

Synergistic Effect of Cetuximab in Combination with Bee Venom: A Novel Approach for the Treatment of Colon Cancer

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Abstract: Anti-EGFR monoclonal antibody (cetuximab) is a promising new targeted therapy for cancer treatment currently approved to treat colon cancer. The synergistic effect of (Ce/BV) on two colon cancer cell lines (HCT116 and Caco-2) was investigated in this work to get the lowest lethal dosages of cetuximab. MTT assay was used to evaluate the cytotoxic effect of cetuximab, bee venom, and their combination. Flow cytometry analysis was used to look at cell cycle distribution and apoptosis induction. RT-PCR can identify changes in apoptotic genes expression. The results show that cetuximab and BV have IC₅₀ against Caco-2 (686 and 10.5 g/ml, respectively) and HCT-116 (740 and 13 g/ml, respectively). According to the results, the combination treatment had a synergistic inhibitory impact on two cell lines (CI < 1). Both cell lines were arrested in the G2/M phase of the cell cycle. In addition, when combination therapy was used instead of cetuximab alone, apoptosis was dramatically increased in both cell lines. In addition, after treatment with (Ce/BV), Bcl2 and p53 were shown to be down-regulated and up-regulated, respectively, in two cell lines. As a result, the combination of cetuximab and BV showed preclinical efficacy in the treatment of colon cancer

Keywords: colon cancer; cetuximab; bee venom; MTT assay; apoptosis; cell cycle.

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1. Introduction

Colon cancer is one of the most common malignancies worldwide and is the third leading cause of cancer death in both men and women. Colon cancer has an estimated incidence of over 1,85 million new cases and 850 000 deaths annually [1, 2]. The treatment of colon cancer necessitates a multi-pronged approach that includes surgical removal of the tumor followed by chemotherapy and/or radiation therapy, or a combination of these treatments [3]. Despite significant progress in colon cancer therapy, there is still a need for better treatments and creative concepts, such as the targeted modulation of cancer signaling pathways. In recent years, targeted therapy has represented a valid approach for treating colon cancer and a promising area of research that aims to deed molecular mechanisms responsible for tumor progression [4, 5].

Oncology has recently shifted its focus to targeted therapy. Many molecular modulations have been proposed as therapeutic targets. The epidermal growth factor receptor (EGFR) is an example [6, 7]. The EGFR is a member of the ErbB family of transmembrane receptor tyrosine kinases consisting of an extracellular ligand-binding domain, a hydrophobic

transmembrane region, and an intracellular tyrosine kinase domain. The EGFR has been over-expressed in several epithelial malignancies, including head and neck, colorectal, breast, ovarian, prostate, bladder, and lung cancers [8]. Since discovering EGFR as a cancer target, EGFR monoclonal antibodies (mAbs) have been used alone or in conjunction with traditional cytotoxic drugs like chemotherapy or radiotherapy [9]. EGFR-targeting mAbs, like other clinically relevant mAbs, have shown limited efficacy as monotherapies and are therefore usually used in combination with anti-cancer drugs [10, 11]. As a result, EGFR mAbs in combination with chemotherapy may be useful in treating colon cancer [12]. Cetuximab is a chimeric IgG1 an anti-EGFR monoclonal humanized antibody interacting with the extracellular binding site of EGFR to block ligand stimulation, serves as targeted therapy, and has been licensed and widely used in the clinical treatment of colorectal cancer and head and neck malignancies [13].

Furthermore, cetuximab binding causes EGFR internalization and degradation, which results in signal cessation. Cetuximab, unlike EGFR-TKIs, can also cause antibody-dependent cellular cytotoxicity (ADCC) activity, an important immunologic antitumor effect. In combination with chemotherapy, cetuximab has been approved by the FDA for the treatment of metastatic colorectal cancer and locally advanced head and neck cancer [14]. Bee venom (BV) has been known since ancient times for its healing properties. It can be seen in many religious texts, including the Quran, Bible, and Veda [15]. BV is a complex mixture of different peptides, amines, and enzymes, including melittin (a major component of BV), apamin, adolapin, and mast cell degranulating peptide and phospholipase A2 [16]. BV has anti-cancer activity on several types of cancer cells [17]. Melittin, a major polypeptide of BV, is thought to function as a lytic agent that has been used against chronic inflammation and has an antitumor effect against a broad spectrum of cancers, including prostate, breast, liver, and colon cancer [18]. Melittin is a pro-inflammatory cytokine used to treat various ailments, including arthritis, rheumatism, atherosclerosis, and peripheral neurological problems [19]. However, the anti-cancer effect of BV on colon cancer cells has been studied in rare studies. The purpose of this study was to examine the anti-cancer effects of cetuximab, BV on colon cancer cells, assess the synergistic effects of cetuximab and BV, and find the lowest lethal concentration of these drugs and their combination on colon cancer cells

2. Materials and Methods

2.1. Cell lines and reagents.

Two human colon cancer cell lines, HCT-116 and Caco-2, were obtained from the Egyptian holding company for vaccines and Sera (VACSERA) via American type culture collection (ATCC); the cell lines were maintained in RBMI 1640 media (Lonza, Verviers, Belgium) were supplemented with 5% fetal bovine serum (FBS) (Biowest, Nuaille, France) plus 1% penicillin-streptomycin and 1% non-essential amino acids and cultured at 37°C in a humidified incubator with 5% CO₂

2.1.1. Monoclonal antibody.

Cetuximab was originally purchased from (Merck Serono, Frenchs Forest, NSW, Australia) at 5 mg/mL.

2.1.2. Source of bee venom.

Bee venom of Egyptian bee *Apis mellifera* was obtained from the Economic Entomology and Pesticides Department of the Faculty of Agriculture, Cairo university

2.2. Cell viability assay by MTT.

The MTT assay was performed to assess the cytotoxicity of monoclonal antibody products, bee venom, and combination treatment of them. Cells were seeded in 96 tissue culture plates at density 1.6×10^3 cells per well (HCT-116) and 4×10^3 cells per well (Caco-2) in a volume of medium 100 μ supplemented with 5% fetal bovine serum plus 1% penicillin-streptomycin and 1% non-essential amino acids and incubated in a humidified incubator with 5% CO₂ at 37°C to form a confluent sheet. After 24 hours, the cells were treated with 100 μ l of different concentrations (serial dilutions) of cetuximab (Merk). Bee venom dissolved in a serum-free medium, and combinations of them after 48 hours of the treatment medium were removed and replaced by 50 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) to each well (treated and untreated) to investigate the synergistic effect of each treatment and combinations (comb A & comb B) on the growth of the cells after incubation for 2 hours the formazan crystals are dissolved by 100 μ g of dimethyl sulfoxide(DMSO). The absorbance of each well was measured at 570 nm with a microplate ELISA reader (LERX-800 Biotek –USA) to calculate the cell viability percent. The half-maximal inhibitory concentration (IC₅₀) values were determined by using GraphPad Prism 6.0 (Graph pad Software, USA). The optimal concentrations were chosen to complete the experiments based on the results. This experiment was repeated 3 times at least

2.3. Morphological changes.

Morphological changes were detected after HCT-116, and Caco-2 cell lines were treated for 48 hr with different treatments (single and combination) and IC₅₀ values. Cells were investigated by using an inverted microscope (Olympus, Japan) at 10 X magnification

2.4. Calculation of combination index (CI).

Firstly, The combination index (CI) was calculated using the following equation (Chou and Talalay equation) $CI = Ac/Be + Bc / Ae$, where Ae and Be are the concentrations of A and B that inhibit the X% of the proliferation when used alone. Ac and Bc are the concentrations of A and B that produce the same effect when used in combination, respectively[20]. In the present study, the interaction between BV and cetuximab was evaluated by calculating the combination index (CI) that indicates the drug synergism. CI value was analyzed using the CompuSyn 1.0 software using a non-constant ratio (CompuSyn Inc., Paramus, NJ, USA). Drug synergism was analyzed (CI < 1 indicates a synergistic effect, CI = 1 an additive effect, and CI > 1 an antagonistic effect). The level of synergism and/or antagonism of the two-drug combinations could be determined by the generation of fraction affected (Fa) versus CI plots for HCT116 and Caco-2 cell lines at the same concentrations used in the cell growth measurement.

2.5. Flow cytometry analysis.

2.5.1. Apoptosis evaluation.

Cell culture flasks (25 cm²) were cultured with Caco-2 and HCT116 cell suspensions. Cells were seeded at (1.4×10^5). Both Cell lines were treated with half the IC₅₀ value of cetuximab alone or half IC₅₀ of both cetuximab combined with bee venom, while untreated cells were left as the negative control. These concentrations (half IC₅₀ of both Ce&BV) were used because they were less than IC₅₀ with a low cytotoxic effect. Treated and untreated cells were incubated at 37°C for 48 h in 5% CO₂, trypsinized, washed twice with cold phosphate-buffered saline (PBS) (Sigma, USA), and centrifuged at (1500 rpm for 10 min) for each sample. Cells were resuspended in 1 x binding buffer and then stained using Annexin V-FITC Apoptosis Detection Kit (Biovision research, CA, USA) with 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI) and incubated at room temperature for 15 min in the dark. Cells were analyzed by flow cytometry using the FITC signal detector and PI staining by the phycoerythrin emission signal detector. Early apoptotic cells stained positive for Annexin V-FITC and negative for PI. Late apoptotic cells were positive for both Annexin V-FITC and PI. The experiment was performed in triplicate and repeated at least three times.

2.5.2. Cell cycle analysis.

The same process as mentioned above was followed. To adherent cell fractions were then trypsinized and fixed with ice-cold 70 % dropwise ethanol that was added dropwise in stirring condition on vortex and dropwise added. Fixed cells were kept overnight at 4°C.

Finally, the pellets were suspended in PBS, treated with RNase enzyme, and stained with PI for 30 min at 37 °C in the dark. Cell cycle distribution was analyzed using flow cytometry (Becton Dickinson, San Jose, CA, USA). The data were analyzed using the software was available from (Verity Software House, Topsham, ME, USA). The experiment was performed in triplicate and repeated at least three times

2.6. Estimation of apoptotic genes level by RT- PCR.

Total RNA was extracted from untreated and treated HCT-116 and Caco-2 cells using the EZ-10 Spin column total RNA mini – preps super kit (Bio Basic, USA) according to the manufacturer's instruction. The total RNA concentration and A260:A280 ratio of each sample were determined using Nanodrop 1000 spectrophotometer to get a final concentration measured by ng/ µl. cDNA strand was synthesized with 1 µg of total RNA using a Quantitect Reverse Transcription kit (Qiagen, Germany) by the manufacturer's instructions. These samples were subsequently frozen at a temperature of -70°C until used to determine the expression levels of BCL-2 and P53 genes using real-time PCR. Quantitative real-time PCR was formed on a Rotor-Gene Q cycler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Germany) and forward and reverse for each gene. The real-time PCR primers for *BCL-2* were as follows (F:5'-ATCGCCCTGTGGATGACTGAG-3')&(R:5'-CAGCCAGGAGAAATCAAACAGAGG- 3'). The real-time PCR primers for P53 were as follow (F:5'-GTTCCGAGAGCTGAATGAGG-3')and (R: 5'-TCTGAGTCAGGCCCTTCTGT- 3'). β -actin was used as a housekeeping gene(F:5'-TCTGGCACCACCTTCTACAATG-3')and(R-5'-GCACAGCCTGGATAGCAACG3-'). Cycling parameters and amplification conditions were followed by the protocol. All reactions

were repeated three times for each sample. The dissociation stage was added after the amplification one to verify the specificity of the PCR products, and calculable analysis was achieved by measuring the threshold cycle (Ct) values during the exponential phase of amplification. The MX3005P software (Agilent Technologies, GmbH) was used for Ct values measurement and the variations of mRNA genes expression calculated by the " $\Delta\Delta C_t$ " method [21].

2.7. Statistical analysis.

The results are expressed as means $\pm$ standard deviation (SD) of three independent experiments. Statistical analysis was performed with Graph Pad Prism 6.0 (Graph Pad Software, USA). For in vitro assays, statistical significance was reported if the P-value was <0.05 , non-significance if the p-value > 0.05 , and highly significant if the P-value was <0.001 . For in vitro comparative data- way ANOVA and T-test analysis were used

3. Results and Discussion.

3.1. Cytotoxicity assay by MTT.

MTT colorimetric assay method is used to determine cell viability and the cytotoxic activity depending on the activity of viable cell mitochondrial dehydrogenase enzyme, which converts the yellow color of MTT dye into violate formazan crystals that can be dissolved and assayed for color intensity. The cells which are no longer alive lose the ability to convert MTT into formazan [22]. Because of the role of the EGFR signaling pathway in the progression of colorectal cancer, therapeutic use of agents that block the EGFR downregulation has been considered [5].

The half-maximal inhibitory concentration (IC₅₀) is a measure of the effectiveness of anti-cancer agents in inhibiting colon cancer cells proliferation. In this study, The IC₅₀ was first quantitatively measured for each cetuximab (Ce) and bee venom (BV) to determine the amount of treatment needed to reduce cell proliferation by 50% in two colon cancer cell lines. HCT116 and Caco-2 are the colon cancer cells used in our study to assess the inhibitory effect of treatments (cetuximab, BV, and a combination of them). Caco-2, which expresses high levels of EGFR, and HCT-116, which expresses low levels of EGFR [23], this consistent with our results; the cytotoxic effects of cetuximab on two cell lines are shown in Figure 1 in a dose-dependent manner. Cetuximab had IC₅₀ values of 740 $\mu\text{g/ml}$ and 686 $\mu\text{g/ml}$ in HCT116 and Caco-2 cells. Also, the MTT assay verified the cytotoxic effect of BV on colon cancer cell lines. The IC₅₀ value for BV in the Caco-2 cell line was 10.5 $\mu\text{g/ml}$ after 48 hours and 13 $\mu\text{g/ml}$ in the HCT116 cell line, as shown in Figure 2. The results of bee venom matched with a study that showed the effect of BV on colon cancer cell lines HCT116 and SW480 [24]. In addition, Carole *et al.*, 2021 confirm the cytotoxic effects of bee venom and its two main compounds MEL and PLA2 on human colon cancer cells HCT-116 [25]. Previously, several studies showed an effect of the combination of cetuximab with cisplatin in different cancers cell lines. The study reported by Dong Ju Son *et al.* showed that the treatment of cetuximab combined with cisplatin in colon cancer cells exerts synergistic effects on cell growth inhibition and cell apoptosis [26]. Different concentrations of cetuximab mixed with IC₅₀ (Comb A) or 1/2 IC₅₀ (Comb B) of bee venom to see if it could increase the inhibitory effect of cetuximab combined with bee venom on cell proliferation. After 48 hours of treatment, the IC₅₀ was computed for different doses of cetuximab combined with BV on both cell lines, as shown in

Figure 3, which showed a comparison between the IC₅₀ values of cetuximab alone and combination treatments (Comb A&B). Comb A had IC₅₀ values of 133.4g/ml and 170.7g/ml against HCT-116 and Caco-2, respectively, while Comb B had IC₅₀ values of 248.4g/ml and 297.4g/ml. When compared to a single treatment, combination treatment inhibits cell proliferation more, and there is a significant difference ($P > 0.001$) in the two cell lines. According to the findings, cetuximab alone has an inhibitory impact, and BV enhances this inhibitory effect by 50%, lowering the required dose of cetuximab to limit cell proliferation (IC₅₀).

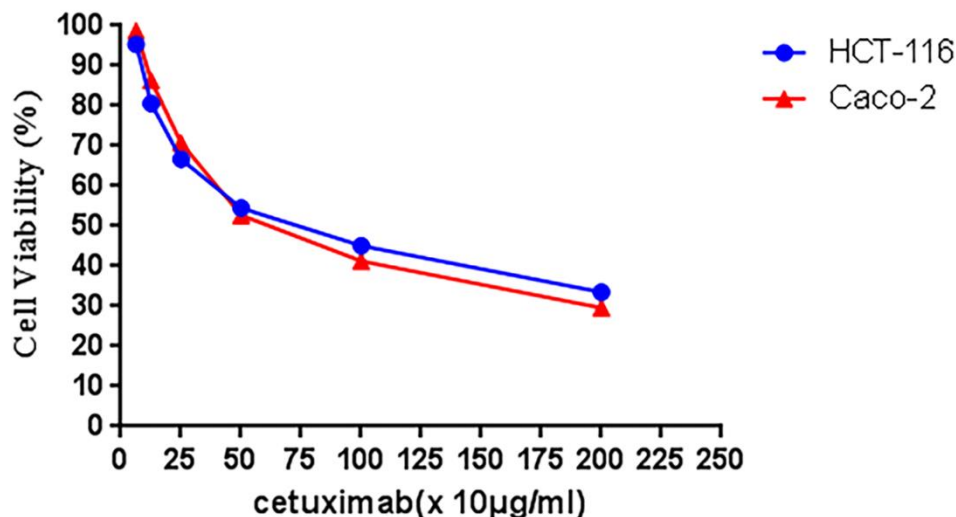


Figure 1. Effect of Cetuximab at different concentrations on the growth of HCT116 and Caco2 cells for 48 hrs.

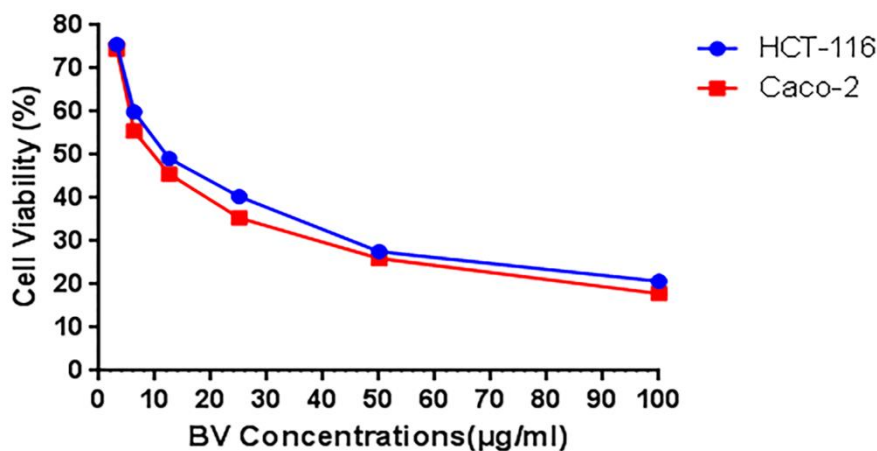


Figure 2. Dose-dependent effects of bee venom (BV) against Caco2 and HCT116 cell lines for 48 hrs.

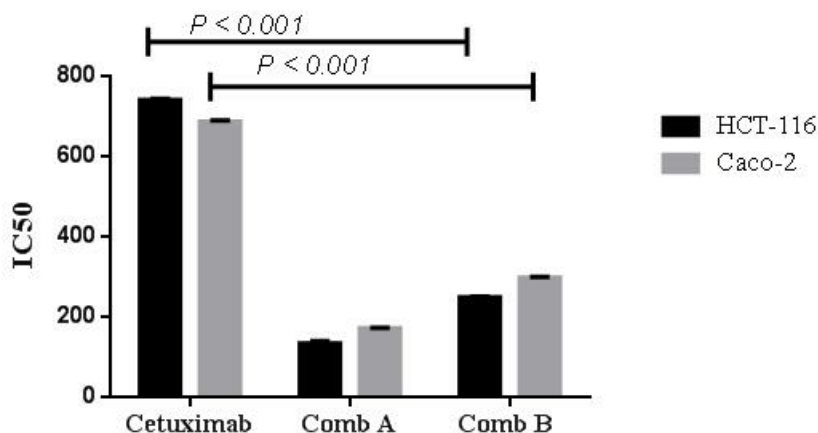


Figure 3. Determination of IC₅₀ and Comparison between the effect of cetuximab alone and combined treatments (A& B) against two cell lines (HCT116 and Caco2).

3.2. The combination index (CI).

The combination index (CI) analysis used the median effect equation of Chou and Talalay to quantify synergism or antagonism at different concentrations to identify the effective combinations of anti-cancer drugs [27]. The Chou-Talalay method can differentiate between two compounds' synergistic effects and additive effects with the constant ratio combination design[28]. CI values are interpreted as follows: <0.1 very strong synergism, 0.1–0.3 strong synergism, 0.3–0.7 synergism, 0.7–0.9 moderate to slight synergism, 0.9–1.1 nearly additive, 1.1–1.45 slight to moderate antagonism, 1.45–3.3 antagonism, and >3.3 strong to very strong antagonism[29]. In the current study, CI values were calculated using compulsion 1.0 software for Comb A and Comb B in using two colon cancer cell lines. CI analysis of the cytotoxicity results determined that in HCT116, the following combinations (4 concentrations) had synergistic effects at cetuximab and bee venom concentration of (740 + 13 or 6.5 µg/ml) and (370 + 13 or 6.5 µg/ml), which had CI value less than 1 as shown in Figure 4A. In contrast, at cetuximab and bee venom concentrations of 46.25 + 13 and 46.25 + 6.5 µg/ml, antagonistic effects were observed, which had a CI value of more than 1. Synergistic effects were observed in Caco2 cells when the cetuximab was combined with bee venom at these concentrations (686 + 10.5 or 5.25 µg/ml) and (343 + 10.5 or 5.25 µg/ml). In contrast, at Cetuximab and bee venom concentration of 86 + 10.5 or 5.25 µg/ml, antagonistic effects were observed and one concentration (686 + 10.5 µg/ml) had strong synergistic effect (CI = 0.22) µg/ml as shown in Figure 4B. Treatment with cetuximab and bee venom combines a synergistic effect compared with cetuximab alone in two cell lines in HCT116 and Caco-2.

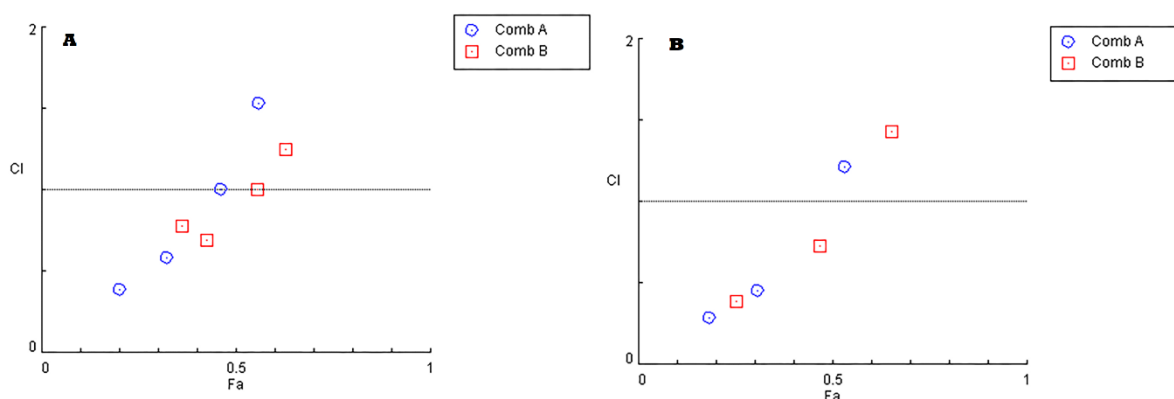


Figure 4. The combination index (CI) analysis for the synergistic effect of cetuximab and bee venom combinations in (A) HCT-116 cell line and (B) Caco 2 cell line.

3.3. Morphological changes.

The microscopic examination demonstrates that combination treatment induces marked changes in the morphology of the cancer cells such as nuclear shrinking and chromatin condensation known as apoptotic morphology, which is proportionate with the results from the cytotoxicity assays and thus confirms that the reduction in cell viability is related to the damage of cellular DNA. Combination treatment of oral cancer CAR cells with cetuximab and curcumin also resulted in cell shrinkage, cytoplasmic membrane blabbing, and cell death[30]. In another study, apoptotic morphology, including cell shrinkage, rounding, detaching from the flask surface, and followed by cell swelling and rupture, were observed in the colon (Caco-2) and breast (MCF7) cancer cell lines treated with bee venom (*Apis mellifera*) [31]. In the present study, the changes in the morphology of human colon cancer cells were examined using a 10X inverted microscope. Untreated HCT-116 and Caco2 cells were well adherent and distributed

in an epithelial form. HCT-116 and Caco-2 cells treated with cetuximab IC50 lost viability, lost their characteristic appearance, and began to shrink. Cells treated for 48 hours with IC50 of bee venom showed swelling and rupture. Finally, cells treated with combination treatment (Ce/BV) displayed apoptotic morphology, which included complete disintegration of the cell sheet, increased cytoplasmic density, and irregular shape. These morphological changes were noticeable in Figure 5 and Figure 6.



Figure 5. Microscopic examination of the effect of IC50 of single and combination treatments on HCT-116 cell line. The cells incubated with different treatments, (A) the untreated cells; (B) IC50 of cetuximab; (C) IC50 of combination (Ce/BV) treatment. The pictures were captured under an inverted microscope 10 X lens.

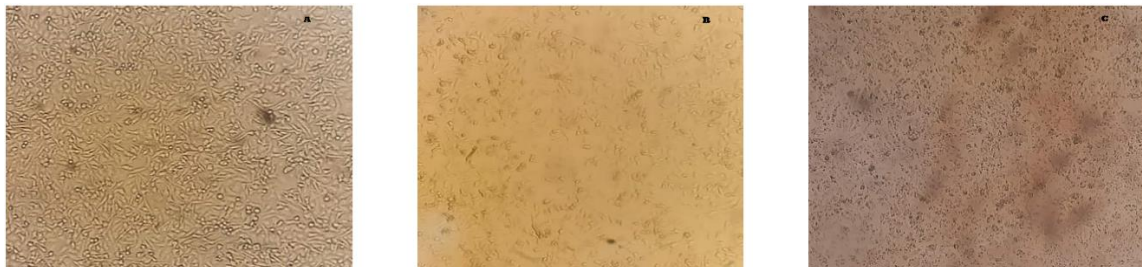


Figure 6. Microscopic examination of the effect of IC50 of single and combination treatments on Caco-2 cell line. The cells incubated with different treatments, (A) the untreated cells; (B) IC50 of cetuximab; (C) IC50 of combination (Ce/BV) treatment. The pictures were captured under an inverted microscope 10 X lens.

3.4. Flow cytometry analysis.

3.4.1. Evaluation of cells apoptosis.

Flow cytometry was used to assess whether or not The rate of apoptosis in both cells increased after treatment with the combination of bee venom and cetuximab compared with control cells (untreated cells) and to examine the effects of Ce/BV on HCT-116 and Caco-2 cell proliferation using 1/2 the IC50 value of cetuximab alone or a combination of them (1/2 IC50 Ce/BV). Annexin V-FITC staining was used to detect the apoptosis and necrosis rate in the cells. As shown in Figure 7,8, the rate of apoptosis was significantly increased in both cells after treatment with cetuximab alone or in combination with BV when compared to the control group also the rate of apoptosis was increased by 6.5% in Caco-2 and by 10 % in HCT-116. The results of cell death analysis by flow cytometry showed that the cell death induced by treatments (single or combined) is apoptosis. This is consistent with morphological changes mentioned above. Most cytotoxic drugs cause apoptosis in most cancer cells. The results of the present study confirm that the combination treatment (ce / BV) increases the rate of apoptosis compared with cetuximab alone these results agree with the results reported by DongJu Son *et al.* [26]

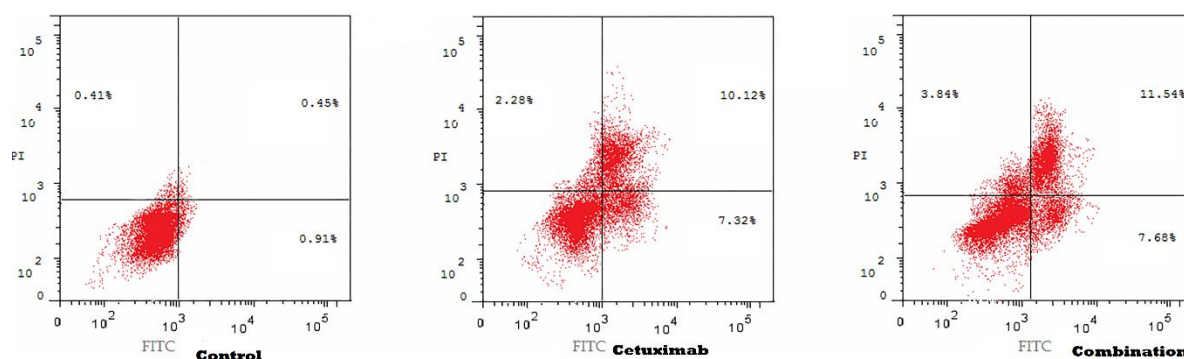


Figure 7. Apoptosis analysis in HCT-116 treated with cetuximab alone or combination treatment of cetuximab with bee venom for 48 hrs by using Annexin V-FITC Apoptosis Detection Kit.

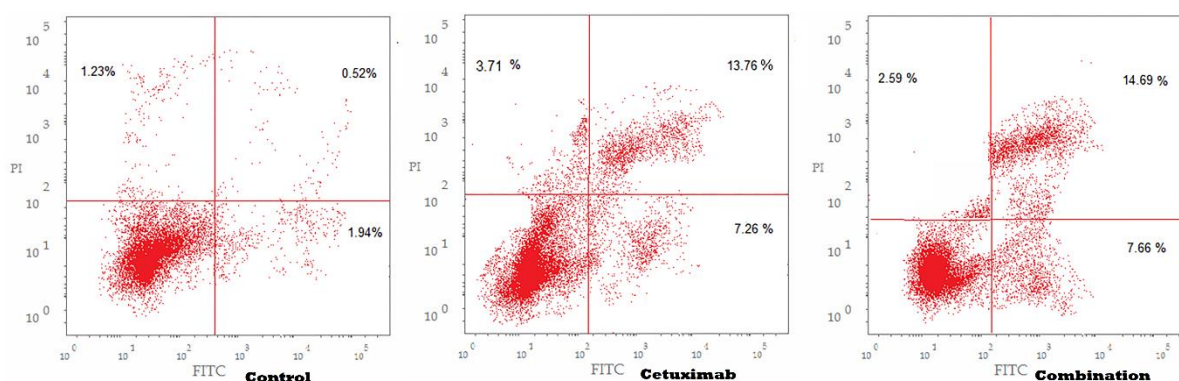


Figure 8. Apoptosis analysis in Caco-2 treated with cetuximab alone or combination treatment of cetuximab with bee venom for 48 hours using Annexin V-FITC Apoptosis Detection Kit.

3.4.2. Cell cycle analysis.

Add to that, the flow cytometry using Propidium iodide (PI) stain was performed to examine combination treatment and single treatment (Ce) on HCT116 & Caco2 cell proliferation with $\frac{1}{2}$ IC₅₀ of each of Ce and BV and combination of them (Comb B). Propidium iodide (PI) staining of the nuclei was carried out to assess the time course for the cell cycle. RNAase was added to eliminate RNA as PI can stain the latter, causing interference in results. Usually, the amount of PI incorporated is proportional to the amount of DNA. Thus, the fluorescence data correlates with the DNA content [32]. The cell cycle is also a main regulatory mechanism of cell growth, and many chemical compounds could lead to apoptosis in cancer cells accompanied by cell cycle arrest [33]. The present study indicated the effect of single and combined treatment on cell cycle arrest in two types of cancer cell lines (HCT 116 and Caco-2) after 48 hours by using flow cytometry. Cetuximab alone or combined with BV induced accumulation of cells in the G2/M phase of the cell cycle. This indicates that cetuximab and bee venom could inhibit cellular proliferation in 2 colon cancer cells. The cell cycle distribution in HCT-116 treated cells was shown in (Figure 9) and in Caco-2 cells as shown (Figure 10). The above-mentioned results agree with Gu Jiajia *et al.*, who demonstrated that cetuximab in combination with cisplatin in NPC cells induced G2/M1 arrest). When cells that have been subjected to the treatments have reached an early stage of apoptosis, these data suggest that BV-addition into cetuximab causes induced apoptosis rather than cell cycle arrest, which would explain the growth-inhibitory impact. It is worth noting that an increase in G2/M arrest has been associated with enhanced apoptosis, an indicator of a better cytotoxicity effect against

cancer cells [34]. So, the current results of our study are a confirmation of the cytotoxicity assay results.

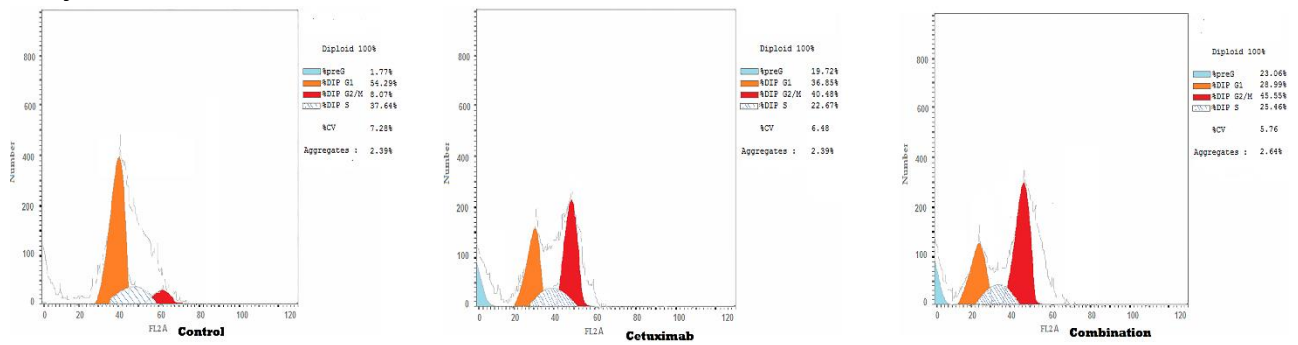


Figure 9. Changes of arrested cell cycle phases in HCT-116 cell line after treatment with cetuximab alone and combination treatment for 48 hrs using flow cytometry.

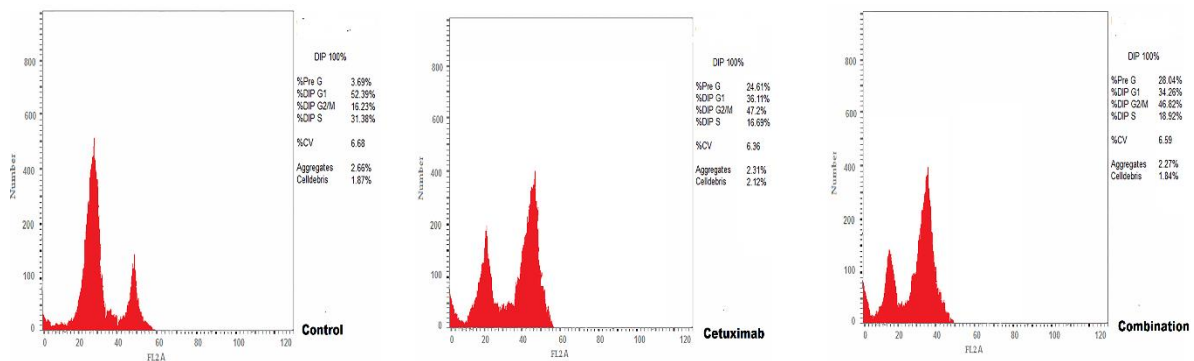


Figure 10. Changes of arrested cell cycle phases in Coca-2 cell line after treatment with cetuximab alone and combination treatment for 48 hrs using flow cytometry.

3.5. Estimation of apoptotic genes level by qRT- PCR.

The regulation and control of the apoptotic mitochondrial pathway (intrinsic pathway) regulation and control occur through pro-apoptotic or anti-apoptotic genes. The tumor suppressor gene p53 is a pro-apoptotic protein that stimulates apoptosis, and it is activated in the nucleus by DNA damage or oxidative stress. The increase in p53 level causes either cell cycle arrest or apoptosis, both of which inhibit tumor growth.[35]Bcl-2 is an anti-apoptotic protein (proliferative protein) that inhibits cell death and apoptosis.BCL2 protein is a major negative regulator in apoptosis. It plays a critical role in inhibiting the influx of adenine nucleotides through the outer mitochondrial membrane, reducing ATP hydrolysis and inhibiting cytochrome-C release. [36]. The initiation of apoptosis is explained by the upregulation of P53 gene expression and down-regulation of Bcl-2 gene expression

The results of gene expression levels for pro and anti-apoptotic P53 and BCL-2 genes in two colon cancer cell lines (Caco-2 and HCT-116 2) are demonstrated in Figures 11 and 12 after treatment with a combination of IC50 cetuximab/bee venom using RT-PCR. The results revealed a significant difference ($P<0.001$) in the expression level of the P53 gene in two cell lines (HCT-116 & Caco-2) between control cells and cells treated with the combination treatment, indicating upregulation of P53 relative to control by about 15-fold in Hct-116 and more than 15-fold in Caco2. There was also a significant difference ($P<0.001$) between cells treated with a single treatment (IC50 of cetuximab or BV) and cells treated with a combination treatment (IC50s of Ce/ BV). The results of the expression level of the BCL-2 gene indicated a strongly significant difference ($P<0.001$) in two cell lines (HCT-116 and Caco-2) between control cells and cells treated with combination treatment, indicating that BCL-2 was down-

regulated by roughly 0.14-fold in HCT-116 and 0.2-fold in Caco2. There was also a significant difference ($P < 0.005$) between cells treated with single IC50 cetuximab or BV treatments and cells treated with combination treatments. The above findings revealed that bee venom increased cetuximab-induced apoptosis in two cells, which was associated with an increase in pro-apoptotic gene expression (P53) and a decrease in proliferative and anti-apoptotic gene expression (Bcl-2)

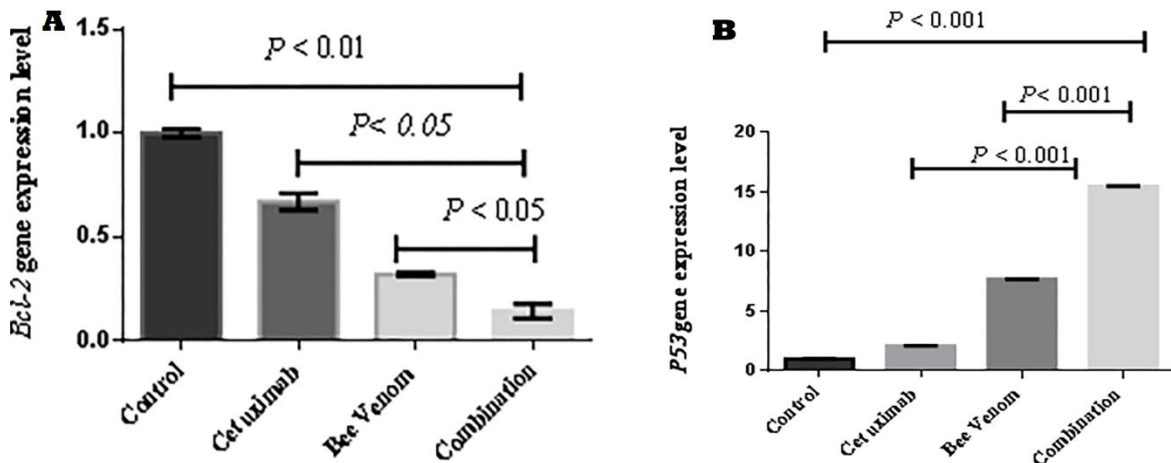


Figure 11. Gene's expression levels of (A) BCL-2 & (B) P53 gene in cell line HCT-116 treated with cetuximab or bee venom alone and combination treatment of them for 48 hrs by using real-time PCR.

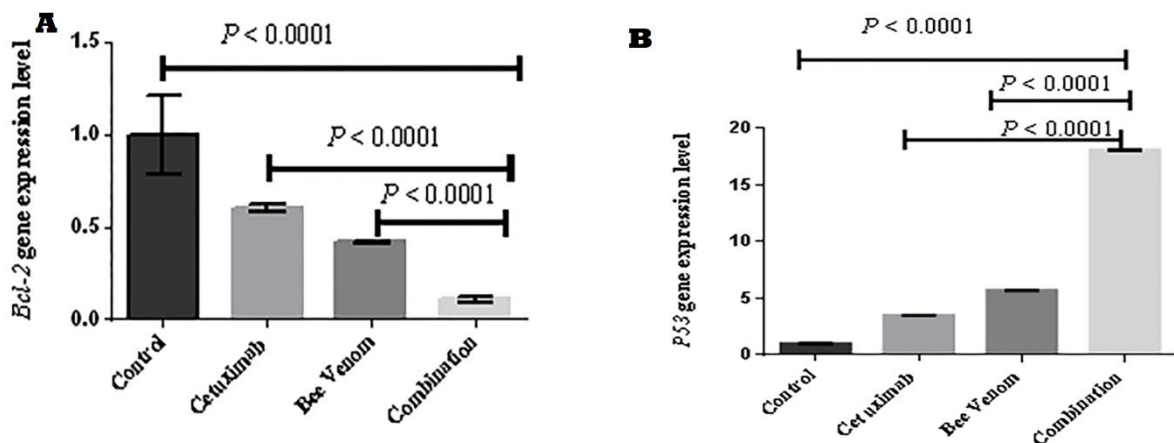


Figure 12. Gene's expression level of (A) BCL-2 gene & (B) P53 gene in cell lines Caco2 treated with cetuximab or bee venom alone and combination treatment of them for 48 hrs by using real-time PCR.

4. Conclusions

Based on the findings of this study, cetuximab combined with bee venom has a potential synergistic effect on cell growth suppression and cell apoptosis induction in human colon cancer cells. As a result, cetuximab and bee venom may be a new beneficial treatment for colon cancer with higher effectiveness and lower cost.

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Conflicts of Interest

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References

1. Kim, S.H.; Jang, S.H.; Jeon, H.J.; Choi, H.S.; Kim, E.S.; Keum, B.; Jeon, Y.T.; Chun, H.J.; Kim, J. Colonic stenting as a bridge to surgery for obstructive colon cancer: is it safe in the long term? *Surgical Endoscopy* **2022**, 1-9, <https://doi.org/10.1007/s00464-021-08789-0>.
2. Biller, L.H.; Schrag, D. Diagnosis and Treatment of Metastatic Colorectal Cancer: A Review. *JAMA* **2021**, 325, 669-685, <https://doi.org/10.1001/jama.2021.0106>.
3. El-Sayes, N.; Vito, A.; Mossman, K. Tumor Heterogeneity: A Great Barrier in the Age of Cancer Immunotherapy. *Cancers (Basel)* **2021**, 13, 806, <https://doi.org/10.3390/cancers13040806>.
4. Wu, F.; Yang, J.; Liu, J.; Wang, Y.; Mu, J.; Zeng, Q.; Deng, S.; Zhou, H. Signaling pathways in cancer-associated fibroblasts and targeted therapy for cancer. *Signal Transduct Target Ther* **2021**, 6, 218, <https://doi.org/10.1038/s41392-021-00641-0>.
5. Luca, T.; Barresi, V.; Privitera, G.; Musso, N.; Caruso, M.; Condorelli, D.; Castorina, S. In vitro combined treatment with cetuximab and trastuzumab inhibits growth of colon cancer cells. *Cell proliferation* **2014**, 47, 435-447, <https://doi.org/10.1111/cpr.12125>.
6. Qiu, Y.; Yin, X.; Li, X.; Wang, Y.; Fu, Q.; Huang, R.; Lu, S. Untangling dual-targeting therapeutic mechanism of epidermal growth factor receptor (EGFR) based on reversed allosteric communication. *Pharmaceutics* **2021**, 13, 747, <https://doi.org/10.3390/pharmaceutics13050747>.
7. Pirker, R. EGFR-directed monoclonal antibodies in non-small cell lung cancer. *Target Oncol* **2013**, 8, 47-53, <https://doi.org/10.1007/s11523-012-0244-7>.
8. Abdullah, M.i.N.; Ali, Y.; Abd Hamid, S. Insights into the structure and drug design of benzimidazole derivatives targeting the epidermal growth factor receptor (EGFR). *Chemical Biology & Drug Design* **2021**, <https://doi.org/10.1111/cbdd.13974>.
9. Nguyen, P.V.; Allard-Vannier, E.; Chourpa, I.; Herve-Aubert, K. Nanomedicines functionalized with anti-EGFR ligands for active targeting in cancer therapy: Biological strategy, design and quality control. *Int J Pharm* **2021**, 605, 120795, <https://doi.org/10.1016/j.ijpharm.2021.120795>.
10. You, B.; Chen, E.X. Anti-EGFR monoclonal antibodies for treatment of colorectal cancers: development of cetuximab and panitumumab. *J Clin Pharmacol* **2012**, 52, 128-155, <https://doi.org/10.1177/0091270010395940>.
11. Mohelnikova-Duchonova, B.; Melichar, B.; Soucek, P. FOLFOX/FOLFIRI pharmacogenetics: the call for a personalized approach in colorectal cancer therapy. *World J Gastroenterol* **2014**, 20, 10316-10330, <https://doi.org/10.3748/wjg.v20.i30.10316>.
12. You, K.S.; Yi, Y.W.; Cho, J.; Park, J.S.; Seong, Y.S. Potentiating Therapeutic Effects of Epidermal Growth Factor Receptor Inhibition in Triple-Negative Breast Cancer. *Pharmaceutics (Basel)* **2021**, 14, 589, <https://doi.org/10.3390/ph14060589>.
13. Bourhis, J.; Stein, A.; de Boer, J.P.; Van Den Eynde, M.; Gold, K.A.; Stintzing, S.; Becker, J.C.; Moran, M.; Schroeder, A.; Pennock, G. Avelumab and cetuximab as a therapeutic combination: An overview of scientific rationale and current clinical trials in cancer. *Cancer treatment reviews* **2021**, 97, 102172, <https://doi.org/10.1016/j.ctrv.2021.102172>.
14. Mendelsohn, J.; Baselga, J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol* **2003**, 21, 2787-2799, <https://doi.org/10.1200/JCO.2003.01.504>.
15. Chand, B.; Kumari, I.; Kumar, R. Evolution of Apiculture, History and Present Scenario. In *Honey*; CRC Press: 2021; pp. 1-22.
16. El-Hameed, A.; Mohamed, A.; Abuelsaad, A.S.; Khalil, A. Bee venom acupuncture therapy ameliorates neuroinflammatory alterations in a pilocarpine-induced epilepticus model. *Metabolic Brain Disease* **2021**, 36, 2047-2058, <https://doi.org/10.1007/s11011-021-00766-9>.

17. Liu, X.; Chen, D.; Xie, L.; Zhang, R. Effect of honey bee venom on proliferation of K1735M2 mouse melanoma cells in-vitro and growth of murine B16 melanomas in-vivo. *J Pharm Pharmacol* **2002**, *54*, 1083-1089, <https://doi.org/10.1211/002235702320266235>.
18. Lv, S.; Sylvestre, M.; Song, K.; Pun, S.H. Development of D-melittin polymeric nanoparticles for anti-cancer treatment. *Biomaterials* **2021**, *277*, 121076, <https://doi.org/10.1016/j.biomaterials.2021.121076>.
19. Tang, F.; Wu, H.; Zheng, J.; Guo, H.; Yan, L.; Lin, Y.; Xu, C.; Hu, S. Effect of melittin on iNOS and NF-kappaB expression induced by IL-1betain C518 cells. *Pak J Pharm Sci* **2021**, *34*, 95-101.
20. Bhandari, A.; Woodhouse, M.; Gupta, S. Colorectal cancer is a leading cause of cancer incidence and mortality among adults younger than 50 years in the USA: a SEER-based analysis with comparison to other young-onset cancers. *Journal of Investigative Medicine* **2017**, *65*, 311-315, <http://dx.doi.org/10.1136/jim-2016-000229>.
21. Yuan, J.S.; Reed, A.; Chen, F.; Stewart, C.N., Jr. Statistical analysis of real-time PCR data. *BMC Bioinformatics* **2006**, *7*, 85, <https://doi.org/10.1186/1471-2105-7-85>.
22. Oh, Y.J.; Hong, J. Application of the MTT-based colorimetric method for evaluating bacterial growth using different solvent systems. *LWT* **2022**, *153*, 112565, <https://doi.org/10.1016/j.lwt.2021.112565>.
23. Sabra, R.; Billa, N.; Roberts, C.J. Cetuximab-conjugated chitosan-pectinate (modified) composite nanoparticles for targeting colon cancer. *Int J Pharm* **2019**, *572*, 118775, <https://doi.org/10.1016/j.ijpharm.2019.118775>.
24. Eichelmann, F.; Schulze, M.B.; Wittenbecher, C.; Menzel, J.; Weikert, C.; di Giuseppe, R.; Biemann, R.; Isermann, B.; Fritsche, A.; Boeing, H.; Aleksandrova, K. Association of Chemerin Plasma Concentration With Risk of Colorectal Cancer. *JAMA Netw Open* **2019**, *2*, e190896, <https://doi.org/10.1001/jamanetworkopen.2019.0896>.
25. Yaacoub, C.; Rifi, M.; El-Obeid, D.; Mawlawi, H.; Sabatier, J.M.; Coutard, B.; Fajloun, Z. The Cytotoxic Effect of Apis mellifera Venom with a Synergistic Potential of Its Two Main Components-Melittin and PLA2-On Colon Cancer HCT116 Cell Lines. *Molecules* **2021**, *26*, 2264, <https://doi.org/10.3390/molecules26082264>.
26. Son, D.J.; Hong, J.E.; Ban, J.O.; Park, J.H.; Lee, H.L.; Gu, S.M.; Hwang, J.Y.; Jung, M.H.; Lee, D.W.; Han, S.B.; Hong, J.T. Synergistic Inhibitory Effects of Cetuximab and Cisplatin on Human Colon Cancer Cell Growth via Inhibition of the ERK-Dependent EGF Receptor Signaling Pathway. *Biomed Res Int* **2015**, *2015*, 397563, <https://doi.org/10.1155/2015/397563>.
27. Alonezi, S.; Tusiimire, J.; Wallace, J.; Dufton, M.J.; Parkinson, J.A.; Young, L.C.; Clements, C.J.; Park, J.K.; Jeon, J.W.; Ferro, V.A.; Watson, D.G. Metabolomic Profiling of the Synergistic Effects of Melittin in Combination with Cisplatin on Ovarian Cancer Cells. *Metabolites* **2017**, *7*, 14, <https://doi.org/10.3390/metabo7020014>.
28. Chou, T.C.; Motzer, R.J.; Tong, Y.; Bosl, G.J. Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. *J Natl Cancer Inst* **1994**, *86*, 1517-1524, <https://doi.org/10.1093/jnci/86.20.1517>.
29. Reynolds, C.P.; Maurer, B.J. Evaluating response to antineoplastic drug combinations in tissue culture models. *Methods Mol Med* **2005**, *110*, 173-183, <https://doi.org/10.1385/1-59259-869-2:173>.
30. Chen, C.F.; Lu, C.C.; Chiang, J.H.; Chiu, H.Y.; Yang, J.S.; Lee, C.Y.; Way, T.D.; Huang, H.J. Synergistic inhibitory effects of cetuximab and curcumin on human cisplatin-resistant oral cancer CAR cells through intrinsic apoptotic process. *Oncology letters* **2018**, *16*, 6323-6330, <https://doi.org/10.3892/ol.2018.9418>.
31. Abdel-Aziz, S.A.A.; Mohamed, A.F.; Zahouk, S.A.M.; Ali, R.A.M. Evaluation of Anticancer Activity of Some Venomous Animal Toxins on Human Breast and Colon Cancer Cell Lines and Related Antioxidant Profile. *International Journal of Advanced Research* **2017**, *5*, 2036-2053, <https://doi.org/10.21474/ijar01/3376>.
32. Wang, F.; Wang, Y.-H.; Wang, J.-J.; Xu, H.-L.; Wang, C.-M. Eriodictyol-induced anti-cancer and apoptotic effects in human hepatocellular carcinoma cells are associated with cell cycle arrest and modulation of apoptosis-related proteins. *Bangladesh Journal of Pharmacology* **2016**, *11*, 285-291, <https://doi.org/10.3329/bjp.v11i2.25549>.
33. Gu, J.; Yin, L.; Wu, J.; Zhang, N.; Huang, T.; Ding, K.; Cao, H.; Xu, L.; He, X. Cetuximab and cisplatin show different combination effect in nasopharyngeal carcinoma cells lines via inactivation of EGFR/AKT signaling pathway. *Biochemistry research international* **2016**, *2016*, <https://doi.org/10.1155/2016/7016907>.

34. DiPaola, R.S. To arrest or not to G2-M Cell-cycle arrest: commentary re: AK Tyagi et al., Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis. *Clinical cancer research* **2002**, *8*, 3311-3314.
35. Kruiswijk, F.; Labuschagne, C.F.; Vousden, K.H. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat Rev Mol Cell Biol* **2015**, *16*, 393-405, <https://doi.org/10.1038/nrm4007>.
36. Zhou, J.D.; Zhang, T.J.; Xu, Z.J.; Gu, Y.; Ma, J.C.; Li, X.X.; Guo, H.; Wen, X.M.; Zhang, W.; Yang, L.; Liu, X.-h.; Lin, J.; Qian, J. BCL2 overexpression: clinical implication and biological insights in acute myeloid leukemia. *Diagn Pathol* **2019**, *14*, 68, <https://doi.org/10.1186/s13000-019-0841-1>.