

RESEARCH ARTICLE

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Synergistic Chemopreventive Effects of Curcumin, Gingerol, and Shogaol on HeLa Cervical Cancer Cells

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Abstract

Objective: Bioactive compounds including curcumin, gingerols, and shogaols possess strong anti-inflammatory and anti-tumour properties. However, their combined efficacy has yet to be fully explored. This study aimed to investigate the chemopreventive potential of individual and combined treatments of 6-/10-gingerols (6-/10-G), 6-/10-shogaols (6-/10-S), and curcumin (Cur) in HeLa cervical cancer cells. **Methods:** HeLa cell viability was evaluated using the MTT assay following a 24-hour treatment with a wide range of concentrations and ratios of the bioactive compounds. The combination index was analysed using the Chou-Talalay method, and apoptotic cells were assessed via flow cytometry with Annexin V/FITC-PI staining. **Results:** We found that the optimal formulation of Cur in combination with all ginger bioactive compounds demonstrated synergistic inhibition of HeLa cancer cell growth at a 3:1 ratio of Cur to ginger bioactive compounds. Furthermore, treatment with the Cur:6-G (45µM: 40µM) and Cur:10-S (45µM: 20µM) combination formulations at a ratio of 3:1 significantly ($p < 0.005$) inhibited cell growth (72-77%) and induced apoptosis (60-87%). These findings highlight the potent anticancer properties of the combined formulations on HeLa cells, surpassing the efficacy of individual compounds which require relatively high concentrations. **Conclusion:** The combination of Cur and ginger bioactive compounds enhances anti-proliferative effects and induces apoptosis in HeLa cell lines.

Keywords: turmeric- ginger- cervical cancer- HeLa- chemopreventive effect

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Introduction

Cervical cancer is one of the most prevalent cancers worldwide, with an estimated 4,310 deaths in 2023 [1]. The incidence and mortality rates of cervical cancer remain high in low- and middle-income countries due to the limited resources allocated to cancer prevention and control [2]. Countries reporting significant health concerns related to cervical cancer, with more than 1,000 cases annually, include Latin America [3], India [4], Indonesia [5], and Malaysia [6]. Notably, infections with high-risk human papillomavirus (HPV) subtype 18 are responsible for 12.6 to 25.7% of cervical cancer cases worldwide [7]. HPV subtype 18 is present in HeLa cell lines and is commonly used in cervical cancer research to study the mechanisms of the disease and to develop potential treatments [8].

The current treatment for cervical cancer typically involves a combination of different approaches, including surgery, radiation therapy, chemotherapy, and targeted therapy. Various side effects such as fatigue, nausea, diarrhea, and memory impairment have been associated with cancer chemotherapy drugs, affecting patients' overall quality of life [9]. Among the commonly employed chemotherapy drugs for the treatment of cervical cancer are cisplatin, paclitaxel, 5-fluorouracil, and vinblastine [10]. Notably that drug resistance has become a significant challenge in cancer treatment. The high cost of treatment with chemotherapy drugs also warrants safer, more effective, and lower-cost natural chemopreventive agents [11].

Owing to their anticancer properties, plants play a pivotal role in the treatment of cancer [12-14]. Over 60% of currently used anticancer agents are derived from

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natural plants, including pepper, ginger, turmeric, cumin, and clove [13, 14]. The high potency of polyphenols in these plants have chemopreventive effects by modulating different biological processes of cancer, including cell cycle progression, inflammation, apoptosis, and cell invasion [11]. Curcumin, resveratrol, quercetin, apigenin, gingerol, and shogaol are examples of bioactive compounds that demonstrate anticancer effects by inducing cell apoptosis, inhibiting cell proliferation, and exhibiting anti-angiogenic and anti-metastatic properties [15-17]. Curcumin (Cur) has shown promising results in the treatment of several types of cancer [18]. It exhibited cytotoxicity against non-small cell lung cancer (NSCLC) and the breast cancer cell lines MCF7 and MDA-MB-231 by inhibiting cell proliferation and promoting apoptosis [19, 20]. Cur has also been shown to suppress the expression of the viral oncogenes E6 and E7 in cervical cancer cell lines, including HeLa, SiHa, CaSki, C33A and SW756, thereby affecting the development and progression of cervical cancer [21].

In addition to Cur, gingerols and shogaols are bioactive compounds that have been reported to have anticancer properties. They have been shown to modulate multiple signaling pathways. For example, 6-gingerol (6-G) inhibited SW-48 and HCT116 colon cancer cells by modulating MAPK signaling pathways without affecting normal colon cells [22]. Moreover, 6-shogaol (6-S) and 10-shogaol (10-S) exhibited anticancer effects by inducing apoptosis in colon cancer cells (HCT-116 and SW480) [23], lung carcinoma cells (A549) [24], and breast cancer cells (MCF-7 and T47D) [25]. Studies using HeLa cells have also reported that 6-S is able to inhibit cell proliferation and activate caspase activity, leading to apoptosis [26, 27].

Combination therapy with anticancer agents can overcome drug resistance, decrease single-drug toxicity, and expand the range of treatment, providing a good therapeutic approach [28]. Combinations of two or more anticancer agents allow the targeting of multiple molecular pathways that are critical for cancer cell survival [29]. Different studies have shown similar results when Cur is combined with quercetin and catechin, it inhibits the proliferation of lung (A549), colon (HCT116), breast (MCF7) and liver (HepG2) cancer cell lines [30, 31].

Since cervical cancer remains a prevalent health concern despite the intervention of vaccination programs, there is an urgent need to find more novel and natural anticancer agents. Therefore, this study was designed to evaluate the anticancer properties of the bioactive compounds Cur, 6-/10-G, and 6-/10-S, in single treatments as well as in combination with Cur in HeLa cervical cancer cell lines with the final aim of determining the best combined formulation to inhibit its growth.

Materials and Methods

Chemicals and reagents

The bioactive compounds 6-gingerol, 10-gingerol, 6-shogaol, 10-shogaol, and curcumin were purchased from Biopurify Phytochemicals (Chengdu, Sichuan, China) and had a purity of >98%. Eagle's Minimum

Essential Medium (EMEM), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent, cisplatin, 0.25% trypsin-EDTA solution, and Dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St Louis, MO, USA). Penicillin-streptomycin (10mg/mL streptomycin sulphate; 10000U/mL penicillin) was purchased from Elabscience (Houston, Texas, USA). Fetal Bovine Serum (FBS) was purchased from EURx (Gdańsk, ul. Przyrodnikow 3, Poland). Annexin V-FITC/PI apoptosis detection kit was obtained from BD Bioscience (556547) (San Diego, CA, USA).

Cell cultures

The human cervical carcinoma cell line HeLa (ATCC, CCL-2) was purchased from ATCC (Manassas, VA, USA) and cultured in EMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37°C and 5% CO₂. Active monitoring of the cell culture by microscopic analysis or through color change observation in the medium serves as a substitute for the routine use of antifungals. The cells were maintained below passage (P) 20 to maintain cell health and reduce the potential for genotypic and phenotypic drift.

Cell viability assay

Cell viability was evaluated with an MTT assay according to Mosmann [32]. HeLa cells were seeded in 96-well plates in triplicate at a density of 5,000 cells per well and incubated for 24 hours before treatment. For single compound treatment, the cells were treated with 6-/10-G, 6-/10-S, Cur, or the drug cisplatin at concentrations ranging from 3-100 µM for 24 hours. For compound combination treatment, the concentrations of 6-/10-G, 6-/10-S, and Cur were determined on the basis of IC₅₀ values of each compound according to Zhang et al. [33]. By using ratios of 1:1, 1:2, 1:3, 2:1, and 3:1, the cells were treated with a combination of Cur:6-G, Cur:10-G, Cur:6-S, and Cur:10-S for 24 hours over a wide concentration range to generate growth curves. The IC₅₀ of Cur increased in the 2:1 and 3:1 ratios, whereas the IC₅₀ of 6-/10-G and 6-/10-S increased in the 1:2 and 1:3 ratios. Following incubation, the cells were incubated with MTT solution (5mg/mL) followed by DMSO to dissolve the formazan salt. The absorbance readings were analysed at 570 nm and 620 nm (as reference wavelength) via a microplate reader (Tecan, Austria). Cell viability was calculated from the optical density (OD) using the following formula (1). IC₅₀ values were determined via a Microsoft Excel plot.

$$\text{Cell Viability (\%)} = \frac{\text{OD of treated groups}}{\text{OD of control groups}} \times 100 \quad (1)$$

Synergy determination

The Chou-Talalay method was applied to determine the combination index (CI) in the analysis of the combination study, as previously described by Chou [34]. The proliferative inhibition rate (%) was calculated and transformed to the fraction affected (Fa: range 0-1) via the following formula (2)

$$\text{Affected fraction (Fa)} = 1 - \frac{\text{treated group}}{\text{control group}} \quad (2)$$

where Fa=0 and 1 represented 100% viability and 0% viability, respectively. The data were entered into Compusyn software (Biosoft, Ferguson, MO, USA) for CI analysis basis of the Chou-Talalay formula below (3).

$$\text{Combination Index (CI)} = \frac{dA}{DA} + \frac{dB}{DB} \quad (3)$$

where dA=IC₅₀ of compound A in combination, DA=IC₅₀ of single compound A, where dB=IC₅₀ of compound B in combination, DB=IC₅₀ of single compound B. The effects of combining the two bioactive compounds were primarily reflected by the CI values, where CI=1 indicates additive effect, CI>1 indicates antagonism, and CI<1 indicates synergism. On the basis of the obtained CI values, GraphPad Prism software was used to create a three-color scale, where synergism, additive and antagonism are represented by green, yellow, and red, respectively. Each CI value's color was interpolated between these limits.

Annexin V-FITC/PI Apoptosis Analysis

Apoptosis was evaluated with an Annexin V-FITC/PI kit according to the manufacturer's protocol. A total of 3 x 10⁵ HeLa cells were seeded in a 6-well plate and then treated with the IC₅₀ concentrations of cisplatin, Cur, 6-/10-G, or 6-/10-S, and the optimum combinations were selected for 24 hours. The floating cells were collected while the adherent cells were detached with 400 µL of trypsin-EDTA and washed twice with 800 µL of ice-cold PBS supplemented with 5% FBS as FBS protects the cells from harmful disruption. The collected cells were subsequently centrifuged (Eppendorf, Germany) for 5 minutes at 500 x g at 4°C. The supernatant was removed, and the cell pellet was resuspended in 150 µL of ice-cold 1X binding buffer. A total of 100 µL of the suspension was transferred into a fluorescence-activated cell sorting (FACS) tube and stained with 5 µL of Annexin V-FITC and PI each, followed by gentle vortexing and incubation for 15 minutes at room temperature in the dark. Another 400 µL of 1X binding buffer was added to the samples which were further analyzed via flow cytometry (BD Bioscience FACSCanto Flow Cytometer, Hampton, NH, USA) within 1 hour. For each sample, 10,000 events were acquired. The analysis was performed via FlowJo software.

Statistical analysis

All the data were statistically analysed via GraphPad software version 8.0.2. and the results are expressed as the means ± standard deviation (SD). Significant differences between and within multiple groups were examined via One-way ANOVA followed by Dunnet's test and Tukey's test. p < 0.05 was considered statistically significant.

Results

Effects of treatment with Cur, 6-/10-G, and 6-/10-S on the viability of HeLa cells

The results of the cell viability assay and the IC₅₀ for each treatment are presented in Table 1. Our results showed that Cur, 6-/10-G, and 6-/10-S inhibited the growth of the HeLa cervical cancer cell line in a concentration-

dependent manner (Supplementary Figure 1). Cur had the greatest cytotoxic effect (p< 0.05) after cisplatin in HeLa cells, whereas 10-S had the greatest cytotoxic effect among all the bioactive compounds in ginger.

Effects of combination treatment with Cur and 6-/10-G, and 6-/10-S on the viability of HeLa cells

We tested the effects of Cur and its combination with 6-G at various concentrations ranging from 5-160 µM at ratios of 1:1, 1:2, 1:3, 2:1, and 3:1 (Cur:6-G). In term of these ratios, we found that a combination treatment is always better than a single treatment at inhibiting the growth of HeLa cancer cells. A ratio of 3:1 (Cur:6-G) has greater cytotoxic effects, whereby a ≥50% reduction in cell viability is observed compared with the other combination ratios at a range of 20-80 µM of 6-G (Figure 1A).

The combination of Cur and 10-G at concentrations from 4-140 µM at similar ratios exhibits varying cell growth effects (Figure 1B). Compared with combination ratios of 1:1, 1:2, 2:1, and 1:3 (Cur:10-G), combination treatment at a ratio of 3:1 (Cur:10-G) substantially decreased cell viability to 27.3% at a concentration of 35 µM 10-G, which indicates greater cytotoxic effects.

We also tested the effects of Cur and its combination with 6-S at concentrations ranging from 4-140 µM at various ratios of 1:1, 1:2, 1:3, 2:1, and 3:1 (Cur:6-S). Among these ratios, 3:1 (Cur:6-S) similarly had better cytotoxic effects than the individual Cur and 6-S treatments on the inhibition of HeLa growth, with 88% and 97% reductions in cell viability observed at concentrations of 35 µM and 70 µM 6-S, respectively (Figure 1C).

The effects of Cur and its combination with 10-S at concentrations ranging from 2-120 µM at ratios of 1:1, 1:2, 1:3, 2:1, and 3:1 (Cur:10-S) are presented in Figure 1D. Compared with single treatments with Cur or 10-S, combination ratios of 2:1 and 3:1 (Cur:10-S) resulted in reductions in cell viability by 46% and 72%, respectively, with a reduction of 13-45% at 20 µM of 10-S. These results indicate that combination ratios of 2:1 and 3:1 (Cur:10-S) have strong anti-proliferative effects on HeLa cells. The combination ratio of 3:1 (Cur:10-S) notably results in a lower concentration-effect curve than the ratio of 2:1 (Cur:10-S), suggesting that HeLa cells are more responsive to earlier combinations.

The results revealed that the combination of 3:1 curcumin and ginger bioactive compounds had greater

Table 1. Half Maximal Inhibitory Concentration Values of Cur, 6-G, 10-G, 6-S, and 10-S in HeLa cells after 24 hours Treatment

Compound	IC ₅₀ (µM)
Cur	31.2 ± 1.7*
6-G	83.3 ± 10.5*
10-G	65.5 ± 1.5*
6-S	68.8 ± 2.4*
10-S	36.3 ± 3.2*
Cisplatin	17.2 ± 1.89

The data are presented as the means ± SDs of three independent experiments. *p< 0.05 compared with cisplatin. Cur, Curcumin; 6-G, 6-gingerol; 10-G, 10-gingerol; 6-S, 6-shogaol; 10-S, 10-shogaol.

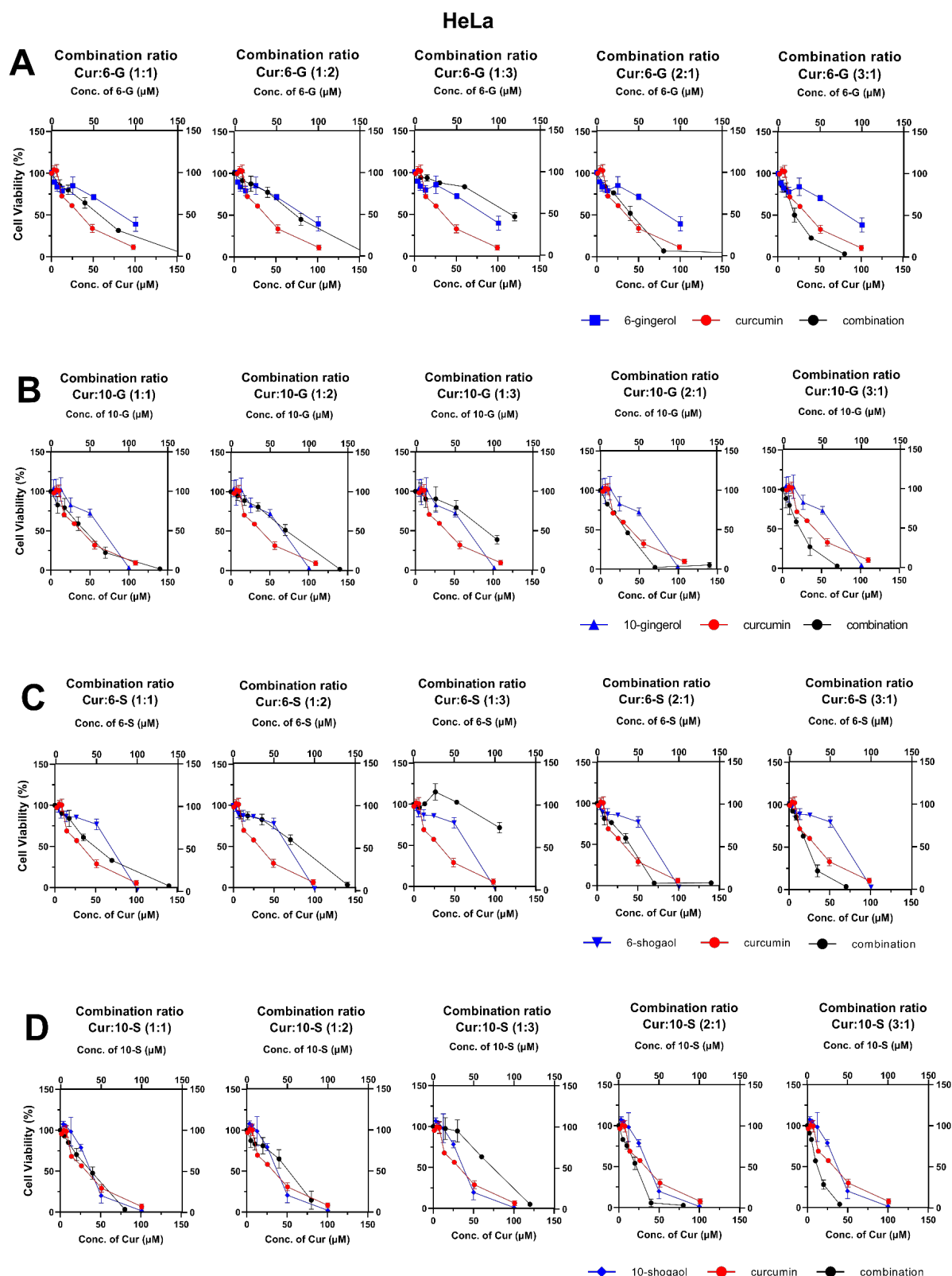


Figure 1. Concentration Effect Curves of Individual Compounds and Their Combination on HeLa Cell Line (A) Cur and 6-G, (B) Cur and 10-G, (C) Cur and 6-S, and (D) Cur and 10-S, at different combination ratios of 1:1, 1:2, 1:3, 2:1, and 3:1. The graphs represent mean $\pm$ SD of three independent experiments.

cytotoxic and anti-proliferative effects than the individual compounds at the same concentration did, suggesting a synergistic effect of these compounds on HeLa cells.

The combination index (CI) of the combined treatment of

Cur with ginger bioactive compounds

To determine whether the combination of Cur with 6-/10-G and 6-/10-S has synergistic or antagonistic effects on cervical cancer cell lines, CI was calculated via the Chou-Talalay method [34].

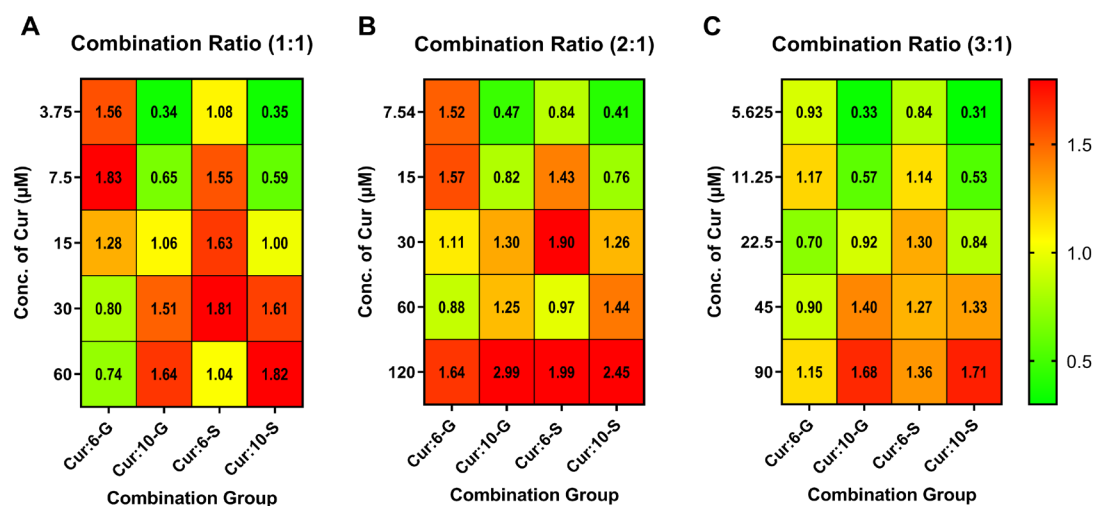


Figure 2. Combination Index (CI) Heatmap of Different Combination Ratios in the HeLa Cell Line. The CI was determined via Compusyn software according to the Chou-Talalay formula, as explained in the Methods section. The concentration of combination therapy in HeLa cells was as follows: Cur (3-120 μM) with 6-G (5-160 μM), 10-G (4-140 μM), 6-S (4-140 μM), or 10-S (2-120 μM) at (A) Combination ratio of 1:1, (B) combination ratio of 2:1, and (C) combination ratio of 3:1. Additivity, CI=1 (yellow); synergism, CI <1 (green); and antagonism, CI >1 (red), where colors correspond to CI values. The greenish surface area demonstrates more synergistic action (lower CI), whereas the orange and reddish surface areas represent more antagonistic action (higher CI).

The results of the CI analysis on HeLa cells, presented in Figures 2A-C, summarize the combinations effects of Cur with 6-/10-G and 6-/10-S at various concentrations and treatment ratios. A significant variation in CI was observed whereby strong synergistic effects were observed for the combination of Cur:6-G, Cur:10-G, and Cur:10-S at a concentration ratio of 3:1 with CI values ranging from 0.3-0.7. These synergistic effects are always observed when both Cur and ginger bioactive compounds are combined at relatively low concentrations.

The synergistic effects of combination treatments using 1:1 ratio of Cur with 6-/10-G and 6-/10-S are presented in Figure 2A. Cur:10-G and Cur:10-S clearly had strong synergistic effects at low Cur concentrations of ≤ 7.5 μM and ≤ 3.75 μM, respectively, with CI values ≤ 0.65 , whereas synergistic effects were observed in the combination group of Cur:6-G at a combination ratio of 1:1 but at high Cur concentrations of 30-60 μM, with CI values of 0.3-0.8. Additive and antagonist reactions are observed in the combination group of Cur:6-S in all ranges of the Cur concentrations, with CI values of 1.0-1.8.

Treatments with a combination ratio of 2:1 showed similar trends of synergism in both Cur:10-G and Cur:10-S combination groups at low Cur concentrations ≤ 15 μM with CI values ranging from 0.4-0.8 (Figure 2B). Additionally, as the Cur concentration increased from 30 to 120 μM, the CI values increased in the range of 1.2-2.9, indicating antagonistic effects. Moreover, a moderate to weak synergism was observed for the combination of Cur:6-G and Cur:6-S at Cur concentrations of 7 μM and 60 μM with CI values ranging from 0.8-0.9. In contrast, the other concentrations presented antagonistic CI values ≥ 1.2 .

The combination ratio of 3:1 had the greatest synergistic

effects on HeLa cells among all the combination groups (Figure 2C). Strong synergistic effects were observed for the combination groups of Cur:10-G and Cur:10-S when Cur was used at concentrations ranging from 5-11 μM, with CI values ranging from 0.3-0.5. Synergistic effects with CI values ranging from 0.7-0.9 were observed in combination with Cur:6-G at various Cur concentrations ranging from 5-45 μM, with weak antagonistic effects observed at Cur concentrations of 11.25 μM and 90 μM, with CI values of 1.1.

On the basis of these results, the combination ratio (3:1) of 22.5 μM and 45 μM curcumin with ≤ 40 μM bioactive compounds from ginger, which have synergistic and antagonistic effects, were tested for their ability to induce apoptosis in HeLa cells.

Effects of single and combination treatments of Cur with 6-/10-G and 6-/10-S on apoptosis of HeLa cells

The effects of single treatments of Cur, 6-/10-G, and 6-/10-S on the apoptosis of HeLa cells as determined by the IC₅₀ concentration of each compound, are presented in Figure 3A. High levels of apoptosis are observed at a late stage. The percentages of total late apoptotic cells observed for Cur, 6-G, 10-G, 6-S, and 10-S were 76.4%, 8.8%, 14.9%, 16.8% and 26.8%, respectively. The highest total percentage of apoptotic cells (98.2%) was observed in the HeLa cells treated with Cur at the IC₅₀. Interestingly, compared with the drug cisplatin, Cur and 10-S resulted in 5.1-fold and 1.7-fold greater numbers of apoptotic cells, respectively.

The combination treatments at 3:1 ratio of Cur with 6-/10-G and Cur with 6-/10-S induced notable effects on the apoptosis of HeLa cells (Figure 3B). The percentage of apoptotic cells increased in a concentration-dependent

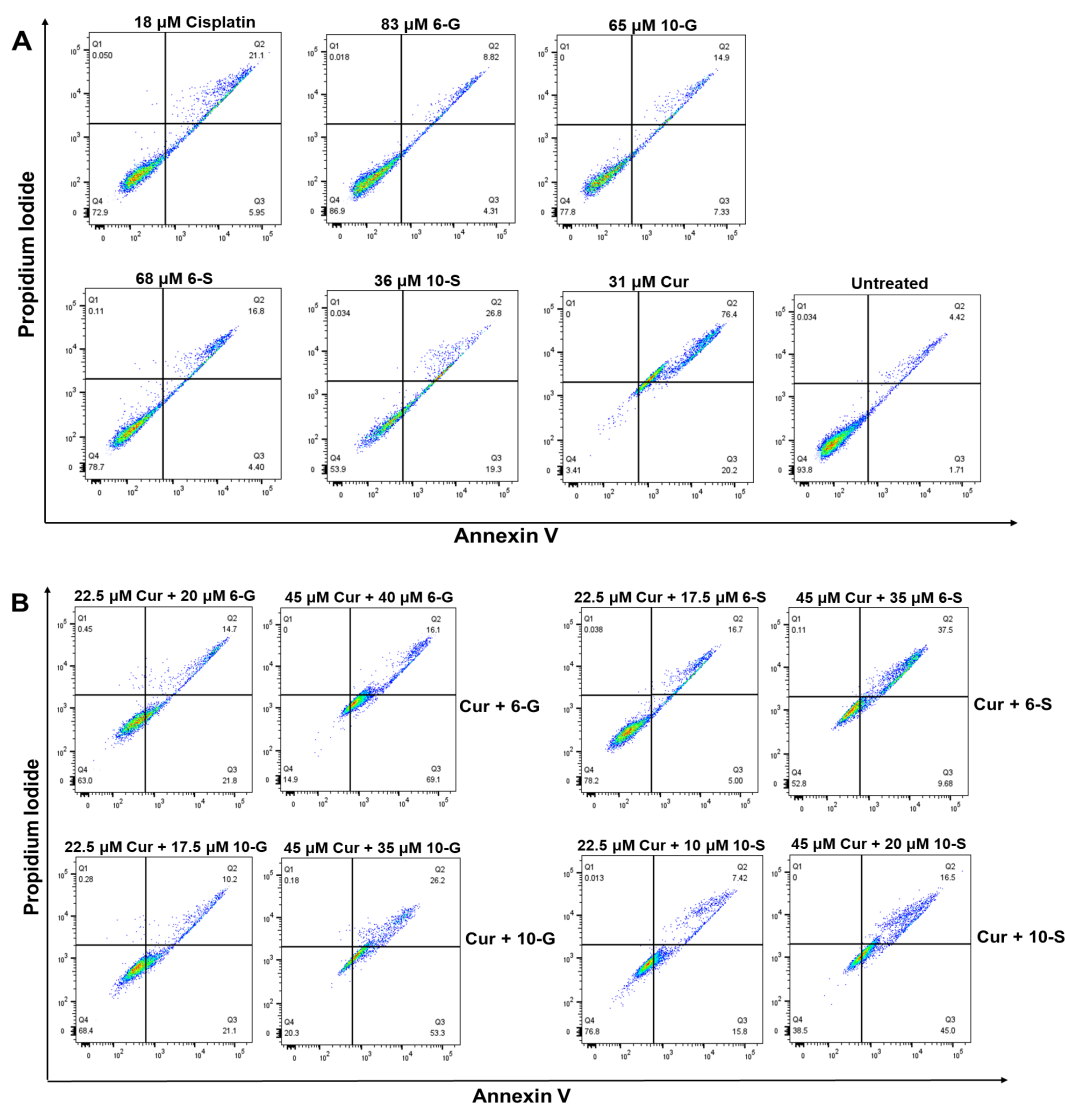


Figure 3. Cytogram Analysis on the Effects of Cisplatin and Bioactive Compounds on Apoptosis of HeLa Cells at 24 hours. (A) Effects of IC₅₀ of single compound treatments, and (B) combined compound treatments at combination ratio (3:1) of Cur and ginger bioactive compounds. Apoptosis was quantified using Flow Cytometry. Q1 represents dead cells/necrosis, AnxV-/PI+, Q2 represents late apoptosis, AnxV+/PI+, Q3 represents early apoptosis, AnxV+/PI-, and Q4 represents viable cells, AnxV-/PI-.

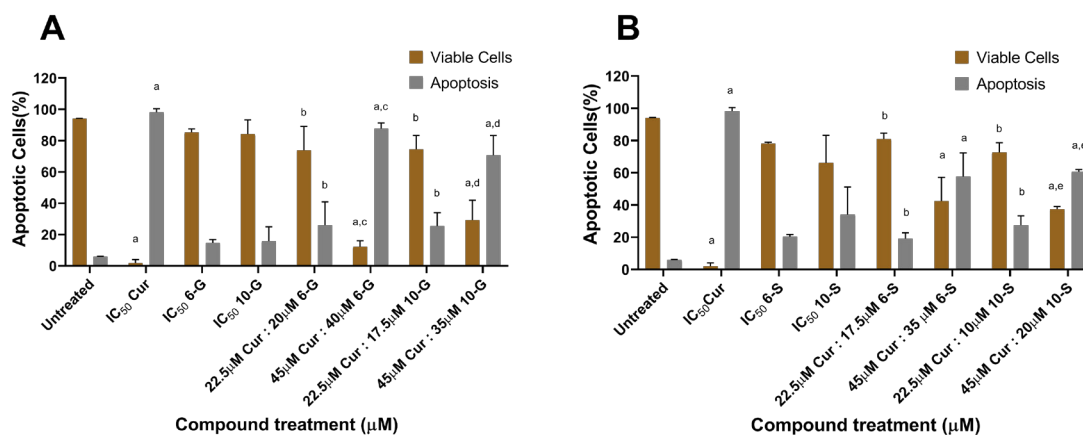


Figure 4. Apoptotic Effects of Single and Combined Treatments with a 3:1 Ratio of Bioactive Compounds from Curcumin and Ginger on HeLa Cells. (A) gingerol and (B) shogaol treatment groups. The viable cells are represented by the lower left quadrant of the cytogram (AnxV-/PI-). The apoptotic cells are represented by early apoptotic and late apoptotic cells in the lower right quadrant (AnxV+/PI+) and upper right quadrant (AnxV-/PI+) of the cytogram, respectively. The data are presented as the means $\pm$ SDs from two independent experiments. ^a($p < 0.05$) compared with the control group, ^b($p < 0.05$) compared with the Cur alone group, ^c($p < 0.05$) compared with the 6-G alone group, ^d($p < 0.05$) compared with the 10-G alone group, and ^e($p < 0.05$) compared with the 10-S alone group.

manner, with a high percentage of apoptotic observed at the early stage. However, combination treatment with Cur:6-S resulted in high apoptotic activity at the late stage.

The number of viable and total apoptotic HeLa cells following single and combined treatments using a combination 3:1 ratio of curcumin, gingerols or shogaols after 24 hours is summarized in Figure 4A-B. Compared with the untreated control, curcumin alone significantly increased the total number of apoptotic cells ($p < 0.05$) and was more effective than gingerols or shogaols in inducing apoptosis. The combination formula of 45 μM : 40 μM (Cur:6-G) induced 87.75% of the cells to be apoptotic, which was higher than the percentage of apoptotic cells induced by the individual 6-G treatment, (14.65%). Similarly, the combination formula of 45 μM : 20 μM (Cur:10-S) had a more significant apoptotic effect, with 60.55% more apoptotic cells than the single 10-S treatment, 33.96%. Overall, with the combination of 45 μM Cur, the percentage of apoptotic HeLa cells was significantly greater (by 2.3- to 6-fold) than with the single treatments of 6-/10-G and 6-/10-S.

Discussion

Cervical cancer is a significant health concern, and many studies have focused on finding new compounds from natural products for treating cervical cancer. Various bioactive compounds derived from natural products have been explored for their chemopreventive effects in cervical cancer [11, 16]. Moreover, combinations of bioactive compounds have shown synergistic effects on certain types of cancer [35].

In the present study, a single treatment with Cur, 6-/10-G, and 6-/10-S inhibited the growth of HeLa cervical cancer cell lines in a concentration-dependent manner. Cur is the most potent anticancer agent, with an IC_{50} value of $31.2 \pm 1.7 \mu\text{M}$, whereas bioactive ginger compounds, 6-/10-G and 6-/10-S, need higher concentrations, $83.3 \pm 10.5 \mu\text{M}$, $65.5 \pm 1.5 \mu\text{M}$, $68.8 \pm 2.4 \mu\text{M}$, and $36.3 \pm 3.2 \mu\text{M}$ to kill 50% of HeLa cancer cells ($p < 0.05$), respectively (Table 1). When we tested the combined effects of the bioactive compounds of Cur and ginger ratios ranging from 1:1, 1:2, 1:3, 2:1, and 3:1, we found that the 3:1 ratio of the bioactive compounds of Cur and ginger had the greatest synergistic effects (Figure 2), as the IC_{50} values were lower than those of the individual compounds. Heatmap analysis revealed that the 3:1 ratio of the bioactive compounds from Cur to ginger had a high synergistic CI value of 0.3-0.9 when the formulation was used at a concentration of 5.6 μM of Cur and 2.5-5 μM bioactive compounds from ginger (Figure 2). A low reduction in cell viability of less than 15% was observed. Conversely, we found that concentrations of 22.5 μM and 45 μM of Cur combined with ginger bioactive compounds at 17–40 μM resulted in greater reductions in cell viability (35–78%). These concentrations were subsequently used for apoptotic analysis. Although the same combination ratio of 3:1 of Cur and ginger bioactive compounds was utilized, synergistic interactions were mostly observed at low concentrations of the combinations and antagonists at higher concentrations against the growth of HeLa cells.

A similar study by Kayacan et al. [36] revealed that the interaction of Cur and apigenin (in chamomile tea) works best at low concentrations, resulting in a synergistic effect. Another study by Santana-Gálvez et al. [37] reported that a synergistic combination of Cur and dihydrocaffeic acid (DA) (in grapes) occurred at a ratio of 9:1 (Cur: DA).

Shogaols are compounds derived from gingerols, therefore, both compounds have structural similarities. However, the results from the cell viability assay performed in this study suggest that their therapeutic mechanistic targets might differ. Gingerol and shogaol differ in the presence of a hydroxyl group and in the location of the double bond. Compared with their 6-carbon counterparts, the increased number of carbon chains in 10-gingerol and 10-shogaol, enhances their free radical scavenging activity against RAS 264.7 cell lines, as reported by Dugasani et al. [38]. Our findings in HeLa cells agree with the notion that the extension of the carbon chain, along with the presence of double bond at C4-C5 in 10-S, is superior to the hydroxyl group in gingerol in enhancing anti-proliferative effects, as observed in individual and combination treatments. Therefore, our proliferation assay suggested that 10-S is superior to 6-/10-G and 6-S because it has a better anticancer effect when combined with Cur at a concentration ratio of 3:1 of Cur and 10-S in HeLa cells. The synergistic effect of 3:1 ratio of Cur to ginger bioactive compounds in this study is possibly due to the modulation of the anti-proliferative, anti-inflammatory, and anti-cytokine mechanisms of action, leading to the inhibition of HeLa cell growth. Zhou et al. [39] reported that the combination of 6-S, 10-S, and Cur synergistically reduced the production of proinflammatory mediators and the downregulation of the MAPK pathway in RAW 264.7 and THP-1 cells. A different study by Zhou et al. [40] identified Cur and 10-S as the leading compounds involved in reducing the levels of cytokines involved in inflammation. The ability of combined treatments to work effectively at specific concentration ratios is crucial in determining their efficacy [29]. By quantifying synergistic effects through CI, we have been able to optimize and assess the degree of synergy between curcumin, gingerol, and shogaol and thus we found a potential combination ratio of 3:1 of 22.5-45 μM of Cur and 17-40 μM of ginger bioactive compounds for enhancing treatment efficacy in cancer cells.

Apoptosis is a natural mechanism by which cancer cells are eliminated by the activation of certain apoptotic genes. In the present study, each individual treatment with ginger bioactive compounds did not significantly increase the total number of apoptotic cells. However, a significant increase of more than 61% in total apoptotic cells was found upon combination treatment at 3:1 ratio of Cur (45 μM) with 10-S (20 μM) and a combination of Cur (45 μM) with 6-G (40 μM) where Cur was shown to potentiate the anticancer activity of 10-S and 6-G compared with that of individual compounds in inducing HeLa cell death ($p < 0.05$) (Figure 4). While our research focused on combination treatments, Cur alone remains the most potent compound for inducing apoptosis in HeLa cells. Our apoptosis analysis revealed that 98.2% of the cells underwent apoptosis following treatment with Cur.

Cur has been well documented to induce apoptosis in various human cancers by causing DNA damage [41]. Kayacan et al. [36] reported that Cur induced cell death via the caspase-3 pathway, FAS death receptor and necrotic cell death factor (TNF) in HeLa cells. While previous studies by Liu et al. [42] and Bawadood et al. [25] demonstrated the induction of apoptosis by gingerols and shogaols in breast and prostate cancer cell lines, our study did not find a significant increase in the number of apoptotic HeLa cells after treatment with individual 6-/10-G and 6-/10-S. This could be attributed to the different responses of the cell lines as well as variations in treatment concentrations which may play a role in the cytotoxicity of the bioactive compounds in ginger to induce apoptosis. However, this study revealed that Cur combined with 6-G and 10-S is able to induce cell death in HeLa cells after combination treatment at a ratio of 3:1 (Cur:6-G) (Cur:10-S). Furthermore, the ability of the combination treatments may be due to the multiple mechanisms of action of the polyphenols in Cur, 6-G and 10-S in producing anti-inflammatory and anticancer effects via the induction of apoptotic activity at multiple signaling levels and their crosstalk in cells [39].

In contrast to the results of our cell viability analysis, we found that the combination treatment with Cur and 6-G at a ratio of 3:1 (Cur:6-G) effectively induced apoptosis. This may be because gene activation in the apoptotic pathway occurs, and a stronger formulation of the compounds is needed to induce the death of HeLa cells, which needs Cur in combination with the bioactive compounds of ginger (CI value of 0.9; synergistic interactions). On the other hand, a combination of Cur and 10-S at the same ratio demonstrated consistent efficacy in inhibiting cell proliferation pathways and inducing apoptotic pathways on the basis of the MTT cell viability and apoptotic analyses, although the CI value of 1.33 was antagonistic. Therefore, further research is needed to elucidate the precise mechanism by which combination treatment with gingerol bioactive compound and Cur induces apoptotic activity in HeLa cells. To the best of our knowledge, no study has reported formulations of 10-S in single and combination treatments with Cur in inhibiting the growth of cervical cancer cells.

In summary, our study shows the best formulation for inhibiting the growth and inducing the apoptosis of HeLa cervical cells is by using Cur: 6-G (45 µM:40 µM) at a ratio of 3:1. Although the combination treatment in this study resulted in significant changes, Cur can be considered effective on its own. Cur enhances the sensitivity of ginger bioactive compounds to induce the apoptosis of the HeLa cancer cells, resulting in improved anticancer effects. We hypothesized that multiple pathways are likely involved, hence, future studies should target specific signaling pathways involved in cervical cancer via the proposed combined formulation for the bioactive compounds Cur and ginger.

Author Contribution Statement

Unwaniah Abdull Rahim: carried out the experiments, data analysis, drafted the manuscript, and revised

the manuscript. Yasmin Anum Mohd: Supervised the experiments and data analysis and revised the manuscript. Marami Mustapa, Armania Nurdin, Nur Aishah Che Roos, Nursiati Mohamad Taridi, Mariam Firdhaus Mad Nordin: supervised the experiments and revised the manuscript, Nik Noorul Shakira Mohamed Shahrin: provided research funding, supervised the experiments and revised the manuscript.

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If any scientific body approved it/ if it is part of an approved student thesis

There is no formal approval from a scientific body and the research does not form part of an approved student thesis.

Ethical approval

This study was approved by the Research and Ethics Committee of the Faculty of Medicine and Defence Health (FPKP), National Defence University of Malaysia (UPNM), under approval number Bil.2/2021.

Availability of data

Data is available on reasonable request from the corresponding authors.

Conflict of interest

The authors declare that they have no conflicts of interest associated with this manuscript.

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