ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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DOES EXCESSIVE CONSUMPTION OF FLAXSEED (LINUM USITATISSIMUM L.) CAUSE A LIVER DAMAGE IN RAT MODELS?

AŞIRI DOZ KETEN TOHUMU (LINUM USITATISSIMUM L.) TÜKETİMİ RATLARDA KARACİĞER HASARINA NEDEN OLUR MU?

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Öz

Amaç
Doğal sağlık ürünleri ve fonksiyonel gıdalar, birçok hastalığın önlenmesi ve tedavisi için tüketilmektedir. Keten tohumu antikanser, antiviral, antibakteriyel ve antifungal özelliklere sahip fonksiyonel gıdalardan biridir. Yararlı etkilerinin yanı sıra bilinçsiz ve aşırı tüketilmesi toksik etkilere neden olabilmektedir. Bu çalışmada, farklı miktarlarda keten tohumu tüketiminin sıçan karaciğeri üzerindeki etkilerinin araştırılması planlanmıştır.

Gereç ve Yöntem
Sıçanlar; her grupta 8 adet olarak; kontrol grubu ve 7 gün süreyle sırasıyla; 1.4, 2.8 ve 5.6 g/kg/gün keten tohumu verilen deney grupları şeklinde dört gruba ayrılmıştır. Deney sonunda doku ve kan örneklerinde histolojik, immünohistokimyasal ve biyokimyasal analizler yapılmıştır.

Bulgular
Serum AST, ALT ve ALP değerleri 5,6 g/kg keten tohumu verilen grupta kontrol grubuna göre anlamlı olarak daha yüksek gözlendi (p değerleri 0.003, 0.012 ve 0.009). Albümin değerleri deney gruplarında kontrol grubuna göre anlamlı olarak düşük gözlendi ($p<0.05$). TOS ve OSI kontrole göre tüm deney gruplarında anlamlı olarak artmış ($p<0.05$), TAS ise azalmıştı ($p>0.05$). Kontrol grubu ile deney grupları arasında histopatolojik bulgular arasında anlamlı farklar bulundu. Özellikle 5.6 g/kg keten tohumu grubunda tüm gruplara göre daha fazla histopatolojik bulgulara rastlandı. İmmünohistokimyasal analizlerde, en fazla boyama yoğunluğu 5,6 g/kg keten tohumu grubunda gözlemlendi. En yüksek pozitif boyanmaya sırasıyla NOX_4, iNOS, TNF-α ve IL-6 boyanmalarında rastlandı.

Sonuç
Aşırı keten tohumu tüketiminin oksidatif stres ve toksisiteye bağlı olarak karaciğerde iltihaplanmaya neden olabileceği ve çalışmanın keten tohumu toksisitesi ile ilgili diğer çalışmalara katkı sağlayacağı düşünülmektedir.

Anahtar Kelimeler: Keten tohumu toksisitesi, Karaciğer toksisitesi, Sıçan

Abstract

Objective
Natural health products and functional foods are frequently consumed for the prevention and treatment of many diseases. Flaxseed is one of the functional

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foods with anticancer, antiviral, antibacterial and antifungal properties. In addition to its beneficial effects, excessive consumption without considering the appropriate dosage can cause toxic effects. In this study, it was planned to investigate the effects of different amounts of flaxseed consumption on rat liver.

Materials and Methods
Rats were randomly divided into four groups with 8 rats in each groups; control group and experimental groups which given flaxseed for 7 days; 1.4, 2.8 and 5.6 g/kg/day, respectively. At the end of the experiment, histological, immunohistochemical and biochemical analyzes were performed on tissue and blood samples.

Results
Serum AST, ALT and ALP values are significantly higher in 5.6 g/kg of flaxseed compared to control group (p values 0.003, 0.012 and 0.009, respectively). Albumin values were significantly lower in experimental groups compared to the control group ($p<0.05$). TOS and OSI increased significantly ($p<0.05$), TAS decreased ($p>0.05$) in all experimental groups compared to control. In histopathological findings, there was significant difference in three groups compared to control group, especially in 5.6 g/kg of flaxseed group compared to others. As a result of immunohistochemical analyzes, staining intensity of the receptors was highest in 5.6 g/kg of flaxseed group. The highest positive staining was observed respectively in NOX_4, iNOS, TNF-α and IL-6.

Conclusion
These results show that excessive consumption of flaxseed can cause oxidative stress and toxicity due to inflammation in the liver and this study can helpful other studies about flaxseed toxicity.

Keywords: Flaxseed toxicity, Liver Toxicity, Rat

Introduction

Functional foods and health products are preferred in many chronic diseases, from cancer to diabetes. It has been observed that many plants, beneficially contribute to diseases (1, 2). However, via the studies conducted, herbal products are reported to be harmful, and even generating toxic effects when consumed unconsciously and in large quantities, regardless of them being beneficial (3-5).

Flaxseed is often called functional food, bioactive food and endocrine active food. Flaxseed, is rich in linolenic acid and quality protein also the natural source of phytochemicals such as flavonoid, phenolic acids, vitamins, omega - 3 fatty acid, omega - 6 fatty acid, α-linoleic acid, minerals such as calcium, magnesium, phosphate, and large amounts of lignan content (5-7). These ingredients has led flaxseed to be recognized as a protective food against heart disease, diabetes, various types of cancer, and many other chronic diseases, and enabled it to be considered among the functional foods (1, 6-9). Flaxseed contains harmful chemicals as well as beneficial chemicals. Cyanogenic glycoside and cadmium, which are a heavy metal, are among the most important causes of poisoning caused by flaxseed (4, 6, 10). The possible negative effect of nutritional components in flaxseed is also associated with the high amount of polyunsaturated fatty acids (6). Therefore, high amounts of flaxseed taken in a diet for a long time might increase the oxidative stress and cause a decrease in antioxidant compounds. In addition, flaxseed might have a toxic effect due to the linamarin, linustatin and neolinustatin it contains, and it is known that more than 100 mg can be fatal for health (5).

While the majority of the studies on flaxseed were based on its protective effect against toxicity (7, 11-15), this study was conducted in order to observe whether or not any toxicity occurred in the liver tissue due to excessive consumption of flaxseed.

Materıals And Methods

Experimental Animals
Thirty-two female Wistar Albino rats weighting 250-300 g were used and were kept in cages under standard humidity, light (12h light/12h darkness) and temperature (22 ± 2°C) conditions during the 7 days. The animals were provided unlimited access to water and food. Study was approved by the Local Ethical Committee of Experimental Animal Ethics of Suleyman Demirel University (SDU) and was performed entirely according to ethical rules (Protocol Number: 14.02.2019, 02/04).

Experimental Protocol
Rats were randomly divided into four groups with 8 rats in each groups.

Group I: Control, not receiving flaxseed
Group II: 1.4g/kg/day flaxseed, by gavage for 7 days
Group III: 2.8g/kg/day flaxseed, by gavage for 7 days

Group IV: 5.6g/kg/day flaxseed, by gavage for 7 days (16).

Sample Collection And Preparation
After experimental process, anaesthesia was produced by using ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneal. And then all animals were sacrificed and blood samples and liver tissues were obtained. Tissue samples were placed in 10% neutral formalin then sectioning 3–4 µm thickness for analyzes.

Collection Of Serum And Liver
Serum was separated from blood samples by centrifugation at 3000g for 10 min and then stored at -20 °C until analysed. A 0.5 g portion of liver was homogenized on ice in 4.5 mL of phosphate buffer saline (PBS, pH 7.0) and centrifuged at 15000g for 20 min. The supernatant was collected and stored at -20 ºC for further analyses.

Biochemical Analysis
Blood Biochemical Markers Assay
The biochemical parameters (Activities of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP) and Albumin (ALB) in serum were measured on an automatic clinical chemistry analyzer (Gesan chem 200, Italy) device in Veterinary Training Hospital of Mehmet Akif Ersoy University.

Measurement of Total Antioxidant Status
Total Antioxidant Status (TAS) kits (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Turkey) using the spectrophotometric protocol developed by Erel (17) were applied to the tissue homogenates obtained from all experimental groups. Antioxidants in the sample cause the reduction of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radicals in the kit and cause the disappearance of the dark blue-green color of ABTS. For this purpose, the total antioxidant amount is determined by reading the absorbance of 660 nm in the spectrophotometer. This analysis is calibrated with Trolox (Vit E analogue), a stable antioxidant solution, and expressed as Trolox equivalent (mmol Trolox Equiv/L).

Measurement of Total Oxidant Status
The total oxidant status (TOS) of the tissue homogenates obtained from all experimental groups were measured using Rel Assay Kit which spectrophotometric protocol developed by Erel (18). This test is a colorimetric method that is measured spectrophotometrically at 530 nm. Oxidants in the sample oxidize the iron ion chelating complex. Ferric ion forms a chromogenic colored complex in an acidic environment. The intensity of the color is directly proportional to the amount of oxidant in the sample. This assay is calibrated with H_2O_2 and the results are shown as H_2O_2 equivalent (µmol H_2O_2 Equiv/L).

Measurement of Oxidative Stress Index
The TOS to TAS ratio was regarded as the oxidative stress index (OSI) which is an indicator parameter of the degree of oxidative stress. The OSI value was calculated as follows:

OSI (AU) = [(TOS, micromoles H_2O_2 equivalent per L)/ (TAS, micromoles Trolox equivalent per liter)] (19).

Histochemical Procedure
The liver tissues were washed in water over night then fixation in 10% neutral formalin. Samples were dehydrated in ethanol (50-60-70-80-90-100%), made transparentinxylol and embedded into paraffin. Samples were sectioned by using a sliding microtome (Leica SM2000R, Germany) from the prepared paraffin blocks and stained by Hematoxylin–Eosin (H–E) than covered with entellan. Later, the histopathological findings were observed in tissue samples belonging to the all groups were analysed and evaluated.

Immunohistochemical Procedure
Tissues of 3–4µm thicknesses were obtained and were stained with NOX_4 primary ab (rabbit anti-NOX_4 antibody, Abcam, Cambridge, USA), iNOS primary ab (rabbit anti-iNOS antibody, Abcam, Cambridge, USA), TNF-α primery ab (rabbit anti-TNF-α antibody, Abcam, Cambridge, USA), IL-6 primary ab (rabbit anti-IL-6-antibody, Abcam, Cambridge, USA) and were covered with entellan. Next, the liver tissue samples were analysed and evaluated and the receptor densities observed were identified using the semi-quantitative evaluation method (20).

Statistical Analysis
Data were analyzed using SPSS 18 software. One-way analysis of variance and Dunnett's two-tailed post hoc t test were employed to evaluate the statistical significance of differences between the control and experimental groups. Data are presented as mean + standard error of mean or standard deviation, as appropriate, and at least in triplicate. All results were considered significant at $p<0.05$.

Results

Biochemical Results
Serum AST, ALT and ALP values, which are used clinically to determine the degree of liver damage, were

evaluated compared to the control. AST and ALT values were higher in group II, III and IV compared to the control group, but significantly higher in Group IV (respectively p= 0.003, p= 0.012) and ALP values were higher in Group II, III and IV compared to the control, but significantly higher in Group III and IV (p= 0.018 and 0.009, respectively) (Table 1). ALB is a plasma protein synthesized in the liver and low levels of albumin suggest underlying liver damage. Albumin values were no significantly lower between in Group II, III and IV, but significantly lower in experimental groups compared to the control (p>0.05).

TOS, which is an indicator of oxidation products, significantly increased in all groups compared to the control group (p<0.0001 in all groups), (Fig 1). TAS, the measure of antioxidant capacity, insignificantly decreased in all groups compared to the control group (p>0.05), (Fig 2). OSI is an indicator of oxidant activity and significantly increased in all groups compared to control (p=0.0001, 0.001 and 0.0001, respectively), (Fig 3).

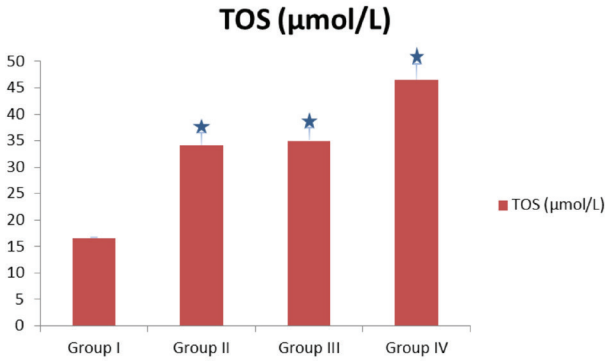

Figure 1
Comparison of total oxidant status (TOS) in groups

Histopathological Results
Normal histological structures were observed in the control group (Fig. 4a-b). When the experimental groups (group II-III-IV) were compared with the control group significantly changes such as; granular and vacuolar degeneration in hepatocytes, sinusodal dilatation, picnotic nucleus, vascular congestion, mononuclear cell infiltration, micro vesicular steatosis were observed, (Fig. 4c-d-e-f-g-h), (Table 2). Significant histological structural changes were higher in group IV compared to group II and III, (Fig. 4c-d-e-f-g-h).

Histological structural and immunohistachemical staining changes were compared with using the semi-qualitative method. Acording to this;

(-) (negative score): No structural changes and no staining
(+) (1 positive score): Light structural changes and light staining
(++) (2 positive score): Middle structural changes and middle staining
(+++) (3 positive score): Serious structural changes and serious staining (Table 2).

Immunohistochemical Results
It was observed that the NOX4, TNF-α, iNOS and IL-6 receptors in the centrilobular areas of the liver sections were stained very lightly in the control group (Fig.5, Table 3), on the other hand, they were more clearly stained in the experimental groups compared to the control group, (Fig 5, Table 3).

The staining intensity of the receptors is highest in group IV, while less and same in group II and III, (Fig. 5, Table 3).

In the comparison of NOX4, iNOS, TNF-α and IL-6 receptor staining in all groups, the highest positive staining was found as NOX4, iNOS, TNF-α, and IL-6, respectively.

Table 1 Changes in the levels of ALT, AST, ALP and total ALB in serum of rats treated with differnt flaxseed

Groups	AST (U/l)	ALT (U/l)	ALP(U/l)	ALB (g/dl)
Group I	75.27±10.12	23.71±10.17	91.12±31.19	3.62±0.25
Group II	79.31±21.93	26.65±5.89	116.75±30.71	3.41±0.38
Group III	77.67±24.69	30.45±4.78	140.00±56.00*	3.49±0.31
Group IV	119.00±39.85*	39.91±18.94*	145.12±30.89*	3.44±0.41

The values represent the mean±SD for eight rats per group. Statistical significance compared to Group I-Control (C).*
p < 0.05 was considered statistically significant

Table 2 Average scoring of structural changes between groups

Groups	Granular And Vacuolar Degeneration in Hepatocytes	Sinusoidal Dilatation	Picnotic Nucleus	Vascular Congestion	Mononuclear Cell Infiltration	Micro Vesicular Steatosis
Group I	-	-	-	-	-	-
Group II	+/++	+/++	+/++	+/++	++	+
Group III	++	++	+/++	++	++	+
Group IV	+++	+++	++	++	+++	+

Table 3 Average scoring of staining NOX_4, TNF- α, iNOS, IL-6 between groups

	GROUP I	GROUP II	GROUP III	GROUP IV
NOX_4	-/+	+/++	+/++	+++
TNF-α	-/+	+/++	+/++	+++
iNOS	-/+	+/++	+/++	+++
IL-6	-/+	+	+	+

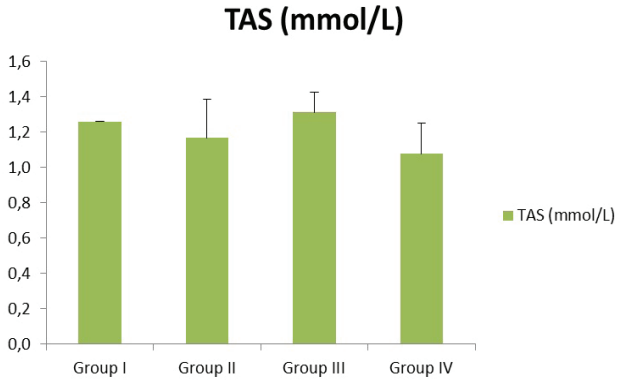

Figure 2
Comparison of total antioxidant status (TAS) in groups

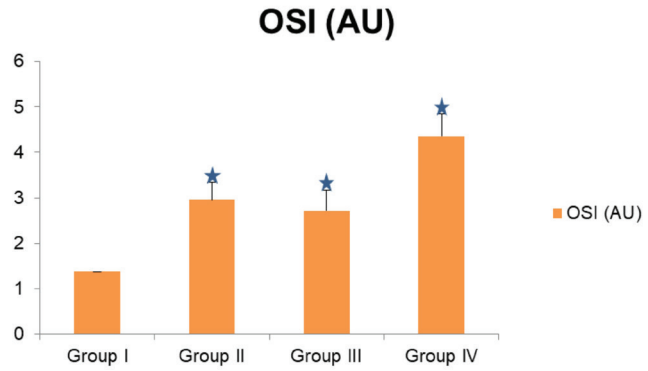

Figure 3
Comparison of oxidative stress index (OSI) in groups

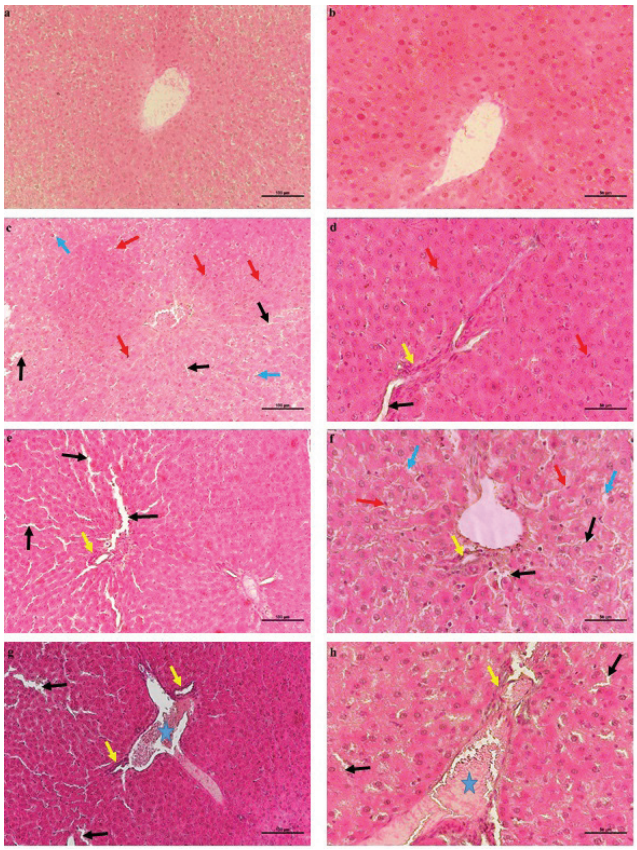

Figure 4
Histopathological findings in liver tissue belonging to control and experimental groups: a–b: control group (group I); no histopathological findings were found. c-d: group given 1.4 g/kg flaxseed (group II) and e-f: group given 2.8 g/kg flaxseed (group III); histopathological findings were found compared to the control group and there was no damage difference between the two groups. g–h: group given 5.6 g/kg flaxseed (group IV); the most damage was observed in this group. Red arrows; picnotic nucleus, black arrows; sinusodal dilatation, yellow arrows; mononuclear cell infiltration, blue arrows; vacuolar degeneration in hepatocytes, blue stars; vascular congestion, a-c-e-g, H-E x20, b-d-f-h, H-E x40.

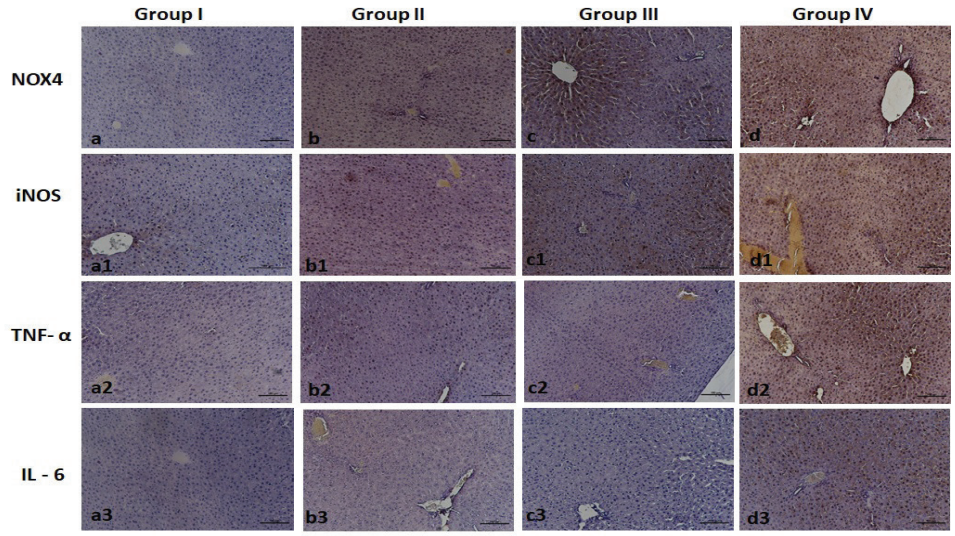

Figure 5
NOX4, TNF-α, iNOS, IL-6 immune stainings in liver tissue in control and experimental groups. a-b-c-d: NOX4, a1-b1-c1-d1: TNF-α, a2-c2-d2: iNOS, a3-b3-d3: IL-6. No positive staining in control group (group I) with NOX_4, TNF-α, iNOS, IL-6 was detected a-a1-a2-a3. While mild stainings in group II and group III with NOX4, TNF-α, iNOS were observed, no positive staining with IL-6 was observed b-b1-b2-b3,c-c1-c2-c3. Intensive staining in group IV with NOX4, TNF-α, iNOS were observed, while no positive staining with IL-6 was observed d-d1-d2-d3, immune staining x40.

Discussion

In addition to the curative effects of herbs, which are indispensable and the most important parts of Alternative and Complementary Medicine, the existence of its negative effects causing organ damage is also known. Therefore, studies have started to be carried out on the damages that may occur (1-5). In this study, was aimed to evaluate the effects of different doses of flaxseed on rat liver by histochemical, immunohistochemical and biochemical analysis.

Flaxseed is known one of functional foods and has lots of benefical properties such a; anticancer, antiviral, antibacterial, antifungal. The negative health effects of phytoestrogens and toxic compounds in flaxseed content cannot be neglected. Flax seeds contain inhibitors such as trypsin, phytic acid, cadmium and cyanogenic glycosides (21). It is known that trypsin inhibitors in the diet reduce the growth in animals by reducing the digestion and thus absorption of proteins by inhibiting proteases (22). Phytic acid, with its strong chelating potential, reduces the absorption of minerals in the intestines and can prevent the digestion of proteins (23). Flaxseed may causes toxic effect after its excessive consumption due to the harmful chemicals like as cyanogenic glycoside and cadmium. Studies have shown that cadmium causes acute hepatotoxicity by binding to sulfhydryl groups in mitochondria, and then activates thiol groups through either oxidative damage or inflammation caused by increased cytokines (24-26). In studies on the toxicity of cadmium, it has been shown that the serum hepatic marker enzymes AST, ALT and ALP activities are increased. Hydrogen cyanide toxicity occurs as a result of enzymatic destruction of cyanogenic glycosides contained in plants such as flaxseed (27-30). The elevation of hepatic markers is also notable in the hepatotoxicity study conducted with linamarin, which is a cyanogenic glycosides (31).

In present study, it was observed that compared to the control group, the levels of AST, ALT and ALP increased due to the increase in the amount of flaxseed given to the groups. In study which carried out by Uzunhisarcikli and Kalender, reported that the amount of albumin decreased in the rats in which they created hepatotoxicity by methyl parathion (32). In this study, it was observed that the amount of albumin decreased in parallel with the literature.

Studies have shown that in rats treated with cadmium, the balance of prooxidant / antioxidant was disrupted, lipid peroxidation increased, and total antioxidant capacity decreased (28, 29). Cyanide hepatotoxicity increases oxidation with a similar effect to cadmium while reducing the antioxidant parameters (33). It's known that while reactive oxygen species (ROS) are produced in metabolic and physiological processes, harmful oxidative reactions can occur in organisms that remove them through enzymatic and non-enzymatic antioxidative mechanisms (29-31). Compared to the control group in this study, the increase in TOS levels and oxidative stress index due to the decrease in TAS levels support the presence of toxic effects.

A study by Zarepoor and et al. showed that flaxseed exacerbates the damage to the colon mucosa, which is caused by cadmium and cyanogenic glycoside detected in flaxseed (3). In a study by Khan et al. investigating the toxicity of flaxseed on mice during pregnancy and lactation, they observed an increase in breast tumors in offspring and attributed the cause to detectable levels of heavy metal cadmium in flaxseed (4).

In studies on liver damage; it was found that TNF-α and IL-6 stained strongly positively as a result of increased inflammation, while iNOS and NOX4 receptors were strongly positively stained as a result of increased oxidative stress and inflammation (34-39). Similarly, immuno-stainings were performed with NOX_4, iNOS, TNF-α, and IL-6 in order to observe whether or not the flaxseed caused inflammation and oxidative stress in the liver.

Many mechanisms, such as the release of proinflammatory cytokines and enzymes from kupffer cells, play a role in liver damage and the formation of the inflammatory response. The proinflammatory cytokines can also manifest their effects through inducing the iNOS release, which produces NO from many cell types (40, 41).

In the study carried out by Faty andet al., they observed an increase in TNF-α and IL-6, and in parallel to that, subsequently in iNOS in case of liver damage. Moreover, proinflammatory cytokines produced after stress were also shown to play a role in the increase of iNOS (41).

In another study conducted by Nascimento and et al., it was observed that flaxseed oil and extra virgin oil had undesirable side effects on ulcerative colitis disease, TNF-α, IL-6, and iNOS values increased (42). In this study, according to the immunohistochemical evaluations of the experiental groups to which flaxseed is over-given (group II – III - IV), the highest immunopositive staining was observed respectively in NOX4, iNOS, TNF-α, and IL-6. As a result of the increase of NOX_4 related to oxidative stress and the

increase of inflammatory cytokines (TNF-α and IL-6), more positive staining was observed in iNOS value than expected. When the groups that were given flaxseed were compared, oxidative stress and inflammation were observed to be highest in group IV, and then equally in group II and group III. In the comparisons of damage in experimental groups, results parallel to immunohistochemical stainings were also found in Hematoxiclen-Eosin stainings.

Conclusion

In this study, it was shown that the flaxseed, which has a protective effect on organ damage at appropriate doses, may also be a toxic on liver when consumed in excessive amounts. The consumption of flaxseeds in the habitual diet does not present a risk of poisoning to consumers, as excessive amounts of flaxseeds will be required to provide the content that is considered toxic to the organism. In addition, previous studies have shown that toxicity is caused by cadmium or cyanogenic glycosides and hepatic markers are increased for this reason, which led us to comment in this sense. The use of large amounts of flaxseed resulted in an increase in oxidative stress and inflammation in the liver. In order to fully understand the mechanism of action of damage caused by flaxseed, it would be useful to do new studies on different paths.

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