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Biological properties of citrus IntegroPectin: insights on the anti-oxidant and anti-proliferative effects

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Declaration of Authorship

I, Chiara Valenza, declare that this thesis titled, “**Biological properties of citrus IntegroPectin: insights on the anti-oxidant and anti-proliferative effects**” and the work presented in it are original:

- This work was done while in candidature for a research degree at this University.
- Where the thesis is based on work done jointly with others, I have made it clear.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given, with the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
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1.

Introduction

1.1 Citrus fruits

Citrus fruits (*Rutaceae* family) are often associated with health-promoting properties. [1,2]

Citrus includes approximately seventeen species of plants that produce some of the most widely cultivated fruits in the world. To date, the Citrus used for eating or processing mainly includes pomelos (*Citrus maxima*), sweet oranges (*Citrus sinensis*), sour oranges (*Citrus aurantium*), mandarins (*Citrus reticulata*), lemons (*Citrus limon*), limes (*Citrus aurantiifolia*), citrons (*Citrus medica*), grapefruits (*Citrus paradisi*), kumquat (*Citrus japonica*), and hybrids [3]. The phytochemical bioactive compounds contained in citrus fruits have been suggested to have a wide variety of biological functions including antioxidant, antiinflammation, antimutagenicity, anticarcinogenicity and anti-aging effects. [2,4,5] Consequently, Citrus fruits may help reduce the risk of risk of metabolic syndrome, neurodegenerative disorders, cardiovascular diseases, and cancer. [4,5,6]

The composition and concentration of these bioactive compounds can vary significantly among different citrus species, fruit parts, and are influenced by factors such as maturity, environmental conditions, and processing methods. [3,4] Most notably, over 60 different flavonoid types have been identified in citrus, predominantly categorized into flavanones, flavones, and polymethoxylated flavones (PMFs). Flavones and PMFs are unique to citrus fruit and include compounds like hesperidin, naringin, eriocitrin, diosmin, nobiletin, and tangeritin. [3,4]

Carotenoids represent another crucial group of bioactive compounds in citrus and include both carotenes (α -carotene, β -carotene, and lycopene) and xanthophylls (β -cryptoxanthin, lutein, zeaxanthin, and violaxanthin) [3,4]. Phenolic acids, comprising hydroxycinnamic acids (such as p-coumaric acid, caffeic acid, and ferulic acid) and hydroxybenzoic acids, further contribute to the bioactive profile of citrus fruits. [3,4] Moreover, citrus fruits are a natural source of limonoids, a class of tetranortriterpenoids that includes compounds like limonin and nomilin. [4,6] Other significant bioactive components include high concentrations of vitamin C (ascorbic acid), and pectins, which are found abundantly in the cell walls, particularly in the peels. [3,4]

Among all the bioactive compounds mentioned before, pectins have emerged as particularly noteworthy subject in literature. [6]

1.2 Pectins

Pectin is a complex polysaccharide found in the cell walls of plants and is particularly abundant in the peel and pomace of fruits. In the plant cell wall, the concentration of pectin decreases gradually from the primary cell wall towards the plasma membrane, with the highest concentration of pectin located in the middle lamella. [3] Processed primarily from fruit industrial waste, such as apple, orange, lemon, lime, pear, papaya, and dragon fruit, pectin extraction is environmentally beneficial, reducing greenhouse gas emissions and microbial degradation. [3,6,7]

Pectin has been recognized as a food supplement by EFSA in 2010 and it holds significant relevance for its thickening, stabilizing, and gelling properties. [1] The gel-like properties of pectin, for instance, are exploited for the development of edible food coatings in the pharmaceutical field providing functional benefits within the gastrointestinal tract based on its ability to reduce post prandial glycaemic responses, maintain normal blood cholesterol concentration and enhance satiety, ultimately leading to a reduction in food intake. [1,4]

Beyond these established nutritional effects, additional biofunctional properties have emerged, including anti-inflammatory, antioxidant, anticancer, and antidiabetic activities. [1,4,5,6,7,8,9]

1.3 Structural characteristics of pectin and interaction with biological molecules

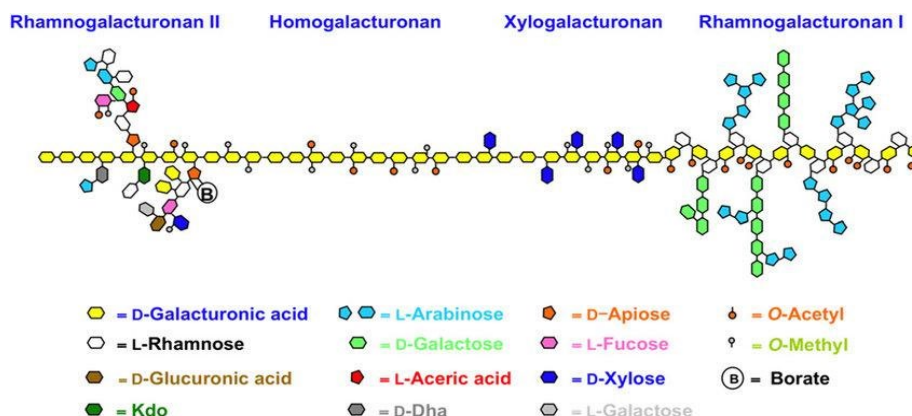


Figure 1 Simplified structure of pectin

As shown in **Figure 1**, pectin is mainly built from D-galacturonic acid units, but its structure can change depending on how it is extracted and the plant source. It is made up of three main types of polysaccharide regions: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) [4,7,8]. The arrangement and modification of these regions—such as methylation, acetylation, and branching—affect pectin properties. The degree of esterification (DE), which measures how many galacturonic acid residues are linked with methoxyl groups, is especially important. A higher DE makes pectin more rigid and better at forming gels [4,7,8,9] while a lower DE makes it more flexible and soluble in water. Enzymes like pectin methylesterase (PME) can change the DE, altering pectin solubility and gelling ability.

The side chains of pectin, made up of sugars like arabinose and galactose, vary in length and composition based on the plant and extraction process. Highly branched pectins are more soluble but gel less easily, which is useful for pharmaceutical and biomedical uses where solubility matters. These branching patterns, especially when rich in galactose as in modified citrus pectin (MCP), influence how pectin interacts with biological molecules. (See **Table 1**)

Structural Backbone Component	Structural Role	Bioactivity	Ref.
Galacturonic acid	Main backbone sugar; forms α -1,4-linked chains (homogalacturonan)	Essential for gelling; binds water; reduces cholesterol; prebiotic effects	[1][3][5]
Rhamnose	Alternates with galacturonic acid in RG-I and RG-II regions	Provides branching points for side chains; structural diversity	[1][4][5]
Arabinose	Neutral sugar in side chains (especially RG-I)	Contributes to solubility and bioactivity; prebiotic potential	[1][4][5]
Galactose	Neutral sugar in side chains (especially RG-I)	Involved in galectin-3 binding; enhances anti-metastatic activity	[1][2][4][5]
Xylose	Present in side chains and xylogalacturonan domains	Adds to structural complexity; may influence gelling and bioactivity	[1][4][5]
Apiose	Found in RG-II side chains	Contributes to RG-II unique structure and cross-linking	[1][4][5]
Fucose	Rare sugar in RG-II side chains	Structural role in RG-II	[4][6]
Homogalacturonan (HG)	Linear region of α -1,4-D-galacturonic acid	Main gelling domain; affects texture and viscosity	[1][4][5]
Rhamnogalacturonan I (RG-I)	Backbone of galacturonic acid and rhamnose, with side chains of arabinose and galactose	Provides branching; increases solubility and bioactivity	[1][4][5]
Rhamnogalacturonan II (RG-II)	Highly branched, complex region with up to 12 different sugars	Cross-linking ability; key for cell wall integrity; minor but highly conserved	[1][4][5]
Xylogalacturonan	Galacturonic acid backbone with xylose side chains	Structural diversity; present in storage tissues	[4][5]
Polyphenols (complexed)	Often associated with pectin, especially in fruit byproducts	Antioxidant, anti-inflammatory, and antiproliferative activities	[4]
Methoxyl groups	Esterifies carboxyl groups of galacturonic acid	Determines degree of esterification (DE); affects gelling and solubility	[1][4][5]
Acetyl groups	May be attached to galacturonic acid residues	Modifies solubility and gelling properties	[1][4][5]

Table 1 – Representative table of the structural backbone components of pectins and their bioactivity

1.4 Major associated bioactive molecules in pectins

The presence of **polyphenols** (including flavonoids) and **terpenes** in pectin highlights its potential not just as a gelling agent but also as a natural polymer capable of delivering bioactive compounds. These phytochemicals contribute to the intrinsic antioxidant capacity of pectin, which is particularly relevant for scavenging free radicals and mitigating oxidative stress, mechanisms closely associated with the prevention of various chronic diseases. [10,11,12,13,14,15]

Polyphenols are a broad category of natural organic compounds characterized by the presence of multiple phenol units. They are one of the most abundant antioxidants in the diet and are found in a wide variety of plant-based foods, such as fruits, vegetables, tea, coffee, wine, and chocolate. Polyphenols can be divided into several classes, including flavonoid, phenolic acids, stilbenes, and lignans, based on their structural characteristics. [10,11] **Flavonoids** are a large group of polyphenolic compounds with a basic structure consisting of two aromatic rings connected by three carbon atoms forming an oxygenated heterocycle. They are categorized into several subclasses, including flavones, flavonols, flavanones, flavan-3-ols (catechins), anthocyanins, and isoflavones, based on the oxidation level of the central 3-carbon chain and the pattern of substitution of the rings. [10,11,12]

Terpenes are a diverse class of organic compounds produced by a variety of plants, especially conifers, and by some insects. They are made up of isoprene (C₅H₈) units and classified based on the number of these units in the molecule: monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), etc. [5,11]

Flavonoids, terpenes, and polyphenols show several neuroprotective effects by targeting oxidative stress, neuroinflammation, and protein aggregation pathways. [16,17] Flavonoids, such as quercetin and luteolin, mitigate endoplasmic reticulum (ER) stress in Alzheimer's disease (AD) models by suppressing markers like CHOP and GRP78, while enhancing synaptic plasticity via Phosphatidylinositol 3-Kinase (PI3K) / protein kinase B (Akt) and mitogen-activated protein kinases (MAPK) pathways [16,17,18]. Resveratrol exhibits anti-inflammatory and antioxidant effects by inhibiting pro-inflammatory cytokines such as TNF- α , IL-1 β , and nitric oxide (NO), thereby reducing neuroinflammation and neuronal damage in microglia. [19] Quercetin also reduces amyloid-beta (A β) aggregation and tau hyperphosphorylation by modulating GSK3 β /CDK5, improving cognitive function in preclinical studies [16,20]. Similarly, baicalein (a flavone from *Scutellaria baicalensis*) inhibits A β fibrillization and neurofibrillary tangle formation, delaying AD progression. [21,22,23] In Parkinson's disease (PD), morin and rutin restore calcium homeostasis and protect dopaminergic neurons by upregulating Binding Immunoglobulin Protein (BiP) and stabilizing mitochondrial function [24,25].

Terpenes like limonene and α -pinene demonstrate neuroprotective activity by scavenging reactive oxygen species (ROS) and suppressing nuclear factor kappa B

(NF- κ B)-mediated neuroinflammation. Limonene reduces ROS production by 30% in neuronal cells exposed to oxidative stress [26], while α -pinene enhances nuclear Nrf2 translocation, boosting antioxidant enzymes, such as catalase and super oxide dismutase (SOD), in PC12 cells [27,28]. β -caryophyllene, a sesquiterpene, binds to CB2 receptors, attenuating microglial activation and neuropathic pain in PD models [29,30,31].

Other polyphenols, such as epigallocatechin gallate (EGCG), enhances mitochondrial biogenesis and scavenges ROS in stroke models [32]. Curcumin, a polyphenolic curcuminoid, reduces fibrillar α -synuclein by 35% in PD models and suppresses amyloid plaque formation in AD via inhibition of β -secretase [33,34]. These compounds also enhance cerebral blood flow and neurogenesis through the vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) upregulation [32,33,34]

These bioactive compounds also exert anticancer effects by inducing apoptosis, inhibiting metastasis, and sensitizing tumors to chemotherapy. Flavonoids like quercetin and apigenin act as pro-oxidants in cancer cells, such as HT-29 colon cancer cells triggering mitochondrial apoptosis via caspase-3 activation and Bcl-2 suppression, with the inhibition of Akt-CSN6-Myc signaling [35,36]. Quercetin induces G2/M cell cycle arrest in colorectal cancer (Caco-2) [37] and synergizes with TRAIL to enhance apoptosis in non-small-cell lung cancer (NSCLC) via p53-dependent pathways [38,39]. EGCG inhibits NF- κ B and VEGF, reducing glioblastoma angiogenesis by 40% in preclinical models [40,41].

Terpenes can target oncogenic signaling pathways: auranthene (a monoterpene) suppresses metastasis in cervical and ovarian cancers by downregulating MMP-2/9 and β -catenin/T-cell factor (TCF) signaling [42,43] while taxol (a diterpene) induces apoptosis in glioblastoma via caspase-3 activation [44]. Sesquiterpene lactones (e.g., ambrosin) arrest the cell cycle in MCF-7 breast cancer cell lines by enhancing p53 expression and ROS generation [45,46].

Resveratrol and curcumin inhibit tumor proliferation and metastasis through multi-target mechanisms. Resveratrol suppresses PI3K/Akt/mTOR, reducing invasiveness in glioblastoma and acute myeloid leukemia [47,48,49], while curcumin inhibits MMP-2/9 secretion and angiogenesis in ovarian cancer xenografts [50,51].

Synergistic combinations (e.g., quercetin with green tea extract) enhance efficacy, reducing tumor growth and metastasis by 50% in preclinical models; while preclinical data are robust, large-scale trials are needed to confirm efficacy. In this context, phase I trials of resveratrol in glioblastoma are promising, extending median survival from 5 to 9 months. [52]

Pectin-bound polyphenols show prebiotic and metabolic modulation by promoting gut microbiota health, enhancing short-chain fatty acid production and systemic antioxidant defenses [53,54]. These biomolecules also showed a promising role in drug delivery and bioavailability: nano formulations (quercetin-loaded nanoparticles) improved blood-brain barrier penetration [55], while pectin matrices protect polyphenols from gastrointestinal degradation and may enhance their bioavailability by modulating gut flora and delaying absorption [56,57]. However poor solubility and rapid metabolism may limit clinical translation, and nano capsulation and structural modifications, like acetylated flavonoids, need more investigation, although supported by promising data. [58]

The activity of polyphenols complexed with pectin is influenced by multiple interrelated factors. The source of pectin must be considered, since citrus and olive-derived pectins retain higher polyphenol content compared to apple pectin, possibly due to structural variations in their polysaccharide matrices [59]. Molecular weight and domain composition (ratio of homogalacturonan to rhamnogalacturonan) further affect antioxidant activity, with lower-weight, HG-rich pectins exhibiting stronger radical-scavenging effects [60].

Subtle changes in pectin composition or processing can amplify or diminish the therapeutic potential of polyphenol complexes.

1.5 Citrus pectin as a bioactive compound with therapeutic potential

Citrus pectin exhibits several therapeutic properties due to its unique structural synergistic interactions with associated flavonoids, terpenes, and polyphenols [61]. Its molecular weight and methoxylation degree contribute to its functional versatility and its applications includes neuroprotection and anticancer properties. [62,63,64,65]

Citrus pectin exhibits indirect antimicrobial activity by modulating gut microbiota and disrupting biofilms. Fermentable pectin fibers promote beneficial bacteria (e.g.,

Bifidobacterium), suppressing pathogenic species like *Clostridium difficile* and inhibiting *Helicobacter pylori* biofilm formation by 50% in vitro, likely through electrostatic interactions with bacterial surface proteins [65].

Citrus pectin reduces serum cholesterol by 15–20% through bile acid sequestration and hepatic low-density lipoprotein (LDL) receptor upregulation. [66] In addition, RG-I-enriched pectin delays postprandial glucose absorption by 25% via carbohydrate digestion slowdown, offering glycemic control in metabolic disorders [82,83].

Modified citrus pectin (MCP), a low-methoxyl derivative, reduces neuroinflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) by suppressing Toll-like receptor 4 (TLR4) /NF- κ B signaling, as demonstrated in rodent models of cerebral ischemia [67].

MCP also enhances immune function through dual mechanisms: natural killer (NK) cell activation and macrophage polarization. In vitro studies demonstrate that MCP increases NK cell cytotoxicity against K562 leukemic cells by 35%, attributed to its low degree of methoxylation and unsaturated oligogalacturonic acids. Concurrently, MCP elevates anti-inflammatory interleukin-4 (IL-4) levels in splenic tissues of Balb/c mice, shifting the immune response toward a T-helper 2 (Th2) profile [68]. In colorectal cancer models, MCP reduces pro-inflammatory M1 macrophages in the tumor microenvironment, attenuating interleukin-1 beta (IL-1 β) and TNF- α secretion [69]. Tumor-associated macrophages (TAMs) tend to accumulate in these hypoxic tumor regions, where they contribute to tumor progression by releasing cytokines that support tumor cell growth, invasion, metastasis, and the formation of new blood vessels. In the same study, MCP curtails the heightened glucose consumption by TAMs under hypoxic conditions by suppressing GLUT-1 expression, thereby diminishing TAMs viability in such environments. [70] Moreover low-methoxyl pectin exert anti-inflammatory effects and reduces colitis severity by 40% in murine models by suppressing cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression [71,72,73].

MCP exerts multitarget anticancer effects through structural and molecular interactions, partly due to its binding to Galectin-3 carbohydrate recognition domain, which inhibits its function. [74]

Galectins are part of a β -galactoside-binding proteins family (**Figure 2**), which is implicated in various physiological and pathological processes, including cell-cell adhesion, cell-matrix interactions, immune and inflammatory responses, apoptosis, and cancer progression. [75,76] Galectins are found in the cytoplasm and can be secreted outside the cell, where they interact with cell surface and extracellular matrix glycoproteins, influencing cell signaling, proliferation, and survival. [77]

Pectins shows anti-cancer properties from the ability of its multiple galactan, but also the α -1,5 arabinan, side chains to bind and inhibit Galectin-3. This interaction enables certain pectin fragments, especially those with abundant and accessible galactose residues, such as RG-I regions, to bind and inhibit galectin-3 activity. MCP, with its reduced molecular weight and low esterification, demonstrates enhanced bioavailability and increased affinity for Galectin-3, allowing it to effectively antagonize galectin-3-mediated cellular functions [74].

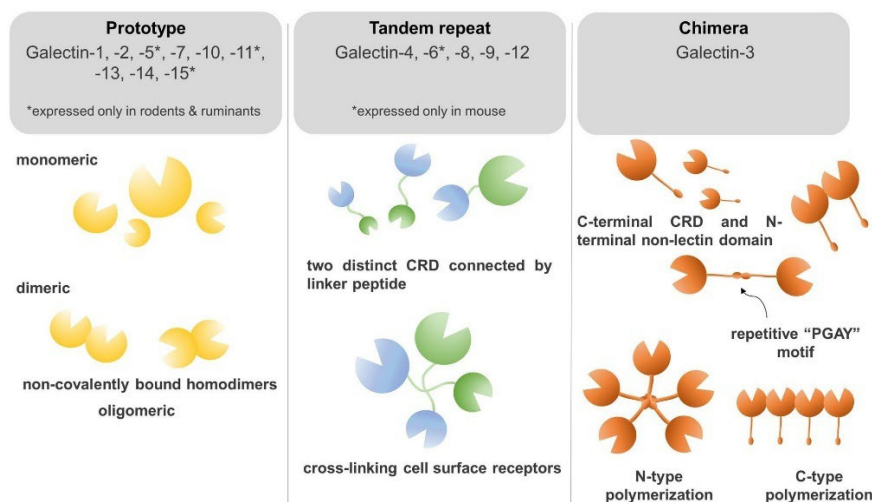


Figure 2 Classification and structural overview of galectins. They are categorized into three structural types based on their domain organization. **Prototype** contains a single carbohydrate recognition domain (CRD), which binds to specific sugar molecules on glycoconjugates, often involved in basic cellular processes like cell-cell adhesion and immune modulation. **Tandem repeat Galectins** are composed of two distinct CRDs connected by a flexible linker peptide. This structure allows them to cross-link cell surface receptors and facilitates the clustering of glycoproteins on cell membranes and enhances receptor signalling or adhesion. **Galectin 3** is the only chimera galectin, which contains a C-terminal carbohydrate recognition domain (CRD) and an N-terminal non-lectin domain. It features repetitive "PGAY" motifs in the N-terminal region that enable polymerization and can form structures through N-type polymerization or C-type polymerization. This galectin has several roles in diverse biological processes such as cell migration, inflammation, and cancer progression.

Galectin-3 is involved in various biological processes, including cell growth, cell adhesion, inflammation, and immune responses, where it contributes to immune cell activation and regulation. This protein has also been implicated in regulation of

normal cellular proliferation and apoptosis, as well as malignant transformation and the metastasis of cancer cells [75,76] and it has received also much attention also for its utility as a diagnostic marker for **thyroid cancer** for its relatively higher sensitivity and specificity in thyroid malignancies, especially in papillary carcinomas. In a large multicenter study, the sensitivity and specificity of Galectin-3 in thyroid carcinomas were 99% and 98%, respectively. Galectin-3 is known to be overexpressed in well-differentiated thyroid carcinomas but not in normal thyrocytes or benign thyroid nodules. This makes it a candidate biomarker for improving thyroid cancer diagnosis. [77,78] Besides Galectin-3, the expression of **Galectin-1** has also been shown to be upregulated in thyroid carcinoma-derived cell lines, Galectin-1 has been identified as a potential biomarker for papillary thyroid cancer (PTC), the most common type of thyroid cancer. [79] Research has shown that Galectin-1 can be targeted by specific peptides, which can then be used to develop imaging probes for non-invasive diagnosis of thyroid cancer. A study demonstrated that peptide-functionalized imaging probes could detect Galectin-1 expression in a mouse model of PTC with high sensitivity (75%) and specificity (100%), suggesting that Galectin-1 could be a reliable biomarker for distinguishing malignant from benign thyroid nodules.[79]

By binding galectin-3 carbohydrate recognition domain (CRD) via RG-I domains, MCP blocks breast cancer cells adhesion, migration, and angiogenesis [75]. Additionally, MCP induces apoptosis in androgen-independent prostate cancer cells via caspase-3 activation and Bim upregulation, inhibiting proliferation through MAPK pathway suppression [76].

MCP cardioprotective effects are also linked to Galectin-3 inhibition, which attenuates myocardial fibrosis and cardiac remodeling in hypertensive rats by blocking TLR4/MyD88/NF- κ B signaling [80,81].

Pectin mucoadhesive properties and biocompatibility enable targeted drug delivery. For example, pectin-doxorubicin nanocomposites enhance chemotherapeutic efficacy by 30% in colorectal tumors due to prolonged intestinal retention [84]. In regenerative medicine, low-methoxyl pectin promotes diabetic ulcer healing by stimulating fibroblast migration and collagen synthesis via transforming growth factor-beta (TGF- β) pathway activation [85].

1.6 Extraction processes of pectin

Extraction methods significantly impact pectin bioactivity: hydrodynamic cavitation preserves polyphenol-pectin bonds more effectively than traditional acidic or thermal methods, enhancing stability. The degree of esterification (DE) modulates interactions, as low-ester pectin ($DE < 50\%$) binds polyphenols more efficiently via hydrogen bonding and hydrophobic interactions, thereby improving radical-scavenging capacity.[85]

The extraction process is influenced by factors like temperature, pH and solvent used, affecting the yield and properties of the extracted pectin. Extracting pectin from apple pomace is more challenging compared to citrus peels due to susceptibility to spoilage by pectolytic enzymes unless promptly dried to reduce moisture content before further processing. These chemical methods are commonly used in industrial processes to extract pectin from citrus fruits and apples. Each method offers unique advantages in terms of efficiency, yield, and product quality.[86]

Acid extraction Some common chemical methods used for pectin extraction include acid extraction, which involves the use of hydrochloric acid or citric acid, to break down the cell walls and release pectin from plant materials. Pectin is extracted in water acidified with 0.2M sulfuric, nitric, phosphoric, acetic, or hydrochloric acid between 89-100°C for with continuous stirring. This method is widely employed in pectin extraction processes due to its effectiveness in isolating pectin.

Enzymatic extraction utilizes enzymes, such as pectinase, to break down the complex structure of pectin molecules and facilitate their release from plant tissues. This method holds high specificity and has a gentle action on the raw materials.

Microwave techniques provide rapid and uniform heating, which can enhance the efficiency of pectin extraction. Microwave assisted extraction involves dielectric heating of plant molecules due to the exposure to microwaves. The irradiation accelerates cell rupture by a sudden temperature rise and internal pressure increase inside the sample. This promotes the destruction of the plant sample surface and promotes the exudation of pectin within the plant cells into the surrounding solvents. [87]

1.7 Alternative processes for extracting pectin

Alternative processes have been developed to extract less degraded pectin using acid-free technologies, aiming to reduce environmental impact and improve product quality. These technologies offer higher purity and lower degradation levels, leading to increased profitability through reduced capital and production costs. [86]

Innovative methods such as **microwave-assisted hydrodistillation**, **enzymatic degradation using recyclable biocatalysts from citrus waste**, and **hydrodynamic cavitation** have been developed for green extraction of pectin with commercial potential. These processes are reproducible, scalable, environmentally friendly, and offer practical applications in the industry. Being reproducible and scalable, these acid free and green extraction processes hold practical and commercial applicative potential. [87,88,89]

Microwave-assisted hydrodistillation: This method utilizes microwave technology to assist in the extraction process, offering a more efficient and controlled extraction of pectin. [88]

Enzymatic degradation with pectin methylesterase: Enzymatic treatment using pectin methylesterase, directly captured as a recyclable biocatalyst from citrus processing waste, enables a more sustainable and environmentally friendly extraction process. [88,90]

Hydrodynamic cavitation: This process involves the use of cavitation in water only, without the need for chemical reactants, to extract pectin efficiently. It can yield pectin alongside valuable compounds like citrus flavonoids and emulsified terpenes. Water is the only dispersion medium. Hydrodynamic cavitation occurs when a liquid experiences rapid pressure changes as it flows through constricted areas, leading to the formation, growth, and collapse of vapor cavities. This green technology transforms mechanical energy into high kinetic energy, which is released in very short bursts that are exploited for green extraction processes. [91,92]

This method not only promotes the sustainable use of industrial waste but also enhances the bioavailability of key bioactive compounds like flavonoids and terpenes. In citrus processing waste, hydrodynamic cavitation facilitates the extraction of pectin and flavonoids, forming novel conjugates like **a new pectin** extracted in 2019 from citrus fruits, and called **IntegroPectin**. [93]

1.8 Citrus IntegroPectin

The focus of this project relies on this new Citrus **IntegroPectin**, initially isolated in 2019 through the application of **hydrodynamic cavitation** to waste orange peel on a semi-industrial scale—processing 30 kg of orange biowaste in 120 liters of water—and subsequently from waste lemon and grapefruit biowaste from the citrus juice industry. This extraction method is conducted solely in water, rendering it a good example of green extraction technology. The innovative extraction method not only enhances the bioavailability of citrus flavonoids but also contributes to the sustainable use of industrial waste. [93]

The starting material consists of citrus peels, which are by-products of the citrus processing industry. This extraction method uses hydrodynamic cavitation, where rapid formation and collapse of vapor bubbles in the liquid create intense shear forces, and water is used as the only extraction medium. The process disrupts the plant cell walls, enhancing the release of pectin from the citrus material. The extracted liquid is filtered to separate solid residues, such as cellulose (CytoCell) from the pectin-rich solution. A filtration system then ensures efficient separation and purification; the filtered solution undergoes lyophilization, a process where water is removed by sublimation under low temperature and pressure. This step preserves the structural integrity and bioactivity of the pectin while converting it into a dry powder form. The resulting product is IntegroPectin. [93] (**Figure 3**)

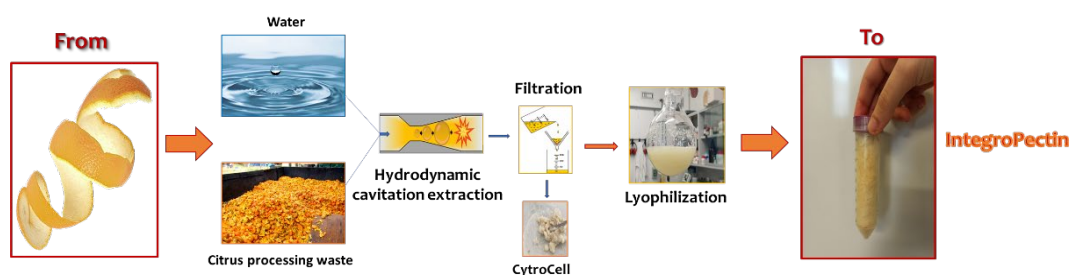


Figure 3 – Extraction process of Citrus IntegroPectin via hydrodynamic cavitation

This new pectic molecule exhibits unique structural properties that contribute to its enhanced bioactivity. One of the key features is the presence of hairy RG-I regions. These regions are particularly bioactive compared to the homogalacturonan portion of pectin, which explains some of the numerous health benefits associated with IntegroPectin [93]. The extraction process preserves the integrity of these RG-I regions and ensures that the resulting IntegroPectin is highly soluble in water.[94]

IntegroPectin is characterized by a low degree of esterification, which is important for its bioactivity. This low esterification allows for more free carboxylic groups, enabling interactions with proteins like galectin-3 and contributing to its antiproliferative and anti-inflammatory effect. The structure also includes citrus flavonoids and terpenes that are either physically adsorbed or chemically bound to the pectin polymer surface. These flavonoids, such as hesperidin, naringin, and eriocitrin, have been previously mentioned for their antioxidant and anticancer properties, and their solubility is enhanced when conjugated with pectin. [95]

So far, several types of IntegroPectin have been extracted from citrus fruits using advanced methods like hydrodynamic cavitation and acoustic cavitation. Bitter Orange IntegroPectin was the first high methoxyl citrus pectin extracted via hydrodynamic cavitation, with unique flavonoid concentrations such as hesperidin and eriocitrin, followed by Red Orange IntegroPectin, which is instead a low methoxyl pectin with a high concentration of adsorbed flavonoids like hesperidin and naringin; it has demonstrated anticancer activity against lung cancer cell. Lemon IntegroPectin, extracted via hydrodynamic cavitation, is rich in RG-I regions, flavonoids, terpenes and exhibits antioxidant, antimicrobial, neuroprotective, and cytoprotective properties. Grapefruit IntegroPectin contains flavonoids like naringin and terpenes concentrated at the polymer surface and showed enhanced antimicrobial and radical scavenging activity. Sweet Orange IntegroPectin was instead extracted via acoustic cavitation; this variant has lower bioactivity compared to lemon and red orange but still exhibits anticancer properties. [93,94,95]

This new family of pectin exhibits significantly enhanced and more diverse biological activity compared to commercially available citrus pectin. The HPLC-MS analysis of terpenes in new lemon and grapefruit IntegroPectin obtained via the hydrodynamic cavitation of industrial lemon and grapefruit processing waste, as seen in **Table 2** reveals the presence of eriocitrin, naringin, hesperidin and kaempferol. The pectic fibers rich in RG-I regions act as chemical sponges adsorbing and concentrating at their outer surface highly bioactive citrus flavonoids and terpenes. [94]

Biophenol	Grapefruit IntegroPectin [mg/g]	Lemon IntegroPectin [mg/g]
Naringenin	0.34	-
Naringin	73.66	0.11
Hesperidin	0.60	0.60
Eriocitrin	-	3.35
Kaempferol	-	0.26
Kaempferol-7-O-glucuronide	0.26	0.26
p-Coumaric acid	-	0.28
Gallic acid	0.31	0.56

Table 2. Flavonoids in lemon and grapefruit IntegroPectin identified by HPLC-MS. [94]

In lemon IntegroPectin, eriocitrin is the predominant flavonoid, together with high amounts of hesperidin, kaempferol and kaempferol 7-O-glucuronide. In Grapefruit IntegroPectin the predominant component is naringin with minor amounts of hesperidin and naringenin concentrations.

The combination of these structural features—low methoxyl content, high RG-I regions, and bound flavonoids—makes IntegroPectin a promising compound with enhanced biological activity compared to commercial citrus pectins.

In a recent study Lemon IntegroPectin showed neuro-and mitoprotective effects resulting from the synergistic action of eriocitrin, hesperidin, α -terpineol and terpinen-4-ol against H₂O₂ induced damage of neuronal cells in an oxidative stress model in SH-SY5Y neuroblastoma cell line. Collectively they exert neuroprotective, antioxidant and anti-inflammatory activities. [96] The antioxidant capacity and the role of Citrus IntegroPectin as a powerful radical scavenger was shown again in another study where Grapefruit IntegroPectin had a significant neuroprotective activity in vitro on SH-SY5Y. [97] In both studies the treatments with

Lemon IntegroPectin and then Grapefruit IntegroPectin indeed hindered the ROS increase induced by the addition of H₂O₂ and counteracted the induced mitochondrial remodelling, by also preserving the mitochondrial membrane potential and morphology. Since mitochondria are severely impaired in neurodegenerative disorders IntegroPectin showed to be a potential therapeutic and preventive agent for the treatment of oxidative stress associated brain disorders. The protective action is synergetic with an anti-proliferative effect. Grapefruit IntegroPectin produced a cell cycle arrest at the G2/M phase. This cell cycle checkpoint prevents cells with damaged DNA from undergoing mitosis by inhibiting the mitotic complex CDK1-cyclin B and activating apoptosis cascade. [96,97] The same treatment with commercial citrus pectin did not produce the same effect

Lemon IntegroPectin also demonstrated markedly superior antimicrobial activity relative to commercially extracted citrus pectin using hot mineral acid. While commercial pectin exhibits antibacterial activity predominantly against Gram-negative bacteria, lemon and grapefruit IntegroPectin exhibited potent antimicrobial effects against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with Grapefruit IntegroPectin displaying bactericidal properties against both strains at low concentrations. [98]

Grapefruit IntegroPectin has also demonstrated cardioprotective effects, potentially through the activation of mitochondrial potassium channels. The primary bioactive compound in Grapefruit IntegroPectin found is naringin, which constitutes 98% of its flavonoid content. Naringin, through its bioactive aglycone naringenin, has demonstrated significant anti-ischemic cardioprotective activity. This effect is attributed to the activation of mitochondrial potassium channels, which play an important role in protecting the heart from ischemic damage. [99]

A recent study compared grapefruit IntegroPectin powders isolated via spray drying and via freeze drying in terms of phenolic content, quantification of the most representative flavonoids and radical scavenging activity. Except for the protein content and the antioxidant power, the two drying methods afford similar pectins. [100]

AIM OF THE STUDY

This thesis aligns with sustainable development goals by exploring the valorization of agricultural byproducts for biomedical applications. Developing sustainable bioactive compounds from agricultural waste streams may offer novel therapeutic applications and IntegroPectin has emerged as a promising candidate due to its reported antioxidant, anti-inflammatory, and anticancer properties. Grapefruit IntegroPectin is an innovative type of Citrus pectin that is very soluble in water. It contains a high number of bioactive compounds such as terpenes and flavonoids, which are recognized for their strong antioxidant as well as anti-inflammatory effects, as this pectin also preserves the **RG-I** backbone. This suggests that this biopolymer has a range of positive effects on **multiple targets**.

The aim of this study is to comprehensively evaluate the bioactive properties and structural characteristics of grapefruit IntegroPectin through complementary experimental and computational approaches, thereby explaining its potential as a therapeutic anti-inflammatory, anti-oxidant, and anti-proliferative agent.

Previous works in literature have demonstrated that grapefruit IntegroPectin exerts protective effects on neuronal cells in an oxidative stress model. Neurodegenerative disorders and cancer represent major global health challenges characterized by complex pathophysiological mechanisms including oxidative stress, inflammation, and dysregulated cellular proliferation.

Therefore, the **first** part of this study focused on testing Grapefruit IntegroPectin properties in a model of oxidative stress in microglial cells.

The **second** part of this study focused on testing anti proliferative properties of Grapefruit IntegroPectin in thyroid cancer cells, which represent an increasing health concern with limited therapeutic options for aggressive variants, necessitating the exploration of novel anti-proliferative compounds.

In order to understand more the structural uniqueness of this IntegroPectin, and how flavonoids are linked to it, the **third** part of the study comprehends a computational approach to understand the structural characteristics of IntegroPectin provided an opportunity to correlate observed bioactivities with molecular features.

To achieve the overarching aim of this study, we followed specific objectives:

1. **Investigate** the antioxidant and anti-apoptotic properties of grapefruit IntegroPectin in human microglial cell line under oxidative stress conditions, thereby evaluating its potential neuroprotective applications.
2. **Assess** the anti-proliferative effects of grapefruit IntegroPectin on thyroid cancer cell lines, examining its cytotoxicity profile and potential mechanisms of action.
3. **Conduct** a computational analysis of the molecular structure and properties of IntegroPectin using Density Functional Theory (DFT) calculations via Gaussian 16, providing insights into the structure-activity relationships underpinning its biological effects.
4. **Integrate** the experimental and computational findings to propose a comprehensive model of IntegroPectin mechanism of action and to identify structure-function relationships guiding its bioactivities.

By combining cellular models of neuroinflammation and cancer with advanced computational chemistry techniques, this study wants to provide an understanding of IntegroPectin bioactivities at both cellular and molecular levels.

2.

MATERIALS AND METHODS

2.1 PART I: Methodology and experimental design on human microglial HMC3 cell line

2.1.1 Production and solubilization of grapefruit IntegroPectin

Grapefruit IntegroPectin was extracted from grapefruit (*Citrus paradisi*) processing waste kindly donated by Campisi Citrus (Siracusa, Italy). IntegroPectin samples were obtained by freeze-drying the aqueous solution collected after the hydrodynamic cavitation-based extraction of grapefruit citrus processing waste using water as the only solvent. The aqueous extract is filtered and the IntegroPectin isolated via lyophilization (freeze-drying). A powder sample of the IntegroPectin was solubilized in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich) at the concentration of 10 mg/mL. The solution was filtered using a 0.45 µm Sartorius filter and stored at 4 °C.

2.1.2 HMC3 Cell culture

We utilized HMC3 cells, a human microglial cell line, generously provided by Professor M. Sortino from the University of Catania. Cells were maintained in T25 tissue culture flasks under controlled environment, set to maintain a humidified atmosphere composed of 95% air and 5% carbon dioxide, with a constant temperature of 37°C. The growth medium consisted of DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, non-essential amino acids and 100 U/mL each of penicillin and streptomycin. The cell culture medium was refreshed on a weekly basis to replenish nutrients and remove metabolic waste products. Subculturing was performed when the cells reached approximately 90% confluence, every 7-10 days. Cells were detached using trypsin-EDTA, followed by reseeding at an appropriate density in fresh culture flasks.

Prior to initiating any experimental treatments, cells were treated after minimum 48 hours post-plating to ensure that the cells were in a stable state before exposure to various experimental conditions.

2.1.3 Cell treatments

For the IntegroPectin concentration-effect studies, cells were exposed to IntegroPectin at concentrations of 1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL for a

duration of 24 hours. This concentration gradient allowed for the assessment of dose-dependent effects of IntegroPectin on HMC3 cells. To first evaluate IntegroPectin's effects, time-course experiments were conducted, with cells being treated with 1 mg/mL IntegroPectin for varying durations: 24 hours, 48 hours, and 72 hours. Tert-Butyl Hydroperoxide (TBH) concentration-response studies were also performed. Cells were subjected to a range of TBH concentrations (500 μ M, 300 μ M, 200 μ M, 100 μ M, and 50 μ M) for 24 hours to establish a dose-response relationship for this oxidative stress inducer. To investigate potential protective effects of IntegroPectin against oxidative stress, cells were co-treated with IntegroPectin (1 mg/mL) and TBH (200 μ M), with IntegroPectin administered immediately prior to the 24-hour TBH treatment.

Signaling pathway inhibition studies were carried out to elucidate the involvement of specific cellular mechanisms. Cells were pre-treated with pathway inhibitors before exposure to IntegroPectin and TBH. PD98059 (1213 Tocris Biotechnique, Milano, Italy), a MAPK inhibitor, was used at a concentration of 30 μ M, while LY294002 (440202 Sigma-Aldrich Merck KGaA, Darmstadt, Germany), a PI3K/Akt inhibitor, was applied at 10 μ M. These inhibitors were administered 1 hour before the introduction of IntegroPectin and TBH.

For experiments focused on assessing changes in mRNA expression and acute modulation of intracellular pathways, cells were treated with 1 mg/mL IntegroPectin for a shorter duration of 4 hours. This approach allowed for the capture of early cellular responses at the molecular level.

In all experimental setups, a control group was included to serve as a baseline for comparison. The control cells received an equivalent volume of cell culture medium, ensuring that any observed effects could be attributed to the specific treatments rather than volume-related changes or handling procedures.

2.1.4 MTT assay

Cells were seeded into 96-well plates at a density of 2.5×10^4 cells per well in a final volume of 100 μ L. MTT assay, a colorimetric method, was employed to evaluate cellular metabolic activity as a proxy for cell viability. This technique relies on the ability of metabolically active cells to reduce the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to purple

formazan crystals. The reduction is primarily carried out by mitochondrial succinate dehydrogenase, an enzyme active only in living, metabolically functional cells.

Following the experimental treatments, the cells were incubated with MTT solution at a concentration of 0.5 mg/mL. The incubation period was set at 3 hours, conducted under standard cell culture conditions of 37°C. This duration allowed sufficient time for the enzymatic reduction of MTT to occur in viable cells. After the incubation period, the water-insoluble formazan crystals were solubilized by adding 100 µL of dimethyl sulfoxide (DMSO) to each well. DMSO effectively dissolves the formazan, creating a homogeneous purple solution, the intensity of which correlates with the number of viable cells. The absorbance of the solubilized formazan was quantified using a spectrophotometer set at a wavelength of 570 nm. To account for any background interference, a background subtraction was performed. This step ensures that the measured absorbance accurately reflects the amount of formazan produced by the cells.

The cell viability was expressed as a percentage, with the control group serving as the baseline and set to 100%. This normalization allows for easy comparison between different experimental conditions and replicates. The percentage of cell viability in treated groups was calculated relative to this control, providing a clear indication of the impact of various treatments on cell metabolic activity and, by extension, cell viability.

2.1.5 Nuclear morphology by 40,6-diamidino-2-phenylindole (DAPI) staining

To investigate nuclear morphology, cells were cultured at a density of 2×10^5 cells per well on coverslips positioned within a 24-well plate. Each well contained a final volume of 400 µL. Following the incubation period, the cells required fixation to preserve their structural integrity for subsequent analysis. A 4% formaldehyde solution was utilized for this purpose, with the cells being treated for 15 minutes at room temperature. This fixation step is crucial as it cross-links proteins and stabilizes the cellular architecture, so that nuclear morphology can be accurately assessed.

After fixation, the cells underwent two washes with phosphate-buffered saline (PBS) to remove excess formaldehyde and prepare the cells for staining. Subsequently, the nuclei were counterstained using 4',6-diamidino-2-phenylindole

(DAPI), a fluorescent dye that specifically binds to DNA. This binding results in a strong fluorescence signal when excited by ultraviolet light, allowing for clear visualization of the nuclei. Following the DAPI staining, an additional wash with PBS was performed to eliminate any unbound dye, thereby enhancing the specificity of the staining and minimizing background fluorescence. Once washed, the coverslips containing the stained cells were carefully mounted onto glass slides.

Cellular images were captured using a fluorescence microscope (DMRBE, Leica Microsystems GmbH, Wetzlar, Germany), which is equipped with a digital video camera (Spot-RT Slider, Diagnostic Instruments, Sterling Heights, MI, USA). The fluorescence microscope allows for high-resolution imaging of DAPI-stained nuclei, enabling detailed analysis of nuclear morphology. The images obtained through fluorescence microscopy provided insights into various aspects of nuclear morphology, including size, shape, and any potential morphological alterations induced by experimental treatments.

2.1.6 Quantification of ROS via Dichlorofluorescein Diacetate (DCFH-DA) assay

HMC3 cells were seeded into 96-well plates at a cell density of 2.5×10^4 cells per well in a final volume of 100 μ L.

Following the experimental treatments, cells were incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) at a concentration of 10 μ M. This incubation was performed in the dark for 10 minutes at room temperature. DCFH-DA is a cell-permeable, non-fluorescent probe that is de-esterified intracellularly and becomes highly fluorescent upon oxidation by ROS.

After the incubation period, cells were washed with phosphate-buffered saline (PBS) to remove any excess, unincorporated DCFH-DA. This washing step is important for reducing background fluorescence and ensuring that the measured signal accurately reflects intracellular ROS levels.

The fluorescence intensity was measured using a GloMax fluorimeter (Promega Corporation, Madison, WI, USA). The excitation wavelength was set at 475 nm, and the emission was detected at 555 nm. These specific wavelengths are optimal for detecting the fluorescence of oxidized DCFH-DA, providing a quantitative measure of intracellular ROS levels. The results of the ROS assay were expressed

as percentages, with the control group set as the baseline at 100%. This normalization allows for easy comparison between different experimental conditions and replicates. The percentage of ROS production in treated groups was calculated relative to this control.

2.1.7 Western Blot

The Western blot analysis was performed to assess the expression levels of several key proteins involved in cellular signaling pathways and apoptosis. We focused on Caspase-3, an important marker of apoptosis, as well as phosphorylated forms of extracellular signal-regulated kinases 1/2 (ERK1/2), Akt, and NF- κ B. These phosphorylated proteins are crucial components of signaling pathways that regulate cell proliferation, survival, and inflammatory responses.

HMC3 cells were seeded at a density of 2.5×10^4 cells per well in 96-well plates, with each well containing a final volume of 100 μ L. Following the experimental treatments, cells from four wells were pooled into a single sample to ensure sufficient protein yield. The cell pellets were homogenized in cold radioimmunoprecipitation assay (RIPA) buffer, supplemented with protease and phosphatase inhibitor cocktails to prevent protein degradation and maintain phosphorylation states. Samples were then sonicated to ensure complete cell lysis and protein extraction. The protein concentration in each sample was determined using the Lowry method, ensuring equal protein loading across all samples for subsequent analysis.

Protein samples (30 μ g per lane) were separated on 8% polyacrylamide gels alongside a molecular weight marker. The separated proteins were transferred onto nitrocellulose membranes for immunodetection.

Membranes were blocked for 1 hour at 4°C in a blocking buffer to prevent non-specific antibody binding. They were then incubated overnight with primary antibodies specific to the proteins of interest: Caspase-3 (1:400, sc-56053 Santa Cruz Biotechnology, Dallas, TX, USA), phosphorylated (p)-ERK1/2 (1:1000, 9101 Cell Signaling Technology, Beverly, MA, USA), p-Akt (1:1000, 4060 Cell Signaling Technology, Beverly, MA, USA), and p-NF- κ B (1:500, sc-136548 Santa Cruz Biotechnology, Dallas, TX, USA). Each antibody was diluted in blocking buffer.

After primary antibody incubation, membranes were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies: goat anti-mouse IgG-HRP (sc-2005 Santa Cruz Biotechnology, Dallas, TX, USA) or mouse anti-rabbit IgG-HRP (sc-2357 Santa Cruz Biotechnology, Dallas, TX, USA) horseradish peroxidase conjugated diluted at a ratio of 1:10,000. The resulting immunocomplexes were visualized using a chemiluminescence reagent. Signal detection was performed using either autoradiography film or the iBright FL1000 Imaging System.

For normalization, membranes were re-probed with a β -Actin antibody as a loading control. Band intensities were quantified using ImageJ software, with results expressed as arbitrary units relative to β -Actin expression.

2.1.8 Annexin V/Propidium Iodide (PI) apoptosis assay

HMC3 cells were seeded at a density of 2.5×10^4 cells per well in 96-well plates, with each well containing a final volume of 100 μ L. Following the experimental treatments, cells were mechanically detached and collected by centrifugation at 1200 rpm for 5 minutes at room temperature.

The cell pellets were washed with cold PBS (without calcium and magnesium) to remove any residual media. After centrifugation and supernatant removal, the cells were resuspended in Annexin-binding buffer at a concentration of 1×10^5 cells per 100 μ L.

To each 100 μ L of cell suspension, 5 μ L of Alexa Fluor 647-conjugated Annexin V (diluted 1:10 in Annexin V binding buffer) was added. An unstained control was also prepared for comparison. The samples were then incubated for 15 minutes at room temperature in the dark, allowing Annexin V to bind to phosphatidylserine exposed on the surface of apoptotic cells. Following incubation, the cells were washed with cold PBS to remove unbound Annexin V, centrifuged, and resuspended in 200 μ L of Annexin-binding buffer. The samples were kept on ice to maintain cell integrity.

Just prior to analysis, 1 μ L of Propidium Iodide (PI) was added per 100 μ L of cell suspension. PI stains the DNA of cells with compromised membrane integrity, typically indicating late apoptotic or necrotic cells. The samples were then analyzed using a CytoFlex S flow cytometer.

The results were visualized and analyzed using CytExpert flow cytometry analysis software. The gating strategy employed first excluded cell doublets based on forward scatter area (FSC-A) and height (FSC-H) parameters. The resulting single-cell population was then analyzed for Annexin V and PI staining, allowing for the differentiation between viable, early apoptotic, late apoptotic, and necrotic cells.

2.1.9 RNA isolation and RT PCR

HMC3 cells were cultured in 96-well plates at a density of 2.5×10^4 cells per well, with each well containing a final volume of 100 μ L. At the end of the experimental treatments, cells from seven wells were pooled for each condition to ensure sufficient RNA yield. The RNA was extracted from the collected cell pellets using the Qiagen RNeasy mini kit, following the manufacturer's instructions. The concentration of the isolated RNA was determined using a MultiskanGO Microplate Spectrophotometer.

For each experimental condition, 1 μ g of total RNA was retro-transcribed into complementary DNA (cDNA). The mastermix contained 5X first strand buffer, random hexamers at a concentration of 2.5 μ M, dithiothreitol (DTT) at 2.5 mM, a dNTP mix at 0.5 mM, an RNase inhibitor (40 U), and Superscript III Reverse Transcriptase (200 U). The reverse transcription reaction was carried out in a total volume of 20 μ L per sample and incubated for two hours at 50°C to allow cDNA synthesis, followed by an additional incubation step at 70°C for 15 minutes to inactivate the reverse transcriptase enzyme.

At the end of the protocol, a complete conversion of RNA into cDNA was obtained, which could then be used for subsequent gene expression analysis.

2.1.10 Real-Time PCR

To provide a quantitative measure of how the experimental treatments affected the expression of pro-inflammatory factors in HMC3 cells, a quantitative gene expression analysis was performed for interleukin-6 (IL-6), interleukin-1 β (IL-1 β), and iNOS using SYBR Green Real-time PCR methodology. The PCR reaction was conducted in a total volume of 20 μ L, with the mixture containing Power Track SYBR Green Master mix, dNTPs, and the SYBR Green fluorescent dye. To this master mix, 2 μ L of cDNA template was added, along with a primer mix at a concentration of 0.6 μ M. Specific primers were designed for each target gene:

Primers	Forward (5'-3')	Reverse (5'-3')
β-actin	TCCCTTGCCATCCT AAAAGCCACCC	CTGGGCCATTCTCC TTAGAGAGAAG
IL-1β	GCCAGTGAAATGA TGGCTTATT	AGGAGCACTTCAT CTGTTTAGG
IL-6	CACTGGTCTTTTGG AGTTTGAG	GGACTTTTGTACTC ATCTGCAC
iNOS	GACTTTCCAAGAC ACACTTCAC	TTCGATAGCTTGAG GTAGAAGC

The β -actin gene was selected as the internal control for normalization purposes, with its own set of forward and reverse primers. The real-time PCR was carried out using the Step-One Real-Time PCR System from Applied Biosystems, with reactions set up in 48-well plates. For data analysis, comparative 2⁻(Delta Delta CT) method was employed. This approach enables relative quantification of gene expression by comparing the CT (cycle threshold) values of the samples of interest with a control or calibrator sample. The CT values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene, in this case, β -actin. Results were expressed as fold changes in gene expression relative to the control samples.

2.1.11 Statistical analysis

The statistical analysis of the experimental data was conducted using GraphPad Prism 9.0.2 software (GraphPad Software, La Jolla, CA, USA). To determine whether the data followed a normal distribution, the Shapiro–Wilk test was applied. Once normality was confirmed, further statistical evaluations were carried out. For comparisons involving more than two groups, a one-way analysis of variance (ANOVA) was performed. This was followed by Tukey's post hoc test to identify specific group differences when the ANOVA indicated statistical significance. For comparisons between two groups, an unpaired t-test was used to evaluate the statistical differences between their means. Results were expressed as the mean $\pm$ standard error (SE) based on at least three independent experiments to ensure

reliability and reproducibility. Statistical significance was defined as a p-value <0.05 .

2.2 Materials and methods PART II: Materials and experimental methodology on primary thyroid cells and thyroid cancer cell lines

2.2.1 Solubilization of Grapefruit IntegroPectin

Even if the Grapefruit IntegroPectin used in this section was extracted from grapefruit (*Citrus paradisi*) processing waste kindly donated by Campisi Citrus (Siracusa, Italy) as before, the Grapefruit IntegroPectin in powder form was obtained via spray-drying, which affords an IntegroPectin identical in structure with higher flavonoid amount and antioxidant activity. Compared to the freeze-dried Grapefruit IntegroPectin, that retains a small amount of oxidative enzymes that slowly by progressively degrade the conjugated biophenols, the spray drying process inactivates these enzymes and protects the bioactive polyphenols from subsequent degradation. A powder sample of the spray dried Grapefruit IntegroPectin was solubilized in Dulbecco's Modified Eagle Medium (DMEM) purchased from Sigma Aldrich at the concentration of 10 mg/mL. The solution was filtered using a 0.45 μ m Sartorius filter and stored at 4 °C.

2.2.2 Human primary thyroid cells and thyroid cancer cell lines culture

Human primary thyroid epithelial cells were purchased from Cell Biologics (Chicago, IL, USA). The cells were cultured in complete human epithelial cell medium with supplements kit (H6621, cell biologics, Chicago, IL, USA), according to the manufacturer's instructions. FTC-133 follicular thyroid cancer cells were cultured in DMEM and F-12 (Ham's) medium (1:1) with 10% FBS and 1% L-glutamine and 1% P/S. ML-1 follicular thyroid cancer cells were cultured in DMEM with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin (P/S). THJ16T anaplastic thyroid cancer cells were cultured in PriGrow with 5% FBS and with 1 mM sodium pyruvate, 1% NEAA, 10 mM HEPES and 1% P/S. C643 anaplastic thyroid cancer cells were cultured in RPMI 1640 with 10% FBS and 1% L-glutamine, 2,0 g/L NaHCO₃ and 1% P/S. Cells were seeded on the gelatin-coated plates as per manufacturer's instructions. The cell cultures were maintained in a water saturated atmosphere supplemented by 5% CO₂ and 95% air at 37 °C in the incubators.

Cells were plated at a 1×10^6 /ml density in a 6-well plate for final volume of 2 ml and treated after 48 hours post-plating to ensure that the cells were in a stable state before exposure to the experimental conditions.

2.2.3 Cell treatments

For the Grapefruit IntegroPectin concentration-effect we tested 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, 2.5 mg/mL, and 5 mg/mL for 24 hours. We then selected the 1 mg/mL concentration for all subsequent experiments in this study. Next, to investigate the potential anti-proliferative effects of IntegroPectin, cells were singularly treated for 24 hours with Grapefruit IntegroPectin (1 mg/mL) and sphingosine 1-phosphate receptor (S1PR) modulator FTY720 (SML070, Sigma Aldrich) and then co-treated. S1PRs promote tumor progression by activating signaling pathways such as PI3K/Akt and MAPK to enhance proliferation. Mechanistically, FTY720 is phosphorylated into FTY720-phosphate (FTY720-P), which serves as a functional antagonist of S1PRs including S1PR1-2. Thus, the binding of FTY720-P activates S1PR1 signaling, leading to the internalization and subsequent degradation of S1PR1. As a result, administration of FTY720 causes long-lasting inhibition of S1PR1 signaling through the depletion of cell surface S1PR1. The treatment with FTY720 was done at an already known concentration of 10 μ M from previous experiments on the same primary cells and cell lines; cells were then co-treated with Grapefruit IntegroPectin administered immediately prior FTY720.

Follicular thyroid cancer cell lines FTC-133 and ML-1 were treated with Grapefruit IntegroPectin 1mg/ml and FTY720 10 μ M in order to investigate their effects on cell signaling pathways. Treatments were applied both individually and in combination to assess potential synergistic interactions. Primary thyroid cells were used as a control group, serving as a physiologically appropriate baseline for comparison.

2.2.4 MTT assay

Cells were plated into 96-well plates at a density of 2.5×10^4 cells per well in a final volume of 100 μ L. MTT assay was employed to evaluate cellular proliferation as a proxy for cell viability. For the IntegroPectin concentration-effect studies, we tested the following concentrations: 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, 2.5 mg/mL, and 5

mg/mL for 24 hours. The incubation period was set at 1 hour and 30 minutes, conducted under standard cell culture conditions of 37°C 5% CO₂. After the incubation period, the water-insoluble formazan crystals were solubilized by adding 100 µL of dimethyl sulfoxide (DMSO) to each well. The absorbance of the solubilized formazan was quantified using a spectrophotometer set at a wavelength of 570 nm. To account for any background interference, a background subtraction was performed. This step ensures that the measured absorbance accurately reflects the amount of formazan produced by the cells.

The cell viability was expressed as a percentage, with the control group serving as the baseline and set to 100%. This normalization allows for easy comparison between different experimental conditions and replicates. The percentage of cell viability in treated groups was calculated relative to this control.

2.2.5 Transwell migration assay

The invasion assays were performed by using Transwell (Corning) inserts method. Briefly, 5×10^5 cells in serum free medium (no FBS) were allowed to migrate towards 10% FBS containing medium for 16 h. Cells were then treated with Grapefruit IntegroPectin at the concentration of 1 mg/ml for 24 hours. Next day, cells were allowed to migrate towards 10% lipid stripped FBS (chemoattractant) for 16 h. The non-migrated cells on the top of migration insert were removed with a cotton swab. The migrated cells on the insert membrane bottom were fixed with 2% paraformaldehyde for 10 min, followed by staining with 0.1% crystal violet (20% methanol) for 5 minutes. Next, the membranes were washed with PBS and water and allowed to dry for overnight. The migrated stained cells were counted using 40× magnification from eight microscopic fields per insert.

2.2.6 [3H] thymidine incorporation assay

The proliferation of primary thyroid cells and follicular thyroid cancer cell lines FTC-133 and ML-1 was investigated using [3H] thymidine incorporation assay method. This technique is based on the principle that actively dividing cells incorporate radioactively labeled thymidine into their newly synthesized DNA during the S phase of the cell cycle. Cells are cultured in the presence of [3H] thymidine, a radioactive isotope of thymidine where one of the hydrogen atoms is replaced with tritium (3H). As cells divide and replicate their DNA, they incorporate

this radiolabeled nucleoside into the newly synthesized DNA strands. The amount of incorporated radioactivity is directly proportional to the rate of DNA synthesis and, by extension, to the rate of cell proliferation.

Briefly, 5×10^4 cells were seeded on 35 mm plates and allowed to grow for 24 h. The cells were then treated with Grapefruit IntegroPectin and FTY720 as above for 24 h, and 0.4 $\mu\text{Ci/mL}$ [^3H] thymidine was added to the cells for the last 4 h of incubation. The cells were washed three times with PBS, incubated for 10 min with 1 mL of 5% trichloroacetic acid, and then for 10 min with 1 mL of 0.1 M NaOH. The samples were transferred into scintillation vials and 3 mL of high sample load scintillation cocktail Optiphase Hisafe 3 was added in each sample. The radioactivity was measured using a Wallac 1414 liquid scintillation counter. The radioactivity data counts per minute (cpm) was normalized, and the results presented as % proliferation.

2.2.7 Western blot

As per protocol, 1×10^6 cells/ml (for a final volume of 2 ml) per condition were seeded in a 6-well plate and treated. 24-hours post treatment cells were washed with ice-cold PBS twice and lysed in RIPA buffer (150mM NaCl, 1% Triton-X-100, 0.5% Sodium deoxycholate, 1% SDS, 50mM Tris-HCl, pH 7.4) supplemented with protease inhibitor (cOmplete, Roche) and phosphatase inhibitor (PhosSTOP, Roche). Cells were lysed on ice for at least 15 minutes following which they were scraped and collected for further processing. Protein concentration measurements were performed with Pierce BCA Protein Assay Kit (ThermoFisher Scientific).

Equal amounts of protein (20 μg per lane) were loaded to SDS-PAGE and blotted onto nitrocellulose membrane filters (Sartorius). The membranes were then incubated with 5% skimmed milk in TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) blocking solution for 1 h and then with the primary antibodies diluted in blocking solution overnight at 4°C with gentle agitation. The primary antibodies used were as follows: anti-pAkt (1:2000, 9271 Cell Signaling Technology), anti-Akt (1:2000, 9272 Cell Signaling Technology), anti-Galectin-1 (1:2000, ab25138 Abcam), anti-Galectin-3 (1:2000, ab2785 Abcam) and anti-GAPDH (1:10000, MAB374 Merck-Millipore). The following day, membranes were further washed with TBS-T and incubated with horseradish peroxidase (HRP)

-conjugated secondary antibody diluted in blocking solution (1:5000, Jackson ImmunoResearch Laboratories) for 1 h at room temperature with gentle agitation. The secondary antibody was removed and the membranes were washed with TBS-T. Protein signals were detected using enhanced chemiluminescence substrate Pierce ECL Western Blotting Substrate (ThermoFisher Scientific). Signal was detected with the Fujifilm TyphoonTM FLA 9000 Image Reader.

Immunoblots were quantified with Fiji-ImageJ (NIH) quantification software. The intensities of each protein were normalized to GAPDH expression. The results are presented as relative band optical intensity.

2.2.8 Statistical analyses

The statistical analysis and graph design was performed using GraphPad Prism 10 software (GraphPad Software, La Jolla, CA, USA). Statistical comparison was performed using one-way/two-way ANOVA, this was followed by Tukey test to identify specific group differences when the ANOVA indicated statistical significance. For comparisons between two groups, an unpaired t-test was used to evaluate the statistical differences between their means. Results were expressed as the mean $\pm$ standard error (SE) based on at least three independent experiments to ensure reliability and reproducibility. A p-value $p < 0.05$ was considered as statistically significant.

2.3 Materials and methods PART III: Computational methods for analysis of Citrus IntegroPectin

2.3.1 Density functional theory

Our computational methodology employed density functional theory (DFT) calculations, specifically the B3LYP functional with the 6-31+G basis set, as implemented in the GAUSSIAN 16 software package. This approach is widely validated for diverse molecular systems due to its optimal balance between accuracy and computational cost compared to semi-empirical and wavefunction-theory-based methods. Frequency calculations were performed to verify the nature of stationary points as minima and to obtain Gibbs free energy corrections. Solvent effects were accounted for using the polarizable continuum model (PCM) with water as the solvent, which utilizes the integral equation formalism variant to create the solute cavity via overlapping spheres. [101]

DFT is a powerful computational method used in quantum chemistry and solid-state physics to investigate the electronic structure of many-body systems, particularly atoms, molecules, and condensed phases. [102,103,104]

For this study, DFT calculations were employed to analyze the molecular interactions and structural characteristics of pectin-flavonoid conjugates in citrus IntegroPectin. DFT is based on the idea that the properties of a many-electron system can be determined using functionals of the electron density, simplifying complex quantum mechanical wavefunctions and making it suitable for studying relatively large molecular systems. By using DFT we can obtain information about the electronic structure, bonding, and energetics of molecular systems, which is important for understanding how pectin and flavonoids interact at the molecular level.

Here, these DFT calculations served several purposes: predicting the optimal geometric arrangement of atoms in the pectin-flavonoid conjugates, estimating the strength of interactions between pectin and flavonoid molecules, revealing information about charge distribution and molecular orbitals, and simulating spectroscopic data for comparison with experimental results. This computational approach complements experimental methods and provides a more comprehensive

picture of this novel pectin extract derived from citrus processing waste, offering insights into its unique properties and potential applications.

To model IntegroPectin, we used a dimer structure consisting of a galacturonic unit linked to a rhamnose unit, forming the Pec-RGI structure. (**Figure 4**)

This model allowed us to simulate the formation of pectin-flavonoid conjugates through esterification reactions between the carboxylic acid group of pectin and the hydroxyl group of flavonoids, with the release of a water molecule. The flavonoids considered were naringenin, eriocitrin, and hesperidin, each with distinct R and R' substituents: R= OH, R'= H for naringenin; R = OCH₃, R'= OH for eriocitrin; and R = OH, R'= OH for hesperidin. This computational framework enabled us to investigate the energetic favorability of forming these conjugates in a solvent environment.

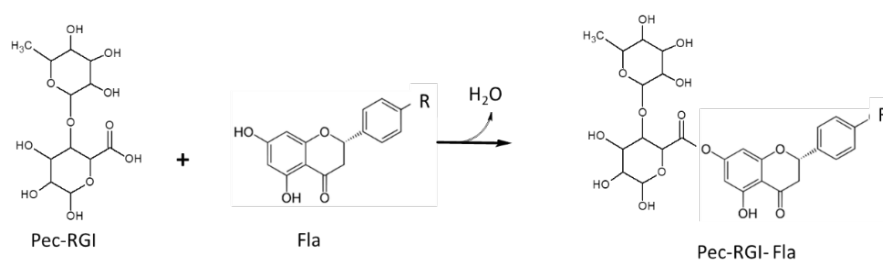


Figure 4. Esterification reaction leading to the formation of the pectin-flavonoid conjugate.

RESULTS

PART I:

Investigating the anti-oxidative and anti-apoptotic effects of Grapefruit IntegroPectin

Contributions: 3.1.1 - 3.1.2 M. Scordino, G. Urone; 3.1.3 C. Valenza; 3.1.4 M. Scordino, G. Urone, C. Valenza; 3.1.5 M. Frinchi.

Neurological disorders (NDs), including neurodegenerative diseases, represent one of the most significant challenges in medicine. NDs are the second leading cause of death worldwide. These disorders are characterized by sharing common pathogenic pathways that include mitochondrial dysfunction, oxidative stress, misfolded protein aggregation, and neuroinflammation. [105]

Microglia, myeloid-derived resident immune cells of the brain, maintain homeostasis in their resting state through ramified morphology, enabling surveillance of neuronal environments via neurotrophic factor secretion, synaptic pruning, and phagocytosis of cellular debris. Chronic pathological stimuli trigger microglial activation, characterized by morphological changes and dysregulated inflammatory responses that disrupt brain homeostasis. Activated microglia drive neuroinflammation and neurodegeneration through excessive release of pro-inflammatory cytokines (e.g., IL-1 β , IL-6), chemokines, prostaglandins, nitric oxide (NO), and activation of NF- κ B and ROS pathways. Oxidative stress from ROS accumulation synergizes with neuroinflammatory cascades, exacerbating neuronal damage and disease progression. [105,106]

Natural compounds demonstrated to have neuroprotective effect against oxidative stress and apoptosis [107,108]

Investigating Grapefruit IntegroPectin effects on microglia cells exposed to oxidative stress conditions was prompted by previous cited findings demonstrating neuroprotective effects against oxidative damage in neuronal-like SH-SY5Y cells. Specifically, IntegroPectin was shown to counteract cell death and apoptotic processes induced by exposure to powerful oxidizing agents, such as hydrogen peroxide. The protective effects were associated with a marked decrease in ROS production and preservation of mitochondrial function.

Given the critical role of oxidative stress in neuroinflammation and neurodegenerative processes, it was hypothesized that IntegroPectin might exert similar beneficial effects on microglial cells under oxidative stress conditions. This hypothesis was the starting point for extending the investigation to human microglial HMC3 cells, aiming to elucidate potential anti-apoptotic and anti-inflammatory properties of grapefruit IntegroPectin in the context of neuroinflammation and oxidative stress-related neurological disorders.

3.1.1 Effects of Grapefruit IntegroPectin on HMC3 cell viability

At first, we assessed the impact of Grapefruit IntegroPectin on the viability of HMC3 cells. We conducted a series of concentration-response experiments, exposing the cells to three different concentrations of IntegroPectin (0.01 mg/mL, 0.1 mg/mL, and 1 mg/mL) for 24 hours. The results, as illustrated in **Figure 5A**, revealed no significant alterations in cell viability across all tested concentrations. To further investigate the long-term effects of Grapefruit IntegroPectin, we performed a time-course experiment using the highest concentration (1 mg/mL). The cells were exposed to this concentration for extended periods, up to 72 hours. As shown in **Figure 5B**, even with prolonged exposure, Grapefruit IntegroPectin did not induce any significant changes in cell viability. Based on these findings, which demonstrated both the safety and stability of IntegroPectin at higher concentrations and over extended periods, we selected the 1 mg/mL concentration for all subsequent experiments in our study.

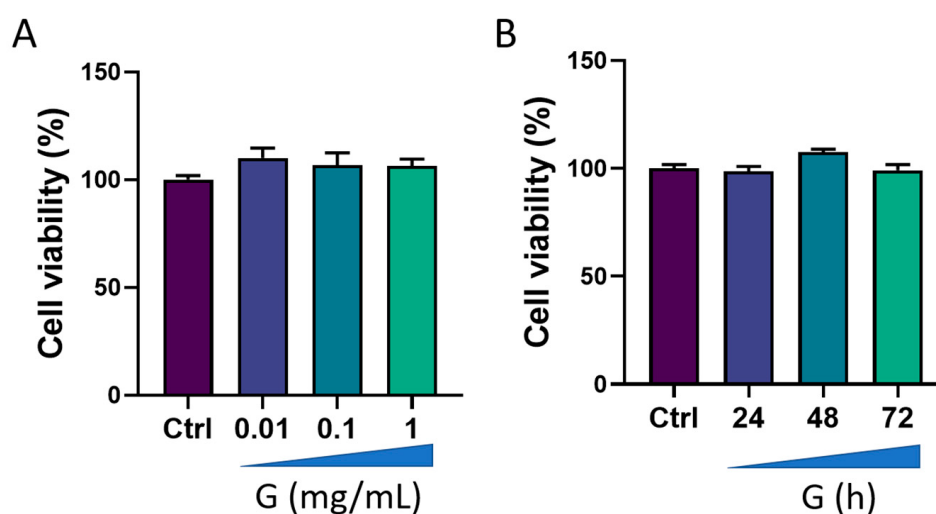


Figure 5. (A) Concentration–effect of Grapefruit IntegroPectin (G) treatment (24 h) on cell viability, evaluated via MTT assay. (B) Time-course of G treatment (1 mg/mL) effects on cell viability assessed via MTT assay. No changes in cell viability were observed following G treatment.

3.1.2 Protective and antioxidant effects of Grapefruit IntegroPectin in HMC3 cells treated with TBH

After the viability assessments, we investigated the potential protective effects of IntegroPectin against oxidative stress. We used tert-butyl hydroperoxide (TBH) as an oxidizing agent to induce cellular stress. HMC3 cells were exposed to various concentrations of TBH for 24 hours, resulting in a concentration-dependent decrease in cell viability (**Figure 6A**). From these results, we identified TBH at 200 μ M as the optimal concentration for inducing significant viability reduction, and this concentration was used in subsequent experiments. To evaluate the protective capacity of IntegroPectin, we pre-treated HMC3 cells with IntegroPectin (1 mg/mL) immediately before exposing them to TBH. IntegroPectin could mitigate the oxidative stress induced by TBH as showed in **Figure 7B**.

To further investigate the protective effects of IntegroPectin against TBH-induced oxidative stress, we employed a DCFH-DA fluorescence intensity assay. This assay serves as a sensitive indicator of intracellular ROS levels. Our findings revealed a significant reduction in intracellular ROS generation in HMC3 cells treated with grapefruit IntegroPectin prior to TBH exposure (**Figure 7C**). This result suggests that IntegroPectin possesses potent antioxidant properties, effectively mitigating the oxidative stress induced by TBH.

We complemented our quantitative analyses with a qualitative assessment of nuclear morphology using DAPI staining. This technique allowed us to visualize the protective effects of IntegroPectin at the cellular level. In cells exposed to TBH alone, we observed distinct morphological changes characteristic of cellular stress and potential apoptosis. These changes included smaller, fragmented, and condensed nuclei with bright fluorescence, markedly different from the normal nuclear morphology seen in controls. Cells pre-treated with grapefruit IntegroPectin before TBH exposure exhibited nuclear morphology more closely resembling that of control cells. This observation provides visual evidence of IntegroPectin's protective effect, preventing the nuclear changes typically associated with oxidative stress-induced cellular damage (**Figure 7D**).

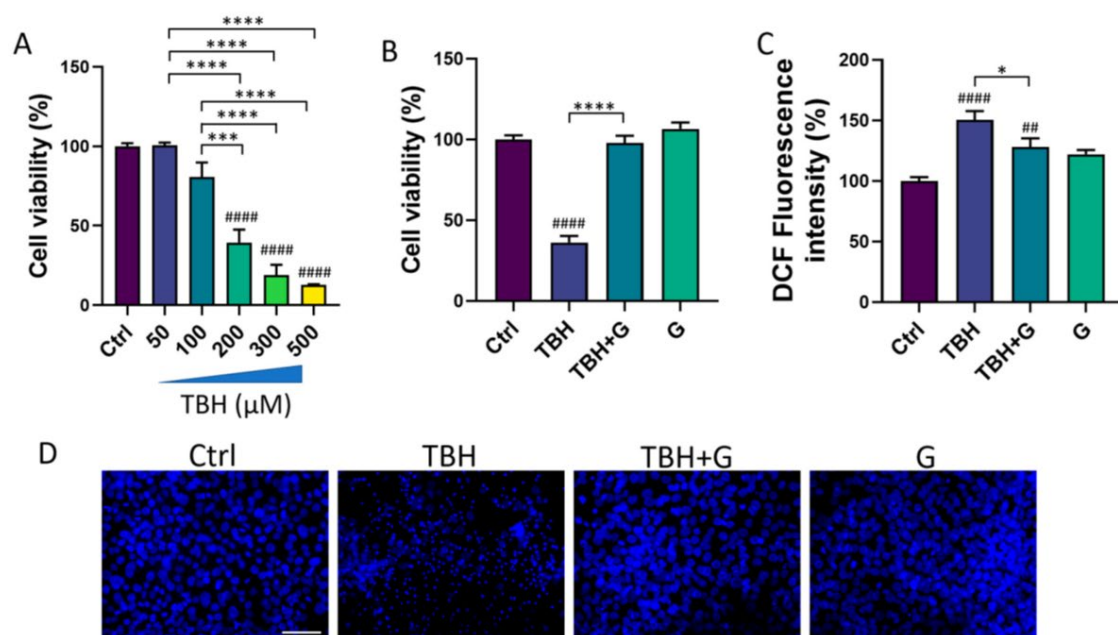


Figure 7 HMC3 exposure for 24 h to TBH led to a concentration-dependent decrease in cell viability, evaluated via MTT assay. TBH 200 μM was the concentration used in all experiments. (A). Grapefruit IntegroPectin (G) treatment fully prevented the cell death induced by the TBH treatment (B). DCFH-DA fluorescence intensity assay also showed reduced intracellular ROS generation (C). The protective effects were also shown by qualitatively comparing the morphology of DAPI-stained cell nuclei. The fluorescent nuclei of TBH-treated cells appear smaller, fragmented, and condensed, as compared to the control nuclei, while treatment with IntegroPectin prevented this morphological change (D). Tukey test: ## $p < 0.01$, ### $p < 0.001$ as compared Ctrl group; ** $p < 0.01$, *** $p < 0.001$.

3.1.3 Anti-apoptotic effects of Grapefruit IntegroPectin in HMC3 cells treated with TBH

The cell death induced by TBH exposure is mainly mediated by the activation of the apoptotic process. To study the ability of grapefruit IntegroPectin to inhibit the apoptosis induced by TBH treatment, cellular apoptosis/necrosis was evaluated via the flow cytometry quantification of the Annexin V binding and PI uptake. In **Figure 8A**, values within the representative density plots indicate the percentage of viable AnnexinV⁺ PI⁺ cells, intermediate apoptotic Annexin V⁺ PI⁺ cells, necrotic Annexin V⁺ PI⁺ cells, and late apoptotic Annexin V⁺ PI⁺ cells. Plots show that cells in the untreated (Ctrl) sample retained high viability (Annexin V⁺ PI⁺, 95.5%) with only a small percentage of Annexin V⁺ PI⁺ cells (4.3%) undergoing intermediate apoptosis. The oxidizing action of TBH affected the viability of the sample (Annexin V⁺ PI⁺, 36%) leading to a higher percentage of Annexin V⁺ PI⁺ cells (63%) indicative of intermediate apoptosis. In a co-treatment with TBH, IntegroPectin exerted a protective action as the sample yielded a high percentage of Annexin V⁺

PI⁺ cells (95%) and a small percentage (4.5%) of Annexin V⁺ PI⁺ cells, thus preventing oxidative stress-induced apoptosis. Treatment with IntegroPectin alone did not induce any change in cell viability and in the percentage of Annexin V⁺ PI⁺ cells (96.7%) and Annexin V⁺PI⁺ cells (3%), as compared to the Ctrl group. No significant percentages of necrotic cells (Annexin V⁺ PI⁺) or secondary necrotic cells (Annexin V⁺ PI⁺) show up in both density plots. Histograms in **Figure 8B, 8C** gather the cumulative percentages of the viable Annexin V⁺ PI⁺ cells and intermediate apoptotic Annexin V⁺ PI⁺ cells in the different experiments. To further characterize the anti-apoptotic role of grapefruit IntegroPectin, the protein levels of Caspase-3, a thiol protease that acts as a major effector caspase involved in the execution phase of apoptosis, were explored. Treatment with the oxidizing agent alone (TBH 200 μ M) induced a significant increase in the cleaved forms of Caspase-3 (25 kDa and 17 kDa), while grapefruit IntegroPectin was able to counteract this effect (**Figure 8D**).

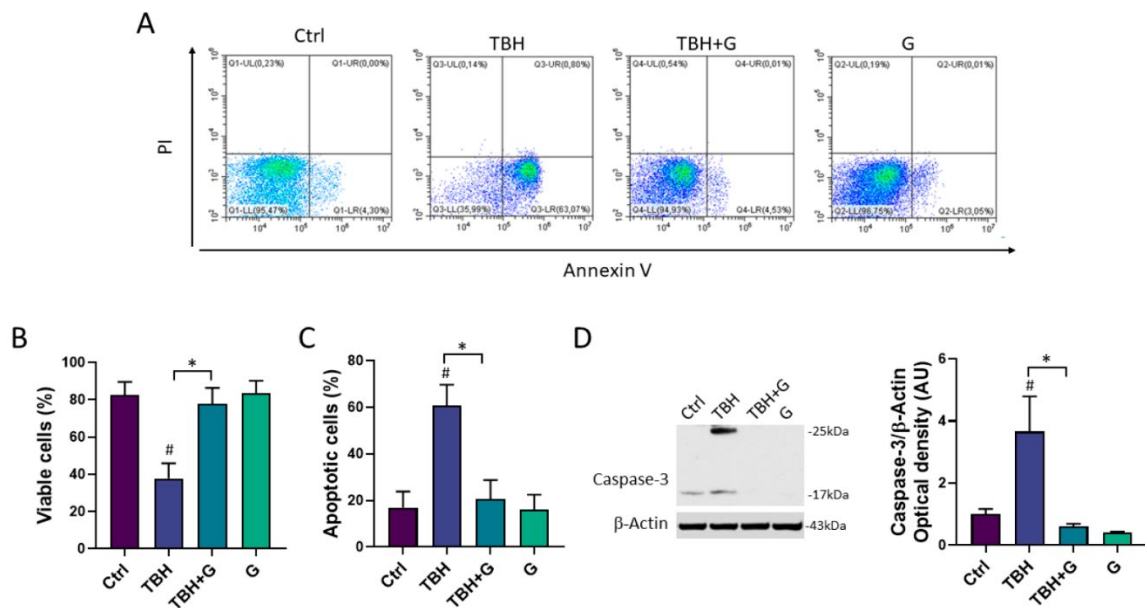


Figure 8 TBH-induced cell death is primarily mediated by apoptosis. To investigate grapefruit IntegroPectin potential to inhibit TBH-induced apoptosis, flow cytometry was employed to quantify Annexin V binding and PI uptake. Density plots (A) display the distribution of viable, intermediate apoptotic, necrotic, and late apoptotic cells based on their Annexin V and PI staining patterns. Untreated (Ctrl) cells exhibited high viability with minimal intermediate apoptosis. TBH treatment significantly reduced cell viability and increased intermediate apoptosis. Co-treatment with IntegroPectin demonstrated a **protective effect** against TBH-induced oxidative stress, with cell viability comparable to the control. Negligible levels of necrotic or secondary necrotic cells were observed across all conditions. Cumulative percentages of viable and intermediate apoptotic cells are presented in histograms (B, C). Moreover, TBH oxidizing action significantly increased cleaved **Caspase-3** forms (25 kDa and 17 kDa), a key effector in apoptosis, while IntegroPectin counteracted this effect (D). Tukey test: # $p < 0.05$ as compared to Ctrl group; * $p < 0.05$. AU (Arbitrary Units)

3.1.4 Modulation of the MAPK/ERK and PI3K/Akt pathways

Our initial focus centered on the MAPK/ERK pathway, a key regulator of cell proliferation and survival. Western blot analysis revealed modulations of this pathway in response to various treatments (**Figure 9**). Exposure of HMC3 cells to tert-butyl hydroperoxide (TBH, 200 μ M) resulted in a significant downregulation of phosphorylated ERK1/2 (p-ERK1/2) levels, indicative of compromised cell survival signaling under oxidative stress conditions. Co-treatment with grapefruit IntegroPectin (1 mg/mL) fully counteracted the TBH-induced suppression of p-ERK1/2. Moreover, IntegroPectin treatment alone significantly enhanced p-ERK1/2 levels compared to untreated control cells. (**Figure 9A**)

To further delineate the specific involvement of the MAPK/ERK pathway in IntegroPectin protective function, we employed a selective ERK1/2 signaling inhibitor, PD98059. Specifically, HMC3 cells were pre-treated with PD98059 (30 μ M) for 1 hour before exposure to grapefruit IntegroPectin (1 mg/mL) and TBH (200 μ M) for 24 hours. The inhibition of ERK1/2 signaling by PD98059 resulted in a modest reduction of IntegroPectin protective effects against oxidative stress-induced cell death (**Figure 9B**). The partial pathway attenuation suggests that while the MAPK/ERK pathway contributes to IntegroPectin protective mechanism, it is likely not the sole pathway involved. The ability of IntegroPectin to both counteract TBH-induced ERK suppression and independently activate ERK suggests a dual mechanism of protection. Furthermore, the upregulation of ERK phosphorylation after treatment with IntegroPectin alone suggests it may prime cells for better stress response.

In parallel with the p-ERK1/2 analysis, we investigated the modulation of the PI3K/Akt pathway. Co-administration of IntegroPectin (1 mg/mL) with TBH (200 μ M, 24 h) led to a complete restoration of phosphorylated Akt (p-Akt) protein levels, which had been significantly diminished by TBH exposure alone (**Figure 9C**). This suggests a potential role for the PI3K/Akt pathway in IntegroPectin protective mechanism. We then employed LY294002 (10 μ M), a selective PI3K/Akt inhibitor. HMC3 cells were pre-treated with LY294002 for 1 hour prior to the administration of grapefruit IntegroPectin (1 mg/mL) and subsequent TBH (200 μ M) exposure for 24 hours. Notably, this inhibition of PI3K/Akt signaling resulted in a complete abrogation of IntegroPectin protective effects (**Figure 9D**). This finding strongly

implicates the PI3K/Akt pathway has a critical role in IntegroPectin cytoprotective action against oxidative stress-induced cellular damage.

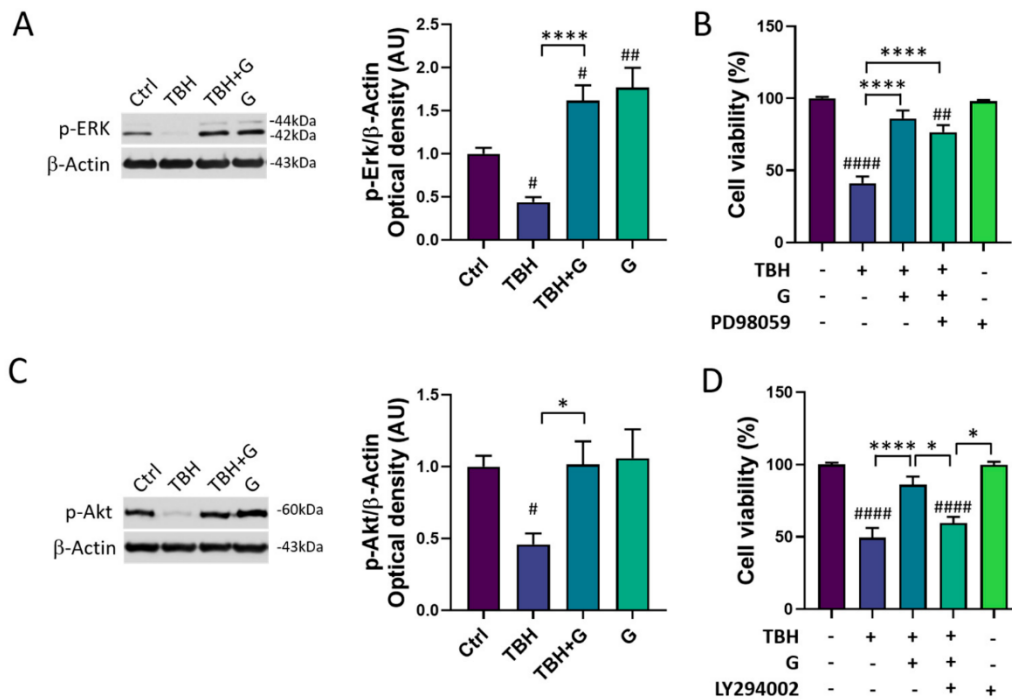


Figure 9 (A) HMC3 cells treated for 24h with TBH show a significant down-regulation of phosphorylated (p)-ERK1/2 levels, which was fully counteracted by grapefruit IntegroPectin co-treatment. IntegroPectin alone was able to significantly enhance p-ERK protein levels, as compared to untreated (Ctrl) cells. To explore the specific involvement of the MAPK/ERK pathway in IntegroPectin's protective function, HMC3 were treated with PD98059 (30 μ M), a selective ERK1/2 signaling inhibitor, 1 h before IntegroPectin and TBH exposure. The treatment with this inhibitor did not significantly affect IntegroPectin's protective effects against oxidative stress-induced cell death (B) Grapefruit IntegroPectin co-treatment was also able to fully recover the phosphorylated (p)-Akt protein levels, down-regulated by TBH exposure. (C). When HMC3 cells were treated with LY294002 (10 μ M), a selective PI3K/Akt inhibitor, 1 h before exposure to Grapefruit IntegroPectin and TBH, the IntegroPectin's protective activity completely vanished (D), suggesting the major involvement of PI3K/Akt signaling. Tukey test: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.001$

3.1.5 Modulation of neuroinflammatory pathways

To evaluate the potential anti-inflammatory properties of grapefruit IntegroPectin (Figure 10), we examined the mRNA expression of three key genes involved in microglial inflammatory response modulation: the pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1 β (IL-1 β), and the downstream inflammatory mediator iNOS. While IntegroPectin treatment did not significantly alter the expression of IL-6 (Figure 10A) or IL-1 β (Figure 10B), it notably reduced iNOS expression compared to untreated control cells (Figure 10C). Furthermore, we checked the time-dependent activation of three crucial intracellular modulators of the neuroinflammatory response: ERK1/2, Akt, and NF- κ B. A short-term 4-hour

exposure to IntegroPectin resulted in a significant upregulation of phosphorylated ERK1/2 (p-ERK1/2) levels (**Figure 10D**), also with a decrease in phosphorylated Akt (p-Akt) (**Figure 10E**) and phosphorylated NF- κ B (p-NF- κ B) (**Figure 10F**) levels, relative to control cells.

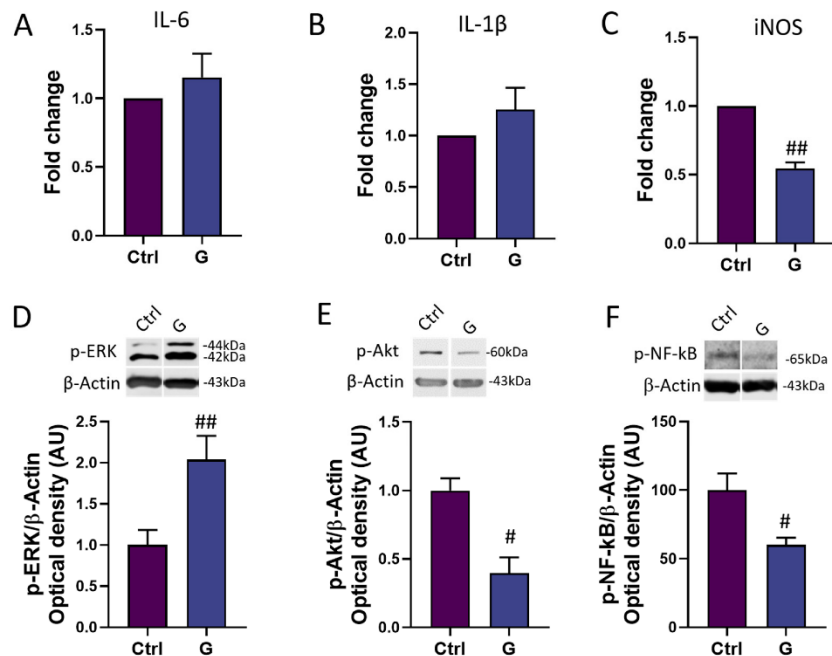


Figure 10 - Modulation of inflammatory mediators and pathways by grapefruit IntegroPectin. Real-time PCR of IL-6 (**A**), IL-1 β (**B**) and iNOS (**C**) mRNA levels in untreated (Ctrl) cells and cells treated with IntegroPectin (G) (1 mg/mL, 4 h). Representative images of phosphorylated (p)-ERK1/2 (**D**), p-A (**E**), p-NF- κ B (**F**) and β -Actin western blotting bands and histogram of p-ERK1/2 (**D**), p-Akt (**E**) and p-NF- κ B (**F**) normalized to β -Actin Optical density in Ctrl cells and cells treated with IntegroPectin (G) (1 mg/mL, 4 h). t-test: # $p < 0.05$, ## $p < 0.01$, as compared to Ctrl group. AU (Arbitrary Units).

Grapefruit IntegroPectin may exert its anti-inflammatory effects primarily through the modulation of iNOS expression and the differential regulation of ERK1/2, Akt, and NF- κ B signaling pathways in microglial cells. The observed upregulation in p-ERK1/2 levels, coupled with the downregulation in p-Akt and p-NF- κ B levels, indicates an interplay of cellular signaling mechanisms that may contribute to the anti-inflammatory properties of IntegroPectin.

3.1.6 Discussion: Investigating the antioxidant and anti-apoptotic properties of grapefruit IntegroPectin in human microglial cell line under oxidative stress conditions

These results show that Grapefruit IntegroPectin had a positive effect on microglia HMC3 cells by blocking the pathways linked to cell death, oxidative stress, and neuroinflammation.

Until now, the impact of IntegroPectin on microglia cells as well as its ability to regulate the neuroinflammatory response had not been investigated. As reported previously, studies using grapefruit as well as lemon IntegroPectin in SH-SY5Y cells showed that grapefruit IntegroPectin on SH-SY5Y cells led to a reduction in cell growth as well as a halt in the cell cycle in the G2/M phase. With HMC3 there was no impact on cell viability or growth, suggesting that this IntegroPectin triggers specific effects depending on the cell type.

By utilizing several experimental methods such as examining nuclei morphology, Annexin V binding PI uptake and measuring Caspase 3 levels, we found that grapefruit IntegroPectin can prevent TBH-induced apoptosis in the human microglial HMC3 cell line. The scavenging ability of grapefruit IntegroPectin against ROS could potentially interfere with both the extrinsic as well as intrinsic apoptotic pathways and help maintain balance in mitochondrial function.

TBH treatment decreases PI3K / Akt as well as MAPK / ERK signalling activation in microglia, but Grapefruit IntegroPectin treatment was able to counter both effects. The PI3K/Akt and MAPK / ERK pathways are generally referred to as pathways that promote cell survival, playing a crucial role in protecting cells from several harmful factors such as oxidative stress. Flavanones, for instance, demonstrate their ability to protect the brain by influencing the apoptosis process via the ERK1/2 and Akt signaling pathways. Additionally, these two cellular pathways play a role in the neuroprotective benefits triggered by ginseng pectin treatment in cortical neuron cells.

The MAPK / ERK as well as PI3K /Akt pathways are keypoints for various cellular processes, such as inflammation. PI3K/Akt pathway plays a crucial role in the activation of microglial cells by lipopolysaccharide (LPS). [109] Additionally, literature suggests that PI3K/Akt activation is also involved in the anti-inflammatory

effects of various biomolecules. ERK plays a crucial role in the activation of microglia because of interferon (IFN), while various anti-inflammatory drugs work by reducing MAPK / ERK signaling. [110] In this study, PI3K/Akt cascade emerged as the central mediator of Grapefruit IntegroPectin anti-apoptotic and antioxidant activity in HMC3. Inhibition of PI3K/Akt with LY294002 completely reduced Grapefruit IntegroPectin ability to counteract TBH-induced cell death, highlighting this pathway role in survival signaling. While MAPK/ERK activation was partially modulated by Grapefruit IntegroPectin, evidenced by p-ERK1/2 levels under basal conditions, its contribution to cytoprotection was secondary, as ERK inhibition (PD98059) only marginally attenuated Grapefruit IntegroPectin efficacy. This highlights the PI3K/Akt pathway as the dominant effector here.

It was interesting to see that just a 4-hour exposure to Grapefruit IntegroPectin can stimulate increased MAPK/ERK activity while reducing activity in the PI3K/Akt and NF-kB pathways.

NF-kB pathway is triggered by various external factors related to inflammation, immune function, cell growth, specialization, and viability, playing a key role in regulating inflammatory and apoptotic responses. This activation occurs by phosphorylating and breaking down the inhibitory I κ B protein. On the contrary, the non - canonical NF-kB pathway is specifically triggered by TNF superfamily receptors, resulting in the activation of NF-kB - inducing kinase (NIK), phosphorylation of p100 by NIK, and the movement of NF-kB into the nucleus. It is possible to theorize that the baseline activation of the NF-kB pathway in HMC3 cells is probably influenced by the PI3K-Akt pathway, which controls NF-kB transcriptional activity by triggering the phosphorylation and breakdown of I κ B. [111,112]

As results shown, Grapefruit IntegroPectin blocked the activation of AKT and suppressed the activity of NF-kB. This confirms what recent research has already shown about modified citrus pectin (MCP) inhibiting the NLR family pyrin domain containing 3 (NLRP3) inflammasome as well as NF-kB pathway activation in microglia following cerebral ischemia. [113] By investigating the activity of a group of genes commonly linked to the triggering of the PI3K-NF-kB pathway [114], we found that Grapefruit IntegroPectin does not result in noticeable changes in the levels of IL-1 β and IL-6. There was a notable reduction in iNOS expression, which

is typically increased by NF- κ B activation. Usually, microglia cells trigger the expression of iNOS following contact with inflammatory triggers such as LPS. The rise in iNOS levels results in continued release of nitric oxide (NO) as well as the creation of RNOS, which can result in various harmful effects, such as disrupting cell metabolism. While NO serves a few important functions, an overproduction of NO through iNOS activation can trigger inflammatory pathways as well as release inflammatory mediators such as histamine as well as cytokines, resulting in cellular damage as well as eventual cell death. [115]

Notably, Grapefruit IntegroPectin downstream suppression of the PI3K/Akt-NF- κ B-iNOS axis not only mitigated TBH-triggered neuroinflammation but also reduced basal microglial activation, suggesting a dual mechanism: acute protection against oxidative stress via Akt-mediated survival signaling and chronic anti-inflammatory action through NF- κ B transcriptional repression.

These findings position IntegroPectin as a multifunctional modulator of microglial pathophysiology, with implications for neurodegenerative disorders where oxidative stress and neuroinflammation converge. [116,117,118]

As mentioned previously, Grapefruit IntegroPectin stands out for its high content of adsorbed naringin (74 mg/g) and its abundance of long and hydrophilic RG I regions. This promotes the bonding of the IntegroPectin chains, which contain hydrophilic carboxylate as well as hydroxyl groups, with the HMC3 cellular membrane. This improves the transportation of bioactive substances, such as naringin, which is typically not very soluble but has notable anti-inflammatory as well as antioxidant properties. Since naringin is highly concentrated in grapefruit IntegroPectin, and because of its ability to scavenge free radicals as well as its impact on inhibiting the NF- κ B signaling pathway and reducing the expression of inflammatory proteins like iNOS REF, it is possible to suggest that naringenin plays a significant role in the protective effects of grapefruit IntegroPectin on microglia cells.

Works published following this research: [Anti-Apoptotic and Anti-Inflammatory Properties of Grapefruit IntegroPectin on Human Microglial HMC3 Cell Line](#)

3.2

RESULTS PART II

Investigating the anti-proliferative effects of Grapefruit IntegroPectin in human thyroid cancer

Contributions: 3.2.1 – 3.2.2 – 3.2.3 M.Y. Asghar; 3.2.4 – 3.2.5 – 3.2.6 – 3.2.7 C. Valenza

Despite many literature entries on its effects in various cancer types [119,120] the impact of citrus pectin on thyroid cancer cells has not been previously explored in an *in vitro* model. This section aims to preliminarily investigate the effects of Grapefruit IntegroPectin on the proliferation of thyroid cancer cells.

We used FTC-133 and ML-1 thyroid cancer cell lines and primary thyroid cells as control. [121] Cell viability assays were conducted to determine any cytotoxic effects of Grapefruit IntegroPectin on these cells.

We also investigated the role of Sphingosine-1-Phosphate receptors (S1PR) signaling in the effects of Grapefruit IntegroPectin treatments, by evaluating how S1PR1 inhibition influences the proliferation and migration of follicular thyroid cancer cell lines co-treated with grapefruit IntegroPectin. S1P is a bioactive lipid mediator involved in key cellular processes like proliferation, migration, survival, and angiogenesis. [122] In thyroid cancer cells, S1PR promotes tumor progression by activating signaling pathways such as PI3K/Akt and MAPK to enhance proliferation [123], regulating cytoskeletal dynamics to facilitate migration and invasion via epithelial-mesenchymal transition (EMT), and acting as a survival factor by inhibiting pro-apoptotic pathway. [124] Proliferation and migration of ML1 follicular thyroid cancer cells are affected by calcium signals by the bioactive lipid S1P and the expressional profile of S1P receptors. [125,126,127,128]

Since Grapefruit IntegroPectin, due to the modified extraction process, has a higher galacturonic acid content, we investigated the expression levels of Galectin-3, this because galactans are structural elements in pectins that directly binds Galectin-, that is known to be overexpressed in differentiated thyroid carcinomas but not in normal thyrocytes or benign thyroid nodules. We also analyzed the expression levels of Galectin-1 since it has also been shown to be upregulated in thyroid cancer cell lines.

3.2.1 Effects of Grapefruit IntegroPectin on the viability of primary thyroid cells and FTC-133 follicular thyroid carcinoma cell line

At first, we assessed the impact of grapefruit IntegroPectin on the viability of primary thyroid cells and only FTC-133 follicular thyroid carcinoma cell line. We conducted a series of concentration-response experiments, exposing the cells to three different concentrations of IntegroPectin (0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, 2.5 mg/mL, and 5 mg/mL) for 24 hours. (**Figure 11**)

The results, as illustrated in **Figure 11A**, revealed no significant alterations in primary thyroid cell viability across all tested concentrations. On the contrary, significant dose-dependent alteration of cell viability was observed in FTC-133 treated with Grapefruit IntegroPectin (**Figure 11B**), as cell proliferation decreased as the concentration went higher. Based on these results, we selected the 1 mg/mL concentration for all the following experiments in this study.

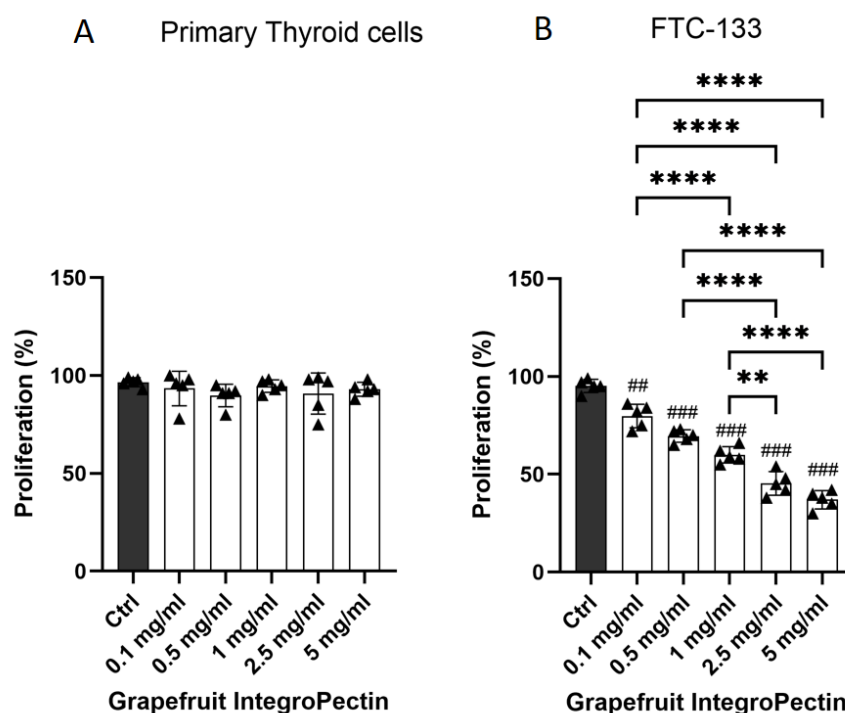


Figure 11 – (A) Concentration–effect of Grapefruit IntegroPectin (G) treatment (24 h) on cell viability of primary thyroid cells, evaluated via MTT assay. Minimal variation and no significant cytotoxic or antiproliferative effects were observed. (B) Concentration–effect of Grapefruit IntegroPectin treatment (24 h) on cell viability of FTC-133 thyroid cancer cells shows a marked, concentration-dependent decrease in proliferation. At the lowest concentration (0.1 mg/ml), proliferation is only slightly reduced relative to the control, but as the concentration of Grapefruit IntegroPectin increases, there is a progressive and substantial reduction in cell proliferation. At the highest concentration (5 mg/ml), proliferation drops to approximately 30% of the control, indicating a strong antiproliferative effect. Tukey test: ## $p < 0.01$, ### $p < 0.001$ as compared Control group; ** $p < 0.01$, **** $p < 0.001$. whereas not indicated it is not significative.

3.2.2 Effects of Grapefruit IntegroPectin on the invasion of primary thyroid cells and FTC-133 follicular thyroid carcinoma cell line

We then conducted Transwell migration Invasion assay on primary thyroid cells and FTC-133 cells after 24 h dose-treatment with Grapefruit IntegroPectin at 1 mg/mL. **(Figure 12)** In primary thyroid cells, Grapefruit IntegroPectin (G) did not significantly impair cell invasion, suggesting that the normal invasive capabilities of healthy thyroid cells were not affected by the treatment. **(Figure 12A)**. Conversely, when the treatment was applied to the FTC-133, Grapefruit IntegroPectin at 1 mg/mL demonstrated a significant inhibitory effect on cell invasion, showing that IntegroPectin treatment was able to reduce the invasive potential of these cancerous thyroid cells. **(Figure 12B)** The differential effect observed between primary and FTC-133 cells is particularly interesting, as it suggests that IntegroPectin might target specific mechanisms involved in cancer cell invasion without affecting normal cell function.

This selective inhibition of invasion in carcinoma cells aligns with existing literature on citrus IntegroPectin, which has shown antiproliferative and protective effects in different cell types. The ability to impair invasion in cancer cells while sparing normal cells could be a valuable point for potential therapeutic applications.

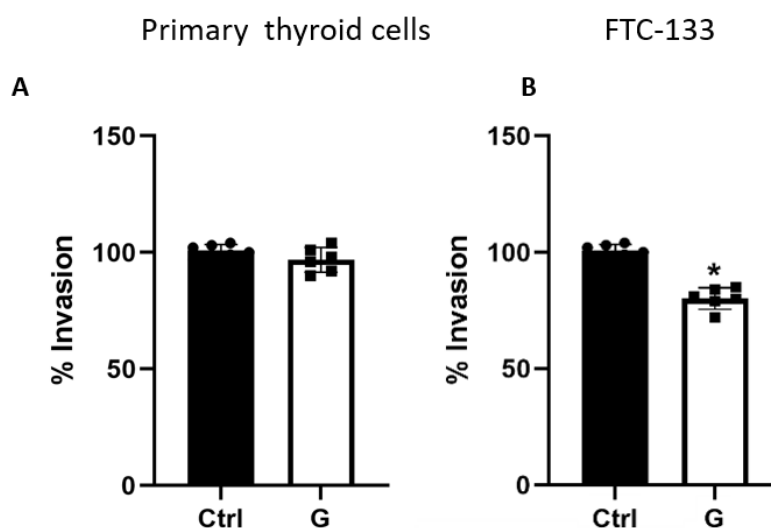


Figure 12 –Transwell migration Invasion assay on primary thyroid cell. **(A)** The percentage of invasion for primary thyroid cells remains essentially unchanged between the control group and the group treated with Grapefruit IntegroPectin (G) 1mg/ml. Both groups exhibit invasion rates close to 100%, with minimal variability and no significant difference, **(B)** 6-hour Transwell migration Invasion assay on FTC-133 cells, showing a significant reduction in invasion for FTC-133 cells treated with G 1mg/ml compared to the control, with invasion dropping to approximately 80% of control levels. Tukey test: * $p < 0.05$ whereas not indicated it is not significant.

3.2.3 Grapefruit IntegroPectin inhibits proliferation and migration of FTC-133 and ML-1 cells

Including ML-1 cells in addition to FTC-133 cells allowed us for a broader investigation into how S1P signalling interacts with Grapefruit IntegroPectin (G) treatment across follicular thyroid cancer cell types. (**Figure 13**)

In **Figure 13A** we can see in primary thyroid cells that treatment with S1P1R inhibitor FTY720 for 24 hours reduces proliferation to approximately 85%, showing significant inhibition compared to control. Unexpectedly, in contrast with the previous MTT results, Grapefruit IntegroPectin treatment significantly decreased proliferation to 73%. The co-treatment with IntegroPectin and FTY720 yields similar results to treatment with IntegroPectin alone (72%), with no additive effect observed. This result will be further discussed in the next Discussion section.

In **Figure 13B** we can see that treatment with FTY720 in FTC-133 cells did not result in a significant reduction in cell proliferation, as determined by the incorporation of [3H]-thymidine. In contrast, Grapefruit IntegroPectin treatment reduces proliferation to 45%, showing highly significant inhibition compared to both control and S1P treatment. The co-treatment with FTY720 and Grapefruit IntegroPectin maintains this strong inhibitory effect, with no apparent enhancement from adding FTY720 (43%). However, results highlight the potential of IntegroPectin to enhance the anti-proliferative effects beyond those achieved by targeting S1P signaling pathway independently. The observed reduction in proliferation could involve synergistic effects that disrupt cancer cell growth.

In **Figure 13C**, treatment with FTY720 alone on ML-1 cells again shows minimal impact on proliferation (97%) compared to control. Treatment with IntegroPectin substantially reduced proliferation to 45%, and the co treatment FTY720 and Grapefruit IntegroPectin enhances this inhibition to 35%. Both treatments with IntegroPectin alone and co-treatments show highly significant effects compared to control and treatment with S1P inhibitor alone. This result further confirms that in ML-1 cells, treatment with Grapefruit IntegroPectin exerts strong antiproliferative effects, which may be slightly enhanced when combined with S1PR1 inhibition.

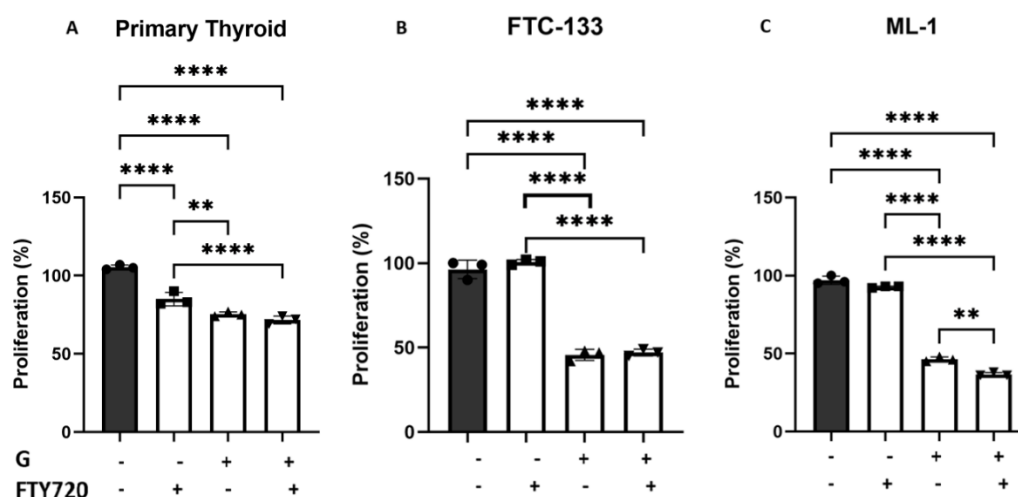


Figure 13 - 3H incorporation assay conducted over cells treated with Grapefruit IntegroPectin (G) and FTY720 S1PR modulator for 24 hours. (A) shows that all the treatments reduced proliferation in primary thyroid cells. (B) and (C) show that treatment with IntegroPectin, alone or in co-treatment with FTY720 markedly inhibits proliferation in FTC-133 and ML-1 thyroid carcinoma cells, while treatment with FTY720 alone has no significant effect. Tukey test: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; whereas not indicated it is not significative.

3.2.4 Modulation of PI3K/Akt pathway

Western blot analysis was performed to evaluate pAkt in Primary T cells, FTC-133 cells, and ML-1 cells under control, FTY720 (10 μ M), Grapefruit IntegroPectin (G) (1 mg/mL), and Grapefruit IntegroPectin and FTY720 co-treatment conditions. (Figure 14) In Primary T cell (Figure 14A) S1P inhibition slightly reduced pAkt levels compared to control while Grapefruit IntegroPectin alone decreased pAkt levels more significantly. Interestingly, the combination of IntegroPectin and FTY720 showed an increase in pAkt levels.

In FTC-133 cells (Figure 14B) we observed different signaling dynamics characterized by substantially higher baseline pAkt levels, with a higher constitutive PI3K/Akt pathway activation. Treatments with Grapefruit IntegroPectin did not affect significantly pAkt expression level, rather we can see that treatment with FTY720 alone showed a slight increase, even if not significative, in pAkt levels. Consistent with the previous result, the co-treatment with Grapefruit IntegroPectin and FTY720 enhanced pAkt level.

Treatments done in ML-1 cells (Figure 14C) showed another different dynamics. Grapefruit IntegroPectin exerted a potent, selective downregulation of pAkt. while FTY720 treatment upregulated pAkt levels compared to untreated controls, which

is consistent with S1PR1 inhibition-induced compensatory PI3K/Akt activation. IntegroPectin alone significantly downregulated pAkt to near-baseline levels and the co-treatment again showed that IntegroPectin managed to lower pAkt expression levels significantly.

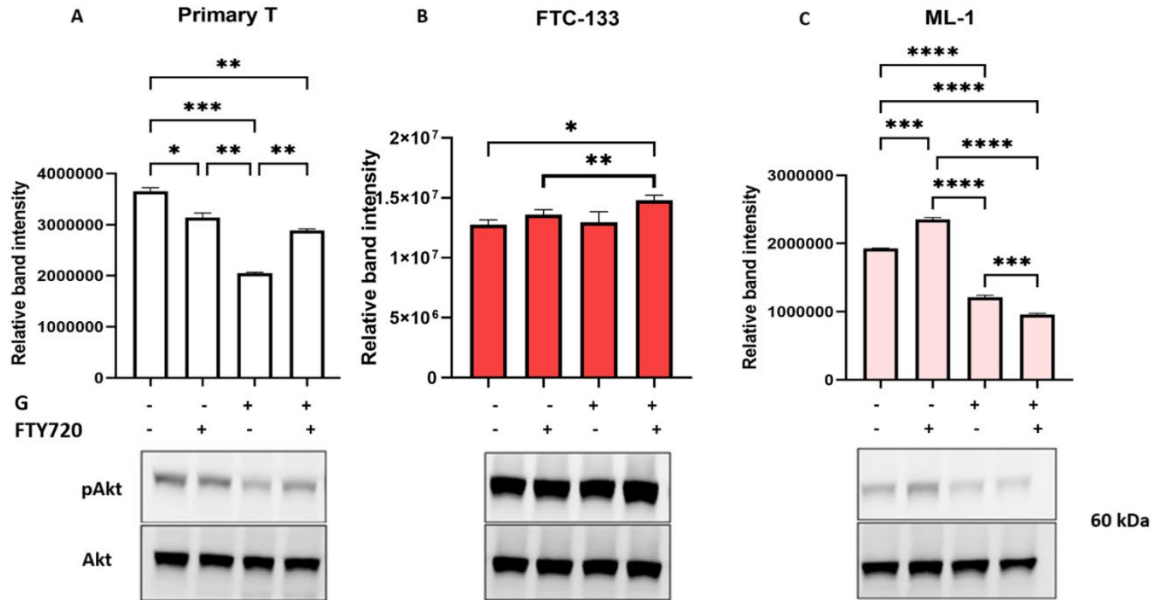


Figure 14 (A) In primary thyroid cells there is a small reduction in pAkt/Akt ratio with FTY720 S1PR1 inhibitor treatment (10 μ M), with maximal inhibition by Grapefruit IntegroPecting (G) 1mg/ml alone, and partial rescue when combined with FTY720. (B) In FTC-133 we observed different signalling dynamics, starting from a higher baseline pAkt levels. Both treatments with FTY720 and G alone slightly upregulated pAkt levels and the co-treatment slightly enhanced pAkt expression. (C) In ML-1 cells there is a marked downregulation of pAKT after treatment with G alone and in combination with FTY720. Tukey test: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; whereas not indicated it is not significant.

3.2.5 Differential effects of Grapefruit IntegroPectin on Galectin-3 expression in thyroid cancer cell lines and primary thyroid cells

We investigated the expression of Galectin-3, across primary thyroid cells and FTC-133 and ML-1 follicular thyroid cancer cell lines. Galectin-3 is known for its role in cell adhesion, migration, and angiogenesis, and its expression levels are often elevated in cancerous tissues.

Western blot analysis was performed to assess Galectin-3 protein levels in response to FTY720 (10 μ M), Grapefruit IntegroPectin (1mg/ml), and co treatment with Grapefruit IntegroPectin and FTY720 across all three cell types (**Figure 15**). In primary thyroid cells, all the treatments resulted in a modulated increase in Galectin-3 levels. This increase in Galectin-3 may reflect a stress response to external stimuli, as primary cells lack the oncogenic drivers that amplify Galectin-3 in cancer. (**Figure 15A**) This will be further pointed out in the Discussion section.

FTC-133 cell line exhibits high baseline levels of Galectin-3 and treatment with FTY720 did not alter Galectin-3 expression compared to control, whereas both Grapefruit IntegroPectin alone and in combination with FTY720 reduced Galectin-3 levels compared to FTY720 treatment and the control group (**Figure 15B**).

The most promising result is showed in (**Figure 15C**). ML-1 cells instead exhibited low baseline levels in Galectin-3 expression compared to FTC-133. Grapefruit IntegroPectin treatment even more significantly reduced Galectin-3 levels compared to control cells, alone and in combination with FTY720

These results indicate that Grapefruit IntegroPectin exerted a marked downregulation in surface Galectin-3 expression even when in co-treatment with S1PR1 modulator in a cell-type-specific manner.

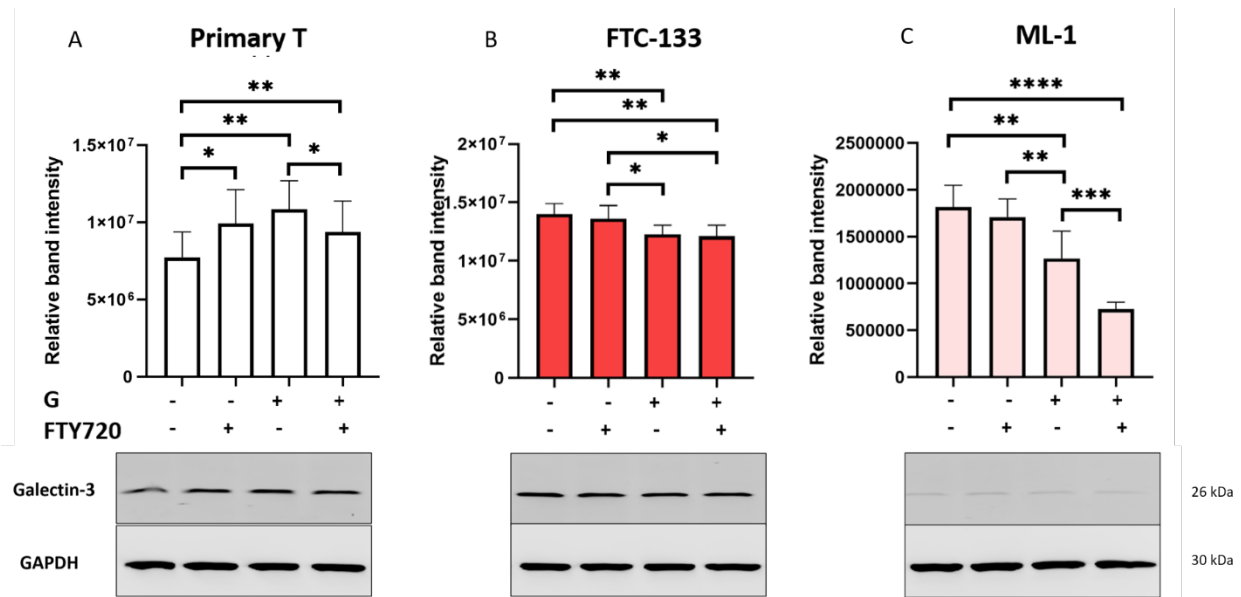


Figure 15 – (A) In primary thyroid cells, Grapefruit IntegroPectin and FTY720 treatment modulated Galectin-3 expression compared to the control group. (B) FTY720 treatment did not affect Galectin-3 expression relative to the control, whereas both Grapefruit IntegroPectin alone and in combination with FTY720 reduced Galectin-3 levels compared to FTY720 treatment and the control group. (C) In ML-1 cells, treatment with Grapefruit IntegroPectin induced a significant downregulation of Galectin 3, that can be seen also in the co treatment with Grapefruit IntegroPectin and FTY720 compared to control. GAPDH serves as a loading control in all panels. Tukey test: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.001$; whereas not indicated it is not significative.

3.2.6 Galectin-1 expression levels are unaffected by S1P receptor modulation and treatment with Grapefruit IntegroPectin

We assessed Galectin-1 protein levels in response to FTY720 (10 μ M), Grapefruit IntegroPectin (1mg/ml), and co treatment with Grapefruit IntegroPectin and FTY720 across all three cell types (**Figure 16**).

As shown, Galectin-1 baseline levels of expression are high across all the cell lines and no significant alterations in Galectin-1 expression were noticeable after any of the treatment conditions in Primary thyroid cells (**Figure 16A**), in FTC-133 (**Figure 16B**) and ML-1 cells (**Figure 16C**). GAPDH was used as a loading control to ensure equal protein loading across all samples.

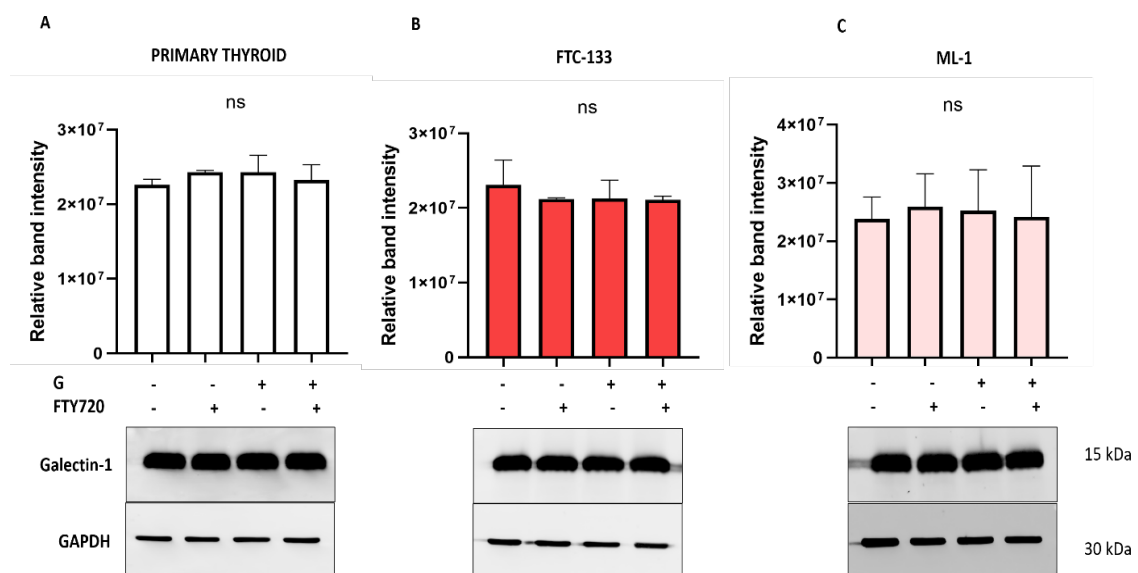


Figure 16 (A) In primary thyroid cells Galectin-1 expression has non-significant modulation across all treatment conditions compared to control. (B) FTC-133 cells show non-significant modulation of Galectin-1 following all treatments. (C) ML-1 cells show uniform Galectin-1 expression levels across all conditions with no statistically significant differences between treatments. GAPDH serves as loading control in all experiments.

3.2.7 Comparing Galectin-3 and Galectin-1 basal levels in primary cells and thyroid cancer cell lines

The further Western blot analysis, as depicted in **Figure 17**, evaluated Galectin-3 baseline expression in Primary Thyroid cells and in a panel of cell lines including follicular cancer cell lines FTC-133 and ML-1, and anaplastic thyroid cancer cell lines THJ16T and C643. Primary thyroid cells show relatively common baseline expression of Galectin-3, while FTC-133 cells exhibits markedly elevated expression compared to the other cell lines, as this confirms the results from previous experiments where Galectin-3 levels in FTC-133 were mostly upregulated in every treatment. This may correlate with their aggressive phenotype. THJ16T, and C643 demonstrate relatively moderate Galectin-3 expression, while ML-1 cells show minimal detectable levels.

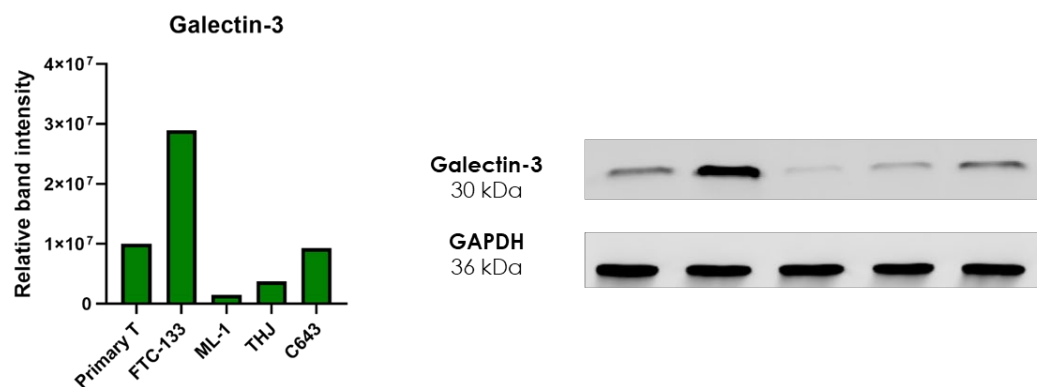


Figure 17 Analysis and quantification of Galectin-3 expression across primary thyroid cells and four thyroid cancer cell lines. The most upregulated expression with FTC-133 thyroid carcinoma cells exhibiting 3-fold higher Galectin-3 levels compared to primary thyroid cells. ML-1 and THJ cell lines demonstrate minimal Galectin-3 expression, while C643 cells show expression levels comparable to primary thyroid cells. GAPDH serves as loading control, confirming equivalent protein loading across all samples.

On the other hand, Galectin-1 expression across the same panel of cell lines shows that, as expected, Galectin-1 expression is steadily high in all the cell lines FTC-133, ML-1, THJ16T, and C643 cells with primary thyroid cells having comparatively lower baseline expression. GAPDH was again used as a loading control to normalize for protein loading variations. (Figure 18)

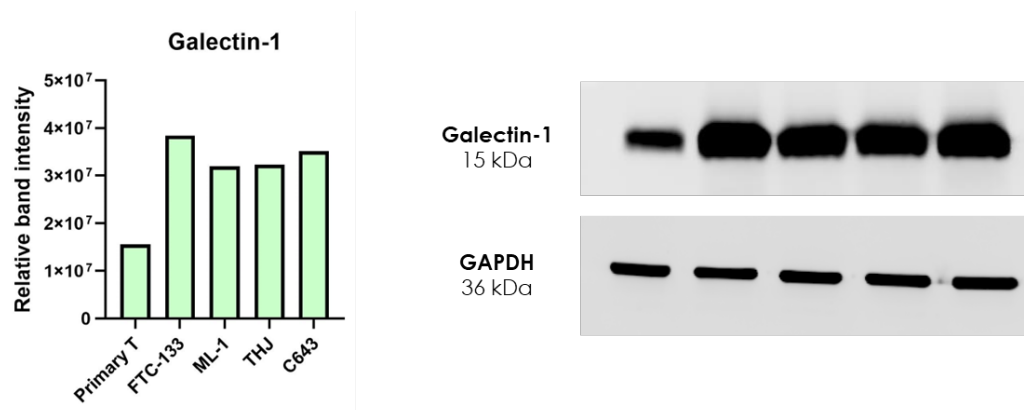


Figure 18 Analysis and quantification of the prominent Galectin-1 baseline expression in FTC-133, ML-1, THJ, and C643 cells, with a less intense baseline expression in primary thyroid cells. GAPDH serves as loading control, confirming equivalent protein loading across all samples

3.2.8 Discussion: the anti-proliferative effects of grapefruit IntegroPectin on thyroid cancer cell lines and examining potential mechanisms of action.

Thyroid cancer is the most common endocrine cancer [129]. The incidence has steadily increased during the past decades throughout the world. There are different types of thyroid cancers ranging from more benign ones with a relatively good prognosis to malignant anaplastic tumours. Although the follicular type of thyroid cancer is relatively benign some of the tumours can spread and be aggressive warranting the development of novel therapeutic approaches [130]

The differential effects observed between primary thyroid cells, FTC-133 and ML-1 cancer cell lines underscore Grapefruit IntegroPectin potential as a therapeutic agent, while raising critical questions about its mechanisms of action and cell type-specific responses.

The absence of cytotoxicity in primary cells suggests IntegroPectin bioactivity affects more cancer cells with dysregulated signaling networks. This aligns with prior studies showing citrus pectins modulate pathways such as NF-κB and

MAPK/ERK, which are hyperactive in malignancies but less critical for normal cell homeostasis.

Grapefruit IntegroPectin exhibited pronounced, concentration-dependent inhibition of FTC-133 proliferation. This aligns with IntegroPectin structural composition, since the RG-I region, enriched in galactose side chains, interact with specific targets such as galectin-3 and β 1-integrins. [131] These targets promote tumour progression via PI3K/Akt and Wnt/ β -catenin pathways. This mechanism is supported by the significant reduction in FTC-133 invasion likely mediated by disrupted galectin-3-dependent cell-matrix interactions, while no effects are seen in primary thyroid cells.

However, we could see contrasting results between MTT assay and 3H-thymidine in primary cells. While MTT assays measures mitochondrial activity, 3H-thymidine quantifies DNA synthesis. The prolonged duration of the incubation and the treatment might have influenced the proliferation and survival of the primary thyroid cells under established culture conditions, leading to assay-specific artifacts. This hypothesis is supported by the lack of cytotoxicity in MTT assays and Transwell assays.

Sphingosine-1-phosphate (S1P) receptors play critical roles in thyroid cancer by mediating the effects of S1P, a bioactive lipid involved in various cellular processes such as proliferation, migration, survival, and angiogenesis. [124] These receptors, part of the G-protein-coupled receptor family, include five subtypes (S1PR1–S1PR5), each with distinct functions depending on their expression patterns and downstream signaling pathways. S1PR1 and S1PR3 are commonly overexpressed in thyroid cancer cells and are associated with enhanced cell proliferation and survival. These receptors activate key oncogenic pathways such as PI3K/Akt, which promotes tumor growth and resistance to apoptosis. [124]

In normal thyroid cells, the Akt pathway regulates various cellular processes, including cell survival, proliferation, and metabolism, acting as a mediator of growth factor signaling, particularly in response to insulin-like growth factor 1 (IGF-1). In thyroid cancer cells, the Akt pathway is often hyperactivated, as showed in FTC-133 contributing to enhanced cell proliferation and survival, increased resistance to apoptosis, promotion of cell migration and invasion, and stimulation

of angiogenesis. [128] The importance of the Akt pathway in thyroid cancer is further underscored by its involvement in drug resistance mechanisms, such as acquired resistance to V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF) inhibitors in some thyroid cancer patients [132].

Here, Akt phosphorylation is differentially regulated by treatments with Grapefruit IntegroPectin and FTY720 across primary thyroid cells and thyroid cancer cell lines. Grapefruit IntegroPectin has the capacity to reduce cell proliferation and migration of ML-1 follicular and of FTC-133 thyroid cancer cells in culture in a concentration dependent manner and sometimes in synergy with the inhibition of S1P receptors. Although, co-treatment FTY720 does not significantly enhance these effects beyond the effects of treatments with Grapefruit IntegroPectin alone, indicating that its anti-proliferative activity is largely independent of S1PR signaling.

The combination of IntegroPectin and FTY720 showed effects in modulating pAkt levels with an evident reduction in ML-1 cells. By inhibiting Akt phosphorylation, IntegroPectin may help suppress the aggressive behaviour of thyroid cancer cells and potentially enhance their sensitivity to other treatments.

Notably, both FTC-133 and ML-1 are follicular thyroid cancer cell lines yet Grapefruit IntegroPectin elicited a differential response in modulating pAkt expression only in the latter. This likely reflects intrinsic molecular and cellular differences between these two cell lines and may arise from variations in genetic mutations, signaling pathway activation, or the tumor microenvironment. Differences in the composition of the extracellular matrix or cell surface receptors could also influence how each cell line responds to IntegroPectin, given that this pectin only exerts its activity outside the cell.

One possible explanation is that ML-1 cells may have a greater dependency on the PI3K/Akt pathway for survival and proliferation compared to FTC-133 cells. [133] This dependency could make ML-1 cells more sensitive to anti proliferative activity of IntegroPectin. In contrast, FTC-133 cells might rely more heavily on alternative pathways, such as the MAPK/ERK pathway [134], for their oncogenic behavior, thereby rendering pAkt levels less susceptible to modulation by IntegroPectin.

Another factor could be differences in the expression or activity of upstream regulators of Akt, such as receptor tyrosine kinases (e.g. EGFR or VEGFR) [135]

or lipid kinases like PI3K [136]. ML-1 cells exhibit higher basal activity of these upstream regulators, leading to elevated pAkt levels that are more effectively inhibited by IntegroPectin. Conversely, FTC-133 cells have lower basal Akt activity or compensatory mechanisms that maintain pAkt levels despite treatment.

The same PI3K/Akt pathway has been shown to upregulate Galectin-3 expression by promoting *LGALS3* transcription. [137] The activation of specific signaling pathways can modulate galectins expression and Galectin-3 and Galectin-1 are sensitive diagnostic markers in thyroid cancer. [138,139] As said, since Grapefruit IntegroPectin exerts its activity outside the cell we looked for cell surface receptors that could explain the anti-proliferative activity. As mentioned earlier, Grapefruit IntegroPectin, due to the modified extraction process, has a higher galacturonic acid content and galactans are structural elements in pectin that directly binds Galectin-3.

Hence, we also wanted to investigate the antiproliferative activity in vitro of grapefruit IntegroPectin on the expression of Galectin-3 and Galectin-1 in thyroid cancer cell lines and primary thyroid cells, since the same naringin-rich IntegroPectin has already shown powerful antiproliferative activity on neuroblastoma cells [96,97]

In line with the data on Akt phosphorylation, we saw that treatment with Grapefruit IntegroPectin resulted in a significant downregulation of galectin-3 expression only in ML-1 thyroid cancer line, which already showed a low level of Galectin-3 expression compared to the levels showed in FTC-133. Conversely, treatment with the FTY720 alone did not prompt a similar downregulation of galectin-3 expression in either of the FTC-133 and ML-1 thyroid cancer cell lines. This indicates that the inhibition of S1P signalling pathway alone may not directly influence Galectin-3 expression in these cell lines and that the effect of S1P inhibition on galectin-3 expression may be context-dependent.

The differential response between the FTC-133 and the ML-1 cell lines may instead reflect inherent variations in their molecular profiles and the mechanisms underlying galectin-3 regulation, since the baseline levels for Galectin-3 in FTC-133 cells in higher.

Partly in contrast to the findings in thyroid cancer cell lines, treatment with Grapefruit IntegroPectin, FTY720, had a modulated effect on Galectin-3 expression in primary thyroid cells. This effect in primary thyroid cells suggests that the regulatory mechanisms modulating galectin-3 expression in response to these treatments could be specific to cancerous cells, potentially due to the altered signalling pathways and molecular environments characteristic of these cancer cells. It may also reflect a stress response to external stimuli, as primary cells lack the oncogenic drivers that amplify Galectin-3 in cancer. It is also important to consider the established culture conditions for these primary cells with growth factors components in medium that may have influenced the outcome.

The observed stability of Galectin-1 levels across all treatments in thyroid cancer cells suggests that these levels are likely elevated and resistant to modulation. This interpretation aligns with existing literature, which has consistently reported overexpression of Galectin-1 in thyroid carcinoma-derived cell lines and tissues compared to normal thyroid cells. [140,141] The inability of S1P inhibition, IntegroPectin, or their co-treatment to alter Galectin-1 expression indicate that the regulatory pathways controlling Galectin-1 expression are not directly influenced by these treatments. Alternatively, high Galectin-1 levels could depend by multiple redundant pathways, rendering them resistant to the applied interventions. The fact that Galectin-1 levels did not decrease despite the treatments indicates its importance in the thyroid cancer cell molecular profile and suggests that more targeted approaches may be necessary to modulate its expression effectively.

Additionally, we extended the analysis of Galectin-3 and Galectin-1 basal expression in more thyroid cancer cell lines without performing further treatments. The variation in Galectin-3 levels across the different thyroid cancer cell lines (FTC-133, ML-1, THJ, and C643) likely reflects the distinct molecular characteristics and tumorigenic pathways active in each cancer subtype. [142] These variations highlight the role of Galectin-3 as a biomarker for specific thyroid cancer subtypes. Its differential expression reflects the underlying biology of each cancer type, including their origin, differentiation status, and reliance on selected oncogenic pathways.

In follicular thyroid carcinoma-derived FTC-133 cells, the high Galectin-3 levels observed are consistent with the known overexpression of this protein in follicular

thyroid cancers. Galectin-3 plays a role in promoting tumor progression and metastasis in this subtype by enhancing cell motility and invasion.

In contrast, ML-1 cells exhibit minimal Galectin-3 expression. ML-1 cells are derived from medullary thyroid carcinoma (MTC), a neuroendocrine tumor type that arises from parafollicular C cells rather than follicular epithelial cells. MTCs typically do not rely on the same mechanisms of Galectin-3 overexpression seen in follicular or papillary thyroid carcinomas. This difference underscores the cell-type-specific regulation of Galectin-3 expression. [143]

THJ16T and C643 cells, which are derived from anaplastic thyroid carcinoma (ATC), show moderate levels of Galectin-3. While ATCs are highly aggressive and poorly differentiated, they may retain some molecular features of their differentiated precursors, such as partial Galectin-3 expression. However, the loss of differentiation in ATCs could explain why their Galectin-3 levels are lower than those observed in FTC-133 cells.

In primary thyroid cells, Galectin-1 expression is relatively low, consistent with their nature and their role as a baseline control for comparison. In contrast, thyroid cancer cell lines (FTC-133, ML-1, THJ16T, and C643) exhibit significantly higher Galectin-1 levels. This elevated expression is likely linked to the pro-tumorigenic functions of Galectin-1 in cancer cells, where it promotes immune evasion by inducing T-cell apoptosis, enhances angiogenesis, and facilitates tumor cell migration and invasion. [139]

The consistently high levels of Galectin-1 across all thyroid cancer cell lines confirms that this protein plays a fundamental role in thyroid cancer progression, regardless of the specific subtype or differentiation status. Unlike Galectin-3, which shows more variability between cell lines, Galectin-1 is uniformly upregulated in thyroid cancers, indicating that Galectin-1 is a core basal component of the tumorigenic phenotype in these cancers and might not be affected by the anti-proliferative activity of Grapefruit IntegroPectin or by the S1PR inhibition.

Unfortunately, the biological replicates were not enough for repeating the experiments thus these sets of data must be considered preliminary, but overall, this data highlights the potential of Grapefruit IntegroPectin as a dietary therapeutic agent in thyroid cancer, capable of modulating the expression of key molecules such

as galectin-3. Furthermore, the combination of Grapefruit IntegroPectin with an S1PR inhibitor may offer enhanced efficacy in reducing Galectin-3 expression, particularly in certain thyroid cancer cell lines.

These findings are also a starting point for further exploration of S1PR inhibitors as potential therapeutic strategies in the management of thyroid cancer, although in literature IU1 was more effective in the ML-1 cells compared with primary thyroid cells. [144] IU1 is an inhibitor of Ubiquitin Specific Peptidase 14 (USP14), a deubiquitinating enzyme associated with the proteasome. [145] IU1 inhibits S1P-induced migration of ML-1 cells, and its effects are mediated by enhanced proteasome and autophagy activity, which suppress both proliferation and migration stimulated by S1P. The inhibition of USP14 by IU1 counteracts S1P-driven cellular behaviours in ML-1 thyroid cancer cells by altering downstream proteostasis and autophagic pathways. [146] Likewise, the expression of USP14, being a target for IU1, is reduced in ML-1 cells compared with control thyroid cells. [144]

In conclusion, the differential responses observed between primary thyroid cells and cancer cell lines pinpoint the importance of considering cellular context in developing targeted therapies for thyroid malignancies. This preliminary study provides new data about anti-proliferative effects of Grapefruit IntegroPectin on cancer cell line and these new insights go through the modulation of signaling pathways in thyroid cancer cells by Grapefruit IntegroPectin and S1PR inhibition.

3.3

RESULTS PART III

Citrus IntegroPectin: a computational insight

Contributions: 3.3.1 – 3.3.2 V. Butera, R. Ciriminna; 3.3.3 V. Butera, R. Ciriminna, C. Valenza.

This last part of the study presents a different, yet complementary, take on Citrus IntegroPectin (and, by extension, Grapefruit IntegroPectin). Compared to what was previously discussed we took a computational approach for the analysis of citrus IntegroPectin, using **density functional theory** (DFT) calculations.

3.3.1 Formation and energetics of conjugates

To determine the potential formation of the Pec-RGI-Fla conjugates, the corresponding Gibbs free energies of formation (ΔG_{form}) have been calculated from Eq.1:

$$\Delta G_{\text{form}} = G(\text{Pec-RGI-Fla}) + G(\text{H}_2\text{O}) - G(\text{Pec-RGI}) - G(\text{Fla}) \quad (1)$$

Where $G(\text{Pec-RGI})$, $G(\text{Fla})$ and $G(\text{H}_2\text{O})$ are the calculated Gibbs energies of the isolated pectin, flavonoids and water, respectively, while $G(\text{Pec-RGI-Fla})$ is the computed Gibbs energy of the Pec-Fla conjugates. Accordingly, negative values of ΔG_{form} , indicate that the formation of the Pec-Fla conjugates is favoured.

The formation of pectin-flavonoid (Pec-RGI-Fla) conjugates was found to be endergonic for all three studied compounds, with ΔG_{form} ranging from 3.3 to 4.9 kcal/mol. These values, while positive, are not very high, suggesting that conjugate formation is feasible under certain conditions. The energy required for conjugate formation is likely provided by the intense localized conditions created during hydrodynamic cavitation, which can generate "hotspots" of up to 5000 K and 1000 bar pressure. This process, utilizing a Venturi tube at low cavitation numbers, produces cavitating flow structures primarily consisting of re-entrant jets flowing upstream in cloud cavities.

Compound (Conjugate)	ΔG_{form} (kcal/mol)
Pec-Nar	4.6
Pec-Hes	3.3
Pec-Eri	4.9

Table 3. Calculated ΔG_{form} , for all the Pec-Fla conjugates

3.3.2 Structural insights into conjugates

Optimized structures of the pectin-flavonoid conjugates reveal distinct hydrogen bonding patterns. (**Figure 19**) Naringenin and eriocitrin conjugates form hydrogen bonds between the O1 oxygen of the RGI monomer and the H1 of the flavonoid units, with distances of 2.24 Å (**Figure 19A** and **Figure 19B**). In contrast, the hesperidin conjugate exhibits a hydrogen bond of 2.22 Å between the O1 of RGI and H2 of hesperidin (**Figure 19C**).

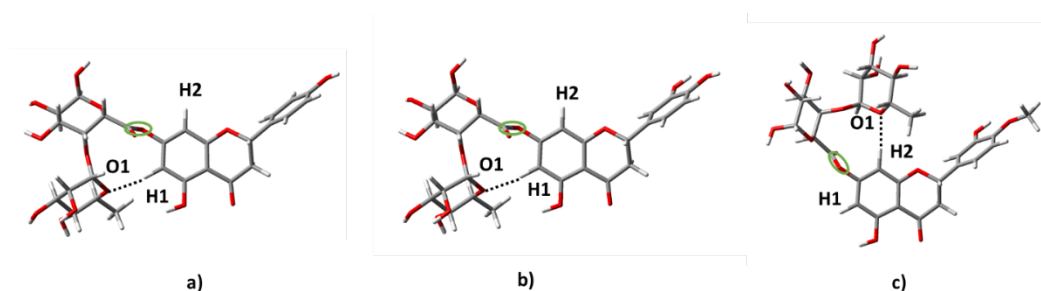


Figure 19 Optimized structures of the pectin conjugates with naringenin (a), eriocitrin (b) and hesperidin (c).

The newly formed ester C—O bond consistently measures 1.35 Å across all three structures. Dihedral angles (α and β) show remarkable similarity among the conjugates, indicating a conserved spatial arrangement. These structural insights provide a molecular basis for understanding the interactions between pectin and flavonoids in IntegroPectin. (**Figure 20**)

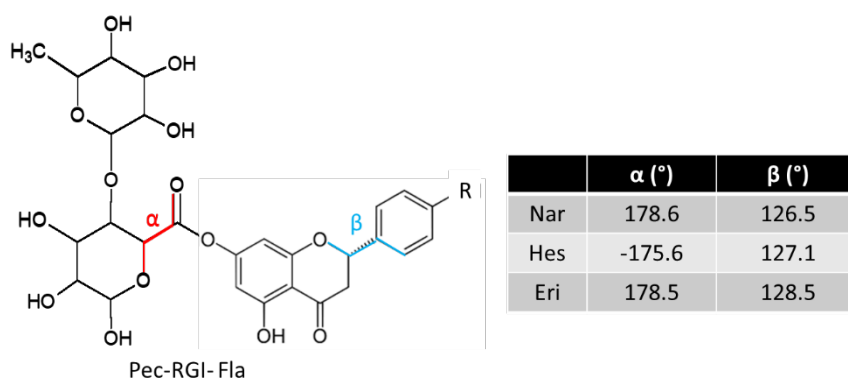


Figure 20 Dihedral angle values computed for the pectin conjugates with naringenin (a), eriocitrin (b) and hesperidin (c).

3.3.3 Experimental validation

The computational findings suggest a molecular association between flavonoids in citrus IntegroPectin and GalA units, likely resulting from esterification during the hydrodynamic cavitation-based extraction of the pectic polymer. Further experimental evidence supporting this hypothesis was obtained through flavonoid analysis of lemon and grapefruit IntegroPectin. 600 mg of pectin powder was dispersed in 50 mL of EtOH/H₂O mixture (4:1 v/v), followed by ultrasonic extraction (150 W, 35 kHz) for 15 minutes. While a significant quantity of flavonoids was successfully extracted, the residual pectin powder retained a less intense yellow coloration, indicating incomplete extraction in the aqueous ethanol medium.

A quantitative study on flavonoid release was performed using lemon and grapefruit IntegroPectin cross-linked films in a buffered solution (pH 7.3, Tryptic Soy Broth medium). The mechanical properties of these films were enhanced by incorporating 1 wt% CytoCell nanocellulose. The films showed sustained, controlled, and prolonged release of flavonoids over 72 hours, with lemon IntegroPectin exhibiting a more extended-release profile. This observation aligns with the higher computed Gibbs energy of the Pec-Eri conjugate determined before.

When immersed in non-buffered water, the cross-linked IntegroPectin films rapidly lowered the pH due to the inherent acidity of the IntegroPectin powder, attributed to its low degree of esterification and molecular weight. Despite the rapid coloration of the aqueous phase, the grapefruit and lemon IntegroPectin films maintained their yellow colour for extended periods: several days for grapefruit IntegroPectin and over a week for lemon IntegroPectin, confirming the strong flavonoid retention.

3.3.4 Discussion: the computational calculations

This density functional theory (DFT) study provides preliminary insights into the molecular interactions underlying the formation of citrus IntegroPectin-flavonoid conjugates. The combination of computational modeling with ongoing experimental inquiries provides some understanding of this complex natural product and its potential applications and evidence that flavonoid actually binds to citrus pectin

DFT analysis was conducted on citrus IntegroPectin modeled (Pec-RGI) as a dimer, comprising a galacturonic unit linked to a rhamnose molecule, representing the RGI hairy regions. This approach provided interesting results for the understanding of the molecular structure and behavior of IntegroPectin. The formation of IntegroPectin-flavonoid (Pec-RGI-Fla) conjugates is endergonic for all three fruit varieties studied. The Gibbs free energy values for these conjugation reactions were found to be relatively low, ranging from 3.3 to 4.9 kcal/mol. This means that while the conjugate formation is not spontaneous under standard conditions, it requires only a modest energy input to proceed. The energy necessary for the formation of these conjugates is hypothesized to be supplied by the large amount of energy released during the implosion of cavitation bubbles in the hydrodynamic cavitation extraction process. This process, since it is characterized by extreme local conditions of temperature and pressure, likely provides the activation energy required to overcome the small endergonic barrier and facilitate the conjugate formation.

Previous solid-state ^{13}C NMR spectroscopic analyses of cross-linked IntegroPectin films were unable to detect ^{13}C resonance peaks attributable to flavonoids. This limitation was attributed to the relatively low concentration of flavonoids compared to the main components of the films. [147] The challenge of detecting flavonoid-pectin interactions in complex systems highlights the importance of complementary computational and experimental approaches.

Moreover, the endergonic nature of the flavonoid-pectin conjugate formation suggests that these interactions may be reversible under certain conditions, potentially explaining the controlled release behaviour observed in previous studies.

Surely, optimizing extraction conditions will control conjugate formation and stability, retaining IntegroPectin bioactive properties and developing more sensitive methods to analyze flavonoid-pectin interactions. More data will expand IntegroPectin structure and how to translate it to its practical applications, exploring the biological and pharmacological effects of these conjugates, especially for potential applications in regenerative medicine and drug delivery.

Recent experimental findings by Zheng and coworkers support our computational results, demonstrating that hesperidin can molecularly bind to citrus pectin, forming

a naturally occurring flavonoid-pectin conjugate in mandarin peel that exhibits enhanced UV absorption and amphiphilicity. [148]

Works published following this research [Citrus IntegroPectin: a computational insight](#)

4. CONCLUSION

The research project, aimed at preserving the ecosystem, reducing the impacts of climate change, and promoting sustainable development, aimed at studying the efficiency of alternative methods for extracting biomolecules from food industry waste, using “green” chemical-physical methods, such as hydrodynamic cavitation, microwaves, ultrasound, or enzymatic complexes. These methodologies, in addition to ensuring the sustainability of the chemical process and a more rational use of natural resources and industrial waste, protecting the functional groups and the structure of the extracted biomolecules, aim to preserve their bioactive healthy properties. In particular, the antioxidant and the anti-proliferative properties of a new citrus pectin, namely Grapefruit IntegroPectin, were object of study in this thesis.

Grapefruit IntegroPectin demonstrated significant neuroprotective properties in **human microglial HMC3 cells**. It counteracted TBH-induced apoptosis, likely through its **potent antioxidant activity** by ROS scavenging, and modulated key signaling pathways including PI3K/Akt and MAPK/ERK. Notably, IntegroPectin also inhibited the PI3K-NF- κ B-iNOS cascade, with a potential role in modulating neuroinflammatory responses.

The IntegroPectin unique structural properties, including low crystallinity, low degree of esterification and high content of adsorbed naringin, enhance its interaction with cell membranes and delivery of bioactive compounds. These findings position grapefruit IntegroPectin as a promising candidate for further investigation **in oxidative stress-neuroinflammatory-based diseases**, including neurodegenerative disorders.

Coupled with the anti-apoptotic and anti-inflammatory properties of Grapefruit IntegroPectin, this demonstrated in the experiments performed in HMC3, with the antiproliferative and antioxidant effects observed in neuroblastoma SH-SY5Y cells, highlights the versatility of IntegroPectin in addressing various types of cancer cell lines models and oxidative stress-related disorders.

The anti-proliferative activity of IntegroPectin was also observed in thyroid cancer cell lines such as ML-1 and FTC-133. The treatments showed that it may modulate key signaling pathways implicated in cancer progression. The effects observed with IntegroPectin and S1P modulator highlight a promising approach for enhancing

therapeutic efficacy, particularly in modulating the PI3K/Akt pathway, which is frequently dysregulated in thyroid carcinomas. The differential responses between cell lines, with IntegroPectin eliciting a significant modulation in pAkt levels in a cell-specific manner, underscore the importance of considering cellular context in developing targeted therapies. This variability reflects intrinsic molecular differences between cell lines, such as differences in genetic mutations, signaling pathway activation or the tumor microenvironment. Differences in the composition of the extracellular matrix or cell surface receptors could also influence how each cell line responds to IntegroPectin, given that this pectin only exerts its activity outside the cell.

IntegroPectin efficacy aligns with its high galacturonic acid content, which competitively binds Galectin-3 CRD, disrupting its interaction with oncogenic ligands like β -catenin and MMPs. The cell-specific modulation of galectin-3 expression by IntegroPectin suggest that its regulation is context-dependent. The computational models further support that IntegroPectin unique phytocomplex stabilizes flavonoid-Galectin-3 interactions, enhancing inhibitory effects.

Galectin-1, on the other hand, was found to be uniformly overexpressed across all thyroid cancer cell lines examined, indicating its potential role as a core component of the tumorigenic phenotype. The inability of IntegroPectin or S1P modulation to affect galectin-1 levels suggests that IntegroPectin may not competitively bind Galectin-1 CRD. Additionally, Galectin-1 over-expression is maintained by redundant pathways, rendering it resistant to these treatments. The high baseline levels in all thyroid cancer cell line examined indicate and confirms Galectin-1 as a strong biomarker for thyroid malignancies.

At last, we wanted to understand how flavonoids, the major bioactive components of IntegroPectin, are structurally conjugated into its structure. The computational model used set the IntegroPectin as a dimer, where a galacturonic unit is linked to a rhamnose molecule, representing the RG-I hairy regions, thereby forming the "Pec-RGI" structure. We utilized DFT calculations to investigate whether the formation of conjugates between pectin and flavonoids is energetically favourable. Specifically, we selected the most abundant flavonoids associated with each citrus fruit: naringenin for grapefruit, hesperidin for orange, and eriocitrin for lemon. Although the model was simplified, the computational outcomes indicate that the

formation of IntegroPectin-flavonoid bioconjugates is endergonic for all three conjugates. Notably, the energy released during the implosion of cavitation bubbles in the hydro cavitation extraction process can provide the necessary energy for conjugate formation.

More extended models and advanced computational protocols could better mimic real systems but are constrained by high computational demands. The balance between method reliability and computational cost is crucial in such studies. However, focusing on the esterification reaction between pectin and flavonoids, the inclusion of more extended models may only marginally influence the reaction. The results from this analysis shed light on how polyphenols are conjugated in the structural characteristics of this phytocomplex, which holds significant potential for nutraceutical and therapeutic applications.

In summary, this combinatorial approach in this thesis study contributes to the growing group of evidence supporting the therapeutic potential of Citrus IntegroPectin in anti-oxidant and anti-cancer treatment. Its ability to modulate key signaling pathways and biomarkers, combined with its antioxidant, anti-inflammatory and anti-proliferative properties, positions IntegroPectin as a promising bioactive molecule for further investigation in the development of novel therapeutic and nutraceutical strategies.

However, the limitations of this study, including the use of simplified models and the need for further biological replicates, necessitate additional research to fully elucidate the molecular mechanisms underlying the effects of Citrus IntegroPectin. Future investigations should focus on exploring the interactions with other bioactive molecules and the potential applications of IntegroPectin in pharmaceuticals and nutraceuticals, taking in consideration green extraction methods for sustainability.

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