INTRODUCTION

Oxidative stress is currently suggested to be a mechanism underlying diabetes and diabetic complications [1]. Reactive oxygen species (ROS)† are generated in biological systems through metabolic processes and through exogenous sources such as food components, drugs, ultraviolet light, ionizing radiation, and air pollution [2]. Under physiological conditions, a wide range of antioxidant defenses protects against the adverse effects of free radical production in vivo [2]. The chronic hyperglycaemia in diabetes enhances the production of ROS from glucose oxidation, protein glycation and glycoxidation [3].

In diabetes, protein glycation and glucose oxidation may generate free radicals, which, in turn, cause lipid peroxidation [4]. Moreover, ROS have also been implicated in the mechanism of damage to the red blood cells (RBC) [5]. The concentration of
ROS is modulated by antioxidant enzymes such as SOD, CAT, and GPx, and by nonenzymatic antioxidants such as GSH [6]. In diabetes, oxidative stress seems to be caused by increased production of ROS, a sharp reduction in antioxidant defenses, and altered cellular redox status [7]. In addition to endogenous mechanisms of quenching ROS, much attention has been focused on the antioxidative roles of many plant extracts. Plant products are considered to be less toxic and freer from side-effects than synthetic ones [8].

Umbelliferone (7-hydroxycoumarin), a derivative of coumarin, is a benzopyrone present in the fruits and roots of Anethum graveolens L and the roots of Ruta graveolens L c9-11. It was noted that several plant-derived phenolic coumarins might play a role as dietary antioxidants through their consumption in the human diet in fruits and vegetables; UMB has been reported to have antioxidant properties [12]. The parent compound coumarin has been reported to reduce blood glucose level [13]. Coumarin may be a prodrug for which 7-hydroxycoumarin is the pharmacologically active agent [14]. Our preliminary studies showed that treatment with UMB effectively reduced blood glucose levels in diabetic rats [15], but no detailed study has been carried out on the effect of UMB on erythrocyte redox status in the diabetic condition. Hence, the present study was designed to investigate the effect of UMB on lipid peroxidation, antioxidants and lipid profile in erythrocytes of STZ-diabetic rats. The structure of UMB is depicted in Figure 1.

**MATERIALS AND METHODS**

**Animals**

Male albino (9-week-old) rats of Wistar strain with a body weight ranging from 180 to 200 g, were procured from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and were maintained in an air-conditioned room (25 ± 1°C) with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum* to all the animals. All studies were conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals [15] and the study was approved by the Ethical Committee of Rajah Muthiah Medical College and Hospital (Reg No.160/1999/CPCSEA), Annamalai University, Annamalainagar.

**Chemicals**

Streptozotocin was purchased from Sigma-Aldrich (St. Louis, Missouri, United States). UMB was procured from Carl Roth GmbH and Company (Germany). All the other chemicals used in our study were of analytical grade obtained from E. Merck and HIMEDIA (India).

**Experimental induction of diabetes**

The animals were rendered diabetic by a single intraperitoneal injection of STZ (40 mg/kg/b.wt) in freshly prepared citrate buffer (0.1 M, pH 4.5) after an overnight fast. STZ injected animals were given 20 percent glucose solution for 24 hours to prevent initial drug-induced hypoglycaemic mortality. STZ-injected animals exhibited massive glycosuria (determined by Benedict’s qualitative test) and hyperglycaemia (by glucose oxidase method) within a few days. Diabetes in STZ rats was confirmed by measuring the fasting blood glucose concentration 96 hours after injection with STZ. The animals with blood glucose above 235 mg/dl were considered to be diabetic and used for the experiment.
**Experimental design**

The animals were randomly divided into five groups of six animals described below. The UMB and glibenclamide were administered intraperitoneally using a vehicle solution (10 percent DMSO).

- **Group I:** Normal control (10 percent DMSO)
- **Group II:** Normal + UMB (30 mg/kg/b.wt in 10 percent DMSO)
- **Group III:** Diabetic control (10 percent DMSO)
- **Group IV:** Diabetic + UMB (30 mg/kg/b.wt in 10 percent DMSO)
- **Group V:** Diabetic + glibenclamide (600 µg/kg/b.wt in 10 percent DMSO)

After 45 days of treatment, the animals were fasted for 12 hours, anaesthetized between 8:00 a.m. to 9:00 a.m. using ketamine (24 mg/kg/b.wt, intramuscular injection), and sacrificed by cervical decapitation. Blood was collected in tubes with a mixture of potassium oxalate and sodium fluoride (1:3) for the estimation of blood glucose. The buffy coat was removed, and the erythrocytes were washed three times with physiological saline. Aliquots of erythrocytes were kept at 4°C until analysis.

**Biochemical analysis**

Blood glucose was estimated by the method of Trinder et al. [16]. Erythrocyte TBARS, HP, and CD were estimated by the methods of Nichans and Samuelson [17], Jiang et al. [18], and Klein [19], respectively. The nonenzymic antioxidants, GSH, vitamins C and E were estimated by the methods of Ellman [20], Roe and Kuether [21], and Baker and Frank [22], respectively. The activities of SOD, CAT, GPx, glucose-6-phosphate dehydrogenase, and the levels of cholesterol and phospholipids were measured by the methods of Kakkar et al. [23], Sinha [24], Rotruck et al. [25], Bergmeyer [26], Siedel et al. [27], and Zilversmit and Davis [28], respectively.

**Statistics**

Values are given as means ± SD for six rats in each group. Data were analysed by one way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT) using SPSS-10. The limit of statistical significance was set at p < .05.

**RESULTS**

Table 1 illustrates the effect of UMB on blood glucose in diabetic rats. Blood glucose was significantly elevated in diabetic rats as compared with normal control rats. In UMB and glibenclamide-treated rats, the blood glucose was reversed to near normal level when compared with diabetic control rats. The changes in the levels of TBARS, HP, and CD in the erythrocytes of normal and diabetic rats are given in the Table 2. Diabetic rats had elevated levels of
Table 2. Effect of UMB on lipid peroxidation markers in erythrocytes of diabetic rats.

| Group                     | TBARS (nmol/mg protein) | HP (µmol/mg protein) | CD (Ratio of 240/214 nm) |
|---------------------------|-------------------------|----------------------|-------------------------|
| Normal control            | 1.93 ± .13b             | 1.15 ± .09b          | 0.72 ± .05b             |
| Normal + UMB (30 mg/kg/b.wt) | 1.72 ± .07a             | .085 ± .05a          | 0.66 ± .04a             |
| Diabetic control          | 4.67 ± .32a             | 1.38 ± .07d          | 0.87 ± .07d             |
| Diabetic + UMB (30 mg/kg/b.wt) | 2.83 ± .23d             | 1.24 ± .04c          | 0.78 ± .03c             |
| Diabetic + glibenclamide (600 µg/kg b.wt.) | 2.39 ± .11c             | 1.21 ± .04c          | 0.75 ± .04b,c           |

Values are given as means ± SD from six rats in each group. Values not sharing a common superscript vertically differ significantly at p < 0.05 (DMRT).

Table 3. Effect of UMB on enzymic antioxidants in erythrocytes of diabetic rats.

| Group                     | SOD (U*/mg Hb) | CAT (U**/mg Hb) | GPx (U***/mg Hb) |
|---------------------------|----------------|-----------------|------------------|
| Normal control            | 7.50 ± .54     | 176.09 ± 11.03b | 14.75 ± 1.04b    |
| Normal + UMB (30 mg/kg/b.wt) | 8.17 ± 1.18a   | 189.86 ± 7.72a  | 16.6 ± .78a      |
| Diabetic control          | 3.52 ± .66e    | 100.54 ± 9.52d  | 8.45 ± .69d      |
| Diabetic + UMB (30 mg/kg/b.wt) | 6.15 ± .78c   | 157.20 ± 4.90c  | 13.4 ± .61c      |
| Diabetic + glibenclamide (600 µg/kg b.wt.) | 6.75 ± .50c   | 164.97 ± 7.81c  | 14.15 ± .91b,c   |

Values are given as means ± SD from six rats in each group. Values not sharing a common superscript differ significantly at p < .05 (DMRT). *One unit of SOD is defined as the enzyme reaction, which gives 50 percent inhibition of NBT reduction in one minute. **One unit of CAT is defined as the µmole of hydrogen peroxide consumed per minute. ***One unit of GPx is defined as the µg of glutathione consumed per minute.

TBARS, HP, and CD in erythrocytes as compared with normal control rats. Diabetic rats treated with UMB and glibenclamide brought back TBARS, HP, and CD to near normal levels.

The levels of nonenzymatic, enzymatic antioxidants, and glucose-6-phosphate dehydrogenase in erythrocytes of normal and diabetic rats are represented in Tables 3 and 4. Diabetic rats showed a significant decrease in erythrocyte levels of vitamin C, GSH, SOD, CAT, GPx, and glucose-6-phosphate dehydrogenase and an increase in vitamin E levels as compared to normal control rats. Diabetic rats treated with UMB and glibenclamide showed a reversal of erythrocyte vitamin E and vitamin C, GSH, and SOD, CAT, GPx, and glucose-6-phosphate dehydrogenase levels when compared to diabetic control rats.

The levels of cholesterol and phospholipids in erythrocytes of normal and
Table 4. Effect of UMB on nonenzymatic antioxidants and glucose-6-phosphate dehydrogenase in erythrocytes of diabetic rats.

| Group                        | Vitamin C (µg/mg of Hb) | Vitamin E (µg/mg of Hb) | GSH (mg/dl) | Glucose-6 phosphate dehydrogenase (IU/g Hb) |
|------------------------------|--------------------------|--------------------------|-------------|---------------------------------------------|
| Normal control               | 1.85 ± .03              | 1.18 ± .08              | 75.76 ± 3.71| 4.65 ± .31                                  |
| Normal + UMB (30 mg/kg/b.wt) | 2.16 ± .01              | 1.39 ± .02              | 80.26 ± 3.84| 5.85 ± .42                                  |
| Diabetic control             | 0.95 ± .06              | 2.74 ± .09              | 48.53 ± 4.37| 3.1 ± .36                                   |
| Diabetic + UMB (30 mg/kg/b.wt)| 1.73 ± .02             | 1.52 ± .10              | 63.2 ± 2.42 | 4.25 ± .35                                  |
| Diabetic + glibenclamide (600 g/kg/b.wt) | 1.78 ± .03           | 1.43 ± .08              | 67.73 ± 2.5 | 4.55 ± .29                                  |

Values are given as means ± SD from six rats in each group. Values not sharing a common superscript differ significantly at p < .05 (DMRT).

Table 5. Effect of UMB on cholesterol and phospholipids in erythrocytes of diabetic rats.

| Group                        | Cholesterol (µg/mg protein) | Phospholipids (µg/mg protein) |
|------------------------------|-----------------------------|-------------------------------|
| Normal control               | 148.5 ± 7.19                | 294.82 ± 17.08               |
| Normal + UMB (30 mg/kg/b.wt) | 165.16 ± 5.16               | 314.07 ± 17.70               |
| Diabetic control             | 108.16 ± 6.73               | 221.72 ± 21.13               |
| Diabetic + UMB (30 mg/kg/b.wt)| 137.86 ± 6.62              | 275.54 ± 26.23              |
| Diabetic + glibenclamide (600 g/kg/b.wt) | 141.16 ± 8.26            | 278.72 ± 24.62              |

Values are given as means ± SD from six rats in each group. Values not sharing a common superscript differ significantly at p < .05 (DMRT).

diabetic rats are represented in Table 5. Diabetic control rats had decreased levels of cholesterol and phospholipids in erythrocytes. Treatment with UMB and glibenclamide brought back cholesterol and phospholipids levels to near normal levels as compared with the diabetic control rats.

**DISCUSSION**

In an earlier report, coumarin was reported to reduce blood glucose levels [13]. In our study, diabetic rats treated with UMB brought blood glucose level to near normal level. A possible mechanism by which UMB brings about its antihyperglycaemic effect is through the elevation of plasma insulin levels.

The tremendous increase in lipid peroxidation in erythrocytes observed in diabetic rats is attributed to chronic hyperglycaemia, which causes increased production of ROS due to autoxidation of monosaccharides, which leads to the production of superoxide and hydroxyl radicals. This, in turn, causes tissue damage by reacting with polyunsaturated fatty acids in membrane [30, 31]. It was noted that several of the plant-derived phenolic coumarins...
might play a role as dietary antioxidants because of their consumption in the human diet in fruits and vegetables, and UMB has also been reported to have antioxidant an property [12]. Diabetic rats treated with UMB brought lipid peroxidation markers back to near normal, which could be a result of improved antioxidant status.

Oxidative stress in diabetes correlates with a reduction in the antioxidant status [32]. The antioxidants vitamins C and E have been shown to reduce oxidative stress in experimental diabetes [33]. In our study, decreased vitamin C and increased vitamin E are found in the erythrocytes of diabetic rats. This is due to increased utilization and increased membrane damage. Since circulating RBCs act as a sink for free radicals, both superoxide radicals and hydrogen peroxide have the ability to penetrate the membrane of the cells [34]. In UMB-treated rats, the reversal of these antioxidants is due to decreased peroxidation of erythrocyte membrane.

Normally, the SOD enzyme works in parallel with GPx, which plays an important role in the reduction of hydrogen peroxides in the presence of GSH-forming oxidized glutathione, (GSSG) thereby protecting cell protein and membrane from oxidative stress [35]. The erythrocyte SOD, CAT, and GPx activities were decreased in diabetic rats. These results are consistent with the report of Skhra et al. [36]. In UMB-treated rats, the reversal of activities of these enzymes in erythrocytes is evidenced by decreased lipid peroxidation markers and improved glycaemic control.

ROS are continuously generated in physiological conditions and eliminated by several intracellular and extracellular antioxidant systems [37]. Decreased activity of antioxidant enzymes in uncontrolled diabetes is due to decreased GSH formation, which requires NADPH and glutathione reductase [38]. The reduced availability of NADPH could be due to reduced synthesis in the HMP shunt due to decreased activity of glucose-6-phosphate dehydrogenase, which plays an important role in the maintenance of the high NADPH/NADP⁺ ratio in the cell and plays a crucial role in the regeneration of GSH from GSSG [39]. In UMB-treated rats, the increased activity of glucose-6-phosphate dehydrogenase is due to enhanced synthesis caused by UMB. The NADPH generated could increase the concentration of GSH observed in our study, which is, in turn, utilized by GPx [40].

In our study, the reduction of TC in erythrocytes of diabetic rats was observed, as reported earlier [41]. The reduction in membrane cholesterol content is known to increase the disordering and hence alter the fluidity of membrane [42]. The diabetic rats treated with UMB reversed membrane cholesterol to near normal, which could be as a result of decreased lipid peroxidation.

Phospholipids are vital components of biomembranes and play an important role in the transport of triglycerides [43]. In our study, the total phospholipid content of erythrocytes was decreased in STZ-diabetic rats, which is in agreement with Jain et al. [44], who reported that choline containing phospholipids, which exists at the outer side of the membrane, were not altered, whereas the phosphatidylethanolamine and phosphatidylserine existing at the inner side of the erythrocyte membrane were reduced. This hypothesis supports the idea that the phospholipid fraction closer to the site of peroxidation reaction was affected. The diabetic rats treated with UMB have reversed the total phospholipids of erythrocytes as a result of reduced membrane lipid peroxidation.

**CONCLUSION**

Our results indicate that UMB has exerted a rapid protective effect against lipid peroxidation by scavenging free radicals and elevating both nonenzymic and enzymic antioxidants and thus alleviating the adverse complications of diabetes mellitus.
REFERENCES

1. Halliwall B and Gutteridge JMC. Free Radicals in Biology and Medicine, 2nd ed, Oxford: Clarendon Press, 1989.
2. Briviba K and Sies H. Nonenzymatic antioxidant defense systems. In: Frei B. Natural antioxidants in Human Health and Disease. San Diego: Academic Press, Inc.; p. 107.
3. Brownlee M. Metabolism: clinical and experimental. 2000;49:9-13.
4. Mullarkey CJ, Edelstein D, and Brownlee L. Free radical generation by early glycation products a mechanism for accelerated atherogenesis in diabetes. Biochem Biophys Res Common 1900:932-9.
5. Scott MD, van den Berg JJ, Repka J, et al. J Clin Invest. 1993;91:1706.
6. Saxena AK, Srivastava P, Kale RK, and Baquer NZ. Impaired antioxidant status in diabetic rat liver. Effects of vanadate. Biochem and Pharmacol 1993;45:539-42.
7. West CJ. Diabetic Medicine. 2000;17:171-80.
8. Morin A. Role of indigenous medicine in primary health. Proceedings of First International Seminar on Unani Medicine, New Delhi, India; 1987, p. 54.
9. Keating GJ and O’ Kennedy R. The chemistry and occurrence of coumarins. In: O’ Kennedy R and Thornes RD, eds. Coumarins: Biology, Applications and Mode of Action. New York: John Wiley & Sons, Inc.; 1997, p. 348.
10. Parmar C and Kaushal MK. Aegle marmelos. In: Wild Fruits. New Delhi: Kalyani Publishers; 1982, pp. 1-5.
11. Wu FJ and Sheu SJ. Analysis and processing of Chinese herbal drugs: The study of Fructus auranti immaturus. Chin Pharm J 1992;44:257-63.
12. Hoult JRS and Paya M. Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. Gen Pharmacol 1996;27:713-22.
13. Marles RJ and Farnsworth N. Antidiabetic plants and their action constituents: An update. Prof J Bot Med. 1996;3:85-135.
14. Ritschel WA, Grummiche KW, Kaul S, and Hardt TJ. Pharm Ind. 1981;43: 271.
15. National Institute of Health. Guide for the Use and Care of Laboratory Animals. DHEW Publication (NIH), 2nd ed., revised. Bethesda, Maryland: Office of Science and Health Reports, DRR/NIH; 1985.
16. Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann Clin Biochem. 1969;6:24.
17. Nichans WG and Samuelson B. Formation of MDA from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem 1968;6:126-30.
18. Jiang ZY, Hunt J, and Wolff SP. Detection of lipid hydroperoxides using the “Fox method.” Anal Biochem 1992;202:384-9.
19. Klein RA. The detection of oxidation in liposome preparation. Biochim Biophys Acta 1979;210:486-9.
20. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-7.
21. Roe JH and Kuether CA. Detection of ascorbic acid in whole blood and urine through the 2,4-DNPH derivative of dehydroascorbic acid. J Biol Chem 1943;147:399-407.
22. Baker H, Frankel O, De Angelis B, and Feingold S. Plasma α-tocopherol in man at various time intervals after ingesting free or acetylated tocopherol. Nutr Rep Int 1980;21:531-6.
23. Kakkar P, Das B, and Viswanathan PN. A modified spectrophotometric assay of SOD. Ind J Biochem Biophys 1978;21:130-2.
24. Sinha KA. Colorimetric assay of catalase. Annal Biochem 1972;47:389-94.
25. Rotruck JJ, Pope AL, Gantter HE, and Swanson AB. Selenium: biochemical role as a component of glutathione peroxidase. Science 1973;179:588-90.
26. Bergmeyer HU. Glucose-6-phosphate dehydrogenase. In: Bergmeyer, HU, ed. Methods of Enzymatic Analysis, Vol. 2. Weihem: Verlag Chemie. 1984, pp. 222-3.
27. Siedel J, Hagele EO, Ziegenhorn J, and Wahlefeld AW. Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. Clin Chem 1983;20:1075.
28. Zilversmit DB and Davis AK. Micro determination of phospholipids by TCA precipitation. J Lab Clin Med 1950;35:155-9.
29. The expert committee on the diagnosis and classification of diabetes mellitus: follow-up report on the diagnosis of diabetes mellitus. Diabetes Care. 2003;26:3160-7.
30. Wolff S and Dean RT. Glucose autooxidation and protein modification. Biochem J 1987;24:243-50.
31. Das S, Vasishf S, Snehalata, Das N, and Srivastava M. Correlations between total antioxidant status and lipid peroxidation in hypercholesterolemia. Curr Sci 2000;78:486.
32. Collier A, Wilson R, Bradley H, Thompson JA, and Small M. Free radical activity in type 2 diabetes. Diab Med 1990;7:27-30.
33. Madhu CG and Devi DB. Protective antioxidant effects of vitamin E and C in
streptozotocin diabetic rats. Ind J Exp Biol. 2000;38:101-4.
34. Arai K, Lizuka S, Tada Y, Oikawa K, and Taniguchi NC. Increase in the glycosylated form erythrocyte Cu-Zn superoxide dismutase in diabetes and close association of the nonenzymatic glycosylation with the enzyme activity. Biochem Biophys Acta. 1989;924:292-6.
35. Jacob RA. The integrated antioxidant system. Nutr Res 1995;15:755-66.
36. Skhra J, Hodinar A, Kvosnicka J, and Hilgertova J. Relationship of oxidative stress and fibrinolysis in diabetes mellitus. Diab Med 1996;13:800-3.
37. Halliwall B and Gutteridge JMC. Free Radicals in Biology and Medicine, 3rd ed. Oxford: Oxford University Press; 1999, p. 936.
38. Garg MC, Ojha S, and Bansal DD. Antioxidant status in streptozotocin diabetic rats. Ind J Exp Biol 1996;34:264-6.
39. Jain SK. Glutathione and glucose-6-phosphate dehydrogenase deficiency can increase protein glycation. Free Radic Biol Med. 1998;24:197-201.
40. Shanthi VP and Ramakrishnan P. Mechanism of antioxidant effect of Bordetella pertussis extract. Ind J Biochem Biophys 1994;31:398-402.
41. Baba Y, Kai M, Kamada T, Setoyama S, and Ostuji S. High levels of erythrocyte membrane microviscosity in diabetes. Diabetes 1979;28:1138-40.
42. Cooper RA, Leslie MH, Fischkoff S, Shinitzky S, and Shattil SJ. Factors affecting the lipid composition and fluidity of red cell membrane in vitro: Production of red cells possessing more than two cholesterols per phospholipid. Biochemistry 1978;17:327-31.
43. Draznin B and Eckel RH. Diabetes and atherosclerosis: molecular basis and clinical aspects. In: Betteridge J, ed. Lipids and Vascular Disease. New York: Elsevier; 1997, pp. 17-38.
44. Jain SK, Levine SN, Duett J, and Holliert B. Elevated lipid peroxidation levels in red blood cells of streptozotocin treated diabetic rats. Metabolism 1989;39:971-5.