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Antidiabetic activity of 2-hydroxy 4-methoxy benzoic acid isolated from the roots of *Hemidesmus indicus* on streptozotocin-induced diabetic rats

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Abstract
2-Hydroxy 4-methoxy benzoic acid (HMBA) isolated from the roots of *Hemidemus indicus* (*H. indicus*) was evaluated for its anti-diabetic activity on streptozoticin (STZ)-induced diabetic rats. HMBA, the active principle of *H. indicus*, was administered (500µg/kg body weight) orally to STZ-induced diabetic and non diabetic rats. The effect of HMBA on plasma glucose, insulin, glycosylated hemoglobin, liver glycogen and serum total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyl transferase (γ-GT) and creatine kinase (CK). After administration of HMBA, the levels of glycosylated hemoglobin, total cholesterol, triglycerides, LDL-cholesterol were normalized in diabetic rats. It also reversed the elevated levels of AST, ALT, ALP, γ- GT and CK to near normal level. The levels of plasma insulin, glycosylated hemoglobin and liver glycogen were also restored after HMBA treatment. Thus the results of our study suggested that HMBA could help in controlling DM owing to their hypoglycemic and hypocholesterolemic effects.

Key words: *Hemidesmus indicus, 2-Hydroxy 4-methoxy benzoic acid hemoglobin sugar, insulin, lipid profile, serum marker enzymes*

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease with increasing prevalence and mortality worldwide. It has been suggested that a total of 300 million people around the world will have diabetes by the year 2025 and the global cost of treating diabetes and its complications could reach US$ 1 trillion annually.[1] The high prevalence and severity of the disease is quite alarming in most of the developing countries including India. India alone has more than 40 million diabetic individuals which represent nearly 20% of the total diabetes population of the world and it is estimated to raise to almost 70 million by 2025.[2] Even now the underlying mechanism of diabetic complications remains unclear. The absolute or relative deficiency of insulin secretion/lack of insulin action results in alterations in carbohydrate, fat and protein metabolism.[3] Although insulin and numerous other oral hypoglycemic agents are available, but still there is no promising therapy available to cure diabetes. Recently much attention has been focused on herbal remedies due to its therapeutic efficacy and safety.[4] The biologically active compounds of most of the medicinal plants having anti-diabetic potential are yet to be identified. Moreover, many of the currently available anti-diabetic agents are reported to produce a number of adverse effects on the body.[5] Therefore, managing diabetes without any side effects is still a challenging task for health care providers.[6] Hence, the search for a more effective and safer herbal remedies has continued to be an important area of study.

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.[7] *H. indicus* is also employed in traditional medicine for gastric ailments.[8] It mainly consists of essential oils and phytosterols such as hemidesmol, hemidesterol and saponins. The pure compound, 2-hydroxy-4-methoxy benzoic acid (HMBA), $C_8H_8O_4$ was isolated and purified from the methanolic root extract of *H. indicus* root bark as described by Alam et al.[9]

HMBA is a white, needle-shaped crystal which is soluble in water, methanol and chloroform and has a melting point of 155-158°C and lambda max at 260 nm.[10] By spectral analysis, the presence of a benzene ring, methoxy group and hydroxyl group was confirmed. The molecular weight of the compound is 168. [9] The concentration of HMBA is in the range of 0.03-0.54% in the root bark of *H. indicus.*[11] HMBA possess potent anti-inflammatory, antipyretic and antioxidant properties.[12] 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.[12] The

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compound also has an adjuvant effect and antiserum potentiation activity against viper venom.[13] American Conference of Governmental Industrial Hygienists (ACGIH), International Agency for Research on cancer (IARC), National Institute for Occupational Safety and Health (NIOSH), National Toxicological Program (NTP) and Occupational safety and Health Administration (OSHA) do not list it for carcinogenicity. The protective effect of *H.indicus* against rifampicin- and isoniazid-induced hepatotoxicity in rats as well as chloroform- and paracetamol-induced hepatic damage is known.[14,15]

In the present investigation, 2-Hydroxy-4-methoxy benzoic acid (HMBA) was isolated from the roots of *H.indicus* and tested for their anti-diabetic activity in STZ-induced diabetic rats.

Materials and methods

Animals

Male albino rats (Wistar strain, weighing 150-200g) were purchased from Tamil Nadu Veterinary Animal Science University, Madhavaram, Chennai and housed under standard husbandry conditions (30°C ± 2°, 60–70% relative humidity and 12h:12h day-night cycle) and allowed standard pellet rat feed and water *ad libitum*. Animal experiments were designed and conducted in accordance with the guidelines of Institutional Animal Ethical Committee (IAEC – VIT University).

Plant material

The root bark of *H. indicus* was collected from the Morappur forest area, Dharmapuri District, Tamil Nadu, India. A voucher specimen was prepared and submitted to VIT University. *H. indicus* bark was washed with distilled water, shade dried, powdered and stored in an air-tight container until further use.

Preparation of methanolic extract

The bark of *H. indicus* (100g) was cut in to small slices, powdered and extracted with petroleum ether (50-60 °C for 72 h) in Soxhlet apparatus. Then it was extracted with methanol by refluxing (50- 60 °C for 72 h) and the extract was concentrated in rotary evaporator and dried in a desiccator at room temperature.

Isolation and purification of the active principle

The active principle (HMBA) of *H. indicus* was isolated and purified as described by Alam et al.[9] and compared with the standard purchased from the Sigma-Aldrich Co (St. Louis, USA). The spectroscopic data of the isolated HMBA matches with the standard HMBA of *H. indicus.*

Induction of diabetes mellitus in rats

Diabetes was developed by injecting streptozotocin (STZ) (Sigma, USA) at a dose of 35mg/kg bodyweight (bw) in 0.1M cold citrate buffer of pH 4.5, interaperitoneally. STZ-injected animals exhibited severe glycosuria and hyperglycemia and rats were stabilized over a period of 7 days.[16] On set of diabetes was confirmed in the experimental rats by measuring blood glucose concentration at 96 h after injection with STZ. The rats with blood glucose level above 250 mg/dl were considered to be diabetic and were used for the experiment. Control rats were given citrate buffer (pH 4.5).

Experimental design

Animals were divided in to six 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 tolbutamide (100 mg/kg). Group IV had the STZ-induced diabetic rats treated with HMBA (500µg/kg bw/day) for 7 weeks, by oral intubation. Rats were sacrificed at the end of 7 weeks and the blood samples were collected to analyze the effect of HMBA on biochemical parameters.

Collection and processing of blood for the estimation of glucose and other biochemical parameters

Plasma insulin concentration was determined by radioimmunoassay kit (Pharmacia, Uppsala, Sweden) with a betamatic counter (Cronex, Dupont, France). The kit included human insulin as standard and ^{125}I labeled human insulin antibody, which cross-reacts similarly with rat insulin. Total hemoglobin was estimated by the cyanomethaemoglobin method[17] and glycosylated hemoglobin (HbA_1c) was estimated by the modified method.[18,19] Serum total cholesterol, triglycerides and serum HDL-cholesterol were determined using commercial kits (Dialab, Austria). Plasma enzymes such as alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1), alkaline phosphatase (ALP; EC 3.1.3.1), γ-glutamyl transferase (γ-GT; 2.3.2.2) and creatine kinase (CK; 2.7.3.2) activity were measured by using Ecoline kits (E. Merck) in autoanalyser (Microlab-2000).

Statistical analysis

Statistical analysis was performed using the 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 of the results were expressed as mean ± SD for six rats in each group and $P<0.05$ was considered as statistically significant.

Results

The yield of HMBA was found to be 0.1 % (w/v). The effect of HMBA on plasma glucose, insulin, total hemoglobulin, glycosylated hemoglobin and liver glycogen is given in Table 1. Oral administration of aqueous solution of HMBA significantly ($F>0.05$; $P<0.001$) increased the levels of liver glycogen, plasma insulin and total hemoglobin and restored glycosylated hemoglobin to near normal level when compared to untreated control rats.

Table 2 presents the levels of serum lipids in normal and in diabetic rats. Total cholesterol, triglycerides and LDL cholesterol levels were significantly ($F>0.05$; $P< 0.001$) increased in diabetic rats with significant decrease in HDL cholesterol level when compared to untreated control rats. Serum lipid levels were restored with mild increase in HDL

Table 1: Effect of HMBA on plasma glucose, insulin, haemoglobin, glycosylated haemoglobin and hepatic glycogen levels in normal and streptozotocin-induced diabetic rats

Groups	Dose (mg/kg bw/day)	Plasma glucose (mg/dL)	Plasma insulin (µU/ml)	Total haemoglobin (g/dL)	Glycosylated hemoglobin (%)	Liver glycogen (mg/g of wet tissue)
Normal	-	69±3.19	15.62±1.3	15.50±0.5	5.5±0.4	10.75±0.74
Diabetic control	-	288±3.28	6.89±1.0	12.95±1.0	7.2±0.5	5.69±0.69
Diabetic + Tolbutamide	100	197±2.98*	13.90±1.4*	14.10±1.1*	4.5±0.3*	8.23±0.12*
Diabetic + HMBA	0.5	75±2.0*	16.0±1.3*	15.95±0.9*	5.4±0.1*	10.4±1.9*

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 $P < 0.05$ Duncan's multiple range test (DMRT).

Table 2: Effect of HMBA on serum total cholesterol, triglycerides, HDL and LDL levels in normal and streptozotocin-induced diabetic rats

Groups	Dose (mg/kg bw/day)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)
Normal	-	80±2.13	84±1.0	57.7±1.1	32.2±1.5
Diabetic control	-	126±2.9*	125±2.5*	36.0±1.5*	46.1±2.1*
Diabetic + Tolbutamide	100	80±1.5	87±1.9	39.5±1.8	33.2±2.5
Diabetic + HMBA	0.5	76±1.4	81±1.4	42.7±2.15	32.1±2.1

Each value is mean ± SD for six rats in each group. *Values 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 AST, ALT, ALP,ɣ- GT and CK in normal and streptozotocin-induced diabetic rats.

Groups	Dose (mg/kg bw/day)	AST (U/L)	ALT (U/L)	ALP (U/L)	ɣ GT (U/L)	CK (U/L)
Normal	-	210±8.0	181±1.5	233±1.7	12.54±1.0	352±1.2
Diabetic control	-	230±8.0	185±1.5	240±1.2	13.95±1.2	630±1.3
Diabetic + Tolbutamide	100	222±7.9*	182±1.2*	231±1.2*	12.65±1.6*	352±1.2*
Diabetic + HMBA	0.5	210±7.2*	181±0.7*	233±2.4*	12.21±1.8*	352±1.5*

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 $P < 0.05$ Duncan's multiple range test (DMRT).

cholesterol level. The effect of HMBA on the activities of serum enzymes is given in Table 3. The elevated levels of AST, ALT, ALP, -ɣGT and CK in diabetic rats were restored to near normal levels on treatment with HMBA.

Discussion

The present investigation was designed to evaluate the efficacy of the HMBA on STZ-induced diabetes and other metabolic changes in diabetic rats. The decreased level of Hb observed in diabetic rats might be due to increased formation of glycosylated Hb. It has been reported that in diabetic subjects the total haemoglobin level is much lower than the normal level[20] and increased levels of HbA_1c.[21] Earlier report states that during diabetes mellitus, excess of blood glucose reacts with hemoglobin leading to the formation of HbA_1c.[22] The level of HbA_1c is always monitored as a reliable index of glycemic control in diabetes.[23] Elevated levels of HbA_1c and reduced levels of Hb observed in our study reveal that diabetic animals had prior high blood glucose level. Administration of HMBA (500µg/kg bw/day) had lowered the elevated HbA_1c levels to near normal level. It has already been reported that decreased liver glycogen level is due to insulin deficiency and associated glycogenolysis.[24] The possibility of restoring liver glycogen level in STZ-induced diabetic rats by the administration of HMBA may be due to increased insulin secretion and reactivation of glycogen synthase enzyme system.

Hypertriglyceridemia and hypercholesterolemia are conditions frequently seen in diabetic patients. In STZ-induced diabetic rats, hypercholesterolemia and

hypertriglyceridemia are well documented.[25] Insulin deficiency leads to increased serum lipids because of increased lipolysis.[26] The elevated levels of serum total cholesterol, triglycerides and LDL cholesterol were significantly decreased after treatment with HMBA. The abnormally high concentration of serum lipids in diabetes mellitus is mainly due to an increase in the mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the hormone sensitive lipase.[27] It has been reported that significant reduction in total cholesterol and triglycerides is considered to be one of the desirable biochemical conditions for the prevention of diabetes and its complications.[27]

In STZ-induced diabetic animals, alterations in the levels of serum enzymes are directly related to changes in the metabolic functions of AST, ALT, and ALP, ɣ- GT and CK. It has been reported that the increased levels of transaminases under insulin deficiency [28] are responsible for increase in gluconeogenesis and ketogenesis in diabetes. The increased levels of serum AST, ALT and ALP have already been reported to be associated with liver dysfunction and leakage of these enzymes from liver cytosol into the blood stream in diabetes.[29] Reduction in the activity of AST, ALT, and ALP, ɣ- GT and CK in HMBA-treated diabetic rats indicates the alleviating role of the compound against STZ-induced hepato-cellular necrotic changes. Our observations are in agreement with the reports of several workers that STZ-induced diabetes mellitus and insulin deficiency leads to increased levels of cholesterol and triglycerides,[30] and increased levels of alkaline phosphatase and transaminases.[31]

From this study, it can be concluded that administration of HMBA is beneficial in normalizing the altered carbohydrate and lipid metabolism in diabetes, and also protects the liver by restoring the levels of liver specific enzymes.

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