

THE EFFECT OF BIOACTIVE COMPOUNDS
OF *Prunus spinosa* LEAVES AND FLOWERS
MIXTURE ON THE ANTIOXIDANT CAPACITY
IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract

Diabetes is a serious and chronic public health problem worldwide. Hyperglycemia leads to the production of free radicals thus impairing oxidant/antioxidant balance and causing various complications in diabetes. Herbs can provide natural remedies against various diseases in traditional medicine. Phytochemicals especially phenolic compounds have some biological properties such as antioxidant and antidiabetic effects. Plant parts of *Prunus spinosa* are used in traditional medicine against various disorders. This study evaluated the mixture of *Prunus spinosa* leaves and flowers extract (PSE) on the antioxidant capacity in diabetes. The phenolic profile of PSE was determined by HPLC method. Antioxidant capacity was evaluated with radical scavenging activities as well as total phenolic and flavonoid content. Seven random groups ($n = 8$) of rats were constituted to evaluate the antioxidant capacity – control, diabetic, PSE25, PSE50, insulin, metformin, and acarbose. Total oxidant status (TOS) and total antioxidant status (TAS) were analyzed in the liver tissues. Chlorogenic acid and ellagic acid were found to be the main compound in the mixture of *P. spinosa* leaves and flowers extract. Antioxidant capacity of PSE exhibited effective radical scavenging activity. Although oxidative stress seriously increased in diabetes, PSE25 and PSE50 supplementation significantly

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recuperated liver antioxidant status. In conclusion, PSE markedly has antioxidant capacity through its high phenolic compounds, thus PSE treatment may have potential antioxidant effects for health benefits in diabetes.

Key words: antioxidant, diabetes mellitus, phenolics, *Prunus spinosa*, TAS, TOS

Introduction. Diabetes is a serious, chronic disease characterized by hyperglycemia that occurs either when the pancreas does not produce enough insulin, or when it cannot effectively use the insulin. Diabetes is an important public health problem of which heart attack, stroke, kidney failure, leg amputation, vision loss and nerve damage are among the major complications [1]. The metabolic abnormalities of diabetes caused by hyperglycemia such as non-enzymatic glycosylation and glucose auto-oxidation lead to the continuous production of free radicals. Increased free radical production causes the activation of some major pathways such as polyol pathway, protein kinase C, increase in the formation of advanced glycation end-products, and overactivity of the hexosamine pathway. At the same time, these pathways lead to the production of free radicals again and further exacerbate diabetic complications [2]. Herbs offer various biological properties such as antioxidant as well as antidiabetic properties, antihyperlipidemic, antimicrobial, and anticarcinogenic effects. Antioxidant and antidiabetic effects of herbs may lead to the reduction of both oxidative stress and hyperglycemia. These dual combination effects can prevent the production of free radicals and diabetic complications.

Prunus spinosa L. (Rosaceae) known as “blackthorn” shows propagation in Europe, West Asia, and Northwest Africa. Its flowers are used for vasoprotective, anti-inflammatory, diuretic, and blood purifying activities as well as its branches having antihypertensive properties [3]. Its fruits are a traditional medicine against inflammation of the oral and pharyngeal mucosa in the form of antiseptic mouthwash. Also, its fruits are used in treatment of gastrointestinal and respiratory tract disorders, and atherosclerosis [3]. Moreover, ethnopharmacological sources indicate that *P. spinosa* is also used as analgesic, antispasmodic, diaphoretic, emmenagogue, against edema and leucorrhea in folk medicine [4]. To the best of our knowledge, despite the wide traditional usage of *P. spinosa*, there are limited experimental studies in the literature. This study aims to evaluate the antioxidant capacity of the mixture of *P. spinosa* flowers and leaves extract (PSE) in experimental diabetes mellitus in rats.

Material and methods. Standards and reagents. All chemicals and reagents used were of analytical grade and also procured from Sigma and Merck (Germany). Insulin (Humulin® N Lilly, Turkey), Metformin (Glifor® Bilim, Turkey), and Acarbose (Glucobay®, Turkey) were procured from a local pharmacy.

Plant material and extraction. *P. spinosa* leaves were picked up from Tekirdag-Hayrabolu province, Turkey, in April 2018 and were cleared of foreign

substances. The plant parts were lyophilized (Freeze dryer, Labconco, Czech Republic, Model: 117) and all dried leaves and flowers were pulverized. The plant parts were blended to 50% and extracted with 75% aqueous ethanol at 50 °C for 3 h by continuous stirring (Wisd WiseStir MSH-20D). The extract was filtered and lyophilized. The lyophilized *P. spinosa* extract (PSE) was stored in amber bottles under nitrogen atmosphere at –26 °C until further analyses.

HPLC analysis of *P. spinosa* extract. The phenolic profiles of samples were determined as previously described by COLARIC et al. [5] with some modifications using the Thermo-Finnigan Surveyor HPLC system (Thermo Fisher, USA). Separations were carried out on a C18 column (Zorbax extend-C18, 4.6 × 150 mm, 5 µm) using a gradient solvent system. Elution was performed at the flow rate of 1.0 mL/min and the injection volume was adjusted to 20 µL using a binary mobile phase mixture of water/acetic acid (98:2 v/v) (A) and acetonitrile/water/acetic acid (50:49.5:0.5 v/v) (B) at 35 °C. The gradient programme was used as follows: 0 min 80% A, 30 min 75% A, 60 min 65% A. The detection wavelengths were set to 254–280 nm using the Thermo Finnigan Surveyor PDA Plus detector and the peak areas were integrated using the ChromQuest software. The identification of each compound was based on their retention times and spectral matching through comparison with external standards.

Elemental analyses of *P. spinosa*. Mineral component in plant sample was analyzed using dry-ashing method. After ashing the leaves and flowers in a porcelain crucible at 500–550 °C in a muffle furnace for 24 h, the ash was dissolved with 5 mL nitric acid (1 N) on a heating plate at 80 °C and was filtered (Whatman No. 41). The solution was diluted with 1 N HNO₃ and used for elemental analysis using an atomic absorption spectrophotometer (Thermo Scientific iCE 3000 Series, UK). Working standard solutions were used to calculate the amount of each element.

Determination of total phenolic and total flavonoid content. Total phenolic content (TPC) in the PSE was determined by the modified Folin–Ciocalteu reagent method using gallic acid as a standard [6]. TPC were calculated as mg gallic acid equivalent (GAE) per 100 g dry weight (dw). Total flavonoid content (TFC) was determined by the AlCl₃ method using quercetin as a standard [7]. TFC was calculated as mg quercetin equivalent (QE) per 100 g dry weight.

DPPH radical scavenging activity. The free radical-scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [8]. Briefly, 0.1 mL diluted extract or trolox standard was mixed with 3.90 mL methanolic solution of DPPH (6×10^{-5} mol L⁻¹) in a tube. The tubes were left for 60 min at room temperature in the dark; thereafter, their absorbance was measured at 517 nm against blank (Boeco-S22 UV-Vis spectrophotometer, Germany). The percentage of DPPH was expressed as half-maximal inhibitory concentration which was graphically calculated.

ABTS assay. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical was dissolved in potassium persulphate (2.45 mM) to generate the ABTS radical cation (ABTS⁺). The ABTS⁺ solution was diluted with distilled water to an absorbance of 0.700 ± 0.020 at 734 nm [9]. Briefly, 20 μ L diluted extract or trolox standard was mixed with 1980 μ L ABTS⁺ solution in microcentrifuge tubes and vortexed. After 6 min at room temperature in darkness, the absorbance was measured at 734 nm. Percent inhibition was graphically calculated and expressed as half-maximal inhibitory concentration. All measurements were performed in triplicate.

Animals and experimental protocol. Experiments were performed on 56 healthy male rats (*Wistar albino*, 200–300 g and 2–3 months of age) obtained from Experimental Application and Research Center, Van Yüzüncü Yıl University (Turkey). The rats were housed in standard plastic rat cages at 22 ± 2 °C, 50% humidity, and 12-h day/night cycles. This study was approved by Van Yüzüncü Yıl University Animal Researches Local Ethics Committee (decision No 2020/01) and procedures applied complied with the Guidelines for the Care and Use of Laboratory Animals.

Streptozotocin (STZ) was administered at 45 mg/kg body weight (bw) i.p. Rats with glucose levels ≥ 200 mg/dL 3 d after STZ injection were considered diabetic. The rats were randomly categorized into seven groups ($n = 8$) as follows: 1) Control group (CG), administered with 1 mL citrate buffer i.p. only; 2) diabetic group (DG), injected with a single dose of 45 mg/kg bw, i.p. STZ; 3) diabetic+PSE-25 (PSE25), where diabetic rats were treated with 25 mg/kg bw PSE daily using an intragastric tube; 4) diabetic+PSE-50 (PSE50), where diabetic rats were treated with 50 mg/kg bw PSE daily using an intragastric tube; 5) diabetic+insulin (Insulin) group, where diabetic rats were treated with 0.5 IU/kg bw insulin (Humulin® N Lilly, Turkey) daily p.o. 6) diabetic+metformin (Metformin) group, where diabetic rats were treated with 100 mg/kg bw metformin (Glifor® tablets Bilim, Turkey) daily using an intragastric tube. 7) diabetic + acarbose (Acarbose) group, where diabetic rats were treated with 50 mg/kg bw acarbose (Glucobay® tablets Bayer, Turkey) daily using an intragastric tube. All rats were provided with standard chow and tap water ad libitum for 21 d. At the end of the experiment, the rats were anesthetized with ketamine and xylazine and then tissue samples were taken.

Total antioxidant and oxidant status evaluations. Rat liver tissue was homogenized in ice-cold phosphate-buffered saline (pH 7.4) for 3 min, using titanium probe homogenizer (Bandelin Sonopuls HD 2200, Germany) and centrifuged at $8570 \times g$ for 30 min at $+4$ °C. The supernatants were used to evaluate total antioxidant status (TAS) and total oxidant status (TOS). TAS and TOS were evaluated using a commercially available kit (Rel Assay Diagnostic, Turkey). The TAS method is based on the conversion of the ABTS⁺ radical into ABTS by the antioxidants in the sample [10]. The TOS method is based on the oxidation of

ferrous (Fe^{+2}) ion complexes to the ferric (Fe^{+3}) form by the oxidants in the sample [11]. The oxidative stress index (OSI) is the ratio of TAS and TOS parameters and is used to express the status of oxidative stress in tissues. OSI was calculated using the following formula:

$$\text{OSI (arbitrary unit)} = (\text{TOS/TAS}) \times 100.$$

Statistical analyses. Data were expressed as mean and standard deviation ($\bar{X} \pm \text{SD}$). Significant differences between groups were assessed using one-way analysis of variance followed by Tukey's test. A value of $p < 0.05$ was accepted as statistically significant.

Results and discussion. Phenolic composition of mixture of *P. spinosa* leaves and flowers extract. The amount of phenolic compounds in *P. spinosa* is shown in Table 1. Chlorogenic acid and ellagic acid are the main compounds in the mixture of *P. spinosa* leaves and flowers extract. *O*-coumaric, protocatechuic, and gallic acid are other dominant phenolic compounds. MARCHELAK et al. [3] detected nearly 60 components, mainly flavonoids and phenolic acids in *P. spinosa* flowers through UHPLC-PDA-ESI-MS analysis. It was reported that 25 polyphenolic compounds in the leaves [12] and 26 in branches [13] were detected. In a previous study, mostly fruit extracts of *P. spinosa* were generally evaluated for phenolic and flavonoid compounds. Rutin, quercetin, chlorogenic acid, and gallic acid were found to be major phenolic compounds in *P. spinosa* fruit [14]. Overall, it can be stated that different parts of *P. spinosa* possess similar phenolic contents.

Elemental compositions of mixture of *P. spinosa* leaves and flowers. The mineral components of *P. spinosa* are summarized to indicate the nutritive and dietetic value (Table 2). K, Ca, and Mg were determined as major minerals in the mixture of *P. spinosa* leaves and flowers. The most abundant mineral content in *P. spinosa* fruits was notified as K, Ca, and P [15]. However, protein content of

T a b l e 1
Amount of phenolic compounds of *Prunus spinosa* extract (mg/kg)

	GA	PCA	ChA	CA	<i>p</i> -Cou	FA
PSE	607.2 $\pm$ 24.1	803.6 $\pm$ 26.4	14 659.7 $\pm$ 256.8	86.4 $\pm$ 0.8	76.1 $\pm$ 1.9	160.4 $\pm$ 0.9
RT of Standards	3.125	4.005	4.873	6.172	9.473	11.033
	<i>o</i> -Cou	Phz	Ru	EA	Qe	
PSE	1066.8 $\pm$ 45.4	115.8 $\pm$ 1.2	nd	1547.8 $\pm$ 32.6	nd	
RT of Standards	13.218	15.437	18.538	20.915	45.070	

Legend: GA: Gallic acid; PCA: Protocatechuic acid; ChA: Chlorogenic acid; CA: Caffeic acid; *p*-CouA: *p*-Coumaric acid; FA: Ferulic acid; *o*-CouA: *o*-Coumaric acid; Phz: Phloridzin; Ru: Rutin; EA: Ellagic acid; Qe: Quercetin; RT: Retention time (min); nd: Not determined

T a b l e 2

Some physicochemical properties and mineral contents of *Prunus spinosa*

	Ash%	Protein%	Mineral content (mg/100 g dw)		
			K	Ca	Mg
Leaf & flower mix	6.9 ± 0.1	20.0 ± 1.9	12 863.9 ± 54.6	10 062.2 ± 26.3	3039.9 ± 26.3
	Mineral content (mg/100 g dw)				
	Na	Fe	Mn	Zn	Cu
Leaf & flower mix	1151.7 ± 10.2	372.9 ± 9.7	51.6 ± 1.4	38.4 ± 1.1	18.1 ± 0.4

fruits (% 3.4) in the same study was poor compared to the mixture of leaves and flowers in the current study. The mixture of leaves and flowers is richer in Fe, Mn, and Cu than the fruit. On the other hand, Cu, Zn, and Mn especially can assist in enhancing the antioxidant status [16]. SOD is an important metal-containing antioxidant enzyme that provides the first line of defense against superoxide radicals by catalyzing their dismutation to oxygen and hydrogen peroxide. Therefore, the role of the abovementioned minerals in diminishing oxidative stress is important.

Antioxidant capacity. Phenolic acids and flavonoids are responsible for most of the antioxidant capacity. They contain –OH groups which scavenge the free radicals and terminate the chain reactions by donating electrons and/or hydrogen atom. DPPH and ABTS radical scavenging activities are frequently used for the evaluation of antioxidant capacity. The antioxidant capacity values, TPC and TFC of PSE are presented in Table 3. HPLC result is in accordance with TPC and TFC findings. In the previous study, TPC was 206.07 ± 10.86 mg GAE/g dw in the methanolic extract of *P. spinosa* flowers [3]. The acetone extract of *P. spinosa* leaf was found with the highest value for TPC (in the range of 116.63–181.19 mg GAE/g) and TFC (in the range of 36.28–80.10 mg QE/g) compared to water and ethanol extracts [17]. The results confirmed the previous literature reports indicating that phenolic acids are the major group of phenolic compounds in *P. spinosa* leaves and flowers. However, previous studies reported relatively high TPC and TFC values compared to the current study. The values of TPC and TFC of different parts of *P. spinosa* have a wide range. The TPC in ethanol

T a b l e 3

DPPH and ABTS radical scavenging IC₅₀ values, TPC and TFC of *Prunus spinosa* extract

Sample	DPPH	ABTS	TPC	TFC
	(mmol TE/g extract)	(mmol TE/g extract)	(mg GAE/100 g)	(mg QE/100 g)
PSE	1.08 ± 0.06	2.90 ± 0.05	4341 ± 114	1754 ± 69

(732.34 ± 6.41 mg/g) and aqueous (499.23 ± 1.99 mg/g) extract from branches were significantly higher than those of fruits (359.11 ± 2.54 mg/g and 327.02 ± 4.66 mg/g) and leaves (228.56 ± 2.22 mg/g and 101.28 ± 1.94 mg/g). Moreover, the highest TFC was found in ethanolic extraction of branches (554.82 ± 4.51 mg/g) followed by leaves (196.88 ± 3.97 mg/g) and fruits (141.80 ± 2.11 mg/g) [13].

In respect to radical scavenging activities, it was noted that the acetone extract of *P. spinosa* leaf was the most effective for DPPH and ABTS with EC₅₀ values 44.57 and 16.12 mg/mL, respectively, in comparison to water and ethanol extracts [17]. It can be stated that the mixture of leaves and flowers of *P. spinosa* in current results is more effective in comparison with previous findings. However, in another study, ethanol extract of *P. spinosa* leaves IC₅₀ value for DPPH was 14.36 ± 1.45 µg/mL [13]. Also, EC₅₀ value of methanol extract of *P. spinosa* flower for DPPH was 15.46 ± 0.38 µg/mL [3]. Nevertheless, a comparison of the current and previous results was quite difficult due to the difference in expression of the concentrations. However, the radical scavenging capacity of *P. spinosa* clearly appears to be effective, whether mg/mL, IC₅₀ and/or EC₅₀.

Oxidant and antioxidant status in the liver tissue. Diabetes causes a depletion of antioxidant molecules and enzymes. Therefore, the impaired antioxidant status in diabetes influences many tissues adversely. The alterations in antioxidant enzymes activities and GSH concentration have been reported in diabetes [2,18]. It was observed that total oxidant status has exacerbated accompanied by a decrement in antioxidant status in diabetes (Fig. 1). Furthermore, OSI more clearly revealed the oxidative stress burden caused by diabetes (Fig. 1c). While PSE25 and PSE50 administration significantly assisted in decreasing TOS, their supplementation also increased TAS. Similarly, it was notified that oxidative stress markers such as TOS, OSI, and MDA increased in the liver of streptozotocin-induced diabetic rats [19].

It has been shown in our previous study that the administration of PSE reduced blood glucose level and decreased lipid peroxidation [18]. Since the decrease in blood glucose level is directly effective in alleviating oxidative stress, it can be stated that PSE reduces TOS with both its antidiabetic and antioxidant effects. However, no references have been found in relation to the oxidant and antioxidant status of *P. spinosa* leaves and flowers. In the previous study, *P. armeniaca* fruit feeding in CCl₄-induced liver damage decreased the oxidative stress and improved antioxidant enzymes [20]. It was asserted that this effect occurred probably due to its high phytochemical content thus exhibiting the high radical scavenging capacity. Current results are in accordance with previous findings. It is seen that plants of Prunus genus are effective in suppressing oxidative stress due to the phenolic compounds they possess.

Conclusion. The mixture of leaves and flowers of *P. spinosa* markedly have phenolic compounds that can be effective in maintaining the oxidant/antioxidant balance that is impaired in diabetes. The results reveal that phytochemicals in

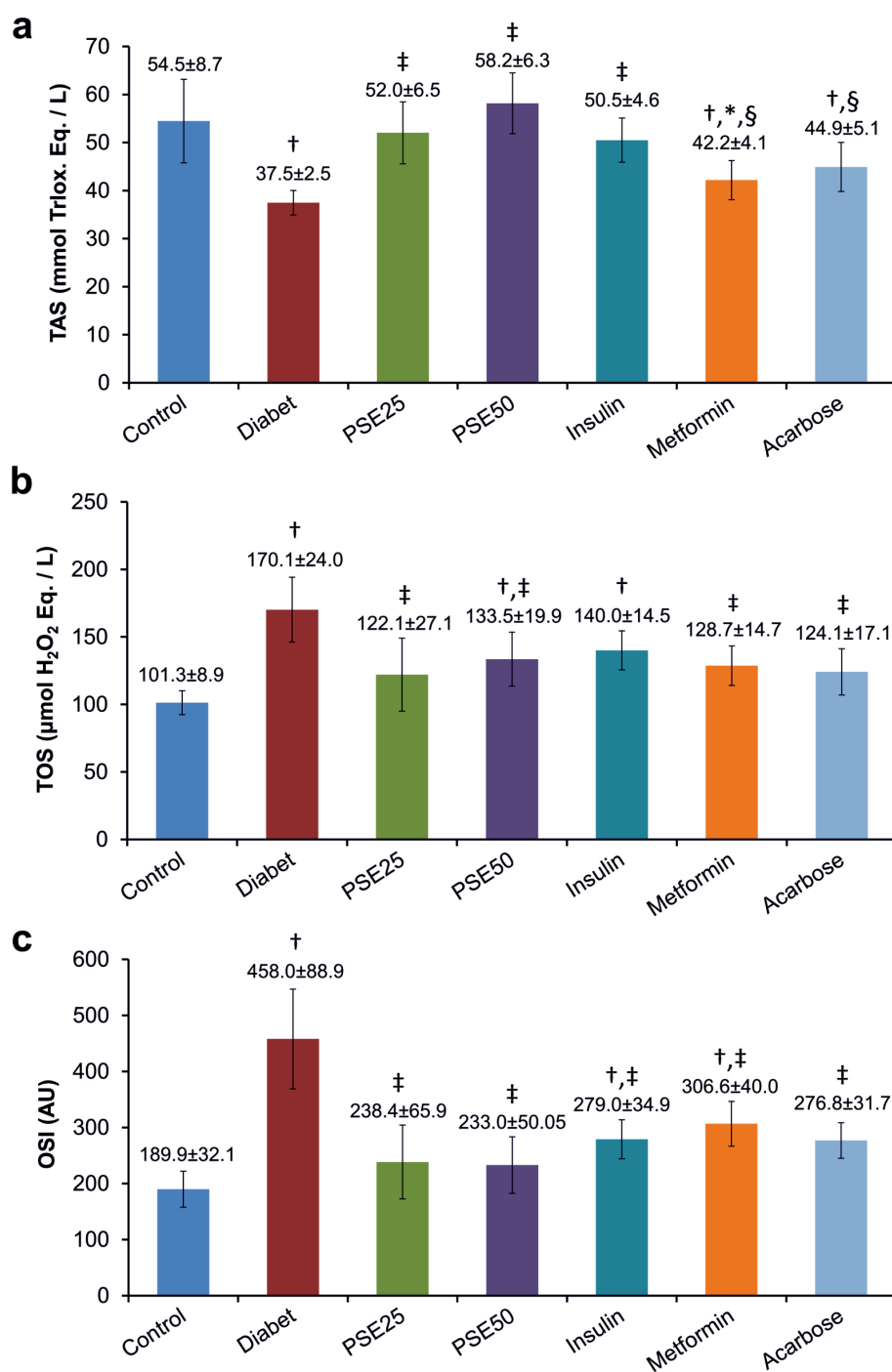


Fig. 1. Effect of mixture of *Prunus spinosa* leaf and flower extract administration on TAS (A), TOS (B), and OSI (C) in streptozotocin-induced diabetic rats. †: Significantly different from Control, ‡: Significantly different from Diabetic, *: Significantly different from PSE25, §: Significantly different from PSE50, $p < 0.05$, $n = 8$

PSE may show liver protective effect by suppressing oxidative stress and boosting antioxidant capacity. Moreover, the mixture of leaves and flowers of *P. spinosa* have dietetic and nutritional value in terms of mineral components.

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