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Investigation of the molecular effects of isatin on HepG2 and AML12 cell lines

İsatinin HepG2 ve AML12 hücre hatları üzerindeki moleküler etkilerinin araştırılması

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Abstract

Isatin is an indole-derived organic compound. It is a natural component of the *Couroupita guianensis* plant. It is also the metabolic derivative of the human body hormone adrenaline. Studies have shown the anti-tumoral effects of isatin derivatives. In this study, the cytotoxic effects of isatin on HepG2, a hepatocellular cancer cell line was investigated. Additionally, its cytotoxic and protective-proliferative effects on AML12, a healthy liver cell line was investigated. This evaluation was conducted using MTT, fluorescent staining, wound healing, and real-time polymerase chain reaction analyses. The IC_{50} values for 48 hours of isatin application were calculated as 186.23 µM for HepG2 and 7.05 mM for AML12. The analysis of wound healing and fluorescent staining at varying doses of HepG2 application revealed suppression of proliferation and triggered apoptosis in HepG2 cells. In contrast, AML12 cells exhibited promoted proliferation under similar conditions. Moreover, the observed upregulation of oxidative stress genes CuZn/Mn-SOD and mitochondrial apoptotic pathway genes Bax, cleaveled-Cas3, APAF1, and p53 in HepG2 cells contrasted with their decreased expression in AML12 cell lines. These results suggest the potential of natural isatin as a promising anti-cancer agent for liver cancer cell lines and as a protective supplement for healthy liver cells.

Keywords: AML12, Anti-cancer, Cytotoxic, HepG2, Isatin

Öz

İsatin, indol türevi olan organik bir bileşiktir. Couroupita guianensis bitkisinin doğal bir bileşenidir. İnsan vücudu hormonu olan adrenalinin de metabolik türevidir. Yapılan çalışmalarda isatin türevlerinin anti-tümöral etkileri gösterilmiştir. Bu çalışmada isatinin hepatoselüler kanser hücre hattı olan HepG2 üzerindeki sitotoksik etkileri ve sağlıklı karaciğer hücre hattı olan AML12 üzerindeki sitotoksik, koruyucu-proliferatif etkileri MTT, floresan boyama, yara iyileşmesi ve gerçek zamanlı polimeraz zincir reaksiyonu analizleri ile araştırıldı. 48 saatlik isatin uygulaması için IC_{50} değerleri HepG2 için 186.23 µM, AML12 için 7.05 mM olarak hesaplandı. Her iki hücre hattı için HepG2 uygulamasının IC_{50} dozuna bağlı olarak, yara iyileşmesi ve floresan boyama analizleri, HepG2 hücrelerinde proliferasyonun baskılandığını ve apoptozun tetiklendiğini, ancak AML12 hücrelerinde proliferasyonun teşvik edildiğini göstermiştir. Ek olarak, oksidatif stres genleri CuZn/Mn-SOD ve mitokondriyal apoptotik yol genleri Bax, kesilmiş-Cas3, APAF1 ve p53'ün ekspresyon seviyeleri HepG2'de artmış ancak AML12 hücre hatlarında azalmıştır. Bu çalışmalar sonucunda isatinin doğal formunun karaciğer kanseri hücre dizileri için iyi bir anti-kanser ajanı ve sağlıklı karaciğer hücreleri için koruyucu bir takviye olabileceği sonucuna varılmıştır.

***Anahtar kelimeler**: AML12, Anti-kanser, Sitotoksik, HepG2, İsatin*

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1. Introduction

According to 2020 data, liver cancer is the sixth most common and third fatal cancer case in the world (Sung et al., 2021). However, it is the second type of cancer with the shortest life expectancy after diagnosis (Ferlay et al., 2021). Although HBV and HCV are the most important risk factors, excessive alcohol consumption, metabolic syndrome, type 2 diabetes, obesity, and non-alcoholic fatty liver disease are among the leading causes of liver cancer (McGlynn et al., 2021; Chuang et al., 2009). In a study on the frequency of liver cancer incidence and death in 2020, according to the order of development in the world countries, it has been reported that 31.600 deaths occurred in 33.100 cases in underdeveloped countries, 95.900 deaths in 100.000 cases in middle-developed countries, 524.300 deaths in 548.900 cases in developed countries, and 178.100 deaths in 223.300 cases in highly developed countries (Rumgay et al., 2022). In line with these data, it is seen that the percentage of deaths due to liver cancer is 95.47% in underdeveloped countries, 95.9% in middle-developed countries, 95.51% in developed countries, and 79.76% in highly developed countries. Although death rates are similar in underdeveloped, middle-income, and developed countries, it is remarkable that this rate decreases by 20% in highly developed countries. After the diagnosis of liver cancer cases, generally two different treatment options are followed. One of them aimed to complete recovery from illness, and the other aimed at prolonging life. The curative treatment methods can be listed as orthotopic liver transplantation, surgical intervention, and ablation. Treatment methods aimed at prolonging the life span are slowing tumor progression or relieving symptoms by administering trans arterial chemoembolization (TACE), trans arterial radio embolization (TARE), stereotactic body radiation therapy (SBRT), systematic chemotherapy, and/or radiotherapy (Marrero et al., 2018).

Undoubtedly, it is much easier to reach these treatment methods in highly developed countries, both in terms of cost and necessary hospital facilities, than in less developed countries. Because of these situations, scientists are intensively striving to discover effective and accessible anti-cancer agents. Especially recently, scientists have been intensively researching the effects of many flavonoids, nanoparticles, hormones, peptides, organic compounds, and natural medicinal plants on cancer to contribute to traditional treatment methods. One of these organic compounds is isatin.

Isatin is an indole-derived organic compound, and its molecular formula is $C_8H_5NO_2$. *Couroupita guianensis* plant is known to contain compounds that can be used in the synthesis or extraction of isatin (Bergman, 1998; Silva et al., 2001). It is also the metabolic derivative of the human body hormone adrenaline (Chiyanzu, 2003). With its use in many areas as an antitumoral, antiviral, anti-HIV, and antituberculosis agent (Sriram, 2005; Mallamo, 2006; Jiang et al., 2006; Abdoul-Fadel & Bin-Jubair, 2010), there have been limited studies on its candidate as an anti-hepatocellular carcinoma agent in recent years. However, in all of these studies on hepatocellular cancer types, the effects of isatinin derivatives such as trimethoxyphenyl (Cao et al., 2016), piperidine, phenyl acetadimide (Eldeeb et al., 2022), and diethynitrosamine (DENA)/2-acetylaminofluorene (2-AAF) (Tawfik et al., 2022) were investigated. Investigating the anti-cancer effects of isatin on the HepG2 hepatocellular cancer cell line due to its natural availability and affordability is quite promising. By focusing on a compound that's easily obtained and cost-effective, this study aims to offer valuable guidance, especially to countries with limited income and healthcare resources. The potential of isatin could contribute to reducing death rates or extending life expectancy in these regions, presenting a hopeful avenue for cancer treatment in less economically developed areas. By investigating the cytotoxic or proliferative effects of isatin on the healthy liver cell line AML12, this research sets itself apart from others. Understanding how isatin impacts healthy liver cells is crucial for assessing its safety and potential side effects. This comprehensive evaluation helps ensure that while targeting cancer cells, isatin doesn't harm healthy cells, which is a crucial aspect in developing safe and effective treatments.

2. Material and method

2.1. Cell culture

Human hepatocellular carcinoma (HepG2) (HB-8065, ATCC) and human healthy liver cell line (AML12) (CRL-9589, ATCC) were maintained in “Dulbecco's Modified Eagle's Medium/ Nutrient F-12 Raw”, 10% fetal bovine serum (SIGMA-ALDRICH), 2 mM L-Glutamine (Thermo-Fisher) and 100 IU/ml penicillin-streptomycin (Thermo-Fisher). Isatin (SIGMA-ALDRICH) was gifted from Trakya University Vocational School of Health Services. Cells were cultured in a humidified incubator at 37°C and 5% carbon dioxide.

2.2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) analyzes

AML12 and HepG2 cell lines were separately seeded in 180 µl volumes with approximately 5×10^3 cells in each well to 96-well spectrophotometric plates (NEST). 1 M stock solution was prepared by dissolving 7.35 gr of isatin in 50 mL of 0.2% ethanol solution. Cells were treated with 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 µM concentrations of isatin for 48-h. After treatments, 20 µl of MTT (5 mg/ mL) solution applied to each well and plates were incubated at 37°C for 3 hours in 95% humidity and 5% CO_2. At the end of the incubation, all the liquid phase on the cells was removed and 180 µl dimethyl sulphoxide (DMSO) was applied for 20 min into each well. The viability of the cells was calculated by reading the absorbance value at 570 nm wavelength in the spectrophotometer device (Thermo Scientific Multiskan GO) (Abel & Barid, 2018).

In MTT analyzes, cells without substance were considered 100% viable. The percentage of cell viability was calculated using the following formula:

Cell viability = (absorbance value of the isatin applied wells / absorbance value of the control wells) × 100 (1)

2.3. Cell fluorescent staining analyzes

AML12 and HepG2 cells were separately seeded in 4 wells of 6-well plates (NEST) with 5×10^4 cells per well. No substance applied to two wells, and they were used as an AML12 and HepG2 controls. 186.23 µM isatin was applied to other two wells to both cell lines for 48 h. At the end of 48 h, each cell line was stained with Annexin V/PI (Elabscience) and microscope images were taken. For the Annexin V/ PI application, at the end of time, the medium on the cells was removed and washed with 1× phosphate buffer saline (PBS). In 2 ml, 1× Annexin V binding buffer, 5 µl of Annexin V-FITC and 5 µl PI were mixed and applied to each well for 20 min at room temperature in the dark. Images were taken on a 5× objective using FITC channel in a Fluorescent (Zeiss Observer Z1) microscope.

2.4. Wound healing assay

AML12 and HepG2 cell lines were separately seeded 2 wells into 6-well plate, with 5×10^4 cells each well. After 24 h to cells adhered to the plate, they were scratched by a 10 µl pipette tip. To remove cell debris from the scratched area each well was washed with 1× PBS. Then application groups were treated with 186.23 µM isatin and culture medium was used for the control group. The scratchered closure was followed by taking microscope images with 5× objective at the end 24 h (Jonkman et al., 2014).

2.5. Total RNA isolation, cDNA synthesis and RT- PCR analyzes

Total RNA was extracted from cells using Column Pure RNA Miniprep Kit (ABM, USA) and OneScript Plus cDNA Synthesis Kit (ABM) was used for cDNA synthesis according to the manufacturer's protocols. RT-qPCR was performed using BlasTaq 2× qPCR MasterMix (ABM). RT-qPCR analyzes applied in the QuantStudio 6 Flex (Applied Biosystems) RT-PCR device. The primer sequences of the genes and RT-qPCR conditions are shown in Table 1 (Hossain et al., 2022).

Table 1. Primer sequences and PCR conditions of the gene regions analyzed by Real Time PCR.

Gene codes and genbank ID	Primer base sequences	Real time PCR conditions
Gapdh **NM_002046**	F: CAATGCCTCCTGCACCACCA R: GATGTTCTGGAGAGCCCCGC	Hold stage: 1 Cycle 50°C 2 minute 95°C 10 minute
APAF1 **NM_001160**	F: GTCACCATACATGGAATGGCA R: CTGATCCAACCGTGTGCAAA	
Akt **NM_005163**	F: TCCCCCTCAGATGATCTCTCCA R: CGGAAAGGTTAAGCGTCGAAAA	

Table 1. Continued

Gene codes and genbank ID	Primer base sequences	Real time PCR conditions
Bax	F: CCCGAGAGGTCTTTTTCCGAG	PCR stage: 40 cycle
NM_138761	R: CCAGCCCATGATGGTTCTGAT	95°C 15 second
Cleaveled_Cas3	F: GGAAGCGAATCAATGGACTCTGG	60°C 1 minute
NM_004346	R: GCATCGACATCTGTACCAGACC	
CD133	F: ACACTGAAAGTTACATCCACAGAA	Melt curve stage: 1 cycle
NM_006017.2	R: GGGTGTATCCAAAACCCGGA	95°C 15 second
CuZn-SOD	F: TCACTGTGGCTGTACCAAGGTG	60°C 1 minute
NM_000454.4	R: CCAGGAAGTAAAAGCATTCCAGC	95°C 15 second
ErbB2	F: CCTCTGACGTCCATCATCTC	
NM_001005862.2	R: ATCTTCTGCTGCCGTCGCTT	
PI3K	F: TTGTCTGTCACACTTCTGTAGTT	
NM_006218.2	R: AACAGTTCCCATTGGATTCAACA	
GSR	F: TATGTGAGCCGCCTGAATGCCA	
NM_000637.5	R: CACTGACCTCTATTGTGGGCTTG	
p53	F: GAGGTTGGCTCTGACTGTACC	
NM_001126118	R: TCCGTCCCAGTAGATTACCAC	
VEGF	F: GCTACTGCCATCCAATCGAG	
NM_001033756	R: TGGTGATGTTGGACTCCTCA	

2.6. Statistical analyzes

For exact IC_{50} doses for isatin application, MTT analyses were re-evaluated with SPSS Regression Probit (IBM, SPSS Statistics 22) analyses (Noufal et al., 2023). Gene expression changes for 3 repetitive doses were calculated with the $2^{-\Delta\Delta CT}$ formula. The relative gene expression levels were normalized to glyceraldeyhde 3-phosphate dehydrogenase (GAPDH) gene. The results were statistically performed with "Paired-Samples T Test" (IBM, SPSS Statistics 22). $P \leq 0.05$ values were considered statistically significant.

3. Results and discussion

The highest dose (100 µM) of isatin application to both AML12 and HepG2 cell lines for 48 hours, 83.31% viability in the AML12 and 53.42% in the HepG2 cells were detected from MTT assay (Figure 1).

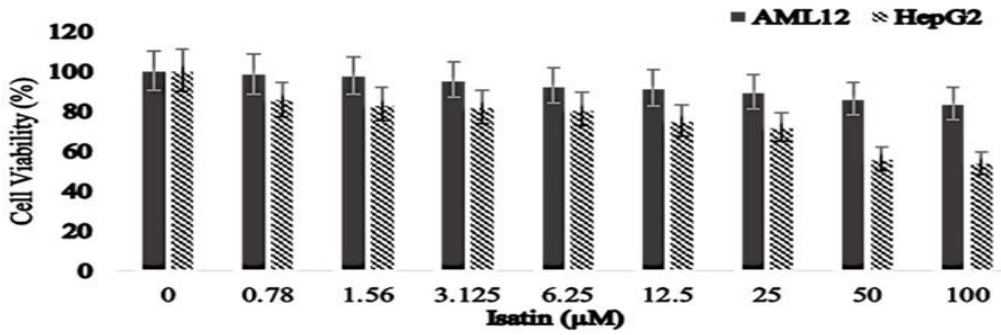

Figure 1. Comparison to cell viability of AML12 and HepG2 cells due to 0-100 µM isatin application.

From MTT analyzes IC_{50} doses could not obtained for both cell lines, so these data were re-evaluated with SPSS Regression Probit analyzes. The IC_{50} value for the AML12 cell line was calculated as 7.05 mM and 186.23 µM for the HepG2 cell line (Figure 2).

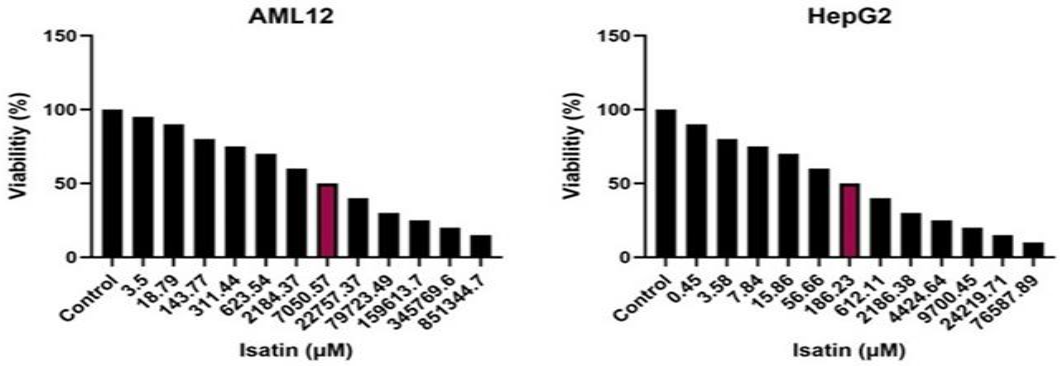

Figure 2. Re-evaluation of isatin-dependent IC values for AML12 and HepG2 cell lines by SPSS Regression Probit analyses (Red column refers to IC_{50} values of each cell line).

The IC_{50} value for AML12 is 37.86 times higher than for HepG2, indicating that isatin may be a good anticancer agent for liver cancer cells. In addition, as a result of the MTT study, even at a low dose of 0.78 µM, isatin suppressed proliferation in the HepG2 cell line by 14.53%, whereas the suppression of cell division was determined to be only 1.91% due to the application of the same dose to the AML12 cell line.

The suppressive effects of isatin on HepG2 cells proliferation was investigated by applying the HepG2 IC_{50} dose to both AML12 and HepG2 cells through wound healing assays. At the end of 48-h, scratched area closured in isatin applied AML12 cells similar to control AML12 and HepG2 cells. This situation proves that isatin has a proliferative effect on healthy liver cells. Contrary to this finding, at the isatin applied HepG2 cell line; it was observed that scratched area expanded. (Figure 3).

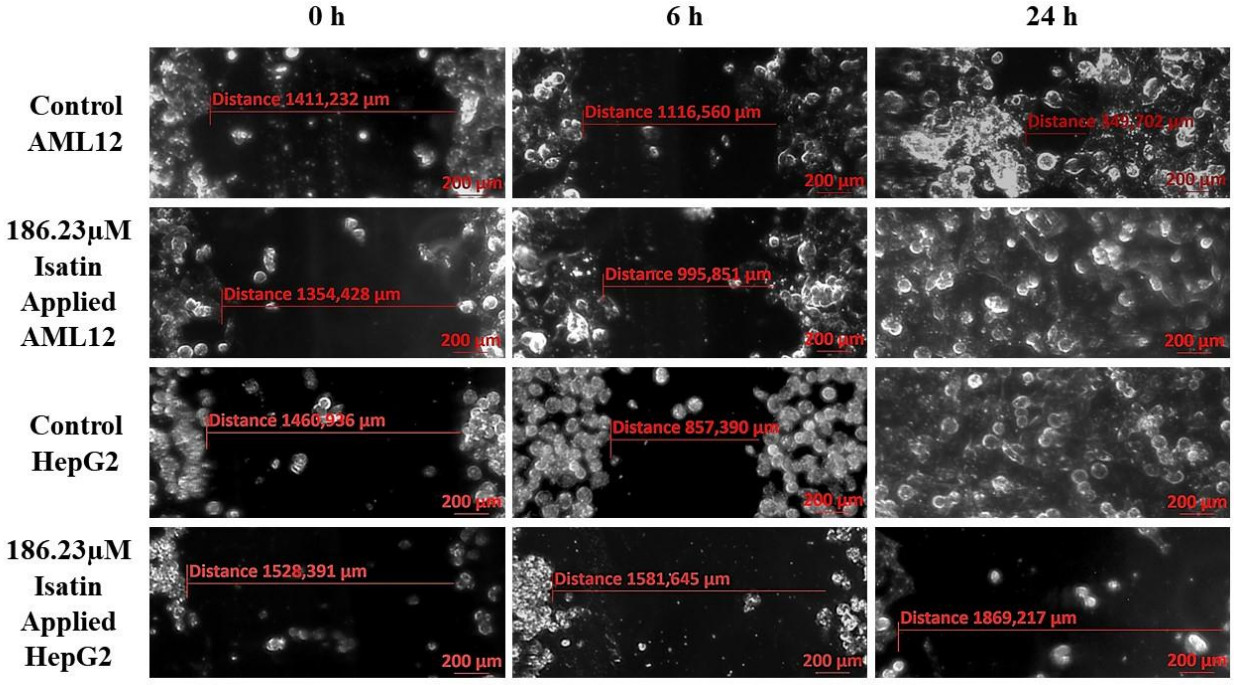

Figure 3. 0- 24 h changes in the scratched areas in control and isatin applied AML12 and HepG2 cells.

At the study, both early and late apoptosis were demonstrated in isatin applied HepG2 cells via Annexin V/PI staining methods, but there has no apoptotic sign in AML12 cell line by the same application. (Figure 4).

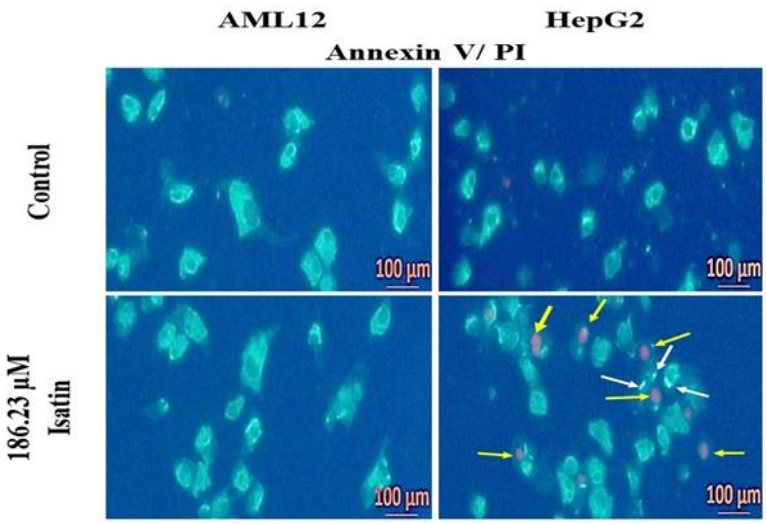

Figure 4. White arrows indicate early apoptotic cells and yellow arrow indicates late apoptotic cells.

In RT-PCR analyzes; compared to the control, some increases were observed at the expression levels of CuZn-SOD, Mn-SOD, Bax, APAF1, cleaveled-Cas3, p53 genes in the isatin applied HepG2 cells. However, PI3K, Akt, ErbB2, CD133 expression levels decreased. Contrary to HepG2 cell line, Akt, VEGF, CD133 expression levels increased, while Mn-SOD, GSR, Bax, APAF1, cleaveled-Cas3 levels were decreased in AML12 cell line (Table 2).

Table 2. In AML12 and HepG2 cell lines, mRNA expression levels of oxidative stress, proliferative genes, oncogenes and mitochondrial apoptotic pathway genes (n=3±SD) (relative change to the control group for each AML12 and HepG2 cell lines; Paired-Samples T Test, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<001$).

Genes	AML12 relative fold changes	P values	HepG2 relative fold changes	P values
CuZn-SOD	1.04±0.09	0.305	2^{**}±0.09	0.006
Mn-SOD	0.5±0.05	0.058	4.2^{**}±0.27	0.006
GSR	0.15^{**}±0.06	0.006	1.06±0.2	0.917
PI3K	0.77^{**}±0.03	0.004	0.64^{*}±0.09	0.035
Akt	1.69^{*}±0.23	0.023	0.47^{*}±0.05	0.031
VEGF	2.64^{*}±0.38	0.02	1.27^{*}±0.17	0.049
ErbB2	1.22±0.11	0.306	0.71±0.14	0.069
CD133	1.58^{*}±0.14	0.015	0.49±0.1	0.132
Bax	0.5±0.1	0.076	1.7^{*}±0.2	0.036
APAF1	0.64±0.17	0.081	1.78^{**}±0.04	0.002
Cleaveled_Cas3	0.52^{*}±0.1	0.016	2.26^{**}±0.33	0.006
p53	0.87±0.08	0.499	1.99^{**}±0.16	0.008

In the isatin applied AML12 cell line, there was a general increase in the expression levels of the angiogenesis pathway genes PI3K/Akt/VEGF and the oncogene CD133, whereas the oxidative stress genes Mn-SOD and GSR, and the mitochondrial apoptosis pathway genes Bax/APAF1/cleaveled-Cas3 significant decreases were observed. Contrary to AML12 cell line, in the isatin applied HepG2 cell line, significant increase in the expression levels of CuZn-SOD and Mn-SOD oxidative stress, Bax/APAF1/cleaveled-Cas3 mitochondrial apoptosis pathway and p53 tumor suppressor genes, and a general decrease in PI3K/Akt/VEGF angiogenesis pathway were detected. In addition, significant decreases were detected in the expression levels of ErbB2 and CD133 oncogenes. Nagarsenkar et al. (2016) showed that intracellular oxidative stress increased due to the application of increasing concentrations of triazole linked 3-benzylidene, which is an isatin derivative, to the DU-145 prostate cancer cell line, resulting in collapse in the mitochondrial membrane structure (Nagarsenkar et al., 2016). This situation is similar to the findings of the present study that, increase in the expression levels of oxidative stress genes and the activation of mitochondrial apoptosis pathway genes in the HepG2 cell line treated with isatin. In addition, the decrease in the expression levels of both oxidative stress genes and mitochondrial apoptotic genes in the AML12 cell line indicates that, the 186.23 µM dose of isatin has not any

cytotoxic effect on the healthy liver cell line. On the contrary, it reduced oxidative stress. Jaksch et al. showed that, cell proliferation stopped in the G0/G1 phase due to the decrease in CD133 gene expression level in the epithelial cancer cell line CaCo2 cell line (Jaksch et al., 2008). In this study, the CD133 gene expression level increased in AML12 cell line due to isatin application, contrary to significantly decreased in HepG2 cell line; it shows that cell division is promoted in isatin applied AML12 cells, whereas HepG2 cells cannot pass the G0/G1 phase and went to apoptosis.

4. Conclusions

In many highly developed countries, chemotherapy drugs are used in the fight against HCC. However, it is more difficult to procure these drugs in undeveloped countries due to financial inadequacies, and accordingly, mortality rates are much higher in undeveloped countries than in developed countries. In this study, the anticancer effects of isatin, which is both cheaper and easier to obtain than other chemotherapeutic agents, in HepG2 cells and protective effects in AML12 cells were investigated. In line with all these data, it is clear that isatin not only has anti-proliferative effects, but also promotes proliferation in healthy liver cell lines, exerts a protective effect by reducing oxidative stress, while it has a selective effect on cancerous cell lines thanks to its apoptotic effects. However, more studies are needed to fully clarify the therapeutic and protective effects of isatin on humans.

Authors contribution

The design, execution, and interpretation of experimental studies, as well as the writing of the article, were done by the corresponding author.

Declaration of ethical code

The author declares that this study do not require ethical committee approval or any legal permission.

Conflicts of interest

The author declare no competing interests.

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