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Preventive role of Pycnogenol® against the hyperglycemia-induced oxidative stress and DNA damage in diabetic rats

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A R T I C L E I N F O

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A B S T A C T

Diabetes mellitus, a complex progressive metabolic disorder, leads to some oxidative stress related complications. Pycnogenol® (PYC), a plant extract obtained from *Pinus pinaster*, has been suggested to be effective in many diseases including diabetes, cancer, inflammatory and immune system disorders. The mechanisms underlying the effects of PYC in diabetes need to be elucidated. The aim of this study was to determine the effects of PYC treatment (50 mg/kg/day, orally, for 28 days) on the DNA damage and biochemical changes in the blood, liver, and kidney tissues of experimental diabetic rats. Changes in the activities of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase enzymes, and the levels of 8-hydroxy-2′-deoxyguanosine, total glutathione, malondialdehyde, insulin, total bilirubin, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, high density lipoprotein, low density lipoprotein, total cholesterol, and triglyceride were evaluated. DNA damage was also determined in the whole blood cells and the liver and renal tissue cells using the alkaline comet assay. PYC treatment significantly ameliorated the oxidative stress, lipid profile, and liver function parameters as well as DNA damage in the hyperglycemic rats. The results show that PYC treatment might improve the hyperglycemia-induced biochemical and physiological changes in diabetes.

1. Introduction

Diabetes mellitus is a rising world health problem especially in developing countries (Whiting et al., 2011). The global prevalence of diabetes among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014 (WHO, 2016) and it is estimated to cross 5.4% by the year of 2025 (Chaturvedi, 2007; King et al., 1998; Shukla et al., 2011). Diabetes, a complex progressive metabolic disorder, is characterized by hyperglycemia with the disturbances of carbohydrate, lipid, and protein metabolism, which results from absolute or relative deficiencies in insulin secretion and/or insulin action (ADA, 2009; Cardinal et al., 2001; Golbidi et al., 2011). The chronic hyperglycemia in diabetes can lead to the long-term damages, dysfunctions, and failures of various organs, especially the blood vessels, heart, kidneys, eyes, and nerves (ADA, 2009).

Oxidative stress has an important role in the pathophysiology of diabetic complications. The glucose autoxidation observed in diabetes is the most important factor, which leads to the production of free radicals. High levels of free radicals and the simultaneous decline of antioxidant defense mechanisms may cause the damages of cellular organelles and macromolecules, and enzymes, the increases in the lipid peroxidation, and the development of insulin resistance (Maritim et al., 2003; Wei et al., 1997). It is suggested that type 2 diabetes (T2D) may be associated with the elevated level of oxidative DNA damage and also the increased susceptibility to mutagens and the decreased efficacy of DNA repair (Blasiak et al., 2004). In a study of Xavier et al. (2014), oxidative DNA damage was reported to be significantly reduced in T2D patients with one-week of glycemic control.

There is a growing interest in the reduction of complications of diabetes through diets including polyphenolic compounds such as flavanols (Dogan et al., 2017; Ola et al., 2018; Parmar et al., 2015; Parveen et al., 2013). Pycnogenol® (PYC), a standardized plant extract

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obtained from the bark of the French maritime pine *Pinus pinaster* containing 65–75% of catechin and epicatechin, is commonly consumed as a dietary food supplement. The radical scavenging activity of PYC against reactive oxygen and nitrogen species has been demonstrated in many studies. It was suggested to be effective in many chronic diseases including obesity, dyslipidaemia, inflammatory and immune system disorders, and diabetes (Gulati, 2015; Parveen et al., 2013). PYC (50 mg/kg body weight (b.w.)/day, orally, 8 weeks) ameliorated diabetes-induced cardiac dysfunction, probably by its metabolic and direct radical scavenging activity without affecting the molecular maladaptations of reactive oxygen species (ROS)-producing enzymes and cytoskeletal components (Klimas et al., 2010). PYC treatment with an intraperitoneal (i.p.) dose of 10 mg/kg b.w./day for 4 weeks protected hyperglycemia-induced oxidative damage in the liver of T2D rats through potentiating the antioxidant defense system (Parveen et al., 2010, 2013). It was also demonstrated to improve the altered parameters of glucose metabolism in 3T3-L1 adipocytes. PYC might be useful in maintaining blood glucose control, since it could stimulate glucose uptake via the PI3K dependent tyrosine kinase pathways involving Akt (protein kinase B) (Lee et al., 2010).

Considering the importance of oxidative stress in the pathophysiology of diabetic complications, PYC may protect tissues from oxidative stress-related damage in diabetes due to its strong antioxidant activity. Taking into account all the available knowledge, the present study was aimed to determine the effects of PYC on oxidative stress, lipid metabolism, and hepatic enzymes as well as its DNA protective effects in streptozotocin (STZ)-induced diabetic rats. Oxidative stress parameters including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) enzyme activities and 8-hydroxy-2′-deoxyguanosine (8-OHdG), total glutathione (GSH), and malondialdehyde (MDA) levels; biochemical parameters including insulin, total bilirubin, total protein; hepatic enzyme levels including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), and lipid profile parameters including high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol, and triglyceride levels were evaluated. DNA damage was determined in the blood, liver and renal tissue cells in the diabetic rats by the alkaline single cell gel electrophoresis (Comet assay) technique.

It is well known that oxidative stress leads to DNA damage, however the protective mechanisms of PYC supplementation on the hyperglycemia-induced genotoxicity are not well clarified (Lee and Chan, 2015; Sifuentes-Franco et al., 2018). Despite the fact that our study seems to be similar to the previous works, we investigated, for the first time, considerably more parameters together as well as determining DNA damage using Comet assay in our study. The results of our study may help to exhibit the role of this bioactive molecule in reducing/improving the diabetes complications due to oxidative stress and DNA damage. Our study may also provide a novel strategy for preventing the hyperglycemia-related complications.

2. Material and methods

2.1. Chemicals, kits and equipment

The chemicals used in the study were purchased from the following suppliers: Pycnogenol® (PYC) standardized to contain 70 ± 5% procyanidins and a registered trade mark of Horphag Research Ltd. (Geneva, Switzerland) was provided by Henkel Corporation (La Grange, IL, U.S.A.); dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), normal melting agarose (NMA), low melting point agarose (LMA), sodium chloride (NaCl), sodium hydroxide (NaOH), phosphate-buffered saline (PBS) tablets, potassium chloride (KCl), thiobarbituric acid, trichloroacetic acid, triton-X-100, ALT assay kit, AST assay kit, GGT assay kit, insulin assay kit, bicinchoninic acid (BCA) protein assay kit, HDL-LDL assay kit, total cholesterol assay kit, triglyceride assay kit, and STZ from Sigma-Aldrich Chemicals (St Louis, Missouri, USA); ethylendiaminetetraacetic acid disodium salt dehydrate (EDTA-Na_2), natriumlauroylsarcosinate, tris, 8-OHdG assay kit, SOD assay kit, CAT assay kit, GR assay kit, GPx assay kit, GST assay kit, and GSH assay kit from Cayman Chemicals Co. (Ann Arbor, MI, USA); ketamin hydrochloride from Eczacıbaşı (Istanbul, Turkey).

2.2. Animals and experimental design

Thirteen-week-old male Wistar albino rats (*Rattus norvegicus*) weighing 200–350 g, housed in stainless steel cages under a controlled temperature (23 ± 2 °C) and humidity (55%–60%) and with a 12-h light/dark cycle, were used in this study. Standard laboratory rat feed containing 21% protein and fresh drinking water were given *ad libitum*. The blood glucose levels of animals were measured at the beginning of the experiment. The animals were treated humanely with regard to alleviation of suffering, and the study protocol was designed according to the ethical standards for animal use and approved by the Ankara University Animal Ethical Committee (2015-12-138).

2.3. Experimental design

A total of 24 healthy adult male rats were randomly divided into four groups:

Group 1: Sham group (n = 6). This group consisted of animals treated only with saline orally for 28 days.

Group 2: Diabetic group (n = 6). This group consisted of animals in which only diabetes was induced and the animals were treated only with oral saline for 28 days.

Group 3: PYC-treated group (n = 6). This group consisted of animals treated orally with PYC at the dose of 50 mg/kg b.w./day (in saline) for 28 days.

Group 4: PYC-treated diabetic group (n = 6). This group consisted of animals treated with PYC orally at the dose of 50 mg/kg b.w./day (in saline) for 28 days following the induction of diabetes.

After 12 h of starvation, the animals subjected to T1D were treated with a single i.p. injection of STZ (60 mg/kg b.w.) in freshly prepared 0.01 M citrate buffer with a pH of 4.30) as previously described (Cumaoğlu et al., 2011).

Two days after the STZ injection, blood samples were obtained by tail prick to measure the blood glucose levels. After one week, diabetes was confirmed by fasting blood glucose value of 250 mg/dL higher using glucometer (Plusmed®). At the beginning of the experiment, the blood glucose levels of each diabetes-induced rat treated with/without PYC were higher than 250 mg/dL (Table 1). After that, the blood glucose levels and the weights of the rats were measured weekly until termination of the experiment (for 28 days). Insulin and other parameters were also measured in fasting state.

PYC dose (50 mg/kg b.w./day per oral) was selected according to the study of Jankyova et al. (2009). The acute toxicity of PYC is very low and the median lethal dose (LD_{50}) was reported to vary between 1000 mg/kg b.w. and 4000 mg/kg b.w. after oral administration in mice, rats, and guinea pigs (Rohdewald, 2002).

At the end of the experimental period, all animals were decapitated under the anesthesia (90 mg/kg b.w. ketamine hydrochloride, i.p.). Blood samples via cardiac puncture were collected into preservative-free heparin tubes for biochemical analysis and DNA damage evaluation. The serum and plasma were immediately separated by centrifugation and stored at −80 °C until being assayed. Liver and kidney tissues were carefully dissected from their attachments and totally excised. These tissues were divided into the parts to evaluate oxidative stress parameters and DNA damage. The organs were examined for changes in size, color, and texture. The liver and kidney tissues were weighted and extracted following the homogenization and sonication procedures (Sier et al., 1996). The blood and tissue samples were kept in the dark at 4 °C and processed within 4 h for Comet assay. The serum,

Table 1
Blood glucose levels and body weights of the study groups weekly.

Day		Sham group	Diabetic group	PYC treated group	PYC treated diabetic group
0^{th}	Glucose (mg/dl)	118.9 ± 13.9	526.0 ± 86.1[a]	122.5 ± 10.5[b]	501.1 ± 22.3[a]
	Body weights (g)	220.9 ± 7.3	248.5 ± 20.9	250.2 ± 21.6	285.1 ± 21.6[a]
7^{th}	Glucose (mg/dl)	124.7 ± 14.0	425.0 ± 92.4[a]	124.9 ± 14.2[b]	426.5 ± 55.4[a]
	Body weights (g)	233.4 ± 11.7	266.0 ± 29.5	263.8 ± 10.9	279.5 ± 25.2
14^{th}	Glucose (mg/dl)	133.4 ± 13.2	465.0 ± 10.0[a]	121.4 ± 12.7[b]	386.3 ± 37.2[a,b]
	Body weights (g)	245.1 ± 12.6	225.0 ± 10.0	258.7 ± 15.9	282.5 ± 48.8
21^{sh}	Glucose (mg/dl)	120.4 ± 14.2	419.9 ± 141.1[a]	123.5 ± 19.9[b]	251.8 ± 25.8[a,b]
	Body weights (g)	249.1 ± 12.8	253.0 ± 33.1	252.4 ± 17.8	298.9 ± 58.2
28^{th}	Glucose (mg/dl)	156.1 ± 29.4	408.5 ± 92.1[a]	132.5 ± 24.5[b]	208.6 ± 38.4[a,b]
	Body weights (g)	259.9 ± 13.6	255.0 ± 33.9	251.9 ± 17.3	301.4 ± 49.3

The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®.

plasma, liver, and kidney homogenates were kept in −80 °C for the determination of oxidative stress parameters, hepatic enzyme levels, and lipid profiles until the time of analysis.

2.4. Determination of plasma biochemical parameters

The determination of insulin, total bilirubin, and total protein levels in the plasma samples were performed spectrophotometrically using assay kits at 450 nm, 530 nm, and 562 nm, respectively. The samples were analyzed in duplicate. The results were given as the mean ± standard deviation for six rats in each group and expressed as μIU/ml, mg/dl, and μg/ml, respectively.

2.5. Determination of liver enzyme levels

The liver enzyme levels in the serum samples were determined spectrophotometrically using assay kits for ALT, AST and GGT at 570 nm, 450 nm, and 418 nm, respectively. The samples were analyzed in duplicate. The results were given as the mean ± standard deviation for six rats in each group and expressed as mU/ml.

2.6. Determination of serum lipid levels

HDL, LDL, total cholesterol, and triglyceride levels in the serum samples were determined spectrophotometrically using assay kits at 570 nm. The samples were analyzed in duplicate. The results were given as the mean ± standard deviation for six rats in each group and expressed as μg/ml for HDL and LDL, mg/dl for total cholesterol.

2.7. Determination of oxidative stress parameters

Oxidative stress parameters were assayed in the plasma samples, the liver and kidney homogenates. The determinations of CAT, SOD, GPx, GR, GST enzyme activities, GSH, and MDA levels in the plasma, liver and renal tissue samples at 540 nm, 440 nm, 340 nm, 340 nm, 340 nm, 420 nm, and 535 nm, respectively and 8-OHdG levels in the plasma samples at 440 nm were performed spectrophotometrically using kits following the manufacturer's procedures. The samples were analyzed in duplicate. The results were given as the mean ± standard deviation for six rats in each group and expressed as nmol/min/ml for enzyme activities, μM for GSH, nmol/g for MDA levels, and pg/ml for 8-OHdG.

2.8. Determination of the DNA damage by the alkaline comet assay

The single cell suspensions from the liver and kidney tissue samples were prepared and the alkaline Comet assay to determine DNA damage was carried out according to the standard procedures (Aydın et al., 2013; Tice et al., 2000). The whole blood samples were used for the evaluation of DNA damage. The concentrations of renal and hepatic tissue cells in the supernatant were adjusted to approximately 2×10^5 cells/ml in HBSS containing 20 mM EDTA/10% DMSO. The concentrations of lymphocytes were adjusted to 2×10^5 cells/ml in PBS.

A fifty micro liter of the cell suspension was mixed with 75 μl of 0.65% LMA. Then the suspensions were embedded on the slides pre-coated with a layer of 1% NMA. The slides were allowed to solidify on ice for 5 min. Then coverslips were removed. The slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium sarcosinate, pH 10.0), with 1% Triton X-100 and 10% DMSO was added just before use for a minimum of 1 h at 4 °C. Then they were removed from the lysing solution, drained and left in the electrophoresis solution (1 mM sodium EDTA and 300 mM NaOH, pH 13.0) for 20 min at 4 °C to allow unwinding of the DNA and expression of alkali-labile damage. Electrophoresis was conducted also at room temperature (20–25 °C) for 20 min using 25 V and adjusting the current to 300 mA by rising or lowering the buffer level. The slides were neutralized by washing 3 times in 0.4 M Tris–HCl (pH 7.5) for 5 min at room temperature. After neutralization, the slides were incubated in 50%, 75%, and 99% of alcohol for 5 min, successively. The dried microscope slides were stained with ethidium bromide (EtBr 20 μg/ml in distilled water) and covered with a cover-glass prior to analysis with a Leica® fluorescence microscope under green light. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel.

In order to visualize DNA damage, one hundred cells from two replicate slides were examined at x400 magnification for each experiment. The results were given as the mean ± standard deviation for six rats in each group and DNA damage was expressed as a percentage of DNA in tail (tail intensity).

2.9. Statistical analysis

Analysis of data was performed using the computer program SPSS 20.0 for Windows. The distribution of the data was checked for normality using the Kolmogorov Smirnov test. The homogeneity of the variance was verified by the Levene test. The differences among the groups with normal distribution were evaluated by the one-way variance analysis (ANOVA) test and post hoc analysis of group differences was performed by the LSD test. The differences among the groups without normal distribution were evaluated by Kruskal Wallis test. The results were given as the mean ± standard deviation. The p-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Blood glucose levels

The fasting blood glucose levels of the study groups are given in

Table 2
Plasma biochemical parameters of the study groups.

	Sham group	Diabetic group	PYC treated group	PYC treated diabetic group
Insulin (μIU/ml)	192.4 ± 51.0	68.1 ± 65.8[a]	287.5 ± 40.0[b]	126.9 ± 35.3[b]
Total bilirubin (mg/ml)	4.4 ± 4.7	7.7 ± 10.8	1.5 ± 1.0	2.4 ± 1.9
Total protein (μg/ml)	3668 ± 69	3727 ± 25	3756 ± 65	3698 ± 48

The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®.

Table 1. The blood glucose levels were significantly increased in the diabetic group compared to sham group ($p < 0.05$). The blood glucose levels in the PYC treated diabetic group were found to be significantly lower than in the diabetic group at the14th, 21st, and 28th days of the treatment ($p < 0.05$). There was no significant difference between the sham group and the PYC treated group in the blood glucose levels ($p > 0.05$).

3.2. Biochemical parameters

The plasma biochemical parameters of the study groups are given in Table 2. The insulin levels in the diabetic group were found to be significantly lower than in the sham group ($p < 0.05$). The insulin levels of the PYC treated diabetic group were significantly increased when compared to the diabetic group ($p < 0.05$). The insulin levels in both the PYC treated group and the PYC treated diabetic group were not found to be different than in the sham group ($p > 0.05$). There were no significant changes in both total bilirubin and total protein levels between groups, although PYC seemed to decrease total bilirubin levels.

3.3. Lipid levels

The serum HDL, LDL, total cholesterol, and triglyceride levels of the study groups are given in Table 3. LDL, total cholesterol, and triglyceride levels in the diabetic group were significantly higher than in the sham group ($p < 0.05$), while HDL levels in the diabetic group were significantly lower than in the sham group ($p < 0.05$). LDL, total cholesterol, and triglyceride levels in the PYC treated diabetic group were significantly decreased when compared to the diabetic group ($p < 0.05$). HDL levels in the PYC treated diabetic group were significantly increased when compared to the diabetic group ($p < 0.05$). Triglyceride levels in the PYC treated diabetic group were found to be significantly higher than in the sham group ($p < 0.05$). The levels of HDL, LDL, and total cholesterol in the PYC treated group and the PYC treated diabetic group were not different than in the sham group ($p > 0.05$).

3.4. Liver enzyme levels

The serum ALT, AST, and GGT levels of the study groups are given in Table 4. ALT, AST, and GGT levels were significantly increased in the diabetic group when compared to the sham group ($p < 0.05$). PYC treatment significantly decreased AST and GGT levels in the diabetic rats ($p < 0.05$), while there were no significant changes in ALT levels ($p > 0.05$). The changes in ALT and AST levels in the PYC treated diabetic group were not found to be different than in the sham group ($p > 0.05$), while GGT levels in the PYC treated diabetic group were found to higher than in the sham group ($p < 0.05$). All the studied liver enzyme levels in the PYC treated group were not significantly different than in the sham group ($p > 0.05$).

3.5. Oxidative stress parameters

The enzyme activities of CAT, SOD, GPx, GR, and GST and the levels of GSH and MDA in the plasma, liver and kidney tissue samples are given in Tables 5–7, respectively. The plasma 8-OHdG levels are also given in Table 5. CAT, SOD, GPx, and GST enzyme activities and GSH levels in the diabetic group were found to be significantly lower than in the sham group for the plasma, liver and kidney tissue samples (Table 5, Table 6, and Table 7, respectively) ($p < 0.05$). GR enzyme activities and MDA levels in the diabetic group were found to be significantly higher than in the sham group for the plasma, liver and kidney tissue samples (Tables 5–7, respectively) ($p < 0.05$). The plasma 8-OHdG levels in the diabetic group were found to be significantly higher than in the sham group (Table 5) ($p < 0.05$).

The CAT, SOD, GPx, GST enzyme activities and GSH levels in the PYC treated diabetic group were significantly increased when compared to the diabetic group for the plasma, liver and kidney tissue samples (Tables 5–7, respectively) ($p < 0.05$). GR enzyme activities and MDA levels were significantly decreased when compared to the diabetic group for the plasma, liver and kidney tissue samples (Tables 5–7, respectively) ($p < 0.05$). The plasma 8-OHdG levels in the PYC treated diabetic group were significantly decreased when compared to the diabetic group (Table 5) ($p < 0.05$).

No significant changes were observed in CAT, SOD, GPx, GR, and GST enzyme activities and GSH and MDA levels in the plasma, liver and kidney tissue samples between the PYC treated group and the sham group ($p > 0.05$). There was also no significant difference between the PYC treated group and the sham group in the plasma 8-OHdG levels ($p > 0.05$).

3.6. DNA damage

DNA damage expressed as DNA tail intensity for the blood, liver and kidney tissue cells of animals are shown in Fig. 1. In all samples, DNA damage in the diabetic rats were found to be significantly higher than in the sham group ($p < 0.05$). DNA damage in the PYC treated diabetic group was found to be significantly decreased when compared to the

Table 3
Serum lipid levels of the study groups.

	Sham group	Diabetic group	PYC treated group	PYC treated diabetic group
HDL (μg/ml)	0.70 ± 0.16	0.34 ± 0.29[a]	0.64 ± 0.10[b]	0.61 ± 0.13[b]
LDL (μg/ml)	0.33 ± 0.05	0.52 ± 0.05[a]	0.33 ± 0.12[b]	0.38 ± 0.16[b]
Cholesterol (μg/ml)	0.28 ± 0.04	0.69 ± 0.05[a]	0.28 ± 0.01[b]	0.27 ± 0.03[b]
Triglyceride (mg/dl)	159.0 ± 26.1	514.5 ± 289.0[a]	148.4 ± 44.7[b]	357.3 ± 232.4[a,b]

The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®; HDL: high density lipoprotein; LDL: low density lipoprotein.

Table 4
Liver enzyme levels in the serum samples of the study groups.

	Sham group	Diabetic group	PYC treated group	PYC treated diabetic group
ALT (mU/ml)	1984 ± 2348	8457 ± 8684[a]	1309 ± 418[b]	6195 ± 6038
AST (mU/ml)	79.2 ± 124.9	1536 ± 1925[a]	117.2 ± 183.1[b]	53.6 ± 107.1[b]
GGT (mU/ml)	1926 ± 1696	4213 ± 4158[a]	991 ± 1886[b]	2903 ± 2607[a,b]

The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®; ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: Gamma-glutamyl transpeptidase.

diabetic group in all samples (Fig. 1A, B, 1C) ($p < 0.05$). The reduction in DNA damage of the blood and liver cells in the PYC treated diabetic group was found to be at the sham group level (Fig. 1A and B) ($p > 0.05$). However, the DNA tail intensity of renal cells in the PYC treated diabetic group was found to be significantly higher than in the sham group (Fig. 1C) ($p < 0.05$). In all samples, there were no statistically significant differences in DNA tail intensity between the sham group and the PYC treated groups (Fig. 1A, B, 1C) ($p > 0.05$).

4. Discussion

The possible ameliorative effects of PYC administration (50 mg/kg b.w./day, orally, 28 days) on the diabetes-induced oxidative damage and genotoxicity in the blood, liver and kidney tissues of rats were investigated in our study. The most notable result of the present research was the fact that PYC improved all oxidative stress parameters and DNA damage in the rats with STZ-induced T1D.

Diabetes mellitus is a complex progressive metabolic disorder that occurs in a genetically susceptible human population as a result of the loss of the insulin-producing pancreatic β cells (Cardinal et al., 2001; Whiting et al., 2011). STZ, a highly selective cytotoxic agent on pancreatic islet β-cell of Langerhans, causes a prominent reduction in insulin release, thereby inducing hyperglycemia. It is widely used experimentally to achieve a model of T1D (Cumaoğlu et al., 2011; Furman, 2015; Okamoto, 1985). STZ is actively transported into pancreatic β cells via the Glut-2 glucose transporter. It reacts at many sites in DNA but in particular at the ring nitrogen and exocyclic oxygen atoms of the DNA bases and a single high dose of STZ also produces a large number of DNA strand breaks.

Hyperglycemia is commonly followed by hyperlipidemia in diabetes (Poitout and Robertson, 2008). The glucotoxicity and lipotoxicity in diabetes should be considered, since hyperglycemic and hyperlipidemic abnormalities leads destructive effects in different tissues of the organism, hence the serious health complications such as liver disease, renal failure, cardiovascular problem, blindness, neuropathy are emerged (Chaturvedi, 2007; Gite et al., 2017). Hyperglycemia results in the over production of reactive oxygen/nitrogen species in various tissues, which leads to tissue damage with lipid peroxidation and protein oxidation, along with disruption in cellular homeostasis and accumulation of damaged molecules. The decreases in antioxidant defenses and the increases in oxidative stress are generally responsible for the development of diabetic complications via glucose autoxidation, mitochondria dysfunction, polyol pathway, and protein glycation (Cumaoğlu et al., 2011; Dymkowska, 2016; Maritim et al., 2003; Negre-Salvayre et al., 2009; Valko et al., 2007).

In our experimental diabetes model on the day of 28th, STZ treatment caused a decrease in insulin release and an increase in blood glucose levels and the changes in the lipid profile (increases in the serum levels of LDL, cholesterol, and triglyceride and decreases in HDL) in Wistar albino rats, which indicates significant hyperglycemia and dyslipidemia in diabetes (Olatunji et al., 2017; Ramachandran et al., 2017; Sato et al., 2017). Consistent with our study, the high plasma glucose levels and low insulin levels were demonstrated in the diabetic rats (Murali et al., 2013). The increases in the serum total cholesterol, triglyceride, and LDL levels and the decreases in the serum HDL levels and liver GSH levels were also reported in diabetic rats on the 14th day of experimental diabetes model (Júnior et al., 2017).

The cellular macromolecules including lipids, proteins, and DNA are the common targets of oxidation. Of these biological targets of oxidative stress, lipids are the prominent of most involved class of biomolecules. Various secondary products including MDA are produced followed by lipid oxidation. MDA is one of the many reactive electrophile species that cause damage to cells and form covalent protein adducts, called advanced lipo-oxidation end products. It forms mutagenic DNA adducts when it reacts with deoxyadenosine and deoxyguanosine in DNA (Del Rio et al., 2005). The elevations in lipid peroxidation in the blood and tissues of diabetic rats have been demonstrated in many studies (Maritim et al., 2003; Parmar et al., 2015; Ramesh and Pugalendi, 2006; Shamsaldeen et al., 2018; Valko et al., 2007). In diabetic mice, the significant increases in MDA content in the liver, spleen, and kidney tissues of diabetic mice were observed on the 7th day of diabetes. In that study, peroxidative damage was suggested to be the reason for the diabetes-related hepatic and renal dysfunctions (Oršolić et al., 2013). In another study, MDA, protein carbonyl, and 8-OHdG levels in the plasma and the liver homogenates of diabetic rats on the 8th week of diabetes were found to be higher than controls. The reduction of the total thiol and GSH levels in plasma and liver tissue were also observed in the diabetic rats (Korkmaz et al., 2012). The levels of 8-OHdG on the 4th week of diabetes in rats were higher than the untreated control (Kushwaha and Jena, 2013). Consistent with these

Table 5
Plasma oxidative stress parameters of the study groups.

	Sham group	Diabetic group	PYC treated group	PYC treated diabetic group
CAT (nmol/min/ml)	153.7 ± 13.3	89.3 ± 34.2[a]	158.2 ± 11.2[b]	160.7 ± 4.7[b]
SOD (U/ml)	0.96 ± 0.66	0.40 ± 0.31[a]	1.32 ± 0.18[b]	0.97 ± 0.35[b]
GPx (nmol/min/ml)	114.4 ± 2.4	42.4 ± 9.4[a]	110.1 ± 5.4[b]	95.4 ± 5.4[a,b]
GR (nmol/min/ml)	2.47 ± 0.48[b]	9.10 ± 3.51[a]	3.12 ± 0.91[b]	3.42 ± 0.41[b]
GST (nmol/min/ml)	7.68 ± 0.76	3.74 ± 2.04[a]	9.02 ± 0.57[b]	5.72 ± 0.49[a,b]
GSH (μM)	4.16 ± 1.28	2.11 ± 0.16[a]	4.51 ± 1.03[b]	3.24 ± 0.12[a,b]
MDA (nmol/g)	11.4 ± 4.1[b]	26.4 ± 4.4[a]	12.1 ± 3.1[b]	13.5 ± 3.4[b]
8-OHdG (ng/ml)	11.1 ± 5.9[b]	20.1 ± 7.0[a]	11.9 ± 7.0[b]	4.8 ± 3.3[a,b]

The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®; CAT: catalase, SOD: superoxide dismutase; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione S transferase; GSH: glutathione; 8-OHdG: 8-hydroxydeoxyguanosine; MDA: malondialdehyde.

Table 6
Hepatic oxidative stress parameters of the study groups.

	Sham group	Diabetic group	PYC treated group	PYC treated diabetic group
CAT (nmol/min/ml)	15.1 ± 2.2[b]	10.5 ± 4.7[a]	12.9 ± 5.4[b]	17.1 ± 0.9[b]
SOD (U/ml)	0.45 ± 0.30[b]	0.24 ± 0.15[a]	0.38 ± 0.21[b]	0.33 ± 0.21[b]
GPx (nmol/min/ml)	124.1 ± 2.5[b]	56.5 ± 5.4[a]	126.8 ± 5.4[b]	91.1 ± 7.7[a,b]
GR (nmol/min/ml)	3.74 ± 1.28[b]	11.38 ± 6.26[a]	4.67 ± 3.14[b]	7.49 ± 1.51[a,b]
GST (nmol/min/ml)	62.6 ± 19.8	44.5 ± 2.9[a]	70.3 ± 10.9[b]	73.9 ± 8.8[b]
GSH (μM)	9.94 ± 3.62	5.42 ± 0.58[a]	9.58 ± 2.84[b]	6.42 ± 0.73[b]
MDA (nmol/g)	13.4 ± 5.1	24.3 ± 4.7[a]	13.8 ± 8.5[b]	16.2 ± 2.7[b]

The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®; CAT: catalase, SOD: superoxide dismutase; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione S transferase; GSH: glutathione; MDA: malondialdehyde.

findings, our study also showed that the levels of MDA in plasma, liver, and kidney and the levels of 8-OHdG in plasma of diabetic rats were higher than the untreated control, which indicates the increased free radical generation. The association between genotoxicity and diabetes has been also well demonstrated. Oxidative stress and DNA damage were increased in diabetic patients, (Al-Aubaidy and Jelinek, 2011; Binici et al., 2013; Tatsch et al., 2015). The elevations in metabolic abnormalities such as insulin resistance and inflammatory activation, as well as ROS generation factors may be responsible for the oxidative DNA damage in diabetes (She et al., 2017; Tatsch et al., 2015).

The changes in the activities of antioxidant enzymes including CAT, SOD, GPx, and GR in diabetes have been documented in many studies. However, there are conflicting results. In diabetic patients, both increases and decreases in the activities of key antioxidant enzymes including CAT, SOD, GPx, and GR have been observed (Godin et al., 1988; Kumawat et al., 2013; Miranda-Díaz et al., 2016). The increases in the levels of lipid peroxidation markers (thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and conjugated dienes), and the decreases in non-enzymatic antioxidants (vitamin C and GSH in the plasma and liver) and antioxidant enzymes (SOD, CAT and GPx in the liver) were determined in the diabetic rats on the 45th day of diabetes (Ramesh and Pugalendi, 2006). In our experimental model, the increases in MDA levels and GR and GST enzyme activities and the decreases in GSH levels and CAT, SOD, and GPx enzyme activities in the plasma, liver, and kidney samples were also observed.

The liver is an important metabolic organ and insulin and other metabolic hormones regulate its metabolic activity. It is an insulin dependent tissue that plays a pivotal role in glycolysis, gluconeogenesis, and lipid homeostasis. The diabetic process can disturb this metabolic homeostasis (Gupta et al., 1999; Hiroshi et al., 1989). The prognostic values of AST, ALT, and GGT as a biomarker of the impaired glucose metabolism have been demonstrated in numerous studies on patients with diabetes with conflicting results (Monami et al., 2008; Nannipieri et al., 2005). In a two-year cohort study, in which ALT and GGT enzyme levels were examined for the prediction of diabetes and cardiovascular disease, high rates of elevated ALT and GGT were reported to be associated with diabetes (Monami et al., 2008). High rates of elevated GGT levels were also noted among diabetic patients (Whitfield, 2001). In a systematic review and a meta-analysis of 21 prospective, population-based studies from the adult-women with diabetes, ALT and GGT levels were reported to be increased in diabetes and GGT was found to be a better diabetes predictor than ALT (Fraser et al., 2009). Consistent with the previous studies, in our study the liver function parameters in the diabetic rats were significantly altered when compared to the sham group. The elevated levels of ALT, AST, and GGT were noticed in diabetic rats, which may be possibly by STZ-mediated hepatic damage. We also showed that there were no significant differences in the total bilirubin and total protein levels in the plasma samples between diabetic rats and healthy controls.

There is a growing interest in the prevention of diabetes through dietary supplementation with antioxidant compounds such as flavanols due to their proven benefits on health and their low toxicity and cost (Martin et al., 2017; Rotimi et al., 2018). Polyphenolic compounds present in many plants and natural products are considered to be potential therapeutic natural antioxidant agents against a wide range of disorders such as cancer, neurodegenerative and inflammatory diseases, ageing, and diabetes (Anlar et al., 2018; Bacanlı et al., 2017; Kim et al., 2016; Oršolić et al., 2013; Parmar et al., 2015; Srinivasan et al., 2007; Taner et al., 2014).

PYC, a standardized extract of French maritime pine bark, has been widely consumed as a dietary supplement due to its probable antioxidant activity and radical scavenging properties based primarily on its procyanidin content (Parveen et al., 2010, 2013; Siler-Marsiglio et al., 2004; Virgili et al., 1998; Wei et al., 1997). The components of PYC are reported to be highly bioavailable and it has been suggested that the extract itself has more potent effects than its individual components (Chen et al., 2009). The flavonoids in PYC, which are composed of one or more aromatic rings bearing one or more hydroxyl groups, can readily combine with free radicals forming resonance-stabilized phenoxyl radicals (Rice-Evans et al., 1996). The multiple hydroxyl groups and catechol rings are suggested to be responsible for its radical scavenging effects (Ki et al., 2011; Kim and Yokozawa, 2009; Ordoudi and Tsimidou, 2006). PYC is commonly used as food supplement in various food and beverages. There is no specific dosage

Table 7
Renal oxidative stress parameters of the study groups.

	Sham group	Diabetic group	PYC treated group	PYC treated diabetic group
CAT(nmol/min/ml)	166.5 ± 8.2	65.2 ± 3.8[a]	168.1 ± 11.3[b]	116.3 ± 25.3[a,b]
SOD (U/ml)	0.86 ± 0.55	0.35 ± 0.07[a]	0.91 ± 0.41[b]	0.72 ± 0.17[b]
GPx (nmol/min/ml)	149.5 ± 9.5	64.5 ± 1.5[a]	148.4 ± 9.4[b]	129.4 ± 8.4[b]
GR (nmol/min/ml)	4.46 ± 1.47	12.03 ± 3.47[a]	4.13 ± 1.42[b]	6.13 ± 2.45[b]
GST (nmol/min/ml)	7.61 ± 0.67	3.67 ± 2.02[a]	8.42 ± 1.44[b]	6.12 ± 2.34[b]
GSH (μM)	8.37 ± 0.57	5.28 ± 0.11[a]	8.47 ± 1.29[b]	7.52 ± 0.78[b]
MDA (nmol/g)	12.4 ± 4.0	23.9 ± 6.1[a]	12.8 ± 2.4[b]	16.1 ± 5.4[b]

The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®; CAT: catalase, SOD: superoxide dismutase; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione S transferase; GSH: glutathione; MDA: malondialdehyde.

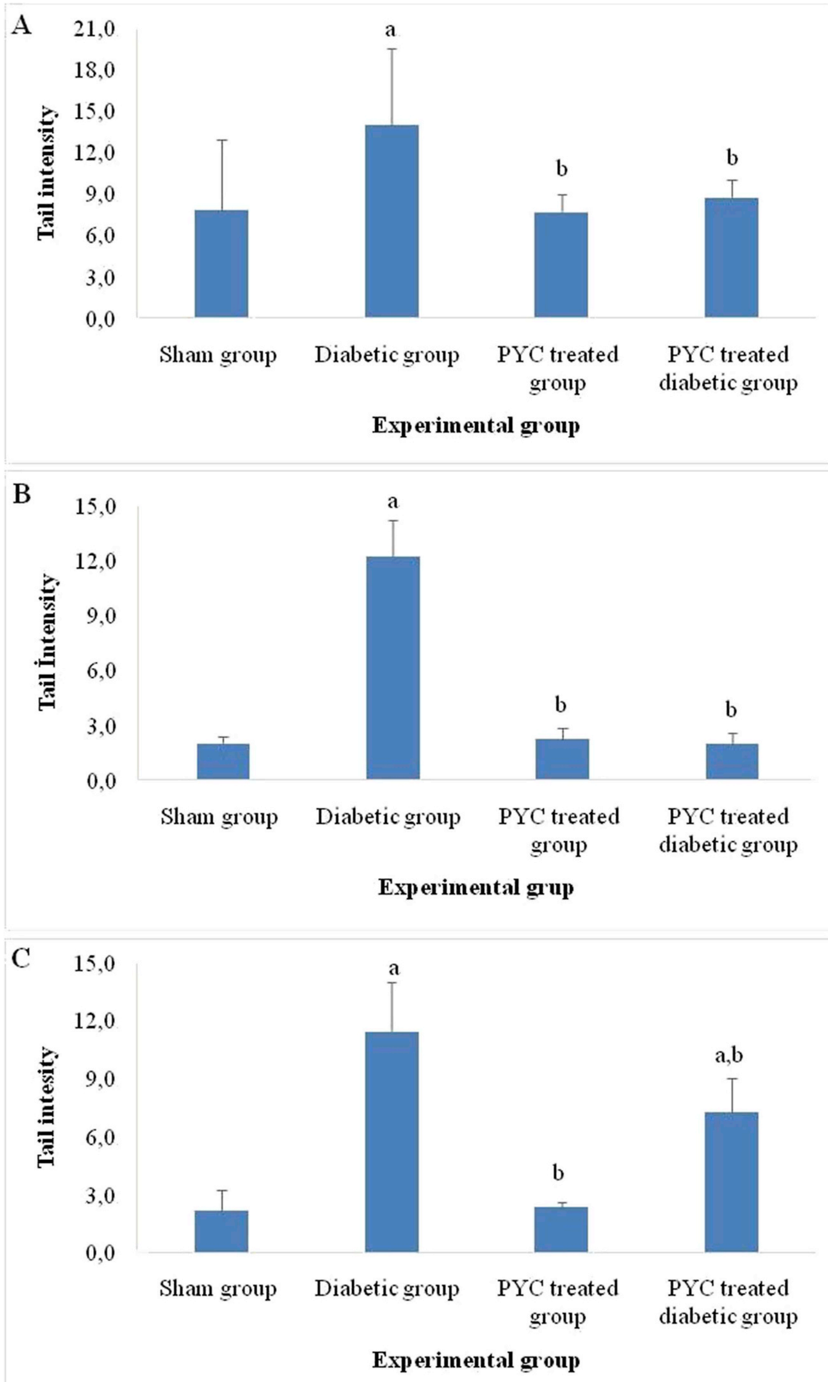

Fig. 1. Effects of pycnogenol® administration on DNA damage in (A) the blood; (B) the liver cells; (C) the renal cells of the experimental groups. The results are expressed as the mean ± standard deviation for six rats in each group. [a]Statistically different from sham group ($p < 0.05$); [b]Statistically different from diabetic group ($p < 0.05$). PYC: pycnogenol®.

recommendation and dosages can vary between supplements. The oral dose of PYC is generally around 1.5 and 3 mg/kg b.w./day, which represents a daily dose of 100–200 mg for an adult (Masquelier, 1987; Mülek et al., 2017; Rohdewald, 2002).

PYG has been suggested to protect tissues from oxidative stress-related damage in diabetes due to its strong antioxidant activity. In the study of Parveen et al. (2013), PYC treatment (10 mg/kg b.w./day, orally, 4 weeks) significantly decreased TBARS and protein carbonyl levels and increased GSH levels, GST and CAT activities in the liver and pancreas of the diabetic rats. The elevated levels of serum nitric oxide, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β were also decreased in diabetic rats with PYC treatment. Maritim et al. (2003) observed that GSH levels, GSH/disulphide reductase (GSSG-R) ratio, the activities of endogenous antioxidant enzymes (SOD, CAT, GPx, GSSG-R), and the activity of GGT an enzyme in the pathway of glutathione synthesis were increased in diabetic rats treated with PYC. Decreased retinal γ-GT activity in diabetic rats and elevated activity of SOD in diabetic retina due to the oxidative stress were normalized by PYC (Dene et al., 2005; Berryman et al., 2004). Considering its preventive potential in diabetes, it is concluded that PYC supplementation might reduce or prevent diabetic complications.

It was reported to inhibit α-glucosidase, an enzyme of the intestinal brush border. The inhibition of α-glucosidase decreases glucose reabsorption and postprandial hyperglycemia (Kim et al., 2005). It has been concluded that the mechanism of PYC to produce antidiabetic effect is through the inhibition of α-glucosidase independent of increased insulin secretion (Gulati, 2015; Schafer and Hogger, 2007).

There are limited studies on the combination of PYC with antidiabetic drugs, which should be concerned. Jankyova et al. (2016) investigated the effects of PYC as add-on drug to metformin therapy in diabetic rats. They reported that PYC and metformin improved the blood glucose levels, vascular reactivity, left ventricular hypertrophy, expression of AMP-activated protein kinase, glucose transporter 4, and calcium/calmodulin-dependent protein kinase II in left ventricle of the hearts. Nevertheless, the combination of these interventions did not have a higher efficacy (Jankyova et al., 2016).

In a double blind control study, 100 mg/day b.w./day orally dose of PYC for 12 weeks were given to diabetic patients (n = 77, age 45–66 years) on-going their antidiabetic medication. PYC significantly decreased plasma glucose higher than did placebo at all-time intervals. Glycated haemoglobin (HbA1c) was reduced until one month (Liu et al., 2004). In a study of Cesarone et al. (2006), on-going their antidiabetic medication were given to the patients with severe diabetic microangiopathy (n = 60, age 55–68 years) at 150 mg/day orally dose of PYC for 4 weeks. The findings of microcirculation at rest, venoarteriolar response, and ankle swelling were significantly improved compared with baseline or untreated control group.

In accordance with the previous studies, we revealed the ameliorative effects of 50 mg/kg b.w./day orally doses of PYC treatment for 28 days against the hyperglycemia-associated changes in diabetic rats. PYC alone did not significantly change blood glucose levels, plasma biochemical parameters (insulin, total bilirubin, and total protein), liver functions (ALT, AST, and GGT), serum lipid levels, all oxidative stress parameters, and DNA damage in the blood, liver, and kidney of Wistar albino rats when compared to the sham group. We demonstrated that PYC regulated the diabetes-induced alterations. The administration of PYC provided a significant improvement in insulin levels. This treatment also reduced blood glucose level, despite being not at the control level. Without changes in ALT levels, serum AST and GGT levels in the PYC treated diabetic rats decreased to control levels. PYC treatment improved serum lipid levels including HDL, LDL, and total cholesterol in diabetic rats and it also lowered triglyceride levels even though it was not at control level. Clinical findings on PYC in healthy volunteers have also shown that PYC significantly reduced blood LDL levels and increased HDL levels based on its antioxidant activity (Devaraj et al., 2002; Durackova et al., 2003). We also demonstrated that PYC treatment improved all oxidative stress parameters in blood, liver, and kidney of rats. It increased CAT, SOD, GPx, and GST activities and GSH levels and decreased GR activities, MDA and 8-OHdG levels in the plasma, liver and kidney tissue samples of diabetic rats. It is estimated that PYC administration may reduce oxidative stress-related damage in diabetes and therefore improve or delay diabetes complications.

In our previous study, the preventive effects of PYC (100 mg/kg b.w./day, i.p., 28 days) on oxidative stress parameters (SOD, and GPx activities and GSH and MDA levels), an inflammatory cytokine (TNF-α level) and DNA damage were demonstrated in the septic rats. PYC decreased TNF-α level in plasma, MDA levels and increased GSH levels, SOD and GPx activities in the liver and kidney tissues of septic rats (Taner et al., 2014).

It seems that the molecular mechanisms of PYC on diabetes should be put forward by more experiments. The novelty of our study is that the protective effects of PYG administration on the hyperglycemia-induced oxidative stress parameters were evaluated together with DNA damage. Our study may clarify the mechanisms of how PYC supplementation prevents the tissue damages in diabetes. In the study of Parveen et al. (2013), the effect of PYC (10 mg/kg b.w./day, i.p., 4 weeks) on diabetes was partially demonstrated, although no enough studies have been performed on the diabetes-induced DNA damage of PYC administered orally. In the lymphocytes, kidney, and liver tissue cells of the septic rats, PYC treatment significantly decreased oxidative DNA damage using Comet assay (Taner et al., 2014). Chovanová et al. (2006) reported that PYC supplementation reduced urine 8-OHdG levels in the children suffered from the attention-deficit-hyperactivity-disorder excreting significantly enhanced 8-OHdG in urine, which indicates that PYC protects DNA from damage.

In the present study, diabetes-induced DNA damage was found to be improved by PYC administration (50 mg/kg b.w./day orally, 28 days) in the blood, liver, and kidney of rats, despite being not at control levels. We suggest that the hyperglycemia-induced oxidative stress might be the reason for the DNA damage. PYC treatment may have a preventive role on the oxidative damage by ameliorating the antioxidant status and DNA damage in rats through its free radical scavenging property.

5. Conclusion

In conclusion, the treatment of PYC at the orally dose of 50 mg/kg b.w./day for 28 days was found to be protective against tissue injury through reducing oxidative stress and genotoxicity in the blood, liver, and kidney of diabetic rats. This is the important finding providing evidence that PYC administration might be protective against diabetes-induced DNA damage, which might light the way for the possible beneficial effects of PYC against diabetes related disorders in the clinic and follow-up process of patient. Considering its preventive potential in diabetes, PYC supplementation may reduce or prevent diabetic complications by ameliorating the hyperglycemia-induced changes. Finally, the molecular mechanisms for the various biological activities of PYC are yet to be determined, and need to be confirmed for its therapeutical potential.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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