Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Therapeutic potential of hesperidin: Apoptosis induction in breast cancer cell lines

Gozde Ozge ONDER (gozdeozgekorkmaz@hotmail.com)
Erciyes University
Ozge GOKTEPE
Erciyes University
Munevver BARAN
Erciyes University
Nazmiye BITGEN
Erciyes University
Funda AYDIN
Van Yuzuncu Yil University
Arzu Yay
Erciyes University

Research Article

Keywords: Apoptosis, Hesperidin, MCF-7, MDA-MB-231

Posted Date: January 25th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2493328/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Purpose

Hesperidin is a flavonoid commonly found in citrus fruits. Studies have shown that hesperidin has anti-inflammatory, analgesic, and antimicrobial properties, as well as its effectiveness in carcinogenesis. In this paper, we aim to investigate the molecular mechanisms of hesperidin-induced apoptosis in MCF-7 and MDA-MB-231 cancer cells.

Methods

Hesperidin's 50% inhibitory concentrations in the cells were evaluated by the MTT assay. Cell cycle analysis of hesperidin-treated cells was then performed, as well as immunocytochemical analysis of the effect on the apoptosis pathway (TUNEL, Bax, and Bcl-2 expression).

Results

Moreover, hesperidin induced cell apoptosis in MCF-7 breast cancer cells by inhibiting Bcl-2 and enhancing Bax expression at protein levels. On the other hand, hesperidin caused apoptosis in the MDA-MB-231 breast cancer cell line, but it did not activate the Bax/Bcl-2 pathway. Hesperidin also induced cell cycle arrest at the S phase in the MCF-7 and MDA-MB-231 cell lines.

Conclusion

These findings showed that hesperidin is a potential therapeutic candidate for preventing the progression of breast cancer. In addition, hesperidin could significantly stimulate the death mechanisms in ER/PR (+) MCF-7 cells by changing the expression balance of Bax and Bcl-2 proteins, but lead ER/PR (-) MDA-MB-231 breast cancer cells to apoptosis in a different way.

Introduction

One of the most prevalent malignancies, breast cancer accounts for roughly 11.6% of cancer incidence and 6.6% of cancer-related deaths globally (Bray et al. 2018). Currently, treatment of breast cancer involves a combination of a number of different methods, including surgery, chemotherapy, immunotherapy, and radiation therapy (Bange et al. 2001), nevertheless, there is currently no fully effective treatment for breast cancer (Khan et al. 2018). Novel, effective medicines and treatment approaches are desperately needed as the prevalence of breast cancers increases worldwide.

Plant-derived products are gaining global interest for use in a variety of treatments as they have fewer side effects, even at high doses (Wang et al. 2012). Due to its anti-cancer characteristics, about 40–50%

of cancer patients include plant components and their derivatives in their diets (Donaldson 2004). The flavonoid hesperidin is one of the most significant bioactive substances with promising anti-cancer potential (Ahmadi and Shadboorestan 2016). Hesperidin (HES) was first extracted from orange peels in 1828 (Li and Schluesener 2017). HES is the glycoside form of hesperetin. Once intestinal bacteria have deglycosylated dietary HES to hesperetin, it is absorbed (Ameer et al. 1996). Thus, HES might be seen as a pro-drug, which turns into hesperetin (Lee et al. 2004).

This polyphenolic glycoside can be abundantly found in various citrus fruits. Numerous studies have focused on herbal medicines over the past years as an alternative treatment for various cancers, because of their minimal effects on healthy cells. HES' anticancer effects have been established against human cancers such as endometrial cancer, ovarian cancer, lung cancer, breast cancer, and many others (Xia et al. 2018; Zhao et al. 2017; Cincin et al. 2018; Lee et al. 2009).

MCF-7 (estrogen- and progesterone-receptor positive, ER and PR (+)) and MDA-MB-231 (ER and PR (-)) cells are two frequently preferred subtypes among breast cancer cells. MCF-7 was derived from metastatic sites in adenocarcinomas, so it is called a tumorigenic adherent mammary epithelial cell line. However, the MDA-MB-231 cell line is an aggressive, metastatic, and invasive breast cell line (Razak et al. 2019). There's a lot of uncertainty about the function of HES in cancer biology and metabolism. Hence, the current study's objective was to examine how HES affected the cell proliferation and apoptosis of MCF-7 and MDA-MB-231, two extremely aggressive and proliferative cell lines.

Materials And Methods

Cell culture

The human breast epithelial cell lines MCF-7 and MDA-MB-231 were used in this study. Both were grown in Dulbecco's Modified Eagle's Medium (Gibco BRL, MD). The medium was enhanced with heat-inactivated 10% fetal bovine serum (Gibco, South America), antibiotics (1% penicillin and streptomycin; Capricorn, Germany), and 1% L-glutamine (Thermo, ABD). Cells were then routinely propagated at 37°C and 5% CO_2 atmosphere.

Preparation Of Hesperidin

HES powder (Sigma, MO, USA) was suspended in dimethyl sulfoxide (Sigma, MO, USA). The 1 mM stock solution was prepared.

Cell Viability Assay

Cell growth was assessed in 96-well plates at the 24, 48, and 72 h points using the methylthiazolyl tetrazolium (MTT) assay (Onder et al. 2022). 100 ml of complete medium containing 5x103 cells were seeded in each well. The following day (day 0), cells were treated with different concentrations of HES

(20, 40, 60, 80 and 100 µM). Cell growth was assessed at the 24, 48, and 72 h points using the methylthiazolyl tetrazolium (MTT) assay (Onder et al. 2022). HES's half maximal inhibitory concentration (IC50) was determined. After the IC50 dose was determined, five groups were identified: control, cells that received the IC50 dose of HES were identified as HES1, cells that received half the IC50 dose of HES were identified as HES2, cells that received the ratio of DMSO used in the preparation of HES in the HES1 group were identified as D1, cells that received the ratio of DMSO used in the preparation of HES in the HES2 group were identified as D2.

Cell-cycle Analysis

Muse Cell Cycle Kit (Luminex, TX, USA) was used to analyze the cell cycle. HES-treated cells were trypsinized from the culture medium, centrifuged at 300 g for 5 min. Then phosphate-buffered saline (PBS) was used to wash the cells. 70% cold ethanol were used to fixed the cells in at -20°C. The cells were then centrifuged and exposed to cell cycle reagents for 30 minutes without light. A Muse cell analyzer and Muse analysis software (Merck, Darmstadt, Germany) were used to analyze the data.

Immunocytochemical Analysis For Detecting Apoptosis

To assess HES-mediated apoptotic cell death, the ApopTag Fluorescein In Situ Apoptosis Detection Kit (EMD Millipore, Darmstadt, Germany) was as previously described (Baran et al. 2020). An Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) was used to observe the apoptotic cells (magnification, x400). A total of 10 fields of view were randomly selected for analysis.

Immunocytochemical Analysis Of Bax And Bcl-2

To determine Bax and Bcl-2 expression profiles, an immunofluorescence staining was used as previously described (Onder et al. 2022). Cells were incubated on coverslips in a 24-well plate. After cells were treated with HES for 24 hours, they were fixed in 10% formaldehyde. Then, the procedure of immunofluorescence staining was applied. Cells were treated with Bax (1:100, primary antibody, Novus Biologicals, Littleton, USA) and then Bcl-2 (1:30, primary antibody, Thermo, Rockford, USA). For each group, 400x images were obtained from ten separate microscopic fields using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). The intensity of Bax and Bcl-2 primary antibodies' immunoreactivity was measured using Image J software (Bethesda, USA).

Statistical analysis

GraphPad Prism 9.4.1 (CA, USA) was used to statistically analyze the data. The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to determine whether the data were in accordance with the normal distribution. The one-sample t test was used to compare viability percentage values between dosage groups. The Kruskal-Wallis test and one-way analysis of variance (ANOVA) were employed to evaluate the

quantitative variables in more than two groups. The multiple comparisons were done using the Tukey and Bonferroni tests. P values that were less than 0.05 were regarded as statistically significant.

Results

Effect of HES Treatment on the Proliferation

We first assessed, by the MTT test, the potential of HES to prevent the proliferation of two breast cancer cell lines of different molecular subtypes. The cells were incubated for 24, 48, and 72 h with a wide range of HES concentrations (5, 10, 15, 20, 25, 30, and 40 μM). We found that the cell viability was reduced in a dose-dependent manner. The IC50 doses for the MCF-7 cells (Fig. 1A) and the MDA-MB-231 cells (Fig. 1B) were calculated to be 30 μM and 20 μM, respectively.

Effect Of Hes Treatment On Apoptosis

Apoptosis triggered by HES in cells was confirmed by the TUNEL method. Figure 2 shows the findings of TUNEL investigation on MCF-7 breast cancer cells. Treating cells with HES caused a statistically significant activation of apoptosis, resulting in a dose-dependent increase in the cells ($p < 0.001$) (Fig. 4A).

Figure 3 shows the findings of TUNEL analysis on MDA-MB-231 breast cancer cells. Compared with control cells, it was demonstrated that HES treatment significantly induced concentration-dependent apoptosis ($p < 0.001$) (Fig. 4B).

Effect Of Hes Treatment On Bax/bcl-2 Expression

Thus, to further demonstrate that the underlying mechanism is apoptosis in HES-mediated cell death, we analyzed Bax and Bcl-2 expression in cells. HES upregulated the expression of pro-apoptotic Bax protein in MCF-7 breast cancer cells in a dose-dependent manner when compared to the control group ($p < 0.001$) (Fig. 5). When the groups' Bcl-2 expression levels were assessed, there was no statistically significant difference between them ($p > 0.05$) (Fig. 6).

The results of MDA-MB-231 cells' Bax/Bcl-2 expression are displayed in Fig. 7. It was noted that there was no increase in the Bax and Bcl-2 proteins' expression. Therefore, the expressions of Bax and Bcl-2 did not differ in a statistically meaningful way (Fig. 8).

Effect Of Hes Treatment On Cell Cycle Phases

In both cancer cell lines, When the experimental groups were assessed, it was found that the S phase was statistically significantly lower in the experimental group in contrast with the control group ($p < 0.001$)

(Fig. 9A-B). It was shown that HES prevented cell division in the S phase in the both of breast cancer cell lines.

Figure 9 Cell cycle analysis in MCF-7 **(A)** and MDA-MB-231 **(B)** breast cancer cell lines treated with HES (ns: non-significant $p > 0.999$, $^{**}p < 0.01$)

Discussion

In the current study, we report on the findings that HES induces apoptosis both of cell lines. According to epidemiological studies, people who regularly consume flavonoid-containing foods have a lower risk of developing cancer and other chronic diseases. Therefore, it has led to increased interest in using these substances as dietary supplements to treat cancer cases (Mazza 2007). It has been reported that HES, a flavanone extracted from citrus fruits, has important curative effects on human health (Del et al. 2017). HES has significant antioxidant and anti-inflammatory properties, according to both in vivo and in vitro clinical studies (Li and Schluesener 2017). In addition, HES has various anti-cancer activities, including those against breast cancer (Lee et al. 2009). HES can suppress breast cancer by causing apoptosis and cell cycle arrest (Kabala-Dzik et al. 2018). However, the exact role and mechanisms of HES in human breast cancer cell lines remain unknown.

The main target of drugs used in cancer treatment is to activate cell death pathways like apoptosis in these cells to kill cancer cells. Apoptosis is an important step in malignant tumor metastasis, such as in breast cancer. Apoptosis is a programmed cell death mechanism that involves the participation of a series of genes. This process maintains an organism's homeostasis and health (Nikoletopoulou et al. 2013). Family of Bcl-2 proteins, which is found on the mitochondrial membrane, regulates the activity of apoptosomes (Frias et al. 2008). The Bcl-2 protein family consists of two protein groups that act in pro-apoptotic and anti-apoptotic roles in cell biology (Gorji and Malekshah 2013). Bad, Bax, and Bid are pro-apoptotic proteins that induce the cells to undergo apoptosis. They increase the release cytochrome c from the mitochondria into the cytosol. On the other side, Bcl-2 and Bcl-xl are two anti-apoptotic proteins that prevent the release of cytochrome c into the cytoplasm (Golstein 1997). In numerous studies, the antioxidant HES's anti-apoptotic effects have been shown (Celik et al. 2016; Thenmozhi et al. 2017). HES's anticancer role is also well identified. It has been shown that it has tumor-suppressive properties against several forms of human cancer (Xia et al. 2018; Zhao et al. 2017; Cincin et al. 2018). Additionally, studies have shown that in various types of cancer as well as MCF-7 and MDA-MB-231 breast cancer lines, HES induces apoptosis in a dose- and time-dependent manner (Kabala-Dzik et al. 2018; Park et al. 2008; Nazari et al. 2011; Meiyanto et al. 2012). It has been indicated that the administration of HES can ameliorate injury, thereby increasing the expression of the Bcl-2 protein and reducing caspase-3 activity (Siddiqi et al. 2015). Khamis et al. analyzed the effectiveness of HES co-administration with tamoxifen in the MCF-7 cell line on apoptosis-related genes such as Bcl-2 and Bax and showed that the expression of these genes changed (Khamis et al. 2018). The findings of the current investigation showed that HES induced apoptosis through the activation of the Bax/Bcl-2 pathway in the MCF-7 breast cancer cell line.

However, in the MDA-MB-231 breast cancer cell line, HES induced apoptosis, but the Bax/Bcl-2 pathway wasn't triggered.

Apoptosis and cell cycle regulation are intertwined processes (Evan et al. 1995). In tumor development, avoiding cell cycle arrest is the most common phenomenon observed. There are several checkpoints that act as control mechanisms during the cell cycle. Upon DNA damage or cellular stress, these checkpoints cause cells to activate repair systems, induce apoptosis, or arrest the cell cycle (Hartwell and Kastan 1994; Kastan and Bartek 2004). It has been shown that HES induces G0/G1 cell cycle arrest and apoptotic cell death in A549 non-small lung cancer cell lines (Xia et al. 2018). To determine HES's inhibitory effects against prostate cancer cells, studies on their cell cycle and apoptosis were made. It has been shown that HES arrests the cell cycle at the G2/M stage (Banjerdpongchai et al. 2016). In this work, it was found that HES prevented S phase cell division in the MCF-7 and MDA-MB-231 cell lines.

Conclusion

Our investigation demonstrated that HES, as an antioxidant component, increased the rate of programmed cell death in both breast cancer cell lines. According to the findings of the current study, HES upregulated the Bax/Bcl-2 pathway in the MCF-7 breast cancer cell line but downregulated it in the MDA-MB-231 breast cancer cell line. Also, HES leads to cell cycle arrest at the S phase, which increases HES-induced apoptosis. These findings may contribute to an understanding of the mechanisms by which HES regulates breast cancer and provide a starting point for antineoplastic therapy.

Declarations

Ethical Approval

Ethics committee approval is not required as the study was performed with cell culture.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Funding

This research was supported by the Scientific Research Coordination Unit of Erciyes University, Turkey (EUBAP, TKB-2020-10305).

Availability of data and materials

Not applicable.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Gözde Özge Önder, Özge Göktepe, Münevver Baran, Nazmiye Bitgen and Arzu Hanım Yay. The first draft of the manuscript was written by Gözde Özge Önder, Arzu Hanım Yay and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

1. Ahmadi A, Shadboorestan A (2016) Oxidative stress and cancer; the role of hesperidin, a citrus natural bioflavonoid, as a cancer chemoprotective agent. Nutrition and cancer 68(1): 29-39. https://doi.org/10.1080/01635581.2015.1078822
2. Ameer B, Weintraub RA, Johnson JV, Yost RA, Rouseff RL (1996) Flavanone absorption after naringin, hesperidin, and citrus administration. Clin Pharmacol Ther 60: 34–40. https://doi.org/10.1016/S0009-9236(96)90164-2
3. Bange J, Zwick E, Ullrich A (2001) Molecular targets for breast cancer therapy and prevention. Nat Med 7 (5): 548–552. https://doi.org/10.1038/87872
4. Banjerdpongchai R, Wudtiwai B, Khaw-on P, et al (2016) Hesperidin from Citrus seed induces human hepatocellular carcinoma HepG2 cell apoptosis via both mitochondrial and death receptor pathways. Tumor Biol 37: 227- 237. https://doi.org/10.1007/s13277-015-3774-7
5. Baran M, Ozturk F, Canoz O, et al (2020) The effects of apoptosis and apelin on lymph node metastasis in invasive breast carcinomas. Clin Exp Med. 20(4):507-514. doi:10. 1007/s10238-020-00635-2
6. Bray F, Ferlay J, Soerjomataram I, et al (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer J. Clin. 68(6): 394–424. https://doi.org/10.3322/caac.21492
7. Celik E, Oguzturk H, Sahin N, et al (2016) Protective effects of hesperidin in experimental testicular ischemia/ reperfusion injury in rats. Arch Med Sci 12, 928. https://doi.org/10.5114/aoms.2015.47697
8. Cincin ZB, Kiran B, Baran Y, et al (2018) Hesperidin promotes programmed cell death by downregulation of nongenomic estrogen receptor signalling pathway in endometrial cancer cells. Biomed Pharmacother 103: 336-345. https://doi.org/10.1016/j.biopha.2018.04.020
9. Del Río JA, Díaz L, Ortuño A (2017) Properties and Use of Citrus Flavonoids. Phytochemicals in Citrus. Applications Function Foods CRC Press, USA, pp 323-331.
10. Donaldson MS (2004) Nutrition and cancer: a review of the evidence for an anti-cancer diet, Nutrition journal, 3(1): 1-21. https://doi.org/10.1186/1475-2891-3-19
11. Evan GI, Brown L, Whyte M, et al (1995) Apoptosis and the cell cycle. Curr Opin Cell Biol 7: 825–834. https://doi.org/10.1016/0955-0674(95)80066-2

12. Frias MA, Somers S, Gerber-Wicht C, et al (2008) The PGE2-Stat3 interaction in doxorubicin-induced myocardial apoptosis. Cardiovasc Res 80: 69–77. https://doi.org/10.1093/cvr/cvn171
13. Golstein P (1997) Controlling cell death. Science 275: 1081–1082. https://doi.org/10.1126/science.275.5303.1081
14. Gorji SM, Malekshah AAK (2013) Effect of doxorubicin on Bcl2 and Bax expression in Rat heart. J Gorgan Univ Med Sci 15: 19–24
15. Hartwell LH, Kastan MB (1994) Cell cycle control and cancer. Science. 266: 1821-1828. https://doi.org/10.1126/science.7997877
16. Kabala-Dzik A, Rzepecka-Stojko A, Kubina R, et al (2018) Flavonoids, bioactive components of propolis, exhibit cytotoxic activity and induce cell cycle arrest and apoptosis in human breast cancer cells MDA-MB-231 and MCF-7-A comparative study. Cell Mol Biol 64: 1–10. http://dx.doi.org/10.14715/cmb/2018.64.8.1.
17. Kastan MB, Bartek J (2004) Cell-cycle checkpoints and cancer. Nature 432: 316-323. https://doi.org/10.1038/nature03097
18. Khamis AA, Ali EM, Abd El-Moneim MA, et al (2018) Hesperidin, piperine and bee venom synergistically potentiate the anticancer effect of tamoxifen against breast cancer cells. Biomedicine & pharmacotherapy 105: 1335-1343. https://doi.org/10.1016/j.biopha.2018.06.105
19. Khan FA, Akhtar S, Almofty SA, et al (2018) FMSP-nanoparticles induced cell death on human breast adenocarcinoma cell line (MCF-7 cells): morphometric analysis. Biomolecules 8 (2): 32. https://doi.org/10.3390/biom8020032
20. Lee CJ, Wilson L, Jordan MA, et al (2009) Hesperidin suppressed proliferations of both Human breast cancer and androgen-dependent prostate cancer cells. Phytother. Res. 24: 15–19. https://doi.org/10.1002/ptr.2856
21. Lee NK, Choi SH, et al (2004) Antiallergic activity of hesperidin is activated by intestinal microflora. Pharmacology 71: 174–180. https://doi.org/10.1159/000078083
22. Li C, Schluesener H (2017) Health-promoting effects of the citrus flavanone hesperidin. Crit Rev Food Sci Nutr 57: 613–631. https://doi.org/10.1080/10408398.2014.906382
23. Mazza G (2007) Anthocyanins and heart health. Annali-Istituto Superiore Di Sanita 43(4): 369-374
24. Meiyanto E, Hermawan A, Anindyajati A (2012) Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. Asian Pacific Journal of Cancer Prevention 13(2): 427-436. http://dx.doi.org/10.7314/APJCP.2012.13.2.427
25. Nazari M, Ghorbani A, Hekmat-Doost A, et al (2011) Inactivation of nuclear factor-κB by citrus flavanone hesperidin contributes to apoptosis and chemo-sensitizing effect in Ramos cells. European journal of pharmacology 650(2): 526-533. https://doi.org/10.1016/j.ejphar.2010.10.053
26. Nikoletopoulou V, Markaki M, Palikaras K, et al (2013) Crosstalk between apoptosis, necrosis and autophagy. *Biochim Biophys Acta* 1833(12):3448–3359. doi: http://dx.doi.org/10.1016/j.bbamcr.2013.06.001

27. Onder GO, Sezer G, Ozdamar S, et al (2022). Melatonin has an inhibitory effect on MCF-7 and MDA-MB-231 human breast cancer cell lines by inducing autophagy and apoptosis. Fundamental & Clinical Pharmacology *36*(6): 1038-1056. https://doi.org/10.1111/fcp.12813
28. Park H, Kim MJ, Ha E, et al (2008) Apoptotic effect of hesperidin through caspase3 activation in human colon cancer cells, SNU-C4. Phytomedicine 15(1): 147–151. https://doi.org/10.1016/j.phymed.2007.07.061
29. Razak NA, Abu N, Ho WY, et al (2019) Cytotoxicity of eupatorin in MCF-7 and MDA-MB-231 human breast cancer cells via cell cycle arrest, anti-angiogenesis and induction of apoptosis, Sci Rep 9(1): 1-12. https://doi.org/10.1038/s41598-018-37796-w
30. Siddiqi A, Nafees S, Rashid S, et al (2015) Hesperidin ameliorates trichloroethylene-induced nephrotoxicity by abrogation of oxidative stress and apoptosis in wistar rats. Mol Cell Biochem 406: 9–20. https://doi.org/10.1007/s11010-015-2400-8
31. Thenmozhi JA, William Raja TR, Manivasagam T, et al (2017) Hesperidin ameliorates cognitive dysfunction, oxidative stress and apoptosis against aluminium chloride induced rat model of Alzheimer's disease. Nutr Neurosci 20: 360–368. https://doi.org/10.1080/1028415X.2016.1144846
32. Wang H, Khor TO, Shu L, et al (2012) Plants vs. Cancer: a review on natural phytochemicals in preventing and treating cancers and their druggability, in: Anti-Cancer Agents in Medicinal Chemistry 12(10): 1281-1305. https://doi.org/10.2174/187152012803833026
33. Xia R, Sheng X, Xu X, et al (2018) Hesperidin induces apoptosis and G0/G1 arrest in human non-small cell lung cancer A549 cells. Int J Molec Med 41: 464-472. https://doi.org/10.3892/ijmm.2017.3250
34. Zhao J, Li Y, Gao J, et al (2017) Hesperidin inhibits ovarian cancer cell viability through endoplasmic reticulum stress signaling pathways. Oncol Lett 14: 5569-5574. https://doi.org/10.3892/ol.2017.6873

Figures

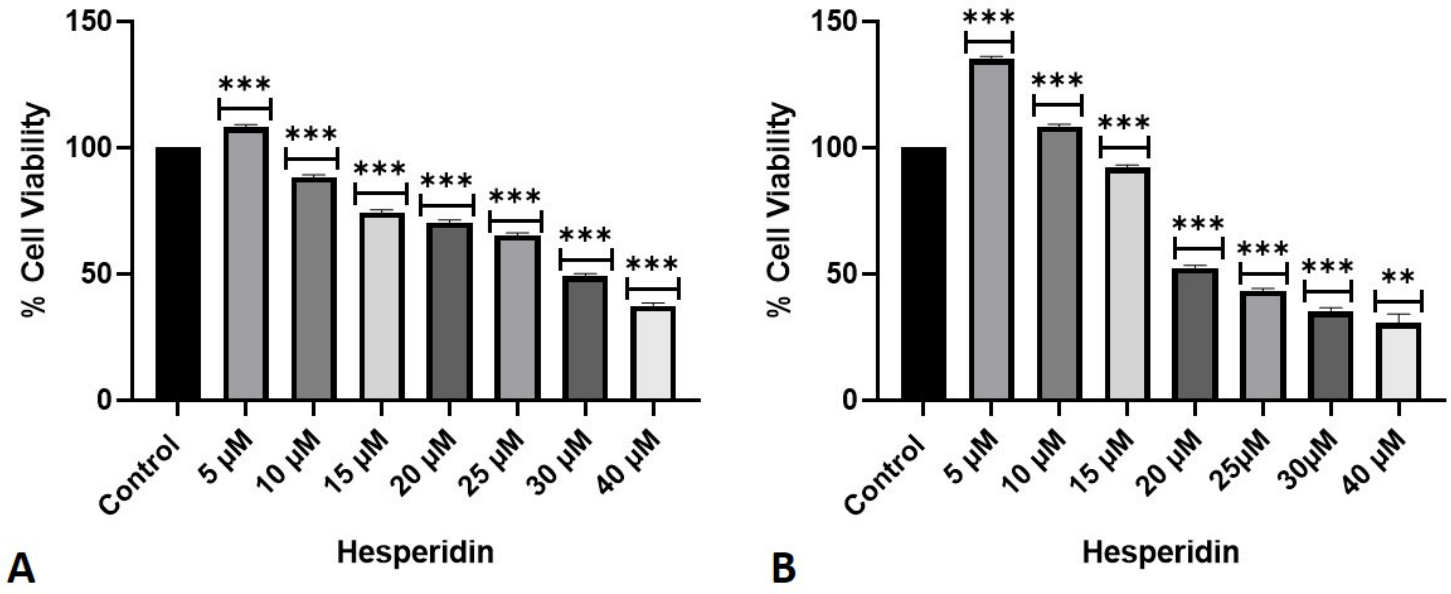

Figure 1

Cell viability of the HES-treated MCF-7 **(A)** and MDA-MB-231 **(B)** breast cancer cell line (**p<0.01, ***p<0.001)

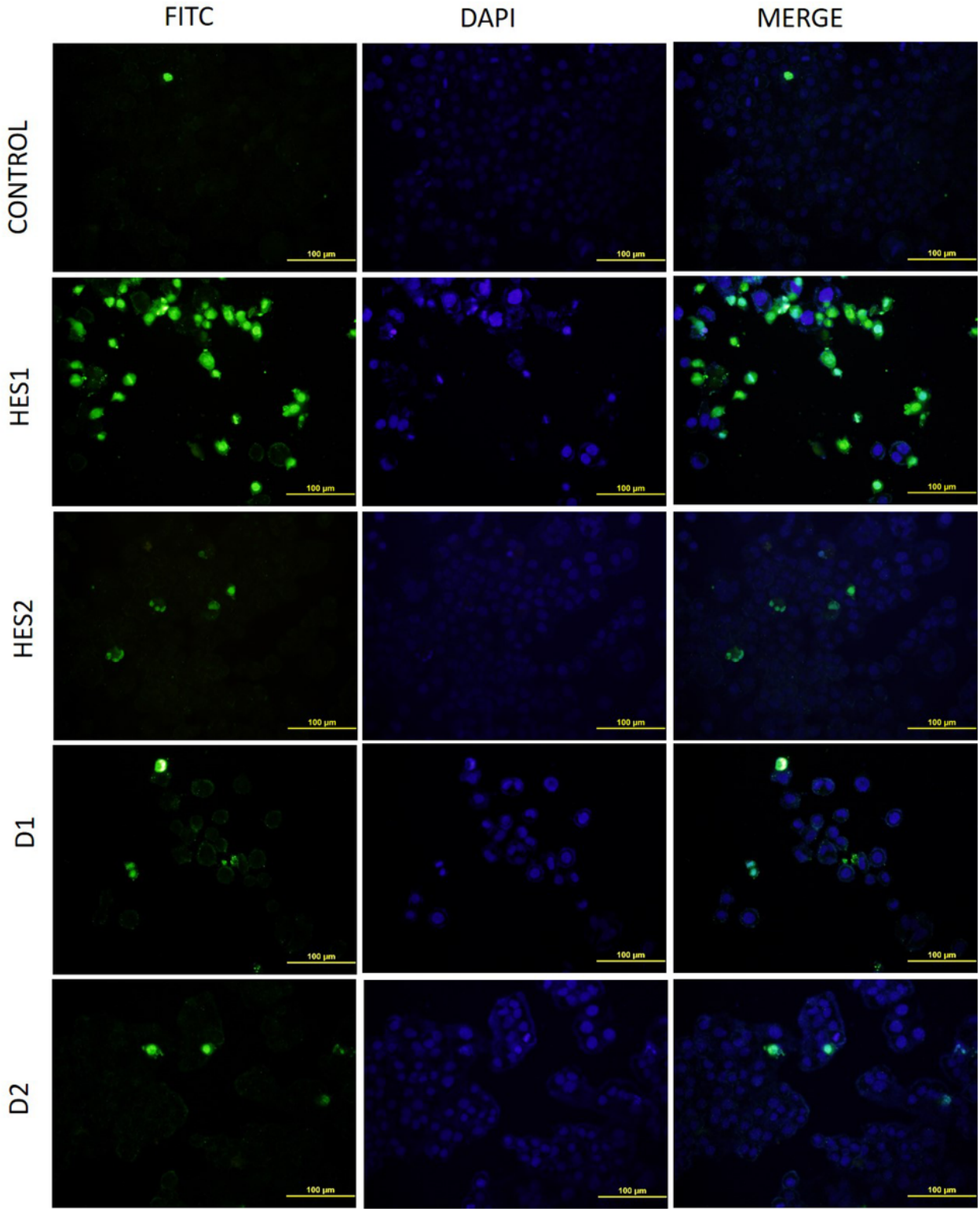

Figure 2

TUNEL images of experimental groups after HES application in the MCF-7 breast cancer cell line. Under a fluorescent microscope, apoptotic bodies formed in cells are visible as green fluorescent reflections

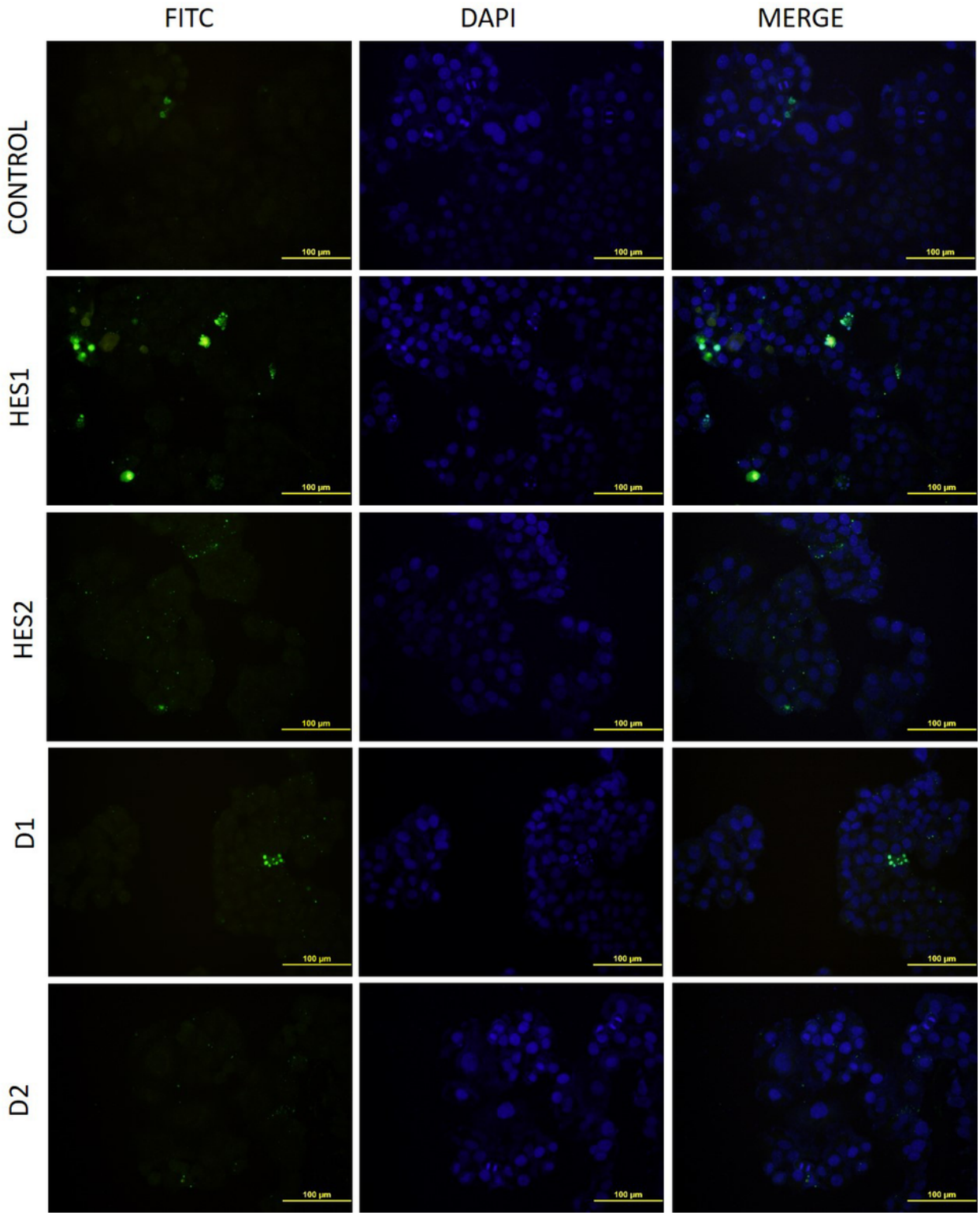

Figure 3

TUNEL images of experimental groups in the HES-treated MDA-MB-231 breast cancer cell line. Under a fluorescent microscope, apoptotic bodies formed in cells are visible as green fluorescent reflections

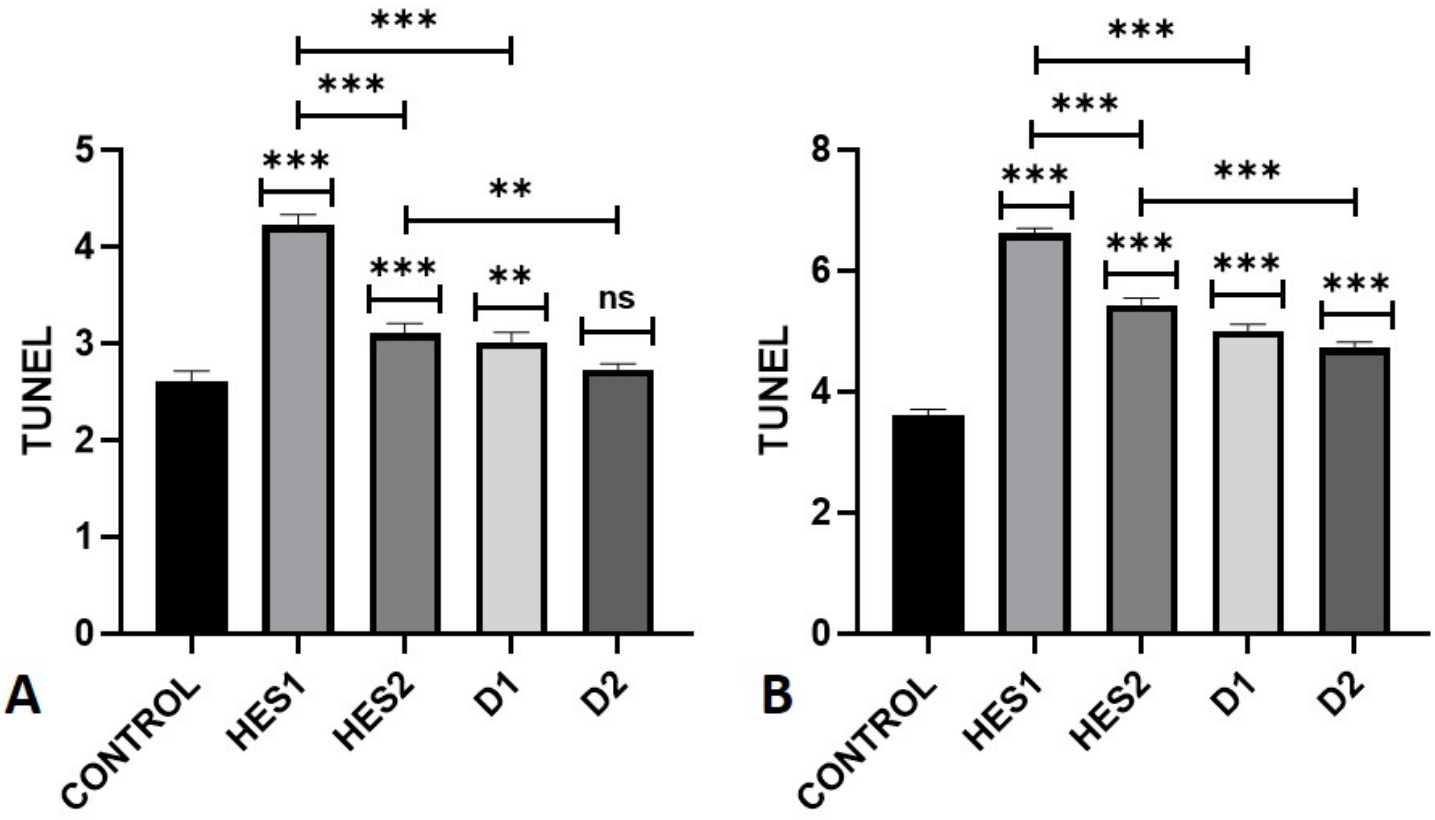

Figure 4

TUNEL statistical analysis results of MCF-7 **(A)** and MDA-MB-231 **(B)** breast cancer cell lines treated with HES (ns: non-significant $p>0.999$, *$p<0.05$, **$p<0.01$, ***$p<0.001$)

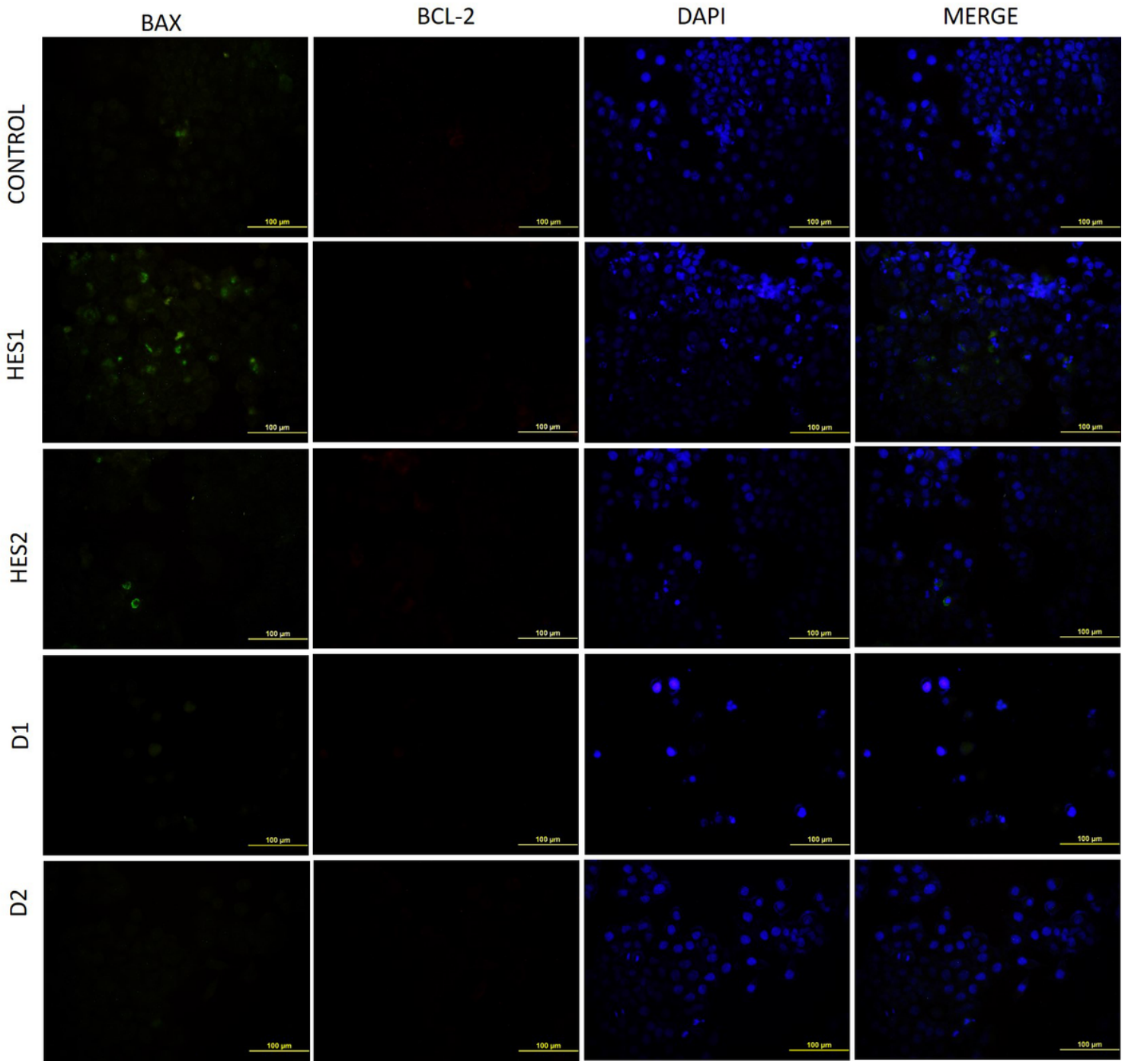

Figure 5

Bax/Bcl-2 expressions of the MCF-7 breast cancer cell line treated with hesperidin. Bright green reflections in the first column indicate Bax expression, while bright red reflections in the second column represent Bcl-2 expression

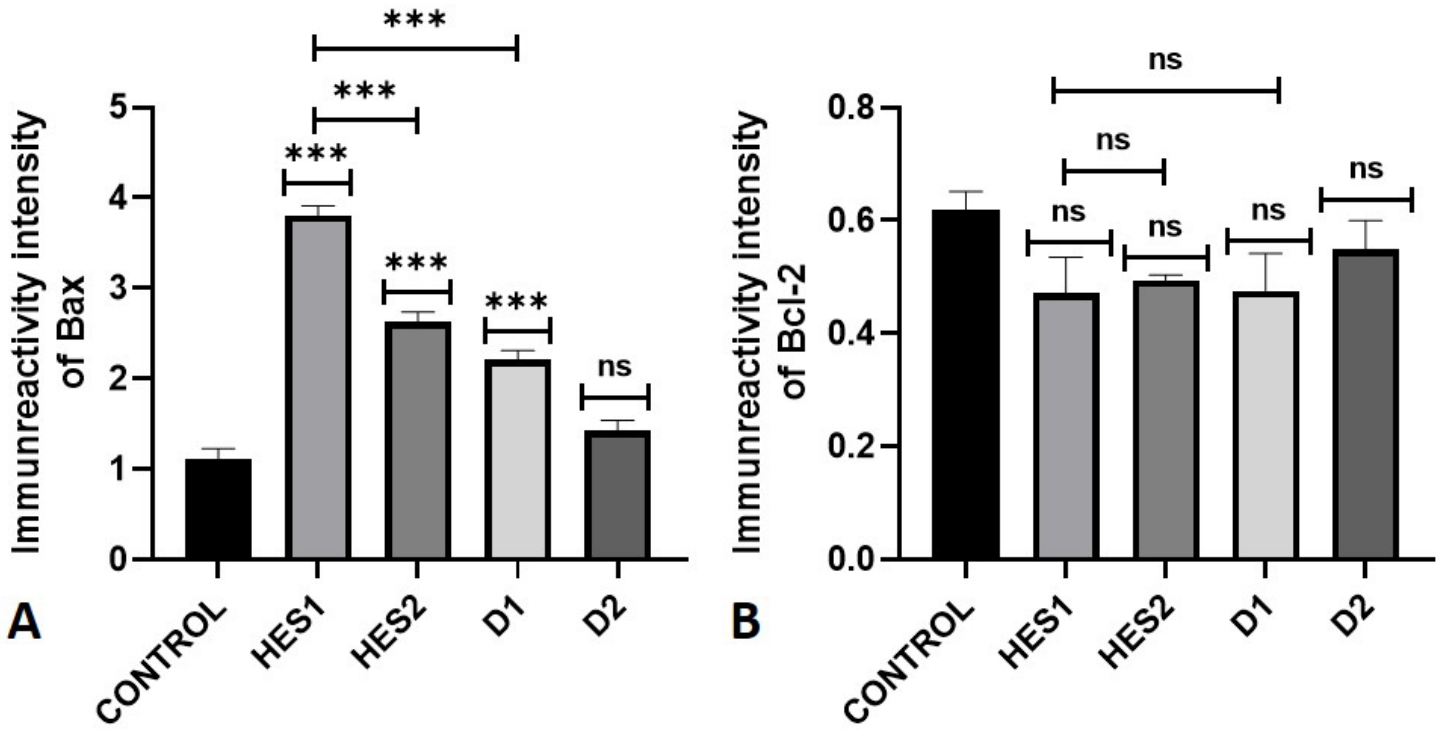

Figure 6

Statistical analysis results of Bax **(A)** and Bcl-2 **(B)** expression in the MCF-7 breast cancer cell line treated with HES (ns: non-significant $p>0.999$, ***$p<0.001$)

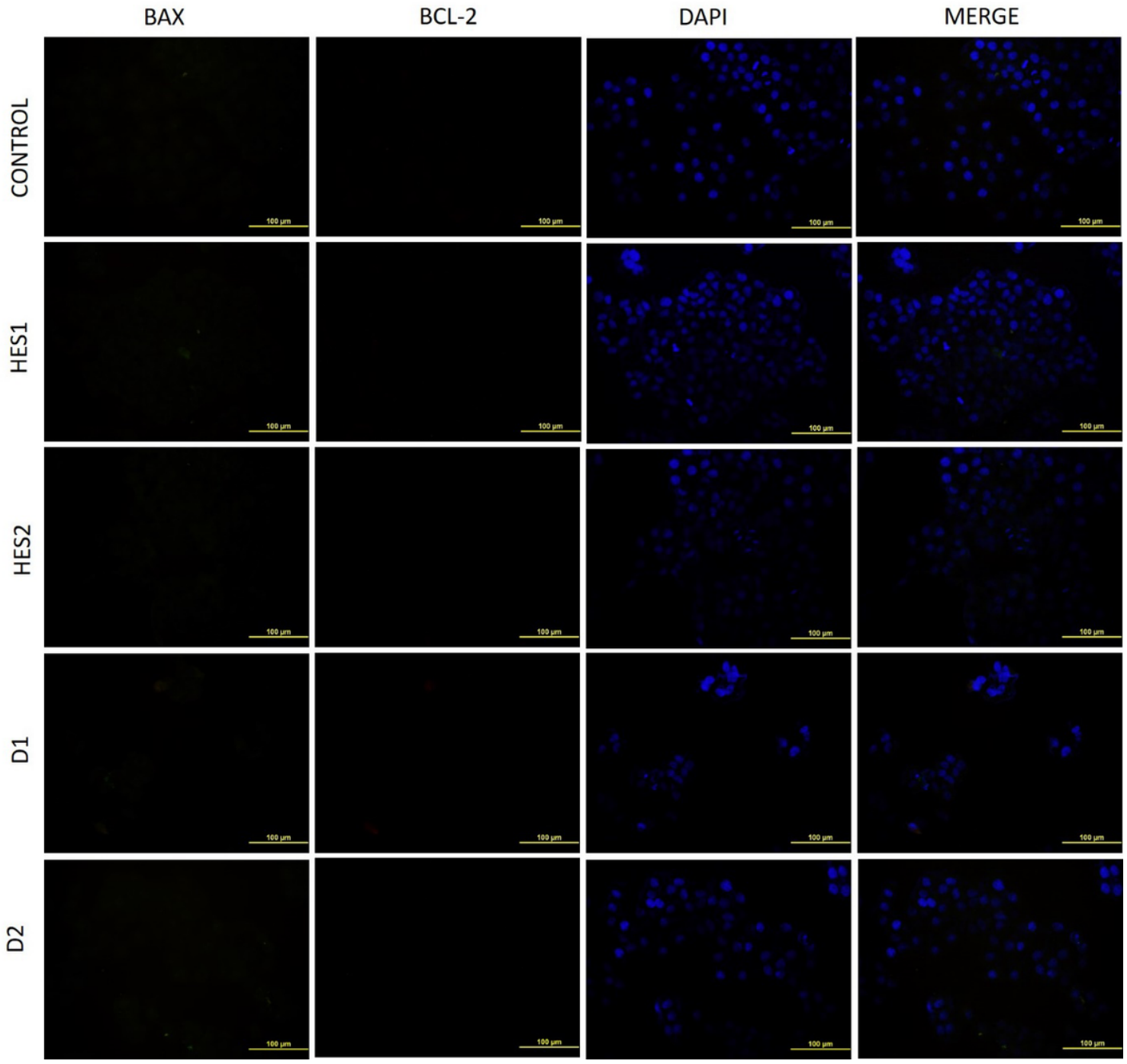

Figure 7

Bax/Bcl-2 expressions of the MDA-MB-231 breast cancer cell line treated with hesperidin. Bright green reflections in the first column indicate Bax expression, while bright red reflections in the second column represent Bcl-2 expression

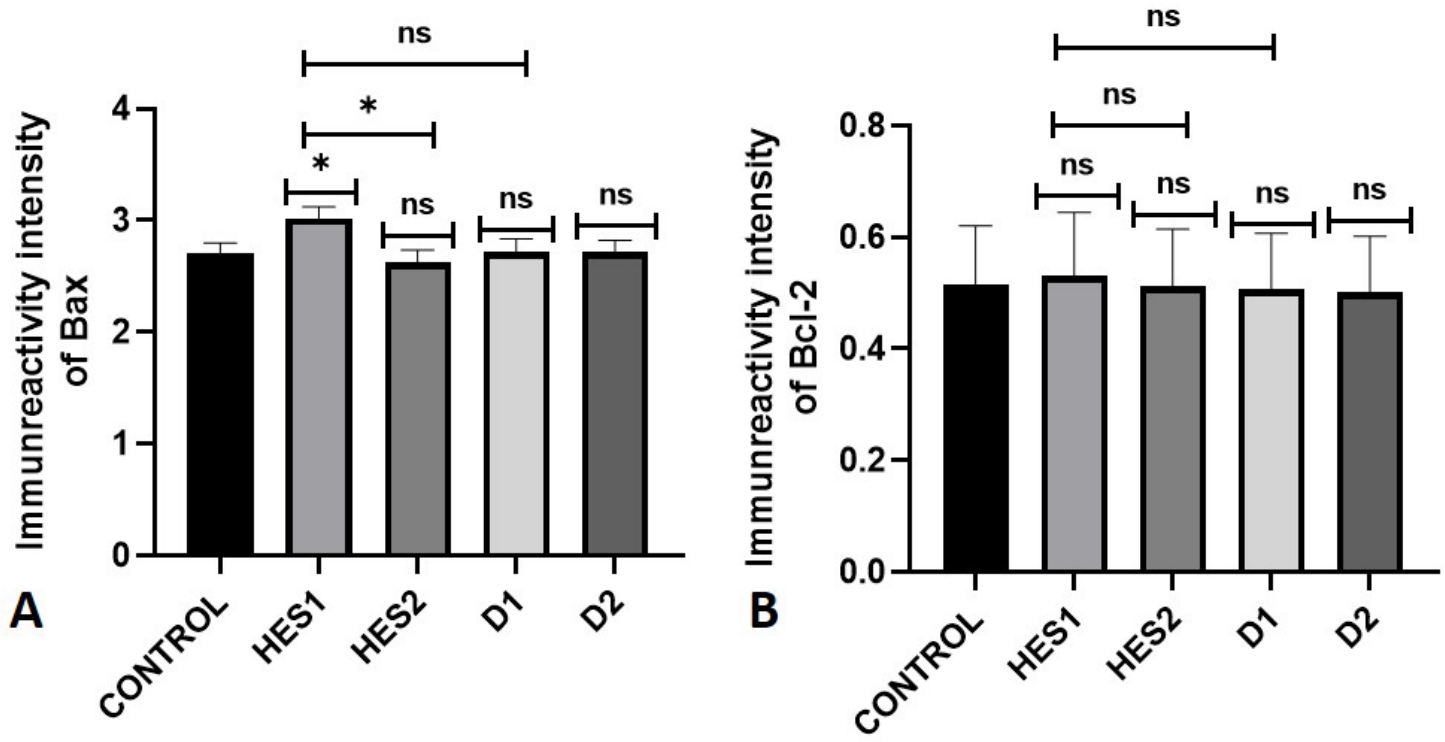

Figure 8

Statistical analysis results for Bax **(A)** and Bcl-2 **(B)** expression in the MDA-MB-231 breast cancer cell line treated with HES (ns: non-significant p>0.999, *p<0.05)

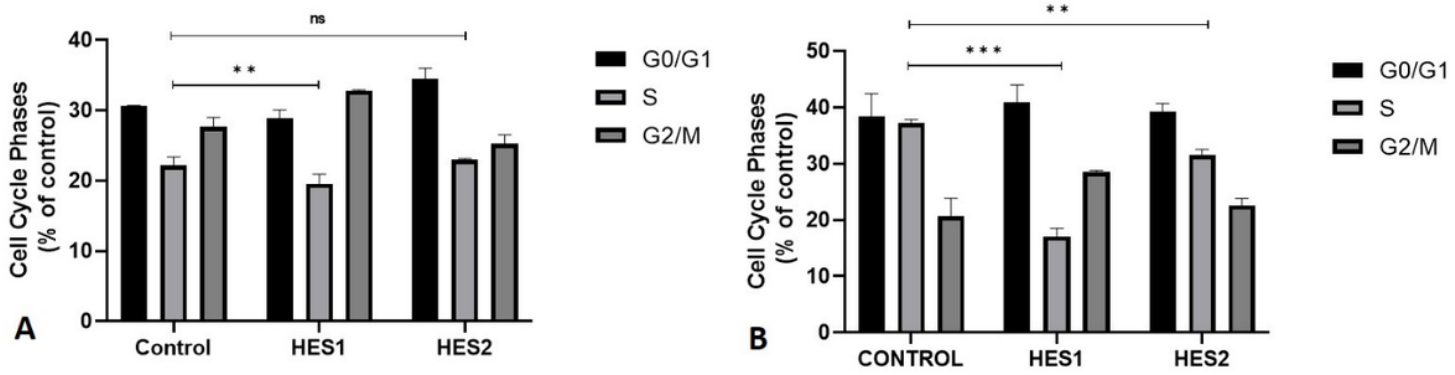

Figure 9

Cell cycle analysis in MCF-7 **(A)** and MDA-MB-231 **(B)** breast cancer cell lines treated with HES (ns: non-significant p>0.999, **p<0.01)