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Original scientific article

CONTRARY TO KNOWN SILIBININ IS NOT AN ANTICANCER AGENT AND LIVER PROTECTIVE SUPPLEMENT

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Silibinin is a flavinoid that is the main component of the milk thistle plant and is widely used around the world, thought to have liver protective and anticancer effects. In this study, contrary to what is generally known, it was aimed to show that silibinin is neither a healthy liver protective supplement nor an anticancer agent for hepatocellular cancer cells. For this purpose, molecular effects of silibinin were investigated in both HepG2 (hepatocellular cancer) and AML-12 (healthy liver) cells. The cytotoxic concentrations of silibinin investigated by MTT analysis. Cell proliferation by wound healing assays, intracellular apoptosis and cell division events demonstrated by fluorescent microscopy imaging via Annexin V/ PI and Hoechst 34580 staining. Gene expression level changes were investigated by real-time polymerase chain reaction. In the study, IC50 values were calculated as 739.9 mM for AML-12 and 1.35 M for HepG2 in 24 h, 529.23 µM for AML-12 and 15.51 mM for HepG2 in 48-h of silibinin administration. From these data, IC50 value of 48 h of silibinin administration for the AML-12 cell line resulted a decrease in AML-12 cell quantity, whereas an increase in HepG2 cells. Fluorescent staining studies show that, there was an intense proliferation in the HepG2 cells, whereas an important apoptotic effect induced in the AML-12 cell line as a result to 529.23 µM silibinin application. Also, all proliferation and oncogene expression levels were incrased in HepG2 cells, but expression levels of Akt, ErbB2 were decreased in AML-12 cells, whereas APEX1 DNA repair, CuZn-SOD oxidative stress gene expression levels were increased. As a result, application of 529.23 µM silibinin was found to be cytotoxic for AML-12 cells as well as proliferative effect on HepG2.

Keywords: AML-12, hepatocellular carcinoma, HepG2, silibinin, MTT.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common and the third most death rate cancer type among all cancer types in the world (SUNG *et al.*, 2021). Although the criteria for the

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diagnosis of HCC are currently under development, information about the course of the disease is not clear yet (HILEY *et al.*, 2014). After the diagnosis of HCC, many treatment methods are applied. These treatment methods are divided into two groups; as curative and life-prolonging by the American Association for the Study of Liver Diseases. Curative treatment methods classified as an orthotopic liver transplantation, surgical intervention and ablation. Life-prolonging treatment methods are; slowing the development of the tumor or relieving symptoms by applying trans arterial chemoembolization (TACE), trans arterial radio embolization (TARE), stereotactic body radiation therapy (SBRT), systematic chemotherapy and/or radiotherapy (MARRERO *et al.*, 2018). Because HCC rapidly angio invades and metastasizes even in the very early stages, it is often late for curative treatment modalities such as surgery or liver transplantation (FENOGLIO *et al.*, 2013). For this reason, the average life expectancy of patients with HCC is 6-20 months after diagnosis. According to studies in USA, 2-year and 5-year survival after diagnosis is less than 50% and 10% respectively for total HCC cases (MCGLYNN *et al.*, 2015). Because of high mortality ratio of HCC, today many researchers are competing with each other to develop alternative treatment methods. The effects of many flavinoids, nanoparticles, hormones, peptides and their derivatives on cancer are intensively investigated in order to increase the efficiency of conventional treatments. Silibinin is one of these flavinoids.

Silibinin is an antioxidant flavonoid, and its chemical properties were first discovered in 1959 (BIEDERMANN *et al.*, 2014). Silibinin is the main component of "silymarin", which is the active ingredient of milk thistle (GAZAK *et al.*, 2007; KROLL *et al.*, 2007). It has been claimed that silibinin has antitumor effects in many cancer types such as prostate, colon, skin, bladder, lung (CHU *et al.*, 2004; MALLIKARJUNA *et al.*, 2004; SANGEETHA *et al.*, 2009; DEEP *et al.*, 2012). VARGHESE *et al*. (2005) and LAH *et al*. (2007) stated that silibinin has anti-proliferative effects on HCC cells. In addition, GU *et al*. (2015) investigated the synergistic anticancer effects of gefitinib, sorafenib and silibinin on Hep3B, HepG2, Huh7, PLC/PRF5, SNU387, SNU398, SNU449, SNU475 and SNU761 human HCC cell lines. However, none of these studies investigated the effects of silibinin in healthy cell lines. BIJAK *et al*. (2017) reported that silibinin up to 100 μM was safe and did not show genotoxicity and cytotoxicity on blood platelets. In a study about teratogenicity and reproductive toxicity of silymarin in female mice; 50- 200 mg/ kg/ day slymarin caused teratogenic effects and fetal weights were lower than the control group. Some anomalies were observed in the face, vertebrae and skull in these mice (GHOLAMI *et al.*, 2016). To interpret these observations, the molecular effects of silibinin on AML-12 healthy liver cells and HepG2 hepatocellular carcinoma cells were investigated in this study.

MATERIALS AND METHODS

Culturing cells

HepG2 (HB-8065, ATCC) and healthy liver AML-12 (CRL-9589, ATCC) cell lines were used to elucidate the molecular effects of silibinin (Cayman Chemical, USA). "Dulbecco's Modified Eagle's Medium/ Nutrient F-12 Raw", 10% fetal bovine serum (SIGMA-ALDRICH, USA), 2 mM L-Glutamine (Thermo-Fisher, USA) were used as medium. 100 IU/ ml penicillin-streptomycin (Thermo-Fisher) antibiotic was used to prevent bacterial contamination. Cells were seeded into 75 cm^3 flasks (NEST, China) with 10 ml of medium and incubated in an incubator at 37°C containing 5% carbon dioxide (Panasonic MCO-18ACL-PA, Japan) for culturing.

Analysis of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

AML-12 and HepG2 cell lines were seeded in 3 replicates in sterile 96-well spectrophotometric plates (NEST). Cells were dispensed into wells with 180 µl volumes of medium, approximately 5x 10^3 cells in each well. Before starting the silibinin application for 24 h waited for cells to adhere to the inner bottom surfaces of the wells. In order to determine the optimal conditions, 12.5, 25, 50, 100 µM silibinin was applied to HepG2 and AML-12 cell lines in 96-well plates for 24 and 48 h. Since silibinin dissolves well in 1% ethanol, cells in other wells were treated with 1% ethanol, and these cells were used as controls for viability comparisons. To calculate the effective inhibition concentration 50 (IC50) from these treatments, 20 µl of MTT (Biomatik; A3338-5GM, USA) solution (0.5 mg of MTT powder dissolved in 1 mL DNase- RNase free water) was applied to all wells. Cells treated with MTT solution were incubated for 3 h in an incubator (Panasonic Incusafe, Japan) with 95% humidity and 5% CO_2 at 37°C. At the end of the incubation, all the liquid phase on the cells was removed. 180 µl of dimethyl sulfoxide (DMSO) was applied to all wells for 20 minutes. Cell viabilities were calculated by reading the absorbance values at 570 nm wavelength in the spectrophotometer device (Thermo Scientific Multiskan GO, USA). Cells treated with 1% ethanol were considered 100% viable.

Fluorescent Microscopy Analysis

AML-12 and HepG2 cells were seeded separately in 4 wells of 6-well plates (NEST) with 5x 10^4 cells per well. After the cells adhered to the plate, 1% ethanol was applied to two wells for both cell lines and these cells were used as a control. To examine its molecular effects, 529.23 µM silibinin was applied to the other two wells. After 48 h, one group stained with Annexin V/ PI (Elabscience, USA) and the other group with Hoechst 34580 (Santa Cruz, USA) fluorescent dyes.

For the Annexin V/ PI application, at the end of 48 h, 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. At the end of 20 min mixture on the each well removed and washed with 1× PBS. 1× PBS added to each well for protecting to cells from drying and images were taken on a 5× objective using DAPI and FITC channels in a Fluorescent (Zeiss Observer Z1) microscope.

25 µg of Hoechst 34580 dye dissolved in 50 mL of 1× PBS to show degraded nuclei structure and proliferation under fluorescent microscope. The medium on the cells was removed and 2 mL of Hoechst 34580 solution was applied to each well for 20 minutes at room temperature in the dark. At the end of the application period, the cells were visualized under a fluorescent microscope using a 20× objective via the DAPI channel.

Wound healing assays

AML-12 cells and HepG2 cells were seeded into 2 wells of a 6-well plate at 5× 10^4 cells per well. After waiting for 24 h for the cells to adhere to the plate, the cells scraped by a 10 µl pipette tip (Interlab, Turkey). In order to completely clean the cells from the wound area, the medium was removed from the wells and washed with 1× PBS. Then 2 mL of medium was

added to the cells in the wells to be used as control, 2 mL of medium containing 500 µM silibinin was added onto the cells for silibinin application. Imaging was performed under the microscope using a 5× objective immediately for 0 hours. Scraped area changes in the cells followed by taking microscope images at the end of 3, 6, 24 and 48 h.

Total RNA isolation, cDNA synthesis and real time PCR analysis

25 cm³ 6 flasks of AML-12 and HepG2 cells were inoculated as 3 repeat of control and 3 repeat of silibinin administrations. After 24 h from 529.23 µM of silibinin application, total RNAs were isolated from each flask separately according to the protocol of the Column Pure RNA Miniprep kit (ABM Good, Canada). The amounts of total RNAs obtained from each flask were determined spectrophotometrically (OPTIZEN NanoQ, Republic of Korea). After the total RNA amounts were equalized with dH_2O, the cDNA synthesis reaction was created following the OneScript Plus cDNA Sytnhesis kit (ABM Good) protocol. The cDNA synthesis process was performed in thermal cycler (Applied Biosystems, USA) following the protocol of Step 1: 10 minutes at 25°C, Step 2: 120 minutes at 37°C, Step 3: 5 minutes at 85°C. Obtained cDNAs were stored at -20°C until Real Time PCR (RT-PCR) analysis. In RT-PCR analysis change of; MRP1 multidrug resistance, APEX1 DNA repair mechanism, CuZn-SOD oxidative stress, PI3K/ Akt/ VEGF angiogenesis pathway, CD133, ErbB2 oncogenes, CycD1 cell cycle and proliferation genes expression levels were investigated. For this purpose, the protocol applied in the QuantStudio 6 Flex (Applied Biosystems) RT-PCR device and the primer sequences of the genes are shown in Table 1.

Table 1. Gene codes, primer sequences and PCR conditions of the analyzed gene regions by RT-PCR.

Gene Codes	Primer Base Sequences	Real Time PCR Conditions
GAPDH	F: CCTGCCAAGTATGATGACATCAA	Hold Stage: 1 Cycle
	R: AGCCCAGGATGCCCTTTAGT	50°C 2 minute
APEX1	F: CGACTCAGCGACCTTCTTGC	95°C 10 minute
	R: GTTTACAGTTGTTTTCAGGCCAC	
AKT	F: ATGAGCGACGTGGCTATTGTGAAT	PCR Stage: 45 Cycle
	R: GAGGCCGTCAGCCACAGTCTGGATG	95°C 15 second
CycD1	F: GATCAAGTGTGACCCGGACT	60°C 1 minute
	R: TCCTCCTCTTCCTCCTCCTC	
CD133	F: CAATGACCCTCTGTGCTTGGT	Melt Curve Stage: 1 Cycle
	R: GTGGAAGCTGCCTCAGTTCAG	95°C 15 second
CuZn-SOD	F: GTTCGGTGACAACACCATG	60°C 1 minute
	R: GGAGTCGGTGATGTTGACCT	95°C 15 second
ERBB2	F: CCTCTGACGTCCATCATCTC	
	R: ATCTTCTGCTGCCGTCGCTT	
PI3K	F: CCTCTGACGTCCATCATCTC	
	R: ATCTTCTGCTGCCGTCGCTT	
MRP1	F: GATGGGTTCTTATCAGGAGCTG	
	R: TGGAGAATCGGTTCACTAGGTT	
VEGF	F: AGGAGGGCAGAATCATCACG	
	R: CAAGGCCCACAGGGATTTTCT	

Statistical analysis

Cell amount calculations due to silibinin application were made according to the formula "Cell Viability%: (absorbance value of the substance applied wells / absorbance value of the

control wells) × 100". The IC50 doses calculated using the SPSS Regression Probit (IBM SPSS Statistics 22) application. Changes in gene expressions in the both cell lines due to the 3 repetitive application of 529.23 µM silibinin were calculated with the formula $2^{-\Delta\Delta cT}$. The relative gene expression levels normalized to glyceraldeyhde 3- phosphate dehydrogenase (GAPDH) gene. The results statistically performed with "SPSS Independent Samples T Test" (IBM SPSS Statistics Data Editor), one of the independent group comparision test. $P < 0.05$ values considered statistically significant.

RESULTS

In the study, 12.5-100 µM silibinin was applied to AML-12 and HepG2 cell lines for 24 h and 48 h. Depending on the application of 12.5 µM, 25 µM, 50 µM, 100 µM silibinin to AML-12 cells for 24 h, 83.41%, 79.17%, 77.24% and 74.46% viability were detected, respectively. Under the same conditions in the HepG2 cells 82.45%, 78.21%, 77.46%, 76.86% viability were determined (Figure 1). These data were evaluated by SPSS Regression Probit analysis and the IC50 values were calculated as 7.76 mM for AML-12 and 322.37 mM for HepG2 (Table 2). On the other hand, 91.77%, 89.99%, 80.90%, 75.96% viability were determined for same concentrations of silibinin application to the AML-12 cells for 48 h. In HepG-2 cells at same conditions; 92.11%, 86.65%, 85.71%, 84.76% viability were determined (Figure 1). When these values re-evaluated by Probit analysis, the IC50 value of silibinin was determined as 529.23 µM for AML-12 and 15.51 mM for HepG2 (Figure 2). Among these data, the lowest IC50 value obtained from 48 h silibinin application in AML-12 cells (Figure 1 and Table 2). For this, 529.23 µM silibinin was accepted as a reference application for both AML-12 and HepG2 cells in the next steps of the study.

Table 2. Re-evaluation to AML-12 and HepG2 cells viability with SPSS Regression Probit statistical analyzes.

Probability		95% Confidence Limits for Silibinin			
		AML-12 24-h	HepG2 24-h	AML-12 48-h	HepG2 48-h
PROBIT	IC10 (µM)	1.34	0.16	19.07	18.57
	IC15 (µM)	7.05	2.37	37.87	71.02
	IC20 (µM)	26.29	21.87	52.31	186.98
	IC25 (µM)	81.34	147.13	104.20	294.53
	IC30 (µM)	224.28	814.83	158.54	653.52
	IC35 (µM)	574.04	3987.28	233.91	1640.36
	IC40 (µM)	1416.25	17938.08	328.57	3848.64
	IC45 (µM)	3327.16	76910.23	427.09	8644.66
	IC50 (µM)	7764.42	322374.47	529.23	15510.50
	a. Logarithm base = 10.				

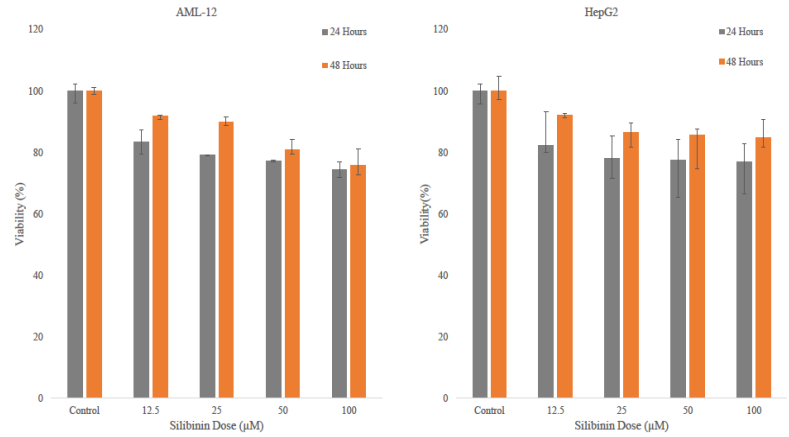


Figure 1. Viability analysis of 12.5- 100µM silibinin applied AML-12 and HepG2 cells by MTT

In the application of 529.23 µM silibinin to AML-12 and HepG2 cell lines for 0-48 h, a decrease in the number of AML-12 cells and an increase in the number of HepG2 cells were observed depending on time. On the other hand, a time-dependent increasing in the amount of AML-12 and HepG2 control cells were observed (Figure 2).

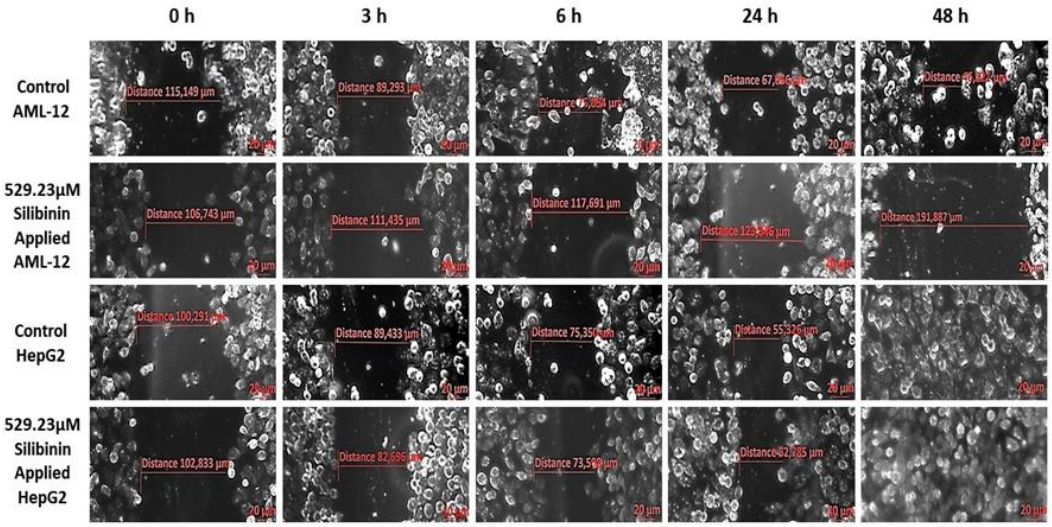
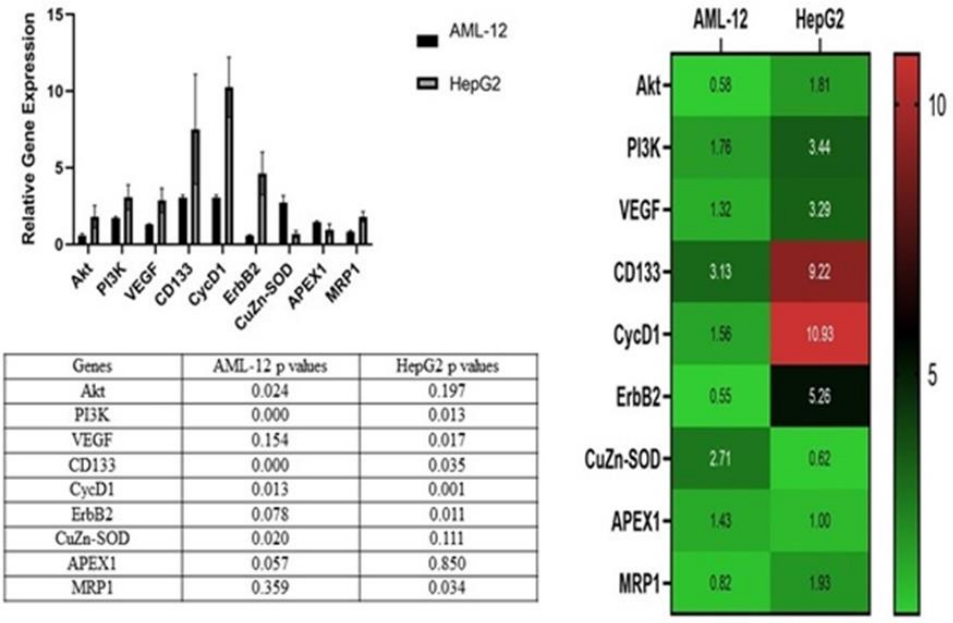


Figure 2. Wound healing microscope images of 529.23µM silibinin applied in AML-12 and HepG2 cells for 0 - 48 h.

Considering the wound healing tests, it was determined that there was a rapid decrease at the amount of AML-12 cells at the 24 h and after silibinin application (Figure 1). Because of this situation, total RNA isolations for RT-PCR analysis were performed by this time. Depending on the silibinin application; MRP1 multi-drug resistance gene, Akt and ErbB2 oncogenes expression levels decreased in AML-12 cells, while in HepG2 cells; PI3K/ Akt/ VEGF, ErbB2, CycD1, CD133, proliferation and MRP1 expression levels increased. Expression levels of APEX1 and CuZn-SOD levels were increased in AML-12 cells, but CuZn-SOD level decreased while APEX1 level remained stable in HepG2 cells. These changes are shown in Figure 3.



Genes	AML-12 p values	HepG2 p values
Akt	0.024	0.197
PI3K	0.000	0.013
VEGF	0.154	0.017
CD133	0.000	0.035
CycD1	0.013	0.001
ErbB2	0.078	0.011
CuZn-SOD	0.020	0.111
APEX1	0.057	0.850
MRP1	0.359	0.034

Figure 3. Relative gene expressions changes of AML-12 and HepG2 cells for 24 h 529.23μM silibinin application ($p<0.05$ significant).

Dependent on silibinin application, apoptotic events not observed in HepG2 cells, but proliferation promoted whereas apoptosis promoted in AML-12 cell line. These apoptotic/ cell proliferation events demonstrated in Figure 4.

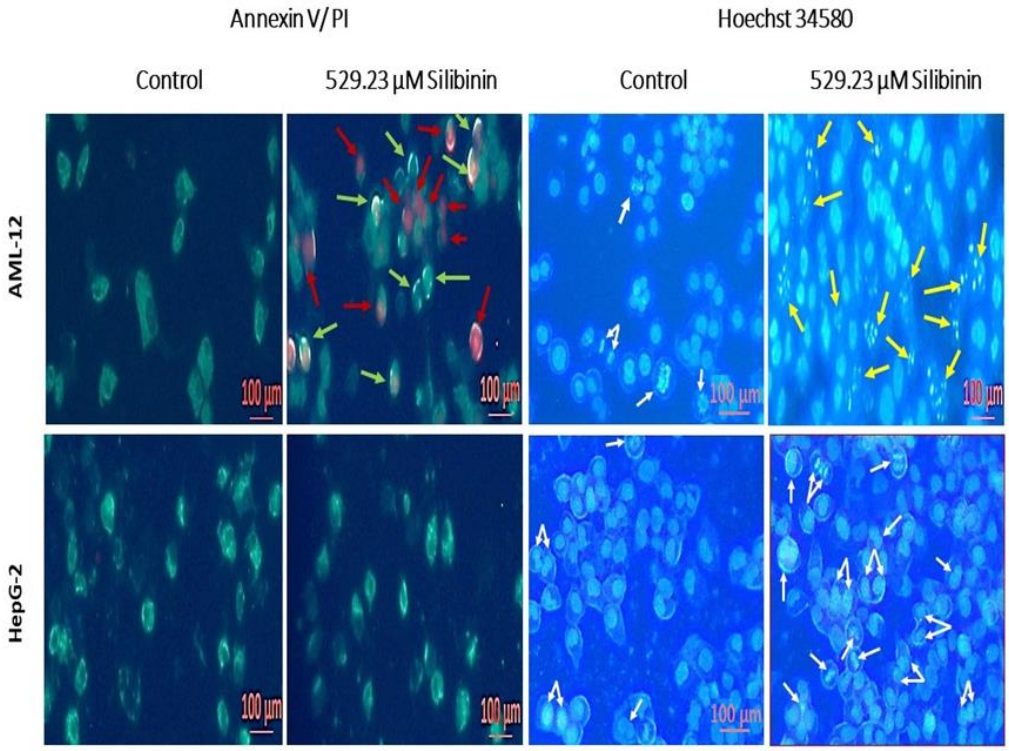


Figure 4. 529.23 µM silibinin causes apoptosis in healthy AML-12 cell line (green arrows indicate to early apoptosis via accumulation of Annexin V-labeled phosphatidyl serines below the cell membrane, red arrows indicate to late apoptosis via PI binds to degrade nuclei and yellow arrows indicate to degraded nuclei), but promote to cell division in HepG2 cells (white arrows indicate to cell division).

DISCUSSION

With the claim that silibinin has positive effects on fatty liver, non-alcoholic steatohepatitis and some types of cirrhosis (SALLER *et al.*, 2008), it has become popular to conduct cancer research on this flavinoid over time. In addition to the liver protective effects of silibinin, GAZAK *et al*. (2007) suggested that it has anti-cancer, prostate, lung, kidney, pancreatic and skin protective properties. KROLL *et al.* (2007) stated that the application of 100- 1600 µM silibinin for 24 h on SK-BR-3 and BT-474 breast cancer cells triggered to cell death. In a study by DEEP *et al.* (2012) on DU-145 prostate cancer mice, 30 µM silibinin having important roles for suppressing to expression of angiogenesis; VEGF, VEGFR1, VEGFR2, phospho-Akt, HIF-1α genes and cell cycle/ cell division; CD31, CyclinD1, CyclinD3, CDK2, CDK4, Cyclin A, Cyclin B1, Cdc 25 A, Cdc 25 C genes. Based on these data, they suggested that silibinin has

important anti-cancer effects. LAH *et al.* (2007) found the IC25 value of silibinin as 120 µmol/ L and the IC50 value as 240 µmol/ L depending on the 24 h application in the HUH7 HCC cell line. They also applied these IC25 and IC50 values to HepG2, Hep3B, PLC/PRF5 HCC cell lines, and they found that the CycD1/ CDK4 complex expression was significantly suppressed, whereas the expression levels of p21, p27, Cas3, Cas9 apoptotic pathway genes were significantly increased. LAMA *et al.* (2019) stated that 68 µM silibinin significantly inhibited cell division due to its application to HepG2 cells for 48 hours. However, the exact value of IC50 was not specified in the study. In addition, this team also stated that there was a significant decrease in Akt and CycD1 expressions levels in HepG2 cells with the application of silibinin. VARGHESE *et al.* (2005) stated that the application of 100, 200, 300 µM silibinin to HepG2 cells for 12, 24, 48 and 72 h suppressed cell division by 40%- 75% depending on the application time. Similar to LAMA *et al.* (2019), Varghese *et al.* suggested that silibinin administration has a CycD1 suppressive effect on the HepG2 cells. Unlike other studies, GU *et al.* (2015) stated that, depending on the application of 1- 200 µM silibinin for 72 h; low doses had a proliferation suppressive effect on Huh7, Huh-BAT and SNU761 cells, but the same doses promoted cell division in HepG2 and SNU475 cells. However, they also stated that high doses of silibinin suppressed cell division of HepG2 and SNU475. The low and high concentration range in this study refers to 1- 100 µM, and silibinin combined with 1.77-13.31µM sorafenib. However, none of these studies on the cytotoxic effects of silibinin on cancer cells have been compared with any healthy cell line or animal model, and they have been limited to cancerous cell lines only. Contrary to previous studies, GHOLAMI *et al.* (2016) stated that 50-200 mg/ kg silymarin caused teratogenic effects on the face, spine and skull of mice.

To resolve these contradictions, the present study, investigated the 24 h and 48 h molecular effects of silibinin on both healthy AML-12 and HepG2 HCC cells. Unlike other studies, in this study, it was determined that silibinin has more cytotoxic effects on AML-12 cells than on HepG2 cells. As a result of Probit analysis, the IC50 value for AML-12 cells was determined as 739.9 mM and 1.35 M for HepG2 cells, depending on the 24 h silibinin administration. These IC50 values are 10.000- 25.000 times higher than compared to KROLL *et al*. (2007), LAH *et al*. (2007) and VARGHESE *et al*. (2005) findings for the HepG2 cells. IC50 values for 48 h of silibinin applications were calculated as 529.23 µM for AML-12 and 15.51mM for HepG2.

It is noteworthy that the IC50 value of silibinin for the AML-12 cell line was 29.3 times lower than for the HepG2 cell line, due to 48 h of administration. This raises the question whether high silibinin concentration is more cytotoxic in healthy cells than in cancerous cells. Moreover; The IC50 of 48 h silibinin administration obtained for healthy AML-12 cells in this study is considerably higher than the IC50 values obtained from the studies of LAH *et al.* (2007), LAMA *et al.* (2019), GU *et al.* (2015), VARGHESE *et al.* (2005) in HepG2 and other HCC cell lines. To answer this question, the IC50 (529.23 µM) value was chosen as the reference IC50 value for all subsequent steps of the study.

Depending on the application of 529.23 µM silibinin, a time-dependent decrease was observed in the amount of AML-12 cells, while an increase was observed in HepG2 cells in the wound healing assays (Figure 2). Although no significant apoptotic event was observed in HepG2 cells, it was well understood from Hoechst 34580 staining assay, 529.23 µM silibinin

promote to HepG2 cells proliferation. Contrast to HepG2 cells, abundant apoptotic cells were detected in the AML-12 cell line due to the same application via both Annexin V/ PI and Hoechst 34580 staining assays (Figure 4).

When gene expression levels were compared by RT-PCR analysis, a 1.81-fold increase in MRP1, a multi-drug resistance gene, was detected in HepG2 cells. The 1.43-fold increase in gene expression of APEX1, a DNA repair mechanism gene, indicates that DNA damage has occurred in AML-12 cells. Therefore, there was a 2.71-fold increase in the expression level of the CuZn-SOD oxidative stress gene in order to suppress the free radicals, which cause DNA damage in AML-12 cells. Contrary to the findings of GU *et al.* (2015), LAH *et al.* (2007), LAMA *et al*. (2019) and VARGESE *et al.* (2005), in this study, in HepG2 cells gene expression levels of PI3K 3.44-fold/ Akt 1.81-fold/ VEGF 3.29-fold, CycD1 10.93-fold, CD133 9.22-fold, ErbB2 5.26-fold increased. 0.58-fold decrease in the expression level of Akt and 0.55-fold decrease in the expression level of ErbB2 genes indicate that silibinin has a proliferation suppressive effect for this cell line (Figure 3).

This study shows that, the application of 529.23 μM silibinin has a suppressive effect in AML-12 cells, and while the 529.23 μM value is much higher than the IC50 values in other studies, it promotes cell growth and proliferation, unlike suppressing proliferation of HepG2 cells. Silibinin is widely used as herbal and dietary supplement around the world. It is believed that, silibinin has a strong antihepatotoxic activity against a lot of human liver damage and toxicity (DEHMLOW *et al*., 1996). May be lower than 100 μM of silibinin has protective effects for healthy liver as stated from BIJAK *et al.* (2017). However, further studies are needed to determine the consequences of using silibinin at concentrations lower than 100 μM or high concentrations such as 529.23 μM in individuals with cancer progenitor cells in their livers.

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SUPROTNO RAŠIRENOM MIŠLJENJU, SILIBININ NIJE SREDSTVO PROTIV KANCERA NITI SUPLEMENT ZA ZAŠTITU JETRE

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Izvod

Silibinin je flavinoid koji je glavna komponenta biljke čička i široko se koristi širom sveta, jer se smatra da ima zaštitno i antikancerogeno dejstvo na jetru. U ovoj studiji, suprotno onome što je opšte poznato, cilj je bio da se pokaže da silibinin nije ni zdrav dodatak za zaštitu jetre, niti sredstvo protiv raka za ćelije hepatocelularnog raka. U tu svrhu, molekularni efekti silibinina su ispitivani u ćelijama HepG2 (hepatocelularni kancer) i AML-12 (zdrava jetra). Citotoksične koncentracije silibinina ispitane su MTT analizom. Proliferacija ćelija testovima zarastanja rana, intracelularna apoptoza i deoba ćelija demonstrirani su fluorescentnim mikroskopskim snimanjem putem Aneksina V/PI i Hoechst 34580 bojenja. Promene nivoa ekspresije gena su ispitivane RT-PCR metodom. U studiji, IC50 vrednosti su izračunate 739,9 mM za AML-12 i 1,35 M za HepG2 za 24 h, 529,23 µM za AML-12 i 15,51 mM za HepG2 za 48 h primene silibinina. Iz ovih podataka, vrednost IC50 od 48 h primene silibinina za AML-12 ćelijsku liniju je rezultirala smanjenjem količine AML-12 ćelija, dok je povećanje HepG2 ćelija. Studije fluorescentnog bojenja pokazuju da je došlo do intenzivne proliferacije u ćelijama HepG2, dok je važan apoptotički efekat izazvan u ćelijskoj liniji AML-12 kao rezultat primene 529,23 µM silibinina. Takođe, svi nivoi ekspresije proliferacije i onkogena su povećani u HepG2 ćelijama, ali nivoi ekspresije Akt, ErbB2 su smanjeni u AML-12 ćelijama, dok su nivoi ekspresije gena za popravku APEKS1 DNK, CuZn-SOD oksidativni stres povećani. Kao rezultat, utvrđeno je da je primena 529,23 µM silibinina citotoksična za ćelije AML-12, kao i proliferativni efekat na HepG2.

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