ANTIOXIDANT PROPERTIES OF *Rubus discolor* L. EXTRACTS AND PROTECTIVE EFFECTS OF ITS FLOWER EXTRACT AGAINST HYDROGEN PEROXIDE-INDUCED OXIDATIVE STRESS IN WISTAR RATS

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Abstract: In the present study, *in vitro* antioxidant activities of ethanol and water extracts of *Rubus discolor* L. leaves, flower, unripe and ripe fruits were investigated. Additionally, the antioxidant and protective properties of *R. discolor* flower (RD) water extract on some important biochemical parameters against hydrogen peroxide (HP)-induced oxidative stress in rats were determined. The phytochemical profiles and antioxidant properties (scavenging against $DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2, O_2^-, metal chelating, inhibition of lipid peroxidation and reducing power) of extracts were determined by spectrophotometric methods and HPLC. The antioxidant potential of RD flower extract supplementations were evaluated by measuring total protein, glutathione (GSH), malondialdehyde (MDA), vitamin A and E, cholesterol, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) levels in various tissues. The flower extracts were the most active extracts for *in vitro* antioxidant assays. In HP administered groups, MDA level was increased, GSH and total protein levels were decreased. In RD treated group, these substances levels were protected. In RD plus HP treated group, SOD and GSH-Px levels were increased. These results show that RD flower extract have the protective effects against H_2O_2 via its free radical scavenging activity, and this extract affected the level of antioxidant enzymes, vitamin A and E, cholesterol, GSH, total protein and MDA.

Keywords: *Rubus discolor*, MDA, GSH, SOD, hydrogen peroxide, cholesterol.

Rubus discolor L. EKSTRELERİNİN ANTİOKSİDAN ÖZELLİKLERİ VE ÇİÇEK EKSTRESİNİN HİDROJEN PEROKSİT-NEDENLİ OKSİDATİF STRES OLUŞTURULMUŞ WISTAR SIÇANLARDA KORUYUCU ETKİLERİ

Özet: Sunulan çalışmada, *Rubus discolor* L. yaprak, çiçek, ham meyve ve olgun meyvelerinin etanol ve sulu ekstrelerinin *in vitro* antioksidan aktiviteleri incelenmiş ve *R. discolor* çiçek su (RD) ekstresinin hidrojen peroksit (HP)-nedenli oksidatif stres oluşturulmuş sıçanlarda bazı önemli biyokimyasal parametreler üzerindeki antioksidan ve koruyucu etkileri saptanmıştır. Ekstrelerin fitokimyasal profili ve antioksidan özellikleri ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, H_2O_2, O_2^- yok edici, metal şelatlama, lipit peroksidasyonun inhibisyonu ve indirgeme kuvveti aktiviteleri)

spektrofotometrik metotlar ve HPLC cihazıyla belirlendi. RD çiçek su ekstresi uygulamasının antioksidan potansiyeli çeşitli dokularda total protein, glutatyon (GSH), malondialdehit (MDA), A ve E vitaminleri, kolesterol, glutatyon peroksidaz (GSH-Px), süperoksit dismutaz (SOD) seviyelerinin ölçülmesiyle değerlendirildi. *In vitro* antioksidan testlerde çiçek ekstrelerinin en aktif ekstreler olduğu belirlendi. HP uygulanan gruplarda, MDA seviyesi artmışken, GSH ve total protein seviyeleri azalmıştır. RD uygulanan grupta ise bu maddelerin seviyeleri korunmuştur. RD + HP uygulanan grupta SOD ve GSH-Px seviyeleri artmıştır. Bu sonuçlar göstermiştir ki RD çiçek ekstresi H_2O_2'e karşı serbest radikal yok etme aktivitesi sayesinde koruyucu etkilere sahiptir ve bu ekstre antioksidan enzimlerle, vitamin A ve E, kolesterol, GSH, total protein ve MDA seviyelerini etkilemiştir.

Anahtar Kelimeler: *Rubus discolor*, MDA, GSH, SOD, hidrojen peroksit, kolesterol.

INTRODUCTION

Rubus discolor L., is a plant belonging to the Rosaceae family, genus *Rubus*. The geographical distribution of *Rubus* covers a wide range from Europe to northern Asia and most temperate areas. *Rubus* species have been cultivated for centuries. *Rubus* species and their fruits have been used traditionally for therapeutic purposes in many countries to treat wounds, colic pain and some other diseases such as diarrhea and renal disease as well as nutritional purposes (1-3). They are characterized by their capability of synthesizing and accumulating ellagitannins, a major class of phenolic compounds largely responsible for the astringent and antioxidant properties of raspberries and blackberries (4,5). They have found to metabolize several phenolic carboxylic acids, such as ellagic acid, and phenyl propenoids, particularly caffeic acid (6,7).

The reactive oxygen species (ROS) play a major role in either the initiation or progression of carcinogenesis by inducing oxidative stress. Peroxides and superoxide anion produce cytotoxicity/genotoxicity in cellular system (8-10). ROS and nitrogen species are formed in the human body, and endogenous antioxidant defenses are not always sufficient to counteract them completely. A large number of studies have been supported the oxidative stress caused the DNA, lipid and protein damages, therefore this fact may contribute to the development of cardiovascular disease, cancer and neurodegenerative diseases (11,12). Diet-derived antioxidants may play an important role to prevent the chronic diseases (13). Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells (14), and it together with superoxide radical anion can damage many cellular components (15). Recently, the hydrogen peroxide was viewed mainly as a toxic cellular metabolite. The generation of hydrogen peroxide is increased in response to the various stresses, implicating it as a key factor mediating the phenomena of acclimation and cross-tolerance, in which previous exposure to one stress can induce tolerance of subsequent exposure to the same or different stresses (16-19).

In this paper, we evaluated the antioxidant and radical scavenging activities of *R. discolor* flower, leaves, unripe and ripe fruit water and ethanol extracts by several different *in vitro* antioxidant test systems, such as the DPPH, ABTS and superoxide radical scavenging, inhibition of lipid peroxidation, H_2O_2 scavenging, the reducing power and the metal chelating activity assays. We also investigated the possible protective effects of the *R. discolor* flower water extract against H_2O_2-induced oxidative damage in Wistar rats. To achieve this aim, rats were given *R. discolor* flower water extract by oral gavage for ten weeks, then malondialdehyde (MDA), glutathione (GSH) levels, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activities, retinol, tocopherol, and cholesterol levels in their serum, liver, kidney, muscle, heart, brain, lung and spleen tissues were assessed.

EXPERIMENTAL

Chemicals and Standards

All chemical compounds and standards were obtained from Sigma-Aldrich (Germany).

Plant Materials and Extraction Procedures

Rubus discolor L. flowers, leaves, unripe fruits and ripe fruits were collected from Bingol/Turkey in spring and summer of 2011. Voucher specimen number is Turkoglu 4820. All samples were dried in air and at dark. For extraction, 25 g of sample was mixed with 100 mL solvent (water and ethanol). Extraction continued until the extraction solvents became colorless. The obtained extracts were filtered and the filtrate was collected, and then solvent was removed. All extraction processes were repeated three times. The dried real extract and standard antioxidants were dissolved at µg/mL (100-1000) concentration (20).

In vitro Studies

Determination of Antioxidant Properties and Total Phenolic Compounds of R. discolor Extracts

The spectrophotometric analysis of 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical scavenging capacity was determined according to the method of Re et al (21). $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM $K_2S_2O_8$, and it was stored for 2 h at room temperature in the dark. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH=7.4). Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of extracts at 100 µg/mL concentrations. After 0.5 h, absorbance was recorded at 734 nm. The extent of decolourization was calculated as percentage reduction of absorbance.

The 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\bullet}$) scavenging capacity was measured by using the method of Shimada et al (22). Briefly, 0.1 mM solution of $DPPH^{\bullet}$ in ethanol was prepared and 1 mL of this solution was added to 3 mL of extracts solution at 100 µg/mL concentration. Absorbance at 517 nm was measured after 0.5 h against a blank solution containing the ethanol. Lower absorbance of the reaction mixture indicates the higher DPPH radical scavenging activity.

The measurements of superoxide anion scavenging capacity were based on the method described by Liu et al (23) with slight modification. One milliliter of nitroblue tetrazolium (NBT) solution (156 mmol/L NBT in 100 mmol/L phosphate buffer, pH=7.4), 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 mmol/L in 100 mmol/L phosphate buffer, pH=7.4) and 100 µL of sample solution of extracts were mixed. The reaction was started by adding 100 µL of phenazine methosulphate (PMS) solution (60 mmol/L PMS in 100 mmol/L phosphate buffer, pH=7.4) to the mixture. The mixture was incubated at 25 ºC for 5 min and the absorbance was measured at 560 nm. Decreased absorbance of the reaction mixture shows the increase in superoxide anion scavenging capacity.

The chelating of ferrous was estimated by the method of Dinis et al (24). Briefly, 100 µg/mL extracts were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was started by addition of 5 mM ferrozine (0.2 mL). The mixture was shaken vigorously and then it was kept at room temperature for 10 min. Absorbance of the solution was measured at 562 nm.

The reducing power activities were determined by the method of Oyaizu (25). Briefly, 100 µg/mL of extract in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH=6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50 ºC for 20 min. Trichloroacetic acid (TCA) (2.5 mL, 10%) was added to the mixture, and then

centrifuged for 10 min at 1000×g. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm.
The hydrogen peroxide scavenging activity of extracts was determined according to the method of Ruch et al (26). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH=7.4). Extracts in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40 mM). Absorbance of extracts was measured 10 min later against a blank solution containing the phosphate buffer without hydrogen peroxide at 230 nm.
Inhibition of lipid peroxidation was determined according to the ferric thiocyanate method in linoleic acid emulsion (27). With this method peroxide formation occurred during the oxidation of linoleic acid oxidation.
All test and analyses were repeated three times and average values were calculated. The antioxidant or antiradical activities of samples were estimated by the following equation:
% Scavenging Activity = $[(A_0 – A_1)/A_0] \times 100$
where A_0 is the absorbance of control, and A_1 is the absorbance of the sample in the presence of extracts or standards.

Determination of Polyphenolic Contents
Total polyphenolic contents in the extracts were determined with Folin–Ciocalteu's reagent according to the method of Slinkard and Singleton (28) using pyrocatechol and quercetin as standard phenolic compounds. Briefly, 1 mg/mL of the extract solution in a volumetric flask was diluted with distilled water (46 mL). One milliliter of Folin–Ciocalteu's reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2%) was added and then it was intermittent shaken for 2 h. The absorbance was measured at 760 nm. The total concentrations of phenolic contents in the extracts were determined as milligram of pyrocatechol and quercetin equivalents by using an equation that was obtained from standard pyrocatechol and quercetin calibration curves:
Absorbance= 0.00053 × total phenols [quercetin equivalent (mg)] + 0.00019.
Absorbance= 0.00198 × total phenols [pyrocatechol equivalent (mg)] + 0.00158.

Chromatographic Conditions for Flavonoid Analysis
Chromatographic analysis was carried out using PREVAIL C 18 reversed-phase column (150 x 4.6 mm x 5 μm) diameter particles. The mobile phase was methanol/water/acetonitrile (46/46/8, v/v/v) containing 1.0% acetic acid (29). This phase was filtered through a 0.45 μm membrane filter (millipore), then deaerated ultrasonically prior to use. Naringin, rutin, apigenin, myricetin, naringenin and quercetin were quantified by DAD following RP-HPLC separation at 280 nm for naringin and naringenin, 254 nm for rutin and myricetin, 306 nm for apigenin and 265 nm for quercetin. Flow rate and injection volume were 1.05 mL/min and 10 μL, respectively. The peaks in the chromatograms of the extracts were confirmed by comparing to their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using the external standard method. All chromatographic operations were carried out at 25 °C.

Animal Studies
Animals
Rats (Wistar albino male) 4 months of age with an average weighing 200–250 g were provided from the Experimental Animal Research Center, Firat University, and were housed in four groups, and each group contained ten rats. The animals were housed at 20 ± 2 °C in a daily light/dark cycle. All animals were fed a group wheat-soybean meal-based diet and water ad libitum in stainless cages, and received humane care according to the criteria outlined in

the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the National Academy of Science and published by the National Institutes of Health (WMA, 2000). The ethic regulations were followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments. This study was approved by The Ethic Committee of Firat University, Faculty of Medicine. The ethical approval date, certification and decision number are 26.03.2009, 20/29, respectively. These treatments were continued for ten weeks, after this process each experimental rat was decapitated and tissue samples were collected and stored in -85 °C prior to biochemical analysis (30).

Experimental Design

The rats were randomly divided into four groups each containing ten rats. It was done preliminary experiments before starting to the study for minimize possible hitches.

Group C (Control): The rats received tap water and fed with standard pellet diet as ad libitum.

Group HP (Hydrogen Peroxide): The rats were injected intraperitoneally with hydrogen peroxide at a concentration of 20 mg/kg in physiologic saline buffer two times per week and fed with standard pellet diet as ad libitum.

Group RD (*Rubus discolor*): The rats received 250 mg/kg *R. discolor* flower water extract orally by gavage four times per week and fed with standard pellet diet as ad libitum.

Group RD+HP (*Rubus discolor* + Hydrogen Peroxide): The rats were injected intraperitoneally with hydrogen peroxide at a concentration of 20 mg/kg in physiologic saline buffer two times per week and the rats received 250 mg/kg *R. discolor* flower water extract orally by gavage four times per week and fed with standard pellet diet as ad libitum.

Each experimental rat was decapitated one week after the last injection HP and the last intake RD flower extract (31).

Biochemical Analysis

MDA concentration in tissues was determined using the method described by Jain et al. (32) based on thiobarbituric acid (TBA) reactivity. GSH concentration in tissues was measured using the method described by Beutler et al (33). The total protein amount was determined using the method described by Lowry et al (34). GSH-Px activity was assayed according to Paglia and Valentine (35) based on that of GSH-Px catalyses the oxidation of GSH. SOD activity was measured at 505 nm by calculating inhibition percentage of formazan dye formation (36).

Determination of Vitamin A and E Levels in Tissue Samples

Tissue samples were homogenized in 3 mL acetonitrile/methanol/isopropyl alcohol (2:1:1, v/v/v) containing tubes and the samples were vortexed for 30 s and centrifuged at 6000×g for 10 min at 4 °C. Supernatants were transferred to autosampler vials of the HPLC instrument. For lipophylic vitamins, the mixture of acetonitrile/methanol (3:1, v/v) was used as the mobile phase and the elution was performed at a flow-rate of 1 mL/min. The temperature of column was kept at 40 °C. Supelcosil™ LC 18 DB column (250 x 4.6 mm, 5 µm; Sigma, USA) was used as the HPLC column and detection was performed at 320 nm for retinol (vitamin A), and 215 nm for α-tocopherol, α-tocopherol acetate (37). Identification of the individual vitamins was performed by frequent comparison with authentic external standard mixtures analyzed under the same conditions. Quantification was carried out by external standardization using Class VP software. The results of analysis were expressed as µg/g tissue (38).

Total Cholesterol Analysis in Tissue Samples

The tissue samples were homogenized in 3 mL acetonitrile/isopropyl alcohol (70:30, v/v)-containing tubes and the mixture were vortexed for 30 s and centrifuged at 6000×g for 10 min

at 4 °C. Supernatants were transferred to autosampler vials of the HPLC instrument. Acetonitrile-isopropyl alcohol (70:30 v/v) was used as mobile phase at a flow rate of 1 mL/min (39). Supelcosil LC 18™ DB column (250 x 4.6 mm, 5 µm) was used as the HPLC column. Detection was performed by UV at 202 nm and 40 °C column oven (40). Quantification was carried out by external standardization using Class VP software. The results were expressed as µg/g wet weight tissue (38).

Statistical Analysis

The experimental results were reported as mean ± S.D. Statistical analysis was performed using SPSS 15.0 software. Analysis of variance (ANOVA) and an LSD test were used to compare the experimental groups with the controls.

RESULTS

In vitro Studies

In this study, we firstly evaluated the *in vitro* antioxidant properties of RD leaf, flower, unripe and ripe fruits using the ABTS, DPPH, superoxide, H_2O_2 scavenging, chelating of ferrous ions, reducing activity, inhibition of lipid peroxidation, determination of total phenolic compounds and flavonoid contents. Table 1 shows ABTS, DPPH, superoxide, H_2O_2 scavenging, chelating of ferrous ions, reducing activity, inhibition of lipid peroxidation results of RD water and ethanol extracts. The most effective extracts are, flower ethanol extract for ABTS, H_2O_2 scavenging and reducing power tests; ripe fruit water extract for superoxide scavenging test; flower water extract for metal chelating and DPPH scavenging tests; unripe fruit water extract for inhibition of lipid peroxidation test.

Table 2 shows total phenolic contents of RD water and ethanol extracts as quercetin and pyrocatechol equivalents. 93.58, 152.05, 74.54, 92.75, 69.96, 72.06, 70.85 and 91.08 mg quercetin equivalent of phenols was detected in 1 g of extracts of RD flower water, flower ethanol, leaf water, leaf ethanol, unripe fruit water, unripe fruit ethanol, ripe fruit water and ripe fruit ethanol, respectively. 23.12, 26.47, 20.25, 18.72, 19.30, 18.34, 18.82 and 18.57 mg pyrocatechol equivalent of phenols was detected in 1 g of extracts of RD flower water, flower ethanol, leaf water, leaf ethanol, unripe fruit water, unripe fruit ethanol, ripe fruit water and ripe fruit ethanol, respectively.

Flavonoid contents of RD extracts shown in Table 3. Rutin, apigenin, naringin, naringenin, myricetin and quercetin were determined in the RD extracts. 856, 44, 261, 809, 41 and 2011 µg rutin, apigenin, myricetin, naringin, naringenin and total flavonoid (respectively) were detected in 1 g of extract of RD leaf. 310, 15, 2682, 2962, 22, 5 and 5996 µg rutin, apigenin, myricetin, naringin, naringenin, quercetin and total flavonoid (respectively) were detected in 1 g of extract of RD flower. 336, 8, 86, 238 and 668 µg rutin, apigenin, myricetin, naringin and total flavonoid (respectively) were detected in 1 g of extract of RD unripe fruit.

Animal Studies

Following the exposure of experimental groups; the effects of RD flower extract, hydrogen peroxide and RD plus HP, on oxidative stress and antioxidative role were evaluated as enzymes (SOD and GSH-Px), MDA content, GSH, total protein, cholesterol, vitamin A and E levels of serum, liver, kidney, heart, muscle, brain, lung and spleen tissues samples from control and treated rats (Table 4-Table 11).

Malondialdehyde (MDA) Levels

When the HP treated group was compared with the control group at the end of 10^{th} week, the MDA levels were significantly increased in kidney, spleen and lung tissues. MDA levels were

not changed muscle tissue of HP and control groups, but significantly decreased in liver and brain tissues. In the RD flower extract treated group, the MDA levels were statistically and significantly decreased in the muscle, brain and lung tissues. There is no difference in the MDA levels of liver, kidney, and spleen tissues compared to control group. When the RD flower extract plus HP treated group was compared with the control group, there is a decrease in the MDA levels of liver, kidney, brain, and lung tissues. Additionally an increase in the MDA levels of muscle and spleen tissues was observed (Table 5-Table 11).

Superoxide dismutase (SOD) Activity
In the serum, compared to the control group, there were statistically significantly increase in the SOD activity in the RD treated and RD plus HP treated groups at the end of 10^{th} week. However, there was not any difference for SOD activity in the HP treated group compared with the control group (Table 4).

Glutathione peroxidase (GSH-Px) Activity
A significant increase in GSH-Px activity was observed at the end of the 10^{th} week in the RD plus HP treated group compared with the control group, but no difference was observed in the serum samples of HP treated and RD treated groups compared with the control group (Table 4).

Triglyceride and Lipoprotein Levels
A significant decrease in triglyceride level was observed in the HP treated group compared with the control group, and a significant increase was observed in the RD plus HP treated group in the serum. There was not any difference in triglyceride level in the RD treated group. The RD plus HP treated group significantly increased in the HDL, LDL and VLDL levels in comparison to the control group. Nevertheless, the HDL and LDL levels were not changed statistically in the HP treated and RD treated groups compared to the serum samples of control group. LDL level was increased in the RD treated group, but it was not any difference in the HP treated group compared with the control group (Table 4).

Reduced Glutathione (GSH) Levels
When the HP treated group was compared with control group, there were a significant decrease in the liver, kidney, muscle, heart, brain and lung tissues, GSH levels were increased in the spleen tissue. In the RD treated group, GSH levels were increased in the kidney, heart and brain tissues, and there is no statistical difference in the liver and muscle tissues. It was decreased in the spleen and lung tissues compared to control group. When the RD plus HP treated group was compared with the control group, there was a significant decrease in the liver, muscle, brain, spleen and lung tissues, and there was a significant increase in the GSH levels of kidney and heart tissues (Table 4-Table 11).

Total Protein Levels
In the liver, kidney, muscle, heart, spleen and lung tissues, total protein levels were significantly decreased in the HP treated group in comparison to the control group. In the brain tissue, there was not any statistically difference in comparison to control group in the same group. In the RD treated group compared to the control group, total protein levels were significantly increased in the liver, heart and brain tissues, there is no statistically difference in the spleen tissue, and there were a significantly decrease in the kidney, muscle and lung tissues. In the liver and heart tissues, total protein levels did not differ in the RD plus HP treated group compared to the control group. In the kidney and brain tissues, its level was

increased, but it was decreased in the muscle, spleen and lung tissues when compared with the control group (Table 4-Table 11).

Cholesterol Levels

A significant increase was observed in the cholesterol level of the liver, heart, muscle, brain and lung tissues in the HP treated group compared with the control group. A significant decrease was determined in the kidney and spleen tissues, and also there was any statistically difference between control and HP treated group in the serum. A significant increase in the cholesterol level was observed in the liver, muscle, brain and lung tissues in the RD treated group compared with the control group. While its level was significantly decreased in the kidney and spleen tissues, there was not any statistical difference between control and RD treated group in the serum and heart tissues. A significant increase was observed in the cholesterol level of the liver, muscle, serum, brain and lung tissues in the RD plus HP treated group compared with the control group. A significant decrease was determined in the heart and spleen tissues, and any difference was not observed between control and RD plus HP treated group in the kidney tissue (Table 4-Table 11).

Vitamin A (Retinol) Levels

In comparison to the control group, there were an increase in the retinol level in the liver and heart tissues in the HP treated group at the end of the 10^{th} week. However, the retinol level was decreased in the serum and lung tissues, but its level did not show any difference in the kidney, muscle and brain tissues in the HP treated group compared with the control group. When the RD treated group was compared with the control group, there was a significant increase in the retinol level in the liver tissue. The retinol level was decreased in the serum and brain tissues, but its level was not statistically different in the kidney, heart, muscle and lung tissues when compared with the control group. Compared to the control group, the retinol level in the liver tissue in the RD plus HP treated group was significantly increased. However, the retinol level was decreased in the kidney and lung tissues, while its level was not different in the serum, heart, muscle and brain tissues compared to the control group (Table 4-Table 11).

Vitamin E (α-tocopherol and α-tocopherol acetate) Levels

The α-tocopherol level was significantly decreased in the serum and spleen tissues in the HP treated group compared to the control group. A significant increase in the α-tocopherol level was observed in the brain and lung tissues, while its level did not show any statistically difference in the liver, kidney, heart and muscle tissues in the HP treated group compared with the control group. The α-tocopherol level was significantly increased in the liver, kidney, muscle and brain tissues in the RD treated group in comparison to the control group. However, its level was significantly decreased in the serum, heart and spleen tissues, but it is not statistically different between control and the RD treated group in the lung tissue. A significant increase in the α-tocopherol level was observed in the liver, muscle and brain tissues in the RD plus HP treated group in comparison to the control group. The α-tocopherol level was decreased in the kidney, heart, spleen and lung tissues, and there was no statistically difference between control and RD plus HP treated group in the serum (Table 4-Table 11).

In comparison to the control group, there was a decrease in the α-tocopherol acetate level in the kidney, heart, muscle and spleen tissues in the HP treated group. Nevertheless, the α-tocopherol acetate level was increased in the brain tissue, but its level was not statistically different in the liver and lung tissues in the HP treated group compared with the control group. When the RD treated group was compared with the controls, there was a significantly increase in the α-tocopherol acetate level in the liver and brain tissues. On the other hand, the

α-tocopherol acetate level was decreased in the kidney, heart, spleen and lung tissues, but its level was not statistically different in the muscle tissue in the RD treated group compared to the control group. Compared with the control group, there was a significantly increase in the α-tocopherol acetate level in the brain and lung tissues in the RD plus HP treated group. The α-tocopherol acetate level was decreased in the heart and spleen tissues, but its level did not show any statistically difference in the liver, kidney and muscle tissues in the RD plus HP treated group in comparison to the control group (Table 4-Table 11).

DISCUSSION

Different antioxidant compounds may act through different mechanisms; consequently, one method alone can not be utilized to fully evaluate the antioxidant capacity of herbal extracts and does not reflect *in vitro* antioxidant capacity of pure compounds. For this reason, different *in vitro* antioxidant tests were carried out using different approaches and mechanisms.

$ABTS^{\bullet+}$ radical scavenging capacity

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and biological systems. The improved technique for the generation of $ABTS^{\bullet+}$ described here involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through reaction between ABTS and potassium persulfate (41). In our study, RD extracts showed scavenging activity on ABTS radical in the range of 24.64-97.30%. ABTS scavenging activities of RD leaf ethanol, leaf water and flower ethanol are higher than BHT and tocopherol.

Superoxide anion scavenging capacity

In this method, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the blue NBT formation (42,43). The decrease in the absorbance value at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. In the present study, the scavenging activity of superoxide anion for RD extracts was found to in the range of 28.83-90.67%. Ripe fruit water, flower water and leaf water extracts showed higher superoxide scavenging activity.

Ferrous ion chelating activity

Among the transition metals, iron is known as the most important pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals *via* the Fenton reaction. Fe^{3+} ion also produces radicals from peroxides, although the rate is ten fold less than that of Fe^{2+} ion (44). Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions (45). Chelation of the ferrous ions by *R. discolor* extracts was estimated by the ferrozine assay. Ferrozine can quantitatively form complexes with Fe^{2+}. The complex formation is inhibited and the red colour of the complex fades in the presence of chelating agents. By measuring the colour reduction, therefore, it is possible to estimate the chelating activity of the co-existing chelator (46). In this assay, the natural compound interfered with the formation of the ferrozine-Fe^{2+} complex, suggesting that it has chelating activity and captures ferrous ions before ferrozine. In this study, ferrous ion chelating of RD extracts were in the range of 6.70-54.58%. All extracts showed lower activity of metal chelating than BHA, BHT and tocopherol.

$DPPH^{\bullet}$ radical scavenging capacity

The effect of antioxidants on $DPPH^{\bullet}$ radical scavenging was presumed to be due to their hydrogen donating ability. $DPPH^{\bullet}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of $DPPH^{\bullet}$ radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. It is visually noticeable as a discolouration from purple to yellow (41). In our study, the scavenging activities of DPPH radical for RD extracts were found to in the range of 21.65-84.42%. Flower water, flower ethanol, leaf water and leaf ethanol extracts showed higher scavenging activity than BHT.

Hydrogen peroxide scavenging and reducing power capacity

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic for the cells because it may give rise to hydroxyl radical in the cells (14). Thus, the removing of H_2O_2 is very important for antioxidant defense in living organisms. In our study, the results for RD extracts in H_2O_2 scavenging and reducing power assays were in the range of 7.88-98.77% and 0.110-1.589 (absorbance), respectively. Flower ethanol extract showed higher activity than BHA and tocopherol for these assays.

Inhibition on Lipid Peroxidation

Antioxidant activity is defined as the ability of a compound to inhibit oxidative degradation, such as lipid peroxidation (47). The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which are the primary products of oxidation. In the present study, RD extracts were in the range of 90.10-95.66% for inhibition of lipid peroxidation. Activities of all extracts were higher than tocopherol for inhibition of lipid peroxidation.

Total Phenolic Compounds

Phenols are very important plant constituents because of the radical scavenging ability of their hydroxyl groups (48). In the present study, the highest phenolic compounds in the RD flower ethanol and flower water extracts were 152.05 mg/g, 93.58 mg/g quercetin, 26.47 mg/g, 23.12 mg/g pyrocatechol, respectively.

Total Flavonoid Contents

Flavonoids are plant phenolic compounds with strong antioxidant properties found in many dietary sources such as tea, onion, broccoli, apple and green beans (49). Flavonoids can prevent oxidative damage as a result of their ability to scavenger against reactive oxygen species such as hydroxyl radical and superoxide anion (50) and metal chelating (51). In our study, highest total flavonoid contents were observed in the RD flower extract. The flower extracts showed antioxidant activity at least up to the standard antioxidants or higher than these standards in all *in vitro* tests.

Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids and its occurrence in biological membranes causes impaired membrane fluidity and inactivation of a several membrane bound enzymes (52). MDA is one of the major oxidation products of peroxidized polyunsaturated fatty acids, and thus increased MDA content which is an important indicator of lipid peroxidation (53). Previous studies have shown that treating of *Rubus* sp. extracts were decreased and/or prevented MDA levels in various tissues (54-58). In our study, MDA levels were decreased or prevented in the RD treated and RD plus HP treated groups in the liver, kidney, muscle, brain, spleen and lung tissues. So, this might be due to inhibitory effect of *R. discolor* on lipid peroxidation.

SOD is the most important defense mechanism against toxic effects of ROS. It accelerates the dismutation of H_2O_2, also termed as a primary defence, as it prevents further generation of

free radicals. Catalase (CAT) helps in the removal of H_2O_2 formed during the reaction catalyzed by SOD (59). It has been reported that the increase of SOD activity can be known to serve as protective responses to eliminate reactive free radicals (60). In some studies, it has been indicated that superoxide radicals can inhibit CAT activity and the increase of H_2O_2 resulting from CAT inhibition could finally inhibit SOD activity (61). In this study, SOD activity was increased significantly in the serum of RD treated and RD plus HP treated groups. Similarly, in the previous studies, it has been reported that SOD activity was increased in various tissues by *Rubus* sp. extracts treated different organisms, such as mice, hamster, human, rat (55,57,58,62,63). The increase of SOD activity might be associated with increasing effect of RD extracts on rat serum, because, it was observed that SOD activity increased in the RD treated and RD plus HP treated groups.

Glutathione peroxidase (GSH-Px) is an antioxidant selenoenzyme and is present in the cytosol of cells or plasma. The kidney secretes the GSH-Px into plasma. The major function of this enzyme, used in the glutathione as a substrate, is to reduce soluble hydrogen peroxide and alkyl peroxidases (53,64). GSH-Px converts hydrogen peroxide into H_2O in the presence of oxidized glutathione (GSSG) (65). It has been reported that *Rubus* sp. extracts were significantly effected GSH-Px activity in various organism tissues (62,63,66). In the present study, the increase of GSH-Px activity might reflect cellular oxidative stress due to H_2O_2 exposure or increasing effect of RD extracts in the RD plus HP treated in the rat serum. Kumar et al. (67) have been reported that GSH-Px activity was significantly increased in the rat liver tissues due to H_2O_2 treatment.

High levels of free radicals or active oxygen species create oxidative stress which leads to a variety of biochemical and physiological lesions often resulting in metabolic impairment and cell death. SOD catalyzes the breakdown of O_2^- to O_2 and H_2O_2, removes singlet oxygen as well as O_2^-, prevents formation of OH^- (68), and has been implicated as an essential defense against the potential toxicity of oxygen (69). GSH-Px may be responsible for scavenging H_2O_2, catalyzing the peroxidation of reduced glutathione (GSH), and forming the oxidized disulfide form of glutathione (GSSG) as a product. Glutathione is the major low molecular weighted thiol compound in most plants and exists in both a reduced form (GSH) and an oxidized form, glutathione disulfide (GSSG). The reduced form of glutathione plays an important role in the stabilization of many enzymes. It also reacts directly with free radicals including hydroxyl radical to prevent the inactivation of enzymes by oxidation of the essential thiol group. The majority of glutathione in the cell is maintained in the reduced state (70). In this study, GSH levels were increased significantly in the liver, kidney, heart and brain tissues of RD treated and RD plus HP treated groups. Similarly, in the previous study, GSH levels were increased in the rat serum by treating of *Rubus* sp. extracts (66). The increase in the GSH levels might be associated with increasing effect on the antioxidant potential of RD extracts on rat serum. However, its level was significantly decreased in the liver, kidney, heart, muscle, brain and lung tissues of HP treated group. This increase can be attributed to toxicity and oxidized effects of H_2O_2. Kumar et al. (67) determined that GSH level was decreased in the rat liver tissue due to H_2O_2 treatment. GSH is central to the cellular antioxidant defenses and acts as an essential cofactor for antioxidant enzymes such as GSH-Px. Under oxidative stress, GSH is consumed by the GSH reductase to detoxify peroxides produced due to increase of lipid peroxidation.

In this study, total protein levels were significantly decreased in the liver, kidney, heart, muscle, spleen and lung tissues of HP treated groups. The decrease of total protein levels might be associated with toxicity and oxidized effects of H_2O_2 on the living organism.

In the present study, we have observed that the triglycerides, HDL, LDL, VLDL and cholesterol levels were increased in the RD plus HP treatment group without influencing in the HP treated and RD treated groups in the rat serum. In addition, cholesterol levels were

decreased in the kidney, heart and spleen tissues of RD treated groups. However, its level was significantly increased in the liver, muscle, brain and lung tissues of RD treated groups. In the previous studies, *Rubus* sp. extracts have decreased cholesterol levels in the rat and hamsters (56,71).

Vitamin E is a common term used for tocopherols and tocotrienols. It is an important antioxidant that is directly involved in scavenging oxygen free radicals and quenching lipid peroxidation chain reactions that occur during oxidation reactions with poly unsaturated fatty acids (PUFA) (72). Vitamin E reactions result in the formation of tocopheroxyl radicals that react with other antioxidants to regenerate the active molecule (73,74). Ghalayini et al. (57) showed that treating of *R. idaeus* water extract was increased the vitamin E level in kidney tissue of mice. In our study, vitamin E level increased in the RD treated groups in the liver, kidney and muscle tissues. It can be said that treatment of RD flowers extract increased vitamin E levels in these tissues.

Vitamin A plays a vital role in the development and homeostasis of almost every vertebrate tissue by regulating embryogenesis, cell differentiation, proliferation, metabolism, and apoptosis (75,76). Vitamin A (retinol) is obtained in the diet in the form of retinyl esters or through the ingestion of β-carotene, which is converted to two molecules of retinol. The carboxylic acid form of vitamin A (all-*trans*-retinoic acid) has important effects on the development of the cardiovascular system (76). Epidemiologic evidences have suggested that vitamin A is an important dietary factor for decreasing the incidence of heart disease (77,78). In our study, while vitamin A level was decreased in the RD treated group in the serum, brain and spleen tissues, its level was increased in the RD treated group in the liver tissue. It can be speculated that treatment of RD flowers extract affected vitamin A levels in these tissues. Keser et al. (79) have indicated the *R. discolor* flower water extract significantly affect the vitamin D and K levels against hydrogen peroxide administered Wistar rat tissues.

In previous studies, it has been determined that antioxidant properties of other species in the genus *Rubus* as *in vivo* and/or *in vitro*. However, we did not find any study about antioxidant properties of *R. discolor* extracts in literatures. As a result of this study for the first time the antioxidant properties of *R. discolor* water and ethanol extracts were determined *in vitro* the antioxidant effects of RD flower water extract in Wistar rats were investigated. In tissues of hydrogen peroxide treated groups, while MDA level was increased, GSH and total protein levels were decreased; in RD flower extract given group, these substances levels were protected. In hydrogen peroxide and RD flower extract treated groups, SOD and GSH-Px levels were increased. In addition, it was observed that vitamin A and E, and cholesterol levels were affected by the administration of hydrogen peroxide and RD flower extract. The present study results showed the *R. discolor* extract have the protective effects against H_2O_2 *via* its free radical scavenging activity and it affected levels of antioxidant enzymes, lipophylic vitamins, cholesterol, GSH, total protein and MDA.

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Table 1. ABTS, superoxide, DPPH scavenging, metal chelating, inhibition of lipid peroxidation, hydrogen peroxide scavenging and reducing power results of *R. discolor* extracts and some standard antioxidants (100 µg/mL)

Samples	% ABTS Scavenging	% Superoxide Scavenging	% Metal Chelating	% DPPH$^{\bullet}$ Scavenging	% Inhibition of Peroxidation	% H_2O_2 Scavenging	Reducing Power
Control	0	0	0	0	0	0	0.027±0.001
Flower water	96.20±0.35^{a}	87.16±0.21^{b}	54.58±0.77^{b}	84.42±0.83^{b}	92.60±0.09^{b}	42.07±0.64^{a}	0.600±0.012
Flower ethanol	96.89±0.43^{a}	82.11±0.94^{c}	52.82±0.88^{b}	81.61±0.31^{b}	90.20±0.12^{b}	98.77±0.05^{b}	1.589±0.052
Leaf water	96.47±0.28^{a}	84.83±0.46^{c}	40.35±0.75^{c}	80.80±0.28^{b}	93.67±0.05^{b}	28.57±0.54^{c}	0.312±0.011
Leaf ethanol	97.30±0.11^{a}	80.33±0.44^{c}	44.94±0.84^{c}	81.34±0.66^{b}	90.41±0.21^{b}	38.69±0.48^{a}	0.444±0.018
Unripe fruit water	60.17±1.58^{c}	82.50±0.81^{c}	48.35±1.05^{b}	38.13±1.41^{e}	95.66±0.02^{b}	20.82±0.95^{d}	0.121±0.009
Unripe fruit ethanol	81.17±1.09^{b}	28.83±2.58^{d}	15.17±1.54^{d}	49.82±0.87^{d}	90.51±0.29^{b}	12.57±0.25^{e}	0.184±0.010
Ripe fruit water	24.64±2.14^{d}	90.67±0.24^{b}	41.88±0.99^{c}	21.65±1.26^{f}	92.09±0.04^{b}	7.88±0.09^{f}	0.045±0.001
Ripe fruit ethanol	64.04±1.58^{c}	80.83±0.95^{c}	6.70±0.54^{e}	41.30±0.81^{e}	90.10±0.05^{b}	17.69±0.54^{c}	0.110±0.008
BHA	98.70±0.05^{a}	94.83±0.09^{a}	66.23±0.54^{a}	nt	nt	39.26±0.45^{a}	0.820±0.017
BHT	96.41±0.44^{a}	82.67±0.35^{c}	61.52±0.65^{a}	76.72±0.59^{c}	nt	nt	0.610±0.012
Tocopherol	95.05±0.68^{a}	96.33±0.58^{a}	63.52±0.67^{a}	nt	40.48±0.08^{a}	44.58±0.09^{a}	0.450±0.013
Trolox	nt	nt	nt	90.31±0.09^{a}	nt	nt	nt

nt: not tested

The experimental results were reported as mean ± S.D (standard deviation). There is not statistically difference among the same letter groups in the vertical column ($p<0.05$).

Table 2. Total phenolic contents of *R. discolor* extracts

Extracts (1 g)	Quercetin Equivalent (mg)	Pyrocatechol Equivalent (mg)
Flower water	93.58±1.25	23.12±0.58
Flower ethanol	152.05±1.36	26.47±0.08
Leaf water	74.54±0.89	20.25±0.25
Leaf ethanol	92.75±1.02	18.72±0.36
Unripe fruit water	69.96±0.12	19.30±0.47
Unripe fruit ethanol	72.06±0.25	18.34±0.29
Ripe fruit water	70.85±0.09	18.82±0.22
Ripe fruit ethanol	91.08±0.22	18.57±0.09

The experimental results were reported as mean ± S.D (standard deviation). Total phenolic compounds as expressed as mg quercetin equivalent /g extract and mg pyrocatechol equivalent / g extract

Table 3. Flavonoid contents in *R. discolor* water extracts (µg/g)

Flavonoids	*R. discolor* leaf water extract	*R. discolor* flower water extract	*R. discolor* unripe fruit water extract
Rutin	856	310	336
Apigenin	44	15	8
Myricetin	261	2682	86
Naringin	809	2962	238
Naringenin	41	22	Trace
Quercetin	Trace	5	Trace

Total	2011	5996	668

Table 4. The biochemical parameters in serum of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
Retinol (mg/mL)	0.411±0.007	**0.271±0.014[c]**	**0.324±0.015[c]**	0.390±0.005
α-tocopherol (mg/mL)	4.03±0.02	**2.74±0.12[c]**	**3.54±0.20[a]**	4.28±0.08
Cholesterol (mg/dL)	50.00±1.80	45.10±1.80	51.00±1.60	**63.80±1.60[c]**
Triglyceride (mg/dL)	66.50±12.20	**61.00±6.30[a]**	69.10±10.40	**76.10±7.10[c]**
HDL (mg/dL)	32.50±2.70	27.80±1.40	30.30±0.80	**39.00±2.40[c]**
LDL (mg/dL)	12.60±0.80	14.80±1.10	**16.60±1.30[c]**	**20.60±1.60[c]**
VLDL (mg/dL)	13.00±2.40	12.10±1.20	13.60±2.10	**15.30±1.40[c]**
GSH-Px (U/mL)	0.250±0.011	0.247±0.016	0.263±0.015	**0.320±0.012[c]**
SOD (U/mL)	6.79±0.24	6.92±0.45	**8.39±0.58[a]**	**9.57±0.60[c]**

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** $p<0.05$ **b:** $p<0.01$ **c:** $p<0.001$

Table 5. The biochemical parameters in liver of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
Retinol (µg/g)	224.05±11.35	**251.47±7.07[a]**	**254.83±8.09[a]**	**271.53±6.48[c]**
α-tocopherol	4.24±0.19	4.39±0.08	**6.45±0.10[c]**	**6.57±0.12[c]**

(µg/g)				
α-tocopherol acetate (µg/g)	0.120±.026	0.116±0.011	**4.251±0.131^{c}**	0.088±0.018
Cholesterol (µg/g)	950.03±8.89	**1053.93±13.85^{c}**	**1024.01±11.57^{c}**	**1191.95±9.53^{c}**
GSH (µg/g)	1860.63±38.32	**997.84±15.06^{c}**	1924.62±16.87	**1479.00±17.80^{c}**
Total Protein (mg/g)	81.81±0.94	**65.50±2.27^{c}**	**86.13±1.33^{a}**	84.90±0.89
MDA (nmol/g)	4.33±0.09	**3.48±0.14^{c}**	4.14±0.15	**2.13±0.048^{c}**

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** $p<0.05$ **b:** $p<0.01$ **c:** $p<0.001$

Table 6. The biochemical parameters in kidney of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
Retinol (µg/g)	2.16±0.14	2.49±0.12	2.21±0.09	**1.40±0.06^{c}**
α-tocopherol (µg/g)	21.40±0.14	21.82±0.24	**22.56±0.18^{b}**	**17.91±0.21^{c}**
α-tocopherol acetate (µg/g)	3.184±0.190	**0.864±0.077^{c}**	**0.305±0.053^{c}**	2.842±0.154
Cholesterol (µg/g)	1682.51±18.86	**1575.19±12.43^{c}**	**1606.21±14.11^{b}**	1636.20±17.83
GSH (µg/g)	331.40±12.53	**131.14±1.51^{c}**	**393.31±15.79^{b}**	**733.10±16.15^{c}**
Total Protein (mg/g)	51.52±0.93	**41.86±0.42^{c}**	**42.68±0.24^{c}**	**61.27±0.31^{c}**
MDA (nmol/g)	4.13±0.18	**5.18±0.18^{c}**	3.88±0.18	**0.55±0.04^{c}**

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** p<0.05 **b:** p<0.01 **c:** p<0.001

Table 7. The biochemical parameters in heart of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
Retinol (µg/g)	0.167±0.012	**0.219±0.018[a]**	0.167±0.013	0.170±0.015
α-tocopherol (µg/g)	5.66±0.24	6.02±0.16	**5.13±0.13[a]**	**3.88±0.05[c]**
α-tocopherol acetate (µg/g)	2.458±0.110	**0.076±0.004[c]**	**0.103±0.018[c]**	**0.579±0.100[c]**
Cholesterol (µg/g)	801.58±3.16	**906.56±1.09[c]**	790.12±3.95	**660.08±6.03[c]**
GSH (µg/g)	23.16±0.41	**21.73±0.17[a]**	**25.21±0.61[b]**	**28.42±0.15[c]**
Total Protein (mg/g)	32.55±0.47	**31.37±0.24[a]**	**38.89±0.26[c]**	32.07±0.17

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** p<0.05 **b:** p<0.01 **c:** p<0.001

Table 8. The biochemical parameters in muscle of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
Retinol (µg/g)	0.153±0.063	0.099±0.008	0.188±0.014	0.117±0.005
α-tocopherol (µg/g)	2.48±0.09	2.82±0.13	**5.83±0.05[c]**	**14.96±0.13[c]**
α-tocopherol acetate (µg/g)	2.32±0.15	**1.89±0.12[a]**	2.35±0.08	2.06±0.12
Cholesterol (µg/g)	335.57±7.77	**439.16±9.47[c]**	**470.16±3.93[c]**	**432.67±4.18[c]**

GSH (µg/g)	138.55±1.32	**118.06±1.43[c]**	138.87±1.25	**117.16±1.11[c]**
Total Protein (mg/g)	45.20±0.56	**32.51±0.78[c]**	**32.63±0.70[c]**	**34.15±0.40[c]**
MDA (nmol/g)	26.08±0.28	25.78±0.39	**14.50±0.11[c]**	**30.34±0.19[c]**

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** $p<0.05$ **b:** $p<0.01$ **c:** $p<0.001$

Table 9. The biochemical parameters in brain of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
Retinol (µg/g)	0.044±0.006	0.038±0.001	**0.032±0.002[a]**	0.039±0.001
α-tocopherol (µg/g)	9.11±0.19	9.69±0.30	9.59±0.17	8.71±0.07
α-tocopherol acetate (µg/g)	3.31±0.14	**7.59±0.15[c]**	**7.99±0.15[c]**	**6.40±0.12[c]**
Cholesterol (µg/g)	1511.37±17.00	**1641.87±12.75[c]**	**1638.71±9.24[c]**	**1658.58±7.24[c]**
GSH (µg/g)	33.97±0.25	**28.60±0.25[c]**	**34.81±0.20[b]**	**25.41±0.07[c]**
Total Protein (mg/g)	16.36±0.16	15.94±0.14	**17.60±0.08[c]**	**16.90±0.17[a]**
MDA (nmol/g)	2.860±0.159	**1.246±0.154[c]**	**0.397±0.094[c]**	**0.118±0.031[c]**

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** $p<0.05$ **b:** $p<0.01$ **c:** $p<0.001$

Table 10. The biochemical parameters in spleen of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
α-tocopherol (µg/g)	1.96±0.11	**1.37±0.07[c]**	**1.27±0.06[c]**	**0.85±0.11[c]**

α-tocopherol acetate (µg/g)	6.93±0.19	**4.13±0.04[c]**	**4.04±0.18[c]**	**2.93±0.15[c]**
Cholesterol (µg/g)	1961.36±11.97	**1691.99±20.91[c]**	**1840.63±11.66[c]**	**1063.06±11.94[c]**
GSH (µg/g)	48.40±0.82	**50.69±0.45[a]**	**41.28±0.57[c]**	**32.25±0.79[c]**
Total Protein (mg/g)	60.51±0.40	**54.29±1.48[c]**	58.35±0.51	**53.31±0.59[c]**
MDA (nmol/g)	68.28±0.45	**95.86±0.77[c]**	65.14±0.93	**91.58±1.74[c]**

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** $p<0.05$ **b:** $p<0.01$ **c:** $p<0.001$

Table 11. The biochemical parameters in lung of Wistar rats

Biochemical Parameters	Control	HP	RD	RD+HP
Retinol (µg/g)	4.66±0.12	**3.14±0.11[c]**	4.42±0.10	**3.81±0.17[c]**
α-tocopherol (µg/g)	4.34±0.15	**5.47±0.15[c]**	4.77±0.17	**3.08±0.17[c]**
α-tocopherol acetate (µg/g)	5.94±0.18	5.46±0.10	**3.25±0.18[c]**	**7.50±0.14[c]**
Cholesterol (µg/g)	1122.10±11.58	**1281.48±14.26[c]**	**1390.07±28.42[c]**	**1300.38±11.56[c]**
GSH (µg/g)	14.79±0.28	**10.92±0.29[c]**	**13.56±0.19[c]**	**13.13±0.18[c]**
Total Protein (mg/g)	30.00±0.44	**23.04±0.42[c]**	**25.86±0.61[c]**	**25.93±0.43[c]**
MDA (nmol/g)	43.58±0.50	**45.90±0.59[b]**	**39.76±0.62[c]**	**38.31±0.09[c]**

Data are mean ± SD values for ten rats in each group. There is not statistically difference among in the same letter groups.
All the groups were compared with control group. **a:** $p<0.05$ **b:** $p<0.01$ **c:** $p<0.001$