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Investigation of Histopathological and Biochemical Changes in Rats Feeding Roasted and Raw Sunflower (*Helianthus Annuus* L.) Seed Extract

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ABSTRACT

Today, sunflower seeds, which are in first place in the consumption of nuts, have many benefits. However, sunflower seeds are consumed mainly by roasting. In this study, the effect of roasted and raw sunflower seeds on the liver of rats fed with experimentally roasted and raw sunflower seeds was investigated histopathologically and biochemically. For this purpose, a total of 24 rats were with eight rats in each group; the control, roasted sunflower seed (ROSS), and raw sunflower seed (RASS) groups. At the end of the experiment, blood and tissue samples were taken from the rats for histopathological and biochemical examinations. While degenerative-necrotic changes were detected in the livers of rats fed with roasted sunflower seeds, no significant morphological changes were detected in the raw sunflower and control groups. Furthermore, there was no significant difference between the groups in the biochemical parameters AST, ALT, CAT, GSH, BcL-2 and SOD. However, TAS levels in the ROSS group were significantly lower than in the other groups, while LDH, Bax and caspase-3 levels were high. Data showed that ROSS decreased antioxidant status in liver tissue and increased LDH levels compared to RASS and could damage liver tissue.

Keywords: Sunflower seeds, Helianthus annuus, Histopathology, Oxidative stress, Rat

Introduction

Sunflower (*Helianthus annuus* L.) is an agricultural plant from the Asteraceae family, grown for its oil and seeds (1). The sunflower seed extract is also used to create raw materials for products such as soap, paint, plastic and cosmetics. Also, stalks, seed coats, and heads are used as fuel or in papermaking in the paper industry.

Sunflower seeds, also consumed as snacks, are heat treated before consumption (2). Roasting, baking, frying, grilling, cooking and drying processes, which are among the heat treatments, are generally applied at 150-250 °C (3). These heat treatments render certain pathogenic microorganisms and enzymes inactive and reduce the content of dew. Thus, it is ensured to be preserved for a more extended period (4,5). With the effect of technological developments today, many processes are applied. However, changes in the structure of foods occur with packaging, storage, and different food storage techniques (6).

The heating process causes the food to shrink and changes the density of the extract. This can change sunflower seeds' chemical and physical properties (7,8). In a study on oilseeds, an increase in the fatty acid profile and saturated fatty acid content of heat-treated sunflower seeds, which are rich in polyunsaturated fatty acids, was observed. The heat treatment methods used in the study also reveal a decrease in the unsaturated fatty acid content in sunflower seeds (9). The use of heat treatment to obtain the desired colour, structure and taste-like qualities in foods can lead to the emergence of toxic chemical components that can negatively affect the nutritional aspect of the nutrient (10,11). Some natural substances in the composition of foods can transform into forms such as acrylamide, which threatens human health after heat treatment (12,13). The International Agency for Research on Cancer has named

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acrylamide a "possible human carcinogen". In addition, its toxic effects have been observed, and in vivo studies are continuing (14,15).

We have not seen any studies investigating the histopathological and biochemical effects of feeding with raw and roasted sunflower seeds on the liver. Therefore, this study will provide new information to the literature to investigate the histopathological and biochemical effects of feeding with raw and roasted sunflower seeds on the liver. Therefore, this study aimed to investigate the histopathological changes and levels of aspartate transaminase (AST), lactate dehydrogenase (LDH), and alanine transaminase (ALT) from liver function tests, lipid profile, and oxidative stress and apoptosis markers in the livers of rats fed with sunflower raw and roasted diets.

Materials and Methods

Plant Material and Preparation of Extracts: The Roasted and raw versions of sunflower seeds were used as plant material. Raw sunflower seeds were roasted in the oven at 140±5 ºC for 10 min (16). Raw and roasted sunflower seeds were ground in an electric mill. Then 50 g were weighed and placed in a glass beaker. It was mixed with 250 mL of distilled water and 250 mL of ethyl alcohol. The beaker was covered with aluminium foil and homogenized in a shaker for two h. After the homogenized mixture was filtered, the pulp was discarded, and the homogenate was pipetted into 10 mL falcon tubes and centrifuged at 3500 *xg* for 5 min. All supernatants were placed in the same container. Next, 400 mL of the supernatant was left in the evaporator, and the solvent was removed by evaporation at +37 °C for approximately one h and 45 min. At the end of the process, the concentrated extract was placed in falcon tubes and incubated at -80°C for 48 h. Then, the frozen samples were dried in a lyophilizer at 0.03 mBar pressure and -54°C for three days. The resulting lyophilized saline fraction was stored at -20 °C until the experimental days.

Animals and Experimental Design: The protocol of this study was performed with the revised Declaration of Helsinki in 2000. This study was carried out with the decision of the Van Yuzuncu Yil University Animal Experiments Local Ethics Committee (Dated: 30.05.2019, approval number: 2019/05). Experimentals were obtained from Van Yuzuncu Yil University Experimental Animals Unit. Twenty-four Wistar Albino male 2-month-old rats were used. The rats were fed with tap water, and standard pellet rat chow in rooms lit at a rhythm of 12 hours of light and 12 hours of dark, at a temperature of 22 ± 2 ºC and a humidity of 60%. Feed and water intake were free (adlibitum) for all the groups. A total of 24 rats were divided into three groups (n=8).

Control: The rat was fed with standard pellet feed and drinking water. No application was made.

Raw Sunflower Seeds (RASS): In addition to standard pellet feed and water, one mL (one mL/kg) roasted sunflower seed extract was given orogastrically every day for 4 weeks.

Roasted Sunflower Seeds (ROSS): In addition to standard pellet feed and water, one mL (one mL/kg) raw sunflower seed extract was given orogastrically every day for 4 weeks.

At the end of the application, the rats were anaesthetised with a dose of 50 mg/kg Ketamine hydrochloride + 10 mg/kg Xylazine hydrochloride and sacrificed by the cervical dislocation method. Serum and plasma samples were obtained from the intracardiac blood of the rats. Afterwards, systemic necropsies of the rats were performed, the liver tissue was macroscopically examined and photographed, and tissue samples were taken for histopathological examination.

Preparation of Serum Samples: Intracardiac blood samples were born into a non-heparin biochemistry tube and centrifuged at 3500 *xg* for 10 min. The remaining serum samples were transferred to another Eppendorf tube and kept at -80 °C until they were studied.

Measurement of Biochemical Parameters: Serum AST, ALT, LDL, HDL, LDH and Triglyceride levels were measured in a biochemical autoanalyzer (Abbott Architect c16000, U.S.A) using the spectrophotometric method.

Measurement Method of Apoptosis, Oxidative Stress, and Antioxidant Parameters: TOS, TAS, SOD, CAT, GSH, Bax, BcL-2 and caspase-3 levels in serum were studied in the BioTek EL&800 device with commercial ELISA kits (YL Biotech Co., Ltd., Shanghai, China) following the instructions in the kit procedure. The results were determined with the help of the standard curve by doing an end-point reading at 450 nm. Then, quantitative values were calculated by comparing the obtained absorbance values with the standard curve equation.

Histopathological Examination: At the end of the 4-week trial period, all the animals were necropsied, and macroscopic findings observed in the liver were recorded. In addition, tissue samples cut from livers were fixed in 10% buffered formalin

and embedded in paraffin blocks; 4 μm sections were taken with a microtome, stained with hematoxylin-eosin (H&E) for histopathological examination and examined under a light microscope.

Statistical Analysis: The SPSS package program (Version 21) was used for statistical analysis. First, descriptive statistics of the biochemical parameters in all the groups were given as mean and standard deviation. Next, the Shapiro-Wilk test was used to determine whether the data were normally distributed. Since the groups were usually distributed, the One-way ANOWA test determined whether there were significant differences between the groups within the same parameter. Finally, post-hoc analysis was performed to determine which group the differences originated from. Results with a p-value of 0.05 or less were considered significant.

Results

Data of Oxidative Stress and Apoptosis Markers in Serum Samples: TAS levels were examined in serum samples, and the TAS values of the RASS and ROSS groups were significantly lower than in the control ($p< 0.05$). In addition, the ROSS group's TAS level was considerably lower than the RASS group ($p< 0.05$, Fig. 1a).

When serum SOD values were examined (Fig. 1b), it was seen that the lowest results belonged to the ROSS group. However, the difference between the groups was not statistically significant ($p>0.05$). Finally, when the CAT and GSH levels measured in serum samples were examined, no significant difference was found between the groups ($p> 0.05$, Fig. 1c and d).

When the Bax and caspase-3 values in serum samples were examined, no difference was found between the control and RASS groups ($p> 0.05$). However, the Bax and caspase-3 levels of the ROSS group were found to be dramatically higher than the control and RASS groups ($p< 0.05$, Fig.1e and f). The situation was slightly different at BcL-2 levels. Although BcL-2 levels in the RASS group were slightly higher than in the other groups, their difference was insignificant ($p> 0.05$, Fig. 1g).

Data of Liver Function Tests and Lipid Profile in Serum Samples: When the AST values were examined, the highest activities belonged to the ROSS group compared to the other groups. However, the group's difference was insignificant ($p> 0.05$, Fig. 2a). There was a similar situation with ALT levels, and there was no significant difference between these activities ($p> 0.05$, Fig. 2b). However, there was a different situation at LDH levels. LDH values of the RASS and ROSS groups were significantly higher than the control. However, this increase in LDH levels was much higher in the ROSS group ($p< 0.05$, Fig. 2c).

The triglyceride values of the RASS and ROSS groups were dramatically lower than in the control ($p< 0.05$). In addition, the ROSS group's triglyceride level was considerably lower than in the RASS group ($p< 0.05$, Fig. 2d). When the LDL levels measured in serum samples were examined, the LDL values of the RASS and ROSS groups were significantly lower than in the control ($p=0.001$). Also, the ROSS group's LDL level was significantly higher than the RASS group ($p< 0.05$, Fig. 2e). In terms of HDL levels, there was no significant difference between the groups ($p> 0.05$, Fig. 2f).

Histopathological Findings: Microscopically, a normal histological appearance of the livers was seen in the control. In the livers of this group of rats, hepatocytes and the structures of the portal areas were normal, and hepatocytes formed regular remark cords around the vena centralis. The sinusoids between the remark cords had a normal appearance (Fig. 3A).

The histopathological examination of the RASS group showed that the livers of all the rats in this group had an almost similar morphological appearance to the control (Fig.3B).

In the histopathological examination of the ROSS group, almost similar morphological changes were observed in the livers of all the rats in this group. These changes were characterized by coagulation necrosis in hepatocytes, especially in the periacinar and sometimes intermediary regions of the lobules (Fig. 3C). The cytoplasm and nucleus of the necrotic hepatocytes were stained in dark colour and could be easily distinguished from the surrounding hepatocytes. In these regions, parenchymal degeneration was also observed in some hepatocytes. In addition, inflammatory reactions consisting of focal mononuclear cell infiltrations were also observed in some portal areas and sometimes in the parenchyma. However, no significant increase in connective tissue was found in these regions. There was congestion in some vena centralis and sinusoids (Fig. 3C).

Discussion

Sunflower is an important plant grown worldwide to meet edible vegetable oil needs. In addition,

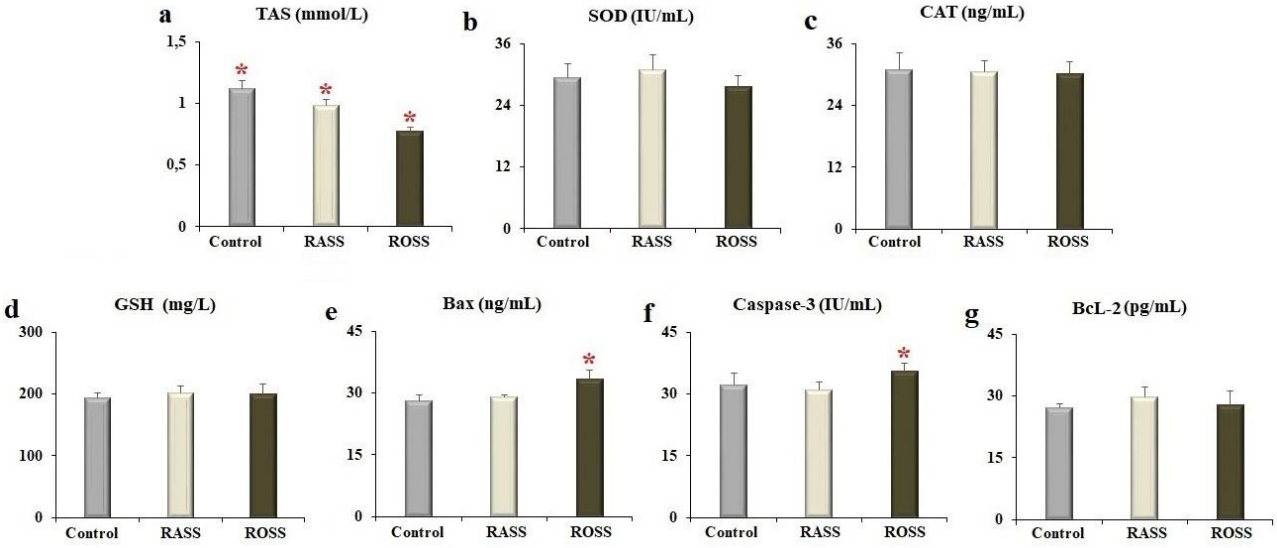

Fig. 1. Comparison of oxidative stress indicators and apoptosis markers measured in serum samples of all the groups. Values were given as mean ± SD. *p: It is significant compared to the other groups ($p<0.05$)

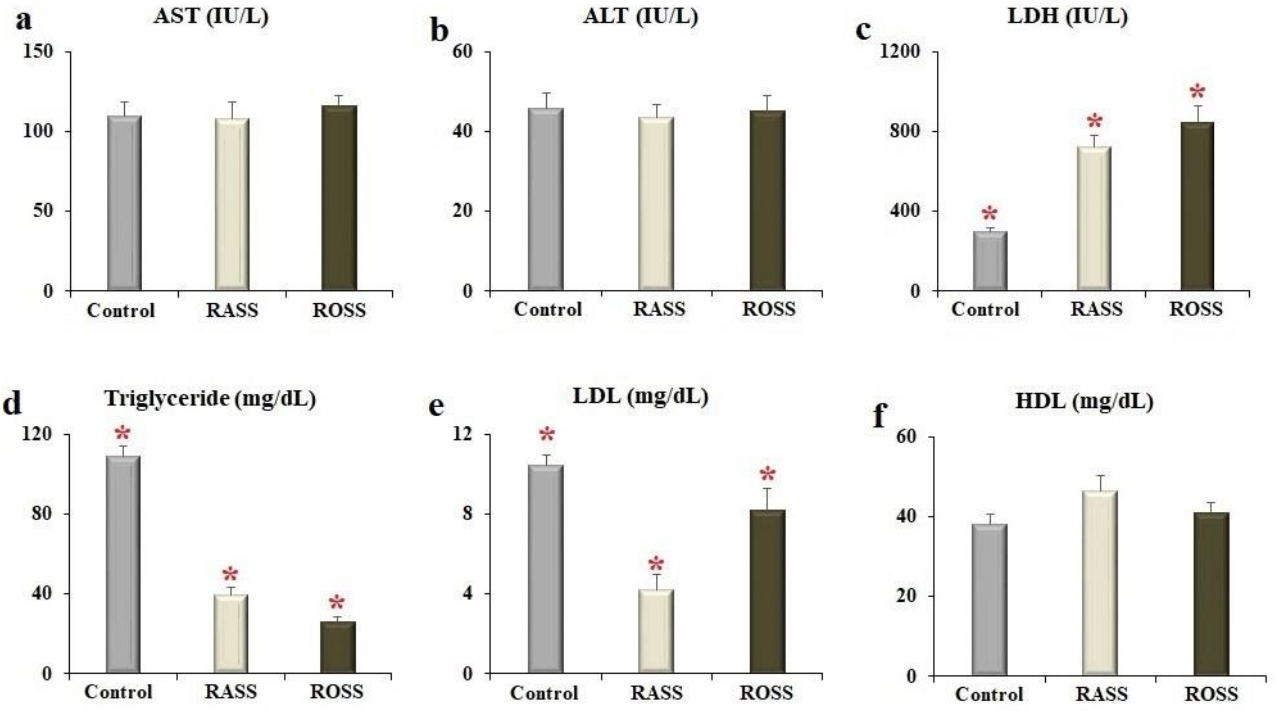

Fig. 2. Comparison of AST, ALT, LDH, triglyceride, LDL and HDL levels measured in serum samples of all the groups. Values were given as mean ± SD. *p: It is significant compared to the other groups ($p<0.05$)

sunflower seeds obtained from it are often consumed as a snack. Sunflower seed has been used as a snack for a long time and is also used in foods such as bakery products, ice cream and chocolate (17). However, sunflower seeds used as snacks are often consumed roasted after heat treatment (18,19). The purpose of preserving and processing foods is to extend their shelf life. Heat treatments are the most commonly used among the various preservation methods in the food industry for this process (20). Interestingly, an increase in the fatty acid profile and saturated fatty acid content of sunflower seeds, which are rich in polyunsaturated fatty acids, was observed in a study on oilseeds (9). A study found that roasting induced allergenic properties in some sunflower seed proteins (21). Heat treatments are generally applied at 90-220°C to process and preserve food products. Similar high temperatures may lead to the formation of carcinogenic and toxic

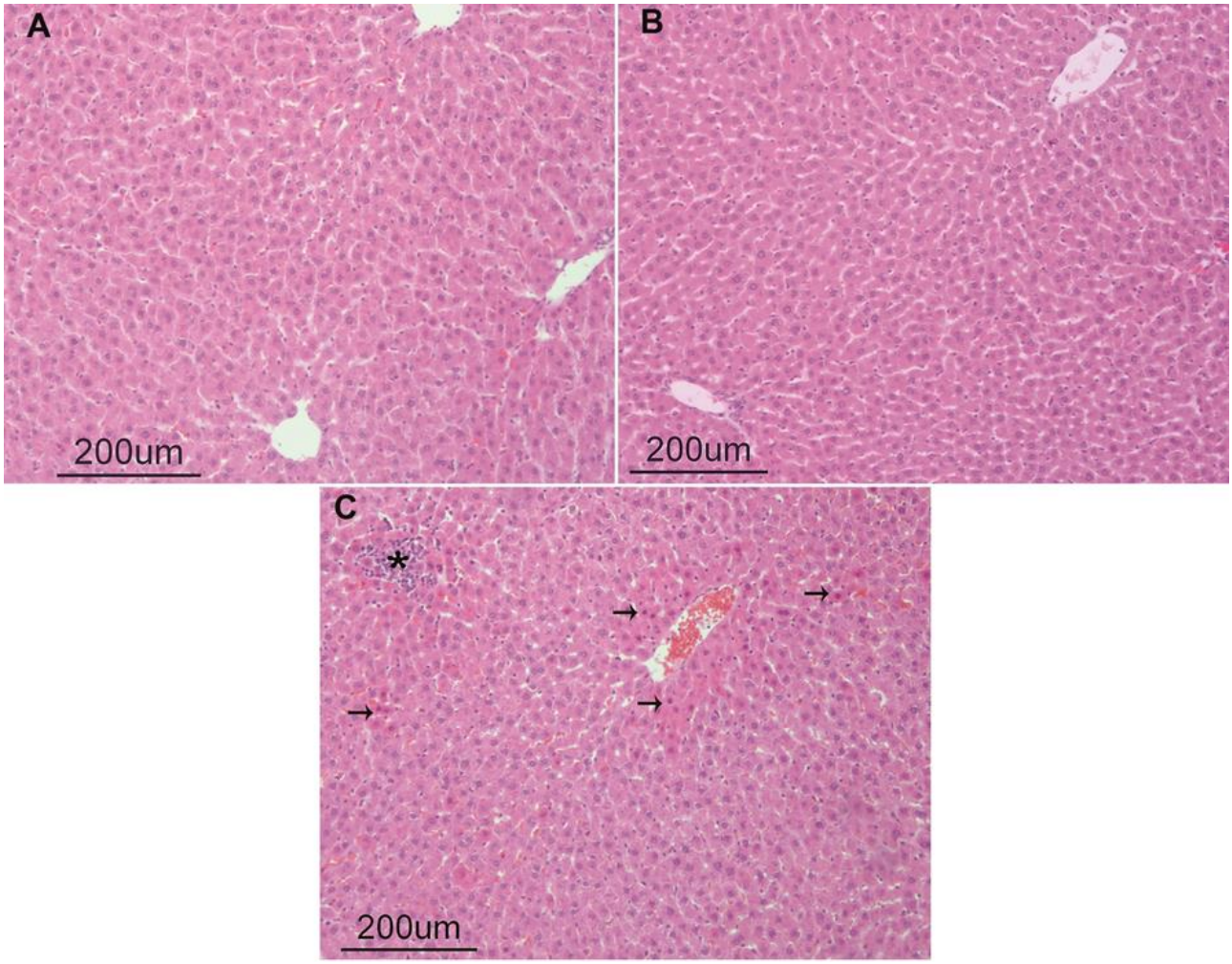

Fig. 3. Histopathological photomicrographs of liver sections of Control, ROSS and RASS groups rats, H&E staining, Bar; 200 µm. (A-B) Liver sections of the control and RASS groups rats showed a normal histological appearance. (C) Liver section of the ROSS group rat showed coagulation necrosis in hepatocytes (arrows), especially in the periacinar region and inflammatory reactions (*) consisting of focal mononuclear cell infiltrations in the parenchyma

compounds such as acrylamide. These components can reduce the food safety and nutritional properties of foods (22). This study aimed to investigate the histopathological changes and levels of liver function tests, lipid profile, oxidative stress and apoptosis markers in the livers of rats fed with sunflower raw and roasted diets.

The endogenous antioxidant activity of enzymes serves as the main line of defence in protection against free radicals. Evaluation of antioxidant enzyme activities is an important point to examine the changes that may occur in the liver tissue (23). TAS is used to learn the general antioxidant status of the body (24). Giada et al. argued that sunflower seeds have excellent antioxidants (25). Our study examined TAS levels in serum samples of the rats, and the TAS values of the RASS and ROSS groups were significantly lower than in the control. In addition, the TAS level of the ROSS group was found to be considerably lower than in the RASS group (Fig. 1a).

Ağır et al., in an experimental study they conducted, reported that SOD, CAT, GSH-Px and GSH levels were decreased in liver injury (26). Roghani et al. observed a decrease in SOD, CAT, GSH and TAS levels in parallel with the increase in oxidative stress in methotrexate-induced hepatotoxicity, while they noted an increase in MDA, NO, IL-6 and TNF-a levels (27). This study showed no significant change between groups in SOD levels, an important indicator of antioxidant defence. However, the lowest SOD values belonged to the ROSS group, while the highest SOD belonged to the RASS group. Considering this result, it can be concluded that consuming raw sunflower seeds may positively affect SOD levels. It has been noted that the aqueous extract of sunflower seeds has a high antioxidant capacity, and consumption of sunflower seeds can prevent

oxidative reactions responsible for inducing cancer and various diseases (25). This supports the results of our study partly in terms of SOD values and TAS values at a high level (Fig. 1b). GSH is an intracellular protein that determines the redox state in liver cells. Also, GSH is an antioxidant protein with essential functions as a metabolic regulator and detoxification (28,29). In addition, antioxidants can prevent or delay the occurrence of free radical reactions and lipid peroxidation(30). Our study showed no significant difference between the groups regarding both CAT and GSH values (Fig. 1c and e).

Considering ELISA kits results of apoptotic markers of this study, it can be said that roasted sunflower seeds induced apoptosis in the liver tissue of rats. Because the Bax and caspase-3 values of the ROSS group were considerably higher than in the other groups. The most important factor causing the activation of mitochondria in the initiation of apoptosis is the BcL-2 family (31). Some BcL-2 proteins (such as Bax and BcL-Xs) are pro-apoptotic. On the other hand, some are anti-apoptotic (such as BcL-2, BcL-xL, and BcL-1). If the balance of pro and antiapoptotic proteins expressed by the BcL-2 gene is disturbed in favour of proapoptotic proteins, the cell becomes apoptotic (31,32). In this study, the BcL-2 levels of the groups were similar, and there was no significant difference between them. Still, the ROSS group's Bax levels were higher than in the control and RASS groups. In addition, the higher levels of Bax and caspase-3 in the ROSS group compared to the other groups indicated that sunflower seeds exposed to heat treatment can induce apoptosis of the liver cells of rats (Fig. 1e, f, and g). These results were supported by the determination of histopathologically significant degenerative-necrotic changes in liver sections of the ROSS group only.

Aminotransferases are enzymes that are frequently used in the diagnosis of liver diseases. Aminotransferases pass from the cells to the plasma in the inflammatory process that occurs for any reason in the liver and increases their levels in the serum. It is known that the AST enzyme is found in many tissues and organs, especially in the liver and kidney. In case of damage to the tissues, serum activities of AST and ALT enzymes increase (33,34). LDH enzyme is found in almost all tissues in the body (35). As with aminotransferases, LDH passes into the blood in liver damage, and their's levels increase in serum (36). In this study, although the AST activities in serum samples were similar in all the groups, the AST activities of the ROSS group were partially higher than the other groups. In addition, this study showed that especially roasted sunflower seed extract dramatically increased LDH enzyme activities in serum samples (Fig. 2a-c).

Triglyceride, a type of lipid found in the blood, is essential for detecting heart health risks. Nutrients taken in excess of the body's needs are stored in the adipose tissue as triglycerides (37). Studies have shown that polyunsaturated fatty acids in sunflower seed oil positively affect lipid metabolism (38,39). In addition, according to studies, sunflower seed oil is a well-known nutritional component and has been shown to have a triglyceride-lowering effect (40,41). Our study showed that sunflower seed extract dramatically reduced triglyceride levels, in line with the literature. However, the use of raw sunflower seed extract was expected to lower triglyceride levels compared to roasted sunflower seed extract, while these values were higher (Fig. 2d).

Cholesterol is an important cell membrane regulator and a substrate for synthesizing steroid hormones such as androgen and estrogen. Lipoproteins transport cholesterol and other fats between tissues and the blood. Two cholesterol transporters are LDL and HDL. LDL carries cholesterol from the liver to the extrahepatic tissues, while HDL carries cholesterol from the extrahepatic tissues to the liver. Both play an important role in cholesterol homeostasis. While high LDL cholesterol invites cardiovascular diseases, increased HDL cholesterol suggests a positive development (42). Studies have suggested that reducing the cholesterol value of sunflower seeds is one of their essential properties (43). Our study showed that raw sunflower seed extract significantly reduced LDL levels and partially increased HDL levels. In addition, this study revealed that the use of roasted sunflower seeds dramatically increased LDL levels (Fig. 2e and f).

In histopathological examinations of liver tissue, it was observed that the histological appearances of liver tissue of the control and RASS groups were normal and similar. However, degenerative-necrotic changes and inflammatory reactions were observed in the ROSS group's histological appearance of liver sections. As a result, it has been determined that consuming raw sunflower seeds in recommended doses does not cause any adverse effects. Still, the consumption of roasted sunflower seeds may adversely affect liver health.

Because it extends the shelf life and is more preferred by the consumer, dried nuts, especially sunflower seeds, are mostly exposed to high temperatures and sold in roasted form. However, many consumers consume roasted foods without being aware of the adverse effects of heat treatment. However, it has been understood in our study that the roasting process not only reduces the food safety and nutritional properties of sunflower seeds but can also cause various harm to the human body. Our study showed that recommended dose of roasted sunflower seed extract reduces TAS and partially SOD levels, increases and especially LDH levels from liver function tests, and can induce Bax and caspase-3 levels, which are apoptosis factors. It was also observed that the recommended dose of roasted sunflower seed extract caused an increase in LDL levels, which increases the risk of coronary heart disease, coagulation necrosis, and inflammatory reactions in liver tissue. Our study will shed light on new molecular-based studies on raw or roasted sunflower seed consumption at different doses and times.

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Data Availability: Data will be made available on request.

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