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Caffeic Acid's Influence on the Viability and Apoptosis of a Diverse Array of Cancer Cell Lines

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Abstract − Natural phytochemicals, such as caffeic acid (CA), are emerging as promising candidates for cancer therapy due to their bioactive properties. This study investigated the cytotoxic potential of CA across ten cancer cell lines—HuH7 (hepatocellular carcinoma), PC3 (prostate adenocarcinoma), HeLa (cervical carcinoma), CaCo2 and HT29 (colorectal adenocarcinoma), SW48 (colon carcinoma), MG63 (osteosarcoma), A673 (Ewing's sarcoma), 2A3 (pharyngeal squamous cell carcinoma), and CARM-L12-TG3 (lung carcinoma)—alongside the healthy HMC3 (microglial) cell line. Cell viability was evaluated via MTT assays, while apoptosis induction and cell cycle modulation in CA-treated CaCo2 cells were analyzed using flow cytometry. Results revealed heightened sensitivity to CA in HT29, 2A3, and A673 cells, which exhibited reduced viability at lower concentrations than other cancer and healthy cell lines. CA induced apoptosis and inhibited proliferation in CaCo2 colorectal cells, with pronounced effects observed in cancer types directly exposed to dietary components (e.g., colon and pharynx) and bone-related malignancies (Ewing's sarcoma and osteosarcoma). This study provides novel insights into CA's efficacy against less-studied cancers, such as pharyngeal squamous cell carcinoma (2A3) and Ewing's sarcoma (A673). These findings underscore CA's potential as a targeted cytotoxic agent, particularly for diet-associated and bone cancers. Further research is warranted to elucidate its mechanisms, optimize therapeutic applications, and validate safety and efficacy in preclinical and clinical settings, positioning CA as a viable candidate for preventive and adjunctive cancer therapies.

Keywords − *Caffeic acid, cancer cell lines, cytotoxicity, apoptosis, phytochemicals*

1. Introduction

Cancer persists as a leading cause of global mortality, driving the urgent need for novel therapeutic strategies that circumvent the adverse effects of conventional treatments like chemotherapy and radiotherapy. In recent years, the interplay between diet and cancer pathogenesis has emerged as a critical area of investigation, with phytochemicals—naturally occurring bioactive compounds in plant-based foods—garnering attention for their potential to mitigate cancer risk and progression [1-4]. Among these compounds, phenolic acids, particularly caffeic acid (CA), have demonstrated significant biological activity, including anti-inflammatory, immunomodulatory, and anticancer properties [5-8]. CA, a hydroxycinnamic acid derivative, is ubiquitously distributed in plant tissues (e.g., leaves, seeds, and fruits) and is notably abundant in coffee, one of the world's

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most consumed beverages [9-11]. Its esterified forms are present in cereals such as barley, rice, oats, and herbs like oregano and sage [2,9]. Epidemiological studies associate moderate coffee consumption with reduced cancer incidence and mortality, particularly in postmenopausal women, underscoring CA's potential role in these protective effects [12-16]. While 95% of ingested CA is absorbed in the small intestine, its bioavailability and bioactivity depend on factors such as dietary source, brewing methods, and intestinal metabolism [11,17]. Beyond its antioxidative effects, CA has been shown to modulate DNA methylation, reduce genotoxicity, and inhibit tumorigenic pathways, positioning it as a multifaceted candidate for cancer therapy [13,18,19].

Despite these advances, gaps persist in understanding CA's cell-type-specific cytotoxicity, particularly in cancers affecting anatomical regions directly exposed to dietary components (e.g., colon, pharynx) and bone-related malignancies. To address this, we evaluated CA's cytotoxic effects across ten cancer cell lines representing diverse malignancies—including colorectal adenocarcinoma (CaCo2, HT29), pharyngeal squamous cell carcinoma (2A3), Ewing's sarcoma (A673), and others—alongside healthy HMC3 microglial cells. Using MTT assays, we determined IC50 values (the concentration required for 50% growth inhibition) and further investigated CA's mechanism of action via apoptosis and cell cycle analysis in CaCo2 cells.

2. Materials

2.1. Chemicals, Reagents, and Kits

Caffeic acid (CA) was purchased from Sigma Aldrich (#C0625). CA was dissolved in ethanol (50 mg/ml) and then diluted with the medium. DMEM (Dulbecco's modified Eagle's medium) (#DMEM-HA), RPMI 1640 (Roswell Park Memorial Institute) (#RPMI-A) medium, and Dulbecco's phosphate-buffered saline (dPBS) (#PBS-1A) were purchased from Capricorn Scientific. 3-(4,5-Dimethylthiazol-2)-yl)-2,5-diphenyl (MTT) (#M2128) and Trypan blue (#T8154-20ML) were purchased from Sigma. Penicillin and streptomycin (#PS-B), as well as fetal bovine serum (FBS) (#10500-064), were purchased from GIBCO. Trypsin–EDTA (#P10-019100) and sodium pyruvate (#P04-43100) were purchased from PANbiotech. Dimethyl sulfoxide (DMSO) (#1264ML500) was purchased from Neofroxx GmbH. The Annexin V apoptosis detection kit with 7-AAD (#30060) was purchased from Biotium.

2.2. Cancer Cell Lines and Culture Conditions

In vitro cell culture studies used a laminar flow cabinet from Nüve (Nüve, Turkiye). Ten cancer cell lines and one healthy cell line were tested. Human liver hepatocellular carcinoma cells (HuH7) were obtained from Thermo Fisher Scientific. Human prostate cancer cells (PC3 #CRL-1435), human cervical carcinoma cells (HeLa #CRM-CCL-2), colorectal adenocarcinoma cells (Caco2 #HTB-37™; HT29 #HTB-38; SW48 #CCL-231), osteosarcoma cells (MG63 #CRL-1427™), Ewing's sarcoma cells (A673, #CRL-1598), pharynx carcinoma squamous cells (2A3 #CRL-3212), and microglial health cells (HMC3 CRL-3304™), and malignant mesothelioma cells (CARM-L12-TG3) were obtained from ATCC® (American Type Culture Collection, Manassas, VA, USA). Complete DMEM (cDMEM, 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% sodium pyruvate) was used to cultivate all cell lines. The environment was humidified with 5% CO2 and maintained at 37°C for the cells.

3. Methods

3.1. Determination of Cytotoxicity by MTT Assay

CA was dissolved in ethanol before use. In 96-well plates, cancer cells were seeded at a density of 4,000 cells per well. The cells were incubated for 48 and 72 hours, respectively, in media with a higher CA concentration following a 24-hour initial incubation. Additionally, cells were cultured in media containing caffeic acid dissolved in ethanol and increasing amounts of doxorubicin for control. Each well was incubated for four hours

with an MTT solution (0.5 mg/ml) added after 48 and 72 hours at 37°C. Due to the reaction, the viable cell mitochondrial dehydrogenases reduced MTT to a purple formazan product. The quantity of MTT formazan product was determined by measuring its absorbance at 570 nm using a microplate reader after dissolving it in DMSO.

3.2. Flow Cytometry Analysis

The cells intended for apoptosis analysis by flow cytometry were seeded into 24-well tissue culture plates. Following a 24-hour incubation period, the culture media were aspirated and replaced with fresh media containing CA. The cells were then allowed to incubate for an additional 48 hours. For analysis, cells were detached from the plate by trypsinization and harvested. In accordance with the manufacturer's guidelines for the apoptosis kit, the cells were centrifuged to separate them from the medium and subsequently stained with FITC Annexin V and 7-AAD. Flow cytometry was used to examine at least 30.000 cells immediately following staining for each measurement.

3.3. Statistical Analysis

Cytotoxicity experiments were conducted in triplicate, and the IC50 values were determined using the GraphPad Prism software program (GraphPad Software, San Diego, USA). The mean ± standard deviations (SD) of the experiments were determined. To assess significant differences among the IC50 values of the cells, a one-way analysis of variance (ANOVA) was performed, with statistical significance defined at $p < 0.05$.

4. Results

4.1. Cytotoxic Effects of Caffeic Acid (CA) Across Cell Lines

Cell viability dose-response curves for PC3 (Figure 1A), HeLa (1B), HuH7 (1C), CaCo2 (1D), MG63 (1E), HT29 (1F), A673 (1G), SW48 (1H), 2A3 (1I), CARM-L12-TG3 (1J), and healthy HMC3 (1K) cells treated with caffeic acid (CA) for 48 and 72 hours are shown in Figure 1. Curves were generated using GraphPad Prism to determine IC50 values across the tested cell lines.

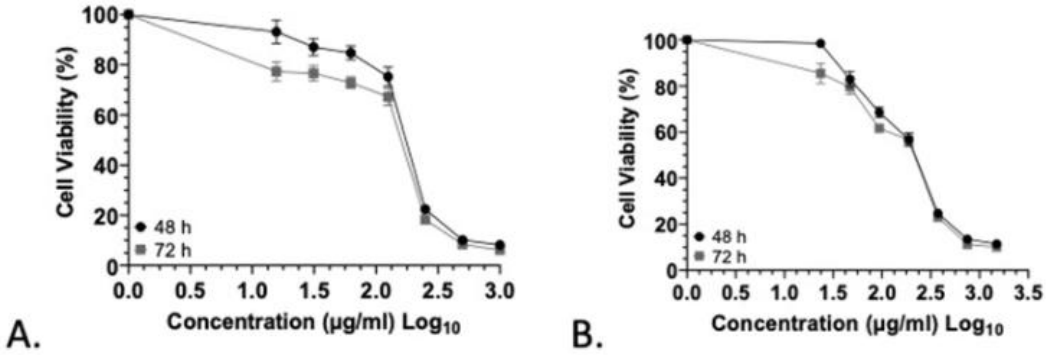

Figure 1. Dose- and time-dependent effects of CA on cell viability. A) Viability percentages of PC3 cells, B) Hela cells – exposed to different CA concentrations ranging from 23.44-1500 µg/ml (0 - 3.5 Log10) for 48 hours and 72 hours

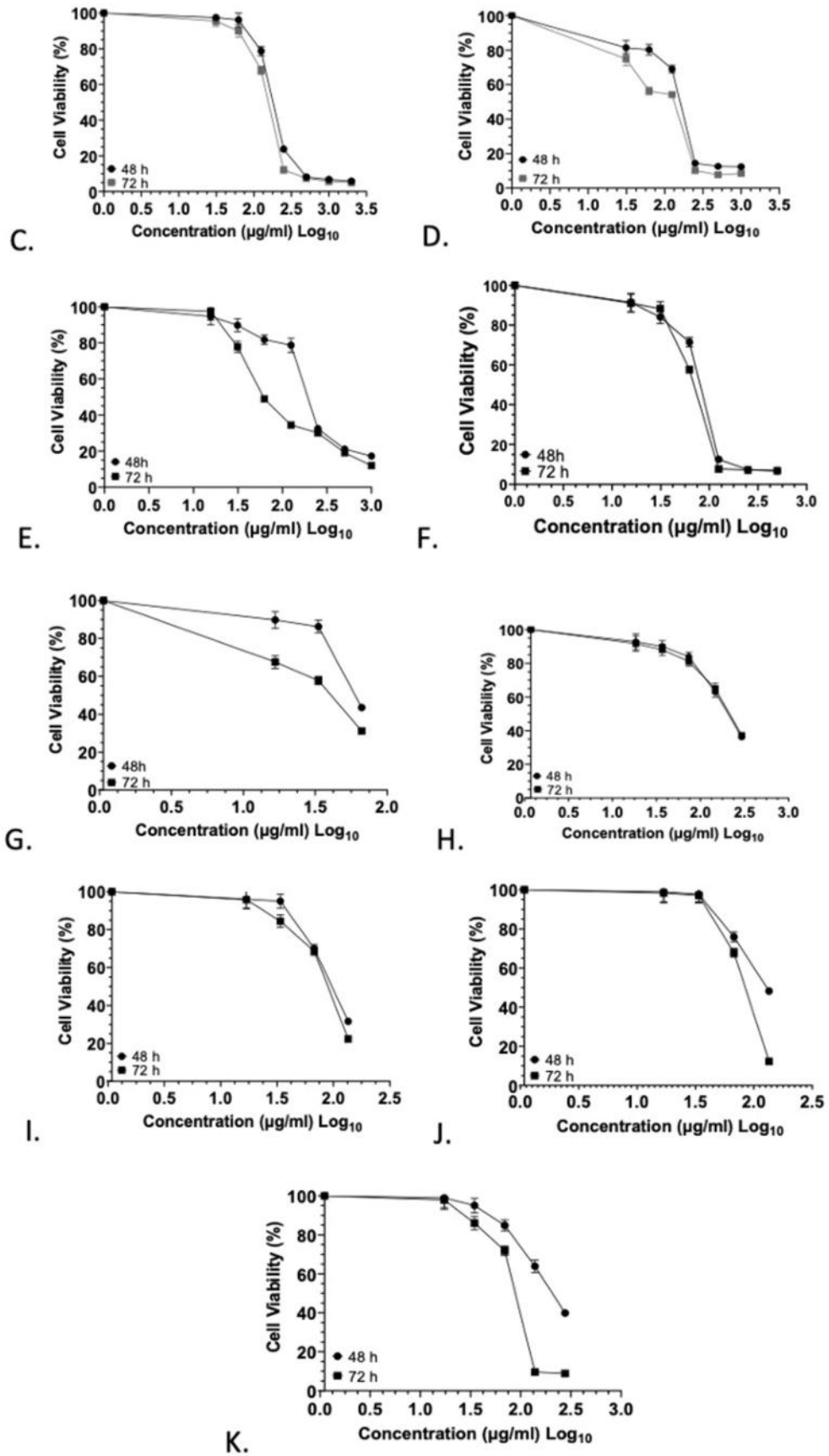

Figure 1. (Continued) Dose- and time-dependent effects of CA on cell viability. C) HUH7 cells, D) CaCo2 cells, E) MG63 cells, F) HT29 cells, G) A673 cells, H) SW48 cells, I) 2A3 cells, J) CARM-L12-TG3, K) HMC3 health cells – exposed to different CA concentrations ranging from 23.44-1500 µg/ml (0 - 3.5 Log10) for 48 hours and 72 hours

IC_{50} values were calculated for each cancer cell line at specified treatment durations, ranked by sensitivity, and compiled in Table 1.

Table 1. IC_{50} values of caffeic acid (CA) on different cell lines

Tissue Sources	Cell Lines	IC_{50} value ± SD (mg/ml)	
		48 h	72 h
Ewings sarcoma	A673	59.21 ± 0.71	45.36 ± 1.07
Colorectal adenocarcinoma	HT29	81.05 ± 4.92	67.63 ± 4.10
Pharynx carcinoma	2A3	93.52 ± 1.66	88.64 ± 1.19
Malign mesothelioma	CARM-L12-TG3	120.16 ± 3.36	82.53 ± 0.86
Osteosarcoma	MG63	215.02 ± 13.92	75.86 ± 4.72
Colorectal adenocarcinoma	CaCo2	152.24 ± 10.38	97.19 ± 5.92
Liver hepatocellular carcinoma	HUH7	177.06 ± 9.12	141.17 ± 5.96
Prostate cancer	PC3	188.35 ± 11.17	150.25 ± 12.34
Colorectal adenocarcinoma	SW48	183.26 ± 8.56	193.71 ± 8.39
Cervical cancer	HeLA	195.4 ± 17.22	176.65 ± 16.12
Health microglial cell	HMC3	185.98 ± 10.52	77.52 ± 2.35

Cancer cells were treated with various concentrations (23.3 – 1500 µg/ml) of CA and assessed for cell viability using the MTT assay at 48 and 72 hours. The IC50 values were calculated using GraphPad Prism 5 software. The IC_{50} values for CA against each cell line represent the means of three independent experiments (n=3) and are presented as mean ± SD.

Significant decreases in cell viability were observed in the HT29, A673, 2A3, and CARM-L12-TG3 cell lines with increasing CA concentrations. When these cell lines are compared with the healthy cell line HMC3, it is evident that the CA concentration, which does not affect the healthy cell line, exerts a cytotoxic effect on these cancer cell lines (Figure 2).

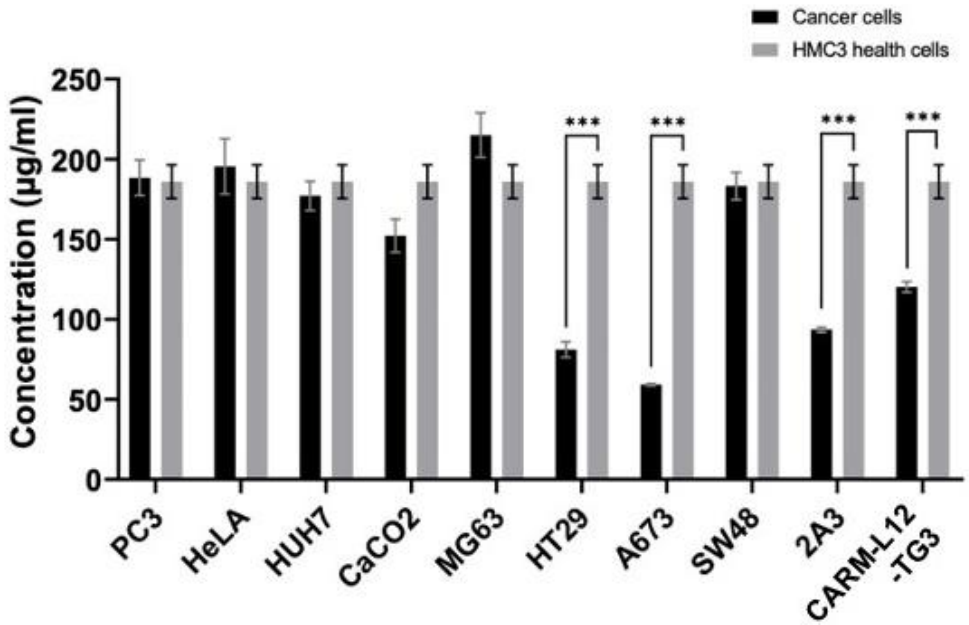

Figure 2. Comparative analysis of CA cytotoxicity in cancer versus healthy cells. IC50 values of caffeic acid (CA) across cancer cell lines (PC3, HeLa, HUH7, CaCo2, MG63, HT29, A673, SW48, 2A3, CARM-L12-TG3) and the healthy HMC3 cell line are indicated by graphical representation with statistically significant differences in IC50 (***$p < 0.05$).

4.2. CA Induces Apoptosis in CaCo2 Colorectal Cancer Cells

We evaluated the early apoptotic, late apoptotic, and necrotic effects of caffeic acid (CA) using flow cytometry after staining with Annexin V and 7-AAD. Annexin V staining was observed in both early and late stages of apoptosis, while 7-AAD staining was present only in cells at the late apoptosis stage. This differentiation allowed us to distinguish late apoptotic or necrotic cells from those in early apoptosis. Flow cytometry analysis represented early apoptotic cells as positive for Annexin V and negative for 7-AAD in the lower right quadrant, while late apoptotic cells were positive for both Annexin V and 7-AAD in the upper right quadrant. As depicted in Figure 3, negative area (Figure 3A), the control group without CA exhibited 14.6%±1.8% for early apoptosis and 1.3%±0.3% for late apoptosis (Figure 3B), following treatment with 125 µg/ml of caffeic acid (CA) for 48 hours, the percentage of early and late apoptotic CaCo2 cells was 37.0%±3.8% and 32.8%±2.9%, respectively (Figure 3C). When treated with doxorubicin for 48 hours and used as a drug control, the percentage of early and late apoptotic CaCo2 cells was 26.9%±2.6% and 29.9%±2.1% (Figure 3D).

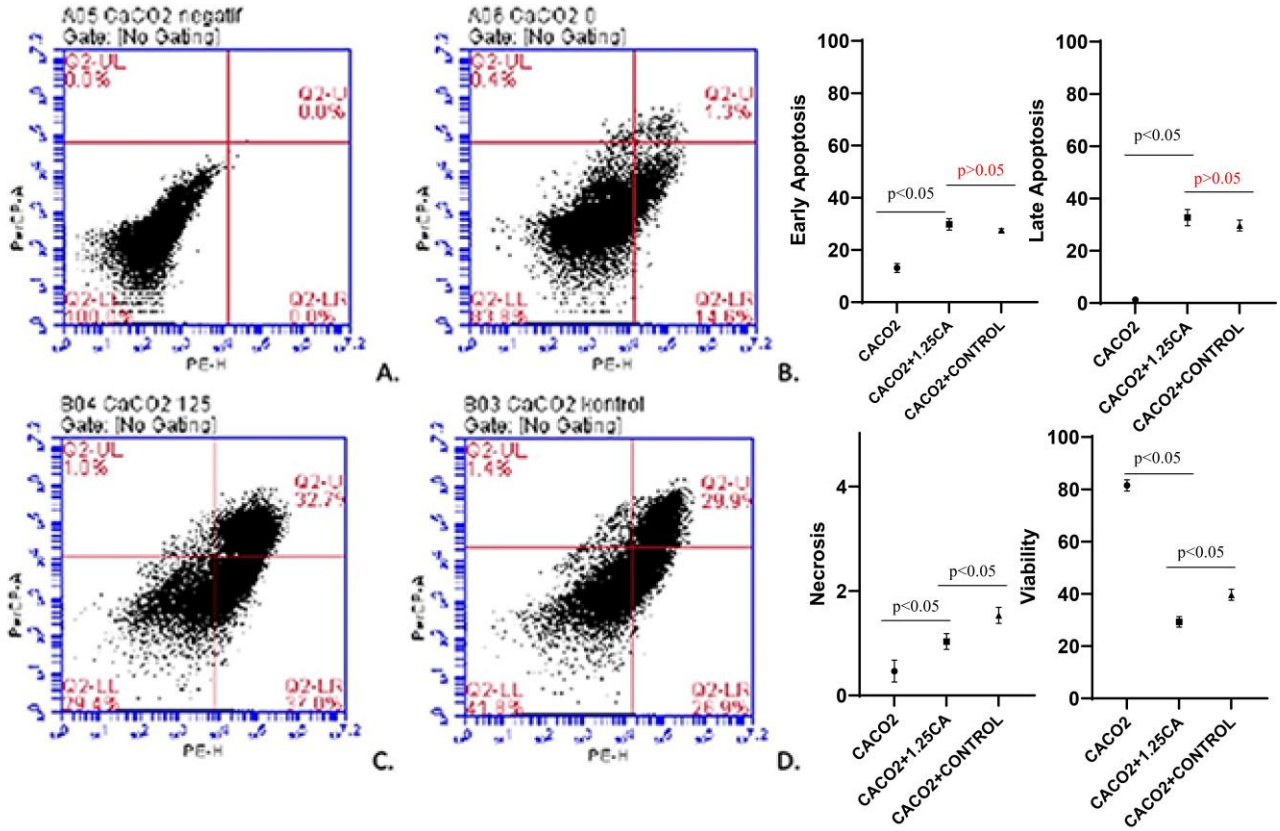

Figure 3. Flow cytometry apoptosis assay following Annexin V fluorescein isothiocyanate/7-AAD staining. CaCo2 cells were treated with CA (125 µg/ml) (C), doxorubicin (drug control) (D), without CA (B) for 48 h, and negative area (A). Representative scatter plots of 7-AAD (y-axis) vs. Annexin V (x-axis)

Statistical analysis revealed that untreated CaCo2 cells exhibited significantly higher cell viability compared to cells treated with 125 µg/mL caffeic acid (CA) or doxorubicin (positive control) ($p<0.05$). Furthermore, untreated cells displayed elevated necrosis levels relative to CA- and doxorubicin-treated groups ($p< 0.05$). CA and doxorubicin treatments robustly induced apoptosis: early and late apoptosis rates were significantly higher in treated cells than in untreated controls ($p<0.05$). Notably, CA-treated cells showed greater necrosis ($p<0.05$) but lower overall viability ($p<0.05$) compared to doxorubicin-treated cells, underscoring CA's potent cytotoxic activity.

5. Discussion

Cancer remains one of the leading causes of mortality worldwide, despite significant advances in medical science. Current research is focused on discovering novel compounds with enhanced therapeutic efficacy and reduced side effects compared to conventional treatments [20-23]. Caffeic acid (CA), a naturally occurring polyphenol found in various dietary sources, has garnered attention due to its potential anticancer properties. In our study, we evaluated the cytotoxic effects of CA on 10 different cancer cell lines representing seven distinct cancer types.

Caffeic acid is a prominent bioactive component of coffee, one of the most widely consumed beverages worldwide. Coffee is a pharmacologically active beverage nearly ubiquitous in daily life [24]. The influence of coffee consumption on health has been the subject of extensive research. Coffee's impact on human health has been extensively studied, with evidence suggesting both beneficial and adverse effects. The majority of these studies have indicated that coffee consumption has a beneficial impact on various aspects of health [25,26]. Recent meta-analyses have reinforced the potential protective effects of coffee consumption against various cancers, including colorectal and liver cancer, due to its antioxidant and anti-inflammatory properties [27] and metabolic disturbances [28,29]. The presence of bioactive compounds such as CA in coffee has been proposed as one of the contributing factors to these effects.

In our study, we analysed the cytotoxic impact of CA on multiple cancer cell lines and compared these findings to a healthy cell line (HMC3). The HT29, A673, 2A3, and CARM-L12-TG3 cell lines exhibited statistically significant differences in viability compared to the healthy cell line (Table 2). In particular, CA demonstrated a pronounced effect on the HT29 colon cancer cell line, reducing its viability by 50% at a concentration of 81.05 µg/ml, which did not adversely affect the healthy cell line. This finding aligns with recent research highlighting the anticancer potential of CA and other phenolic acids in colorectal cancer models [21, 30-32].

Table 2. Listing significant changes by statistically comparing the IC50 values of cancer cell lines versus healthy cell lines against caffeic acid (CA)

	Significant Differences	***P* value**	**Mean of Group A**	**Mean of Group B**	**Difference**	**SE of difference**	**t ratio**	**df**	**q value**
PC3	No	0.803	188.30	186.00	2.367	8.860	0.267	4	0.48635
HeLA	No	0.464	195.40	186.00	9.426	11.650	0.809	4	0.35133
HUH7	No	0.330	177.10	186.00	-8.917	8.040	1.109	4	0.28534
CaCo2	No	0.017	152.20	186.00	-33.740	8.531	3.955	4	0.02029
MG63	No	0.045	215.00	186.00	29.040	10.070	2.883	4	0.04533
HT29	Yes	0.000	81.05	186.00	-104.900	6.704	15.650	4	0.00023
A673	Yes	0.000	59.21	186.00	-126.800	6.087	20.820	4	0.00019
SW48	No	0.746	183.30	186.00	-2.722	7.829	0.348	4	0.48635
2A3	Yes	0.000	93.52	186.00	-92.450	6.149	15.040	4	0.00023
CARM-L12-TG3	Yes	0.000	120.20	186.00	-65.810	6.376	10.320	4	0.00075

Their digestive systems come into direct contact with food components. Therefore, it can be expected that cells in these systems would be the most affected by changes in diet. Colon cancer cells are directly influenced by dietary components, making them particularly susceptible to bioactive compounds. Prior studies have demonstrated that phenolic acids such as 3-O-methylgallic, gallic, p-coumaric, and ferulic acid can reduce cell viability, induce apoptosis, and regulate cell cycle progression in colon cancer models [33-36]. Our results corroborate these findings, reinforcing that dietary polyphenols like CA may exert chemopreventive effects by modulating key cellular pathways. In this study, we observed a similar effect on colon cancer cell lines. Specifically, we evaluated and compared three different colon cancer cell types in our research. Among these

three colon cancer cell lines, it was found that CA had the most significant impact on the HT29 cell line. At a CA concentration of 81.05 µg/ml, which did not affect the HMC3 healthy cell line, the viability of the HT29 colon cancer cell line decreased by 50% (Figure 4).

Beyond the digestive tract, bioactive food components interact with cells in the oral cavity, tongue, and esophagus. Syringic acid, another phenolic compound, has been reported to induce cell cycle arrest and promote apoptosis in cervical, tongue, and mouth carcinomas [9, 37]. Consistent with these observations, our study identified the 2A3 pharynx cancer cell line as one of the most responsive to CA treatment, suggesting a potential therapeutic application in upper gastrointestinal cancers (Figure 2).

Additionally, we found that the Human Ewing's sarcoma A673 cell line exhibited the greatest sensitivity to CA exposure, with IC50 values of 59.21 ± 0.7 mg/ml at 48 hours and 45.36 ± 1.07 mg/ml at 72 hours (Figure 2). These findings expand on prior research indicating that phenolic compounds, including CA, may disrupt sarcoma cell proliferation via oxidative stress modulation and apoptosis induction [37,38].

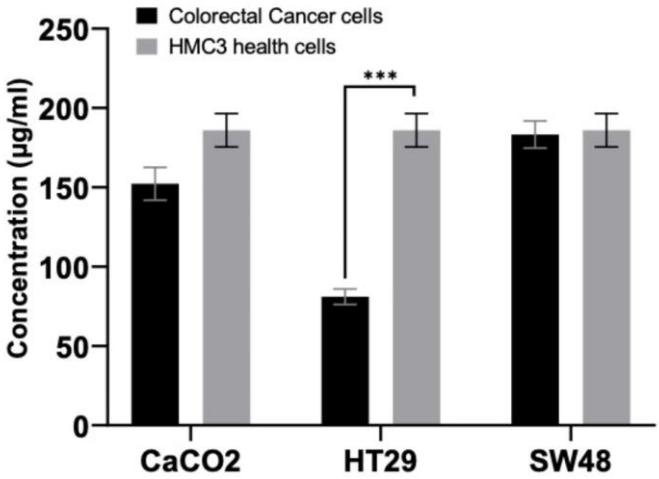

Figure 4. Comparison of CaCo2, HT29, SW48 IC50 caffeic acid (CA)concentrations of colon cancer cells and IC50 caffeic acid (CA) concentrations of HMC3 healthy cell line.

Lastly, diet plays a pivotal role in bone health, with studies indicating that coffee consumption may influence bone mineral density. Hirata et al. [39,40] previously reported positive effects of coffee intake on lumbar spine bone density . Recent investigations [41,42] have further elucidated the relationship between polyphenol-rich diets and osteoporosis risk reduction, emphasizing the potential osteoprotective effects of CA and other coffee-derived compounds .

Overall, our study contributes to the growing body of evidence supporting the anticancer properties of CA. However, further research is warranted to elucidate its precise mechanisms of action and to determine its potential for clinical applications. Future studies should focus on in vivo models and combinatorial approaches with existing chemotherapeutic agents to enhance therapeutic efficacy while minimizing toxicity.

6. Conclusion

Caffeic acid (CA) demonstrated significant cytotoxic effects on various cancer cell lines, particularly those associated with body regions directly exposed to bioactive food components, such as the colon, pharynx, and bone. CA exhibited selective cytotoxicity toward cancer cells while sparing healthy cells, suggesting its potential as a targeted treatment for specific cancer types. Different cancer cell lines displayed varying sensitivities to CA, with some cell lines, like HT29, A673, 2A3, and CARM-L12-TG3, being more affected by CA concentrations that did not impact healthy cells. This study underscores the potential of phytochemicals, such as CA, found in commonly consumed foods and coffee, as natural and effective treatments for certain cancers. It suggests that dietary components may play a role in cancer prevention and treatment. The paper

compiled by Pavlíková in 2023 highlighted studies demonstrating that caffeic acid possesses anticancer properties across various cancer types [43]. The review reveals that the mechanisms of action of CA vary depending on the cancer type, emphasizing the need for further research to fully understand its effectiveness and safety in cancer prevention and treatment. Our study also focused on cancer cell lines that had not been previously explored in the literature, offering new insights into how caffeic acid affects cancer cells in anatomical regions directly influenced by its bioactive properties. The study emphasizes the need for additional research to investigate the underlying mechanisms of CA's cytotoxic effects, its potential as a complementary therapy, and its safety and efficacy in clinical applications. Future studies should consider incorporating additional experiments, such as survival assays and wound healing assays, to further validate the findings and enhance the robustness of the study. These supplementary analyses could provide deeper insights into the underlying mechanisms and strengthen the overall conclusions. Overall, the findings suggest that CA holds promise as a cytotoxic agent against specific cancer types and warrants further investigation as a potential therapeutic option in cancer treatment.

Author Contributions

The author read and approved the final version of the paper. They all read and approved the final version of the paper.

Conflict of Interest

All the authors declare no conflict of interest.

Ethical Review and Approval

No approval from the Board of Ethics is required.

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