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## Investigation of antioxidant effect of silibinin molecule on U-2 OS cells induced by hydrogen peroxide

Kubra Gunduz<sup>a</sup>, Hasret Ecevit<sup>b</sup>, Emre Dirican<sup>c</sup>, Meral Urhan Kucuk<sup>b,\*</sup>

<sup>a</sup>Hatay Mustafa Kemal University, Institute of Health Sciences, Department of Molecular Biochemistry and Genetics, Hatay, Turkey  
<sup>b</sup>University of Health Sciences, Institute of Hamidiye Health Sciences, Department of Medical Biology, Istanbul, Turkey

<sup>b</sup>Hatay Mustafa Kemal University, Faculty of Medicine, Department of Medical Biology, Hatay, Turkey

<sup>c</sup>Hatay Mustafa Kemal University, Faculty of Medicine, Department of Biostatistics and Medical Informatics, Hatay, Turkey

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### Abstract

**Aim:** Silibinin is one of the active constituent of silymarin, an extract of the *Silybum marianum L.* seeds. There are previous studies that have reported the antioxidant, anti-inflammatory, anti-apoptotic and anti-carcinogenic effects of silibinin. However, the antioxidant effect of silibinin on bone cancer have been not evaluated before. In our study, we aimed to evaluate the effect of silibinin on U-2 OS cell proliferation and oxidative stress parameters catalase enzyme activity and MDA level.

**Materials and Methods:** U-2 OS cells were exposed to H<sub>2</sub>O<sub>2</sub> (0-800 μM H<sub>2</sub>O<sub>2</sub>, for 24 and 48 hours) to form an oxidative damaged cell model. Moreover, the cells were exposed to silibinin (0-100 μM) for 24 hours. Malondialdehyde (MDA) level and antioxidant enzyme catalase (CAT) activity were determined by spectrophotometric analysis.

**Results:** CAT activity and MDA level were observed to be higher in both H<sub>2</sub>O<sub>2</sub> exposure groups compared to control group ( $p < 0.05$ ). Moreover, CAT activity and MDA level were observed to be lower in 500 μM H<sub>2</sub>O<sub>2</sub>+silibinin groups in comparison with 500 μM H<sub>2</sub>O<sub>2</sub> group ( $p < 0.05$ ). Similarly, MDA level was determined to be lower in 650 μM H<sub>2</sub>O<sub>2</sub>+10 μM silibinin group in comparison to 650 μM H<sub>2</sub>O<sub>2</sub> group ( $p < 0.05$ ).

**Conclusion:** In conclusion, it was observed that silibinin molecule has anti-proliferative effect and decreases catalase enzyme activity and MDA level in a concentration dependent manner.



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### Introduction

Osteosarcoma is one of the most common primary malignant bone tumor in children and adolescents [1]. They are the primary mesenchymal tumors that are characterized histologically osteoid production by malignant cells. These tumors classified in solid tumors and named as primary bone tumors since they originate directly from bone tissue [2]. These tumors are generally locally aggressive and tend to produce early systemic metastases. They are kept separate from other malignant tumors and metastases that form in other organs. Most osteosarcoma grows and spreads quickly which causes patient to die if an effective treatment is not provided.

In normal cells, reactive oxygen species (ROS) containing superoxide anions ( $\cdot\text{O}_2^-$ ), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and high reactive hydroxyl radical (OH $\cdot$ ) are produced as

by-products of cellular metabolism and are found in the balance of cellular redox with biochemical antioxidants. Oxidant / antioxidant balance plays an important role in the onset and progression of cancer [3]. This vital balance is impaired in many cancers as a result of ROS accumulation which result with oxidative stress condition [4]. ROS damage macromolecules such as DNA, RNA and proteins which contribute to the development of many diseases including cancer [5]. The human body has several mechanisms to prevent oxidative stress condition. Antioxidants are produced naturally in situ (endogenous) or can externally supplied through foods and supplements (exogenous). Endogenous and exogenous antioxidants act as free radical scavengers by preventing and repairing the damage caused by ROS. Therefore, it can increase immune defense and reduce the risk of many diseases such as cancer [6].

*Silybum marianum L. Gaertn.* is a member of *Asteraceae* family which has been used as a medicinal plant in the treatment of various diseases for thousands of years [7]. It naturally grows in southern Europe, southern Russia, Asia

\*Corresponding author:

Email address: [meralurhan@hotmail.com](mailto:meralurhan@hotmail.com) Meral Urhan Kucuk

Minor and northern Africa and is naturalized in North and South America as well as in South Australia [7, 8]. Silymarin is an isomeric mixture of flavonoid complexes representing 1.5-3% of the dry weight of the *silybum marianum* L. seed [9]. It consists of silibinin, isosilibin, silicristin, silidianin, dehydrosilibin and other phenolic compounds [10] Silibinin is the most abundant compound in the structure of silymarin with a percentage about 50-70%. There are previous studies indicating the antioxidant [11], anti-inflammatory [12], anti-apoptotic [13], anti-carcinogenic [14] and anti-angiogenic effects [15] of silibinin.

Flavonoids (Fl-OH) are a group of natural compounds found in plants which has various phenolic structures. There are previous studies that have been shown the antioxidant activity and bio-protective properties of flavonoids [16]. Silibinin which has been known to be a powerful antioxidant has flavonoid properties [17]. It inhibits free radical formation and lipid peroxidation in membranes which mediates the modulation of membrane permeability [18].

There are previous studies that have reported the antioxidant [11], anti-inflammatory [12], anti-apoptotic [13], and anti-carcinogenic [14] effects of silibinin. The effect of silibinin on various cancer types also have been reported. However, the antioxidant effect of silibinin on bone cancer have been not evaluated before. In our study, we aimed to evaluate the effect of silibinin on U-2 OS cell proliferation and oxidative stress parameters catalase enzyme activity and MDA level.

## Materials And Methods

### Cell culture

In our study, human bone cancer cell line (U-2 OS) was used which supplied from American Type Culture Collection (ATCC<sup>®</sup> HTB-96<sup>™</sup>). U-2 OS cells were produced in Dulbecco's Modified Eagle Medium (DMEM) (Cat no: 41966-029, Gibco, USA) which contain % 10 Fetal Calf Serum (FCS) (Cat no: 10270106, Gibco, USA) and % 1 penicillin streptomycin (Cat no: 15140122, Gibco, USA) and in 95 % humidity, 5 % CO<sub>2</sub> and 37 °C incubator conditions (NuAire NU-5830, USA). The culture medium was changed twice a week and was used in experiments after they cover the bottom of the wells by 70-80 %.

### Cell viability

U-2 OS cells were seeded in 24 well cell culture plates (Cat no: NC-930186, Thermo, USA) (50.000 cell/mL) with. The cells were incubated in serum-free medium for 24 hours after they cover the bottom of the wells by 70-80 %. Then the cells were exposed to H<sub>2</sub>O<sub>2</sub> (Cat no: 7722841, Sigma, USA) (0, 500, 650, 750 and 800 μM concentrations) for 24 and 48 hours and silibinin (Cat no: S0417-1G, Sigma, USA) [dissolved in dimethyl sulfoxide (DMSO) (Cat no: 41640-2.5L, Sigma, USA) and 0, 5, 10, 20, 30, 50, 75 and 100 μM concentrations] for 24 hours prepared in serum-free medium to determine the appropriate concentration and exposure time to create an oxidative stress model. Cell viability was evaluated by MTT (Cat no: M2128-1G, Sigma, USA) assay. Suitable H<sub>2</sub>O<sub>2</sub> concentrations to create an oxidative stress model was determined as 500 and 650 μM conditions whereas it was 5 and 10

μM for silibinin. The exposure time of experiments was determined as 24 hours.

### Oxidative stress parameters

U-2 OS cells were seeded in 75 cm<sup>2</sup> cell culture flasks (Cat no: NC-130190, Thermo, USA) (200.000 cell/mL) with appropriate culture medium. The cells were incubated in serum-free medium for 24 hours after they cover the bottom of the wells by 70-80 %. Then U-2 OS cells were exposed to H<sub>2</sub>O<sub>2</sub> (500 and 650 μM) and silibinin (5 and 10 μM) for 24 hours. U-2 OS cells were classified into 7 groups as;

Control group without exposure to H<sub>2</sub>O<sub>2</sub> and silibinin,

500 and 650 μM H<sub>2</sub>O<sub>2</sub> group,

5 and 10 μM silibinin group,

500 μM H<sub>2</sub>O<sub>2</sub>+5 μM silibinin group,

500 μM H<sub>2</sub>O<sub>2</sub>+10 μM silibinin group,

650 μM H<sub>2</sub>O<sub>2</sub>+5 μM silibinin group,

650 μM H<sub>2</sub>O<sub>2</sub>+10 μM silibinin group.

After exposure under the above conditions the cells were scraped from flasks with cold Hank's Balanced Salt Solution (HBSS) (Cat no: 14175053, Gibco, USA) and collected into a separate tube. They were stored at -80 °C until experiments. After homogenization process [TissueLysorLT (Qiagen, Germany)] the cells were used for catalase enzyme activity, MDA and total protein measurements.

### Measurement of lipid peroxidation

The method reported by Jain JK et al. (19) was modified according to cell culture and used for MDA measurement. The final product of lipid peroxidation, MDA, forms a pink colored complex as a result of its incubation with thiobarbituric acid at 95 °C and pH: 3.5 under aerobic conditions. The amount of MDA was determined by measuring this complex spectrophotometrically at 532 nm.

### Measurement of catalase enzyme activity

Catalase enzyme activity was measured according to the method suggested by Aebi H. et al. (20) and which was developed by Lartillot S. et al. (21). The method modified according to cell culture. Enzymatic activity determination was performed according to the decrease of H<sub>2</sub>O<sub>2</sub> absorbance value at 240 nm over time as a result of its interaction with catalase enzyme.

### Statistically analyses

In this study, the statistically evaluation of cell viability analyses was performed by GraphPad Prism 5 (GraphPad Software Inc. USA) software. Comparison between groups were carried out by One-way analysis of variance ANOVA and Tukey test.

The evaluation of oxidative stress parameters was performed by IBM SPSS Statistics 20 software. Comparison between groups were carried out by Mann-Whitney U test.  $p < 0.05$  values were considered as statistically significant for all analyses.

**Table 1.** Catalase enzyme activity levels in U-2 OS cells as a result of 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  exposure.

GROUPS	Mean $\pm$ Standard Deviation	Median	p value (Compared to control)	p value (Compared 500 $\mu\text{M}$ )
Control group (without $\text{H}_2\text{O}_2$ treatment)	13.66 $\pm$ 1.24	13.68	-	0.034*
500 $\mu\text{M}$ $\text{H}_2\text{O}_2$	41.71 $\pm$ 9.78	38.32	0.034*	-
5 $\mu\text{M}$ silibinin	32.03 $\pm$ 14.12	30.73	0.014*	0.297
10 $\mu\text{M}$ silibinin	22.32 $\pm$ 9.34	21.96	0.050*	0.053
500 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 5 $\mu\text{M}$ silibinin	22.17 $\pm$ 4.38	21.39	0.034*	0.050*
500 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 10 $\mu\text{M}$ silibinin	19.88 $\pm$ 8.80	21.08	0.480	0.050*

**Table 2.** Catalase enzyme activity levels in U-2 OS cells as a result of 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$  exposure.

GROUPS	Mean $\pm$ Standard Deviation	Median	p value (Compared to control)	p value (Compared 650 $\mu\text{M}$ )
Control group (without $\text{H}_2\text{O}_2$ treatment)	13.66 $\pm$ 1.24	13.68	-	0.021*
650 $\mu\text{M}$ $\text{H}_2\text{O}_2$	23.92 $\pm$ 0.77	24.17	0.021*	-
5 $\mu\text{M}$ silibinin	32.03 $\pm$ 14.12	30.73	0.014*	0.624
10 $\mu\text{M}$ silibinin	22.32 $\pm$ 9.34	21.96	0.050*	0.221
650 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 5 $\mu\text{M}$ silibinin	24.57 $\pm$ 1.95	25.01	0.021*	0.564
650 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 10 $\mu\text{M}$ silibinin	25.46 $\pm$ 6.97	23.35	0.021*	1.000

**Table 3.** MDA level in U-2 OS cells as a result of 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  exposure.

GROUPS	Mean $\pm$ Standard Deviation	Median	p value (Compared to control)	p value (Compared 500 $\mu\text{M}$ )
Control group (without $\text{H}_2\text{O}_2$ treatment)	4.30 $\pm$ 0.36	4.18	-	0.050*
500 $\mu\text{M}$ $\text{H}_2\text{O}_2$	7.74 $\pm$ 1.40	8.42	0.050*	-
5 $\mu\text{M}$ silibinin	4.43 $\pm$ 0.70	4.22	0.827	0.050*
10 $\mu\text{M}$ silibinin	4.15 $\pm$ 0.98	3.74	0.513	0.050*
500 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 5 $\mu\text{M}$ silibinin	4.94 $\pm$ 0.45	4.78	0.127	0.050*
500 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 10 $\mu\text{M}$ silibinin	4.74 $\pm$ 0.91	4.27	0.513	0.050*

**Table 4.** MDA level in U-2 OS cells as a result of 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$  exposure.

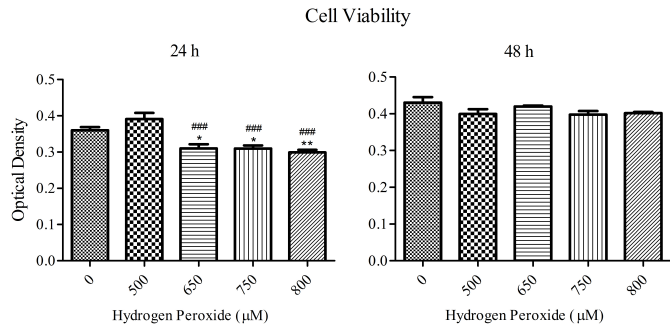
GROUPS	Mean $\pm$ Standard Deviation	Median	p value (Compared to control)	p value (Compared 650 $\mu\text{M}$ )
Control group (without $\text{H}_2\text{O}_2$ treatment)	4.30 $\pm$ 0.36	4.18	-	0.050*
650 $\mu\text{M}$ $\text{H}_2\text{O}_2$	5.88 $\pm$ 0.44	6.14	0.050*	-
5 $\mu\text{M}$ silibinin	4.43 $\pm$ 0.69	4.22	0.827	0.050*
10 $\mu\text{M}$ silibinin	4.15 $\pm$ 0.98	3.74	0.513	0.050*
650 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 5 $\mu\text{M}$ silibinin	5.85 $\pm$ 0.07	5.81	0.050*	0.513
650 $\mu\text{M}$ $\text{H}_2\text{O}_2$ + 10 $\mu\text{M}$ silibinin	5.16 $\pm$ 0.11	5.18	0.050*	0.050*

## Results

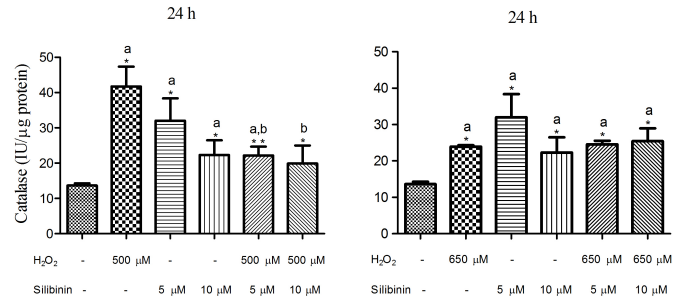
There was statistically significant difference between control and 650-800  $\mu\text{M}$   $\text{H}_2\text{O}_2$  groups ( $p < 0.05$ ) in terms of cell viability as a result of 24 hour exposure whereas there were not any difference between control and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  group ( $p > 0.05$ ). In addition, cell viability was observed to be significantly lower in 650, 750 and 800  $\mu\text{M}$   $\text{H}_2\text{O}_2$  groups when compared to 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  group ( $p < 0.05$ ) (Figure 1). There was not any significant difference between groups after 48 hour  $\text{H}_2\text{O}_2$  exposure.

There were statistically significant difference between control and 20-100  $\mu\text{M}$  silibinin groups as a result of 24 hour

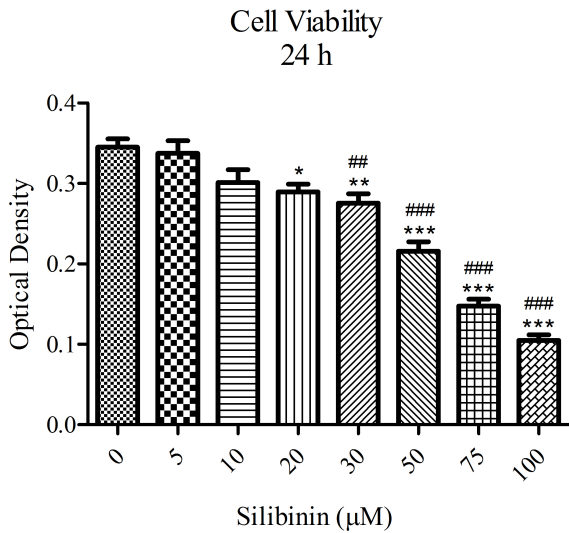
silibinin exposure ( $p < 0.05$ ) whereas there was not any significant difference between control 5 and 10  $\mu\text{M}$  silibinin group ( $p > 0.05$ ). Cell viability was observed to be significantly lower in 30-100  $\mu\text{M}$  silibinin groups compared to 5  $\mu\text{M}$  silibinin group ( $p < 0.05$ ) (Figure 2). Moreover, in 50-100  $\mu\text{M}$  silibinin groups, cell viability was shown to decrease significantly when compared to both 10, 20 and 30  $\mu\text{M}$  silibinin groups separately. In addition, there were statistically significant decrease in 75-100  $\mu\text{M}$  silibinin groups according to 50  $\mu\text{M}$  silibinin group (not shown).



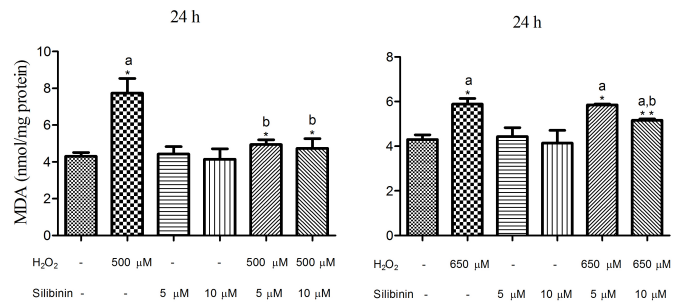
**Figure 1.** The evaluation of cell viability of U-2 OS cells as a result of H<sub>2</sub>O<sub>2</sub> exposure for 24 and 48 hours. Comparison of all groups versus control group; \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.00$ . Comparison of all groups versus 500 µM H<sub>2</sub>O<sub>2</sub> group; # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$ .



**Figure 3.** Catalase enzyme activity levels in U-2 OS cells after 500 and 650 µM H<sub>2</sub>O<sub>2</sub> exposure. The comparison between control and other groups indicated as ‘a’ whereas between 500 and 650 µM H<sub>2</sub>O<sub>2</sub> and other groups as ‘b’. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$



**Figure 2.** The evaluation of cell viability as a result of silibinin exposure for 24 hour in U-2 OS cells. Comparison of all groups versus control group; \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.00$ . Comparison of all groups versus 5 µM Silibinin group; # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$ .



**Figure 4.** MDA level in U-2 OS cells after 500 and 650 µM H<sub>2</sub>O<sub>2</sub> exposure. The comparison between control and other groups indicated as ‘a’ whereas between 500 and 650 µM H<sub>2</sub>O<sub>2</sub> and other groups as ‘b’. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$

### Catalase enzyme activity

Catalase enzyme activity was found to be significantly higher in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 5  $\mu\text{M}$  silibinin, 10  $\mu\text{M}$  silibinin and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin groups compared to control group ( $p < 0.05$ ) whereas there was not any significance between control and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin group ( $p > 0.05$ ) (Figure 3 and Table 1). Moreover, catalase enzyme activity was found to be significantly lower in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin groups compared to 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  group ( $p < 0.05$ ) (Figure 3 and Table 1).

In addition, catalase enzyme activity was found to be significantly higher in 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 5  $\mu\text{M}$  silibinin, 10  $\mu\text{M}$  silibinin, 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin groups compared to control group ( $p < 0.05$ ) (Figure 3 and Table 2). However, there were not any significance between 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and other 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +silibinin groups (650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin) ( $p > 0.05$ ) (Figure 3 and Table 2).

### MDA level

In our study MDA level was found to be significantly higher in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  group compared to control group ( $p < 0.05$ ) whereas there were not any difference in 5  $\mu\text{M}$  silibinin, 10  $\mu\text{M}$  silibinin, 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin groups ( $p > 0.05$ ) (Figure 4 and Table 3). Moreover, MDA level was found to be significantly lower in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin groups when compared to 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  group ( $p < 0.05$ ) (Figure 4 and Table 3).

In addition, MDA level was found to be significantly higher in 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin group compared to control group ( $p < 0.05$ ) whereas there was not any difference in 5  $\mu\text{M}$  silibinin and 10  $\mu\text{M}$  silibinin groups ( $p > 0.05$ ) (Figure 4 and Table 4). Moreover, MDA level was found to be significantly lower in 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin group when compared to 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$  group ( $p < 0.05$ ) whereas there were not any significant difference in terms of 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin group ( $p > 0.05$ ) (Figure 4 and Table 4).

### Discussion

The basic treatment for osteosarcoma, one of the most common malignant bone tumor in childhood and youth, is surgical and systemic chemotherapy. However, the five-year survival rate after surgical treatment is only about 20%. Therefore, new treatment approaches and the discovery/research of new therapeutic agents and/or supplements have become very important.

Flavonoids are a large family of polyphenolic compounds synthesized by plants and have many biological effects mainly due to their antioxidant properties. The antioxidant [11], hepato-protective [22] and anti-cancer [23] properties of silibinin, one of the popular dietary supplement, has been studied extensively. However, the antioxidant effects of this substance on bone cancer have not been studied before.

In this study, we aimed to investigate the effects of silibinin on the cell proliferation and its antioxidant activity

on the human bone cancer cell line U-2 OS cells. For this purpose, U-2 OS cells were exposed to  $\text{H}_2\text{O}_2$  at different doses to create an oxidative stress condition and simultaneously appropriate doses of silibinin to investigate its effect of on cell proliferation, oxidative stress parameter MDA level and antioxidant catalase enzyme activity.

In our study, cell viability was found to decrease significantly at 10, 20, 30, 50, 75 and 100  $\mu\text{M}$  silibinin conditions compared to control group whereas there was not any significant difference at 5  $\mu\text{M}$  silibinin condition as a result of exposure for 24 hour.

In our study we used two separate control groups to determine the antioxidant effect of silibinin on MDA level and catalase enzyme activity. The first one was the cell group that untreated with  $\text{H}_2\text{O}_2$  and the second one was the group treated with 500 or 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . The first control group was used to determine MDA level and catalase enzyme activity between  $\text{H}_2\text{O}_2$  treated group and untreated group whereas the second one was used to determine the antioxidant effect of silibinin.

In previous studies silibinin was shown to have powerful antioxidant effect against various malignant cell lines such as human pancreatic carcinoma [24], human bladder carcinoma [25], human prostate adenocarcinoma [26] and human colon cancer [27] cells.

Catalase, which catalyzes the conversion of hydrogen peroxide to water and molecular oxygen very effectively, shows optimum activity in cases where the concentration of hydrogen peroxide increases. In our study, significant difference had been shown in terms of catalase enzyme activity between  $\text{H}_2\text{O}_2$  exposed (500  $\mu\text{M}$  and 650  $\mu\text{M}$ ) and untreated groups.

$\text{H}_2\text{O}_2$ , which occurs in a wide variety of physiological and pathological processes, can increase cell damage as well as it can also be a regulatory molecule as a signal molecule and activate signal pathways. Redox balance which can be linked with oncogenic stimulation is known to impair in cancer cells. In many cancers, changes in signal pathways related to antioxidant enzymes such as SOD, catalase, glutathione peroxidase are clearly known [28]. In this study, catalase enzyme activity was reported to increase in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin concentration as a result of exposure with  $\text{H}_2\text{O}_2$  and silibinin simultaneously according to control group in U-2 OS cells. Moreover, catalase enzyme activity was shown to decrease in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin groups according to 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  concentration group. Vecchione G et al. [29] were reported catalase enzyme activity to decrease in steatotic hepatic cells as a result of exposure with 50  $\mu\text{mol/L}$  silibinin according to control group similar with our results [29].

Catalase enzyme activity was reported to increase in PC12 cells exposed to 0.4 mM  $\text{H}_2\text{O}_2$  and 1.2 mM PCA, a phenolic compound isolated from *Alpinia oxyphylla* beans, whereas PCA and  $\text{H}_2\text{O}_2$  did not affect the enzyme activity when treated separately [30]. Moreover, Luo et al. [31] reported catalase enzyme activity to decrease in MRC-5 cells as a result of 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  exposure for 6 hours whereas it was reported to increase in a dose-dependent manner as a result of F2 (radish leaf extract) exposure. Catalase en-

zyme activity was reported to increase 1.52, 2.57 and 3.45 fold as a result of 12.5, 25 and 50  $\mu\text{g}/\text{mL}$  F2 exposure, respectively in their study in which they have suggested that it retain redox potential [31].

There are previous studies about the investigation of antioxidant effects of flavonoids in the treatment and prevention of many diseases, especially in cancer and cardiovascular diseases [32]. Phenolic compounds that function as antioxidants act as terminators of free radical chains and as chelators of redox-active metal ions that can catalyze lipid peroxidation [33]. Lipid peroxidation is a common result of oxidative stress and MDA occurs as a last product of lipid peroxidation process. Flavonoids protect lipids against oxidative damage by various mechanisms [34].

In our study, MDA level was shown to increase in  $\text{H}_2\text{O}_2$  exposure groups (500  $\mu\text{M}$  and 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ) compare to control group. Moreover, it was reported to increase in 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin exposures whereas there was not any difference in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin groups. In addition, MDA level was shown to decrease in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin concentrations compared to 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  dose group. There was not any difference in 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +5  $\mu\text{M}$  silibinin group whereas MDA level was reported to decrease in 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ +10  $\mu\text{M}$  silibinin group compared to 650  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . Therefore, it was observed that silibinin showed antioxidant effects in a dose-dependent manner.

Jiang et al. [35] were reported significantly increased MDA level in  $\text{H}_2\text{O}_2$ -induced HepG2 cells depending on the amount of intracellular ROS. When they exposed HepG2 cells to an antioxidant N-acetyl-serotonin (NAS), they observed that intracellular ROS level significantly changed and the amount of MDA level was shown to decrease. Therefore, they demonstrated that  $\text{H}_2\text{O}_2$  can stimulate ROS accumulation in HepG2 cells by disrupting the balance of endogenous antioxidant defense mechanisms, and NAS can effectively reduce  $\text{H}_2\text{O}_2$ -induced ROS production [35]. In another study, MDA level was reported to decrease in steatotic hepatic cells as a result of exposure to 50  $\mu\text{mol}/\text{L}$  silibinin for 24 hours compared to control group similar with our results [29]. In addition, MDA level was shown to increase in another study that performed in  $\text{H}_2\text{O}_2$ -induced different cell line which exposed to plant extract containing antioxidant flavonoid according to control group without  $\text{H}_2\text{O}_2$  exposure [31].

## Conclusion

In conclusion, silibinin was reported to have anti-proliferative effect in U-2 OS cells and decreased the catalase enzyme activity and MDA level in a dose-dependent manner. Therefore, it can be used as a useful antioxidant and anti-carcinogen supplement.

## Limitations

Since the commercial cell lines consist of only a single type of cell, not having complicated coordination mechanisms in their microenvironment and does not interact with the factors such as the extracellular matrix and don't have the same genotypic and/or phenotypic characteristics as in the

human body, the studies in which commercial cell lines are used may be misleading [36-41]. Moreover, the absence of recurrence and support of our experiments in terms of in vivo conditions is a limitation of our study.

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