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• Commentary

Homeopathic potencies of *Arnica montana* L. change gene expression in a Tamm–Horsfall protein–1 cell line *in vitro* model: the role of ethanol as a possible confounder and statistical bias

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ABSTRACT

Marzotto et al. showed that homeopathic preparations of *Arnica montana* L. acted directly on gene expression of Tamm-Horsfall protein-1 (THP-1) monocyte/macrophage cell lines activated with phorbol12-myristate13-acetate and interleukin-4 (IL-4). *A. montana* homeopathic dilutions are used in complementary and alternative medicine to treat inflammation disorders and post-traumatic events as well as for wound repair. The *French Pharmacopoeia* of these remedies uses 0.3% ethanol in each centesimal dilution. In this paper, we discuss how ethanol-containing *A. montana* homeopathic centesimal dilutions can change gene expression in IL-4-treated monocyte/macrophage THP-1. We assessed the role of ethanol in the *Arnica* homeopathic dilutions containing this alcohol by investigating its action on gene expression of THP-1 cell. Evidence would strongly suggest that the presence of ethanol in these remedies might play a fundamental role in the dilutions ability to affect gene expression, particularly for doses from 5c to 15c. Where, rather than playing a major role in the mesoscopic structure of water, the ethanol might have a chemical-physical role in the induction of THP-1 gene expression, apoptosis, and deoxyribonucleic acid function. This evidence generates a debate about the suggestion that the use of a binary-mixed solvent in homeopathic chemistry, used by Hahnemann since 1810, may be fundamental to explain the activity of homeopathy on cell models.

Keywords: *Arnica*; homeopathy; bias; statistics; gene expression; Tamm-Horsfall protein; cell line

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1 Introduction

A recent very interesting paper reported that a series of homeopathic dilutions from a sesquiterpene lactone-containing mixture of *Arnica montana* L., in 30% v/v ethanol (EtOH)/water (Boiron Laboratoires, France) were able to change gene expression in the human monocyte/macrophage Tamm-Horsfall protein-1 (THP-1) cell line which was stimulated with interleukin-4 (IL-4) for 24 h.[1] The paper met the Agreement of the Department of Medicine of the University of Verona, as it gained award and congratulation from the same. The present

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work re-analysed the original data[1] without performing any further adjunctive experiment, in order to evaluate the role of ethanol as a possible confounder and to evaluate the statistical tools and approaches used to evaluate these data.[1] Fundamentally, we based our calculations on the read per kilobase of exon model per million mapped reads (RPKM) data reported in the Supplementary Tables S1 and S2 of the cited paper.[1] Marzotto et al.[1] demonstrated that homeopathic preparations of *A. montana* acted directly on gene expression despite the negligible amount of sesquiterpene lactones; however, in this commentary, we introduced the possibility that ethanol plays a major role in the result. The conclusion that *Arnica* homeopathic dilutions, containing millimolar concentrations of ethanol, were able to change gene expression prompted our group to verify whether ethanol could have been a confounding factor in the experiment, affecting the nano-sized structures described by the authors.[1]

2 *A. montana* homeopathic dilutions and the experimental setting

At a first glance, the authors established an outstanding experimental setting, as they isolated poly(A)-mRNAs to prepare a directional Illumina RNA-Seq library and used Next-Generation Sequencing (NGS) technology; then they measured gene expression as RPKM, using GenBank (rel. 80) as a reference.[1,2] Ribonucleic acids from cells treated with *A. montana* 2c, which contained about 10 nmol/L sesquiterpene lactones, according to the evaluation made by the authors,[1] were subjected to this analysis procedure. The authors did not report any chemical analysis of the *A. montana* ethanol extract, which they called mother tincture (MT). These data would have given the readers more insight into the study and its results.[1] A deeper consideration of the MT, following the existing literature in the field, would suggest that the ethanol extract did not contain mainly helenalin and 11α,13-dihydrohelenalin as the authors assumed. It likely contained at least six ester derivatives of these two compounds (acetyl-, meth acryloyl-, isobutyl-, tygloyl-, isovaleryl- and 2-methylbutryl-); further helenalin and 11α,13-dihydrohelenalin components might not have been the dominant components in this mixture. Despite these considerations, the authors estimated their theoretical molarity of MT based on a calculation from Staneva et al.,[3] who identified at least eight components related to helenalin and dihydrohelenalin in an *A. montana* extract using ^{1}H-NMR spectroscopy; and assumed an average molar mass of dihydrohelenalin-derived compounds as 340.41 a.m.u. The calculation done by Marzotto et al.,[1] which did not report any chromatographic output data, would be an approximation to the estimation done by Staneva et al.;[3] they reported possible errors introduced by using only the average molar mass, particularly for compounds such as methacryloyl-helenalin. They further determined that the molar mass that they calculated by summing the molar weights of single lactones, particularly for isobutyryl-helenalin, 6-*O*-(2-methylbutyryl)-helenalin and 2-methylbutyryl dihydrohelenalin, which could not be separately evaluated, was most likely lower than reported.[3] The molar estimation calculated by Marzotto et al.[1] in *A. montana* 1c actually refers to the main sesquiterpene lactones present in *A. montana* (i.e. a mixture of helenalin and 11α,13-dihydrohelenalin esters) giving the reported theoretical molarity.[1,3]

Furthermore, helenalin and 11α,13-dihydrohelenalin have been reported to have different effects in cell culture models, particularly for cancer cells. In these results, growth was inhibited by helenalin, while 11α,13-dihydrohelenalin had lost these properties.[4] These esters also have solubilities that are affected by the concentration of ethanol in the solution. In addition to lactones, extracts of *A. montana* flowers contain flavonoids, phenolic acids, essential oils, monoterpenes, derivatives of thymol, polyacetylenes, etc.; all of these compounds may have biological effects, though Marzotto et al.[1] and Staneva et al.[3] did not address this point. Thus, it is not completely correct to assign a specific study outcome to the lactones, which were considered to be the primary active ingredients, because they can interact synergistically or antagonistically with other compounds present in the extracts, and the tincture contains a mixture of components.[1] One way to clarify the role of helenalin would be to evaluate the effect of the maximum dissolved concentration (1.05 mg/L) of pure helenalin in water as a control. Due to the fact that the sesquiterpene lactones are lipophilic substances, having a low water solubility of 1 mg/L and of 10 mg/mL in dimethyl sulfoxide, ethanol is therefore the only perfect solvent for them, which considerably limits the possibilities of devising a simple control for them.

3 The experimental setting: an introduction

Aside from these important considerations about the molecular composition of *A. montana* ethanol extracts, the authors claim that beyond the *Arnica* 5c, it is unlikely that any molecules of *Arnica* are present, due to the Avogadro-Loschmidt's threshold.[1] When the amount of *A. montana* remaining in the solution is theoretically negligible (much less than 1.0 nmol/L), the effect of solvents might be fundamental.

The $\log_2$ of the ratio between the RPKM of each gene in treated and control samples, called the $\log_2$ fold change, gave the effect of treatment as differentially expressed genes (DEGs), with positive values for up-regulated and