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Original Article

Polyherbal Dietary Supplement MD-1 Ameliorates Severity of Type 2 Diabetes Mellitus in High-fat Diet-fed C57BL/6J Mice by Attenuating Adipose Tissue Inflammation

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Abstract

Background and objectives: MD-1 is a time-tested polyherbal diabetes supplement in Tamil Nadu, India. It is composed of dried powdered herbs: *Phyllanthus amarus* Schum. & Thonn, *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms, *Emblica officinalis* Gaertn., *Eugenia jambolana* Lam., *Gymnema sylvestre* R. Br. Ex, and *Cassia auriculata* Linn. This study aimed to investigate the *in vivo* effects of MD-1 in high-fat diet (HFD)-induced diabetes mellitus in C57BL/6J mice.

Methods: After 10 weeks of HFD induction, diabetic mice (n = 60) were randomized to 21-day treatments with MD-1, metformin, or left untreated on a standard pellet diet. Fasting blood glucose, triacylglycerol (TAG), total cholesterol, and liver tissue markers including superoxide dismutase, glutathione peroxidase, glutathione, thiobarbituric acid reactive substance, glucokinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase expressions were measured. Adipose tissue tumor necrosis factor (TNF)-α infiltration and messenger RNA expression of peroxisome proliferator-activated receptor γ (PPAR-γ) and glucose transporter type 4 (Glut4) were also analyzed.

Results: MD-1 treatment significantly reduced elevated fasting blood glucose, TAG, and total cholesterol in HFD-fed mice and countered HFD-induced weight gain despite unchanged caloric intake. Improved adipose tissue function was evidenced by reduced TNF-α infiltration and increased messenger RNA expression of PPAR-γ and Glut4. MD-1 attenuated HFD-induced fatty liver disease by reducing oxidative stress and TAG accumulation, suggesting a possible two-hit mechanism.

Conclusions: MD-1 administration primarily targets adipose tissue TNF-α signaling in HFD mice, restoring function via PPAR-γ/Glut4 expression. These findings support its glycemic intervention potential and justify its supplementation in diabetes.

Introduction

Diabetes is a current epidemic with rapidly changing dynamics, with major prevalence in developing nations. According to the International Diabetes Federation (2021), 537 million adults are diabetic globally, and this number is expected to reach 643 million by 2030 and 783 million by 2045. Globalization and economic development in low- and middle-income countries have spurred nutritional transitions. Traditional dietary patterns are being lost as people adapt to more industrialized and urban food environments.[1] The failure of conventional medicine to provide a cure and the emergence of diabetes as a global epidemic have encouraged the use of ethnomedicine in diabetes mellitus (DM).[2] Nearly 400 herbal formulations are reportedly used in the management of DM.[3] Given the intrinsic pharmacological properties of the phytochemicals present in dietary supplements,[4] understanding their mechanisms of action is salient for healthcare practitioners, especially considering their potential to

Keywords: Adipose tissue; Herbal supplement; Oxidative stress; Peroxisome proliferator-activated receptor γ; PPAR-γ; Type 2 diabetes mellitus.

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interfere with the pharmacodynamics of prescription medicines.[5]

MD-1 is a herbal formulation developed in South India for type 2 diabetes mellitus (T2DM), drawing from time-tested traditional dietary principles in the management of diabetes-related symptoms. Formulated as a 500 mg hard gelatin capsule, it contains 100 mg of aerial parts of *Phyllanthus amarus* Schum. & Thonn. (Keezhanelli - Euphorbiaceae), 75 mg of stems and leaves of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms. (Guduchi - Menispermaceae), 75 mg of *Emblica officinalis* Gaertn. (Amla - Euphorbiaceae) fruits, 75 mg of *Eugenia jambolina* Lam (Naval Pazham - Myrtaceae) fruits, 100 mg of *Gymnema sylvestre* R. Br. Ex (Sirukurinjan - Apocynaceae) leaves, and 75 mg of *Cassia auriculata* Linn (Avarampoo - Caesalpiniaceae) flowers. The hypoglycemic activity of these herbs is well documented, and the clinical use of MD-1 by traditional practitioners at a dose of three capsules per day in Tamil Nadu, India, presupposes its effective glycemic intervention in diabetes.[6]

Preliminary pharmacognostical and phytochemical parameters of MD-1 were established in our earlier studies. Residual analysis ruled out heavy metals, aflatoxins, pesticide residues, and microbial contamination in MD-1.[7] High-performance thin-layer chromatography quantified biomarkers including phyllanthin, berberine, gallic acid, ellagic acid, and gymnemic acid.[8] *In vitro* analysis demonstrated that the hydroalcoholic extract of the polyherbal formulation improved insulin-stimulated glucose uptake and significantly reduced triacylglycerol (TAG) accumulation in 3T3L-1 adipocytes. MD-1 also exhibited weak peroxisome proliferator-activated receptor γ (PPAR-γ) agonistic activity in messenger RNA (mRNA) expression studies.[7] These *in vitro* findings led us to hypothesize beneficial *in vivo* effects of MD-1 on obesity-associated type 2 diabetes (T2D). The objective of the present work was to investigate the therapeutic mechanisms of MD-1 and its potential effects on whole-body metabolism in high-fat diet (HFD)-induced T2D in C57BL/6J mice.

Materials and methods

Drugs and reagents

MD-1 capsules were commercially manufactured by ISHA AROGYA manufacturing unit, Chennai, Tamil Nadu, India. Three batches of the capsules (B20/2014, B21/2014, B22/2015) were procured to evaluate the antidiabetic activity. Samples from twenty capsules of each batch were collected and stored in amber-colored containers at room temperature. HFD (D12492) was purchased from Research Diets, Inc., New Brunswick, USA. Metformin hydrochloride was procured from Sigma Aldrich, India. All other chemicals and solvents were of analytical grade and obtained from SISCO Research Laboratories Pvt. Ltd., India. A 70% methanolic extract of MD-1 was prepared as per our previously reported method.[7]

In vitro studies — effect of MD-1 extract on lipid metabolism

Cell culture

3T3L-1 preadipocytes were procured from the National Centre for Cell Science, Pune, and maintained in Dulbecco's Modified Eagle's Medium (DMEM) high-glucose medium supplemented with 10% fetal bovine serum, penicillin (5 units/mL), and streptomycin (5 µg/mL) in 5% CO_2 incubator at 37°C.

Effect of MD-1 on TAG accumulation[9]

Briefly, two days post-confluency, cells grown in 6-well plates were induced to differentiate using a medium containing 0.25 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 1 mg/L insulin in DMEM with 10% fetal calf serum (day 0). After 72 h, the medium was replaced with maintenance medium containing 1 mg/mL insulin for 48 h (day 5), followed by fresh culture medium (DMEM with 10% fetal calf serum) for two days. Cells were incubated with MD-1 extract at concentrations of 62.5 µg/mL, 125 µg/mL, and 250 µg/mL from day 0 to day 7 to study its effect on TAG accumulation. Post-differentiation, cells were washed with phosphate-buffered saline (PBS), lysed in 500 µL of 0.1% sodium dodecyl sulfate, and the lysate centrifuged at 3,000 × g for 5 m at 25°C. Supernatants were assayed for TAG (Accurex kit, India) and protein content.[10] TAG accumulation was normalized to cellular protein content.

Effect of MD-1 on lipolysis[11]

Differentiated 3T3L-1 adipocytes were treated with MD-1 extract (62.5 µg/mL, 125 µg/mL, and 250 µg/mL) for 48 h. Post-incubation, culture media were collected and centrifuged at 3,000 × g for 5 m at 25°C. Supernatants were assayed for glycerol using a free glycerol reagent (Sigma Aldrich, USA) according to the manufacturer's instructions.

In-vivo studies

Experimental animals

Male C57BL/6J mice, four weeks old, were procured from the National Institute of Nutrition, Hyderabad, India. Animals were housed at the Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research (DU), under controlled temperature (23 ± 1°C) and a 12 h light/12 h dark cycle. All experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of Sri Ramachandra Institute of Higher Education and Research (DU), constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (IAEC No: IAEC/XXXXV/SRU/439/2015). All animal experiments were conducted by the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize animal suffering.

Before the experiment, serum parameters such as fasting blood glucose (FBG), TAG, and total cholesterol (TC) were estimated for all animals on a normal pellet diet. Animals were allocated to two dietary regimens: standard pellet diet or 60% Kcal HFD for 10 weeks. After 10 weeks of HFD feeding, animals with FBG above 175 mg/dL were considered diabetic and assigned to study groups. The experimental design consisted of six groups, each comprising 10 animals (n = 10). Group 1: normal diet control (NC); group 2: HFD control (vehicle control: 0.5% sodium carboxymethyl cellulose); group 3: HFD+MD-1 (100mg/kg., b.wt); group 4: HFD+MD-1 (300 mg/kg., b.wt); group 5: HFD+MD-1 (500 mg/kg., b.wt); group 6: HFD+Metformin (500 mg/kg., b.wt) for 21 days. The criteria recommended by the U.S. Food and Drug Administration, incorporating body surface area, were used to calculate equivalent doses across species to select experimental MD-1 doses.[12] MD-1 was suspended in 0.5% sodium carboxymethyl cellulose in distilled water and administered orally. Feed intake (daily) and body weight (weekly) were measured throughout the study. Animals were anesthetized using diethyl ether, and blood samples were collected from 6 h-fasted mice at weeks 0, 10, and 13. The plasma was analyzed for FBG, TAG, and TC using commercially available kits (Accurex, India)

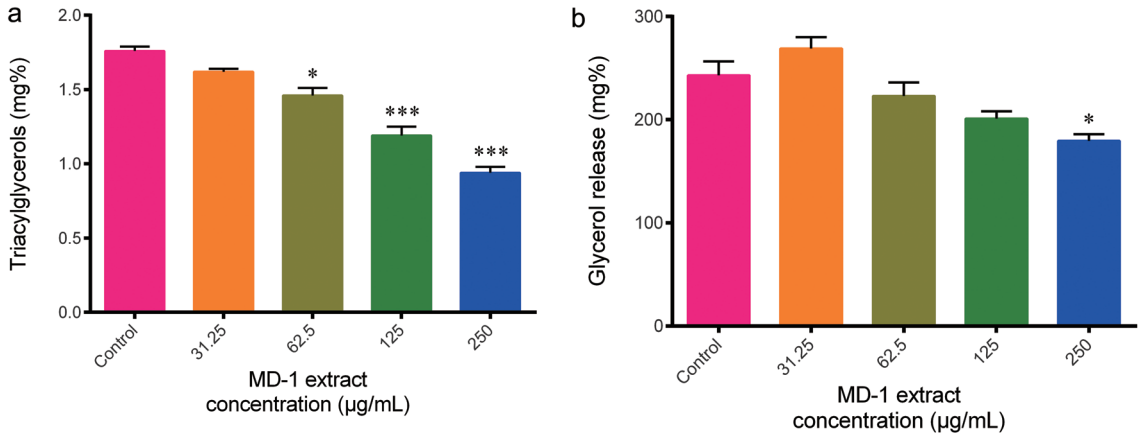

Fig. 1. Effect of MD-1 extract on lipid metabolism in 3T3L-1 adipocytes. (a) Effect of MD-1 extract on triacylglycerol accumulation in 3T3L-1 preadipocytes after differentiation induction. Two days post confluency, 3T3L-1 cells were induced for differentiation according to the protocol in the presence of MD-1 methanolic extract. After eight days of induction, cells were assayed for the estimation of triacylglycerol; (b) Effect of MD-1 extract on lipolysis in 3T3L-1 adipocytes. Post differentiation, cells were incubated with MD-1 extract. After 48 h of incubation, the supernatant was assayed for glycerol content. Data are represented as means ± SEM of triplicates of three independent experiments. *$P < 0.05$, ***$P < 0.001$ (One-way ANOVA followed by Dunnett's post hoc test) as compared with adipocyte control. ANOVA, analysis of variance; SEM, standard error mean.

per manufacturer instructions. Animals were euthanized and organs harvested on day 22.

Biochemical analysis

A 10% (w/v) liver homogenate was prepared in appropriate buffer (pH 7.0). Tissue antioxidants, including superoxide dismutase (SOD),[13] glutathione peroxidase (GPx),[14] and non-enzymatic reduced glutathione (GSH),[15] were measured using reported methods. Lipid peroxidation was estimated as thiobarbituric acid reactive substances.[16] Hepatic enzymes involved in carbohydrate metabolism, glucokinase (GK),[17] fructose-1,6-bisphosphatase (F16Pase),[18] and glucose-6-phosphatase (G6Pase),[19] were also evaluated. Enzyme activities were normalized to tissue protein content. Tissue lipid content was estimated as described.[20]

Total RNA isolation and mRNA expression studies

Total RNA was isolated from white adipose tissue (WAT) using TRIZOL reagent, and reverse transcriptase polymerase chain reaction (PCR) was performed using Amplicon master mix according to the manufacturer's protocol. Primer sequences used for PCR analysis were as follows: PPAR-γ:5′-ACCTGAAGCTCCAAGAATACCA-3′(forward) and 5′-TAAGCTTCAATCGGATGGTTCT-3′(reverse), Glut4:5′-GGACCTGTAACTTCATTGTCG-3′(forward) and 5′-TCTGTACTGGGTTTCACCTCCT-3′(reverse), GAPDH: 5′-ACCACAGTCCATGCCATC-3′(forward) and 5′-TCCACCAC CCTGTTGCTG-3′(reverse). The reaction mixture was subjected to denaturation (95°C for 30 s), annealing (57°C for 30 s), and extension (72°C for 1 m) for 35 cycles in a Mastercycler gradient (Eppendorf, Germany). PCR products were analyzed by electrophoresis on 1% agarose gel at 80 V and visualized with SYBR Safe DNA gel stain (ThermoFisher Scientific). PCR bands were quantified using ImageJ software. Gene expression was shown as the ratio of the densitometry value of target mRNA to that of GAPDH.

Histopathology and immunohistochemistry

Liver, WAT, kidney, and skeletal muscle tissue sections were observed for histological changes using hematoxylin and eosin staining. The effect of MD-1 on tumor necrosis factor (TNF)-α infiltration was studied in epididymal fat pads of HFD-fed mice. Briefly, paraffin-embedded sections of WAT were deparaffinized by heating at 60°C for 1 h, followed by xylene washes. After rehydration with graded alcohol, slides underwent heat-mediated antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 10 m. Slides were allowed to reach room temperature and rinsed with PBS containing 0.025% Triton X-100 to facilitate reagent spreading. Tissue sections were blocked in 10% serum containing 1% BSA in PBS for 1 h at room temperature, followed by incubation with rabbit polyclonal anti-TNF-α antibody (1:100) diluted in PBS with 1% BSA for 20 m at 25°C. Slides were rinsed twice in PBS containing 0.025% Triton with gentle agitation and blocked with 0.3% H_2O_2 in PBS to suppress endogenous peroxidase activity. Slides were then incubated with horse radish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody for 1 h at room temperature, rinsed in tap water, and counterstained with hematoxylin.

Statistical analysis

Data were analyzed using GraphPad Prism software version 6 (GraphPad Software, USA). Data are presented as mean ± standard error mean. One-way analysis of variance (ANOVA) followed by post hoc Dunnett's test was used to compare means between groups. Two-way repeated measures ANOVA with Bonferroni correction was used to analyze time-course data within groups. A *P*-value of <0.05 was considered statistically significant.

Results

MD-1 extract reduced TAG accumulation and lipolysis in 3T3L-1 adipocytes

3T3L-1 preadipocytes treated with MD-1 extract along with differentiation media showed a significant reduction ($P = 0.0072$, Fig. 1a) in TAG accumulation in a dose-dependent manner. At a dose of 31.25 µg/mL, MD-1 showed a 7.8% ± 0.52% decrease, whereas at 250 µg/mL, the MD-1 extract showed a 66.43% ± 2.10% reduction in TAG accumulation compared to adipocyte controls. After 48 h of incubation with MD-1 extract, differentiated adipocytes demonstrated a considerable reduction in lipolysis. At 250 µg/mL, MD-1

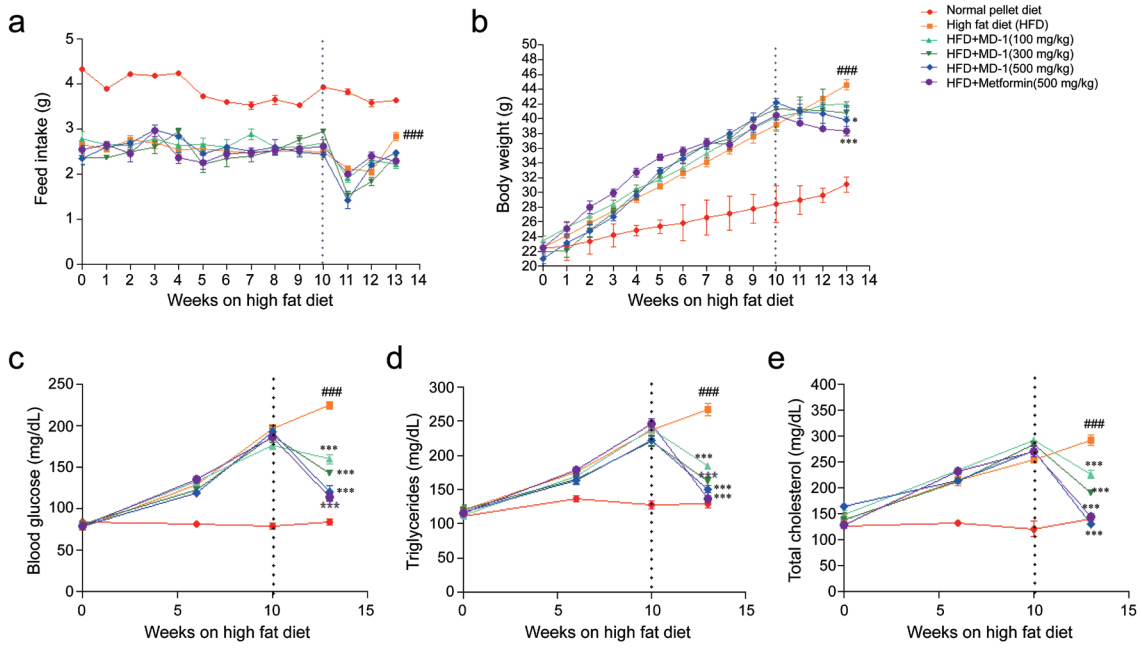

Fig. 2. Effect of MD-1 on body weight and serum parameters. (a) Feed intake; (b) Body weight; (c) Fasting blood glucose; (d) Triacylglycerol; (e) Total cholesterol. The vertical line indicates the week of MD-1 treatment. Data are represented as means ± SEM (n = 6 per group). ###$P < 0.001$ vs. Normal control; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. High-fat diet (HFD) control as measured by Two-way ANOVA followed by Bonferroni correction. ANOVA, analysis of variance; SEM, standard error mean.

extract showed a 30.46% ± 3.69% decrease ($P = 0.028$) in glycerol release compared to control adipocytes (Fig. 1b).

Anti-diabetic activity in HFD-induced diabetic C57BL/6J mice

Effect on feed intake and body weight

Feed intake in normal diet-fed mice was 4.09 ± 0.25 g/day, whereas it was 2.56 ± 0.27 g/day in HFD-fed mice. No change in caloric intake was observed throughout the study period, except for a transient decrease at week 11 immediately after MD-1 treatment. During the third week after the introduction of HFD, body weight increased significantly more in HFD-fed mice (1.58 ± 0.1 g) than in normal diet-fed mice (0.35 ± 0.1 g). The growth curves showed similar patterns in both groups, with greater body weight gain over the 10-week induction period (Fig. 2a). The weight gain thereafter continued to be progressively higher in HFD mice (Fig. 2b). The growth rate in normal diet-fed mice during the first 10 weeks was 0.56 ± 0.03 g/week, which increased to 1.65 ± 0.04 g/week in HFD–fed mice ($P = 0.0086$). The growth rate was significantly reduced during the treatment period, i.e., from week 11, in MD-1-treated groups. In MD-1-treated groups, at 100 mg/kg b.wt, the growth rate was reduced to 0.09 ± 0.01 g/week, whereas at both 300 and 500 mg/kg b.wt doses, HFD-induced weight gain was completely suppressed by MD-1. In the last week of treatment (week 13), at 500 mg/kg b.wt, a body weight reduction of 0.83 ± 0.05 g/week was observed.

MD-1 treatment reduced elevated FBG, TAG, and TC

Six weeks of HFD feeding showed a significant rise in FBG, TAG, and TC levels compared to normal controls. At week 10, FBG, TAG, and TC increased to 195 ± 5.27, 184 ± 6.6, and 223.94 ± 7.65 mg/dL, respectively, in HFD-fed animals, whereas no significant changes were observed in NC animals. After three weeks of MD-1 administration, a progressive improvement was observed in FBG, TAG, and TC levels in HFD animals (Fig. 2c–e). MD-1 reduced glucose levels to 120.71 ± 6.73 mg/dL, while TAG and TC were reduced to normal levels (HFD + MD-1 500 mg/kg b.wt, $P = 0.001$). The beneficial improvement in the metabolic profile mediated by MD-1 treatment in HFD animals is comparable to that of standard metformin.

MD-1 treatment reduced TNF-α signaling in adipose tissue

Histological observations of adipose tissue in HFD-fed animals as seen in Figure 3, showed adipocyte hypertrophy and cell necrosis with macrophage infiltration (Fig. 3b). Mohallem *et al*.[21] reported the involvement of adipose tissue-derived TNF-α in the etiology of insulin resistance. Immunohistochemical staining of adipose tissue showed increased TNF-α expression in HFD animals. Three weeks of MD-1 treatment significantly reduced macrophage infiltration and TNF-α expression in a dose-dependent manner in the adipose tissue of HFD-fed animals. At 500 mg/kg b.wt (Fig. 3f), there was a substantial decrease in adipose tissue inflammation with complete restoration of normal adipose architecture, and TNF-α infiltration was reduced to basal levels.

MD-1 treatment restored mRNA expression of PPAR-γ and Glut4 in epididymal fat pads

PPAR-γ activation in mature adipocytes is associated with transcriptional activation of many genes involved in the insulin signaling cascade. WAT tissue expression of mRNA for PPAR-γ and its target gene Glut4 showed significant downregulation after 13 weeks of HFD feeding. Figure 4 shows significantly enhanced ($P < 0.001$) expression of PPAR-γ mRNA by MD-1 at 300 mg/kg b.wt and 500 mg/kg b.wt doses compared to HFD controls. Similarly, MD-1-treated animals showed significantly increased Glut4 mRNA expression at 300 mg/kg b.wt ($P = 0.008$ and 500 mg/kg b.wt ($P = 0.001$) doses compared to HFD mice, while standard metformin

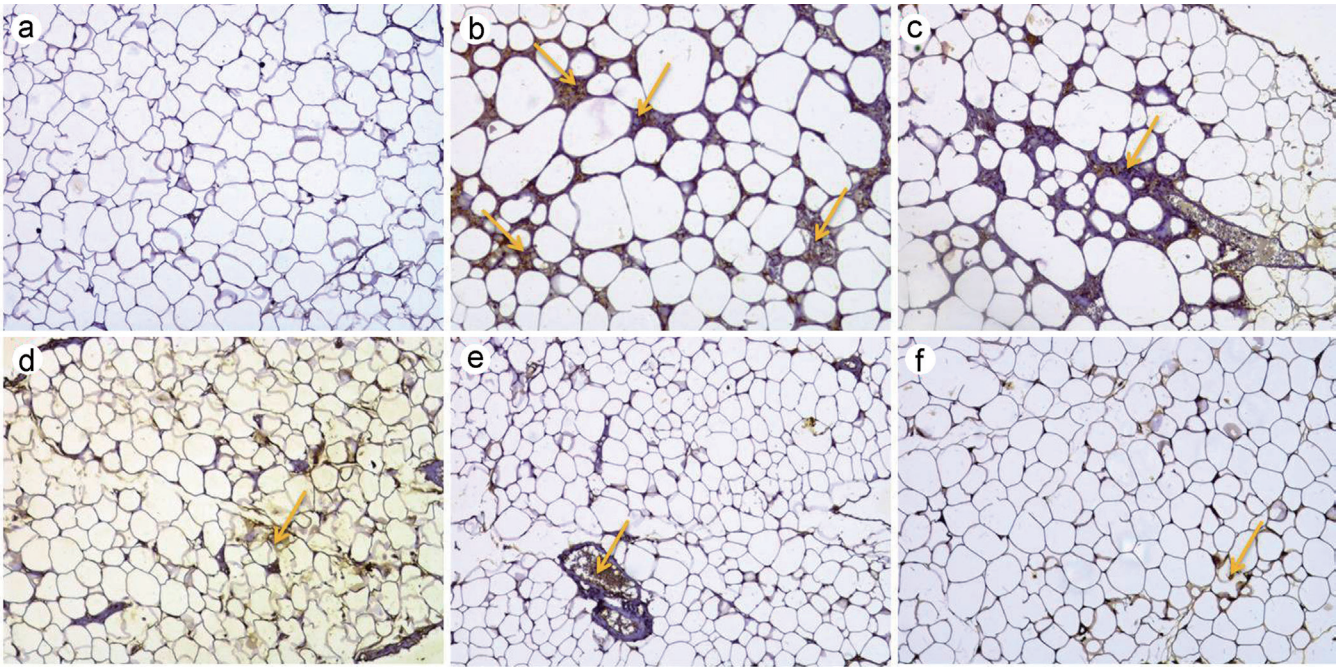

Fig. 3. Effect of MD-1 on TNF-α infiltration in epididymal fat pads. Tissue sections were incubated with rabbit polyclonal anti-TNF-α antibody, followed by HRP-conjugated goat anti-rabbit polyclonal secondary antibody. The protein was visualized by hematoxylin counterstain. Representative photomicrographs were shown at ×100. (a) Normal pellet diet control- Normal adipose tissue architecture; (b) HFD control – Presence of adipose tissue hypertrophy and increased TNF-α infiltration; (c) HFD+MD-1(100 mg/kg b.wt) – Presence of adipose tissue hypertrophy and increased TNF-α infiltration; (d) HFD+MD-1(300 mg/kg b.wt) – Moderate decrease in adipose tissue hypertrophy and TNF-α infiltration; (e) HFD+MD-1 (500 mg/kg b.wt)- Normal adipose tissue architecture and decreased TNF-α infiltration; (F) HFD + Metformin (500 mg/kg b.wt) – Normal adipose tissue architecture and no TNF-α infiltration. ANOVA, analysis of variance; HFD, high-fat diet; HRP, horse radish peroxidase; SEM, standard error mean; TNF-α, tumor necrosis factor alpha.

treatment ($P = 0.001$) restored expression completely.

MD-1 reduced hepatic TAG accumulation and regulates the activity of enzymes involved in carbohydrate metabolism

Assessment of hepatic TAG (Fig. 5a) levels and activities of GK (Fig. 5b), F16Pase (Fig. 5c), and G6Pase (Fig. 5d) in HFD-fed mice after three weeks of MD-1 treatment showed an increase (approximately three-fold, $P = 0.00027$) in hepatic TAG content and a significant enhancement in F16Pase and G6Pase activities (approximately three-fold) relative to NC animals. GK activity was

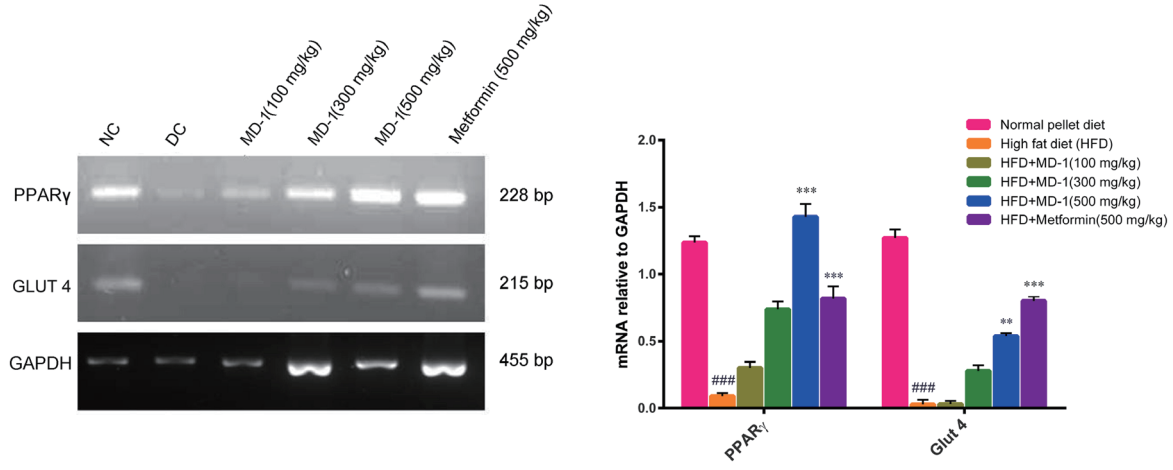

Fig. 4. Effect of MD-1 on mRNA expression of PPAR-γ and Glut4 in epididymal fat pads. mRNA was extracted from adipose tissues and subjected to RT-PCR analysis with indicated primers. PCR bands were quantified using Image J software. Data represent mean ± SEM (n = 6 per group). $^{**}P < 0.01$, $^{***}P < 0.001$ (One-way ANOVA followed by Dunnett's post hoc test) in comparison with HFD control. $^{###}P < 0.01$ vs. Normal pellet diet control. ANOVA, analysis of variance; DC, drug control lysate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Glut4, glucose transporter 4; HFD, high-fat diet; mRNA, messenger RNA; NC, negative control lysate; PPAR-γ, peroxisome proliferator-activated receptor gamma; RT-PCR, real time -polymerase chain reaction; SEM, standard error mean.

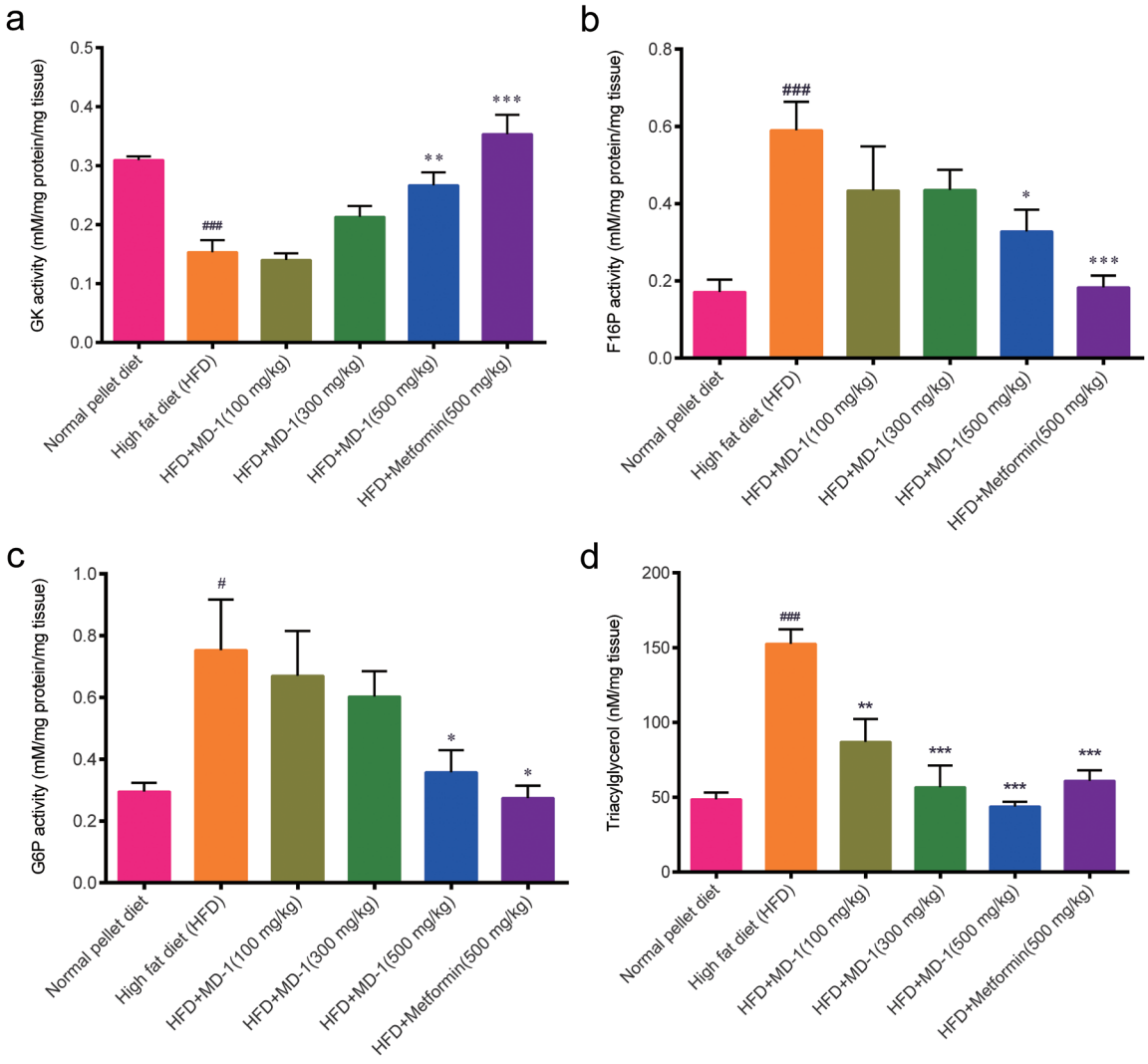

Fig. 5. Effect of MD-1 on enzymes regulating glucose metabolism in the liver and lipid accumulation. (a) Glucokinase; (b) Fructose-1,6-bisphosphatase (F16P); (c) Glucose 6 phosphatase (G6P); (d) Triacylglycerol. Data represent mean ± SEM (n = 6 per group). *P < 0.05, **P < 0.01, ***P < 0.001 (One-way ANOVA followed by Dunnett's post hoc test) in comparison with HFD control. ###P < 0.01 vs. Normal pellet diet control. ANOVA, analysis of variance; HFD, high-fat diet; SEM, standard error mean.

significantly reduced (approximately 1.5-fold, P = 0.00027) in HFD mice compared to NC mice. MD-1 administration for three weeks significantly (P = 0.001) reduced hepatic TAG deposits, with marginal reductions in GK, F16Pase, and G6Pase activities (HFD + MD-1 500 mg/kg b.wt, P = 0.028). The metformin-treated group showed significant restoration of these parameters to normal values.

MD-1 attenuated hepatic lipid peroxidation and enhanced antioxidant enzyme activities

Tissue antioxidants GSH (Fig. 6a), GPx (Fig. 6b), and SOD (Fig. 6c) levels were significantly reduced (P = 0.004), while lipid peroxidation (Fig. 6d) increased by 55% in the HFD-fed group compared to normal controls. Three weeks of MD-1 treatment restored SOD, GPx, and GSH levels to normal, and significantly reduced lipid peroxidation (HFD + MD-1 500 mg/kg b.wt, P < 0.001). These results establish the protective effect of MD-1 against HFD-induced oxidative stress.

Effect of MD-1 on liver, WAT, kidney, and skeletal muscle tissue

Hepatic steatosis and chronic oxidative stress can progress to non-alcoholic fatty liver disease (NAFLD) as observed in histological examination of liver sections (Fig. 7) from HFD-fed mice after 13 weeks (Fig. 7b). After three weeks of MD-1 treatment, steatosis and macrophage infiltration were reduced in a dose-dependent manner (Fig. 7c–e). Chronic inflammation and moderate macrophage infiltration were observed in skeletal muscle and kidney sections of HFD mice, which were reduced to normal levels in MD-1-treated animals (Figs. 8 and 9).

Discussion

MD-1, constituted of traditional medicinal herbs popularly used

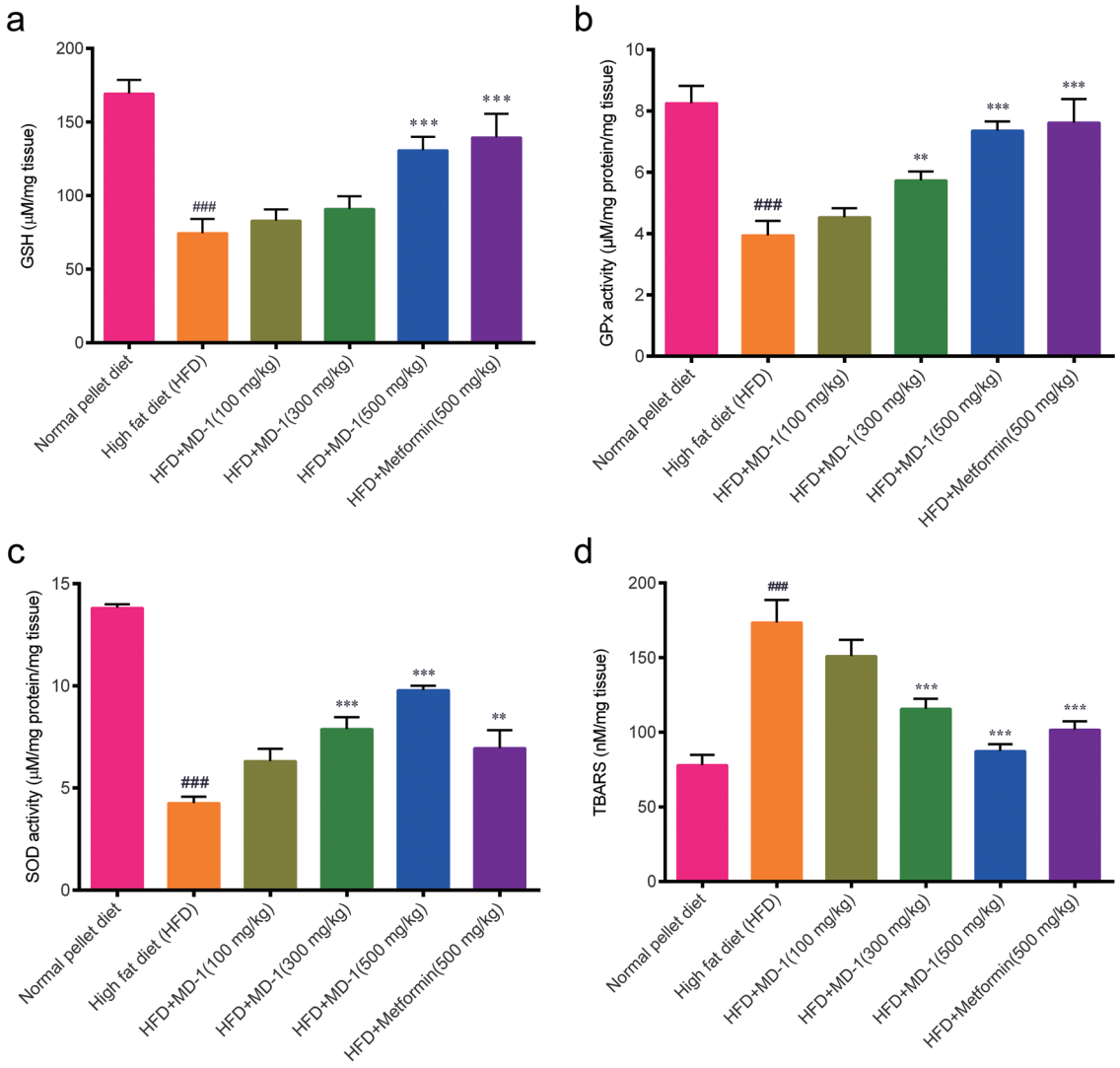

Fig. 6. Effect of MD-1 on oxidative stress parameters in liver. (a) GSH; (b) GPX; (c) SOD; (d) Lipid peroxidation. Data represent mean ± SEM (n = 6 per group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (One-way ANOVA followed by Dunnett's post hoc test) in comparison with HFD control. ###*P* < 0.01 vs. Normal pellet diet control. ANOVA, analysis of variance; GPX, glutathione peroxidase; GSH, glutathione; HFD, high-fat diet; SEM, standard error mean; SOD, super oxide dismutase.

in the treatment of diabetes, has been shown to ameliorate HFD-induced diabetes in C57BL/6J mice, primarily by improving adipose tissue function. Following 10 weeks of HFD feeding, diabetes induction was confirmed by a significant increase in FBG levels. The accompanying increases in body weight and circulating TAG and TC levels further confirmed the development of metabolic syndrome. Notably, a three-week administration of MD-1 mediated a decrease in FBG without a change in body weight or calorie intake. However, all treatment groups showed a reduction in progressive growth rate during the treatment period. Animals treated with MD-1 at 500 mg/kg b.wt showed a slight decrease in body weight, suggesting that prolonged administration of MD-1 could potentially reduce excess body weight. Reduced TAG accumulation in MD-1-treated 3T3L-1 adipocytes further supports the observed body weight reduction in HFD-fed mice. The rich gallic acid content of MD-1 is noteworthy, given literature supporting gallic acid's protective effect against HFD-induced weight gain without changes in calorie intake.[8,22]

TNF-α infiltration induces lipolysis in adipose tissue and increases fatty acid mobilization to the liver.[23] Excess lipid storage in the liver, accompanied by macrophage infiltration and oxidative stress, implicates NAFLD in HFD-fed mice. Histological observations revealed a clear reduction of macrophage infiltration in the liver sections of MD-1-treated HFD mice.[24] Hepatic steatosis observed in HFD mice improved dose-dependently after three weeks of MD-1 treatment, and at the higher dose of 500 mg/kg b.wt, normal liver architecture was completely restored. Biochemical analysis of liver tissue homogenates also showed reduced TAG content and lipid peroxidation following MD-1 treatment in HFD mice. Additionally, tissue antioxidants SOD, GPx, and GSH were significantly restored to normal levels in MD-1-treated HFD mice at 500 mg/kg b.wt. The observed improvements in HFD-induced steatosis and oxidative stress suggest a possible two-hit mechanism of action. The considerable phenol and flavonoid content of

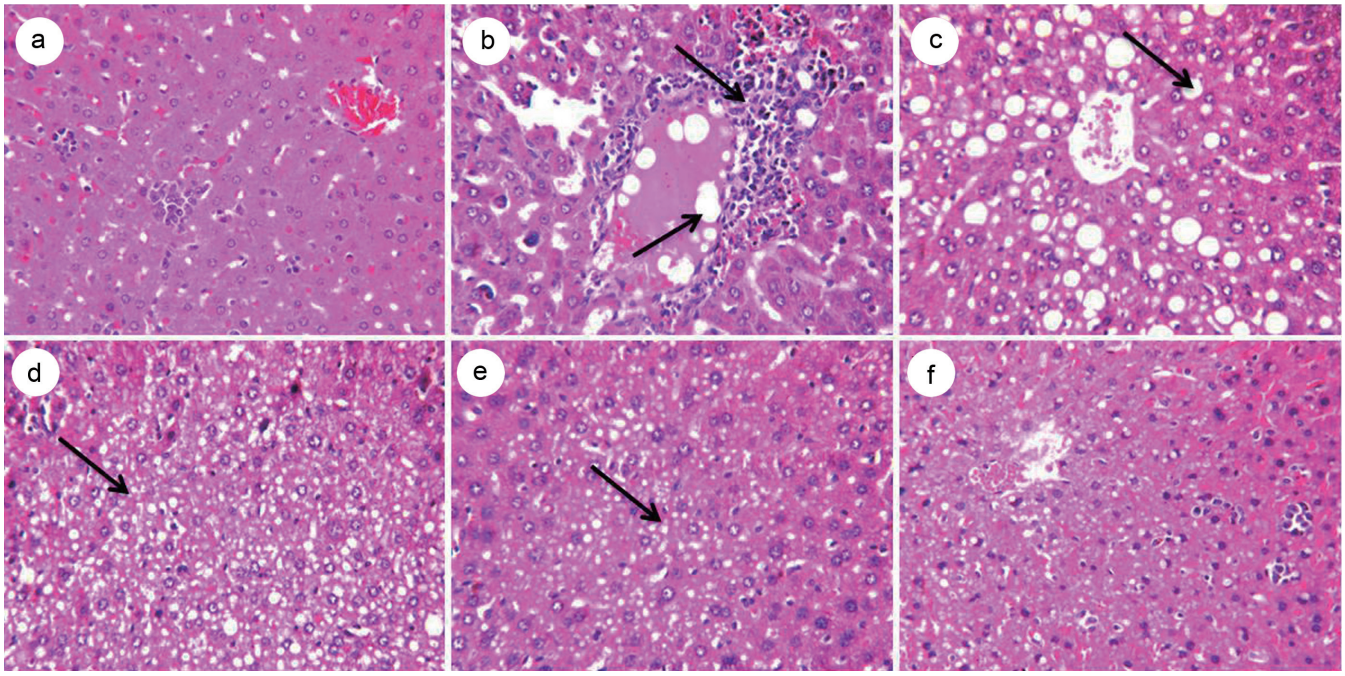

Fig. 7. Effect of MD-1 on hepatic steatosis. Representative photomicrographs of H&E staining in liver tissues are shown at a magnification of ×400. (a) Normal pellet diet control – Normal tissue architecture; (b) HFD control – Presence of fatty liver and infiltration; (c) HFD+MD-1(100 mg/kg b.wt) – Presence of fatty liver and infiltration; (d) HFD+MD-1 (300 mg/kg b.wt) – Presence of fatty liver with reduced infiltration; (e) HFD+MD-1 (500 mg/kg b.wt) – Normal tissue architecture with reduced infiltration; (f) HFD + Metformin (500 mg/kg b.wt) – Normal tissue architecture. HFD, high-fat diet; H&E, hematoxylin & eosin.

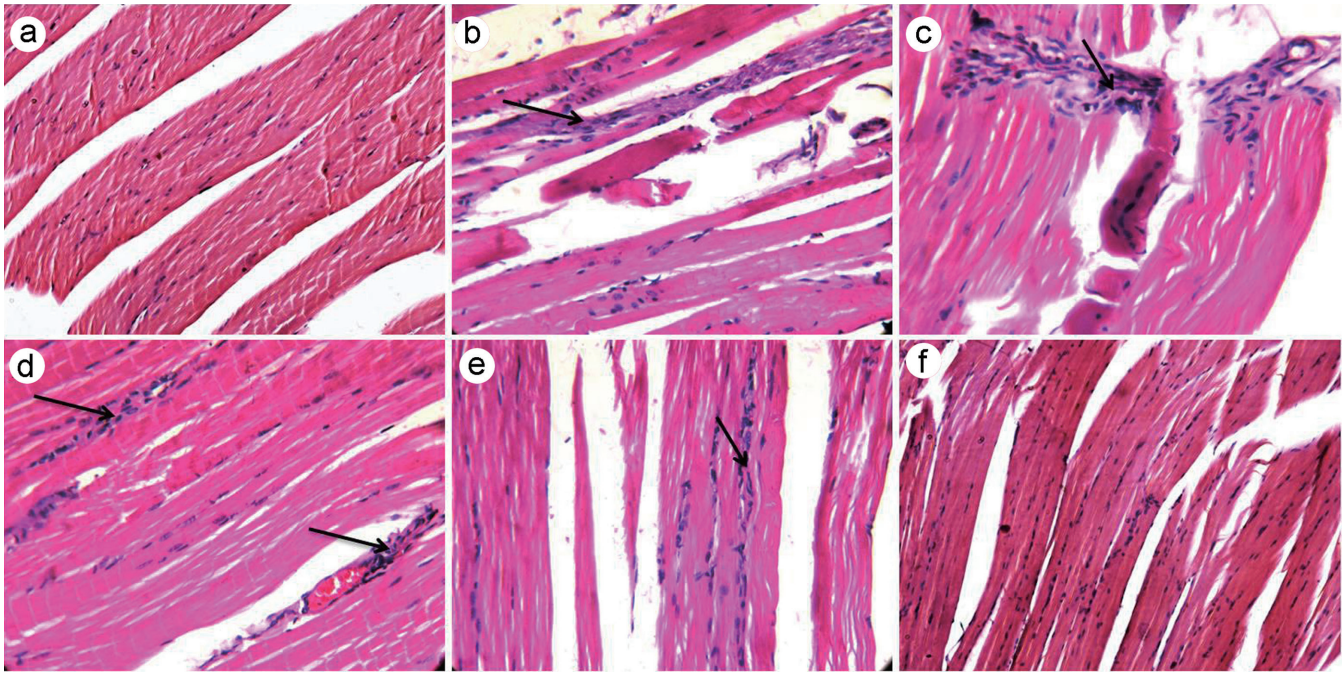

Fig. 8. Effect of MD-1 on skeletal muscle. Representative photomicrographs of H&E staining in liver tissues are shown at a magnification of ×400. (a) Normal pellet diet control – Mild mono nuclear cell infiltration; (b) HFD control – Moderate mono nuclear cell infiltration; (c) HFD+MD-1(100 mg/kg b.wt) – Mild mono nuclear cell infiltration; (d) HFD+MD-1 (300 mg/kg b.wt) – Mild mono nuclear cell infiltration; (e) HFD+MD-1 (500 mg/kg b.wt) – Mild mono nuclear cell infiltration; (f) HFD + Metformin (500 mg/kg b.wt) – Mild mono nuclear cell infiltration. HFD, high-fat diet; H&E, hematoxylin & eosin.

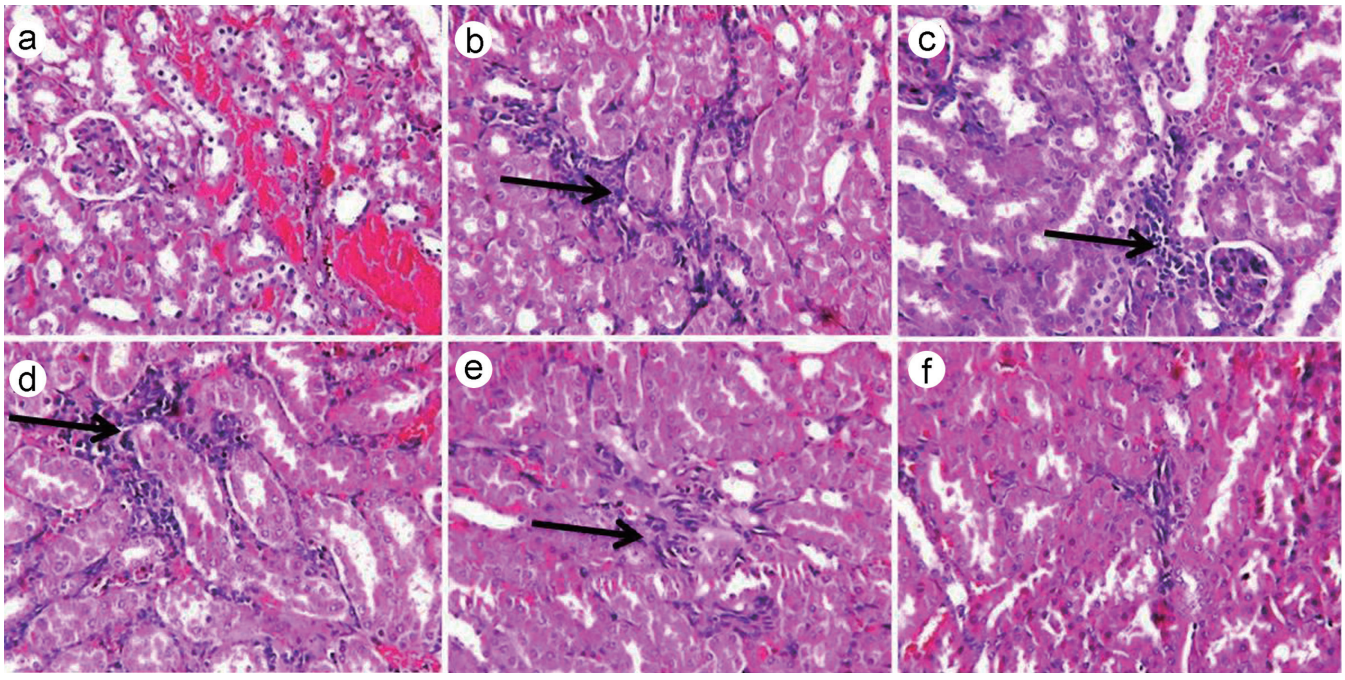

Fig. 9. Effect of MD-1 on the Kidney tissue. Representative photomicrographs of H&E staining in kidney tissue sections were shown at a magnification of ×400. (a) Normal pellet diet control - Normal architecture; (b) HFD control – Severe mono nuclear cell infiltration in the interstitium; (c) HFD+MD-1(100 mg/kg b.wt) – Severe mono nuclear cell infiltration; (d) HFD+MD-1 (300 mg/kg b.wt) – Moderate mono nuclear cellinfiltration; (e) HFD+MD-1 (500 mg/kg b.wt) – Mild mono nuclear cell infiltration; (f) HFD + Metformin (500 mg/kg b.wt) – Mild mono nuclear cell infiltration. HFD, high-fat diet; H&E, hematoxylin & eosin.

MD-1 likely supports the favorable modulation of oxidative stress induced by HFD.

Excess TAG deposits can induce gluconeogenesis and promote insulin resistance in the liver.[25] However, three weeks of MD-1 treatment did not significantly affect glucose-metabolizing enzymes GK, F16Pase, or G6Pase compared to standard metformin, suggesting that MD-1 may exert its effects peripherally on lipid and glucose homeostasis. The reduced lipolysis observed *in vitro* in differentiated adipocytes treated with MD-1 further supports *in vivo* findings of decreased hepatic TAG in HFD mice.

TNF-α signaling primarily targets the mRNA expression of the adipogenic master regulator PPAR-γ and its transcriptional activity in WAT tissue, leading to tissue remodeling and impaired adipogenesis.[26] Enhanced PPAR-γ transcriptional activity, as evidenced by the increased expression of its target gene Glut4 in WAT tissue, suggests that MD-1 reverses HFD-induced T2D by targeting adipose tissue.

Activation of the AMPK pathway is attributed to several phytochemicals, and the reduction in adipose tissue lipolysis caused by MD-1 suggests a similar phytoremediation effect due to its multi-constituent composition.[27] Quantification of major markers of MD-1 by HPLC could not be performed due to the physicochemical nature of the total extract, which could potentially clog the expensive column. Additionally, Glut4 translocation assays, which could have added clarity to the study findings, were not conducted, representing another limitation.

Conclusions

MD-1 administration primarily targeted TNF-α signaling in WAT tissue of HFD mice, thereby restoring adipose tissue function. The concomitant reduction in hepatic TAG, accompanied by decreased macrophage infiltration, suggests protection against NAFLD in HFD mice. Reduction of blood glucose levels from diabetic to pre-diabetic levels and a considerable decrease in body weight (HFD + MD-1 500 mg/kg b.wt) after three weeks of MD-1 administration indicate its protective role in obesity-associated DM. The beneficial effects of MD-1 supplementation in diet-induced metabolic syndrome reinforce the scientific validity of traditional knowledge systems predating the modern scientific era.

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Conflict of interest

The authors declare no conflicts of interest or financial disclosures.

Author contributions

Study concept (TS, MK), study design (TS, MK), preclinical experiments, data analysis, drafting of the manuscript (TS), supervision of data collection, review (MK, SLJ, CJ), revision (MK), conception and analysis of biochemical observations (SLJ), conception and performance of cell culture studies, data analysis, and

data interpretation (CJ). All authors read and approved the final manuscript.

Ethical statement

All experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of Sri Ramachandra Institute of Higher Education and Research (DU), constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (IAEC No: IAEC/XXXXV/SRU/439/2015). All animal experiments were conducted by the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize animal suffering.

Data sharing statement

The Supplementary Data, used in support of the findings of this study are available from the corresponding author at kvmanga@yahoo.com upon request.

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