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Investigation of the Cytotoxic Effect of Ethyl Pyruvate on Various Cancer Cell Lines

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ABSTRACT

Ethyl pyruvate (EP) is a simple aliphatic ester derived from pyruvic acid which is an endogenous metabolite. Although various studies have investigated the antioxidant and anti-inflammatory properties of EP, there has been only limited research into the cytotoxic effect of EP on cancer cells. The aim of this study was to determine the cytotoxic effects of EP on cells representing common cancer types. EP was purchased commercially and intermediate stock solutions were prepared with phosphate buffer saline. The cytotoxic effect of EP on human melanoma (VMM917), cervix (HeLa), breast (MCF-7), lung (A549), liver (HepG2), colon (WiDr) cancer and normal fibroblast (BJ) cells was determined using the MTT assay. Cisplatin was used as a positive control in cytotoxicity experiments. The results showed that EP exhibits selective cytotoxic effect on VMM917 (10.1-fold) and HeLa (3.04-fold) cells compared to BJ cells. This study shows for the first time that EP has a highly selective cytotoxic effect, especially on melanoma and cervix cancer cells. The mechanism of this effect needs to be elucidated by more extensive studies.

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Etil Piruvatın Çeşitli Kanser Hücre Hatları Üzerindeki Sitotoksik Etkisinin İncelenmesi

ÖZET

Etil piruvat (EP), endojen bir metabolit olan pirüvik asitten türetilen basit bir alifatik esterdir. Çeşitli çalışmalar EP'nin antioksidan ve anti-inflamatuar özelliklerini ortaya koymuş olmasına rağmen, EP'nin kanser hücreleri üzerindeki sitotoksik etkisi hakkında sınırlı sayıda araştırma bulunmaktadır. Bu çalışmanın amacı, EP'nin yaygın kanser türlerini temsil eden hücreler üzerindeki sitotoksik etkilerini belirlemektir. Çalışmada kullanılan EP ticari olarak satın alınmış ve ara stok çözeltiler fosfat tamponu ile hazırlanmıştır. EP'nin insan melanoma (VMM917), serviks (HeLa), meme (MCF-7), akciğer (A549), karaciğer (HepG2), kolon (WiDr) kanseri ve normal fibroblast (BJ) hücreleri üzerindeki sitotoksik etkisi MTT testi kullanılarak belirlenmiştir. Sitotoksisite deneylerinde pozitif kontrol olarak sisplatin kullanılmıştır. Sonuçlar EP'nin BJ hücrelerine kıyasla VMM917 (10.1 kat) ve HeLa (3.04 kat) hücreleri üzerinde seçici sitotoksik etki gösterdiğini ortaya koymuştur. Bu çalışma, EP'nin özellikle melanoma ve serviks kanseri hücreleri üzerinde oldukça seçici bir sitotoksik etkiye sahip olduğunu ilk kez göstermektedir. Bu etkinin mekanizmasının daha kapsamlı çalışmalarla açıklanması gerekmektedir.

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INTRODUCTION

Cancer is a term that generally refers to more than 277 types of diseases, and is the second leading cause of death worldwide (Hassanpour and Dehghani, 2017). Overall, the prevalence of cancer has been increasing steadily on a yearly basis and it is estimated that 18.1 million people were diagnosed with cancer and 9.1 million cancer-related deaths occurred in 2018 in the world (Bray et al., 2018). The formation of cancer is explained with the theory that cancer formation

develops as a result of mutations and that physiological cells are transformed into cancer cells programmed for continuous reproduction (Sever and Brugge, 2015). In general, the process of cancer formation occurs as a result of disruption of cellular signaling, which creates a negative effect on the control of the cell cycle (Hassanpour and Dehghani, 2017). Proto-oncogenes are responsible for cell division under normal conditions, but their becoming oncogene as a result of genetic mutations causes tumor formation. In addition, the ineffectiveness of tumor suppressor genes further triggers this uncontrolled cell division (Wang et al., 2018). Today, various methods, such as chemotherapy, radiotherapy, surgical resection and immunotherapy are used in the cancer treatment. Although chemotherapy is one of the widely used treatment method against cancer, the emergence of side effects and drug resistance over time decreases the percentage of success (Demir et al., 2018a). To overcome these problems, scientists have stepped up new chemotherapeutic discovery studies (Kilinc et al., 2020).

Pyruvate, an important metabolite in cellular energy metabolism, is produced as a result of glycolysis. The pyruvate is used as a substrate for the tricarboxylic acid cycle, and as a result of this cycle, ATP and electron-rich compounds (NADH and $FADH_2$) are produced (Liang et al., 2009; Demir et al., 2020a). Pyruvate is not only an end product of the glycolysis metabolic pathway in cells, but also an endogenous antioxidant and free radical scavenger. The determination that pyruvate is an effective reactive oxygen species (ROS) scavenger encouraged many researchers to try using it as a therapeutic agent for the treatment of various pathological conditions that are thought to mediate redox-dependent phenomena (Sappington et al., 2003). Despite the promising findings from further studies, the utility of pyruvate as a therapeutic agent was found to be limited by its poor stability in solution. When pyruvate dissolves in water, it can undergo condensation and cyclization reactions spontaneously, and some intermediates formed may exhibit toxic effects. Due to these disadvantages of pyruvate, different analogs, such as sodium pyruvate (SP), methyl pyruvate (MP) and ethyl pyruvate (EP) have been produced in time (Sappington et al., 2003; Vyawahare et al., 2012). EP is a simple aliphatic ester derived from pyruvic acid which is the endogenous metabolite (Vyawahare et al., 2012). The effective ROS scavenger, anti-inflammatory, cardioprotective and neuroprotective properties of EP have been determined through *in vitro* and *in vivo* studies (Vyawahare et al., 2012; Turkmen et al., 2016a; Turkmen et al., 2016b). Although these studies have described the protective roles of EP in cells, tissues and organs, its cytotoxic effect has so far been demonstrated only in some cancer models, such as lung, pancreas and malignant mesothelioma (Park et al., 2011; Li et al., 2012a; Pellegrini et al., 2017). Recently, Zhou and Sakamoto (2019) reported that EP inhibits the proliferation of mouse melanoma (B16F10) cells through inhibiting tyrosinase activity and modulating ROS-ERK signaling pathway. Human melanoma (VMM917), cervix (HeLa), breast (MCF-7) and colon (WiDr) cancer cell lines are frequently used in *in vitro* experiments as melanoma, cervix, breast and colon cancer models, respectively (Narisawa-Saito et al., 2008; Berg et al., 2017; Liu et al., 2017; Montagner and Sahai, 2020). However, there is no study that determines the cytotoxic effect of EP on these four cell lines. The aim of this study was to determine the cytotoxic effects of EP on these cell lines for the first time.

MATERIALS and METHOD

Chemicals

Ethyl pyruvate ($CH_3COCOOC_2H_5$, purity: 98%, Cat No: E47808) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and serial dilutions were prepared with phosphate buffer saline (PBS). All other chemicals used in cytotoxicity studies were purchased from Lonza (Verviers, Belgium), Biological Industries (Kibbutz Beit Haemek, Israel) and Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

Human melanoma (VMM917), cervix adenocarcinoma (HeLa), breast adenocarcinoma (MCF-7), lung carcinoma (A549), hepatocellular carcinoma (HepG2), colon adenocarcinoma (WiDr) and normal foreskin fibroblast (BJ) cells were supplied by the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% heat inactivated fetal bovine serum and 1% gentamicin solution and the cells were incubated at 37°C with 5% CO_2 supply (Demir et al., 2018a).

Cytotoxicity Experiments

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to determine the cytotoxic effect of EP on various cell lines as described earlier with slight modifications (Mosmann, 1983; Turan et al., 2017). All cancer cells were seeded into 96-well microplates with 10.000 cells per well, while BJ cells were seeded with 2.500 cells per well (Demir et al., 2019a; Demir et al., 2019b). All cells were then incubated with varying concentrations of EP (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 mM) for 72-h (Park et al., 2011; Cheng et al., 2014). Cisplatin (CDDP) was used as a positive control in cytotoxicity experiments to show that the experimental setup was working correctly (Demir et al., 2018b). At the end of the period,

the plate contents were removed and 10 µL of MTT (0.25 mg mL^{-1}) dye was added to the wells and the cells were incubated with this dye for 3 h (Turan et al., 2018). The formazan crystals formed at the end of the incubation were dissolved with dimethyl sufoxide (DMSO) and the resulting optic density was measured using a microplate reader (Molecular Devices Versamax, California, USA) at 570 nm. Cell viability values corresponding to each concentration were calculated according to the negative control using the obtained absorbance values. Dose-response curves were drawn using %logarithmic concentrations against cell viability and the IC_{50} value of EP and CDDP were calculated for each cell line (Aliyazicioglu et al., 2019; Demir et al., 2020b). IC_{50} values calculated for EP and CDDP in all cell lines were used to determine the selectivity index value with the following formula (Turan et al., 2019):

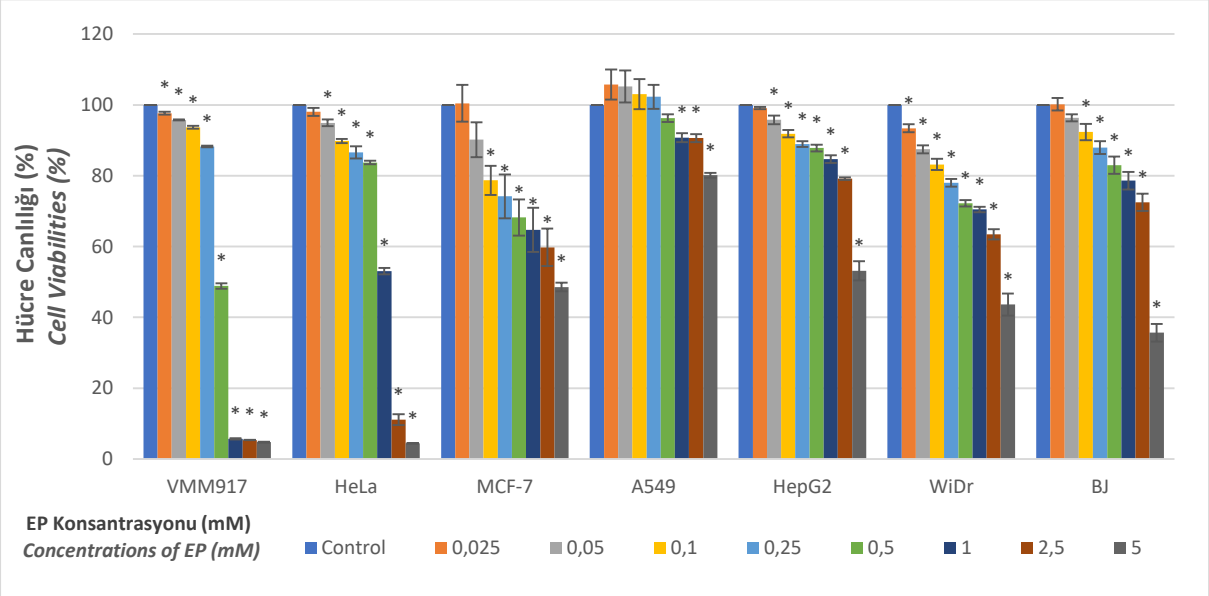
Selectivity index = BJ cells IC_{50} / Cancer cells IC_{50}

Statistical Analysis

All cytotoxicity experiments were performed four times. The distribution of the data was examined with the Kolmogorov-Smirnov test. Data showing normal distribution were expressed as arithmetic mean±standard deviation. Statistical analyzes between the groups were revealed by ANOVA and post-hoc Tukey tests. $p<0.01$ was regarded as significant.

RESULTS and DISCUSSION

Ethyl pyruvate is a stable and lipophilic ester derived from the endogenous metabolite pyruvic acid (Pellegrini et al., 2017) and it has been reported that it has many beneficial biological properties, such as antioxidant, anti-inflammatory, cardioprotective and neuroprotective (Vyawahare et al., 2012; Turkmen et al., 2016b; Turkmen et al., 2016b). However, the cytotoxic effect of EP has so far been demonstrated only in some cancer models, such as lung, pancreas, malignant mesothelioma (Park et al., 2011; Li et al., 2012a; Pellegrini et al., 2017). Therefore, this study aimed to determine the cytotoxic effect of EP on cell lines, including VMM917, A549, HepG2, HeLa, WiDr and MCF-7, representing common cancers in the world. The cytotoxic effect of EP on these cell lines was determined using the MTT assay and the the growth curves of the cells are shown in Figure 1. When all cells are evaluated together (except on A549 cell line), statistically significant cytotoxic effect of EP was emerged starting at a concentration of 0.1 mM. Although, the growth curves showed that EP exhibited cytotoxic effect in all studied cancer cells in a dose-dependent manner, the most strong cytotoxic effect was determined in VMM0917 and HeLa cells.

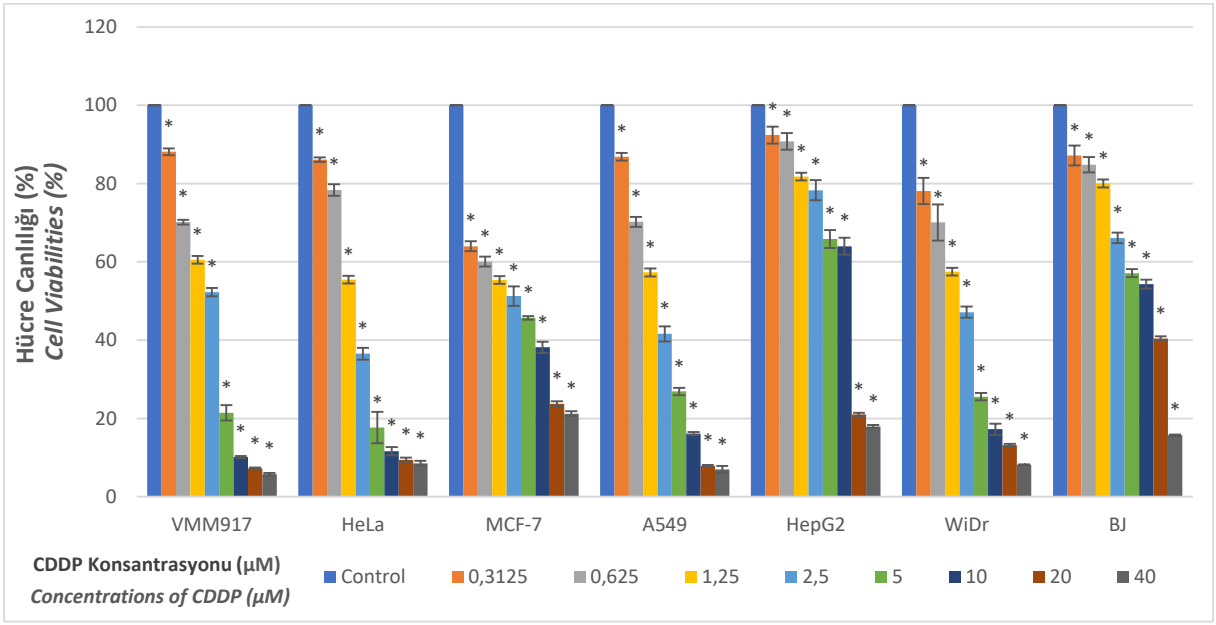


Şekil 1. EP'nin kanser ve BJ hücre hatları üzerindeki sitotoksik etkisi. *Kontrol ile kıyaslandığında istatistiksel olarak anlamlı farkı belirtir ($p<0.01$).

*Figure 1. The cytotoxic of EP on cancer and BJ cell lines. *Denotes statically significant differences in comparison with control ($p<0.01$).*

Cisplatin is a metallic (platinum) coordination compound with square planar geometry (Dasari and Tchounwou, 2014). It was approved by the U.S. Food and Drug Administration (FDA) in 1978 for use in cancer chemotherapy (Makovec, 2019). CDDP is used in the clinical treatment of different types of cancer, such as lung, ovarian, breast, brain, head and neck (Dasari and Tchounwou, 2014). The main target molecule of CDDP in eukaryotic cells is DNA. It binds to the N7 reactive sides of purine residues which

causes DNA damage in cancer cells, blocks cell division and causes apoptotic cell death (Makovec, 2019). The other mechanisms of cytotoxic effect of CDDP are described with its ability to induce oxidative stress, modulate the intracellular calcium level and proliferation signaling pathways (Dasari and Tchounwou, 2014). Chemotherapeutic drugs, such as CDDP and paclitaxel are used *in vitro* cytotoxicity studies to demonstrate that the experimental setup is working properly (Demir et al., 2020b; Misir et al., 2020). CDDP was therefore employed as a positive control in cytotoxicity experiments and the concentration dependent cytotoxic effect of CDDP was shown in Figure 2.



Şekil 2. CDDP'nin kanser ve BJ hücre hatları üzerindeki sitotoksik etkisi. *Kontrol ile kıyaslandığında istatistiksel olarak anlamlı farkı belirtir ($p<0.01$).

*Figure 2. The cytotoxic of CDDP on cancer and BJ cell lines. *Denotes statically significant differences in comparison with control ($p<0.01$).*

In order to make the results more understandable, the IC_{50} values of EP and CDDP in all studied cell lines were calculated and presented in Table 1. When Table 1 is examined, it is seen that IC_{50} values for EP ranged from 0.34 to 3.79 mM. The concentration range of 0.025-5 mM prepared by serial dilution for ethyl pyruvate was used in the study. IC_{50} values could not be calculated for A549 and HepG2 cells since 50% growth inhibition was not also observed even in the highest concentration of EP (5 mM).

Çizelge 1. Farklı hücre hatlarında EP ve CDDP için hesaplanan IC_{50} değerleri (n=4)

Table 1. IC_{50} values calculated for EP and CDDP on different cell lines (n=4)

Hücre Serileri *(Cell Lines)*	**EP (mM)**	**CDDP (µM)**
VMM917	0.34±0.01	2.34±0.05
HeLa	1.13±0.01	2.06±0.14
MCF-7	3.51±0.53	3.73±0.27
A549	>5	2.13±0.12
HepG2	>5	9.14±0.16
WiDr	3.79±0.26	2.76±0.22
BJ	3.44±0.24	12.07±0.34

In consistent with these results, Cheng *et al.* (2014) reported that EP inhibits the proliferation of liver cancer cell lines (SMMC-7721, HepG2, and HCC-LM3) in a dose-dependent manner and the IC_{50} values were 24.7, 29.7, and 20.4 mM, respectively. Michel *et al.* (2019) demonstrated that the concentrations of 1, 5, 10, 15 and 20 mM EP exhibits dose dependent antiproliferative effect on HepG2 cells. Lim *et al.* (2007) demonstrated that EP inhibits high mobility group box protein 1 (HMGB1) release through inducing the necrosis-to-apoptosis switch in A549 lung adenocarcinoma cells, while Liu *et al.* (2019) reported that the concentration of 30 mM EP inhibits the growth of A549 cells through HMGB1/receptor for advanced glycation end products (RAGE) axis and the nuclear factor kappa-B (NF-κB)/signal transducer and activator of transcription 3 (STAT3) pathway. No literature comparison has been made since there is no study showing the cytotoxic effect of EP on VMM917, WiDr, HeLa and MCF-7 cell lines. However, cytotoxic effects of EP have been also reported in different cell lines, such as colon adenocarcinoma (MC38) (Liang et al., 2009), gallbladder carcinoma (GBC-SD and SGC-996) (Li et al., 2012b), gastric carcinoma (SGC-7901) (Zhang et al., 2012), leukemia (THP-1 and K562) (Birkenmeier et al. 2016), prostate adenocarcinoma

(PC-3 and CWR22RV1) (Huang et al. 2018) and diffuse large B-cell lymphoma (Su-DHL-4, Su-DHL-8, and Su-DHL-10) (Zhang et al., 2019). The mechanism of this cytotoxic effect of EP is explained by its property to increase the rate of apoptosis and autophagy (Liang et al., 2009), to arrest the cell cycle (Birkenmeier et al. 2016; Huang et al. 2018; Zhang et al., 2019) and to decrease the expression of some proteins related the formation of tumor microenvironment, such as HMGBl, RAGE, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) proteins (Li et al., 2012b; Zhang et al., 2012).

When all cells are evaluated together, statistically significant cytotoxic effect of CDDP was emerged starting at a concentration of 0.3125 µM. In order to make the results more understandable, the IC_{50} values (µM) of CDDP in all studied cell lines were calculated and presented in Table 1. When Table 1 is examined, it is seen that IC_{50} values for CDDP ranged from 2.06 to 9.14 µM in cancer cell lines. Consistent with these results, it is reported that the IC_{50} values of CDDP on HeLa and A549 cell lines vary between 0.75 to 8.6 µM (Gumus et al., 2009; Casagrande et al., 2013; Singh et al., 2015) and 1.12 to 7 µM (Adach et al., 2016; Jin et al., 2019; Zhang et al., 2020), respectively. Similarly, it is reported that the IC_{50} values of CDDP on MCF-7 and HepG2 cell lines vary between 3.09 to 12.5 µM (Aung et al., 2007; Gumus et al., 2009; Mansouri-Torshizi et al., 2016) and 7.75 to 24.1 µM (Sakinah et al., 2007; Adach et al., 2016; Hashiesh et al., 2018), respectively. In parallel with these results, it is reported that the IC_{50} values of CDDP on WiDr and BJ cell lines vary between 1.2 to 6 µM (Temmink et al., 2007; Turan et al., 2018; van Zweeden et al., 2018) and 13 to 20 µM, (Adach et al., 2016; Col Ayvaz et al., 2017; Varbanov et al., 2019), respectively. Since there is no study in the literature investigating the cytotoxic effect of CDDP on the VMM917 cell line, no direct comparison could been made.

Selectivity is one of the most important criteria for a compound to be evaluated as a chemotherapeutic (Demir et al., 2019b). For this reason, one normal BJ cell line was used along with six cancer cells in the study. The selectivity index of the EP and CDDP for all studied cancer cells were calculated using the formula described in the "Materials and Method Section" of the IC_{50} values obtained for each cell and results were presented in Table 2. Since the IC_{50} value cannot be calculated in the A549 and HepG2 cells for EP, the selectivity index value could not be calculated in these two cell lines. Other results showed that EP exhibited a highly selective cytotoxic effect, especially in the VMM917 and HeLa cell lines. In fact, the selectivity index value of EP obtained for VMM917 is higher than the CDDP which was used as positive control. Malign cervical tumors are one of the most common malignancies in the female population. More than half a million women are diagnosed with cervical cancer each year, and approximately 300.000 cervical cancer-related deaths occur each year (Cohen et al., 2019). Melanoma represents the most aggressive and deadliest form of skin cancer and according to WHO data, about 132.000 new cases of melanoma are diagnosed globally each year. (Domingues et al., 2018). Although chemotherapy is one of the most used treatment method of melanoma and cervical cancer, the development of drug resistance over time and the occurrence of side effects negatively affect the continuity and success percentage of the treatment. For this reason, studies are continuing to discover chemotherapeutics with selective cytotoxic effects (Demir et al., 2018b; Domingues et al., 2018). In this regard, we think that the results of this study, which demonstrated the selective cytotoxic effect of EP on VMM917 and HeLa cells for the first time, are important. The mechanism of the selective cytotoxic effect of EP, especially on these two cell lines, should be determined and the results should be supported by *in vivo* studies.

Çizelge 2. EP ve CDDP'nin seçicilik indeks değerleri
Table 2. Selectivity index values of EP and CDDP

Hücre Serileri *(Cell Lines)*	**Test Bileşikleri** *(Test Compounds)*	
	EP	**CDDP**
VMM917	10.1	5.16
HeLa	3.04	5.86
MCF-7	0.98	3.24
A549	Not determined	5.67
HepG2	Not determined	1.32
WiDr	0.91	4.38

CONCLUSION

This is the first study to determine the cytotoxic effect of EP on VMM917, HeLa MCF-7 and WiDr cell lines. It was determined that the extract had selective cytotoxic effect especially against VMM917 and HeLa cells. The determination of the mechanism of this cytotoxic effect through more extensive studies is thought to contribute to the usability of EP as a potential therapeutic agent in melanoma and cervix cancer.

Researchers Contribution Rate Declaration Summary

The authors declare that they have contributed equally to the article.

Conflicts of Interest Statement

None of the authors had any financial or personal relationships with other individuals or organizations that might inappropriately influence their work during the submission process.

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