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Investigating the Impact of Hibiscus Extracts on Paraoxonase and Antioxidant Activities in Diabetic Rats

Diyabetik Sıçanlarda Hibiskus Ekstraktlarının Paraoxonase ve Antioksidan Aktiviteleri Üzerindeki Etkisinin Araştırılması

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ABSTRACT

Objective: Diabetes mellitus (DM) accelerates oxidative stress beyond its broad effects on metabolic function, which has been linked to various chronic complications. This study investigated the antioxidative and therapeutic potential of *Hibiscus syriacus* (HSE) and *Hibiscus trionum* extracts (HTE), focusing on their effects on paraoxonase (PON) and arylesterase enzymes activity in diabetic rat models.

Material and Methods: This study evaluated PON and ARE activities in 36 Wistar albino rats divided into the following groups: control (C), C+HSE, C+HTE, Diabetes (D), D+HSE, and D+HTE. The total phenolic content of HSE and HTE was determined using the Folin- Ciocalteu method, and their antioxidant activities were assessed using DPPH and CUPRAC tests.

Results: HSE and HTE extracts have demonstrated significant increases in paraoxonase and arylesterase activities, which are crucial for cardiovascular protection and reducing oxidative stress in diabetes.

Conclusions: This study highlights the potential of natural extracts in managing oxidative stress-related complications associated with diabetes and underscores the need to integrate such phytotherapeutic agents into broader diabetes care strategies. Future research should focus on confirming these findings in clinical settings and investigating the molecular processes responsible for the observed effects, potentially paving the way for innovative interventions for diabetes management.

Keywords: Antioxidant activity, Diabetes mellitus, *Hibiscus syriacus*, *Hibiscus trionum*, Paraoxonase

ÖZ

Amaç: Diyabetes mellitus (DM), metabolik işlevler üzerindeki geniş etkilerinin ötesinde, çeşitli kronik komplikasyonlarla bağlantılı olan oksidatif stresi hızlandırır. Bu çalışma, diyabetik sıçan modellerinde paraoksonaz (PON) ve arilesteraz enzim aktiviteleri üzerindeki etkilerine odaklanarak *Hibiscus syriacus* (HSE) ve *Hibiscus trionum* ekstraktlarının (HTE) antioksidatif ve terapötik potansiyelini araştırmaktadır.

Materyal ve Metot: Bu çalışma, kontrol (C), C+HSE, C+HTE, Diyabet (D), D+HSE ve D+HTE olmak üzere ayrılan 36 Wistar albino sıçanda PON ve ARE aktivitelerini değerlendirmiştir. HSE ve HTE'nin toplam fenolik içeriği Folin-Ciocalteu metodu ile belirlenmiş, antioksidan aktiviteleri DPPH ve CUPRAC testleri kullanılarak değerlendirilmiştir.

Sonuç: HSE ve HTE ekstraktları, diyabetik kardiyovasküler koruma ve oksidatif stresi azaltmada kritik öneme sahip olan paraoksonaz ve arilesteraz aktivitelerinde önemli artışlar göstermiştir. Sonuç: Bu çalışma, diyabetle ilişkili oksidatif stres komplikasyonlarının yönetiminde doğal ekstraktların potansiyelini vurgulamakta ve bu tür fitoterapötik ajanların geniş kapsamlı diyabet bakım stratejilerine entegre edilmesinin gerekliliğini ortaya koymaktadır. Gelecek çalışmalar, bu sonuçları klinik ortamlarda doğrulamayı ve gözlemlenen etkilerin altında yatan moleküler mekanizmaları araştırmayı hedeflemelidir, bu da diyabet yönetimi için yenilikçi müdahalelerin yolunu açabilir.

Anahtar Kelimeler: Antioksidan aktivite**,** Diyabetes mellitus, *Hibiscus syriacus*, *Hibiscus trionum*, Paraoksonaz

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by consistently high blood glucose levels owing to inadequate insulin production or reduced insulin sensitivity. This can disrupt the metabolism of carbohydrates, fats, and proteins, which may lead to broader health problems. Factors contributing to DM include genetic predispositions, diets high in sugars and fats, obesity, sedentary lifestyles, and oxidative stress that affects lipid metabolism.[1]

Discovered by Abraham Mazur in 1946 the enzyme Paraoxonase (PON1) was further characterized in 1953 by Aldridge W.N. as an A-esterase that breaks down p-nitrophenyl acetate, propionic acid, and butyric acid. Initially, this enzyme was extensively studied in toxicology because of its ability to hydrolyze the organophosphate substrate paraoxon, and thus it was named paraoxonase (EC 3.1.8.1). Later, it was discovered that the enzyme also had an activity that could hydrolyze aromatic esters such as phenyl acetate, which led to it being named arylesterase activity (EC 3.1.1.2). The term "A esterase" was introduced to describe an enzyme capable of hydrolyzing both compounds. However, it was concluded that both paraoxonase and arylesterase activities are characteristic of the PON1 enzyme.[2,3]

For many years, the only recognized function of paraoxonase 1 (PON1) has been to break down organophosphate compounds. However, recent studies have revealed that PON1's physiological role extends to the hydrolysis of lipolactones, which are cyclical esters found in damaged oxidized lipoproteins. Three types of paraoxonases have been identified: PON1, PON2, and PON3, with PON1 being the most thoroughly studied. It is primarily found in the serum. The lipolactonase activity of PON1 plays a crucial role in cardiovascular health by breaking down oxidized lipoproteins that can lead to atherosclerosis. PON1 has been shown to shield lipoproteins from oxidation mediated by free radicals. Additionally, it can break down oxidized cholesterol esters and lipid peroxides.[4]

Studies have shown that PON1 activity is markedly reduced in a broad range of human conditions associated with oxidative stress, including cardiovascular diseases, diabetes mellitus (DM), obesity, renal disease, liver cirrhosis, non-alcoholic steatohepatitis, and various mental disorders.[5]

In DM, it has been observed that PON1 activity decreases, and significant alterations in different blood metabolites also occur.[6] Multiple factors could contribute to the decrease in PON1 activity that has been observed. The lower PON1 activity is thought to be due to the lowering of its specific activity by non-enzymatic glycation because of elevated blood glucose.[7]

Globally, numerous plants with antidiabetic properties are used in traditional medicine for their hypoglycemic, affordable, and antioxidant benefits. These plants support conventional diabetes treatment and offer a holistic management approach. Among these, Hibiscus, from the Malvaceae family, has anti-inflammatory, antidiabetic, and antioxidant properties.[8]

Hibiscus sabdariffa may enhance serum PON1 levels in a dose-dependent manner, potentially mitigating oxidative stress in diabetes. The long-term use of *Hibiscus syriacus* and *Hibiscus trionum* extracts has demonstrated potential antidiabetic, antilipidemic, and hepatoprotective effects in diabetic rat studies. These results highlight the potential of hibiscus and similar antioxidant-rich plants to manage oxidative stress and boost enzyme activities linked to disease resistance.[9,10]

This study aimed to explore the effects of *Hibiscus syriacus* (HS) and *Hibiscus trionum* (HT) extracts on the activities of PON and ARE enzymes in diabetic rats and to evaluate their antioxidative properties to better understand their therapeutic potential.

MATERIALS AND METHODS

The animals used in this study were approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (Date:17.04.2018, decision no: 2018-06/07). All animal studies were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) and other guidelines.

Plants and Extraction Methods: Aerial parts of *Hibiscus syriacus* were collected from Kahramanmaraş and *Hibiscus trionum* from Bursa, Türkiye and necrotic sections were discarded. The voucher specimen was deposited at the Herbal Products Laboratory at Bursa Uludağ University with *Hibiscus tyrionum* specific code 45707 and *Hibiscus syriacus* specific code 47614. The plant materials were treated with 30% ethanol for ten minutes to remove contaminants and then washed with tap and distilled water. After air-drying in a shaded area, the samples were ground using a blender. Five grams of this powder were mixed with 50 ml of methanol and repeated three times for consistency. This mixture was then incubated at 40°C for 24 h, centrifuged at 4,500 rpm, and the clear supernatant was extracted using an organic solvent evaporator and stored at +4°C for stability.

Determination of Total Phenolic Content: The total phenolic content of the extract was determined using the Folin-Ciocalteu method, according to Aybastier.[11] Fresh Lowry A, B, and C solutions were prepared using Folin-Ciocalteu reagent diluted 1:3 in

water. Lowry C solution was prepared by combining 50 mL of Lowry A with 1 mL of Lowry B. The assay involved mixing 0.10 mL of the extract, 1.90 mL of water, and 2.50 mL of Lowry C solution, followed by the addition of 0.25 mL of Folin-Ciocalteu reagent. After 30 min, the absorbance was measured at 750 nm using a spectrophotometer. Total phenolic content was reported in mg of gallic acid equivalent (GAE) per g of dry weight (DW) of the plant.

Determination of Antioxidant Capacity:

DPPH Assay: Developed by Brand-Williams et al.,[12] the DPPH assay measures the antioxidant capacity of molecules against the DPPH free radical, a stable molecule with an unpaired electron.[13] In brief, samples were pipetted into 96-well microplates in 100 µL volumes at two-fold dilutions from 2.4 to 10,000 µg/ml. Trolox and ascorbic acid served as reference antioxidants. Each well was treated with 100 µL of DPPH in methanol for a final 0.1 mM concentration. The microplates were incubated at 37 °C for 30 min, and the absorbance was measured at 517 nm using an ELISA microplate reader. The IC50 value, where 50% of the DPPH radicals were neutralized, was derived from the linear progression of the curve. Antioxidant activity was reported in milligram Trolox equivalents per gram of freeze-dried sample.

CUPRAC Assay: Developed by Apak et al.,[14] the CUPRAC method measures the reduction of copper (II) ions by polyphenols in a Cu (II)-neocuproine solution, indicating "Copper Reducing Antioxidant Capacity." Samples were added to 50 µL volumes at two-fold dilutions from 2.4 to 10,000 µg/ml into wells. Freshly prepared CuCl2, NH4Ac, and neocuproine were added in 150 µL volumes. Cu(I)–Nc chelates were spectrophotometrically measured at 450 nm. Antioxidant capacity was expressed in mg Trolox equivalents per gram of lyophilized sample.

Animals: Thirty-six male Wistar rats, weighing 350–400 g, were housed under standard conditions with free access to food and water at a steady temperature of 25 ± 2°C and 55 ± 5% humidity with a 12-hour light-dark cycle. Ethical standards were followed to ensure animal welfare. Rats were administered daily morning doses of *Hibiscus trionum* extract (HTE) and *Hibiscus syriacus* extract (HSE) via gavage.

Induction of Diabetes: Type 1 diabetes was induced in overnight-fasted rats via a single intraperitoneal injection of 65 mg/kg streptozotocin (STZ; Sigma). The control group received a citrate buffer injection. Blood glucose levels were measured two days post-STZ injection, with inclusion in further research requiring levels of 200 mg/dL or higher.

Grouping of animals: Animals were randomly assigned and separated into six different groups as follows:

1.The healthy rats (control group) “C” (n=6),

2.The healthy rats administered with *Hibiscus syriacus* extract “C+HSE” (n=6),

3. The healthy rats administered with *Hibiscus trionum* extract “C+HTE” (n=6),

4.The diabetic rats “D” (n=6),

5.The diabetic rats administered with *Hibiscus syriacus* extract “D+HSE” (n=6),

6.The diabetic rats administered with *Hibiscus trionum* extract “D+HTE (n=6).

The rats in the "C+HSE", "C+HTE", "D+HSE", and "D+HTE" groups received *H. syriacus* and *H. trionum* extracts (100 mg/kg/day) simultaneously over four weeks via gavage. This method involves administering the extracts directly into the stomach through a tube passed down the throat, ensuring precise dosage and absorption. This is important for the accuracy and consistency of the dose used in the study.

Biochemical Analysis Procedures: At the end of the study, after a 12-hour fast, the animals were euthanized via cardiac puncture under anesthesia. Blood was collected in serum-specific tubes, centrifuged at 1500 rpm for 10 min to extract serum, and stored at -20 °C. Kidney and liver tissues were immediately preserved at -20 °C, rinsed with saline, and suspended in 1.15% potassium chloride (KCl). The tissues were homogenized using a T-line laboratory mixer (model No: 136-2), then centrifuged at 3000 rpm for 15 minutes at +4 °C. The supernatant was used for further analyses.

Serum Paraoxonase Activity: To assess PON activity, 15.62 µL of serum was combined with 2.5 mL of a solution containing 1.0 mM paraoxon, 1.0 mM CaCl2, and 0.05 M glycine-sodium hydroxide buffer at pH 10.5. PON's release of p-nitrophenol from paraoxon at 25 °C was measured at 412 nm using a spectrophotometer. The spontaneous non-enzymatic hydrolysis rates from the blank control were subtracted to determine the true absorbance. PON activity was defined as the production of 1 µmol p-nitrophenol per minute, reported as units per liter (U/L).[15]

Serum Arylesterase Activity: The enzyme activity of serum PON1 arylesterase was measured by the hydrolysis of phenylacetate into phenol and acetate, with the absorbance of phenol at 270 nm indicating the arylesterase activity. A substrate solution of 20 mM Tris-HCl buffer (pH 8.00), 1 mM CaCl2, and 1 mM phenylacetate was prepared immediately before use. Measurements were conducted at 25°C using a 270 nm wavelength over 2 min against a blank. The change in absorbance was recorded, and enzyme activity was calculated from this change and expressed in units per liter (U/L).[15]

Statistical Analysis: Statistical evaluations were conducted using SPSS (version 28.0) for Windows, with data presented as mean ± Standard Error. Fol-

lowing normality test outcomes, the Kruskal-Wallis non-parametric test was used for analysis. Differences between groups were assessed using Tamhane's T2 test for post hoc comparisons, with a significance threshold set at $p \leq 0.05$.

RESULTS

The total phenolic content of the Hibiscus species, expressed in milligrams of gallic acid equivalent per gram of dried weight, was determined using the Folin-Ciocalteu method and is presented in Table 1. The total phenolic contents in *Hibiscus syriacus* and *H. trionum* were 7.79 ± 0.06 and 7.22 ± 0.13 mg GAE/g, respectively. The total phenolic content was slightly higher in the HSE than in HTE.

The DPPH assay results of Hibiscus species are shown in Figure 1A. An absorbance/concentration graph was plotted, and the values where a linear increase occurred were determined first. According to the curve equation created from these values, the concentration at which the extract scavenges half of the DPPH free radicals (IC50) was calculated.

These antioxidant activities were quantified using Trolox-equivalent antioxidant capacity (TEAC) values, HSE IC50: 973 ug/ml, HTE IC50: 886 ug/ml and Trolox IC50: 3,7 ug/ml. Both Hibiscus species showed low DPPH scavenging activity. Compared to Trolox (3,7 ug /ml), statistically non-significant results were obtained in terms of the IC50 value of DPPH free radical scavenging activity between both HSE and HTE extracts (Figure 1A). The CUPRAC assay results of Hibiscus species are shown in Figure 1B. An absorbance/concentration graph was plotted, and the values where a linear increase occurred were determined first. A curve equation was developed based on these values. The absorbance value corresponding to a 1 mg/ml solution containing Trolox was calculated, and the corresponding plant extract solution concentrations required to obtain this value were determined using the respective curve equations. Thus, the CUPRAC antioxidant capacity was determined as the activity of the extract solution corresponding to the activity of 1 µg Trolox, expressed in µg (µg Trolox equivalents/µg extract).

The antioxidant capacities were measured and expressed in terms of Trolox-equivalent antioxidant capacity (TEAC) values. HSE Trolox eq: 14.13 mg Teq /g dry weight HS and HTE Trolox eq: 21.29 mg Teq /g dry weight HT and Trolox eq: 59.60 ug/ml (Figure 1B).

Table 1. The total phenolic content of *H. syriacus* and *H. trionum* extracts.

Extracts	Total Phenolic Content mg/gallic acid/g plant
H. syriacus extract (HSE)	7.79±0.06
H. trionum extract (HTE)	7.22±0.13

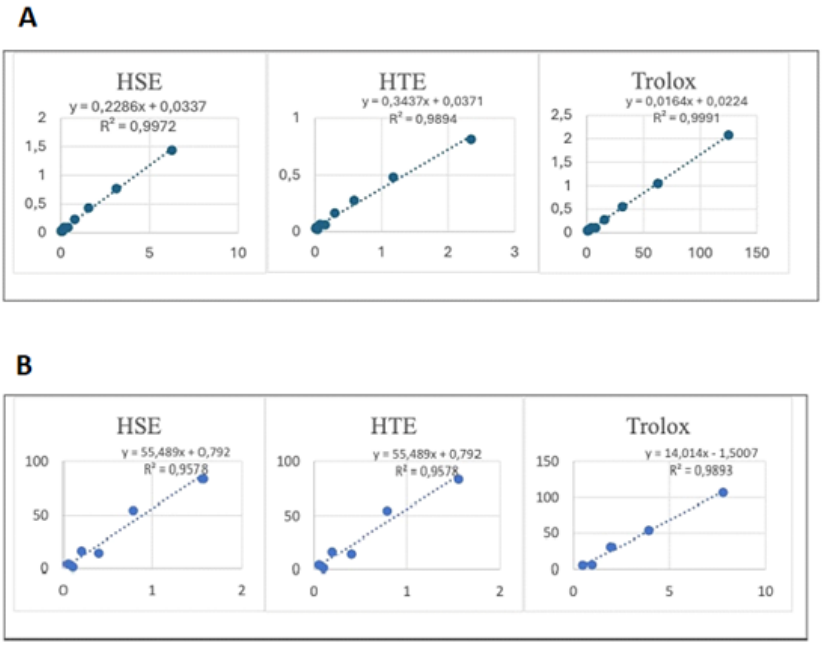

Figure 1. The DPPH (A) and CUPRAC (B) assay results of Hibiscus species. HSE: *Hibiscus syriacus* extract; HTE: *Hibiscus trionum* extract.

Comparisons of the antioxidant activity of *H. syriacus* and *H. trionum* species from different tests are shown in Figure 2.

The PON activities of diabetic and healthy rats treated with *H. syriacus* and *H. trionum* extracts in the serum, liver, and kidney tissues are shown in Table 2. Both serum and tissue (liver, kidney) PON activity were significantly lower in the diabetic group versus the control group ($p < 0.05$, 0.01). Both *Hibiscus syriacus* extract (HSE) and *Hibiscus trionum* extract (HTE) caused significantly increased PON activities in the C + HSE, C + HTE, D + HSE, and D + HTE groups compared to the C and D groups (Respectively, $p < 0.05$, 0.01; Table 2).

The arylesterase activities of diabetic and healthy rats treated with *H. syriacus* and *H. trionum* extracts in the serum are shown in Figure 3. ARE activity was notably reduced in the diabetic group (58 U/L) compared to the control group (154 U/L) ($p < 0.05$, 0.01). *Hibiscus syriacus* extract (HSE) and *Hibiscus trionum* extract (HTE) significantly enhanced ARE activities in the C + HSE (185 U/L), C + HTE (220 U/L), D + HSE (121 U/L), and D + THE (138 U/L) groups relative to the C and D groups, respectively ($p < 0.05$, 0.01).

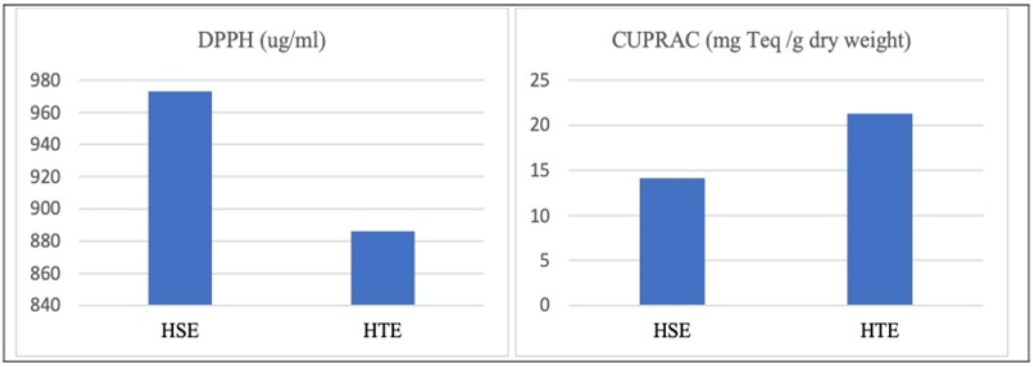

Figure 2. The antioxidant activity of *Hibiscus syriacus* and *Hibiscus trionum* species.

Table 2. The PON Activities in Diabetic and Healthy Rats Treated with *H. syriacus* and *H. trionum* Extracts.

Extracts	PON Activity		
	Serum (U/L)	Liver (U/g/tissue)	Kidney (U/g/tissue)
C	120±1	80±1	35±2
C +HSE	142±2 ^{a*}	92±1	41±2
C +HTE	162±4 ^{a*}	108±2 ^{a*}	56±2 ^{a*}
D	48±1 ^{a**}	41±1 ^{a*}	22±1 ^{a*}
D + HSE	116±2 ^{b*}	61±2	30±2
D + HTE	118±3 ^{b*}	78±3 ^{b*}	33±2 ^{b*}

a: Compared with control. b: Compared with diabetes group. Statistical significance: *p<0.05, **p< 0.01; PON: Paraoxonase; C: Control; C +HSE: Control + *Hibiscus syriacus* extract; C +HTE: Control + *Hibiscus trionum* extract; D: Diabetes; D+HSE: Diabetes + *Hibiscus syriacus* extract; D+HTE: Diabetes + *Hibiscus trionum* extract.

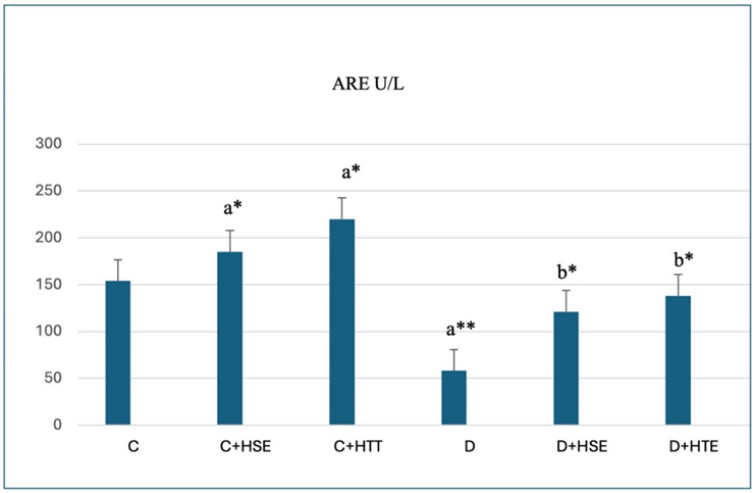

Figure 3. The arylesterase enzyme activities in control and experimental groups of rats treated with *H. syriacus* and *H. trionum* extracts. ^{a}: Compared with control; ^{b}: Compared with diabetes group. Statistical significance: *: p<0.05, **: p< 0.01. ARE: Arylesterase; C: Control; C +HSE: Control + *Hibiscus syriacus* extract; C +HTE: Control + *Hibiscus trionum* extract; D: Diabetes; D+HSE: Diabetes + *Hibiscus syriacus* extract; D+HTE: Diabetes + *Hibiscus trionum* extract.

DISCUSSION AND CONCLUSION

Historically, natural products have served therapeutic and preventive roles in the treatment of diseases, such as diabetes. Over time, these drugs have evolved into specific medications; however, today, synthetic drugs are often replaced. Despite this, interest in herbal treatments remains strong, especially in East and Southeast Asia and other regions with diverse flora, where natural remedies are considered viable diabetes treatments. Research indicates that natural products generally have fewer side effects than synthetic drugs, promoting their use in the management of chronic diseases. Moreover, their use to reduce the side effects of conventional diabetes medications has gained popularity.[16]

Despite their popularity, the lack of precise dosage and usage studies of plants and herbal products can result in reliability issues and adverse outcomes. In phytotherapy, metabolites, such as phenolics and flavonoids, which are crucial in fighting diabetes, are well studied for their high antioxidant levels and effectiveness against oxidative stress, providing protective benefits against bodily damage.[17]

The DPPH assay, a method that uses a stable free radical soluble in methanol or ethanol, changes color upon neutralization by antioxidants. Substances are categorized based on their antioxidant strength, with IC50 or EC50 values below 50 µg/ml considered very strong antioxidants, between 50-100 µg/ml strong, 101-150 µg/ml moderate, and above 150 µg/ml weak.[18]

The CUPRAC method detects hydroxyl radicals (OH) and measures OH scavenger activity. The hydroxyl radical, the most reactive of all reactive oxygen species (ROS), exhibits significant reactivity. In this assay, a sample is considered to have antioxidant capabilities if it can reduce Cu (II) to Cu (I), indicating a strong reduction potential.[14] In the present study, in the antioxidant activity tests comparing the two methods, the DPPH method displayed weaker antioxidant activity with a higher IC50 value, highlighting a significant difference compared with the CUPRAC method. CUPRAC exhibited the highest antioxidant activity of Hibiscus species (Fig 1-2). The antioxidant capacity of plants is often associated with the presence of phenolic compounds and flavonoids. Phenolic compounds are known to possess antioxidant effects owing to their oxidation-inhibiting properties. These compounds function as reducing agents, hydrogen donors, singlet oxygen quenchers, and chelators. Flavonoids neutralize free radicals by donating hydrogen atoms, thereby acting as antioxidants.

Despite their popularity, imprecise dosing and usage guidelines for plants and herbal products can lead to reliability concerns and negative effects. In phytotherapy, key metabolites such as phenolics and flavonoids, which are essential for combating diabetes, are noted for their strong antioxidant properties and efficacy against oxidative stress, offering protection against bodily damage.[19]

PON enzyme activity is linked with various diseases, including inflammation, stroke, myocardial infarction, obesity, hypercholesterolemia, renal failure, hypertension, diabetes, and Alzheimer's disease. Reduced PON activity has been identified as a cause of inflammation, particularly in cancer and diabetes patients. Studies indicate that expressing human PON1 in mice prevents diabetes by enhancing its antioxidant properties and promoting insulin secretion from pancreatic β-cells.[20,21]

This study found that the administration of hibiscus extracts to diabetic rats improved PON1 and ARE activity. Our research has observed a reduction in PON activity within the diabetic group; in the diabetic groups, treatment with HSE and HTE led to significant increases in paraoxonase activity; overall activity increased by 109% and 137%, liver activity by 48% and 99%, and kidney activity by 36% and 50%, respectively (Table 2). In the control groups receiving HSE and HTE, arylesterase activity increased by 20% and 43%, respectively, whereas in the diabetic groups receiving HS and HT, the increase was 109% and 137%, respectively (Fig. 4). In this decrease of the diabetic group is thought to be associated with hyperglycemia and oxidative stress. Treatment led to significant increases in paraoxonase and arylesterase activities in both healthy and diabetic groups, suggesting beneficial effects on metabolic functions. The decrease in PON activity in diabetic groups is thought to be associated with hyperglycemia and oxidative stress.

These findings align with those of Bahlil et al.,[22] who reported increased PON1 levels in hypercholesterolemic diabetic rats supplemented with *Zygophyllum album*. PON1, an enzyme associated with HDL, possesses antioxidant and anti-inflammatory properties that can mitigate the oxidation of atherogenic LDL, offering protection against it.[23] The significant increase in PON1 activity could be a result of enhanced synthesis and/or secretion of HDL-C.

This research confirms that plant-based antioxidants can effectively modulate PON1 activity, as supported by strong evidence from animal models. According to the results of this study, *H. trionum* is more effective than *H. syriacus* in terms of antioxidative activity and treatment of complications caused by diabetes. This can be explained by the presence of different and more effective bioactive molecules in *H. trionum* than in *H. syriacus.* However, the variability in antioxidant effects due to environmental factors calls for further methodological research, especially to verify the impacts of *Hibiscus syriacus* and *Hibiscus trionum* at physiological doses. This

study highlights the necessity of using FDA-recommended doses in clinical trials to maximize benefits while ensuring safety. Future research is needed to explore the metabolic effects of polyphenols on human health, emphasizing the importance of natural antioxidants in clinical settings and their regulatory compliance.

In conclusion, ongoing exploration of the role of natural antioxidants in clinical applications is crucial. It not only helps to refine our understanding of their therapeutic potential but also ensures that the interventions are both effective and safe, aligning with regulatory standards, and addressing the variability introduced by biological and environmental factors.

Ethics Committee Approval: Our study was approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (2018-06/07).

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – SZD; Supervision – SZD; Materials – SZD; Data Collection and/or Processing – SZD; Analysis and/or Interpretation – SZD; Writing – SZD.

Peer-review: Externally peer-reviewed.

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