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Correspondence

Critical comments and methodological variations in Arnica montana's research studies

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Dear Editor,

We read the article entitled "*Bias and misleading concepts in an Arnica research study*" [1], that criticizes our recent paper published in PlosONE [2]. The commentary claims the existence of "*biases and misleading concepts*" in the experimental line of *Arnica montana* (*Arnica*) studies. However, these arguments are based on incorrect interpretations of the data we reported.

In their section 1, the authors criticize the molar estimation of the sesquiterpene lactones in the *Arnica* dilutions. However, the content of sesquiterpene lactones in Ref. [2] was determined by liquid chromatography in conformity with the European Pharmacopoeia Guidelines, which expresses the active constituents of *Arnica* as dihydrohelenalin tiglate. This is considered the most abundant type, even if a variety of chemical ester variants could be present depending on the cultivar, the part of the plant, the time and methods of harvesting. The correctness of calculation of standard sesquiterpene lactones was already illustrated [3] in response to a previous letter of the same authors [4].

The authors calculate that we used 51.1 mM ethanol in the assay system [2], a dose that they consider toxic for the cells. However, the final ethanol in cell culture was 5.1 mM, not 51.1 mM. Consequently, all the considerations about the purported confounding effects ascribed to ethanol are wrong, since they refer to ethanol doses ten times higher. Moreover, the same small dose of ethanol was used in control samples, and there was no toxicity at all. Note that a refutation of the toxicity of ethanol in homeopathic medicines was already published [5].

The cited paper [1] casts doubt as to the UV–vis spectrum of the *Arnica* solution, suggesting that this may be due to ethanol. Actually, as clearly indicated in the methods, the spectrum was obtained using a double-beam spectrophotometer procedure, using as reference-blank sample the same solvent control, so it is impossible that the published profile [2] corresponds to ethanol. Concerning the preparation of samples and the problem of filtering that is raised, all the procedural handlings were conducted in parallel, including filtering of the first 1c solution of the *Arnica* or the vehicle ethanol. Control and *Arnica* samples were matched in each experimental step from the cell cultures to RNA-seq and bioinformatics. Again, the authors assert: "*Batch-derived biases were even reported for gene microarray, particularly in pooling the RNA samples*". This sentence is off-topic, since it does not refer to possible "biases" in RNA-sequencing (used by us), but to RNA quality in a different technique, DNA-microarray hybridization. Pooling of equal amounts of carefully-quantized RNAs from replicated experiments with the same cells is a conventional procedure in RNA-seq.

In section 3, the cited paper [1] criticizes the statistical approach, asserting the need to increase the number of replicates and requiring a more stringent test. However, the significant genes differentially expressed upon *Arnica* 2c treatment derived from the statistical analysis of five independent experiments. We calculated the p values by DESeq2, one of the most modern and restrictive statistical methods, specifically designed and applied to RNA-seq datasets experiments [6]. The few genes that emerged from our analysis as targets of *Arnica* action have a high biological meaning and internal coherence, and the most strongly-expressed of them (fibronectin) was confirmed by the secreted protein. The same genes were expressed also in a pool of cell extracts from LPS-treated macrophages [7].

The authors [1] declare that we used only the Friedman test, but a paired comparison using the signed rank test (as a post-hoc) was also applied. They then seek to recalculate the p values, but they give an incomplete description of the original data used. As a matter of facts, Marzotto's Supplementary Tables S1 and S2 contain data from different lists of genes [2], but the criticizing commentary does not specify which ones were used. This re-analysis is fallacious for two further reasons: i) The Wilcoxon-U Mann–Whitney test is not correct, because this test must be applied to independent samples, and the gene set expression profiles are matched samples, ii) The paper [2] compared the profiles of biologically coherent gene sets, i.e. the down-regulated genes separately from the upregulated ones, and not the entire list that includes genes with opposite expression trends.

The criticism about the presumed irregular homoscedasticity is pointless. Bartlett's test is used to simply assess for non-normality of the distributions, but this was already known! We don't have a normal distribution in the gene set, since the genes were selected because they were significantly up-regulated or down-regulated when compared to control. To verify if the direction of change was maintained throughout the increasing dilutions of *Arnica,* non-parametric tests were correctly applied in the original paper [2]. Moreover, the authors cite their own previous commentaries [8] stating that the distribution of standard error media may lead to artifactual statistical significance, due to

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alpha error. However, this hypothesis was already rejected in a previously published paper [3].

In section 4, the authors expound and discredit the graphics of Nanoparticle tracking analysis of *Arnica* 1c solution. The authors probably don't know that one of the outputs of the NanoSight software is the Excel format: the Figure is simply the result of the average and standard error representation of 10 readings, as clearly described in the text. Other purported "bullet points" in table 3 of Chirumbolo and Björklund's paper [1] are naive recommendations to any researcher in homeopathy, and are all too easy to write down. In any case, these bullets do not apply to our research.

We hope that these clarifications provide correct and truthful information to readers on the action mechanisms of *Arnica*, a medicine plant with promising clinical applications [9]. Basic research in homeopathy is a new field; it is fascinating but challenging, because it deals with the difficult-to-solve technical issues of the physicochemical nature of high dilutions and their action mechanism, including even gene expression changes. One would therefore expect the related questions to be addressed not through theoretical opinions or antagonistic allegations, but rather through patient and critical comparison of data and results obtained by different laboratories. There is a need for experiments rather than disputes.

Conflict of interest

None.

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