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Department of Biochemistry, Bharathidasan University Constituent College for Women, Orathanadu - 614 625, Tamil Nadu, India

<u>**Research Article**</u>

Effect of *Macrotyloma uniflorum* on free radicals and antioxidants in tissues of high fructose-fed rats

[1]Veerappan Ramanathan, [2]Malarvili Thekkumalai*

[1]Department of Biochemistry, Enathi Rajappa Arts and Science College, Pattukkottai, Tamilnadu, India

[2*]Bharathidasan University Constituent College for Women, Orathanadu, Tamilnadu, India

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Abstract

Feeding rats with high fructose, induces insulin resistance, hyperinsulinaemia, elevation of blood glucose level and impaired glucose tolerance. Oxidative stress plays a vital role in pathology associated with insulin resistance. The present study aimed to investigate the effects of *Macrotyloma uniflorum* (*M. uniflorum*) on the oxidant-antioxidant status in liver, kidney and heart of high fructose-fed diet (HFFD) rats. Male albino Wistar rats (160-180 g) were divided into six groups. Groups I and II received control diet (Group I served as normal control, group II received *M.uniflorum* (1000 mg/kg). Groups III-IV received HFFD (groups IV-VI received 250, 500 and 1000 mg/kg of *M. uniflorum* respectively). The HFFD fed rats showed increased levels of glucose, thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), lipid hydroperoxides (HP) and impaired antioxidant defense as evidenced by decreased in the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) glutathione reductase (GR) and the levels of reduced gluatathione (GSH). Treatment with *M.uniflorum* to the fructose-fed rats mitigated these alterations. *M. uniflorum* administration for 15 days decreased glucose levels; lipid peroxidation and restored the antioxidant potential. These findings support and strengthen the utility of *M. uniflorum* in the management of IR and associated pathology during diabetes.

Keywords: Antioxidants; fructose diet, insulin resistance, lipid peroxidation, liver, *M. uniflorum***.**

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies; in which chronic hyperglycemia is caused by defect or alterations in either the discharge or action of insulin results in disturbances in carbohydrate, fat and protein metabolism [1]. Insulin resistance (IR) is common with obesity and predisposes to a variety of diseases, including diabetes, dyslipidemia, hypertension and cardiovascular problems [2]. Increased consumption of fructose-enriched food products is associated with prevalence of metabolic disorders, including weight gain, IR and hyperlipidemia in both animals and humans [3,4]. IR represents a collection of disorders that include dyslipidaemia, obesity, impaired glucose tolerance and hypertension, and predisposes one to type 2 DM [5,6].

An increase in high fructose corn syrup, as well as total fructose, consumption over the past 10 to 20 years has been linked to a rise in obesity and metabolic disorders [7]. Fructose is rapidly absorbed from the intestine and delivered to the liver via the portal vein [8]. Increased fructose consumption can lead to increase in blood lipids [9], development of IR [10], increase in inflammatory biomarkers and oxidative stress, risk on development of obesity, and comorbidities such as hypertension and DM type II [11]. A diet high in fructose (> 60/100 g) induces IR in animals are fed a high dose of fructose are considered in forming a nutritional model for IR [12,13].

Several human diseases have been closely associated with oxidative stress [15], including aging [15], metabolic syndrome and diabetes [16]. Reactive oxygen species (ROS) cause damage of several macromolecules (proteins, lipids, DNA) and leading to oxidative stress. Antioxidants and other chemical bioactive compounds detoxify ROS and prevent from damage of the cellular macromolecules and organelles through various mechanisms [17]. Studies show that 3 weeks of fructose intake to normal rats is associated with a state of oxidative stress due to an imbalance between ROS production and antioxidant capacity [18], while others showed that chronic consumption of fructose may lead to an overwhelmed of endogenous antioxidants and, consequently, promoting change in redox state by increasing ROS production, promoting oxidative stress [19].

In the modern era also most of the people believe the plants and phytoconstituents are better choice to treat diseases than the allopathic drugs, even most of the drugs used in primitive medicine were instigated from plants [20,21]. The health benefits of horse gram are being recognized in the western world recently, but have been known for its ability to prevent and cure various diseases by Indian "Ayurvedic" system since centuries. Horsegram (*Macrotyloma uniflorum* (Lam.) Verdc) is an important rainfed minor pulse crop. It is a potential grain legume having excellent nutritional and remedial properties with better climate resilience to adapt harsh environmental conditions, grown almost all over the world including East and Northeast Africa, India, China, Philippines, Bhutan, Pakistan, Sri Lanka and Queensland in Australia [22,23]. The seeds have been used for the treatment of heart conditions, asthma, kidney stones, bronchitis, leukoderma, urinary discharges, hepatoprotective role and obesity [24,25]. Therefore the present study aimed to investigate the role of *M. uniflorum* on lipid peroxidation and antioxidant defense HFFD induced diabetic rats.

Materials and Methods

Animals: Thirty six male adult Wistar strain albino rats, weighing 160-180g were purchased from "Sri Venkateswara Enterprises", Bangalore, Karnataka, India. They were housed in clean sterile polypropylene cages under the constant environmental and nutritional conditions throughout the period of experiment. During the course of the experiments, the temperature was maintained between 22ºC ± 2ºC. The rats were fed on a standard pellet diet and HFFD during experimental period and water *ad libitum*. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Chemicals: Fructose, bovine serum albumin, G-6-P, γ-glutamyl paranitroaniline, nicotinamide adenine dinucleotide (NAD^+, NADH) nicotinamide adenine dinucleotide phosphate ($NADP^+$, NADPH), reduced glutathione, oxidized glutathione, adenosine triphosphate, adenosine monophosphate and 1,2,4-aminonaphthol sulphonic acid were obtained from Sigma Chemical Company, ST. Louis, MO, USA. All other chemicals and reagents used were of highest purity and of analytical grade marketed by Glaxo Laboratories, Mumbai, SD Fine Chemicals, Mumbai and Sisco Research Laboratories, Pvt. Ltd., India.

After one week of acclimatization the animals were divided into two batches. One batch was provided with a control diet containing starch as the source of carbohydrate (groups I and II) and the other was fed a fructose-enriched diet for 45 days (Groups III-VI). Different composition of diet (Table. 1) given to all the rats for 45 days followed by *M. uniflorum* was given orally for 15 days. Blood samples from all the groups of animals were collected from the tail vein on the 10th, 20th and 30th days and estimated glucose levels to ensure diabetic status.

Experimental Design: The rats were divided into six groups, each group consisting of six rats. *M.*

uniflorum seed powder dissolved in water and given to rats twice daily for a period of 15 days, after 45 days of control and HFFD.

Group I : Normal control rats (for 45 days)

Group II : Control rats treated with *M. uniflorum* seeds (1000 mg/kg) twice daily for 15 days

Group III: High Fructose fed rats (>60% fructose for 45 days)

Group IV : HFFD + *M. uniflorum* seeds (250 mg/kg) twice daily for 15 days

Group V : HFFD + *M. uniflorum* seeds (500 mg/kg) twice daily for 15 days

Group VI: HFFD + *M. uniflorum* seeds (1000 mg/kg) twice daily for 15 days

Collection of Samples: At the end of the 45th day, all the rats were fasted overnight and sacrificed by cervical decapitation under mild ether anesthesia. Blood was collected in a tube with heparin and plasma was separated by centrifugation. The liver, heart and kidney tissues were immediately removed and washed in ice-cold saline to remove blood. The tissues were sliced and homogenized in 0.1 M Tris-HCl buffer (pH 7.0). The homogenates were centrifuged at 1000 rpm for 10 min at 4ºC in a cold centrifuge.

Analytical Method: An estimation of plasma glucose level was assayed by the method of Sasaki *et al*., [26]. The levels of TBARS [27] and Lipid hydroperoxides (HP) [28] were determined in tissue homogenates. Conjugated dienes (CD) was measured by the method of Rao and Recknagel [29]. The clear supernatant thus obtained was used for the assay of SOD[30], catalase [31], glutathione peroxidase [32], reduced glutathione (GSH) [33] and glutathione reductase [34].

Statistical analysis: Values or mean ± SD for six rats in the each group and statistically significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by DMRT values of P <0.05 was considered to be significant. Statistical Package for Social Studies (SPSS Inc., Chicago, IL)) 19.0 versions were used for this analysis.

Table I: Composition of diets fed to rats for the determination of insulin resistance

Ingredient (g/100 g)	Control diet	High-fructose diet
Corn starch	60	-
Fructose	-	60
Casein	20	20
Methionine	0.7	0.7
Groundnut oil	5	5
Wheat bran	10.6	10.6
Salt mixture†	3.5	3.5
Vitamin mixture‡	0.2	0.2

†Composition of the mineral mix (g/kg): $MgSO_4.7H_2O$, 30.5; NaCl, 65.2; KCl, 105.7; KH_2PO_4, 200.2; $3MgCO_3.Mg(OH)_2.3H_2O$, 38.8; $FeC_6H_5O_7.5H_2O$, 40.0; $CaCO_3$, 512.4; KI, 0.8; NaF, 0.9; $CuSO_4.5H_2O$, 1.4; $MnSO_4$, 0.4; and $CONH_3$, 0.05.

‡One kilogram of vitamin mix contained: thiamine mononitrate, 3 g; riboflavin, 3 g; pyridoxine HCl, 3.5; nicotinamide, 15 g; d-calcium pantothenate, 8 g; folic acid, 1 g; d-biotin, 0.1 g; cyanocobalamin, 5 mg; vitamin A acetate, 0.6 g; α-tocopherol acetate, 25 g; and choline chloride, 10 g.

Results

Table 2 shows the level of plasma glucose in control and experimental rats. The increased level of plasma glucose in fructose-fed rats as compared to control rats has been observed. Administration of *M. uniflorum* (250, 500 and 1000 mg/kg respectively) daily for a period of last 15 days were significantly decreased the level of glucose in HFFD rats as compared to group diabetic rats.

Table 3 presents the levels of TBARS, CD and HP in plasma, liver, kidney and heart of normal and experimental rats. Rats induced with HFFD, showed a significant increase in the levels of TBARS, Conjugated dienes (CD) and Lipid hydroperoxides (HP) in plasma and tissues (liver, kidney and heart) when compared to normal control rats. Treatment with *M. uniflorum* (250, 500 and 1000 mg/kg) to HFFD-induced rats significantly decreased the levels of TBARS, CD and HP in plasma and the heart when compared with HFFD-alone induced rats.

The activities of SOD, CAT, GPx, in the liver, kidney and heart were significantly lowered in fructose fed rats than normal control rats shown in Table 4. In High fructose rats treated with *M. uniflorum*, the enzymatic antioxidants were significantly increased as compared to high fructose fed rats.

Table 5 illustrates the effect of *M. uniflorum* on the activities of Glutatione reductase (GR) and the levels of GSH in plasma, liver, kidney and heart in normal and HFFD-induced rats. Rats induced with HFFD, showed a significant decrease in the activity of GR and the levels of GSH on comparison with normal control rats. Oral administration of *M. uniflorum* (250, 500 and 1000 mg/kg) to HFFD-induced rats significantly increased the activities of these antioxidant enzymes.

Table 2. Effect of *M. uniflorum* on glucose in plasma of control and Experimental rats

Parameters	Control	Control (1000 mg/ kg)	HFFD control	HFFD+MUF (250 mg/ kg)	HFFD+MUF (500 mg/ kg)	HFFD+MUF (1000 mg/ kg)
Glucose (mg/dL)	84.7±5.26[a]	82.0±6.32 [a]	214.8±11.3 [b]	128.1±6.36[c]	110.8±8.37 [d]	95.7±5.05 [d]

Each value is mean ± S.D. for six rats in each group. Values not sharing a common superscripts (a, b, c and d) differ significantly at $p < 0.05$ (DMRT).

Table 3. Effect of *M. uniflorum* on TBARS, CD and HP in plasma and tissues of control and Experimental rats

Parameters	Control	Control (1000 mg/ kg)	HFFD control	HFFD+MUF (250 mg/ kg)	HFFD+MUF (500 mg/ kg)	HFFD+MUF (1000 mg/ kg)
TBARS						
Plasma (m moles/L)	0.71±0.06[a]	0.70±0.05[a]	2.49±0.21[b]	1.65±0.06[c]	0.95±0.06[d]	0.81±0.05[d]
Liver	1.48± 0.12 [a]	1.42±0.11[a]	2.47 ±0.21[b]	1.63±0.14[c]	1.78±0.14[d]	1.61±0.12[d]
kidney	1.70±0.16[a]	1.68±0.14[a]	2.32±0.21[b]	1.94±0.15[c]	1.82±0.15[d]	1.78±0.13[d]
Heart	0.99±0.07[a]	0.96±0.07[a]	1.59±0.17[b]	1.23±0.07[c]	1.17±0.07[d]	1.11±0.06[d]
CD (A 233)						
Plasma (m moles/L)	0.92±0.08[a]	0.89±0.07 [a]	1.42±0.11[b]	1.23±0.11[c]	1.12±0.11[d]	1.02±0.06[d]
Liver	0.68±0.06[a]	0.67±0.06[a]	0.91±0.07[b]	0.81±0.06[c]	0.76±0.06[d]	0.74±0.05[d]
kidney	0.64±0.05[a]	0.63±0.06[a]	0.88±0.07[b]	0.78±0.06[c]	0.72±0.06[d]	0.66±0.06[a]
Heart	0.55 ±0.04[a]	0.54 ±0.04[a]	0.74 ±0.05[b]	0.67 ± 0.05[b]	0.63 ± 0.05[d]	0.57 ± 0.05[a]
HP						
Plasma (m moles/L)	0.96±0.08[a]	0.94±0.08[a]	2.84±0.21[b]	1.59±0.10[c]	1.24±0.10[d]	1.18±0.08[a]
Liver	1.51±0.19[a]	1.50±0.12[a]	2.18±0.21[b]	1.79±0.12[b]	1.65±0.12[c]	1.63±0.12[d]
kidney	1.69±0.16[a]	1.68±0.15[a]	2.21±0.21[b]	1.88±0.14[c]	1.84±0.14[d]	1.78±0.12[d]
Heart	1.26±.0.11[a]	1.25±0.02[a]	1.86±0.02[b]	1.52 ±0.12[c]	1.43 ±0.15[d]	1.38 ±0.12[d]

Each value is mean ± S.D. for six rats in each group. Values not sharing a common superscripts (a, b, c and d) differ significantly at $p < 0.05$ (DMRT). Tissue TBARS: μmol/mg protein; Tissue HP: μmol/mg protein;

Table 4. Activities of antioxidant enzymes in the liver, kidney and heart of control and experimental animals.

Parameters	Control	Control (1000 mg/ kg)	HFFD control	HFFD+MUF (250 mg/ kg)	HFFD+MUF (500 mg/ kg)	HFFD+MUF (1000 mg/ kg)
SOD (Units) Liver	3.98±0.32[a]	3.99±0.31[a]	2.54±0.25[b]	3.23±0.32[c]	3.75±0.31[d]	3.86±0.31[a]
kidney	4.05±0.41[a]	4.06±0.38[a]	3.11±0.29[b]	3.68±0.31[c]	3.90±0.39[d]	3.9±0.39[a]
Heart	3.64 ± 0.31 [a]	3.69 ±0.31 [a]	2.98 ±0.22 [b]	3.52 ± 0.34 [c]	3.58 ± 0.31 [d]	3.60 ± 0.31 [a]
CAT Liver	54.21±4.56[a]	55.1±4.39[a]	34.9±2.91[b]	46.15±4.14[c]	49.28±5.14[d]	52.28±5.14[a]
Kidney	58.1±4.81[a]	59.6±4.87[a]	39.99±3.6[b]	49.10±3.89[c]	53.48±4.91[d]	56.48±4.91[a]
Heart	53.15 ± 5.1[a]	54.1 ± 4.9[a]	40.8 ±4.31[b]	47.55 ± 4.5[c]	49.6 ± 4.9[d]	51.6 ± 4.9[a]
GPx Liver	6.09 ± 0.58 [a]	6.10±0.46 [a]	4.48 ±0.39 [b]	5.92±0.54 [c]	6.01±0.51 [d]	6.07±0.51 [a]
Kidney	5.24±0.57 [a]	5.25±0.49 [a]	4.07±0.35 [b]	5.12±0.41 [c]	5.16±0.48 [d]	5.22±0.48 [a]
Heart	5.18 ± 0.48 [a]	5.21 ± 0.5 [a]	4.12 ± 0.3 [b]	5.02 ± 0.41 [c]	5.09 ± 0.49 [d]	5.14 ± 0.49 [a]

Each value is mean ± S.D. for six rats in each group. Values not sharing a common superscripts (a, b, c and d) differ significantly at p < 0.05 DMRT). SOD: Amount of enzyme, which gave 50% inhibition of NBT reduction/mg protein; CAT: µmol of H_2O_2 consumed/min/mg protein; GPx: µmol of GSH consumed/min/mg protein

Table 5. Activities of antioxidant enzymes GSH and GR in the liver, kidney and heart of control and experimental animals.

Parameters	Control	Control (1000 mg/ kg)	HFFD control	HFFD+MUF (250 mg/ kg)	HFFD+MUF (500 mg/ kg)	HFFD+MUF (1000 mg/ kg)
GSH liver	162.1±15.7[a]	163.4±15.9[a]	103.2±9.7[b]	138.1±9.8[c]	152.3±11.5[d]	155.3±11.5[d]
kidney	106.4±9.87 [a]	107.8±9.2 [a]	64.1±5.2 [b]	85.4±7.9 [c]	97.6±9.67 [d]	101.6±9.67 [a]
Heart	104±9.1[a]	105.8±8.8[a]	59.1±3.6[b]	92.16±8.5[c]	88.1±9.5[d]	96.1±9.5[d]
GR Liver	22.8±1.89[a]	23.74±2.8[a]	11.32±1.0[b]	18.1±1.5[c]	1.96±2.4[d]	21.4±2.4[a]
Kidney	24.5±1.9[a]	25.4±0.2[a]	16.41±1.7[b]	19.21±2.4[c]	21.65±2.2[d]	23.65±2.2[a]
Heart	19.8±2.4[a]	20.0±0.30[a]	13.8±1.2[b]	17.5±1.6[c]	18.1±1.7[d]	18.9±1.7[a]

Each value is mean ± S.D. for six rats in each group; Values not sharing a common superscripts (a, b, c and d) differ significantly at p < 0.05 DMRT). GSH: µmol/mg protein; GR: µmoles/h/mg protein

For all the parameters studied, administration of *M. uniflorum* (1000 mg/kg) to normal rats for a period of 15 days showed a minor effect but it was not statistically significant. Treatment with *M. uniflorum* (250, 500 and 1000 mg/kg) to HFFD rats significantly minimized the alterations in all the parameters studied in a dose dependent manner. *M. uniflorum* 1000 gm/kg showed a better effect than the other two doses (250 and 500 mg/kg) in HFFD rats.

Discussion

Consumption of HFFD promotes the development of pathological characteristics, which is associated with metabolic syndrome. It is an accepted fact that, food ingredient high in fructose content potentially increases oxidative stress [35]. IR is not only an early and major feature in development of non-insulin-dependent DM, but also associated with hyperlipidemia, hypertension, obesity, enhanced oxidative stress, endothelial dysfunction and cardiovascular disease [36]. It has been shown that rats fed with 60% fructose diet for 60 days exhibit higher insulin and glucose levels [37].

In our study, the level of plasma glucose significantly increased in HFFD rats (group 3) as compared to control rats (group 1). High levels of dietary fructose and severe hyperglycemia may have interactive effects, which contribute to the progression and development of pathology. HFFD can certainly cause IR. Lavau *et al*.,[38] pointed that high-fructose diet could lower the activity of the intracellular enzymes associated with fatty acid synthesis and decrease the intra cellular capacity to utilize glucose, which in turn resulted in a blunted glucose metabolism response to insulin. Treatment with *M. uniflorum* twice daily for 15 days to HFFD rats significantly reduced the glucose levels, could be due to positively alter the enzymes involved in glucose metabolism.

In our study, the lipid peroxidation markers such as TBARS, CD, and HP were significantly increased in the plasma, liver, kidney and heart of HFFD rats. Enhanced lipid peroxidation in fructose-fed rats could be associated with high circulating glucose, which enhances free radical production from glucose autoxidation and protein glycation. Fructose feeding can induce free radical formation by down regulation of HMP shunt enzymes that generate reduced environment in the form of NADPH and NADH [39]. The measurement of TBARS contents are an index of lipid peroxidation. Very high positive correlation show that fasting glucose and insulin levels were significant determinants of TBARS levels, suggesting a role of IR increased lipid peroxidation. CD is also linked to several steps of lipid peroxide degeneration. Around 30-35% of lipid peroxidation is actually detected by diene measurements[40]. LHP oxidize ferrous ion to ferric ion, which depends not only on the rate of initiation of peroxidation but also their decomposition to other products. Although the TBA test and CD measurement are very non-specific, they can offer an empirical window on the complex process of lipid peroxidation [41].

Moreover administration of the crude powders of *M. uniflourm* to the HFFD rats reduced the oxidative stress and enhanced the insulin sensitivity. The phytochemical constituents of *M. uniflourm* were also established for their potent effect to inhibit/decrease the generation of ROS by reducing the oxidative stress. From the results of the present study, it could be observed that supplementation of *M. uniflourm* to HFFD rats had effectively decreased the formation of O^2.- in the liver, kidney as well as in heart which is reflected through scavenging of lipid peroxidative products like TBARS, CD, and HP.

The decreased activities of SOD, CAT in the liver, kidney and heart were shown in HFFD rats. High levels of free radicals and the simultaneous decline in endogenous antioxidants can lead to damage of cellular organelles, and development of IR [42]. ROS can themselves reduce the activity of antioxidant enzymes such as SOD, CAT and GSH. SOD is a ubiquitous chain breaking antioxidant and a metalloprotein, plays an important protective role against oxidative damage induced by ROS. CAT detoxifies hydrogen peroxide in to molecular oxygen and water, due to this conversion the toxic hydrogen peroxide is converted in to non-toxic one. Moreover this herbal powders may serve as an effective free radical scavenger and/or neutralizes the free radicals and increase the activities of SOD and CAT, which is due to the phytoactive constituents (polyphenols) of *M. uniflourm* seed.

GSH is an important reducing agent in the cell, where it protects against the toxic effects of free radicals, peroxides and other toxic components. The increase in ROS and the decrease of GR activity lead to depletion of GSH concentration [43]. The decreased GSH levels in HFFD could be due to increased utilization to trap free radicals, and/or decreased regeneration as evident with the lower activity of GR [44]. GSH, being a potent free radical scavenger, is also a cofactor of GPx and plays the essential role in the antioxidant defense of the body. The decrease in the activity of hepatic GPx might also be due to increased turnover of the enzyme. GR is a protective mechanism to reduce the peroxide toxicity where in GSSG formed is actively reduced to GSH [45].

Glutathione levels and activities of glutathione dependent enzymes were increased in rats treated with *M. uniflorum*. GPx and GR are essential for maintaining the constant ratio of GSH to GSSG in cells. Decreased glutathione levels on fructose fed rats may be due to increased utilization for protecting sulfhydryl group of proteins from lipid peroxides. *M. uniflorum* feeding restores the glutathione level and increased the activities of GPx and GR. In our studies administration of *M. uniflorum* extract to rats with high-fat diet induced oxidative stress, showed improvement in antioxidant enzymes such as SOD, CAT. Hence, all the medicinal properties of *M. uniflorum* could be responsible for reducing glucose, lipid peroxidation and increasing antioxidant levels in HFFD rats.

Seeds of *M. uniflorum* contain phenolic compounds like, 3,4-dihydroxy benzoic acid, vanillic acid, caffeic acid, p-cumaric acid, ferulic acid, chlorogenic acid, syringic acid and sinapic acid. Also contain flavonoids and tannins like, quercetin, kaempferol and myricetin, stigmasterol, β-sitosterol, anthocyanidins and saponins. These active constituents possess strong glucose lowering, free radical scavenging, and antioxidant properties, which suppress the ROS mediated oxidative stress in HFFD rats. In addition, the seeds have the ability to slow down the carbohydrate digestion and insulin resistance by inhibiting protein tyrosine phosphatase 1 beta enzyme [46].

Conclusion

Our results show that HFFD rats results in development of oxidative stress in plasma, liver, kidney and heart. This oxidative stress may play a role in pathology associated with fructose feeding such as IR. In uncontrolled diabetes, oxidative stress results from increased free radical production and depletion of antioxidants like SOD, CAT, GPx, GR and GSH. Administration of *M. uniflorum* significantly reduced the free radical mediated lipid peroxidation, preserved the activities of antioxidant enzymes and maintained the levels of non-enzymic antioxidant. The finding of the present study shows that utility of *M. uniflorum* could be considered as therapeutic tool for the management of diabetic complications in which induction of oxidative stress is the major contributing mechanism.

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