RESEARCH ARTICLE

Editorial Process: Submission:04/21/2024 Acceptance:09/03/2024

Effect of Crocin and Crocetin Compared to Cyclophosphamide on the Expression Level of *miRNA-16-1* in a B Cell Transformed with EBV Virus Cell Line

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Abstract

Introduction: Crocin and Crocetin are compounds that have shown promising therapeutic potentials in various medical contexts. To date, the effect of crocin and crocetin on the expression level of *miRNA-16-1* in Epstein Barr Virus (EBV)-induced lymphoma has not been investigated. This research delved into a comparative analysis of the cytotoxic effects of crocin and crocetin compared to cyclophosphamide, the main drug used in the treatment of lymphoma and PTLD, on B-cell lymphoma infected with EBV (cell line CO 88BV59-1). Additionally, the study examines the changes in *miRNA-16-1* expression following these treatments in this cell line. **Materials and methods:** CO 88BV59-1 LCL cells were treated with crocin, crocetin (0.2 to 200 μM), and cyclophosphamide (0.05 to 50 μM) for 72 hours. Cell viability and apoptosis were assessed using resazurin and Annexin V/PI techniques, respectively. Additionally, the expression of *miRNA-16-1-3p* and *miRNA-16-1-5p* was determined using the Real-Time PCR method. The data were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post-hoc test. **Results:** Crocin and crocetin inhibited the proliferation and apoptosis caused by EBV-infected cells in a dose- and time-dependent manner ($P<0.05$). The expression levels of *miRNA-16-1-3p* and *miRNA-16-1-5p* remained unchanged in cells treated with crocin and crocetin. **Conclusion:** The study found that the cytotoxic effect of Crocin, Crocetin, and Cyclophosphamide on CO 88BV59-1 LCL is independent of the expression level of *miRNA-16-1*. The results showed a reduction in cell survival and an increase in cell death.

Keywords: CO 88BV59-1 LCL- Epstein Barr Virus- B-cell lymphoma- crocin- crocetin

Asian Pac J Cancer Prev, **25 (9)**, 3179-3185

Introduction

The prevalence of Epstein Barr Virus (EBV), a member of the herpes gamma virus family, in communities exceeds 90% [1]. EBV is known to cause lymphoproliferation and is associated with B cell malignancies in immune deficient conditions. The virus alters the expression of microRNA (miRNA), a group of small non-coding RNAs, in infected cells, which in turn changes the expression of cellular genes. This can lead to the infected cell becoming immortal or cancerous [2].

miRNAs are non-coding RNAs that regulate gene expression in the post-transcriptional stage. They are 18-23 nucleotides in size and are closely linked to various diseases, including cancer. Approximately one-third of human gene expression is regulated by miRNAs [3].

miRNAs play a crucial role in regulating various biological processes, such as organ development [4], stem cell self-replication, cell differentiation [5], growth, apoptosis, immune system regulation [6], tumorigenesis, tumor suppression, metastasis, and drug resistance [7]. The miRNAs *miRNA-16-1-3p* and *miRNA-16-1-5p* are located in the 13q14 gene locus and act as negative regulators of B-cell lymphoma protein 2 (Bcl-2). They are considered tumor suppressor miRNAs [8].

In many cancer cells infected with EBV, a decrease in the activity or deletion of miRNA-16-1 has been observed. As a result, the activity of the BCL-2 gene increases, leading to the uncontrolled proliferation of tumor cells. This decreases apoptosis and increases cell immortality

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[9].

Post-transplantation lymphoid disorders (PTLD) are caused by the transmission of EBV from an EBV-positive transplant donor to a transplant recipient. Alternatively, PTLD may occur due to the activation of the virus in a transplant recipient who was latently infected with EBV before receiving the transplant, as a result of immune system weakening. Preventing PTLD in transplant patients is challenging due to the high prevalence of EBV in the community [10].

Antiviral drugs that are used to treat other diseases caused by EBV are ineffective in treating PTLD caused by EBV. Cyclophosphamide is one of the chemotherapy drugs used to treat lymphoproliferative disorders, lymphomas, and PTLD by apoptotic effect, but its use has many side effects [11]. PTLD can be treated with immunotherapy, such as rituximab, but it is expensive and only effective when combined with chemotherapy [12]. Reducing the prescription of immunosuppressive drugs is also recommended to avoid transplant rejection. However, there is currently no ideal treatment for PTLD [13].

Due to the adverse effects of conventional cancer treatment drugs [14, 15] and drug resistance [16], herbal compounds have recently been noticed for cancer treatment [17]. Plant derivatives, such as carotenoids, are significant in cancer treatment and provide a valuable source of anti-cancer agents. Evidence suggests that crocin and crocetin, carotenoids isolated from the saffron plant (Crocus sativus L), have anticancer effects and do not exhibit cytotoxic effects on normal cells [18, 19]. Crocin (C32H44O14, Molecular Weight: 652.68) and Crocetin (C20H24O, Molecular Weight: 328.40) are compounds that have shown promising therapeutic potentials in various medical contexts. Previously, reported that crocin and crocetin have inhibitory properties against various tumor cells [20-23].

By elucidating the impacts of these treatments on miRNA-16-1 regulation, this study aims to provide valuable insights into potential therapeutic strategies for EBV-associated diseases. To date, the effect of crocin and crocetin on the expression level of miRNA-16-1 in EBV-induced lymphoma has not been investigated. Given the high prevalence of organ transplantation and PTLD, as well as the lack of proper treatment for PTLD, it is important to explore the potential of these compounds in treating this condition. This research aims to investigate the cytotoxic effects of crocin and crocetin compared to cyclophosphamide, the main drug used in the treatment of lymphoma and PTLD, on B-cell lymphoma infected with EBV (cell line CO 88BV59-1). Additionally, the study examines the changes in miRNA-16-1 expression following these treatments in this cell line.

Materials and Methods

Cell line and reagents

This experimental study was done on CO 88BV59-1 cell line. The 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). Additionally, Sigma-Aldrich (USA) provided 7-hydroxy-3H-phenoxazin-3-one-10-oxide (resazurin), crocin (>95%) CAS No.: 55750-85-1, crocetin (>95%) CAS No.: 27876-94-4, cyclophosphamide (>95%) CAS No: 6055-19-2, Fluorescein isothiocyanate (FITC) annexin V antibody, and propidium iodide (PI). TRIzol was obtained from Invitrogen (USA), while a real-time PCR Platinum SYBR Green qPCR Super Mix-UDG and a cDNA synthesis Kit were purchased from Invitrogen (USA) and Roche Diagnostic (Switzerland) and Fermentas (Lithuania), respectively.

Cell culture

The CO 88BV59-1 cells were cultured in RPMI medium with 10% (v/v) FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained at 37 ºC in a humidified atmosphere (90%) containing 5% CO2. The cells were incubated with varying concentrations of crocin, crocetin (0.2-200 µM), and cyclophosphamide (0.05-50 µM) for up to 72 hours. All treatments were performed in triplicate.

Cell viability assay

Cell viability was determined using the resazurin reagent. To achieve this, CO 88BV59-1 cells (1×105) were added to each well in 96-well culture plates treated with crocin, crocetin (0.2-200 µM), and cyclophosphamide (0.05-50 µM) for up to 72 hours. Afterwards, 20 µl of the resazurin reagent was added to each well, and the plates were incubated for 4 hours. The fluorescence intensity of the product resorufin, which is proportional to the number of viable cells per well, was measured using a fluorescence Victor X5 2030 Multilabel Plate Reader (Perkin Elmer, Shelton, Connecticut) with excitation at 530 nm and emission at 590 nm.

Cell apoptosis assay

The apoptosis effects of crocin, crocetin, and cyclophosphamide on CO 88BV59-1 cells were assessed by FITC annexin V/PI staining. The cells were treated with crocin (177.4, 119.5, and 53.2 µM), crocetin (158.1, 105.7, and 13.1 µM), and Cyclophosphamide (142.5, 38.3, and 4.7 µM) for different durations (24, 48, and 72 h) based on their respective IC_{50} values.

Following treatment, the cells were incubated with FITC annexin V/PI and analyzed using a flow cytometer (BD Biosciences, USA). Data analysis was performed using FlowJo software (TreeStar Inc.).

Real-time PCR quantification

Real-time PCR quantification was carried out using SYBR Green. The CO 88BV59-1 cells were treated with crocin (177.4, 119.5, and 53.2 µM), crocetin (158.1, 105.7, and 13.1 µM), and cyclophosphamide (142.5, 38.3, and 4.7 µM) for up to 72 hours. RNA extraction was performed using TRIzol according to the manufacturer's instructions. RNA concentration and purity were evaluated using spectrophotometry. Complementary DNA (cDNA) was synthesized for each sample using a cDNA synthesis kit with the universal step loop (USTL) primer [24] (Table 1).

The primers for *miRNA-16-1-3p* and *miRNA-16-1-5p* were designed using the Beacon software (Applied Biosystems; see Table 1).

Gene expression changes for *miRNA-16-1-3p* and *miRNA-16-1-5p* were determined using Platinum SYBR Green qPCR Super Mix-UDG from Invitrogen and the Applied Biosystems Step One Plus Detection System (ABI, USA). The reaction mixture consisted of 1.5 µl of forward primers (10 µmol), 1.5 µl of Universal Reverse primer (10 µmol), 1 µl of cDNA (250-400 ng), 25 µl of SYBR mix, and 21 µl of dH2O. The thermocycler was optimized with a short hot-start at 95 °C for 15 min, followed by 40 cycles, each consisting of denaturing at 95 °C for 15 secs, annealing at 60 °C for 45 secs, and extension at 72 °C for 30 sec [24] (Table 1).

Gene expressions were normalized to the housekeeping miRNA, mi-RNA U6. 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 [24].

Statistical analysis

The data 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). The results were presented as mean ± SE. A p-value of less than 0.05 was considered statistically significant.

Results

Effect of Crocin, crocetin and cyclophosphamide of the viability on CO 88BV59-1 LCL

Crocin, crocetin and cyclophosphamide reduced cell viability in a dose- and time-dependent manner. The percentage of cell survival decreased with increasing concentration. The lowest cell viability was observed at a concentration of 200 µM for crocin and crocetin, and at a concentration of 50 µM for cyclophosphamide after 72 hours of incubation (P>0.001) (Figure 1). Table 2 shows the IC_{50} values at different incubation times.

Apoptotic effect of Crocin, crocetin and cyclophosphamide on CO 88BV59-1 LCL

To investigate the apoptotic effects of crocin, crocetin, and cyclophosphamide on the CO 88BV59-1 LCL cell line, the cells were treated with the compounds for 24, 48, and 72 hours, according to the obtained IC_{50} values. The apoptotic effects were then determined by flow cytometry using Annexin V and PI (Figure 2A). Figure 2B shows that crocin, crocetin, and cyclophosphamide had a significant apoptotic effect on the cell line compared to the control group's cells (untreated) (P<0.001).

Effect of Crocin, crocetin and cyclophosphamide on miRNA-16-1 in CO 88BV59-1 LCL

Crocin, crocetin, and cyclophosphamide did not affect

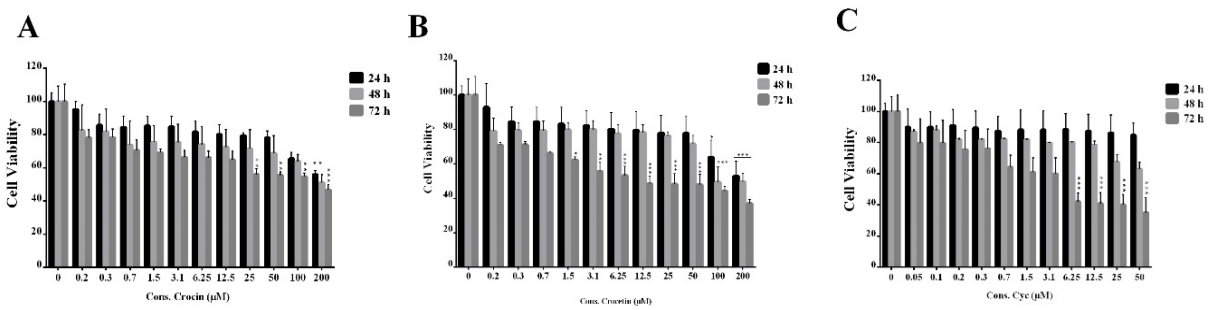

Figure 1. The Effects of Crocin (A), Crocetin (B), and Cyclophosphamide (C) on the Viability of CO 88BV59-1 Cells were Investigated. The cells were treated with different concentrations of crocin, crocetin (0.2-200 µM), and cyclophosphamide (0.05-50 µM) for up to 72 hours. Cell viability was determined using the resazurin assay. However, the effect of cyclophosphamide on cell viability was not significant. The results show that treatment with crocin and crocetin had a significant effect on cell viability compared to untreated control cells (concentration of 0) (*p < 0.05, **p < 0.01, ***p < 0.001).

Table 1. Primers were Used to Determine the Expression of *hsa-miR-16-1*

mi-RNA	(5′>3′) sequence	(C º)
USTL primer	5-GAAGGCGAGGAGCAGATCGAGGAAGAAGACGGAA	90
	GAATGTGCGTCTCGCCTTCTTTCNNNNNNNN-3	
hsa-miR-16-1-3p	5-CCAGUAUUAACUGUGCUGCUGA-3	
hsa-miR-16-1-3P-F primer	5-ACACTCCAGCTGGGCCAGTATTAACTGTGCTGCTG-3	60
hsa-miR-16-1-5p	5-UAGCAGCACGUAAAUAUUGGCG-3	
hsa-miR-16-1-5P-F primer	3-5-ACACTCCAGCTGGGTAGCAGCACGTAAATATTGGC	60
Universal Revers primer	5-TGGTGTCGTGGAGTCG-3	59/8
U6 internal control F primer	5-AACGCTTCACGAATTTGCGT-3	59/1
U6 internal control R primer	5-CTCGCTTCGGCAGCACA-3	59/1

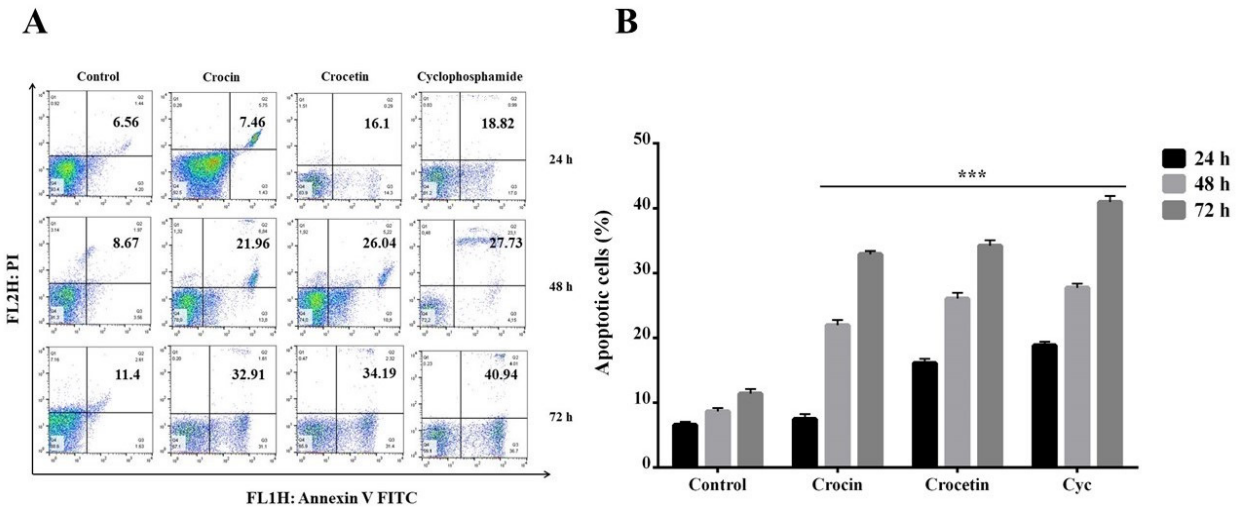

Figure 2. The Effects of Crocin, Crocetin, and Cyclophosphamide on the Apoptosis of CO 88BV59-1 Cells were Evaluated Using Annexin V and Propidium Iodide Double-Staining. The fluorescence intensity of annexin V and PI double-stained cells treated with crocin (177.4, 119.5, and 53.2 µM), crocetin (158.1, 105.7, and 13.1 µM), and cyclophosphamide (142.5, 38.3, and 4.7 µM) based on their IC_{50} value for 24, 48, and 72 h (based on table 2), was represented in a dot blot (2A). Quantitative analysis was performed using Flow Jo software. The data is presented as the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis showed a significant difference between treated and untreated control cells (concentration of 0) (***$p < 0.001$) (2B).

the expression of *miRNA-16-1-3p* and *miRNA-16-1-5p* in CO 88BV59-1 cells. Figure 3 shows the effects of these treatments on the expression of *miRNA-16-1-3p* and *miRNA-16-1-5p* genes in CO 88BV59-1 cells up to 72 h. The expressions of *miRNA-16-1-3p* and *miRNA-16-1-5p* genes were not significantly altered in these cells treated with crocin, crocetin, and cyclophosphamide compared to control (untreated) cells ($p > 0.05$).

Table 2. The IC_{50} Values of Crocin, Crocetin, and Cyclophosphamide were Determined in the CO 88BV59-1 Cell Line after up to 72 hours of Incubation.

	24 h	48 h	72 h
Crocin (µM)	177.4±0.08	119.5±0.13	53.2±0.12
Crocetin (µM)	158.1±0.11	105.7±0.09	13.1±0.13
Cyclophosphamide (µM)	142.5±0.20	38.3±0.08	4.7±0.12

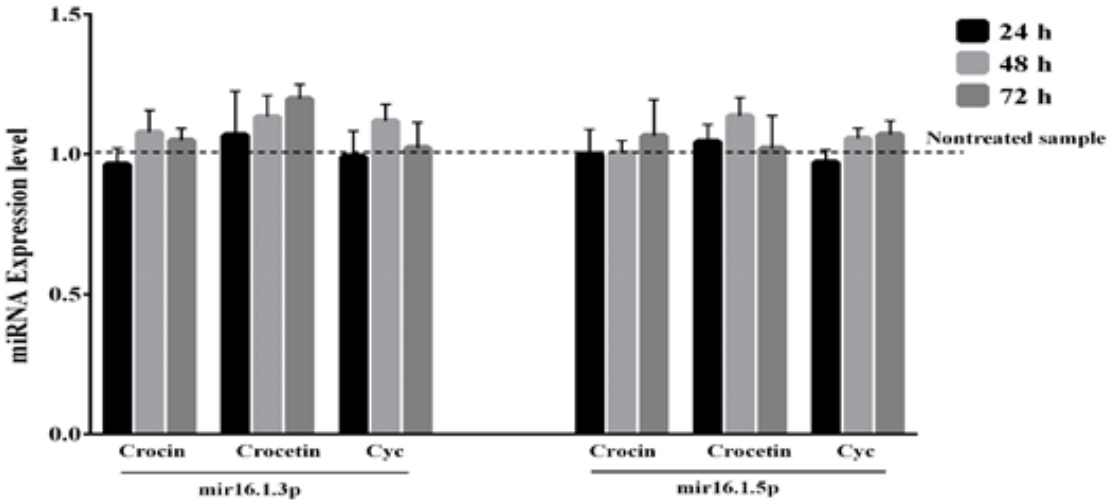

Figure 3. Effects of Crocin, Crocetin, and Cyclophosphamide on the Expression of *miRNA-16-1-3p* and *miRNA-16-1-5p* Genes in CO 88BV59-1 Cells were Investigated. The cells were treated with crocin (177.4, 119.5, and 53.2 µM), crocetin (158.1, 105.7, and 13.1 µM), and cyclophosphamide (142.5, 38.3, and 4.7 µM) based on their IC_{50} values for 24, 48, and 72 h (based on table 2). The expression levels of miRNA-16-1-3p and miRNA-16-1-5p genes were determined by real-time PCR. The data represent the mean ± SEM of three independent experiments performed in triplicate. Comparisons were made to untreated control cells (concentration of 0).

Discussion

The study aimed to investigate the effect of crocin and crocetin, compared to cyclophosphamide as a standard drug, on the expression level of miRNA-16-1 in B-cell lymphoma in vitro. The study found that crocin and crocetin had a significant effect on the expression level of miRNA-16-1 on CO 88BV59-1 LCL cell line, suggesting their potential as a treatment option. The study is the first of its kind to investigate this effect. In this study, the cytotoxicity and apoptotic effect of crocin and crocetin in different concentrations were evaluated in comparison with cyclophosphamide against CO 88BV59-1 LCL cell line, which is a type of transformed B cell infected with EBV virus.

The study results indicate that cyclophosphamide significantly reduces the viability of CO 88BV59-1 LCL cells, which is consistent with previous reports on the inhibitory and therapeutic properties of cyclophosphamide against various tumor cells [25]. Crocin and crocetin were found to significantly reduce the viability of CO 88BV59-1 LCL cells, which is consistent with previously published reports on their inhibitory properties against various tumor cells [20-23].

The present study demonstrates that treatment with crocin, crocetin, and cyclophosphamide inhibits the growth of the CO 88BV59-1 LCL cell line in a time-dependent manner. This result is consistent with previous research investigating the induction of early and delayed apoptosis in lymphoma cells treated with cyclophosphamide [25].

The apoptotic effects of crocin and crocetin are consistent with previous research, although those studies were conducted on cell lines other than lymphoma. Crocin has been shown to be safe in in-vivo models and can induce significant apoptosis in the breast cancer cell line MCF-7 [26]. Research has demonstrated that crocin can induce apoptosis in chemotherapy-resistant cervical cancer cells [27]. In a separate study, it was found that crocin exhibits antitumor properties and can improve melanoma tumors in vivo [28]. The anticancer properties of crocetin have been confirmed in in vitro tests by several studies [20, 21, 29, 30]. The study evaluated the expression levels of miRNA-16-1 in cells treated with crocin, crocetin, and cyclophosphamide. The results showed no significant changes in the expression level of *miRNA-16-1-3p* and *miRNA-16-1-5p* in CO 88BV59-1 cells treated with the aforementioned substances compared to untreated cells.

Numerous studies have investigated the cytotoxic effects of crocin [19, 23, 26], crocetin [20, 21], and cyclophosphamide [25] on tumor cells. It is important to note that one miRNA can control the expression of several proteins at the translational level [31, 32], while conversely, the expression of one protein can be controlled by several miRNAs [32, 33]. Therefore, the lack of effect of crocin, crocetin, and cyclophosphamide on the expression level of *miRNA-16-1-3p* and *miRNA-16-1-5p* in CO 88BV59-1 LCL cell line can be interpreted as follows:

The initial interpretation posits that crocin, crocetin, and cyclophosphamide augmented apoptosis in the CO 88BV59-1 LCL cell line via an independent mechanism involving miRNA [31, 32].

The second interpretation suggests that crocin, crocetin, and cyclophosphamide may have increased the expression of other miRNAs that control the Bcl-2 protein, such as miRNA-206 [34], miRNA-181b [35], miRNA-216a [36], and miRNA-34a [37]. This increase in miRNA expression may have led to a decrease in the expression of the Bcl-2 protein and an increase in the production of P53 [32, 33], ultimately resulting in the apoptosis of the CO 88BV59-1 LCL cell line.

The third interpretation suggests that crocin, crocetin, and cyclophosphamide may have reduced the levels of EBV miRNA, including miR-BART16, BHRF1, and BART7-3p. These miRNAs inhibit the expression of proteins such as BAX [38], P27 [39], and P-10 [40], respectively, which are involved in the intrinsic pathway of apoptosis [41]. By increasing the production and expression of apoptotic proteins, these compounds may have induced apoptosis in the CO 88BV59-1 LCL cell line.

However, there has been no research conducted on the effect of crocin, crocetin, and cyclophosphamide on the expression of *miRNA-16-1-3p* and *miRNA-16-1-5p* in cancers, making it impossible to compare the present results with previous studies.

The study's limitations include the lack of examination of apoptotic and anti-apoptotic genes, other miRNAs involved in the process of apoptosis, and the expression of genes and miRNA of the EBV virus. These limitations were due to budget constraints.Furthermore, the immune system is of great significance in the prevention and control of cancers, with cytokines playing a pivotal role in this function [42-44], It is recommended that the impact of crocin and crocetin on the cells of the immune system and the expression of cytokines be evaluated. Moving forward, further exploration of these compounds' efficacy and safety profiles in the context of B cell transformation could pave the way for novel treatment approaches with potential clinical applications.

In conclusions, crocin and crocetin induce apoptosis in a dose- and time-dependent manner, similar to cyclophosphamide, in the CO 88BV59-1 LCL cell line. These preclinical studies suggest evaluating the effect of crocin and crocetin in EBV-associated B-cell lymphoma.

Author Contribution Statement

M. M., M.K., A. SJ; Contributed to the conception and study design. M.R. H., A.SJ., M. HF; Contributed to lab experiments and the data interpretation. M.R. H., A.SJ., K R., Contributed to statistical analysis. A. SJ., S. E., K R; Drafted the manuscript, and then it was revised by M. M., M.K., M.R. H., M. HF; All authors read and approved the final manuscript.

Acknowledgements

Ethical consideration

The ethics committee of Jahrom University of Medical Sciences approved this research work with code IR.JUMS.

REC.1399.026.

Conflict of interest

The authors have no relevant conflicts of interest to disclose.

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