

# The Protective Effect of Kefir on Carbon Tetrachloride-induced Histopathological Changes in the Livers of Rats

Şule Yurdağül ÖZSOY<sup>1</sup> 

<sup>1</sup> Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Pathology, TR-31001 Alahan, Antakya/Hatay - TURKEY

Article Code: KVFD-2015-14825 Received: 07.12.2015 Accepted: 12.01.2016 Published Online: 11.01.2016

## Abstract

The aim of the study was to investigate the protective effects of kefir in the liver damage of rats, at experimental carbon tetrachloride (CCl<sub>4</sub>) intoxication by histologically and immunohistochemically. During the 45 days trial period, 18, female, Wistar albino rats were used. One of them was control, three experimental group was created. Twice a week 0.5 cc carbon tetrachloride (CCl<sub>4</sub>) + olive-oil (1:1) suspension was injected subcutaneously to the second and third group. At third group additionally to this administration 30 ml kefir was given daily by oral gavage. CCl<sub>4</sub>-induced hepatocellular damage and apoptosis was observed but these adverse findings reduced with kefir administration. These findings indicate that kefir may have a protective role at liver damage.

**Keywords:** Apoptosis, Carbon tetrachloride, Histopathology, Liver, Rat

## Karbon Tetraklorüre Bağlı Ratların Karaciğerinde Oluşan Histopatolojik Değişikliklere Karşı Kefirin Koruyucu Etkisi

## Özet

Çalışmada deneysel karbon tetraklorür (CCl<sub>4</sub>) toksikasyonu oluşturulan ratlarda kefirin karaciğer hasarına karşı koruyucu etkilerinin histolojik ve immunohistokimyasal olarak araştırılması amaçlandı. Kırkbeş günlük deneme boyunca, 18 adet, dişi, Wistar albino rat kullanıldı. Biri kontrol olmak üzere, üç deneme grubu oluşturuldu. Grup 2 ve 3'e haftada 2 kez 0.5 cc karbon tetraklorür (CCl<sub>4</sub>) + zeytinyağı (1:1) süspansiyonu subkutan yolla verildi. Grup 3'e buna ek olarak günlük 30 ml kefir oral gavaj yoluyla verildi. CCl<sub>4</sub>'e bağlı hepatoselüler dejenerasyon ve apoptosis gözlemlendi, ancak kefir eklenmesi ile bu olumsuz değişiklikler azaldı. Bu bulgular kefirin karaciğer hasarında koruyucu rolü olabileceğini gösterdi.

**Anahtar sözcükler:** Apoptosis, Karbon tetraklorür, Histopatoloji, Karaciğer, Rat

## INTRODUCTION

Carbon tetrachloride is obtained by the chlorination of carbondisulfide or reacting of the same compound with sulfur monochloride. This material absorbed by respiration, skin and gastrointestinal tract. They are used as anthelmintic, against parasites in veterinary medicine [1]. When carbon tetrachloride used in high doses the accumulation of it causes damage in liver even cirrhosis can be created. It also makes degeneration in many other organs in the body [1-3].

In regard to FAO/WHO; probiotics means 'for life' organisms, are useful for humans and animals [4]. The probiotics include some yeast such as; *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus*

*helveticus*, *Lactobacillus lactis*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bifidobacterium spp.*, *Escherichia coli* and *Saccharomyces*. Some of them also content *Bacillus subtilis* [5,6]. It has been reported that probiotic applications might be protective against to the urogenital, gastrointestinal and surgical infections [7,8].

A fermented milk product kefir drink, obtained from kefir grains, is Caucasian origin and ethyl alcohol and lactic acid fermentation are shaped together in it [9,10]. The polysaccharide structure, white-yellowish color kefir grains contains microorganisms such as, lactobacilli, lactococci, leuconostocs, acetobacteri and fungi (*Kluyveromyces marxianus*, *Torulasporea delbrueckii*, *Saccharomyces cerevisiae*, *Candida kefir*) [11-13]. It was reported that kefir has antioxidant,



İletişim (Correspondence)



+90 326 2455845



suleozsoy@yahoo.com

antifungal <sup>[14]</sup>, antibacterial, antitumor, immunological <sup>[15,16]</sup>, cholesterol-lowering <sup>[17]</sup> and anti- the apoptotic effect <sup>[18]</sup>.

Both physiological and pathological inducible cell death mechanism apoptosis known as programmed cell death or cell suicide. Organism refers to apoptosis during organogenesis of multicellular organisms or completed the development in living, damaged or the removal of cells that potentially tumor predisposed <sup>[19,20]</sup>. Apoptosis occurs by one of two pathways: (1) a death receptor pathway, and (2) the mitochondrial pathway <sup>[21]</sup>. It was reported that in cultured rat hepatocytes, the hydrophobic bile acid glycochenodeoxycholate, GCDC, at pathophysiological relevant concentrations (20-100  $\mu\text{M}$ ) induces apoptosis, as documented by cell shrinkage, nuclear condensation and lobulation, caspase activation, DNA fragmentation, and phosphatidylserine externalization <sup>[22]</sup>.

In the study, it was aimed that detection the protective effect of kefir at histologic and apoptotic changes with TUNEL method, induced by carbon tetrachloride in the livers of rats.

## MATERIAL and METHODS

A total of 18 female Wistar albino rats were used in the study. Three experimental group (one of them was control) formed and each group consisting 6 animals. All groups were fed with pellet (standart commercial rat chow) and drinking water was given *ad libitum*. The research project and animal housing conditions were approved by the Mustafa Kemal University Ethical Committee for Animal Studies (approval 2014-01/11). Rats were obtained from the Mustafa Kemal University Laboratory Animal Breeding Unit. The rats were assigned randomly to three groups. The first group was the control group, were fed only rat chow and drinking water. Twice a week 0.5 cc carbon tetrachloride ( $\text{CCl}_4$ ) + olive-oil (1:1) suspension was injected subcutaneously to the second and third group. A total of 12 injections applied for 45 days. At third group additionally to this administration 30 ml kefir was given daily by oral gavage. Kefir drink was prepared as; kefir grain to sterile milk, 3% (w/v) and fermenting at 30°C for 24 h. After fermentation kefir was diluted 1:3, before given to rats.

At the end of the 45-day experimental period animals were sacrificed by decapitation under anesthesia [intramuscular injection of ketamine (50 mg/kg) and xylazine (20 mg/kg)]. Necropsy was performed and liver tissues took out and routine process was done. Initially tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin then were cut in 5-6  $\mu\text{m}$  for hematoxylin and eosin (H & E) staining <sup>[23]</sup> and for in situ detection of apoptotic cells.

*In-situ Detection of Apoptotic Cells by TUNEL Assay:* DNA fragmentation was assessed in situ in liver sections using the terminal deoxynucleotidyl transferase (TdT)-mediated

dUTP-digoxigenin nick endlabelling (TUNEL) method used as catalog procedure (*In Situ Cell Death Detection Kit, POD, Roche, Mannheim, Germany*). In summary sections were de-waxed and rehydrated using routine methods. Firstly sections were held in 3%  $\text{H}_2\text{O}_2$  for 20 min later in proteinase K (20 mg/ml; Roche, Mannheim, Germany) for 15 min at room temperature. As following step; sections initially washed with Phosphate buffer solution (PBS, pH 7.4) for 3 times for 5 min, later 50  $\mu\text{l}$  TUNEL reaction mixture (including TdT & dUTP) was dropped and incubated in 37°C humid camera for 1 h. Again washed with PBS. Later sections were incubated with conjugate anti-fluorescein-POD for 40 min at room temperature and washed three times with PBS. To visualize reaction products, samples were incubated with AEC (*3-Amino 9-Ethyl Carbasole, Dako, Glostrup, Denmark*) for 5 min, and counterstained with Mayer's hematoxyline stain. As a control, samples were treated with labeling solution instead of TdT.

Finally all sections were examined by light microscopy (*Olympus CX31*) and microphotographed (*Olympus DP12*).

## RESULTS

At control group liver was normal in colour and consistency, any macroscopical change was observed. Liver sections of the control group showed a normal histological appearance of the sinusoids and hepatic central vein, any fatty degeneration was observed (*Fig. 1*).

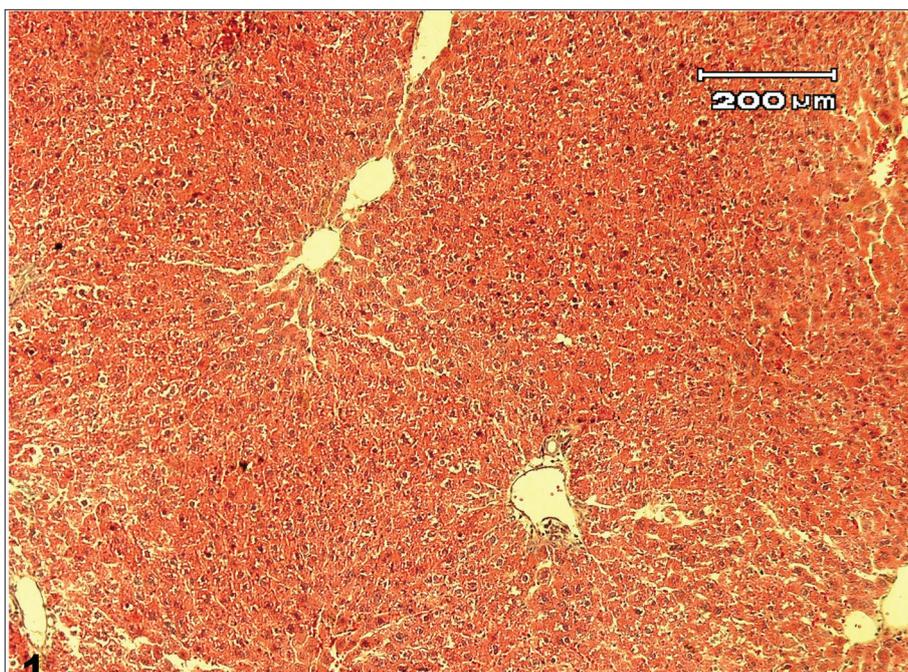
At  $\text{CCl}_4$  treated group livers yellowish pigmentation and crumbly-fatty consistency noticed at macroscopical examination. Liver histopathology revealed centrolobular lipid accumulation with necrosis in the hepatocytes (*Fig. 2*). Also sinusoidal congestion, locally yellowish-green gall pigmentation, increase in the number of kupffer cells and inflammatory cell infiltration around the necrotic tissue was noted. With TUNEL staining DNA fragmentation was observed at some liver epithelium cells. TUNEL reaction in cell cytoplasm was demonstrated as granular staining (*Fig. 3*).

At kefir added group macroscopical appearance of the liver was resembled to control group, normally in colour and pigmentation. The parenchymal structure of the liver was preserved via kefir administration. Kefir significantly reduced fatty degeneration, hepatocytes necrosis, sinusoidal congestion and inflammatory cell infiltration (*Fig. 4*). Compatible with this histopathological results any staining was observed with TUNEL reaction test.

At positive and negative control sections dropping terminal transferase-free solution instead of TUNEL reaction mixture, test gave negative result in all sections.

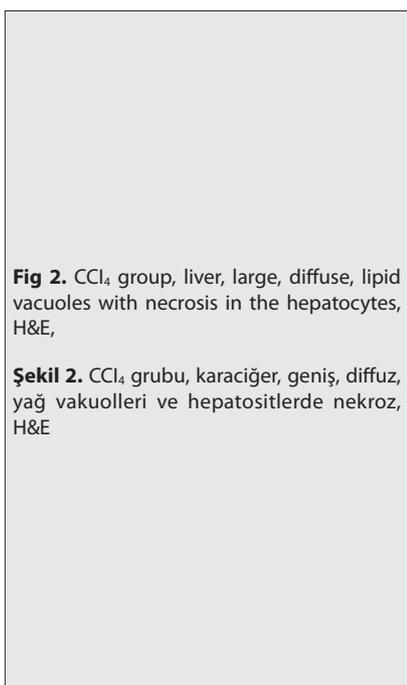
## DISCUSSION

Due to chemicals or infectious agents the liver



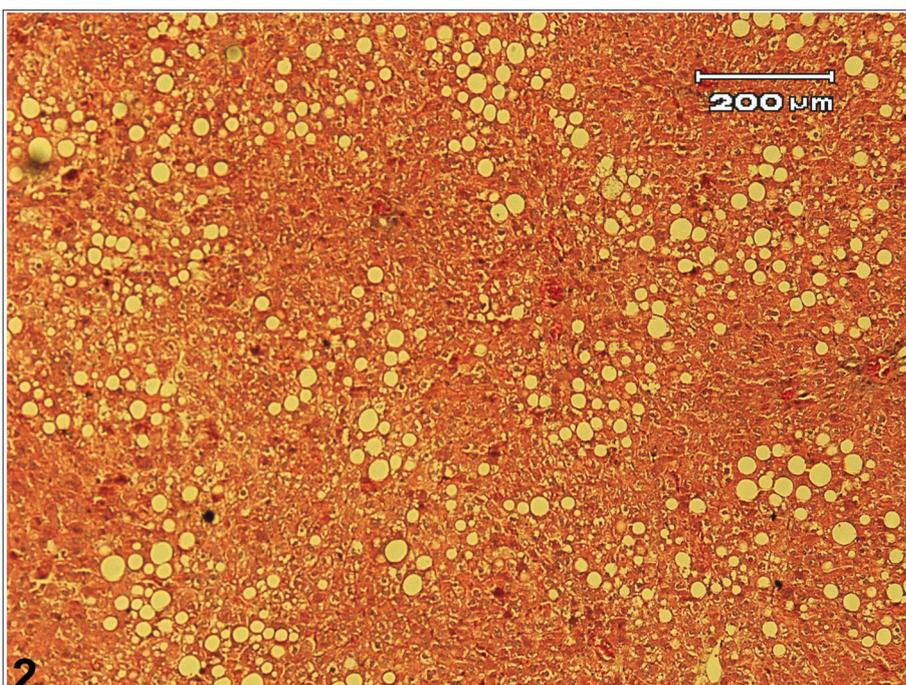
**Fig 1.** Control group, liver, normal histological appearance of the sinusoids and hepatic central vein, H&E

**Şekil 1.** Kontrol grup, karaciğer, sinuzoidler ve hepatic sentral damarın normal histolojik görüntüsü, H&E



**Fig 2.** CCl<sub>4</sub> group, liver, large, diffuse, lipid vacuoles with necrosis in the hepatocytes, H&E,

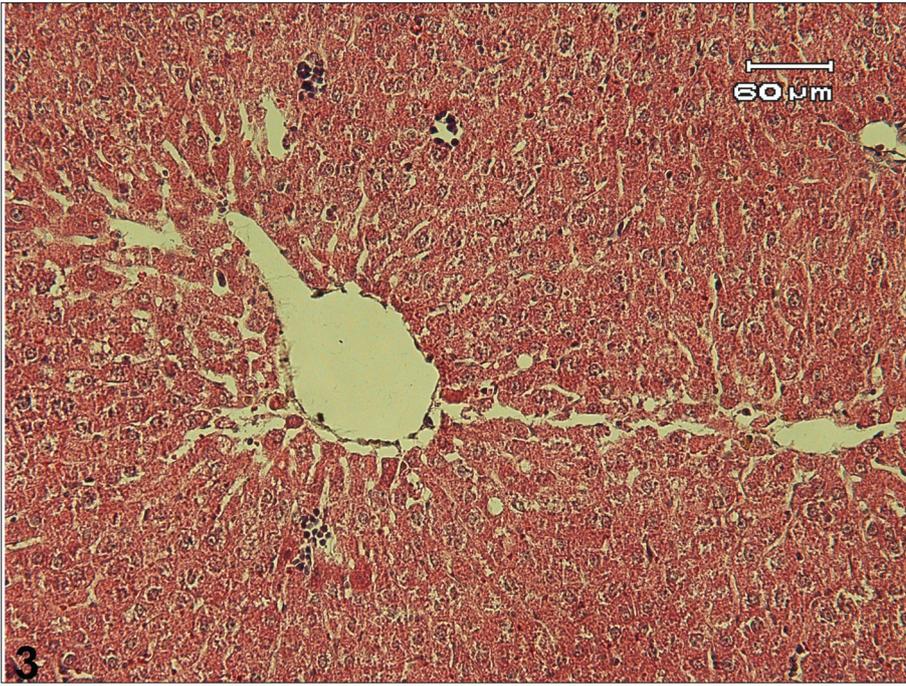
**Şekil 2.** CCl<sub>4</sub> grubu, karaciğer, geniş, diffuz, yağ vakuelleri ve hepatositlerde nekroz, H&E



disorders are one of the world problems and unfortunately no effective treatment that prevent disease damage, progression and complications has yet been found [24,25]. But each passing day investigators studied with new agents to prevent liver damage. Many hepatotoxin agent was found in the environment [26,27], carbon tetrachloride is one of them [28]. Carbon tetrachloride and other halogenated hydrocarbons are used as liquid cleaner (detergents) and oil-repelling substances immemorial. In veterinary medicine they are used against the parasitic anthelmintic [2]. Low doses of CCl<sub>4</sub> caused fatty degeneration of the liver cells, while high doses caused the necrosis of liver cells has

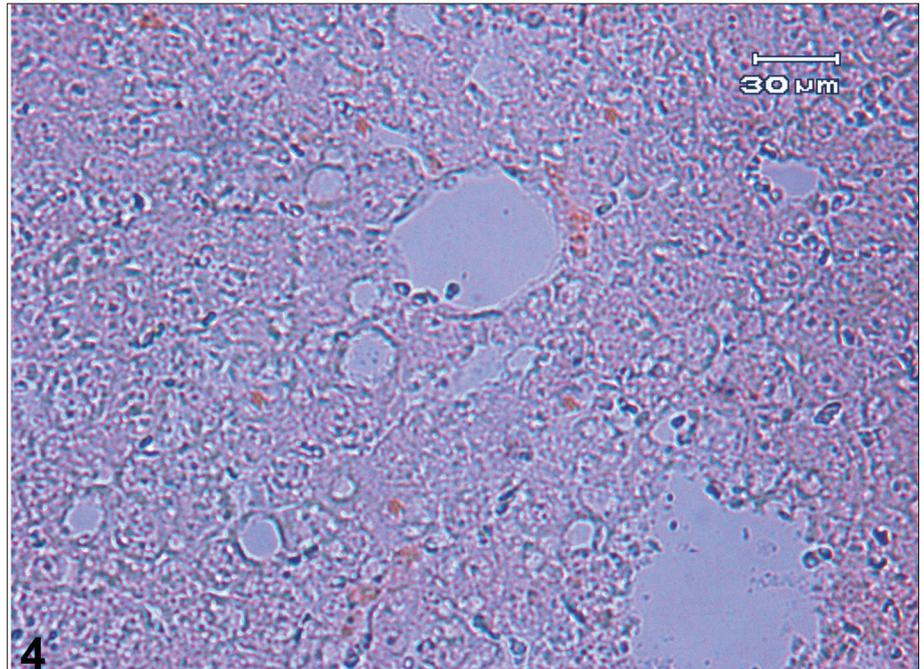
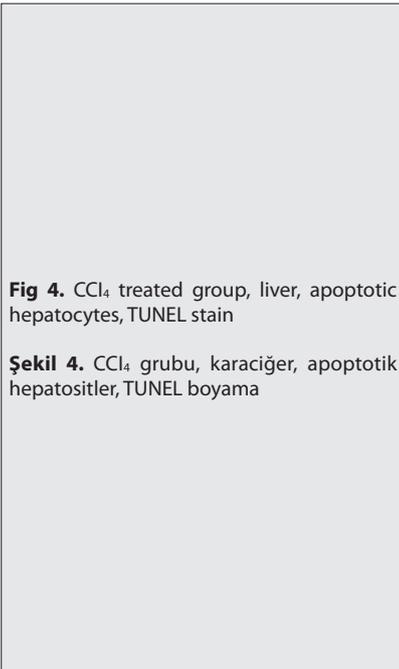
been reported [1,29]. In the our study due to 45 day, low doses CCl<sub>4</sub> administration; hepatocellular degeneration, necrosis and lipid accumulation was observed as described before.

Human and animal beings can encounter with many hepatotoxic agents during their life because of this each passing day both humans, pet owners and of course investigators use a lot of functional foods such as kefir. In the developing world kefir drink increasingly become as popular. Its known that Caucasian origin acidic and midly alcoholic fermented milk kefir drink contain beneficial microorganisms and can treat some diseases [9] and has



**Fig 3.** Kefir group, liver, only few lipid vacuoles, H&E

**Şekil 3.** Kefir grubu, karaciğer, birkaç yağ vakuölü, H&E



an effect on natural immune system it modulates the immune system [30] and also previous studies reported that kefir including many beneficial general health properties such as antioxidant features [14,31]. In the study its clearly observed that affiliated to CCl<sub>4</sub> administration severe liver damage was formed but kefir drink significantly reduced pathological changes. Initial positive change observed at macroscopical appearance of the liver. While in CCl<sub>4</sub> treated group yellowish pigmentation and crumbly-fatty consistency noticed, with kefir administration nearly to normally colour and consistency was observed. Due to CCl<sub>4</sub> toxication histopathologically diffuse, big lipid vacuoles

were replaced with few and small lipid vacuoles with kefir addition. Also kefir protect the paranchymal structure of the liver. This situation was explained at previous studies by this; kefir can able to inhibite the adipocyte differentiation due to its ability to eliminate lipid accumulation at mature adiposites. GPDH (gliserol 3 fosfat dehidrogenaz) is an enzyme that converse glycerole to triglyceride and regular kefir consumption also can be significantly reduced GPDH activity [32]. Kefir has other beneficial effect on decreasing chlosterol levels [33-35]. By apoptosis dying cells are promptly removed by phagocytosis and replaced by new cells generated by mitosis, also apoptosis is an essential feature

of a wide variety of acute and chronic diseases, including liver diseases [36]. In the study cytoplasmic reactions that were detected with TUNEL test, were associated with apoptotic bodies that including nucleous residuals. We observed apoptosis in some hepatocytes depending on CCl<sub>4</sub> exposed. But although severe lipid accumulation, hepatocellular degeneration and inflammatory reaction; apoptotic changes were very mild. Based on kefir supplementation microscopically nearly to control group liver paranchymal structure was observed. Otherwise any reaction that was related to apoptosis was determined.

In conclusion, our results indicate that CCl<sub>4</sub> induce histopathological changes and apoptosis at hepatocytes. Kefiran intake decreased these adverse alterations and did not show any negative effects in the liver of rats. As a results the study shows that kefir is a healthy food that protect liver from CCl<sub>4</sub> toxication and inhibits hepatocellular degeneration, lipid accumulation and apoptosis.

#### ACKNOWLEDGMENT

We are thankful to *Antgen Medical* for chemical material supply.

#### REFERENCES

- Sanlı Y:** Veteriner Farmakoloji. *Ankara Üniv. Vet. Fak. Yay*, No: 412, Ankara, 1988.
- Vural N:** Toksikoloji. *Ankara Üniv. Eczacılık Fak. Yay*, No: 56, Ankara, 1984.
- Özsoy N, Okyar A, Arda-Pirinçi P, Can A, Bolkent Ş, Akev N:** Evaluation of *Smilax excelsa* L. Use in Experimentally Induced Nephrotoxicity. *Kafkas Univ Vet Fak Derg*, 19, 807-814, 2013. DOI: 10.9775/kvfd.2013.9253
- FAO/WHO:** Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria; *FAO/WHO: Amerian Córdoba Park Hotel, Córdoba, Argentina*, 1-34, 2001.
- Fuller R:** Probiotics in man and animals. *J Appl Bacteriol*, 66, 365-378, 1989. DOI: 10.1111/j.1365-2672.1989.tb05105.x
- Robert P, Fedor RN, Madsen KL:** Probiotics and nutraceuticals: Non-medical treatments of gastrointestinal diseases. *Curr Opin Pharma*, 5, 596-603, 2005. DOI: 10.1016/j.coph.2005.06.009
- Falagas ME, Betsi GI, Athanasiou S:** Probiotics for prevention of recurrent vulvovaginal candidiasis - A review. *J Antimicrob Chemother*, 58, 266-272, 2006. DOI: 10.1093/jac/dkl246
- Szymanski H, Pejcz J, Jawien M, Chmielarczyk A, Strus M, Heczko PB:** Treatment of acute infectious diarrhoea in infants and children with a mixture of three *Lactobacillus rhamnosus* strains - A randomized, double-blind, placebo-controlled trial. *Aliment Pharmacol Ther*, 23, 247-253, 2006. DOI: 10.1111/j.1365-2036.2006.02740.x
- Korolev NS:** Starters for fermented milks. Sections 4, Kefir and Kumys Starters. *Bulletin of the IDF* 227, Chapter 2. International dairy Federation, Brussels, Belgium, 1988.
- Zubillaga M, Weill R, Postaire E, Goldman C, Caro R, Boccio J:** Effect of probiotics and functional foods and their use in different diseases. *Nutr Res*, 21, 569-579, 2001. DOI: 10.1016/S0271-5317(01)00281-0
- Kubo M, Odani T, Nakamura S, Tokumaru S Matsuda H:** Pharmacological study on kefir a fermented milk product in *Caucasus I. On antitumor activity. Yakugaku Zasshi*, 112, 489-495, 1992.
- Duitschaever CL, Kemp N, Emmons D:** Pure culture formulation and procedure for the production of kefir. *Milchwissenschaft*, 42, 80-82, 1987.
- Neve H:** Analysis of kefir grain starter cultures by scanning electron microscopy. *Milchwissenschaft*, 47, 275-278, 1992.
- Hoolihan LK:** Prophylactic and therapeutic use of probiotics: A review. *J Am Diet Assoc*, 101, 220-238, 2001. DOI: 10.1016/S0002-8223(01)00060-8
- Furukawa N, Matsuoka A, Takahashi T, Yamanaka Y:** Effects of orally administered yogurt and kefir on tumor growth in mice. *J Jpn Soc Nutr Food Sci*, 43, 450-453, 1990.
- Zacconi, C, Parisi MG, Sarra PG, Dallavalle P, Bottazzi V:** Competitive exclusion of *Salmonella kedougou* in kefir fed chicks. *Microbiol Alim Nutr*, 12, 387-390, 1995.
- St-Onge MP, Farnworth ER, Jones PJ:** Consumption of fermented and nonfermented dairy products: Effects on cholesterol concentrations and metabolism. *Am J Clin Nutr*, 71, 674-681, 2000.
- Matsuo M, Shichijo K, Okaichi K:** The protective effect of fermented milk kefir on radiation-induced apoptosis in colonic crypt cell of rats. *J Radiat Res*, 44, 111-115, 2003. DOI: 10.1269/jrr.44.111
- Cohen GM:** Caspases: The executioners of apoptosis. *Biochem J*, 326, 1-16, 1997. DOI: 10.1042/bj3260001
- Everett H, McFadden G:** Apoptosis: An innate immune response to virus infection. *Trends Microbiol*, 7, 160-165, 1999. DOI: 10.1016/S0966-842X(99)01487-0
- Green DR:** Apoptotic pathways: The roads to ruin. *Cell*, 94, 695-698, 1988. DOI: 10.1016/S0092-8674(00)81728-6
- Patel T, Bronk, SF, Gores, GJ:** Increases of intracellular magnesium promote glycodeoxycholate-induced apoptosis in rat hepatocytes. *J Clin Invest*, 94, 2183-2192, 1994. DOI: 10.1172/JCI117579
- Luna LG:** Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. McGraw-Hill Book Co., NewYork. p.32, 1968.
- Bruck RR, Hershkoviz O, Lider, H Aeed, L Zaidel, Z Matas, J Berg, Helpert Z:** Inhibition of experimentally induced liver cirrhosis in rats by a nonpeptidic mimetic of the extracellular matrix associated Arg-Gly-Asp epitope. *J Hepatol*, 24, 731-738, 1996.
- Anand BS:** Cirrhosis of liver. *West L Med*, 171, 110-115, 1999.
- Mutlu N, Ersan Y, Nur G, Koç E:** Protective effect of caffeic acid phenethyl ester against lead acetate-induced hepatotoxicity in mice. *Kafkas Univ Vet Fak Derg*, 17 (Suppl. A): S1-S5, 2011. DOI: 10.9775/kvfd.2010.2717
- Seven İ, Gülbaykalır, Tatlıseven P, Dağoğlu G:** The ameliorative effects of propolis against cyclosporine A induced hepatotoxicity and nephrotoxicity in rats. *Kafkas Univ Vet Fak Derg*, 20, 641-648, 2014. DOI: 10.9775/kvfd.2013.10643
- Güven A, Maraşlı N, Kaya N:** Karbontetraklorür (CCl<sub>4</sub>) ve etil alkolün fare eritrosit antioksidan ve plazma lipid peroksidasyonuna etkisi. *Kafkas Univ Vet Fak Derg*, 9 (1): 1-4, 2003.
- Abraham P, Wilfred G, Catherine SP:** Oxidative damage to the lipids and protein in the lungs, testis and kidney of rats during carbon tetrachloride intoxication. *Clin Chim Acta*, 289, 177-179, 1999. DOI: 10.1016/S0009-8981(99)00140-0
- Eliş Yıldız S, Yiğit F, Duman Aydın B, Karadağ Sarı E, deprem T, Koral Taşçı S:** Effects of kefir, koumiss, milk and yoghurt administration on distribution of plasma cells and mast cells in mice spleen. *Kafkas Univ Vet Fak Derg*, 21, 195-201, 2015. DOI: 10.9775/kvfd.2014.12015
- Lin MY, Change FJ:** Antioxidative effect of intestinal bacteria *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ATCC 4356. *Dig Dis Sci*, 45, 1617-1622, 2000.
- Ho JN, Jae-Woo C, Won-Chul L, Mi-Kyoung K:** Kefir inhibits 3T3-L1 adipocyte differentiation through down-regulation of adipogenic transcription factor expression. *J Sci Food and Agricult*, 93, 485-490, 2013. DOI: 10.1002/jsfa.5792
- Kiessling G, Schneider J, Jahreis G:** Long-term consumption of fermented dairy products over 6 months increases HDL cholesterol. *Eur*

---

*J Clin Nutr*, 56, 843-849, 2002. DOI: 10.1038/sj.ejcn.1601399

**34. Xiao JZ, Kondo S, Takahashi N, Miyaji K, Oshida K, Hiramatsu A, Iwatsuki K, Kokubo S, Hosono A:** Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. *J Dairy Sci*, 86, 2452-2461, 2003. DOI: 10.3168/jds.S0022-0302(03)73839-9

**35. Güven A, Güven A:** Hiperkolesterolemi oluşturulmuş tavşanlarda kefirin total kolesterol, trigliserit, HDL-kolesterol, LDL-kolesterol ve lipit peroksidasyonu üzerine etkisi. *Kafkas Univ Vet Fak Derg*, 11 (2): 127-131, 2005.

**36. Guicciardi ME, Gores:** Apoptosis: A mechanism of acute and chronic liver injury. *Gut*, 54, 1024-1033, 2005. DOI: 10.1136/gut.2004.053850