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RESEARCH ARTICLE

Effects of Pycnogenol and Its Combinations with Cisplatin on Hepatocellular Carcinoma Cell Viability

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SUMMARY

Many challenges of hepatocellular carcinoma treatment, such as side effects and drug resistance, still remain. Therefore, new improvements with high pharmaceutical function and low toxicity are needed. Recently, the efficacy of the combination therapy of antineoplastic drugs with natural products like pycnogenol has garnered attention.
Pycnogenol® is a standardized extract from the bark of Pinus pinaster and consists of phenolic compounds. Pycnogenol is considered complementary in the treatments of some cancer besides its good tolerability and high safety. This study aims to reveal the synergistic effects of pycnogenol combination with cisplatin and its relation to human hepatocellular carcinoma (HepG2) cell viability. Effects of single and combined treatment on cell viability were evaluated in Chinese hamster lung fibroblasts (V79) and HepG2 cells using MTT assay. In HepG2 cells, this combined treatment showed a more cytotoxic effect than single-dose groups. Pycnogenol increased the cytotoxicity of cisplatin at 500 µM for 24 h; at 250-500 µM for 48 h in V79 cells; and also, at 125-500 µM for 24 h; at 62.5-500 µM for 48 h in HepG2 cells ($p<0.05$). Our study is the first study to show that pycnogenol treatment with cisplatin has been combined in the HepG2 cell line. As a result, pycnogenol induced cisplatin cytotoxicity via combined treatment on HepG2 cells and exhibited a synergistic effect with cisplatin. In conclusion, pycnogenol may play a role in the chemotherapy of hepatocellular carcinoma; however, further studies are required to confirm their interactions with cisplatin.

Key Words: *Pycnogenol, cisplatin, MTT, cancer, hepatocellular carcinoma, HepG2 cells.*

Piknogenol ve Sisplatin ile Kombinasyonlarının Hepatoselüler Karsinom Hücre Canlılığı Üzerine Etkileri

ÖZ

Hepatoselüler karsinom tedavisinin yan etkiler ve ilaç direnci gibi birçok zorluğu devam etmektedir. Bu nedenle, farmasötik fonksiyonu yükseltecek ve toksisiteyi düşürecek yeni iyileştirmelere ihtiyaç vardır. Son zamanlarda, antineoplastik ilaçların piknogenol gibi doğal ürünlerle kombinasyonel tedavisinin etkinliği dikkat çekmektedir. Pycnogenol®, Pinus pinaster kabuğundan standartlaştırılmış bir ekstrakt olup fenolik bileşiklerden oluşur. Pycnogenolün, iyi tolere edilebilirliği ve yüksek güvenliği yanı sıra bazı kanserlerin tedavisinde de tamamlayıcı olduğu düşünülmektedir. Bu çalışmanın amacı, sisplatin ile piknogenol kombinasyonunun sinerjik etkilerini, bu etkilerin insan hepatosellüler karsinom (HepG2) hücre canlılığı ile nasıl bir ilişki olduğunu ortaya çıkarmaktır. Tek başlarına ve kombine olarak tedavinin hücre canlılığı üzerindeki etkileri, Çin hamsteri akciğer fibroblastlarında (V79) ve HepG2 hücrelerinde MTT kullanılarak değerlendirildi. HepG2 hücrelerindeki bu kombinasyon tedavi, tek doz gruplarından daha fazla sitotoksik etki gösterdi. Piknogenol, sisplatinin sitotoksisitesini V79 hücrelerinde 24 saatlik inkübasyonda 500 µM, 48 saatlik 250-500 µM konsantrasyonda ve ayrıca HepG2 hücrelerinde 24 saat için 125-500 µM, 48 saat için 62.5-500 µM'de anlamlı artırdı ($p <0.05$). Çalışmamız HepG2 hücre hattında piknogenolün sisplatin ile tedavisinin kombine edildiğini gösteren ilk çalışmadır. Sonuç olarak, Pycnogenol HepG2 hücreleri üzerinde kombine tedavi ile sisplatin sitotoksisitesini indükledi ve sisplatin ile sinerjistik bir etki gösterdi. Sonuç olarak, piknogenol, hepatosellüler karsinomun kemoterapisinde rol oynayabilir; bununla birlikte, sisplatin ile etkileşimlerini doğrulamak için daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: *Piknogenol, sisplatin, MTT, kanser, hepatoselüler karsinoma, HepG2 hücresi.*

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INTRODUCTION

Hepatocellular carcinoma, the most frequently occurring liver neoplasm, is still characterized by an important mortality rate. Even if there have been significant developing new therapeutic alternatives in the treatment of cancer, many challenges, such as side effects and drug resistance, still remain (Grazie et al., 2017). In order to overcome these challenges, new improvements with high pharmaceutical function and low toxicity are needed. Recently, the efficacy of the combination of antineoplastic drugs with natural products like pycnogenol has garnered attention, however knowing how to develop the effect of the combination treatment is of significance (Belcaro et al., 2008; Florea & Büsselberg, 2011; D'Andrea, 2010)

As an effective chemotherapeutic agent, cisplatin has been widely used in the treatments of various malignancies. The cytotoxic properties of cisplatin have not been completely understood. Increasing evidence, however, indicates that cisplatin induces apoptosis through DNA cross-linking and leads to nuclear and mitochondrial DNA damage. Despite its effectiveness, cisplatin often causes significant dose-limiting toxicity, including ototoxicity, neurotoxicity, nephrotoxicity, and cardiotoxicity. Oxidative stress, mitochondrial dysfunction, nuclear and mitochondrial DNA damage, activation of apoptotic pathways, and induction of inflammation have been associated with cisplatin-induced toxicity (Dugbartey et al., 2016). It has been proposed new therapeutic strategies, including combination therapy with antioxidant agents, which could mitigate or prevent these toxicities and improve anticancer effects. However, current studies are inadequate (Cheng et al., 2018).

Pycnogenol® (Horphag Research Ltd) is a standardized extract from the bark of *Pinus pinaster.* The standardized extract consists of phenolic compounds, divided between monomers (catechin, epicatechin, and taxifolin), condensed flavonoids (classed as procyanidins/proanthocyanidins), and phenolic acids (cinnamic acids and other glycosides) (Rohdewald, 2005; Simpson et al., 2019). As numerous studies report that pycnogenol components are highly bioavailable, pycnogenol is now consumed throughout the world as a nutritional supplement for various diseases in tablet or capsule forms in doses varying from 20 mg to 100 mg. Due to its multiple phenolic components, the most obvious feature of pycnogenol is its strong antioxidant activity (D'Andrea, 2010; Simpson et al., 2019). It has demonstrated good tolerability with few adverse effects, including gastrointestinal discomfort, dizziness, headache, and nausea. The adverse effects reported in the clinical trials were unrelated to the duration of use or dose of pycnogenol. Furthermore, pycnogenol has a high level of safety (Rohdewald, 2005; Simpson et al., 2019). Based on a recent update by the American Botanical Council of the Scientific and Clinical Monograph for Pycnogenol (The American Botanical Council, 2019), it is reported that an independent panel of toxicology experts has classified pycnogenol as generally recognized as safe (GRAS) based on clinical safety and preclinical toxicology data. Additionally, it is recommended that it should not be taken on children under 6 years of age and during the first 3 months of pregnancy due to limited study (Rohdewald, 2005; Simpson et al., 2019).

Many studies have suggested that it could possibly have anticancer activity in various human carcinoma cell lines by multiple mechanisms: prevention the reactive oxygen species formation, suppression of neoplastic transformation, augmented apoptotic activity (Yang et al., 2016). Up to now, the effect of pycnogenol against hepatocellular carcinoma cells has remained unknown. Also, there are not enough data about the interaction between antineoplastic drugs and pycnogenol. Furthermore, the underlying cellular and molecular processes on cancer are not clear. Therefore, new and advanced studies are needed to clarify the effects of pycnogenol and combinatorial therapy.

The purpose of this study was to evaluate the synergistic effects of cisplatin with the combination of pycnogenol, how this effect was related to cell viability in hepatocellular carcinoma cells. After the incubation with cisplatin and pycnogenol either alone or combination, the cell viability was evaluated in Chinese hamster lung fibroblasts (V79) and human hepatocellular carcinoma (HepG2) cells using

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MATERIALS AND METHODS

Materials

The chemicals were purchased from the suppliers: cisplatin (Koçak Farma, Turkey); dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), ethanol, fetal bovine serum (FBS), MTT, penicillin-streptomycin, trypan blue, trypsin–EDTA, RPMI 1640 medium, Dulbecco's phosphate-buffered saline (PBS) from Sigma (St. Louis, MO, USA); millipore filters from Millipore (Billerica, MA, USA), all other plastic materials from Corning (Corning Inc., NY, USA). Pycnogenol® was purchased from Horphag Research Ltd., (UK, Geneve, Switzerland). The quality of standardized pycnogenol extract is specified in the United States Pharmacopeia (USP). The stock solution of pycnogenol was freshly prepared in PBS and filtered with Millipore filters (0.20 µm). Negative control experiments were carried out with the culture medium containing only PBS (1%).

Cell culture

V79 and HepG2 cells were provided from the American Type Culture Collection (ATCC; Rockville, MD, USA). V79 cells were grown in RPMI-1640 medium, and HepG2 cells were grown in DMEM containing low glucose (1 g/L) and sodium pyruvate. Both types of media were supplemented with 10% FBS, 2mM L-glutamine, and 1% penicillin-streptomycin solution (10000 units of penicillin and 10 mg of streptomycin in 0.9 % NaCl) at 37°C in 5% CO2 incubators. The cells were subcultured in 75 cm^2 cell culture flasks. The culture medium was changed every 3 days. The passage numbers used in our study for both cell lines were between passage 8 and 10.

Determination of cytotoxicity

The effects of pycnogenol and cisplatin and their combination on cell viability were determined by MTT assay (van Meerloo, 2011). According to the cell viability data, IC50 was estimated. The cytotoxic profiles of pycnogenol on the IC50 of cisplatin were evaluated in a wide range of doses in V79 cells and HepG2 cells. Cells were seeded in 96-well plates containing 200 µL medium at a density of 1 × 104 cells/well and incubated to adhere to the plate for 24 h. The cells were treated with cisplatin (0.49-500 µM), pycnogenol (1.95-2000 µM), or the combination at the related culture medium for 24 h and 48 h. At the end of the incubation, 5 mg/mL MTT solution was added to each well and incubated for another 4 h at 37°C in the dark. Then the medium was discarded. The formazan crystals were dissolved in 200 µL of DMSO, and absorbance of each sample was detected at 570 nm using the microplate reader (SpectraMax M2, Molecular Devices Limited, Berkshire, UK). The percentage of cell viability was calculated using the formula: "Percentage of cell viability = (The absorbance of sample/ control) x 100" The cytotoxic concentration that killed cells by 50% (IC50) was determined from absorbance versus concentration curve.

Statistical analysis

All experiments were carried out in quadruplicate. The results were given as the mean ± standard deviation. The statistical analysis was performed with software programs "SPSS 10.5" (Statistical Package for the Social Sciences, Chicago, IL, USA). The distribution of the data was checked for normality using the Kolmogorov-Smirnov test. The means of data were compared by the one-way variance analysis test and post hoc analysis of group differences was performed by the least significant difference (LSD) test. A p values less than 0.05 were considered statistically significant.

RESULTS and DISCUSSION

Cytotoxic effects of pycnogenol and cisplatin in V79 cells and HepG2 cells

Cells were treated with the increasing concentrations of pycnogenol (1.95-2000 µM) and cisplatin (0.49-500 µM) alone for 25 h and 48 h. The results of pycnogenol and cisplatin cytotoxicity are given in Figure 1 and Figure 2, for V79 cells and HepG2 cells, respectively.

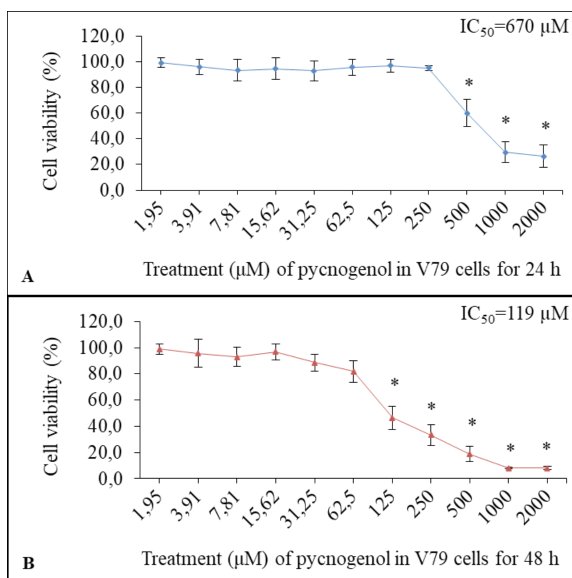

Figure 1. Cytotoxic effects of pycnogenol on the viability of V79 cells for 24 h (A) and 48 h (B). The values were given as the mean ± standard deviation (n=4). *p < 0.05, compared to negative control (PBS).

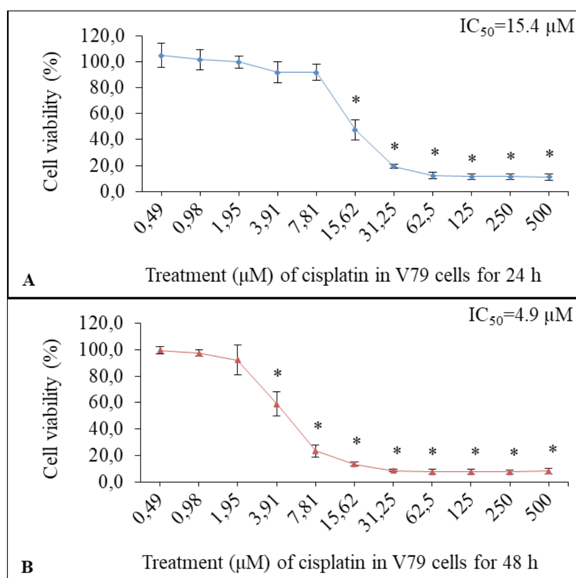

Figure 2. Cytotoxic effects of cisplatin on the viability of V79 cells for 24 h (A) and 48 h (B). The values were given as the mean ± standard deviation (n=4). *p < 0.05, compared to negative control (PBS).

In V79 cells, pycnogenol did not cause a significant cytotoxic effect at the concentrations of 1.95-250 µM and 1.95-62.5 µM when compared to the negative control for 24 h and 48 h incubation, respectively; however, the cell viabilities were significantly decreased above 500 µM and 125 µM of pycnogenol for 24 h and 48 h incubation, respectively, in a dose-dependent manner ($p < 0.05$). The IC50 value of pycnogenol was found to be 670 µM and 119 µM for 24 h and 48 h, respectively (Figure 1A and Figure 1B) (Table 1). Cisplatin did not cause a significant cytotoxic effect at the concentrations of 0.49-7.81 µM and at the concentrations of 0.49-1.95 µM when compared to the negative control for 24 h and 48 h, respectively; however, the cell viabilities were significantly decreased above 15.62 µM and 3.91 µM of cisplatin for 24 h and 48 h incubation, respectively, in a dose-dependent manner ($p < 0.05$). The IC50 value of cisplatin was found to be 15.4 µM and 4.9 µM for 24 h and 48 h, respectively (Figure 2A and Figure 2B) (Table 1).

In HepG2 cells, pycnogenol did not cause a significant cytotoxic effect at the concentrations of 1.95-62.5 µM and 1.95-15.6 µM when compared to the negative control for 24 h and 48 h, respectively; however, the cell viabilities were significantly decreased above 125 µM and 31.25 µM of pycnogenol for 24 h and 48 h incubation, respectively, in a dose-dependent manner ($p < 0.05$). The IC50 value of pycnogenol was found to be 192 µM and 51.5 µM for 24 h and 48 h, respectively (Figure 3A and Figure 3B) (Table 1). Cisplatin did not cause a significant cytotoxic effect at the concentrations of 0.49-7.81 µM and at the concentrations of 0.49-3.91 µM when compared to the negative control for 24 h and 48 h, respectively; however, the cell viabilities were significantly decreased above 15.62 µM and 3.91 µM of cisplatin for 24 h and 48 h incubation, respectively, in a dose-dependent manner ($p < 0.05$). The IC50 value of cisplatin were found to be 25.5 µM and 7.7 µM for 24 h and 48 h, respectively (Figure 4A and Figure 4B) (Table 1).

Table 1. The IC_{50} values of pygnogenol and cisplatin in V79 and HepG2 cells

Pygnogenol	**V79 cells**	**HepG2 cells**
IC_{50} (24 h)	670 µM	192 µM
IC_{50} (48 h)	119 µM	51.5 µM
Cisplatin	**V79 cells**	**HepG2 cells**
IC_{50} (24 h)	15.4 µM	25.5 µM
IC_{50} (48 h)	4.9 µM	7.7 µM

Effects of pycnogenol on cisplatin cytotoxicity in V79 cells and HepG2 cells

In V79 cells, the effects of pycnogenol on cisplatin cytotoxicity at the concentrations range of 15.6-500 µM are shown in Figure 5, for 24 h and 48 h. As depicted in Figure 5A, pycnogenol did not change the IC50 value of cisplatin (15 µM, approximately) at the concentrations of 15.6-250 µM for 24 h incubation; however, the IC50 value of cisplatin was significantly reduced in V79 cells treated with 500 µM of pycnogenol (1,75 fold; vs. positive control) for 24 h incubation and 1000 µM (of pycnogenol (4,04 fold; vs. positive control) ($p < 0.05$). As depicted in Figure 5B, pycnogenol did not change the IC50 value of cisplatin (5 µM, approximately) at the concentrations of 15.6-62.5 µM for 48 h incubation; however, the IC50 value of cisplatin was significantly reduced at concentrations of 125 µM, 250 µM, 500 µM and 1000 µM of pycnogenol (2.26, 2.92, 6.96, and 12.43 fold; vs. positive control) ($p < 0.05$). As a result, pycnogenol did not change the IC50 value of cisplatin at non-cytotoxic doses (15.6-250 µM pycnogenol for 24 h and 15.6-62.5 µM pycnogenol for 48 h) ($p > 0.05$).

In HepG2 cells, the effects of pycnogenol at the concentrations range of 15.6-500 µM on cisplatin cytotoxicity are shown in Figure 6, for 24 h and 48 h. As depicted in Figure 6A, pycnogenol did not change the IC50 value of cisplatin (25 µM, approximately) at the concentrations of 15.6-62.5 µM for 24 h incubation; however, the IC50 value of cisplatin was significantly reduced at the concentration of 125 µM, 250 µM, and 500 µM of pycnogenol (1.32 and 2.50 and 4.38 fold; vs. positive control) for 24 h incubation ($p < 0.05$). As depicted in Figure 6B, pycnogenol did not change the IC50 value of cisplatin (10 µM, approximately) at the concentrations of 15.6-31.25 µM for 48 h incubation; however, the IC50 value of cisplatin was significantly reduced at concentrations of 62.5 µM, 125 µM, and 250 µM and 500 µM of pycnogenol (1.29 and 1.63 and 2.96 and 6.56 fold; vs. positive control) ($p < 0.05$). As a result, pycnogenol did not change the cytotoxicity of cisplatin at non-cytotoxic doses (15.6-62.5 µM pycnogenol for 24 h and 15.6 µM pycnogenol for 48 h) ($p > 0.05$).

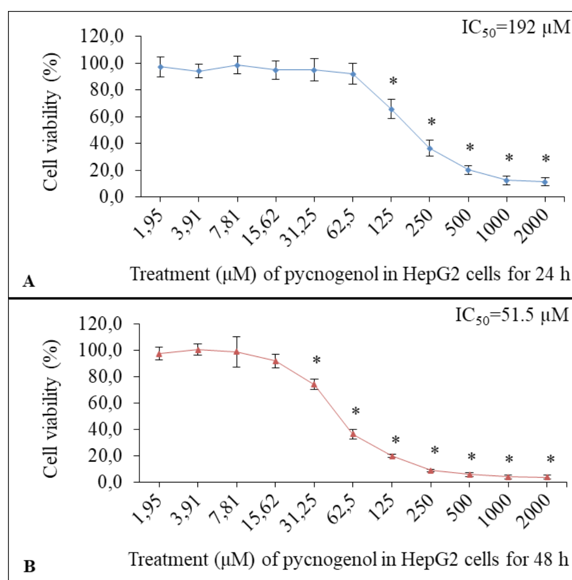

Figure 3. Cytotoxic effects of pycnogenol on the viability of HepG2 cells for 24 h (A) and 48 h (B). The values were given as the mean ± standard deviation (n=4). *p < 0.05, compared to negative control (PBS).

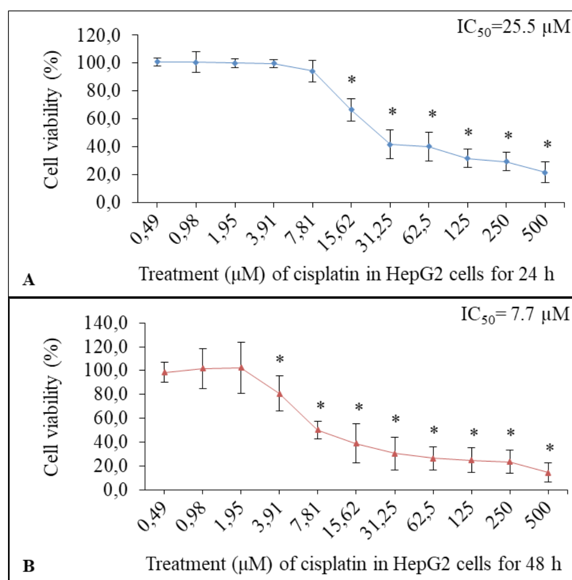

Figure 4. Cytotoxic effects of cisplatin on the viability of HepG2 cells for 24 h (A) and 48 h (B). The values were given as the mean ± standard deviation (n=4). *p < 0.05, compared to negative control (PBS).

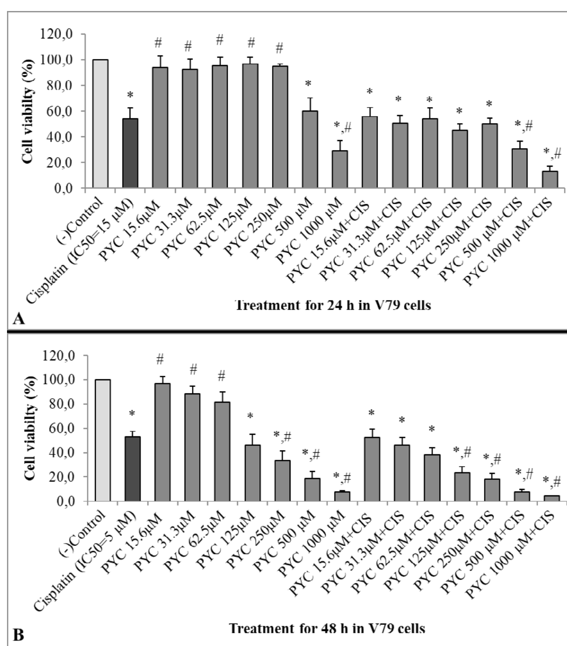

Figure 5. Effects of pycnogenol on the cisplatin cytotoxicity in V79 cells for 24 h (A) and 48 h (B). The values were given as mean ± standard deviation (n=4). *p<0.05, compared to negative control (PBS); #p<0.05, compared to IC_{50} value of cisplatin (15 **µM** for 24 h treatment and 5 **µM** for 48 h treatment). PYC: pycnogenol; CIS: cisplatin.

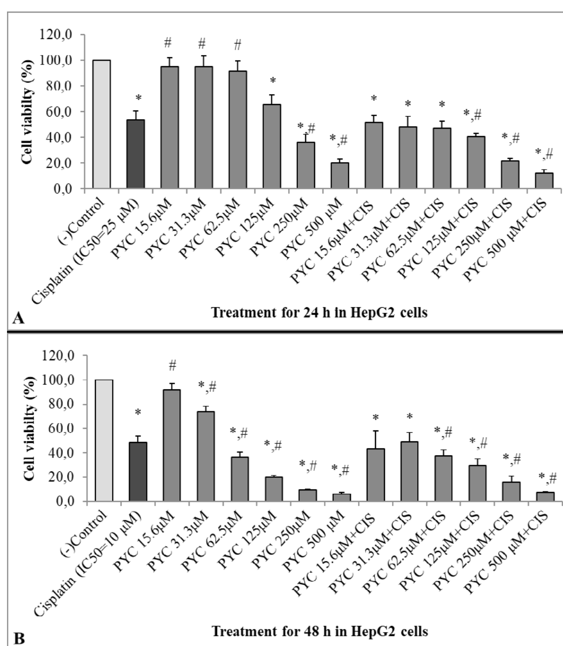

Figure 6. Effects of pycnogenol on the cisplatin cytotoxicity in HepG2 cells for 24 h (A) and 48 h (B). The values were given as mean ± standard deviation (n=4). *p<0.05, compared to negative control (PBS); #p<0.05, compared to IC_{50} value cisplatin (25 **µM** for 24 h treatment and 10 **µM** for 48 h treatment). PYC: pycnogenol; CIS: cisplatin.

DISCUSSION

Many challenges, such as side effects and drug resistance, still remain in the treatment of hepatocellular carcinoma (Grazie et al., 2017). In order to overcome these challenges, new improvements with high pharmaceutical function and low toxicity are needed. For this reason, the synergistic effects of the combination of antineoplastic drugs with natural products like pycnogenol, which has many different properties besides its good tolerability and high level of safety, has garnered attention. (Belcaro et al., 2008; Florea & Büsselberg, 2011; D'Andrea, 2010; Simpson et al., 2019).

Numerous reports have claimed that pycnogenol could possibly have anticancer activity in various human carcinoma cell lines, including mammary (MCF-7) (Huynh & Teel, 2000), promyeloid leukemia (HL- 60, K562 and U937), (Huang et al., 2005), ovarian (Buz'Zard & Lau, 2007), mucoepidermoid carcinoma (MC-3) (Yang et al., 2014), fibrosarcoma (HT1080) (Harati et al., 2015), oral squamous (HSC-3) (Yang et al., 2016) cancer cells. Harati et al. (2015) investigated the apoptotic effects of pycnogenol and its constituents on human fibrosarcoma cells (HT1080) and also its metabolites from healthy subjects who intake a single dose of 300 mg pycnogenol orally, using flow cytometric analysis and RNA microarray. It was reported that pycnogenol induced apoptosis in HT1080 cells. Therefore, the results provide experimental support for in vivo trials assessing the anticancer effect of pycnogenol. These studies have revealed that pycnogenol acts by multiple mechanisms: prevention the reactive oxygen species formation, suppression of neoplastic transformation, augmented apoptotic activity in a dose-dependent manner (Yang et al., 2016). Yang et al. (2016) also informed that pycnogenol at higher concentrations clearly produced reactive oxygen species, and it may be a pro-oxidant in human oral squamous carcinoma (HSC-3) cells. It also suggests that the pro-oxidant action of pycnogenol might be a critical mechanism of its anticancer potential. These studies may help understand the underlying mechanism of the synergistic cytotoxic effects of combination therapy on cell viability, at least in part. Despite a few studies,

little has been discovered relating to the effects of pycnogenol human cancer on cancer yet. Up to now, the effect of pycnogenol against hepatocellular carcinoma cells remains unknown. Considering the importance of oxidative stress in the pathophysiology of cancer, pycnogenol may be a potential candidate for chemoprevention or chemotherapy due to its strong antioxidant activity. It is suggested that a combination consisting of pycnogenol may increase antitumor effects and reduce the toxicity associated with oncologic treatment (Belcaro et al., 2008).

As an effective chemotherapeutic agent, cisplatin has been widely used in the clinical treatments of various malignancies. Increasing evidence indicates that cisplatin induces apoptosis through DNA cross-linking and leads to nuclear and mitochondrial DNA damage. Despite its effectiveness, cisplatin often causes significant dose-limiting toxicity, including ototoxicity, neurotoxicity, nephrotoxicity, and cardiotoxicity against normal cells and tissues. Oxidative stress, mitochondrial dysfunction, nuclear and mitochondrial DNA damage, activation of apoptotic pathways, and induction of inflammation is associated with cisplatin-induced toxicity (Dugbartey et al., 2016). It has been proposed new therapeutic strategies, including combination therapy with antioxidant agents, which could mitigate or prevent these toxicities and improve anticancer effects (Cheng et al., 2018).

The combination of cisplatin with pycnogenol, which targets ROS generation, inflammatory and apoptotic pathways in cisplatin toxicity may offer clinically meaningful (Dugbartey et al., 2016; Florea & Büsselberg, 2011). Numerous researches have reported the ameliorative and preventing effects of pycnogenol in cisplatin-induced toxicity including hepatotoxicity (Ko et al., 2014), ototoxicity (Eryılmaz et al., 2016), acute kidney injury (Lee et al., 2017), optic nerve injury (Icel et al., 2018). However, current studies are inadequate about the interaction between cisplatin and pycnogenol.

In our previous study, we reported that pycnogenol might increase the cisplatin cytotoxicity in human cervix cancer (HeLa) cells at its non-genotoxic doses, which suggests that pycnogenol might contribute to the anticancer effect of cisplatin in cervical carcinoma (Becit et al., 2020).

In our current study, we aimed to investigate the combined synergistic effects of cisplatin with pycnogenol upon cell viability in HepG2 cells and offer a new approach for hepatocellular carcinoma treatment. After the incubation with cisplatin and pycnogenol either alone or combination, the cell viabilities were evaluated in normal and cancerous cell lines, V79 and HepG2 cells, respectively, using MTT assay.

We determined the effects of pycnogenol (1.95-2000 μM) and cisplatin (0.49-500 μM) on the viabilities of V79 cells and HepG2 cells, for 24 h and 48 h. The results showed that the cytotoxicity profiles of pycnogenol and cisplatin alone were different in terms of exposure time and dose. In V79 cells, the IC50 values of pycnogenol were found to be 670 μM and 119 μM; in HepG2 cells, the IC50 values of pycnogenol were found to be 192 μM and 51.5 μM, for 24h and 48h, respectively. Pycnogenol cytotoxicity (IC50 value) increased approximately ~5.6-fold in V79 cells, ~3.7-fold in HepG2 cells for 48 h when compared to 24 h incubation. Additionally, in HepG2 cells, the pycnogenol cytotoxicity was found to be ~3.5-fold and ~2.3-fold lower, for 24 h and 48 h, respectively, when compared to V79 cells. The IC50 values of cisplatin were found to be 15.4 μM and 4.9 μM for 24 h and 48 h, respectively, in V79 cells; the IC50 values of cisplatin were found to be 25.5 μM and 7.7 μM for 24 h and 48 h, respectively, in HepG2 cells. Cisplatin cytotoxicity increased approximately ~3.1-fold in V79 cells, ~3.3-fold in HepG2 cells for 48 h when compared to 24 h incubation.

The IC50 value of pycnogenol was observed differently in several studies listed. In a study, the IC50 values in HL-60, U937, and K562 were detected as 150 μg/ml (~516.8 μM), 40 μg/ml (~137.8 μM), and 100 μg/ml (~344.5 μM), respectively, for 24 h incubation, by propidium iodide exclusion method. Huang et al. (2005) also reported that pycnogenol inhibited cell proliferation, dose, and time-dependently. It seems that pycnogenol may be hopeful about the treatment and prevention of human leukemia. In another study, the IC50 value was determined to be 285 μg/ml (~982

µM) for 24 h incubation, in Chinese hamster ovary (CHO) cells, by NRU test (Taner et al., 2013). Yang et al. (2016) reported that the IC50 value was 20 µg/ml (~ 68.9 µM) in HSC-3 cells for 24 h using MTS assay. (Yang et al., 2016).

Similarly, there are studies reporting the IC50 value of cisplatin differently. The IC50 value of cisplatin in the selected human cancer cells was reported to be 14.87 µM and 77.89 µM in hepatocellular carcinoma (Hep-G2 and SK-HEP-1) cells, respectively; 97.20 µM and 85.66 µM in pancreatic cancer (MIA PaCa-2 and BxPC-3) cells, respectively; 54.07 µM and 96.38 µM in cervical cancer (HeLa and Caco-2) cells, respectively; for 24 h incubation, using MTT method (Nurcahyanthi et al., 2016).

The IC50 values obtained from the current studies on pycnogenol cytotoxicity seem to be inconsistent. It is thought that IC50 dose differences between our results and literature results may arise from the cytotoxicity method, exposure time, selected cells, and their properties. However, as shown by our teams and others, pycnogenol has gained increased interest in both the prevention and treatment of various cancers by multiple mechanisms.

After the determination of IC50 values, we investigated the effects of pycnogenol on cisplatin cytotoxicity in V79 cells and HepG2 cells. In V79 cells, pycnogenol significantly reduced the IC50 value of cisplatin (15 µM for 24 h treatment and 5 µM for 48 h treatment) above 500 µM for 24 h and at the concentrations of 125-1000 µM for 48 h($p<0.05$), which indicates the synergistic cytotoxic effects of pycnogenol with cisplatin. In HepG2 cells, pycnogenol significantly reduced the IC50 value of cisplatin (25 µM for 24 h treatment and 10 µM for 48 h treatment) at the concentrations of 125-500 µM for 24 h, at the concentrations of 62.5-500 µM for 48 h incubation, respectively ($p<0.05$). Based on our findings, the combination of cisplatin with pycnogenol showed a synergistic effect by inhibiting cell viability in a time and dose manner. To our knowledge, this study reported for the first time that the combination showed to synergistic cytotoxic effect in HepG2 cells. Our results suggest pycnogenol is a potential candidate in combination therapy with cisplatin in the treatment of hepatocellular carcinoma.

As consistently, according to a study that shows the synergistic anticancer effect of combination therapy with doxorubicin and grape seed extract in human breast carcinoma (MCF-7 and MDA-MB468) cells; these results suggest promising effects of the combination of antineoplastic drug and phenolic compound for breast cancer treatment (Sharma et al., 2004).

CONCLUSION

In the scope of this study, the combined treatment of cisplatin with pycnogenol in HepG2 cells showed anticancer effect more than single-dose groups. On the basis of our findings, we conclude that pycnogenol may be a promising candidate for chemoprevention or chemotherapy of hepatocellular carcinoma and may develop new therapeutic approaches. Further researches and studies are needed to determine the molecular mechanisms of pycnogenol in tumor cells, the appropriate dose, and treatment methods for this combination. In this regard, our study shows for the first time that combined treatment of cisplatin and pycnogenol in the HepG2 cell line. Hence, it can be a source by providing a basis for further researches.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Idea (Becit M., Aydın Dilsiz S., Başaran N.), design (Aydın Dilsiz S.), supervision (Becit M., Aydın Dilsiz S., Başaran N.), data collection and processing (Becit M., Aydın Dilsiz S.), analysis (Becit M., Aydın Dilsiz S., Başaran N.), data interpretation (Becit M., Aydın Dilsiz S., Başaran N.), literature review (Becit M., Aydın Dilsiz S.), writing article (Becit M., Aydın Dilsiz S., Başaran N.), critical review (Aydın Dilsiz S., Başaran N.), materials (Becit M., Aydın Dilsiz S.), funding (Aydın Dilsiz S.).

REFERENCES

Becit, M., & Aydın, S. (2020) An In Vitro Study on the Interactions of Pycnogenol® with Cisplatin in Human Cervical Cancer Cells. *Turkish Journal of Pharmaceutical Sciences, 17*(1), 1-6.

Belcaro, G., Cesarone, MR., Genovesi, D., Ledda, A., Vinciguerra, G., Ricci, A., & et al. (2008). Pycnogenol may alleviate adverse effects in oncologic treatment. *Panminevra Med,50*(3), 227-34.

Buz'Zard, AR., & Lau, BHS. (2007). Pycnogenol® reduces Talc-induced Neoplastic Transformation in Human Ovarian Cell Cultures. *Phytotherapy Research, 21*(6), 579-586.

Cheng, Y., Zhao, P., Wu, S., Yang, T., Chen, Y., Zhang, X., & et al. (2018). Cisplatin and Curcumin Co-loaded Nano-liposomes for the Treatment of Hepatocellular Carcinoma. *International Journal of Pharmaceutics, 545*(1-2), 261-273.

D'Andrea, G. (2010). Pycnogenol: A blend of procyanidins with multifaceted therapeutic applications? *Fitoterapia*, *81*(7), 724-36.

Dugbartey, GJ., Peppone LJ. & Graaf, IAM.de. (2016). An integrative view of cisplatin-induced renal and cardiac toxicities: molecular mechanisms, current treatment challenges and potential protective measures. *Toxicology, 371,* 58-66. doi.org/10.1016/j.tox.2016.10.001

Eryilmaz, A., Eliyatkin, N., Demirci, B., Basal, Y., Kurt Omurlu, I., Gunel, C., & et al. (2016). Protective effect of Pycnogenol on cisplatin-induced ototoxicity in rats. *Pharmaceutical Biology, 54*(11), 2777-2781.

Florea, AM., & Busselberg D. (2011). Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. *Cancers (Basel), 3*(1), 1351-71.

Grazie, ML., Biagini, MR., Tarocchi, M., Polvani, S., & Galli, A. (2017). Chemotherapy for hepatocellular carcinoma: The present and the future. *World J Hepatol*, *9*(21), 907-20.

Harati, K., Slodnik, P., Chromik, AM., Behr, B., Goertz, O., Hirsch, T., & et al. (2015). Pro-apoptotic effects of pycnogenol on HT1080 human fibrosarcoma cells. *International Journal of Oncology*, *46*(4), 1629-36.

Huang, WW., Yang, JS., Lin, CF., Ho, WJ., & Lee, MR. (2005). Pycnogenol induces differentiation and apoptosis in human promyeloid leukemia HL-60 cells. *Leukemia Research*,*29*(6),685-92.

Huynh, H.T., & Teel, R.W. (2000), Selective induction of apoptosis in human mammary cancer cells (MCF- 7) by pycnogenol, *Anticancer Res, 20* (4), 2417-20.

Icel, E., Uçak, T., Agcayazi, B., Karakurt, Y., Yilmaz, H., Keskin Çimen, F., & et al. (2018). Effects of Pycnogenol on cisplatin-induced optic nerve injury: an experimental study. *Cutaneous and Ocular Toxicology, 37*(4), 396-400.

Ko, JW., Lee, IC., Park, SH., Moon, C., Kang, SS., Kim, SH., & et al. (2014). Protective effects of pine bark extract against cisplatin-induced hepatotoxicity and oxidative stress in rats. *Laboratory Animal Research, 30*(4), 174-180.

Lee, IC., Ko, JW., Park, SH., Shin, NR., Shin, IS., Kim, YB., & et al. (2017). Ameliorative effects of pine bark extract on cisplatin-induced acute kidney injury in rats. *Ren Fail. 39*(1), 363-371.

van Meerloo, J., Kaspers, GJ., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Methods in Molecular Biology, 731,* 237-45.

Nurcahyanti, AD., & Wink, M. (2016). L-Canavanine potentiates the cytotoxicity of doxorubicin and cisplatin in arginine deprived human cancer cells. *J-PEER | Purdue University Press Open Access Journals, 4*:e1542.

Rohdewald, P. (2005). "Pycnogenol®, French maritime pine bark extract," in Encyclopedia of dietary supplements. Eds. P. Coates, M. Blackman, G. Cragg, M. Levine, J. Moss, and J. White (New York: Marcel Dekker), 545–553. doi: 10.1201/b13959-57.

Simpson, T., Kure, C., & Stough, C. (2019). Assessing the Efficacy and Mechanisms of Pycnogenol® on Cognitive Aging From in Vitro Animal and Human Studies. *Frontiers in Pharm, 10*, 694.

Sharma, G., Tyagi, AK., Singh, RP., Chan, DC., & Agarwal, R. (2004). Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. *Breast Cancer Research and Treatment, 85*(1), 1-12.

Taner, G., Aydin, S., Aytac, Z., Basaran, AA., & Basaran, N. (2013). Assessment of the cytotoxic, genotoxic and antigenotoxic potential of Pycnogenol in in vitro mammalian cells. *Food and Chemical Toxicology, 61*, 203-208.

Taner, G., Aydin, S., Bacanli, M., Sarigol, Z., Sahin, T., Basaran, AA., & et al. (2014) Modulating effects of pycnogenol on oxidative stress and DNA damage induced by sepsis in rats. *Phytotherapy Research, 28*(11), 1692-700.

The American Botanical Council (2019). Scientific and clinical monograph for Pycnogenol (French maritime pine bark extract) Pinus pinaster Aiton subsp. *atlantica* [Fam. Pinaceae].

Yang, IH., Shin JA., Cho, SD. (2014). Pycnogenol Induces Nuclear Translocation of Apoptosis-inducing Factor and Caspase-independent Apoptosis in MC-3 Human Mucoepidermoid Carcinoma Cell Line. *Journal of Cancer Prevention, 19*(4):265-72.

Yang, IH., Shin, JA., Kim, LH., Kwon, KH., & Cho, SD. (2016). The caspase 3-dependent apoptotic effect of pycnogenol in human oral squamous cell carcinoma HSC-3 cells. *Journal of Clinical Biochemistry and Nutrition*, *58*(1), 40-7.