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RESEARCH ARTICLE

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Study of the mechanisms of crocetin-induced differentiation and apoptosis in human acute promyelocytic leukemia cells

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Abstract

Crocetin, the major carotenoid in saffron, exhibits potent anticancer effects. However, the antileukemic effects of crocetin are still unclear, especially in primary acute promyelocytic leukemia (APL) cells. In the current study, the potential antipromyelocytic leukemia activity of crocetin and the underlying molecular mechanisms were investigated. Crocetin (100 μM), like standard anti-APL drugs, all-trans retinoic acid (ATRA, 10 μM) and As_2O_3 (arsenic trioxide, 50 μM), significantly inhibited proliferation and induced apoptosis in primary APL cells, as well as NB4 and HL60 cells. The effect was associated with the decreased expressions of prosurvival genes Akt and BCL2, the multidrug resistance (MDR) proteins, ABCB1 and ABCC1 and the inhibition of tyrosyl-DNA phosphodiesterase 1 (TDP1), while the expressions of proapoptotic genes *CASP3*, *CASP9*, and *BAX/BCL2* ratio were significantly increased. In contrast, crocetin at relatively low concentration (10 μM), like ATRA (1 μM) and As_2O_3 (0.5 μM), induced differentiation of leukemic cells toward granulocytic pattern, and increased the number of differentiated cells expressing CD11b and CD14, while the number of the immature cells expressing CD34 or CD33 was decreased. Furthermore, crocetin suppressed the expression of clinical marker

Abbreviations: ABC, ATP-binding cassette membrane transporters; APL, acute promyelocytic leukemia; As_2O_3, arsenic trioxide; ATRA, all-trans retinoic acid; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GUSB, glucuronidase β; HDACs, histone deacetylases; MDR, multidrug resistance protein; NBT, nitro blue tetrazolium; PBS, phosphate-buffered saline; PI, propidium iodide; PMA, phorbol myristate acetate; PML/RARα, promyelocytic leukemia/retinoic acid receptor-α; RARs, retinoic acid receptors; TDP1, tyrosyl-DNA phosphodiesterase 1; TFRC, transferrin receptor.

promyelocytic leukemia/retinoic acid receptor-α (*PML/RARα*) in NB4 and primary APL cells, and reduced the expression of histone deacetylase 1 (*HDAC1*) in all leukemic cells. The results suggested that crocetin can be considered as a candidate for future preclinical and clinical trials of complementary APL treatment.

KEYWORDS
acute promyelocytic leukemia, apoptosis, crocetin, differentiation, multidrug resistance, tyrosyl-DNA phosphodiesterase 1

1 | INTRODUCTION

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia, which is associated with the accumulation of immature promyelocytes in the peripheral blood and the bone marrow. Cytogenetically, it is characterized by chromosomal translocation t(15;17)(q22;q21) in the majority of patients. This translocation leads to the fusion between the retinoic acid receptor-α (RARα) and promyelocytic leukemia (PML) genes.[1] Both RARα and PML play key roles in hematopoiesis, and the gain-of-function PML-RARα fusion protein prevents the antiproliferative and proapoptotic effects of PML. This fusion also recruits histone deacetylases (HDACs) resulting in the repression of the differentiating role of retinoic acid.[1-3] Therefore, HDAC inhibitors are suggested as valuable therapeutic agents for this form of leukemia.[4]

APL needs early diagnosis and treatment because it can cause severe coagulopathy and life-threatening bleeding.[5] Introducing all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) for the treatment of APL patients could dramatically improve their survival rate. However, despite improvements in the prognosis of the majority of APL patients, relapse of disease and resistance to ATRA and/or As_2O_3 are still a critical problem.[6,7] The overexpression of multidrug resistance (MDR)-associated proteins including ATP-binding cassette (ABC) membrane transporters (eg, ABCB1 and ABCC1) in leukemic cells is an important contributor to chemotherapy resistance.[8,9]

Crocetin, the major carotenoid in saffron, has been shown to induce significant anticancer effects in the breast, liver, lung, pancreas, skin, and colorectal cells.[10] Saffron, a spice derived from the flower of *Crocus sativus*, is widely used as a natural dietary spice and also in the traditional medicine for centuries.[11,12] The mechanisms underlying the anticancer effect of carotenoids include antioxidant activity, inhibition of cell proliferation, induction of apoptosis, stimulation of cell differentiation, and regulation of retinoid-dependent signaling.[13,14] Tarantilis reported that crocetin inhibits growth and induces differentiation of human promyelocytic leukemia (HL60) cell line.[15] Studies have also shown that crocetin is far less potent genotoxin than ATRA.[16] To date, however, no study had evaluated the antileukemic effect of crocetin in freshly isolated APL cells and the underlying molecular mechanisms. In this study we designed a series of experiments to examine the effects of crocetin on proliferation, apoptosis, and differentiation of primary APL cells isolated from newly diagnosed APL patients, as well as in NB4 (which express PML-RARα) and HL60 (which are null for PML-RARα) cell lines. It was also aimed to determine whether antileukemic effects of crocetin are associated with altering the activity of tyrosyl-DNA phosphodiesterase 1 (TDP1) and HDAC1 and the expressions of, PML-RARα, and ABC membrane transporters.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

High-glucose Roswell Park Memorial Institute (RPMI 1640) medium and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). TRIzol was purchased from Invitrogen (Carlsbad, CA). Penicillin-streptomycin solution, ATRA, As_2O_3, propidium iodide (PI), nitro blue tetrazolium (NBT), phorbol myristate acetate, 7-hydroxy-3*H*-phenoxazin-3-one-10-oxide (resazurin; 300 μM of resazurin, 78 μM of methylene blue, 1 mM of potassium hexacyanoferrate III, and 1 mM of potassium hexacyanoferrate II [all from Sigma-Aldrich]), and antibody against β-actin were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of crocetin and ATRA were prepared in dimethyl sulfoxide (DMSO). The solutions were protected from light and stored at −20°C. Before being added to cells, the stock solutions were diluted with the culture medium to prepare the working solutions. The final DMSO concentrations in the treatment and control groups were all less than 0.1%. The enhanced chemiluminescence (ECL) detection kit and FITC annexin V kit were obtained from GE Healthcare (Chicago, IL) and BD Biosciences (Franklin Lakes, NJ), respectively. Giemsa-staining solution was purchased from Merck (Darmstadt, Germany). Human promyelocytic leukemia cell lines, HL60

and NB4, were obtained from cell bank of Pasteur Institute (Tehran, Iran). Human lymphoblastoid TK6 cells were maintained at Professor Sherif F El-Khamisy lab (Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, UK). HRP-conjugated goat anti-rabbit IgG and antihistone H3 were bought from Bio-Rad (Hercules, CA) and Abcam (Cambridge, UK), respectively. Crocetin was kindly provided by School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. It was prepared from crocin (the main water soluble pigment in saffron stigmas) using acidic hydrolysis and its purity was evaluated using spectrophotometry, thin layer chromatography and high performance liquid chromatography (HPLC), as described previously.[17] Representative HPLC chromatogram of purified crocetin was shown in Figure 1. Fluorescein isothiocyanate-conjugated antibodies against CD14 and CD33, and phycoerythrin-conjugated antibodies against CD11b and CD34 were obtained from Novus Biologicals (Littleton, CO). Real-time PCR Master Mix and cDNA Synthesis Kit were obtained from Roche Diagnostic (Risch-Rotkreuz, Switzerland) and Fermentas (Vilnius, Lithuania), respectively.

2.2 | Clinical samples

Primary APL cells were obtained from heparinized bone marrow samples from 15 untreated patients (9 men and 6 women 37 ± 3 years old) with newly diagnosed APL at the Oncology Department of Ghaem Hospital (Mashhad University of Medical Sciences, Iran). Eligibility criteria for newly diagnosed APL included demonstration of the t(15;17) or PML/RAR rearrangement,[18] and a signed informed consent. All patients showed >80% of promyelocytic leukemic cells in bone marrow aspirate. Immunophenotyping was also performed by flow cytometry for verifying promyelocytes in AMLM3 patients, mainly CD13, CD33, CD117, and MPO-positive and HLA-DR-negative blast cells. The APL cells of bone marrow were isolated under sterile conditions by Ficoll-Hypaque density-gradient centrifugations. Normal polymorph nuclear cells were prepared from bone marrow of three complete remission APL patients (two men and one woman 40 ± 2 years old) by two consecutive Ficoll-Hypaque density-gradient centrifugations.[19] The normal cells were more than 97% pure, as judged by morphological examination of Wright-stained smears. Informed consent was obtained in accordance with the Declaration of Helsinki, and all study protocols were approved by the Ethics Committee of the Mashhad University of Medical Sciences.

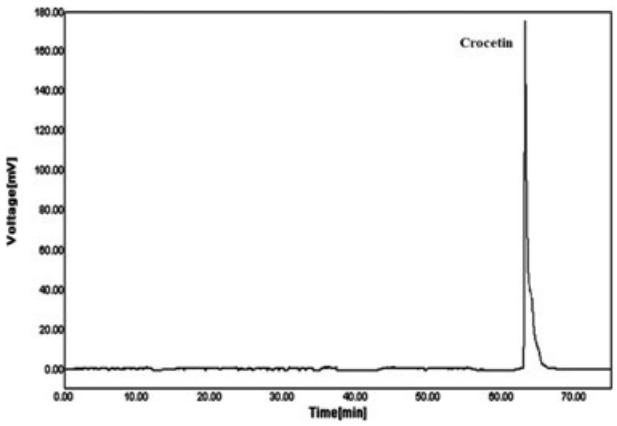

FIGURE 1 Chromatogram of crocetin (20 μg/mL) in acetone at 440 nm. The composition of mobile phase: methanol-water (20/80, v/v) for 2 minutes; then, was changed to methanol-water (70/30, v/v) in 50 minutes; and then changed to 100% methanol in 5 minutes and continued with 100% methanol for 15 minutes

2.3 | Cell culture and treatment

Primary APL cells, normal polymorph nuclear cells, NB4 cell line, HL60 cell line, and human lymphoblastoid TK6 cells were grown in RPMI 1640 medium supplemented with 1 mM of sodium pyruvate, 1% L-glutamine, 20% FBS, 100 U/mL of penicillin, and 100 U/mL of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The cells were plated in 96-well plates at the density of 1×10^4 cells/well (for cell proliferation assay) or six-well plates at the density of 1×10^5 cells/well (for apoptosis and differentiation assays) and treated for 3 or 5 days with different concentrations of crocetin (2.5 to 100 μM), ATRA (0.25 to 10 μM), or As_2O_3 (0.01 to 5 μM).[15,20-24]

2.4 | Cell proliferation assay

Cell proliferation was determined using resazurin reduction by live cells to resorufin, a highly fluorescent compound. After treatment of NB4, HL60, primary APL cells, and normal polymorph nuclear cells with crocetin (5 to 100 μM), ATRA (0.5 to 10 μM), or As_2O_3 (0.5 to 5 μM), 20 μL of resazurin reagent was added to each well. After 4 hours, fluorescence intensity was measured by a fluorescence Victor X5 2030 Multilabel Plate Reader (PerkinElmer, Shelton, CT) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.[25]

2.5 | Apoptosis assay

HL60, NB4, and primary APL cells were treated with crocetin (5 to 100 μM), ATRA (10 μM), or As_2O_3 (5 μM) for 5 days, and then the rate of apoptosis and necrosis were evaluated using the fluorescent microscopy, sub-G1 cells analysis, and annexin V/PI staining methods, as described previously. The cell images were photographed using a fluorescent microscope (Eclipse TS-100, Nikon, Tokyo, Japan) equipped with Canon camera. The

quantification sub-G1 cell population and the rate of apoptosis and necrosis were carried out by FACS Calibur (BD Biosciences, Franklin Lakes, NJ) flow cytometer followed by analysis using Flowjo software (TreeStar Inc., Ashland, OR).[26]

2.6 | Differentiation assay

Cell differentiation was evaluated by morphological observation using Giemsa staining, NBT reduction, and cell surface differentiation antigens expression assays. To perform NBT assay, the treated HL60, NB4, and primary APL cells with crocetin (2.5 to 50 μM), ATRA (0.25 to 5 μM), or As_2O_3 (0.01 to 0.1 μM) were washed with PBS and suspended in NBT solution (2 mg/mL) containing 200 ng/mL of phorbol myristate acetate. After 25 minutes incubation at 37°C in the dark, cytospin slides were prepared and stained with Giemsa. Differentiated cells were recognized by their intracellular reduced dark blue formazan granules (300 cells were scored for the presence of the granules).[27]

Cell-surface markers of monocytes/granulocytes (CD11b and CD14) and immature cells (CD33 and CD34) were evaluated by immunophenotyping analysis.[28] The treated HL60, NB4, and primary APL cells were washed with PBS and incubated with the antibodies against these markers for 15 minutes. Then, the cells were subjected to flow cytometric analysis using FACS Calibur (BD Biosciences) flow cytometer.

2.7 | Real-time PCR quantification

2.7.1 | Real-time PCR quantification with Taq-Man probes

The treated HL60, NB4, and primary APL cells with crocetin (5 to 100 μM), ATRA (1 and 10 μM), or As_2O_3 (0.05 and 5 μM) were subjected to RNA extraction using TRIzol according to manufacturer's instruction. The RNA concentration and purity were determined using spectrophotometry. Complementary DNA (cDNA) was synthesized from total RNA (100 ng) of each sample using cDNA synthesis kit with random hexamer primer. Primers and probes were designed using Beacon software (Applied Biosystems; Table 1). Gene expression changes were measured using Taq-Man-based real-time PCR Technology by Applied Biosystems Step One Plus Detection System (ABI). As a brief, the reaction mixture (20 μL) contained 2 μL of cDNA (250 to 400 ng), 1 μL of each primer (900 nM), 10 μL of 2× master mix, and 0.4 μL of the probe (250 nM). The optimized parameters used for the thermocycler were: short hot-start at 95°C for

TABLE 1 Sequences of primer and probe selected for real-time PCR quantification using Taq-Man probes

Gene	Forward primer	Reverse primer	Probe	Product size, bp
ABCB1	TAGCGAAACATTGAAAATAC	AGTCGGAGTATCTTCTTC	CAGTTGGTTTCTTTTCCTTCTTATCTT	189
ABCC1	GGAAGAGCATCAGTAACTAA	CTCCCAAGTATTACCAGTG	AAGTTCTCCACAATGCTGCC	150
HDAC1	CTGCTGCTTATTAAGTTTC	GCGATGACTACATTAAATTC	CACAGAACCACCAGTAGACAA	179
PML-RAR	CCG ATG GCT TCG ACG AGT T	GCT TGT AGA TGC GGG GTA GAG	AGT GCC CAG CCC TCC CTC GC	147
GUSB	GGCTTCTGATACTTCTTATACCA	TCGCTCACACCAAATCCTT	ACTACTCTTGGTATCACGACTACGGG	205
TFRC	ACAGTCTCCTTCCATATTCCCAAA	CCTTCCTTCAATCACACTCAGTT	ACCATCTCGGTCATCAGGATTGCC	120

TABLE 2 Sequences of primer selected for real-time PCR quantification using SYBR Green

Gene	Forward primer	Reverse primer	Product size, bp
P53	GGAACTCAAGGATGCCCAG	CAAGAAGTGGAGAATGTCAGTC	155
P21	AACGGCGGCAGACCAGCAT	GAGACTAAGGCAGAAGATGTAGAGCG	150
PTEN	AGTAGAGGAGCCGTCAAATC	ATCAGAGTCAGTGGTGTCAG	109
CASP3	AGAACTGGACTGTGGCATT	GCTTGTCGGCATACTGTTT	191
CASP9	CTTTGTGTCCTACTCTACTTTCC	AACAGCATTAGCGACCCTA	151
CASP8	TGTTGGAGGAAAGCAATCTG	CCTGGTGTCTGAAGTTCCCT	124
PI3K	TGCGGAAACTGACGGACGATGA	CGGAGCGGAGGTGCCAGAA	162
AKT	GCACCTTCATTGGCTACA	CCGCTCCGTCTTCATCAG	104
BCL2	CCAAGAAAGCAGGAAACC	GGATAGCAGCACAGGATT	170
BAX	GCCTCCTCTCCTACTTTG	CTCAGCCCATCTTCTTCC	102
GAPDH	GAAGTCAGGTGGAGCGAGG	TGGGTGGAATCATATTGGAACAT	200
BACTIN	GCCTTTGCCGATCCGC	GCCGTAGCCGTTGTCG	90

15 minutes, followed by 40 cycles, each consisting of denaturing at 95°C for 15 seconds, annealing at 60°C for 1 minute and extension at 72°C for 20 seconds. Samples were run in triplicate and the fold difference of expression in treated and untreated samples were calculated using the $2^{-\Delta\Delta C_t}$ method.[29] The expressions of genes related to differentiation (PML-RARα, HDAC1), and ABC membrane transporters were normalized to glucuronidase β (GUSB) and transferrin receptor (TFRC) as housekeeping genes.

2.7.2 | Real-time PCR quantification with SYBR Green

After synthesis of cDNA, genes were amplified using primer and real-time PCR Master Mix by Applied Biosystems StepOneplus Thermocycler 7900 (Applied Biosystems, Foster City, CA). The reaction mixture consisted of 2 μL of cDNA (250 to 400 ng), 1 μL of the primers (100 pmol), 10 μL of 2× SYBR Green master mix (Sigma-Aldrich, St. Louis, MO), 0.4 μL of ROX, and dH_2O to bring the volume to 20 μL. The optimized parameters used for the thermocycler were: short hot-start at 95°C for 10 minutes followed by 40 cycles, each consisting of denaturing at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds. As the final step of real-time PCR, melting curves were incorporated from 60 to 90°C rising by 0.3°. Samples were run in triplicate and the fold difference of expression in treated and untreated samples were calculated using the $2^{-\Delta\Delta C_t}$ method.[29] The expressions of genes related to apoptosis pathways (PI3K, Akt, Bcl2, Bax, p53, p21, PTEN, CASP3, CASP8, and CASP9) were normalized to GAPDH and β-actin as housekeeping genes (Table 2).

2.8 | Western blot analysis

Following treatment with crocetin (5 and 10 μM), ATRA (1 μM), or As_2O_3 (0.05 μM), HL60, NB4, and primary APL cells were lysed using RIPA buffer. The lysates were then centrifuged at 18 000 g for 1 hour at 4°C to precipitate the particulates. Then, equal amounts of total protein extracts were separated by 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. After blocking with 2% BSA, the blots were exposed to antihistone H3 (1:5000 dilution) or β-actin (1:3000 dilution) for 3 hours at room temperature. Then, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:20 000) for 1 hour at room temperature. The membranes were visualized using an ECL detection kit. The reactions were revealed and documented by Gel-Doc (Syngene, Cambridge, UK). Images were quantified using NIH ImageJ software (version 1.46, National Institutes of Health, Bethesda, MD).[30]

2.9 | TDP1 fluorescence assay

TDP1 protein was selected on the basis of the probability of binding of crocetin, given by the Swiss Target Prediction web interface.[31] Human lymphoblastoid TK6 cells extract was treated with crocetin (5 to 100 μM). The in vitro TDP1 inhibitory activity of crocetin was determined using a gel-based 3′-tyrosyl-DNA phosphodiesterase activity assay. Biochemical assays were performed in 10 μL of reaction volumes containing TDP1 reaction buffer (50 mM of Tris HCL (pH 7.5), 50 mM of KCl, 1 mM of DTT, 10 M of EDTA, and 100 μg/mL of BSA), cell lysate (15 μg, TDP1), and 50 nM of Cy5.5-labeled substrate oligomer

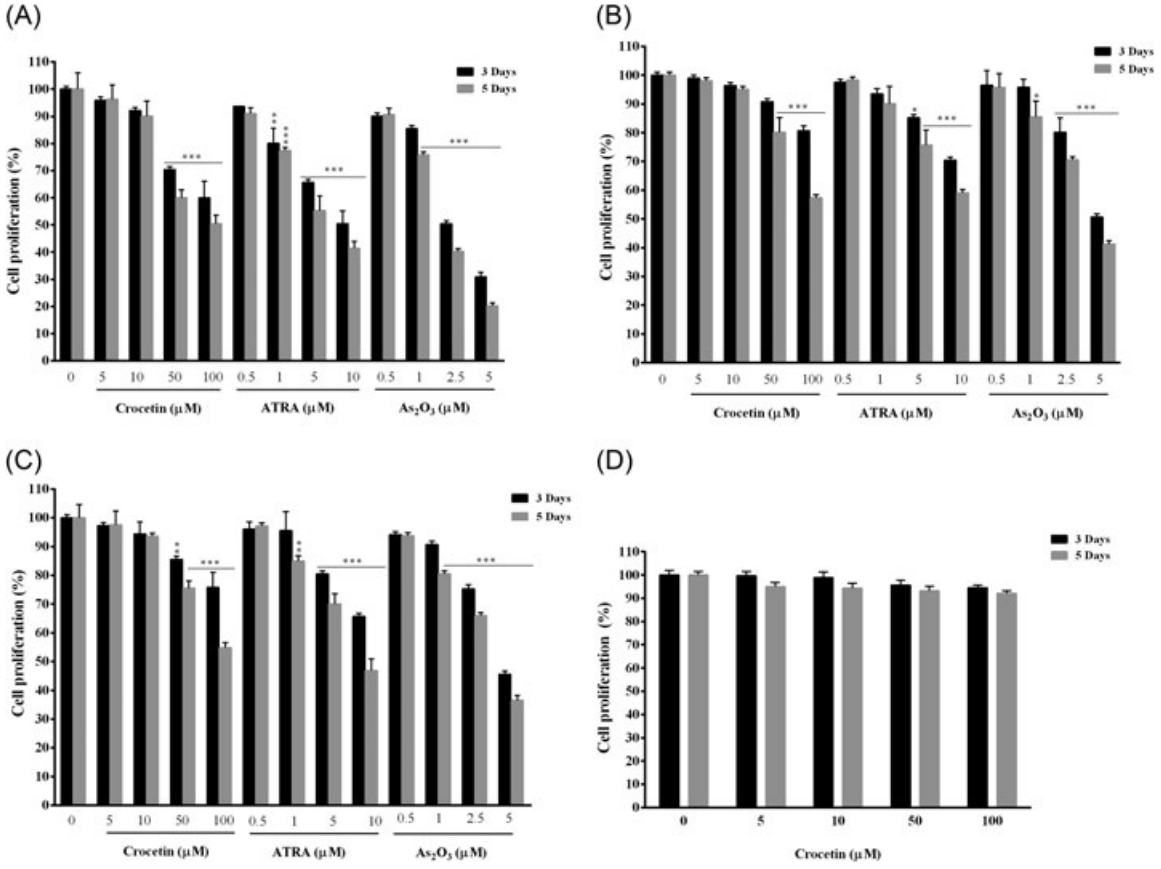

FIGURE 2 Effects of crocetin on proliferation of leukemic cells. A, HL60 cells, B, NB4 cells, C, primary APL cells, and D, normal polymorph nuclear cells. The cells were treated for 3 or 5 days with different concentrations of crocetin, all-trans retinoic acid (ATRA), or arsenic trioxide (As_2O_3). Cell proliferation was determined using resazurin assay. Data are expressed as the mean ± SEM of five independent experiments performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding untreated control cells (concentration of 0). SEM, standard error of the mean

5′-(Cy5.5) GATCTAAAAGACT (pY)-3′ (Midland Certified Reagent Company, TX). The reaction progressed at 37°C for 2 hours and was quenched with 10 μL of loading buffer (44% deionized formamide, 2.25 mM of Tris-borate, 0.05 mM of EDTA, 0.01% xylene cyanol, and 1% bromophenol blue). Samples were then heated at 90°C for 10 minutes before separation on a 20% urea SequaGel by gel electrophoresis at 100 V for 30 minutes. Reaction products were visualized by gel imaging using ChemiDoc MP Imaging System (Bio-Rad).[17]

2.10 | Statistical analysis

Data are presented as the mean ± SEM and analyzed by one-way analysis of variance and Tukey multiple comparisons post-test. The *P*-value less than 0.05 was considered as statistically significant. Statistical analysis was made using Graph Pad PRISM software (version 6, Graph Pad Software, La Jolla, CA).

3 | RESULTS

3.1 | Crocetin inhibited proliferation of leukemia cells

As shown in Figure 2, crocetin at concentrations of 50 and 100 μM significantly reduced proliferation of HL60 cells at 3 and 5 days ($P < 0.001$). Such a decrease in proliferation was seen in NB4 and primary APL cells after 5 days. Similarly, a significant decrease in proliferation was seen in NB4, HL60, and primary APL cells incubated for 5 days with 5 and 10 μM of ATRA ($P < 0.001$) and 1 to 5 μM of As_2O_3 ($P < 0.05$). Crocetin had no effect on the proliferation of normal polymorph nuclear cells at concentrations of 5 to 100 μM (Figure 2D).

3.2 | Crocetin enhanced apoptosis of leukemia cells

Figure 3 shows the effects crocetin on sub-G1 population percent of HL60, NB4, and primary APL cells as

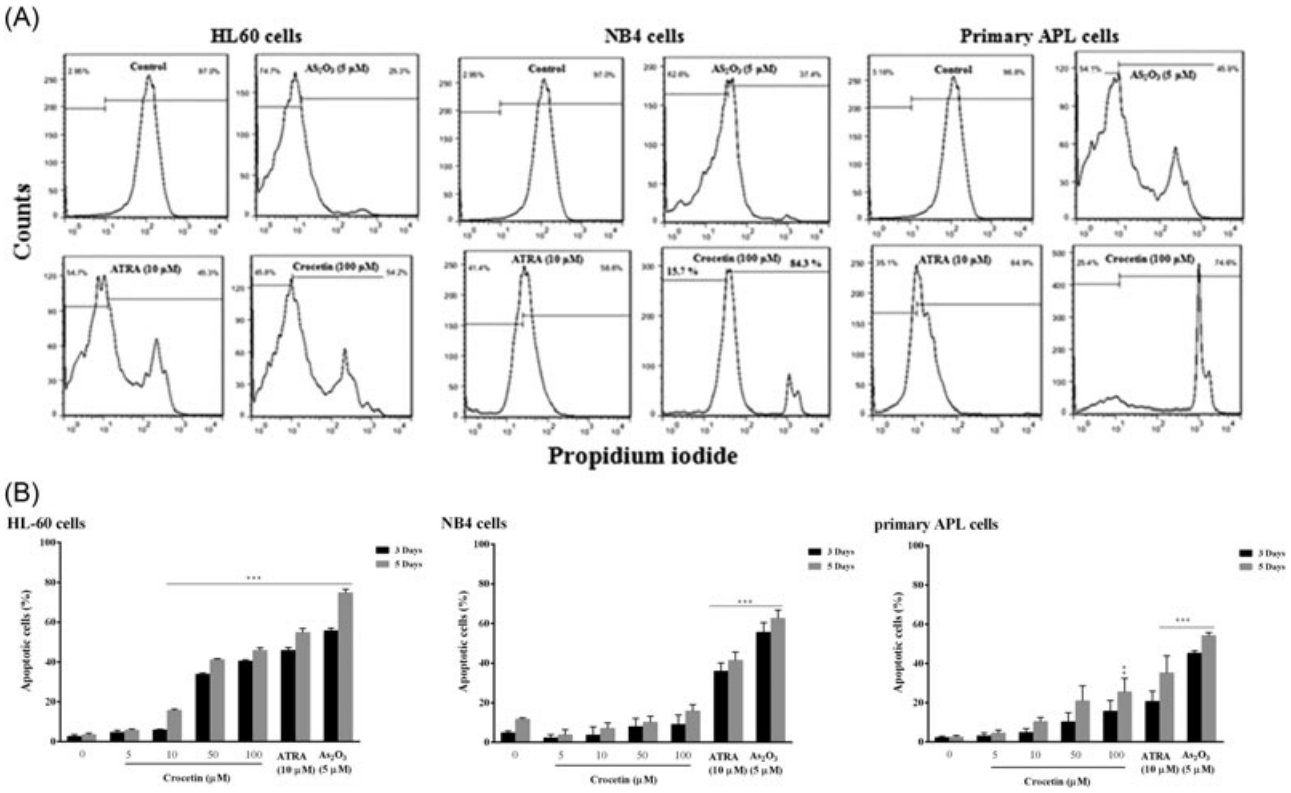

FIGURE 3 Effects of crocetin on sub-G1 population of PI-stained leukemic cells. The HL60, NB4, and primary APL cells were treated for 3 or 5 days with crocetin, all-trans retinoic acid (ATRA), or arsenic trioxide (As_2O_3). A, Representative histogram of the fluorescence intensity of PI-stained leukemic cells. The sub-G1 region is made by cells with reduced DNA content (apoptotic cells). B, Quantitative analysis of apoptosis as shown in (A). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus untreated control cells (concentration of 0). SEM, standard error of the mean

evaluated by PI staining. In the presence of 50 and 100 μM of crocetin, the percent of HL60 cells in sub-G1 phase was increased at 3 and 5 days ($P < 0.001$). Crocetin also increased the percent of primary APL cells in sub-G1 phase at concentration of 100 μM after 5 days (Figure 3A and 3B; $P < 0.01$).

Figure 4 shows the effects of crocetin on apoptosis of leukemic cells as evaluated by annexin V and PI double staining. Like ATRA and As_2O_3, crocetin at concentrations of 50 and 100 μM significantly increased the apoptosis rate of HL60, NB4 and primary APL cells (Figure 4A-C; $P < 0.001$).

3.3 | Crocetin modulated genes involved in survival and apoptosis in leukemia cells

Figure 5 shows the effects of 5 days incubation with crocetin on the expression of genes involved in survival (PI3K, Akt, and Bcl2) and apoptosis (p53, p21, PTEN, Bax, CASP3, CASP8, and CASP9). In HL60 cells, crocetin at concentration of 100 μM significantly increased the expressions of p21, PTEN, CASP3, CASP9, and Bax while decreased the expression of Akt ($P < 0.01$; Figure 4A). Similarly, crocetin (100 μM) significantly caused increased p21, p53, PTEN, CASP3, CASP9, and Bax while decreased Akt expression in NB4 and primary APL cells ($P < 0.05$ for both, Figure 4B and 4C). The expression of CASP8 gene did not increase in three leukemic cells upon treatment with the drugs. As illustrated in Figure 5D, it was demonstrated that the Bax/Bcl2 ratio was significantly increased in HL60, NB4, and primary APL cells after treatment with crocetin, ATRA, and As_2O_3.

3.4 | Crocetin-induced differentiation of leukemia cells

Morphological analysis of the leukemic cells by Giemsa staining is shown in Figure 6A. Untreated primary APL cells had promyelocytic characteristics containing a large nucleus and granules in the cytoplasm. After treatment with crocetin (less than 10 μM), the cells began to morphological changes toward having hollow nuclei and larger zones of clear cytoplasm. After 5 days of treatment, the granulocytic maturation pattern was seen in crocetin- and ATRA-treated cells, so that ratio of nuclei to cytoplasm was decreased and nuclei

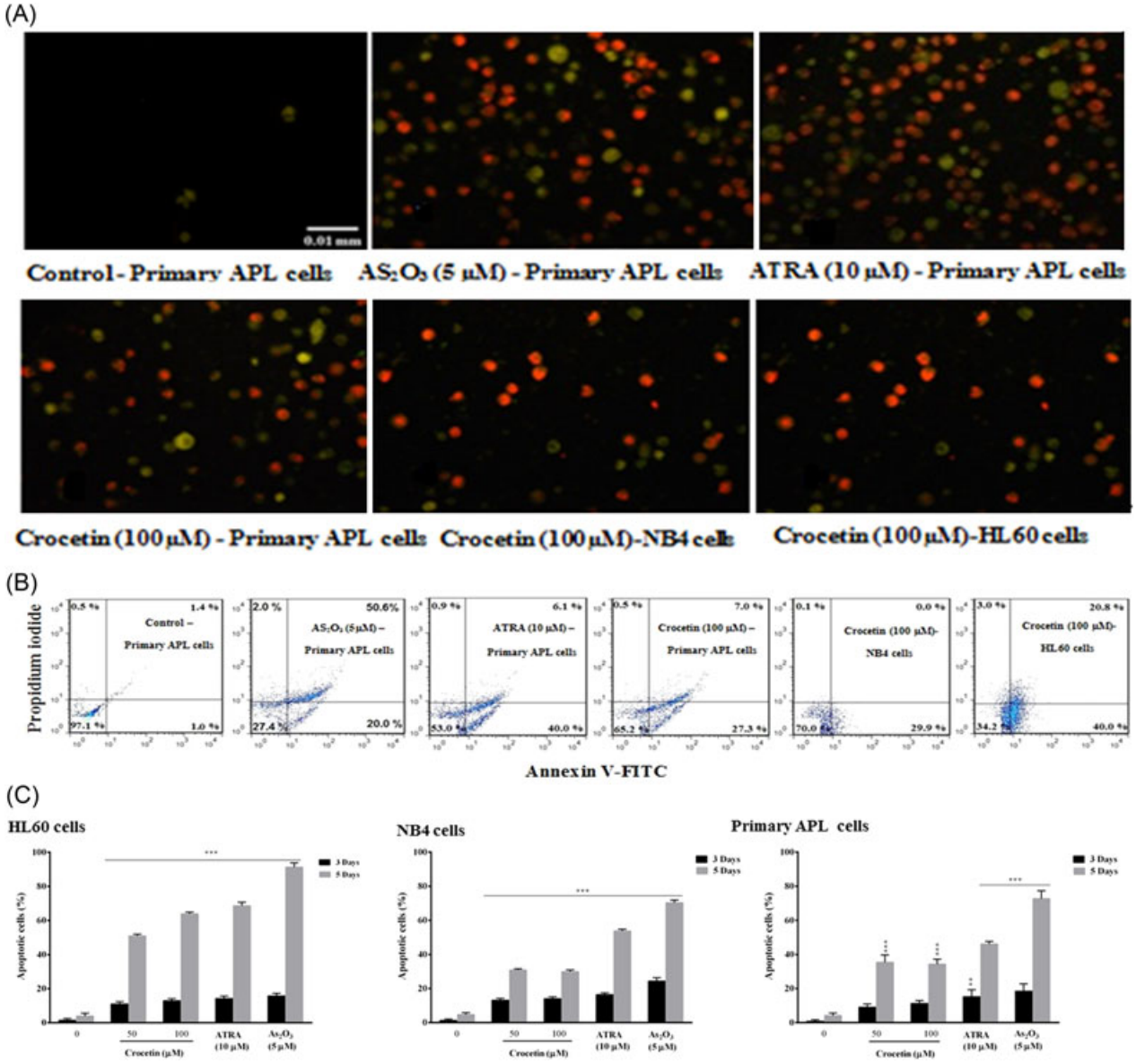

FIGURE 4 Effects of crocetin on apoptosis of leukemic cells as evaluated by annexin V and propidium iodide double-staining. The HL60, NB4, and primary APL cells were treated for 3 or 5 days with crocetin, all-trans retinoic acid (ATRA), or arsenic trioxide (As_2O_3). A, Representative fluorescence photomicrographs of the cells treated for 5 days with the tested drugs (bar represents 0.01 mm). B, Representative histogram of the fluorescence intensity of annexin V and PI double-stained cells after 5 days treatment (*x*-axis: green fluorescence of annexin V-FITC showing apoptotic cells; *y*-axis: red fluorescence of PI showing necrotic cells). C, Quantitative analysis of apoptosis as shown in (B). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus untreated control cells (concentration of 0). PI, propidium iodide; SEM, standard error of the mean

demonstrated a range of remodeling from simple indentations to polylobular nuclei.

In all HL60, NB4, and primary APL cells, com pared to untreated control cells, crocetin-treated cells displayed increased NBT reduction ability (the hallmark of granulocytic maturation) similar to the positive control and ATRA-treated cells (Figure 6B and 6C). The effect of crocetin on NBT reduction capacity of HL60, NB4, and primary APL cells was comparable to ATRA and more than that of As_2O_3. Table 3 shows the EC_{50} values of crocetin, ATRA, and As_2O_3 in HL60, NB4, and primary APL cells at day 5 incubation.

3.5 | Crocetin increased the expressions of granulocyte and monocyte surface membrane markers in leukemia cells

Flow cytometric analysis of the expressions of cell-surface markers associated with monocytic-granulocytic

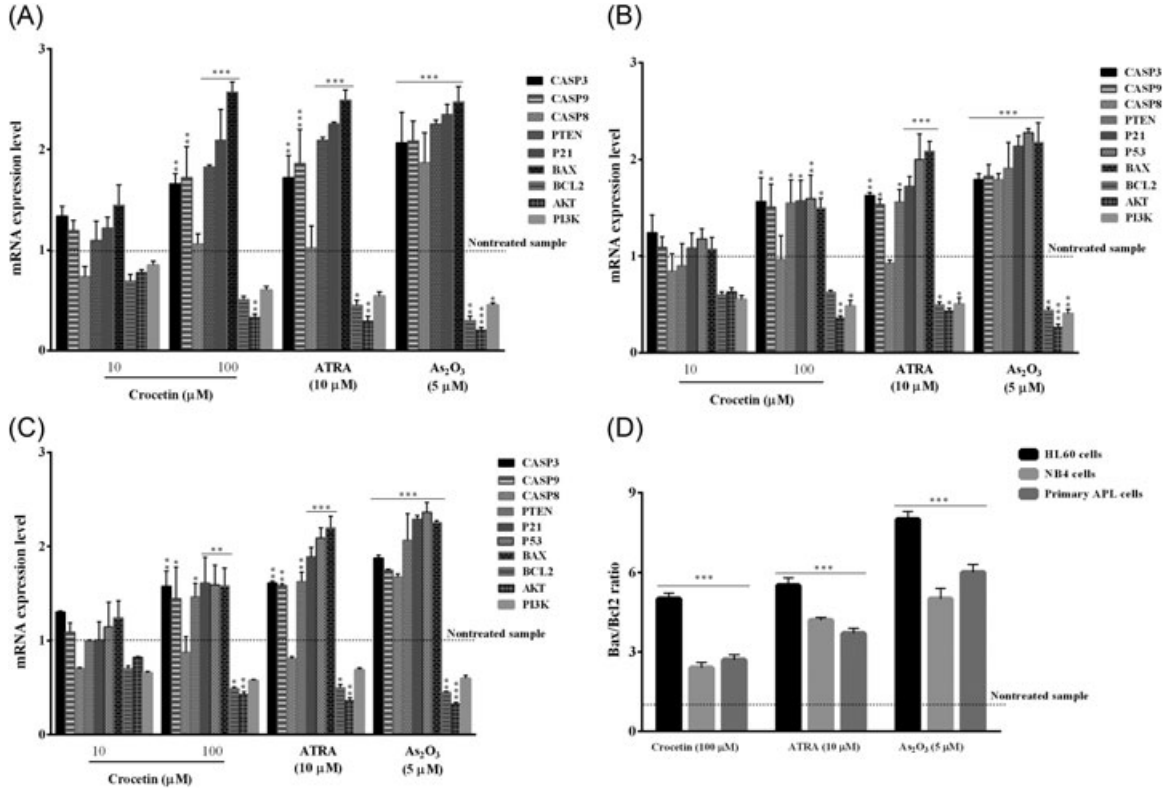

FIGURE 5 Effects of crocetin on the expression of apoptotic and antiapoptotic genes in leukemic cells. The HL60, NB4, and primary acute promyelocytic leukemia (APL) cells were treated for 5 days with crocetin, all-trans retinoic acid (ATRA), or arsenic trioxide (As_2O_3), and the expression of apoptotic (P53, P21, PTEN, CASP3, CASP9, CASP8, and Bax) and antiapoptotic (PI3K, AKT, and Bcl2) genes were determined by real-time PCR. A, HL60 cells, B, NB4 cells, C, primary APL cells, and D, Ratio of Bax/Bcl2 in the cells treated with crocetin, ATRA, and As_2O_3. Data are expressed as the mean ± SEM of three independent experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus nontreated control cells

differentiations is shown in Figure 7A. In comparison with control cells, the numbers of CD11b positive cells were increased significantly after 3 and 5 days treatment with 10 μM of crocetin, 1 μM of ATRA, and 0.05 μM of As_2O_3 ($P < 0.001$). Similar results were found regarding the expression of CD14 in the HL60, NB4, and primary APL cells treated with 10 μM of crocetin, 1 μM of ATRA, and 0.05 μM of As_2O_3. On the other hand, the expressions of immature cells CD markers (CD33 and CD34) were significantly decreased during 5 days treatment with crocetin in all leukemic cells (Figure 7B).

3.6 | Crocetin decreased the expressions of PML-RARα and HDAC1 genes in leukemia cells

Figure 8 shows that 5 days treatment with 10 μM of crocetin, 1 μM of ATRA, and 0.05 μM of As_2O_3 significantly decreased the expressions of PML-RARα gene ($P < 0.001$) in NB4 and primary APL cells. It is noteworthy to mention that HL60 cells were checked for PML-RARα expression and found to be null. Also, crocetin and ATRA significantly reduced the expression of HDAC1 gene in all NB4, HL60, and primary APL cells when compared to untreated control cells ($P < 0.001$). To further assess the effect of crocetin on HDAC1, the level of its substrate, histone H3K4ac (acetyl-H_3K_4), was evaluated by Western blot analysis. The levels of acetyl-H_3K_4 protein were significantly increased in all NB4, HL60, and primary APL cells treated with 10 μM of crocetin, with respect to untreated control cells ($P < 0.05$), confirming the inhibitory effect of crocetin on HDAC1. A similar increased was also observed in the cells incubated with 1 μM of ATRA (Figure 8C).

3.7 | Crocetin decreased the expression of MDR genes in leukemia cells

Real-time PCR assay showed that 5 days incubation with crocetin (100 μM) decreased the expressions of ABCB1 and ABCC1 genes in all HL60, NB4, and primary APL cells (Figure 9). The effect was found to

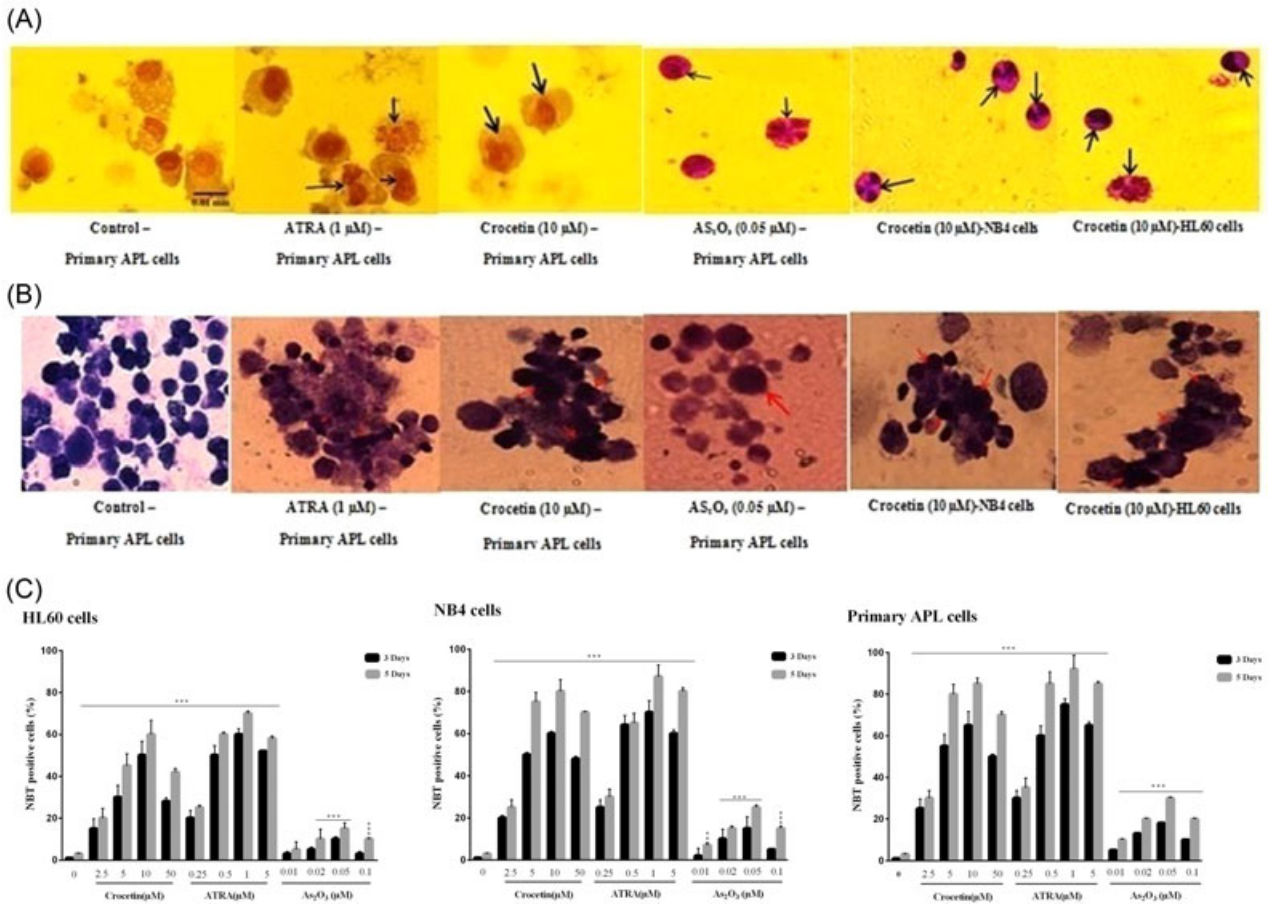

FIGURE 6 Effects of crocetin on granulocytic morphology and differentiation of leukemic cells as determined by Giemsa staining and nitro blue tetrazolium (NBT) assays, respectively. A, The HL60, NB4, and primary acute promyelocytic leukemia (APL) cells were treated for 5 days with crocetin, all-trans retinoic acid (ATRA), or arsenic trioxide (As_2O_3). Control APL cells show promyelocytes characteristic with cytoplasmic granules. Arrows in the treated cells demonstrate polymorphonuclear morphology of granulocyte (bar represents 0.01 mm). B, Representative photomicrographs of the NBT stained HL60, NB4, and primary APL cells treated 5 days with crocetin, ATRA, or As_2O_3. Arrows showed dark blue formazan deposits in the differentiated cells (bar represents 0.01 mm). C, Quantitative analysis of differentiated cells as shown in (B). A minimum of 300 cells were scored. Results are means ± SEM of three independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ versus untreated control cells (concentration of 0). SEM, standard error of the mean

be concentration-dependent. On the contrary, no significant decrease in ABCB1 and ABCC1 expressions were observed in the cells treated with 5 μM of As_2O_3. Also, ATRA had no effect on ABCC1 expression, and only significantly decreased the ABCB1 expression in NB4 cells ($P < 0.05$).

TABLE 3 EC_{50} values of crocetin, ATRA, and As_2O_3 against HL60, NB4, and primary APL cells at 5 d

	Cell lines		
Treatments, μM	NB4 cells	HL60 cells	Primary APL cells
ATRA	0.32 ± 0.04	0.46 ± 0.03	0.21 ± 0.05
Crocetin	3.30 ± 0.2	7.05 ± 0.3	2.70 ± 0.1
As_2O_3	0.14 ± 0.03	0.26 ± 0.09	0.11 ± 0.03

3.8 | Crocetin inhibited TDP1 activity in TK6 cells

Whole cell extracts were generated from TK6 cells and then incubated with crocetin (5 to 100 μM) and enzyme activity determined by TDP1 fluorescence assay.[32] As shown in Figure 10, the TDP1 activity was inhibited in TK6 cells at high concentrations (50 and 100) of crocetin.

4 | DISCUSSION AND CONCLUSION

Introducing ATRA and As_2O_3 for the treatment of APL could significantly increase the survival rate of the leukemic patients. However, resistance to these drugs and their toxicity are major problems in the treatment of

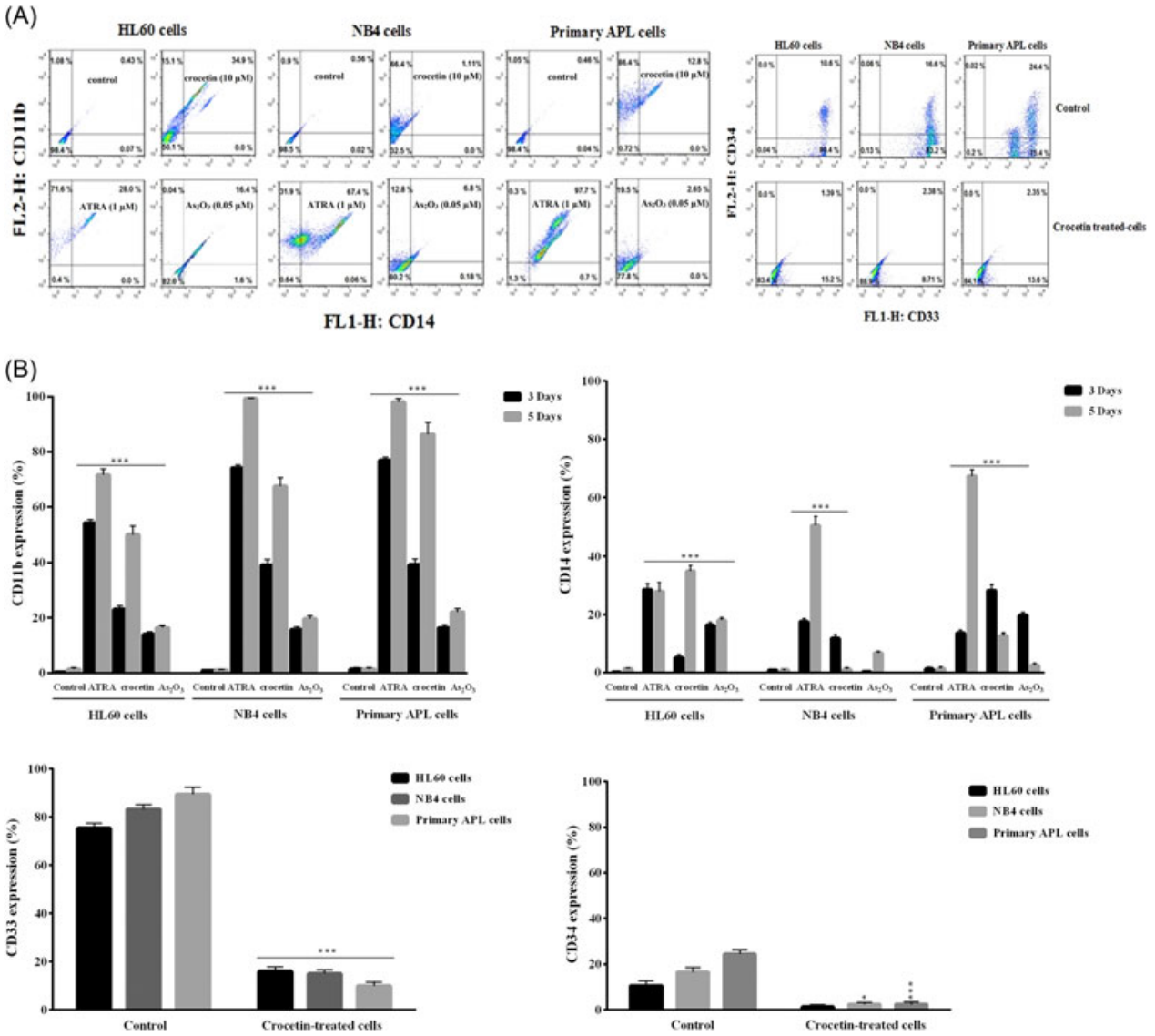

FIGURE 7 Effects of crocetin on the expression of cell-surface markers associated with differentiation of leukemic cells. A, Flow cytometric diagrams of monocytic-granulocytic (CD14, CD11b) and myeloidy (CD33, CD34) expressions from the representative experiment. The HL60, NB4, and primary APL cells were treated for 5 days with 10 μM of crocetin, 1 μM of all-trans retinoic acid (ATRA), or 0.5 μM of arsenic trioxide (As_2O_3). B, Quantitative analysis of the markers expression as shown in (A). Results are mean ± SEM of three different experiments. $^*P < 0.05$, $^{***}P < 0.001$ versus corresponding untreated control cells. SEM, standard error of the mean

APL.[6,7] In the recent years, natural compounds are major subjects for investigating the anticancer drugs with low toxicity and high efficacy.[33-35] Currently, some of the plant-derived compounds are widely used for chemotherapy of cancerous patients. For example, taxol analogs, vinca alkaloids (vincristine and vinblastine), and podophyllotoxin analogs have played an important role in the treatment of cancer patients.[31] The presence of several documents related to the antitumor action of crocetin encouraged us to test whether it had antileukemic effect in freshly isolated human APL cells and to reveal the underlying mechanisms. Our data showed that crocetin decreased proliferation, induced apoptosis, and promoted differentiation in the isolated cells and also promyelocytic leukemia cells via inhibition of MDR/TDP1 and PML-RARα/HDAC1.

Uncontrolled cell proliferation is the main feature of APL cells which is the result of t(15;17)(q22;q21) translocation and formation of PML-RARα protein, which prevents the antiproliferative and proapoptotic actions of PML.[1,2] PML-RARα fusion protein recruits the nuclear corepressor (N-CoR)-histone deacetylase complex through the RARα CoR box. At a pharmacological dose of RA, PML-RARα releases the HDAC complex and activates transcription, thus mimicking RARα.[36] Molecular monitoring for residual leukemia by quantitative

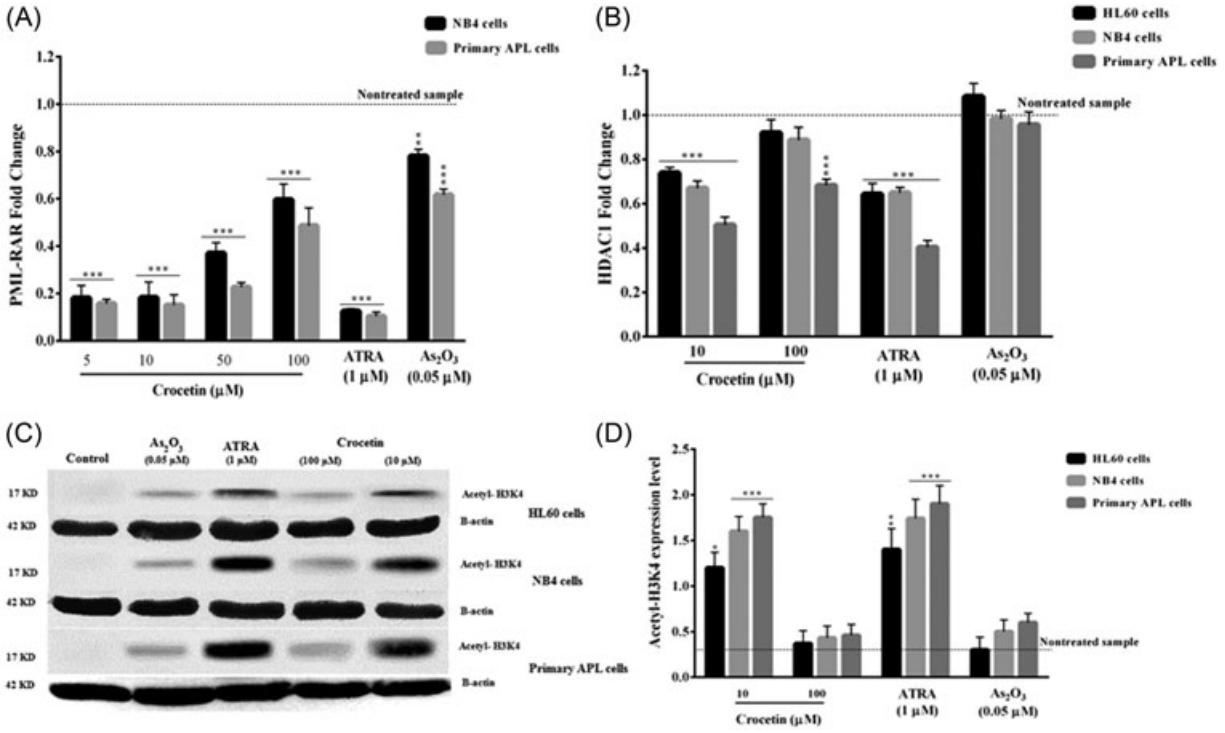

FIGURE 8 Effects of crocetin on the expression of PML-RARα and HDAC1 genes in leukemic cells. The HL60, NB4, and primary APL cells were treated for 5 days with crocetin, all-trans retinoic acid (ATRA), or arsenic trioxide (As_2O_3) and the expressions of A, PML-RARα and B, HDAC1 genes were determined by real-time PCR. C, HDAC1 substrate (acetyl-H3K4) determined by Western blot. D, Quantitative analysis of the bands as shown in (C). Results are mean ± SEM of three different experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus untreated control cells. SEM, standard error of the mean

RT-PCR technique has now provided important information about the effectiveness of treatment and the risk of recurrent disease as shown by minimal residual disease analysis in the APL patients.[32] Crocetin was able to decrease the proliferation rate of APL cells and this effect is consistent with previous reports on growing inhibitory property of crocetin against various cancerous cells.[10] Although the IC_{50} values of crocetin for all HL60, NB4, and primary APL cells were higher than ATRA and As_2O_3, its antiproliferative effect was observed at the concentrations, which had no obvious toxic effect on normal polymorph nuclear cells, suggesting specificity for leukemic cells. Results of PI and annexin V staining indicated that, similar to ATRA and As_2O_3, the

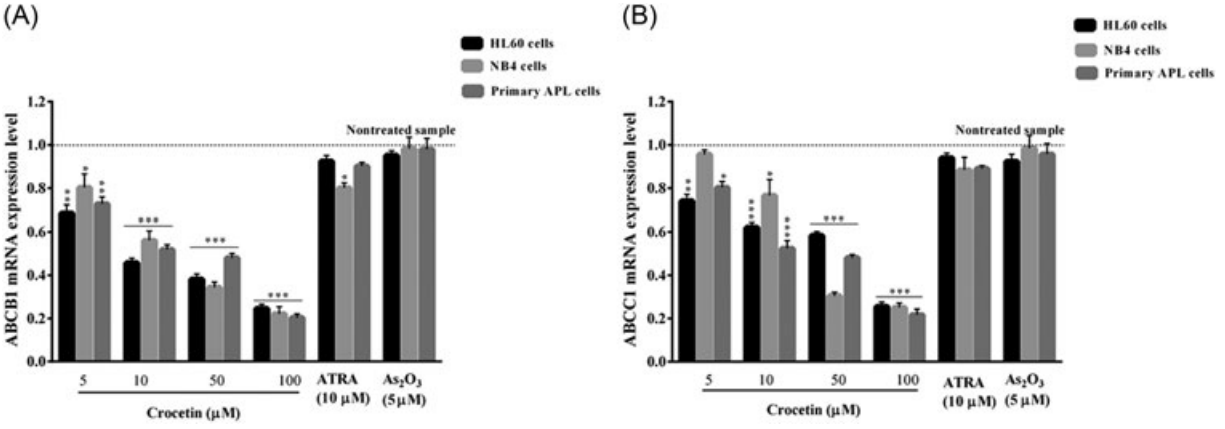

FIGURE 9 Effects of crocetin on the expression of ATP-binding cassette (ABC) membrane transporters genes in leukemia cells. The HL60, NB4, and primary APL cells were treated for 5 days with crocetin, all-trans retinoic acid (ATRA), or arsenic trioxide (As_2O_3) and the expressions ABCB1 (A) and ABCC1 (B) genes were determined by real-time PCR. Results are mean ± SEM of three different experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus untreated control. SEM, standard error of the mean

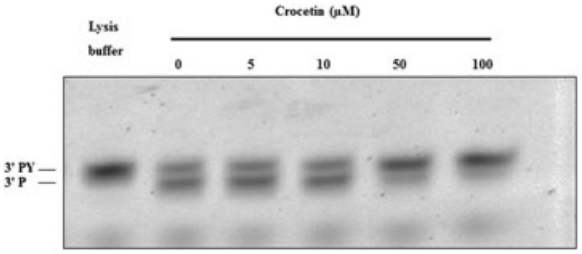

FIGURE 10 Effects of crocetin on TDP1 activity in human lymphoblastoid cells (TK6). TK6 cells were treated with crocetin (5 to 100 μM) for 5 days. Reaction mixture was fractionated on denaturing PAGE. Arrows point at the position of substrate (PY) and cleaved product (P). TDP1, tyrosyl-DNA phosphodiesterase 1

antiproliferative effect of crocetin was associated with increased
sub-G1 population and enhanced of apoptosis.

Apoptosis can be initiated by two main signaling pathways, known as extrinsic and intrinsic pathways. The former is triggered by binding of Fas and other plasma membrane death receptors with their ligands, which activate caspase 8 and then induces apoptosis via stimulating caspase 3. The latter pathway is initiated by several intracellular signals including calcium, reactive oxygen species, hypoxia and chemotherapeutics. These signals lead to the release of cytochrome *c* from mitochondria into the cytosol which recruits pro-caspase-9 and Apaf-1 to stimulate a caspase 9/3 signaling cascade leading to DNA fragmentation and apoptosis.[37,38] The ratios of proapoptotic and antiapoptotic proteins of Bcl2 family, most importantly Bax/Bcl2 ratio, control the resistance or sensitivity of cells to apoptotic stimuli.[38,39] Also, the tumor suppressor p53 governs apoptosis by increasing the expression of several proapoptotic proteins involved in intrinsic pathway (eg, Bax and BID) and by upregulating membrane death receptors (eg, Fas and DR5). The p21, a cyclin-dependent kinase inhibitor, is a major effector of p53 and leads to cell cycle arrest in the G1 phase and prevents cell proliferation in response to oncogenes, cell stress, and DNA damage.[37,38,40] On the other hand, activation of prosurvival pathways such as PI3K/Akt signaling cascade help cells to resist against apoptosis triggers. Some tumor suppressors like PTEN block the PI3K/Akt signaling pathway, resulting in inhibition of cell growth and apoptosis. In the present work, results of real-time PCR showed that crocetin (at 100 μM), similar to ATRA and As_2O_3, could increase the expression of proapoptotic gene Bax and decreased the expression of antiapoptotic genes Akt and Bcl2 in primary APL cells. In all HL60, NB4, and primary APL cells, the expressions of CASP3, CASP9, and Bax/Bcl2 ratio were significantly increased after treatment with crocetin. These data suggest that crocetin induces its antiproliferative and proapoptotic effects on the leukemic cells by inhibiting Akt-mediated prosurvival cascade and by stimulating intrinsic pathway of apoptosis.

Tyrosyl-DNA phosphodiesterase I (TDP1) belongs to the phospholipase D superfamily of phospholipids hydrolyzing enzymes[41] and participates in the repair of a variety of 3′ adducts/base damages of DNA. It is well documented that inhibition of TDP1 decreases cell survival signals and promotes apoptosis in a variety of cancerous cells. Therefore, proapoptotic effects of crocetin may be also mediated through TDP1 inhibition.[42]

Failure to differentiate is one of the main characteristics of the promyelocytes in APL patients.[43] Results of Giemsa staining and NBT assay showed that crocetin, like ATRA and As_2O_3, induces morphological changes in leukemic cells toward granulocytic pattern, and increases their NBT reduction ability, which is a hallmark of granulocytic maturation. Also, in all HL60, NB4, and primary APL cells, crocetin increased the number of differentiated cells expressing CD11b and CD14, and reduced the number of immature cells expressing CD34 or CD33. In comparison to its antiapoptotic property, prodifferentiating action of crocetin was observed at lower concentrations suggesting that effects of this carotenoid depend on its concentration: at concentrations of $>50\,\mu M$ induces apoptosis in leukemic cells and at concentrations of $\leq 10\,\mu M$ induces their differentiation to granulocytes. The inhibitory effect of crocetin on the level of PML-RARα expression may describe the prodifferentiating action of crocetin on NB4 and primary APL cells. It is well known that fusions between PML and RAR recruit HDACs resulting in the suppression of differentiation-related genes.[3,44] In agreement with this, our data showed that treatment with crocetin and ATRA reduced the expression of HDAC1 in the leukemic cells. Interestingly, crocetin was able to induce differentiation in HL60, which is PML-RARα null cells, suggesting that the HDAC1 inhibition could be the major mechanism responsible for myeloid differentiation in such cells.

Leukemic cells often express high levels of MDR-associated proteins such as ABCB1 and ABCC1, which is a challenge to the successful treatment of some patients with ATRA and/or As_2O_3.[8,9] Overexpressions of ABCB1 and ABCC1 have also been shown following chronic use of As_2O_3 in other cancers such as liver and gastric tumors.[45] Here, we have shown that crocetin at low concentrations is able to decrease the expression of ABCB1 and ABCC1 in all HL60, NB4, and primary APL cells, while ATRA and As_2O_3 failed to show such meaningful effects. This effect is in agreement with previous findings of Mahdizadeh et al,[46] who showed that crocin, another carotenoid from saffron, decreased ABCB1 and ABCC2 genes expressions at the mRNA

level in ovarian cancer cell line, and supports the view that crocetin may suppress drug resistance via down regulation of ABC transporters.

In conclusion, the present results demonstrated that crocetin has antiproliferative, proapoptotic, and prodifferentiating effects on human leukemia cells by inhibiting Akt-mediated prosurvival cascades, increasing intracellular Bax/Bcl2 ratio, and decreasing TDP1 enzyme activity and the expressions of PML-RARα, HDAC1, and MDR-associated proteins. These findings suggest that crocetin may be considered as a candidate for future preclinical and clinical trials of complementary APL treatment.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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