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The cucurbitacins D, E, and I from *Ecballium elaterium* (L.) upregulate the *LC3* gene and induce cell-cycle arrest in human gastric cancer cell line AGS

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ABSTRACT

Objective(s): Cucurbitacins exhibit a range of anti-cancer functions. We investigated the effects of cucurbitacins D, E, and I purified from *Ecballium elaterium* (L.) A. Rich fruits on some apoptotic and autophagy genes in human gastric cancer cell line AGS.

Materials and Methods: Using quantitative reverse transcription PCR (qRT-PCR), the expression of *LC3*, *VEGF*, *BAX*, *caspase-3*, and *c-MYC* genes were quantified in AGS cells 24 hr after treatment with cucurbitacins D, E, and I at concentrations 0.3, 0.1 and 0.5 µg/ml, respectively. Cell cycle and death were analyzed by flowcytometry.

Results: Purified cucurbitacins induced sub-G1 cell-cycle arrest and cell death in AGS cells and upregulated *LC3* mRNA effectively, but showed a very low effect on *BAX*, *caspase-3*, and *c-MYC* mRNA levels. Also after treatment with cucurbitacin I at concentration 0.5 µg/ml, *VEGF* mRNA levels were increased about 4.4 times. Pairwise comparison of the effect of cucurbitacins D, E, and I on *LC3* mRNA expression showed that the cucurbitacin I effect is 1.3 and 1.1 times that of cucurbitacins E and D, respectively; cucurbitacin D effect is 1.2 times that of cucurbitacin E (*P-value* <0.05). *In silico* analysis showed that among autophagy genes, *LC3* has an important gastric cancer rank relation.

Conclusion: Cucurbitacins D, E, and I purified from *E. elaterium* fruits upregulate *LC3* and induce sub-G1 cell-cycle arrest and cell death in human gastric cancer cell line AGS. Cucurbitacin I effect on *LC3* mRNA expression is significantly more than that of cucurbitacins E and D.

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Introduction

Due to a wide variety of biologically effective chemicals in medicinal plants, there is a growing interest in their use as therapeutics (1). The *Ecballium elaterium* (L.) A. Rich is a wild medicinal herb from the Cucurbitaceae family, which produce cucurbitacins, a family of highly oxygenated tetracyclic triterpenes (2). The role of cucurbitacins in Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway inhibition, MAP kinase (MAPK) pathway regulation, and cytoskeleton disruption, suggests their excellent efficacy for cancer treatment and prevention (3, 4).

Recent studies have shown that many anticancer drugs induce both autophagy and apoptosis in various cancer cells. Autophagy is a dynamic multi-step phenomenon in which double-membrane autophagosomes enclose damaged cellular proteins, lipids, and organelles and subsequently deliver them to lysosomes for degradation (5-7). Under normal physiological conditions, autophagic activity is low. However, a range of stimuli can induce autophagy to protect cells from stress (8, 9). The role of autophagy in *tumorigenesis* is complicated, its role in tumor promotion and suppression, as well as its contribution to therapeutic resistance, has been reported (10-12).

There are several genes that contribute to autophagy and apoptosis. Among them, microtubule-associated protein light chain 3 (LC3) is the key factor in autophagosome formation (13, 14). Also, *c-MYC* potently induces different types of regulated cell death, including apoptosis (15), and autophagy (16). And *BAX* and *caspase-3* are among main regulators of apoptosis (17). In addition, it is reported that *VEGF* is one of the participant genes in autophagic cell death (18). Thus, studying *LC3*, *VEGF, BAX, caspase-3,* and *c-MYC* genes' contribution to autophagy could be a valuable goal for anticancer investigations.

Gastric cancer is considered as the fifth most common cancer in the world and the third leading cause of cancer mortality and morbidity (19). Our previous MTT assay using purified cucurbitacins D, E, and I from *E. elaterium* showed that these chemicals have cytotoxic effects on human stomach adenocarcinoma cell line AGS (20). The aim of this study was to

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investigate the effects of cucurbitacins D, E, and I purified from *E. elaterium* fruits on the expression of *BAX*, *caspase-3*, *LC3,* and *VEGF* and *c-MYC* genes in the AGS cell line.

Materials and Methods

Cell culture

In this research, human stomach adenocarcinoma cell line AGS was provided from Iranian Biological Resources Center's Cell Bank (Tehran, Iran). Cells were cultured in Ham's F-12 nutrient mix with L-glutamine and sodium bicarbonate (Cat. No. 10-FN1-500, G. Innovative Biotech Co, Iran) medium supplemented with 10% FBS (Cat. No. FB-st 500, Pasteur Institute of Iran) and were incubated at 37 °C in a water-saturated atmosphere of 5% CO2 and 95% air until confluence.

Cucurbitacins

We obtained cucurbitacins D, E and I from the stock of our previous purification study (20). 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 (20).

RNA extraction

AGS cells (5×10^5 cells/well) were seeded into 6-well plates and were grown to 80% confluency. 24 hr after treatment with cucurbitacins D, E, and I at concentrations 0.3, 0.1 and 0.5 µg/ml, respectively, cells were harvested and total RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Synthesis of cDNA

The cDNA was synthesized using Easy cDNA Synthesis Kit (Cat. No. A101161, pars tous biotechnology, Iran) according to the manufacturer's instructions.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Using quantitative polymerase chain reaction (q-PCR), expression of *LC3*, *VEGF, BAX*, *caspase-3*, and *c-MYC* genes was quantified in AGS cells 24 hr after treatment with cucurbitacins D, E, and I. All experiments were performed at least in duplicate using a 48 well StepOne Real-Time PCR Systems (Applied Biosystems, USA) and specific primers (Table 1) with the following conditions: 95 °C for 15 min, 40 amplification cycles consisting of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 60 sec. Melting curves were then determined with temperature ranging from 60 to 95 °C. *GAPDH* was chosen as an internal control. SYBR Green reagents were used for all real-time PCR reactions. The expression of the genes was analyzed based on the cycle threshold (Ct) and relative expression levels were determined as $2^{-[\Delta\Delta C(t)]}$.

Cell cycle and cell death analysis by flow cytometry

For cell cycle and cell death analysis, AGS cells (5×10^5cells/well) were seeded into 6-well plates and were grown to 80% confluency. Two wells were chosen as control and received no treatment. Cells were collected 24 hr after treatment with cucurbitacins D, E, and I at concentrations 0.3, 0.1, and 0.5 µg/ml, respectively and were washed with phosphate buffered saline (PBS) and trypsinized with 0.025% trypsin-EDTA to yield single cell suspension. Two groups of cells were used for flowcytometric analysis using a BD FACSCalibur flowcytometer (BD Biosciences, USA). For cell cycle analysis, cells were then fixed in ice-cold 70% ethanol and stained with 50 µg/ml propidium iodide (PI) solution containing 10 µg/ml RNaseA (Takara, Japan). For cell death analysis, cells were stained with AnnexinV/PI. Experiments were repeated at least twice and cell cycle profiles were analyzed using the FlowJo Software (ver. 7.6.1).

In silico analysis: Gene prioritization

After harvesting the results of the experimental part we performed an *in silico* analysis to show the validity of findings. The hypothesis of *LC3* contribution in gastric cancer cell autophagy was tested using a bioinformatics approach: gene prioritization. Using statistical methods, this approach scores and ranks a set of test genes through complex comparison with a set of training genes based on their average similarity in function and interactions. *LC3* is one of the main autophagy-associated genes. We obtained the list of these genes from HADb (http://autophagy.lu/clustering/), which is the first human autophagy-dedicated database. The autophagy genes list, containing *LC3*, was used as test genes set to find *LC3* priority among them in gastric cancer. For training gene set, we used the SNPs3D server (http://www.snps3d.org/) to get the list of candidate genes associated with gastric cancer. After that, prioritizing servers: ToppNet (https://toppgene.cchmc.org/network.jsp), which prioritize and rank candidate genes based on topological features in protein-protein interaction network (21), and Endeavour (https://endeavour.esat.kuleuven.be/), which works based on similarity of a candidate gene with a profile derived from genes already known to be involved in the disease (22), were used for finding the *LC3* rank among autophagy genes in relation to gastric cancer.

Statistical analysis

One way ANOVA followed by Tukey's *post-hoc* test was used to compare the expression level of desired

Table 1. Primers used in quantitative reverse transcription polymerase chain reaction (qRT-PCR) and amplicon sizes (bp: base pair)

Target	Forward primer	Reverse primer	Amplicon size (bp)
BAX	5'GCCCTTTTGCTTCAGGGTTTC3′	5'CATCCTCTGCAGCTCCATGT3′	168
caspase-3	5'GCGGTTGTAGAAGAGTTTCGTG3′	5'CTCACGGCCTGGGATTTCAA3′	101
c-MYC	5'CCCTCCACTCGGAAGGACTA3′	5'GCTGGTGCATTTTCGGTTGT3′	96
GAPDH	5′GACCCCTTCATTGACCTCAACTAC3′	5′TCGCTCCTGGAAGATGGTGATGG3′	138
LC3	5'GGACATCTACGAGCAGGAGAAAGACGAG3′	5'TCAGAAGCCGAAGGTTTCCTGGGAG3′	79
VEGF	5'TGTCTAATGCCCTGGAGCCT	5'GCTTGTCACATCTGCAAGTACG	175

genes. The *P-values* lower than 0.05 were considered as significant.

Results

Effect of cucurbitacins D, E, I on the AGS cell cycle and cell death

The accumulation of the sub-G1 population is considered as a biomarker of DNA damage. As shown in Figure 1, the sub-G1 accumulation of AGS cells treated with cucurbitacins D, E, and I, was increased in comparison to the untreated control cells. Moreover, treatment of AGS cells with cucurbitacins D, E, and I induced cell death (Figure 1).

Effect of the cucurbitacins on LC3, VEGF, BAX, caspase-3, and c-MYC mRNA expression

We evaluated the effects of cucurbitacins D, E, and I on the mRNA expression levels of *LC3*, *VEGF*, *BAX*, *caspase-3*, and *c-MYC* genes using qRT-PCR. As shown in Figure 2, based on *P-value* ≤ 0.05 *LC3* mRNA levels were increased about 23, 20, and 25 times after treatment with cucurbitacins D, E, and I at concentrations 0.3, 0.1, and 0.5 µg/ml, respectively, for 24 hr. Also, after treatment with cucurbitacin I at concentration 0.5 µg/ml, *VEGF* mRNA levels were increased about 4.4 times. However, *BAX*, *caspase-3*, and *c-MYC* mRNA levels were not considerably changed after treatment with cucurbitacins D, E, and I at aforementioned concentrations. Effect of cucurbitacin I treatment on *LC3* mRNA expression levels was significantly more than that of cucurbitacins E and D. Comparing the effect of cucurbitacin D on *LC3* mRNA expression with that of cucurbitacin E showed that cucurbitacin D's effect is significantly more than that of cucurbitacin E. It was LC3 among studied genes which showed significant induction under the cucurbitacin treatments.

In silico analysis: Gene prioritization

SNPs3D introduced 567 possible gastric cancer-related genes. Also, 232 genes were obtained from HADb as human autophagy-associated genes. Since ToppNet and Endeavour servers apply different approaches to compare training and test gene sets and prioritize candidate genes importance in relation to a disease, the ranks of candidate genes in the result sheets of these servers were different. ToppNet server identified 185 genes from the test genes set and located the *LC3* gene in rank 38 among them. It means

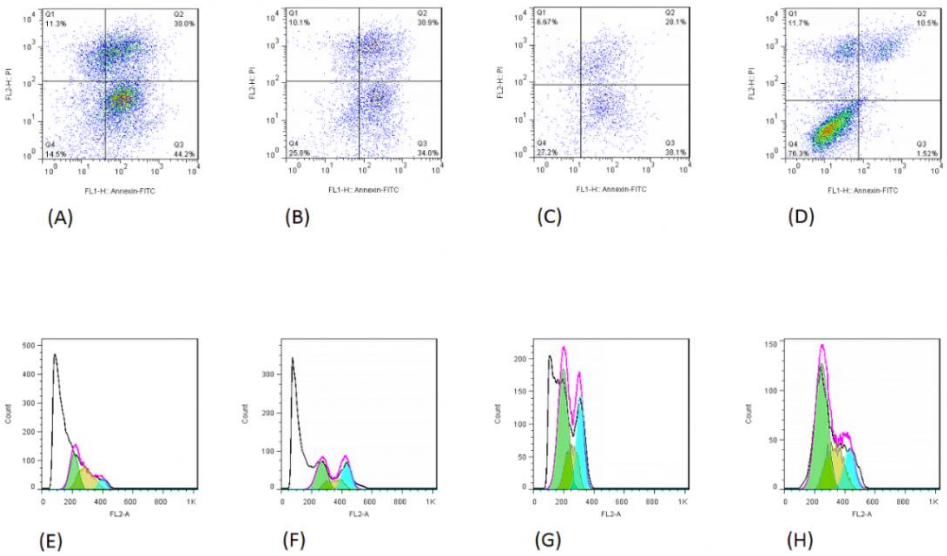


Figure 1. The death of AGS cells treated with cucurbitacins D (panel A), E (panel B), I (panel C), and untreated control cells (panel D). Panels A-D: Top left corner shows the necrotic cells, top right corner shows late apoptotic cells, bottom left corner shows healthy live cells, and bottom right corner shows early apoptotic cells. Panels E-H: Sub-G1 accumulation of AGS cells treated with cucurbitacins D (panel E), E (panel F), and I (panel G), in comparison to the untreated control cells (panel H)

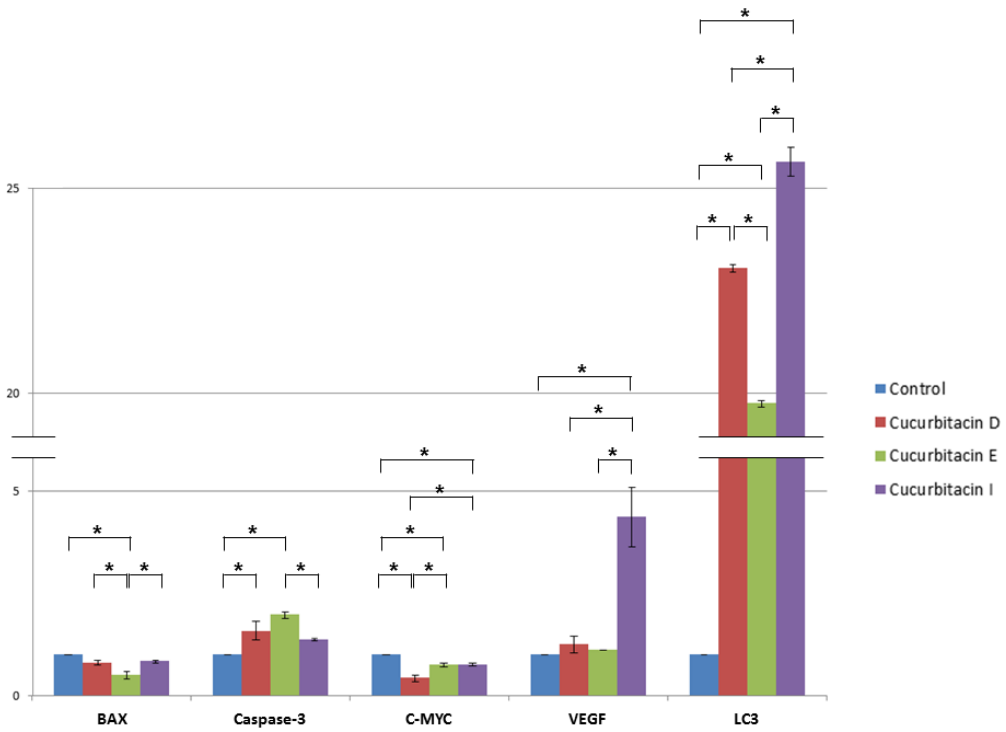


Figure 2. Effects of cucurbitacins D, E, and I on the mRNA expression levels of *LC3*, *VEGF*, *BAX*, *caspase-3*, and *c-MYC* genes (*P-value* ≤ 0.05). *GAPDH* was chosen as an internal control. The expression of the genes was analyzed based on the cycle threshold (Ct) and relative expression levels were determined as $2^{-[\Delta\Delta C(t)]}$

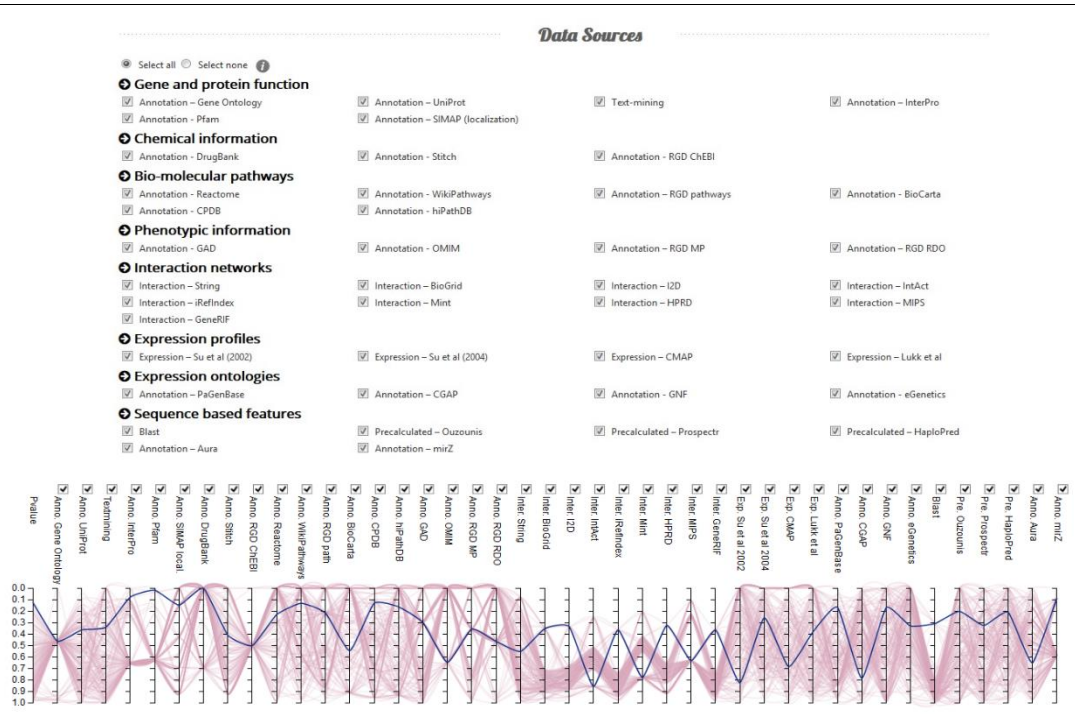
Training gene set	*P-value* diagram of test gene set (human autophagy-associated genes) and selected data source in Endeavour
Gastric cancer-associated genes reported by SNPs3D server	

Figure 3. Endeavour prioritization diagram of autophagy genes in relation to gastric cancer. The bold line in the diagram corresponds to LC3 gene's *P-value*. The other lines belong to other autophagy-associated genes.

that according to ToppNet, among 185 autophagy genes *LC3* is the 38th gene in ranking, which probably implicates it in gastric cancer. Also, the Endeavour server identified 182 test genes and compared them with 565 identified training genes. Endeavour ranked *LC3* as the 92nd gene among 182 analyzed autophagy genes that could be related to gastric cancer. Endeavour provides a diagram for test genes' *P-values* in numerous databases, however, the server stated that this is not a real statistical *P-value*. But, it could be used as a comparative item to show the place of a special gene among the candidates list. The *LC3* Endeavour *P-values* among autophagy genes are depicted in Figure 3.

Discussion

There is a 10-fold variation in gastric cancer incidence internationally, with high rates seen in many countries of Eastern Asia, Central and Eastern Europe, and Central and South America, and much lower rates reported from North America, and Africa (19). Indeed, stomach cancer is the third leading cause of cancer death worldwide (23).

Natural therapies based on medicinal plants are becoming increasingly important means of disease treatment. Indeed, more than 70 percent of new pharmaceuticals that have been approved since 1981 have directly or indirectly been derived from natural products (24). The *E. elaterium* is a wild toxic herb from the Cucurbitaceae family, which produce cucurbitacin molecules and has medicinal importance in traditional treatment prescriptions (25).

Cucurbitacins are highly oxidized tetracyclic triterpenoids, which have cytotoxic effects on various cancer cell lines. Cucurbitacins are claimed to be inhibitors of the JAK/STAT pathway, however, their cytotoxic effects may involve other mechanisms including the MAPK pathway, PARP cleavage, expression of active caspase-3, decreasing pSTAT3 and JAK3 levels, as well as decreasing various STAT3 downstream targets such as MCL-1, BCL-2, BCL-x_L, and CYCLIN D3 (26). However, the anticancer effects and underlying mechanisms of cucurbitacins in human gastric cancer are still elusive.

There are many instances of cell death similar to classical apoptosis that show no evidence of caspase activation. Likewise, increased amounts of phosphatidylserine in the plasma membrane exoplasmic leaflet as detected by Annexin V binding is not a definite marker of apoptosis. Similarly, extensive DNA fragmentation is frequently assumed a specific marker of apoptosis. However, there are increasing examples of apoptotic or apoptotic-like cell death proceeding without internucleosomal DNA degradation. In these cases, the intensity of cell labeling in TdT-mediated dUTP nick end labeling (TUNEL) assay will not be sufficient to positively identify apoptotic cells (27). It is suggested to restrict the term apoptosis to only the conventional cascade displaying all canonical hallmarks of apoptotic cell death such as (i) activation of caspases as a definite marker of cell death; (ii) high degree of compaction of chromatin; (iii) activation of endonucleases(s) causing internucleosomal DNA cleavage and extensive DNA fragmentation; (iv) appearance of characteristic cellular morphology with maintenance of organelles, (v) cell shrinkage, (vi) plasma membrane blebbing, and (vii) nuclear fragmentation followed by formation of apoptotic bodies (27).

In the present study, we show that cucurbitacins D, E, and I purified from *E. elaterium* inhibit AGS gastric cancer cell growth by inducing sub-G1 cell-cycle arrest and cell death. We evaluated the effects of cucurbitacins D, E, and I on the mRNA expression levels of *LC3*, *VEGF, BAX*, *caspase-3*, and *c-MYC* genes using qRT-PCR. As shown in Figure 2, *LC3* mRNA levels were significantly increased after treatment with cucurbitacins D, E, and I.

Increased expression of the *LC3* gene may be indicative of autophagic activity. Upon induction of autophagy, cytosolic LC3-I is cleaved and conjugated to phosphatidylethanolamine to form LC3-II, which associates with the phagophore (28). The use of autophagy markers such as LC3-II requires assays to estimate overall autophagic flux or flow, to permit an accurate interpretation of the results. Therefore, autophagic substrates should be followed dynamically over time to confirm that they have reached the lysosome, and, when appropriate, are degraded. Also, our *in silico* analysis revealed that among autophagy genes in humans, *LC3* is a promising candidate for studying and analyzing in gastric cancer cellular and molecular studies.

Cucurbitacins D, E, and I induced *LC3* mRNA expression, but *BAX*, *caspase-3*, and *c-MYC* mRNA levels were not considerably changed and effect of cucurbitacin I was significantly more than that of cucurbitacins E and D. Researchers (29) reported that cucurbitacin E induced autophagy at least partly via downregulation of mTORC1 signaling and upregulation of AMPK activity. Likewise, another study (30) reported that cucurbitacin B and I induced autophagy by the production of mitochondrial-derived reactive oxygen species (ROS) through a STAT3-independent process. Thus, another potential mechanism(s) may also contribute to cucurbitacin-induced autophagy. Research (31) showed that GBM cells treated with cucurbitacin I for 48 hr significantly upregulated Bax and cleaved caspase-3 (p17) but decreased antiapoptotic proteins such as Bcl-2 and Bcl-xL in a dose-dependent manner. Our findings that cucurbitacins show a very low effect on *BAX* and *caspase-3* mRNA levels in AGS cells, disagree with these results. However, consistent with these results, *LC3* mRNA was upregulated. This discrepancy may be explained by the fact that there is a complicated connection between autophagy and apoptosis. These two processes can mutually regulate and interconvert to determine

the fate of a cell, depending on the context. Deng *et al.* (32) showed that cucurbitacin I markedly inhibits gastric cancer cell growth by inducing G2/M phase cell cycle arrest and apoptosis at low nanomolar concentrations via a STAT3-independent mechanism. Our study showed that cucurbitacins D, E, and I inhibit AGS gastric cancer cell growth by inducing sub-G1 cell-cycle arrest at concentrations 0.58, 0.18, and 0.97 μM, respectively. It seems that cucurbitacins inhibit gastric cancer cell cycle progression through different mechanisms at different concentrations. Furthermore, comparing our results with the Deng *et al.* results suggests that cucurbitacins may preferentially induce autophagy at a particular concentration range but induce apoptosis at other concentration ranges. Common upstream signals may trigger both autophagy and apoptosis resulting in the mutual activation of autophagy and apoptosis. Under certain conditions, they may be also mutually exclusive. Recent research has implied that MAPK, particularly p38 MAPK and c-Jun NH2-terminal kinase (JNK), plays a key role in crosstalk between apoptosis and autophagy induced by genotoxic stress (31). Sun *et al.* reported that JNK activation is essential for up-regulation of *LC3* during ceramide-induced autophagy in human nasopharyngeal carcinoma cells. Furthermore, their findings suggest that c-Jun is essential for *LC3* transcription after ceramide treatment (33). Ishdorj *et al.* (18) demonstrated that in B Leukemic Cells, cucurbitacin I activates the JNK/c-Jun signaling pathway independent of apoptosis and cell cycle arrest, leading to increased *VEGF* expression. Comparing our results with Sun *et al.* and Ishdorj *et al.* results suggests that cucurbitacin I from *E. elaterium* may activate the JNK/c-Jun signaling pathway in human gastric cancer cell line AGS leading to increased *LC3* and *VEGF* expression.

It is reported that autophagosomal membranes serve as platforms for intracellular death-inducing signaling complex (iDISC) mediated caspase-8 activation and apoptosis. Upon the formation of iDISC, procaspase-8 is recruited to the phagophore by two mechanisms: (1) through Atg12-Atg5 complex interaction with FADD; and (2) LC3-II: p62 interaction. iDISC mediates apoptosis independent from death receptor signaling and requires LC3-positive autophagic membranes (34). Purified cucurbitacins D, E, and I induced cell death in AGS cells but showed the negligible effect on *BAX*, *caspase-3*, and *c-MYC* mRNA levels. One possibility is that cucurbitacins trigger non-canonical apoptosis. This could be of great importance as the induction of non-canonical apoptosis in cancer cells, could represent a novel and effective strategy for elimination of cancer cells. Another possibility is that cucurbitacins induce autophagy to limit caspase-dependent apoptosis. Yet another possibility is that cucurbitacins limit caspase-dependent apoptosis and at the same time induce non-canonical apoptosis. Since cucurbitacins D, E, and I showed no considerable effect on *BAX*, *caspase-3,* and *c-MYC* genes expression but increased *LC3* gene mRNA level in AGS cells, they may preferentially induce autophagy. However, these and other possibilities need further investigation. Also, it must be noted that the concentrations used in the current study were achieved by an *in vitro* study and the possibility of extrapolating these concentrations to clinical practice needs more investigation.

Conclusion

Treatment with cucurbitacins D, E, and I purified from *E. elaterium* fruit resulted in AGS cancer cell line death. Cucurbitacins increased expression of the *LC3* gene, but *BAX*, *caspase-3*, and *c-MYC* mRNA levels were not considerably changed. Notably, cucurbitacin I effect on expression of the *LC3* gene is significantly more than that of cucurbitacins E and D. The present study provides new insights into the molecular mechanisms underlying cucurbitacin-mediated cell death in gastric cancer.

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