

Toros Göknarı (*Abies cilicica* (Antoine & Kotschy) Carrière) 'ndan Elde Edilen Reçinenin *In vitro* Antioksidan ve Sitotoksik Aktivitelerinin Belirlenmesi

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ÖZET

Bu çalışmada, Toros Göknarı bitkisinden elde edilen reçinenin antioksidan ve sitotoksik aktivite değerleri değerlendirilmiştir. Bu reçine, 2,2'-Azino-bis-(3-etilbenzotiazolin-6-sülfonik asit) (ABTS) radikal süpürme testine göre antioksidan aktiviteye sahiptir. Reçinenin *in vitro* sitotoksik aktivitesi, bir dizi insan kanser hücresine (MDA-MB-231, Hep G2, PC-3, U-87, MCF-7, HT-29) karşı MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromid) analizi ile 48 saat boyunca araştırıldı. Sağlıklı hücreler olarak normal insan akciğer fibroblast hücreleri (WI-38) kullanıldı. Sonuçlar, reçinenin *in vitro* sitotoksik aktivitesinin hücre hattı tipine ve numunenin konsantrasyonuna bağlı olduğunu göstermiştir. IC₅₀ değerlerine göre, reçine diğer kanser hücrelerine kıyasla endometriyal adenokarsinom kanser hücreleri üzerinde en fazla sitotoksik aktiviteye sahiptir (IC₅₀ = 8.94 ± 0.03 µg mL⁻¹). Sonuçlar ayrıca seçicilik indeks (SI) değeri > 2 olan Ishikawa endometriyal adenokarsinom hücrelerinin reçineye karşı en yüksek hassasiyete sahip olduğunu göstermiştir. Bu çalışma, reçinenin farklı kanser hücrelerinin büyümesini engellediğine dair ilk kanıtları sunmaktadır.

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Anahtar Kelimeler

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Sitotoksikite

The Determination of *In vitro* Antioxidant and Cytotoxic Activities of Resin Obtained from Cilician Fir (*Abies cilicica* (Antoine & Kotschy) Carrière)

ABSTRACT

In this study, the antioxidant and cytotoxic activities of resin obtained from the Cilician Fir plant were evaluated. This resin has antioxidant activity according to 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay. The *in vitro* cytotoxic activity of the resin was investigated against a panel of human cancer cells (MDA-MB-231, Hep G2, PC-3, U-87, MCF-7, HT-29) with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for 48 h. Normal human lung fibroblast cells (WI-38) were used as healthy cells. The results indicated that the *in vitro* cytotoxic activity of the resin depends on the cell line type and concentration of the resin. According to the IC₅₀ values, the resin has the most cytotoxic activity on endometrial adenocarcinoma cancer cells (IC₅₀=8.94 ± 0.03 µg mL⁻¹) compared to other cancer cells. The results also indicated that Ishikawa endometrial adenocarcinoma cells, which have Selectivity Index (SI) value >2, have the most sensitivity against the resin. This study provides the first evidence that the resin inhibits the different cancer cells' growth.

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GİRİŞ

Abies belong to the Pinaceae family, and this genus has

approximately 50 species worldwide (Fujita et al. 1995, Singh et al. 2000). Some *Abies* species are used in folk

medicines such as colds, bronchitis, stomach-ache, dyspepsia, and tuberculosis (Fujita et al. 1995, Yeşilada et al. 1995, Singh et al. 2000, Wu et al. 2016). It is also known to have antioxidant, antibacterial and antitumor activities (Handa et al. 2013, Hasegawa et al. 1987, Lavoie et al. 2013, Li et al. 2015, Wang et al. 2015). *Abies cilicica* is one of the native fir species in Turkey, with two subspecies of *A. cilicia* subsp. *isaurica* and *A. cilicia* subsp. *cilicica*. *Abies cilicica* subsp. *cilicica* is known as Cilician Fir in the Mediterranean region of Turkey. *Abies cilicica* subsp. *cilicica* does not have resinous buds and has hairy young shoots (Davis 1967).

Plant medicines and natural products are currently widely used as traditional medicine, and because of the side-effects of some chemical drugs are increasing in importance and attracting more attention (Pohanka 2011). It has been known for decades that plant extracts obtained from medicinal and aromatic plants have antioxidant, antimicrobial and anticancer effects (Yeomans 1996, Do 2004, Kunyanga et al. 2012). Some active components of plant extracts can prevent oxidative tissue damage caused by oxygen free radicals (Waris and Ahsan 2006, Evans et al. 2004, Silva et al. 2006) and in this regard, the antioxidant uptake into the body plays an important role in preventing various diseases such as cancer and cardiovascular diseases and in delaying the aging process (Albayrak et al. 2010).

Plant chemicals have primary and secondary metabolites. Although primary metabolites are needed for plants because of their role in basic cell metabolism, secondary metabolites have no effect on the plant's primary metabolism. Secondary metabolites are generally produced for defense against ecological conditions. Medicinal and aromatic plants often have organic compounds such as oils, resins, tannins, natural rubbers, waxes and dyes (Camarda et al. 2011).

Currently and in the future, it is expected that there will be more research into the chemical or biologically active constituents. The aim of this study was to focus on the antioxidant and cytotoxic activities of the resin obtained from Cilician Fir (*Abies cilicica* (Antoine & Kotschy) Carrière) as a biologically active component to find a new active ingredient against cancer.

MATERYAL ve METOD

This study was conducted in the laboratories of CUTAM (Cumhuriyet University Advanced Technology Research Center), Cumhuriyet University, Sivas in 2019. Fir plants were collected from the wild flora of Kahramanmaraş (Göksun district, Tekir plateau (1400m)) in Turkey. The experiments were carried out in completely randomized design with three replications.

The Chemical Components and Determination of Biological Activity

Preparation of Extracts

The resin that accumulating and collected on tree bark, was used as the study material (Figure 1). 10 gr resin was soaked in 20 mL ethanol for 24 h with intermittent agitation. At the end of this process, a uniform solution was obtained. The obtained extracts were analyzed by GC-MS



Figure 1. Image of resin accumulating on tree bark
Şekil 1. Ağaç kabuğu üzerinde biriken reçine görüntüsü

Antioxidant Assay

DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract was examined using the Clarke method (2013). This method was slightly modified as follows. 20 µL of test solution was mixed with 180 µL of DPPH solution and placed in a 96-well plate. The plates were left in the dark for 15 min, then absorbance was read with an ELISA reader at 540 nm. Gallic acid solution prepared with DMSO instead of test resin as standard and DMSO as the control were run in parallel. All the experiments were made in parallel in three groups and the results were evaluated. The standard average error (SEM) was calculated. The results are expressed as % DPPH sweeping effect using the following equation.

2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay

The method of Chun et al. (2005) was used to determine the ABTS scavenging activity of the plant extract. On a 96-well plate, 50 µL resin solution was mixed with 100 µL ABTS solution. Then, the mix of ABTS solution and resin solution was kept for 10 minutes (room temperature), and absorbance was read at 734 nm.

Total phenol content

The total amount of phenol was performed according to Clarke method (2013). After experiment, the total phenol quantities were calculated from the absorbance values of the samples (Clarke et al. 2013).

Total flavonoid content

The aluminum chloride colorimetric method was used to determine total flavonoid content in the extract. The quantities of total flavonoids were calculated as mg equivalent of quercetin over dry weight of extract (Yang et al. 2011).

Cell culture

The MTT assay was done against MDA-MB-231 and MCF-7 breast, HepG2 liver, Ishikawa endometrial, PC-3 prostate, U-87 glioblastoma, HT-29 colon cancer cells to determine the *in vitro* cytotoxic activity of the resin. The resin was also applied to WI-38 healthy cells to investigate selectivity of the resin between cancer cells and healthy cells. MDA-MB-231, MCF-7, HT-29 and PC-3, cells were cultured in the DMEM medium. HepG2, Ishikawa, U-87, and WI-38 cell lines were cultured in EMEM medium. 10% FBS and 1% antibiotics solution were added in DMEM and EMEM medium. All cells were plated of 1×10^5 cells mL^{-1} (each well 100 μL) in 96-well plates and incubated one day. The resin was dissolved in DMSO and 1 μL of the different concentration ($1-10\ 000\ \mu\text{g}\ \text{mL}^{-1}$) of the resin was added and the incubated of 48 h of incubation. Culture medium and sterile DMSO (0.5% v/v) were added controls and negative control wells. After 48 hrs, 10 μL of MTT (5 mg mL^{-1} in PBS) in PBS was added and the plates were incubated for 2-3 h. After, 100 μL of DMSO was added to each well and the plates were incubated for 15 min at RT with agitation. The

absorbance values were at 570 nm on an ELISA reader (Biotek, Epoch, USA).

Statistical analysis

All *in vitro* cytotoxic activity experiments were carried out in triplicate (n=9) and the results were expressed as mean \pm SEM. Data were analyzed using one-way analysis of variance and Dunnett's multiple comparisons test. Differences were considered statistically significant at *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001. The IC₅₀ were determined by statistical software, GraphPad Prism7 (GraphPad Software, San Diego, CA, USA).

BULGULAR ve TARTIŞMA

Chemical composition

The Cilician fir ethanol extracts were analyzed with GC-MS and the chemical composition of the plant was evaluated. According to the data obtained, the major component of the Cilician fir was determined to be α -pinene (50.43%). The other components determined were 2- β -pinene (20.17%), 1-limonene (11.53%), 3,4-Dihydrothenyl-[3,4,B]-5-Carboxythiol (9.22%) and Butanoic acid, 3,7-dimethyl-2,6-octadienyl ester (8.64%) (Table 1). Other researchers have also determined α -pinene to be the major component (Kilic Pekközlü and Ceylan 2018, Dayısoylı and Mehmet 2009).

Table 1. The chemical composition of ethanol extract of resin from Cilician Fir

Tablo 1. Toros Göknarı'ndan elde edilen etanol reçine ekstraktının kimyasal kompozisyonu

No	Chemical components	RT	Ethanol extract (%)
1	Alpha pinene	9.08	50.43
2	2- β pinene	10.53	20.17
3	3,4-Dihydrothenyl-[3,4,B]-5-Carboxythiol	1.94	9.22
4	1-limonene	12.32	11.53
5	Butanoic acid, 3,7-dimethyl-2,6-octadienyl ester	11.09	8.64
Total			99.99

Antioxidant activity

DPPH and ABTS radical scavenging activity

The *in vitro* antioxidant activities such as DPPH and ABTS radical scavenging activities of Cilician fir in ethanol extract were determined and the results were compared with the standard antioxidants. The percentage DPPH and ABTS radical scavenging capabilities of the Cilician fir resin extract are illustrated in Figure 2. According to the data obtained, the scavenging effect of the extract on ABTS radical increased linearly with increasing concentration from 0.1 to 2.0 mg ml^{-1} , although lower than the gallic acid that was the standard. According to Broznic et al. (2018) and Albanese et al. (2019) *Abies alba* Mill. plant has antiradical activity. Vasincu et al. (2013) reported that the antioxidant activity of *Abies alba* were

investigation with different radical scavenging activity methods. They obtained that in the DPPH method showed lower effect than other methods. In this study, DPPH method showed lower activity compared to ABTS method.

Total phenol and flavonoid content

Phenolic compounds are generally used for protection from oxidative damage for living creatures (Duthie et al., 1997; Skaper et al., 1997). There is a relationship between antioxidants and flavonoids/phenolic acids. In this respect, these components are important for antioxidant activity properties (Saddiqe et al. 2010). In this study, flavonoids and phenolic acids were examined and the results are given in Figure 3. According to the results obtained from the ethanol extract of Cilician fir, while the total flavonoid content

was determined as $94,85054 \pm 4.67 \mu\text{g}$, the total phenolic content was found to be $308,8282 \pm 4.83 \mu\text{g}$.

The results showed that the ethanol extract of the resin of Cilician fir has phenolic substances.

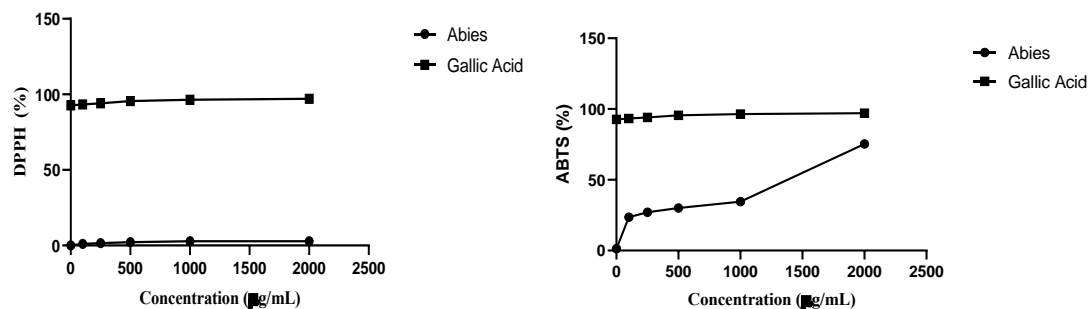


Figure 2. *In vitro* antioxidant activities (DPPH and ABTS radical scavenging activity (%)) of ethanol extracts of resin from Cilician Fir

Şekil 2. *Toros Göknarı'ndan elde edilen reçinenin etanol ekstraktının (DPPH ve ABTS radikal süpürücü aktivitesi (%)) in vitro antioksidan aktivitesi*

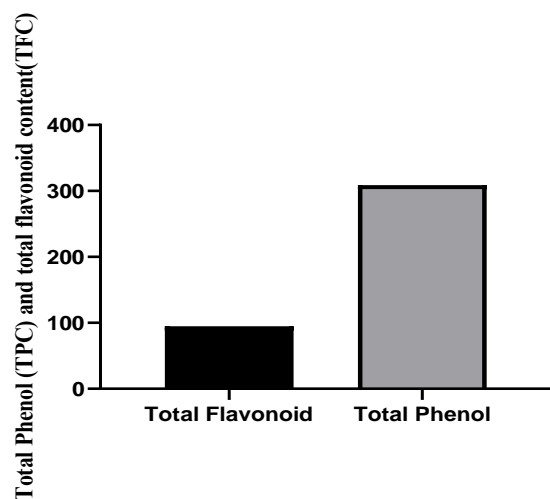


Figure 3. *In vitro* total phenol and flavonoid content of ethanol extracts of resin from Cilician Fir

Şekil 3. *Toros Göknarı'ndan elde edilen reçinenin etanol ekstraktının in vitro toplam fenol ve flavonoid aktivitesi*

Cell culture

The *in vitro* cytotoxicity of the resin on MDA-MB-231, Hep G2, Ishikawa, PC-3, U-87, MCF-7, and HT-29 cancer cells and WI-38 human healthy cells was assessed by the ability to alter MTT assay for 48 h. Figures 4a-h show the cell type and dose-dependent *in vitro* cytotoxic activities of the resin on MDA-MB-231, Hep G2, Ishikawa, PC-3, U-87, MCF-7, and HT-29 cancer cells, respectively. WI-38 cells were used as for the toxicity of the compound against healthy cells. The IC_{50} values (concentration that causes a 50% reduction in cell viability) of the resin for 48 h against all cell lines are listed in Table 2. The IC_{50} values of the resin varied among different cell-lines. The IC_{50} values of the resin changed from 18.32 to $430.1 \mu\text{g mL}^{-1}$ in the human cell line series for 48 h. Lower IC_{50} values indicate higher cytotoxic activity. As the resin had the highest IC_{50} value ($430.1 \pm 0.06 \mu\text{g mL}^{-1}$) against

HepG2 cell line, this demonstrated that it had the lowest cytotoxic activity against Hep G2 cell lines.

According to Table 2, the resin had similar cytotoxic activity toward PC-3, U-87 and MCF-7 cancer cells with IC_{50} values of 23.0 ± 0.05 , 30.6 ± 0.02 , and $38.4 \pm 0.02 \mu\text{g mL}^{-1}$, respectively. The results also indicated that the resin was more cytotoxic on HT-29 colorectal adenocarcinoma cells than on MDA-MB-231 breast cancer cells.

3 These results also showed that Ishikawa endometrial
4 adenocarcinoma cells had the lowest IC_{50} value ($8.94 \pm$
5 $0.03 \mu\text{g mL}^{-1}$). As the resin did have larger IC_{50} values
6 for healthy cells ($18.32 \pm 0.03 \mu\text{g mL}^{-1}$) compared to the
7 Ishikawa endometrial adenocarcinoma cells ($8.94 \pm$
8 $0.03 \mu\text{g mL}^{-1}$), the resin was seen to be more effective
9 on endometrial adenocarcinoma cancer cells.

In order to select the most sensitive cancer cell line, the selectivity index (SI) of the resin was calculated. The calculated SI value for the resin was found to be >2 toward endometrial adenocarcinoma cancer cells,

Table 2. Cytotoxic activities^a of the resin against cancer cells^b and WI-38 healthy cells^c

Tablo 2. *WI-38 sağlıklı hücreler ve kanser hücrelerine karşı reçinenin sitotoksik aktiviteleri*

Cell lines	$IC_{50} (\mu\text{g mL}^{-1})^a$
MDA-MB-231 ^b	82.8 ± 0.03
Hep G2 ^b	430.1 ± 0.06
Ishikawa ^b	8.94 ± 0.03
PC-3 ^b	23.0 ± 0.05
U-87 ^b	30.6 ± 0.02
MCF-7 ^b	38.4 ± 0.02
HT-29 ^b	62.8 ± 0.01
WI-38 ^c	18.32 ± 0.03

^aCell viability after treatment for 48 h were determined by MTT assay as described in the Experimental section ($\mu\text{g mL}^{-1}$). ^bCancer cells, ^cHealthy cells. Each IC_{50} value represents the mean \pm SEM of three independent experiments (n=9).

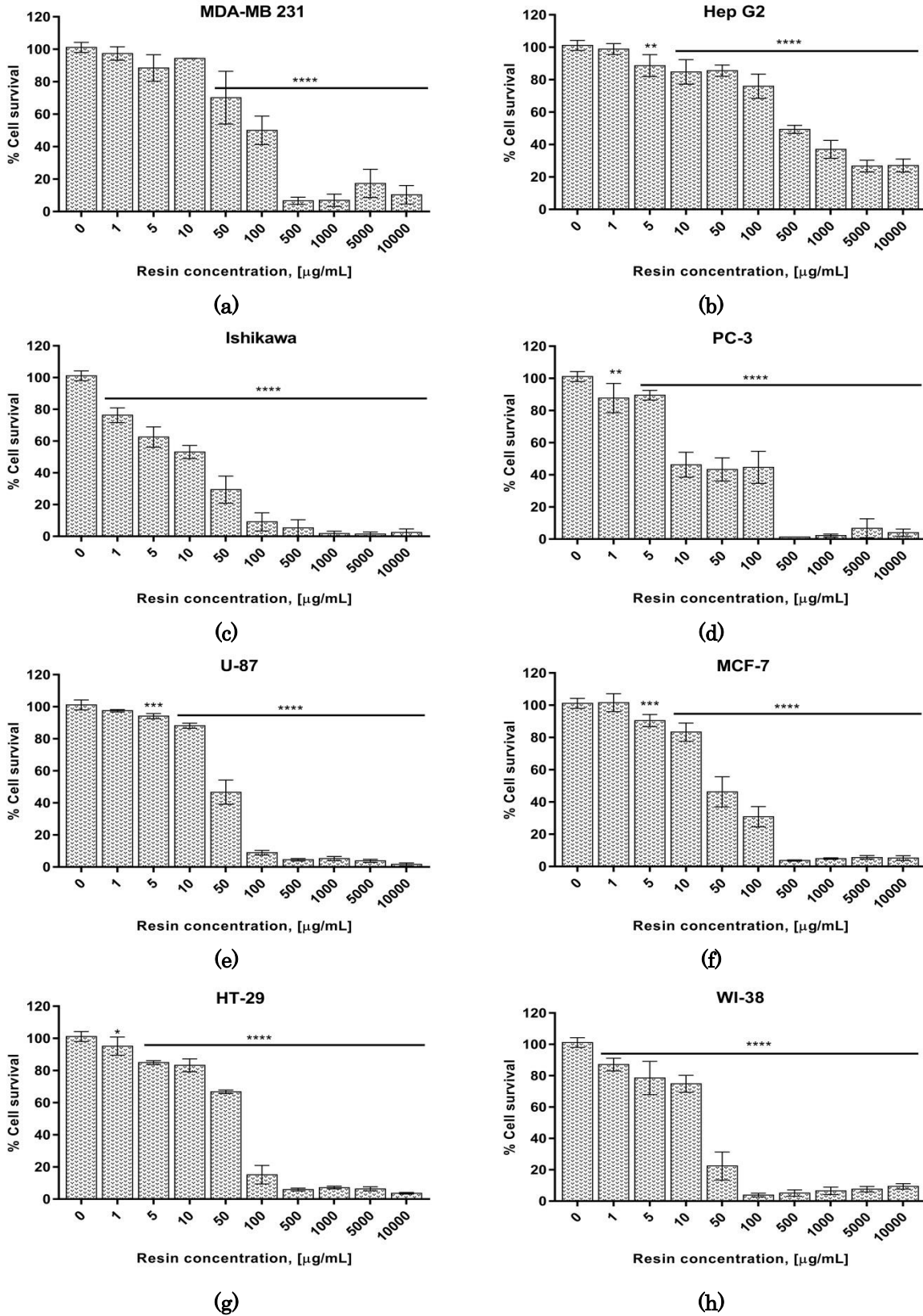


Figure 4. The *in vitro* cytotoxic activities of the resin against MDA-MB-231, Hep G2, Ishikawa, PC-3, U-87, MCF-7, HT-29 cancer cells and WI-38 healthy cells at 48 h (a-h). (n=9 with \pm SEM) (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ kontrole göre)

Şekil 4. Reçinenin Hep G2, Ishikawa, PC-3, U-87, MCF-7, HT-29 kanser hücreleri ve WI-38 sağlıklı hücrelere (a-h) karşı 48 saat için *in vitro* sitotoksik aktiviteleri (n=9, \pm SEM) (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ vs kontrol)

indicating that Ishikawa endometrial adenocarcinoma cells were the most susceptible to the resin. The results showed that the *in vitro* cytotoxic activity of the resin changed according to the cell line type and concentration of the resin.

There are very few studies on Cilician Fir (*Abies cilicica* subsp. *cilicica*). Tümen et al., investigated the anti-inflammatory and *in vivo* wound healing activities essential oil from four different fir species, *Picea orientalis* and *Cedrus libani* (Tümen et al., 2011).. They were found that the essential oils from *Abies cilicica* subsp. *cilicica* and *Cedrus libani* demonstrated the anti-inflammatory activities and also has highest activities on the wound models. It has been investigated that the anti-inflammatory capacity of resin extract of *Abies cilicica* tumor necrosis factor alpha (TNF- α) induced inflammation models and in glucose dependent inflammation (Arslan et al., 2019). It has been measured that the effects on gene expression levels of ICAM-1, P-selectin, VCAM1, monocyte adhesion, and trans endothelial migration for the two *in vitro* models. They were also determined the total phenolic and total flavonoid contents of the extract. Another study, researchers found to be essential oil of the resin from *Abies cilicica* subsp. *cilicica* effective the inhibition of different bacteria growth with MICs at 0.5 g mL⁻¹ (Dayisoglou et al. 2009). Kızıl et al., evaluated the antimicrobial capacity of *Abies cilicica* resin obtained from stems and roots. and against a few bacteria and fungi and found encouraging results (Kızıl et al., 2001).

CONCLUSION

Despite many researches on cancer, which is one of the deadliest diseases of today, there is still no complete treatment method. For these reasons, the antioxidant and cytotoxic activity values of resin obtained from the Cilician Fir plant were evaluated. According to obtained data, the resin has been found to have cytotoxic activity on endometrial adenocarcinoma cancer cells (IC₅₀=8.94 \pm 0.03 μ g mL⁻¹) and antioxidant activity.

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Author contributions

Concept – E.U., S.Ş.B., H.A.A.; Design – E.U., S.Ş.B., Supervision – E.U., S.Ş.B.; Resources – E.U., S.Ş.B. H.A.A; Materials – H.A.A.; Data Collection and/or

Processing – E.U., S.Ş.B.; Analysis and/or Interpretation – E.U., S.Ş.B., M.U.; Literature Search – E.U., S.Ş.B., M.U., H.A.A.; Writing – E.U., S.Ş.B.; Critical Reviews – E.U., S.Ş.B., H.A.A., M.U.

Conflict of interest statement

The authors declared no conflict of interest.

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