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MORUS LATIFOLIA (BC2-59) LEAF EXTRACT AGAINST OXIDATIVE STRESS IN STREPTOZOTOCIN INDUCED DIABETES RAT MODELS

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ABSTRACT

Natural bioactive compounds play a significant role in curing various diseases related to oxidative stress. Mulberry leaves (*Morus alba*) are abundant in several bioactive constituents such as polyphenolic compounds that can combat oxidative stress in diabetes. The present study investigated the antioxidant capacity of *Morus latifolia* leaf extract against oxidative damage caused by diabetes. Streptozotocin induced diabetic rats were subjected to a treatment with *M.latifolia* leaf extract (250 and 500mg/kg) over a course of 21 days. Treatment with *M. latifolia* leaf extract brought about a significant reduction in the levels of lipid peroxides (LPO) and catalase (CAT) activity in serum and liver tissue in streptozotocin treated diabetic rats. A considerable increase in superoxide dismutase (SOD) activity and improvement of reduced glutathione (GSH) in serum and liver tissue of *M. latifolia* treated diabetic rats was also observed. Total polyphenols and flavonoids are found to be 46.06% and 12.75% respectively. HPLC analysis of the extract identified chlorogenic acid, caffeic acid, coumaric acid, rutin and quercetin as active principles. This study establishes that *M.latifolia* leaf extract produces an ameliorating effect on oxidative stress in diabetic rats through the activity of polyphenolic compounds inherent in *M.latifolia* leaf extract.

Keywords: ***Diabetes, polyphenols, HPLC, antioxidant enzymes, oxidative stress, lipid peroxidation***

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INTRODUCTION

Diabetes Mellitus is a malady stemming from a number of different causes or influences wherein intensified oxidative stress performs a critical pathogenic function. There is an unequal formation of free radicals caused by glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins. This large-scale rise in the presence of free radicals specifically reactive oxygen species (ROS) in diabetes may be resultant of their enhanced production and/or diminished decimation by non-enzymatic and enzymic catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) antioxidants. Such imbalance between free radical formation and cellular defense mechanism can lead to serious abnormalities in the functioning of the cell as well as its complete obliteration bringing about tissue injury.[1] It is observed that a reduction in oxidative stress and rate of complications arising in diabetes occurs through augmented presence of antioxidants. The ingestion of natural antioxidants is a hopeful nutritional approach towards constraining oxidative stress brought about by hyperglycemia in diabetes. There has been a great deal of focus and interest in natural plants as suppliers of biologically active substances including antioxidants, antimutagens and anticarcinogens.[2] The total antioxidant activity of plant foods is attributed to the distinct activities of every antioxidant compound that are inherent- namely, vitamin C, tocopherols, carotenoids, and phenolic compounds, with the last being the foremost phytochemicals causative for antioxidant activity in plants. Furthermore, these compounds execute their influence through various means like radical scavenging, metal chelation, inhibition of lipid peroxidation, quenching of singlet oxygen and the like to function as antioxidants. The mulberry plant is a deciduous tree which quickly sprouts in various climatic environments.[3] Greatly valued for its foliage, which forms the principal feed for silkworms, the mulberry is a part of the family *Moraceae* and genus *Morus.* In addition to its function as feed for animals and insects, the mulberry has been proven to possess numerous biological and physiological effects along with hypoglycemic, hypotensive and diuretic effects due to the plenteous presence of anthocyanins and phenolics. It has been proposed that a mulberry leaf extract diet can act as a deterrent against an STZ-induced diabetic occurrence in rats.[4] However, the correlation of the mechanism of their action to their antioxidant properties has been made of late. The phytochemical study shows mulberry leaves as comprising polyphenolic compounds like rutin, quercetin, isoquercitrin, polyhydroxylated alkaloids and other flavonoids.[5] While there are a few reports revealing the antioxidant effects of the leaves of *Morus indica* species of mulberry[6], no studies have been obtained yet in *M.latifolia* (BC2-59) variety of mulberry with respect to its *in vivo* antioxidant activity. In light of this, the objective of the current study is to validate the antioxidant effect of *M.latifolia* leaf extract in STZ induced diabetic rat models.

MATERIALS AND METHODS

Chemicals and assays

Reference standards of chlorogenic acid, caffeic acid, coumaric acid, rutin, quercetin and Streptozotocin (STZ) was procured from Sigma-Aldrich Co., USA. All other chemicals were purchased from HIMEDIA, Mumbai, India.

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).

Collection of Plant Material

The leaves of Mulberry (*M.latifolia* L.cv.BC2-59) were collected from Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore.

Preparation of crude extract from Mulberry leaf

Mulberry leaves of BC259 were dried overnight at 70°C and 20gm was powdered and extracted with 70% ethanol using soxhlet apparatus. The extract obtained was further separated with chloroform and ethyl acetate using separating funnel. Polyphenolic ethyl acetate layer finally acquired was evaporated using rotary evaporator[7], diluted and was taken for its quantification of polyphenolic constituents using HPLC.[8]

Determination of polyphenol content in M. latifolia leaf extract

One ml of the diluted sample was added with 0.5ml of Folin-Ciocalteu's reagent and 2ml of 20% sodium carbonate. Absorbance was read for the blue colour developed at 650nm against the reagent blank in a UV-Vis spectrophotometer. Quantum of polyphenols present in extract was computed using the standard calibration curve derived from known concentrations (10 to 50ppm) of gallic acid and the results were expressed as gallic acid equivalents according to Malick and Singh.[9]

Determination of flavonoid content in M.latifolia leaf extract

One ml of the diluted sample was added with 0.3ml of sodium nitrite (1:20). 0.3ml of Aluminium chloride (1:10) was added 5 minutes later. After 6 minutes, 2ml 1M sodium hydroxide was added. The solution was mixed well again and the absorbance was measured against a blank at 510nm with a UV-Vis spectrophotometer. Catechin was used as the standard for a calibration curve. The flavonoid content was calculated using the standard calibration curve derived from known concentrations (10 to 50ppm) of catechin and the results were expressed as catechin equivalents according to Zhuang *et al.*[10]

HPLC analysis of polyphenolic compounds in M.latifolia leaf extract

The phenolic constituents were determined using the HPLC method described by Rodriguez-Delgado *et al.*[11] The *M.latifoilia* leaf extract obtained after extraction with 70% ethanol was further analysed for the identification and quantification of polyphenolic constituents. The chromatographic conditions include separation in Waters

HPLC (Model: 515) fitted with Photodiode Array detector (Model: 2998) and ODS column of size 250mm × 4.6mm, 4µm (Hi Chrom, USA), solvents methanol- acetic acid-water in the proportion of 10:2:88 and 90:2:8 were used as mobile phase A and B respectively for binary gradient elution. Measurements were made at 280nm. Identification of phenolic acids was based on retention time and UV spectrum with those of standards. The solutions of standards at various concentrations from 1 to 40µg/mL were injected into the HPLC-DAD system and the calibration curves were established for each standard compound. The concentrations of the compounds were calculated from peak area according to the respective calibration curves.

Animal study

Thirty male wistar rats, with body weight (150-200gm) procured from Thrissur Veterinary College, Kerala were housed in individual cages at a temperature of 25±2°C with a relative humidity of 45-55% in a 12 h light: 12 h dark cycle. Animals were fed with standard laboratory diet and water *ad libitum*. The rats used in the study were kept in accordance with guidelines of the Institutional Animal Ethical Committee of the University. The Institutional Animal Ethical Committee (IAEC) approval was obtained under experiment No. IAEC/KU/BT/13/02 for the present study.

Experimental design

The rats were separated into five groups with six rats in each group and treated as follows: Group I: control rats with distilled water; Group II: Diabetic rats with distilled water; Group III: Diabetic rats with standard drug Glibenclamide (120mg/kg of body weight): Group IV-V: Mulberry extract at doses of 250 and 500mg/kg/day were given orally to diabetic rats for 21 days.

Induction of Diabetes Mellitus using Streptozotocin

Diabetes mellitus was induced in rats that had previously been kept on a fast overnight through a single intraperitoneal injection of Streptozotocin (45mg/kg body weight). After 3 days, fasting glucose levels were measured and the animals showing blood glucose level of 225mg/dl and above were used. The treatment process continued for 3 weeks.

Biochemical Analysis

After 3 weeks of experiment, overnight fasted rats were sacrificed by euthanasia and blood was collected in vials by cardiac puncture for analysis. SOD[12], CAT[13], lipid peroxidation[14], Reduced Glutathione[15] were assayed. The liver obtained from the rats was chilled on cracked ice and a 10% (w/v) tissue homogenate was prepared in 0.15mol/L potassium chloride at 0°C[14]. The homogenate was used for the estimation of lipid peroxidation CAT, SOD and GSH.

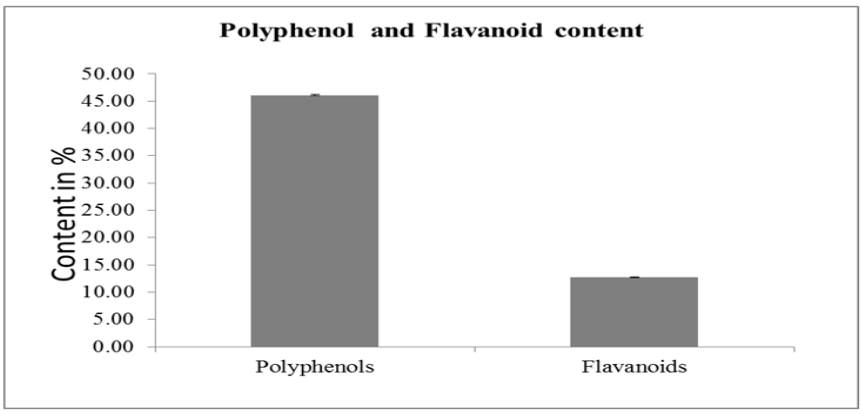
Statistical analysis

Data generated was expressed as mean ± SD for all experimental groups. Statistical analysis was performed using SPSS version 16.0 to conduct the 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

Polyphenolic and flavonoid content in M.latifolia crude extract

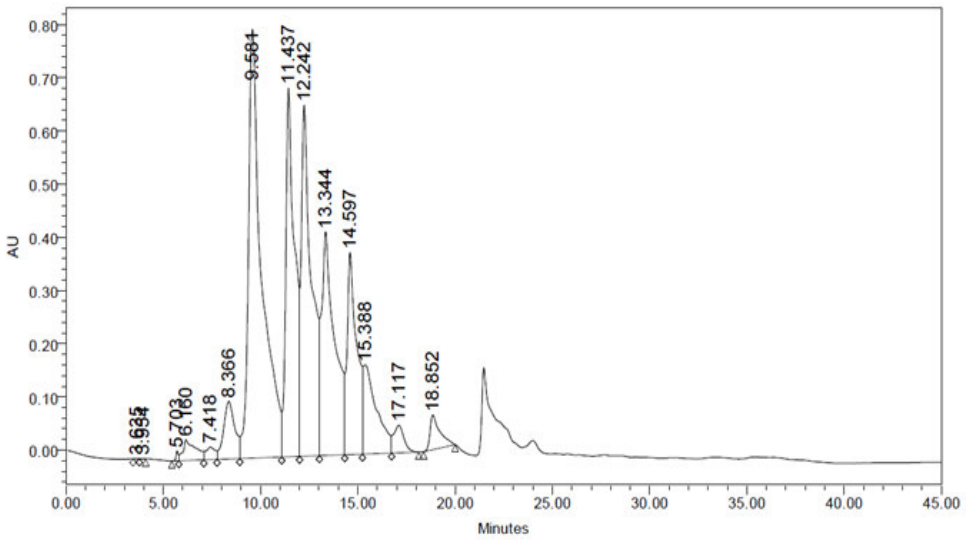
The polyphenolic content in *M.latifolia* leaf extract was found to be 46.06% and flavonoid content 12.75% (Graph 1).



Graph 1
Polyphenol and Flavonoid content in M.latifolia

Polyphenolic compounds in M.latifolia crude extract

Chromatographic separation identified the major polyphenolic compounds to be chlorogenic acid, caffeic acid, coumaric acid, rutin and quercetin (Graph 2). Among the identified polyphenolic compounds, chlorogenic acid is found to be relatively prominent followed by rutin and quercetin. *M.latifolia* registered higher values for chlorogenic acid (360.50mg/gm dry wt.). Rutin and quercetin ranges from 185.50 and 161.67mg/gm. Caffeic acid and coumaric acid was found to be in the range of 15.05mg/100gm and 40.64mg/gm (Table 1).



Graph 2
HPLC Chromatogram of M.latifolia leaf Extract

Table 1
Phenolic Constituents in M.latifolia crude extract

Compound	Retention time[a]	Content[b]
Chlorogenic acid	9.581	360.50±0.25e
Caffeic acid	11.437	15.05±0.15a
Coumaric acid	12.242	40.64±0.20b
Rutin	13.344	185.50±0.35d
Quercetin	14.597	161.67±0.45c

Values are presented as mean ± S.D. (n=3);Different lower-case letters within the column are statistically significant by DMRT at p<0.001[a] Min; [b] mg/gm

Effect of M.latifolia leaf extract on antioxidant enzymes SOD and CAT

The effect of *M.latifolia* on the serum antioxidant system is shown in Table 2. Diabetic control rats showed increased level of catalase activity compared to normal control rats. The serum and liver catalase enzyme of *M.latifolia* treated diabetic rats showed significant decrease in their activity (p<0.01) compared to diabetic control rats. In contrast to CAT, the level of SOD in diabetic control rats decreased in comparison to normal control rats. After administration of *M.latifolia* leaf extract (250mg/kg and 500mg/kg), the level of SOD significantly reduced in serum (p<0.01) and liver (P<0.001).

Effect of M.latifolia leaf extract on non-enzymatic antioxidants (Reduced glutathione)

In streptozotocin induced diabetic rats (Group II), the level of reduced glutathione decreased considerably compared with that of the normal control group. The levels of reduced glutathione in serum (P<0.001) and liver tissue increased significantly (P<0.05) after administration of *M.latifolia* leaf at the concentration of 250mg/kg and 500mg/kg respectively. An increased level of GSH activity was observed in serum and liver tissue (P<0.001) of Glibencalamide (120mg/kg body weight) treated rats compared to diabetic control rats (Table 2).

Effect of M.latifolia leaf extract on Lipid peroxidation levels

The serum and liver peroxide levels in the mulberry leaf extract were depicted in Table 2. In STZ diabetic rats, the level of lipid peroxides in serum and hepatic tissue increased compared with normal control group. After administration with *M.latifolia* extract, there was a significant decrease in concentrations of Lipid peroxides in serum and liver tissue of diabetic rats (P<0.01). Glybencalamide treated group also showed a decreased level of peroxides in serum (P<0.05) and liver tissue (0.001) when compared to diabetic control group.

Table 2
In vivo antioxidant activity of M.latifolia leaf extract on Streptozotocin induced diabetic rat models

Groups	Super Oxide Dismutase (SOD) Activity [a]		Catalase (CAT) Activity [b]		Glutathione (GSH) Activity [c]		Lipid Peroxidation (LPO) Activity [d]	
	Serum	Liver Tissue	Serum	Liver Tissue	Serum	Liver Tissue	Serum	Liver Tissue
Normal Control	8.77± 0.32	8.93 ± 0.05	5.93 ± 0.01	6.25 ± 0.34	6.11 ± 0.07	6.23± 0.15	0.312 ± 0.02	0.25 ± 0.03
Diabetic control (STZ) (45 mg/kg)	1.96 ± 0.63	2.13 ± 0.04	11.53 ± 0.04	12.58 ± 0.60	3.18 ± 0.10	2.48 ± 0.32	1.52 ± 0.06	1.62 ± 0.04
Glibenclamide Control (120 mg/kg)	7.94 ± 0.66**	8.67± 0.05***	6.84 ± 0.06***	7.11 ± 0.09***	5.09± 0.08***	5.46 ± 0.27***	0.43 ± 0.24*	0.32 ± 0.02***
Mulberry leaf extract (250 mg/kg)	4.44 ± 0.38***	4.70 ± 0.02***	9.91 ± 0.02***	9.12 ± 0.19**	4.09 ± 0.14***	3.18 ± 0.36*	0.96 ± 0.24*	0.92± 0.02**
Mulberry leaf extract (500 mg/kg)	6.94 ± 0.60**	6.78 ± 0.05***	7.14 ± 0.05***	8.41 ± 0.46**	5.55 ± 0.05***	4.3 ± 0.07***	0.63 ± 0.04***	0.52 ± 0.02***

n=6 in each group; values are presented as mean ± S.D, *P<0.05, **p<0.01, ***p<0.001 compared with the diabetic control.
[a] units/mg of protein; [b] µM H_2O_2 consumed/min/mg of protein; [c] µM of glutathione oxidized/mg of protein;
[d] nano moles of MDA released/mg of protein

DISCUSSION

Phenolic compounds are the most active antioxidant derivatives in plants[16] and have generated much interest as a result of their free radical scavenging abilities that can bring about substantial improvement in human health.[17,18] In the current study the total polyphenolic content in *M.latifolia* leaf extract was found to be 46.06%. This is the first report on polyphenolic content in *M.latifolia* leaf extract. Plant flavonoids are an important part of our diet because of their impact on human nutrition.[19] They are known to possess the following properties: free radical scavenging, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes. The quantity of total flavonoids (catechin equivalents) is 3.6% in 70% ethanolic leaf extract. Earlier, the flavonoid contents of mulberry leaves of 19 varieties of species reported in terms of rutin equivalents varied from 11.7 to 26.6mg/g in spring leaves and 9.84 to 29.6mg/g in autumn leaves.[5] HPLC analysis showed that the main polyphenolic constituents in *Morus latifolia* (BC259) leaf extract were chlorogenic acid, caffeic acid, coumaric acid, rutin and quercetin. The *in vivo* antioxidant effect of *M.latifolia* leaf extract in STZ induced diabetic rats is due to the cumulative effect of these identified polyphenolic compounds. The activities of antioxidant enzymes such as SOD, CAT and GPx has significantly increased by the administration of p-coumaric acid in liver and kidney tissues of diabetic rats.[20] According to Jung *et al.*, caffeic acid is beneficial against oxidative stress by significantly increasing the superoxide dismutase, catalase, and glutathione peroxidase activities while lowering the lipid peroxidation products in the erythrocyte and liver of db/db mice.[21] The *in vitro* and *in vivo* effect of Quercetin on the expression of superoxide dismutase, glutathione peroxidases, quinone reductase were reported.[22] These earlier reports strongly suggest that these compounds have strong antioxidant potential to evade the stress induced complications associated with diabetes. A significant role is performed by enzymatic antioxidants in combating oxidative damage. In the current study, a decline in the activity of superoxide dismutase and catalase activity in liver and serum of untreated diabetic rats demonstrates diabetes-induced stress. A similar kind of action has been reported for these enzymes earlier by Stanely and Menon.[23] GSH were registered low in the untreated diabetic animals indicating its augmented consumption as a result of oxidative stress while a substantial promotion of GSH activity in *M.latifolia* leaf extract treated diabetic rats corresponded with a substantial waning in oxidation of lipids. These results are in par with the findings of Andallu and Varadacharyulu.[6] The peroxidation of lipids in biological systems has drawn huge interest in recent times as it results in impairment to tissues *in vivo*, producing atherosclerosis and numerous complications associated with diabetes. The enormous rise in lipid peroxides observed in rats having diabetes is due to hyperglycemic condition which instigates an enhanced generation of highly reactive oxygen molecules which pave a way to the oxidation of glucose molecules thereby enhancing the production of superoxide anions and hydroxyl radicals which are the key factors that bring about damage to tissues by interacting with polyunsaturated fatty acids in membranes.[24] The progress of diabetes brings about a decrease in the antioxidant potential thereby resulting in an increase of lipid peroxides.[25] Administration of *M.latifolia* leaf extract reduced the lipid peroxidation to a maximal extent in diabetic rats. These results are in accordance with Andallu *et al.*[26] The data contained in this work suggests that *M.latifolia* leaf extract averts oxidative damage due to diabetes by protecting oxidation of lipids through antioxidant defence mechanisms.

CONCLUSION

The study has concluded that the leaf extract of *M.latifolia* showed significant antioxidant potential against oxidative stress caused by diabetes. The antioxidant properties of the leaf of *M.latifolia* identify this plant to be used for therapeutic purposes apart from being used as fodder for silkworms. Our findings can pave the way in future for these varieties to be explored by herbal druggists in India as potential health supplements in the pharmaceutical industry thus making moriculture a highly profitable enterprise.

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CONFLICT OF INTEREST

Conflict of interest is declared none.

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