Redox Modulatory Anti-Inflammatory Potential of Hempseed (*Cannabis sativa*) based Green Selenium Nanoparticles

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ABSTRACT

Biomolecules mediated synthesis of nanoparticles (NPs) has revolutionized the nanotechnology research field due to its eco-friendly and non-toxic nature. The green approaches of NPs synthesis using plant extracts offer an effective and better alternative than other synthesis methods. Metal-based NPs have emerged at the forefront of biomedicine due to their versatility in health and therapeutics. In this context, Selenium (Se), a versatile trace element is known for its critical role in various pathophysiological processes through regulation of cellular redox status and inflammatory pathways besides others. However, these beneficial effects of Se are limited by its narrow physiologically relevant concentration range. Deficiency or excess of Se is associated with numerous pathologies and toxicities. Thus, in current study we have synthesized and characterized Hempseed (*Cannabis sativa*) based novel biogenic Se nanoparticles (SeNPs) using UV visible spectroscopy, DLS, EDX, FE-SEM, and FT-IR. Further, the phytochemical profiles and antioxidant as well as anti-inflammatory potential of these SeNPs were evaluated to ascertain their physiological benefits. Results indicated the spherical shaped SeNPs of average size 140–150 nm with favourable zeta potential of –45.0 mV. The phytochemical and Se analysis validated the redox modulatory anti-inflammatory potential of these hempseed based green SeNPs.

Keywords: Hempseed, Green synthesis, Selenium nanoparticles, Antioxidant activity, Anti-inflammatory potential

1. Introduction

Metal-based nanoparticles (NPs) due to their broad spectrum of applications ranging from optoelectronics, catalysis, biological probes, and drug delivery have emerged as the forefront of therapeutic approaches [1, 2]. In terms of their biological and pathophysiological implications, various metal NPs like gold, silver, copper, etc. have been used due to their enhanced biological activities and lower side effects [3]. Relevant to redox biology, NPs extending up to 200 nm can successfully eliminate free radicals [4] are considered efficient agents in therapeutics [1]. Gold NPs, for instance, show excellent catalytic properties due to its large surface area in redox reactions [5], whereas Silver NPs, due to its anti-inflammatory, anti-oxidant and anticancer properties, possess tremendous biomedical applications [6, 7]. Similarly, trace elements-based NPs, due to their diverse properties are generally used as a therapeutic agent.

Previous studies suggested that metal NPs have enhanced bioavailability and low toxicity effects on biochemical and hematological assays; thus, they can be considered as a better alternative for the drug delivery system [8]. In this regard, Selenium (Se), an interesting and essential trace element, demonstrates its beneficial antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic effects [9] through incorporation into various selenoproteins [10]. Though at adequate levels, it plays a crucial role in disease resistance and has immune modulatory activity [11]. The beneficial effects of Se are seen at a very narrow concentration range. Any physiological deficiency or excess of Se

is associated with pathologies and toxicity of Se respectively [1, 12, 13]. Thus, to overcome the drawbacks of high dosage of Se and at the same time maintaining its biological effects Se nanoparticles (SeNPs) are taken into consideration [14]. Based on the high catalytic efficiency, absorbing ability, and surface activity [15] SeNPs are reported to be more effective compared to the Se or selenite [16]. SeNPs regulate thyroid hormones [17], anti-atherosclerotic activity [18], anti-leukemia activity [19], and many others. In terms of the redox regulatory role, SeNPs also play a defensive role against DNA oxidation due to its anti-hydroxyl radicals features [20].

Despite these plausible benefits, the harsh chemical and expensive physical methods [13] involved in the NPs synthesis such as UV radiation [16], evaporation or laser ablation technique [21], use of harmful solvents, additives, stabilizers, acid decomposition, and reductants limit their use [1]. Whereas the chemical methods of NPs synthesis yield toxic wastes and are not eco-friendly [22], the physical methods of NPs synthesis require high-cost instrumentation [4]. Apart from this, high temperatures, acidic pH, and other harsh conditions can hinder the use of NPs as therapeutics for biomedical applications [23]. Thus, biological methods using microorganisms, enzymes, and plant extract are emerging as a new way to synthesize NPs [24, 25].

Amongst the biological methods too, the use of microorganisms and enzymes to synthesize NPs have some limitations, as they require aseptic conditions, which are not only difficult to maintain but are also time-consuming [26]. Thus, biosynthesis of NPs by plant extract has the edge over the other biological methods as it is cost-efficient,

less time consuming, and does not require any special conditions [27]. Studies over the decade have also demonstrated the enhanced potential benefits of many plant extracts-based metal NPs [24, 27–29]. Various reports indicate green synthesized SeNPs using different types of plant derived extract such as *Emblica officinalis* fruit extract [23], leave extract of *Withania somnifera* [30] and dried *vitis vinifera* (Raisin) extract [15].

Considering the exceptional nutritional value and numerous health benefits of hemp (*Cannabis sativa*) such as cardioprotection, preventing platelet aggregation, improving atopic dermatitis, and stabilizing blood sugar levels [31, 32]; currently, we have synthesized and characterized hemp-based green SeNPs. Further, we have reported the superior redox modulatory and anti-inflammatory effects of these SeNPs. These phytochemicals based green SeNPs are quick to synthesize, safe, cost-effective, environment friendly, and can be exploited as therapeutic interventions against various pathological conditions, where redox regulation of key pathways of inflammation are involved [15].

2. Experimental details

Plant collection and material:

Cannabis sativa (*C. sativa*) seeds were purchased from the Mystique Hills-Organic Living (Amazon, India). Source of selenium, Sodium Selenite (Na_2SeO_3) was procured from LobaChemie Pvt Ltd. (Mumbai, India). All the solutions were prepared in deionised double distilled water.

Preparation of plant extract:

The seeds were rinsed with sterile distilled water to clear contaminants and dried under shade. The desiccated seeds were crushed with a mortar and pestle. To prepare the extract, 5 g of powdered seeds were solubilised in 30 ml of deionised water. The solution was heated at 100 °C for 20 min, cooled down to ambient temperature, and then filtered (Whatman no.1). The final extract was obtained by the centrifugation of the filtrate at 8000 rpm for 15 min. The extract was refrigerated at 4 °C.

Biosynthesis of SeNPs:

For the eco-friendly green synthesis of SeNPs, an aqueous solution of sodium selenite (10 mM) was prepared and mixed with fresh plant extract of *C. sativa* at a ratio of 15:1 (two ml of hemp seed extract was added to 10 mM sodium selenite together with 30 ml of water). Finally, the reaction solution was autoclaved at 121 °C for 1 h at 1.5 bar for the synthesis of SeNPs, as described earlier [33].

Characterization of SeNPs:

UV-Vis spectrum analysis: The bioreduction of selenite to its elemental form was analyzed by UV-Vis spectroscopic analysis between 200 and 700 nm (PerkinElmer UV Vis spectrophotometer, Lambda-19) and the spectrum was plotted.

Size distribution analysis and zeta potential: Average particle size, polydispersity index (PDI), particle distribution and zeta potential of synthesized SeNPs was measured by particle size analyzer (Malvern Particle Size Analyzer MS2000).

Field Emission Scanning Electron Microscopy (FE-SEM) and EDX: The morphology of synthesized SeNPs was studied by FE-SEM (SU 8010 Series, Hitachi, Japan) with the accelerating voltage of 15 kV. Elemental analysis was also performed to determine the elemental composition of nanoparticles.

Fourier transform-Infrared (FT-IR) analysis: The major bio-active compound of the *C. sativa* that can be involved in the formation of nanoparticle process was determined by a spectral scan analysis at wave number ranging from 400 to 4000 cm^{-1} using FT-IR spectrophotometer (Model Spectrum RX-I, Perkin Elmer) with the resolution of 0.15 cm^{-1} to examine functional groups of hemp seed extract and SeNPs.

Free radical scavenging antioxidant activity of SeNPs:

DPPH assay: The DPPH solution (1 ml) was added to the mixture containing 200 μl of sample and 800 μl of 0.1 M Tris-HCl buffer (pH

7.4) into a test tube and kept at room temperature in the dark conditions. The absorbance of the final solution was measured at 517 nm. For blank, the sample solution was replaced with 1.2 ml ethanol and 800 μl Tris-HCl buffer [34].

The inhibition ratio (%) was calculated as following:

$$\text{Inhibition ratio (\%)} = \frac{A_c - A_s}{A_c} \times 100. \quad (1)$$

The absorbance of sample was expressed as A_s and the absorbance at addition of ethanol as A_c .

ABTS assay: To prepare the stock solution of ABTS, 7 mM ABTS aqueous solution was added to 2.4 mM potassium per sulphate and maintained in the dark conditions for 13–14 h at room temperature. The stock solution was diluted in ethanol (about 1:89 v/v) to attain a final absorbance of 0.700 ± 0.02 at 734 nm. 15 μM solution of ascorbic acid was used as standard and ethanol was used as Blank [35]. The percentage inhibition of various dilutions of sample extracts was calculated as :

$$\text{Inhibition ratio (\%)} = \frac{A_c - A_s}{A_c} \times 100. \quad (2)$$

Phytochemical screening:

Total phenol content: Spectrophotometric analysis was carried out to determine the phenolic content in aqueous extract of *C. sativa* and SeNPs as described by Barreira *et al.* [36]. The total phenolic content was revealed in terms of microgram of gallic acid equivalents (GAEs) per millilitre of extract.

Total flavonoid content: Spectrophotometric analysis was carried out to determine the flavonoid content in aqueous extract of *C. sativa* and SeNPs in the aqueous extract of *C. sativa* and SeNPs as described by the method of Kathirvel and Sujatha [37]. The total flavonoid content was revealed in terms of microgram of quercetin equivalents (QEs) per millilitre of extract.

Selenium estimation by 2,3-diaminonaphthalene (DAN):

Selenium concentration in NPs was estimated using fluorometric method [38]. For digestion of sample HNO_3 was added followed by HClO_4 . After sample hydrolysis with 9 % HCl, the digest was reacted with DAN 2,3-diaminonaphthalene under acidic condition. The selenodiazole so formed was extracted with cyclohexane. EDTA and hydroxylamine hydrochloride was used as masking-reducing agent. Subsequently fluorometric Se estimation (PC spectrofluorophotometer) was performed at 376 nm and emission wavelength at 518 nm. Sodium selenite was used as standard.

In-vivo anti-inflammatory assessment:

Anti-inflammatory activity of the SeNPs solution at 0.1 mg/kg concentration were ascertained by the carrageenan-induced hind paw edema model. 16 Balb/C female mice were divided into four groups with four animals per group ($n = 4$). The animals were subjected to 16 h starvation before the experiment. Inflammation was induced by injecting 0.1 ml of 1 % w/v carrageenan suspension in the right hind foot pad of mice. The animals in the first two groups received subcutaneous injection of SeNPs (0.1 mg/kg) and sodium selenite (0.2 mg/kg body weight) just after carrageenan treatment. 0.5 % DMSO was used as negative control and Indomethacin (20 mg/kg body weight) was used as positive control. The diameter of the inflamed paw was checked until four hours using thread and scale. The percent edema inflammation (%) is defined as follows:

$$\text{Edema} = \left(1 - \frac{V_c}{V_t}\right) \times 100, \quad (3)$$

where V_c denotes edema volume in control and treated groups or Indomethacin [7].

Statistical analysis: The data were analysed using one-way analysis of variance (ANOVA) and multiple posthoc test (Tukey) to compare various treatment groups using the GraphPad Prism 8.0 program

(GraphPad Software, San Diego, CA). The significance was set at $p < 0.05$. All data were expressed as mean $\pm$ SD of at least ($n = 6$) independent observations (for anti-inflammatory studies $n = 4$).

3. Results and discussion

Se and Se based compounds have gained substantial attention due to their promising pathophysiological and chemotherapeutic potential. However, oxidative pathologies and toxicity associated with Se at deficient or excess concentration, respectively, restrict its biomedical and therapeutic potential. Chemically synthesized SeNPs due to their low toxicity and excellent bioavailability have a extensive of biomedical applications including chemoprevention, antimicrobial effect, antifungal activity as well as reducing oxidative stress [9]. However, the chemical synthesis of SeNPs uses expensive methods, harsh chemicals, and generates harmful and toxic substances. Thus, recently the usage of plant extracts has become an interest due to its clean and simpler approaches. Different green synthesized SeNPs have been explored for various bio-potential applications such as anti-inflammatory activity [39] anti-oxidant activity [40] and anti-cancer activity [41]. SeNPs synthesis employing plant extracts is not only eco-friendly and safe but also has tremendous advantages and wide applicability in the field of nanomedicine [42–46]. Considering the unique and balanced nutritional composition as well as therapeutic and pharmacological properties of hempseeds (*C. sativa*) [47, 48] currently, we report the green synthesis of SeNPs from its aqueous seed extracts.

Synthesis and characterization of SeNPs: Green synthesis of SeNPs was performed by the reduction of sodium selenite solution (10 mM Na_2SeO_3) into elemental selenium with the addition of aqueous seed extract of *C. sativa*. The final reaction mixture was subjected to the autoclave to obtain SeNPs, as described earlier [33]. The concentration of Se was first analyzed by the spectrofluorometric method using DAN. The SeNPs thus obtained were characterized by UV-visible spectroscopy, FE-SEM, EDX, and FT-IR.

Metal nanoparticles possess unique optical properties, which change proportionally with the shape and size of nanoparticles. The conversion of selenite (Se^{+4}) to elemental selenium (Se^0) was accompanied by the color change from colorless to orange, indicating the successful formation of SeNPs [Fig. 1(a)]. In accordance with the previous studies, this color change can be attributed to the excitation of the surface plasmon resonance [49]. The synthesis of these biogenic SeNPs was ascertained by UV-Vis spectroscopy. The electronic spectra demonstrated a wide peak around 300 nm representing the formation

Figure 1. Illustration of color change on the reduction of sodium selenite by aqueous *C. sativa* seed extract: (a) before synthesis and after SeNPs formation, (b) UV-Vis spectra of (i) selenium nanoparticles, (ii) aqueous extract of hemp seed, and (iii) solution of sodium selenite, and (c) EDX spectrum of SeNPs synthesized using *C. sativa* seed extract.

Figure 2. (a) Histogram of SeNPs size distribution obtained by DLS, (b) Zeta potential of SeNPs synthesized by aqueous seed extract of *C. sativa*, and (c) FT-IR spectra of synthesized SeNPs and seed extract of *C. sativa* were measured in % transmittance in the wavenumber frequency range of 4000–400 cm^{-1} .

of SeNPs [Fig. 1(b)]. These results are in concordance with various reported studies concerning the synthesis of SeNPs [11, 50].

The morphological characterization of SeNPs was performed by measuring size, size distribution, and PDI using DLS (dynamic light scattering) analysis. DLS histogram revealed that the mean size of SeNPs is 183.8 nm [Fig. 2(a)]. PDI value is the indicator of the homogeneity of nanoparticle size. In the current study, the PDI of the SeNPs was 0.032, which indicates a highly monodisperse nanoparticle system with negligible aggregation [1]. Further, the stability of colloidal SeNPs was ascertained by zeta potential analysis (electric potential surrounding the particle). A sharp peak of zeta potential for negatively charged SeNPs was noticed at -45.0 mV [Fig. 2(b)], which confirmed repulsion between particles to prevent agglomeration. The nanoparticles, with a higher magnitude of zeta potential exhibits, increased stability due to greater electrostatic repulsion between nanoparticles [1]. The negative value of zeta potential indicates that the capping of NPs might be responsible for their observed charge and long-term stability [51].

Further, the size and morphology of the green SeNPs were confirmed by FESEM analysis. The FESEM images of biogenic SeNPs suggest that the particles maintain uniformity and are well dispersed without any aggregation. Synthesized SeNPs were spherical in shape with an average size of 140–150 nm [Fig. 3]. The observed difference between FESEM and DLS size distributions is due to the fact that size measured by DLS is representative of the size of hydrodynamic diameter, which represents the hypothetical sphere that diffuses at the same rate as the particles being measured, while the size measured from FESEM observations informed the exact physical size of the particle [52].

The chemical composition of SeNPs evaluated using EDX revealed

Figure 3. FE-SEM micrographs of SeNPs synthesized by using aqueous *C. sativa* seed extract.

the presence of two strong signals of Se at 1.4 and 11.2 keV [53], indicating the presence of substantial Se concentration in SeNPs. The peak of carbon and oxygen in the sample solution indicates the presence of stabilizers [15], whereas the peak of sodium suggests the presence of sodium selenite in the solution. The lack of other elemental peaks and strong signals of selenium metal in the spectra confirms the purity of the SeNPs [Fig. 1(c)].

FT-IR spectroscopic analysis was performed to determine the major functional groups present on the surface of phytochemical SeNPs and in the *C. sativa* aqueous seeds extract and their possible involvement in the synthesis and stabilization of SeNPs. Figure 2(c) depicts spectral changes in the FT-IR profile for extract and SeNPs with slight peak shifts due to the interactions between functional groups and metals. These functional groups are known to mediate the reduction of nanoparticles [54]. The electronic spectral transmittance peak at 3339.4 cm^{-1} was attributed to O-H stretching vibration assigned to -OH group of phenols and alcohols which indicated the presence of cannabinoids [55]. A very low-intensity peak of the SeNPs spectrum at 2153.4 cm^{-1} corresponds to the $\text{C}=\text{C}=\text{O}$ stretching mode. The centered peak at 1643.1 cm^{-1} was associated with the protein (amides I) [33]. The report also confirms the presence of flavonoids in the same region acquired by the above peak [56]. The results suggest the involvement of phenols and flavonoids as reducing agents from seed extract in the formation of nanoparticles [57]. The spectral peak at 1408.5 cm^{-1} was allocated to C - C stretching of the aromatic ring [30]. The strong peak at 1088.6 cm^{-1} was safely assigned to C - N stretching vibration of aromatic amines.

SeNPs demonstrate enhanced antioxidant properties:

To explore the biological functions of these SeNPs *in vitro* antioxidant activity assay and total phenolic and flavonoid contents were evaluated. The DPPH and ABTS based free radical scavenging assays were performed to assess the antioxidant activity of synthesized SeNPs using ascorbic acid as a positive control. As depicted in Figs. 4(a) and 4(b), phytochemical SeNPs show increased antioxidant activity in a dose-dependent manner. On similar lines, the potential antioxidant activities of chemically and green synthesized SeNPs have also been reported in the literature [40, 58]. The increased ABTS and DPPH radical scavenging activity of biogenic SeNPs compared to ascorbic acid could be attributed to the synergistic free radical scavenging effect of SeNPs and the bioactive capping agents. Various reports confirm that the phytochemicals present in the plant extract act as capping constituents on the surface of nanoparticles [51].

The phytochemical screening of SeNPs validated these results. Total phenolic and flavonoid content of *C. sativa* aqueous seeds extract and synthesized SeNPs were evaluated to confirm the presence of these phytochemicals (Table I). The results revealed that SeNPs have a substantial concentration of flavonoids and phenolic compounds, which could be mainly responsible for the reduction and stabilization of metal ions. A plethora of studies, revealed the formation of metal NPs by the aid of phytoconstituents present in the plant extract possesses diverse medicinal properties [59]. The concentration of Se in the nanoparticles was measured by the fluorometric (DAN) assay. The total concentration of Se revealed by spectrofluorometric analysis using DAN was $403.8 \pm 34.54\text{ ppm}$.

Paw edema/anti-inflammatory potential of Se nanoparticles:

Upon switching from *in-vitro* to *in-vivo* systems, carrageenan-induced acute inflammation mice model was used to assess the anti-inflammatory

Figure 4. The increase in concentration (a) the % decay of DPPH radical and (b) the % decay of ABTS was more in the case of SeNPs compared to standard (Ascorbic acid). Data is expressed as mean $\pm$ SD of at least 7 and 11 independent observations respectively and analyzed using paired t-test represents $p < 0.05$. (c) Anti-inflammatory effect on groups based on carrageenan-induced paw edema. Data is expressed as mean $\pm$ SD of at least 4 independent observations and analyzed using one-way ANOVA (Tukey's multiple comparison method) representing $p < 0.05$.

potential of synthesized SeNPs. Carrageenan, a high molecular weight sulfated polysaccharide has been widely used to evaluate the antiedematous effect of the compounds while producing a high degree of reproducibility [60]. Due to the increased vascular permeability, the subcutaneous administration of carrageenan in the subplantar region of mice causes swelling along with the edema formation [61]. Carrageenan induced edema is a triphasic response. The initial stage is mediated by the release of histamine and serotonin, while the second phase is linked with the release of kinins (2 h), and the next phase is attributed to prostaglandins and cyclooxygenase substances which peaks at 5 h [60]. The subcutaneous administration of 0.1 mg/kg (body weight) SeNPs resulted in a significant decrease in the inflammation as compared to the vehicle (0.5 % DMSO). The maximum inflammation percentage obtained were 10 (2 h), 15 and 20 % (4 h) for the doses of 0.1 mg/kg SeNPs and sodium selenite (0.2 mg/kg) as described in Fig. 4(c). It is evident from the current study that the anti-inflammatory effect of SeNPs (0.1 mg/kg) is almost comparable with sodium selenite (0.2 mg/kg) group and Indomethacin, a standard non-steroidal anti-inflammatory drug used against inflammatory conditions.

Studies indicate that inflammation causes the dilation of blood vessels and contraction of endothelial cells that allows extravasations of nanoparticles (in the size range 20–200 nm) into the tissues in the inflamed areas through enhanced permeability and retention (EPR) effect [62]. In the current study, the anti-inflammatory potential of SeNPs in the edema region may be attributed to enhanced permeability and retention effect. The subcutaneous administration of SeNPs may lead to site-specific accumulation of SeNPs in the impaired region that eventually resulted in attenuation of inflammation. Reduction in inflammation percentage by nano form of Se proves to be more effective than the inorganic form of Se because of its free radical scavenging activity and lower toxicity [63]. Previous reports demonstrate that plant derived SeNPs possess anti-inflammatory activity [39]. Various studies have shown that phytochemicals prove to be successful in mediating the anti-inflammatory response [64]. Flavonoids have been found to inhibit the release of histamine, cytokine, and prostaglandins [65, 66], and phenolic compounds are effective in treatment against inflammatory disorders [67]. As validated earlier in the FT-IR analysis, the presence of a substantial amount of phenols and flavonoids along with Se present in SeNPs might be responsible for aiding anti-inflammatory action. The obtained results suggest that green synthesized SeNPs provides a better antioxidant alternative in antagonizing the effect of the inflammatory mediators as compared to the sodium

Table I. Values are expressed as mean $\pm$ SD, $n = 3$ independent observations ^a. Total phenolic content in terms of Gallic acid equivalent per milliliter; ^bTotal flavonoid content in terms of quercetin equivalent per milliliter.

Bioactive compound	Phenolic content ($\mu\text{g GAE/ml}$) ^a	Flavonoid content ($\mu\text{g QE/ml}$) ^b
Extract	3.058 ± 1.355	69.44 ± 36.66
SeNPs	4.240 ± 3.289	33.21 ± 12.32

selenite.

4. Conclusions

The green approach towards the synthesis of nanoparticles has been proposed as cost-effective and environment friendly alternative to chemical and physical methods. The current study elucidates the phytofabrication of SeNPs using aqueous seed extract of *C. sativa*, deploying a simple and reproducible method. Seed extract rich in phytochemicals such as phenols and flavonoids act as capping constituents in the bioreduction of SeNPs. The antioxidant potential of biogenic SeNPs could be attributed to the synergistic free radical scavenging effect of SeNPs and the bioactive capping agents present in the seed extract. Furthermore, *in-vivo* studies conclude that biogenic SeNPs exhibits anti-inflammatory activity. These SeNPs hold the potential to pave the path towards developing nutraceuticals and biologically active compounds based facile nanoparticle against various debilitating diseases.

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