HYPOGLYCEMIC EFFECT OF 2-HYDROXY 4-METHOXY BENZOIC ACID ISOLATED FROM THE ROOTS OF *HEMIDESMUS INDICUS* ON STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Summary

The active principle, 2-Hydroxy 4-methoxy benzoic acid (HMBA) isolated from the roots of *Hemidemus indicus* was investigated for its antidiabetic activity in streptozoticin (STZ)-induced diabetic rats. HMBA of *H. indicus* was administered (500 µg/kg body weight) orally to fed, fasted and glucose-loaded diabetic and non diabetic rats. The effect of HMBA on liver and kidney glycolytic, glyconeogenic enzymes and serum marker enzymes of diabetic rats was also studied. The blood glucose level was reduced significantly ($F>0.05$ (ANOVA) and $P< 0.05$ (DMRT)) in fed, fasted, and glucose loaded diabetic rats. The increased activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase in diabetic rats was significantly ($F>0.05$; $P< 0.001$) decreased to near normal level in the liver and kidney of HMBA treated (7 weeks) diabetic rats. The decreased levels of hexokinase and phosphoglucoisomerase in the liver and kidney of diabetic rats were restored to normal level when diabetic rats were fed with HMBA. A significant reduction in glucose, glucose-6-phosphatase, and fructose-1, 6-bisphosphatase activities in diabetic rats indicate the role of HMBA in suppressing the gluconeogensis in diabetic rats. Alternatively normalization of hexokinase and phosphoglucoisomerase activities indicates the role of HMBA in inducing glycolysis in diabetic rats. On the basis of our findings, HMBA could be used as an antidiabetic agent for prevention and management of diabetes mellitus.

Keywords: Diabetes mellitus, 2-Hydroxy 4-methoxy benzoic acid, glucose-6-phosphatase, hexokinase

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Introduction

Diabetes mellitus is a major global health problem by 2025 it is suggested that 300 million people will have diabetes world wide. India has more than 40 million diabetic people which represent nearly 20% of total diabetes population of the whole world. Antidiabetic allopathic medicines are often overprescribed, found to be dangerous on long term use due to its toxicity and adverse effects in the body. World Health Organization (WHO) as recommended the evaluation of traditional plant treatments for diabetes (1). Medicinal plants are traditionally used in many countries to control diabetes mellitus (2, 3). Plant based remedies are considered to be natural, safe and remains as one of the most popular and complementary treatments for diabetes mellitus. Traditionally many plants are used in Ayurveda, Sidha and folklore systems of medicines to treat diabetes mellitus (4). The hypoglycemic effect of more than 800 plant species has been reported (2). But the pharmacognostic and pharmacological studies on many of these plants are yet to be explored. There are more than 200 pure compounds representing numerous chemical compounds from plant sources have already been reported with their possible use in diabetes mellitus as glucose lowering agent (4). The need of new chemical entities (NCEs) for diabetic health care is explored and served through the plant sources (5). The plant phytochemicals such as alkaloids, glycosides, galactomannan gun, polysaccharides, peptidoglycons, hypoglycans, guanidine, steroids, carbohydrates glycopeptides, terpenoids, amino acids and inorganic ions have been used to control diabetes (2).

Hemidesmus indicus (Asclepiadaceae) is one of the indigenous Ayurvedic medicinal plants commonly available and widely distributed throughout India. The root bark of this plant has been used as a traditional medicine in the treatment of biliousness, blood diseases, diarrhoea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism (6). *H. indicus* is also employed in traditional medicine for gastric ailments (7). It mainly consists of essential oils and phytosterols such as hemidesmol, hemidesterol and saponins. Isolation of the pure compound 2-hydroxy-4-methoxy benzoic acid (HMBA) having the molecular formula $C_8H_8O_4$ from the methanolic root extract of *H. indicus* root bark was reported (8). HMBA has been shown to possess potent anti-inflammatory, antipyretic and antioxidant properties (9). The compound effectively neutralizes viper-venom-induced changes in serum phosphatase and transaminase activity in male albino rats and is also known to reduce free radical formation as estimated by thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) activity (10). The compound also has an adjuvant effect and antiserum potentiation activity against viper venom (11). HMBA has not been reported as toxic and carcinogen by ACGIH, IARC, NIOSH, NTP and OSHA. The protective effect of *H.indicus* against rifampicin- and isoniazid induced hepatotoxicity in rats (12), as well as CCl_4 and paracetamol-induced hepatic damage (13), is known. In the present investigation HMBA was isolated from the roots of *H.indicus* and tested for their antidiabetic activity in STZ-induced diabetic rats.

Materials and methods

Animals

Male Wister rats weighing 150-200 g were used. They were housed in standard environmental conditions (as per Institutional Animal Ethical Committee norms) and fed with standard pellet diet and water *ad libitum.*

Plant material

The roots of *H. indicus* were collected from the Morappur forest area, Dharmapuri District, Tamil Nadu, India. The roots were identified with help of botanist and the voucher specimen was submitted to the VIT University. Roots of *H. indicus* was washed with distilled water, shade dried, powdered and stored in an air- tight container until for further use.

Extraction, isolation and purification of the pure compound

The root powder of *H. indicus* (100 g) was extracted with methanol using soxhlet apparatus and concentrated in rotary evaporator and then purified by silica gel (Merck,100 to 200 mesh) column chromatography using benzene-chloroform as eleuant (8) and the purity was checked by thin layer chromatography. The purity of the isolated compound was confirmed with the standard.

Induction of diabetes

Diabetes was induced experimentally in rats by a single intraperitoneal injection of freshly prepared solution of streptozotocin (STZ) (Sigma, USA) at a dose of 35 mg/kg, bodyweight in 0.1M citrate buffer, pH 4.5. The STZ treated animals were considered to be diabetic, if the blood glucose values were above 250 mg/dl and stabilized for a period of 7 days and those animals alone were selected for this study.

Experimental design

Animals were divided in to four groups of six animals each. Group I served as a control; group II had STZ- treated surviving diabetic rats; group III served as a positive control and received a standard hypoglycemic agent, talbutamide (100 mg/kg bw); group IV diabetic rats treated with the HMBA (500 µg/kg bw) by oral intubation method. Blood samples were collected in heparinised vials in each time from the tail using aseptic precautions at the end of 1 hr, 3 hr and 5 hr and blood glucose and plasma insulin levels were measured. Blood glucose levels in rats were estimated by glucose oxidase method (14). Administration of HMBA was continued for 7weeks and at the end of treatment period the animals were sacrificed and the liver and kidney were dissected and washed in ice-cold saline and stored at 4 °C.

Assay of glycolytic and gluconeogenic enzymes

The liver and kidney tissues were used for enzyme assays. The enzymes hexokinase (15), phosphoglucoisomerase (16), aldolase (17), glucose-6-phosphatase (King, 18) and fructose-1,6-disphosphatase (19) were assayed in the tissue homogenates.

Assay of serum marker enzymes

Serum marker enzymes, glycogen synthase (GS), glucokinase (GK), lactate dehydrogenase (LDH), succinate dehydrogenase (SD) and malate dehydrogenase (MD) were estimated by using commercial kits purchased from Bayer Diagnostics India Ltd.

Statistical analysis

Statistical analysis was performed using SPSS software package, version 9.05. The values were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). All the results were expressed as mean ±SD for six rats in each group, *P* values <0.05 were considered as statistically significant.

Results

The HMBA isolated is a white needle-shaped crystal, soluble in water, methanol and chloroform and has a melting point of 155–158°C and lambda max 260 nm as reported (Alam et al., 1994). Spectral analysis revealed the presence of a benzene ring, methoxy group and hydroxyl group and the molecular weight of the compound were found to be 168.

STZ treatment increased the level of blood glucose significantly ($F>0.05$; $P< 0.001$) in experimental rats when compared to control rats. Administration of HMBA decreased the glucose level in blood significantly ($F>0.05$; $P< 0.001$) at 1 h, 3 h and 5 h in STZ–induced diabetic rats in the fasted, fed and glucose- loaded models [Table 1].

The metabolic enzymes of glucose, hexokinase and phosphoglucoisomerase of liver and kidney were significantly decreased ($F>0.05$; $P< 0.001$) in STZ-induced diabetic rats when compared to normal control rats [Table 2]. Oral administration of HMBA had a significant effect ($F >0.05$; $P< 0.001$) in restoring the levels of glycolytic enzymes to near-normal levels.

Table 3 shows the effect of HMBA on serum carbohydrate metabolizing enzymes in normal and STZ induced-diabetic rats. The decreased activities of GS, GK, LDH, SD and MD in diabetic rats were restored significantly ($F >0.05$; $P< 0.001$) to near normal level on HMBA administration for 7 weeks. The activities of gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1, 6-diphosphatase increased in the liver and kidney of diabetic rats when compared with normal rats. A significant ($F >0.05$; $P< 0.001$) reduction in glucose-6-phosphatase and fructose-1, 6-bisphosphatase activities were observed in the groups treated with HMBA and tolbutamide when compared with diabetic controls [Table 4].

Table 1: Effect of HMBA on plasma glucose levels in normal and streptozotocin- induced diabetic rats

Groups	Experimental Condition	Dose (mg/kg bw/day)	1h (mg/dl)	3h (mg/dl)	5h (mg/dl)
Normal	-	-	167±2.09	167±2.09	167±2.09
Diabetic control	-	-	378±2.28	378±2.28	378±2.28
Diabetic + Tolbutamide	-	100	295±2.98*	282±2.96*	274±2.52*
Diabetic + HMBA (Fasted Model)	ND	0.5	169±2.48	163±2.45	156±2.98
	D	0.5	272±1.23*	267±2.61*	246±2.65*
Diabetic + HMBA (Fed model)	ND	0.5	189±2.36	160±2.15	165±1.84
	D	0.5	296±2.56*	260±2.45*	259±1.54*
Diabetic + HMBA (Glucose loaded Model)	ND	0.5	180±1.52	170±2.01	156±1.65
	D	0.5	287±1.84*	253±1.95*	246±2.03*

ND – Nondiabetic rats; D – Diabetic rats.
Each value is mean ± SD for six rats in each group.
*Values are statistically significant when compared to diabetic control at $F>0.05$ (ANOVA) and $P< 0.05$ (DMRT).

Table 2. Effect of HMBA on liver and kidney glycolytic enzymes in streptozotocin-induced diabetic rats

Gpoups	Liver			Kidney		
	Hexokinase	Aldolase	Phosphogluco-isomeras	Hexokinase	Aldolase	Phosphogluco-isomeras
Normal	412±30.12	165±6.8	53.75±0.97	312±30.12	225±6.8	32.75±0.97
Diabetic control	83.9±0.05	265.8±10.8	28.9±1.05	69.9±10.05	285.8±10.7	16.9±1.05
Diabetic + Tolbutamide (100 mg/kg bw/day)	418±20.0*	168±8.27*	56.8±0.04*	290±22.0*	235±8.27*	37.8±0.04*
Diabetic + HMBA 500 µg/kg bw/day)	415±10.02*	169±9.5*	53.85±0.04*	320±0.02*	225±9.5*	37.85±0.04*

Each value is mean ± SD for six rats in each group.
Hexokinase: (nmoles of glucose-6phosphate/min/mg/protein); Aldolase: (nmole of glyceraldehyde formed/min/mg protein); Phosphoglucoisomerase (nmoles of fructose formed/min/mg protein)
*Values are statistically significant when compared to diabetic control at $F>0.05$ (ANOVA) and $P< 0.05$ (DMRT).

Table 3: Effect of HMBA on serum marker enzymes GS, GK, LDH, SD and MD in normal and streptozotocin-induced diabetic rats

Groups	Dose (mg/kg bw/day)	GS (U/L)	GK (U/L)	LDH (U/L)	SD (U/L)	MD (U/L)
Normal	-	8.07±1.25	9.07±2.52	92.74±2.13	4.84±125	5.85±1.42
Diabetic control	-	5.55±1.36	4.12±2.31	60.84±1.54	3.14±1.45	1.87±1.32
Diabetic + Tolbutamide (100 mg/kg bw/day)	100	6.17±1.58*	8.65±1.95*	89.40±1.45*	6.74±1.28*	4.32±1.62*
Diabetic + HMBA (500 µg/kg bw/day)	500	8.5±2.42*	9.0±2.34*	92.85±1.5*	4.8±1.5*	5.0±2.1*

Each value is mean ± SD for six rats in each group..

GS - Glycogen synthase; GK – Glucokinase; LDH - Lactate dehydrogenase; SD - Succinate dehydrogenase; MD - Malate dehydrogenase.

*Values are statistically significant when compared to diabetic control at $F>0.05$ (ANOVA) and $P<0.05$ (DMRT).

Table 4. Effect of HMBA on liver and kidney gluconeogenic enzymes on streptozotocin-induced diabetic rats

Groups	Liver		Kidney	
	Glucose-6-phosphatase (nmoles of Pi liberated/min/mg protein)	Fructose-1 ,6 diphosphatase (nmoles of Pi liberated/min/mg protein)	Glucose-6-phosphatase (nmoles of Pi liberated/min/mg protein)	Fructose-1 ,6 diphosphatase (nmoles of Pi liberated/min/mg protein)
Normal	39±0.12	25±0.8	52±1.12	25±0.8
Diabetic control	169.9±1.05	385.8±1.7	189.9±1.05	218±0.7
Diabetic + Tolbutamide (100 mg/kg bw/day)	43±2.0*	35±0.27*	53±1.0*	35±1.27*
Diabetic + HMBA (500 µg/kg bw/day)	39±0.02*	25±90.5*	53±0.02*	29±0.5*

Each value is mean ± SD for six rats in each group.
*Values are statistically significant when compared to diabetic control at $F>0.05$ (ANOVA) and $P< 0.05$ (DMRT).

Discussion

Our observations are in well agreement with the reports by several workers that STZ-induced diabetes mellitus and insulin deficiency leads to increased blood glucose (20). Administration of HMBA (500 µg/kg bw/day) decreased the elevated blood glucose level within 5h and prolonged administration might have stimulated the β-cells of islets of Langerhans to produce insulin. From the results it is assumed that HMBA could be responsible for stimulation of insulin release and the observed reduction in blood glucose. Further the observed blood glucose-lowering effect of HMBA in STZ-induced diabetic rats could also possibly be due to the increased peripheral glucose utilization. A number of phytochemicals isolated from plants have also been shown to exert hypoglycemic activity through stimulation of insulin release (2). The antihyperglycemic activity of HMBA was comparable with tolbutamide, a standard hypoglycemic drug. Tolbutamide has long been used to treat diabetes and is known to act by stimulating insulin secretion through the action on the pancreatic β-cells. Alterations in glucose metabolism in diabetes are frequently accompanied by change in the activities of the enzymes that control glycolysis and gluconeogenesis in liver and kidney (21). Persistent hyperglycemia is a major contributor to such metabolic alterations that lead to the pathogenesis of diabetic complications, especially microvascular diseases (22).

One of the key enzymes in the catabolism of glucose is hexokinase, which phosphorylates glucose to glucose-6-phosphate (23). The activity of hexokinase, the rate limiting enzyme of glycolysis was found to be increased significantly (F>0.05; P< 0.001) on HMBA treatment. Insulin has been shown to be a potentiator of hexokinase/ glucokinase (24). The restoration of hexokinase activity in HMBA administered diabetic rats indicate the role of HMBA on insulin release followed by insulin mediated restoration of hexokinase activity. In our study, the hexokinase activity was decreased in the liver and kidney of diabetic rats, which may be due to the diabetes mediated deficiency of insulin. Partial or total deficiency of insulin in diabetes also results in derangement of carbohydrate metabolism causing a decrease in the activity of regulatory enzymes of glycolysis and glycogen synthesis (25, 26). The key enzymes regulating the glycolytic metabolite pools and glycolytic pools are very important to maintain the normal blood glucose levels by keeping a balance between glucose production and its utilization in the body. The reduction in blood glucose in HMBA treated diabetic rats may be due to the insulinomimetic action islet cells of pancreas and restoration of hexokinase activity.

Insulin decreases gluconeogenesis by decreasing the activities of key enzymes, such as glucose-6-phosphatase, fructose-1, 6-bisphosphatase, phosphoenolpyruvate carboxykinase, and pyruvate carboxykinase (27). Glucose-6- phosphatase is an important enzyme in homeostasis of blood glucose as it catalyzes the terminal step both in gluconeogenesis and glycogenolysis (28) Fructose-1, 6-bisphosphase is one of the key enzymes of gluconeogenic pathway. It is present in liver and kidney but absent from heart, muscle, and smooth muscle. In our study, the increased activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase in liver and kidney of diabetic rats may be due to insulin deficiency. In HMBA administered diabetic rats, the activities of these two enzymes were significantly reduced, which is responsible for the improved glycemic control.

The decreased levels of enzymes such as glycogen synthase, glucokinase, lactate dehydrogenase, succinate dehydrogenase and malate dehydrogenase may be due to decreased insulin levels in diabetic rats. Restoration of the levels of glycolytic enzymes after oral administration of HMBA might be due to insulinomimetic action of HMBA. Hence hyperglycemia could be repressed by administering the HMBA to diabetic rats. Moreover HMBA can be considered for supplementation for the control and management of diabetes mellitus.

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