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ANTIOXIDANT AND ANTI-DIABETIC POTENTIAL OF MR-1 MULBERRY VARIETY

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ABSTRACT

The current study was aimed to analyze an antioxidant activity and anti-diabetic effect of mulberry leaf extract. Free radical quenching activity of MR-1 mulberry leaf was determined by in vitro and anti-diabetic activity of mulberry leaf extract was analyzed through in vivo using STZ diabetic male mice strain. The in vitro antioxidant studies revealed that the extract of MR-1 mulberry leaf exhibited highest antioxidant content (67.44 % in methanol solvent) and (73.45 % in ethanol solvent) at 400 µl concentration than ascorbic acid (62.37 % in methanol solvent) and (72.89 % in ethanol solvent) at 400 µl concentration. The MR-1 mulberry variety significantly (<0.05) decrease the glucose concentration and enzyme activity such as SGOT and SGPT, CAT, whereas the G-6PDH activity increases. The study revealed MR-1 mulberry variety has been potential for quenching of free radicals and capacity to reduce the blood glucose levels.

KEY WORDS

Antioxidant, Anti-diabetic, DPPH, Mulberry, Streptozotocin

1.0 INTRODUCTION

In the human body system, a number of physiological and biological processes take place which generates oxygen center free radicals, molecules, and their byproducts. Excess production of free radicals leads to oxidative stress in the human body system. Antioxidant compounds bind with free radical, neutralize the effect of free radicals inside the body system by the action of the different mechanism.[1] The antioxidant is a molecule which has a property of prevention or postponement of oxidation of other molecules by inhibiting the initiation or propagation of oxidative chain reactions.[2] Humans who consume leaves and vegetables rich with phenolic compounds have relatively decreased the risk of emerging chronic diseases through reduction of oxidative stress and inhibition of macromolecular oxidation.[3] Oxidation is a chemical reaction which has the ability to produce free radicals which undergo a series of reactions that may damage essential biomolecules (protein, lipid, and DNA) in the form of tissue injury and cell death. If they exceed a certain limit, their harmful effects can be avoided by their regular removal from the body with the help of natural anti-oxidative defense system. They may be enzymatic and non-enzymatic in the human body, which continuously and proportionally neutralizes free radicals by scavenging through substances such as ascorbic acid (vitamin C) or thiols and other compounds like polyphenols and carotenoids which terminate the oxidation chain reaction.[4]

Diabetes is a chronic disorder characterized by the rise in blood glucose levels or concentration inside the body resulting in body system disorders. It is a condition mainly attributed to the levels of hyperglycemia which give rise to a risk of microvascular damage like central vein, central vein membrane, the sheet of hepatocytes and retinopathy, nephropathy and neuropathy. It is allied with reduced life anticipation; significant injury

due to precise diabetes linked microvascular problems, and increased risk of macrovascular problems like peripheral vascular disease.[5] Several anti-diabetic drugs currently available in the market manage to keep the blood glucose levels under normal range by supplementing insulin. There are many types of glucose-lowering drugs, including secretagogues (sulfonylureas, meglitinides), insulin sensitizers (biguanides, metformin, thiazolidinediones) and alpha-glucosidase inhibitors (miglitolacarbose). New peptide analogs, such as exenatide, liraglutide and dipeptidyl peptidase (DPP)-4 Inhibitor, increases glucagon-like peptide (GLP-1) serum concentration and slow down the gastric emptying. Most of the glucose-lowering drugs cause side effects, like insulin targeting the liver, muscle, and fat which shows adverse reaction leading to hypoglycemia, weight gain etc. Other drugs such as Acarbose, miglitol target the alpha-glucosidase that has intestine as the site of action, shows adverse effects like gastrointestinal disturbances, lactic acidosis.[6] Antioxidant compounds including ascorbic acid, carotenoids, flavonoids, tannins play an important role in the prevention of many chronic diseases. [7] Some of the plants that are medically identified as rich resources of antioxidant compounds will be more useful to treat the diabetes disorder without side effects. These natural antioxidants will be helpful to human health. Therefore, the present study was aimed to screen the antioxidant and anti-diabetic activity of MR-1 mulberry variety.

1.1 MATERIALS AND METHODS

1.1.1 Chemicals

2, 2-diphenyl-1-picryl-hydrazyl (DPPH), 2, 4, 6-Tripridyl-s-triazine (TPTZ), Ascorbic acid, sodium acetate buffer (pH-3.6), glacial acidic acid, sodium acetate, were used. All other chemicals used were of analytical grade. Glucose-6-phoshtate dehydrogenase (G6PDH), β-nicotinamide adenine dinucleotide phosphate (β-NADP) was purchased from Sisco Research Laboratory Pvt., Mumbai, India. Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were obtained from ARKRAY Healthcare Pvt. Ltd., India. STZ was bought from Hi-Media, Mumbai, India. Haematoxylin and Eosin were purchased from Sigma-Aldrich, USA. All other chemicals used were of analytical grade.

1.1.2 Collection of materials

MR-1 variety of mulberry leaf sample was collected from the Central Sericultural Germplasm Resources Centre (CSGRC), Hosur, Tamil Nadu, India. The plant species was identified by Dr. P. Sarasvati, Division of Mulberry, CSGRC, Hosur.

1.1.3 Preparation of Ascorbic acid (standard)

Standard of ascorbic acid (10 mg) was dissolved in 10 ml of both methanol and ethanol solvents separately and final concentration was made from the stock solution of ascorbic acid.

1.1.4 Preparation of test samples (Mulberry leaf) extracts

Leaf powder sample of selected mulberry variety was dissolved in both the solvents of methanol and ethanol. The powdered plant material (50 mg) was soaked in methanol and ethanol solvents for 48 hours. Further, the extract was filtered using Buchner funnel and Whatman No.1 filter paper and a freshly prepared extract were used for analysis of antioxidant activity through 2-2-Di-Phenyl-1-1Picry-Hrazyl (DPPH)and Ferric Reducing Antioxidant Power (FRAP) assays. The final concentration of mulberry extract was taken 1mg/ml.

1.1.5 Preparation of 2-2-Di-Phenyl-1-1Picry-Hrazyl (DPPH) solution

DPPH solution was prepared by using a method of[8]Gulluce *et al.,* 2003, 4 mg of DPPH reagent was dissolved in 100 ml solvents of methanol and ethanol respectively and final concentration 3 ml of 0.004 % DPPH was taken and it was protected from light by covering the bottle with aluminum foil.

1.1.6 Determination of free radical scavenging activity using DPPH method

The free radical scavenging activity of the selected mulberry leaf extract was measured by the spectrophotometric method for the assay of hydrogen donating free radical.[8] Different concentrations (50, 100, 200, 300, 400 µl/ml) of mulberry leaf extracts and ascorbic acid as standard were prepared in both methanol and ethanol solvents separately. Subsequently, 3 ml of 0.004 % DPPH reagent was added. The compound was mixed thoroughly and left for incubation at room temperature in dark. The absorbance was measured at 517 nm using a spectrophotometer and antioxidant activity was expressed as percentage inhibition.

Where $Abs_{control}$ is the absorbance of the control (without test material) and Abs_{sample} is the absorbance of the test material. The assay was carried out in

triplicate and results are expressed as mean % inhibition.

1.1.7 Experimental animal model

Male strains mice of 4-6 weeks old (25±5gm) were used for the anti-diabetic test. The mice were housed in an individual cage in an air- habituated room with 12 hr light/dark cycle at a temperature of 25±2 ^{0}C with free access to food and water. All the mice were acclimatized to the laboratory environment for 7 days prior to the experiment.

1.1.8 Preparation of test sample (mulberry leaf)

Preparation of test sample was done following the method of [9] (Yihai *et al.* 2013). Mulberry leaves were washed with distilled water, left to dry naturally at room temperature and powdered with a grinder. The powder leaf material (100 mg) was dissolved in 10 ml of 20 % ethanol solvent for 2 hours and then filtered. The filtered extract was used for *in vivo* study with diabetic mice.

1.1.9 Test animals and induction of diabetes

Inductions of diabetes in mice were carried out with minor modification as per [10] (Hua *et al.,* 2014). 1 % Fresh solution of streptozotocin (STZ) prepared in 0.1 M citrate buffer (pH 4.5) was injected to 18 mice (overnight fasted) single injection (dose of STZ 125 mg/kg body weight) through intraperitoneal injection for inducing diabetes. Subsequently, after 72 hours, assessments were made for Fasting Blood Glucose levels (FBG) of the mice from the tail vein. Mice with FBG values >226mg/dl were considered to be hyperglycemic. A total number of 84 such mice were divided into five groups (Six animals in each group) as follows:

Group I: Normal group of mice

Group II: Diabetic Group of mice (FBG> 226 mg/dl) - Control

Group III: Diabetic mice treated with Insulin (4U/dl)

Group IV: Diabetic mice treated with MR-1 mulberry leaf extracts

1.1.10 Feeding schedule

Standard feed of laboratory diet was given to Groups IV and V, orally administering through mulberry leaf extracts (4U/dl) two times (7 am and 7 pm) in 24 hours. [11] Group III mice were provided at the same dose of insulin at the same time.[12]

1.1.11 Measuring the changes in body weight and Hepatic Enzyme Activity

The effect of mulberry leaf extract in different groups of diabetic mice was measured by changes in body weight of mice and FBG level at 7, 14, and 21 days. After 21 days of the experiment, where mulberry leaf extracts were administrated to different groups of diabetic mice, they were anesthetized with diethyl ether. Excised the liver tissue and washed with phosphate buffer saline solution at PH-7.4 and homogenate the tissue of cold buffer (1x PBS) containing protease inhibitor, then centrifuged at 10,000 rpm for 15 min and the supernatant was collected in another test tube and stored at -80 ^{0}C for further use in the assayed activities of various enzymes such as Catalase, Glucose-6-phosphate dehydrogenase, Serum glutamic oxaloacetic transaminase (SGOT) and Serum glutamic pyruvate transaminase (SGPT).

1.1.12 Measuring Catalase (CAT), Glucose-6-phosphate dehydrogenase (G6PDH), Serum Glutamic oxaloacetic transaminase and Serum glutamic pyruvate transaminase

The catalase activity was measured according to the method of Beers &Sizer (1952) whereas Glucose-6-phosphate dehydrogenase was measured according to Worthington enzymes manual. The activities of SGOT and SGPT in liver tissue of each group of mice were assayed by commercial span kit.[13-16,27,28]

1.1.13 Histopathological Study of Liver Tissue

Effects of Mulberry leaf extract on liver tissue of different groups of diabetic mice were observed through a histopathological study by staining of Haematoxylin and Eosin stain. After 21 days treatment of mulberry leaf extract, each group of mice was anesthetized with diethyl ether and excised the fresh portion of liver tissues and fixed in a fixative of aqueous bouins**.** Further, tissue was dehydrated in an ethanol series of ascending concentrations (30%, 50%, 70%, 90%, and absolute alcohol) in order to ensure dehydration of the tissue it was rinsed in xylene, cleared cedarwood oil and embedded in paraffin wax. Serial sections were cut at a thickness of 6 µm using a Leica Rotary Microtome (Model RM 2125RT; Leica Microsystems, Bensheim, Germany). Sections were deparaffinized in xylene, hydrated in a descending ethanol series (absolute alcohol, 90%, 70%, 50%, 30%) and were stained with Ehrlich's hematoxylin and eosin (H/E) [17].The stained sections were dehydrated in an ascending ethanol series, cleared in xylene and mounted in dibutyl phthalate xylene (DPX).

1.3 Ethics Clearance

All experiments were conducted according to the ethical norms approved by the *CPCSEA*,

India Government of India, in the Department of Zoology, Banaras Hindu University.

1.4 Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA) - one-way technique and later Dunnett's post hoc test was applied for significance testing. Dunnett's test is applied as each comparison has the same control in common. The results are expressed as the mean ± standard deviation (SD). Significance was tested at the level of $p<0.05$.

1.5 RESULTS

1.5.1 Antioxidant scavenging activity of DPPH

The DPPH radical scavenging activity was recorded in terms of % Inhibition in methanol and ethanol solvents. Ascorbic acid is a standard (control) and compared with different tested mulberry genotypes. Values on percent inhibition of free radical scavenging activity of ascorbic acid and mulberry leaves with methanol and ethanol are shown in Table 1.

Table 1 DPPH radical scavenging activity of mulberry leaf extract and ascorbic acid

Concentration	Ascorbic acid In methanol	Ascorbic acid In ethanol	MR-1 In methanol	MR-1 In ethanol
50 µl/mL	13.70 ±0.8	12.46 ± 1.2	33.27* ± 1.8	14.79 ± 2.2
100 µl/mL	27.46 ± 1.2	26.56 ± 2.6	39.17* ± 1.4	25.46 ± 1.0
200 µl/mL	39.99 ±0.3	43.41 ± 1.8	51.74* ± 1.9	46.51 ± 1.1
300 µl/mL	50.84 ±0.2	59.29± 2.1	57.80* ±0.8	67.55* ± 2.1
400 µl/mL	62.37 ±0.6	72.90 ± 2.6	67.44* ±0.8	73.45 ±0.7

The percent inhibition of DPPH free radicals scavenging activity of the ascorbic acid in methanolic solvent was measured at different concentrations are, 50 µl/mL, 100 µl/mL, 200 µl/mL, 300 µl/mL, 400 µl/mL. The values ranged from 13.70 % to 62.37 % in respect of Ascorbic acid under methanol solvent under different concentrations as shown in Table 1 while the respective values in ethanol solvent for the same ascorbic acid ranged from 12.46 % to 72.89 % at the same concentrations (Table 1). The radical activity has progressively increased with increase in concentrations in respect of both the solvents. This trend is similar to both ascorbic acid as well as all the mulberry varieties. The response of free radicals scavenging activity increases 67.44 % in methanol and 73.45 in ethanol solvent respect of the MR-1 mulberry variety (Table-1 and Figure 1A & 1B).

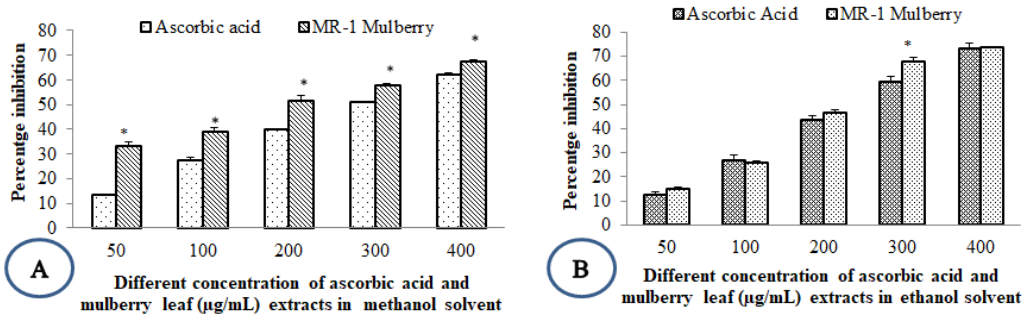

Figure 1 DPPH radical scavenging activity at different concentration of MR-1 mulberry leaf extract and ascorbic acid in methanol (Fig 1A) and Ethanol (Fig 1B) Solvent. Values are mean ± standard deviation. *On bars indicates significant difference from control. Mulberry leaf extracts compared with the ascorbic acid (control). Level of significant was tested at the level of $P < 0.05$ by one-way analysis of variance analysis.

1.5.2 *In vivo* study

Leaf extract of MR-1 mulberry variety experimented on diabetes-induced mice and the body weight, fasting blood glucose levels were measured at intervals of 7, 14, 21 days and the average readings are tabulated in Table 2 & 3. However, measurements of the enzyme activities were also taken and the average readings in respect of Catalase, G6PDH, SGPT, and SGOT are presented in Tables 4 & 5 below.

Table 2 Effect of mulberry leaf extract (ML) administration on weight (gm/kg) in chronic diabetic mice

Treatments	First Week	% ↑↓over Dia group	Second Week	% ↑↓ over Dia group	Third Week	% ↑↓ over Dia group
Normal mice (Non-diabetic mice)	25.8±0.96	-3.01	24.4*±1.14	14.02	25.7*± 1.53	30.46
Diabetic mice (STZ treaded mice)-Control	26.6±1.78	-	21.4±1.14	-	19.7± 3.06	-
Diabetic mice + insulin treated	25.58±3.09	-3.01	26.0*±1.76	21.50	26.2*±1.75	32.99
Diabetic mice + mulberry (MR-1) treated	24.8±2.64	-6.77	25.0*±1.26	16.82	26.6*±1.53	35.03

Note: Data expressed as mean± SD (r =3). Minus sign indicates reduction over the control. Figures in bold with* indicate statistically significant values below the control (diabetic mice) at p<0.05.

Table 3 Effect of mulberry leaf extract (MLE) administration on fasting blood glucose (mg/dl) in chronic diabetic mice.

Treatments	First Week	% ↑↓ over Dia group	Second Week	% ↑↓ over Dia group	Third Week	% ↑↓ over Dia group
Normal mice (Non-diabetic mice)	134.0*±2.6	-74.49	138.8*±5.0	-76.85	145.5*±5.0	-74.31
Diabetic mice (STZ treaded mice)-Control	525.2±39.0	-	596.2±4.8	-	566.3±36.9	-
Diabetic mice + insulin treated	374.7*±51.1	-28.66	172.2*±9.9	-71.12	167.7*±5.0	-70.39
Diabetic mice + mulberry (MR-1) treated	321.2*±17.7	-38.84	284.3*±13.6	-52.31	148.7*±8.5	-73.74

Note: Data expressed as mean± SD (r =3). Minus sign indicates reduction over the control. Figures in bold with* indicate statistically significant values below the control (diabetic mice) at p<0.05.

Table 4 Effect of mulberry leaf extract (MLE) administration on Enzymes of chronic diabetic mice

Treatments	Catalase (mmol /mg)	% ↑↓over Dia group	G-6-PDH (mmol /mg)	% ↑↓over Diab group
Normal mice (Non-diabetic mice)	109.0*±1.5	-27.86	417.2*±16.9	133.76
Diabetic mice (STZ treaded mice)-Control	151.1±2.4	-	178.9±9.4	-
Diabetic mice + insulin treated	116.9*±3.0	-22.63	342.0*±10.6	91.17
Diabetic mice + mulberry (MR-1) treated	108.8*±3.0	-27.99	374.9*±5.2	109.56

Note: Data expressed as mean± SD (r =3). Minus sign indicates reduction over the control. Figures in bold with* indicate statistically significant values below the control (diabetic mice) at p<0.05.

Table 5 Effect of mulberry leaf extract (MLE) administration on Enzymes of chronic diabetic mice

Treatments	SGPT (Unit/ml)	% ↑↓over Diab group	SGOT (Unit/ml)	% ↑↓over Diab group
Normal mice (Non-diabetic mice)	29.2*±1.6	-57.25	25.3*±0.4	-54.50
Diabetic mice (STZ treaded mice)-Control	68.3±0.6	-	55.6±0.6	-
Diabetic mice + insulin treated	41.6*±5.6	-39.09	26.3*±0.5	-52.70
Diabetic mice + mulberry (MR-1) treated	28.5*±1.6	-58.27	24.6*±0.7	-55.76

Note: Data expressed as mean± SD (r =3). Minus sign indicates reduction over the control. Figures in bold with* indicate statistically significant values below the control (diabetic mice) at p<0.05.

All the experimental values were compared with the Diabetic mice group considering it as the control. This is because the effect of mulberry has to be observed in case of mice severely affected with diabetes so as to see whether the mulberry treatment decreases the glucose levels and maintain the related body system normally. The treatments such as Normal mice and the diabetic mice + insulin-treated groups serve us as the normal standards for comparison of the readings. It can be expected that the readings of mulberry treated groups have to be in the close vicinity of these two groups. Statistical comparison with the Diabetic mice group (control) will help us to know whether the mulberry treatments are really effective in curing the acute diabetic cases or not. For augmenting the examination of the treatment effects more precisely, increase and decrease in the experimental values over the control are also given in percentages to assist instant grasp.

1.5.3 Effect of mulberry leaf extract (MLE) on the body weight

From the body weight of the mice as tabulated in Table 2, it is observed that during the first week of the experiment it was 26.6 g followed by 21.4 and 19.7 g. in case of diabetic mice. From week after week, the weight has decreased. In the experimental groups, the body weight has increased in certain cases and decreased in certain cases over the three-week period. The cases where it has increased are by and large the ones which have shown a statistically significant increase in the diabetic mice group (control). We may observe that in case of the insulin-treated and MR-1 mulberry treated groups the body weight has gained. This weight gain is quite significant (p<0.05) over the diabetic group. These values are marked with * in Table 2. In the third week, the body weight gain is to an extent of 35.03 % (26.6 g.) in MR-1 treated group. This variety has shown consistent performance over the three-week period expressing the effect to be well over the control group. These responses are reflected in the bar diagram (Fig. 2A) for an easy glance.

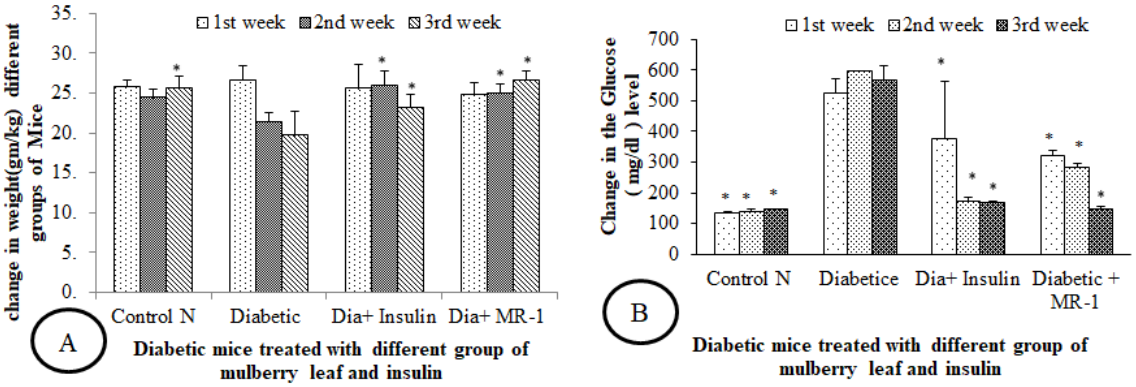

Figure 2 Effect of mulberry leaf extract on Weight (Fig 2A) and Glucose level (Fig 2B) in different group at 21 days of mice. Values are mean ± standard deviation. (x ±SEM, n=6 in each group), *On bars indicates significant difference from control. Level of significant was tested at the level of $P < 0.05$ by one-way analysis of variance analysis. Aberration-Control N-Normal Group, Dia-Diabetic mice, Dia + I- Diabetic treated with insulin, Dia +MR-1-Diabetic group treated with mulberry leaf of MR-1 variety.

1.5.4 Effect of mulberry leaf extract (MLE) on fasting blood glucose

Measurement of glucose level in the blood is an important variable to decide about the presence of diabetic disorder. From the Table 3, we see the measurements of glucose levels (fasting) under influence of STZ and the other mulberry treatments. The measurements are made over a period of three weeks. Induction of STZ has increased the glucose levels to a surprising level of 566 mg/dl at the end of three weeks which is known to be quite high. Now the mulberry treatments have to reduce this level substantially. The insulin treatment which is a well-known anti-diabetic has reduced the glucose level to 167.7 mg/dl from 566.3 mg/dl during the third week - a significant reduction of 70.39 % which is statistically significant at $p<0.05$. Continuing the observation, we notice that some of the mulberry treatments have reduced the glucose level to a level even better than(less) the normal. The variety MR-1 have contributed very significantly to the reduction of glucose levels to 148.7 (-73.74 %) compared to the diabetic group. The values 148.7 mg/dl in respect of MR-1 variety has been contributed so significantly for reduction of the glucose level in the blood compared to insulin treatment even. It is also noticed that the reduction in blood glucose has gradually taken place over a period of 21 days (Table 3 and Fig. 2B).

1.5.5 Effect of mulberry leaf extract (MLE) on Enzymes

1.5.5.1 Catalase

The response of catalase enzyme activity was measured in normal and experimental mice treated with MLE and insulin in the liver tissue and the mean values so obtained are tabulated in Table 4. Under the present study in the STZ- induced mice the activity of catalase has significantly ($p< 0.05$) increased to 151.1 mmol/mg. After 21 days of treatment with mulberry leaf extracts to different groups of mice, significant positive change was observed in the activity of catalase. The values have shown predominant reductions in different groups of mice which was treated with mulberry leaf extracts. MR-1 mulberry variety shown reduction in catalase activity compared to the diabetic group. As against 151.1 mmol/mg the values observed in case of 108.8 mmol /mg. in MR-1 variety. This value is significantly lower than the values of Diabetic mice with $p < 0.05$. This interesting variation can also be glanced from the Figure 3A.

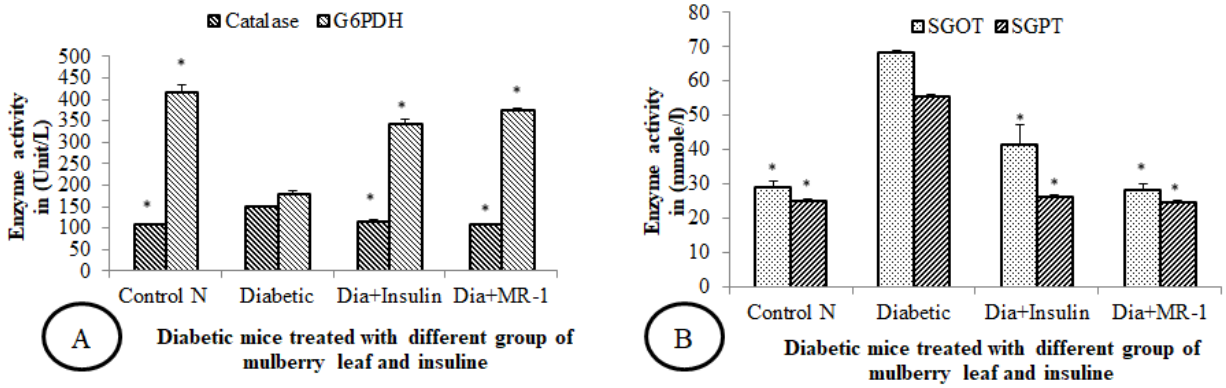

Figure 3 Effect of mulberry leaf extract on hepatic antioxidant enzyme of G6PDH glucose 6 phosphate dehydrogenase and catalase activity (Fig 3A) and SGOT (Serum glutamic oxaloacetic transaminase) and SGPT (serum glutamic-pyruvic transaminase) activity (Fig 3B) in liver tissue of different group of mice at 21 days. (x ±SEM, n=6 in each group), Values are mean ± standard deviation. *On bars indicates significant difference from control. Level of significant was tested at the level of $P < 0.05$ by one-way analysis of variance analysis. Aberration- Control N-Normal Group, Dia-Diabetic mice, Dia + I- Diabetic treated with insulin, Dia +MR-1- Diabetic group treated with mulberry leaf of MR-1 variety.

1.5.5.2 G6-PDH

Effect of mulberry extracts through G-6-PDH in units of mmol/mg is shown in Table 4. After 21 days of treatment with mulberry extracts for the Diabetes-induced mice, the mean values have shown an interesting increase. In fact, it is a welcome increase. Under the normal mice the value noticed is 417.2 mmol/mg. whereas it was dropped to 178.9 mmol/mg. in the Diabetic Mice. For a normal health this value needs to increase. Hence it has increased to 342.0 mmol/mg. in the insulin-treated mice. With anti-diabetic treatment the value has to increase to a level of

400 mmol/mg. This kind of significant increase ($p<0.05$) is observed in case of MR-1 mulberry variety. The variations are depicted in Fig 3A.

1.5.5.3 SGPT and SGOT

The average values obtained after 21 days of treatment with the mulberry leaf extracts in respect of SGPT (Serum Glutamic Pyruvate Transaminase) and SGOT (Serum Glutamic Oxaloacetic Transaminase) Levels are presented in table 5 and also shown in Fig.3B. Here the Normal value in respect of SGPT is 29.2 units/ml. When the mice are induced with the Diabetes, the value has gone up to 68.3units/ml. This means the higher glucose level in blood increases the value of SGPT. It is seen that with the treatment of mulberry leaf extracts this variable level has significantly gone down to almost the normal level in case of Diabetic Mice. Treatment with MR-1mulberry variety has significantly ($p < 0.05$) contributed for lowering the SGPT values. The value in respect of the above is 28.5 (Table 5 and Fig. 3B).

1.5.6 Histopathological Variation

Light microscopic examination of the liver of control mice demonstrated regular and compact architecture with well-organized hepatic cell and central vain (Fig. 4A). whereas, (Fig 4B), the section of treated STZ diabetic mice showed different histopathological alteration. Diabetic mice treated with Insulin showed (Fig 4C) moderate enlargement of sinusoids, vacuole formations in hepatocytes, congestion in the central vein (this results in dilatation of central veins and pooling of blood in the sinusoids towards the center of the liver lobule) and mild hemorrhage in hepatic tissues. The diabetic mice treatment with MR-1 indicated granular repaired of sheet hepatocytes, central vein and healthy nucleus (Fig 4D)

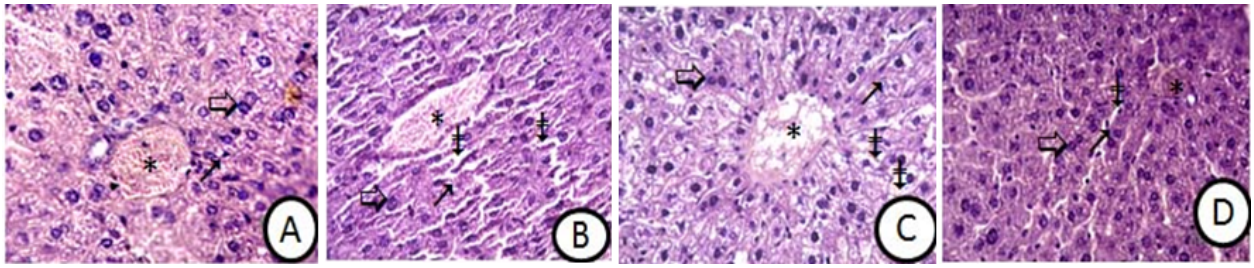

Figure 4 (A-D) Photomicrograph of liver section of mice showing the changes in cellular organization after three week of experiment (H/E stain), Scale bar = 40µm abbreviation marks- * (stick) central vein, ˅ (open arrow) Nucleus, ↗ (arrow) sheet of hepatocytes, ‡(barred arrow) sinusoids. (A) Normal control group showed normal central vein, sheet of hepatocytes, (B) Diabetic group representing destruction of central vein, de-leafing of sheet of hepatocyte and sinusoids cells. (C) Insulin treated diabetic group appears almost similar to normal control mice but in central vein showed regaining of normal structure. (D) Mulberry leaf of MR-1 variety treated with diabetic group was shown almost as like control.

1.6 DISCUSSION AND CONCLUSION

An antioxidant molecule is a natural bioactive compound which has the ability to neutralize the oxidative free radical. Among the natural plant material that has rich antioxidant compounds mulberry is known to be highly useful. Therefore, the free radical-scavenging activities of mulberry leaf along with standard compound such as ascorbic acid was tested by the DPPH radical scavenging assay method. The response of control and experimental mulberry varieties under methanol solvent as tabulated in table 1 indicate that the mulberry variety MR-1, expressed higher percentage of inhibition over control under methanol solvent which is also found to be statistically significant at a probability level of >0.95 (considered as $p<0.05$). Results obtained under the ethanol solvent are tabulated in Table 1. The MR-1 variety has also performed well except for one lower value in respect of 100µl/mL. Allowing some margin for this value which is closer to the control value and considering that as on par with control, the variety MR-1 can also be roped into the promising ones under ethanol solvent. It is found that the potential of the mulberry varieties get increased with increased concentration of leaf extract. This is because of the richness of mulberry leaves with secondary metabolites like alkaloid, saponin, tannin, phenol hydroquinone, flavonoid flavonol (quercetin, kaempferol, myricetin) that are present which are acting as strong antioxidant molecules[18].

1.6.1 Effect on the body weight

The mean values obtained from the normal group where the mice were not treated with anything serve as the standards for comparison. Generally, the body weight in case of acute diabetes deteriorates over a period of time. With the treatment for diabetes the body has to gain weight, and this signifies improvement in the health condition. The diabetic mice treated with selected variety of mulberry leaf extracts have shown significant weight gain compared to the control (Diabetic mice). The increased body weight in case of diabetic mice in the experiment is a clear indication of the effect of mulberry leaf on the cellular mechanism of the organism. The diabetic group treated with insulin- a well-known drug for controlling the blood glucose levels also serves as a reference point in the experiment. From the experimental data in Table 2, it observed that over a period of 21 days, the body weight of the sampled mice has decreased from 26.6g to 19.7g. While that of the normal mice remained almost the same at about 25g. In the insulin-treated normal mice group, the body weight gain has shown improvement from 25.6g to 26.2g. This is of course the effect of insulin in controlling the blood glucose level and 27g. (35%) in case of MR-1. Diabetes Mellitus (DM) is a known chronic disorder caused by overproduction of excessive hepatic glycogenolysis and gluconeogenesis, resulting in decreased body weight and inept utilization of glucose by tissues[19].

1.6.2 Effect on Fasting Blood Glucose (FBG) levels:

The present study has clearly indicated the process of creation of lot of stress inside the body with obstruction in gluconeogenesis pathway of liver and raising the glucose levels in blood serum due to induction of STZ drug. It is also found that treatment with mulberry leaf extract has been effectively significant ($p<0.05$) in lowering the high FBG, to a normal level within a period of three weeks. The diabetic group treated with insulin drug has also been found to be effective significantly ($p< 0.05$). It is found that the glucose level which was 525.2mg/dl in the first week has shot up to 566.3mg/dl at the third week in case of the Diabetic mice group (Table 3).If the reduction rate is found to be around 70% in just three weeks times, the treatment with mulberry extracts of selected variety should work phenomenally over a period of time. The ultimate performance over longer run will matter much and hence MR-1 variety also stood successful over the test. The variations could also be seen more vividly in the bar diagram (Fig. 2B). This study revealed that the mulberry leaf extracts will impact the pancreas for production of insulin and liver cells for removal of free radicals of tissue; hence it is more effective in hyperglycemia treatment. This observation supports the finding of [20], that administration of mulberry leaf of *Morus indica* decreases lipid peroxidation and helps reduction of hyperglycemia.

1.6.3 Catalase and G-6-PDH

The levels of Catalase and G-6-PDH as measured through the experiment after 21 days of duration are expressed in Table 4. The oxidative stress resulting in higher glucose levels in the blood is reflected in the diabetic mice group. The levels of catalase in the liver have shown increased value of 151.1mmol/mg. in diabetic mice group. In the normal category the value is 109.0mmol/mg. The insulin treatment has reduced the value of this variable to 116.9mmol/mg. which means that this score should not be higher in the healthy group. Therefore, with the treatment of mulberry leaf extract, the value of catalase has been brought down substantially with statistical significance at $p<0.05$ compared to control. The activity related to G6PDH is with a difference. Here, the mean value of the normal group is 417.2mmol/mg. and the corresponding score in respect of diabetic group is 178.92mmol/mg. This indicates that the mean values in respect of the mulberry treated groups must rise to the normal level of around 417.2mmol/mg. It is also seen that this value in respect of insulin-treated group has gone up to 342.02mmol/mg. The glutathione (GSH) redox system are connected by the G6PDH enzyme, which controls the GSH level is well known to play a key role in free radical and peroxide metabolism.

1.6.4 SGOT and SGPT

The data in respect of the said enzyme activities with respect to SGPT and SGOT under diabetic and treated mice groups is presented in Table 5 and depicted in Fig. 3B. Here the normal value in respect of SGPT is 29.2unit/ml. and that in respect of SGOT is 25.3unit/ml. Due to induction of diabetes the values have shot up to 68.3 and 55.6unit/ml respectively. The activity of the enzymes has increased in diabetic mice massively after induction of drug STZ. This is indicated through the pathogenic condition of mice. This increased enzyme activity is due to the effect of STZ on liver cells which has raptured the liver cell membrane, sheet hepatocytes cells and central vein. As a result, the SGOT and SGPT are released in high quantities from the liver into blood

serum and these high quantities caused stress and damaged the cell membrane. Now the need is to reduce the quantity of release of SGPT and SGOT. Mulberry consisting of phytochemical compounds have the potential to regulate this and contain the oxidative stress. The insulin treatment will be taking care of this issue. Thus, it has reduced the value of 68.3 and 55.6 to a level of 41.6 and 26.3unit/ml. respectively. Similar effects in various other medicinal plants have been reported by some group of scientist [21-23]. Therefore, Mulberry leaf extracts are also supposed to do the job of reduction of the levels of Enzyme activity.

1.6.5 Histopathological Variations

In the liver tissue of mice, injuries in the form of vacuolation and necrosis were mainly demonstrated in the peripheral zones of hepatic lobules. The pathological changes are extended to involve the central zones, and this might be explained by the type of blood circulation inside the hepatic lobule. Normally, the direction of blood flow proceeds from the periphery of the lobule toward the central vein, where the flow of blood, is centripetal. Blood percolates within the sinusoids to the central vein and is exposed to the activities of the hepatocytes around the sinusoids. Plasma flows freely through the sinusoidal wall into the sinusoidal spaces where it is exposed to the various activities of the hepatocytes and then flows back into the bloodstream described by [24]. Most of the injected STZ drug reached the liver through the portal vein and finally in the terminal portal venules in the portal tracts. Thus, the peripheral hepatocytes became exposed to a higher concentration of the STZ drug have quite effectively reduced the glucose levels and repaired the injuries of liver tissue, resulting in the mice regaining normalcy after 21 days. This is because of the mulberry leaf containing high antioxidant activity treating the oxidative free radicals and other associated problem by the synergetic action of MLE. The Overall findings of histopathological studies suggests that the MLEs are quite effective in improving the health status of tissue and maintain the enzymatic level inside the body system along with treating the cellular structure due to the antioxidant properties of the leaf setting right the oxidative free radicals. Similar observations were also reported in case of *Morus indica* and Morus alba by [25,26], respectively. In conclusion, both the solvents (methanol and ethanol) extracts of mulberry leaf showed concentration-dependent variable degree antioxidant activity under the both the assays (DPPH) methanol was found to be most efficient solvent for extraction of antioxidant MR-1 from mulberry leaves the related extracts exhibited the strongest antioxidant capacity in all the assay used. Further, MR-1 also proven to be extraordinary with anti-diabetic ingredients and thus would stay longer to benefit the human.

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CONFLICT OF INTEREST

The authors declare that there are no financial or other conflicts of interest associated with this work.

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