

Polyphenolic constituents in mulberry leaf extract (*M. latifolia* L. cv. BC259) and its antidiabetic effect in streptozotocin induced diabetic rats

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Abstract: Mulberry (*M. alba* L.) has prominent use in traditional Chinese medicine since ancient times but its therapeutic properties have not been sufficiently explored in India. Present study was designed to isolate and identify the polyphenolic constituents present in mulberry leaf (*M. latifolia*) using HPLC and to evaluate its antihyperglycemic and antihyperlipidemic properties in streptozotocin (STZ) induced diabetic wistar rat models. HPLC analysis identified chlorogenic acid (103mg/100gm), caffeic acid (4.3mg/100gm), coumaric acid (11.61mg/kg), rutin (53mg/100gm) and quercetin (46.19mg/100gm) as the major constituents of crude polyphenolic extracts in *M. latifolia*. STZ induced diabetic rats administered with mulberry leaf extract at doses 250 and 500mg/kg after 4 weeks of treatment significantly improved their glycemic control ($p < 0.001$). Body weight increased significantly ($p < 0.001$) after administration of BC259 extract at a dose of 500mg/kg. Results also showed that there was a significant decrease in serum urea ($p < 0.001$) and creatinine level ($p < 0.01$). Significant decline was observed in the levels of serum triglycerides ($p < 0.01$), total cholesterol ($p < 0.001$), LDL-cholesterol ($p < 0.01$) and VLDL-cholesterol ($p < 0.01$), while the serum HDL-cholesterol ($p < 0.01$) significantly increased. Results revealed that the leaf extract of *M. latifolia* (var.BC259) causes significant antidiabetic and antihypercholesterolemic activity. Evidence of identified bioactive polyphenolic compounds present in *M. latifolia* leaf extract strengthens its antidiabetic property.

Keywords: HPLC, blood glucose, polyphenols, diabetes, streptozotocin, cholesterol.

INTRODUCTION

Under-utilization of glucose leads to Diabetes mellitus (DM), a typical metabolic disorder characterized by hyperglycemia (Zhang *et al.*, 2003). It is reported to be the third most life-threatening disease whose mortality is next to cancer and cardiovascular disease (Gong *et al.*, 2012). It is estimated that approximately 438 million people of the adult population will suffer from diabetes by 2030 (IDF Diabetes Atlas, 2009). India leads this race with the largest number of diabetic subjects when compared with any given country. Predictions are that the total number of people with diabetes will rise to 87.0 million by 2030 (Ramachandran *et al.*, 2010).

Treatment of diabetes mellitus in clinical practice has normally been confined to the use of oral hypoglycemic agents and insulin. However, insulin cannot be used orally and use of synthetic drugs for long periods cause serious side effects and can be toxic. It has also been seen that none of the existing practices offer complete glycemic control. As a result, investigations with antihyperglycemic agents derived from medicinal plants have become more attractive. Furthermore, due to their effectiveness, negligible side effects in clinical experience and relatively low cost, they are prescribed widely when compared to other synthetic hypoglycemic agents (Rajesh *et al.*, 2005).

Mulberry, which belongs to the family *Moraceae* and genus *Morus*, is one of the conventional herbs widely used in medicine from centuries ago due its chemical composition and pharmacological function (Ramesh *et al.*, 2014). India has planted mulberry for sericulture and mulberries have been a valuable resource. The herb is known for its antiphlogistic, diuretic, expectorant and antidiabetic properties in traditional Chinese medicine (Nomura, 1998). Various parts of the mulberry plant such as fruit, bark, leaf and root have attracted interest owing to their potential in treating diabetes, with the bark and root especially used to reduce hyperglycemia (Memon *et al.*, 2010). By means of several experimental models, many constituents of mulberry leaf including iminosugars, polysaccharides, alkaloids, flavonoids and related compounds were found to contribute to antidiabetic activity (Singab *et al.*, 2005).

India remains home for many species or natural variants of mulberry plants and hence there is an urgent need to explore the therapeutic potential of these plants. There are several varieties of mulberry being cultivated in different states of India. Most of the cultivated varieties belong to either *M.alba* or *M.indica* sps. There have been a few reports showing antidiabetic effects of *Morus alba* and *Morus indica* species of mulberry.

M.latifolia (BC259) being a high yielding variety of mulberry with wider adaptability is cultivated in the hills

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of Eastern India (Vijayan *et al.*, 2004) for commercial silkworm rearing with better cocoon yield as well as for sustainable growth and development in sericulture industry with nutritional superiority (Sharma *et al.*, 2015). No findings have been obtained yet in *M. latifolia* (BC259) variety of mulberry with respect to their therapeutic effects. Major interventions have focused on identification and extraction of functional components of plants that hold therapeutic potential. In light of plant based medicine, an attempt has been made to isolate and identify the polyphenolic constituents present in the leaves of *M. latifolia* with an aim to evaluate the antihyperglycemic and antihypercholesterolemic activity of *M. latifolia* (BC259) leaf extract and identify the bioactive compounds responsible for these effects in STZ induced diabetic rat models.

MATERIALS AND METHODS

Chemicals and assays

Reference standards of chlorogenic acid, caffeic acid, coumaric acid, rutin and quercetin were purchased from Sigma-Aldrich Co., USA. Methanol and acetic acid (HPLC grade) were obtained from Merck, Germany. Streptozotocin (STZ) was procured from Sigma-Aldrich Co., USA. Glucose, total cholesterol (TC), high-density lipoprotein (HDL) and triglyceride (TG) were assayed using standard kits obtained from ERBA Diagnostics, Mannheim, Germany.

Instruments and equipment

The extracts were concentrated with Superfit-ROTAVAP (PBV-7D). All UV-Vis analyses were carried out with UV-Vis double beam spectrophotometer (Hitachi, U2910). Blood glucose was analyzed with One-touch gluco-meter (Accu-Chek) of Roche Diagnostics, Mannheim, Germany.

Collection of plant material

The leaves of Mulberry (*Morus latifolia* L.cv.BC259) were collected from the germplasm maintained by the Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore, India.

Preparation of polyphenols from mulberry leaf

Mulberry leaves of BC259 were dried overnight at 90°C and 20gm was powdered and extracted with 70% ethanol using soxhlet apparatus. Extract obtained was further separated with chloroform and ethyl acetate using separating funnel. Polyphenolic ethyl acetate layer finally acquired was evaporated using rotary evaporator and was taken for its quantification of polyphenolic constituents profile using HPLC (Jibu Thomas *et al.*, 2006).

Identification of polyphenolic compounds by HPLC and its quantification

Phenolic constituents were determined using the HPLC method described by Rodriguez *et al.*, 2001.

Chromatographic conditions include separation in HPLC (Waters, Model: 515) fitted with Photodiode Array detector (Model: 2998) and ODS column (250mm × 4.6mm, 4µm) (Hi Chrom, USA). Binary elution was performed with solvents (methanol- acetic acid- water) in the proportion of 10:2:88 and 90:2:8 as mobile phase A and B respectively. Measurements were made at 254 and 280 nm. Phenolic acids were identified based on the retention time and UV spectrum of reference standards. Reference standards at concentrations (1-40µg/mL) were injected into the HPLC-DAD system and the calibration curves were generated. Concentrations of the compounds were calculated from peak area according to the generated calibration curves.

In vivo animal studies

Animal studies were performed with 30 male wistar rats weighing (150-200gm) procured from Veterinary College, Thrissur, Kerala, India. Animals were acclimatized at a temperature of 25±2°C with a relative humidity of 45-55% in a 12 hour light dark cycle and were fed with standard laboratory diet where water was given *ad libitum*. The rats used in the study were maintained in accordance with guidelines of the Institutional Animal Ethical Committee of the University. Institutional Animal Ethical Committee (IAEC) approval was obtained under experiment No.IAEC/KU/BT/13/02 for the present study.

Experimental design

Rats were divided into five groups with six rats in each and treated as- Group I: Normal control rats; Group II: Streptozotocin (45mg/kg body weight) induced diabetic rats; Group III: Diabetic rats with standard drug glibenclamide (0.6mg/kg/day) and Group IV: Diabetic rats with *Morus latifolia* leaf extract at doses of 250mg/kg/day; Group V: Diabetic rats with *Morus latifolia* leaf extract at doses of 500mg/kg/day respectively. The treatment was continued orally for 28 days.

Induction of diabetes mellitus using streptozotocin

Type 2 diabetes mellitus was induced in rats that had previously been kept on a fast overnight through a single intra peritoneal injection of streptozotocin (45mg/kg body weight). After 3 days, fasting glucose levels were monitored and the animals showing blood glucose level of 225 mg/dl and above were used in the treatment groups accordingly (Pushparaj *et al.*, 2000).

Collection of blood sample for biochemical analysis

At the end of the experimental treatment (28 days), body weight of the rats in each group was noted. Blood was withdrawn by cardiac puncture under mild ether anesthesia from each treatment group and the serum glucose (Trinder, 1969), serum creatinine (Slot, 1965), serum urea (Wybenga *et al.*, 1971) were estimated.

Based on the report that uncontrolled type 2 diabetes mellitus leads to an increase in TC, LDL VLDL cholesterol and triglycerides with decrease in HDL cholesterol that contributes to coronary artery disease (Arvind *et al.*, 2002), the serum triglyceride (Fossati and Prencipe, 1982), Cholesterol, HDL-Cholesterol (Castelli, 1977), LDL and VLDL were also estimated in the study period.

STATISTICAL ANALYSIS

Data generated was expressed as mean \pm S.E.M. for all experimental groups. Statistical analysis was performed using SPSS version 16.0 to conduct one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test to find out the significant differences among the various treatment groups. Values corresponding to $p < 0.05$ were considered statistically significant.

RESULTS

Identification of polyphenolic compounds by HPLC

HPLC analysis showed that the main polyphenolic constituents in *Morus latifolia* (BC259) leaf extract were chlorogenic acid, caffeic acid, coumaric acid, rutin and quercetin (fig.1). Among the identified polyphenolic compounds, chlorogenic acid (103mg/gm dry.wt) is found prominent in relative distribution followed by rutin (53mg/gm dry.wt), quercetin (46.19mg/gm dry.wt), coumaric acid (11.61mg/gm dry.wt) and caffeic acid (4.3mg/gm dry.wt) (table 1).

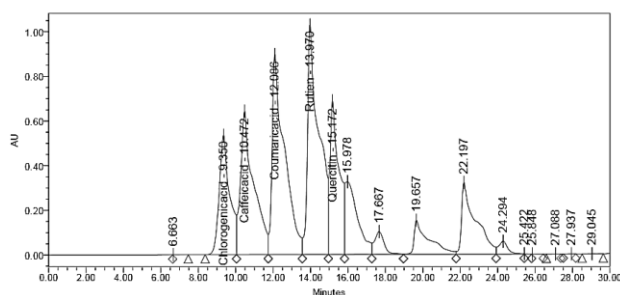


Fig 1: HPLC profile of phenolic constituents in *M. latifolia* (BC259) extract.

Influence of mulberry leaf extract on blood glycemc parameters in streptozotocin induced diabetic rats

STZ induced diabetic rats administered with standard drug glibenclamide revealed a significant decline in serum glucose level to a value of 105 mg/dl ($P < 0.001$) when compared with diabetic control rats. Similarly, the treatment with mulberry leaf extract 250mg/kg or 500mg/kg body weight showed a significant decline in serum glucose level to a value of 143mg/dl and 132mg/dl respectively ($P < 0.001$) as compared with diabetic control rats (table 2).

Effect of mulberry leaf extract on body weight of treated rats

The intra peritoneal injection with streptozotocin (45mg/kg b.wt) in rats resulted in the decrease of body weight from 193 to 158 gm as compared with the normal control rats. The administration of glibenclamide or mulberry leaf extract (500mg/kg) for 28 days to STZ induced diabetic rats significantly restored this loss in body weight to reach a value of 177 ($P < 0.05$) and 172gm ($P < 0.001$) respectively as compared with diabetic control rats (table 2).

Table 1: Identification and quantification of phenolic constituents

Identified Compound	Retention time (min)	Content (mg/g)
Chlorogenic acid	9.350	103.00
Caffeic acid	10.472	4.30
Coumaric acid	12.086	11.61
Rutin	13.970	53.00
Quercetin	15.172	46.19

Effect of mulberry leaf extract on urea and creatinine levels

Diabetic control group registered an increased level of urea and creatinine as compared to normal control rats. Administration of standard drug glibenclamide (0.6mg/kg) had a significant reduction in both urea and creatinine levels ($P < 0.01$) as compared to diabetic control rats (table 2). A significant reduction in the levels of urea ($P < 0.001$) and creatinine ($P < 0.01$) were also observed in diabetic rats treated with 250mg/kg *M. latifolia* (BC259) leaf extract. Similar effect in the levels of urea and creatinine ($P < 0.001$) was noticed in diabetic rats treated with extract at 500mg/kg.

Effect of mulberry leaf extract on lipid profile

The serum lipid levels of rats at the end of the experiments are shown in table 3. Serum cholesterol, triglyceride, LDL and VLDL except HDL have shown an increase in diabetic control rats when compared to normal control. A significant reduction in the levels of serum cholesterol, triglyceride, LDL and VLDL was observed in diabetes induced rats on administration with glibenclamide or mulberry leaf extract at 250mg/kg or 500mg/kg when compared to the diabetic control rats. A significant improvement in HDL was noticed in rats fed with standard drug or rats fed with mulberry leaf extract at 250mg/kg or 500mg/kg.

DISCUSSION

The mulberry tree has been an integral part of traditional medicine since ancient times. The presence of several active ingredients with tremendous therapeutic potential has rendered it a plant of great medicinal worth. *M.*

Table 2: Effect of the *M. latifolia* leaf extract on the Serum glucose, body weight, Urea and Creatinine level in treated rats

Treatment	Serum glucose (mg/dl)	Body weight (gm)	Urea (mg/dl)	Creatinine (mg/dl)
Normal Control	89.837±4.241	193.41±12.18	23.658±2.96	0.808±0.082
Diabetic Control	274.678±0.12	158.46±8.40	46.87±1.795	3.397±0.34
Diabetic + Glibenclamide Control (0.6mg/kg)	105.098±8.453 ^{***}	177.90±8.82 ^{**}	37.062±1.675 ^{**}	2.39±0.27 ^{**}
Diabetic + Mulberry leaf extract (250mg/kg)	143.098±6.885 ^{***}	166.2±6.97 ^{NS}	29.173±2.2 ^{***}	2.17±0.02 ^{**}
Diabetic + Mulberry leaf extract (500mg/kg)	132.912±3.63 ^{***}	172±8.36 ^{***}	23.60±2.78 ^{***}	1.69±0.18 ^{***}

Table 3: Effect of the *M. latifolia* leaf extract on the lipid profile in streptozotocin induced diabetic rats

Groups	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal Control	148±3.09	65.59±5.46	47.70±4.06	125.79±3.01	13.11±1.09
Diabetic Control	235±5.56	177.95±11.39	17.04±2.35	196.13±7.03	35.59±2.27
Diabetic + Glibenclamide control (0.6mg/kg)	159.72±4.33 ^{***}	72.24±6.16 ^{**}	44.74±2.28 ^{***}	136.32±5.37 ^{**}	14.44±1.23 ^{***}
Diabetic + Mulberry leaf Extract (250mg/kg)	182.94±5.25 ^{***}	93.26±4.98 ^{***}	37.68±2.80 ^{**}	146.21±4.72 ^{**}	18.65±0.99 ^{**}
Diabetic + Mulberry leaf Extract (500mg/kg)	172.48±5.10 ^{***}	80.25±5.71 ^{***}	44.97±2.51 ^{**}	127.91±5.36 ^{**}	16.05±1.14 ^{**}

n=6 in each group; values are presented as mean± S.E.M.

NS- Not significant, **p<0.01, ***p<0.001 compared with the diabetic control

latifolia (BC259) is one such species which holds promise as a prospective drug.

Presence of polyphenolic compounds such as chlorogenic acid, caffeic acid, coumaric acid, rutin and quercetin in *M. latifolia* (BC259) leaf extract strengthens its antidiabetic property. The antidiabetogenic action of some of these compounds in other plants have already been reported. Chlorogenic acid (5mg/kg) exerts antidiabetic potential in nicotinamide induced diabetic rats (Karthikesan *et al.*, 2010). Caffeic acid is known to have antidiabetic effect in STZ induced diabetic rats (Park and Min, 2006). Rutin has antihyperglycaemic and antioxidant effects (Kamalakkannan and Prince, 2006). Quercetin is reported to have antidiabetic effect in STZ induced diabetic wistar rats (Vessal *et al.*, 2003).

STZ treated diabetic rats stimulate a drastic rise in serum glucose thereby indicating the damage of their pancreatic β -cells. It was observed that diabetic rats treated with *M. latifolia* leaf extract experienced a substantial decrease in fasting blood glucose concentrations thus demonstrating a good glycemic control. These results are in accordance with other findings of Andallu and Varadacharyulu (2003).

Diabetic animals exhibited high levels of serum creatinine and urea indicating that they suffer from an impaired renal

function. A high level of urea is generally witnessed during increased protein breakdown and also in renal disorders. Group treated with BC259 leaf extract showed a major reduction in serum urea and serum creatinine levels indicating a healthy recovery of renal function as well as an inhibition of oxidative stress caused by diabetes. In the present investigation, elevated levels of blood urea in the diabetic group were restored to the control group level after treatment with mulberry leaf extract. These results are in accordance with the reports of Mohammadi and Naik (2008).

Tan *et al.* (2005) have observed earlier that hypercholesterolemia and hypertriglyceridemia occurs in STZ induced diabetic rats. Under normal circumstances, insulin activates the enzyme lipoprotein lipase which hydrolyses triglycerides. However, in diabetic state, lipoprotein lipase is not activated due to deficiency of insulin secretion resulting in hypertriglyceridemia (Taskinen, 1987). The present study showed the improvement in glycemic parameters by treatment with BC259 leaf extract in STZ induced diabetic rats followed by a fall in serum cholesterol (P<0.001), triglycerides (P<0.001), LDL (P<0.01) and VLDL (P<0.01) after 28 days of treatment. The study also showed a significant rise in HDL (P<0.01) which was a desirable feature. These results are in par with the findings of Andallu *et al.*, 2001.

CONCLUSION

From the study we conclude that leaves of *M. latifolia* (BC259), apart from being used as a feed for silkworm, can serve as a promising source of dietary antioxidants such as polyphenols which can be further used in nutraceutical industry. The present study has also concluded that the polyphenolic extract of *M. latifolia* causes significant antidiabetic and antihypercholesterimic activity. The evidence of identified bioactive polyphenolic compounds present in *M. latifolia* leaf extract strengthens these properties. Further purification of these identified polyphenolic compounds from mulberry germplasm will be a major breakthrough towards developing new natural drugs for human ailments.

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