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Letter to Editor

The Effect of Homeopathic Arnica on Macrophage Gene Expression

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An interesting paper recently published in the renowned journal PLoS One claims the ability of homeopathic *Arnica* to change gene expression in the THP-1 monocyte-macrophage cell line previously treated for 24 hrs with IL-4 [1]. The paper elicited also a press release from the web newspaper of the local university and apparently sounds as an excellent manuscript in the field. For a long time, I was interested in the strange phenomena underlying homeopathy and for a short period I even believed that water might bear something new in the crowded world of molecules and proteins. But I was wrong.

The paper from Bellavite's team assessed that all the high diluted preparations of an alcoholic extract from *Arnica montana* L, provided by Boiron Laboratoires, Lyon (France) and experimentally tested, always affect the genetic expression of an *in vitro* macrophage cell line, as the effect, with $p<0.05$, was observed indifferently for *Arnica* 2c, 3c, 5c, 9c and 15c. The authors rely on this effect to the presence of sesquiterpene lactones (SLs), which are the *Arnica* active principle, mainly represented by helenalin, 11α, 13-dihydrohelenain and their esters [1].

However, starting from the value of SLs in the 1c given by the authors, i.e. 1.05×10^{-5} M, the molar mass calculation of each dilutions should be the following: *Arnica* 2c has 1.05×10^{-7} M SLs, *Arnica* 3c 1.05 nM SLs, *Arnica* 5c 0.105 pM SLs, *Arnica* 9c 1.05 zM, *Arnica* 15c none (as out of the Avogadro's threshold). Each dilution contains 0.03% v/v ethanol into water, namely 51.43 mM of the alcohol (EtOH). In each homeopathic dilution, the ratio SLs/EtOH is therefore:

Arnica 2c	1: 50,000
Arnica 3c	1: 50,000,000
Arnica 5c	1: 50,000,000,000
Arnica 9c	1: 5,000,000,000,000,000,000
Arnica 15c	practically only 51.43 mM EtOH

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From an Avogadro's point of view, which cannot be the simple opinion of a buried chemist who deserves a Requiem, the presence of ethanol in each dilution is huge and cumbersome and makes very hard to trust the idea that SLs are the causing agents of the reported evidence.

Ethanol and controls

Surely ethanol may be a confounder, but the most accepted opinion is that if this molecule is present both in controls and in tested dilutions (cases), its confounder effect should be statistically null. Does ethanol cause effects on *in vitro* cells?

While analysing their first dilution, the authors of the paper in PLoS One reported an UV-VIS spectra, which interestingly showed exclusively the UV-VIS peak of ethanol at 205 nm, [1]. Ethanol may cause mitochondrial injury [2] and even mitochondrial DNA damage [3] and in doses as low as 50 mM is able to cause mitochondria damage, oxidative stress and apoptosis in several cell models [4-7], as 50 mM ethanol may cause 2.03% apoptosis in cardiomyocytes and 4.32% apoptosis in 24 hrs treated endothelial cells [5, 6]. The first effect of EtOH 50 mM is apoptosis and mitochondria damage and interestingly the authors did not perform a TUNEL or AnnexinV/PI test [1].

Furthermore, the paper presented some statistical bias. The intra-series and inter-series variance of controls should be homogeneously dispersed and a Bartlett's test should be given a $p>0.05$ in a H_0 null hypothesis. Taking into account data from Tables S1 and S2 in ref 1, a reappraisal of the statistics was recently accomplished. The Bartlett's test on the control distribution showed that this variability was highly significant ($p<0.0001$, $\chi^2=409.19452$). This would mean that controls used in the report were not homogeneously dispersed.

When revising the results expressed in table 1 of ref 1, the overall RPKM evaluation of the signed rank comparison between all averaged controls and means for each tested dilution, gave the following statistics:

[*A. montana* 2c] p=0.13622; [*A. montana* 3c] p=0.23404; [*A. montana* 5c] p=0.21498; [*A. montana* 9c] p=0.21499; [*A. montana* 15c] p=0.17702, which should suggest for the existence of a possible bias in the distribution used to evaluate the dilution activity on THP-1 cells, as these comparisons would indicate the complete absence of effects on the gene expression of macrophages by *A. montana* alcoholic extracts. This evidence seems to contradict the conclusive remark forwarded by the authors about the activity *of Arnica*. Goodness of fit test, performed with a Shapiro-Wilk test and a Lilliefors-van Soers test assessed that any distribution was non parametric. The number of outliers in a Rosner's extreme studentized deviate test ($p<0.00001$, ≥ 10 out of values) was 2.25 times higher for controls than for any test solutions [8-10].

The huge amount of EtOH in any dilution made negligible the percentage of SLs in each of them. From a chemical point of view, this occurrence transforms *de facto* cases into a type of controls. Therefore, another fundamental confounder is control handling and management.

Table 1. Wilcoxon-Mann Whitney test of *A. montana* effects on IL-4 treated THP-1 gene expression (RPKM) [1]

Sample	Statistics	Test 1	Test 2	W-Value	Mean Difference	Sum of Pos Ranks	Sum of Neg Ranks	Z-value	Kolmogorov -Smirnov (P)	p value (2-Tailed)
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 3c	34	686.74	176	34	-2.6506	P = 0.98314	**0.00804**
		2 CTRL		55	505.79	155	55	-1.8666	P = n.s.	0.06148
		3 CTRL		45	637.04	165	45	-2-24	P =0.98314	0.0251
		4 CTRL		12	672.29	198	12	-3.4719	P =0.98314	**0.00052**
		5 CTRL		33	640.64	177	33	-2.688	P = n.s.	**0.00714**
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 5c	35	688.14	175	35	-2.6133	P = n.s.	**0.00906**
		2 CTRL		43	507.19	43	167	-2.3146	P = n.s.	**0.02088**
		3 CTRL		51	638.44	159	51	-2.016	P = n.s.	**0.04338**
		4 CTRL		15	673.69	195	15	-3-3599	P =0.98314	**0.00078**
		5 CTRL		36	642.04	174	36	-2.576	P = n.s.	**0.00988**
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 9c	31	689.82	179	31	-2.7626	P = n.s.	**0.00578**
		2 CTRL		36	508.87	174	36	-2.576	P = n.s.	**0.00988**
		3 CTRL		48	640.12	162	48	-2.128	P = n.s.	**0.03318**
		4 CTRL		14	675.37	196	14	-3.3973	P = 0.98314	**0.00068**
		5 CTRL		37	643.72	173	37	-2.5386	P = n.s.	**0.01108**
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 15c	38	687.89	172	38	-2.5013	P = n.s.	**0.001242**
		2 CTRL		0	506.94	0	210	-3.9199	P = n.s.	**0**
		3 CTRL		62	638.19	148	62	-1.6053	P = n.s.	0.1074
		4 CTRL		11	673.44	199	11	-3.5093	P = n.s.	**0.00044**
		5 CTRL		**40**	**641.79**	**170**	**40**	**-2.4266**	**P = n.s.**	**0.0151**

1.- Cluster 01- Controls. [1 vs 2] p = 0.00026; [2 vs 3] p=0.00116; [3 vs 4]; p=0.00068; [4 vs 5] p=0.01016; [1 vs 3] p=0.0151 [1 vs 4] p = 0.05238; [1 vs 5] p=0.07346; [2 vs 4] p=0.00005 [2 vs5] p=0.10044; [3 vs 5] p=0.24604, bold letter: biased or critical values. About 70% control matches are biased

Ribonucleic acids from cells treated with *A. montana* 2c, which accounted for about 10 nM sesquiterpene lactones and mainly represented by helenalin and 11α,13-dihydrohelenalin, underwent the Next Generation Sequencing (NGS) technology. The $\log_2$ of the ratio between RPKM of each gene either in treated or control samples, expressed as the $\log_2$ fold change, gave the effect of treatment as differentially expressed genes (DEGs), with positive values for up-regulated and negative ones for down-regulated genes [1]. This process was performed for at least 5 separate experiments, while for the following dilutions (from 3c to 15c) RNAs were pooled from each separate experiment and arranged as a single test for each dilution, in order to reduce the variability. Although this approach was induced by technical constraints [1], pooling RNAs in gene microarray might lead to bias of the test performance [11]. The authors adopted RNA pooling due also to concerns in the availability of sample volumes, and yet both RNA quality and quantity can affect the performance of a genomic assay [1, 12]. Statistics was performed using a Friedman sign test, which is less powerful than other non

parametric rank tests, such as the Wilcoxon-Mann Withney test [13]. This evidence resembles previous reported data, with quantitative real-time polymerase chain reaction [14].

In this experimental settling, controls should be treated in a blinded fashion. Furthermore, they should be processed as sham dilutions, i.e. made with the same, identical handling of test dilutions. Moreover, controls and dilutions should not undergo different procedural steps, as 0.22 µm filtration, which was performed only on dilutions [1]. If a control and a dilution are only chemically made by 51.43 mM EtOH, then the authors should have compared two "controls" with each other. An inhomogeneously dispersed variance in controls might bear a $p<0.05$ in a non parametric rank test or in a sign test, as these statistical approaches evaluate only the difference between two distributions, not the acknowledgment of the control respect to a dilution.

Tables 1 and 2 summarized the reappraised statistics comparing data from dilutions vs controls and using both Kolmogorov-Smirnov and Wilcoxon Mann Whitney test.

Table 2. Wilcoxon-Mann Whitney test of *A. montana* effects on IL-4 treated THP-1 gene expression (RPKM)[2]

Sample	Statistics	Test 1	Test 2	W-Value	Mean Difference	Sum Of Pos Ranks	Sum Of NEG Ranks	Z-value	Kolmogorov-Smirnov (P)	p value (2-tailed)
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 3c	72	-27.47	72	118	-0.9296	P = n.s.	0.35238
		2 CTRL		239	18.55	239	257	-0.1764	P = 0.97184	0.85716
		3 CTRL		90	-24.47	90	100	-0.2012	P = n.s.	0.84148
		4 CTRL		75	-25.9	75	115	-0.8048	P = n.s.	0.42372
		5 CTRL		74	-27.34	74	116	-0.8451	P = n.s.	0.39532
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 5c	75	-29.73	75	115	-0.8048	P = n.s.	0.42372
		2 CTRL		40	-31.79	40	150	-2.2133	P = 0.97808	**0.0271**
		3 CTRL		86	-26.73	86	104	-0.3622	P = n.s.	0.71884
		4 CTRL		74	-28.16	74	116	-0.8451	P = n.s.	0.39532
		5 CTRL		76	-29.6	76	114	-0.7614	P = n.s.	0.44726
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 9c	91	-22.81	91	99	-0.161	P = n.s.	0.87288
		2 CTRL		53	-26.26	53	118	-1.4154	P = 0.97184	0.1556
		3 CTRL		90	-19.81	90	100	-0.2012	P = n.s.	0.84148
		4 CTRL		81	-21.24	81	109	-0.5634	P = n.s.	0.57548
		5 CTRL		71	-22.68	71	119	-0.9658	P = n.s.	0.33204
20	Wilcoxon-U-Mann Whitney	1 CTRL	pooled 15c	85	-23,71	105	85	-0.4024	P = n.s.	0.68916
		2 CTRL		50	-25.77	50	140	-1.8109	P = 0.97908	0.0703
		3 CTRL		83.5	-20.71	83.5	106.5	-0.4628	P = n.s.	0.64552
		4 CTRL		84	-22.14	84	106	-0.4427	P = 0.97908	0.65994
		5 CTRL		**80**	**-23.58**	**80**	**110**	**-0.6036**	**P = n.s.**	0.5485

2.- Cluster 02- Controls**.** [1 vs 2] p = 0.25848; [2 vs 3] p = 0.14706; [3 vs 4] p = 0.90448 [4 vs 5] p = 0.27572 [1 vs 3] p = 0.68916 [1 vs 4] p = 0.63122; [1 vs 5] p = 0.4965 [2 vs 4] p = 0.29372 [2 vs5] p = 0.68916 [3 vs 5] p = 0.0703, Bold letter: biased or critical values. No control match is biased
Bartlett's tests on controls p =0 $\chi^2 = 409.19452$

Conclusion

Obviously, we do not know what in the conundrum of homeopathy is hidden. However, as it was reported in the previous papers [14], a reappraisal has major importance to address the actual effect of *Arnica* on gene expression. Ethanol, as a possible confounder should be virtually removed by introducing the same amount of ethanol in paralleled matched controls. Controls and cases (i.e. tested dilutions) should be treated in a blinded or double blinded fashion, having the same procedural handling and matching the same experimental running [9, 10, 15]. The Wilcoxon-Mann Whitney test or a sign test such as the Friedman's one, are able only to evaluate if the differences in the ranks between the controls and cases (dilutions) are significant and assess the H_0 hypothesis. The test is unable to indicate if the sample 1 is a control or not. Therefore, if controls bear such differences (as assessed by the Bartlett's test) they may give to a $p<0.05$, generating a misleading interpretation of the results, which should occur simply for ethanol, in this case. Statistics performed on the results reported in the paper should not match with the conclusions addressed by the authors and this would encourage for a thorough reappraisal of the study.

Conflict of Interest

Contrarily to the many attempts arranged by further colleagues to turn the scientific debate onto personal views and personal outbursts, the only purpose of this comment is to highlight the possible bias and/or misleading conclusions existing in homeopathy. Despite some comment managed in order to dampen any fair criticism and claiming further awkward allegations, the only, simple effort is to elucidate the issue and try to publish this research on the community journals. Without any personal affair. Truth can be earned only by sharing our scientific studies and comments in the widest way within the community. This is the main purpose of mine, which clarify the absence of conflicts of interest, as in discussing these issues I gained much more career disadvantages than advantages.

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