



Anticancer Impacts of *Terminalia catappa* Extract on SW480 Colorectal Neoplasm Cell Line

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Published online: 10 December 2019

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Abstract

Purpose Colorectal cancer (CRC) is one of the most lethal and prevalent cancers throughout the world. Despite the remarkable advance in the field, drug resistance still remains as an unresolved problem in cancer. Hence, finding effective compounds with minimal side effects to fight cancer is of central priority. Herbal products have been traditionally used to prevent and treat a variety of diseases.

Methods In the present study, the antitumor effect of *Terminalia catappa* plant ethanolic extract (TCE) was assessed on SW480 CRC model cell line. In this regard, effects of TCE were evaluated on the proliferation, apoptosis, and migration of SW480 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Annexin V/PI flow cytometry, and scratch tests, respectively. Furthermore, changes in the expression of genes involved in these events including Bax, Bcl-2, Caspase 3, Caspase 8, Caspase 9, MMP-13, miR-21, and miR-34a were measured by quantitative real-time PCR (qRT-PCR).

Results According to the MTT results, TCE reduced the proliferation of SW480 cells significantly. The flow cytometry test also revealed a notable rate of apoptosis induction after TCE treatment. An inhibitory effect on cell migration was also evident in scratch test. Expression patterns of the assessed genes also changed subsequent to TCE treatment.

Conclusion The results of this study indicated that *T. catappa* could be considered as a potential source of anticancer compounds and a candidate for further investigations.

Keywords Colorectal cancer · *Terminalia catappa* · SW480 · Apoptosis · Metastasis

Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers with high rates of mortality and morbidity. Both environmental factors and genetics play important roles in initiation and development of CRC [1, 2]. Despite recent advances in

understanding the molecular biology of CRC and introduction of new therapeutic agents, only 14% of patients could survive for 5 years. Accordingly, researchers of the field continuously try to find new substances and methods to cure or control the CRC and other cancers [3]. Herbal products have been long utilized for prevention and treatment of various diseases. Likewise, the antitumor properties of herbal compounds are currently under investigation. *Terminalia catappa*, which is native to India, belongs to the family Combretaceae. In South America, its derivatives are being used for production of ornamental and reforestation products [4]. It has shown that compounds from *T. catappa* exhibit antioxidant [5] and anti-inflammatory activities [6]. Furthermore, derivatives of *T. catappa* have been shown to have inhibitory effects on various cancer cell lines including anti-metastatic effects on lung cancer [7], anti-mutagenicity effects on human hepatoma cells [8], and the inhibitory effects on metastasis-associated proteases in oral cancer [9]. The present study was aimed to

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evaluate the cytotoxic, apoptotic, and anti-migratory activity of ethanolic extract from *Terminalia catappa* on SW480 cell line. Moreover, expression patterns of the genes possibly involved in the mentioned events were assessed.

Materials and Methods

Preparation of *Terminalia catappa* Leaves Extract (TCE)

The leaves that are used in this study were collected from Rodan City (Hormozgan Province, Iran). Leaves of *Terminalia catappa* were healthy and green, with uniform color and no pests or disease contaminations. Then, 30 g of the leaves were air-dried, ground, and dissolved in ethanol 50% and extracted by maceration method [10]. The extraction procedure was repeated three times. Then, solvent was removed from the combined extract with a vacuum rotary evaporator to obtain TCE. Afterward, 5 mg of TCE was dissolved in 100 μ l DMSO and then mixed with 900 μ l of complete culture medium for subsequent treatments.

Cell Culture

The SW480 colorectal cancer cell line was purchased from Pasteur Institute (Tehran, Iran) and grown in Dulbecco's Modified Eagles Medium (DMEM) (GIBCO/BRL Life Technologies, USA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotics (100 IU/ml penicillin, 100 μ g/ml streptomycin) (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were incubated at 37 °C in a CO₂ incubator. In all of the tests, control cells were treated with DMSO.

Cytotoxicity Assay

The effect of TCE on cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The SW480 cells were seeded in 96-well culture plates for 48 h with an initial concentration of 10⁴ cells in 200 μ l medium. The cells were treated in several concentrations (1, 10, 20, 50, 100, and 200 μ g). After 48 h incubation period, 50 μ l of a 5 mg/ml MTT solution was added to each well, and the plate was further incubated at 37 °C for 4 h. Then, the medium was aspirated and the wells washed twice with PBS. Subsequently, 200 μ l of DMSO and 25 μ l of Sorenson's buffer were added to the wells. The plate was located on a shaker for 30 min to dissolve the dye. After the formazan crystals were dissolved, the absorbance was determined by ELX800 UV universal microplate reader (Bio-Tek Instruments) at 570 nm. Furthermore, the half inhibitory

concentration (IC₅₀) was calculated using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA).

Annexin V/Propidium Iodide Double-Staining Assay to Determine the Apoptotic Cells

SW480 cells were seeded at a density of 10⁵ cell/well in six-well plates. The cells were treated in aforementioned concentrations. After 24 h, the cells were harvested by EDTA 2%, washed once with phosphate-buffered saline (PBS, pH 7.4), and centrifuged. Subsequently, the cells were suspended in 200 μ g binding buffer (Invitrogen CO. USA). Then the FITC-Annexin V reagent was added into the plate and incubated on ice for 10 min (in dark). Thereafter, the cells were resuspended in another 200 μ l binding buffer, and 10 μ l of propidium iodide (PI) was added to detect the apoptosis occurrence by flow cytometry.

Scratch (Wound Healing Assay)

Migration of SW480 cells was measured by using wound healing assay. SW480 cells (4 × 10⁵ cells/well) were seeded in 6-well plates, and when the cultured cells reached >80% confluence, a scratch was created by a sterile yellow pipette tip across the cell monolayer. The detached cells were removed by washing with serum-free medium. Then, the plates were incubated for 72 h at 37 °C with 5% CO₂ to allow the cells to migrate into the wound area. Images of the wound area were captured at 0, 24, 48, and 72 h using a light microscope. Rate of migration was calculated by measuring the width of the gap area.

RNA Isolation, cDNA Synthesis, and qRT-PCR

Extraction of total RNA was carried out 24 h after TCE treatment using an extraction kit (Takara Biotechnology Inc.). Complementary DNA (cDNA) was synthesized from 2 μ g of total RNA by use of MMLV reverse transcriptase (Takara, cat.No:RR037Q) with random hexamer and oligo dt primers according to the manufacturer's instructions. The quality and quantity of the extracted RNA was determined by NanoDrop 2000c instrument (Thermo Scientific). The PCR reaction contained 10 μ l of SYBR green Premix (Takara, RR820L), 0.5 μ M of each primer, 1 μ l of cDNA template, and 8 μ l of nuclease-free water. The initial enzyme activation step at 95 °C for 1 min was followed by 45 cycles at 95 °C for 10 s, 59–63 °C for 30 s, and 72 °C for 20 s for different primers. qRT-PCR was performed in the Applied Biosystems StepOne instrument. The PCR product sizes were confirmed by 2% agarose gel (Bio Basic, Canada) electrophoresis. The primers used in the study were designed by Oligo 7 software (Table 1). Relative Bax, Bcl2, Caspase 3, Caspase 8, Caspase 9, MMP-13, vimentin, miR-34a, and miR-21 expressions were calculated with the 2^(- $\Delta\Delta$ Ct) method (Livak and Schmittgen, 2001) using β -actin as housekeeping gene.

Table 1 The primer sequences

Primers	Forward and reverse	Sequences
Bax	Forward	5'- GACTCCCCCGAGAGGTCTT-3'
	Reverse	5'- ACAGGGCCTTGAGCACCAAGTT -3'
Bcl-2	Forward	5'- GAGCGTCAACCGGGAGATGTC -3'
	Reverse	5'- TGCCGGTTCAGGTACTCAGTC-3'
Caspase 3	Forward	5'-ATGGTTTGAGCCTGAGCAGA-3'
	Reverse	5'-GGCAGCATCATCCACACATAC-3'
β-actin	Forward	5'-TCCCTGGAGAAGAGCTACG-3'
	Reverse	5'-GTAGTTTCGTGGATGCCACA-3
Caspase 9	Forward	5'-GCAGGCTCTGGATCTCGGC-3'
	Reverse	5'-GCTGCTTGCCTGTTAGTTCGC-3'
Caspase 8	Forward	5'-ACCTTGTGTCTGAGCTGGTCT-3'
	Reverse	5'--GCCACTGGTATTCTCAGGC -3'
miR-21	Exiqon	Product number: 202,007
miR-34a	Exiqon	Product number 202,860
MMP-13	Forward	5' TGCAGAGCGCTACCTGAGATCATAC-3'
	Reverse	5'- GGAGCTTGCTGCATTCTCCTTCA-3'

Assessment of DNA Damage Following TCE Treatment

Topaz gene™ total DNA extraction kit (Iran, TGK1003) was utilized for this purpose. A number of 5×10^5 cells were seeded in a 6-well culture plate. After 24 h, cells were treated with IC50 concentration of TCE, and DNA extraction was performed 48 h later. Agarose gel electrophoresis and UV light visualization were employed for assessment of the integrity of the extracted DNA.

Statistical Methods

All the results of this study were presented as the mean ± standard deviation (SD). Statistical significance of differences between groups was evaluated by analysis of variance (ANOVA) followed by Bonferroni’s and Sidak’s multiple comparisons and for two groups was done by unpaired Student’s t test. “p values” less than 0.05 were considered significant. GraphPad Prism 6.01 software was employed for statistical analyses.

Results

Cytotoxic Effect of Terminalia catappa Leaf Extracts (TCE) on SW480 Cell Line

To evaluate the effect of TCE on cell viability, the SW480 cells were treated with different concentrations of TCE. After 24 h, the cell viability was analyzed by MTT assay. The results

showed that the TCE decreased the cell survival rate in a dose-dependent manner. To such an extent, 200 µg of TCE lowered the viability to 22.21%, compared to the control group ($p < 0.05$; Fig. 1). Furthermore, an IC50 of 46.79 µg was calculated by GraphPad Prism software for SW480 cell line.

Effects of TCE Treatment on Apoptosis Induction

In this study, Annexin V/PI double staining was performed to quantify the percentage of apoptotic cells. The population of live cells is located in lower left (LL)

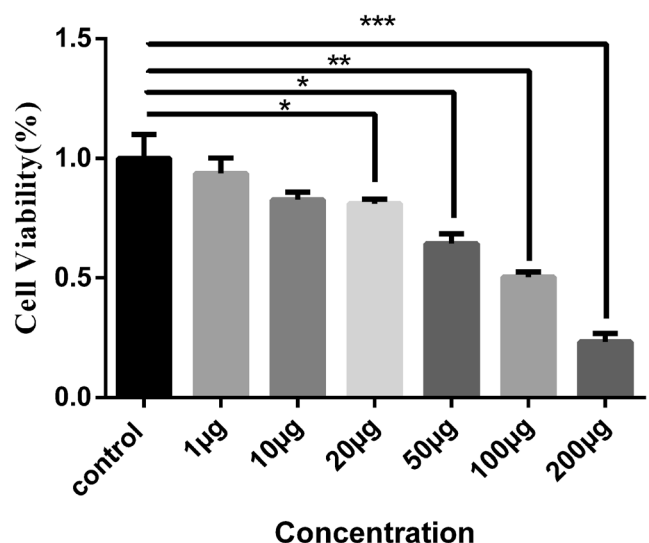


Fig. 1 Effects of IC50 concentration of TCE on SW480 cell proliferation: TCE decreased the cell survival to 22.21%, compared to the control cells ($p < 0.05$)

quadrant, the cell population in the early stage of apoptosis is shown in lower right (LR) quadrant, upper left (UL) quadrant represents the population of cells in the necrosis stage, and upper right (UR) quadrant is considered as the population of the cell at late apoptosis stages (Fig. 2) and (Table 2).

TCE Prohibits the Migration of SW480 Cell Line

Migration is a common feature of metastatic cancer cells. To evaluate the impact of TCE on SW480 cell migration, the potential of the treated cells to close the wound was assessed and compared to that of the controls over the experiment time. The results of wound healing assay showed a remarkable decrease in the capability of the TCE-treated cells in migration to the gap area (Fig. 3).

Downregulation of Bcl-2 and MMP-13 Expression and Upregulation of Bax, Caspase 3, Caspase 9, and miR-34a in SW480 Cells after Treating by TCE

The effects of the TCE treatment on the expression of Bcl-2, Caspase 9, Bax, Caspase 3, Caspase 8, miR-34a, miR-21, Vimentin, and MMP-13 in SW480 cell line were evaluated by qRT-PCR. In the TCE-treated group, 24 h after treatment, the level of Bcl-2 and MMP-13 mRNA decreased to 72.4 and 81.2%, respectively. Also, after treatment by TCE, the level of Bax, Caspase 3, Caspase 9, and miR-34a increased to 141%, 145%, 147%, and 119%, respectively. There was no significant difference between TCE-treated cells and untreated cells in the expression of Caspase 8, miR-21, and Vimentin mRNAs (Fig. 4).

DNA Damage Test

Total DNA was extracted from the SW480 cells treated with IC50 concentration of TCE. Visualization of the DNA on agarose gel indicated a damaged DNA in cells treated with TCE. In the test group, majority of the DNA formed a smear pattern whereas there was a distinct band in the control lane (Fig. 5).

Discussion

Herbal products have long been used to prevent and treat various diseases, including chronic diseases and cancer. Emergence of advanced strategies like RNA interference [11–13] and genome editing technologies [14] has not been able to detract from the importance of natural products as source of therapeutic agents.

Plant-derived antitumor compounds have attracted a great deal of interest for finding safer therapeutics with lowered side effects [15]. Inhibitory effects of *T. catappa* extract on cancer cell migration and invasion have been reported in different types of human cancers, including lung cancer [7], oral cancer [9], and hepatocellular carcinoma [16]. In accordance with these works, the current study revealed that treatment with TCE could suppress proliferation and metastasis of SW480 CRC cell line. Likewise, Yeh CB and co-workers demonstrated the inhibitory effects of *T. catappa* extract on hepatocellular carcinoma [8].

In 2018, Chung Yuan Lee and co-workers found that TCE have low cytotoxicity on human HeLa and SiHa cervical cancer cells. However, there was a significant negative impact on cellular migration and invasion by inhibiting matrix metalloproteinase-9 and MAPK pathway [17, 18]. In the

Fig. 2 Flow cytometry results indicating the effect of TCE treatment on the apoptosis rate of SW480 CRC cells

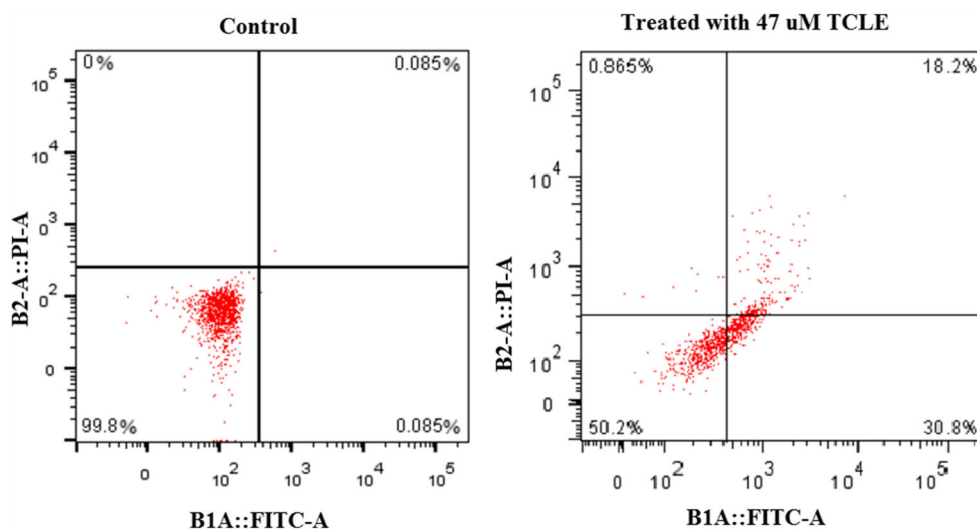


Table 2 Flow cytometry analysis of SW480 cells treated with TCE

Treatment	LL	LR	UL	UR	LR + UR
Control	99.8%	0.085%	0.0%	0.085 0%	0.17%
Treated with 47 µg of TCE	50.2%	30.8%	0.865%	18.2%	49%

current study, TCE exhibited significant inhibitory effects on both viability and cell motility of SW480 cell line. Most of the therapeutic methods for cancer treatment like chemotherapy and radiotherapy are expected to induce apoptosis as the mechanism of choice [3]. According to flow cytometry results, a notable range of apoptosis occurrence was evident in the treated cells.

Apoptosis-related genes are important players in several malignancies especially cancer. In the present study, upregulation of Caspase 9 as the indicator of intrinsic apoptosis was evident after treating by TCE. However, caspase 8 which is a mediator of extrinsic apoptosis induction did not show a significant increase in the treated cells. Caspase 3, an important member of Caspase family which is involved in both mitochondrial and extrinsic pathways, indicated a significant up-regulation after treatment.

Bcl-2 and Bax are important genes in regulating apoptosis [4]. According to qRT-PCR results, Bcl-2 as negative regulator of apoptosis was downregulated in the test group. The expression of Bax gene in the treated cells was upregulated which interferes with the Bcl2 structure and function. It has indicated that Bax/Bcl2 ratio could be an indicator of susceptibility to apoptosis [19]. In addition to desired changes in the expression of Bax and Bcl2

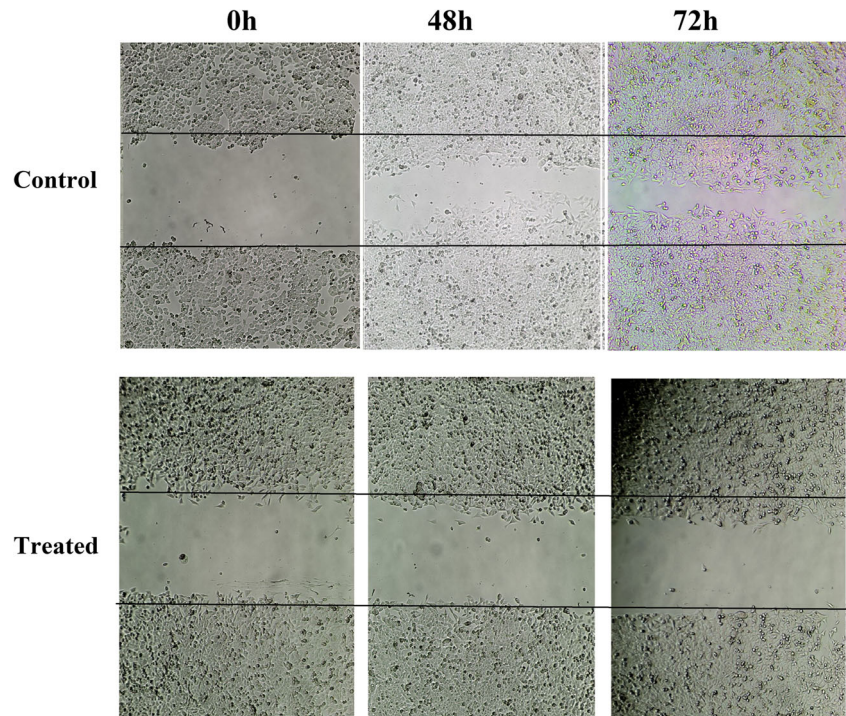
mRNAs, Bax/Bcl2 ratio showed a notable increase in comparison to the untreated cells (fig. 3).

Furthermore, MMP-13 as key member of matrix metalloproteinase family (which are responsible for cell motility and metastasis) was significantly downregulated. Yang et al. demonstrated that TCE could suppress migration and invasion of SCC-4 oral cancer by inhibiting MMP-2, MMP-9, and u-PA both in mRNA and protein level [9]. Recent studies suggest that MMP-13 may have a key role in the extracellular MMP activation cascade [19]. In another study, Shu-Chen Chu and co-workers show that *T. catappa* extract has dose-dependent inhibitory impact on the motility of highly metastatic and invasion in A549 and Lewis lung carcinoma (LLC) cells [7].

Moreover, expression of miR-34a as a tumor suppressor miRNA in CRC increased in the TCE-treated group. miR-34a exerts inhibitory effects on the development and invasion of cancer cells by positive feedback on p53 [20].

miRNA-34a could also target Bcl-2 mRNA which is important factor for the malignancy of cancer cells. In addition, expression levels of Vimentin (as a metastasis relating gene) and miR-21 (a well-known onco-miR) were decreased after TCE treatment. However, the rate of the changes was not statistically significant.

Fig. 3 Results of wound healing assay shows that untreated cells (control group) have a significant further migration in comparison with cells treated with TCE



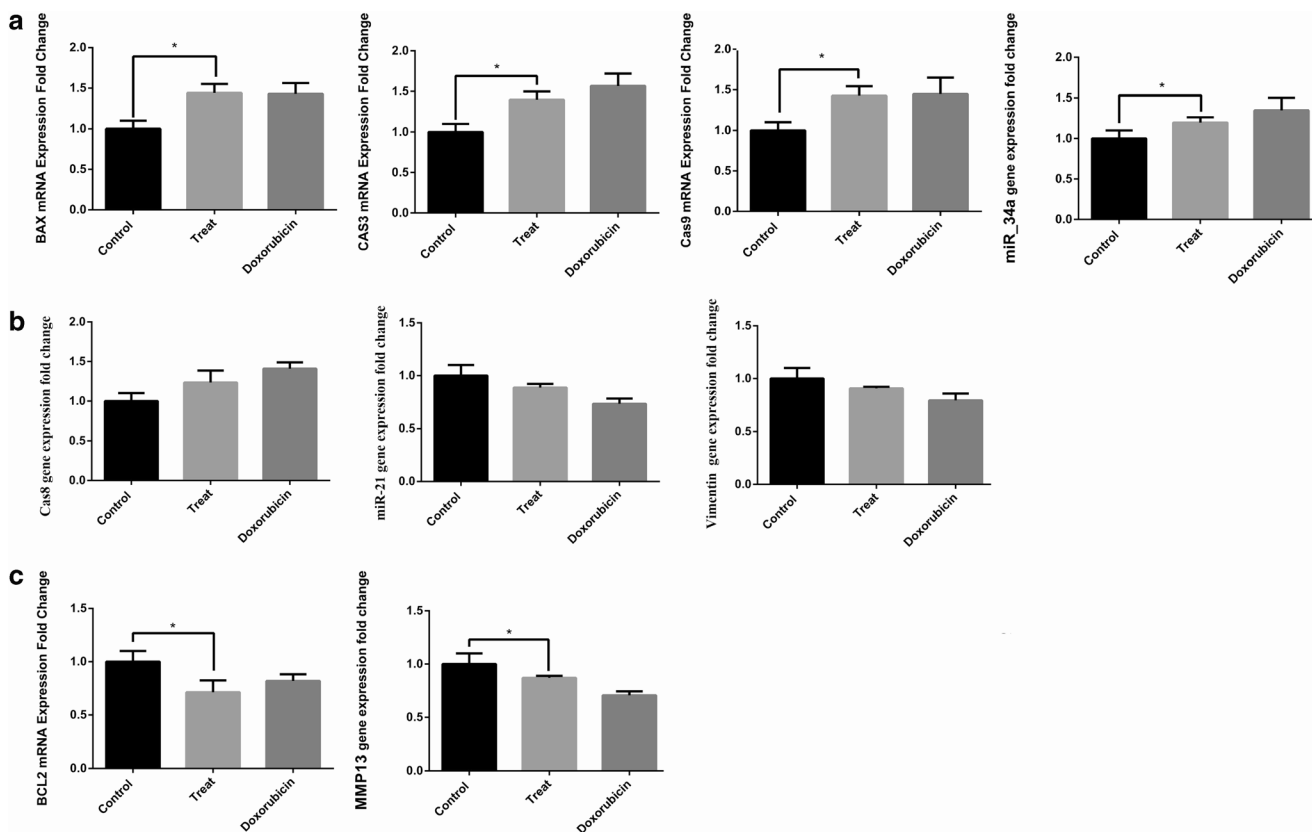


Fig. 4 Effects of TCE treatment on the expression of BAX, Bcl-2, MMP-13, miR-21, miR-34a, Cas-3, Cas-8, Cas-9, and Vimentin in SW480 cell line. The data was presented as mean \pm SD (N = 3) (* = $P < 0.05$)

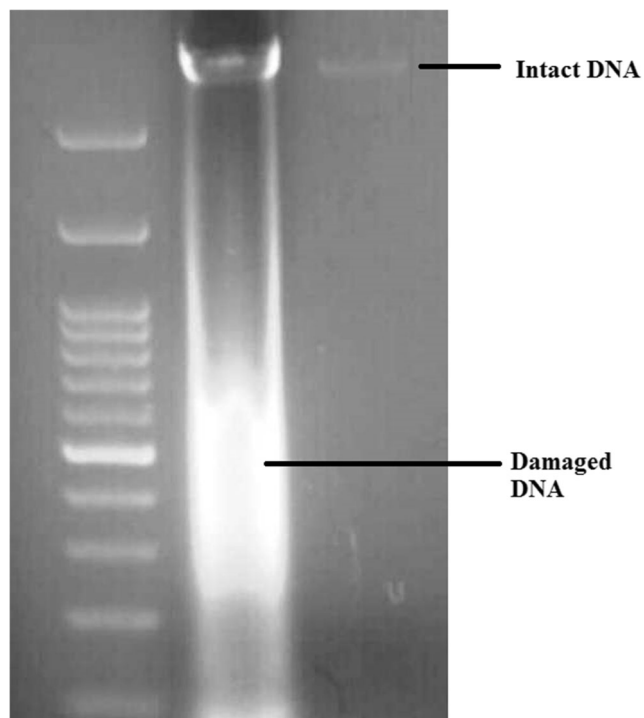


Fig. 5 DNA damage was evident after TCE treatment in SW480 cells. Left to right: lane 1, size marker (M); lane 2, damaged DNA of the treated cells (with DNA smear); and lane 3, indicating the DNA extracted from control cells (with good integrity)

Based on the whole data, TCE extract was able to harness the malignancy and induce the mitochondrial apoptosis in CRC cell line.

Conclusions

TCE exerts growth inhibitory and apoptotic effect on SW480 cell line and reduces the migration rate by regulating the expression of genes related with apoptosis and metastasis. According to the results, TCE may be considered as a potential candidate for developing anti-cancer agents for CRC.

Funding Information This study was funded by the Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Science, Tabriz, Iran.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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