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Post-Synthesis Size Segregation of Bio-Fabricated Gold Nanoparticles Prepared from Homeopathic Mother Tincture (*C. officinalis*) and Evaluation of their Antioxidant Bio-Efficacy

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ABSTRACT

The plant-mediated synthesis of noble metal nanoparticles is widely used for the preparation of allopathic nano drugs with enhanced bio-efficacy. However, little attention has been paid towards the use of such green nanotechnological approach for the bio-fabrication of noble metal nanoparticles using homeopathic mother tincture (highly dilute plant extracts) for the enhancement of pharmacological activities. The enhancement in pharmacological bio-efficacy chiefly depends on the shape and size of the nanoparticles. Among the various methods used for size segregation of nanoparticles, post-synthesis segregation of nanoparticles using sucrose density gradient centrifugation is considered a fast, simple and green method for improving solution-phase heterogenous metal nanoparticles to homogenous nature with desirable size.

The present communication warrants the synthesis of gold nanoparticles using *Calendula officinalis* homeopathic mother tincture in a single-pot green process, accompanied by thorough characterization for their optical property, surface morphology, crystalline lattice, particle size and zeta potential. Post synthesis size segregation has been carried out using sucrose density gradient centrifugation. The fractionated nanoparticles sized in the narrow range (Z-average 28.13 to 13.24 nm) have been monitored to study the effect of nano-sizing on the enhancement in the free radical scavenging activity (*DPPH* assay). The nanosized fractionated gold nanoparticles (Z-average 13.24 nm) exhibited elevated free radical scavenging activity (28.17%) compared to native *Calendula officinalis* homeopathic mother tincture (Z-average 8217 nm). A tentative mechanism has been put forward explaining the observed enhancement.

Keywords: Density gradient centrifugation, *Calendula officinalis*, Homeopathic mother tincture, Gold nanoparticles, DPPH assay

Abbreviations: Cm@AuNp: Gold Nanoparticles Embedded with *Calendula officinalis* Homeopathic Mother Tincture; UV-Vis Spectroscopy: Ultra-Violet-Visible Spectroscopy; SEM: Scanning Electron Microscope; XRD: X-Ray Diffraction; DLS: Dynamic Light Scattering; RPM: Revolutions per Minute; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl Radicals; JCPDS: Joint Committee on Powder Diffraction Standards

INTRODUCTION

The growing popularity of complementary medicine has been accompanied by a call for controlled clinical studies to examine the efficacy and cogency of its mode of action [1]. Homeopathy is one of the important complementary medicine and a time-tested two-century-old empirical system of healing. Homeopathic medicines are prepared through a characteristic process known as potentization, where serial dilutions are performed with strong strokes at each step of dilution [2]. Homeopathy is controversial because its most medicines do not contain one single

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molecule of the corresponding starting substance and earlier has been labeled as the placebo effect. However, homeopathy elicits much more than placebo responses.

It is an empirical puzzle and a challenge to its orthodox scientific models. In recent perspectives, with the advent of new tools supplied by nanoscience and nanotechnology, novel research pathways of homeopathic medicines are to be opened [3].

Size and shape are important parameters for nanoparticle-based drugs [4] that control the kinetics of internalization [5], biodistribution [6], cellular membrane deformability [7] and cargo loading efficiency [8]. It has been shown that smaller nanoparticles escape natural body clearance mechanisms more efficiently and hence circulate longer in the blood [9]. In addition, the shape plays an important role in demonstrating that cylindrical nanoparticles interact with cells very differently to spherical ones, resulting in dramatic changes in the bio-availability [10]. The preparation of nanoparticles with the well-defined size is essential for accurate control of chemical and physical properties [11]. Size-controlled nanoparticles find its application in medical science, biotechnology self-assembly, electronics, diagnostics, and sensing [12,13]. The various influencing factors and different synthetic methods make it challenging to synthesize nanoparticles with uniform size and morphology. Therefore, post-synthesis separation methods remain a more feasible way to procure monodisperse nanoparticles [14]. Several techniques have been employed to separate nanoparticles of desired size and shape. Techniques such as magnetic field flow fractionation filtration [15], size exclusion chromatography [16], size selective precipitation [17], density gradient centrifugation [18] and cross-flow filtration [19] have been utilized to produce certain nanoparticle fractions with narrow shape and size distributions. Recently, centrifugation has been proven to be a more effective and easy method to segregate nanoparticles based on their size due to its high efficiency, capability of scalable production, and free of nanoparticle aggregation [20,21]. Density gradient centrifugation is cost-effective, fast, easy and highly versatile method used for separation of nanoparticles according to their size [22].

In continuation of our work pertaining to the green synthesis, characterization and enhancement of various pharmacological bio-efficacy [23-27], the present piece of work demonstrates the single pot green synthesis of gold nanoparticles (*Cm@AuNp*) using homeopathic mother tincture *Calendula officinalis*, thorough characterization and post-synthesis size segregation of bio-fabricated gold nanoparticles using sucrose density gradient centrifugation. Further, the effect of size of segregated gold nanoparticles on the antioxidant bio-efficacy has been studied.

MATERIALS AND METHODS

Calendula officinalis homeopathic mother tincture was purchased from the homeopathic store of SBL India. The synthesis of gold nanoparticles was carried out using different dilutions of *Calendula officinalis* homeopathic mother tincture, keeping the concentration of $NaAuCl_4.2H_2O$ solution constant as a function of pH.

Characterization of *Cm@AuN*p

The *Cm@AuNp* was characterized for optical properties using UV-Vis spectrophotometer (Lab India, India). The morphology of *Cm@AuNp* was studied using *SEM* (Table tops SNE-3200M, USA). The X-ray diffraction pattern of *Cm@AuNp* was recorded using *XRD* (Bruker AXS D8 Advance, Germany) over 35°-75° with scan run 40/min, step size of 0.02° and Cu Kα radiation of λ=1.54Å. The hydrodynamic size distribution with poly-dispersity index (*PDI*) and zeta potential was analyzed using zeta sizer (Nano ZS90 model Malvern, Germany).

Density gradient centrifugation (DGC)

Sucrose density gradient method was lucratively used to fractionate anisotropic *Cm@AuNp* having different sedimentation rates. A discontinuous density gradient of sucrose solutions (5%, 10%, 15% and 20%) was developed upon one another. Colloidal solution of *Cm@AuNp* (5 mL) was added to the capacity vial and centrifuged at 5000 *RPM* for 15 min. Nanoparticles depending upon the size were confined to different layers of the sucrose density gradient. Each layer was separated using a sterile syringe and was further monitored for the determination of the size of separated nanoparticles using zeta sizer. Sedimentation coefficient S was calculated for each layer using the equation: $S = (\rho_p - \rho_m)d^2 /18\eta$

DPPH radical scavenging activity

The anti-oxidative effect of sucrose gradient concentrations (5%, 10%, 15%, 20%) containing fractionated *Cm@AuNp* and native homeopathic mother tincture was determined using *DPPH* assay. Ascorbic acid (1 mM) was used as a positive control. The test samples (2 ml) were incubated with *DPPH* ethanolic solution (1 ml; 1 mM). The contents after vigorous mixing were allowed to stand for 30 min at room temperature. The absorbance was measured at 517 nm and the free radical scavenging activity was calculated using the formula: (C-T)/C × 100

Where, C is absorbance of control and T is the absorbance of the test sample.

RESULTS AND DISCUSSION

Optimized experimental conditions of bio-fabricated gold nanoparticles were as follows: *Calendula officinalis* homeopathic mother tincture with 40% dilution (1 ml), sodium tetrachloroaurate dehydrates solution (1 ml; 1 mM) and sonication (15 min; 20 KHz) at pH 8. The conversion

of Au (III) to its elemental form (Au°) may be ascribed to the presence of flavonoidal moieties present in *Calendula officinalis* homeopathic mother tincture which is nothing but an ethanolic extract of the plant *Calendula officinalis*. The visual change in color from pale yellow to ruby red indicated the bio-fabrication of target nanoparticles. *Calendula officinalis* extract has been reported to contain a series of polyphenolics flavonoidal compounds namely quercetin, isorhamnetin, isoquercetin, narcissin, isorhamnetin-3-O-β-D-glycoside, calendoflaside, calendoflavoside, calendoflavobioside, rutin, isoquercitrin, neohesperidoside, isorhamnetin-3-O-neohesperidoside, isorhamnetin-3-O-2G-rhamnosyl rutinoside, isorhamnetin-3-O-rutinoside, quercetin-3-O-glucoside and quercetin-3-O-rutinoside [28-30]. The bio-fabrication of *Cm@AuNp* using polyphenolics (flavonoids) present in the homeopathic mother tincture can be explained by the following reaction:

$$AuCl^{4-} + 3R\text{-}OH \xrightarrow[(flavonoid)]{} Cm@Au^{0}Np + 3R{=}O + 3H^{+} + 4Cl^{-}$$

Flavonoidal content not only reduce Au^{3+} to Au^{0} but also get coated (capping) on the freshly generated *Cm@AuNp* and thus adding their medicinal properties [24].

Characterization of gold nanoparticle UV-Vis spectrophotometer

Different dilutions of *Calendula officinalis* homeopathic mother tincture (20%, 40%, 60% and 80%) and native mother tincture were mixed with $NaAuCl_4.2H_2O$ solution respectively in the ratio 1:1 and analyzed by UV-Vis spectrophotometer **(Figure 1)** for confirming the formation of gold nanoparticles. The absorption spectrums of bio-fabricated *Cm@AuNp* were found to have a maximum absorption band in the range of 534-541 nm. However, it was found that 40% diluted *Calendula officinalis* homeopathic mother tincture and $NaAuCl_4.2H_2O$ solution in 1:1 ratio had a maximum absorption band at 536 nm and further dilution doesn't show any marked increase in the absorbance.

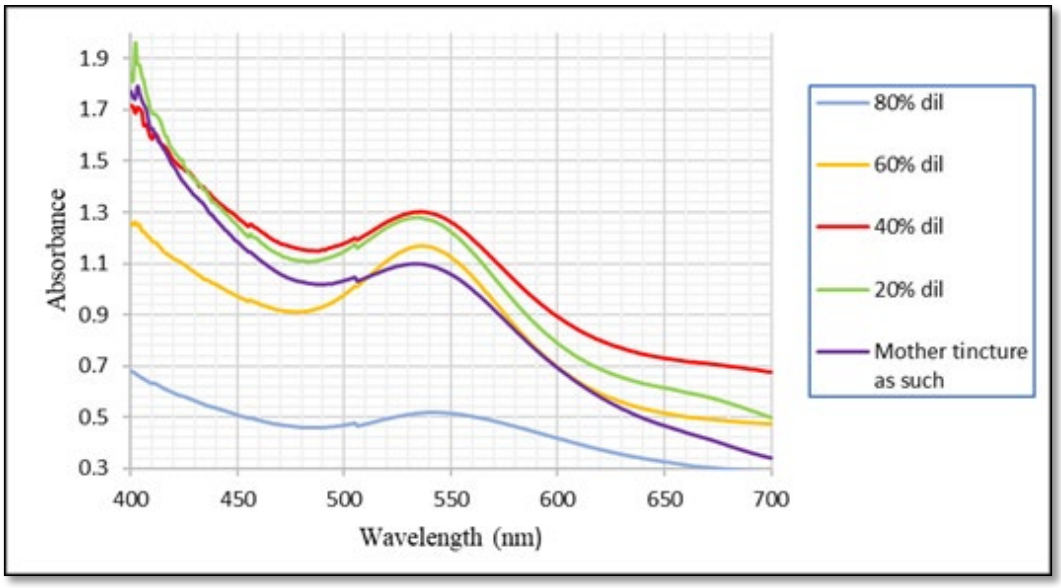

Figure 1. UV-Vis spectra of *Cm@AuNp* at different ratios of dilution.

X-ray diffraction

Bragg diffraction peaks 2θ appeared at 38.17°, 44.23° and 64.29° in the bio-fabricated *Cm@AuNp* **(Figure 2)**. It could be indexed to (111), (200) and (220) having lattice planes of face-center cubic compared with (JCPDS file 04-0784). The intensity of the diffraction peaks (200) and (220) were found lower than the corresponding crystallographic plane (111). The fact established that the lattice plane (111) is the transcendent crystallographic plane and is more reactive because of its high atom density [31].

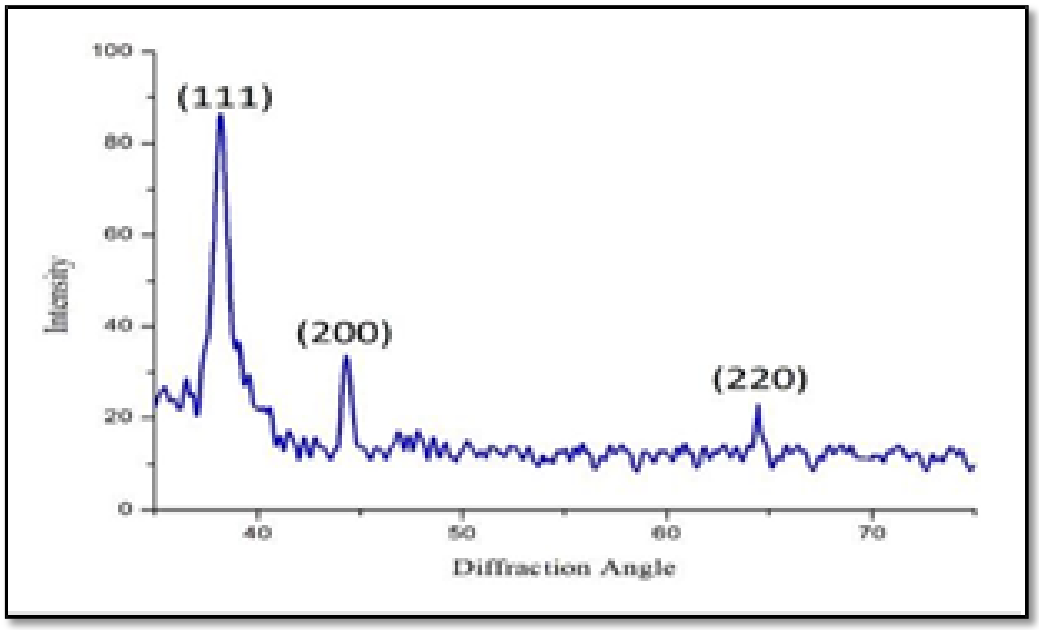

Figure 2. XRD graph of *Cm@AuNp*.

SEM

SEM images **(Figure 3)** acquired from the drop-coated film of nanoparticles indicated their polydisperse spherical shaped morphology.

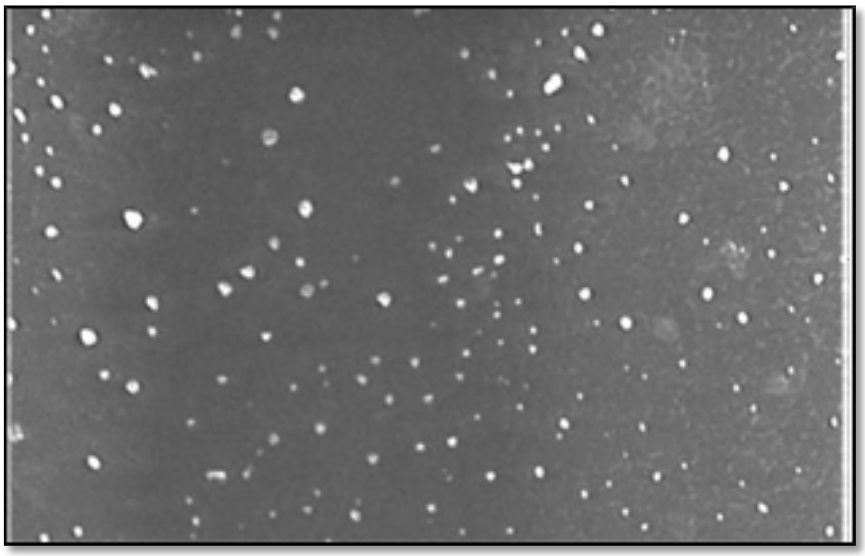

Figure 3. SEM image of *Cm@AuNp*.

Dynamic light scattering

The dynamic light scattering (*DLS*) spectrum highlighted the asymmetric distribution of nanoparticles mainly in the range (21 to 1281 nm) with poly-dispersive index 0.664 and intercept 0.922 **(Figure 4a)**. However, a little population was extended in the range of 6000 nm. The average hydrodynamic size (Z-Average) of *Cm@AuNp* was found to be 265.6 nm. Zeta potential of *Cm@AuNp* determined in water medium as a dispersant was -20.8 mV **(Figure 4b)**. The magnitude of observed high negative charge on the bio-fabricated nanoparticles might be acting as a repulsive barrier, avoiding aggregation of nanoparticles.

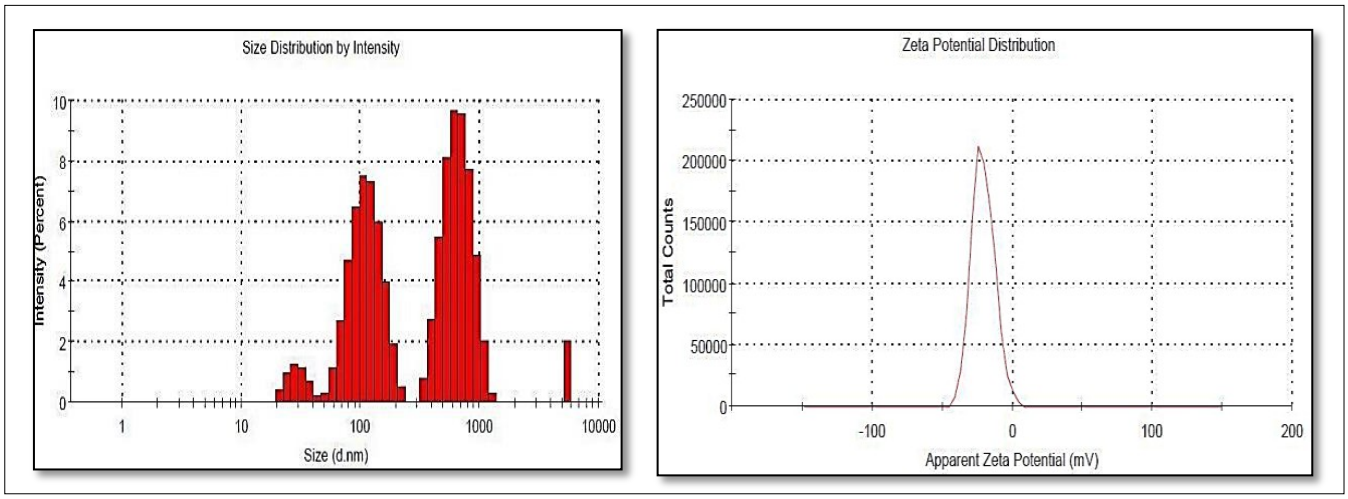

Figure 4. (a) Zeta size and (b) Zeta potential graph of synthesized *Cm@AuNp*.

Density gradient centrifugation (DGC)

The size fractionation of *Cm@AuNp* centrifuged at 5000 RPM for a fixed time (15 min) are shown in **(Figure 5)**. Different bands obtained after the run indicated successful segregation of *Cm@AuNp* as per their sedimentation rates.

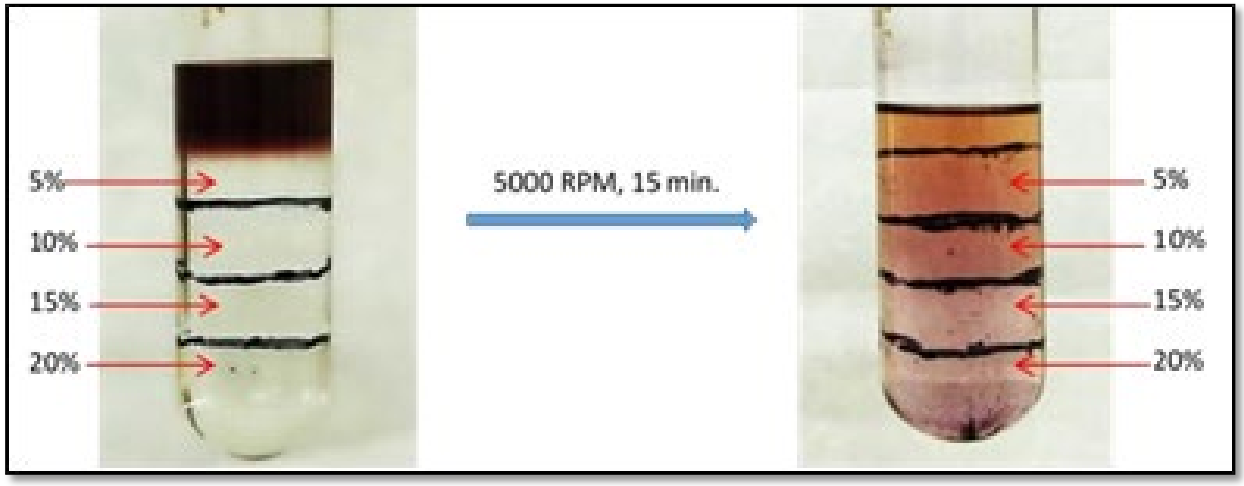

Figure 5. Optical images showing the sucrose density gradient separation of anisotropic *Cm@AuNp*. Images of the as-prepared parent gold nanoparticle mixture (left) and the color image (right) of the individual fractions upon successful size fractionation.

Histograms of *Cm@AuNp* at different sucrose concentration gradient are shown in **Figures 6a-6d**. It represents the distribution of the nanoparticles confined at 5%, 10%, 15% and 20% sucrose concentration gradient. **Table 1** indicates that particle size decreased in a concentration-dependent manner with larger particles occurring at higher sucrose density gradient (20%) while smaller at lower density gradient (5%). This reflects that the decrease in the size of the particle takes place with a dilution of a sucrose concentration gradient.

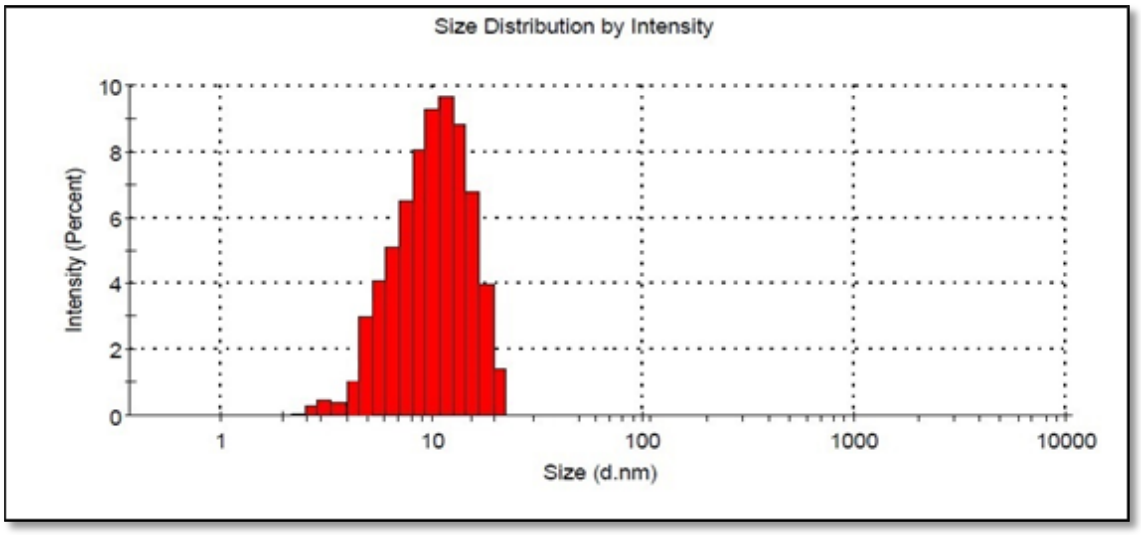

Figure 6a. Zeta size graph of 5% sucrose concentration gradient.

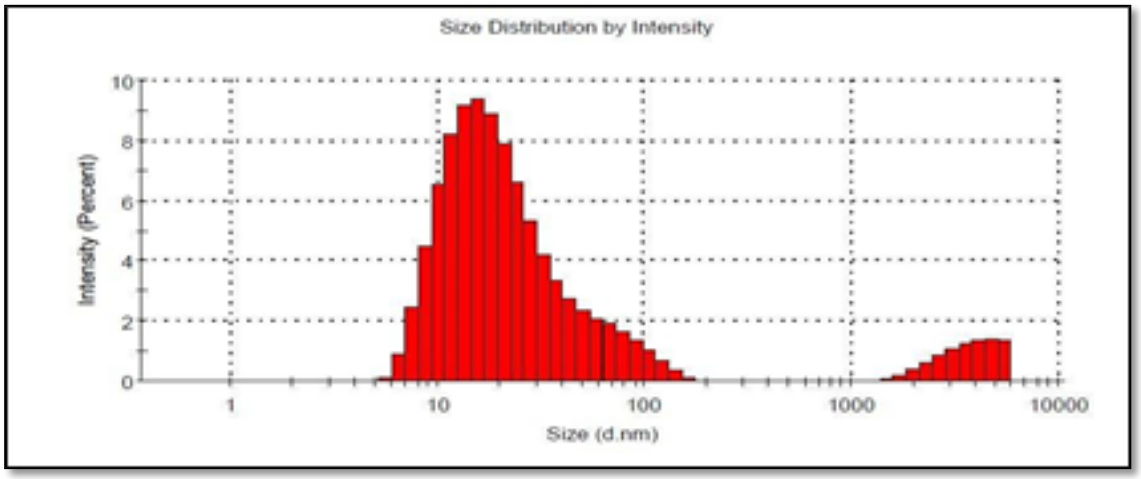

Figure 6b. Zeta size graph of 10% sucrose concentration gradient.

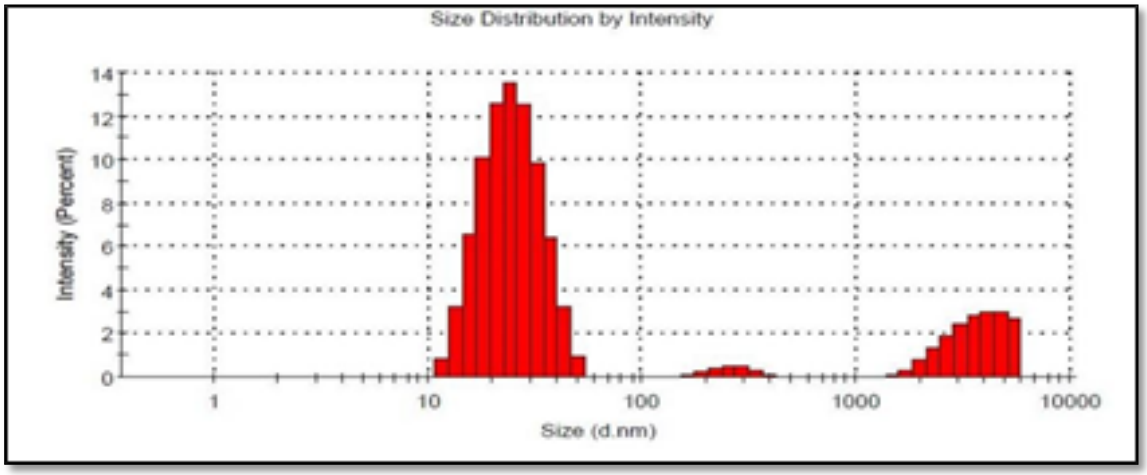

Figure 6c. Zeta size graph of 15% sucrose concentration gradient.

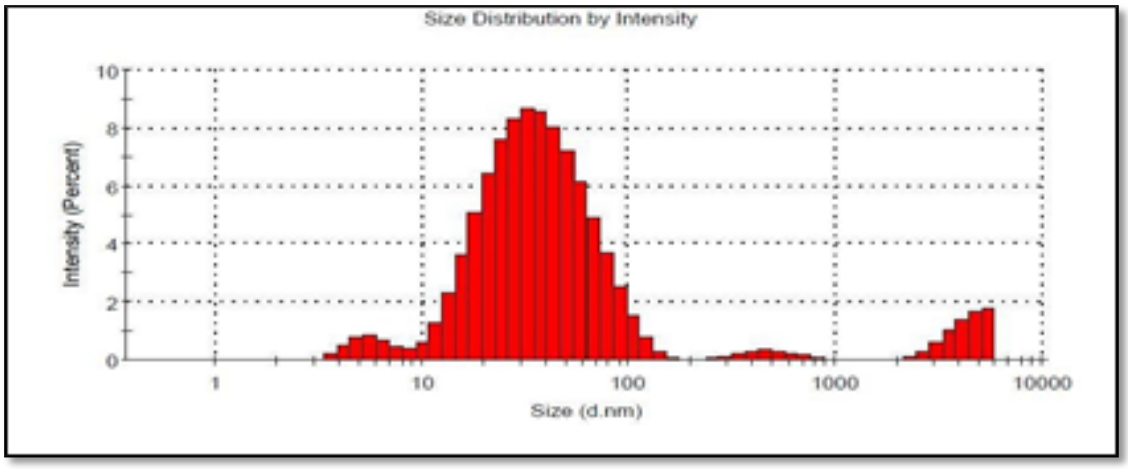

Figure 6d. Zeta size graph of 20% sucrose concentration gradient.

Sedimentation coefficients for *Cm@AuNp* at 5%, 10%, 15% and 20% sucrose concentration gradient **(Figure 7)** were 95.1 × 10^{-3} s, 261.02 × 10^{-3} s, 1121.47 × 10^{-3} s and 1717.22 × 10^{-3} s, respectively indicating faster sedimentation rate of heavier particles. Overall results obtained demonstrate the utility of density gradient technique to separate mixtures of different size distributions of nanoparticles to near monodispersity.

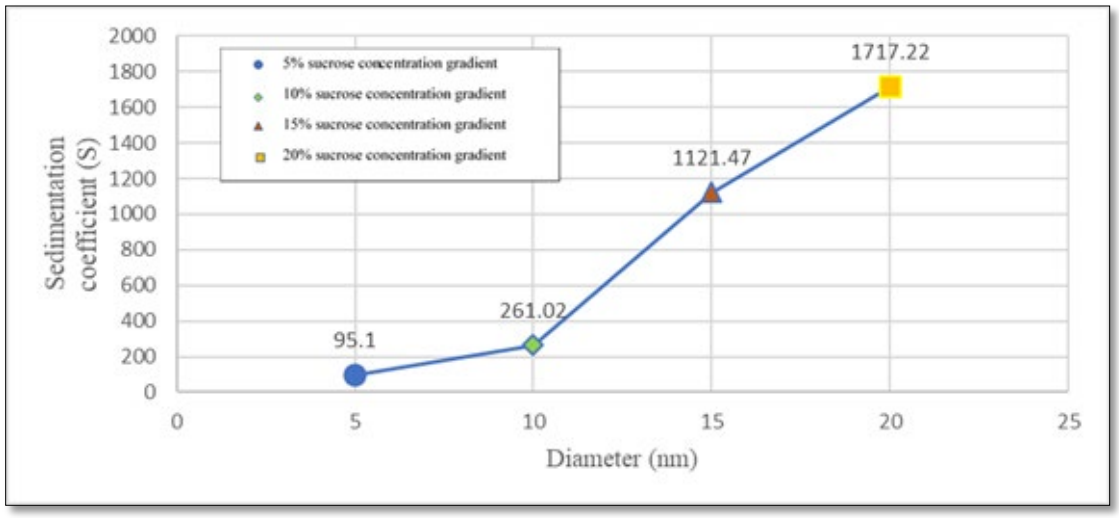

Figure 7. Variation in sedimentation coefficient with diameter of *Cm@AuNPs*.

DPPH radical scavenging activity

DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon the reduction by either the process of hydrogen or electron donation. Substances that perform this reaction can be considered as antioxidants and, therefore, radical scavengers [32]. Natural antioxidants (secondary metabolites of plants) are limited in their radical scavenging action; therefore, recent efforts are focused on the enhancement of the free radical scavenging activity of the natural antioxidants by synthesis of noble metal nanoparticles prepared from different plant extracts. In the present study *Calendula officinalis* homeopathic mother tincture was used to bio-fabricate gold nanoparticles. The free radical scavenging activity of different sucrose gradient fractions (20%, 15%, 10%, 5%) containing *Cm@AuNp* 5 ml each and native mother tincture was tested. As the size of *Cm@AuNp* decreases (20% to 5% sucrose gradient fraction) free radical scavenging activity increases which can be visualized by the change in color from dark brown to yellow exhibiting size dependent free radical scavenging activity **(Figure 8)**. The fact finds support **(Table 1)** from the higher free radical scavenging activity of fractionated *Cm@AuNp* (Z-average (28.13 to 13.24 nm) compared to native *Calendula officinalis* homeopathic mother tincture (Z-average 8217 nm). A perusal of the **Figure 9** further highlights that with the decrease of the size of *Cm@AuNp* (Z-average to 13.24 nm), the free radical scavenging activity is increased from (67.07% to 79.3%). However, sharp increase in the free radical scavenging activity has been observed with the change in the size of the nanoparticle (Z-

average 26.25 to 15.51 nm). Free radical scavenging activity of segregated *Cm@AuNp* (5% sucrose gradient fraction), prepared from native *Calendula officinalis* homeopathic mother tincture overall reports 28.17% enhancement **(Table 1)**. The observed enhancement in free radical scavenging activity may be assigned to the biocompatibility, astonishing optical properties related to surface plasmon resonance and high surface area to volume ratio (nanosizing) overall improving permeability in the cell membrane and higher accumulation of the *Cm@AuNp*.

The bio-fabricated gold nanoparticles embedded with flavonoids *(Cm@AuNp)* possibly might release 3 electrons for scavenging of 3 free radicals (enhancement) as depicted in the following reaction [33].

$$Cm@Au^{0}Np \longrightarrow Au^{3+} + 3e$$

The simultaneous coating of polyphenolic flavonoids of *Calendula officinalis* extract on freshly prepared nanoparticles also contributes in the enhancement of free radical scavenging activity [24,26].

Table 1. Sucrose density gradient based segregation of size of *Cm@AuNp* and their free radical scavenging activity.

Test samples	Distribution of the particle (nm)	Z-average (nm)	% Free radical scavenging activity
20% sucrose gradient fraction	3.61-164.24	28.13	67.07 ± 3
15% sucrose gradient fraction	11.71-50.75	26.25	67.49 ± 5
10% sucrose gradient fraction	6.54-164.0	15.51	78.99 ± 1
5% sucrose gradient fraction	2.6-40.34	13.24	79.3 ± 2
Native mother tincture	6025-9754	8217	61.61 ± 2

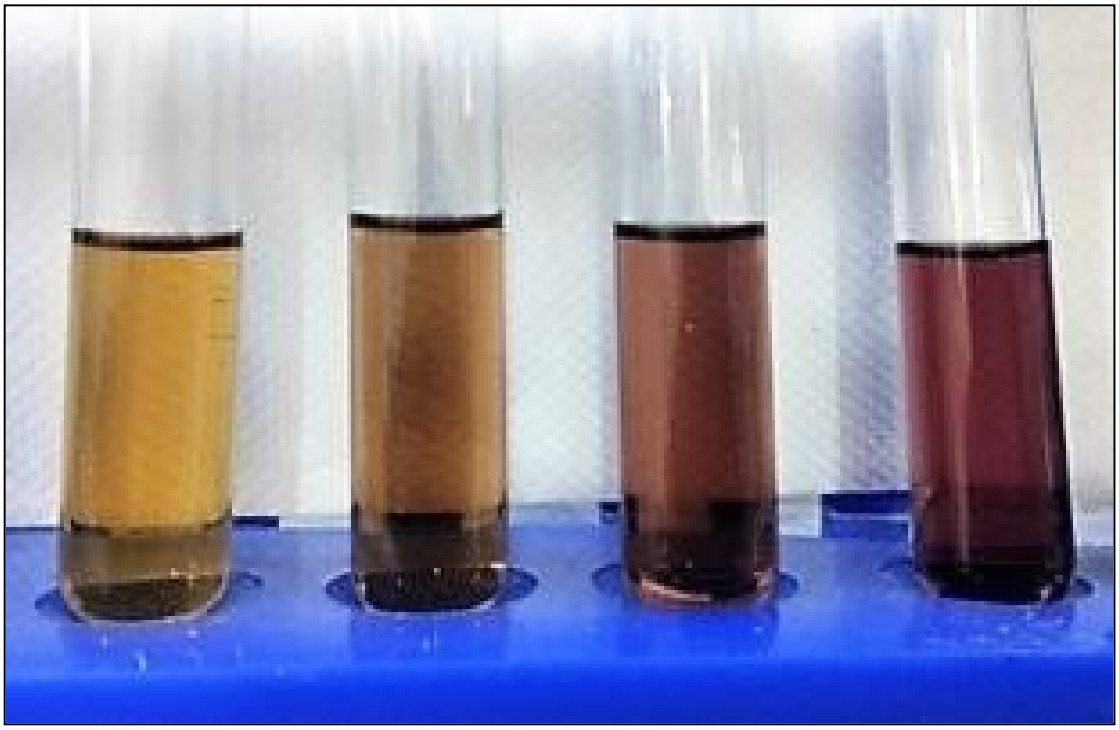

Figure 8. Optical images showing the free radical scavenging activity of fractionated *Cm@AuNp*. Images from left to right: 5% sucrose gradient fraction, 10% sucrose gradient fraction, 15% sucrose gradient fraction, 20% sucrose gradient fraction.

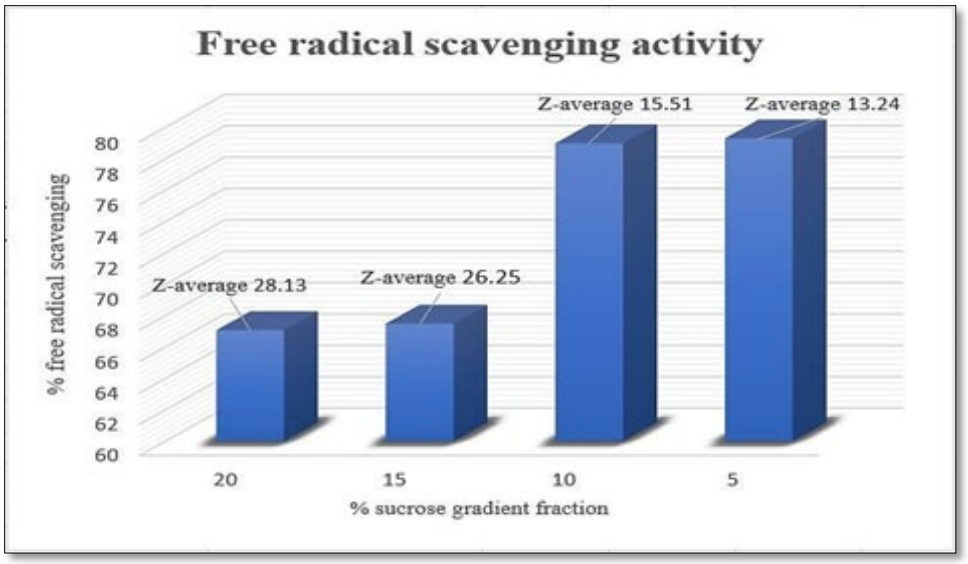

Figure 9. % Free radical scavenging of different sucrose gradient fraction.

CONCLUSION

The present communication reports the bio-fabrication of *Cm@AuNp* using homeopathic mother tincture *Calendula officinalis* in a single step green method. After thorough characterization of bio-fabricated gold nanoparticles embedded with biomolecules of *Calendula officinalis* mother tincture (*Cm@AuNp*) have been fractionated into smaller sized gold nanoparticles using sucrose density gradient centrifugation method. The fractionated nanoparticles ranging in the size (Z-average 28.13 to 13.24 nm) are monitored for the effect of size of nanoparticles on the enhancement in the free radical scavenging activity (DPPH assay). The enhancement in free radical scavenging activity (28.17%) of minimum sized fractionated (*Cm@AuNp:* 5% sucrose gradient fraction) having Z-average 13.24 nm compared to native *Calendula officinalis* homeopathic mother tincture (Z-average 8217 nm) have been recorded.

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