five standard solutions in the 0.023–0.192 mg/mL ($R^2 = 0.9982$) concentration range. All analyses were conducted in triplicate, and phenolic

acid or flavonoid content was expressed as mg/g of each IntegroPectin, reported as mean ± standard deviation (SD, $n = 3$).

2.4. DPPH assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Carlo Erba (Milan, Italy). A 2 mL aliquot of DPPH stock solution (40 μg/mL) was introduced into a quartz cuvette. A sample (20 mg) of each IntegroPectin was suspended in 5 mL of aqueous ethanol (EtOH:H_2O, 4:1, v/v), treated in a sonication bath for 3 min followed by filtration through a 0.22 μm nylon membrane filter. A 100 μL aliquot of the resulting solution was added to the DPPH solution for the spectrophotometric analysis conducted with a UV-Vis 1800 spectrophotometer (Shimadzu, Kyoto, Japan).

The reduction of DPPH was monitored at room temperature at 5 min intervals over a period of 1 h. A calibration curve was constructed using five DPPH standard solutions in MeOH with the following parameters: maximum absorbance at $\lambda = 515$ nm, linearity range of 2.6–42 μg/mL, regression equation Abs = 0.0383 + 0.0293x [concentration in μg/mL], and R^2 of 0.999. All experiments were performed in triplicate, and results expressed as the percentage of residual DPPH over time ± SD. To contextualize the findings, data were compared with the activity of GA as reference antioxidant molecule. Standard solutions of GA in MeOH ranging in concentration from 0.0069 to 0.0554 mg/mL were used. The residual DPPH percentage at two selected times (30 and 60 min) was used to generate calibration plots. Results are presented as the mean equivalent amount of GA of each IntegroPectin sample ± SD ($n = 3$).

2.5. Folin–Ciocalteu assay

The total phenolic content was assessed by the Folin-Ciocalteu method. A 25 mg aliquot of the IntegroPectin solution was dissolved in 5 mL of ultrapure water. Subsequently, 50 μL of the prepared sample was transferred into a 15 mL plastic tube containing 2 mL of ultrapure water. To this mixture, a 130 μL sample of 2 M Folin-Ciocalteu reagent purchased from Merck (Darmstadt, Germany) was added. The solution was thoroughly mixed and incubated in the dark for 5 min. Following this step, 370 μL of a sodium carbonate solution (200 mg/mL in ultrapure water) was introduced in each tube.

The reaction mixture was homogenized and maintained at ambient temperature in the dark for 2 h. Upon completion of the reaction, the samples were analyzed using UV–vis spectrophotometry. A calibration curve was generated using as standard five standard solutions of GA in ultrapure water, with concentrations ranging from 31.2 to 500 μg/mL. The calibration curve parameters were determined as follows: maximum absorbance at 760 nm, calibration equation $y = -0.0041 + 2x$, with $R^2 = 0.999$. Results were reported as GA equivalent mg/g of IntegroPectin ± SD ($n = 3$).

2.6. Cell cultures

Human NSCLC cell lines, A549, a lung adenocarcinoma cell line, were cultured in RPMI-1640 medium supplemented with heat-deactivated (56 °C, 40 min) 10 % FBS, streptomycin and penicillin, 1 % nonessential amino acids and 2 mM L-glutamine (all purchased from Euroclone, Pero, Italy). The cells were maintained as adherent monolayers in an incubator at 37 °C with a humidified atmosphere with 5 % CO_2. The cells were grown in polystyrene flasks (BD Falcon, Franklin Lakes, New Jersey) to 90 % confluence and passaged by trypsin/EDTA.

2.7. Effects of IntegroPectin from lemon, red orange and sweet orange on A549 cell viability

To assess the right concentration of the stimuli to add to the culture, cell viability was evaluated by CellTiter 96 AQueous One Solution Cell Proliferation Assay (PROMEGA, Madison WI USA). A549 cells were plated in 96-well plates and were treated for 24 h in quadruplicate with IntegroPectin bioconjugates dissolved in PBS (phosphate-buffered saline, pH 7.4, purchased from Gibco Invitrogen, New York USA) at different concentrations (0.25, 0.5, 1.0, 5.0 and 10 mg/mL). Then 20 μL of One Solution reagent containing MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethox-yphenyl)-2-(4-sulfopheyl)2H-tetrazolium] was added to each well, and incubated at 37 °C, 5 % CO_2. The absorbance was read at 490 nm on the Microplate reader. Results were calculated as percentage relative to no treated (NT) cells. Comparison between different experimental conditions was evaluated by ANOVA corrected with Fisher's test (*$p < 0.05$ was accepted as statistically significant).

2.8. Effects of IntegroPectin from lemon, red orange and sweet orange on colony formation ability in A549 cell line

A549 cell line was cultured for 24 h with each of the three IntegroPectin samples dissolved in PBS at 0.5 and 1.0 mg/mL concentration. Then, the cells were harvested and seeded in a six well plate at a density of 50 cells/cm^2 and were maintained in fresh medium at 37 °C in an atmosphere containing 5 % CO_2 to form colonies [17]. After 1–3 weeks the cells were fixed in 100 % methanol and stained with 0.5 % crystal violet in 20 % methanol. Then, the plates were air dried. The colonies were photographed using a digital camera and counted with countPHICS (count and Plot HIstograms of Colony Size) software, a macro written for ImageJ [18].

Data obtained as mean ± SD ($n = 3$) are expressed as colonies number. The comparison between different experimental conditions was evaluated by ANOVA corrected with Fisher's test (*$p < 0.05$ was accepted as statistically significant).

2.9. Effect of IntegroPectin from lemon, red orange and sweet orange on A549 cell line motility

A549 cells were grown in a 6-well plate until the confluence and three circular wounds were done in each well, using a 200 μL pipette tip. Wells were washed with PBS to remove debris and after one hour, cells were stimulated with IntegroPectin bioconjugates at 0.5 and 1 mg/mL concentration. Using the scratch migration assay with the area method, we evaluated whether the stimulation with each of the three different citrus IntegroPectin bioconjugates reduced cell migration.

During the experiment, a "wound" (a cell-free zone in the cell monolayer) is made and recolonization of the scratched region is monitored to quantify cell migration area. In detail, the wound area is tracked and the ratio between wound area at time t A(t) and the initial area A(0) expressed as the percentage wound area at a specific time point indirectly allows to evaluate the migration rate [19]. Images were obtained using a digital camera connected to an inverted phase-contrast optical microscope at 0, 24 and 48 h after wound creation.

ImageJ program was used to measure the area of remaining wound size and wound closure rates. The results were expressed as percentage of area reduction at time 24 h and 48 h compared to $t = 0$ h. Comparison between different experimental conditions was evaluated by ANOVA corrected with Fisher's test (*$p < 0.05$ was accepted as statistically significant).

3. Results and discussion

The photographs of vividly colored lemon, red orange, and sweet orange sourced via AC from fresh CPW in Fig. 1 show visual evidence of the abundant amount of flavonoids contained in these flavonoid-pectin bioconjugates.

3.1. Effects of IntegroPectin from lemon, red orange and sweet orange on A549 cell viability

Fig. 2 shows that the cell viability of A549 cells cultured with citrus

Fig. 1. Sweet orange (A), red orange (B), and lemon (C) IntegroPectin sourced from industrial citrus processing waste by acoustic cavitation.

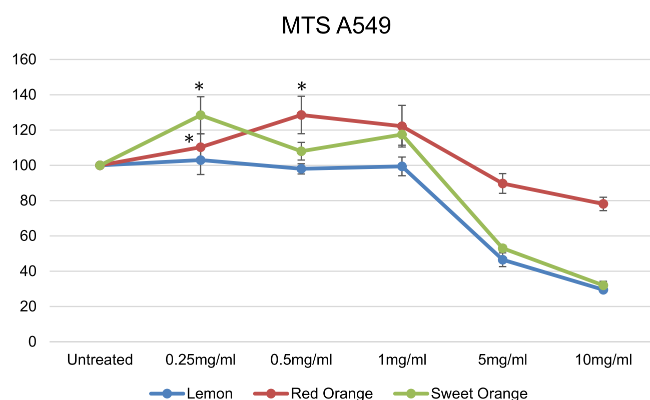

Fig. 2. Effect of different citrus fruit IntegroPectins on A549cell viability. Cells were cultured for 24 h with different citrus IntegroPectin bioconjugates at different concentration (0.25, 0.5, 1.0, 5.0 and 10 mg/mL). Viability data (mean ± SD, $n = 3$) are expressed as % of untreated cells.

IntegroPectin in solution at increasing concentration (0.25, 0.5, 1.0, 5.0 and 10 mg/mL) started to decrease at 5 mg/mL IntegroPectin concentration. Especially for lemon and sweet orange bioconjugates, which at a concentration of 5 mg/mL, reduced cell viability below the 60 %, until viability dropped below 40 % at 10 mg/mL concentration.

3.2. Effects of IntegroPectin from lemon, red orange and sweet orange on colony formation ability in A549 cell line

Plots in Fig. 3 show that a significant reduction in A549 cell colony number was observed after treatment with lemon IntegroPectin at 1 mg/mL concentration, and with red orange IntegroPectin at 0.5 and 1.0 mg/mL concentration.

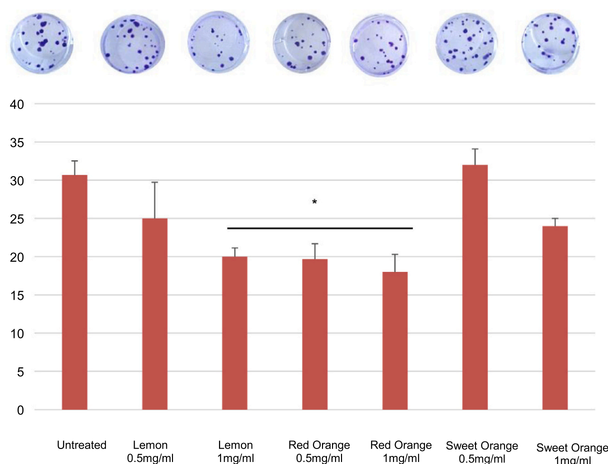

Fig. 3. Effect of different citrus fruit IntegroPectin bioconjugates at 0.5, and 1.0 mg/mL concentration on colony formation ability in A549 cell line. Value of untreated cells is also plotted.

3.3. Effects of IntegroPectin from lemon, red orange and sweet orange on A549 cell line motility

Increased cancer cell motility is a feature of tumor aggressiveness [20]. To evaluate the stimulation effect of the three IntegroPectin on A549 NCSLC cell motility, we used the scratch migration assay. The scratch assay is a widely used method to assess cell migration even though it may not fully replicate the complexity of *in vivo* processes. However, its simplicity, reproducibility, and ability to provide real-time data on cell migration are significant strengths. To improve the reproducibility, we replicated three times the experiments, and the obtained data were statistically analyzed using ANOVA corrected with Fisher's test. As highlighted in the Experimental section, values of $^{*}p < 0.05$ were considered statistically significant.

Photographs and histograms in Fig. 4 show that all three citrus IntegroPectin bioconjugates tested significantly reduced cell motility.

The most effective was sweet orange IntegroPectin that at 1.0 mg/mL concentration limited the wound area reduction to just 10 % *vs.* 18 % of the untreated cells, and to less than 15 % after 48 h *vs.* ~35 % for the untreated cells.

Lemon IntegroPectin required the use of a 1.0 mg/mL concentration because at 0.5 mg/mL concentration it actually increased motility after 24 h and reduced it by just 2 % at 48 h.

Red orange IntegroPectin was highly effective in reducing cell motility after 24 h at 0.5 mg/mL concentration, and decreased motility from sweet orange bioconjugate pectins at 0.5 and 1.0 mg/mL were able to significantly reduce cell migration.

After 48 h treatment of the cells with red orange IntegroPectin the original cell motility was significantly reduced, with a wound area reduced to 20 % *vs.* 35 % for the untreated cells.

Results reported in this study unveil that the three IntegroPectin bioconjugates were able to decrease both proliferation (colony-forming ability) and cell migration. Beforehand, however, we tested the effects on cell viability and found that at a concentration of 5 mg/mL, cell viability decreased (albeit to varying degrees among the three IntegroPectin). As a result, we decided to use concentrations of 0.5 and 1.0 mg/mL for the subsequent experiments.

Concerning late anti-cancer events, the colony forming ability was significantly reduced by lemon IntegroPectin at 1.0 mg/mL, and by the red orange bioconjugate at 0.5 and 1.0 mg/mL concentrations. Sweet orange IntegroPectin at 1 mg/mL was able to reduce colony formation, although not significantly.

This assay provides information on the ability of a cell to maintain its proliferation and survival, which are essential for primary tumor development and metastasis formation. Data suggest that the three IntegroPectin bioconjugates were able to affect growth and survival capacity of A549 cancer cells. This first finding is very important because for example cellular hypoxia, found in up to 80 % of NSCLC tumors, causes radioresistance with radiotherapy with X-rays increasing tumor invasiveness in surviving hypoxic A549 cells [21].

T=0 T=2 T=4

Untreated

Lemon 0.5 mg/mL

Lemon 1 mg/mL

Red orange 0.5 mg/mL

Red orange 1 mg/mL

Sweet orange 0.5 mg/mL

Sweet orange 1 mg/mL

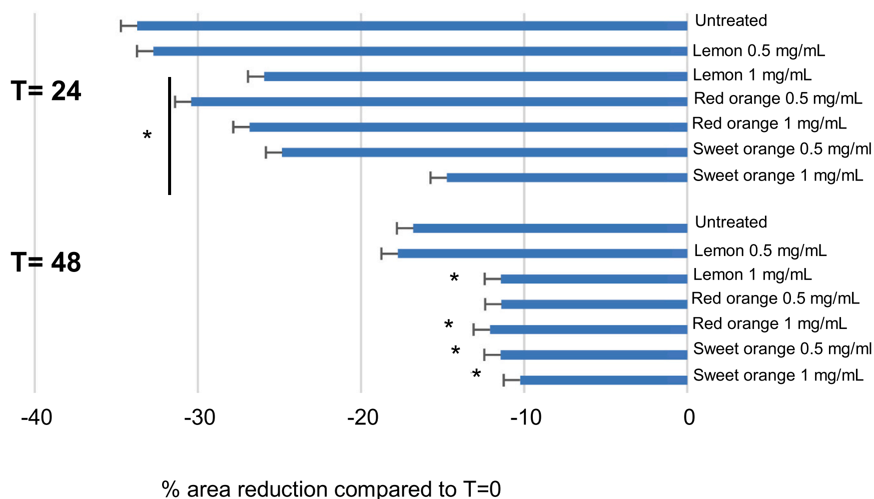

Fig. 4. Qualitative (photographs in the upper part) and quantitative (plots in the bottom part) effect of citrus IntegroPectin bioconjugates on cell migration in A549 cell line. Cells were stimulated with different citrus fruit IntegroPectin bioconjugates at 0.5 and 1 mg/mL concentration, for 24 (T = 24) and 48 (T = 48) hours.

We investigated also the anticancer effects of three *Citrus* IntegroPectin from lemon, red orange and sweet orange evaluating the effect of treatment with IntegroPectin on A549 cell motility. In this case, sweet orange IntegroPectin bioconjugate showed an exceptional ability to reduce cell motility both after 24 h and 48 h of contact with the cells both 0.5 and 1.0 mg/mL concentrations investigated. This is another most promising result in sight of practical applications.

Cell migration (the ability of cells to move through tissues) indeed is a key step in the metastatic process, the prevalent cause of death from cancer. Though not yet fully understood, metastasis of lung cancer cells is controlled by many factors, including the tumor microenvironment (acid, hypoxia, and inflammation creating a site for cancer cells to quickly metastasize); stromal cells positively assisting the invasion and migration of lung cancer cells; and epithelial-mesenchymal transition (transformation, and metastasis of cancer cells through blood vessels and lymphatics) [22].

Following demonstration of antiproliferative activity *in vitro* of grapefruit IntegroPectin (against SH-SY5Y neuroblastoma cells [23]) sourced via HC from fresh grapefruit processing waste, these findings demonstrate that IntegroPectin sourced via AC from sweet orange, lemon and red orange processing waste possess significant anticancer activity *in vitro* against A549 lung cancer cells, inhibiting both proliferation and cell motility.

Reviewing the applications of pectin in cancer therapy in 2016, Zhang and co-workers concluded that maintaining structural consistency in scalable processes is another challenge [24]. Citrus pectin as such does not show anticancer activity against lung cancer cells. However, pointing to the relevance of the pectin's structure, heat-modified Citrus pectin at of 3 mg/mL concentration induces apoptosis-like cell death and autophagy in A549 cancer cells [25]

Said heat-stressed citrus pectin does not induce classical apoptosis but rather a form of cell death that does not require caspase-3 activation. Obtained via heating commercial Citrus pectin dissolved in water at 123 °C for 1 h under 1.5 atm pressure, said heat-modified pectin holds great promise in the treatment of cancer because, as noted by Michiels and co-workers [25], a new therapeutic treatment driving a caspase-independent cell death pathway could enhance the efficiency of current cancer chemotherapeutic treatment relying on caspase-dependent apoptosis.

3.4. Structural insight into IntegroPectin bioconjugates

Table 1 shows the content of selected flavonoids and phenolic acids in the three IntegroPectin bioconjugates obtained, analyzed via high performance liquid chromatography. Flavonoids are naringin (NAR), kaempferol (KAE), and hesperidin (HESP), whereas the phenolic acids are gallic acid (GA), and *p*-coumaric acid (CA).

Red orange IntegroPectin contains the highest concentration of the analyzed flavonoids and phenolic acids, with hesperidin being the predominant compound exceeding the 7.2 mg/g concentration. Red orange IntegroPectin had the highest concentration of kaempferol (nearly 0.12 mg/g). Sweet orange IntegroPectin also contains plentiful hesperidin, beyond 6.8 mg/g, an nearly 1.0 mg/g of naringin. Lemon IntegroPectin contains nearly 3.5 mg/g of hesperidin but is the only IntegroPectin containing a substantial (2.6 mg/g) amount of *p*-coumaric acid, and nearly 2 mg/g (1.88 mg/g) of naringin.

Table 1
Flavonoid concentration in IntegroPectin bioconjugates.

IntegroPectin	GA (mg/g)	CA (mg/g)	NAR (mg/g)	KAE (mg/g)	HESP (mg/g)
Lemon	0.089 ± 0.023	2.607 ± 0.030	1.885 ± 0.028	0.019 ± 0.003	3.48 ± 0.04
Sweet orange	0.061 ± 0.002	0.142 ± 0.055	0.903 ± 0.102	0.018 ± 0.002	6.84 ± 0.50
Red orange	null	0.201 ± 0.084	0.550 ± 0.213	0.116 ± 0.015	7.23 ± 0.61

Sweet orange and red orange IntegroPectin exhibited markedly lower concentrations of CA, though the amounts of GA and NAR were comparable to those observed in the lemon IntegroPectin. These results are in agreement with the fact that hesperidin is by far the most abundant flavonoid in the peel of orange fruits, being particularly abundant in red orange cultivars [26]. Due to the very solubility in water, hesperidin precipitates and enriches the CPW waste formed during citrus fruit processing from which it is readily recovered for commercial applications. Hesperidin is also the most abundant flavonoid found in the sweet orange IntegroPectin sourced via HC [27].

The kinetic curves in Fig. 5 of 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) quenching assay show that lemon IntegroPectin exhibited the highest radical scavenging activity, and sweet orange IntegroPectin the lowest radical scavenging activity. Red orange IntegroPectin displayed a kinetic profile similar to that of lemon IntegroPectin. Similarly to what happens for lemon and grapefruit IntegroPectin sourced via hydrodynamic cavitation [28], the antioxidant power of all the IntegroPectin bioconjugates, showing steep curves rather than a curve reaching a *plateau* as it happens for single flavonoids, indicates into an antioxidant power that is growing with time.

To standardize and compare the results, data were expressed as milligrams of gallic acid equivalents (GAE) per gram of IntegroPectin. The percentage of residual DPPH at 30 and 60 min was used as a benchmark, corresponding to the initiation of the *plateau* phase (indicating completion of the reaction between the sample and the DPPH radical) and the conclusion of the experiment, respectively.

Results in Table 2 include the total phenolic content (TPC) assessed by the Folin-Ciocalteu method [29].

The TPC of lemon and red orange IntegroPectin were exceptionally high, ranging from 24.1 mg GAE/g in the case of sweet orange IntegroPectin to 33.6 mg GAE/g in the case of lemon IntegroPectin. For comparison, the TPC of lemon IntegroPectin sourced via HC and similarly dried via freeze-drying analyzed two years after its isolation was 0.88 mg GAE/g [30].

Even though being high also in the latter case (for comparison the TPC of lemon peel varies, depending on the cultivar, between 5.12×10^{-3} and 8.30×10^{-3} mg GAE/g) [31], the TPC of citrus IntegroPectin sourced via AC and analyzed two months after isolation is nearly 40 times higher than the TPC of IntegroPectin sourced via HC and aged for more than 24 months. We ascribe such decline in the TPC to polyphenol oxidase residual at the surface of the freeze-dried IntegroPectin that progressively degrade the flavonoids at the surface of the

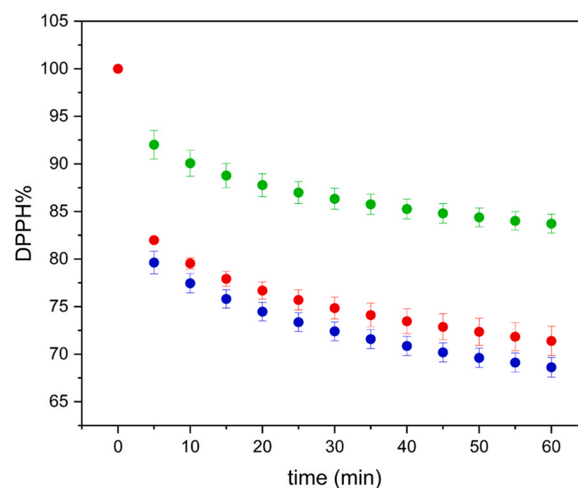

Fig. 5. Kinetic curves of DPPH (2,2-diphenyl-1-picrylhydrazyl) quenching in the presence of lemon (blue), sweet orange (green) and red orange (red) IntegroPectin sourced via acoustic cavitation.

Table 2
DPPH residual and TPC of IntegroPectin bioconjugates.

IntegroPectin	DPPH (%) (mg GAE/g) after 30 min	DPPH (%) (mg GAE/g) after 60 min	TPC (mg GAE /g)
Lemon	3.85 ± 0.19	4.24 ± 0.20	33.61 ± 0.71
Sweet orange	1.24 ± 0.19	1.56 ± 0.17	24.16 ± 0.64
Red orange	3.44 ± 0.21	3.90 ± 0.28	30.91 ± 0.84

IntegroPectin.

Indeed, when the IntegroPectin is isolated via spray-drying, the TPC is slightly higher than in the case of IntegroPectin isolated via freeze-drying, whereas the amount of proteic nitrogen in the latter case in vanishingly low due the high temperature reached in the spray-drying process [28].

Using energy made available during the cavitation process, flavonoids abundant in citrus processing waste (chiefly present in the peel and residual pulp) can overcome the relatively low energy barrier and chemically bind to the galacturonic acid residues of pectin. Recently shown via a computational study [32], this explains the unique abundance of phenolics concentrated at the surface of citrus IntegroPectin.

Ranging from –14.6 ± 3.60 mV for lemon IntegroPectin to −22.7 ± 3.70 mV for red orange IntegroPectin (Table 3), the ζ potential values of the pectic bioconjugates sourced from fresh CPW via AC are high and negative, due to the presence of anionic carboxylate groups of pectin galacturonate moieties [15]. The ζ-potential values are consistent with the TPC. In particular, the lowest ζ-potential observed in lemon integroPectin is likely due to higher DE of galacturonic acid residues with flavonoids, which likely influences its surface charge. The esterification reaction, indeed, is catalyzed by citric acid which is more abundant in lemon and red orange CPW when compared to sweet orange processing biowaste.

The FTIR spectra of all IntegroPectin bioconjugates (Fig. 6) are similar and clearly indicate highly de-esterified pectins rich in citrus flavonoids and phenolic acids.

In detail, the signals at 1720 cm^{-1} and 1630 cm^{-1} correspond to the stretching vibrations of the carbonyl group of esters and the carboxylate group of the pectin homogalacturonan (HG) galacturonic acid chain, respectively [33]. Notably, the ratio between these two peaks varies among the IntegroPectin samples. This variation is particularly evident when comparing the spectra of lemon and red orange bioconjugate samples. In the case of lemon IntegroPectin, the two peaks exhibit similar intensities, whereas in the red orange IntegroPectin spectrum, the signal at 1720 cm^{-1} is barely visible. This difference can be attributed to the degree of esterification, with the red orange sample showing the lowest DE. This observation is further supported by the ζ-potential values, as the red orange sample exhibits a greater number of free –COOH groups, resulting in the most negative zeta potential among the analyzed IntegroPectin bioconjugates. Finally, the signal at 2931 cm^{-1} is due to the stretching vibrations of C–H bonds of CH and CH_2 groups of polysaccharide rings, whereas the broad band centered at 3406 cm^{-1} is due to the O–H stretching vibration of the pyranose ring and adsorbed water [33].

Besides the peaks of residual crystalline fructose, the XRD spectra (not shown) for all citrus IntegroPectin bioconjugates confirm that the different citrus phytocomplexes obtained via AC are comprised of amorphous pectin polymer. In contrast to commercial citrus pectin (showing many diffraction peaks between 12.4° and 40.2° due to partly crystalline arrangement of the HG chains) [34], the IntegroPectin pectin sourced from fresh sweet and red orange as well as from lemon processing waste shows a broad peak centered around 18.5°. This indicates complete decrystallization of the HG regions, as it happens for lemon and grapefruit IntegroPectin sourced via HC. In either case (HC or AC), cavitation destroys the "fringed-micellar" structure of the crystalline regions of the semicrystalline pectin biopolymer [35].

Table 3
ζ-Potential of IntegroPectin bioconjugates.

IntegroPectin	ζ-potential (mV)
Lemon	–14.6 ± 3.60
Sweet orange	−18.8 ± 4.87
Red orange	−22.7 ± 3.70

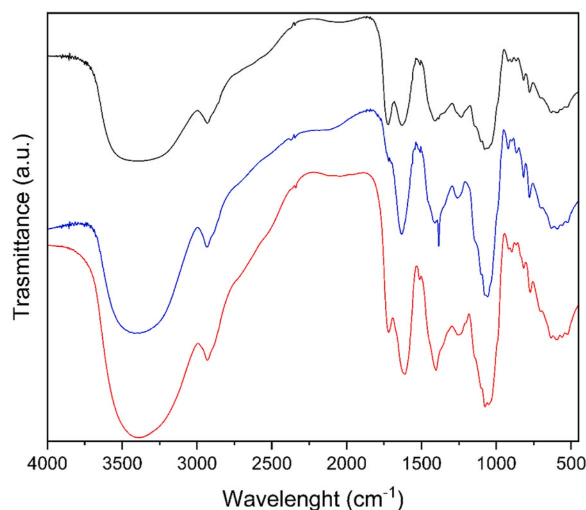

Fig. 6. FTIR spectra of lemon (black line), red orange (red line), and sweet orange (blue line) IntegroPectin.

The low degree of esterification of all citrus IntegroPectin is crucially important from the bioactivity viewpoint, as modified citrus pectin or commercial lemon pectins with DM values of 5 % and 18 % employ free carboxylic groups to bind galactin-3 to exert antiproliferative [36], mitoprotective and anti-inflammatory [37] activity.

These structural features suggest a suitable mechanism explaining the powerful lung anticancer activity *in vitro* of citrus IntegroPectin sourced via AC and isolated via freeze-drying. Alongside the intrinsic activity of modified citrus pectin rich in RG-I regions and free carboxylic acids, the highly soluble IntegroPectin uniquely rich in flavonoids bound to the molecular structure of pectin delivers the otherwise poorly soluble citrus flavonoids such as hesperidin, naringin, kaempferol and eriocitrin within the cell membranes, where these flavonoids can ultimately unleash their anticancer activity so far limited by their vanishingly low solubility in water. This is the case for naringenin, hesperidin, eriocitrin and kaempferol.

Naringenin (the aglycone of naringin) inhibits migration of A549 lung cancer cells via the inhibition of matrix metalloproteinase-2 and metalloproteinase-9 [38]. Eriocitrin, inhibits the epithelial-mesenchymal transition of cancer cells, a key process in metastatis, in lung adenocarcinoma (the most prevalent pathological subtype of NSCLC) cells, by induction of ferroptosis in cancer cells [39]. Hesperidin inhibits A549 NSCLC cell proliferation and increases caspase-3 and other apoptosis-related activities [40]. It also decreases mitochondrial membrane potential activities, and inhibits inflammatory response pathways. Kaempferol induces apoptosis of A549 cells via cleavage of caspase-7 and poly ADP-ribose polymerase, and concomitant activation of MEK-MAPK pathways [41].

4. Conclusions

In summary, we have discovered that citrus IntegroPectin bioconjugates obtained through acoustic cavitation in water only of lemon, red orange, and sweet orange industrial processing waste show substantial anticancer action *in vitro* against human non-small cell lung

cancer cells of line A549.

All IntegroPectin bioconjugates tested affected long-term proliferation and cell migration, although IntegroPectin from lemon and red orange were more effective than sweet orange in reducing colony formation activity. These results indicate that citrus IntegroPectin bioconjugates exhibit antitumor effects in A549 cells by reducing cancer cell progression, providing a basis for further investigation in lung cancer therapy. The use of IntegroPectin is an unconventional approach. Most *in vitro* studies on the anti-cancer effects of citrus pectin, indeed, were conducted using either commercial citrus pectin, or citrus pectin sourced from the fruit peels using conventional acid hydrolysis of citrus peel at high temperature. The latter process heavily degrades the molecular structure the native heteropolysaccaride by removing most "hairy" RG regions of the polymer leaving most of the "smooth" HG regions with a few neutral sugar units bound to the HG chain [42].

Based also on previous results on *in vitro* antimicrobial, anticancer neuroprotective, and *in vivo* cardioprotective properties of lemon and grapefruit IntegroPectin sourced from CPW via hydrodynamic and acoustic cavitation [14], we ascribe said pronounced biological activity to the unique structure of pectin sourced via cavitation in water only; and to the uniquely high amount of flavonoids bound and adsorbed at the surface of the IntegroPectin pectic polymeric chain. For comparison, the total phenolic content of lemon and red orange IntegroPectin ranges from 24.1 mg GAE/g in the case of sweet orange IntegroPectin to 33.6 mg GAE/m in the case of lemon IntegroPectin, namely four orders of magnitude higher than the TPC of lemon peel varying, depending on the cultivar, between 5.12×10^{-3} and 8.30×10^{-3} mg GAE/g.

Results are particularly promising because citrus pectin and citrus flavonoids are both health beneficial substances whose application in medicine and nutraceutics so far has been hindered by the conventional extraction process in the case of pectin, and by the low solubility of citrus flavonoids [43].

Carried out in water only directly on semi-industrial scale using CPW from organic agriculture followed by freeze-drying of the aqueous extract to isolate the pectic bioconjugate, the process to produce citrus IntegroPectin is both reproducible and readily scalable. Sourcing IntegroPectin from untreated (not dried, in this case) agro-industrial waste via the CytroCav circular economy process based on cavitation of CPW conducted in water only converts biowaste into two new valued bioproducts (IntegroPectin bioconjugate and CytroCell nanocellulose) thereby promoting a more efficient and economically viable approach to pectin (and nanocellulose) production, aligning with global sustainability goals [44].

In conclusion, given the frequency and high mortality of lung cancer, *in vivo* and clinical studies to test the activity of citrus IntegroPectin in the treatment of lung cancer should be urgently conducted. IntegroPectin could work as such, or included in a multifaceted therapeutic strategy for lung cancer aimed at boosting immune function, reducing inflammation, inhibiting tumor growth and spread. Translating *in vitro* results to clinical practice is a complex and multifaceted process. Several key factors need to be addressed such as bioavailability and absorption, dosage and safety, formulation and delivery. It is encouraging and further supporting the aforementioned need for preclinical and clinical studies that citrus pectin is already used in various clinical applications. For example in a pilot study, orally administered MCP (modified citrus pectin) improved quality of life and clinical benefits in patients with solid tumors. Trial NCT02575404 investigating "belapectin", a galectin-3 inhibitor, combined with pembrolizumab, showed no dose-limiting toxicities and an objective immune response, with increased T-cell activation and reduced suppressor cells [45].

CRediT authorship contribution statement

D'Anna Claudia: Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Giovanna Li Petri:** Visualization, Investigation, Data curation. **Caterina Di Sano:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization. **Francesco Meneguzzo:** Writing – review & editing, Visualization, Methodology, Data curation. **Rosaria Ciriminna:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Mario Pagliaro:** Writing – original draft, Formal analysis, Conceptualization. **Giuseppe Angellotti:** Visualization, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prenap.2025.100313.

Data availability

Data will be made available on request.

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