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Investigating the Influence of 3,4-Dihydroxyphenylethanol (DHPE) on Apoptotic Genes in HCT-116 Cells: An *In Vitro* Study

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A B S T R A C T

Introduction: 3,4-Dihydroxyphenylethanol (DHPE) is a strong antioxidant that has activities such as controlling oxidative stress, inhibiting cell proliferation, and inducing apoptosis. Toxicological studies have shown that this substance has no mutagenic or genotoxicological effects that support its long-term use. This study investigated DHPE's role in regulating key apoptotic genes (*Bax*, *Bcl2*, *Caspase3*, and p53) in HCT116 cells, with a particular focus on its p53-independent effects, offering new insights into alternative apoptosis pathways in colorectal cancer (CRC).

Materials & Methods: The human colorectal cancer cells (HCT-116) were treated with 50, 100, 150, and 200 µM of DHPE for 24 hours. Then, the expression level of *Bax*, *Bcl2*, *Caspase3*, and p53 genes was evaluated by real-time PCR.

Results: Analysis of gene expression results showed that DHPE increased gene expression of *Bax*, *Bcl2*, *caspase3*, and the *Bax*/*Bcl2* ratio ($P<0.05$), but there were no significant changes in p53 gene expression in treated groups compared to the control group in HCT116 cells.

Conclusion: This study provides evidence that DHPE induces apoptosis in HCT116 cells without altering p53 mRNA levels, suggesting potential activity through p53-alternative pathways. These findings highlight DHPE's promise as a novel therapeutic candidate for CRC, particularly in tumors with p53 dysfunction.

Keywords: HCT116 cells, 3,4-Dihydroxyphenylethanol, Apoptosis

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Introduction

Among Iranian males, colorectal cancer ranks third, and among Iranian women, it ranks fourth, making it the most frequent kind of gastrointestinal cancer in Iran (1). A number of epidemiological studies have shown that sedentary lifestyles, obesity, inflammatory bowel diseases (such as Crohn's disease), and bad dietary habits, smoking, excessive alcohol consumption, and lack of physical activity are important risk factors, in addition to age, genetics, and heredity. There may be an increased risk of colorectal cancer in those whose diets are heavy in red and processed meat, saturated fat, and low in fiber, folate, and calcium (2). Surgery and chemotherapy have been the mainstays of colorectal cancer treatment up until recently. Natural chemicals that potentially cure colorectal cancer and enhance a person's life have been the subject of much research in recent years (3).

There are chemicals in olive oil that may help prevent and treat colorectal cancer. The phenolic chemicals found in olive oil have promising therapeutic potential, particularly in the treatment of colorectal cancer (CRC), according to mounting data. Inflammation, oxidative stress, the cell cycle, immunological modulation, apoptosis, and epigenetic alterations are just a few of the carcinogenesis processes that olive oil's phenolic components impact. Intestinal issues, including colorectal cancer (CRC), may be better avoided by altering the microbiota of the intestines. In contrast, olive oil has anti-inflammatory properties in the intestines and may boost the development of good bacteria like lactic acid bacteria while decreasing the number of harmful bacteria. Consequently, a crucial therapeutic or preventative approach against CRC (2) might be preserving or reestablishing gut microbial balance.

Among the several polyphenols present in olive oil, 3,4-Dihydroxyphenylethanol (DHPE) stands out as the most potent antioxidant (4). This phenolic molecule is one of the primary components of olive oil and its leaf extract. According to research, this compound protects against some chronic illnesses, lowers plasma cholesterol and leptin levels (6), and boosts the body's natural defenses (5). It seems that DHPE activates molecular signaling pathways that produce apoptosis and cell cycle arrest (8), as this chemical displays antioxidant, pro-apoptotic, anti-proliferative, anti-inflammatory, and anti-proliferative actions (7). Researchers have demonstrated that this compound may induce cell death in some cell lines, including the MCF-7 breast cancer cell line (9), but the exact mechanism by which it does this remains unclear. Thus, it seems that more research is necessary to validate 3,4-DHPE's ability to induce apoptosis as a potential drug for cancer prevention and therapy. Consequently, this research set out to examine how 3,4-dihydroxyphenylethanol affected the expression of apoptosis-related genes in the HCT-116 colorectal cancer cell line, including p53, Bax, Bcl-2, and caspase 3.

Materials and Methods

Study Design

Here, we used RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin to grow the HCT-116 cell line. To find the best DHPE exposure period and dosage, the MTT test was used. This study followed five groups, including a control group and four treatment groups, as determined by MTT. After DHPE treatment, we used real-time PCR to compare the treated and control groups' RNA levels for Bax, Bcl2, Caspase3, and p53 expression.

Setting and Participants

The HCT-116 cell line was purchased from Encito Pasteur, Iran. The cells were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin antibiotics and kept in an incubator containing 5% CO_2 and 95% humidity at 37°C.

Measurements & Validity and Reliability

MTT Test

We used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test to find the best dosage and exposure duration for DHPE. The 96-well plates were seeded with 5×10^3 cells/well of HCT-116 cells and left to adhere for 24 hours. Afterward, cells were subjected to a DHPE concentration gradient ranging from 5 to 300 µM for three distinct incubation durations (24, 48, and 72 hours). Each well was treated, and then 20 µL of MTT solution (5 mg/mL in PBS) was added. The mixture was then

incubated at 37°C in 5% CO_2 for 4 hours. After the growth media was carefully removed, the formazan crystals that had grown were dissolved in 100 µL of DMSO. With a reference wavelength of 630 nm, the microplate reader measured the absorbance at 570 nm. The proportion relative to the untreated control was used to determine cell viability.

Gene Expression Analysis

Following DHPE treatment, RNA was extracted from both the treated and control groups of cells using the protocol provided by the kit (Jena Bioscience, Germany). One microgram of total RNA was used for cDNA synthesis following the directions of the Jena Bioscience commercial kit after quantitative and qualitative examination of the isolated RNA using nanodrops and electrophoresis. A SYBER Green Real-Time kit (Jena Bioscience, Germany), 1 µL of cDNA product, and specific primers for each gene were used in a real-time PCR experiment to verify the expression of the genes under study and the reference gene (β2 microglobulin). An automated real-time polymerase chain reaction (PCR) instrument developed by Corbett Life Science and manufactured by Qiagen in Germany was used to conduct the quantitative PCR. The experimental setup consisted of a 15-minute activation stage at 95°C, 40 cycles of 25 seconds of denaturation at 95°C and 60 seconds of concomitant extension at 60°C. After running the real-time PCR process, we drew the gene melting curve and used electrophoresis on an agarose gel to control the PCR products, ensuring their specificity.

This study followed five groups, including a control group and four treatment groups, as determined by MTT. With no treatment in the control group, the HCT-116 cell line was subjected to varying doses of DHPE (50, 100, 150, and 200 µM) for 24 hours in each of the four treatment groups.

Statistical and Data Analysis

Using the Relative Expression Software Tool (REST 2009), this research conducted gene expression analysis by real-time PCR. The data was normalized using the ΔΔCt technique, with β2 microglobulin (β2M) acting as the endogenous reference genes. Across all treatment groups, cell viability was evaluated using one-way ANOVA followed by Tukey's post hoc test. For all analyzes, a p-value less than 0.05 was used as a significant criterion.

Results

At all dosages tested, our tests demonstrated that DHPE decreased the survival of colorectal cancer cells. Starting at 50 µM ($p < 0.05$), the impact became statistically significant. Even though the cell killing was greater at the highest dosages (250-300 µM), we chose to conduct our follow-up experiments in the 50-200 µM range since these concentrations are more likely to have therapeutic use, and the higher levels may not be applicable to real-world therapy situations.

A one-way analysis of variance revealed that increasing the quantity of DHPH decreased the cell viability percentage. Figure 1 shows the data that were used to choose DHPE doses of 50, 100, 150, and 200 µM for cell therapy (Fig. 1).

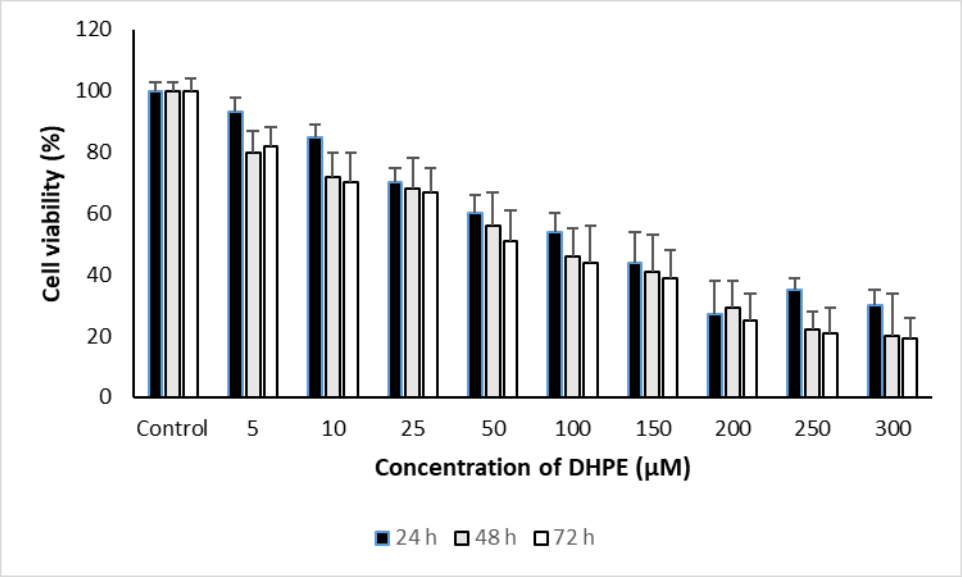

Figure 1. Results from a one-way ANOVA regarding cell viability percent of HCT-116 cells according to different concentrations of DHPE and times of 24, 48, and 72 h. The results are presented as mean ± SD of three separate measurements. The decrease in cell viability following DHPE treatment became statistically significant at concentrations starting from 50 µM ($p < 0.05$).

Table 1. Primer Sequences used in this study.

Genes	Sequences(5′-3′)	Size(bp)	Ref
Bcl2	F: TCGCCCTGTGGATGACTGA	134	(10)
	R:CAGAGACAGCCAGGAGAAATCA		
Bax	F:TGGCAGCTGACATGTTTTCTGAC	195	(10)
	R: TCACCCAACCACCCTGGTCTT		
Caspase3	F: TACCTGTTCTCCATGGATACCT	134	(11)
	R: TCAGTGTTCTCCATGGATACCT		
p53	F: GGACATTTGCGTTCGGG	118	(11)
	R: CTAGGATCTGACTGCGGCTC		
β_2M	F:ACTGAATTCACCCCCACTGA	167	(12)
	R:AAGCAAGCAAGCAGAATTTGGA		

A substantial loss in viability ($p < 0.05$) in the absence of plateau effects reported at prolonged durations supports the selection of the 24-hour timepoint to examine primary pharmacological mechanisms before secondary effects appear. In order to assess possible anticancer drugs, this experimental design adheres to well-established procedures. In comparison to the control group, the findings demonstrated that Bax gene expression was enhanced when cells were treated with DHPE at all doses (Fig. 2A, $P < 0.05$). In comparison to the control group, Bcl2 gene expression increased across all DHPE treatment doses (Fig. 2B) ($P < 0.05$). In comparison to the control group, the groups treated with DHPE exhibited a substantial rise in the Bax/Bcl2 ratio (Fig. 2C, $P < 0.05$). Compared to the control group, all groups treated with DHPE showed an increase in caspase-3 gene expression. This is seen in Figure 2D with a p-value less than 0.05. The DHPE-treated groups did not differ significantly from the control group with respect to p53 gene expression ($P > 0.05$) (Fig. 2E).

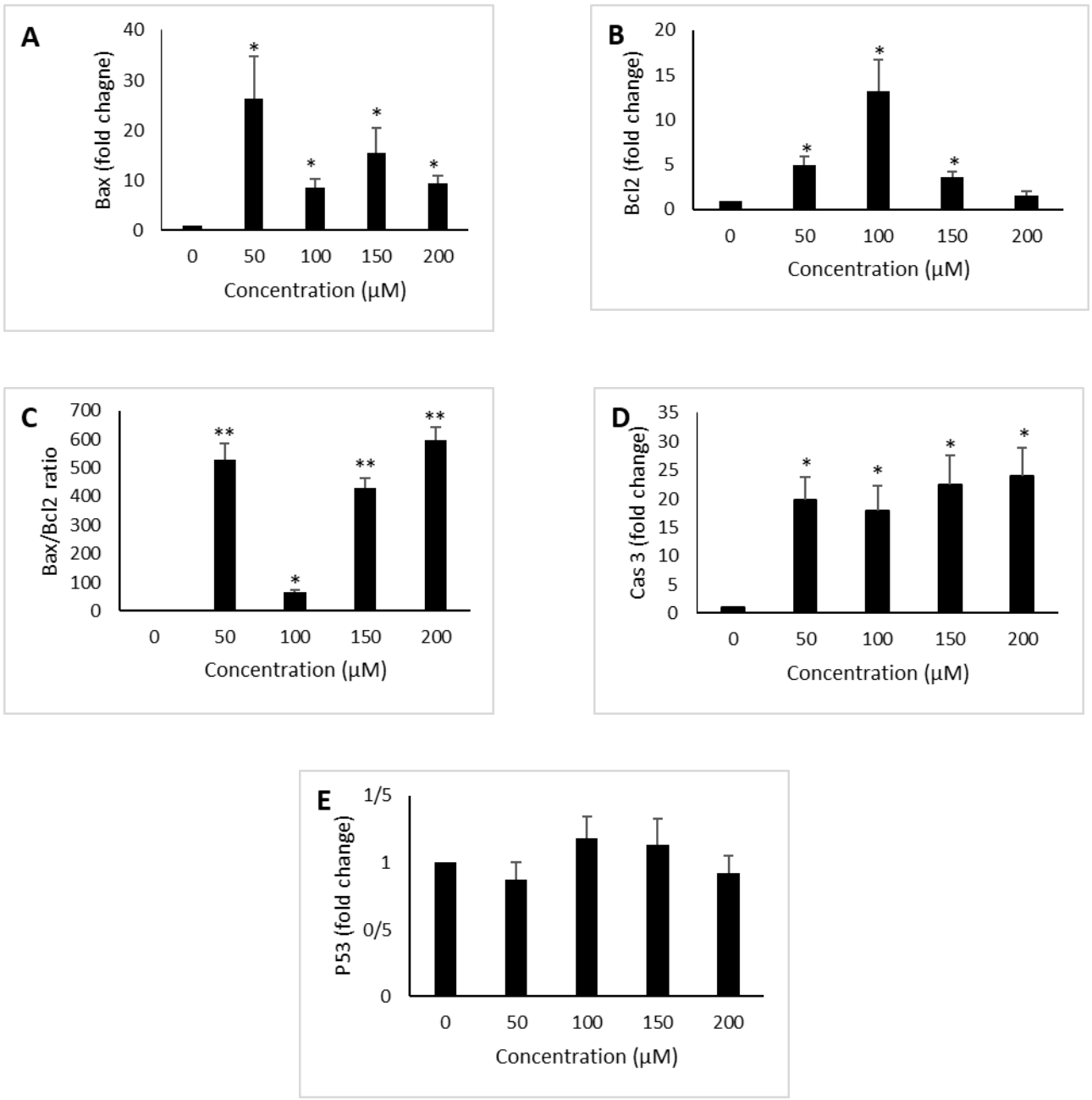

Figure 2. Expression level of *Bax* (A), *Bcl2* (B), *Bax*/*Bcl2* ratio (C), Cas3 (D), and P53 (E) in HCT-116 cells treated with 50, 100, 150, and 200 µM DHPE compared to the untreated control group. Data are presented as mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test, with *P < 0.05 (*) and **P < 0.01 (**) considered significant.

Discussion

This research found that in the HCT-116 cell line, DHPE considerably increased the expression of Bax, Bcl2, caspase 3, and Bax/Bcl2 ratios, but had no discernible effect on the expression of p53. CRC ranks high among the most significant malignancies globally. It develops from the gut's epithelial cells in response to environmental and genetic variables like poor nutrition, lack of exercise, inflammatory bowel illness, and an imbalance of the microbiota in the intestines (3). There have been a number of research looking at the link between food and colorectal cancer, but the results are still inconclusive. A number of research have shown that dietary changes

may lower the disease's prevalence (4, 14). Finding natural products that help combat CRC has also been a major focus in recent years. Phytochemicals are a class of plant-based chemicals that include phenolic acids, phytoalexins, flavonoids, and polyphenols; they have biological activities. Some of the polyphenols included in olive oil have anti-inflammatory and anti-cancer effects, according to research. A powerful antioxidant and naturally occurring phenolic compound, DHPE is one of these substances. This potent antioxidant has several biological functions, including regulating oxidative stress, promoting multicellular competence, and triggering apoptosis in various tumor cell lines, according to recent research (2). Multiple cancer cell lines, including those from breast cancer, leukemia, and melanoma, have validated this substance's anti-tumor activity. Research has shown that this compound suppresses cell growth and produces G2/M cell cycle arrest. Furthermore, it suppresses the AKT and NFKB pathways, which leads to cell death in cancer cells (13). While DHPE was shown to inhibit cell growth and proliferation, halt the cell cycle, and trigger apoptosis in HL-60 (promyelocytic leukemia) and HT-29 (colon adenocarcinoma) cells, no such impact was seen in human colon cancer Caco-2 cells (14). This data suggests that the cellular phenotype may determine the substance's effects. The control of cell death is mediated by a number of proteins. A family of proteins known as Bcl2 regulates this process in fundamental ways; its members include both pro- and anti-apoptotic proteins (15). In contrast to Bcl2, which is mostly found within the mitochondrial membrane and forms a heterodimer with Bax protein, Bax protein exists as a monomer in the cytosol or connected to the membrane. The pro- and anti-apoptotic actions of the two proteins are mutually neutralized by this heterodimer. In order to activate the mitochondrial route of apoptosis, which regulates the integrity of the mitochondrial outer membrane, the balance between the expression levels of Bax and Bcl2 is involved (16). The findings showed that DHPE greatly upregulated caspase-3 gene expression in the HCT-116 cell line. Therefore, it seems that the Bax/Bcl-2 ratio is a better indicator to use when studying apoptotic induction.

By downregulating cyclin D1 mRNA and protein expression and upregulating p21 expression, DHPE was shown to decrease the survival of thyroid and uterine cancer cells. In these cell lines, this chemical has also boosted caspase-3 and PARP-1 expression (17). This compound promotes apoptosis, lowers Bcl2 and PPAR levels, and raises caspase-3, caspase-9, and Bax levels, according to a research conducted on human cholangiocarcinoma and bladder cancer cell lines (18). Evidence suggests that this chemical may also work by downregulating EGFR expression; for instance, in colorectal cancer (CRC) tumor cells, it ubiquitinates and kills receptors, which in turn reduces their proliferation. takes on a cellular form. Another research shown that DHPE inhibits the ERK1/2 signaling pathway, which in turn promotes death in ovarian cancer cell lines. Research has also shown that DHPE raises levels of caspase-3 and Bax, two molecules that promote cell death, while lowering levels of Bcl-2, a protein that inhibits cell death (19).

The p53 gene is well-known for its function in tumor suppression and genomic stability; it is altered in the vast majority of human malignancies. Tumor suppression is primarily accomplished by triggering apoptosis, pausing the cell cycle, and preventing angiogenesis. One of the pharmacological targets for cancer therapy is this gene, which is thought to play a crucial role. The overarching objective of these therapies is to either cause cancer cells to undergo apoptosis by activating p53 or to temporarily render healthy cells immune to radiation and chemotherapy by inactivating p53 (20).

At the transcriptional level, the p53 protein promotes cell death in tumors by lowering anti-apoptotic gene expression and raising apoptotic gene expression. In a transcription-independent route, p53 proteins are released from the nucleus and attach to the outer membrane of mitochondria following DNA damage. This process directly targets mitochondria. The process of mitochondrial permeability, cytochrome C

release, and apoptosis induction is initiated when mitochondrial p53 binds to Bcl2 and neutralizes its inhibitory action on Bax protein (21). Research using the MCF-7 breast cancer cell line demonstrated that oleuropein induced p53-dependent apoptosis by increasing p53 expression (20). Moreover, a different investigation on thyroid and uterine cancer cells found that DHPE causes apoptosis and raises p53 and BAD expression (18). Even though the p53 protein is a critical transcription factor that helps cells die, cells that have problems with the p53 pathway may still die. These findings point to the existence of non-p53 dependent apoptotic mechanisms (21). In reaction to DNA damage, ribosomes halt at the uncommon leucine codon UAA, as recently discovered by Boon et al. Apoptosis is activated when this stalling causes ribotoxin stress (22). Paclitaxel caused apoptosis and a substantial DNA-damage response in bladder cancer cells without triggering the p53 pathway, as shown by Drosos et al. (23).

The complicated illness known as cancer involves several alterations at the genetic and epigenetic levels. Understanding the specific genetic and epigenetic alterations associated with a cancer is crucial for developing effective combination treatments, as these alterations can vary greatly across cancers, even when they originate from the same tissue. molecular targets and changed pathways.

Regardless of the dose of DHPE tested, the findings demonstrated no change in p53 gene expression. It might explain why apoptosis-inducing genes are being expressed at higher levels via a mechanism unrelated to p53. It seems that a mechanism independent of p53 is responsible for the upregulation of apoptosis-inducing genes.

We acknowledge that apoptotic pathways have dynamic temporal patterns, however our data reflect the first pro-apoptotic effects of DHPE. Typically, important indicators such as Bax and Caspase-3 reach their peak within 48 to 72 hours. The effects of DHPE may be due to transitory stress reactions or prolonged apoptotic commitment; further study into these delayed responses may shed light on the matter and point researchers in the direction of the specific molecular targets within pathways that do not include p53.

At 24 hours, our results mainly describe the early apoptotic processes caused by DHPE. On the other hand, it was noted that apoptotic pathways commonly display dynamic temporal patterns, with important indicators like as Bax and Caspase-3 often displaying their peak activity at later time periods, typically between 48 and 72 hours. Further exploration into these delayed reactions would provide a more comprehensive knowledge of the apoptotic potential of DHPE, while our findings clearly show that it may induce apoptosis within the first 24 hours. To distinguish between brief stress reactions and prolonged apoptotic commitment, future research should investigate these later time periods.

Limitations and Strengths

The strengths of this study include the focus on p53-independent mechanisms (an innovation in the treatment of tumors with defective function of this gene), the use of a standard colorectal cancer cell model (HCT116), and the examination of several key markers of apoptosis in a dose-dependent manner. However, the most important limitation of this study is that it is limited to the mRNA level and does not confirm the results at the protein level and cellular function (such as viability and apoptosis assays). Also, the lack of identification of the exact molecular mechanism and the lack of testing in other cell lines limit the generalizability of the results. Overall, this study provides a promising basis for the application of DHPE as an anticancer agent, but its findings require further studies to confirm.

Conclusion

Our findings demonstrate that DHPE significantly upregulates key apoptotic genes in HCT-116 cells, indicating its potent pro-apoptotic activity. Notably, this effect occurred independently of p53 activation, suggesting that DHPE may induce programmed cell death through alternative pathways—a promising trait for targeting p53-resistant cancers. Given the

critical role of apoptosis evasion in tumor progression, DHPE's p53-independent mechanism underscores its potential as a novel therapeutic agent, particularly for malignancies harboring p53 mutations or dysregulation. Further in vivo studies and mechanistic investigations are needed to validate its efficacy and safety, paving the way for clinical translation as either an adjunctive or standalone anticancer therapy.

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CRediT authorship contribution statement

Conceptualization, Methodology, Validation, Formal Analysis: AM, Investigation, Resources, Software, Data Curation, Writing–Original Draft Preparation, Writing–Review & Editing, Visualization, Supervision, Project Administration: AM, HZ.

Declaration of Generative AI and AI-assisted technologies in the writing process

No AI writing assistance was utilized in the production of this manuscript.

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical considerations

Ethical concerns included acquiring the ethics code (IR.LUMS.REC 1396.315), ensuring integrity in library collection and data reporting in accordance with the Declaration of Helsinki.

Data availability statement

The data that support the findings of this study are available on request.

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