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Research Paper

Ameliorative Effect of *Calendula persica* Against Zinc Oxide Nanoparticle-induced Cytotoxicity in Human Hepatoma HepG2 Cells

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ABSTRACT

Background: The extensive application of zinc oxide nanoparticles (ZnO NPs) in a wide range of consumer and industrial products has increased human exposure to these nanomaterials. *Calendula persica* is a traditional medicinal plant that has been used in folk medicine for the treatment of various diseases.

Materials and Methods: In the present study, we investigated the potential ameliorative effect of *C. persica* extract against ZnO NP-induced cytotoxicity in HepG2 cells. The cytotoxic effects of different concentrations of ZnO nanoparticles, as well as the protective role of *C. persica* extract, were evaluated in HepG2 cells. Morphological alterations were first assessed using microscopic examination. Subsequently, cell viability was determined using neutral red uptake (NRU) and MTT assays.

Results: Exposure to higher concentrations of ZnO NPs resulted in pronounced morphological alterations and significantly increased cell mortality in HepG2 cells. In contrast, treatment with *C. persica* markedly attenuated ZnO NP-induced morphological damage and reduced cell mortality, suggesting a protective effect against NP-mediated cytotoxicity.

Conclusion: *C. persica* extract exhibits significant protective effects against ZnO NP-induced cytotoxicity in HepG2 cells, as evidenced by its ability to attenuate cellular damage and improve cell viability. These findings suggest that *C. persica* may serve as a promising natural agent for mitigating nanoparticle-induced cellular toxicity.

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Introduction

Nanomaterials (NMs) are used in different fields, such as biomedicine, food, and cosmetics, due to their unique properties [1-4]. However, the increasing production of NMs boosted the chance of their release into the environment, leading to unavoidable exposure among humans and other biological systems [4, 5]. It has been demonstrated that some NMs induce toxicity in biological systems through various processes and mechanisms [4, 6]. Some NMs cross the cell membrane and interact with intracellular metabolic processes. Therefore, assessing the toxicity of nanoparticles (NPs) in cells is a valuable tool for identifying their toxicity mechanisms. This approach is cost-effective, rapid, and associated with minimal ethical concerns [4, 7]. Also, other tests, such as proliferation assays, apoptosis assays, oxidative stress assays, DNA damage assays, and in vivo tests, are used to assess toxicity [7-12].

Zinc oxide nanoparticles (ZnO NPs) are among the most widely used NPs in different industries, such as cosmetics, antibacterial and anti-fungal products, food industry, photocatalysis, biosensing, and bioimaging [13]. Recently, ZnO NPs have been used in biomedicine as anticancer, antidiabetic, and antibacterial agents [13, 14]. On the other hand, the results of some studies have demonstrated that these NPs induce toxicity in animal and cellular models [15-19]. ZnO NPs enter the human body through different routes, including inhalation, skin penetration, the digestive system, and parenteral injection [18]. Studies have demonstrated that ZnO NPs induce adverse effects in different organs, such as the kidneys, liver, brain, stomach, pancreas, eyes, and prostate gland [19-21]. Also, ZnO NPs induce cytotoxicity in different cell lines, such as A172, GC-1, Caco-2, T47D breast cancer, PC12, and LT97 cells [19, 22].

Given the extensive use of ZnO NPs in various industries and the lack of sufficient information regarding their safety, conducting extensive research on their safety is urgent and constitutes a cornerstone of sustainable development. In this field, a new approach involves adding compounds to ZnO NP-based products, such as sunscreens, facial creams, and body lotions, to reduce the side effects of NPs. One such component is medicinal plants, which contain many beneficial ingredients with minimal side effects. One of these plants is *Calendula persica*, which has traditionally been used in Iran for the treatment of many health problems. *Calendula* flowers have been used to treat liver obstructions, snake bites, heart problems, headache, jaundice, red eyes, as well as measles and smallpox [23, 24]. Pharmacological studies have demonstrated that different species of *Calendula* plant have antibacterial, antiviral, anti-inflammatory, anti-tumor, antioxidant, antimutagenic and hemolytic activities [24]. Different species of *Calendula* contain several classes of phytochemicals, such as carbohydrates, amino acids, phenolic compounds, lipids, steroids, tocopherols, terpenoids, coumarins, quinones, volatile oils, and carotenoids. The major active ingredients of the *Calendula* are triterpendiol esters, saponins, and flavonoids, such as rutin and hyperoside [25, 26]. This study aimed to assess the potential ameliorative role of *C. persica* extract against cytotoxicity induced by ZnO NPs in HepG2 cells.

Materials and Methods

Nanoparticles

ZnO NPs with a diameter of 20 nm and a purity of 99% were purchased from Pishgaman Company (Iran) and used in the present investigation without further purification or sieving. Ultraviolet light was used for 24 hours to sterilize the ZnO NPs (Figure 1).

Preparation of *C. persica* extract

The *C. persica* plant was purchased from Zarin Giah Company (Iran), and the plant samples were taxonomically identified by a botanist through morphological analysis. Then, 100 g of plant powder was mixed with double-distilled water and ethanol for 72 hours. Then, the hydroethanolic extract was filtered, and the filtrate was concentrated through evaporation under a vacuum at 40 ˚C. Then, the extract was kept at -20 ˚C until the experiments were conducted.

Human HepG2 cell culture

The cell line was purchased from the Cell Bank of the Pasteur Institute (Iran). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM medium) supplemented with fetal bovine serum (10%, v/v) and antibiotics (penicillin and streptomycin 1%) and incubated at 37 ˚C in a 5% CO_2 atmosphere with 95% humidity for 24 h. Then, the cells were removed from the culture flasks by enzymatic digestion with trypsin/EDTA, and the cell suspension was centrifuged at 200 g for 5 min. The cells were resuspended in culture medium, and a cell suspension containing 1×10^5 cells/mL was prepared. Using an 8-channel pipette, 100 µL of the 1×10^5 cells/mL cell suspension was distributed into the wells of a 96-well microtiter plate. The cells were then incubated for 24 h at 37 ˚C in a 5% CO_2 atmosphere with humidity >90% to form a semi-dense monolayer.

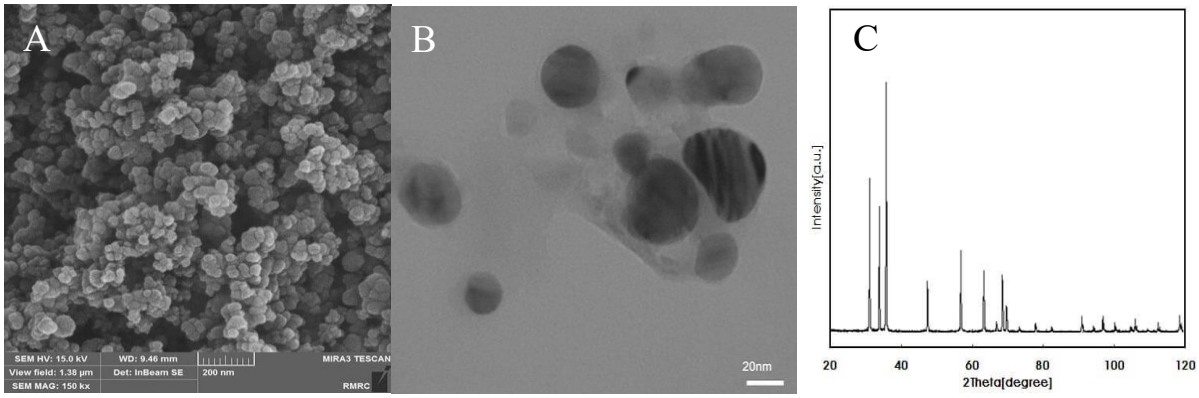


Figure 1. SEM image (A and B) and XRD pattern (C) of zinc oxide nanoparticles

Treatment of HepG2 cells with ZnO NPs and *C. persica* extract

In the first group, 100 µL of culture medium containing different concentrations of ZnO NPs (10, 20, 40, and 80 µg/mL) was added to each well in triplicate. An untreated cell population in 100 µL of growth medium was used as the negative control in triplicate, while 100 µL of Triton X-100 was added to three wells as the positive control. In the second group, different concentrations of ZnO NPs (10, 20, 40, and 80 µg/mL), together with one concentration of *C. persica* extract (100 µg/mL) were added to the cells in triplicate. The cells were then incubated for 24 h at 37 ˚C in a 5% CO_2 atmosphere with humidity >90%. In this study, different concentrations of the plant extract were tested to determine a nontoxic but biologically effective dose. A range of concentrations from 50 to 200 µg/mL was used for the preliminary screening of the plant extract. Among the tested concentrations, 100 µg/mL showed the most appropriate profile in terms of cell compatibility without inducing cytotoxic effects. Therefore, 100 µg/mL was selected as the optimal nontoxic concentration for subsequent experiments evaluating the protective effect against ZnO NP-induced toxicity.

Morphological assessment

The wells were examined using an inverted phase-contrast microscope to identify systematic errors in cell culture and to assess the growth characteristics of the control and treated cells following treatment with ZnO NPs and *C. persica* extract. Morphological changes in the cells caused by the toxic effects of the NPs were examined and recorded using a microscope (Hund, Germany).

Neutral red uptake assay

In the first step, the culture medium was removed from the plate. The cells were then washed with 100 µL of prewarmed PBS, and the washing solution was removed by gentle washing. Then, 100 µg of neutral red medium was added, and the cells were incubated at 37 ˚C in a 5% CO_2 incubator for 3 hours. The neutral red culture medium was then removed, and the cells were washed with 100 µL of phosphate-buffered saline (PBS). After incubation, the PBS was slowly removed, and 100 µL of neutral red-absorbing solution (ETOH/acetic acid) was added to all wells. Then, the microtiter plate was shaken rapidly for 10 min on a microtiter plate shaker until the neutral red was extracted from the cells and a uniform solution was formed. Finally, the absorbance of the colored solution was measured at 540 nm using a plate spectrophotometer (BioTek, USA) [27].

MTT assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay is a rapid colorimetric method in which mitochondrial dehydrogenases convert MTT into formazan crystals, thereby providing an estimate of the metabolic activity of viable cells. For cytotoxicity analysis, exponentially growing HepG2 cells were collected and cultured (1×10^4 cells/well) in a 96-well microtiter plate, and then incubated for 24 hours in a 5% CO_2 atmosphere before treatment. After 24 hours of treatment, each plate was examined under an inverted phase-contrast microscope to identify any systematic errors in cell culture and to assess the growth characteristics of the control and treated cells. Subsequently, 50 µL of MTT solution (5 mg/mL) was added to each well and incubated at 37 ˚C for 4 hours. After incubation, the supernatants were removed, and 50 µL of isopropanol was

added to each well to solubilize the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (BioTek, USA). The generated data were recorded in a raw data file, and the percentage of cell viability was calculated using the Equation 1:

$$\text{Cellular viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of negative control}} \times 100 \quad (1)$$

Statistical analysis

All experiments were performed in triplicate to ensure the reliability of the results. Data are presented as Mean±SD. Statistical comparisons among the groups were performed using one-way analysis of variance (ANOVA). Data analysis was conducted using GraphPad Prism software (version 10), and differences were considered statistically significant at $P<0.05$.

Results

Morphological changes in HepG2 cells

A concentration of 10 µg/mL of ZnO NPs d caused no morphological changes in the HepG2 cells. At this concentration, the exposed cells were completely attached to the bottom of the well and maintained their integrity. No cells were rounded, and no cell membranes were disrupted. All organelles, such as mitochondria, lysosomes, and nucleus were intact. However, higher concentrations of ZnO NPs (20 to 80 µg/mL) induced morphological alterations in the cells. These concentrations caused cell death, cell rounding, cell detachment, and nuclear fragmentation. All organelles, such as the nucleus, lysosomes, and mitochondria were destroyed (Figure 2).

Protective effect of *C. persica* extract against the cytotoxicity of ZnO NPs

C. persica extract (100 µg/mL) had no cytotoxic effect on HepG2 cells. It protected the cells and reduced the cytotoxicity of high concentrations of ZnO NPs (20 to 80 µg/mL). As can be observed in Figure 3, there were no morphological changes in the cells following *C. persica* extract treatment. Furthermore, this plant could decline morphological changes, such as cell detachment, nuclear fragmentation, and cell rounding, and could reduce cell death at higher concentrations of ZnO NPs. Considering that the lower concentration of ZnO NPs (10 µg/mL) caused no morphological alterations in the cells, and no changes were observed following co-exposure to *C. persica* extract and ZnO NPs.

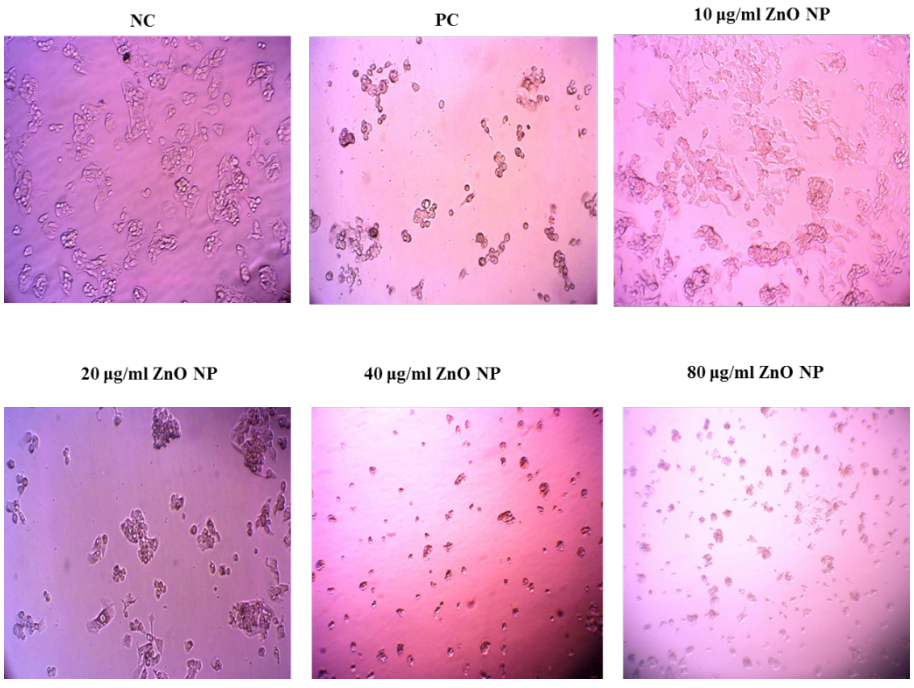


Figure 2. Inverted microscopy images of cytotoxic effects of different concentrations (10, 20, 40, 80) µg/mL of ZnO NPs on HepG2 cells (Magnification ×4)

NC: Negative control; PC: Positive control.

Neutral red uptake assay

did not significantly affect cell viability. In the combined

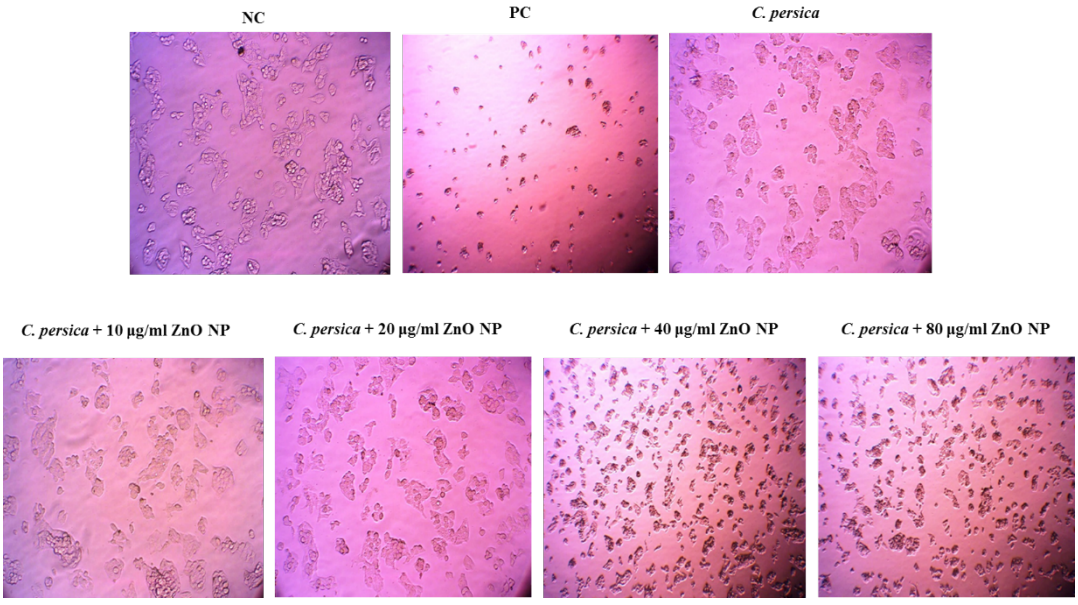


Figure 3. Inverted microscopy images of protective effect of *C. persica* extract against cytotoxicity of different concentrations (10, 20, 40, 80) µg/mL of ZnO NPs on HepG2 cells (Magnification ×4)

NC: Negative control, PC: Positive control.

The viability of HepG2 cells after 24 h exposure to ZnO NPs varied in a concentration-dependent manner. As shown in Figure 4, a significant decrease in cell viability was observed with increasing ZnO NP concentrations, decreasing from 87% at 10 µg/mL to 9% at 80 µg/mL. In contrast, treatment with *C. persica* extract alone treatment group, *C. persica* extract significantly improved cell viability at different concentrations of ZnO NPs. Specifically, cell viability increased from 87%, 57%, 32%, and 9% to 94%, 81%, 59%, and 35%, respectively (Figure 5).

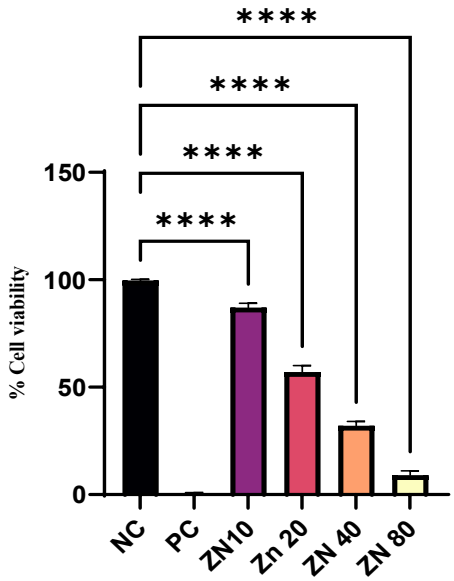


Figure 4. Cell viability of HepG2 cells treated with ZnO NPs (10, 20, 40, 80 µg/mL)

Abbreviations: ZN: ZnO NPs; NC: Negative control; PC: Positive control.

****P<0.01, significant difference compared to the negative control.

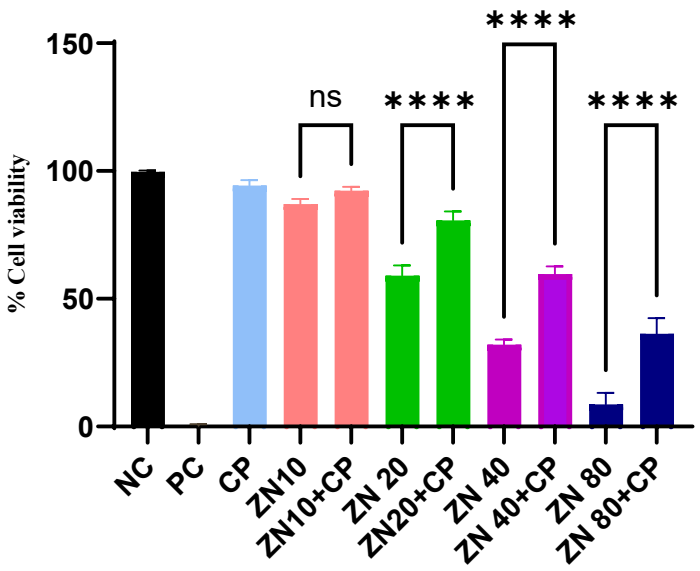


Figure 5. Cell viability of HepG2 cells treated with *C. persica* extract (100 µg/mL) and different concentrations of ZnO NPs (10, 20, 40, 80) µg/mL plus *C. persica* extract (100 µg/mL)

Abbreviations ZN: ZnO NPs; CP: *C. persica*; NC: Negative control, PC: Positive control.

****P<0.01, significant difference compared to the ZnO NPs treated group.

MTT assay

In the first group, the highest percentage of cell viability was observed at a ZnO NP concentration of 10 µg/mL, whereas the lowest percentage was observed at a concentration of 80 µg/mL. On the other hand, *C. persica* extract did not alter cell viability. In the second group, *C. persica* extract significantly protected cell viability at different concentrations of ZnO NPs (Table 6).

Discussion

ZnO NPs are among the most widely used engineered NMs in various industries due to their unique physicochemical properties. Consequently, their extensive application in consumer and industrial products makes human exposure inevitable, raising concerns regarding their potential health risks that require careful evaluation [19]. ZnO NPs are commonly incorporated into cosmetic and hygiene products because of their ultraviolet (UV) absorption, optical transparency, and antibacterial properties [13]. Following systemic exposure, ZnO NPs can be distributed and metabolized primarily in the liver, where they may accumulate and disrupt hepatic function. Evidence suggests that these NPs can exert particle-specific toxic effects on antioxidant defense systems and energy metabolism pathways in hepatic tissue [28]. Accordingly, the present study investigated the cytotoxic effects of ZnO NPs in human hepatoma HepG2 cells. Given the growing interest in identifying protective agents against NP-induced toxicity, medicinal plants have gained attention as complementary therapeutic sources due to their natural origin, low side effects, and rich antioxidant constituents. Therefore, this study also evaluated the protective effect of *C. persica* extract against ZnO NP-induced cytotoxicity in HepG2 cells.

Our results demonstrated that high concentrations of ZnO NPs exerted marked cytotoxic effects on HepG2 cells, leading to morphological alterations, such as cell rounding and reduced viability. Severe structural damage was observed, including disruption of cellular organelles, such as the nucleus, lysosomes, and mitochondria, along with a loss of membrane integrity. These findings are consistent with previous reports. For instance, Pinho et al. [22] demonstrated that ZnO NPs induce oxidative stress, increase ROS production, and cause DNA damage, cytoskeletal alterations, and cell death in GC-1 cells. Similarly, reduced viability has been reported in A172 glial cells following ZnO NP exposure [19]. Other studies have also shown that ZnO NPs induce mitochondrial dysfunction, apoptosis, and lipid peroxidation at specific concentrations [29].

Table 1. MTT assay in HepG2 cells treated with different concentrations of ZnO NPs (10, 20, 40, and 80) µg/mL plus one dose of *C. persica* extract (100 µg/mL)

Treatment	Concentration	Cell Viability (%)
ZnO NPs	10 µg/mL	92±5.3
ZnO NPs	20 µg/mL	69±4.1*
ZnO NPs	40 µg/mL	29±1.6*
ZnO NPs	80 µg/mL	12±0.9*
ZnO NPs + *C. persica* extract	10 + 100 µg/mL	97±4.8
ZnO NPs + *C. persica* extract	20 + 100 µg/mL	79±3.2**
ZnO NPs + *C. persica* extract	40 + 100 µg/mL	52±2.7**
ZnO NPs + *C. persica* extract	80 + 100 µg/mL	35±2.2**
C. persica extract	100 µg/mL	99±4.1
Negative control	100 µL	100
Positive control	100 µL	0

NC: Negative control, PC: Positive control.

*P<0.05, significant difference compared to the negative control. **P<0.05, significant difference compared to the ZnO NPs treated group.

Oxidative stress is considered one of the primary mechanisms underlying NP-induced cytotoxicity [17, 30]. Excessive reactive oxygen species (ROS) generation leads to damage to lipids, proteins, DNA, and carbohydrates. In particular, lipid peroxidation compromises membrane integrity, ultimately contributing to cell dysfunction and death [31, 32]. Moreover, NPs can disrupt intracellular calcium homeostasis, activate transcription factors, and alter cytokine expression, thereby interfering with normal cellular signaling and gene regulation [30]. Inflammatory responses mediated by macrophages and neutrophils further amplify ROS and reactive nitrogen species production, exacerbating cellular injury [32, 33]. Importantly, a strong correlation has been reported between the oxidative stress-inducing capacity of metal NPs and the extent of cellular damage [30, 32]. Additionally, NP toxicity is influenced by multiple factors, including concentration, exposure time, particle size, shape, surface chemistry, and cell type [34]. In the present study, *C. persica* extract exhibited a strong cytoprotective effect against ZnO NP-induced cytotoxicity. To assess cell viability, both neutral red uptake (NRU) and MTT assays were employed. The NRU assay, recommended by OECD guideline TG 432, evaluates lysosomal integrity as an indicator of cell viability [35, 36]. In contrast, the MTT assay reflects mitochondrial metabolic activity and cellular energy status [36, 37].

The use of these complementary assays provided a more comprehensive assessment of cytotoxicity and cell viability. Due to the limited literature on the protective effects of *C. persica*, comparisons were made with other species of the *Calendula* genus. *C. persica,* belonging to the Asteraceae family, is one of approximately 15 species within the genus. Traditionally, *Calendula* species have been used for the treatment of burns, nervous disorders, insomnia, liver diseases, ocular inflammation, and skin and digestive conditions [25, 38, 39]. Furthermore, various pharmacological activities have been reported, including anti-inflammatory, antimicrobial, antioxidant, anticancer, hepatoprotective, neuroprotective, and cardioprotective effects [25]. These biological activities are attributed to different phytochemicals, such as flavonoids, coumarins, terpenoids, carotenoids, quinones, and fatty acids [24, 25, 38, 40-42]. Several studies support the cytoprotective potential of *Calendula* species. For example, Nowak-Terpiłowska et al. reported that ethanol extracts of *Calendula officinalis* reduced alcohol-induced cytotoxicity in human gingival fibroblasts without causing morphological abnormalities or affecting cell proliferation [42]. Similarly, Alnuqaydan et al. demonstrated that *Calendula* extracts exhibit strong free radical scavenging activity, protecting human skin cells against hydrogen peroxide-induced oxidative stress [43]. In addition, Abutaha et al. reported that *Calendula arvensis* exerts anticancer effects by inducing apoptosis and mor-

phological changes, such as cell shrinkage, nuclear fragmentation, and chromatin condensation in breast cancer cell lines [44].

The cytoprotective effects of *C. persica* observed in this study are likely mediated through multiple mechanisms. Oxidative stress, primarily driven by excessive ROS generation, is a key pathway in ZnO NP-induced toxicity. Phytochemical constituents of *C. persica*, particularly flavonoids and phenolic compounds, may directly scavenge ROS through electron donation and radical neutralization. In addition, these bioactive compounds may enhance endogenous antioxidant defense systems by upregulating enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Furthermore, activation of the Nrf2/HO-1 signaling pathway, along with modulation of MAPK (ERK, JNK, and p38) and inhibition of NF-κB signaling, may contribute to the observed protective effects [43, 44].

Conclusion

The findings of this study demonstrate that *C. persica* extract exerts a significant cytoprotective effect against ZnO NP-induced toxicity in HepG2 cells, as evidenced by improved cell viability and reduced morphological damage. Therefore, *C. persica* may represent a potential hepatoprotective agent against ZnO NP-induced toxicity and could be considered a complementary therapeutic approach for hepatic disorders.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Author's contributions

Study design, supervision, data analysis, and writing the original draft: Ehsan Zayerzadeh; Review and editing: Mohammad Kazem Koohi; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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