RESEARCH ARTICLE

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Synergistic Effect of Caffeic Acid Phenethyl Ester and Deuterium Oxide in Colon Cancer: An *In Vitro* Evaluation

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Abstract

Objective: Caffeic acid phenethyl ester (CAPE) and deuterium oxide (D_2O), known as heavy water, have anticancer properties. However, their combined effects in colon cancer have not been addressed yet. This study aimed to evaluate the synergistic effects of CAPE and D_2O in colon cancer using in vitro techniques. **Methods:** The cancer cells (HCT-116) were treated with CAPE and D_2O alone and in combination to evaluate their cell viability (CV), induction of apoptosis, expression levels of tumor proliferative (AKT, NF-κB, CCND1), tumor suppressor (*p16, BAX, TP53, p21*) genes, and colony-forming ability, respectively. **Result:** The combined treatment of 10 μM CAPE and 30% D_2O in human colon cancer cells (HCT-116) were significantly: (i)cytotoxic on the CV; (i)induced apoptosis, (iii)downregulated the expression of AKT, NF-κB and CCND1 while upregulated the expression of *p16, BAX*, and *TP53*, without altering *p21* level, and (iv)suppressed the cells' colony forming ability, respectively ($p<0.05$). **Conclusion:** Overall, CAPE and D_2O may be an effective adjuvant for enhancing therapeutic efficacy in colon cancer. However, further research is needed to determine whether their synergistic effects are also selective in other malignancies, as well as with their toxicokinetic potential.

Keywords: Caffeic acid phenethyl ester- deuterium oxide- heavy water- colon cancer

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Introduction

Amongst the cancer types, colon cancer is the third most common one at the global level [1]. In terms of cancer-associated fatalities, it ranks fourth and results in 700k fatalities annually [2]. From 1990 to 2012, there was a yearly increase in the occurrence of colon cancer, with Western countries accounting for 55% of the cases [3]. Currently, the standard treatments for colon cancer are surgery, chemotherapy and radiotherapy [4]. However, the effectiveness of these methods can be limited by various adverse effects. [5]. Therefore, naturally occurring products with potent and low-toxic properties, including curcumin, resveratrol, epigallocatechin gallate, flavonoids and many others, have gained significant interest as novel therapeutic agents in this context [6].

Honeybee propolis has been widely studied in cancer types due to its anti-inflammatory, antioxidant, and anticancer effects [7]. A novel formula containing propolis exhibited clear-cut cytotoxic effects on colon cancer [8]. Of all the components that make-up propolis, the caffeic acid phenethyl ester (CAPE) has been the subject of the most research with a broad spectrum of therapeutic effects, including antitumor, anti-inflammatory, immunomodulatory, hepatoprotective, neuroprotective, and cardioprotective effects [9, 10], explicitly targeting the genes involved in cell death, cell cycle regulation, angiogenesis, and metastasis [11], due to its selective toxicity on cancer cells and lack of toxic effects on healthy cells [12].

Deuterium (D) is the isotope of hydrogen (H) first discovered in 1934 by Harold Urey, which is found in low proportions in natural environments, making up 0.0156% of total H. Deuterium oxide (D_2O), also known as heavy water, has recently gained interest in pharmaceutical, biological and spectroscopic applications [13]. Its mass is 11% > than that of regular water (H_2O), resulting in different physicochemical properties than those of normal water [14]. For instance, it can inhibit tumour cells of hepatic, pancreatic, gastric and colon cancer [15, 16].

In literature, although a restricted number of studies have approached the effects of CAPE and D_2O alone in colon cancer [17], their combined effects have not yet been investigated. Based on this fact, this study aimed to

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evaluate the synergistic anticancer potential of CAPE and D_2O in colon cancer using in vitro techniques, whether both can be an effective adjuvant of chemotherapy for enhancing therapeutic efficacy.

Materials and Methods

Chemicals, Reagents and Cell Line

The human colon cancer cell line (HCT-116) was obtained from the American Type Culture Collection (ATCC® CCL-247, Manassas, USA). CAPE (Sigma Aldrich 104594-70-9, St. Louis, MO, USA), deuterium oxide (99.9 Atom %D - Sigma Aldrich 151882-250G, Darmstadt, Germany), Dulbecco's Modified Eagle's Medium (DMEM) (Gibco 11965092, Massachusetts, USA), fetal bovine serum (FBS) (Gibco10500), 1% penicillin/streptomycin/amphotericin (PSA) (Capricorn Scientific GmbH, Ebsdorfergrund, Germany), BioLegend's FITC Annexin V Apoptosis Detection Kit (BioLegend 640914, San Diego, USA), PureLink RNA Isolation Kit (PureLink 12183020, Massachusetts, USA), cDNA kit (Applied Biosystems 01019730, Massachusetts, USA), and SYBR qPCR Kit (GeneMark QPSY01-25, Atlanta, USA) were purchased. The primers (f/r) were designed by Ex-im Life Sciences Ltd. Şti. (Istanbul, Türkiye).

In vitro Cell Viability (MTS) Test

The cell line was cultured in DMEM medium (4.5 g/L D-glucose,10% FBS, and 1% PSA) supplemented with 10% FBS and 1% PSA and kept at 37 °C in a 5% CO2 environment. Upon achieving confluence, the cells were detached from the culture surface via trypsinization, centrifuged, and counted by a hemocytometer. The 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay examined the effects of the CAPE and D_2O on the cells. The HCT-116 cells were seeded at 5 × 10^3 cells per well in 96-well plates. After 24 h of incubation at 37 °C with 5% CO_2, the cells were first treated with different concentrations of the CAPE and D_2O (10, 20, 30, 40 and 50%) for 24 h. The absorbance was measured at 490 nm by an Agilent BioTek 800 TS microplate reader (Santa Clara, USA). Based on the results, 30% of D_2O was chosen for further experiments. The cells were treated with 5, 10, and 40 µM of CAPE for combination treatment, and 10 µM CAPE was selected for subsequent testing. Finally, alone and combined doses of 10 µM CAPE and 30% D_2O were applied to the cells. The dose was evaluated as cytotoxic if the cell viability (CV) was ≤70%, and noncytotoxic if CV was>70%. The CV values were statistically analyzed using one-way ANOVA with the Tukey Post Hoc test ($p < 0.05$) [18, 19].

The Induction of Apoptosis

The induction of apoptosis in the cell line (HCT-116) was conducted following the instructions of a BioLegend fluorescein-5-isothiocyanate (FITC) annexin V apoptosis detection kit. The cells were seeded in 6-well plates (1.5 × 10^5 cells/well) and incubated at 37 °C for 24 h. Followingly, 10 µM CAPE and 30% D_2O alone and in combination were added to the cells and allowed to incubate at 37 °C for 24 h. After that, the cells were harvested in the Falcon tube, and 500 µL of the staining buffer was added. The tubes were then centrifuged at 1300 rpm for 5 min. The supernatant was discarded. A volume of 200 µL binding buffer was added to the control group, and a volume of 100 µL to the treatment groups. The suspensions were transferred to the flow cytometry tubes, 3 µL of FITC dye was added to each tube, and the tubes were incubated in dark conditions for 15 min. Then, 3 µL of propidium iodide (PI) dye and 400 µL of binding buffer were pipetted into each tube. Finally, the flow cytometry analysis was performed using a Beckman Coulter flow cytometry instrument (Indianapolis, USA).

Determination of Gene Expression Level by qPCR

The expression levels of the tumour proliferative genes AKT, NF-κB, and CCND1, tumour suppressor genes *p16* (CDKN2A), *BAX*, and *TP53*, and *p21* (CDKN1A) in the regulation of apoptosis were quantitatively determined using a Bio-Rad real-time PCR (California, USA). The total RNA was isolated from the cells treated with 10 µM CAPE alone, 30% D_2O alone, and 10 µM CAPE and 30% D_2O in combination following the manufacturer's instructions of a PureLink RNA isolation kit. The isolated RNA was then converted to cDNA using the Applied Biosystems cDNA kit. The gene-specific primers were obtained from Exim Life Sciences (Istanbul, Türkiye) (Table 1). Finally, qPCR was performed using GeneMark SYBR qPCR Kit. Amplification was performed at 95 °C for 10s, 59 °C for 10s and 72 °C for 20s for 39 cycles, respectively. The sample was run in triplicate, and the relative expression of the gene was calculated using the comparative threshold cycle method (ΔΔCt method), normalized to the housekeeping gene (GAPDH).

Colony-Forming Ability (CFA)

The cells (HCT-116) were initially seeded at a density of 3×10^2 cells per well in 6-well plates and incubated for 24 h. Subsequently, the incubated cells were treated with

Table 1. Primer Sequences for Gene Expression Analysis

Primer*		Sequences	Tm (°C)
AKT	f	AGCGACGTGGCTATTGTGAA	51.78
	r	CACGTTGGTCCACATCCTG	53.25
NF-κB	f	AGCACAGATACCACCAAGAC	51.78
	r	TGGTCCCGTGAAATACACCT	51.78
CCND1	f	GCGGAGGAGAACAAACAGAT	55.00
	r	GAGGGCGGATTGGAAATGA	55.10
p16 (CDKN2A)	f	AATTAGACACCTGGGGCTTG	54.90
	r	AGGGCGATAGGGAGACTCAG	58.00
BAX	f	AAGAAGCTGAGCGAGTGTCT	55.45
	r	GTTCTGATCAGTTCCGGCAC	55.45
TP53	f	GCCCCTCCTCAGCATCTTAT	56.45
	r	AAAGCTGTTCCGTCCCAGTA	56.45
p21 (CDKN1A)	f	GCTTCATGCCAGCTACTTCC	51.80
	r	CCCTTCAAAGTGCCATCTGT	50.90
GAPDH	f	ACACCCACTCCTCCACCTTT	56.50
	r	TACTCCTTGGAGGCCATGTG	56.70

10 µM CAPE alone, 30% D_2O alone, 30% D_2O and 10 µM CAPE in combination. After that, the treated and untreated control samples were allowed to incubate again at 37 °C for 14 days with 5% CO_2. After the incubation and removal of the medium, the wells were washed with 500 µL PBS, and the cells were fixed with 500 µL of 100% methanol at 4 °C for 20 min, after which the methanol was discarded. Each well was stained with 500 µL crystal violet solution for 5 min, followed by washing with 500 µL distilled water for 2 min. Finally, the CFA was evaluated under an inverted light microscope [20].

Statistical Evaluation

The level of correlation between the gene expression levels of the focal genes in terms of 10 µM CAPE, in 30% D_2O, and 10 µM CAPE and 30% D_2O in combination treatments was statistically tested with Pearson Correlation Analysis using the SPSS 26.0 Software (SPSS Inc., Chicago, USA). A p level <0.05 was considered statistically significant.

Results

In vitro Cell Viability (MTS)

Figure 1 shows the in vitro dose-dependent cytotoxic effects of the regular water (H2O), D_2O, 10 µM CAPE and 30% D_2O on the CV (%) of the colon cancer cells (HCT-116) after a 24 h of incubation, respectively. The MTS assay demonstrated that a treatment with H_2O (50%) H_2O caused a significant toxicity in the cells due to medium deprivation (Figure 1A). At the same time, an exposure to D_2O (30 to 50%) significantly reduced the CV down < 70% (Figure 1B). The highest concentration of CAPE, with 40 µM, significantly exhibited a cytotoxic effect on the HCT-116 cells relative to the control group (Figure 1C), indicating potential safety for specific therapeutic applications ($p<0.05$). Accordingly, 10 µM CAPE and 30% D_2O in combination were selected as the optimal dose for subsequent in vitro assays.

The Induction of Apoptosis

The ability of CAPE (10 µM) and D_2O (30%) alone and in combination to induce apoptosis in HCT-116 cells was evaluated using an apoptosis detection testing kit (Figure 2). The results demonstrated that the combined 10 µM CAPE and 30% D_2O treatment at 24 h has the highest proportion of apoptotic cells (early + late apoptosis) (46.2 ± 8.2%), followed by 10 µM CAPE alone (39.9 ± 5.7%) and 30% D_2O (44.4 ± 8.0%) alone, compared to the control group with the lowest proportion (9.6 ± 7.6%), respectively ($p<0.05$).

Gene Expression Levels by qPCR

The expression levels of the 7 genes to the CAPE (10 µM) alone, D_2O (30%) alone and CAPE (10 µM) and D_2O (30%) in combination were determined using qPCR. The AKT is an important regulator of cell proliferation, whose expression was significantly downregulated in all treatment groups, especially in the combined dose, relative to the control ($p<0.05$). The remaining proliferative- and cell cycle–related genes, including NFKB and CCND1, were also significantly suppressed in the combined dose ($p<0.05$). In contrast, CAPE and D_2O alone did not significantly affect their expression levels ($p>0.05$).

The apoptosis-related genes showed distinct changes: *p16*, *BAX* and *TP53* were significantly upregulated in the combination group but remained unaffected by the individual treatments of CAPE and D_2O. The *TP53* expression was also elevated under the combined treatment. Interestingly, CAPE alone reduced *TP53* levels, which may explain the lack of cytotoxic effect observed in the CV assay. Finally, the *p21* expression remained unchanged under all treatment conditions, whether alone or in combination (Figure 3).

Colony-Forming Ability (CFA)

The CFA is a hallmark of many cancer cells, and one of the principal aims of chemotherapy is to inhibit this process. To assess the CFA of D_2O and CAPE, alone

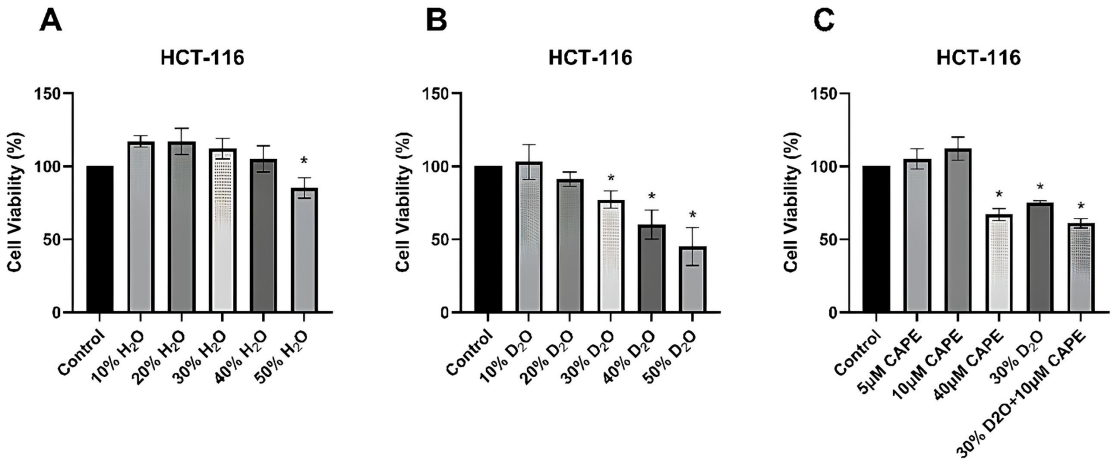

Figure 1. Cell Viability Results on HCT-116 Colon Cancer Cell Line to Alone and Combined Doses of CAPE and D_2O: (A) H_2O, (B) D_2O alone, and (C) CAPE alone and D2O in combination. One-way ANOVA with Tukey Post Hoc test (*$p<0.05$). A treatment with H_2O (50%) H_2O caused a significant toxicity on the cells due to medium deprivation (Figure 1A). At the same time, an exposure to D_2O (30 to 50%) significantly reduced the CV down < 70% (Figure 1B). The highest concentration of CAPE with 40 µM significantly exhibited a cytotoxic effect on the HCT-116 cells relative to the control group (Figure 1C), indicating potential safety for specific therapeutic applications ($p<0.05$)

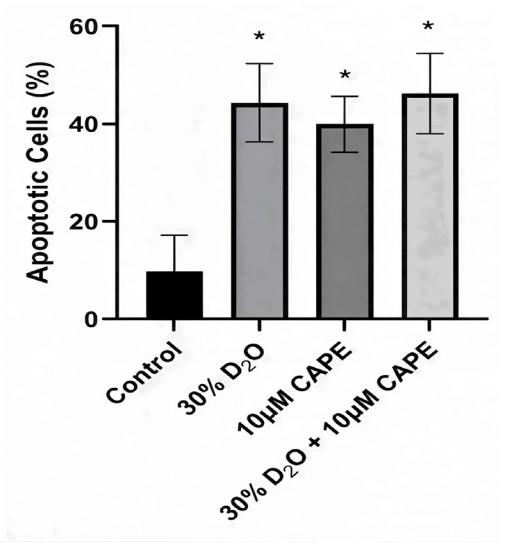

Figure 2. The Proportion of Apoptotic Cells (early + late apoptosis) in the HCT-116 Cells under Control and Different Treatment Conditions. The combined 10 μM CAPE and 30% D_2O treatment at 24 h has the highest proportion of apoptotic cells (early + late apoptosis) (46.2 ± 8.2%), followed by 10 μM CAPE alone (39.9 ± 5.7%) and 30% D_2O (44.4 ± 8.0%) alone, compared to the control group with the lowest proportion (9.6 ± 7.6%), respectively ($p<0.05$).

and in combination, a small number of HCT-116 cells were treated under each condition. Numerous large colonies were observed in the control group, whereas the exposure to D_2O or CAPE alone resulted in the formation of markedly smaller colonies. The most pronounced effect was seen in the combination group of CAPE and D_2O, in which colony formation was almost completely suppressed. These CFA results strongly support the synergistic cytotoxic effect of D_2O and CAPE, as demonstrated in the previous experiments (Figure 4).

Discussion

This study evaluated the synergistic anticancer potential of CAPE and D_2O in the human colon cancer cells using in vitro techniques. The results showed that CAPE and D_2O could be an effective adjuvant of chemotherapy for enhancing therapeutic efficacy in colon cancer, suggesting further research into their synergistic effects selectively in other malignancies, as well as their toxicokinetic potential in cancer treatment. In recent years, the therapeutic potential of bioactive natural compounds in colon cancer has been extensively investigated as promising candidates for anticancer treatment, with mainly positive outcomes [21]. These naturally occurring

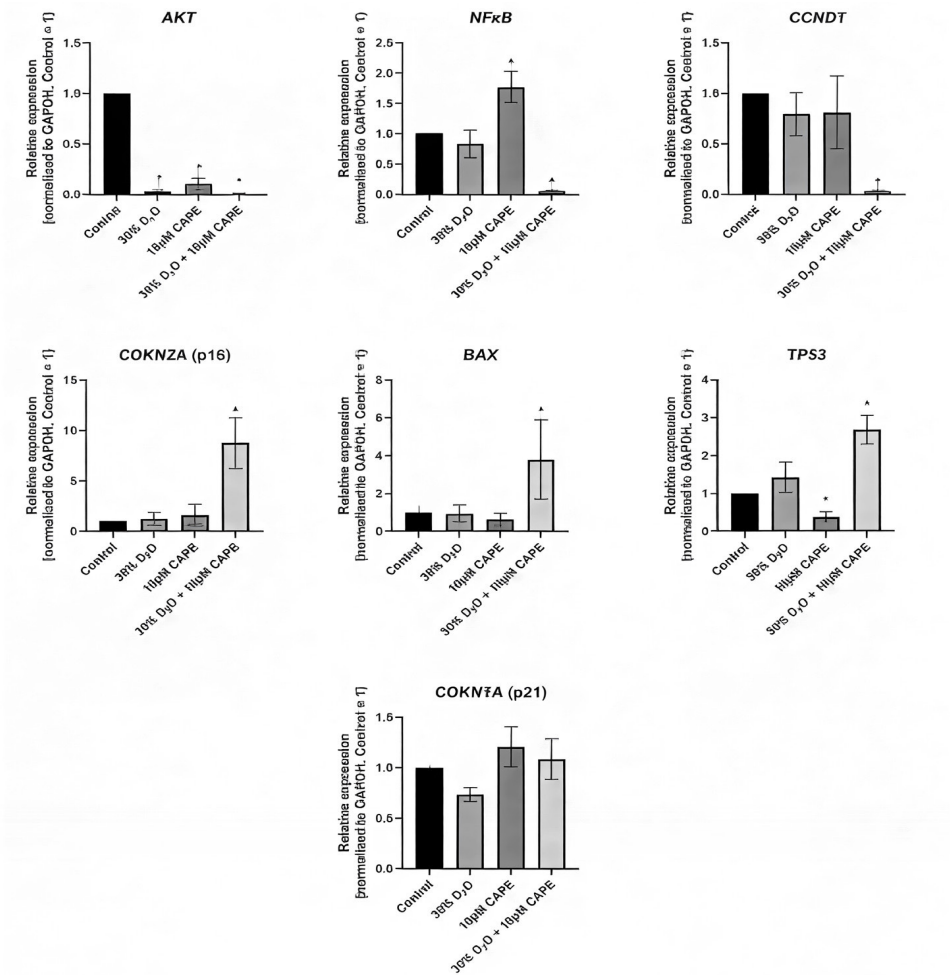

Figure 3. Gene Expression Profiles of HCT-116 Cells in Response to CAPE and D_2O, Administered Individually or together. AKT, NFKB, and CCND1 were significantly downregulated at the combined CAPE and D_2O dose relative to the control group. However, the apoptosis-related genes *p16, BAX*, and *TP53* were significantly upregulated in the combined treatment, indicating that CAPE alone lacked cytotoxicity. Finally, the *p21* expression remained unchanged under all treatment conditions.

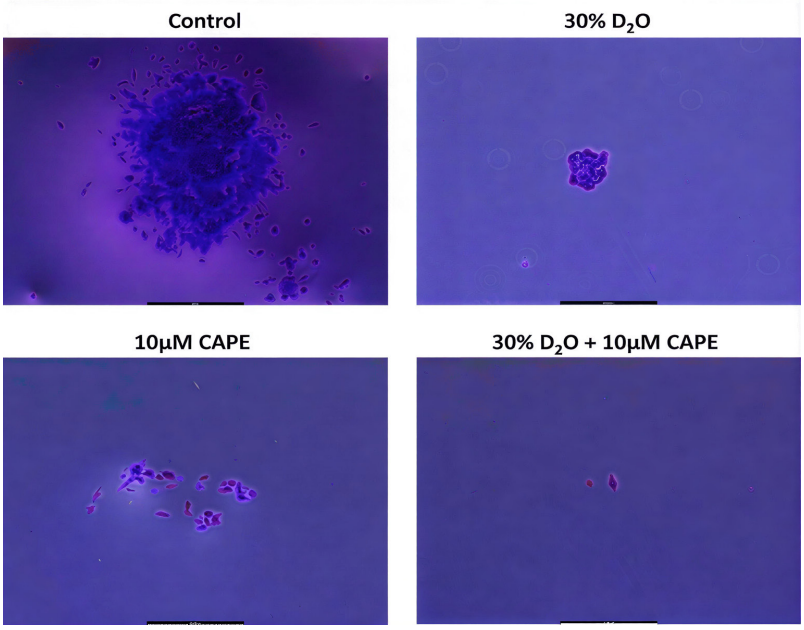

Figure 4. Colony-Forming Ability of HCT-116 Cells Following Treatment with CAPE and D_2O, Alone and in Combination. The most pronounced effect was observed in the combined CAPE and D_2O treatment, in which colony formation was almost completely suppressed.

substances have been reported to induce apoptosis, inhibit oxidative stress, cellular progression and signalling in cancer treatment, mitigate drug resistance, and minimize adverse effects associated with chemotherapy, especially in combination with other drugs [22, 23]. Amongst them, the CAPE from honeybee propolis has gained a significant attention with its antitumor and chemotherapy-sensitizing effect for inhibiting autophagy [24]. Besides, D_2O, also known as heavy water, has gained interest in pharmaceutical (i.e., therapeutic agent against cancer, deuterated drugs, antibiotics), biological (i.e., cell development, metabolism, tissue homeostasis, drug resistance, and ageing), and spectroscopic (i.e., mass spectrometry, nuclear magnetic resonance spectroscopy, Fourier transform infrared spectroscopy) applications [13]. Furthermore, it has also been reported to suppress hepatic, pancreatic, gastric and colon cancer cells [16]. However, the anticancer effect of CAPE and D_2O in combination has not been assessed as a novel therapeutic approach in colon cancer yet. With respect to this lack of evidence and gap in the literature, our study contributed to the literature, suggesting a novel perspective, potentially in combination with chemotherapeutics for anti-colon cancer treatment.

In this study, the MTS assay was used to study the cytotoxic effects of CAPE and D_2O alone and in combination within the colon cancer cell line (HCT-116), which is a highly tumour-aggressive and cell-invasive type, as previously emphasized by Zhou et al. [25]. Our cytotoxicity-based data showed that the co-administration of CAPE (10 µM) and D_2O (30%) significantly reduced the CV of HCT-116 cells, compared to the other treatment groups ($p<0.05$). In literature, some works reported the in vitro dose- and time dependent inhibitory effect of CAPE within HCT-116 cells: for instance, 2.5, 5 and 10 mg/L for 24 h [26], 47.2 µM for 48 h [27], 66.86 µM for 72 h [28], 0.5-500 µM for 72 h [29], 826.59 µM for 24 h [30], and 51.74 µM for 24 h [31], respectively. In a study, a 10 µM concentration of CAPE did not suppress HCT-116 cells for 24 h. It was therefore taken as the co-administration concentration, which precisely aligned with our optimal dose of CAPE for the further tests after the in vitro cytotoxic MTS assay. Regarding D_2O, some cancer types could not notably grow in a D_2O-rich medium [32]. For instance, Somlyai et al. suggested 90 ppm of D_2O on prostate cancer, followed by Cong et al. as 25 to 150 ppm on lung cancer, Yavari & Kooshesh as 40 to 100% on malignant melanoma cancer, and Kleemann et al. as 30-100 ppm on breast cancer during 24 to 72 h, respectively [16, 33-35], whereas a concentration of 40-150 ppm did not impose an inhibitory effect on HCT-116 cells, as previously reported by Kovács et al. [36]. However, a work determined the cytotoxic concentration of $D_2O \geq 30\%$ in Colo205 colonic cancer cells [37], which precisely aligns with our data. Overall, although most of the studies on the cytotoxic effect of D_2O within certain cancer types, including colon cancer, our experimental data demonstrated significant evidence, which appears to be overwhelmingly in support of D_2O's anti-proliferation activity. To our knowledge, this study has been a pioneering one within the investigation of CAPE and D_2O's therapeutic potential in colon cancer, providing a preliminary basis to better clarify the tentative mode of action in the induction of apoptosis.

This study used the flow cytometry analysis to determine the ability of the induction of apoptosis of CAPE and D_2O in the cancer cells (HCT-116), as the proportion of apoptotic cells (early + late apoptosis). The results revealed that the 10 µM CAPE and 30% D_2O in combination at 24 h has the highest proportion of apoptotic cells (46.2 ± 8.2%), followed by 10 µM CAPE alone (39.9 ± 5.7%) and 30% D_2O (44.4 ± 8.0%) alone,

compared to the control group (9.6 ± 7.6%), respectively ($p<0.05$). The apoptosis can be described as a phenomenon of "self-killing" [38]. The induction of apoptosis is another basic stage towards the success of cancer therapy, maintaining the balance between cell death and division, as well as controlling the proliferation and growth of cells, leading to cancer development [39]. In literature, some works reported that 10 μM of CAPE induced apoptosis in HCT-116 cells as 25.5% at 24 h, and 10-20% at 48 h, respectively [26, 27]. Nevertheless, in another study, 10 μM CAPE did not show an induced apoptosis in the HCT-116 cell line [31]. Similarly, in literature, a concentration of 30% D_2O raised the number of apoptotic cells to 22% in pancreatic cancer [40, 41]. Moreover, D_2O improved the synergistic anticancer effect of some chemotherapeutic agents such as 5-fluorouracil, bleomycin, methotrexate, and gemcitabine [42, 43]. In this study, the concentrations ≥ 30% alone significantly induced apoptosis in HCT-116 cells by 35.6%, while 10 μM CAPE and 30% D_2O in combination exhibited the highest apoptotic rate (40.4%), compared to the other treatment groups. Overall, we can note that despite many studies on CAPE and D_2O's biological and pharmacological activities in cancer cases [12], their synergistic effects have not been investigated yet. That is why our study suggests extending the research on CAPE and D_2O as synergistic enhancers in tumour cells' apoptosis modes, despite some discrepancies between our work and previously conducted studies.

In this study, the expression levels of the seven target genes in response to the doses of CAPE (10 μM) and D_2O (30%) alone and in combination were investigated using qPCR. The findings demonstrated that AKT, NFKB and CCND1 were significantly downregulated in the combined dose of CAPE and D_2O, relative to the control group ($p<0.05$). However, the apoptosis-related genes *p16*, *BAX* and *TP53* were significantly upregulated in the combined treatment, indicating the lack of cytotoxic effect of CAPE alone ($p<0.05$). Finally, the *p21* expression remained unchanged under all treatment conditions. Cancer cells proliferate rapidly because of inactivation or mutation of suppressor genes, as well as overexpression or amplification of oncogenes [44], playing a critical role in the progression of colon cancer [45]. In literature, some in vitro studies mainly focused on blocking AKT, NF-KB and CCND1 for preventing colon cancer [46, 47], aligning with our work. The findings obtained in literature revealed that CAPE could downregulate AKT, NF-KB and CCND1 [48, 49] and could upregulate *p16*, p53, *p21* and *BAX* [27]. With respect to D_2O, it could suppress AKT, NF-KB and CCND1 [50, 51]. However, there is no evidence on *p16*, p53, *p21* and *BAX* with D_2O. Overall, this study is noteworthy for providing scientific evidence and data on the tumour suppressive genes with D_2O, and the capability of both CAPE and D_2O in regulating the gene-mediated anti-apoptotic pathways in colon cancer cells.

This study performed the test of CFA to unravel the inhibitory effect of both CAPE and D_2O within the colon cancer cells (HCT-116). The findings indicated that the most pronounced effect was observed in the combined CAPE and D_2O treatment, in which colony formation was almost completely suppressed. The CFA is a suitable technique for assessing the toxicity of chemicals [52]. In the literature, there is insufficient evidence about the CFA of CAPE and D_2O in colon cancer cells. However, several works demonstrated that CAPE alone significantly suppressed the CFA of colon adenocarcinomas by 47.8% at 10, 25, 50 and 100 μM concentrations, as noted by Ozturk et al. and Wu et al. [53, 54], matching our CAPE concentration (10 μM) alone. However, this study emphasized the co-treatment of CAPE and D_2O with a significant visible suppressive effect on the colon cancer cells to survive and form colonies.

In conclusion, to the best of our knowledge, this is the first study to evaluate the combined effects of CAPE and D_2O in colon cancer, indicating potentially an effective therapeutic adjuvant in colon cancer. Our data suggest further clinical research on whether their synergistic effects are also selective in other malignancies, as well as with their underlying mechanisms of action, with toxico-kinetic effects, to obtain specific clinically relevant conclusions.

Author Contribution Statement

GD, FB and ÖB performed the experiments and collected the data; LM and SA analyzed and interpreted the results; IHT and HA conceptualized and designed the study, prepared the initial manuscript, reviewed and approved the final version of manuscript.

Acknowledgements

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Declaration

Not applicable because this study does not involve experiments on animals or human subject.

Conflict of Interest

None to declare.

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