Appendices
APPENDIX-I

1. **Qualitative study of urine sugar** (Benedicts, 1911)

   About 5 ml of Benedict’s solution was heated to boiling, then 0.5 ml (8 drops) of urine was added. The change in color was observed and the approximate urine sugar was determined as follows:
   
   - Green - +
   - Yellow - ++
   - Reddish brown - +++

   The animals, which survived the alloxan injection became diabetic was confirmed by weight loss, presence of glycosuria (+++) and hyperglycemia.

2. **Estimation of blood glucose** (Sasaki et al., 1972)

   0.1 ml of freshly drawn blood was immediately mixed with 1.9 ml of 10% TCA to precipitate the proteins and then centrifuged, 3000 rpm, 10 min. 1 ml of the supernatant was mixed with 4 ml of O-toluidine reagent and was kept in boiling water bath for 15 min. The green color developed was read at 620 nm against reagent blank. A set of standard glucose solutions (20-100 µg) was treated simultaneously. Glucose concentration was expressed as mg/dl.

3. **Quantitative determination of plasma insulin** (Anderson et al., 1993)

   The plasma insulin was assayed by ELISA method (Enzyme Linked Immunosorbent Assay) using Boehringer-Mannheim kit (Boehringer analyzer ES 300). 0.1 ml of plasma was loaded into the plastic wells coated with monoclonal anti-insulin antibodies. Phosphate buffer (40 mM, pH 7.0) and anti-insulin peroxidase conjugate were added to form anti-insulin antibody-POD conjugate. Substrate-chromogen solution (ABTS solution) was then added to form indicator reaction. A set of standards was also treated in the similar manner. After the development of color, the absorbance was read at 420nm. The values were expressed as µU/ml.

4. **Estimation of haemoglobin** (Drabkin and Austin, 1932)

   To 0.02 ml of blood 5 ml of Drabkin’s reagent was added and mixed. The reaction mixture was kept at room temperature for 5 min and read at 540 nm against a reagent blank. The haemoglobin content was expressed as g/dl.
5. **Estimation of glycosylated haemoglobin (HbA1C)** (Sudhakar Nayak and Pattabiraman, 1981)

After the separation of plasma, the buffy coat enriched in white cells was removed and the remaining erythrocytes were washed three times with physiological saline. The contents were centrifuged (3000 rpm for 10 min) and the supernatant was discarded. To 0.5 ml of packed cell, 0.5 ml of saline was added, mixed and processed for the estimation.

To an aliquot, 4 ml of oxalate (0.1 M) in HCl solution (2 N) was added, mixed, heated at 100°C for 4 h, cooled and precipitated with 2 ml of 40% TCA. The mixture was centrifuged, and to an aliquot, 0.05 ml of 80% phenol and 3 ml of Conc. H2SO4 were added. A set of fructose stock standard (10-50 mg) was also treated in the similar manner. The color developed was read at 480 nm after 30 min. The values were expressed as mg/g haemoglobin.

6. **Activity of hepatic hexokinase** (ATP: D-hexose-6-β phosphotransferase; E.C.2.7.1.1) (Brandstrup et al., 1957)

To 1 ml of glucose solution (0.005 M), 0.5 ml of ATP solution (0.072 M), 0.1 ml of magnesium chloride (0.05 M), 0.4 ml of 0.0125 M potassium dihydrogen phosphate, 0.4 ml of 0.0125 M potassium chloride, 0.4 ml of 0.5 M sodium fluoride and 2.5 ml of Tris-HCl buffer (0.01 M, pH.8.0) were added, mixed and incubated at 37°C for 5 min. The enzymatic reaction was initiated by the addition of 2 ml of tissue homogenate. 1 ml of the reaction mixture was immediately removed to the tubes containing 1 ml of 10% TCA that was considered as zero time. A second aliquot was removed after 30 min incubation at 37°C. The protein precipitate was removed by centrifugation (3000 rpm for 10 min) and the residual glucose in the supernatant was estimated by the method of O-toluidine as described previously. The enzyme activity was expressed as μmoles of glucose phosphorylated/min/g protein.

7. **Activity of hepatic glucose-6-phosphate dehydrogenase** (D-glucose-6-phosphate: NADP oxido reductase; E.C.1.1.1.49) (Ellis and Kirkman, 1961)

To 0.5 ml of the tissue homogenate, 1 ml of Tris-HCl buffer (0.05 M, pH.7.5), 0.1 ml of magnesium chloride (0.1 M), 0.1 ml of 0.1 M NADP+, 0.5 ml of phenazine methosulphate (0.005%) and 0.4 ml of 0.01% 2,6-dichlorophenol indo phenol solution were added, mixed and allowed to stand at room temperature for 10 min to permit completion of the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 ml of glucose-6-phosphate (0.02 M).
The absorbance was read at 640 nm against water blank at one min intervals for 3-5 min. The activity of the enzyme was calculated in units by multiplying the change in OD/min by the factor 17.6, which is the molar extinction co-efficient of the reduced co-enzyme.

\[
\text{Change in OD/min} \times \text{molar extinction co-efficient} \times \text{Temperature correction factor (Tf)}
\]

Tf at 37°C is 0.76 and molar co-efficient of NADPH is 17.6

The activity of enzyme was expressed as mlU/mg protein.

8. **Activity of hepatic glucose-6-phosphatase** (Glucose-6-phosphate phosphohydrolase; E.C.3.1.3.9) (Baginsky et al., 1974)

To 0.3 ml of tissue homogenate, 0.7 ml of citrate buffer (0.1 M, pH 6.5) and 0.3 ml of 0.01 M of glucose-6-phosphate were added. The reaction mixture was incubated at 37°C for 1 h. Addition of 1 ml of 10% TCA to the reaction tubes terminated the reaction of the enzyme. The suspension was centrifuged (3000 rpm for 10 min) and the phosphorus content of the supernatant was estimated. The supernatant was made up to 1 ml and to this 1 ml of ammonium molybdate (2.5%) was added followed by 0.4 ml of ANSA (0.25%). The blue color developed after 20 min was read at 680 nm. The enzyme activity was estimated as μmoles of inorganic phosphorus liberated/min/mg protein.

9. **Activity of hepatic fructose-1, 6-bisphosphatase** (Fructose-1, 6-bisphosphate phosphohydrolase; E.C.3.1.3.11) (Gancedo and Gancedo, 1971)

To 0.2 ml of tissue homogenate, 1.2 ml of Tris-HCl buffer (0.1 M, pH 7.0), 0.1 ml of substrate (0.05 M of fructose-1, 6-bisphosphate), 0.25 ml of magnesium chloride (0.1 M), 0.1 ml of potassium chloride solution (0.1 M), 0.25 ml of 0.001 M EDTA solution were added to make to 2 ml and incubated at 37°C for 15 min. The reaction was terminated by the addition of 10% TCA. The suspension was centrifuged (3000 rpm for 10 min) and the supernatant was used for phosphorus estimation. The supernatant was made up to 1 ml and to this 1 ml of ammonium molybdate was added followed by 0.4 ml of ANSA (0.25%). The blue color developed after 20 min was read at 680 nm. Enzyme activity was expressed as μmoles of inorganic phosphorus liberated/h/mg protein.
10. **Extraction of lipids** (Folch et al., 1957)

Lipids were extracted from tissues using chloroform - methanol mixture (CHCl₃ : MeOH; 2:1 v/v). A known weight of tissue was homogenized in 7 ml of methanol using Potter Elvehjem homogenizer. The contents were filtered into a previously weighed side arm flask, residue on the filter paper was scrapped off and homogenized with 14 ml of CHCl₃ : MeOH mixture. This was again filtered into the side arm flask and the residue was successively homogenized in CHCl₃ : MeOH and each time this extract was filtered. The pooled filtrate in the flask was evaporated to dryness to a constant weight.

The dried residue of lipid was dissolved in 5 ml of CHCl₃: MeOH mixture (2:1 v/v) and transferred into a centrifuge tube. To which 2 ml of 0.1 M potassium chloride was added, shaken well and centrifuged. The upper aqueous layer containing gangliosides was discarded. The chloroform layer was mixed with 1 ml of chloroform-methanol-potassium chloride mixture (1:10:10 v/v) and then centrifuged (3000 rpm for 10 min). This washing was repeated thrice and each time, the upper layer was discarded. The lower layer was made up to 5 ml and used for the analysis of total cholesterol, triglycerides, free fatty acids and phospholipids. The serum was also treated similarly for the estimation of lipids. The dry defatted tissues were used for the estimation of tissue glycoproteins.

11. **Estimation of glycoproteins**

To the dry defatted tissues remaining after lipid extraction, 2 ml of 0.1 N H₂SO₄ was added and hydrolyzed at 80°C for 1 h, cooled and an extract was used for sialic acid estimation. To the remaining solution, 5 ml of 0.1 N NaOH was added and kept in an ice bath for 1 h. The aliquots were used for fucose, total hexose and hexosamine estimation.

12. **Estimation of sialic acid** (Warren, 1959)

To 0.5 ml of extract/plasma, 0.5 ml of water and 0.25 ml of periodic acid (0.025 M) were added and incubated at 37°C for 30 min. To this 0.2 ml of sodium meta arsenate (4%) and 2 ml of TBA (1.44%) were added and heated in a boiling water bath for exactly 6 min, cooled and 5 ml of acidified butanol was added. The absorbance was read at 540 nm against reagent blank. Sialic acid was expressed as mg/100 g in defatted tissue and mg/dl in plasma.
13. **Estimation of hexose** (Niebes, 1972)

To 0.5 ml of the aliquot/plasma, 0.5 ml of 5% phenol and 2.5 ml of conc. H$_2$SO$_4$ were added. For blank, 0.5 ml of 0.1 N NaOH was treated in the same way. The tubes were then heated in a boiling water bath for 20 min and the absorbance was read at 490 nm. Total hexose was expressed as mg/100 g in defatted tissue and mg/dl in plasma.

14. **Estimation of fucose** (Dische and Shettle, 1948)

To 0.5 ml of the aliquot/plasma, 4.5 ml of conc. H$_2$SO$_4$ was added and heated in boiling water bath for 3 min. The samples were cooled and 0.1 ml of cysteine hydrochloride reagent was added. 0.5 ml of 0.1 N NaOH was also treated in the same way for blank. After 75 min in dark, the absorbance was read at 430 nm. Fucose was expressed as mg/100 g in defatted tissue and mg/dl in plasma.

15. **Estimation of hexosamine** (Elson and Morgan, 1979)

To 0.5 ml of plasma/0.1 ml of aliquot, 2.5 ml of 3 N HCl was added and boiled for 6 h in a boiling water bath and then neutralized with 6 N NaOH. To 0.8 ml of the neutralized sample 0.6 ml of acetyl acetone reagent was added, and the tubes were heated in boiling water for 30 min. After cooling, 2 ml of Ehrlich’s reagent was added and mixed well. Blank and standards were processed similarly. The color developed was read at 540 nm. Hexosamine was expressed as mg/100 g in defatted tissue and mg/dl in plasma.

16. **Estimation of total cholesterol** (Zlatkis et al., 1953)

To 0.1 ml of the serum/lipid extract, 9.9 ml of ferric chloride-acetic acid reagent was added and allowed to stand for 15 min and then centrifuged (3000 rpm for 10 min). To 5 ml of the supernatant, 3 ml of conc. H$_2$SO$_4$ was added. The color developed was read after 20 min at 560 nm against a reagent blank. A set of cholesterol standards was also treated in the similar manner. The values were expressed as mg/100g in tissue and mg/dl in serum.

17. **High density lipoprotein-cholesterol (HDL-C)** (Burnstein et al., 1970)

To 1 ml of serum 0.18 ml of heparin-manganese chloride reagent was added, mixed, allowed to stand at 4°C for 30 min and then centrifuged in a refrigerated centrifuge at 3000 rpm for 10 min. The supernatant represents the HDL-C fraction. An aliquot of supernatant was used for cholesterol estimation. The values were expressed as mg/dl.
18. **Very low density lipoprotein-cholesterol (VLDL-C)** (Friedward et al., 1972)

VLDL-cholesterol was calculated using the equation: VLDL-C = TG/5. The values were expressed as mg/dl.

19. **Low density lipoprotein-cholesterol (LDL-C)**

LDL-C was calculated using the equation: LDL-C = Total cholesterol - (HDL-C + VLDL-C). The values were expressed as mg/dl.

20. **Estimation of triglycerides** (Foster and Dunn, 1973)

An aliquot of serum/lipid extract was evaporated to dryness and 0.1 ml of methanol was added followed by 4 ml of isopropanol. 0.4 g of alumina was added and shaken well for 15 min, centrifuged (3000 rpm for 10 min) and then 2 ml of the supernatant was transferred to labeled tubes. The tubes were placed in a water bath at 65°C for 15 min for saponification after adding 0.6 ml of the saponification reagent followed by 0.5 ml of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65°C for 1 h, the contents were cooled and absorbance was read at 420 nm. A series of standards (8-40 μg triolein) was treated similarly along with a blank containing only the reagents. The triglyceride content was expressed as mg/100g in tissue and mg/dl in serum.

21. **Estimation of phospholipids** (Zilversmit and Davis, 1950)

To 0.1 ml of serum/lipid extract, 1 ml of 5 N H₂SO₄ and 1 ml of conc. HNO₃ were added and digested to a colorless solution. The phosphorus content in the extract was determined. The values were expressed as g/100 g in tissue and mg/dl in serum.

22. **Estimation of free fatty acids** (Falholt et al., 1973)

0.1 ml of serum/lipid extract was evaporated to dryness. 1 ml of phosphate buffer (pH 6.4), 6 ml of extraction solvent and 2.5 ml of copper reagent were added and shaken vigorously. 200 mg of activated silicic acid was added and left aside for 30 min. The tubes were centrifuged (3000 rpm for 10 min) and 3 ml of the copper layer was transferred to another tube containing 0.5 ml of diphenyl carbazide (1.5 M) and mixed carefully. The absorbance was read at 550 nm immediately. The values were expressed as mg/100g in tissue and mg/dl in serum.
23. **Indirect assessment of β-hydroxy-β-methylglutaryl-co-enzyme A (HMG-CoA) reductase activity** (E.C.1.1.34) (Philipp and Shapiro, 1979)

The ratio between HMG-CoA and mevalonate in hepatic tissue was taken as an index of the activity of HMG-CoA reductase.

Equal volumes of fresh 10% tissue homogenate and diluted perchloric acid (50 ml/l) were mixed. After 5 min it was centrifuged at 2000 rpm for 10 min. To 1 ml supernatant, 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG-CoA) was added, mixed and after 5 min, 1.5 ml of ferric chloride (5.2 %) was added and shaken well. Readings were taken after 10 min at 540 nm against a similarly treated saline-arsenate blank. The ratio of HMG-CoA to mevalonate was calculated. Lower ratio indicates higher enzyme activity and vice-versa.

24. **Gas Chromatographic (GC) analysis of fatty acid composition**

(Morrison and Smith, 1964)

For GC analysis of fatty acids, lipids in the samples were extracted and evaporated to dryness using a water bath and subjected to saponification. To the test tubes, 1.0 ml of saponification reagent was added and vortexed for 5-10 seconds and kept in a boiling water bath. After 5 min, the tubes were removed from the water bath and again vortexed for 5-10 seconds. Again kept in a boiling water bath for 25 min, the tubes were removed and cooled with water. To the above tubes, 2.0 ml of methylation reagent was added and heated at 80°C in a water bath for 10 min and removed quickly to water. The fatty acid methyl esters thus formed were removed from the aqueous phase and transferred to an organic phase by liquid-liquid extraction procedure. To the above test tubes, the extraction solvent was added and mixed well and the aqueous lower phase was discarded. To the upper solvent phase 3.0 ml of base wash solution was added, mixed well and subjected to centrifugation. The upper solvent layer was transferred to a GC bottle sealed and kept in refrigerator till analysis.

The fatty acid esters thus obtained were analysed on a Tracer 540 - gas chromatograph (Tracor Instruments, Austin, TX) equipped with a flame ionisation detector (FID) under the following conditions: inlet temperature - 200°C; detector temperature - 220°C; separating column - 2 m long x 2 mm internal diameter, packed with 10% Cilar on chromosorb W, 80/100 mesh. Fatty acids separated were identified by the comparison of retention times with those obtained by the
separation of a mixture of standard fatty acids. Individual fatty acids were expressed as percentage of total fatty acids of 100 mg tissue.

25. **Estimation of thiobarbituric acid reactive substances (TBARS)**
   (Nichans and Samuelson, 1968)

   1 ml of the tissue homogenate/0.5 ml of plasma was treated with 2 ml of TBA-TCA-HCl reagent, mixed thoroughly and kept in a boiling water bath for 15 min. After cooling, the tubes were centrifuged at 3000 rpm for 10 min and the supernatant was taken for measurement. A series of standard solution (1, 1', 3, 3' tetramethoxy propane) was also treated in a similar manner. The absorbance of chromophore was read at 535 nm against the reagent blank. The values were expressed as mM/100g in tissue and mM/dl in plasma.

26. **Lipid peroxidation – *in vitro* analysis** (Cynamon and Isenbery, 1985)

   After the separation of plasma, the buffy coat enriched in white cells was removed and the remaining erythrocytes were washed three times with physiological saline. The contents were centrifuged (3000 rpm for 10 min) and the supernatant was discarded. One aliquot of 0.2ml of packed cells was suspended in 3.8 ml of phosphate buffered saline. A second aliquot of 0.2 ml of packed cells was suspended in 3.8 ml of phosphate buffer to which sodium azide (26.0 mg/100 ml) had been added. 1.0 ml of 3 % H2O2 was added to the first tube and 1.0 ml of 0.75 % was added to the second aliquot. A third aliquot containing 0.2 ml of packed cells with 4.8 ml of phosphate buffer and a reagent blank were incubated for one hour along with test samples.

   Following incubation 2.0 ml of TBA reagent was added to all the tubes and the tubes were then centrifuged. Then placed in boiling water bath for 15 min and the pink color formed was read at 540nm. The percent maximal malondialdehyde (MDA) release was calculated according to the following equation

   \[
   \text{% Maximal release} = \frac{\text{TBARS release (3 % H}_2\text{O}_2)}{\text{TBARS release (0.75 % H}_2\text{O}_2 + \text{azide})} \times 100
   \]

26. **Estimation of lipid hydroperoxides** (Jiang et al., 1992)

   1.8 ml of the Fox reagent was mixed with 0.2 ml of the tissue homogenate/0.2 ml of plasma. Then incubated for 30 min at room temperature
and read at 560 nm. The lipid hydroperoxides was expressed as mM/100 g in tissue and $10^{-5}$ mM/dl in plasma.

27. **Assay of superoxide dismutase** (SOD; EC 1.15.1.1) (Kakkar et al., 1984)

0.5 ml of tissue homogenate was diluted to 1 ml with distilled water. Then 2.5 ml of ethanol and 1.5 ml of chloroform (all reagents chilled) were added. This mixture was shaken for 1 min at 4°C and then centrifuged at 3000 rpm for 10 min. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 μM PMS, 0.3 ml of 30 μM NBT, 0.2 ml of 780 μM NADH, appropriately diluted enzyme preparation and distilled water in a total volume of 3 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 sec the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control.

One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units/mg protein.

28. **Assay of catalase** (EC 1.11.1.6) (Sinha, 1972)

To 0.9 ml of phosphate buffer (0.01M, pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 0.2 M H$_2$O$_2$ were added. After 60 sec, 2 ml of dichromate acetic acid reagent was added. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620 nm. Standards (H$_2$O$_2$) in the range of 2-10 μmols and blank containing reagents alone were processed as test. The activities were expressed as μmoles of H$_2$O$_2$ consumed/min/mg protein.

29. **Assay of glutathione peroxidase** (GPX; EC 1.11.1.9) (Rotruck et al., 1973)

To 0.2 ml of tris buffer (0.4 M, pH 7.0), 0.2 ml of 0.4 mM EDTA, 0.1 ml of 10 mM sodium azide and 0.5 ml of tissue homogenate were added. To the mixture, 0.2 ml of 2 mM glutathione followed by 0.1 ml of 20 mM H$_2$O$_2$ was added. The contents were mixed well and incubated at 37°C for 10 min along with a tube containing all the reagents except sample. After 10 min the reaction was arrested by the addition of 0.5 ml of 10% TCA, centrifuged and the supernatant was
assayed for glutathione. The activities were expressed as μg of GSH consumed/min/mg protein.

30. **Assay of glutathione-S-transferase** (GST; E.C.2.5.1.18) (Habig et al., 1974)

The reaction mixture contained 1 ml of phosphate buffer (0.3 M, pH 6.5), 0.1 ml of CDNB (30 mM), 0.1 ml of tissue homogenate and 0.7 ml of distilled water. The reaction mixture was incubated at 37°C for 5 min and then the reaction was started by the addition of 0.1 ml of 30 mM glutathione. The absorbance change was read at 340 nm for 5 min. Reaction mixture without the enzyme was used as the blank.

\[
\text{Activity} = \frac{\text{OD} \times 3}{9.6 \times 5 \times 100} \frac{\text{mg protein}}{\text{min/mg protein}}
\]

9.6 was the difference in the micromolar extinction co-efficient between CDNB-GSH conjugate formed per min/mg protein. The activity of GST was expressed as μmoles of CDNB-GSH conjugate formed/min/mg protein.

31. **Estimation of reduced glutathione** (Ellman, 1959)

Protein in 0.5 ml of homogenate/plasma was precipitated with 2 ml of 5% TCA. 1 ml of the supernatant was taken after centrifugation at 3000 rpm for 10 min and added to it 0.5 ml of Ellman’s reagent and 3 ml of phosphate buffer (0.2 M, pH 8.0). The yellow color developed was read at 412 nm. A series of standard glutathione solution (10 μg – 100 μg) was treated in a similar manner along with a blank containing 3.5 ml of buffer. The values were expressed as mg/100g in tissue and mg/dl in plasma.

32. **Estimation of protein** (Lowry et al., 1951)

Protein in 0.5 ml of tissue homogenate was precipitated with 0.5 ml of 10% TCA, centrifuged at 3000 rpm for 10 min, and the precipitate was dissolved in 1 ml of 0.1 N NaOH. 0.1 ml of aliquot was taken and made up to 1 ml with distilled water. Then 4.5 ml of alkaline copper reagent was added and allowed to stand at room temperature for 10 min. After incubation, 0.5 ml of folins-ciocalteau reagent was added and the blue colour developed was read after 20 min at 620 nm. A standard curve was obtained using bovine serum albumin. The protein levels were expressed as mg/g tissue.
APPENDIX-II

1. 1, 1', 3, 3' tetramethoxy propane standard - 0.16 ml of 3 M solution of standard tetramethoxy propane was made up to 100 ml with double distilled water. 1 ml of this was taken and made up to 100 ml with double distilled water, which served as working standard.

2. 1-chloro-2, 4-dinitrobenzene (CDNB) - 30 mM was prepared in 95% ethanol.

3. 2,6 - dichlorophenol indo phenol - 0.01% in distilled water (fresh).

4. ABTS solution - 5 ml of 7 mM ABTS and 88 µl of 140 mM potassium persulphate were mixed and allowed to stand in the dark at room temperature for 12-16 h before use.

5. Acetyl-acetone reagent - 0.75 ml of acetyl acetone was dissolved in 60 ml of distilled water and 40 ml of isopropanol.

6. Acidified butanol - 5% HCl in n-butanol.

7. Acridine orange/ethidium bromide mixture (1:1) - Dissolve 40 µg in 0.5 ml of 95% ethanol and add 9.5 ml distilled water.

8. Adenosine tri phosphate (ATP) solution - 0.396 g of ATP was dissolved in 10 ml of phosphate buffer (pH 7.4).

9. Alkaline copper reagent - Solution A: 2% sodium carbonate in 0.1N sodium hydroxide; Solution B: 0.5% copper sulphate in distilled water; Solution C: 1% sodium potassium tartarate in distilled water; 50 ml of solution A was mixed with 0.5 ml of solution B and 1 ml of solution C, just before use.

10. Amino naphthol sulphonatic acid (ANSA) - 500 mg of 2-amino-4-naphthol sulphonatic acid was dissolved in 195 ml of 15% sodium bisulphite and 5 ml of 20% sodium sulphite solution was added for complete solubilization. The solution was filtered and stored in the brown bottle.

11. Ammonium molybdate solution - 2.5 g of ammonium molybdate was dissolved in 100 ml of 3N H₂SO₄.

12. Base wash solution - 10.8 g of NaOH dissolved in 900 ml of deionised distilled water.

13. Benedict's reagent - 173 g of sodium citrate and 100 g of anhydrous sodium carbonate were dissolved in 600 ml of hot distilled water in a beaker. Into this with constant stirring, a solution of copper sulphate (CuSO₄. 5H₂O) (17.3 g is dissolved in 100 ml of cooled water) was run slowly and then made up to 1 liter with distilled water.

14. Bovine serum albumin (BSA) standard - 100 mg of BSA was dissolved in 100 ml of distilled water in a standard flask. Small quantities of alkali could be added to make complete dissolution of BSA. 10 ml of the stock was diluted to 100 ml to get a working standard concentration 100 µg/ml.

15. Cholesterol working standard - 40 µg/ml in ferric chloride-acetic acid reagent.
16. Citrate buffer (0.1 M, pH 6.5) - 2.94 g of sodium citrate and 1.92 g of citric acid in 100 ml of distilled water and adjust pH to 6.5 if needed.

17. Copper reagent - 10 ml of copper solution was mixed with 10 ml of triethanolamine and 6 ml of sodium hydroxide and diluted to 100 ml, then 33 g of sodium chloride was added and the pH was adjusted to 8.1.

18. Cyanmethaemoglobin standard - The solution was obtained commercially and had a concentration of 16 g/dl.

19. Cysteine hydrochloride reagent - 3% cysteine hydrochloride in distilled water.

20. DAPI solution - 100 µg of DAPI was dissolved in 10 ml of DMSO.

21. Developing solution for silver staining - 3 ml of 0.02% sodium thio sulphate and 75 µl of formaldehyde was added with 4.5 grams of sodium carbonate and the volume made up to 150 ml with distilled water.

22. Dichromate-acetic acid reagent - 1:3 ratio of potassium dichromate was mixed with glacial acetic acid. From this 1 ml was diluted again with 4 ml acetic acid.

23. Diphenyl carbazide solution (1.5 M) - 120 mg diphenyl carbazide in 30 ml ethanol and 0.3 ml triethanolamine solution was added just before use.

24. Disodium hydrogen phosphate (0.3 M) - 4.26 g of Na$_2$HPO$_4$ of 100 ml distilled water.

25. DPPH solution - 2.5 mg DPPH were solved in 5 ml methanol (1.27 mM) as stock.

26. Drabkin's reagent - This reagent contains 0.05 g of potassium cyanide, 0.20 g of potassium ferricyanide and 1 g of sodium bicarbonate in 1 L of distilled water (pH 9.6).

27. EDTA (0.4 mM) - 11.68 g of EDTA was dissolved in 100 ml of distilled water.

28. Ehrlich's reagent - 320 mg of p-dimethyl amino benzaldehyde was dissolved in 21 ml of iso-propanol and 4 ml of conc. HCl.

29. Electrophoresis buffer - 300 mM NaOH and 1 mM EDTA-Na (pH > 13).

30. Ellman's reagent - 19.8 mg of 5, 5'-dithiobis (2-nitrobenzoic acid) in 100 ml of 1% sodium citrate solution.

31. Equilibration buffer-I - 6 M urea, 2% SDS, 1% DTT, 10% glycerol, and 1.5 M Tris-HCl (pH 8.8).

32. Equilibration buffer-II - 6 M urea, 2% SDS, with 2.5% iodoacetamide, 10% glycerol, and 1.5 M Tris-HCl (pH 8.8).

33. EtBr (10 µg/ml) - 1 mg of EtBr was dissolved in 100 ml of deionized water, stirred and stored in dark at 4°C.

34. Extraction solvent: - Chloroform : Heptane : Methanol (5 : 5 : 1)
35. Ferric chloride-acetic acid reagent - 250 mg of ferric chloride was dissolved in 500 ml of glacial acetic acid

36. Ferric chloride reagent - 5.2 g of TCA and 10 g for ferric chloride were dissolved in 50 ml of 0.65 N HCl and diluted to 100 ml with distilled water.

37. FeSO₄ (15 mM) - 22.8 g of FeSO₄ was dissolved in 10 ml of distilled water

38. Fixative solution - 40% methanol and 10% acetic acid

39. Folin - Ciocalteau reagent: The following substances were mixed together and refluxed for 10 h. Sodium tungstate - 100 mg; Sodium molybdate - 25 g; Distilled water - 700 ml; Phosphoric acid 85% - 15 ml; Conc. HCl - 100 ml. After refluxing, 150 g of lithium sulphate and 50 ml of distilled water were added along with few drops of bromine. The mixture was boiled for 15 min to remove excess of bromine. The contents were cooled, diluted to 1 liter and filtered. This reagent was diluted 1:2 with distilled water before use.

40. Fox reagent - 88 mg of BHT, 7.6 mg of xylenol orange and 9.8 mg of ammonium ion sulphate were added to 90 ml of methanol and 10 ml of 250 mM H₂SO₄.

41. Fructose stock standard (1 mg/ml) - 100 mg of fructose was dissolved in 100 ml of distilled water.

42. Fructose-1, 6-bisphosphate substrate (0.05 M) - 0.2 g in 10 ml of distilled water.

43. Fucose stock standard - 20 mg of fucose was dissolved in 100 ml of distilled water.

44. Galactosamine hydrochloride stock standard (100 µg/ml) - 10 mg of galactosamine hydrochloride was dissolved in 100 ml of distilled water.

45. Galactose-Mannose standard (0.2 mg/ml) - 0.1 mg/ml of each galactose and mannose.

46. Glucose (0.005 M) - 90 mg of glucose was dissolved in 100 ml of distilled water.

47. Glucose standard (stock) - 1 mg/ml: 100 mg of anhydrous D-glucose was dissolved in 100 ml of distilled water containing 0.01% benzoic acid.

48. Glucose-6-phosphate Substrate (0.01 M) - 0.028 g of Glucose-6-phosphate was dissolved in 10 ml of distilled water.

49. Glutathione solution (2 mM) - 30.7 mg of glutathione was dissolved in 5 ml distilled water.

50. Glutathione standard solution - 10 mg of reduced glutathione was dissolved in 100 ml of distilled water.

51. Glycine buffer (0.1 M, pH 10.5) - Dissolve 3.75 g of glycine buffer salt in 500 ml and adjust the pH 10.5 by adding 0.1 M "NH₂CH₂COO" or 0.1 M NaOH.
52. $\text{H}_2\text{O}_2$ (0.2 M) - 0.68 ml of $\text{H}_2\text{O}_2$ 100 ml of distilled water.
53. $\text{H}_2\text{SO}_4$ (85 %) - 212.5 ml of $\text{H}_2\text{SO}_4$ in 37.5 ml of distilled water.
54. $\text{H}_2\text{SO}_4$ (0.1N) - 3.6 ml made up to 100 ml with distilled water.
55. HCl (0.5N) - 4.424 ml HCl was made up to 100 ml with distilled water.
56. HCl (3N) - 66.37 ml of HCl in 250 ml of distilled water.
57. Heparin-manganese chloride solution - 3.167 g of manganese chloride was added to 1 ml solution of heparin containing 20,000 units. The mixture was made up to 8 ml with distilled water.
58. Hydroxylamine hydrochloride reagent for HMG CoA - Equal volumes of hydroxylamine hydrochloride (2 M/litre) and sodium hydroxide (4.5 M) solution were mixed freshly before use.
59. Hypo solution (0.02%) - 20 mg of sodium thio sulphate was dissolved in 100 ml of distilled water.
60. Ice-cold lysis solution - 2.5 M NaCl, 100 mM EDTA-Na, 10 mM Tris, 10% DMSO, 1% Triton X-100 and adjust the pH to 10.
61. KCl (0.15 M) - 3.772 g was dissolved in 500 ml distilled water.
62. Magnesium chloride (0.05 M) - 0.476g in 100 ml of distilled water.
63. Methylation reagent - 325 ml of 6 N HCl mixed with 275 ml methanol.
64. MTT - Dissolve 5 mg of MTT in 1 ml of DMSO and the resulting solution should appear bright yellow in color.
65. NADH (780 µM) - 5 mg in 10 ml of phosphate buffer.
66. NADP⁺ (0.1 M) - 0.765 g in 10 ml of phosphate buffer.
67. Nitroblue tetrazolium (NBT) - 300 µM - 3 mg in 10 ml of distilled water.
68. Agarose normal melting point (1%) - 1 g of agarose was dissolved in 100 ml of double distilled water.
69. O-toluidine reagent - 12.5 g of thiourea and 12 g of boric acid were dissolved in 50 ml of distilled water by heating over a mild flame. 75 ml of redistilled O-toluidine and 375 ml of acetic acid were mixed and the total volume was made up to 500 ml with distilled water. The reagent was left in a refrigerator overnight and filtered.
70. Oxalate (0.1 M) - 134 mg of sodium oxalate was dissolved in 10 ml of 2 N HCl.
71. Palmitic acid standard - A solution containing 2 mg/ml of palmitic acid in chloroform was used as stock. Working standard containing 200 µg/ml was prepared by diluting 1 ml of stock to 10 ml with chloroform.
72. Perchloric acid diluted - 50 ml of Perchloric acid was made up to 1 litre using distilled water.
73. Periodic acid (0.025 M) - 0.569 g in 100 ml of 0.1N H$_2$SO$_4$.

74. Phenazine methosulphate (PMS) - 186 μM - 5 mg in 10 ml of distilled water.

75. Phosphate buffer (0.01M, pH 7.0) - 156 mg NaH$_2$PO$_4$ and 142 mg of Na$_2$HPO$_4$ in 100 ml of distilled water and adjust the pH to 7.0.

76. Phosphate buffer (0.2 M, pH 8.0) - 3.12 g NaH$_2$PO$_4$ and 2.84 g of Na$_2$HPO$_4$ in 100 ml distilled water and adjust the pH to 8.0.

77. Potassium dichromate - 5 g of Potassium dichromate solution was dissolved in 100 ml of distilled water.

78. Potassium dihydrogen phosphate (0.0125 M) - 0.170g in 100 ml of distilled water.

79. Phosphate buffer pH 7.4 (10 mM) - Add 8 ml of K$_2$HPO$_4$ and 2 ml of KH$_2$PO$_4$ and made up to 1000 ml using distilled water and adjust the pH to 7.4.

80. Phosphate buffer pH 8.2 (10 mM) - Add 9.4 ml of K$_2$HPO$_4$ and 0.6 ml of KH$_2$PO$_4$ and made up to 1000 ml using distilled water and adjust the pH to 8.2.

81. Reduced glutathione (30 mM) - 460.5 mg of glutathione was dissolved in 5 ml of distilled water.

82. Rehydration buffer - 8 M urea, 20 mg Bio-lytes, 2% CHAPS and 77 mg DTT and a trace of bromophenol blue to a total volume of 350 μl in distilled water.

83. Saponification reagent - 5 g of potassium hydroxide was dissolved in 60 ml of distilled water and 40 ml of isopropanol was added to it.

84. Sialic acid standard - 179 mg of orosomucoid was dissolved in 100 ml of distilled water.

85. Silver nitrate solution - 200 mg was dissolved in 100 ml of distilled water.

86. Sodium azide (10 mM) - 65 mg of sodium azide in 100 ml distilled water.

87. Sodium fluoride (0.5 M) - 0.419g in 100 ml of distilled water.

88. Sodium hydroxide (1 M) - 1g NaOH in 25 ml distilled water.

89. 4 % Sodium meta arsenate - 4 g of Sodium meta arsenate in 0.5N HCl (4.424 ml HCl made upto 100 ml with distilled water).

90. Sodium meta periodate reagent - 77 g of anhydrous ammonium acetate was dissolved in about 700 ml of distilled water, 60 ml glacial acetic acid was added to it followed by 650 mg of sodium meta periodate. The mixture was diluted to 1 litre with distilled water.

91. Sodium pyrophosphate buffer (0.025 M, pH 8.3) - 1.38g of sodium pyrophosphate in 100 ml 0.24 ml of HCl + 49.76 ml of distilled water.
92. Stacking buffer - 0.1% SDS, 0.25 mol/L Tris, 0.192 mol/L glycine, supplemented with bromophenol blue as a tracking dye.

93. Standard phosphorus solution - 35.1 mg of potassium dihydrogen phosphate was dissolved in distilled water. To this 1 ml of 10 N H₂SO₄ was added and made up to 100 ml with distilled water. 10 ml of this solution was diluted to 100 ml, to prepare a working standard containing 8 μg phosphorus/ml.

94. Standard triolein solution - 1 g of triolein was dissolved in 100 ml isopropanol. 1 ml of stock standard was diluted to 100 ml to prepare a working standard 100 μg of triolein/ml.

95. Stop solution - citric acid (2.3M) - 22.1 g of citric acid was dissolved in 50 ml of the developing solution.

96. TCA (10 %) - 10 g of TCA in 100 ml distilled water.

97. TCA-TBA-HCl reagent - 15 % TCA - 15 g of TCA in 100 ml distilled water: 0.25N HCl - 0.25N-0.275 ml made up to 100 ml with distilled water: Thiobarbituric acid (TBA) - 0.38% - 38g in 100 ml of hot distilled water: TCA - TBA - HCl reagent - (1 : 1 : 1 v/v)

98. Triethanolamine (1 M) - 2.67 ml of Triethanolamine was dissolved in 20 ml distilled water.

99. Tris-HCl buffer - 0.1 M, pH 7.0 - 0.157 g in 100 ml of distilled water.
Effect of *Gymnema montanum* leaves on red blood cell resistance to oxidative stress in experimental diabetes

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**Abstract** The aim of the present study was to evaluate the protective effect of *Gymnema montanum* on red blood cell (RBC) membrane in diabetic rats during lipid peroxidation. Ethanol extract of *G. montanum* leaves (GLEt) was administered orally to alloxan-induced diabetic rats for 3 weeks, and the effects on blood glucose, insulin, lipid peroxidation markers, thiobarbituric acid reactive substances, hydroperoxides in plasma and antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase activities in erythrocytes were studied. Administration of GLEt to diabetic animals at doses of 50, 100, and 200 mg/kg body weight lowered elevated blood glucose levels by 24, 35, and 66%, respectively, relative to untreated diabetic rats. In comparison, treatment with the known antidiabetic drug, glibenclamide (600 μg/kg body weight) decreased blood glucose concentrations by 51%. Plasma insulin concentrations were increased in the diabetic rat by 73% with GLEt (200 mg/kg body weight) and 45% with glibenclamide (600 μg/kg body weight). Although a significant decrease in the lipid peroxidation markers was observed in plasma on treatment with GLEt and glibenclamide, the RBC antioxidant levels were increased significantly in diabetic rats. Furthermore, erythrocytes from the GLEt-treated animals were found to be more resistant to H₂O₂-induced peroxidation than that of untreated diabetic animals. The chemical characterization of the polyphenolics of the extract showed the presence of gallic acid (5.29% w/w), resveratrol (2.2% w/w), and quercetin (16.6% w/w). The results of this study suggest that *G. montanum* may be useful for the control, management, and prevention of oxidative stress associated with diabetes.

**Keywords** *Gymnema montanum* • In vitro lipid peroxidation • Erythrocytes • Diabetes • Antioxidants • Free radicals

**Introduction**

Oxidative stress has been suggested to be a common pathway-linking diverse mechanism for the pathogenesis of complications in diabetes (Bandyopadhyay 2004; Baynes 1991). The increase in oxygen free...
radicals (OFR) in diabetes could be because of elevated blood glucose levels, which upon autoxidation generates free radicals and damages the cell membrane through peroxidation of membrane lipids (Baynes and Thrope 1999) and protein glycation (Hunt et al. 1988). The lipid peroxidation of the cell membrane has been associated with a number of pathologic phenomena such as increased membrane rigidity, decreased cellular deformability, and lipid fluidity in erythrocytes (Selvam and Anuradha 1988).

Alloxan is used to induce diabetes in laboratory animals, because of its structural similarity to glucose as well as the β-cell’s highly efficient uptake mechanism. The mechanism of action has been intensively studied, predominantly by generating reactive free radicals that have been shown to be cytotoxic to the β-cells of the pancreas, thereby leading to hyperglycemia (Szkudelski 2001). Thus, alloxan-induced diabetes could elicit the antioxidant defense system in response to increased oxidative stress.

The disturbances of antioxidant defense systems in diabetes were shown to cause alteration in antioxidant enzymes (Strain 1991), impaired glutathione metabolism (Mc Lennan et al. 1991), and decreased ascorbic acid levels (Jennings et al. 1987). Administration of antioxidants has been shown to reduce the oxidative stress in diabetic animals (Sabu and Kuttan 2002). As several epidemiological studies show a significant inverse relationship between the dietary intake of antioxidants and the risk of various diseases (review of Kinsella et al. 1993), an intensive search for novel type of antioxidants is being carried out. Many plant extracts and plant products have been shown to have significant antioxidant activity (Anjali and Manoj 1995).

In recent times, many traditionally used medicinally important plants were proved for their antidiabetic potential by various investigators (Grover et al. 2002, Sabu and Kuttan 2002). In our earlier studies, Gymnema montanum Hook (Asclepiadaceae), an endemic plant species found mainly in Western Ghats, India (Vajravelu and Bhargavan 1983), was found to have modulatory effect on rate-limiting enzymes of glycolysis and gluconeogenesis (Ananthan et al. 2003a), antihyperlipidemic effect (Ananthan et al. 2003b), and antiperoxidative effect in diabetic rats (Ananthan et al. 2004; Ramkumar et al. 2004). It has also been demonstrated for preventing the cholinergic neural and retinal complications of hyperglycemia in diabetes (Ramkumar et al. 2005). However, no other information about the pharmacological properties of this plant has yet appeared. These facts justify our interest in further studies of this plant. Hence, this study was designed to investigate the protective effect of G. montanum leaf extract on erythrocytes against oxidative stress in alloxan diabetic rats. The ethanol extract of G. montanum leaves (GLEt) was also analyzed for the major phenolic components using high-performance thin layer chromatography (HPTLC).

**Methods**

Experimental animals

Male albino Wistar rats of 170–200 g body weights (b.w.) were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai Nagar, India and were maintained at Bharathidasan University under a constant 12-h light and dark cycle at 21–23°C. The animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and approved by the ethical committee, Bharathidasan University, Tamilnadu. Throughout the experimental period, the animals were fed with a balanced commercial diet (Hindustan Lever Ltd., Mumbai, India; composition of the diet: 5% fat, 21% protein, 55% nitrogen free extract, and 4% fiber (w/w) with adequate mineral and vitamin contents) and water ad libitum.

Preparation of GLEt

G. montanum leaves were collected freshly from the Shola forests of Western Ghats, Gudalur, The Nilgiri Biosphere Reserve at an altitude of 900–1,500 m above the mean sea level. The plant was identified at the Herbarium of Botanical Survey of India, Southern Circle, Coimbatore, India (accession no. 32561-65) and was deposited in the Department of Biotechnology, Bharathidasan University. To prepare the extract (GLEt), 500 g of fresh leaves of G. montanum were chopped into small pieces and soaked overnight in 1.5 l of 95% ethanol. This suspension was filtered, and the residue was resuspended in an equal volume of 95% ethanol for 48 h and filtered. The two filtrates were pooled, and the solvents were evaporated in a
rotavapor at 40–50°C under reduced pressure. The greenish-black residue (approximately 25 g) was obtained, lyophilized, and stored at 0–4°C until further use. When needed, the residue was suspended in distilled water and used.

Induction of experimental diabetes

Rats were injected intraperitoneally with freshly prepared solution of alloxan monohydrate in normal saline at a dose of 150 mg/kg b.w. (Al-Shamaony et al. 1994). To prevent fatal hypoglycemia associated with alloxan treatment, rats were treated with 20% glucose solution (5–10 ml) orally after 6 h for the next 24 h. Then, the rats were provided with 5% glucose solution in their cages. After 2 weeks, rats with moderate diabetes that exhibited glycosuria and hyperglycemia (i.e., blood glucose concentration 200–300 mg/dl) were included in the experiment.

Experimental design

A total of 42 rats (30 diabetic rats and 12 normal rats) were divided into 7 groups of 6 animals each. The treatment groups were summarized as follows: group 1, normal untreated rats; group 2, normal untreated rats given GLEt (200 mg/kg b.w.); group 3, diabetic control rats; group 4, diabetic rats given GLEt (50 mg/kg b.w.); group 5, diabetic rats given GLEt (100 mg/kg b.w.); group 6, diabetic rats given GLEt (200 mg/kg b.w.); group 7, diabetic rats given glibenclamide (600 μg/kg b.w.). The plant extract and the drug glibenclamide were prepared in an aqueous solution and given daily using an intragastric tube for 3 weeks. Fasting blood glucose was monitored every 7 days throughout the experiment.

At the end of the experimental period, the animals were deprived of food overnight and then killed by decapitation. Blood was collected into heparinized tubes. Plasma was separated by centrifugation at 4°C and stored at −20°C until determination of insulin, thiobarbituric acid reactive substances (TBARS), and hydroperoxides.

The red blood cells (RBCs) that remained after the removal of plasma were washed with isotonic saline (0.89% NaCl), and buffy coat was removed. The RBCs were washed again with isotonic saline and further processed for the preparation of hemolysate and the erythrocyte membrane.

Biochemical assays

Fasting blood glucose was estimated by O-toluidine method (Sasaki et al. 1972). Plasma insulin was estimated using enzyme-linked immunosorbent assay kit (Boehringer Mannheim, Germany; Anderson et al. 1993).

Oral glucose tolerance test (OGTT) was performed according to the method of Du Vigneaud and Karr (1975). After overnight fasting, ‘0’ minute blood sample was taken from the rats by cyanacular puncture in normal and experimental rats. Without delay, a glucose solution (2 g/kg b.w.) was administered by gavage. Then, blood samples were taken at 30, 60, 90, and 120 min after glucose administration. The blood samples were collected with potassium oxalate and sodium fluoride and analyzed for their glucose content by O-toluidine method (Sasaki et al. 1972).

Lipid peroxidation in plasma was assessed by analyzing the formation of TBARS spectrophotometrically by the method of Nichans and Samuelsion (1968) and of lipid hydroperoxides by the method of Jiang et al. (1992). Oxidation of ferrous ion in the presence of xylene orange leads to the formation of chromophore, which was measured at 560 nm.

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities were determined in hemolysate. SOD was assayed by the method of Kakkar et al. (1984), based on the inhibition of the formation of nicotinamide adenine dinucleotide (reduced form)–phenazine methosulfate–nitro blue tetrazolium (NBT) formazan. The amount of enzyme that gave 50% inhibition of NBT reduction/mg hemoglobin was taken as one unit of the enzyme activity. CAT (Sinha 1972) and GPx (Rotruck et al. 1984) activities were assayed by measuring the amount of the substrate consumed (hydrogen peroxide and glutathione, respectively).

Erythrocyte lipid peroxidation was determined according to the method of Cynamon et al. (1985). Malondialdehyde (MDA), a product of polyunsaturated fatty acid peroxidation, was measured as a thiobarbituric acid derivative in the supernatants after incubation of erythrocytes with two different concentrations of hydrogen peroxide. When normal RBCs were exposed to peroxides in vitro, oxidation of antioxidants results in the release of TBARS that can be detected. Sodium azide, an inhibitor of CAT, prevents the destruction of hydrogen peroxide. The
release of lipid peroxides without CAT inhibition can be considered as a reflection of erythrocyte membrane antioxidant protection and that with CAT inhibition as the maximum release possible. The percentage of maximal MDA release was calculated according to the following equation:

\[
\text{percent maximal release (\%MDA)} = \frac{\text{MDA release}(3\%H_2O_2)}{\text{MDA release}(0.75\%H_2O_2 + \text{azide})} \times 100.
\]

Estimation of polyphenolics using HPTLC

The GLEt was dissolved in ethanol at a concentration of 1 mg/ml, and 10 µl of the aliquot was spotted as bands of 10 mm length over HPTLC silica gel plate with a 100 µm silica coating (E. Merck KGaA-silica gel 60F 254, Darmstadt, Germany) at 12 mm from the lower edge using an automatic TLC Sampler III apparatus (WinCATS Planar Chromatography Manager). After 10 min, the chromatogram was developed with toluene/ethyl acetate/glacial acetic acid (12.5:7.5:0.5 v/v) as mobile phase. The chromatogram was run at 48°C until the mobile phase travels approximately 16 cm in distance. Then the plates were air dried for 10 min and scanned at 260 nm (Shrikumar et al. 2005). Gallic acid, resveratrol, and quercetin were used as standards and run along with the sample. The compounds were quantified using a calibration curve obtained by plotting the concentration of standards against the peak area on the scanned chromatogram.

Statistical analysis

All data were expressed as mean±SD of number of experiments. The Kolmogorov–Smirnov goodness-of-fit test was used to control whether the distribution of parameters was normal. Groups of data were compared by analysis of variance (ANOVA; one-way ANOVA) followed by Duncan’s multiple range test (DMRT) using SPSS version 7.5 (SPSS, Cary, NC, USA). Results were regarded as being significant when \( p \) values were lower than 0.05.

Results

When compared with the diabetic control group, the mean body weight in the GLEt-treated group rats increased significantly \((p<0.001)\) at the end of third week (data not shown). The food and water intake was significantly less in GLEt-treated groups than diabetic control rats. There was no significant difference observed between the GLEt-treated groups and glibenclamide-treated group. In addition, the oral administration of the plant extract modified the animal food intake, which is evidenced by feed conversion ratio (food intake/weight gain; data not shown).

In all animals before alloxan administration, the basal levels of blood glucose were not significantly \((p<0.05)\) different. However, 15 days after alloxan administration, blood glucose levels were significantly higher \((p<0.05)\) in the experimental rats that were selected for the study. Table 1 shows the effect of GLEt on blood glucose levels. Although a significant antihyperglycemic effect was evident from the first week onward, it was maximum on the third week. A significant increase in plasma insulin level was observed in animals receiving GLEt in a dose-dependent fashion. As the increase in plasma insulin was maximum at 200 mg/kg b.w., further biochemical studies were conducted on rats that received 200 mg GLEt/kg b.w. only. Normal rats treated with 200 mg/kg b.w. of GLEt did not show any variation in blood glucose level with that of untreated control rats.

Results of OGTT are presented in Fig. 1. In diabetic rats, blood glucose levels reached a peak 1 h after glucose administration, although it started to decline but remained higher even after 2 h. However, in GLEt-treated animals, blood glucose level remained low at all sampling time points. Normal rats treated with GLEt also showed a significant decrease in glucose levels at 2 h.

The extent of lipid peroxidation in plasma is presented in Table 2. TBARS and hydroperoxides were significantly \((p<0.05)\) increased in diabetic rats. Treatment with GLEt significantly \((p<0.05)\) brought down the levels of these lipid peroxide markers to near normal levels. No significant alteration was observed in normal rats treated with GLEt.

For studying the effect of GLEt on free radical production, the activities of SOD, CAT, and GPx were measured in hemolysate of the control and experimental animals (Table 3). The activities of all the three enzymes were significantly increased in the GLEt treatment group when compared to the diabetic control rats. GLEt administration to normal rats did not alter the levels of these antioxidant enzymes.
Table 1  Changes in the levels of blood glucose and plasma insulin of normal and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood glucose (mg/dl)</th>
<th>Plasma insulin (μU/ml) after 3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'0' day</td>
<td>15 days after alloxan injection</td>
</tr>
<tr>
<td>Normal</td>
<td>79±3.03</td>
<td>84±5.14 *</td>
</tr>
<tr>
<td>Normal + GLEt (200 mg kg⁻¹)</td>
<td>84±5.59</td>
<td>83±4.66 *</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>81±4.94</td>
<td>265±19.45 *</td>
</tr>
<tr>
<td>Diabetic + GLEt (50 mg kg⁻¹)</td>
<td>80±0.57</td>
<td>255±16.3 *</td>
</tr>
<tr>
<td>Diabetic + GLEt (100 mg kg⁻¹)</td>
<td>83±6.59</td>
<td>248±14.02 *</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg kg⁻¹)</td>
<td>79±5.09</td>
<td>258±18.61 *</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 mg kg⁻¹)</td>
<td>77±4.48</td>
<td>245±13.99 *</td>
</tr>
</tbody>
</table>

One-way ANOVA

F 38.09  56.63  56.64  42.85  32.62
p <0.01 <0.01 <0.01 <0.01 <0.01

Values are given as mean±SD for six rats in each group. Statistical evaluation was done by ANOVA followed by DMRT.

Values not sharing a common superscript letter differ significantly at p<0.05.

In the in vitro lipid peroxidation studies, in the presence of 3% H₂O₂, erythrocytes of the diabetic control and GLEt-treated animals showed significantly greater release of lipid peroxides than the untreated animals. The level of lipid peroxidation was higher in the presence of sodium azide in untreated diabetic rats. Lipid peroxidation in RBCs incubated with and without H₂O₂ was significantly lower in GLEt-treated rats. Erythrocytes of the diabetic rats showed significantly higher release of lipid peroxides than the untreated rats. The percent maximal release of lipid peroxides was also significantly less in GLEt-treated rats than in the diabetic rats. No remarkable changes were observed in normal rats treated with GLEt (Table 4).

The preliminary phytochemical testing of GLEt indicated the presence of high amount of phenolics along with flavonoids and alkaloids (unpublished data). The HPTLC analysis of GLEt showed six major peaks having Rf values 0.03, 0.08, 0.37, 0.39, 0.41, and 0.95 (Fig 2). The Rf values of the three peaks coincide with that of gallic acid (0.08), resveratrol (0.37), and quercetin (0.41). The content of gallic acid was found to be 5.29% w/w, resveratrol 2.2% w/w, and quercetin 16.6% w/w. The other three compounds remained to be identified.

Discussion

Erythrocytes are the most sensitive configuration that is getting damaged by the action of OFR species. It has been stated that the damaging action of OFR...
Table 2 Change in the levels of plasma TBARS and hydroperoxides in normal and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS (mmol dl⁻¹)</th>
<th>Hydroperoxides (10⁻⁵ mmol dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.15±0.011a</td>
<td>9.95±0.65a</td>
</tr>
<tr>
<td>Normal + GLEt (200 mg kg⁻¹)</td>
<td>0.17±0.015a</td>
<td>10.08±1.18a</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>0.36±0.024b</td>
<td>14.68±1.99b</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg kg⁻¹)</td>
<td>0.20±0.014c</td>
<td>10.75±1.30a</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 µg kg⁻¹)</td>
<td>0.24±0.022d</td>
<td>12.96±1.76e</td>
</tr>
<tr>
<td>One-way ANOVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>45.86</td>
<td>11.89</td>
</tr>
<tr>
<td>df</td>
<td>4.25</td>
<td>4.25</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are given as mean±SD for six rats in each group. Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript letter differ significantly at p<0.05.

could occur by membrane lipid oxidation leading to lysis of RBCs (Simic et al. 1989). Previous evidence suggested that the free radical generation was increased during diabetes (Feillet-Coudray et al. 1999; Datta et al. 2000). Therefore, the present study was designed to evaluate the protective property of an antidiabetic plant G. montanum, against oxidative challenge in RBCs of diabetic rats.

Administration of G. montanum significantly reduced blood glucose levels in diabetic rats as observed earlier (Ananthan et al. 2003a). The fall in blood glucose levels was dose dependent and was highly significant at 200 mg/kg. The capacity of GLEt to decrease the elevated blood glucose is an essential trigger for the liver to revert to its normal homeostasis. Furthermore, the plasma insulin level was found to increase in GLEt-treated diabetic animals. The possible mechanism by which GLEt exerts its hypoglycemic action in diabetic rats may be because of stimulating the insulin release. The active constituents of G. montanum that are responsible for the blood glucose lowering and insulin stimulatory effect need to be clarified.

GLEt might enhance glucose utilization, as it significantly reduces blood glucose in diabetic rats. From the data obtained with the OGTT, it is clear that blood glucose levels reached a peak and returned to fasting values after 2 h in both normal and GLEt-treated diabetic rats. In untreated diabetic rats, blood glucose levels remained high even after 2 h. GLEt administration effectively prevented the increase in blood glucose without causing a hypoglycemic state and possibly because of the restoration of the delayed insulin response.

Lipid peroxide-mediated damage in erythrocytes has been observed in the development of both Type I and Type II diabetes mellitus. It has been reported that reactive oxygen species, superoxide anions, and hydroxyl radicals enhance the lipid peroxidation that damages the cell membrane and its physiological functions leading to disturbances in membrane integrity (Datta et al. 2000). Elevation of lipid peroxidation

Table 3 Activities of enzymatic antioxidants in hemolysate of normal and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (Units/mg Hb)</th>
<th>CAT (µmol/min/mg Hb)</th>
<th>GPx (µg/min/mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.18±0.25a</td>
<td>60.44±5.48a</td>
<td>10.14±1.67a</td>
</tr>
<tr>
<td>Normal + GLEt (200 mg kg⁻¹)</td>
<td>2.36±0.015b</td>
<td>63.58±7.37a</td>
<td>10.89±1.95a</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>1.51±0.17c</td>
<td>45.72±6.42b</td>
<td>6.52±1.53b</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg kg⁻¹)</td>
<td>1.98±0.14d</td>
<td>57.16±4.30a</td>
<td>9.24±1.32c</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 µg kg⁻¹)</td>
<td>1.99±0.22d</td>
<td>58.94±5.76d</td>
<td>9.89±1.46c</td>
</tr>
<tr>
<td>One-way ANOVA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>30.25</td>
<td>7.62</td>
<td>9.22</td>
</tr>
<tr>
<td>df</td>
<td>4.25</td>
<td>4.25</td>
<td>4.25</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are given as mean±SD for six rats in each group. Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript letter differ significantly at p<0.05.
Table 4 Susceptibility of RBC to H$_2$O$_2$ induced lipid peroxidation in vitro in normal and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cells untreated</th>
<th>3% H$_2$O$_2$</th>
<th>0.75% H$_2$O$_2$ + sodium azide</th>
<th>Maximal release (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.86±0.32</td>
<td>4.62±0.38</td>
<td>48.45±1.52</td>
<td>9.53±0.42</td>
</tr>
<tr>
<td>Normal + GLEt (200 mg kg$^{-1}$)</td>
<td>3.18±0.18</td>
<td>4.10±0.26</td>
<td>41.26±1.76*</td>
<td>9.94±0.56</td>
</tr>
<tr>
<td>Diabetic</td>
<td>4.94±0.28</td>
<td>7.28±0.49*a</td>
<td>56.16±2.32*a</td>
<td>12.96±0.84*a</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg kg$^{-1}$)</td>
<td>4.04±0.12</td>
<td>5.72±0.26$b$</td>
<td>51.84±2.78$b$</td>
<td>10.34±0.76*b</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 µg kg$^{-1}$)</td>
<td>4.36±0.22</td>
<td>6.68±0.38$^{ab}$</td>
<td>61.88±3.10$^{ab}$</td>
<td>10.79±0.82$^{ab}$</td>
</tr>
</tbody>
</table>

$F$ 51.58  1078  64.12  36.42
$df$ 4.25  4.25  4.25  4.25
$p$  <0.05  <0.05  <0.05  <0.05

Values given as nmol/mg Hb. Values are given as mean±SD for six rats in each group.

Statistical evaluation was done by ANOVA followed by DMRT ($p<0.05$).

1 Significantly different from control group.
2 Significantly different from diabetic control.

$^{*}$%Maximal release = \( \frac{\text{TBARS release}(\text{3%H}_2\text{O}_2)}{\text{TBARS release}(0.75\% \text{H}_2\text{O}_2 + \text{sodium azide})} \times 100 \)

Levels in erythrocytes is one of the characteristic features of chronic diabetes (Selvam and Anuradha 1988). In the current study, a significant increase in TBARS and hydroperoxides levels was observed in plasma of diabetic rats. The administration of GLEt and glibenclamide tend to bring the peroxides back to near normal level. This indicates that GLEt may inhibit oxidative damage and protect the membrane.

Associated with the changes in lipid peroxidation, diabetic erythrocytes showed decreased activity of antioxidant enzymes SOD, CAT, and GPx, which play an important role in scavenging toxic free radicals. These results are consistent with the previous findings, and the decreased erythrocyte membrane bound enzyme activities can be attributed to altered membrane fluidity and enhanced lipid peroxidation in diabetic rats (Selvam and Anuradha 1988). GLEt administration resulted in significant increase in activities of these antioxidant enzymes in erythrocytes of diabetic rats. The excess availability of antioxidants upon GLEt supplementation facilitates RBCs to enrich the antioxidant content, which could prevent the membrane lipid peroxidation and protect from toxic effects because of the free radicals. The antioxidant properties of GLEt could be directly linked to both a direct reaction with free radicals as
scavengers or indirectly by increasing the levels of enzymic antioxidants.

Our studies revealed that the erythrocytes and erythrocyte membranes of the diabetic rats are found to be more susceptible to H_2O_2 attack and consequently release higher amount of MDA when compared to the normal rats. In this context, it can be suggested that the RBCs incubated with sodium azide, an inhibitor of CAT, resulted in higher release of TBARS in erythrocytes, and this reflects that the erythrocytes isolated from diabetic rats were already damaged by oxidative stress. The enhanced percent maximal release of lipid peroxides from erythrocytes of diabetic rats might be attributed to impairment of antioxidant protection and loss of functional integrity of membrane. The fluidity of erythrocyte membrane derived from diabetic rats is lower, and the membrane was easily susceptible against hemolysis induced by peroxyl radicals (Selvam and Anuradha 1988).

Administration of GLEt and glibenclamide results in the reduction of RBC lipid peroxidation and was brought down to near normal levels that suggests the protective property of these substances on the cell membrane composition and cellular antioxidant potential. Any compound, natural or synthetic, with antioxidant properties might contribute toward the partial or total alleviation of this damage. Therefore, removing OH· and OH* is probably one of the most effective defenses against diseases (Halliwell and Gutteridge 1984). Results of this study clearly show that GLEt contains a free radical scavenging activity and exerts a beneficial action against pathologic alterations caused by the free radicals. The strong radical scavenging activity of GLEt may be because of the presence of polyphenolic compounds. Furthermore, the results demonstrate the dose-dependent protective effect of polyphenols from GLEt on alloxaan-induced RBC lipid peroxidation.

Numerous epidemiological studies have shown the relationship between phenolics and various diseases mediated by oxidative stress. Many phenolic phytochemicals have antioxidative, anticarcinogenic, antimicrobial, antiallergic, antimutagenic, antidiabetic, and anti-inflammatory activities (Ho 1992). Among the numerous polyphenolics, gallic acid, resveratrol, and quercetin are widely distributed in the plant kingdom and are reported to possess antioxidant, antidiabetic, and anticancer properties (Lopez-Velez et al. 2003). The GLEt was found to contain 5.29% w/w of gallic acid, 2.2% w/w of resveratrol, and 16.6% w/w of quercetin. Based on these results, the phenolic composition present in GLEt possibly plays a significant role in protecting RBCs from oxidative insult caused by hyperglycemia.

In conclusion, the above observations show that the ethanol extract of G. montanum leaves possesses strong antioxidant activity possibly because of the presence of polyphenols such as gallic acid, resveratrol, and quercetin, which could exert a beneficial action against pathological alterations, caused by superoxide and hydroxyl radicals in alloxaan-induced diabetes.

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Ethanol extract of *Gymnema montanum* leaves reduces glycoprotein components in experimental diabetes

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**Abstract**

In the present study, the modulatory effects of *Gymnema montanum* leaves on glycoprotein levels in alloxan-induced diabetic rats were examined. The ethanol extract of *G. montanum* leaves was administered orally (200 mg/kg of body weight) for 3 weeks. The effect of *G. montanum* leaves on blood glucose, plasma insulin, and plasma/tissue glycoproteins was compared with that of a reference drug, glibenclamide (600 μg/kg of body weight). The levels of blood glucose and plasma glycoproteins were increased significantly, whereas the levels of plasma insulin and sialic acid were decreased in diabetic rats. The levels of hexose, hexosamine, and fucose in the liver and kidney of alloxan-diabetic rats increased significantly. Oral administration of the ethanol extract of *G. montanum* leaves to diabetic rats reversed these hyperglycemia-induced biochemical changes. The observed antihyperglycemic effect was found to be positively correlated with the total phenolic content of the extract. The present study indicates that the *G. montanum* leaves possess a significant beneficial effect on glycoprotein moiety in addition to its antidiabetic effect. Thus, the results of the present study indicate a positive role of *G. montanum* as a therapeutic agent for diabetes.

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**Keywords**

*Gymnema montanum*, Alloxan diabetes, Glycoproteins, Glucose, Total phenolics, Rats

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**1. Introduction**

Medicinal plants continue to provide valuable therapeutic agents, both in modern and in traditional medicine. The uncertainty about the efficacy and safety of the currently available oral hypoglycemic drugs has prompted a search for safer and more effective agents in the treatment of diabetes [1]. A wide variety of the traditional herbal remedies are used by diabetic patients, especially in the third world countries [2], and may, therefore, represent new avenues in the search for alternative hypoglycemic drugs. Because numerous research findings suggested that the phenolic compounds present in plants largely contribute to their protective properties, the correlation between total phenolic content and their role in diabetes was studied [3].

Diabetes mellitus (DM) is widely recognized as one of the leading causes of death in the world [4]. It is defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated, resulting in elevated blood glucose levels. The long-term effects of DM include dysfunction and failure of various organs. The development of diabetic complications such as diabetic retinopathy, diabetic neuropathy, diabetic nephropathy, and diabetic ketoacidosis are the major causes of morbidity and mortality in diabetic populations [5]. Impaired metabolism of glycoproteins plays a major role in the pathogenesis of DM [6]. It has been reported that alterations occur in the concentrations of various membrane-bound carbohydrates (glycoproteins) in human diabetes [7].
Glycoproteins are rich in extracellular matrix, and they contribute a major source to the structure of the matrix [8]. These groups of macromolecules carry out numerous biologic functions including lipid and hormone transport, hemoglobin binding, and blood coagulation. In the diabetic state, glucose is used by the insulin-independent pathways, leading to the synthesis of oligosaccharide moieties of glycoprotein, hexose, hexosamine, fucose, and sialic acid have an important role in protein stability, function, and turnover [6]. Raised levels of glycoproteins in diabetics may also be a predictor of angiopathic complications [9]. In recent times, many traditionally medicinal important plants have been tested for their efficacy against impaired glycoprotein levels in diabetes [10,11].

Gymnema montanum Hook, an Asclepiadaceae family member, is an endemic plant species found mainly in Western Ghats, India [12]. Several researchers have reported that Gymnema extracts (Gymnema sylvestre, Gymnema inodorum, Gymnema yunnanense) may actually help to repair or regenerate the pancreas beta cells that are responsible for insulin secretion [13-15]. In traditional systems of Indian medicine, Gymnema sp is used for diabetes treatment, as a diuretic, and a digestive stimulant [16]. In our previous studies, we reported the effect of G. montanum on rate-limiting enzymes of glycolysis and gluconeogenesis in the liver and circulation of diabetic rats [17] and its antihyperlipidemic and antioxidant effects in the liver and kidney [18-21]. In addition, it has been shown to prevent the cholinergic neural and retinal complications of hyperglycemia in diabetes [22]. To our knowledge, there are no available reports on the effect of this plant on glycoprotein levels in diabetic rats. Hence, the present study was carried out to determine the effect of ethanol extract of the G. montanum leaves (GLEt) on plasma and tissue glycoproteins in alloxan-induced diabetic rats. In this investigation, glibenclamide was used as the reference drug.

2. Methods and materials

2.1. Animals and diet

Male albino Wistar rats (body weight, 170-200 g) were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalainagar, India, and were maintained under a constant 12-hour light-dark cycle at 22°C to 23°C. The animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The study was approved by the institutional ethical committee, Bharathidasan University, Tamil Nadu, India. Throughout the experimental period, the rats were fed a balanced commercial pellet diet (Hindustan Lever Ltd, Mumbai, India) with a composition of 5% fat, 21% protein, 55% nitrogen-free extract, and 4% fiber (wt/wt) with adequate mineral and vitamin levels for the rat. Diet and water were provided ad libitum.

2.2. Drugs and chemicals

All the chemicals used in this experiment were obtained from Sigma Chemical Company (St Louis, Mo), Hi Media (Mumbai, India), or SD-Fine Chemicals (Mumbai, India). All chemicals used were of analytical grade.

2.3. Plant material

G. montanum leaves were collected from the Shola forests of the Nilgiri Biosphere Reserve in Western Ghats, Gudalur, at an elevation of 900 to 1500 m above sea level. The plant was identified at the Herbarium of Botanical Survey of India, Southern Circle, Coimbatore, India (accession no. 32561-65) and was deposited in the Department of Botany, Bharathiar University, Coimbatore, India.

2.4. Preparation of GLEt

To prepare the extract (GLEt), 500 g of fresh leaves of G. montanum was chopped into small pieces and soaked overnight in 1.5 L of 95% ethanol. This suspension was filtered, and the residue was resuspended in an equal volume of 95% ethanol for 48 hours and filtered. The 2 filtrates were pooled, and the solvent was evaporated in a rotavapor at 40°C to 50°C under reduced pressure. The greenish-black residue (≈ 25 g) was lyophilized and stored at 0°C to 4°C until further use. When needed, the residue was suspended in distilled water and used in the study.

2.5. Induction of experimental diabetes

Rats were injected intraperitoneally with a freshly prepared solution of alloxan monohydrate in normal saline at a dose of 150 mg/kg of body weight [23]. Because alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution (5-10 mL) orally after 6 hours for the next 24 hours to prevent hypoglycemia. Then, rats were provided with 5% glucose solution in their cages. Neither death nor any other adverse effect was observed at the tested concentration throughout the study. After 2 weeks, rats with moderate diabetes (ie, blood glucose concentration, 200-300 mg/dL) that exhibited glycosuria and hyperglycemia were selected for the experiment.

2.6. Experimental design

Groups of 6 diabetic rats were treated with graded concentrations of GLEt (25, 50, 100, and 200 mg/kg of body weight) for 3 weeks. A positive control group of 6 diabetic animals was treated with glibenclamide (600 µg/kg of body weight) [24]. A group of 6 diabetic rats was maintained without any treatment. Two groups of 6 normal
Table 1
Changes in levels of blood glucose and plasma insulin in normal and experimental rats.

<table>
<thead>
<tr>
<th>Blood glucose (mg dL)</th>
<th>Plasma insulin (μU mL) after 1 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Groups</strong></td>
<td><strong>0 d</strong></td>
</tr>
<tr>
<td>Normal</td>
<td>79.66 ± 3.03</td>
</tr>
<tr>
<td>Normal + GLEt (200 mg/kg)</td>
<td>84.60 ± 5.59</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>81.66 ± 4.94</td>
</tr>
<tr>
<td>Diabetic + GLEt (25 mg/kg)</td>
<td>85.45 ± 5.71</td>
</tr>
<tr>
<td>Diabetic + GLEt (50 mg/kg)</td>
<td>80.00 ± 5.71</td>
</tr>
<tr>
<td>Diabetic + GLEt (100 mg/kg)</td>
<td>83.66 ± 6.59</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>79.50 ± 5.09</td>
</tr>
<tr>
<td>Diabetic + glibenclamide (600 μg/kg)</td>
<td>77.48 ± 4.48</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 rats in each group. Diabetic control was compared with normal. Experimental groups were compared with corresponding values after alloxan injection (15 days). Values not sharing a common superscript letter differ significantly at P < 0.05 Duncan multiple range test for levels 2.89, 3.03, 3.13, 3.20 and 3.25.

* P < 0.01
** P < 0.001

rats were treated with either distilled water (vehicle control) or GLEt (200 mg/kg of body weight).

The plant extract and the drug glibenclamide were given in aqueous solution daily using an intragastric tube for 3 weeks at a dosage level of 10 mL/kg of body weight. Fasting blood glucose was monitored every 7 days throughout the experiment. An oral glucose tolerance test (OGTT) was carried out on all animals on the 21st day of the experiment. At the end of the experimental period, the rats were deprived of food overnight and then euthanized by exsanguination. Blood was collected in tubes containing potassium oxalate and sodium fluoride solution for the estimation of blood glucose, and the plasma was separated for the estimation of insulin and glycoproteins. The liver and kidney were removed, washed in ice-cold saline, patted dry, weighed, and subjected for glycoprotein estimation.

2.7 Biochemical analysis

2.7.1 Determination of blood glucose, OGTT and plasma insulin

Blood glucose was determined by the O-toluidine method by Sasaki et al. [25]. Blood (0.1 mL) was precipitated with 1.9 mL of 10% TCA (tricholoro acetic acid) and centrifuged. One milliliter of supernatant was mixed with 4 mL of O-toluidine reagent, kept in a boiling water bath for 15 minutes, and cooled. The absorbance was read at 620 nm. Glucose was expressed as milligrams per deciliter of blood.

The OGTT was performed according to the method of Du Vigneaud and Karr [26]. After overnight fasting, a "0"-minute blood sample (0.2 mL) was taken from the normal and experimental rats by cyancutural puncture. Without delay, a glucose solution (2 g/kg of body weight) was administered by gavage. Then, blood samples were taken at 30, 60, 90, and 120 minutes after glucose administration. All the blood samples were collected with potassium oxalate and sodium fluoride, and their glucose content was estimated by the O-toluidine method [25] as described previously.

Plasma insulin was estimated using an enzyme-linked immunosorbent assay kit (Boehringer, Mannheim, Germany) using human insulin as the standard [27].

2.7.2 Extraction of lipids

Lipids were extracted from tissues by the method of Folch et al. [28] using a chloroform methanol mixture (2:1 vol/vol). The defatted tissues were used for the estimation of tissue glycoproteins.
### Table 2

Changes in levels of plasma, liver, and kidney hexose, hexosamine, sialic acid, and fucose in normal and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal</th>
<th>Diabetic control</th>
<th>Diabetic + GLEt (200 mg/kg)</th>
<th>Diabetic - glibenclamide (600 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hexose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (mg/dL)</td>
<td>92.0 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>130.1 ± 8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.2 ± 5.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>106.5 ± 7.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (mg/g defatted tissue)</td>
<td>29.2 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.2 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.5 ± 2.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.2 ± 3.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney (mg/g defatted tissue)</td>
<td>23.1 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.8 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.8 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.1 ± 2.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Hexosamine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (mg/dL)</td>
<td>71.0 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.4 ± 5.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.1 ± 3.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86.6 ± 4.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (mg/g defatted tissue)</td>
<td>8.0 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.1 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.1 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney (mg/g defatted tissue)</td>
<td>12.1 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.1 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.2 ± 12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.1 ± 13&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Sialic acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (mg/dL)</td>
<td>51.1 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.2 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.0 ± 2.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60.5 ± 3.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (mg/g defatted tissue)</td>
<td>7.8 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.1 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.7 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney (mg/g defatted tissue)</td>
<td>8.0 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.9 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Fucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (mg/dL)</td>
<td>27.0 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.3 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.5 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.2 ± 2.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (mg/g defatted tissue)</td>
<td>14.2 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.3 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.3 ± 12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.6 ± 15&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney (mg/g defatted tissue)</td>
<td>11.3 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.8 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.4 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.6 ± 13&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 rats in each group. Values not sharing a common superscript letter differ significantly at P < 0.05 (Duncan multiple range test for levels 2.89, 3.03, 3.13, and 3.20).

### 2.7.3 Determination of hexose, hexosamine, sialic acid, and fucose

To the dry defatted tissues, 2 mL of 0.1 N H₂SO₄ was added and hydrolyzed at 80°C for 1 hour and cooled, and an aliquot was used for sialic acid estimation. To the remaining solution, 2 mL of 0.1 N NaOH was added and kept in an ice bath for 1 hour. The aliquots were used for total hexose, fucose, and hexosamine estimation.

Hexose was estimated by the method of Niebes [29]. Briefly, 0.5 mL of the aliquot/plasma, 0.5 mL of 5% phenol and 2.5 mL of concentrated H₂SO₄ were added. For a blank, 0.5 mL of 0.1 N NaOH was treated in the same way. The tubes were then heated in a boiling water bath for 20 minutes, and the absorbance was read at 490 nm.

Hexosamine was estimated by the method of Elson and Morgan [30]. To the plasma/aliquot (0.5 mL/0.1 mL), 2.5 mL of 3N HCl was added and boiled for 6 hours in a boiling water bath and then neutralized with 6N NaOH. To 0.8 mL of the neutralized sample, 0.6 mL of acetyl acetone reagent was added and heated in boiling water for 30 minutes. After cooling, 2 mL of Ehrlich reagent was added and mixed well. The color developed was read at 540 nm.

Sialic acid was estimated by the method of Warren [31]. Briefly, to 0.5 mL of extract/plasma, 0.5 mL of water and 0.25 mL of 0.025 mmol/L periodic acid were added and incubated at 37°C for 30 minutes. To this, 0.2 mL of 4% sodium meta-arsenate and 2 mL of thiobarbituric acid were added, heated in a boiling water bath for exactly 6 minutes, and cooled, and 5 mL of acified butanol was added. The absorbance was read at 540 nm against the reagent blank.

Fucose was estimated by the method of Dische and Shettle [32]. To 0.5 mL of the aliquot/plasma, 4.5 mL of H₂SO₄ (6:1 vol/vol in distilled water) was added and heated in a boiling water bath for 3 minutes. The samples were cooled, and 0.1 mL of cysteine hydrochloride reagent was added. For the blank, 0.5 mL of 0.1 N NaOH was also treated in the same way. After 75 minutes in dark, the absorbance was read at 393 and 430 nm.

The glycoprotein levels were expressed as mg/100 g for defatted tissue and milligrams per deciliter for plasma.

### 2.7.4 Total phenolic assay

The phenolic content of the plant extract was determined according to the method of Shetty [33]. Suitable aliquots of the extract were taken in a test tube and made up to the volume of 1 mL with distilled water. Then, 0.5 mL of Folin-Ciocalteau reagent and 2.5 mL of sodium carbonate solution (20%) were added sequentially in each tube. The tubes were vortexed and placed in the dark for 40 minutes, and the absorbance was recorded at 725 nm. A standard curve was established for each assay using 50 to 500 µg of gallic acid in 95% ethanol and expressed as gallic acid equivalent.
(milligram of gallic acid equivalent per gram of the leaf extract) [34,35]

2.8 Statistical analysis

All data are expressed as mean ± SD. The data were subjected to a 1-way analysis of variance using SPSS, version 7.5 (SPSS, Chicago, III), and Duncan multiple range test. P < 0.05 was considered to indicate a significant difference between means [36].

3. Results

The body weight of rats treated with GLEt increased significantly (P < 0.01) at the end of the third week compared with the diabetic control group (data not shown). The food and water intake were significantly less in GLEt-treated groups than in diabetic control rats. There was no significant difference between the GLEt- and glibenglamide-treated groups. In addition, the oral administration of the plant extract modified food intake, which is evidenced by the feed-efficiency ratio (food intake per weight gain) (data not shown).

In all the groups, before alloxan administration, the basal levels of blood glucose were not significantly different. However, 15 days after alloxan administration, blood glucose levels were significantly increased, and only animals that exhibited such were selected for the study. Table 1 shows the effect of GLEt on blood glucose levels of alloxan-diabetic rats. From the first week onwards, although a significant antihyperglycemic effect was evident, the reduction in blood glucose was maximum on the third week, with a significant increase in plasma insulin in rats receiving 200 mg/kg of body weight of GLEt, and the results were comparable with that of glibenglamide. Hence, the group receiving GLEt only (200 mg/kg of body weight) was used for further studies. No significant modulation was observed in blood glucose levels in normal rats treated with GLEt at 200 mg/kg of body weight.

Although diabetic rats treated with a higher concentration (400 mg/kg) of GLEt exhibited a significant decrease in hyperglycemia (data not shown), the histopathologic examination showed evidence of damage to the liver, kidney, and pancreatic tissues. Therefore, further studies were limited to rats given GLEt at 200 mg/kg of body weight.

Results of OGGT are presented in Fig 1. In diabetic rats, blood glucose levels reached a peak 1 hour after glucose administration, although they started to decline, they remained higher even after 2 hours. However, in GLEt-treated rats, blood glucose levels remained low at all sampling time points. Normal rats treated with GLEt also showed a significant decrease in glucose levels at 2 hours.

The levels of plasma and tissue glycoproteins in normal and experimental rats are shown in Table 2. In diabetic rats, plasma levels of all 4 tested glycoproteins were significantly increased. Whereas in the liver and kidney, significant increases were observed in the levels of hexose, hexosamine, and fucose (P < 0.05), the sialic acid level was significantly decreased (P < 0.05). Oral administration of GLEt significantly reversed the changes in plasma, liver, and kidney glycoproteins of diabetic rats. The modulatory effect of GLEt on glycoprotein levels was observed to be better than that of the reference drug, glibenglamide.

Fig 2 shows correlations between the phenolic content and the percentage of glucose reduction. The total phenolic content in GLEt was found to be 156 mg of gallic acid equivalent per gram of leaf extract, which showed a positive relationship (r = 0.98) with antidiabetic efficacy in terms of glucose level reduction.

4. Discussion

Diabetes is possibly the world's fastest growing metabolic disease, and as knowledge of the heterogeneity of this disorder increases, so does the need for more appropriate therapies [37]. Currently, available drug regimens for management of DM have certain drawbacks such as vascular complications and hepatotoxicity [38]. Traditional plant medicines are used throughout the world for a range of diabetic conditions. The study of such medicines might offer a natural key to unlock a diabetologist's pharmacy for the future [39]. Various epidemiological surveys have shown an inverse relationship between the intake of polyphenolics and incidence of various diseases, and more than 8000 phenolic compounds are currently known [3].

In the present study, we estimated the effect of GLEt on glycoprotein metabolism in alloxan-induced diabetic rats. The capacity of an antidiabetic drug such as GLEt to decrease the elevated blood glucose to a normal level is an essential trigger for the liver to revert to its normal homeostasis during experimental diabetes. In GLEt-treated animals, a decrease in the blood glucose levels was dose dependent and was very significant at 200 mg/kg of body weight. The possible mechanism for the observed antihyperglycemic effect of GLEt in diabetic rats may be due to the stimulation of insulin release and the insulin-mimetic action of compounds present in the extract. However, administration of GLEt did not cause any significant alteration in plasma insulin levels in normal rats. Hence, the observed rise in plasma insulin levels in GLEt-administered diabetic rats may be due to the insulin-stimulatory effects rather than insulin-mimetic effects.

Our findings coincide with those of earlier studies which reported that the extract of G. sylvestre and a number of other plants reduced the blood glucose and increased the plasma insulin levels in diabetic rats. G. sylvestre is reported to be rich in gymnemic acid and gymnemic acids that are responsible for the antihyperglycemic effect [40, 41]. Because the test plant belongs to the same genus, the presence of such active constituents in G. montanum also may be envisaged.

Ethanol extract of the G. montanum leaves might enhance glucose use because it significantly reduces blood.
glucose in diabetic rats. From the data obtained with the OGTT it is clear that blood glucose levels reached a peak and returned to fasting values after 2 hours in both normal and treated rats (diabetic + GLEt). In diabetic rats, blood glucose levels remained high even after 2 hours Administration of GLEt effectively prevented the increase in blood glucose without causing a hypoglycemic state. In this context, other medicinal plants such as Scoparia dulcis and Phaseolus vulgaris have also been reported to have a similar effect [42,43].

Generalized abnormalities in glycoprotein metabolism are observed in both naturally occurring and experimental diabetes [44,45]. Recently, we have reported the modulatory effect of an aqueous extract of Scoparia dulcis on plasma and tissue glycoproteins within 3 weeks of treatment in streptozotocin-induced diabetic rats [10]. Berenson et al. [46] reported that streptozotocin-diabetic rats exhibited a significant modification in the connective tissue macromolecule. Insulin has been shown to increase the incorporation of glucose in the rat submaxillary gland [7]. The requirement of insulin for the biosynthesis of the carbohydrate moiety of mucopolysaccharides from glucose is thus evident. In diabetes, synthesis of glycoproteins was decreased because of reduced incorporation of glucose caused by insulin deficiency.

Previous reports suggest that serum concentrations of glycoproteins are significantly increased in DM [10,11,44]. This increase in plasma glycoproteins has been reported to be associated with the severity and duration of diabetes. Glycoproteins found in a variety of tissues including the arterial wall are very similar in structure and composition to those in plasma [47]. Therefore, vascular complications that involve complex protein-carbohydrate molecules could contribute to an increase in plasma glycoproteins.

The biosynthesis of the carbohydrate moieties of glycoproteins forms the insulin-independent pathways for the use of glucose 6-phosphate. But the deficiency of insulin during diabetes produces derangement of glycoprotein metabolism, resulting in the thickening of the basal membrane of pancreatic beta cells. The increased availability of glucose in the hyperglycemic state accelerates the synthesis of basement-membrane components, that is, glycoproteins [48]. This is because of depressed use of glucose by insulin-dependent pathways, thereby enhancing the formation of hexose, hexosamine, and fucose for the accumulation of glycoproteins [49].

The synthesis of fibronectin, which contains sialic acid residues in the core structure, was reported to increase significantly in various tissues of diabetic patients and animals [50]. The decrease in sialic acid content in tissues observed in diabetic rats may be due to its increased use for the synthesis of fibronectin. Administration of GLEt to diabetic rats significantly reversed these changes to normal levels. The antihyperglycemic action of GLEt, which is mediated via an enhancement of insulin action, as evidenced by the increased level of insulin in GLEt-treated diabetic rats, may be responsible for the reversal of glycoprotein changes associated with diabetes.

Numerous epidemiological studies have shown the relation-ship between phenolics and various diseases. Many phenolic phytochemicals have antioxidant, anticarcinogenic, antimicrobial, antiallergic, immunomodulatory activity, and antiinflammatory activities [3]. In the present study, G. montanum was found to be rich in phenolics which may also play a role in the modulation of glycoprotein components in diabetic rats.

In conclusion, administration of GLEt demonstrated a beneficial effect on carbohydrate moieties of glycoproteins as well as a protective effect against alloxan challenge and thus provides a scientific rationale for the use of G. montanum as an antidiabetic drug. The isolation of bioactive compounds in the extract would certainly help to ascertain the medicinal value of the extract, which could be further explored for use by the food and pharmaceutical industries.

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Amelioration of Metabolic Complications Associated with Diabetes by Gymnema montanum in Alloxan Induced Rats

K.M Ramkumar¹, P. Rajaguru¹, M. Latha² and R. Ananthan³

Abstract

The effect of Gymnema montanum leaves on alloxan induced diabetic complications was studied in male wistar rats. Ethanolic extract of G montanum leaves (GLEt) was administered orally at doses of 50, 100, 200 mg/kg body weight for 3 weeks and the effect on blood glucose, plasma insulin, glycogen, carbohydrate metabolic enzymes such as hexokinase and glucose-6-phosphatase, lipid profile, lipid peroxidation markers such as thiobarbituric acid-reactive substances (TBARS) and hydroperoxides and level of antioxidants such as reduced glutathione (GSH) and activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) in alloxan-induced diabetic rats were studied. Administration of GLEt reversed the biochemical changes in diabetic rats. Antidiabetic effects of GLEt at 200 mg/kg body weight were found to be comparable to glibenclamide, a reference drug. These data strongly evidenced the antidiabetic, antiperoxidative, antioxidant, antihyperlipidemic properties of G. montanum and are suggestive of the potential use of G montanum leaves extract for the management of diabetes and its complications.

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Introduction

Diabetes mellitus is a non-communicable metabolic disorder, which is considered as one of the five leading causes of death in the world (Ugochukwu and Babady, 2003; Devendra et al., 2004). The development of diabetic complications such as diabetic retinopathy, neuropathy, nephropathy and ketoacidosis are major causes of morbidity and mortality in diabetic population (Ferris et al., 1999). The worldwide prevalence is expected to rise from 135 million patients in 1995 to 300 million by the year 2025 (Wild et al., 2004).

Oxidative stress plays an important role in chronic complications of diabetes and is postulated to be linked with increased lipid peroxidation. The lipid peroxidation of the cell membrane has been associated with a number of pathologic phenomena such as increased membrane rigidity, decreased cellular deformability and lipid fluidity in erythrocytes. On the other hand, our body system possess defense against oxidative stress through endogenous and exogenous antioxidant substances. The elevated level of blood glucose in diabetes cause disturbances in defense mechanism such as alteration in antioxidant enzymes, impairment in glutathione metabolism and decreased ascorbic acid levels.

Nowadays, the drugs used for diabetes are divided into insulin secretagogues, aldose reductase inhibitors and α-glucosidase inhibitors. The drugs commonly available are Insulin, Sulphonyl urea and Biguanides. In most cases, these drugs cause side effects such as hypoglycaemia, lactic acid intoxication and gastrointestinal upsets (Krische, 2000).

Due to the adverse effects of the presently available synthetic drugs, there is an urgent need to identify indigenous natural resources for the effective medicine (Eisenberg et al., 1993). The World Health Organization has also recommended the evaluation of the effective plants, in conditions where safe modern drugs are lacking (WHO report, 1980). Recently, an intensive search for novel types of antioxidants has been carried out from numerous plant materials (Grover et al., 2002). India holds one of the biggest heritages in plant diversity since ancient times where plant based drugs were practiced for the treatment of major diseases such as diabetes, cancer etc. The goals and strategies of the herbal treatment for diabetes focus the treatment and prevention of diabetes without any side effects.

Gymnema montanum Hook. (Hooker, 1883), belonging to the family Asclepiadaceae, is an endemic plant species of India found mainly in Western Ghats (Vajravelu and Bhargavan, 1983; Subbarayalu and Velmurugan, 1999). A few members of the Gymnema genus (Gymnema sylvestre,
Gymnema inodorum and Gymnema yunnanense) have been reported to involve in repair or regenerate the pancreatic β-cells, which play a crucial role in the production and secretion of insulin (Persaud et al., 1999; Shimizu et al., 2001; Xie et al., 2003).

In the present study the antidiabetic effects especially antioxidant status, antilipidemic effects and lipid peroxidation of ethanolic extract of G. montanum on alloxan induced rats were characterized.

Methods

Animals

Male albino Wistar rats, body weight of 180 - 200g bred in Central Animal House, Rajah Muthiah Medical College, Annamalai University, were used in this study. The animals were fed on a pellet diet (Hindustan Lever Ltd., India) and water *ad libitum*. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with a 12 hr alternating light and dark cycle. The animals used in the present study were maintained in accordance with the guidelines prescribed by the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and approved by the ethical committee, Annamalai University.

Drugs and chemicals

All drugs and chemicals used in this research were purchased from Sigma Chemical Co. (St. Louis, MO). The chemicals were of analytical grade.

Plant materials

Fresh leaves of G. montanum were collected from the Shola forests of Western Ghats, Gudalur, Nilgiri Biosphere Reserve at an altitude of 900-1500 MSL. The plant was identified with the Herbarium of Botanical Survey of India, Southern Circle, Coimbatore, India (Accession No. 32561-65) and was deposited in the Department of Botany, Bharathiar University.

Preparation of plant extract

The ethanolic extract of the leaves was prepared as described earlier (Hossain et al., 1992). Briefly, about 500 g of fresh leaves of G. montanum were chopped into small pieces, soaked overnight in 1.5 L of 95 % ethanol. This suspension was filtered and the residue was resuspended in an equal volume of 95 % ethanol for 48 h and filtered again. The two filtrates were pooled and the solvents were evaporated in a rotavapor at 40°- 50°C under reduced pressure and lyophilized. A greenish-black residue was obtained (20-30 g) and was stored at 0-4 °C until further use. When needed, the residue extract was dissolved in distilled water and used.
Experimental induction of diabetes

Rats were injected intraperitoneally with freshly prepared solution of alloxan monohydrate in normal saline at a dose of 150 mg/kg body weight (Al-Shamaony et al., 1994). Since alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution (5-10 ml) orally after 6 h. The rats were then kept for the next 24 h on 5% glucose solution to prevent hypoglycaemia (Gupta et al., 1994). After 2 weeks, rats with moderate diabetes having glycosuria and hyperglycaemia (i.e. with blood glucose of 200-300 mg/dl) were chosen for the experiment.

Experimental procedure

A total of 36 rats (30 diabetic surviving rats, 6 normal rats) were used and they were divided into 6 groups of 6 rats each.

Group 1: Normal untreated rats.
Group 2: Diabetic control.
Group 3: Diabetic rats treated with GLEt (50 mg/kg b.w.)
Group 4: Diabetic rats treated with GLEt (100 mg/kg b.w.)
Group 5: Diabetic rats treated with GLEt (200 mg/kg b.w.)
Group 6: Diabetic rats treated with glibenclamide (600 µg/kg b.w.) (Pari and Uma, 2000).

The animals were treated daily for 3 weeks. At the end of 3rd week, the animals were deprived of food overnight and sacrificed by decapitation. Plasma was separated from the blood collected in tubes containing potassium oxalate and sodium fluoride solution for the estimation of blood glucose. The erythrocytes (RBCs) remaining after the removal of plasma were washed with isotonic saline (0.89% saline) and the buffy coat was removed. The RBCs were washed again with physiological saline and proceeded for the preparation of hemolysate and erythrocyte membrane. Liver, kidney and brain tissues were dissected out washed with ice-cold saline, patted dry and weighed. The tissues were homogenized and further subjected to biochemical analysis.

Analytical methods

Blood glucose was determined by the O-toluidine method (Sasaki et al., 1972). Plasma insulin was assayed by ELISA method, using Boehinger-Mannheim Kit with a Boehinger analyser ES300 (Anderson et al., 1993). Hexokinase (E.C 2.7.1.1) and Glucose-6-phosphate dehydrogenase were assayed according to the method of Brandstrup et al. (1957) and Ellis and Kirkman (1961) respectively and the glycogen content was estimated by the method of Morales et al. (1973).
The extent of lipid peroxidation was estimated colorimetrically by thio-
barbituric acid reactive substances (TBARS) and hydroperoxides by
the method of Nichans and Samuels (1968) and Jiang et al. (1992)
respectively.
Superoxide dismutase (SOD) was assayed according to the method of
Kakkar et al. (1984). One unit of enzyme was expressed as 50 % inhibi-
tion of NBT (Nitroblue tetrazolium) reduction / min. / mg protein. Catalase (CAT) was
assayed colorimetrically and expressed as μmoles of H₂O₂ consumed / min. / mg protein as described by Sinha (1972). Glutathione peroxidase (GPx) activity
was measured by the method described by Rotruck et al. (1984) and expressed
as μg of GSH consumed / min. / mg protein. Reduced glutathione (GSH) was
determined by the method of Ellman (1959). The glutathione-S-transferase
(GST) activity was determined spectrophotometrically by the method of Habig
et al. (1974) and expressed as mmoles of GSH-CDNB conjugate formed / min. / mg protein using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹.
Lipids were extracted from serum and tissues by the method of Folch
et al. (1957). Total cholesterol and triglycerides were estimated by the
method of Zlatkis et al. (1953) and Foster and Dunn (1973) respectively.
Free fatty acids and phospholipids were analysed by the method of Falholt
et al. (1973) and Zilversmit and Davis (1980) respectively. Protein was
determined by the method of Lowry et al. (1951) using Bovine Serum
Albumin (BSA) as standard, at 660 nm.

In vitro antilipidperoxidation assay
The extend of lipid peroxidation in rat homogenate was measured in vitro
in terms of formation of TBARS according to the method of Ohkawa et al.
(1979). The percentage inhibition of lipid peroxidation was calculated by
comparing the results of the test with those of controls (not treated with
the extract) as per the formula.

\[
\text{Inhibition (\%)} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100
\]

Total phenolic assay
Total phenolics were determined according to the method of Shetty et al.
(1995). A standard curve was established for each assay using 50-500 μg
of gallic acid in 95 % ethanol.

Statistical analysis
All data were expressed as Mean ± S.D (n = 6). The statistical significance
was evaluated by one-way analysis of variance (ANOVA) using SPSS version
7.5 (SPSS, Cary, NC, USA) and the individual comparisons were obtained
by Duncans’ Multiple Range Test (DMRT) (Duncan, 1957).
Fig. 1. Body weight (A), food (B) and fluid (C) intake in alloxan diabetic rats before and after oral treatment with Gymnema montanum leaf extract for 3 weeks.

Groups 1-Normal, 2 -Diabetic control, 3 -Diabetic + GLEt (50 mg kg⁻¹), 4 -Diabetic + GLEt (100 mg kg⁻¹), 5 -Diabetic + GLEt (200 mg kg⁻¹), 6 -Diabetic + Glibenclamide (600 μg kg⁻¹).

Values are given as mean ± S D from six rats in each group. Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT) Duncan Procedure. Ranges for the level 2.95, 3.09, 3.20, 3.22. Diabetic control was compared with normal, ‘#’ - P < 0.001. Experimental groups were compared with diabetic control '*' - P < 0.001.
Results

The changes in the body weight, water and food intake in normal and drug induced diabetic rats (experimental) are represented in Fig.1. The body weights in the GLEt treated and glibenclamide treated groups increased significantly at the end of third week when compared with the diabetic control group. The food and water intake was significantly reduced in GLEt and glibenclamide treated group than diabetic control rats.

Before alloxan administration, the basal levels of blood glucose of rats in all groups were not significantly different. However, 15 days after alloxan administration, blood glucose levels were significantly increased and only such rats were selected for the study. Table 1 shows the effect of GLEt and glibenclamide on blood glucose and plasma insulin levels. Although a significant antihyperglycaemic effect was evident from the 1st week onwards, it attained maximum on 3rd week with significant increase in plasma insulin in group receiving 200 mg kg⁻¹ body weight of G. montanum leaf extract. The antihyperglycaemic effect of GLEt at a dose of 200 mg kg⁻¹ was more significant as compared to that of animals received 50 mg kg⁻¹ and 100 mg kg⁻¹ and therefore the higher dose group was subjected for further biochemical analysis.

The changes in the activities of hepatic hexokinase, glucose-6-phosphate dehydrogenase and glycogen are given in Table 2. The hepatic...
### Table 1. Changes in levels of blood glucose and plasma insulin of normal and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 day</th>
<th>15 days after alloxan injection</th>
<th>I week (after treatment)</th>
<th>II week</th>
<th>III week</th>
<th>Plasma Insulin (µU/ml) after III week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>79.66 ± 3.03</td>
<td>84.16 ± 5.14</td>
<td>82.16 ± 5.92</td>
<td>80.66 ± 6.04</td>
<td>81.45 ± 5.98</td>
<td>13.62 ± 5.52*</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>81.66 ± 4.94</td>
<td>265.00 ± 19.45**</td>
<td>279.66 ± 12.93**</td>
<td>285.50 ± 12.95**</td>
<td>298.00 ± 15.77**</td>
<td>5.50 ± 2.75*</td>
</tr>
<tr>
<td>Diabetic + GLEt (50 mg/kg)</td>
<td>80.00 ± 5.71</td>
<td>255.50 ± 16.36</td>
<td>231.66 ± 15.45*</td>
<td>207.54 ± 8.73**</td>
<td>163.75 ± 8.08**</td>
<td>6.70 ± 0.94*</td>
</tr>
<tr>
<td>Diabetic + GLEt (100 mg/kg)</td>
<td>83.66 ± 6.59</td>
<td>248.00 ± 14.02</td>
<td>215.83 ± 13.04*</td>
<td>180.40 ± 9.83**</td>
<td>161.83 ± 13.25**</td>
<td>9.16 ± 0.62*</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>79.50 ± 5.09</td>
<td>258.00 ± 18.61</td>
<td>200.00 ± 12.58**</td>
<td>120.21 ± 6.39**</td>
<td>86.35 ± 9.25**</td>
<td>12.06 ± 4.32*</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 µg/kg)</td>
<td>77.48 ± 4.48</td>
<td>245.58 ± 13.99</td>
<td>219.29 ± 7.05*</td>
<td>191.58 ± 10.8**</td>
<td>118.22 ± 4.48**</td>
<td>10.08 ± 3.13*</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D for 6 rats in each group. Values in parentheses indicated the percentage lowering of blood glucose in comparison to basal reading after alloxan administration (15 days).

Diabetic control was compared with normal. Experimental groups were compared with corresponding values after alloxan injection (15 days). * - P < 0.01, ** - P < 0.001.

Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT). Duncan procedure, Range for the level 2.89, 3.03, 3.13, 3.20, 3.25.
Table 2. Changes in activities of hexokinase, glucose-6-phosphate dehydrogenase and glycogen in liver of normal and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hexokinase (units/g protein)</th>
<th>Glucose-6-phosphate dehydrogenase (× 10⁶ IU/mg protein)</th>
<th>Glycogen (mg / 100 g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>142 ± 4.89</td>
<td>4.46 ± 0.34</td>
<td>33.06 ± 1.43</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>109 ± 4.71</td>
<td>2.58 ± 0.13</td>
<td>20.66 ± 1.07</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>139 ± 8.09</td>
<td>3.91 ± 0.011</td>
<td>30.53 ± 0.85</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 μg/kg)</td>
<td>137 ± 9.40</td>
<td>3.71 ± 0.14</td>
<td>28.25 ± 0.76</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD for 6 rats in each group
Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT)
Duncan procedure, Range for the level 2.95, 3.09, 3.20
A - μ moles of glucose phosphorylated/ min
glycogen content was reduced significantly in diabetic control rats as compared to the normal rats. Treatment with GLEt and glibenclamide prevented this alteration in glycogen content but could not restore the normal level. The activity of hexokinase significantly decreased in the liver of diabetic rats, whereas glucose-6-phosphate dehydrogenase levels were increased. Administration of GLEt to diabetic rats resulted in significant reversal in enzyme activities.

TBARS and hydroperoxides (Table 3) from plasma, liver, kidney and brain homogenates were decreased significantly with GLEt and glibenclamide treatment, compared to diabetic control rats.

For studying the effect of GLEt on free radical production, the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and reduced glutathione were measured and presented in Table 4, 5 and 6. They showed significant increase in GLEt treatment when compared with diabetic control rats.

The effect of GLEt on serum and tissue lipids of normal and experimental rats is summarized in Table 7. A marked increase in the levels of cholesterol, free fatty acids, triglycerides and phospholipids were observed in diabetic control rats. Treatment with GLEt significantly reduced the lipid levels.

In all the biochemical parameters, there was no significant difference was observed between GLEt treated groups and glibenclamide treated groups.

Discussion

The rising incidence of non-communicable diseases such as cancer, diabetes, heart diseases and hypertension is becoming a serious public health problem. Despite recent advancements made in drug research, physicians and patients are in the need of effective therapeutic agents with low incidence of side effects. Plant derived substances continue to serve as exclusive source of drugs for the majority of the world population and several plant based drugs are in extensive clinical use (Roja and Rao, 2000). This study was therefore undertaken to assess the antihyperglycaemic, antiperoxidative and antilipidemic properties of G. montanum, an Indian endemic plant.

It is clearly evident that the antihyperglycaemic effect of GLEt is dependent upon the dose and this probably an indicative of the presence of active principles in the plant extract. Moreover, it indirectly indicates that, part of the antihyperglycemic activity of this plant is through release of insulin from the pancreas. Our findings coincide with those of earlier studies on the same genus, which reported that, the extract of Gymnema sylvestre reduced the blood glucose and increased the plasma insulin levels in diabetic rats (Persaud et al., 1999). G. sylvestre is reported to be rich
Table 3. Changes in levels of TBARS, hydroperoxides in plasma and tissues of normal and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS</th>
<th>Hydroperoxides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>0.15 ± 0.011*</td>
<td>0.76 ± 0.02*</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>0.36 ± 0.024*</td>
<td>1.72 ± 0.10*</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>0.20 ± 0.014*</td>
<td>1.23 ± 0.03*</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 µg/kg)</td>
<td>0.24 ± 0.022*</td>
<td>1.30 ± 0.04*</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D for 6 rats in each group.
Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT)
Duncan procedures, range for the level 2.95, 3.09, 3.20.
Values are represented by mM/dl for plasma and mM/100 g tissue for liver, kidney and brain.
Table 4. Changes in activities of superoxide dismutase (SOD) and catalase (CAT) in haemolysate and tissues of normal and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Superoxide dismutase&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Catalase&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC haemolysate</td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>2.17 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.10 ± 0.62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>1.48 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.65 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>2.11 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.86 ± 0.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 μg/kg)</td>
<td>2.02 ± 0.19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.56 ± 0.34&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RBC haemolysate</td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>6.65 ± 5.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.50 ± 6.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>45.89 ± 3.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.35 ± 2.52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>62.17 ± 5.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67.40 ± 4.44&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 μg/kg)</td>
<td>56.49 ± 5.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>58.73 ± 3.86&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. Values are represented by U/mg Hb for RBC haemolysate and U/mg of protein for liver, kidney and brain.
2. Values are represented by mM/mn/mg Hb for RBC haemolysate and mM/mn/mg of protein for liver, kidney and brain.

Values are given as mean ± S D for 6 rats in each group.
Values not sharing a common superscript letter differ signifi cantly at P < 0.05 (DMRT).
Duncan procedure, Range for the level 2.95, 3.09, 3.20.
Table 5. Changes in activities of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) in haemolysate and tissues of normal and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glutathione peroxidase</th>
<th>Glutathione-S-transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC haemolysate</td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>8.51 ± 0.64*</td>
<td>6.16 ± 0.47*</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>6.38 ± 0.67*</td>
<td>3.21 ± 0.13*</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>8.22 ± 0.92*</td>
<td>5.84 ± 0.45*</td>
</tr>
<tr>
<td>Diabetic + Gbenesamide (600 µg/kg)</td>
<td>7.84 ± 0.69*</td>
<td>4.96 ± 0.41*</td>
</tr>
</tbody>
</table>

1. Values are represented by U/mg Hb for RBC haemolysate and U/mg of protein for liver, kidney and brain.
2. Values are represented by µM/min/mg Hb for RBC haemolysate and µM/min/mg of protein for liver, kidney and brain.
3. Values are given as mean ± S.D for 6 rats in each group.
4. Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT).
5. Duncan procedure, range for the level 2.95, 3.09, 3.20.
in gymnemagenin and gymnemic acids that are responsible for the antihyperglycaemic effect (Murakami et al., 1996).

**Carbohydrate metabolism**

The hepatic glycogen content was reduced significantly in diabetic control rats as compared to the normal rats. Treatment with GLEt and glibenclamide prevented reduction in liver glycogen content which is possibly due to the stimulation of insulin release from existing β cells and subsequent enhanced glycolysis (Lolitkar and Rao, 1996).

Hexokinase plays an important role in the maintenance of glucose homeostasis (Saxena et al., 1992). The increased activity of hexokinase causes the increase in glycolysis and utilization of glucose for energy production. Administration of GLEt has been observed to decrease the concentration of blood glucose in alloxan diabetic rats, which may be due to increased level of plasma insulin that in turn favored glycolysis.

Significant increase in glucose-6-phosphate dehydrogenase activity in diabetic rat liver caused by GLEt treatment suggests that the hydrogen shuttle system and the redox state of the cells become more oxidized which results in the increased formation of NADPH for increased utilization and in turn activates the enzyme, as NADPH is a strong inhibitor of glucose-6-phosphate dehydrogenase (Baquer et al., 1998).

A sequential metabolic correlation between increased glycolysis, decreased gluconeogenesis, increased hydrogen shuttle reactions and normoglycaemia stimulated by GLEt suggests the possible biochemical mechanism through which glucose homeostasis is regulated.

**Oxidative stress**

Elevated levels of lipid peroxidation in tissues and plasma as observed in alloxan diabetic rats is one of the characteristic features of chronic diabetes (Feillete et al., 1999; Venkateswaran et al., 2002; Prince and Menon, 1998). Our study shows that administration of GLEt and glibenclamide tend to bring the liver and kidney peroxides back to near normal. This indicates that GLEt may inhibit oxidative damage of the system.

Under *in vivo* conditions, GSH acts as an antioxidant and its decrease was reported in diabetes mellitus (Baynes and Thrope, 1999). We have observed a significant decrease in GSH levels in liver, kidney and brain tissues of diabetic rats. The decrease in GSH levels represents increased levels of its utilization due to oxidative stress. Depression in GPx, GST activities were also observed in tissues during diabetes. GPx has been shown to be an important adaptive response to condition of increased peroxidative stress (Matcovis et al., 1982). The increased GSH content in the tissues of the rats treated with GLEt and glibenclamide may be one of the factors responsible for inhibition of lipid peroxidation.
Table 6. Changes in level of tissue glutathione of normal and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Reduced glutathione (mg/100 mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Normal</td>
<td>46.76 ± 3.9a</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>23.20 ± 1.86b</td>
</tr>
<tr>
<td>Diabetic + GLEt (200 mg/kg)</td>
<td>40.00 ± 2.88b</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (600 μg/kg)</td>
<td>34.78 ± 2.91b</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D for 6 rats in each group. Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT). Duncan procedure, range for the level 2.95, 3.09, 3.20.

SOD and CAT are the two major scavenging enzymes that remove toxic free radicals in vivo. Reduced activity of SOD and CAT in RBC hemolysate and tissues have been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of superoxide radicals (O₂⁻) and hydrogen peroxide (Searle and Wilson, 1998). Administration of GLEt and glibenclamide results in the activation of SOD and CAT returning to near normal levels. The result clearly shows that GLEt contains a free radical scavenging activity, which could exert a beneficial action against pathological alterations caused by the presence of O₂⁻ and OH⁻.

Lipid metabolism

In alloxan treated rats, lower insulin mediated glucose disposal causes excess of free fatty acids in serum and promotes conversion of excess fatty acids into phospholipids and cholesterol in liver. These two substances along with excess triglycerides formed in liver may be discharged into blood in the form of lipoprotein (Bopanna et al., 1997). Both increased hepatic production of triglycerides and decreased peripheral removal have been demonstrated. Hypercholesteremia and hypertriglyceridemia have been reported to occur in diabetic rats (Pushparaj et al., 2000). The antilipemic effect of GLEt may be due to the down regulation of NADPH and NADH, the cofactors in the fat metabolism. In addition, GLEt may down regulate the lipogenesis and lower risk of the tissues for oxidative stress and high resistance for diabetes.

It has been suggested that free radical species responsible for toxicity is the hydroxyl radical formed via the metal catalyzed Haber-Weiss reaction.
**Table 7.** Changes in levels of cholesterol, free fatty acid, triglycerides and phospholipids in serum and liver of normal and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol</th>
<th>Free fatty acid</th>
<th>Triglycerides</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Serum</td>
<td>Liver</td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td>(mg/100 g</td>
<td>(mg/dl)</td>
<td>(mg/100 g</td>
<td>(mM/L)</td>
</tr>
<tr>
<td></td>
<td>wet tissue)</td>
<td></td>
<td>wet tissue)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>330 ± 2.62(^a)</td>
<td>77 ± 3.41(^a)</td>
<td>606 ± 3.98(^a)</td>
<td>2757 ± 342.0(^a)</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>513 ± 4.57(^b)</td>
<td>95 ± 7.51(^b)</td>
<td>911 ± 8.29(^b)</td>
<td>3366 ± 727.3(^b)</td>
</tr>
<tr>
<td>Diabetic + GLEt</td>
<td>417 ± 4.11(^c)</td>
<td>81± 4.30(^c)</td>
<td>769 ± 3.11(^c)</td>
<td>3030 ± 417.0(^c)</td>
</tr>
<tr>
<td>(200 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic + Glibenclamide</td>
<td>438 ± 4.87(^d)</td>
<td>90 ± 4.86(^d)</td>
<td>800 ± 6.17(^d)</td>
<td>3124 ± 611.4(^d)</td>
</tr>
<tr>
<td>(600 µg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D for 6 rats in each group. Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT). Duncan procedure, range for the level 2.56, 3.09, 3.20.
or Fenton reaction (Lubec \textit{et al.}, 1996). In this process, the ferric iron is reduced by superoxide, with subsequent oxidation of ferrous iron by $\text{H}_2\text{O}_2$ forming hydroxyl radical thereby initiating the series of oxidative reactions. The results obtained from the present study may be attributed to few reasons which includes, the scavenging of OH or superoxide radical or by changing the ratio of $\text{Fe}^{3+}/\text{Fe}^{2+}$, reducing the rate of conversion of ferrous to ferric or by chelation of iron (Brauggler \textit{et al.}, 1986). The antioxidant property of the plant is well correlated with the concentration of the extract, which showed the presence of active principals in the extract. Phenolic compounds are known as powerful antioxidants. It has been reported that polyphenolic antioxidants have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka \textit{et al.}, 1998). The total amount of phenolic content in the GLEt is plotted against the percentage inhibition of lipid peroxidation, which suggested that the scavenging activity of the extract may be due to the phenolic constituents present in the plant \textit{G. montanum}.

The overwhelming evidence demonstrated above, indicates that hyperglycaemia coupled with hyperlipidemia increases the risk for cardiovascular diseases. GLEt significantly reduces the levels of serum and tissue lipids, which are actively raised in alloxan diabetic rats. GLEt has beneficial effect on plasma insulin, carbohydrate metabolic enzymes and lipid peroxidation. These findings strengthen the observation that naturally occurring compounds of plant origin have effective action against diabetes and its complications.

\textbf{References}


Metabolic Complications with Diabetes by Gymnema montanum


Basic & Clinical Pharmacology & Toxicology

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Date submitted: 2008-03-14
Decision date: 2008-03-14
Decision: Accept

Decision letter:

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