BÜSAD 2024; 5(1): 11 - 18 **DOI: 10.58605/bingolsaglik.1436057** **Araştırma Makalesi**
BUSAD 2024; 5(1): 11 - 18 **BİNGÖL ÜNİVERSİTESİ SAĞLIK DERGİSİ** Original Article

Evaluation of *in vitro* antioxidative and protective effects of kefir on cyclophosphamide-upon oxidative stress and lung damage in rats

Kefirin sıçanlarda siklofosfamid-nedenli oksidatif stres ve akciğer hasarı üzerine antioksidan ve koruyucu etkilerinin *in vitro* olarak değerlendirilmesi

Songul CETIK YILDIZ [1], Cemil DEMIR[1], Mustafa CENGİZ[2], Betul PEKER CENGIZ[3], Adnan AYHANCI[4]

ÖZ

Doğal bir probiyotik olan kefir, antioksidatif ve antitümör özelliklerinin yanı sıra mikrobiyal türler ve fermantasyondan kaynaklanan yararlı özelliklere de sahiptir. Siklofosfamid (SF), kanser tedavisinde oldukça tercih edilen ancak hem humoral hem de hücresel bağışıklığı baskılayabilen sitotoksik bir ilaçtır. SF'nin immunosupresif etkisinin olması ve akciğer gibi çoklu organ toksisitesine neden olması nedeniyle etkili yüksek dozda kullanımı sınırlıdır. Wistar albino sıçanlar 6 gruba ayrıldı. Kontrol, 150 mg/kg SF, 5 mg/kg kefir, 5 mg/kg kefir +150 SF, 10 mg/kg kefir, 10 mg/kg kefir+150 SF. Ratlara 1., 2. ve 3. günlerdeki fermente kefirler karıştırılarak 12 gün süreyle verilirken, deneyin 12. gününde SF tek doz ve i.p. olarak verildi. Alınan kan ve doku parametreleri değerlendirildi. Oksidatif stresi gösteren OSI değerinin SF uygulanan grupta arttığı, SF+kefir gruplarında ise bu düzeyin kontrole yaklaştığı görüldü. Ayrıca SF uygulanan sıçanların akciğer parankiminde dejenerasyon, interalveoler bölgede yoğun inflamasyon, alveollerde kollaps ve bronşiyol çevresinde yoğun inflamasyon gözlenirken, kefir verilen gruplarda bu etkilerin etkin bir şekilde düzeldiği gözlendi. Sonuç olarak deneysel çalışmamızda kefirin SF kaynaklı oksidatif stres, konjesyon, alveolar hasar ve inflamasyon üzerinde antioksidatif ve koruyucu etkiler gösterdiği gözlendi.

Anahtar kelimeler: Kefir, siklofosfamid, antioksidan, oksidatif stres, akciğer hasarı, sıçan

ABSTRACT

Kefir, a natural probiotic, has antioxidative and antitumor properties, as well as potential beneficial properties resulting from microbial species and fermentation. Cyclophosphamide (CYP) is a cytotoxic drug that is highly preferred in cancer therapy but can suppress both humoral and cellular immunity. The use of effective doses is limited because CYP has an immunosuppressive effect and causes multiple organ toxicity such as the lungs, and the use of effective high doses is restricted. Wistar albino rats were divided into 6 groups. As; control, 150 mg/kg CYP, 5 mg/kg kefir, 5 mg/kg kefir +150 CYP, l0 mg/kg kefir and, l0 mg/kg kefir+150 CYP. Fermented kefirs from the 1st, 2nd, and 3rd days were mixed and given to the rats for 12 days, while CYP was given as a single dose and ip on the 12th day of the experiment. The received parameters were evaluated. It was observed that the OSI value, which indicates oxidative stress, increased in the CYP-administered group, and this level approached control in the CYP+kefir groups. In addition, while degeneration, intense inflammation in the interalveolar area, collapse of the alveoli, and intense inflammation around the bronchioles were observed in the lung parenchyma of CYP-administered rats, it was observed that these effects were effectively improved in the groups given kefir+CYP. In conclusion, in our study, it was observed that kefir showed antioxidative and protective effects on CYP-induced oxidative stress, congestion, alveolar damage, and inflammation.

Keywords: Kefir, cyclophosphamide, antioxidant, oxidative stress, lung damage, rats

This study was financed by Mardin Artuklu University BAP Coordination Office (MAU.BAP.20.SHMYO.004). And this study was approved by the Ethics Committee of Eskisehir Osmangazi University Animal Experiments Local Ethics Committee (784-145 / 2020). Apart of this study was presented at IV. International Siirt Conference on Scientific Research Siirt University, November 17-18, 2023.

[1]Songul CETIK YILDIZ, Department of Medical Services and Techniques, Health Services Vocational School, Mardin Artuklu University, Mardin, Turkiye songulcetik@gmail.com, ORCID No: 0000-0002-7855-5343
[1]Cemil DEMIR, Department of Medical Services and Techniques, Health Services Vocational School, Mardin Artuklu University, Mardin, Turkiye cemildemir@ymail.com, ORCID No: 0000-0002-6365-0196
[2]Mustafa CENGIZ, Department of Elementary Education, Faculty of Education, Siirt University, Siirt, Turkiye. mustafacengizogu@gmail.com, 0000-0002-6925-8371
[3]Betul PEKER CENGIZ, Eskisehir Yunus Emre State Hospital, Eskisehir, Turkey, Eskişehir, Turkiye
betip76@yahoo.com, ORCID No: 0000-0002-2503-7446
[4]Adnan AYHANCI, Department of Biology, Science Faculty, Eskisehir Osmangazi University, Eskisehir, aayhanci@ogu.edu.tr, ORCID No: 0000-0003-4866-9814
İletişim/Corresponding Author: Songul CETIK YILDIZ
E-posta/E-mail: songulcetik@mail.com

Geliş Tarihi/Received : 13.02.2024
Kabul Tarihi/Accepted: 22.02.2024
Yayın Tarihi/Published: 30.04.2024

INTRODUCTION

Kefir slows down tumor development as a result of oral intake of water-soluble polysaccharides found in kefir grains. Selenium in kefir composition; Vitamin E, together with catalase and superoxide dismutase enzymes, has an antioxidative effect on cells. This is considered an anticarcinogenic factor. Linoleic acid found in kefir has been reported to reduce the risk of cancer in studies (1). At the same time, lactic acid, antibiotics and various bactericides found in kefir play a role in the destruction and inhibition of proliferation of pathogens (2). An experimental study has shown that microorganisms in kefir can significantly affect the immunoregulatory properties of rats (3). Regular consumption of kefir has antioxidant, anticarcinogenic, immune system regulating, cholesterol regulating, antiallergic, antidiabetic, antibacterial, lactose intolerance reducing, and blood pressure lowering effects (4). Studies have also reported that kefir exhibits activities such as antioxidative, antimicrobial, anticarcinogenic properties and protection against apoptosis (5,6). Kefir has an anticarcinogenic effect by slowing the growth of cancer cells and accelerating apoptosis. According to the results of the research examined, kefir is a natural therapeutic agent for cancer (7). Kefir accelerates apoptosis in cancerous cells by reducing mutation and DNA damage, decreasing the activities of enzymes (ß-glucuronidase, nitroreductase, azoreductase) that predispose to cancer formation, neutralizing mutagens, increasing the production of short-chain fatty acids and acidity, and provides an anticarcinogenic effect.

Cyclophosphamide (CYP), which is widely preferred in cancer treatment, is a cytotoxic drug that can suppress both humoral and cellular immunity, and its effective use in high doses is restricted because it causes multiple organ damage (8). CYP, an alkylating-type prodrug, is metabolized by liver cytochrome P450 enzymes, namely CYP3 - CYP2B6, which display active cytotoxic and therapeutic metabolites and are released from hepatocytes into plasma (9). CYP diffuses throughout the body, and produces two active metabolites acrolein (ACR) and phosphoramide mustard (PAM) (10). ACR causes high production of reactive oxygen species (ROS) and oxidative stress in hepatocytes (11). Thus, it damages the tissue antioxidant defense mechanism as it interacts with proteins and causes changes in the structure and functions of enzymes (12). The toxic side effects of acrolein in CYP treatment must be avoided by utilizing certain antioxidants. In our experimental study conducted for this purpose, the cytoprotective effects of kefir on CYP-induced toxicity in rats were examined.

MATERIAL and METHODS

This study was financed by the Mardin Artuklu University BAP Coordination Office (MAU.BAP.20.SHMYO.004). This study was approved by the Ethics Committee of Eskisehir Osmangazi University Animal Experiments Local Ethics Committee (784-145 / 2020).

Kefir fermentation

In our study, commercially supplied and freeze-dried kefir yeast and 1-liter cow's milk (Dost, 1 L golden full-fat pasteurized milk) were preferred for kefir fermentation. For fermentation, Marshall et al. (1984) It was based on the method used by (13). Three groups of kefirs were created, with fermentation at 24-26 °C temperature at intervals of 24, 48, and 72 hours and days 1, 2, and 3. It was kept at +4 °C ready for use. It was given to rats by gavage method for 12 days. Kefirs from the 1st, 2^{nd,} and 3rd days were mixed and given by gavage method for 12 days.

Chemicals and injections

Cyclophosphamide (CYP) (Sigma-Aldrich) was commercially available. 500 mg CYP was dissolved in 25 ml bidistilled water to prepare for injection of 150 mg/kg CYP. The injection was performed as a single dose intraperitoneally (i.p.) / body-weight (b.w.) on the 12th day, that is, the last day of the experiment, using sterile disposable syringes.

Experimental setup

In our experimental study, healthy, males, 200±20 gr, about 3 months age Wistar albino rats were used. During the experiment, the animals were kept in rooms with 12;12 light/dark lighting, 45-50% humidity, and 22±2 C˚ temperature. And were given tap water and normal pellet feed. The 42 rats used in this study were divided into 6 groups, each group including 7 rats. As; Control, a single dose of 150 mg/kg/b.w CYP, 5 mg/kg/b.w kefir, 5 mg/kg/b.w kefir+150 mg/kg/b.w, 10 mg/kg/b.w kefir and 10 mg/kg/b.w kefir + 150 mg/kg/b.w CYP was given group. Kefir was given to rats by gavage method for 12 days. A single dose of CYP was given i.p. on the last day of the experiment, namely the 12th day. At the end of the experiment, biochemical parameters and lung tissues were taken under anesthesia.

Oxidative stress index (OSI)

The TOS/TAS ratio was used to calculate the OSI value. To do this, the Troloxequivalent/L type unit of measurement was converted from mmol to μmol.

OSI= [(TOS, μmol H2O2 equivalent/L) / (TAS, μmol Trolox equivalent/L) x 100].

Histopathology

Before being examined under a light microscope, tissue samples were preserved in a 10% Neutral Buffer formaldehyde solution. Following identification, tissue samples were put into cassettes and given a two-hour rinse under running water. Tissues were run through a succession of increasing alcohol concentrations (60, 70%, 80%, 90%, 96%, 100%) to extract water. The tissues were then polished by passing them through xylene before being implanted in melted paraffin. For each group, 4-micron-thick slices were cut from paraffin blocks and stained with hematoxylin-eosin stain. Using the Leica Q Vin 3 program on the Leica DCM 4000 computer-aided imaging system (Germany), the sections were assessed and captured on camera. A Criteria table was created as a result of the evaluations made with Hematoxylin-Eosin staining.

RESULTS and DISCUSSION

Since different microbial flora formed around kefir grains after fermentation, fermented kefirs on different days were tested. However, since no significant change was observed between the kefirs of the 1st, 2nd, and 3rd days, the kefirs of the three days were mixed and used. Studies show that kefirs are used in very different doses and durations. In our study, we gave kefir to rats by gavage method for 12 days, as in Cooper, (1986) and Matsuu et al., (2003) studies (14, 15).

In our study, the possible protective effect of kefir's known antioxidant and antitumor effects against CYP-induced oxidative stress and lung damage was investigated; Considering the congestion, alveolar damage, and inflammation conditions in the lung, a severe change was observed in the 150 mg/kg CYP group compared to the control (score 3). Compared to the group given CYP, the severe change turned into a slight change in the group given 150 mg/kg CYP+5 kefir (score 1). This severe change turned into a moderate change in the group given 150 mg/kg CYP+10 kefir (score 2) (Table 1).

Table 1: Control, 150 mg/kg CYP, 5 mg/kg kefir, 5 mg/kg kefir+150 CYP, 10 mg/kg kefir, 10 mg/kg kefir+150 CYP applied experimental groups' lung congestion, alveolar scoring according to damage and inflammation status

Groups	Score
Control	0*
150 mg/kg CYP	3****
5 mg/kg kefir	1**
l0 mg/kg kefir	2***
5 mg/kg kefir +150 CYP	1
10 mg/kg kefir +150 CYP	2

Score *0: *No change* ***Score **1:*** *slight change* ***Score ***2:*** *moderate change* ***Score ****3:*** *severe change*

In Figure 1, where the OSI findings are shown, it is seen that the OSI value indicating oxidative stress is high in the 2nd group given CYP. The OSI value in groups 3 and 4, which were given kefir, was found to be significantly lower than in group 2. When the 2nd group given CYP and the CYP+kefir given groups are compared, it is seen that this situation is approaching control in the 5th and 6th groups given 5 and 10 mg/kg despite CYP application (Figure 1).

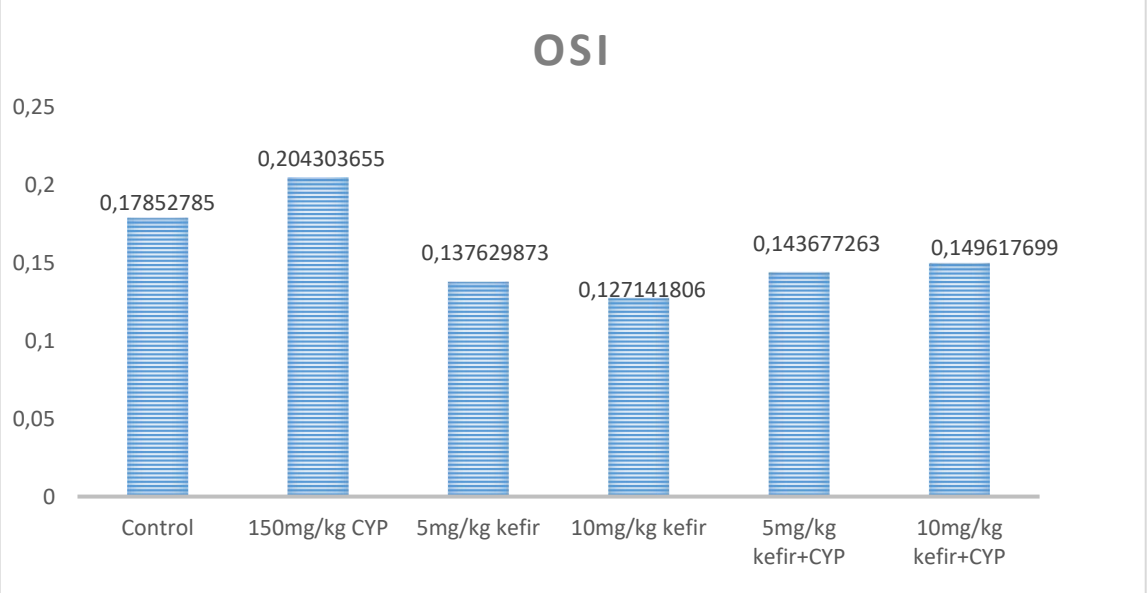

Figure 1: Comparison of OSI values of experimental groups administered Control, 150 mg/kg CYP, 5 mg/kg kefir, 5 mg/kg kefir +150 mg/kg CYP, l0 mg/kg kefir, l0 mg/kg kefir+150 mg/kg CYP.

According to Figure 2-a, the lung parenchyma, alveoli (blue asterisk), and bronchioles (blue arrow) of the rats in the control group can be seen. In 150 mg/kg, CYP administered to group 2, degeneration in the lung parenchyma, intense inflammation in the interalveolar area (black asterisk), the collapse of the alveoli (blue asterisk), and intense inflammation around the bronchioles (blue arrow) are observed (Fig. 2-b). There are bronchioles (blue arrow) and alveoli

(blue asterisk) in the lung parenchyma of rats administered 5 mg/kg kefir (Fig. 2-c). In the lung parenchyma of rats administered 150 mg/kg CYP + kefir, inflammatory cells (black asterisk) in the interalveolar area and destruction (blue arrow) due to inflammatory cells in the bronchiole are observed (Fig. 2-d). Alveoli (blue asterisk), bronchioles (blue arrow), and congested and dilated vascular structures (yellow arrow) are observed in the lung parenchyma of rats administered 10 mg/kg kefir (Fig. 2-e). Alveoli (blue asterisk), bronchioles (blue arrow), and congested vascular structures (yellow arrow) were observed in the lung parenchyma of rats administered 10 mg/kg kefir+150 mg/kg CYP. (Fig. 2-f).

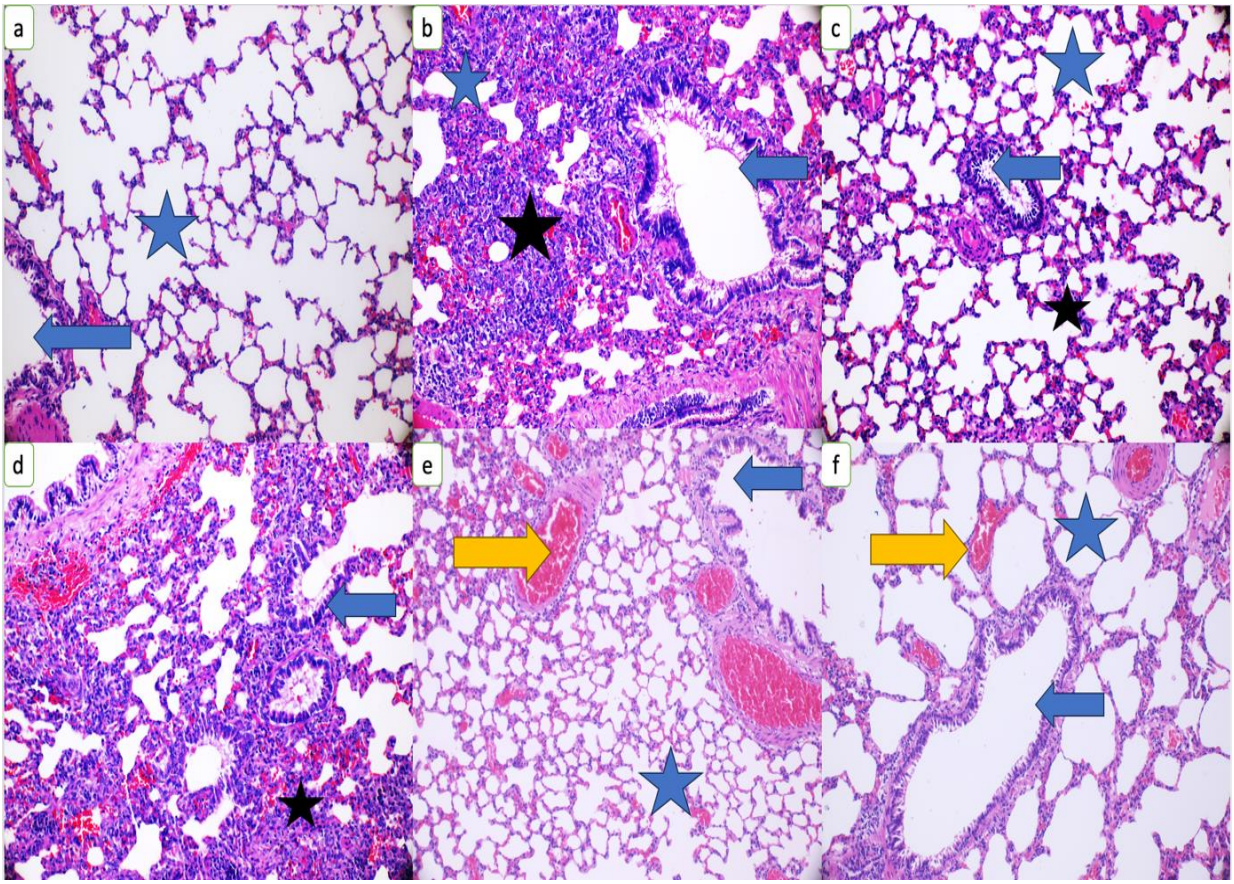

Figure 2: a; Lung parenchyma of rats in the control group alveoli (blue asterisk) and bronchioles (blue arrow) **b;** Degeneration in the lung parenchyma of CYP-treated rats, intense inflammation in the interalveolar area (black asterisk), collapse in the alveoli (blue asterisk), intense inflammation around the bronchioles (blue arrow) **c;** Bronchioles (blue arrow), alveoli (blue asterisk) in the lung branch of rats administered kefir. **d;** Inflammatory cells in the interalveolar area (black asterisk) in the lung parenchyma of rats administered CYP and kefir, destruction due to inflammatory cells in the bronchiole (blue arrow). **e;** Alveoli (blue asterisk), bronchioles (blue arrow), congested and dilated vascular structures (yellow arrow) in the lung parenchyma of rats administered kefir. **f;** Alveoli (blue asterisk), bronchioles (blue arrow), and congested vascular structures (yellow arrow) in the lung parenchyma of rats administered CYP and kefir (H&E, x200).

The absence of two key detoxifying enzymes in lung tissue, namely aldehyde oxidase and aldehyde dehydrogenase, is considered one of the main causes of selective CYP-induced lung toxicity (16). Acrolein, which is produced during drug metabolism and causes excessive ROS production, lipid peroxidation, suppression of the antioxidant defense system, and severe inflammatory response due to neutrophil recruitment, is linked to CYP cytotoxicity. ROS plays

an important role in the pathogenesis of acute and chronic lung injury. In their study, Naraoka et al., (2021) reported that neutrophils, monocytes, and macrophages, which are pulmonary defense cell types, seem particularly prone to convert molecular oxygen into ROS (17). The findings of our study show that the OSI value is high in the CYP group. High OSI values in the CYP group indicate CYP-induced oxidative stress. Because it is seen that the OSI value is close to control in the 3rd and 4th groups given kefir (Figure 1). Similarly, a study suggests that the acute effect of CYP on rat lungs involves severe inflammatory reactions including acrolein formation during drug metabolism, accumulation of neutrophils, ROS formation, and increases in lipid peroxidation (18). When we look at the groups given kefir together with CYP, it is seen that this ratio has improved significantly and is closer to control. This result shows that kefir reduces oxidative stress by having an antioxidant effect. Similarly, there is also a study showing that kefir positively affects antioxidant parameters and reduces lipid peroxidation in carbon tetrachloride toxicity in mice (19). In the study investigating the antimutagenic and antioxidant effects of kefir, it was determined that kefir had a significantly more pronounced antimutagenic effect than unfermented milk and soymilk. Likewise, when looking at the antioxidant effect, it has been reported that milk kefir and soy milk kefir scavenge more free oxygen radicals on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and show a greater inhibition effect on linoleic acid peroxidation (20). Superoxide dismutase and catalase enzymes, vitamin E, and selenium in kefir have antioxidative properties and become an anticarcinogenic factor (21). In other studies, it has been reported that kefir consumption has antioxidant and anticarcinogenic effects, in parallel with the results of our study (22-24). It has been previously shown that Immune activities have been observed in humans and various animals after ingestion of lactic acid bacteria found in kefir, and it has been observed that lactic acid bacteria increase non-specific resistance against tumors or infections in humans or animals or have a strengthening effect on specific immune reactions (25).

Apoptosis, which is carried out by acrolein via mitochondria, can be detected mostly in alveolar macrophage cells in the lungs. Considering the results of our study, degeneration in the lung parenchyma of CYP-treated rats, intense inflammation in the interalveolar area, collapse of the alveoli, and intense inflammation around the bronchioles were observed (Fig.2.b). Similarly, in an experimental study where the lung toxicity of CYP was determined, it was reported that it showed pneumonia at the alveolar level, type-1 pneumocystis cell injury and unnatural type-2 cell formation, specialized fibrosis with an increase in the amount of collagen, and deterioration of alveolar elasticity (26). In a similar study, histopathological examination of lung tissues in the CYP-treated group reported alveolar cell injuries, alveolar septa thickness, polymorphonuclear cells, and the presence of erythrocytes in the alveolar lumen (27). In another study, as a result of immunohistochemical and electron microscopic evaluations in CYP-treated groups, it was observed that along with the deterioration of the alveolar structure, the alveolar tension decreased and the alveolar cells lost their normal cytoplasmic properties, the lamellae disappeared in the lamellar bodies, the bodies became vacuoles, and collagen fiber deposits symbolizing fibrosis were observed in the lung tissue. has been distinguished (28). Likewise, Pouzet and Travis (1992), in their studies with mice, reported that CYP acts rapidly in lung tissue and that this effect can last up to a year (29). As seen in the kefir groups in our study, it is seen that this bad situation improved in the groups given CYP + kefir, especially in the 6th group given 10 mg/kg kefir + 150 mg/kg CYP (Fig. 2. e, f). Parallel to our results, there are studies that kefir has an anticarcinogenic effect by slowing the growth of cancer cells and accelerating apoptosis (25,30). It is suggested that this anticarcinogenic effect of kefir is due to the amino acid groups containing sulfur in its structure (31). The effects of the water-soluble and water-insoluble polysaccharide fractions of kefir grains on Lewis lung cancer and B16 melanoma cells in mice were examined by Furukawa et al. in 2000. According to their findings, the water-insoluble fraction prevented melanoma metastases while the water-soluble fraction shielded against pulmonary metastases (32). In a study on the antitumor effect of kefir, mice transplanted

with fusiform cancer cells were given 0.5 ml of kefir daily intraperitoneally for 20 days, and as a result, a significant reduction in tumor size was observed. It has also been found that kefir is effective in eliminating tumoral necrosis (33). Kefir also has antitumor properties with its immunotherapeutic effect. It has been reported that methods developed to prevent the growth of tumors and to treat cancer by stimulating the immune response against tumor cells are important in cancer treatment (34).

CONCLUSION

An important way to ensure the effectiveness of cancer treatment is to regulate the microbiota through probiotic consumption. Kefir is a very effective agent both in reducing the side effects of CYP, an antineoplastic drug whose toxic effect on the lungs has been determined by *in vivo* and *in vitro* experiments, and in providing cancer immunotherapy that uses the power of the patient's immune system to destroy cancerous cells. Our findings strengthen the idea that kefir, which has antioxidant and antitumor effects, may be a potentially effective combination in the prevention and treatment of CYP-induced damage.

Conflict of interest: No conflict of interest was declared by the authors. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions: SCY: Writing – original draft, Review & editing, Methodology, Visualization. **CD:** Conceptualization, Methodology, Review & editing. **MC:** Visualization, Conceptualization, Review & editing. **BPC:** Formal analysis, Methodology **AA:** Conceptualization, Review & editing, All authors reviewed the results and approved the final version of the manuscript.

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