Pterostilbene protects cochlea from ototoxicity in streptozotocin-induced diabetic rats by inhibiting apoptosis

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Abstract

Diabetes mellitus (DM) causes ototoxicity by inducing oxidative stress, microangiopathy, and apoptosis in the cochlear sensory hair cells. The natural anti-oxidant pterostilbene (PTS) (trans-3,5-dimethoxy-4-hydroxystilbene) has been reported to relieve oxidative stress and apoptosis in DM, but its role in diabetic-induced ototoxicity is unclear. This study aimed to investigate the effects of dose-dependent PTS on the cochlear cells of streptozotocin (STZ)-induced diabetic rats. The study included 30 albino male Wistar rats that were randomized into five groups: non-diabetic control (Control), diabetic control (DM), and diabetic rats treated with intraperitoneal PTS at 10, 20, or 40 mg/kg/day during the four-week experimental period (DM + PTS10, DM + PTS20, and DM + PTS40). Distortion product otoacoustic emission (DPOAE) tests were performed at the beginning and end of the study. At the end of the experimental period, apoptosis in the rat cochlea was investigated using caspase-8, cytochrome-c, and terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL). Quantitative real-time polymerase chain reaction was used to assess the mRNA expression levels of the following genes: CASP-3, BCL-associated X protein (BAX), and BCL-2. Body weight, blood glucose, serum insulin, and malondialdehyde (MDA) levels in the rat groups were evaluated. The mean DPOAE amplitude in the DM group was significantly lower than the means of the other groups (0.9–8 kHz; P < 0.05 for all). The Caspase-8 and Cytochrome-c protein expressions and the number of TUNEL-positive cells in the hair cells of the Corti organs of the DM rat group were significantly higher than those of the other groups (0.9–8 kHz; P < 0.001 for all). A dose-dependent increase of the mean DPOAE amplitudes was observed with PTS treatment (P < 0.05 for all). The Caspase-8 and Cytochrome-c protein expressions and the number of TUNEL-positive cells in the hair cells of the Corti organs of the DM rat group were significantly higher than those of the PTS treatment and control groups (DM > DM + PTS10 > DM + PTS20 > DM + PTS40 > Control; P < 0.05 for all). PTS treatment also reduced cell apoptosis in a dose-dependent manner by increasing the mRNA expression of the anti-apoptosis BCL2 gene and by decreasing the mRNA expressions of both the pro-apoptosis BAX gene and its effector CASP-3 and the ratio of BAX/BCL-2 in a dose-dependent manner (P < 0.05 compared to DM for all). PTS treatment significantly improved the metabolic parameters of the diabetic rats, such as body weight, blood glucose, serum insulin, and MDA levels,
consistent with our other findings ($P < 0.05$ compared to DM for all). PTS decreased the cochlear damage caused by diabetes, as confirmed by DPOAE, biochemical, histopathological, immunohistochemical, and molecular findings. This study reports the first in vivo findings to suggest that PTS may be a protective therapeutic agent against diabetes-induced ototoxicity.

**Introduction**

Diabetes mellitus (DM) is a common metabolic disorder that affects the metabolism of proteins, fats, and carbohydrates. DM is caused by the pancreas’s failure to secrete insulin and/or the deterioration of the tissue’s response to insulin [1–3]. Globally, diabetes is currently projected to affect 425 million people, and that number that is expected to reach 629 million by 2045 [4]. Diabetes causes complications in many organs, such as the kidneys, liver, nervous system, reproductive system, and eyes [5–8].

Sudden or gradual hearing loss has been observed in DM patients [6–9]. DM has been reported to cause various histological changes and hearing loss in the cochlea, VIII nerve, and the temporal bone [10,11]. The distortion product otoacoustic emission (DPOAE) is a low-level sound that can be measured in the external ear canal as a reflection of active processes in the cochlea [12]. In diabetic patients, DPOAE amplitudes have been reported to decrease functionally, especially at high frequencies [13].

In DM, metabolic stress and oxygen reduction have been shown to increase free oxygen radical production and to decrease the protective capacity of the antioxidant defense system [14]. DM contributes to the development of complications such as blindness, heart-vessel disease, and kidney disease, leading to a series of cascade reactions that cause the excessive production of free radicals [15].

Pterostilbene (PTS) (trans-3,5-dimethoxy-4-hydroxystilbene) is a natural antioxidant derived from the *Pterocarpus marsupium* (leguminasae) tree, which is also known as Indian kino or bijasar [16]. *P. marsupium* has been used traditionally in public medicine to treat diabetes and has been shown to control blood sugar levels in diabetic experimental animals [17,19]. This study aimed to investigate the effects of dose-dependent PTS on the cochlear cells of streptozotocin (STZ)-induced diabetic rats.

**Materials and methods**

**Animals and care**

Thirty male Wistar albino rats were purchased from a commercial company (Kobay DHL A. S., Ankara, Turkey) and the rats had an average weight of 250–300 g. All the animals were housed in separate cages, in conditions of 50–60% humidity and $22 \pm 2 \degree C$ heat, and fed standard pellet feed (in the form of seasonal fresh vegetables and fruits) and tap water during 4-week experimental period [17–19]. The lighting in the room was rotated between 12 hours bright and 12 hours dark. The numbers of animal were determined by the G-power calculator (G*Power 3.1) [20]. All the rats underwent otoscopic examinations, and only the animals who passed the Distortion Product Otoacoustic Emission (DPOAE) test were included in the study. Rats with ear disease, a tympanic membrane anomaly, or who did not pass the DPOAE test were excluded from the study.
Ethics statement

The study was handled according to the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act, and the NIH’s guidelines. The study protocol was approved by the Ethics Committee of Celal Bayar University, Manisa, Turkey (ID: 700/2019).

Chemicals

STZ, ethylenediamine tetra-acetic acid (EDTA), dimethyl sulfoxide (DMSO), sodium citrate, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). PTS was supplied as a free sample from Sabinsa Corporation, USA, and its 99% purity was determined by high performance liquid chromatography (HPLC).

STZ-induction of DM

The STZ rats were starved for 16 hours, and their water intake was restricted. Fresh STZ was prepared in a cold 20 mM solution of sodium citrate (pH 4.5). Within 15 minutes of the preparation, 45 mg/kg (a single dose) of STZ was administered intravenously (IV) through the tail vein of each rat [21]. After 72 hours, tail vein blood samples were obtained from the rats. The glucose levels of the samples were ≥ 300 mg/dL (16.7 mmol/l), indicating that the rats were considered diabetic [22]. However, any rats with excessive weight loss, weak body resistance, or blood glucose levels above 500 mg/dL were excluded from the study; any rats that were ill or deceased were also excluded from the study [23].

Experimental design

This study used 30 rats (6 normal control rats and 24 diabetic rats). After STZ-induced DM was established, the rats were divided into five groups, with each group containing 6 rats. PTS was administered intraperitoneally (IP) each day to the diabetic rats in a buffer solution that contained 10% DMSO. The study design is provided below.

Control (n = 6); non-diabetic control rats treated with 0.1 M sodium citrate buffer, IV)  
DM (n = 6); diabetic control rats treated with STZ (45 mg/kg/day bodyweight, IV)  
DM+PTS10 (n = 6); diabetic rats treated with PTS (10 mg/kg/day bodyweight, per day, IP)  
DM+PTS20 (n = 6); diabetic rats treated with PTS (20 mg/kg/day bodyweight, per day, IP)  
DM+PTS40 (n = 6); diabetic rats treated with PTS, (40 mg/kg/day bodyweight, per day, IP)

DPOAE test

DPOAE are low-level acoustic signals that can be measured from the ear canal in the presence of an external acoustic stimulus [24]. All the rats were twice tested for DPOAE, both at the beginning of the study and at the end of the 4-week experimental period. Sodium pentobarbital (75 mg/kg; Akorn, Lake Forest, IL, United States) was administered IP for sedation before each measurement. The DPOAE were measured using a Neuro-Audio/OAE device (version 2010, Neurosoft, Ivanovo, Russia) that was set at 500–8,000 Hz and recorded in DP gram. The DPOAE stimulus intensity was set to 55 for the L1 and L2 levels; the ratio of the f1 frequency (65 dB SPL) and the f2 frequency (55 dB SPL) (f2 / f1) was set to 1.22. The signal-to-noise ratio values were recorded at different frequencies (0.988, 2.222, 2.963, 5.714, and 8.000 kHz).

Anesthesia and tissue preparation

After the DPOAE test was repeated at the end of the 4-week experimental period, all the animals were anesthetized with a combination of ketamine hydrochloride (15 mg/kg; Pfizer,
Walton Oaks, United Kingdom) and xylazine hydrochloride (5 mg/kg; Bioveta, Komenskeho, Czech Republic), decapitated, and sacrificed. The cochleas were removed from the temporal bones and dissected in cold PBS. For the histological examination, the cochleas were preserved with neutral formalin.

**Histopathological examination**

The cochlear tissues were fixed in neutral formalin for 24 hours. The tissues were then placed in a 0.1 mol/L EDTA solution to decalcify the osseous portions. This procedure was followed by an overnight washing under flowing water. The tissues were dehydrated with an alcohol series, and transparency was achieved using xylene. Serial sections (5 μm in thickness) were mounted to polylysine-coated slides. Images were obtained from the basal turn of each cochlea.

**Immunohistochemistry**

For immunohistochemical staining, the remaining serial sections were assigned, incubated at 60˚C overnight, and the slides were deparaffinized by xylene and dehydrated through an alcohol series. The sections were boiled for 15 min in citrate buffer (10 mM, pH 6.0) using a microwave oven to retrieve antigen. Furthermore, for preventing endogenous peroxidase activity, they were placed in hydrogen peroxidase for 15 min. The sections were incubated in a blocking serum (Ultra V Block, TP-060-HL; NeoMarker, Fremont, CA) for 10 min and then with primary antibodies, including Caspase-8 (1:100, LabVision, USA) and Cytocrome c (1:100, LabVision, USA) in a moist environment at room temperature for 60 min. The antigen-antibody complex was fixed with biotinylated secondary antibodies and streptavidin-peroxidase for 20 min. Labeling was performed using DAB, and background staining was achieved using Mayer’s hematoxylin and covered by mounting medium. The images were captured using a camera attached to an Olympus microscope (CX31, Germany). Except for omission of the incubation period with the primary antibody, control samples were similarly processed. Two pathologists, blinded to the study, independently evaluated the immunolabeling scores. Scores of the staining intensity of the slides was semi-quantitatively assigned. The staining intensity was decided weak, moderate, or strong and valued 1, 2, or 3, respectively. The score was obtained from the following: \[ \text{SCORE} = \sum \Pi (i+1), \] where \( i \) is the intensity of staining, and \( \Pi \) is the percentage of stained cells for each intensity, varying from 100% to 0%.

**Terminal deoxribonucleotidy transferase-mediated dUTP-biotin end labeling (TUNEL)**

A specific kit was used for detecting DNA fragmentation and apoptotic cell death in situ (Apoptag, S7101, Chemicon, CA, USA). Sections were stored at 60˚C heat overnight in an oven in order to facilitate deparaffinization. To complete deparaffinization, the sections were reacted with xylol for 2 rounds of 15 min each. The sections were then placed into an alcohol series of 100%, 96%, and 80% by 10-min intervals. After 2 washings in distilled water for 5 min, the sections were incubated with 20 μg/mL proteinase K. Next, the endogenous peroxidase activity was blocked in the sections, and they were allowed to react with 3% hydrogen peroxide (TA-015-HP; Lab Vision, Fremont, CA) after washing with PBS. Subsequently, the sections were incubated in a balanced buffer for 15 min and then incubated with a TdT enzyme (77 μL reaction buffer+33 μL TdT enzyme, 1-μL TdT enzyme) at 37˚C for 60 min. Further, the sections were placed in pre-warmed stopping/washing buffer at room temperature for 10 min before incubating them with anti-digoxigenin for 45 min. PBS washing was performed after every step. After the washing, DAB staining was used for detecting TUNEL-positive cells. For
background staining, methyl green was applied for 5 min. The stained slides were dehydrated using an alcohol series and placed in xylol for 20 min. The slides were then covered by entellan and thin glass. Finally, the slides were observed using a photolight microscope equipped with a computer. Two pathologists, who were blinded to the experiment, independently evaluated the TUNEL scores. The average number was determined by counting the TUNEL-positive apoptotic cells in randomly selected fields of each case. A total of 100 TUNEL-positive or -negative cells were counted in each case, and the TUNEL-positive cells were provided as a percentage. The cells in necrotic regions and those having a poor morphology and borders between sections were excluded.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The cochlear tissue was dissected in cold-PBS and homogenized with the Qiazol lysis reagent (Qiagen, Hilden, Germany). A high pure RNA isolation kit (Roche Diagnostic, GmbH, Germany) was used to isolate the total RNA in the homogenate according to the manufacturer’s protocol. The RNA concentration was evaluated with a spectrophotometer (Shimadzu UV-mini 1240, Gmbh, Germany). The complementary DNA (cDNA) was synthesized using a transcriptor high fidelity cDNA synthesis kit (Roche Applied Science, Penzberg, Germany). Each prepared sample used 20 μL of the SYBR green qPCR reaction kit (Roche Applied Science), which contained 2 μL of cDNA for qRT-PCR using the following primer pairs: CASP-3, forward 5’-GGAGCAGCTTTGTGTGTGTG-3’ and reverse 5’-CTTTCCAGTCAGACTCCGGC-3’; BAX, forward 5’-GTTTCATCCAGGATCGAGCAG-3’ and reverse 5’-CATCTTCTTCAGATGTTGA-3’; BCL-2, forward 5’-GCTGTGGATGACTGAGTACC-3’ and reverse 5’-GAGACAGCCAGGAAAATCA-3’; GAPDH, forward 5’-GTCCTGGTTTCTTACCCCAATGT-3’ and reverse 5’-GCCCTGGTCCACCACCTTCTTGATG-3’. The GAPDH sequences were used for normalization, as it represents a housekeeping gene. The rotor-gene Q 5plex HRM platform (Qiagen, Hilden, Germany) was used for the qRT-PCR, and the data were analyzed using the comparative Ct method (ΔΔCt).

Body weight and biochemical investigations

During the experiment, the animals’ blood glucose levels and body weights were measured once a week [25]. The blood samples were taken from the rats’ tail veins. The blood glucose levels were measured using a glucometer (Accu-Chek Performa Nano, Roche Diagnostics, Mannheim, Germany). The rats’ insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Hangzhou Eastbiopharm Co., Ltd., Hangzhou, China) and a spectrophotometer (Biotek Epoch™ Microplate Spectrophotometer, United States). Malondialdehyde (MDA) levels were measured at an absorbance of 532 nm (Shimadzu UV-mini 1240, Gmbh, Germany) [26].

Statistical analysis

The Statistical Package for the Social Sciences (SPSS version 20.0, IBM, Inc., Chicago, IL, United States) software program was used to analyze the data. The data from the DPOAE tests, the TUNEL assays, the immunohistochemical staining, and the biochemical tests were compared between the groups with the one-way analysis of variance (ANOVA) test, followed by Tukey’s post hoc test. The Mann–Whitney U test was used to compare the means of the groups for the qRT-PCR data. All the data are presented as the mean ± the standard error of the mean. P values < 0.05 were considered statistically significant.
Results

**DPOAE measurements**

The DPOAE amplitudes were recorded, and the averages were compared against the 0.9–8 kHz frequencies of each rat group (Table 1). The means of the DPOAE amplitudes in the DM group were significantly lower at all frequencies compared to both the Control group and the other treatment groups (P < 0.001 for all). In all the DM + PTS treatment groups, a significant increase was observed in the DPOAE amplitude averages at every frequency compared to the untreated diabetic rats (P < 0.05 for all). The highest amplitude increase was observed in the diabetic rats that received 40 mg/kg/day of PTS (P = 0.05, P = 0.001, P = 0.003, P < 0.001, respectively). In addition, a significant increase was observed in the mean DPOAE amplitudes compared to the other diabetic treatment groups that received PTS treatments of 20 and 40 mg/kg/day compared to the mean of the DPOAE amplitudes at every frequency (P < 0.05 for all). However, the mean values of the DPOAE amplitudes of the healthy Control group animals were similar to those of the diabetic rats that received the PTS treatment of 40 mg/kg/day at each frequency (P = 0.998, P = 0.995, P = 0.998, P = 0.914, P = 0.934, respectively).

**Immunohistochemical and histopathological examinations**

The cells in all the study groups were stained and counted using the Caspase-8, Cytochrome-c, and TUNEL assays (Fig 1).

While poor expression was observed in the Control group’s Corti organ in the Caspase-8 immune staining (Fig 2A), significant staining was noted in the DM group (Fig 2B). A moderate-to-severe Caspase-8 immunoreaction was observed in the Corti organ of the diabetic rats that had been treated with PTS at 10 mg/kg/day (Fig 2C). In the group of diabetic rats that had been treated with PTS at 20 mg/kg/day, a moderate Caspase-8 immunoreaction was observed (Fig 2D). Poor expression was detected in the diabetic rats that were treated with PTS at 40 mg/kg/day (Fig 2E).

The Cytochrome-c immunodeficiency dye had a weak reaction in the Corti organs of the healthy Control group (Fig 3A). In the DM group, an increased dye reaction was observed in the Corti organ, especially in the outer hair cells, which ranged from medium to high reaction levels (Fig 3B). In the diabetic rats that were treated with PTS at 10 and 20 mg/kg/day, cytochrome-c expression ranged from medium to weak in the Corti organ cells (Fig 3C and 3D). Weak staining was observed in the diabetic rats treated with PTS at 40 mg/kg/day (Fig 3E).

In the tissues from the healthy Control group that were stained using the TUNEL assay, there were few or no TUNEL-positive cells in the Corti organ (Fig 4A). In the DM group,

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Table 1. The DPOAE amplitude (dB SPL) measurement results of the rat groups.

| Groups            | 0.988 kHz | 2.222 kHz | 2.963 kHz | 5.714 kHz | 8.000 kHz |
|-------------------|-----------|-----------|-----------|-----------|-----------|
| Control (n = 6)   | 5.80 ± 4.1| 5.83 ± 3.5| 5.13 ± 2.4| 10.28 ± 5.4| 12.63 ± 9.7|
| DM (n = 6)        | 4.83 ± 5.6| 4.28 ± 3.3| 4.20 ± 3.6| 7.66 ± 5.0 | 9.63 ± 5.5 |
| DM+PTS10 (n = 6) | 6.68 ± 5.8| 5.63 ± 5.7| 6.37 ± 4.5| 10.28 ± 4.7| 12.73 ± 5.7|
| DM+PTS20 (n = 6) | 6.35 ± 5.9| 5.46 ± 0.1| 5.41 ± 6.4| 10.19 ± 5.2| 12.57 ± 4.4|
| DM+PTS40 (n = 6) | 5.70 ± 4.9| 5.91 ± 2.6| 5.20 ± 2.2| 10.52 ± 4.2| 12.93 ± 6.8|
| P value           | < 0.001   | < 0.001   | < 0.001   | < 0.001   | < 0.001   |

DPOAE: The Distortion Product Otoacoustic Emission; DM: Diabetes Mellitus; PTS: Pterostilbene

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Fig 1. The cells counted staining using Caspase-8, Cytochrome-c, and TUNEL method in the rat groups after STZ-application to end of the experimental period. (A) For Caspase-8; * significantly different from DM (P < 0.001 for all), # different from DM+PTS10, DM+PTS20 (P < 0.001 for all). (B) For Cytochrome-c; * Significantly different from DM (P < 0.001 for all), # different from DM+PTS10, DM+PTS20 (P < 0.001 for all). (C) For Tunnel-method; * Significantly different from DM (P < 0.05 for all), # different from DM+PTS10 (P = 0.033), and † similar to Control.

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strong TUNEL reactions were seen in the outer hair cells of the Corti organ, as well as the other supporting cells of the Corti organ (Fig 4B). In the diabetic rats treated with PTS at 10 mg/kg/day, fewer outer hair cells had TUNEL reactions, while TUNEL-positive reactions were observed in the support cells (Fig 4C). In the diabetic rats that were treated with PTS at 20 mg/kg/day, the outer hair cells were TUNEL-negative, and the support cells were partially TUNEL-positive (Fig 4D). In the diabetic rats that were treated with PTS at 40 mg/kg/day, the TUNEL reactions were minimal in both the outer hair cells and the support cells (Fig 4E).
The mRNA expressions of the \textit{CASP3}, \textit{BAX}, \textit{BCL-2} and \textit{BAX/BCL2} genes by qRT-PCR

The mRNA levels of the \textit{BAX}, \textit{CASP-3}, \textit{BCL-2} and \textit{BAX/BCL-2} genes were assessed with qRT-PCR to investigate whether PTS affected apoptosis (Fig 5). In the untreated diabetic rats, compared to the rats in the healthy Control group and all the PTS treatment groups, the mRNA levels of the \textit{BAX} and \textit{CASP-3} genes were significantly higher ($P < 0.001$ for all). When

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\textbf{Fig 3. Cytocrome-c immunohistochemistry staining in the cochlear Corti organ of the rat groups.} →: Inner hair cells, ↔: Outer hair cells, Sm: Spiral limbus, Slg: Spiral ligament, BM: Basilar membrane, Control (A), DM (B), DM +PTS10 (C), DM+PTS20 (D), DM+PTS40 (E). Corti organ X40 (1), Corti organ X100 (2). Methyl-green ground staining.

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the diabetic rats that received PTS were compared to the untreated diabetic rats, the mRNA levels of the *CASP-3* and *BAX* genes of the rats that received 20 and 40 mg/kg/day of PTS were significantly lower, and the *BCL-2* levels were higher (*P* < 0.05 for all). In addition, the diabetic rats were received 20 and 40 mg/kg/day of PTS, the ratio of the *BAX/BCL-2* the mRNA levels were significantly decreased with the dose dependent (the mean ratio 3 ± 0.37, and 1.62 ± 0.58, respectively; *P* < 0.001 for all).

Fig 4. TUNEL staining in the cochlear Corti organ of the rat groups. ←: Inner hair cells, ➡: Outer hair cells, Slm: Spiral limbus, Slg: Spiral ligament, BM: Basilar membrane, Control (A), DM (B), DM+PTS10 (C), DM+PTS20 (D), DM+PTS40 (E). Corti organ X40 (1), Corti organ X100 (2). Methyl-green ground staining.

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Body weight and biochemical measurements

The rats were evaluated for weight loss during the 4-week experiment. Significant weight loss was observed in the untreated diabetic rats compared to the healthy Control group and the diabetic groups that received PTS ($P < 0.001$). While weight gain was observed in the PTS treatment groups compared to the untreated diabetic rats, no significant differences were observed between the rats that were treated with PTS at 10 and 20 mg/kg/day ($P = 0.978$, $P = 0.514$). However, a significant weight gain was detected in the group that received 40 mg/kg/day of PTS compared to the groups that received the lower doses, and similarities were observed between the group that received the highest PTS level and the Control group ($P < 0.001$ for the other doses, $P = 0.822$ for the Control group) (Fig 6).

The rats’ blood glucose values were measured after the STZ application. 3. daily measurements were taken during the first week and were continued until the end of the fourth week. The blood glucose values were then compared between the rat groups. The blood glucose values of the rat groups were reported with ± standard error of values on average, and a statistically significant difference was observed between all groups ($P < 0.001$ for all). The blood glucose levels were significantly higher in the diabetic rats that received PTS treatment compared to the healthy Control group ($P < 0.001$). A significant decrease in blood glucose levels was observed in the rats that were treated with 20 and 40 mg/kg/day of PTS compared to the untreated diabetic rats ($P = 0.003$, $P < 0.001$, respectively). In addition, the diabetic rats that received 20 and 40 mg/kg/day of PTS had similarly reduced blood glucose levels, and their blood glucose levels were lower than the diabetic rats that received 10 mg/kg/day of PTS ($P = 0.038$ and $P < 0.001$ for others) (Fig 7).
The serum insulin levels were significantly lower in the untreated diabetic rats compared to the healthy Control group and all the PTS treatment groups ($P < 0.001$ for all). The diabetic rats that received PTS had increased insulin levels after 10, 20, and 40 mg/kg/day of PTS compared to the untreated diabetic rats ($P < 0.001$ for all). However, 20 and 40 mg/kg/day of PTS treatment were more effective in increasing serum insulin levels than the 10 mg/kg/day PTS dose ($P < 0.001$ for all) (Fig 7).

The serum insulin levels were significantly lower in the untreated diabetic rats compared to the healthy Control group and all the PTS treatment groups ($P < 0.001$ for all). The diabetic rats that received PTS had increased insulin levels after 10, 20, and 40 mg/kg/day of PTS compared to the untreated diabetic rats ($P < 0.001$ for all). However, 20 and 40 mg/kg/day of PTS treatment were more effective in increasing serum insulin levels than the 10 mg/kg/day PTS dose ($P < 0.001$ for all) (Fig 7).

![Fig 6. Body-weight (g) change of the rat groups after STZ-application to end of the experimental period.](https://doi.org/10.1371/journal.pone.0228429.g006)

| Body-weight (g) | Mean |
|----------------|------|
| Control        | 268.96 |
| DM             | 243.79 |
| DM+PTS10       | 234.71 |
| DM+PTS20       | 245.13 |
| DM+PTS40       | 263.67 |

![Fig 7. The serum MDA, insulin and blood glucose level change of the rat groups.](https://doi.org/10.1371/journal.pone.0228429.g007)

| Serum MDA, Insulin and Blood Glucose Levels |
|--------------------------------------------|
| MDA level (nmol/mL)                        | 0.95 | 5 | 3 | 2 | 2 |
| Insulin level (mIU/mL)                     | 13   | 5 | 11 | 2 | 2 |
| Blood Glucose (mg/dL)                      | 106.29 | 360.29 | 290.12 | 200.13 | 181.29 |
Serum MDA levels were significantly higher in the healthy Control group and in all the PTS treatment groups compared to the diabetic rats ($P < 0.001$ for all). The diabetic rats that received PTS (in all the treatment groups) had decreased serum MDA levels compared to the untreated diabetic rats ($P < 0.001$ for all). However, the PTS administrations of 20 and 40 mg/kg/day were more effective in lowering serum MDA levels in the diabetic rats than the lower treatment dose ($P < 0.001$ for all). The serum MDA levels of the healthy Control group rats were similar to those of the rats that received 40 mg/kg/day of PTS ($P = 0.014$) (Fig 7).

**Discussion**

Our study aimed to investigate the effects of PTS, an antioxidant, on the cochleas of diabetic rats. Using an STZ-induced diabetic rat model, we found that PTS reduced serum MDA and blood sugar levels and increased insulin levels; in addition, PTS increased cell viability by inhibiting apoptosis in the cochleas. Our results showed that PTS protected the cochleas from DM-induced ototoxicity by increasing the DPOAE amplitude averages in a dose-dependent manner. Our study confirmed the anti-apoptotic effect of PTS in cochleas isolated from diabetic rats using the expression profiles of factors associated with apoptotic pathways. This data provides a basis for further studies.

DM is a metabolic disease with multiple etiologies. DM is characterized by metabolic disorders that are related to defects in insulin secretion and/or insulin resistance, and it is characterized by chronic hyperglycemia. DM is also associated with increased oxidative stress [1]. DM induces oxidative and nitrosative stress, damaging DNA. Over time, DM becomes involved in the pathogenesis of cancer, the nervous system, and cardiovascular diseases [3]. DM is a significant global public health problem, and there is a need for alternative prevention and treatment strategies [1,2].

Recent research has shown that polyphenolic compounds have anti-diabetic potential and may prevent hyperglycemia [27]. The polyphenolic-derivative PTS is a therapeutic agent with a high bioavailability, as its hydrophobic structure facilitates intracellular uptake [28,29]. Research has shown that PTS's antioxidant properties have protective and therapeutic effects in many diseases, including neurological, cardiovascular, diabetic, dyslipidemic, and hematological disorders [17,30]. In addition, PTS has been reported to have anti-inflammatory effects [31], restorative immune system effects [32] and potent anti-cancer effects [33]. In experiments conducted by Erkan et al., the effects of resveratrol (10 and 20 mg/kg-body weight) were evaluated on the cochlea of STZ-induced rats, and it was found that the resveratrol reduced DM-induced ototoxicity in a dose-dependent manner [19]. Moreover, in recent studies, it is thought that resveratrol shows antioxidant effect at low doses and pro-oxidant effect at high doses [34,35]. PTS is a new promising antioxidant and a natural methoxylated derivative of resveratrol [29]. Although studies have demonstrated the benefits of PTS for DM, the reports in the literature regarding the effect of PTS on the cochlea are so far inadequate [30,36]. In considering the literature, effects on diabetic cochlea were investigated with low doses (10, 20 and 40 mg/kg-body weight) of PTS.

Sensorineural hearing loss is associated with several etiological factors, including congenital diseases, ototoxic agents, infectious diseases, and metabolic diseases, such as DM [9]. Increased oxidative stress induced by DM can cause hearing disorders by accelerating neuron loss and demyelination in neuronal tissues, leading to peripheral endotelial dysfunction and microvascular complications [4,5,10]. Studies have reported that DM causes various histopathological changes in the cochlea [10]. Wackym, Linthicum and Smith et al. reported that spiral ganglion neurons in the cochlea deteriorated due to diabetic microangiopathy [37,38]. In a human temporal bone study, Fukushima et al. reported that DM causes the loss of outer hair cells, a
thickening in the basal membrane of the stria vascularis and the Corti organ, and atrophy in the stria vascularis [39]. In a study with genetically diabetic rats, Nakae et al. observed stria vascularis degeneration, edema, and the loss of inner-outer hair cells on the basilar membrane [40]. However, Tachibana-Nakae and Nageris et al. reported that there was no difference between the controls and the diabetic animals in terms of cochlear histopathology [41,42].

The TUNEL assay provides an objective indicator of non-specific cell deterioration [43]. Our histopathological analysis revealed significant TUNEL staining in the inner and outer hair cells of the Corti organs of the diabetic rats compared to the rats in the Control group. However, the rats in the PTS treatment group had decreased dose-dependent TUNEL-reactivity, likely because the PTS treatment protected the cochlea from DM-induced ototoxicity. Intrinsic- and extrinsic-pathways are main apoptosis signalling-pathways. The intrinsic-apoptotic pathway is controlled by the mitochondrial permeability of the pro-apoptotic factor Bax and the anti-apoptotic factor Bcl-2 that is bound to Caspase-3 [44, 45]. Therefore, the ratio of the Bax/Bcl-2 expression is an essential indicator of the intrinsic-apoptotic pathway and initiated apoptosis [45]. Moreover, increased mitochondrial permeability and depolarization triggers Cytochrome-c release, activating the apoptotic pathway related to Caspase-3. However, in the extrinsic-apoptotic pathway various apoptotic factors activate Caspase-8 causing the cleavage of Caspase-3 and induce apoptosis [44]. In our study, there was significant Caspase-8 and Cytochrome-c immunostaining in the inner and outer hair cells of the Corti organs of the diabetic rats compared to those of the Control group. Also, 20 and 40 mg/kg/day of PTS decreased Caspase-8 and Cytochrome-c expression in the same cell types consistent with data of TUNEL. In current study, PTS therapy in diabetic rats caused a dose-dependent decrease in the mRNA expression of pro-apoptotic BAX and its effector CASP-3 and an increase in the mRNA expression of anti-apoptotic BCL2. Also, in our study, the ratio of the BAX/BCL-2 the mRNA levels in PTS treatment group decreased significantly with dose-dependent. Interestingly, it was observed that 10 mg/kg/day of PTS was not significantly altered expression of the apoptotic factors related to intrinsic-pathway while increased in TUNEL-reactivity in the diabetic rats. These data suggest that low dose of PTS is likely to induced apoptosis in cochlea cells primarily by the extrinsic-signalling pathway. Advanced further studies are necessary to elucidate this intriguing observation. The mRNA expression data of these pro- and anti-apoptotic genes correlate with the histopathological and immunohistochemical data, suggesting that an anti-apoptotic molecular mechanism is associated with the antioxidant PTS.

DPOAE is an important objective test used to determine hearing loss caused by damage to the outer hair cells in the cochlea. Cochlear deterioration may be caused by metabolic diseases, such as hyperlipoproteinemia, and DM has been reported to cause changes in DPOAE amplitudes [25,46]. In our study, the mean DPOAE amplitudes were low in the diabetic rats and increased in a dose-dependent manner with PTS treatment. These data indicate that PTS may protect the outer hair cells, a finding that is consistent with our histopathological, immunohistochemically, and molecular findings, which all demonstrated decreased cochlear damage related to DM. However, exhaustive comprehension for the protective effect of PTS the cochlea from ototoxicity in STZ-induced diabetic rats is still essential. Therefore, further studies with functional analyses are needed to elucidate the molecular-mechanisms underlying the its cytoprotective role in cochlea cells and assess the impact of PTS on the retrocochlea-cochlea in the auditory pathway of diabetic animals.

The animal model for STZ-induced DM is characterized by elevated blood sugar levels, decreased serum insulin levels, and increased weight loss [47]. Our study showed increased weight loss in untreated diabetic rats and decreased weight loss in the diabetic rats that received PTS treatment. Although the molecular action mechanism of PTS is not known in detail, it has been reported in some studies that pterocarpus marsupium containing PTS causes
re-granulation of pancreatic beta cells [48]. This situation can be regarded as one of the reasons explaining the anti-diabetic mechanism of PTS. Compared to the other doses, 40 mg/kg/day of PTS was also associated with the least amount of weight loss. In experiments conducted by Manickam et al., the anti-glycemic effects of PTS isolated from pterocarpus marsupium were evaluated in STZ-induced rats, and it was found that oral dose of 20 mg/kg PTS reduced body weight loss by 20% [18]. Our findings are also in accordance with those of Korusu et al. and Sun et al. [49,50]. We believe that PTS reduces weight loss by promoting glycolysis, improving glycemic control, and maintaining adipose-muscle tissue. In our study, serum insulin levels were significantly lower in the diabetic rats compared to the healthy Control group and the treatment groups, likely due to the destruction of pancreatic beta-cells in the STZ-induced diabetic rats [47,51]. In accordance with the literature, PTS treatment increased serum insulin levels in a dose-dependent manner. In experiments performed by Amarnath et al., it was observed that oral 40 mg/kg PTS treatment for 6-week caused a 56.5% decrease in plasma glucose levels and an increase in plasma insulin levels [17]. While 15 mg/kg PTS application has beneficial effects on glycemic control with the contribution of both muscle and liver, it was indicated that only skeletal muscle was responsible for 30 mg/kg PTS application. PTS has been reported to improve glycemic control in rats showing insulin resistance caused by an obesogenic diet. In addition to hepatic glucokinase activity, an increase in skeletal muscle glucose uptake appears to play a role in the anti-diabetic effect of this phenolic compound [52]. PTS may increase insulin sensitivity and increase hepatic hexokinase activity in glycolysis [48,53]. In our study, PTS treatment caused a significant decrease in the blood glucose levels of the diabetic rats, particularly at doses of 20 and 40 mg/kg/day. These data are consistent with the work of Pari et al. and Manickam et al. and can be explained by the cytoprotective action of PTS, which promotes pancreatic beta-cell granulation [10,18]. Also, our findings showed that the PTS treatment at doses of 20 and 40 mg/kg/day significantly increased insulin levels and body-weight of the diabetic rats. However, PTS treatment at 10 mg/kg/day increased insulin levels but not body-weight of the diabetic rats. It is assumed that PTS has similar interactions with resveratrol, which is structurally similar [54]. Also, it is stated that the bioavailability of PTS (80%) is higher than RSV (20%) [55]. Based on this similarity, it is predicted that PTS may also have an antioxidant effect at low doses and pro-oxidant effects at high doses such as resveratrol. On the other hand, more molecular investigations are needed to be performed in order to explain such differences. Previous studies have reported that MDA, a byproduct of lipid peroxidation, is an important oxidative-stress marker, and that serum MDA levels increase in diabetic rats [56,57]. Similarly, in this study, all PTS treatment doses decreased serum MDA levels, and this decrease was more pronounced at increased PTS doses. In our study, the decreased otoxicity and increased cytoprotection of PTS in the cochlea of STZ-induced diabetic rats demonstrated the remedial effect of PTS on metabolic parameters.

The amounts of PTS taken naturally vary according to the type of product consumed daily. For instance, PTS acquired by consuming blueberries (depending on the type) is thought to be between 99 ng and 520 ng/gram [58,59]. There are also studies showing that PTS has preventive or therapeutic effects in various human diseases, including DM, cardiovascular, neurological, metabolic and hematological disorders. It has been stated in pre-clinical studies that it can be used as a potential anti-cancer agent in some malignancy patients [60–62]. Also, Hougee et al., in his study with the participation of healthy-human-volunteers, it was found that a daily two-week dose of 450 mg of Pterocarpus marsupium extract including PTS can be beneficial in inflammatory diseases [63]. Furthermore, in a randomized double-blind placebo-controlled human clinical trial conducted by Riche et al., it has been reported that PTS has no adverse drug reactions (ADRs) on hepatic, renal and glucose markers, and is generally safe for use in humans up to 250 mg daily [64]. However, no human clinical studies showing the effects of
PTS doses used in this study on the cochlea of diabetic patients were found. We hope that this study, which we carried out experimentally in animals and obtained positive results from them, will inspire clinical research.

**Conclusions**

PTS can be used to control blood glucose and serum insulin levels and to reduce oxidative stress in STZ-induced diabetic rats. There was a significant increase in the dose-dependent mean DPOAE values of the PTS-treated diabetic rats compared to the untreated diabetic rats. There was also a significant decrease in the number of apoptotic cells in the cochleas of the PTS-treated diabetic rats compared to those of the untreated diabetic rats. PTS had a significant dose-dependent, protective effect against DM-induced ototoxicity by inhibiting the intrinsic apoptotic pathway in the treatment group. The biochemical data in our study, showing that PTS has a dose-dependent effect in STZ-induced diabetic rats, are consistent with our cochlear histopathological, immunohistochemical, molecular, and auditory findings. This study provides the first findings indicating that PTS can prevent DM-induced ototoxicity and suggests that PTS should be considered as a potential therapeutic agent.

**Supporting information**

S1 File. (PDF)

**Acknowledgments**

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References

1. Baquer NZ, Gupta D, Raju J. Regulation of metabolic pathways in liver and kidney during experimental diabetes: effects of antidiabetic compounds. Indian J Clin Biochem. 1998; 13: 63–80. https://doi.org/10.1007/BF02867866 PMID: 23105185

2. Olokoba AB, Obateru OA, Olokoba LB. Type 2 diabetes mellitus: a review of current trends. Oman Med J. 2012; 27: 269–273. https://doi.org/10.5001/omj.2012.68 PMID: 23071876

3. Kahn CR. Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes. 1994; 43: 1066–1084. https://doi.org/10.2337/diab.43.8.1066 PMID: 8039601

4. International Diabetes Federation. IDF Diabetes Atlas, 8th Edition. Available at: http://www.diabetesatlas.org/. Accessed July 29, 2019.

5. Bhatt JK, Thomas S, Nanjan MJ. Resveratrol supplementation improves glycemic control in type 2 diabetes mellitus. Nutr Res. 2012; 32: 537–541. https://doi.org/10.1016/j.nutres.2012.06.003 PMID: 22901562

6. Aimon C, Bianchini C, Borin M, Ciorba A, Fellin R, Martini A, et al. Diabetes, cardiovascular risk factors and idiopathic sudden sensorineural hearing loss: A case-control study. Audiol Neurotol. 2010; 15: 111–5. https://doi.org/10.1159/000231636 PMID: 19657186

7. Axelsson A, Siroth K, Vertes D. Hearing in diabetics. Acta Otolaryngol Suppl. 1978; 356: 1–23. PMID: 30083527

8. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am. 2010; 39: 481–497. https://doi.org/10.1016/j.ecl.2010.05.011 PMID: 20723815

9. Bagai A, Thavendiranathan P, Detsky AS. Does this patient have hearing impairment? JAMA. 2006; 296: 425–426. https://doi.org/10.1001/jama.296.4.425 PMID: 16434632

10. Akinpelu OV, Ibrahim F, Waissbluth S, Daniel SJ. Histopathologic changes in the cochlea associated with diabetes mellitus-a review. Otol Neurotol. 2014; 35: 764–74. https://doi.org/10.1097/MAO.000000000000293 PMID: 24686289

11. Cullen JR, Cinnamond MJ. Hearing loss in diabetics. J Laryngol Otol. 1993; 107: 179–182. https://doi.org/10.1111/j.2042-7158.1994.tb05722.x

12. Wennberg PE, Tomlinson L, Kuller LH, Pfeifer MA, Guralnik JM, Kritchevsky SB, et al. Frailty and diabetes: the importance of bone status and sarcopenia in the elderly. J Am Geriatr Soc. 2010; 58: 1477–84. https://doi.org/10.1111/j.1532-5415.2010.03232.x PMID: 20883028

13. Cilenkic del Rosario M, Pachon-Ortiz G, Maldonado-Rodriguez CE, Aquino-Torres L, Ramirez del Riego-von der Veer P, et al. The effect of metformin on echocardiographic parameters in diabetic patients with hypertension. Rev Bras Cardiol Clin. 2015; 40: 142–50. https://doi.org/10.1016/j.rbrc.2015.03.004 PMID: 26144586

14. Botelho CT, Carvalho SA, Silva IN. Increased prevalence of early cochlear damage in young patients with type 1 diabetes detected by distortion product otoacoustic emissions. Int J Audiol. 2014; 53: 402–408. https://doi.org/10.3109/14992027.2013.879341 PMID: 24564623

15. Ceriello A, Bortolotti N, Falleti E, Taboga C, Tonutti L, Crescentini A, et al. Total radical-trapping antioxidant parameter in NIDDM patients. Diabetes Care. 1997; 20: 194–7. https://doi.org/10.2337/diacare.20.2.194 PMID: 9118773

16. Bortoli S, Amessou M, Collinet M, Desuquoi B, Lopez S. Vanadate, but not insulin, inhibits insulin receptor gene expression in rat hepatoma cells. Endocrinology. 1997; 138: 4821–4829. https://doi.org/10.1210/endo.138.11.5521 PMID: 9348211

17. Warrier PK, Nambiar VPK, Ramankutty C, In: Indian Medicinal Plants. Orient Longman Limited, Madras. 1995; 381–383. https://doi.org/10.1111/j.2042-7158.1994.tb05722.x

18. Satheesh A, Pari L. The antioxidant role of pterostilbene in streptozotocin nicotinamide-induced type 2 diabetes mellitus in Wistar rats. JPP. 2006; 58: 1483–1490. https://doi.org/10.1211/jpp.58.11.0009 PMID: 17132211

19. Manickam M, Ramanathan M, Jahromi MA, Chansouria JP, Ray AB. Antihyperglycemic activity of phenolics from Pterocarpus marsupium. J Nat. 1997; 60: 609–610. https://doi.org/10.1021/np9607013 PMID: 9214733

20. Erkan SO, Türkün B, Guven SG, Ozgen aT, Tastekin B, Peltt A, et al. The effect of resveratrol on the histologic characteristics of the cochlea in diabetic rats. Laryngoscope. 2019; 129(1): E1–E6. https://doi.org/10.1002/lary.27253 PMID: 30284252

21. Faul F, Erdfelder E, Lang AG, Buchner A. A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007; 39: 175–91. https://doi.org/10.3758/BF03193146 PMID: 17695343

22. Brosius F. High-Dose Streptozotocin Induction Protocol (Mouse); Animal Models of Diabetic Complications Consortium. AMDCC Protocols.
23. Pyhu HE, Irwin JC, Vella RK, Fenning AS. Resveratrol shows neuronal and vascular-protective effects in older, obese, streptozocin-induced diabetic rats. Br J Nutr. 2016; 115: 1911–1918. https://doi.org/10.1017/S0007114516001069 PMID: 27153202

24. Hao J, Fu X, Zhang C, Zhang X, Zhao S, Li Y. Early detection of hearing impairment in patients with diabetes mellitus with otoacoustic emission. A systematic review and meta-analysis. Acta Oto-laryngologica. 2017; 137: 179–185. https://doi.org/10.1080/00016489.2016.1223344 PMID: 27632340

25. Klimes I, Sebokova E, Gasperikova D, Miltova A, Kuklova S, Bohov P. Search for extra pancreatic effects of new oral hypoglycemic agent A-1466: 1. Oral glucose tolerance tests in normal and hereditary insulin resistant rats. Endocrinology Regulations. 1998; 32: 115–123. PMID: 10196508

26. Yuksel Y, Guven M, Kaymaz B, Sehitoglu MH, Aras AB, Akman T, et al. Effects of Aloe Vera on Spinal Cord Ischemia-Reperfusion Injury of Rats. J Investig Surg. 2016; 29: 389–398. https://doi.org/10.1080/08941939.2016.1178358 PMID: 27142763

27. Kim Y, Keogh JB, Clifton PM. Phenolics and Glycemic Control. Nutrients. 2016; 8: 17. https://doi.org/10.3390/nu8010017 PMID: 26742071

28. Jagadeb M, Konkimala VB, Rath SN, Das RP. Elucidation of the inhibitory effect of phytochemicals with Kir6.2 wild-type and mutant models associated in type-1 diabetes through molecular docking approach. Genomics Inform. 2014; 12: 283–288. https://doi.org/10.5808/GI.2014.12.4.283 PMID: 25705171

29. Rimando AM, Cuendet M, Desmarchelier C, Mehta RG, Pezzuto JM, Duke SO. Cancer chemopreventive and antioxidant activities of pterostilbene, a naturally occurring analogue of resveratrol. J Agric Food Chem. 2002; 50: 3453–3457. https://doi.org/10.1021/jf0116855 PMID: 12033810

30. Bhakkiyalakshmi E, Shalini D, Sekar TV, Rajaguru P, Paulmurugan R, Ramkumar KM. Therapeutic potential of pterostilbene against pancreatic beta-cell apoptosis mediated through Nrf2. Br J Pharmacol. 2014; 171: 1747–1757. https://doi.org/10.1111/bph.12577 PMID: 24417315

31. Liu J, Fan C, Yu L, Yang Y, Jiang S, Ma Z, et al. Pterostilbene exerts an anti-inflammatory effect via regulating endoplasmic reticulum stress in endothelial cells. Cytokine. 2016; 77: 88–97. https://doi.org/10.1016/j.cyto.2015.11.006 PMID: 26551859

32. McCormack D, McFadden D. A review of pterostilbene antioxidant activity and disease modification. Oxid Med Cell Longev. 2013; (15–16)575482. https://doi.org/10.1155/2013/575482 PMID: 23691264

33. Chakraborty A, Gupta N, Ghosh K, Roy P. In vitro evaluation of the cytotoxic, anti-proliferative and anti-oxidant properties of pterostilbene isolated from Pterocarpus marsupium. Toxicol In Vitro. 2010; 24: 1215–1228. https://doi.org/10.1016/j.tiv.2010.02.007 PMID: 20152895

34. Crowell JA, Korytko PJ, Morrissey RL, Booth TD, Levine BS. Resveratrol-associated renal toxicity. Toxicol Sci. 2004; 82(2): 614–619. https://doi.org/10.1093/toxsci/kfh263 PMID: 15329443

35. Dudley J, Das S, Mukherjee S, Das DK. Resveratrol, a unique phytoalexin present in red wine, delivers either survival signal or death signal to the ischemic myocardium depending on dose. J Nutr Biochem. 2009; 20(6): 443–452. https://doi.org/10.1016/j.jnutbio.2008.05.003 PMID: 18798672

36. Pari L, Satheesh MA. Effect of pterostilbene on hepatic key enzymes of glucose metabolism in streptozotocin- and nicotinamide-induced diabetic rats. Life Sci. 2006; 79: 641–5. https://doi.org/10.1016/j.lfs.2006.02.036 PMID: 16616938

37. Wackym PA, Linthicum FH Jr. Diabetes mellitus and hearing loss: clinical and histopathological relationships. Am J Otol. 1986; 7: 176–82. PMID: 3717308

38. Smith TL, Raynor E, Prazma J, Buenting JE, Pillsbury HC. Insulin-dependent diabetic microangiopathy in the inner ear. Laryngoscope. 1995; 105: 236–240. https://doi.org/10.1289/00005537-19950500-00002 PMID: 7877409

39. Fukushima H, Cureoglu S, Schachem PA, Paparella MM, Harada T, Oktay MF. Effects of type 2 diabetes mellitus on cochlear structure in humans. Arch Otolaryngol Head Neck Surg. 2006; 132: 934–8. https://doi.org/10.1001/archotol.132.9.934 PMID: 16982969

40. Nakae S, Tachibana M. The cochlea of the spontaneously diabetic mouse. II. Electron microscopic observations of non-obese diabetic mice. Arch Otorhinolaryngol. 1986; 243: 313–6. https://doi.org/10.1007/BF00460208 PMID: 3819968

41. Nageris B, Hadar T, Feinmesser M, Elidan J. Cochlear histopathologic analysis in diabetic rats. Am J Otol. 1998; 19: 63–5. PMID: 9455951

42. Tachibana M, Nakae S. The cochlea of the spontaneously diabetic mouse. I. Electron microscopic observation of KK mice. Arch Otorhinolaryngol. 1986; 243: 238–41. https://doi.org/10.1007/BF00464437 PMID: 3778298

43. Lee SK, Kim DI, Kim SY, Kim DJ, Lee JE, Kim JH. Reperfusion cellular injury in an animal model of transient ischemia. AJNR Am J Neuroradiol. 2004; 25: 1342–47. PMID: 15466329
44. Roy S, Nicholson DW. Cross-talk in cell death signaling. J Exp Med. 2000; 192: F21–5. PMID: 11034612
45. Liu J, Huang RW, Lin D, Peng J, Wu XY, Lin Q, et al. Expression of survivin and bax/bcl-2 in peroxisome proliferator activated receptor-y ligands induces apoptosis on human myeloid leukemia cells in vitro. Ann Oncol. 2005; 16: 455–459. https://doi.org/10.1093/annonc/dmi077 PMID: 15642706
46. Erdem T, Ozturan O, Miman MC, Ozturk C, Karatas E. Exploration of the early auditory effects of hyperlipoproteinemia and diabetes mellitus using otoacoustic emissions. Eur Arch Otorhinolaryngol. 2003; 260: 62–6. https://doi.org/10.1007/s00405-002-0519-1 PMID: 12582780
47. Roh S, Kwon OJJ, Yang H, Kim YS, Lee SH, Jin JS, et al. Allium hookeri root protects oxidative stress induced inflammatory responses and -cell damage in pancreas of streptozotocin-induced diabetic rats. BMC Complement Altern Med. 2016; 16: 63. https://doi.org/10.1186/s12906-016-1032-1 PMID: 26888412
48. Grover JK, Yadav S, Vats V. Medicinal plants of India with anti-diabetic potential. J Ethnopharmacol. 2002; 81(1): 81–100. https://doi.org/10.1016/s0378-8741(02)00059-4 PMID: 12020931
49. Kosuru R, Singh S. Pterostilbene ameliorates insulin sensitivity, glycemic control and oxidative stress in fructose-fed diabetic rats. Life Sci. 2017; 182: 112–121. https://doi.org/10.1016/j.lfs.2017.06.015 PMID: 28629731
50. Sun H, Liu X, Long SR, Teng Wang, Ge H, Wang Y, et al. Antidiabetic effects of pterostilbene through PI3K/Akt signal pathway in high fat diet and STZ-induced diabetic rats. Eur J Pharmacol. 2019; 859: 172526. https://doi.org/10.1016/j.ejphar.2019.172526 PMID: 31283935
51. Rizvi SI, Mishra N. Traditional Indian medicines used for the management of diabetes mellitus. J Diabetol Res. 2013; 2013: 712092. https://doi.org/10.1155/2013/712092 PMID: 23481105
52. Gómez-Zorita S, Fernández-Quintela A, Aguirre L, Macarrúa MT, Rimando AM, Portillo MP. Pterostilbene improves glycemic control in rats fed an obesogenic diet: involvement of skeletal muscle and liver. Food Funct. 2015; 6(6): 1968–1976. https://doi.org/10.1039/c5fo00151j PMID: 25998070
53. Tsai HY, Ho CT, Chen YK. Biological actions and molecular effects of resveratrol, pterostilbene, and 3&#x2032; hydroxypteroistilbene. J Food Drug Anal. 2017; 25(1): 134–147. https://doi.org/10.1016/j.jfda.2016.07.004 PMID: 28911531
54. Bhakkiyalakshmi E, Sireesh D, Sakthivelavimal S, Sivasubramanian S, Gunasekaran P, Ramkumar KM. Anti-hyperlipidemic and anti-oxidative role of pterostilbene via Nrf2 signaling in experimental diabetes. Eur J Pharmacol. 2016; 777: 9–16. https://doi.org/10.1016/j.ejphar.2016.02.054 PMID: 26921755
55. Elango B, Dornadula S, Paulmurugan R, Ramkumar KM. Pterostilbene Ameliorates Streptozotocin-Induced Diabetes through Enhancing Antioxidant Signaling Pathways Mediated by Nrf2. Chem Res Toxicol. 2016; 29: 47–57. https://doi.org/10.1021/acs.chemrestox.5b00378 PMID: 26700463
56. Li W, Chen S, Zhou G, Li H, Zhong L, Liu S. Potential role of cyanidin 3-glucoside (C3G) in diabetic cardiomyopathy in diabetic rats: an in vivo approach. Saudi J Biol Sci. 2018; 25: 500–506. https://doi.org/10.1016/j.sjbs.2016.11.007 PMID: 29686513
57. Wojnar W, Kaczmarczyk-Siedlik I, Zych M. Diosmin ameliorates the effects of oxidative stress in lenses of streptozotocin-induced 1 diabetic rats. Pharmaco Rep. 2017; 69: 995–1000. https://doi.org/10.1016/j.pharep.2017.04.005 PMID: 28846859
58. Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR. Resveratrol, pterostilbene, and piceatannol in vaccinium berries. J Agric Food Chem. 2004; 52(15): 4713–9. https://doi.org/10.1021/jf040095e PMID: 15264904
59. Rodríguez-Bonilla P1, López-Nicolás JM, Méndez-Cazorla L, García-Carmona F., Development of a reversed phase high performance liquid chromatography method based on the use of cyclodextrins as mobile phase additives to determine pterostilbene in blueberries. J Chromatogr B Analyt Technol Biomed Life Sci. 2011; 879(15–16): 1091–7. https://doi.org/10.1016/j.jchromb.2011.03.025 PMID: 21482204
60. McCormack D. and McFadden D., Pterostilbene and cancer: current review. J Surg Res. 2012; 173(2): S3–61. https://doi.org/10.1016/j.sjs.2011.09.054 PMID: 22099605
61. Philip S. Flexible dose open trial of Vijayasar in cases of newly-diagnosed non-insulin-dependent diabetes mellitus. Indian J Med Res. 1998; 108: 24–9. PMID: 9745215
62. Patidar A, Tonpay SD, Agrawal N. Hypoglycemic activity of Pterocarpus marsupium in patients with Type 2 diabetes mellitus. Int J Basic Clin Pharmacol. 2015; 4(6): 1189. 1.http://dx.doi.org/10.18203/2319-2003.ijbcp20151356
63. Hougee S, Faber J, Sanders A, de Jong RB, van den Berg WB, Garssen J, et al. Selective COX-2 inhibition by a Pterocarpus marsupium extract characterized by pterostilbene, and its activity in healthy human volunteers. Planta Med. 2005; 71(5): 387–92. https://doi.org/10.1055/s-2005-864130 PMID: 15931573
64. Riche DM, McEwen CL, Riche KD, Sherman JJ, Wofford MR, Deschamp D, et al. Analysis of Safety from a Human Clinical Trial with Pterostilbene. J Toxicol. 2013; 2013: 463595. https://doi.org/10.1155/2013/463595 PMID: 23431291