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Research Article

The effect of *Crocetin* on cholesterol depletion-mediated lipid raft disruption-induced apoptosis in breast cancer cells

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Abstract: The purpose of this study was to determine the effect of lipid raft function loss due to depletion of cholesterol in the lipid raft structure of cell membrane by crocetin and Methyl β cyclodextrin (MβCD) on cell viability and lipid raft-associated gene and apoptotic gene expressions of breast cancer cell (MCF-7). For this purpose, MCF-7 cells were treated with different concentrations of MβCD and crocetin. Cell viability was evaluated by WST-1 at 24 and 48 hours. The mRNA expressions of caveolin 1, LRP 6, survivin, Bcl2, Bax, and Caspase3 were assessed in the MβCD-treated group; crocetin-treated group; mixed-treated group MβCD+ crocetin MCF-7 cells by reverse transcription polymerase chain reaction at 24 h exposure. Cell viability indicated that all concentrations of MβCD decreased the viability of MCF-7 cells compared with control; reduction in cell viability was greatest with 1 mM. Additionally, exposure to all crocetin concentrations significantly reduced the cell viability of MCF-7 in a time-dependent manner. There was statistically significant down-regulation of caveolin 1, LRP-6, survivin, Bcl2 in response to MβCD, and crocetin at 24 h but Bax ve caspase 3 expressions were increased compared to control at 24h. These results indicated that crocetin application to MCF-7 in addition to MβCD regulated mRNA expression of lipid raft-associated genes and apoptotic genes. These findings suggest that crocetin affects MCF-7 function via cholesterol depletion-related deterioration in the lipid raft structure, which is critical for the induction of apoptosis in MCF-7 cells.

1. INTRODUCTION

Breast cancer, primarily due to its heterogeneous cell diversity (Yeo *et al*., 2017), is a complex disease that encompasses various clinical presentations, diagnoses, and treatment approaches (Badana *et al*., 2018). Lacking specific target markers for diagnosis, treatment, and clinical monitoring, breast cancer presents a challenge in the medical field. When compared to healthy cells, many cancer cells including breast cancer cells show an enhanced absorption of glucose. The metabolization of this acquired glucose occurs through both anabolic and catabolic pathways, leading to the excessive production of precursor molecules, heightened glutamine levels, altered lipid metabolism, and a highly proliferative and acidic microenvironment. This complexity contributes to the diverse clinical manifestations observed in breast cancer and other cancer types (Hanahan & Weinberg, 2011; Mathupala *et al*., 2006; Murai, 2015). It has been established that lipid rafts, characterized by a high content of cholesterol, within the cell

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membrane serve as pivotal signaling entities in various cancer types, notably breast cancer. These lipid rafts are implicated in fostering an augmented accumulation of cholesterol within malignant tissues as opposed to their healthy counterparts. This phenomenon is thought to have a significant impact on the initiation and progression of tumors(Hirsch *et al*., 2010; Li *et al*., 2017; Shah *et al*. 2016). Lipid rafts represent dynamic microdomains, characterized by the dense packing of fatty acid chains measuring approximately 10-20 nm in size. These microdomains are situated within the cell membrane, exhibiting an enrichment of cholesterol and sphingolipids (Li *et al*., 2017; Pike *et al*., 2006; Sezgin *et al*., 2017; Varshney *et al*., 2016).

These molecules, characterized by a highly dynamic structure, particularly within the cell membrane, intricately participate in the regulation of diverse membrane proteins through protein-protein interactions. They play a pivotal role in executing numerous cellular functions, including apoptosis and adhesion, by modulating the phosphorylation cascade through activation or inactivation mechanisms. In the context of breast cancer cells, lipid rafts establish molecular connections among dynamic membrane microdomains, contributing significantly to the process of carcinogenesis. Leveraging proteomic datasets, Shah *et al*. (2016) conducted an empirical investigation that quantitatively delineated the association between breast cancer and the abundance of lipid rafts (Shah *et al*., 2016). The study revealed a statistically significant correlation between the developmental progression of breast cancer and the intricate interplay involving cell cytoskeleton dynamics and membrane rafts. To achieve this objective, lipid raft-centric research employs the cholesterol deprivation model. Agents such as filipin, nystatin, or methyl beta cyclodextrin (MβCD) are utilized to extract cholesterol from the lipid raft structure, inducing a temporary loss of microdomain functionality. Notably, MβCD stands out as one of the most frequently employed specific agents in research endeavors aimed at effecting cholesterol deprivation from lipid raft (Zidovetzki & Levitan, 2007). Moreover, lipid rafts emerge as pivotal regulators of signaling pathways associated with diminished apoptosis, concurrent with heightened proliferation and invasion common hallmarks in the diagnosis of breast cancer cells. Notably, the fluidity and transitivity of cholesterol within the cell membrane, its intricate association with receptor functionality, ion channel activity, and its substantial presence within lipid rafts collectively underscore its profound implications in the modulation of apoptosis within cancer cells (Burger *et al*., 2000). In conjunction with these considerations, a multitude of phytochemicals, including species such as saffron (*Crocus sativus*), are presently incorporated into traditional therapeutic regimens for their discernible chemopreventive and therapeutic attributes in the realms of breast cancer diagnosis and treatment, owing to their characteristic low toxicity and heightened efficacy (Bolhassani *et al*., 2014; Patel *et al*., 2017). Saffron is a spice derived from the flower of *Crocus sativus*, renowned for its abundant carotenoid content. The flower encompasses two primary carotenoids responsible for its coloration, namely crocin and crocetin (Bathaie *et al*., 2014). Among these, crocin stands out as a crucial carotenoid of significant importance, serving as the precursor molecule to crocetin. Derived from the stigmas of the saffron plant, crocin is characterized by a short carbon chain length. This yellow-hued primary component emerges as a distinctive molecule in traditional medical applications, showcasing unique properties as an antioxidant, anti-inflammatory, and anti-tumorigenic agent (Li *et al*., 2017; Umigai *et al*., 2011). Chryssanthi *et al*. (2007) investigated the impact of four distinct *Crocus* species extracts on the viability of MCF-7 and MDA-MB-231 breast cancer cell lines through MTT analysis (Chryssanthi *et al*., 2007). The results elucidated that among the four *Crocus* species incorporated in the study, *Crocus sativus* demonstrated the most pronounced efficacy as a chemopreventive and anti-cancer agent (Chryssanthi *et al*., 2007). In our study, we chose this structure to examine the lipid raft structure and the functional effects of disruption of this structure in MCF-7 cells, especially to investigate the effects of Crocetin and Methyl β cyclodextrin. Lipid rafts are known as dynamic microdomains that play an important role in cell membrane organization and cellular signal transduction. These regions have been associated with cancer progression by providing platforms for growth factor receptors,

signaling proteins and proteins involved in apoptosis processes cancer cells including breast cancer. In particular, lipid rafts have been shown to affect signal transduction, proliferation and metastasis processes in cancer cells and it is known that disruption of these structures can directly affect the survival and proliferation potential of cancer cells. Our study aims to better understand the functional effects of lipid raft structure on MCF-7 cells and to reveal the potential effects of Methyl β cyclodextrin administration together with Crocetin on this process. Therefore, lipid raft structure was chosen as an important research topic in terms of its impact on cancer cell functions. Therefore, we propose that changes in the associated cellular functions that may result from disruption of the lipid raft structure localized in the cell membrane will play a key role in understanding the therapeutic potential of Crocetin on breast cancer cells.

In this study, the potential influence of crocetin carotenoid and methyl beta cyclodextrin (MβCD) on cell viability, along with their impact on the mRNA expression levels of lipid raft structure-associated genes (caveolin 1, LRP 6) and apoptotic genes (survivin, Bcl2, Bax, Caspase3), was investigated in MCF-7 cells (estrogen receptor-positive [+], non-metastatic, epithelial-like breast cancer cell line). The examination focused on the induction of lipid raft function loss attributable to the depletion of cholesterol within the cell membrane's inherent lipid raft structure.

2. MATERIAL and METHODS

2.1. Breast Cancer Cell Line and Culture

The MCF-7 cell line, which is an epithelial-like, non-metastatic, estrogen receptor-positive [+] cell line, was employed as a breast cancer model in this investigation. The cells were defrosted and cultured in 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% glutamine in a laminar flow cabinet supplemented with Dulbecco's Modified Eagle Medium (DMEM) after being removed from their -150°C frozen storage. After that, the cells were grown in a CO2 incubator at 37°C and 5% CO2 until they attained the confluence needed for the experiment. The cell culture media was replaced every other day after their morphological development was seen under an inverted microscope.

2.2. The Preparation of Methyl Beta Cyclodextrin (MβCD) Concentrations

Methyl Beta Cyclodextrin (MβCD) (332615, Sigma Aldrich) utilized in the experiments was commercially procured. The working concentrations employed in the experiments (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM) were prepared from the master stock and administered to the cells without delay before each application.

2.3. The Preparation of Crocetin Concentrations

The saffron carotenoid utilized in the study, Crocetin (C20H24O4, molecular weight: 328.4, Biomedicals, Santa), was commercially obtained. After dissolving the compound in dimethyl sulfoxide (DMSO) and preparing primary stock solutions, the experimental groups were further diluted to concentrations of 250 µM, 500 µM, and 1000 µM, according to the experimental requirements (Mir *et al*., 2020).

2.4. The Experimental Design of Study Groups

The cell viability experiments were performed with the following groups;

A) 0, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM MβCD (24h and 48h),

B) 250, 500, 1000 µM Crocetin (24h and 48h).

The total RNA isolation experiments were performed with the following groups;

1) MCF-7 cells+ 0 mM MβCD (only) (24h)
2) MCF-7 cells + 0.1 mM MβCD (only) (24h)
3) MCF-7 cells + 0.2 mM MβCD (only) (24h)
4) MCF-7 cells + 0.3 mM MβCD (only) (24h)

5) MCF-7 cells + 0.4 mM MβCD (only) (24h)
6) MCF-7 cells + 0.5 mM MβCD (only) (24h)
7) MCF-7 cells + 1 mM MβCD (only) (24h)
8) MCF-7 cells + 500 µM Crocetin (24h)
9) MCF-7 cells+ 0 mM MβCD+ 500 µM Crocetin (24h)
10) MCF-7 cells + 0.1 mM MβCD+ 500 µM Crocetin (24h)
11) MCF-7 cells + 0.2 mM MβCD+ 500 µM Crocetin (24h)
12) MCF-7 cells + 0.3 mM MβCD+ 500 µM Crocetin (24h)
13) MCF-7 cells + 0.4 mM MβCD+ 500 µM Crocetin (24h)
14) MCF-7 cells + 0.5 mM MβCD+ 500 µM Crocetin (24h)
15) MCF-7 cells + 1 mM MβCD+ 500 µM Crocetin (24h)

2.5. Cell Viability Analysis

Within the framework of the research, the impact of Crocetin carotenoid on cell viability, specifically about methyl beta cyclodextrin (MβCD)-mediated cholesterol depletion-induced lipid raft function loss in MCF-7 breast cancer cells, was analyzed using WST-1 at 24 and 48 hours (Sarı *et al*., 2021). MCF-7 cells were subjected to several dosages of MβCD (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM) and crocetin (250, 500, 1000 µM) in the experimental setup. For every group, six wells with 5000 cells each (n = 6) were created. On the 24th hour, 200 µL of WST-1 reagent (20 µL/well) was added to the cells after MβCD and Crocetin doses were applied to the designated experimental groups. After two hours of incubation at 37°C with 5% CO2, the cell culture was automatically agitated for one minute. Then, using an ELISA-reader spectrophotometer, the 96-well cell culture plate comprising the experimental groups was read at 480 nm to calculate dose-dependent cell viability ratios. For the 48th-hour cell viability examination, the identical experimental procedures were carried out again.

2.6. mRNA Expression Experiments

2.6.1. *Total RNA isolation*

Based on the effective concentrations determined for MβCD and crocetin according to the results of cell viability experiments, the following groups were established, and total RNA isolation was conducted at 24-hour:

1. MCF-7 cells were subjected to MβCD applications at concentrations of 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM,
2. MCF-7 cells were subjected to the application of 500 µM crocetin,
3. Following the application of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM MβCD to MCF-7 cells, 500 µM crocetin was administered to each group at 6 h.

Specifically, MCF-7 cells were seeded at a density of 25,000 cells/cm² in 60 mm cell culture dishes following the specified experimental groups. After evaluating the cell morphologies using an inverted microscope the next day, MCF-7 cells were treated according to the assigned experimental groups with 500 µM of crocetin and 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM of MβCD. After 24 hours of MβCD and crocetin treatments, the cells were washed with Phosphate-Buffered Saline (PBS), and aspirated using DMEM containing 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% glutamine. Subsequently, the cells were added to 500 µL of EZ-RNA A solution, which is a part of the RNA isolation kit, for lysis. After adding 500 µL of EZ-RNA B solution—an additional part of the RNA isolation kit to the mixture, it was centrifuged for 15 minutes at 4°C at 12,000 rpm to help separate the protein-DNA-RNA phases inside the tubes. After transferring the RNA phase to a different tube and adding 500 µL of isopropanol, the RNase-DNase-free polypropylene tubes were centrifuged for 15 minutes at 12.000 rpm and 4°C to extract the RNA pellets. The extracted total RNA samples were stored at -80°C before being used in subsequent mRNA expression assays.

2.6.2. *Complementary DNA (cDNA) synthesis*

The synthesis of Complementary DNA (cDNA) was conducted under the protocol of the cDNA synthesis kit. For this purpose, from each RNA sample, an amount containing 1 µg of RNA was utilized, and the reaction volume was adjusted to 20 µL by adding 1 µL 20XRT Enzyme Mix, 10 µL 2XRT Buffer Solution, and dH2O. The temperature cycle was applied via polymerase chain reaction (PCR) equipment, which ran for 50 minutes at 95°C and 60 minutes at 37°C. Before being used in real-time polymerase chain reaction (RT-PCR) investigations, the resultant cDNA was kept at -20°C.

2.6.3. *Real-time polymerase chain reaction (RT-PCR) experiments*

The mRNA expression levels of lipid raft-associated genes of caveolin 1, LRP 6, and apoptotic genes (survivin, Bcl2, Bax, caspase 3), as well as the housekeeping gene (GAPDH) (Table 1) were measured by RT-PCR using the SYBR Green Master Mix and analyzed with Stratagene MX3000P.

Table 1. Primer sequences of human-origin lipid raft and apoptosis-associated genes, 5'-3'

Primers	Forward (5'-3')	Reverse (3'-5')
Caveolin-1	CATGTCTGGGG-GCAAATACG	GAACTTGAAATTGGCACCAGG
LRP-6	GATTATCCAGAAGGCATGGCAG	TCCCATCACCATCTTCCA
survivin	ACCACCGCATCTCTAC	TCCTCTATGGGGTCGT
Bcl-2	CCGGGAGATCGTGATGAAGT	ATCCCAGCCTCCGTTATCCT
BAX	AGTGGCAGCTGACATGTTTT	GGAGGAAGTCCAATGTCCAG
Caspase 3	TTAATAAAGGTATC	CATGGAGAACACT
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

2.7. Statistical Analysis

In the evaluation of mRNA expressions, the comparative Ct method was employed to normalize the results (Giulietti *et al*., 2001; Livak & Schmittgen, 2001; Pfaffl 2001). Subsequently, the normalized values were compared using a one-way analysis of variance (ANOVA). Increases and decreases in the expression of target genes relative to GAPDH were presented with 95% confidence intervals, and statistically, a p-value < 0.05 was considered significant. One-way analysis of variance (ANOVA) was also utilized in the analysis of cell viability results.

3. RESULTS

3.1. Cell Viability Analysis Results

WST-1 was used to measure the effects of crocetin and MβCD concentrations on the viability of MCF-7 cells after 24 and 48 hours. The obtained findings showed that at MβCD doses (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM) (p<0.05), there was a substantial, dose- and time-dependent reduction in cell viability in comparison to the control group (Figure 1A, B). Furthermore, treatment with 250 µM, 500 µM, and 1000 µM crocetin led to a substantial reduction in cell viability that was dose- and time-dependent when compared to the control group. The largest loss was noted at a dosage of 500 µM (p<0.05) (Figure 1C, D).

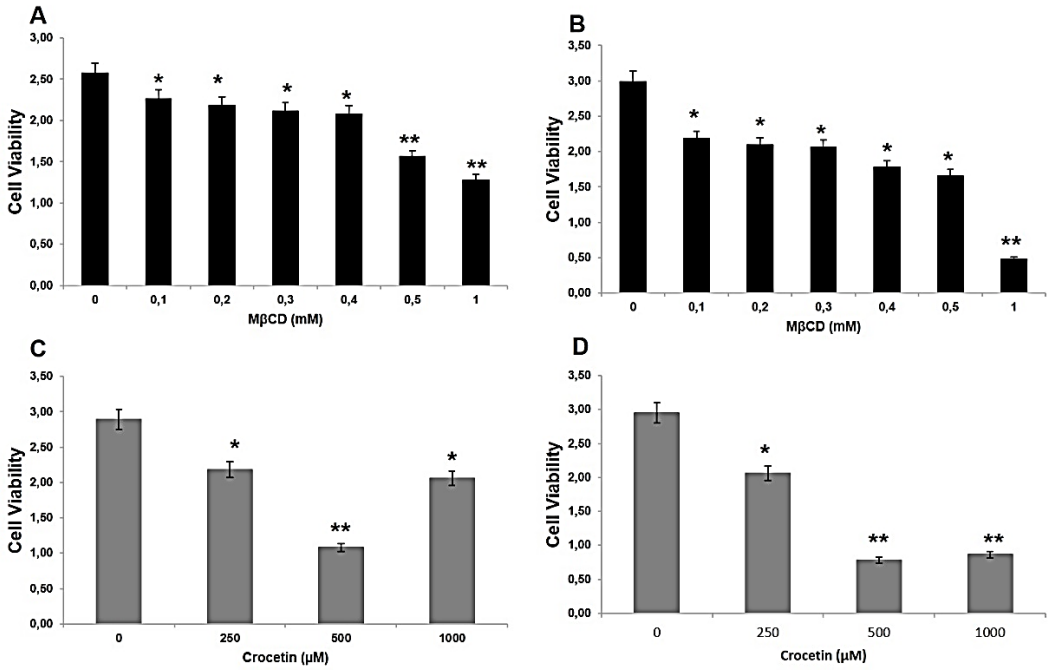

Figure 1. Cell viability analysis results from 24 hours (A) and 48 hours (B) after different concentrations of MβCD application to MCF-7 cells and 24 hours (C) and 48 hours (D) after *crocetin* application.

3.2. Lipid Raft Structure mRNA Expression Findings

Total RNA isolation was carried out at the 24-hour mark of the experiment after the cells were exposed to MβCD concentrations (0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM), crocetin concentration (500 µM), and 6 hours after the application of 500 µM crocetin following MβCD concentrations (0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM). The mRNA expression levels of caveolin 1 and LRP-6 in the lipid raft structure were next confirmed by RT-PCR assays. After applying MβCD doses to MCF-7 cells, the findings showed a dose-dependent substantial drop in caveolin 1 and LRP-6 expression levels compared to the control group, with the most significant reduction shown at concentrations of 0.5 mM and 1 mM (p<0.05) (Figure 2A, B). Additionally, it was shown that the administration of 500 µM crocetin to cells significantly decreased the levels of caveolin 1 and LRP-6 mRNA expression in comparison to the control group (p<0.05) (Figure 3). Moreover, it was shown that, six hours after MβCD application, adding 500 µM crocetin to each group significantly decreased the levels of caveolin 1 and LRP-6 mRNA expression in comparison to the control group (p<0.05). (Figure 4A, B). Additionally, the cumulative effect of adding crocetin to cells after MβCD application was found to significantly further reduce caveolin 1 and LRP-6 expressions compared to their individual applications, indicating a synergistic effect (p<0.05) (Figure 4A, B).

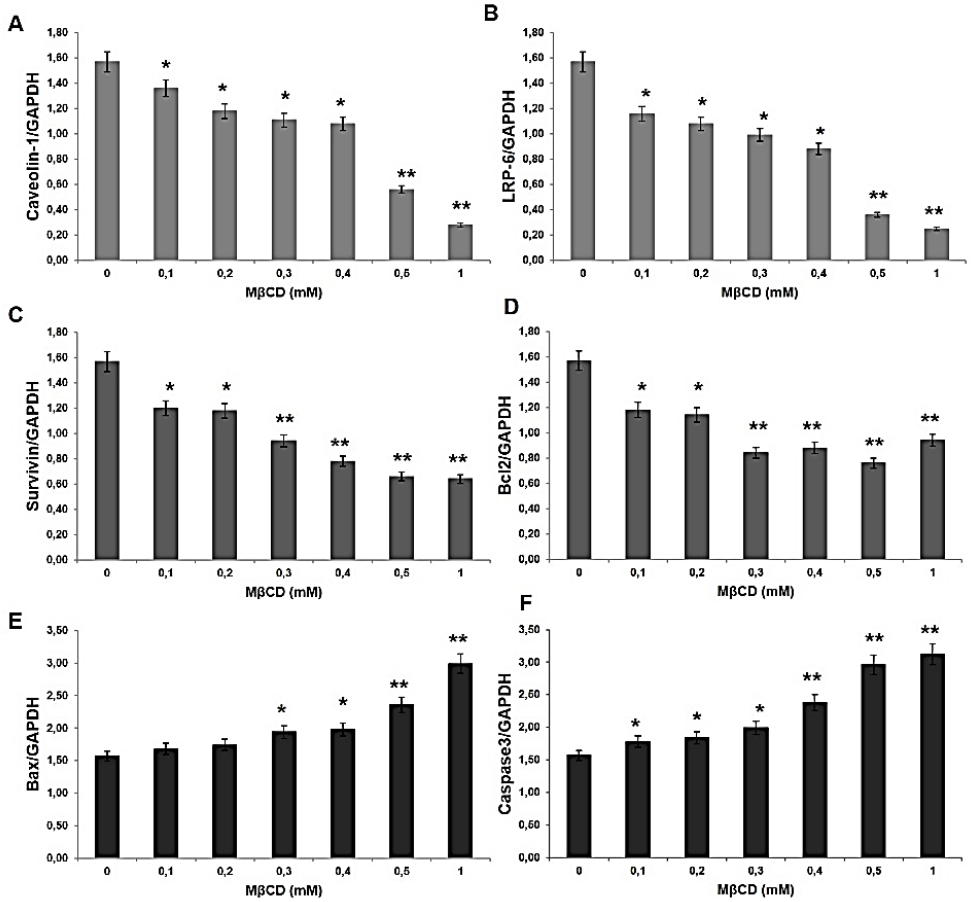

Figure 2. Caveolin 1 (A), LRP 6 (B), survivin (C), Bcl2 (D), Bax (E), caspase3 (F) mRNA expression results after only MβCD application at different concentrations in MCF-7 cells.

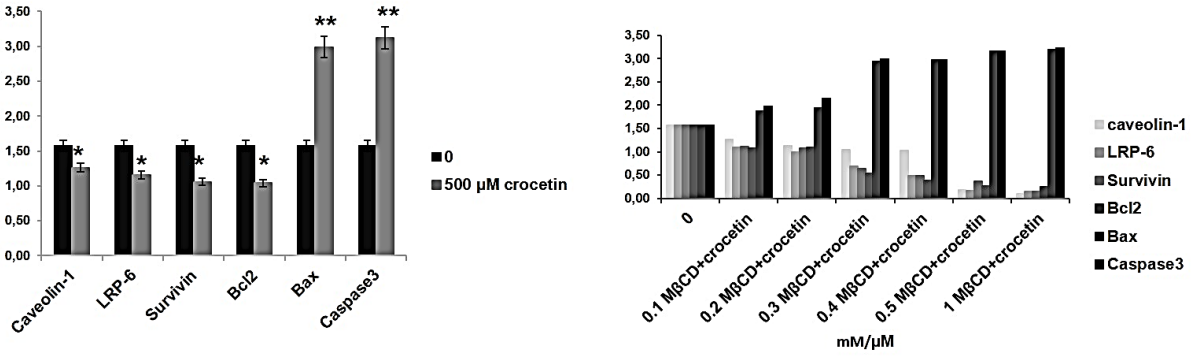

Figure 3. Caveolin 1, LRP 6, survivin, Bcl2, Bax, caspase3 mRNA expression results after only 500 µM *crocetin* application of MCF-7 cells.

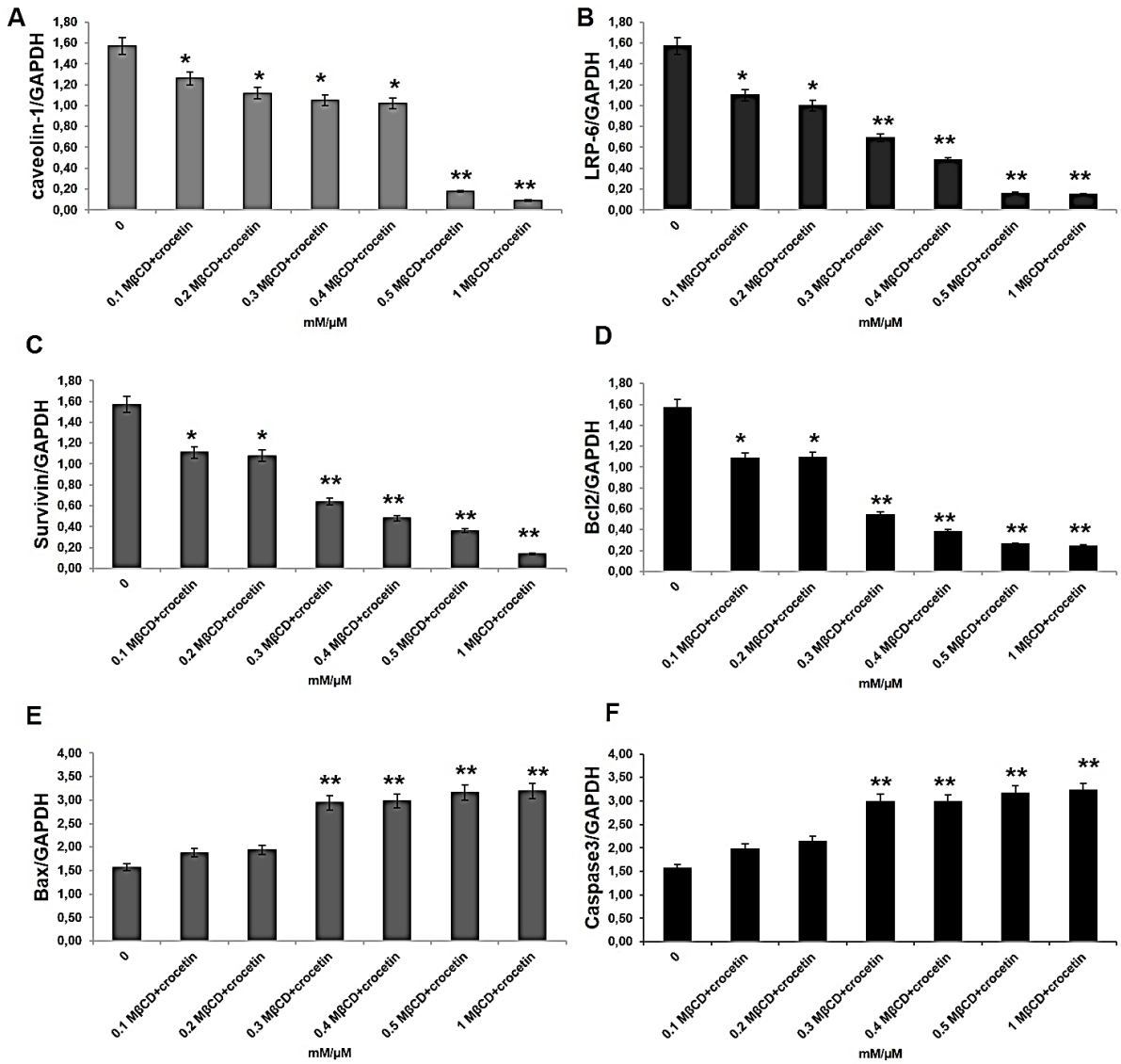

Figure 4. MCF-7 cells mRNA expression results of caveolin 1 (A), LRP 6 (B), survivin (C), Bcl2 (D), Bax (E), caspase3 (F) after application of 500 µM *crocetin* with different concentrations of MβCD.

3.3. Apoptotic Gene Expression Findings in MCF-7 Cells following MβCD and Crocetin Application

In our experiments utilizing MCF-7 cells as a breast cancer cell model under in vitro conditions, applications of only MβCD, only crocetin, and MβCD+crocetin were performed, and RT-PCR was used to examine the mRNA expressions of the apoptotic genes survivin, Bcl2, Bax, and caspase 3. According to Figures 2C and D, the administration of only MβCD resulted in a substantial drop in Bcl-2 expression and an increase in survivin expression (p<0.05) when compared to the control group. Furthermore, it was discovered that Bax expression increased at doses of 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM and that the expression of caspase 3 increased significantly in a dose-dependent manner in comparison to the control group (p<0.05) (Figure 2 C, D, E, F). Similarly, the application of crocetin alone significantly reduced survivin and Bcl2 expressions while significantly increasing Bax and caspase 3 levels compared to the control group (p<0.05) (Figure 3). Additionally, crocetin was added to MCF-7 cells six hours after MβCD was applied, and this significantly decreased the expressions of survivin and Bcl2 in a dose-dependent manner when compared to the control group (p<0.05) (Figure 4C, D). At concentrations of 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM, however, Bax and caspase 3 levels were significantly increased when compared to the control group (p<0.05) (Figure 4E, F).

Specifically, the administration of MβCD+crocetin resulted in a substantial rise in the Bax/Bcl2 ratio, indicating that it promoted apoptosis in MCF-7 cells when compared to the control group.

4. DISCUSSION and CONCLUSION

The regulation of membrane proteins in cholesterol-rich lipid rafts, which are essential structural microdomains, and variations in lipid metabolism (Hirsch *et al*., 2010), including triglycerides, phospholipids, cholesterol, and cholesterol esters in cancer cells (Luo *et al*., 2017), have a major effect on the aggressive progression of tumors through a variety of cellular pathways, including adhesion, apoptosis, and regulation. The disruption and dysfunctionality of microdomain structure through cholesterol depletion are crucial for the aggressive progression of tumors (Li *et al*., 2017; Shah *et al*., 2016). In the current study, we investigated the effects of crocetin carotenoid and MβCD on MCF-7 cell viability and expression of lipid raft structure-associated genes and apoptotic genes. The estrogen receptor-positive (+), non-metastatic, epithelial-like breast cancer cell line MCF-7 had its vitality levels examined in this study utilizing WST-1 following the application of several doses (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM) of MβCD and crocetin (250, 500, 1000 μM). The results demonstrated that both crocetin and MβCD significantly reduced cell viability in a dose- and time-dependent manner ($p<0.05$). Previous studies by Badana *et al*. (2018) demonstrated that using MTT assay, MβCD doses ranging from 0.1 to 1 mM decreased the viability of breast cancer cell lines (MDA-MB 468) as well as normal breast epithelial cells (MCF-12A) (Badana *et al*., 2018). KB cells are an oral squamous cell carcinoma cell line lipid raft structures and are a frequently used in vitro model for the study of lipid raft regulation and signaling pathways as they play critical roles in cellular functions such as signal transduction, cell-cell communication, and apoptosis. Another research team treated the human oral squamous cell line KB with 20 mM MβCD for 2 hours, testing cell viability with WST-1, and demonstrated the strong cytotoxicity of MβCD on cell viability (Onodera *et al*., 2013). Maja *et al*. (2022) investigated whether cholesterol contributes to the spread of cancer cells, whether the effects are specific to cancer cells and the underlying mechanism using MCF10A cell line series (non-tumorigenic) and MDA-MB-231 cell lines (as a model of breast cancer progression and the highly invasive). Their results demonstrated that that partial membrane cholesterol depletion specifically and reversibly decreased invasion of the malignant cell lines. Also, dorsal cholesterol-enriched domains can be endocytosed and reach the cell ventral face where they were involved in invadopodia formation and extracellular matrix degradation. In contrast, non-malignant cells showed low cell invasion, low surface cholesterol exposure and cholesterol-dependent focal adhesions (Maja *et al*., 2022). The results of our study, showing a dose-dependent reduction in MCF-7 cell viability with MβCD concentrations at both 24 and 48 hours, are important in the context of similarities with existing literature findings. Crocetin is a significant carotenoid derived from the stigmas of the saffron plant, characterized by a short carbon chain length. Known for its anti-inflammatory, anti-tumor, and antioxidant qualities, this yellow-colored main component is a special chemical used in traditional medicine (Li *et al*., 2017; Umigai *et al*., 2011). By utilizing the MTT test to treat MCF-7 and MDA-MB-231 breast cancer cell lines with extracts from four distinct *Crocus* species, Chryssanthi *et al*. (2007) examined existing dose-dependent inhibition. In their research, they identified *Crocus sativus* as the most effective species with chemopreventive and anti-cancer properties among the four *Crocus* species included in the study (Chryssanthi *et al*., 2007). The bioactive components of saffron, crocin, and crocetin molecules, were found to inhibit the proliferation of cancer cells and induce apoptotic processes in cells, as evidenced by the results of the conducted study on breast cancer (Gutheil *et al*., 2012; Zhang *et al*., 2013; Zheng *et al*., 2016). Moreover, Sajjadi *et al*. (2017) through their investigation on a breast cancer model induced in Wistar albino rats using three different doses of N-methyl-N-nitrosourea, explored the anticancer effects of crocetin and crocin active molecules through different mechanisms. According to their research, crocetin and crocin molecules both work well on breast cancer cells, however, crocetin is more potent than crocin

during the beginning and advanced phases of the disease (Sajjadi *et al*., 2017). Applying 250, 500, and 1000 µM crocetin to the estrogen receptor-positive (+), non-metastatic, epithelial-like breast cancer cell line MCF-7 as a breast cancer model showed dose- and time-dependent reductions in cell viability, corroborating the body of research showing the anticancer effects of crocetin carotenoid on cancer cell viability.

In our study, the effect of 500 µM crocetin addition to cells treated with only MβCD, only crocetin concentrations, and MβCD-applied cells was investigated on the expression of lipid raft-related genes (caveolin 1, LRP 6) and apoptotic genes (survivin, Bcl2, Bax, caspase3) in MCF-7 cells using RT-PCR. The findings showed that whereas Bax and caspase 3 were upregulated, the expression of caveolin 1, LRP 6, survivin, and Bcl2 was decreased by both MβCD and crocetin treatments. Furthermore, it was noted that the target genes' expression levels were further elevated ($p<0.05$) by the addition of crocetin after the administration of MβCD. The caveolin-1 protein is an essential member of the caveolin family that plays a vital role in regulating intracellular signals, found in the cell membrane of breast cancer cells. Research findings indicated a decrease in Caveolin-1 levels in breast cancer cells due to the disruption of lipid raft structure (Badana *et al*., 2018; Elsheikh *et al*., 2008; Raghu *et al*., 2010). LRP6, a receptor in the Wnt pathway, exhibits a lipid raft localization associated with caveolin-1. Furthermore, depending on the disruption and malfunction of the lipid raft structure in breast cancer cells, the survivin gene, an inhibitor of the apoptosis family, plays a dual role in the proliferation and apoptosis cycles (Chen *et al*., 2016). Badana *et al*. (2018) used MβCD to induce cholesterol depletion in triple-negative breast cancer cell lines MDA-MB-231, MDA-MB-468, and breast epithelial cell line MCF-12A. The mRNA expressions of caveolin-1, LRP6, survivin, Bcl2, ki67, and c-myc were found to be decreased, whereas the levels of BAX and caspase-3 were increased (Badana *et al*., 2018). The Bcl-2 family, known to regulate apoptotic signals, includes the Bax gene, which, along with the p53 gene, accelerates the cell's progression to apoptosis (Naseri *et al*., 2015). The increase in the Bax/Bcl-2 ratio within the cell indicates the initiation of the apoptotic cycle associated with an increase in caspase 3. The findings demonstrated that when given in conjunction with MβCD, crocetin carotenoid both raised the Bax/Bcl-2 ratio and improved the Bax/Bcl-2 response. Although there aren't many research findings about crocetin's use in breast cancer cells in the literature, this study, which shows how crocetin works by depriving lipid raft cholesterol to have anti-proliferative and apoptosis-supporting effects, can act as a roadmap for future research. A limitation of this study is that Caveolin-1, LRP-6, survivin, Bcl-2, BAX, and Caspase-3 study results could not be included at the protein level

Cholesterol depletion in the lipid raft structures of the breast cancer cell membrane using MβCD has been shown to affect cell viability and activate genes involved in the apoptosis pathway.Following the administration of crocetin carotenoid to MCF-7 cells, a comparable and more significant impact was seen when MβCD and crocetin were applied together. The potent anti-proliferative and anti-tumor carotenoid, crocetin, derived from the saffron plant, is believed to be a potential biological agent, especially in cancer cells, highlighting lipid raft-focused cholesterol depletion through in vitro and in vivo studies that would elucidate pharmacokinetic interactions.

The findings of this study show that crocetin carotenoid decreases cell viability and promotes the apoptosis process in MCF-7 cells by suppressing MβCD-induced lipid raft dysfunction. Lipid rafts are cholesterol-rich structures in the cell membrane and play critical roles in signal transduction and cell-cell interactions (Simons & Ikonen, 2000). Signaling pathways through lipid rafts may promote cell proliferation, metastasis, and resistance mechanisms in breast cancer (Hirsch *et al*., 2010). Given that crocetin induces apoptosis by enhancing lipid raft dysfunction, this may lead to the suppression of cholesterol-dependent proliferation and signaling pathways of breast cancer cells. This feature may support the evaluation of crocetin as a potential biological agent to inhibit the progression of breast cancer cells, particularly

through cholesterol depletion occurring in lipid raft structures. Moreover, given the role of crocetin in inhibiting lipid raft-dependent cell signaling, it may contribute to the development of novel tumor progression suppressive approaches for clinical treatment strategies.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). The study was approved by the Ethics Committee of the Faculty of Medicine (2021/112) of Niğde Ömer Halisdemir University.The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Niğde Ömer Halisdemir University, 2021-112.

Authorship Contribution Statement

Şerife Buket Bozkurt Polat: Concept-Design-Supervision-Materials- Data Collection or Processing -Analysis or Interpretation- Literature Search-Writing **Esma Ozmen**: Materials-Data Collection or Processing-Analysis or Interpretation-Writing.

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