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OPEN Zein-sodium caseinate-diosmin nanoparticles as a promising anticancer agent with targeted efficacy against A2780 cell line

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This research investigated the potential of zein-sodium caseinate-diosmin nanoparticles (ZCD-NPs) as an anti-cancer agent against the A2780 cell line. Dynamic light scattering (DLS) analysis showed that ZCD-NPs have an average size of 265.30 nm with a polydispersity index of 0.21, indicating good uniformity suitable for pharmaceutical applications. Fourier transform infrared spectroscopy (FTIR) confirmed the successful incorporation of diosmin into the NPs and highlighted the interactions between the components. Field emission scanning electron microscopy (FESEM) images showed spherical NPs with smooth surfaces, suggesting stability and high production quality. Encapsulation efficiency was remarkably high, at 93.45%. Cytotoxicity assays showed a dose-dependent effect of ZCD-NPs, with A2780 cells showing significant sensitivity compared to normal HDF cells, indicating selective targeting of cancer cells. Flow cytometry analysis confirmed that ZCD-NPs induced apoptosis and necrosis in A2780 cells, as evidenced by increased expression of apoptotic genes such as p53 and caspases 8 and 9. In addition, ZCD-NPs exhibited potent antioxidant activity, effectively scavenging free radicals. These results suggest that ZCD-NPs have promising properties for targeted cancer therapy and antioxidant applications, which warrant further exploration in clinical settings.

Keywords Diosmin, Zein-caseinate, Sodium caseinate, A2780, Nanoparticles

Pharmaceutical nanocarriers are considered drug delivery systems (DDSs) for cancer treatment. Nanocarriers are biocompatible and non-toxic vehicles that deliver therapeutic agents to cancerous tissues with minimal impact on healthy cells¹. They improve drug pharmacokinetics and biodistribution, enhance stability and solubility, and enable controlled site-specific drug delivery^{2,3}. Nanoparticles (NPs), nanotubes, dendrimers, and polymeric or lipid-based carriers like liposomes are essential for improving anti-cancer drugs and having fewer side effects⁴. Conventional treatments often damage healthy cells and cause serious side effects. However, nanocarriers such as NPs, with sizes ranging from 1 to 100 nm, can be engineered to deliver drugs directly to the malignancy by binding to specific markers of cancer cells⁵. In addition, NPs can be precisely targeted to cancerous tissues using imaging technologies⁶. Another advantage of NPs is overcoming drug resistance, one of the biggest challenges in cancer treatment⁷. Once in the bloodstream, NPs acquire a protein corona that affects their interactions with biological environments. This corona affects the pharmacokinetics and biodistribution of NPs. Protein corona, such as casein, can enhance cellular uptake and stability, improving therapeutic effects while reducing side effects⁸.

In this context, zein-sodium caseinate composites (ZC-composites) have emerged as a promising DDS in nanomedicine⁹. In various studies, ZC-composites have demonstrated efficacy in delivering anti-cancer drugs, antibiotics, and bioactive compounds, showing promise in treating cancer, infectious diseases, and inflammatory conditions¹⁰⁻¹³. These NPs leverage the unique properties of zein, a hydrophobic corn protein, and casein, an amphiphilic milk protein. Zein enhances the encapsulation of hydrophobic compounds, while casein improves dispersion in water and offers binding sites for hydrophilic drugs^{14,15}. In addition, casein, a milk protein, effectively encapsulates bioactive compounds, enhancing solubility and controlled release. Research indicates that casein-based formulations can improve drug dissolution and bioavailability¹⁶. Curcumin-loaded

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casein NPs show enhanced cytotoxicity against cancer cells¹⁷. Casein NPs enhance the solubility and stability of poorly soluble compounds like apigenin, significantly boosting oral bioavailability¹⁸. ZC-composite can be easily functionalized to target specific cells, improving the therapeutic index of encapsulated drugs. Their natural origin also addresses the toxicity and immunogenicity associated with synthetic nanocarriers. ZC-composite's versatility, biocompatibility, and efficacy make them valuable tools for targeted therapy.

Given the advantages of ZC-composite, we used these nanocarriers to deliver diosmin to ovarian cancer cells in an in vitro model. Diosmin (3',5,7-trihydroxy-4'-methoxyflavone-7-rhamnoglucoside) is a citrus flavonoid glycoside with antioxidant, anti-inflammatory, and vasoprotective properties^{19,20}. Recent evidence has also revealed diosmin's anti-cancer potential. Studies suggest that diosmin has antiproliferative effects against several cancer cell lines, including colon²¹, breast²², and prostate cancer²³. It was demonstrated that diosmin can inhibit tumor growth and metastases by blocking angiogenesis by downregulating vascular endothelial growth factor (VEGF)²⁴. The restriction of angiogenesis is an essential strategy for controlling cancer progression²⁵. Also, diosmin acts on several key signaling pathways associated with cancer progression and apoptosis. It inhibits the STAT3/c-Myc pathway, promoting apoptosis and reducing tumor growth, particularly in osteosarcoma²⁶. In addition, diosmin induces apoptosis by modulating apoptotic markers such as Bcl-2, Bax, and caspases, enhancing its potential as a chemotherapeutic agent. It also regulates the NF-KB signaling pathway, potentially alleviating inflammation associated with cancer development²¹. By modulating the Wnt signaling pathway, diosmin may inhibit tumor growth and metastasis. In addition, it affects the PI3K/Akt pathway, leading to decreased tumor cell viability and increased apoptosis while also affecting the JAK/STAT pathway to inhibit cancer cell proliferation and survival²⁷. However, the bioavailability of diosmin is an essential factor affecting its therapeutic efficacy^{28,29}. Diosmin has relatively low bioavailability when administered orally, mainly due to its poor water solubility and limited gastrointestinal absorption^{30,31}. Therefore, we hypothesized that encapsulation with the ZC-composite could improve the bioavailability of diosmin by increasing its solubility and stability. Consequently, we evaluated the effects of diosmin loaded in ZC-composite (ZCD-NPs) on A2780 ovarian cancer cell lines in an in vitro model.

Materials and methods

Preparation of ZCD-NPs First, zein powder (1 g) and diosmin po

First, zein powder (1 g) and diosmin powder (0.1 g) were dissolved in 20 mL ethanol (80%). The solution was diluted with 50 mL of distilled water containing sodium caseinate (25 mg/mL) and stirred continuously at 25 °C. The dispersion was then desolvated under vacuum at 45 °C and centrifuged at 2000 ×g for 10 min to remove the large aggregates. Finally, the dispersion was lyophilized for 24 h, and the powder obtained was stored at -20 °C.

Characterization of ZCD-NPs

Dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR), and field emission scanning electron microscopy (FESEM) were used to characterize ZCD-NPs. DLS was used to determine the hydrodynamic size and distribution of ZCD-NPs in an aqueous solution using a Malvern Zetasizer Nano ZS (UK). Samples were diluted, and experiments were performed at room temperature with a scattering angle of 90°. In addition, zeta potential was measured to assess the surface charge and stability of the ZCD-NPs. The zeta potential was determined based on the frequency shift of a laser at 28 °C and light scattering at -14 °C. FTIR analysis was performed to identify functional groups and confirm the chemical structure of the particles. ZCD-NPs were dispersed (10 mg) in 100 mg KBr and then scanned in the 400 to 4000 cm⁻¹ range at 20 °C using an infrared spectrophotometer (Nicolet 5700; USA). For morphological analysis, FESEM (TESCAN MIRA 3 LMU) was performed by depositing the particles on a silicon wafer, coating them with gold, and imaging them at an accelerating voltage of 5 kV.

Cytotoxicity assay

The toxic effects of ZCD-NPs and free diosmin on A2780 cells purchased of Ferdowsi University were investigated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. HDF (normal human dermal fibroblasts) purchased of Ferdowsi University was also considered a control group. The cells were seeded at a density of 1×10^4 in 96-well plates and incubated for 24 h prior to exposure to ZCD-NPs (7.8, 15.6, 31.2, 62.5, 125, 250, and 500 µg/mL). Following a 48-hour incubation period, 20 µL of MTT reagent (5 mg/mL) was added to each well, and the plates were incubated in the dark at 37 °C for four hours. Subsequently, the medium was removed, and 200 µL of dimethyl sulfoxide (DMSO, Sigma) was added for one hour to dissolve the formazan crystals. Subsequently, the absorbance was quantified at 570 nm using a microplate reader, and the cell viability was calculated as a percentage relative to the control group.

Annexin V-FITC/PI assay

The Annexin V-FITC apoptosis staining detection kit (Abcam, ab14085) was employed to assess apoptosis. Following exposure of A2780 cells to ZCD-NPs at 5, 25, and 125 μ g/mL concentrations for 48 h, the trypsinized cells were rinsed with serum-containing media and centrifuged. After that, 1×10^5 cells were resuspended in 500 μ L of binding buffer (1X). Subsequently, the cell suspension was incubated with 5 μ L of annexin V-FITC and 5 μ L of propidium iodide (PI) at 25 °C for 5 minutes in the dark. The cell suspension was centrifuged, resuspended in 400 μ L of binding buffer, and analyzed by flow cytometry to determine total apoptosis in each sample.

Acridine orange/propidium iodide (AO/PI) staining

A2780 cells were initially seeded at a density of 1×10^4 cells per well in 6-well plates and cultured for 24 h. Subsequently, the cells were treated with ZCD-NPs (5, 25, and 125 µg/mL) for 48 h. Following the designated treatment period, the cells were trypsinized and washed with PBS. Subsequently, the cells were placed in a PBS

solution containing AO (1 μ g/mL) and PI (5 μ g/mL) and incubated at room temperature for 10 min. Following staining, the cells were washed and observed under a fluorescence microscope (Carl Zeiss, Jena, Germany).

DAPI staining

To evaluate the efficacy of ZCD-NPs (5, 25, and 125 μ g/mL) treatment on apoptosis in A2780 cells, we employed the DAPI staining method. After treatment, the trypsinized cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Afterward, the cells were permeabilized with 0.1% Triton X-100 for 10 min and then rinsed with PBS. The cells were then incubated with a DAPI solution (1 μ g/mL) for 5 min in a dark place. Finally, the excess dye was removed by washing the cells with PBS, and the stained cells were examined under a fluorescence microscope to ascertain the impact of the treatment.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was employed to evaluate the expression of P53, caspase 8, and caspase 9 genes in A2780 cells treated with ZCD-NPs. RNA was isolated using a QIAGEN total RNA extraction kit. The quality and quantity of the extracted RNA were assessed using a ThermoFisher Nanodrop ND-1000 spectrophotometer at 260 nm. Subsequently, the isolated mRNA was converted into complementary DNA (cDNA) using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega). Subsequently, a real-time PCR reaction was conducted using a Corbett thermal cycler with specific primers (Table 1) and Bio-Rad SYBR Green Master Mix. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the comparative (Ct) method. The mean Ct values from the triplicate samples were employed to ascertain the expression level of the target gene via the $2^{-\Delta\Delta Ct}$ formula.

Antioxidant activity of ZCD-NPs

The antioxidant activity of ZCD-NPs was evaluated using ABTS and DPPH free radical assays. To perform the ABTS assay, a mixture of equal volumes of ABTS solution (7 mM) and potassium persulfate (2.45 mM) was incubated in the dark at 25 °C for 12 h to prepare ABTS radical solution. The solution was then diluted with ethanol to absorb 0.70 at 734 nm. Then, different concentrations of ZCD-NPs were mixed with a diluted ABTS solution in a 96-well plate, and the absorbance was recorded after 6 min. For the DPPH assay, a solution of DPPH (0.1 mM) in methanol was prepared. After a 30-minute incubation period in the dark, different concentrations of ZCD-NPs were added, and the absorbance was measured at a wavelength of 517 nm. Radical scavenging activity was calculated for both assays using the following formula:

scavenging $(\%) = [(\text{control absorbance} - \text{sample absorbance})/(\text{control absorbance}) \times 100.$

Statistical analysis

The statistical analysis was performed using the statistical software package SPSS. The normality of the data was evaluated using the Shapiro-Wilk test. A one-way analysis of variance (ANOVA) was employed to compare multiple groups. Subsequent comparisons were conducted using Tukey's test to identify specific group differences. A significance level of P < 0.05 was employed, and all data were presented as standard deviations (SD).

Results

This study's objective was to evaluate the effects of ZCD-NPs on A2780 cell lines, focusing on the NPs' encapsulation efficiency, cytotoxicity, and antioxidant capacity.

DLS assay

As illustrated in Fig. 1A, the DLS analysis of the ZCD-NPs exhibited a Z-average particle size of 265.30 nm, signifying the mean hydrodynamic diameter of the particles within the sample. The polydispersity index (PDI) of 0.21 indicates a relatively narrow size distribution, suggesting that the NPs are uniform. A PDI value below 0.3 is typically acceptable for NP systems intended for pharmaceutical applications. The mean intensity diameter of 301.46 nm represents the size distribution, with the weighting applied being that of the scattered light intensity. The mean volume diameter of 340.23 nm offers insight into the volume-weighted size distribution. The mean number diameter of 157.56 nm is smaller than the Z-average and other mean diameters, indicating that a significant portion of the particles are small. However, larger particles contribute more to the intensity and volume distributions due to their more robust light-scattering properties. These findings collectively indicate

Primer	Sequence
P53	F: 5'-TGGAAGTAAGCCTCCGATA-3' R: 5'-CGACGAACTACGCAATGTAA-3'
Caspase-8	F 5'-TTGAGGAGGAGTGCAGGTCTAG-3' R 5'-TCGCACTGAACTAGCAAGTAA-3'
Caspase-9	F 5'- ACACCACAACACGCTGG-3' R 5'- AGCACGCATTAGCAACCAA-3'
GAPDH	F 5'- CATTCAAGACCGGACAGAGG-3' R 5'- ACATACTCAGCACAGCATCACC-3'

Table 1. Specific primer sequences for real-time PCR.



Fig. 1. Characterization of zein–sodium caseinate–diosmin nanoparticles (ZCD-NPs). (**A**) The DLS assay shows a Z-average particle size of 265.30 nm, a polydispersity index (PDI) of 0.21, and an average number diameter of 157.86 nm. (**B**) Field emission scanning electron microscopy (FESEM) shows spherical nanoparticles with an average diameter of 157.86 nm, smooth surfaces, and uniform sizes.

that the ZCD-NPs have been successfully formulated with a nanoscale size range and good uniformity. These characteristics are conducive to potential applications in drug delivery or food technology.

FESEM

The FESEM image provides information regarding the surface structure of ZCD-NPs, including their size, shape, and distribution mode (Fig. 1B). The photograph depicts spherical nanoparticles with an average diameter of 157.86 nm, smooth surfaces, and uniform sizes, indicating the proper quality of their production process. The distribution of the NPs is random, with no formation of large agglomerates, which indicates the stability of the colloidal system of NPs. The spherical morphology and uniform size distribution of these NPs can potentially enhance drug delivery efficiency.

FTIR spectroscopy

The FTIR spectrum of diosmin (Fig. 2) reveals several characteristic peaks that provide insight into the compound's molecular structure. The broad peaks at 3525.99, 3460.61, and 3408.99 cm⁻¹ indicate O–H stretching vibrations likely to originate from hydroxyl groups. The peaks at 2986.61, 2921.6, and 2843.59 cm⁻¹ can be attributed to C–H stretching vibrations of alkyl groups. A strong peak at 1661.23 cm⁻¹ indicates the potential existence of a C=O stretching vibration, which may originate from a carbonyl group. The 1610.59 and 1566.81 cm⁻¹ peaks may correspond to C=C stretching in aromatic rings. The peak at 1500.52 cm⁻¹ may be attributed to C–C stretching vibrations. The peaks observed at 1098.62 cm⁻¹ may be attributed to a range of C–H bending and C–O stretching vibrations. The peaks observed at lower wavenumbers (981.97 to 522.59 cm⁻¹) are likely attributable to out-of-plane C–H bending and other skeletal vibrations. In conclusion, the observed peaks are in accordance with the established structure of diosmin, which comprises multiple hydroxyl groups, aromatic rings, and a carbonyl group.

Furthermore, the FTIR spectrum of the ZCD-NPs (Fig. 2) exhibits a broad peak at 3314.39 cm⁻¹, which can be attributed to O–H and N–H stretching vibrations. These are likely derived from diosmin hydroxyl groups and zein and sodium caseinate amino groups. The peaks at 3068.33, 2957.67, 2929.40, and 2872.19 cm⁻¹ are attributed to all components' C–H stretching vibrations of alkyl groups. A pronounced peak at 1663.14 cm⁻¹ indicates C=O stretching vibrations, which may originate from the amide I band of proteins (zein and sodium caseinate) and the carbonyl group of diosmin. The peak at 1516.13 cm⁻¹ may be associated with the amide II bands of proteins and C=C stretching in the aromatic rings of diosmin. The peak at 1449.59 cm⁻¹ may be attributed to C–H bending vibrations. The peaks observed between 1260.54 and 1069.28 cm⁻¹ may be attributed to various C–O stretching and C–N stretching vibrations, which are characteristic of proteins and diosmin. The peaks observed at lower wavenumbers (981.23 to 679.24 cm⁻¹) are likely attributable to out-of-plane C–H bending and other skeletal vibrations. These peaks indicate the presence of all three components (zein, sodium caseinate, and diosmin) and suggest the potential for interactions, including hydrogen bonding and electrostatic interactions.

Encapsulation efficacy

The encapsulation efficacy was determined using the spectrophotometric method at 355 nm. The high encapsulation efficiency of 93.45% indicates the successful incorporation of a substantial quantity of diosmin into the ZCD-NPs. These findings validate the effective conjugation of diosmin with ZC-composite.



ZCD NPs



Fig. 2. FTIR spectra of ZCD-NPs and its constituents. The graph shows transmittance (%) versus wavelength for the crystal structures.

Cytotoxicity assay

The MTT assay results indicate a dose-dependent cytotoxic effect of ZCD-NPs on both HDF and A2780 cell lines. HDF cells demonstrated excellent resistance to treatment with ZCD-NPs, maintaining a viability of over 95% at concentrations of up to $31.2 \mu g/mL$. Only at higher concentrations was a notable decline in HDF viability evident, with 46.7% viability at 500 $\mu g/mL$, as observed in Fig. 3A (p < 0.001). In contrast, A2780 cancer cells demonstrated markedly higher sensitivity to ZCD-NPs, with a notable decline in viability even at low concentrations. At a



Fig. 3. A cytotoxicity assay was performed using the MTT assay to evaluate the effects of ZCD-NPs on HDF (human dermal fibroblasts) cells (controls) (**A**), ZCD-NPs on A2780 (ovarian cancer) cells (**B**), free diosmin on HDF (**C**), and free diosmin on A2780 (**D**) after 48 h of treatment. The MTT assay shows that ZCD-NPs exhibit selective cytotoxicity, significantly reducing the viability of A2780 cancer cells at low concentrations. In contrast, HDF cells remain mainly resistant to higher doses, indicating a preferential effect on cancer cells. Additionally, free diosmin was more toxic to normal cells than ZCD-NPs, and ZCD-NPs were more harmful to cancer cells than free diosmin. Data are presented as mean ± standard deviation (SD), and statistical significance is denoted as ***P<0.001.

concentration of 7.8 μ g/mL, the viability of A2780 cells decreased to 66.9%, and a further decline was observed at 500 μ g/mL, with a viability of 4.1% (Fig. 3B; *P*<0.001). This differential response indicates that ZCD-NPs have a selective cytotoxic effect on cancer cells while exhibiting relatively lower toxicity on normal cells. The IC50 for A2780 cells appears to be between 7.8 and 15.6 μ g/mL, whereas it is above 500 μ g/mL for HDF cells. As shown in Fig. 3C,D, free diosmin was more toxic to normal cells than ZCD-NPs, while ZCD-NPs were more harmful to cancer cells. These findings suggest that ZCD-NPs could potentially be developed as an anti-cancer agent with a favorable therapeutic window, effectively targeting cancer cells while sparing normal tissues.

Annexin V-FITC/PI assay

The flow cytometry analysis of A2780 cells treated with ZCD-NPs revealed a significant increase in the total apoptosis rate in a dose-dependent manner. As shown in Fig. 4, the scatter plot classifies cells as live cells (Q4), early apoptotic (Q3), late apoptotic (Q2), and necrotic cells (Q1), reflecting different stages of cell health and apoptosis. The untreated A2780 cells exhibited a total apoptosis rate of 3.08%. However, treatment with ZCD-NPs at concentrations of 5, 25, and 125 μ g/mL resulted in total apoptosis rates of 27.6%, 52.98%, and 67.54%, respectively. Notably, the number of cells observed in Q2 and Q3 quarters increased with growing concentrations. It suggests that necrosis also occurs in these cells. The results demonstrate that ZCD-NPs can dose-dependently induce apoptosis and necrosis in A278 cells.

AO/PI and DAPI staining

AO/PI and DAPI staining are standard methods for evaluating cell viability and morphological changes. As illustrated by the AO/PI staining results (Fig. 5A), most untreated A2780 cells appear green, indicating high viability. Nevertheless, treatment with 5, 25, and 125 μ g/mL of ZCD-NPs resulted in a marked reduction in green fluorescence while red fluorescence increased. It suggests that the NPs have induced cell death. The appearance of red fluorescence indicates damage to the cell membrane and the subsequent uptake of PI into the cell, a hallmark of advanced necrosis or apoptosis. Therefore, higher concentrations of ZCD-NPs elicit potent cytotoxic effects. Moreover, DAPI staining corroborated the AO/PI staining findings by demonstrating a dose-dependent





enhancement in nuclear condensation and fragmentation (Fig. 5B). Changes such as the shrinking of cell nuclei, increased density, or chromatin fragmentation indicate typical variations associated with apoptosis. The results demonstrate that ZCD-NPs effectively induce apoptosis in A2780 cells, indicating their potential as a therapeutic intervention for ovarian cancer.

Apoptotic gene expression

Figure 6 illustrates the mRNA expression levels of the p53, caspase 8, and caspase 9 genes in A2780 cells treated with ZCD-NPs (5, 25, and 125 μ g/mL). The results demonstrated that p53 expression was upregulated at 25 and 125 μ g/mL of ZCD-NPs in comparison to the control (untreated cells). Nevertheless, this increase was statistically significant only at 125 μ g/mL (*P*<0.01). Furthermore, caspase 8 expression was significantly upregulated at concentrations of 25 and 125 μ g/mL of ZCD-NPs compared to the control (*P*<0.01). Additionally, the expression of caspase 9 was significantly upregulated compared to the control at 125 μ g/mL of ZCD-NPs



Fig. 5. Fluorescence graph of A2780 cells stained with AO/PI (**A**) and DAPI (**B**). AO/PI staining analyses show that ZCD-NPs induce significant cytotoxic effects in A2780 cells, as evidenced by decreased green and increased red. DAPI staining further confirms a dose-dependent increase in nuclear condensation and fragmentation, characteristic of apoptosis.



Fig. 6. Real-time PCR analysis of apoptotic gene expression in A2780 cells treated with ZCD-NPs. The control group was set as a baseline, and gene expression in other groups was presented as a fold change to controls. The results show a significant upregulation of p53 at 125 μ g/mL, along with a significant increase in caspase-8 at 25 and 125 μ g/mL and caspase-9 at 125 μ g/mL (P<0.001). These results suggest that ZCD-NPs may induce apoptosis in A2780 cells by activating the p53 pathway. Data are presented as mean ± standard deviation (SD), *P<0.05, *P<0.01, and ***P<0.001.



Fig. 7. Antioxidant capacity of ZCD-NPs assessed by DPPH and ABTS free radical scavenging assays. At a 2000 µg/ml concentration, ZCD-NPs exhibit 8.9 ± 2.3% inhibition of DPPH radicals and 64.4 ± 1.8% inhibition of ABTS radicals, demonstrating a significantly higher efficacy in scavenging ABTS free radicals. Data are presented as mean ± standard deviation (SD), *** P < 0.001.

(P < 0.001). The concomitant increase in P53 and caspase gene expression indicates that ZCD-NPs may induce the death of A2780 cancer cells by activating the P53 pathway and triggering apoptosis.

Antioxidant capacity assay

The antioxidant capacity of ZCD-NPs was determined by evaluating its ability to scavenge ABTS and DPPH free radicals (Fig. 7). The DPPH free radical scavenging activity of ZCD-NPs increases with concentration, reaching about $8.9\pm2.3\%$ inhibition at 2000 µg/ml. In contrast, the ABTS scavenging activity is significantly higher, showing around $64.4\pm1.8\%$ inhibition at the same concentration. These results indicate that ZCD-NPs are more effective in scavenging ABTS free radicals, particularly at higher concentrations. These results suggest that the ZCD-NPs may be more effective in scavenging certain free radicals.

Discussion

In light of the rising prevalence of cancer and the inherent limitations of conventional therapies, there is a pressing need for DDSs that can enhance therapeutic efficacy while minimizing adverse effects. In this context, the principal objective of the present study is to develop and evaluate ZCD-NPs as a therapeutic approach for treating ovarian cancer. Our results demonstrate the potential of ZCD-NPs for pharmaceutical applications. The DLS assay revealed a Z-average particle size of 265.30 nm with a PDI of 0.21, indicating good uniformity and stability for drug carriers. FESEM images also confirmed the spherical morphology and smooth surfaces of the NPs, providing further evidence of their quality and stability. Likewise, FTIR spectroscopy confirmed the presence of critical functional groups, indicating the successful conjugation of the components. Furthermore, the encapsulation efficiency for diosmin was 93.45%, thereby validating the effective incorporation of this compound into the NPs. On the other hand, cytotoxicity assays demonstrated that ZCD-NPs reduce cell viability and promote programmed cell death in A2780 cells. The MTT assay indicated a significant decrease in cell viability at 7.8 µg/mL concentration, with a prominent decline observed at higher concentrations. The results of the flow cytometry analysis further confirm that the reduction in cell viability correlates with increased apoptosis, particularly at higher concentrations of ZCD-NPs. This mechanism of action-decreasing cell viability and enhancing apoptosis—indicates that ZCD-NPs may be a promising therapeutic strategy for targeting ovarian cancer cells while minimizing toxicity to normal cells, as demonstrated by the relative resistance of HDF cells in the MTT assay. Besides, real-time PCR analysis revealed the upregulation of p53, caspase 8, and caspase 9 genes. These findings suggested that ZCD-NPs induce apoptosis by activating the p53 pathway. In addition, ZCD-NPs exhibited considerable antioxidant capacity and effectively scavenged free ABTS radicals. These findings emphasize the potential of ZCD-NPs as a promising platform for targeted cancer therapy and antioxidant applications.

The present study offers insights into the anti-cancer potential of ZCD-NPs against A2780 ovarian cancer cells. The encapsulation of diosmin with ZC-composite enhanced its bioavailability, significantly improving therapeutic outcomes. While ZCD-NPs are larger than 200 nm, various factors can enhance their internalization. Research shows that cells can still effectively take up NPs in the 100–500 nm range, especially when their surface properties are optimized for interactions with cell membranes. The unique composition of ZCD-NPs, derived from zein and sodium caseinate, improves their biocompatibility and bioactivity, facilitating cellular recognition and uptake. Additionally, surface modifications with specific ligands or polymers can promote endocytosis, enhancing internalization even for larger NPs^{32–34}.

Zein is a plant-derived protein with excellent mechanical strength, biodegradability, and the ability to encapsulate hydrophobic compounds. Its hydrophobic nature allows for effective drug loading and controlled release, rendering it an optimal vehicle for the delivery of anti-cancer agents³⁵. Likewise, sodium caseinate reinforces zein's structural integrity, enhancing the encapsulated compounds' solubility³⁶. Combining these two proteins creates NPs that protect bioactive substances from degradation and enhance their therapeutic effects. Our finding on the ability of ZC-composite to effectively encapsulate diosmin confirms previous research emphasizing the use of natural polymers in the fabrication of DDSs. For example, a study by Zhang et al. demonstrated the successful encapsulation of curcumin in ZC-composite, achieving an encapsulation efficiency of over 91.18%. The study demonstrated improved stability, increased resistance to gastric digestion, and effective delivery of hydrophobic bioactive compounds³⁷. This finding is consistent with our finding of 93.45% for diosmin, highlighting the effectiveness of ZC-composite in encapsulating hydrophobic compounds. Similarly, Hou et al. (2018) successfully synthesized zein-paclitaxel NPs. These NPs effectively released paclitaxel (80–90% in 5 min) and demonstrated selective cytotoxicity against HeLa cells while sparing NIH/3T3 fibroblast normal cells. In an animal model, zein-paclitaxel NP administrations led to a 50% reduction in tumor size after treatment³⁸.

Based on these findings, the anti-cancer properties of diosmin further highlight the potential of ZCD-NPs to target specific cancer cells. In this regard, the advantage of ZCD-NPs in our study was their selective cytotoxicity against A2780 cancer cells. The induction of apoptosis, as demonstrated by Annexin V-FITC/PI and PCR assays, is consistent with other studies investigating the anti-cancer properties of diosmin. This flavonoid glycoside has shown dose-dependent apoptotic effects in various cancer types. For example, several studies have revealed that diosmin could induce apoptosis in breast cancer cell lines. However, MCF-7 cells were the most responsive compared to other breast cancer cell lines like MDA-MB-231 and SKBR-3²⁷. In addition, the pro-apoptotic activity of diosmin was confirmed in the androgen-independent prostate cancer cell line DU145. Diosmin has induced oxidative stress, changes in mitochondrial membrane potential, and apoptotic cell death in DU145 cells. It also exhibited potent genotoxicity, which induced DNA double-strand breaks and micronuclei formation²³. Buddhan et al. demonstrated the cytotoxicity of diosmin in A431 skin cancer cells in an in vitro study. Their results indicated that diosmin reduced the viability of A431 cells in a dose-dependent manner. Diosmin treatment leads to excessive generation of reactive oxygen species (ROS), increased DNA fragmentation, and altered expression of genes related to apoptosis. These included upregulation of p53, caspases 3, and caspases 9 and downregulation of Bcl-2 and matrix metalloproteinases 2 and 9^{39} . In another study, Devika et al. aimed to investigate the effects of diosmin on DLD-1 human colon cancer cells. The results showed that diosmin induced dose-dependent apoptosis, as confirmed by AO/EtBr double staining and generated high levels of ROS. Flow cytometry analysis revealed that diosmin-induced S-phase cell cycle arrest and Annexin V staining indicated significant cell death. In addition, diosmin treatment upregulated the mRNA expression of p53 and p21, suggesting that the p21/ p53 pathway partially mediates the induction of S-phase arrest and apoptosis⁴⁰. Zeya et al. also found that the combination of diosmin and naringenin (DiNar) induced cytotoxicity in colon cancer cell lines HCT116 and SW480, leading to increased chromatin condensation, DNA fragmentation, and G0/G1 phase cell cycle arrest²¹. In addition, diosmin exhibited protective effects against oral carcinogenesis⁴¹, urinary bladder carcinogenesis⁴², esophageal⁴³, and hepatocellular⁴⁴ cancers in animal models.

Regarding diosmin's mechanisms of action, there is evidence that it can induce apoptosis in cancer cells such as glioblastoma and HepG2 by activating caspase pathways^{45,46}. Our results also show significant upregulation of caspases 8 and 9 in ZCD-NPs-treated A2780 cells, further supporting the idea that diosmin can activate intrinsic apoptotic pathways. Apoptosis induction is a critical mechanism in cancer therapy, allowing for the selective elimination of malignant cells⁴⁷. In addition, the role of p53 in mediating apoptosis is particularly relevant. Our finding of the upregulation of p53 expression in response to ZCD-NPs treatment is consistent with studies by Lewinska et al., which showed that diosmin caused an increase in p53, p21, and p27 levels in MCF-7 breast cancer cells, leading to G2/M cell cycle arrest and stress-induced premature senescence²². Devika and colleagues also showed that diosmin upregulates the mRNA levels of p53 and p21 in DLD-1 colon cancer cells, suggesting that diosmin-induced S-phase arrest and apoptosis are partially controlled by the p53 pathway⁴⁰. These results indicate that diosmin can modulate p53 expression in various cancer cell lines, leading to cell cycle arrest and apoptosis. Upregulation of p53 and its downstream targets, such as p21, is a crucial mechanism by which diosmin exerts its anti-cancer effects. In addition, Ning et al. showed that diosmin significantly inhibited cell proliferation and induced cell cycle arrest at the G2/M phase through suppression of the STAT3/c-Myc signaling pathway in human osteosarcoma Saos-2 and U2OS cells. The proportion of cells in the G2/M phase increased significantly when treated with diosmetin²⁶. Diosmin also promoted cell cycle arrest in the G2/M phase in HepG2 liver cancer cells⁴⁸. Bioinformatic analysis and molecular studies also showed that diosmin targets cell cycle regulatory proteins in triple-negative breast cancer (TNBC), including CDK1, KIF11, and other mitotic regulators, inducing cell cycle arrest and mitotic catastrophe⁴⁹. Collectively, diosmin exerts anti-cancer effects by inducing cell cycle arrest at G2/M and G1 in various cancer types. The underlying mechanisms involve the upregulation of tumor suppressors like p53, p21, and p27 and targeting cell cycle regulatory proteins. This arrest prevents cancer cells from proliferating and inhibits tumor growth.

In addition, in the present study, we have demonstrated the antioxidant capacity of ZCD-NPs through their ability to scavenge ABTS and DPPH free radicals, which adds another layer of therapeutic potential. The dual role of antioxidants in cancer therapy has been extensively discussed in the literature. For example, Wójciak et al. showed that diosmin protects endothelial cells against hydrogen peroxide-induced oxidative stress. According to their results, pretreatment with diosmin improved cell viability, reduced intracellular reactive oxygen species, restored antioxidant enzyme activity, and decreased malondialdehyde levels⁵⁰. In contrast, in studies of human lung cancer cells, diosmin treatment led to increased generation and cytoplasmic condensation of ROS, which is associated with the induction of apoptotic pathways⁵¹. Thus, the antioxidant properties of diosmin may protect normal tissues and sensitize cancer cells to treatment.

Conclusion

In summary, the study highlights the promising potential of ZCD-NPs as an effective therapeutic strategy for ovarian cancer. It demonstrates their ability to enhance the bioavailability of diosmin, induce selective cytotoxicity, and activate apoptotic pathways while exhibiting significant antioxidant properties that may further contribute to their therapeutic efficacy. We recommend that future studies include ROS and mitochondrial membrane potential (MMP) analyses to evaluate mitochondrial-based apoptosis in cancer cells. Assessing ROS can offer insights into oxidative stress and the mechanisms of cancer cell death, while changes in MMP indicate mitochondrial dysfunction, which is essential for apoptosis. Future research should also investigate other cancer cell lines and conduct in vivo studies to validate these findings and further explore the underlying mechanisms. Additionally, more research is necessary to optimize the formulation for specific cancer types, enhance targeting efficiency, and evaluate long-term stability and safety for human applications.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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References

- 1. Dang, Y. & Guan, J. Nanoparticle-based drug delivery systems for cancer therapy. Smart Mater. Med. 1, 10-19 (2020).
- Sohail, M. et al. Nanocarrier-based drug delivery system for cancer therapeutics: A review of the last decade. Curr. Med. Chem. 28(19), 3753–3772 (2021).
- Namvar, F. et al. Nanosized silver-palm pollen nanocomposite, green synthesis, characterization and antimicrobial activity. Res. Chem. Intermed. 42, 1571–1581 (2016).
- 4. Raj, S. et al. (eds) Specific targeting cancer cells with nanoparticles and drug delivery in cancer therapy. *Seminars in cancer Biology*. (Elsevier, 2021).
- Dadwal, A., Baldi, A. & Kumar Narang, R. Nanoparticles as carriers for drug delivery in cancer. Artif. Cells Nanomed. Biotechnol. 46(sup2), 295–305 (2018).
- 6. Gindy, M. E. & Prud'homme, R. K. Multifunctional nanoparticles for imaging, delivery and targeting in cancer therapy. *Expert Opin. Drug Deliv.* **6**(8), 865–878 (2009).
- 7. Hu, C-M-J. & Zhang, L. Therapeutic nanoparticles to combat cancer drug resistance. Curr. Drug Metab. 10(8), 836-841 (2009).
- 8. Li, H. et al. The protein Corona and its effects on nanoparticle-based drug delivery systems. Acta Biomater. 129, 57-72 (2021).
- 9. Zhang, S. & Han, Y. Preparation, characterisation and antioxidant activities of rutin-loaded zein-sodium caseinate nanoparticles. *PloS One* **13**(3), e0194951 (2018).
- 10. Asrorov, A. M. Zein-Based Nanoparticles for Drug Delivery and Targeting 579-620 (Elsevier, 2023).
- 11. Luo, Y. & Wang, Q. Zein-based micro-and nano-particles for drug and nutrient delivery: A review. J. Appl. Polym. Sci. 131(16) (2014).
- 12. Chen, H. et al. Multi-frequency ultrasound-assisted dialysis modulates the self-assembly of alcohol-free zein-sodium caseinate to encapsulate curcumin and fabricate composite nanoparticles. *Food Hydrocoll.* **122**, 107110 (2022).
- Li, K-K., Yin, S-W., Yang, X-Q., Tang, C-H. & Wei, Z-H. Fabrication and characterization of novel antimicrobial films derived from thymol-loaded zein–sodium caseinate (SC) nanoparticles. J. Agric. Food Chem. 60(46), 11592–11600 (2012).
- Patel, A. R., Bouwens, E. C. & Velikov, K. P. Sodium caseinate stabilized Zein colloidal particles. J. Agric. Food Chem. 58(23), 12497–12503 (2010).
- Luo, Y., Teng, Z., Wang, T. T. & Wang, Q. Cellular uptake and transport of Zein nanoparticles: Effects of sodium caseinate. J. Agric. Food Chem. 61(31), 7621–7629 (2013).
- Elzoghby, A. O., El-Fotoh, W. S. A. & Elgindy, N. A. Casein-based formulations as promising controlled release drug delivery systems. J. Controlled Release 153(3), 206–216 (2011).
- 17. Somu, P. & Paul, S. Protein assisted one pot controlled synthesis of monodispersed and multifunctional colloidal silver–gold alloy nanoparticles. *J. Mol. Liq.* 291, 111303 (2019).
- 18. Wang, L., Jia, W., Yang, Q., Cai, H. & Zhao, X. Casein nanoparticles as oral delivery carriers for improved bioavailability and hypoglycemic activity of apigenin. *Food Hydrocoll.* **146**, 109194 (2024).
- 19. Horowitz, R. Flavonoids of citrus. I. Isolation of diosmin from lemons (Citrus limon). J. Org. Chem. 21(10), 1184–1185 (1956).
- 20. Om, H. et al. Combating atherosclerosis with targeted diosmin nanoparticles-treated experimental diabetes. *Investig. New Drugs* 38, 1303–1315 (2020).
- 21. Zeya, B. et al. Diosmin in combination with naringenin enhances apoptosis in colon cancer cells. Oncol. Rep. 47(1), 4 (2022).
- Lewinska, A., Adamczyk-Grochala, J., Kwasniewicz, E., Deregowska, A. & Wnuk, M. Diosmin-induced senescence, apoptosis and autophagy in breast cancer cells of different p53 status and ERK activity. *Toxicol. Lett.* 265, 117–130 (2017).
- Lewinska, A., Siwak, J., Rzeszutek, I. & Wnuk, M. Diosmin induces genotoxicity and apoptosis in DU145 prostate cancer cell line. *Toxicol. In Vitro* 29(3), 417–425 (2015).
- Choi, J., Lee, D-H., Park, S-Y. & Seol, J-W. Diosmetin inhibits tumor development and block tumor angiogenesis in skin cancer. Biomed. Pharmacother. 117, 109091 (2019).

- Soltani, M., Parivar, K., Baharara, J., Kerachian, M. A. & Asili, J. Transcriptional analysis of VEGF-D and TGFβ genes in MCF7 cells exposed to saponin isolated from holothuria leucospilota (sea cucumber). *Rep. Biochem. Mol. Biol.* 4(1), 25 (2015).
- 26. Ning, R. et al. Diosmetin inhibits cell proliferation and promotes apoptosis through STAT3/c-Myc signaling pathway in human osteosarcoma cells. *Biol. Res.* 54(1), 40 (2021).
- 27. Huwait, E. & Mobashir, M. Potential and therapeutic roles of diosmin in human diseases. Biomedicines 10(5), 1076 (2022).
- 28. Russo, R., Chandradhara, D. & De Tommasi, N. Comparative bioavailability of two diosmin formulations after oral administration to healthy volunteers. *Molecules* 23(9), 2174 (2018).
- 29. Udapurkar, P. P., Bhusnure, O. G. & Kamble, S. R. Diosmin phytosomes: Development, optimization and physicochemical characterization. *Indian J. Pharm. Educ. Res.* 52(4), S29-S36 (2018).
- Vrbata, P., Berka, P., Stránská, D., Doležal, P. & Lázníček, M. Electrospinning of diosmin from aqueous solutions for improved dissolution and oral absorption. *Int. J. Pharm.* 473(1–2), 407–413 (2014).
- 31. Gerges, S. H., Wahdan, S. A., Elsherbiny, D. A. & El-Demerdash, E. Pharmacology of diosmin, a citrus flavone glycoside: An updated review. *Eur. J. Drug Metab. Pharmacokinet.* 1–18 (2022).
- 32. Lesniak, A. et al. Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. J. Am. Chem. Soc. 135(4), 1438–1444 (2013).
- 33. Zhang, W. et al. Effects of morphology and size of nanoscale drug carriers on cellular uptake and internalization process: A review. RSC Adv. 13(1), 80-114 (2023).
- Hoshyar, N., Gray, S., Han, H. & Bao, G. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. Nanomedicine 11(6), 673–692 (2016).
- Jaski, A. C. et al. Zein-a plant-based material of growing importance: new perspectives for innovative uses. Ind. Crops Prod. 186, 115250 (2022).
- Parker, E. A., Donato, L. & Dalgleish, D. G. Effects of added sodium caseinate on the formation of particles in heated milk. J. Agric. Food Chem. 53(21), 8265–8272 (2005).
- 37. Zhang, X. et al. Ternary nanoparticles of sodium caseinate-zein-hexaglycerol monooleate: Fabrication, characterization, and curcumin encapsulation. *LWT* 203, 116376 (2024).
- Hou, H. et al. Zein-paclitaxel prodrug nanoparticles for redox-triggered drug delivery and enhanced therapeutic efficiency. J. Agric. Food Chem. 66(44), 11812–11822 (2018).
- Buddhan, R. & Manoharan, S. Diosmin reduces cell viability of A431 skin cancer cells through apoptotic induction. J. Cancer Res. Ther. 13(3), 471–476 (2017).
- 40. Devika, M., Vysakh, A., Vijeesh, V., Jisha, N. & Ruby, P. M. Flavonoid glycoside diosmin induces apoptosis and cell cycle arrest in DLD-1 human colon cancer cell line. J. Biol. Act. Prod. Nat. **12**(3), 232–242 (2022).
- Rajasekar, M., Suresh, K. & Sivakumar, K. Diosmin induce apoptosis through modulation of STAT-3 signaling in 7,12 dimethylbenz (a) anthracene induced harmster buccal pouch carcinogenesis. *Biomed. Pharmacother.* 83, 1064–1070 (2016).
- 42. Yang, M. et al. Chemopreventive effects of diosmin and hesperidin on N-butyl-N-(4-hydroxybutyl) nitrosamine-induced urinarybladder carcinogenesis in male ICR mice. *Int. J. Cancer* 73(5), 719–724 (1997).
- Tanaka, T. et al. Modulation of N-methyl-N-amylnitrosamine-induced rat oesophageal tumourigenesis by dietary feeding of diosmin and hesperidin, both alone and in combination. *Carcinogenesis* 18(4), 761–769 (1997).
- Perumal, S. et al. Effect of diosmin on apoptotic signaling molecules in N-nitrosodiethylamine-induced hepatocellular carcinoma in experimental rats. Mol. Cell. Biochem. 449, 27–37 (2018).
- 45. Soares, J. M. et al. Diosmin Induces caspase-dependent apoptosis in human glioblastoma cells. Anais da Academia Brasileira de Ciencias 91, e20191031 (2020).
- 46. Cheng, C. et al. Anti-cancer effects and mechanisms of diosmin in HepG2 cells in vitro revealed by network. *Pharmacol. Mol. Dock.* (2021).
- 47. Carneiro, B. A. & El-Deiry, W. S. Targeting apoptosis in cancer therapy. Nat. Rev. Clin. Oncol. 17(7), 395-417 (2020).
- Ma, A. & Zhang, R. Diosmetin inhibits cell proliferation, induces cell apoptosis and cell cycle arrest in liver cancer. *Cancer Manag. Res.* 3537–3546 (2020).
- Musyayyadah, H., Wulandari, F., Nangimi, A. F., Anggraeni, A. D. & Meiyanto, E. The growth suppression activity of diosmin and PGV-1 co-treatment on 4T1 breast cancer targets mitotic regulatory proteins. *Asian Pac. J. Cancer Prevent. APJCP* 22(9), 2929 (2021).
- 50. Wójciak, M. et al. Antioxidant potential of diosmin and Diosmetin against oxidative stress in endothelial cells. *Molecules* 27(23), 8232 (2022).
- Naso, L. et al. Antioxidant, anticancer activities and mechanistic studies of the flavone glycoside diosmin and its oxidovanadium(IV) complex. Interactions with bovine serum albumin. *Bioorg. Med. Chem.* 24(18), 4108–4119 (2016).

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Declarations

Competing interests

The authors declare no competing interests.

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