Materials & Methods
Materials:

Chemicals:
Sephadex G-25, Sephadex G-100, Streptozotocin (STZ), Guanidine hydrochloride, 2,4-Dinitrophenyl hydrazine (2,4-DNPH), Thiobarbituric acid (TBA), Desferrioxamine (DFO), Deoxyribose, Mannitol, Ferrozine, Arachidonic acid, Malondialdehyde (MDA), 4-Trimethyl amino-diphenyl hexatriene (TMA- DPH), Dimethyl formamide (DMF), Pyrogallol, para-Nitrophenyl phosphate (p-NPP), para-Nitrophenol (p-NP), Nitroblue tetrazolium (NBT), Deoxymorpholinofructose (DOMF), Bovine serum albumin (BSA), Hydroxylamine hydrochloride, Cacodylic acid, Phosphatidyl choline, Cholesterol, Ethidium bromide, Phosphotungstic acid, Pentobarbital, Hematoxylin, Eosin, Phloxine B, Pelargonidin chloride, poly(lactide-co-glycolide) (PLGA) and Didodecyldimethyl ammonium bromide (DMAB) were purchased from SIGMA chemicals (USA). Proteinase-K was purchased from Banagalore Genei, India. Other reagents were of analytical grade and purchased locally.

Diagnostic kits:
Plasma glucose, triglyceride and cholesterol estimation kits were purchased from Span Diagnostics Ltd (India). Rat ultra sensitive insulin kit was purchased from DRG Diagnostics, Germany. Glycohemoglobin kit was purchased from Transasia Bio-Medicals Ltd, India.
Animal experiments:

**Maintenance of animals**

Animal experiments were performed in accordance with regulations specified and monitored by the Institutional Ethics Committee. Male Wister rats obtained from University approved local animal supplier weighing 100-120g were maintained at 24-26°C, 60-80% relative humidity and on a 12 h light/dark cycle and were fed a standard rat chow (Hindustan Lever, India) and had access to water ad libitum.

**Experimental design:**

**Pelargonidin treatment**

The experiments were designed in 3 sets with 32 rats in each set. Each set was subdivided into 4 groups, 8 rats in each group.

- Group 1: Normal untreated control (C)
- Group 2: STZ treated diabetic control (D)
- Group 3: Normal control treated with pelargonidin (CP)
- Group 4: Diabetic treated with pelargonidin (DP).

**Induction of diabetes:**

The rats were randomly divided primarily into two groups – normoglycemic and diabetic. Diabetes was induced by a single i.p injection of STZ (60 mg/kg body weight) in 0.01 M citrate buffer, pH 4.5. The normoglycemic rats received an equal volume of vehicle. Rats receiving STZ were given 10% glucose in ringer solution first 48 hours to prevent hypoglycemia [Brian et al. 2006]. The glucose concentration in blood taken from tail vein was determined by conventional GOD-POD method using glucose estimation [Trinder, P. 1969]. After two weeks of STZ injection, stable fasting blood glucose levels at ≥ 200 mg/dl were considered diabetic at and treatments were started.

**Mode of treatment:**

The diabetic rats were divided into two groups: Group D - untreated diabetic rats; Group DP - diabetic rats treated with one time i.p injection of freshly prepared pelargonidin in 60% ethanol at a dose of 3 mg/kg body weight. Normoglycemic control rats were divided
into two groups: Group CP - rats received same dose of pelargonidin as in diabetic treated group DP; Group C and Group D - rats received vehicle 60% ethanol.

The experimental design and parameters studied have been presented in Fig. 14.

Figure 14: Diagrammatic representation of the experimental design and parameter studied in pelargonidin treatment (i.p. injection at a dose 3 mg/Kg body wt) in STZ-induced diabetic rats.

**Liposome encoded pelargonidin treatment**

Preparation of liposome-encoded pelargonidin:

Multilamellar liposomes were prepared with phosphatidylcholine, cholesterol and pelargonidin in a molar ratio 7:2:1 [Das et al., 1987]. Briefly, phosphatidylcholine, cholesterol and pelargonidin (0.3mg / kg body weight) were dissolved in chloroform and methanol mixture (2:1, v/v) in a round-bottomed flask. A thin layer of lipid film containing drug was made by constant swirling during gradual evaporation of the organic solvent and was desiccated overnight. The thin film was swollen in PBS (pH 7.2) for 1 h and sonicated in a probe type sonicator for 30secs.
The experiments were designed in 2 sets with 40 rats in each set. Each set was subdivided into 5 groups, 8 rats in each group.

Rats were divided into following groups:

- Group 1: Normal control (C),
- Group 2: STZ treated diabetic control (D),
- Group 3: Diabetic rats treated with liposome-encoded pelargonidin (DP₁),
- Group 4: Diabetic treated with free pelargonidin (FD) at the dose used for liposome-encoded pelargonidin preparation,
- Group 5: Diabetic treated with free liposome (FL).

Mode of treatment:
For liposome-encapsulated pelargonidin preparation, 0.3mg pelargonidin/ kg body weight of rats was used. Liposomes were injected (i.v) to the diabetic rats every 3 days interval, until the glucose level of treated group (DP) dropped down to normal. Glucose level came to normal level after 5th dose of injection. FD group of rats received identical dose of pelargonidin (i.e., 0.3 mg / kg body weight with same number of administration) and FL group of rats received only empty liposome suspension (same number of administration).

The experimental design and parameter studied have been presented in Figure 15.
Materials & Methods

Liposome encoded pelargonidin injection (total 5 injection and 3 days interval between each injection till blood suger reaches normal, blood suger estimation done 2 day after each dose). Free pelargonidin injection or vehicle injection was given identically following equal doses maintaining same interval as of liposome encoded pelargonidin injection.

Period (week) after treatment (Liposome encoded pelargonidin, free pelargonidin or vehicle)

Figure 15: Diagrammatic representation of the experimental design and parameter studied in liposome encoded pelargonidin treatment (i.v. injection; dose 0.3 mg/kg body wt in each injection, total 5 injection) in STZ-induced diabetic rats.

PLGA-nanoparticle encoded pelargonidin treatment

Preparation of PLGA-pelargonidin nanoparticle:

Pelargonidin was encapsulated into PLGA loaded nanoparticles by emulsion-diffusion-evaporation method employing DMAB as stabilizer. A modified emulsion-diffusion-evaporation method [Hariharan et al., 2006] was used to make the pelargonidin-PLGA

Parameters → Glucose Glucose Glucose Glucose
Estimated
IPGTT, Insulin GHb
SOD, Catalase, Fructosamine
MDA
Free iron in Hb
Deoxyribose degradation
DNA degradation by Hb
Carbonyl Content in Hb
Lipid profiles
nanocapsule. In brief, 36 μmol of PLGA was dissolved in 2.5 ml of ethyl acetate at room temperature. Pelargonidin in three different doses (0.3mg/kg body weight; 0.6mg/ kg body weight; 1.5mg/kg body weight dissolved initially in DMSO) was added in the above solution drop wise at room temperature with constant stirring. The organic solution was then emulsified with 5 ml of an aqueous phase containing 0.01 % DMAB. The resulting oil/water emulsion was stirred at room temperature for 3 h before sonication in a probe type sonicator for 120secs. The organic solvent was removed by constant stirring on a water bath set at 40°C. Water was added to this emulsion to reach the final drug concentration and just before injection the preparation was sonicated again in a probe type sonicator for 30secs.

The experiments were designed in 2 sets with 48 rats in each set. Each set was subdivided into 6 groups, 8 rats in each group.

Rats were divided into following groups:

- Group 1: Normal control (C),
- Group 2: STZ treated diabetic control (D),

Here three different doses of pelargonidin were used in diabetic rats for treatment. The doses were as follows:

- Group 3: Diabetic rats treated with nanoparticle-encoded pelargonidin $DT_{NI} = 0.3$ mg/kg body weight;
- Group 4: Diabetic rats treated with nanoparticle-encoded pelargonidin $DT_{N2} = 0.6$mg/ kg body weight;
- Group 5: Diabetic rats treated with nanoparticle-encoded pelargonidin $DT_{N3} = 1.5$mg/kg body weight of pelargonidin encoded nanoparticle.

In initial experiments three doses were used to study the efficacy of pelargonidin-nanoparticle treatment in diabetic rats. 0.6mg/ kg body weight was selected in subsequent experiments.

- Group 6: Diabetic rats treated with free pelargonidin (FD) at the dose used for nanoparticle encoded pelargonidin preparation (0.6mg/ kg body weight).
PLGA-pelargonidin nanoparticle were injected (i.v) to the diabetic rats every 2 day interval, until the glucose level of diabetic treated groups (DT$_{N1}$, DT$_{N2}$, DT$_{N3}$) dropped down to normal. For DT$_{N1}$ group of rats three consecutive doses were required. While for DT$_{N2}$ two consecutive doses and for DT$_{N3}$ groups only one dose was sufficient. The FD groups of rats received two consecutive doses of pelargonidin (0.6mg/ kg body weight).

The experimental design and parameter studied has been presented in Figure 16.

Figure 16: Diagrammatic representation of the experimental design and parameter studied in PLGA-nanoparticle encoded pelargonidin injection (dose 0.6 mg/kg body wt in each injection, total 2 injection) in STZ-induced diabetic rats.
Materials & Methods

For characterization of nanoparticles (size, shape, drug encapsulation efficiency and surface morphology determination) following techniques were used.

Transmission Electron Microscopy (TEM):
TEM measurements of the PLGA-loaded pelargonidin nanoparticles were performed on a Microscope (Techna FEII, Philips Co., Netherlands) operated at an accelerating voltage of 120 kV. Samples for TEM analysis were prepared by placing drops of the PLGA-pelargonidin nanoparticle suspension on carbon-coated TEM copper grids. The mixtures were allowed to dry for 1 min, after which the extra solution was removed using blotting paper and was loaded in the TEM.

Scanning electron microscopy (SEM):
SEM measurements of the PLGA-pelargonidin nanoparticles were performed on a Scanning Electron Microscope (FEI Quanta-200MK2, Philips Co., Netherlands.). 50 µl of PLGA-pelargonidin nanoparticles suspension were mixed with 50 µl of 2% gluteraldehyde (in 0.1 M PBS) for 30 min. Then the resultant solution was centrifuged at 15,000 rpm for 5 min. The pellet was washed in 0.1 M PBS. The cells were resuspended in PBS and a thin film of the suspension was applied on a thin cover slip. The cover slip was air-dried. 5-6 nm of gold was sputtered onto the cell samples and their images observed under the microscope.

Atomic Force Microscopy (AFM):
Samples for AFM imaging were prepared by placing a drop of PLGA-loaded pelargonidin nanoparticles suspension on a clean glass cover slip and allowing it to dry in the air for overnight in a desiccators. AFM imaging was performed using a Nanoscope IIIa system (Vecco multimedia, USA) in a tapping mode at room temperature.
Measurement of hydrodynamic radii, zeta potential (\(\zeta\)) and polydispersity index (PDI) by Dynamic light scattering (DLS):
The hydrodynamic size and polydispersity index (PI) were measured using a Malvern Instruments NanoZS (Worcestershire, UK) at a temperature of 25°C and the scattering angle was fixed at 175° angle for these measurements. The polydispersity index measures the size distribution of the nanoparticle population (Koppel, D. E. 1972). The pelargonidin encoded PLGA-nanoparticle's water dispersions were diluted 1:10 with distilled water before analysis.

The same instrument was used to measure the zeta potential of the pelargonidin encoded PLGA-nanoparticle's colloidal suspension. The zeta potential is a purely electrokinetic property of the electrical double layer surrounding the subject but not the surface of the subject itself. This technique efficiently measures the velocity of tiny particles within the fluid streams moving at the velocity of the fluid. Determining the electrophoretic mobility and then applying the value in Henry's equation measures this quantity. The velocity of a particle in an electric field is known as electrophoretic mobility (UE). Now, applying this value to Henry's equation we obtain the value of the zeta potential. Each value was measured in triplicate determination and pure water was used as a reference dispersing medium. The results are showed as mean ± standard distribution.

Differential scanning calorimetry (DSC):
The thermograms of pure pelargonidin, PLGA-nanoparticle (ghost) and lyophilized pelargonidin encoded PLGA-nanoparticle were determined using a differential scanning calorimetry (DSC-7, Perkin-Elmer, Norwalk, CT). Approximately 1mg of each sample was heated in sealed aluminum pans from 50-400°C and the scanning rate was conducted at 10 °C min⁻¹.

Drug entrapment efficiency:

UV-spectroscopy:

The amount of the drug in the water phase of the suspension was analyzed by filtering the nanoparticle sample through a 0.22-µm filter (Millipore) and by measuring the concentra-
Materials & Methods

The amount of drug in the filtered sample in a UV-spectrophotometer. The amount of drug inside the particles was calculated by subtracting the amount of drug present in the aqueous phase of the suspension from the total amount of the drug used in the nanoparticle formulation. The entrapment efficiency (%) of drug was calculated by the following equation:

\[
\text{Drug Entrapment (\%)} = \left( \frac{\text{Amount of the drug in Nanoparticles}}{\text{Amount of the drug used in Nanoparticles formulation}} \right) \times 100
\]

HPLC analysis:
The reversed phase chromatographic system was composed of a pump and UVD-10AT detector (Shimadzu corporation, Japan). The analytical column used was the Luna (250mm×4.6mm i.d., particle size- 5μm) and the temperature maintained at 25°C. The mobile phase was composed of methanol. The flow rate was set at 0.5 ml/min. The wavelength of UV detector was kept at 520 nm. The calibration curve of pelargonidin was linear (r = 0.9999) within range 1–100 μg/ml. The relative standard deviations of the interlay were less than 5% (n = 5).

Methodology:
Methods of different experiments are presented in brief in the following sections.

Collection of blood:
Blood samples (fasting) both heparinized and nonheparinized were collected from lateral tail veins. For estimation of serum insulin level, blood samples were also drawn from the retro-orbital plexus using heparinized capillary tubes [Schrijvers et al., 2004]. Plasma or serum was separated from the whole blood by centrifugation at 5000 rpm.

Preparation of hemoglobin:
Hemolysates were prepared from RBC after washing with normal saline (0.9% NaCl) and hypotonic lysis with distilled water. Hemoglobin (Hb) was isolated and purified by
Materials & Methods

sephadex G-100 column chromatography pre-equilibrated with 50 mM potassium phosphate buffer, pH 7.4 (Bhattacharyya et al., 1998). The concentration of hemoglobin was measured from the Soret absorbance with extinction coefficient, ε415 as 125 mM⁻¹cm⁻¹ (monomer basis) [Bhattacharyya et al., 1994].

Estimation of blood glucose by glucose oxidase /peroxidase (GOD/POD) method:
Glucose was estimated in fasting blood samples of the rats by glucose estimation kit by glucose oxidase reaction as first proposed by Trinder, P. (1969).

Estimation of serum total cholesterol:
Total cholesterol in serum was estimated by cholesterol estimation kit (Span Diagnostics Ltd, India).

Estimation of triglyceride in serum:
Triglyceride level was estimated in blood serum by commercially available kit (Span Diagnostics Ltd, India).

Intraperitoneal glucose tolerance test (IPGTT):
A sterile solution of 20% glucose was injected (i.p) at a dose of 2 gm /kg body weight to overnight fasting animals for IPGTT. Tail vein bloods were collected before glucose administration (0 min) and 30, 60, 90 and 120 min after administration for glucose estimation by GOD-POD method.

Serum insulin estimation:
Serum insulin was estimated after 1 h of i.p injection of 20% glucose solution at a dose of 2 g/Kg body weight using Rat insulin ELISA kit (DRG diagnostics, Germany).

Estimation of protein content in serum:
The protein content of serum was estimated by the method of Lowry (1951) using BSA as the standard. The principle of the procedure is that protein reacts with the Folin-Ciocalteau reagent to give a colored complex. The color so formed is due to the reaction
of the alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the color depends on the amount of these aromatic amino acids present and thus varies for different proteins. The protein concentration was calculated against BSA standard.

Estimation of antioxidative enzymes namely superoxide dismutase and catalase activity in plasma:

SOD activity:
Superoxide dismutase activity (SOD) in plasma was estimated following the method of Marklund and Marklund, 1974 using pyrogallol as substrate. The method involves quantification of pyrogallol autooxidation and expressed as units/mg protein. One unit of enzyme activity was defined as the amount of the enzyme necessary for inhibiting the reaction by 50%.

Catalase activity:
Catalase activity was estimated following the method of Aebi, H. 1984. In this method, the rate of H₂O₂ (10 mM) degradation was followed spectrophotometrically at 240 nm and expressed as units/mg serum protein where the unit was the amount of enzyme that liberates molecular oxygen from due to degradation of 10 mM H₂O₂ per min per mg of serum protein.

Estimation of serum malondialdehyde (MDA):
Serum MDA was estimated following the method of of Yagi, K. (1987). Briefly, the reaction mixture containing 0.4 ml serum was diluted with 0.1 ml distilled water. 2.5 ml of TCA was (1.22 M dissolved in 0.6 M HCl) was added in the serum sample and the mixture was left at room temperature for 15 mins. 1.5 ml of 0.76% TBA containing 0.05 M NaOH was then added to the mixture and incubated in boiling water bath for 30 mins. The reaction mixture was cooled and 4 ml of n-butanol was added. The resultant chromophore was extracted from butanol phase. The generation of MDA was measured from the fluorescence emission intensity of the resultant chromagen 553 nm by excitation at 515 nm and the results were expressed as arbitrary fluorescence unit.
Materials & Methods

Estimation of serum alkaline phosphatase:
Alkaline phosphatase activity in serum was measured using p-NPP as the substrate and measuring the amount of p-NP released at 405 nm (Thambidorai & Bachawat, 1977). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of p-NP at 37°C under standard assay condition. Specific activity of the enzyme was expressed as μmole p-NP liberated/mg of serum protein/min. Standard curve of p-NP under same reaction condition was used to determine the enzyme content.

Estimation of glycohemoglobin concentration in whole blood:
Glycohemoglobin concentration was determined in 0.1 ml of freshly collected whole blood using commercially available kits (Transasia BioMedicals Ltd, India). The glycohemoglobin procedure employs a weakly binding cation-exchange resin for rapid separation of glycohemoglobin (fast fraction) from nonglycylated hemoglobin.

Estimation of serum fructosamine:
Fructosamine in serum was determined by NBT reduction assay according to the method of Johnson et al. (1982). Briefly, 1 ml of NBT reagent (0.5 mM NBT in 0.2 M sodium carbonate buffer, pH 10.35) was added to serum (0.3 mg protein content) and the mixture was incubated at 37°C for 1 h. The absorbance of the monoformazan produced from NBT reduction was measured at 530 nm against a reagent blank. Formazan derivative formed was estimated from a standard curve of DOMF and the result was expressed as pmol of formazan formed/mg protein.

Estimation of free iron level in hemoglobin samples:
Free iron in hemoglobin sample was estimated according to the method of Panter S. S. (1994). A 50 μl cold 20%TCA solution was added to the hemoglobin solution containing 50 μM Hb. After centrifugation at 3000 rpm for 10 mins, the supernatant (50 μl) was transferred to a test tube containing 50 μl distilled water, 0.5 ml iron buffer reagent (1.5% hydroxylamine hydrochloride in 50 mM acetate buffer, pH 4.5) and 10 μl iron color
Materials & Methods

reagent (0.85% ferrozine in iron buffer reagent). The color was developed for 30 mins at 37°C and read at 560 nm. Free iron level in Hb was calculated from the standard curve using a standard iron solution and expressed as µg iron/g of Hb. Results were expressed as µg of iron/gm of Hb.

Assay of hemoglobin-catalyzed iron-mediated free radical reactions:

Lipid peroxidation:
Aldehydes are produced when lipid hydroperoxides break down in biological system. Measurement of these compounds is an index of lipid peroxidation caused by free radical reaction. Hb-mediated lipid (arachidonic acid) peroxidation was measured essentially according to the method of Sadrzadeh et al. (1984) with modifications as described by Kar & Chakraborti, (2001) except without addition of H$_2$O$_2$. 1 ml sample containing Hb (80 µM), arachidonic acid (160 µM) in 50 mM phosphate buffer, pH 7.4 was incubated at 37°C for 1 h. The reaction was stopped by 20% TCA. Then 0.5 ml TBA (1%) was added. The protein was precipitated by centrifugation and the supernatant was incubated in a boiling water bath for 30 mins. The absorbance was measured at 530 nm and the value was corrected for endogenous TBA reactive substances present in arachidonic acid. The values were corrected for endogenous TBARS present in arachidonic acid solution. The results were calculated from a standard obtained with MDA treated similarly. The result was expressed as nmol MDA/ mg Hb/h.

Deoxyribose degradation:
Deoxyribose degradation was measured in presence of Hb samples and H$_2$O$_2$ according to the method of Gutteridge (1986). Deoxyribose (0.67 mM) in phosphate buffer, 50 mM, pH 7.4 was incubated for 1 hr at 37°C with hemoglobin (4 µM), H$_2$O$_2$ (0.67 mM) and other additions (DFO or mannitol) as required. TBA reactivity was developed by adding 0.5 ml each of TBA (1%) and TCA (2.8%), and then heated for 30 mins in a boiling water bath. The resulting chromogen was extracted with n-butanol. The product was estimated from fluorescence emission at 553 nm by exciting at 532 nm.
Materials & Methods

DNA degradation:
The experiment was done essentially following the procedure of Sen et al. (2005). However, \( \text{H}_2\text{O}_2 \) was not added to the reaction mixtures. Approximately 450 ng plasmid (pGEM) DNA (3 kb) was incubated without or with Hb (25 \( \mu \text{M} \)) at 37°C for 1 h. DFO (10 mM) or mannitol (15 mM) was included in reaction mixture as indicated. The reaction was stopped with 10\% (v/v) glycerol (Dixon et al., 1991). Different forms of DNA were separated by agarose (1\%) gel electrophoresis and visualized by ethidium bromide (0.5 \( \mu \text{g/ml} \)) staining.

Carbonyl content in Hemoglobin:
Protein carbonyl formation in hemoglobin was measured using 2,4-DNPH following the method of Levine et al. (1990) with modifications, in which 0.10 mg protein was used. The reaction mixture (500 \( \mu \text{l} \)) containing 50 \( \mu \text{M} \) hemoglobin sample and to it 500 \( \mu \text{l} \) DNPH (10 mM) was added, thoroughly mixed and incubated at 37°C for 1 h. After addition of 250 \( \mu \text{l} \) TCA (20\%) and centrifugation, the pellet was collected and washed three times with 1 ml of ethanol:ethyl acetate (1:1) mixture. The pellet was then dissolved in 0.6 ml 6 M guanidine hydrochloride and incubated at 37°C for 15 min. After centrifugation, the supernatant was collected and carbonyl content was estimated from the absorbance at 370 nm using a molar absorption coefficient 22,000 \( \text{M}^{-1} \text{cm}^{-1} \). The value was expressed as \( \mu \)moles/mg Hb.

Fluorescence depolarization measurements of the fluidity of membrane of red blood cells:
The fluorescence depolarization, associated with the hydrophobic fluorescence probe, 4-Trimethyl amino-diphenyl hexatriene (TMA-DPH), was used to monitor the changes in the fluidity of the lipid matrix of RBC membranes. RBC (7.5 \( \times 10^4 \) cells) was incubated with TMA-DPH (50 \( \mu \text{M} \)) dissolved in DMF (2 mM) (Schachter & Shinitzky, 1977). The excitation was kept at 358 nm and emission was measured at 428 nm, respectively. The
fluorescence anisotropy was calculated by using the equation, $r = \frac{(I_{II} - I_{I})}{(I_{II} + 2I_{I})}$ where $I_{II}$ and $I_{I}$ are the fluorescence intensities in parallel and perpendicular to the direction of polarization of the excited light.

Comet assay on lymphocyte

Lymphocytes were isolated from blood using Histopaque 1077 and the cells were resuspended in 100mM phosphate buffer saline. Comet assay was done under alkaline solution according to the method of Singh et al. (1998) with modification. In brief, 100 μl 1% normal melting agarose (NMA) was applied to fully frosted slide precoated with 50 μl of 1% NMA for firm attachment and the slides were allowed to solidify with cover slips in the refrigerator for 5min. After solidification of the gel, the cover slips were removed in and lymphocytes 50 μl mixed with 1% low melting agarose (LMA) were added. The cover slips were placed on the layer and the slides were allowed to solidify in the refrigerator for 5min. After removing the cover slips, 100 μl of 0.05% LMA was applied and the slides with the cover slips were placed again within the refrigerator for 5 min. The slides were submerged in the lysing solution (2.5M NaCl, 100 mM EDTA-Na, 10 mM Tris-HCl, pH-10, 1% Triton-X 100 and 10% DMSO, pH-10 were added fresh) for 1h. The slides were then placed in an unwinding buffer (1mM EDTA and 300mM NaOH, pH-13) for 20 mins and electrophoresis was carried out with the same solution for 20 minutes at 20V and 300mA (0.8V/cm). After electrophoresis the slides were neutralized by washing three times in a neutralizing buffer (400 mM Tris-HCl, pH-7.4) for 5 mins each and were stained with 50μl of 10μg/mL of ethidium bromide. For each treatment group two slides were prepared in lots of 50 randomly chosen cells (a total of 100 cells). were scored manually. DNA damage was scored visually as described by Collins et al. (1997) where values are attributed according to DNA damage from a range of 0-3 starting from no damage to full comet formation.

Histological studies:

After 2 months of pelargonidin treatment animals were anesthetized with pentobarbital (50 mg / kg, i.p). Liver, kidney and pancreas were promptly removed and immediately
Materials & Methods

processed for histological examination. Liver and pancreas were fixed in Boiun's fluid (12-24 h). Kidney was fixed in 10% formalin (12-24 h). The tissues were dehydrated in a series of ethanol solution [70, 80, 90, 100 % (v/v)], cleared in xylene and embedded in paraffin wax (56-58°C). After paraffin embedding, 5 μm sections were prepared.

Staining of pancreatic sections:
The pancreatic sections were stained by Gomori's Chrome Alum hematoxylin (Luna, 1968). The sections were treated with potassium permanganate (0.3 g %) for 1 min. Decolorized in sodium bisulphite (5 %) and rinsed in tap water for 10 mins. Then the sections were stained in chromium hematoxylin (1%) at 60°C for 1 h, followed by rinsing in acid-alcohol (1% acetic acid:70% alcohol) for 1 min and rinsed in tap water for 10 mins. The sections were then stained in Phloxine B (0.5%) at 60°C for 2-3 h. Rinsed in a tilled water, treated with phosphotungstic acid (5%) for 1 min and finally differentiated in 95% ethyl alcohol for approximately 1 min. Violet colored cells were designated as β-cells.

Staining of kidney sections:
The kidney sections were stained with Harris hematoxylin (Preece,1972) for 10 mins, rinsed in tap water and differentiated in acid-alcohol(1% acetic acid:70% alcohol) for 2-3 dips. The sections were washed in tap water and put in ammonia water until they appeared bright blue. The excess blue colour was removed by washing in running tap water for 10-20 mins. The sections were then stained in eosin for 2-5 mins at room temperature and dehydrated in 95% alcohol until the excess stain was removed.

Staining of liver sections:
The liver sections were stained with Harris Hematoxylin and eosin as mentioned above. The slides were examined under optical microscope.
Iron staining in liver tissue:

After sacrificing the rats, liver tissues were promptly removed and immersed in 10% formalin. 5 μm thick sections were prepared from paraffin-embedded tissues, and were stained with Perl’s prussian blue for presence of iron (Bunting, H. 1949).

Studies on pelargonidin-RBC and pelargonidin-hemoglobin interactions:

Study of pelargonidin-RBC interaction:

*Phase contrast microscopy*

The effect of pelargonidin on human RBC was studied using Phase-contrast microscope. RBCs (4.8 × 10^7 cells) in the absence or presence of pelargonidin (10 μM, 25 μM, 50 μM, 100 μM) were incubated at 25°C for 30 mins. 20 μl of the cells were taken on a clean glass slide, covered with a cover slip and observed under the microscope.

*Scanning electron microscopy (SEM)*

RBC (6.8 × 10^7 cells) were incubated both in absence and presence of pelargonidin (10 μM, 25 μM, 50 μM, 100 μM) for 30 mins at 25°C. The cells were fixed by putting 50 μl cells in 2% gluteraldehyde (in 0.1 M PBS) for 30 mins. Then the resultant cell suspension was centrifuged at 15,000 rpm for 5 min. The pellet was washed in 0.1 M PBS. The cells were resuspended in PBS and a thin film is applied on a thin cover slip. The cover slips containing sample were air-dried. 5-6 nm of gold was sputtered onto the cell samples and their images observed under the microscope (FEI Quanta-200MK2).

*Osmotic fragility of RBC*

RBC (1.3 × 10^6) were incubated in absence and presence of pelargonidin (10, 20 μM) at 25°C for 30 mins (total volume: 1 ml) and exposed to 50 mM PB containing different concentration of NaCl. RBC osmotic fragility was determined by the method
0.02, 0.03, 0.05, 0.10 and 0.15 M; total volume: 1 ml) and allowed to stand for 30 mins. Then the test tubes were gently shaken for approximately 2 mins and centrifuged at 2000 rpm for 5 mins. The resulting supernatants contained hemoglobin from lysed cells. The absorbance of the supernatants was analyzed using a wavelength of 540 nm. The following equation was used to determine the percent of lysis:

% Hemolysis = \left[ \frac{OD_X - OD_{0.15 \text{ NaCl}}}{OD_0 - OD_{0.01 \text{ NaCl}}} \right]

where $OD_X$ is the absorbance of the test solution, $OD_{0.15}$ is the absorbance of the supernatant containing 0.15 M NaCl solution, and $OD_{0.01}$ is the absorbance of the supernatant containing 0.01 M NaCl solution and $OD_0$ is the absorbance of the supernatant without containing NaCl solution.

Studies on pelargonidin-hemoglobin interactions:

**Preparation of pelargonidin stock solutions**

Pelargonidin was dissolved in spectrograde ethanol containing 0.01% HCl. The concentration was measured using extinction coefficient ($\varepsilon_{520\text{nm}}$) of 32000M$^{-1}$cm$^{-1}$. Small aliquots from the stock solution were added to the reaction mixture to get the required final concentration of pelargonidin so that final concentration of ethanol in reaction buffer (always <1%).

**Spectroscopic studies**

Spectrophotometric, spectrofluorimetric and circular dichroic (CD) studies were done to understand pelargonidin and hemoglobin interaction.

**Absorption spectroscopic (250 – 650 nm) measurement**:

The UV–VIS spectrum was recorded on UV–VIS, spectrophotometer (Hitachi U2000) using 1 ml quartz cuvette of pathlength 1cm. The reaction mixture consists of Hb (10 µM) and gradual increasing pelargonidin concentrations 2.5–15 µM. The reaction blank also contains gradual increasing pelargonidin concentrations of 2.5–15 µM. The reaction was done at room temperature. The binding parameters (K) for the Hb-pelargonidin
complex had been calculated at room temperature (25°C) data using Kapp's equation (Kapp et al., 1990).

\[
\frac{\Delta A}{\Delta A} = \frac{\Delta A}{\Delta A_{\text{max}}} + \frac{\Delta A}{\Delta A_{\text{max}}} \frac{1}{K L_t}
\]

where \(\Delta A=A_0-A\), \(A_0\) and \(A\) are respective absorbance intensity of the protein in absence and presence of pelargonidin, \(L_t\) is the total pelargonidin concentration (μM). Binding constant \(K\) was obtained from the linear plot of \(A_0/\Delta A\) vs \(1/L_t\). \(\Delta A_{\text{max}}\) is the maximum change in absorbance intensity. The intercept of the plot on the \(A_0/\Delta A\) axis corresponding to \(1/L_t=0\) gives \(A_0/\Delta A_{\text{max}}\) and slopes measures the affinity constant, \(K\).

**Fluorimetric study:**

i) Fluorescence quenching measurement:
Fluorescence emission spectra were measured with a Hitachi F-3010 spectrofluorimeter in a 1-cm quartz cell using 5 nm/5 nm slit widths. The excitation wavelength was 285 nm, and the emission wavelengths were monitored from 300 - 500 nm.

A quantitative analysis of the interaction between pelargonidin and Hb was performed by fluorimetric titration. 1.0 ml of 10 μM Hb was titrated by successive addition of pelargonidin solution to reach a final concentration of 25 μM. The binding parameter (K) for the Hb-pelargonidin interaction has been calculated at room temperature from fluorescence quenching data using Kapp's equation (Kapp et al., 1990) and from Stern-Volmer equation. In case of Kapp's equation,

\[
\frac{F_0}{\Delta F} = \frac{F_0}{\Delta F_{\text{max}}} + \frac{F_0}{\Delta F_{\text{max}}} \frac{1}{K L_t}
\]

where \(\Delta F=F_0-F\), \(F_0\) and \(F\) are respective fluorescence intensity of the protein in absence and presence of pelargonidin, \(L_t\) is the total pelargonidin concentration (μM). \(K\) was obtained from the linear plot of \(F_0/\Delta F\) vs \(1/L_t\). \(\Delta F_{\text{max}}\) is the maximum change in
fluorescence intensity. The intercept of the plot on the $F_0/\Delta F$ axis corresponding to $1/L_C=0$ gives $F_0/\Delta F_{\text{max}}$ and slopes measures the affinity constant, $K$.

The Stern-Volmer equation is as followed,

$$\frac{F_0}{\Delta F} = 1 + K_{sv}Q$$

where $\Delta F = F_0 - F$, $F_0$ and $F$ are respective fluorescence intensity of the protein in absence and presence of pelargonidin, $Q$ is the pelargonidin concentration and from the slope of the plot of $F_0/\Delta F$ vs, $Q$ gives the Stern-Volmer constant $K_{sv}$.

In the calculation of fluorescence data inner filter effect correction was incorporated. For drug O.D$>0.05$ the following correction is done

$$F_{\text{corr}} = F_{\text{obs}} \times \text{Antilog} \left[ \frac{(A_{\text{ex}} + A_{\text{em}})}{2} \right]$$

Where $F_{\text{corr}} =$ corrected emission intensity, $F_{\text{obs}} =$ observed emission intensity, $A_{\text{ex}}$ and $A_{\text{em}}$ are the absorbance of the dye at the excitation and emission wavelength respectively.

ii) **Thermodynamic analyses of the binding:**

Temperature dependence of the binding constant ($K$) was done spectrofluorimetrically from the emission spectra of hemoglobin in the presence of pelargonidin in the temperature range 15°C -35°C using a circulating waterbath, the temperature of which was controlled within ±0.1°C. Thermodynamic analysis of the temperature dependence of binding was done by plotting $\ln K$ vs. $1/T$ following van’t Hoff’s equation (Martin et al., 1991). Considering that the enthalpy change ($\Delta H^o$) does not vary significantly over this temperature range, then its value and that of entropy change ($\Delta S^o$) was calculated using the van’t Hoff equation:

$$\ln K = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R}$$
where $K$ is the binding constant at the corresponding temperature and $R$ is the gas constant. The plot of $\ln K$ against $1/T$ is a straight line, the equation of which provides the enthalpy ($\Delta H^0$) and entropy ($\Delta S^0$) changes on binding. The free energy of binding is estimated from the following relationship:

$$
\Delta G^0 = \Delta H^0 - T\Delta S^0
$$

This calculation was performed in case of Hb-pelargonidin interaction.

**iii) Effect of different NaCl molarity on the binding process:**

To determine the nature of Hb-pelargonidin interactions, binding studies were done at different molarities of NaCl solution (0.05 M-0.30 M). In this experiment, 1.0 ml of 10 μM Hb was titrated by successive addition of pelargonidin solution to reach a final concentration of 25 μM in presence each NaCl concentration. The binding data at different NaCl concentrations were analyzed from the plot of $\ln K$ vs $\ln [NaCl]$ following the relation of Record et al. (1978).

$$
\ln K_{obs} = \ln K_T + z\Psi^1 \ln \gamma \pm \delta - z\Psi \ln [NaCl]
$$

where $K_T$ is the binding constant at NaCl concentration 1M, $z$ = number of charged group per binding site, $\gamma$ = the activity coefficient and $\Psi$ = the thermodynamic counter ion association parameters. Within the range of NaCl used in the experiment the value of $\gamma$ was practically 1 thereby reducing the term $z\Psi^1 \ln \gamma \pm \delta$ to zero. Slope of the plot gave the estimate of total bound charge ($z\Psi$).

**Circular dichroic (CD) measurements (200 – 600 nm):**

CD spectroscopy was done in Jasco-600 spectropolarimeter using 1 mm path length cuvettes. Molar ellipticity [$\theta$] values were obtained using the relation (Geraci and Parkhurst, 1981)

$$
[\theta] = [\text{M}_r \text{W}] 0/10.1c
$$

where $c$ is the concentration of the proteins in gm/ml, $\theta$ (obtained directly from the CD chart) is the observed rotation in degrees (mdeg), $l$ is the path length in cm and $[\text{M}_r \text{W}]$ the mean residual molecular weight of the amino acid is 110. The $\alpha$-helical contents of Hb
Materials & Methods

and pelargonidin interaction were determined according to the relationship (Chen, et al., 1972):

\[
\text{fraction of } \alpha\text{-helix} = [\theta]_{222} + 2340/-30,300,
\]

where \([\theta]_{222}\) is the molar specific ellipticity at 222 nm. In the reaction mixture pelargonidin concentrations of 10–50 \(\mu\text{M}\) and constant protein concentration of 10 \(\mu\text{M}\) was maintained at 25°C. k2d server was used to calculate protein secondary structure. In this server an algorithm was used for the estimation of the percentages of protein secondary structure from UV circular dichroism spectra using a Kohonen neural network with a 2-dimensional output layer.

**Isothermal Titration Calorimetry (ITC):**

Isothermal Titration Calorimetric study was performed to monitor the nature of temperature upon binding nature of pelargonidin-Hb interaction. An OMEGA, ITC, microcalorimeter of Microcal, Northampton (USA), was used. A 1.325 ml of 10 \(\mu\text{M}\) Hb solutions was taken both in the reaction and reference cells. The injection syringe (350 \(\mu\text{l}\)) was filled pelargonidin (100 \(\mu\text{M}\)), which was injected after equal time intervals of 300 secs in multiple steps to the solution in the calorimeter cell under constant stirring (350 rpm) condition. The heat released at each step of interaction of the drug with protein solution was recorded and the enthalpy per mole of drug was calculated using the ITC software. The experiment of protein dilution was also performed with the same injection matrix as that of interaction experiments. All measurements were taken at thermo stated condition maintained by a Neslab RTE 100 circulating water bath. Titrating drug solution in the cell by its own solution assessed the dilution error in the experiments. The error was found to be ±0.5 % and hence neglected.
Materials & Methods

Assay of peroxidase activities:
Hemoglobin possesses enzyme-like activities. It interacts with H₂O₂ to yield a potent oxidant ferrylhemoglobin which is capable of oxidizing a wide variety of electron donors resembling peroxidase-like activities (Everse et al., 1994).

The reaction mixture (1 ml) contained 50 mM citrate buffer, pH 5.4, 1.5 µM Hb, pelargonidin concentration (0.1-2 µM and 0.002% o-dianisidine. The reaction was initiated by adding 17.6 mM H₂O₂. The absorbance at 450 nm was followed at 25°C for 2 min (Everse, et al., 1994).

Statistical analysis
Results were expressed as mean ± SE, and statistical significance was determined using unpaired two-tailed student’s t test and one-way analysis of variance (ANOVA). The level of significance was established at p<0.05.
Molecular modeling study

1. Preparing the ligand and macromolecule files for AutoDock

The PDB files obtained from the Protein Data Bank (PDB) repository were not perfect for docking study. Using the Graphic User Interface (GUI) of AutoDockTools (ADT) the files were prepared as follows.

The downloaded PDB file of hemoglobin is (PDB ID – 2DN1). Since 2DN1 is human dimeric oxyhemoglobin, using crystal symmetry, the corresponding tetrameric oxyhemoglobin was generated. The ligand file pelargonidin data was obtained from Cambridge Crystallographic Data Centre (CCDC) and the structure of pelargonidin was generated by using Mercury CSD 2.0 (Macrae et al., 2006) and viewed AutoDockTools version 4 (Morris et al., 1998). AutoDock (Huey and Morris, G.M., 2007, Huey et al., 2009) is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known three dimensional structure.

AutoDock consists of two main programs:

- AutoGrid - calculates the sampling of the conformation to be explored. It also helps to find the optimum fit between protein and ligand. Thus the conformational space is sampled discretely in the form of a grid.
- AutoDock then performs docking by positioning both the protein and the ligand in a specified relative geometry according to the precalculated grid coordinates.

a) The Macromolecule file

The downloaded PDB files were first read in ADT, added waters removed and polar hydrogens added. ADT then checked if the molecule has charges, if not it added Kollman charges (by default, ADT adds Kollman charges for a peptide, determined by checking whether all of its residues' names appear in the standard set of 20 commonly occurring amino acids, and gasteiger charges if not so). Finally solvation parameters were added and the files saved as molecule.pdbqs (where 'q' and 's' represent charge and solvation respectively).
Materials & Methods

b) The Ligand file

In a similar procedure, the ligand files were read in ADT, non-polar hydrogens merged and charges added. ADT then determined the best root (the best root is the atom in the ligand with the smallest largest subtree, in case of a tie, if either atom is in a cycle, its picked as the root and if neither or both are in a cycle, the first found is picked). Next rotatable bonds in the ligand was defined, making all amide bonds non rotatable and set the number of active torsions set to fewest atoms. The ligand file was then saved with ligand.out.pdbq extension (q representing charge)

2. Preparing the grid parameter file

For the calculation of docking interaction energy it is necessary to create a three-dimensional box (grid) in which the protein molecule is enclosed. The grid volume should be large enough to allow the ligand to rotate freely, even when the ligand is in its most fully extended conformation. The parameters required to create such a grid are stored in the Grid Parameter File molecule.gpf.

3. AutoGrid 4

Then AutoGrid 4 job was run to create one map for every atom type in the ligand and create the corresponding macromolecular file with the extension molecule.glg.

4. Preparing the docking parameter file

The docking parameter file, which instructs AutoDock about the ligand to move, the map files to use, and other properties defined for the ligand, was created. AutoDock’s search methods include the Monte Carlo simulated annealing (SA) method, the Genetic Algorithm (GA), local search (LS) and the hybrid genetic algorithm with local search (GA-LS). The latter is also referred to as the Lamarckian genetic algorithm (LGA) because offsprings are allowed to inherit the local search adaptations of their parents and this was the chosen algorithm for our analysis.
5. *AutoDock4*

Finally the AutoDock job was run from the GUI of ADT and the docked ligand files (.dlg extension) were used for study. The dlg files were read in ADT as well as in PyMol to calculate the inter-atomic distances in the docked ligand-protein complexes. The entire procedure is illustrated in the flowchart provided.
Materials & Methods

1. The pdb file is opened in the GUI of ADT, added waters removed and polar hydrogens added with no bond order.
2. ADT then checks if the molecule is a peptide and adds Kollman charges if so, and Gasteiger charges if not so.
3. Merged non-polar hydrogens

Ligand.pdbq (q represents charge)

1. Rigid root is defined automatically.
2. Rotable bonds are defined.
3. Number of active torsions is set. (To default)

Ligand.out.pdbq

1. Selected Macromolecule file and ligand for docking runs
2. Search Parameters set.
3. Docking Run Parameters set

Ligand.dpf (docking parameter file)

AutoDock3 launched

Output dlg (docked Ligand file) is analyzed in both ADT and PyMol.
Entire process repeated with the lowest energy docked conformation of ligand till docking energy is not further minimized.

Flow chart of the docking procedure
Action of Pelargonidin in STZ-induced diabetic rats
Introduction:

Diabetes mellitus, the most common non communicable disease of the today's world, results hyperglycemia and chronic oxidative stress mediated biochemical alterations and pathophysiological complications towards of several tissue sites. Free radicals and oxidative stress have long been implicated in eliciting complications of the diabetic condition (Rosen et al., 2001; Baynes & Thorpe, 1999; Baynes, J.W. 1991). In diabetes hyperglycemia, protein glycation and glucose auto-oxidation have been suggested to induce free radical generation (King and Loken., 2004; Adachi et al., 1991; Wolf et al., 1991; Wolf and Dean, 1987). Glycation of proteins especially heme proteins like hemoglobin, myoglobin and their subsequent modifications have been ascribed to play significant roles in different pathological complications of this disease, particularly because of their association with oxidation reactions involving reactive oxygen generation and AGE formation. At the same time disturbances in antidiabetic defense systems including scavenging enzymes like superoxide dismutase (SOD), glutathione reductase and deficiencies of antioxidants like vitamin C and E have been reported (Abu-Seif and Youseef, 2004; Price et al., 2001; West, 2000).

There is suggestive evidence that overload of iron plays a pathogenic role in diabetes and its complications. A link has been established between development of diabetes and increased dietary iron intake (Rajpathak et al., 2006; Jiang et al., 2004; Schulze et al., 2003) and increased body iron stores (Jehn et al., 2007; Chen et al., 2006; Bozzini et al., 2005). The exact mechanism of iron-induced diabetes is not clear, but is likely to be mediated by insulin deficiency, insulin resistance and hepatic dysfunction (Mendler et al., 1999; Dandona et al., 1983). However, iron overload may not be a prerequisite for iron to induce the disease. Its pathophysiology may be associated with the availability of so-called catalytic iron or iron that is available to participate in free radical reactions (Swaminathan et al., 2007). In a vicious cycle, diabetes may lead to formation of more catalytic iron with further aggravation of the situation.

Even today there is no full proof therapy of diabetes which can cure the disease completely along with its multiple associated complications. The focus of current research is based upon to find an appropriate drug from herbal origin which can cure the
Flavonoids are ubiquitously present in common plant based food items and beverages. Rusznyak and Szent-Gyorgyi (1936) first drew attention to the therapeutically beneficial role of dietary flavonoids. Many flavonoids are known to possess powerful antioxidant properties (Rice-Evans, 2001; van Acker et al., 1996). Since diabetes mellitus is considered as a free radical-mediated disease, there has been renewed interest in the use of flavonoids in diabetes research, which includes quercetin, a flavonol (Vessal et al., 2003; Sanders et al., 2001), (-) epicatechin, a flavan-3-ol (Chakravarty et al., 1981), G-rutin, a glycoside of quercetin (Nagasawa et al., 2003), puerarin, an isoflavone (Hsu et al., 2003) etc. Anthocyanins and their aglycone derivatives anthocyanidins are important groups of flavonoids having strong antidiabetic potential. Of these groups, pelargonidin-3-O-rhamnoside has been reported to exhibit an antidiabetic activity in diabetic rats (Cherian et al., 1992). Pelargonidin-3-galactoside and its aglycone stimulate insulin secretion in rodent pancreatic β cells in vitro in presence of glucose (Jayaprakasam et al., 2005). Since these findings are very significant, but only in a seminal state, we have undertaken the present study, in which we have used the simplest anthocyanidin pelargonidin in streptozotocin (STZ)-induced diabetic rats.

The common way to study diabetes is to create an appropriate animal model, which can mimic its complications, and there by help to identify underline processes involved in this disease scenario. The most common substances used to induce diabetes mellitus in experimental animals are alloxan and STZ. STZ-induced diabetes provides a suitable case of chronic oxidative stress, hyperglycemia and other biochemical alterations that mimic those of human diabetics to a great extent.

Here in this study the focus is based upon to explore the role of pelargonidin, an anthocyanidin in alleviating hyperglycemia and oxidative stress in streptozotocin-induced experimental rats. Simultaneously the aspect of hemoglobin glycation and its associated pathophysiological complications in model diabetes and the role of pelargonidin treatment in this context are studied.
Results:

Glycemia status and insulin level in diabetes: Effect of pelargonidin

Fasting blood glucose was monitored in different groups of rats at different time intervals and presented in Fig. 17. Blood glucose levels of untreated diabetic rats (D) were significantly higher compared to those of control group (C). Treatment of diabetic rats with one dose of pelargonidin (DP) reduced the glucose levels to normal levels within one week and it maintained throughout the five-week experiment period. Results up to three weeks have been shown (Fig. 17a). The drug had no effect on blood glucose concentrations of normoglycemic rats (CP). The extent of reduction of fasting blood glucose during first week after administration of pelargonidin in diabetic rats (DP) has been shown in Fig. 17 b. The levels were significantly reduced within two days and were almost normal after five days of treatment.

Fig 17 a. Blood glucose-time profile in different groups of rats. Plasma concentration of glucose was measured at different time intervals after i.p administration of pelargonidin in diabetic and control rats (DP and CP) together with untreated diabetic (D) and normal groups (C). Bars represent mean ± SE of eight experiments for each group (n = 8). p<0.01 for C vs. D, and for D vs. DP.

b. Blood glucose changes at different time intervals during first week after pelargonidin treatment.
IPGTT was done in different groups of rats after two weeks of pelargonidin treatment (Fig. 18a). Glucose tolerance curves of untreated diabetic rats (D) exhibited characteristic glucose intolerant behavior in comparison with control rats (C). Pelargonidin exerted no effect on blood glucose tolerance curves of normoglycemic animals (CP). However, it normalized the glucose tolerance curves of diabetic rats (DP).

Onset of diabetes in rats (D) resulted in significant reduction in serum insulin levels, and was found to increase significantly after two weeks of pelargonidin treatment (DP) as shown in Fig. 18 b. On the other hand, normal rats (C) and pelargonidin-treated normal rats (CP) exhibited almost similar levels of insulin.
Induction of diabetes in rats (D) caused significant decrease in free radical scavenging enzymes like serum SOD and catalase over the normal levels (C) as shown in Table 2. Increased levels of serum MDA and fructosamine, index of formation of free radicals and early glycation end products, respectively, were evident in diabetic rats. All these changes enhanced oxidative stress condition, which was counteracted by pelargonidin. Serum SOD and catalase activities as well as serum MDA level were found to be normal after two weeks of pelargonidin treatment in diabetic rats (DP). Serum fructosamine level, measured after five weeks of pelargonidin injection, was found to be comparable to the normal level. The drug did not have any marked change on all these oxidative stress markers in normal rats (CP).

Table 2: Effect of pelargonidin on scavenging enzymes (serum SOD and catalase) and oxidative markers (serum MDA and fructosamine levels) in different groups of rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (Units/mg protein)</th>
<th>Catalase (Arbitrary fluorescence unit)</th>
<th>MDA level (pmol/mg protein)</th>
<th>Fructosamine level (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.400 ± 0.020</td>
<td>0.244 ± 0.024</td>
<td>74.3 ± 11.3</td>
<td>204 ± 15.4</td>
</tr>
<tr>
<td>D</td>
<td>0.290 ± 0.026*</td>
<td>0.058 ± 0.010*</td>
<td>154.6 ± 3 0.0*1</td>
<td>315 ± 22.4*2</td>
</tr>
<tr>
<td>DP</td>
<td>0.422 ± 0.030*</td>
<td>0.260 ± 0.032*</td>
<td>97.8 ± 17.0*2</td>
<td>225 ± 17.1*3</td>
</tr>
<tr>
<td>CP</td>
<td>0.470 ± 0.050</td>
<td>0.256 ± 0.030</td>
<td>74.6 ± 17.3</td>
<td>207 ± 10.2</td>
</tr>
</tbody>
</table>

Serum enzymes and MDA levels assayed after two weeks and serum fructosamine levels assayed after five weeks of pelargonidin treatment have been presented.

Values represent mean ± SE. of eight experiments (n = 8).

*p <0.01 for C vs. D; *p <0.05 for D vs. DP; *p <0.01 for D vs. DP; *p <0.01 for C vs. D; *p <0.01 for D vs. DP; *p <0.01 for C vs. D; *p <0.05 for D vs. DP.
GHb levels were measured in different groups of rats (Fig. 19 a), and were found to be significantly higher in diabetic rats (D) than in control animals (C). Experiments were done after seven weeks of STZ administration i.e. five weeks of pelargonidin treatment in DP group of rats. In diabetic treated group (DP), GHb levels in diabetic treated group were found to be comparable with those of normal group of rats (C). The treatment did not change the level in control rats (CP).

Fig 19 a. GHb levels in different groups of rats after five weeks of pelargonidin treatment. Results are mean ± SE of six experiments (n = 6). p<0.05 for C vs. D and for D vs. DP.

b. Free iron levels in Hb samples isolated from different groups of rats after two weeks of treatment. Results are mean ± SE of eight experiments for each group (n = 8). p<0.01 for C vs. D and for D vs. DP.

Ferrozine-detected iron levels were estimated in Hb samples isolated from different groups of rats and presented in Fig. 19 b. Free iron levels in Hb of diabetic rats (D) were significantly higher in comparison with those of control group of rats (C). The results presented are from rats after two weeks of pelargonidin treatment together with
Action of Pelargonidin in STZ-induced diabetic rats

respective control groups. The treatment normalized free iron level in diabetic rats (DP). However, pelargonidin had no effect on control rats (CP).

Iron deposition in liver of diabetic rats: effect of pelargonidin

Iron staining by prussian blue in liver sections has been shown in Fig. 20, which demonstrated significant stains in liver sections of diabetic rats (D). For this experiment, rats were sacrificed seven weeks after diabetes induction. Iron stain was not visible in control rats (C). In pelargonidin treated diabetic rats (DP), iron stains after five weeks of treatment were comparable with those of control group of rats (C). Similar results were obtained in three additional experiments.
Fig 20. Histological examination of Perl's prussian blue-stained liver sections of different groups of rats. a: normoglycemic rats (C), b: diabetic rats (D), c: pelargonidin-treated diabetic rats (DP). Arrowheads indicate positive staining areas. Representative sections are from four separate experiments in each group (magnification 40x).
Action of Pelargonidin in STZ-induced diabetic rats

Hb-induced oxidative stress in diabetes: inhibitory function of pelargonidin

To understand iron-mediated free radical insult, lipid peroxidation and DNA breakdown by Hb samples were studied in absence of exogenously added H$_2$O$_2$. Aldehydes are produced during lipid hydroperoxide breakdown in biological systems. MDA is the major product in this process, and its determination by TBA is used as an index of the extent of lipid peroxidation. As shown in Fig. 21, arachidonic acid peroxidation by Hb of diabetic rats (D) was much more efficient than that of normal rats (C).

![MDA (nmol/mg Hb/h) vs Groups]

Fig 21. Hb-mediated lipid peroxidation. Experiments were done two weeks after pelargonidin treatment. Bars represent mean ± SE of eight experiments in each case (n = 8). p<0.01 for C vs. D and for D vs. DP.

Pelargonidin treatment almost normalized the enhanced level of peroxidation reaction as shown in DP group of animals. The results shown were from rats after two weeks of pelargonidin treatment. Control treated group (CP) did not exhibit any significant change.

Hb-mediated DNA (plasmid) damage was monitored by agarose gel electrophoresis followed by ethidium bromide staining. A representative experiment has been shown in Fig. 22, in which Hb samples were collected from DP group after two weeks of treatment.
and respective control groups (C and D). Plasmid DNA alone had slight form II along with form I (lane 1), form I and form II being intact supercoiled DNA and nicked DNA, respectively. Hb from diabetic rats (D) led significantly enhanced DNA degradation (lane 3) in comparison with control (C) Hb-induced degradation (lane 2). DNA breakdown catalyzed by Hb of diabetic rats was significantly inhibited by DFO, an iron chelator (lane 4) and mannitol, an OH radical quencher (lane 5). In presence of Hb from pelargonidin-treated diabetic rats (DP), DNA breakdown was significantly inhibited as shown in lane 6 in comparison with diabetic (D) Hb-induced degradation (lane 3). Similar results were obtained in three additional independent experiments.

![DNA electrophoresis](image)

Lane 1 2 3 4 5 6
DNA + + + + + +
Hb - C D D D DP
DFO - - - + - -
Mannitol - - - - + -

Fig 22. Hb-mediated DNA (plasmid) breakdown studied after two weeks of pelargonidin treatment. Hb samples were isolated from different groups of rats (C, D, DP) and treated with DNA. DFO or mannitol was added to the reaction mixture as indicated. Different forms of DNA were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in three additional experiments.

**Carbonyl formation in Hb: prevention by pelargonidin**

Carbonyl formation in proteins due to increased oxidation is another marker of oxidative stress. Experiments were done to detect the extent of carbonyl formation in Hb samples isolated from different groups of rats. As shown in Fig. 23, carbonyl contents in Hb
samples from diabetic rats (D) were significantly higher in comparison with those from normal rats (C). Pelargonidin efficiently improved this stress condition by decreasing carbonyl content to control level as found in DP groups of rats. The results obtained with Hb after two weeks of treatment have been presented.

Fig 23. Levels of carbonyl contents in Hb samples from control and treated rats. Experiments were done two weeks after pelargonidin treatment. Values represent mean ± SE of eight experiments in each case (n=8). p<0.01 for C vs. D and for D vs. DP.

Serum triglyceride and total cholesterol levels
Effect of pelargonidin treatment in serum triglyceride and serum cholesterol levels were studied in different groups of rats (Fig. 24 a and b). The experiment was done for three weeks after drug treatment. Both triglyceride and serum cholesterol levels in diabetic rats increased throughout the experimental period. After 7 days of drug treatment, increased level of serum triglyceride and serum cholesterol level in D group of rats were decreased significantly in DP group and these levels came to the respective normal levels of group C. Pelargonidin thus have hypolipidemic action in diabetes.
Fig 24. Levels of serum a: triglyceride concentration (mg/dl) and b: total cholesterol concentration (mg/dl) during different time intervals after pelargonidin treatment. Values represent mean ± SE of eight experiments in each case (n=8). *p<0.01 for C vs. D and for D vs. DP.

Fluorescence depolarization measurements of the fluidity of RBC membranes:
Measurement of RBC membrane anisotropy was done five weeks after drug treatment using TMA-DPH probe. Diabetes led to increased anisotropy indicating alteration of RBC membrane organization (Table-3), while pelargonidin treatment reversed the membrane fluidity.

Table 3: RBC membrane fluidity in C, D, DP, and CP rats

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>DP</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorescence</td>
<td>Anisotropy (r)</td>
<td>Fluorescence</td>
<td>Anisotropy (r)</td>
</tr>
<tr>
<td></td>
<td>0.250±0.006</td>
<td>0.375±0.008</td>
<td>0.279±0.007</td>
<td>0.247±0.01</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. of 5 rats. C vs D, p<0.05 and D vs DP, p<0.05.
**Effect of pelargonidin on serum alkaline phosphatase (ALP) activity:**

Serum ALP activity was estimated by using p-NPP as substrate. The serum ALP activity significantly increased in diabetic control rats while treatment with pelargonidin resulted in significant reduction in alkaline phosphatase activity in DP group when monitored 2 week after drug treatment. (Table 4). The serum ALP activities of normal control and treated diabetic rats were comparable.

**Table 4: Effects of pelargonidin treatment on serum ALP level in normal and diabetic rats 2 weeks after treatment:**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>DP</th>
<th>NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ALP activity (U/mg serum pr)</td>
<td>76.0 ± 7.0</td>
<td>170.0 ± 4.0</td>
<td>43.0 ± 8.0</td>
<td>23.4 ± 8.0</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. of 3 sets of experiments having 8 rats in each group of each set. One unit of activity was taken as μmoles of p-nitro phenol formed per min. NC vs DC, P <0.05, DC vs DT, P<0.01 and DC vs DTgl, P<0.05.

**Comet assay on lymphocyte:**

The comet assay is a sensitive and rapid method for DNA damage detection at individual cell levels. In the assay, under alkaline conditions DNA loops containing breaks lose supercoiling, unwind and are released from the nucleus forming a 'comet tail'. After gel electrophoresis, DNA strand breaks are thus visualized as 'comets'. Comet assay was performed to identify DNA damage level in lymphocytes of different groups of rats to detect whether diabetes leads to elevate level of DNA damage as found in case of diabetic rats (D) (Fig.25 b) compared to that of control rats (C) (Fig.25 a). Pelargonidin therapy in diabetic rats effectively prevents DNA damage in lymphocyte as evident from in Fig.25 c, d and comet score Fig 25e.
Fig 25. Single cell gel electrophoresis on lymphocytes showing comet (40 x) on different group of rats. a: normoglycemic rats (C), b: diabetic rats (D), c: pelargonidin - treated diabetic rats (DP), d: pelargonidin - treated normal rats (CP), e: diabetic rats (D) at 100X, f: DNA damage (comet score). Scoring was done according to the method of Collins et al., (1997) where values are attributed according to DNA damage from a range of 0-3 starting from no damage to full comet formation. Comparison of comet scores was done on different group of rats. Values represent mean ± SE of eight experiments in each case (n=8). p<0.01 for C vs. D and for D vs. DP. 500 cells counted in each case.
Action of Pelargonidin in STZ-induced diabetic rats

Histological studies in pancreas, liver and kidney

Histology of pancreas:

The pancreatic sections were stained with Gomori's Chrome Alum hematoxylin. The slides were visualized under light microscope at ×20 magnifications. The Phloxine B stained violet coloured cells were identified as β-cells. Light microscopic examination of pancreatic section of diabetic rats in Fig. 26 b revealed morphological changes including atrophy of pancreatic islets, pancreatic necrosis and significant reduction in pancreatic β cells (due to vacuolization) compared to the normal control. A significant increase in the number of pancreatic islets and improvement of pancreas morphology was seen in drug treated animals Fig. 26 c. There was considerably higher number of micro islets formation in pelargonidin treated diabetic rats in comparison with diabetic group.

Histology of liver:

Liver tissue was subjected to histological analysis after staining with hematoxylin and eosin. The slides were visualized under light microscope at × 20 magnification. In normal control rats C, polyhedral hepatocytes were observed to have a central nucleus and visible nucleolus; the cytoplasm was well stained with basophilic bodies shown in Fig. 27a. By contrast, the hepatocytes of D rats (Fig. 27 b) exhibited nuclear chromatin with coarse granules, nucleus with diffused nucleolus, weak staining of the cytoplasm and fibrosis. The abnormalities were prominently milder after treatment of diabetic rats with pelargonidin (Fig. 27 c). Normoglycemic rats treated with pelargonidin (CP) shows morphology similar to that of normal control rats (C).

Histology of kidney:

Histological analysis of kidney sections showed diffuse glomerulosclerosis and thickening of glomerular basement membrane in diabetic rats as shown in Fig.28 b. Morphological changes in the diabetic rats were accompanied with randomly placed
nuclei and vasodilatation of capillaries. Next to the proximal convoluted tubules clusters of cells with clear cytoplasmic structure like duct but without membrane were found. Glomerulosclerosis and glomerular basal membrane thickening of diabetic animal treated with pelargonidin (DP) were comparable to that of normal rats (C) in Fig.28 and C. Normal rats treated with pelargonidin showed identical morphology as that of control groups.
Fig 27. Liver sections stained with hematoxylin and eosin visualized at 20 magnification. a: normoglycemic rats (C), b: diabetic rats (D), c: pelargonidin-treated diabetic rats (DP), d: normoglycemic rats treated with pelargonidin (CP). Magnification: 20. Black arrows indicate nucleus with a distinct nucleolus. White arrows indicate nucleus diffused with the nucleolus.
Fig 28. Kidney sections stained with hematoxylin and eosin visualized at magnification 40X. a: normoglycemic rats (C), b: diabetic rats (D), c: pelargonidin-treated diabetic rats (DP), d: normoglycemic rats treated with pelargonidin (CP). Black arrow indicates enhanced glomerular basement membrane thickening and green arrows indicate diffused mesangium in diabetes as shown in Fig. B.
**Discussion:**

Enhanced of blood glucose level in diabetic control (D) rats are compared to that in normal control animals (Fig. 17) supports STZ-induced diabetogenesis in animal model. STZ is taken up by pancreatic β-cells via glucose transporter (GLUT-2) (Schnedl *et al*., 1994). It destroys pancreatic β cells by different mechanisms like DNA damage by alkylation (Yamamoto *et al*., 1981), depletion of NAD⁺ (Schein *et al*., 1973), generation of reactive oxygen and nitrogen species (Takasu *et al*., 1991) etc to induce type 1 diabetes. Diminution of functional β-cells associated with lowered insulin secretion thus leads to hyperglycemia due to STZ injection. Blood glucose lowering activity of pelargonidin in diabetic rats (Fig. 17) indicates hyperglycemic activity of the flavonoid. In this perspective reports by other workers with different flavonoids like quercetin (Vessal *et al*., 2003, Coskun *et al*., 2005), rutin (Prince and Kamalakkannan, 2006), genistein, diadzein (Choi *et al*., 2008) and tea flavonoids (-) epicatechin also show glucose lowering action in diabetic rats. In all these study multiple doses of flavonoids have been used and doses of flavonoids required are also higher than that used in present study. Treatment with various anthocyanin rich sources e.g. black chockoberry extract, tart cherry, purple corn, anthocyanin dyes from grapes etc also reveal their glucose lowering effect in both type 1 and type 2 diabetes when administered in multiple dosage for a long time (Jurgonski *et al*., 2008, Seymour *et al*., 2008, Tusuda *et al*., 2003, Jankowski *et al*., 2000). Normalization of intraperitoneal glucose tolerance curves in diabetic animals treated with single dose of pelargonidin as shown in Fig. 18 a is further supported by enhancement of serum insulin level in Fig. 18 b. Reports by Jayaprakasam, *et al*., (2006) indicates that anthocyanins from Cornelian Cherry normalizes glucose intolerance nature in high fat feed C57BL/6 mice by preserving islet structure and insulin content. They have also shown pelargonidin-induced stimulation of insulin in rodent pancreatic β cells in presence of glucose (Jayaprakasam, *et al*., 2005). Pelargonidin 3-rhamnoside obtained from Ficus bengalensis normalizes glucose tolerance curve in diabetic rats (Cherian *et al*., 1992). Report by Tusuda *et al*., (2003) suggests that dietary cyanidin3-O-b-D-glucoside rich purple corn extract normalizes free glucose and
Action of Pelargonidin in STZ-induced diabetic rats ameliorates insulin resistance mediated by IKKβ activation and suppresses TNFα expression. Our study together with existing reports thus suggests that pelargonidin possesses hypoglycemic activity. However, the exact mechanism of its hypoglycemic activity is still not clear. Pelargonidin may protect and stimulate pancreatic β cells by its antioxidant activity and/or by other activities like modulation of gene expression, interaction with signal transduction pathways etc, leading to insulin release in diabetic treated rats (DP). However, pelargonidin does not change insulin level and other biochemical parameters in control rats (CP) to maintain their physiological balance. Similar results have been reported with flavonoids quercetin and rutin, which have almost no effects on glycemic status and oxidative stress markers in normal rats, but have significant effects on these parameters in STZ-treated rats (Prince and Kamalakkannan, 2006; Vessal et al., 2003).

Table 2 revealed action of pelargonidin treatment upon several oxidative stress markers like serum SOD, catalase, MDA and fructosamine. Oxidative stress has been suggested to be the potential contributor to the development of complications in diabetes (Rosen et al., 2001; Baynes and Thorpe, 1999; Baynes, 1991). Increased free radical production as well as reduced antioxidant defense responses may give rise to increased oxidative stress in diabetic condition. (Abu-Seif and Youseef, 2004; Price et al., 2001; West, I.C. 2000). Catalase and SOD are scavenging enzymes, which are responsible for removing the toxic free radicals in vivo (Levy et al., 1999). The decrease in serum SOD activity in D group of rats could at least in part, result from inactivation of the enzyme by H₂O₂ or by glycation, which are known to occur during diabetes (Sozmen et al., 2001). Human erythrocyte Cu/Zn-SOD undergoes a gradual decrease in activity on incubation with glucose and the proportion of glycated SOD is considerably higher in diabetic patients (Sakurai et al., 1987, Arai et al., 1984, Adachi et al., 1991). Glycation has been suggested to cause loss of antigenicity and consequent inactivation of SOD (Yan & Harding, 1997). The decreased SOD activity in serum of STZ-induced diabetic rats found in the present study is in agreement with reports of Montilla et al., 2005 and Godin et al., 1988. Catalase is responsible for removal of intracellular H₂O₂, which itself is the inhibitor of SOD activity. In vitro incubation of catalase (bovine hepatic) with 10 mM glucose at 37°C for 8 days results in enzyme inactivation and loss of antigenicity (Yan & Harding,
Inactivation of catalase leads to oxidative damage directly through \( \text{H}_2\text{O}_2 \) and indirectly through inhibition of SOD resulting in increased level of superoxide radicals. Pelargonidin treatment can efficiently restore the depleted status of the antioxidant enzymes SOD and catalase, thereby indicating antioxidative function of pelargonidin. The lowering of blood glucose in treated diabetic rats (DP) may reduce the potential glycation of these antioxidative enzymes and thereby increasing their activity.

Lipid peroxidation is a free radical-mediated propagation of oxidative insult to polyunsaturated fatty acids, and is increased in diabetic rats as evident from elevation of lipid peroxidation product MDA in serum, which are in agreement with previous studies on hyperglycemia-induced oxidative stress in STZ-induced diabetogenesis (Montilla et al., 2005, Godin et al., 1988). Pelargonidin treatment in diabetic rats effectively reverts serum MDA to normal level, suggesting antioxidative function of the flavonoid.

The Amadori product or fructosamine is the first stable product of protein modification by glucose, and its level in serum increases in diabetes (Cohen & Wu, 1994). Fructosamines can undergo oxidative cleavage resulting in the formation of advanced glycation end products (AGEs), which are thought to contribute to long-term complications of diabetes (Giardino et al., 1994). Serum fructosamine, the early glycation end product is found to be increased in diabetic rats (D). The increased level has also been reported in Montilla et al., 1988. Pelargonidin treatment in diabetic rats is highly effective in normalizing fructosamine content of serum (Table 2). The prevention of formation of fructosamines or removal of these modified proteins is an efficient way to interrupt the glycation cascade and prevent the potential pathological consequences of glycation in diabetes. Studies with anthocyanin rich source black chokeberry as well as anthocyanin dyes from grapes suggest that daily dietary supplementation of these extracts causes marked decrease oxidative stress and prevent free radical generation in diabetes (Jurgoński et al., 2008, Jankowski et al., 2000). Other flavonoids catechin also increases total plasma antioxidant activity (Yokozawa et al., 2002, Skrzydlewska et al., 2002). It is also effective in enhancing SOD activity in serum, catalase activity in aoata (Skrzydlewska et al., 2002, Negishi et al., 2004) and prevents against oxidative stress, malondialdehyde concentration (Yokozawa et al., 2002, Yokozawa 1999). Quercetin alleviates oxidative stress in STZ-induced diabetic rats by increasing free radical...
scavenging enzyme levels (Coskun et al., 2005). Rutin and G-rutin have antidiabetic potential as dietary supplementation via reducing TBA reactive substances, protein carbonyl content and AGES formation in diabetic rats. (Prince and Kamalakkannan 2006, Nagasawa et al., 2003).

Fig 19a, b demonstrates the profiles of glycated hemoglobin and free iron release from hemoglobin in different groups of rats. The enhanced glycated hemoglobin levels in D group of rats compared to that in C group of rats, are in agreement with findings of Vasques et al., 2003. Pelargonidin treatment normalizes enhanced level of GHb in diabetic rats (Fig. 23 a).

The enhanced free iron level in hemoglobin as well as deposition of iron in liver tissues have been demonstrated in diabetes rats. Earlier studies by our group suggested that HbA1c might be a source of free radicals and oxidative stress in diabetes (Kar & Chakraborti, 1999; Kar & Chakraborti, 2001; Sen et al., 2005). Because of hazardous nature of free iron, elaborate defense mechanisms have been developed so that more than 99% of the iron in the body is sequestered in specialized cells (hemoglobin in red cells), transport or storage proteins (transferrin or ferritin), in which iron is not redox active (Goswami et al., 2002; Crichton & Charloteaux-Wauters, 1987). Ferrous iron with six coordination states is bound in heme pocket of hemoglobin. However, under specific circumstances iron can be liberated from the heme and ligated to another moiety, probably distal histidine (E7) near heme pocket. This iron, termed 'mobile reactive iron' (Panter, 1994) can catalyze Haber-Weiss reaction producing free radicals, particularly hydroxyl (OH) radicals, which may damage different cell constituents (Gutteridge, 1986). Free reactive iron level in purified Hb, isolated from blood of diabetic patients, is proportionately increased with increased level of blood glucose (Kar and Chakraborti, 1999). In another report, heme-globin linkage has shown to be weaker in HbA1c than in HbA0, and heme is released more easily from the glycated protein (Kar et al., 2006). Cussimamio et al., (2003) have demonstrated that hemoglobin and myoglobin are extremely susceptible to damage by glucose in vitro through a process that leads to complete destruction of heme group. The iron released from heme destruction enhances AGE formation and level of free radicals, which have been suggested to be involved in pathological complications of diabetes mellitus. Iron released from Hb of diabetes rats
Action of Pelargonidin in STZ-induced diabetic rats

(Fig. 19b) may be deposited in liver tissues (Fig. 20). Farhangkhoee et al., (2003) have reported increased iron deposition in cardiomyocytes of diabetic rats due to increased heme oxygenase (HO) activity. HO induction in diabetes may directly lead to iron release from heme proteins with formation of H$_2$O$_2$ as a byproduct. According to Takasu et al., (1991), H$_2$O$_2$ generation is stimulated in STZ-induced diabetic rats. H$_2$O$_2$ induces iron release from Hb (Halliwell & Gutteridge, 1990; Gutteridge, 1986). According to Lau et al., (1994) the iron content of the kidney tissues significantly increases in STZ-induced diabetic Sprague Dawley rats. Similar changes in tissue metal accumulation are seen in the kidneys of Long-Evans rats exposed to STZ for 21 days (Johnson & Evans 1984). In previous study by our group H$_2$O$_2$ has been shown to promote more iron release from HbA1c than from HbA$_0$ (Kar & Chakraborti, 2001). In vitro-glycated myoglobin is also found to be more susceptible to H$_2$O$_2$-induced iron release than the non-glycated protein (Roy et al., 2004). Thus several factors namely, extent of glycation in Hb, weaker heme-globin linkage in the heme protein, release of heme, induction of HO activity and increased formation of H$_2$O$_2$ may be responsible for enhanced iron level in diabetic condition. Pelargonidin treatment in diabetic rats normalizes the elevated iron level in Hb (Fig. 19b) as well as demonstrates reversal effect on iron deposition in liver (Fig. 20). These effects are not due to iron chelation by pelargonidin. Unlike many flavonoids, it is not an iron chelator due to absence of an ortho 3’, 4’-dihydroxy substitutions in B ring of the compound (Fig. 11). The possession of these substituents is thought to be important for chelation of divalent (Brown et al., 1998, van Acker et al., 1996) and trivalent cations (George et al., 1999). Effect of pelargonidin in reversing iron level in Hb as well as iron deposition in liver of diabetic rats may be associated with controlling glucose and GHB levels by the antioxidant or other activity of the flavonoid. Hb is known to be a source of OH$^-$ radicals through iron-dependent Fenton reaction Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + OH$^-$ + OH$^-$ (Halliwell & Gutteridge, 1990; Kar and Chakraborti, 2001; Sen et al., 2005; Gutteridge, 1986), Hb-mediated different oxidative reactions have been studied to understand the oxidative stress in experimental diabetes as well as to find the role of pelargonidin in this respect. In these oxidative reactions H$_2$O$_2$ has not been included to rule out the involvement of Fenton reaction. Hb from diabetic rats degrades arachidonic acid and DNA (plasmid) more efficiently than that from normal rats (Figs. 21 and 22). Increased
level of free iron present in Hb of diabetic rats having higher level of GHb may be associated with enhanced lipid or DNA break down (lane 3) by this Hb, suggesting pronounced iron-mediated oxidative stress in diabetic condition. Significant arrest of DNA breakdown by DFO and mannitol (lane 4 and lane 5) further indicates that this is an iron-mediated, hydroxyl radical-catalyzed reaction. DNA damage involving transition metal ion-induced reaction modifies the chemical structure of DNA subunits (nucleobases and deoxyribose moieties), and marks the onset of subsequent biochemical and biological effects in OH'-generating systems. Our findings on enhanced lipid and DNA breakdown by Hb having higher level of free iron from diabetic rats in absence of exogenously added H$_2$O$_2$ are quite significant. According to Flemmig and Arnhold (2007), FeCl$_2$ is capable of strand breaks in the pBR322 (plasmid) DNA in absence of H$_2$O$_2$. Auto-oxidation of Fe$^{2+}$ forming a yet unknown reactive species has been suggested to be involved in this process. Fenton reaction is therefore, not essential for iron-mediated oxidative damages. Glycated proteins are known to auto-oxidize in the presence and sometimes absence (Mossine et al., 1999; Jiang et al., 1990) of redox metal ions, thus generating different reactive oxygen species namely, H$_2$O$_2$, O$_2^*$ and OH' radicals (Hunt et al., 1988), and may be responsible for the observed effects of enhanced lipid or DNA breakdown by Hb of diabetic rats even in absence of exogenously added H$_2$O$_2$.

Pelargonidin treatment in diabetic rats (DP) appears to be very effective in reversing arachidonic acid degradation (Fig.21) and DNA breakdown (Fig.22), which are quite comparable with reactions by Hb of normal rats (C). The findings may be related to the lower levels of GHb and free iron in these Hb samples. Although the sequence of events for the observed effects of pelargonidin in diabetes is still not clear, the flavonoid is quite effective in combating hyperglycemia as well as oxidative stress including Hb-induced iron-mediated oxidative reactions. Cyanidin and cyanidin 2-O-βD-glucoside have been reported to act as a DNA cleavage protector along with their antioxidative function (Acquaviva et al., 2003).

Metal-catalyzed oxidation may cause covalent modification of proteins by introducing carbonyl groups into amino acid residues of proteins (Stadtman et al., 1989). Such oxidative modification is an index of oxidative stress and may be significant in several physiological and pathological processes (Oliver et al., 1987). In Fig. 23 enhanced level
Action of Pelargonidin in STZ-induced diabetic rats

of hemoglobin mediated carbonyl formation was observed in diabetic groups (D). This finding is consistent with the previous report on enhancement of protein carbonyl content in STZ-induced diabetes (Nagasawa et al., 2003). Pelargonidin appears to be effective in preventing such modification of Hb and associated oxidative damages in diabetic rats (Fig. 23).

The increased serum total cholesterol and triglyceride concentrations in diabetic rats (Figs. 24a and b) are well in agreement with several reports on STZ-induced diabetogenesis (Vessal et al., 2003, Willsky et al., 2006, Vasques et al., 2003). Alteration of lipid profile in the serum of diabetic patients was also appeared to be a significant factor in the development of premature atherosclerosis (Orchard, 1990. Bierman,v1992). Inadequate utilization of glucose in diabetic models or in diabetic patients stimulates the mobilization of lipid stores in the organisms, thus leading to increase the plasma triglyceride and total cholesterol concentrations (Vasques et al., 2003). Treatment with pelargonidin results in normalization of total cholesterol and triglyceride concentrations (Figs. 24a and b). Reports by other groups suggest lipid-lowering activity of dietary cyanidin 3-O-β-D-glucoside in high fat feed diabetic mice (Tusuda et al., 2003) and anthocyanins from Cornelian Cherry in high fat feed diabetic C57BL/6mice (Jayaprakasam et al., 2005).

RBC membrane fluidity study (Table 3) indicates inhibition of membrane fluidity in D group of rats in comparison to C group of rat. According to Watala and Winocaur, (1992) oxidative damage and non-enzymatic glycation in altering the membrane fluidity of the RBCs under diabetic condition. Increase in surface polarity and stronger ionic interactions or by steric effects via distortion of the surface caused by increased glycation might be responsible for decrease in RBC fluidity. In addition, peroxidation of membrane bound unsaturated fatty acids (PUFA) containing phospholipids can also result in reduction of RBC fluidity (Watala & Winocaur, 1992). Further, cross-linking of proteins by the end products of lipid peroxidation, MDA and 4-hydroxy-nonenal (HNE) resulting in lipoxidation products can influence membrane fluidity (Buko et al., 1996). Pelargonidin treatment in diabetes rats normalizes altered fluidity of RBC membrane.

Table 4 represents serum ALP activity in control, diabetic and pelargonidin treated diabetic rats DP. Diabetes is characterized by high circulating level of alkaline
phosphatase (Hough et al., 1981). Some studies have also attributed to the rise of serum ALP level to the toxic effects of STZ on liver (Voss et al., 1988). Serum ALP activity is also found to increase in alloxan-induced diabetic rats (Awadallah et al., 1977). Treatment with pelargonidin results in significant improvement of ALP activity in serum (Table 4). Mori et al., (2003) have shown that treatment with insulin causes lowering of ALP activity in STZ-treated diabetic rats. The enhanced level of serum insulin in pelargonidin -treated diabetic rats may be responsible for lowering of serum ALP activity and better liver functioning. Rise in serum ALP activity in the untreated diabetic rats (D) (Table 4) is in accordance with abnormalities in liver histology (Fig 27 b). Previous study indicates that treatment of STZ-induced diabetic rats with insulin results in significant decline of serum ALP activity along with normalization of the liver histological abnormalities (Fig 27 c) (Mori et al., 2003). Thus increase of insulin level in serum of diabetic rats treated with pelargonidin may be a contributory factor for normalization of the histological abnormalities and its function was induced by STZ.

Elevated level of DNA degradation is founding diabetic rats as found by increased comet tail formation (Fig 25 b, e) and comet scoring table (Fig 25 f). Previous report suggests DNA breakdown in STZ-induced diabetic rats due to genotoxic nature of the drug (Bolzan & Binachi, 2002). Prevention of lymphocyte DNA breakdown in diabetic rats (DP) by pelargonidin treatment may be due to its antioxidative function and hypoglycemic properties. Different anthocyanins and anthocyanidins from different origins have been reported to possess protective effect upon DNA damages (Duthie et al., 2005, Abraham et al., 2007, Lazze et al., 2003).

Histological analysis of the pancreatic sections reveals β-cell loss and atrophy of the islets in diabetic rats as shown in Figs. 26 b. Pelargonidin treatment causes significant enhancement of β-cell number and partial reversal to normal cellular morphology (Fig. 26c). Many polyphenolic compounds and flavonoids of plant origin acting as antioxidants and free radical scavengers have been reported to prevent autopoly (ADP-ribosyl)-ation of PARP, thereby stabilizing Reg gene transcriptional complex and resulting in the regeneration of β-cell and protection of pancreatic islets against STZ (Szkudelski, T. 2001).
Action of Pelargonidin in STZ-induced diabetic rats

Histological study of kidney sections reveal a significant enhancement of glomerular basement membrane thickening and diffused mesangium in diabetic control rats (D) (Figs. 28 a, b). In diabetes mellitus nephropathy is a common feature that occurs due to accumulation of proteins in the mesangial basal membrane and free radicals derived from self-oxidation of glucose or from the formation of advanced glycation end products (AGEs) (Trachtman et al. 1995). Lesser thickening of glomerular basal membrane with normal cytoskeletal architecture of the kidney sections of diabetic rats treated with pelargonidin (Fig 28 c) suggests that the flavonoid may have some role in prevention of diabetic nephropathy, probably through antihyperglycemic and antioxidative functions.
Action of liposome encoded pelargonidin treatment in STZ-induced diabetic rats
Introduction:

Liposomes are mono or bilayer phospholipid vesicles, non-toxic, non-immunogenic, non-thrombogenic, biodegradable in nature and used as potent drug carriers. The importance of liposome therapy is that they can solubilize water-insoluble drugs, provide site avoidance and reduced toxicity through minimized uptake in sensitive tissues, and facilitate sustained release thereby increases the biostability of the drug. Anthocyanidins are known to be stable under acidic conditions, but rapidly broken down under neutral conditions (Brouillard, 1988) such as those encountered in vivo. Anthocyanidins degrade to form different phenolic acids. p-Hydroxybenzoic acid has been reported to be an important metabolite of pelargonidin (Mohsen et al., 2006). The main problem encountered during pelargonidin treatment was low stability and quick biodegradation of the drug. So the administration of liposome-encapsulated pelargonidin in experimental diabetic rats becomes important in order to minimize biodegradation, if any and for sustained drug release within the body for a prolonged duration.

The reports available so far regarding liposome based treatment of flavonoids and polyphenolic compounds mostly involve in cancer therapy. Efficient suppression of solid tumors occurred in murine models by liposomal quercetin (Yuan et al., 2006). Pegylated nanoliposomal quercetin inhibits formation of malignant ascites of hepatocellular carcinoma (Yuan et al., 2006). Liposome encapsulation of tea catechins (EGCG and its derivative, (+)-catechin) accumulates and thereby resulting protective effect in basal cell carcinomas (Fang et al., 2006). Protective action of liposome-encapsulated quercetin results due to its effect on DNA synthesis and lactate production and cyclic adenosine 3':5'-monophosphate level in Ehrlich ascites tumor cells (Podhajcer et al., 1980). It has also been reported that liposome-mediated administration of different polyphenolic compounds derived from plant origin such as bacopasaponin (Sinha et al., 2002) and quercetin (Mandal and Das 2005) cause prevention of leshmaniasis and CCL4-induced hepatotoxicity in rat model and manocylated quercetin can combat age-related ischemia-reperfusion induced oxidative damage in rat brain (Sarkar and Das, 2006). Targeting of hepatocytes by liposomal-insulin preparation for treatment of diabetes mellitus is used
Action of liposome encoded pelargonidin treatment in STZ-induced diabetic rats

considerably, nowadays. Still now there is no report available for liposome based plant flavonoid treatment in diabetes.

In this chapter pelargonidin encoded liposome treatment was studied in STZ-diabetic animal and the efficiency of the treatment was compared with that of free pelargonidin.

Results:

Glycemia status, hemoglobin glycation and insulin level in diabetes: Effect of Liposome encapsulated pelargonidin

Liposome-encapsulated pelargonidin or free pelargonidin was injected to diabetic rats. Glucose concentration in blood was monitored 2 days after administration of each dose. Liposome-encoded pelargonidin treatment was found to be effective in normalizing blood glucose level in diabetic treated animals (DP₁ group), after introduction of the 5th dose as shown in Fig. 29 and maintained at that level during the experimental period. This treatment also efficiently improved glycated hemoglobin level in treated groups measured after 2 weeks of last drug treatment (Table-5).

![Graph](image)

Fig 29. Time profile of glucose concentration (mg/dl) in blood serum of different group of rats after free and liposome-encapsulated pelargonidin treatment. Each dose was given at 3 days interval and blood glucose was monitored 2 days after each injection. Results are mean ± SE for 8 rats (n = 8).
Blood glucose level appeared normal in FD group of rats after 3rd dose of free pelargonidin administration, whereas identical effect was observed in case of liposomal drug treated DP group of rats after 5th dose of injection as shown in Fig. 29. It apparently suggests that free drug treatment was more effective in normalizing blood glucose level in comparison with liposome-encoded drug treatment. However, blood glycohemoglobin level, the index of diabeticity, was better controlled by liposome-encoded drug treatment than by free drug treatment as shown in Table 5. Glycohemoglobin levels measured after 2 weeks of last drug treatment found to be 7.39 ± 0.45% and 4.88 ± 0.59 % in case of free pelargonidin treated diabetic rats (FD) and liposome encoded diabetic rats (DP_l) respectively.

Pelargonidin treatment (free or liposome-encoded in diabetic rats) showed normal like IPGTT curve (Fig. 30). The experiments were done 2 weeks after 5th dose of pelargonidin treatment. Diabetic rats (group D) showed elevated glucose level at each point of the experiment.

Fig 30. Glucose tolerance curves of normoglycemic (C), diabetic (D) and treated (DP_l, FD) rats. Experiments were done after two weeks of pelargonidin encoded liposome treatment. Results are mean ± SE of six experiments in each case (n = 6).
Action of liposome encoded pelargonidin treatment in STZ-induced diabetic rats

Even at the end of the experiment, glucose level in D group of rats was much higher than normal. In case of free drug treated FD group of rats, blood glucose level increased significantly after 30 min of glucose injection and it took 120 min to come to the normal level. In case of liposome-encapsulated drug treatment (DP_L group), blood glucose level was increased to much less extent after 30 min of glucose administration and became almost normal after 60 min, which was more or less similar with the nature of curves in control rats (C). Liposome-encoded drug treatment might therefore be more effective than free drug treatment in diabetic rats.

Serum insulin levels in diabetic rats (D) were drastically reduced by STZ-treatment when the levels were measured after 2 weeks of last liposome treatment. The levels were good to increase significantly in DP_L group of rats. However there has been very insignificant increase in insulin level in FD group of rats.
Table 5: Effect of pelargonidin encoded liposome on blood glucose, insulin level, glycohemoglobin content and lipid profiles in different group of rats

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<thead>
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<th>C</th>
<th>D</th>
<th>DP_L</th>
<th>FD</th>
<th>p values</th>
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<tr>
<td>Blood Glucose (mg/dl)</td>
<td>111.5±2.9</td>
<td>309.9±6.3</td>
<td>103.2±3.3</td>
<td>108.4±9.4</td>
<td>C vs D; p≤0.01</td>
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<td>D vs DP_L; p≤0.01</td>
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<td>n = 8</td>
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<td>Serum Insulin (mU/l)</td>
<td>44.47±0.12</td>
<td>2.02±0.08</td>
<td>23.21±0.37</td>
<td>3.8±0.62</td>
<td>C vs D; p≤0.01</td>
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<td>n = 8</td>
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<td>GlycoHb (%)</td>
<td>4.49±0.56</td>
<td>10.52±1.78</td>
<td>4.88±0.59</td>
<td>7.39±0.45</td>
<td>C vs D; p≤0.01</td>
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<td>D vs DP_L; p≤0.01</td>
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<td>n = 8</td>
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<tr>
<td>Serum Cholesterol (mg/dl)</td>
<td>87.57±9.0</td>
<td>204.26±1.95</td>
<td>83.67±3.09</td>
<td>130.06±1.38</td>
<td>C vs D; p≤0.01</td>
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<td>D vs DP_L; p≤0.01</td>
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<td>n = 8</td>
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<tr>
<td>Serum Triglyceride (mg/dl)</td>
<td>58.05±2.61</td>
<td>197.44±4.14</td>
<td>65.92±1.81</td>
<td>115.023±2.22</td>
<td>C vs D; p≤0.01</td>
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<td>D vs DP_L; p≤0.01</td>
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**Serum triglyceride and total cholesterol levels**

Serum triglyceride and serum cholesterol levels were studied in different groups of rats (Table 5). These levels were significantly higher in group D rats compared to that of control groups. After 7 days of liposome-encoded drug treatment, increased levels of both serum triglyceride and serum cholesterol level in diabetic rats treated with liposome...
encoded pelargonidin (DP_l) were normalized. Free pelargonidin treatment reduced the levels significantly. However, it was not as effective as liposome-encoded formulation.

Iron release from the heme protein and Hb-induced oxidative stress in diabetes: reversal effect of liposomal pelargonidin

Free iron level in hemoglobin samples from different groups of rats was estimated after 2 weeks of 5th dose of drug treatment. Diabetic rats (D) exhibited significantly increased levels of iron in hemoglobin in comparison with that of normal rats (C) (Table 6). Both liposome-encapsulated pelargonidin treatment as well as free pelargonidin treatment was effective in normalizing elevated free iron levels in treated diabetic rats (DP_l and FD). The elevated free iron in diabetic condition may be a source of free radicals and oxidative stress.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>DP_l</th>
<th>FD</th>
<th>p values</th>
</tr>
</thead>
</table>
| Carbonyl Content in Hb (µM/mg of Hb) | 25.06±0.7 | 79.37±3.4 | 23.26±1.1 | 45.86±4.1 | C vs D; p<0.01
|                      |     |     |      |      | D vs DP_l; p<0.01 n = 8     |
| Free Iron level in Hb (µg/g of Hb)   | 59.56±3.56 | 228.8±1.97 | 56.09±2.11 | 62.54±3.29 | C vs D; p<0.01
|                      |     |     |      |      | D vs DP_l; p<0.01 n = 8     |

Experiments were done to understand the iron-mediated free radical reaction in diabetic condition to find the role of liposome-encapsulated pelargonidin in combating them. Hemoglobin-mediated deoxyribose degradation was studied. Hemoglobin from diabetic
Action of liposome encoded pelargonidin treatment in STZ-induced diabetic rats

Animals (group D) caused elevated deoxyribose degradation compared to that of normal rats of group C as in Fig. 31a. Free as well as liposome-encapsulated pelargonidin effective in combating this stress associated complication in DP_L and FD group of rats. However, liposome-encapsulated pelargonidin treatment was found to treatments were found to be more protective compared to free drug treated group.

In hemoglobin-mediated plasmid DNA breakdown study, hemoglobin of liposome-encapsulated pelargonidin treated rats was found to inhibit hemoglobin-mediated plasmid DNA breakdown (lane 4) just 2 weeks after last treatment by reduced formation of form II DNA (nicked DNA), compared to diabetic hemoglobin-mediated breakdown (lane 3) as shown in Fig. 31b.

Carbonyl formation in Hb: prevention by liposomal pelargonidin

Experiments were done to detect the extent of carbonyl formation in Hb samples isolated from control, diabetic and treated groups of rats. As presented in Table-6, carbonyl contents in Hb samples from diabetic rats (D) were significantly higher in comparison with those from normal rats (C). Pelargonidin treatment in liposome encapsulated form efficiently improved this stress condition by decreasing carbonyl content to control level as found in DP_L and groups of rats. However, hemoglobin samples from free drug-treated animals (FD) exhibited significantly lower carbonyl contents than diabetic group of rats, but still higher than normal group (C). The results obtained with Hb after two weeks of treatment have been presented.
Fig 31. a: Hb-mediated deoxyribose degradation. Experiments were done two weeks after pelargonidin encoded liposome treatment. Bars represent mean SE of eight experiments in each case (n = 8). *p* < 0.05 for C vs. D and for D vs. DP.

b: Hb-mediated DNA (plasmid) breakdown studied two weeks after pelargonidin encoded liposome treatment. Hb samples were isolated from different groups of rats (C, D, DP, FD). Different forms of DNA were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in three additional experiments.
Oxidative stress in diabetes: protective role of liposomal pelargonidin

Liposome encoded pelargonidin also have protective effect in improving antioxidant enzymes status like serum SOD, serum catalase levels antioxidant markers like serum MDA and fructosamine levels in treated diabetic animals as presented in Table-7. Antioxidant enzymes and serum MDA levels were monitored 2 weeks after last dose of drug treatment while serum fructosamine level were estimated 5th weeks after last dose. Free drug treatment (FD) was also found to be effective in controlling these markers but liposome encoded treatment was found to be much more protective.
Table-7: Effect of pelargonidin encoded liposome on antioxidant scavenging enzymes (serum SOD and catalase) and oxidative markers (serum MDA and fructosamine levels) in different groups of rats.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>DP_L</th>
<th>FD</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum SOD (U/mg of protein)</td>
<td>0.410 ± 0.02</td>
<td>0.296 ± 0.03</td>
<td>0.437 ± 0.03</td>
<td>0.316 ± 0.02</td>
<td>C vs D; p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D vs DP_L; p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n = 8</td>
</tr>
<tr>
<td>Serum Catalase (U/mg of protein)</td>
<td>0.238 ± 0.03</td>
<td>0.053 ± 0.01</td>
<td>0.239 ± 0.05</td>
<td>0.097 ± 0.07</td>
<td>C vs D; p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D vs DP_L; p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n = 8</td>
</tr>
<tr>
<td>Serum MDA (Arbitrary fluorescence unit)</td>
<td>79.3 ± 5.3</td>
<td>167.6 ± 9.5</td>
<td>87.7 ± 7.4</td>
<td>124.6 ± 7.3</td>
<td>C vs D; p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D vs DP_L; p&lt;0.05</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n = 8</td>
</tr>
<tr>
<td>Serum Fructosamine (mmol/mg protein)</td>
<td>207 ± 11.6</td>
<td>337 ± 21.7</td>
<td>212 ± 13.1</td>
<td>273 ± 14.2</td>
<td>C vs D; p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D vs DP_L; p&lt;0.05</td>
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<td></td>
<td></td>
<td></td>
<td>n = 8</td>
</tr>
</tbody>
</table>


**Discussion:**

In the previous chapter we have discussed about the blood glucose lowering effect of pelargonidin treatment in STZ-induced diabetic rats. The effect of liposome encapsulated pelargonidin on fasting blood glucose further supports the antidiabetic potential of pelargonidin. Fig. 29 represents the effect of liposomal-pelargonidin monitored at 2 day after each dose and different time period after injection on blood glucose level on different groups of rats namely C, D, DP<sub>L</sub>, and FD. Liposome-encoded pelargonidin treatment was found effective in reducing blood glucose level in diabetic treated animals (DP<sub>L</sub> group), compared to diabetic untreated rats (D group) after the introduction of 5<sup>th</sup> dose in Fig. 29 and maintained at that level during the experimental period and blood glucose level 2 week after last injection was presented in Table-5. The results indicate the effectiveness and safe alternative use of liposome-pelargonidin in treatment of diabetes.

The antidiabetic potential of liposomal pelargonidin was further strengthened by the result of glycohemoglobin concentration of different groups of rats as presented in Table-5. Liposome encapsulated pelargonidin treatment in diabetic rats (DP<sub>L</sub>) resulted normalization of the glycated hemoglobin level compared to that of diabetic rats (D). Though, free drug treated group (FD) also showed partial reduction of glycated hemoglobin level suggesting protective effect of pelargonidin treatment even in low dose (compared to that mentioned in previous chapter) but it is not fully protective as compared to that of liposomal formulation.

IPGTT curve pattern was almost normalized in liposome encapsulated pelargonidin treatment in diabetic rats (DP<sub>L</sub>) (Fig. 30) indicating the normalized glucose tolerance nature. This data was further supported by significantly enhanced level of serum insulin in DP<sub>L</sub> group of rats compared to that in D or FD group of rats as presented in Table-5. Liposomal-pelargonidin treatment was effective in ameliorating hyperlipidemia in diabetic treated rats (DP<sub>L</sub>) evidenced from significant reduction in serum triglyceride and total cholesterol levels compared to those in diabetic rats, as represented in Table-5.

To elucidate the role of liposome encapsulated pelargonidin treatment upon the interplay between glycated hemoglobin, hemoglobin mediated free iron release (Table-6) and
subsequent oxidative stress reactions like hemoglobin-catalysed deoxyribose degradation (Fig. 31a), Plasmid DNA degradation (Fig. 31b) and carbonyl formation (Table-6) by hemoglobin studies were performed 2 week after 5th dose of liposomal pelargonidin treatment. Liposomal drug treatment was found to be more effective in normalizing free iron release and iron mediated oxidative stress conditions in diabetic treated group (DP₁) compared to free drug treated diabetic rats (FD). These results revealed the ameliorative effect of liposomal-pelargonidin in iron-catalyzed free radical-mediated reaction. Liposomal-pelargonidin formulation was also protective in normalizing serum antioxidant enzymes (SOD, Catalase) and oxidative stress marker serum MDA and index of early glycation of serum proteins like serum fructosamine to their respective levels compared to that of diabetic group (D) as presented in Table-7.

The present findings, thus, explain the antidiabetic mode of action of liposomal-pelargonidin and its role in ameliorating the hemoglobin glycation-induced oxidative stress associated complications in experimental diabetes. The efficacy of the liposomal formulation is much better compared to that of free drug. Diminution of free iron release from hemoglobin and iron-catalyzed free radical-mediated in diabetic rats treated with liposomal-pelargonidin due to its antihyperglycemic and/or free radical scavenging property thus suggesting its strong potential as therapeutic agent and also in the better understanding and management of diabetes mellitus.
Action of PLGA-nanoparticle encoded pelargonidin treatment in STZ-induced diabetic rats
Nanoparticle mediated drug delivery is an important approaches in modern drug delivery technique. Biodegradable particles (0.1–1000μm) prepared from poly (lactide-co-glycolide) polymers (PLGA) have generated considerable interest in recent years for their use as a delivery vehicle for various pharmaceutical agents. PLGA is by far the most common biodegradable polymer used for the controlled delivery of drugs due to its early use and approval as a compatible biomaterial in humans (Perrin & English, 1997). By varying the molecular weight and lactide/glycolide ratio, the degradation time of the PLGA, and therefore the release kinetics of the active agent, can be controlled (Lewis, D.H. 1990). The size of the particle plays a major role in the release of the active agent. PLGA-microparticles (diameters >1μm) and nanoparticles (diameters <1μm) have been investigated for the controlled release of hormones, proteins and anti-tumor compounds in the treatment of growth deficiencies, diabetes, and various types of cancer (Ciftci et al., 1997; Cleland et al., 1997; Itoi et al., 1996; Kyo et al., 1995; Matsumoto et al., 1997; Menei et al., 1996; Schally & Comaru-Schally, 1997; Wada et al., 1988). In this chapter pelargonidin encoded PLGA nanoparticle was first characterized. The therapeutic effectiveness in STZ-diabetic rat was studied in comparison with that of free pelargonidin.
PLGA-Nanoparticle encoded pelargonidin characterization:

*Results:*

- *Morphology and size measurement by Transmission electron microscopy (TEM):*

To determine the shape and morphology of the pelargonidin encoded PLGA-Nanoparticle TEM was used. The TEM images (Fig. 32 a, b, c) of different magnification showed that the particles are spherical in nature. The picture also showed no visible aggregates or adhesion. The mean sizes obtained by measuring the TEM images are in Table-8.

- *Morphology by Scanning electron microscopy (SEM):*

To determine the shape and morphology of the pelargonidin encoded PLGA-Nanoparticle SEM was also used. For the visualization of the nanoparticle in SEM gold sputter coating 5-6 nm were done. The SEM image (Fig. 32 d) also revealed spherical nature of the nanoparticles.

- *Morphology and size measurement by Atomic force microscopy (AFM):*

Determination of the shape and morphology of the pelargonidin encoded PLGA-Nanoparticle was used also Atomic Force Microscopy (AFM). The AFM images (Fig. 33 a, b, c, d) of different magnification further showed that the particles were spherical in was with smooth surface morphology. The sizes of several nanoparticles were measured by AFM are shown in Fig 33 e, f. AFM 3-D reconstructed images are shown in Fig. 33 c, d. The mean diameter of the particles is presented in in Table-8.
Fig 32. a, b, c TEM images of Pelargonidin encoded PLGA-Nanoparticle; bar corresponded to a: 500 nm, b: 200 nm, c: 100 nm. d: SEM images of Pelargonidin encoded PLGA-Nanoparticle (mag: bar corresponded 30,000X) bar 1 μM.
<table>
<thead>
<tr>
<th>Data type</th>
<th>Height</th>
<th>Z range 50.00 nm</th>
<th>Amplitude</th>
<th>Z range 0.5000 V</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tbody>
</table>

**Fig 33.a, b:** Surface topology and c, d: corresponding 3-D images and e, f: radius measurement from section analysis of pelargonidin encoded PLGA-Nanoparticles obtained by tapping-mode AFM and visualized in amplitude mode.
Action of PLGA-nanoparticle encoded pelargonidin treatment in STZ-induced diabetic rats

- Measurement of Hydrodynamic Radii, Zeta Potential ($\zeta$) and Polydispersity Index (PI) by Dynamic light scattering (DLS):

The DLS measurements of the three independently produced batches done were not appreciably different from each other (Table 8). This accounted for the robustness and reproducibility of the procedure and the ternary system suitability. Representative plots of Hydrodynamic Radii, Zeta Potential ($\zeta$) measurements are shown in Fig. 34 a and b respectively and their data along with Polydispersity Index (PI) are shown in Table 8.

- Thermal denaturation study by Differential scanning calorimetry (DSC):

Thermal denaturation studies by DSC of pelargonidin, pelargonidin encoded PLGA-Nanoparticle and PLGA-Nanoparticle (ghost) were undertaken to find if alteration of thermal stabilities occurred due to formation of pelargonidin encoded PLGA-Nanoparticle. A representative experiment is shown in Fig. 34c. The thermograms (Heat flow vs. temperature) exhibited respective melting temperatures ($T_m$) for pelargonidin, pelargonidin encoded PLGA-Nanoparticle and PLGA-Nanoparticle (ghost) as 216.66 °C, 359.93 °C and 348.41 °C depicting higher thermostable nature of the pelargonidin encoded PLGA-Nanoparticle.
Fig 34 a: Particle size distribution and b: zeta potential measurement of pelargonidin encoded PLGA-Nanoparticles by DLS. C: Differential scanning calorimeter thermograms (heat flow versus temperature) with respective Tm values of pelargonidin, pelargonidin encoded PLGA-Nanoparticle and PLGA-Nanoparticle (ghost).
Determination of Entrapment Efficiencies:

Spectrophotometric estimation:

Entrapment efficiency of the, pelargonidin encoded PLGA-Nanoparticle was measured by UV-vis spectrophotometer. The UV-vis spectrum of the pelargonidin, pelargonidin encoded PLGA-Nanoparticle and PLGA-Nanoparticle (ghost) was measured by UV-VIS spectrophotometer is presented in Fig. 35 and the entrapment efficiency of the, pelargonidin encoded PLGA-Nanoparticle is presented in Table 8.

HPLC:

Entrapment efficiency of the, pelargonidin encoded PLGA-Nanoparticle was measured by reversed phase-HPLC. The HPLC chromatogram of PLGA-nanoparticle encoded pelargonidin and free pelargonidin was presented in Fig. 35 b. The retention time (RT) for free pelargonidin was found to be 5.43 min. The entrapment efficiency calculated from HPLC is presented in Table 8.
Fig 35a. The UV-VIS spectrum of the pelargonidin (Pel), pelargonidin encoded PLGA-Nanoparticle (Nano-pel) and PLGA-Nanoparticle (ghost)(Nano-PLGA).b. HPLC Chromatogram of Pelargonidin and PLGA-pelargonidin nanoparticle ($\lambda_{max}$=520nm).
Table-8: Physical Properties of Pelargonidin encoded PLGA-Nanoparticle

<table>
<thead>
<tr>
<th>Parameters Studied</th>
<th>Pelargonidin encoded PLGA-Nanoparticle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm) from TEM</td>
<td>91.47 ± 2.89</td>
</tr>
<tr>
<td>Size (nm) from AFM</td>
<td>92.29 ± 3.04</td>
</tr>
<tr>
<td>Hydrodynamic Radii ((z)) average by DLS (nm)</td>
<td>141.8</td>
</tr>
<tr>
<td>Zeta Potential ((\zeta)) by DLS</td>
<td>+91.1mV</td>
</tr>
<tr>
<td>Polydispersity Index (PI) by DLS</td>
<td>0.148</td>
</tr>
<tr>
<td>Melting Temperatures ((T_m)) by DSC</td>
<td>359.93 °C</td>
</tr>
<tr>
<td>Encapsulation Efficiency (%) (UV-Vis Spectroscopy)</td>
<td>96.34%</td>
</tr>
<tr>
<td>Encapsulation Efficiency (%) (HPLC)</td>
<td>100%</td>
</tr>
</tbody>
</table>
Treatment in diabetic animals:

Glycemia status, hemoglobin glycation and insulin level in diabetes: Effect of Pelargonidin encoded PLGA-Nanoparticle

Three different doses (DP_{N1}=0.3 mg/kg body wt; DP_{N2}=0.6 mg/kg body wt; DP_{N3}=1.5 mg/kg body wt) were used for pelargonidin encoded PLGA-Nanoparticle preparation. All three doses along with free drug dose (FD=0.6 mg/kg body wt) (comparable to the middle dose) were injected the intravenously to diabetic rats.

Fig 36.a: Time profile of glucose concentration (mg/dl) in blood serum of different group of rats after free and pelargonidin encoded PLGA-Nanoparticle treatment. Each dose (applicable in case DP_{N2} and DP_{N1} and in case of FD) was given at every 3 days interval and blood glucose was monitored 2 days after each injection.

b: Plasma concentration of glucose was measured at different time intervals after i.p administration of free pelargonidin and Pelargonidin encoded PLGA-Nanoparticle treatment Bars represent mean ± SE of eight experiments for each group (n = 8).

p values: C vsD; \( p \leq 0.01 \), D vsDP_{N1}; \( p \leq 0.01 \), D vsDP_{N2}; \( p \leq 0.01 \), D vsDP_{N3}; \( p \leq 0.01 \), D vsFD; \( p \leq 0.05 \).

Glucose concentration in blood was monitored at different time period after injection. Nanoparticle-encoded pelargonidin treatment was found effective in reducing blood glucose level in diabetic treated animals DP_{N3} group after 1st dose of injection and DP_{N2}
Action of PLGA-nanoparticle encoded pelargonidin treatment in STZ-induced diabetic rats

group 2nd dose of injection and in case of DPNI group after 3rd dose, compared to diabetic untreated rats (D group) as shown in Fig. 36a. As the free drug dose (FD=0.6mg/kg body wt) is comparable to the medium dose (DPN2=0.6mg/kg body wt) it was also injected twice intravenously to diabetic rats. In case of FD treated group the glucose level did not normalize fully. After normalization of blood glucose the level was maintained at the normal level during the whole experimental period in case of all three nanoparticle treated groups and data up to 3 weeks after treatment (after 1st dose) was presented in Fig. 36b.

Glycohemoglobin content in blood of all groups of rats were monitored after 5th week of the last dose. Diabetic group of rats (D) showed elevated glycohemoglobin content. Glycohemoglobin, the index of diabeticity, was successfully controlled by three different dose of nanoparticle-encoded drug treatment than by free drug treatment as shown in Table-9. In case of FD treated group reduction of glycohemoglobin level occurred compared to diabetic rats (group D) though it was not normalized fully.

All three doses of pelargonidin encoded PLGA-Nanoparticle treatment in diabetic rats showed normal like IPGTT curve just 2 week after treatment (Fig. 37), where as of diabetic rats (group D) showed characteristic elevated glucose level throughout the experiment. In case of free drug treated group (FD), the blood glucose level was not fully normalized as exhibited in the IPGTT curve.

Serum insulin levels in diabetic group of rats (D) were significantly lower in comparison with control group and were found to increase significantly after two weeks of Pelargonidin encoded PLGA-Nanoparticle treatment in all three doses in rats (DPN1, DPN2, DPN3) as shown in Table-9. However insulin level did not improve in free drug treated group (FD).

Serum triglyceride and total cholesterol levels

Effect of nanoparticle encapsulated pelargonidin treatment in serum triglyceride and serum cholesterol levels were studied in different groups of rats (Table-9). Diabetic rats (D) showed elevated levels of serum triglyceride and serum cholesterol. After 7 days of drug treatment in DPN1, DPN2, DPN3 levels of serum triglyceride and serum cholesterol were decreased significantly. The levels became almost normal as that of group C. The results of 2 week after last dose of nanoparticle treatment were presented in Table9.
Pelargonidin in nanoparticle encapsulated form thus posses hypolipidemic action in diabetes.

Fig 37. Glucose tolerance curves of normoglycemic (C), diabetic (D) and treated (DPN1, DPN2, DPN3, FD) rats. Experiments were done after two weeks of Pelargonidin encoded PLGA-Nanoparticle treatment. Results are mean ± SE of six experiments in each case (n = 6).
Table-9: Effect of pelargonidin encoded nanoparticle on blood glucose, glycohemoglobin content, serum insulin level and lipid profiles (Serum triglyceride and total cholesterol, and HDL-cholesterol levels)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>DPN3</th>
<th>DN2</th>
<th>DPN1</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Glucose (mg/dl)</td>
<td>96.97±3.55</td>
<td>266.07±3.9</td>
<td>95.77±0.9</td>
<td>102.4±5.87</td>
<td>103.2±3.3</td>
<td>151.58±8.71</td>
</tr>
<tr>
<td>GHb(%)</td>
<td>4.49±0.56</td>
<td>10.52±1.78</td>
<td>6.69±0.99</td>
<td>7.23±0.46</td>
<td>7.29±0.36</td>
<td>7.97±0.215</td>
</tr>
<tr>
<td>Serum Insulin (mU/l)</td>
<td>44.47±0.012</td>
<td>2.02±0.08</td>
<td>19.41±33</td>
<td>15.76±1.13</td>
<td>14.06±1.58</td>
<td>2.07±0.36</td>
</tr>
<tr>
<td>Serum Cholesterol (mg/dl)</td>
<td>87.57±9.0</td>
<td>204.26±1.95</td>
<td>59.36±1.72</td>
<td>74.48±2.53</td>
<td>98.91±9.61</td>
<td>163.3±3.43</td>
</tr>
<tr>
<td>Serum TG (mg/dl)</td>
<td>58.05±2.61</td>
<td>202.3±4.14</td>
<td>50.82±2.23</td>
<td>82.27±2.5</td>
<td>110.77±2.59</td>
<td>140.4±1.96</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl)</td>
<td>84.05±6.07</td>
<td>23.12±3.64</td>
<td>79.23±5.36</td>
<td>45.94±0.67</td>
<td>33.23±1.886</td>
<td>-</td>
</tr>
</tbody>
</table>

\#1 p values: C vsD; p<0.01, D vsDPN3; p<0.01, D vsDPN2; p<0.01, D vsDPN1; p<0.01, D vsFD; p≤0.05
\#2 p values: C vsD; p<0.01, D vsDPN3; p<0.05, D vsDPN2; p<0.05, D vsDPN1; p<0.05, D vsFD; p<0.05.
\#3 p values: C vsD; p<0.01, D vsDPN3; p<0.01, D vsDPN2; p<0.01, D vsDPN1; p<0.01, D vsFD; p= non significant
\#4 p values: C vsD; p<0.01, D vsDPN3; p<0.01, D vsDPN2; p<0.05, D vsDPN1; p<0.05, D vsFD; p<0.05.
\#5 p values: C vsD; p<0.01, D vsDPN3; p<0.01, D vsDPN2; p<0.01, D vsDPN1; p<0.01, D vsFD; p≤0.05.
\#6 p values: C vsD; p<0.01, D vsDPN3; p<0.01, D vsDPN2; p<0.01, D vsDPN1; p<0.01.
Iron release from the heme protein, carbonyl formation in Hb and Hb-induced oxidative stress in diabetes: reversal effect of pelargonidin encoded PLGA-Nanoparticle

Nanoparticle-encoded pelargonidin treatment was found to be effective in reducing elevated free iron status and carbonyl content of hemoglobin in three different groups of diabetic treated rats compared to the elevated levels in case of diabetic (D group) rats just 2 weeks after treatment (Table 10). Free drug treatment also normalized these parameters but the efficacy of the nanoparticle treatment was higher than the free drug treatment.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>DP_N3</th>
<th>DP_N2</th>
<th>DP_N1</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Free iron level in Hb (µg/g of Hb)</strong>&lt;sup&gt;#1&lt;/sup&gt;</td>
<td>59.56±3.56</td>
<td>228.8±1.97</td>
<td>52.94±3.11</td>
<td>66.04±1.72</td>
<td>73.88±2.48</td>
<td>84.17±3.88</td>
</tr>
<tr>
<td><strong>Carbonyl Content in Hb (µM/mg of Hb)</strong>&lt;sup&gt;#2&lt;/sup&gt;</td>
<td>27.26±2.99</td>
<td>70.19±1.99</td>
<td>18.9±0.89</td>
<td>21.48±0.24</td>
<td>29.75±3.97</td>
<td>39.35±2.82</td>
</tr>
</tbody>
</table>

<sup>#1</sup>p values: C vs D; p≤0.01, D vs DP_N3; p≤0.01, D vs DP_N2≤0.01, D vs DP_N1; p≤0.01, D vs FD; p≤0.01.

<sup>#2</sup>p values: C vs D; p≤0.01, D vs DP_N3; p≤0.01, D vs DP_N2; p≤0.01, D vs DP_N1; p≤0.01, D vs FD; p≤0.05.
**Action of PLGA-nanoparticle encoded pelargonidin treatment in STZ-induced diabetic rats**

In hemoglobin-mediated plasmid DNA breakdown study, hemoglobin of nanoparticle-encapsulated pelargonidin treated rats was found to inhibit hemoglobin-mediated plasmid DNA breakdown (lane 4, 5 and 6) 2 weeks after last dose of nanoparticle treatment as exhibited by reduced formation of form II DNA (nicked DNA), compared to diabetic hemoglobin-mediated breakdown (lane 2) as in Fig. 38 a, b.

_Oxidative stress in diabetes: protective role of Pelargonidin encoded PLGA-Nanoparticle_

Nanoparticle encoded pelargonidin treatment showed protective effects in improving antioxidant enzymes status like serum SOD and catalase levels, and antioxidant marker serum fructosamine in treated diabetic animals as presented in Table-11. Antioxidant enzymes were measured 2 weeks after last dose of nanoparticle treatment while serum fructosamine level was monitored after 5 week. Treatment with different doses was found to be effective in normalizing the parameters. Free drug treatment was also effective to a certain extent in controlling these markers but its efficiency was lower compared to that of nanoparticle treatment.
Fig 38. Hb-mediated DNA (plasmid) breakdown studied after two weeks of pelargonidin treatment. Hb samples were isolated from different groups of rats: a: (C, D, DP_N1, DP_N2, DP_N3), b: (C, D, DP_N2, FD). Different forms of DNA were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in three additional experiments.
Table-11: Effect of pelargonidin encoded PLGA-Nanoparticle treatment on scavenging enzymes (serum SOD and catalase) and on antioxidative marker (serum fructosamine levels):

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>D</th>
<th>DP₁</th>
<th>DP₂</th>
<th>DP₃</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum SOD</td>
<td>0.400±</td>
<td>0.290±</td>
<td>0.648±0.0</td>
<td>0.461±0.03</td>
<td>0.329±0.04</td>
<td>0.297±0.07</td>
</tr>
<tr>
<td>(U/mg of protein)</td>
<td>0.02</td>
<td>0.03</td>
<td>1</td>
<td>4</td>
<td>0.185±0.09</td>
<td></td>
</tr>
<tr>
<td>Serum Catalase</td>
<td>0.238±</td>
<td>0.249±</td>
<td>0.236±</td>
<td>0.228±</td>
<td>0.219±</td>
<td>-</td>
</tr>
<tr>
<td>(U/mg of protein)</td>
<td>0.03</td>
<td>0.01</td>
<td>0.07</td>
<td>0.06</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Serum Fructosamine</td>
<td>207±</td>
<td>337±</td>
<td>195.7±</td>
<td>203±</td>
<td>219.7±</td>
<td>-</td>
</tr>
<tr>
<td>(μmol/mg protein)</td>
<td>11.6</td>
<td>21.7</td>
<td>3.5</td>
<td>4.2</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

#1 p values: C vsD; p<0.01, D vsDP₁; p<0.01, D vsDP₂; p<0.01, D vsDP₃; p<0.01, D vsFD; p<0.05.
#2 p values: C vsD; p<0.01, D vsDP₁; p<0.01, D vsDP₂; p<0.01, D vsDP₃; p<0.01, D vsFD; p<0.01.
#3 p values: C vsD; p<0.05, D vsDP₁; p<0.05, D vsDP₂; p<0.05, D vsDP₃; p<0.05.
**Discussion:**

In our study we prepared PLGA-nanoparticle encoded pelargonidin by a modified emulsion-diffusion-evaporation method (Hariharan et al., 2006) employing didodecyldimethyl ammonium bromide (DMAB) as stabilizer. Several physical parameters are presented in Table-8.

Spherical natures of the particles are revealed by TEM, SEM and AFM (Fig. 32, 33). AFM also revealed smooth nanoparticle surface without any noticeable pinholes or cracks and indicate that the nanoparticles surface is rigid and has sufficient mechanical resistance to collapse.

The mean size obtained from DLS (Fig. 34 a) is ~34% greater than that obtained from AFM, TEM Since DLS measurement is performed in aqueous environment, where several water molecules surround the nanoparticle surface thereby increasing its size compared to that in AFM or TEM which performed in dried state without any aqueous environment.

The polydispersity index (PI), as measured by the NanoZS (Worcestershire, UK), ranges from 0–9, with PI increasing with increasing index number (PI=0–1 monodisperse, PI = 8–9 ratio of largest to smallest particle 4–5). Higher the polydispersity index greater will be the chance of aggregate formation. In our preparation, the PI values (0.148) (Table-8) suggests monodisperse nature of the formulation i.e. no characteristic aggregate formation ability, which is supported by the TEM, SEM, and AFM data in Fig. 32, 33 respectively.

Particle charge is a stability determining parameter in aqueous nanosuspensions. Generally, zeta potential of ±30 mV is required as a minimum for a physical stable nanosuspension solely stabilized by electrostatic repulsion (Muller et al., 1999). The zeta potential of the pelargonidin PLGA- nanoparticles prepared in the present study was about +91.1 mV (Fig. 34b and in Table-8). The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in a dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta
potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate as outlined in the table below.

<table>
<thead>
<tr>
<th>Zeta Potential [mV]</th>
<th>Stability behavior of the colloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>from 0 to ±5,</td>
<td>Rapid coagulation or flocculation</td>
</tr>
<tr>
<td>from ±10 to ±30</td>
<td>Incipient instability</td>
</tr>
<tr>
<td>from ±30 to ±40</td>
<td>Moderate stability</td>
</tr>
<tr>
<td>from ±40 to ±60</td>
<td>Good stability</td>
</tr>
<tr>
<td>more than ±61</td>
<td>Excellent stability</td>
</tr>
</tbody>
</table>

So the pelargonidin PLGA- nanoparticles prepared in our study was highly stable particles.

Differential scanning calorimetry curves of pelargonidin, PLGA-Nanoparticles (ghost) and pelargonidin PLGA-nanoparticles are displayed in Fig. 34 c. The endothermic peak of pelargonidin PLGA-nano is obtained at 359.93 °C, corresponding to its melting point. The pelargonidin and PLGA-Nano (ghost) also showed endothermic peak at 216 °C and 348.41°C. In all lyophilized samples of pelargonidin PLGA-nano, we found that pelargonidin PLGA-nano the endothermic peak of pelargonidin is completely disappeared. These results suggested that the drug was dispersed throughout the polymers forming a high-energy amorphous state. Similar findings were also observed by studies using piroxicam in polyvinylpyrrolidone nanopatice and quercetin PLGA nanoparticle. (Tantishaiyakul. et al., 1999 and Wu. et al., 2008).

The encapsulation efficiency (%) of the pelargonidin PLGA-nanoparticles suspension was measured by both UV-vis spectroscopy (spectrum nature shown in Fig. 35 a) and by
HPLC (Fig. 35 b). The encapsulation efficiency (%) calculated from UV-Vis spectroscopy as presented in Table-8 was 96.34% and in case of HPLC 100%.

In our study we used pelargonidin PLGA-nanoparticle suspension to treat STZ-induced diabetes rats. For this purpose three different doses (DPN1=0.3mg/kg body wt; DPN2=0.6mg/kg body wt; DPN3=1.5 mg/kg body wt) were used for pelargonidin encoded PLGA-nanoparticle preparation. All three doses along with free drug dose (FD=0.6mg/kg body wt) were injected the intravenously to diabetic rats.

The pelargonidin PLGA-nanoparticle suspension in all three doses showed prominent hyperglycemic action as evidenced from blood glucose lowering action, normalization of IPGTT pattern, glycohemoglobin content along with improvement in serum insulin secretion. The nanoparticle suspensions are also found to be effective in normalizing hyperlipidemia, free iron release from hemoglobin and hemoglobin mediated oxidative stress conditions. Antioxidant enzymes and oxidative stress markers are also protected in diabetic condition as a result of nanoparticle treatment. Free drug treatment in this context is also found to be effective in certain aspects though its efficiency is much lower than the nanopartieie mediated treatment. The present findings, thus, explain the antidiabetic mode of action of pelargonidin PLGA-nanoparticle formulation and its role in ameliorating the hemoglobin glycation-induced oxidative stress associated complications in experimental diabetes. These findings also projected the fact that pelargonidin PLGA-nanoparticle formulation was an efficient and better therapeutic approach compared to free pelargonidin treatment in diabetic management.
Interaction study of pelargonidin with RBC and hemoglobin
Interaction study of pelargonidin with RBC and Hb

Introduction:
Red blood cells (also referred to as erythrocytes) are the most common type of blood cell and the vertebrate body's principal means of delivering oxygen to the body tissues via the blood. Erythrocytes consist mainly of hemoglobin, a complex metalloprotein containing heme groups whose iron atoms temporarily link to oxygen molecules (O2) in the lungs and release them throughout the body. Oxygen can easily diffuse through the red blood cell's cell membrane.

A typical human erythrocyte disk has a diameter of 6–8 μm and a thickness of 2 μm, much smaller than most other human cells [Hillman et al., 2005]. Mammalian erythrocytes are biconcave disks: flattened and depressed in the center, with a dumbbell-shaped cross section. This shape (as well as the loss of organelles and nucleus) optimizes the cell for the exchange of oxygen with its surroundings. The cells are flexible so as to fit through tiny capillaries, where they release their oxygen load.

The plasma membrane of RBC consists of a complex, ordered array of lipids and proteins stretched over the outer surface of the cell in the form of a lipid bilayer punctuated by penetrating or attached proteins. Plasma membranes are noncovalent assemblies of billions of molecules, yet they tend to be self-assembling, self-sealing, and stable. Plasma membranes are fluid structures, yet they are highly ordered with respect to the distribution of molecules both across the bilayer and within the plane of the bilayer. Plasma membrane of the RBC functions as a barrier, yet they readily pass ions (some against a concentration gradient), nutrients, and signals between the cytoplasm and the extra cellular environment.

Reports suggested that red wine anthocyanins as well as different flavonoids like quercetin, fisetin, chrysin, morin exerts protective effect upon RBCs against oxidative damage [Tedesco et al., 2001, Chaudhuri et al., 2007, Pawlikowska-Pawlega et al., 2003].

Human normal adult Hb, a heterotetrameric protein, that transports oxygen from the lungs to tissues, has served as an excellent model for investigating the structure-function relationship in multimeric, allosteric proteins. Hb consists of four subunits, i.e., two identical α-chains of 141 amino acid residues each and two identical β-chains of 146
amino acid residues each; each subunit contains a heme group leading to a complex with a molecular weight of 67000 Da.

It is important to study the interaction of the drug with the protein because protein–drug binding plays an important role in pharmacology and pharmacodynamics. The effectiveness of drugs depends on their binding ability. Investigating the interaction of drugs to Hb can be used as a model for elucidating the properties of drug–protein complex, as it may provide useful information of the structural features that determine the therapeutic effectiveness of drugs, and it has become an important research field in life sciences, chemistry, and clinical medicine. Current knowledge suggests that factors such as protein binding may impair flavonoid absorption and bioavailability and even mask their antioxidant activity [Sengupta et al., 2002, Arts et al., 2002]. A number of biochemical and molecular biological investigations have revealed that proteins (including enzymes) are frequently the “targets” for therapeutically active flavonoids of both natural and synthetic origin [Takahama 1983]. Protein–flavonoid association is a well-known phenomenon; however, it is only relatively recently that any considerable information has been obtained in the area of how the structure of either the protein or the flavonoid may affect the interaction at the molecular level. Flavonoids have a high affinity for proteins and bind to them by hydrophobic interactions, hydrogen bonds, and covalent bonds (Loomis, W. D, 1974). This binding, however, cannot be generalized because protein-phenol complexes depend heavily on individual structures. Polyphenolic compounds like flavonoids are known to bind several proteins [Bate-Smith, E. C, 1973, Okuda et al., 1985], and they may be transported remaining bound to proteins to different proteins.

Since interaction of pelargonidin with RBC and Hb has not been studied, it seemed worthwhile to study their interaction to have better insight into the action of the herbal drug.
Results:

Effect of Pelargonidin on RBC

RBC size and shape by microscopy:

- **Light Microscopy (phase contrast):**

  Normal human RBC were observed under light microscope (phase contrast) both in absence (Fig. 39a) and presence of various concentration of pelargonidin (10-100µM) (Fig. 39b-e). The untreated RBCs exhibited their normal discoid shapes but pelargonidin treated cells tend to undergo a diminution in size with surface creanation. The percentage of deformed cells 50 randomly selected untreated and treated cells were counted, measured cross-diagonally and averaged, (Fig. 39 f and 42b). The extent of reduction in cell size along with increase in percentage of deformed cell was dependent on the concentration of pelargonidin.

- **Scanning Electron Microscopy (SEM):**

  Visualization of the changes in the size and shape of RBC due to treatment with pelargonidin becomes more prominent using scanning electron microscopy. Measurements were carried out on 50 cells incubated with or without pelargonidin. The result showed a reduced diameter and with higher number of irregular cells with numerous extrusion on their surface or cells with ruffled edges (Fig. 40a-e, 41a-d). The percentage of change in diameter of erythrocytes incubated with pelargonidin at concentration range of (10-100 µM) is presented in Fig40 f along with the number of deformed erythrocytes in and 42a.
Fig 39. Phase contrast microscopic image of control and pelargonidin treated RBC of different magnifications. a: control RBC, b: RBC treated with pelargonidin (10 μM), c: RBC treated with pelargonidin (25 μM), d: RBC treated with pelargonidin (50 μM), e: RBC treated with pelargonidin (100 μM), f: % of deformed RBC.
SEM of Control and pelargonidin treated RBC of different magnifications. 
a: control (3000x), b: pelargonidin (10μM) (4000x), c: pelargonidin (25μM) (4000x) 
d: pelargonidin (50 μM) (3000x) e: pelargonidin (100 μM) (3000x),f: % of deformed RBC from SEM images due to pelargonidin treatment.
Fig 41. SEM of images of pelargonidin treated RBC of different magnifications. a: RBC (18000x) with measurements, b: RBC treated with pelargonidin (25 µM) (20000x), c: RBC treated with pelargonidin (50 µM) (20000x), d: RBC treated with pelargonidin (100 µM) (20000x).
Fig 42. a: Sizes of control and pelargonidin treated RBC from SEM images due to pelargonidin treatment,
b: Sizes of control and pelargonidin treated RBC from Phase contrast microscopic images.
Effect of pelargonidin on Osmotic fragility of RBC:

To find if alteration in morphology of pelargonidin treated RBC is associated with change in osmotic fragility of the cells experiments were done. Osmotic fragility measures RBC's resistance to hemolysis when exposed to a series of increasingly dilute saline solutions. The sooner hemolysis occurs, higher the osmotic fragility of the cells. The osmotic fragility was studied in RBCs treated without and with pelargonidin (25, 50 μM) in PB buffer containing increasing NaCl concentrations (0-0.15 M). Fig.43, illustrates that the osmotic fragility of pelargonidin-treated RBCs was markedly lower compared to that of control.

Fig 43. Measurement of osmotic fragility of control and pelargonidin treated (25,50μM) RBC in gradually increasing NaCl concentration (0-.15M).
Studies of (in vitro drug-protein interactions and molecular modeling) of pelargonidin-hemoglobin interactions:

Spectroscopic studies

Spectrophotometric, spectrofluorimetric and CD studies were done to understand interaction of pelargonidin with human Hb.

a. Spectrophotometric studies

Absorption spectra of Hb (10 µM) and in absence and presence of different concentration of pelargonidin (2.5-25 µM) were recorded from 250-650 nm (Fig.44) The interaction of pelargonidin with hemoglobin in gradual increasing concentration led to gradual decrease in the characteristic soret peak of hemoglobin at 415nm. The plot 1/ΔA vs 1/L_A for Hb is shown in Fig 45 respectively and the binding parameters is calculated using Kapps equation (Kapp et al., 1990) and presented in Table- 12.

b.i) Spectrofluorimetric analysis:

Emission spectra (300-500 nm) of Hb (10 µM) in absence and presence of different concentration of pelargonidin (2.5-25 µM), were recorded with excitation at 285 nm. Hb exhibited emission peak at 339 nm, while in presence of pelargonidin concentration there was red shift of the emission peaks (Fig. 46). Addition of increasing concentration of pelargonidin to like Hb caused gradual decrease in arbitrary fluorescence emission. The binding parameters were calculated using Kapps equation (Kapp et al., 1990) after correction of Inner filter effect. Representative plot of 1/ΔF vs 1/L_A from Kapps equation has been shown in in Fig 47 a. Binding affinity constant was also estimated from Stern-Volmer equation shown in Fig 47 b The binding constant values obtained were presented in Table-12.
Fig 44. Representative absorption spectra of 10 μM Hb in absence and presence of pelargonidin (0-15 μM) in the region 250-650 nm. Inset: The spectra in 350-450 nm and 500-600nm ranges with enlarged scale.

Fig 45. The plot for 1/A vs 1/L, from Kapps equation for Hb- pelargonidin interaction. Hb concentration used was 10μM and pelargonidin concentration used were from 0-15 μM.
Interaction study of pelargonidin with RBC and Hb

Fig 46. A representative emission spectra of 10 μM Hb in absence and presence of pelargonidin (0-25 μM) in the wavelength region 300-500 nm.

Fig 47. The plot for a: 1/ΔF vs 1/Lt from Kapps equation and b: F₀/F vs Q from Stern Volmer equation for Hb- pelargonidin interaction. Hb concentration used was 10μM and pelargonidin concentration used were from 0-25 μM.
ii) Thermodynamic analysis of the binding from Spectrofluorimetric Study:

Temperature dependence of the binding constant (K) was studied from the emission spectra of (2.5-25 μM) in the temperature range 15°C-35°C. Thermodynamic analysis of the temperature dependence of binding was determined by plotting lnK vs 1000/T following Van’t Hoff’s equation (Fig 48a). The enthalpy ($\Delta H^\circ$), entropy ($\Delta S^\circ$) and free energy of binding ($\Delta G^\circ$) of pelargonidin-Hb complex formation were respectively 15.79 kcal/mole, 75.2 cal/mole/°K, -6.772 kcal/mole at 30°C respectively.

iii) Effect of different NaCl molarity on the binding process:

To determine the nature (electrostatic/ non electrostatic) of Hb-pelargonidin interaction, the binding was studied spectrofluorimetrically in presence of different concentration of NaCl (0.05 M-0.30 M) for each of Hb (10 μM) in absence and presence of different concentration of pelargonidin (2.5-25 μM). The binding affinity constant K for pelargonidin interaction with Hb declines with an increase in NaCl concentration revealing electrostatic mode of interaction as a major governing factor. The binding data at different NaCl concentrations were further analyzed from the plot of lnK vs ln[NaCl] (Fig 48 b) following the relation of Record et al. (1978). Slope of the line in Fig 48 b gave the estimate of total bound charge ($z\Psi$), which was found to be in case of Hb-pelargonidin interaction 0.82.
Interaction study of pelargonidin with RBC and Hb

Fig 48: The plot of Hb- pelargonidin interaction a: lnK vs 1000/T for thermodynamic analysis of the binding and b: lnK vs ln[NaCl] for the effect NaCl molarity on the binding process of from spectrofluorometric study.

c. CD experiments

Conformational changes of Hb (10μM) due to interaction with pelargonidin (5-50 μM) were studied by CD spectral analysis (Fig 49).

Fig. 49 a exhibits spectra of Hb in absence and presence of different concentration of pelargonidin (5-50 μM) at far UV region (200-250 nm). In this region of wavelength, CD measurement of a protein gives information about its secondary structure. Compared to Hb alone, interaction of pelargonidin showed an increase in negative ellipticity of the protein in the region 210-225 nm. Molar ellipticity [θ] values were obtained using the relation of Geraci and Parkhurst (1981) and the α-helical contents of Hb and pelargonidin interaction were determined according to the relationship of Chen, et al., 1972 and by using k2d server. The α-helical contents of Hb were found to be approximately 73% and Hb-pelargonidin exhibited a decreased ellipticity and gradual a diminution of α-helical content with increased pelargonidin concentration. The decrease was compensated by in gradual random coil formation and β-sheet of the Hb-pelargonidin complex (Fig. 49 c) by using k2d server. The CD spectra of Hb in absence and presence of different concentration of pelargonidin (5-50 μM) in

![Graph showing thermodynamic analysis of Hb-pelargonidin interaction](image)
Interaction study of pelargonidin with RBC and Hb

near UV region (250-350 nm) were more or less identical (data not shown). The CD spectra in the Soret region (400-500 nm) are sensitive to the position of the heme in relation to the aromatic residues lining the heme pocket. Hb-pelargonidin interaction caused a spectral change in this region as shown in Fig 49b.

Fig 49. a: CD spectra of Hb (10pM) in absence and presence of different concentration of pelargonidin (5-50 pM) far UV region (200-250 nm) and b: visible region (400-600 nm), c: Plot of % of α-Helix content sheet and random coil from k2Dd server.
Isothermal Titration Calorimetry:

Isothermal titration calorimetry was performed to measure the thermodynamic parameters for Hb-pelargonidin interaction at 25°C. The enthalpy of binding, binding constant and number of binding sites obtained by fitting the data in simple 1 binding model (Fig. 50) were found to be as follows $\Delta H^o = 777.4 \text{ kJ/ mol}$, $K=1.99 \times 10^6 \text{ M}$ and $N=0.0112$ respectively.

![Fig 50. Plot of $\Delta H$ vs [Pelargonidin] /mM from ITC data for measurement of the thermodynamic parameters for Hb-pelargonidin interaction. Hb (10μM) was used in absence and presence of different concentration of pelargonidin (5-250 μM).](image-url)
Table 12: Binding constant (K) of Hb-pelargonidin interaction estimated by using different experimental techniques.

<table>
<thead>
<tr>
<th>Binding Parameters</th>
<th>Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>By Fluorescence Spectroscopy (Kapp eqn.)</td>
<td>3.012X10^4 ± 0.15 ;(n= 6)</td>
</tr>
<tr>
<td>By Fluorescence Spectroscopy (Stern Volmer eqn.)</td>
<td>9.945X X10^2±0.20;(n= 6)</td>
</tr>
<tr>
<td>By absorption spectroscopy (Kapp eqn.)</td>
<td>1.5 X10^4±0.07;(n= 6)</td>
</tr>
<tr>
<td>By Isothermal Titration Calorimetry</td>
<td>1.99 x 10^6±0.91;(n= 3)</td>
</tr>
</tbody>
</table>

Effect of pelargonidin on functional properties of Hb:

Peroxidase activity

Hb possesses peroxidase-like activity. It interacts with H₂O₂ to yield a potent oxidant ferrylhemoglobin, which is capable of oxidizing a wide variety of electron donors resembling peroxidase-like activities [Everse et al., 1994]. Peroxidase activities of Hb (1.5 μM) in absence and presence of pelargonidin (0.5-2 μM) was assayed. The reaction rate was followed at 25°C just after addition of H₂O₂. The peroxidase activity of Hb was found to be lowered by addition of pelargonidin. The activity decreased gradually with increase concentration of pelargonidin. The result was given in Fig 51.
Interaction study of pelargonidin with RBC and Hb

Fig 51. Peroxidase-like activity of a: Hb (1.5 µM) in absence and presence of pelargonidin (0.5-2 µM). The results are mean ± SEM of four experiments.

**Molecular modeling studies**

Using the software AutoDock 4 [Morris et al., 1998, Huey and Morris 2003.], docking was performed to identify the possible protein ligand interaction site. Docking studies (Fig.52) show that binding of pelargonidin to hemoglobin was possible due to hydrogen bonding with amino acids residues of ALA130, SER131 of chain A, TYR35 of chain B and TYR140, SER138, THR137, ARG141, LYS139 of chain C. The highly negative Gibbs energy of binding values (-7.31 kcal/mol) reflects a strong interaction between hemoglobin and pelargonidin. Binding constant was calculated from binding energy was 2.38X10^{-5}. Docking reveals 10 clusters of sites for pelargonidin on hemoglobin. The minimal docking energy estimated was -7.71 kcal/mol (Table: 13 a). Average bonding distance of pelargonidin from neighboring residues was given in Table: 13 b.
Table 13

a: Cluster histogram for pelargonidin and hemoglobin docking.

<table>
<thead>
<tr>
<th>Clus</th>
<th>Lowest Energy (kcal/mol)</th>
<th>Run</th>
<th>Mean Energy (kcal/mol)</th>
<th>Docked</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-7.71</td>
<td>12</td>
<td>-7.31</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-7.68</td>
<td>32</td>
<td>-7.37</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-6.92</td>
<td>18</td>
<td>-6.82</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-6.57</td>
<td>22</td>
<td>-6.45</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-6.57</td>
<td>49</td>
<td>-6.41</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-6.47</td>
<td>42</td>
<td>-6.47</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-6.44</td>
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<td>-6.37</td>
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</tr>
<tr>
<td>8</td>
<td>-6.40</td>
<td>45</td>
<td>-6.40</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-6.28</td>
<td>7</td>
<td>-6.28</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-6.22</td>
<td>10</td>
<td>-6.22</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>-6.05</td>
<td>2</td>
<td>-6.05</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-5.59</td>
<td>29</td>
<td>-5.59</td>
<td></td>
</tr>
</tbody>
</table>

b: Neighboring residues of pelargonidin and hemoglobin docking with their distance.

<table>
<thead>
<tr>
<th>Chain</th>
<th>Residue</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>TYR140</td>
<td>3.9</td>
</tr>
<tr>
<td>C</td>
<td>SER138</td>
<td>7.4</td>
</tr>
<tr>
<td>C</td>
<td>THR137</td>
<td>5.7</td>
</tr>
<tr>
<td>C</td>
<td>ARG141</td>
<td>6.3</td>
</tr>
<tr>
<td>C</td>
<td>LYS139</td>
<td>4.0</td>
</tr>
<tr>
<td>B</td>
<td>TYR35</td>
<td>3.6</td>
</tr>
<tr>
<td>A</td>
<td>ALA130</td>
<td>5.0</td>
</tr>
<tr>
<td>A</td>
<td>SER131</td>
<td>5.2</td>
</tr>
</tbody>
</table>
Fig 52. Human tetrameric oxyhemoglobin (PDB ID: 2DN1) (Ribbon diagram) complexed with pelargonidin shown as: a: stick (red), b: mesh (red), c: stick (red) with neighboring residues in view using autodock version 4. Pymol was used in composing M3.
Interaction study of pelargonidin with RBC and Hb

Discussion:

The cytoskeleton proteins are responsible for the shape of red blood cells [Kwiatkowska, J. 1989, Sato et al., 1987]. The untreated RBC exhibits their normal discoid shapes but pelargonidin treated RBC cells tend to undergo a diminution in size as well as deformed shape like irregular cells with ruffled edges are found specially in SEM (Fig.40, 41). Previous report by Fiorani et al., (2001) suggested that flavonoids like quercetin rapidly penetrates the rabbit RBC membrane and that as much as 90% of the flavonoid is found within the cell after only 5 min of exposure to 50 μM quercetin. Similar results were previously obtained in another report Ferrali et al. (1997) in which it was found that quercetin is almost quantitatively taken up by mouse erythrocytes after 60 min of exposure. Other flavonoids, however, may have different abilities to enter the cell (Fiorani et al., 2002). Incubation of pelargonidin with RBC also leads to enter the drug within the cell rapidly. The changes in the RBC morphology may take place due to intercalation of pelargonidin into the membrane and interaction with the membrane cytoskeletal proteins.

In the bloodstream, RBC encounters a variety of oxidant stressors which can be both endogenous, from cellular generation of superoxide and hydrogen peroxide [Van Dyke et al., 1996], and exogenous in areas of inflammation. At the same time RBC is also susceptible to oxidative damage as a result of high concentrations of Hb, a promoter of oxidative process. It is clearly evident from Fig. 43 that incorporation of the flavonoids in erythrocyte enhances the stability of intact erythrocytes by reducing its osmotic fragility. This observation suggests that erythrocyte membrane may becomes more ordered in presence of pelargonidin, which can rationalize their antihemolytic activities. Probably reduction in osmotic fragility might be due the antioxidative action of pelargonidin and free radical scavenging ability thereby protecting membrane phospholipids [Bilto and Abdalla, 1998] or due to changed permeability of the membrane or increase in surface area/volume ratio of the cell [Abe et al., 1991] are behind the effect. The increased proportion of cell surface to its volume due to intercalation of pelargonidin into the membrane or cell shrinkage resulted the increase in surface area/volume ratio of the cell. Reports on quercetin action upon erythrocyte membrane [Pawlikowska-Pawlega et al., 2003], red wine action in normal and catalase-inactive
human erythrocytes [Tedesco et al., 2001], interaction of flavonoids with red blood cell membrane lipids and proteins: antioxidant and antihemolytic effects (Chaudhuri et al., 2007) support our observations.

The absorption spectroscopic study reveals that pelargonidin interacts with Hb in the ground state as shown in Fig. 44 and binding affinities constant in Table 11.

Hb is a tetrameric protein of approximately molecular weight 67,000 containing two α and two β subunits, each of which has one redox iron heme as its prosthetic group, the heme is located in crevices at the exterior of the subunit. Hemoglobin molecule has six tryptophan residues, one each at 14th position of the α-chains and two each at 15th and 37th position of the β-chains. These hydrophobic residues are partly exposed in quaternary conformation of the hemoglobin molecule. Due to presence of these tryptophans (Trp), Hb shows an emission peak at 339 nm when excited at 285 nm. Intrinsic fluorescence study is performed in presence of drug to evaluate the changes in the tertiary structure of Hb caused by reaction with pelargonidin in Fig. 46. The fluorescence intensity of hemoglobin gradually decreases in presence of increasing drug concentration with a red shift of the peaks indicating binding of pelargonidin with hemoglobin in excited state. The strong quenching of the fluorescence indicates that the binding of the drug to Hb changes the microenvironment of tryptophan residues and the tertiary structure of the protein. The gradual red shift in fluorescence emission intensity in presence of pelargonidin is indicative of the fact that the conformation of Hb is changed and the polarity around the tryptophan residues is increased and the hydrophobicity is decreased leading a shift towards a more polar environment. The quantitative analysis of the binding of pelargonidin to Hb was carried out using the Kapps equation and stern Volmer equation as shown in Fig. 47 a, b. The binding constants were summarized in Table 12.

A decrease in fluorescence intensity upon binding was also observed in case of hemoglobin binding study with quercetin, rutin [Xi & Guo, 2007]. In case of (-) Epigallocatechin-3-gallate, a constituent of green tea binding study with HSA indicates lateral red shift (Maiti et al., 2006).

Hydrogen bonds, van der Waals, hydrophobic and electrostatic interactions are the major interactions that have a key role in protein-ligand binding. [DeLano, 2004, Hubbard &
The thermodynamic parameters, free energy ($\Delta G^\circ$), enthalpy ($\Delta H^\circ$), and entropy ($\Delta S^\circ$) changes of interaction provide an insight into the binding mode. For this purpose, the temperature dependence of the binding constants is studied. Experiments were conducted at 25-35°C considering that because Hb does not undergo gross structural change in this temperature range. The binding parameters of the Hb-pelargonidin complex at the different temperatures were estimated from a van’t Hoff plot (Fig. 48 a). The $\Delta H^\circ$ and $\Delta S^\circ$ values are obtained from the slope and intercept of van’t Hoff plot respectively. The formation of Hb-pelargonidin complex is accompanied by a positive enthalpy change $\Delta H^\circ = 15.786$ kcal/mole and a positive entropy change $\Delta S^\circ = 75.2$ cal/mole·°K, which indicates that the binding is endothermic and entropy driven process. The negative values for $\Delta G^\circ = -6.772$ kcal/mole at 30°C indicates the spontaneity of binding of pelargonidin to Hb. The net $\Delta G^\circ$ for the complete association process is essentially determined by the relative magnitude of $\Delta S^\circ$ and $\Delta H^\circ$ [Timaseff, S. N. 1972, Ross & Subramanian, 1981]. For the Hb–pelargonidin system, the main source of the $\Delta G^\circ$ value is derived from a substantial contribution from the $\Delta S^\circ$ factor as $\Delta H^\circ$ is positive. The partial immobilization of the protein and the ligand occurs in an initial step involving hydrophobic association that results in a positive $\Delta S^\circ$. In the subsequent interacting complex, the positive $\Delta S^\circ$ contribution to the overall $\Delta G^\circ$ may be associated with van der Waals interactions and hydrogen bonding [Ross & Subramanian, 1981]. Similar type of binding nature was also obtained in case of hemoglobin binding studies with quercetin, rutin [Xi and Guo, 2007], and resveratrol [Lu et al., 2007]. HSA interacting with (–)-Epigallocatechin-3-gallate [Maiti et al., 2006] and isoflavonoids [Mahesha et al., 2006] also appear to exhibit similar thermodynamic parameters.

To determine the nature (ionic and non ionic) of pelargonidin–Hb interaction, the ionic strength of the buffer is increased by the addition of NaCl 0.05 M–0.30 M. In presence of increasing NaCl concentration a lateral red shift of the emission peaks were observed. The binding constant decreases with increasing ionic strength (Fig. 44b), establishing the role of ionic interaction in the binding. Using the plot of lnK vs ln[NaCl] (Fig 48 b) the total bound charge $z^\Psi = 0.82$ of Hb-pelargonidin interaction has been estimated.
following the relation of Record et al., (1978). Similar types of binding characteristic is also obtained in interaction study of isoflavonoids with HSA [Mahesha et al., 2006].

The CD spectral analysis and measurement of α-helices shown that binding of pelargonidin to Hb decreases the α helical content in protein with increase in gradual random coil formation and β-sheet of the drug-protein complex as shown in Fig. 49 c. The CD spectra of Hb in the presence and absence of flavonoids are similar in shape, indicating that the structure of Hb after the addition of drug is also predominantly α-helical. The CD spectra of Hb and Hb + pelargonidin (with increasing concentration) in near UV region (250-350 nm) are more or less identical indicating no change in the tertiary structure. The CD spectra in the Soret region (400-500 nm) are sensitive to the position of the heme in relation to the aromatic residues lining the heme pocket. Hb-pelargonidin interaction caused a spectral change in this region as shown in Fig 47 b. Decrease in a helical content was also obtained in case of hemoglobin binding study with quercetin, rutin [Xi & Guo, 2007] and resveratrol [Lu et al. 2007]. HSA interacting with (-)-Epigallocatechin-3-gallate [Maiti et al., 2006] and isoflavonoids [Mahesha et al., 2006] also reveals to exhibit similar findings.

Through the recent advancements both in instrumentation and theoretical approaches underlying the data analysis, isothermal titration calorimetry (ITC) has become an important tool in protein–ligand studies, and the technique has been applied to different proteins in a number of studies on the binding of drugs and toxins [Barbosa et al., 2003, Lunda et al., 2006, Perry et al., 2003, Savas et al., 2004]. ITC is the only technique which in addition to measuring the binding affinity directly detects the magnitude of its enthalpic and entropic contributions, which is endemic to ligand binding processes [Gilli et al. 1994]. This is an important advantage in elucidating structure–energy relationships since the enthalpy–entropy compensation. The directly measured enthalpy change, ΔH, is often precise enough to enable reasonable estimates of the heat capacity change, ΔCp = dΔH/dT, of the process, when binding is studied at different temperatures (within the narrow range allowed by other limitations). Detailed information on enthalpy, entropy and heat capacity changes are particularly important for ligands with large non-polar groups since these functions are highly sensitive to hydrophobic hydration. A second principal advantage of ITC is that it directly detects the displacement of protein–ligand
Interaction study of pelargonidin with RBC and Hb

equilibria upon a small increase in the total concentration of one of the components. It follows that the raw data (the so-called enthalpogram) reflects the slope of the binding isotherm rather than the isotherm itself which is endemic to ligand binding processes. Hb-pelargonidin interaction at 25°C leads to 1;1 complex formation as revealed by ITC study.

Heme proteins Hb exhibits peroxidase activity. The peroxidase activity of hemoglobin occurs through the formation of a ferryl intermediate (Hb\(^{+}\)-Fe\(^{4+}\) or Hb-Fe\(^{4+}\)) with \(H_2O\). The ferryl hemoglobin resembles Compound I and Compound II of the peroxidases, and is capable of withdrawing an electron from a suitable substrate. The decreased peroxidase activity, of pelargonidin-Hb (Fig. 51), may be associated with a modulation mechanism linked to structural change of the protein. This structural change may cause reduced ferryl formation or may interfere with entry of the substrate molecule o-dianisidine to heme pocket leading to decreased peroxidase activity of the protein.

Autodock, in molecular modeling study provides a comprehensive view of the available ligand docking sites and additionally generates quantitative data on fit reliability by assigning an affinity to each docking position. The validity of projected docking sites depends on the three-dimensional template of the protein [Salas-Burgos et al., 2004] and the docking methodology of Autodock [Morris et al., 2009]. Molecular modeling using Autodock gives an insight of the possible sites of binding of -pelargonidin with Hb. Docking studies (Fig. 52) show that the binding of pelargonidin to hemoglobin is possible due to hydrogen bonding with amino acid residues of ALA130, SER131of chain A, TYR35 of chain B and TYR140, SER138, THR137, ARG141, LYS139 of chain C. The distances with respective residues are listed in Table 13 b. [The four chains of Hb are represented as A=\(\alpha_1\), B=\(\beta_1\), C=\(\alpha_2\), D=\(\beta_2\)] The highly negative Gibbs energy of binding values (-7.31kcal/mol) reflects a strong interaction between hemoglobin and pelargonidin.