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Experimental Research Article

Gallic acid mitigates DEHP-Induced oxidative stress by upregulating the Nrf2-NFkB pathway in rat bone marrow mesenchymal stem cells

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ABSTRACT

Introduction: Di-2-ethylhexyl phthalate (DEHP), a chemical used in medical bags and tubing, can leach into the bloodstream and bone marrow, causing oxidative stress in bone marrow mesenchymal stem cells (BMSCs). This study investigates the potential of gallic acid (GA), a potent antioxidant, to counteract DEHP-induced oxidative effects in vitro.

Methods: BMSCs were exposed to various GA concentrations (0.063, 0.125, 0.25, 1, and 10 µM) for 4 days, identifying 0.25 µM GA as the optimal concentration based on cell viability. Cells were then treated with 100 and 500 µM DEHP, with or without 0.25 µM GA, for 4 or 8 days to assess viability, population doubling number, total protein levels, malondialdehyde levels, total antioxidant capacity, catalase and superoxide dismutase activity, cellular morphology, and expression of Nrf2 and NFκB genes.

Results: Treatment with DEHP led to a significant, dose- and time-dependent reduction in viability, proliferation rate, total protein levels, total antioxidant capacity, and the activity of antioxidant enzymes. In contrast, GA treatment significantly increased these parameters. Additionally, DEHP resulted in an increase in malondialdehyde levels and caused morphological changes as well as down-regulated NFκB and up-regulating Nrf2 expression. In the co-treatment group, GA counteracted the toxic effects of DEHP at 100 µM compared with control, whereas it only partially mitigated the toxic effects at 500 µM.

Conclusion: Consuming low concentrations of GA over time may reduce DEHP-induced oxidative stress. Further animal studies with GA-rich foods are essential to confirm its preventive benefits in vivo.

Keywords:
Gallic acid
Di-2-ethylhexylphthalate
Bone marrow
Mesenchymal stem cells
Oxidative stress

Introduction

Di-2-ethylhexyl phthalate (DEHP), among many different members of its family, is the most commonly used plasticizer (Ito, et al., 2019). DEHP, a colorless viscous and lipophilic liquid, has almost no odor, and as plasticizer, it is added to various polyvinylchloride

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(PVC) products such as plastic covering, pastes, coloring agents, coating materials, adhesives, wall coverings, plastic tablecloths, furniture upholstery, shower curtains, plastic shoes, dolls, car upholstery, medical tubing, and blood storage bags (Ito, et al., 2019; Horne, et al., 2009). Since DEHP does not chemically bond to PVC, it easily diffuses into the environment, especially following temperature variations, leading to ubiquitous contamination of the surrounding environment (Henkel, et al., 2023). Considering its omnipresence, the general population is exposed to this environmental pollutant through contaminated water, foods (especially high-fat diets), plastics, or other products containing DEHP. This environmental pollutant can enter the human body even during certain medical procedures such as, blood products in plastic bags that may contain 5-38 mg/L of DEHP (Lozano and Cid, 2013; Münch, et al., 2020) and the plastic tubing, used in dialysis procedures or respiratory aids in kidney failure or lung malfunctioning. Therefore, this medical equipment, containing DEHP causes this chemical to enter patients' bodies via blood or respiratory airways (Kim, et al., 2018).

Gallic acid (GA) is a potent antioxidant, abundantly found in various plant sources such as berries, grapes, pomegranates, oak, and chestnut (Wianowska, et al.,2023). Additionally, both green and black tea serve as rich sources of GA. Upon ingestion, GA is readily absorbed, and detectable in human blood serum at micromolar concentrations (Yang, et al., 2020). Studies indicate that an oral dosage of 900 mg/kg GA administered over 28 days does not induce significant adverse effects, with an established LD50 of more than 200 mg/kg/day, underscoring its safety for consumption (Variya, et al., 2019). GA finds applications across multiple fields including medicine, cosmetics, and the food industry. Medically, it has shown efficacy in managing allergies through the modulation of inflammatory mediators (Kim, et al., 2006) and possesses bacteriostatic properties (Tian, et al., 2022). Its most notable role is as an antioxidant (Delfanian , et al., 2021), where it interrupts free radical chain reactions by scavenging reactive oxygen species (ROS) such as hydroxyl radicals (HO.), hydroxide ions (HO-.), singlet oxygen (O22.), superoxide anions (O2.-), peroxide ions (O22-.), hydroperoxyl radicals (ROOH), and nitric oxide (NO.) (Chaudhary, et al., 2023). While ROS naturally occur within cellular environments as signaling molecules, their excessive accumulation, often due to exogenous toxicants, can lead to significant cellular and molecular dysfunction. The endogenous antioxidant defense system, comprising enzymatic components like catalase, superoxide dismutase, and glutathione peroxidase, along with non-enzymatic entities such as vitamin C and glutathione, works to mitigate ROS-induced damage (Yang, et al., 2022). Exogenous antioxidants from plant sources, including GA, play a critical role in fortifying this defense system, thereby mitigating oxidative stress and preventing subsequent biological impairments.

In our previous cytotoxic investigation, it was revealed, that DEHP reduces the viability of rat bone marrow mesenchymal stem cells (BMSCs) in a concentration-dependent manner, when treated with 100 to 2500 μM for 48 hours. While a concentration of 100 μM showed no significant reduction in viability, nucleus diameter, cell metabolism, and oxidative stress, concentration of 500 μM and highercaused a significant reduction inall analyzed parameters due to oxidative stress induced by this chemical (Abnosi and Aliyari Babolghani, 2022; Abnosi and |Aliyari Babolghani, 2020). We also found, that the mechanism of DEHP toxicity was due to the induction of caspase-dependent apoptosis and G1stage cell cycle arrest, resulting from elevatedof P53 gene expression and the induction of oxidative stress (Abnosi, et al., 2023).

Given the widespread use of DEHP in various medical products and its oxidative effects, BMSCs,which play a critical role in supporting osteoblast populations,are inevitably exposed to this chemical. This exposure raises significant concerns regarding the potential negative impacts on bone health and overall cellular function. Consequently, there is an urgent need for further investigation into safer alternatives and the implementation of stricter regulations to ensure the protection of bone health. Therefore, this study aims to utilize GA as a potent natural antioxidant to protect BMSCs and alleviate the toxic impacts of DEHP. By demonstrating GA's expanded potential in its preventive capacity, we hope to highlight its importance in mitigating the harmful effects of DEHP exposure.

Methods and Materials

Extraction and rat bone marrow cells culture

Wistar rats from Pasteur Institute (Tehran,Iran) were housed in the animal facility at Arak University (Arak,

Iran) and provided with standard food at normal temperature and lighting conditions. After one week, the rats were euthanized by cervical dislocation, and their tibias and femurs were surgically removed, with the surrounding flesh cleaned. Both ends of each bone were cut, and the marrow was extracted by injecting 2 ml of culture media (DMEM containing 15% fetal bovine serum and 1% penicillin/streptomycin). The bone marrow content was centrifuged at 250 g for 5 minutes, and the cells were re-suspended in a T25 flask with fresh culture media and placed in a CO_2 incubator. The culture media was replaced every 3 days until a monolayer of cells covered the flask bottom. The cells were then harvested using trypsin-EDTA, washed with phosphate-buffered saline (PBS) (20mM, pH 7.2), and subcultured in a new T25 flask with fresh media. The subculturing process was repeated twice, and the purity of the cells was assessed using a flow-cytometer (Germany, PARTEC (PAS)) before further analysis. All culture materials were purchased from the Gibco company (Germany), and the entire procedure was conducted under sterile conditions.

viability and proliferation of BMSCs

Since trypan blue dye only passes through damaged cell membranes, dead cells appear blue and are counted relative to live cells. Therefore, the trypan blue exclusion method was used to check the viability of the cells in the presence of varying concentrations of GA (0.063, 0.125, 0.25, 1, and 10 µM), and compared to the control group. After 4 days, cells were separated using trypsin-EDTA and homogenized in the culture media. Then, the homogenized cells (50 µL) were mixed with an equal amount of trypan blue (Sigma-Aldrich, USA) (40 mg/ml in phosphate buffer). After 2 minutes of incubation, cell counting was carried out using a hemocytometer chamber, and the percentage of vability was reported. Additionally, the proliferation ability of the BMSCs was calculated using the population doubling number (PDN) formula, i.e., PDN = log N/N0 + 3.32. In the formula, N0 is the initial number of cells cultured, and N stands for the final number of harvested cells.

Based on the viability and proliferation test mentioned above, a concentration of 0.25 µM of GA was selected and used for further analysis in the presence or absence of 100 and 500 µM of DEHP (chosen based on a previous study) (Abnosi and Aliyari Babolghani, 2022; Abnosi and Aliyari Babolghani, 2020; Abnosi et al., 2023) following 4 and 8 days of treatment.

Morphological analysis

Nuclear and cytoplasmic morphology of the cells treated with GA and DEHP individually, as well as in combination, was investigated using Hoechst (5 mg/mL in PBS) and Acridine orange (1mg/mL in PBS), respectively. The plates were washed with PBS, and then 10 µL of each fluorescent dye was added to separate wells containing 100 µL of PBS. The plates were incubated for 5 minutes and observed with a fluorescent inverted microscope (Olympus, Japan, IX70) equipped with a digital camera (Olympus, DP72). The analysis of nuclear diameter (µm) and cytoplasmic area (µm) was carried out using Motic software (Micro Optical Group Company version 1.2).

Extraction of cell content

After 4 and 8 days of treatment, the cells were collected with the aid of trypsin-EDTA, washed and suspended in PBS (50 mM, pH=7.2). Then the cell membrane was lysed by freeze-thawing and centrifuged at 12000g for 10 minutes. The Lowry method was used to determine the concentration of protein, and a standard graph was plotted using bovine serum albumin as a standard. The concentration of total protein was estimated using the linear formula $Y=0.0056X+0.0382$ with $R^2=0.9854$ where Y is absorbance and X is the protein concentration (µg). Further analysis of the biochemical factors was performed considering each sample having the same quantity of protein.

Determination of SOD activity

The activity of superoxide dismutase (SOD) was assessed using nitro-blue tetrazolium (NBT) (Sigma-Aldrich, N6876). Briefly, 50 µL of extracted samples containing same amount of protein were mixed with 1 ml of a reagent containing NBT (6.1 mg), methionine (1.9 mg), riboflavin (7.9 mg), and EDTA (3.3 mg) all dissolved in potassium phosphate to make a final volume of 10 ml. After incubating the mixture in a light box for 10 minutes, the absorption was taken at 560 nm. A separate blank and control were also prepared without sample extract. The blank tube was kept away from light while the control and other sample tubes were placed in the light box for 10 minutes. Using the blank tubes, the

TABLE 1: Sequences of primer sets used for gene expression analysis.

Gene	Size (bp)	Annealing temp. (°C)	Sequence
		54.8	R: GCACCTCCAGGGAAAAC
GAPDH	136 bp	56.4	F: TCGTCTCATAGACAAGATGG
		59.4	R: GTAGTTGAGGTCAATGAAGGG
Nrf2	177 bp	58.1	F:GGACCTAAAGCACAGCCAACACAT
		57.7	R:TCGGCTTGAATGTTTGTCTTTTGTG
NF-κβ	201 bp	61.8	F: ACCTTTGCTGGAAACACACC
		60.5	R: ATGGCCTCGGAAGTTTCTTT
		59.4	R: TTGGGATGGAGGGAGTTTA

T80^{+}-spectrophotometer (PG company, England) was adjusted to zero, and measurements were carried out. To calculate the activity of the enzyme, the absorption of the control and samples was subtracted and divided by the absorption of the control. The activity of the enzyme was reported as units per minute per mg of protein required to cause 50% inhibition.

Catalase activity estimation

To estimate the activity of catalase (CAT), a reaction mixture was prepared by combining 300 µL of H2O2 and 200 µL of 25 mM potassium phosphate buffer (pH 7.0). The absorption of the solution was adjusted to 0.4 before the measurement. The CAT activity was determined by adding 50 µL of samples containing an equal amount of protein to the mixture, and the reduction in absorption was measured after 2 minutes at 240 nm using the T80+ spectrophotometer (PG company, UK). The CAT activity was calculated using an extinction coefficient of 39.4 mM−1cm−1 after 1 minute.

Determination of lipid peroxidation

Malondialdehyde (MDA) levels were estimated to determine the level of lipid peroxidation. To 1 ml of the reaction mixture (containing thiobarbituric acid (0.5%) and trichloroacetic acid (20%) in HCl), 100 µL of samples containing an equal amount of protein was added, and the tube was boiled for 30 minutes. Then the tube was kept in an ice bath for 15 minutes and centrifuged for 15 minutes at 10000g. Using a T80^{+}spectrophotometer (PG Instrument Company, England), sample absorption was measured at 523 nm and then at 600 nm. After subtracting the values using the extinction coefficient (155 $mM^{-1}cm^{-1}$), the MDA concentration was determined and reported as µM/ml.

Estimation of total antioxidant content

Based on an equal concentration of protein, the Total Antioxidant Content (TAC) was estimated by mixing 150 µL of the sample and 1700 µL of a reagent containing sodium acetate buffer (300 mM, pH 6.3), 2,4,6-Tri(2-pyridyl)-s-triazine (10 mM) (Sigma-Aldrich, USA) (which was dissolved in 40 mM Hydrochloric Acid) and Iron chloride (20 mM). Then 850 µL of double-distilled water was added to the above solution and after incubating the mixture for 10 minutes, (direct light was avoided), absorbance was measured at 593 nm with the help of T80^{+}-spectrophotometer (PG Instrument Co. England). By plotting a standard graph with various concentrations of iron sulfate (FeSO4.7H2O) from Merck company, the TAC levels in the samples were estimated using the formula Y= 0.0072X+0.0011 with R^2= 0.9965, where Y is absorption and X is the concentration.

Gene expression analysis

Total RNA extraction was carried out using a commercial kit (Super RNA extraction kit YT9080) and the cDNA was synthesised with the help of the BioFACT kit (BR631-096). Amplification of Nuclear factor erythroid 2-related factor 2 (Nrf2), Nuclear factor kappa B (*NFkB*) and glyceraldehyde dehydrogenase (*Gapdh))* genes was carried out using a PCR machine (Eppendorf master cycler gradient, Eppendorf Co. Hamburg, Germany) in triplicate with their specific primers (Table 1).

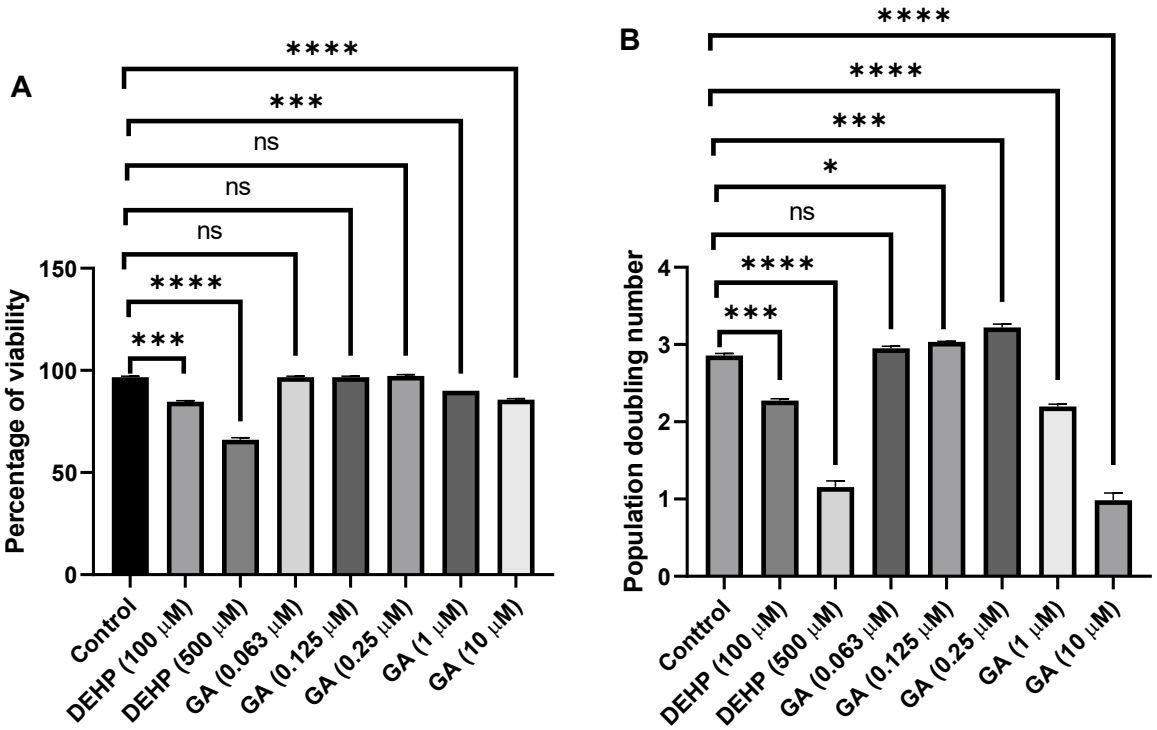

FIGURE 1. Selection of effective concentration based on cell toxicity analysis of BMSCs from different concentration of DEHP and GA after 4 days of incubation: A) cell viability using trypan blue assay B) cell proliferation using PDN calculation. Control group (no treatment with any of the tested compounds), DEHP (100 µM) group (cells treated with 100 µM of DEHP), DEHP (500 µM) group (cells treated with 500 µM of DEHP), GA (0.063 µM) group (cells treated with 0.063 µM of gallic acid), GA (0.125 µM) group (cells treated with 0.125 µM of gallic acid), GA (0.25 µM) group (cells treated with 0.25 µM of gallic acid) GA (1 µM) group (cells treated with 1 µM of gallic acid), and), GA (10 µM) group (cells treated with 10 µM of gallic acid). All the mean values were compared with the control, and the data are presented as mean ± SD with the level of significance as (ns) $p>0.05$, (*) $p<0.05$, (***) $p<0.001$ and (****) $p<0.0001$.

The PCR program was carried out at 95° C for 5 minutes as an initial stage followed by 35 cycles consisting of 95° C for 1 minute, an annealing temperature specific to the primer for 1 minutes, and 72° C for 1 minute. The final stage included an elongation temperature of 72°C for 7 minutes. The PCR product was checked using agarose gel (1.5%) then the results were visualized and photographed using Gel documentation (Gel flash, Syngene bio imaging, England) and further analyzed by Gel Quant software (Gel Quant: 1.8.2).

Statistical analysis

Data analysis was performed using SPSS software (version 20). One-way analysis was used with Tukey's test as the post hoc test. GraphPad Prism was used to create the graphs. Results were presented as mean ± SD, and a significance level of $p < 0.05$ was considered.

Results

Effect on viability and proliferation

Concentrations of GA from 0.063 to 0.25 µM showed no significant change ($p>0.05$) in cell viability compared to the control group (Figure 1A). However, in terms of cell proliferation, 0.063 µM of GA did not show any change, while 0.125 and 0.25 µM significantly increased cell proliferation, with the highest effect observed at 0.25 µM (Figure 1B). GA at concentrations of 1 and 10 µM showed significant ($p<0.0001$) cell toxicity in terms of viability and proliferation (Figure 1A and B). Data analysis revealed that the selected concentrations of DEHP (100 and 500 µM) caused a highly significant ($p<0.001$) reduction in the viability and proliferation ability of BMSCs compared to the control group (Figure 1A and B).

Ameliorating effect of GA

Treatment of the cells with 100 and 500 µM of DEHP for a period of 4 and 8 days showed, a highly significant decrease in viability ($p<0.0001$) (Figure 2A). It was revealed that treatment of the cells with GA and DEHP

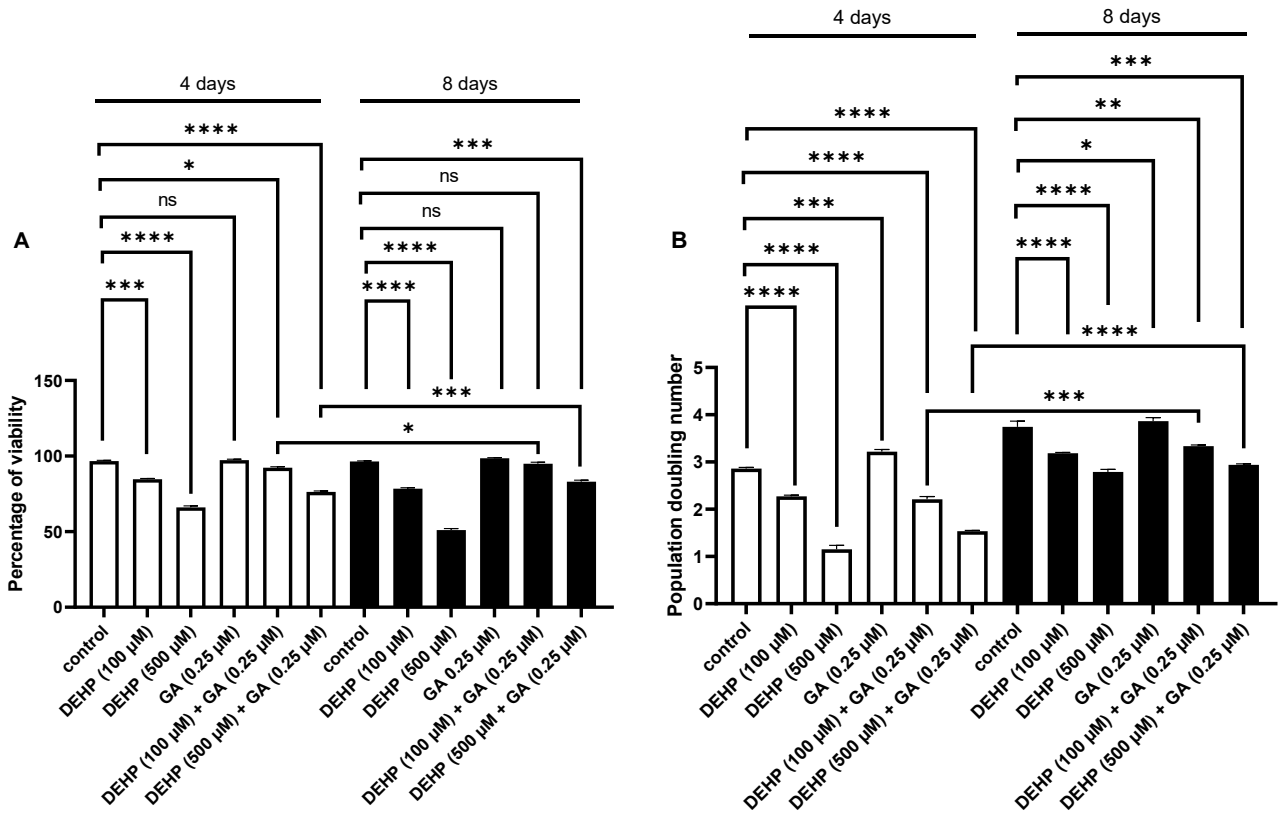

FIGURE 2. Ameliorating effect of 0.025 µM of GA on 100 and 500 µM of DEHP following 4 and 8 days of incubation: A) cell viability using trypan blue assay B) cell proliferation using PDN calculation. Control group (no treatment with any of the tested compounds), DEHP (100 µM) group (cells treated with 100 µM of DEHP), DEHP (500 µM) group (cells treated with 500 µM of DEHP), GA (0.25 µM) group (group of cells treated with 0.25 µM of gallic acid), DEHP (100 µM) + GA (0.25 µM) group (cells treated with 100 µM of DEHP and 0.25 µM of gallic acid simultaneously) and DEHP (500 µM) + GA (0.25 µM) group (cells treated with 500 µM of DEHP and 0.25 µM of gallic acid simultaneously. All the mean values were compared with control and data is presented as mean ± SD with the level of significance as (ns) $p>0.05$, (**) $p<0.01$, (***) $p<0.001$ and (****) $p<0.0001$.

improved viability, especially with increased treatment time. As shown in Figure 2A, the viability of cells treated with 0.025 µM of GA and 100 or 500 µM of DEHP for 8 days significantly improved ($p<0.05$) and highly significantly improved ($p<0.001$) respectively compared to the same group treated for 4days. The same improvement in viability was also observed in terms of cell proliferation ability based on PDN of the BMSCs (Figure 2B).

Cell morphology analysis

Data analysis showed that the nuclear diameter as well as cytoplasmic area of BMSCs significantly decreased ($p<0.0001$) when treated with different concentrations of DEHP after 4and 8 days. In co-treatment groups, GA could significantly ($p<0.05$) mitigate the toxic effects of 100 and 500 µM of DEHP on nuclear diameter and cytoplasmic area after 4 and 8 days, respectively compared to the groups treated with DEHP only. It is worth mentioning that the toxic effect of DEHP (100 µM) was neutralized by GA in 4 and 8 days, while only after 8 days of treatment the nuclear diameter was compensated compared to the control group (Figure 3A and B). The microscopic observation of groups treated for 4 and 8 days also showed that the nuclei of the cells treated with 100 and 500 µM of DEHP were smaller and more condensed in comparison with the control group (Figure 4B and C). Additionally, cytoplasmic shrinkage was observed in the DEHP-treated groups (Figure 4B and C). In the groups treated simultaneously with GA and DEHP, most of the nuclei and cytoplasm of the cells exhibited normal morphology with the same appearance as the control group (Figure 4E and F).

Analysis of protein concentration

The concentration of extracted protein from BMSCs treated with 100 and 500 µM of DEHP decreased sig-

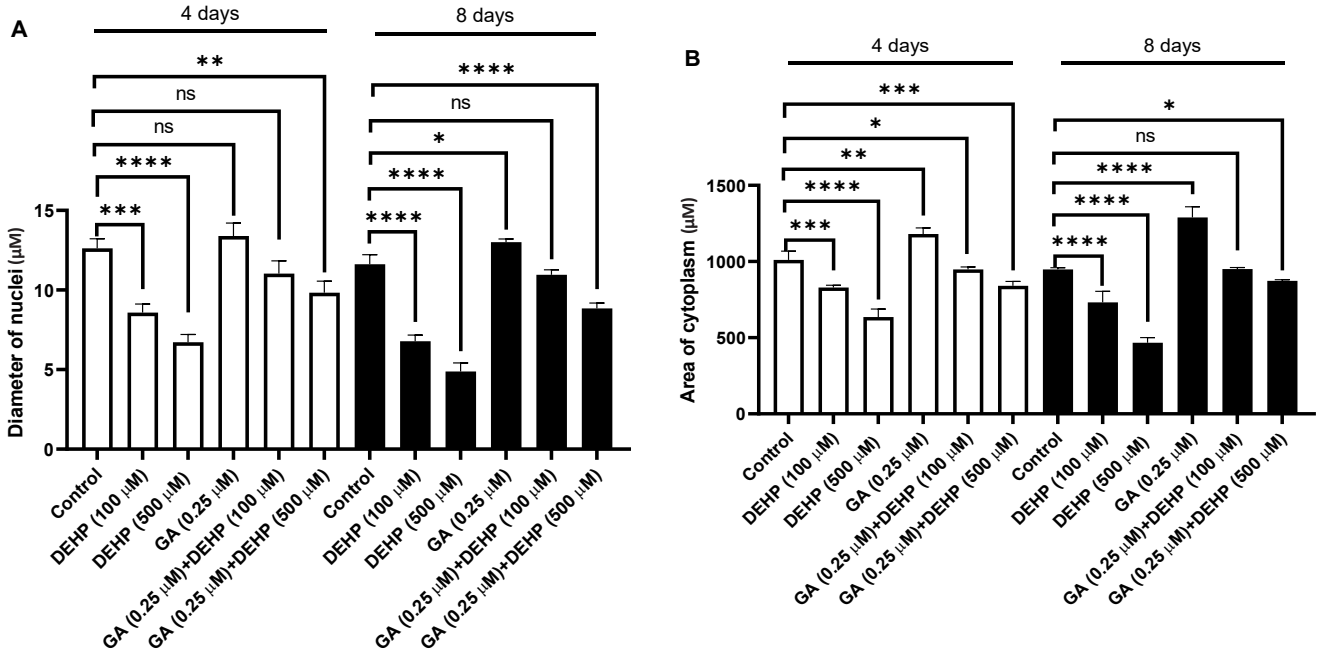

FIGURE 3. Mean A) nuclear diameter and B) cytoplasmic area of BMSCs treated with 0.025 μM of GA as well as 100 and 500 μM of DEHP following 4 and 8 days of incubation: A) cell viability using trypan blue assay B) cell proliferation using PDN calculation. Control group (no treatment with any of the tested compounds), DEHP (100 μM) group (cells treated with 100 μM of DEHP), DEHP (500 μM) group (cells treated with 500 μM of DEHP), GA (0.25 μM) group (cells treated with 0.25 μM of gallic acid), DEHP (100 μM) + GA (0.25 μM) group (cells treated with 100 μM of DEHP and 0.25 μM of gallic acid simultaneously) and DEHP (500 μM) + GA (0.25 μM) group (cells treated with 500 μM of DEHP and 0.25 μM of gallic acid simultaneously. All the mean values were compared with control, and data are presented as mean ± SD with the level of significance as (ns) $p>0.05$, (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ and (****) $p<0.0001$.

nificantly ($p<0.0001$) after 4 and 8 days of treatment compared to the control group. Co-treatment of the cells with 0.025 μM of GA and DEHP (100 and 500 μM) for 4 and 8 days, showed that GA significantly ($p<0.001$) compensated for the toxic effects of this environmental pollution compared to the groups treated with DEHP only. It was observed that only after 8 days, the toxic effect of 100 μM DEHP was compensated for with 0.025 μM of GA (Figure 5).

Estimation of total antioxidant capacity and malondialdehyde

In the cells treated with 100 and 500 μM of DEHP for 4 and 8 days, the concentration of malondialdehyde (MDA) (Figure 6A) increased significantly ($p<0.0001$), while the total antioxidant capacity (TAC) (Figure 6B) significantly decreased ($p<0.0001$) compared to the respective control groups. Although, co-treatment of the cells compensated for the oxidative effect of DEHP (100 μM) at 4 and 8 days compared to the control, the compensation was not fully recovered at 500 μM when compared with the group treated only with DEHP (500 μM) (Figure 6A).

As mentioned earlier, TAC decreased significantly in the groups treated with DEHP after 4 and 8 days. However, treatment of the cells with GA resulted in a highly significant ($p<0.0001$) compensation for the DEHP (100 and 500 μM) after 4 and 8 days of treatment. By day 8, the compensating effect of GA in the presence of 100 μM of DEHP had reached the control level (Figure 6B).

Antioxidant enzymes activity

The cells exposed to 100 and 500 μM of DEHP showed a highly significant ($p<0.0001$) reduction in catalase (CAT) activity and superoxide dismutase (SOD) following treatment for 4 and 8 days (Figure 7A and B). Co-treatment of the cells with 0.25 μM of GA and 100 as well as 500 μM of DEHP individually for 4 and 8 days, caused highly significant ($p<0.0001$) elevation of enzyme activity compared to the groups treated with 100 μM and 500 μM, respectively. The compensation of GA was more effective in 8 days, as the activity of these

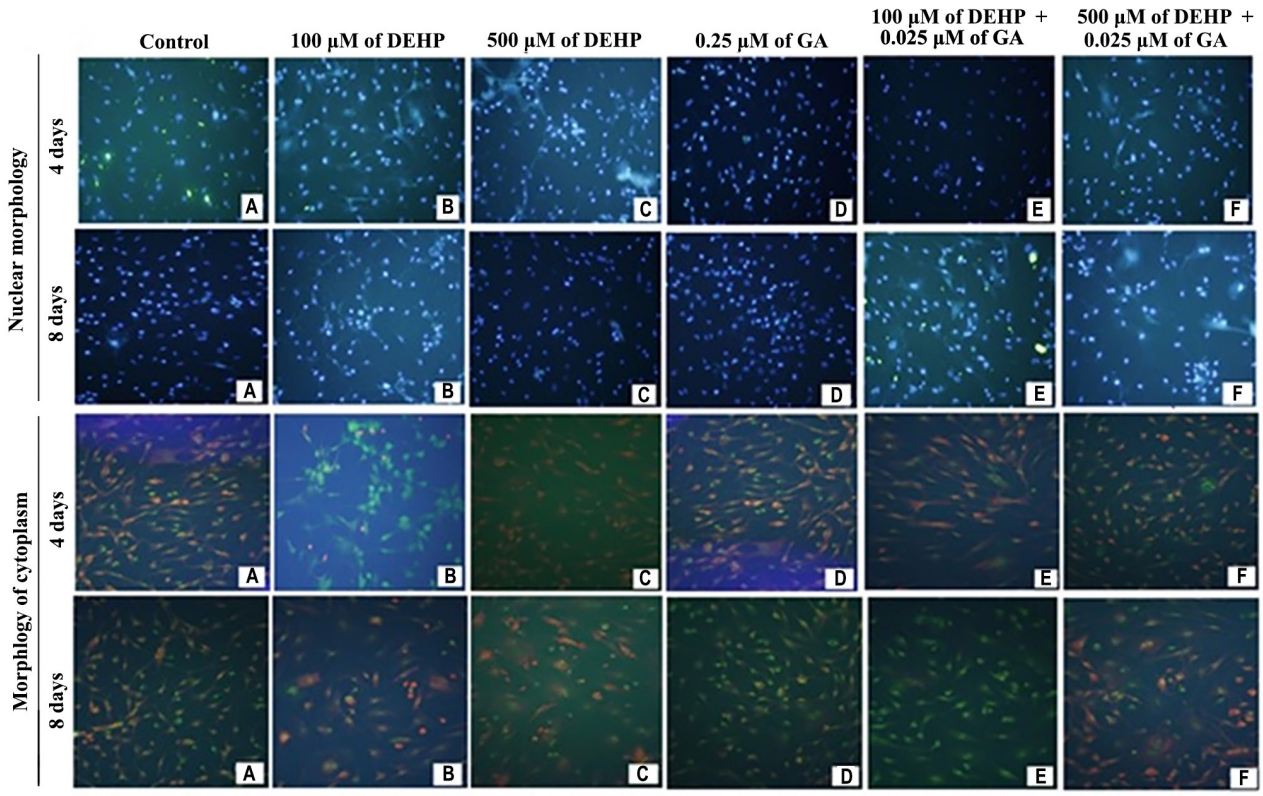

FIGURE 4. Morphological analysis: Photographs show nuclear morphology and cytoplasm morphology of the BMSCs after4 and 8 days of treatment. A) control, B) treated with100 µM of DEHP, C) treated with 500 µM of DEHP, D) treated with 0.25 µM of GA, E) treated with 0.25 µM of GA and 100 µM of DEHP, F) treated with 0.25 µM of GA and 500 µM of DEHP. (Microscope magnification 200 ×).

enzymes was the same as the control groups, respectively (Figure 7A and B).

Gene expression analysis

The expression of Nrf2 was significantly down-regulated ($p<0.0001$), and NFkB was highly up-regulated ($p<0.001$) following treatment with 100 and 500 µM of DEHP for 4 and 8 days compared to the control group. However, in the co-treated groups, GA caused a significant elevation of Nrf2 and reduction of NFkB expression after 4 and 8 days respectively when compared with the groups treated with 100 and 500 µM only (Figure 8A). The results indicated that after 8 days of treatment , GA could completely compensate ($p>0.05$) for the effect of DEHP (100 µM) on Nrf2 and NFkB when compared with the control group. The gel electrophoresis photograph also showed the same result (Figure 8A and B). The expression of GAPDH as a housekeeping gene did not show any variation (Figure 9).

Discussion

Research has shown that DEHP, a common component in PVC products, can infiltrate the bloodstream (Lozano and Cid, 2013; Larsson, et al., 2021). In vitro studies also indicate that this chemical significantly diminishes the viability and proliferation of BMSCs (Abnosi and Aliyari Babolghani, 2022; Abnosi and Aliyari Babolghani, 2020; Abnosi, et al., 2023). As BMSCs are essential for the generation of osteoblasts, their toxicity directly impacts bone regeneration and remodeling (Zhang, et al., 2022). Abnosi et al. (2022) found that a DEHP concentration of 100 µM did not affect the viability of BMSCs after 48 hours. However, prolonging the exposure to four days markedly reduced cell proliferation. Furthermore, the study revealed that DEHP concentrations ranging from 500 to 2500 µM reduced both viability and proliferation of BMSCs even after just 12 hours of treatment (Abnosi and Aliyari Babolghani, 2022). Another study by Abnosi et al. (2020) further corroborated the toxic effects of DEHP, demonstrating

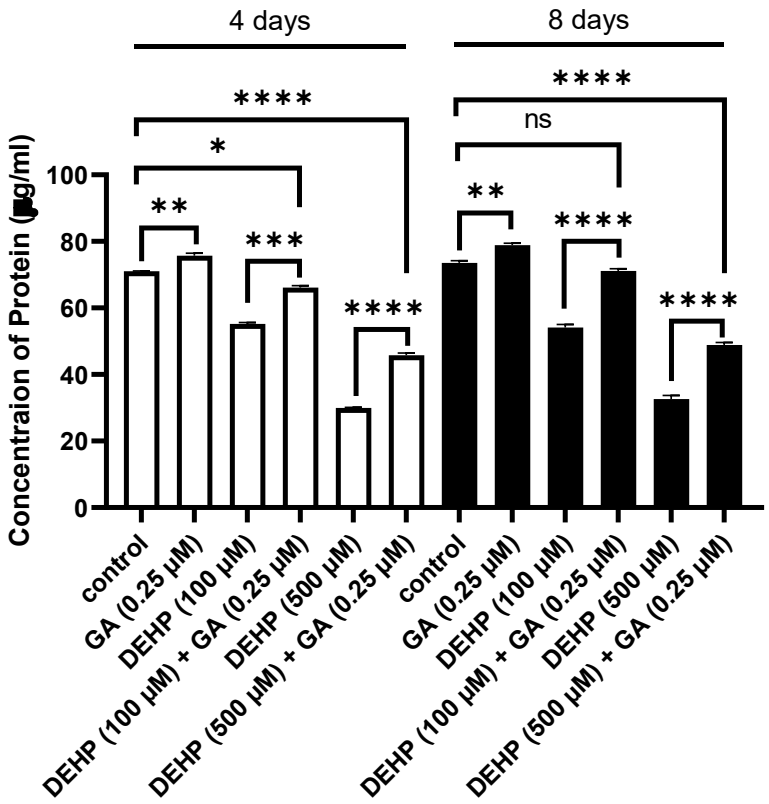

FIGURE 5. Mean protein concentration extracted from BMSCs treated with 0.025 µM of GA as well as 100 and 500 µM of DEHP following 4 and 8 days of incubation. The control group (no treatment with any of the tested compounds), DEHP (100 µM) group (group of cells treated with 100 µM of DEHP), DEHP (500 µM) group (cells treated with 500 µM of DEHP), GA (0.25 µM) group (cells treated with 0.25 µM of gallic acid), DEHP (100 µM) + GA (0.25 µM) group (cells treated with 100 µM of DEHP and 0.25 µM of gallic acid simultaneously) and DEHP (500 µM) + GA (0.25 µM) group (cells treated with 500 µM of DEHP and 0.25 µM of gallic acid simultaneously. All the mean values were compared with control, and data are presented as mean ± SD with the level of significance as (ns) $p>0.05$, (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ and (****) $p<0.0001$.

its detrimental impact on the viability of osteoblasts derived from BMSCs (Abnosi and Aliyari Babolghani, 2020). It appears that DEHP is hazardous even at short-term exposure when at high concentrations, while lower concentrations require longer exposure to pose a threat. Both of the aforementioned studies align with the findings of the present study, as treatment of BMSCs with 100 µM of DEHP for 4 and 8 days also significantly reduced the viability and proliferation of these cells.

In the present study, we also found that treating BMSCs with a low concentration of DEHP (100 µM) for 4 and 8 days affected the nuclear and cytoplasmic morphology of the cells, a finding corroborated by another study (Abnosi, et al., 2023). Additionally, Abnosi et al. (2022) showed, that the cell membrane of the BMSCs is damaged by oxidative stress induced by DEHP (Abnosi and Aliyari Babolghani, 2022), which was considered to be the main reason for viability reduction and morphological changes. In the present study, the same factor was analyzed and confirmed the results of the previous investigation. Meanwhile, we found that the total protein content of the cells decreased significantly. Although we suggest detailed investigation to be carried out on microtubules and microfilaments in presence of DEHP, but reduction of protein content of the cell might be an important biochemical factor responsible for morphological changes since the cytoskeleton arrangements are critical for normal shape of the cell (Nurmagambetova, et al., 2023).

DEHP is a lipophilic compound (Henkel, et al., 2023) that can accumulate in the lipid portions of tissues, such as bone marrow (Rendina-Ruedy and Rosen, 2020). Our present study demonstrated that prolonged treatment (8 days) with DEHP enhances the toxicity of this environ-

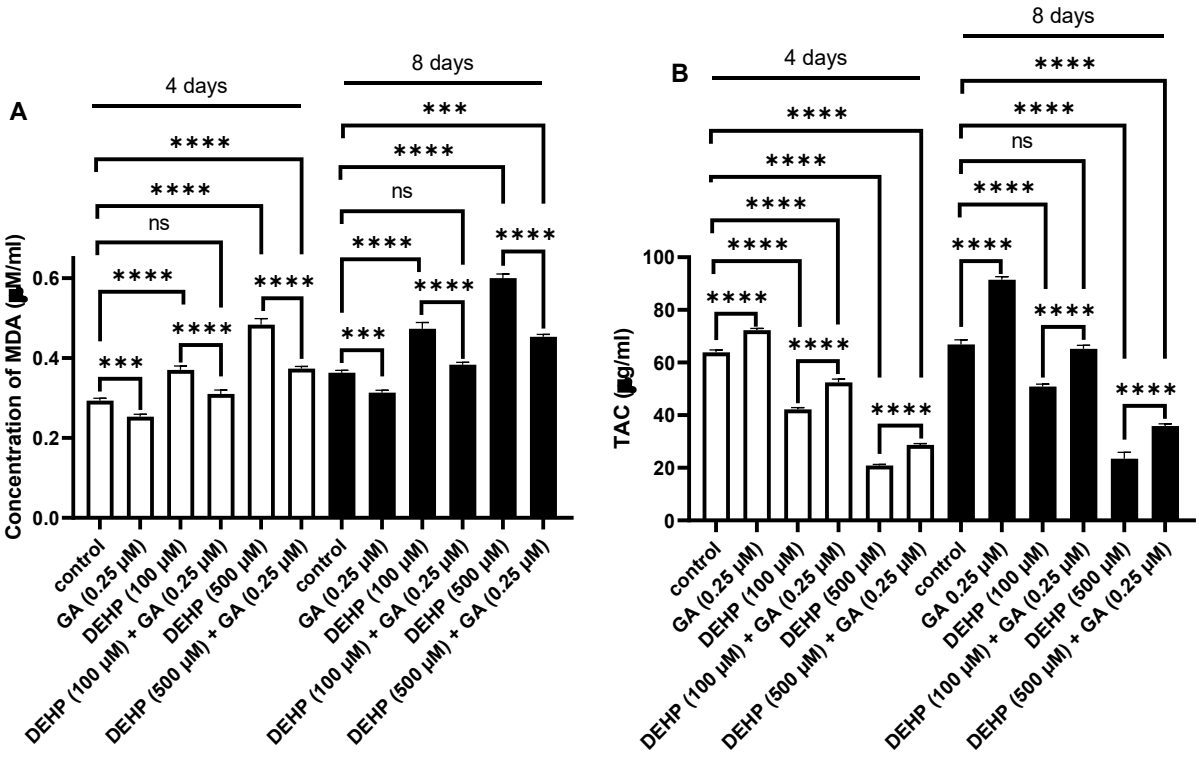

FIGURE 6. Mean A) concentration of MDA and B) concentration of total antioxidant capacity (TAC) in BMSCs treated with 0.025 µM of GA as well as 100 and 500 µM of DEHP following 4 and 8 days of incubation. Control group (no treatment with any of the tested compounds), DEHP (100 µM) group (cells treated with 100 µM of DEHP), DEHP (500 µM) group (cells treated with 500 µM of DEHP), GA (0.25 µM) group (cells treated with 0.25 µM of gallic acid), DEHP (100 µM) + GA (0.25 µM) group (cells treated with 100 µM of DEHP and 0.25 µM of gallic acid simultaneously) and DEHP (500 µM) + GA (0.25 µM) group (cells treated with 500 µM of DEHP and 0.25 µM of gallic acid simultaneously. All the mean values were compared with the control, and the data are presented as mean ± SD with the level of significance as (ns) $p>0.05$, (***) $p<0.001$ and (****) $p<0.0001$.

mental pollutant, confirming that its harmful effects intensify with continuous exposure. Given the widespread use of PVC, human exposure to DEHP is unavoidable, making it crucial to mitigate the adverse effects of this pollutant, particularly during medical procedures.

Gallic acid (GA), a natural plant compound, is highly soluble in water, and following its oral consumption, approximately 70% of GA is absorbed (Bhuia, et al., 2024). After consumption of foods containing GA, the micromolar concentration of this antioxidant is measured in human blood serum (Yang, et al., 2020), indicating its fast absorption. No side effects have been reported following the consumption of 900 mg/kg of GA for 28 days, and the LD50 for this natural compound was reported to be far more than 2000 mg/kg/day (Variya, et al., 2019). Therefore, GA consumption is safe and a variety of its applications in the medicine, cosmetics, food industries, and many others have been reported (Kim, et al., 2006; Tian, et al., 2022; Delfanian, et al., 2021). Thus, continuous consumption of GA through fruits, vegetables, or even some woody parts of plants, may help alleviate the toxic effects of DEHP.

The present study revealed, that treating the cells with gallic acid (GA) ameliorated the cytotoxic effects of DEHP (100 µM) on morphology and protein levels, particularly after 8 days of treatment. It was also observed that the impact of DEHP at 500 µM improved with GA treatment, although not entirely compensated. GA is a potent antioxidant (Velderrain-Rodriguez, et al., 2018; Geo, et al., 2019), while DEHP has been shown to induce oxidative stress (Abnosi and Aliyari Babolghani, 2022; Abnosi and Aliyari Babolghani, 2020). Therefore we observed that GA was effective in reducing the concentration of malondialdehyde (MDA), a lipid peroxidation marker, and additionally enhanced the activity of antioxidant enzymes, especially after 8 days of treatment. Since the toxic effects of DEHP are largely due to reactive oxygen species (ROS) production, we recommend the prolonged consumption of low concentrations of GA, particularly through daily food intake

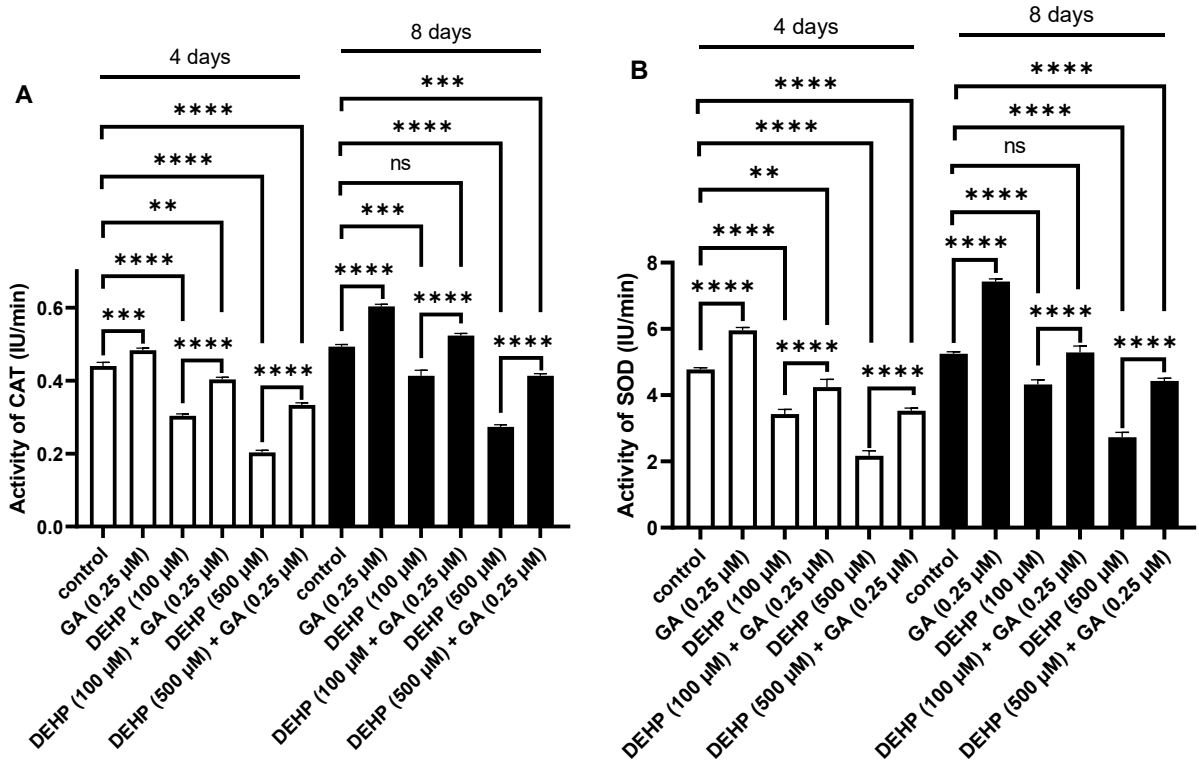

FIGURE 7. Mean A) activity of catalase (CAT) and B) activity of superoxide dismutase (SOD) in BMSCs treated with 0.025 µM of GA as well as 100 and 500 µM of DEHP following 4 and 8 days of incubation. Control group (no treatment with any of the tested compounds), DEHP (100 µM) group (cells treated with 100 µM of DEHP), DEHP (500 µM) group (cells treated with 500 µM of DEHP), GA (0.25 µM) group (cells treated with 0.25 µM of gallic acid), DEHP (100 µM) + GA (0.25 µM) group (cells treated with 100 µM of DEHP and 0.25 µM of gallic acid simultaneously) and DEHP (500 µM) + GA (0.25 µM) group (cells treated with 500 µM of DEHP and 0.25 µM of gallic acid simultaneously. All the mean values were compared with the control, and data are presented as mean ± SD with the level of significance as (ns) $p>0.05$, (**) p,0.01, (***) $p<0.001$ and (****) $p<0.0001$.

of tea leaves extract and fruits. This might serve as a crucial supplementary measure for individuals exposed to DEHP, especially patients undergoing medical treatments.

Studies have shown that the antioxidant properties of phenolic compounds, along with their carboxy group, depend on the number of hydroxy groups attached to the phenolic ring (Chen, et al., 2020; Rajan, et al., 2017) which play a crucial role in donating electrons to ROS. Among the various forms of phenolic compounds, such as caffeic acid, ferulic acid, protocatechuic acid, coumaric acid, ellagic acid, resveratrol, quercetin, and rosmarinic acid, GA is the most abundant. It has a simple molecular formula with three hydroxyl groups and can be found in a variety of plant products, particularly tea, without any restrictions on its use (Hadidi, et al., 2024). As proposed, the hydroxyl group of GA neutralizes free radicals and prevents further production of ROS (Nourah, et al., 2020); therefore, it ameliorated the oxidative damage induced by DEHP and improved the osteogenic ability of BMSCs.

SOD and CAT are the enzymes that nullify the ROSs in a concert mechanism by first converting the superoxide to hydrogen peroxide and reducing the H_2O_2 to water and molecular oxygen (Jing, et al., 2020) which synergistically reduces the molecular oxidation caused by oxidative stress. The activity of these enzymes is regulated by the Nrf2-ARE pathway when Nrf2 released from Kelch-like ECH-associated protein-1 and translocated to the nucleus to bind to antioxidant response elements (AREs) which finally initiates the expression of antioxidant related genes (Ali, et al., 2022). There is a strong cross-talk between Nrf2 and NFkB in opposite direction, where elevation of NFkB inhibits the activity of Nrf2 by forming complex with transcriptional co-activator CBP at promoter region of the genes, leading to

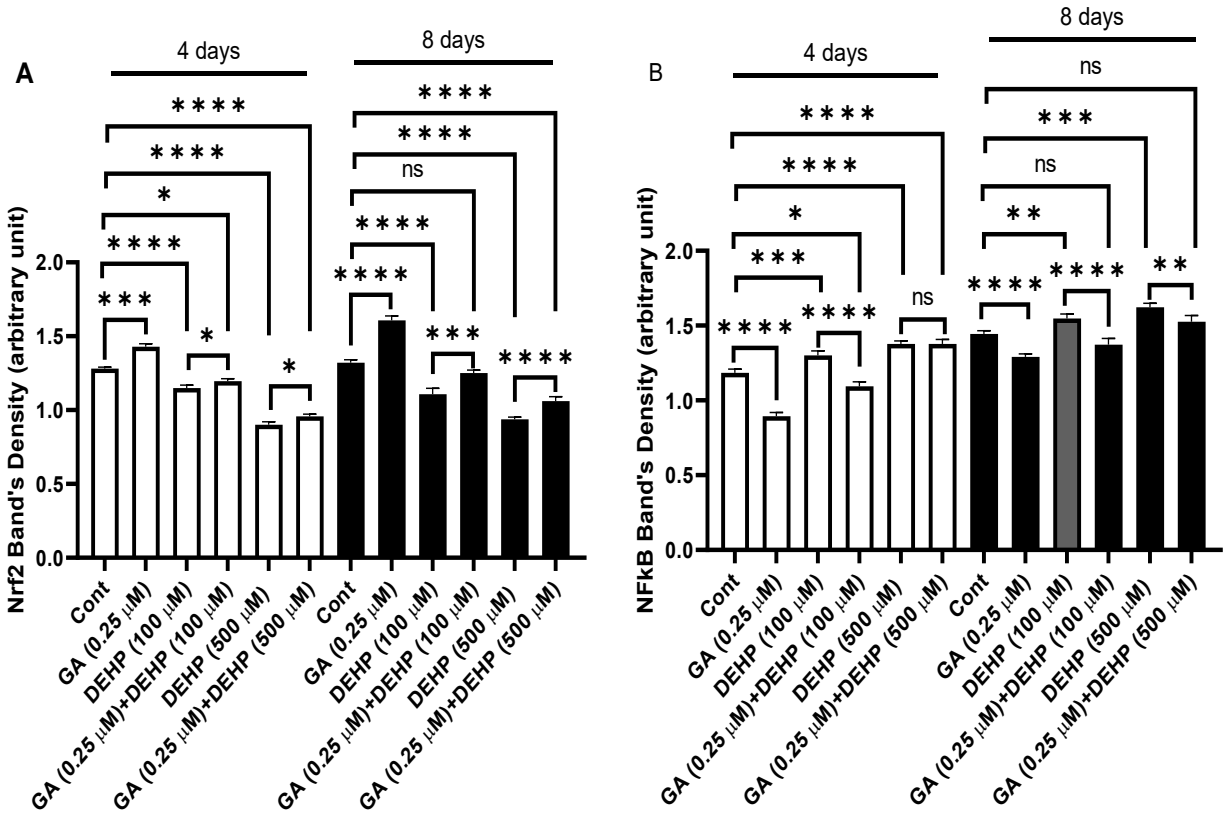

FIGURE 8. Mean A) expression of Nrf2 and B) expression of NFkB in BMSCs treated with 0.025 μM of GA as well as 100 and 500 μM of DEHP following 4 and 8 days of incubation. Control group (no treatment with any of the tested compounds), DEHP (100 μM) group (cells treated with 100 μM of DEHP), DEHP (500 μM) group (cells treated with 500 μM of DEHP), GA (0.25 μM) group (cells treated with 0.25 μM of gallic acid), DEHP (100 μM) + GA (0.25 μM) group (cells treated with 100 μM of DEHP and 0.25 μM of gallic acid simultaneously) and DEHP (500 μM) + GA (0.25 μM) group (cells treated with 500 μM of DEHP and 0.25 μM of gallic acid simultaneously. All the mean values were compared with control, and data are presented as mean ± SD with the level of significance as (ns) $p>0.05$, (*) $p<0.05$, (**) p,0.01, (***) $p<0.001$ and (****) $p<0.0001$.

inactivation of Nrf2-ARE pathway (Gao, et al., 2022). Our results showed, that GA elevated the expression of Nrf2 and down regulated the NFkB gene expression to control the oxidative stress caused by DEHP in a longer time period of its treatment. Consistent with our findings, research has demonstrated that gallic acid inhibits NF-κB activation and enhances Nrf2 activity (Tanaka, et al., 2018; Ho, et al., 2010; Sohrabi, et al., 2021).

Conclusion

The results of the present study showed that GA, a strong antioxidant found in different plant products, can ameliorate the oxidative effects of DEHP by regulating the activity of antioxidant enzymes through the upregulation of the Nrf2-ARE pathway. The preventive effect of GA inhibited cell membrane lipid peroxidation and improved the viability, proliferation and morphology of the BMSCs, which serve as the cellular back up for osteoblasts in the bone marrow. Date analysis showed that the effect of GA is more substantial when used in low concentrations for a longer period. Although further in vivo investigations are recommended, this plant-derived antioxidant shows potential as an alternative therapeutic approach to mitigate DEHP toxicity.

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Conflict of interest

The authors declare no conflicts of interest, financial or otherwise.

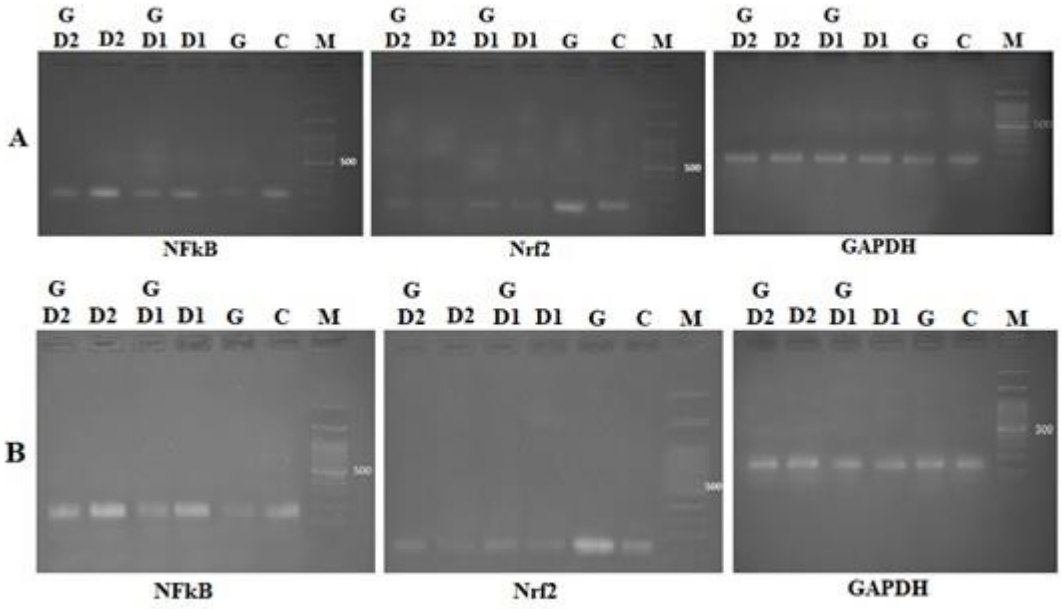

FIGURE 9. Representative agarose gel electrophoresis images showing the expression of NFκB and Nrf2 genes in bone marrow mesenchymal stem cells (BMSCs) following treatment with 0.25 µM gallic acid (GA), 100 µM and 500 µM di(2-ethylhexyl) phthalate (DEHP). Panel A: Gene expression after 4 days of treatment. Panel B: Gene expression after 8 days of treatment (NFκB only). Lanes are arranged in the same order across both gels and correspond to the following experimental groups: M: DNA marker, C: Untreated control, G: Treated with 0.25 µM gallic acid, D1: Treated with 100 µM DEHP, GD1: Treated with 0.25 µM gallic acid + 100 µM DEHP, D2: Treated with 500 µM DEHP, GD2: Treated with 0.25 µM gallic acid + 500 µM DEHP. GAPDH was used as an internal control due to its consistent expression as a housekeeping gene.

Ethics approval

The present study was conducted based on the protocols recommended by Guide for Care and Use of Laboratory Animals of the National Institutes of Health and approved by Committee on the Ethics of Animal Experiments of Arak University (Protocol Number IR.ARAK-MU.REC.1401.078). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

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