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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF ANTIOXIDANT ACTIVITY OF *HIPPOPHAE RHAMNOIDES* L. GROWING IN TURKEY AND INVESTIGATION OF ITS EFFECTS ON THE LIVER TOXICITY IN RATS

TÜRKİYE'DE YETİŞEN HİPPOPHAE RHAMNOİDES L. BİTKİSİNİN ANTİOKSİDAN AKTİVİTESİNİN BELİRLENMESİ VE SIÇANLARDA KARACİĞER TOKSİSİTESİ ÜZERİNE ETKİLERİNİN ARAŞTIRILMASI

Metin ÇAPA[1]*, Yücel KADIOĞLU[2], Elif ÇADIRCI[3], Mevlüt ALBAYRAK[4]

[1]Tokat Gaziosmanpaşa University, Faculty of Pharmacy, Department of Analytical Chemistry, 60400, Tokat, Türkiye
[2]Atatürk University, Faculty of Pharmacy, Department of Analytical Chemistry, 25240, Erzurum, Türkiye
[3]Atatürk Univesity, Medical Faculty, Department of Pharmacology, 25240, Erzurum, Türkiye
[4]Atatürk University, Vocational School of Health Services, Department of Pharmacy Services, 25240, Erzurum, Türkiye

ABSTRACT

Objective: *The goal of the present work was to investigate the antioxidant properties of the extracts of Hippophae rhamnoides L. and to determine their effects on liver toxicity in rats.*
Material and Method: *Metal chelation, reducing power and DPPH radical scavenging methods were used in the antioxidant activity analysis of extracts. The total phenolic content was determined using the folin-ciocalteu reagent. Plant extracts were administered orally to the rats at doses of 500 µg/kg for 2 days. Each animal group was composed of six female Albino Wistar rats with an average weight of 250 g. Microscopic examination was carried out to observe any pathological changes in the rat livers.*
Result and Discussion: *Water extract showed the highest radical scavenging activity (48.65%), reducing power (0.291 absorbance at 700 nm) and metal chelating (35.40%) at 1 mg/ml concentration. Histopathological studies showed that especially water extract reduced the severity of CCl_4-induced intoxication. Hippophae rhamnoides L. extracts were found to have antioxidant activity, and also Hippophae rhamnoides L. water extract was shown to be particularly effective in preventing liver damage.*
Keywords: *Antioxidant activity, DPPH, Hippophae rhamnoides L., rat liver toxicity, reducing power*

ÖZ

Amaç: *Bu çalışmanın amacı Hippophae rhamnoides L. meyve ekstraktlarının antioksidan özelliklerini araştırmak ve sıçanlarda oluşturulan karaciğer toksisitesi üzerindeki etkilerini belirlemektir.*
Gereç ve Yöntem: *Ekstraktların antioksidan aktivite analizinde metal iyonu kelatlama, indirgeme kuvveti ve DPPH radikal tutuklama yöntemleri kullanıldı. Toplam fenolik bileşik miktarları folin-ciocalteu ayıracı ile tespit edildi. Bitki ekstraktları 2 gün süre ile 500 µg/kg doz olacak şekilde*

* **Corresponding Author / Sorumlu Yazar:** Metin Çapa
e-mail / e-posta: metin.capa@gop.edu.tr, **Phone / Tel.:** +903562521616

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ratlara oral olarak verildi. Her bir hayvan grubu ortalama ağırlığı 250 g olan altı adet dişi Albino Wistar sıçanlardan oluşturuldu. Sıçanların karaciğerlerinde herhangi bir patolojik değişiklik olup olmadığını gözlemlemek için mikroskobik inceleme yapıldı.

Sonuç ve Tartışma: *Su ekstraktı, 1 mg/ml derişimde en yüksek radikal giderme aktivitesini (%48.65), indirgeme gücünü (700 nm'de 0.291 absorbans) ve metal iyonu kelatlamayı (%35.40) gösterdi. Histopatolojik çalışmalar özellikle su ekstraktının CCl_4 kaynaklı intoksikasyonun şiddetini azalttığını göstermiştir.*

Anahtar Kelimeler: *Antioxidan aktivite, DPPH, Hippophae rhamnoides L., indirgeme kuvveti, rat karaciğer toksisitesi*

INTRODUCTION

The use of plants for therapeutic purposes dates back to the earliest civilizations. About 5-10% of the 250.000 flowering plants grown worldwide have been studied for their active ingredients [1]. Some natural chemicals found in plants are gaining more attention and increasing demand for antioxidants that are not harmful to the human organism, as these plants have been consumed by humans and animals since ancient times [2]. *Hippophae rhamnoides* L. (HR) is a plant species belonging to the Elaeagnaceae family. HR contain more than 200 bioactive constituents, many vitamins, sterols, carotenoids, flavonoids, tocopherols, phenolics, lipids, citric acid, ascorbic acid, and more than 15 microelements (including Mn, Fe, B, F, Al, Ti, K, and so on) [3]. These biological compounds, particularly tocopherols and tocotrienols, would have an extensive range of biological activities, such as antioxidants [4,5]. Bioactive compounds in plants exert antioxidant activity through various mechanisms such as chain initiation prevention, transition metal chelation, peroxide decomposition, reducing capacity, and radical scavenging [6]. The active oxygen species hydroxyl radical ($HO^{\bullet}$), superoxide ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) are by-products of normal metabolism. These reactive oxygen species are known to have a detrimental effect. DNA damage is one of the most important factors in cancer formation [7]. Peroxidation of lipids is associated with diabetes, cancer, cardiovascular disease and aging as well as many degenerative disorders [8-11]. In conclusion, antioxidants are essential blockers of lipid peroxidation both in foods and in living cells. They are regarded as an important protection against oxidative damage. Synthetic antioxidants such as propyl gallate (PG), tertiary-butyl hydroquinone (TBHQ), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used in the food industry to prevent oxidation. They are cheaper and more effective. However, synthetic antioxidants such as BHA and BHT is considered to be carcinogenic for use in the protection of these nutrients is limited [12]. Therefore, especially in recent years, the use of plant sources of antioxidants has increased greatly [13]. These natural antioxidants have started to take an important place in scientific research [14]. Some herbs are known to have the ability to synthesize the well-known antioxidant compounds α-tocopherol (vitamin E), ascorbic acid (vitamin C) and carotenoids. A number of lipophilic compounds, including vitamin E, free fatty acids and retinol (vitamin A), have been shown to contribute to antioxidant protection [15]. These natural antioxidants can further contribute to human health by protecting DNA, proteins and membrane lipids from oxidizing damage in biological systems and preventing disease [16]. However, a very small amount of natural flora plants have been studied in terms of antioxidant determination. In this research it has been aimed to determine the antioxidant properties of the extracts of *Hippophae rhamnoides* L. (HR) plant in various solvent systems and the effects on liver toxicity in rats.

MATERIAL AND METHOD

Plant Material

The fruits of HR were collected from Tortum region (Eastern Anatolia sub. 750 m). The plant was described by Botanical Institutes, Ataturk University, Erzurum, Turkey. After the fruit parts of the plants were dried in a room environment, they were ground in a blender. Then the soxhlet apparatus was set up for extraction with diethyl ether and the extraction continued in the water bath until it became colorless. The plant residue remaining after extraction with ether was extracted with ethanol at 37ºC 3

times for 9 hours in a shaking water bath. Then, the remaining plant residue from ethanol extraction was extracted with distilled water at 37ºC 3 times for 9 hours in a shaking water bath. The resulting ether and ethanol extracts were filtered and the solvent was evaporated in the evaporator until thickening, dried in a nitrogen atmosphere and the residue was stored in a vacuum desiccator. After the water extracts were filtered, they were dried in a lyophilizer under 5μm-Hg pressure at -50ºC and stored in the freezer part of the refrigerator.

Animal Groups

The experiments were performed in accordance with the ethical guidelines approved by the Ethics Committee of the Laboratory Animal Facility (No. B.30.2.ATA.0.23.85-126-10-74). This study used a total of 30 Albino Wistar rats 12 weeks old. For the animal number calculation, a power analysis was performed using the G-Power program (v.3.1.9.7). In the power analysis of the study, it was calculated that a total of at least 10 experimental animals for 5 groups should be studied with a 5% margin of error (α err probe) and 80% power (1-β err probe). The number of animals for experiment in the groups was planned to be 6. Study of Abdel-Moneim et al. was used as a reference for the analysis [17]. Animals were provided from Medicinal and Experimental Application and Research Centre, Erzurum, Turkey (ATADEM). The animals were kept under standard laboratory conditions. They were given a regular cycle of 12 hours light and 12 hours dark at 24°C. Water and standard chow were provided to the animals. The experimental animals were Albino Wistar rats, all of which were female and their average weight was 250 g. CCl_4 was administered intraperitoneally to the animals in whom liver toxicity would be established, as 0.6 ml/kg subject [18]. Plant extracts HR was given orally as 500 μg/kg subjects. The HR extract doses to be applied were within the dose range determined according to the method of Ting et al. [19].On the first day, plant extracts or provender were given to the animals according to their diet. On the 2nd day, CCl_4 was given to the groups and 30 minutes later, plant extracts or provender were given according to their diet. On the 3rd day, excitation was performed with anesthesia under carbon monoxide (CO) gas. For pathological evaluation after excitation, tissue samples were taken in 10% formalin and transferred to Atatürk University Medical Faculty Pathology Laboratory. Liver tissues were collected and stored at -80°C until the assay was performed. In addition, blood samples were taken to be given to the Biochemistry Laboratory of Atatürk University Research Hospital for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination. Grouping of experimental animals is given below.

Group 1 was given a normal diet and served as a control.
Group 2 was given CCl_4 and served as a CCl_4 control.
Group 3 was given 0.5 mg/kg HR ether extract orally for 2 days and on the 2nd day CCl_4 was applied.
Group 4 was given 0.5 mg/kg HR ethanol extract orally for 2 days and on the 2nd day CCl_4 was applied.
Group 5 was given 0.5 mg/kg HR water extract orally for 2 days and on the 2nd day CCl_4 was applied.

Determination of Total Phenolics

Total phenolic content quantification in the extracts was carried out by Folin-Ciocalteu Reagent (FCR) [20]. 200 μl of sample (extract) solution was taken and the volume was completed to 500 μl with 0.3% HCl. 200 μl was taken from the mixture and added to 4 ml of 2% Na_2CO_3 solution. After 2 minutes, 200 μl FCR was added and after 30 minutes, the absorbance was measured at 750 nm. 2% Na_2CO_3 solution was used as a blank. The results are given as the equivalent amount of gallic acid per extract (EAG/g extract).

DPPH Radical Scavenging Activity

Free radical scavenging activities of HR plant extracts, α-tocopherol, BHA and ascorbic acid were performed using DPPH [21]. DPPH ethanol solution at a concentration of 100 μg/ml was prepared. In the method study, after taking 1 ml of plant extract solution and putting it in a test tube, 3 ml DPPH solution was added on it and kept in the dark for 30 minutes. The mixture was vortexed and its absorbance was read against ethanol at a wavelength of 517 nm. The antioxidant-free solution was used

as a control. The lower absorbance indicates the higher radical scavenging ability of the extracts. The formula shown below was used to compute the percent of radical scavenging activity.

% Radical Scavenging Activity: [(A_0-A_1/A_0) x 100]
A_1: Absorbans of mixture.
A_0: Absorbans of DPPH Solution.

Determination of Reducing Power

The extracts' reducing power was determined employing the Oyaizu method [22]. 1 ml of sample was placed in the test tube, and 2 ml of 0.2 mM phosphate buffer (pH 6.6) and 2 ml of potassium ferri cyanide [1% $K_3Fe(CN)_6$] were added and vortexed. For 20 minutes, the mixture was maintained in a 50 °C water bath. 2 ml of a 10% solution of trichloroacetic acid (TCA) was added, and the mixture was then centrifuged for 10 minutes at 2500 rpm. At the end of the centrifuge, 2 ml was taken from the clear part and 2 ml of pure water was added. Soon after waiting for 10 minutes, the absorbance of the mixture at 700 nm was measured by adding 0.5 ml $FeCl_3$ (0.1%). An increase in absorbance indicates an increased reducing power. Vitamin E and vitamin A were used as a control.

Ferrous Chelating Activity

Fe^{2+} chelating activity of the extracts was performed according to the method of Dinis et al. [23]. 10 mg/ml stock solution of the extracts was prepared in ethanol. Working solutions were prepared from this solution. 1 ml of each working solution was taken and 3.7 ml of ethanol was added on it. Then, 0.1 ml $FeCl_2$ (2mM) and 0.2 ml 5mM ferrosine were added, respectively. After 10 minutes, each sample's absorbance was compared to a $FeCl_2$ and ferrosine-free blank at 562 nm. The absorbance of each sample was measured against the $FeCl_2$ and ferrosine-free blank at 562 nm after 10 minutes. Ethylenediaminetetraacetic acid (EDTA) solution was used as reference. % Metal ion chelation was calculated by the formula below.

Chelating %: (A_0-A_1 / A_0) x100
A1: Absorbance value of the sample; A0: Absorbance value of the control

Histopathological Studies

Normal saline (physiologic saline 0.9%) was used to wash the excised liver tissue pieces. These sections were then reserved for histopathological studies. The tissues were fixed in buffered neutral formalin (%10). Then dehydrated in gradual ethanol, cleared in xylene and embedded in paraffin. Hematoxylin and eosin (H-E) stains were used to stain the prepared sections. Histopathology changes such as mononuclear cell infiltration, necrotic area, hemorrhage, dilation of sinusoids, hepatocellular degeneration and vascular congestion were studied microscopically in the sections [24].

ALT (alanine amino transaminase) and AST (aspartate amino transaminase) Analyzes of Experimental Groups

Blood samples taken from experimental animal groups were delivered to Atatürk University Research Hospital biochemistry laboratory. Results were expressed in International Units per liter (IU/l).

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Where appropriate, the differences between the groups were evaluated using the Student t test an alpha level of 0.05. Pearson correlation coefficients and p-values were used for correlations and their significance. G-Power software (v3.1.9.7) was used for Power Analysis.

RESULT AND DISCUSSION

Determination of Total Phenolics

In the determination of phenolic compounds, the results are given as equivalent amount of gallic acid. For this, the concentration-absorbance graph of the standard gallic acid was used (Figure 1). The

order of phenolic compound amount of HR plant extracts was 62.43 mg for ether extracts, 41.94 mg for water extracts and 18.94 mg for ethanol extracts (Table 1). Phenolic compounds are secondary metabolites commonly found in plants [25]. The factors caused by phenolic compounds are antioxidant properties, nutritional values and colors of foods. Phenolic compounds in plants exhibit antioxidant activity, protecting cells from free radical oxidative damage [26]. Phenolic compounds are also known to have antimutagenic, antitumor and antibacterial properties [27]. Previous studies have shown that polyphenolic compounds are compatible with antioxidant activity and have an important role in stabilizing the oxidation of lipids [28]. In the studies of Varshneya et al. [29], Phenolic compounds in extracts of HR pulp were determined as mg gallic acid equivalent, and these values for methanol, AME (70:30 water:methanol, v/h) and water extracts were 78.12, 84.28 and 19.96 mg EAG/g extract, respectively. In our study, it was determined that the water extract value of this plant was higher (41.94) and the ethanol extract value was lower (18.94).

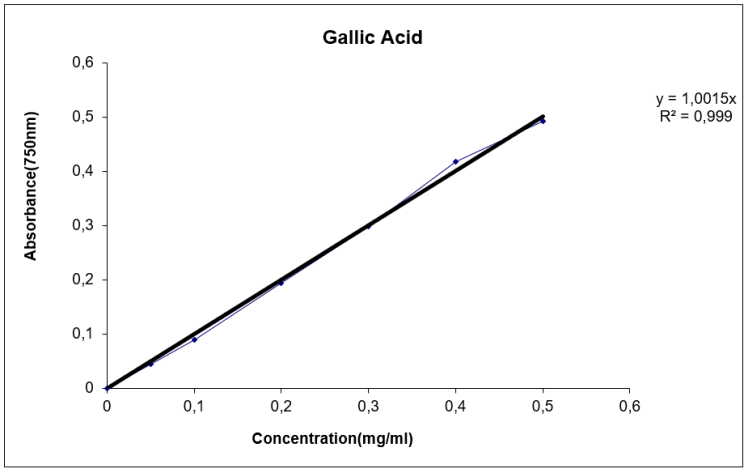

Figure 1. Standard curve of gallic acid

Table 1. Phenolic compound amounts of HR extracts as equivalent gallic acid

Plant Extract	HR Ether Extract	HR Ethanol Extract	HR Water Extract
Total Phenolics (mg EAG/g extract)	62.73±0.927	18.94±0.412	41.94±0.854

*Values were given as the mean ± SD (n=6)

DPPH Radical Scavenging Activity

Ether, ethanol and water extracts of *Hippophae rhamnoides* L. (HR) were utilized at concentrations ranging from 100 to 1000 g/ml. The results were presented in Table 2. The highest value was HR water extract of the plant (51.42%). BHA, ascorbic acid and α-tocopherol produced radical scavenging activities of 95.19%, 93.81% and 83.43%, respectively, at 100 µg/ml concentration.

DPPH radical scavenging activity was found in a concentration-dependent manner in all extracts ($p<0.05$). Finally, the % DPPH radical scavenging activity of the different extracts was calculated and presented in Table 2. The DPPH scavenging method applied in our study is very common among spectrophotometric methods used to determine the antioxidant capacity of extracts and pure compounds. This method is fast, sample analysis requires little time and does not require expensive reagents and devices. DPPH, which is a resistant free radical, takes electron or hydrogen radical from the reaction medium and turns into a durable diamagnetic molecule. For this reason, the DPPH radical is used as a substrate to determine the antioxidative properties of antioxidants. This durable chromogen free radical has high sensitivity [30]. DPPH method is a modern method that allows analysis of samples with different characteristics. A large number of pure compounds, juices, wines, tea and other extracts were

analyzed by applying this chromogen radical [31,32]. DPPH method is a modern method that allows analysis of samples with different characteristics. A large number of pure compounds, juices, wines, tea and other extracts were analyzed by applying this chromogen radical. With a DPPH concentration of 0.0004%, the IC_{50} value, which is the concentration that inhibits the DPPH radical by 50%, was determined to be 70.91 µg/ml in the study of Shivapriya et al. [33]. DPPH concentration in this study is 25 times lower than in our study. In another DPPH (0.2mM) scavenging activity study conducted by Yogendra Kumar et al. [34]. In HR leaf extracts, an activity of 47.25% was found at 0.2 mg/ml extract concentration. This value is higher than our study values (23.5-28.7%). In the studies of Varshneya et al. [29], the IC_{50} value of DPPH (0.1mM) radical removal of the water-methanol extract of the plant was determined as 143.33 µg/ml. The DPPH concentration in this study is 2/5 of that in our study. However, in our study, the IC_{50} value of the water extract of the plant was calculated by linear regression analysis and was found to be 872.56 µg/ml. Total phenolic content and DPPH radical scavenging activity were found in this increasing order, HR ethanol < HR water extract. This finding suggested that phenolic content and the ability to scavenge DPPH radicals may be closely related.

Determination of Reducing Power

The Oyaizu method was used to determine the reducing power of HR extracts [22]. The amounts of the extracts were taken in the concentration range of 100-1000 µg/ml. In our study, it was determined that all extracts have a certain reducing force activity. Results are given in Table 2. The highest activity is HR plant was detected in water extract. Retinol and α-tocopherol showed a reducing power of 0.619 and 0.245 respectively, at 100 µg/ml concentration.

Table 2. Radical scavenging activity (%) and reducing power of HR extracts

	Radical Scavenging Activity (%)			**Reducing Power (Absorbance at 700 nm)**		
Simples Concentrations	**Ether exctract**	**Ethanol exctract**	**Water exctract**	**Ether exctract**	**Ethanol exctract**	**Water exctract**
100 µg/ml	21.85±0.29	25.21±0.51	27.14±0.42	0.021±0.001	0.006±0.001	0.093±0.001
200 µg/ml	23.51±0.33	25.46±0.43	28.73±0.31	0.026±0.001	0.058±0.001	0.102±0.002
400 µg/ml	25.32±0.28	27.63±0.56	35.25±0.44	0.070±0.001	0.069±0.001	0.146±0.003
600 µg/ml	26.46±0.41	30.09±0.60	42.11±0.75	0.104±0.002	0.085±0.001	0.195±0.004
800 µg/ml	28.64±0.51	30.54±0.49	45.37±0.51	0.122±0.002	0.097±0.002	0.244±0.003
1000 µg/ml	28.39±0.42	30.61±0.59	48.65±0.94	0.154±0.002	0.109±0.002	0.291±0.005

*Values were expressed as the mean ± SD (n=6)

The reducing power of plant extracts was determined by evaluating the conversion of Fe^{3+} to Fe^{2+} using the Oyaizu method [22]. The reducing power values exhibited by the compounds are a measure of their electron donation ability [35,36]. It is known that polyphenolic compounds of plant origin have good electron and hydrogen atom donating properties. For this reason, polyphenols in plant extracts have the ability to terminate or slow down chain reactions with free radical mechanisms. Yen and Duh showed that there is a consistent correlation between the reducing forces and antioxidant activity values of methanol extracts containing yen and duh polyphenols [28]. On the other hand, Gordon stated that reductants show antioxidant activity by giving hydrogen atoms to the free radical chain [37]. The values of the reducing forces of HR extracts, ascorbic acid and tocopherol determined depending on the concentration are given in Table 2. The reducing power of the extracts at a concentration of 1 mg/ml are as follows: HR water extract (0.291)> HR ether extract (0.154)> HR ethanol extract (0.109) ($p<0.05$). Retinol and tocopherol showed reducing power of 0.619 and 0.245 respectively, at a concentration of 0.1 mg/ml. It was observed that HR water extracts showed higher reducing power at 1 mg/ml. The reduction force results of the supercritical carbon dioxide extracts of Hung et al. [19]. HR plants were measured at 700 nm in the 0.42-8.32 mg/ml concentration range and the absorbance values were recorded. The absorbance value is 0.11 at 0.42 mg/ml concentration. In our study, the absorbance value (0.146) of HR water extract at a concentration of 0.4 mg/ml is higher. For this reason, it can be said that these extracts tested have the ability to donate electrons, interact with free radicals and turn

them into more stable products, and have the ability to terminate the radical chain reaction. In addition, since the reducing power depends on the ability to deliver protons, it can be thought that there is a relationship between radical scavenging activity and reducing power. In this study, it is seen that there is a strong relationship between reducing power and radical scavenging activity (r>0.952).

Ferrous Chelating Activity

Fe^{2+} ions chelating activity of ether, ethanol and water extracts of the HR plant was performed using to the method of Dinis et al. [23]. Ferrous chelating ability of the extracts were investigated depending on the concentration. The ethanol extract demonstrated the highest ion chelating activity (56.68%) among HR plant extracts. Results are given in Table 3 and Figure 2. The Ferrous chelating activity of EDTA at 5 mg/ml concentration was 99.8%.

Table 3. Fe^{2+} Ions Chelating Activity (%) of HR extracts

Simples/Concentrations	HR Ether exctract	HR Ethanol exctract	HR Water exctract
1 mg/ml	6.53±0.12	13.56±0.29	35.40±0.61
2 mg/ml	14.07±0.18	16.58±0.36	37.88±0.74
3 mg/ml	17.28±0.24	33.86±0.37	38.13±0.55
4 mg/ml	19.19±0.35	37.48±0.59	40.58±0.60
5 mg/ml	22.31±0.49	56.68±0.86	42.81±0.81

*Values were expressed as the mean ± SD (n=6)

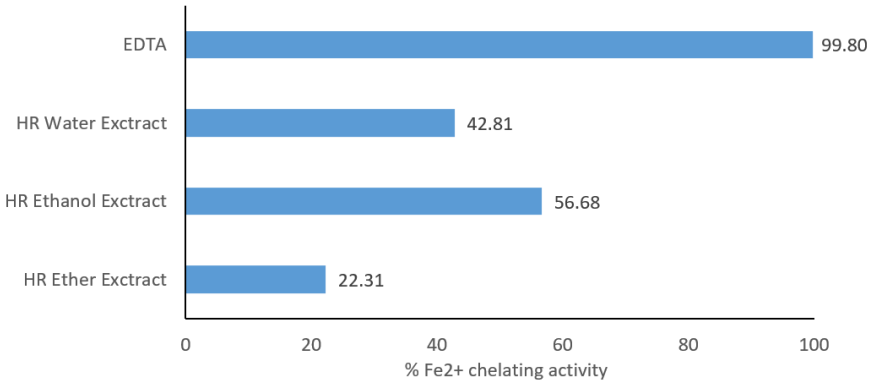

Figure 2. HR extracts and EDTA Ferrous chelating abilities at 5 mg/ml concentration

The chelating activity of metal ions is an important factor in antioxidant effect. Because Fe^{+2} ions are the most effective prooxidants [38]. Polyphenols can chelate metal ions with prooxidant properties such as iron and thus prevent the formation of free radicals caused by prooxidants [39]. Iron ions can increase lipid peroxidation by converting lipid peroxides to peroxyl and alkoxyl radicals via the Fenton process and can promote lipid peroxidation. By accepting hydrogen, these radicals can initiate lipid peroxidation chain reactions [40]. The metal ion chelating activity of the extracts is very important as they enable the capture of transition metals acting as catalysts in lipid peroxidation. It is known that even traces of metal ions accelerate the oxidation process. Therefore, compounds showing low chelating effect also have an importance in the formation of antioxidant activity [41]. It was determined that ether, ethanol and water extracts of HR plant at concentrations of 1 and 5 mg/mL showed 6.53-22.31%, 13.56-56.68% and 35.40-42.81% chelating effect, respectively. EDTA was used as a standard metal chelator in our study. At a concentration of 1 mg/ml, EDTA showed 99.5% activity. The metal chelating effect of HR extracts and EDTA decreased in the order: EDTA >HR ethanol extract> HR water extract > HR ether extract. Among HR extracts HR ethanol extract resulted the most active one (56.68%). Hung et al. found the metal ion chelation activity of the core parts of the HR plant as 7.74-38.50%, respectively, at concentrations of 0.92-18.3 mg/ml [19]. In our study, all values except HR ether extract were higher

(13.56-56.68%). The scavenging properties of phenolic compounds against radicals and reactive oxygen species such as singlet oxygen, hydroxyl radicals and free superoxide radicals make them highly effective antioxidants. [42]. A positive correlation between metal ion chelation activity with radical scavenging activity and was found, as indicated by the coefficient of determination (r=0.991). A positive correlation was found between metal ion chelation activity with radical scavenging activity and reducing power. The coefficients of correlation (r) were 0.991 and 0.889, respectively.

Histopathological Studies

Histopathology revealed liver damage in control and CCl_4-treated rats. Photomicrographs of hematoxylin-eosin-stained liver tissues are shown in Figure 3 a-f. The livers were cut into sections and stained with hematoxylin-eosin using standard techniques (200X). Histopathologic examinations such as hepatocellular degeneration, hemorrhage, mononuclear cell infiltration, vascular congestion, dilation of sinusoids, and necrotic area were recorded and scored in Table 4.

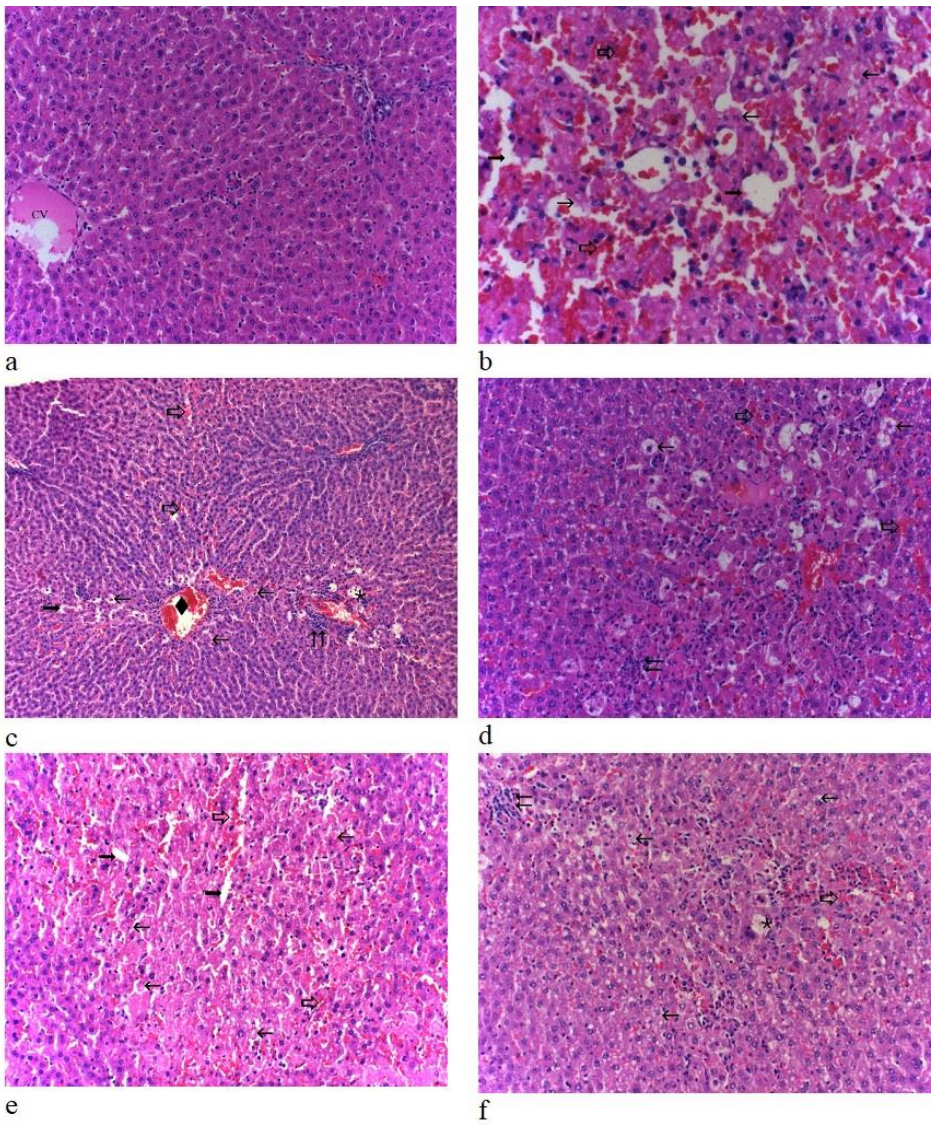

Figure 3. Effect of HR extracts on rat liver toxicity with CCl_4. (a) Control; (b-c) CCl_4 control; (d) HR Ether extract (0.5 mg/kg) + CCl_4; (e) HR Ethanol extract (0.5 mg/kg) + CCl_4; (f) HR Water extract (0.5 mg/kg) + CCl_4. Photomicrographs showing central vein (cv), hemorrhage(⇨), mononuclear cell infiltration (⇆), vascular congestion(♦), dilation of sinusoids (➞), hepatocellular degeneration (←) and necrotic area (✶)

Table 4. Histopathologic changes grading in liver sections of HR extract treated rats

Groups	Mononuclear cell infiltration	Hemorrhage	Hepatocellular degeneration	Dilation of sinusoids	Vascular congestion	Necrotic area
Control	-	-	-	-	-	-
CCl_4 Control	++	+++	+++	+++	+++	++
HR Ether Extract + CCl_4	+	++	++	++	++	++
HR Ethanol Extract + CCl_4	++	+++	++	+++	++	++
HR Water Extract + CCl_4	+	+	+	+	+	+

*Features were rated in these categories: severe (+++), moderate (++), mild (+) and none

Liver damage of CCl_4 treated rats were revealed by histopathological examinations. Photomicrographs of liver tissues stained with hematoxylin-eosin are shown in Figure 3. In the control group (Group 1), hepatocytes had normal architecture. Severe infiltration, hemorrhage, hepatocellular degeneration, dilation of sinusoids, vascular congestion and necrotic area were found in rats (Group 2) 24 hours after CCl_4 administration (Figure 3b-c). According to these results, it was determined that CCl_4-induced liver toxicity was reduced to a certain extent in rats fed with all HR extracts. Specially pretreatment of 0.5 mg/kg body weight of HR water extract reduced the severity of liver intoxication caused by CCl_4 (Figure 3f). These results clearly demonstrate the protection provided by HR water extract. It was observed that rats treated with HR water extract were less affected by CCl_4-induced toxicity.

ALT and AST Analyzes of Experimental Groups

Blood samples taken from animal groups were given to Ataturk University Research Hospital Biochemistry Laboratory for ALT and AST determination from serum. The results are presented in IU/l in Table 5.

Table 5. Effects of diets containing HR extracts on alanine amino transaminase (ALT) and aspartate amino transaminase (AST)

Item	ALT [a] (IU/l)	AST [b] (IU/l)
Group 1	45.12 ± 7.94	163.14±8.45
Group 2	516.31±24.14	2858.26±156.17
Group 3	936.72±48.36	2316.35±120.51
Group 4	841.77±39.61	3849.68±189.56
Group 5	318.48±17.97	1002.39±64.88

*Values were expressed as the mean ± SD (n=6)
[a] $p<0.05$
[b] $p<0.05$

Serum AST and ALT activities were evaluated to detect liver damage. ALT and AST values are very high in all animal groups treated with CCl_4 except the positive control group. There was a significant difference between the CCl_4 control group and the serum samples taken from the groups fed with HR extracts and administered CCl_4 ($p<0.05$).

To sum up, the data of the present study submits that extracts of HR have antioxidants. Our findings indicate that the hepatoprotective effects of HR water extracts can be attributed to the fact that they contain several components with potentially healthy biological properties, such as unsaturated fat, α-tocopherol, retinol, and carotene. Therefore, HR water extract may be effective as a hepatoprotective substance against chemically induced hepatotoxicity *in vivo*.

AUTHOR CONTRIBUTIONS

Concept: M.C., Y.K.; Design: M.C., Y.K.; Control: M.C., Y.K.; Sources: Y.K.; Materials: M.C.,

Y.K., E.C.; Data Collection and/or Processing: M.C., E.C., M.A.; Analysis and/or Interpretation: M.C., E.C., M.A.; Literature Review: M.C., Y.K.; Manuscript Writing: M.C.; Critical Review: M.C., Y.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The experiments were performed in accordance with the ethical guidelines approved by the Atatürk University Ethics Committee of the Laboratory Animal Facility (No. B.30.2.ATA.0.23.85-126-10-74).

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