Protective effect of *Morus rubra* L. leaf extract on diet-induced atherosclerosis in diabetic rats

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The antiatherosclerotic effect of aqueous leaves extract of *Morus rubra* was studied in streptozotocin-induced diabetic rats fed with atherosclerotic (Ath) diet [1.5 ml olive oil containing 8 mg (3, 20,000 IU) vitamin D2 and 40 mg cholesterol] for 5 consecutive days. A short-term toxicity assessment was also conducted in healthy rats to examine toxic effects of the extract. Oral administration of extract to diabetic rats (100, 200 and 400 mg/kg body weight per day for a period of 30 days) produced significant (*p*<0.001) fall in fasting blood glucose (FBG) in a dose-dependent manner. Treatment with the extract (400 mg/kg) showed significant (*p*<0.001) improvement in body weight and serum lipid profile i.e., total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol, when compared with diabetic control. Endothelial dysfunction parameters (sVCAM-1, Fibrinogen, total NO levels and oxidized LDL), apolipoprotein A and apolipoprotein B were significantly (*p*<0.001) reversed to near normal, following treatment with the extract. Thus, our study shows that aqueous leaf extract of *Morus rubra* (400 mg/kg) significantly improves the homeostasis of glucose and fat and possesses significant anti-atherosclerotic activity.

Keywords: *Morus rubra*, Streptozotocin, Atherosclerosis, Diabetes mellitus

Diabetes mellitus is going to become an epidemic disease all over the world. India, China and USA will be the countries with the largest number of diabetic patients in the year 2025. Diabetes is characterized by a loss of glucose homeostasis resulting in high blood glucose level, accompanied by an alteration of lipid parameters. The persistence of hyperglycemic state causes enhanced oxidative stress, leading to the development of atherosclerosis, coronary artery disease (CAD) and other diabetic complications.

In the recent years, there is growing interest in herbal medicine all over the world, as they have little or no side effects. Traditional medicinal plants having antidiabetic property can prove to be useful source for the development of new oral hypoglycemic agents as pharmaceutical entities or simple dietary adjuvant to existing therapies. Ethnopharmacological surveys indicate that more than 1200 plants are used in traditional medicine for the hypoglycemic activity. The hypoglycemic activity of a number of plants/plant products has been evaluated and confirmed in animal models, as well as in human beings. In India, several indigenous plants and their products have been used by the practitioners of the Ayurvedic system to treat diabetes.

*Morus rubra* L., or Red Mulberry, belonging to the family Moraceae is a deciduous, fast growing, small to medium-sized tree up to 15-20 m tall. The leaves are generally 8-15 cm long, cordate at the base and acuminate at the tip and serrated on the margin. The fruit is a compound cluster of several small drupes, red ripening dark purple, edible and very sweet with a good flavour. Several *Morus* spp. have been reported to possess medicinal properties, including antibacterial, astringent, hypoglycemic, anti-atherosclerotic, ophthalmic and diuretic. *Morus* spp. are known to contain a variety of antidiabetic phytochemical constituents, such as flavonoids (particularly rubraflavones A, B, C, D), tannins, triterpenes, saponins, anthocyanins, anthroquinones, sterols and phenolic compounds.

The leaves from several mulberry varieties are reported to contain a high concentration of sugar-mimic glycosidase inhibitors known to have
antidiabetic properties, such as 1,4-dideoxy-1, 4-imino-D-arabinitol, 1-deoxynojirimycin and 1,4-dideoxy-1,4-imino-dribitol. The presence of these compounds shows the potential of the Morus species in the treatment of diabetes. Earlier, the antidiabetic activity of leaves of M. alba and M. indica has been demonstrated. In our previous study, we have reported the antidiabetic activity of M. rubra leaves. In the present study, we have evaluated the antiatherosclerotic potential of aqueous extract of M. rubra leaves. In addition, extract has also been studied for toxicity assessment.

Materials and Methods

Plant material and preparation of extract

Leaves of Morus rubra were obtained from GTB Enclave, Dilshad Garden, Delhi. The identification was done by the Scientist-Incharge, Botanic Garden of Indian Republic (BGIR), Noida (India) and the specimen (no: MRL A/09) was kept there for further reference. The fresh leaves were dried in shade and machine ground to moderately coarse powder. The leaf powder (250 g) was then suspended in 500 ml of distilled water overnight and filtered to remove the residue. The dark brown colored filtrate was lyophilized.

Experimental animals

Male wistar albino rats (weighing 160-200 g) were procured from Central Animal House of University College of Medical Sciences (UCMS), Delhi, India. The animals were housed in standard conditions of temperature (22 ± 2°C) and at 12 h light-dark cycle and fed with commercial diet (Hindustan Lever Ltd., Mumbai) and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of UCMS, Delhi, India. All experimental procedures were conducted in accordance to the ethical guidelines of International Association for the Study of Pain.

Induction of diabetes

A freshly prepared solution of streptozotocin or STZ (45 mg/kg in 0.1 M citrate buffer, pH 4.5) was injected intraperitonially to overnight fasted rats. STZ injected animals exhibited hyperglycemia within 48 h. The rats having fasting blood glucose (FBG) values of 250 mg/dl or above were considered for the study.

Experimental procedure

The experiment was carried out on following groups of five rats in each group. Atherosclerotic (Ath) diet included 1.5 ml olive oil containing 8 mg (3, 20,000 IU) vitamin D and 40 mg cholesterol and was given for 5 consecutive days. Group 1 served as healthy control. Group 2 served as diabetic control (Diabetic + Ath diet). Group 3 was subdivided into three groups as follows: subgroups A, B and C. Diabetic rats fed with Ath diet and treated with aqueous extract of M. rubra leaves (100, 200 and 400 mg/kg body weight, respectively). Group 4: diabetic rats fed with Ath diet treated with glibenclamide (600 µg/kg) which is a standard antidiabetic drug. Control rats received vehicle i.e., distilled water and treated rats received M. rubra or glibenclamide in 1 ml of distilled water. The treatment was given daily for a period of 30 days using standard orogastric cannula. Blood was drawn from retro-orbital plexus of overnight fasted rats by using micro-capillary technique. FBG was measured at 0, 6, 10, 20 and 30 days while other parameters were determined at day 0, 6 and 30.

Biochemical assays

Blood glucose, serum triglycerides (TG), total serum cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) were assayed. Low-density lipoprotein-cholesterol (LDL-C) and very low-density lipoprotein-cholesterol (VLDL-C) were calculated as described previously. Apolipoprotein A (Apo-A1) and apolipoprotein B (Apo-B) were estimated by imunoturbidimetry method using commercially available kits from Giesse diagnostics. Nitric oxide (NO) end products and oxidized LDL (Ox-LDL) were also assayed. Plasma sVCAM-1 and fibrinogen levels were estimated by ELISA using commercially available kits from Diaclone France and Hyphen Biomed, France respectively.

Toxicity studies

To study the effect of high dose of the aqueous extract of M. rubra leaves, rats (five animals per group) were administered orally with the dose of 2, 4 and 6 g/kg b. wt. (5, 10 and 15-times of the effective dose) of the extract. The rats were observed during first 4 h and then after every 24 h up to 30 days for any gross behavioural changes or mortality. Liver function tests, such as serum glutamate phosphotransferase (SGPT) and alkaline phosphatase (ALP) as well as kidney function tests i.e., urea and creatinine were performed at the end of the study using standard kits. Body weight was also recorded prior to treatment and then post-treatment.
Statistical analysis

Values were expressed as the mean ± SEM for five animals in each group. The data were analyzed by using repeated measure analysis of variance (ANOVA), followed by Dunnett’s multiple comparison test and one-way ANOVA, followed by Tukey’s multiple comparison test. The results were considered significant at *P*<0.05.

Results

Antihyperglycemic activity

There was a significant decrease (*p*<0.001) in the levels of FBG in diabetic rats treated with aqueous extract of *M. rubra* leaves (100, 200 and 400 mg/kg) from day 6 to 30; diabetic control rats showed marked hyperglycemia throughout the experimental period (Table 2). As extract showed dose-dependent fall in blood glucose level up to 400 mg/kg b. wt. dose, this was considered as effective dose and further studies were carried out with this dose. The diabetic rats fed with the extract and glibenclamide exhibited remarkable glycemic control as evident by significant decrease (*p*<0.001) in FBG levels.

Body weight

Diabetic rats grew poorly and had significant (*p*<0.001) lower body weight as compared with healthy control (Table 1). The diabetic rats treated with the extract (400 mg/kg) and glibenclamide (600 µg/kg) had their body weight comparable to healthy control.

Lipidemic control

The serum lipid profile of normal, diabetic control, diabetic treated with *M. rubra* leaf extract and glibenclamide is summarized in Table 3. Diabetic control animals showed significant increase (*p*<0.001) in the levels of TG, TC and LDL-C and decrease (*p*<0.001) in HDL-C compared with normal group. These changes were reversed towards normal in extract-treated diabetic animals. The effect of extract was comparable to that of glibenclamide.

Endothelial dysfunction parameters

Tables 4 and 5 demonstrate the effect of aqueous extract of *M. rubra* leaves on the endothelial dysfunction parameters in diabetic rats. A significant elevation (*p*<0.001) in the levels of Ox-LDL, sVCAM-1 and serum fibrinogen was observed in diabetic control rats as compared with normal rats. The Ox-LDL was found to be significantly decreased (*p*<0.001) in extract-fed diabetic rats. The supplementation with the extract reverted back the sVCAM-1 and serum fibrinogen levels to near normal in diabetic rats (*p*<0.001). Glibenclamide-treated diabetic rats also produced significant reduction (*p*<0.001) in the levels of oxidized LDL, sVCAM-1 and fibrinogen.

NO levels in diabetic control animals were found to be significantly decreased (*p*<0.001) as compared to normal control group. The protective effect of extract was indicated by the significant increase (*p*<0.001) in levels of total NO levels of extract-fed diabetic rats. Moreover, there was significant improvement in the levels of Apo- A, Apo-B and Apo-B/Apo-A ratio. The effect was comparable to glibenclamide on endothelial dysfunction parameters.

Toxicity studies

Toxicity studies revealed that administration of graded doses of *M. rubra* aqueous leaf extract (up to a
dosage of 6 g/kg) produced no adverse effect on the general behaviour. All the rats survived during the experimental period. No significant change was observed in the levels of SGPT and ALP (data not shown) in the treated rats, when compared with control group. Blood urea and creatinine were also not significantly changed in treated animals compared to control.

**Discussion**

As prevalence of diabetes is globally increasing, its management and control is an urgent priority. The major classes of oral hypoglycemic agents currently available for the treatment of diabetes include sulphonylureas, biguanides, thiazolidinediones, α-glucosidase inhibitors etc. Glibenclamide used as the reference antidiabetic drug in this study is a...
member of sulphonylureas. Sulphonylureas have been proposed to produce their hypoglycemic effect through increased insulin release from pancreatic β-cells, and enhanced insulin’s action on target tissues. Thus, any plant secondary metabolite or chemical constituent that is capable of affecting the pancreatic β-cells and/or insulin action will be a good mimicker of sulphonylureas. Due to least or no side effects, healers still use plant drugs and herbal formulations for the treatment of several diseases, as the synthetic drugs are known to have side effects.

STZ, a β cytoxin is commonly used for induction of experimental diabetes. It acts through rapid depletion of pancreatic β-cells which leads to reduction in insulin secretion. STZ-induced diabetic animals represent a good experimental diabetic state with residual or remnant insulin production by the β-cells. The diabetic state in these animals is, therefore, not the same as that obtained by total pancreatectomy, as daily administration of insulin is not required for survival of STZ-induced diabetic rats. Further, the STZ-induced diabetic animals may exhibit most of the diabetic complications.

The aqueous extract of M. rubra leaves like glibenclamide produced significant reduction in blood glucose level in STZ-treated diabetic rats. The antihyperglycemic effect of the extract appears to be exerted via a mechanism similar to that of glibenclamide. A number of other plant extracts have been reported to have antihyperglycemic activity through a stimulatory effect on insulin secretion. The possibility also exists that plant extracts mimic or improve insulin action and/or may have extra pancreatic mechanism of action.

Since hyperlipidemia is one of the major risk factors for the development of atherosclerosis, it is associated with high rate of cardiovascular morbidity and mortality. Various agents, which affect hyperlipidemia are still not used for prevention or cure of atherosclerosis because of their potential toxicity and intolerance. In the present study, we investigated whether the M. rubra leaf extract has any effect on lipids as well as on endothelial dysfunction parameters. The altered lipid and lipoprotein profile i.e. increase in TG, TC and LDL-C with fall in HDL-C was reversed towards normal level after oral administration of the extract at the end of experimental period in STZ-induced diabetic rats. The increased TG and TC levels and decreased HDL-C are known factors associated with coronary heart disease (CHD). The atherogenic index (TG/HDL-C ratio) used to predict risk of CHD and marker of small, dense LDL-C (an atherogenic lipoprotein) were significantly reduced following treatment with the extract, suggesting the beneficial effect of extract in cardiovascular diseases.

Recent studies suggest that TG itself is an independent risk factor related to CHD. Most of the antihypercholesterolemic drugs do not decrease TG levels, but M. rubra leaf extract significantly lowered TG level to near normal after 30 days of treatment. Its strong effect on diabetic hypertriglyceridemia could be through its control on hyperglycemia. This was in agreement with the fact that (a) the level of glycemic control is the major determinant of TG and VLDL concentrations and (b) improved glycemic control following sulphonylurea therapy decreases levels of serum TG and VLDL. Although the antihyperglycemic and hypolipidemic effect aqueous extract of M. rubra leaves was comparable to that of glibenclamide, the effect of the extract on endothelial dysfunction parameters was found to be significant, as compared to glibenclamide.

The rats which were orally administered a single dose of up to 6 g/kg of the extract survived and appeared active and healthy. No signs of abnormal behavior, adverse toxicological effect on liver and kidney and/or mortality were observed during 30 day post-treatment. Therefore, the extract can be considered relatively safe at the dose of 6 g/kg body wt.

In conclusion, the aqueous extract of M. rubra leaves exhibited strong antihyperglycemic and antiatherosclerotic activity in diabetic animals. Hence, the data of present study provide impetus for further molecular and mechanistic studies on the therapeutic action of M. rubra leaves extract, before it can be considered as a possible insulin replacement or adjuvant in the management of diabetes mellitus.

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References