

Embryonic Zebrafish Model - A Well-Established Method for Rapidly Assessing the Toxicity of Homeopathic Drugs

- Toxicity Evaluation of Homeopathic Drugs Using Zebrafish Embryo Model -

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Key Words

homeopathic medicine, homeopathy, traditional medicine, therapeutic use, transmission electron microscope, zebrafish toxicity

Abstract


Objectives: Advancements in nanotechnology have led to nanoparticle (NP) use in various fields of medicine. Although the potential of NPs is promising, the lack of documented evidence on the toxicological effects of NPs is concerning. A few studies have documented that homeopathy uses NPs. Unfortunately, very few sound scientific studies have explored the toxic effects of homeopathic drugs. Citing this lack of high-quality scientific evidence, regulatory agencies have been reluctant to endorse homeopathic treatment as an alternative or adjunct treatment. This study aimed to enhance our insight into the impact of commercially-available homeopathic drugs, to study the presence of NPs in those drugs and any deleterious effects they might have, and to determine the distribution pattern of NPs in zebrafish embryos (*Danio rerio*).


Methods: Homeopathic dilutions were studied using high-resolution transmission electron microscopy with selected area electron diffraction (SAED). For the toxicity assessment on Zebrafish, embryos were exposed to a test solution from 4 - 6 hours post-fertilization, and embryos/larvae were assessed up to 5 days post-fertilization (dpf) for viability and morphology. Toxicity was recorded in terms of mortality, hatching delay, phenotypic defects and metal accumulation. Around 5 dpf was found to be the optimum developmental stage for evaluation.

Results: The present study aimed to conclusively prove the presence of NPs in all high dilutions of homeopathic drugs. Embryonic zebrafish were exposed to three homeopathic drugs with two potencies (30CH, 200CH) during early embryogenesis. The resulting morphological and cellular responses were observed. Exposure to these potencies produced no visibly significant malformations, pericardial edema, and mortality and no necrotic and apoptotic cellular death.

Conclusion: Our findings clearly demonstrate that no toxic effects were observed for these three homeopathic drugs at the potencies and exposure times used in this study. The embryonic zebrafish model is recommended as a well-established method for rapidly assessing the toxicity of homeopathic drugs.

Received: Sep 07, 2016 Reviewed: Dec 01, 2016 Accepted: Dec 01, 2016

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 This paper meets the requirements of KS X ISO 9706, ISO 9706-1994 and ANSI/NISO Z39.48-1992 (Permanence of Paper).

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1. Introduction

Although in recent years we have witnessed a new beginning of interest in homeopathy, the reliability of its main principles, the 'simile' and the 'dilution' of medicines, is yet to be demonstrated experimentally, and few studies have been conducted to understand its underlying mechanism(s) [1-3]. These few efforts have borne fruit in that they have generated several hypotheses, but none of them have been sufficiently validated empirically, with results ranging from the scientifically preposterous "memory of water" to the prodigious hand waving around quantum physics [4]. With the emergence of new tools provided by nanoscience and nanotechnology, new research options have been opened. The latest such attempts using these new tools found that extremely diluted homeopathic solutions did not contain the solute, but in fact contained the starting material in the forms of nanoparticles (NPs) and aggregates that were detectable by using electron microscopy, atomic spectroscopy, fluorescence spectroscopy, and cyclic voltammetry [5, 6]. Based on the results of the above studies, homeopathy is, in fact, a form of nanopharmacology [7]. Through a specific pharmacological process of potentization/tituration, medicinal substances are broken down in a grinding machine into NPs. However, whether the homeopathic effect depends on the presence of the NPs is still not known. Recent research has shown that remedies can carry numerous NPs, even up to a 200CH potency, and that homeopathy is not "just water". Another hypothesis says that solutions of homeopathic drugs are ultra-molecular dilutions of the starting material (above Avogadro's number). However, as above, whether these NPs are completely responsible for homeopathy's therapeutic effects is still unknown. Perhaps, they are. Although many people believe that highly diluted homeopathic medicines, which are recommended by professionals, may be safe and unlikely to produce any adverse action, drawing precise conclusions from the reported literature in this field is difficult. This could be due to the low methodological quality of the reports claiming possible adverse effects [8] or to the many comments coming from the uproar over a critical controversy [9]. That the raw materials for many homeopathic medicines are known to be poisons and that other studies have found that homeopathic drugs containing NPs exert biological effects on the body are both true. Even though homeopathic medicines are generally not likely to cause or lead to harm when dispensed appropriately, the toxicological outlook should not be ignored, specifically when using low concentrations of a risky starting material. Every toxicologist knows that a substance is poisonous only in a certain dose.

In the past few years, the number of *in vitro* and *in vivo* studies to evaluate the pharmacological kinetics or efficacy of some homeopathic remedies has been increasing [10, 11]. Despite the increasing popularity of homeopathy in biological applications, *in vitro* and *in vivo* toxicity studies on homeopathic drugs are still lacking. So far, no studies have investigated the toxicity of homeopathic drugs by using the zebrafish model. Our general working hypothesis is that using the zebrafish model should help to clarify some of the mechanisms of action of these tradi-

tional medicines.

Over the last decade, the zebrafish (*Danio rerio*) model has come to be recognized as a unique vertebrate model for assessing drugs *in vivo* with respect to a wide range of toxicological and safety pharmacological evaluation, because of its sequenced genome and highly-conserved genetic pathway between zebrafish and human [12-14]. This model is utilized for high turnout toxicity screening of diverse compounds. This whole-animal model may be useful as a rapid, high-throughput, low-cost assay for toxicity screening of compounds in the early developmental stages of Zebrafish [15]. Recent studies reported that the zebrafish embryo model has good predictivity for the toxicity and the teratogenicity of compounds in rodents [16-19].

The aim of this study was to investigate the possible existence of NPs in homeopathic medicines, and if possible, to examine whether this suffices to qualify homeopathy as a form of nanomedicine. Another aim was to illustrate the embryonic toxicity in zebrafish embryos exposed to homeopathy drugs for 4 - 96 hours post fertilization (hpf). To investigate the toxic effects in zebrafish embryos exposed to homeopathy drugs, we conducted a sequence of assessments, including embryonic mortality, the hatching rate, the presence of malformations, and whole-embryo cellular death. Taking embryonic toxicity as the indicator for evaluating homeopathic toxicity will be beneficial for studies on homeopathy and safety evaluations.

2. Materials and Methods

Medicines (potentized hydro alcoholic solutions) with different potencies (30CH and 200CH) based on *Calcearia carbonica*, *Argentum metallicum* and *Sepia* were obtained from Schwabe India (authorized producing house in Mumbai certified by Good Manufacturing Practices (GMP) and International Organization for Standardization (ISO)). The procured drugs were colorless, odorless, pre-sterilized and endotoxin free. The remedies were stored in brown-colored glass containers at room temperature away from sunlight.

For the ultraviolet-visible spectrometry (UV-Vis) characterization of the homeopathic drugs, their particle sizes were evaluated by using an Epoch Microplate Spectrophotometer (BioTek-SN-141215F) to monitor the visible absorption band. Data reported encompassed the wavelength range from 190 to 1100 nanometers (nm) [20]. For the nanoform characterization of the homeopathic drugs, the sizes and the morphologies of the homeopathic drugs were determined by using field emission gun - transmission electron microscopy (FEG-TEM) (Tecnai G2, F30 magnification 58 X 1 million X (300 kV)). Carbon-coated grids were immersed in the sample and left to dry prior to observation. The selected area electron diffraction (SAED) ring patterns were examined using high-resolution TEM (HR-TEM).

All animal experiments were conducted with prior approval from Mahatma Gandhi Missions's Medical College's Institutional Animal Ethics Committee. All procedures for the maintenance and care of the zebrafish were

in accordance with procedures described in the zebrafish book [21, 22]. Zebrafish embryos were collected 4 - 5 hpf from the zebrafish aquarium at the zebrafish facility of MGM Central Research Laboratory, MGMIHS, Navi Mumbai, and were staged according to standard procedures. For the toxicity studies, six groups were homeopathically treated as follows: *Calcarea carbonica* at 30CH and 200CH, *Argentum metallicum* at 30CH and 200CH, and *Sepia* at 30CH and 200CH. An potentized hydro alcoholic solution and an untreated control group of fertilized eggs were used as a control.

For the treatments with the above drugs, three sets of freshly prepared fertilized eggs were used, with 10 eggs being placed in ten individual wells of a 24-well plate. The homeopathic drugs treatment began at 24, 48 and 72 hpf for all 3 sets of embryos respectively. For the 24 hpf exposure set, eggs in the wells were exposed to the above-mentioned drugs at 24 hpf when the eggs had developed normally through different stages like blastula, gastrula and segmentation. For the 48 hpf and the 72 hpf exposure sets, the embryos in individual wells were exposed to the aforesaid concentrations of the homeopathic drugs at 48 and 72 hpf when the eggs/embryos had developed normally past the pharyngula stage and hatching period, respectively. As a control, an untreated control group of 30 fertilized eggs and potentized hydro-alcoholic solution were examined in parallel. Observations for any adverse effects due to treatments with the homeopathic drugs were made on zebrafish embryos and larvae and were documented at specified time points ($t = 6, 12, 24, 48, 60, 72, 84$ and 96 hours). The endpoints used to assess developmental toxicity comprised embryo/larva survival and hatching rate. Malformations among the embryos and the larvae from both the control and the treated groups were described and documented using a stereo microscope (Motic SM3-168 series). Dead embryos/larvae were removed immedi-

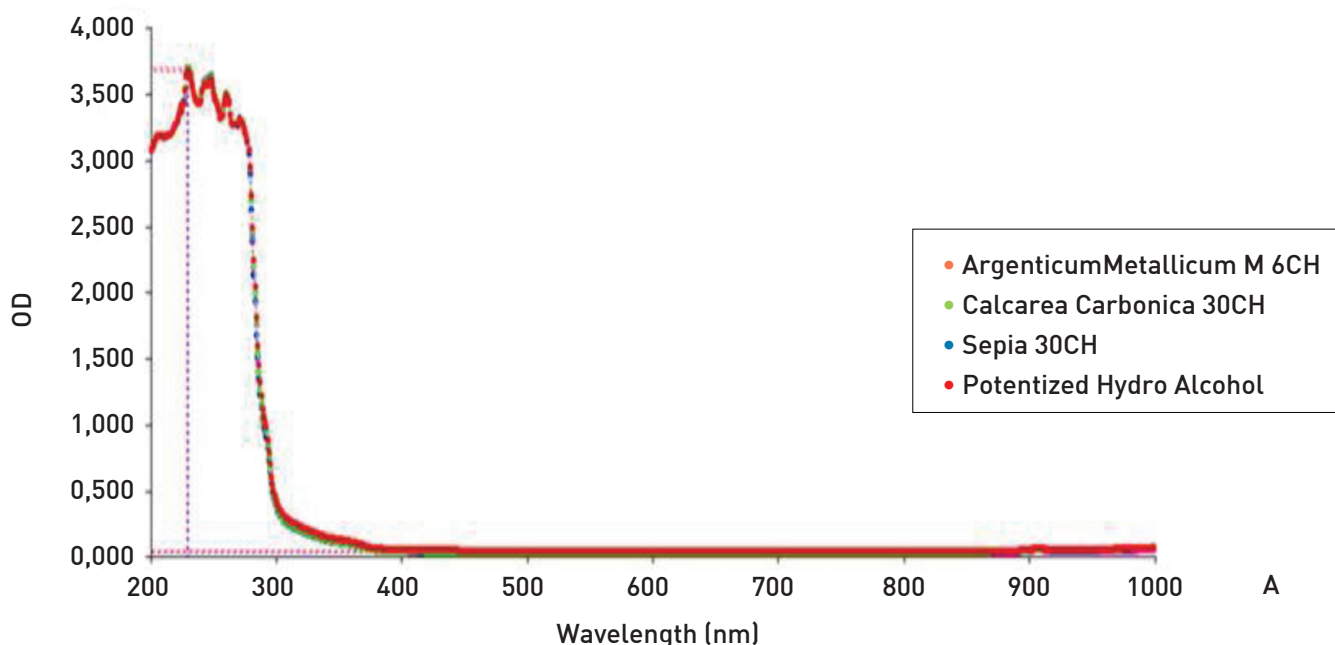
ately once they had been observed. Tests were performed in triplicate.

To investigate the role of apoptosis in the toxicity of homeopathy drugs at different strengths, we used acridine orange (AO) staining of the drug-treated and -untreated embryos [23]. AO stains cells with disturbed plasma membrane permeability, so it preferentially stains necrotic or late apoptotic cells whereas normal cells are impermeable to AO. Then, embryos were rinsed three times with phosphate buffer solution (PBS) and incubated in a solution of AO at 5 $\mu\text{g}/\text{mL}$ for 30 minutes in darkness at 28°C, which was followed by three rinses in PBS. Embryos were examined using fluorescence microscopy (EVOL FL Auto-Life Technologies, AMAFP1000).

3. Results

The properties of NPs change, depending upon their size. Thus, the size-dependent evolution of semiconducting NPs must be known to evaluate the properties of the material properly. UV-visible absorption spectroscopy is extensively used to observe the optical properties of nano-sized particles, and as a result, new evidence is emerging on the quality and the properties of homeopathic medicines or their remedies. For these reasons, we determined the UV-V absorption spectra for three homeopathic drugs with different potencies (30CH, and 200CH), and the spectra are shown in Fig. 1. Specifically, the UV-Vis spectral graphs revealed a large peak absorbance signal close to a wavelength of 230 nm, with smaller peaks through approximately 350 nm in wavelength, which were notable in all samples, as well as in the potentized hydro alcohol control.

Calcarea carbonica, *Argentum metallicum*, and *Sepia* at dilutions of 30CH and 200CH were analyzed using bright-field TEM. The electron micrographs clearly showed the



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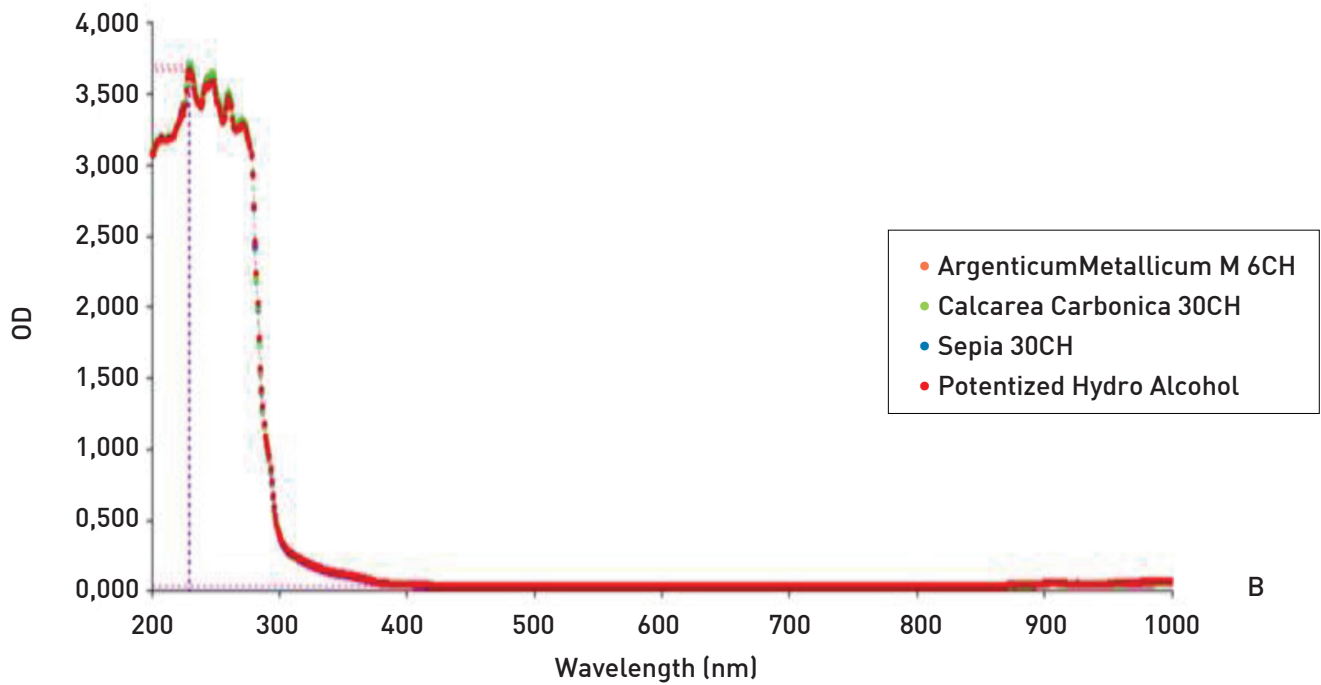


Figure 1 UV-Vis absorption spectra for homeopathic drugs with (A) 30CH and (B) 200CH potencies.

UV-Vis, ultraviolet-visible spectrometry.

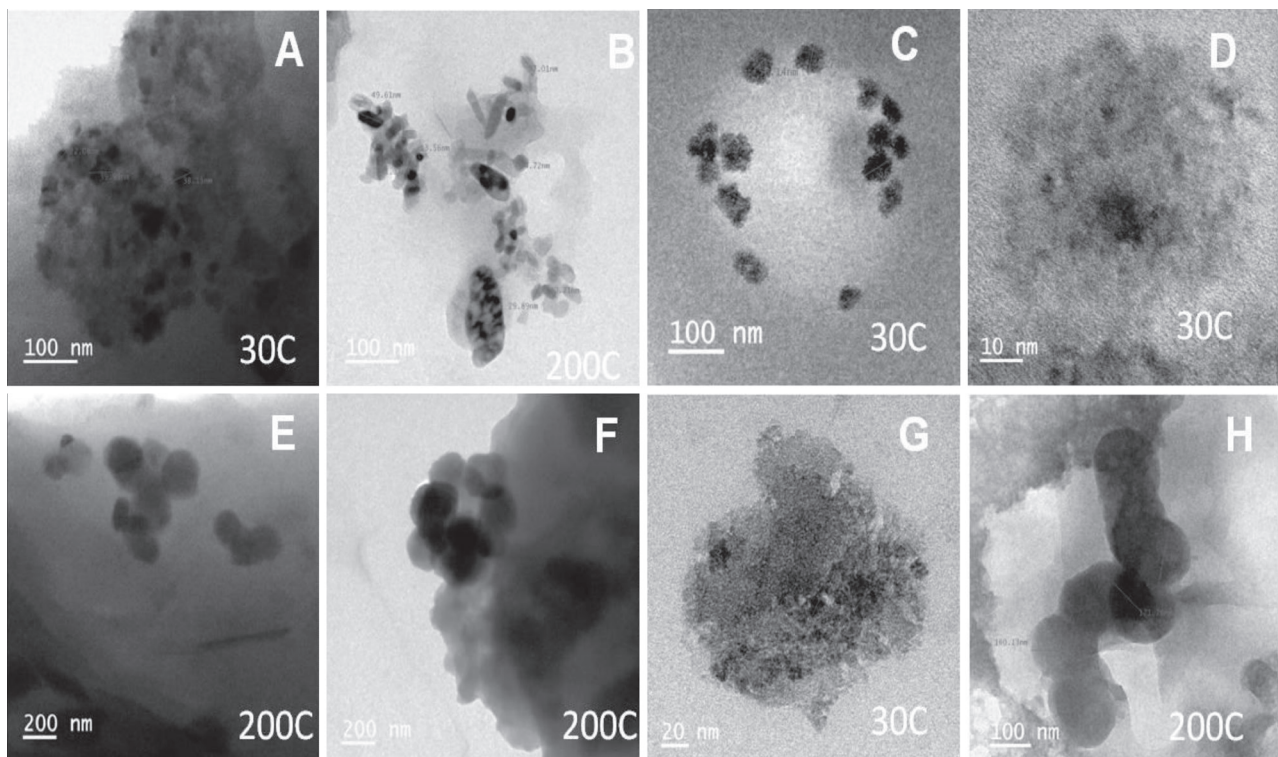


Figure 2 TEM of homeopathic medicines showing NPs and aggregates: (A) *Calcarea Carbonica* at 30CH, (B) *Calcarea Carbonica* at 200CH, (C, D) *Argentum Metallicum* at 30CH, (E, F) *Argentum Metallicum* at 200CH, (G) *Sepia* at 30CH, and (H) *Sepia* at 200CH.

TEM, transmission electron microscopy; NPs, nanoparticles.

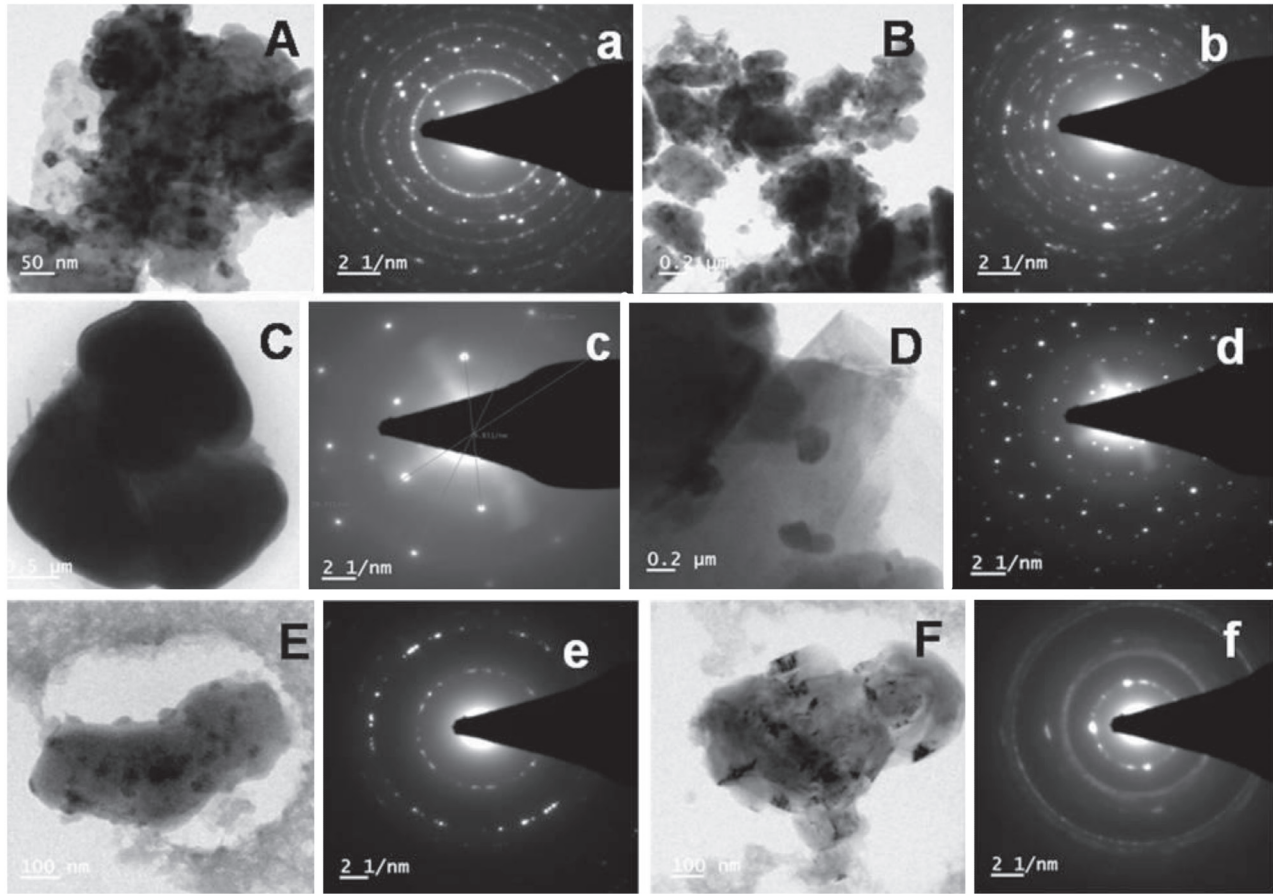


Figure 3 Bright-field TEM image and corresponding SAED patterns of NPs and conglomerates: (A, a) *Calcarea Carbonica* at 30CH, (B, b) *Calcarea Carbonica* at 200CH, (C, c) *Argentum Metallicum* at 30CH, (D, d) *Argentum Metallicum* at 200CH, (E, e) *Sepia* at 30CH, and (F, f) *Sepia* 200CH.

TEM, transmission electron microscopy; SAED, selected area electron diffraction; NPs, nanoparticles.

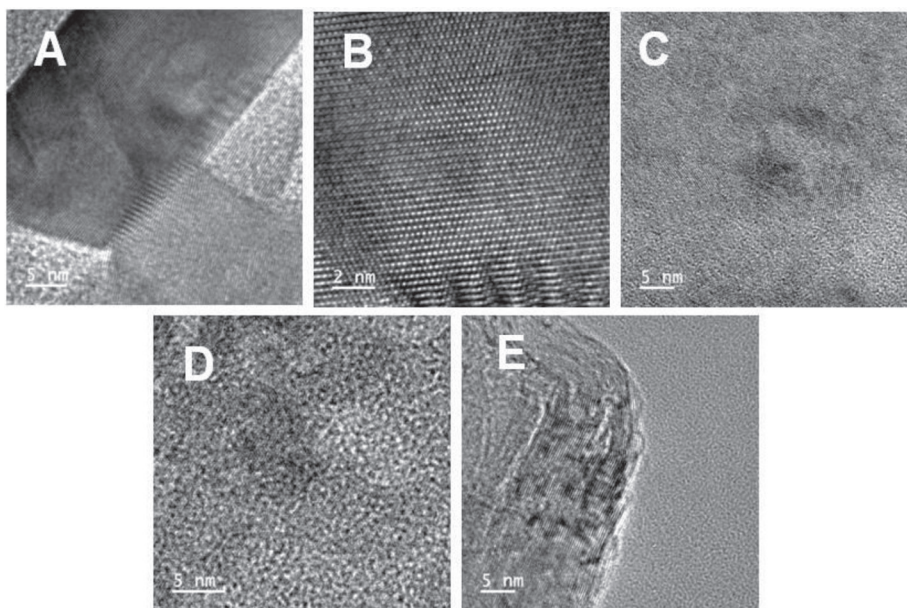


Figure 4 HR-TEM images of the homeopathic medicines showing various degrees of crystallinity: (A, B) *Calcarea Carbonica*, (C) *Argentum Metallicum*, and (D, E) *Sepia*.

HR-TEM, high-resolution transmission electron microscopy.

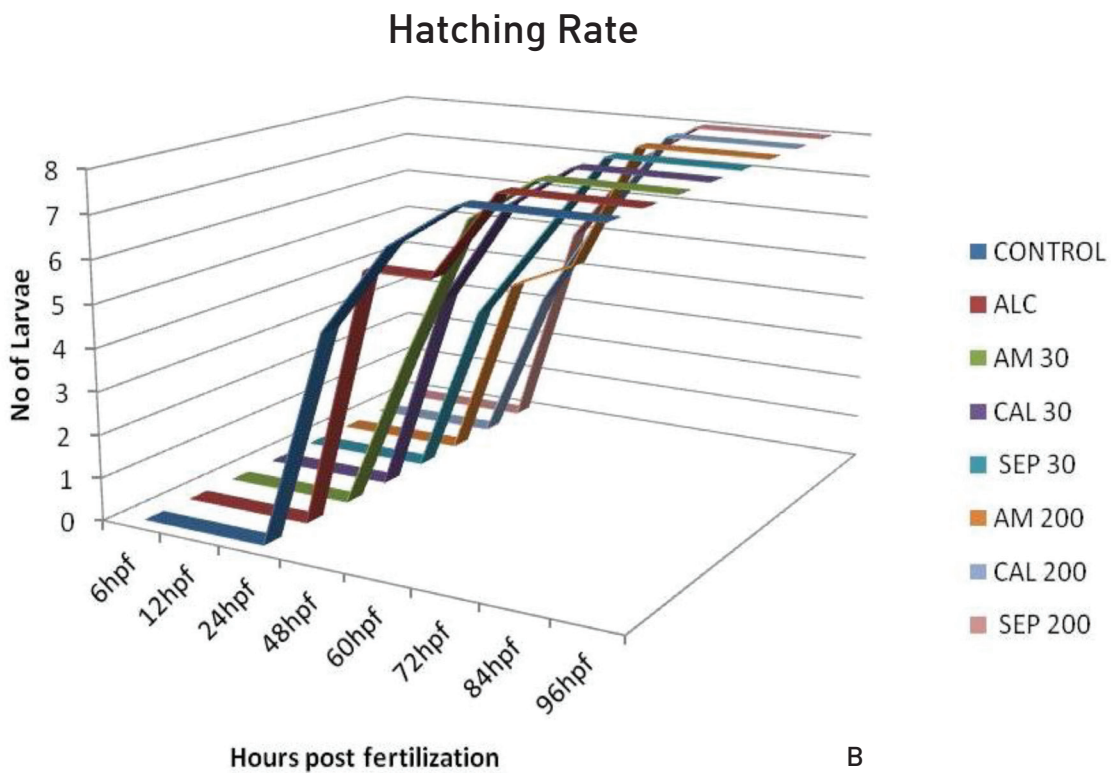
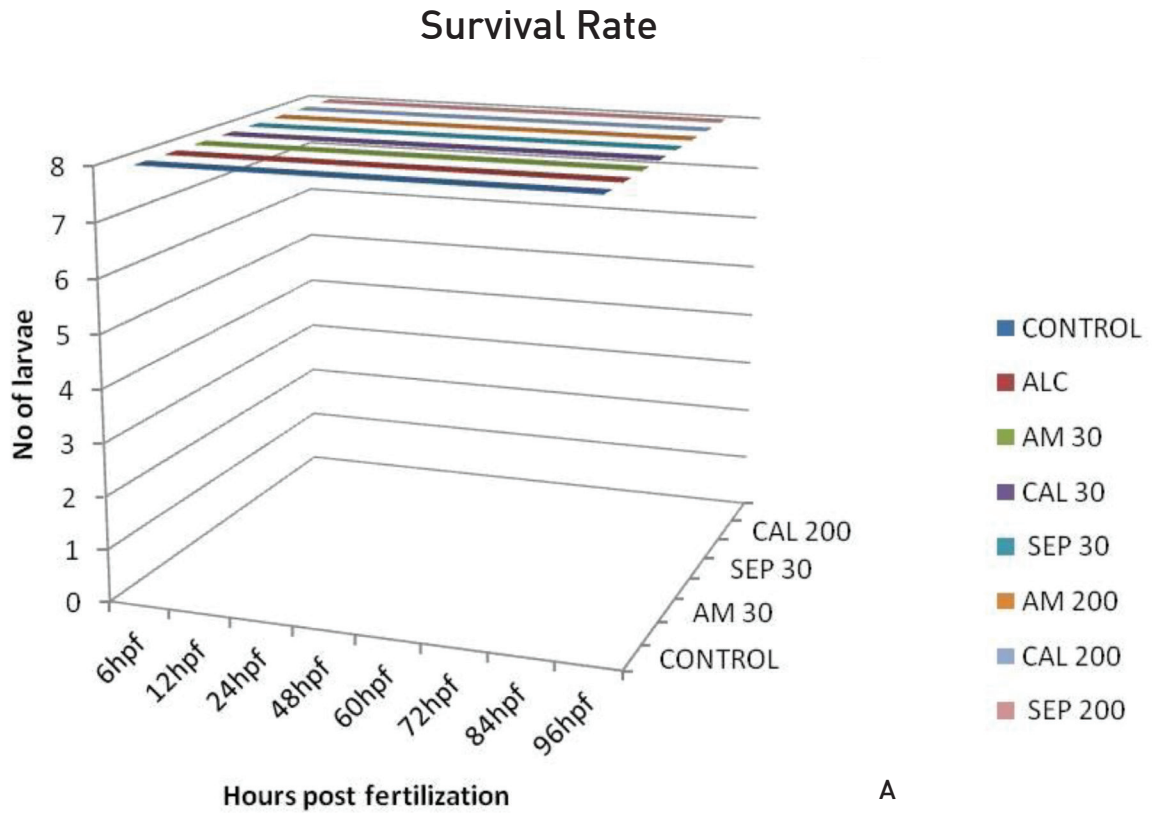


Figure 5 (A) Survival and (B) hatching rate of zebrafish embryos exposed to different potencies of three homeopathic drugs at 96 hours post-fertilization.

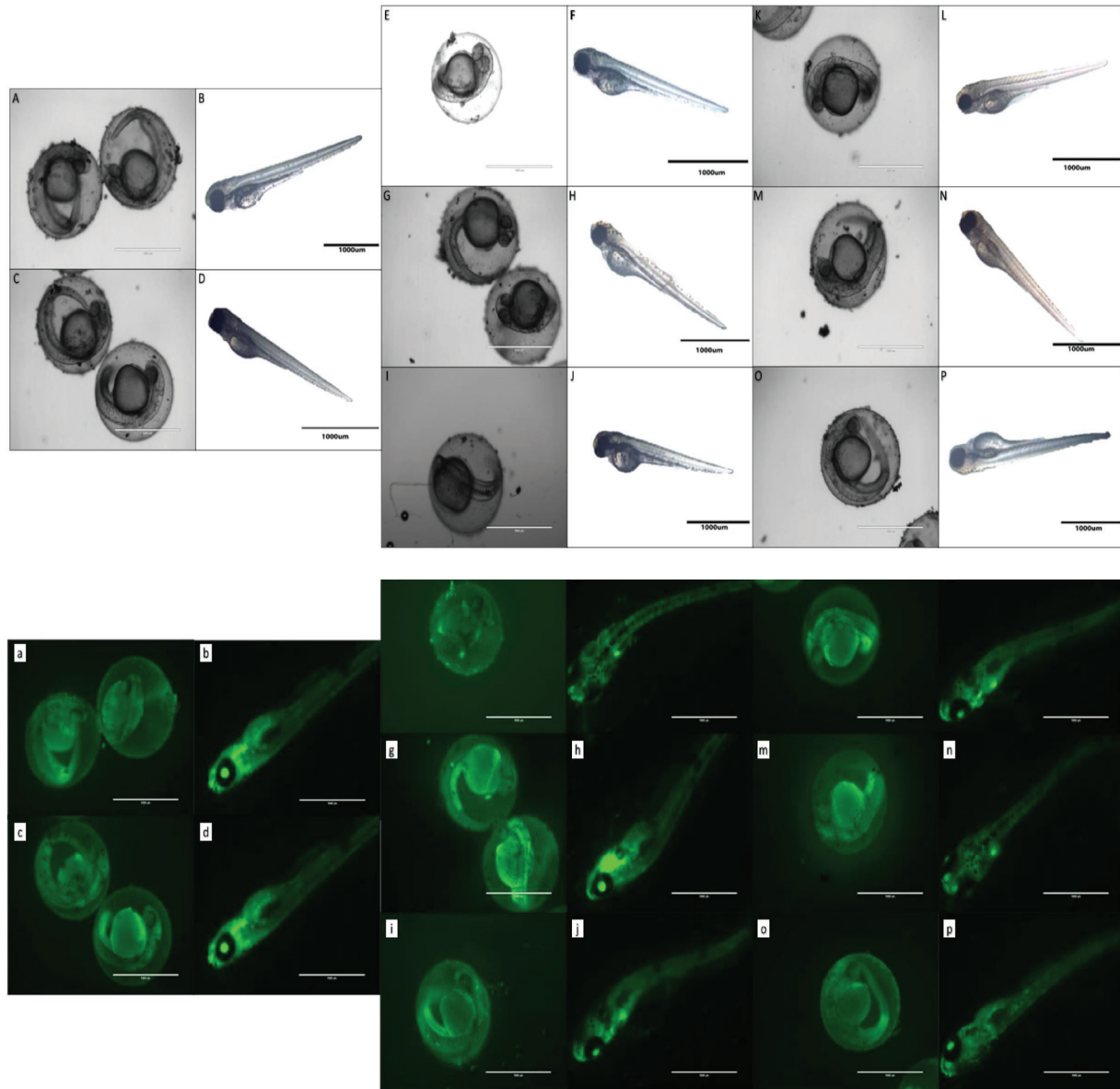


Figure 6 Phenotypic observation and apoptotic pattern seen over the time course of exposures to homeopathic drugs at different potencies from 4 hour to 96 hour post-fertilization.

Bright field images of (A) an untreated embryo at 24 hour (B) untreated larvae at 96 hour and (C) a potentized hydro alcoholic solution at 24 hours; (D) larvae exposed to a potentized hydro alcoholic solution observed after 96 hour; embryos treated with 30CH potency of (E) *Calcaria Carb*, (G) *Argentium Metallicum*, and (I) *Sepia* observed after 24 hour; (F, H, J) larvae post 30 CH exposure at 96 hpf (K, M, O) embryos treated with 200CH potency post 24 hours. (L, N, P) larvae post 200 CH exposure at 96 hpf.

Fluorescent images of an untreated embryo at (a) 24 hour and (b) 96 hour. untreated embryo and larvae. Fluorescent potentized images of the hydro alcoholic solution at (c) 24 hour and (d) 96 hour. Fluorescent images of embryos treated with a 30CH potency of (e) *Calcaria Carb*, (g) *Argentium Metallicum*, and (i) *Sepia* observed after 24 hour and (f, h, j) larvae at 96 hpf, embryos treated with 200CH potency of (k) *Calcaria Carb*, (m) *Argentium Metallicum*, and (o) *Sepia* observed at 24 hours and (l, n, p) larvae at 96 hpf.

hpf, hours post fertilization.

presence of NPs and conglomerates in all three homeopathic medicines at both dilutions. We also observed diversity in the shapes and the sizes of the NPs in the various medicines analyzed as well as in their different potencies. TEM analysis of *Calcareo carbonica* showed spherically shaped NPs of 20 - 40 nm in diameter at 30CH whereas at 200CH, it showed elliptical NPs with sizes in the range from 20 to 50 nm (Figs. 2A, 2B). In the case of *Argentum metallicum*, the shapes appeared to be spherical for both potencies, but the sizes varied (Figs. 2C-2F). The lower dilution of *Argentum metallicum* had smaller particles (30 - 60 nm) as compared to the higher dilution (50 - 100 nm). Similarly, *Sepia* at a dilution of 200C showed the presence of bigger spherical NPs of 100 - 200 nm in diameter (Figs. 2G, 2H).

The bright field TEM images confirmed the presence of NPs and conglomerates in all three homeopathic medicines. The conglomerates appeared as NPs clustered in the mesh of some matrix, which was further analyzed by using SAED to confirm the elemental composition. The SAED patterns of *Calcareo carbonica* and *Sepia* clearly revealed their polycrystalline nature (Figs. 3A, 3B, 3E, 3F) whereas the SAED pattern of the *Argentum metallicum* nanocluster consistently showed a crystalline pattern (Figs. 3C, 3D). The polycrystalline and the crystalline properties of *Calcareo carbonica*, *Argentum metallicum* and *Sepia* were found to be consistent for the two potencies that were studied. The crystalline lattices of these homeopathic medicines were further analyzed using HRTEM to study their atomic structures. HRTEM images of these medicines showed various degrees of crystallinity from nanograins, dot and line array patterns, to lattice fringes (Figs. 4A-4D). This evidence indicates that the science of homeopathy is a form of NP nanomedicine [20].

To assess the toxicity of the above-mentioned homeopathic drugs at dilutions of 30CH and 200CH, we used the zebrafish embryo model to determine whether and how homeopathic medicines, which contain NPs, influence embryonic development. In this study, zebrafish embryos were exposed to homeopathic drugs for 96 hours, as shown in Fig. 5A. Here, drugs with 30CH and 200CH potencies had no toxic effects on zebrafish embryos or larvae. This result suggests that the development of toxicity in zebrafish due to homeopathic drugs is neither time nor dose dependent.

The hatching rates among surviving embryos exposed to different potencies of the homeopathic drugs in each of the three sets showed no significant differences; at high dilution, as well as less dilution, these three homeopathic drugs with different CH values had no toxic effects on the zebrafish embryos. As shown in Fig. 5B, no toxic effect of the homeopathic drugs was detected when observing the mortality among zebrafish embryos. Mortality was found to be neither dose nor time dependent (data not shown). As compared with the control group, none of the three homeopathic drugs had any significant effect on mortality in the zebrafish embryos. All the embryos survived after exposure to the homeopathic drugs at dilutions of 30CH and 200CH for 96 hours.

Zebrafish embryos exposed to homeopathic drugs developed normal morphologies, and zebrafish larvae showed a range of phenotypic effects after exposure. Uninflated

swim bladder, yolk sac oedema, and dispersed pigment cells were the phenotypes most commonly observed. Bent tail, brachial arch hypoplasia, and bent body axis were the least recorded phenotypes. The six compounds did not produce any significant effects possibly because these compounds are not teratogenic according to the criteria used here. Simultaneously, we determined whether these homeopathic drugs induced apoptosis in zebrafish embryos and larvae by performing acridine orange staining to detect cellular death in live embryos. In the control group, a low, natural fluorescence was observed, which was similarly observed in the treated samples, as shown in Fig. 6.

4. Discussion

Surely, homeopathy must be free of undesirable effects. The typical homeopathic medicines at higher dilutions contain no active molecules, so they cannot cause any undesirable effects. The raw materials for most homeopathic medicines are known to be poisons. Still, every toxicologist knows that a substance is poisonous only in a certain dose. One could even go one step further and argue that the generally acknowledged absence of side effects has made homeopathy as popular as it is today. In recent decades, homeopathy has developed a growing basic science and is providing clinical and health services, with the research literature showing unique physicochemical properties, biological activities *in vitro* and *in vivo* and exceptional safety.

The physicochemical properties of homeopathic remedies show that they contain low, but measurable, amounts of NPs [5, 6, 20, 24]. Advancements in the fields of nanoscience and nanotechnology have resulted in countless possibilities for consumer product applications, many of which have already migrated from laboratory benches onto store shelves. From the results of the present study, the correlation of the UV-VIS spectral results with those derived from the analysis of the HR-TEM images provides further insights into the morphologies of homeopathic drugs. Clearly, the FEG-TEM images of the three different homeopathy preparations with two potencies showed varying size distributions of NPs. Interestingly, they contained significant proportions of NPs. Furthermore, despite the wide differences regarding the amounts of dilution- from 30CH to 200CH, no significant differences in the shapes and the sizes of the particles from those in the original material were observed. In addition, recent studies indicated that homeopathically-prepared medicines (HMs) contain source NPs, silicates [5, 6, 11, 20, 24-28], and other less well-characterized nanostructures. However, the TEM images in this study did not confirm that these NPs were NPs of the starting material. In this study, we only investigated the possibility of NPs existing in homeopathic medicines and examined whether homeopathy as a form of nanomedicine could cause any adverse effects or toxicity.

Over 100 studies have evaluated the protective and the therapeutic effects of homeopathic doses of normally toxic substances [29]. A team of doctors from German research institutions and America's Walter Reed Hospital performed

a meta-analysis of this study [31]. The zebrafish model is a vertebrate model that is gaining attention for preclinical drug-toxicity and drug-screening applications. Zebrafish embryos develop most of the major organ systems present in mammals, including the cardiovascular, nervous and digestive systems, in less than one week. Their early stages of life are susceptible to the adverse effects of drugs and chemicals [30]. In the present study, categorical lethal and sub-lethal endpoints were recorded from 24 hpf onwards for the three homeopathic drugs at the two different potencies (30CH and 200CH) that were tested. No delayed (72 hpf or 96 hpf) lethal endpoints were recorded for any of the three drugs at either of the two potencies. The observations recorded in this study were in accordance with those in the zebrafish embryo toxicity test described earlier [21, 22]. We should note that the proposed zebrafish embryo model for toxicity testing is easy to perform and that the data obtained from this study will be helpful in assessing the potential risk of various homeopathic drugs having different components such as organic, metallic, plant-based components.

5. Conclusion

Therapeutically, homeopathic medicines can cause clinical toxicity if the medications are improperly used. The few available clinical studies showed insufficient evidence of toxicity. However, insufficient clinical evidence proves neither a lack of toxicity assessment nor a lack of a scientific basis for treatment. The scientific evidence presented here has shown that homeopathic drugs have no proven toxicity in zebrafish embryos (*in vivo*) at either lower or higher potencies. Most conventional treatments continue to be associated with severe and sometimes fatal adverse effects while homeopathic treatments have been found to be free from such adverse effects.

Acknowledgment

The authors gratefully acknowledge the technical support from Bhabha Atomic Research Centre (BARC) and Indian Institute of Technology (IIT), Mumbai. We also wish to thank MGM Institute of Health Sciences for providing the facility to carry out the research work.

Authors' contributions

HG and YR performed the experiments and helped in data acquisition. DS interpreted the electron microscopy results. MT designed the experiments and gave intellectual input. HG, DS, and MT wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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