

## ORIGINAL CONTRIBUTION

# Impact of Umbelliferone on Erythrocyte Redox Status in STZ-diabetic Rats

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Oxidative stress is currently hypothesized to be a mechanism underlying diabetes. The present study was designed to evaluate the effect of umbelliferone (UMB), a derivative of coumarin, on erythrocyte lipid peroxidation, antioxidants, and lipid profile in normal and streptozotocin (STZ) diabetic rats. Diabetes was induced in adult male albino rats of Wistar strain, weighing 180 to 200 g, by the administration of STZ (40 mg/kg/b-wt) intraperitoneally. The normal and diabetic rats were treated with UMB in 10 percent dimethyl sulfoxide (DMSO) dissolved in water for 45 days. The diabetic rats had elevated levels of blood glucose and lipid peroxidation markers such as thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), and lipid hydroperoxide (HP) and decreased levels of nonenzymatic antioxidants (Vitamin C and reduced glutathione [GSH]), elevated levels of vitamin E, and elevated levels of enzymatic antioxidants (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx]), elevated glucose-6-phosphate dehydrogenase activity, and altered lipid profile (cholesterol and phospholipids) in erythrocytes. These changes were reversed by treatment with UMB. Thus, our results indicate that the administration of UMB shows promising potential for the restoration of normal blood glucose levels, erythrocyte lipid peroxidation, antioxidants, and lipid profile in STZ-diabetic rats.

## INTRODUCTION

Oxidative stress is currently suggested to be a mechanism underlying diabetes and diabetic complications [1]. Reactive oxygen species (ROS)<sup>†</sup> 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 glycooxidation [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

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<sup>†</sup>Abbreviations: CAT, catalase; CD, conjugated dienes; DMSO, dimethyl sulfoxide; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; HP, lipid hydroperoxide; RBC, red blood cells; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, thiobarbituric acid reactive substances; UMB, umbelliferone.

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.

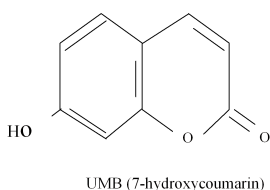


Figure 1. Structure of UMB.

## 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 ( $25 \pm 1^\circ\text{C}$ ) 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.

**Table 1. Effect of UMB on blood glucose in diabetic rats.**

Group	Blood glucose (mg/dl)	
	0 day	45th day
Normal control	79.60 ± 5.25	82.44 ± 2.68 <sup>b</sup>
Normal + UMB (30 mg/kg/b.wt)	82.14 ± 3.19	74.29 ± 4.17 <sup>a</sup>
Diabetic control	240.47 ± 5.82	289.28 ± 3.18 <sup>d</sup>
Diabetic + UMB (30 mg/kg/b.wt)	244.63 ± 6.29	114.28 ± 5.71 <sup>c</sup>
Diabetic + glibenclamide (600 µg/kg/b.wt)	242.85 ± 5.04	107.23 ± 7.23 <sup>c</sup>

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

### 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	Haemolysate		
	TBARS ( nmol/mg protein)	HP ( $\mu$ mol/mg protein)	CD (Ratio of 240/214 nm )
Normal control	1.93 $\pm$ .13 <sup>b</sup>	1.15 $\pm$ .09 <sup>b</sup>	0.72 $\pm$ .05 <sup>b</sup>
Normal+UMB (30 mg/kg/b.wt)	1.72 $\pm$ .07 <sup>a</sup>	.085 $\pm$ .05 <sup>a</sup>	0.66 $\pm$ .04 <sup>a</sup>
Diabetic control	4.67 $\pm$ .32 <sup>e</sup>	1.38 $\pm$ .07 <sup>d</sup>	0.87 $\pm$ .07 <sup>d</sup>
Diabetic+ UMB (30 mg/kg/b.wt)	2.83 $\pm$ .23 <sup>d</sup>	1.24 $\pm$ .04 <sup>c</sup>	0.78 $\pm$ .03 <sup>c</sup>
Diabetic+ glibenclamide (600 $\mu$ g/kg b.wt.)	2.39 $\pm$ .11 <sup>c</sup>	1.21 $\pm$ .04 <sup>c</sup>	0.75 $\pm$ .04 <sup>b,c</sup>

Values are given as means  $\pm$  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	Hemolysate		
	SOD (U*/mg Hb)	CAT (U**/mg Hb)	GPx (U***/mg Hb)
Normal control	7.50 $\pm$ .54	176.09 $\pm$ 11.03 <sup>b</sup>	14.75 $\pm$ 1.04 <sup>b</sup>
Normal + UMB (30 mg/kg/b.wt)	8.17 $\pm$ 1.18 <sup>a</sup>	189.86 $\pm$ 7.72 <sup>a</sup>	16.6 $\pm$ .78 <sup>a</sup>
Diabetic control	3.52 $\pm$ .66 <sup>e</sup>	100.54 $\pm$ 9.52 <sup>d</sup>	8.45 $\pm$ .69 <sup>d</sup>
Diabetic + UMB (30 mg/kg/b.wt)	6.15 $\pm$ .78 <sup>c</sup>	157.20 $\pm$ 4.90 <sup>c</sup>	13.4 $\pm$ .61 <sup>c</sup>
Diabetic + glibenclamide (600 $\mu$ g/kg/b.wt)	6.75 $\pm$ .50 <sup>c</sup>	164.97 $\pm$ 7.81 <sup>c</sup>	14.15 $\pm$ .91 <sup>b,c</sup>

Values are given as means  $\pm$  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  $\mu$  mole of hydrogen peroxide consumed per minute. \*\*\*One unit of GPx is defined as the  $\mu$ g 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	Hemolysate			
	Vitamin C ( $\mu\text{g}/\text{mg}$ of Hb)	Vitamin E ( $\mu\text{g}/\text{mg}$ of Hb)	GSH (mg/dl)	Glucose-6 phosphate dehydrogenase (IU/g Hb)
Normal control	$1.85 \pm .03^b$	$1.18 \pm .08^b$	$75.76 \pm 3.71^b$	$4.65 \pm .31^b$
Normal + UMB (30 mg/kg/b.wt)	$2.16 \pm .01^a$	$1.39 \pm .02^a$	$80.26 \pm 3.84^a$	$5.85 \pm .42^a$
Diabetic control	$0.95 \pm .06^d$	$2.74 \pm .09^e$	$48.53 \pm 4.37^e$	$3.1 \pm .36^d$
Diabetic + UMB (30 mg/kg/b.wt)	$1.73 \pm .02^c$	$1.52 \pm .10^e$	$63.2 \pm 2.42^d$	$4.25 \pm .35^c$
Diabetic + glibenclamide (600 g/kg/b.wt)	$1.78 \pm .03^c$	$1.43 \pm .08^c$	$67.73 \pm 2.5^c$	$4.55 \pm .29^c$

Values are given as means  $\pm$  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	Hemolysate	
	Cholesterol ( $\mu\text{g}/\text{mg}$ protein)	Phospholipids ( $\mu\text{g}/\text{mg}$ protein)
Normal control	$148.5 \pm 7.19^b$	$294.82 \pm 17.08^b$
Normal + UMB (30 mg/kg/b.wt)	$165.16 \pm 5.16^a$	$314.07 \pm 17.70^a$
Diabetic control	$108.16 \pm 6.73^d$	$221.72 \pm 21.13^d$
Diabetic + UMB (30 mg/kg/b.wt)	$137.86 \pm 6.62^c$	$275.54 \pm 26.23^c$
Diabetic + glibenclamide (600 mg/kg/b.wt)	$141.16 \pm 8.26^{b,c}$	$278.72 \pm 24.62^c$

Values are given as means  $\pm$  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 autooxidation 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 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<sup>+</sup> 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.

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