



Whole transcriptome expression array analysis of human colon fibroblasts culture treated with *Helichrysum italicum* supports its use in traditional medicine

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ABSTRACT

Ethnopharmacological relevance: *Helichrysum italicum* (HI) is a Mediterranean plant with well-reported use in traditional medicine for a wide range of applications, including digestive and liver disorders, intestinal parasitic infections, wound healing, stomach ache and asthma. However, little is known about the global mechanism behind its pleiotropic activity.

Aim of the study: The aim of this study was to explain the mechanism behind the previously demonstrated effects of HI and to justify its use in traditional medicine.

Materials and methods: A microarray-based transcriptome analysis was used to discover the global transcriptional alterations in primary colon fibroblasts after exposure to HI infusion for 6 h and 24 h. In addition, quantitative real-time PCR was used to verify the microarray results.

Results: Altogether we identified 217 differentially expressed genes compared to non-treated cells, and only 8 were common to both treatments. Gene ontology analysis revealed that 24 h treatment with HI infusion altered the expression of genes involved in cytoskeletal rearrangement and cell growth, whereas pathway analysis further showed the importance of interleukin signaling and transcriptional regulation by TP53. For the 6 h treatment only the process of hemostasis appeared in the results of both enrichment analyses. In functional assays, HI infusion increased cell migration and decreased blood clotting and prothrombin time.

Conclusions: With the careful evaluation of the role of individual genes, especially *SERPINE1*, *ARHGAP1*, *IL33* and *CDKN1A*, represented in the enriched pathways and processes, we propose the main mode of HI action, which is wound healing. In addition to its indirect prevention of diseases resulting from the impaired barrier integrity, HI also effects inflammation and metabolic processes directly, as it regulates genes such as *LRPPRC*, *LIPA*, *ABCA12*, *PRKAR1A* and *ANXA6*.

1. Introduction

The plant usage in treatment of various diseases is a practice known since ancient times, and even nowadays, around 80% of people worldwide rely on herbal medicinal products for some part of primary healthcare (Ekor, 2014). The plant *Helichrysum italicum* (Roth) G. Don is an aromatic subshrub native to the Mediterranean region. The plant is well adapted to environments that lack water as it naturally grows on

alkaline, dry, sandy and poor soil at the altitude from the sea level up to 2200 m (Galbany-Casals et al., 2011). As a result, its yellow fade-resistant inflorescences and green aerial parts are a treasury of bioactive secondary metabolites with possible therapeutic potential. The health-beneficial potential of *H. italicum* (hereinafter abbreviated as HI) has been reported in ethnopharmacological surveys and supported by numerous *in vitro* and *in vivo* experiments. Traditional uses of the plant are related mostly to respiratory, digestive and dermatologic conditions, which include treatments of respiratory or intestinal infections, asthma,

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List of abbreviations:

CM	Conditioned Media
DEG	Differentially Expressed Gene
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
GEO	Gene Expression Omnibus
GO	Gene Ontology
HI	<i>Helichrysum italicum</i>
MIAME	Minimum Information About a Microarray Experiment
PBS	Phosphate Buffer Saline
RMA	Robust Multi-array Average
RT-PCR	Real Time Polymerase Chain Reaction

gall and bladder disorders, wounds and skin inflammation as well as pain management (Antunes Viegas et al., 2014). While *in vitro* studies report mostly antimicrobial, antioxidant and anti-inflammatory properties of HI extracts or its isolated substances, *in vivo* studies highlight also photoprotective, anti-erythematous, anti-spasmodic and hypoglycemic activity (Kramberger et al., 2021a). In phytotherapy research the cumulative extract activity should be investigated preferentially, since plant extracts are complex mixtures of bioactive substances that often exhibit additive or synergistic effects.

Based on the ethnopharmacological reports on HI use, the most common application is ingestion of the infusion or decoction. The gastrointestinal tract is most likely to be affected by ingestion of such beverages, since it has direct contact with the herbal solution and its components, usually in high concentrations, irrespective of whether they are absorbed, retained or re-circulated to the gut tissues (Koo and Cho, 2004). Fibroblasts are cells with spindle-shaped morphology that reside in human connective tissue and play variable roles within organs with different functions. In colonic tissue, subepithelial fibroblasts are known to contribute to the maintenance of epithelial cell homeostasis, wound healing, and immune responses (Higuchi et al., 2015), the latter two functions being especially relevant for HI activity. In addition, fibroblast have been extensively used for a wide range of cellular and molecular studies, as they are among the most accessible normal mammalian cell types and still the most amenable to culture *in vitro* (Chang et al., 2002). Therefore, primary colon fibroblasts were chosen as a cell model for testing the intestinal activity of HI infusion.

In our previous studies the phytochemical profile of HI prepared with different extraction procedures was examined and one hundred compounds were identified. Our key observation was that the hot water extracts (i.e., infusions) of HI proved to be comparably active as alcoholic ones in terms of radical scavenging potential (Kramberger et al., 2020). Activity of HI infusion was further tested in cellular and non-cellular *in vitro* tests where it has proved efficient in protecting different cell lines from induced oxidative stress and in inducing oxidative stress-related genes superoxide dismutase 1 (SOD1), catalase (CAT), and glutathione reductase 1 (GR1) (Kramberger et al., 2021b). Moreover, the HI infusion has also proved to be effective in increasing energy expenditure and fat oxidation in healthy male subjects. *In vivo* these effects were acute, whereas in cell culture they were detectable also at the transcriptional level, as we found that a 24 h treatment with HI infusion upregulated the expression of carnitine palmitoyl-transferase 1 and 2 (CPT1 and CPT2), and downregulated acetyl-CoA carboxylase (ACACA), suggesting increased β -oxidation of fatty acids (Kenig et al., 2021).

Despite extensive research on the bioactivity of HI, the mechanisms beyond its action on certain enzymes or dysregulation of individual genes that would explain such broad range of application reported in ethnopharmacological surveys, are not well known. This study aims to enhance the understanding of potential biological processes induced by

HI infusion exposure within a 24 h time frame. Using a microarray-based transcriptome analysis of primary colon fibroblasts, as the cell model of the intestinal milieu, the global transcriptional alterations after exposure to HI infusion were discovered and major pathways affected by this herbal beverage identified. The function of the key dysregulated genes is discussed in attempt to explain the mechanism behind the previously demonstrated effects of HI and to justify its use in traditional medicine.

2. Material and methods

2.1. Plant material and preparation of the infusion

Plant material used in this study was *Helichrysum italicum* ssp. *italicum* (also called immortelle or everlasting) harvested from Ex Situ experimental collection of the University of Primorska located near Ankaran (45°34'19.3"N 13°46'33.2" E) in June 2019. The material was morphologically characterized based on the presence of axillary leaf fascicles, caulinar leaf length, leaf margin and number of capitula per synflorescence (Kramberger et al., 2020). Herbarium specimen is deposited at University of Primorska, Faculty of mathematics, natural sciences and information technologies, Slovenia, under the accession number Hla1_UP21.

HI infusions were prepared by infusing 250 mg of the dried and milled plant material in 50 mL of boiling water. After a 10-min incubation, infusions were filtered through Whatman paper No. 41 and passed through a sterile 0.2 μ m cellulose acetate syringe filter (Macherey-Nagel GmbH & Co KG, Düren, Germany). Infusion prepared from the same plant material and following the same protocol was previously characterized using high performance liquid chromatography-mass spectrometry analysis (Kramberger et al., 2020). The main identified polyphenolic compounds were hydroxycinnamic acids, such as caffeic and caffeoylquinic acids and their derivatives, pyrones including micropyrone and italyprone, arzanol and its derivatives and hydroxybenzoic acids. Of the flavonoids the most abundant were quercetin and myricetin derivatives, there were also several isobenzofuranones and acetopenones (Kramberger et al., 2020).

2.2. Cell culture and treatment with the infusion

Primary colon fibroblasts CCD112CoN (ATCC® CRL1541™) were purchased from American Type Culture Collection (ATCC®, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS). Caco-2 colorectal cancer cells (ATCC® HTB37™) were cultured in DMEM supplemented with 20% FBS. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

CCD112CoN cells were seeded in a density of 0.15 million cells/well in a 6-well plate and cultured for 24 h to allow attachment. On the next day the cell culture media was replaced with media containing 0.1 v/v% of the freshly prepared and sterile filtered HI infusion or sterile water in the same concentration. The HI infusion was at that concentration not cytotoxic as was confirmed by PrestoBlue Assay (Invitrogen™, Carlsbad, CA, USA). Cells were treated with HI infusion in triplicate for 6 h and 24 h, since most expression changes were observed after 24 h in our previous studies, whereas in our recent study (Kenig et al., 2021) more acute effects were measured, thus implying that expression changes could occur even earlier.

2.3. RNA extraction

Total RNA was isolated from primary colon fibroblasts using the TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA was next quantified with the nanodrop ND-1000 spectrophotometer (Nano-Drop Technologies, Rockland, DE, USA), and its quality evaluated based on A_{260/280} and A_{260/230} ratios. RNA integrity was assessed with RNA 6000 Nano kit

using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA). Only RNA with an RNA integrity number >8.5 was used for further experiments.

2.4. Microarray analysis

500 ng of total RNA was amplified and labelled using the Gene Chip™ WT PLUS Reagent Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). For each sample (6 h-treated, 24 h-treated and control), ss-cDNA was hybridized to Human Clariom™ S GeneChip (Thermo Fisher Scientific, Waltham, MA, USA). Arrays were immediately scanned, and images were quality controlled for hybridization artefacts.

2.5. Data normalization and statistical filtration of the differentially expressed genes

Analysis and pre-processing of data were performed with the R/Bioconductor package (Gentleman et al., 2004; R Core Team, 2021). First, we used oligo package (function read.celfiles) to import the CEL files (Carvalho and Irizarry, 2010). Next, the RMA algorithm was applied to our data to background-correct, normalize and summarize data. The limma package (Ritchie et al., 2015) was used to identify the differentially expressed genes between control and HI infusion-treated samples. Non-adjusted p-value < 0.005 was set as the threshold for identifying differentially expressed genes. The genefilter package (Gentleman et al., 2021) and corresponding function genefinder was implemented to find a background set of genes that are similar in expression to the differentially expressed genes. In other words, for every differentially expressed gene, we tried to find genes with a similar expression. Gene expression data have been deposited in the NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) under the accession number GSE188456, according to the Minimum Information About a Microarray Experiment (MIAME) standards (Barratt and Edgar, 2006).

2.6. Functional annotation of the differentially expressed genes

A set of background and differentially expressed genes were used for gene ontology term and pathway enrichment analysis. Gene ontology-based enrichment analysis was performed with topGO package (Alexa and Rahnenfuhrer, 2021) using the Fisher-classic test (p-value < 0.01). The analysis was performed on top three ontologies: cellular component, biological processes, and molecular function. A pathway enrichment analysis was done with package ReactomePA (Yu and He, 2016) using function enrichPathway (p-value < 0.05), which can be used to test the enrichment of specific pathways using the Reactome pathway database (Croft et al., 2014; Fabregat et al., 2018). The visualization of the enriched pathways was made using igraph package (Csardi and Nepusz, 2006).

2.7. RT-qPCR assay

150 ng of the remaining ss-cDNA from the microarray experiment was used as the template for the quantitative RT-qPCR reactions. qPCR assays were performed with QuantStudio® 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using SYBR™ Green PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA) and gene-specific primers selected from PrimerBank (Spandidos et al., 2010) in a 0.5 µM final concentration. The list of the primer sequences used is shown in Supplementary file (Table S6). Reaction conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were inspected to ensure primer specificity and the amplification curves of the PCR products were analyzed with the QuantStudio™ Design and Analysis software v1.5.0 using the comparative cycle threshold method. C_t values of all the targeted genes were

normalized based on the endogenous housekeeping gene of *GAPDH* as previously described (Dydenborg et al., 2006; Radonić et al., 2004). Furthermore, microarray data exhibited no significant change in *GAPDH* expression upon both treatments with HI infusion compared to control. The results are presented as the fold-change ($-\Delta\Delta C_t$) compared to non-treated cells.

2.8. Cell migration assay

Wound healing assay was performed to test cell migration. Caco-2 cells were plated into cell culture inserts for gap creation (Ibidi, Gräfelting, Germany) in high density and left to attach for 24 h to form a monolayer. Inserts were removed, cells were washed in PBS to remove damaged and floating cells. HI infusion was prepared at 0.1 v/v% in DMEM with FBS reduced to 2% to prevent cell proliferation. Cells were treated immediately after removal of the inserts and photos were taken under 4× magnification at times 0, after 6 h and after 24 h using Olympus IX51 TH4-200 microscope, equipped with a digital camera and cellSens Dimension software. Experiment was performed in triplicate; a representative image is shown.

2.9. Coagulation tests

The experiment was conducted in accordance with the Declaration of Helsinki and the procedure was approved by the Slovenian National Medical Ethics Committee under protocol number 0120–557/2017/4. Five healthy volunteers of both sexes were between 18 and 40 years old and without any known medical condition; taking any anticoagulation agent in the preceding two weeks was an exclusion criterion. All participants signed the informed consent. Full blood withdrawn into 6 mL serum vacutainers (BD, Franklin Lakes, NJ, USA) was used to test clotting time. For the prothrombin test, 2 mL of venous blood was collected into 2 mL sodium citrate vacutainers, plasma was separated by centrifugation at 2500g for 10 min, stored at 4 °C, and used within 2 h.

Blood clotting time was measured using a modified Lee and White method (Osoniyi and Onajobi, 2003; Ayodele et al., 2019). To test the effect of HI, HI infusion was diluted at 0.1 v/v% and 0.5 v/v% in PBS. To test the effect of conditioned media, CCD112CoN cells were exposed to cell culture media alone or containing 0.1 v/v% or 0.5 v/v% HI for 24 h. Conditioned media were collected and centrifuged at 1500g for 10 min to remove floating cells. 0.5 mL of PBS and conditioned media samples were incubated at 37 °C in water bath and 0.5 mL of fresh blood was added. Stopwatch was started simultaneously and stopped when clot formation was observed.

For prothrombin test, 100 µL of plasma samples were mixed with 100 µL of PBS samples containing 0.1 and 0.5 v/v% HI or with conditioned media of HI-treated and control cells (Osoniyi and Onajobi, 2003). Mixtures were prewarmed to 37 °C. Thromboplastin (Sigma-Aldrich, ST. Louis, MO, USA) was reconstituted in 3.2 mM CaCl₂ and 100 µL was added to the samples. Times of clot formation were recorded.

2.10. Statistical analysis

Statistical analysis related to the microarray data was performed with the R/Bioconductor package. The results shown with bar graphs are presented as mean values ± standard error of the mean (for clotting and prothrombin time) and as mean values ± standard deviation of duplicate or triplicate experiments (for log fold change in gene expression). Data on clotting time and prothrombin time were analyzed using SPSS software, version 23; paired sample *t*-test was used to compare differences between controls and HI samples.

3. Results

3.1. Identification of the differentially expressed genes

To investigate the underlying mechanism of HI infusion leading to the potential biological effects in human colon fibroblasts (CCD112CoN), gene expression profiling was performed. Of the 21448 genes screened we were able to identify a total of 217 differentially expressed genes (DEGs) following 0.1 v/v% HI infusion treatments for 6 h and 24 h compared to untreated cells and only 8 were common to both treatments (*GOLGA8N*, *RHBG*, *NAA16*, *PLEKHH2*, *OR2T12*, *ANKRD36B*, *PAFAH1B3* and *ICT1*). Among the DEGs, 34 genes were up-regulated and 10 down-regulated at a 6 h treatment (Fig. 1A), whereas at a 24 h treatment 96 genes were up-regulated and 85 down-regulated (Fig. 1B). Table 1 lists the top ten dysregulated genes in each treatment, whereas the full list of DEGs is available in Supplementary file (Tables S1 and S2).

Among the genes listed in Table 1, five up- and five down-regulated genes in each treatment were chosen for validation with RT-qPCR (Fig. 2A and B). The direction of changes in gene expression determined with RT-qPCR matched the microarray data for all the genes tested, except for *JPH3*, which rather showed an up-regulated expression. For the gene *HTR3E* we could not determine the expression, as threshold cycle has not been reached. In general, changes in expression levels determined with microarrays were higher compared to RT-qPCR ones.

Similarly, the direction of changes in gene expression after 24 h treatment matched the microarray data for all the genes tested, except for *ABCA12*, which showed a slightly down-regulated expression in RT-qPCR experiment. We also could not determine the expression of the gene *DEFB115*, as no replication was observed. Overall changes in expression levels determined with microarrays were similar or higher than RT-qPCR ones for the up-regulated genes, whereas for the down-regulated genes the opposite was true. Pearson correlation coefficient was 0.733 ($p = 0.025$) for the 6 h-treatment and 0.803 ($p = 0.009$) for the 24 h treatment.

3.2. Gene ontology term enrichment analysis

With the Gene Ontology (GO) analysis we found that tested conditions altered the expression of genes sorted in several categories within “Biological processes”, “Cellular Compartment” and “Molecular Function” domains but mostly after 24 h treatment with HI infusion. In Fig. 3 top ten ($p < 0.01$) biological processes and all cellular compartment and molecular functions affected by HI infusion treatment are presented in order of statistical significance for this time point. Biological processes that were the most affected by 24 h-HI infusion exposure included cellular processes involved in cell-substrate junction assembly, cell-

matrix adhesion, cell growth and its regulation, and developmental processes such as tissue morphogenesis and epithelium development. Other affected biological processes included muscle contraction and response to stimulus. Cellular compartments affected were actin cytoskeleton, supramolecular complex (fiber and polymer), Golgi cisterna membrane and adherens junction. Molecular functions associated with HI infusion exposure were cytoskeletal protein binding (microtubule, tubulin) and enzyme nucleoside-triphosphatase regulator activity. For the 6 h treatment we only got hits within the “Biological processes” domain. The only affected biological process was hemostasis ($p < 0.01$). However, with the significance set to $p < 0.05$ additional biological processes appeared as significantly affected. These were blood coagulation, coagulation, wound healing, regulation of body fluids and response to wounding. The set of genes associated with these terms was the same for all these pathways. The full list of GO categories significantly affected by HI infusion exposure ($p < 0.01$) along with the symbols of the assigned genes is available in Supplementary file (Table S3, S4 and S5).

At the first glance, overall GO results point out to modulation of epithelium in terms of its interaction with extracellular matrix and assembly of signaling complexes resulting in cell growth, presumably in response to external stimulus. The processes involved are important in maintaining the intestinal barrier integrity and this could be a mode of action that would explain a lot of HI activity.

3.3. Pathway enrichment analysis

To gain better understanding of the interaction between the identified DEGs and to put them in a biological context, which would ease the interpretation of the results, a pathway enrichment analysis using Reactome pathway database was performed on the entire set of identified genes in each sample. Analysis of the 6 h treatment samples revealed one significantly ($p < 0.05$) enriched pathway, and for 24 h treatment samples 12 pathways were identified (Table 2). For the 6 h treatment this was the hemostasis pathway, which is the same biological process that was identified through GO analysis and even involved the same four genes. Pathways affected in the 24 h treatment were associated predominantly with cell cycle or cytokine signaling in immune system, especially by interleukins. Other important pathways were transcriptional regulation of mostly metabolic genes by TP53, or activation of AKT signaling by PIP3. Remaining identified pathways belonged to signal transduction pathway involving Rho GTPases and muscle contraction. Among the identified pathways after 24 h treatment, muscle contraction and interleukin-12 mediated signaling were the two processes that also appeared in GO results. Other results are concordant as well, as the cell cycle and transcriptional regulation are processes involved in cell growth and Rho GTPases are known as actin

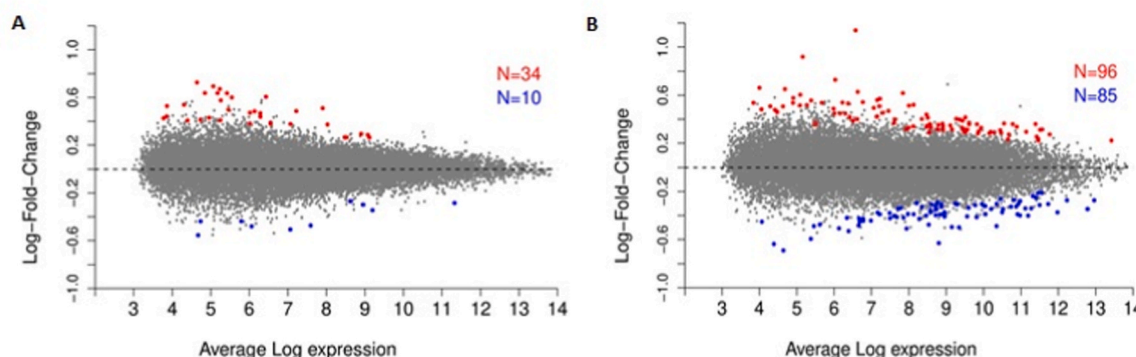


Fig. 1. Scatter plots showing the average Log expression values of each gene and Log-fold change in expression after HI infusion exposure for 6 h (A) and 24 h (B), compared to control. Red and blue color indicate significantly up-regulated and down-regulated genes, respectively (p -value < 0.005 , $n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

List of the top ten differentially expressed genes upon both treatments (for 6 h and 24 h) with 0.1 v/v% HI infusion.

Up-regulated genes		Down-regulated genes	
Gene symbol	Gene name	Gene symbol	Gene name
6 h treatment			
<i>LIPA</i>	lipase A, lysosomal acid type	<i>APBA1</i>	amyloid beta (A4) precursor protein-binding, family A, member 1
<i>DYNLL2</i>	dynein light chain LC8-type 2	<i>ICT1</i>	immature colon carcinoma transcript 1
<i>PRNT</i>	prion protein (testis specific)	<i>SPX</i>	spexin hormone
<i>HTR3E</i>	5-hydroxytryptamine (serotonin) receptor 3E, ionotropic	<i>MAFK</i>	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K
<i>MYO1E</i>	myosin IE	<i>LINC02347</i>	long intergenic non-protein coding RNA 2347
<i>PROX2</i>	prospero homeobox 2	<i>JPH3</i>	junctophilin 3
<i>CCDC121</i>	coiled-coil domain containing 121	<i>HIST1H3G</i>	histone cluster 1, H3g
<i>PDE4DIP</i>	phosphodiesterase 4D interacting protein	<i>PAFAH1B3</i>	platelet-activating factor acetylhydrolase 1b, catalytic subunit 3
<i>GOLGA8N</i>	golgin A8 family, member N	<i>PRKAR1A</i>	protein kinase, cAMP-dependent, regulatory, type I, alpha
<i>RHBG</i>	Rh family, B glycoprotein (gene/pseudogene)	<i>HDGF</i>	hepatoma-derived growth factor
24 h treatment			
<i>IL33</i>	interleukin 33	<i>ROPN1L</i>	rhophilin associated tail protein 1-like
<i>HGF</i>	hepatocyte growth factor	<i>DEFB115</i>	defensin, beta 115
<i>CCDC191</i>	coiled-coil domain containing 191	<i>MRPL49</i>	mitochondrial ribosomal protein L49
<i>ABCA12</i>	ATP binding cassette subfamily A member 12	<i>USP17L19</i>	ubiquitin specific peptidase 17-like family member 19
<i>EFCAB13</i>	EF-hand calcium binding domain 13	<i>CECR5</i>	cat eye syndrome chromosome region, candidate 5
<i>GOLGA6B</i>	golgin A6 family, member B	<i>TUFT1</i>	tuftelin 1
<i>NAA16</i>	N(alpha)-acetyltransferase 16, NatA auxiliary subunit	<i>TP53I13</i>	tumor protein p53 inducible protein 13
<i>CLASP2</i>	cytoplasmic linker associated protein 2	<i>OXTR</i>	oxytocin receptor
<i>XPNPEP1</i>	X-prolyl aminopeptidase 1	<i>FHL3</i>	four and a half LIM domains 3
<i>PLEKHH2</i>	pleckstrin homology domain containing, family H member 2	<i>LHPP</i>	phospholysine phosphohistidine inorganic pyrophosphate phosphatase

cytoskeleton regulators. Pathways enriched upon 24 h treatment with HI infusion are also presented graphically in Fig. 4.

3.4. Functional assays

Since the analysis of transcriptome data collectively points to the role of HI infusion in wound healing, we further selected functional *in vitro* assays to test the consequences of the identified gene expression changes. HI infusion increased the migration potential of Caco-2 cells. As it is visible from Fig. 5A, HI-treated cells completely closed the gap 24 h after the removal of inserts, whereas non-treated cells did not. Conditioned media of CCD112CoN cells that were exposed to 0.5 v/v% HI decreased blood clotting time in comparison to control conditioned media or HI infusion alone (Fig. 5B). Also, prothrombin time was shorter when plasma was mixed with conditioned media of 0.1 v/v% or 0.5 v/v%

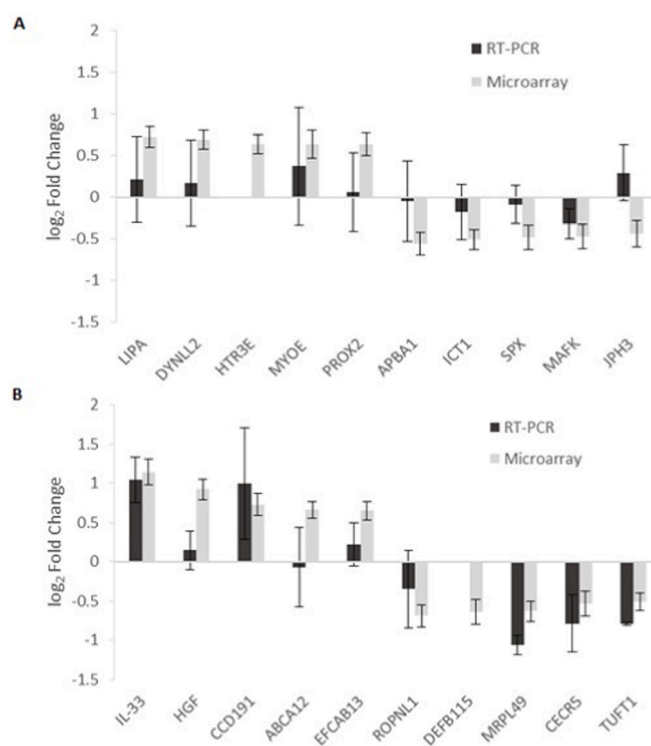


Fig. 2. Log fold change in expression levels of the top five up-regulated and the top five down-regulated genes in CCD112CoN cells treated with 0.1 v/v% HI infusion for 6 h (A) and for 24 h (B). Results are presented as average values $\pm$ standard deviation. Experiments were conducted in triplicates for microarray and duplicates for RT-qPCR.

HI-treated CCD112CoN cells. Again, HI infusion alone at same concentrations had no significant effect on the prothrombin time (Fig. 5C).

4. Discussion

4.1. Identification of the DEGs and validation by RT-qPCR

The aim of the present study was to characterize the global transcriptional response of primary colon fibroblasts to *H. italicum* infusion exposure. This study provides the first insight into the biological processes and canonical pathways that underlie the effects of HI infusion in human CCD112CoN cells. Our microarray data indicated that a 24 h treatment of primary colon fibroblasts with HI infusion resulted in 181 significantly dysregulated genes, whereas 6 h treatment resulted in only 44 DEGs. The degree of overlap between DEGs after both treatments was surprisingly low, meaning that 82% of DEGs in the 6 h treatment, were no longer differentially expressed after additional 18 h. The total number of DEGs corresponds to about 1% of the analyzed genes, but we cannot exclude that other treatment conditions in terms of timing and dosage could lead to an amplified effect in primary fibroblasts. Validation by RT-qPCR assays showed that the majority of the most dysregulated transcripts identified by our microarray experiments were confirmed to be differentially expressed in colon fibroblasts upon HI infusion treatment, therefore demonstrating the reliability of the transcriptomic data. However, microarray and qPCR data are often inconsistent, especially for the genes exhibiting small degrees of change or genes with low expression levels (Morey et al., 2006). Together with the fundamental differences in data normalization between the both methods, this could be the reason why oxidative stress-related genes (*SD1*, *GR1* and *CAT*) which were previously identified in the same cell model as dysregulated with RT-qPCR (Kramberger et al., 2021b), did not appear among the DEGs identified through the analysis of the

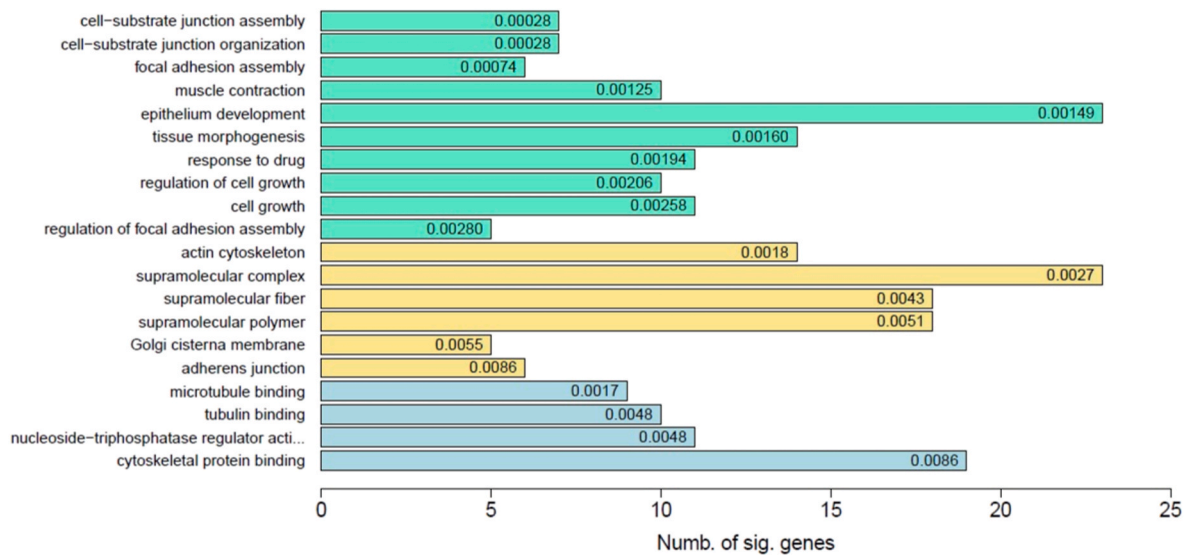


Fig. 3. Graph representing a ranked list of the gene ontology terms categorized in Biological process (turquoise bars), Cellular compartment (yellow bars) and Molecular function (blue bars) domains of the differentially expressed genes upon treatment with 0.1 v/v% HI infusion for 24 h. The numbers at the end of each bar are the statistical values of the Fisher-classic test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

List of the enriched Reactome pathways and corresponding genes identified upon both treatments (for 6 h and 24 h) with 0.1 v/v% HI infusion. Up-regulated genes are marked in red and down-regulated genes are marked in blue color. Affected pathways are written in bold and sub-pathways (subsets of genes) are not bolded.

Pathway	Genes
6 h treatment	
Hemostasis	<i>MAFK, PRKAR1A, HIST1H3G, SERPING1</i>
24 h treatment	
Cell Cycle	<i>RBL1, NCAPG, LEMD3, CENPE, NDC80, CENPF, POLR2G, PSME2, RAD50, CDKN1A, RBBP8, VRK2, CENPK, SMC4, HAUS6</i>
Cytokine Signaling in Immune system	<i>IL18R1, HLA-B, IL33, CNTF, PSME2, MTAP, UBE2L6, CNN2, CFL1, IFI6, CDKN1A, JUN, CHUK</i>
Signaling by Interleukins	<i>IL18R1, IL33, CNTF, PSME2, MTAP, CNN2, CFL1, CDKN1A, JUN, CHUK</i>
Interleukin-12 family signaling	<i>PSME2, MTAP, CNN2, CFL1</i>
Interleukin-12 signaling	<i>PSME2, MTAP, CNN2, CFL1</i>
Transcriptional Regulation by TP53	<i>RBL1, POLR2G, SESN2, DDIT4, RAD50, CDKN1A, RBBP8, JUN, TSC2, LRPPRC</i>
TP53 Regulates Metabolic Genes	<i>SESN2, DDIT4, TSC2, LRPPRC</i>
PIP3 activates AKT signaling	<i>SUZ12, IL33, PSME2, CDKN1A, JUN, TSC2, CHUK</i>
RHOG GTPase cycle	<i>LEMD3, ARHGAP1, VRK2</i>
RAC3 GTPase cycle	<i>LEMD3, ARHGAP1, VRK2</i>
RND2 GTPase cycle	<i>LEMD3, ARHGAP1, KIF14</i>
Muscle contraction	<i>ACTG2, VCL, ANXA6, ACTA2, CACNG7</i>

microarray data.

4.2. Functional annotation of the differentially expressed genes indicates involvement of the wound healing process

Beyond the identification of individual genes, our analysis also focused on their functional annotation and the identification of the canonical signaling pathways enriched after HI infusion treatment of colon fibroblasts. The GO term analysis revealed that DEGs were predominantly enriched in hemostasis after 6 h of HI infusion treatment, whereas biological processes related to cytoskeletal rearrangement and epithelial development were prominent after 24 h of treatment. In addition,

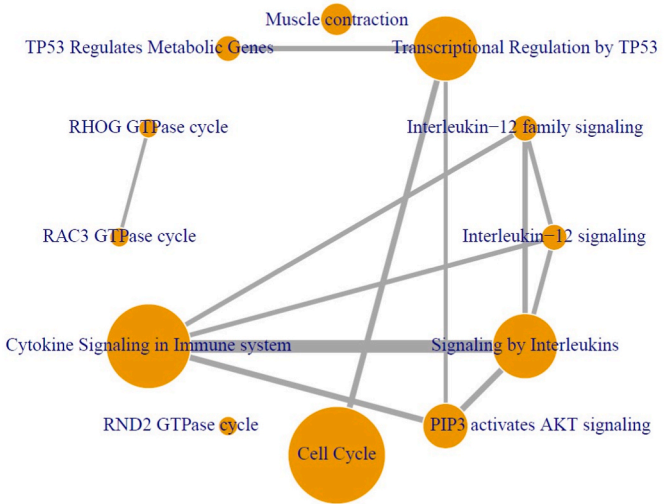


Fig. 4. Illustration of the enriched Reactome pathways upon treatment with 0.1 v/v% HI infusion for 24 h. In the graph line thickness is proportional to the number of genes shared by nodes and node size is proportional to the number of genes contained in a set.

Reactome pathway analysis identified signaling by interleukins and transcriptional regulation of metabolic genes by TP53 as the two major enriched pathways. Observed changes in global gene expression point out to previously observed activity of HI, especially towards its wound healing properties (Rivera et al., 2008). Not only the skin, but also the intestinal epithelial barriers play a pivotal role in protecting underlying tissues from harsh external environments (Brazil et al., 2019). Together with the chemical barrier of the mucosal layer and the cellular immune system, intestinal epithelium also maintains a symbiotic relationship with commensal bacteria that have a vital role in digestion and the development of the immune system (Chelakkot et al., 2018). In response to damage, epithelial wounds repair through a series of events that integrate epithelial responses with those of resident and infiltrating immune cells (Brazil et al., 2019). Impaired barrier integrity, in turn, leads to an enhanced immune response and contributes to several

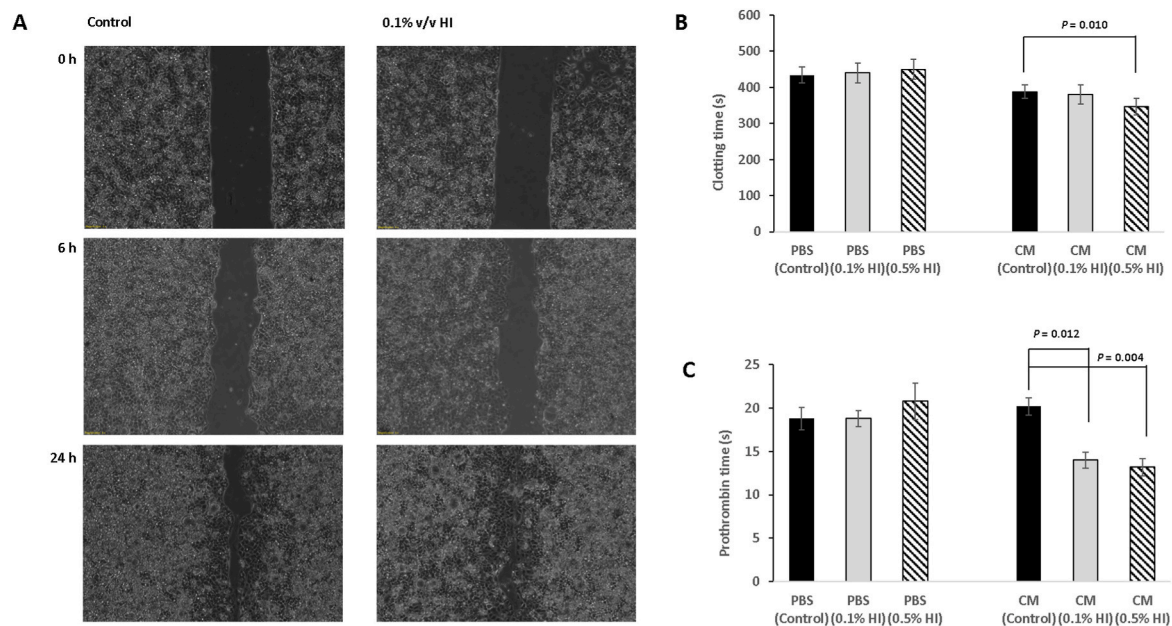


Fig. 5. Wound healing assays: (A) Microscopy images of wound closure of untreated (left panels) and 0.1 v/v% HI infusion-treated (right panels) Caco-2 cells at 0, 6, and 24 h after culture insert removal. (B) *In vitro* clotting time of normal human blood treated with PBS (control), 0.1 v/v% HI infusion (in PBS), 0.5 v/v% HI infusion (in PBS), conditioned media from CCD112CoN cell line (CM, control), conditioned media from CCD112CoN cells treated with 0.1 v/v% HI infusion for 24 h (CM, 0.1% HI), and conditioned media from CCD112CoN cells treated with 0.5 v/v% HI infusion for 24 h (CM, 0.5% HI). Results are Mean $\pm$ SE values ($n = 5$). (C) Prothrombin time of human plasma mixed with PBS (control), 0.1 v/v% HI infusion (in PBS), 0.5 v/v% HI infusion (in PBS), conditioned media from CCD112CoN cell line (CM, control), conditioned media from CCD112CoN cells treated with 0.1 v/v% HI infusion for 24 h (CM, 0.1% HI), and conditioned media from CCD112CoN cells treated with 0.5 v/v% HI infusion for 24 h (CM, 0.5% HI). Results are Mean $\pm$ SE values ($n = 5$). Abbreviations: CM, conditioned media; HI, *Helichrysum italicum*; PBS, phosphate buffered saline.

pathological conditions including inflammatory bowel disease and metabolic disorders such as obesity and diabetes (Chelakkot et al., 2018). In the following paragraphs the identified processes and function of the key associated genes are discussed to support this hypothesis.

4.3. The process of hemostasis and its role in wound healing and viral infections

The initial response to intestinal epithelial injury involves hemostasis, which limits blood loss and seals damaged tissue (Brazil et al., 2019). Hemostasis was indeed the process identified as enriched by both GO and pathway analysis, but only after 6 h treatment. Since the hemostasis occurs immediately after injury and is followed within 24 h by the next phase of the wound healing process – inflammation (Schultz et al., 2011), our results seem reasonable. The up-regulated gene in the process of hemostasis was *SERPING1*, which encodes C1 esterase inhibitor – the only regulator of classical complement activation as well as the major regulator of the contact system and coagulation (Schoenfeld et al., 2016). Functional assays performed here also support the involvement of HI in the process of hemostasis, as the conditioned medium of primary colon fibroblasts exposed to HI infusion decreased blood clotting and prothrombin time. HI infusion alone had no significant effect in these experiments, suggesting that the bioactive compounds of HI have no direct effect. Instead, the colon cells seem to respond to HI and secrete substances that promote hemostasis, consistent with the observed gene expression changes.

C1 inhibitor is also associated with viral infections as viruses activate the complement system which enhances their infectivity (Sanfilippo et al., 2017). It has been proposed that the HIV-1 protease production reduces the functional *SERPING1* and consequently its function to switch-off the HIV-1 mediated inflammation (Sanfilippo et al., 2017) and that deficiency in C1 inhibitor caused by CoV-2 or -1 proteins primes the intrinsic coagulation pathway and leads to a pro-coagulant state (Thomson et al., 2020). In addition, replenishment of C1 inhibitor

prompted the recovery of patients with severe COVID-19 (Thomson et al., 2020). It has been previously demonstrated that HI possesses antiviral activity *in vitro* and *in silico*. Pre-treatment of Jurkat cells with its main bioactive substance arzanol reduced the viral replication upon infection with HIV-1 (Appendino et al., 2007). In a recent study, among more than 300 medicinal compounds, arzanol showed significant interaction with the SARS viral proteins. Together with its good pharmacokinetic properties and low acute toxicity, this makes it a promising antiviral candidate against SARS-CoV-2 virus (Salman et al., 2020).

4.4. The cytoskeletal reorganization and wound healing

During wound repair, epithelial cells undergo morphologic changes in shape, modify cell-cell contacts, and migrate collectively to reseal the barrier. Collective epithelial cell migration during wound healing requires cytoskeletal remodeling and active crosstalk between cell matrix and cell-cell junction proteins. To facilitate migration, integrin-containing focal adhesive complexes are dynamically remodeled in concert with intracellular F-actin-rich extrusions at the leading edge that adhere to the extracellular matrix to propel epithelial sheet migration (Brazil et al., 2019). GO results perfectly support the hypothesis of HI involvement in these processes. Furthermore, small GTPases of the Rho family are considered to be master regulators of actin cytoskeleton rearrangements (Spiering and Hodgson, 2011), and several such signaling pathways were identified as enriched in our experiment. *ARHGAP1*, which encodes one of the key members of the Rho GTPase-activating proteins (RhoGAPs), was downregulated in all three pathways. RhoGAPs enhance intrinsic GTPase activity, leading to an inactive state of the GTPase (Tcherkezian and Lamarche-Vane, 2007), thus their downregulation promotes cytoskeletal reorganization. Several additional genes encoding proteins associated with actin binding (*CNN2*, *CFL1*, *ACTG2*, *VCL*, *ACTA2*, *MYO1*, *PLEKHH2*) or microtubule dynamics (*DYNLL2*, *PDE4DIP*, *CLASP2*) were dysregulated, further supporting the enrichment of biological processes involved in cell

motility. The fact that in a wound healing assay cells treated with the HI infusion closed the gap faster than control cells is in line with the proposed influence of HI on cell migration potential.

4.5. Enrichment of muscle contraction pathway explains effects of *H. italicum* on intestinal motility and blood pressure

Actin filaments are also involved in other cellular processes such as muscle contraction, which was included among the enriched processes in both GO and pathway analysis. It can be seen from Table 2 that all genes involved in this pathway were down-regulated. ACTA2 (Actin alpha 2), also known as alpha smooth muscle actin is a pivotal marker of smooth muscle cells in fibrosis and engaged in vascular contractility and blood pressure homeostasis. In smooth muscle α -actin null mice, compromised vascular contractility, lowered blood pressure, and reduced blood flow were observed (Schildmeyer et al., 2000). Similarly, spexin hormone encoded by *SPX*, when administrated intracerebroventricularly induces an increase in arterial blood pressure and contraction of smooth muscles in the stomach (Kim et al., 2019). It has been demonstrated before that HI exerts intestinal antispasmodic action (Rigano et al., 2013) and a slight decline in diastolic blood pressure was observed also in our study (Kenig et al., 2021). However, both studies indicate more direct action of HI, probably involving enzyme activity, since gene expression changes take longer to manifest. The latter mechanism would only gain importance with chronic use of HI infusion. Regarding the study by Rigano et al. (2013) it might be worthwhile to mention, that HI inhibited intestinal motility in the model of intestinal inflammation at doses which were ineffective in healthy mice. This finding, together with the well-established anti-inflammatory effect of HI, suggests that HI could be indicated for inflammatory bowel disease.

4.6. Interleukin signaling and the role of IL33 in tissue homeostasis, wound healing, parasitic and microbial infections

Interleukins are low molecular weight proteins that have pleiotropic effects on cells which bind them, impacting processes such as tissue growth and repair, hematopoietic homeostasis, and multiple levels of the host defense against pathogens where they are an essential part of the immune system (Voßhenrich and Santo, 2002). The most up-regulated gene in our study was *IL33*, encoding a nuclear-associated cytokine of the IL-1 family. IL-33 signals via the receptor ST2, which is highly expressed on group 2 innate lymphoid cells (ILC2) and T helper 2 cells. ILC2s, some regulatory T cells, and mast cells are the primary tissue-resident cells that constitutively express high levels of ST2, positioning these cells as initial targets of IL-33 and hereby suggesting its role also in tissue homeostasis and repair (Molofsky et al., 2015). Studies in genetically obese diabetic (ob/ob) mice show that administration of recombinant IL-33 resulted in reduced adiposity, decreased fasting glucose, and improved glucose and insulin tolerance, suggesting a protective role of IL-33 in the development of adipose tissue inflammation during obesity (Miller et al., 2010). It is proposed that IL-33 sustains the architecture and function of ST2⁺ resident cells, which limit the infiltration of adipose tissue by inflammatory lymphocytes and myeloid cells leading to insulin resistance and metabolic syndrome (Molofsky et al., 2015). These data suggest that the action of HI in metabolic disorders is not only indirect by maintaining the integrity of the intestinal barrier but may also influence other processes leading to the manifestation of the disease. Increasing evidence indicates that IL-33 accelerates cutaneous wound healing as it facilitates the development of alternatively activated macrophages in incisional wound tissue (Yin et al., 2013). In fact, type 2 immunity plays active roles in both wound healing and helminth immunity, as helminths cause considerable tissue damage which induces type 2 immune responses that mediate host protection through enhanced tissue repair, the control of inflammation and worm expulsion (Gause et al., 2013). The pleiotropic functions of IL-33 are tightly associated with many activities ascribed to HI, such as

antibacterial, antiviral, anti-inflammatory and antidiabetic, and in turn to its application in treatment of skin inflammatory and infectious conditions, respiratory infections, intestinal parasitic infections and metabolic disorders.

4.7. Transcriptional regulation by TP53 affects energy metabolism

The p53 transcription factor (encoded by the human gene *TP53*) stands out as a key tumor suppressor and a master regulator of various signaling pathways involved in tumorigenesis (Rivlin et al., 2011). While the function of p53 is best understood in response to genotoxic stress, increasing evidence suggests that p53 also plays a key role in the regulation of metabolic homeostasis. Here, several genes with function in lipid metabolism were found to be dysregulated. Leucine-rich PPR motif-containing protein (LRPPRC) is a multifunctional protein involved in energy metabolism and human diseases (Cui et al., 2019), which was shown (Lei et al., 2016) to promote palmitate uptake and its oxidation by hepatocytes in mice. *LIPA*, encoding lipase A, which catalyzes the hydrolysis of cholesteryl esters and triglycerides in lysosomes, was the most up-regulated gene after 6 h treatment, whereas *ABCA12*, encoding an ATP-binding cassette transporter involved in lipid transportation, cholesterol homeostasis, and insulin secretion, was among the most up-regulated genes after 24 h treatment. On the other hand, *PRKAR1A* and *ANXA6* were among the down-regulated genes. Protein kinase A is an enzyme with central role in the regulation of lipid and glucose metabolism and is even thought to be involved in obesity resistance (London et al., 2020). *PRKAR1A* encodes type 1 α regulatory subunit of cAMP-dependent protein kinase that inhibits kinase activity of the catalytic subunit (Bossis and Stratakis, 2004). *ANXA6* (Annexin A6) is a lipid-binding protein highly expressed in the liver, regulating cholesterol homeostasis and signaling pathways with a role in liver physiology (Meier et al., 2016). Cubells et al. (2007) demonstrated that increased expression of *ANXA6* results in a sequestration and reduced export of cholesterol from late endosomes. Lysosomal cholesterol accumulation is an important target for intervention in metabolic diseases that are characterized by the presence of an inflammatory response (Hendriks et al., 2014), therefore administration of HI could likely prove beneficial in this context. In our previous study (Kenig et al., 2021) we found that HI infusion indeed promotes β -oxidation both *in vitro* and in human, which can now be explained also by the dysregulation of above-mentioned genes.

4.8. Transcriptional regulation by TP53 and wound healing

Another gene, that is tightly controlled by the tumor suppressor protein p53 and was down-regulated upon HI infusion exposure is *CDKN1A*. This gene encodes a potent cyclin-dependent kinase inhibitor p21, that has been implicated in DNA damage-induced p53-mediated G1 arrest, as well as in physiological processes, such as cell differentiation and senescence, that do not involve p53 function (Wu et al., 1996). The inhibitory effect of p21 knockout on transforming growth factor- β signaling and collagen production directly leads to reduced collagen deposition and fibrosis, which results in reduced scar formation (Papismadov et al., 2017). Down-regulation of *CDKN1A* could thus partially explain wound healing (Rivera et al., 2008) and anti-ageing (Granger et al., 2020) properties of HI. Studies have also investigated the role of p21 in regeneration of the mammalian liver. Up-regulation of p21 led to impaired regeneration in fatty livers (Torbensohn et al., 2002), and inversely, repression of the p53/p21 pathway by c-Jun/AP-1 was shown to enhance liver regeneration (Stepniak et al., 2006). In discordance, *JUN* was rather down-regulated in our study. Anyhow, *HGF*, which encodes for hepatocyte growth factor that is a polypeptide with potent mitogenic activity toward hepatocytes in culture and has been implicated as a mediator of hepatic regeneration *in vivo* (Maher, 1993), was among the top up-regulated genes upon HI infusion treatment. This suggests that HI infusion could also stimulate liver regeneration in

addition to eliminating steatosis in the liver. Moreover, in relation to earlier discussed vascular contractility and ACTA2, a study showed that inhibition of ACTA2 leads to reduced cellular motility and contraction of myofibroblast during hepatic wound healing *in vivo* (Rockey et al., 2013), which demonstrates that several processes identified in the present study are connected in a way and contribute to collective activity of HI.

5. Conclusions

With the present study we identified several important biological processes and signaling pathways enriched after *H. italicum* infusion treatment. We were able to logically explain the observed alteration of the identified processes by understanding the function of some key dysregulated genes and linking them to previously demonstrated activity of HI. Our data of the whole transcriptome analysis supported by functional assays, suggests that the major global mode of HI action, derived from the gene expression changes in colon fibroblasts, is the wound healing process, characterized by hemostasis, cytoskeletal reorganization, and cellular growth. By maintaining the integrity of the intestinal barrier, HI is able to prevent or ameliorate pathological conditions such as inflammatory bowel disease and metabolic disorders. In addition, HI also acts on inflammation, lipid, and glucose metabolism directly, by altering the expression of relevant anti-inflammatory and metabolic genes. This study provides a more thorough understanding of the main biological processes affected by HI that contribute to its observed activity and explain its traditional use in treatment of several ailments.

CRediT authorship contribution statement

Katja Kramberger: performed the experiments, wrote and revised the manuscript. Investigation, Writing - original draft, review and editing, Visualization. **Darja Barlič-Maganja:** acquired the resources and funding and administered the project, Resources, Funding acquisition, Project administration. **Zala Jenko Pražnikar:** analyzed, visualized and validated the data, and acquired the funding, Formal analysis, Visualization, Validation, Funding acquisition. **Tadeja Režen:** Conceptualization, Supervision, conceptualized the experiments and supervised them. **Damjana Rozman:** Funding acquisition, Resources, acquired the resources and funding. **Jure Pražnikar:** Software, Formal analysis, Data curation, programmed the software for data analysis and curated the data. **Saša Kenig:** Conceptualization, Supervision, Investigation, designed and supervised the experiments. All authors have reviewed the manuscript and agreed to the submitted version of the manuscript.

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

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