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Short Review

Experimental neuropharmacology of *Gelsemium sempervirens*: Recent advances and debated issuesPaolo Bellavite^{*}, Clara Bonafini, Marta Marzotto

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ABSTRACT

Gelsemium sempervirens L. (*Gelsemium*) is traditionally used for its anxiolytic-like properties and its action mechanism in laboratory models are under scrutiny. Evidence from rodent models was reported suggesting the existence of a high sensitivity of central nervous system to anxiolytic power of *Gelsemium* extracts and Homeopathic dilutions. *In vitro* investigation of extremely low doses of this plant extract showed a modulation of gene expression of human neurocytes. These studies were criticized in a few commentaries, generated a debate in literature and were followed by further experimental studies from various laboratories. Toxic doses of *Gelsemium* cause neurological signs characterized by marked weakness and convulsions, while ultra-low doses or high Homeopathic dilutions counteract seizures induced by lithium and pilocarpine, decrease anxiety after stress and increases the anti-stress allo-pregnanolone hormone, through glycine receptors. Low (non-Homeopathic) doses of this plant or its alkaloids decrease neuropathic pain and c-Fos expression in mice brain and oxidative stress. Due to the complexity of the matter, several aspects deserve interpretation and the main controversial topics, with a focus on the issues of high dilution pharmacology, are discussed and clarified.

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1. Introduction

Gelsemium sempervirens L. (here referred as *Gelsemium*) (Loganiaceae) is a medicinal plant used for the treatment of various neuroses in both Homeopathic and Ayurveda systems [1–3]. All parts of the plant contain the major active principle gelsemine as well as other strychnine-related alkaloids, such as gelseminine and sempervirine [3–5]. In Homeopathic *Materia Medica*, *Gelsemium* is described as a remedy for a variety of neurological and behavioral symptoms including general prostration, drowsiness, tiredness, mental apathy, lack of muscular coordination, and discomfort when confronted with novelty or unfamiliar situations; these symptoms are alleviated by motion and aggravated by emotion and excitement [6] and the practical experience of homeopaths confirms this suggestion [7,8]. However, evidence of clinical efficacy of *Gelsemium* and other Homeopathic remedies in humans is still controversial [1,9,10]. Our group working at Verona University has explored the action mechanism of *Gelsemium* on *in vivo* and *in vitro*

model systems [11–15]. Evidence from both animal and cellular studies suggested a high sensitivity of central nervous system to this plant effects, that were detected even at extremely low doses and high Homeopathic dilutions (e.g. 5th and 9th centesimal dilution). These studies were never disproved experimentally, but sparked a debate and were the object of comments or critical commentaries concerning the action mechanisms of *Gelsemium* and the methods of its investigation [16–22]. In the meantime, the effect of *Gelsemium* or its alkaloids in neurological and behavioral models were reported by several independent laboratories [1,23–28]. Due to the complexity and novelty of the topic, several aspects deserve clarification and the main issues of this debate are here summarized.

2. Animal models

Bellavite and co-workers have demonstrated that low doses and high dilutions of extract of *Gelsemium* – traditionally used for patients with anxiety-like symptoms – reduce anxiety and fear in validated mice behavioral models [11,29]. These effects confirm previous reports [30,31] and were followed by other similar evidence by other's laboratories [24,32].

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A critical commentary of the studies of the Verona research group was sent in 2011 to *Frontiers in Neurology* by S. Chirumbolo [16], who ironically belonged to the same university department. He claimed the existence of purported “biases” in Magnani et al. paper [11] related to the question of a possible toxicity of the plant and of gelsemine. However, this assertion is highly debatable, since toxicity of *Gelsemium* components is well-known in literature [5], but has no bearing on the question of the pharmacological effect of Homeopathic medicines, which were accomplished through administration of extremely low doses (less than 10^{-12} mol/L) that are absolutely non-toxic to animals [17]. Also *Gelsemium* extract fractions tested by others [33] were non-toxic for mice at the doses that exerted anxiolytic activity. The only study that tested both anxiolytic (low dose) and lethal effects (high dose) of *Gelsemium* alkaloids (from *Gelsemium elegans* L.) in animals showed that the lethal dose produced convulsions that led to respiratory failure [5].

The same commentary [16] then raised the question of the methods used and criticized the lack of testing anxiety with elevated plus-maze, but actually dozens of tests for animal behavior exist, which are thought to reflect different facets of emotionality [34]. Our evidence of anxiolytic-like effect in mice was obtained with open-field (OF) and light–dark (LD) tests, with five different behavioral variables extracted, that are demonstrative enough of the claimed psychopharmacological effect [11–13]. Then, the commentary raised the question of possible biases due to placebo or nocebo effects. The authors replied that the work in question, which adopted rigorous double blind controls, consisting of the same solvent as the drug but without the active principle, precisely to rule out these types of effects [17]. Even if we can exclude placebo effects since we carried out suitable controls, the problem of high dilution effects (beyond Avogadro–Loschmidt constant) poses a challenge to conventional pharmacologic wisdom. From the data of our experiments, there emerged clear and consistent evidence indicating that lower dilutions (4c) were less effective than higher dilutions (9c and 30c). However, the pattern was more complex since the dilutions with maximum effects varied in different test assays.

One and half years after the publication of our paper in *Psychopharmacology* [11] a letter was sent to the same journal by V. Cervo and L. Torri, belonging to Mario Negri Institute of Pharmacological Research (Milano) [19], whose director S. Garattini expressed strong opposition to Homeopathy elsewhere [35,36]. This letter claimed that data were not reproduced in another preliminary paper on the same subject and that they were implausible. The authors responded that the charge of non-reproducibility was unfounded because in both series of experiments *Gelsemium* worked in two well-validated models to the same direction, even with different statistical significance [20]. For further confirmation, we also performed a pooled analysis of the two papers' results and found a highly significant effect of *Gelsemium* in both models [13]. In Mario Negri researchers' letter there was a claim concerning the alleged lack of activity of diazepam in OF parameters (diazepam was used as control drug). This evidence was previously reported by others [37] and may suggest that the effect of *Gelsemium* in OF test concerned the exploratory behavior and the decrease in neophobia, instead of the classic anxiolytic-like effect. *Gelsemium* did not alter the locomotion assessed in OF in any series, indicating that the effect was not sedative [38].

In January 2013, a lengthy letter to *Journal of Medicine and the Person* contained a series of criticisms to experimental studies on *Gelsemium*, arguing that previously advanced critical comments did not elicit any serious reappraisal [39]. However, most technical points raised in the cited critical letter were thoroughly addressed in previous publications and confirmed the validity of their findings and interpretations [40]. The response also explained that, contrary

to allegations, the original papers already provided evidence of reproducibility, absence of alcohol interference, rationale of test employed, power of statistics; they discussed toxicity and placebo issues, animal housing, physico-chemical theories on highly diluted remedies, including nanoscience and hormesis (inverse effects according to high versus low doses). In the same letter [39], Homeopathic medicine was considered an example of the dark times of the Middle Ages. However this comment was out of place and incoherent, given that Homeopathy has developed in the nineteenth century and a growing number of research works are published in peer-reviewed journals during the XXI century (a total of 6038 papers concerning Homeopathy are cited in PubMed, accessed December 23, 2016).

A further letter from S. Chirumbolo in *Journal of Clinical Practice* [41] again criticized Bellavite's group papers on *Gelsemium* effects in experimental animals, repeating the same allegations as previously reported [39]. Contrary to what the cited letter asserts, the concerns relating to the applications of anxiety models in Homeopathic research, the lack of interference from minimal doses of alcohol and the effects of *Gelsemium* have been thoroughly addressed and the journal published a detailed table on these aspects [42]. Again, the letter [41] and a following commentary [43] raised the suspect of possible toxicity of Homeopathic medicines and the risk of adverse effects. However, it is well-known that Homeopathic remedies – according to the U.S., European, and Italian pharmacopoeias and legislation – are used in dilutions such that they can't have direct toxic effects. Moreover, others have shown that adverse effects of Homeopathic and Ayurvedic medicines are rare and these therapies are well tolerated [44,45]. Direct evidence from placebo-controlled studies of *Gelsemium* and of *Ignatia amara* [46] showed that used dilutions have anxiolytic-like properties without weakening locomotion and without adverse or sedative effects. The difficult-to-solve technical issues of high dilutions, hormesis, and paradoxical reversal of the effects of drugs should be addressed not through subjective opinions and jeering, but rather on the experimental ground, through patient and critical comparison of data and results.

In summary, current literature from other's [1,2,24,47–49] and our [11–13] laboratories offers reliable evidence in animal models that *Gelsemium* exerts anxiolytic, analgesic and anti-depressive effects in a wide range of doses and dilutions.

3. Neurocyte models

In March 2014, the Verona group published two scientific papers on the effects of *Gelsemium* in a laboratory model made with SH-SY5Y neurocytes, where we showed a prevalent down-regulating effect of this plant on gene expression [14,15]. Searching for a possible mechanism of action, we found that very low doses and Homeopathic dilutions of *Gelsemium* modulate the expression of genes involved in neuronal functions (G-protein coupled receptor signaling pathways, calcium homeostasis, inflammatory response and receptors). Following these publications, again criticisms and comments appeared first in PubMed under the citation of our papers, then were reported in a further commentary in *Frontiers in Neuropharmacology* [50] by S. Chirumbolo, expressing doubts about the methods used and suggesting “bias” in the results. First criticism was that the search for an involvement of neural genes related to anxiety/depression or mood disorders were biased by the expression of human genes having no orthologs/homologs in mice. However this type of experimental procedure, which addresses various knowledge gaps using both animal studies and *in vitro* models, is very common in pharmacological studies [51]. The commentary [50] then claimed that concentration of gelsemine was calculated from previous spectrometry investigations and new

preparations, from ethanol draw extracts, were not further quantified by analytical chemistry. The authors replied that the concentration of gelsemine which they reported (0.021 g/100 ml, corresponding to 6.5×10^{-4} mol/L) [51] was precisely determined by liquid chromatography in the mother tincture from which exactly the samples used in their investigation. The same commentary casted doubts on the possible interference of ethanol used in original drug solutions. However, it was clearly reported [51] that the final ethanol concentration was 0.03% v/v, and no significant differences in cell viability were observed between cells treated with the ethanol control solution 0.03% (v/v) and untreated cells. Furthermore, as other researchers have found that cell viability of neurocytes is unaffected by doses of ethanol up to 10 mmol/L (0.06%) [52].

The most recent commentary on *Gelsemium* studies [22] repeats several previous “critical issues” and “comments” concerning purported “biases”, summarized in a full table. This further comment maintained that herbal medicine is widely used in anxiety and mood disorders, often with contradictory evidence, and this is quite obvious. However, the author claimed that the use of “negligible doses” of *Gelsemium* hydroalcoholic extracts to affect gene expression is questionable. However, there is confusion on what are considered as “negligible doses” of *Gelsemium*, while there is redundant claim of interference by ethanol, whose role is however negligible at the used doses, as already demonstrated [15,51]. Table 1 reports a point-by-point reply to all the issues and claims reported in the cited commentary [22].

4. Discussion

In the recent years the pleiotropic effects of *Gelsemium* and/or of its major alkaloids gelsemine and koumine have been investigated in several models and a complex picture of action mechanisms is emerging (Table 2). The *in vitro* cellular models confirmed the neurotropic action of *Gelsemium* previously observed in animals.

In the cited laboratory studies with *Gelsemium* a wide range of dilutions and dosages or concentrations were used. Strongest and more consistent evidence of beneficial effects in anxiety models and neuropathic pain models were obtained by most researchers with higher doses (or low Homeopathic dilutions) [14], but we also reported that drug activity could be detected also in high dilutions both in mice [11,13] and in neurocytes [15]. These latter figures await confirmation by independent laboratories.

The Verona research group used anxiety and behavior models in mice to test the effects of low (4th and 5th centesimal, i.e. 4c and 5c), medium (7c and 9c), and high Homeopathic dilutions (30c) of the plant extract [11,13]. Since the principal alkaloid gelsemine (molar mass = 322 g/mol) was 0.021% (w/v) in the mother tincture and this value corresponded to a concentration of 6.5×10^{-4} mol/L, we could calculate that dilutions such as the 4c and 5c (where the dilution factor from the mother tincture is 10^8 – 10^{10} times respectively) are expected to contain low concentrations of the purported active ingredient, but still falling within the molecular range (approximately 10^{11} – 10^{13} mol/L). A 9c dilution should contain a theoretical concentration of 10^{-22} mol/L, that is near the limit of Avogadro–Loschmidt constant, and the 30c should be far below that limit [57]. Boustia et al. [30] reported that *Gelsemium* at 5c and 15c dilutions caused a significant increase in steps climbed in the staircase test in stressed mice and in the light/dark box test caused a significant increase of transitions at 5c, 9c and 15c. Interestingly, the authors observed concomitant variation of some immunological parameters like an increase of lymphocytes and neutrophils. Venard et al. [32] observed a stimulatory action of *Gelsemium* at 5c and 9c Homeopathic dilutions on the production of anti-stress neurosteroids in rat brain, but not at 15c. Studies in rodents from other laboratories were done with non-Homeopathic doses: a single intrathecal injection of 0.5–0.6 µg gelsemine produced antinociception effects in rats [58]. Meyer et al. [24] reported an anxiolytic-like effect in rats with injections of 500 µl of 10^{-10} mol/L

Table 1

Fundamental issues in the research about *Gelsemium* and anxiety, raised by a commentary in the Journal Neurology and Therapy [22] and clarifications of corresponding points.

Issues from commentary [22]	Reply	
Active principles and toxicity	Gelsemine was supposed to be the only active principle working in <i>Gelsemium</i> extracts Adverse effects not evaluated	Gelsemine is known as the major active principle, but the effect of further components was suggested [12] Adverse effects were evaluated with Open-Field test and found to be null with <i>Gelsemium</i> while some sedation was noted with buspirone [11]
Ethanol in solvent and test samples	Final dilution contained ~500 µM EtOH, still biologically active	The used dose was not toxic on the cells, as reported [15] and in any instance the control (placebo) contained the same small amount of ethanol [11,17,38]
Experimental setting: animal model	Behavioral tests performed did not include specific tests on anxiety, depression, sedation	Behavioral test used (Light–dark and Open-field) are among the best validated test for anxiety [53,54], and sedation was excluded [11]. Depression was not object of this research
Experimental setting: <i>in vitro</i> cell model	Operators treating animals performed behavioral tests Criticism in gene expression performing and interpretation	This is a normal procedure in behavioral pharmacology to make the animals accustomed to a single person This generic comment, devoid of precise consistency, already received a response [51]
Pharmacological interpretation	Associated exclusively with gelsemine and considering the allopregnanolone/GABAR pathway. The anxiolytic activity of <i>Gelsemium</i> may derive from other alkaloids besides gelsemine Pharmacological interpretation may be hindered by diluted test solutions with negligible amounts of active principles	This is untrue. Several possible pathways of <i>Gelsemium</i> action were outlined, including glycine receptors [12], prokineticine receptor [14] and signal transduction [15] Nobody denied that point and it was actually discussed [12]
Statistics	Pooling data projected to retrieve positive evaluation of the mechanism Blinded confounders with the same operator in treating and performing test with animals	A pharmacological interpretation of diluted test solutions was widely reported in discussion of relevant papers and in this review This allegation is without foundation. The data analysis was performed by independent statistician and published by a peer-reviewed Journal [13] On the contrary, the procedure of double-blinding, rarely done in animal research, prevented any possible confounding factors, as already pointed out [11,42]

Table 2
Reported effects *Gelsemium* and its alkaloids in laboratory models.

Issue	Model	Dose/dilution	Evidence
Toxicity	Goats	Ingestion of whole plant	Neurological signs characterized by marked progressive weakness and convulsions culminating in death [55]
Toxicity	Mice	Crude extract, high dose (<i>Gelsemium elegans</i>)	Convulsions, respiratory failure [5]
Toxicity	Mice and neurons	Homeopathic 5c, 9c, 15c, 30c dilutions	No evidence of toxicity <i>in vivo</i> and <i>in vitro</i> [11–13,15]
Epilepsy	Mice experimental seizures	Low dilution of mother tincture	Counteracts seizures induced by lithium and pilocarpine [31]
Anxiety	Mice behavior	Homeopathic 5c, 9c, 15c dilutions	Decreases anxiety after stress [30]
Anxiety	Mice behavior	Homeopathic 7c, 9c, 30c dilutions	Decreases anxiety parameters, no locomotion unspecific effects [11–13]
Anxiety	Rat behavior	<i>Gelsemine</i> (alkaloid of <i>Gelsemium</i> plants) 10^{-6} to 10^{-10} M	Decreases anxiety parameters [24]
Pain	Mice allodynia and thermal hyperalgesia	Koumine (0.28–7.0 mg/kg) and <i>Gelsemine</i> (2 and 4 mg/kg)	Decreases neuropathic pain and sleep disturbances [26,49]
Anxiety	Mice behavior	<i>Gelsemine</i> , koumine, and <i>gelsevirine</i> 0.4–10 mg/kg (biphasic effects)	Anxiolytic effects [47]
Dementia	Scopolamine-induced dementia in mice	Homeopathic mother tincture of <i>Gelsemium</i> (1 mg/kg)	Protects against scopolamine-induced cognitive discrepancies [2]
Action mechanism	Rat nervous tissue	Homeopathic <i>Gelsemium</i> and <i>gelsemine</i> 5c and 9c	Increases the anti-stress allopregnanolone hormone, through glycine receptors [32]
Action mechanism	Rat neuropathic pain	Koumine (alkaloid of <i>Gelsemium</i> plants) 0.28–7.0 mg/kg	Increases allopregnanolone and the key synthetase enzyme 3 α -hydroxysteroid [26,56] oxidoreductase (3 α -HSDR)
Action mechanism	Intracerebral administration of strychnine	<i>Gelsemine</i> , koumine, and <i>gelsevirine</i> 0.4–10 mg/kg	Anxiolytic effects antagonized by strychnine, suggesting involvement of glycine receptor in the brain [47]
Action mechanism	Neurocyte SH-SY5Y cell line	<i>Gelsemium</i> 2c	Decrease of the prokineticin receptor 2 gene expression, whose ligand is a neuropeptide involved in nociception, anxiety and depression-like behavior [14]
Action mechanism	Neurocyte SH-SY5Y cell line	<i>Gelsemium</i> 2c (corresponding to a <i>gelsemine</i> concentration of about 10^{-10} M) and higher dilutions until 9c and 30c with lower effect	Decreases the expression of 49 genes involved in cell excitability [15]
Action mechanism	Transfected HEK293 cells and cultured spinal neurons	<i>Gelsemine</i> 10^{-6} – 10^{-5} M	Directly modulates the function of glycine receptors (biphasic effects) [28]
Action mechanism	Immunohisto-chemical study	<i>Gelsemine</i> (4 mg/kg)	Decreases c-Fos expression in mice brain [49]
Action mechanism	Scopolamine-induced dementia in mice	Homeopathic mother tincture of <i>Gelsemium</i> (1 mg/kg)	Decreases beta-secretase and oxidative stress in brain [2]

Gelsemine; assuming a 1/1000 dilution in the body, this should correspond to a concentration as low as approximately 10^{-13} mol/L. Dutt et al. [33] found a significant anxiolytic activity in mice of a methanol extract of *Gelsemium* at the dosage of 150 mg/kg and in a partially purified fraction at the dose level of 10 mg/kg. In the last mentioned study the molar concentration of active principles was not indicated.

Besides the cited studies with *Gelsemium*, other examples of biological activity of medicinal plants, even in dilutions beyond Avogadro–Loschmidt constant, have been reported in recent years. *Toxicodendron pubescens* (known in Homeopathy as *Rhus toxicodendron*) exhibited anti-inflammatory effect on rat arthritis [59]; the maximum protective effect was evident in the crude form at 10 mg/kg/day, but significant results were obtained also with Homeopathic dilutions 3c, 6c, 30c, and 200c. *Terminalia chebula*, which is a component of the Ayurvedic formulation *Triphala* [60], showed a dose-dependent anti-proliferative activity against cancer cell lines when tested as low dilutions or crude ethanolic extract [61,62] but also as Homeopathic preparations 6c and 30c [63]. It has been suggested that *T. chebula* extract and other Indian plants with anticancer properties may act by enhancing Fenton reaction-mediated damage to deoxynucleoside triphosphates, thus slowing down DNA replication in rapidly dividing cancer cells [64]. Evidence of anti-proliferative effects of high dilutions of other plants like *Ruta graveolens*, *Thuja occidentalis* and *Phytolacca decandra* was reported [65–67]. Also in these cases the effects were greatest with the crude extracts, but persisted in the ultra-diluted molecular preparations, although to a lesser degree. Interestingly, *Gelsemium* also exhibits anti-proliferative properties, but only in crude extracts or nanoparticulate preparations [68,69].

Recent evidence from our laboratory suggests that *Arnica montana* stimulates the gene expression of chemokines and of connective tissue components by macrophages *in vitro* [70,71]; in our experiments the best effects required a pre-sensitization with interleukin-4 and were obtained with the low dilution 2c, but significant gene expression persisted also after treatment with 5c, 9c, and 15c *A. montana*.

A number of observations and theoretical models suggest that in high dilution pharmacology there is no linear or proportional relation between the molecular concentration of active substances and the therapeutic effect. Although many conventional physicians find such notions implausible [19,72,73], in science theoretical doubts should vanish in the face of strong experimental evidence. Thus, till now, there is no satisfactory or unifying theoretical explanation for these claims, though some hypotheses suggest a role of the nano-heterogenous structures and dynamics of water: the Homeopathic serial dilutions and succussions (shaking) might induce a process of increasing physical structuring or dissipative structures in water or water/ethanol solutions, with the possible participation of other solvent components like silica released by the glass containers [57,74–80]. Moreover, hypotheses of the pharmacodynamics of such extremely diluted solutions and paradoxical dose–response requires assimilation of modern self-organization theories, including systems biology, networks, and the phenomena of hormesis and epitaxy [38,75,81–83]. Recent evidence suggests that beneficial actions of *Gelsemium* are dependent on glycine receptors, which are inhibitory neurotransmitter-gated ion channels of the CNS [28,32,84]. In general, gene expression studies have started to provide new insights on action mechanisms of Homeopathy at cellular and molecular level [14,15,21,83,85].

5. Conclusions

We hope these explanations are useful to correctly represent and clarify the scientific advancements concerning Homeopathic basic research and in particular the neurotropic action of *Gelsemium* on *in vivo* and *in vitro* experimental models. As suggested also by others [24], these studies open new perspectives for the development of safe and effective gelsemine- or *Gelsemium*-based strategies against pathological anxiety.

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