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Walnut Seed Coat (*Juglans regia* L.), a Plant Effective in Human Health: Antioxidant Activity and in Rats Nephroprotective Effect

İnsan Sağlığında Etkili Bir Bitki Ceviz Tohumu Kabuğu (*Juglans regia* L.): Antioksidan Aktivitesi ve Sıçanlarda Nefroprotektif Etkisi

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ABSTRACT

In the study, the seed coat (WSC) of Posof (Ardahan/Türkiye) walnuts was extracted to determine their phytochemical components and antioxidant capacities. The effects of bioactive components in the ethanol extract of WSC (E-WSC) on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors were investigated. Additionally, antioxidant enzyme activity parameters were measured in the kidney tissues of Triton WR-1339-induced hyperlipidemic rats. Bioactive compounds in WSC were identified by GC-MS system. The antioxidant properties of WSC were measured using Fe^{+3}, Cu^{+2} and Fe^{+3}-2,4,6-tripyridyl-s-triazine (TPTZ) reducing agent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'- azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activities. In this analysis, using 30 male Wistar rats (300 ± 30 g) randomly divided into five groups were treated as follows; K1: Healthy control group, K2: E-WSC (150 mg) o.d., K3: E-WSC (300 mg) o.d., K4: Hyperlipidemic group i.p., K5: Hyperlipidemic group i.p. + E-WSC (300 mg) o.d. Superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) analyzes were performed in kidney tissues. Based on these results, it was clearly determined that E-WSC has significant antioxidant activity due to its bioactive components, has an inhibitory effect on AChE and BChE enzymes, and has a protective effect against oxidative stress by improving hyperlipidemia-related kidney damage.

Keywords: Antioxidant activity, kidney, oxidative stress, Triton WR-1339; walnut seed coat

ÖZ

Çalışmada, Posof (Ardahan/Türkiye) cevizlerinin tohum kabuğu (WSC), fitokimyasal bileşenlerinin ve antioksidan kapasitelerinin belirlenmesi için ekstrakte edilmiştir. WSC'nin (E-WSC) etanol ekstraktındaki biyoaktif bileşenlerin asetilkolinesteraz (AChE) ve butirilkolinesteraz (BChE) inhibitörleri üzerindeki etkileri araştırılmıştır. Ayrıca Triton WR-1339 ile indüklenen hiperlipidemik sıçanların böbrek dokularında antioksidan enzim aktivite parametreleri ölçülmüştür. WSC'deki biyoaktif bileşikler GC-MS sistemi ile tanımlanmıştır. WSC'nin antioksidan özellikleri, Fe^{+3}, Cu^{+2} ve Fe^{+3}-2,4,6-tripiridil-s-triazin (TPTZ) indirgeyici ajan, 1,1-difenil-2-pikrilhidrazil (DPPH) ve 2,2'- azino-bis (3-etilbenzotiyazolin-6-sülfonik asit) (ABTS) temizleme aktiviteleri kullanılarak ölçülmüştür. 30 adet erkek Wistar sıçanı (300 ± 30 g) kullanılarak yapılan bu analizde, rastgele beş gruba ayrılan sıçanlara aşağıdaki gibi uygulama yapılmıştır; K1: Sağlıklı kontrol grubu, K2: E-WSC (150 mg) o.d., K3: E-WSC (300 mg) o.d., K4: Hiperlipidemik grup i.p., K5: Hiperlipidemik grup i.p. + E-WSC (300 mg) o.d. Böbrek dokularında Süperoksit dismutaz (SOD), katalaz (CAT) ve malondialdehit (MDA) analizleri yapılmıştır. Bu sonuçlara göre E-WSC'nin biyoaktif bileşenlerine bağlı olarak önemli antioksidan aktiviteye sahip olduğu, AChE ve BChE enzimleri üzerinde inhibitör etkiye sahip olduğu ve hiperlipidemiye bağlı böbrek hasarını iyileştirerek oksidatif strese karşı koruyucu etkiye sahip olduğu açıkça belirlenmiştir.

Anahtar Kelimeler: Antioksidan aktivite, böbrek, oksidatif stres, Triton WR-1339, ceviz tohumu kabuğu

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Introduction

Studies carried out to provide valuable products in terms of bioactive components against health problems that may occur due to the consumption of products with high amounts of harmful chemical substances have become the focus of attention recently. (Soccol et al., 2017; Zhang et al., 2016). Therefore, metabolites obtained from plants emerge as natural products of great importance of developing most of the existing drugs (Xu et al., 2019). The codeine alkaloid found spontaneously in Papaver spp. (Dicpinigaitis et al., 2014), quinine, a component of the Cinchona genus (Achan et al., 2011), and aspirin derived from the Salix genus are examples of these metabolites (Oketch-Rabah et al., 2019).

Clinical and epidemiological studies have revealed that consuming foodstuffs, especially those with herbal ingredients, protects by producing solutions against some problems, especially cardiovascular diseases and immune system problems (Aune et al., 2016). Phytochemicals such as carotenoids and polyphenols, when used alone or in combination, act as an effective defense mechanism in stopping disease formation and progression as they prevent the inflammatory state and possible oxidation (Hever & Cronise, 2017). Polyphenols, a wide-ranging secondary metabolite group containing important contributors such as phenolic acid and flavonoids, provide a great advantage to human health with important activities such as cytotoxic, anti-inflammatory and, cardioprotective, with many studies (Di Mauro et al., 2019; Fraga et al., 2019). In addition to these, it also eliminates the risk of many diseases with its antioxidant properties (Shahidi & Ambigaipalan, 2015; Silinsin and Bursal 2018). Plant polyphenols, which have radical scavenging, hydrogen donor functions, reducing and metal chelating activity and antioxidant properties against reactive nitrogen and oxygen species in the system can also balance energy in enzymatic pathways. (Bjørklund & Chirumbolo, 2017; Limmongkon et al., 2017). Many studies have confirmed that some macro and micronutrients of shell foods have positive effects on health through various mechanisms (Pei & Lu 2011; Rusu et al., 2019). To determine the antioxidant compounds in the plant, the extraction process is performed using a solvent. Ethanol, water, methanol, ethyl acetate (Hazli et al., 2019; Rasera et al., 2019), chloroform (Fernández-Agulló et al., 2013), and N-butanol are among the most commonly used solvents. Depending on the solvent used, the antioxidant activity and yield of the plant vary (Lateef et al., 2012).

Hyperlipidemia, which develops as a result of variable lipid metabolism, is defined as elevated serum triglyceride (TC), total cholesterol (TG), and low-density lipoprotein-cholesterol (LDL-C) levels (Nie et al., 2018). This disorder is also the main risk factor for the development of cardiovascular diseases including atherosclerosis and coronary heart disease among the most important health and economic problems (Cicero & Colletti, 2016). Additionally, hyperlipidemia can cause solid organ damage, including kidney damage (Zong-liang et al., 2006). Experimental and epidemiological studies have shown that high-density lipoprotein-cholesterol (HDL-C) acts as an anti-atherogenic in the prevention of atherogenic diseases triggered by atherosclerotic plaques formed by the accumulation of excess LDL-C on the arterial wall (Alissa & Ferns 2017). Oxidative stress disorders and disorders in the antioxidant defense system, which play a key role in the pathogenesis of hyperlipidemia and these critical diseases, are associated with excessive increase in reactive oxygen species in the biological system (Nijhawan et al., 2019) as these radicals cause modifications in LDL-C molecules (Yang et al., 2017). While oxidation of lipids causes the formation of many toxic products such as oxidized LDL-C and malondialdehydes (MDA) (Alissa & Ferns 2017), it also inhibits antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT).

Triton WR-1339, a nonionic detergent, is frequently used to establish an acute hyperlipidemia model in animals to evaluate potential hyperlipidemic drugs (Bertges et al., 2011). It inhibits the uptake of circulating lipoproteins by extrahepatic tissues, resulting in a high plasma lipid profile and in increased blood lipoprotein levels (Da Rocha et al., 2009). In other words, by inhibiting lipoprotein lipase (LPL) activity, it causes the accumulation of very low-density lipoprotein (VLDL) and TGs and reduces HDL-C production (Zarzecki et al., 2014). Studies have shown that a single intraperitoneal administration of Triton WR-1339 to adult rats produces hyperlipidemia within 24 hours (Palabiyik et al., 2022; Sheikha et al., 2018). Statins are a group of synthetic drugs used as a primary step in regulating lipid metabolism and reducing LDL-C levels (Nelson, 2013). The use of these traditional pharmacological drugs used in the treatment of hyperlipidemia has been limited due to the side effects they may cause (Chien et al., 2019; Chiu et al., 2019). Therefore, search for a cheap, safe, and easily available functional food in hyperlipidemia studies is an important and scientific area of interest (Chiu et al., 2019). Medicinal plants and natural components are considered excellent alternative medicine sources and may show anti-hyperlipidemic activity of treating experimentally induced diseases (Abdallah et al., 2020).

Walnut (*Juglans regia* L.), a very valuable product in terms of economic importance, belongs to the Juglandaceae family. It has a worldwide production, especially in mild climate conditions. It contains good nutritional and nutraceutical components (Acquaviva et al., 2021; Bernard et al., 2018) and is rich in unsaturated fatty acids (Linolenic, Oleic,

Palmitic acid, etc.), proteins, tocopherols (g-tocopherol), phytocetrols, vitamin E and polyphenols (Ellagic, Tannins, Gallic acid) (Ros et al., 2018). It is considered as a nutraceutical in terms of this content. In addition to the antioxidant effect protecting the living body from reactive oxygen species and free radicals (Nunes et al., 2012), its antibacterial, anti-inflammatory, cytotoxic and prebiotic effects have paved the way for its use in various industries such as food, medicine and cosmetics, especially phytotherapy (Alasalvar & Bolling, 2015; Jahanban-Esfahlan et al., 2019). It has been observed that the results similar to these positive bioactivities of walnut on health and the results of the analysis made on other parts of the walnut (leaves, green peel, shell, skin etc.) (Cosmulescu et al., 2015; Vieira et al., 2020) support each other. In addition, it has been reported that the septum exhibits hypoglycemic, hematological regeneration and anti-aging activities (Rusu et al., 2020).

The main aim of this study is to determine the bioactive components and antioxidant activity of WSC with an experimental setup. While GC-MS analysis was used to detect phenolic bioactive components, different methods such as Fe^{3+}, Cu^{2+}, DPPH•, $ABTS^{\bullet+}$ and FRAP were employed to evaluate antiradical and antioxidant activity. Besides, few studies have investigated the use of E-WSC to determine the protective effect in the hyperlipidemic state in Triton WR-1339-induced rats. Therefore, our study is significant as it is one of the few studies evaluating the effect of E-WSC on oxidative stress parameters in the kidney tissues of Triton WR-1339-induced hyperlipidemic Wistar (male) rats.

Methods

Reagents and Chemicals

Triton WR-1339 was purchased from Santa Cruz Biotechnology (California, USA). Kits used for oxidative stress (MDA) and antioxidant enzyme activity (SOD and CAT) measurements were obtained from SunRed Biological Technology Co., Ltd (Shanghai, China). Ethanol (C_2H_5OH) was obtained from isolab (Wertheim, Germany). Sevorane, AbbVie Medical Pharmaceuticals Industry, and Trade Ltd. Co. was obtained from İstanbul/Türkiye.

Plant materials

Walnut seed coats were extracted from walnuts obtained from Posof (Ardahan/Türkiye). The samples were dried at room temperature to avoid exposure to sunlight. The powdered substance was stored at -18 °C.

Extraction of WSC with Ethanol

50 g of WSC dissolved in 1000 mL of ethanol was stirred in a magnetic stirrer (Heidolph MeiR H-Standard, Schwabach, Germany) for 3 days and then extracted at 60-80°C for 4 hours. After separating the waste material with a sieve, the solvent was evaporated (Heidolph, Schwabach, Germany) at 155 rpm and 50 °C. The extract was preserved at +4°C by drying in an oven (Binder, Tuttlingen, Germany).

Determination of Chemical Compound Content of WSC

Chemical content of WSC was determined with ChemStation (Agilent Technologies, Palo Alto, CA, USA) software after using Agilent 7820A gas chromatography-mass spectrometry (GC-MS) instrument, 5977 mass spectroscopy detector and 7673 series autosampler. Compounds were separated with a 0.25 µm, 30m x 0.25 mm diameter HP-5 MS column. Both the injection temperature and the detector temperature were set to 250°C. The injection capacity was determined as 1µl indivisible injection mode, the transporter gas was helium, the flow rate was 1ml/min, and the ionization energy was 70 eV. The oven temperature was programmed to increase 50°C for 1 minute, increasing 20°C per minute for 1 minute at 100°C, increasing by 10°C per minute for 1 minute at 180°C, increasing by 5°C per minute for 5 minutes at 220°C by 10°C for 5.5 min at 300°C. Extract components chromatograms and mass spectra were evaluated with reference standard substance values. In addition, the chemical content was defined by comparing it with the NIST MS (National Institute of Standards and Technology) library values.

Reducing Power Protocols

Fe^{3+}–Fe^{2+} Reducing Power Analysis

Reducing capacity was studied by rearranging the Oyaizu process (Oyaizu, 1986). Compounds with antioxidant properties, which can negatively affect oxidants and render them dysfunctional, have reducing power (Li et al., 2020; Gulcin,2020). The Prussian blue color of Perl, which appears with the addition of Fe^{3+} ions to the experimental medium, forms the $Fe_4(Fe(CN)_6)_3$ complex with an effective absorbance by measuring 700 nm in the spectrophotometer (Göçer & Gülçin, 2011; Gulcin et al., 2010). A raised absorbance value is an indicator of reducing power.

CUPRAC Reducing Capacity

The CUPRAC method, that is copper ion reduction analysis, shows the Cu^{2+} reducing power of phenolic compounds (Apak et al., 2006). A strong absorbance at 450 nm is defined as an indication of an increase in reducing capacity (Gülçin & Daştan, 2007).

FRAP Analysis

It is a method based on the reduction of iron ions (Fe^{3+}) to iron ions (Fe^{2+}) under acidic conditions (Göçer & Gülçin, 2011). It is a blue-colored complex (Fe^{3+}-TPTZ complex) formed by iron ions (Fe^{3+}) and trippyridyl triazine (TPTZ), showing max absorbance at 593 nm (Köse et al., 2015).

Radical Scavenging Protocols

DPPH• Scavenging Activity

Radical scavenging is one of the most effective antioxidant methods for removing free radicals. Since the situations triggered by these radicals are dangerous for biological systems, it is very important to remove the radicals. DPPH• analysis, which is the most common method in which the electron or hydrogen donating abilities of the extracts are determined and accurate and reliable results are obtained, was carried out based on the Blois principle (Bursal et al., 2019; Blois, 1958). The radical scavenging power gives spectrophotometric absorbance at 517 nm. The decrease in absorbance seen in the extracts meant that the DPPH• radical scavenging activity was strong (Gülçin et al., 2007).

$ABTS^{\bullet+}$ Scavenging Assay

$ABTS^{\bullet+}$ scavenging capacity is widely used to measure the radical scavenging activities of various extracts or pure substances. DPPH• is a very effective method like radical scavenging activity (Re et al., 1999). Percent inhibition of $ABTS^{\bullet+}$ was measured at 734 nm (Sujayev et al., 2016). The decrease in absorbance with the formation of discoloration was calculated. $ABTS^{\bullet}$ radical scavenging activity was performed by the procedure specified by Re et al (1999).

AChE and BChE Enzymes Inhibition Assays

AChE and BChE enzyme inhibition analyses were performed by modifying the Ellman's method. Three replications of the analyses were taken, IC_{50} values were calculated and standard deviations were added to the obtained findings (Ellman et al., 1961).

Supply and Adaptation of Experimental Animals

Male Wistar rats (300 ± 30 g), obtained through the decision of Atatürk University Medical Experimental Application and Research Center ethics committee (ATADEM) dated 28.12.2020 and numbered 236643897-000-EBYS-1, were kept at a controlled temperature (22-24°C) and dark-light cycle (12:12 hours). After a seven-day adaptation period with a regular diet and free access to ad-libitum water, the animals were randomly divided into 5 groups and placed in (n=30) marked polycarbonate cages. The entire application and care process was carried out by the national animal care directive (Cinar et al., 2019).

Preparation of Substances to be Applied

1200 g of E-WSC extract was made up to 20 cc with distilled water, and 1 cc was applied to the animals in the relevant group. Triton WR-1339 (400 mg/kg) was dissolved in normal saline (pH 7.4) and injected once i.p. to rats fasted for 12 hours. After 24 hours, hyperlipidemia was induced (Baldissera et al., 2017).

Experimental Design

The applications to the rats divided into five groups were followed for five days. The application doses and the forms of administration of the substances are as follows: K1: Healthy control group. Physiological water only (2.5 mL/kg, intraperitoneally (i.p) administered group (Kumar et al., 2013). K2: E-WSC (150 mg). The group was given E-WSC (150 mg/kg, oral dose (o.d)) 30 minutes before physiological water (2.5 mL/kg, i.p) administration (Beigh et al., 2017). K3: E-WSC (300 mg). The group was given E-WSC (300 mg/kg, o.d) 30 minutes before physiological water (2.5 mL/kg, i.p) administration (Cintesun et al., 2023). K4: HL - Hyperlipidemic group. The group that received physiological water 30 minutes before Triton WR-1339 (400 mg/kg, 2.5 mL/kg, i.p) administration (Baldissera et al., 2017). K5: HL + E-WSC (300 mg). Group that received E-WSC (300 mg/kg, o.d) 30 minutes prior to administration of Triton WR-1339 (400 mg/kg, 2.5 mL/kg, i.p).

The experimental animals were anesthetized with sevoflurane (sevorane) one day after the basic inductions were made and the experimental period was completed. Using sterile materials, the abdominal region was carefully opened with a vertical incision, and the kidney tissue was removed with a surgical procedure. The excised tissues were washed in salt water and purified, then dried and treated with liquid nitrogen, consequently stored at -80 °C until analysis.

Oxidative Stress Indicators

The 0.2 g was taken from the kidney tissues heated to +4°C before analysis. Ceramic beads of different sizes and 1 mL homogenization buffer (0.1 M $K_2H_2PO_4$ – 10 mM EDTA) were added to the tissues taken into the screw tube and placed in the homogenizer device (4000 rpm, 5 cycles, 1 min shaking and 10 sec holding). Then, the supernatants were taken by centrifugation (4°C, 13000 rpm, 30 min), and the homogenate was ready.

SOD, CAT, and MDA activities were determined using freshly prepared kidney tissue homogenates. SOD analysis was conducted using the Rat (SOD) ELISA Kit (Catalog No: 201-11-0169, SunRed), CAT analysis was performed with the Rat (CAT) ELISA Kit (Catalog No: 201-11-5106, SunRed), and MDA analysis was carried out according to the protocol outlined in the Rat (MDA) ELISA Kit (Catalog No: 201-11-057, SunRed).

Statistical Analysis

The data from the treatment groups were analyzed using Analysis of Variance (ANOVA). Differences in the means of these groups were further assessed using Duncan's Test, with p-values calculated via the Unpaired T-Test. A significance level of $p < .05$ was used for all mean comparisons.

Results

GC-MS Analysis Results

In the GC-MS analysis performed on the ethanol extract of walnut seed coats; it was determined that there are many bioactive compounds such as phenolic compounds, mono and polyunsaturated fatty acids, alcoholic compounds, phytosterols and esters (Table 1).

Table 1.
Identification of phytocomponents isolated from the ethanolic extract of WSC.

Ret. time	Compound name	Peak area (%)
21.044	Lineoleic acid	0.564
32.523	1- Heptatriacotanol	1.067
33.199	Oleic acid amide	21.211
34.707	Ethyl iso-allocholate	0.614
35.525	Hexadecanoic acid	4.065
35.667	Cinnamic acid	3.225
37.675	Brassicasterol acetate	4.717
38.860	B- Sitosterol	61.071

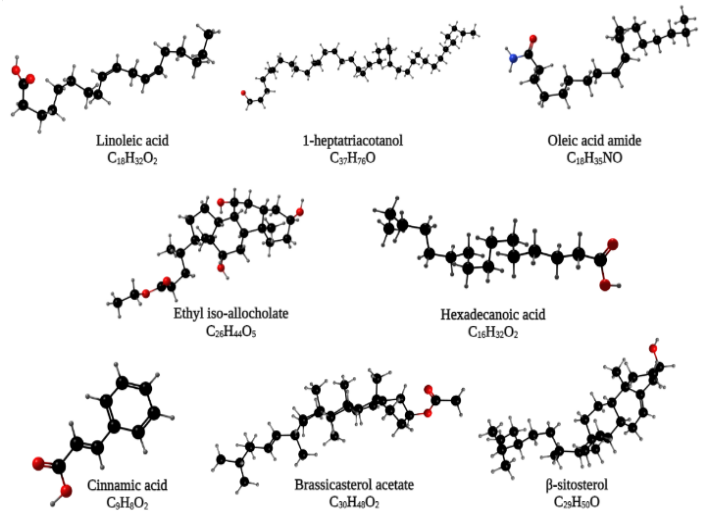

Figure 1.
Structures of bioactive compounds determined by GC-MS analysis (KingDraw).

The structure of the compounds identified by GC-MS is given in Figure 1. Among these compounds, β-Sitosterol (61.071%) has the highest percentage, while Linoleic acid (0.564%) has the low WSC percentage.

It is known that herbal extracts have many beneficial effects on health and is actively used in the prevention and cure of many diseases. In general, around 25% of medicines worldwide are derived from plants. This is largely due to the richness of phenolic compounds, which serve as secondary metabolites in these plants. Consuming foods rich in phenolic compounds is very important in terms of providing biological benefits (Mutlu et al., 2023). Of the eight identified compounds, Cinnamic acid is antibacterial, antifungal, anti-inflammatory (de Almeida Lima et al., 2018), neuroprotective (Lan et al., 2017), anticancer (Gießel et al., 2019) and antidiabetic (Adisakwattana et al., 2013), Linoleic acid is antihyperlipidemic (Mozaffarian et al., 2010; Mensink & WHO, 2016) and antidiabetic (Imamura et al., 2018), Oleic acid amidine antimicrobial (Ahmad et al., 2021), Hexadecanoic acid is anticytotoxicity, apoptotic (Harada et al., 2002) and anticancer (breast-colorectal) (Nazarudin et al., 2020), 1-Heptatriacotanol's antimicrobial, antioxidantve anti-inflammatory (Hadi et al., 2016), Ethyl isoallocholat's anti-inflammatory (Johnson et al., 2020), β-Sitosterol's anti-inflammatory (Kmiecik et al., 2011; von Holtz et al., 1998), anticancer activity (breast, liver, colon, stomach) (Sanches et al., 2005; Zhao et al., 2017) and antihyperlipidemic (Luo et al., 2015), and Brassicasterol acetate's immunomodulatory and antioxidant (Çakmakçı et al., 2015) effects have been reported in studies.

Antioxidant Results

Antioxidant activity of WSC was tested using DPPH• scavenging and $ABTS^{\cdot+}$ scavenging activities. Three separate analysis methods were used to determine the reduction ability of WSC: CUPRAC, FRAP and Fe^{3+} (Figure 3 and Table 2). Reduction potential affects the capacity of plant extracts to exhibit biological activity. These extracts neutralize reactive oxygen species, oxidants and reducing agents (Çakmakçı et al., 2015). The reduction capacity of WSC is measured by the Fe^{3+} reduction test system. Fe^{3+} ions addition to WSC occur blue colored complex of $Fe_4(Fe(CN\text{-})_6)_3$ (Tohma et al., 2016). The absorbance value of the $Fe_4(Fe(CN\text{-})_6)_3$ complex was determined as 700 nm (Cakmak & Gülçin, 2019). As a result of the formation of the complex, a yellow color appears in the sample. However, the color may vary from green to blue, which depends on the effect of the test compounds (Gülçin et al., 2012). Accordingly, it was determined that WSC, which uses the reduction properties of $Fe(Fe(CN\text{-})_6)_3$, $Cu^{2+,}$ and Fe^{3+}-TPTZ, has a very effective and valuable reduction potential. The reduction potential of WSC was determined by the Oyaizu method. For this measurement, Fe^{3+}- Fe^{2+} reduction was achieved (Oyaizu, 1986). The Fe^{3+} reducing capacity of WSC is shown in Table 2 and Figure 3A. The radical scavenging and reduction properties of WSC will not be detailed in the discussion section as the structure-activity relationship is relatively low. The Fe^{3+} reducing effects of 30 µg/mL of WSC and standards declined as following orders: BHA (1.471, r^2: 0.9699) > WSC (1.894, r^2: 0.9839) > Trolox (1.826, r^2: 0.9746) > BHT (1.173, r^2: 0.9601) > α-Tocopherol (1.004, r^2: 0.9749). Various antioxidant compounds serve as standards for determining the antioxidant capacity of samples. These

standards are chosen based on important features such as stability in the solvent environment, price, and solubility (Han et al., 2018). The formation of complexes and the increase in the reducing effect are associated with the rise in absorbance values. The results clearly demonstrate that WSC has the ability to effectively reduce Fe^{3+} and neutralize free radicals and ROS. Therefore, WSC may serve as a potential agent against oxidative stress-induced damage in biochemical and biological systems.

Table 2.
Fe^{3+}, Cu^{2+}, and Fe^{3+}-TPTZ reducing ability of WSC and standards (30 µg/mL)

Antioxidants	Fe^{3+} reducing		Cu^{2+} reducing		Fe^{3+}-TPTZ reducing	
	λ_{700}	r^2	λ_{450}	r^2	λ_{593}	r^2
BHA	2.471	0.9699	2.598	0.8705	1.501	0.8576
BHT	1.173	0.9601	1.519	0.8807	0.772	0.8044
α-Tocopherol	1.004	0.9749	1.324	0.8710	0.949	0.9417
Trolox	1.826	0.9746	1.523	0.7630	1.619	0.9110
WSC	1.894	0.9839	1.245	0.8624	1.247	0.8857

Table 2 and Figure 3B show the Cu^{2+} reduction values of WSC. A positive correlation was observed between the Cu^{2+} reducing and WSC as concentration-dependently (10-30 µg/mL). At the concentration of 30 µg/mL, Cu^{2+} reducing capability of WSC and standards were declined as following orders (Table 2 and Figure 3B): The Fe^{3+} reducing effects of WSC and standards declined as following orders: BHA (2.598, r^2: 0.8705) > Trolox (1.523, r^2: 0.7630) > BHT (1.519, r^2: 0.8807) > α-Tocopherol (1.324, r^2: 0.8710) > WSC (1.245, r^2: 0.8624). Cuprac analysis is capable of testing both hydrophilic and lipophilic antioxidants. Since the optimal pH of the method is close to physiological pH, it will not carry the risk of underestimation (under acidic conditions) or overestimation (under basic conditions) of the total antioxidant capacity due to the protonation of antioxidants or proton dissociation of phenolic compounds, respectively. In addition, it is a low-cost, fast and stable test (Taslimi et al., 2020).

WSC determined to have Fe^{3+} and Cu^{2+} reduction potential was also observed to have an effective reduction capacity in FRAP analysis (Figure 3C and Table 2). Reducing ability of WSC was found to be in descending order of Trolox (1.619, r^2: 0.9110) > BHA (1.501, r^2: 0.8576) > WSC (1.247, r^2: 0.8857) > α-Tocopherol (0.949, r^2: 0.9417) > BHT (0.772, r^2: 0.8044). As similar Cu^{2+} reducing of WSC, a positive correlation was observed between the Cu^{2+} reducing and WSC as concentration-dependently (10-30 µg/mL). As in the previous reduction analysis, it was revealed that the strong reducing absorbance value is related to the effective reducing ability in the complex. The FRAP method is performed in acidic environment to maintain the solubility of iron ions (Sehitoglu et al., 2015).

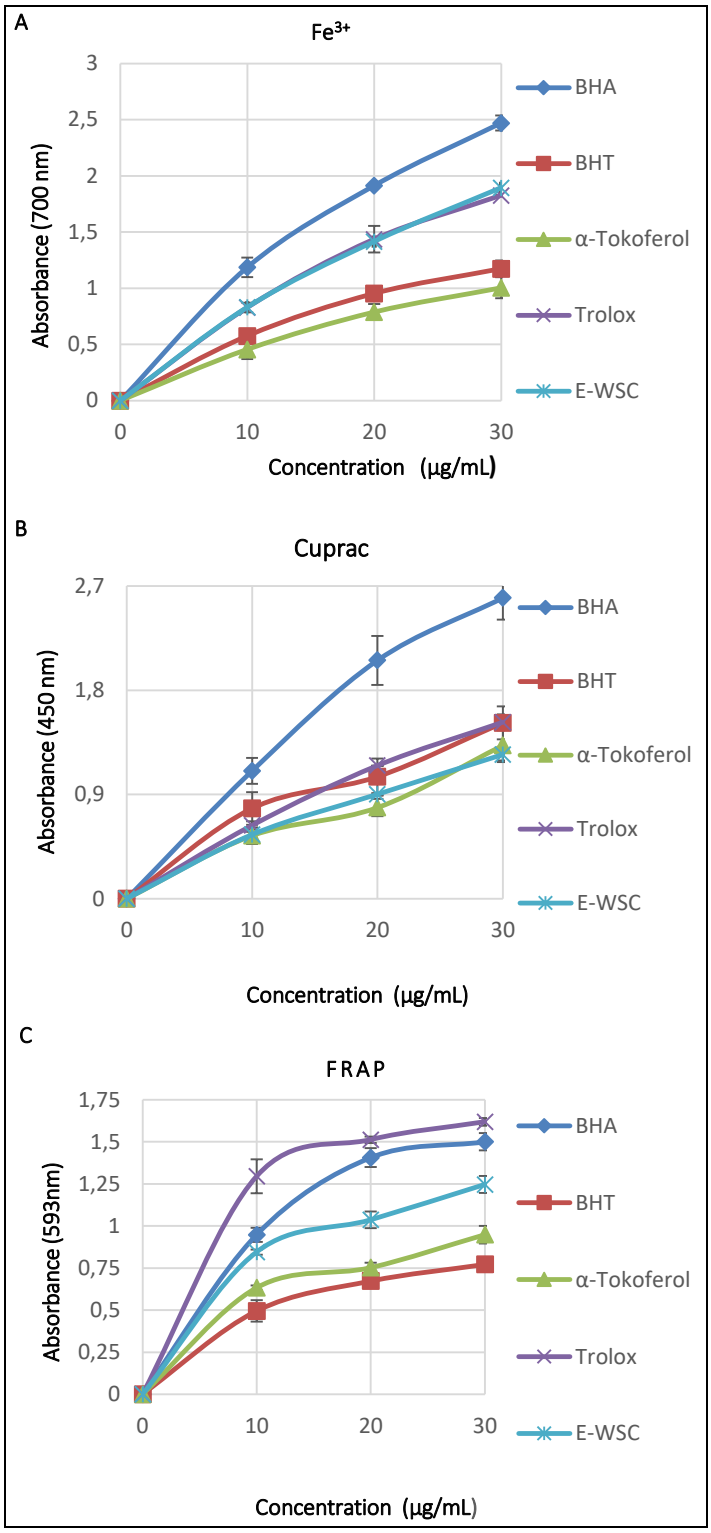

Figure 3.
*Different antioxidant assay for WSC: **A.** Fe^{3+} reducing method, **B.** Cu^{2+} reducing method, **C**. Fe^{3+}-TPTZ reducing method.*

DPPH• and $ABTS^{\cdot+}$ removal analyzes are most commonly used as spectrophotometric reduction methods. These two tests used to measure antioxidant and radical scavenging potential in plant sample extracts were also used in this study (Gülçin et al., 2020). Some parameters stand out for

inhibition percentage measurements., which are concentrations of antioxidants and radicals, solvent and reagent ratios, incubation time and temperature. In addition, the presence of hydrogen, water, and metal in antioxidant test systems is another important parameter used for measurement (Gulcin et al., 2019). Another element used to evaluate antioxidant properties is the IC50 (Half-maximum scavenging concentration) value. This value is defined as the concentration that plays an effective role in removing 50% of oxidant agents (Türkan et al., 2020). For DPPH radical scavenging activities of WSC and standards were found to be in the following order: Trolox (IC_{50}: 8.59 µg/mL, r^2: 0.9704) < BHA (IC_{50}: 11.59 µg/mL, r^2: 0.9451) < WSC (IC_{50}: 13.39 µg/mL, r^2: 0.9704) < α-Tocopherol (IC_{50}: 13.78 µg/mL, r^2: 0.9221) < BHT (IC_{50}: 26.55 µg/mL, r^2: 0.9794) (Table 3 and Figure 4A). A lower EC_{50} value demonstrates a higher DPPH• scavenging ability (Tohma et al., 2019).

Table 3.
DPPH• and $ABTS^{•+}$ IC50 (µg/mL) values of WSC and standards

Compounds	DPPH• scavenging		$ABTS^{•+}$ scavenging	
	IC_{50}	r^2	IC_{50}	r^2
BHA	11.59	0.9451	4.50	0.9334
BHT	26.55	0.9794	5.94	0.9033
α-Tocopherol	13.78	0.9221	8.43	0.9985
Trolox	8.59	0.9214	5.13	0.9557
WSC	13.39	0.9704	8.18	0.9687

Besides these, WSC showed a strong $ABTS^{•+}$ scavenging potential. As given in Table 3 and Figure 4B, WSC effectively scavenged ABTS radicals as concentration-dependently (10-30 µg/mL, $p < .001$). EC_{50} values of WSC in $ABTS^{•+}$ scavenging assay were found to be in descending order of BHA (IC_{50}: 4.50 µg/mL, r^2: 0.9451) < Trolox (IC_{50}: 5.13 µg/mL, r^2: 0.9214) < BHT (IC_{50}: 5.94 µg/mL, r^2: 0.9794) < WSC (IC_{50}: 8.18 µg/mL, r^2: 0.9704) < α-Tocopherol (IC_{50}: 8.43 µg/mL, r^2: 0.9221). The EC50 value indicates a high $ABTS^{•+}$ scavenging potential, as seen in radical scavenging tests. As previously reported, the ABTS radical scavenging capacity of antioxidants has been associated with the H-donating effect (Artunc et al., 2020; Balaydın et al., 2010).

Enzyme Inhibition Results

This study revealed the relationship between AChE/BChE enzymes and E-WSC. This extract effectively inhibited AChE and BChE with IC50 values of 5.75 µg/mL (r2: 0.9783) and 30.26 µg/mL (r2: 0.9191), respectively. On the other hand, tacrine was used as a positive control for AChE and BChE inhibition. The IC50 value of tacrine was found to be 0.044 µM for AChE and 0.0102 µM for BChE. When the results were examined, E-WSC inhibited the AChE enzyme 5.26 times more than the BChE enzyme (Table 4, Figures 5 and 6).

Table 4.
IC_{50} values of E-WSC for AChE and BChE enzymes

	AChE		BChE	
	IC_{50}	R^2	IC_{50}	R^2
E-WSC	5.75103 µg/mL	0.9783	30.262 µg/mL	0.9191
Tacrine	0.0441 µM	0.9805	0.0102 µM	0.9830

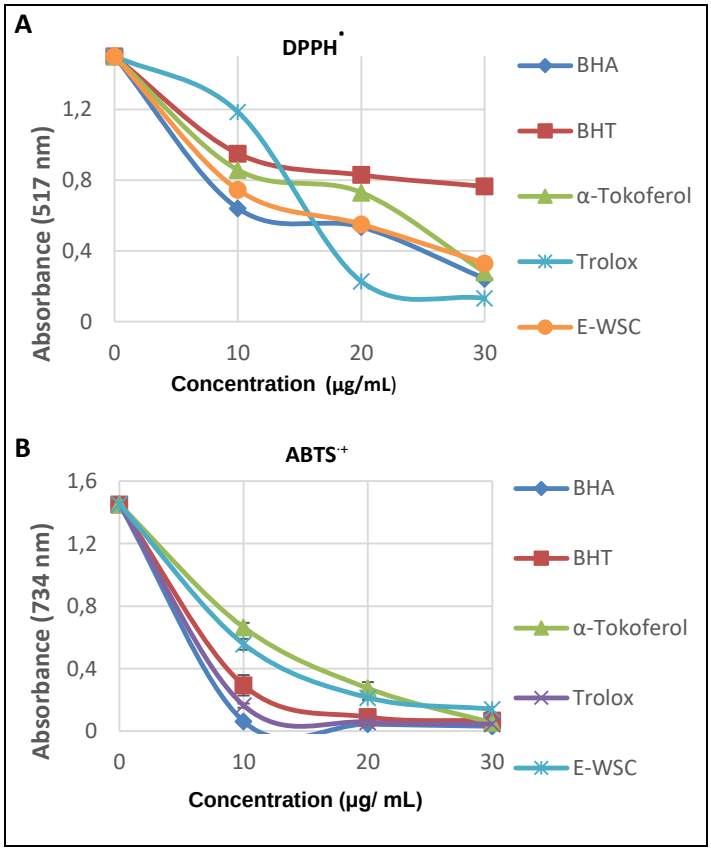

Figure 4.
*Radical scavenging assays for WSC. **A.** DPPH• scavenging method, **B.** $ABTS^{•+}$ scavenging method*

Enzyme inhibition study is seen as an effective step in the treatment of neurodegenerative diseases, especially Alzheimer's (AD), Parkinson's (PD) and senile dementia (Tohma et al., 2019). These diseases include many issues such as oxidative stress, misfolded proteins, protein aggregation, excitotoxicity, neuroinflammation, and neuron loss (Ramsay et al., 2016). Here, especially the oxidative stress factor has a very important effect on the oxidative damage to occur in cellular components such as lipids, proteins and DNA in AD and PD (Lezi & Swerdlow, 2012). The importance of phenolic antioxidants in preventing or delaying the onset of these damages is well documented (Bayrak et al., 2020; Ceylan et al., 2019; Demir et al., 2019; Palabıyık et al., 2023). When looking at the neuropathological features of AD and PD, dysfunctions in the cholinergic and dopaminergic systems are striking. AChE and BChE function at this point, slowing down and terminating nerve impulse transmission by hydrolyzing acetylcholine (ACh) and

butyrylcholine (BCh). Inhibition of these two enzymes has a great effect on the duration of disease mechanisms such as AD, PD and dementia (Artunc et al., 2020). Although the drugs currently used in treatment (Tacrine, Donepezil and Rivastigmine) work to repair this disorder, they may cause some side effects, including gastrointestinal disorders (Artunc et al., 2020; Mathew et al., 2019).

Therefore, it is inevitable that E-WSC, which is both a natural product and has a strong antioxidant effect, helps increase ACh and BCh levels in synapses by inhibiting ChEs and improving cholinergic function.

Lipid Peroxidation and Antioxidant System Modulation of E-WSC in Hyperlipidemic Rats

The results of SOD activity, involved in the removal of superoxide radicals from the kidney tissue of the study groups, CAT activity, an antioxidant enzyme influencing cell life, and MDA activity, a biomarker of oxidative stress, are presented in Table 5.

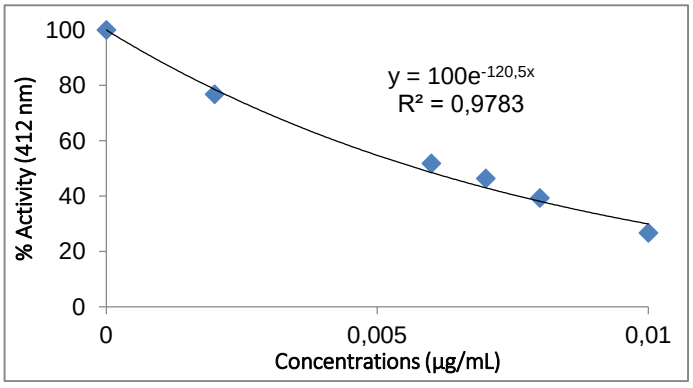

Figure 5.
AChE concentration-activity graph

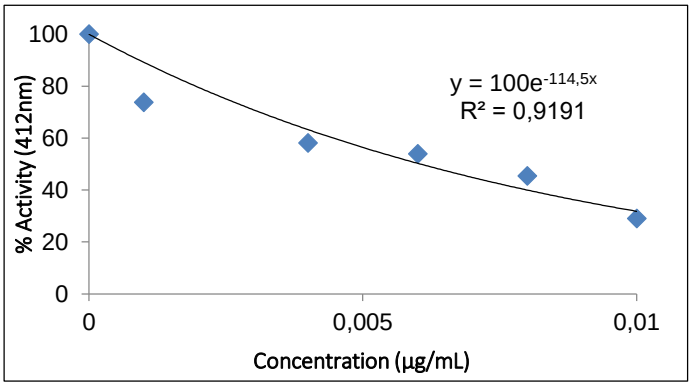

Figure 6.
BChE concentration-activity graph

Table 5.
SOD, CAT and MDA Level Values in Kidney Tissues (Mean ± SD, n=6)

Groups	Kidney Tissue		
	SOD (ng/mL)	CAT (ng/mL)	MDA (nmol/mg protein)
K1 (Control)	30.8553±0.95896^{a,b}	57.046±4.46961^{a,b}	2.33065±0.095218^{b}
K2 (E-WSC-150mg)	28.0468±0.42904^{a,b}	49.6262±2.54587^{b}	2.06552±0.091717^{b}
K3 (E-WSC-300mg)	27.1279±0.50183^{b}	59.267±3.12407 ^{a,b}	2.03018±0.064749^{b}
K4 (HL with Triton WR-1339)	27.4672±1.8076^{b}	52.678±5.97322^{a,b}	2.65356±0.256121^{a}
K5 (E-WSC-300mg+ Triton WR-1339)	31.8495±4.37602^{a}	62.075±10.2431^{a}	2.72167±0.217631^{a}

* While the difference between the group means with the same letter is not significant ($p > .05$), the difference between the group means with different letters is significant ($p < .05$).

There was a significantly decrease in SOD activities (27.4672±1.8076 ng/mL) compared to the control group (30.8553±0.95896) due to Triton WR-1339 applied to create hyperlipidemia in the kidney tissue. The dose-related decrease (28.0468±0.42904/27.1279±0.50183 ng/mL) and deterioration in SOD activity in the groups administered E-WSC-150 mg and E-WSC-300 mg alone, E-WSC-300 mg given with Triton WR-1339 eliminated with. These changes are shown in Figure 7. CAT activity decreased in the hyperlipidemia group (52.678±5.97322 ng/mL) compared to the control group (57.046±4.46961 ng/mL). In the group in which E-WSC-300 mg was administered alone (59.267±3.12407 ng/mL), an increase in activity was detected, but this increase was not statistically significant. E-WSC-300 mg (62.075±10.2431 ng/mL) given with Triton WR-1339 caused a critical increase in activity compared to the hyperlipidemia group. Renal tissues showed tremendous improvement in CAT activities, especially in the groups administered E-WSC 300 mg. (Figure 8) MDA levels were measured to determine lipid peroxidation as a result of in vivo induced hyperlipidemia. Accordingly, a statistically significant increase in MDA levels occurred in the hyperlipidemia group (2.65356±0.256121 nmol/mg protein) developed with Triton WR-1339 induction compared to the control group (2.33065±0.095218 nmol/mg protein). E-WSC-300 mg (2.72167±0.217631 nmol/mg protein) given with Triton WR-1339 statistically significantly reduced MDA levels (Figure 9). However, E-WSC-300 mg showed a significant protective effect against lipid oxidation in kidney tissue when administered alone (2.03018±0.064749 nmol/mg protein).

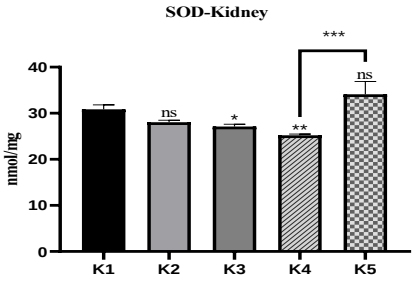

Figure 7.
*Effect of ethanol extraction (E-WSC) of Walnut Seed Coat on superoxide dismutase (SOD) activity in kidney tissue in hyperlipidemic rats induced by Triton X-1339. Results are expressed as Mean ± SD (n=6). The degree of importance compared to the control group was given as * p < .05.*

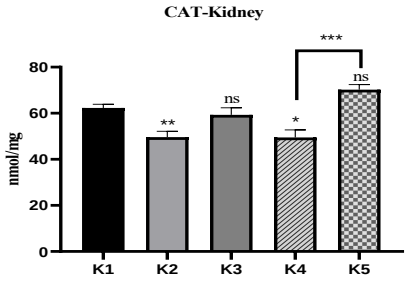

Figure 8.
*Effect of ethanol extraction (E-WSC) of Walnut Seed Coat on catalase (CAT) activity in kidney tissue in hyperlipidemic rats induced by Triton X-1339. Results are expressed as Mean ± SD (n=6). The degree of importance compared to the control group was given as * p < .05.*

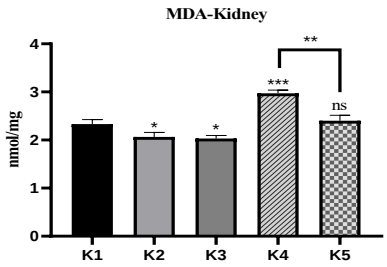

Figure 9.
*Effect of ethanol extraction (E-WSC) of Walnut Seed Coat on malondialdehydes (MDA) activity in kidney tissue in hyperlipidemic rats induced by Triton X-1339. Results are expressed as Mean ± SD (n=6). The degree of importance compared to the control group was given as * p < .05.*

Hyperlipidemia has occurred due to the deterioration of lipid and lipoprotein metabolism and has caused deaths in the world (Monguchi et al., 2017). Triton WR-1339, which is used to induce hyperlipidemia by changing the physicochemical properties of lipoproteins (Venkadeswaran et al., 2014), is an ionic group-free detergent used to prevent the uptake of plasma lipoproteins by peripheral tissues by inhibiting the activity of LPL (Surya et al., 2017). While this enzyme is involved in the lipolytic process, it plays a role in the reuptake of TG from chylomicrons and VLDL to peripheral tissues (Tooulia et al., 2015). A model of acute hyperlipidemia was created in rats within 24 hours with Triton WR-1339, which we used in our current study. Our model was supported by using this induction in the study in which the anti-hyperlipidemic properties of *Campomanesia xanthocarpa* leaves (De Sousa et al., 2019) and N-(Benzoylphenyl)-Carboxamide derivatives were determined (Sweidan et al., 2022). With the application of Triton WR-1339 by Attia et al. (2020), TGs, TC, LDL-C and lipid peroxidation (LPO) increased and HDL-C decreased (Attia et al., 2020). Experimental studies suggest that there is a relationship between the development of renal impairment and dyslipidemia. Increases in cholesterol and triglyceride levels are shown as independent risk factors for the development of kidney disease (Kovesdy et al., 2017).

Hyperlipidemic induction with Triton WR-1339 caused an increase in MDA level with inhibition of antioxidant enzymes (SOD and CAT) activities in rat kidney tissues. These resulting changes and disorders manifests in the kidney tissue with an increased production rate of reactive oxygen species (ROS) or a gap in the antioxidant system (Beyegue et al., 2012) as lipid peroxidation is a free radical that triggers protein oxidation, disrupts cell membrane structure and function, and produces MDA (Gaschler & Stockwell, 2017). The formation of the foam cell is the first step in the onset of the atherosclerotic state. The reason for this is LDL oxidation due to oxidative stress (Winklhofer-Roob et al., 2017). In other words, oxidative stress poses a problem for many related diseases such as hyperlipidemia, atherosclerosis and cardiovascular (Chiu et al., 2019).

The data obtained from our research revealed that E-WSC exhibited antihyperlipidemic activity in Triton WR-1339-induced hyperlipidemia. As expected, the SOD and CAT values, indicative of antioxidant activity, decreased in the hyperlipidemic group compared to the control group. However, it was observed that E-WSC at a dose of 300 mg, both alone and in combination with Triton WR-1339, stimulated activities, resulting in a significant increase in SOD and CAT values (Figures 1 and 2). In a similar study, it was found that the myocardial infarction group, induced by exposure to Isoproterenol (ISO) in rat hearts, exhibited significantly lower activity of SOD and CAT enzymes compared to control rats. Walnut kernel application, on the other hand, cleaned the superoxide and hydrogen peroxides produced by ISO and improved SOD and CAT activities (Sun et al., 2019). In other studies, it has been reported that walnut intake prevents the decrease in SOD and CAT activity. In rats, scopolamine-induced cognitive impairment, SOD and CAT activities were regulated by walnut consumption (Haider et al., 2018). Another significant finding in our study was the measurement of MDA, an indicator of lipid peroxidation. With the induction of hyperlipidemia and the subsequent disruption in lipid metabolism, high levels of MDA were observed. However, our measurements revealed a significant decrease in MDA content following the administration of E-WSC-300 mg (Fig. 3). Additionally, in line with our findings, walnut supplementation in a high-fat diet (HFD) significantly reduced hepatic levels of lipid peroxidation (LPO) and cytosolic MDA levels (Choi et al., 2016). Another study demonstrated that oxidative stress

induced by abnormal lipid profiles in pregnant rats, attributed to the polyunsaturated fatty acid (PUFA) content of walnuts, was alleviated due to the reduction in MDA content (Sun et al., 2020).

Statins and their derivatives, anti-hyperlipidemia drugs, react by inhibiting the HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA reductase) reductase enzyme, which is responsible for cholesterol synthesis in the liver. HMG-CoA activates a mevalonate pathway, which is the rate-limiting step in hepatic cholesterol synthesis, and ensures the synthesis of cholesterol. However, inhibiting HMG-CoA reductase reduces cholesterol synthesis in the liver and reduces the level of LDL-C in the bloodstream (Mo et al., 2019). This drug group and its derivatives, which have an anti-hyperlipidemic effect by inhibiting cholesterol synthesis, can cause some toxic effects, although they are used as a therapeutic in the first step (Abu-Raghif et al., 2015; Hashem et al., 2021).

Although the safety of plant-based drugs is of concern due to the high risk of contamination (Kosalec et al., 2009), natural products may inhibit lipid biosynthesis by showing a similar mechanism of action to statins (Shattat, 2015). For this reason, the use of natural substances to regulate and control hyperlipidemia has recently attracted attention. Plants, especially rich in flavonoid content, are associated as anti-hyperlipidemic agents because they can deactivate HMG CoA reductase in the cholesterol formation pathway. In a previous in vivo study on Wistar rats, it was reported that walnut septum extracts did not show subacute or acute toxic effects at doses of 1000 mg/kg (body weight) (Ravanbakhsh et al., 2016). E-WSC, which is the active ingredient of our study and has a rich content, greatly relieves kidney damage due to hyperlipidemia (Palabiyik et al., 2022) and the total flavonoids extracted from the leaves of *Actinidia kolomikta* improve hyperlipidemia through this mechanism (Yu et al., 2017) supports this thesis.

Oxidative stress has a great impact on the progression of diseases such as Alzheimer's, Patkinson's and Dementia, as it damages cholinergic neurons and causes loss of function (Chen et al., 2012). Free radicals such as ROS play a critical role, especially in AD pathology (Cheignon et al., 2018). It affects important pathways such as oxidative stress, macromolecule peroxidation, Aβ metal ion redox potential, and mitochondrial dysfunction. This interaction, in turn, induces cell homeostasis, ROS production, and upregulation of Aβ and p-tau formation (Chen et al., 2012; Gella & Durany, 2009; Hawking, 2016). As a result, oxidative stress can cause disease progression and even death in patients suffering from neurogenerative diseases such as AD, PD and Dementia. However, no effectiveness has been achieved in the treatments accepted by the FDA (Food and Drug Administration). Therefore, new approaches should be developed to improve cholinergic neurons. Especially the use of antioxidant drugs is extremely important in terms of providing positive results. Antioxidants obtained from polyphenolic compounds found in medicinal plants and natural products found in nature are a more valuable alternative to synthetic compounds. Because they are more harmless, easily cross the blood-brain barrier and improve cognitive decline (Cassidy et al., 2020).

The phytochemical content and strong antioxidant ability of E-WSC may have the capacity to reduce oxidative stress in individuals with AD. However, this should be clarified in more detailed studies. In addition, the fact that our extract is cheap and easily accessible and has no toxic effects increases its potential for treatment.

Conclusion and Recommendations

New developments in pharmacotherapy are of great importance to ensure the effective continuity and development of the healthcare system. Drug potentials obtained from bioactive components and successfully completing the testing phases day by day should be taken into consideration. Because these potentials may have a percentage that will increase success in chronic diseases. Mitigation of chronic diseases, mutagenesis, DNA damage, carcinogenesis and inhibition of pathogenic bacterial growth are generally associated with the scavenging of ROS and propagation of free radicals in living systems. At the same time, the presence of compounds with antioxidant properties is also very important to eliminate the undesirable side effects of some drugs used in the treatment of AD and PD. Antioxidant plants and extracts are, therefore, widely used in different food and medicinal applications. In this study, it was observed that WSC had effective antioxidant ability compared to standard and powerful antioxidants BHA, α-Tocopherol, BHT, and Trolox. Considering that E-WSC is a powerful antioxidant, it was thought that it could be a potentially effective source to treat AD and PD as its anticholinesterase properties were reported for the first time, its ethnopharmacological use against neurodegenerative disorders was, in a sense, confirmed. In the acute hyperlipidemia model caused by Triton WR-1339, lipid peroxidation caused by the increase in reactive oxygen species caused an increase in MDA levels. When E-WSC was administered to the application group, it was observed that the damage was largely repaired and there was a significant increase in antioxidant enzyme activities. These inhibitory activities are thought to be due to β-sitosterol, a phytochemical found in significant amounts in the WSC ethanol extract.

Analyses in our study proved that WSC has a renal protective

effect against hyperlipidemia and is also a therapeutic candidate for treatment. To be evaluated as a combined drug in future studies, analyses must be made to determine drug dosages and application procedures of these drugs, reduce undesirable effects, adapt to the patient and provide maximum benefit. Thus, we are hopeful that it will make a great contribution to the areas that need to be developed.

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