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Commentary

Arnica montana experimental studies: Confounders and biases?

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ABSTRACT

Arnica montana is a popular traditional remedy widely used in complementary and alternative medicine, in part for its wound-healing properties. The authors recently showed that this plant extract and several of its homeopathic dilutions are able to modify the expression of a series of genes involved in inflammation and connective tissue regeneration. Their studies opened a debate, including criticisms to the "errors" in the methods used and the "confounders and biases". Here the authors show that the criticisms raised on methodology and statistics are not consistent and cannot be considered pertinent. The present comment also updates and reviews information concerning the action of *A. montana* dilutions in human macrophage cells while summarizing the major experimental advances reported on this interesting medicinal plant.

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1. Introduction

Experimental homeopathy is a field that has attracted the interest, curiosity and doubts of the scientific world for years. Research at the laboratory level on homeopathy investigates various areas, from physical–chemical studies on highly diluted medicines to biological studies with *in vitro* and *in vivo* models, and the results support evidence of an effect that is not merely placebo [1–5]. All the experiments conducted to verify the activity of the homeopathic preparations require scrupulous settings that make possible precise measures of even small events with high reproducibility and discrimination against any systematic or random errors. Careful design of controlled experiments with adequate biological replicates is important, especially given the current lack of a unique physicochemical description of high dilutions or of the factors that may affect the transmission of information at the biological level.

Our research team has been engaged in homeopathic research for a long time. Together with other research groups worldwide, we have made theoretical contributions to homeopathic concepts such as the "simile" mechanism of action [6–9] and to experimental studies in animals [10,11] and in cell lines (neurons and macrophages) [12–15]. Today, the availability of modern, high-throughput laboratory technologies, based on the study of gene expression, has enabled us to demonstrate a biological basis, both cellular and molecular, for the medicinal action, completing certain pieces of a very complex mosaic. The ability of highly diluted compounds to modulate gene expression in human/animal cells and unicellular organisms has been reported previously by a number of authors [9,16–24]. Ribonucleic acid (RNA)-seq technique, in particular, is a reliable and well established procedure, both for preparing libraries of transcripts and for bioinformatic analysis and statistics. In addition, collaboration among researchers with advanced technical expertise and skill in setting up biological studies with high dilution medicines ensures reliable results. This appears to be a good direction for future homeopathic experimental research [25,26].

Starting from year 2011, our publications have been the subject of several critical annotations by Dr. Salvatore Chirumbolo [27–30], and a summary of our technical responses was reported immediately afterward [31]. More recently, we used real-time polymerase chain reaction to investigate the effects of *Arnica montana* on gene expression of the THP-1 myelomonocytic cell line, differentiated by phorbol-myristate acetate and interleukin-4 in the wound-healing phenotype [32]. These findings also drew critical commentary by Chirumbolo and Bjørklund [33], based on some recalculations and extrapolations from the values of standard errors of the mean. Our reply, accepted by the journal *Frontiers in Immunology*, showed that those recalculations were wrong [15].

A subsequent series of our studies on the effects of *A. montana*, based on RNA-seq methodology, were published by PlosOne in

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November 2016 [14]. This article was the subject of a recent commentary by Chirumbolo and Bjørklund [34], published in the *Journal of Integrative Medicine*.

Such debate has enabled us to define in more detail the experimental methods applied, providing a useful tool for experimental homeopathic research.

2. *A. montana* drug composition

Chirumbolo and Bj∅rklund [34] criticize the molar estimation of sesquiterpene lactones in *A. montana* dilutions. We do not agree with this remark. In actual fact, in our study, *A. montana* mother tincture is chemically characterized in accordance with the European Pharmacopoeia Guidelines [35], which conventionally measure the quality of *A. montana* preparation in terms of the content of sesquiterpene lactones, considered to be the active ingredients of reference, even if a complex variety of chemical ester variants and other chemical compounds could be present as a function of the cultivar, the part of the plant and the timing and methods of harvesting. Sesquiterpene lactones are determined by liquid chromatography and expressed as dihydrohelenalin tiglate. It follows that estimating the average molar weight of sesquiterpene lactone esters as 340 g/mol [36] is adequate for converting chromatographic quantification into molar concentration. Note that we had already published a rebuttal reaffirming the validity of our calculation of standard sesquiterpene lactones in our samples [15] in response to a previous letter from Chirumbolo and Bjørklund [33].

A major argument of Chirumbolo and Bjørklund [34] is that they think we used 51.1 mmol/L ethanol in our assay system, a dose which they consider to be too high and toxic for cells. However, the two authors made an error in their calculations: as is clearly stated in the methods of our paper, all the dilutions tested in cells (2 c and higher) were prepared in 0.3% ethanol and then further diluted 1/10 in culture medium. Since ethanol in final cell culture was 0.03%, and the molar mass of ethanol is 46.07 g/mol, with a density of 0.7893 g/cm³, this corresponds to 5.1 mmol/L and not 51.1 mmol/L.

Taking into consideration the presence of factors that may alter cell viability is a mandatory step, especially in *in vitro* experiments. It was precisely for this reason that we chose to dilute both the medicinal product and its vehicle (control), originally hydroalcoholic solutions at 30% v/v ethanol, as a large part of homeopathic medicinal products, a total of 1000 times the concentration present in cell cultures. This operation guarantees the non-toxicity of the ethanol, whether in the *verum* or placebo, as shown by suitable cell viability assays [13–15]. The residual ethanol (0.03%) could have an effect on the background activity of the cells, but it would be identical in the medicine and in the control. Consequently, all considerations about the purported confounding effects on gene expression and cell viability ascribed to ethanol in control and in *A. montana*-treated samples should be considered to be wrong and misleading, since they refer to ethanol doses ten times higher than the actual ones. Note that a refutation of Chirumbolo's erroneous opinions on the toxicity of ethanol in homeopathic medicines has already been published [12].

Throughout the text, Chirumbolo and Bjørklund [34] suggested a possible biasing effect on the control samples due to a supposed difference in the handling of the *A. montana* dilutions and of the control vehicle (the preparations and the treated samples). This is an important point in the design of experiments with highly diluted medicine, giving us the opportunity to emphasize how these controls were properly performed in our experiments. Actually, as described by Marzotto et al. [14], the control solutions (solvents) in all the reported experiments were prepared under the same procedure as the drug dilutions, just without the plant extract, as indicated. All the procedural handling was conducted in parallel, including filtering of the first 1c solution of the *A. montana* or of the ethanol vehicle (the same batch of the *verum*, provided by the pharmaceutical manufacturer). The time of conservation was exactly the same. Control and *A. montana* samples were subjected to matching experimental steps, from the cell cultures to RNA-seq and bioinformatic pipeline.

3. Clarification of protocol and statistics

Chirumbolo et al. [34] hint at purported "biases" due to the pooling of RNA samples. Actually, the pooling of equal amounts of carefully quantized RNAs from replicated experiments in the same cell line—as we did in our experiments—is a conventional procedure for evaluating the presence of general trends in gene expression, in cases where the number of test samples must be minimized. As confirmation of the reliability of the results from the pooled samples, in our RNA-seq study (not DNA-microarray), we analyzed the gene expression values of *A. montana* 2 c and of the control-treated samples, both as five separate samples and as a pool, and the values matched very well, as reported in Fig. 5 and Table S1 [14]. Moreover, in a recent paper [37], it was reported that *A. montana* 2c up-regulated the same gene set both in THP-1 cells activated with lipopolysaccharide 10 ng/mL and in cells in a resting condition. These data confirmed that such differentially expressed genes (DEGs) are the true target of *A. montana* 2 c and excluded the possibility of control biases. In any case, the significant genes differentially expressed upon *A. montana* 2 c treatment, as reported by Marzotto et al. [14], were derived from a statistical analysis of five independent experiments and not from pooled samples. We calculated the *P* values of DEGs with *A. montana* 2 c by DESeq2, one of the most modern and rigorous statistical methods, specifically designed and applied to RNA-seq dataset experiments [38]. The few genes that emerged from our analysis as targets of *A. montana* action on human macrophages have an elevated biological significance and internal coherence; moreover, increased fibronectin secretion was observed at the protein level as well. Therefore, the criticism of Chirumbolo and Bjørklund [34] regarding the statistical approach we used to infer the significance of DEGs, claiming that a greater number of replicated samples was necessary and a more rigorous test should have been used, is unwarranted.

The paper cited [34] also criticized the statistical test used to compare expression trends of the set of 20 DEGs after treatment with higher *A. montana* dilutions (2 c, 3 c, 5 c, 9 c, 15 c) and the control. The authors wrongly declared that the criticized paper used only the Friedman test (defined as not effective), while Marzotto et al. [14] applied the Friedman test (as a non-parametric analysis of variance), followed by the Wilcoxon-signed rank test (as a paired comparison post-hoc test). Chirumbolo and Bjørklund [34] purport to recalculate *P* values with the Wilcoxon–Mann–W hitney and Kolmogorov–Smirnov tests, concluding with the contention that most of our results are false and not meaningful. This re-analysis is erroneous for a series of reasons:

(1) The Wilcoxon–Mann–Whitney and Kolmogorov–Smirnov tests are not appropriate for this analysis, because those tests, unlike the Wilcoxon-signed rank test, must be applied to independent samples, but the geneset expression profiles of treated and control samples found in Tables S1 and S2 are absolutely matched or dependent samples. This means that, in their own statistical tests, Chirumbolo and Bjørklund [34] compared expression profiles without any matching of genes between the treated and the control, i.e., the

expression value of gene A in the treated sample is not matched with the value of the same gene A in the control, and this obviously results in a misleading procedure.

(2) Moreover, Marzotto et al. [14] compared profiles of a biologically coherent geneset, i.e., down-regulated genes separately from up-regulated ones, and not the entire list, which includes genes with opposite expression trends.

(3) Marzotto et al. [14] clearly stated that the list reported in Table S2 included other candidate DEGs that were analyzed to understand the role of *A. montana* in the extracellular matrix network. Even if genes were apparently modulated by *A. montana* 2 c, most of those were not modulated by higher *A. montana* dilutions and their role deserved further investigation. So the re-analysis of Chirumbolo and Bjørklund [34], as reported in their Table 2, was not carried out correctly, nor does it add anything to the results already presented. Notably, the genes on Tables S1 and S2 are different populations, and thus cannot be merged to perform a new statistical analysis, as reported in the text on "summing all data reported in (a) and (b)".

The criticism regarding a presumably irregular homoscedasticity of RNA-seq dataset is surprising. The Bartlett's test suggested by Chirumbolo and Bjørklund [34] simply assesses for non-normality of the distributions, but this was already known, and clearly reported by Marzotto et al. [14] Actually, the distribution of the expression values of the genome, as in the case of a restricted gene list, is not normal, and other models are used to prepare the data for a statistical analysis [38]. The RNA-seq dataset obtained by Marzotto et al. [14] was submitted to quality control analysis, as is customarily required for publication in public databases (as requested by PlosOne). Cluster analysis of samples, using Euclidean distance, confirmed that all datasets from replicated experiments had high similarity scores (>0.85) and were not identified as outliers. Moreover, different genes have intrinsically different variability, depending on their level of expression and on different regulation mechanisms, but this cannot be due to bias in the handling of control samples. Notably, a criticism based on the analysis of standard error of mean distribution and the presence of outliers was already fully refuted as being totally worthless by Olioso et al. [15].

In chapter 7, the authors introduce the themes of hydroalcoholic solution inhomogeneity and the possible presence of nanobubbles, issues that have recently drawn the interest of scientists working with theoretical and experimental chemo-physics in the field of high dilutions. As a general suggestion for further investigation of the characteristics of nanostructures, an area of research still in the preliminary phase, this is acceptable. In fact, we reported the presence of nanostructures in the *A. montana* 2 c dilution, without further defining the nature of these structures. To a certain extent, the presence of nanostructures in *A. montana* 1 c is to be expected, as the method of preparation involves the plant material being macerated, triturated and vortically mixed in an ethanol solution in glass containers [39]. The control solution itself might contain nanostructures originating from glass contaminants, but in the case of the control preparation used in this study, they were not detected.

4. Conclusions

We hope that these clarifications are welcome, in the interests of providing correct and truthful information to readers on the action mechanisms of *A. montana,* which we discovered and published [14,15,37].

Our results are complemented by a series of laboratory and clinical studies that, in recent years, have confirmed the potential of *A. montana* as a plant capable of regulating mechanisms of inflammation and the healing of wounds (Table 1).

Certainly, the vastness of the subject, and above all the variety of medicinal formulations and dilutions employed by the different authors, make it difficult to draw conclusions on the efficacy and therapeutic capabilities of the plant, and further studies are desirable.

Basic research in homeopathy is a new field. It is fascinating but challenging, because it deals with difficult-to-solve technical issues

Table 1
Summary of laboratory and clinical studies on *Arnica montana*.

Study type	Tested formulation	Setting	Evidence	References
In vitro	*A. montana* 30 c	*Escherichia coli* exposed to ultraviolet irradiation	Up-regulation of repair genes and amelioration of the oxidative stress	[40]
	A. montana 2–15 c (nonlinearity)	Cultivated macrophages differentiated by interleukin (IL)-4	Increase in gene expression of some CXC chemokines and bone morphogenetic protein, decrease in MMP-1	[32]
	A. montana 2–15 c (nonlinearity)	Cultivated macrophages differentiated by IL-4	Increase in gene expression and production of fibronectin	[14]
	Pure helenalin, dihydrohelenalin and chamissonolid	T-cells, B-cells and epithelial cells	Inhibition of activation of transcription factor NF-κB	[41]
	Pure helenalin	CD4$^+$ T-cells	Immunosuppressive effects	[42]
	Whole extract	Murine macrophages challenged with lipopolysaccharide	Inhibition of nitric oxide production without cytotoxicity	[43]
Laboratory animals	*A. montana* 6 c	Rat paw edema induced by carrageenan	Treated rats presented less intense edema. Animals that presented precocious edema were less responsive to *A. montana*	[44]
	A. montana 6 c	Rat paw edema induced by nystatin	Reduction in edema (post-induction effect)	[45]
	Traumeel S, a homeopathic formulation containing *A. montana* and other plant extracts	Rat paw edema induced by blood	Reduction in edema (post-induction effect)	[46]
	A. montana 4 x	Rat paw edema induced by carrageenan	No effect	[47]
	A. montana 30 c	Rat	Protects against hepatic mitochondrial peroxidation	[48]
	Whole *A. montana* extract (75 mg/kg)	Rat paw arthritis induced by collagen	Improvement of arthritis index, decrease in pro-inflammatory cytokines	[49]

Table 1 (continued)

Study type	Tested formulation	Setting	Evidence	References
	Whole *A. montana* extract (50 mg/kg)	Citric-acid-induced cough reflex in guinea pig	Reduction in the cough efforts by and bronchodilator activity of *A. montana* complex	[50]
	A. montana 3D gel combined with microcurrent	Rat (linear incision wound model)	Improved wound healing	[51]
Clinical studies	*A. montana* gel plus hydroxyethylsalicylate	Ankle sprains	Alleviates pain	[52]
	A. montana gel	Hand osteoarthritis	*A. montana* gel probably improves symptoms as effectively as a gel containing non-steroidal anti-inflammatory drug	[53]
	A. montana 30 x	Knee surgery	Reduced pain and swelling after cruciate ligament surgery (not after arthroscopy)	[54]
	Various (Review)	Rhinoplasty	Eyelid edema and ecchymosis during the first 7 days postoperatively showed a statistically significant decrease in the *A. montana* administration groups versus the control group	[55]
	A. montana 1 M and *A. montana* 12 c	Rhinoplasty	Reduced edema during the early postoperative period	[56]
	A. montana 1 M and 12 c	Rhinoplasty	*A. montana* seemed to accelerate postoperative healing	[57]
	A. montana 1 M and 12 c	Rhytidectomy	Reduction in ecchymosis	[58]
	Topical *A. montana* cream	Rhinoplasty	Regression of postoperative edema and ecchymosis in treated patients	[59]
	A. montana 1000 Korsakovian dilution	Mastectomy	The per-protocol analysis revealed a lower mean volume of blood and serum collected in drainages with *A. montana*	[60]
	Topical hydrogel pads (OcuMend, Cearna Inc., Chicago, IL) containing *A. montana* 50 M 50% and Ledum 50 M	Oculofacial procedures, including blepharoplasty, browpexy, and rhinoplasty	Subjective physician-patient rating scores showed accelerated healing in *A. montana*-treated patients compared with expected healing	[61]
	Homeopathic complex (*A. montana* and other components, each at 6c dilution)	Osteoarthritis	Treatment group significantly outperformed the control group with regard to pain, daily functioning, and motion	[62]
	A. montana 4 x	Hallux valgus surgery	Equivalent of diclofenac in wound healing, lower efficacy in pain scores	[63]
	A. montana 200 c	Wisdom teeth removal	No effects on pain and swelling	[64]
	A. montana 30 c, 6 c	Carpal tunnel syndrome	No effects on pain and swelling	[65]
	A. montana 30 c	Tonsillectomy	Reduction in pain, not of analgesic consumption	[66]
	A. montana 30 c	Hysterectomy	No effects on pain and recovery rate	[67]

The units of homeopathic dilutions cited are: "c", diluted at a ratio 1:100; "x" diluted at a ratio 1:10; "M", diluted at a ratio 1:100 for 1000 times. MMP-1: matrix metalloproteinase-1; NF-κB: nuclear factor-kappa B.

of the physicochemical nature of high dilutions, their action mechanisms, and the paradoxical reversal of the effects of drugs.

Competing interests

The authors declare no conflict of interest.

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