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RESEARCH ARTICLE

Hepatoprotective effect of pomegranate (Punica granatum l.) in a rabbit model of steatohepatitis

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ABSTRACT

The study aimed to exhibit liver protection, activity, and probable side effects of LPJE on a steatohepatitis rabbit model. In this study, 48 rabbits were used. The first group (Group 1) rabbits were fed a standard diet (SD), while the second group (Group 2) rabbits were fed a high-fat diet (HFD). In two groups, three different doses of LPJE (25 mg/kg, 50 mg/kg, and 100 mg/kg) were given simultaneously to the rabbits for eight weeks. Results showed that the serum albumin concentration was significantly different in the LPJE group (100 mg/kg) than in the positive control group. The cholesterol concentration of the LPJE (50mg/kg) administered subgroup of Group 2 (96.71 ± 12.03) was found to be the lowest. According to histopathological examination results, Group 2 did not display a statistical difference; however, the lowest liver fat deposition degree was 26.49 ± 8.64% in the LPJE (50 mg/kg) subgroup. As a result of this study, the lyophilized pomegranate extract has hepatoprotective activity. The most effective dose was 50 mg/kg on the non-alcoholic steatohepatitis (NASH) rabbit model. No side effects were determined up to 100 mg/kg pomegranate juice extract on rabbits.

Keywords: Antioxidant activity, bioactivity, non-alcoholic steatohepatitis, punica granatum.

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Bir tavşan steatohepatit modelinde narın (Punica granatum l.) hepatoprotektif etkisi

ÖZ

Bu çalışmada, bir steatohepatit tavşan modelinde Liyofilize nar suyu ekstresinin (LPJE) karaciğer koruması, aktivitesi ve olası yan etkilerinin gösterilmesi amaçlandı ve 48 tavşan kullanıldı. Birinci grup (Grup 1) tavşanlara standart bir diyet (SD), ikinci grup (Grup 2) tavşanlara ise yüksek yağlı bir diyet (HFD) verildi. Her iki gruptaki üçer alt grup tavşanlara sekiz hafta boyunca eş zamanlı olarak üç farklı dozda LPJE (25 mg/kg, 50 mg/kg ve 100 mg/kg) verildi. Pozitif ve standart kontrol grubu tavşanlara ise 1 ml cmc solüsyonu içirildi. Sonuçlar; serum albümin konsantrasyonunun LPJE grubunda (100 mg/kg) pozitif kontrol grubundan önemli ölçüde farklı olduğunu gösterdi. En düşük kolesterol konsantrasyonu, Grup 2'nin (96.71 ± 12.03) LPJE (50mg/kg) uygulanan alt grubunda bulundu. Histopatolojik inceleme sonuçlarına göre Grup 2 istatistiksel olarak farklılık göstermedi; ancak en düşük karaciğer yağlanma derecesi LPJE (50 mg/kg) alt grubunda %26.49 ± 8.64 olarak ölçüldü. Sonuç olarak, liyofilize nar ekstresinin hepatoprotektif aktivite gösterdiği ve alkolsüz steatohepatit (NASH) tavşan modelinde en etkili dozun 50 mg/kg olduğu saptandı. Ayrıca tavşanlarda 100 mg/kg nar suyu ekstresine kadar herhangi bir yan etki tespit edilmedi.

Anahtar Kelimeler: Alkolsüz steatohepatit, antioksidan aktivite, biyoaktivite, punica granatum

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a deterioration of the liver functions without alcohol and histopathological alcohol-induced hepatic impairment (Ludwig et al. 1980). The golden standard for the diagnosis of NASH is liver biopsy (Marchesini and Marzocchi 2007, Neuschwander-Tetri 2005). Steatosis, inflammatory infiltration, ballooning degeneration in hepatocytes, and fibrosis are the main histopathological findings of NASH (Brunt 2002, Sonsuz and Baysal 2011). The primary objective in the treatment of NASH is to prevent and treat metabolic syndrome. Presently, there are no effective drugs used in NASH treatment (Adorini et al. 2012, Pfohl et al. 2020). However, lipid/cholesterol reducers, insulin sensitizers, alpha-glucosidase inhibitors, non-steroidal anti-inflammatory drugs, and cholinesterase inhibitors are used for NASH treatments. (Scarpini et al. 2003, Takahashi et al. 2015).

Pomegranate is used for protection from diseases in folk medicine, particularly in the Middle East countries (Gurib-Fakim 2006). Pomegranate juice contains active compounds such as vitamin C, anthocyanins, punicalagin, ellagic and gallic acids (El-Nemr et al. 1990). Previous evidence suggests that pomegranate had chemopreventive, cardioprotective, antihyperlipidemic, and hepatoprotective effects (Afaq et al. 2005, Sumner et al. 2005, Fuhrman et al. 2005, Osman et al. 2011). Moreover, the phenol content of pomegranate products, such as extracts and juice, is abundant. Thus, dietary pomegranate products showed protective effects against inflammation-related disorders in different human clinical trials (Pfohl et al. 2020). A clinical trial showed that a pomegranate extract reduced complications linked with obesity in overweight and obese individuals (Hosseini et al. 2016). Pomegranate is considered to be effective against NASH owing to its antihyperlipidemic activity (Fuhrman et al. 2005, Pagano et al. 2002, Çolak and Tuncer 2010).

In the present study, the hepatoprotective effect of lyophilized pomegranate fruit extract obtained from fresh pomegranate on the livers of rabbits fed with HFD was investigated. Also, its antioxidant properties and possible side effects were evaluated.

MATERIALS AND METHODS

Plant Material

Pomegranate extracts were prepared from the fresh pomegranate whole fruits growing in the Mediterranean Region of Turkey. Fresh pomegranate fruits were squeezed with a juicer, and the juice was lyophilized. "Lyophilized pomegranate juice extract" was obtained and administered per os to the test animals in three different doses [(25 mg/kg; 50 mg/kg; and 100 mg/kg in 1 ml 0.5% carboxymethyl cellulose (CMC)].

Animals

Forty-eight non-pathogenic, five-month-old, male New Zealand breed rabbits were used in the present study. The research was conducted on a total of two groups. One of them was the main control group (Group 1), and the other was the experimental group (Group 2). Each group was formed by four subgroups (n=6). The experiments in all groups were initialized after a one-week acclimatization process. The rabbits in Group 1 were fed a Standart diet (SD) ad libitum, while the ones in Group 2 were fed a high-fat diet (HFD), for all groups were free to reach the water. The animals were kept under standard conditions for animal care.

Standard Control Group (SCG) in Group 1 and Positive Control Group (PCG) in Group 2 were formed for intergroup comparisons. Instead of pomegranate extracts, 1 mL of 0.5% CMC was given daily to the rabbits in those groups (placebo).

Group 1 was formed by a total of four subgroups, one of which was the standard control groups (1 mL of 0.5% CMC/day) and lyophilized pomegranate juice extract at 25 mg/kg, 50 mg/kg, and 100 mg/kg doses were given daily to the animals in the other three subgroups.

For setting the steatohepatitis model, an HFD (20% of corn oil and 1.25% (w/w) of cholesterol addition to the SD) was given ad libitum to the experimental group (Group 2) for eight weeks (Ogawa et al. 2010). During the model formation, pomegranate extracts were given simultaneously to the rabbits in group 2 in three doses (25, 50, and 100 mg/kg) for eight weeks.

During the experiment, daily live weight gain and food intake of the rabbits were recorded.

Sampling and Analyses

At the end of the experiment, blood samples from central ear arteries were obtained with 22-gauge intravenous catheter from the rabbits into serum tubes and anticoagulant tubes from all the groups. Serum samples were stored at -20°C until the measurement time.

In the serum samples, Alanine Transaminase (ALT), Aspartate Transaminase (AST), Gamma-Glutamyl Transferase (GGT), Albumin (ALB), Total Protein (TP), Total Bilirubin (TBIL), Glucose (GLU), Cholesterol (TC), Triglycerides (TG), High-Density Lipoprotein (HDL), and Low-Density Lipoprotein (LDL) values were measured with a Mindray BS 120 biochemistry analyzer. NEFA (Diametra, REF: FA115, LOT: 288862) and insulin levels (Diametra, REF: DKO076, LOT: 3118) were measured with ELISA kits. Very Low-Density Lipoprotein (VLDL) value was calculated with TG/5 formula (Civelek et al. 2011).

For the evaluation of the antioxidant activity (AoA), Glutathione (GSH), Malondialdehyde (MDA), and

Nitric Oxide (NO) levels were measured. MDA and NO were examined in the liver tissue samples.

After the procedure, both groups (Group 1 and 2) were euthanized under anesthesia (Xylazine 5 mg/kg + Ketamine 35 mg/kg) by administering 150 mg/kg of intravenous thiopental sodium. Then, the rabbit's tissues were collected, livers were removed, and wet weights were measured. To standardize body weight on the liver scale, the formula of body weight/liver weight was used. The samples were sent to the laboratory in 10% Ca-formaldehyde for histopathological examination.

The tissue samples obtained from the liver were kept in neutral buffered formaldehyde solution for 48 hours. Sections in 6-8 μm thickness were made using Cryostat and stained with oil red O staining technique (Lillie and Ashnburn 1943). The preparation was closed with aqueous adhesives and examined under a light microscope.

Statistical Analyses

For Group 1 and Group 2, the animals' live weights were taken as covariate variables in the last week. The covariance analysis (ANCOVA) method was utilized in the comparisons within the groups. The Mann-Whitney U test was employed in the comparison of the control groups (SCG and PCG). To analyze the disparities between live weight gain and food intake of the subgroups of Group 1 and Group 2, the Kruskal-Wallis H test was utilized. For those statistical analysis methods, MedCalc 15, SPPS 13 for Windows, and Microsoft Excel 2010 packages were used.

The research procedure was carried out with the approval of the Institutional Ethic Committee of the Faculty of Veterinary Medicine, Afyon Kocatepe University (No. 2012/234).

RESULTS

Biochemistry, oxidant/antioxidant parameter measurements, and pathologic evaluation results of the present study were showed in Table 1-4.

Table 1. Biochemical analysis results (Group 1 and Group 2)

Groups	Mean ± SEM (n=6)												
	Dose (mg/kg)	**ALB (g/dL)**	**AST (U/L)**	**ALT (U/L)**	**GGT (U/L)**	**TBIL**	**TP (g/dL)**	**TG (mg/dL)**	**TC (mg/dL)**	**HDL (mg/dL)**	**LDL**	**NEFA**	**VLDL (mg/dL)**
Standard Control Group (SD)	-	4.32 ±0.086	41.01 ±4.60	35.40 ±6.73	12.76 ±1.82	0.15 ±0.012	6.46 ±0.21	61.15 ±11.59	59.37 ±11.25	32.20 ±5.18	23.21 ±7.79	0.08 ±0.01	12.22 ±2.32
P. granatum liyophilized extract (LPJE)	25	4.50 ±0.079	47.22 ±4.20	37.00 ±6.15	11.17 ±1.66	0.17 ±0.011	6.67 ±0.19	55.36 ±10.58	52.13 ±10.27	27.06 ±4.73	23.72 ±7.11	0.07 ±0.01	11.07 ±2.12
	50	4.59 ±0.079	51.88 ±4.20	44.00 ±6.15	12.19 ±1.66	0.16 ±0.011	6.96 ±0.19	45.87 ±10.58	60.73 ±10.27	33.46 ±4.73	26.19 ±7.11	0.08 ±0.01	9.17 ±2.12
	100	4.47 ±0.079	46.72 ±4.20	44.67 ±6.16	12.08 ±1.66	0.15 ±0.011	6.58 ±0.19	34.92 ±10.60	44.14 ±10.29	29.71 ±4.74	15.20 ±7.12	0.08 ±0.01	6.98 ±2.12
P	**-**	**0.176**	**0.408**	**0.648**	**0.931**	**0.505**	**0.336**	**0.374**	**0.664**	**0.789**	**0.723**	**0.912**	**0.374**
Positive Control Group (HFD)	-	4.46 $\pm 0.10^{b}$	53.28 ±26.18	49.75 ±11.24	32.32 ±4.19	0.17 ±0.016	7.04 ±0.23	104.66 ±22.58	118.52 ±12.03	43.56 ±4.86	57.44 ±7.86	0.11 ±0.014	20.93 ±4.52
P. granatum liyophilized extract (LPJE)	25	4.16 $\pm 0.10^{ab}$	41.13 ±26.96	61.00 ±11.57	26.70 ±4.31	0.19 ±0.016	6.46 ±0.24	162.89 ±23.25	125.47 ±12.39	42.21 ±5.00	51.36 ±8.09	0.12 ±0.014	32.57 ±4.65
	50	4.39 $\pm 0.10^{ab}$	69.82 ±26.17	69.18 ±11.23	23.49 ±4.19	0.20 ±0.016	6.66 ±0.23	94.37 ±22.58	96.71 ±12.03	44.90 ±4.86	40.46 ±7.85	0.07 ±0.014	18.87 ±4.52
	100	4.04 $\pm 0.10^{a}$	96.95 ±26.39	64.07 ±11.33	24.99 ±4.22	0.18 ±0.016	6.10 ±0.23	92.14 ±22.76	114.95 ±12.13	44.53 ±4.90	49.45 ±7.92	0.08 ±0.014	18.42 ±4.55
P	**-**	**0.019**	**0.504**	**0.662**	**0.483**	**0.626**	**0.057**	**0.146**	**0.406**	**0.981**	**0.509**	**0.109**	**0.146**

[a]and [b] describe difference between groups.

Table 2. Biochemical analysis results (Group 1 and Group 2)

Groups	Mean ± SEM (n=6)												
	Dose (mg/kg)	**GLU (mg/dL)**	**İnsulin (U/L)**	**Liver weight (BW/LW)**	**LS (%) Mean**	**Food Consumption (g)**	**Live weight gain(g)**	**MDA (nmol/mL)**	**dMDA (nmol/mL)**	**NO (µmol/L)**	**dNO (µmol/L)**	**GSH (mmol/L)**	**AoA (mmol/L)**
Standard Control Group (SD)	-	107.46 ±7.99	0.14 ±0.08	2.51 ±0.19	0.01 ±3.62	7732.20 ±1646.24^{ab}	780.40 ±177.75	3.95 ±0.29	2.20 ±0.19	11.57 ±2.01	7.53 ±1.30^{ab}	26.62 ±3.82	6.37 ±0.24
P. granatum liyophilized extract (LPJE)	25	111.23 ±7.30	0.31 ±0.07	2.61 ±0.18	3.38 ±3.31	8536.50 ±804.73^{a}	793.00 ±331.22	4.05 ±0.26	2.59 ±0.18	14.67 ±1.84	7.26 ±1.19^{a}	26.41 ±3.48	6.65 ±0.22
	50	113.86 ±7.30	0.22 ±0.07	2.88 ±0.18	3.37 ±3.31	7394.33 ±969.32^{ab}	702.50 ±320.13	3.48 ±0.26	2.18 ±0.18	14.02 ±1.84	11.90 ±1.19^{b}	19.40 ±3.48	6.45 ±0.22
	100	119.69 ±7.30	0.17 ±0.07	2.35 ±0.18	6.57 ±3.31	6885.66 ±364.77^{b}	617.50 ±230.90	3.22 ±0.26	2.44 ±0.18	16.03 ±1.84	10.53 ±1.19^{ab}	22.51 ±3.49	6.40 ±0.22
P	**-**	**0.713**	**0.406**	**0.231**	**0.627**	**0.047**	**0.764**	**0.134**	**0.347**	**0.449**	**0.036**	**0.440**	**0.805**
Positive Control Group (HFD)	-	125.86 ±6.64	0.03 ±0.06	2.73 ±0.13	49.50 ±9.51	4720.33 ±529.31	619.33 ±231.69	4.75 ±0.40	2.46 ±0.26	11.64 ±1.57^{ab}	12.58 ±1.83	13.08 ±2.85	7.01 ±0.15
P. granatum liyophilized extract (LPJE)	25	132.91 ±6.84	0.22 ±0.07	2.46 ±0.14	44.44 ±8.94	4187.16 ±402.27	508.00 ±250.06	4.90 ±0.41	2.73 ±0.27	9.85 ±1.62^{b}	8.64 ±1.88	19.65 ±2.93	6.88 ±0.16
	50	117.24 ±6.64	0.11 ±0.06	2.68 ±0.13	26.49 ±8.64	4637.50 ±971.37	589.16 ±275.93	4.95 ±0.40	2.81 ±0.26	10.31 ±1.57	15.82 ±1.83	23.24 ±2.85	6.83 ±0.15
	100	109.16 ±6.69	0.09 ±0.06	2.56 ±0.13	27.81 ±8.70	4992.16 ±616.94	569.66 ±191.50	4.60 ±0.40	3.16 ±0.26	16.14 ±1.58^{a}	12.05 ±1.84	15.53 ±2.87	7.07 ±0.15
P	**-**	**0.112**	**0.277**	**0.519**	**0.148**	**0.209**	**0.904**	**0.928**	**0.335**	**0.046**	**0.092**	**0.093**	**0.690**

[a]and [b] describe difference between groups.

Table 3. Biochemical analysis results (Standard Control Group and Positive Control Group)

Groups		Mean ± SEM (n=6)											
	Dose (mg/kg)	**ALB (g/dL)**	**AST (U/L)**	**ALT (U/L)**	**GGT (U/L)**	**TBIL**	**TP (g/dL)**	**TG (mg/dL)**	**TC (mg/dL)**	**HDL (mg/dL)**	**LDL**	**NEFA**	**VLDL (mg/dL)**
Standard Control Group (SD)	-	4.32 ±0.31	41.00 ±8.46	35.40 ±6.99	12.76 ±1.99	0.15 ±0.019	6.46 ±0.64	61.10 ±37.13	59.24 ±28.99	32.12 ±7.61	23.16 ±24.86	0.084 ±0.042	12.22 ±7.42
Positive Control Group (HFD)	-	4.47 ±0.23	51.33 ±17.35	48.67 ±13.97	31.92 ±13.35	0.17 ±0.050	7.04 ±0.76	104.53 ±25.74	118.23 ±24.64	43.12 ±11.56	59.79 ±17.75	0.108 ±0.037	21.55 ±5.47
P	**-**	**0.360**	**0.201**	**0.143**	**0.006**	**0.169**	**0.201**	**0.045**	**0.018**	**0.201**	**0.047**	**0.709**	**0.047**

Table 4. Biochemical analysis results (Standard Control Group and Positive Control Group)

Groups	Mean ± SEM (n=6)												
	Dose (mg/kg)	**GLU (mg/dL)**	**Insulin (U/L)**	**Liver weight (BW/LW)**	**LS (%) Mean**	**Food Consumption (g)**	**Live weight gain(g)**	**MDA (nmol/mL)**	**dMDA (nmol/mL)**	**NO (µmol/L)**	**dNO (µmol/L)**	**GSH (mmol/L)**	**AoA (mmol/L)**
Standard Control Group (SD)	-	107.40 ±27.13	0.14 ±0.11	2.51 ±0.22	0.00 ±0.00	7732.20 ±1646.24	780.40 ±177.75	3.95 ±0.94	2.20 ±0.50	11.56 ±3.62	7.52 ±0.66	26.60 ±7.83	6.37 ±0.66
Positive Control Group (HFD)	-	126.50 ±23.34	0.04 ±0.00	2.73 ±0.38	50.00 ±29.15	4720.33 ±529.31	619.33 ±231.69	4.77 ±0.60	2.45 ±0.45	11.55 ±3.24	12.61 ±6.07	13.20 ±8.23	7.01 ±0.17
P	**-**	**0.784**	**0.006**	**0.170**	**0.005**	**0.009**	**0.329**	**0.100**	**0.410**	**1.000**	**0.017**	**0.036**	**0.094**

In biochemical measurements, no statistical difference was determined in the ALB value of Group 1. At the same time, a statistically significant decrease was detected in LPJE (100 mg/kg) subgroup when compared to the LPJE (25 mg/kg) subgroup of Group 2 in intergroup comparisons ($p < 0.05$). In the comparison of the control groups, no statistical difference was found.

When ALT, AST, GGT, TBIL, and TP levels were evaluated, no statistically significant differences were detected in Group 1 and Group 2. In comparing the control groups, a statistical difference was seen only in the GGT value (SCG, 12.76 ± 1.99; PCG, 31.92 ± 13.35, $p = 0.006$).

Although no statistically significant difference was detected between Group 1 and Group 2 in terms of TG values, a numerical difference was found between the SCG and LPJE (100 mg/kg) subgroup of Group 1. In comparing control groups, the increase of TG in Group 2 was considered statistically significant (SCG, 61.10 ± 37.13, PCG, 104.53 ± 25.74, $p = 0.045$).

In the statistical evaluation of the TC measurements, no statistical difference was detected in Group 1 and Group 2. In comparing the control groups, the increase of TC in Group 2 was found to be statistically significant (SCG, 59.24 ± 28.99; PCG, 118.23 ± 24.64, $p = 0.018$).

No statistical differences were found in HDL, LDL, NEFA, and VLDL values in Group 1 and Group 2. In the comparison of the control groups, no statistically significant differences were detected in HDL and NEFA values, but the increase in LDL (SCG, 23.16±24.86; PCG, 59.75±17.75 $p = 0.047$) and VLDL (SCG 12.22 ± 7.42; PCG, 21.55 ± 5.47, $p = 0.047$) values in Group 2 were considered as significantly detected.

Even though there were no statistical differences in serum GLU and insulin levels between Group 1 and Group 2, a numerical difference was detected in serum insulin concentration in SCG when compared to the LPJE (25 mg/kg) subgroup of Group 1 and in PCG compared to LPJE (25 mg/kg) subgroup of Group 2. Where there was not a statistical difference in GLU level in the comparison of the control groups, the decrease in the insulin level in Group 2 (SCG, 0.14 ± 0.11; PCG, 0.04 ± 0.00, $p = 0.006$) was found to be statistically significant.

In evaluating wet liver weight (LWW) and liver steatosis rates (LS%) in Group 1 and Group 2, no statistically significant differences were found in none of the groups. Moreover, although no statistical difference was detected in Group 2 in terms of LS%, a numeric difference was found in LPJE (50 mg/kg) subgroup (26.49 ± 8.64) and LPJE (100 mg/kg) subgroup (27.81 ± 8.70) of Group 2 when compared to PCG (49.50 ± 9.51). In comparing the control groups (SCG, 0.00 ± 0.00; PCG, 50.00 ± 29.15, $p = 0.005$), a statistically significant difference in liver steatosis was detected.

In terms of the food consumption during the study, there was a statistically significant difference between the LPJE (25 mg/kg) subgroup of Group 1 (8536.50 ± 804.73) and the LPJE (100 mg/kg) subgroup of Group 1 (6885.66 ± 364.77) ($p = 0.047$). In comparing the control groups (SCG, 7732.20 ± 1646.24; PCG, 4720.33 ± 529.31, $p = 0.009$), the food consumption of Group 2 was dramatically lower than Group 1. No difference was detected in the food consumption rate within Group 2.

During the statistical evaluation of the differences in the animals' live weights between the beginning and the end of the study, no statistical difference was determined in Group 1 and Group 2.

In comparing oxidant and antioxidant concentrations of tissue and serum, a statistical difference was detected in NO level in Group 1 ($p = 0.036$). No statistical difference was detected in any other oxidant and antioxidant concentrations of tissue and serum. In Group 2, only the NO value increased at a statistically significant PCG rate compared to the LPJE (100 mg/kg) subgroup of Group 2 ($p = 0.046$). In the comparison of the control groups, there was a statistical difference in dNO (SCG, 7.52 ± 0.66; PCG, 12.61 ± 6.07, $p = 0.017$) and GSH (SCG, 26.60 ± 7.83; PCG, 13.20 ± 8.23, $p = 0.036$) values.

DISCUSSION

HFD, obesity, insulin resistance, and oxidative stress are the main factors of NASH. Unlike many other studies, an HFD was preferred in the present study rather than the chemicals to induce the experimental model. Due to its rich antioxidant content and preliminary studies on its bioactivity, the hepatoprotective effect of pomegranate was investigated in the present study.

The first symptom of NASH is the increase in liver enzymes. However, high levels of these enzymes do not always indicate liver fibrosis (Angulo et al., 1999; Gören & Fen, 2005). When positive and standard control groups were compared in the study, although a numerical increase was detected in the liver enzymes in Group 2, only the increase in GGT was statistically significant. The present research findings were found to be in parallel with the previous studies (Ogawa et al., 2010; Bayan et al., 2004). The rise in GGT suggests that damage in bile ducts might develop due to HFD (Ideo et al. 1972). It was reported in a study by Patel et al. (2019) that the use of pomegranate extract enriched with punicalagin even in such high doses like 600 mg/kg/day did not lead to an increase in ALT, AST, and GGT values. Haber et al. (2007) reported that pomegranate extract given in the dose of 1420 mg/day did not increase liver enzymes. Like the other studies, it was determined in the present study that the pomegranate extract given to the rabbits in Group 1 did not cause an increase in AST, ALT, and GGT levels in any of the doses. The researchers have reported that

different pomegranate extracts dramatically lower the rises in ALT, AST, and GGT in liver injury (Osman et al. 2011, Celik et al. 2009, Ashoush et al. 2013, Zou et al. 2014). Unlike those studies, in the present report, no statistically significant differences were detected in ALT, AST, and GGT levels in Group 1 and Group 2. Unlike the study presented in these studies, while different pomegranate components were used, the extract of pomegranate fruit was used in our research.

A toxicity study demonstrated that pomegranate extract did not affect serum ALB level in male rats. On the other hand, pomegranate in the dose of 60 mg/kg/day led to a statistically significant increase in female rats' serum albumin level (Patel et al. 2008). In the present study, male rabbits were used, and similarly, there was no significant difference found in serum ALB level. In several other studies, a decrease in the ALB level in blood due to liver injury was reported to bring to the normal range by administering the pomegranate extract (Osman et al. 2011, Shaban et al. 2013). In this report, although 100 mg/kg pomegranate extract given to Group 2 decreased serum ALB limits compared to the control group, the results were within the reference limits.

In the present research, it was discovered that pomegranate did not change serum TP levels in Group 1 and Group 2. A statistical difference was not detected in comparing the control groups (SCG and PCG), yet a numerical increase was observed in Group 2. In the subgroups of Group 1, no statistically significant difference was seen. Some other research reports are similar to Vidal et al. (2003) and different from Patel et al. (2008) our findings. Evaluating the concentrations of serum TP in Group 2, a decrease was observed in all subgroups compared to the positive control group numeric decrease was in the LPJE (100 mg/kg) subgroup of Group 2. However, this difference was only numerical and not statistically significant ($p = 0.057$). In another study, pomegranate was reported to increase the low level of serum TP. Since ALB consists of 50% of plasma proteins (Kaysen 1998), the present study's decrease could be due to the reduction of the ALB level.

In the present study, pomegranate extract did not significantly affect the TG level in Group 1 and Group 2. However, a numerical decrease was found in the serum TG concentration of LPJE (100 mg/kg) subgroup of Group 1 compared to SCG. In our opinion, it seems a dose-dependent effect. The TG level of Group 2 was higher than the TG levels of control rabbits (SCG and PCG 250mg). The previous reports are consistent with our results (Zou et al. 2014, Lei et al. 2007). Lei et al. (2007) reported that pomegranate peel extract (at 400 and 800 mg/kg/day doses) provided a statistically dramatic decrease in the animals' TG level fed an HFD. Despite not being statistically significant in the present study, LPJE (100 mg/kg) numerically decreased the TG level. According to the previous studies, pomegranate peels' TG lowering effect could be due to the tannin content (Zou et al. 2014, Squilacci and Di Maggio 1946).

Although cholesterol is used in many functional tasks in the body, high levels significantly increase heart and blood vessel diseases (Mamurekli et al. 2000). When the control groups (SCG and PCG) were compared, TC level increased significantly in Group 2, although there was no statistical difference. The results of the present study were found to be in accord with previous results (Ogawa et al. 2010, Zou et al. 2014, Zhang et al., 2010). In their research on obese rats, Huang et al. (2005) determine that pomegranate lowered plasma TC level. Similarly, concentrated pomegranate juice was reported to decrease the TC level in patients with hyperlipidemia and diabetes (Zhang et al. 2010, Esmaillzadeh et al. 2006). It was reported that the pomegranate extract enriched with punicalagin led to statistically significant decreases in the values of the liver and serum TC, particularly in high doses (150 mg/kg) in the Non-alcoholic fatty liver disease (NAFLD) model (Zou et al. 2014). Lei et al. (2007) reported that pomegranate extract lowered serum TC level in the doses of 400 and 800 mg/kg. However, the decrease was not statistically significant. In the mentioned studies in which a decrease was observed in TC, pomegranate extracts were enriched with punicalagin. The decrease in TC level was not significant might have resulted from the punicalagin amount of the extract.

Our study determined that pomegranate extract affects the levels of HDL in Group 1 and Group 2. However, compared with the control groups (SCG and PCG), a numerical increase was detected in the serum HDL concentrations in the animals fed an HFD. The mentioned results may be related to the high fat and cholesterol levels of the diet. The researcher reported no significant dietary pomegranate effect on the lipid profile, consistent with our results (Esmaillzadeh et al. 2006, Rashidi et al. 2013). Zhang et al. (2010) reported that pomegranate extract given in the dose of 400 mg/kg lowered serum HDL concentration, but no difference occurred between the groups in 800 mg/kg.

The present report determined that pomegranate extracts did not affect the LDL levels in any of the groups. Compared with the control groups, it was observed that the LDL levels were statistically significant in the rabbits fed an HFD than the ones on an SD. Thus, our findings were compatible with the outcome of a previous study by Ogawa et al. (2010). It was reported in the conducted studies that various pomegranate extracts lowered the level of serum LDL when compared to the control group. Concentrated pomegranate extracts were used in both of the studies (Zou et al. 2014, Esmaillzadeh et al. 2006). The present study determined that pomegranate did not affect the level of serum LDL for treatment groups. It has been considered that this

might be related to the different active substance contents of different extracts.

In our research, no statistical difference was detected in the VLDL levels in Group 1 and Group 2. Comparing with the control groups (SCG and PCG), VLDL levels of the rabbits on an HFD were measured higher than those on SD, and a statistically significant difference was found. The obtained results were parallel with the findings of Ogawa et al. (2010). In another study conducted on patients with steatohepatitis, no change in the patients' VLDL levels was reported (Bayan et al. 2004). Aviram et al. (2000) did not find a difference in serum VLDL levels of the healthy people who drank 50 ml/day of pomegranate juice every day for two weeks.

Huang et al. (2005) gave pomegranate juice to the standard and diabetic rats for five weeks and reported that the value of NEFA dropped in both of the groups. On the contrary, in our study, no statistical difference was detected in the level of NEFA in Group 1 and Group 2.

In other previously published studies investigating the toxicity of pomegranate in healthy animals, it was reported that pomegranate did not affect blood glucose (Patel et al. 2008, Xu et al. 2009). Herein, the pomegranate extract raised the GLU level, but that rise was statistically insignificant. Although several studies suggest that pomegranate does not affect the blood glucose level (Huang et al. 2005a, Rashidi et al. 2013, Huang et al. 2005b, Jelodar et al. 2007, Rock et al. 2008), some other researchers have stated that it has hypoglycemic activity (El-Nemr et al. 1990; Parmar and Kar, 2008, Hontecillas et al. 2009). In our study, while there was a decrease in the serum glucose level in the LPJE (100 mg/kg) subgroup of Group 2 compared to PCG, it was statistically insignificant. It was thought that these differences might be due to the different sugar contents of the pomegranate fruit.

The HFD leads to insulin resistance (Hancock et al. 2008). It has been reported that pomegranate does not affect hepatic insulin sensitivity, yet it increases peripheral tissues' insulin sensitivity (Vroegrijk et al. 2011). In the present study, when we compared Group 1 and Group 2, serum insulin level decreased significantly in Group 2. Similarly, Pagano et al. (2002) reported that the patients' insulin levels with NASH were lower than the control group in a statistically significant manner. In another study by Gonzalez-Ortiz et al. (2011), it was reported that pomegranate did not affect insulin level. This was similar to the findings of the present study. The intergroup examination results demonstrated that pomegranate did not affect the level of insulin in either of the groups.

Zou et al. (2014) reported that pomegranate extract enriched with punicalagin lowered liver weight. On the contrary, in the present study, no statistical difference was detected in the comparisons of Group 1, Group 2, and the control groups (SCG and PCG) in terms of the wet liver weights.

The results suggest that pomegranate did not side affect the level of liver in any of the doses in Group 1. While the level of steatosis in SCG was 0%, it was 6.57% in the LPJE (100 mg/kg) subgroup of Group 1. No previous literature about the effects of pomegranate on liver histopathology was revealed. The present study is original in that sense. We suggest that the numerical increase in dose-dependent steatosis ratio might be associated with the high glucose/energy level. The results of the histopathologic examination in Group 2 demonstrated no statistical difference within the groups. Besides, numerical differences were detected within the groups in our research. While the ratio of steatosis was 49.50% in PCG, the ratio was 44.44% in LPJE (25 mg/kg) subgroup, 26.49% in LPJE (50 mg/kg) subgroup and 27.81% in LPJE (100 mg/kg) subgroup. Although a numerical decrease was observed in the rates of hepatic steatosis, no statistically significant difference emerged that might have resulted from the small number of samples. Zou et al. (2014) reported that hepatic steatosis dramatically lowered in the group with 150 mg/kg of pomegranate extract than the animals fed an HFD. In another study, it was reported that pomegranate extract decreased the accumulation of liver TG at a statistically significant rate. However, it did not affect the TG level of the liver (Xu et al. 2009). The pomegranate's lowering effect on hepatic steatosis might be a result of its inhibitory effect on the pancreatic lipase (Zhang et al. 2010). It was also reported that pomegranate significantly decreased TNF-α, IL1-β, IL-4, and IL-6, playing an important role in the pathogenesis of NASH (Non-alcoholic steatohepatitis) and NAFLD (Non-alcoholic fatty liver disease) (Zou et al. 2014). Examining the rates of hepatic steatosis in our study, a statistically significant increase was observed in the group fed an HFD compared to the group fed an SD.

That increase confirms the accuracy of the model. There are several studies conducted on the effects of pomegranate on live weight (Patel et al. 2008, Zou et al. 2014, Xu et al. 2009, Vroegrijk et al. 2011). Along with the studies reporting that pomegranate lowers live weight gain (Patel et al. 2008, Zou et al. 2014, Vroegrijk et al. 2011), other studies suggest that it does not affect the live weight (Ashoush et al. 2013, Xu et al. 2009). In this study, no effect of the pomegranate on live weight was detected.

In their study on pomegranate's oxidant/antioxidant paramaters on rats, Moneim (2012) determined a statistically insignificant numeric increase in serum NO level compared to the control group. In our study, there was a statistically significant increase in serum NO in Group 1 and the level of dNO in Group 2. Combining with O2 (superoxide) which has a higher oxidant property than itself, NO converts to ONO2− e (peroxynitrite), which was less harmful (Besson-Bard et al. 2008). Kaur et al. (2006) reported that pomegranate extract swept O2- anion up to 53%.

The increase seen in our study might be related to pomegranate's lowering effect on the level of O2-.
Several studies have investigated the effects of pomegranate juice and pomegranate extracts in various polarities on the levels of antioxidant parameters in the serum and tissues. However, the results obtained from these studies are not consistent (Osman et al. 2011, Celik et al. 2009, Ashoush et al. 2013, Moneim 2012; Faria et al. 2007, Yüce and Aksakal 2007, Türk et al. 2008). In our study, no statistically significant changes were determined in the levels of MDA, GSH, and AoA in Group 1 and Group 2. This change might be related to pomegranate juice, whole pomegranate extract, or pomegranate extracts in various polarities and different preparation methods (Osman et al. 2011, Celik et al. 2009, Ashoush et al. 2013, Moneim 2012, Faria et al. 2007, Yüce and Aksakal 2007, Türk et al. 2008).

CONCLUSION

Consequently, depending on the Group 1 data, it can be stated that the use of lyophilized whole pomegranate extract in various doses has no adverse effects on the general metabolism. In the present study, lyophilized whole pomegranate extract was evaluated using the NASH rabbit model for hepatoprotective activity. The active dose was found to be 50 mg/kg. It was ascertained that the use of pomegranate up to the dose of 100 mg/kg had no significant side effects on the parameters examined in this study.

Conflict of Interest: The authors declare a no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical permisson: The study was approved by Afyon University Animal Experiments Local Ethics Committee.

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