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The Anti-oxidant and Anti-inflammatory Properties of Cerium Oxide Nanoparticles Synthesized Using *Origanum majorana* L. Leaf Extract

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Abstract

Introduction: Free radicals have singlet electron in their outer layer rendering them high reactivity against biomolecules (i.e., DNA, carbohydrates, proteins, and lipids). Oxidative stress is created when the production of free radicals exceeds their removal by antioxidant systems and is involved in the pathogenesis of several diseases such as diabetes, arthritis, inflammatory conditions, and various cancers. Regarding the therapeutic potential of nanoparticles (NPs) in human diseases, the purpose of this study was to synthesize cerium oxide NPs using *Origanum majorana* leaf extract.

Methods: Cerium oxide nanoparticles (CeO_2-NPs) were synthesized using aqueous leaf extract of *O. majorana*. The sizes of NPs were characterized by a particle size analyzer. The antioxidant properties of the CeO_2-NPs were determined by Ferric-reducing antioxidant power (FRAP) assay. The anti-inflammatory effects of the NPs were also determined by measuring gene expressions of IL-1β and IL-10 using real-time polymerase chain reaction (PCR).

Results: The CeO_2-NPs were successfully synthesized using *O. majorana* leaf extract. The results of FRAP assay showed that the anti-oxidant activities of CeO_2-NPs at concentrations of 50, 100, and 400 µg/mL were 75%, 77.1%, and 94.5%, respectively. Moreover, interleukin 10 (IL-10) gene expressions increased by 4.6 folds while the expression of IL-1β gene decreased by 0.75-fold in HUVECs.

Conclusion: The CeO_2-NPs synthesized using the aqueous extract of *O. majorana* demonstrated antioxidant and anti-inflammatory properties. Therefore, these NPs can be used as potential therapeutic agents in medicine.

Keywords: Cerium oxide nanoparticle, Green synthesis, *Origanum majorana*, Interleukin, Anti-inflammatory

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Introduction

Free radicals have one or more unpaired electrons in their outer electron layers. These molecules inflict the structure and function of various biological molecules such as nucleic acids, proteins, and lipids.[1,2] Living organisms benefit from various antioxidant mechanisms to neutralize these free radicals.[3] An imbalanced ratio of production and removal of free radicals triggers oxidative stress affecting cellular metabolism and multiple biological processes such as signal transduction, gene expression, cellular proliferation, and programmed cell death.[4] Oxidative stress is involved in the pathogenesis of many disorders such as neurological diseases, diabetes, arthritis, inflammation, and cancer.[5]

Inflammation is a complex host defense mechanism against invading microorganisms. Nevertheless, chronic inflammatory conditions can increase the risk of cancer and malignant transformation and modulate tumor angiogenesis and metastasis by suppressing anti-cancer immune responses.[6]

Nanoparticles (NPs) are materials with variable sizes (1-100 nm) and surface to volume ratios [7], giving them unique physical, chemical, and biological properties.[8-10] In previous studies, NPs have shown antioxidant,[11] anti-bacterial, anti-inflammatory,[12] anti-cancer and many other biological properties.[13] Accordingly, NPs have considerably widespread

applications in medicine, as well as pharmaceutical, food safety, and other industries.[14] Cerium oxide (CeO_2) is a lanthanide metal oxide with anti-oxidative properties.[15] CeO_2 synthesized NPs (CeO_2-NPs) have commonly been used in biomedicine as anti-cancer[16] and wound healing agents.[17]

Due to the presence of biologically active substances such as phenols, medicinal plants have been used for synthesizing NPs.[18-20] *Origanum majorana* is a globally available medicinal plant grown in many regions of the world. In this study, the aqueous leaf extract of *O. majorana* was used to synthesize CeO_2-NPs (i.e., green synthesis). The synthesized NPs were further characterized using the particle size analyzer. In addition, the anti-oxidant and anti-inflammatory properties of the CeO_2-NPs were evaluated by Ferric-reducing antioxidant power (FRAP) assay and real-time PCR, respectively.

Materials and Methods

Chemicals and Reagents

The PCR Master Mix, SYBR green PCR master mix, RNeasy Mini Kit, and cDNA Synthesis Kit were purchased from Qiagen GmbH, Hilden (Germany). Other reagents not mentioned here were from Merck (Germany).

Preparation of Plant Extract and Synthesis of CeO_2 NPs

In order to prepare the aqueous extract, 10 g of dried *O. majorana* leaf powder was added to 100 mL distilled water, heated up to 100°C and incubated for 10 minutes. For the biosynthesis of CeO_2-NPs, 8.68 g of $Ce(NO_3)_3.6H_2O$ was allowed to react with 200 mL of aqueous leaf extract of *O. majorana*. In the next step, the CeO–*O. majorana* mixture was dried at 100°C for 48 hours. Finally, the green-synthesized CeO_2-NPs were purified by heating at 450°C for 4 hours to obtain brownish pellets.

Characterization Procedures

The synthetized CeO_2-NPs were characterized using a particle size analyzer.[21] In brief, the size distribution of NPs was analyzed using Zetasizer instrument (Malvern, UK). The diameter of CeO_2-NPs was determined using Nano-ZS90 dynamic light scattering instrument (Malvern, UK) at a 90° fixed-angle and room temperature.

FRAP Assay

The ferric reducing capacity of the CeO_2-NPs was determined as described by Ozgen et al.[22] One milliliter of different concentrations of CeO-*O. majorana* was mixed with 2.5 mL potassium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1 g/100 mL). The mixture was incubated at 50°C for 25 minutes. Then, trichloroacetic acid (10%) was added to the mixture to stop the reaction. An equal volume of distilled water was subsequently added followed by addition of 0.5 mL ferum chlorate (0.1 g/100 mL) ($FeCl_3$). The procedure was carried out in triplicate. The mixture was allowed to stand for 30 minutes before measuring absorbance at 700 nm.

Expression of Anti-inflammatory and Pro-inflammatory Genes

The expressions of interleukin 10 (IL-10) and IL-1β genes were determined in human umbilical vein endothelial cell (HUVEC) lines treated with the synthesized NPs. The cells were seeded at 5×10^3 cells/mL concentration in a 6-well plate supplemented with RMPI 1640, FBS 10%, and Pen-Strep 0.5%. The cells were then treated with different concentrations of NPs (i.e., 0, 50, 100, and 200 µg/mL) and incubated for 48 hours. At the end of incubation, the treated cells were washed with phosphate-buffered saline (PBS, 0.1 M, pH 7.2) twice and scraped with Trypsin. The gene expression of IL-10 and IL-1β was assessed using real-time PCR and primers mentioned in Table 1.

RNA Extraction

After 48 hours of incubation with the NPs, total RNA was extracted from the HUVECs. Briefly, 1 mL of the ice-cold RNX-plus solution was added to homogenized cells and mixed by vortexing. Then, 200 µL chloroform was added and the solution was centrifuged at 12000× g for 15 minutes at 4°C. An equal volume of isopropyl alcohol was added to the aqueous phase, and the mixture was centrifuged again. Afterwards, 75% ethanol (1 mL) was added to the supernatant. The solution was finally centrifuged to extract RNA. The RNA concentration was calculated using NanoDrop UV-Vis spectrophotometer followed by denaturing 1% agarose gel electrophoresis.

cDNA Synthesis

The cDNA was synthesized from the total extracted RNA using fermentase Kit according to the manufacturer's instructions. The mixture was incubated in thermal cycler for one cycle at 37°C for 15 minutes, one cycle at 85°C for 5 seconds and one cycle at 4°C for 5 minutes. In addition, samples without RT enzymes were used to detect contamination in the samples.

Real-Time Polymerase Chain Reaction

SYBR green-based real-time PCR (Qiagen Rotor-Gene Q, Hilden, Germany) was used to assess the expression of *IL-1β* and *IL-10* genes. Amplification conditions were set as follows: an initial step at 95°C for 2 minutes followed by 30 cycles of 95°C for 15 seconds, 56.4°C for 20 seconds, and 72°C for 30 seconds. Melting curves were generated

Table 1. Primer Sequences for Analysis of IL-10 and IL-1β Gene Expression

Gene	Sequences (5′ to 3′)
IL 10	F TGGAGGACTTTAAGGGTTAC
	R GATGTCTGGGTCTTGGTT
IL 1-B	F GCTTATTACAGTGGCAATGA
	R GTGGTCGGAGATTCGTAG
GAPDH	F CGTGCTGAATGAGGAACAGA
	R AGTCAGGGTGGACCTCAGTG

by monitoring the fluorescence of SYBR green signal from 65°C to 95°C. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as the reference gene. Negative control contained ddH2O.

Statistical Analysis

The obtained data were analyzed by ANOVA test using SPSS software version 22.0. Significant results were confirmed by Duncan's multiple range test. *P* value of less than 0.05 was considered statistically significant. All tests were performed in triplicate, and the results were expressed as mean values ± standard deviations (mean ± SD).

Results

Nanoparticles Sizes

The results of particle size analysis are shown in Figure 1. The size of CeO_2-NPs ranged from 10 to 70 nm. The average particle size was about 25 nm.

Antioxidant Activity Assessments

The antioxidant activity was investigated using FRAP assay by determining free radical scavenging capacity of the CeO_2-NPs (Figure 2). The CeO-*O. majorana* mixture actively reduced Fe^{3+} to Fe^{2+}. At concentrations of 50, 100, and 400 µg/mL, the antioxidant capacities of CeO_2-NPs were 75%, 77.1%, and 94.5%, respectively, indicating dose-dependent antioxidant activity of the synthesized CeO_2-NPs.

Gene Expression of IL-10

The anti-inflammatory activity of CeO_2-NPs was evaluated by measuring *IL-10* gene expression as the main biomarker of anti-inflammatory immune response. HUVECs treated with 0, 50, 100, and 200 µg/mL CeO_2-NP for 24 hours showed significant up-regulation of IL-10 ($P<0.001$) as compared to untreated cells (Figure 3).

IL-1β Gene Expression

The pro-inflammatory activity of CeO_2-NPs was evaluated by assessing IL-1β gene expression as a pro-inflammatory biomarker. HUVECs treated with 50, 100, and 200 µg/mL CeO_2-NPs for 24 hours were analyzed. Only at the 200 µg/mL concentration, CeO_2-NPs significantly decreased the expression of IL-1β gene compared with untreated control cells ($P<0.001$, Figure 4).

Discussion

Several studies have reported potent antioxidant properties for CeO_2-NPs. In this study, the green-synthesized CeO_2-NPs (using *O. majorana* leaf extract) also revealed high antioxidant activity. At 400 µg/mL concentration, the synthesized CeO_2-NPs reduced more than 94% of ferric (Fe^{3+}) ions to ferrous (Fe^{2+}). In another study, ROS content significantly decreased in H9C2 cells exposed to CeO_2-NPs with sizes of 1, 10, and 100 nm for 24 hours.[23]

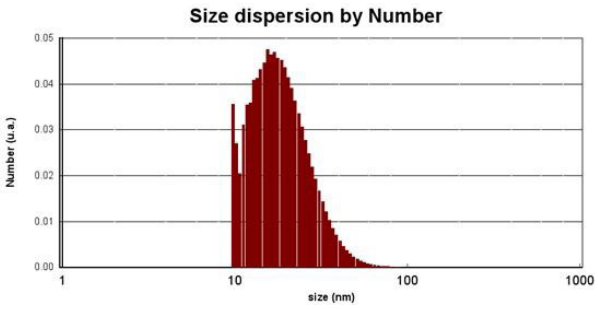

Figure 1. The Particle Size Distribution Analysis of CeO-NPs.

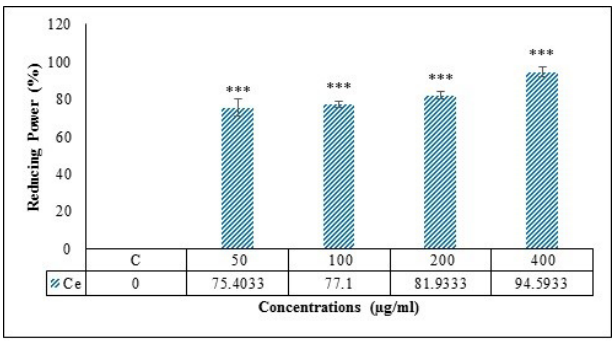

Figure 2. Ferric Reducing Antioxidant Activity of CeO-NPs Synthesized Using *O. majorana L.* Leaf Extract. The experiment was conducted in triplicate. *** $P<0.001$; significant difference as compared to the control (i.e., no CeO_2-NP treatment).

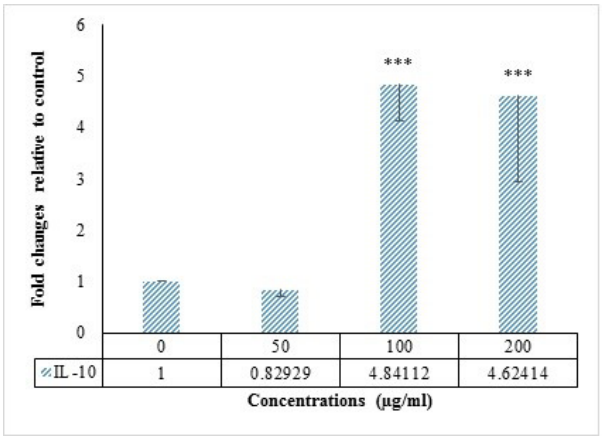

Figure 3. The Expression of *IL-10* Gene in HUVECs Upon Treatment With 50, 100, and 200 µg/mL of CeO_2-NPs for 24 Hours. *** $P<0.001$ indicated significant difference compared with the control (i.e., no CeO_2-NP treatment).

In another study, CeO_2-NPs synthesized using the extract of *Hyssopus officinalis* plant effectively scavenged DPPH free radicals.

We here investigated the effects of CeO_2-NPs on the gene expressions of IL-10 and IL-1β by real-time PCR. Accordingly, the expression of IL-10 (anti-inflammatory) significantly increased in a dose-dependent (50, 100, and 200 µg /mL CeO_2-NPs) manner. In addition, the expression of IL-1β (pro-inflammatory) significantly

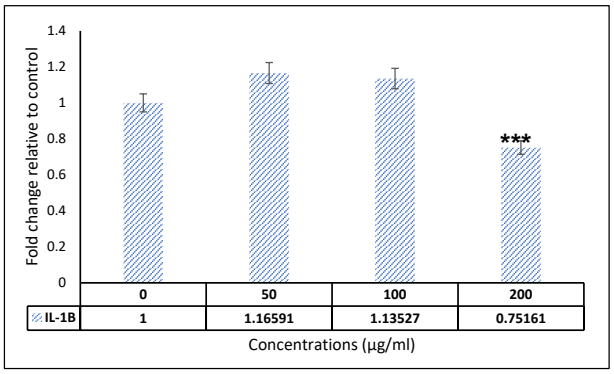

Figure 4. The Gene Expression of IL-1β in HUVECs Upon Treatment With 50, 100, and 200 µg/mL CeO_2-NPs for 24 Hours. *P<0.05, and ***P<0.001 indicated significant differences compared with the control (i.e., no CeO_2-NP treatment).

decreased at 200 µg/mL concentration of CeO_2-NPs.

Inflammation is a defensive mechanism protecting the body against various pathogens. Nevertheless, chronic inflammation can lead to various tissue damages and pathologic conditions.[24,25] Chronic inflammatory conditions may be triggered by genetic mutations, autoimmune diseases, or multiple environmental factors.[26] In some cases, chronic inflammation may even lead to cancer.[27]

CeO_2-NPs (Ce^{3+}/Ce^{4+}) can have many therapeutic applications due to their potent antioxidative and anti-inflammatory properties.[28] Both anti-inflammatory and free-radical scavenging properties of CeO_2-NPs have been shown in numerous studies.[29-31] In another study, CeO_2-NPs represented anti-inflammatory properties in the brain tissue of mouse accompanied by increased expression of *iNOS* gene.[29] In BALF lymphocytes, anti-inflammatory properties of CeO_2-NPs have been investigated by measuring pro-inflammatory genes expression (i.e., TNF-α, IL-1β, MIP-2, IL-13, and IFN-γ).[32]

The anti-inflammatory properties of *O. majorana* extract have also been investigated. In this study, we also assessed the anti-inflammatory effects of *O. majorana* extract in HUVECs. Our results showed that the expression of pro-inflammatory genes such as IL-1β decreased, while the expression of anti-inflammatory genes such as IL-10 increased in HUVECs. The anti-inflammatory properties of *O. majorana* extract can be attributed to anti-inflammatory signaling pathways triggered by its ingredients.[33]

Conclusion

In this study, CeO_2-NPs were synthesized using *O. majorana* L. leaf extract and a bio-reduction method. The synthesized NPs exhibited all characteristic features of functional NPs. Our results confirmed the antioxidant and anti-inflammatory properties of the CeO_2-NPs. Our results suggested that these NPs can be used as anti-inflammatory and anti-oxidative agents in biomedical fields. However, further in vivo studies are required.

Ethical Approval

Not applicable.

Competing Interests

The authors have no conflict of interest to declare.

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