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Arnica montana Stimulates Extracellular Matrix Gene Expression in a Macrophage Cell Line Differentiated to Wound-Healing Phenotype

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Abstract

Arnica montana (*Arnica m*.) is used for its purported anti-inflammatory and tissue healing action injuries, but its cellular and molecular mechanisms are largely unknown. This work tested *Arnic* using an *in vitro* model of macrophages polarized towards a "wound-healing" phenotype. The m cell line was cultured and differentiated with phorbol-myristate acetate and Interleukin-4, then e centesimal (c) dilutions 2c, 3c, 5c, 9c, 15c or Control. Total RNA was isolated and cDNA librarie NextSeq500 sequencer. Genes with significantly positive (up-regulated) or negative (down-regu differentially expressed genes (DEGs). A total of 20 DEGs were identified in *Arnica m*. 2c treate up-regulated and 13 were down-regulated. The most significantly up-regulated function concern epidermal growth factor-like region ($p<0.001$) and three genes of proteinaceous extracellular m proteoglycan 2 (HSPG2), fibrillin 2 (FBN2), and fibronectin (FN1) ($p<0.01$). Protein assay confir increase of fibronectin production ($p<0.05$). The down-regulated transcripts derived from mitoch components of electron transport chain. The same groups of genes were also regulated by incr 9c, 15c), although with a lower effect size. We further tested the healing potential of *Arnica m*. 2 closure based on the motility of bone marrow-derived macrophages and found evidence of an a in this system. The results of this work, taken together, provide new insights into the action of *A* and identify extracellular matrix regulation by macrophages as a therapeutic target.

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Data Availability: All relevant data are within the paper and its Supporting Information files. has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO S GSE77381 and GSE77382 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77381 /geo/query/acc.cgi?acc=GSE77382).

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Introduction

Arnica montana L. (referred to here as *Arnica m*.) is one of the most popular medications in con employed in tincture, ointment, cream, gel, and tablet form. It is a herb native to the mountains has been used to treat various pathological conditions, including pain, stiffness and swelling ass clinical conditions (including contusions and sprains) and for symptomatic relief in osteoarthritis *Arnica m*. is generally applied only topically on the skin, while as a homeopathic formulation it is —since it is not considered dangerous owing to the high dilution. The literature on *Arnica m*. in homeopathic preparations is rapidly increasing, but the knowledge of its action mechanism(s) re

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

There is some experimental evidence, in laboratory animals, of an anti-inflammatory action of *A* ethanolic extract [10] or at the 6^{th} centesimal homeopathic dilution (6c) [11,12]. Additionally, *Arn* in inducible NO synthase and cyclooxygenase-2 protein levels, a reduction in TNF-α, and preve in J774 murine macrophage cells without cytotoxicity *in vitro* [13]. Furthermore, oral treatment w 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 hyp targets of *Arnica m*. action is the macrophage, and accordingly decided to evaluate this plant's cell line, a widely used model for immune modulation [15,16]. This cell line is widely used in lab macrophage biochemistry and molecular biology. The advantage of a cell line resides essentiall experiments in the same conditions, avoiding the variations due to individual sensitivity of differ doses of drugs—even with the highest *Arnica m*. 2c dilution, in assay medium the sesquiterpen dose—we expected small effect sizes and so preferred to use a highly reproducible model. THP monocytes, but when treated with low doses of phorbol esters (PMA) they differentiate to cells v functional features of tissue macrophages. On the basis of environmental cues and molecular n to either a pro-inflammatory type (M1) or to an anti-inflammatory or pro-reparatory type (M2) [17 macrophages polarized by interleukin-4 (IL-4) treatment to a phenotype that takes on character

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 p immune response [22]. Among the tested genes, CXCL1 in particular exhibited the most substa CXCL2, CXCL8, BMP2 and NFKB1 were slightly up-regulated, suggesting a positive influence o recruitment and on angiogenesis. The most pronounced effects were noted in IL-4 polarized ma treatment. Therefore, we decided to re-investigate the same cell extracts with the most high-thr (RNA-seq), designed to evaluate the whole transcriptome. We assessed RNA samples from a s *Arnica m*. at the 2nd centesimal homeopathic dilution (*Arnica m*. 2c) compared with vehicle (Co identify all the potential genomic targets of the plant's regulating action, through bioinformatics a of genes (*Arnica m*. 2c vs Control) and using statistical methods correcting for false discovery ra 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 Control solutions using an *in vitro* model of wound healing, in which macrophages migrate throu cell monolayer. A major advantage of this method is that it mimics, to some extent, the migratio 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). Growt 20mM solution and DMEM with l-glutamine were purchased from Lonza (Belgium). Foetal bovir 12-myristate 13-acetate (PMA), pure ethanol and ultra-pure water (W3500) were purchased fro USA). Human interleukin-4 (IL-4) was purchased from Macs-Miltenyi Biotec (Germany). Murine (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 (Upps

Test solutions

Arnica m. was produced by Boiron Laboratoires (Lyon, France) according to the French Homeo as a first centesimal dilution (*Arnica m*. 1c) of the hydroalcoholic extract (Mother Tincture, MT) i content of sesquiterpene lactones of the MT was determined by liquid chromatography and the pharmacopoeia standards was checked by thin layer chromatography. UV-visible absorption sp performed with a Jasco V-650 double-beam spectrophotometer using quartz cuvettes with 1cm 30% v/v as the reference-blank sample. Nanoparticle content was determined by nanoparticle t NanoSight LM10 (Malvern) instrument equipped with laser at 532nm and the NanoSight NTA 3. 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 ultra-pure water. Therefore, 2c corresponds to 10^{-4} of the MT. This solution was filtered with a 0 to vigorous succussion with a Dyna-A mechanical shaker delivering 20 strokes/second with an test solution was prepared using 30% ethanol/distilled water (same batch of the *Arnica m*. 1c di ultra-pure water and succussed as described for *Arnica m*. 2c sample. Final ethanol concentrati the test medicines were added as 10% of the final culture volume. This dose did not affect cell v experiments.

Higher dilutions of *Arnica m*. were prepared as described previously [22]. Starting from a 1c sol prepared in 5ml of 30% ethanol/distilled water solvent followed by filtering and succussion. Stoc ethanol/distilled water were wrapped in aluminium foil, stored at room temperature in the dark, a

preparation. The last centesimal dilution step was always performed immediately before each e
the *Arnica m*. dilutions prepared in this way, those tested with the cells were: 2c, 3c, 5c, 9c, and

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% CO_2 in a humidified incubator as described [22]. Briefly, i
the cells were seeded at a density of $2.5x10^5$ cells/mL in 24-well plates in 1ml medium with 2ml
day 2 all the cell cultures were supplemented with 20 ng/mL of PMA and on day 3 the cultures v
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 ex
every treatment was performed in triplicate wells.

Bone marrow–derived macrophages

For the scratch test, bone marrow–derived macrophages (BMDM) were isolated from femurs ar
C57BL/6J mice as described by Suen et al. (1999) [24] and Baruzzi et al. [25]. Briefly, cells were
(Lonza) supplemented with 15% FBS, 10% L929-cell conditioned medium (LCM) as a source of
U/ml penicillin, and 100 µg/ml streptomycin (BMDM complete medium), and cultured at 37°C/5%
the non-adherent cells were removed, counted, plated on bacteriological (non tissue-culture-tre
concentration of $1×10^5$/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 seed
in 96-well plates and differentiated with IL-4 as described above. After 24h of treatment with *Arn*
(v/v) pre-warmed WST-1 solution was added to the cells and the plate was incubated for 20 min
samples was measured using a Victor3 multilabel reader (PerkinElmer, Shelton, CT, USA) at 45
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 qua
using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA samples
using an RNA 6000 Nano Kit (Agilent, Wokingham, UK). The samples with RNA integrity numbe
adequate for library preparation. RNA aliquots (2.5µg) were used to isolate poly(A) mRNA for th
Illumina RNA-Seq library using the TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, C
before the generation of clusters, was checked with High Sensitivity DNA Kit (Agilent, Wokingha
quantified by qPCR using the KAPA Library Quantification kit (Kapa Biosystems Inc., Woburn, M

Libraries were sequenced with a NextSeq500 sequencer (HighOutput flow cell with 75 sequenc
sequences. The reads were aligned to the human reference genome (GRCh38) using the TopH
expression value of known and novel genes was quantified as reads per kilobase of exon mode
using the human working gene set (Ensembl release 80) as reference annotation. The effect of
calculating Log_2 of the ratio between RPKM of each gene in *Arnica m*.-treated samples and Co
Change). Genes with Log_2 Fold Change values that were significantly positive (up-regulated) or
defined as differentially expressed genes (DEGs).

Sequences of DEGs in the “protein coding” category were functionally annotated using Blast2G metabolic pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG) to the query sec 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 On through GEO Series accession numbers GSE77381 and GSE77382 **(https://www.ncbi.nlm.ni /acc.cgi?acc=GSE77381; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7738**

Protein quantification by ELISA

Matrix-related proteins (fibronectin, fibrillin, and heparan sulfate proteoglycan 2) were quantifiec cells using ELISA assays fibronectin human Elisa kit (Abcam), human Fibrillin-2 ELISA Kit and 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 BMD On day 4, a portion of the cell cultures was supplemented with 20 ng/ml murine IL-4 while anoth day 5 the monolayers were wounded by transversely scratching the well with the tip of a 200 µl The detached cells were carefully aspirated and the wells washed with phosphate-buffered salir migration test was DMEM (Glutamax, pen-strep) with 2% FBS, either with or without 20 ng/ml IL solutions were added 24 h before wounding and maintained, during the migration time, at the c culture volume. Three sets of experiments were performed with triplicate wells for each conditio field of the wound were acquired by means of contrast phase microscopy using an Olympus IX5 magnification to assess cell migration. The experiments were evaluated by examining microsco and using a grid composed of 500 small frames to calculate the % of wounded area occupied b

Statistics

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

The evaluation of differential gene expression between the *Arnica m*.-treated and Control samp 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-valu Comparisons were done by testing, for each gene and each experiment (N = 5), the null hypoth exactly zero, i.e., that the gene was not at all affected by the treatment.

Effect	HGC Nomenclature		RPKM/IL-4 Control		RPKM/IL-4 + *Arnica m.*		Log_2 Fold Change			Description
	Symbol	ID	Mean	SE	Mean	SE	Mean	SE	P value(corrected for FDR)	
Up-regulated	CR1	2334	1.6	0.4	2.0	0.5	**0.3**	0.07	0.0225	Complement component (3b/4b) receptor 1
	LRP1	6692	19.4	2.0	23.6	2.0	**0.29**	0.05	< 0.0001	Low density lipoprotein receptor-related protein 1
	FN1	3778	426.3	30.8	509.5	23.4	**0.27**	0.05	0.0007	Fibronectin 1
	FBN2	3604	3.0	0.2	3.6	0.1	**0.27**	0.09	0.0418	Fibrillin 2
	HSPG2	5273	9.5	1.3	11.3	1.5	**0.25**	0.09	0.0332	Heparan sulfate proteoglycan 2
	KMT2D	7133	6.3	0.4	7.5	0.3	**0.24**	0.06	0.0037	Lysine (K)-specific methyltransferase 2D
	MACF1	13664	2.5	0.1	2.9	0.1	**0.24**	0.08	0.0379	Microtubule-actin crosslinking factor 1
Down-regulated	COX3	7422	529.6	23.7	448.8	18.0	**-0.24**	0.05	0.0418	Mitochondrially encoded cytochrome c oxidase III
	ND2	7456	4766.7	289.3	3982.6	184.5	**-0.25**	0.04	0.0038	Mitochondrially encoded NADH dehydrogenase 2
	COX1	7419	1798.3	60.5	1506.3	46.7	**-0.25**	0.07	0.0418	Mitochondrially encoded cytochrome c oxidase I
	ND6	7462	85.2	4.4	70.2	1.8	**-0.27**	0.04	0.0018	Mitochondrially encoded NADH dehydrogenase 6
	ATP6	7414	404.9	15.1	334.1	11.3	**-0.28**	0.04	0.0008	Mitochondrially encoded ATP synthase 6
	ND5	7461	392.7	24.5	321.2	8.1	**-0.28**	0.06	0.002	Mitochondrially encoded NADH dehydrogenase 5
	ND1	7455	2081.5	112.5	1690.4	68.2	**-0.3**	0.061	0.0008	Mitochondrially encoded NADH dehydrogenase 1
	ND4	7459	819.0	41.2	659.1	16.6	**-0.31**	0.06	0.0006	Mitochondrially encoded NADH dehydrogenase 4
	MTRNR2L8	37165	45.6	4.1	36.3	1.4	**-0.31**	0.068	0.0176	MT-RNR2-like 8
	CYTB	7427	35.1	2.2	28.1	1.5	**-0.32**	0.057	0.0001	Mitochondrially encoded cytochrome b
	CCDC88B	26757	5.4	0.5	4.3	0.3	**-0.32**	0.068	0.0078	Coiled-coil domain containing 88B
	ATP8	7415	1530.0	85.2	1218.2	38.6	**-0.32**	0.094	0.0135	Mitochondrially encoded ATP synthase 8
	ND4L	7460	102.6	7.4	79.5	2.0	**-0.36**	0.068	0.0006	Mitochondrially encoded NADH dehydrogenase 4L

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Table 1. Gene expression of IL-4 differentiated THP-1 macrophages, treated with Control solvent or *Arnica*
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The statistical significance of the differences between expression profiles of gene groups (Up-re genesets) from cells treated with various *Arnica m*. dilutions (2c, 3c, 5c, 9c, 15c) was calculatec followed by the Wilcoxon signed-rank test using the SPSS software, version 17 (SPSS Inc., Chi is a nonparametric test for multiple related samples (in this case, the multiple genes– 7 up-regu cells treated with five *Arnica m*. dilutions and control solution) that checks the null hypothesis th terms of RPKM come from the same population. After verifying the significance of the Friedmar signed-rank test for paired data to evaluate the differences between RPKM of genes after treatr and mean RPKM of Control-treated cells, and to check whether such differences were prevalen distributed between the two signs. The differences were accordingly ranked, and the positive ar summed and statistically compared using the specific Wilcoxon tables. The logic of this approac the absence of treatment effects: if treatment has no effect the differences between gene expre samples and Control-treated samples should approach zero in all considered genes of the grou be modified by chance, the number of up- and down- regulated genes should be approximately significantly different). Log_2 Fold Changes were lower than or equal to ±0.05 (-0.05<FC<0.05) v

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

Statistical evaluation of the scratch assay was done using the Friedman test. It is used to test fc this case the series of time points for the Treated and Control samples) when the dependent va The null hypothesis is that the time series for two compared treatments (*Arnica m*. and Control

Results

Characterization of *Arnica m*.

The *Arnica m*. 1c used the starting material for this series of experiments was analysed by phys shows the UV-VIS absorption spectrum. This was characterized by a large UV peak around 22(280nm and 340nm. No substances absorbing at wavelength > 500 nm were detected in our pre

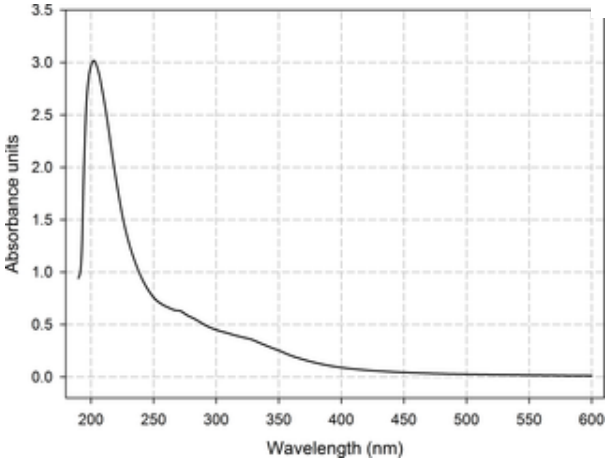

Fig 1. Absorption spectrum of *Arnica m*. 1c used as starting material.
https://doi.org/10.1371/journal.pone.0166340.g001

NTA analysis of the original *Arnica m*. 1c sample revealed the presence of a heterogeneous an nanoparticles (9.3 ± 1.0 particles/frame) corresponding to a concentration of 1.83 x 10^8 ± 1.88 x spectrum (Fig 2) showed a profile with about 6 peaks with a hydrodynamic diameter ranging fro 274.4 ± 100.4 nm. Zeta potential of these nanoparticles was -25.54 ± 9.50 mV (n = 12 determin

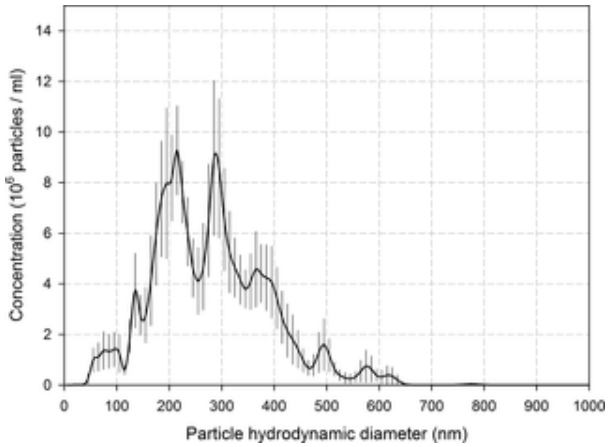

Fig 2. Nanoparticle spectrum of *Arnica m*. 1c. used as starting material.
The line is the average of 10 replicate measurements and vertical bars indicate SD.
https://doi.org/10.1371/journal.pone.0166340.g002

The amount of total sesquiterpene lactones in the original Mother Tincture was 36 mg/100ml. S *Arnica m*. sesquiterpene lactones is 340 g/mol [31], this amount is equivalent to 1.05 x 10^{-3} Mo of these active substances is 1.05 x 10^{-5} Mol/L in *Arnica m*. 1c and 1.05 x 10^{-7} Mol/L in *Arnica* 1.05 x 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, bu vehicle was not statistically significant. Since the WST-1 assay depends on the level of NADH p shows that mitochondrial NADH-producing activity was not significantly impaired by *Arnica m*. I higher in IL-4 differentiated macrophages, irrespective of the presence of *Arnica m*., suggesting basal metabolism of macrophages.

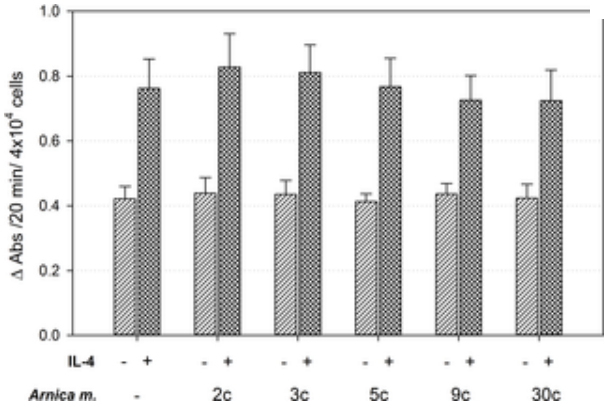

Fig 3. Cell viability of macrophages.
THP-1 macrophages in the resting state (diagonal bars) or after differentiation with IL-4 (cros hours in the presence of *Arnica m*. at various dilutions or Control solvent. The histograms rep separate wells of a typical experiment. There are no significant differences between any *Arni* (p>0.05)
https://doi.org/10.1371/journal.pone.0166340.g003

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 w incubation by comparison with Control. The basic RNA-seq analysis were done in cells treated dilution and highest dose that could be used since 1c contained a dose of ethanol incompatible reproduced in 5 different biological replications. Approximately 25 million valid reads obtained fo unambiguously annotated on 60434 gene transcripts. No arbitrary filtering of expression level w Differential gene expression analysis was performed to identify significant target genes of *Arnic* statistically significant DEGs was thus obtained as shown in Table 1. The RPKM and Log_2 Fold separate experiments 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 *A* shows that FN1 (fibronectin) was by far the most expressed gene and its RPKM values increas 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, Cyt Cytochrome oxidases of Complex 4, and two ATP synthases of Complex V. In eukaryotes, the h Complex I are encoded by the mitochondrial genome [33] and are normally highly expressed. W expression in Control (e.g. 529.6 RPKM for cytochrome c oxidase III, 4766.7 RPKM for NADH d caused a slight but reproducible decrease of the expression of all the indicated genes (Table 1 a

Mean fold changes, calculated as the average of the Log_2 Fold Change of the 5 replicates, rang

up-regulation) to -0.36 (maximum down-regulation). The 7 up-regulated genes included low-der protein 1 (LRP1), fibronectin 1 (FN1), lysine (K)-specific methyltransferase (KMT2D), compleme heparan sulfate proteoglycan (perlecan, HSPG2), microtubule-actin crosslinking factor 1(MACF down-regulated DEGs (13 genes) were mitochondrial genes coding for proteins of the mitochor

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

Geneset	Database	Function ID	Description	Fold Enrichment	P value	Genes
Up-regulated	INTERPRO	IPR013032	EGF-like region, conserved site	32.49	<0.001	LRP1, HSPG2, FBN2, FN1
	GOTERM_CC	GO:0005578	Proteinaceous extracellular matrix	17.12	<0.01	HSPG2, FBN2, FN1
	GOTERM_MF	GO:0005509	Calcium ion binding	7.06	<0.05	LRP1, MACF1, FBN2
Down-regulated	GOTERM_CC_FAT	GO:0005747	Mitochondrial respiratory chain complex I	101.44	<0.001	ND1, ND4L, ND4, ND5, ND2, ND6
	KEGG_PATHWAY	hsa00190	Oxidative phosphorylation	26.08	<0.001	ND1, ND4L, ND4, COX3, ND5, COX1, ND2, CYTB, ATP8, ND6, ATP6

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Table 2. Functional classification and gene enrichment analysis.
https://doi.org/10.1371/journal.pone.0166340.t002

Protein release in supernatants

To confirm the function of up-regulated genes, we measured the release of some relevant prote these, HSPG2 and fibrillin were detected only in traces, while fibronectin was identified in consi protein was increased in IL-4 macrophages as compared with non-polarized cells and was incre

		Normal Macrophages			IL-4-polarized Macrophages		
		Arnica m. 2c	Control	p	*Arnica m.* 2c	Control	p
HSPG2(Perlecan)	Mean	0.43	0.42	n.s.	0.35	0.31	n.s.
	SD	0.12	0.18		0.09	263	
Fibrillin	Mean	0.002	0.005	0.065	0.0004	0.0004	n.s.
	SD	0.001	0.0003		0.0004	0.0004	
Fibronectin	Mean	1.62	1.41	0.063	22.91	17.49	0.031
	SD	0.65	0.51		22.21	15.50	

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Table 3. Proteins detected in supernatant of THP-1 macrophages cultivated 24 h in the presence and abse
https://doi.org/10.1371/journal.pone.0166340.t003

PMA-differentiated THP-1 macrophages were polarized with IL-4 as described in Methods or m without IL-4 (Normal Macrophages), then both cultures were incubated for 24 h in the absence *Arnica m*. 2c. N = 6 complete experiments, assay in technical duplicates (HSPG2 and fibrillin) o micrograms/million cells. Note that HSPG2 and fibrillin in some experiments were under the det

Fig 4 shows the amount of fibronectin detected in the supernatants in the 6 separate experimer *m*. 2c effect was almost null in one experiment only, while in the other 5 it ranged from 13.9% tc

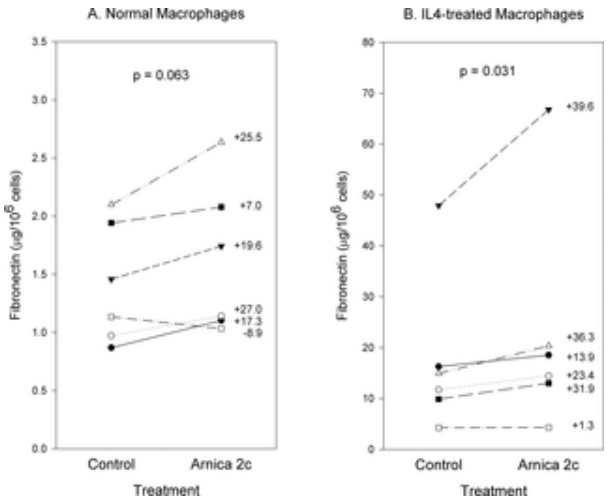

Fig 4. Fibronectin detected in supernatants of cell cultures in the absence and in the presence of *Arnica n*
Symbols indicate the fibronectin values of the same experiments in the two conditions of pola
percent effect as compared with Control of the same experiment.
https://doi.org/10.1371/journal.pone.0166340.g004

Testing higher *Arnica m*. dilutions

We then investigated the changes induced by increasingly higher *Arnica m*. dilutions in the sam
significant alterations after treatment with 2c test solution (Table 1). These effects are reported i
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
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 err
these reasons, Fig 5 (panels B-F) does not include the error bars for individual genes, but only
each group of genes (7 up-regulated in red, 13 down-regulated in blue). Since most of genes in
belong to similar functional groups, calculating the mean of the various genes provided a first a
major effects across different dilutions.

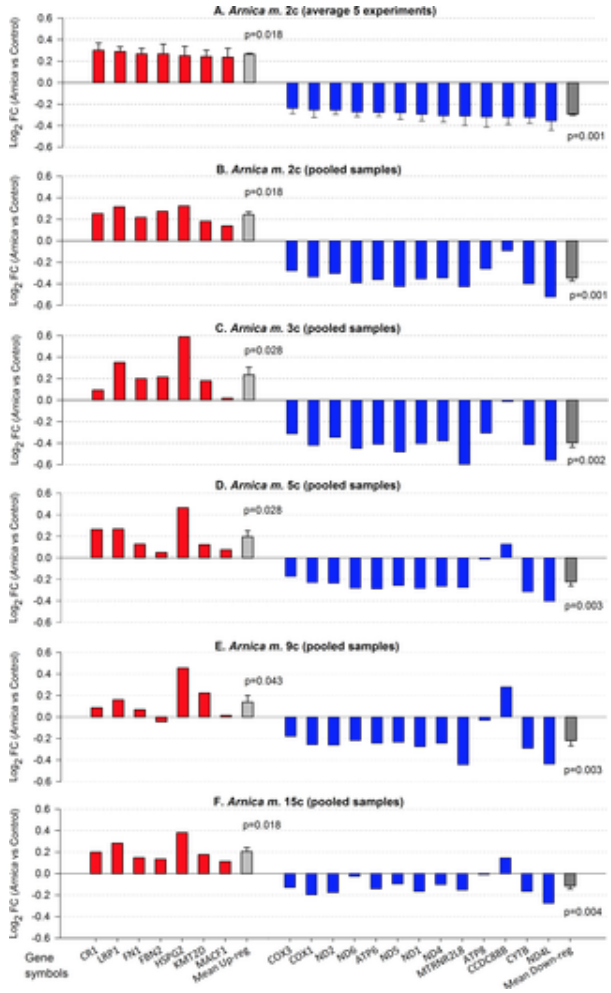

Fig 5. Effects induced by increasing dilutions of *Arnica m*. on gene expression in THP-1 cells.
DEGs described in Table 1 were divided in the two groups as upregulated (red bars) and dov genesets. Grey bars report the mean fold changes ± SE of the two genesets at each dilution Log_2 fold change values 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 https://doi.org/10.1371/journal.pone.0166340.g005

The gray bars in Fig 5 show the means and standard errors of the *Arnica m*. effects for each ge down-regulated), with the p values of the differences between treatments and Control solvent. E the null hypothesis that treatment has no effect: in such a case the mean fold change values fo 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 ger or down-regulation—should be approximately the same.

Considering the *Arnica m*. 2c dilution (Fig 5, top two panels) we can see that the up-regulated a responded in the pooled analysis (Panel B) roughly in the same direction as they did in the sepa 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-regulated) we see that all 13 of these genes are likewise down-regulated in this pooled a values of pooled samples were reliable also if done with a single RNA-seq assay. The differenc

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 di The changes due to *Arnica m*. 2c were highly significant both in the mean of 5 experiments (Pa (Panel B). 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 sti up-regulated geneset: 6 genes out of a total of 7 (the exception was MACF1) were here also be genes of the previously down-regulated geneset were actually down-regulated also here (the ex changes of each geneset (grey bars) were approximately of the same magnitude as those indu statistically significant. *Arnica m*. 5c (Panel D) instead stimulated all genes of the up-regulated g 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 e variable in different genes, so that the global analysis was at limit of statistical significance thres On the downregulated geneset *Arnica m*. 9c had a faint but statistically significant effect, with th CCDC88B. *Arnica m*. 15c slightly up-regulated 7 genes out of a total of 7, the strongest effect w downregulated 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 maintained across the increasing dilutions, while for many considered genes a non-linear trend

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-1errors but also the probability of discovering true positive effects of *Arnica m*. on other ca ECM. In point of fact, a large list of proteins with different roles are involved in the ECM change phases of wound healing and remodeling and includes various cell types. To explore the possib 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 wi database (http://www.reactome.org/) and retrieved a list of 291 genes that represent the pathwa organization (identifier R-HSA-1474244.1). By matching these 291 genes with the 476 differenti treatment, we retrieved 22 genes, 13 of which were upregulated and 9 downregulated. The upr 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 DE already discovered with application of FDR and cited in Table 1) are reported in S2 Table, left pa important proteins—e.g. periostin, osteopontin or tissue inhibitor metallopeptidases–does not a

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 i the same direction could be noted, most genes changed from up to down regulation or did not c Only the genes ADAMTS2 and ITGAD responded to *Arnica m*. treatment in the same way (upre respectively) in cells treated by all dilutions. Although this supplemental analysis should be cons indication of further genes involved, it suggests that in *Arnica m*. may affect ECM organization i

In a previous study, which analysed a panel of inflammatory genes by RT-array, *Arnica m*. stimu 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 expres those genes, albeit with values that do not attain statistical significance (data not shown). Of the only ones whose trend was not confirmed by RNA-seq were NFKB1 and TNFA, which did not c according to the present RNA-seq analysis. It should be noted that RNA-seq is a high-throughp range compared to gene-focused RT-PCR, a factor that may explain a partial discrepancy betw case 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, a 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 ga 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 assa stable monolayers and showed more consistent motility [25]. The left panels of Fig 6 show som assay.

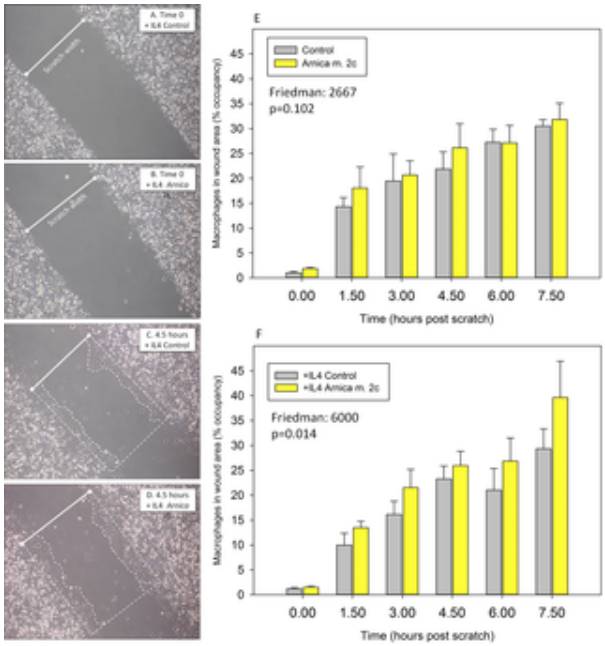

Fig 6. Wound closure effect of *Arnica m*.

Light microscope images of *in vitro* wound closure using a confluent monolayer of BMD mac show one representative experiment of cell migration into the created wound area in the abs presence (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 v The bar charts (E and F) report the gap width of the wound area before and after cell migrati wound occupancy in the absence (E) and in the presence (F) of 20 ng/ml IL-4. Gray bars: Co *m*. 2c. Means±SE of three replicate wells of an experiment representative of the three perfor 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 macropha a few hours the cells started to fill the gap sufficiently to allow the front line to be easily determir 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 v
higher than that obtained with the Control solvent.

Discussion

Preparations from traditional medicinal plants are often used as alternative remedies aimed at f
repair [34,35]. However, according to the available literature in medical databases, the mechani
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 targe
treat various pathological conditions such as bruises, swelling associated with trauma, pain, infl
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 renc
different dilutions of the whole plant extract in THP-1 human cells, differentiated into an IL-4 act
healing and tissue remodelling. RNA sequencing of whole transcriptome allowed the identificati
expression was significantly altered following the treatment.

The physicochemical features of the *Arnica m*. used as a starting material to prepare further wo
through NTA, which provided a quantitative and morphological analysis of nanostructures in the
naturally during preparation of herbal extracts in liquid solution, and the presence of traces of si
been observed to help nanoparticle nucleation [39]. Moreover, exosomes or lipidic plant debris
[40]. In this work, for the first time, *Arnica m*. preparations were found to exhibit polydispersed n
400 nm in size. These nanosized formations might represent a bioactive form of diluted herbal e
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 prot
whose increase was confirmed also by protein assay in culture supernatants. This evidence sug
of this plant in wound-healing processes. In fact, most of the up-regulated genes which emerge
extracellular matrix (ECM), and their enrichment as a functional group of genes is highly signific
collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, fibrillin, and severa
components bind to each other as well as to cell adhesion receptors, forming a complex networ
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 f
three-dimensional tissue architecture and for regulating cellular processes including adhesion, s
migration [47]. Fibronectin supports efficient platelet aggregation and pro-coagulant activity [48]
regulating the neovascularisation of granulation tissue during the resolution of tissue injury. Fibr
important component of the early tendon repair process [49,50]. FN-1 gene is overexpressed in
phase of inflammation, suggesting it has important role in ECM deposition and tissue remodelin
and in chronic inflammatory diseases [51]. Based on these considerations, we can formulate th
increase (20–30%) in the production of fibronectin induced by treatment with *Arnica m*. could gr
the wound 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
significant association of these four genes into this functional group defined by enrichment anal
the described changes of gene expression identify specific *Arnica m*. targets and can not be a r
an evolutionary conserved domain, which derives its name from the epidermal growth factor, wh
growth and tissue repair. Most occurrences of the EGF-like domain are found in the extracellula
proteins or in proteins known to be secreted, such as components of the extracellular matrix. Th

is important in protein-protein interactions and the proteolytic release of this domain from memb on erbB receptors involved in cell growth and survival [52]. The interaction between the EGF-lik 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 endocytos signaling. LRP-1 binds and internalizes numerous, structurally diverse ligands, delivering most l lysosomes for degradation [53]. LRP-1 also controls the plasma membrane proteome by regula proteins in the secretory pathway [54]. Recently, its role as a regulator of inflammation has eme to bind extracellular matrix proteins including fibronectin and thrombospondin [55] and to clear r of macrophage LRP1 to modulate endocytosis and protein degradation confers on it a role in re after wound and inflammation—eg. adhesion and deadhesion processes, cell movements, clea proteases (e.g. MMP) that are generated in an inflammatory environment. In addition, LRP1 reg modulating levels of connective tissue growth factor [57]. Finally, LRP-1 affects macrophage po development of an anti-inflammatory M2 functional phenotype [58].

Heparan sulfate proteoglycan 2 (HSPG2, Perlecan) is a protein that in humans is encoded by th 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 bon vivo [59].

The protein encoded by KMT2D is a histone methyltransferase that methylates the Lys-4 positio 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 modificatior markers for M2 phenotype in IL-4 treated macrophages [60]. The gene MACF1 (microtubule an likewise slightly but significantly overexpressed by *Arnica m*. 2c treatment. This protein has the crosslink microtubules and F-actin networks, thereby directing microtubule organization. Directi 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 puzz phenomenon was accompanied by a decreased synthesis of related proteins, one would envisa 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 increa statistically significant, and to be confirmed by further studies) could suggest an increased level consequence of decreased consumption in the mitochondrial respiratory chain. Furthermore, si free radicals in conditions of lack of oxygen and reperfusion [62], it is possible that a moderate d could have a cytoprotective effect in conditions of lack of oxygen, such as those presumably en tissue. This hypothesis is in agreement with the finding that a 30c dilution of *Arnica m*., administ 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 reports an inhibitory action of *Arnica m*. on nitric oxide and TNF-α production by murine macrop 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 regulati inflammation [64]. Interestingly, this gene showed an unusual behaviour in cells treated with diff decreased in samples from cells treated with low dilutions of *Arnica m*. (2c and 3c) while it increa dilutions.

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

Our findings provide a firm molecular explanation for previous experimental observations report
on inflammation and wound healing processes. In a randomized double-blind study conducted
homeopathic dilution (4x) reduced wound irritation (redness, swelling and heat) after hallux valg
postoperative conditions in fewer days than did diclofenac [37]. An anti-inflammatory effect of *A*
carrageenan-induced foot oedema in rats has been reported by some authors [12] and a compl
Arnica m. plus other compounds at low dilutions (4x-6x) was effective for blood-induced experim
as a topical gel improved the healing of surgically-induced wounds in Wister rats, but significant
the drug was delivered together with microcurrent application [67]. A commercial homeopathic
(4^{th} decimal, 4x) of *Arnica m*., *Calendula* and *Hypericum* promoted fibroblast growth in a scratch
[68]; the present work confirms this healing capacity of *Arnica m*. 2c (which corresponds approx
authors) also in macrophages, using a scratch-test model to evaluate cellular migratory events
healing process. This same model has previously been proven a valuable tool for assessing the
remedy, Calendula officinalis 3c, and of low-level laser therapy on human skin fibroblasts [69]. T
after treatment with *Arnica m*. can be due to many factors, including the augmented synthesis o
the ability to adhere to the surface of the well and to each other, also in virtue of the increased s

At the present state of our knowledge, the mechanism of action of homeopathic medicines is ex
without the help of a suitable working hypothesis. On the basis of all the evidence available, the
originally proposed by Khuda-Bukhsh [70] offers an acceptable logical explanation of the molec
biological action of diluted homeopathic remedies in living organisms—whether plants or anima
microbiological models ultradiluted *Arsenicum* 30c or *Arnica m*. 30c modified the expression of
arsenite and UV irradiation injury, respectively [72–74]. Our studies [75,76] proved the extremel
neurocyte gene network to centesimal dilutions (2c, 3c, 4c, 5c, 9c and 30c) of *Gelsemium s*. Ot
non-neoplastic epitelial prostate cell lines treated with Apis mellifica (honey bee) homepathic pr
expression of hundreds of genes after 24h incubation even with high dilutions 9c, 12c, 15c and
regulatory networks [78,79] may be the target of subtle messages by virtue of their flexibility in r
These findings support the hypothesis that homeopathic remedies could turn some important g
gene actions to correct the gene expression that has gone wrong and produced the disorder or
relevant target gene should be sensitive to similar stimuli and exert a pleiotropic transcriptional
related functions.

The slight effect in this *in vitro* model does not mean that the modulating effect will also be smal
Whereas conventional anti-inflammatory drugs are designed to suppress the underlying enzym
(e.g. prostaglandins, cytokines) and act at considerably high doses, homeopathic treatment is d
pathological aspects and malfunctioning tissues, because the inflammatory process in itself is s
healing dynamics. In these conditions, even a 20–30% increase of macrophage activity in produ
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 tha
more complex and could involve modulation of different cells and further pathways. The field of
connective tissue and cell matrix by natural and chemical compounds is open to further studies

Conclusions

The results of this work indicate that *Arnica m*. acts on macrophages by modulating a number c
motility. RNA-seq analysis allowed the identification of several genes which are particularly sen
dilutions of this plant extract. Molecular analysis of gene expression suggests that a primary act
stimulation of tissue matrix synthesis. These findings provide new insights into wound-associate
point to macrophage fibronectin production as a potential therapeutic target of *Arnica m*. for the

Supporting Information

**S1 Table. Expression values (RPKM) of Control and *Arnica m*.-treated cells and differential expression (Log_2 I
reported in Table 1.**

IL-4-differentiated THP-1 macrophages were treated with Control solvent or with *Arnica m*. 2c, 3
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 15 c were pooled,
Change calculated comparing their RPKM with the mean RPKM of Control values.
https://doi.org/10.1371/journal.pone.0166340.s001
(XLSX)

**S2 Table. Expression values (RPKM) and differential expression (Log2 Fold Change) of a series of extracellul
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 re
values < 0.05 are reported in Table 1. Samples from Control solvent and *Arnica m*. 2c were ana
experiment of five performed. RNA samples from 5 experiments of cells treated with *Arnica m*. c
were pooled, analysed with RNA-seq and Fold Change calculated comparing their RPKM with t
https://doi.org/10.1371/journal.pone.0166340.s002
(XLSX)

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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