Crocin Promotes Apoptosis in Human EBV-Transformed B-Lymphocyte via Intrinsic Pathway

Abdolreza Sotoodeh Jahromi¹, Mohammad Kargar1*, Farshid Kafilzadeh1, Marzieh Jamalidoust2 and Maliheh Moradzadeh3*

¹ Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran
² Department of Clinical Virology, Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Namazi Hospital, Shiraz, Iran.
³ Golestan Rheumatology Research Center, Sayad Shirazi Hospital, Golestan University of Medical Sciences, Gorgan, Iran.

* Both corresponding authors contributed and supervised equally to this research work and manuscript.

Competing interests: The authors declare no conflict of Interest.

Abstract. Background: As a major carotenoid in saffron, crocin demonstrates potent anti-cancer impacts. However, its anti-lymphoma effects remain vague, especially in the human EBV-associated B-cell lymphoproliferative disorders. This study examined crocin’s apoptogenic potential and its underlying mechanism in CO 88BV59-1 cell line vs. normal human peripheral blood B cells.

Methods: CO 88BV59-1 cells were treated with crocin alone or in combination with vincristine for up to 72 h. The cell viability was examined using a resazurin assay. Flow cytometry using annexin V and propidium iodide labeling was performed to detect apoptotic cells. Also, the expression levels of genes and proteins involved in apoptosis (CASP3, CASP8, CASP9, P53, Bax, and Bcl-2) were respectively determined via real-time PCR and Western blot analysis.

Results: Crocin concentration-dependently reduced cell viability in CO 88BV59-1 cells with no significant toxicity toward normal B cells. Similar to vincristine, crocin significantly increased apoptosis in these cells during 72 h of incubation. Furthermore, the combination of crocin (80 μM) and vincristine (1 μM) enhanced apoptosis in CO 88BV59-1 cells. Therefore, this synergistic effect was detected in human EBV-transformed B-lymphocyte. CASP3, CASP9, P53, and Bax/Bcl-2 ratio expressions were significantly raised in CO 88BV59-1 cells, whereas CASP8 was unaltered. It was proposed that crocin promoted apoptosis in CO 88BV59-1 cells in a time- and concentration-dependent manner via the induction of the intrinsic pathway.

Conclusion: The results suggest that crocin may serve as a good alternative/coadjuvant to vincristine in EBV-associated B-cell lymphoproliferative disorders.

Keywords: Crocin; CO 88BV59-1 cells; EBV-associated B-cell lymphoproliferative disorders; Apoptosis.

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Correspondence to: Mohammad Kargar, Department of biology, Jahrom Branch, Islamic Azad University, Jahrom, Iran Tel: 09173149203 Fax: 071 5437 2001 Email: microkargar@gmail.com
Maliheh Moradzadeh, Golestan Rheumatology Research Center, Sayad Shirazi Hospital, Gorgan, Iran Telefax: + 98 17 32239791 Email: Moradzadeh63@yahoo.com
Introduction. B-cell lymphomas associated with Epstein-Barr virus (EBV) infection are common in patients who have immunodeficiency states, e.g., organ transplantation and human immunodeficiency virus (HIV) infection. Reports show that 1.8% of global cancer deaths in 2010 are related to EBV-attributable malignancies (Approximately 142,979). The three main types of B cell malignancy associated with EBV are the Burkitt, Hodgkin, and diffuse large B-cell lymphomas (respectively BL, HL, and DLBCL). These pre-neoplastic/neoplastic lesions appearing in transplant patients are collectively referred to as post-transplant lymphoproliferative disorders (PTLD). The majority of PTLD cases are B cell, but 5-10% T and NK cell lymphomas have been described. EBV inflicts >95% of the world’s population and leads to lifelong asymptomatic infection. Its ability to induce oncogenesis may occur because of the suppression of the immune system or uncontrolled proliferation. The virus increases the activity of the B cell lymphoma 2 (Bcl-2) gene, which promotes cell proliferation by curbing apoptosis. The upregulation of the Bcl-2 gene can decrease the activity of tumor suppressor genes, including P53. Apoptosis is an evolutionary conserved, intrinsic program of cell death that occurs in various physiological and pathological states. The underlying mechanisms for initiating an apoptosis response upon cytotoxic therapy may depend on the individual stimulus and damage to DNA; however, damage to DNA or other critical molecules is considered a common initial event propagated by the cellular stress response. Apoptosis pathways can be initiated through different entry sites, for example, at the plasma membrane by death receptor ligation (named receptor or extrinsic pathway) or at the mitochondria (mitochondrial or intrinsic pathway). Bcl-2, P53, Bax, Caspase-9 (CASP9) are involved in the intrinsic pathway, and CASP8 in the extrinsic pathway.

Introducing rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) to treat B-cell lymphoma can markedly improve patients’ survival rate. Nevertheless, resistance to these drugs and their toxicity are significant obstacles to the course of treatment. Therefore, there is an urgent need for the development of novel therapeutic medications.

Researchers held that dietary phytochemical agents might affect chemotherapy and contribute to the treatment of cancer patients. According to some studies, phytochemicals isolated from medicinal plants inhibited cell proliferation and induced apoptosis. At present, some of these plant-derived compounds are widely applied in chemotherapy for cancer treatment. For instance, taxol analogs, vinca alkaloids (vincristine and vinblastine), and podophyllotoxin analogs have greatly contributed to cancer treatment. Plant-derived compounds such as carotenoids play a pivotal role against cancer, providing a valuable source of anti-cancer agents. Increasing evidence suggests that crocin, a carotenoid isolated from the saffron plant (Crocus sativus L.), has anti-cancer effects in various types of cancer. Like vincristine, crocin’s anti-proliferative activity involves targeting microtubules or p53-dependent and -independent mechanisms in cancer cells.

Our laboratory has been interested in crocin’s application in lymphoma and leukemia. Still, the exact mechanism of its action against EBV-associated B-cell lymphomas remains unknown. Thus, herein, a series of experiments were designed to examine crocin’s apoptogenic potential and its underlying mechanisms in CO 88BV59-1 EBV-transformed B-lymphocyte vs. normal human B cells.

Materials and Methods

Cell line and reagents. Human CO 88BV59-1 EBV-transformed B-lymphocyte (CRL-10624™) was purchased from ATCC (USA). The high-glucose Roswell Park Memorial Institute medium (RPMI 1640), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from Gibco BRL Life Technologies (USA). Moreover, 7-hydroxy-3H-phenoxyamin-3-one-10-oxide (resazurin), crocin (>95%), vincristine (>95%), Fluorescein isothiocyanate (FITC) annexin V antibody, and propidium iodide (PI) were procured from Sigma-Aldrich (USA). TRizol was obtained from Invitrogen (USA). A real-time PCR Master Mix and a cDNA synthesis Kit were also purchased from Roche Diagnostic (Switzerland) and Fermentas (Lithuania). Moreover, an enhanced chemiluminescence (ECL) detection kit and polyvinylidene difluoride (PVDF) membranes were purchased from GE Healthcare (UK) and Millipore (USA), in that order. Primary antibodies for β-actin, Bcl-2, Bax, P53, cleaved caspases (3, 8, and 9), and secondary antibodies were obtained from Cell Signaling Technology (USA). Fluorescein isothiocyanate-conjugated antibody against CD19 was also purchased from BioLegend (USA). Finally, a human B cell isolation kit was obtained from Miltenyi Biotec (Germany).

Human normal B cell isolation and cell culture. Human peripheral blood mononuclear cells (PBMCs) were obtained from fresh blood samples taken from healthy...
volunteers by Ficoll-density (Pharmacia, Sweden) gradient centrifugations. Subsequently, B-cells were isolated from PBMC via a B-cell isolation kit. Then, the purity of the cellular preparation was tested via FITC-conjugated anti-human CD19 antibody staining in the MACS analysis, and it was found to be >97% pure (Figure 1).\(^\text{15}\) CO 88BV59-1 and normal B cells were also cultured in the RPMI medium containing 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin maintained at 37 ºC in a humidified atmosphere (90%) containing 5% CO\(_2\). The cells were subsequently incubated with different concentrations of vincristine (0.05-50 μM) and crocin (0.2-200 μM) up to 72 h. All the treatments were conducted in triplicate.

Cell viability assay. We determined cell viability by the resazurin reagent. For this purpose, CO 88BV59-1 and normal B cells (1×10\(^5\)) were added to each well in 96-well culture plates treated with vincristine (0.05-50 μM) and crocin (0.2-200 μM) up to 72 h. Next, 20 μl of the resazurin reagent was added to each well, and the plates were incubated for 4 h. The fluorescence intensity of the product resorufin, proportional to the number of viable cells per well, was measured via a fluorescence Victor X5 2030 Multilabel Plate Reader (Perkin Elmer, Shelton, Connecticut) with excitation at 530 nm and emission at 590 nm.\(^\text{16}\)

Cell apoptosis assay. Crocin's apoptosis effect on CO 88BV59-1 cells was assessed by FITC annexin V/PI staining. The cells were treated with crocin (175, 112, and 80 μM) and vincristine (35, 23, and 1 μM) according to IC50 values for different durations (24, 48, and 72 h), respectively. Also, we assessed the combination of crocin (80 μM) and vincristine (1 μM) on these cells for 72 h. After the treatment, the cells were incubated with the FITC annexin V antibody and analyzed by a flow cytometer (BD Biosciences, USA). The FlowJo software (TreeStar Inc.) was employed for data analysis.\(^\text{17}\)

Real-time PCR quantification with SYBR Green. The CO 88BV59-1 cells were treated with crocin (175, 112, and 80 μM) alone or in combination with vincristine (35, 23, and 1 μM) up to 72 h, and then RNA extraction was done using TRizol according to the manufacturer's instruction. RNA concentration and purity were evaluated via spectrophotometry. For each sample, the complementary DNA (cDNA) was synthesized from the total RNA (100 ng) via a cDNA synthesis kit with the random hexamer primer. Primers (Bcl-2, Bax, P53, CASP3, CASP8, and CASP9 genes) were designed using the Beacon software (Applied Biosystems; Table 1). Gene expression changes were determined using SYBR Green-based real-time PCR technology by the Applied Biosystems Step One Plus Detection System (ABI, USA). The reaction mixture comprised 1 μl of the primers (100 pmol), 2 μl of cDNA (250-400 ng), 10 μl of 2x master mix, and dH\(_2\)O to bring the volume to 20 μl. The optimized parameters utilized for the thermocycler included a short hot-start at 95 ºC for 15 min, followed by 40 cycles, each consisting of denaturing at 95 ºC for 15 secs, annealing at 60 ºC for 1 min, and extension at 72 ºC for 20 sec. Melting curves were used from 60 to 90 ºC rising by 0.3 º, as the final step of the SYBR Green

![Figure 1](image-url) **Figure 1.** Normal B cells purification using MACS technique as evaluated by flow cytometry. A) Representative histogram of the fluorescence intensity of CD19-labeled normal B cells. In a series of three experiments, B cells were enriched from PBMC, to give populations of 94-98% CD19-positive B cells. B) IgG isotype control was used as a negative control to differentiate the background noise of the flow cytometry analysis.
real-time PCR. Gene expressions were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. The samples were run in triplicate, and the fold difference of expression in the treated and untreated samples was calculated using the $2^{-\Delta\Delta CT}$ method. 

**Western blot analysis.** Following 72 h of treatment with crocin (80 μM) alone or in combination with vincristine (1 μM), the cells were lysed using the lysis buffer and centrifuged at 18000 g, at 4 °C for one h, and then the supernatant was collected. Next, the lysates were run on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred onto nitrocellulose membranes. After blocking with 2% Bovine serum albumin (BSA), the blots were exposed to the primary antibody for one h at room temperature. In the next step, they were washed and incubated with the corresponding horseradish peroxidase-conjugated to the secondary antibody for two h. Finally, membrane visualization was performed using an ECL detection kit. The reactions were revealed and documented by Gel-Doc (Syngene, Cambridge, UK), and the images were quantified using the Image J software (version 1.46). 

**Statistical analysis.** The data are represented as mean ± SD and were analyzed using one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons post-hoc test in the Graph Pad PRISM software (Version 6, Graph Pad Software, CA). A p-value <0.05 was considered statistically significant.

**Results.**

*Crocin concentration-dependently reduced the viability of CO 88BV59-1 cells.* Crocin at concentrations of 100 and 200 μM significantly decreased the viability of CO 88BV59-1 cells at 48 and 72 h (p < 0.05; Figure 2A). Similarly, a significant drop in viability was observed in these cells incubated for 48 and 72 h with 25 and 50 μM of vincristine (p < 0.05). However, crocin did not affect the viability of normal B cells at concentrations of 0.2-200 μM (Figure 2B). **Table 2** presents the IC50 values of crocin and vincristine in CO 88BV59-1 cells for 24, 48, and 72 h of incubation.

**Table 2:** IC50 values of crocin and vincristine in CO 88BV59-1 cell line up to 72 h incubation.

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![Figure 2](image2.png)

**Figure 2.** Effects of crocin on the viability of CO 88BV59-1 cells. **A** The cells were treated with various concentrations of crocin (0.2-200 µM) and vincristine (0.05-50 µM) up to 72 h. **B** Normal B cells were treated with various concentrations of crocin (0.2-200 µM) up to 24 h. Cell viability was determined using the resazurin assay. The data are expressed as the mean ± SEM of three independent experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 vs. untreated control cells (concentration of 0).

![Figure 3](image3.png)

**Figure 3.** Effects of crocin alone or in combination with vincristine on the apoptosis of CO 88BV59-1 cells as evaluated by annexin V and propidium iodide double-staining. The representative dot blot of the fluorescence intensity of annexin V and PI double-stained cells treated with crocin (175, 112 and 80 µM) and vincristine (35, 23 and 1 µM) based on their IC50 value for 24, 48, and 72 h. Also, these cells were treated with combination of crocin (80 µM) and vincristine (1 µM) for 72 h. Quantitative analysis was performed in the Flow Jo software. The data are presented as the mean ± SEM of three independent experiments performed in triplicate. ***p < 0.001 vs. untreated control cells (concentration of 0).
Crocin time-dependently induced apoptosis in CO 88BV59-1 cells. Figure 3 displays the impacts of crocin on the apoptosis of CO 88BV59-1 cells, assessed by annexin V and PI double staining. Similar to vincristine, crocin (175, 112, and 80 µM) significantly and time-dependently spiked the apoptosis rate of these cells (p < 0.001). Furthermore, the combination of crocin (80 µM) and vincristine (1 µM) remarkably enhanced apoptosis by up to 68% in CO 88BV59-1 cells (p < 0.001).

Crocin modulated genes involved in survival and apoptosis in CO 88BV59-1 cells. Figure 4 depicts the effects of crocin alone or in combination with vincristine on the expression of genes involved in survival (Bcl-2) and apoptosis (P53, Bax, CASP3, CASP8, and CASP9) in CO 88BV59-1 cells up to 72 h. The expressions of P53, CASP3, CASP8, and CASP9 were significantly raised in these cells treated with either crocin or vincristine (p < 0.001 compared with control cells). In addition, crocin alone or in combination with vincristine significantly increased the Bax/Bcl-2 ratio to 4.4 ± 0.16 and 5.7 ± 0.20-fold in the cells during 72 h, respectively (p < 0.001 compared with control cells; Figure 4).

To further assess the impact of crocin on apoptotic genes, their protein levels were evaluated by western blot analysis (Figure 5). Treatment of cells with crocin increased the protein expressions of P53, Bax, CASP3, and CASP9 but reduced the Bcl-2 expression. On the contrary, the expression of CASP8 protein did not change in CO 88BV59-1 cells following treatment with crocin (Figure 5); however, crocin combined with vincristine raised the expression of CASP8 (p < 0.001). The levels of Bax/Bcl-2 protein was significantly increased in crocin (80 µM) alone or in combination with vincristine (1 µM)-treated cells compared to untreated cells (4.09 ± 0.18 and 5.4 ± 0.11, respectively; p < 0.001), thereby confirming the synergistic effect of crocin on CO 88BV59-1 cells. A similar surge was also observed in the cells incubated with 1 µM of vincristine (4.2 ± 0.11, p < 0.001). It was demonstrated that crocin significantly increased Bax/Bcl-2 ratio, approximately equal to vincristine.

Discussion. This study was the first to examine the mechanism of apoptotic cell death induced by crocin in EBV-transformed B-lymphocyte (CO 88BV59-1 cell line), compared to the standard anti-lymphoma drug, vincristine, during short-term treatment. Crocin effectively inhibited cell proliferation and induced apoptosis at high concentrations in CO 88BV59-1 cells during 72 h of treatment. The cytotoxic effect of crocin was more noticeable against CO 88BV59-1 cells than against normal human B cells, and these effects were comparable with those of vincristine. Furthermore, crocin significantly up-regulated the expression P53, CASP3, CASP9, and Bax/Bcl-2 ratio in CO 88BV59-1 cells at the mRNA and protein level, whereas the CASP8 protein remained unchanged. The present study's
findings revealed that crocin induces apoptosis via the intrinsic pathway in a concentration- and time-dependent manner in CO 88BV59-1 cells. Interestingly, the combination of crocin (80 μM) and vincristine (1 μM) induced apoptosis in these cells via both pathways (intrinsic and extrinsic). Therefore, this synergistic apoptotic effect of crocin and vincristine was detected in these cells.

Patients who have acquired or inherited immune incompetence demonstrate a high incidence of lymphoma. A common factor in these patients seems to be the impairment of immunoregulatory mechanisms involved in neoplastic and/or viral surveillance. The incidence of EBV among B-cell lymphoma patients is <5% in the United States and European countries, but 10-15% in Latin American and Asian countries. However, EBV-related B-cell lymphomas in transplant recipients show some additional characteristics; most notably, a large proportion of these tumors tend to regress spontaneously upon immunosuppression withdrawal or reduction, even though they are almost universally fatal if they remain untreated.

Several studies reported that nontoxic natural agents could be useful either alone or in combination with conventional therapeutics to prevent tumor progression and/or treat human malignancies.

The fact that crocin is abundantly available in large quantities in food products and is reportedly nontoxic makes its anti-cancer effect even more attractive. Furthermore, since it also possesses immunosuppressive characteristics, it can exert potent anti-inflammatory effects in autoimmune diseases via inhibiting cytokines. Consistent with our findings, a study explored the effect of crocin on the proliferation and differentiation of HL-60 cells during long-term (5 days) exposure. In another research, crocin inhibited proliferation and induced apoptosis in leukemic cell lines (K562, HL-60, L1210, and P388): crocin also inhibited the proliferation of Jurkat and HL60 cells by reducing cell growth and induced apoptosis by raising the Bax/Bcl-2 ratio.

Some studies showed that the anti-proliferative activity of crocin like vincristine involves targeting microtubules and p53-dependent and -independent mechanisms in colon cancer cells. Crocin triggers apoptosis by increasing the Bax/Bcl-2 ratio and caspase activation in human gastric adenocarcinoma without affecting human normal fibroblast skin cells. Also, reported that the combination of crocin with cisplatin exerts growth suppression and apoptosis in gastric carcinoma cells. Crocetin (hydrolyzed crocin) induced p53-mediated cell death in functional p53-expressing cancer cells through Bax and P53-induced protein with a death domain (PIDD) caspase-2-t-BH3 interacting-domain death (BID) pathway. Another study showed that dimethyl-crocetin and crocin induced cytotoxicity on HL60 cells but did not affect K562 cells. They suggested that dimethyl-crocetin could disrupt DNA–protein interactions (e.g., topoisomerase II) and inhibit nucleic acid synthesis. Controversially, the other study showed that crocin, unlike silymarin, retinoic acid, and other drugs, was unable to prevent the neoplastic transformation of rat tracheal epithelial cells by Benzoyprene. Another study presented that crocin suppressed multiple human myeloma growth through inhibition of STAT3-mediated gene products, including BAX, Bcl-2, vascular endothelial growth factor (VEGF), CXC Chemokine Receptor 4 (CXC4), and cell cycle regulator (cycclin D1). Xu et al. showed that crocin could block HL-60 cells in the G0/G1 phase and inhibit their proliferation. The suggested mechanism in these cells may be related to the inhibition of Bcl-2 and activation of Bax.

The studies investigated the effect of crocin on the proliferation and immune function of dendritic cells (DC) derived from the bone marrow of children with acute leukemia. They concluded that crocin could synergically promote the maturity of DC cooperating with recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), recombinant human IL-4 (rhIL-4), and recombinant human TNF-α (rhTNF-α). The DC induced by crocin can particularly enhance the proliferation of T cells.

According to Molnar et al., crocin and crocetin were ineffective in reversing multidrug resistance of lymphoma cells but inhibited the early tumor antigen expression of adenovirus-infected mouse lymphoma cells. It is noteworthy that crocin demonstrates a significant antiviral activity against HSV-1 and also HIV-1. Another study also showed that crocin concentration- and time-dependently inhibited the proliferation and prolonged the lifespan of Dalton's lymphoma-bearing animals through significant effects on hematological parameters. On the other hand, Khavari et al. concluded that a combination of DNA vaccine with crocin did not potentiate protective and therapeutic effects compared to mono-therapies for controlling papillomavirus-infected tumors. Based on another study, crocin exhibited low cytotoxic effects on the MOLT-4 cell line, which might be mediated through the escalation of DNA fragmentation. Also, crocin significantly and concentration-dependently promoted cell proliferation and IL-2 and IL-4 secretion. Crocin itself caused no significant damage to T cells but curbed DNA damage in T cells treated with cytarabine. Clinical experiments reported that healthy volunteers treated with saffron tablets (200 mg/kg) did not demonstrate hematological or biochemical toxicity. In addition, a pharmacokinetic study suggested that orally administered crocins are hydrolyzed to crocetin before or during intestinal absorption. The LD50 of crocin has
been reported to be >3 g/kg.\(^1\)

The present study had some limitations partially due to a reduced financial budget. This study was carried out on only one EBV-transformed B cell, and only six genes and proteins (apoptotic and anti-apoptotic) were evaluated in this research work. It should be better to utilize more than two EBV-transformed B cells and study other apoptotic and anti-apoptotic genes.

**Conclusions.** The results showed that crocin promotes apoptosis in CO 88BV59-1 cells in a time- and concentration-dependent manner via the induction of the P53-dependent intrinsic pathway. Furthermore, crocin and vincristine have a synergistic effect on these cells. Thus, it is suggested from these preclinical studies to evaluate the effect of crocin alone or in combination with vincristine in EBV-associated B-cell lymphoproliferative disorders.

**Ethical consideration.** This study was approved by the Research Ethics Committee of Jahrom University of Medical Sciences (ethic code: IR.JUMS.REC.1399.026).

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