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Nanoparticle Characterization of Traditional Homeopathically-Manufactured *Gelsemium sempervirens* Medicines and Placebo Controls

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Abstract

Multiple studies have observed nanostructures in traditionally-manufactured homeopathic medicines. Homeopathy is a 200-year-old system of complementary and alternative medicine used worldwide. The nature of homeopathic medicines has historically stimulated much debate. The present placebo-controlled study extended previous work to characterize nanoparticles (NPs) in homeopathically-prepared *Gelsemium sempervirens (GELS)*, a natural botanical source with previously-documented anxiolytic, analgesic, and anticancer properties. An ethanolic GELS herbal extract was serially diluted and succussed (agitated) in a 95% ethanol-water diluent solvent in glass vials, following Homeopathic Pharmacopoeia of the U.S. guidelines. GELS (VERUM, at homeopathic potencies of 6C, 30C, 200C, each n=3 vials), succussed controls (SUCC-CONT, at homeopathic potencies of 6C, 30C, 200C, each n=3), and one set of unsuccussed solvent control vials also used natural cork (*Quercus suber*) stoppers (UNSUCC-cork, n=3). A final set of unsuccussed solvent controls used silicone stoppers (UNSUCC-silicone, n=3). Analytical methods included nanoparticle tracking analysis (NTA), zeta potentials, and UV-Visible spectroscopy. NTA revealed >4 x 10^8 nanoparticles per milliliter in all VERUM, SUCC-CONT, and UNSUCC-cork vials, significantly more than the UNSUCC-silicone controls. Particle sizes were polydisperse, significantly larger in the VERUM 30C at 129.8 nanometers versus SUCC-CONT at 6C, 30C, 200C and the UNSUCC-cork controls. Zeta potentials consistent with greater particle stability were significantly most negative in the VERUM GELS 200C (-47.75 mV). Within the UV-vis wavelength range 300-400 nm, the SUCC-CONT 30C exhibited significantly higher, whereas UNSUCC-silicone stopper controls had significantly lower, mean absorbance than all other samples. Taken together, the data suggest that traditional homeopathic methods involving succussions release not only the previously-shown silica from glassware walls, but also *Quercus suber* materials from natural cork stoppers to stabilize NPs in solution. With verum source material *Gelsemium*, additional NP size growth and surface stabilization can occur. Further study of homeopathic manufacturing materials and methods and their biological correlates is indicated.

Keywords: Nanoparticles; *Gelsemium sempervirens*; Silica; *Quercus suber*; Homeopathy; Nanoparticle tracking analysis; Zeta potential

Introduction

Within both conventional bulk pharmacology and nanopharmacology, natural source botanical extracts used in traditional forms of complementary and alternative medicine (CAM) have emerged as potential direct therapeutic agents for conditions including cancer [1-5], infectious diseases [6-8], diabetes mellitus [9,10],and stress-related disorders [11,12]. In conventional nanomedicine, various botanical extracts have also demonstrated the capacity to generate bioactive silver [2,13-17], gold, [18-20] and silica [21,22] nanoparticles (NPs) from their respective precursor materials. Merely agitating a solution of sodium chloride will also generate NPs and embed them into glassware in contact with the solution [23].

Within systems of CAM, *Gelsemium sempervirens* (GELS) from the Loganiaceae plant family is one of the more widely studied botanical agents. GELS is used clinically in both traditional Ayurvedic herbal medicine and homeopathy [24,25]. *Gelsemium* (GELS) botanical extracts prepared as a homeopathic medicines (HMs) reportedly exhibit anticancer [26,27], analgesic [28], and anxiolytic effects [29,30]. *Gelsemium* encapsulated in poly (lactide-co-glycolide) (PLGA) nanoparticles (NPs) exhibits enhanced cellular uptake and pro-apoptotic effects in a skin cancer cell line [27]. *Gelsemium*–generated silver nanoparticles also exert anticancer effects *in vitro* [2]. Finally, homeopathically-prepared GELS per se in potencies up to 30C can modify gene expression patterns of human neuronal cells in culture [31,32].

In part to reduce the risks of herbal toxicity from some of the plant's constituent alkaloids in their more concentrated plant extract form, some investigators have focused on the far less toxic homeopathic rather than herbal extract forms of *Gelsemium* for clinically-relevant investigation. Potencies of GELS are widely used in homeopathic clinical care for treatment of acute conditions such as symptoms of influenza infections [33] or public speaking anxiety [34]. In addition to

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other evidence for analgesic and anxiolytic effects from the *Gelsemium* herbal extract [25,35,36], recent research has demonstrated that homeopathically-prepared *Gelsemium* has effects on not only human neuronal cell gene expression [32,37], but also neuronal function [28]. Modulatory effects of homeopathically-prepared medicines on patterns of gene expression appear to generalize across not only *Gelsemium* HMs, but also HMs from other sources [38-40]. Homeopathy is an over 200-year-old system of CAM used worldwide [41,42], the nature of whose medicines has historically stimulated vigorous debate.

To manufacture *Gelsemium* in traditional homeopathic potencies, the raw plant source is typically first prepared as an ethanolic herbal extract of the botanical material. Next, the botanical extract is put as a liquid into a process of serial dilutions in solvent (ethanol-water or water). The ratio of source material to solvent is usually 1:10 (X potencies) or 1:100 parts (C potencies). In classical homeopathic manufacturing, as outlined by German physician-chemist founder Samuel Hahnemann MD in the 1800's [43], each dilution step is followed by intense mechanical agitation (succussions) either manually (e.g., 10-20 or more repeated succussions/step) or, now sometimes by vortexing, within a stoppered glass container. By standardized protocols dictated by national regulatory agencies such as the German or United States pharmacopoeias, each serial dilution-succussion step generates a consecutively higher homeopathic potency.

Both source nanoparticles [44-52] and various forms of silica from inside walls of glass containers [48,49,53,54] result. Gas nanobubbles formed by succussions may also play a role in the manufacturing process by surrounding and facilitating transfer of the solute particles from a given preparation step to the next, in glass or plastic vials [51,55,56]. Thus, as in mainstream NP generation techniques [23,57-63], mechanical milling and/or liquid agitation/attrition methods contribute to formation of the final product in HM manufacturing [4,64-66].

Against the validity of homeopathy, skeptics usually object that the serial dilutions ultimately remove any remaining bulk source molecules past 24X or 12C (where Avogadro's number is 6.023×10^{23}). As noted elsewhere, however, the dilution controversy may apply only to removal of bulk but not of nanoscale forms of materials [51]. Multiple laboratories in different countries have demonstrated the presence of not only nanostructures in homeopathically-prepared medicines [44-47,49,51,52,67-69], but also unique electromagnetic [70,71] and optical properties [72,73] of HMs at high potencies (highly diluted and extensively succussed) versus controls.

The initial NP report from Chikramane et al. [44] showed transmission electron microscopic (TEM) and inductively-coupled plasma-atomic emission spectroscopy (ICP-AES) evidence for source metal nanoparticles (gold, zinc, copper, tin, platinum, silver) in HMs. Sizes ranged from 5 nm or less for individual NP up to aggregates of 2000 nm sizes at 6C, 30C, and 200C potencies. Using nanoparticle tracking analysis, we recently replicated and extended NP findings in samples of traditionally-made homeopathically-prepared silver (*Argentum metallicum 6C, 30C, 200C*) versus succussed and unsuccussed controls [69]. In the latter study, however, we found evidence consistent with the possible release of not only silicates from glassware, but also natural cork stopper organic materials (*Quercus suber* oak tree bark botanical extract) into solution during succussion procedures in verum and succussed control samples.

Quercus extract and quercus-generated metal NPs are biologically active with antioxidant and anti-cancer effects [16-18,74-76]. Another type of plant extract, e.g., *Equisetum telmateia*, also used in herbal and homeopathic medicine, can generate silica NPs from silicate precursors in solution [21,22]. Similarly, other investigators have demonstrated the ability of *Gelsemium sempervirens* plant extract to generate silver NPs with anti-cancer properties [2]. Taken together, the findings raise a question as to the relative contributions of verum source materials from plants, e.g., from *Gelsemium sempervirens,* in addition to and apart from *Quercus suber in* traditionally-used natural cork stoppers for making biologically-active HMs.

Not all modern homeopathic manufacturers use cork stoppers. However, the physician-chemist founder of the field, Samuel Hahnemann MD, developed homeopathy in the late 1700's to mid-1800's [43], when glass or natural cork stoppers would have been the main material available for bottle closure during agitation procedures, storage, and transport [77].

The purpose of the present study was to extend previous empirical findings of nanostructures in homeopathically-manufactured medicines to *Gelsemium sempervirens*, using nanoparticle tracking analysis (NTA). For further characterization, we also examined nanoparticle zeta potentials and ultraviolet visible spectroscopy patterns in all samples. In specific, we evaluated (a) verum *Gelsemium* HMs versus succussed controls at potencies below (6C) and above (30C and 200C) Avogadro's number for bulk form dilution; and (b) compared two different sets of unsuccussed controls with natural cork versus silicone stoppers. Because of our prior nanoparticle findings with cork-stoppered samples [69], an additional exploratory hypothesis was that control vials with modern silicone stoppers would not contain the same concentrations, sizes, and/or zeta potentials of NPs as did those with traditionally-used cork stoppers.

Materials and Methods

Design

As recommended by previous investigators [78], the study design was randomized and blinded for the analytic laboratories involved. Analytic laboratories were not informed of hypotheses related to the study. Study design included making triplicate samples in separate vials of 8 different types of samples. That is, samples included not only verum homeopathically-prepared *Gelsemium sempervirens* 6C (V6, n=3), 30C (V30, n=3), and 200C (V200, n=3) potencies; but also succussed solvent controls made at 6C (SC6, n=3), 30C (SC30, n=3), and 200C (SC200, n=3) potencies; unsuccussed solvent controls with natural cork stoppers (UC-Cork or UC-C, n=3); and unsuccussed solvent controls with silicone stoppers (UC-Silicone or UC-S, n=3).

To control for any effects of the shipment process itself, the total number of vials per box shipped by overnight courier from the manufacturer (Hahnemann Laboratories, San Rafael, CA) in the same packaging together to each of the two different analytic laboratories was thus 24. Each number-coded sample vial from the same box of samples was run through the analytic nanoparticle tracking analysis (NTA) and zeta potential tests in triplicate at Northwestern University (Evanston, IL). A separate box of samples made at the same time as those shipping to Northwestern University was shipped and evaluated using UV-vis spectroscopy at Nanocomposix (San Diego, CA).The manufacturer made all placebo samples before making the verum samples to minimize risk of cross-contamination. Each test vial was assigned and labelled with a randomized unique code number (see below).

Randomization and blinding

The first author (IB) created a spreadsheet of vial contents and materials for the study and assigned a unique random vial code number to each sample using the list randomizer program at http://random.org. The manufacturing pharmacy implemented the code assignments with code numbered labels without any other identifying information on each vial. Only the PI and manufacturing pharmacy had access to the list of the actual contents of each randomized, number-coded vial.

Previously, using a similar design, we observed nanoparticles in homeopathically-prepared, silver-derived verum medicines (*Argentum metallicum*) at 6C, 30C, and 200C with some higher particle concentrations, larger sizes, and more negative zeta potentials, different from those of succussed control solvent and/or unsuccussed solvent [69]. In the latter study, verum and control samples also showed complex UV-vis spectroscopic data that raised the possibility of contaminants in solution from organic materials such as the natural cork stoppers *(Quercus suber)* used during traditional manufacturing and storage. As a result, we expanded our previous design from the *Argentum metallicum* study to include not only the traditional cork stoppers in one set of unsuccussed controls, but also non-cork silicone stoppers in a different set of unsuccussed solvent controls.

Test materials

The *Gelsemium sempervirens* verum homeopathic medicines and the succussed and unsuccussed placebo solvent controls were made by Hahnemann Laboratories (San Rafael, CA, http://hahnemannlabs.com/), a commercial FDA-regulated U.S. homeopathic pharmacy specializing in supplying custom-made HMs to practitioners. Manufacturing procedures were done under clean-room procedures with HEPA air filters at room temperature and pressure in accord with the Homeopathic Pharmacopoeia of the United States (HPUS). The manufacturing methods followed the historical original methods and materials outlined by the physician-chemist founder of the field, Samuel Hahnemann, MD [43,79].

Diluent solvent for all samples was 95% v/v pharmaceutical grade ethanol (Pharmco-AAPER, USA, phenol-capped jug) in double-distilled water. Because of industry-standard manufacturing procedures for lower potencies (e.g., 6C) versus higher potencies (e.g., 30C, 200C), the current samples were made using (a) a different new glass vial for each serial dilution and succussion step up to 6C (Hahnemannian method); but (b) the same glass vial for each serial dilution and succussion step beyond 15C to make 30C and 200C potencies (Korsakovian method). This pharmacy also uses a standardized mechanical arm (Quinn Potentizer) to perform repeated successions of potentized medicines and succussed controls.

We chose this pharmacy because of our previous clinical and electroencephalographic studies in human subjects showing detectable differences in effects between multiple participant-individualized verum and control agents [80,83] as well as verum-placebo differences for two other of their custom-made products (mineral and plant sources), in Raman and UV-vis spectroscopic patterns as part of a basic science exploratory study [84]. However, none of our previous studies had specifically investigated homeopathic *Gelsemium sempervirens*.

To make and store samples, the manufacturer used clear borosilicate pharmacy quality glass 8 ml vial containers (Acme Bottle and Glass Co., Inc, Paso Robles, CA USA) with natural cork stoppers (size 3, Zandur, Nottingham, PA USA) for their sample production of each 5.5 milliliter sample of verum, succussed controls, and one set of unsuccussed controls. The remaining set of 3 unsuccussed controls used the same source for vials and solvent but were closed with silicone stoppers. Each vial was covered with parafilm and packed securely prior to shipment to prevent leakage.

Analytic procedures

Core laboratories at Northwestern University (Evanston, IL) performed the nanoparticle tracking analysis (NTA) and zeta potential testing. Separately, Nanocomposix (San Diego, CA) carried out UV-vis spectroscopy on a parallel set of samples number-coded, made, packaged, and shipped to them at the same time as the full set sent to Northwestern University. Both analytical laboratories received 3 randomized samples of each of the 8 types of samples. As a result, all analytic tests were performed under blinded conditions. At the laboratories, in accord with homeopathic manufacturer recommendations, samples were stored at room temperature away from direct sunlight [85]. Based on clinical standards and past research [86], laboratories were asked to minimize the exposure of the samples to any extraneous electromagnetic sources during storage.

Nanoparticle tracking analysis: As in our previous study [69], NTA was performed in triplicate on each of the samples using the NanoSight LM 10-HS, with software version 2.3 (NanoSight/Malvern, Malvern Worcestershire, UK) at the Northwestern University Keck Biophysics Core Facility, Evanston, IL USA. Particle size range detection is 10-1000 nm or larger per the manufacturer's manual. Advantages of NTA technology include (a) reducing the risk of sizing artifacts from evaluation of polydisperse mixtures of large and small particles, e.g., a problem to which dynamic light scattering is prone [87]; and (b) avoiding drying artifacts, e.g., to which transmission electron microscopy is prone, by retaining samples in as-manufactured liquid form for analysis.

Zeta potential measurements: The samples at Northwestern University were then locally moved from the NTA Keck Biophysics Core Laboratory directly to the Equipment Core Facility of the Simpson Querrey Institute at Northwestern University for additional characterization. The zeta potential assessments were performed in triplicate using a Zetasizer Nano ZSP (Malvern Instruments, Inc, USA) at 25°C with a scattering angle of 173°. Limits of particle size detection for this instrument per the manufacturer manual range from 0.3 nm to 10 μm.

Ultraviolet visible spectrometry (UV-Vis): Nanocomposix (San Diego, CA) performed the UV-Vis absorbance testing on all sample vials. The instrument was an Agilent 8453 UV-visible spectrometer. Testing was performed in a quartz cuvette with a 1 cm path length. Data reported encompassed the wavelength range of 190 to 1100 nanometers (nm).

Statistical analysis

Statistical analyses used Statistica Academic 12.5 software and included analyses of variance over all 8 possible types of samples (verum 6C, 30C, 200C; succussed controls 6C, 30C, 200C; unsuccussed controls/cork stoppers; unsuccussed controls/silicone stoppers), with post-hoc Tukey tests for subgroup comparisons when indicated. Planned comparisons were to compare triplicate tests on all verum and succussed control samples at each potency against unsuccussed controls as well as comparing verum and succussed control samples with one another at each of their respective three different homeopathic potencies.

Results

Nanoparticle tracking analysis

NTA revealed polydisperse nanoparticles at detectable average concentrations (particles/milliliter (ml)) in most samples other than the unsuccussed controls with silicone stoppers (Figure 1). To facilitate reporting the post-hoc tests, the following abbreviations are used: V6=verum *Gelsemium sempervirens* (Gels) 6C, V30=verum *Gelsemium sempervirens* 30C, V200=verum *Gelsemium sempervirens* 200C; SC6=succussed (Succ) control 6C; SC30=succussed control 30C; SC200=succussed control 200C; UC-C=unsuccussed controls with natural cork stoppers; UC-S=unsuccussed controls with silicone stoppers (Unsucc), where E=exponent of 10.

Over all samples for concentration of nanoparticles per milliliter, vial contents and stopper type samples were significantly different (F(7,58)=3.7, p=0.00225). Post-hoc Tukey tests showed that the UC-S had significantly fewer NPs than the succussed controls with cork stoppers at all 3 potencies (6C (p=0.01), 30C (p=0.008), 200C (p<0.01), with a trend toward fewer NPs in the UC-S than the verum Gelsemium 200C (with cork stoppers) (p=0.076) or the unsuccussed controls with cork stoppers (p=0.059).

Particle sizes also differed between types of contents and stoppers (Figure 2), with an overall mean of 95.99 SD 31.57 nm and a range over all samples between 73.56 nm (Unsuccussed Control with Cork stoppers) to 129.78 nm (Verum Gels 30C).

Over all samples for NP mean sizes, vial contents and stopper types were significantly different (F(7,58)=4.19, p=0.00085). Among the natural cork stoppered type of samples, post-hoc Tukey tests revealed that the Verum 30C samples contained significantly larger concentration-weighted mean size particles than did Succussed Controls at all potencies [SC6 (p=0.01), SC30 (p=0.02), SC200 (p=0.008)], and the UC-CORK stopper (p=0.0008) samples. There was a trend for Verum 200C samples to contain larger NPs than the UC-CORK stopper (p=0.08) samples. Compared with Verum 30C, the Verum 6C samples showed a trend toward being smaller sized (p=0.10).

To illustrate the polydispersity of the NPs, Figure 3 shows exemplar NTA particle sizes/relative intensity 3D plots for one of each type of sample and stopper vial tested.

Particle surface stability as indicated by negative zeta potential mean values was poorest in the UC-Silicone stopper samples (-5.6 mV) and best in the Verum Gels 200C (V200, -47.8 mV) (overall F (7,58)=18.7, p=0.00000) (Figure 4).

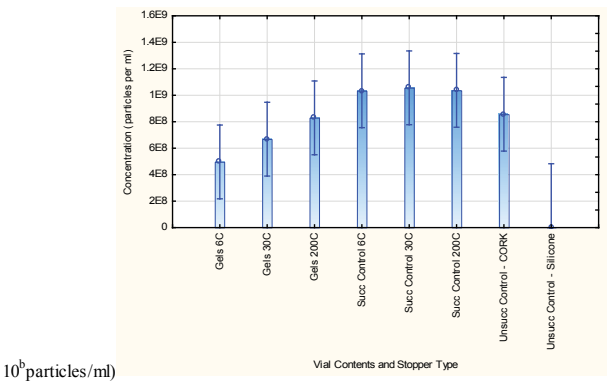

Figure 1: Particle concentration by vial contents and stopper type (F(7,58)=3.7, p=0.00225). All verums and succussed controls had natural cork stoppers. (Note: a x Eb= a x).

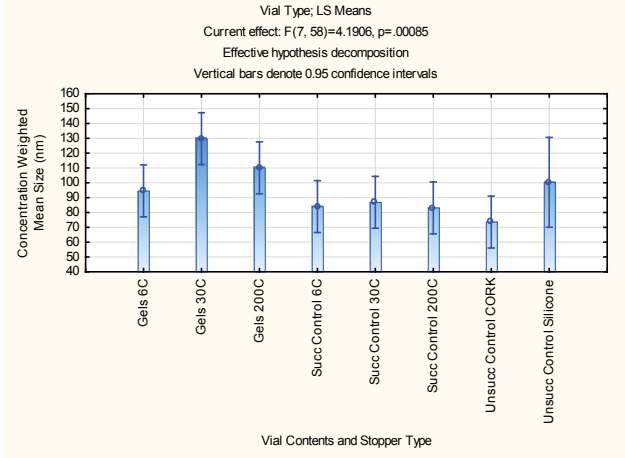

Figure 2: Concentration Weighted Mean Particle Size (nanometers, nm).

Post-hoc Tukey tests on the zeta potential data indicated that particles in the Unsuccussed Controls with Silicone stoppers (UC-Silicone) were significantly less stable than those in all of the sample types with cork stoppers, including V6, V30, v200, SC6, SC30, SC200, and UC-CORK (all comparisons, p<0.001). Zeta potentials for the Verum Gels 200C samples were significantly more negative than those in most of the other samples (p<0.05) except for a trend versus the Verum Gels 6C (p=0.09) and no difference from the SC30 (p=0.22, ns). Within the succussed controls, the SC30 exhibited a trend toward more negative zeta potentials than the SC6 samples (p=0.058).

Finally, for additional perspective to compare with the test vials described above, the contents of the same large glass ethanol jug source with phenol cap that provided the ethanol diluent used in the test vial samples were evaluated. The jug ethanol's particle concentration by NTA was 0.061E8; mean particle size was larger at 172 nm than the test vial particle contents; and jug ethanol's zeta potential exhibited poor stability (-5.58 mV) (similar to the NC-Silicone stopper samples' mean zeta potential).

Ultraviolet visible spectroscopy

All succussed and unsuccussed samples with cork stoppers exhibit absorbance in the 280-380 nm regions with similar integrated absorbance values. This absorbance is most likely due to organic compounds being extracted from the cork (*Quercus suber*) stoppers employed, as supported by the absence of absorbance in this region when silicone stoppers were used in one set of unsuccussed controls. The apparent peak around 202 nm in most samples likely reflects an artifact due to subtraction of a high solvent blank absorbance value from high sample absorbance values, as all samples showed high absorbance in the far ultraviolet region.

On statistical analysis, UV-vis (Figure 5) revealed that analytic laboratory dilution-corrected mean absorbance in the wavelength range of 200-400 nm was significantly higher for the succussed control 30C samples (with cork stoppers) as greater outliers than for all other types of vials (Overall F(7,4768)=200.5 p<0.00001; paired comparisons between SC30 and each other type of vial were all significant at p=0.000032; in addition, V30>SC6, p=0.026; SC200>Unsucc Control-CORK stopper,

p=0.03). Most samples had absorbance maxima at approximately a 200 nm wavelength. For the narrower wavelength band of 200-300 nm, the overall comparison across all types of contents and stoppers was again highly significantly, but with only SC30 samples exhibiting greater absorbance than all other samples (overall $F(7,2368)=161.4$, $p<0.0001$; SC30>all other sample types and stoppers, p=0.000032).

Within the narrower but higher wavelength band of 300-400 nm, Figure 6 shows that absorbance remained significantly greater for the Succussed Controls 30C (overall $F(7,2368)=189.59$, $p<0.0001$; with SC30> all other sample types (post-hoc Tukey tests, p=0.000032). The V30 also had significantly higher absorbance than did the SC6 and the UC-CORK (respectively, p=0.0004; p=0.008).

In addition, the Unsuccussed Controls with Silicone stoppers exhibited significantly lower UV-vis absorbance between 300-400 nm wavelengths, compared with all other types of samples (p=0.009 or less). On further subanalysis, the lower UV-vis absorbance between 300-400 nm wavelengths for the UC-Silicone samples was significant in comparison with all or most other types of samples, especially for wavelengths from 300-325 nm (UC-Silicone<all other sample types except SC6), 325-350 nm (UC-Silicone< all other sample types), and to a lesser extent, 350-375 nm (UC-Silicone<all except V6, SC6, and UC-CORK). Within the 375-400 nm wavelength band, the latter type of finding was significant only for UC-Silicone showing lower absorbance versus the SC30 (p=0.000032). Examining absorbance for wavelengths in 100 nm increments from 400-1000 nm, the highest absorbance for the SC30 samples and the lowest absorbance for the UC-Silicone samples persisted as significant findings against all other types of samples.

Although smaller in magnitude than the pattern for succussed controls across the three different homeopathic potencies, there was a similar low-high-low pattern of highest absorbance within the verum samples for V30 versus V6 and V200. Within the verum samples, the pattern first became significant at wavelengths of 400-500 nm (V30>V6, p=0.007 and V30>V200, p=0.005) and persisted in the 100 nm wavelength analyses through 900-1000 nm (V30>V6, p=0.000032; V30>V200, p=0.000042).

Figure 7 illustrates exemplar UV-vis spectroscopic graphs by wavelength for one of each type of sample and stopper vial tested. Apart from the maximum peaks close to 200 nm wavelengths in the different sample types, many samples exhibited smaller shoulders at wavelengths of approximately 290 nm and 330 nm. In contrast, the unsuccussed controls with silicone stoppers showed almost no absorbance between 300-400 nm, consistent with absence of the organic material otherwise seen in all of the verum *Gelsemium*, succussed control, and unsuccussed cork- stoppered samples.

Discussion

As in previous studies across multiple laboratories [44-47,49-52,67-69], the current data again demonstrate that traditional homeopathic materials (glass containers, natural cork stoppers, ethanol-water diluents) and/or manufacturing methods (serial dilutions followed by multiple succussions) generate nanostructures in liquid ethanol-water solutions (verums, succussed controls, and unsuccussed controls with cork stoppers). Succussions per se, even in ethanol-water solvent-only controls in glassware with corks (Succussed Controls), appear to generate the largest number of NPs, albeit with smaller, less stable particles compared with verum *Gelsemium* samples. Such findings could have biological relevance. For instance, other investigators have

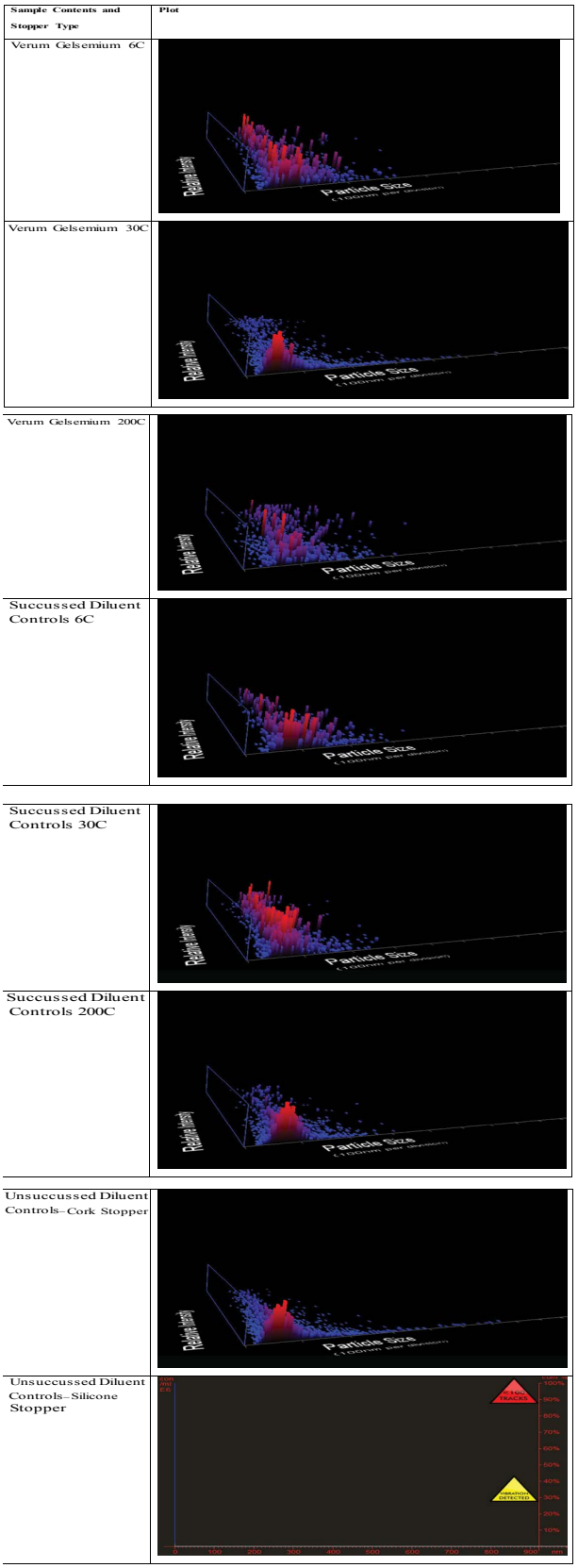

Figure 3: Particle Size/Relative Intensity 3D Plots for Exemplar Samples of Each Type Zeta Potentials.

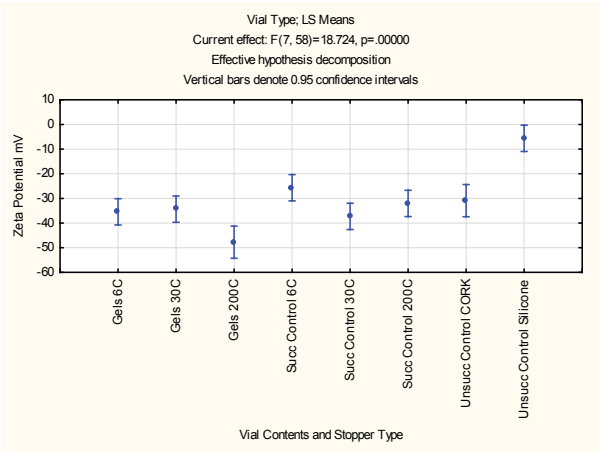

Figure 4: Zeta Potentials (mV, millivolts) by Vial Contents and Stopper Type.

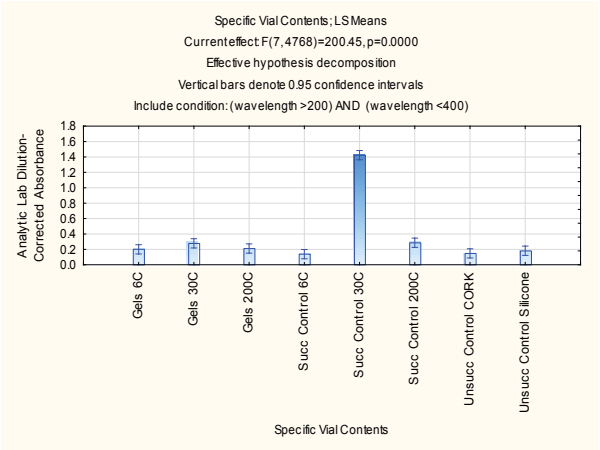

Figure 5: Analytic Laboratory Dilution-Adjusted UV-Vis Mean Absorbance in wavelength range of 200-400 nm.

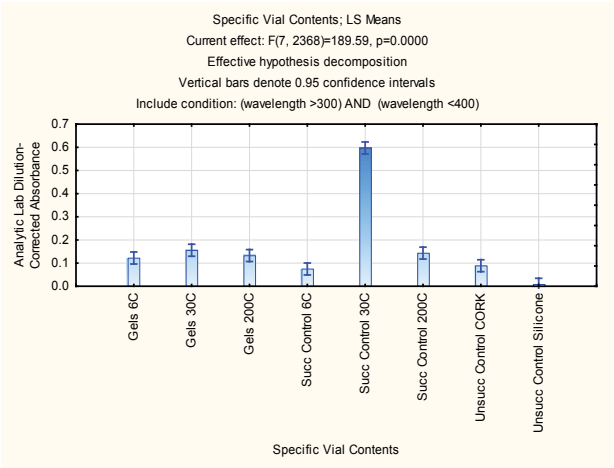

Figure 6: Maximum UV-vis Absorbance by Vial Contents and Stopper Type in the Wavelength Range 300-400 nm. Dilution correction was for sample dilution by analytic lab at the time of the UV-vis testing, not original manufacturer's preparation.

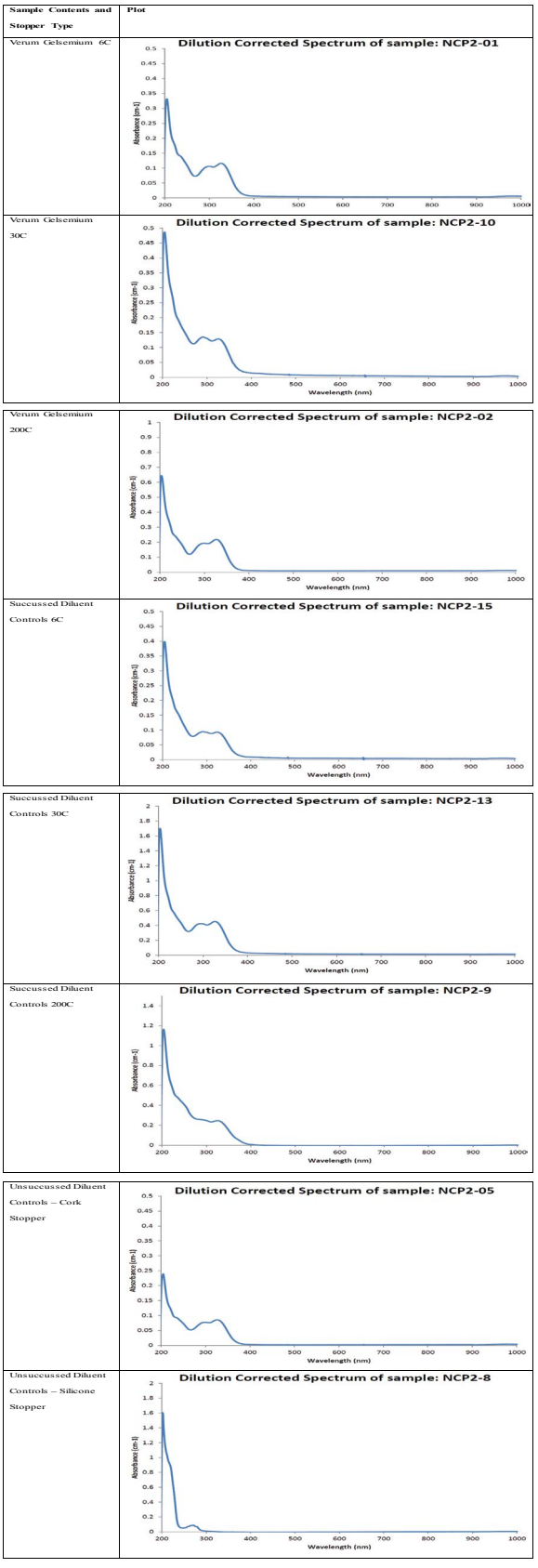

Figure 7: UV-Vis Spectroscopy Exemplar Graphs by Sample Contents and Stopper Type.

observed biological wound healing activity in fibroblast cell cultures for succussed solvent controls at a magnitude higher than that of unsuccussed controls, though less than that of verum combination botanical source HMs [88].

The verum *Gelsemium* HM samples in the present study were unique, even if not globally different from all of the control samples. NPs in the present verum *Gelsemium* samples differed in specific characteristics from the control samples. Such differences included (a) larger NP mean sizes (130 nm) in the verum Gels 30C potency compared with all of the succussed controls and the unsuccussed controls with natural corks; and (b) the most stable negative zeta potentials for the verum Gels 200C potency (-47.75 mV) compared with other types of samples. Both of the latter findings suggest the possibility that the verum botanical material and the natural cork stopper contaminants from *Quercus suber* could have served to foster formation of larger, specific, stable verum nanostructures than in the controls [16-18]. Plant extracts including some from oak tree sources, are well established agents for generating nanoparticles from metal or silica precursors in solution [2,16,18,22,76,89-92].

For context, in our previous study [69] of homeopathically-prepared silver (*Argentum metallicum*) at 6C, 30C, and 200C, the 200C verum NPs were highest in particle counts. All of the silver-source verum potencies were significantly larger sized, with more negative zeta potential values than the unsuccussed controls with natural corks (no silicone stopper condition was done in the prior study). In fact, the present patterns of zeta potentials across the 6C to 30C to 200C potencies in the current *Gelsemium* study for verums versus succussed controls were similar to those in our previous study of homeopathic silver verum medicine potencies and their respective succussed controls [69].

The primary difference in manufacturing methods between *Argentum metallicum* silver- and *Gelsemium*-derived HMs is that the bulk silver, but not the bulk *Gelsemium*, was triturated in dry lactose (mechanical grinding or milling) extensively to make the initial potency prior to introduction into the homeopathic serial dilution-succussion process. Trituration or grinding may have generated a given sized core silver NP onto whose surface the other materials, including triturated lactose, in solution adsorbed. In contrast, plants like *Gelsemium* enter into a liquid phase immediately as ethanolic herbal extracts (mother tinctures) for subsequent dilution-succussion processing into higher potencies. Core NPs from plant materials might be more variable in sizes and shapes, susceptible to further size reductions with additional succussions [50] compared with lactose-stabilized metal source core silver NPs. At the same time, intense ball milling or agitation in liquids can generate increasingly smaller silica NPs from its larger silicate precursors or from rice husk ash [59,60,93].

The present findings on botanical *Gelsemium* source HMs raise testable hypotheses for further study. For example, does the HM verum source material seed specific NP formation, generating a core for unique hybrid nanostructures? If so, silver and other metal-derived particles may be more size-consistent [44] than *Gelsemium* exosomes in serving as such a core across homeopathic potencies. Then the NP hybrid shell would form from adsorption of silica [94] made from silicates released over successive potencies by repeated succussions of glassware [49,53,95] and any other materials in the ethanol-water diluent solution, [51,96] e.g., including cork (*Quercus suber* extract) proteins and nucleic acids from natural cork stoppers (if used during manufacturing and storage [97]. At the same time, silica NPs could also form from the silicates succussed into solution, [21,98] with their nanostructures templated [99] and/or directed in part by the *Gelsemium* extract in the verums [2,21] as well as by the *Quercus suber* extract from the corks in vials with cork stoppers, cf. [17].

The more stable negative zeta potential values for the *Gelsemium* verums in this study and in our previous study of silver HMs versus controls [69] are consistent with adsorption of botanical and related organic materials [2] and perhaps silica [94] onto the nanoparticle surfaces. In turn, the resulting hybrid nanomaterials may serve as the solute-induced nanostructures and aggregates reported in homeopathic medicines by independent laboratories [49,67,100]. Any subsequent effects of these nanostructures on the structure of surrounding water at the nanoscale deserves additional study [45,100]. Studies using various technologies suggest a complex ordering of the surrounding fluid medium in contact with homeopathically-prepared medicines [73,101,102]. Compared with controls, disrupting the order within verum homeopathic materials in liquid form releases a measurable excess of heat and/or light [70,73,103].

Organic materials, e.g., food sources (herbs such as ginger, vegetables such as carrots, fruits such as grapes, or milk), can generate their own nanoscale structures, i.e., exosomes, with biological effects on animal tissues [104-106]. Notably, one study of HMs from botanical extracts found that repeated homeopathic succussions can progressively reduce the size of the plant herbal extract particles in solution into nanosizes as small as 14 nanometers [50]. Such an observation overlaps in part findings from studies in conventional nanotechnology on the ability of another method of agitation of solutions, i.e., sonication, to generate nanoparticles from organic materials [59] or salt solutions (NaCl, KI) [23]. In the present study, exosomes from the ethanolic extract of *Gelsemium* could have formed and transferred within the low potencies (6C) from the verum extract and from the *Quercus suber* materials released into solution of all cork-stoppered vials.

For research on HMs, zeta potential is emerging as a key variable indicating particle stability from surface properties of a given type of nanoparticle. The surface properties of NPs can change with adsorption of proteins in solution [107-109]. Consequently, adsorption of plant proteins from verum *Gelsemium sempervirens* and/or from *Quercus suber* released by the cork stoppers into verums and the controls stoppered by natural corks could have modified the NP zeta potentials measured. Ives et al. [53] demonstrated that multiple succussions of plain solvent per se in borosilicate glassware can change the pH of the solution, initially in an alkaline direction by releasing sodium that forms sodium bicarbonate. Further succussions later stabilized the solution closer to neutral pH [53]. Protein adsorption, ionic environment, and changes in pH are known to modulate NP zeta potential values [107-110] as well as foster transitions between gel and nanoparticle states [111]. Quartz (silica-based glassware), but not polystyrene, cuvettes reportedly participate in propagating and amplifying the homeopathic "signal" in HM potencies [112].

If HMs involve adsorption of mixed types of nanomaterials onto one another as previously proposed [64], zeta potential measurements may be one useful technology for assessing successful generation of stable NPs in HMs from particular source and manufacturing materials. Zeta potentials, pH measurements, and other fundamental variables that change or reflect changes on NP surfaces may also assist in studying the impact of additional variations of the container and stopper materials [95,113] and solvent reagents [114,115] in making HMs.

The ability of glass, rubber, plastic and other packaging materials to

introduce bioactive particles and nanostructures into liquid medicines is well known, even in conventional pharmacy [54,95,113,116,117]. Silica NPs from glassware can induce protein aggregation from any proteins they contact in solution to mobilize immune reactivity [54,95,113]. Notably, silica nanostructures can survive drying [94,118-120]. At the same time, most homeopathic manufacturers ultimately pour or spray and dry their final liquid potency onto lactose sugar pellets for longer-term storage and administration. Lactose is a known reducing and capping agent for nanoparticle generation from precursor materials as well as carrier for other nanostructures [67,121-127].

Prior UV-vis spectroscopy tests on *Gelsemium* botanical extract at an extremely low potency such as 1C apparently reveals an absorption maximum around 210 nm, with absorption shoulders at 280 and 330 nm [32]. However, even by a greater dilution factor of *Gelsemium* extract at 3C potency (still a low potency in homeopathy), the UV-vis absorbance signal becomes greatly attenuated. Of note, the *Gelsemium* botanical extract source materials began as an ethanolic herbal extract in the present study as compared with the lactose-triturated (milled) silver source materials in our previous study of HMs [69]. Thus, lactose was not involved in making the current verum samples.

Ethanol per se could be a factor in some of the UV-vis peak absorbance findings. The 95% ethanol solvent per se reportedly has an absorbance lower limit of 205; while water has an absorbance lower limit of 190 (http://www.chem.ucla.edu/~bacher/UV-vis/uv_vis_tetracyclone.html.html). On the other hand, the unsuccussed control vials with silicone stoppers in this study, which contained the same ethanol-water solvent as other sample types, had significantly lower average UV-vis absorbance at wavelengths between 300-400 nm versus most other samples, including the unsuccussed controls with cork stoppers. Ethanol concentration can govern particle sizes of silica NPs formed in simple synthesis systems with sonication [93,128].

As plant material released by the corks [2,21,22,129,130], *Quercus suber* extract could also serve as a botanical reducing and capping agent [16,17] at more potencies than could the serially-diluted HM source botanical *Gelsemium* to generate silica nanoparticles from precursor materials such as silicates or silver NPs from any ionic silver released from the source verum *Argentum Metallicum* NPs in our previous study. As a result, we had added the set of unsuccussed controls with silicone stoppers to the present design to compare with the natural corked vials in the present study.

However, as the verum source material *Gelsemium sempervirens* in the present study was, like the natural corks, also a plant-derived extract rather than a metal, we anticipated greater difficulty in distinguishing plant medicine verums from succussed controls with cork stoppers. Identifying a specific biological assay for activity of the different samples might help distinguish between *Gelsemium* versus *Quercus suber*-related effects on recipient living systems [16,17,28,29,32,35,74,75]. Including silicone stoppered controls in future NP characterization studies of other HMs, including metal, animal, and other plant sources, would be helpful.

The present set of findings for the unsuccussed solvent controls with silicone stoppers is revealing. In the current study, the unsuccussed controls with silicone stoppers had the fewest (almost no) nanoparticles. Like the phenol-capped jug ethanol diluent, particle zeta potentials in the UC-silicone vial samples were closest to 0 on average (-5.63 mV), with the poorest stability of any type of sample vials in this study. The UV-vis spectroscopic absorbance magnitude for wavelengths in the 300-400 nm region and up to 900 nm was significantly lower for the UC-silicone vials than for most other types of samples, including the UC-CORK vials, suggesting the presence of different materials in solution for the UC-silicone controls versus the corked samples of verums, succussed and unsuccussed controls.

The observation of greater UV-vis absorbance in the V30 (verum 30C), versus both lower V6 (6C) and higher V200 (200C) verum potencies is notably similar to the more exaggerated pattern of greater absorbance in the SC30, succussed controls 30C, versus both lower SC6 (6C) and higher SC200 (200C) succussed control potencies. These data suggest that there is a larger release of bulk and other sizes of materials from the glassware inner walls and corks when they have undergone more total succussions (i.e., 30C vs 6C) and/or are newer and earlier in the Korsakovian re-use of the same container and stopper materials (i.e., 30C vs 200C).

Overall, the current data indicate that homeopathic manufacturing methods (multiple succussions or intense agitation) and traditional materials (GELS plant extract, ethanolic solvent, glass vials, natural cork stoppers) can generate nanomaterials not seen in unsuccussed silicone-stoppered glass vials of ethanolic solvent alone. Contrary to claims of skeptics, such findings indicate that HMs made with materials and methods described by Hahnemann, are not "just" the same as placebo, i.e., not just plain solvent (water or ethanol) in a bottle.

Biotherapeutic implications

At the same time, do the findings have implications for biological effects and therapeutics of HMs? The present data create a nanotechnology context for the extensive prior research literature on biological and behavioral effects of homeopathic *Gelsemium sempervirens* [26-32]. In homeopathic clinical practice, choice of a specific HM such as GELS is based upon clinical pattern matching the integrative biopsychosocial symptom picture of the affected patient to the previously-documented effects of specific source materials on living systems [42]. The goal is to induce adaptive bioplasticity responses that reverse the direction of pre-existing acute or chronic disease processes [131-136]. Hahnemann also focused on eliciting the organism's "counter-action" to the correctly-matched HM [43]. He explicitly tried to minimize the drug toxicity of his day by using low doses while still eliciting the counter-action responses, i.e., biological adaptation in contemporary scientific terms. The clinical approach of homeopathy relies upon the reaction of the organism or cell to the agent to evolve across the system, not upon the direct local actions or higher dose-response effects typical of conventional pharmaceutical agents.

For lower potency (less diluted, less succussed) HMs made from plants such as GELS or other botanical agents, multiple succussions could generate nanosized structures as small as 14 nm [50]. As noted above, nanosized plant exosomes might play a direct biological signaling role with associated adaptive responses. Exosomes can directly engage cell signaling mechanisms in the body [104,137-139]. However, higher homeopathic potencies such as 30C or 200C (i.e., more diluted and succussed forms) of GELS or other HMs may need to involve source-derived electromagnetic or optical signaling by the homeopathic agent to the cell danger response pathways of the recipient to elicit adaptive responses [66,71,72,140].

In terms of possible biotherapeutic mechanisms for both lower and higher potencies, investigators have proposed low-dose models of nanoparticle-initiated hormesis, biological signaling, and other endogenous bioamplification phenomena in cells or organisms as complex adaptive systems [64,140-143]. Hormesis is a now widely-

recognized low-dose elicited phenomenon that includes low dose stimulation and high dose inhibition, i.e., a marker of biological plasticity [144-148]. Nanoparticles of many different types are capable of initiating hormesis [132-134, 149].

Moreover, others have demonstrated thermal changes [70,100,150], as well as optical [72,73,151] and electromagnetic [70,71,100,152] signal emissions from HMs. Such HM-related information could provide its own unique signal to set endogenous adaptive events into motion. Still others propose quantum macro-entanglement [153,154] or aqueous nanodomains [155] to account for effects of the higher homeopathic potencies in HMs.

Similar properties are consistent with well-known emergent properties of nanomaterials of various sources, sizes, shapes, and surface chemistries, including thermal, electromagnetic, optical, and even quantum mechanical properties for certain very small NPs (<10nm in size) [156-161]. Furthermore, small NPs (e.g., 10-150 nm size range of viruses) [162] can serve as virus-like particles and individually-salient cell danger signals to activate immune system and other biological responses of the endogenous cell defense response network [66,163,164].

Conclusions

The current data support the conclusion that (a) homeopathic manufacturing materials and methods generate nanoparticles; (b) the verum *Gelsemium* HMs contained NPs different for specific particle characteristics from those in control samples (e.g., largest size NPs were in verum *Gelsemium* 30C; most stable negative zeta potential value was in verum *Gelsemium* 200C); (c) compared with silicone-stoppered unsuccussed controls, the natural cork stoppers may have added varying amounts of organic plant material (presumably unfiltered bulk *Quercus suber* extract) into solutions of the verums, succussed controls, and unsuccussed controls-CORK. Successions per se generated the largest number of nanoparticles in the succussed controls within this study, possibly related to release of not only cork extract, but also silicates from the glass container inside walls. Unsuccussed solvent in glass vials with silicone stoppers differed from most of the other types of samples with the findings of fewer NPs and poorer zeta potential-assessed stability of particles that were present.

The present study requires replication and extension to additional HM source materials [165]. Nonetheless, these data represent further evidence for the presence of relatively stable nanostructures in HMs prepared in borosilicate glassware with successions and traditional cork stoppers, as compared with unsucussed diluent controls with silicone stoppers [69]. Future studies should further examine from a nanotechnology perspective the relative contributions of each of the (a) reagents (source material, lactose when used for mechanical milling and mixing, ethanol, water), (b) processing/packaging materials (container sizes, shapes, materials (borosilicate glass, soda-lime glass, plastic) [53], stopper materials (natural cork [69], silicone, rubber)), and (c) methods (trituration/milling and serial dilutions followed by succussions) involved in traditional homeopathic manufacturing and their impact on the biological activity [38,65,88,141,166-168].

Variability in particle surface properties and materials involved in NP stabilization might contribute to previous findings of variability over time in UV-vis findings on HMs versus controls [85,169,170]. Finally, subsequent research should also evaluate the nanostructure and surface stability characteristics of HMs as correlates of their biological activity [26,27,31,32,132,133,171-174] in comparison with succussed and unsuccussed controls [65,78,141,175]. Prior research has demonstrated low-dose hormetic effects triggered in recipient organisms by NPs of various source materials [132-134,149,171]. Consequently, examining NPs in various HMs, especially at higher homeopathic potencies such as 30C and 200C (i.e., low conventional doses), for these types of low-dose-dependent, nonlinear dose-response patterns, rather than direct pharmacological ligand-receptor effects, merits further investigation [31,131,142,176].

Disclosure

Dr. Bell is a consultant to Standard Homeopathic/Hylands Inc, a U.S.-based manufacturer of homeopathic medicines. None of the company's products were evaluated in this study, and the company did not provide any financial assistance for the completion of the project or the publication of this paper. Drs. Muralidharan and Schwartz have no conflicts of interest to report.

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