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Hypotheses and findings on the action mechanism(s) of homeopathic drugs

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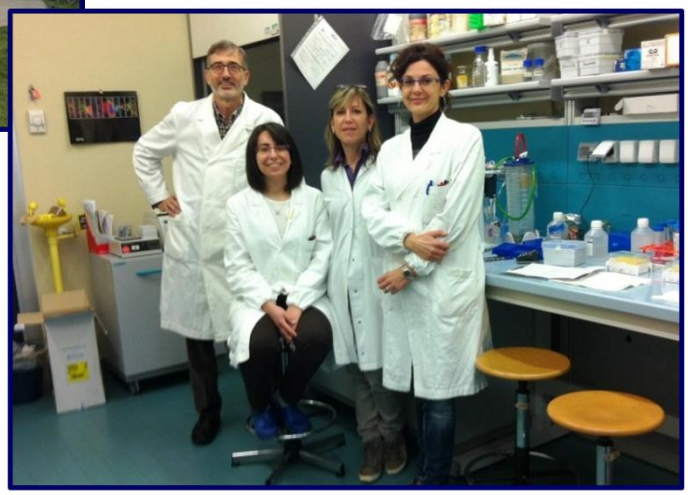


Authors' research group (2015)

Verona University laboratories and research group. From left: Paolo Bellavite, Clara Bonafini, Marta Marzotto, Debora Olioso. Ongoing collaborations also with Anita Conforti and Elisabetta Zanolin (University of Verona) and with three Brazilian Universities groups led by Leoni Bonamin (Sao Paulo), Carla Holandino Quaresma (Rio Janeiro), Silvana Marquez de Araujo (Maringa).

ABSTRACT

While drugs at low dilutions work at molecular level exploiting high sensitivity of biological systems and amplification pathways, drugs at high dilutions require new models and new approaches. A major question is how these medicines may work in the body. The possible answers concern the identification of biological targets, the means of drug-receptor interactions, the mechanisms of signal transmission and amplification, and the models of inversion of effects according to the traditional "simile" rule. Laboratory models developed in our laboratory (mice and in vitro basophils, neurocytes, macrophages) to test homeopathic

drugs are currently used to explore and develop these topics and partially confirm traditional homeopathic ideas and findings. Despite the major puzzling claims of homeopathy are far from to be clarified, there are several plausible hypotheses of the nature and action mechanism of homeopathic drugs, and some experimental evidence confirms their effects in rigorous laboratory models.

INTRODUCTION: IS HOMEOPATHY SCIENTIFIC?

Despite several progresses in the last twenty years, acceptation of homeopathy into the mainstream medicine remains very difficult. There are three major chapters in this debate:

1. A first difficulty is due to the structure of contemporary scientific thought inherited from positivism :
   a) everything is material,
   b) everything object can be reduced to its single parts,
   c) quantitative relation cause-effect.
   Clearly, this intellectual position, which is dominant in the so called "conventional medical system" makes difficult to appreciate the very nature of homeopathy which is an approach based on different standpoints expressed since the time of Hahnemann
2. Second, there are different OPINIONS on the evidence of clinical efficacy. I mean, what is the grade of evidence, which is the sufficient evidence. The detractors of homeopathy believe that the evidence is insufficient and that the quality of evidence is not sufficient. Of course this is an OPINION, based on subjective and not objective parameters. Related with this discrepancy of opinions is the problem of suitable methods to prove homeopathy. As is well known, the so called Evidence-Based-Medicine trusts only in rigorous double-blind clinical trials and on meta-analyses, but there are questions that this method alters the normal clinical setting of homeopathic cure, particularly in chronic cases. Moreover, the criteria of evidence are often based on parameters that do not comply with the clinical approach which is typical of homeopathic approach.
3. Different OPINIONS on the plausibility of action mechanisms in terms of current pharmacological theories. In this case, the detractors of homeopathy maintain that the theories are not plausible and incompatible with scientific knowledge. I think that this is a wrong position and we will see why.
   Point 1 belongs to philosophy (and history) of science
   Points 2 and 3 are scientific matter!
   In my presentation I will speak especially on the latter point. But I want to express only one idea concerning the first point, the philosophical one.

Sir Karl Raimund Popper (28 July 1902 – 17 September 1994) was an Austrian-British philosopher and professor. He is generally regarded as one of the greatest philosophers of science of the 20th century. He wrote*: "We must not look upon science as a "body of knowledge", but rather as a system of hypotheses, or as a system of guesses or anticipations that in principle cannot be justified, but with which we work as long as they stand up to tests. . ."*
In conclusion, a theory should be considered scientific if, and only if, it is falsifiable.
For this very reason, Homeopathy is a science.

Hahnemann expressed the major principle of homeopathy in these terms: "The majority of substances have more than one action; the first is a direct action, which gradually changes into the second, which I call its indirect secondary action. The second is generally the opposite of the first" (*Essay on a new principle*, 1896)
This principle can actually be verified or falsified, so is the object of scientific investigation
The other more controversial principle is that of dilution: "A medicine whose selection has been accurately homoeopathic must be all the more salutary the more its dose is reduced to the degree of minuteness appropriate for a gentle remedial effect..." (Organon par. *277)*
Also this principle can actually be verified or falsified, so is the object of scientific investigation.

So, we have two general working hypotheses

1. The same substance or similar substances can have opposite (inverse) effects in different conditions

2. Some pharmacological power of the original substance is retained in successive dilutions/dynamizations

These concepts are the object of hundreds of studies published in major scientific literature, concerning either clinical evidence and basic research.

This evidence is very strong. Of course, when we translate these hypotheses into experimental work, not all evidence was positive but this is NORMAL in every field of science and especially in medicine. In any case, the science of homeopathy is growing, especially in the last twenty years, and the Mumbai world homeopathic summit is a testimony of this undisputable development. Several Indian research groups have given and are giving a strong support to this growth.

The detractors of homeopathy, in order to continue their attacks, are forced to negate the reality and to dismiss the evidence in favour of their theories, a procedure that has nothing to do with scientific attitude.

In my group we have used laboratory animals (rats and mice) and cells like neutrophils, platelets, neurocytes and recently macrophages. We have no experience on physical models but we are just beginning to investigate the presence and the role of nanoparticles in homeopathic solutions.

ANIMAL AND PLANT MODELS

Animal and plant models are particularly useful for the so called pre-clinical research (efficacy) in controlled trials that can be reproduced over a number of different experimental conditions, but also for studying the action mechanism.

Table 1. Examples of inverse effects of various agents when used at homeopathic doses

System	Agent	"Conventional" effect	"Homeopathic" effect	Ref.
Rat, Guinea pig	*Histamine Lung Histam. Apis mellifica*	Pro-inflammatory agent	Histamine (30x), *Lung histamine* (18c) and *Apis mellifica* (7c/10c) reduce inflammation symptoms	Bastide 1975,Poitevin 1988, Bildet 1990 Conforti 1993
Rat, Mouse	Arsenic	Whole body and liver toxicity	*Arsenicum* high dilutions (7c-30c) protect from intoxication	Lapp 1955; Wurmser 1955; Cazin1987-1991; Banerjee,P, Khuda-Bukhsh 1998-2000
Rat	*Nux vomica*	Neurohinibition (strychnine)	Reduces alchool-indced sleeping time	Sukul et al., 1999
Rat	Aspirin	Antithrombotic	Aspirin 10^{-30} g/kg (15c) has pro-thrombotic effects	Beulogne-Malfatti,Doutreme-puich , Eizayag et al. 1998-2012
Rat	*Phosphorus*	Hepatotoxicity	*Phosphorus* high dilutions (30x) protects from toxic hepatitis	Bildet 1984, Guillemain 1987 Palmerini 1993
Tadpoles	Thyroxine	Increases the rate of metamorposis	Thyroxine high dilutions (up to 30x) inhibit metamorphosis	Endler 1990-2014, Lingg 2008, Weber 2008, Guedes 2011, Harrer 2013
Rat, Mouse	*Gelsemium*	Toxic and convulsivant	Anxiolytic effect (2c-30c) of *Gelsemium*	Magnani 2010, Venard 2011, Bellavite 2012
Wheat	Arsenic	Cell toxicity	*Arsenicum* high dilutions (45x) stimulate vitality	Betti et al. 1997-2014

Results in an animal behavioural model

Here some results with psychopharmacological effects of homeopathic drugs are reported.

Research in anxiety and psychopharmacology has a long history of development of animal models. The measurement of anxiety-related behaviour in animal models is based on the assumption that anxiety in animals is comparable to anxiety in humans.
Although it cannot be proven that animals experience anxiety in the same way as human beings, it is generally undisputed that certain behaviours of rodents in experimental conditions correspond to central and peripheral emotional responses to acute or protracted stress. Most importantly, drug responses in animals are in many cases predictive of the response of the average population in human clinical studies, or can suggest novel pharmacological approaches. They have also helped to elucidate the mechanisms of absorption, distribution, transformation and excretion of drugs, thereby allowing the active ingredients of medicinal plants to be identified.
We used essentially two validated models for studying animal behaviour, namely the Open field and the Light dark test.

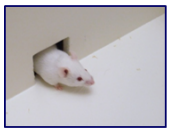
The OF test involves placing an animal in an unfamiliar environment consisting of a wooden platform, with 25-cm high surrounding walls. The total distance travelled in 10 minutes indicates the general capability to move and vitality. The OF arena is virtually divided into two parts, with a square central zone having an area corresponding to 25% of the total area. The percentage time spent in this central zone is considered indicative of exploratory behaviour, and may reflect a decrease in anxiety.
The LD exploration test is based on the innate aversion of rodents to brightly lit areas, and their spontaneous exploratory behaviour in response to mild stressors such as novel environments and light. The test apparatus consists of a small, secure dark compartment and a large, aversive illuminated compartment. The two compartments are separated by a partition with an opening through which the animal can pass from one compartment to the other. Mice tend to prefer dark, enclosed spaces to large, well-lit areas, and the amount of time spent in the lit zone indicates decrease of anxiety. The transitions between two compartments indicate the exploratory attitude of the animal and is sensitive to anxiolytics like benzodiazepines.
All observation was done in automatic and recorded way, using videotracking system. Studies were performed in double blind.
Animals were not exposed to painful or electric shock or other kind of stress, just observed in their behavior in ethically approved and ethological way.

We started testing a series of Homeopathic medicines with supposed activity in behavioral symptoms, using the knowledge of Materia Medica: *Aconitum napellus, Atropa Belladonna, Gelsemium sempervirens, Nux vomica, Argentum nitricum, Tabacum* and their control solvent hydroalcoholic (30%) solution.
We started with 5C potencies because they are high enough to be surely non-toxic and low enough in order to contain some (a few) molecules of active principle.
So, their possible action seemed more plausible to pharmacology colleagues who were available to collaboration.
From this screening, *Gelsemium sempervirens (Gelsemium)* emerged as the most promising drug.
Subsequently we also demonstrated behavioural effects of *Ignatia amara* (Marzotto et al. 2012), but *Gelsemium* was largely the most interesting for our model systems.
Gelsemium sempervirens L. is a traditional medicinal plant mainly distributed in the Southeast of the United States, employed in phytotherapy and homeopathy as nervous system relaxant to treat various types of anxiety, pain, headache and other ailments. Although animal models showed its effectiveness, the mechanisms by which it might operate on the nervous system are largely unknown.
In Materia Medica there are characteristic symptoms indicating *Gelsemium*, concerning movement, space, air, fear, irritation and so on. In our working hypothesis, similar symptoms could characterize the behaviour of mice in our model systems.

We carried out a 5-years long study (2007-2011) with a large series of experiments. This was necessary in order to reach the statistical power in conditions where there is high variability of animal responses to stress.

After a first report at the LIGA conference in Ostende (Magnani et al. 2008), we published a major paper in the conventional pharmacology journal "Psychopharmacology" in 2010 (Magnani et al. 2010), then a pooled data analysis of 14 experiments where we tested several different potencies. This complete report was published in the Journal Evidence-Based Complementary and Alternative medicine in 2012 (Bellavite et al. 2012).

Open field



Light Dark

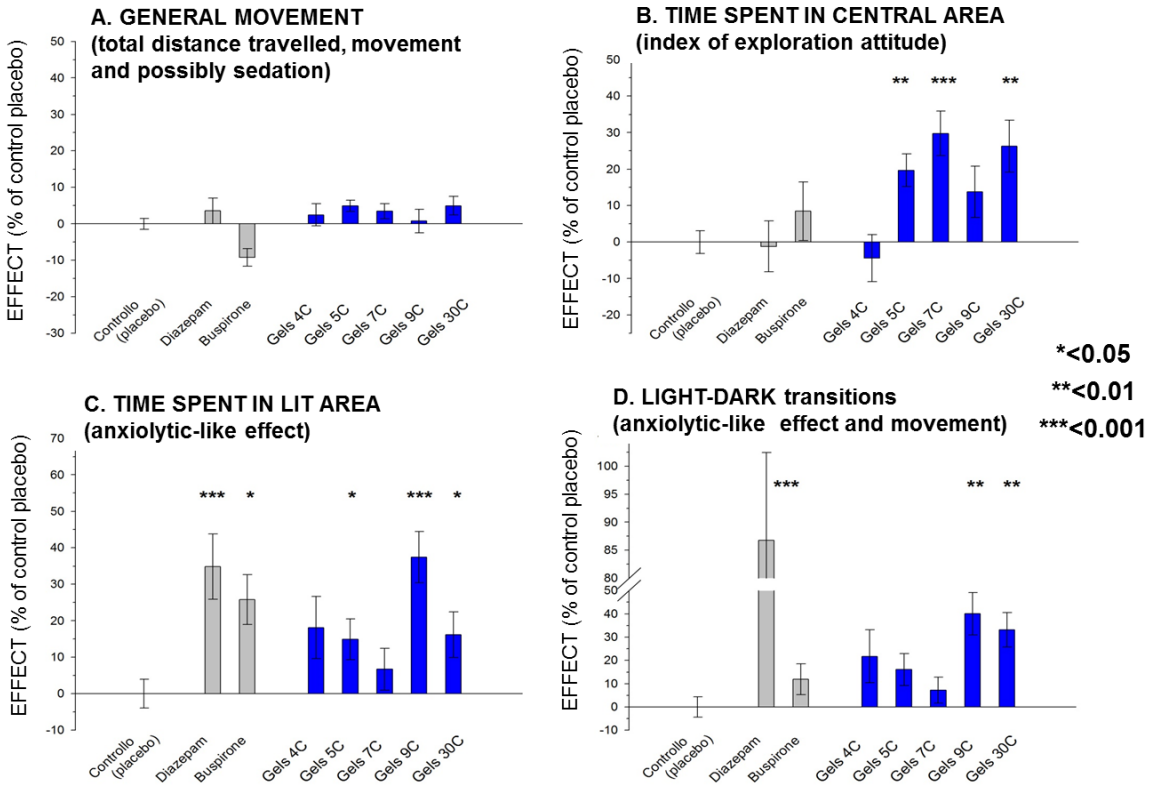


Figure shows the main results of pooled data analysis of *Gelsemium* effects in mice behavioural models

Open field test

The results of pooling all the tests performed with OF test is reported in the above panels.The total distance travelled by the mice in the arena showed no significant differences between various groups, although a small inhibitory effect was found in buspirone-treated vs. solvent-treated animals, suggesting a possible sedative effect instead of anxiolytic-like effect. This phenomenon was not present with diazepam and *Gelsemium*, suggesting that these drugs did not affect the unspecific locomotor activity of the mice.

In the variable "Time spent in centre", there were highly significant differences between groups. All *Gelsemium* samples except for 4C showed a stimulatory activity as compared with control solvent, with a statistically significant difference for the 5C, 7C and 30C dilutions/dynamizations. Equally apparent is the lack of effect of the two standard drugs diazepam and buspirone on these parameters, suggesting that this model system in these experimental conditions was not suitable for detecting a conventional anxiolytic effect, and hence that the effect of *Gelsemium* on mouse behaviour in the OF is qualitatively different from that of standard drugs.

Light-Dark test

As shown in bottom panels, the time spent in the open, illuminated (white) compartment of the LD test arena increased in all the *Gelsemium*-treated groups and in the groups treated with diazepam and buspirone. Considering the whole of this large population of animals, the effects of *Gelsemium* 5C, 9C and 30C proved highly statistically significant in post-hoc analysis, with a peak at 9C dilution/dynamization. Similar results were obtained by measuring the number of transitions between compartments, with the difference, as compared with the permanence time, that here only the effects of 9C and 30C dilutions/dynamizations proved to be statistically significant. Moreover, in this test parameter buspirone

was less effective as positive control. Since this parameter is likewise linked to physical motility, this may be due to the slight inhibitory effect of buspirone on unspecific locomotion already noted in OF.

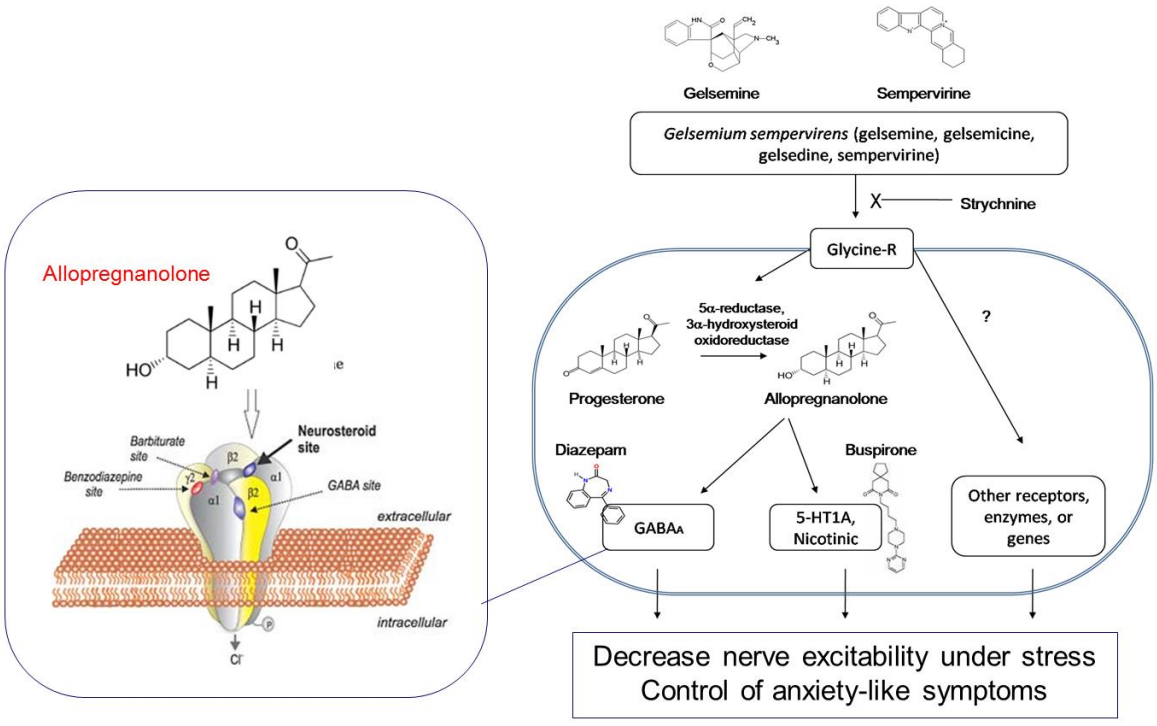
The effects of *Gelsemium* displayed marked non-linearity with dilution/dynamization, and were different in the OF and LD assessments. In the OF, the 4C was inactive and showed significantly lower effects than the 5C, 7C and 30C. In the LD, the activity of the 7C dilution/dynamization was very low, while peak activation was noted using the 9C. In the OF test there was a significant effect of *Gelsemium* (peak 7C) but not of the conventional drugs, while in the LD test both *Gelsemium* (peak 9C) and the conventional drugs showed significant effects. These discrepancies strongly suggest that the two test paradigms explore different behavioural symptoms which respond differently to conventional and homeopathic drugs.

Gelsemine content in *Gelsemium*

Gelsemine is the major alkaloid of *Gelsemium* and is considered as the active principle with strong neurotoxicity at ponderable doses. Since we knew the dose contained in Mother Tincture, we could calculate the molecular concentration of this active principle in the drug dilutions used. In 9C this dose corresponded to 1 molecule/mouse and was 10,000,000,000,000,000 (ten millions of billions) times less molecules than standard dose of buspirone or diazepam. In 30 C of course no molecular doses of Gelsemine could be calculated. Several control experiments excluded that the observed behavioural effects could be due to the small content of alcohol in the homeopathic and placebo solutions (0.3 ml of 0.3% alcohol/99.7% water solution).

***Gelsemium* activity on the spinal cord and limbic system of rats**

A very interesting finding was reported in the same years by other investigators working on *Gelsemium* action mechanism (Venard et al. 2011; Venard et al. 2008). This french group showed that *Gelsemium* and gelsemine at dilutions 5C, 9C stimulate allopregnanolone production in the rat limbic system (hippocampus and amygdala or H-A) and spinal cord (SC).



Scheme of a working model of the action of *Gelsemium* on allopregnanolone metabolism

The stimulatory action of *Gelsemium* and gelsemine (5C) on 3,5-THP production was blocked by strychnine, the selective antagonist of glycine receptors. These results allow to draw a working model of the mechanism of action of *Gelsemium* based on allopregnanolone production in neurocytes.
It is well known that anxiolytic drugs like benzodiazepines act by enhancing the inhibitory effects of the neurotransmitter GABA. The neurosteroid allopregnanolone is produced from progesterone and acts both on the GABA receptor - at a binding site different from benzodiazepines, thus reducing the impulse generation in postsynaptic neurons. Allopregnanolone of cellular mechanisms involved in anxiety, pain, depression and neurodegeneration.
By fine tuning inhibitory transmission through glycinergic system and allopregnanolone synthesis, *Gelsemium* 5C may help the nervous system adapt to adverse situations. Due to the multicomponent nature of *Gelsemium* and to our finding of preferential effects on OF paradigm even at high dilutions/dynamizations, the existence of other neurosteroid-independent mechanisms may be hypothesized.
The anxiolytic-like effects of *Gelsemium* and its alkaloids in rodent models have been confirmed by several laboratories (Liu et al. 2011; Meyer et al. 2013; Jin et al. 2014).

Key-notes on animal models

- **Reproducible and significant effects produced by *Gelsemium* in mice, concerning a subset of "symptoms" which have been tested in Open field and Light-dark: aversion to open space, amelioration with movement, feeling in a danger, aversion to light. No adverse effects on general locomotion (an effect shown by buspirone in chronic treatment)**
- **NON-LINEARITY (various activity peaks) with increasing potencies, BUT in general different potencies have the same trend of effects (important for practical purposes: our results comparing different potencies of the same substance in the same conditions suggest that the selection of the exact potency may be the best objective but is not determinant for obtaining a desired effect)**
- **First working hypothesis of action mechanism: stimulation of glycine receptors and thus neurosteroid synthesis with consequent increase of GABA inhibitory effects.**
- **Consistent evidence that high dilutions (even beyond Avogadro) have reproducible effects different from control (placebo) solutions: "end of placebo hypothesis for explaining homeopathy"**
- **Confirmation of the "similia principle": homeopathic dilutions counteract toxicity of ponderal doses (e.g. *Arsenic*, *Phosphorus*)**
- **Confirmation in animals of some symptoms reported by Materia Medica (e.g. *Gelsemium, Apis, Histaminum*)**

***IN VITRO* CELL MODELS**

Cell models allow to investigate drug action at the level of receptors, signal transduction and gene expression. In other words, they can help to identify some specific targets in the most elementary particle of life, that is the cell.
Also in this case a summary of some typical findings in literature is followed by a report of original evidence from our laboratory.

Basophils

The human basophil model was followed by several investigators and is one of the most consistent and reproducible models.
These cells are stimulated by anti-IgE antibodies and respond with histamine release and expression a series of markers on the plasmamembrane
An important series of experiments done by B. Poitevin in collaboration with E. Davenas and J. Benveniste was published in 1988 by a pharmacological journal. There they showed that the homeopathic *drugs Lung Histamine* and *Apis* significantly inhibit the degranulation induced by Anti IgE.

Table 2. Typical effects of homeopathic drugs or highly diluted substances in laboratory models

System	Agent	Dilution	Effect	Ref.
Human basophils	Histamine	12C-16C $10^{-24} \rightarrow 10^{-32}$	Inhibition of activation markers	Belon 1999-2009 and other groups
Human basophils	Adrenaline	12C-16C $10^{-24} \rightarrow 10^{-32}$	Inhibition of activation markers	Mannaioni et al. 2010
Cicken embrio	Bursin	15 C (10^{-27}g)	Immunomodulatory and endocrine activity	Bastide, Youbicier-Simo 1993-97
Human neutrophils	*Phosphorus*	12 D to 30 D	Inhibition of superoxide production	Chirumbolo and Bellavite 1993
Wheat germination	Arsenic Silver nitrate	26 D (10^{-45})	Protect from toxicity Enhances growth	Betti 1997/2015 Pongratz 1998
Rat neurons	Glutamate	$10^{-18} \rightarrow 10^{-30}$	Protection from glutamate toxicity	Jonas et al., 2001
Neurocytes	Cycloheximide	10^{-27}	Increases viability	Marotta 2002
Bacteria	*Arsenicum*	30C	Protects from toxicity	Das et al 2011, De et al 2012
Neurocytes	*Gelsemium*	2-30 C	Prevalent gene down-regulation	Marzotto 2014, Olioso 2014
Colon cancer cells	*Ruta grav.*	MT-30C	Decrease cell viability, apoptotic gene expression	Arora and Tandon 2015

The inhibitory effect is particularly interesting because, when released at normal doses into a tissue, both histamine and bee venom have pro-inflammatory powers and irritant properties. This experiment therefore illustrates the application of the principle of similarity in an experimental model: a substance known to stimulate inflammation at conventional doses can, at different doses, inhibit the cells responsible for many of the phenomena of the inflammatory process.
Subsequently, the group of J. Sainte-Laudy and P. Belon has reported other data confirming that high dilutions of histamine (pure histamine chloride) significantly inhibit the degranulation of basophils . The addition of pharmacological doses of cimetidine (an antagonist of histamine H2 receptors) abolished the effect of all of the active dilutions. The authors therefore tend to believe that the action of high dilutions involves an effect of the solvent (water) on H2 receptors.
Furthermore, similar results have been obtained in a multicentre study by three of the four laboratories involved in a multicentre collaboration in 1999.
However, uncertainties about the nature of the phenomenon and its reproducibility mean that further, rigorously controlled studies are necessary.

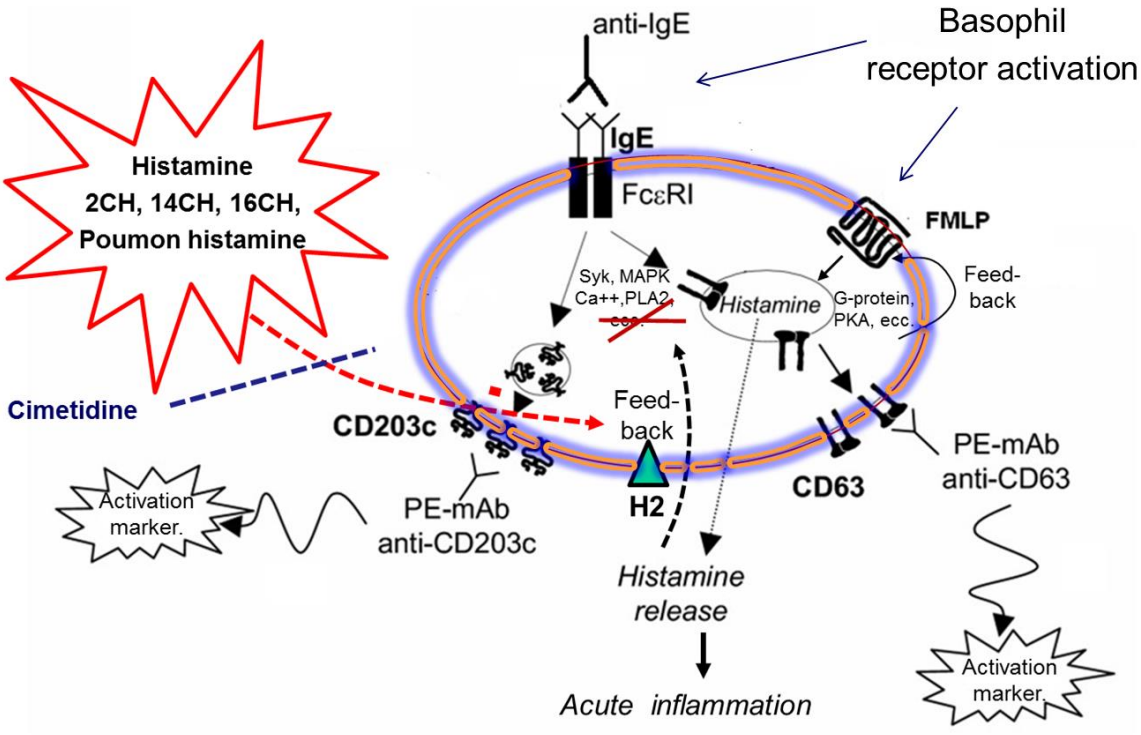
Replication experiments

We also checked this model using our laboratory equipment.
1:100 (v:v) histamine dilutions (centesimal dilutions, C) and pure water controls were tested on human basophil responsiveness to anti-IgE monoclonal antibodies, using flow cytometry. Basophil-enriched buffy coats from healthy blood donors were incubated with 10^{-4} mol/L histamine (2C) and with serially diluted preparations from 10^{-20} mol/l (10C) to 10^{-32} mol/l (16C), then incubated for 30 min with monoclonal anti-human IgE and basophils stained for immunophenotyping.
Results: Membrane up-regulation of CD203c, was significantly inhibited in samples treated with histamine at the dilutions of 2C (P=0.001), 12C (P=0.047), 14C (P=0.003), 15C (P=0.036) and 16C (P=0.009). Separate experiments, in which basophils were incubated with control water dilutions, did not show any significant effect. *Conclusion:* Using a strictly standardized flow cytometry protocol and a new dilution/succussion

procedure, we have independently confirmed that low and high dilutions of histamine inhibit CD203c up-regulation in anti-IgE stimulated basophils.

IN SUMMARY, the studies of effects of high dilutions of histamine or adrenaline are at present:
14 publications (2 with multicentre studies)
4 independent laboratories involved
12 papers with positive results
1 negative
2 uncertain
The amount of positive evidence is much higher than that usually accepted for conventional drugs.

Scheme of Histamine action
So in this case we can envisage the inverse effects of histamine high dilutions.

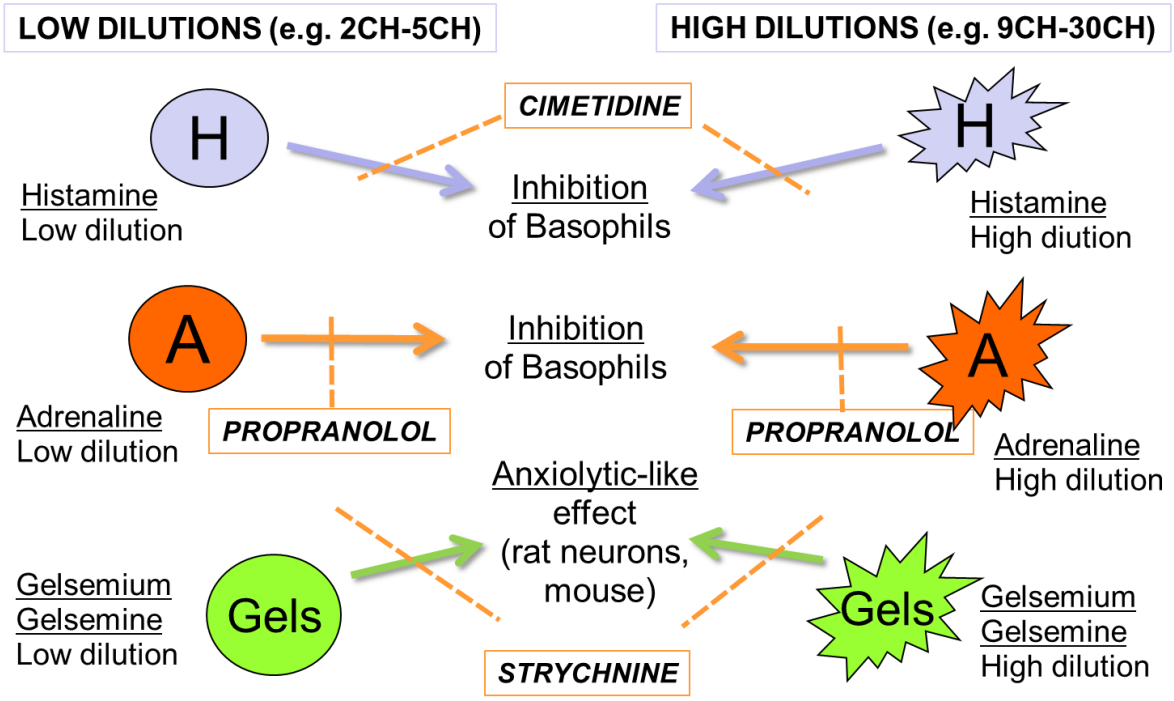


Drawing of the hypothetical action mechanism of high dilutions of histamine on human basophils

Basophils are activated by membrane IgE and other receptors in presence of their agonists. Histamine is released from granules and provokes acute inflammation.
Basophils and mast cells also have Histamine H2 receptors that are used at ponderable doses by histamine as an internal feed-back. In other words, the same histamine is able to inhibit its own release when in excess.
However, data the described experiments show that the same mechanism can be activated by low doses and high dilutions. The involvement of H2 receptors is indirectly suggested by the action of the specific inhibitor Cimetidine.

ACTION ON CELL RECEPTORS

An interesting concept emerging from studies in cellular models is that high homeopathic dilutions have often shown the same trend of effect as low homeopathic dilutions.



Drawing of the similar actions of low dilutions (substantial molecular doses) and high dilutions on three typical models of investigation and similar inhibitory effects of receptor antagonists.

When homeopathic drugs were tested in the same assay system at increasing dilutions, in most cases the greatest effects were observed at the lowest dilutions and also in Mother Tinctures (high molecular concentrations), but the same activity remained even at high dilutions. In other cases, the effect was noted only in the low potencies. In many instances, when the dilution exceeded the Avogadro constant, the effects appeared as pseudo-sinusoidal curves, with peaks of activity at certain dilutions/dynamizations, followed by inactive or less active dilutions (for a review see (Bellavite et al. 2015)).

Many of these effects have also been explained mechanistically as modifications of receptors, transduction mechanisms and gene expression changes. As there are many levels of cell regulation, there is no single mechanism explaining homeopathic effects, just as there is no single mechanism explaining the effects of conventional drugs.

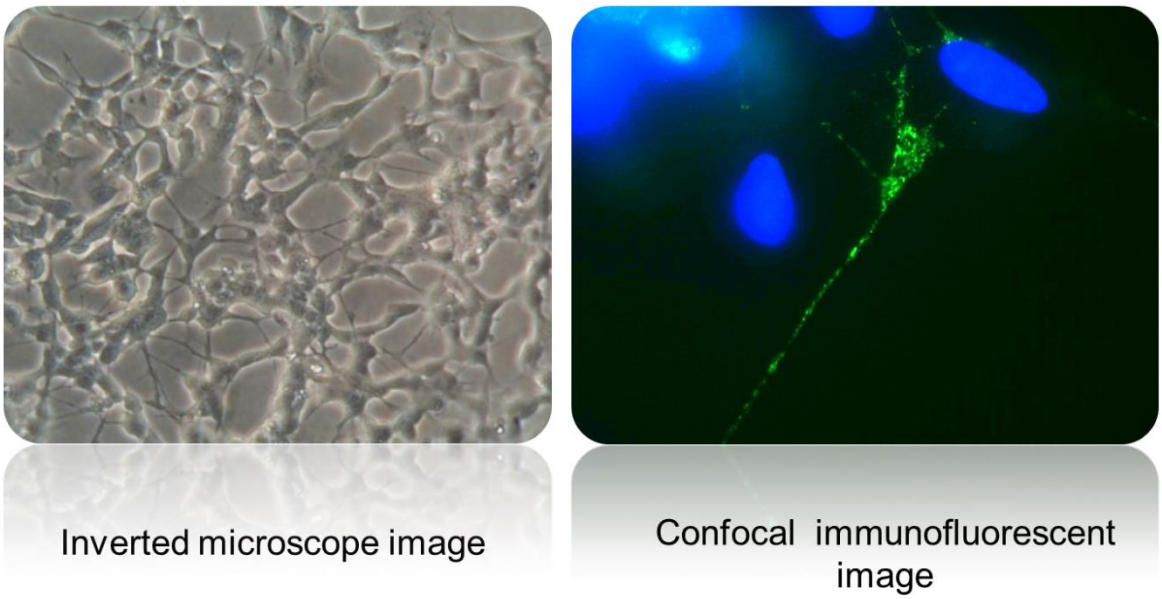
The effect of specific inhibitors suggest that also the effects of high dilutions are mediated by the same receptor system as low dilutions and ponderable doses.

High sensitivity to external regulations and nonlinear responses are frequently reported also with non-dynamized dilutions. It is worth pointing out that not all homeopathic effects can be reduced to the cellular level. A clear example is the recent finding that *Calcarea carbonica* induced regression of Ehrlich's ascites carcinoma in mice in vivo, but failed to induce any significant cell death when administered to cancer cells in vitro.(Saha et al. 2013) This apparent discrepancy was explained considering that *Calcarea carbonica* employs the immuno-modulatory circuit to assert its anti-tumor effects.

***GELSEMIUM* IN A NEUROCYTE MODEL**

Our model system are human neurocytes SHSY5Y, a cell line derived from human neuroblastoma.

This study investigated for the first time by a real-time PCR technique (RT-PCR Array) the gene expression of a panel of human neurotransmitter receptors and regulators, involved in neuronal excitatory signaling, on a neurocyte cell line.
Exposure to the G. sempervirens 2c dilution, containing a nanomolar concentration of active principle gelsemine, induced a down-regulation of most genes of this array. In particular, the treated cells showed a statistically significant decrease of the prokineticin receptor 2, whose ligand is a neuropeptide involved in nociception, anxiety and depression-like behaviour. Overall, the results indicate a negative modulation trend in neuronal excitatory signalling, which can suggest new working hypotheses on the anxiolytic and analgesic action of this plant (Olioso et al. 2014).

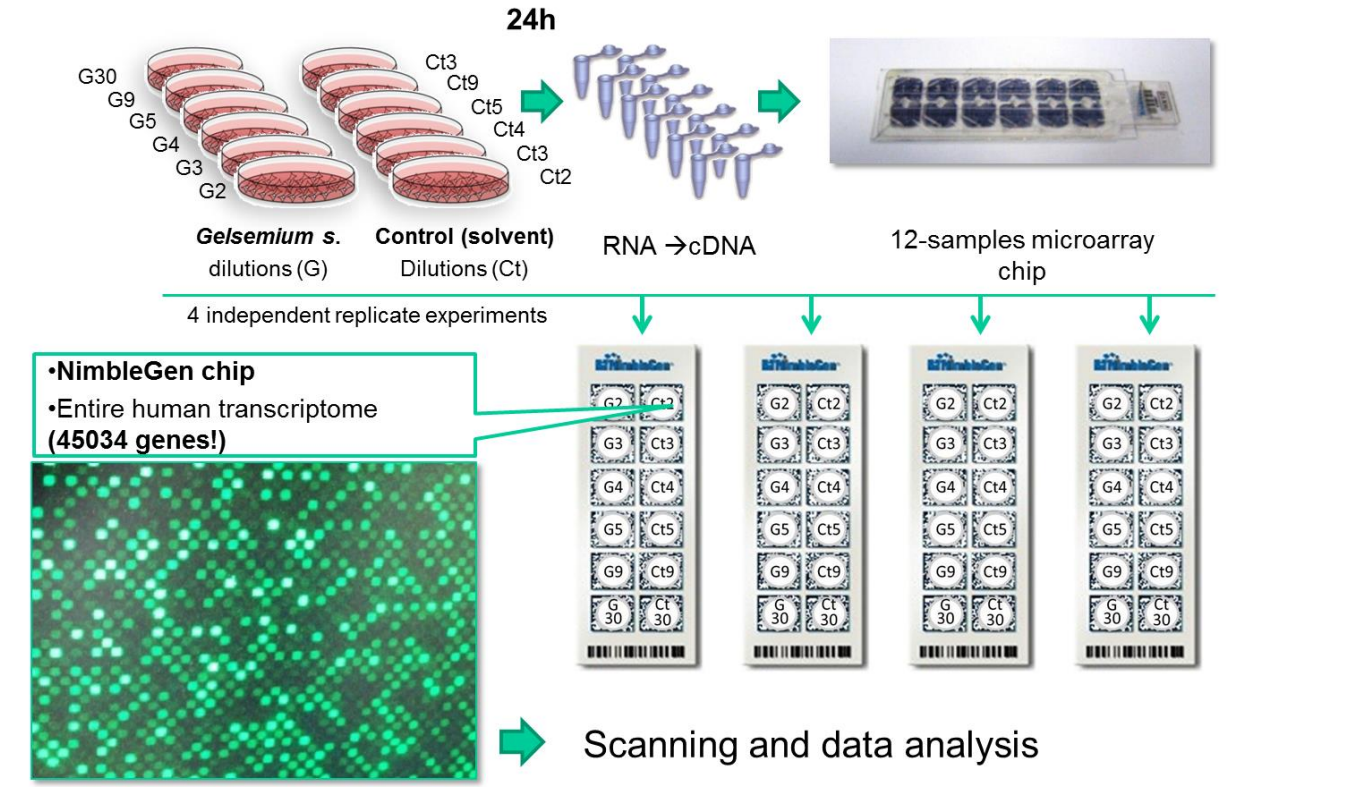


Micrographs of the cells used in in vitro test of *Gelsemium* In the left picture we observe a culture of SH cells with a neuron-like shape with visible axons and synaptic junctions. On the right the image of a cell with the cytoplasmatic localization of Synaptophysin antibodies (green), a marker of mature neurons.

The most significant down regulated gene in this array was the G-protein coupled receptor PROKR2 of prokineticin 2 (PK2). The decrease of the receptor and associated transduction pathways may eventually lead to increase of GABA-A receptor function, thus decreasing cell excitability.

Microarray analysis of gene expression changes in human neurocytes

In a detailed study we investigated the gene expression in the whole human genome using microarray technique(Marzotto et al. 2014). A brief description of the protocol, that required extremely careful standardization of each passage and it is distributed in a lot of weeks of work.



Schematic drawing of the microarray analysis of SHSY5Y neurocytes treated with different dilutions/dynamizations of *Gelsemium* (G) and of Control solvent solution (Ct)

We have seeded a standard number of cells in 12 culture plates and incubated for 24h with *Gelsemium* dilutions or controls. After that RNA was extracted and cDNA synthesized and labeled with fluorescence for further hybridization on the microarray chip. Including cell colture, treatment, extraction and data analysis, each experiment took over one month of work.

We used NimbleGen chips that are portioned in 12 subarrays each containing spotted on the surface thousands of probes that recognized the entire set of human expressed genes (exactly 45 thousands transcripts).

We analyzed 4 biological replicates, in a total of 4 chips. After scanning we obtained images of thousands of spots, in which the intensity of fluorescence of each spot is roughly function of the gene expression level.

Further data analysis allow to measure the expression change of a gene exposed to Gelsemium compared to the control, indicated as fold-change.

Results

Exposure to the *Gelsemium* 2C promoted the significant down-expression of 49 genes while 7 genes were overexpressed.

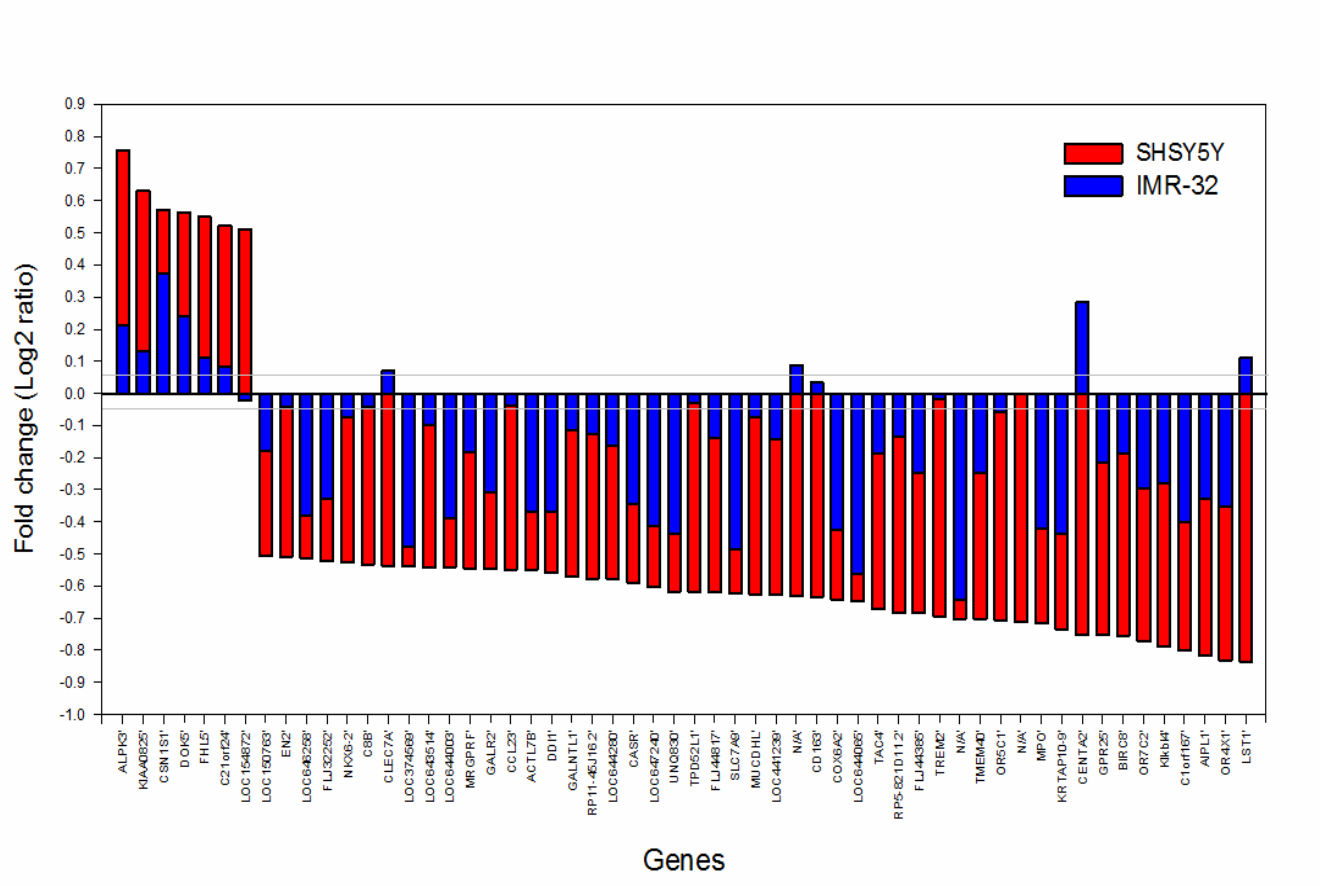
Many of these genes regulated by *Gelsemium* belong to:

- neuropeptide/receptor systems
- calcium signalling
- G-protein coupled transduction systems
- inflammatory pathways

Effects of *Gelsemium* increasing dilutions/dynamizations on the expression of 49 Gels C2-down-regulated genes

Figure shows the distribution of the fold changes in the 49 down-regulated genes for all the dilutions tested. All those genes were inhibited by *Gelsemium* 2C as shown in the right part of previous figure.

In human neurons 49 Genes among 45034 tested are down-regulated by high dilutions of *Gelsemium.*



Representations of genes significantly Up-regulated (Fold change >0.5) and Down-regulated (Fold change <0.5) in SHST5Y neurocytes (red) and their respective changes in another neurocytes cell line (IMR-32). Although with less intensity, the same trend was observed in this second type of cells, with the exception of only 6 genes.

A net inhibitory effect in all genes but two was observed with all dilutions. In subsequent centesimal dilutions, the number of genes with negative fold change (indicating down regulation of those genes), was systematically higher than the number of genes with positive fold change. In particular, the frequency of down-regulated vs up-regulated genes was 47 vs. 2 (96% vs. 4%) in 3c, 42 vs. 3 (86% vs. 6%) in 4c, 38 vs. 3 (78% vs. 6%) in 5c, 30 vs. 9 (61% vs. 18%) in 9c, 27 vs 7 (55% vs. 14%) in 30c. By applying Fisher exact test, the exact probability of the distributions, under the null hypothesis of indifference, was calculated and significant *p* values resulted for all dilutions ($p < 0.001$ for 3c, 4c and 5c treatments, $p = 0.0035$ for 9c and $p = 0.004$ for 30c). The absence of an equal scattering between the two signs (positive and negative fold changes) suggests that *Gelsemium* at high dilutions affects the expression of a significant portion of these genes. This conclusion is reinforced by a separate Fisher exact test carried out on a list of 49 genes randomly selected by the SPSS software from the 45033 transcripts; no significantly different distribution of down-regulated or up-regulated genes in this random gene-set was observed with any *Gelsemium* dilution.
This study provides evidence that *Gelsemium* exerts a prevalently inhibitory effect on a series of neurocyte genes across a wide dose-range. The effect decreases with increasing dilutions, but whole genome expression analysis allowed to detect statistically significant changes even at the highest dilutions tested (9c and 30c).

The results suggest the extreme sensitivity of human gene expression to regulation by ultra-low doses and high dilutions/dynamizations of a plant remedy and encourage further efforts in research on this field.

Studies using “omic-based” approaches and systems biology should be particularly worthy at generating new hypotheses on mechanisms for the effects of highly diluted natural compounds.

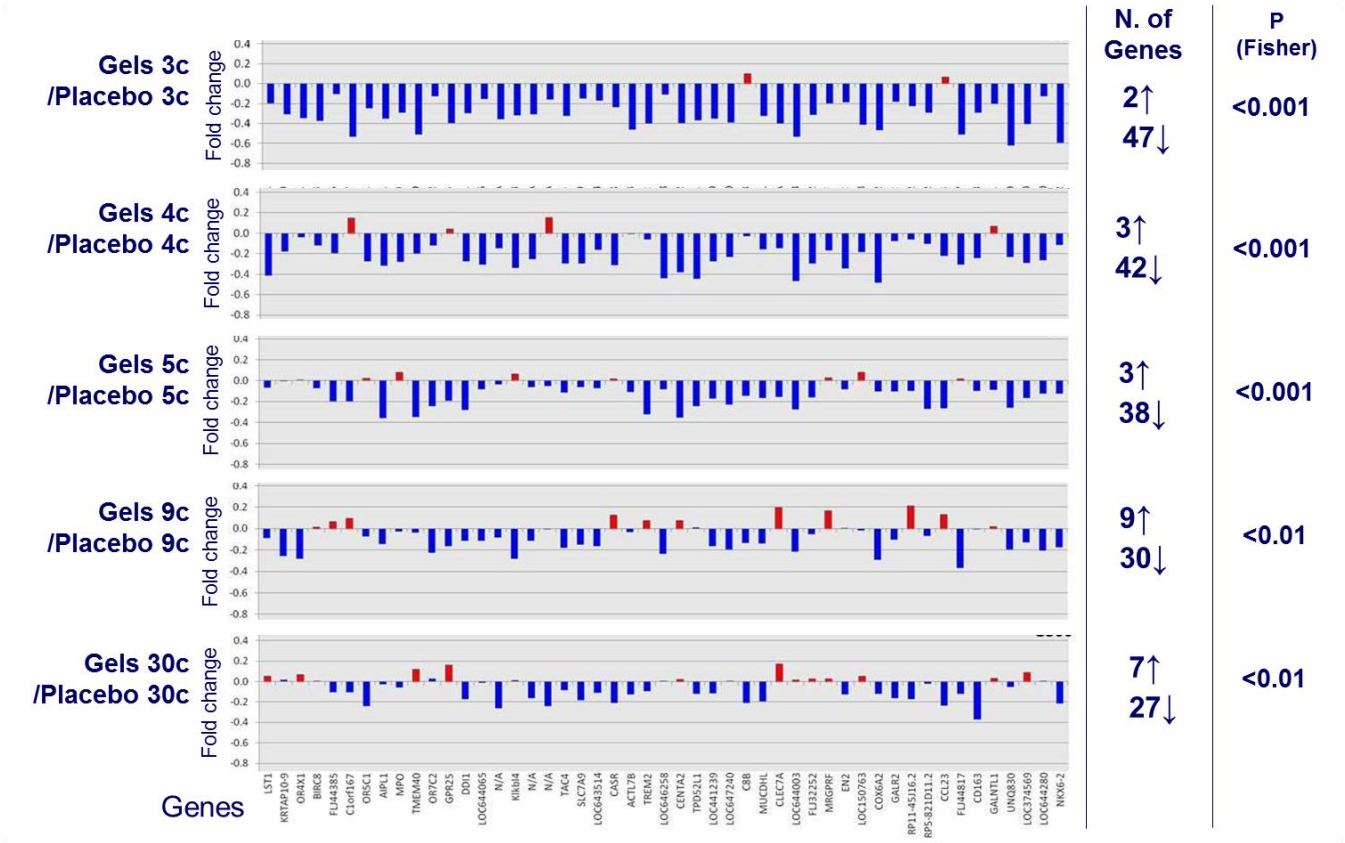


Figure of the distribution of the fold changes in the 49 down-regulated genes for the dilutions 3CH, 4CH, 5CH, 9CH and 30CH. Even though the size of the differences was distributed in a small range, the number of genes with negative fold change (Log2 *Gelsemium* < Log2 control, blue in Figure) was systematically higher than the number of genes with positive fold change (pink).

OTHER MODELS OF GENE EXPRESSION STUDIES

Several recent studies also document the ability of highly diluted compounds to modulate gene expression in human/animal cells and unicellular organisms (Bellavite et al. 2015), confirming early findings and general hypotheses put forward by others (Khuda-Bukhsh 1997). In experiments conducted in microbiological models, ultradiluted *Arsenicum album* 30C or *Arnica montana* 30C modified the expression of specific genes that are the targets of arsenite and UV irradiation injury, respectively. A long series of studies promoted by Lucietta Betti showed that ultrahigh diluted arsenic applied to wheat seedlings, poisoned with a sub-lethal dose of arsenic, produced a strong gene modulating effect compared to pure water (control)(Marotti et al. 2014). Authors have suggested that diluted arsenic treatment induced a re-equilibration of those genes that were upregulated during poisoning. Mother tinctures and highly diluted drugs were tested in human tumor cell lines or cells challenged with carcinogens; they affected the modulation of the expression of specific mRNA markers . These findings support the hypothesis that homeopathic remedies could turn some important genes on or off, initiating a cascade of gene actions to correct the gene expression that has gone wrong and produced the disorder or disease. In this hypothesis the relevant target gene should be sensitive to similar stimuli and exert a pleiotropic transcriptional regulation on a battery of genes with related functions. Needless to say, these in vitro effects are very

interesting but cannot support the use of high dilutions in the treatment of cancer. In several studies cited above, not all the cells used, nor all the dilutions tested, gave positive results.
Further experiments conducted on resting epithelial cell lines treated with *Apis mellifica* MT, 3C, 5C and 7C dilutions reported modified expression of hundreds of genes after 24h incubation (Bigagli et al. 2014). The main reduced functions were cytokine expression and inflammatory processes, while anti-oxidative responses and proteasome degradation were up-regulated.

Table 3. High dilutions and molecular biology (citations in review of Bellavite et al. 2015)

Test compound	Potencies	Cell type	Effect	REF.
Carcinosinum	MT, 30C, 200C	DLA cells	↑ specific gene expression (p53 pro-apoptotic)	Sunila et al. 2009
Arsenicum alb.	30C	Saccharomyces cerevisiae, E. coli	↑ Resistance to arsenicum toxicity ↓↑ expression of specific genes (apoptotic gene, stress response proteins)	Das et al. 2011; De et al. 2012
Carcinosinum, Hydrastis, Ruta or Thuja	200C	DLA cells	↑ Apoptosis , ↓↑ Gene expression (whole genome analysis)	Preethi et al. 2012
Gelsemium	2C, 3C, 5C, 9C, 30C	Human neurocytes SHSY5Y	7 genes ↑ 49 genes ↓ expression (whole genome analysis) ↓ gene expression (RT-Array, 2C)	Marzotto et al. 2014; Olioso et al. 2014
Apis mellifica	3C, 5C, 7C	Human prostate RWPE-1	↑↓ expression of different groups of genes (whole genome analysis)	Bigagli et al. 2014
Rhus tox.	30X	Primary cultured mouse chondrocytes	↑ specific gene expression (COX-2), ↓ specific gene expression (collagen II; de-differentiation role)	Huh et al. 2013
Arsenicum alb.	45X	Arsenic-intoxicated wheat seeds	↑ Germination ↓ Gene expression levels	Marotti et al. 2014
Condurango	30C	H460-non-small-cell lung cancer (NSCLC) cells	↓↑ expression of specific genes (apoptotic markers), ↑ Apoptosis, oxidative stress, mitochondrial depolarization	Sikdar et al. 2014

UP-TO-DATE KEY FINDINGS IN CELL MODELS.

- **Multicentre confirmation of high dilution effects (even beyond Avogadro) in rigorous cell models (e.g. *Histamine in basophils),* incompatible with placebo hypothesis**
- **According to different models, high dilutions may have protective effects (e.g. Arsenic), prevalent inhibitory (e.g. *Gelsemium*) or apoptotic (e.g. *Carcinosinum*)**
- **Dilution-effect studies show that the same trend is nonlinearly obtained with 2-3-5-9-12 CH (*Histamine*, *Arnica, Gelsemium*) with various peaks**
- **High dilutions act through membrane cell receptors as as shown by studies with inhibitors: cimetidine, propanolol, strychnine**
- **Homeopathic high dilutions have effects on the expression of a number of genes, revealed at the best by molecular biology high-throughput techniques**
- **The scenery of homeopathic drug actions at the level of gene expression reveals multiple and extremely complex actions that may partially explain some *in vivo* effects**

We do not enter in the discussion of the high-dilutions and homeopathic "doses", a topic which is actively investigated (Bellavite et al. 2014; Fisher 2015; Demangeat 2015), while wish to finish this presentation with a citation from Hahnemann (Organon, par. 278):

"How small, in other words, must be the dose of each individual medicine, homeopathically selected for a case of disease, to effect the best cure?"
"Is, as may easily be conceived, not the work of theoretical speculation. (...) Pure experiment, careful observation of the sensitiveness of each patient, and accurate experience can alone determine this in each individual case"

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