

Cucurbitacin-B Triggers Fas/FasL-dependent Apoptosis via the Death Receptor Signalling Pathway in Human Breast Cancer Cell Line - MCF-7

Jawaher Amer Alsaiari, Abdulraheem A. Almalki, Jawaher Ayidh Alosaimi, Raja Mohammed Al-Qurashi, Bedour Abdullah Al-Qurashi, Fatima Anwar Dhafer, Ahad Amer Alsaiari*

Department of Clinical Laboratory Science, College of Applied Medical Science, Taif University, Taif, SAUDI ARABIA.

ABSTRACT

Background: Globally, Breast Cancer (BC) ranks as the second most common cause of cancer-related death for women. The high mortality and recurrence rates contribute substantially to its fatality. **Objectives:** This research focused to investigate the anti-cancer potential of Cucurbitacin-B (Cu-B) against the human breast cancer cell line MCF-7, focusing on the Fas/FasL-mediated death receptor signalling pathway. **Materials and Methods:** The anti-proliferative effect of Cu-B was assessed using the tetrazolium-based MTT assay, while morphological alterations were observed through phase-contrast microscopy. Apoptotic features were further examined using Acridine Orange/Ethidium Bromide (AO/EtBr) dual staining, and intracellular Reactive Oxygen Species (ROS) levels were quantified with DCFH-DA fluorescence assay. Nuclear alterations were investigated by DNA fragmentation analysis, and FasL gene expression was quantified by qPCR. **Results:** MTT assay revealed that Cu-B exhibited an IC_{50} value of $74.836 \mu\text{g/mL}$. Morphological assessment demonstrated pronounced cellular shrinkage and structural disintegration. AO/EtBr staining confirmed an increased proportion of apoptotic cells in Cu-B-treated groups, which was corroborated by the elevated ROS of 7750 ± 901.38 in the treatment group compared to 1333.33 ± 125.83 in the control group, as indicated by DCFH-DA assay. DNA laddering further confirmed nuclear damage, and qPCR analysis showed significant upregulation of FasL expression in treated cells, supporting the activation of death receptor-mediated apoptosis. **Conclusion:** Cucurbitacin-B demonstrated potent anti-cancer activity against MCF-7 cells. The findings suggest that Cu-B induces apoptosis primarily through the extrinsic pathway by activating Fas/FasL-mediated death receptor signalling.

Keywords: Breast Cancer, Cucurbitacin B, Death Receptor Signal, Extrinsic Apoptosis Pathway, Fas/FasL, MCF 7.

Correspondence:

Dr. Ahad Amer Alsaiari

Department of Clinical Laboratory Science, College of Applied Medical Science, Taif University, P.O. Box 11099, Taif-21944, SAUDI ARABIA.
Email: ahadamer@tu.edu.sa

Received: 09-03-2026;

Revised: 15-05-2026;

Accepted: 29-07-2026.

INTRODUCTION

Breast Cancer (BC) ranks second among other cancers in women, and its occurrence and lethality rates continue to rise each year. In 2022, 2.3 million cases were recorded, with approximately 600,000 deaths worldwide.^{1,2} Despite the availability of advanced therapeutic options, the five-year survival rate remains limited, particularly in Asian and African countries, where it is reported at about 66% and 40%, respectively. According to recent statistics by the WHO, BC cases are expected to increase by 40% in 2050.³ The late diagnosis is the primary cause of the complexity of the disease, which leads to incurability. Focusing on proper nutrition,

early detection, and precise treatment strategies is therefore critical.⁴ However, conventional therapies are often associated with severe side effects, which compromise patients' quality of life and recovery.⁵ To overcome these challenges, phytoactive compounds with strong pharmacological profiles have been increasingly investigated for their potential in cancer therapy over the past few decades.⁶

Cucurbitacin B (Cu-B) is an oxygenated triterpenoid derived from the Cucurbitaceae family, traditionally used in Chinese medicine for its hepatoprotective effects. Among the various derivatives of this family, Cu-B is recognized as a prominent bioactive compound with a broad pharmacological profile, including antioxidant, anti-inflammatory, hypoglycaemic, hepatoprotective, antiviral, anticancer and neuroprotective.^{7,8} The anticancer potential of Cu-B has been demonstrated in several *in vitro* models, including myeloma, liver, breast, pancreatic, osteosarcoma, and bladder cancer cells.^{6,9,10} Previous studies have shown that Cu-B modulates multiple apoptotic signalling pathways across different cancer types. For instance, it suppresses JAK/STAT3, MAPK and Raf/



DOI: 10.5530/ijper.20264032

Copyright Information :

Copyright Author (s) 2026 Distributed under
Creative Commons CC-BY 4.0

Publishing Partner : Manuscript Technomedia. [www.mstechnomedia.com]

MEK/ERK signalling in leukemic, pancreatic, and osteosarcoma cells.^{8,9,11-14} In breast cancer (MCF-7) cells, Cu-B has been reported to inhibit proliferation and instigate apoptosis through mechanisms involving the Wnt/ β -catenin pathway, the MAPK pathway (via EGFR), and JAK/STAT3 signalling.^{15,16} However, to date, no study has specifically investigated whether Cu-B also induces apoptosis in MCF-7 cells through the extrinsic death receptor pathway mediated by Fas/FasL.

Fas (Factor-associated suicide) and its ligand, FasL, play a key role in initiating apoptosis. The binding of Fas receptors with FasL on the cell surface activates downstream signaling events that trigger a cascade of caspase activations.^{17,18} In mammalian cells, apoptosis occurs through two main pathways: the extrinsic pathway, mediated by transmembrane death receptors such as TNFR1, Fas, and TRAIL, and the intrinsic pathway, regulated by mitochondria. Key caspases, including 3, 6, 7, 8, 9, and 10, serve as effectors and executioners in Fas/FasL-mediated apoptosis.^{19,20} Importantly, there is cross-talk between these pathways, as activation of caspase-8 can cleave Bid into truncated Bid (tBid), thereby linking the extrinsic pathway with mitochondrial-mediated apoptosis through caspase-3.^{17,21,22} Based on this, the present dissertation is framed to exploit the potential of Cucurbitacin-B (Cu-B) to activate the Fas/FasL receptor-ligand complex and induce death receptor-mediated apoptosis in human breast cancer MCF-7 cells. This objective was achieved through the investigation of antiproliferation potency of Cu-B by MTT assay, apoptosis by dual staining assay such as AO/EtBr, ROS release assay determined by DCFH-DA fluorophore dye, DNA fragmentation analysis and FasL gene expression by qPCR method.

MATERIALS AND METHODS

Materials

Cucurbitacin-B was purchased from Sigma Aldrich, Catalog number - PHL82226, Dulbecco's Modified Eagle's Medium (DMEM - Sigma Aldrich, USA), sodium bicarbonate (Merck, Germany), Acridine Orange (AO) and Ethidium Bromide (EtBr) (Sigma, USA), and DCFH-DA from Sigma Aldrich, USA.

Methods

Cell line procurement

Human Breast cancer cell line MCF-7 was acquired from ATCC. Cells were maintained and cultured using 10% Dulbecco's modified Eagles' medium supplemented with Sodium bicarbonate and L-Glutamine. Penicillin (100 U/mL) /Streptomycin (100 μ g/mL) - Amphotericin B (2.5 μ g/mL) solution was added to prevent contamination. The cultured cells were incubated at 37°C in a CO₂ incubator.

Cytotoxicity assessment

Cytotoxicity assessment of Cucurbitacin B (Cb-B) is the primary step to confirm its extended anti-cancer potency. For this purpose, the MTT method was employed from Laura B. Talarico *et al.* (2004) with slight modifications.²³ The cells were seeded in a 96-well cell culture plate, with each well containing 5000 cells, and incubated to reach a confluency of 70 - 80%. The Cb-B drug was prepared as a 1 mg/mL stock in 0.1% DMSO, vortexed, and filtered through a 0.22 μ m syringe filter to ensure sterility. From the stock solution, the compound was diluted to 6.25, 12.5, 25, 50, and 100 μ g/mL and added to the wells as triplicates, then incubated for 24 hr. The treated and control cells were observed for the adverse effect after 24 hr of incubation, then the media with the drug was aspirated and freshly prepared 2% media containing 5 mg/mL of MTT reagent was introduced to each well. The plate was incubated for 2 - 4 hr at 37°C in a CO₂ incubator in the dark. The media with MTT solution was discarded and the purple-blue stained cells were observed. Absolute DMSO of 200 μ L was added to each well for the purpose of solubilization of the purple-blue complex by pipetting up and down. The colored solution was read at 540 nm using an ELISA reader and the results were expressed as % viability using the formula below:

$$\% \text{ Viability} = (\text{Mean OD value of sample} / \text{Mean OD value of control}) \times 100$$

Morphometric analysis

The cells of appropriate count were seeded in a 6-well plate, and the cells of required confluence were treated with different concentrations as given in the MTT assay, such as 6.25, 12.5, 25, 50 and 100 μ g/mL, to observe the morphometric deterioration of the cells. The treated and untreated groups were incubated for 24 hr. The cells were observed under a Phase contrast inverted microscope (Olympus CKX41 with Optika Pro5 CCD camera) and the images were captured.²⁴

AO/EtBr double staining method

Acridine Orange and Ethidium Bromide stains were used in this procedure, where both dyes were DNA-binding dyes. Acridine Orange will be taken up by viable and non-viable cells, whereas Ethidium Bromide will be taken up only by non-viable cells by intercalating with DNA. This double staining method was employed to analyse the cell's apoptotic and necrotic response upon treatment with Cb-B. The method was followed by Liu K *et al.*, 2015 with brief changes.²⁵ Cells were seeded as required, as per the standard protocol and incubated until confluency. The cells were treated with Cu-B, such as 74.83 μ g/mL (IC₅₀ dosage), and untreated cells were kept as a control. After 24 hr of incubation, the cells were washed with ice-cold PBS (Phosphate-Buffered Saline) twice. Then stained with AO and EtBr stains of 100 μ g/mL for both the dye and incubated for 10 - 15 min at RT in the dark. The stained cells were subjected to washing with PBS twice

for the removal of excessive unbound stains, then subjected to observation with a fluorescence microscope (Olympus CKX41 with Optika Pro5 camera) in blue filter, and the images were captured.

DNA fragmentation assay

Cells of the required count were seeded in a T25 flask and incubated to reach the maximum confluency. The IC_{50} concentration of Cb-B (74.83 $\mu\text{g/mL}$) was added and incubated for 72 hr, and the untreated group was maintained in parallel. DNA isolation was performed by the protocol followed by Yadav *et al.*, 2022.²⁶ Treated and control cells were trypsinised and a pellet was obtained by centrifugation. The pellet was washed with ice-cold 1X PBS by centrifugation. The finally obtained cell pellet was resuspended with digestion buffer (sodium chloride - 100 mM, Tris - 10 mM (pH 8.0), EDTA - 25 mM (pH 8.0), SDS - 10% and Proteinase K - 0.1 mg/mL) and incubated for 12 -18 hr at 50°C with constant rotation. Digested pellet extracted with Phenol:Chloroform:Isoamyl alcohol of 25:24:1 by centrifugation at 12000 rpm for 10 - 15 min. The aqueous layer was transferred to the new tube and added with 7.5 M ammonium acetate of 50 μL and twice the volume of absolute ethanol. The contents were centrifuged for 2 min at 12,000 rpm. The pellet was washed with 70% ethanol by centrifugation, and residual ethanol was decanted. Then, the pellet was dried for 5 min, followed by resuspension with TE buffer. The DNA suspension was confirmed by Agarose gel electrophoresis. For this, 1% agarose was prepared with 1X TAE buffer by boiling, and 5 μL of 10 mg/mL Ethidium Bromide was added when the temperature of the agarose was hand bearable. The sample and the DNA ladder were loaded with 1X gel loading buffer once the gel solidified. The Agarose gel was initiated to run at 50 V. The process was terminated once the gel loading dye reached 3/4th of the gel. Then the gel was visualised under a gel documentation system (E-Gel Imager, Invitrogen) for the qualitative observation of DNA bands, and the image was captured.

ROS determination by the DCFH-DA method

The cells were seeded as required by the standard protocol in a clear aqueous layer in a 96-well cell culture plate and incubated until 80% confluency. Cells were treated with the IC_{50} concentration of Cb-B (74.83 $\mu\text{g/mL}$) for 24 hr; an untreated control was also maintained. A 10 mM stock solution of DCFH-DA was prepared in methanol. Then, the working solution of 20 mM was prepared by diluting the 10 mM stock solution with Hank's Balanced Salt Solution (HBSS) at a 500-fold dilution without serum and other additives. The treated and untreated cells were washed twice with HBSS to remove the media and drug. The cells received 0.1 mL of 20 mM DCFH-DA working stock in each well and were incubated for 30 min at 37°C. Then, the cells were observed under a fluorescence microplate reader at the excitation and emission

wavelengths of 485 nm and 520 nm, respectively. The data obtained were compiled and interpreted.²⁴

Gene expression by qPCR

Cells were seeded in a T25 flask at 5×10^5 cells and incubated for 24 hr to attain maximum confluency. The flasks were treated with an IC_{50} concentration of Cb-B as 74.83 $\mu\text{g/mL}$ for 24 hr, a control/untreated flask was also maintained in parallel. Treated and untreated cells were collected by TRIzol reagent (Total RNA isolation kit - Invitrogen - Product code: 10296010) by centrifugation. 200 μL of chloroform was added to the pellet, followed by centrifugation at 14,000 rpm for 15 min at 4°C. The Clear aqueous layer was collected in a new tube, and absolute isopropanol 0.5 mL was added, and incubated for 10 min, then centrifuged under the same conditions. The pellet obtained was washed with 200 μL of 75% ethanol, followed by centrifugation. The final pellet was dried and resuspended with TE buffer. Qualitative analysis was done using a 1.5% agarose gel by agarose gel electrophoresis and quantitative determination was also performed to check its purity and concentration. cDNA synthesis was carried out using the iScript cDNA synthesis kit (Bio-Rad, Cat.No: 1708891). 5X iscript reaction mix - 4 μL , Reverse transcriptase enzyme - 1 μL , RNA template - 0.5 μg of total RNA were added and the final volume was made up to 20 μL with nuclease-free water and mixed gently. The steps for cDNA synthesis were followed: Priming (25°C, 5 min), cDNA synthesis (46°C, 20 min) and inactivation (95°C, 1 min) each for one cycle. Further, the cDNA synthesised was used for gene expression analysis by qPCR. SYBR Green Master Mix (G Biosciences, Product code: 786-5062) was utilised in the process of qPCR analysis performed using LightCycler 96 (Roche). The sequence of the gene of interest is given in Table 1. PCR conditions - 2 min of initial denaturation at 95°C, followed by 10 sec of denaturation at the same temperature. Then, Annealing for 1 min at 58°C, extension for 1 min at 72°C and the cycles set for 40 counts. All reactions were triplicated, and data were recorded and calculated using the $\Delta\Delta C_t$ method with LightCycler 96 SW 1.1 software.

Statistical assessment

Graphpad PRISM software version 10 was used to analyse the statistical significance of the data obtained. All the experiments were run in triplicate, and the values are provided as Mean $\pm$ SE. One-way ANOVA was used for the statistical analysis, and Dunnett's multiple comparison test was performed to compare the significance between the treated and control groups. $p < 0.01$ is considered significant.

RESULTS

Cytotoxicity assessment

Cytotoxicity potential of Cb-B was analyzed with the help of the MTT reagent method. Figure 1 shows the result of the MTT assay.

The figure confirms that the cytotoxic effect of Cb-B was highly dose-dependent. The viability percentage decreased significantly as the concentration of the drug increased. A negligible effect was observed at 6.25 µg/mL (98.24±0.41% viability), which was not significantly different from the control group. The remaining groups (12.5, 25, and 50 µg/mL) showed a gradual decrease in viability, with values of 86.62±1.40, 76.32±0.56, and 63.56±0.42, respectively, and a highly significant difference from the control group ($p < 0.001$). The sudden decrease was observed at 100 µg/mL, as 36.25±1.24 was also highly significant compared with the control group. Therefore, the dosage responsible for the 50% viability (IC_{50} of Cb-B) was determined using ED₅₀ PLUS V1.0 software as 74.83 µg/mL. The vehicle (DMSO) exhibits no cytotoxicity effect, while the standard anticancer drug Doxorubicin exhibited an IC_{50} value at 0.531 µg/mL (Data not provided).

Morphometric analysis

The wells of treated and untreated cells were observed under a microscope, and Figure 2 depicts the anti-cancer effect of Cb-B on the MCF-7 cell line. Control cells exhibited a distinct morphology, whereas the higher dosages demonstrated cell shrinkage, granulation, vacuolization in the cytoplasm, cell detachment, and cell aggregation, which indicates the apoptosis of cells. The deterioration scale range was increased in a dose-dependent manner.

AO/EtBr - Double staining method

The dual staining AO/EtBr-based assay was performed to confirm the strong confirmation of apoptosis and necrosis response of MCF-7 cells once treated with the Cb-B drug. The resulting images are shown in Figure 3. Image (A) depicted the control/Untreated group, where the cells emit highly green fluorescence, indicating the high percentage of viable cells. In contrast, image (B), the treated group showed green fluorescence - living cells with normal nucleus, green fluorescence (bright) - early apoptotic cells (with condensed or fragmented nucleus), orange fluorescence - late apoptotic cells (with condensed or fragmented nucleus) and orange fluorescence (bright) - necrotic cells. The dual staining method once again confirmed the anti-cancer efficiency of the Cb-B drug.

DNA Fragmentation analysis

DNA Fragmentation analysis was carried out on treated and untreated MCF-7 cells using a conventional protocol. Figure 4 depicts the DNA bands obtained in the process of DNA isolation from the treated and control groups. The gel image indicated that the control group exhibited a well-defined DNA band. Cb-B-treated groups showed multiple DNA fragments, confirming the nucleus condensation and deterioration. The result substantiated the previous results.

ROS determination - DCFH-DA method

Reactive oxygen species estimation is one of the major checkpoints in the case of apoptosis. The imbalance and elevated ROS will collapse almost all the basic cellular phenomenon. To analyze the ROS level, DCFH-DA stain was utilized. The values obtained in the fluorescence reader are shown in Figure 5. From the figure, it is evident that the ROS level was elevated to 7750±901.38 in the Cb-B-treated group, whereas the control group showed 1333.33±125.83. The elevated ROS level demonstrated an imbalance in cellular homeostasis, which will lead the cells towards the apoptotic pathway. The treated group showed a significant difference with the control group at-value 0.000129.

Gene expression by qPCR

The anticancer efficacy of Cb-B was confirmed at the molecular level by measuring gene expression of the key activator gene FASL. FASL is the key player in the initiation of the caspase-dependent pathway, mainly the extrinsic pathway. The analysis of FASL gene expression is depicted in Figures 6A and 6B. 6A shows increased FASL gene expression, as evidenced by the brighter band in the treated group (Lane ii) compared to the control group (Lane i). A similar pattern was also observed in the qPCR output shown in Figure 6B, where FASL gene expression was significantly higher than in the untreated group, as assessed by the housekeeping gene GAPDH. Hence, the apoptosis was further highlighted at the molecular level in the MCF-7 cell line.

DISCUSSION

Breast Cancer (BC) commonly originates in the milk-producing lobules, and while early detection significantly improves curability, delayed diagnosis often results in metastasis, making the disease life-threatening.² BC is the second most prevalent cancer among women worldwide, with 2.3 million new cases and approximately 670,000 deaths reported in 2022. Worryingly, global projections

Table 1: Gene sequence.

Sequences	Forward (5' ->3')	Tm	Reverse (5' ->3')	Tm
Gene of interest				
FASL	ATGTTTCAGCTCTTCCACCTACAGA	61.3	CCAGAGAGAGCTCAGATACGTTGAC	64.6
GAPDH	ACTCAGAAGACTGTGGATGG	57.3	GTCATCATACTTGGCAGGTT	55.3

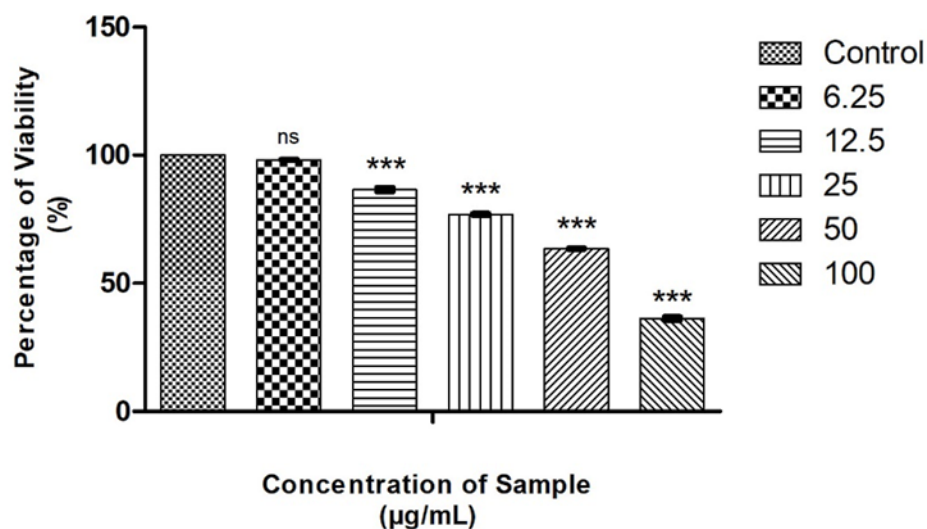


Figure 1: MTT assay: Figure depicts the anti-cancer effect of Cb-B, where the X axis - concentration of Cb-B at µg/mL, Y axis - % Viability. All the experiments were triplicated and expressed as Mean±SE. The data were subjected to one-way ANOVA and comparison was performed by Dunnet's multiple comparison test. Low dosage 6.25 µg/mL exhibited non-significant with the control group, whereas other increased dosages were highly significant with the control group (** $p < 0.001$). ns: non-significant.

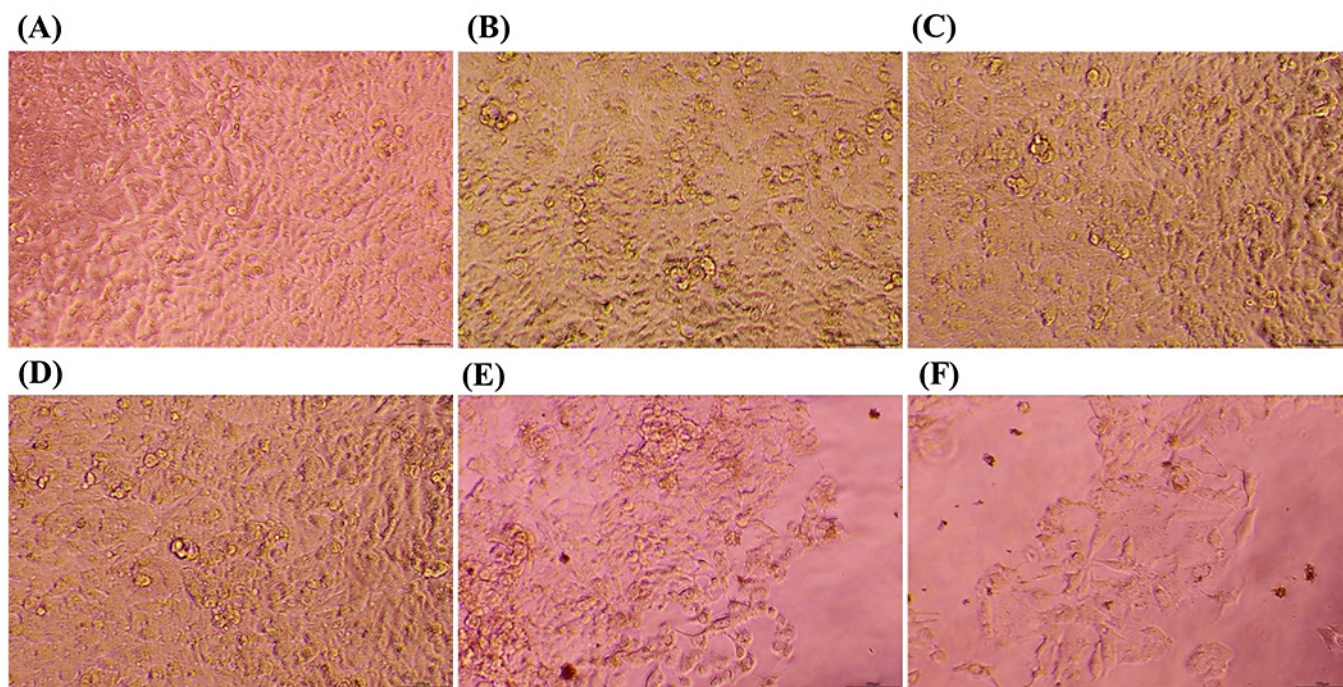


Figure 2: Morphometric observation: Pictorial representation of the morphometric deterioration of Cb-B on MCF-7 cells. Control - (A), 6.25 µg/mL - (B), 12.5 µg/mL - (C), 25 µg/mL - (D), 50 µg/mL - (E) and 100 µg/mL - (F).

estimate a 38% increase in incidence and a 68% rise in mortality by 2050. To address this, the World Health Organisation (WHO) has set a target of a 2.5% annual reduction in BC-related deaths.¹ Current treatment strategies include chemotherapeutic agents such as doxorubicin and paclitaxel, hormonal therapies including tamoxifen and aromatase inhibitors, targeted drugs like trastuzumab and palbociclib, and various immune-based

approaches.²⁷ Molecularly, Estrogen Receptor (ER) and Human Epidermal growth factor Receptor 2 (HER2) are critical in BC development and progression; however, around 20% of cases are negative for both receptors, rendering them unresponsive to hormonal therapy.²⁸ Furthermore, conventional therapies are frequently associated with drug resistance and toxicity, which compromise survival and recovery. These limitations emphasize

the urgent need for improved diagnostic strategies and the exploration of novel therapeutic options with higher efficacy and reduced side effects.

In recent years, herbal-derived therapeutics have gained considerable attention in cancer management due to their efficacy and relatively minimal side effects. Cucurbitacin B (Cu-B), a triterpenoid compound long utilised in traditional Chinese medicine, exhibits an extended pharmacological profile including anti-inflammatory, antiviral, hepatoprotective, antioxidant, and anticancer activities. The oxygenated carbon framework of Cu-B contributes to its strong antioxidant potential, which is critical to its anticancer action. Extensive studies have demonstrated the anticancer effects of Cu-B in various malignancies, such as hepatocarcinoma, myeloma, osteosarcoma, bladder, pancreatic, and breast cancers, primarily through the modulation of key signalling pathways that drive apoptosis. However, the role of Cu-B in activating the Fas/FasL-mediated death receptor pathway in breast cancer remains largely unexplored. Therefore, the present study focuses on elucidating the involvement of Fas/FasL signalling in Cu-B-induced apoptosis in human breast cancer MCF-7 cells.

Cucurbitacin B (Cu-B)- treated cells subjected to cytotoxicity assessment with the MTT reagent showed an increased inhibition in a dose-related manner. IC_{50} value was determined as 74.836 μ g/mL. The results indicated that Cu-B has the potential to inhibit the progression of MCF-7 cells. Morphological observation showed the detached and shrunken cells upon treatment with Cu-B, which also confirms the deformities of the cells. Previous studies have also highlighted the cytoskeletal alterations, morphological changes and microtubule aggregations in melanoma and glioblastoma cells treated with Cu-B.⁸ Wakimoto *et al.*, 2008 noticed rapid morphological changes in breast cancer cell lines such as cell refractility, clumping, disruption of microtubules, cell shrinkage and detaching. This disruption of the cytoskeletal

framework leads to growth inhibition due to loss of cell-to-cell communication, cell invasiveness, and cell cycle arrest, leading to apoptosis.²⁹

Dual staining was performed for the treated and untreated group by (AO/EtBr) dye through fluorescence microscopic examination, which revealed the augmented count of apoptotic cells in the treated group compared to the control group, suggesting the anti-proliferative property of Cu-B at IC_{50} concentration. Similarly, reactive oxygen species release was elevated in the treatment group, confirming a homeostatic imbalance in BC cells upon treatment with Cu-B. The release of unstable free radicals indicates that the imbalances in oxidative stress lead to disturbances in all cellular events. ROS is the primary biomarker; the increase is directly or indirectly involved in the dilation of nucleic acids, lipids, and peptides, leading to apoptosis. Alafnan *et al.*, 2022 reported that the ROS-mediated oxidative stress noticed in the DCFH-DA fluorescence method impacted on the apoptosis of PC3 cells.³⁰ An augmented ROS level also disrupts the mitochondrial membrane potential, paving the way for DNA damage to lead to apoptosis.⁷

DNA was isolated from the treated and untreated groups; in the treated group, fragmented DNA was visualized, while the control exhibited a distinct DNA band. This further confirms that Cu-B treatment disturbs and damages the DNA, which may be due to the massive release of ROS as stated before. Excessive ROS may interrupt all cellular components and cellular events, ultimately leading to apoptosis. Previous research with Cu-B on BC cells also reported DNA damage and identified the causative factor as excessive ROS release. Free radicals have a pronounced impact in the induction of DNA damage and lead to cell cycle arrest.¹⁵ Similarly, the other triterpenoids such as Ganoderic acid, oleanolic acid and urosolic acid instigate the DNA fragmentation in MCF-7 cell line resulted in mitochondrial membrane potential loss which will further generate pro apoptotic signals via

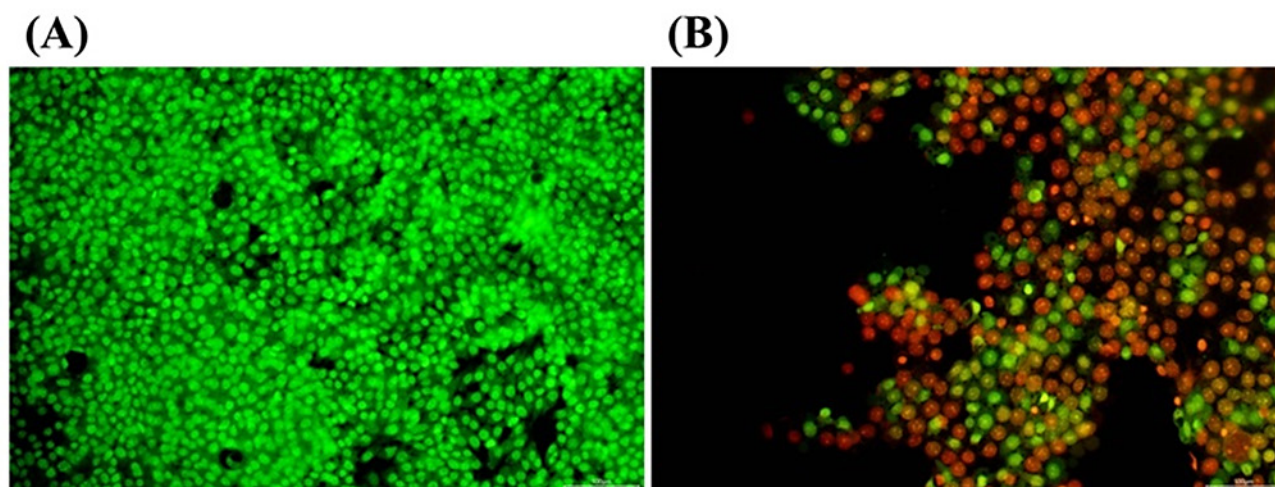


Figure 3: AO/EtBr staining: The images depicted was apoptotic and necrotic cells of MCF 7 by AO/EtBr dual staining method. (A) - control/untreated and (B) IC_{50} value of Cb-B.

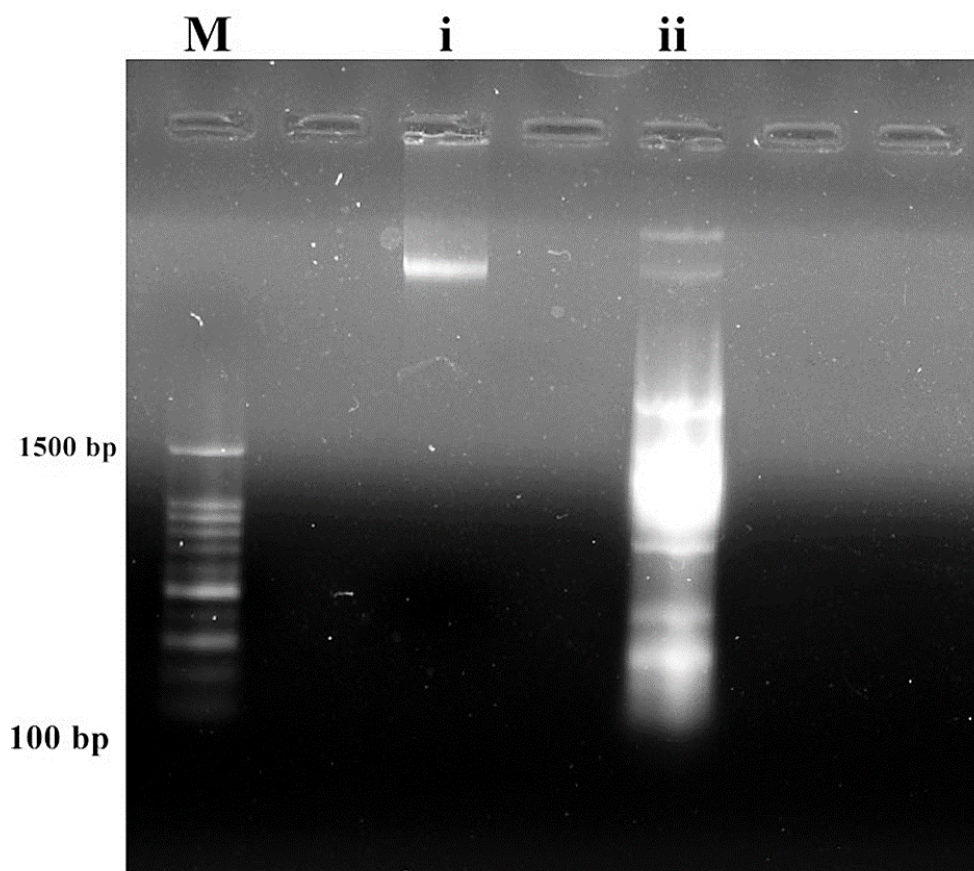


Figure 4: DNA fragmentation: Electrophoresis gel image of DNA isolated from MCF-7 cells. Lane 1 - DNA marker, Lane 2: Control cells, Lane 3: Cb-B treated cells

releasing cytochrome C.^{31,32} Total plant-derived flavonoids and Icariside II exhibited DNA damage in BC cells, and stated that plant-based bioactive compounds tend to induce ROS in cancer cells, influencing DNA injury, which obviously generates cell cycle arrest.^{5,33}

A molecular-level investigation is essential to assess the depth of the evidence for the anti-cancer potency of Cu-B in MCF-7 cells. FasL gene analysis demonstrated the elevated expression in the Cu-B-treated group compared to the control group. The FasL gene will be upregulated when the Fas receptor is activated and transmits the binding signal for Fas Ligand (FasL) on the cell surface. The Fas-FasL pair further forms a complex called DISC (Death-inducing Signaling Complex) consisting of Fas-FADD (Fas-Associated Death domain) and procaspase 8 (cysteine proteases), which activates caspases 8 and 10 as upstream executioners, leading to the activation of downstream effector caspases such as caspases 3 and 7.^{5,21,34,35} The Fas/FasL-mediated pathway is the extrinsic pathway. The cleavage of pro-caspase 8 expresses the protein called Bid, which is converted into Truncated Bid (tBid) by caspase 8 signals and then activates or is interlinked with the intrinsic pathway. tBid instigates the mitochondrial apoptosis signal by inducing the release of cytochrome C, Apaf-1,

and other apoptotic promoters, leading to apoptosis.^{18,20,22,36} So, the increased FasL gene expression manifested that the apoptosis of MCF-7 by Cu-B was mediated through the Fas/FasL pathway.

Previous studies have demonstrated that several phytochemicals can regulate the Fas/FasL apoptotic pathway in breast cancer cells. For instance, fraxetin and silibinin were shown to upregulate FasL gene expression in MCF-7 cells in a dose-dependent manner, as confirmed by qPCR analysis.^{20,37} Similarly, other phytoactive compounds, including Icariside II, Gambogenic acid, β -sitosterol, lycorine, and pulveraven A, have been reported to activate Fas/FasL-mediated apoptosis in breast cancer cells. These effects were validated through western blot analyses of key signalling molecules such as Fas, FasL, FADD, procaspase-8, procaspase-3, caspase-8, caspase-3, caspase-9, Bid, and tBid, along with evidence of cross-talk with linked apoptotic signalling pathways.^{18,20,33,35,38} In contrast, ethanolic extracts of fenugreek seeds were found to induce Fas/FADD-dependent apoptosis in MCF-7 cells through a FasL-independent mechanism.²² In line with these findings, the present study substantiates that Cucurbitacin B (Cu-B) exerts its anticancer activity in MCF-7 breast cancer cells through the possible activation of the Fas/FasL death receptor signalling pathway.

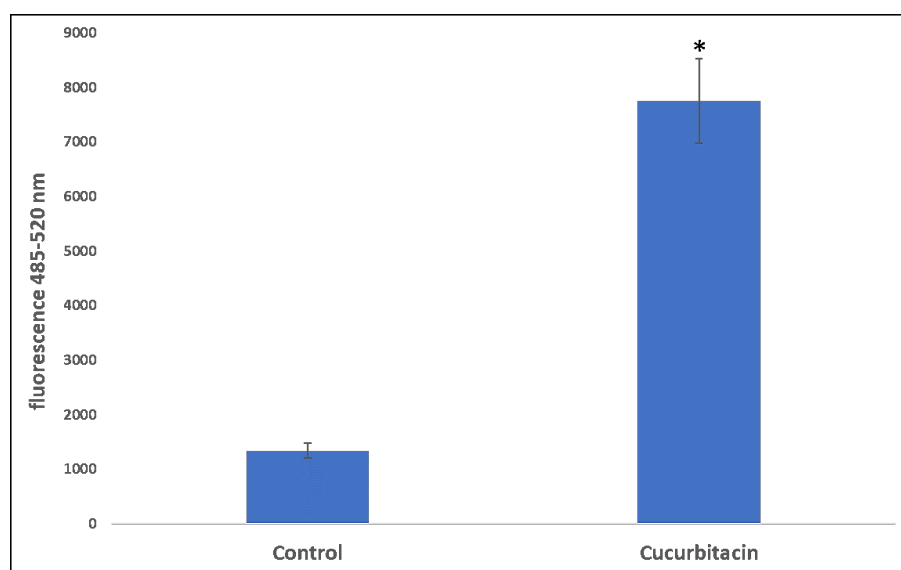


Figure 5: ROS analysis: Graphical representation of ROS determination by the DCFH-DA method. The control group showed low ROS liberation, treated group exhibited elevated level of ROS release with low significant with control group. * indicates $p < 0.001$.

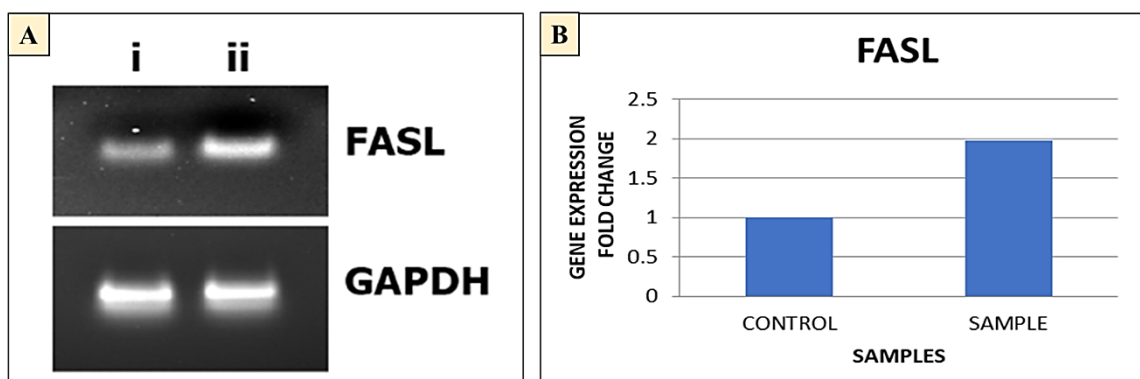


Figure 6: Gene expression by qPCR: 6 (A) indicates the gel image of agarose gel (1.5%) depicting the gene expression of FASL gene and GAPDH a reference gene expression in control/untreated (i) and treated (ii) MCF 7 cells. The gel image was taken by ChemiDoc imaging system, Biorad. 6 (B) denotes the graphical representation of the expression pattern of FASL gene with respect to the housekeeping gene GAPDH.

Limitations and Future Directions

This study is confined to a single breast cancer cell line, MCF-7, limiting the generalizability of the findings. Future investigations will incorporate multiple breast cancer models to elucidate FADD-dependent apoptotic signaling. In addition, while the current work is restricted to *in vitro* analysis, subsequent studies are planned to assess the pharmacokinetic and bio-distribution profiles of Cucurbitacin B in an *in vivo* model. These investigations will also aim to provide a comprehensive mechanistic insight into the cross-talk between intrinsic and extrinsic apoptotic pathways, including the comparative evaluation of structurally related Cu-B analogs.

CONCLUSION

The present study provides novel evidence that Cucurbitacin B (Cu-B) induces apoptosis in human breast cancer (MCF-7) cells, possibly through the Fas/FasL-mediated death receptor pathway. Treatment with Cu-B disrupted cellular integrity and promoted

apoptotic features, accompanied by oxidative stress and genomic instability, ultimately leading to programmed cell death. Importantly, molecular analysis revealed upregulation of the FasL gene, confirming the engagement of the death receptor pathway in Cu-B-induced apoptosis. These findings provide strong evidence that Cu-B possesses significant anti-cancer potential and may serve as a promising candidate for the development of novel therapeutic strategies against breast cancer.

ACKNOWLEDGEMENT

The researchers would like to acknowledge the Deanship of Scientific Research, Taif University for funding this work.

ABBREVIATIONS

AO/EtBr: Acridine Orange/Ethidium Bromide; **BC:** Breast Cancer; **Cu-B:** Cucurbitacin-B; **DCFH-DA:** 2',7'-Dichlorofluorescein Diacetate; **DISC:** Death-inducing Signaling Complex; **DMEM:** Dulbecco's modified Eagles

medium; **DMSO**: Dimethyl Sulfoxide; **ER**: Estrogen Receptor; **FADD**: Fas-Associated Death domain; **Fas/FasL**: Fas/Fas Ligand; **GAPDH**: Glyceraldehyde-3-Phosphate Dehydrogenase; **HBSS**: Hank's Balanced salt Solution; **HER2**: Human Epidermal Growth Factor Receptor 2; **IC₅₀**: Half Maximal Inhibitory Concentration; **MTT**: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **PBS**: Phosphate Buffered Saline; **qPCR**: Quantitative Polymerase Chain Reaction; **ROS**: Reactive Oxygen Species; **RT**: Room Temperature; **SE**: Standard Error; **TAE**: Tris-Acetate-EDTA; **WHO**: World Health Organization.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

Ethical approval was not required because the study involved only in vitro experiments and did not include any human or animal subjects.

FUNDING

The researchers would like to acknowledge the Deanship of Scientific Research. Taif University for funding this work.

SUMMARY

Breast cancer is a major global health threat, with rising incidence and mortality rates. This study explores Cucurbitacin-b (Cu-B), a triterpenoid from cucurbitaceae, for its anticancer effects on MCF-7 cells via the Fas/FasL-mediated extrinsic apoptosis pathway. Using the MTT assay, Cu-B showed IC₅₀ of 74.83 µg/mL, inducing dose-dependent cytotoxicity. morphological changes included cell shrinkage and detachment. AO/Etbr staining revealed increased apoptotic cells, while DCFH-DA assay detected elevated ROS (7750±901 vs. 1333±126 in controls). DNA fragmentation confirmed nuclear damage, and qPCR demonstrated upregulated FASL expression. Cu-B disrupts cellular homeostasis, triggers oxidative stress, and activates death receptor signaling, linking to caspase cascades. These results position Cu-B as a promising natural therapeutic for breast cancer.

REFERENCES

- Kim J, Harper A, McCormack V, *et al.* Global patterns and trends in breast cancer incidence and mortality across 185 countries. *Nature Medicine*. 2025;31(4):1154-1162. doi:10.1038/S41591-025-03502-3;SUBJMETA
- Zhang Y, Ji Y, Liu S, *et al.* Global burden of female breast cancer: new estimates in 2022, temporal trend and future projections up to 2050 based on the latest release from GLOBOCAN. *Journal of the National Cancer Center*. 2025;5(3):287-296. doi:10.1016/J.JNCC.2025.02.002
- Bunnell N. Breast Cancer Awareness month. *The Journal of the Kentucky Medical Association*. 2005;103(10):517-518.
- Bate J, Rasmussen E. Key Facts. *Macbeth*. Published online 2009: 21-22. doi:10.1007/978-1-137-00443-7_3
- Wang S, Tian Q, An F. Growth inhibition and apoptotic effects of total flavonoids from *trilolius chinensis* on human breast cancer MCF-7 cells. *Oncology Letters*. 2016;12(3). doi:10.3892/ol.2016.4898
- Wu D, Wang Z, Lin M, *et al.* *In vitro* and *in vivo* antitumor activity of cucurbitacin C, a novel natural product from cucumber. *Frontiers in Pharmacology*. 2019; 10. doi:10.3389/fphar.2019.01287
- Alafnan A, Khalifa NE, Hussain T, Osman ME. Cucurbitacin-B instigates intrinsic apoptosis and modulates Notch signaling in androgen-dependent prostate cancer LNCaP cells. *Frontiers in Pharmacology*. 2023; 14. doi:10.3389/fphar.2023.1206981
- Dai S, Wang C, Zhao XT, *et al.* Cucurbitacin B: A review of its pharmacology, toxicity, and pharmacokinetics. *Pharmacological Research*. 2023; 187. doi:10.1016/j.phrs.2022.106587
- Varela C, Melim C, Neves BG, *et al.* Cucurbitacins as potential anticancer agents: new insights on molecular mechanisms. *Journal of Translational Medicine*. 2022;20(1). doi:10.1186/s12967-022-03828-3
- Yin D, Chen H, Lin S, *et al.* Recent Advances in the Application of Cucurbitacin B as an Anticancer Agent. *International Journal of Molecular Sciences* 2025, Vol 26, Page 8003.2025; 26(16): 8003. doi:10.3390/IJMS26168003
- Zhang ZR, Gao MX, Yang K. Cucurbitacin B inhibits cell proliferation and induces apoptosis in human osteosarcoma cells via modulation of the JAK2/STAT3 and MAPK pathways. *Experimental and Therapeutic Medicine*. 2017;14(1). doi:10.3892/etm.2017.4547
- Li Y, Li Y, Yao Y, *et al.* Potential of cucurbitacin as an anticancer drug. *Biomedicine and Pharmacotherapy*. 2023; 168. doi:10.1016/j.biopha.2023.115707
- Duangmano S, Sae-lim P, Suksamrarn A, Domann FE, Patmasiriwat P. Cucurbitacin B inhibits human breast cancer cell proliferation through disruption of microtubule polymerization and nucleophosmin/B23 translocation. *BMC Complementary and Alternative Medicine*. 2012; 12. doi:10.1186/1472-6882-12-185
- Aiswarya SUD, Vikas G, Haritha NH, *et al.* Cucurbitacin B, Purified and Characterized From the Rhizome of *Corallocarpus epigaeus* Exhibits Anti-Melanoma Potential. *Frontiers in Oncology*. 2022; 12. doi:10.3389/fonc.2022.903832
- Ren G, Sha T, Guo J, Li W, Lu J, Chen X. Cucurbitacin B induces DNA damage and autophagy mediated by reactive oxygen species (ROS) in MCF-7 breast cancer cells. *Journal of Natural Medicines*. 2015;69(4). doi:10.1007/s11418-015-0918-4
- Dakeng S, Duangmano S, Jiratchariyakul W, U-Pratya Y, Böglar O, Patmasiriwat P. Inhibition of Wnt signaling by cucurbitacin B in breast cancer cells: Reduction of Wnt-associated proteins and reduced translocation of galectin-3-mediated β-catenin to the nucleus. *Journal of Cellular Biochemistry*. 2012;113(1). doi:10.1002/jcb.23326
- Huang WW, Yang JS, Lin MW, *et al.* Cucurbitacin e Induces G 2/M Phase Arrest through STAT3/p53/p21 Signaling and Provokes Apoptosis via Fas/CD95 and Mitochondria-Dependent Pathways in Human Bladder Cancer T24 Cells. *Evidence-based Complementary and Alternative Medicine*. 2012; 2012. doi:10.1155/2012/952762
- Ji Y, Yu M, Qi Z, *et al.* Study on apoptosis effect of human breast cancer cell MCF-7 induced by lycorine hydrochloride via death receptor pathway. *Saudi Pharmaceutical Journal*. 2017;25(4). doi:10.1016/j.jsps.2017.04.036
- An W, Lai H, Zhang Y, Liu M, Lin X, Cao S. Apoptotic pathway as the therapeutic target for anticancer traditional Chinese medicines. *Frontiers in Pharmacology*. 2019; 10. doi:10.3389/fphar.2019.00758
- Awad AB, Chinnam M, Fink CS, Bradford PG. β-Sitosterol activates Fas signaling in human breast cancer cells. *Phytomedicine*. 2007;14(11). doi:10.1016/j.phymed.2007.01.003
- Strasser A, Jost PJ, Nagata S. The Many Roles of FAS Receptor Signaling in the Immune System. *Immunity*. 2009;30(2). doi:10.1016/j.immuni.2009.01.001
- Alshatwi AA, Shafi G, Hasan TN, Syed NA, Khoja KK. Fenugreek induced apoptosis in breast cancer MCF-7 cells mediated independently by fas receptor change. *Asian Pacific Journal of Cancer Prevention*. 2013;14(10). doi:10.7314/APJCP.2013.14.10.5783
- Talarico LB, Zibetti RGM, Faria PCS, *et al.* Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsia* and *Cryptonemia crenulata*. *International Journal of Biological Macromolecules*. 2004;34(1-2):63-71. doi:10.1016/J.IJBIOMAC.2004.03.002
- Moongkarndi P, Kosem N, Kaslungka S, Luanratana O, Pongpan N, Neungton N. Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell line. *Journal of Ethnopharmacology*. 2004;90(1):161-166. doi:10.1016/J.JEP.2003.09.048
- Liu K, Liu P cheng, Liu R, Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. *Medical science monitor basic research*. 2015;21:15-20. doi:10.12659/MSMBR.893327
- Yadav S, Sadique MA, Pal M, Khan R, Srivastava AK. Cytotoxicity and DNA fragmentation-mediated apoptosis response of hexagonal ZnO nanorods against human prostate cancer cells. *Applied Surface Science Advances*. 2022;9:100237. doi:10.1016/J.APSADV.2022.100237
- Cheng GJ, Leung EY, Singleton DC. *In vitro* breast cancer models for studying mechanisms of resistance to endocrine therapy. *Exploration of Targeted Anti-tumor Therapy*. 2022;3(3). doi:10.37349/etat.2022.00084
- Duangmano S, Sae-Lim P, Suksamrarn A, Patmasiriwat P, Domann FE. Cucurbitacin B causes increased radiation sensitivity of human breast cancer cells via G2/M cell cycle arrest. *Journal of Oncology*. Published online 2012. doi:10.1155/2012/601682
- Wakimoto N, Yin D, O'Kelly J, *et al.* Cucurbitacin B has a potent antiproliferative effect on breast cancer cells *in vitro* and *in vivo*. *Cancer Science*. 2008;99(9). doi:10.1111/j.1349-7006.2008.00899.x

30. Alafnan A, Alamri A, Hussain T, Rizvi SMD. Cucurbitacin-B Exerts Anticancer Effects through Instigation of Apoptosis and Cell Cycle Arrest within Human Prostate Cancer PC3 Cells via Downregulating JAK/STAT Signaling Cascade. *Pharmaceuticals*. 2022;15(10). doi:10.3390/ph15101229
31. Chakravarti B, Maurya R, Siddiqui JA, *et al.* *In vitro* anti-breast cancer activity of ethanolic extract of *Wrightia tomentosa*: Role of pro-apoptotic effects of oleanolic acid and urosolic acid. *Journal of Ethnopharmacology*. 2012;142(1). doi:10.1016/j.jep.2012.04.015
32. Smina TP, Nitha B, Devasagayam TPA, Janardhanan KK. Ganoderma lucidum total triterpenes induce apoptosis in MCF-7 cells and attenuate DMBA induced mammary and skin carcinomas in experimental animals. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*. 2017; 813. doi:10.1016/j.mrgentox.2016.11.010
33. Huang C, Chen X, Guo B, *et al.* Induction of apoptosis by icariside II through extrinsic and intrinsic signaling pathways in human breast cancer MCF7 cells. *Bioscience, Biotechnology and Biochemistry*. 2012;76(7). doi:10.1271/bbb.120077
34. Sharma U, Sahni PK, Sharma B, *et al.* Silymarin: a promising modulator of apoptosis and survival signaling in cancer. *Discover Oncology*. 2025;16(1):1-12. doi:10.1007/S12672-025-01800-3/TABLES/2
35. Zhou J, Luo YH, Wang JR, Lu B Bin, Wang KM, Tian Y. Gambogenic acid induction of apoptosis in a breast cancer cell line. *Asian Pacific Journal of Cancer Prevention*. 2013;14(12). doi:10.7314/APJCP.2013.14.12.7601
36. Lahiry L, Saha B, Chakraborty J, *et al.* Theaflavins target Fas/caspase-8 and Akt/pBad pathways to induce apoptosis in p53-mutated human breast cancer cells. *Carcinogenesis*. 2010;31(2). doi:10.1093/carcin/bgp240
37. Liu G, Liu Z, Yan Y, Wang H. Effect of fraxetin on proliferation and apoptosis in breast cancer cells. *Oncology Letters*. 2017;14(6). doi:10.3892/ol.2017.7143
38. Lee D, Yu JS, Ryoo R, *et al.* *Pulveraven A* from the fruiting bodies of *Pulveroboletus ravenelii* induces apoptosis in breast cancer cell via extrinsic apoptotic signaling pathway. *Journal of Antibiotics*. 2021;74(10). doi:10.1038/s41429-021-00435-0

Cite this article: Alsaieri JA, Almalki AA, Alosaimi JA, Al-Qurashi RM, Al-Qurashi BA, Dhafer FA, *et al.* Cucurbitacin-B Triggers Fas/FasL-dependent Apoptosis via the Death Receptor Signalling Pathway in Human Breast Cancer Cell Line - MCF-7. *Indian J of Pharmaceutical Education and Research*. 2026;60(4s):s1553-s1562.