Investigation of the Anticancer Mechanism of Isoorientin Isolated from *Eremurus Spectabilis* Leaves via Cell Cycle Pathways in HT-29 Human Colorectal Adenocarcinoma Cells

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ABSTRACT

Objective: Isoorientin (ISO) is a flavonoid compound extracted from plant species. The goal of this study was to determine the potential antiproliferative effects of ISO in HT-29 human colorectal adenocarcinoma cell line *in vitro*, specifically on cell viability, apoptosis, and cell cycle pathways.

Materials and Methods: The cytotoxic effect of ISO isolated from *E. spectabilis* was measured using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay in HT-29 cell lines. Total RNA was isolated using Tri-Reagent protocol. The effects of ISO on apoptosis-related gene were detected using real-time polymerase chain reaction (RT-PCR). The findings were analyzed using "Delta-Delta CT" ΔΔCT method and evaluated using a computer program. Volcano plot analysis was used for comparing groups and the data obtained were statistically analyzed using Student t test.

Results: According to XTT result analysis, the 50% inhibitory concentration (IC50) value of ISO was 125 μM at the 48th h in HT-29 cells. The RT-PCR analysis in HT-29 cells showed that *Cyclin D1* (*CCND1*), Cyclin-dependent kinase 6 (*CDK6*), *BAX, BCL-2*, Checkpoint kinase 1-2 *(CHEK1, CHEK2)* and Excision repair cross-complementing 1 (*ERCC1*) expressions were reduced in ISO-treated cells compared with those in the control group of cells. P53, P21, Caspase-3 *(CASP-3)*, Caspase-8 *(CASP-8)*, and Caspase-9 (*CASP-9*) gene expressions were increased Ataxia Telengiectasia and Rad-3 related (*ATR*) was activated in the ISO-treated group of cells compared with those in the control group of cells ($p<0.05$).

Conclusion: ISO affected the proliferation of colorectal cancer (CRC) cells via cell cycle pathways. It also altered apoptosis gene expression. These results demonstrated that ISO can be a therapeutic agent for CRC treatment; however, more studies are needed to investigate its mechanism of actions.

Keywords: Isoorientin, colorectal cancer, cell cycle pathway

Introduction

Cancer is a serious health problem worldwide, particularly with rising life expectancy and changing environmental conditions as well as lifestyle. Moreover, cancer causes genetic alterations following failure of homeostatic mechanisms [1]. A large number of changes in gene expression leading to unregulated control of cell proliferation may result in tumor formation. An unregulated cell growth can metastasize and cause significant morbidity and mortality [2]. Thus, altered gene expression, particularly of oncogenes and tumor suppressor genes, plays a key role in carcinogenesis [3].

Colon cancer originates either from the colon or rectum. Colorectal cancers (CRCs) are the most common cancers after lung and breast cancers in women and after lung and prostate cancers in men. CRC is the more prevalent form of lower gastrointestinal cancer, and about 75% of CRC cases are associated with environmental factors and nutrition. The remaining 25% cases have a hereditary CRC association among family members [4]. CRC, with more than a million of new cases per year, has few treatment options especially for advanced and metastatic patients.

The cell cycle is defined as experimentally determined intervals, wherein the cells are prepared and the genomes are copied equally between the two daughter cells. There are four consecutive phases in cell division: G1 phase, during which cells accumulate mass and metabolites necessary for DNA replication; S phase, when DNA replicates; G2, a gap phase in which accuracy of DNA replication controls; and M phase, in which DNA splicing is successfully completed [5].

In eukaryotes, cell cycle continuity depends on the sequential activation of the *cyclin-dependent kinase (CDK)* involved in cyclin-dependent activation [6]. The CDKs are cyclically linked to cell cycle continuity enzymes, and these complexes are activated. Cyclin D1 (CCND1) overexpression occurs as a result of gene amplification or disorder of growth factors in many types of cancer [7].

Apoptosis, which is also known as type I programmed cell death, shows nuclear fragmentation, chromatin condensation, and generation of apoptotic bodies. Apoptosis is mainly regulated by extrinsic and intrinsic pathways, which are known as death receptor and mitochondria-mediated pathways, respectively. Both of these pathways depend on the activation of caspases [8]. Activation of apoptotic pathways is a novel mechanism for preventing the development and progression of cancer [9].

While most dietary constituents can enhance cancer risk, evidence-based studies have reported that there is a negative correlation between the routine consumption of any nutrients (food and vegetables) and the development of certain cancer types. Nowadays, natural compounds have garnered great interest because of their potential effects on cancer cells; because they are thought to be naturally safe, chemopreventive, and chemotherapeutic; and because they decrease the mutagenicity of cells [10]. Phytochemicals, which are known as the nonnutritive part of a plant-based diet, exhibit substantial antimutagenic and anticarcinogenic features. The natural populations of *Eremurus*, which are classified into 50 different species, are found in different territories such as dry and stony grazed hillsides, particularly in Asia and the Middle East. *Eremurus spectabilis* is found in the east side of Turkey and is widely distributed. The leaves of *E. spectabilis* are accepted as a vegetable and consumed as a meal. Moreover, *E. spectabilis* has been traditionally used for treating some conditions such as hemorrhoids and diabetics and also used as antihypertensive and antidysuria agent [11]. One of the active components of *E. spectabilis* is isoorientin (ISO), which has antiproliferative and antinociceptive effects.

Isoorientin is a C-glycosyl flavone; it is called 3',4',5,7-tetrahydroxy-6-C-glucopyranosyl flavone and abbreviated as ISO. It can be extracted from different plant species, such as *Phyllostachys pubescens, Patrinia, Fagopyrum esculentum, Eremurus spectabilis,* and *Drosophyllum lusitanicum* (Figure 1) [12].

Isoorientin has been reported to have many pharmacological activities. Studies performed with mice have shown that ISO has antinociceptive and anti-inflammatory activities [13]. Additionally, ISO levels significantly increased in apoptotic neurotoxicity induced by 6-hydroxydopamine [14] and reduced the proliferation of HepG2 cells [15]. However, in HT-29 human colorectal adenocarcinoma cells, the effects of ISO-induced apoptosis and anticancer effects are not known.

There is increasing evidence that dietary plant extracts, particularly from fruits, grains, and herbs, play a protective role against colon cancer. The chemopreventive properties of these compounds include inhibition of cell proliferation, induction of apoptosis, and scavenging of free radicals [16].

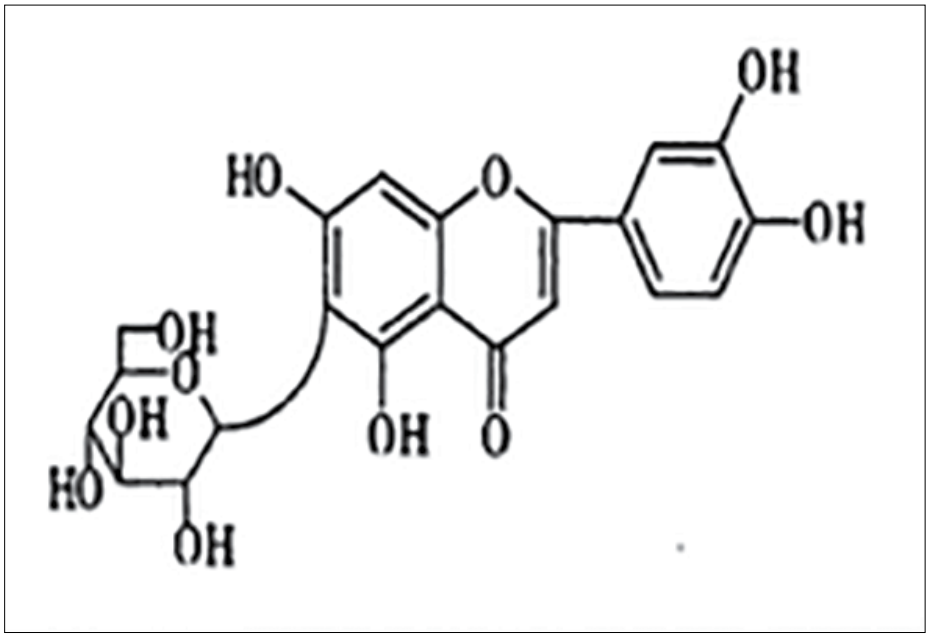

Figure 1. Chemical structure of isoorientin.

The main goal of this study was to investigate the potential antiproliferative properties of ISO including cell viability, apoptosis, and cell cycle-related gene expressions in HT-29 human colorectal adenocarcinoma cell line *in vitro*.

Materials and Methods

This article contains only *in vitro* cell culture study. Therefore, this study does not require the Ethics Committee approval and information on informed consent.

Reagents and Solutions

Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine (Gibco, USA), fetal bovine serum (FBS) (Gibco, USA), penicillin/streptomycin (Gibco, USA), phosphate buffered saline (Gibco, USA), Trizol reagent (Invitrogen, USA), cell proliferation 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) kit (Biological Industries, USA), and cDNA synthesis kit (Roche, Germany) were used. ISO as an authentic sample was isolated in pure form from *E. spectabilis*.

Cell Culture

In the current study, experiments were performed on human colorectal adenocarcinoma cell line HT-29 (ATCC, USA). HT-29 human colorectal adenocarcinoma cells were cultured in the RPMI-1640 medium containing 2 mM L-glutamine, 10% FBS, penicillin (20 units/mL), and streptomycin (20 µg/mL) at 37 °C with 5% CO_2 and 95% saturated humidity. ISO was dissolved in dimethyl sulfoxide (DMSO). The concentrations of ISO ranging from 7.81 µM to 1 mM were applied to HT-29 cells for 72 h, where time and dose were the dependent variables.

XTT Proliferation Assay

The cytotoxic effect of ISO in HT-29 cells and the concentration of ISO that decreased the cell viability by 50% (IC_{50}) were determined using trypan blue dye exclusion test and XTT assay based on the kit protocol. Briefly, HT-29 cells were cultured in 96-well plates at a concentration of 10^3 and incubated for 24 h (at 37 °C and 5% CO_2) without ISO. After 24 h of incubation, the cells were exposed with ISO concentrations as mentioned earlier and incubated for 24, 48, and 72 h. For the XTT assay, a reaction solution for one plate (96 wells) was prepared by adding 0.1 mL activation solution to 5 mL XTT reagent. Then, 50 µL of the reaction mixture was applied to the wells and the plate was incubated in an

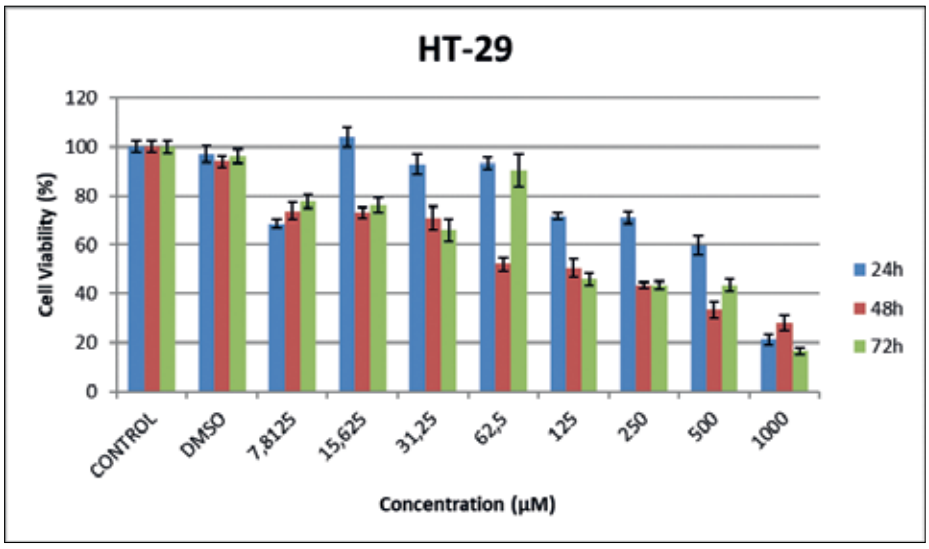

Figure 2. Effects of ISO on the viability of HT-29 cells. Isoorientin induced cells at different concentrations and time intervals and XTT assay results.

Table 1. Difference in the mRNA expression of genes related to beta-actin mRNA expression was determined using real-time polymerase chain reaction in HT-29 cells ($p<0.05$)

HT-29 cells	Gene Name	Fold Regulation
1	Beta-actin	1
2	CCND1	−12.6527*
3	p21	14.579*
4	p53	2.0702*
5	Casp-3	4.6072*
6	Casp-9	1.0916
7	Casp-8	2.4713*
8	Bax	−1.296
9	Bcl-2	−2.5902*
10	ATM	−4.4324*
11	ATR	1.394
12	CHECK1	−1.6646*
13	CHECK2	−1.7279*
14	ERCC1	−1.0277
15	URG-4	3.3917*
16	CDK6	−6.3755*

CCND1: cyclin D1; CDK6: cyclin-dependent kinase 6; CASP-3: caspase-3; CASP-8: caspase-8; CASP-9: caspase-9; CHEK1: checkpoint kinase 1; CHEK2: checkpoint kinase 2; ERCC1: excision repair cross-complementing 1; ATM: ataxia telengiectasia mutated; ATR: ataxia telengiectasia and rad-3 related.

incubator for 2–5 h. Formation of formazan crystals was spectrophotometrically measured at 450 nm (reference wavelength, 630 nm). The percentage of viable cells was quantified using the following formula:

$$\text{Viability (\%)} = \text{Absorbance}_{(\text{dose well})} / \text{Absorbance}_{(\text{control well})} \times 100$$

Isolation of RNA, cDNA Synthesis, and Real-time polymerase chain reaction (RT-PCR)

HT-29 cells were seeded on a six-well plate at a concentration of 3×10^5 cells/well. After a 24-h incubation, the IC_{50} dose of ISO was applied to each well excluding the control well. Total RNA was isolated according to the Trizol reagent protocol. cDNA synthesis was conducted using Transcriptor First Strand cDNA Synthesis kit (Roche, Germany) as per the manufacturer's instructions. *CCND1*, *CDK6*, *p21*, *p53*, *Caspase-3 (CASP-3)*, *Caspase-8 (CASP-8*, *Caspase-9 (CASP-9)*, *BCL-2*, Ataxia Telangiectasia Mutated (*ATM*), Ataxia Telangiectasia and Rad-3 Related (*ATR*) , *BAX*, Checkpoint kinase 1-2 (*CHEK1*, *CHEK2*) and Excision repair cross-complementing 1 (*ERCC1*) gene expression analyses were performed using the StepOnePlus quantitative RT-PCR system (Applied-Biosystems, USA) with respect to SYBR Green (Thermo-Scientific, USA) method. The RT-PCR analysis was performed using specific primers for the gene. To identify gene expressions, the results of the selected gene's expressions were normalized to the beta-actin housekeeping gene.

Statistical Analysis

The analyses of RT-PCR results were performed using computer program according to ΔΔCT. The groups were compared using Volcano plot analyses from RT^2 Profiles^{TM} PCR Array Data Analysis with Student t test. A p value of <0.05 value was considered significant.

Results

XTT Results

The cytotoxic effect of ISO was determined in HT-29 cells using XTT assay. Various concentrations (7.81–1000 µM) were used to determine the cytotoxic effect of ISO on HT-29 human colon cancer cells as shown in the Figure 2. In this study, IC_{50} value of ISO was detected to be 125 µM at the 48th h in HT-29 cells using XTT assay because no significant result was observed on 24- and 72-h incubations. As seen in the Figure 2, DMSO did not show any cytotoxic effect on HT-29 human colorectal adenocarcinoma cells.

RT-PCR Results

Control and IS-induced cells were observed in terms of gene expression analysis using RT-PCR after total RNA isolation; cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis kit.

Effects of ISO on Cell Cycle-associated Gene Proteins

RT-PCR analysis in the present study demonstrated that ISO-treated HT-29 cells significantly decreased *CCND1* and *CDK6* expressions with respect to the control group cells ($p<0.05$). Furthermore, both p21 and p53 expressions were significantly increased in ISO-treated HT-29 cells compared with those in control group cells ($p<0.05$; Table 1).

Effects of ISO on ATM and ATR Kinases

In our study, while the ATM- and Rad3-related (*ATR*) pathway was activated, ataxia-telangiectasia (*ATM*) expression was significantly decreased ($p<0.05$; Table 1).

Effects of ISO on Apoptosis-related Genes and Proteins

In our study, the PCR analysis demonstrated that the expression of antiapoptotic gene *Bcl-2* was significantly lowered in ISO-treated HT-29 cells compared with that in control group cells ($p<0.05$; Table 1).

Effects of ISO on Caspase-3, and -8 Activities

We determined that ISO has an effect on protease activities of *CASP-3* and *CASP-8*. There was a significant increase in *CASP-3* and *CASP-8* activation in ISO-treated HT-29 cells compared with that in control group cells ($p<0.05$; Table 1).

Effects of ISO on Excision repair cross-complementing 1 gene (ERCC1)

In the present study, ERCC1 gene expression was reduced in ISO-treated HT-29 cells compared with that in control group cells (Table 1).

Effects of ISO on CHK1 and CHK2

In our study, expressions of CHK1 and CHK2 were reduced in ISO-treated HT-29 cells compared with those in control group cells (Table 1).

Discussion

Natural compounds are novel therapeutic agents because of being safe and having a lower risk of mutagenicity in normal cells [10]. ISO is a common flavonoid found in human diet and has been isolated from many plant species [13]. Several studies have reported a pharmacologic effect of ISO on cancer cells [17].

Isoorientin exerts cytotoxic effects on HT-29 human colorectal adenocarcinoma cells in a both time- and dose-dependent manner. This conclusion is based on XTT assay result and it was detected that IC_{50} value of ISO was 125 µM at the 48th h on HT-29 human colorectal adenocarcinoma cells. In this study, we demonstrated for the first time the effects of ISO causing decreased cell viability and proliferation of HT-29 human colorectal adenocarcinoma cells. ISO also showed similar effects on cell viability and proliferation in different cancer cells such as MCF-7, the human breast adenocarcinoma cells [18], and HepG2, the human hepatocellular carcinoma cells [13]. Various studies have demonstrated that apoptotic and cell cycle arrest mechanisms are important targets for cancer therapeutic strategy [9, 13]. These studies determined the molecular mechanism of ISO and its therapeutic activity on HT-29 human colorectal adenocarcinoma cells. In addition, the effects of ISO on cell cycle control genes and apoptosis were studied.

Recently, apoptosis has garnered a lot of interest in oncogenic research. The lifetime of healthy and cancerous cells in alive systems is mainly affected by the rate of apoptosis. Apoptosis is a different cell death pathway that diverges from necrotic cell death and is assumed to be an optimal way of cell destruction. Thus, apoptosis which is the main target in cancer treatment can be modulated by the chemopreventive agents [19]. Treatment with such agents can lead to the arrest of the cell cycle, thereby causing cessation of cancerous cell growth and proliferation, and may also affect the malign transition. It was claimed that tumor growth is related with normal control mechanisms of cell cycle and caused by unprogrammed cells [20]. In fact, apoptosis involves both physiologic and pathologic process and it functions as a crucial mechanism for tissue homeostasis.

In the present study, we detected the underlying molecular mechanisms of effects of ISO on HT-29 human colorectal adenocarcinoma cells by evaluating the mRNA expression change of genes related to programmed cell death and cell cycle arrest. Yuan et al. [13] showed that in HepG2 cancer cells, ISO stimulates mitochondria-mediated apoptosis in human hepatoblastoma cancer cells and induces apoptosis with the dysfunction of mitochondria and inhibits PI3K/Akt signaling pathway.

Cell cycle transitions are mediated by cyclins and CDK regulatory proteins in the cell. The *CCND1* protein is a major regulator in the cell cycle's G1-S checkpoint, and it has an important role in cell cycle arrest, tumor suppressor inhibition, or oncogene activation because it is related to CDK4 and *CDK6* [21]. The folding of *CCND1/CDK6* or *CCND1/CDK4* complexes causes inhibition of pRB pathway and activation of transcriptional E2F [22]. Oncogenic functions and roles of *CCND1* in numerous cancer types have been determined. Studies on different cancer cell lines have shown that overexpression or amplification of *CCND1* cause tumor cell growth [23]. RT-PCR demonstrated that *CCND1* and *CDK6* expressions were significantly reduced in HT-29 human colorectal adenocarcinoma cells exposed to ISO compared with those in the control group cells. Many studies on different cancers have indicated that the overexpression or amplification of *CCND1* cause tumor cell growth.

The tumor suppressor protein and transcription factor p53 play a key role in the response of normal mammalian cells to DNA damage and other stresses [24, 25]. Two prominent functions of p53 to date have been identified: implementation of growth arrest and execution of apoptotic cell death [25]. The p53 protein shows its effects on apoptosis by inducing transcription of apoptosis-related genes and binding apoptosis-related target genes. In our study, the IC_{50} dose of ISO significantly increased in mRNA expression of p53 in HT-29 human colorectal adenocarcinoma cells. Furthermore, mRNA expression of p21 was increased in ISO-treated colorectal adenocarcinoma cells compared with that in the control group cells. With respect to apoptosis- and DNA repair-related genes, there was no significant expression change in ISO-treated cells. These results indicated that ISO has potential impacts on cell cycle control in human colorectal adenocarcinoma cells by downregulating *CCND1* and *CDK6*. The effect of ISO on cell cycle of human colorectal adenocarcinoma cells has been shown for the first time in our study.

We studied activated *CASP-3* for exploring the mechanism of ISO-induced apoptosis in HT-29 cells. *CASP-3* is one of the most important mediators of caspases involved in apoptosis, which is found in the cytosol as a zymogen under normal circumstances. *CASP-3* is either exactly or relatively responsible for the proteolytic cleavage of some substrates such as poly (ADP-ribose) polymerase, called as the death substrate, during the activation of apoptosis [8]. The apoptotic pathways are divided into two main pathways. *CASP-3* activation can affect both intrinsic and extrinsic pathways of apoptosis [8]. In addition, mitochondria can cause caspase-independent apoptosis by releasing endonuclease G, death-modulating flavoprotein, inhibitor of apoptosis protein, and Omi protein [26]. Therefore, ISO-induced apoptosis is caspase dependent, and ISO significantly increases expression levels of *CASP-3* and *CASP-8* in colorectal adenocarcinoma cells compared with those in the control group cells.

Bax is a type of protein that shares homology with Bcl-2. Bax is similar to Bcl-2 in several highly conserved regions. The Bcl-2 proteins play a role in the regulation of apoptosis, causing promotion or suppression of cell death. Cell death is promoted by the overexpression of Bax. Bax forms homodimers that can cause heterodimerization with other Bcl-2-related proteins. Bcl-2 genes were first identified in a translocation point (14:18) of human B follicular lymphoma. Bcl-2 overexpression induces cell survival in spite of the wide range of apoptosis-inducing factors such as chemotherapeutic agents and radiation [27]. In our study, ISO decreased the expression of Bcl-2 in ISO-induced HT-29 cells in accordance with the controls. This result suggest that ISO can induce apoptosis by disrupting the cell cycle.

ATM and *ATR* kinases, which are members of the phosphatidylinositol-3-kinase-like kinase family, are the most upstream DNA damage-related kinases [26]. Many proteins are phosphorylated at the Ser/Thr-Glu sites and at additional sites in an *ATM*- or *ATR*-dependent manner in response to DNA damage. Both *ATM* and *ATR* are activated at the activation of *CHK1* and *CHK2* protein kinases when the second wave of phosphorylation occurs [28]. In our study, ISO affected *ATM*, *ATR*, *CHK1*, and *CHK2* expressions. While the ATR pathway was actiavted, *ATM*, *CHK1*, and *CHK2* expressions were decreased in HT-29 human colorectal adenocarcinoma cell line. It suggested that ISO exerts effects on cell cycle control.

ERCC1 is a member of nuclear excision repair genes, and it encodes a protein that works with other members of repairing complex to provide genomic integrity through the repair of constructional aberrations and chemical nucleotide differences [29]. The DNA damage repair function of *ERCC1* is considered to affect the tumor behavior. The role of *ERCC1* in cancer regarding

cancer genesis, disease progression, and clinical management is becoming increasingly explicit. *ERCC1* expression level is predictive of cell survival. This can be secondary to the lowered accumulation of genomic differences due to the repair of DNA damage [30]. In the present study, ISO decreased the expression of *ERCC1* gene. Thus, low *ERCC1* expression means poor survival of cells. Hence, ISO shortened the life span of HT-29 adenocarcinoma cells.

The present study has some limitations. Financial limitations and lack of equipment prevented the evaluation of cell viability and protein expression analyses, such as Western blotting. The lack of financial support also prevented researchers from confirming the apoptotic effect of ISO using further assessments, such as terminal deoxynucleotidyl transferase dUTP nick end-labeling assay.

In conclusion, numerous researches exploring new anticancer agents and biomarker molecules considered beneficial for cancer treatment should be focused specifically on the molecular–cellular mechanisms such as inducing programmed cell death, DNA damage, and cell cycle arrest. According to our results, ISO inhibits the viability and proliferation of colorectal adenocarcinoma cells affecting cell cycle arrest mechanisms. We explained for the first time the molecular mechanisms of action of ISO in HT-29 human colorectal adenocarcinoma cells. Thus, ISO is a potential agent for treatment of human colorectal adenocarcinoma. More *in vitro* and *in vivo* studies should be conducted to determine the optimum safe dose and effects of ISO.

Ethics Committee Approval: N/A

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

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Conflict of Interest: Authors have no conflicts of interest to declare.

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