Madhuca-longifolia-hydro-ethanolic-fraction reverses mitochondrial dysfunction and modulates selective GLUT expression in diabetic mice fed with high-fat-diet

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Abstract

Metabolic disorders are characterized by chronic mild inflammation that elevates circulatory inflammatory markers. The proposed hypothesis behind this includes the occurrence of mitochondrial dysfunction, oxidative stress, and hypoxia due to a high-fat diet (HFD). In the present study, the effect of madhuca-longifolia-hydro-ethanolic-fraction (MLHEF) on HFD-induced obesity and diabetes through mitochondrial action and selective GLUT expression was studied. In this study, it was observed that chronic administration of HFD (50% of diet) aggravated metabolic problems by reducing ATP production, imbalanced oxidative stress, and altered GLUT expression. Chronic HFD administration significantly reduced ($p < 0.001$) the superoxide dismutase (SOD) and catalase (CAT) levels, as well as elevated the liver function markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT). MLHEF administration reduces oxidative stress. HFD administration also decreased the ATP/ADP ratio owing to mitochondrial dysfunction and elevated lactose dehydrogenase (LDH) levels. This imbalance leads to altered GLUT expression in hepatocytes, skeletal muscles, and adipose tissue. HFD significantly ($p < 0.001$) upregulated in the GLUT 1 and 3 expression while significant downregulation ($p < 0.001$) was observed in GLUT 2 and 4 expression in the liver, skeletal muscles, and adipose tissue. Administration of MLHEF significantly ($p < 0.001$) reduced LDH levels and mitochondrial dysfunction. The imbalance in GLUT levels was significantly reversed to maintain GLUT expression in tissues following MLHEF administration.

Introduction

Metabolic syndrome is an agglomeration of disorders like obesity, diabetes and cardiovascular diseases. Increase in obesity level commonly enhances the risk of metabolic abnormalities and diabetes. Obesity is characterized as chronic mild inflammation which elevates the circulatory inflammatory markers like tumour necrosis factor (TNF-α),C- reactive protein (CRP-1), interleukins (IL 4, IL6) and plasminogen activating factor (PAI-1) [1, 2]. These changes are viewed as the primary factor in the development and progression of metabolic disorders. The proposed hypothesis behind the development and progression of metabolic disorders includes, endoplasmic stress (ER stress), mitochondrial dysfunction, oxidative stress and hypoxia [3-6]. Increase in lipid availability in obesity leads to altered fatty acid metabolism, mitochondrial dysfunctions and GLUT (glucose transporter) expression [7-9]. According to endocrine mechanism explained by several studies fatty acids, adipokines and leptins are the major causes of insulin resistance in which pivotal role is also played by released inflammatory markers (TNF-α, CRP-1, IL 4, IL6, PAI-1, SOCS proteins). Fatty acids and its metabolites also activates protein kinase C, Jun kinase, NF kB which acts by impairing insulin signalling [7, 8, 10]. Cell intrinsic mechanism explains that, increased free fatty acids (FFA) and lipids cause ectopic fat accumulation in liver and muscle as triglycerides which leads to insulin resistance. This is achieved by activation of reactive oxygen species (ROS), mitochondrial dysfunction, ER stress and hypoxia [11].

Hypoxia and ROS damages and alters the facilitative GLUT transporter (suppression in expression of GLUT 2 and 4 while increased expression of GLUT 1 and 3) responsible for glucose uptake across the

cells. This also elevates the LDH level which finally causes suppressed insulin sensitivity and induces insulin resistance [7, 11]. Excess production of ROS and oxidative stress due deposition of triglyceride causes mitochondrial dysfunction in tissues. Thus decrement in mitochondrial oxidative capacity and ATP/ADP ratio in liver, skeletal muscles and white adipose tissue, also mediates selective class 1 GLUT transporter expression in turn causing insulin resistance [12-14]. In case of HFD induced metabolic disorder, Role of GLUT 1, 3 and 4 in liver, GLUT 1, 2 and 3 in muscles and Glut 1,2 and 3 in white adipose tissue are yet to be explored and exhaustive study is needed. In this study expression of these Glucotransporter is also observed in liver, skeletal muscles and adipose tissue. LDH and mitochondrial dysfunction help in establishing the link between the occurrences of metabolic stress due to HFD.

In recent years use of traditional medicine for therapeutic approach has been raised immensely because of its wide acceptance and lower side effects. Presence of different bioactive constituents are responsible for its role in oxidative homeostasis and can be used for the treatment and prevention of diseases like diabetes, cardiovascular and renal related disorders [15-17]. Bark, flowers and seed parts of *M. longifolia* has shown the effect on the treatment of epilepsy, diabetes, inflammation, bronchitis, ulcer and other diseases [18-21]. Leaves of this plant has antioxidant properties and are used in Cushing's disease and bronchitis [22, 23]. However no mechanistic approach of *M. longifolia* has been reported on its action against the high fat diet induced metabolic disorder via GLUT transporters, mitochondrial dysfunction and LDH. In the present work our aim was to elucidate the possible mechanism of action of hydroethanolic fraction of *M. longifolia* leaves against the metabolic disorder.

Results and discussion

LCMS analysis of MLHEF suggest following tentative major compounds:

LCMS analysis of *M.longifolia* hydro ethanolic extract and structure of tentative compounds. LCMS finger printing analysis of MLHEF shows the presence of high amount of sugar moiety i.e D Arabinose (RT 1.08), and flavonoids i.e myricetrin (RT 7.65), quercetin (RT 10.82). 10- Shogaol was also identified at RT 8.30. Two more compound were partially characterized 3-O- 28-O-(R-L-rhamnopyranosyl-(1f3)-β-D-xylopyranosyl-(f4) [R-L-rhamnopyranosyl-(1f3)]-R-L-rham-nopyranosyl-(1f2)-â-D xylopyranosyl) protobassic acid and β-D-glucopyranosyl protobassic acid at RT 9.61 and 11.90 respectively (shown in figure.1 and table.1).

Table 1
LCMS analysis MLHEF suggest following tentative compounds.

S.no	RT	Tentative Compound	Mol. Wt. (M+)	References
1	1.08	D Arabinose	151.3	[24]
2	7.65	Myricetrin	319.3	[25]
3	8.30	10- Shogaol	333.3	[26]
4	9.61	3-O- 28-O-(R-L-rhamnopyranosyl-(1f3)-β-D-xylopyranosyl-(f4)[R-L-rhamnopyranosyl-(1f3)]-R-L-rham-nopyranosyl-(1f2)-â-D xylopyranosyl) protobassic acid,	1367.9	[27]
5	10.82	Quercetin	302.6	[25]
6	11.90	β-D-glucopyranosyl protobassic acid	684.7	[27]

Table 1

LCMS analysis of *M.longifolia* hydro ethanolic extract and structure of tentative compounds. LCMS finger printing analysis of MLHEF shows the presence of high amount of sugar moiety i.e D Arabinose (RT 1.08), and flavonoids i.e myricetrin (RT 7.65), quercetin (RT 10.82). 10- Shogaol was also identified at RT 8.30. Two more compound were partially characterized3-O-28-O-(R-L-rhamnopyranosyl-(1f3)-β-D-xylopyranosyl-(f4)[R-L rhamnopyranosyl-(1f3)]-R-L-rham-nopyranosyl-(1f2)-â-D xylopyranosyl) protobassic acid and β-D-glucopyranosyl protobassic acid at RT 9.61 and 11.90 respectively.

MLHEF attenuates physiological changes in diabetes mice.

The MLHEF treatment minimizes the hepatic oxidative stress elevated due to administration of HFD + STZ and is expressed in Table 2. There was significant decrease ($p < 0.001$) whereby 50% reduction was observed in SOD and CAT level while increase in MDA level in high fat fed group and HFD + STZ group was observed as compared to normal control group. Treatment with MLHEF significantly reduced ($p < 0.001$) the hepatic oxidative stress in liver.

Table 2

Effect of MLHEF treatment on liver oxidative stress profile altered due to HFD and HFD + STZ. All values are mean ± SD; n = 6; [a]p≤0.001 compared to control, [b]p≤0.001 compared to obesity control, [c]p≤0.001 compared to diabetes control [One-way ANOVA followed by Tukey's Multiple Comparison].

S.no	SOD (U/mg protein)	CAT (nmoles of H_2O_2 consumed /min/mg protein)	MDA (nmol/g)	AST (IU/L)	ALT (IU/L)
Normal Control	40.03 ± 1.89	45.78 ± 3.01	13.11 ± 1.46	16.45 ± 1.11	26.22 ± 3.21
Obesity Control	20.11 ± 1.32[a]	25.45 ± 2.33[a]	24.45 ± 2.44[a]	39.65 ± 1.75[a]	53.56 ± 3.16[a]
Diabetic Control	24.42 ± 2.01[a]	23.97 ± 1.98[a]	26.89 ± 2.16[a]	45.66 ± 1.36[a]	57.14 ± 2.89[a]
Treated (400 mg/kg)	37.45 ± 1.41[b,c]	37.89 ± 2.13[b,c]	16.87 ± 1.84[b,c]	23.47 ± 1.41[b,c]	35.37 ± 2.13[b,c]
Standard	41.84 ± 3.10	43.86 ± 3.11	14.77 ± 3.11	20.54 ± 2.66	30.42 ± 3.42

HFD and HFD + STZ caused oxidative imbalance by altering the oxidising enzymes. In order to examine the chronic HFD associated hepatic injury was determined by the activity of liver enzymes such as AST and ALT. There was twofold elevation ($p < 0.01$) in AST and ALT level in HFD and HFD + STZ group. There was significant decrease ($p < 0.01$) in AST and ALT level was observed in MLHEF treatment group (see supplementary material Table 1).

MLHEF administration mitigates GLUT (1–4) expression in liver, adipose tissue, and skeletal muscles.

In liver (Fig. 2), GLUT 2 and GLUT4 protein content was observed to be initially lower in obesity control and diabetic control as compared to normal control. MLHEF (400 mg/kg) treatment reprogramed the GLUT 2 and GLUT 4 protein expression in diabetic group towards the balanced glucotransporter protein expression. On the other hand GLUT 1 and GLUT 3 protein was found to be significantly increased ($p < 0.001$) in disease control groups (obesity control and diabetic control) as compared to normal control group which was further reduced by the MLHEF treatment (400 mg/kg).

In skeletal muscle tissue (Fig. 2), GLUT 2 and GLUT4 protein expression was shown to be lesser in obesity control and diabetic control as compared to normal control. MLHEF (400 mg/kg) treatment modulated the GLUT 2 and GLUT 4 protein expression in diabetic group towards the balanced gluco transporter protein expression in skeletal muscle. However, GLUT 1 and GLUT 3 protein expression was found to be elevated in disease control groups (obesity control and diabetic control) ascompared to normal control group which was reprogrammed by the MLHEF treatment to diabetic mice.

In adipose tissue (Fig. 2), GLUT 2 and GLUT4 protein content was shown to be reduced in obesity control and diabetic control as compared to normal control. After the treatment with MLHEF (400 mg/kg), the GLUT 2 and GLUT 4 protein expression were upregulated in diabetic animals towards the balanced glucose transporter protein expression. Contrarily it was found that GLUT 1 and GLUT 3 protein was found be significantly downregulated ($p < 0.001$) in disease control groups (obesity control and diabetic control) as compared to normal control group and was restored by the MLHEF treatment to diabetic group

In this study, the effect on prolonged treatment with MLHEF on Class 1 GLUT transporter (GLUT 1, GLUT2 GLUT 3, and GLUT 4) protein expression in the liver, skeletal muscles and adipose tissue in normal control mice, obesity control, type 2 diabetic control and MLHEF treated group were studied. In liver, skeletal muscles and adipose tissue (Fig. 2), GLUT 2 and GLUT4 protein content was shown to be initially lower in obesity control and diabetic control as compared to normal control. MLHEF (400mg/kg) treatment induced the GLUT 2 and GLUT 4 protein expression in diabetic group towards the balanced glucose transporter protein expression. While GLUT 1 and GLUT 3 protein was found to be increase in disease control group (obesity control and diabetic control) as compared to normal control group which was reduced by the MLHEF treatment (400mg/kg).

MLHEF administration mitigates LDH level in liver, adipose tissue, and skeletal muscles.

Lactate a cytosolic product of glycolytic pathway, is considered as a modulator of energy homeostasis. Figure 3 and Table 3 illustrates the effect of MLHEF treatment on HFD and HFD + STZ induced alteration in LDH activity in different organs (a) liver, (b) white adipose tissue and (c) skeletal muscles, respectively. One-way ANOVA showed that there were significant differences in liver ($p < 0.05$), skeletal muscle ($p < 0.05$), and white adipose tissue ($p < 0.05$) activity among the groups. The HFD and HFD + STZ significantly reduced the respiratory enzyme activities in liver tissues compared to control animals. MLHEF (400 mg/kg) attenuated HFD induced decrease in the activity of LDH activity in liver, skeletal muscle and white adipose tissue as compared to disease control (Obesity control and diabetic control) groups.

MLHEF administration restores mitochondrial complex activity in diabetes mice.

Figure 4 illustrates the effect of MLHEF treatment on HFD and HFD + STZ induced alteration in the mitochondrial complex-I (a), mitochondrial complex-II (b), mitochondrial complex-IV (c), and mitochondrial complex-V (d) activities of liver tissue. One-way ANOVA showed that there were significant difference in complex-I ($p<0.05$), complex-II ($p<0.05$), complex-IV($p<0.05$), and complex-V ($p<0.05$) activity among the groups. The HFD and HFD + STZ significantly reduced the respiratory enzyme activities in liver tissues compared to control animals. MLHEF attenuated HFD induced decrease in the activity of complex-I, II, IV, and complex-V in liver tissues as compared to disease control (Obesity control and diabetic control) groups.

Figure 4

Describes the effect of MLHEF treatment on HFD and HFD + STZ induce alteration in the mitochondrial complexes- I(a), II (b), IV (c), and V(d) activities of liver tissue. One way ANOVA analysis indicated the significant difference in complex I ($NADH^{+}$) ($F(4, 24) = 1.97$, $p < 0.001$), complex II (SDH) ($F(4, 24) = 18.18$, $p < 0.001$), complex-IV ($F(4, 24) = 23.45$, $p < 0.001$), and complex-V ($F(4, 24) = 9.8$, $p < 0.001$) activity. The HFD and HFD + STZ significantly decreased the activity of mitochondrial respiratory enzymes as compared to normal control. MLHEF reversed the HFD associated decrease in the activity of mitochondrial complexes in liver tissues as compared to disease control (Obesity control and diabetic control) group mice. All values are mean ± SD; n = 6; $^{a}p \leq 0.001$ compared to control, $^{b}p \leq 0.001$ compared to obesity control, $^{c}p \leq 0.001$ compared to diabetes control [One-way ANOVA followed by Tukey's Multiple Comparison].

Discussion

LCMS analysis of MLHEF showed the presence of some biologically active compounds which exhibits many pharmacological properties like anti-obesity, anti-diabetic and activity against metabolic disorder. Compounds like myricetrin and quercetin has already been established as drug for metabolic disorder like obesity, diabetes and cardiovascular diseases and also as hepato-protectant [28–31]. While 10- Shogaol has been proved scientifically as anti-oxidant and anti-inflammatory agent [32, 33]. Two more compounds were partially characterized through LCMS. However further studies pertaining to their isolation and charazterization needs to be performed.

Fat induced hepatic injury is triggered due to fatty acid overconsumption leading to suppressed hepatic ability to fat breakdown. Triglycerides accumulation is considered as crucial factor in non-alcoholic fatty liver disease (NAFLD) occurrence. On administration of HFD prevalence of oxidative stress is observed in hepatocytes [34, 35]. Which is confirmed by quantifying the oxidative markers SOD, MDA, CAT while change in hepatic function is evaluated by the level of AST and ALT. HFD on high consumption causes elevated ALT, triglycerides and cholesterol thus decrease in AST / ALT ratio. AST/ALT ratio was found to be less than 1, suggesting that hepatic injury is occurred due to HFD as value greater than 2 suggest alcoholic hepatic injury [36–38]. Administration of MLHEF significantly reduced the ALT and AST level thus reversing the effect of HFD. Previous reports also suggest the *M.longifolia* exhibits hepatoprotective activity. In this study MLHEF also preserved the normal hepatic architecture. The excess lipid burden on hepatocytes due to HFD leads to excess ROS generation leading of mitochondrial dysfunction. Along with this FFA also induces insulin resistance which also triggers the release of ROS and proinflammatory markers [39, 40]. The elevated oxidative stress marker SOD, MDA, CAT was also observed to be normalised on administration of MLHEF.

Mitochondria plays important role in maintenance of ATP supply (OXPHOS pathway), ROS generation and cell apoptosis. Prolong HFD administration causes reduction in mitochondrial fatty acid oxidation in hepatocytes, leading to FFA accumulation and elevation in *de novo* liver lipogenesis [41, 42]. This results in altered insulin signalling and reduced insulin stimulation along with elevated liver gluconeogenesis. “Two hit” model is accepted pathophysiological model for HFD induced hepatic disease. First hit suggest

initiation of accumulation of TAG and FFA in liver resulting in change in influx and oxidation of fatty acids. This triggers the second hit causing oxidative imbalance, liver insulin resistance, reduced hepatic ATP production (ATP/ADP ratio) and release of proinflammatory factors resulting in mitochondrial dysfunction [43, 44]. This mitochondrial dysfunction is associated with ROS excess and hypoxia. Hypoxia hampers OXPHOS pathway as there is decrease in O_2 level thus leading to decrease in ATP/ADP ratio thus resulting in cellular damage. Active Hypoxia inducible factor-1 (HIF-1) is considered as main factor in mitochondrial damage as it alters cell energy homeostasis by activation of anabolism and anaerobic glycolysis and also mitochondrial aerobic metabolism inhibition [11, 41, 45, 46]. Vail et al suggested that HFD administration resulted in reduced mitochondrial quinine and imbalanced mitochondrial lipid composition causing the obstruction in fatty acid oxidation and ROS generation in liver [47]. In this study, HFD associated reduction in mitochondrial complexes (I, II, IV and V) was observed indicating hepatic mitochondrial dysfunction. Inhibition of complex-I ($NADH^+$) reduces the ATP production. Decreased activity of enzyme SDH (complex II) was also observed which is associated with enhanced peroxide levels causing oxidative stress. This ultimately leads to disturbance in electron transport chain (ETC) on mitochondria. All such changes would presumably lead to a decrease in substrate oxidation. This dysfunction was attenuated by MLHEF treatment. Electrons generated by mitochondrial electron transport chain are used by complex-V for generation of ATP which is decreased due to oxidative stress caused by HFD and is reversed by MLHEF treatment.

HFD administration to rodents leads to severe molecular changes leading to defects in cells and tissue especially liver, skeletal muscles and adipocytes due to lipid accumulation. This alteration causes in change in glucose homeostasis which may be because of altered membrane proteins like GLUT transporters [52, 53]. Molecular defects in glucose disposal leading to diabetic states are suggested to reside within the glucose or insulin-signalling pathways. Although cause and effect are not yet well established it is of considerable interest to elucidate possible regulators of GLUT expression, as well as their mode of action, for the development of new therapies to stimulate glucose storage in the liver and its uptake by adipocytes and muscle [53–55]. The two glucotransporter isoforms GLUT2 and GLUT4 play central roles in the complex pathways mediating whole-body glucose disposal, in which dysregulation of their controlling mechanism can result in the pathophysiologic states associated with diabetes. High-fat diet results in not only increased body weight gain and over time a stable hyperglycaemia but a progressively increased hyperinsulinemia, indicating progressive worsening of insulin resistance. HFD further reduces the GLUT 2 and 4 expression in hepatocytes, adipocytes and muscles which may be due to mitochondrial dysfunction and oxidative stress in cells. GLUT 4 is responsible for glucose uptake in different tissue via activation of PKC or PI3K pathway which may be altered due HFD. Several Scientists had reported reduced hepatic GLUT4 in rodent fed with HFD, which is linked to hepatic insulin resistance [55–57]. Feeding of high fat diet to rodents significantly showed negative shift to the muscle and adipose tissue GLUT 2 and GLUT 4 protein expression which is attenuated by MLHEF.

In NASH (HFD induced), several GLUTs (GLUT 1 and 3) were upregulated in as compared to normal hepatocytes. Specifically, GLUT 1 and – 3 level were significantly elevated in disease condition [56–58].

Rodents on exposure of high fat diet for longer time, resulted in upregulated GLUT-1 and downregulated GLUT-2 proteins in hepatocytes which presumably reflects changes in energy metabolism in response to the dietary changes. This increase in GLUT expression may be due to effect of diet induced insulin resistance or insulin sensitivity [54, 55, 59]. The enhanced GLUT 1 and GLUT 3 also occurred due to imbalance in glucose homeostasis leading to insulin resistance. Some studies also suggested that hypoxia raised due HFD is also responsible for elevation in GLUT 1and 3 expression in tissues [59–62]. In the contrary GLUT 1 and GLUT 3 showed reverse effect that is protein expression was elevated in disease state which was modulated downward due to MLHEF activity.

Material and methods

LC-MS fingerprint analysis of Madhuca longifolia extract

The hydroalcohlic extract of *M. longifolia* leaf was prepared by successive soxhlation and its analysis was carried out by LC-MS fingerprinting. LCMS fingerprint analysis was performed in a HPLC system (Waters Alliance 2695 pump, 2424 ELSD detector) coupled with a mass spectrophotometer (XEVO-TQD#QCA1232). Separation was performed on a Sunfire C_{18} column (4.6 × 250 mm, 5µm). Briefly, 1mg/mL stock solution of *Madhuca longifolia* extract was prepared by dissolving in HPLC grade methanol and filtered through 0.22 um PTFE syringe filter. Data acquisition was done in positive mode. Separation of extract was achieved by stepwise gradients of mobile phase containing ACN (solvent A) and 0.1% formic acid (solvent B). Gradient was programmed as 0–1 min 5% ACN, 1–6 min 5–30% ACN, 6–12 min 30–60% ACN, 12–16 min 60% ACN, 16–20 min 60–80% ACN, 20–24 min 80%, 24–26 min 80 – 5%. Flow rate of mobile phase was kept 1.5 ml/min with a 1:1 split before the ESI source. Molecular ions (M + 1) were detected in a full scan mode and mass range of 50–2000 m/z. Separated metabolites present in ML leaf extract were tentatively identified based on their m/z ratio and literature reports.

Animals:

Healthy male albino mice (Swiss strain 20–25 g) were obtained from the animal facility of Birla Institute of Technology (621/02/ac/CPCSEA). Animals were housed in propylene cages with free access to food and water under temperature (27 ± 2ºc) and relative humidity of 60% for acclimatization for 7 days. All the experiments were approved by IAEC as per approval no. (1972/PH/BIT/18/17/IEAC).

Induction of obesity and diabetes by High fat diet – Streptozotocin (HFD- STZ) in experimental mice

Procured animals for experimentation were fed high fat diet for continuously for a period of 2 months and intermittently Body weight and BMI assessment were done for the confirmation of obesity. The animals having higher BMI were included for the study and divided into four groups having six animals in each group. Group 1 was kept as normal control group having normal diet and untreated animals. The group 2 was taken as obesity control (HFD 60 Days). Remaining three group was injected multiple low

dose STZ for 5 days (dose 30mg/kg bw, i.p) after 15 days of HFD administration [63, 64]. The group 3 was kept as diabetic group. Further group 4 were taken for treatment group followed by group 5 as standard treated group for 45 days. Blood glucose levels were checked at regular interval for confirmation of induction of diabetes up to 60 days and BMI was subsequently calculated for each group. Animals having blood glucose level consistently > 200mg/dl were considered as diabetic and included in the study. Concurrently the obese group were checked for BMI and blood glucose level for its confirmation. Animals were euthanised at day 60. Treatment protocol is as follows:

Group 1: Normal Control- (untreated animals with normal diet and water)

Group 2: Obesity control (treated with high-fat diet for 60 days)

Group 3- Diabetic control -Treated with STZ (multiple low dose 30mg/kg bw, i.p.) and high fat diet

Group 4-MLHEF (400mg/kg, p.o) (Diabetes control treated with MLHEF)

Group 5-Pioglitazone, (10mg/kg, p.o) (Diabetes control treated with Pioglitazone)

Assessment of physiological function:

At end of experiment blood was withdrawn in EDTA tubes for detection of glycosylated haemoglobin (HbA1C) by commercially available kit (HbA1C estimation kit, Coral Surat). For serum AST (alkaline transaminase) and ALT (alkaline transaminase) estimation blood was collected in non-coagulant containing tubes. Liver was dissected out immediately washed followed by homogenisation in ice cold 0.1 M phosphate buffer (PBS) (7.4 pH) at 10,000g for 20 min at 4ºC. Then the obtained supernatant was used for quantification of antioxidant enzyme (MDA, SOD and CAT).

Assessment of Mitochondrial function:

Isolation of Mitochondria from Mice liver:

Liver was dissected out and mitochondria were extracted by the described method of Pederson *et al*. [65]. The total hepatic mitochondrial protein concentration was quantified through Lowry method [66].

Estimation of Mitochondrial function:

The NADH dehydrogenase activity (Complex I) was performed via enzymatic oxidation of NADH in presence of artificial electron acceptor potassium ferricyanide. The wavelength for NADH excitation and emission were 350nm and 470nm respectively as described by Shapiro *et al*. NADH dehydrogenase activity was expressed as nmol NADH oxidized /min / mg protein [67].

The mitochondrial complex II was performed by the described method of Sally and Margaret [68]. In this succinate dehydrogenase was estimated by formation of insoluble coloured compound diformazan on progressive reduction of NBT (nitro blue tetrazolium) which was detected at 570nm. The SDH activity was expressed as micromole formazone formed /min per mg protein.

The complex IV (cytochrome oxidase) activity was measured in isolated mitochondrial fraction in presence of reduced cytochrome C for 3min at 550 nm by reported technique of Storrie and Amadden (1990) [69]. The complex IV activity was measured as nanomole cytochrome-C oxidized /min per mg protein.

The complex – V (F1- F0 synthase) was estimated by incubation of mitochondrial suspension in ATPase buffer by reported method of Griffiths et al. (1974), and content of phosphate was measured as reported by Fiske and Subbarow (1925) [70, 71]. Results were expressed as nanomole ATP hydrolyzed per minute per mg protein.

Determination of lactate dehydrogenase (LDH):

Liver, adipose tissue and skeletal muscles tissues were taken from mice immediately after treatment time and tissues were stored at -20ºC. Samples was prepared as described in Faddah et al. (2007) with modifications [72]. Briefly, tissue were homogenized in a solution of 0.9% w/w NaCl, 5 mM TRIS-HCl buffer (pH 7.4). Supernatant was collected after centrifugation for 30 minutes at 20 ºC, 15000xg, and clear supernatant was used for analyzing LDH activity. The protein in samples was estimated by using Lowry's method, and LDH estimation was done by the reported method of Wroblewski and Ladue (1955) [73]. The reaction mixture was prepared by mixing of phosphate buffer (0.1M NaH_2PO_4 and 0.1M Na_2HPO_4, pH7.2), sodium pyruvate (1.5mM), and 100 µg of protein. This reaction mixture was incubated at 37˚C for 30 min. The reaction was initiated after addition of 0.5mM NADH. Absorbance was measured by a spectrophotometer (Gen5; BioTek, Winooski, VT, USA) at 340nm for 60s at an interval of 15s. LDH enzyme activity was quantified in terms of substrate utilized by LDH/mg of protein/min.

Effect on Glut transporter by Western blot analysis:

After euthanasia tissue samples namely liver, white adipose tissue and skeletal muscle were collected from following groups: (1) normal control, (2) obesity control, (3) diabetic control (4) treatment group and (5) standard samples were prepared for western blot analysis.

Protein expression of class 1 GLUT transporter (GLUT – 1, GLUT-2, GLUT-3, GLUT-4 and β-actin) were done by western blotting. The liver, adipose tissue and skeletal muscles tissues from mice were freeze thawed and triturated in liquid nitrogen, and protein lysates were prepared in mammalian cell Lysis buffer (G-biosciences) supplemented with phosphatase and protease inhibitors. Protein was estimated using Lowry's [66] method followed by loading of protein on SDS-PAGE. The proteins were separated and then transferred on to polyvinylidene difluoride (PVDF) membrane. Blocking was performed using 5% BSA solution for 2 h, and membranes were incubated with primary antibody rabbit anti GLUT1, GLUT2, GLUT3 antibodies (1:500) (from santachruz), mouse anti GLUT4 (1: 1000) from (cellular signaling technology) and mouse anti β-actin (1:1000) (from sigma) kept overnight at 4ºC. Further, membranes were incubated at room temperature with appropriate secondary antibodies for 2h after washing with PBS-T (phosphate buffered saline with 1% Tween 20). Membranes were developed and detected by chemiluminescence using luminata substrate in chemidoc system (BioRad XRS+). Relative optical density of developed

bands were evaluated through Image J software. Comparison between groups of different treatments was carried out by densitometric analysis based on mean intensity of the protein band after normalization with β-actin.

Data Analysis

All the obtained results were expressed as mean ± SD (standard deviation). One way ANOVA followed by Tukey's test has been used to determine the significance between the respective data of different groups with help of Graph pad prism (v5.1). Values $p \leq 0.05$ were significant. All the disease control group (obesity control group and diabetic control) were compared to normal control group.

Conclusion

Thus, concluding this work it can depicted that chronic HFD and HFD + STZ results in alteration of several cellular mechanism which leads to provocation of hypoxia, oxidative stress and mitochondrial dysfunction. This anomalies also triggers inflammatory markers which simultaneously results in insulin resistance and decreased insulin sensitivity. Thus terminating in diabetic and other metabolic disorder through extra pancreatic pathway i.e. through altering liver, skeletal muscle and adipose tissue functions. The treatment with MLHEF not only reduces the oxidative stress, but also helps in normalisation of hepatic, adipocyte and muscular function. It also helps in diminishing the mitochondrial dysfunction is tissues and normalising GLUT transporters functioning in liver, adipose and skeletal muscles. The graphical summary of this work is shown below.

Abbreviations

HFD : high fat diet, STZ : streptozotocin, GLUT : glucose transporter, LDH: lactate dehydrogenase, IL: interleukins, CAT : catalase, SOD: superoxide dismutase, MDA : malondialdehyde, AST : aspartate transaminase, ALT : alanine transaminase , HIF : hypoxia inducible factor , ROS: reactive oxygen species, OXPHOS: oxidative phosphorylation, FFA : free fatty acids, TNF : tumour necrosis factor, SDH : succinate dehydrogenase. WAT: white adipose tissue, NBT: nitro blue tetrazolium. NAD: nicotinamide adenine dinucleotide, i.p: intraperitoneal, ATP : adenosine triphosphate, ADP: adenosine diphosphate, SD : standard deviation.

Declarations

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and finalized the manuscript. The authors state that all data were generated internally and that no paper mill was used.

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Ethical statement

All animal experiments were performed in accordance with the National Institutes of Health Guidelines (publication number 85-23, revised 2013). Experiments on animals were approved by the Institutional Animal Ethical Committee, (approval no. 1972/PH/BIT/18/17/IEAC).

Availability of data and material

Data will be provided on request.

Consent to Participate: Not Applicable

Consent for publication: All the authors consent for publication.

Declaration of interest

Declaration from the author that there is no conflict of interest.

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Figures

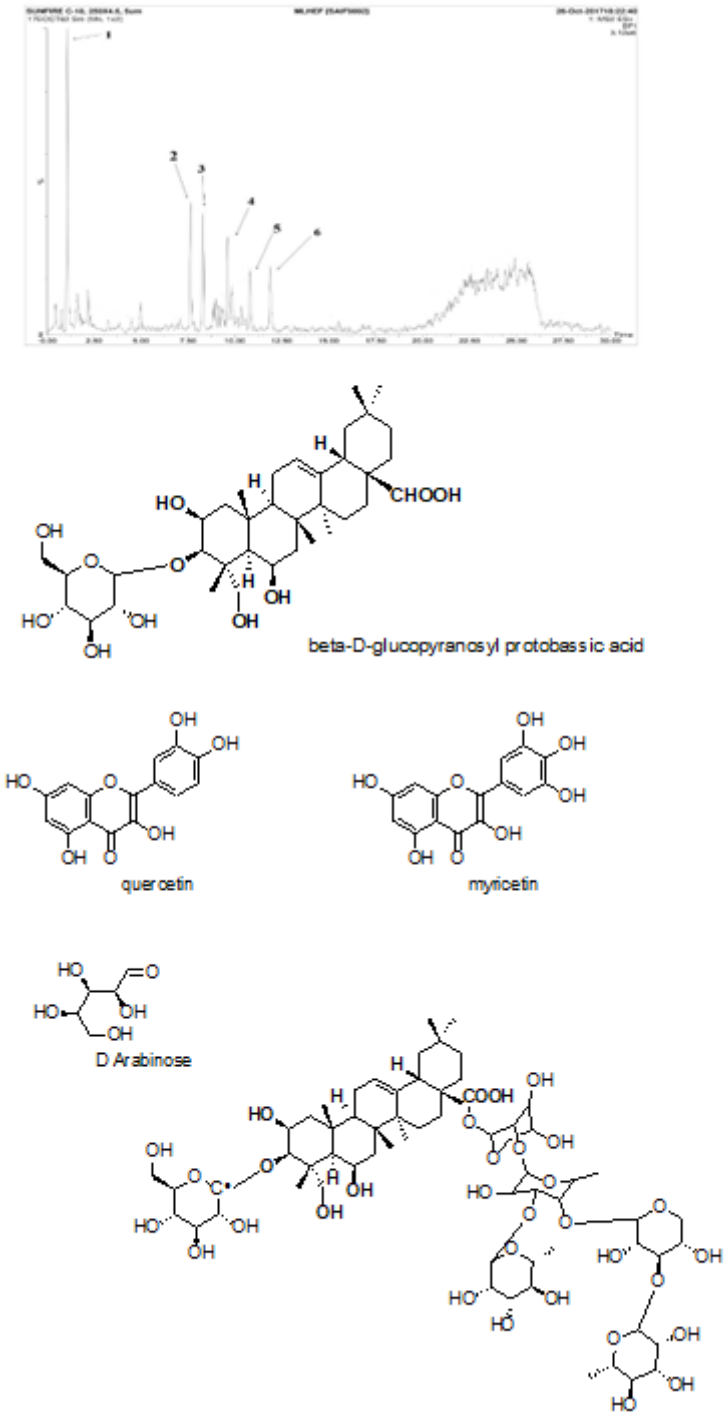

Figure 1

LCMS analysis of *M.longifolia* hydro ethanolic extract and structure of tentative compounds.

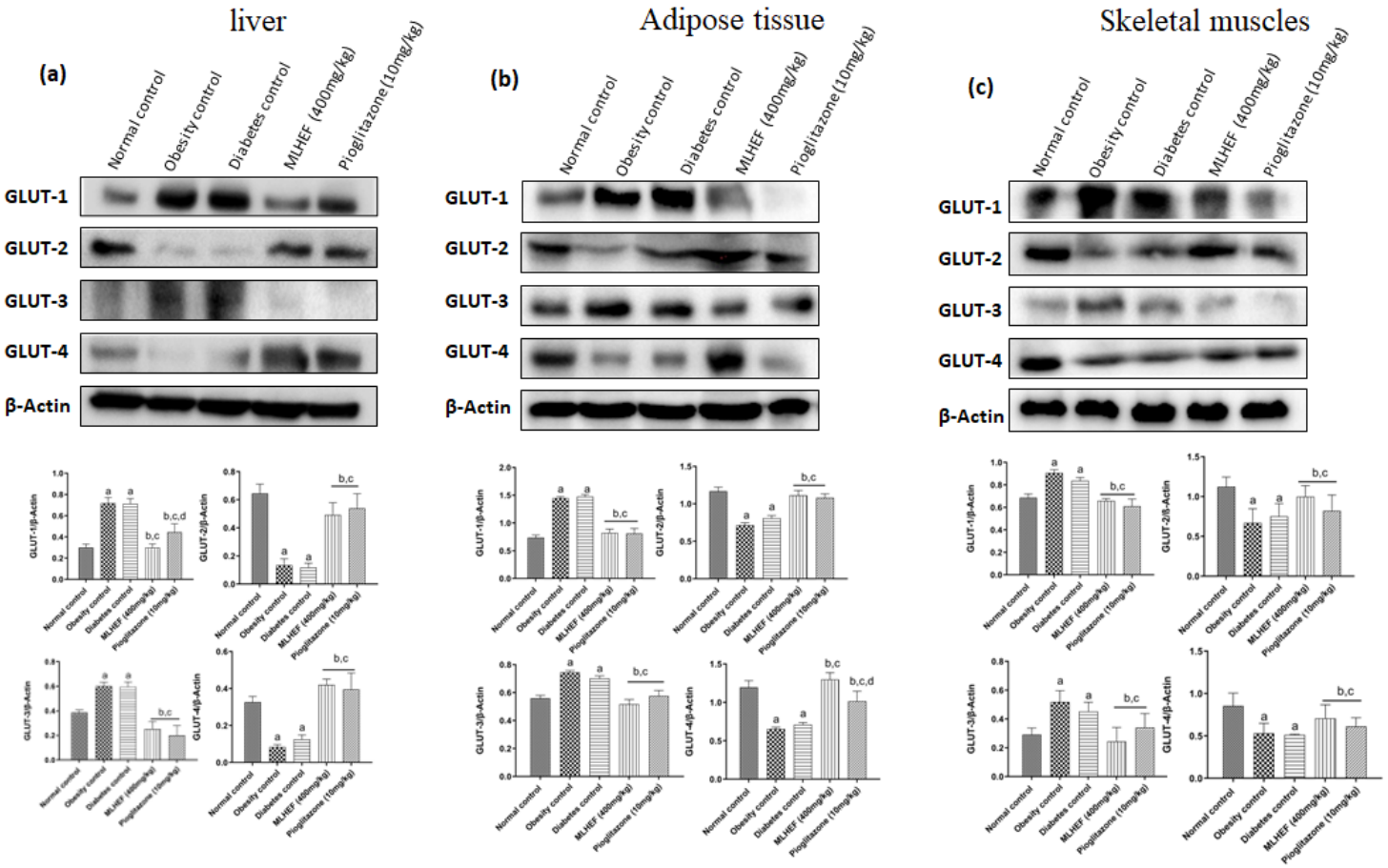

Figure 2

Protein extracts were isolated from liver, adipose tissue and skeletal muscles of each animal and class 1 GLUT transporter were examined by western blot analysis. (MLHEF enhances the GLUT 2 and GLUT4 protein expression and reduces the GLUT 1 and GLUT 3 expression in HFD and HFD + STZ induced alteration in liver, adipose tissue and skeletal muscles, respectively of mice. All values are mean ± SD; n=6; $^{a}p \leq 0.001$ compared to control, $^{b}p \leq 0.001$ compared to obesity control, $^{c}p \leq 0.001$ compared to diabetes control [One-way ANOVA followed by Tukey's Multiple Comparison].

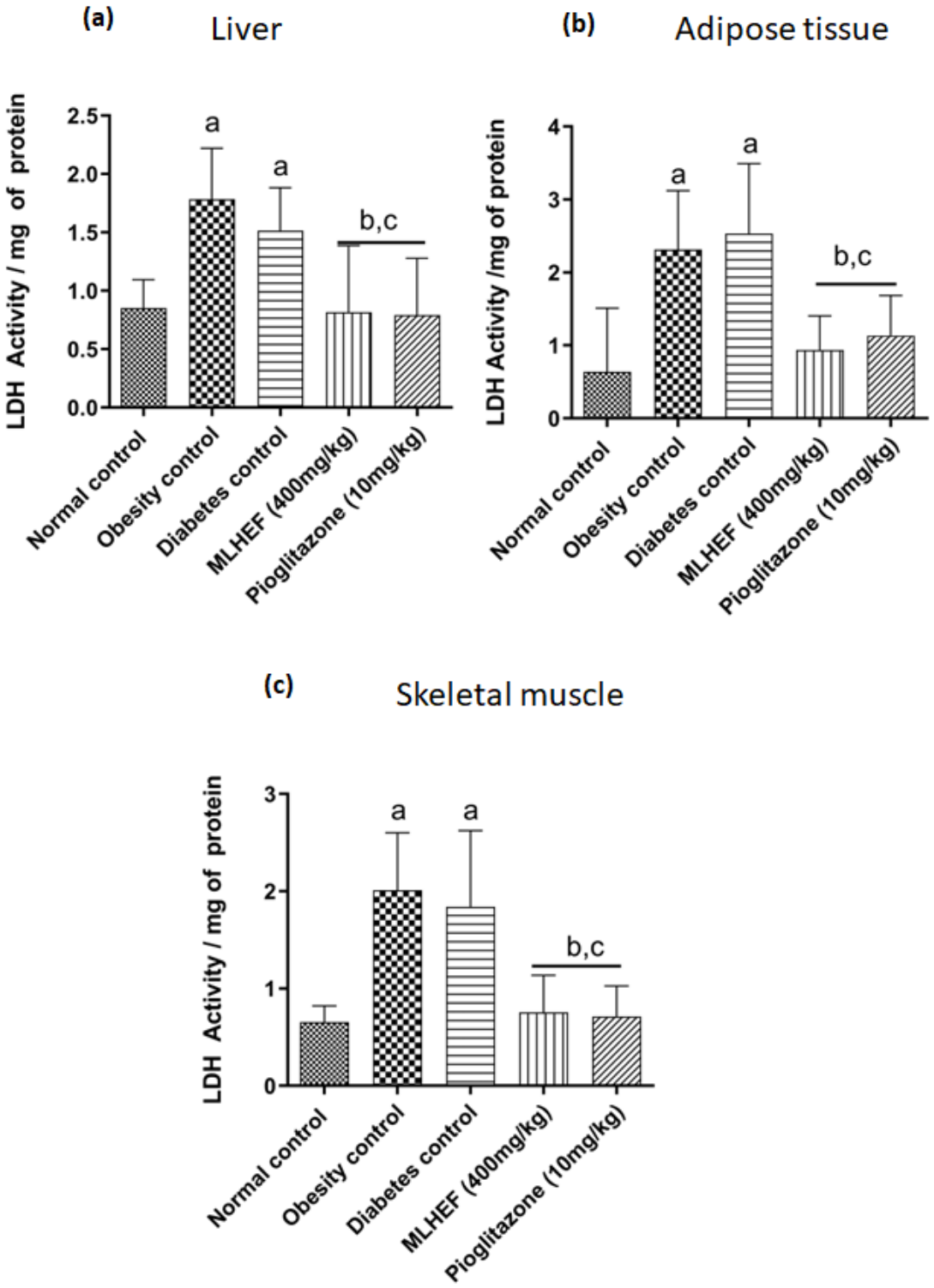

Figure 3

illustrates the effect of MLHEF treatment on HFD and HFD + STZ induce alteration in LDH activity of different organs (a) Liver, (b) Skeletal muscles and (c) White adipose tissue. One-way ANOVA showed that there were significant difference in Liver ($F(4, 24) = 3.299$, $p < 0.001$), Skeletal muscle ($f(4, 24) = 2.707$, $p < 0.001$), and White adipose tissue ($F(4, 24) = 4.393$, $p < 0.001$ activity among the groups. The HFD and HFD + STZ significantly suppressed the respiratory enzyme activities in liver tissues as

compared to control animals. MLHEF reversed the HFD induced decrease in the activity of LDH activity in Liver, Skeletal muscle and white adipose tissue as compared to disease control (Obesity control and diabetic control) group mice. All values are mean ± SD; n=6; [a]p≤0.001 compared to control, [b]p≤0.001 compared to obesity control, [c]p≤0.001 compared to diabetes control [One-way ANOVA followed by Tukey's Multiple Comparison].

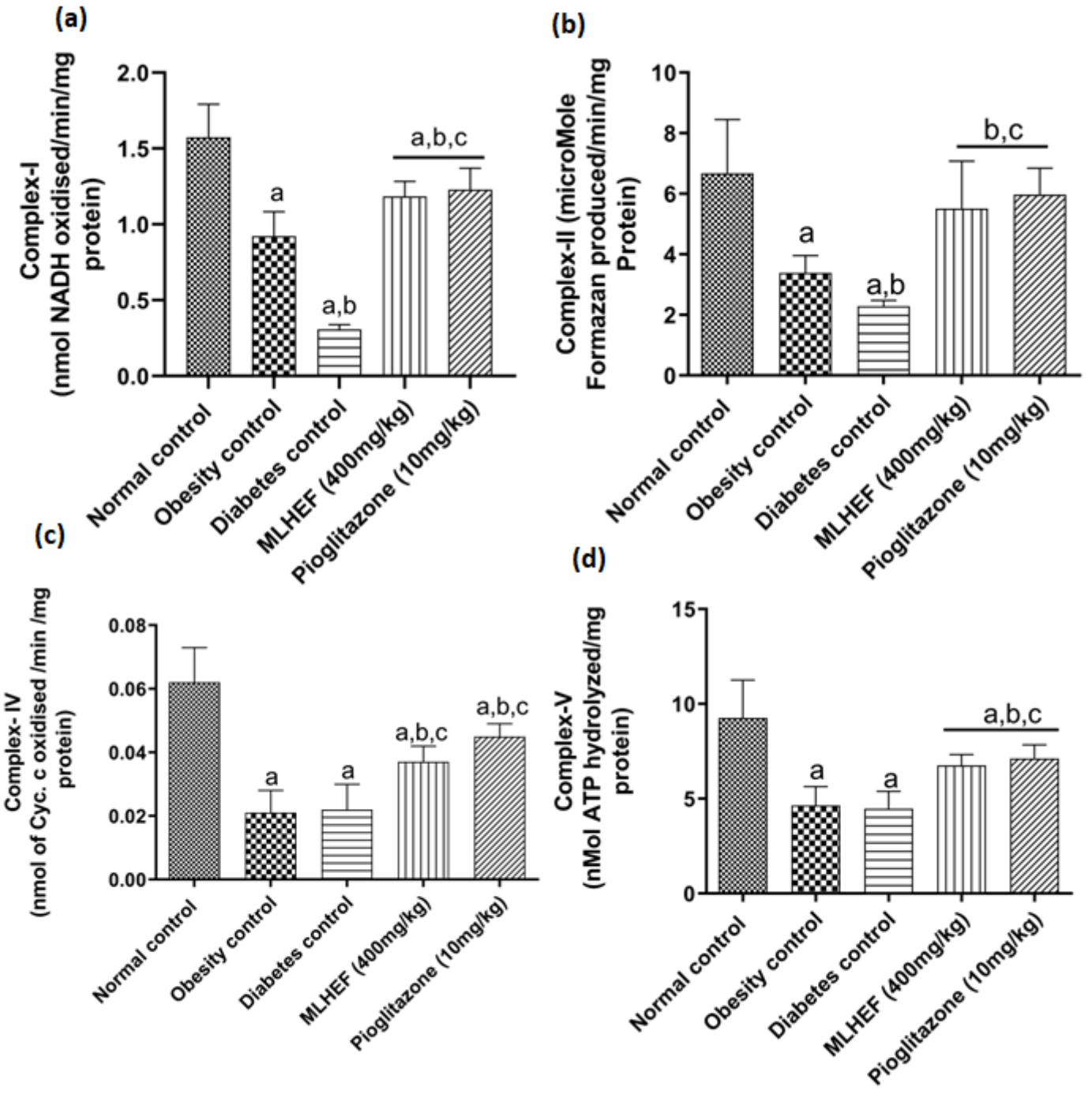

Figure 4

illustrates the effect of MLHEF treatment on HFD and HFD + STZ induced alteration in the mitochondrial complex-I (a), mitochondrial complex-II (b), mitochondrial complex-IV (c), and mitochondrial complex-V (d) activities of liver tissue. One-way ANOVA showed that there were significant difference in complex-I ($p<0.05$), complex-II ($p<0.05$), complex-IV($p<0.05$), and complex-V ($p<0.05$) activity among the groups. The HFD and HFD + STZ significantly reduced the respiratory enzyme activities in liver tissues compared to control animals. MLHEF attenuated HFD induced decrease in the activity of complex-I, II, IV, and complex-V in liver tissues as compared to disease control (Obesity control and diabetic control) groups.

Supplementary Files

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