

Effects of Cucurbitacins E, D and I on the Gene Expressions of Apoptotic, Autophagic and AKT-Mtor Pathways in SW480 Colorectal Cancer Cells

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Abstract

Background: Many studies have reported the anticancer effects of cucurbitacins. However, related molecular events need to be described. The current study aims at evaluating the impact of cucurbitacins D, E, and I on death and survival pathways in colorectal cancer (CRC).

Methods: Cell viability was determined by MTT assay in both SW-480 and HT-29 (only cucurbitacin-E). Total RNA was extracted, and the expression of mRNA was quantified by real-time RT-PCR analysis.

Results: The MTT results for SW-480 revealed that cucurbitacins E and I had almost similar potency in different doses, but cucurbitacin-D only had up to almost 50% lethality as a function of dose. Cucurbitacin-E reached IC50 in a very lower dosage at 6 micromolar in HT-29. The expression levels of BAX mRNA were significantly decreased or remained almost unaltered (HT-29) and that of BCL-2 mRNA witnessed a considerable increase ($p < 0.05$), which is a non-canonical paradigm. What is more, while the expression levels of p53 and AIF mRNA was increased in all treatments of cucurbitacins in SW-480, they were suppressed in HT-29 cells treated with cucurbitacin-E. Caspase-3 expression increased in both colon cell lines. According to expression pattern, only cucurbitacin-I had the possibility of suppressing the AKT-mTOR pathway. While autophagy genes were increased in cucurbitacins, cucurbitacin-I decreased the ATG5 expression level.

Conclusion: We noticed that cucurbitacins have the potential to reveal more about both non-canonical interactions of death pathway and BAX/BAK independent apoptosis. These results indicate that cucurbitacins might contribute to BAX/BCL-2-independent cell death in CRC cells.

Keywords: Colorectal cancer; Cucurbitacin; Apoptosis; Autophagy; Gene expression; BAX/BCL-2-independent cell death; AKT-mTOR pathway; Non-canonical death pathway

Introduction

Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer-related mortality worldwide [1]. Colon carcinogenesis is the result of a gradual transformation of colonic epithelial cells, which accumulates genetic as well as epigenetic changes that both increase their growth and alter their phenotypes [2]. Cucurbitacins are a class of highly oxidized tetracyclic triterpenoids and the various degrees of substitution and saturation allows cucurbitacins to have a variety of chemical compounds; however, they have several common characteristics like presenting a double bond between C-5—C-6, and many of them showing a double bond at C-1 (E and I) and/or C-23 (E, D and I). Cucurbitacin E represents C-25 acetoxy while cucurbitacins D and I have C-25 hydroxyl. Natural and semi-synthetic cucurbitacins have demonstrated promising anticancer activities ranging from anti-proliferation, cell cycle arrest to induction of apoptosis [3,4].

Some cucurbitacins have been shown to have cytotoxic effects [5,6]. The most critical mechanisms relating to the apoptotic effects of cucurbitacins are their ability to change transcriptional activities (nuclear factors or genes), and also their capability of activating or inhibiting pro- or anti-apoptotic proteins [7,8].

Studies have demonstrated that both classical and non-classical apoptosis pathways exist. Previous findings have indicated that under specific conditions, anti-apoptotic BCL2 family members can be cleaved and thereby converted into pro-apoptotic molecules directly facilitating cytochrome c release [9-11]. What is more, mitochondria-mediated apoptosis can be activated in the absence of BAX/BAK.

This situation is significant because in several tumors, resistance to chemotherapy is due to the downregulation of BAX and BAK [12] that reflect a substantial clinical challenge. As such, it is essential to identify novel apoptosis inducers that bypass BAX/BAK.

Some exterior factors were reported that forced cells a non-classical death pathway [13-16]. For instance, Mullauer, F. B., et al. reported that BAX/BAK double-deficient mouse embryonic fibroblasts displayed the release of cytochrome c, caspase activation, DNA fragmentation, and PARP cleavage upon betulinic acid treatment. This result designates that BetA does not induce a classical mitochondrial pathway to apoptosis [15].

It seems counter-intuitive, but several studies have shown that high levels of anti-apoptotic factors correlated with better prognosis in specific cancers. It has shown that a high level of expression of anti-apoptotic BCL-2 is associated with favorable results in some human cancers. Notably, the increase in anti-apoptotic BCL-2 does not necessarily lead to a decline in apoptotic sensitivity, and indeed the

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opposite can hold true. In contrast, a high level of expression of pro-apoptotic BAX can correlate with poor results. Studies demonstrated that high levels of BAX can be associated with decreased survival and increased risk of relapse in all kinds of cancers [17-22]. As we described above, findings challenge the dogma and suggest this viewpoint that anti/pro-apoptotic factors can be served in an unusual direction to inhibit cancer.

The mutant TP53 is in approximately 50% of human cancers. The modified TP53 loses its tumor-suppressive function and obtain new oncogenic activities whether through transcriptional effects on various genes or by protein-protein interactions. Thus, it turns into an active antithetical protein having its own "social network" of interacting proteins and transcriptional targets which endows it with a gain of function (GOF) activities. Tumor cells gain resistance to cell death and become chemoresistance by recruiting the mutant TP53 interacting with proteins such as caspase 3, P300, P73, VDR, etc. [23,24]. SW480 and HT-29 are primary colorectal adenocarcinoma cell-lines with mutant TP53 [25], from which can benefit binding caspase-3 and inhibits its activation.

Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome [26]. Autophagy is activated in response to multiple stresses during cancer progressions, such as nutrient starvation, the unfolded protein response (ER stress), and hypoxia; besides, it is observed upon treatment of cancers with a broad spectrum of cytotoxic and targeted chemotherapeutic agents [27].

The mammalian kinase target of rapamycin (mTOR) is a primary regulator of the autophagic process and is regulated by starvation, growth factors, and cellular stressors [28]. Upstream of mTOR the AKT/PEN pathway modulates mTOR activity. The interplay between the AKT/PEN/mTOR pathway and the autophagic process is complex, and disruption of the molecular effectors of the negative feedback loop of the AKT/PEN/mTOR pathway may unbalance the effects towards cell death with several outcomes [29].

In the extensive number of oncological researches, the isolation and purification of biologically active compounds from plants have been increased due to the discovery of potent antitumor drugs with high biodiversity and minimum side effects [30]. We, therefore, set out to compare the cytotoxic effects of the cucurbitacins E, D and I through measuring their IC₅₀ and also by evaluating the expression of some prominent genes in death (apoptotic and autophagic) and survival (Akt/mTOR) pathways to infer (if feasible) the cause of the differences in cytotoxicity effects of these three types of cucurbitacins. The candidate genes were *BCL-2*, *BAX*, *p53*, *Aif* and *caspase-3* for apoptosis pathway, and *LC3*, *Beclin* and *ATG5* for autophagy as well as *Akt*, *mTOR* and *PTEN* for survival in the signaling pathway.

Materials and Methods

Reagents

Fetal bovine serum (FBS) and RPMI 1640 were bought from Gibco (Denmark). RPMI 1640 supplemented with 2 g/l NaHCO₃, 30 mg/l L-glutamine, 100 U/ml penicillin and 10 µg/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA) in pH 7.2 was prepared and after sterilization by 0.2 µm filter kept at 4°C before use. Cucurbitacins D, E, and I were obtained from the stock of our previous purification study [30]. The methanolic extract of *E. elaterium* fruits was fractionated to petroleum ether, chloroform, and ethyl acetate fractions. The chloroform fraction was chosen for further purification with column chromatography. Finally, cucurbitacins D, E, and I were isolated by

column chromatography and identified by NMR spectroscopy. MTT and propidium iodide were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Cell culture and drug treatment

Human colorectal carcinoma cell lines, SW-480 and HT-29 were obtained from the Iranian Biological Resources Center's Cell Bank (Tehran, Iran). The cells were maintained as monolayers in RPMI medium containing 10% heat inactivated FBS and kept in a fully humidified atmosphere at 37°C with 5% CO₂ in the air. The cells (1 × 10⁶) were seeded for 12 h and then treated with various concentrations (0-60 µM) of cucurbitacins, which were dissolved in RPMI medium. After 24 h cells were detached by trypsinization, pooled by centrifugation, and washed twice with sterile PBS.

MTT assay

Survival evaluation of SW-480 cells was performed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells (2 × 10⁴ cells/well) were seeded into a 96-well plate (Nunclon, Denmark) in the absence and presence of different concentrations of the drug (0-60 µM). 10 µl of MTT reagent (5 mg/ml in H₂O) was added, and the plates were incubated at 37°C with 5% CO₂ in the air for four hours in the dark. The violet formazan crystals were dissolved entirely in 150 µl DMSO, and the absorbance measured at 570 nm using a microplate reader (Model Power Wave XS2, Bio Tek, USA). The percentage of surviving cells was calculated from the ratio of the absorbance between treated and untreated cells. The proliferation of the cells is presented as an average (±SD) of at least three independent experiments.

RNA purification, reverse transcription, and real-time RT PCR amplification

After 24 hours of drug treatment, total RNA was isolated from SW-480 cells using TRIzol (Invitrogen) as recommended by the manufacturer and cDNA synthesized using an M-MuLV reverse transcriptase kit (Vivantis, Malaysia) at 42°C for 60 min, followed by 85°C for 5 min. Duplicate PCR reactions were performed using the real-time PCR kit (Jena Bioscience, Germany) according to the manufacturer's instructions, using *GAPDH* as normalizers for genes. The cycling program was as a pre-incubation at 95°C for 5 min followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 secs and 72°C for 30 secs which was followed by a final extension step of 72°C, 5 min. Thermal amplification was carried out on a Rotor-Gene 6000 instrument (Corbett, Sydney, Australia). All reactions were run in triplicate, and the expression of the genes was analyzed based on the cycle threshold (Ct) and relative expression levels were determined as 2^{-ΔΔCt} (t). The sequences of primers are listed in Table 1.

Statistical analysis

All data were expressed as means ± SD. The student t-test was used to determine statistical significance at p<0.05. SPSS 22.0 and GraphPad Prism 7 software was used for the statistical analyses.

Results

Cytotoxic effect of Cucurbitacins D, E and I on SW-480 and HT-29 cells

The potential effect of cucurbitacins on the survival rate of colorectal cancer cells was analyzed with MTT assay. Cells in the absence of cucurbitacins D, E, I show more than 90% viability (e.g., 98%, 96% and 97% viability for the absence of cucurbitacins D, E and

Table 1: Nucleotide sequences of the primers used for real-time RT-PCR.

Gene	Forward primer	Reverse primer
<i>GAPDH</i>	5'-GACCCCTTCATTGACCTCAACTAC-3'	5'-TCGCTCCTGGAAGATGGTATGG-3'
<i>BAX</i>	5'-GCCCTTTTGCTTCAGGGTTTC-3'	5'-CATCCTCTGCAGCTCCATGT-3'
<i>LC3</i>	5'-GGACATCTACGAGCAGGAGAAGACGAG-3'	5'-TCAGAAGCCGAAGGTTTCTGGGAG-3'
<i>caspase-3</i>	5'-GCGGTTGTAGAAGATTTTCGTG-3'	5'-CTCACGGCTGGGATTTCAA-3'
<i>BCL-2</i>	5'-CAGGATAACGGAGGCTGGGATG-3'	5'-AGAAATCAAACAGAGGCCGCA-3'
<i>p53</i>	5'-CCCAAGCAATGGATGATTTGA-3'	5'-GGCATTCTGGGAGCTCATCT-3'
<i>AIF</i>	5'-GATTGCAACAGGAGGTACTCCAAGA-3'	5'-GATTTGACTTCCCGTAAATCTTCTC-3'
<i>ATG5</i>	5'-GACTTTTATTGTTGGGTTTC-3'	5'-TCAATCTGTTGGCTGTGG-3'
<i>Beclin 1</i>	5'-TGTCACCATCCAGGAAGTCA-3'	5'-CTGTTGGCATTCTGTGGA-3'
<i>mTOR</i>	5'-CTG GGA CTC AAA TGT GTG CAG TTC-3'	5'-GAA CAA TAG GGT GAA TGA TCC GGG-3'
<i>AKT</i>	5'-ATCCCCTCAACAACCTTCTCAGT-3'	5'-CTTCCGCTCAACTTCTCTTTC-3'
<i>PTEN</i>	5'-CATTGCCTGTGTGGTGATA-3'	5'-AGGTTTCCTCTGGTCTGGTA-3'

I respectively). Upon addition of cucurbitacins D, E and I, the cell viability was reduced to 78%, 61% and 62% at 10 μ M of cucurbitacins until it reaches 61%, 20% and 25% at 50 μ M, respectively. There was a significant decrease ($p < 0.01$) in the cell viability after cucurbitacins E and I exposure, especially at high concentrations ($\geq 80 \mu\text{g/ml}$). The behavior of cucurbitacins E and I are similar, and at approximately 20 μ M of cucurbitacins E and I cells reach to IC50; however, cucurbitacins D can only reduce the viability of the cells by 60 % (Figure 1).

Cytotoxic effect of Cucurbitacin-E on HT-29 cells

Cells in the absence of cucurbitacins-E showed more than 90% viability. Cell viability was reduced to 50% at 6 μ M, and it reached to 60%, at about 50 μ M. There was not a significant decrease in the cell viability at higher concentrations ($\geq 30 \mu\text{g/ml}$) (Figure 1).

Effect of Cucurbitacins on BCL2, BAX, p53, Caspase-3 and AIF mRNA expression in SW-480 cells

Upon addition of E and I cucurbitacins, p53 mRNA level was increased, but the effect of cucurbitacin D on p53 mRNA expression levels was significantly ($p < 0.001$) more than that of cucurbitacins E and D with the approximately 7-fold increase in mRNA level. The expression of AIF mRNA in SW-480 cells was increased after exposure to all types of cucurbitacins (Figure 2). The caspase-3 mRNA level of SW-480 cells was significantly increased ($p < 0.001$) by cucurbitacin treatments in a concentration-dependent manner. Interestingly, the level of BAX mRNA was significantly decreased in SW-480 cells. By contrast, the expression of BCL-2 mRNA was significantly increased (Figure 3).

Effect of Cucurbitacin-E on BCL2, BAX, p53, Caspase-3 and AIF mRNA expression in HT-29 cells

We evaluated just apoptotic gene expressions for only HT-29 cells treated with cucurbitacin-E to have a comparative apoptotic gene analysis in a different primary cell line. Upon addition of cucurbitacins E, p53 mRNA was downregulated. The expression of AIF mRNA was also decreased after exposure to cucurbitacin-E (Figure 4). The expression of caspase-3 was considerably increased ($p < 0.05$), while that of BAX significantly dropped. Conversely, BCL-2 mRNA was significantly increased (Figure 4).

Effect of Cucurbitacins on ATG5, LC3 and Beclin-1 mRNA expression in SW-480 cells

After the treatment of the SW-480 cells with cucurbitacins, the expression of LC3 and Beclin-1 mRNA was increased. Upon addition of cucurbitacin D and E, the expression of ATG5 mRNA increased, but

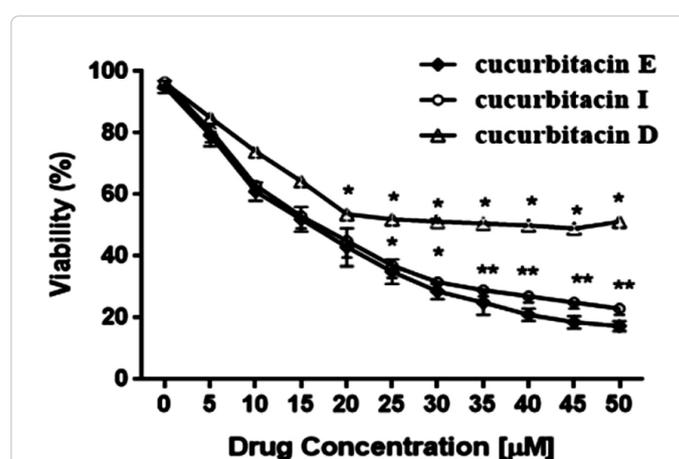


Figure 1: Effect of cucurbitacins on the viability of SW-480 cells analyzed by MTT assay after exposure to various concentrations of cucurbitacins. The viability of cells was significantly decreased in a dose-dependent manner. The cells at a density of 10^6 cells/ml were cultured with various concentrations of cucurbitacins. Data are presented as means \pm SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus control.

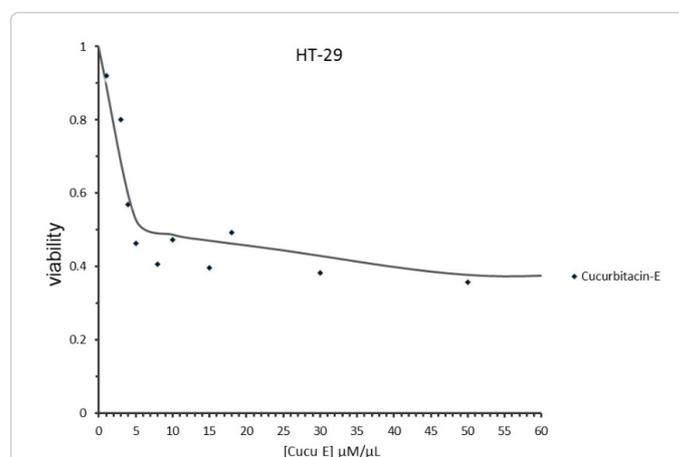
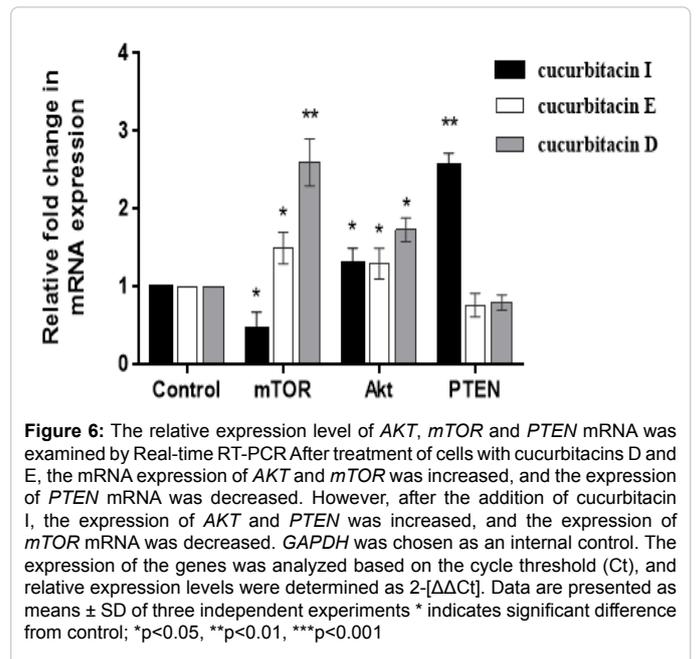
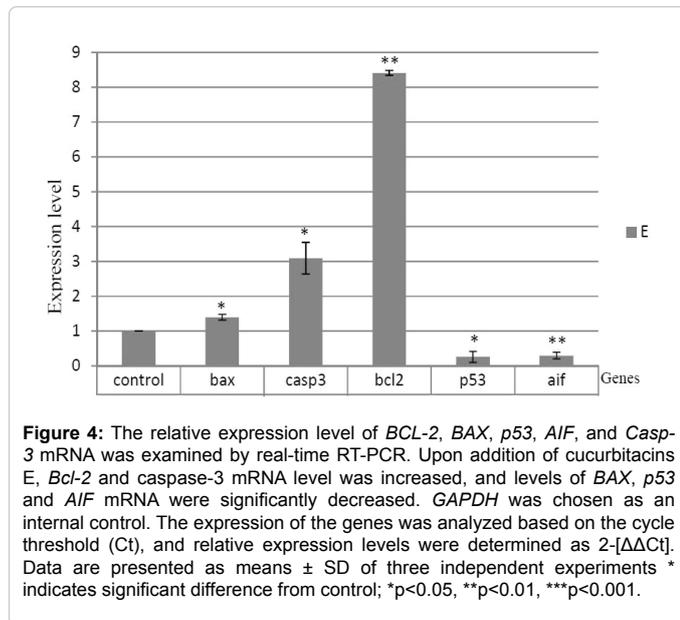
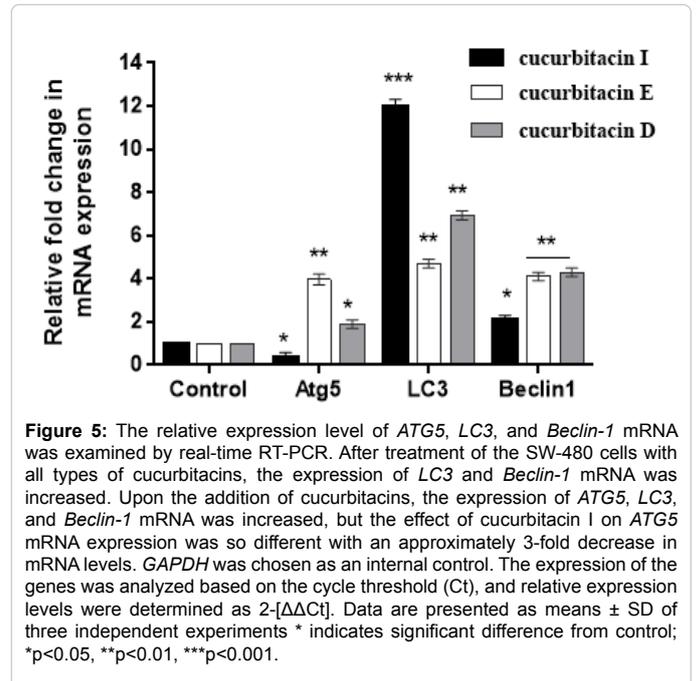
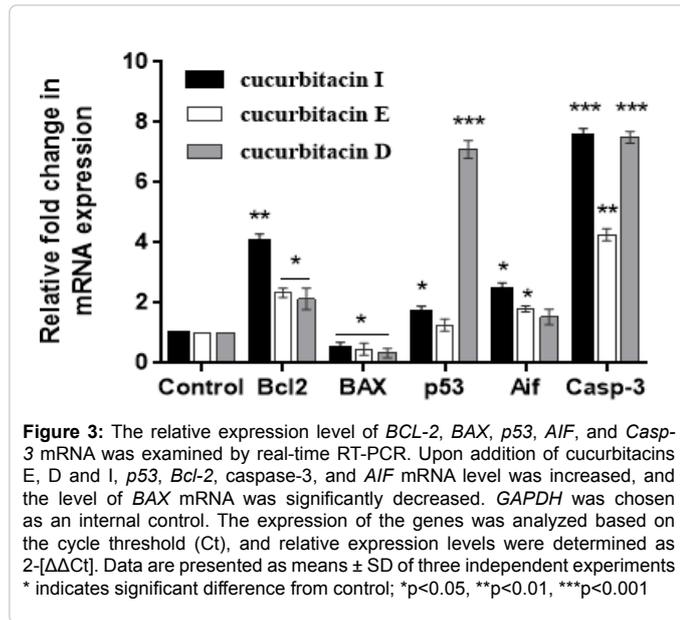


Figure 2: Effect of cucurbitacin-E on the viability of HT-29 cells analyzed by MTT assay after exposure to various concentrations. The viability of cells was significantly decreased in a dose-dependent manner. The cells at a density of 10^6 cells/ml were cultured with various concentrations of cucurbitacins. Data are presented as means \pm SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus control.



the effect of cucurbitacin I on *ATG5* mRNA expression was different with an approximately 3-fold decrease in mRNA levels (Figure 5).

Effect of Cucurbitacins on AKT, mTOR and PTEN mRNA expression in SW-480 cells

After treatment of cells with cucurbitacins D and E, the mRNA expression of *AKT* and *mTOR* was increased, and the expression of *PTEN* mRNA was decreased. However, the expression pattern of *AKT* and *PTEN* was different after the treatment of SW-480 cells with cucurbitacin I. Upon addition of cucurbitacin I, the expression of *AKT* and *PTEN* was increased and the expression of *mTOR* mRNA was decreased (Figure 6).

Discussion

MTT results demonstrated that the IC_{50} for cucurbitacins E, I and

D in SW-480 were 20, 20 and 40 μ M respectively. Cucurbitacins E and I had almost similar cytotoxicity effect as a function of increasing their doses and also were more fatal to SW-480 CRC cells than cucurbitacin D. Unlikely, with increasing the dosage of cucurbitacin D, lethality went only up to 50% compared to 70% (cucurbitacin E) and 65% (cucurbitacin I). One reason why cucurbitacin D was less potent, would be the increase in the expression of *AKT* (1.9-fold) and *mTOR* (2.6 fold), and fall in the expression of *PTEN* (0.8 fold) which are in favor of the survival pathway.

Wild-type TP53 plays a significant role in suppressing tumorigenesis by inducing genomic stability, cell cycle arrest, or apoptosis while

mutant TP53 resists to cell death and apoptosis through binding and interacting with various proteins like caspase-3 [23,24]. P53 is mutant in SW-480 and the expression level of P53 (7 fold) in the cucurbitacin D treated cells was much more than E (expression levels was similar to control) and I (1.6 fold) treated cells, so this could be another explanation why cucurbitacin-D was less potent. Thus, it implicates that although cucurbitacin D treated cells induced caspase-3 gene expression 5 fold compared to control, this treated cells became resistant to cucurbitacin D through suppressing of caspase-3 by mutant p53. To study the cytotoxicity effects of the cucurbitacins and the influence of p53 in a different scenario, we exposed another colorectal primary cancer cell line HT-29 to cucurbitacin E, which similarly has mutant p53. we noticed that although the expression levels of caspase-3 in HT-29 was lower than that of SW-480 under the influence of cucurbitacin E, it reached to IC50 in a lower dosage (6 μ M), and interestingly, evaluation of the gene expression of p53 indicated that it was nearly suppressed with the 0.25 of expression level compared to non-treated HT-29 cells. Thus, the HT-29 cell line might miss the function of mutant p53 as a survival factor. Assessing the AKT-mTOR survival pathway demonstrated that cucurbitacin-I almost suppressed the expression of mTOR (just under 0.5), and AKT was expressed nearly in a similar quantity of control (1.28), while PTEN was upregulated 2.5-fold which overall is against the survival pathway. In cucurbitacin E, there was not any considerable tendency in favor or against of survival pathway compared to control.

Apoptosis pathway examination in SW-480 showed an unusual gene expression model. It was with the characteristics of upregulating BCL-2 and downregulating BAX, while caspase-3 and AIF were upregulated. As we explained earlier, the overexpression of BCL-2 and downregulation or suppression of BAX can be against cancer cell survival (9-22). The result for HT-29 cell line treated with cucurbitacin-E is in agreement with this idea, since even though the BCL-2 expression status was higher (8 fold), the IC50 was at a lower dosage (about 6 μ M), and surprisingly the expression levels of caspase-3, on the other hand, was lower (3 fold) compared to the results of all three types of cucurbitacins in the SW-480 cell line. Our previous flow cytometry results demonstrated that purified cucurbitacins D, E and I induced apoptotic cell death in the human gastric cancer cell line (AGS). However, they showed a negligible effect on the BAX mRNA level [31].

The autophagy gene expressions showed that cucurbitacin E and D treated cells in SW-480 upregulated the expression levels of LC-3, Becline-1 and ATG5 which is in favor of autophagic cell death. On the other hand, ATG5 was suppressed in cucurbitacin-I treated cells, and therefore autophagic cell death seemed unlikely to happen. This might be one reason that although caspase-3 expression was the highest in treatment with cucurbitacin-I, and conversely cucurbitacin-E had the lowest expression levels of caspase-3 (just over 4), there were no considerable differences in their MTT results.

AIF is a mitochondrial protein, which can participate in caspase-independent apoptosis. AIF gene is a transcriptional target of p53 [32,33]. The effect of cucurbitacin-E on the HT-29 cell line caused the suppression of p53 expression, and thus the AIF gene had no expression. Conversely, the p53 gene was expressed in all treatments of SW-480, which was followed by the expression of AIF. These results illustrate that SW-480 capable of recruiting AIF to respond to caspase-independent apoptosis while HT-29 was not able to make it.

Conclusion

A better understanding of the mechanisms of BAX/BCL-2-

independent cell death is crucial because various tumor cell lines have been shown to resist classical mitochondrial death pathways, as they lacking BAX or p53, or harboring mutations of these proteins which fail to respond to chemotherapeutic drugs and death ligands. Agents that overcome drug resistance in this type of cancer are of particular interest in drug development and cancer therapy. What is more, these results and other findings challenge the viewpoint that a pro- or anti-apoptotic factor serves solely to inhibit or promote cancer, arguing instead that the factors in the apoptosis pathway have a dark side that can actually be served in their opposite direction. Thus, fundamental research on this unusual and specific network of interactions could be promising in clinical settings. Cucurbitacins seem to have the potential to understand more about unusual interactions of apoptotic factors in cellular pathways and could also be more investigated for BAX/BAK independent apoptosis.

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Declaration of interest

This work was a thesis subject confirmed by the University of Tehran. The author(s) declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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