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Investigating the effects of different ratios of *Citrus limon*, *Citrus sinensis*, and *Vitis vinifera* extracts on insulin resistance in HepG2 cells

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ABSTRACT

This study aimed to investigate the molecular mechanisms by which Lemotrin™, a nutraceutical formulation based on *Citrus limon* (L.) Osbeck, *Citrus sinensis* (L.) Osbeck, and *Vitis vinifera* L. extracts, improves insulin resistance in an *in vitro* model of insulin-resistant human liver cells (HepG2/IR). Three different blends of these plant extracts (Mix1, Mix2 and Mix3) were tested to evaluate their efficacy. The cellular cytotoxicity of each formulation was first assessed. The hypoglycemic effect of each blend was analyzed by measuring glucose uptake, intracellular glycogen content, and the modulation of IRS1 and pro-inflammatory cytokines expression. Mix 1 and Mix 2 of Lemotrin™ significantly increased glucose uptake and intracellular glycogen content, enhanced IRS1 expression and reduced its inhibitory phosphorylated form at Serine 307. TNF-α and IL6 were reduced following treatment with Lemotrin™. Our findings suggest that Lemotrin™ is a promising nutraceutical candidate for the prevention and management of insulin resistance-related conditions.

1. Introduction

Diabetes is a severe chronic disease characterized by elevated blood glucose levels, mostly due to insulin resistance (IR) and destruction of pancreatic beta cells. Insulin resistance occurs when the body's tissues- mainly the liver, skeletal muscles and adipose tissue- fail to respond properly to insulin, impairing glucose uptake. As a result, beta cells in the pancreas compensate by producing more insulin, leading to hyperinsulinemia. Generally, insulin facilitates the entry of glucose into cells, where it is used as an energy source. At the cellular level, insulin binds to its receptor, triggering a phosphorylation cascade that activates insulin receptor substrates (IRS). IRS is activated through tyrosine (Tyr) phosphorylation and is inhibited when phosphorylated at serine (Ser) residues. In particular, phosphorylation of IRS1 at Serine 307 (Ser^{307}), has an inhibitory effect on the insulin signaling pathway, reducing its activity. This makes Ser^{307} phosphorylation an ideal therapeutic target (Martínez Báez et al., 2024).

The liver plays a major role in blood glucose homeostasis by balancing insulin-mediated glucose uptake and storage. Loss of insulin signaling in hepatocytes leads to severe IR with consequent elevation of blood glucose levels. To improve hepatic IR, several drugs, such as the Metformin, are commonly prescribed. Metformin is considered as the first-line treatment for hyperglycemia and diabetes (Alaaeldin et al., 2021). Besides conventional therapies, research is ongoing to identify and characterize valuable alternatives that might be free from side effects, such as liver toxicity and drug resistance associated with long-term drug use. In this context, a variety of natural products, including flavonoids, have been shown to positively affect IR. Flavonoids extracted from various plants display a wide range of biological activities, including potential antioxidant, anti-inflammatory, antiviral, anti-atherosclerotic, anti-diabetic and antitumor properties (Ullah et al., 2020).

Both *in vitro* and *in vivo* studies support the beneficial effects of flavonoids and anthocyanins in counteracting IR and diabetes. Among these bioactive molecules, hesperidin, commonly found in citrus fruits, has gained attention for its significant antioxidant and anti-diabetic properties (Mosqueda-Solıs et al., 2018). Peng and colleagues demonstrated the protective effect of hesperidin on type 2 diabetes in rats with IR induced by alloxan and High Fat Diet (HFD) (Peng et al., 2021a, b). Hesperidin has also been shown to upregulate IRS1 and glucose transporter 2 (GLUT2) while downregulating Toll-like receptor 4 (TLR4) and NF-kB expression in IR HepG2 cells, thereby enhancing glucose uptake and reducing IR (Hu et al., 2019a, b).

Eriocitrin, another abundant flavonoid in lemons, improves insulin

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sensitivity by reducing gluconeogenesis and proinflammatory responses in the liver (Kwon & Choi, 2020). It has also been found to reduce hyperglycemia and improve diabetes-related parameters in patients with elevated blood glucose levels (Cesar et al., 2022a, b).

Additionally, anthocyanins found in berries, currants, grapes, and some tropical fruits, display anti-inflammatory, antioxidant, anti-obesity and anti-diabetic effects (Babu et al., 2013; Belwal et al., 2017a, b; Mena et al., 2014). In murine 3 T3-L1 adipocyte model, anthocyanins reduce inflammation and palmitic acid-induced IR (Muscara et al., 2019). Mulberry anthocyanin extract ameliorates IR in HepG2 cells by increasing glucose consumption, glucose uptake, and glycogen storage. Anthocyanins from *Lycium ruthenicum* activate IRS1/AKT signaling, prevent HFD-induced gluconeogenesis and IR by improving inflammation and oxidative stress (Juan et al., 2024). Furthermore, a dedicated clinical trial demonstrated that blueberry anthocyanins reduce insulinemia and glucose levels (Curtis et al., 2022).

The aim of the present study was to evaluate the ability of a nutraceutical compound based on *Citrus limon*, *Citrus sinensis* and *Vitis vinifera* (Lemotrin™) to ameliorate insulin resistance in an *in vitro* model of insulin-resistant HepG2 cells. Although previous studies have examined the anti-diabetic properties of individual flavonoids and anthocyanins, this is the first study to evaluate, in the HepG2 cell model, the combined effects of these specific natural extracts formulated within a single compound. This integrated approach may uncover synergistic interactions that improve insulin sensitivity and glucose metabolism, thereby suggesting a novel, natural therapeutic strategy for the management of insulin resistance and type 2 diabetes.

2. Materials and methods

2.1. Chemicals for preparation of extracts and HPLC-DAD analysis

All solvents and reagents used in this study were high purity laboratory solvents.; HPLC grade water, acetonitrile, methanol and DMSO were obtained from Carlo Erba (Milano, Italy) and formic acid from Sigma Aldrich s.r.l. (reagent grade $\geq$ 95 %) (Sigma Aldrich-St Louis, MO). Delphinidin, Cyanidin, Petunidin, Ponidin, Malvidin-3-O-glucoside, Eriocitrin and Hesperidin standards were purchased from Sigma Aldrich s.r.l. The purity of the standards was $\geq$98 %.

2.2. Preparation of Citrus limon, Citrus sinensis and Vitis vinifera extracts (Lemotrin™)

Two hundred and fifty mg of dry extract of *Citrus limon* or *Citrus sinensis* were solubilized with 10 g of DMSO, vortexed and sonicated in an ultrasonic bath for 15 min. The bath was adjusted not to exceed the temperature of 25–30 °C. Six hundred mg of dry extract of *Vitis vinifera* were solubilized with 3 mL of a mixture of MeOH/HCOOH/H_2O (50:2:48 *v*/v/v), vortexed and sonicated in an ultrasonic bath for 15 min. The bath was adjusted not to exceed the temperature of 25–30 °C. The solutions were filtered through a 0.45 μm PVDF filter and then injected into column.

2.3. HPLC-DAD analysis of Citrus limon, Citrus sinensis and Vitis vinifera (Lemotrin™)

The qualitative and quantitative analysis of the extract was carried out by HPLC-DAD (Agilent Technologies, Waldrom, Germany). *Citrus limon or Citrus sinensis* extract was mixed with DMSO, vortexed and sonicated in an ultrasonic bath. The solution was filtered through a 0,45 μm PVDF filter. Chromatographic analysis for identification of the standard were carried out on a Luna C18 column (250 × 4,6 mm, 5 μm particle size; Phenomenex). Water/formic acid 0,03 % (Solvent A) and acetonitrile/formic acid 0,03 % (Solvent B) were used for chromatographic separation in an Agilent 1260 Infinity series equipped with a photodiode array detector. The equipment consisted of quaternary pump mod. 1260 Infinity 600 bar with 4-channel degassing system, an autosampler mod. 1620 equipped with flow-through design up to 600 bar, thermostat column compartment mod. 1100 and PDA-Uv-vis diode array detector mod. 1200 Infinity. The HPLC system was controlled by OpenLab CDS Workstation software. The flow rate was 1 mL/ ⟦min⟧ ^ (−1) using the linear gradient scheme (t in min; B); (0;5 %), (50,28 %), (60,43 %) and (65;5 %). The flavonoids were detected at 280 nm. Quantitative analyses were performed by means calibration curves using Eriocitrin e Hesperidin as standards. The HPLC profile of the *Citrus limon* and *Citrus sinensis* formulation is reported in Supplementary Fig. 2 A, *B. vitis Vinifera* extract was mixed with solution of methanol/formic acid/ water (50:2:48 *v/v*/v), vortexed and sonicated in an ultrasonic bath. The solution was filtered through a 0,45 μm PVDF filter. Chromatographic analysis for identification of the standard were carried out on a Luna C18 column (250 × 4,6 mm, 5 μm particle size; Phenomenex). Water/formic acid (99:1 v/v) (Solvent A) and acetonitrile/formic acid (99:1 v/v) (Solvent B) were used for chromatographic separation in an Agilent 1260 Infinity series equipped with a photodiode array detector. The equipment consisted of quaternary pump mod. 1260 Infinity 600 bar with 4-channel degassing system, an autosampler mod. 1620 equipped with flow-through design up to 600 bar, thermostat column compartment mod. 1100 and PDA-UV–Vis diode array detector mod. 1200 Infinity. The HPLC system was controlled by OpenLab CDS Workstation software. The flow rate was 1 mL/min^{-1} using the linear gradient scheme (*t* in min; B); (0;5 %), (15,15 %), (30,30 %), (40;50 %), (45;95 %) and (64;5 %). The anthocyanins were detected at 520 nm. Quantitative analyses were performed by means calibration curves using Delphinidin, Cyanidin, Petunidin, Ponidin and Malvidin-3-O-glucoside as standards. The HPLC profile of the *Vitis vinifera* extract is reported in Supplementary Fig. 1.

2.4. Determination of total polyphenol content of the extracts

Total polyphenols content was determined using Folin-Ciocalteu colorimetric method. An aliquot of 5 μL of the extracts was mixed with 120 μl of water and 125 μl of Folin-Ciocalteu reagent. The mixture was allowed to stand for 5 min, then 1.25 mL of sodium carbonate was added to the solution and the mixture was kept at room temperature and in the darkness for 90 min. Absorbance was measured at 760 nm using a UV/Visible spectrophotometer (Genesys 180, ThermoFisher Scientific, Waltham, MA, USA). All measurements were carried out in triplicate and results were expressed as gallic acid equivalents per 100 g (mgGAE/100 g). The results were reported in Supplementary Table 1.

2.5. Human HepG2 cell culture

HepG2 human hepatocellular carcinoma cells (RRID: CVCL_0027) were purchased from European Collection of Cell Cultures (ECACC, Sigma Aldrich-St Louis, MO). The cells were cultured in 75 cm^2 flasks as monolayer at 37 °C in a humidified atmosphere containing 5 % CO_2. The medium was DMEM High Glucose containing 2 mM glutamine, 1 % non-essential amino acids, 1000 U/mL penicillin, 10 mg/mL streptomycin and 10 % FBS (Aurogene, Italy). For treating cells, each natural mix (containing *Citrus limon*, *Citrus sinensis* and *Vitis vinifera*, 0.1 mg) was dissolved in sterile-filtered water to a stock concentration of 5 mg/mL and filtered through a 0.22 mm polyvinyl difluoride (PVDF) Primo® Syringe Filters, PVDF (Euroclone, Milan, Italy). Different mixes were prepared for treating human cells *in vitro* by combining the extracts of *Citrus limon* (L.) Osbeck, *Citrus sinensis* (L.) Osbeck and *Vitis vinifera* L. as follows: Mix 1 = 2,5:1,5:0,5; Mix 2 = 3:1:1; Mix 3 = 3:1:0,2″.

2.6. Induction of the insulin-resistance (IR) phenotype in HepG2 cells

To develop and characterize an insulin-resistant (IR) HepG2 cellular model, the effect of different concentrations of insulin was assessed in HepG2 cells in terms of intracellular glucose uptake (Curtis et al., 2022;

Ullah et al., 2020). In brief, cells were cultured in 96-well plates (2×10^4 cells/well) in FBS-free medium for 24 h, then treated with insulin (Sigma-Aldrich Inc., St. Louis, MO, USA) at concentrations of 0.000005, 0.00005, 0.0005, 0.005, 0.05, 0.5, 5 and 50 μM for further 24 h. The intracellular glucose uptake was assayed at the end of the 24 h treatment using the Glucose Uptake-Glo™ Assay kit (Promega, Madison, WI, USA), as detailed in the following paragraph. The experiment was performed in triplicate.

2.7. Analysis of the intracellular glucose uptake

Cells were seeded in 96-well plates at 2×10^4 cells/well for 24 h in DMEM High glucose and then incubated with the specific set of treatments, as described in each result section. After incubation, the rate of glucose uptake was evaluated utilizing Glucose Uptake-Glo™ Assay kit (Promega), according to the manufacturer's instructions and the luminescence was measured using a Glomax® Discover Microplate Reader (Promega). The rate of glucose uptake was calculated as follows:

Rate of glucose uptake (fmol/cell/min) = ([2DG6P] × (volume of sample)) ÷ ((number of cells) × (time of uptake).

The experiments with either insulin plus mixes or insulin plus Metformin were performed in triplicate.

2.8. Assessment of cell viability in HepG2 cells

Cell viability was analyzed *via* Cell Counting Kit (CCK)-8 (Sigma-Aldrich) assay, which is based on the conversion of water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl) − 3-(4-nitrophenyl)-5-(2,4-disulfophenyl) − 2H-tetrazolium, monosodium salt] to a water-soluble formazan dye upon reduction in the presence of an electron carrier by dehydrogenases. Briefly, the HepG2 cells were plated in a 96-well plate at a density of 5×10^3 cells/well; twenty-four hours after plating, cells were treated with each mixture (50, 100, 200, 300, 500, 1000, 2000 μg/mL) or with Insulin (50, 5, 0.5, 0.05, 0.005, 0.0005, 0.00005, 0.000005 μM) in 100 μL of medium for 24 h. Untreated cells (negative control) were included in the analysis. After treatment, 10 μl of CCK-8 solution was added to each well of the plate and the plate was incubated at 37 °C for 4 h. The absorbance of each well was measured at 450 nm using a Glomax® Discover Microplate Reader (Promega, Madison, WI, USA). All experiments were performed in triplicate and the cell viability (%) was expressed as the mean absorbance, from three independent experiments performed in triplicate, of the experimental group/the mean absorbance of the negative control.

2.9. Determination of the intracellular glycogen content

Cells were seeded into 96-well plate at a density of 2×10^4 cells/well and treated with 100 μg/mL of each mix or with Metformin (100 μg/mL) for 24 h. The intracellular glycogen content was detected using Glycogen Detection Assay (Promega). The luminescence was measured using a Glomax® Discover Microplate Reader (Promega) and the concentration was measured using a standard curve, according to manufacturer's instructions. All experiments were performed in quadruplicate and expressed as fold change as compared to control (untreated cells).

2.10. RNA extraction, reverse transcription and gene expression analysis

HepG2 cells were plated in a 6-well plate at a density of 8×10^5 cells/well; twenty-four hour after plating, cells were treated with 0.000005 μM of Insulin for 24 h, then administered with each mix (100 μg/mL) or with the positive control Metformin (100 μg/mL) for further 24 h. Total RNA from treated and untreated cells was isolated using Quick-RNA Microprep kit (Zymo Research, Irvine, CA, U.S.A) according to manufacturer's instructions. The amount and purity of the extracted RNA was evaluated by fiber optic spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) calculating the 230/260 and the 260/280 absorbance ratios. One microgram of total RNA was reverse transcribed into cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline Meridian BioScience, Cincinnati, Ohio, US). mRNA levels were analyzed by real-time PCR using a SensiFAST™ SYBR No-ROX Kit (Bioline Meridian BioScience, Cincinnati, Ohio, US). Real-time PCR was performed in a QuantStudio 5 Real-Time PCR (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) using Taqman Assay: IRS1 (Hs00178563_m1), IL6 (Hs00174131_m1), TNFα (Hs00174128_m1) and GAPDH (Hs03929097_g1). All reactions were run in triplicate and the relative abundance of the transcripts was calculated by normalizing to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, applying the $2^{-\Delta\Delta Ct}$ method.

2.11. Protein extraction and immunoblot analyses

HepG2 cells were plated in a 6-well plate at a density of 8×10^5/well; twenty-four hours after plating, cells were first treated with 0.000005 μM of Insulin for 24 h, then with each mix (100 μg/mL) or Metformin (100 μg/mL) for further 24 h. After treatments, cells were placed in ice-cold lysis buffer (Cell Extraction Buffer, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) containing 1 mM phenylmethylsulphonyl fluoride and protease/phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA, USA), homogenized and incubated in ice for 30 min. Samples were centrifuged at 13,000 rpm for 10 min and supernatants were collected, quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA) and analyzed by western blot. 30 μg of total proteins were fractionated by precast polyacrylamide gels Bolt™ 4–12 % Bis-Tris Plus (Invitrogen, ThermoFisher Scientific), transferred on iBlot™ 2 Transfer Stacks nitrocellulose membrane (Invitrogen, ThermoFisher Scientific) and blocked with TBS-T (Tris-buffered saline with Tween-20) containing 5 % non-fat dry milk. The antibodies Anti-IRS1 (phospho S307) (1:1000; AbClonal, Woburn, MA, USA), anti-IRS1 (1:1000; AbClonal, Woburn, MA, USA) and anti-β-actin (1:5000; Sigma-Aldrich) were diluted in TBS-T containing 3 % non-fat dry milk and incubated overnight at 4 °C. Membranes were washed in TBS-T, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA), washed in TBS-T, and developed with ECL-Plus (GE Healthcare, Life Science, Uppsala, Sweden). Densitometrical analyses of the blots were performed using iBright Analysis Software (Invitrogen).

2.12. Statistical analysis

All experiments were repeated from three to four times ($N \geq 3$, specified in each figure caption). Data were given as mean ± standard deviation (SD). All statistical analyses were performed with GraphPad PRISM 9.0 software (GraphPad Software, San Diego, CA, USA). Comparison among experimental groups was performed using the Mann–Whitney *U* test or non-parametric ANOVA ordinary test (Kruskal–Wallis Test). Multiple comparisons post-test was carried out with Dunn's test. A p level of <0.05 was considered significant for all tests.

3. Results

3.1. Mix 1 and Mix 2 revert insulin resistance by enhancing glucose uptake and glycogen content in HepG2 cells

To evaluate the potential protective effect of Mix 1, Mix 2 and Mix 3 on glucose metabolism in response to insulin, an insulin-resistant (IR) experimental model was first induced and characterized using HepG2 cells. To this purpose, HepG2 cells were treated with various non-cytotoxic insulin concentrations (50, 5, 0.5, 0.05, 0.005, 0.0005, 0.00005, 0.000005 μM, Fig. S3) selected according to existing literature (Teng et al., 2018; Ullah et al., 2020) for 24 h, and the intracellular glucose uptake was assessed by Glucose Uptake-Glo™ Assay kit

(Fig. S4). The lowest glucose uptake occurred at 0.000005 μM, which was then selected as the concentration to induce IR in the HepG2 cells.

Subsequently, cell viability was assessed in response to different doses of each mix (50, 100, 200, 300, 500, 1000 and 2000 μg/mL) for 24 h in HepG2 cells to determine the not-cytotoxic dose-range to be used in further experiments. Cell viability was measured *via* the CCK-8 assay and revealed impairment at 2000 μg/mL for all mixes compared to untreated cells ($P < 0.05$) as reported in Fig. 1 A, C. Based on these experimental findings and *in vitro* literature data (Baek et al., 2024; Choi et al., 2022), a dose of 100 μg/mL dose was selected for subsequent experiments on IR HepG2 model.

The effects of the natural extracts on glucose metabolism were tested in the IR HepG2 cells treated with 100 μg/mL of Mix 1, Mix 2, or Mix 3 for 24 h. Glucose uptake and intracellular glycogen content were measured, with metformin (100 μg/mL for 24 h) serving as a positive control. As shown in Fig. 2 A, B, Mix1 and Mix 2, but not Mix 3, triggered a statistically significant increase of both glucose uptake and glycogen content in IR cells.

3.2. Mix 1 and Mix 2 enhance hepatic IRS1 expression and reduce Ser^{307} phosphorylation in IR HepG2 cells

To elucidate the molecular mechanism underlying the improvement of the IR phenotype, IRS1 expression was evaluated in cells treated with Mix 1 and Mix 2. As reported in Fig. 3, IRS1 gene (Fig. 3A) and protein expression (Fig. 3B) were both impaired in IR cells if compared to control and underwent a significant stimulation by both natural extract mixtures. Moreover, as phosphorylation at Serine^{307} residue of IRS1 protein has been correlated with attenuation of the insulin pathway activity, the impact of Mix 1 and Mix 2 on this post-translational modification was evaluated using immunoblotting. Fig. 4 reports the ratio of phosphorylated IRS1 Ser^{307} to total protein content and demonstrates that phospho-IRS1 was significantly impaired in IR HepG2 following treatment with both natural blends.

3.3. Mix 1 and Mix 2 exert an anti-inflammatory effect in the IR HepG2 cells

Increased production of inflammatory cytokines, such as TNF-α and IL-6, is positively associated with obesity, higher insulin levels and insulin resistance (Al-Mansoori et al., 2022). To verify whether Mix 1 and Mix 2 were able to decrease inflammation in hepatic cells, IR HepG2 cells were treated for 24 h with the extracts and cytokines expression was evaluated through real time PCR analysis. Fig. 4 shows that gene expression levels of IL-6 and TNF-α is significantly diminished in IR cells treated either with Mix 1 or Mix 2, compared to untreated IR cells.

4. Discussion

We found that Lemotrin™, a nutraceutical formulation based on *Citrus limon* (L.) Osbeck, *Citrus sinensis* (L.) Osbeck, and *Vitis vinifera* L. extracts exerts a hypoglycemic effect in an *in vitro* model of insulin-resistance (IR). We demonstrated that different combinations of natural extracts positively impact IR, by (i) increasing glucose uptake and glycogen storage within liver cells, (ii) stimulating IRS1 expression while concomitantly impair its phosphorylation at Ser^{307}, and (iii)

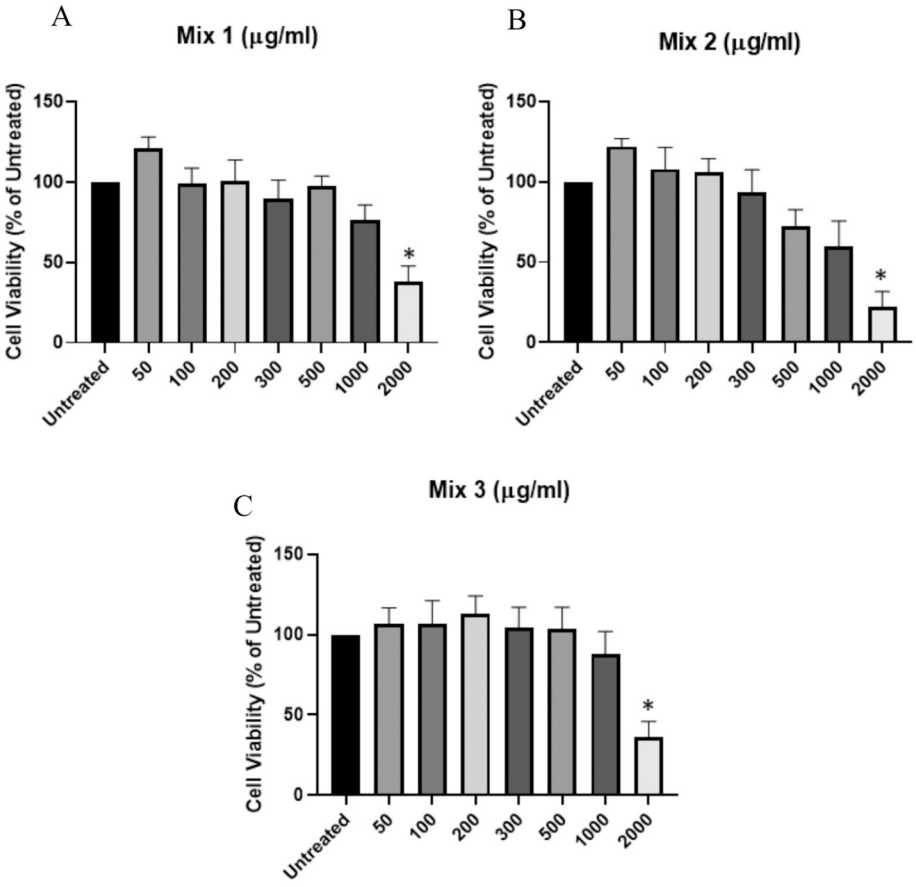

Fig. 1. Cell viability assay. The viability of HepG2 cells was evaluated after treatment for 24 h with an extract containing *Citrus limon*, *Citrus sinensis* and *Vitis vinifera* in the following ratios: (Mix1) 2,5:1,5:0,5; (Mix2) 3:1:1; (Mix3) 3:1:0,2. The three extracts were used at the following concentrations: 50, 100, 200, 300, 500, 1000 and 2000 micrograms/mL. Experiments were performed in triplicate, the mean +/− standard deviation calculated and the results expressed as the percentage of viable cells compared to untreated cells. *P* values ≤ 0.05 were considered statistically significant and indicated with * $P \leq 0.05$.

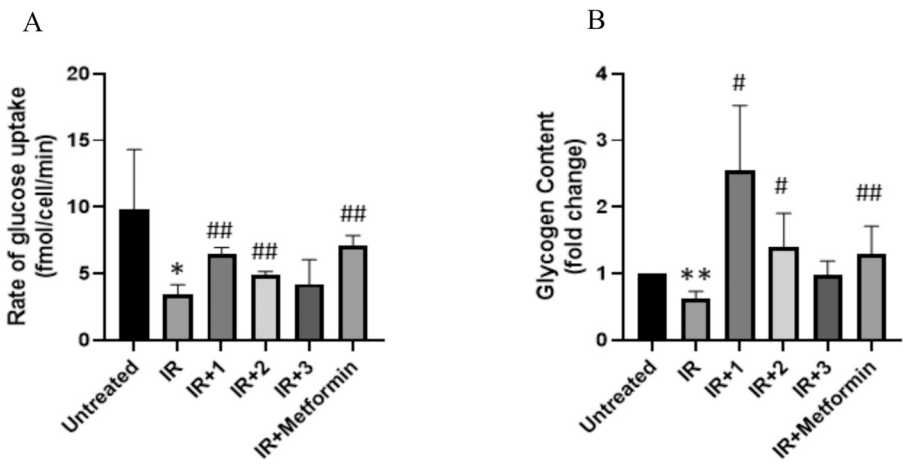

Fig. 2. Effect of extracts on glucose uptake and intracellular glycogen content in HepG2/IR cells.
HepG2 were first treated with insulin (0.000005 microM, for 24 h) to induce the insulin-resistant (IR) phenotype. Subsequently, they were treated with the three blend of extracts containing *Citrus limon*, *Citrus sinensis* and *Vitis vinifera* for 24 h in the following ratios: 2,5:1,5:0,5 (IR + 1), 3:1:1 (IR + 2), 3:1:0,2 (IR + 3). At the end of treatment, both glucose uptake (panel A, $N = 3$) and intracellular glycogen concentration (B, $N = 4$) were tested. Metformin was used as a positive control. # $P \leq 0.05$ and ## $P \leq 0.01$ indicates the comparison of IR+ 1, IR+ 2 and IR + Metformin to IR cells. *P ≤ 0.05 and **P ≤ 0.01 indicate the comparison between IR cells and the untreated ones. Results are expressed as mean ± SD of N = 3 or N = 4 independent experiments.

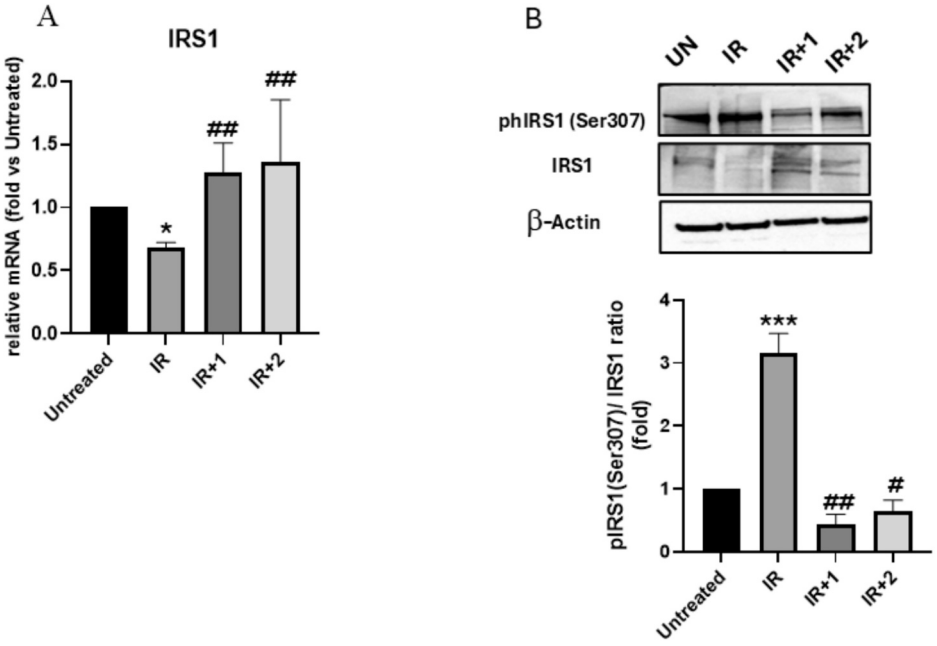

Fig. 3. Evaluation of total IRS1 gene and its phosphorylated form (Ser307) expression. After induction of the IR phenotype by insulin administration for 24 h, the cells were treated for a further 24 h with the two extracts containing *Citrus limon*, *Citrus sinensis* and *Vitis vinifera* in the following ratios: 2,5:1,5:0,5 (IR + 1), 3:1:1 (IR + 2). At the end of treatment, gene and protein expression were evaluated by real time (A) and western blot analysis (B), respectively. (B) Densitometric analysis of phosphorylated/total IRS1 with representative image of western blot. *P ≤ 0.05, ***$P < 0.001$ indicates comparison of IR *versus* untreated cells. #P ≤ 0.05, ##P ≤ 0.01 indicates comparison with IR cells.
Results are expressed as mean ± SD of N = 3 independent experiments.

inhibiting the expression of pro-inflammatory mediators IL6 and TNF-α. Although the correlation between IR and chronic pathologies -such as type 2 diabetes mellitus, metabolic syndrome, dyslipidemia and cardiovascular diseases- has been well established (Al-Badrani & Al-Sowayan, 2022), the molecular mechanisms underlying IR remain incompletely understood. Evaluating the modulation of the expression of certain genes involved both in the IRS1 pathway and in the IR-mediated inflammatory processes could provide new insights into the molecular mechanisms underlying IR.

Plant-based products contain a plethora of bioactive molecules with a wide range of applications for human health protection. *Citrus* fruits and grapes, in particular, are rich in flavonoids, including anthocyanins primarily found in grapevines, which exhibit antioxidant and anti-inflammatory properties in both *in vitro* and *in vivo* models. Diets rich in flavonoids are promoted for the prevention of diabetes, obesity, cardiovascular disease and neurodegenerative disorders. Flavonoids improve IR not only by interacting with intracellular insulin signaling pathway, but also through their antioxidant and anti-inflammatory activities. In this context eriocitrin and hesperidin, a subclass of flavanone molecules particularly abundant in lemon and orange, have demonstrated anti-diabetic potential and the ability to improve IR. Peng and co-authors evaluated the protective effect of hesperidin against type 2

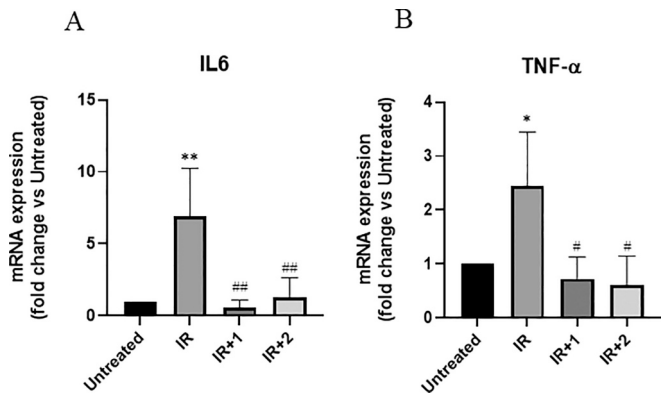

Fig. 4. Effect of extracts on the IL6 and TNF-α gene expression. After insulin administration for 24 h to induce insulin-resistance, HepG2 were treated for a further 24 h with the two extracts containing *Citrus limon*, *Citrus sinensis* and *Vitis Vinifera* in the following ratios: 2,5:1,5:0,5 (IR + 1), 3:1:1 (IR + 2). Real time PCR analysis was used to evaluate the gene expression of IL6 (A) and TNF-α (B). *P ≤ 0.05, **P ≤ 0.01 indicates comparison of IR cells *versus* untreated. #P ≤ 0.05, ## ≤ 0.01 indicates comparison of IR + 1 and IR + 2 with IR cells. Results are expressed as mean ± SD of N = 3 independent experiments.

diabetes in rats with IR induced by alloxan and HFD. They reported that hesperidin (100 mg/kg/day) improves fasting blood sugar levels and insulin sensitivity, preventing IR and diabetes (Peng et al., 2021). In IR HepG2 cells, hesperidin increases the expression of IRS1, which is involved in glucose uptake and the modulation of IR (Hu et al., 2019). Eriocitrin improves insulin sensitivity in the liver by reducing gluconeogenesis and pro-inflammatory responses. Ferreira and co-authors demonstrated that eriocitrin supplementation (administered at a dose of 25 mg/kg body weight for 4 weeks in animal models) significantly increases insulin sensitivity and reduces hepatic triglycerides (Ferreira et al., 2020). Moreover, administration of 200 mg/day of eriocitrin for 12 weeks was shown to reduce hyperglycemia and improve diabetes-related parameters in patients with hyperglycemia higher than 110 mg/dL (Cesar et al., 2022).

The phytochemical characterization of our extracts revealed the presence of eriocitrin, hesperidin and anthocyanins. The rationale for combining these extracts in different proportions was based on the need to evaluate any potential synergistic or additive effect on insulin resistance pathways in human hepatic cells *in vitro*. This approach allowed us to identify the optimal combination ratio for potential nutraceutical application. Indeed, consistent with literature data (Belwal et al., 2017), our findings showed that extracts containing the highest proportion of *Vitis vinifera* were the most effective at stimulating intracellular glucose uptake and increasing intracellular glycogen content, likely due to their anthocyanins content.

At the molecular level, we demonstrated that a specific and balanced blend of *Citrus limon* (L.) Osbeck, *Citrus sinensis* (L.) Osbeck, and *Vitis vinifera* L. extracts (Lemotrin™) reduced the expression of phosphorylated IRS1 (p-IRS1) at Ser307 while increasing total IRS1 expression, supporting the involvement of this signaling pathway in improving insulin sensitivity.

TNF-α has been shown to promote serine phosphorylation of IRS1 and to alter the interaction between the insulin receptor and IRS1 by inhibiting signal transduction (Hotamisligil et al., 1996) The extracts used to treat insulin-resistant cells decreased the expression levels of these pro-inflammatory cytokines and, therefore, might serve as useful candidates as therapeutic adjuvants for controlling inflammation in IR-related diseases. Compared to Metformin, which is commonly used to pharmacologically treat IR pathological conditions (Alaaeldin et al., 2021), we demonstrated that the extracts induced a comparable biological effect in terms of glucose uptake and glycogen content. Interestingly, our nutraceutical formulations also modulated both IRS-1 pathway and cytokines expression, whereas Metformin did not affect these pathways (data not shown). This difference is likely due to the distinct mechanisms of action of these molecules. Combining natural biomolecules that target multiple molecular pathways may reduce the required drug dosage, potentially lowering side effects.

The extract concentrations used *in vitro* (100 μg/mL) provided valuable insights but we acknowledge that *in vivo*, many polyphenolic compounds—including flavonoids (such as eriocitrin and didymin) and anthocyanins from lemon, orange, and red grape extracts-undergo extensive metabolism (*e.g.*, phase I/II biotransformation and gut microbiota-mediated metabolism) before reaching target tissues. However, our primary objective, in this study, was to evaluate the initial cellular effects of the parent extracts on insulin resistance pathways in HepG2 cells before exploring metabolite-specific mechanisms, future studies investigating the major metabolites (*e.g.*, hesperetin, eriocitrin metabolites, didymin conjugates, and anthocyanin-derived metabolites) would be highly valuable to deepen our understanding of the bioactive mechanisms involved insulin resistance modulation.

According to the findings of this study and literature evidence, suggesting a potential synergistic effect on insulin resistance, a Lemotrin ™ formulation was specifically developed to reflect the ratio defined in Mix 1 (2.5 parts lemon extract, 1.5 parts orange extract and 0.5 parts red grape extract).

Furthermore, based on the available literature and our own clinical data (not yet published), a recommended daily dose of Lemotrin ™ - delivering up to 750 mg of lemon extract, 450 mg of orange extract and 150 mg of red grape extract-has demonstrated a favorable safety profile without any significant side effects. These finding reinforce the tolerability of the selected extract combination and supports its potential use in clinical practice.

Lemotrin™ could therefore be a promising candidate for preventing diabetes mellitus and metabolic syndrome, likely through its anti-inflammatory properties.

5. Conclusions

The experimental findings demonstrate that Lemotrin™, a nutraceutical formulation based on *Citrus limon* (L.) Osbeck, *Citrus sinensis* (L.) Osbeck, and *Vitis vinifera* L. extracts, effectively enhances insulin sensitivity in an *in vitro* hepatic model of insulin resistance. This specific blend increases intracellular glucose uptake while stimulating the expression of key genes involved in insulin-mediated signal transduction. Additionally, Lemotrin™ exhibits a strong anti-inflammatory effect, significantly downregulating the expression of pro-inflammatory cytokines TNF-α and IL-6. Further *in vivo* and clinical studies will be necessary to confirm and validate these findings ensuring their potential applicability in the prevention and management of insulin resistance-related metabolic disorders.

CRediT authorship contribution statement

M. Pierdomenico: Writing – original draft, Methodology, Investigation, Conceptualization. **M. Telesca:** Investigation. **C. Riccioni:** Writing – review & editing, Funding acquisition. **E. Tordi:** Methodology. **I. Sagrafena:** Writing – review & editing. **B. Benassi:** Writing – original draft, Validation, Methodology, Investigation, Conceptualization.

Ethics statement

This study does not require an ethical statement.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2025.106984.

Data availability

Data will be made available on request.

References

Alaaeldin, R., Abdel-Rahman, I. A. M., Hassan, H. A., Youssef, N., Allam, E., AE, … Fathy, M. (2021). Carpachromene ameliorates insulin resistance in HepG2 cells via modulating IR/IRS1/PI3k/Akt/GSK3/FoxO1 pathway. *Molecules, 26*, 7629. https://doi.org/10.3390/molecules26247629

Al-Badrani, S. M., & Al-Sowayan, N. S. (2022). Consequences of insulin resistance long term in the body and its association with the development of chronic diseases. *Journal of Biosciences and Medicines, 10*, 96–109. https://www.scirp.org/journal/jbm.

Al-Mansoori, L., Al-Jaber, H., Shoaib Prince, M., & Elrayess, M. A. (2022). Role of inflammatory cytokines, growth factors and Adipokines in Adipogenesis and insulin resistance. *Inflammation, 45*(31–44), 21. https://doi.org/10.1007/s10753-021-01559-z

Babu, P. V. A., Liu, D. M., & Gilbert, E. R. (2013). Recent advances in understanding the anti-diabetic actions of dietary flavonoids. *The Journal of Nutritional Biochemistry, 11*, 1777–1789. https://doi.org/10.1016/j.jnutbio.2013.06.003

Baek, J. Y., Kwak, J. E., & Ahn, M. R. (2024). Eriocitrin inhibits angiogenesis by targeting VEGFR2-mediated PI3K/AKT/mTOR signaling pathways. *Nutrients, 16*, 1091. https://doi.org/10.3390/nu16071091

Belwal, T., Nabavi, S. F., Nabavi, S. M., & Habtemariam, S. (2017). Dietary anthocyanins and insulin resistance: When food becomes a medicine. *Nutrients, 9*, 1111–1132. https://doi.org/10.3390/nu9101111

Cesar, T. B., Ramos, F. M. M., & Ribeiro, C. B. (2022). Nutraceutical Eriocitrin (Eriomin) reduces hyperglycemia by increasing glucagon-like peptide 1 and downregulates systemic inflammation: A crossover-randomized clinical trial. *Journal of Medicinal Food, 25*, 1050–1058. https://doi.org/10.1089/jmf.2021.0181

Choi, S. S., Lee, S. H., & Lee, K. A. (2022). A comparative study of Hesperetin, hesperidin and hesperidin glucoside: Antioxidant, anti-inflammatory, and antibacterial activities in vitro. *Antioxidants, 11*, 1618. https://doi.org/10.3390/antiox11081618

Curtis, P. J., Berends, L., van der Velpen, V., Jennings, A., Haag, L., Chandra, P., … Cassidy, A. (2022). Blueberry anthocyanin intake attenuates the postprandial cardiometabolic effect of an energy-dense food challenge: Results from a double blind, randomized controlled trial in metabolic syndrome participants. *Clinical Nutrition, 41*, 165–176. https://doi.org/10.1016/j.clnu.2021.11.030

Ferreira, P. S., Manthey, J. A., Nery, M. S., Spolidorio, L. C., & Cesar, T. B. (2020). Low doses of eriocitrin attenuate metabolic impairment of glucose and lipids in ongoing obesogenic diet in mice. *Journal of Nutritional Science, 14*, Article e59. https://doi.org/10.1017/jns.2020.52

Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F., & Spiegelman, B. M. (1996). IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science, 271*, 665–668. https://doi.org/10.1126/science.271.5249.665

Hu, X. G., Tian, A. F., Liu, T., Zhou, L. Y., Bei, W. J., & Guo, J. (2019). Hesperidin ameliorates insulin resistance by regulating the IRS1-GLUT2 pathway via TLR4 in Hepg2 cells. *Phytotherapy Research, 33*, 1697–1705. https://doi.org/10.1002/ptr.6358

Juan, L., Hongwei, X., Yang, L., Shuyang, L., Tao, L., Zimian, D., & Xi, C. (2024). Anthocyanins from Lycium ruthenicum Murray mitigate cadmium-induced oxidative stress and testicular toxicity by activating the Keap1/Nrf2 signaling pathway. *Pharmaceuticals (Basel), 17*, 322. https://doi.org/10.3390/ph17030322

Kwon, E. Y., & Choi, M. S. (2020). Eriocitrin improves adiposity and related metabolic disorders in high-fat diet-induced obese mice. *Journal of Medicinal Food, 23*, 233–241. https://doi.org/10.1089/jmf.2019.4638

Martínez Báez, A., Ayala, G., Pedroza-Saavedra, A., González-Sánchez, H. M., & Chihu Amparan, L. (2024). Phosphorylation codes in IRS-1 and IRS-2 are associated with the activation/inhibition of insulin canonical signaling pathways. *Current Issues in Molecular Biology, 46*, 634–649. https://doi.org/10.3390/cimb46010041

Mena, P., Dominguez-Perles, R., Girones-Vilaplana, A., Baenas, N., Garcia-Viguera, C., & Villano, D. (2014). Flavan-3-Ols, anthocyanins, and inflammation. *IUBMB Life, 11*, 745–758. https://doi.org/10.1002/iub.1332

Mosqueda-Solıs, A., Sanchez, J., Reynes, B., Palou, M., Portillo, M. P., Palou, A., & Pico, C. (2018). Hesperidin and capsaicin, but not the combination, prevent hepatic steatosis and other metabolic syndrome-related alterations in western diet-fed rats. *Scientific Reports, 8*, 15100. https://doi.org/10.1038/s41598-018-32875-4

Muscara, C., Molonia, M. S., Speciale, A., Bashllari, R., Cimino, F., Occhiuto, C., … Cristani, M. (2019). Anthocyanins ameliorate palmitate-induced inflammation and insulin resistance in 3T3-L1 adipocytes. *Phytotherapy Research, 7*, 1888–1897. https://doi.org/10.1002/ptr.6379

Peng, P., Jin, J., Zou, G., Sui, Y., Han, Y., Zhao, D., & Liu, L. (2021). Hesperidin prevents hyperglycemia in diabetic rats by activating the insulin receptor pathway. *Experimental and Therapeutic Medicine, 21*, 53–59. https://doi.org/10.3892/etm.2020.9485

Teng, W., Yin,W., Zhao, L., Ma, C., Huang, J., Ren, F. (2018). Resveratrol metabolites ameliorate insulin resistance in HepG2 hepatocytes by modulating IRS-1/AMPK, RSC Advances,8,36034. doi:https://doi.org/10.1039/C8RA05092A.

Ullah, A., Munir, S., Badshah, S. L., Khan, N., Ghani, L., Poulson, B. G., … Jaremko, M. (2020). Important flavonoids and their role as a therapeutic agent. *Molecules, 22*, 5243. https://doi.org/10.3390/molecules25225243