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In Vitro Activity of Citrus IntegroPectin Against Breast Cancer and Colon Cancer

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ABSTRACT

We investigate the ability of IntegroPectin bioconjugates obtained via acoustic cavitation of lemon, red orange, and sweet orange industrial processing waste to inhibit the migratory features of Caco-2 colon cancer and MCF-7 breast cancer cells. All tested flavonoid-pectin conjugates dissolved in aqueous phase substantially reduced cell migration of both tumor cell lines at 0.5 and 1.0 mg/mL concentration. Red orange and lemon IntegroPectin bioconjugates at 5 and 10 mg/mL load reduced cell viability for both cell lines. Adding to recent promising results against lung cancer, these outcomes support further investigation of citrus IntegroPectin bioconjugates for the treatment of cancer.

1 | Introduction

Abundant in fruits and plants, pectin is a galacturonic acid polymer comprising homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), arabinogalacturonan, and xylogalacturonan regions [1]. Its primary structure consists of repeating (1 → 4)-α-D-GalA (galactopyranosyluronic acid residues, partly methyl-esterified at *O*-6 position (and to a 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. Knowledge of its secondary structure, particularly in water, is expanding [2].

Commercially sourced as high methoxyl (HM) pectin (degree of methyl esterification DE>50%) from dried (or fresh, for pectin extraction plants close to citrus juice plants) citrus peel (or apple pomace) via prolonged hydrolysis using dilute mineral acid at 70°C–80°C, pectin is purified via vacuum evaporation and alcohol precipitation using isopropyl alcohol to precipitate pectin [3]. Low methoxyl (LM) pectin having DE < 50% is commercially produced by pectin manufacturers by controlled hydrolysis of HM pectin.

Pectin is a bioactive and health-beneficial heteropolysaccharide [4]. Its modified version rich in galactose residues present in RG-I galactan and arabinogalactan side chains obtained by hydrolysis at high pH of citrus pectin at 123°C for 1 h under 1.5 atm pressure (the process affords lower molecular weight pectin by β-elimination and reduction in DE) binds to lung cancer cells galactoside-binding galactin-3 protein [5]. Subsequent research found that said modified citrus pectin (MCP) is an anti-metastatic agent capable of inhibiting proliferation and metastasis for numerous cancers [6].

Extracted via hydrodynamic or acoustic cavitation in water only of industrial citrus processing waste (CPW) of different organically grown citrus fruits, IntegroPectin is a LM pectin showing antioxidant, anti-inflammatory, cardioprotective, neuroprotective, mitoprotective, antimicrobial and anticancer properties [7]. Its broad-scope bioactivity is ascribed to unique molecular

structure (ultralow degree of methylation and abundant RG-I regions), enriched in citrus flavonoids (and terpenes).

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 [8], explaining the unique abundance of phenolics concentrated at the surface of citrus IntegroPectin.

Recently, we reported the in vitro activity of citrus IntegroPectin bioconjugates sourced via acoustic cavitation (AC) of lemon, red orange and sweet orange CPW against lung cancer evaluating both cell proliferation and long-term proliferation [9]. 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 development of effective therapies requires to prevent metastatic spread [10].

In this study we report the outcomes of in vitro investigation of the anti-cancer properties of the aforementioned three citrus IntegroPectin bioconjugates using Caco-2 colon cancer and MCF-7 breast cancer cells, as representative models of breast and colorectal cancer, due to the high global prevalence and clinical relevance of these tumor types.

Originally sourced from a human colon adenocarcinoma, the human intestinal Caco-2 cell line is frequently used in biomedical studies as a model of the intestinal barrier [11]. MCF-7 is the most studied human breast cancer cell line in the world, and results from this cell line have had a fundamental impact upon breast cancer research [12]. Breast cancer is a significant public health problem that requires further research at the molecular level in order to define its prognosis and specific treatment [13]. Similarly, colon cancer is a major cause of mortality in industrialized countries [14]. Citrus flavonoids, amid natural products researched as new therapies for both types of cancers, are promising potential therapeutic agents [15]. Their practical employment in the treatment of cancer and of many other pathologies, however, has been hindered by their poor solubility in water and limited bioavailability.

2 | Materials and Methods

2.1 | IntegroPectin Isolation, Flavonoid Quantification, DPPH Assay, and Folin–Ciocalteu Assay

Lemon, red orange, and sweet orange IntegroPectin bioconjugates were prepared via AC of fresh citrus processing waste kindly provided by Citrus Campisi (Siracusa, Italy) as recently reported [9]. The same study included details on flavonoid quantification, DPPH assay, and Folin–Ciocalteu assay.

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 NY, 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 conduct the biological tests

2.3 | Cell Cultures

MCF-7 and Caco-2 cells were cultured in D-MEM and RPMI medium, respectively. Both medium were supplemented with heat-deactivated (56°C, 30 min) 10% fetal bovine serum (FBS), streptomycin and penicillin, 1% nonessential amino acids and 2 mM L-glutamine (all purchased from Euroclone, Pero, Italy). The cells grew as adherent monolayers. When they reached approximately 80% of confluence, cells were treated with IntegroPectin from lemon red orange and sweet orange at concentrations of 0.5 and 1 mg/mL and incubated for 24 h in a humidified ambient (5% air CO_2) at 37°C.

2.4 | Effect of IntegroPectin From Lemon, Red Orange, and Sweet Orange on Caco-2 and MCF-7 Cell Viability

Cell viability was assessed with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (PROMEGA, Madison WI, USA), in accordance with the manufacturer's guidelines. Cells were cultured in 96-well plates and treated for 24 h in quadruplicate with IntegroPectin from lemon red orange and sweet orange at different concentrations (0.25, 0.5, 1.0, 5.0 and 10 mg/mL). Absorbance was measured at 490 nm using a microplate reader. The results were expressed as a percentage relative to the untreated (NT) control and the data are presented as the average of quadruplicate wells ± standard deviation (SD). Differences between the various experimental conditions were assessed using ANOVA, with Fisher's test applied for corrections ($*p < 0.05$ was considered statistically significant).

2.5 | Effect of IntegroPectin From Lemon, Red Orange, and Sweet Orange on Caco-2 and MCF-7 Cell Line Motility

During the experiment, a "wound" (a cell-free zone in the cell monolayer) was made and recolonization of the scratched region is monitored to quantify cell migration area. In detail, the wound area was 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 allowed to evaluate the migration rate [16].

Briefly, MCF-7 and Caco-2 cells were grown in a 6-well plate until they reached confluence. Three circular wounds were created in each well using a 200-µL pipette tip. Wells were washed with PBS to discharge any debris, and after 1 h, IntegroPectin from lemon, red orange and sweet orange added (0.5 and 1 mg/mL concentrations). Images were performed at 0, 24, and 48 h post-wounding, with a digital camera integrated into an inverted phase-contrast microscope.

To analyze wound area and closure rate, we used the ImageJ software, presenting results as percentage of area reduction at 24 h and 48 h, relative to time zero (0 h). Differences between the various experimental conditions were assessed using ANOVA, with Fisher's test applied for corrections ($*p < 0.05$ was considered statistically significant) using free software for analyzing numbers and sizes of cell colonies [17].

3 | Results

3.1 | Citrus IntegroPectin Bioconjugates

The vivid colors of lemon, red orange, and sweet orange IntegroPectin sourced via AC from fresh CPW (Figure S1) show visual evidence of the large amount of flavonoids contained in these bioconjugates.

Recently reported [9], the outcomes of the analyses of selected flavonoids and phenolic acids (naringin, kaempferol, hesperidin, gallic acid, and *p*-coumaric acid) in the three IntegroPectin employed indicate that red orange IntegroPectin contains the highest concentration of the analyzed flavonoids and phenolic acids, with hesperidin exceeding the 7.2 mg/g concentration, and kaempferol amounting to nearly 0.12 mg/g. Sweet orange IntegroPectin also contains plentiful hesperidin, beyond 6.8 mg/g, and nearly 1.0 mg/g of naringin, 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 [18]. 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.

The values of total phenolic content (TPC) assessed by the Folin–Ciocalteu method, expressed as milligrams of gallic acid equivalents (GAE) per gram of IntegroPectin, are exceptionally high for all three IntegroPectin phytocomplexes (33.6 mg GAE/mg for lemon, 30.91 mg GAE/mg for red orange, and 24.16 for sweet orange bioconjugates). For comparison, the TPC of lemon IntegroPectin sourced via HC and similarly dried via freeze-drying analyzed two years after its isolation is 0.88 mg GAE/g [19], whereas the TPC of lemon peel varies (depending on the cultivar) between 5.12×10^{-3} and 8.30×10^{-3} mg GAE/g) [20].

The radical scavenging activity of these bioconjugates assessed by the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay shows pronounced and unique activity for all three IntegroPectin pectic materials. Indeed, as in the case of lemon and grapefruit IntegroPectin sourced via hydrodynamic cavitation [21], 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.

The XRD spectra (Figure 1) for all citrus IntegroPectin bioconjugates between 5° and 30° diffraction angle (2θ) displaying a broad peak centered around 18.5° show evidence that all citrus IntegroPectin phytocomplexes obtained via AC of citrus processing waste are comprised of amorphous pectin polymer.

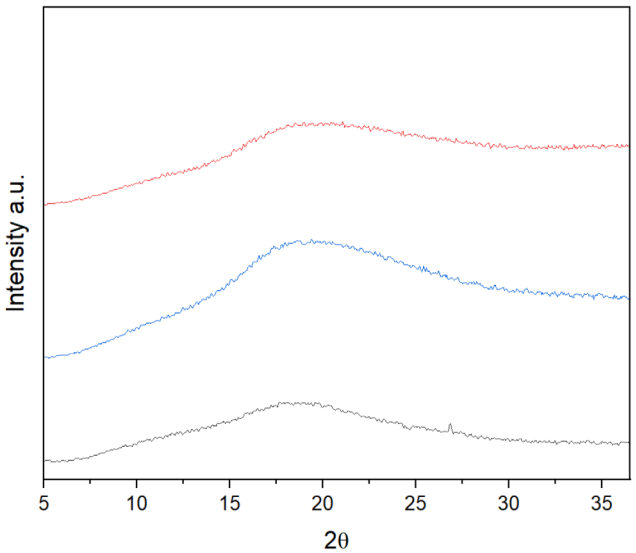

FIGURE 1 | XRD spectra of lemon (blue line), sweet orange (red line), and red orange IntegroPectin (black line).

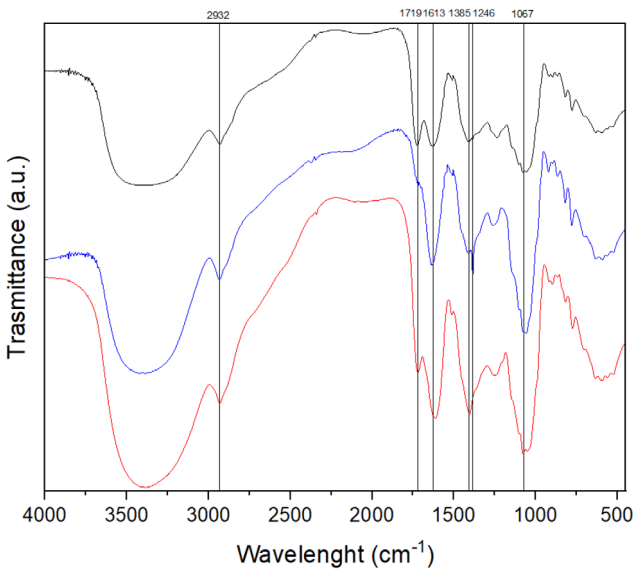

FIGURE 2 | FTIR spectra of lemon (black line), red orange (red line), and sweet orange (blue line) IntegroPectin.

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 [22]. For comparison, the XRD spectrum of commercial citrus pectin sourced via acid hydrolysis of citrus peel shows many diffraction peaks between 12.4° and 40.2° due to partly crystalline arrangement of the HG chains [23].

The FTIR spectra of the three IntegroPectin bioconjugates investigated (Figure 2) are similar and clearly indicate highly de-esterified pectins rich in citrus flavonoids and phenolic acids [24]. Signals at 1720 and 1630 cm^{-1} correspond to the stretching vibrations of the carbonyl group of esters and to carboxylate group of the pectin HG chain, respectively.

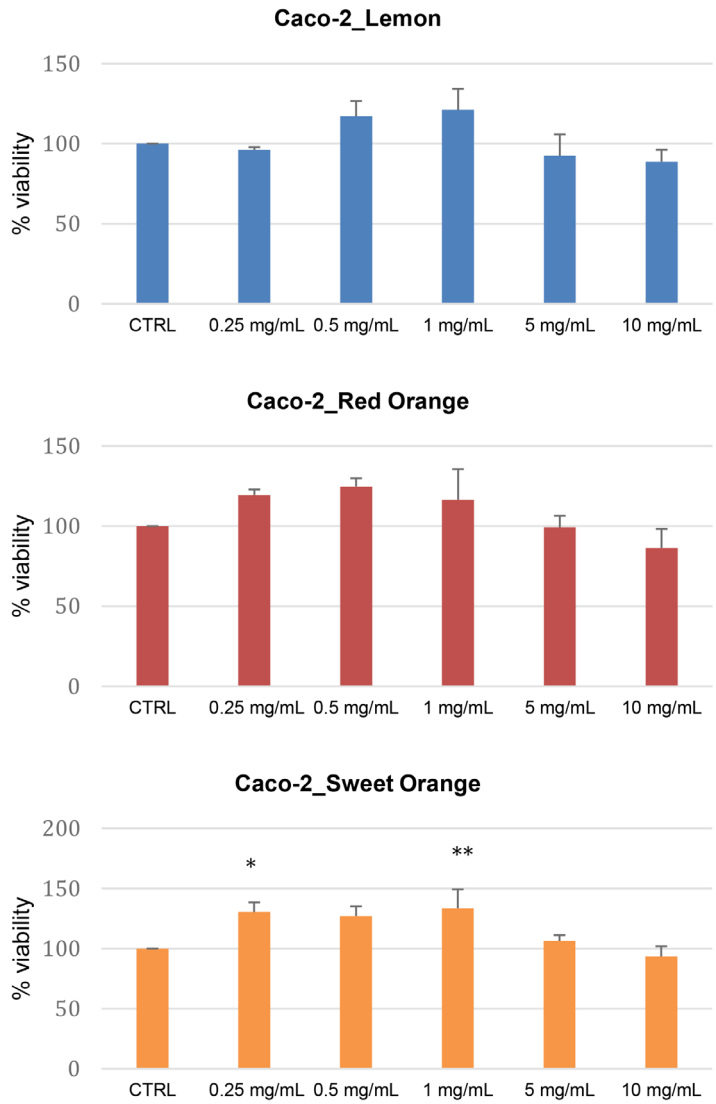

FIGURE 3 | Effect of different citrus IntegroPectin bioconjugates on Caco-2 cell viability. Cells were cultured for 24 h with different citrus IntegroPectin bioconjugates (0.25, 0.5, 1.0, 5.0 and 10 mg/mL). Data are expressed as % of untreated and represent mean ± SD ($n = 4$). Differences between the various experimental conditions were assessed using ANOVA, with Fisher's test applied for corrections (*$p = 0.0386$, **$p = 0.0228$ vs Control).

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 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.

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.

3.2 | Effect of IntegroPectin From Lemon, Red Orange, and Sweet Orange on Caco-2 Cell Viability

Figure 3 shows evidence that the cell viability of Caco-2 cells cultured with citrus IntegroPectin bioconjugaes in aqueous solution at increasing concentration (0.25, 0.5, 1.0, 5.0 and 10 mg/mL) for 24 h started to decrease at 5 mg/mL IntegroPectin concentration.

Cell viability was measured through the MTS cell viability test, in which reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) is due to dehydrogenases enzymes present in mitochondria, with this endpoint being a good biomarker of the damage induced in this organelle. Added to the medium, MTS is reduced by cells into colored formazan which is measured spectrophotometrically at 490 nm after 30 min of incubation in the dark [25].

In the case of lemon and red orange bioconjugate a concentration of 5 mg/mL reduced cell viability below 90%. The lowest cell viability was observed for red orange IntegroPectin at 10 mg/mL concentration, when Caco-2 cell viability dropped to 86.3%. The IntegroPectin sourced from sweet orange was the least active in reducing cell viability, that reached 93.4% at 10 mg/mL load but increased beyond 100% for all loads comprised between 0.25 and 5 mg/mL.

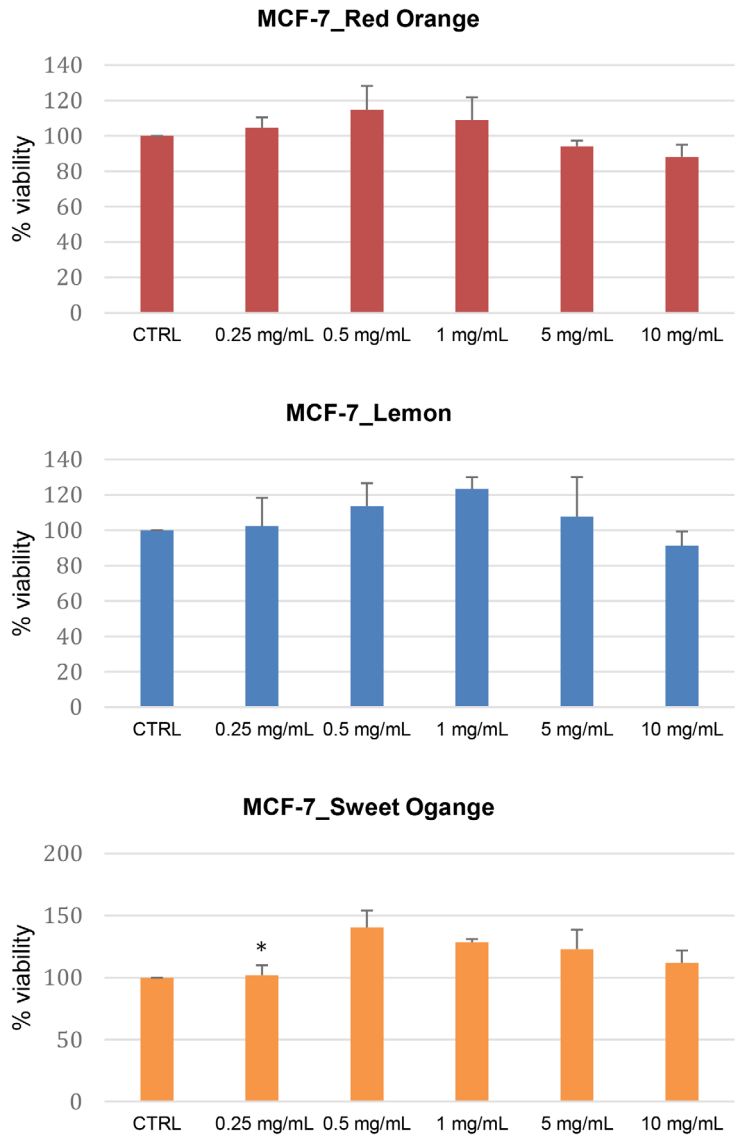

FIGURE 4 | Effect of different citrus IntegroPectin bioconjugates on MCG-7 cell viability. Cells were cultured for 24 h with different citrus IntegroPectin bioconjugates (0.25, 0.5, 1.0, 5.0 and 10 mg/mL). Data are expressed as % of untreated and represent mean ± SD ($n = 4$). Differences between the various experimental conditions were assessed using ANOVA, with Fisher's test applied for corrections (*$p = 0.0170$ vs. Control).

3.3 | Effect of IntegroPectin From Lemon, Red Orange, and Sweet Orange on MCF-7 Cell Viability

Plots in Figure 4 show that a significant reduction in MCF-7 cell colony number was observed after treatment of the cells for 24 h with red orange IntegroPectin at 10 mg/mL concentration when cell viability was 88.2%. Viability reduction below 100% (94.1%) was observed already at 5 mg/mL concentration. Similarly, in the case of lemon IntegroPectin, reduction of cell viability to 91% was observed at 10 mg/mL concentration. In the case of sweet orange IntegroPectin, on the other hand, cell viability increased at all tested loads.

Concerning the cell shape in the presence of IntegroPectin bioconjugates in solution, photographs in Figures 5 and 6 show that whereas at loads between 0.25 and 1.0 mg/mL the morphology of both Caco-2 and MCF-7 cells was not significantly affected, at higher loads tested of 5 and 10 mg/mL, the cell shape significantly changed for both cell lines.

In the case of Caco-2 cells in the presence of each (lemon, red orange, and sweet orange) IntegroPectin phytocomplex at 10 mg/mL load, the cells darkened, detached from each other and contracted pointing to changes in the structure of the cholesterol-rich membrane and thus to endocytosis.

It may be relevant here to notice that in Caco-2 cells in contact with curcumin-loaded zein/pectin nanoparticles, endocytosis takes place via caveolae-dependent endocytosis, clathrin-dependent endocytosis, and micropinocytosis, indicating that nanoparticle size, composition and hydrophilicity/hydrophobicity all affect the nanoparticle internalization and transport across the cells [26].

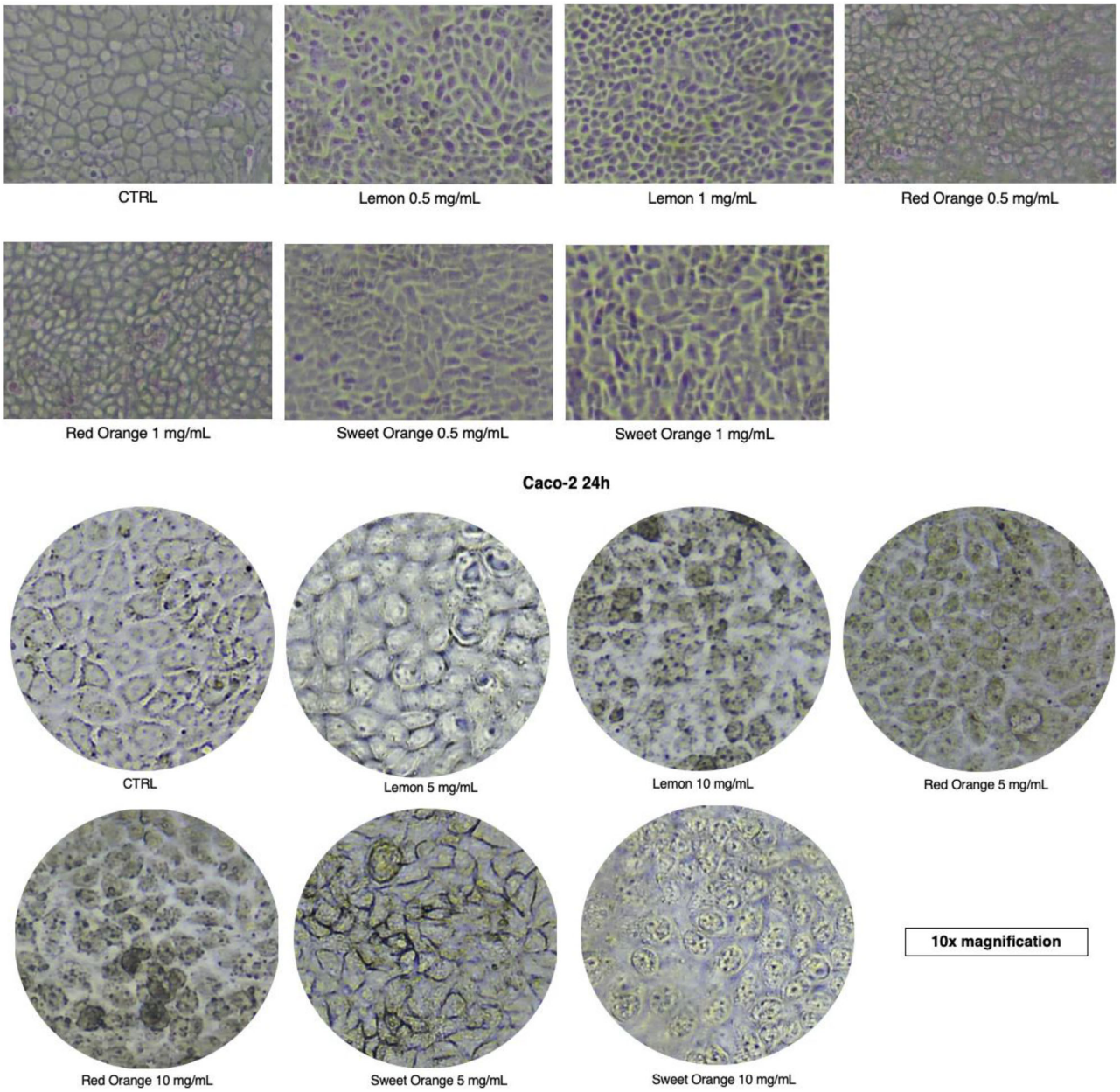

FIGURE 5 | Morphology of Caco-2 cells in the presence of different citrus IntegroPectin bioconjugates at 0.5 and 1.0 mg/mL load after 24 h (*top*); and in the presence of the same IntegroPectin bioconjugates at 5 and 10 mg/mL load (*bottom*).

3.4 | Effect of IntegroPectin From Lemon, Red Orange, and Sweet Orange on Caco-2 and MCF-7 Cell Line Motility

Photographs and histograms in Figure 7 show that Caco-2 cells stimulated with different citrus IntegroPectin bioconjugates at 0.5 and 1 mg/mL concentration for 24 and 48 h significantly reduced cell motility. Increased cancer cell motility is a feature of tumor aggressiveness [27].

The most effective cancer cell motility inhibitors were lemon and red orange IntegroPectin conjugates that at 1.0 mg/mL concentration that after 48 h limited the wound area reduction to just 55% versus 77% of the untreated cells. Contrary to what observed for cell viability in the MTS test, the sweet orange IntegroPectin, too, significantly reduced cell migration, with 56% wound area reduction after 48 h versus 77% in the case of untreated cells. This outcome is in agreement with the previously reported ability of sweet orange IntegroPectin bioconjugate to reduce cell motility also in the case of A549 lung cancer cells [9].

As shown by photographs and plots in Figure 8, in the case of MCF-7 cells too, all three IntegroPectin bioconjugates were able to significantly decrease cell migration. Now, the most effective citrus bioconjugates cancer cell motility inhibitors were red orange and sweet orange IntegroPectin, that at 1.0 mg/mL concentration after 48 h respectively limited the wound area reduction to just 40.2% vs. 75.9% of the untreated cells, and to 42.2% in the case of sweet orange IntegroPectin.

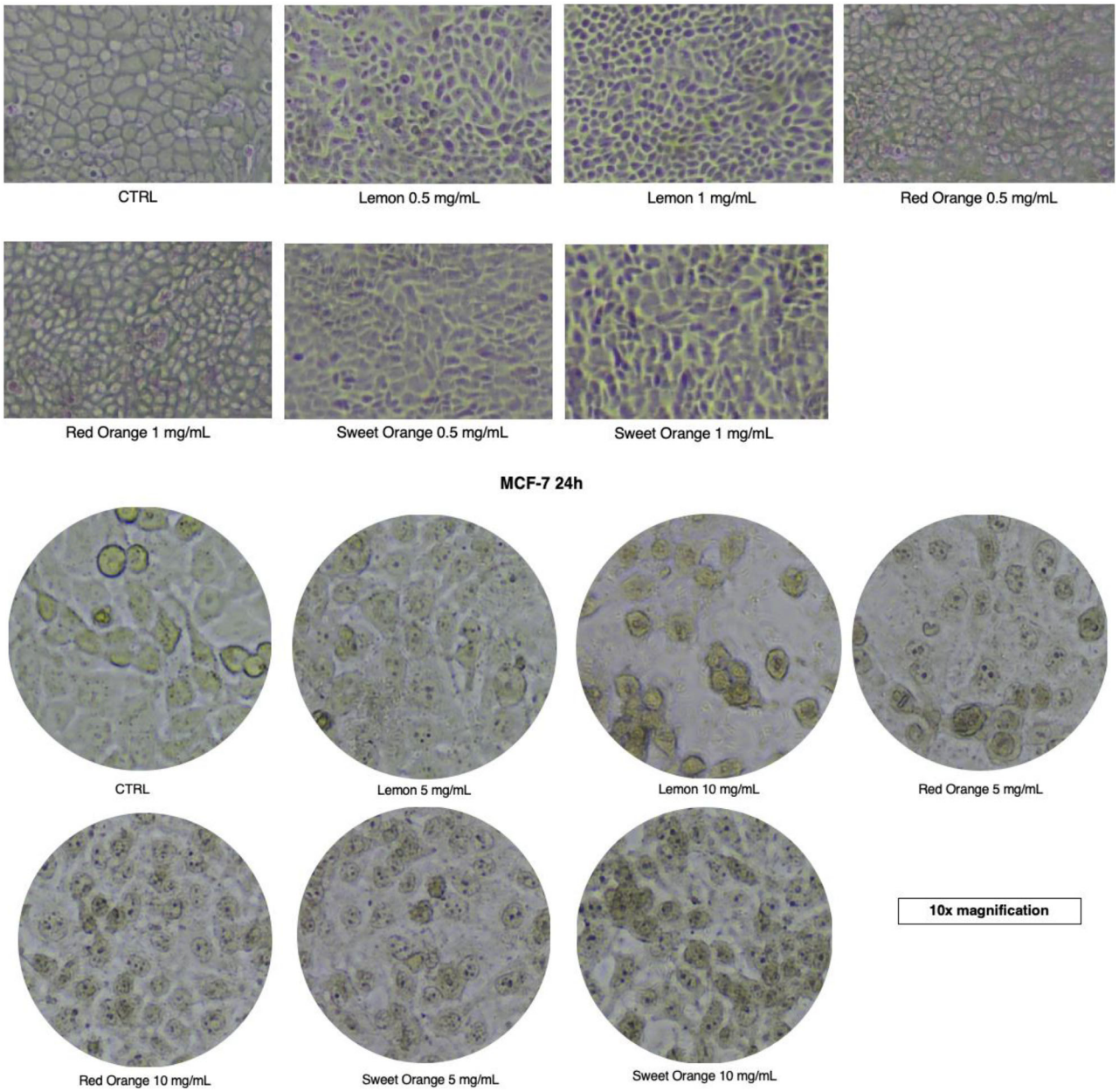

FIGURE 6 | Morphology of MCF-7 cells in the presence of different citrus IntegroPectin bioconjugates at 0.5 and 1.0 mg/mL load after 24 h (*top*), and in the presence of the same IntegroPectin at 5 and 10 mg/mL load (*bottom*).

Lemon IntegroPectin at 1.0 mg/mL concentration after 48 h limited the wound area reduction to 43.2%, but in this case even more pronounced limited wound area reduction to 40.3% was observed at 0.5 mg/mL concentration. Due to enhanced ability to detach and plasticity driven by epithelial-mesenchymal transition (which endows the cells with increased invasiveness and motility [28], tumor cell circulation is a key step in the metastatic process.

4 | Discussion

Following demonstration of in vitro antiproliferative activity of lemon and red orange IntegroPectin bioconjugates against lung cancer cells [9], these findings demonstrate that these new citrus pectins possess significant anticancer activity in vitro against MCF-7 breast cancer and Caco-2 colon cancer cells, inhibiting both proliferation and cell motility.

We ascribe said enhanced bioactivity to the unique molecular structure of the IntegroPectin bioconjugates having low degree of esterification, enriched in RG-I regions, and rich in molecularly bound citrus flavonoids (and adsorbed terpenes) [29, 8]. Accordingly, the significant improvement in biological activity and bioavailability of MCP have both been ascribed to its low DE and low molecular weight, translating into higher number of free carboxylic acid groups binding and inhibiting galactin-3 [30].

These structural features suggest a suitable mechanism explaining the broad-scope anticancer activity in vitro of citrus IntegroPectin bioconjugates. Alongside possessing the intrinsic

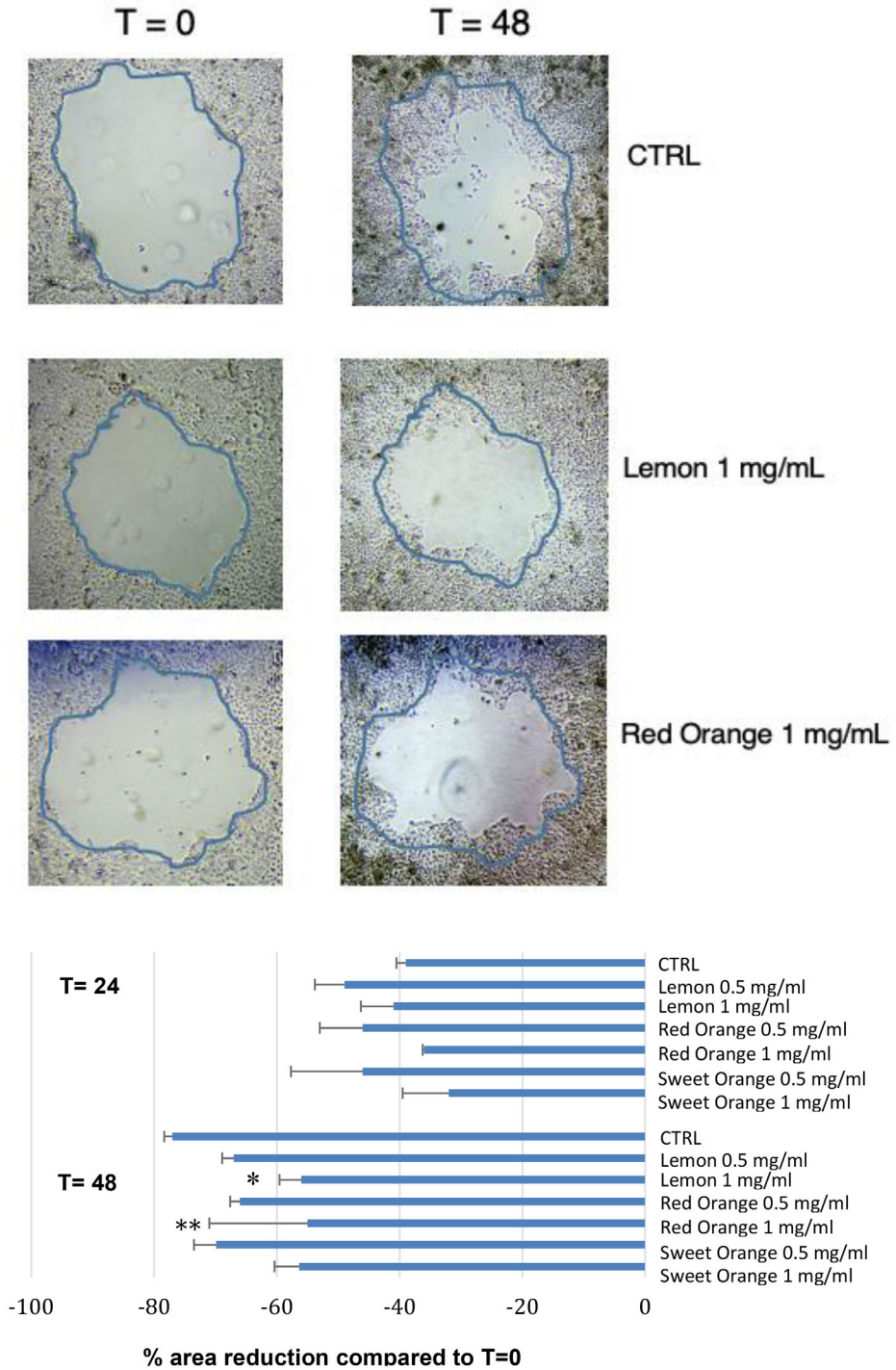

FIGURE 7 | Effect of different citrus IntegroPectin bioconjugates on cell migration, in Caco-2 cell line. Cells were stimulated with different Citrus IntegroPectin bioconjugates at 0.5 and 1 mg/mL concentration, for $t = 24$ h and $t = 48$ h. Results expressed as percentage of area reduction at $t = 48$ h compared to $t = 0$ h ($n = 3$). Differences between the various experimental conditions were assessed using ANOVA, with Fisher's test applied for corrections (*$p = 0.0425$, **$p = 0.0349$ *vs.* Control).

anticancer activity of citrus pectin rich in RG-I regions and free carboxylic acids, the highly soluble IntegroPectin bioconjugates uniquely rich in molecularly bound flavonoids deliver 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 limited by their lack of solubility in water. Indeed, the poor oral bioavailability of these and other dietary biophenols has limited for decades their development as therapeutic agents [31, 32].

For example, the in vivo plasma concentrations of flavonoids are typically in the range of 0.01–0.1 μM, significantly lower than the half maximal inhibitory concentration or half maximal effective concentration values of 5–50 μM commonly reported for their

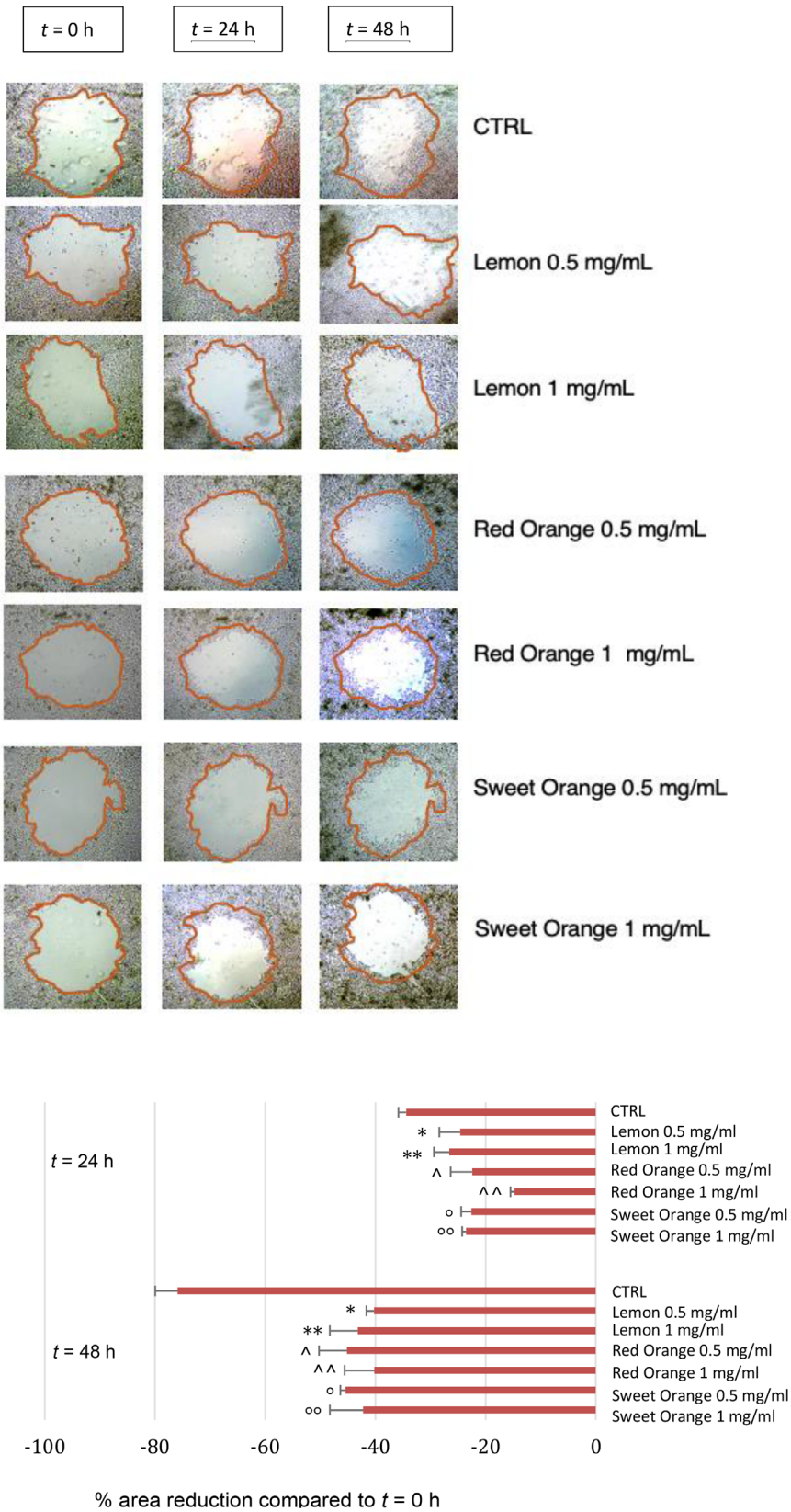

FIGURE 8 | Effect of different citrus IntegroPectin bioconjugates on cell migration, in MCF-7cell line. Cells were stimulated with different citrus IntegroPectin bioconjugates at 0.5 and 1 mg/mL concentration, for 24 and 48 h. Results expressed as percentage of area reduction at $t = 24$ h and $t = 48$ h compared to $t = 0$ h ($n = 3$). Differences between the various experimental conditions were assessed using ANOVA, with Fisher's test applied for corrections (T = 24: *$p = 0.0152$, **$p = 0.0436$, ^$p = 0.0045$, ^^$p = 0.0003$, °$p = 0.0430$, °°$p = 0.0083$ vs. Control; T = 48: *$p = 0.0002$, **$p = 0.0004$, ^$p = 0.0007$, ^^$p = 0.0002$, °$p = 0.0008$, °°$p = 0.0003$ vs. Control).

anticancer, anti-inflammatory effects and other effects in vitro [33]. Indeed, studying the cell viability of Caco-2 cells treated with 40 μM flavonoids, Xu and coworkers found no significant difference between the Caco-2 cell viability of the control and each flavonoid [34]. This outcome shows that flavonoids (including citrus flavonoids such as quercetin, rutin, naringenin, kaempferol and hesperetin) at the tested concentration show no cytotoxicity to Caco-2 cells [34]. However, when a quercetin-DHA ester derivative (DHA is docosahexaenoic acid) is covalently bound to pectin, the bioconjugate becomes able to inhibit migration and cell viability of Caco-2 cells [35].

The fact that sweet orange IntegroPectin increases Caco-2 cell viability up to a concentration of 1 mg/mL (Figure 1) is in agreement with previous findings that the viability of Caco-2 cells incubated with different hesperidin concentrations ranging from 0 to 100 μM remained above 90% compared with that of untreated control cells, namely cell viability values considered non-cytotoxic [36].

The different effects observed at low concentrations between sweet orange and red orange IntegroPectin may be due to the phytochemical composition, including anthocyanins, flavonoids, volatile constituents, and phenolic compounds. Such compositional variations, together with potential variations in pectin structure and degree of esterification, could influence cellular responses and contribute to the distinct biological effects observed in Caco-2 cells.

In brief, the use of IntegroPectin in cancer treatment provides a new approach, alternative to in vitro and in vivo studies on the anti-cancer effects of citrus pectin conducted using either commercial citrus pectin or citrus pectin sourced from the fruit peels via conventional acid hydrolysis at 70°C–80°C [37]. The approach is based at improving the bioavailability of flavonoids via covalent binding to pectin, as well as to improve the antitumor properties of pectin through its fragmentation, decrystallization and reduction of DE.

The former approach to flavonoid-pectin bioconjugates has been successfully investigated also to enhance the bioavailability of numerous flavonoids with the resulting conjugate showing significantly enhanced, antioxidant, antimicrobial and anti-cancer activity [38].

Although no mechanistic investigations were performed in the present study, pectin's anticancer and antimetastatic properties, especially when modified via alkaline hydrolysis at high temperature into bioavailable MCF with low molecular mass and low DE, chiefly arise from the ability to inhibit tumor growth, induce apoptosis and suppress metastasis by binding galectin-3 inhibiting its anti-apoptotic function [39].

In addition, citrus flavonoids such as catechin, kaempferol, naringenin, and quercetin owe their breast antitumor activity to a multi-targeted mechanism involving strong binding affinities with highly interconnected hub-genes EGFR, SRC, PIK3CA, and ESR1 showing the strongest associations with other nodes in protein-protein interaction network in breast carcinoma [40]. Naringenin, furthermore, is a potential therapeutic agent for colorectal cancer based on a multi-targeted mechanism involving apoptosis promotion by increasing the expression of proapoptotic genes (Bax, caspase9, and p53) and downregulation of the anti-apoptotic gene Bcl2, and inhibition of enzymes involved in tumor cell survival and proliferation [41]. Similarly, hesperidin dose-dependently reduces colon cancer cell proliferation, migration, and invasion by suppressing SLC5A1 glucose transporter and phosphorylated EGFR expression [42]. Coordinating signaling pathways that regulate cell survival, migration, and growth, the epidermal growth factor receptor (EGFR) is overexpressed in many solid tumors, including breast and colon cancers [43].

Similarly, a number of preclinical studies have shown that kaempferol inhibits the growth, migration and invasion of breast cancer cells, driving apoptosis [44]. Yet, poor bioavailability of citrus flavonoids in general, including kaempferol, requires the development of drug delivery systems capable to enhance delivery and thus activity and efficacy in biomedically relevant conditions [45].

Cavitation of citrus processing waste directly affords the one-pot concomitant extraction and structural modification of pectin, converted into a modified pectin enriched in RG-I regions, having very low DE and containing a high amount of flavonoids bound to the pectin backbone [7, 29]. Remarkably, Zheng and co-workers lately reported first experimental evidence that hesperidin molecularly binds to pectin in citrus peel [46], as anticipated in a computational study [8].

Commercial citrus pectin does not show anticancer activity against breast cancer cells. However, pointing once again to the crucial relevance of citrus pectin's structure, modified citrus pectin (MCP) at 3 mg/mL concentration inhibits breast cancer development in mice by targeting tumor-associated macrophage (TAM) survival in hypoxic microenvironment [47]. Abundant in hypoxia area of the tumor tissue and associated with metastasis of tumor including breast cancer, TAMs are concentration-dependently suppressed by MCP treatment through inhibiting galectin-3 expression leading to inhibition of glucose transporter-1 expression and glucose uptake.

Finally, looking to forthcoming practical applications, citrus IntegroPectin sourced via acoustic or hydrodynamic cavitation has shown lack of toxicity when in contact with healthy human cells even at high dosage. For example, studies on human neuroblastoma (SH-SY5Y) and microglial (HMC3) cell lines show that IntegroPectin exerts no cytotoxic effects, even at high dosages of up to 1 mg/mL [48, 49].

5 | Conclusions

In summary, we have discovered that citrus IntegroPectin bioconjugates obtained from lemon and red orange industrial processing waste through acoustic cavitation conducted in water only followed by IntegroPectin isolation via freeze drying show substantial anticancer action in vitro against human colon (Caco-2) and breast (MCF-7) cancer cells.

Red orange and lemon IntegroPectin phytocomplexes affected both long-term proliferation and cell migration. These results indicate that citrus IntegroPectin bioconjugates exhibit antitumor

effects on said cell lines by reducing cancer cell progression, providing a basis for further investigation in cancer therapy.

Although limited to in vitro models, these results support further investigation into the biological activity and mechanism of action of citrus IntegroPectins in cancer-related cellular systems. Preclinical studies to test the activity of citrus IntegroPectin bioconjugates in the treatment of these cancers will hopefully be conducted soon.

Author Contributions

Caterina Di Sano: conceptualization, investigation, methodology, writing – review and editing, formal analysis, data curation. **Claudia D'Anna**: conceptualization, methodology, writing – review and editing, formal analysis, data curation, investigation. **Giuseppe Angellotti**: investigation, methodology, data curation, writing – review and editing. **Mario Pagliaro**: conceptualization, writing – original draft, writing – review and editing, formal analysis, supervision, methodology. **Rosaria Ciriminna**: conceptualization, methodology, formal analysis, writing – review and editing, funding acquisition. **Giovanna Li Petri**: investigation, methodology, data curation, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting file 1: cfch70030-sup-0001-SuppMat.docx