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Araştırma Makalesi / Research Article

Determination of Antioxidant Effect of Walnut (*Juglans regia* L.) on Lung and Muscle Tissue

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Abstract

This study was to investigate the antioxidant role of Juglans regia L. (walnut) against ethanol-induced oxidative stress in lung and muscle tissues. Female Albino 36 rats were divided into six groups and treated as follows: I (control), II (20% ethanol), III (10%walnut), IV (20% ethanol + 10%walnut), V (5% walnut) and VI (20% ethanol + 5% walnut). The rats were sacrificed after 50 days of administration and the tissues were analyzed after isolation. According to the results, it was found that the increased malondialdehyde (MDA) content owing to the alcohol-induced oxidative stress in both tissues decreased in the walnut treated tissues. In addition alcohol and alcohol + walnut treatment of nutritional supplemented rats changed catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and superoxide dismutase (SOD) content and antioxidant defense system (ADS) components compared to control rats. As a result, ethanol has been shown to cause fluctuations in ADS components as a result of oxidative stress in rats and it can be said that walnut has a curative effect on the this complications caused by oxidative stress.

Keywords: *Juglans regia* L., Antioxidant defense system, Malondialdehyde, Rat

Akciğer ve Kas Dokularında Cevizin (*Juglans regia* L.) Antioksidan Etkisinin Belirlenmesi

Özet

Bu çalışma akciğer ve kas dokularında etanolün neden olduğu oksidatif strese karşı cevizin (*Juglans regia* L.) antioksidan rolünü araştırmak amacıyla gerçekleştirilmiştir. Bu amaçla 36 dişi albino sıçan 6 gruba ayrılarak gruplar şu şekilde oluşturulmuştur: I (kontrol), II (%20 etanol), III (%10 ceviz), IV (%20 etanol + %10 ceviz), V (%5 ceviz) ve VI (%20 etanol +%5 ceviz). 50 günlük uygulama sonrası sıçanlar sakrifiye edilerek dokuları alındı. Elde edilen sonuçlara göre ceviz uygulanan gruplarda her iki dokuda alkol kaynaklı oksidatif stres sebebiyle artan MDA içeriğinin azaldığı belirlenmiştir. Ayrıca besin takviyesi yapılmış sıçanların alkol ve alkol + ceviz muamelesi, kontrol sıçanlarına kıyasla CAT (katalaz), GPx (glutatyon peroksit), GST (glutatyon S-transferaz) ve SOD (süperoksit dismutaz) içeriği ve ADS (antioksidan savunma sistemi) bileşenlerini değiştirdiği gözlenmiştir. Sonuç olarak, etanolün sıçanlarda oksidatif stresin bir sonucu olarak ADS bileşenlerinde dalgalanmalara neden olduğu, cevizin oksidatif stresin neden olduğu bu komplikasyonlar üzerinde iyileştirici bir etkisi olduğu söylenebilir.

Anahtar Kelimeler: Ceviz, Antioksidan savunma sistemi, Malondialdehit, Sıçan

Introduction

Sies (1991) first described oxidative stress as "proxidance deterioration, potential damage pathway for the antioxidant balance in favor of the oxidant species." In addition, oxidative stress is also defined as the disruption of the balance between

reactive oxygen / nitrogen species (ROS / RNS) and the capacity of the organism to prevent the effectiveness of antioxidant defense systems (Persson et al., 2014). Free radicals cause an improved ROS / RNS production or antioxidant-protective property expressed in reduced endogenous system capacity against oxidative attack in target biomolecules. Oxidative stress is related to various pathological conditions such as cardiovascular, cancer and aging. (Lopez-Alarcona and Denicola, 2013; Sies, 1985). The formation of free radicals leads to the formation of oxidative stress. Many diseases such as oxidative stress, Parkinson's, Alzheimer's, amyotrophic lateral sclerosis, emphysema, cardiovascular and inflammatory diseases are caused by pathogenesis and pathophysiology (Toda, 2011; Lopez-Alarcona and Denicola, 2013). Oxidative stress has been associated with 100 or more diseases that are directly or indirectly caused (Halliwell et al., 1992; Gutteridge, 1993). The irreversible progression of the oxidative degradation caused by reactive oxygen species has an adverse effect on the state of aging biology, such as impaired physiological functions, progression of disease progression and reduction of life span (Maulik et al., 2013). Oxidative stress is a consequence of the deterioration of the balance between the production and consumption of ROS in excess. That is, the decrease in ADS activity is due to the increased rate of free radical formation (Poljsak et al., 2013). Antioxidants are molecules that play a role in clearing these reactive species that cause oxidative stress. They are defined as substances that can prevent oxidation of the substrate at low concentrations (Halliwell and Gutteridge, 1995). Oxidative stress and harmful effects can be prevented by taking naturally occurring antioxidants. Antioxidants act as free radical scavengers and can prevent oxidative reactions leading to various diseases. The majority of exogenous antioxidants come from plants, phytochemicals. There are several classes of antioxidant potential phytochemicals and their unique structural rearrangement (Xu et al., 2017). Antioxidants have a wide range of effects in various disease conditions and help prevent the onset of such diseases. Natural antioxidants that occur in an organism could fight against the oxidative stress that occurs through various physiologic processes. These include antioxidant enzymes which are endogenous antioxidants such as catalase, superoxide dismutase, glutathione peroxidase and reduced glutathione. In particular, glutathione (GSH) plays a very important and central role in defense against oxidative stress. GSH, channels through enzymes such as glutathione peroxidase and glutathione reductase to neutralize ROS through its oxidized form of oxidized glutathione. In addition, antioxidants taken from diets such as vitamin C, vitamin E and vitamin A are taken as exogenous for their protective effect against ROS. Antioxidants have a number of injured effects. One of these is the role of cellular signalling as well as the ability to scavenging free radicals.

Some foods with hydrophilic and lipo or physicochemical properties can be examined as complex systems consisting of various substances dispersed in different microphases. They are among the most interesting fruits to investigate the interactions that can occur between walnut, almond-like fruit, oxidant species, oxidizable phytochemicals, antioxidants and factors that determine the oxidation of foods. Oxidation process that takes place in the components can not only partially or completely change the nutritive and other properties depending on the loss of essential fatty acids or vitamins but also cause discoloration with taste and pigment destruction (Venkatachalam and Sathe, 2006; Salcedo et al., 2010). It has been reported that walnuts contain large amounts of polyunsaturated fatty acids (PUFAs), particularly α-linolenic acid (ALA) and linoleic acid (LA), and a large number of polyphenols, which

have been shown to promote body health and function (Poulose et al. 2014). 100 g of walnut (*Juglans regia* L.) were mixed with 38 g of linoleic acid (LA 18: 3n-6) and 9 g of α-linolenic acid (ALA; 18: 3n-3) as well as 4.4 g of saturated palmitic acid (C16: 0) and 8.7 g of monounsaturated (oleic acid, C18: 1 n-9) fatty acids (Willise et al., 2010; Poulose et al., 2014). In addition, it has been found that the detectable polyphenol levels in walnut extracts can be improved by the addition of hydroxybenzoic acids such as chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, shirinic acid, hydroxycinnamic acids such as elagic and other such as acid and gallic acid, juglone and syringaldehyde compounds (Willise et al., 2010; Poulose et al., 2014). In humans, ALA taken from the walnut by humans is converted to PUFAs such as eicosapentaenoic acid (EPA;20: 5n-3) and docosahexaenoic acid (DHA; 22: 6n-3) in the liver by a series of reactions (Willise et al., 2010; Poulose et al., 2014). In some animal studies, walnut consumption is associated with a marked increase in antioxidant capacity, leading to a decrease in oxidative stress markers (Torabian et al., 2009; Willise et al., 2010; Thangthaeng et al., 2018). Therefore, in this study, we aimed to determine the antioxidant role of walnut using in vivo model. For this purpose, feeds with 5% and 10% walnut contents were prepared and oral feeding of live material was carried out. Because walnut, which is a functional food, is consumed widely both in our country and in the world.

In many studies, it has been determined that oxidative stress caused by consumption of ethyl alcohol causes damage on various tissues. (Dogan and Celik, 2012; Turan and Celik, 2016; Turan et al., 2018) Since there are not many studies on lung and muscle tissue in the literature search, these two tissues were preferred in order to determine the antioxidant effect of walnuts on these tissues. Antioxidant defense elements in the lung and muscle tissues, reduced glutathione (GSH), glutathione reductase (GR), superoxide dismutase (SOD), glutathion-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx) activities and malondialdehyde (MDA) content it was evaluated.

Materials and Methods

Chemical

Sodium chloride (NaCl), reduced glutathione (GSH), trihydroxymethyl aminomethane (Tris), butylated hydroxytoluene (BHT), β-Nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA), Trichloroacetic acid (TCA), thiobarbituric acid (TBA) metphosphoric acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), oxidized glutathione (GSSG), potassium dihydrogen phosphate (KH_2PO_4) and sodium dihydrogen citrate anhydrous ($C_6H_7NaO_7$). The technical materials used in this study were obtained from Sigma Chemical (St. Louis, MO, USA) and standard enzyme kits for enzyme analysis were obtained from Randox Laboratories Ltd.

Experimental animals

In our study, 36 Wistar albino female rat ranging from 150-200 g obtained from Van Yuzuncu Yil University Experimental Animals Unit were divided into 6

groups as 6 rats in each group. Rats (22±2 °C) were fed at room temperature for 12 h light / dark light period for 50 days. It was fed with a standard laboratory diet and walnut-containing diet. During the experiment, rats were treated according to the rules of ethics. This study was carried out with the approval of the Van Yuzuncu Yil University Ethics Committee.

Extracts administration and animal grouping

This work lasted for a total of 50 days. A total of 6 groups were formed in the study.

- Group I (Control): The rats were fed the standard pellet diet.
- Group II (Ethyl Alcohol): The rats received 20% alcoholic water and standard pellet diet.
- Group III (10% walnuts): The rats were fed with 10% walnut-containing feed.
- Group IV (10% Walnuts + 20% Ethyl alcohol): The rats received 20% alcohol water and fed with 10% walnut-containing feed.
- Group V (5% Walnuts): The rats were fed with 5% walnut-containing feed
- Group VI (5% Walnuts + 20% Ethyl alcohol): The rats received 20% alcohol water and fed with 5% walnut-containing feed.

Preparation of foods

The walnut used in this study was collected in the natural habitats of Gevas district of Van. The walnut to be used for the study was first powdered then, with the standard rat feed, the walnut content was adjusted to be 5% and %10.

Preparation of tissues supernatant

At the end of the 50 day study, the rats were anesthetized with 10% ketamine and sacrificed. The lung and muscle tissues were washed with saline water and then kept at -80 °C until the day of the analysis. Both tissues were weighed at 500 mg and crushed with a glass baguette, then homogenized in an ultrasonic homogenizer for 3-5 minutes. The homogenate was then centrifuged at 9500 rpm for 30 minutes at +4 °C. Biochemical analyzes were performed on the obtained supernatants (MDA, GSH, SOD, GPx, GR, CAT, GST) (Yurt and Celik ,2011; Dogan et al., 2012; Celik et al., 2015; Turan and Celik, 2016).

Biochemical analysis

Malondialdehyde (MDA), which is one of the peroxidation products of fatty acids, is measured by the formation of a colored image with thiobarbituric acid (TBA) (Jain et al., 1989). It is measured by the formation of yellow color caused by the reaction of sulfhydryl groups in clear liquid obtained from GSH with DTNB in tissue supernatants using phosphate buffer (Beutler et al., 1963). Glutathione S-transferase catalyzes the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and the glutathione-SH group. Enzyme activity is determined by measuring the intensity of glutathione conjugation with CDNB at 37 °C at 340 nm (Mannervik and Guthenberg 1981). The GR activity is measured by calculating the amount of NADPH consumed

per minute at a wavelength of 340 nm at 37 ºC (Carlberg and Mannervik 1975). GPx activity is measured according to the method defined by Paglia and Valentine (Paglia and Valentine 1967). GPx catalyses the oxidation of glutathione by cumene hydroperoxide. SOD activity is measured by the method described by McCord and Fridovich (McCord and Fridovich 1969). The activity of the CAT enzyme is determined by the method based on the consumption of H_2O_2 at 240 nm at 37 °C (Aebi, 1974).

Analysis of data

Mean and standard deviation (X ± SD) are calculated according to standard methods using Minitab package program; The difference between the group averages was calculated using the One Way ANOVA-Tukey test. Values of $p \leq 0.05$ were considered statistically significant.

Results

The body weight obtained from the rats during the experiment is given in Figure 1.

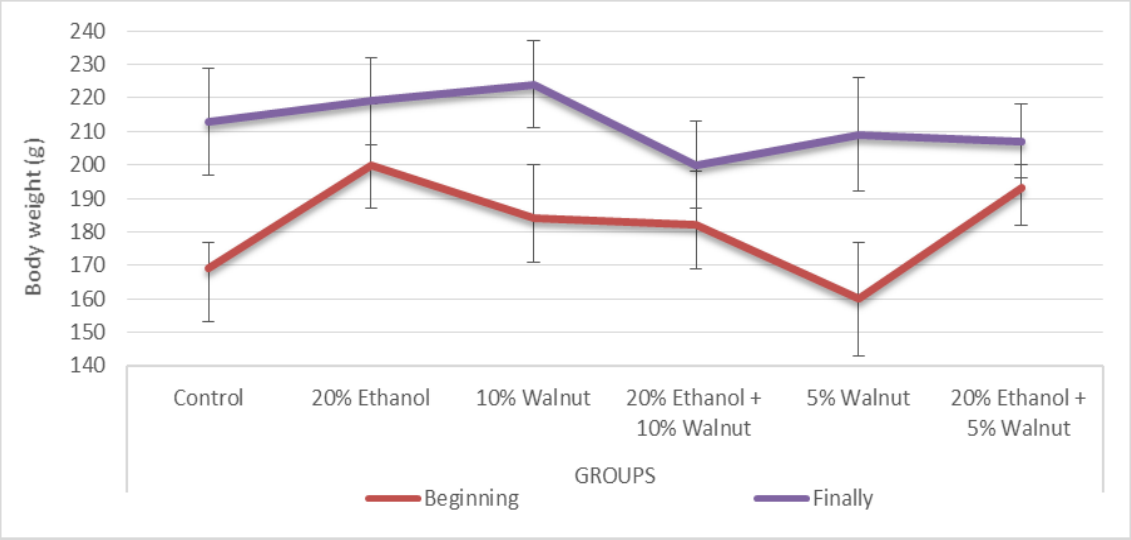

Figure 1. Weight gain in rats during the experiment (Mean ± SD).

The effects on the lung and muscle index and antioxidative role of alcohol and walnut supplementation following exposure of the experimental group were evaluated on ADS and MDA content of lung and muscle tissue samples from control and treated rats. Post treatment results showed that the rats fed alcohol + walnut and walnut supplementation had a change in the MDA content and ADS components compared to the control group. Along with these results, oxidative stress caused by alcohol consumption in both lung and muscle tissues led to increased MDA content and decreased in walnut treated tissues. That is, the content of MDA increases significantly in both tissues exposed to alcohol, while it decreases in walnut treated groups. Alcohol and alcohol + walnut treatment of rats with dietary supplement changed CAT, GPx, GST and SOD contents and ADS components compared to control rat. On the other hand, although ethanol has caused fluctuations in the components of ethanol antioxidant

defense system as a consequence of oxidative stress in rats, it can be determined that the response of walnut is a positive effect on these fluctuations (Table 1).

Table 1. Changes in lipid peroxidation and activities of antioxidant enzymes in rat lung and muscle of experimental group (Mean ± SD).

Parameters	Tissues	GROUPS					
		Control	20% Ethanol	10% Walnut	10% Walnut +20% Ethanol	5% Walnut	5% Walnut+ 20% Ethanol
CAT (U/ml)	Lung	50,6±5,36	64,9±10,4	34,6±11,2^{ab}	36,6±4,5^{b}	37,5±10,8^{b}	38,5±7,5^{b}
	Muscle	413,2±129,1	2224,2±752^{a}	2845,9±603,9^{a}	2531,3±617^{a}	2313,5±764,1^{a}	1852±770,3^{a}
GPX (U/ml)	Lung	128,7±15,2	118,5±17,3	179,6±15,2^{ab}	196,4±4^{ab}	163,2±26,5^{ab}	159,7±10,2^{ab}
	Muscle	169,8±6,7	169,3±2,7	159,2±14,7	156±5^{a}	153,1±5,3^{ab}	151±6,6^{ab}
GR (U/ml)	Lung	19,3±2,5	19,3±1,3	18,8±2,4	19,2±0,8	20,4±3,7	20,5±1,8
	Muscle	6,2±1,2	6,3±1	5,9±1,8	5,6±1,1	5,7±0,7	4,3±0,6^{ab}
GSH (mg/ml)	Lung	30,4±7,8	34,8±4	39,3±4,4	31,3±8,7	30,5±6	38,9±9,3
	Muscle	16,8±4,4	16,4±2,5	16±4	19,1±1,9	16,5±1,8	17±4,6
GST (U/ml)	Lung	41,4±4,7	49,6±8,3	58,5±6,9^{a}	66,1±10,5^{ab}	56,3±10,2	56,3±11,6
	Muscle	28±4,11	35,4±4^{a}	34,5±4,2^{a}	33,3±3,3	21,7±1,2^{ab}	25,6±2,4^{b}
MDA (nmol/ml)	Lung	52,8±13,8	182,1±31,3^{a}	86,8±13,5^{ab}	148,18±11,61^{ab}	78,8±9,9^{b}	151,09±16,64^{ab}
	Muscle	29,6±8,26	68,2±27,2^{a}	32,2±9,6^{b}	44,11±4,09^{b}	25,3±3^{b}	44,33±3,83^{b}
SOD (U/ml)	Lung	2169±76,09	2181±29,5	2194,3±39,1	2188,6±81,2	2182,3±42,4	2131±56,2
	Muscle	2077,2±33,3	2124±26,4	2045,4±57	2049,4±39,3	2040,2±82,4	2214,7±69,9^{ab}

a: The difference according to the control group is statistically significant ($p<0.05$).
b: The difference according to the %20 ethanol group is statistically significant ($p<0.05$).

Discussion

It is known that bioactive compounds in plants prevent diabetes, cancer, obesity, neurodegenerative and cardiovascular disease and chronic disease risk. Phytochemicals such as flavonoids and terpenes in ethanolic extracts have various therapeutic effects such as antioxidant, antidiabetic and anti-Alzhemic (Xi et al., 2008; Erukainure et al., 2014). Many studies have shown that the formation and progression of tissue damage is due to oxidative stress.

Reactive oxygen species can cause oxidative stresses in cells (Gallagher et al., 1995). In the study conducted, it was stated that lipid peroxidation is primarily caused by superoxide anion and hydroxyl radical (Abdollahi et al., 2004). Malondialdehyde occurs as a result of lipid peroxidation. For this reason MDA can be used to determine the damage of the oxidative stress in the body (Cynamon et al., 1985). Both enzymatic and non-enzymatic antioxidants play an important role in the protection of organisms against oxidative damage (Bhor et al., 2004). Catalase enzyme catalyzes the conversion of hydrogen peroxide to water and oxygen. Catalase enzyme is one of the essential antioxidants against the hydroxyl radical (Bagnyukova et al., 2005). Glutathione is both the most important and the most common endogenous antioxidant defense system. Glutathione has an important role in the prevention of oxidative stress caused by singlet oxygen molecules and hydroxyl radicals. (Meister and Anderson, 1983). Previous studies have shown a decrease in the antioxidant defense system as a result of oxidative damage (Jiang et al., 2015; Turan and Celik, 2016; Wu et al., 2017). According to the results of this study, MDA showed that content was reduced to lung and muscle tissues

compared to the alcohol group. Thus it showed that walnuts effectively protected against rat alcohol induced tissue damage.

As shown in Table 1, this study has shown that walnuts may have an antioxidant role in rats. We observed that the MDA concentration at the tissue was different from the alcohol exposed group as a result of the additional treatment of walnuts in vivo. In the direction of the results obtained, MDA content in the lungs and muscles of alcohol-treated rats increased markedly, whereas in the walnut-added group, the MDA content of the tissues decreased significantly compared to the alcohol group. The causes for the inclusion of this type of alcohol and walnut are not identified at this time. The formation of ROS in rats receiving ethyl alcohol from the other side may naturally have caused an increase in the MDA content in the tissues. According to the study results, alcohol consumption increased the formation of lipid peroxide and ROS causing oxidative stresses (Poljsak et al., 2013; Erukainure et al., 2014; Jiang et al., 2015; Xu et al., 2017). Increased oxidative stress due to alcohol consumption depends on ethanol metabolism (Wu et al., 2017). The polyphenolic compounds found in plant foods have antioxidant activity and these compounds play a role in preventing the formation of lipid peroxidation, which results in free radicals. Because, as a result of the researches, phenolic, flavonoid and terpene containing compounds found in plants prevent the damage that can be caused by ROS (Chu et al., 2002; Oboh and Rocha, 2007). Bioactive compounds such as d-fructose, piperazine, octadine, glycidol, 2-hydroxy-gamma-butyroacetone, n-decanoic acid, 9,12-octadecenoic acid and 6-octadecenoic acid in the extracts play an important role in removing free radicals. While there is little report of antioxidant activities of these compounds, their synergistic effects may contribute to free radical scavenging activities of the extract. Although the work on the antioxidant activities of these compounds is limited, the synergistic effects between the compounds may be effective in scavenging free radicals. These phytochemicals and secondary metabolites can decompose OH and form complexes with Fe^{+2}, inhibiting lipid peroxidation. In addition, many of these bioactive compounds are natural aromatic sources, with antioxidant properties and having hepatoprotective effects (Christaki et al., 2012; Erukainure et al., 2017). Meanwhile, in rats treated with alcohol CAT, SOD, GR, GPx and GST activity have significant levels fluctuate (Table 1). It can be argued that walnuts have no significant effect on these fluctuations. Oxidative stress in rats exposed to alcohol can affect ADS activities in organisms. An increase in GPx and GST activities from ADS may indicate a change in lipid peroxidation due to alcohol consumption (Aykac et al., 1985; Sonde et al., 2000; Turan and Celik, 2016). However, it is known that increased GST activities produce protective responses to eliminate xenobiotics (Smith and Litwack, 1980). For this reason, the result of stimulation of the antioxidant defense system may indicate the compliance stage of the organisms. The antioxidant components in the walnut content have a synergistic effect with increased plasma antioxidant capacity in rats (Halvorsen et al., 2002; Bati et al., 2015) and have strong antioxidants in the walnut and these components protect the cells from the negative effects of free oxygen radicals. Many lung diseases are generally associated with the ex-pression of various antioxidant systems as an adaptive response, and in adaptive antioxidant responses, genetic alterations in some antioxidant enzymes and / or defects may contribute to lung pathologies and warrant therapeutic approaches to correct these deficits (Van Der Vliet, 2015). It has been found that compounds such as ellagic acid monomers, polymeric tannins, phenolic and flavonoid, which are present in walnut, are important inhibitors of in vitro plasma and LDL oxidation (Persson et al.,

2014) and reduce the adverse effects of reactive oxygen species in diabetic mice (Fukuda et al. 2004). Walnut can protect cells and tissues from negative effects of ROS due to its strong antioxidant property. Oxygen is indispensable for all eukaryotic life, but the oxidative stress produced by ROS is connected with the development of many diseases. Since antioxidant treatments may be inadequate in the treatment of oxidative stress responsible for many diseases, there is a clear need to implement strategies to better understand and expedite oxidation chemistry in complex biological systems such as lungs (Fukuda et al., 2004). Oxidative stress often appears to increase with age in the muscle, and antioxidant treatments are often not effective in reducing in situ muscle oxidative stress. The response of muscle proteolytic activities to antioxidant support is an interesting parameter for assessing the effect of antioxidants in the muscle because oxidative stress modulates proteolytic activities (Aebi, 1974). In a study conducted, it was reported that the effect of antioxidant defense system on oxidative stress endurance muscle tissue was not very effective (Mosoni et al., 2010). Lack of correlation between plasma and skeletal MDA concentrations may be due to different production sources. High diversity in in vitro and in vivo analysis, and time and style of treatment, study and species tissue differences, etc. Because of the presence of inconsistent factors such as the presence, the present data are the characteristics of the difficulty to compare to different investigations of the chemopreventive method. This study shows compliance with the results of the study mentioned above despite the duration of application and different working conditions.

As a result, it was found that the increased MDA content due to the alcohol-induced oxidative stress in both tissues decreased in the walnut treated lung and muscle tissues. Alcohol and alcohol + walnut treatment of nutritional supplemented rats changed CAT, GPx, GST and SOD content and ADS components compared to control rats. Besides, oxidative stress resulting from alcohol consumption in rats has been shown to cause fluctuations in ADS components and walnut has been found to be a healing effect on the complications caused by oxidative stress. Conclusions suggest that regular removal of beneficial foods can be helpful in preventing chronic degenerative tissue damage.

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