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Experimed 2026, 16 (2): 100–106

https://doi.org/10.26650/experimed.1892531

Submitted: 19.02.2026
Revision Requested: 02.04.2026
Last Revision Received: 08.04.2026
Accepted: 22.04.2026
Published Online 12.08.2026

Experimed

Research Article

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The Effect of Verbascoside on DNA Damage and Oxidative Stress in 3T3-L1 Adipocytes

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Abstract

Objective: Obesity involves excessive lipid accumulation in adipose tissue, triggering oxidative stress and DNA damage. This study evaluated the effects of verbascoside doses (50 µM: V50 and 100 µM: V100) on DNA damage and oxidative stress in mature adipocytes.

Materials and Methods: 3T3-L1 cells were differentiated into mature adipocytes and treated with V50 or V100. Oil Red O staining quantified the lipid accumulation. DNA damage was evaluated using the Comet assay. We assessed oxidative stress by calculating the oxidative stress index (OSI) based on total oxidant status (TOS) and total antioxidant status (TAS) measurements.

Results: V100 reduced lipid accumulation compared with the control group. Furthermore, while V50 increased DNA damage (tail intensity and moment), V100 significantly lowered it compared to V50. Additionally, V100 improved oxidative stress markers (increased TAS, decreased TOS, and OSI), whereas V50 had no significant effect on these parameters.

Conclusion: Verbascoside showed dose-dependent effects on mature adipocytes. While V50 increased DNA damage independently of oxidative stress, V100 improved oxidative status and reduced DNA damage. These findings demonstrate that at appropriate doses, verbascoside is a candidate for preventing metabolic disorders, though further molecular studies are needed.

Keywords Adipocyte · DNA Damage · Obesity · Oxidative Stress · Verbascoside · 3T3-L1 Cells

Citation: Tunç-Ata, M., Akan, G., Güvenç, Ş. S., Çelebi, D., Şafak, S., Ünal, Z. A., Korkmaz, S. & Kılıç-Toprak, E. The effect of verbascoside on DNA damage and oxidative stress in 3T3-L1 adipocytes. Experimed 2026; 16(2): 100-106. DOI: 10.26650/experimed.1892531

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INTRODUCTION

Obesity is a widespread health problem worldwide and is associated with structural and functional changes in adipose tissue that affect metabolic diseases (1). As the number and size of fat cells increase, dysfunctional adipocytes develop, and numerous inflammatory processes are triggered (2, 3). This excessive fat accumulation disrupts the hormonal functions of adipose tissue while creating an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defense mechanisms, thereby increasing oxidative stress (4, 5). This oxidative environment within the adipocyte causes significant cellular damage and plays a key role in the pathogenesis of metabolic disorders (6). In particular, excessive lipid accumulation can trigger mitochondrial stress, leading to excessive ROS production and, consequently, the accumulation of DNA damage. This interrelated state of lipid overload and oxidative stress highlights adipocyte dysfunction and elevates the potential for genotoxic risk due to the increased markers of DNA damage and reduced repair capacity observed in obese individuals (7).

To mitigate these obesity-related metabolic disorders, polyphenols have emerged as promising biological molecules due to their potent metabolic regulatory properties (8, 9). Within this group, verbascoside (acteoside)—a polyphenol belonging to the phenolic acid subclass—is of particular interest because of its multifaceted biological activity (10). The primary reason for selecting verbascoside in this study is the multi-targeted biological activity exhibited by this natural polyphenol against obesity and associated metabolic disorders. These activities include anti-adipogenic effects and a protective role against oxidative stress and DNA damage. Verbascoside has been shown to inhibit the adipogenesis process by suppressing key transcription factors (peroxisome proliferator–activated receptors [PPAR] γ and CCAAT/enhancer-binding protein [C/EBP] α) in the 3T3-L1 cell model (7). While this makes it a strong candidate for reducing lipid accumulation, verbascoside's ability to scavenge free radicals and enhance antioxidant enzyme capacity (total antioxidant status [TAS]/total oxidant status [TOS] balance) has also been documented in the literature (11). Additionally, it has been reported that verbascoside possesses genoprotective effects by reducing DNA breaks associated with oxidative stress (12). Despite these known individual effects of verbascoside, its dose-dependent effect on DNA integrity in lipid-rich mature adipocytes has not yet been fully elucidated. In this context, the aim of our study was to evaluate the effect of different verbascoside doses on DNA damage in mature 3T3-L1 fat cells and to investigate the relationship between these effects and cellular oxidative stress markers.

MATERIALS AND METHODS

Differentiation of the 3T3-L1 Adipocyte

This study used the 3T3-L1 cell line (ATCC® CL173™, USA), a fibroblast cell line derived from a mouse (*Mus musculus*) embryo, recognized as a preadipocyte. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Waltham, USA) with 25 mM glucose, supplemented with 10% heat-inactivated calf serum (Cegrogen, Germany) and antibiotics (penicillin and streptomycin; Wisent, Saint-Jean-Baptiste, Canada) at 37°C in a humidified atmosphere of 5% CO_2 to establish the experimental group. Upon reaching approximately 80% confluence in the culture dishes, $1x10^5$ cells were subsequently seeded in a 6-well plate following cell counting. The culture medium was subsequently replaced with a differentiation medium containing 3-isobutyl-1-methylxanthine (IBMX, Sigma I5879, USA), dexamethasone (DEX, Sigma D4902, USA), insulin (INS, Sigma I6634, USA), and fetal bovine serum (FBS) (Biowest, South America) to induce the differentiation of preadipocytes into adipocytes. The cells were incubated in this medium (MD1) for 48 h. After the incubation period, the medium containing high glucose, FBS (Biowest, South America), and INS (MD2) was renewed every 2 days, and the cells matured within 8 days. Experiments were conducted on adipocytes that matured on the 10th day after completing differentiation (13). Figure 1 illustrates the details of the experimental timeline.

Lipid Accumulation Evaluation

The lipid staining kit was used for microscopic analysis to confirm the transformation of preadipocytes into mature cells (BioVision Oil Red O, Catalogue No. K580-24; BioVision, Milpitas, CA, USA). Mature adipocytes were fixed in 10% formalin in phosphate-buffered saline (PBS, Wisent, Saint-Jean-Baptiste, Canada) for 1 h. Formalin was

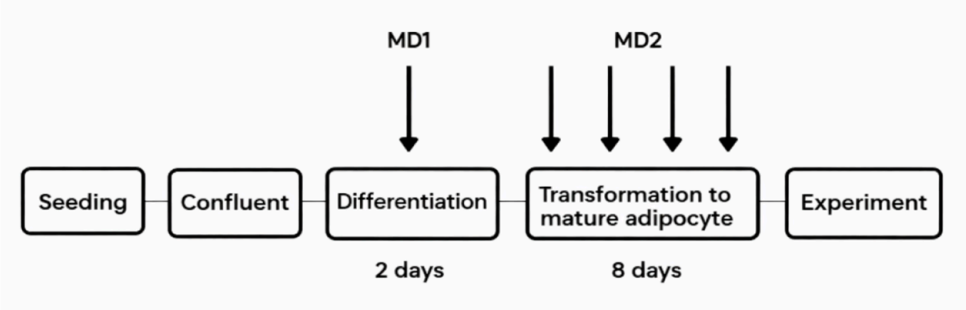


Figure 1. Demonstration of the experimental design. Verbascoside was applied at 50 µM and 100 µM doses. MD1: medium 1, MD2: medium 2.

removed and cells were carefully washed twice with distilled water; after that, add 60% isopropanol (BioVision, K580-24, USA) to each well and incubate for 5 minutes. The isopropanol was eliminated, and the Oil Red O working solution was applied for 30 minutes. Subsequently, the Oil Red O working solution was discarded and washed 2-5 times with distilled water. The optical intensity of the Oil Red O solution was measured at 490 nm using a Thermo Fisher Scientific™ Multiskan™ GO microplate spectrophotometer (Waltham, MA, USA) after being eluted with 100% isopropanol. A microscope (Olympus CKX41, Tokyo, Japan) was used to observe the lipid accumulation at 40X magnification (14).

Administration of Verbascoside

Verbascoside (V4015 ≥99% (HPLC grade), CAS 61276-17-3, $C_{29}H_{36}O_{15}$, Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO) (CAS No: 67-68-5, ChemCruz, USA) to prepare a stock solution, from which intermediate working solutions were prepared. We obtained final concentrations of 50 µM and 100 µM by dilution in the culture medium (15, 16). Mature 3T3-L1 adipocytes were exposed to verbascoside for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2.

Oxidative Stress Parameters

Following the 24 h treatment of 3T3-L1 mature adipocytes with 50 µM and 100 µM verbascoside, the TAS and TOS were determined in the collected culture media. TAS and TOS levels were measured using a Thermo Fisher Scientific™ Multiskan™ GO microplate spectrophotometer (Waltham, MA, USA) at 660 nm and 560 nm, respectively, following the manufacturer's specific instructions regarding reagent incubation and colorimetric reaction. TAS levels were measured based on the antioxidants ability to inhibit the formation of the dianisyl radical, produced through the Fenton reaction-mediated oxidation of o-dianisidine; results were expressed as mmol Trolox Equiv/L (11). TOS levels were quantified colorimetrically by monitoring the oxidation of the ferrous ion-o-dianisidine complex to ferric ions, which subsequently formed a colored complex with xylenol orange in an acidic medium. TOS values were reported as µmol hydrogen peroxide (H_2O_2) Equiv/L (12). Calibration procedures for TAS and TOS were performed using commercially available kits containing Trolox and H_2O_2 standards, respectively. Results for TAS measurements were reported in "mmol Trolox Equiv/L". For TOS measurements, H_2O_2 standards provided ready-to-use by the kit were used, and results were reported as "µmol H_2O_2 Equiv/L". To determine the overall oxidative state, the Oxidative Stress Index (OSI) was calculated using the following formula: OSI (arbitrary unit) = TOS (µmol/L) / TAS (mmol/L) × 100.

Comet Assay

To evaluate DNA integrity, mature adipocytes were rinsed with cold PBS and harvested by centrifugation. The resulting cell pellet was embedded in 0.5% low-melting-point agarose and layered onto slides pre-treated with 1% normal-melting-point agarose, followed by a final overlay of low-melting-point agarose. For proteolysis, slides were incubated overnight in a chilled lysis buffer (pH 10) at +4°C. DNA unwinding was performed in an alkaline electrophoresis buffer for 30 min, followed by electrophoresis at +4°C (21 V and 300 mA) for an additional 30 min. Post-electrophoresis, the slides underwent neutralization, were rinsed with distilled water, and stained using ethidium bromide (CAS No: 1239-45-8, Sigma, USA). Comets were visualized via fluorescence microscopy (Olympus CKX41, Tokyo, Japan, equipped with a fluorescence attachment) and quantified using Comet Assay IV (AutoComet) software (Perceptive Instruments, United Kingdom). DNA damage levels were characterized using head length (µm), tail length (µm), head intensity (AU), tail intensity (AU), and tail moment (AU) (17).

Statistical Analyses

All experimental measurements were performed in triplicate (n=3). Statistical analyses were performed using SPSS 25.0 software (IBM Corporation, Armonk, NY, USA). Continuous variables are presented as mean ± standard deviation (Mean ± SD). Group differences among Control (C), V50, and V100 were evaluated using the Kruskal–Wallis test (KW). A p value <0.05 was considered statistically significant.

RESULTS

The V100 group significantly reduced lipid accumulation in mature 3T3-L1 cells compared to the control group (C: 0.44 ± 0.028 OD; V100: 0.277 ± 0.012 OD; p=0.0001). The V50 group showed a tendency to reduce lipid accumulation compared to the control, but this difference was not statistically significant (Figure 2).

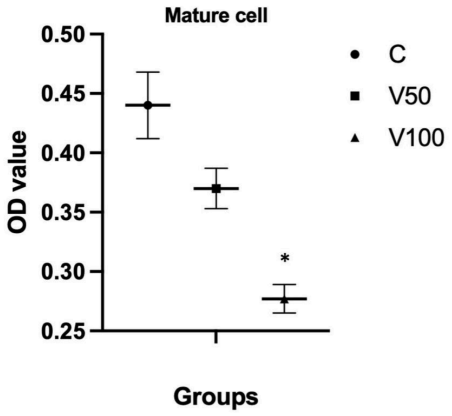


Figure 2. Lipid accumulation results of verbascoside in mature adipocytes. Mean ± SD were used to express the results. Group differences were evaluated using the Kruskal–Wallis test. p<0.05 was considered statistically significant. *: Significantly different from the control group. C: control group; V50: group treated with 50 µM verbascoside; V100: group treated with 100 µM verbascoside.

The V50 group significantly increased DNA damage compared to the control. Figure 3A represents the images of the comet assay parameters. When parameters supporting this general finding were examined, the V50 group significantly reduced head intensity compared to the control (C: 60.249 ± 12.562 AU; V50: 36.416 ± 0.505 AU; p=0.0001). The V50 group significantly increased tail intensity and moment compared to the control (C: 39.744 ± 12.562 AU; 6.722 ± 2.841 AU; V50: 63.91 ± 2.555 AU; 14.222 ± 1.005 AU; p=0.0001, respectively). The V100 group significantly reduced DNA damage compared to the V50 group. When examining the parameters supporting this general finding, head length and intensity were significantly increased in the V100 group compared to the V50 group (V50: 26.546 ± 1.749 µm; 36.416 ± 0.505 AU; V100: 31.545 ± 0.809 µm; 67.383 ± 0.779 AU; p=0.002 and p=0.0001, respectively). Tail length, tail intensity, and tail moment were significantly reduced in the V100 group compared to the V50 group (V50: 48.294 ± 0.964 µm; 63.91 ± 2.555 AU; 14.222 ± 1.005 AU; V100: 34.691 ± 0.978 µm; 32.61 ± 0.797 AU; 4.512 ± 1.178 AU; p=0.006, p=0.0001, and p=0.0001, respectively). No significant difference was observed between the V100 group and the control group in terms of all DNA damage parameters investigated (p>0.05). When examining the parameters in detail, head length and intensity showed a slight non-significant increasing trend in the V100 group compared to the control (C: 29.078 ± 3.387 µm; 60.249 ± 12.562 AU; V100: 31.545 ± 0.809 µm; 67.383 ± 0.779 AU, respectively). Conversely, tail length, tail intensity, and tail moment values in the V100 group were found to be statistically similar to the control levels (C: 41.972 ± 9.091 µm; 39.744 ± 12.562 AU; 6.722 ± 2.841 AU; V100: 34.691 ± 0.978 µm; 32.610 ± 0.797 AU; 4.512 ± 1.178 AU, respectively) (Figure 3A-B).

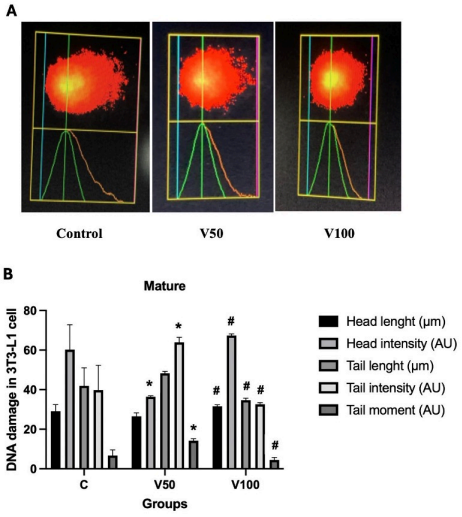


Figure 3. Comet assay images and the determined comet parameters for 3T3-L1 cells. (A) Representative comet assay images for the C, V50, and V100 groups. (B) Determined comet parameters. Mean ± SD were used to express the results. Group differences were evaluated using the Kruskal–Wallis test. p<0.05 was considered statistically significant. *: Significantly different from the control group. #: Significantly different from the V50 group. C: control group; V50: group treated with 50 µM verbascoside; V100: group treated with 100 µM verbascoside; AU: arbitrary unit.

In the V50 group treated with mature 3T3-L1 cells, no significant changes were observed in TAS, TOS, and OSI levels compared to the control. However, in the V100 group, oxidative stress decreased compared to the control. When the parameters supporting this general finding were examined, TAS increased in the V100 group compared to the control (C: 411.471 ± 16.35 mmol Trolox Equiv/L; V100: 861.296 ± 14.587 mmol Trolox Equiv/L; p=0.0001) (Figure 4A). When TOS and OSI values were examined, TOS and OSI values in the V100 group were decreased compared to the control group (C: 72.791 ± 0.716 µmol H_2O_2 Equiv/L; 15.172 ± 1.847 AU; V100: 47.044 ± 0.267 µmol H_2O_2 Equiv/L; 10.284 ± 1.366 AU; p=0.0001, respectively) (Figure 4B, Figure 4C).

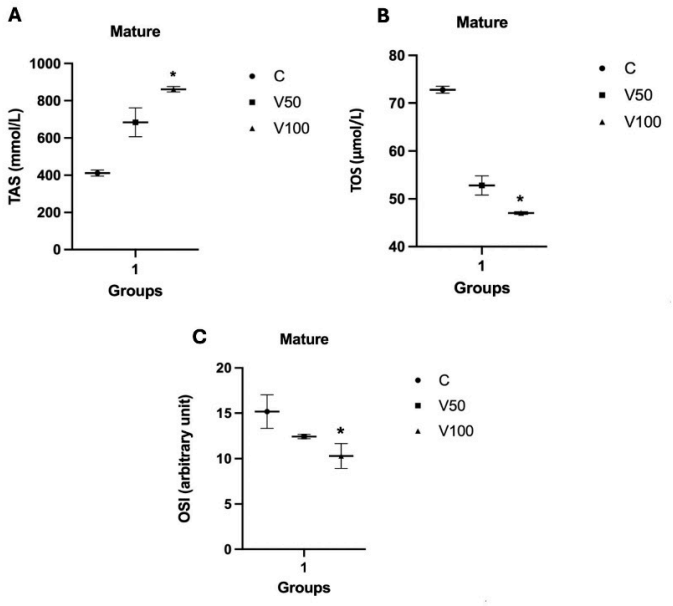


Figure 4. Effects of verbascoside on the oxidative stress status of mature 3T3-L1 adipocytes. (A) TAS, (B) TOS, and (C) OSI levels. Mean ± SD were used to express the results. Group differences were evaluated using the Kruskal–Wallis test. p<0.05 was considered statistically significant. *: Significantly different from the control group. C: control group; V50: group treated with 50 µM verbascoside; V100: group treated with 100 µM verbascoside.

DISCUSSION

Lipid accumulation in adipose tissue, oxidative stress, and DNA damage are interrelated processes, and investigating the effects of verbascoside on these mechanisms is important for understanding the dose-dependent biological responses of polyphenols.

In our study, although a 50 µM dose of verbascoside did not cause a significant change in oxidative stress parameters, DNA damage comet parameters increased markedly. This reveals that low doses of verbascoside may induce genotoxicity at the cellular level through mechanisms independent of oxidative stress. Possible reasons include the disruption of intracellular redox balance at the local level by metabolites of phenolic compounds or the creation of inhibitory effects on DNA repair mechanisms (18, 19).

In the literature, verbascoside (0.05 mM and 0.1 mM) increases chromosomal damage in human lymphocytes. This effect has been reported to be related to pathways involving poly ADP-ribose polymerase-1 (PARP-1) and p53, which are involved in DNA repair. Furthermore, it has been noted that verbascoside can be broken down into caffeic acid, and this metabolite may negatively affect DNA repair by disrupting the intracellular redox balance (19). Therefore, the DNA damage observed at a dose of 50 µM may be due to either the direct genotoxic effect of the polyphenol or the adverse mechanisms caused by the resulting metabolites. Additionally, a study using Barleria (0.43 mg/g) and verbascoside (1.02 mg/g) observed DNA damage in different cell types, particularly in PC3 cancer cells, using the Comet assay (20). These findings support the effect demonstrated by 50 µM verbascoside in our study. Beyond its dose-dependent variations, verbascoside is widely recognized as a potent polyphenolic antioxidant due to its ability to scavenge free radicals and enhance cellular antioxidant enzyme activities. This robust antioxidant capacity plays a crucial role in mitigating oxidative stress-induced damage in various metabolic disorders (21).

Furthermore, the fact that changes observed in DNA damage parameters appear before oxidative biomarkers demonstrates that they may be independent of these effects. This is because the comet assay has the capacity to reveal early and local DNA damage in cells before systemic biomarkers (22). Oxidative stress markers (TAS, TOS, and OSI), on the other hand, indicate more general, systemic responses.

In contrast, the V100 dose reduced oxidative stress and DNA damage compared to the V50 dose. This highlights that verbascoside has not been able to stimulate sufficient antioxidant defense systems at low doses. The 50 µM dose may have posed a threat to the cells; however, as this threat was not sufficiently strong, antioxidant defense mechanisms (e.g., activation of nuclear factor erythroid 2–related factor 2 [Nrf2]) may not have been stimulated to an adequate level. The 'hormetic response' has been frequently described in the literature for polyphenols, whereby low doses stress the cell but do not fully trigger defense responses, whereas higher doses exert a protective effect through the activation of Nrf2/antioxidant response element-mediated antioxidant enzymes. This U-shaped dose-response curve has been demonstrated for numerous phenolic compounds in the literature (23, 24). Therefore, the DNA damage observed at a 50 µM dose may be related to the insufficient stimulation of cellular defense mechanisms. The Nrf2/ARE/HO-1 pathway plays a critical role in maintaining cellular redox homeostasis. Under basal conditions, Nrf2 is sequestered in the cytoplasm by the Keap1 protein and targeted for proteasomal degradation. Upon exposure to antioxidant compounds, critical cysteine residues on Keap1 undergo modification, allowing Nrf2 to escape degradation and translocate from the cytoplasm into the nucleus. In the nucleus, it binds to the ARE sequences located in the promoter regions of target genes. This binding triggers the expression of Phase II antioxidant enzymes, primarily heme oxygenase-1 (HO-1), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (25). In our study, the administered V50 dose may have been insufficient to activate the Nrf2/ARE/HO-1 signaling pathway.

In our study, the inhibitory effect of the V100 dose on lipid accumulation and its role in enhancing antioxidant capacity are consistent with those reported in the literature across both cellular and animal models. Herranz-López et al. reported that verbascoside significantly reduced triglyceride accumulation and ROS generation in hypertrophic adipocytes, a process associated with 5' adenosine monophosphate-activated protein kinase (AMPK) activation and PPAR-α upregulation (26). The systemic manifestation of these cellular defense mechanisms was further validated by Hao et al. in a diabetic mouse model; the researchers demonstrated that acteoside (verbascoside) effectively prevented hepatic steatosis and oxidative stress by activating the Nrf2/HO-1 signaling pathway (27).

In summary, a 50 µM dose of verbascoside can cause genotoxic effects by disrupting intracellular redox balance or suppressing DNA repair mechanisms, whereas at a 100 µM dose, this damage is suppressed by the stimulation of antioxidant defenses and the activation of repair mechanisms. This indicates that verbascoside exhibits a hormetic effect and may elicit dose-dependent biological responses. These results indicate that verbascoside, at an appropriate dose, may be a potential candidate for the prevention of metabolic diseases and support the existence of dose-dependent biological responses to polyphenols.

CONCLUSION

In the present study, the V100 dose exhibited a favorable metabolic effect by suppressing lipid accumulation in mature adipocytes. Furthermore, this concentration augmented TAS while simultaneously reducing TOS and OSI. This outcome fortified the cellular antioxidant capacity and subsequently mitigated DNA damage. Conversely, the observation that the V50 dose intensified DNA damage demonstrates that relatively lower concentrations may trigger divergent metabolic pathways. By unveiling the "Janus-faced" (bi-modal) impact of verbascoside on adipocyte metabolism, this research underscores the critical necessity of precise dose calibration for treating obesity and its associated cellular pathologies. These findings illustrate that a single molecule can function as either a protective shield or a source of cellular stress depending on the dosage, thereby providing a foundational premise for future investigations. A primary limitation of this study is the lack of direct molecular quantification for Nrf2/ARE signaling pathways, leaving the proposed 'hormetic response' mechanism as a hypothetical framework to be validated in future research. Another

limitation of this study is the absence of cytotoxicity assays for the determined doses.

Acknowledgments The authors thank Asst. Prof. Hande Senol for her help in statistical analyses. Part of this work was presented as poster at the 50th National Physiology Congress, 12-16 November 2025, in Antalya, Türkiye.

Ethics Committee Approval Ethical approval was not required, as the study involved only commercial cell lines in an *in vitro* model with no human or animal subjects.

Peer Review Externally peer-reviewed.

Author Contributions Conception/Design of study: E.K.T., M.T.A., G.A.; Data Acquisition: E.K.T., M.T.A., G.A., S.K., Ş.S.G., D.Ç., S.Ş., Z.A.Ü.;Data Analysis/Interpretation: E.K.T., M.T.A., G.A.; Drafting Manuscript: E.K.T., M.T.A., G.A., S.K., Ş.S.G., D.Ç., S.Ş., Z.A.Ü.; Critical Revision of Manuscript: E.K.T., M.T.A., G.A.; Final Approval and Accountability: E.K.T., M.T.A., G.A., S.K., Ş.S.G., D.Ç., S.Ş., Z.A.Ü.

Conflict of Interest The authors have no conflict of interest to declare.

Financial Disclosure This study was supported by TUBITAK under the 2209/A - Research Project Support Programme for Undergraduate Students. Project numbers; 1919B012335425 and 1919B012326675.

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