MicroRNA-34b/c is involved in the response of human liver cells to the Corylus avellana L. hazelnut extract

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Research Article

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Abstract

Background Epigenetic regulation by microRNAs (miRs) underlies liver tissue biology and affects the hepatocytes response to pathogens, drugs, and food-derived molecules.

Methods and results In this study we characterized the epigenetic role driven by an ethanolic extract of hazelnut (*Corylus Avellana L*, HZN) in both THLE-2 human primary hepatocytes and HepG2 human hepatocarcinoma cells (HCC), in terms of microRNA-34b/c involvement. Both the precursor transcript and mature molecules (miR-34b and miR-34c) were found hyper-expressed in primary cells if compared to HCC ($P<0.05$). When treated with HZN (0.04-0.4 mg/ml) for 72h, microRNA-34b/c underwent significant stimulation (≥ 2-fold change, $P<0.05$) exclusively in primary hepatocytes, whereas 144h of continuous HZN treatment were required to induce such an increase in HCC, demonstrating a different kinetics of miR-34 stimulation in liver cells, according to their either cancer or non-cancer phenotype. In THLE-2 cells, miR-34b/c stimulation plays a significant antioxidant role; when challenged with oxygen peroxide (H_2O_2 1000-2000 μM, 24h), cells are significantly protected from oxidative stress if pre-treated with HZN, the H_2O_2-driven cytotoxicity and reactive oxygen species generation being recovered by hazelnut. In HCC cells, prolonged exposure (144h) to high HZN concentration (0.4 mg/ml) triggers cell growth arrest and cell death, demonstrating a promising potential anti-neoplastic function of the biomolecules composing the hazelnut extract.

Conclusions Overall, our experimental findings demonstrate the epigenomic effect of the *Corylus Avellana L*. extract in human liver cells *in vitro*, highlighting the great potential of tree nuts consumption for personalized nutrition and of its bioactive molecules for possible therapeutic options.

Introduction

The epigenetic profile of human hepatic cells is a tissue-specific molecular signature which significantly affects liver morphogenesis, metabolism, response to both injury and external stimuli, ranging from toxins to food-derived biomolecules [1, 2].

MicroRNAs (miRs) are epigenetic modulators that regulate most biological processes in tissues, including liver, and –if deregulated- correlate with several hepatic diseases such as alcoholic and nonalcoholic steatohepatitis, hepatitis, autoimmune liver disease, drug-induced liver injury and cancer [3–5].

In this context, the experimental evidence addressing the specific role of microRNA-34b and microRNA-34c (miR-34b/c) in liver physiology, response to toxicants and eventually disease etiology, are not univocally understood. In human cells, miR-34b and miR-34c form a cluster on chromosome 11q23. They are transcribed from the same primary transcript (pri-miR-34b/c) to subsequently undergo specific pathways of processing to release mature single miRs molecules (miR-34b and miR-34c), that can each affect different functional pathways [6]. In hepatocellular carcinoma (HCC), molecular studies have characterized the functional single nucleotide polymorphism (SNP) rs4938723 C > T in the promoter region of pri-miR-34b/c gene -located in a typical CpG island- that affects its expression by genetic and

epigenetic mechanisms and triggers primiR-34b/c gene silencing [7, 8]. Epidemiological findings have further shown the association between the incidence of miR-34b/c rs4938723 C alleles in Chinese and Korean populations and the higher risk of developing HCC [9–11]. This evidence is consistent with the higher frequency of DNA methylation, thereby silencing, of miR-34b/c gene reported in HCC tissues if compared to adjacent non-tumor tissues [12, 13]. In accordance, miR-34c-3p down regulation correlates with advanced tumor stage and metastasis [13], and its over-expression in HepG2 and Huh7 HCC cells has been shown to reduce cell proliferation, migration and invasion by specifically targeting the myristoylated alanine-rich protein kinase c substrate (MARCKS) [13], in support of the hypothesis of miR-34b/c acting as tumor suppressor gene in liver cancer cells [14–16].

MicroRNA-34b/c family is also involved in the response to oxidative stress, especially in the central nervous system [17–19]. In liver cells, the response to free nitrogen and oxygen radicals represents a key event in the metabolism as well as in the toxin-induced damage and detoxification pathways [20]. Recently, miR expression changes have been also reported in hepatic cells in response to nutrients and natural compounds, highlighting the epigenetic basis of the nutrition metabolism [22]. The epigenetic impact on such mechanism(s) has been mostly described in terms of miR-34a involvement [23–26], whereas sparse data have been provided to define the role of miR-34b/c family in hepatic cells response to oxidative damage and on the potential protective role triggered by nutrients in liver stress.

The purpose of the present experimental study is to investigate the epigenetic effects of an ethanolic extract of hazelnut (*Corylus Avellana L*, HZN) in both human HepG2 hepatocarcinoma cells and THLE-2 primary hepatocytes, specifically in terms of miR-34b/c involvement. We demonstrate that hazelnut administration stimulates both miR-34b and miR-34c at transcription level in both cell lines, although with different kinetics and at different dose-range. The HZN-driven miR-34b/c stimulation is responsible for a significant cytotoxic effect in HCC cells, whereas it is associated to a significant antioxidant ability in primary hepatocytes.

As overall, our findings support the key role played by the miR-34 family in the response of human liver cells to hazelnut, and strengthen the functional ability of food-derived biomolecules through stable epigenetic signature that might be tuned and exploited to optimize the nuts-derived health benefits.

Materials And Methods

Chemicals

Phosphate Buffer Saline (PBS), Hank's balanced salt solution (HBSS), DMEM (Dulbecco's Modified Eagle's Medium) culture medium, culture supplements, and trypsin-EDTA (ethylenediaminetetraacetic acid) were obtained from Euroclone (Milan, Italy). LHC-8 culture medium was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). The fetal bovine serum was obtained from Hyclone (Logan, UT, USA). The bovine serum albumin (BSA), collagen, epidermal growth factor (EGF), ethylene-diamine-tetra-acetic acid (EDTA), fibronectin, hydrogen peroxide (H_2O_2), phosphoethanolamine, propidium

iodide (PI), RNAse, trypan blue solution (0.4%) and Triton X-100 were obtained from SIGMA-Aldrich (St Louis, MO, USA). Ethanol was purchased from CARLO ERBA Reagents (Milan, Italy).

Preparation of the hazelnut liquid extract (HZN)

Preparation of the ethanolic extract of hazelnut (Corylus avellana L., cultivar Tonda Gentile Romana, Coopernocciola srl, Vico Matrino, VT, Italy) was carried out as previously described [27]. Briefly, 20 grams of raw materials were homogenized, and added to 60% aqueous ethanol solution at liquid:solid ratio of 1:5 (v/w). After 1h of continuous stirring, the extraction process was carried out in closed bottle at room temperature (20-22 ºC), in dark conditions, for 30 days. The hazelnut suspension was mixed by hand each three days. At the end of the maceration procedure, the hazelnut liquid extract (HZN) was collected, passed through a filter of 0.2µm, aliquoted and stored at -80ºC for all experiments. The complete metabolomic characterization of the HZN ethanolic liquid extract used in this study was performed by liquid chromatography-high resolution mass spectrometry, as previously detailed [28].

Cell cultures and treatments

HepG2 human hepatocellular carcinoma cells (RRID: CVCL_0027) were purchased from the European Collection of Cell Cultures (ECACC, Sigma-Aldrich), maintained as sub-confluent monolayers in DMEM, with 10% heat-inactivated foetal bovine serum, 2mM glutamine, 1% non-essential amino acids and 1% penicillin-streptomycin (10,000 U/ml), at 37°C in a 5% CO2 atmosphere in air.

THLE-2 cells (RRID: CVCL_3803), derived from SV40-immortalized normal human liver cells, were purchased from the American Type Culture Collection, ATCC (Manassas, VA, USA). They were cultured in LHC-9 medium, supplemented with 70 ng/ml phosphoethanolamine, 5 ng/ml EGF, 10% fetal bovine serum (Hyclone) and antibiotics as above. Flasks and dishes for THLE-2 cultures and experiments were pre-coated with collagen (2.9 mg/ml), fibronectin (1 mg/ml) and BSA (1 mg/ml), according to ATCC guidelines.

In all the experiments, cells were seeded in 35mm Petri-dishes at 2x105 cells/dish. Twenty-four hours after plating, cells were treated with either the Hazelnut (HZN) ethanolic extract or the corresponding aqueous ethanol solution, as previously detailed [27-29]. The solutions were freshly prepared before each experiment in culture media.

The effect on cell proliferation and viability was assessed by counting viable cells at the hemocytometer, following trypan blue staining (0.4% solution). For inducing oxidative stress, THLE-2 cells were administered with H_2O_2 (doses ranging from 100 to 2000 mM) for 24 hours. In HZN-H_2O_2 combination experiments, cells were washed twice in PBS at the end of the 72 h HZN treatment, before adding hydrogen peroxide.

RNA extraction, reverse transcription and gene expression analysis

Total RNA was extracted by Trizol® (Invitrogen, Thermo Fisher Scientific) followed by spin-column elution, also including a DNAse I digestion step (Direct-zolTM RNA miniPrep, Zymoresearch, Irvine, CA, USA), as previously described [27]. 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 260/280 absorbance ratios.

TaqMan® Reverse Transcription Reagent (Applied Biosystems, Thermo Fisher Scientific) was used to perform retro-transcription of total RNA (200 ng) with random primers, according to manufactures' indications. Analysis of the Pri-miR-34b/c was carried out as previously described [19], with 1 µL of cDNA using SYBR Green master mix (Applied Biosystems) and analyzed on an Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA). All reactions were run in quadruplicate, and the relative abundance of the specific mRNA levels was normalized to the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) expression, applying the $2^{-\Delta\Delta Ct}$ method. PCR primers were designed by NCBI-Primer Blast free software (https://www.ncbi.nlm.nih.gov/tools/primerblast), according to gene sequences available in the UCSC database (https://genome.ucsc.edu), and selected to amplify an exon-intron-exon region (≤ 200bp) to exclude genomic contamination. PCR primers were synthesized by Eurofins Scientific (Luxembourg). Sequences were as follows: GAPDH forward (5'-GCACCGTCAAGGCTGAGAAC-3'); GAPDH reverse (5'-GAGGGATCTCGCTCCTGGA-3'); pri-miR34 forward (5'-GCTCTTTGTCCCTCCTGCTAGA-3'); pri-miR34 reverse (5'-GTGGGCGGTCCCTGAAG-3').

Mature microRNA expression analysis

The analysis of mature miRNA-34b and miRMA-34c expression was carried out on total RNA, as previously described [19]. Ten nanograms were retro-transcribed by the miRcury LNA universal RT microRNA kit (Exiqon, QIAGEN, Hilden, Germany); cDNA was diluted 1:80 and amplified by the miRcury LNA Sybr green master mix and miR-specific LNAPCR primer sets (Exiqon), according to the manufacturer's instructions. All reactions were run in quadruplicate and the relative abundance of each specific microRNA was normalized to RNU1A1 small nucleolar RNAs, by applying the 2−ΔΔCt method.

Flow cytometric analysis

A FACScan flow cytometer (Becton Dickinson, Bedford, MA, USA) equipped with a 488 nm argon laser, was used for all flow cytometric analyses.

The evaluation of the DNA content for the assessment of the percentage of the apoptotic cells (sub-G1 index) was carried out as previously described [27]. Adherent cells were harvested by trypsinization and collected with floating ones; the pool was washed twice in PBS, then fixed in ice-cold ethanol 70% (1×106 cells/ml) over-night. An aliquot of the suspension (at least 5×105 cells) was then washed twice in PBS, and stained with PI (50 µg/ml) in a mix containing RNAse A (50 µg/ml), Triton X-100 (0.1%), EDTA (0.1 mM) in PBS, in the dark, for 60 min at room temperature, then immediately analyzed. The evaluation of sub-G1 DNA content was performed by the FlowJo software®.

To quantify oxidative stress, cells were stained with the 2',7'-dichlorofluorescin diacetate (H2DCF-DA, Molecular Probes, Thermo Fisher Scientific), and analyzed as previously described [19]. Cells were quickly scraped on ice, washed twice in cold PBS, and re-suspended in 5 μM H2DCF-DA (30', 37 °C in the dark, in HBSS). After a final wash in PBS, cells were immediately transferred into a tube on ice for flow cytofluorometric analysis. For each measure, a minimum of 20,000 events were evaluated. The mean fluorescence intensity (MFI) was measured as the mean fluorescence value in the channel of the H2DCF-DA-labeled cells minus the mean fluorescence value of the unstained cells.

Statistical analysis

The variations of the samples values are reported as Mean ± S.D., calculated in N = 3 independent experiments. The statistical differences were analyzed trough the KailedaGraph software (Synergy Software, Reading PA, USA) by applying the one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. P values <0.05 were considered statistically significant and indicated as follows: *P <0.05.

Results

The administration of the hazelnut extract stimulates the miR-34b/c gene expression in human liver cells

We evaluated the expression level of both pri-miR-34b/c and mature miR-34b and miR-34c in THLE-2 human primary hepatocytes compared to HepG2 carcinoma cells. As reported in Fig. 1, basal primiR-34b/c gene level was significantly higher in THLE-2 than HepG2 cancer cells (Fig. 1A); consistently, mature forms of both miR-34b and 34c were hyper-expressed in primary if compared to hepatocarcinoma cells (Fig. 1B).

We then screened the response of both liver models to 72h of continuous challenge with the ethanolic extract of *Corylus Avellana L.* (HZN). According to our previous characterization, we selected 0.04 and 0.4 mg/ml hazelnut concentrations, as they were both demonstrated to induce no change in cell viability, integrity and proliferation in THLE-2 and HepG2 cells [27, 29]. The 0.06% ethanol solvent solution (corresponding to the highest HZN dose) was selected as control treatment [27, 29]. Figure 2A shows that 72h exposure to HZN triggers a significant dose-dependent increase in pri-miR-34b/c transcript level exclusively in THLE-2 primary cells, whereas no modulation is reported in HZN-treated HepG2 cells. In terms of mature miRs molecules, we demonstrate a slight divergence between 34b and 34c stimulation in response to HZN in primary hepatocytes; miR-34b expression level was significantly increased by HZN administration at both tested doses (Fig. 2B), while miR-34c is stimulated after 72h treatment with the highest hazelnut concentration (0.4 mg/ml) (Fig. 2C). No change in both mature miRs was reported in HepG2 cells (Fig. 2B-C).

We verified whether longer exposure to hazelnut extract might affect miR-34b/c level in HepG2 cells. As reported in Fig. 3, the stimulation of pri-miR-34b/c (Fig. 3A) as well as both miR-34b and miR-34c mature forms (Fig. 3B), was achieved in cancer cells by HZN only if administered for 144 h (6 days) at the highest dose applied (0.4 mg/ml). Concurrently, cell proliferation (Fig. 3C), cell viability (Fig. 3D) and percentage of apoptotic cells (as measured by sub-G1 population) (Fig. <link rid="fig3">3</link>D-3) were all impaired at 144 h of continuous exposure to 0.4 mg/ml HZN, thus demonstrating a cytotoxic effect at high concentrations of the ethanolic HZN extract in HCC cells, associated to a significant miR-34 deregulation.

The Hazelnut Ethanolic Extract Protects Thle-2 Cells From Ho-driven Oxidative Damage

To further investigate the functional significance of such HZN-driven miR-34b/c change in primary liver cells, we selected the hydrogen peroxide (H_2O_2)-induced oxidative stress as a model of cell injury. THLE-2 response to H_2O_2 was preliminarily characterized following the exposure to increasing concentrations of H_2O_2 (100–2000 μM dose range) administered for 24 h (Supplementary Fig. 1). Doses higher than 1000 μM H_2O_2 significantly impair both cell growth (Suppl. Figure 1A) and cell viability (Suppl. Figure 1B) of primary liver cells, concomitantly to a rise in intracellular reactive oxygen species (ROS) (Suppl. Figure 1C). Oxidative stress in THLE-2 is also characterized by a statistically significant decline in pri-miR-34b/c expression levels (Suppl. Figure 1D).

To test the potential antioxidant ability of the hazelnut extract in H_2O_2-treated cells, we applied the administration schedule reported in Supplementary Fig. 3A. THLE-2 cells were given 0.4 mg/ml HZN (or control ethanol 0.06% solution) for 72 h, then washed and treated with the highest hydrogen peroxide doses (1000, 1500, 2000 μM) for the following 24h. Both H_2O_2-induced proliferation and viability impairment (at 1000 and 1500 μM) was partially restored in HZN-pretreated cells (Fig. 4A-B), whereas hazelnut pre-treatment was unable to maintain its potential antioxidant effect at the highest H_2O_2 dose tested (2000 μM).

In accordance, ROS generation was significantly reduced in HZN-pretreated cells exclusively at 1000 and 1500 μM H_2O_2 concentrations (Fig. 4C and Supplementary Fig. 3B), the antioxidant effect being accompanied by a concomitant rise in pri-miR-34b/c expression driven by hazelnut stimulation (Fig. 4D).

Discussion

We here demonstrate that the microRNA-34b/c cluster is specifically stimulated by an ethanolic extract of hazelnut (*Corylus Avellana L.)* in liver cells (Fig. 5).

Epigenetic modulation -including miR-regulated pathways- is a hallmark of liver physiology and underlies the response to environmental agents, toxicans and stressors as well as aging [1, 2, 17]. Recently, miR

expression changes have been reported in hepatic cells also in response to nutrients and natural compounds, highlighting the epigenetic basis of the nutrition metabolism [22]. There is a growing piece of experimental evidence supporting the potential application of food and plant-derived active biomolecules in personalized nutrition, nutraceutics and also -if efficacy supported by preclinical studies- in innovative therapeutic options. The basis for such applications is the ability to specifically tune the epigenetic mechanisms in a tissue-specific fashion.

In HCC cells, licorice roots have been proved to activate apoptosis and cell cycle arrest through the upregulation of miR-34c-5p [30]. Treatment of HepG2 and Huh7 HCC with nanosome-encapsulated curcumin specifically regulates liver epigenetic machine, by both stimulating miR-34 family and downregulating the DNA methyltransferase expression [31]. We here prove and support the key role of the miR-34 family in the response of liver cells to food compounds, by studying the biological effects of an ethanolic extract of *Corylus Avellana L*. in terms of miR-34b/c expression. To the best of our knowledge, this is the first *in vitro* experimental evidence of a microRNA-based functional property of hazelnut related to human health. We demonstrate that a hazelnut stimulates miR-34b/c in both hepatocytes and HCC, although with a different kinetics and at different dose-range. Basal level of both the precursor (pri-miR-34b/c) and mature miR-34 forms significantly differs in HepG2 cells if compared to THLE-2 hepatocytes. This is consistent to what reported in literature and clinical studies, where miR-34b/c acts as tumor suppressor gene (TSG) in liver cancer cells [9, 14, 16]. We previously reported that HepG2 and THLE-2 cells also differ in DNA methylation pattern [27, 29], strengthening the difference occurring -at different epigenetic levels- in cancer cells if compared to hepatocytes. Based on this miR-34b/c TSG hypothesis, the reactivation of miR-34b/c might drive beneficial effect in cancer cells, as proven for licorice roots and curcumin [30, 31]. In HepG2 cells, we demonstrated that long exposure (144h), to the highest HZN dose tested, can stimulate miR-34b/c expression at transcription level. MicroRNA-34b/c over-expression drives a significant functional effect in HCC, the proliferation rate slowing down after long time exposure to HZN, together with a significant reduction of cell viability and concomitant induction of apoptosis at 144 h of HZN treatment, as proved by the increased sub-G1 percentage. These data demonstrate the cytotoxic ability of the whole ethanolic extract of *Corylus Avellana L.* in HepG2 cells *in vitro*, paving the way for further experimental studies aimed at identifying the mechanism(s) -and mainly the specific biomolecules- responsible for such effect, in the perspective of targeting miRNAs with natural/food-derived molecules as a promising tool for cancer therapy or adjuvant strategies. The metabolic profile of the ethanolic extract used in this study has been already characterized [28], highlighting a complex composition of bioactive molecules, including vitamins, alkaloids and phenylpropanoids. These biomolecules might be in future singularly studied and correlated to the HZN health beneficial properties to identify those specifically responsible for the promising anti-neoplastic effect here depicted.

In primary hepatocytes, the administration of *Corylus Avellana L* exerts a biological effect at lower doses than in HCC, as far as microRNA-34b/c expression is concerned. The treatment is effective in stimulating the miR-34b/c precursor as early as after 72 h of administration; moreover, there is a dose-dependent increase from 0.04 mg/ml to 0.4 mg/ml HZN dose in terms of pri-miR-34b/c. Interestingly, the maturation steps after the pri-miR transcription undergo a divergent destiny according to the type of final miR form:

mature miR-34b is sensitive to HZN treatment at both doses applied, whereas miR-34c rises up exclusively in 0.4 mg/ml-treated cells. This result discloses a well-documented molecular mechanism characterizing miR biogenesis, i.e. the occurrence of different levels of post-transcriptional regulation steps. Similarly to the regulation of gene expression, miRs molecules can undergo a further level of regulation of their expression after pri-miR transcription by RNA polymerase II [32, 33]. This is particularly relevant when poly-cistronic miR genes are involved, like the miR-34b/c locus. Mature microRNAs target and bind to different mRNA molecules according to their sequence, it is thus necessary to selectively regulate each mature miR to affect a specific gene/protein/pathway set under the epigenetic control. The evidence of a miR-specific effect in THLE-2 cells strengthen the functional properties of the hazelnut extract, that can finely tune a specific epigenetic pathway. MicroRNA-34b and 34c can indeed share gene targets and cooperate in regulating key biological processes, mainly in caner [34], but also silence different specific mRNAs and pathways [35]. A combination of both events might be activated in primary liver cells when exposed to the HZN extract.

In our experimental model, the miR-34b/c stimulation is involved in the protection of primary hepatocytes from the hydrogen peroxide (H_2O_2), selected as a model of cell injury. Oxidative stress is triggered by high concentrations of H_2O_2, as revealed by the massive ROS generation and concomitant cytotoxicity; interestingly, it is also associated to a significant decline in miR-34b/c expression levels. By pre-loading cells with the hazelnut extract for 72 h (at the highest 0.4 mg/ml HZN concentration that we proved to stimulate miR-34b/c), the oxidative damage induced by the hydrogen peroxide is recovered. This is in line with the experimental data obtained in animal models, where miR-34b protects against focal cerebral-induced oxidative stress (36), and improves the oxidative damage in substantia nigra of Parkinson's Disease animals [18]. We here demonstrate that the miR-34b/c-dependent antioxidant property is specifically activated by *Corylus Avellana L* in liver cells, although the protective effect is efficient over a defined window of oxidative damage. At the highest H_2O_2 concentration applied, the treatment with the nut is unable to help cells recovering from the oxidative stress, suggesting the occurrence of an irreversible damage to cell biological structures that overcomes the protective ability stimulated by the natural extract.

However, as the accumulation of oxidative damage, together with inflammation, mitochondrial dysfunction and telomere shortening, characterizes aging and age-related disorders, the hazelnut-dependent antioxidant function via epigenetic regulation is a promising field for further experimental investigation.

Conclusions

Overall, our experimental findings demonstrate the epigenomic power of the ethanolic extract of *Corylus Avellana L.* in human liver cells *in vitro*, that occurs via miR-34b/c stimulation. Given the role exerted by miRs in the hepatic response to both drugs and food-derived molecules, these data highlight the great potential of tree nuts consumption for personalized nutrition and of its bioactive molecules for possible therapeutic options.

Abbreviations

DNMT, DNA methyltransferase

H_2O_2, hydrogen peroxide

HCC, hepatocellular carcinoma

HZN, hazelnut

miR/miRNA, microRNA

SNP, nucleotide polymorphism

TSG, tumor suppressor gene

Declarations

Author contribution B.B. and F.P. conceived the study concept and design. B.B. performed and supervised the whole experimental study and led the interpretation and critical revision of the manuscript. L.B. prepared and characterized the *C. Avellana L.* extract. All authors revised and approved the final version of the manuscript.

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Availability of data and material All data generated and/or analyzed during this study are available from the corresponding author on reasonable request.

Conflict of interest The authors have no financial or non-financial interests to disclose.

Ethical approval Not applicable

References

1. Brosch M, Kattler K, Herrmann A et al (2018) Epigenomic map of human liver reveals principles of zonated morphogenic and metabolic control. Nat Commun 9(1):4150
2. Aloia L (2021) Epigenetic Regulation of Cell-Fate Changes That Determine Adult Liver Regeneration After Injury. Front Cell Dev Biol 9:643055
3. Szabo G, Bala S (2013) MicroRNAs in liver disease. Nat Rev Gastroenterol Hepatol 10(9):542–552
4. Wang X, He Y, Mackowiak B, Gao B (2021) MicroRNAs as regulators, biomarkers and therapeutic targets in liver diseases. Gut 70(4):784–795

5. Xu F, Jiang L, Zhao Q, Zhang Z, Liu Y, Yang S, Yu M, Chen H, Zhang J, Zhang J (2021) Whole-transcriptome and proteome analyses identify key differentially expressed mRNAs, miRNAs, lncRNAs and circRNAs associated with HCC. Oncogene 40(29):4820–4831
6. Agostini M, Knight RA (2014) miR-34: from bench to bedside. Oncotarget 5(4):872–881
7. Han Y, Pu R, Han X et al (2013) Associations of pri-miR-34b/c and pre-miR-196a2 polymorphisms and their multiplicative interactions with hepatitis B virus mutations with hepatocellular carcinoma risk. PLoS ONE 8:e58564
8. Xu Y, Liu L, Liu J, Zhang Y, Zhu J, Chen J, Liu S, Liu Z, Shi H, Shen H, Hu Z (2011) A potentially functional polymorphism in the promoter region of miR-34b/c is associated with an increased risk for primary hepatocellular carcinoma. Int J Cancer 128:412–417
9. Wang Z, Wu J, Zhang G, Cao Y, Jiang C, Ding Y (2013) Associations of miR-499 and miR-34b/c Polymorphisms with Susceptibility to Hepatocellular Carcinoma: An Evidence-Based Evaluation. Gastroenterol Res Pract 2013:719202
10. Son MS, Jang MJ, Jeon YJ et al (2013) Promoter polymorphisms of pri-miR-34b/c are associated with hepatocellular carcinoma. Gene 524(2):156–160
11. Hashemi M, Moazeni-Roodi A, Bahari G, Taheri M, Ghavami S (2019) Association between miR-34b/c rs4938723 polymorphism and risk of cancer: An updated meta-analysis of 27 case-control studies. J Cell Biochem 120(3):3306–3314
12. Xie K, Liu J, Chen J, Dong J, Ma H, Liu Y, Hu Z (2014) Methylation-associated silencing of microRNA-34b in hepatocellular carcinoma cancer. Gene 543(1):101–107
13. Song J, Wang Q, Luo Y, Yuan P, Tang C, Hui Y, Wang Z (2015) miR-34c-3p inhibits cell proliferation, migration and invasion of hepatocellular carcinoma by targeting MARCKS. Int J Clin Exp Pathol 8(10):12728–12737
14. He L, He X, Lim LP et al (2007) A microRNA component of the p53 tumour suppressor network. Nature 447(7148):1130–1134
15. Wang Y, Wang CM, Jiang ZZ et al (2015) MicroRNA-34c targets TGFB-induced factor homeobox 2, represses cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma. Oncol Lett 10(5):3095–3102
16. Sun YY, Xiao L, Wang D, Ji YC, Yang YP, Ma R, Chen XH (2017) Triptolide inhibits viability and induces apoptosis in liver cancer cells through activation of the tumor suppressor gene p53. Int J Oncol 50(3):847–852
17. Liu N, Landreh M, Cao K, Abe M, Hendriks GJ, Kennerdell JR, Zhu Y, Wang LS, Bonini NM (2012) The microRNA miR-34 modulates ageing and neurodegeneration in Drosophila. Nature 482(7386):519–523
18. Li Y, Fang J, Zhou Z, Zhou Q, Sun S, Jin Z, Xi Z, Wei J (2020) Downregulation of lncRNA BACE1-AS improves dopamine-dependent oxidative stress in rats with Parkinson's disease by upregulating microRNA-34b-5p and downregulating BACE1. Cell Cycle 19(10):1158–1171

19. Consales C, Cirotti C, Filomeni G et al (2018) Fifty-Hertz Magnetic Field Affects the Epigenetic Modulation of the miR-34b/c in Neuronal Cells. Mol Neurobiol 55(7):5698–5714
20. Muriel P (2009) Role of free radicals in liver diseases. Hepatol Int 3(4):526–536
21. Ross SA, Davis CD (2011) MicroRNA, Nutrition, and Cancer Prevention. Adv Nutr 2(6):472–485
22. Gracia A, Fernández-Quintela A, Miranda J, Eseberri I, González M, Portillo MP (2017) Are miRNA-103, miRNA-107 and miRNA-122 Involved in the Prevention of Liver Steatosis Induced by Resveratrol? Nutrients 9,36
23. Li N, Muthusamy S, Liang R, Sarojini H, Wang E (2011) Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1. Mech Ageing Dev 132(3):75–85
24. Wan Y, Cui R, Gu J, Zhang X, Xiang X, Liu C, Qu K, Lin T (2017) Identification of Four Oxidative Stress-Responsive MicroRNAs, miR-34a-5p, miR-1915-3p, miR-638, and miR-150-3p, in Hepatocellular Carcinoma. Oxid Med Cell Longev 2017:518913
25. Torres LF, Cogliati B, Otton R (2019) Green Tea Prevents NAFLD by Modulation of miR-34a and miR-194 Expression in a High-Fat Diet Mouse Model. Oxid Med Cell Longev 4:2019:4168380
26. Cossiga V, Lembo V, Nigro C et al (2021) The Combination of Berberine, Tocotrienols and Coffee Extracts Improves Metabolic Profile and Liver Steatosis by the Modulation of Gut Microbiota and Hepatic miR-122 and miR-34a Expression in Mice. Nutrients 13(4):1281
27. Benassi B, Santi C, Santangeli S, Grollino MG, Raschellà G, Bacchetta L, Pacchierotti F (2019) Modulation of LDL receptor expression and promoter methylation in HepG2 cells treated with a Corylus avellana L. extract. J Funct Foods 53:208–218
28. Cappelli G, Giovannini D, Basso AL, Demurtas O, Diretto G, Santi C, Girelli G, Bacchetta L, Mariani F (2018) A Corylus avellana L. extract enhances human macrophage bactericidal response against Staphylococcus aureus by increasing the expression of anti-inflammatory and iron metabolism genes. J Funct Foods 45:499–511
29. Bacchetta L, Pacchierotti F, Santangeli S, Benassi B (2021) Amplification of the Hazelnut-Induced Epigenetic Modulation of LDLR Gene Expression in THLE-2 Human Primary Hepatocytes Compared to HepG2 Hepatocarcinoma Cells. Austin J Nutr Metab 8(2):1103
30. Abdel-Wahab AA, Effat H, Mahrous EA, Ali MA, Al-Shafie TA (2020) A Licorice Roots Extract Induces Apoptosis and Cell Cycle Arrest and Improves Metabolism via Regulating MiRNAs in Liver Cancer Cells. Nutr Cancer 24:1–12
31. Chamani F, Sadeghizadeh M, Masoumi M, Babashah S (2016) Evaluation of MiR-34 Family and DNA Methyltransferases 1, 3A, 3B Gene Expression Levels in Hepatocellular Carcinoma Following Treatment with Dendrosomal Nanocurcumin. Asian Pac J Cancer Prev 17(S3):219–224
32. Obernosterer G, Leuschner PJ, Alenius M, Martinez J (2006) Post-transcriptional regulation of microRNA expression. RNA 12(7):1161-7
33. Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM (2006) Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev

20(16):2202–2207. doi:10.1101/gad.1444406

34. Corney DC, Flesken-Nikitin A, Godwin AK, Wang W, Nikitin AY (2007) MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res 67(18):8433–8438
35. Kim JS, Kim EJ, Lee S et al (2019) MiR-34a and miR-34b/c have distinct effects on the suppression of lung adenocarcinomas. Exp Mol Med 51:1–10
36. Huang R, Ma J, Niu B, Li J, Chang J, Zhang Y, Liu P, Luan X (2019) MiR-34b Protects Against Focal Cerebral Ischemia-Reperfusion (I/R) Injury in Rat by Targeting Keap1. J Stroke Cerebrovasc Dis 28(1):1–9

Figures

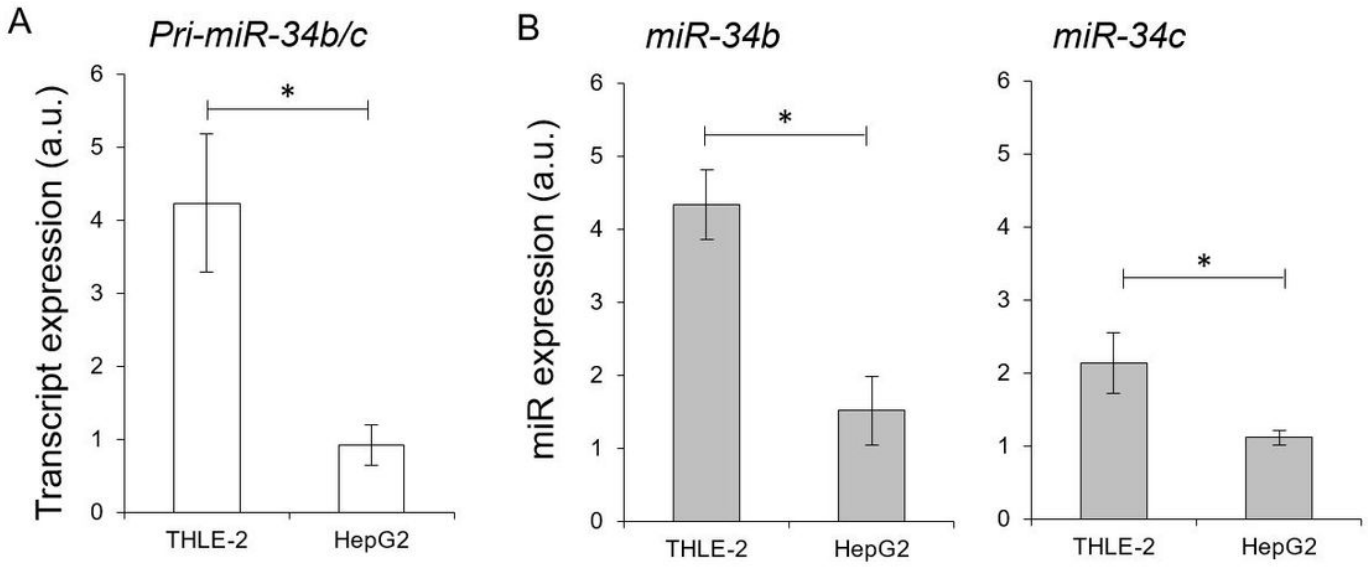

Figure 1

MicroRNA-34b/c basal expression level differs in HepG2 cells if compared to THLE-2 primary hepatocytes. Evaluation of (**A**) pri-miR-34b/c gene expression, (**B**) mature miR-34b and (**C**) mature miR-34c levels, carried out by real-time PCR in THLE-2 human primary hepatocytes and HepG2 human hepatocarcinoma cells. Values are expressed as Mean ± S.D. calculated in N=3 independent experiments. *P <0.05, calculated in HepG2 versus THLE-2 cells.

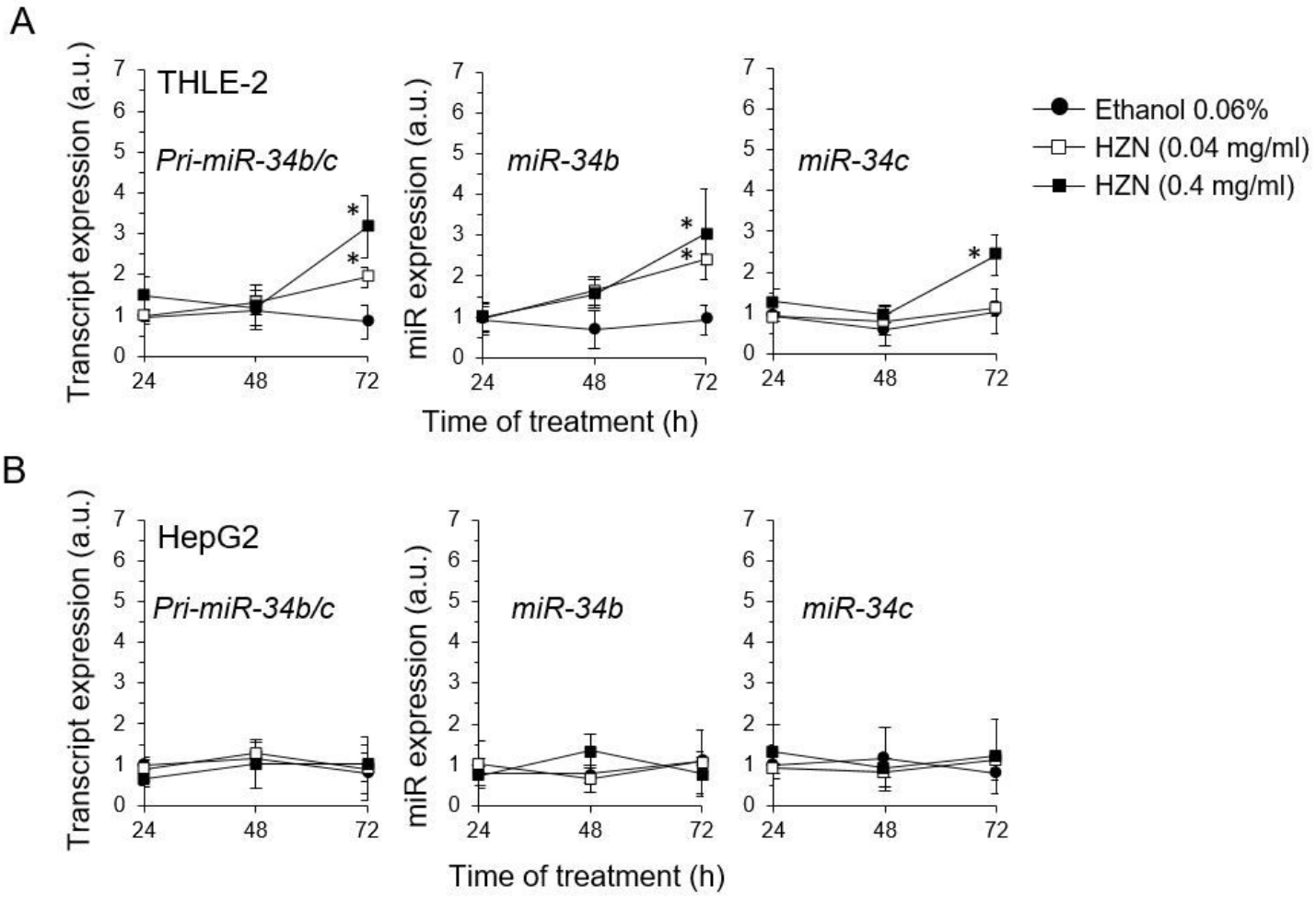

Figure 2

Seventy-two hours treatment with the ethanolic extract of *Corylus Avellana L.* (HZN) stimulates miR-34b/c expression level exclusively in THLE-2 human primary hepatocytes. Analysis of pri-miR-34b/c gene expression, mature miR-34b and mature miR-34c levels, carried out by real-time PCR in (**A**) THLE-2 human primary hepatocytes and (**B**) HepG2 hepatocarcinoma cells. Both cell lines have been treated with either HZN (0.4-0.04 mg/ml) or control ethanol (0.06%) up to 72h. Values are expressed as Mean ± S.D., calculated in N=3 independent experiments. *P <0.05 refers to HZN-treated cells compared to control ethanol-exposed cells.

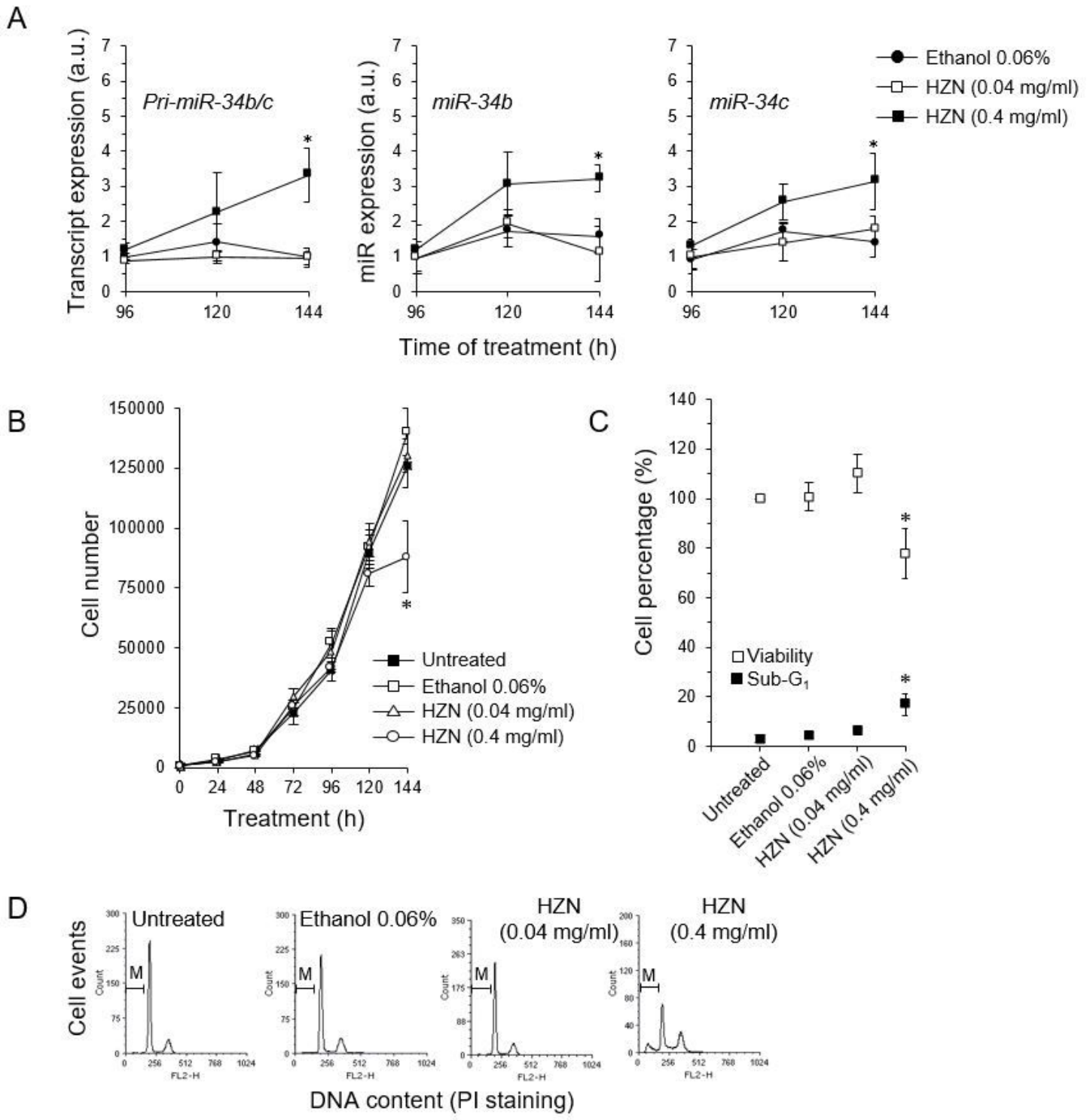

Figure 3

Long exposure (144h) to *Corylus Avellana L.* (HZN) stimulates miR-34b/c expression and drives cytotoxic effects in HepG2 hepatocarcinoma cells. Evaluation of (**A**) pri-miR-34b/c gene expression and mature miR-34b and miR-34c levels, carried out by real-time PCR in HepG2. Cells were treated with either HZN (0.4-0.04 mg/ml) or control ethanol (0.06%) up to144h. Values are expressed as Mean ± S.D., calculated in N=3 independent experiments. *P <0.05 refers to HZN-treated cells compared to control ethanol-

exposed cells. (**B**) Cell number evaluation performed in cells treated with HZN (0.4-0.04 mg/ml) or control ethanol (0.06%) up to 144h. Untreated cells have been also included in the study. Values are expressed as Mean ± S.D., calculated in N=3 independent experiments. *P <0.05 refers to HZN-treated cells compared to control ethanol-exposed cells. (**C**) Analysis of both viability and apoptosis (percentage of sub-G1 cells) carried out in HepG2 cells at 144h of continuous treatment with either HZN or control ethanol solution. Untreated cells have been also included in the study. Values are expressed as Mean ± S.D., calculated in N=3 independent experiments. *P <0.05 refers to HZN-treated cells versus control ethanol-exposed cells. (**D**) Representative flow cytometry histograms of cells undergoing 144h of treatment with the indicated agents. The images report the DNA content of cells, describing the cell cycle distribution and sub-G1 content (indicated by the M marker).

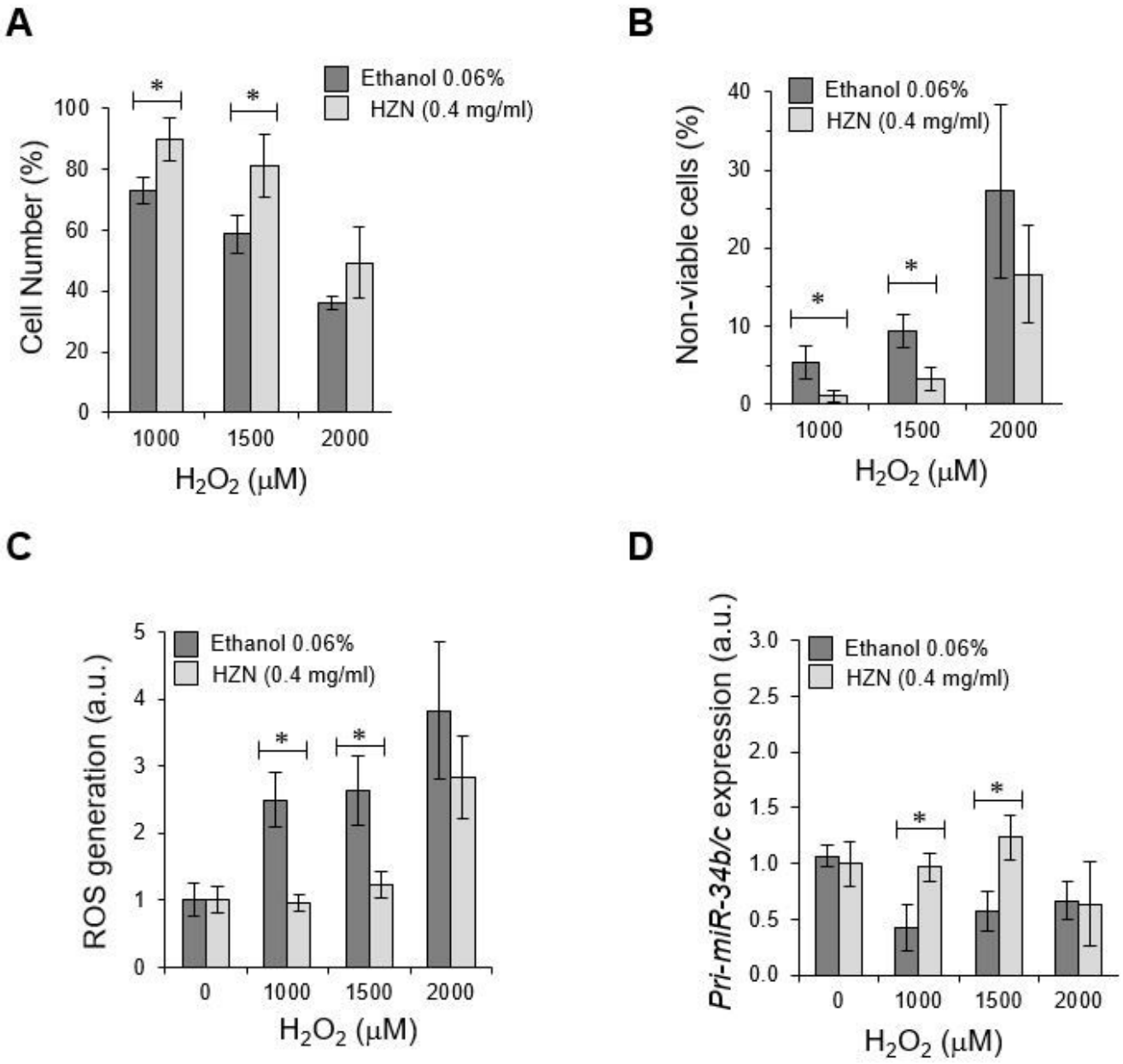

Figure 4

Corylus Avellana L. protects THLE-2 human primary hepatocytes from H_2O_2-induced oxidative stress. Evaluation of (**A**) cell number (%), (**B**) viability (%), (**C**) ROS generation, and (**D**) pri-miR-34b/c gene expression, carried out in THEL-2 cells undergoing HZN+ H_2O_2 combination treatment. Values are expressed as Mean ± S.D., calculated in N=3 independent experiments. *P <0.05 refers to HZN-treated cells versus control ethanol-exposed cells at any given H_2O_2 concentration.

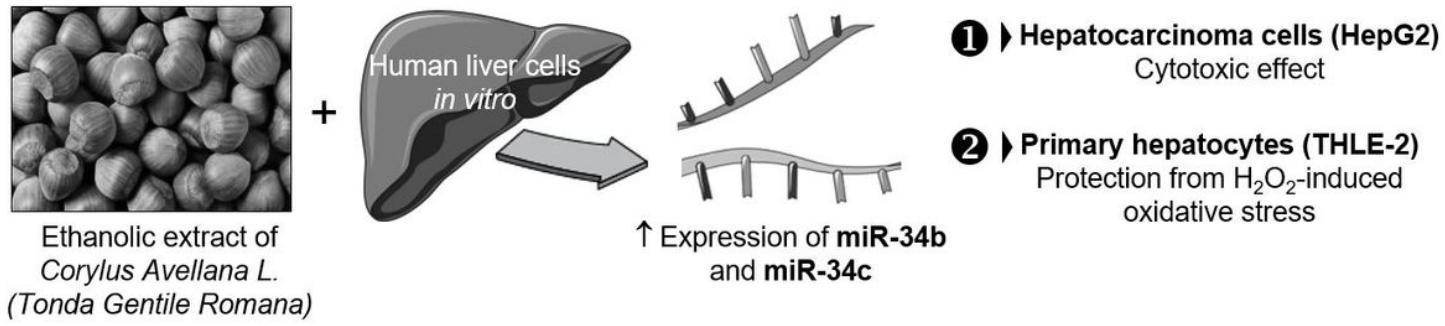

Figure 5

Graphical Abstract. The figure summarizes the molecular pathways tuned by *Corylus Avellana L* treatment in both human HCC and primary hepatocytes, according to the experimental findings here demonstrated. Hazelnut stimulates miR-34b/c expression level in both cell types and (1) triggers a specific cytotoxic effect in cancer cells; (2) drives a significant antioxidant function in H H_2O_2-challenged primary hepatic cells. (Hazelnut image downloaded from the Coopernocciola srl. Artworks taken and modified from https://smart.servier.com/smart_image).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SI1.pdf
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