Abstract

*Arnica montana* (*Arnica m.*) is used for its purported anti-inflammatory and tissue healing actions after injuries, but its cellular and molecular mechanisms are largely unknown. This work tested *Arnica m.* using an *in vitro* model of macrophages polarized towards a “wound-healing” phenotype. The monocyte-macrophage cell line was cultured and differentiated with phorbol-myristate acetate and Interleukin-4, then exposed for 24h to centesimal (c) dilutions 2c, 3c, 5c, 9c, 15c or Control. Total RNA was isolated and cDNA libraries were sequenced with NextSeq500 sequencer. Genes with significantly positive (up-regulated) or negative (down-regulated) fold changes were differentially expressed genes (DEGs). A total of 20 DEGs were identified in *Arnica m.* 2c treated cells. Of these, 7 genes were up-regulated and 13 were down-regulated. The most significantly up-regulated function concerned 4 genes with a conserved epidermal growth factor-like region (p<0.001) and three genes of proteinaceous extracellular matrix, including heparin proteoglycan 2 (HSPG2), fibrillin 2 (FBN2), and fibronectin (FN1) (p<0.01). Protein assay confirmed a statistically significant increase of fibronectin production (p<0.05). The down-regulated transcripts derived from mitochondrial genes coding for components of electron transport chain. The same groups of genes were also regulated by increasing dilutions of 9c, 15c, although with a lower effect size. We further tested the healing potential of *Arnica m.* 2c in a scratch model of closure based on the motility of bone marrow-derived macrophages and found evidence of an accelerating effect on closure in this system. The results of this work, taken together, provide new insights into the action of *Arnica m.* and identify extracellular matrix regulation by macrophages as a therapeutic target.


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Introduction

*Arnica montana* L. (referred to here as *Arnica m.*) is one of the most popular medications in complementary medicine employed in tincture, ointment, cream, gel, and tablet form. It is a herb native to the mountains of Siberia and Central Europe, and has been used to treat various pathological conditions, including pain, stiffness and swelling associated with trauma, post-surgical clinical conditions (including contusions and sprains) and for symptomatic relief in osteoarthritis [1]. *Arnica m.* is generally applied only topically on the skin, while as a homeopathic formulation it is given orally or as a 6c dilution since it is not considered dangerous owing to the high dilution. The literature on *Arnica m.* in phytotherapeutic and homeopathic preparations is rapidly increasing, but the knowledge of its action mechanism(s) remains scant.

The chemical composition of *Arnica m.* depends on the part of the plant that is used, principally the flowers and roots, and the most pharmacologically active compounds are sesquiterpene lactones, thymol derivatives, flavonoids, acid polysaccharides and their glycoconjugates [6–8]. The ability of *Arnica m.* to inhibit activation of the nuclear factor of activated T cells (NFAT) and pro-inflammatory cytokines IL-1 and TNF-α correlates with the qualitative content of sesquiterpene lactones [9].

There is some experimental evidence, in laboratory animals, of an anti-inflammatory action of the ethanolic extract [10] or at the 6th centesimal homeopathic dilution (6c) [11,12]. Additionally, *Arnica m.* in inducible NO synthase and cyclooxygenase-2 protein levels, a reduction in TNF-α, and prevents nuclear translocation in J774 murine macrophage cells without cytotoxicity *in vitro* [13]. Furthermore, oral treatment with *Arnica m.* experimental animals (rats) against hepatic mitochondrial membrane permeabilization induced to the attack by reactive oxygen species [14].

Given the central role of macrophages in tissue repair and regeneration, we formulated the hypothesis that one of the targets of *Arnica m.* action is the macrophage, and accordingly decided to evaluate this plant’s effects on a cell line, a widely used model for immune modulation [15,16]. This cell line is widely used in laboratories for the study of macrophage biochemistry and molecular biology. The advantage of a cell line resides essentially in the easier reproduction of experiments in the same conditions, avoiding the variations due to individual sensitivity of different donors. Since we used doses of drugs—even with the highest *Arnica m.* 2c dilution, in assay medium the sesquiterpene lactones were in the 10^3–10^4 dose—we expected small effect sizes and so preferred to use a highly reproducible model. THP-1 monocytes, but when treated with low doses of phorbol esters (PMA) they differentiate to cells with functional features of tissue macrophages. On the basis of environmental cues and molecular mediators, macrophages polarized by interleukin-4 (IL-4) treatment to a phenotype that takes on characteristic properties functional features of tissue macrophages. On the basis of environmental cues and molecular mediators, macrophages polarized by interleukin-4 (IL-4) treatment to a phenotype that takes on characteristic properties functional features of tissue macrophages.
regulation, wound healing, and tissue remodelling [16,21].

In a preliminary study, we used RT-PCR analysis to investigate changes in the expression of a panel of 28 genes focusing on immune response [22]. Among the tested genes, CXCL1 in particular exhibited the most substantial increase of expression, suggesting a positive influence on leukocyte recruitment and on angiogenesis. The most pronounced effects were noted in IL-4 polarized macrophages. Therefore, we decided to re-investigate the same cell extracts with the most high-throughput method, RNA-seq, designed to evaluate the whole transcriptome. We assessed RNA samples from a series of experiments with Arnica m. at the 2nd centesimal homeopathic dilution (Arnica m. 2c) compared with vehicle (Control). To identify all the potential genomic targets of the plant’s regulating action, through bioinformatics analysis of genes (Arnica m. 2c vs Control) and using statistical methods correcting for false discovery rate, we also tested pooled samples of the experiments, namely 3c, 5c, 9c and 15c. Lastly, to further investigate the potential therapeutic capacity of this plant, we tested Control solutions using an in vitro model of wound healing, in which macrophages migrate through a scratch made in a cell monolayer. A major advantage of this method is that it mimics, to some extent, the migration of cells suitable for studies on the effects of cell–matrix and cell–cell interactions during wound healing.

Materials and Methods

Materials

The human monocytic leukaemia cell line THP-1 was purchased from DSMZ (Germany). Growth media RPMI 1640, DMEM with l-glutamine were purchased from Lonza (Belgium). Foetal bovine serum (FBS), phorbol 12-myristate 13-acetate (PMA), pure ethanol and ultra-pure water (W3500) were purchased from Sigma-Aldrich Co. (USA). Human interleukin-4 (IL-4) was purchased from Macs-Miltenyi Biotec (Germany). Murine IL-4 was purchased from R&D Systems (UK). Cell proliferation reagent WST-1 was purchased by Roche Diagnostics GmbH (Germany) (CA, USA). Ficoll-Hypaque and Percoll were purchased from GE Healthcare Life Science (Uppsala, Sweden).

Test solutions

Arnica m. was produced by Boiron Laboratoires (Lyon, France) according to the French Homeopathic Pharmacopoeia as a first centesimal dilution (Arnica m. 1c) of the hydroalcoholic extract (Mother Tincture, MT) by dilution and succussion with 100x of 30% v/v as the reference-blank sample. Nanoparticle content was determined by nanoparticle tracking analysis (NTA) performed with a Jasco V-650 double-beam spectrophotometer using quartz cuvettes with 1cm optical path and the NanoSight NTA 3.0 analysis software. Zein content was measured by Zetasizer Nano (Malvern) using disposable capillary cells (Malvern).

Arnica m. 1c was used to prepare the second centesimal dilution (Arnica m. 2c) by adding 50µl of ultra-pure water. Therefore, 2c corresponds to 10^{-4} of the MT. This solution was filtered with a 0.22µm filter, succussed with a Dyna-A mechanical shaker delivering 20 strokes/second with an amplitude of 11mm and the NanoSight NT 3.0 was measured by Zetasizer Nano (Malvern) using disposable capillary cells (Malvern).

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Higher dilutions of Arnica m. were prepared as described previously [22]. Starting from a 1c solution prepared in 5ml of 30% ethanol/distilled water solvent followed by filtering and succussion. Stock ethanol/distilled water were wrapped in aluminium foil, stored at room temperature in the dark, and used at several doses in clinical settings [5], we also tested pooled samples of the experiments, namely 3c, 5c, 9c and 15c. Lastly, to further investigate the potential therapeutic capacity of this plant, we tested Control solutions using an in vitro model of wound healing, in which macrophages migrate through a scratch made in a cell monolayer. A major advantage of this method is that it mimics, to some extent, the migration of cells suitable for studies on the effects of cell–matrix and cell–cell interactions during wound healing.
preparation. The last centesimal dilution step was always performed immediately before each experiment. The dilutions prepared in this way, those tested with the cells were: 2c, 3c, 5c, 9c, and 15c. All procedures for drug preparation and cell treatments were done in sterile conditions.

**Cell cultures and treatments**

The THP-1 cell line was cultured in RPMI 1640 medium, supplemented with FBS 10% and 2mM Ultraglutamine (Lonza), at 37°C in 5% CO₂ in a humidified incubator as described [22]. Briefly, the cells were seeded at a density of 2.5x10⁵ cells/mL in 24-well plates in 1ml medium with 2ml of cell culture. Day 2 all the cell cultures were supplemented with 20 ng/mL of PMA and on day 3 the cultures were treated with IL-4 a concentration of 50 ng/mL for 24h. On day 4 the plates were washed twice with culture medium supplemented with 50 ng/mL IL-4 and incubated for 24h. Macrophages were exposed for 24h to solvent (1ml cell culture + 110µl test solutions). We performed a total of 5 complete separate experiments, every treatment was performed in triplicate wells.

**Bone marrow–derived macrophages**

For the scratch test, bone marrow–derived macrophages (BMDM) were isolated from femurs and tibias of 8 week-old C57BL/6J mice as described by Suen et al. (1999) [24] and Baruzzi et al. [25]. Briefly, cells were cultured in DMEM with 15% FBS, 10% L929-cell conditioned medium (LCM) as a source of growth factors, 100 U/ml penicillin, and 100 µg/ml streptomycin (BMDM complete medium), and cultured at 37°C/5% CO₂ in 75 cm² flasks. The non-adherent cells were removed, counted, plated on bacteriological (non tissue-culture-treated) plastic dishes at a concentration of 1×10⁵/ml, and cultured in BMDM complete medium.

**Evaluation of cell viability**

Cell viability was checked by the Cell proliferation reagent WST-1 assay. THP-1 cells were seeded in 96-well plates and differentiated with IL-4 as described above. After 24h of treatment with Arnica m (v/v) pre-warmed WST-1 solution was added to the cells and the plate was incubated for 20 min. Cell viability was measured using a Victor3 multilabel reader (PerkinElmer, Shelton, CT, USA) at 450nm. Total proteins of cell lysates were quantified by Bradford assay according to the manufacturer’s instructions.

**RNA sequencing**

Total RNA from cultured THP-1 cells was isolated using the RNeasy mini Kit (Qiagen). RNA quality was assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA samples were isolated using an RNA 6000 Nano Kit (Agilent, Wokingham, UK). The samples with RNA integrity number (RIN) of 8 or greater were used for library preparation. RNA aliquots (2.5µg) were used to isolate poly(A) mRNA for the Illumina RNA-Seq library using the TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, CA). Before the generation of clusters, was checked with High Sensitivity DNA Kit (Agilent, Wokingham, UK) and the library quantified by qPCR using the KAPA Library Quantification kit (Kapa Biosystems Inc., Woburn, MA). Libraries were sequenced with a NextSeq500 sequencer (HighOutput flow cell with 75 sequencer sequences). The reads were aligned to the human reference genome (GRCh38) using the TopHat expression value of known and novel genes was quantified as reads per kilobase of exon model using the human working gene set (Ensembl release 80) as reference annotation. The effect of treatment was measured by calculating Log₂ of the ratio between RPKM of each gene in Arnica m.-treated samples and Control-treated samples (Log₂ Fold Change). Genes with Log₂ Fold Change values that were significantly positive (up-regulated) or negative (down-regulated) were defined as differentially expressed genes (DEGs).
Sequences of DEGs in the “protein coding” category were functionally annotated using Blast2GO metabolic pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG) to the query sequence classification and enrichment analysis were performed by DAVID Bioinformatics Resources 6.7 [27]. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus through GEO Series accession numbers GSE77381 and GSE77382 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77381; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77382).

Protein quantification by ELISA

Matrix-related proteins (fibronectin, fibrillin, and heparan sulfate proteoglycan 2) were quantified in conditioned medium cells using ELISA assays fibronectin human Elisa kit (Abcam), human Fibrillin-2 ELISA Kit and heparan sulfate 2 ELISA Kit (Cusabio), respectively.

In vitro wound-healing (scratch) assay

The capacity of macrophages to close an artificial “wound” was assessed using a culture model BMDM monolayer was denuded of cells by scraping it with a pipette tip, after which the number space was assessed microscopically [23]. Macrophages were cultured in 12-well plates in BMDM On day 4, a portion of the cell cultures was supplemented with 20 ng/ml murine IL-4 while another portion was left untreated by scratching the well with the tip of a 200 μl. The detached cells were carefully aspirated and the wells washed with phosphate-buffered saline migration test was DMEM (Glutamax, pen-strep) with 2% FBS, either with or without 20 ng/ml IL-2 solutions were added 24 h before wounding and maintained, during the migration time, at the cell culture volume. Three sets of experiments were performed with triplicate wells for each condition. The field of the wound were acquired by means of contrast phase microscopy using an Olympus IX51 magnification to assess cell migration. The experiments were evaluated by examining microscopically and using a grid composed of 500 small frames to calculate the % of wounded area occupied by macrophages.

Statistics

RNA-seq analysis was performed separately on 5 experiments for Arnica m. 2c and Control solvent cells treated with higher dilutions, RNA samples from 5 experiments carried out with Arnica m. solvent were pooled and sequenced.

The evaluation of differential gene expression between the Arnica m.-treated and Control sample performed using the DESeq2 package with a paired design [29]. Where indicated (Table 1), the False Discovery Rate (FDR) with the Benjamini and Hochberg method using an adjusted p-value. Comparisons were done by testing, for each gene and each experiment (N = 5), the null hypothesis exactly zero, i.e., that the gene was not at all affected by the treatment.
The statistical significance of the differences between expression profiles of gene groups (Up-regulated and Down-regulated genesets) from cells treated with various Arnica m. dilutions (2c, 3c, 5c, 9c, 15c) was calculated followed by the Wilcoxon signed-rank test using the SPSS software, version 17 (SPSS Inc., Chicago, IL, USA). The Friedman test is a nonparametric test for multiple related samples (in this case, the multiple genes from 7 up-regulated or 13 down-regulated cells treated with five Arnica m. dilutions and control solution) that checks the null hypothesis that multiple ordinal responses (the RPKM of the genes) come from the same population. After verifying the significance of the Friedman signed-rank test for paired data to evaluate the differences between RPKM of genes after treatment and mean RPKM of Control-treated cells, and to check whether such differences were prevalently distributed between the two signs. The differences were accordingly ranked, and the positive or negative ranks were summed and statistically compared using the specific Wilcoxon tables. The logic of this approach is to test the null hypothesis that treatment has no effect; the differences between gene expression (RPKM) of samples and Control-treated samples should approach zero in all considered genes of the group. Moreover, since some differences may be modified by chance, the number of up- and down-regulated genes should be approximately significantly different. Log2 Fold Changes were lower than or equal to ±0.05 (-0.05<FC<0.05) were considered to be non-significant. The statistical significance of the differences in protein release in Arnica m. 2c and Control samples was done with the Sigma Plot statistical package using the paired t-test, or the Wilcoxon Signed Rank Test when data were not normally distributed (as preliminarily evaluated by normality test). Cell viability data were evaluated by ANOVA followed by Dunnet post-hoc test, using the SPSS statistical software.

Comparison of protein release in Arnica m. 2c and Control samples was done with the Sigma Plot statistical package using the paired t-test, or the Wilcoxon Signed Rank Test when data were not normally distributed (as preliminarily evaluated by normality test). Cell viability data were evaluated by ANOVA followed by Dunnet post-hoc test, using the SPSS statistical software.

Statistical evaluation of the scratch assay was done using the Friedman test. It is used to test for differences between the series of time points for the Treated and Control samples) when the dependent variable is a rank score. The null hypothesis is that the time series for two compared treatments (Arnica m. and Control) is the same.

Results

Characterization of Arnica m.

The Arnica m. 1c used the starting material for this series of experiments was analysed by physicochemical approaches. The UV-VIS absorption spectrum shows the UV-VIS absorption spectrum. This was characterized by a large UV peak around 220, 280nm and 340nm. No substances absorbing at wavelength > 500 nm were detected in our preliminary studies.

Table 1. Gene expression of IL-4 differentiated THP-1 macrophages, treated with Control solvent or Arnica m. dilutions

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>KEGG</th>
<th>HUGO</th>
<th>RPKM Control</th>
<th>RPKM Arnica</th>
<th>FDR</th>
<th>Log2 Fold Change</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPP4</td>
<td>1565</td>
<td>1565</td>
<td>1.0</td>
<td>1.8</td>
<td>0.01</td>
<td>0.67</td>
<td>Complement component 3 (C3) receptor</td>
</tr>
<tr>
<td>IL12</td>
<td>3703</td>
<td>3703</td>
<td>3.0</td>
<td>3.0</td>
<td>0.05</td>
<td>0.067</td>
<td>Lysyl oxidase related protein</td>
</tr>
<tr>
<td>IFNG</td>
<td>3142</td>
<td>3142</td>
<td>3.0</td>
<td>3.0</td>
<td>0.05</td>
<td>0.067</td>
<td>IFN gamma 1</td>
</tr>
<tr>
<td>TNF</td>
<td>4050</td>
<td>4050</td>
<td>3.0</td>
<td>3.0</td>
<td>0.05</td>
<td>0.067</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>GMCSF</td>
<td>3652</td>
<td>3652</td>
<td>3.0</td>
<td>3.0</td>
<td>0.05</td>
<td>0.067</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>IL10</td>
<td>3905</td>
<td>3905</td>
<td>3.0</td>
<td>3.0</td>
<td>0.05</td>
<td>0.067</td>
<td>Interleukin 10</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pone.0166340.t001
NTA analysis of the original *Arnica m.* 1c sample revealed the presence of a heterogeneous and polydisperse quantity of nanoparticles (9.3 ± 1.0 particles/frame) corresponding to a concentration of $1.83 \times 10^8 \pm 1.88 \times 10^7$ particles/frame. The spectrum (Fig 2) showed a profile with about 6 peaks with a hydrodynamic diameter ranging from 274.4 ± 100.4 nm. Zeta potential of these nanoparticles was $-25.54 \pm 9.50$ mV ($n = 12$ determinations).

The amount of total sesquiterpene lactones in the original Mother Tincture was 36 mg/100ml. Since the mean molecular weight of *Arnica m.* sesquiterpene lactones is 340 g/mol [31], this amount is equivalent to $1.05 \times 10^{-3}$ Mo of these active substances is $1.05 \times 10^{-5}$ Mol/L in *Arnica m.* 1c and $1.05 \times 10^{-7}$ Mol/L in *Arnica m.* 1c and $1.05 \times 10^{-8}$ Mol/L).

Cell viability
The WST assay of cell viability (Fig 3) showed that the metabolic activity of macrophages, both differentiation, was slightly increased after 24h incubation with Arnica m. dilutions 2c and 3c, but vehicle was not statistically significant. Since the WST-1 assay depends on the level of NADH produced by the cells shows that mitochondrial NADH-producing activity was not significantly impaired by Arnica m. It higher in IL-4 differentiated macrophages, irrespective of the presence of Arnica m., suggesting basal metabolism of macrophages.

![Cell viability of macrophages.](https://doi.org/10.1371/journal.pone.0166340.g003)

**Fig 3. Cell viability of macrophages.**

THP-1 macrophages in the resting state (diagonal bars) or after differentiation with IL-4 (crossed bars) were cultivated hours in the presence of Arnica m. at various dilutions or Control solvent. The histograms represent separate wells of a typical experiment. There are no significant differences between any Arnica m. (p>0.05)

Changes in gene expression after Arnica m. treatment

The effects of Arnica m. treatment on the global gene expression of IL4-polarized THP-1 cells was incubation by comparison with Control. The basic RNA-seq analysis were done in cells treated with highest dose that could be used since 1c contained a dose of ethanol incompatible with cell culture—were reproduced in 5 different biological replications. Approximately 25 million valid reads obtained for unambiguously annotated on 60434 gene transcripts. No arbitrary filtering of expression level was applied to the data. Differential gene expression analysis was performed to identify significant target genes of Arnica statistically significant DEGs was thus obtained as shown in Table 1. The RPKM and Log2Fold changes were performed, plus the original values of pooled samples from assays done S1 Table.

Mean RPKM is an indicator of the absolute amount of RNA in samples from cells treated with Arnica shows that FN1 (fibronectin) was by far the most expressed gene and its RPKM values increased most expressed gene was LRP1 (from 19.4 to 23.6), and the third was HSPG2 (from 9.5 to 11.3), included 6 mitochondrially-coded NADH dehydrogenases which are subunits of Complex I, Cytochrome oxidases of Complex 4, and two ATP synthases of Complex V. In eukaryotes, the Complex I are encoded by the mitochondrial genome [33] and are normally highly expressed. We confirmed this high expression in Control (e.g. 529.6 RPKM for cytochrome c oxidase III, 4766.7 RPKM for NADH caused a slight but reproducible decrease of the expression of all the indicated genes (Table 1).

Mean fold changes, calculated as the average of the Log2 Fold Change of the 5 replicates, ranged...
up-regulation) to -0.36 (maximum down-regulation). The 7 up-regulated genes included low-density lipoprotein-receptor 1 (LRP1), fibronectin 1 (FN1), lysine (K)-specific methyltransferase (KMT2D), complement heparan sulfate proteoglycan (perlecan, HSPG2), microtubule-actin crosslinking factor 1 (MACF1), and fibrillin 2 (FBN2).

Functional gene enrichment analysis (Table 2) was performed by analysing international databases and using the DAVID software. Among the genes stimulated by *Arnica m.*, a statistically significant enrichment of HSPG2, FBN2, FN1 and Calcium ion binding motifs (LRP1, MACF1, FBN2) emerged (Fisher p < 0.001). Most notably, a clearly up-regulated function concerned the proteinaceous extracellular matrix (ECM) (FBN2, FN1 (p<0.01)). The down-regulated genes converge into the common pathway of oxidative phosphorylation into the cell component gene ontology of mitochondrial complex I as expected (p<0.001).

### Table 2. Functional classification and gene enrichment analysis.

https://doi.org/10.1371/journal.pone.0166340.t002

<table>
<thead>
<tr>
<th>Gene</th>
<th>Database</th>
<th>Function</th>
<th>Description</th>
<th>Fold Enrichment</th>
<th>P value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRP1, HSPG2, FBN2, FN1</td>
<td>INTERPRO</td>
<td>PR0 0002</td>
<td>EGF-like region, conserved domains</td>
<td>22.40</td>
<td>&lt;0.001</td>
<td>LRP1, HSPG2, FBN2, FN1</td>
</tr>
<tr>
<td>HSPG2, FBN2</td>
<td>GO:000571</td>
<td>7D050557</td>
<td>Proteoglycan, extracellular matrix</td>
<td>17.12</td>
<td>&lt;0.01</td>
<td>HSPG2, FBN2, FN1</td>
</tr>
<tr>
<td>LRP1, MACF1, FBN2</td>
<td>GENEINFO</td>
<td>3D050558</td>
<td>Calcium binding</td>
<td>7.06</td>
<td>&lt;0.05</td>
<td>LRP1, MACF1, FBN2</td>
</tr>
<tr>
<td>MACF1, FBN2</td>
<td>GO:000571</td>
<td>5D050571</td>
<td>Mitochondrial respiratory chain complex</td>
<td>10.15</td>
<td>&lt;0.001</td>
<td>MACF1, FBN2, NQO, NQ, N2, NOS</td>
</tr>
<tr>
<td>MACF1, FBN2</td>
<td>GO:000571</td>
<td>5D050571</td>
<td>Mitochondrial respiratory chain complex</td>
<td>10.15</td>
<td>&lt;0.001</td>
<td>MACF1, FBN2, NQO, NQ, N2, NOS</td>
</tr>
<tr>
<td>FN1</td>
<td>GO:000571</td>
<td>5D050571</td>
<td>Mitochondrial respiratory chain complex</td>
<td>10.15</td>
<td>&lt;0.001</td>
<td>MACF1, FBN2, NQO, NQ, N2, NOS</td>
</tr>
<tr>
<td>FBN2</td>
<td>GO:000571</td>
<td>5D050571</td>
<td>Mitochondrial respiratory chain complex</td>
<td>10.15</td>
<td>&lt;0.001</td>
<td>MACF1, FBN2, NQO, NQ, N2, NOS</td>
</tr>
</tbody>
</table>

Protein release in supernatants

To confirm the function of up-regulated genes, we measured the release of some relevant proteins of ECM. In these, HSPG2 and fibrillin were detected only in traces, while fibronectin was identified in considerable amounts. The fibronectin protein was increased in IL-4 macrophages as compared with non-polarized cells and was increased by 13.9% to 39.6% (p<0.05). The effect was almost null in one experiment only, while in the other 5 it ranged from 13.9% to 39.6% (p<0.05).

### Table 3. Proteins detected in supernatant of THP-1 macrophages cultivated 24 h in the presence and absence of *Arnica m.*

https://doi.org/10.1371/journal.pone.0166340.t003

PMA-differentiated THP-1 macrophages were polarized with IL-4 as described in Methods or maintained in the same medium without IL-4 (Normal Macrophages), then both cultures were incubated for 24 h in the absence of *Arnica m.*. N = 6 complete experiments, assay in technical duplicates (HSPG2 and fibrillin) of micrograms/million cells. Note that HSPG2 and fibrillin in some experiments were under the detection limit of the assay.

Fig 4 shows the amount of fibronectin detected in the supernatants in the 6 separate experiments. The IL-4 effect was almost null in one experiment only, while in the other 5 it ranged from 13.9% to 39.6%.
Fig 4. Fibronectin detected in supernatants of cell cultures in the absence and in the presence of Arnica m
Symbols indicate the fibronectin values of the same experiments in the two conditions of polarization. The reported percent effect as compared with Control of the same experiment.
https://doi.org/10.1371/journal.pone.0166340.g004

Testing higher Arnica m. dilutions

We then investigated the changes induced by increasingly higher Arnica m. dilutions in the same genes that had shown significant alterations after treatment with 2c test solution (Table 1). These effects are reported in comparison with the mean of Controls and the RPKM of all samples are given in S1 Table, right (availability of sufficient volumes) and the high costs of RNA-seq, we could not separately assay the five experiments at all the various dilutions. Therefore, to decrease experimental variability, we treated with the same Arnica m. dilution, using extracts from all the five experiments performed. Variation possibly due to biological replicates, but meant we could not evaluate the standard error. For these reasons, Fig 5 (panels B-F) does not include the error bars for individual genes, but only the mean of the various genes provided a first and preliminary evaluation of the major effects across different dilutions.
DEGs described in Table 1 were divided in the two groups as upregulated (red bars) and downregulated genes sets. Grey bars report the mean fold changes ± SE of the two genesets at each dilution Log2 fold change values calculated from 5 experiments; Panels B-F *Arnica m*. 2c, 3c, 5c, 9c, pooled RNAs of 5 experiments. P values of Wilcoxon signed-rank test statistics are reported near the mean of each.

The gray bars in Fig 5 show the means and standard errors of the *Arnica m*. effects for each gene (down-regulated), with the p values of the differences between treatments and Control solvent. If the null hypothesis that treatment has no effect: in such a case the mean fold change values for approximate zero, and eventually (since some genes may be modified by chance), the mean of approach zero. Finally, if the null hypothesis is true, the number of up- and down-regulated genes—should be approximately the same.

Considering the *Arnica m*. 2c dilution (Fig 5, top two panels) we can see that the up-regulated genes responded in the pooled analysis (Panel B) roughly in the same direction as they did in the separate analysis (Panel A). Generally, looking at the red bars (which denote the genes previously found to be up-regulated) 7 were also up-regulated in this pooled analysis. Conversely, looking at the blue bars (denoting down-regulation) we see that all 13 of these genes are likewise down-regulated in this pooled analysis. Values of pooled samples were reliable also if done with a single RNA-seq assay. The differences with the Control were
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statistically only for each entire geneset (up-regulated or down-regulated groups of genes) by a test for paired data, testing whether the differences are mainly positive or negative, or evenly distributed.

The changes due to Arnica m. 2c were highly significant both in the mean of 5 experiments (Panel A). Absolute fold changes less than or equal to 0.05 were considered null.

For what concerns the higher dilutions, Arnica m. 3c (Panel C) similarly showed a prevailing stirring up-regulated geneset: 6 genes out of a total of 7 (the exception was MACF1) were here also up-regulated genes of the previously down-regulated geneset were actually down-regulated also here (the expression of each geneset (grey bars) were approximately of the same magnitude as those induced statistically significant. Arnica m. 5c (Panel D) instead stimulated all genes of the up-regulated group and way and the prevailing inhibiting effect was also confirmed on the down-regulated geneset, with unchanged and of CCDC88B that showed an inversion of effect. Arnica m. 9c has a very faint effect in different genes, so that the global analysis was at limit of statistical significance three times.

On the downregulated geneset Arnica m. 9c had a faint but statistically significant effect, with the CCDC88B. Arnica m. 15c slightly up-regulated 7 genes out of a total of 7, the strongest effect was down-regulated geneset, Arnica m. caused a slight but consistent downregulation of 10 genes, h and upregulated CCDC88B as seen with the lower dilutions. In summary, both upregulating and downregulated genes were maintained across the increasing dilutions, while for many considered genes a non-linear trend was observed.

Other candidate DEGs

The results presented thus far concern significant alterations of a series of genes identified thro and analysis under very stringent statistical tests—that is, after adjusting for FDR. By so doing, type-1 errors but also the probability of discovering true positive effects of Arnica m. on other cells in ECM. In point of fact, a large list of proteins with different roles are involved in the ECM changes phases of wound healing and remodeling and includes various cell types. To explore the possibility of genes in the Arnica m. effects on macrophages, we performed a further analysis which included without applying the correction (n = 476). We then searched for the list of proteins interacting with the database (http://www.reactome.org/) and retrieved a list of 291 genes that represent the pathways of wound healing organization (identifier R-HSA-1474244.1). By matching these 291 genes with the 476 differentially treated genes, we retrieved 22 genes, 13 of which were upregulated and 9 downregulated. The upregulated and HSPG2 as expected, plus fibrillin 1, nidogen, osteonectin, dystonin, MMP2 and ADAMTS2. metallopeptidases and the proteolytic enzyme calpain 3 resulted among the down-regulated DEGs already discovered with application of FDR and cited in Table 1) are reported in S2 Table, left part.

We also checked whether the same genes were affected by increasing drug dilutions (S2 Table), less regular than those observed with the group of genes described in Table 1 and Fig 5. Even if a general tendency to the same direction could be noted, most genes changed from up to down regulation or did not change at all. Only the genes ADAMTS2 and ITGAD responded to Arnica m. treatment in the same way (upregulation, respectively) in cells treated by all dilutions. Although this supplemental analysis should be considered only as a preliminary indication of further genes involved, it suggests that in Arnica m. may affect ECM organization in a way and the prevailing inhibiting effect was also confirmed on the down-regulated geneset, with unchanged and of CCDC88B that showed an inversion of effect. Arnica m. 9c has a very faint but statistically significant effect, with the CCDC88B. Arnica m. 15c slightly up-regulated 7 genes out of a total of 7, the strongest effect was down-regulated geneset.

In a previous study, which analysed a panel of inflammatory genes by RT-array, Arnica m. stimulated CCL2 (MCP-1), CXCL1, CXCL2, CXCL8, MRC1, NFkB1 and inhibited the expression of MMP1 analysis, carried out on whole transcriptome with RNA-seq, confirmed the same trend of expression of those genes, albeit with values that do not attain statistical significance (data not shown). Of the many genes whose trend was not confirmed by RNA-seq were NFkB1 and TNFA, which did not change according to the present RNA-seq analysis. It should be noted that RNA-seq is a high-throughput tool compared to gene-focused RT-PCR, a factor that may explain a partial discrepancy between the interested genes have low expression values.
Effectiveness on a wound healing model

In order to investigate the possible functional implications of the observed molecular changes, cells treated with *Arnica m. 2c* comparing them with untreated cells. The scratch assay is an ea method for measuring cell migration *in vitro* [23]. The test is based on the observation that, whe “scratch”–is created on a confluent cell monolayer, the cells on the edge of the newly created get close the gap. To test *Arnica m.* in this system we used primary mouse bone marrow derived m. This because in the previous experience of our department laboratory, and in our own test assay, stable monolayers and showed more consistent motility [25]. The left panels of Fig 6 show some assay.

![Image](https://doi.org/10.1371/journal.pone.0166340.g006)

**Fig 6. Wound closure effect of *Arnica m.***

Light microscope images of *in vitro* wound closure using a confluent monolayer of BMD macrophage show one representative experiment of cell migration into the created wound area in the absence (B and D) of *Arnica m. 2c*. Images A and B show the wound area immediately after the wound area after 4.5 h. Pictures were acquired by means of contrast phase microscopy. The bar charts (E and F) report the gap width of the wound area before and after cell migration wound occupancy in the absence (E) and in the presence (F) of 20 ng/ml IL-4. Gray bars: Control solvent, yellow bars: *Arnica m. 2c*. Means±SE of three replicate wells of an experiment representative of the three performed. The result of the Friedman test comparing the whole series of changes in cells treated with drug or with Control solvent https://doi.org/10.1371/journal.pone.0166340.g006

Standardized scratches initially caused complete removal of the monolayers of BMD macrophages a few hours the cells started to fill the gap sufficiently to allow the front line to be easily determin quantified. In the presence of *Arnica m.* (Fig 6D) the filling of the gap was slightly faster than in the presence of IL-4. The same field was completely full of macrophages after 24 hours of incub (98.8±0.7% occupancy with Control cells and 99.3± 0.1% with *Arnica m.*, treated cells, n.s.) whi part of the gap was filled (63.2±3.9% occupancy with Control cells and 62.3±7.0% with *Arnica m.* shown in figure). The bar charts on the right of Fig 6 represent the time-course of cell migration (F) of IL-4. *Arnica m. 2c* promoted a faster cell migration in both conditions but the difference be
and statistically significant only in the presence of IL-4 (p = 0.014). The level of wound closure was higher than that obtained with the Control solvent.

Discussion

Preparations from traditional medicinal plants are often used as alternative remedies aimed at facilitating wound healing [34,35]. However, according to the available literature in medical databases, the mechanisms of homeopathic remedies have yet to be fully understood. The application of whole plant extracts, formulations, may be beneficial because herbaceuticals can have multiple and pleiotropic targets to treat various pathological conditions such as bruises, swelling associated with trauma, pain, inflammation, and post-operative clinical conditions [3,5,37,38]. Investigations of its cellular and molecular targets pathways, but little is known about its possible action on the stages of tissue formation and remodeling in different dilutions of the whole plant extract in THP-1 human cells, differentiated into an IL-4 activated phenotype involved in healing and tissue remodelling. RNA sequencing of whole transcriptome allowed the identification of several genes whose expression was significantly altered following the treatment.

The physicochemical features of the Arnica m. used as a starting material to prepare further working dilutions were investigated through NTA, which provided a quantitative and morphological analysis of nanostructures in the solution. Nanoparticles naturally form during preparation of herbal extracts in liquid solution, and the presence of traces of silica from the glass container has been observed to help nanoparticle nucleation [39]. Moreover, exosomes or lipidic plant debris may be present in plant extracts [40]. In this work, for the first time, Arnica m. preparations were found to exhibit polydispersed nanosized formations with sizes ranging from 100 to 400 nm in size. These nanosized formations might represent a bioactive form of diluted herbal extract, as suggested [41–44], and this interesting hypothesis deserves further investigation.

The main and novel finding was the increased expression of several genes of tissue matrix proteins whose increase was confirmed also by protein assay in culture supernatants. This evidence suggests a new and relevant role of this plant in wound-healing processes. In fact, most of the up-regulated genes which emerge from extracellular matrix (ECM), and their enrichment as a functional group of genes is highly significant for collagen, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, fibrillin, and several other components bind to each other as well as to cell adhesion receptors, forming a complex network of tissue and epithelial cells bind to basement membrane and fibrillar components.

Fibronectin is a multi-domain protein with an essential role in the ECM since it binds to both cell fibres. Cell receptors for fibronectin transduce signals which regulate diverse functions, such as whose regulation is vital during normal and pathological conditions [45,46]. This protein is vital for three-dimensional tissue architecture and for regulating cellular processes including adhesion, spreading, migration [47]. Fibronectin supports efficient platelet aggregation and pro-coagulant activity [48]; regulating the neovascularisation of granulation tissue during the resolution of tissue injury. Fibronectin is an important component of the early tendon repair process [49,50]. FN-1 gene is overexpressed in the early phase of inflammation, suggesting it has important role in ECM deposition and tissue remodeling [51]. Based on these considerations, we can formulate the hypothesis that the increase (20–30%) in the production of fibronectin induced by treatment with Arnica m. could greatly facilitate the wound closure and the migration of epithelial cells over the granulation tissue.

Other up-regulated genes included LRP1, HSPG2, and FBN2, which have an EGF-like domain in common with FN1. The significant association of these four genes into this functional group defined by enrichment analysis stands out as an evolutionary conserved domain, which derives its name from the epidermal growth factor, with growth and tissue repair. Most occurrences of the EGF-like domain are found in extracellular proteins or in proteins known to be secreted, such as components of the extracellular matrix.
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is important in protein-protein interactions and the proteolytic release of this domain from membr
on erbB receptors involved in cell growth and survival [52]. The interaction between the EGF-like calcium-dependent, a feature that is in keeping with the enrichment of calcium-binding group of

Low-density lipoprotein-receptor-related protein-1 (LRP1) is a receptor that mediates endocytosis signaling. LRP-1 binds and internalizes numerous, structurally diverse ligands, delivering most l
ysosomes for degradation [53]. LRP-1 also controls the plasma membrane proteome by regulaproteins in the secretory pathway [54]. Recently, its role as a regulator of inflammation has ene
to bind extracellular matrix proteins including fibronectin and thrombospondin [55] and to clear r
croplage LRP1 to modulate endocytosis and protein degradation confers on it a role in re
 Af

Heparan sulfate proteoglycan 2 (HSPG2, Perlecan) is a protein that in humans is encoded by the protein in basement membrane. HSPG2 is a key component of the cortical bone and serves as the osteocyte cell body to the bone matrix. A reduction in perlecan secretion interferes with bone vie[v[59].

The protein encoded by KMT2D is a histone methyltransferase that methylates the Lys-4 position regulates chromatin accessibility of adjacent genes and is associated with positive regulation of modifications induced by this protein, which are slight even after Arnica m. 2c treatment, may ha
be identified. In fact, many genes are expressed under the presence of this histone modification markers for M2 phenotype in IL-4 treated macrophages [60]. The gene MACF1 (microtubule an
ike slightly but significantly overexpressed by Arnica m. 2c treatment. This protein has the crosslink microtubules and F-actin networks, thereby directing microtubule organization. Directio
wound repair, and MACF1 has been observed to play a role in wound healing and epidermal mi
required for rapid and efficient formation of a hyperproliferative epithelium in response to injury, on epidermal migration rather than proliferation [61].

The decreased expression of several mitochondrially-coded genes of respiratory chain is a puzzling phenomenon was accompanied by a decreased synthesis of related proteins, one would envis
chain and oxidative phosphorylation. Certainly, this expression change was not associated with damage, since cell viability was not changed upon Arnica m. treatment. Instead, the slight incre
statisitically significant, and to be confirmed by further studies) could suggest an increased level consequence of decreased consumption in the mitochondrial respiratory chain. Furthermore, sio
free radicals in conditions of lack of oxygen and reperfusion [62], it is possible that a moderate d
have a cytoprotective effect in conditions of lack of oxygen, such as those presumably en
issue. This hypothesis is in agreement with the finding that a 30c dilution of Arnica m., adminis
decreased oxygen consumption of isolated liver mitochondria and protected from oxidative dam
[14]. The authors of that work interpreted this effect as a defence against oxidative stress. Furth
whole extract of Arnica m. showed antioxidant activity and a cytoprotective effect against oxidat
report an inhibitory action of Arnica m. on nitric oxide and TNF-α production by murine macroph
wound, treatment with Arnica m. 2c increased cell motility, confirming that the drug does not infl
metabolism of the cell.

Among the down-regulated genes, the only non-mitochondrial gene was CCDC88B, coding for protein 88b, that is expressed in lymphocytes and myeloid cells and may have a role in regulat
inflammation [64]. Interestingly, this gene showed an unusual behaviour in cells treated with dif
decreased in samples from cells treated with low dilutions of Arnica m. (2c and 3c) while it incre

dilutions.
One of the major components of *Arnica m.* with an acknowledged biological activity is the sesquiterpene lactones for its anti-inflammatory properties. In a lymphoid cellular model, helenalin was found to inhibit the NF-kappaB—a central mediator of human immune response—through the alkylation of p65 subunit binding to DNA [6]. However the cited studies did not evaluate the contribution of the whole plant concentration of $10^{-5}$ Mol/L, much higher than that present in *Arnica m.* 2c used in this investigation. The concentration of $1.05 \times 10^{-8}$ Mol/L. In this RNA-seq investigation, NFKB1 and RELA gene expression were significantly upregulated by *Arnica m.* but, the reported effects of helenalin on NF-kappaB were due to the inhibition of proteolytic enzymes at the transcription level. The role of the NF-kb system and other transduction factors in the regulation of fibronectin expression remains to be further investigated.

Our findings provide a firm molecular explanation for previous experimental observations report of inflammation and wound healing processes. In a randomized double-blind study conducted on Sprague-Dawley rats, the homeopathic dilution (4x) reduced wound irritation (redness, swelling and heat) after hallux valgus surgery [37]. An anti-inflammatory effect of *A.* carrageenan-induced foot oedema in rats has been reported by some authors [12] and a complete remission of the disease was obtained in some cases treated with *Arnica m.* plus other compounds at low dilutions (4x-6x) was effective for blood-induced experiments [38]. As a topical gel improved the healing of surgically-induced wounds in Wister rats, but significant drug was delivered together with microcurrent application [67]. A commercial homeopathic complex (4th decimal, 4x) of *Arnica m.*, *Calendula* and *Hypericum* promoted fibroblast growth in a scratch test [68]; the present work confirms this healing capacity of *Arnica m.* 2c (which corresponds approximately to the 4x used in these studies) also in macrophages, using a scratch-test model to evaluate cellular migratory events during healing process. This same model has previously been proven a valuable tool for assessing the effects of honeys [69].

At the present state of our knowledge, the mechanism of action of homeopathic medicines is expected to be complex and could involve modulation of different cells and further pathways. The field of homeopathy is open to further studies.

The effect in this in vitro model does not mean that the modulating effect will also be small in vivo. Whereas conventional anti-inflammatory drugs are designed to suppress the underlying enzymatic and cellular mechanisms (e.g. prostaglandins, cytokines) and act at considerably high doses, homeopathic treatment is designed to modulate pathological aspects and malfunctioning tissues, because the inflammatory process in itself is self-healing. In these conditions, even a 20–30% increase of macrophage activity in producing fibronectin may have a decisive positive outcome of tissue healing and repair. Moreover, given the multiplicity of its alkaloids, flavonoids, and sesquiterpene lactones [80], it is conceivable that these compounds may act more complex and could involve modulation of different cells and further pathways. The field of homeopathy is open to further studies.
Conclusions

The results of this work indicate that *Arnica m.* acts on macrophages by modulating a number of genes and by increasing motility. RNA-seq analysis allowed the identification of several genes which are particularly sensitive to ultra-low doses of this plant extract. Molecular analysis of gene expression suggests that a primary action of this medicinal plant is the stimulation of tissue matrix synthesis. These findings provide new insights into wound-associated molecular events and point to macrophage fibronectin production as a potential therapeutic target of *Arnica m.* for the treatment of wound repair.

Supporting Information

S1 Table. Expression values (RPKM) of Control and *Arnica m.*-treated cells and differential expression (Log2 Fold Change) of the genes reported in Table 1.

IL-4-differentiated THP-1 macrophages were treated with Control solvent or with *Arnica m.* 2c, 3c, 5c, 9c and 15c dilutions. Samples from Control solvent and *Arnica m.* 2c were analysed by RNA-seq in each experiment from 5 experiments of cells treated with *Arnica m.* dilutions 2c, 3c, 5c, 9c and 15c were pooled, and Fold Change calculated comparing their RPKM with the mean RPKM of Control values.

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(XLSX)

S2 Table. Expression values (RPKM) and differential expression (Log2 Fold Change) of a series of extracellular matrix genes selected from the Reactome database as described in the text.

The genes with FDR-adjusted p values > 0.05 and FDR unadjusted p values < 0.05 are here reported, while those with p values < 0.05 are reported in Table 1. Samples from Control solvent and *Arnica m.* 2c were analysed by RNA-seq in each experiment of five performed. RNA samples from 5 experiments of cells treated with *Arnica m.* dilutions 2c, 3c, 5c, 9c and 15c were pooled, analysed with RNA-seq and Fold Change calculated comparing their RPKM with the mean RPKM of Control values.

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Author Contributions

Conceived and designed the experiments: PB MM DO CB.

Performed the experiments: MM DO CB AB LB EG.

Analyzed the data: MM LB FDL.

Contributed reagents/materials/analysis tools: MM AB EG LB.

Wrote the paper: PB MM.

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