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ORIGINAL ARTICLE

Tocopherol from seeds of *Cucurbita pepo* against diabetes: Validation by *in vivo* experiments supported by computational docking

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Background/purpose: Tocopherol from raw pumpkin seeds has been reported to be effective in the alleviation of diabetes through its antioxidant activities. This study evaluates the antidiabetic activities of the tocopherol fraction of raw seeds of *Cucurbita pepo* L. (CPSE) in a diabetic rat model. In addition, the putative action mechanisms of its botanicals were computationally investigated.
Methods: Seed water activity (A_w) was assessed. Tocopherol was extracted and quantified from raw seed oil. The effect of CPSE was studied in poloxamer-407 (PX-407)-induced type 2 diabetic Wistar rats. Glycemic, insulinemic, and lipid profiles, as well as lipid peroxidation status, were evaluated. Glucagon like peptide-1 (GLP-1) content in the cecum was evaluated and

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histopathological analysis of the pancreas was performed. Further, HYBRID and FRED docking were performed for 10 documented CPSE botanicals, for putative action mechanisms concerning three proteins [protein-tyrosine phosphatase 1B (PTP-1B), peroxisome proliferator-activated receptor gamma (PPAR-γ), and dipeptidyl peptidase IV (DPP-IV)] known to have diabetic therapeutic potential.

Results: The A_w of raw seeds was found to be 0.544 ± 0.002. Using tocopherol standards, HPLC determination of CPSE revealed the presence of tocopherol isomers (α, β, γ, and δ). The tocopherol content was found to be 107.4 ± 2.9 mg/100 g of CPSE. When compared to diabetic control (DC) rats, the CPSE-treated diabetic rats presented a significant amelioration of glycemia, insulinemia, and lipid dysmetabolism. A remarkable reduction in oxidative markers and improved cecal and pancreatic characteristics were also observed. Tocopherol isomers have shown a considerable interaction potential with the aforesaid proteins in docking.

Conclusion: The results provide pharmacological evidence of CPSE as an antihyperglycemic mediated by the interaction of various botanicals with multiple targets operating in diabetes mellitus (DM).

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by abnormalities of glucose homeostasis by various organs. It accounts for about 85—95% of all diagnosed cases of diabetes and is associated with considerable morbidity and mortality. Several treatment strategies are being adopted to ameliorate T2DM by therapeutic intervention.[1—3] Synthetic drugs, despite having excellent potencies, had offered unwanted therapeutic profiles marked by fluid retention, drug-induced hypoglycemia, liver malfunctioning, weight gain, and cardiac dysfunction.[4]

Extensive research has been carried out worldwide on rational drug design,[5] to identify and optimize new leads for molecular targets of T2DM, such as nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ),[6,7] human incretin-degrading enzyme dipeptidyl peptidase IV (DPP-IV),[5,8] and protein-tyrosine phosphatase 1B (PTP1 B).[5,9] The PPAR-γ regulates glucose metabolism and fatty acid storage by enhancing the insulin action.[5—7] The DPP-IV is a membrane-bound, serine protease ectoenzyme responsible for the degradation and inactivation of a number of glucose regulating incretin hormones, like glucagon like peptide-1 (GLP-1). GLP-1 causes an increase in the amount of insulin released from pancreatic β-cells.[5—8] Thus, diabetic conditions can be reduced either by injecting an excess of GLP-1 or its analogues, inhibiting the action of DPP-IV.[10] PTP-1B inhibitors have emerged as a promising potential therapeutic target for T2DM, as they are negative regulators of the insulin signaling pathway and regulate lipogenesis and hypertriglyceridemia.[5,9]

These therapeutic targets may improve the efficacy-to-safety ratio, resulting in durable maintenance of glycemic control in the majority of people with diabetes. Numerous structurally unrelated natural products, including alkaloids and flavonoids, have been described to interact with these target proteins in micromolar concentrations.[5,11] Therefore, there is much scope for herbal medicines, which have emerged as a safe and relatively economical therapy found to be effective as add on therapy, in the long-term management of T2DM.[11]

Pumpkin [*Cucurbita pepo* (*C. pepo*)] seed oil (about 11—31% of total seed content) has been implicated as a nutraceutical due to its macro- and micro-constituent composition. The seed oil is a rich source of proteins, diverse fatty acids, and antioxidant vitamins, such as carotenoids and tocopherols.[12—14] It has been documented that the tocopherol content of the oil ranged from 27 μg/g to 75 μg/g of oil for α-tocopherol, from 75 μg/g to 493 μg/g for γ-tocopherol, and from 35 μg/g to 1110 μg/g for δ-tocopherol.[14,15] So far, several pharmacological properties of pumpkin have been reported including lipid-lowering,[16,17] hepatoprotective,[17] anticarcinogenic,[18] antimicrobial,[19] and treatment of urination disorders,[20] in addition to antidiabetic and antioxidant activities.[20—25]

Despite of the folkloric use of seed extract of *C. pepo*, there is insufficient supporting scientific data forming the basis of the present investigation. It systematically evaluates the beneficial effects of the tocopherol fraction of pumpkin seed oil *in vivo* in poloxamer-407 (PX-407)-induced diabetic Wistar rats. Intraperitoneal administration of the nonionic copolymer PX-407 in rats induces hyperglycemia with impaired response and loss of β-cell sensitivity to glucose, which is considered as a useful diabetic model to study the activity of antihyperglycemic agents.[26] In addition, *in silico* molecular docking was performed using HYBRID[27] and FRED[28] programs, to propose the interaction potential of 10 botanicals from *C. pepo* seeds, documented hitherto concerning three proteins (PTP-1B, PPAR-γ, and DPP-IV) known to have diabetic therapeutic potential.

Methods

Chemicals

PX-407, heparin, glucose oxidase, peroxidase, o-dianisidine, bovine serum albumin, Coomassie brilliant blue G-250, cysteine hydrochloride, thiosemicarbazide, and diacetyl monoxime were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tocopherol isomers were purchased as follows: α-tocopherol — Fluka (Sigma-Aldrich, St. Louis, MO, USA), β-tocopherol — Matreya, LLC (Matreya LLC, 168 Tressler St, Pleasant Gap, PA 16823, United States), γ-tocopherol and δ-tocopherol — Supelco (Sigma-

Aldrich, St. Louis, MO, USA). The blood glucose diagnostic kit was purchased from Span Diagnostic Limited, Surat, India and the glycosylated hemoglobin (HbA_1c) diagnostic kit was from Monozyme India Limited, Secunderabad, India. The biochemical kits for measuring the lipid profiles were procured from Biosys, Bangalore, India and ketamine was from Neo Pharmaceuticals, Bangalore, India. The enzyme-linked immunosorbent assay (ELISA) kit for insulin assay and GLP 1 determination was purchased from Millipore Corporation (Linco Research, Inc., St. Charles, MO, USA, 63304). Casein (refined grade) was procured from Nimesh Corporation (Mumbai, India). The salt mixture was procured from SISCO Research Laboratories Pvt. Ltd. (Mumbai, India). The standard pellet diet (SPD) was purchased from Lipton Rat Feed Ltd. (Pune, India). All other chemicals and solvents used in this study were obtained from Merck, India and were of analytical grade.

Collection and preparation of plant materials

The seeds of *C. pepo* L. were collected from Hajipur in northern India, in 2009. It was hermetically sealed in plastic packaging, and purchased from a local folk medicinal plant dealer in Patna, Bihar, India. The plant was identified and botanically authenticated at source and a voucher specimen (CAS#106/2011) was deposited in the postgraduate department of biochemistry, Patna University, Patna for future reference. No fungal development was assured by determining the water activity (A_w) with an Aqualab A_w meter (model CX-2; Decagon Devices, Inc., Hopkins Court Pullman, WA 99163- USA).[30] The plastic cap of the Aqualab was filled with ground seeds and the analysis was performed in two replicates at $24 \pm 1^{\circ}C$. Below a threshold of $A_w = 0.62$, there is usually no notable fungal development, which is a good indicator of the preservation potential of the product and its microbiological stability with time.

Tocopherol extraction from pumpkin seeds

The procedure used was the one described by Murkovic et al (2004).[31] Briefly, ground pumpkin seeds were added with anhydrous sodium sulfate (1:5 ratio) to remove the water and subsequently tocopherol was extracted with *n*-hexane in an ultrasonic bath for 30 minutes. To prevent oxidation, 80 mg of butylated hydroxytoluene were added. At the end of the extraction, the extract was filtered and its volume was reduced on a rotary evaporator, brought to 1000 mL with *n*-hexane, and was named CPSE.

Analysis and quantification of tocopherol in CPSE by HPLC

Tocopherol quantification was carried out for four external tocopherol standards and CPSE by the standard method of ISO 9936 (2006).[32] The standard solutions were prepared and preserved in HPLC grade hexane in flasks without actinic activity. High-performance liquid chromatography (HPLC) was performed on an Agilent 1100 Series HPLC system equipped with a BinPump G1312 A binary pump, a thermostatted ALS G1329 A auto sampler, an ALSTherm G1330 B auto sampler thermostat, a COLCOM G1316 A thermostatted column compartment, an FLD G1321 A fluorescence detector (Agilent Technologies, Hewlett-Packard-Strasse 8, Waldbronn, Germany) operating at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. The analytical column used was a Phenomenex (Torrance, CA, USA) Luna (250×4.60 mm), packed with 5 μm silica, S/N 00G-4274-E0. The mobile phase used was heptane-THF ($1000 + 40$ v/v) at a constant flow rate of 1.0 mL/minute. The injection volume was in the range of 5—100 μL in order for all the tocopherol isomers to elute in the calibration range. The retention times of α-tocopherol and γ-tocopherol were in the range of 11.0—11.5 minutes and 18.0—19.0 minutes, respectively. The elution order was as follows: α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol. These tests were repeated four times to obtain the most representative possible equations calculated by linear correlation coefficient (R^2). This was found to be greater than 0.9998 for all tocopherol isomers (Table S1). The detection of tocopherols was done using a fluorometer, which made it possible to inject and proportion the oil directly in hexane. Excitation was set at 290 nm, thus inducing fluorescence emission by tocopherols at 330 nm.

Experimental animals and induction of diabetes

The healthy male albino Wistar rats (150—160 g) were kept under good hygienic conditions and fed on SPD and *ad libitum* water throughout the experimental period. Rats had undergone multiple administrations of freshly prepared PX-407 solution (10 mg/kg body weight once a day for 6 weeks) followed by 1 hour of fasting. They were allowed to access the food and water *ad libitum* for 6 weeks. Rats with fasting blood glucose level of 200 mg/dL or higher were considered to be diabetic and were used in the study. During the experimental period, the animals were observed within cages daily for mortality, or signs of toxic effects (see also supplemental text). The experimental study was approved by the Institutional Animal Ethics Committee of Department of Health, Patna, Bihar, India.

Experimental design

The rats were divided into four different groups having six animals each. These were the DC group (diabetic control rats), NC group (nondiabetic control rats), DT_2 group (diabetic rats treated with 2 g of CPSE/kg body weight), and DT_5 group (diabetic rats treated with 5 g of CPSE/kg body weight). Body weights were recorded weekly during the experimental period. Treatment with CPSE was started from the 3rd week of PX-407 injection, which was considered as the 1st day of treatment.

Analytical parameters

Glycemic, insulinemic, and lipidic profile

Blood samples were collected from the caudal vein by means of a small incision at the end of the tail. Plasma glucose levels were measured using a glucose oxidase commercial kit (Span Diagnostic Limited). Taking into account the variability of serum glucose levels in rats,

HbA1c levels were used as an index of glucose control (Monozyme India Limited, Secunderabad, India). Serum insulin (SI) was estimated by a radioimmunoassay method using the kit from Bhabha Atomic Research Centre, Mumbai, India. Insulin sensitivity of individual animals was evaluated using the previously validated homeostasis model assessment (HOMA) index[33] using the formula: $\mathrm{HOMA}-R=\frac{FI\ (\mu U/ml)\times FBG\ (mg/dl)}{405}$. The values used (insulin and glucose) were obtained after overnight food deprivation. The serum was separated and analyzed spectrophotometrically for triglyceride (TG) and total cholesterol (TC) using a diagnostic reagent kit (Nicholas Piramal India Ltd., Mumbai, India).

Endogenous enzymatic and nonenzymatic antioxidant levels

At the end of the experimental period, rats were fasted overnight and sacrificed under anesthesia (i.p. ketamine, 150 mg/kg body weight). The whole liver was dissected out subsequent to saline water perfusion, followed by preparation of the 10% liver homogenate with ice-cold saline-EDTA. The protein content was measured with the Folin phenol reagent.[34] The homogenate was further subjected to the evaluation of nonenzymatic [reduced glutathione (GSH) and total thiols] and enzymatic antioxidant [catalase, glutathione-S-transferase (GST), and superoxide dismutase (SOD)] status using spectrophotometric methods.[35] Lipid peroxidation levels of the liver homogenates were also determined.[36]

GLP-1 content in the cecum

The cecal contents from the excised cecum of sacrificed rats were removed. The cecal tissue was thoroughly washed with cold phosphate buffer saline (PBS; pH 7.4) and homogenized at 4°C in PBS containing DPP-IV inhibitor (20 μL/mL PBS). The homogenate was then centrifuged at 10,000 × *g* for 10 minutes, followed by the supernatant collection and GLP-1 level determination by ELISA kit, (Millipore Corporation, Linco Research, Inc., St. Charles, MO, USA).

Histological studies

The pancreas of animals from different experimental groups were examined *in situ*, excised, and perfused with 10% neutral formalin. They were then embedded in paraffin, thinly sectioned using a microtome, stained with hematoxylin-eosin (HE), mounted in neutral DPX medium, and examined under a light microscope. Histopathological studies were performed to estimate the beta cell mass, increase in the islet number, and to check the quality of insulitis.

Molecular docking studies

- Data collection and ligand preparation

To gain further insight in to the interaction of fruit extracts with diabetic proteins, 10 active botanicals of CPSE, reported in various studies in the literature[12,14,20,21] were selected for docking studies. Two-dimensional structures of these botanicals were either retrieved from PubChem[29,37] or drawn using the MSketch tool (v-5.8.0) available from ChemAxon (Graphisoft Park, Budapest, Hungary).[38] The correct protonation states were enumerated by the tautomer enumeration program.[39] The partial charges were assigned by MMFF94[40] force-field available in the molcharge[39] program. Further, stereocenters were enumerated using flipper[39] in those molecules where a stereocenter does not have a specified stereochemistry. The physicochemical properties, as well as the 3D similarity of 10 botanicals and a native ligand used in this study were calculated by MolProp[41] and OEChem shape toolkits.[42] The 3D conformers were generated using OMEGA[39] and used for docking to three diabetic proteins, namely DPP-IV, PPAR-γ, and PTP1 B. The numbers of conformers were determined by counting the rotatable bonds present in the individual ligand.

- Protein preparation and self-docking

The receptors for three selected proteins, namely DPP-IV, PPAR-γ, and PTP1 B, were prepared using the FRED-receptor preparation tool.[39] There are 72, 113, and 120 structures available in the PDB database[37] for DPP-IV, PPAR-γ, and PTP1 B, respectively. Of these, PDB IDs 4AS5 (1.62 Å resolution),[43] 2HWQ (1.97 Å resolution),[44] and 2F71 (1.55 Å resolution)[45] were selected, respectively, for DPP-IV, PPAR-γ, and PTP1 B. The active site was identified on the basis of bound ligand to the receptors. The volume of active sites (outer contour) of DPP-IV, PPAR-γ, and PTP1 B are 2057, 2016, and 1623 cubic angstroms, respectively. Protein constraints were set on critical residues, the interaction of which is necessary for ligand binding of the aforesaid proteins. These hydrogen bonding constraints are set on residues E205, E206, Y631, Y547 in DPP-IV,[43] H449, Y473 in PPAR-γ,[46,47] and D181, S216, A217, R221 in PTP1 B[48,49] active sites. The protein-based docking constraints are interactions between ligands and a heavy atom of the protein-amino acid residues. This does not affect how a given pose scored, but affects the process of selection of poses to score. The botanicals satisfying at least one per set of constraint are successfully docked in order to minimize the rate of false-positive detection. Any pose that does not match the docking constraints is rejected and replaced by the next best scoring pose. The correctness of the protein preparation step was checked by a self-docking process, in which the co-crystallized ligand was re-docked in the receptor. For acceptable self-docking, the root mean square deviation (RMSD) was kept <2.00 Å compared to the reference structure.[50] The HYBRID[27] and FRED[28] docking program was used to dock multi-conformer molecules into a receptor using a two-step process: (1) *Systematic exhaustive search*: This performs systematic exhaustive search by enumerating every possible rotation and translation of each conformer of the ligand being docked within a box enclosing the active site. Poses are discarded based on either clash with the protein or extending too far from the binding site (using the receptor's negative image outer contour). Further, two optional poses rejection steps were employed: (a) based on the receptor's negative image inner contour

and (b) based on (docking) constraint. Poses that do not have at least one heavy atom falling within the inner contour are discarded. A pose that does not match the docking constraints will be rejected and replaced by the next best scoring pose. In the case of FRED, the selected poses are then scored using the Chemgauss3 scoring function. HYBRID additionally considers a similarity of the ligand to the receptor bound ligands. The similarity is calculated based on the match of the 3D shape and chemical features of the docking ligand and bound ligand. It is evident from Table 6 that some of the ligands have low 2D Tanimoto[51] coefficients, calculated based on Extended-Connectivity Fingerprints (ECFP4) keys based on molecular fingerprints[52] than 3D Tanimoto coefficients. The selected poses are scored using the scoring function. All the selected poses are sorted by score and the top scoring poses are passed to the optimization step. (2) *Optimization and final scoring*: The top scoring poses obtained after exhaustive searching are optimized and scored by enumerating the nearby positions of each pose having the initial pose. One positive and one negative step for each translational and rotational degree of freedom are taken. The poses are finally scored with Chemgauss4 and the Chemical Gaussian Overlay (CGO) scoring function in FRED and HYBRID, respectively. Ten botanicals from *C. pepo* were docked to three proteins, namely 2F71 (PTPB1), 2HWQ (PPAR-γ), and 4A5 S (DPP-IV) and molecular interactions were plotted using PoseView.[53]

Statistical analysis

Data were expressed as the mean $\pm$ S.E.M. For statistical analysis of the data, the group means were compared by one-way ANOVA with *post hoc* analysis. The Tukey-Karmer *post hoc* test was applied to identify the significance among groups. Graphs were plotted using MATLAB version 7.8.0 R2009a, Natick, MA, USA: The Mathworks Inc. 2009. A p value of 0.001 was considered to be statistically significant.

Results

Characterization of CPSE

The A_w of raw seeds was found to be 0.544 $\pm$ 0.002. This showed that the seeds were stable with respect to the potential growth of microorganisms.[30]

Tocopherol analysis of CPSE by HPLC

The HPLC analysis of tocopherol standards is shown in Fig. 1A and that of the CPSE with respect to a standard one, in Fig. 1B. The seeds had a tocopherol content of 107.4 $\pm$ 2.9 mg/100 g of CPSE.[14]

Effect of CPSE on body weight and lipid profile

The DC rats demonstrated a higher body weight (+107%; $p < 0.001$) when compared with the NC rats (Table 1). No significant changes in body weight gain were encountered between the treated groups of diabetic rats (DT_2 and DT_5) *versus* the DC rats (Table 1). The DC rats also presented higher TC (+173%; $p < 0.001$) and TGs (+589%; $p < 0.001$) values compared to NC rats. These changes in biochemical indices are expected as the diabetic condition progresses. The treated diabetic rats (DT_2 group) showed significantly

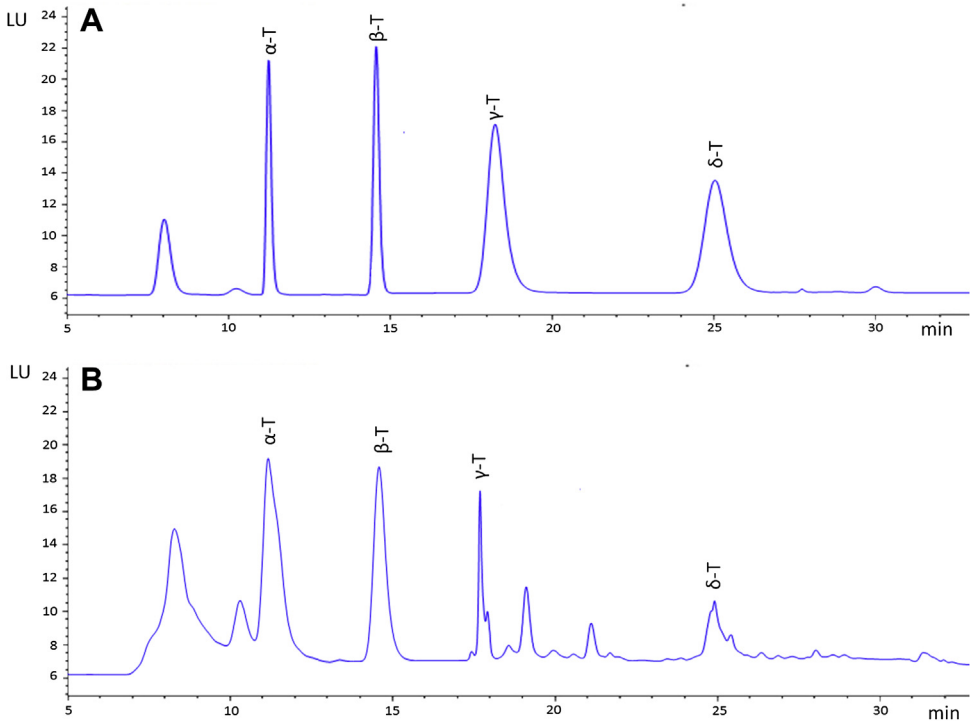

Figure 1 HPLC analysis of (A) standard tocopherols and (B) tocopherols from CPSE with their main peaks. T = tocopherol.

Table 1 Effects of CPSE on body weight, glucose, HbA1c, insulin, HOMA-IR, TC, and TGs on different rat groups.

	Control rats			Treated rats			
	NC	DC	Effect	DT_2	Effect	DT_5	Effect
Body weight (g)	157.13 ± 2.30	325.50 ± 2.73**	⇑	342.00 ± 3.23	⇔	329.42 ± 2.34	⇔
Glucose (mg/dL)	133.72 ± 2.08	614.85 ± 3.84**	⇑	569.43 ± 17.66*	⇓	398.93 ± 5.53*	⇓
HbA1c (%)	3.38 ± 0.12	12.59 ± 0.12**	⇑	11.39 ± 0.25	⇓	12.46 ± 0.07	⇔
Insulin (pmol/L)	50.31 ± 0.99	184.85 ± 1.28**	⇑	174.98 ± 0.36*	⇓	154.62 ± 1.21*	⇓
HOMA-IR	2.34 ± 0.98	40.35 ± 2.02**	⇑	32.13 ± 1.33*	⇓	24.31 ± 1.06*	⇓⇓
TC (mg/dL)	75.95 ± 1.17	208.87 ± 1.37**	⇑	178.79 ± 2.32*	⇓	206.68 ± 0.95	⇔
TGs (mg/dL)	60.25 ± 0.71	418.06 ± 3.19**	⇑	357.65 ± 0.98*	⇓	413.98 ± 1.23	⇔

Results are mean ± SEM of six rats per group.
* Statistically significantly different versus the normal control rat group ($p < 0.001$).
** Statistically significantly different versus the diabetic control rat group ($p < 0.001$).

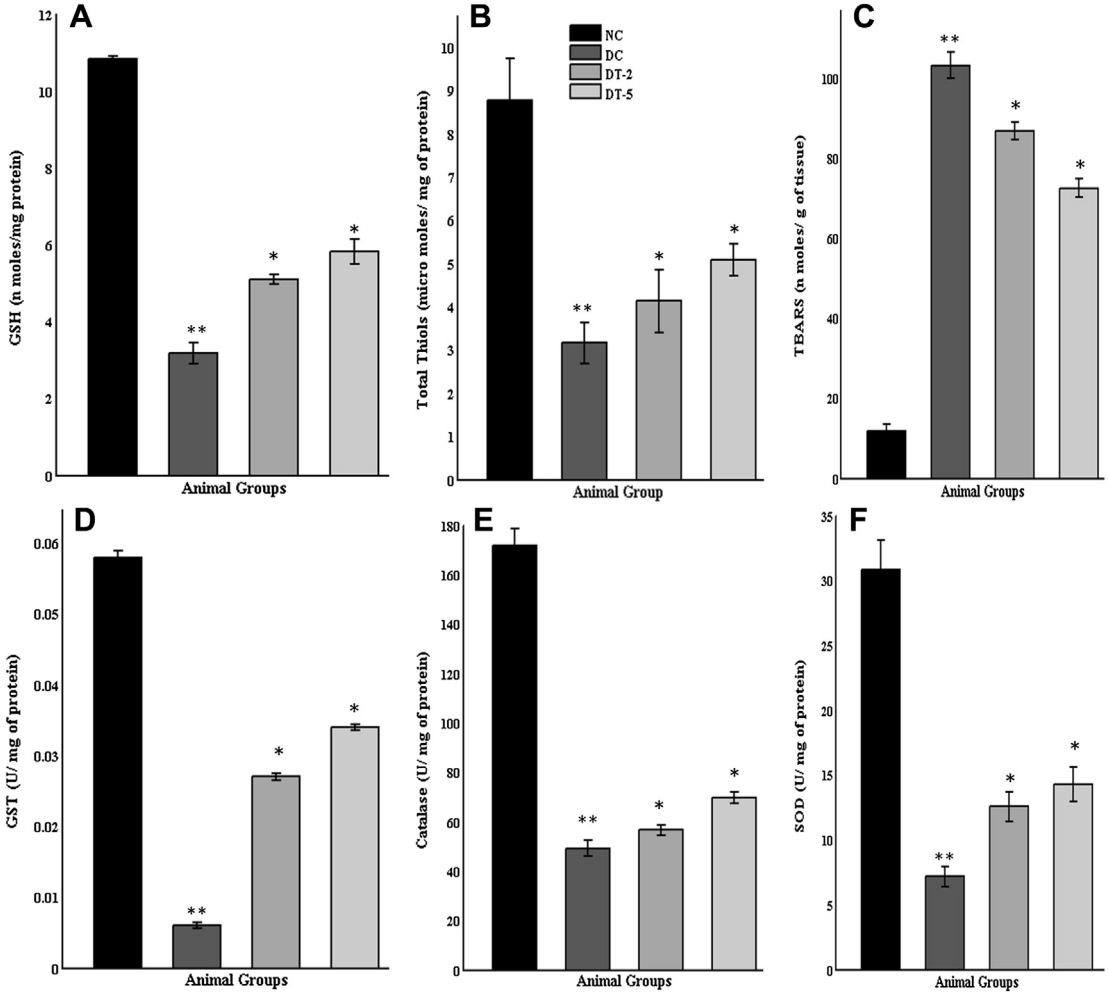

Figure 2 Effect of treatment with CPSE on nonenzymatic antioxidants (A) glutathione (GSH) and (B) total thiols as well as (C) thiobarbituric acid reactive substances (TBARS) and enzymatic antioxidants (D) glutathione-S-transferase (GST), (E) catalase and (F) superoxide dismutase (SOD) in the liver homogenates of different rat groups. Each bar represents the mean ± SE ($n = 6$). DC = diabetic control animals; DT_2 = diabetic animals treated with a low CPSE dose (2 g/kg body weight); DT_5 = diabetic animals treated with a high CPSE dose (5 g/kg body weight); NC = untreated normal control animals. *$p < 0.001$, compared to the diabetic control rats. **$p < 0.001$, compared to the normal control rats.

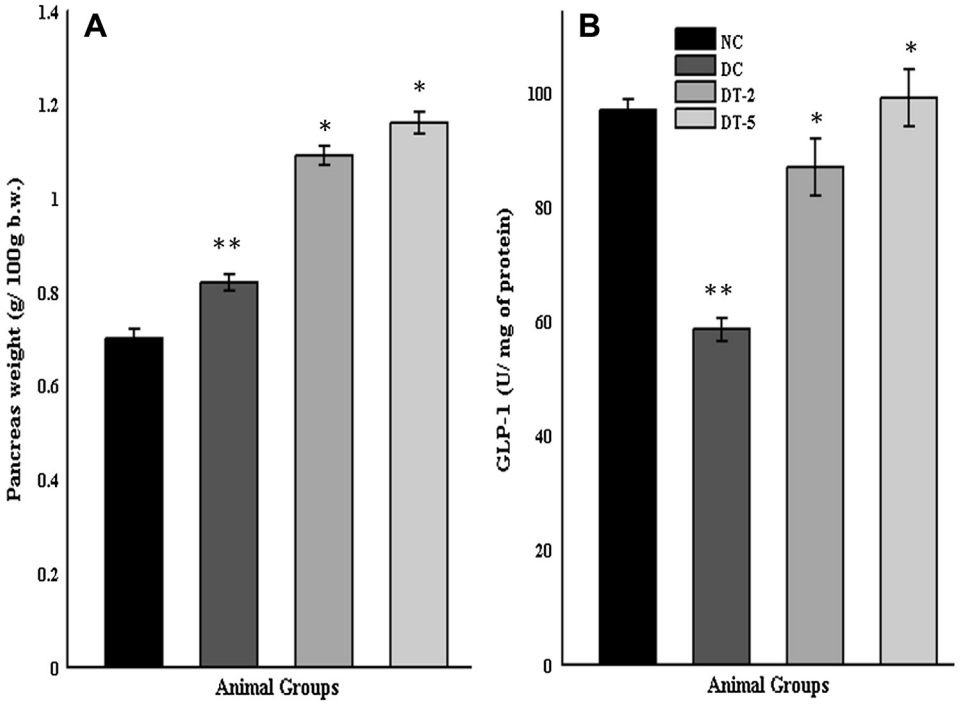

Figure 3 Effect of CPSE treatment on (A) pancreas weight and (B) glucagon like peptide-1 (GLP-1) contents. Each bar represents the mean ± SE ($n = 6$). DC = diabetic control animals; DT_2 = diabetic animals treated with a low CPSE dose (2 g/kg body weight); DT_5 = diabetic animals treated with a high CPSE dose (5 g/kg body weight); NC = untreated normal control animals. *$p < 0.001$, compared to the diabetic control rats. **$p < 0.001$, compared to the normal control rats.

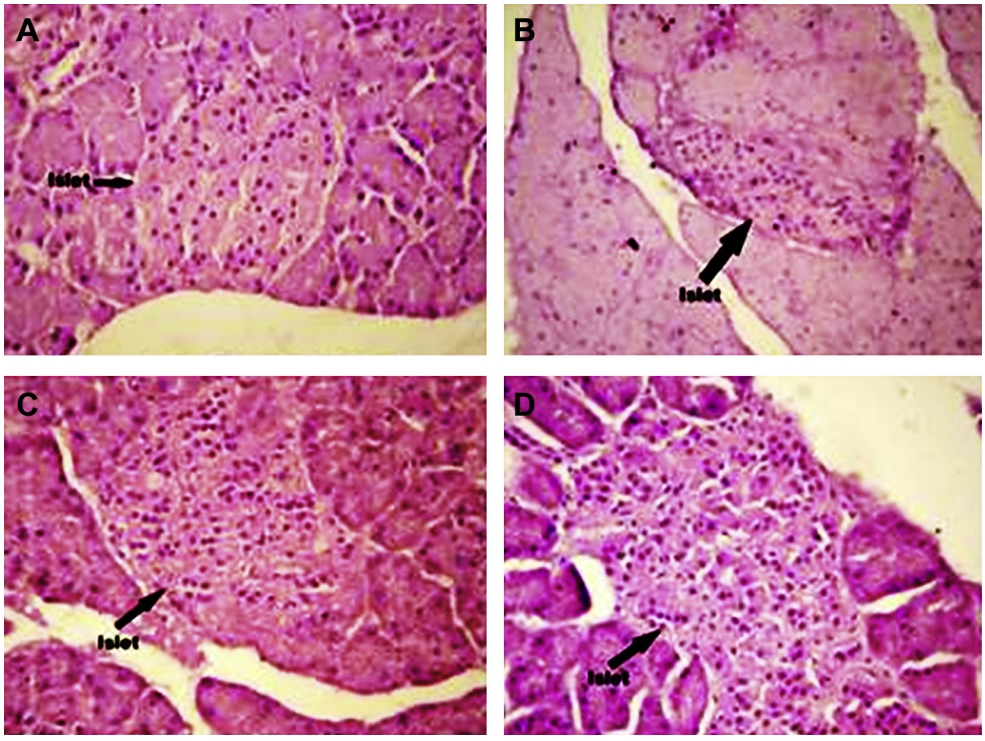

Figure 4 Histopathology of pancreas showing variation in islet number, size, and grade of insulitis in each rat group. (A) NC = untreated normal control animals; (B) DC = diabetic control animals; (C) DT_2 = diabetic animals treated with a low CPSE dose (2 g/kg body weight); (D) DT_5 = diabetic animals treated with a high CPSE dose (5 g/kg body weight).

lower values of serum TC (+21.7%; $p < 0.001$) and TGs (+30.9%; $p < 0.001$), when compared with the DC counterparts. The DT_5 treatment had no lowering effects on TC or on TGs levels (Table 1).

Effect of CPSE on glycemia, HbA1c, insulinemia, and insulin resistance

As expected, the DC rats showed significantly ($p < 0.001$) higher values of glucose (+370%), HbA1c (+280%), and insulin (+275%), together with higher insulin resistance (HOMA-IR), when compared with their treated counterparts (Table 1). Diabetic rats of both groups (DT_2 and DT_5) showed a reduction in glucose levels, when compared to the DC ones; nevertheless, the reduction was particularly evident in the DT_5 rats (-57.5%; $p < 0.001$). When comparing the glucose levels of the DT_5 *versus* the DT_2 group rats, a significantly lower value in the first was found (−55.0%; $p < 0.001$) (Table 1). The 12-week treatment program also significantly diminished the HbA1c levels (−8.3%; $p < 0.01$) in the diabetic rats, although no changes were detected in the HbA1c levels of the DT_5 group rats. The CPSE treatments were able to significantly reduce ($p < 0.001$) insulin levels in diabetic rats (Table 1). In agreement with the abovementioned results, CPSE treatments in diabetic rats (DT_2 and DT_5) significantly reduced ($p < 0.001$) the insulin levels as well as insulin resistance, evaluated by HOMA-IR index (Table 1). Nevertheless, this drop in insulin resistance was more evident in the DT_5 rats (−60.6%) than in the DT_2 rats (−26.8%).

Effect of CPSE on endogenous enzymatic and nonenzymatic antioxidant levels

Reduced GSH

NC rats showed basal GSH levels of about 10.86 ± 0.065 nmoles/mg of protein. PX-407 induction for 6 weeks led to significant reduction ($p < 0.001$) in GSH levels (3.18 ± 0.271 nmoles/mg of protein). Treatment with higher doses of CPSE to diabetic rats (DT_2 and DT_5) produced a significant ($p < 0.001$) improvement in GSH levels (Fig. 2A).

Total thiols

Basal total thiol levels in NC rats were found to be 8.78 ± 0.96 μmoles/mg of protein. Diabetic rats showed significantly decreased ($p < 0.001$) levels of total thiols (3.16 ± 0. 476 μmoles/mg of protein). Moreover, treatment of diabetic rats with CPSE (DT_2 and DT_5) showed significantly ($p < 0.01$) increased levels (Fig. 2B).

Lipid peroxidation

NC rats showed basal thiobarbituric acid reactive substances (TBARS) levels of about 11.83 ± 1.79 nmoles/g of liver tissue. Diabetic rats showed significantly increased ($p < 0.001$) TBARS levels (103.18 ± 3.245 nmoles/g of tissue). Treatment with CPSE (DT_2 and DT_5) significantly ($p < 0.001$) abolished the increase in TBARS levels induced by PX-407 (Fig. 2C).

GST

Normal basal GST activity in NC rats was found to be 0.058 ± 0.0009 U/mg of protein. Diabetic rats exhibited significantly reduced ($p < 0.001$) levels of GST (0.0062 ± 0.0004 U/mg of protein). The two tested doses (DT_2 and DT_5) of CPSE significantly ($p < 0.001$) increased the levels of GST (Fig. 2D).

Catalase

Normal basal level of catalase activity in NC rats was found to be 171.83 ± 6.779 U/mg of protein. Diabetic rats showed significantly decreased ($p < 0.001$) levels of catalase by about more than threefold (i.e., 49.15 ± 3.265). However, treatment with CPSE (DT_2 and DT_5) showed significantly ($p < 0.001$) increased levels of catalase to the near normal values (Fig. 2E).

SOD

NC rats showed basal SOD levels of about 30.83 ± 2.273 U/mg of protein. Diabetic rats exhibited significantly reduced

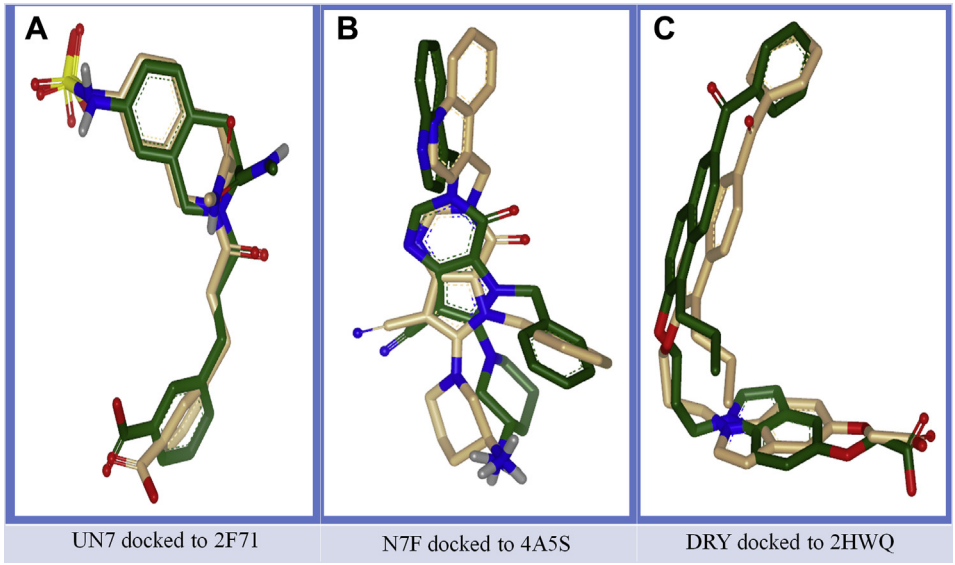

Figure 5 Self-docking results compared with interactions present in PDB (3D). Green docked compounds and yellowish co-crystallized ligand. (A) UN7 docked to 2F71; (B) N7 F docked to 4A5 S; (C) DRY docked to 2HWQ.

Table 2 Botanicals documented for docking from seeds of the *C. pepo* plant.

Compound no.	Name	Pub-chem CID
1	γ-Tocopherol	92729
2	α-Tocopherol	14985
3	δ-Tocopherol	92094
4	β-Tocopherol	6857447
5	β-Carotene	5280489
6	Lutein	448437
7	Cucurbitine	442634
8	β-Sitosterol	222284
9	Campesterol	173183
10	Squalene	638072

($p < 0.001$) levels of SOD by about one fourth (7.18 ± 0.766 U/mg of protein). Treatment with CPSE (DT_2 and DT_5) showed significantly ($p < 0.001$) increased levels of SOD to the near normal values (Fig. 2F).

Effect of CPSE on pancreas weight

The pancreatic weight of diabetic rats was found to be lower than those of the NC rats ($p < 0.001$). However, CPSE-treated diabetic rats (DT_2 and DT_5) had enlarged pancreases compared with DC rats (NC: 2.46 ± 0.08 g, DC: 1.32 ± 0.04 g, DT_2: 1.69 ± 0.21 g, DT_5: 1.91 ± 0.19 g). However, when the biochemical parameters were normalized with respect to body weight, the CPSE-treated diabetic rats showed an increase in pancreatic weight compared to NC rats (Fig. 3A).

Effect of CPSE treatment on GLP-1 levels in cecum tissue

To investigate whether CPSE treatment affected GLP-1 production, the incretin level was measured in intestinal tissue. In the cecum, the diabetes (DC group) showed a significant decrease ($p < 0.001$) in GLP-1 concentration, which was 70% of NC rats. The CPSE treatment significantly increased GLP-1 concentration. Due to the important cecal tissue enlargement in these rats, GLP-1 content expressed in picomoles per total cecum was 1.4-fold higher in DT_2 and 1.6-fold higher in DT_5 than in DC rats (Fig. 3B).

Histopathological analysis

The effect of CPSE on the number of pancreatic islets, its size and the grade of insulitis on different rat groups can be found in the online supplementary material. In the DC group, the number and size of islets decreases significantly ($p < 0.001$) and the grade of insulitis is also very high, when compared to NC rats. In DC_2 and DC_5 rats there was a significant improvement in the number of islets, as well as the grade of insulitis (Fig. 4; Table S2).

LD_{50}

The behavior of the treated rats appeared normal. No toxic effect was reported up to the effective dose of the CPSE, as there was no death in any of these groups.

Molecular docking studies

- Self-docking

In the case of PTP1 B and DPP-IV, the self-docking RMSD was observed within 1 Å, while for PPAR-γ it was within 1.45 Å, indicating acceptable quality of docking preparation. The 3D overlay of the co-crystal bound and docked ligands is shown in Fig. 5 and the protein structures studied along with interacting residues and type of interaction is given in Table 3.

- CPSE botanicals docked to PTP1 B, PPAR-γ, and DPP-IV

a. Docking with constraints

None of the 10 active CPSE compounds (Table 2) were capable of docking to PTP1 B, while the number of botanicals docked to PPAR-γ and DPP-IV were six and five,

Table 3 Protein structures studied.

PDB ID	Name	Ligand ID	RMSD (Å)	Interacting residues in PDB	Interacting residues in self-docking	Major interactions
4A5 S	Dipeptidyl peptidase IV (DPP-IV)	N7 F	0.85	Tyr547 A; Tyr662 A	Tyr547 A	Pi—Pi
				Tyr662 A; Tyr631 A; Glu206 A; Glu205 A	Tyr666 A; Tyr631 A; Lys554 A; Glu206 A; Glu205 A	Hydrogen bond
				Ser630 A; Trp629 A; Gly628 A; Tyr547 A	Ser630 A; Trp629 A; Trp627 A	Hydrophobic
2HWQ	Peroxisome proliferator-activated receptor gamma (PPAR-γ)	DRY	1.45	His449 A; Tyr473 A	Tyr473 A; Ser342 A	Hydrogen bond
2F71	Protein tyrosine phosphatase 1B (PTP1 B)	UN7	0.97	Phe182 A; Tyr46 A	Phe182 A; Tyr46 A	Pi—Pi
				Arg254 A; Arg221 A; Gly220 A; Ala217 A; Ser216 A	Arg254 A; Arg221 A Ala217 A; Cys215AAsp181 A	Hydrogen bond
				Ile219 A; Ala217 A; Phe182 A	Ile219 A	Hydrophobic

Table 4 Interaction of botanicals docked to PPAR-γ and DPP-IV.

Compound no.	Pub chem CID	Hybrid score	FRED score	Interacting residues	Major interactions
Botanicals docked to DPP-IV; bioactive ligand has FRED score (−10.24) and hybrid score (−10.24)					
3	92094	−7.056698	−7.056698	E205, Y662	Hydrogen bond
				S630, Y547, W629	Hydrophobic
1	92729	−6.769999	−6.709151	E205, Y662	Hydrogen bond
				S630, Y547, W629	Hydrophobic
2	14985	−6.014852	−6.014852	Y662, Y666, Y631	Hydrogen bond
				S630, Y547, W629	Hydrophobic
				Y666	Pi—Pi
4	6857447	−5.745492	−5.543730	E205, E206, Y662	Hydrogen bond
				Y547, W627, W629	Hydrophobic
7	442634	−3.490675	−3.758782	Y662, H740	Hydrogen bond
Botanicals docked to PPAR-γ; bioactive ligand has FRED score (−18.90) and hybrid score (−18.02)					
3	92094	−12.337214	−12.901058	H449, C285	Hydrogen bond
				C285, I341	Hydrophobic
1	92729	−11.677748	−12.260074	H449	Hydrogen bond
				G284, I281, C285, Y327, L330, I341	Hydrophobic
4	6857447	−11.559639	−11.897834	H449	Hydrogen bond
				I281, C285, Y327, L330, I341	Hydrophobic
2	14985	−11.270902	−12.379365	H449	Hydrogen bond
				I281, C285, Y327, L330, M361, I341	Hydrophobic
9	173183	−6.870636	−6.424315	Y473	Hydrogen bond
				I341, I330, C285, R288	Hydrophobic
8	222284	−6.145874	−5.925000	H449, Y473	Hydrogen bond
				L333, L330, H449, C285, R288	Hydrophobic

respectively. Table 4 showed that tocopherols (IDs 1, 2, 3, and 4) are commonly docked in DPP-IV and PPAR-γ with conserved binding interactions (Fig. 6). Based on this conservation of interactions with important residues in the active site of PPAR-γ, the modulating role of the above-mentioned botanicals can be proposed (Table 4). Different conformers of botanicals determined by counting the rotatable bonds, present in individual ligand were docked in the active sites of three proteins (Table 5).

DPP-IV contains two sub-pockets S1 and S2. The hydrophobic S1 pocket is composed of residues V656, Y631, Y662, W659, Y666, and V711, while the hydrophobic S2 pocket is determined by the side chains of residues of R125, F357, Y547, P550, Y631, and Y666. The five botanicals (IDs 1, 2, 3, 4 and 7) docked to DPP-IV, bound to DPP-IV in the S1 sub-pocket with H-bonding (Glu205 and/or Y662 and/or Y666) as well as residues in the S1 sub-pocket (Table 4; Fig. 6). Moreover, botanicals with IDs1, 2, and 3 had hydrophobic interactions with S630, Y547, and W629 residues. Similarly, six botanicals (IDs 1, 2, 3, 4, 8, and 9) docked to PPAR-γ formed hydrogen bonds with H449 and hydrophobic interactions with C285 (Table 4).

Most of the H-bonds and hydrophobic interactions were found during tocopherol-target protein interactions. However, with DPP-IV, α- and β-tocopherols interacted through additional H-bonds compared to γ- and δ-tocopherols. Similarly, with PPAR-γ, δ-tocopherol interacted with one additional hydrogen bond when compared to the other three tocopherols. Some residues contributing to hydrophobic interactions also differed during the interaction of tocopherols with DPP-IV and PPAR-γ. The α-, β-, δ-, and γ-tocopherols had similar/identical numbers of H-bond acceptors and donors, however, they differed marginally with each other in terms of XlogP values (Fig. 7). Although, α-tocopherol *versus* β-, δ-, and γ-tocopherols shared a high similarity in 2D, they differed with each other (averaged over 25 conformers of each tocopherol) in their 3D shape similarity, which was calculated by the OEChem shape toolkit[42] (Table S6). The α-, β-, δ-, and γ-tocopherols had differential docking behaviors (with respect to scores of poses), despite being similar/identical with respect to H-bond donors and acceptors, probably due to the difference in 3D shape similarity among these tocopherols.

b. Docking without constraints

In nonconstraint docking, all of the tocopherols showed interaction in the active sites of the proteins. Out of 10 compounds, eight, eight and nine botanicals showed interactions with DPP-IV, PPAR-γ, and PTP1 B, respectively (Tables S3—S5; Fig. S1—S3).

Discussion

DM is a chronic metabolic disorder, characterized by glucose overproduction and glucose underutilization. Although medical nutrition therapy and exercise are important components of a diabetes management plan, most patients need medication to normalize glucose levels. Therapeutic options include sulfonylureas and other insulin secretagogues, biguanides, α-glucosidase inhibitors, thiazolidinediones, and insulin.[1] Extensive research has been carried out worldwide on molecular targets for T2DM,

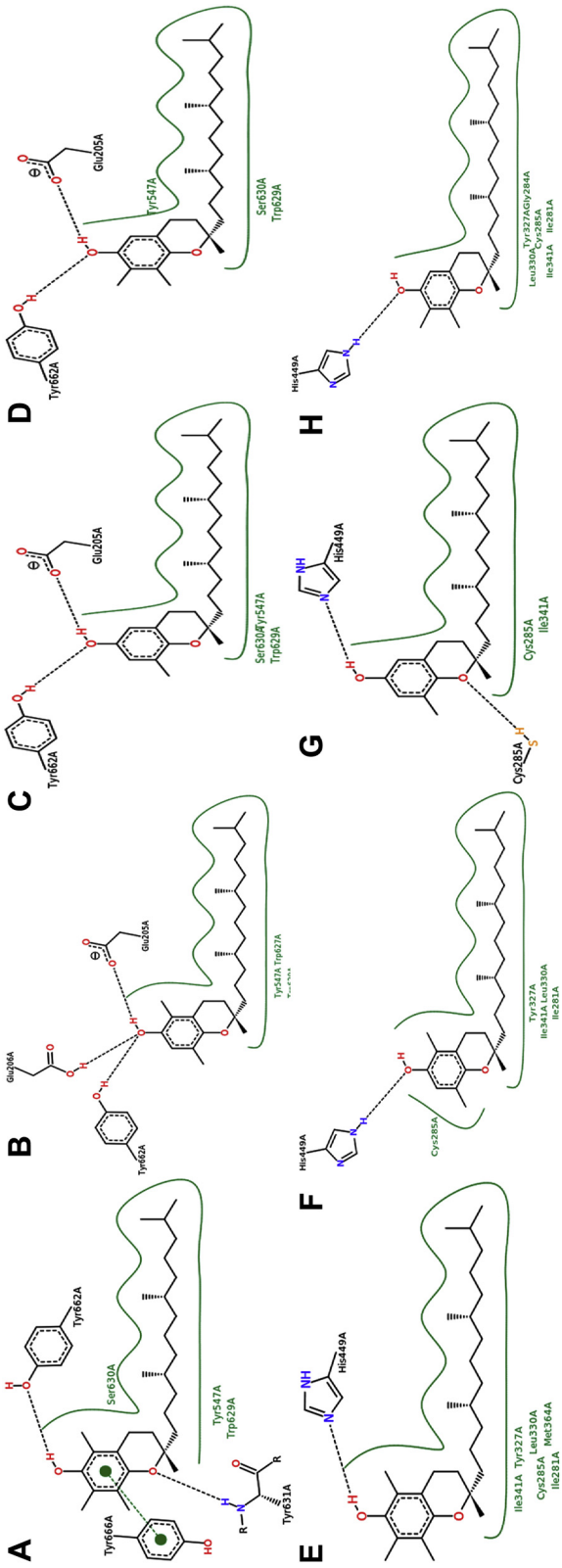

Figure 6 2D docked plots for compounds shown to have maximum interactions for the ligand to dipeptidyl peptidase IV (DPP-IV): (A) γ-tocopherol; (B) α-tocopherol; (C) δ-tocopherol; (D) β-tocopherol and with active sites of peroxisome proliferator-activated receptor (PPAR)-gamma; (E) γ-tocopherol; (F) α-tocopherol; (G) δ-tocopherol; (H) β-tocopherol.

Table 5 Number of conformers generated with respect to number of rotatable bonds.

No. of rotatable bonds	No. of max. conformers
0—2	200
3—5	300
6—8	500
9—11	1000
12—14	1500
15—17	2000

including PPAR-γ, PTP-1B, DPP-IV, etc. These therapeutic targets are quite suitable for *in silico* analysis and rational drug design in the development of newer antidiabetic agents. Therefore, many molecular modeling and informatics studies, such as molecular docking, pharmacophore mapping, virtual screening, and pharmacoinformatics studies have been performed on the drugs/leads/targets associated with T2DM.[5]

PX-407 is a nonionic copolymer, commonly used as a surfactant, emulsifying agent, and solubilizing agent in pharmaceutical formulations. In rats, intraperitoneal administration of PX-407 increases body weight, and manifests hyperglycemia with a low degree of toxicity ($LD_{50} \geq 1.8$ g/kg body weight). It causes impaired response in the glucose tolerance test and a loss of β-cell sensitivity to glucose. It is considered as a useful experimental model to study the pathophysiological aspects, as well as therapeutic influences, of antihyperglycemic agents.[26,54]

The effects of medicinal plants/extracts in diabetic organisms are of great interest, due to their beneficial effects on the metabolic profile. In the present study, the higher dose of CPSE (DT_5 group) presented a significant glucose-lowering effect in diabetic rats, accompanied by a reduction of insulinemia and a decrease in insulin resistance, evaluated by HOMA-IR. Therefore, we hypothesize that CPSE administration at a higher dose was capable of improving peripheral insulin resistance, albeit having an insignificant effect on hepatic resistance, and propose that hyperinsulinemia might be the spontaneous effect of insulin resistance in the liver. These favorable effects of CPSE may be attributed to the action of their antioxidant botanicals, particularly tocopherols, which may therefore regulate the hyperglycemia, lipogenesis, and hypertriglyceridemia associated with T2DM.[21,22] The pumpkin polysaccharides have also been found to increase the SOD and glutathione peroxidase activity and reduce the malonaldehyde in mouse serum, which shows an increase in antioxidant capacity.[55]

Despite the amelioration in glucose metabolism, CPSE treatment was unable to induce beneficial modifications in the lipid profile (TGs and TC). This was supported by our docking results, in which the CPSE botanicals showed poor interactions with the PPAR-γ ligand. However, it has been proven that the product resulting from the metabolism of γ-tocopherol plays an important role in the prevention of cardiac diseases and in the regulation of blood pressure, probably by controlling the drainage of water and of different metabolites in the body. The presence of unsaturated fatty acids, such as oleic acid and linoleic

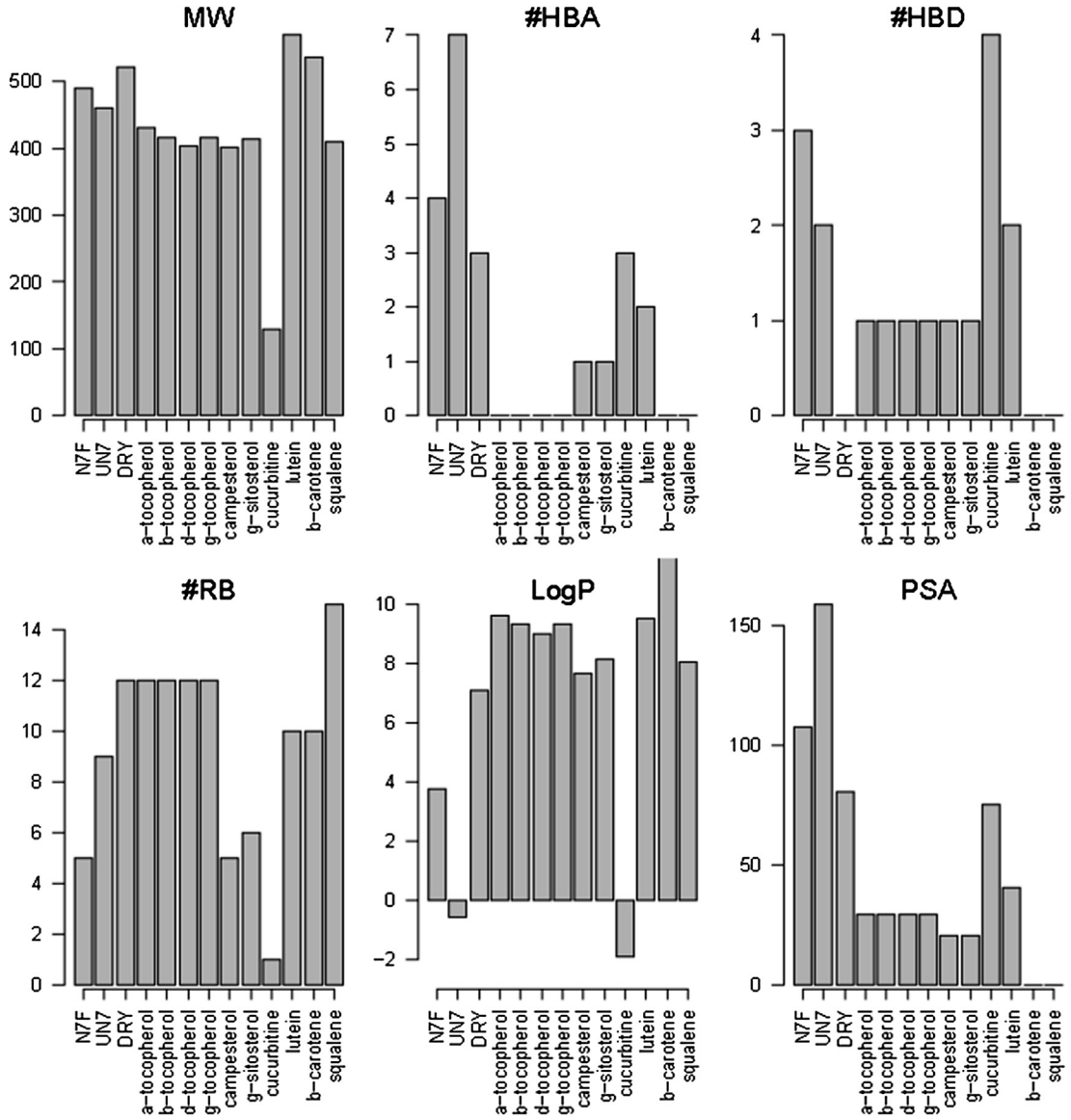

Figure 7 Comparison of physicochemical properties of 10 CPSE botanicals and native ligands bound to crystal structures of dipeptidyl peptidase IV (DPP-IV), peroxisome proliferator-activated receptor gamma (PPAR-γ), and protein-tyrosine phosphatase 1B (PTP-1B) calculated by the MolProp tool. Four parameters, namely molecular weights (MW), number of hydrogen bond acceptors (HBA), number of hydrogen bond donors (HBD), and XlogP were used to evaluate the botanicals with the likelihood of orally active drugs in humans according to 'The rule of 5′ of Lipinski.[69] However, natural products remain bioavailable and are often cited as an exception to Lipinski's rules.[70,71] The number of rotatable bonds (RB) and polar surface area (PSA)[72] of these compounds were also calculated (see also Table S6).

acid in pumpkin seeds reduces cholesterol levels in rats.[13,16,17,21,24–26]

Hyperglycemia and hypertriglyceridemia are known to increase the generation of reactive oxygen species (ROS) and subsequent lipid peroxidation, which are known to accelerate the pathogenesis of T2DM, as evidenced by the glucose and lipid toxicity theories.[1] The catalytic actions of antioxidant enzymes are important for the effective removal of oxygen radicals. It has been reported that tocopherols are antioxidant molecules which prevent the damage caused by free radicals on tissues via a mechanism of membrane transfer.[13,56] This is achieved by donating a hydrogen atom to a peroxide radical, which results from the degradation of unsaturated lipids and as a consequence, tocopherols are minor but omnipresent components in membranes.[57] An increased generation of free radicals by PX-407 induction to rats reduces the activities of antioxidant enzymes, such as catalase, GST, and SOD, as observed in the DC group rats.

Our study showed that CPSE treatment significantly increased GLP-1 content in the cecum of diabetic rats, accompanied by cecal tissue enlargement. This result was also supported by our docking results, in which some of the botanicals strongly inhibited DPP-IV. It has been documented that GLP1 has a number of important biological effects that include, among others, release of insulin,

Table 6 2D and 3D similarity between native ligands and CPSE phytochemicals. 2D similarity is calculated from ECFP4 while 3D similarity was calculated by TanimotoCombo.

Pub chem ID	222284	173183	92094	92729	14985	6857447	638072	5280489	448437	442634
N7 F										
3D similarity	0.765	0.760	0.678	0.671	0.668	0.618	0.589	0.441	0.433	0.395
2D similarity	0.1047	0.1064	0.1064	0.1053	0.1042	0.1053	0.0211	0.0826	0.0721	0.0606
DRY										
3D similarity	0.556	0.573	0.710	0.706	0.704	0.709	0.607	0.348	0.371	0.299
2D similarity	0.0714	0.0518	0.1658	0.1538	0.1421	0.1538	0.0923	0.0628	0.0617	0.0438
UN7										
3D similarity	0.571	0.561	0.713	0.709	0.702	0.705	0.626	0.359	0.389	0.417
2D similarity	0.1053	0.1071	0.1667	0.1529	0.1163	0.1412	0.0824	0.101	0.1188	0.0893

inhibition of glucagon and somatostatin, and maintenance of β-cell mass.[58] We also found that the insulin-positive cell mass was increased after CPSE treatment, which could explain the significant increase in fasting plasma insulin levels, although this increase was not sufficient to normalize fasting glycemia. Probably, these effects are proportional to the duration of the treatment and are potentially beneficial in the context of long-term treatment.

PX-407 partially damages the pancreatic islets and so decreases the number of β-cells and insulin globules.[54] This damage leads to an inflammation in the pancreas which is known as insulitis. Our results showed that, in CPSE-treated diabetic rats, there was a significant ($p < 0.001$) improvement in the number of islets, as well as the grade of insulitis, in agreement with other studies.[59,60] Antioxidant compounds also increase the number of β-pancreatic cells by enhancing the repair and restoration of these cells. It has been well documented that administration of antioxidants to diabetic rats significantly increases the number of β-cells.[61] Studies have indicated that administration of quercetin to β-cells *in vitro* causes an increase in the number of these cells, which is due to an increase in DNA replication in pancreatic islet cells.[61] It has been found that saponin extracted from *Cucurbitaceae* plant colocynth[62] (*Citrullus colocynthis*) and flavonoid compounds, including quercetin, with antioxidant activity possess antihyperglycemic effects in diabetic rats.[63] The increased β-cell mass would increase the secretion of insulin, which may increase the peripheral utilization of glucose.[64] Hence, one of the reasons behind the observed antihyperglycemic activity of CPSE may be the repair and restoration of pancreatic tissue to near normal values.

Pumpkin contains various biologically active components, such as polysaccharides, para-aminobenzoic acid, fixed oils, sterols, proteins, peptides, carotenoids, γ-aminobutyric acid, and vitamins.[65–67] These botanicals exert antidiabetic effects, probably synergistically. With the possibility that botanicals may interact with the constituents to potentiate their antidiabetic effects, further investigation regarding their interactions would be rewarding.[68]

Further, in order to understand the mechanism of action, constrained and nonconstrained docking studies were performed by HYBRID and FRED programs for 10 CPSE botanicals with respect to three antidiabetic proteins, namely PPAR-γ, PTP1 B, and DPP-IV. The 3D conformers of these botanicals were used for docking with three proteins. In the case of PPAR-γ, it has been reported that the direct interaction between a ligand and the C-terminal helix in the ligand-binding domain (LBD), constituting the activation function-2 (AF-2), has a crucial function in the ligand-induced receptor activation.[6] Full agonists of PPAR-γ form hydrogen bonds with H499 on the AF-2 helix H12,[46] whereas partial agonists did not always interact with this helix to activate PPAR-γ.[47] For DPP-IV inhibition, H-bonding with Glu205, as well as extension of the hydrophobic part in the S1 sub-pocket has been reported to be important. We observed that α-tocopherol approximately formed a U-shape while binding to PPAR-γ, observed as critical for agnostic activity.[46] Moreover, in the case of DPP-IV, α-tocopherol matched the shape of bound ligands present in the crystal structure.

Conclusion

The *in vivo* experiment against PX-407-induced diabetic rats, fully supported by molecular docking, proves that *C. pepo* seed extract is antioxidant and thus antidiabetic in nature. Consequently, this study submits that *C. pepo* seed extract is a supplement to the existing oral antidiabetic drugs and it minimizes the transformation of pre-diabetics into diabetics.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfma.2013.08.003.

References

1. Nyenwe EA, Jerkins TW, Umpierrez GE, Kitabchi AE. Management of type 2 diabetes: evolving strategies for the treatment of patients with type 2 diabetes. *Metabolism* 2011; **60**:1—23.
2. Vats RK, Kumar V, Kothari A, Mital A, Ramachandran U. Emerging targets for diabetes. *Curr Sci* 2005;**88**:241—9.
3. Chang CH, Jiang YD, Chung CH, Ho LT, Chuang LM. National trends in antidiabetic treatment in Taiwan, 2000—2009. *J Formos Med Assoc* 2012;**111**:617—24.
4. Rosen CJ. Revisiting the rosiglitazone story — lessons learned. *N Engl J Med* 2010;**363**:803—6.
5. Bharatam PV, Patel DS, Adane L, Mittal A, Sundriyal S. Modeling and informatics in designing anti-diabetic agents. *Curr Pharm Des* 2007;**13**:3518—30.
6. Waku T, Shiraki T, Oyama T, Maebara K, Nakamori R, Morikawa K. The nuclear receptor PPARγ individually responds to serotonin- and fatty acid-metabolites. *EMBO J* 2010; **29**:3395—407.
7. Bharti SK, Krishnan S, Kumar A, Rajak KK, Murari K, Bharti BK, et al. Antidiabetic activity and molecular docking of fructooligosaccharides produced by *Aureobasidium pullulans* in poloxamer-407-induced T2DM rats. *Food Chem* 2013;**136**: 813—21.
8. Bharti SK, Krishnan S, Kumar A, Rajak KK, Murari K, Bharti BK, et al. Antihyperglycemic activity with DPP-IV inhibition of alkaloids from seed extract of *Castanospermum australe*: investigation by experimental validation and molecular docking. *Phytomedicine* 2012;**20**:24—31.
9. Combs AP. Recent advances in the discovery of competitive protein tyrosine phosphatase 1B inhibitors for the treatment of diabetes, obesity, and cancer. *J Med Chem* 2010;**53**: 2333—44.
10. Sutton JM, Clark DE, Dunsdon SJ, Fenton G, Fillmore A, Harris NV, et al. Novel heterocyclic DPP-4 inhibitors for the treatment of type 2 diabetes. *Bioorg Med Chem Lett* 2012;**22**: 1464—8.
11. Heinrich M, Barnes J, Gibbons S, Williamson EM. *Fundamentals of pharmacognosy and phytotherapy*. Edinburgh: Churchill Livingstone; 2004.
12. Applequist WL, Avula B, Schaneberg BT, Wang YH, Khan IA. Comparative fatty acid content of seeds of four *Cucurbita* species grown in a common (shared) garden. *J Food Comp Analy* 2006;**19**:606—11.
13. Xanthopoulou MN, Nomikos T, Fragopoulou E, Antonopoulou S. Antioxidant and lipoxygenase inhibitory activities of pumpkin seed extracts. *Food Res Inter* 2009;**42**:641—6.
14. Stevenson DG, Eller FJ, Wang L, Jane JL, Wang T, Inglett GE. Oil and tocopherol content and composition of pumpkin seed oil in 12 cultivars. *J Agric Food Chem* 2007;**55**:4005—13.
15. Glew RH, Glew RS, Chuang LT, Huang YS, Millson M, Constans D. Amino acid, mineral and fatty acid content of pumpkin seeds (*Cucurbita* sp.) and *Cyperus esculentus* nuts in the Republic of Niger. *Plant Foods Hum Nutr* 2006;**61**:51—6.
16. Al-Zuhair H, Abdel-Fattah AA, Abd-el-Latif HA. Efficacy of simvastatin and pumpkin-seed oil in the management of dietary-induced hypercholesterolemia. *Pharm Res* 1997;**35**: 403—8.
17. Makni M, Fetoui H, Gargouri NK, Garoui EM, Jaber H, Makni J, et al. Hypolipidemic and hepatoprotective effects of flax and pumpkin seed mixture rich in ω-3 and ω-6 fatty acids in hypercholesterolemic rats. *Food Chem Toxicol* 2008;**46**:3714—20.
18. Hong LH. Effect of pumpkin extracts on tumor growth inhibition in S180-bearing mice. *Pract Prev Med* 2005;**12**:745—7.
19. Park SC, Lee JR, Kim JY. Pr-1, a novel antifungal protein from pumpkin rinds. *Biotechnol Lett* 2010;**32**:125—30.
20. Fruhwirth GO, Hermetter A. Seeds and oil of the Styrian oil pumpkin: Components and biological activities. *Eur J Lipid Sci Technol* 2007;**109**:1128—40.
21. Caili F, Huan S, Quanhong L. A review on pharmacological activities and utilization technologies of pumpkin. *Plant Foods Hum Nutr* 2006;**61**:73—80.
22. Xia T, Wang Q. Antihyperglycemic effect of *Cucurbita ficifolia* fruit extract in streptozotocin-induced diabetic rats. *Fitoterapia* 2006;**77**:530—3.
23. Kwon YI, Apostolidis E, Kim YC, Shetty K. Health benefits of traditional corn, beans, and pumpkin: *in vitro* studies for hyperglycemia and hypertension management. *J Med Food* 2007;**10**:266—75.
24. Tarhan L, Kayali HA, Urek RO. *In vitro* antioxidant properties of *Cucurbita Pepo* L. male and female flowers extracts. *Plant Foods Hum Nutr* 2007;**62**:49—51.
25. Parry JW, Cheng Z, Moore J, Yu LL. Fatty acid composition, antioxidant properties, and antiproliferative capacity of selected cold-pressed seed flours. *J Am Oil Chem Soc* 2008; **85**:457—64.
26. Wasan KM, Subramanian R, Kwong M, Goldberg IJ, Wright T, Johnston TP. Poloxamer 407-mediated alterations in the activities of enzymes regulating lipid metabolism in rats. *J Pharm Pharm Sci* 2003;**6**:189—97.
27. McGann M. FRED and HYBRID docking performance on standardized datasets. *J Comp-Aided Mol Des* 2012;**26**:687—99.
28. McGann M. FRED pose prediction and virtual screening accuracy. *J Chem Informa Model* 2011;**51**:578—96.
29. Bryant SH, Bolton E, Wang Y, Thiessen PA. PubChem: Integrated platform of small molecules and biological activities. In: *Ann Rep in Comp Chem 4, chap12*. Washington, DC: Am Chem Soc; 2008.
30. Guiraud J. In: *"Microbiologie alimentaire — Rappels de microbiologie générale"*. France: Dunod; 1998.
31. Murkovic M, Piironen V, Lampi AM, Kraushofer T, Sontag G. Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (Part 1: Non-volatile compounds). *Food Chem* 2004;**84**:359—65.
32. ISO 9936. *Animal and vegetable fats and oils—determination of tocopherol and tocotrienol contents by high-performance liquid chromatography*; 2006.
33. Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere M, et al. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 2000;**23**:57—63.
34. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265.
35. Prabhakar KR, Veerapur VP, Parihar KV, Priyadarsini KI, Rao BS, Unnikrishnan MK. Evaluation and optimization of radioprotective activity of *Coronopus didymus* Linn. in gamma-irradiated mice. *Int J Radiat Biol* 2006;**82**:525.
36. Gelvan D, Saltman P. Different cellular targets of Cu- and Fe-catalyzed oxidation observed using a Cu-compatible thiobarbiturate acid assay. *Biochim Biophys Acta* 1990;**1035**:353.
37. Berman HM. The protein data bank. *Nucleic Acids Res* 2000; **28**:235—42.
38. Chemaxon. http://www.chemaxon.com [Online; accessed 09.9.12].
39. Openeye. http://www.eyesopen.com [Online; accessed 04.3.12].

40. Halgren TA. Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. *J Comput Chem* 1996;**17**:490—519.
41. OEChem MolProp ToolKit (version 2.1.5), www.eyesopen.com.
42. OEChem Shape ToolKit (version 1.9.0), www.eyesopen.com.
43. Aertgeerts K, Ye S, Tennant MG, Kraus ML, Rogers J, Sang BC, et al. Crystal structure of human dipeptidyl peptidase IV in complex with a decapeptide reveals details on substrate specificity and tetrahedral intermediate formation. *Protein Sci* 2004;**13**:412—21.
44. Mahindroo N, Peng YH, Lin CH, Tan UK, Prakash E, Lien TW, et al. Structural basis for the structure-activity relationships of peroxisome proliferator-activated receptor agonists. *J Med Chem* 2006;**49**:6421—4.
45. Klopfenstein SR, Evdokimov AG, Colson AO, Fairweather NT, Neuman JJ, Maier MB, et al. 1,2,3,4-Tetrahydro-isoquinolinyl sulfamic acids as phosphatase PTP1B inhibitors. *Bioorg Med Chem Lett* 2006;**16**:1574—8.
46. Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, et al. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* 1998;**395**:137—43.
47. Oberfield JL, Collins JL, Holmes CP, Goreham DM, Cooper JP, Cobb JE, et al. A peroxisome proliferator-activated receptor gamma ligand inhibits adipocyte differentiation. *Proceed Natl Acad Sci USA* 1999;**96**:6102—6.
48. Zhang X, Li X, Wang R. Interpretation of the binding affinities of PTP1B inhibitors with the MM-GB/SA method and the X-score scoring function. *J Chem Inf Model* 2009;**49**:1033—48.
49. Tuzmen C, Erman B. Identification of ligand binding sites of proteins using the Gaussian Network model. *PLoS One* 2011;**6**: e16474.
50. http://www.ccdc.cam.ac.uk/products/life_sciences/gold/case_studies/flexible_sidechains/.
51. David JR, Tanimoto TT. A computer program for classifying plants. *Science* 1960;**132**:1115—8.
52. David R, Mathew H. Extended-connectivity fingerprints. *J Chem Inform Model* 2010;**50**:742—54.
53. Stierand K, Rarey M. PoseView—molecular interaction patterns at a glance. *J Cheminform* 2010;**2**:50.
54. Johnston TP, Goldberg IJ. Inhibition of pancreatic lipase by poloxamer 407 may provide an adjunct treatment strategy for weight loss. *J Pharm Pharmacol* 2006;**58**:1099—105.
55. Xu GH. A study of the possible antitumour effect and immune-competence of pumpkin polysaccharide. *J Wuhan Prof Med Coll* 2000;**28**:1—4.
56. Murkovic M, Mulleder U, Neunteufl H. Carotenoid content in different varieties of pumpkins. *J Food Comp Anal* 2002;**15**: 633—8.
57. Kamal-Eldin A, Andersson R. A multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils. *J Am Oil Chem Soc* 1997;**74**:375—6.
58. Kielgast U, Holst JJ, Madsbad S. Antidiabetic actions of endogenous and exogenous GLP-1 in type 1 diabetic patients with and without residual β-cell function. *Diabetes* 2011;**60**: 1599—607.
59. Sedigheh A, Jamal MS, Mahbubeh S, Somayeh K, Mahmoud R, Azadeh A, et al. Hypoglycaemic and hypolipidemic effects of pumpkin (*Cucurbita pepo* L.) on alloxan-induced diabetic rats. *Afr J Pharm Pharmacol* 2011;**5**:2620—6.
60. Mohajeri D, Ghafour M, Doustar Y. Antihyperglycemic and pancreas-protective effects of *Crocus sativus* L. (saffron) stigma ethanolic extract on rat with alloxan-induced diabetes. *J Biol Sci* 2009:1—9.
61. Hii CS, Howell SL. Effects of epicatechin on rat islets of Langerhans. *Diabetes* 1984;**33**:291—6.
62. Abdel-Hassan IA, Abdel-Barry JA, Mohammeda ST. The hypoglycemic and antihyperglycaemic effect of *Citrullus colocynthis* fruit aqueous extract in normal and alloxan diabetic rabbits. *J Ethnopharmacol* 2000;**71**:325—30.
63. Rauter AP, Martins A, Borges C, Mota-Filipe H, Pinto R, Sepodes B, et al. Antihyperglycaemic and protective effects of flavonoids on streptozotocin-induced diabetic rats. *Phytother Res* 2010;**24**:133—8.
64. Bonner-Weir S. Islet growth and development in the adult. *J Mol Endocrinol* 2000;**24**:297—302.
65. Murkovic M, Pfannhauser W. Stability of pumpkin seed oil. *Eur J Lipid Sci Technol* 2000;**102**:607—11.
66. Matus Z, Molnár P, Szabó LG. Main carotenoids in pressed seeds (*Cucurbitae semen*) of oil pumpkin (*Cucurbita pepo*). *Acta Pharm Hung* 1993;**63**:247—56.
67. Bombardelli E, Morazzoni P. *Curcubita pepo* L. *Fitoterapia* 1997;**68**:291.
68. Wagner H, Ulrich-Merzenich G. Synergy research: approaching a new generation of phytopharmaceuticals. *Phytomedicine* 2009;**16**:97—110.
69. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery. *Adv Drug Deliv Rev* 1997; **23**:3—25.
70. Lipinski CA. Chris Lipinski discusses life and chemistry after the Rule of Five. *Drug Discov Today* 2003;**8**:12—6.
71. Ganesan A. The impact of natural products upon modern drug discovery. *Curr Opin Chem Biol* 2008;**12**:306—17.
72. Kelder J, Grootenhuis PD, Bayada DM, Delbressine LP, Ploemen JP. Polar molecular surface as a dominating determinant for oral absorption and brain penetration of drugs. *Pharm Res* 1999;**16**:1514—9.