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Homeopathic Mother Tinctures can Precipitate Nanoparticles from Inorganic Salt and Demonstrate Subtle Differences in their Biological Effects: A Commentary Based on our Earlier Publication

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Abstract

In this study, homeopathic mother tinctures derived from crude ethanolic extracts of the medicinal plants -*Phytolacca decandra*, *Gelsemium sempervirens*, *Hydratis canadensis* and *Thuja occidentalis* - were used for the first time for biosynthesizing silver nanoparticles from silver nitrate solution in a cost effective, environment-friendly and non-toxic manner, and their possible anti-proliferative and anti-bacterial effects, if any, tested against A375 cells *in vitro*, and *E. coli*, respectively, to ascertain its possible therapeutic value. The nanoparticles produced by different plant extracts were physico-chemically characterized by spectroscopic analysis, particle size determination, zeta potential measurements and atomic force microscopy. Circular dichroism spectrophotometric data and melting temperature profiles using calf thymus DNA (CT DNA) as target were also analyzed. The silver nanoparticles of different origin were found to interact differently with the DNA, showing differences in their binding capacities with CT DNA and their time for entry into cells also varied because of their size difference.

Keywords: Homeopathic mother tinctures; Silver nanoparticles; A375 cells; *E. coli*; Circular dichroism; Cell cycle arrest; Anti-proliferation

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Introduction

In recent years, technological innovation to produce nanoparticles of well-defined size, shape and marked stability made their use possible in biomedical field, particularly in therapeutic oncology. Biological method of metal nanoparticle synthesis with the aid of plant extracts has proved to be much better than chemical reduction methods, in being cost effective as well as environment friendly [1], and is commonly called “green synthesis” [2]. The use of coriander leaf [3], henna leaf [4] and edible mushroom [5] in the biosynthesis of gold and silver nanoparticles has been reported. This commentary is based on our published research [6] in which we produced environmentally benign and biosynthesized small sized silver nanoparticles that had been reported earlier by other workers [7,8] to easily penetrate across membranes. However, the novelty of our study was that we for the first time deployed some homeopathic mother tinctures in precipitating silver nanoparticles from silver nitrate solution, and characterized the nanoparticles of different origin physico-chemically with some scientifically accepted protocols; we also tested certain biological activities related to anti-proliferative action against A375 cells (melanoma cells) and anti-bacterial activities against *E. coli*, which had not been attempted earlier.

In this study we addressed several issues like i) whether homeopathic mother tinctures, namely, *Gelsemium Sempervirens*, *Thuja Occidentalis*, *Phytolacca Decandra*, *Hydratis Canadensis* could precipitate silver nanoparticles from aqueous solution of silver nitrate at ambient conditions ii) if they produced, whether they belonged to the size in the nano range with significant variation in their shape, size, stability and zeta potential; iii) if these nanoparticles could interact with calf thymus DNA *in vitro* and iv) if these nanoparticles precipitated by different mother tinctures had any subtle differences in their anti-proliferative action against A375 cancer cells (melanoma) and anti-bacterial effect against *Escherichia coli* model.

Silver nanoparticles precipitated by each of the homeopathic mother tinctures available in the market as *Gelsemium Sempervirens*, *Thuja Occidentalis*, *Phytolacca Decandra* and *Hydratis Canadensis* (method described in detail in the original paper [6]) were separated from their

respective colloids by centrifugation and demarcated as SG (meaning Silver-nano from Gelsemium), ST, SP and SH, respectively.

For the physico-chemical characterization and measurement of size, spectroscopic analysis of UV-visible spectra and FTIR spectra, dynamic light scattering (DLS), polydispersity indexes (PDI), and Zeta potential data were critically analyzed and the surface properties determined through atomic force microscopy (AFM). The melting temperature profile™ of DNA was also determined by following the standard technique. Circular dichroism spectroscopy was used to study the changes in CD spectra, if any, caused due to silver nanoparticles and DNA interaction.

AFM results depicted spherical shape of nano-particles with smooth surface, without any pinholes or cracks. DLS data showed that the mean diameter of the silver nanoparticles, PDI and zeta potential varied among the four different nanoparticles of different origin. UV-visible spectra of the nanoparticles also showed subtle differences in their band width.

FTIR study revealed intense IR bands also differing to some extent among them. The same was true in regard to CD spectra that showed ability of the nanoparticles to form strong bonds with DNA bringing conformational changes. The melting temperature profiles also differed between the values of DNA alone and DNA plus nanoparticles of different origin. Further, subtle differences also existed among the four different nanoparticles treated DNA.

An increase in free radical scavenging activity of the nanoparticles was also recorded. The Cell viability test revealed that SP and SG showed maximum cytotoxic effects against A375 cells at IC 50 dose. Fluorescence microscopy demonstrated that different concentrations of treatment of silver nanoparticles of each of the four extracts, appeared to increase in number cells with chromatin condensation; this was more clearly discernible in case of SP and SG treatment. In SH and ST treatments, the change was not very distinguishable.

Results of DNA fragmentation study revealed that all nano-silver treated cells showed DNA laddering confirming clear apoptotic effect of nanoparticles. SP and SG showed better results with effective laddering in DNA compared to SH and ST.

As compared to the nuclei being intact and round, without any fragmented DNA in the control, there was linear increase in comet length with gradual increase in concentration in the SP treated cells. SG also showed increased comet tail lengths, as compared to less tail lengths in SH and ST.

That the cause of A375 cell death induced by silver nanoparticles was apoptosis, could also be confirmed by flow cytometric analysis. The expression patterns of mRNA by RT-PCR were analyzed in different sets of experiments which revealed the expression of caspase 3 to be upregulated in SP, SG, SH, ST treated cells.

Results of antibacterial activity of the silver nanoparticles was, however, not conclusive, as inhibition of bacterial growth was found only in case of SP treatment, whereas in case of SH the inhibition started only at a greater concentration, while both SG and ST treatments showed negligible or no anti-bacterial effects.

In consideration of the overall results, it could be suggested that the crude extracts of all the homeopathic mother tinctures, namely, *Phytolacca Decandra*, *Hydrastis Canadensis*, *Gelsemium Sempervivens* and *Thuja Occidentalis* were capable of synthesizing

silver nano particles from aqueous solution of silver nitrate at ambient conditions. Among them, SP possessed the best quality as a nano-drug, because they had a smaller particle size, suitable shape, negative zeta potential, and greater ability to interact with DNA, bringing about better stability, better drug bioavailability, and conformational changes in the DNA. Results on interaction with melanoma cells also indicated that nano of SP, SG SH and ST had anti-proliferative properties, and SP had the best ability. These shape-dependent properties of silver nanoparticles have different behaviours and make them suitable for therapeutic utilization [9]. Non-regular shape of silver nanoparticles can readily be absorbed in to different proteins and DNA and thus can promote biological activity.

SP had the smallest particle size followed by SH, SG and ST, all with negative zeta potential and a polydispersity index quite ideal for its application in the biomedical field. FTIR analysis was also performed to identify the possible biomolecules present in extracts responsible for capping leading to efficient stabilization of the nanoparticles. FTIR data also revealed the presence of freely water-soluble flavonoids in these homeopathic drugs. These flavonoids can be adsorbed on the cell surface and stimulate or suppress the immune system due to the presence of ^{-}OH groups. It is believed that presence of such phenolic moieties may have synergistic effect for the anti-proliferative activities of these bio-adsorbed metal nanoparticles [10]. The little variation in their contents of bio-flavonoids in these four nano-silvers could be responsible for the small differences observed in their anti- proliferative property in the study under focus. SP demonstrated to have much better stability and better capping efficacy than the nanoparticles of the other plant origin. Usually, the commonly practiced chemical reduction methods require the use of stabilizers in order to prevent agglomeration of the colloids [11], but the biological method adopted in this study did not need any stabilizer, thereby precluding the use of hazardous chemicals.

The most important factors that decide the therapeutic potentials and efficiency of biologically active molecules are essentially their properties that help them identify their bio-target and active sites. The spectral data analysis revealed that there was potential binding of SP, SG, SH, ST with CT DNA, bringing about a corresponding change in helicity of DNA. The thermal denaturation profiles of DNA due to incorporation of the nano-silver interaction also supported DNA conformational changes. The data showed an increase in T_m value of DNA by 3.5°C to 6°C when incubated with the different silver nanoparticles. Thus silver nanoparticles appeared to have a role in significantly changing the helicity of DNA, thereby stabilizing its conformational change in structure as well.

Interestingly, SP nanoparticles that showed greater anti-proliferative effects on skin melanoma A375 cells, also had the strongest inhibitory effects followed by SG, SH and ST, respectively, in skin melanoma cells through induction of apoptosis. This could be due to their strong antioxidative property, rendering them ability to scavenge free radicals. Further, analysis of the results suggested that silver nanoparticles induced apoptosis presumably through fragmentation of DNA, cell cycle arrest at G2/M phase and then, through activation of caspase 3. However, although SP nanoparticles appeared to show evidences of antibacterial effect, SH and SG nanoparticles had relatively less effects while ST did not show any anti-bacterial effect at all. Pal et al. [8] also reported anti-bacterial activity of silver nano particles produced by chemical reduction method.

DNA fragmentation and caspase activation are known to act as important mediators of apoptosis. Consistent with the results of their smaller particle size, better capping efficiency and stability, SP nanoparticles showed a more efficient entry into the cell, compared to other nanoparticles. Cell-cycle checkpoints at G2/M are critical in maintaining DNA integrity and regulating the passage of cells through the cell cycle. It is well known that loss of these checkpoints is involved in the transformation into and progression of cancer cells. In the focussed study, treatment of A375 cells with adequate doses of silver nanoparticles SP, SG, SH and ST resulted in inhibition of DNA synthesis and cell proliferation through G2/M cell cycle arrest.

Overall, the findings would reveal that the homeopathic mother tinctures procured from the plant extracts are capable of precipitating nanoparticles from inorganic salts would also provide a clue as to one of the probable ways by which the homeopathic mother tinctures may act inside the living systems. Thus, it seems quite possible that if we extrapolate the results of the present investigation, SP, SG, SH, ST may also be capable of synthesizing nanoparticles from some other inorganic salts available in the living system and which in turn could have some role in eliciting the medicinal responses to remove the disease/disease symptoms. Looking into all these aspects, it is reasonable to infer that the biosynthesis of irregular-shaped nanoparticles hopefully might reach this aim because they display novel properties. The biological methods of synthesizing nanoparticles have proved to be one of the best methods so far. This is due to its environment-friendly nature and slower kinetics which offer better manipulation and control over crystal growth and stabilization.

However, further in-depth studies will be necessary to verify if some other homeopathic mother tinctures also have similar properties of nano-precipitation from other salts, that may have specific biological/medical applications.

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