

Article

Modulation of Glucose Metabolism Along the Intestine–Pancreas–Liver In Vitro Axis by Mulberry, Bilberry, and Black Currant Extracts: A Mechanistic Approach

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Abstract

Background: The regulation of glucose metabolism is contingent on a multifaceted interaction between intestinal absorption, pancreatic endocrine function, and the hepatic response to insulin. Axis disruption contributes to insulin resistance and type 2 diabetes. **Methods:** This study tested mulberry, bilberry, and black currant extracts individually and in combination in an integrated in vitro gut, pancreas, and liver model. The extracts were phytochemically characterised and tested at optimal concentrations selected through dose–response studies. **Results:** The combined treatment preserved and enhanced the intestinal barrier, as evidenced by increased tight-junction levels and reduced oxidative stress. In the pancreas, the combination significantly improved cell viability, enhanced insulin and C-peptide secretion, and increased glucokinase expression, indicating improved glucose-sensing function. In the liver, the combined treatment synergistically activated insulin signalling, increasing the expression of IRS1, GLUT2, AMPK, AKT, and PGC-1 α . This resulted in increased glucose absorption, glycogen synthesis, and a marked reduction in extracellular glucose levels under hyperglycaemic conditions. The results show that combining mulberry, bilberry, and blackcurrant produces additive benefits for intestinal barrier integrity and synergistically modulates key elements of hepatic insulin signalling. **Conclusions:** These findings support a mechanistic rationale for exploring multi-targeted nutraceutical formulations as complementary approaches to modulating processes involved in glycaemic regulation.

Keywords: 1-deoxynojirimycin; insulin signalling pathway; glycaemic control; C-peptide secretion; glucokinase expression; IRS1 / AKT; AMPK



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1. Introduction

Glucose serves as the main energy source for humans, with its metabolism regulated by coordinated processes including intestinal absorption, pancreatic insulin secretion, and hepatic regulation of glucose production and storage [1]. Glycaemic balance relies on the pancreas–liver axis, where insulin is essential for promoting glucose utilisation, supporting glycogen synthesis, and inhibiting hepatic gluconeogenesis [2]. Changes in glucose metabolism drive insulin resistance and type 2 diabetes mellitus (T2DM) [3], as defective pancreatic β -cells reduce insulin secretion, and hepatic insulin resistance increases glucose output and reduces

uptake, leading to hyperglycaemia [4]. These disruptions alter fasting and postprandial glucose, and are aggravated by oxidative stress and chronic inflammation, impairing pancreatic and hepatic function [5,6]. In addition, insulin sensitivity in extrahepatic tissues, particularly skeletal muscle and adipose tissue, plays a pivotal role in systemic glycaemic control and in the progression of T2DM; however, the present study focuses on pancreatic and hepatic mechanisms as central and early regulators of glucose homeostasis [7].

At the pancreatic level, β cells rely on glucose-sensing mechanisms to detect increases in blood glucose and appropriately regulate insulin secretion. Insulin production and equimolar C-peptide release serve as key indicators of β -cell function [8–11]. The disruption of these processes leads to insufficient insulin secretion and is closely linked to type 2 diabetes progression and poor glycaemic control [12].

At the hepatic level, insulin regulates glucose metabolism primarily through the insulin receptor and its intracellular substrate IRS1, stimulating the PI3K/AKT pathway to enhance glucose uptake, stimulate glycogen synthesis, and suppress gluconeogenesis [13]. Impaired AKT activation reduces hepatic glucose uptake and contributes to hyperglycaemia, a hallmark of hepatic insulin resistance [14]. Additionally, AMP-activated protein kinase (AMPK) senses cellular energy status, enhancing mitochondrial function, glucose uptake, and insulin sensitivity, with PGC1- α as a key downstream effector. Dysregulation of the AMPK–PGC1- α axis is associated with impaired glucose metabolism in insulin-resistant conditions [15].

Beyond pancreatic and hepatic dysfunction, the intestine contributes substantially to systemic glycaemic load by absorbing dietary glucose. Excessive and rapid systemic glucose availability has been demonstrated to overload the pancreas–liver axis, promoting compensatory hyperinsulinemia and progressive metabolic dysfunction [16,17]. Consequently, the modulation of glucose metabolism necessitates an integrated approach that encompasses both pancreatic function and hepatic response to insulin.

Recent research highlights that plant-derived bioactive compounds can modulate glucose metabolism through multiple complementary mechanisms. Polyphenols, flavonoids, and anthocyanins have been shown to reduce glucose absorption, improve pancreatic β -cell function, enhance hepatic insulin signalling, and provide antioxidant and cytoprotective effects [18,19].

In this context, *Morus alba* L. (mulberry) is studied for its flavonoids and 1-deoxynojirimycin (DNJ), which reduce glucose absorption and support pancreatic function by enhancing insulin secretion and β -cell viability [20,21]. *Vaccinium myrtillus* L. (bilberry), rich in anthocyanins, improves insulin sensitivity, protects pancreatic cells from oxidative stress, and promotes hepatic glucose utilisation [22,23]. Similarly, *Ribes nigrum* L. (black currant) contains anthocyanins and polyphenols that modulate insulin signalling, enhance hepatic glucose uptake, reduce circulating glucose, and support pancreatic function under metabolic stress [24,25]. Despite differences in the phytochemical content of these extracts, there is growing evidence that they influence common regulatory pathways involved in glucose homeostasis.

Beyond their overall impact on glycaemic control, increasing evidence indicates that mulberry, bilberry, and black currant extracts target specific regulatory points in glucose metabolism, directly affecting pancreatic and hepatic function [26–28].

At the pancreatic level, *Morus alba*, *Vaccinium myrtillus*, and *Ribes nigrum* extracts improve pancreatic β -cell glucose sensing, partly by modulating glucokinase activity, a key enzyme for glycolysis and insulin secretion [24,29–31]. These effects increase insulin and C-peptide release, while the antioxidant properties of anthocyanins and flavonoids protect β cells from hyperglycaemia-induced oxidative stress, preserving their function and viability [32].

Many studies show that polyphenol and anthocyanin-rich extracts can modulate hepatic insulin signalling via IRS1 and the PI3K/AKT pathways, thereby supporting glucose uptake and glycogen synthesis [15]. These extracts also reduce hepatic glucose

production and improve overall hepatic glucose metabolism, effects that are particularly important in hepatic insulin resistance, a key contributor to persistent hyperglycaemia in type 2 diabetes [33].

Despite the availability of data on individual extracts, evidence on their use in combination remains limited, particularly for the integrated assessment of the molecular mechanisms involved in the pancreas–liver axis. However, the combinatorial approach demonstrates considerable promise, as it facilitates the integration of complementary effects on insulin secretion, activation of the hepatic insulin signalling pathway, and regulation of glucose metabolism.

In consideration of the foregoing points, this study focuses on analysing the effects of mulberry, bilberry, and black currant extracts, whether administered individually or in combination, on glucose metabolism in an *in vitro* model, with particular attention to pancreatic and liver function. This approach provides new mechanistic evidence supporting the use of multifactorial nutraceutical formulations to improve glycaemic control and metabolic health.

2. Materials and Methods

2.1. Natural Extract Characterisation

Based on current research, the phytochemical analyses specifically targeted key bioactive constituents identified as the main mediators of each extract's anti-hyperglycaemic effects, rather than conducting an exhaustive or comparative assessment of all phytochemical classes.

2.1.1. Determination of 1-Deoxynojirimycin (DNJ) by HPLC

The concentration of 1-deoxynojirimycin (DNJ) was quantified with high-performance liquid chromatography (HPLC) using a validated analytical method commonly used for plant-derived samples [34]. Dried and powdered Mulberry extract samples were extracted with ultrapure water or a mildly acidic buffer (pH 4–5) under agitation at room temperature. Following centrifugation, the extracts were filtered through 0.22 µm membranes and subjected to pre-column derivatisation with 9-fluorenylmethyl chloroformate (FMOC-Cl) to enhance chromatographic detection.

Separation was carried out using a C18 reversed-phase column (250 × 4.6 mm, 5 µm) under an acetonitrile/water gradient containing 0.1% trifluoroacetic acid, with a flow rate of 1 mL/min at 30 °C. Quantification of DNJ was performed through external calibration with derivatised standards, following detection at 254 nm. The results were expressed as micrograms of DNJ per gram of dried plant material (%).

2.1.2. Total Polysaccharide Phenol—Sulfuric Acid Assay

Total polysaccharides were assessed employing the phenol–sulfuric acid colourimetric procedure [35]. Mulberry extract was dissolved in filtered water and diluted to fit the assay's linear range. Each sample (1.0 mL) was mixed with 1.0 mL of 5% phenol and 5.0 mL of concentrated sulfuric acid, and the mixture was added to the tube wall. After 30 min at room temperature for colour development, absorbance was measured at 490 nm against a blank using a UV–Vis spectrophotometer. Using a D-glucose reference curve, polysaccharide levels are presented as mean (%) ± SD from five independent replicates.

2.1.3. Determination of Anthocyanins by HPLC-DAD

Anthocyanins in bilberry and blackcurrant extracts were measured using HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA), following the method described in [36]. Dried extract was dissolved in 50:48:2 methanol/water/formic acid, sonicated for 10 min, centrifuged, and filtered through a 0.45 µm PTFE membrane. Compounds were separated

on a C18 column (30 °C, 1 mL/min) and identified at 520 nm by retention time and UV-Vis spectra against standards. Cyanidin-3-O-glucoside served as a reference for quantification at 1 mg/g in the dry extract, and the measurement was performed in triplicate.

2.1.4. Determination of Anthocyanidins by HPLC-DAD

Anthocyanidins in bilberry extract were measured as described in the literature [37]. After acid hydrolysis with 2 M HCl in methanol (1:1) at 90 °C for 60 min, samples were cooled, diluted with mobile phase, and filtered. HPLC analysis, using the same conditions as for anthocyanins, detected compounds at 520 nm, identified by standards. Results were quantified as mg cyanidin equivalents per g dry extract (%), and experiments were performed in triplicate.

2.1.5. Folin–Ciocalteu Assay

Total polyphenol content (TPC) was determined by the Folin–Ciocalteu assay with minor protocol adjustments [38]. Blackcurrant extract reacted with the reagent for 3–5 min, then developed colour in the dark for 30–60 min after the addition of sodium carbonate. Absorbance at 760 nm was measured by UV–Vis spectrophotometry using a gallic acid calibration; results are mean $\pm$ SD (%) from five replicate assays.

2.2. Agents' Preparation

Mulberry, bilberry, and black currant extracts (from Nutra Futura srl, Legnano, Italy) were evaluated individually and in combination for effects on intestinal barrier and glycaemic parameters. Extracts were tested at various concentrations: mulberry (0.5–2 mg/mL) [39], bilberry (100–500 μ g/mL) [40], and black currant (50–200 μ g/mL) [41].

All compounds were prepared in phenol red-free DMEM (Merck, Milano, Italy) supplemented with 0.5% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin. Each substance was initially dissolved at 10 $\times$ the final concentration and then diluted to the desired test concentrations. Optimal, non-toxic concentrations (mulberry 2 mg/mL, bilberry 100 μ g/mL, black currant 50 μ g/mL) were identified and used for subsequent assays to ensure consistency. This method allowed for the accurate assessment of the individual and combined effects of each extract.

2.3. Cell Culture

Human colorectal carcinoma cells, specifically the Caco-2 cell line, were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA) to ensure the use of a well-characterised and reliable model for intestinal epithelial studies. These cells were maintained under standard cell culture conditions in Advanced Dulbecco's Modified Eagle Medium (Adv-DMEM; GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), which was supplemented with 2 mM L-glutamine to provide an energy substrate and to support key cellular metabolic processes, 1% penicillin-streptomycin to prevent bacterial contamination, and 10% FBS to provide essential growth factors and nutrients (purchased from Merck Life Science, Rome, Italy). The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂, creating optimal conditions for cell growth and proliferation [40]. For experimental procedures, a precise number of cells was prepared to ensure consistency and reproducibility. For viability assays, 1 $\times$ 10⁴ cells were plated per well in 96-well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis of mitochondrial activity. In parallel, 2 $\times$ 10⁴ cells were seeded on 6.5 mm Transwell® inserts with 0.4 μ m membranes (Corning® Costar®, Merck Life Science, Rome, Italy) in 24-well plates to assess absorption and barrier integrity.

Media on both sides of the Transwell® inserts were refreshed every other day for 21 days (apical pH 6.5, basolateral pH 7.4) [42].

EndoC- β H5 cells were generated from human foetal pancreatic tissue following previously reported procedures, with minor modifications [43]. Cells were maintained on plastic culture dishes coated with β Coat[®] and grown in Opti- β 1 medium. Subculturing was performed every seven days using trypsin (0.05%, ThermoFisher Scientific, Waltham, MA, USA). To select successfully transduced cells, cultures were expanded for three weeks in the presence of puromycin (1 μ g/mL). Subsequently, to promote cellular maturation, EndoC- β H5 cells were maintained in culture for an additional three weeks and treated with tamoxifen (5 μ M) and ganciclovir (0.5 μ M) to induce excision of the immortalising genes and enable selection of excised cells [44]. Cultures were maintained at 37 °C and 5% CO₂. The cells were seeded for the ensuing experiments: The experiment involved the cultivation of 1×10^4 cells in 96-well plates, with the objective of conducting an MTT test to assess cell viability and a Cytochrome C assay to measure reactive oxygen species (ROS) production and also SOD, CAT and GSH-Px levels (Figure A1 in Appendix B). Additionally, 1.5×10^5 cells were seeded on Transwell[®] inserts to evaluate insulin production, C-peptide levels, and Glucokinase levels following intestinal absorption of the agents under investigation.

Human HepG2 cells, acquired from ATCC (Manassas, NA, USA), were cultured under standard laboratory conditions to ensure optimal growth and viability. The cells were maintained in Adv-DMEM (GIBCO, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, providing essential growth factors and nutrients (purchased from Merck Life Science, Rome, Italy). Furthermore, 2 mM L-glutamine was incorporated as an energy substrate to facilitate essential cellular metabolic processes, including protein and nucleotide synthesis. In addition, antibiotics were included to prevent bacterial contamination. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂, creating a physiologically relevant environment for the cells [45]. Once the cells reached approximately 80–90% confluence, they were carefully seeded at various densities depending on the experimental requirements: 1×10^4 cells in 96-well plates to study cell viability by MTT test and ROS production; 2.5×10^5 cells were seeded in the basolateral compartment to measure the IRS1 levels and the glucose reduction using an ELISA assay Kit (NovusBio, Centennial, CO, USA).

2.4. Experimental Protocol

The study included intestinal, pancreatic, and hepatic phases (see Figure 1) to assess the impact of mulberry, bilberry, and black currant extracts on glycaemic control. Caco-2 cells were first treated with different concentrations of each extract—mulberry (0.5–2 mg/mL), bilberry (100–500 μ g/mL), and black currant (50–200 μ g/mL)—to determine optimal dosing.

Optimal extract concentrations were tested in a Transwell[®] barrier model to evaluate their effects on monolayer integrity, both individually and in combination. Cell viability was assessed through an MTT assay, ROS generation through cytochrome C reduction, and integrity through TEER and tight junction measurements. Absorption was monitored with a fluorescent probe over 1–6 h to track intestinal function transit.

At the intestinal level, the optimal extract concentrations were examined in a Transwell[®] barrier model. This aimed to assess their ability to preserve the monolayer's integrity when given alone or together. Cell viability was analysed using the MTT test. ROS production was measured by reducing cytochrome C. Cell integrity was evaluated through TEER and TJ levels. Lastly, the absorption of test substances and their combination was assessed with a fluorescent probe over 1–6 h, covering intestinal filling and emptying.

In the final phase of the experiment, a co-culture of pancreatic and liver cells was set up using the Transwell[®] system. As a preliminary step, the first set of pancreatic cells (EndoC- β H5 cells) was cultivated in the apical compartment of the Transwell[®]. This was done to assess

various parameters, including cell viability, ROS production, insulin production, the presence of C-peptide, and glucokinase. Moreover, SOD, CAT and GSH-Px levels were reported in Appendix B (Figure A1). Furthermore, in the basolateral compartment, liver cells were seeded and exposed to 30 mM glucose to mimic hyperglycaemic conditions. At the conclusion of the incubation period, analyses were conducted on the liver cells to investigate cell viability, ROS production, IRS1 levels (using an ELISA kit), glucose reduction, and glucose uptake (using an assay kit). These evaluations were conducted using ELISA kits following treatment with all the study agents for approximately six hours. Additionally, the expression of GLUT2, AMPK, and PGC-1 α was examined using Western blot analysis, and pAKT levels were measured using the AKT ELISA kit (Thermo Fisher, Waltham, MA, USA).

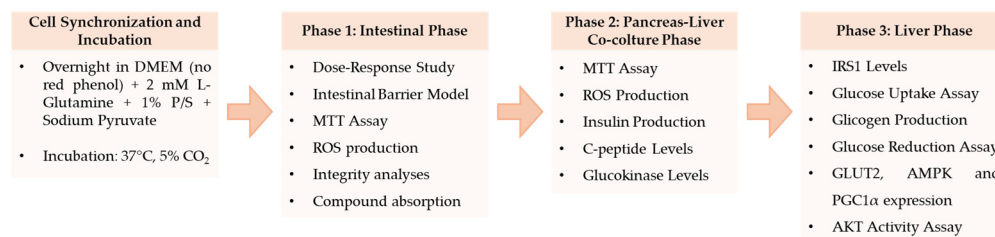


Figure 1. The experimental protocol scheme is divided into phases.

Before stimulation, cells were synchronised overnight with phenol red-free DMEM (Merck Life Science, Milan, Italy) and FBS (Merck Life Science, Rome, Italy), with the supplements of 1% penicillin/streptomycin (Merck Life Science, Rome, Italy), 2 mM L-glutamine (Merck Life Science, Rome, Italy), and 1 mM sodium pyruvate. The cells were then cultivated in a 37 °C, 5% CO₂, 95% humidity incubator.

All ELISA measurements used commercial kits per manufacturer instructions; assay details, sensitivities, and data analyses are provided in the ELISA assays section.

2.5. Intestinal In Vitro Model

A complete intestinal barrier model was established with Transwell® inserts and the Caco-2 cell line (EMA/FDA-approved) to simulate oral absorption. Caco-2 cells were cultured on inserts for 21–28 days to achieve epithelial polarisation and differentiation with tight junctions and microvilli. TEER was checked every other day using an EVOM3 meter until $\geq 400 \Omega \cdot \text{cm}^2$ was reached before stimulation [46–51]. Prior to testing, apical and basolateral media were set to pH 6.5 and 7.4, respectively, to mimic the intestinal lumen and blood [40]. TEER was also measured before the experiments. TEER was measured after 15 min at 37 °C and 5% CO₂ to confirm barrier stability.

Fluorescein (0.04%, Merck Life Science, Rome, Italy) [52] was added at threshold, and basolateral fluorescence was measured at 490/514 nm with a spectrophotometer (Infinite 200 Pro-MPlex, Tecan, Männedorf, Switzerland).

Results indicate cell entry percentage. The permeation rate [nmol min (mg protein)], J, was calculated using a research-validated formula [52]:

$$J = (J_{\max} \times [C]) / (K_t + [C])$$

where

C = starting concentration of fluorescein.

J_{max} = maximum permeation rate.

K_t represents the Michaelis–Menten constant.

Results are shown as mean $\pm$ SD (%) relative to the control sample (untreated cells).

Cell-free negative controls were examined to rule out Transwell® membranes.

2.6. Pancreas–Liver Co-Culture

An in vitro pancreas–liver axis was modelled using the Transwell® system with pancreatic β -cell and HepG2 organoids. About 1.5×10^5 pancreatic cells were plated in the apical compartment and differentiated for 4 days at 37 °C, 5% CO₂ [53]. Then, 2.5×10^5 hepatocytes were seeded basolaterally in parallel. After 4 days, the pancreatic cell insert was placed over the hepatocytes and exposed to 30 mM glucose to induce hyperglycaemia [54]. Co-cultures were maintained in high-glucose DMEM without phenol red or FBS.

2.7. Cell Viability

Cell viability following stimulation was evaluated using the MTT assay [40]. Cells were incubated for 2 h at 37 °C in DMEM lacking phenol red and FBS, with 1% MTT. Absorbance was recorded at 570 and 690 nm. Results are expressed as mean $\pm$ SD (%) relative to untreated controls, based on five independent experiments performed in triplicate.

2.8. Reactive Oxygen Species (ROS) Detection

ROS generation after stimulation [40] was evaluated by monitoring superoxide anion production through absorbance readings at 550 nm. Data represent mean $\pm$ SD percentages compared to untreated controls, calculated from five separate experiments performed in triplicate.

2.9. SOD Levels Analysis

SOD levels in pancreatic cell lysates were determined according to the manufacturer's instructions (Cayman Superoxide Dismutase Assay Kit, Tallinn, Estonia) [40], using a standard curve (0.05–0.005 U/mL) for quantification. Absorbance was measured at 440–460 nm. Results are expressed as percentages relative to untreated controls, based on five independent experiments, each performed in triplicate.

2.10. Glutathione Peroxidase Examination

GPX levels in pancreatic lysates were measured using Cayman's assay kit (Tallinn, Estonia) according to the manufacturer's protocol [40]. Absorbance was read at 340 nm with a spectrometer. Results are presented as % of control from five duplicate experiments.

2.11. Catalase (CAT) Levels Analysis

Human CAT in pancreatic lysates was measured using ELISA (Cusabio, Houston, TX, USA) following the protocol. After lysis and centrifugation, absorbance at 450 nm was recorded. Results (five triplicate assays) are presented as pg/mL (derived from 5.6 to 1000 pg/mL standard curve) and expressed as mean $\pm$ SD (%) compared to control.

2.12. Claudin-1 Levels Determination

Claudin-1 in Caco-2 lysates was quantified by ELISA (Cusabio, USA) according to the manufacturer's instructions [55]. After lysis in cold PBS and centrifugation (1500 $\times$ g, 10 min, 4 °C), absorbance at 450 nm was recorded. Results from five triplicate experiments are reported as pg/mL (0–1000 pg/mL standard curve) and mean $\pm$ SD (%) versus control.

2.13. Occludin Levels Determination

Occludin levels in Caco-2 lysates were measured using ELISA (MyBioSource, San Diego, CA, USA) according to the manufacturer's protocol [55]. After PBS lysis and ELISA processing, absorbance at 450 nm was recorded. Data from five triplicate experiments are presented as pg/mL (0–1500 pg/mL standard curve) and mean $\pm$ SD (%) versus control.

2.14. Zonula Occluden-1 (ZO-1) Levels Determination

ZO-1 levels in Caco-2 lysates were quantified using ELISA (MyBiosource, San Diego, CA, USA) following the manufacturer's protocol [55]. After freeze–thaw lysis, PBS wash, and centrifugation ($5000\times g$, 5 min, 4 °C), absorbance at 450 nm was measured. Results from five triplicate experiments are reported as pg/mL (0–1000 pg/mL standard curve) and are shown as mean $\pm$ SD (%) relative to control.

2.15. Insulin ELISA Kit

Insulin levels were measured using the Human HS-INS Accquant[®] ELISA kit (FineTest, Wuhan, China) according to the manufacturer's protocol [56]. Absorbance at 450 nm was read with a microplate reader. Values were derived from a standard curve ranging from 7.813 to 500 pg/mL and are presented as mean $\pm$ SD (%) compared to untreated cells from five triplicate experiments.

2.16. C-Peptide ELISA Kit

C-peptide was quantified by ELISA (FineTest, China) following the manufacturer's protocol [56]. Absorbance at 450 nm was measured, and values were calculated from a 31.2–2000 pg/mL standard curve. Data are reported as mean $\pm$ SD (%) versus untreated cells from five triplicate assays.

2.17. Glucokinase ELISA Kit

The Human GCK (Glucokinase) ELISA Kit (FineTest, Wuhan, China) measured glucose levels per the manufacturer's instructions. A Tecan Infinite 200 Pro MPlex reader (Männedorf, Switzerland) measured absorbance at 450 nm. Glucose kinase was quantified using a 0.781–50 ng/mL standard curve; results are mean $\pm$ SD (%) vs. untreated cells from five triplicate assays.

2.18. Western Blot

Cells were lysed using ice-cold Complete Tablet buffer (Roche, Basel, Switzerland) containing 1 mM PMSF, a 1:100 dilution of Protease Inhibitor Cocktail, and 2 mM sodium orthovanadate. For each sample, 35 μ g of protein was loaded onto 8% or 15% SDS-PAGE gels and transferred to PVDF membranes (GE Healthcare, Chicago, IL, USA). Membranes were incubated overnight at 4 °C with primary antibodies against GLUT2, AMPK, and PGC-1 α (all at 1:500; Santa Cruz, Dallas, TX, USA), and β -actin (1:5000; Sigma, St. Louis, MO, USA) served as a loading control. Protein expression was assessed through densitometry following enhanced chemiluminescence, and results were normalised to β -actin and compared to untreated controls.

2.19. pAKT ELISA Kit

pAKT activity in HepG2 lysates was measured using ELISA (Thermo Fisher, Waltham, MA, USA, Cat. KHO0111) per manufacturer's protocol, with absorbance at 450 nm. Results were calculated from the standard curve and reported as mean $\pm$ SD (%) versus control, based on five independent duplicate experiments [57].

2.20. IRS1 ELISA Kit

IRS1 in HepG2 lysates was quantified using ELISA (NovusBio, Centennial, CO, USA). Absorbance at 450 nm was measured; results are shown as ng/mL (0.16–10 ng/mL standard curve) and mean $\pm$ SD (%) vs. untreated cells from five triplicate assays.

2.21. Glucose Reduction

The Glucose Assay Kit (Merck, Milano, Italy) was used to assess glucose levels according to the supplied protocol. Standard solutions were prepared from a glucose stock

solution, ranging from 5 μ M to 300 μ M, to construct a calibration curve for the colorimetric assay. Samples and standards were prepared in a 96-well plate, incubated with the reagent for 30 min, and the absorbance at 570 nm was measured. Glucose was calculated from the standard curve and expressed as % of the control (five triplicate assays).

2.22. Evaluation of Glucose Uptake

Glucose absorption in HepG2 lysates was assessed using the Merck kit (Merck, Milano, Italy) [58], measuring absorbance at 412 nm every 5 min. Results (five triplicate assays) were calculated from the standard curve and expressed as % of control.

2.23. Glycogen Measurement

Glycogen in HepG2 lysates was measured with the Glycogen Assay Kit (Merck, Milano, Italy) [58]. Absorbance at 570 nm was recorded, and values were calculated from a 0–200 μ g/mL standard curve. Data from five triplicate experiments are reported as % of the untreated control.

2.24. Statistical Analysis

The data presented herein were obtained from a minimum of five independent experiments, each of which was performed in triplicate. These experiments were analysed using Prism GraphPad 10.6.1 software. Results are presented as mean $\pm$ SD. Statistical significance ($p < 0.05$) was determined using one-way ANOVA with Bonferroni post hoc test. Synergy was assessed post hoc using the Bliss independence model. Expected additive effects were calculated from single-agent responses and compared with the observed combined effect.

3. Results

3.1. Phytochemical Composition Analysis of Natural Extract

Before the biological assays, chemical analyses were carried out to assess the quality and bioactive compound content of each extract (Table 1). Initially, the mulberry extract was found to contain 1-DNJ and polysaccharides, with the analytical data indicating a 1-DNJ content of approximately 5.01%.

Regarding the polysaccharide content, it was found to be approximately 11.7% of the total extract weight. Subsequently, the anthocyanin and anthocyanidin content of the mulberry extract was analysed. The analyses revealed that in the mulberry extract under study, the anthocyanin content constituted approximately 36% of the total extract weight, while the anthocyanidins comprised approximately 25% of the total extract weight.

It was determined that the anthocyanin content of the black currant extract was approximately 20% of its total weight, while the polyphenol content constituted 30% of the total extract weight.

Table 1. Principal bioactive constituents in plant extracts. Values are reported as mean $\pm$ SD (%) from five independent triplicate experiments.

Sample	Methods	Components	Content (%)
Mulberry Leaf extract	HPLC	1-DNJ	5.01 $\pm$ 0.21
	Phenol-sulfuric acid	Total polysaccharides	11.7 $\pm$ 0.89
Bilberry extract	HPLC	Anthocyanins	36 $\pm$ 0.33
	HPLC	Anthocyanidins	25 $\pm$ 0.15
Black currant extract	Folin-Ciocalteu	Total polyphenols	30 $\pm$ 1.02
	HPLC	Anthocyanins	20 $\pm$ 0.44

3.2. Dose–Response Study on Intestinal Cells

A preliminary screening was carried out on Caco-2 cells over a 6 h period, approximating intestinal transit time, to identify the optimal concentrations for the integrated axis model. The Mulberry extract exhibited a distinct, concentration-dependent profile (Figure 2A). Compared to control, lower concentrations (1 and 1.5 mg/mL) led to a moderate reduction in cell viability, reaching a maximum decrease of 37% at 6 h ($p < 0.05$). The 2 mg/mL concentration, on the other hand, showed better cellular resilience; after an initial adaptation period, a notable rebound in viability was seen beginning at 3 h. At 4 h, 2 mg/mL significantly increased viability by 1.76- and 1.23-fold versus 1 and 1.5 mg/mL, respectively ($p < 0.05$). Given the observed cell viability and lack of cytotoxicity, 2 mg/mL was chosen for further experiments.

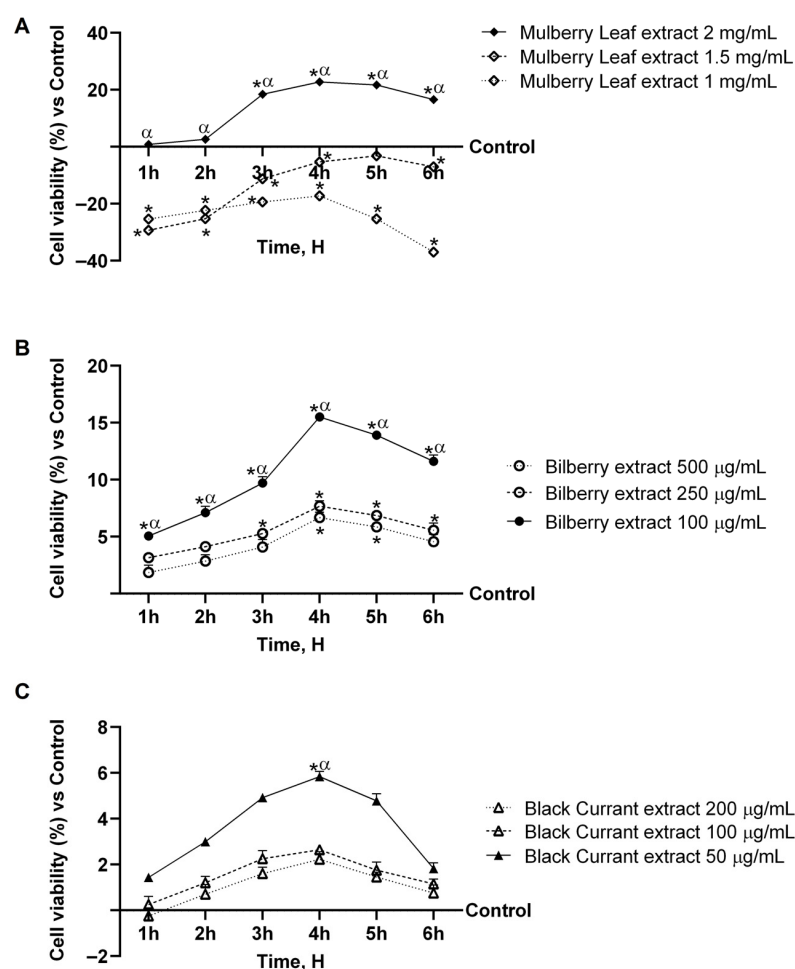


Figure 2. Dose–response and time-dependent analysis of natural extracts on Caco-2 cell viability. Cell viability was assessed using the MTT assay over a 6 h period following treatment with different concentrations of natural extract. In (A), a mulberry extract dose–response study (1–2 mg/mL); in (B), a bilberry extract dose–response study (100–500 µg/mL); and in (C), a black currant extract dose–response study (50–200 µg/mL). Results are presented as mean $\pm$ SD (%) from five triplicate experiments, normalised to untreated control (0%). * $p < 0.05$ vs. control; α $p < 0.05$ vs. single agents.

For bilberry extract (Figure 2B), cell viability significantly increased at all tested concentrations compared to the control ($p < 0.05$). The most prominent effect was observed at 100 µg/mL, with peaks at 4 h showing roughly a 57% increase over 500 µg/mL ($p < 0.05$) and approximately a 50.5% increase over 250 µg/mL ($p < 0.05$). A dose–response assessment of blackcurrant extract was performed to identify the optimal concentration. As shown in Figure 2C, this extract's concentrations correlated with increased cell viability relative to the

control, with only 50 µg/mL reaching statistical significance ($p < 0.05$) across all conditions. Notably, 50 µg/mL had the greatest impact, peaking at 4 h with about a 62% increase compared to 200 µg/mL ($p < 0.05$) and about a 55% increase compared to 100 µg/mL ($p < 0.05$). The optimal non-toxic concentrations selected for subsequent experiments were 2 mg/mL for mulberry, 100 µg/mL for bilberry, and 50 µg/mL for blackcurrant extracts.

3.3. Impact of Natural Extracts on Intestinal Barrier Integrity

For assessing the safety and functional relevance of the chosen concentrations of the extracts (2 mg/mL of mulberry extract, 100 µg/mL of bilberry extract, and 50 µg/mL of black currant extract) on the integrity of the epithelia, the polarised Caco-2 monolayer model was employed. Barrier integrity was assessed using TEER measurements in combination with the expression of the major tight junction proteins.

As illustrated in Figure 3A, all conditions either preserved or improved the electrical monolayer resistance. Indeed, the significant rise in TEER values compared to the control, induced not only by the combination but also by the mulberry extract alone (about +18% and +12%, $p < 0.05$, respectively), is consistent with a barrier-preserving effect on the intestinal epithelium.

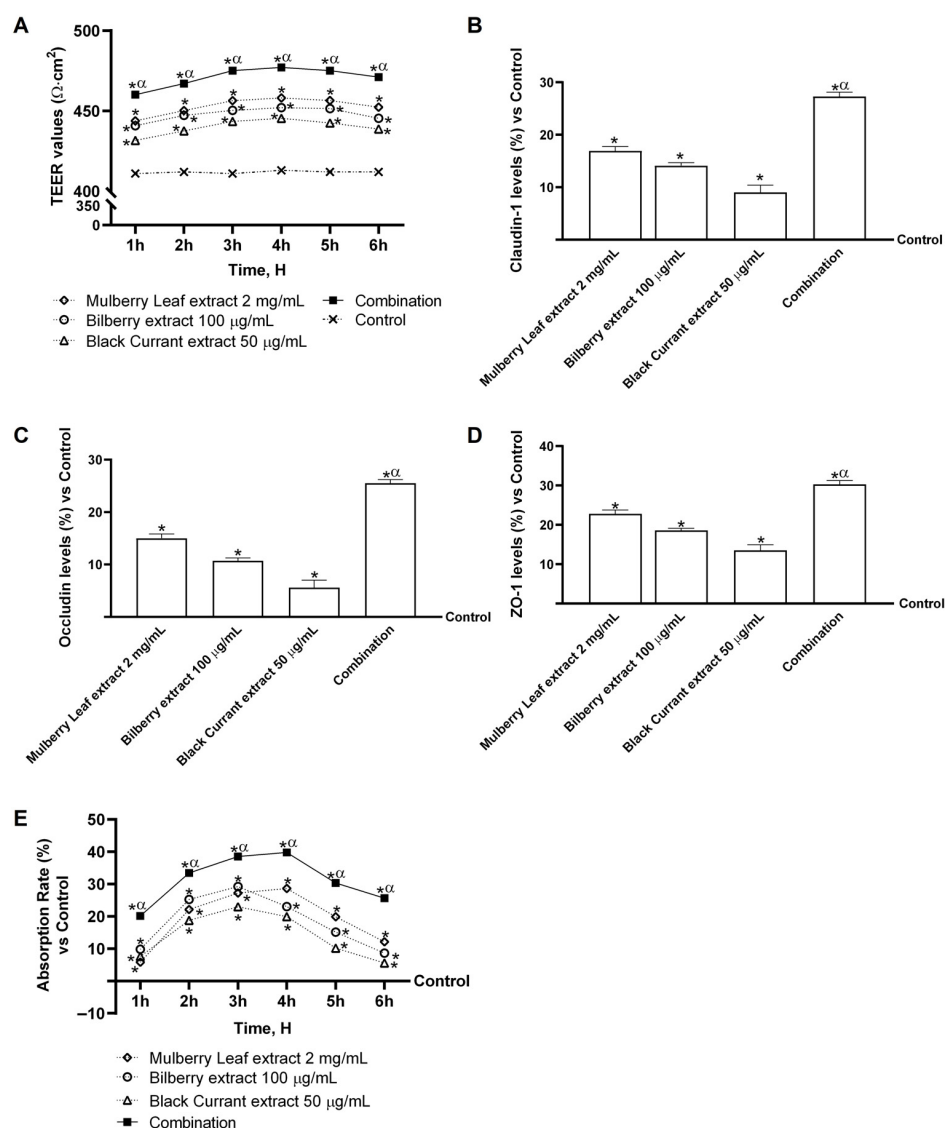


Figure 3. Effect of natural extracts and their combination on the integrity of the intestinal barrier. In (A), TEER analysis in polarised Caco-2 monolayers for a duration of 6 h; in (B), Claudin-1; in

(C), Occludin; and (D), ZO-1 assessed using an ELISA kit; in (E), absorption analysis performed with a fluorescent probe. Results are shown as mean $\pm$ SD (%) of five experiments in triplicate, normalised to control (untreated sample, 0%). * $p < 0.05$ vs. control; $\alpha p < 0.05$ vs. single agents.

Barrier-preserving effects were shown by analysing TJ proteins in cell lysates. Claudin-1, Occludin, and ZO-1 levels (Figure 3B–D) were significantly increased after treatment with individual extracts and their combination compared to controls ($p < 0.05$). The combined treatment increased all three proteins, especially Claudin-1 (about 25% above control; $p < 0.05$), demonstrating that the plant extract mixture helps maintain TJ function in the intestinal barrier.

In conclusion, intestinal absorption was assessed after treatment, with results shown in Figure 3E. All individual agents showed moderate absorption over the treatment period compared to the control ($p < 0.05$), with peak absorption varying by extract. Notably, bilberry and blackcurrant extracts reached maximum absorption rates of about 30% and 22%, respectively, at 3 h, compared to the control ($p < 0.05$). Similarly, mulberry extract reached a peak absorption of approximately 28% at 4 h, which was significantly higher than the control ($p < 0.05$). Additionally, absorption patterns changed when the substances were combined; the mixture showed a higher absorption profile than the individual agents ($p < 0.05$), with peaks of about 15% for mulberry, 20% for bilberry, and 22% for blackcurrant (all $p < 0.05$).

Overall, the data indicate that the tested extracts did not impair intestinal barrier integrity under the experimental conditions used.

3.4. Modulation of Pancreatic Function and Glucose Sensing

To evaluate the extracts' protective effects on pancreatic function, cell viability, ROS production, and insulin levels were measured. Each extract significantly enhanced cell viability versus control ($p < 0.05$): mulberry by 23.1%, bilberry by 7%, and black currant by 10% (Figure 4A). The combination treatment boosted cell viability more than individual extracts. These effects coincided with a significant reduction in oxidative stress (Figure 4B). All single agents maintained ROS levels similar to control, while the combination reduced ROS production by about 62% vs. mulberry, 1.26-fold vs. bilberry, and 1.05-fold vs. black currant ($p < 0.05$). The data pertaining to ROS production were corroborated by the analysis of SOD, CAT and GSH-Px levels, thereby substantiating the antioxidant efficacy of the agents in question, particularly in the context of their combined application (Figure A1 in Appendix B).

Glycaemic modulation was evaluated by measuring multiple markers of pancreatic β -cell function in cells treated with the intestinal metabolite. The investigation focused specifically on measuring insulin production, which is essential for maintaining glucose homeostasis, and C-peptide levels. Specifically, the data presented in Figure 4C,D demonstrate that the individual agents showed a limited ability to enhance insulin and C-peptide production compared to the control group, with no significant differences. In contrast, the combination has been shown to enhance insulin production and C-peptide levels to a greater extent than either agent alone. The magnitude of this enhancement was found to be approximately 64% and 62% greater than that observed with mulberry extract ($p < 0.05$), 95% and 95% greater than that observed with bilberry extract ($p < 0.05$), and 91% and 89% greater than that observed with black currant extract ($p < 0.05$), supporting a synergistic effect. A post hoc Bliss independence analysis, reported in Appendix A (Table A1), indicated that the observed combined effects exceeded the expected additive responses for selected metabolic endpoints.

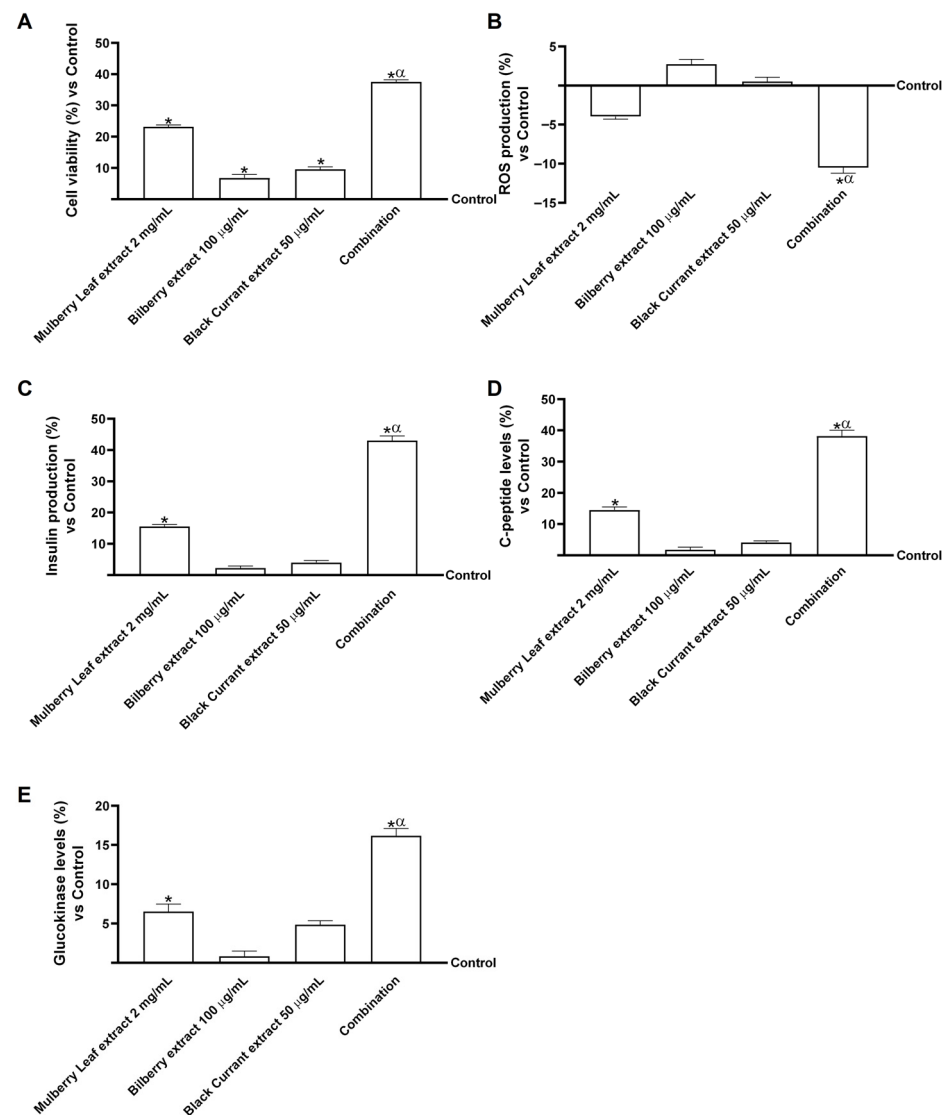


Figure 4. Protective and functional effects of natural extracts on pancreatic cells. In (A), cell viability was performed using MTT test; in (B), ROS production was measured by reduction in Cytochrome C; in (C), insulin production was quantified using ELISA kit; in (D), C-peptide levels were determined using ELISA kit; and in (E), GCK levels were evaluated using ELISA kit. Cells were treated with intestinal metabolites of mulberry, bilberry, black currant and their combination. Results are expressed as mean $\pm$ SD (%) of five independent experiments performed in triplicate and normalised on control (untreated sample, 0%). * $p < 0.05$ vs. control; $\alpha p < 0.05$ vs. single agents.

Finally, Figure 4E shows glucokinase levels after treatment with all examined agents. Each agent raised glucokinase levels compared to the control without significant effects. The combination increased levels by about 60% (vs. mulberry extract, $p < 0.05$), 95% (vs. bilberry extract, $p < 0.05$), and 70% (vs. black currant extract, $p < 0.05$).

Overall, the combination produced larger effects on the measured pancreatic endpoints than individual extracts.

3.5. Regulation of Hepatic Glucose Homeostasis and Insulin Signalling

Following pancreatic treatment, the pancreatic digest of all agents was utilised to treat liver cells under hyperglycaemic conditions. Consequently, IRS1 levels, glucose uptake, glycogen accumulation, and reduction in circulating glucose were analysed in this model. Indeed, IRS1 protein levels in response to the individual extracts were examined in Figure 5A, where protein levels remained within control values. Conversely, the

combination was shown to increase IRS1 protein levels more than either agent alone by approximately 69% (vs. mulberry extract, $p < 0.05$), approximately 93% (vs. bilberry extract, $p < 0.05$), and approximately 76% (vs. black currant extract, $p < 0.05$), suggesting a possible synergistic effect (as reported in Appendix A, Table A2) of the extracts in the study; in particular, the post hoc Bliss independence analysis indicated that the observed combined effects exceeded the expected additive responses for selected metabolic endpoints.

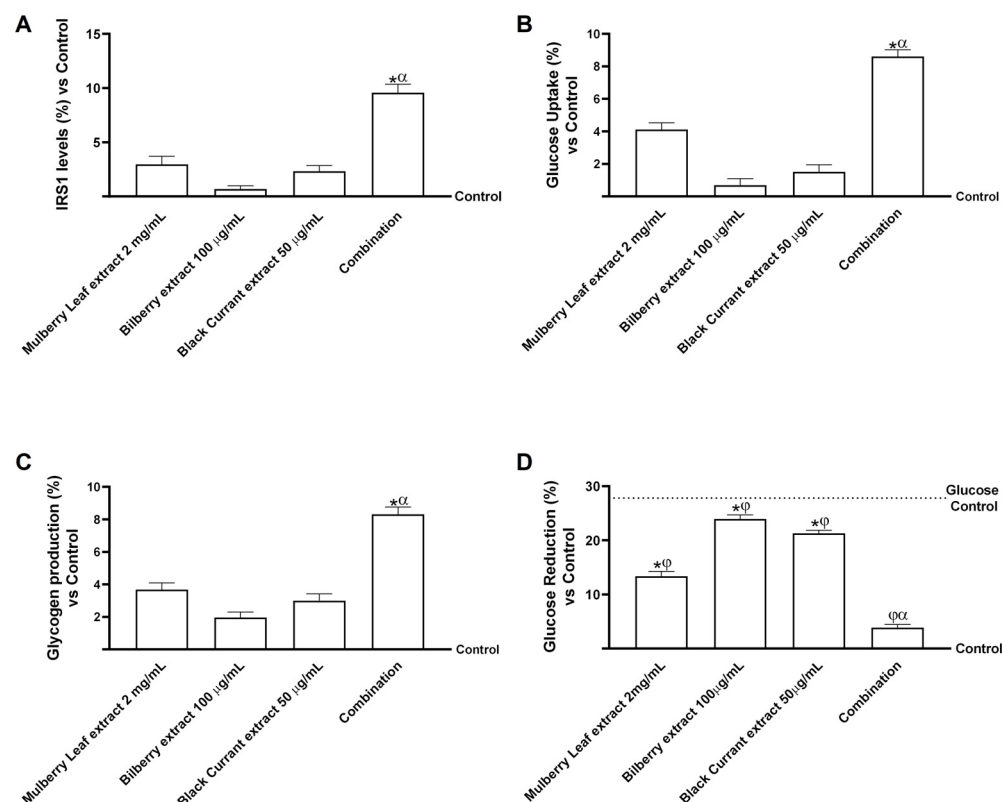


Figure 5. Effects of plant extracts on glucose metabolism in HepG2 cells. In (A), IRS1 protein levels were determined using an ELISA kit; in (B), Glucose uptake was evaluated using an ELISA kit; in (C), glycogen production was quantified using an ELISA kit; and in (D), glucose reduction was measured using an assay kit. Results are expressed as mean $\pm$ SD (%) of five independent experiments performed in triplicate and normalised on control (untreated sample, 0%). From (A–C), * $p < 0.05$ vs. control; α $p < 0.05$ vs. single agents. In (D), * $p < 0.05$ vs. control; α $p < 0.05$ vs. single agents; ϕ $p < 0.05$ vs. glucose control.

This enhanced signalling led to significant changes in glucose utilisation and storage, particularly in glucose uptake and glycogen accumulation. Figure 5B,C show that individual agents slightly regulated these in hepatocytes compared to controls, with mulberry extract showing no significant effect. When combined, the agents' effects increased, suggesting synergy. The combination raised glucose uptake by about 52% vs. mulberry, 92% vs. bilberry, and 82% vs. black currant ($p < 0.05$). It also increased glycogen by about 56% vs. mulberry, 76% vs. bilberry, and 64% vs. black currant ($p < 0.05$).

Finally, the reduction in glucose in the culture medium was measured to reflect the overall glycaemic effect (see Figure 5D). While treatments with mulberry, bilberry, and black currant extracts reduced glucose levels compared to the positive control ($p < 0.05$), they did not restore levels to control values. The combination treatment reduced extracellular glucose more than single treatments (71% vs. mulberry, $p < 0.05$; 84% vs. bilberry, $p < 0.05$; 82% vs. black currant, $p < 0.05$), restoring levels within control limits and showing synergy in post hoc Bliss analysis (see Appendix A, Table A3).

3.6. Effects of Mulberry Leaf, Bilberry, and Black Currant Extracts on Glucose Metabolism-Related Signalling in HepG2 Cells

To see how the extracts affect metabolic processes within cells, we analysed changes in certain proteins involved in glucose uptake and metabolism in HepG2 cells.

As represented in Figure 6A, the expression level of the GLUT2 varied considerably based on each treatment type. The individual extracts exhibited a modest augmentation in GLUT2 expression, with no statistically significant disparities observed in comparison to the control group. All three together increased expression by 6% versus control ($p < 0.05$). The combo raised GLUT2 by 51% vs. mulberry, 84% vs. bilberry, and 75% vs. black currant ($p < 0.05$).

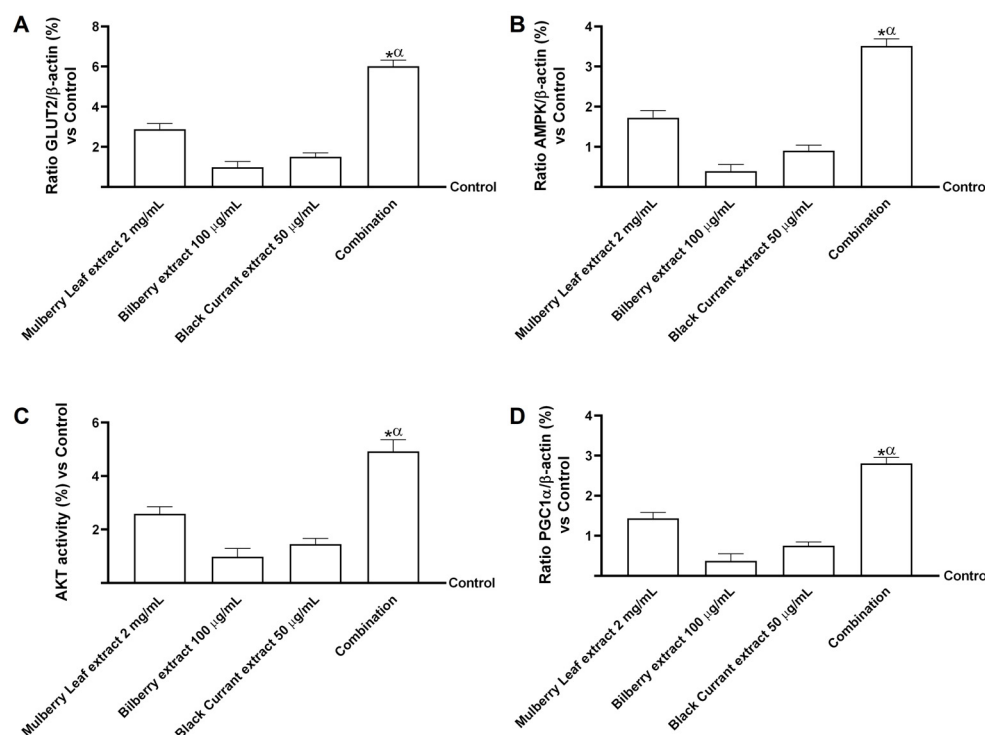


Figure 6. Effect of individual extracts and their combination on glucose metabolism-related markers in HepG2 cells. In (A), GLUT2 expression was evaluated through Western blot analysis; in (B), AMPK expression was evaluated through Western blot analysis; in (C), levels of AKT activity were quantified using an ELISA kit; and in (D), PGC1α expression was evaluated through Western blot analysis. Results are shown as mean $\pm$ SD (%) of five experiments in triplicate, normalised to control (untreated sample, 0%). * $p < 0.05$ vs. control; α $p < 0.05$ vs. single agents.

A similar tendency was also observed in the energy-sensing enzyme AMPK (Figure 6B). Once more, the individual agents provoked a modest elevation in AMPK expression, relative to the control group, yielding no statistically significant outcomes. In particular, the combination increased AMPK expression by approximately 51% vs. mulberry extract ($p < 0.05$), by approximately 89% vs. bilberry extract ($p < 0.05$), and by 74% vs. black currant ($p < 0.05$).

In addition, we also investigated AKT activity as a key mediator of insulin kinase signalling (Figure 6C). Among the individual agents tested, all were able to increase AKT activity compared to the control, with no statistically significant differences. Furthermore, the combination was shown to increase the activity of this marker by approximately 47% compared to mulberry extract ($p < 0.05$), 80% compared to bilberry extract ($p < 0.05$), and 70.5% compared to black currant extract ($p < 0.05$).

Finally, we examined the involvement of PGC1 α , an essential mediator of mitochondrial biogenesis and gluconeogenesis (Figure 6D). The individual extracts exhibited a behaviour analogous to that observed in the previously analysed parameters. Furthermore, the combination demonstrated a higher induction of PGC1 α levels than the individual treatments ($p < 0.05$). To elaborate further, the combination increased PGC1 α levels by approximately 49% in comparison with mulberry extract ($p < 0.05$), by approximately 87% in comparison with bilberry extract ($p < 0.05$), and by approximately 73% in comparison with black currant ($p < 0.05$).

In summary, these data show a coordinated modulation of GLUT2 expression and downstream metabolic signalling pathways following combination treatment, i.e., that the combined plant extracts exert a synergistic effect on GLUT2 expression. This modulation has been consistently associated with an activation of the AMPK/AKT/PGC1 α signalling pathway, a key regulator of cellular energy metabolism. The activation of this pathway has been shown to correlate with a significant improvement in glucose internalisation in liver cells, suggesting that the combined treatment enhances cellular glucose handling more effectively than individual extracts.

4. Discussion

Glucose metabolism is regulated by coordinated processes of intestinal absorption, pancreatic endocrine function, and hepatic insulin sensitivity [59]. Disruption in any component of the glucose regulatory axis may serve as the initiating event for upstream propagation of an abnormality along the glucose metabolic pathway, leading to abnormal glucose tolerance or insulin resistance [60]. In this context, nutraceuticals targeting multiple pathways may offer advantages over individual compounds [61]. An integrated in vitro intestine–pancreas–liver model was employed to evaluate the effects of mulberry leaf, bilberry, and black currant extracts, both individually and combined. The key result was that the combination of extracts produced stronger effects than single extracts across all parts of the model.

At the intestinal level, none of the extracts induced cytotoxicity at the chosen concentration ranges in the MTT cytotoxicity assay. Importantly, the combinatorial approach preserved the integrity of the epithelial cells as a whole and their barrier function, as indicated by TEER measurements and variations in the expression of TJ-associated proteins. Specifically, the increased expression of occludin, claudin-1, and ZO-1 induced by the combinatorial approach implies stronger junctional complexes between cells. Indeed, the deregulation of the tight junction structure, as well as the increased permeability of the intestinal barrier, is considered to underlie excessive glucose absorption into the bloodstream, endotoxemia, and increased systemic inflammation, with concomitant negative impacts on the integrity of the glucose/insulin regulatory axis [62,63].

In addition, the decrease in ROS production in Caco-2 cells indicates that the formulation helps maintain redox balance in the intestine, reducing oxidative stress that could induce metabolic imbalance. At the pancreatic level, metabolic stress triggered a pathological state characterised by reduced cell viability and secretory capacity, as indicated by decreased insulin secretion. This aligns with oxidative stress impairing glucose sensing, insulin production, and secretion, ultimately leading to β -cell exhaustion [64]. Combined treatment significantly reduced ROS levels and markedly increased insulin and C-peptide release. The combination treatment selectively increased glucokinase expression, indicating improved glucose sensing in pancreatic cells and supporting more effective insulin secretion [65]. After intestinal passage, the combination treatment produced a significantly greater increase in secretion markers than either component alone.

Together, the treatment increases both insulin and C-peptide, strongly suggesting improved β -cell function rather than loss of insulin regulation. Also, the increase in glucokinase levels observed only with complete treatment strongly suggests the return of glucose regulation in the cells [66]. Furthermore, the decrease in ROS levels observed suggests the importance of the formulation's antioxidant properties in reducing oxidative stress that may inhibit β -cell glucose regulation or insulin production [67].

The hepatocyte compartment further supports the synergistic benefit of the combined formulation. IRS1 expression, a key regulator of the insulin pathway, was significantly up-regulated only with the combined extract treatment, while individual extracts had minimal impact [68]. The induction of IRS1 expression was associated with a synergistic increase in glucose uptake and glycogen synthesis, supporting the activation of glucose utilisation pathways by insulin [69]. The data on markers of intracellular signalling further support these results, in which the combined treatment showed a dramatic increase in GLUT2 expression, AKT phosphorylation, AMPK activation, and PGC1 α expression. Notably, the combination treatment coordinated the activation of the AMPK, AKT, and PGC-1 α pathways. The AMPK is a key sensor in metabolism [70], AKT is involved in insulin-mediated anabolic functions [71], whereas PGC-1 α is primarily involved in mitochondrial biogenesis and metabolism [72]. The co-activation of these different pathways indicates that this formulation is not targeting metabolism by stimulating a particular body site but appears to induce metabolism by reprogramming glucose metabolism in the liver. This is reflected in enhanced glycogen storage and the controlled reduction in extracellular glucose concentrations [73].

The present study was designed as a mechanistic, proof-of-concept investigation and therefore presents some intrinsic limitations that should be carefully considered when interpreting the results. First, the experimental evidence was generated using an *in vitro* integrated intestine–pancreas–liver model, which, although valuable for dissecting molecular mechanisms and inter-organ communication, cannot fully reproduce the systemic complexity of glucose homeostasis observed *in vivo*. In particular, the absence of hormonal, neural, immune, and endocrine feedback loops limits the direct extrapolation of these findings to whole-body metabolic regulation. Second, hepatic responses were evaluated using the HepG2 cell line, which, despite being a well-established model for studying insulin signalling and glucose metabolism, does not fully recapitulate the metabolic plasticity and insulin sensitivity of primary human hepatocytes. Similarly, although the EndoC- β H5 cell line is among the most advanced human β -cell models available, it remains an *in vitro* system that cannot fully capture the long-term adaptive and pathological changes in pancreatic tissue that occur during insulin resistance or type 2 diabetes. Furthermore, the experimental conditions employed in this study represent an acute exposure model under controlled hyperglycaemic stress and do not address chronic metabolic adaptations, disease progression, or interindividual variability. Consequently, the observed synergistic and additive effects should be interpreted primarily as mechanistic evidence supporting the biological plausibility of multi-target nutraceutical strategies rather than as a direct demonstration of clinical efficacy. Nonetheless, the strength of the present model lies in its ability to link intestinal barrier integrity, pancreatic glucose sensing, and hepatic insulin signalling within a unified experimental framework. By integrating multiple metabolic compartments, this approach provides a robust proof-of-mechanism that supports further validation in more complex preclinical models and, ultimately, in well-designed *in vivo* and clinical studies.

Overall, the data show that the combined formulation acts synergistically, with the intestinal, pancreatic, and hepatocyte pathways functioning in an integrated manner. Indeed, the post hoc Bliss analysis supports the presence of synergistic interactions, particularly

at the level of hepatic insulin signalling. The combined formulation maintained intestinal barrier integrity by regulating junction proteins and redox balance, improved pancreatic β -cell function through improved glucose sensing and insulin secretion, and restored hepatic function by activating glucose and insulin signalling via the coordinated expression of IRS1, AMPK, AKT, GLUT2, and PGC1 α . The fact that the combined formulation showed a positive benefit in each experimental compartment indicates synergistic action.

Although an *in vitro* model, by design, cannot reproduce systemic regulation and glucose homeostasis in the body, the current research offers a powerful tool that links specific molecular targets to their metabolic phenotypes. The results provide a starting point for preclinical and clinical research to validate the potential utility of a comprehensive nutraceutical approach as a complementary method for glycaemic management.

5. Conclusions

This research evaluated how a nutraceutical blend of mulberry, bilberry, and black currant extracts affects the intestine–pancreas–liver axis. The combination modulated multiple pathways involved in glucose homeostasis, including enhanced intestinal barrier integrity and redox balance, amplified pancreatic glucose sensing and insulin secretion, and activated hepatic insulin signalling and glucose utilisation.

These findings suggest that integrated, multi-target nutraceutical strategies may offer advantages over single-component interventions for metabolic disorders characterised by oxidative stress and impaired insulin responsiveness. Despite being confined to *in vitro* experimental systems, the current results provide mechanistic evidence for the modulation of cellular pathways associated with glycaemic regulation by synergistic nutraceutical combinations. However, the physiological relevance of these observations remains to be established through further *in vivo* and clinical studies.

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Conflicts of Interest: Galla R. and Parini F. are employees of Noivita Srls and have no personal commercial interests in this work. Uberti F. is the founder of Noivita Srls. The company supported the purchase of reagents as a university spin-off but had no role in the study design, data collection and analysis, interpretation of results, manuscript preparation, or the decision to publish. Noivita srls has no commercial interest in the exploitation of the related patent. All other authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Adv-DMEM	Advanced Dulbecco's Modified Eagle Medium
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle Medium
DNJ	1-deoxynojirimycin
FBS	Foetal bovine serum
FMOC-C1	9-fluorenylmethyl chloroformate
GCK	Glucokinase
GLUT2	Glucose transporter 2
HPLC	High-performance liquid chromatography
HS-INS	High-sensitivity insulin
IRS1	Insulin receptor substrate-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PMSF	Phenylmethanesulfonyl fluoride
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
T2DM	Type 2 diabetes mellitus
TEER	Trans epithelial electrical resistance
TJ	Tight junction
TPC	Total polyphenol content
ZO-1	Zonula occluden-1

Appendix A

Bliss independence analysis revealed that the observed effects of the combined formulation on insulin secretion, IRS-1 and hepatic glucose uptake were significantly higher than the expected additive values, indicating a synergistic interaction between the extracts.

Table A1. Insulin analysis. Bliss independence analysis revealed that the observed effects of the combined formulation on insulin secretion.

Treatment	E _{exp}	E _{obs}	Interaction
Mulberry + Bilberry	0.174	0.43	Synergistic
Mulberry + BC	0.189	0.43	Synergistic
Bilberry + BC	0.0561	0.43	Synergistic

Table A2. IRS-1 analysis. Bliss independence analysis revealed that the observed effects of the combined formulation on IRS1 levels.

Treatment	E _{exp}	E _{obs}	Interaction
Mulberry + Bilberry	0.014	0.096	Synergistic
Mulberry + BC	0.030	0.096	Synergistic
Bilberry + BC	0.037	0.096	Synergistic

Table A3. Glucose uptake. Bliss independence analysis revealed that the observed effects of the combined formulation on glucose uptake levels.

Treatment	E _{exp}	E _{obs}	Interaction
Mulberry + Bilberry	0.047	0.086	Synergistic
Mulberry + BC	0.056	0.086	Synergistic
Bilberry + BC	0.021	0.086	Synergistic

Appendix B

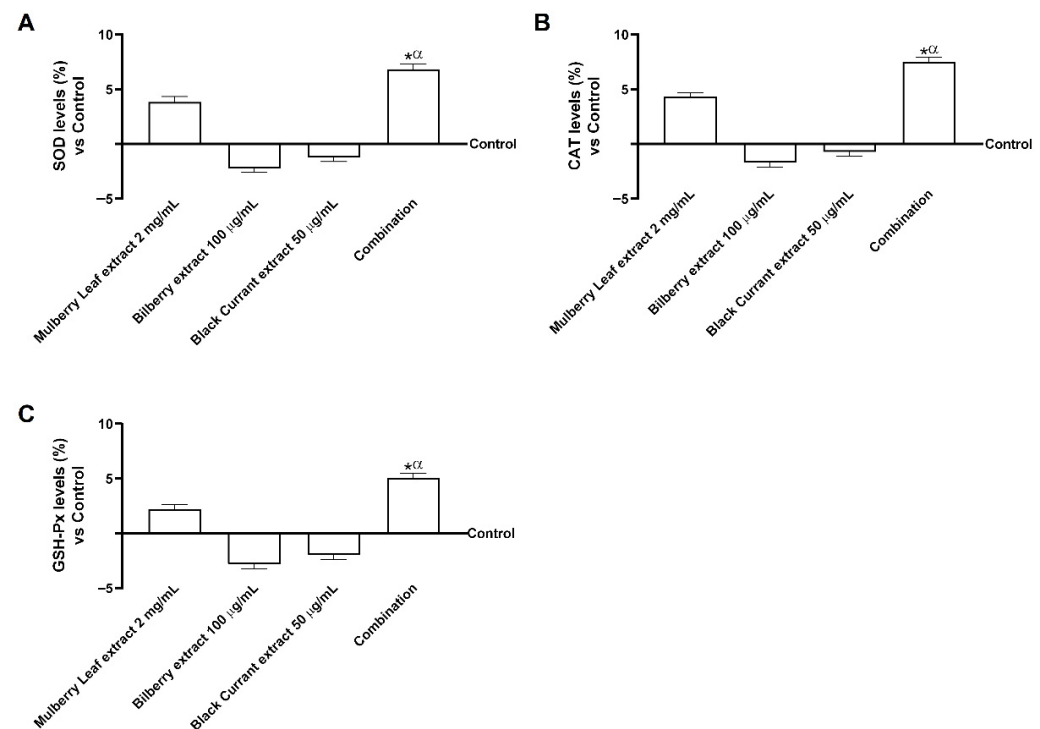


Figure A1. Antioxidant effects of combination. In (A), Superoxide dismutase (SOD) levels, in (B), catalase (CAT) levels, and in (C), glutathione peroxidase (GSH-Px) levels measured using ELISA. * $p < 0.05$ vs. control; $\alpha p < 0.05$ vs. single agents.

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