

## Supplementary data

Cerium nanoparticles synthesized using aqueous extract of *Centella asiatica*:  
characterization, determination of free radical scavenging activity and evaluation  
of efficacy against cardiomyoblast hypertrophy

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### **1. Chemicals used:**

Ammonium ceria nitrate and ascorbic acid were purchased from *Sisco Research Laboratories (SRL, Mumbai)*. 2, 2'-azino-bis- (3-ethylbenzthiazoline-6-sulphonic acid)(ABTS), nitroblue tetrazolium (NBT), phenyl methosulphate (PMS), nicotinamide adenine dinucleotide reduced (NADH), catechin, 2-deoxyribose, thiobarbituric acid (TBA), quercetin, trolox, gallic acid, isoproterenol, (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and dihydroethidium (DHE) were purchased from *Sigma Aldrich* (St Louis, MO). Fura-2 and Hanks balanced salt solution (HBSS) were from *Invitrogen* (Carlsbad, CA). All the other chemicals used were of standard analytical grade. Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-EDTA solution and antibiotics were purchased from *Himedia*, India. Fetal bovine serum (FBS) was purchased from *Gibco* (Langley, OK). The cell culture wares and filters were from *BD Biosciences* (San Jose, CA) and *Millipore* (Billerica, MA), respectively.

### **2. Aqueous extraction of *Centella asiatica*:**

The whole plants of *Centella asiatica* were washed with copious amounts of water and air dried. The dried pieces were powdered using a mechanical grinder. 5g of fine powder were added to 100ml distilled water and heated at 60°C for 15 min. The extract was cooled and filtered through Millipore filter (0.45µm) and the filtrate was collected and kept at 4°C.

### **3. Characterization of CeNPs:**

UV-Vis spectra of CeNPs were recorded using a dual beam spectrophotometer (*Shimadzu UV - 2450*). Absorbance spectra were recorded at wavelengths in 200–800 nm range. The average particle size (hydrodynamic diameter) and zeta potential of CeNPs were determined by photon correlation spectroscopy using *3000 HSA Zetasizer* (*Malvern Instruments, WR, UK*). Additionally, the particle sizes and morphologies of the samples were investigated using high

resolution transmission electron microscope from *FEI Tecnai, Hillsboro, OR, USA* [equipped with EDX and Selected Area Electron Diffraction (SAED) facility] and scanning electron microscope (*JEOL make, model JSM 5600 LV, Peabody, MA, USA*). EDX was carried out to determine the presence of various elements in the sample.

#### **4. Cell viability / MTT assay:**

MTT assay is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase to an insoluble, coloured (dark purple) formazan product. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

The cells were spread in 96-well plates at  $5 \times 10^3$  cells / well. After incubating with different concentrations of CeNP/ CAN for 96 h, the cells were exposed to MTT at a concentration of  $50 \mu\text{g}/\text{well}$  for 2.5 to 3 hrs at  $37^\circ\text{C}$  in  $\text{CO}_2$  incubator. The working solution of MTT was prepared in Hanks balanced salt solution (HBSS). After viewing formazan crystals under the microscope, the crystals were solubilized by treating the cells with DMSO: isopropanol (1:1) for 20 min at  $37^\circ\text{C}$ . Plate was read at an absorbance of 570nm using a Multimode reader (*Biotek, Winooski, VT*). The relative cell viability in percent was calculated as: Absorbance of treated / Absorbance of control  $\times 100$ . Control samples used were cells without CeNP/CAN.

#### **5. Cellular uptake/ internalization:**

Cellular uptake of CeNPs was investigated by flow cytometry and also by fluorescence microscopy.

Flow cytometric analysis was based on side scatter (SSC) of the laser beam recorded for each cell; the intensity of SSC being proportional to the intracellular density.

The cells were seeded at a density of  $5 \times 10^4$  cells / well of 24-well plates for the purpose. After 36 h of seeding, they were exposed to CeNPs (5, 10, 20 and  $30 \mu\text{M}$ ) for 24 h. The cells were then trypsinized and trypsin-inactivated with 10% DMEM, followed by centrifugation at 2300 rpm for 1 min. The pellets were resuspended in HBSS (pH 7.4) for analysis using *BD FACS Aria II* flow cytometer (*BD Biosciences, San Jose, CA, USA*). Acquisition and analysis of flow cytometric data were carried out using *BD FACS Diva* software. SSC intensity was also

determined for bulk cerium (CAN). The SSC intensities of CeNPs were compared with control cells without any treatment and also with CAN.

For fluorescence microscopy, the cells were spread at a density of  $5 \times 10^3$  cells/ well of 96 - well black plates and after 36 h, they were incubated with CeNPs for a 24 h time period. Subsequently, the cells were washed thrice with HBSS and subjected to imaging. Fluorescence images of the cells were collected by high- content spinning disk facility (*BD Pathway 855; BD Biosciences*) using *AttoVision 1.5.3* software. Emission filter used was 435nm LP.

#### **6. Assessment of intracellular superoxide generation:**

Dihydroethidium (DHE) was used to monitor intracellular superoxide production. DHE emits blue fluorescence until it is oxidized to red fluorescent ethidium (Et) that binds to DNA, in the presence of superoxide anion. The ratio of Et-DNA fluorescence to DHE fluorescence was determined after incubation of the cells with different concentrations of CeNPs for 24h. Antimycin ( $5 \mu\text{M}$ ), an inducer of superoxide anion generation, was used as a positive control. Fluorescence was measured using Multimode reader and images were collected using high-content spinning disk facility. For Et-DNA, B548/20nm excitation filter and 570 nm LP emission filter was used.

#### **7. Determination of intracellular calcium levels:**

Effect of CeNPs and ISO on calcium homeostasis was evaluated by loading the cells with Fura 2AM, a fluorescent calcium probe. H9c2 cells were loaded with the probe in HBSS at  $37^\circ\text{C}$  for 30 min and subjected to fluorometric analysis using a multimode reader (*BioTek, Winooski, VT*). Excitation signals were recorded at 340nm and 380nm and emission signal at 510nm. Intracellular calcium concentration was estimated and calculated as:

$$[\text{Ca}]_i = K_d \times \left[ \frac{(R - R_{\min})}{(R_{\max} - R)} \right] \times S_f / S_b$$

Where  $R$  = the ratio of cellular fluorescence emission intensity (510nm) with excitation at 340nm to emission intensity with excitation at 380nm

$R_{\min}$  = ratio at zero free  $\text{Ca}^{2+}$

$R_{\max}$  = ratio at saturating  $\text{Ca}^{2+}$

$S_f/S_b$  = fluorescence values at the denominator wavelengths for the free and bound forms of the dye (ratio of cellular fluorescence emission with excitation at 380nm for  $Ca^{2+}$  free and  $Ca^{2+}$  saturated buffers).

$K_d=225nM/L$  (dissociation constant)

Maximal ( $R_{max}$ ) and minimal ( $R_{min}$ ) fluorescence values were obtained by adding Triton X-100 (0.1%) and EGTA (20mM) sequentially at the end of the experiments. In the case of calcium free condition, HBSS without calcium chloride was used. Amlodipine ( $10\mu M$ ) was used as the positive control. The different experimental groups included control, ISO, CeNP+ISO and Amlodipine +ISO. CeNPs were used at 20 and 30  $\mu M$  concentrations that were found to induce the maximum antihypertrophic effect.

### 8. Statistical Analyses:

A minimum of 3 independent determinations were carried out for each assay. Numerical data are presented as mean  $\pm$  SD. The data were analyzed with 1-way analysis of variance and group differences were detected using a 2-tailed Student t test when initial analysis of variance revealed statistically significant differences. The significance was accepted at probability value,  $P\leq 0.05$ .

### Supplementary figure. 1 (S. Figure 1)

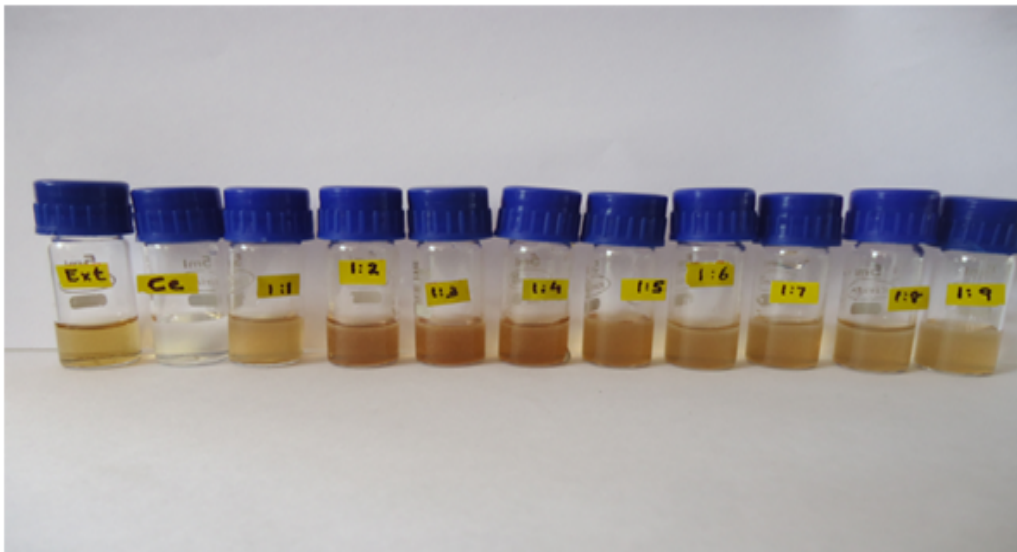


Figure legend: Formation of CeNPs using different volume ratios of CAN and CAE (1:1 to 1:9).