Nanomed Res J 10(2):162-170, Spring 2025

RESEARCH ARTICLE

Protective effect of Iranian *Origanum vulgare* extract against zinc oxide nanoparticles cytotoxicity on L-929 cells

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ARTICLE INFO

Article History:
Received 23 Feb 2025
Accepted 07 May 2025
Published 01 Jun 2025

Keywords:
Zinc oxide nanoparticles
O. vulgare
Cytotoxicity
Neutral red uptake
MTT assay

ABSTRACT

Objective(s): Zinc oxide nanoparticles (ZnO NPs) can induce toxicity in living organisms. *Origanum vulgare* (*O. vulgare*), is a medicinal plant which has antioxidant components and traditionally used to treat different health problems in Iran. In this study, we assessed the potential protective effect of *O. vulgare* extract against ZnO NPs cytotoxicity in L-929 cells.

Methods: In the first group, different concentrations of ZnO NPs, and in the second group, one dose of *O. vulgare* extract plus different concentrations of ZnO NPs were exposed to L-929 cells for 24 hours. After treatment, cytotoxicity of different concentrations of ZnO NPs on the cells and protective effect of *O. vulgare* extract against cytotoxicity of ZnO NPs were examined microscopically. Then, the neutral red uptake and MTT assays were performed to determine cell viability.

Results: Higher concentrations of ZnO NPs showed significant morphological abnormalities on the cells. *O. vulgare* extract had no cytotoxic effect on L-929 cells. It could protect and reduce cytotoxicity of higher concentrations of ZnO NPs in cells. The viability of L-929 cells after 24 hours of exposure to ZnO NPs varied depending on the nanoparticles concentration. The results of MTT and NRU assays showed that different concentrations of ZnO NPs induced cell mortality and *O. vulgare* extract could significantly decline cell mortality induced by ZnO NPs.

Conclusions: It can be concluded that *O. vulgare* extract has potent protective effect against the cytotoxicity induced by ZnO NPs on L-929 cells.

How to cite this article
Zayerzadeh E., Koohi M.K. Protective effect of Iranian Origanum vulgare extract against zinc oxide nanoparticles cytotoxicity on L-929 cells. Nanomed Res J, 2025; 10(2): 162-170. DOI: 10.22034/nmrj.2025.02.007

INTRODUCTION

As nanotechnology progresses, the utilization of nanoparticles has seen significant expansion. These compounds are used in different industries [1-3]. Small size and higher surface area to volume ratio are the most important characteristics of nanoparticles. These characteristics result in enhanced chemical reactivity and greater generation of active oxygen [4]. However, nanoparticles have garnered significant interest because of their toxicity. These particles are released into the environment throughout the creation and disposal of consumer goods and induce toxicity by various processes and mechanisms [5, 6]. These substances can readily pass through the cell membrane and engage with internal metabolic processes. In this context, cellular evaluation of nanoparticle toxicity is among the key approaches. Its benefits comprise reduced expenses, quicker processes, and fewer ethical issues [6, 7]. In addition to the cytotoxicity method, nanoparticles are evaluated by other methods, including proliferation method, apoptosis assay, necrosis assay, oxidative stress assay, DNA damage assay and in vivo tests [7-12]. One of the most applicable nanoparticles is ZnO

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NPs which have extensive applications, including cosmetics and toiletries industry, antibacterial and anti-Fungal, food industry, photocatalytic, biosensing, bioimaging, gas sensing, environmental industry, oil and gas industry, electronics industry, and biomedicine [13]. Over the last twenty years, ZnO NPs have risen to prominence as one of the favored metal oxide nanoparticles in biology, particularly in anticancer, antidiabetic, and antibacterial applications, because of their excellent biocompatibility [13, 14]. On the other hand, the results of some studies showed that zinc oxide nanoparticles could cause toxicity in animal and cellular models [15-19]. These nanoparticles can infiltrate the human body via various routes including inhalation, skin absorption, the digestive tract, and parenteral injection [18]. Given the increasing use of these nanoparticles in various industries in our country and the lack of sufficient information about the safety of nanoparticles used in various products, conducting extensive research on the safety of this compounds is urgent and is a very important step in sustainable development of nanotechnology. In this field, there is a new idea to add new compositions to ZnO NP-based products such as sunscreens, facial creams and body lotions to reduce side effects of nanoparticles. One of this components can be medicinal plants which have many beneficial ingredients. Iran has huge treasure of medicinal plants which many of them are unknown. One of them is *Origanum vulgare (O. vulgare)* that is proved as a strong antioxidant and free radical scavenging agent. The major components of this plant include rosmarinic acid, thymol, luteolin, 7-O-glucoside, eriocitrin, origanol A and B, along with ursolic acid [20-22]. The objective of this study is to evaluate the protective role of *O. vulgare* extract against possible cytotoxicity induced by ZnO NPs in L-929 cells.

MATERIALS AND METHODS

Nanoparticles

Zinc oxide nanoparticles with dimensions of 20 nm and purity of 99%, purchased from Pishgaman Company (Iran), and utilized in this investigation without additional purification or sieving. Ultraviolet light was used for 24 hours to sterilize the zinc oxide nanoparticles (Figure 1).

Preparation of O. vulgare extract

O. vulgare was obtained from Zarin Giah Company in Iran, and a botanist taxonomically identified the plant samples through morphological analysis. Subsequently, 100 grams of plant powder were combined with double distilled water and ethanol for a duration of 72 hours. The hydroethanolic extract was filtered, and the filtrate was concentrated by evaporation under vacuum at 40°C. Subsequently, the extract was stored at -20°C until experiments.

Cell Culture

The L-929 cells were purchased from the Cell Bank of Pasteur Institute (Iran). The cell line was cultured in a minimal essential medium (α-MEM medium), supplemented with fetal bovine serum (10%, v/v) and antibiotics (penicillin and streptomycin 1%) and incubated at 37°C in a 5% CO2 atmosphere with 95% humidity for 24 h. Then, the cells were detached from the culture flasks using enzymatic digestion (trypsin/EDTA), and the cell suspension was centrifuged at 200 g for 5 minutes. The cells were re-suspended in culture medium, resulting in a cell suspension of 1×10^5 cells/ml in the culture medium. An 8-channel pipette was utilized to dispense 100 µl of the 1×10^5 cells/ml cell suspension into the wells of a 96-well microtiter plate. The cells were subsequently incubated for 24

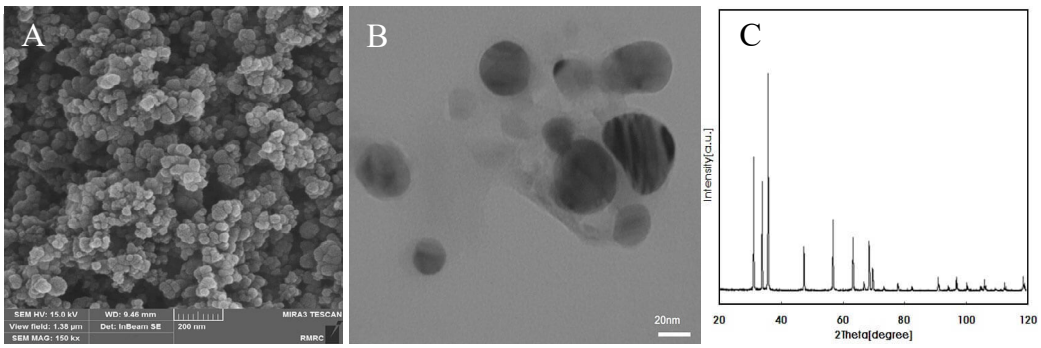

Fig. 1. Scanning electron microscopy (SEM) image (A and B) and XRD pattern (C) of zinc oxide nanoparticles

hours (5% CO2, 37°C, humidity >90%) to develop a semi-dense monolayer. Every well was subsequently inspected using an inverted phase microscope to verify that cell growth progressed uniformly across the microtiter well (Hund, Germany)

Treatment of ZnO NPs and O. vulgare extract onto cells

Following 24 hours of incubation, the culture medium was removed from L-929 cells. In the first group, 100 µl of culture medium with varying concentrations of ZnO NPs (10, 20, 40, 80) µg/ml was added to each well in triplicate. In 100 µl of growth medium, a cell population that was not treated served as the negative control in three replicates, while 100 µl of Triton X-100 was introduced as the positive control in three wells. In the second group, cells were exposed in triplicate to one dose of *O. vulgare* extract (20 µg/mL) along with various concentrations of ZnO NPs (10, 20, 40, 80) µg/mL plus one dose of *O. vulgare* extract (20 µg/mL). Subsequently, the cells were incubated for 24 hours at 37°C, 5% CO2, and humidity greater than 90%.

Neutral red uptake assay

Following treatment, inverted phase microscopy was used to evaluate each well for systematic errors in cell culture and the growth attributes of both control and treated cells. The toxic effects of the nanoparticles were studied, and the resulting morphological changes of the cells were documented. Following the inspection of the wells, the culture medium was taken out from the plate. The cells were subsequently rinsed with 100 µl of pre-warmed PBS. The washing solution was eliminated through gentle rinsing. Subsequently, 100 µg of neutral red medium was introduced and incubated at 37°C in a 5% CO2 environment for 3 hours. Following incubation, the neutral red culture medium was discarded, and the cells were rinsed with 100 µl of PBS. The PBS was subsequently drained gradually. Subsequently, 100 µl of neutral red dye solution (ETOH/acetic acid) was added to each well. Subsequently, the microtiter plate was vigorously shaken for 10 minutes on a microtiter plate shaker until the neutral red was released from the cells, resulting in a homogeneous solution. The absorbance of the colored solution was measured at 540 nm with a plate spectrophotometer (BioTek, USA) [23].

MTT assay

Cell viability was assessed through the MTT assay, a colorimetric technique where mitochondrial dehydrogenases transform MTT into formazan crystals within living cells. For cytotoxicity assessment, L-929 cells in exponential growth were harvested and plated (1×10^4 cells/well) in a 96-well microtiter plate, followed by a 24-hour incubation in a 5% CO2 environment prior to treatment. Following 24 hours of treatment, each plate was inspected under an inverted phase microscope to detect any systematic errors in cell culture and evaluate the growth characteristics of both control and treated cells. Morphological alterations due to the cytotoxic impacts of the test samples were noted. Then, 50 µL of MTT solution (5 mg/mL) was added to every well and incubated at 37°C for three hours. Following incubation, the supernatants were discarded, and 50 µL of isopropanol was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm with a plate spectrophotometer (BioTek, USA). The produced data were saved in a raw data file, and the cell viability percentage was determined.

Statistical Analysis

All data are presented as mean ± SD. The average of all parameters among groups was analyzed using the Student's t-test. Data were examined with the SPSS software (version 19), and a $p<0.05$ is considered statistically significant.

RESULTS

Cytotoxicity of ZnO NPs on L-929 Cells

As can be seen in Figure 2, lower concentrations of ZnO NPs (10 and 20 µg/mL) had no morphological changes on the cells. At these doses, the exposed cells were completely attached to the bottom of the well and maintained their spindle shape and integrity. No cells were rounded and no cell membranes were disrupted. However, higher concentrations of ZnO NPs (40 to 80 µg/mL) showed significant toxic effects on the cells and induced significant morphological changes. Toxic concentrations of ZnO NPs induced cell death and cell rounding. With respect to the exposed dose, the higher the exposure dose, the greater the number of cells that were rounded and detached from the bottom of the well.

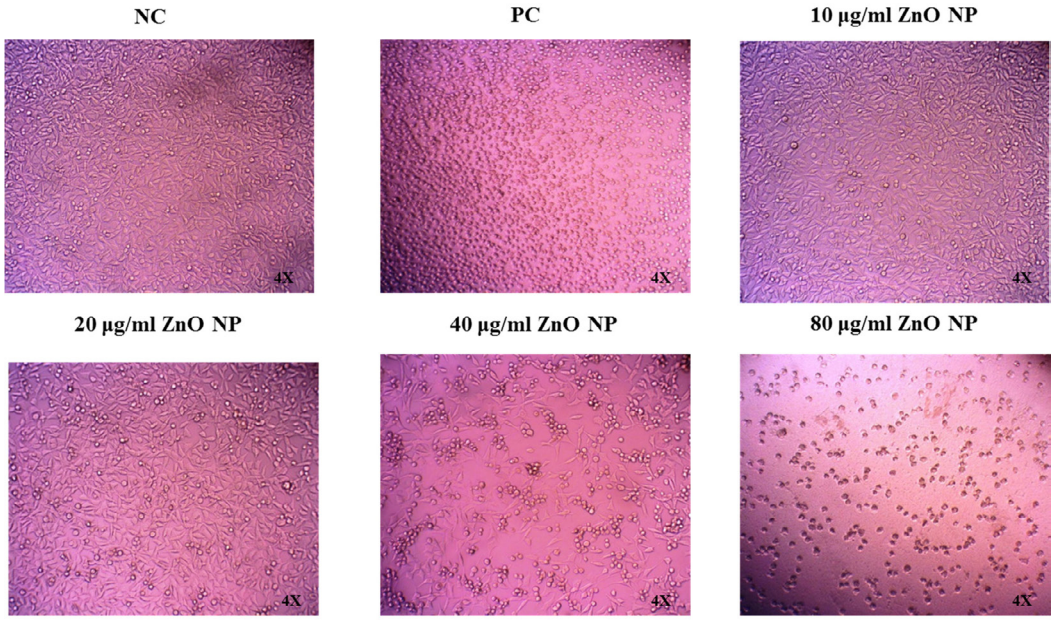

Fig. 2. Inverted microscopy images of cytotoxic effects of different doses (10, 20, 40, 80) µg/ml of ZnO NPs on L-929 cells. NC: Negative control, PC: Positive control. Magnification 4x

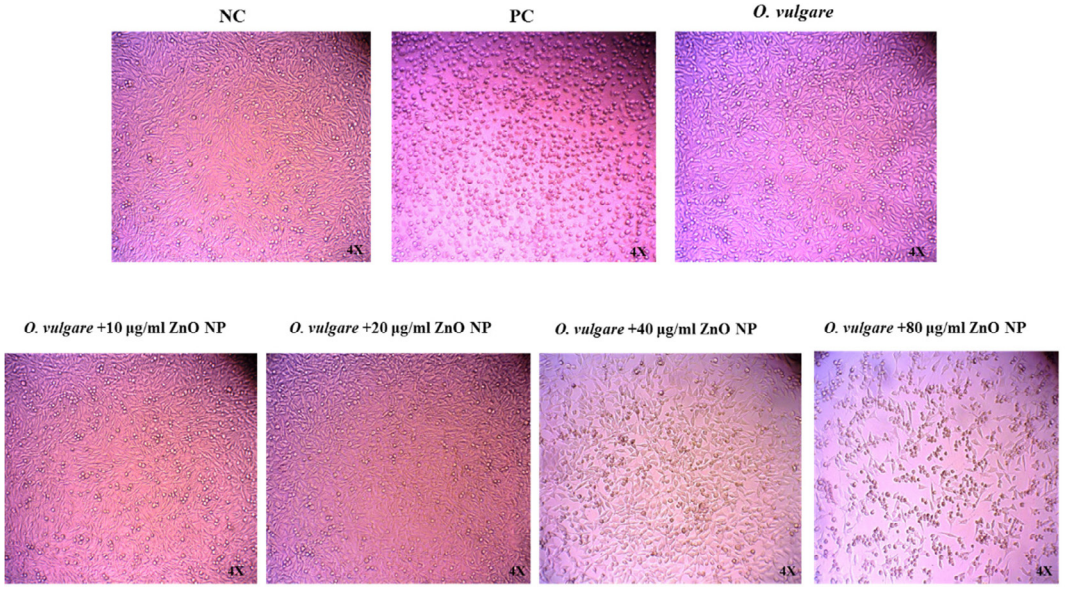

Fig. 3. Inverted microscopy images of protective effect of O. vulgare extract against cytotoxicity of different doses (10, 20, 40, 80) µg/ml of ZnO NPs on L-929 cells. NC: Negative control, PC: Positive control. Magnification 4x

Protective effect of O. vulgare extract against cytotoxicity of ZnO NPs

O. vulgare extract (20 µg/mL) had no cytotoxic effect on L-929 cells. It could protect and reduce cytotoxicity of high concentrations of ZnO NPs (40 and 80 µg/mL) in cells. As can be observed in Figure 3, there were no morphological changes on the cells following *O. vulgare* extract treatment. Furthermore, this plant could decline morphological changes such as rounded cells and could reduce cell death in high doses of ZnO NPs. Considering that lower concentrations of ZnO

NPs (10 and 20 µg/mL) had no morphological alterations in cells, any changes were observed following co-exposure of *O. vulgare* extract plus ZnO NPs. At these concentrations, the exposed cells were completely attached to the bottom of the well and maintained their spindle shape and integrity.

Neutral red uptake assay

The viability of L-929 cells after 24 hours of exposure to ZnO NPs varied depending on the nanoparticle exposure dose. The findings indicated that the greatest level of cell viability occurred at a concentration of 10 µg/mL (90%) and the lowest percentage of cell viability was at a concentration of 80 µg/mL (14%). Thus, based on the findings, an increase in the exposure dose of zinc oxide nanoparticles leads to a decrease in cell viability. As illustrated in Figure 4, a significant decrease in cell viability occurred from a dose of 40 µg/mL, such that the cell viability decreased from 77% at a dose of 20 µg/mL to 39% at a dose of 40 µg/mL. On the other hand, *O. vulgare* extract had no changes in cell viability. In the second group, *O. vulgare* extract could ameliorate cell viability in various concentrations of ZnO NPs, significantly. The results demonstrated that *O. vulgare* extract could alleviate cell viability from 90%, 77%, 39% and 14% to 94%, 89%, 66% and 41%, respectively (figure 5).

MTT assay

As it was depicted in table 1, in the first group, the highest percentage of cell viability was at a concentration of 10 µg/mL ZnO NPs and the lowest percentage of cell viability was at a concentration of 80 µg/mL. On the other hand, *O. vulgare* extract had no changes in cell viability. In the second group, *O. vulgare* extract could protect cell viability in

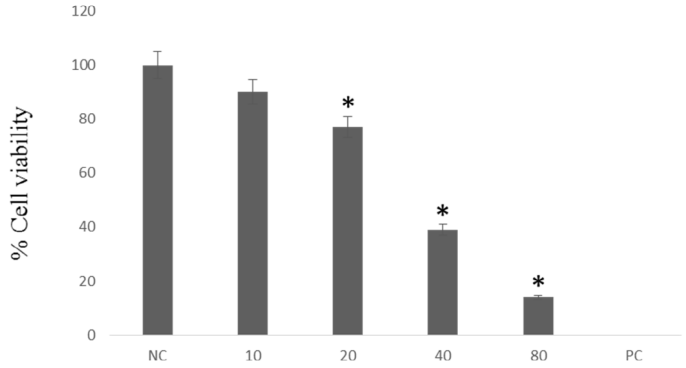

Fig. 4. Results of viability assay (neutral red uptake assay) in L-929 cells treated with ZnO NPs (10, 20, 40, 80 µg/ml) *$P < 0.05$, significant difference compared to the negative control. NC: negative control, PC: Positive control

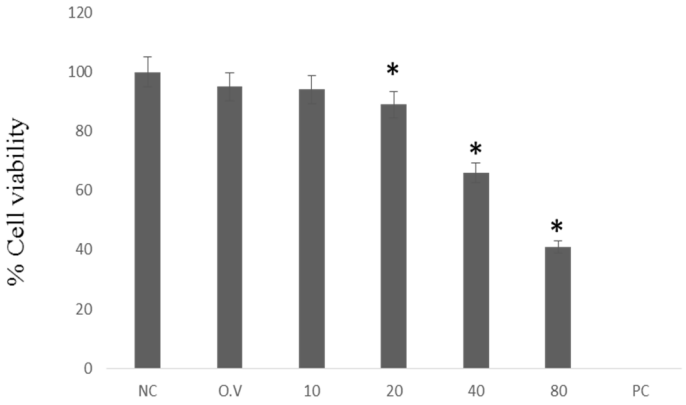

Fig. 5. Results of viability assay (neutral red uptake assay) in L-929 cells treated with one dose of *O. vulgare* extract (20 µg/mL) and different concentrations of ZnO NPs (10, 20, 40, 80) µg/ml plus one dose of *O. vulgare* extract (20 µg/mL). *$P < 0.05$, significant difference compared to the negative control. O.V: *O. vulgare,* NC: negative control, PC: Positive control

Table 1. MTT assay in L-929 cells treated with different concentrations of ZnO NPs (10, 20, 40, and 80) µg/ml plus one dose of *O. vulgare* extract (20 µg/mL)

Treatment	Concentration	Cell viability (%)
ZnO NPs	10 µg/ml	88 ± 4.1
ZnO NPs	20 µg/ml	72 ± 3.4 *
ZnO NPs	40 µg/ml	31 ± 2.1*
ZnO NPs	80 µg/ml	9 ± 0.7*
ZnO NPs + *O. vulgare* extract	10 + 20 µg/ml	94 ± 5.5
ZnO NPs + *O. vulgare* extract	20 + 20 µg/ml	86 ± 4.1**
ZnO NPs + *O. vulgare* extract	40 + 20 µg/ml	60 ± 3.7**
ZnO NPs + *O. vulgare* extract	80 + 20 µg/ml	33 ± 1.9**
O. vulgare extract	20 µg/ml	97 ± 6.3
Negative control	100 µl	100
Positive control	100 µl	0

*$P < 0.05$, significant difference compared to the negative control. **$P < 0.05$, significant difference compared to the first group. O.V: *O. vulgare,* NC: negative control, PC: Positive control

different concentrations of ZnO NPs, significantly. The findings indicated that *O. vulgare* extract was able to improve cell viability in the second group in comparison to the first group.

DISCUSSION

Because of the growing usage of various nanoparticles, including ZnO NPs, in different industries, the possibility of their presence in the environment has increased significantly and it could affect human health following human body exposure [19]. ZnO NPs are utilized in the cosmetic products, particularly in sunscreens and facial creams because it can absorb UV irradiation and optical transparency. Also, it used in various products for its strong antibacterial capability [13]. Therefore, human exposure with this nanoparticle is unavoidable. For this reason, the cytotoxicity of ZnO NPs (20 nm) was investigated in L-929 cells because this cell line is appropriate for cytotoxicity assessment. On the other hand, discovering new ways to protect different cells and as a result, human health against side effects of nanoparticles is urgent. One of this ways is to use medicinal plants to protect cells and human health because many of them have antioxidant and anti-oxidative stress components. Therefore, we assessed protective effect of *O. vulgare* extract against cytotoxicity induced by ZnO NPs on L-929 cells. Our findings indicated that high doses of ZnO NPs with a diameter 20 nm had very toxic effects on L-929 cells. Toxic doses of ZnO NPs caused cell death and cell rounding. In this field, Pinho et al [24] reported that high doses of ZnO NPs (50 nm) had a toxic effect in GC-1 cells. These nanoparticles caused an increase in intracellular reactive oxygen species (ROS) levels, alterations in DNA, changes in cytoskeleton and nucleoskeleton dynamics, and mortality in GC-1 cells, aligning with our findings. In other study Fernández-Bertólez et al reported that ZnO NP reduced viability of A172 glial cells [19]. Czyżowska et al proved that ZnO-NP with a diameter of 100 and 130 nm induced cell mortality at concentrations (12 and 25 mg/L). They observed ZnO-NPs caused mitochondrial damage, cell apoptosis and peroxidation of membrane lipids [25]. In an investigation which had similar results with our study, Maheswaran et al analyzed the toxicity of ZnO NPs produced through a chemical method (C–ZnO NPs) and a green method utilizing *Musa acuminata* leaf aqueous extract (Ma-ZnO NPs) on Vero cells. They noted that C- and Ma-ZnO NPs led to a cytotoxic effect on Vero cells that was dependent on both concentration and duration. They demonstrated that Ma-ZnO NPs exhibited markedly greater cell viability in comparison to C–ZnO NPs [26]. One of the toxicity mechanisms of nanoparticles that has received much attention in recent years is oxidative stress [17, 27]. Oxidative stress leads to harm in lipids, proteins, DNA, and carbohydrates. Lipid peroxidation results in significant damage to the cell membrane, causing substantial alterations in membrane activity that may ultimately result in cell death [28, 29]. Nanoparticles can generate reactive oxygen species, influence intracellular calcium levels, activate transcription factors, and alter cytokine levels. Reactive oxygen species can harm cells in multiple ways, including damaging DNA, disrupting cell signaling pathways, changing gene transcription, and more. Free radicals are transient

intermediate chemical substances that contain one or more unpaired electrons in their outer electron shell. Consequently, they are highly reactive and assault stable nearby molecules to acquire electrons, leading to their oxidation [27]. Nanoparticles may also stimulate inflammatory cells like macrophages and neutrophils in the alveoli, which participate in the phagocytosis of nanoparticles, resulting in the generation of reactive oxygen and nitrogen species [29, 30]. Studies have shown that there is a direct relationship between the ability of metal nanoparticles to induce oxidative stress in cells and the amount of damage caused in target cells [27, 29]. Nonetheless, several factors including dose, duration of exposure, dimensions, form, surface chemistry, and cell types significantly influence the diverse impacts of nanoparticles on cells [31]. Also, our results showed that *O. vulgare* extract is effective to protect cells against cytotoxicity which was induced with ZnO NPs. The Neutral Red Uptake (NRU) assay employed in this study is a validated method recommended by OECD (TG 432) for assessing cytotoxicity based on lysosomal activity [32]. The NRU test offers a measurable assessment of the quantity of living cells in a culture. It is among the most commonly utilized cytotoxicity assays with numerous biomedical and environmental uses. NRU assay assesses lysosomal integrity and activity, indicating the cell's ability to actively take up and retain vital dyes via intact lysosomal membranes. A variety of primary cells and cell lines from different sources can be effectively utilized. The method is less expensive and more precise than alternative cytotoxicity assays [23, 33]. We also performed MTT assay for cell viability assessment. MTT assay measures mitochondrial metabolic activity, reflecting the functional state of mitochondria and cellular energy production [34]. By combining NRU assay and MTT assay, it can obtained a more comprehensive picture of cell health, as each test targets a distinct cellular function. This dual approach increases the sensitivity and reliability of cytotoxicity evaluation and helps distinguish between different mechanisms of cell damage. In a study, Arami et al reported that *O. vulgare* could protect blood lymphocytes from DNA damage and reduced genotoxicity which induced by irradiation. This plant has special components including flavonoids and phenolics which have antioxidant and free radical scavenging characteristics. It has antioxidant components such as carvacrol and thymol which are the two main phenolic compounds. Balusamy et al showed that *O. vulgare* oil triggered mitochondrial-mediated apoptosis in human stomach cancer cell lines and established its effectiveness for treating stomach cancer [35]. In other study, Mashhadi et al [36] reported that *O. vulgare* oil inhibited the growth of BCL-1 cancer cells. They also did not decline normal lymphocytes and macrophages viability which is agreement with our results that proved *O. vulgare* had no toxic effects on L-929 cells. In a study Avola et al proved that *O. vulgare* essential oil is useful to treat inflammation and support human keratinocytes NCTC 2544 cell motility during wound healing [37]. In a separate study, Savini et al demonstrated that *O. vulgare* extract reduced growth and triggered cell death in a dose- and time-dependent manner in colon adenocarcinoma Caco2 cells [38]. Habibi et al also showed that *O. vulgare* could safeguard mouse bone marrow cells from the genotoxic effects induced by cyclophosphamide, suggesting this might be attributable to the scavenging of free radicals resulting from oxidative stress [39]. Therefore, this plant not only is useful for protecting cells against oxidative stress but also is may be effective to treat different cancers. However, further studies are necessary to determine the mechanisms of protective effect of *O. vulgare* extract against cytotoxicity of ZnO NPs. The uniqueness of this study lies in the fact that, to our knowledge, it is the first research evaluating the protective effect of Iranian O. *vulgare* plant on cells that were exposed to ZnO NPs.

CONCLUSION

It can be concluded that *O. vulgare* extract has potent protective effects against the cytotoxicity induced by ZnO NPs on L-929 cells. Therefore, *O. vulgare* extract might help protecting the body against the adverse effects of ZnO NP which are used in cosmetic products such as sunscreens. It may be beneficial to use this plant in such products to reduce side effects of ZnO NPs in human body.

FUNDING

This work was financially supported by Standard Research Institute (project number: 99045)

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest

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