The following manuscript was accepted for publication in Pharmaceutical Sciences. It is assigned to an issue after technical editing, formatting for publication and author proofing.

Citation: Ghaffari M, Shanehbandi D, Sarhadi S, Hanifeh Ahagh M, Maleki Moghaddam M, Dehghan G, Ghodsi R, Ezzati Nazhad Dolatabadi J. Anti-proliferative and apoptotic effect of tetrahydrobenzo[h]quinoline on MCF-7 human breast cancer cell, Pharm Sci. 2021, doi:10.34172/PS.2021.58

Anti-proliferative and apoptotic effect of tetrahydrobenzo[h]quinoline on MCF-7 human breast cancer cell

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Abstract

Background: Quinoline and its derivatives display various biological activities based on versatility in designing a new drug class for medicinal applications. Hence, synthesizing innovative and varied derivatives of quinoline has gained considerable attention among chemists and biologists. This study evaluated the anti-proliferative and apoptotic effect of tetrahydrobenzo[h]quinoline on Michigan Cancer Foundation-7 (MCF-7) human breast cancer cells.

Methods: The anti-proliferative effect of tetrahydrobenzo[h]quinoline was studied via MTT [3 0-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assays. A quantitative and qualitative study of apoptosis was carried out via flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Quantitative real-time PCR (qPCR) and immunoblotting analysis were employed to identify the expression level of genes and proteins involved in the apoptosis signaling pathway.

Results: The synthesized compound reduced 50% of cell growth at concentrations of 10 and 7.5 µM during 24 and 48h, respectively, and induced apoptosis up to 30% in MCF-7 cancer cells. Regarding the gene expression level, *Bcl-2* displayed considerable alleviation, whereas *Bax* expression increased significantly. Despite the remarkable increase in *caspase 9* expression, there was no noticeable difference in the *caspase 8* expression in treated cells compared to the control group. Western blotting data showed that the protein expression level of Bcl-2, pro-caspase *8*, and 9 reduced. The protein content of Bax, cleaved-caspase 8, and 9 increased significantly, of which the protein level of cleaved-caspase 9 exhibited a tremendous rise in the treated group.

Conclusion: The newly synthesized tetrahydrobenzo[*h*]quinoline can be a promising organic compound for cancer treatment if its anti-cancer effect investigates by other types of breast cancer cells. In vivo studies should be used to investigate the anti-cancer efficiency of this compound.

Keywords: Quinoline; MCF-7; Breast cancer; RT-PCR; Cytotoxicity; Apoptosis.

1. Introduction

As a life-threatening malignancy, cancer is an abnormal proliferation of cells in various parts of the body. Lung, breast, colon, uterus and prostate are the main target organs that cancer can frequently occur [1,2]. Breast cancer is considered one of the most prevalent malignancies among the mentioned organs and is the second leading cause of cancer-related death in females worldwide [3-6]. Besides, chemotherapy is considered the most vital and conventional method for cancer treatment [7]. However, the severe side effects of chemotherapeutic agents such as acute toxicity led to failure in cancer treatment [3].

Consequently, the design and discovery of innovative and active agents are carried out to obtain a high therapeutic index and alleviate adverse effects in current cancer treatment approaches[2,8]. Quinoline, termed a parental compound, displays a wide range of anti-cancer activities such as inhibiting cell migration, disruption in angiogenesis, and apoptosis resulting in considerable attention as a heterocyclic compound among newly designed and modified chemotherapeutic agents in medicinal chemistry[9-11]. It must be noted that reducing toxicity is the main advantage of modification carried out on quinoline derivatives, which gives rise to the improvement of biological properties of newly synthesized compounds compared to parental quinoline [12].

Apoptosis, induced via two main intrinsic and extrinsic signaling pathways, is widely considered a vital target for anti-cancer therapies[13]. The intrinsic apoptotic pathway, called the

mitochondrial pathway, is triggered by an imbalance between pro-apoptotic (Bax, Bid, Bad, and Bim) and anti-apoptotic molecules (Bcl-2, Bcl-xl) within mitochondria[14]. Hence, Bcl-2 overexpression leads to tumorigenesis and drug resistance, while high-expression of Bax exhibits the opposite effect and induces cell death[15,16]. Moreover, Bcl-2 proteins are an essential regulator of the intrinsic apoptosis pathway, and Bax protein is determined as a critical effector of apoptosis[17,18].

Along with allosteric change occurring in Bax or Bak, macropores are formed at the mitochondrial surface, resulting in mitochondrial outer membrane permeabilization (MOMP) [19]. Then, the releases of cytochrome c from the inter-membrane of mitochondria cause apoptosome formation and induce caspase activation complex, consequently activating caspase 9 [20,21]. Likewise, caspase 9 activation paves the way for other downstream executioner caspases, including 3, 6, and 7 followed by activation of degrading cytoskeletal and Poly (ADP-ribose) polymerase (PARP) proteins. Finally, it ends with manifestations of different biochemical and morphological changes in apoptotic cells [15,21,22]. It should be noted that Bid, connecting both caspase-dependent and caspase-independent signaling pathways, is activated by caspase 8 and then transfer to mitochondria to activate intrinsic pathway [20,23]. In this study, after synthesizing benzo- and tetrahydrobenzo-[h]quinoline derivatives and also evaluating DNA intercalation potential and anti-apoptotic effect of them[24], we selected the tetrahydrobenzo[*h*]quinoline (Fig. 1) to investigate its anti-cancer activity, apoptosis-inducing capability, and the anti-cancer mechanism by measuring the expression level of genes and proteins involved in both intrinsic and extrinsic apoptosis signaling pathways.

2. Method and Materials

2.1. Materials

MCF-7 cells were supplied from Pasteur Research Institute (Iran). Tetrahydrobenzo[h]quinoline as a quinoline derivative synthesized via Ghodsi et al. in Mashhad University of Medical Sciences [24]. Bax (B-9) sc-7480, Bcl-2 (N-19) sc-492, GAPDH (6C5) sc-32233, m-IgGκBP-HRP sc-516102, and mouse anti-rabbit IgG-HRP sc-2357 were purchased from Santa Cruz Biotechnology (Texas, United States). Caspase-9 (C9) Mouse mAb and Anti-Caspase-8 antibody ab138485 were obtained from Abcam Cell Signaling Technology (Massachusetts, United States). TUNEL Assay Kit (FITC or HRP-DAB) was provided from Elabscience (Texas, United States). MTT reagent dimethyl sulfoxide (DMSO) was supplied from Sigma Aldrich (Missouri, United States). The fetal bovine serum (FBS), penicillin-streptomycin (catalog number: 10566016), and RPMI-1640 (Roswell Park Memorial Institute) media were purchased from Gibco BRL Life Technologies (catalog number: 11995065 Gaithersburg, United States), and the culture plates were gained from SPL Life Sciences Co., Ltd (Gyeonggi-do, Korea).

2.2. MTT assay

Cytotoxicity of newly synthesized quinoline derivative was evaluated on MCF-7 cancer cells in various concentrations employing MTT colorimetric kit. In brief, MCF-7 cells (15×10^3 cell/well) were plated into 96 well plates in RPMI medium supplemented with 10% FBS,100 UI/mL penicillin, 100 µg/mL streptomycin in a humidified atmosphere at 37 °C in 5% CO2 and then incubated in the course of a night. The next step was treated with different concentrations of synthesized components for 24 and 48h. After that, the medium of each well containing a quinoline derivative was replaced with freshly provided MTT solution (concentration of 2mg/ml) and cultured for another 4h. After eliminating the supernatant medium, 200 µl DMSO was added to

dissolve intracellular formazan blue crystals. Eventually, a microplate Reader was used to measure the absorbance at a wavelength of 490 nm. All the trails were fulfilled three times in triplicate [25].

2.3. Flow cytometry assay

Annexin V-FITC /propidium iodide (PI) staining assay investigated the distinction between apoptotic and necrotic cells. First of all, MCF-7 cancer cells were seeded into a 6-well tissue culture plate at a density of 2×10^5 cells/well and then allowed to attach during the night. After eliminating cell culture media, each well was treated with IC50 concentration of quinoline derivative acquired from MTT assay for 48h. Afterward, the cells were harvested and washed twice with ice-cold PBS. Resuspending in binding buffer contains Annexin V-FITC and PI, and cells were incubated for 15 min in the dark at room temperature. The analysis of stained cells was conducted using the Becton Dickinson FACS Calibur System (San Jose, USA). It is worth noting that annexin V and PI double-negative cells were showed as live cells, while annexin V and PI double-positive were considered late apoptotic cells. Moreover, annexin V-positive and PI-negative cells were determined as early apoptotic cells, whereas annexin V-negative and PI-positive were defined as necrotic cells. Intact cancer cells were considered as a negative control.

2.4. DAPI staining assay

Observation of typical signs of apoptosis remarked as nuclear fragmentation, and chromatin condensation was illustrated by microscopic analysis of DAPI stained cells. MCF-7 cells were plated into a 96-well tissue culture plate (15×10^3) and subsequently incubated during the night. Next, each well was treated with a defined IC50 concentration of quinoline derivative and DMSO (5%) as a positive control within 48h. Cancer cells without any treatment was a negative control.

After removing the supernatant media, cells were washed with cold PBS twice. The cells then were fixed with 4% paraformaldehyde for 1h permeabilized in 0.1% (w/v) Triton X-100 for 5 minutes and washed again with PBS. Finally, the fixed cells were stained employing 1 μg/ml DAPI (4',6-diamidino-2-phenylindole) for 20 min. After washing with PBS, stained cells were imaged under a fluorescence microscope to indicate chromatin condensation and marginalization on DAPI staining [26].

2.5. TUNEL assay

TUNEL assay was conducted to investigate further genomic DNA breaks caused by the apoptotic process. Firstly, 2×10^4 cells were seeded into 8 well chamber slide plates, treated with IC50 concentration of tetrahydrobenzo[h]quinoline, and incubated for 48h. The fixation of the treated cells was performed via 4% paraformaldehyde at RT (15-25°C) for 30 min and then 3% H_2O_2 in methanol at 25ºC used as a blocking solution. After 10 min incubation, the blocking solution was removed, washed with PBS three times, and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Ten μl of TUNEL reaction mixture (TdT enzyme and nucleotide) were added to samples and again incubated at 37 ºC for 1 h in a water bath. Next, the slide was washed with PBS (pH 7.4) three times and then added 10 μL DAPI incubated at RT for 5 min, and then a mounting medium was used to seal the slides. Finally, the stained cells were imaged through a light microscope. Untreated cancer cell was considered as a negative control.

2.6. Quantitative real-time-PCR (qPCR) analysis

Real-time PCR was carried out in triplicate to analyze external and internal apoptosis signaling pathways quantitatively. First, MCF-7 cancer cells (5×10^5) were seeded into a 6-well plate and

incubated for 24h, and cultured cells were treated based on IC_{50} concentration for 48h. DMSO with the concentration of 5% was used as a positive control, and untreated cells were considered a negative control. Tripure isolation reagent (Roche, Cat No. 11667165001) as a Total RNA Extraction Kit was used to extract RNA based on the manufacturer's protocol. Then purity and concentration of acquired RNA were evaluated through the NanoDrop spectrophotometer at 260/280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher. Scientific, USA). cDNA syntheses were employed by Universal cDNA Synthesis Kit (Exiqon Cat No. 40023301). Afterward, Exlent SYBR Green Master Mix (Exiqon, Cat No. 400203421) and primer, set for each gene (Table 1), were used to accomplish quantitative real-time PCR to calculate gene expression level. Moreover, GAPDH (housekeeping gene) was considered to normalize the expression level of studied genes. Finally, by measuring the average of the duplicated Ct values, relative mRNA expression level of target genes was defined using the comparative Ct method, explained by Pfaffl [19].

2.7. Western blot analysis

For Western blot analyses, the treated cells were washed first with ice-cold PBS. Then 100 μL lysis buffer was added to lysis the cells and extracted total protein through cold radioimmune precipitation assay (RIPA) (25 mM HEPES, 1% Triton X-100, 2 mM EDTA, 0.1 mM NaCl, 25 mM NaF, 1 mM Sodium Orthovanadate). Next, the equal amount of cell lysates, determined with Bradford protein assay, were loaded into each well of 10%-15% polyacrylamide gels and, after electrophoresis, shifted to nitrocellulose membranes. The membranes were blocked with bovine serum albumin (BSA) 3% in Tris-buffered saline (pH 7.5) for 1 h and probed with primary antibodies (1:500 concentrations) (Santa Cruz Biotechnology, USA) at 4 °C overnight followed by washing with PBS and incubation with HRP-conjugated secondary antibodies for 1 h at room

temperature. Protein expression levels were assessed using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). Finally, Image J software was used to quantified the protein band intensities and normalized via control group (untreated cancer cells) and internal loading control [27].

2.8. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6 Scientific software (GraphPad Software, Inc., La Jolla, CA). The results were expressed as the mean ± standard error of the mean (SEM). A P value of <0.05 was considered to indicate statistical significance. The experimental tests were repeated two times (N=3).

3. Results

3.1. In vitro cell viability

Evaluation of cytotoxic activity of the quinoline derivative on MCF-7 cancer cells was fulfilled via MTT assay to determine the proliferation of cancer cells. Different concentrations of synthesized compound induced cytotoxicity in MCF-7 cells (Fig. 2). Based on obtained results, cell proliferation of the treated groups was reduced in a concentration and time-dependent manner compared to the untreated group. The results showed that tetrahydrobenzo[h]quinoline could reduce the growth of MCF-7 cancer cells by 50 % at concentrations of 10μM and 7.5μM for 24 and 48h, respectively.

3.2. Annexin V-FITC apoptosis

Annexin V-FITC/ PI double staining is the most effective method to investigate cell apoptosis employed based on external phosphatidylserine (PS) detection on the plasma membrane. We measured apoptosis in MCF-7 cells using flow cytometry analysis. As shown in Fig. 3, after

treating with quinoline derivative, 11.53 %, 19.14 %, and 6.27 % of MCF-7 cancer cells endured late apoptosis, early apoptosis, and necrosis, respectively. The result reveals that the studied compound exhibited much more apoptosis than negative and positive controls, which can be considered a significant demonstrative for its cytotoxic activity.

3.3. DAPI staining analysis for morphological changes of the nucleus

Apoptosis induction and cytotoxicity on the nucleus of MCF-7 cancer cells were further proven using a DAPI staining assay. Provided microscopic images in Fig. 4 illustrate the cell nucleus upon staining with DAPI. The results demonstrated various signs of apoptotic cells called nuclear shrinkage, fragmentation in the chromatin, and DNA rings in nucleus of MCF-7 cancer cells upon treatment with quinoline derivative and DMSO (5%) as a positive control within 48h. In contrast, the nucleus morphology of untreated cells did not show any apoptosis induction.

3.4. TUNEL staining for DNA damage detection

The observation of the TUNEL assay was used to confirm that tetrahydrobenzo[*h*]quinoline can produce apoptotic bodies and cause DNA fragmentation due to the anti-proliferative and apoptotic effect of this component in the treated cells compared to the control group (Fig. 6). DNA fragmentation in MCF-7 cancer cells appeared after 48h of exposure to quinoline derivatives at IC50 concentration of 7.5μM obtained from MTT assay.

3.5. Gene expression analysis with RT-PCR

To evaluate the apoptosis mechanism triggering by quinoline derivative, the expression of apoptosis-associated genes (Table 1) was measured by real-time PCR. The obtained results showed that the expression of *Bcl-2* mRNA was significantly downregulated (Fig. 7A $P<0.05$). However,

Bax mRNA expression level was considerably up-regulated in treated cells compared to untreated and control cells (Fig. 7B P<0.01), led to misbalance in *Bcl-2/Bax* ratio (Fig. 7C P<0.001). Moreover, the expression of *caspase-9* remarkably raised in the treated group compared to the negative control (Fig. 7D P<0.001). On the other hand, the *caspase-8* expression did not show detectable differences compared to negative and positive controls (Fig. 7E).

3.6. Protein expression analysis with western blotting

Western blot analysis was carried out to elucidate the apoptosis and anti-proliferative activity (Fig. 8A) induced by tetrahydrobenzo[*h*]quinoline. As shown in Fig. 8 B, the level of pro-apoptotic protein (Bax) showed a considerable reduction in the treated group ($P < 0.01$). In contrast, the level of anti-apoptotic protein (Bcl-2) decreased (Fig. 8C) when it was treated with the mentioned component ($P < 0.001$). Besides, the pro-caspase 8 protein amount displayed gentle alleviation (Fig. 8D) ($P < 0.05$), while after treatment, the protein content of cleaved caspase 8 increased ($P < 0.01$) compared to the control group (Fig. 8E). Besides, there was a remarkable decrease in the level of pro-caspase 9 (Fig. 8F) ($P < 0.001$); however, cleaved caspase 9 protein level depicted a sharp increase ($P < 0.0001$) as a result of releasing cytochrome c from mitochondria (Fig. G).

4. Discussion

In this study, the anti-cancer effect of tetrahydrobenzo[*h*]quinoline was assessed as a synthetic derivative of quinoline. It showed that treatment with this component could reduce cancer cell viability and induce apoptosis in MCF-7 cancer cells. TUNEL, Ladder, and DAPI staining represented further changes in the nucleus of treated cancer cells with tetrahydrobenzo[*h*]quinoline. Furthermore, an imbalance in gene and protein expression level of Bcl-2/Bax induced apoptosis confirmed via several morphological changes in MCF-7 cancer cells.

We analyzed the anti-proliferative activity of tetrahydrobenzo[h]quinoline against the MCF-7 cancer cell for the first step. Based on obtained results, this compound could lessen cell proliferation of treated cells in a dose and time-dependent manner. As mentioned in the previous study, quinolines as an amid derivatives presented more toxicity on all studied cancer cells of which tetrahydrobenzo[h]quinolines showed more potent cytotoxicity than other benzo[h]quinoline derivatives due to lipophilic electron-donating substitution in the para position of the phenyl ring. Besides, our studied compound belongs to saturated quinolines (tetrahydrobenzo[h]quinolines), which were more potent than unsaturated quinolines (benzo[h]quinolines).

On the other hand, the interaction studies in this article revealed a strong interaction between this component and the CT-DNA double helix. Tetrahydrobenzo[h]quinolines as intercalators contain non-intercalating moieties such as sugar and peptides located in the minor or major groove, leading to complex stability. Therefore, unsaturated cyclohexyl group-oriented into the minor or major groove of DNA can be a reason for the extreme cytotoxicity of quinoline derivatives on cancer cells [24,28]. In line with this study, Kuang et al. investigated quinoline derivatives on human liver cancer cells. They found that quinoline derivatives displayed a higher anti-proliferative effect against liver cancer cells than normal cells and 5-FU and cisplatin as conventional anti-cancer drugs [29].

Apoptosis is one of the critical signaling pathway control programmed cell death led to eliminating harmful cells over treatment with anti-cancer drugs in cancer treatment [30].

In addition, nuclear shrinkages, apoptotic body formation, plasma membrane blebbing, and chromatin condensation are several significant morphological signs of apoptosis [31]. In this survey, we showed the potential of tetrahydrobenzo[*h*]quinoline in apoptosis induction by Annexin-V/PI

doubled staining assay and measured the number of apoptotic cells. Besides, DAPI staining and TUNEL assays presented the morphological changes in the apoptotic cells. Acquired results from these assays showed that treatment with quinoline derivatives resulted in a considerable increase in the apoptotic cell population in MCF-7 cancer cells compared to the control group.

The DAPI staining assay showed quite a few changes in the morphology of treated cells, which is evidence for approving the obtained results of the TUNEL assay. In line with this study, Ding et al. studied PQ anticancer effect as a quinoline derivative on breast cancer cells. Flow cytometry results revealed that treated cells with PQ underwent cell death via apoptosis [32].

Both intrinsic and extrinsic apoptosis pathways lead to activating a common set of effector caspases [33]. The extrinsic pathway commences binding death receptors (DRs) to their natural ligands and triggers their trimerization. DRs as members of the tumor necrosis factor receptor (TNFR) are attached to adaptor protein FADD and make death-inducing signaling complex (DISC), which activates caspases 8 and 10, followed by triggering apoptosis [18,22].

To the best of our knowledge, we investigated both intrinsic and extrinsic apoptosis signaling pathways on this newly synthesized quinoline derivative to recognize apoptotic mechanisms and illustrate the morphological change of apoptotic cells for the first time. Real-time PCR was carried out to define the mechanism by which quinoline derivatives induce apoptosis. We measured the expression of genes involved in both signaling pathways. Our observations indicated that quinoline derivatives decreased *Bcl-2* expression level while *Bax* expression experienced a significant increase compared to the control group. In terms of the *Bax* to *Bcl-2* ratio as a vital factor for triggering apoptosis, the results showed remarkable increase in the *Bax*/*Bcl-2* ratio, which is an indicator of cancer cell susceptibility to apoptosis.

Furthermore, *caspase 9* expression level raised upon treatment with quinoline derivatives as a vital result of the change in the ratio of *Bax/Bcl-2* and formation of the apoptosome complex. Moreover, the extrinsic pathway of apoptosis was analyzed by evaluating the expression of *caspase 8* in cells [34], exposing them to quinoline derivatives. The result indicated that *caspase 8* expression did not show detectable change compared to the control group and can acclaim that our synthesized compound could induce apoptosis through the intrinsic pathway.

We conducted a western blotting assay to examine the level of both pro-apoptotic and anti-apoptotic (Bax, Bcl-2) proteins and caspases 8 and 9 to estimate the protein level of mentioned molecules, which are responsible for triggering the apoptosis signaling pathway after treatment with quinoline derivatives. The family of Bcl-2 is of great significance in the molecular mechanisms of apoptosis as these proteins are either in cell membranes or free in cytosol and have a potent function in intracellular membranes to trigger intrinsic apoptosis [35]. It is well known that these two apoptotic proteins are divided into pro-apoptotic proteins, including Bax, Bak, Bok/Mtd, and anti-apoptotic ones such as Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 [36]. *Bcl-2* gene plays a key role in regulating apoptosis based on encoding various molecules responsible for apoptosis regulation. As another protein of the Bcl-2 family member, the *Bax* gene is an enhancer of apoptosis. When transferred from the cytosol to mitochondria, apoptosis is induced via increasing apoptotic stimulant signals production[33].

Further, the reduction of *Bax* expression in breast cancer cells leads to relative drug resistance [37]. Hence, the interaction between *Bcl-2* and *Bax* molecules determines whether the cell experiences cell death or survives[35]. In this study, the results showed that there was a reduction in the protein expression of Bcl-2. In contrast, Bax as a pro-apoptotic protein showed a considerable increase in

protein content following treatment with tetrahydrobenzo[*h*]quinoline, confirming that quinoline derivatives may be a potential component in developing apoptosis.

Protein expression of pro-caspase 8 decreased, whereas the protein amount of cleaved-caspase 8 experienced a remarkable increase in the cells treated with tetrahydrobenzo[*h*]quinoline.

When it comes to caspases, as a group of ICE (interleukin 1 b-converting enzyme)-like proteases are essential molecules in apoptosis mediation [32]. As an initiator caspase, caspase-9 has potent activity in apoptosis, which shows stimulation against chemotherapy, radiation, and stress agents. Based on the caspase activation domain (CARD) motif, caspase 9 is an effective multiprotein that breaks up cells into apoptotic bodies[38]. In this study, the expression of pro-caspase 9 protein declined significantly. However, cleaved-caspase 9, a key protein in the intrinsic pathway of apoptosis and caspase cascade triggering, witnessed a tremendous increase after being treated with quinoline derivatives.

It is shown that forming heterodimers of Bax and Bcl-2 brings about the release of cytochrome c from mitochondria, consequently accelerating apoptosis [39]. Eventually, the results of this study revealed that tetrahydrobenzo[*h*]quinoline could inhibit the proliferation of MCF-7 cancer cells via induction of apoptosis. Besides, this component activates the intrinsic more than extrinsic apoptotic pathway via Bax and Bcl-2 dependent mechanism, which might be a prominent compound as an anti-cancer agent for treating human cancer.

5. Conclusion

This study evaluated the anti-cancer activity of tetrahydrobenzo[h]quinoline in the MCF-7 cancer cell line. The mentioned compound could inhibit cell proliferation and induce apoptosis in MCF-7 cancer cells. Besides, further apoptosis induction was assessed through fluorescent microscopy,

and TUNEL demonstrated that quinoline derivatives induced apoptosis by changing the morphology of cells. Intrinsic pathway triggered considerably than that of extrinsic one over occurring unbalance between *Bcl-2* and *Bax* genes expression. Therefore, protein expression level showed intrinsic pathway as a central mechanism for the induction of apoptosis. The result concludes that tetrahydrobenzo[*h*] quinoline can be considered a therapeutic agent for inducing apoptosis. However, further experiments are needed to approve our observation.

Acknowledgments

The authors would like to thank the Drug Applied Research Center, Tabriz University of Medical Sciences, for supporting this project (grant No: 67394).

Conflict of Interests

The authors declare that there is no conflict of interest.

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Table 1. Primer sequence employed in real-time PCR analysis (5′ to 3′).

Gene	**sequence**
GAPDH	Forward: CAAGATCATCAGCAATGCCTCC
	Reversed: GCCATCACGCCACAGTTTCC
BCL-2	Forward: GAGCGTCAACAGGGAGATGTC
	Reversed: TGCCGGTTCAGGTACTCAGTC
BAX	Forward: CGACGGCAACTTCAACTGGG
	Reversed: CCCATGATGGTCCTGATCAACT
CASP-8	Forward: ACCTTGTGTCTGAGCTGGTCT
	Reversed: GCCCACTGGTATTCCTCAGGC
CASP-9	Forward: GCAGGCTCTGGATCTCGGC
	Reversed: GCTGCTTGCCTGTTAGTTCGC

Figure legends

Figure 1. Structure of tetrahydrobenzo[*h*]quinoline.

Figure 2. Cytotoxicity effect of quinoline derivative. MCF-7 cancer cells were treated with various concentrations of tetrahydrobenzo[*h*]quinoline, ranging from 2.5 to 15μM for 24h and 48h. Untreated cancer cells were control. (n = 3, error bars indicate ± s.d.). *P < 0.05, **P < 0.01, ***P< 0.001 and ****P< 0.0001.

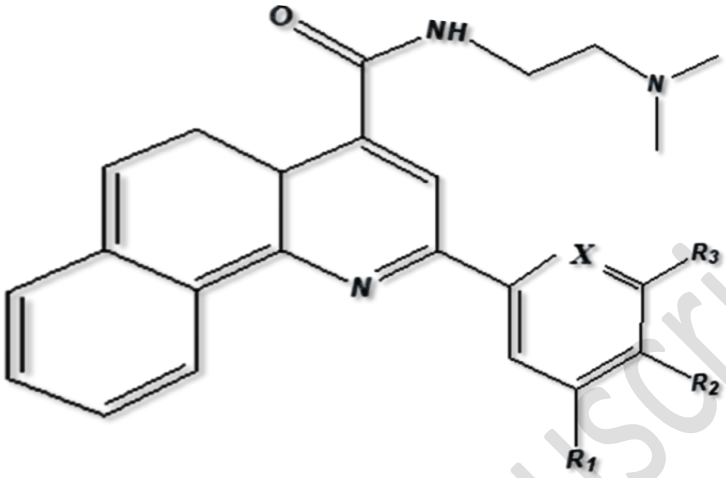
Figure 3. Effects of quinoline derivatives on apoptosis induction. (A) plot and B**)** Bar graph showing the quantification of apoptotic population represented in percentage. (n = 3, error bars indicate ± s.d.). *P < 0.05, **P < 0.01, ***P< 0.001 and ****P< 0.0001.

Figure 4. Displaying MCF-7 cancer cell images for determination of apoptosis by DAPI staining of the nucleus after treatment with quinoline derivatives. A (1-3) light, fluorescent microscopy and merged images of untreated cells as a negative control, B (1-3) light, fluorescent microscopy and merged images of positive control treated by 5% DMSO, and C (1-3) light, fluorescent microscopy and merged images of treated cancer cells with quinoline derivative. Images were captured under a fluorescence microscope.

Figure 5. A representative of DAPI, TUNEL, and Merged images of MCF-7 cancer cells treated with quinoline derivative to evaluate cellular apoptosis of MCF-7 cells pictured via fluorescence microscopy. Untreated cancer cell was considered as a negative control.

Figure 6. RT-PCR analysis to calculate expression level of apoptosis regulation genes 5a) *Bcl-2*, 5b) *Bax*, 5c) the ratio of *Bax* to *Bcl-2*, 5d) expression level of *caspase 9*, 5e) *caspase 3* and 5f) *caspase 8.* (n = 3, error bars indicate ± s.d.). *P < 0.05, **P < 0.01, ***P< 0.001 and ****P< 0.0001.

Figure 7. Western blotting analysis of Pro- and anti-apoptotic proteins including Bcl-2, Bax, caspase 9 and caspase 8 when they were treated with tetrahydrobenzo[*h*]quinoline as a synthetic component in MCF-7 cancer cells. Western blotting images of proteins A), quantification of band density of B) Bax, C) Bcl-2, D) pro-caspase 8, E) cleaved caspase 8, F) pro-caspase 9 and G) cleaved caspase 9 (n = 3, error bars indicate ± s.d.). *P < 0.05, **P < 0.01, ***P< 0.001 and ****P< 0.0001.



R1=R3=H, R2=CH3

Figure 1

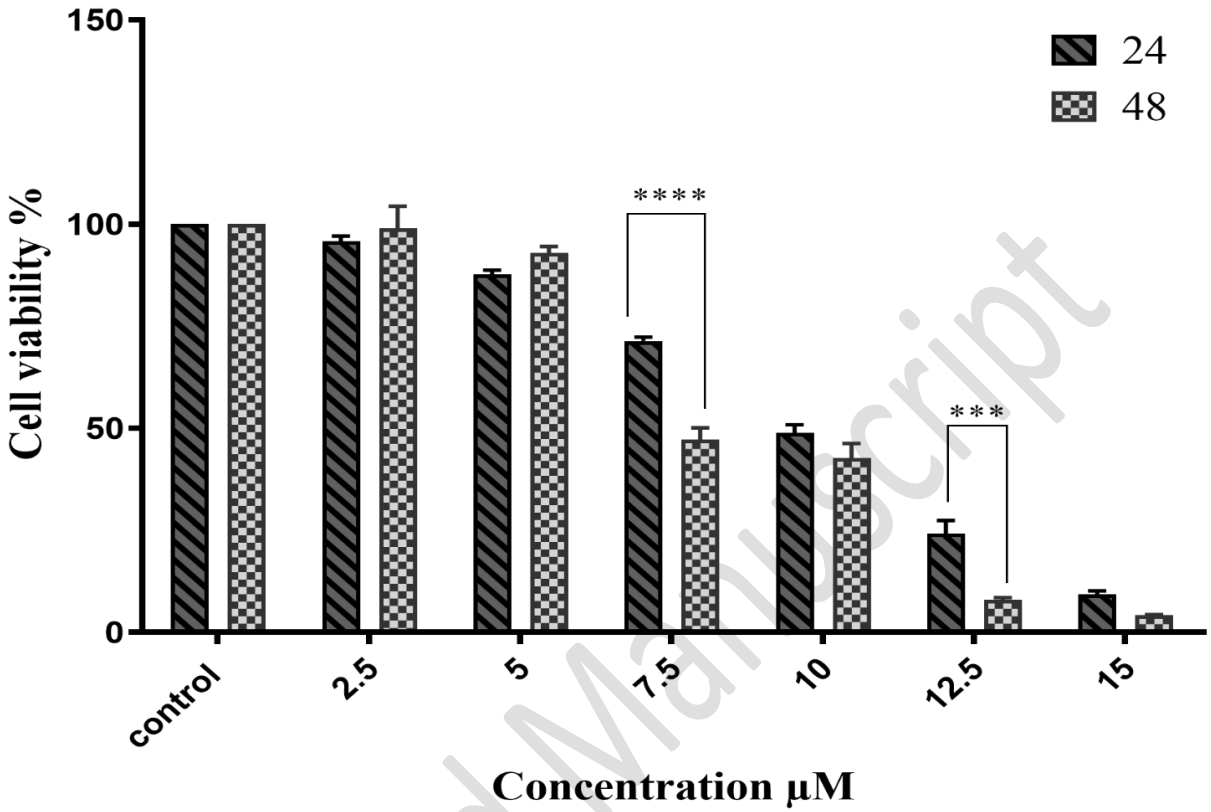


Figure 2

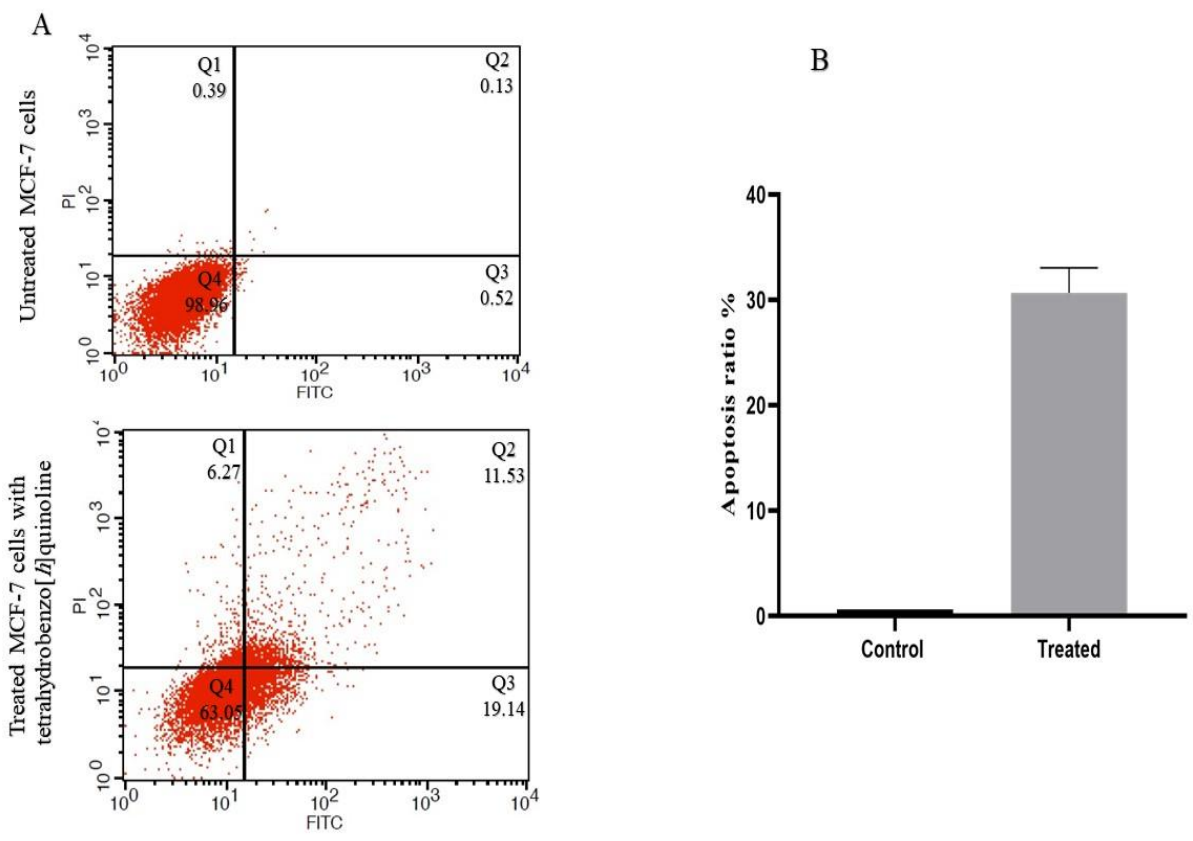

A
Untreated MCF-7 cells
Q1
0.39
Q2
0.13
Q4
98.96
Q3
0.52
PI
FITC
Treated MCF-7 cells with tetrahydrobenzo[h]quinoline
Q1
6.27
Q2
11.53
Q4
63.05
Q3
19.14
B
Apoptosis ratio %
Control
Treated

Figure 3

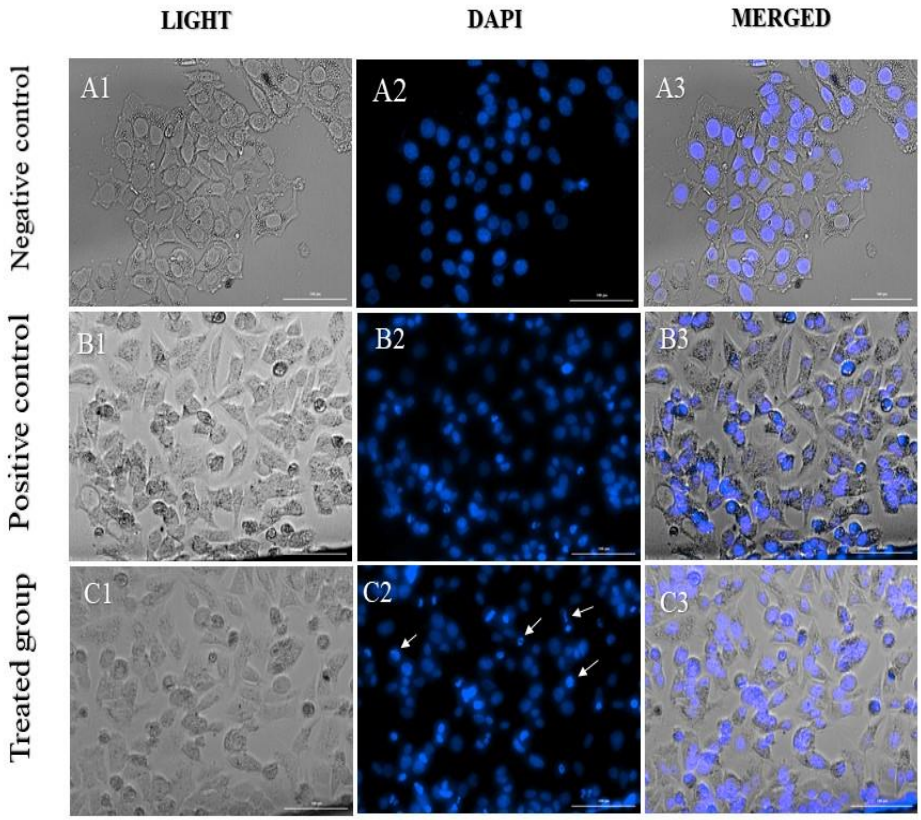


Figure 4

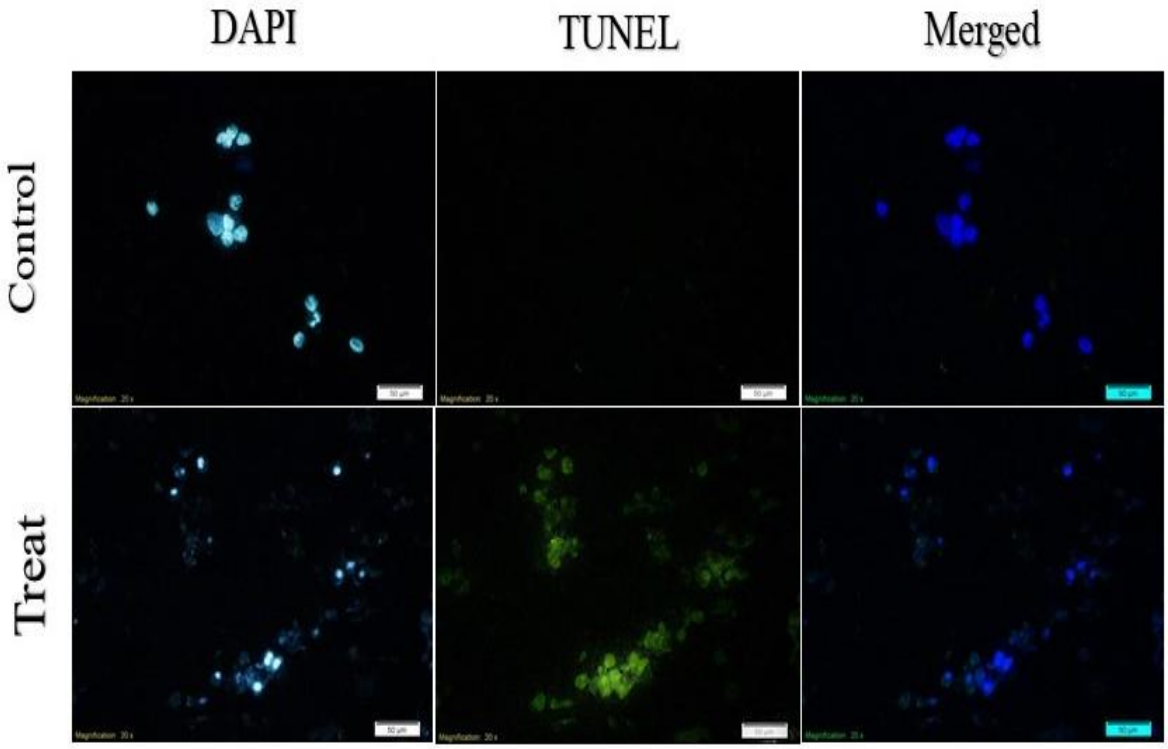


Figure 5

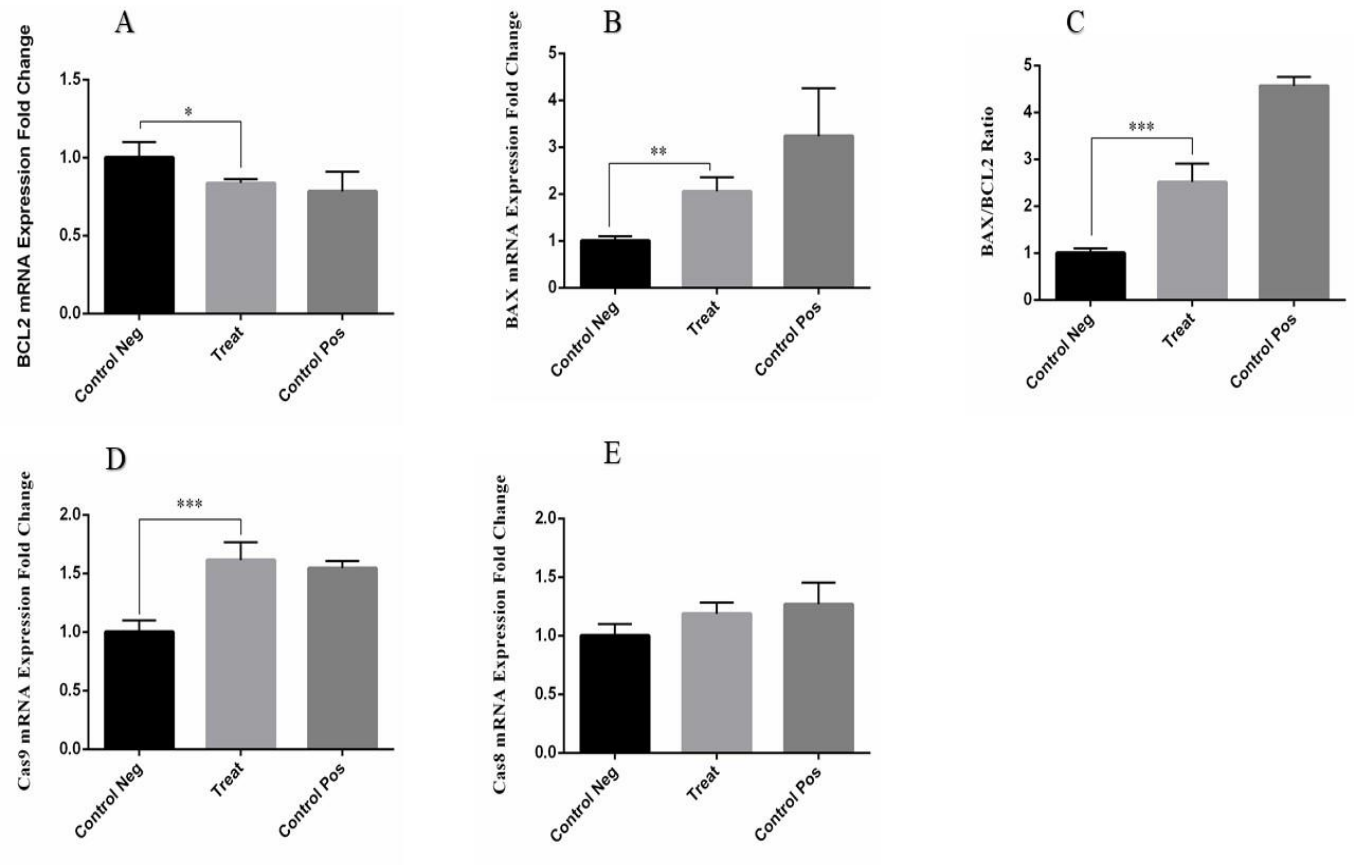


Figure 6

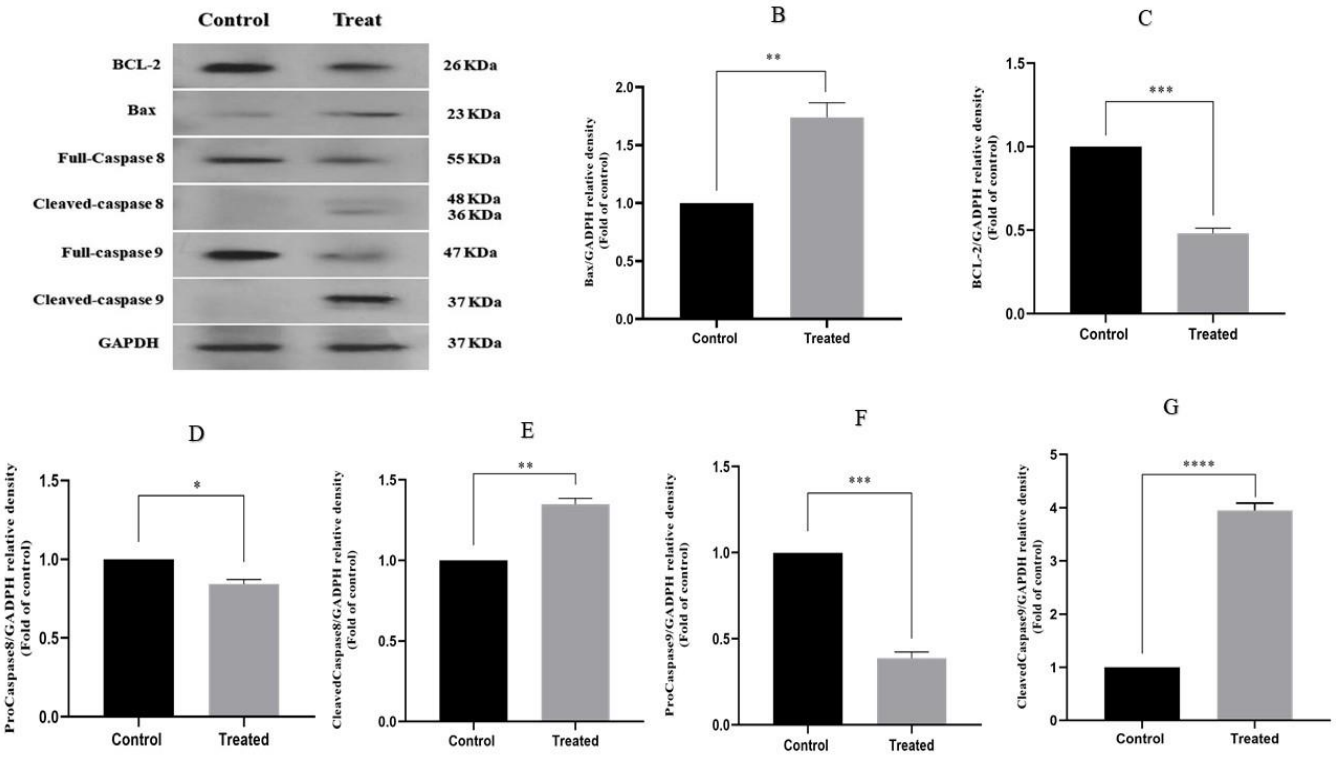


Figure 7