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Honey extracts inhibit PTP1B, upregulate insulin receptor expression, and enhance glucose uptake in human HepG2 cells

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ABSTRACT

Honey is a food known for its medical properties. In this work, we have studied the impact of different types of honey on insulin signalling pathway. We found that honey extracts inhibit the enzyme PTP1B, one of the main negative regulators of insulin receptor signalling. HPLC-MS analysis allowed us to confirm the presence of several natural PTP1B inhibitors in the honey extracts analysed. Statistical analysis methods show a correlation between specific ^{1}H-NMR resonance frequencies/HPLC peaks and the inhibitory power of the samples. This finding will allow the prediction of the biological properties of honey samples applying relative simple analytical methods. Finally, we demonstrated that the treatment of HepG2 cells with honey extracts enhances the expression of insulin receptor, and stimulates glucose uptake. For the first time, our results demonstrate that bioactive components of honey could improve glycaemic control by both inhibiting PTP1B and stimulating the expression of insulin receptor in liver cells.

1. Introduction

Based on data of the last WHO report, to date more than 400 million of people worldwide suffer of diabetes. Type 2 diabetes, the most common diabetes form, is a chronic disease characterized by hyperglycaemia resulting from alterations of physiological mechanisms that contribute to maintain glucose homeostasis. Diabetic patients have to assume daily oral anti-hyperglycaemic drugs, or resort to insulin injections to maintain glycaemia under physiological range. Unfortunately, regardless of the therapeutic approach used, most diabetic people see their health status worsen over time, because of damages on the cardiovascular and nervous apparatus. Only in the 2012, more than 1.5 million of people affected by diabetes aged between 20 and 79 years old, died from complications related to this pathology [1].

It is established that sedentary lifestyle, hyper-caloric diets, and obesity are the main non-genetic risk factors that contribute to the onset of type 2 diabetes in the people aged 15–69 years. Thus, corrective actions aimed to modify the eating habits of people, to promote physical activity, are considered important measures to prevent the onset of type 2 diabetes in all countries [2].

Honey, the product of honeybee, is the most ancient natural sweetener known to mankind. It contains sugars (monosaccharides, disaccharides and oligosaccharides), vitamins, amino acids, proteins, mineral salts, trace elements, and many natural compounds such as flavonoids, phenolic and organic acids. These compounds are responsible for medical properties of honey, such as antibacterial, anti-inflammatory, antioxidant, antiproliferative, anticancer and antimetastatic activity [3,4]. Although it could seem counter-intuitive, several studies published in the last decade demonstrated that a moderate consumption of honey contributes to regulate glycaemia, and to prevent pathological complications in diabetic people [5–12]. Despite all the evidences produced to date, the molecular mechanisms of honey's anti-hyperglycemic activity still need to be clarified.

Recent studies demonstrated that several natural compounds extracted from fruits and vegetables, inhibit PTP1B, showing IC_{50} values in the μM range [13–15]. PTP1B is one of the most important negative

Abbreviations: WHO, World Health Organization; PTP1B, protein tyrosine phosphatase 1B; LC-MS, liquid chromatography- mass spectrometry; PCA, principal component analysis; LDA, linear discriminant analysis; 1H-NMR, proton nuclear magnetic resonance; HPLC-DAD, high performance liquid chromatography-diode array detector; mAU, milli absorption units; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; IC_{50}, the inhibitor concentration that decreases the activity of PTP1B enzyme by 50%; HepG2, liver hepatocellular cell line

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regulators of insulin signalling pathway and it is universally recognized as one of the most promising pharmacological target for the treatment of type 2 diabetes [16]. Since honey contains several phenolic compounds, we decided to investigate whether honey solutions were able to inhibit PTP1B, thereby enhancing insulin signalling pathway. To this aim, we selected twenty Tuscany honey samples belonging to four different floral varieties, such as honeydew, chestnut, wildflowers and acacia, representing some of the most abundant honey types produced in this region. Starting from each sample, we obtained hydroalcoholic solutions that we analysed for their ability to inhibit PTP1B. Moreover, we determined the IC_{50} values of active extracts and we studied the action mechanism of the most active ones. At the same time, HPLC-DAD and ^{1}H-NMR technologies were used to characterize these solutions, while LC–MS technology was used to identify the molecules present in the active extracts. Furthermore, statistical tools, namely PCA and LDA, were applied for correlating biological and chemical results and for creating a predictive model on honey inhibitory activity. Finally, the biological activity of honey extracts was confirmed *ex vivo*, by using human HepG2 cells.

2. Materials and methods

2.1. Materials

General reagents and culture media were purchased from Sigma-Aldrich. All organic solvents used for HPLC analysis were high-performance liquid chromatography grade and were purchased from Sigma-Aldrich. Human insulin was from Eli Lilly and Co. [^{3}H] D-glucose was purchased from Perkin-Elmer. Antibodies: anti p-IRβ (Y 1162–1163), anti IRβ, and *p*-nitrophenyl phosphate were purchased from Santa Cruz Biotechnology Inc. HepG2 cells were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK.

2.2. Methods

2.2.1. Preparation of the honey extracts

For biological assay, honey hydroalcoholic solutions were prepared by dissolving 5 g of honey in 25 mL of ethanol 80% v/v. Samples were left at room temperature under shaking for two hours, and then centrifuged to remove the insoluble residue. Supernatant was collected and stored at 4 °C. The extracts were named with the following symbols: H1-5, honeydew; W1-5, wildflowers; C1-C5, chestnut; A1-5, acacia.

Solutions for ^{1}H-NMR spectra were prepared by dissolving about 100 mg of honey in 0.5 mL of H_2O:D_2O mixture (90:10). 10 μL of maleic acid solution (110 mg/mL) as internal standard (IS) were added. Insoluble residue was removed by centrifugation at 11,000 rpm for 2 min.

For the HPLC analysis, about 100 mg of each honey sample were solubilised in 1 mL of milli-Q water. Honey solubilisation was carried out by shaking with a vortex for 1 min. The obtained solutions were centrifuged (14,000 rpm, rt, 5 min) to remove the undissolved residue before the chromatographic analysis. The same samples were used for the LC-HR-MS analysis.

2.2.2. Enzymatic assay

Enzymatic assays were carried out using recombinant PTP1B enzyme and *p*-nitrophenyl phosphate (*p*-NPP) as synthetic substrate. Recombinant PTP1B was prepared as previously described [17]. All enzymatic assays were carried out in 0.075 M β,β-dimethylglutarate buffer (pH 7.0), containing 0.1 mM DTT, 1 mM EDTA and 2.5 mM *p*-NPP. Each assay was started by adding an aliquot of the enzyme, and was stopped after appropriate time using 2 mL of 0.1 M KOH. The amount of the released *p*-nitrophenolate was determined measuring the absorbance at 400 nm in a 1 cm pathlength glass cuvette. The value of extinction coefficient (ε_{mM}) of *p*-nitrophenolate at pH 7.0 was 18. All assays were carried out in triplicate.

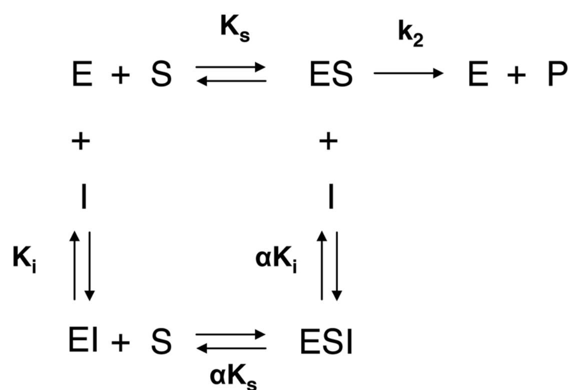
To determine the IC_{50} value of the different honey samples, several assays were carried out using a fixed substrate concentration, corresponding to K_m of the enzyme, and increasing amounts of honey solutions. The obtained data were normalized respect to a control test, and fitted using the following equation:

$$\frac{Vi}{V0} = \frac{Max - Min}{1 + \left(\frac{X}{IC50}\right)^{slope}} + Min$$

where V_i/V_0 represent the ratio between the enzymatic rate calculated in presence of each inhibitor concentration, and the enzymatic rate calculated in absence of inhibitor; "Max" represent the maximum value of activity, while "Min" the minimum value of activity; "x" represents the concentration of the inhibitor; IC_{50} was the concentration of the inhibitor able to decrease up to 50% the enzymatic activity; finally, the term "slope", represents the slope of the curve in the transition zone.

2.2.3. Determination of the mechanism of action of honey extracts

To verify whether the extracts behaved as reversible inhibitors, aliquots of the enzyme were incubated for 1 h at 37 °C in presence of saturating concentration of each extract. After this time, an aliquot of each sample was diluted 400 fold in the assay buffer containing *p*-NPP to determine the enzyme's residual activity. The kinetic parameters, K_m and V_{max}, were determined by measuring the initial hydrolysis rates in the presence of increasing substrate concentrations and by fitting these data with the Michaelis-Menten equation. All tests were carried out in triplicate. To determine the value of the inhibition constant (K_i), the dependence of K_m and V_{max} from the concentration of honey extract was studied. Data obtained from enzymatic assays were reported on a double reciprocal plot, according to Lineweaver-Burk method. Considering that honey extracts behaved as linear mixed type non-competitive inhibitors (see Scheme 1 below), the value of inhibition constant (Ki) was determined using the equations 1 and 2.



$$Slope = (K_m/(V_{max}*Ki))*[I] + (K_m/V_{max}) \qquad \text{eq.1}$$

$$Intercept = (1/(V_{max}*\alpha Ki))*[I] + (1/V_{max}) \qquad \text{eq.2}$$

Scheme 1. Mixed-type non-competitive inhibition. E, free enzyme; ES enzyme-substrate complex; EI, enzyme-inhibitor complex; ESI, enzyme-substrate-inhibitor complex; Ks, enzyme substrate complex dissociation constant; Ki, enzyme-inhibitor complex dissociation constant; $\alpha > 1$. The Ki values were determined applying equations 1 and 2.

2.2.4. Determination of total phenolic and flavonoid content

The total phenolic content was determined by the Folin-Ciocalteau method. The assay was carried out in 96 multiwell plates. Briefly, 20 μL of ethanolic solution were diluted with 100 μL of Folin-Ciocalteau reagent and stored at room temperature for 10 min. After this time, samples were diluted with 80 μL of 7.5% Na_2CO_3 and stored at room temperature for 90 min. Optical density of samples was measured at 595 nm using a Microplate reader (BioRad). Each test was carried out in triplicate. A calibration curve was built using 5, 10, 15, 20, 25, 37.5, and 50 μg/mL solution of gallic acid as external standard.

The total flavonoid content was measured using the colorimetric assay developed by Zhishen et al. [8]. The honey solution (10 μL) was mixed with 4 μL of distilled water. At the baseline, 3 μL of $NaNO_2$ (0.5%, w/v) were added. After 5 min, 3 μL of $AlCl_3$ (1% w/v) were added, followed by the addition of 20 μL of NaOH 1 M (6 min later). The volume was then increased to 100 μL by the addition of distilled water. The mixture was vigorously shaken to ensure adequate mixing, and the absorbance was read at 510 nm A calibration curve was created using a standard solution of catechin (20, 40, 60, 80, and 100 μg/mL; R^2 = 0.998). The results were expressed as mg catechin equivalents (CEQ) per kg of honey.

2.2.5. Color of honey samples

Briefly, 1 mL of the honey extract was transferred in a 1 cm path wavelength grass cuvette before the analysis. The absorbance of the samples was measured at 450 and 720 nm, and the difference in absorbance was expressed as mAU.

2.2.6. ^{1}H-NMR analysis

^{1}H-NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H-NMR) from Bruker Italia (Milan, Italy), operating at 298 K using a 5 mm probe. ^{1}H-NMR spectra were acquired in the following conditions: acquisition time, 1.71 s; spectral width, 4790 Hz; recycling delay, 5 s. Water suppression was performed using the pulse sequence with presaturation "zgpr" (Avance-version 12/01/11).

The peak heights were normalized based on the height of the signal at 6.2 ppm of the internal standard (maleic acid).

2.2.7. HPLC analysis

HPLC-DAD analyses were performed by using a HP-1200 L Liquid Chromatograph provided with a DAD detector all from Agilent Technologies, (Palo Alto, CA, USA). A 150 mm x 3 mm i.d., 2.7 μm Poroshell 120, EC-C18 column equipped with a precolumn of the same phase was used (Agilent Technologies). Solvents for the mobile phase were (A) 0.1% formic acid/water and (B) CH_3CN; flow rate 0.4 mL/min; the multi-step linear solvent gradient used was: 0–15 min 2–15% B; 15–25 min 15–30% B; 25–31 min 30–45% B; 31–35 min 45–100% B; 35–37 min 100% B; 37–43 min 100–2% B; the equilibration time after each analysis was 10 min. The wavelengths simultaneously selected were: 280 nm and 330 nm.

Semi-preparative HPLC analyses were performed using a DIONEX P680-UVD170U system equipped with a Kinetex C18 column, 5 μm, 100 Å, 250 × 4.6 mm (Phenomenex). Elution was performed at a flow rate of 0.8 mL/min applying the same gradient used for the analytical HPLC-DAD; the elution mixture contained acidified water by HCOOH (solvent A) and 10 mM of trifluroacetic acid in acetonitrile (solvent B). For each analysis, 100 μL of sample were injected and detection was based on absorbance at 280 and 330 nm.

2.2.8. LC–MS analysis

LC–MS analyses were carried out by using a qTOF mass spectrometer Impact II coupled to an UltiMate™ 3000 UHPLC System. 20 μL of each sample were used for the analysis. Samples were separated by reverse phase chromatography (RESTEK Ultra C18, 3 μm particle size, 100 mm length), using a flow rate of 0.4 mL/min using a water/CH_3CN gradient. Briefly, the mobile phases were (A) 0.1% formic acid in water and (B) CH_3CN. The applied multi-step linear solvent gradient was: 0–15 min 5–15% B; 15–25 min 15–30% B; 25–31 min 30–45% B; 31–35 min 45–95% B; 35–45 min 95% B; 45–50 min 95–5% B; the equilibration time after each analysis was 8 min.

Each sample was analysed twice in negative and in positive ion mode. Metabolites were ionized by ESI and analysed in the mass spectrometer with the following parameters: ion spray voltage 3000 V (4500 V for positive ion mode), enplate offset 500 V, dry gas 8 L/min, drying temperature 220 °C and nebulizing gas pressure 1.8 bar. The mass spectra were acquired in the *m/z* range 50–1300 and 20 μL were injected for each run.

The calibration ions in the pre-run internal calibration were generated as sodium formiate cluster ions. Theoretical masses of calibration ions were: 158.964069, 226.951493, 294.938917, 362.926341, 430.913765, 498.901189, 566.888613, 634.876037 and 702.863461 in positive ion mode, and 112.985627, 180.973051, 248.960475, 316.947899, 384.935323, 452.922747, 520.910170, 588.897594, 656.885018 and 724.872442 in negative ion mode.

The spectra were calibrated using data analysis 4.4 (Bruker, Bremen, Germany), analysed with profile analysis 2.3 (Bruker, Bremen, Germany), and matched with an analyte list of 117 compounds identified from honey samples using the software Metaboscape (Bruker, Bremen, Germany).

2.2.9. Statistical analysis

Data from ^{1}H-NMR and HPLC were statistically analyzed by PCA and LDA: the values of frequency and the signal intensity from ^{1}H-NMR data, and the peak areas from the chromatographic profiles of HPLC analysis were selected.

PCA was based on ^{1}H-NMR, in which the resonance frequencies were the variables, and the signal intensities were the corresponding values. Each variable was defined as a frequency range of 0.5 ppm, consequently, the related values were the sum of the intensities of the signals belonging to that ranges.

For the PCA applied to HPLC data, the retention times were the variables, and the peak areas were the related values. Each variable was defined as retention time ranges of 1 min, therefore the related values were the sum of the peak areas belonging to these ranges. Chromatograms were recorded at 240, 280 and 330 nm. LDA was applied, in both cases, selecting the three principal components derived by PCA

2.2.10. Ex vivo assay

The activity of the honey extracts was assayed on in cell model system using HepG2 cell line. Cells seeded in P35 plates (150.000 cells/dishes) were routinely grown in DMEM medium, containing 10% FBS, glutamine, streptomycin and penicillin. When the cells reached 70% confluence, the growth medium was withdrawn and substituted with starvation medium (DMEM high glucose without FBS). After 20 h, the cells were incubated with a fresh starvation medium containing 0.02% of honey extracts (1 μL/mL of extract). These cells were incubated for 4 days with honey extracts; each 24 h, the medium was substituted with fresh medium containing fresh extracts. Then, the cells were washed with PBS, and stimulated with 10 nM insulin. Control test were carried out incubating liver cells with an equal amount of 80% ethanol solution. Cells were lysed using 150 μL of 1X sample buffer (the solution contains 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromphenol blue and 0,0625 M Tris HCl, pH 6.8), scraped, collected in an eppendorf tube, and boiled for 5 min. Fifteen microliters of each protein extracts were loaded onto 12% SDS-PAGE. After running, proteins were transferred onto PVDF membrane by western blot technique. Expression levels of insulin receptor and activated form of insulin receptor were evaluated using specific antibodies (IRβ antibodies and p-insulin Rβ Antibody (Tyr 1162/1163, respectively). The antibodies were purchased from Santa Cruz Biotechnology. The obtained values

were normalized with respect to actin expression level.

2.2.11. Glucose uptake

HepG2 cells (30.000 cells/well) were seeded in 24 well plate. After 24 h cells were starved for 20 h and then incubated in the presence of starvation medium containing 0.1 mCi of 2-deoxy-D-1,2-[^{3}H]-glucose (20 Ci/mmol). Cells were incubated at 37 °C for 30 min, then washed with cold PBS and lysed in 0.2 M NaOH. Aliquots of the lysates were counted with a β-scintillation counter, and the data were normalized against protein concentration.

3. Results

3.1. Determination of total phenolic amount and flavonoids content

A preliminary spectrophotometric analysis showed that honey extracts strongly differ in term of natural pigments content (Supplementary Fig. 1A). To estimate the content of phenols and flavonoids in each sample, spectrophotometric tests were carried out. We found that the chestnut and honeydew extracts were richer in phenols and flavonoids than wildflowers and acacia honey samples (Supplementary Fig. 1B, C).

3.2. Evaluation of the inhibitory activity of honey

Enzymatic assays were carried out to evaluate the ability of each extract to inhibit PTP1B. The obtained results showed that the inhibitory power of the extracts differs, depending on the floral origin of honey. Among all, honeydew and chestnut extracts appeared to be the most active samples, while wildflower and acacia extracts showed a weaker inhibitory power (Fig. 1A–E).

To better evaluate the inhibitory power of each honey extract, we determined the IC_{50} values (Table 1). Data reported in Table 1 confirmed that the honeydew extracts were more effective than chestnut, and wildflowers samples (the mean IC_{50} values were 12 μL/mL, 27.8 μL/mL or and 31.7 μL/mL for honeydew, chestnut, and wildflower extracts, respectively), while the acacia extracts behaved as very weak inhibitors. Finally, no correlation was found between the IC_{50} values and the total phenolic content ($R^2 = 0.09$), whereas a weak correlation was verified between IC_{50} values and total flavonoid content ($R^2 = 0.29$) (Fig. 1F). These evidences suggest that flavonoids can be partially responsible for the inhibitory activity of these honey extracts.

3.2.1. Characterization of inhibitory mechanism of honey extracts

Further enzymatic assays were carried out to clarify the action mechanism of honey extracts. Firstly, we evaluated whether the most active samples (H4, C2, and W4) behaved as reversible or irreversible inhibitors. To this scope, aliquots of the enzyme solution were mixed with honey extracts (10:1 v/v was the enzyme/extract ratio), and then incubated at 37 °C for 90 min. After this time, an aliquot of the solution was diluted 400 folds in the assay buffer to evaluate the residual activity of the enzyme. We observed that, in all cases, the recovery of enzyme activity after dilution was almost complete (Supplementary Fig. 2). This finding suggested that honey extracts behaved as PTP1B reversible inhibitors. Moreover, kinetic analyses carried out by using different honey extracts showed that all the samples behaved as mixed-type non-competitive inhibitors towards PTP1B (Fig. 2, Table 2 and Supplementary Figs. 3–5).

3.3. HPLC analysis

The most active honey samples (H4, W4, and C2 extracts) and the A1 extract were analyzed by HPLC-DAD and the detectable peaks were evaluated at 240, 280, 330 and 350 nm (typical wavelengths applied to detect the phenolic compounds). In the chromatograms of the acacia extracts, only few peaks were detectable, suggesting the presence of low amount of potentially bioactive phenolic compounds. Conversely, the chromatographic profiles obtained from honeydew, chestnut and wildflowers honey samples, showed a higher number of constituents. (Supplementary Fig. 6). To identify the active fractions, we repeated the analysis by testing different samples obtained by using a semi-preparative HPLC-column. One hundred microliters of active samples (H4, W4, and C2 honey extracts) were injected, and four fractions were collected (Supplementary Figs. 7–9). All fractions were dried under vacuum, and then solubilized in 100 μL of 80% ethanol to reestablish the original concentration of the analytes, as in the whole honey sample. Finally, all the fractions were assayed to evaluate their inhibitory power on PTP1B. Fractions from H4 sample showed a weak inhibitory activity respect to the whole ethanolic extract (Fig. 3A and Supplementary Fig. 7). This evidence suggested that the compounds responsible for the inhibitory activity were lost during the drying of the sample because were volatile molecules. Conversely, we observed that the fraction 4 derived from C2 (Fig. 3B and Supplementary Fig. 8) and the fractions 3 and 4 from W4 sample (Fig. 3C and Supplementary Fig. 9), showed an inhibitory power comparable to that of the original extract. Considering the retention time of these bioactive compounds, eluted with a high organic solvent percentage, we hypothesize they possess aromatic or aliphatic hydrophobic groups.

3.4. ^{1}H-NMR analysis

Based on the results above, in order to evaluate all the organic components of the honey samples, the ^{1}H-NMR experiments were carried out on water solutions, in which the whole honey was completely dissolved.

From the statistical analysis of our results, the activity resulted associated to molecules of various molecular weights, but having an aromatic group. In particular, we found that the resonance signals at frequencies around 8 ppm correlated with the inhibitory activity. Characteristics of this type suggest the presence of molecules with aromatic structures coplanar to a carbonyl group as those observed in several flavonoids classes (e.g. flavone, flavonols).

3.5. Statistical analysis of data

The final goals was to identify honeys able to inhibit PTP1B and to hypothesize the chemical-physical characteristics of the compounds responsible for the inhibitory activity.

Based on IC_{50} values, the honeys were classified in active samples (symbol A, if $IC_{50} < 40$ μl/mL), or inactive extracts (symbol I, if $IC_{50} > 40$ μl/mL) (Table 1).

PCA based on ^{1}H-NMR, was carried out for the most significant variables, those able to discriminate active and inactive honeys (standard deviation > 20 and CV% > 80) (Fig. 4A). PCA highlighted a good separation of the two groups; in particular, LDA calculated on the three principal components showed that only 10.1% of objects resulted wrongly classified. Frequencies between 1.5 and 3.0 ppm and between 8.0 and 8.5 ppm, strongly affected the first main component (52.8% of the total variance); as consequence, they better explained the differences between the two groups of samples.

It is noteworthy that the sum of peak heights in the frequency range between 1.80–1.90 ppm (Σh), was strongly related with IC_{50} values ($r = -0.79$, Fig. 4B), so indicating that the active molecule(s) has/have protons that resonates in that spectral range.

PCA analysis from HPLC was carried out for the most significant variables, that were those able to discriminate active and inactive honeys (standard deviation > 20, CV% > 80 and area > 100; Fig. 4C). The best results by PCA, in terms of separation between the two groups, were obtained with the area values at 280 nm. LDA calculated on three main components showed that only 10.1% of objects resulted wrongly classified. Finally, a third PCA was generated by using both the variables obtained from ^{1}H-NMR and HPLC (Fig. 4D). Following this latter

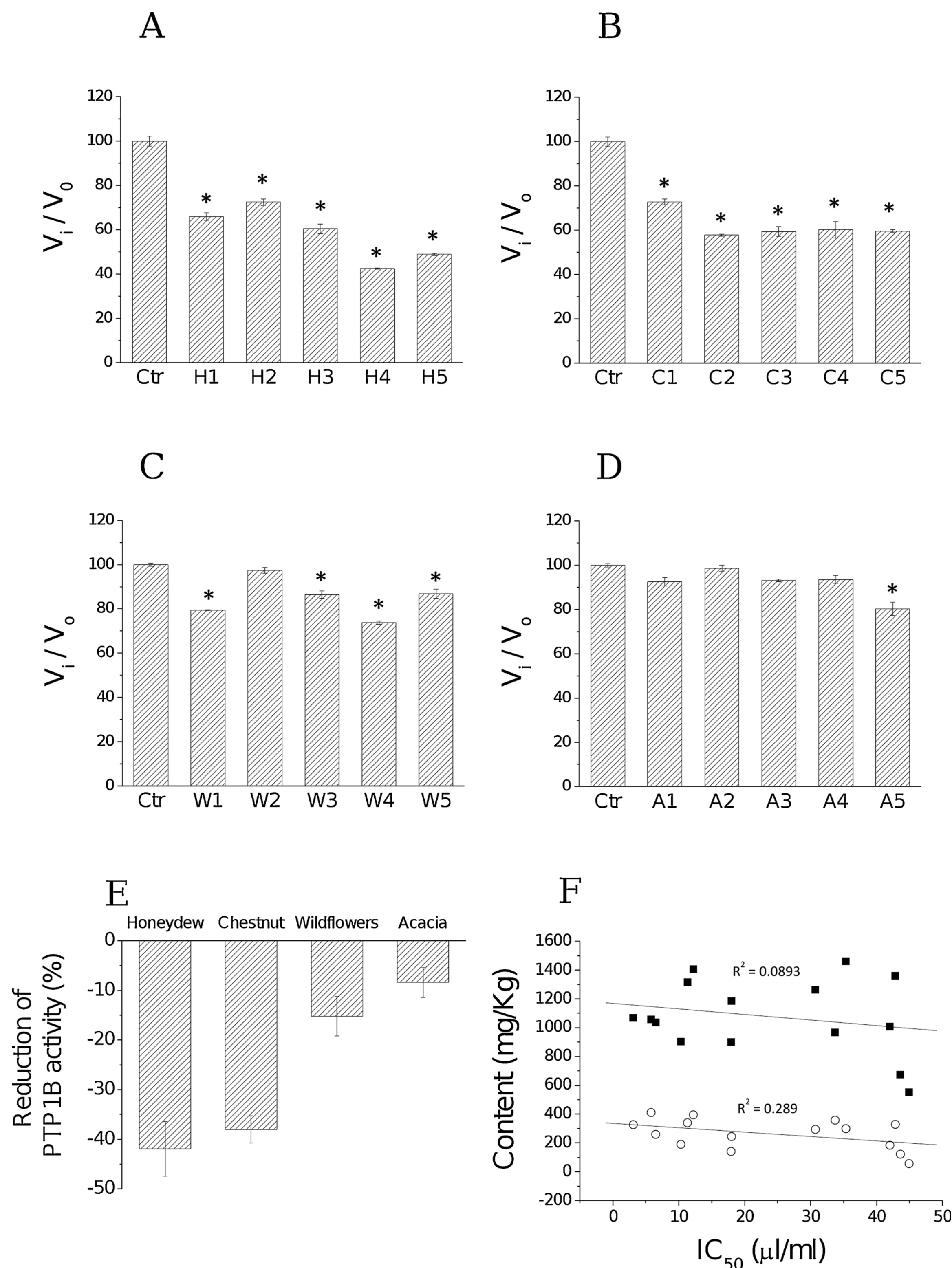


Fig. 1. Evaluation of the inhibitory power of honey extracts. Preliminary screening was carried out by using a fixed amount of each extracts (10 µL/mL), an aliquot of recombinant PTP1B enzyme (5 µL), and *p*-NPP as substrate (2.5 mM final concentration). Data obtained were normalized respect to the control sample. Data reported in the figure represent the mean value +/- S.E.M. (n = 3) (* $p < 0.05$). (A), honeydew; (B), chestnut; (C), wildflowers; (D), acacia. (E), average reduction +/- S.E.M. of PTP1B activity (n = 5); (F), coefficient of determination. The IC_{50} values were plotted versus the total content of polyphenols (full square) and flavonoids (empty circles). The IC_{50} values obtained from acacia extracts were not included in this analysis.

approach the number of objects wrongly classified diminished until 5.6%.

The next step was the chemical characterization of the active honey. To this aim, the different variables (range of resonance frequencies and range of retention times) were correlated by using a correlation matrix, as reported in Table 3. The matrix shows a first correlation between HPLC-peaks with retention times in the range of 7.0–8.0 min, and with ^{1}H-NMR signals in four of the five evaluated ranges of ppm (1.5–2.0;

Table 1

IC_{50} values, and classification of honey samples. The IC_{50} values were determined using PTP1B enzyme (for more details, see materials and method section). Classification of honey samples was carried out setting the cutoff at 40 µL/mL. “A” indicates active honey samples, whereas “I” indicates inactive honey samples.

Honey extracts	IC_{50} (µL/mL))	Classification*
Honeydew		
H1	33.7 ± 1.5	A
H2	5.8 ± 0.3	A
H3	11.3 ± 0.4	A
H4	3.1 ± 0.3	A
H5	6.5 ± 0.3	A
Wildflowers		
W1	17.9 ± 2.3	A
W2	44.9 ± 2.8	I
W3	43.6 ± 1.8	I
W4	10.3 ± 1.1	A
W5	42.0 ± 2.9	I
Chestnut		
C1	35.3 ± 4.2	A
C2	18.0 ± 0.8	A
C3	30.7 ± 1.4	A
C4	12.2 ± 0.8	A
C5	42.8 ± 5.0	I
Acacia		
A1	> 50	I
A2	> 50	I
A3	> 50	I
A4	> 50	I
A5	> 50	I

* A = Active; I = Inactive.

2.0–2.5; 2.5–3.0 and 8.0–8.5 ppm). A second correlation was between HPLC-peaks with retention times of 4.0–5.0 min, and with ^{1}H-NMR signals in three ranges of ppm (1.5–2.0; 2.0–2.5; and 2.5–3.0 ppm). In light of these results, we can predict a honey as active in inhibition of PTP1B when ^{1}H-NMR spectra shown signals at 1.5–3.0 and 8.0–8.5 ppm correlated with HPLC-peaks detected at 280 nm, with retention times in the range of 7.0–8.0 min.

3.6. LC–MS analysis

To identify the compounds responsible for PTP1B inhibition, LC–MS analysis was carried out by using aliquots of H4, C2, and W4 honey extracts, recognized as the most active samples. By comparing the data obtained with those of a library of compounds detected in honey [18–21] (Supplementary material, Table 4), we have identified different masses that can be related to the compounds shown in Table 5. Some of these such as kurarinone [22], acacetin, chrysin [23], pinocembrin [24], naringenin [25], apigenin [26], and caffeic acid, [27] were previously recognized as PTP1B inhibitors. Other compounds, such as Kushenol A and Kushenol K, are structurally similar to lavandulyl flavonoids, which are potent PTP1B inhibitors, suggesting that even these molecules could contribute to the inhibitory power of these honey extracts [17,18]. Finally, we found that fisetin and eriodictyol, flavonoids detected in H4 and W4 samples, behaved as potent PTP1B inhibitors, showing an IC_{50} value of 0.6 ± 0.01, and 33.4 ± 1.8 µM, respectively (Supplementary Fig. 10). Taken together, these results demonstrated that honey extracts contain different natural PTP1B inhibitors.

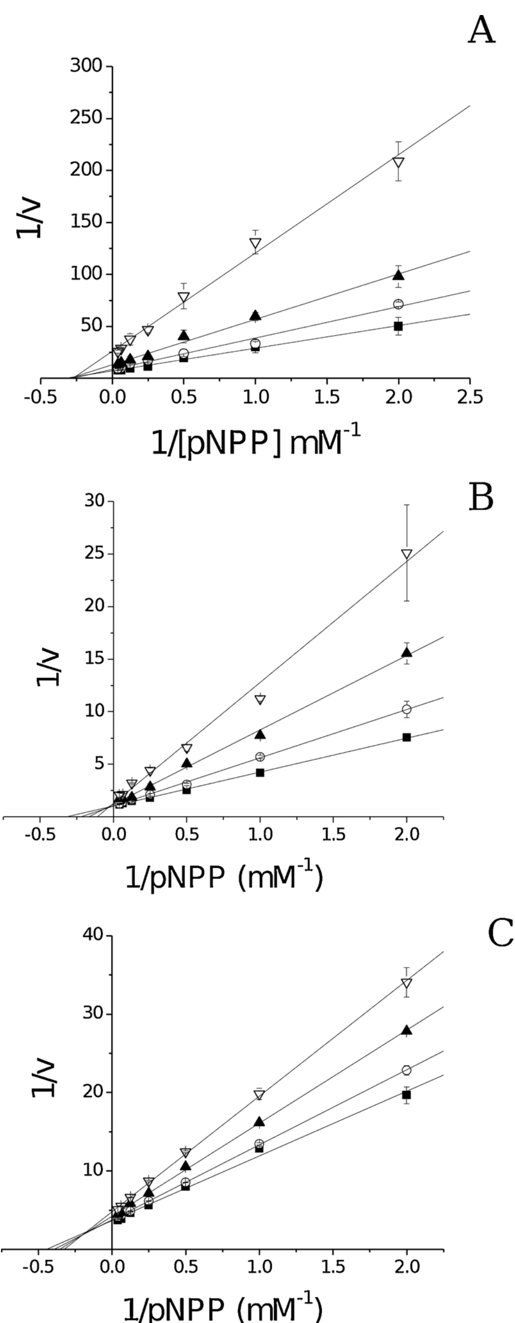


Fig. 2. Double reciprocal plots obtained by using PTP1B and active honey extracts. All data reported in the figures represent the mean values ± S.E.M. (n = 3). Experimental points were fitted by using Microcal Origin softer program (OriginLab Corporation, Northampton, Massachusetts, USA.). (A), honeydew extract (H4). ■, control; ○, 1 µL/mL; ▲, 2 µL/mL; ▽, 4 µL/mL. (B), wildflowers extract (W4). ■, control; ○, 5 µL/mL; ▲, 10 µL/mL ; ▽, 20 µL/mL. (C), chestnut extracts (C2). ■, control; ○, 5 µL/mL; ▲, 10 µL/mL; ▽, 20 µL/mL.

Table 2

Ki values and action mechanism of honey extracts.

Honey extract	Inhibition type	K_i (µL/mL)	α
H4	Linear Mixed type	0.8 ± 0.07	1.3 ± 0.1
W4	Linear Mixed type	6.8 ± 0.2	10.8 ± 2.5
C2	Linear Mixed type	24.7 ± 1.0	2.4 ± 0.3

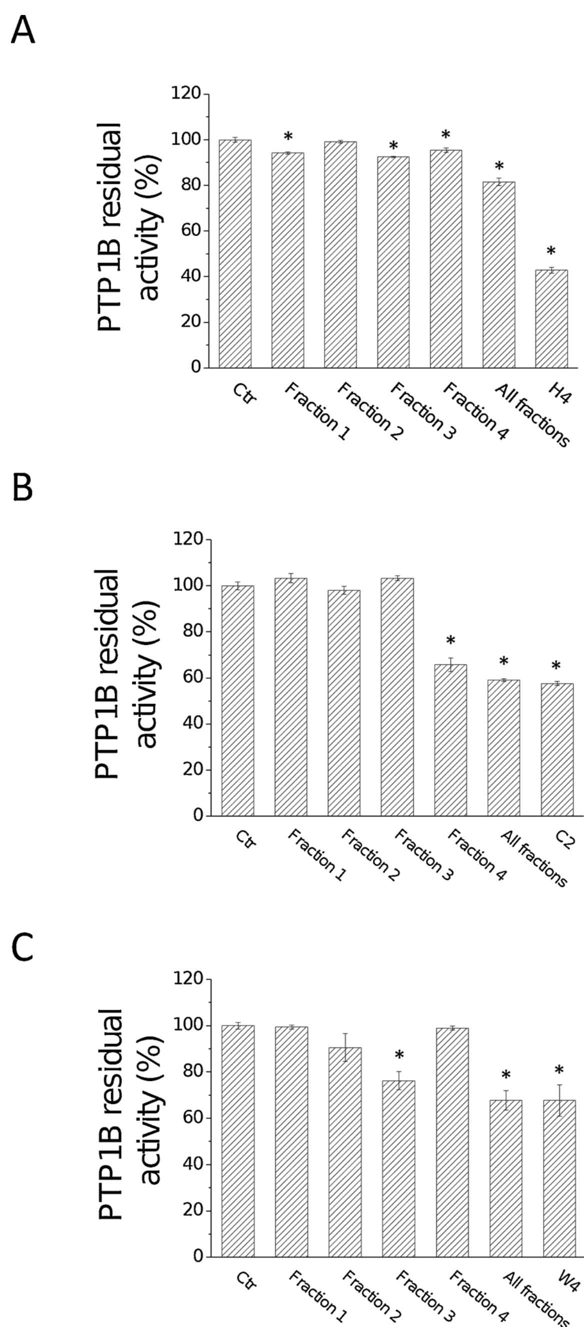


Fig. 3. Evaluation of the inhibitory power of honey fractions eluted from semipreparative HPLC. One hundred microliters of H4, C2, and W4 honey extracts were injected in into C18 column (Kinetex 5 µm C18 100 Å LC Column 250 × 4.6 mm). Elution solvent was collected in 4 fractions: Fraction 1: 9–11 min; Fraction 2: 15–22 min; Fraction 3: 24–28.5 min; Fraction 4: 30–40 min. Fraction were drying under vacuum. Then, the dry residue was solubilized in 100 µL of 80% ethanol solution. Inhibitory power of these solutions was evaluated by enzymatic assay, mixing 10 µL of solution into 1 mL of buffer assay containing substrate of PTP1B enzyme. (A) was for H4 honey extract; (B) was for C2 honey extract and (C) was for the W4 honey extract, respectively. (* $p < 0.05$).

3.7. Ex vivo assays

We used HepG2 cells to assess the effects of honey extracts on liver cells. Preliminary tests carry out with liver cells treated with honey extracts alone showed that these were not able to enhance phosphorylation levels of insulin receptor, neither after 5 days chronic stimulation. This finding suggest that honey extracts do not possess insulin mimetic activity. On the other hand, we know that honey, because of its elevate content in glucose, stimulates the release of insulin [8]. Based on these evidences, we decided to study the effects of honey extracts in co-stimulation with insulin, to evaluate whether the extracts possess insulin-sensitizing activity. Cells were grown in starvation medium for 96 h, in presence or not of H4 honey extract, and then were stimulated with insulin. Phosphorylation levels, as well as total IR expression levels were evaluated by immunoblot. We found that cells treated with honey extracts expressed higher IR levels respect to control cells, improving insulin-signalling transduction, through higher IR phosphorylation levels (Fig. 5). Interestingly, we observed that all the extracts were active in stimulating IR expression in HepG2 cells, even if H4 and W4 samples resulted more active than C2 and A5 extracts (Fig. 6A, B).

Then, to evaluate whether treatment with honey extracts was able to boost insulin signalling, we measured the rate of glucose incorporation in liver cells treated with the honey extracts and stimulated with insulin. We found that glucose incorporation was higher in liver cells pre-treated with C2, W4 and H4 honey extracts than in control cells (Fig. 7). On the contrary, no significant increase of glucose uptake was observed in liver cells treated with the A5 extract respect to control cells. Taken together, these results showed that treatment with honey extracts stimulated IR expression, increased IR phosphorylation levels, and, in some cases, enhanced glucose uptake in liver cells. We suggest that the inactivity of acacia extract (A5) in enhancing glucose uptake could be due to low content of bioactive molecules in this type of honey.

4. Discussion

Honey is a natural food, which possesses medical properties such as antioxidant, anti-inflammatory, antibacterial, antiviral and antitumoral activities [28]. Preclinical and clinical studies conducted in the last decades, demonstrated also that the regular assumption of honey improves glycaemic control in mice and humans affected by diabetes, reduces levels of glycosylated haemoglobin, and favours the loss of body weight [10,29,30]. However, to date the molecular bases of the anti-diabetic activity of honey remain to be clarified.

We focused our study on evaluating the effect of honey on PTP1B, one the most interesting targets for treatment of type II diabetes and obesity [31]. Aim of this study was to evaluate i) whether natural compounds present in this food are able to inhibit this enzyme, and ii) whether honey could have a positive impact on insulin signaling pathway. Moreover, to eliminate confounding elements, we decided to analyze the effects of hydroalcoholic extracts instead of whole honey, and to treat liver cells with small amount of these extracts, thereby simulating the effect of a moderate daily assumption. This experimental approach is different from that used in most of studies conducted on honey to date, which, conversely, evaluated the effects of acute administration of relevant amount of honey [32–34]. Although the use of such an experimental approach would have favoured the achievement of a positive outcome also in our studies, we have excluded it since it would have been impracticable with humans. In fact, is important to remember that honey is rich in glucose, and that chronic assumption of high amount of this food could lead severe side effects both in healthy than in diabetic subjects. Finally, we analyzed the effects of extracts only in co-stimulation with insulin, confident that in humans the natural compounds and insulin cannot act independently after honey assumption.

We showed that honey extracts are able to inhibit PTP1B *in vitro*, being the inhibitory power strictly related to the content of natural compounds. Mass spectrometry analyses revealed that honey extracts

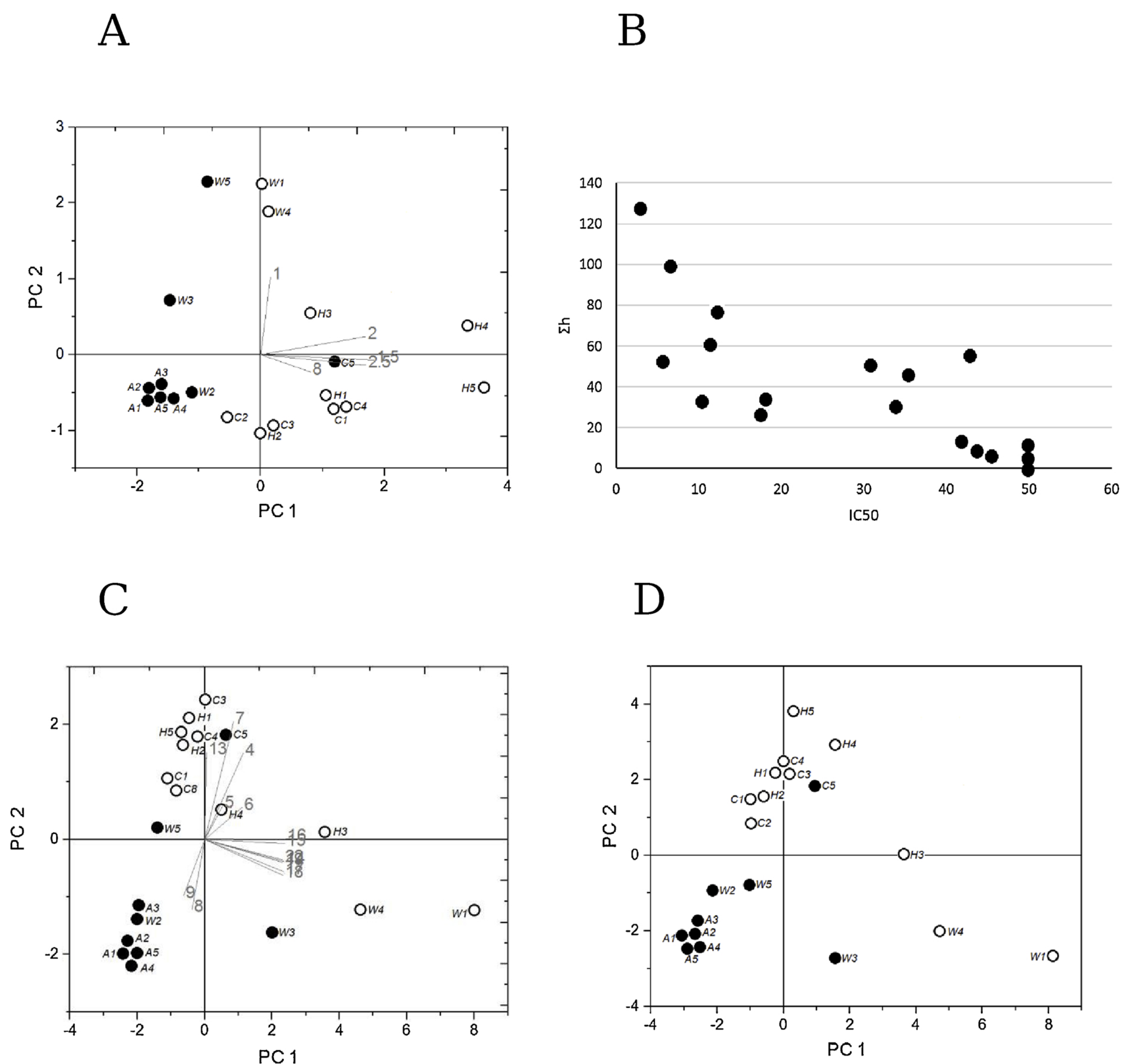


Fig. 4. PCA and LDA data. (A), biplot diagram of PCA obtained from ^{1}H-NMR data. The original variables are indicated with vectors at the end of which the starting range values are shown (e.g. variable "2" is the frequency range 2.0–2.5 ppm). Letters and numbers identified each sample. (B), IC_{50} values vs sum of peak height frequencies range 1.80–1.90 ppm (Σh). (C), biplot diagram of PCA obtained from HPLC data. The original variables are indicated with vectors at the end of which the starting range values are shown (e.g. variable "4" is the range of retentions times 4.0–5.0 min). (D), score plot diagram of PCA obtained from ^{1}H-NMR and HPLC data.

Table 3

Correlation matrix (range of resonance frequencies vs range of retention times). The table highlights the HPLC peaks that correlate, with a significant correlation coefficient, with the greatest number of ^{1}H-NMR signals.

	1.0-1.5 ppm	1.5-2.0 ppm	2.0-2.5 ppm	2.5-3.0 ppm	8.0-8.5 ppm
4-5 min	0.14	0.75	0.62	0.75	0.36
5-6 min	0.26	0.21	0.26	0.48	0.01
6-7 min	0.12	0.54	0.40	0.44	−0.02
7-8 min	0.11	0.72	0.65	0.70	0.70
8-9 min	−0.12	−0.07	−0.10	−0.03	−0.62
9-10 min	−0.22	−0.26	−0.28	−0.30	−0.42
13-14 min	−0.01	0.20	0.19	0.07	0.77
14-15 min	0.69	−0.01	0.34	−0.05	−0.10
15-16 min	0.68	0.08	0.41	0.02	0.04
16-17 min	0.69	0.16	0.42	0.11	0.01
17-18 min	0.71	−0.04	0.34	−0.05	−0.19
18-19 min	0.68	−0.08	0.32	−0.08	−0.21
19-20 min	0.65	−0.06	0.32	−0.08	−0.08
22-23 min	0.68	−0.03	0.35	−0.06	−0.07
36-37 min	0.01	0.46	0.30	0.31	0.05

Table 4
Library of compounds detected in honey. Data reported in the table were obtained from published papers [18–21].

Monoisotopic mass	Molecular Formula	Name of compound
196.0736	C10H12O4	4-methoxyphenyllactic acid
120.05751	C8H8O	Methylated Luteolin
122.03678	C7H6O2	Benzoic Acid
126.03169	C6H6O3	Hydroxymethylfurfural
136.05243	C8H8O2	Phenylacetic acid
138.03169	C7H6O3	3-hydroxybenzoic acid
138.03169	C7H6O3	4-hydroxybenzoic acid
138.03169	C7H6O3	p-Hydroxybenzoic Acid
138.03169	C7H6O3	Salicylic acid
142.02661	C6H6O4	Kojic acid
148.05243	C9H8O2	Cinnamic Acid
148.05243	C9H8O2	Thymol
148.05243	C9H8O2	Trans-cinnamic acid
150.10446	C10H14O	Carvacrol
152.04730	C8H8O3	Mandelic acid
152.04734	C8H8O3	2-methoxybenzoic acid
152.04734	C8H8O3	4-hydroxyphenyl acetic acid
152.04734	C8H8O3	4-methoxybenzoic acid
152.04734	C8H8O3	p-hydroxyphenylacetic acid
154.02661	C7H6O4	2,4-Dihydroxybenzoic Acid
154.02661	C7H6O4	Gentisic acid
154.02661	C7H6O4	Protocatechuic acid
164.04734	C9H8O3	m-Coumaric Acid
164.04734	C9H8O3	o-Coumaric Acid
164.04734	C9H8O3	p-Coumaric acid
164.04734	C9H8O3	p-hydroxycinnamic acid
165.07897	C9H11NO2	Phenylalanine
166.06299	C9H10O3	L-(-)-phenyllactic acid
168.04226	C8H8O4	4-hydroxy-3-methoxybenzoic acid
168.04226	C8H8O4	Homogentisic acid
168.04226	C8H8O4	Vanillic Acid
170.02152	C7H6O5	Gallic acid
180.04226	C9H8O4	Caffeic Acid
182.05791	C9H10O4	DL-p-hydroxy-phenyllactic acid
189.04259	C10H7NO3	Kynurenic acid
194.05791	C10H10O4	Coniferic acid
194.05791	C10H10O4	Ferulic acid
198.05282	C9H10O5	Syringic acid
212.06847	C10H12O5	Methyl Syringate
222.08921	C12H14O4	3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione
222.12559	C13H18O	Dehydrovomifoliol
224.06847	C11H12O5	Sinapic acid
228.07864	C14H12O3	Resveratrol
242.08037	C12H10N4O2	Lumichrome
248.10486	C14H16O4	Dimethylallylcaffeate
254.05791	C15H10O4	Chrysin
256.07356	C15H12O4	Pinocembrin
264.13616	C15H20O4	Abscisic acid
264.13616	C15H20O4	Trans–trans abscisic acid
264.13616	C15H20O4	Trans–Trans-Abscisic Acid
268.07356	C16H12O4	Tecthochrysin
270.05282	C15H10O5	Apigenin
270.05282	C15H10O5	Baicalein
270.05282	C15H10O5	Galangin
270.05282	C15H10O5	Genistein
270.08921	C16H14O4	Pinocembrin 7-Methylether
272.06847	C15H12O5	Naringenin
272.06847	C15H12O5	Pinobankisin
284.06847	C16H12O5	Acacetin
284.06847	C16H12O5	Galangin 3-Methyl Ether
284.06847	C16H12O5	Genkwanin
284.10486	C17H16O4	Phenylethyl Caffeate
286.04774	C15H10O6	Fisetin
286.04774	C15H10O6	Luteolin
286.08412	C16H14O5	Pinobanksin methyl ether
288.06339	C15H12O6	Eriodictyol
290.07904	C15H14O6	Catechin
290.07904	C15H14O6	Epicatechin
300.06339	C16H12O6	Kaempferid
300.06339	C16H12O6	Kaempferol 8-Methyl Ether
300.06339	C16H12O6	Methylbenzaldehyde
302.00627	C14H6O8	Ellagic Acid
302.04265	C15H10O7	Tricetin
302.07904	C16H14O6	Hesperetin
306.07395	C15H14O	Epigallocatechin
306.07395	C15H14O7	Gallocatechin
314.07904	C17H14O6	Pinobanksin acetate
316.05830	C16H12O7	3-Methylquercetin
316.05830	C16H12O7	8-Methoxy Kaempferol
316.05830	C16H12O7	Isorhamnetin
316.05830	C16H12O7	Quercetin-3-methyl ether
318.03757	C15H10O8	Myricetin
330.07395	C17H14O7	Quercetin 3,3-Dimethyl Ether"
330.07395	C17H14O7	Quercetin 3,7-dimethyl ether"
330.07395	C17H14O7	Quercetin 7,3'-dimethyl ether"
330.07395	C17H14O7	Quercetin-3,3'-dimethyl ether
342.11034	C19H18O6	Pinobanksin butyrate
354.09508	C16H18O9	caffeoylquinic acid
354.09508	C16H18O9	Chlorogenic acid
354.14672	C21H22O5	Isoxanthohumol
360.08452	C18H16O8	Rosmarinic Acid
370.14164	C21H22O6	(2R)-3,7,4'-trihydroxy-5-methoxy-8-dimethylallyl flavanone
376.13828	C17H20N4O6	Riboflavin
408.19367	C25H28O5	Andkushenol A
424.18859	C25H28O6	Sophora-flavanone G
438.16785	C25H26O7	Kushenol C
438.20424	C26H30O	Kuraridine
438.20424	C26H30O6	kurarinone
438.20424	C26H30O6	Leachianone A
447.09274	C21H19O11	8-Methoxy kaempferol Glycoside
448.10056	C21H20O11	Kaempferol Glycoside
448.10056	C21H20O11	Luteolin Glycoside
448.10056	C21H20O11	Quercetin
454.19915	C26H30O7	Askushenol H
454.19915	C26H30O7	Kushenol N
456.21480	C26H32O7	Kurarinol
458.08491	C22H18O11	Epigallocatechin gallate
458.08491	C22H18O11	Gallocatechin gallate
464.09548	C21H20O12	Quercetin Glycoside
470.23045	C27H34O7	Neokurarinol
472.20972	C26H32O8	Kushenol K
494.10604	C22H22O13	Myricetin 3,7,4,5-Methyl Ether
580.17921	C27H32O14	Naringin
594.15847	C27H30O15	Kaempferol rutinoside
610.15339	C27H30O16	Quercetin rutinoside
610.15339	C27H30O16	Quercetin-3-o-rutinoside
610.15339	C27H30O16	Rutin

contained small lipophilic molecules containing phenolic groups and aliphatic substituents. It is interesting to underline that several compounds bearing these physical-chemical properties have been already classified as PTP1B inhibitors [15]. Moreover, *in vivo* experiments showed that chronic stimulation with extracts enhanced expression and phosphorylation levels of insulin receptor, and stimulated glucose absorption. Even if the increase of IR phosphorylation is an expected result, as consequence of the PTP1B inhibition, conversely, the increase of insulin-receptor expression levels is a particular interesting result that could open new scenarios in studying the roles of nutraceuticals in insulin signalling. To date, we do not know the mechanisms by which honey extracts regulate the expression of the insulin receptor. We can speculate that some of the extract components could activate specific transcription factors, or could inhibit β-site amyloid precursor protein cleaving enzyme 1 (BACE1) which, cleaving insulin receptor ectodomain, regulates insulin signaling in the liver [35]. Together, data reported in this study reinforce the hypothesis that honey can acts as antihyperglicaemic food, regulating the expression of insulin receptor and enhancing insulin sensitivity.

Table 5
List of masses detected in honey extracts. Molecular weight of compounds present in honey extracts were determined by using a HPLC-MS apparatus. The identification of compounds was carried out by comparing the experimental findings with data reported in the Table 4.

Compounds	rt (min)	M.W.	Measured M.W.	Modality	H4	C2	W4	Ref.
Kushenol A	38.46	408.494	407,1875	neg	–	✓	✓	
Kurarinone, or Kuraridine, or Leachianone A	36.21	438.20	437,1925	neg	✓	✓	✓	[18]
Acacetin, or Galangin 3-Methyl Ether, or Genkwanin	35.96	284.07	283,0625	neg	✓	✓	✓	[19]
Pinobanksin methyl ether	35.96	286.27	285,0775	neg	✓	✓	✓	
Apigenin, or Baicalein, or Galangin, or Genistein	35.71	270.052	269,0475	neg	✓	✓	✓	[19,22]
Chrysin	35.46	254.058	253,0525	neg	✓	✓	✓	[19]
Phenylethyl Caffeate	35.46	284.311	283,097	neg		✓	✓	
Pinocembrin	34.71	256.257	255,06	neg	✓	✓	✓	[20]
Quercetin 3,4'-dimethyl ether	34.21	330.074	331,082	pos	✓	✓	✓	
Dimethylallyl Caffeate	33.96	248.27	247,0975	neg	–	✓	–	
Tectochrysin	32.46	268.073	267,0675	neg	✓	–	✓	
Fisetin, or kaempferol, or luteolin	31.71	286.04	285,0425	neg	✓	–	✓	[19]
Eriodictyol	31.71	288,063	287,0525	neg	✓	–	✓	
Naringenin, or Pinobankisin	29.21	272.06	273,0775	pos	✓	✓	✓	[21]
Abscisic acid	25.21	264.13	263,1275	neg	✓	✓	✓	
Kushenol K	24.71	472.21	473,2175	pos	–	–	✓	
Kaempferol rutinoside	22.71	594.15	593,1525	neg	–	–	✓	
Coniferic acid	19.96	194.057	195,0675	pos	✓	–	✓	
Hesperetin	18.71	302.079	303,0825	pos	–	–	✓	
4-methoxy-phenylactic acid	16.71	166.062	165,0525	neg	–	–	✓	
Caffeic acid	10.96	180.04	179,0325	neg	✓	✓	✓	[23]
Kynurenic acid	8.46	189.04	190,0475	pos	✓	✓	✓	

Despite these results appeared interesting, their relevance in humans remain to be established. For a long time, medicinal plants with antidiabetic potential were the only resource for treating diabetes in humans. In the last decades, several scientific studies have highlighted the molecular basis of antidiabetic activity of medicinal plants, confirming that these contain a large number of natural compounds. To date, we know that most of those have a positive impact in regulating glycaemia in diabetic mouse models, inhibiting glucose absorption in the gut, acting as insulin-mimetic or insulin-sensitizing agents [14], protecting pancreatic beta cells from apoptosis, or to stimulating insulin secretion [36,37]. Taking into account the effects of honey, studies conducted in the last decades both *in vitro* and *in vivo* have generated contrasting results [4]. The reasons are imputable to different kind of experimental protocols used, the kind of honey, the doses administered, and, most probably, to the tentative of evaluate the acute effect of honey. We think that, before to address this kind of studies, some important considerations must be made. First, honey is a food rich in glucose, which excluded the possibility to be administered in high doses to diabetic patients, without the risk to induce severe side effects (i.e. the gradual increase of HbA_{1c}). Latter, there is a limit in the intestinal absorption of most of bioactive constituents, suggesting that the chronic administration of small doses could be more effective and safe procedure than the administration of a relevant amount in a single dose. Unfortunately, in the last decades only two studies has been conducted to evaluate the effects of chronic assumption of honey on diabetic people [10,34]. Nevertheless, it is interesting to highlight that the study conducted by Abdulrhman MM and colleagues, reports that the administration of honey (0.5 mL/Kg) for 12 weeks resulted in significant decreases in fasting serum glucose, and significant increases in both fasting and 2-h postprandial C-peptide levels. More interestingly, patients affected by type 1 diabetes, also after honey withdrawal, maintained reduction in fasting serum glucose, 2-h postprandial serum glucose, and HbA_{1C} levels. These findings reinforced the hypothesis that long-term consumption of honey might have positive effects on glycaemic control in these patients.

Further analyses are necessary to evaluate levels of bioactive compounds absorbed by gut of humans depending on the dose of honey assumed, and their effect on different organs such as liver, muscle and adipose tissue.

5. Conclusions

In summary, the results of this study demonstrate for the first time that honey contains bioactive molecules able to improve insulin signaling. Our results clearly show that several honey extracts are able to inhibit PTP1B enzyme, and upregulate IR expression, thereby increasing insulin sensitivity. We believe these effects can have relevant clinical significance if confirmed also in humans. Indeed, downregulation of insulin receptor has been observed in hepatocytes chronically stimulated with insulin. The reduction of IR expression causes, in turn, the attenuation of insulin signaling pathway, which results in the constitutive activation of gluconeogenesis [38]. In light of the results of this study, we speculate that regular assumption of certain type of honey, by providing bioactive molecules able to simulate IR expression and inhibit PTP1B, could contributes to interrupt this vicious circle, thereby helping in restoring the correct insulin function. Furthermore, it is important to underline that the enhancement of insulin expression was achieved through a chronic stimulation of liver

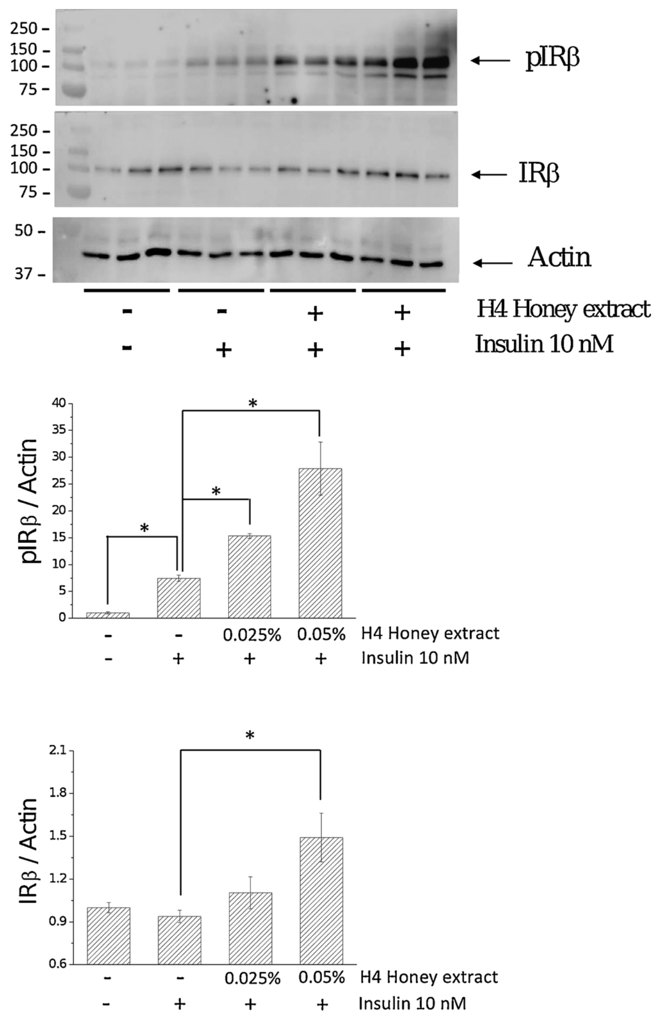


Fig. 5. Effects of honeydew extract (H4) on insulin receptor activation. HepG2 cells were treated with H4 honey extract (final concentration of extract was indicated in the figure) for 96 h and then stimulated with insulin. Phosphorylation levels, and total amount of insulin receptor, were evaluated by immunoblot, using specific antibodies. Quantification of immunoblot bands was carried out using Kodak MI software. Data obtained were normalized respect to control samples. Beta-actin was used as a loading control. Data reported in the figures, represent the mean values ± S.E.M. (n = 3) (* $p < 0.05$). Western blot (top). Quantification of phosphorylation levels of IR (middle). Quantification of expression levels of IR (bottom).

cells with very low amount of honey extracts, a condition that would mimicking a dietary regimen that includes daily assumption of moderate amount of honey.

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Conflict of interest

The authors declare that there are no conflicts of interest.

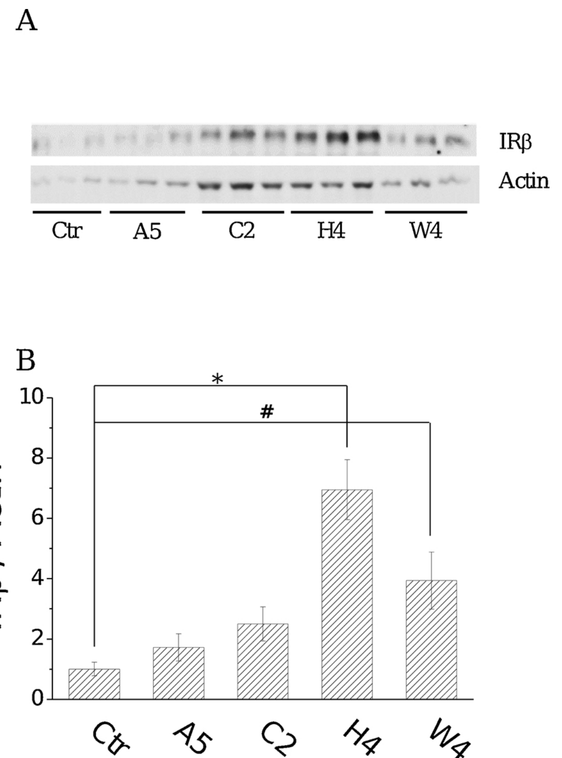

Fig. 6. Expression levels of insulin receptor in HepG2 cells treated with honey extracts. HepG2 cells were incubated with selected honey extracts for 96 h. Final concentration of honey extracts in each samples was 0.02%. After 96 h, total amount of insulin receptor was determined by immunoblot, using specific antibodies. The quantification of samples (B) was carried out using Kodak MI program. Beta-actin was used as a loading control. Data reported in the figure, represent the mean values ± S.E.M. (n = 3) *$p < 0.01$; # $p < 0.05$.

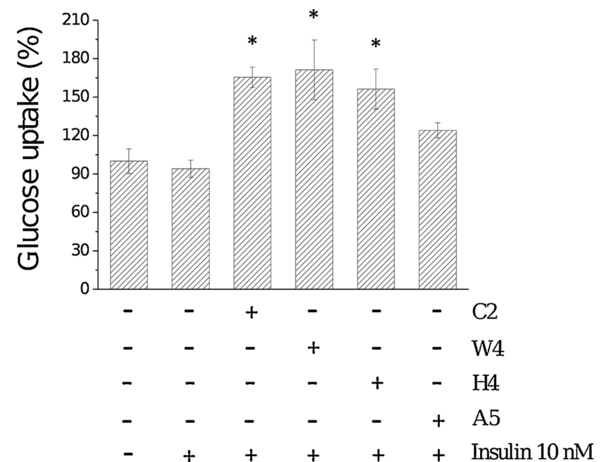

Fig. 7. Glucose uptake in HepG2 cells. Liver cells were treated for 4 days with honey extracts (0.02% final concentration). Every day, mediun was withdrawn and substituted with fresh medium containing fresh honey extracts. After four days, cells were washed with PBS and then incubated in the presence of [^{3}H]-deoxy-D-Glucose (0.5 µCi/mL final concentration) for 15 min. After, the cells were washed twice with ice-cold phosphate-buffered saline, and lysed using 500 µL of 0.1 M NaOH solution and assayed to determine the amount of [^{3}H]-deoxy-D-Glucose incorporated using a liquid scintillation analyzer (Tri-Carb 2800TR, PerkinElmer). Data were normalized respect to protein content. All data were normalized respect to control samples. Data reported in the figure represent the mean value ± S.E.M. *$p < 0.05$ (n = 4).

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.108752.

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