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# ANTIPROLIFERATIVE AND ANTIOXIDANT EFFECTS OF CARNOSIC ACID ON HUMAN LIVER CANCER CELLS* KARNOSİK ASİTİN İNSAN KARACİĞER KANSER HÜCRELERİNDEKİ ANTİPROLİFERATİF VE ANTİOKSİDAN ETKİLERİ

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**ABSTRACT**

The purpose of the study was to investigate the cytotoxic effects of carnosic acid alone and in combination with cisplatin on human liver cancer cells and their capacity to scavenge reactive oxygen species induced in the presence or absence of hydrogen peroxide. Cytotoxic effects of agents on human liver cancer cells for 24 and 48 hours were evaluated by methyl-thiazol tetrazolium-bromide assay. Mitochondrial membrane potential were detected via JC-1 kit. The intracellular reactive oxygen species levels were determined using 2'-7'dichlorofluorescin diacetate assay. According to our findings, both carnosic acid alone and in combination with cisplatin showed cytotoxic effects in human liver cancer cells at 24 and 48 hours of exposure. In particular, it was seen that the cell viability significantly decreased in a dose-dependent manner at 48 hours of exposure, and the combined treatment was found to have a more pronounced cytotoxic effect. In addition, all carnosic acid concentrations alone and in combination with cisplatin were identified to significantly reduce mitochondrial membrane potential. We observed that both carnosic acid alone and in combination with cisplatin lowered intracellular reactive oxygen species levels in the presence or absence of hydrogen peroxide. The results suggested that carnosic acid alone or in combination with cisplatin might be a promising agent in the treatment of liver cancer.

**Keywords:** Antioxidants, HepG2, carnosic acid, reactive oxygen species, cisplatin

**ÖZ**

Bu çalışmanın amacı, karnosik asitin tek başına ve sisplatin ile kombinasyonu insan karaciğer kanseri hücreleri üzerinde sitotoksik etkilerini ve bunların hidrojen peroksit varlığında veya yokluğunda indüklenen reaktif oksijen türlerini temizleme kapasitelerini araştırmaktır. Maddelerin insan karaciğer kanser hücreleri üzerindeki 24 ve 48 saatlik sitotoksik etkileri metil-tiyazol tetrazolyum-bromür testi ile değerlendirildi. Mitokondriyal membran potansiyeli JC-1 kiti ile tespit edildi. Hücre içi reaktif oksijen türlerinin düzeyleri 2'-7'dikloroflоresin diasetat yöntemi kullanılarak belirlendi. Bulgularımıza göre, 24 ve 48 saatlik maruziyette karnosik asitin hem tek başına hem de sisplatin ile kombinasyonu insan karaciğer kanser hücrelerinde sitotoksik etkiler gösterdi. Özellikle, 48 saatlik maruziyette doza bağlı bir şekilde hücre canlılığını önemli ölçüde azalttığı görüldü ve kombine tedavinin daha belirgin bir sitotoksik etkiye sahip olduğu bulundu. Ayrıca, tüm karnosik asit konsantrasyonlarının tek başına ve sisplatin ile kombinasyonlarının mitokondriyal membran potansiyelini önemli ölçüde azalttığı belirlendi. Hem karnosik asitin tek başına hem de sisplatin ile kombinasyonu hidrojen peroksit varlığında veya yokluğunda hücre içi reaktif oksijen türlerinin düzeylerini düşürdüğünü gözlemledik. Sonuçlar, karnosik asitin tek başına veya sisplatin ile kombinasyon şeklinde karaciğer kanserinin tedavisinde umut verici bir ajan olabileceğini düşündürdü.

**Anahtar kelimeler:** Antioksidanlar, HepG2, karnosik asit, reaktif oksijen türleri, sisplatin

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## INTRODUCTION

Cancer continues to be prominent health problem worldwide and the number of cases is expected to increase in the coming years, especially in developing countries.[1] Hepatocellular carcinoma (HCC), a malignant tumor, is seen as the most prevalent cause of cancer-associated mortality and has a high relapse rate,[2] and is resistant to chemotherapy, which makes it difficult the cure the disorder.[3] Due to the continuous increase in the incidence of HCC in recent years, it is very important to determine effective therapeutic agents for the treatment of HCC.[4] Natural products are an important source of bioactive compounds that have both chemopreventive and chemotherapeutic roles against several types of cancer.[5]

Carnosic acid (CA) is a bioactive phenolic diterpene primarily present in *Salvia officinalis* and *Rosmarinus officinalis*[6,7] and displays pharmacological and biological activities such as antioxidant, anticancer activities, antimicrobial, anti-apoptotic, anti-inflammatory, antiproliferative, and neuroprotective.[8-17] Studies on its antitumoral effect, which is among these properties, have focused on this feature of CA in many cancer cells.[15] Among these studies, in addition to inhibiting cell growth in human cervical cancer cells, CA has been stated to induce apoptosis in some cancer cell lines such as HCC, neuroblastoma, and human prostate cancer.[16,17]

Cisplatin (Cis) is one of the most effective and widely used chemotherapeutic drugs for the treatment of some carcinomas and is used in combination with other agents in the treatment of different types of cancer. The mechanism of the impress of Cis therapy is based on DNA damage by interfering with DNA repair mechanisms. This treatment is known to cause many toxic side effects.[18,19]

However, since liver cancer is resistant to chemotherapy, which complicates the treatment of the disease, adjuvant agents are needed to limit the side effects of Cis. It is important to develop new pharmaceutical products with less toxicity, especially products derived from natural sources.[17] Therefore, the purpose of our study was to assess the cytotoxic properties of CA alone and in combination with Cis on human liver cancer (HepG2) cells and their capacity to scavenge reactive oxygen species (ROS) induced in the presence or absence of hydrogen peroxide ($H_2O_2$).

## MATERIALS AND METHODS

### Chemicals

CA was purchased from Santa Cruz Biotechnology. Cis was obtained from Koçak Farma. $H_2O_2$ was supplied from Merck. DMEM culture medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were supplied from Capricorn Scientific GmbH. Dimethyl sulfoxide (DMSO), methyl-thiazol tetrazolium-bromide (MTT), and dichlorodihydrofluorescein-diacetate (DCFH-DA) were obtained from Sigma Chemical. Cells supplemented with 0.1% DMSO alone were reflected as control. All substances were dissolved in DMSO with 99% purity and diluted with medium so that the final concentration of DMSO was 0.1%.

### Cell culture

HepG2 cell was obtained from ATCC (USA) and grown in DMEM containing 10% FBS and supplemented with 1% penicillin-streptomycin. The cells incubated in a humid atmosphere including air (95%), $CO_2$ (5%), and at 37°C were routinely controlled and then treated with trypsin-EDTA followed by treatment with different concentrations of agents. Cells treated with DMSO (0.1%) alone were considered negative control.

### Cell viability assay

The cytotoxicity properties of agents on the HepG2 cell were detected using the MTT test.[20] Firstly, cells were plated in 96-well plates to a final concentration of $10^4$/well. To determine the cytotoxic concentrations and the values of $IC_{50}$, firstly CA (1-500 µM) and Cis (1-40 µM) were applied to the cell for 24 and 48 hours of exposure. The effective concentrations of CA and Cis were determined according to the $IC_{50}$ values obtained. $IC_{50}$ values were calculated using concentration–response curves to express the effects of test substances on cell viability. Cells were then exposed to CA (50, 100, and 150 µM), Cis (10 µM), and their combinations for 24 and 48 h. And then, the HepG2 cell was treated with a dose of 0.5 mg/mL MTT for 3 h and then the medium was removed. To dissolve the formazan-crystal, the DMSO solution (100 µL) was supplemented and the plates were shaken at room temperature for 15 min. The absorbance was read at 570 nm on a reader (Biotek Synergy HT, Gen5, Vermont, USA). The results were expressed as the mean percentage of cell growth.

### Detection of MMP potential

The mitochondrial membrane potential (MMP) was determined in HepG2 cells after exposure to agents using the JC-1 assay kit (Cayman Chemical Company, USA), and the assay was carried out with the manufacturer's instructions. The fluorescence intensities were recorded by using a microplate reader (Biotek Synergy HT, Gen5, Vermont, USA). Monomeric JC-1 (green) was detected by excitation at 485 nm and emission at 535 nm. Aggregated JC-1 (red) was determined by excitation at 535 nm and emission at 595 nm. The ratios of red and green JC-1 fluorescence was calculated.

### Measurement of intracellular ROS production in the presence or absence of $H_2O_2$

ROS levels in HepG2 cells was analyzed by the method of DCFH-DA.[21] Firstly, HepG2 cells (1x $10^4$) were placed in 96-well black plates and held for 24 h. After changing the medium, cells were exposed to agents for 1 h and then exposed to $H_2O_2$ (100 µM) for 2 h. After washing twice with cold PBS, DCFH-D) (5 µM) was supplemented to the cell and held for 45 min at 37 °C in the dark. The fluorescence was read by a microplate reader (Biotek Synergy HT, Gen5, Vermont, USA). The wavelengths of excitation and emission were 485 and 550 nm, respectively.

### Statistical analysis

SPSS 18.0 was applied for statistical evaluation. Data were assessed for normality assumption and homogeneity of variance. The compliance of the data for normal distribution was checked with the "Shapiro-Wilk" test and it was observed that it had a normal distribution ($p$>0.05). The significance was calculated using one-way analysis of variance (ANOVA) with an LSD post-hoc test and $p$ values of <0.05 were regarded as statistically significant. Experiments were repeated three times at different time periods. Experiments were repeated triplicate and values were indicated as the mean ± standard

error.

## RESULTS

### CA and CA+Cis reduced cell viability in HepG2 cell

Firstly, we evaluated the cytotoxic effects of CA in a wide concentration range (1-500 µM) in HepG2 cells with the MTT test for 24 and 48-h incubation periods. $IC_{50}$ values of CA were determined to be 144 µM ($R^2$=0.849) and 87 µM ($R^2$=0.977) for 24 h and 48 h, respectively. After determining the effective concentrations, considering the $IC_{50}$ values; 50, 100, and 150 µM concentrations of CA and 10 µM concentration of Cis were selected for all experiments.

According to the cytotoxicity results of 24-hour exposure, we observed that of CA and their combinations with Cis decreased cell viability in a concentration-dependent manner. In particular, we determined that concentrations of 150 µM CA ($p$<0.001), Cis+100 µM CA ($p$=0.013), and Cis+150 µM CA ($p$=0.004) caused a substantial decrease in % cell viability in comparison to the control (Figure1A).

According to the cytotoxicity results of 48-hour exposure, the decrease in cell viability in a concentration-dependent manner was determined to be quite significant for all CA doses and their combinations with Cis compared to the control cells ($p$<0.001). In addition, when compared with the Cis, the Cis+50 µM CA ($p$=0.014) led to an important reduction in cell viability, while the Cis+100 µM CA ($p$<0.001) and Cis+150 µM CA ($p$<0.001) caused a notably significant decrease in the % cell viability (Figure1B).

### CA and CA+Cis reduced MMP in HepG2 cell

A typical feature of the early stage of apoptosis involving changes in MMP is the disruption of mitochondria and the oxidation-reduction incidental to the mitochondria. JC-1 gathers in the matrix in healthy cells with high MMP and instantly makes up complexes in the form of J-aggregates with intense red fluorescence. In apoptotic or unsanitary cells with low MMP, JC-1 remains in monomeric form, showing green fluorescence. Hereby, the red-to-green reversion of JC-1 fluorescence shows a reduction in MMP.[2]

Our finding indicated that HepG2 cells were exposed to all CA concentrations and their combinations with Cis 24 h resulted in notable reductions in the ratio of red/green fluorescence when compared to the control as demonstrated in Figure2. In particular, we detected that concentrations of 50 µM CA ($p$=0.004), 100 µM CA ($p$=0.004), 150 µM CA ($p$<0.001), Cis+50 µM CA ($p$=0.010), Cis+100 µM CA ($p$<0.001), and Cis+150 µM

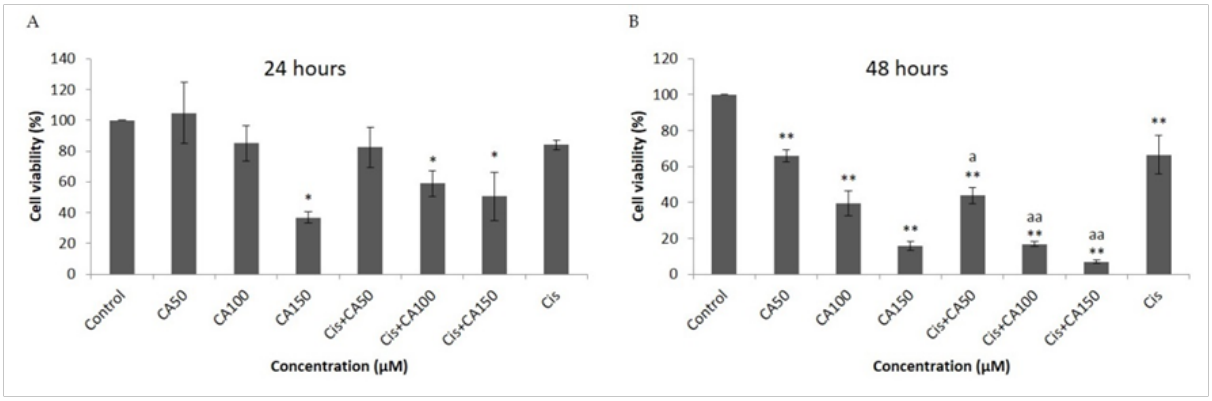


**Figure1:** Cytotoxic effects of CA alone and in combination with Cis on HepG2 cells viability for 24 h (A) and 48 h (B) using MTT assay. Cell viability was plotted as a percent of the control (assuming data obtained from untreated cells as 100%). Differences between the means of data were compared by the one-way analysis of variance (ANOVA) test and post hoc analysis of group differences by the least significant difference (LSD) test (n=8).*$p$<0.05 compared to control;**$p$<0.001 compared to control; a$p$<0.05 compared to Cis; aa$p$<0.001 compared to Cis.

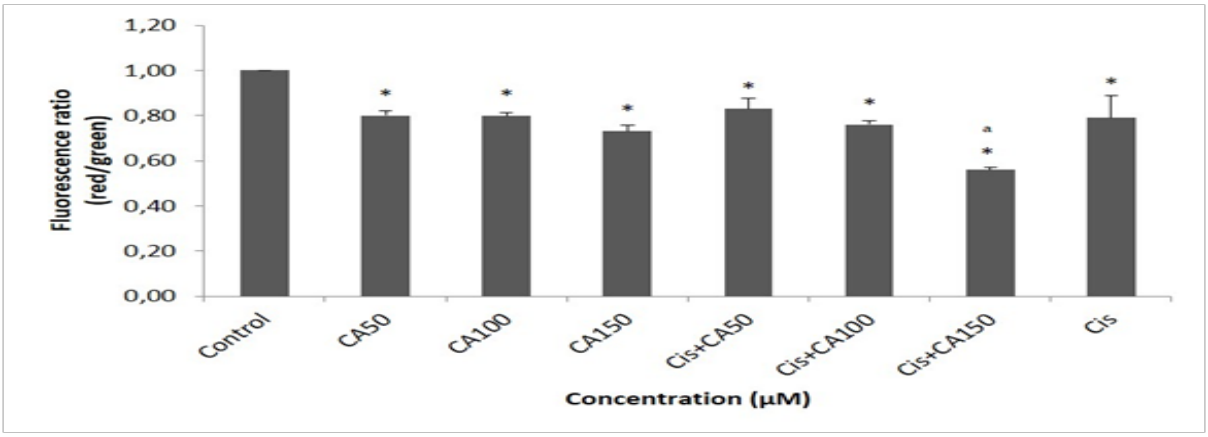


**Figure 2:** MMP changes in HepG2 cells treated with CA alone and in combination with Cis. Differences between the means of data were compared by the one-way analysis of variance (ANOVA) test and post hoc analysis of group differences by the least significant difference (LSD) test (n=8).*$p$<0.05 compared to control; a$p$<0.05 compared to Cis.

CA ($p$<0.001) caused a substantial decrease in MMP in comparison to the control. Furthermore, a decrease in MMP in the Cis+150 µM CA group was found to be substantial in comparison to the Cis-treated cells ($p$=0.001).

**CA and CA+Cis reduced significantly ROS generation in HepG2 cells**

Based on the DCFH-DA assay, we determined that all CA concentrations and their combination with Cis significantly reduced intracellular ROS levels when compared to the control ($p$<0.001). When compared to Cis, the decrease in DCF fluorescence was found to be significant in the Cis+150 µM CA ($p$=0.17) as seen in Figure3A. In the presence of $H_2O_2$, we found that all CA concentrations and their combinations with Cis significantly reduced intracellular ROS levels ($p$<0.001) as shown in Figure3B.

Our data from cytotoxicity results showed that all concentrations of CA (50 µM, 100 µM, and 150 µM) and their combination with Cis decreased cell viability with 24 h exposure in HepG2 cells. In particular, we determined that concentrations of 150 µM CA, Cis+100 µM CA, and Cis+150 µM CA raised a substantial reduction in cell viability compared to the control. Moreover, all concentrations of CA (50, 100, and 150 µM) and their combination with Cis were found to significantly reduce cell viability in a dose-dependently with 48 hours of exposure in HepG2 cells. In addition, when compared with the Cis-treated cells, the Cis+50 µM CA caused a significant reduction in cell viability, while the Cis+100 µM CA and Cis+150 µM CA caused a highly significant decrease in cell viability.

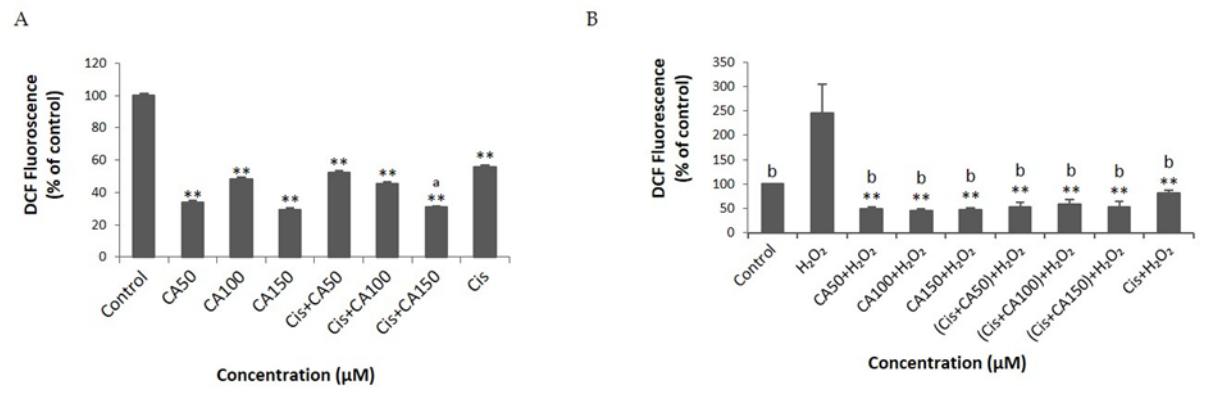


**Figure3:**ROS levels in HepG2 cells treated with CA alone and in combination with Cis in the absence of $H_2O_2$ (A) and the presence of $H_2O_2$ (B) by quantitative analysis of the fluorescent intensity of DCF. Differences between the means of data were compared by the one-way analysis of variance (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test (n=8).[a]$p$<0.05 compared to Cis;[**]$p$<0.001 compared to control; [b]$p$<0.001 compared to the $H_2O_2$ group.

## DISCUSSION

Today, the discovery of new natural products with high anticancer activity but no toxicity on healthy cells come to the fore as a substantial option in cancer therapy. The use of various herbal phenolic compounds with chemotherapeutic drugs is important in terms of increasing the anticancer efficacy of these agents and reducing their possible adverse effects. For this purpose, we investigated the potential cytotoxicity of CA administered at various concentrations alone or in combination with Cis in HepG2 cells.

The results obtained from this study showed that CA, alone or in combination with Cis, potently reduced cell viability in HepG2 cells, induced MMP changes, and significantly reduced intracellular ROS generation in the presence or absence of $H_2O_2$.

As a polyphenol, CA has been suggested to prevent the growth of several human cancer cells as a hopeful dietary supplement in the prevention and treatment of human diseases.[22,23] Cis is an excellent chemotherapy agent for various cancers, but it does cause some side effects. Therefore, the combined treatment of Cis with anticancer natural products may increase its therapeutic potential and reduce its adverse effects.[24] Since there is no data in the literature about the combined effects of CA and Cis on HCC, we evaluated this effect in our study and observed significant dose-dependent cytotoxic effects on HepG2 cells.

In other studies, cytotoxic effects of CA have been observed in various cancer cells. Xiang et al.[2] observed that according to the results of the MTT test, 50 and 100 µM CA reduced dose-dependently cell viability in HepG2 cells at 24 hours of exposure. Zhang et al.[12] have also demonstrated the destructive effects of CA on HCC *in vitro* and *in vivo* studies. *In vitro,* CA significantly reduced cell viability and inhibited cell growth in HepG2 and SMMC-7721 cells. Kaplan et al.[25] established that CA considerably prevents HepG2 cell growth in a dose and time-dependently. Yesil-Celiktas et al.[23] observed a decrease in cell viability of various cancer cell lines exposed to CA at doses of 6.25 to 50 µg/ml for 48 hours. In particular, they observed that CA at 6.25 µg/ml dose led to the least cell viability, resulting in a superior antiproliferative effect. Corveloni et al.[14] found that CA treatment inhibited cell proliferation in non-small-cell human lung carcinoma (NCI-H460) and was only seen at elevated doses (160-320 µM). Su et al.[26] reported that CA (5-100 mM) decreased the cell viability in cervical cancer CaSki and SiHa cells in a time and dose-dependently according to the MTT test results. Tsai et al.[9] stated that CA dose-dependently reduced the cell viability for 24 h and displayed vigorous cytotoxicity against human neuroblastoma IMR-32 cells at an $IC_{50}$ value of about 30 µM. Bai et al.[27] found that CA showed strong antiproliferative effects on HL-60 cells at an $IC_{50}$ value of 1.7 µM and on COLO 205 cells at an $IC_{50}$ value of

32.8 µM.

Recent discoveries have shown that mitochondria, which are known to have a role in cancer development, are targeted by some plant polyphenols in cancer cells.[28] MMP, which reflects the functional state of mitochondria, is thought to be associated with cell differentiation status, malignancy, and tumorigenicity.[29] In our study, we observed that all concentrations of CA and their combinations with Cis significantly reduced MMP in HepG2 cells. Consistent with our result, Xiang et al.[2] observed CA-induced MMP changes in HepG2 cells. They found that exposure of HepG2 to CA (10, 25, and 50 µM) for 24 resulted in significant reductions in the ratio of red-green fluorescence in comparison to control, suggesting that CA may be a hopeful dietary polyphenol in the repress of cancer cell proliferation. Zhang et al.[12] also showed that exposure to 30 µM and 60 µM CA for 6 h led to a reduction in MMP in HepG2 and SMMC-7721 cells.

ROS are quite reactive radicals under the control of intracellular antioxidants and lead to many diseases, including cancer.[30] Excessive ROS production by mitochondria in cancer cells plays an important role in cancer development by leading to oxidative DNA damage.[30,31] $H_2O_2$ is a substantial product in oxidative stress-caused cell death, redox regulation, and signaling.[32,33]

In our study, $H_2O_2$ was used as an intracellular stimulant because it causes cell death through oxidative signaling. Our DCFH-DA assay results showed that intracellular ROS levels were reduced by all CA concentrations and their combination with Cis in the presence or absence of $H_2O_2$. This is because a decrease in the levels of ROS, which has a significant role in the promoter and progress of cancer, may have prevented the proliferation of cancer cells.[34] Having obtained similar results, Kim et al.[33] determined that turmeric leaf extract, which has antioxidant properties, inhibited intracellular ROS formation in Vero cells treated with 600 µM $H_2O_2$. Similarly, Hu et al.[35] examined the level of ROS in $H_2O_2$-treated HepG2 cells to examine whether CA confers protection against oxidative damage. After pretreatment with CA at a dose range of 2.5-10 µM for 2 h, cells were exposed to $H_2O_2$ (3 mM) for 4 h. They determined that cells exposed to $H_2O_2$ showed accumulation of ROS and pretreatment with CA significantly decreased ROS compared to the $H_2O_2$ treatment group. On the other hand, while Cis was expected to increase ROS levels in HepG2 cells, it was a striking result that it decreased ROS levels statistically and significantly in the presence of $H_2O_2$. This suggested that this was probably due to Cis triggering the intracellular antioxidant defense system or through other mechanisms. Therefore, this issue needs to be clarified with further studies.

## CONCLUSION

In conclusion, the main finding of our study revealed that CA, alone or in combination with Cis, potently inhibits HepG2 cancer cell growth, induces changes in MMP, and significantly reduces intracellular ROS generation in HepG2 cells in the presence or absence of $H_2O_2$. Collectively, the results from this study suggested that CA alone or in combination with Cis might be a promising agent in the treatment of liver cancer.

The authors declare no conflict of interest.

**Ethics Committe Approval:** There is no need to obtain ethical approval.

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