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Moringa Oleifera Leaves Extract Ameliorated Obesity Induced Hyperglycemia in Rats via Modulating the Expression of Adipocytokines

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Abstract
The study aimed to investigate the mechanism behind the hypoglycemic effects of *Moringa Oleifera*leaves extract (MOLAE) in rats fed high fat diet (HFD) induced hyperglycemia. A total of 56 male Wister adult albino rats were assigned into seven groups. The control groupwas fed a standard diet. High fat diet group wasfed HFD for 8 weeks. The third group was fed a standard diet supplemented with 400 mg/kg of MOLAE for 8 weeks. The fourth and fifth groups were fed HFD with MOLAE at doses of 200 mg/kg and 400 mg/kg respectively for 8 weeks. The sixth and seventh groups werefed HFD for 8 weeks then fed standard dietsupplemented with 200 mg/kg and 400 mg/kg MOLAE respectively for additional 4 weeks. High fat diet elevated fasting blood sugar, glycated hemoglobin, Complement C1q tumor necrosis factor-related protein 15 (myonectin), and Retinol binding protein 4 (RBP4) levels while decreased serum levels of insulin like growth factor 1 (IGF-1). In addition, it decreased uncoupling protein 1 (UP1) proteinexpression in white and brown adipose tissues, and glucose transporter 4 (GLUT4) gene expression in muscle and white adipose tissue. Conversely, supplementation of HFD fed rats with MOLAE significantly reduced fasting blood sugar, glycated hemoglobin, myonectin, and RBP4 levels while increased serum level of IGF1, UCP1 protein expression in white and brown adipose tissues, and GLUT4 gene expression in muscle and while adipose tissue This study indicated that MOLAE exhibits potential in mitigating HFD-induced hyperglycemia in obese rats, possibly through modulating the expression of adipocytokines that have impacts on blood glucose levels.

Keywords: *Moringa oleifera*leaves extract, HFD, hyperglycemia, myonectin, UCP1 and GLUT4.

1. Introduction

Diabetes mellitus (DM) is a group of metabolic conditions characterized by chronic hyperglycemia, resulting from impaired insulin release, insulin function, or both [1]. It encompasses two primary types: type 1 DM (DM1), which results from the destruction of pancreatic β-cells leading to a complete lack of insulin, and type 2 DM (DM2), primarily associated with insulin resistance, relative insulin insufficiency, and disruptions in insulin secretion [1]. The International Diabetes Federation (IDF) projects that approximately 592 million individuals will be affected by DM by 2035, with Egypt ranking 8th of the top 10 countries for the number of adults with diabetes globally, where 8.2million adults were diabetic in early 2018. That number was predicted to be doubled by 2045, and Egypt will climb to number 6th rank in the top 10 list [2]. The escalating global issue of obesity has long been recognized as a risk factor for prediabetes and diabetes [3]. Obesity, defined by the World Health Organization, refers to an abnormal or excessive accumulation of body fat that can compromise health. Excessive body fat is closely associated with the development of type 2 diabetes, with the risk increasing linearly in correlation with body mass index [4]. Consuming a high-fat diet (HFD) has been linked to an elevated risk of metabolic syndromes, including obesity, type 2 diabetes, and cardiovascular diseases (CVDs) [5]. The path to developing obesity and diabetes is influenced by multiple factors, such as adipokines, inflammatory cytokines, growth factors, insulin resistance, and hyperlipidemia [6]. Retinol binding protein 4 (RBP4), derived from adipocytes and hepatocytes, plays a role in retinol transport to systemic tissues and has been implicated in contributing to insulin resistance, obesity, and metabolic syndrome [7].

The relationship between glucose transporter 4 (GLUT4) expressions and the level of insulin resistance (IR) is well-established [8]. Insulin is responsible for promoting glucose transport in muscle and adipocytes by facilitating the ordered delivery of intracellular GLUT4-containing vesicles to the plasma membrane, leading to increased surface levels of GLUT4 [9]. Insulin-like growth factor I (IGF-I), structurally like insulin, is primarily produced by the liver when stimulated by growth hormone, and it complements the regulation of glucose [10].

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Complement C1q tumor necrosis factor-related protein 15 (myonectin) (CTRP15), a relative of adiponectin, has demonstrated positive effects on glucose and lipid metabolism [11]. Elevated CTRP15 levels in patients and its association with IR and inflammation indicates a potential link between CTRP15 and diabetes pathogenesis [12].

Mammals possess brown and beige thermogenic fat cells, or adipocytes, which are abundant in mitochondria and express uncoupling protein 1 (UCP1) [13]. When activated, brown and beige adipocytes efficiently uptake high levels of glucose and lipids for heat production, acting as a metabolic sink to remove excess nutrients from the bloodstream. This contributes to insulin sensitivity, as well as overall lipid and glucose metabolism, and can aid in weight management. White, brown, and beige adipocytes collaborate to maintain the body's energy balance [14].

A single genetic mutation affects about 3% of diabetic patients ,as more than 20 genes are involved in the function of beta cells. Improved therapy, better prediction of disease prognosis and progression, genetic counseling, and possibly prevention are all benefits of gene diagnosis [15].

Clinical trials investigating the efficacy of herbal remedies for diabetes are necessary [16]. Consequently, there is a pressing need to identify new biomolecules that can effectively contribute to diabetes treatment, given the potential side effects of existing medications [17].

Moringa Oleifera, also known as "drumstick," is a tree belonging to the Moringaceae family and the Moringa genus. It is native to the Himalayas but is now cultivated in numerous tropical and subtropical regions worldwide [18]. Of the three widespread Moringa species, *Moringa Oleifera*, *Moringa Stenopetala*, and *Moringa peregrine*, most parts of the plant, including the bark, roots, leaves, flowers, and seeds, have been utilized for treating various illnesses and for producing drugs to combat bacteria, fungi, viruses, and other pathogens affecting humans. This is because of the significant presence of bioactive phytochemicals such as polysaccharides, alkaloids, flavonoids, isothiocyanates, and glucosinolates. *Moringa Oleifera*has a long history of use as a natural antidiabetic herb in India and other Asian countries, piquing the interest of researchers [19]. While there is strong evidence for the hypoglycemic effects of *Moringa Oleifera*extract in diabetic animal models, its effects on humans remain less clear [20]. Thus, this study aimed to explore the potential mechanisms through which *Moringa Oleifera*leaves extract exert its hypoglycemic effects in rats fed high fat diet induced hyperglycemia.

2. Results

2.1. Moringa Oleifera leaves alcoholic extract ameliorated HFD induced hyperglycemia and altered kidney functions biomarkers in rats.

Rats fed HFD showed a significant increase in blood glucose and HbA1c levels and serum creatinine and urea levels compared to the control group fed the standard basal diet .However administration of rats fed HFD with MOLAE at doses 200 and 400 mg/kg BW from the beginning of the experiment significantly reduced fasting blood glucose , HbA1c ,serum creatinine and urea levels compared withrats fed HFD. Also, treating obese rats that fed HFD with MOLAE at dose of 200 and 400 mg/kg BW for 8 weeks significantly reduced fasting blood glucose , HbA1c , creatinine and urea levels compared with rats fed HFD (Table 1).

In the other hand administration of rats with MOLAE showed no significant changes in the fasting blood glucose and HbA1c levels compared with the control group (Table 1).

Table 1: Effect of high fat diet and/ or *Moringa Oleifera*leaves alcoholic extract on fasting blood glucose, glycated hemoglobin and kidney functions biomarkers level

	Control	MOLAE	HFD group	HFD and MOLAE 200	HFD and MOLAE 400	HFD then MOLAE 200	HFD then MOLAE 400
FBG (mg/dL)	89.21±7.94^{c}	102.06±10.30^{c}	164.47±30.39^{a}	123.38±10.14^{b}	118.75±3.24^{b}	140.75±4.65^{b}	128.63±6.41^{b}
HbA1c(%)	3.46±0.26^{b}	3.41±0.29^{b}	4.29±0.30^{a}	3.71±0.20^{b}	3.51±0.24^{b}	3.73±0.16^{b}	3.64±0.23^{b}
Creatinine (mg/dl)	0.64±0.13^{c}	0.66±0.10^{c}	0.80±0.15^{a}	0.76±0.12^{b}	0.78±0.11^{b}	0.77±0.12^{b}	0.74±0.12^{b}
Urea (Mg/dl)	29.92±4.92^{c}	26.13±5.11^{d}	52.58±6.70^{a}	30.71±3.67^{bc}	32.61±4.35^{bc}	41.00±2.67^{b}	32.14±4.38^{bc}

Values were expressed as means±SD (standard deviation).Values carrying different superscript letters in the same ROW were significantly different at ($P < 0.05$) .

MOLAE, *Moringa Oleifera* leaves alcoholic extract; HFD, high fat diet; HbA1c: glycated hemoglobin; FBG : fasting blood glucose; MOLAE 200, *Moringa Oleifera*leaves alcoholic extract concentration (200 mg/kg BW), MOLAE 400, *Moringa Oleifera* leaves alcoholic extract concentration (400 mg/kg BW)

2.2. Moringa Oleifera leaves alcoholic extract modulated the effects of HFD on serum levels of adipocytokines in rats.

Rats fed HFD exhibited a notable rise in their RBP4 levels compared with the control group. However, the administration of HFD-fed rats with MOLAE at doses of 200 and 400 mg/kg BW from the beginning of the experiment effectively normalized their serum RBP4 levels in contrast to HFD-fed rats. Likewise, treating obese rats fed HFD with MOLAE at these doses for 8 weeks also led to a significant reduction in RBP4 levels compared with HFD fed rats. Notably, the administration of MOLAE to rats alone, without HFD, did not induce significant changes in serum RBP4 levels compared with the control group (Table 2).

Table 2: Effect of high fat diet and/ or *Moringa Oleifera* leaves alcoholic extract on Retinol binding protein 4 , Complement C1q tumor necrosis factor-related protein 15 (myonectin) and Insulin Like Growth Factor 1 serum levels

	Control	MOLAE	HFD group	HFD and MOLAE 200	HFD and MOLAE 400	HFD then MOLAE 200	HFD then MOLAE 400
RBP4 (ng/ml)	4.11 ± 0.52^{b}	4.44 ± 0.47^{b}	52.60 ± 10.22^{a}	4.47 ± 1.05^{b}	4.08 ± 0.30^{b}	4.91 ± 1.23^{b}	3.57 ± 0.25^{b}
CTRP15 (myonectin) (ng/ml)	2.55 ± 1.14^{c}	3.33 ± 0.67^{c}	35.14 ± 13.68^{a}	17.88 ± 2.96^{b}	8.96 ± 3.42^{c}	6.6 ± 2.48^{c}	4.05 ± 0.99^{c}
IGF1 (pg/mL)	43.24 ± 8.58^{b}	54.55 ± 15.16^{a}	4.69 ± 3.82^{d}	14.5 ± 8.4^{c}	8.86 ± 5.89^{c}	7.94 ± 2.4^{c}	8.29 ± 4.98^{c}

Values were expressed as means ± SD (standard deviation), Values carrying different superscript letters in the same ROW were significantly different at ($P < 0.05$).

MOLAE*, Moringa Oleifera leaves alcoholic extract;* HFD, high fat diet; MOLAE 200, *Moringa Oleifera*leaves alcoholic extract concentration (200 mg/kg BW); MOLAE 400*, Moringa Oleifera*leaves alcoholic extract concentration (400 mg/kg BW); RBP4, Retinol binding protein 4; CTRP15 (myonectin), Complement C1q tumor necrosis factor-related protein 15; IGF1, Insulin Like Growth Factor 1.

Furthermore, rats fed HFD experienced a substantial increase in their CTRP15 levels compared with the control group that was on a standard basal diet. Nonetheless, administering HFD-fed rats with MOLAE at doses of 200 and 400 mg/kg BW from the beginning of the study significantly reduced CTRP15 levels compared with rats solely fed HFD. Similarly, treating obese rats fed HFD with MOLAE at these doses for 8 weeks also resulted in a significant decrease in CTRP15 levels in comparison with HFD-fed rats. It's worth noting that the administration of MOLAE to rats in the absence of HFD did not lead to significant changes in CTRP15 levels compared withthe control group (Table 2).
Moreover, rats fed HFD exhibited a significant reduction in their serum levels of IGF1 compared with the control group on a standard basal diet. However, the administration of HFD-fed rats with MOLAE at doses of 200 and 400 mg/kg BW from the beginning of the experiment significantly increased their serum IGF1 levels in comparison with HFD-fed rats. Additionally, treating HFD-fed rats with MOLAE at these doses led to a significant increase in serum IGF1 levels when compared with HFD-fed rats. Furthermore, the administration of MOLAE to rats fed standard basal diet showed a notable increase in serum IGF1 levels compared with the control group (Table 2).

Moringa Oleifera leaves alcoholic extract modulated HFD altered Glucose transporter 4 gene expression in muscles and white adipose tissues:

In the context of HFD, there was a numerical reduction in GLUT4 mRNA expression in muscles compared with the control group fed a standard basal diet. However, the administration of MOLAE to HFD-fed rats at a dose of 400 mg/kg BW from the beginning of the experiment significantly boosted the expression of the GLUT4 gene in muscles compared with HFD-fed rats. Furthermore, treating obese rats with MOLAE at doses of 200 and 400 mg/kg body weight also led to a significant increase in GLUT4 gene expression in muscles compared with HFD-fed rats (Fig. 1A).

In a similar vein, HFD resulted in a significant decrease in GLUT4 mRNA expression in white adipose tissue compared with the control group. Nevertheless, the administration of MOLAE to rats prompted a significant increase in GLUT4 gene expression in white adipose tissue compared with the control group. Furthermore, administering HFD-fed rats with MOLAE at doses of 200 and 400 mg/kg BW from the outset of the experiment led to a significant increase in GLUT4 gene expression in white adipose tissue in comparison with HFD-fed rats (Fig. 1B).

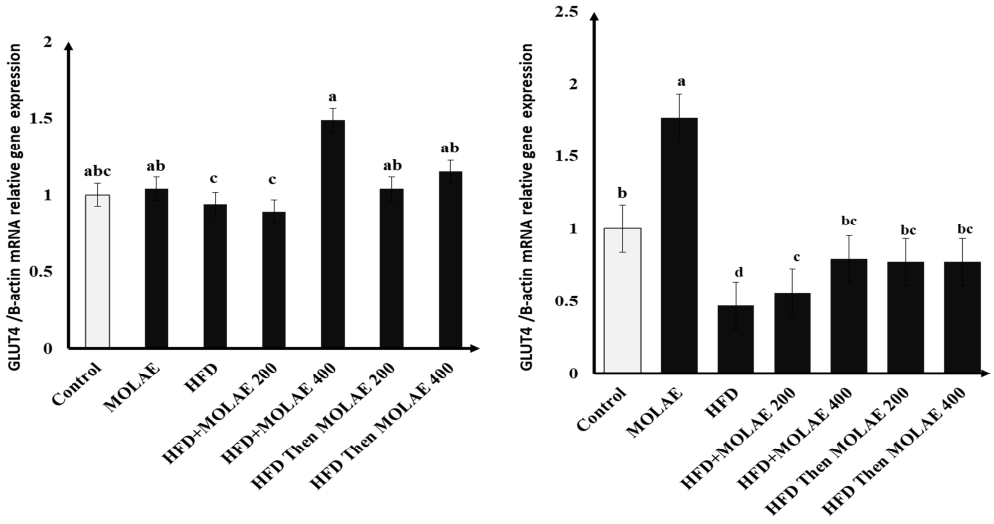

Figure 1: showed the effect of high fat diet and / or *Moringa Oleifera* leaves alcoholic extract on Glucose transporter 4 gene expression in muscles (A) and in white adipose tissues (B) gene expression.

2.3. Moringa Oleifera leaves alcoholic extract increased UCP1 protein expression in white adipose tissues of rats fed HFD:

As depicted in figure 2, the protein expression of UCP1 in white adipose tissue exhibited noteworthy variations across the control and treated groups. Notably, feeding rats HFD led to a significant reduction ($p < 0.05$) in UCP1 protein expression in white adipose tissues compared with the control group fed a standard basal diet (Fig. 2A& H). However, administration of MOLAE to rats at a dose of 400 mg/kg body weight resulted in a significant increase ($p < 0.05$) in UCP1 protein expression in white adipose tissues compared with the control group (Fig. 2B&H). Furthermore, treating obese rats with MOLAE at doses of 200 and 400 mg/kg body weight significantly elevated UCP1 protein expression in white adipose tissuescompared with rats solely fed HFD (Figure 2D, E&H, respectively). Additionally, administration of MOLAE to HFD-fed rats from the beginning of the experiment significantly increased protein expression of UCP1 in white adipose tissues compared with rats fed HFD (Figure 2F, G& H, respectively).

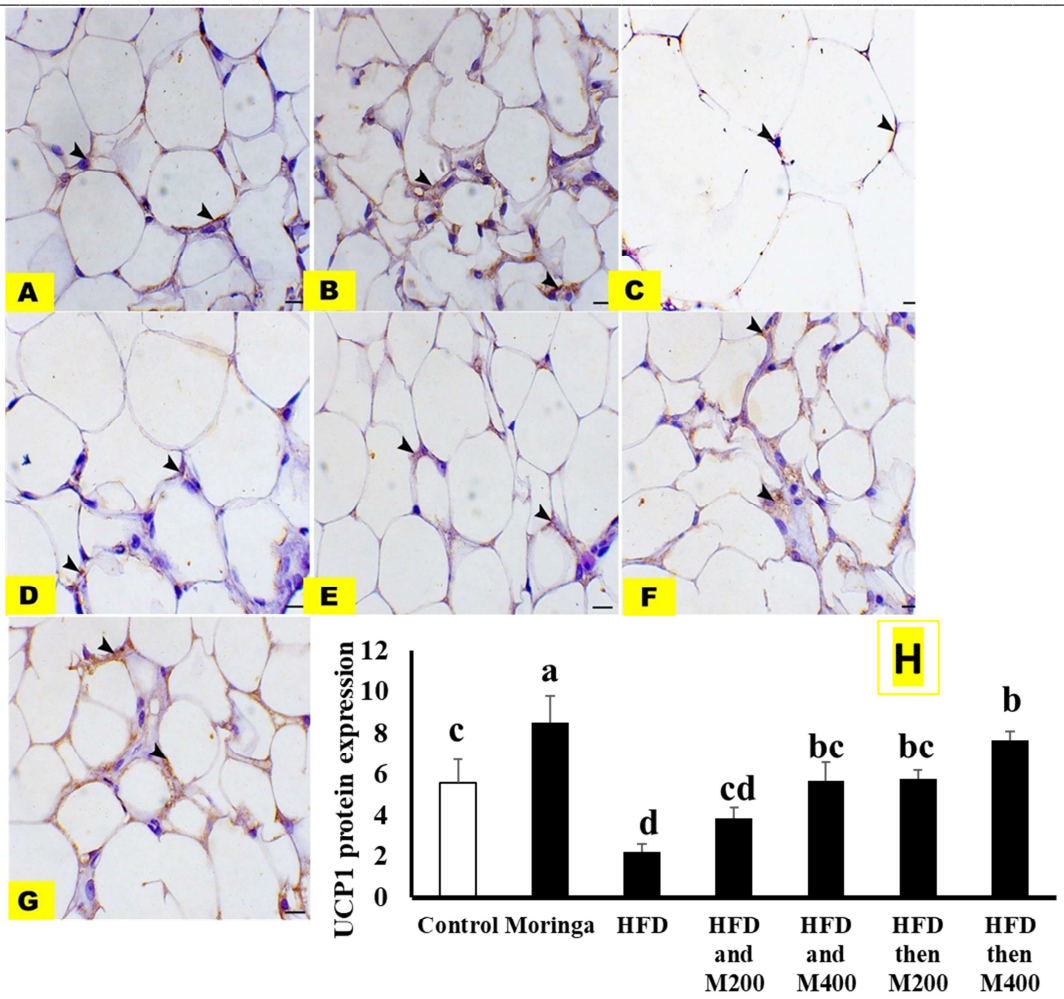

Figure 2: Showed the effect of high fat diet and/ or *Moringa Oleifera*leaves alcoholic extract on uncoupling protein 1in white adipose tissues levels.**A**: White adipose tissue of control animal showing mild cytoplasmic expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**B**: White adipose tissue of normal animal treated with MOLAE showing marked increase the cytoplasmic expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**C**: White adipose tissue of HFD animal showing marked decrease the expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**D**: White adipose tissue of HFD animal treated with MOLAE at a dose of 200 mg/kg showing increased expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**E**: White adipose tissue of HFD animal treated with MOLAE at a dose of 400 mg/kg showing marked increase UCP1 expression within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 **F**: White adipose tissue of HFD animal pretreated with MOLAE at a dose of 200 mg/kg showing marked increase of UCP1 expression within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**G**: White adipose tissue of HFD animal pretreated with MOLAE at a dose of 400 mg/kg showing marked increase the expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**H**: Un coupling protein expression (UCP1) of White Adipose Tissue

2.4. Moringa Oleifera leaves alcoholic extract increased UCP1 protein expression in brown adipose tissues of rats fed HFD

Figure 3 displayed the protein expression of UCP1 in brown adipose tissue within the control and treated groups. Notably, the consumption of HFD led to a significant reduction ($p < 0.05$) in the protein expression of UCP1 in brown adipose tissues compared with the control group fed standard basal diet (Fig. 3A&H). However, administration of MOLAE to rats at a dose of 400 mg/kg body weight resulted in a significant increase ($p < 0.05$) in UCP1 protein expression in brown adipose tissues compared with the control group (Fig. 3B&H). Furthermore, treating obese rats with MOLAE at doses of 200 and 400 mg/kg body weight significantly elevated the protein expression of UCP1 in brown adipose tissues compared with rats solely fed HFD (Fig. 3D,E&H, respectively). Additionally, the administration of MOLAE to HFD-fed rats from the beginning of the experiment significantly increased the protein expression of UCP1 in brown adipose tissues compared with rats on an HFD alone (Fig. 3F, G &H, respectively).

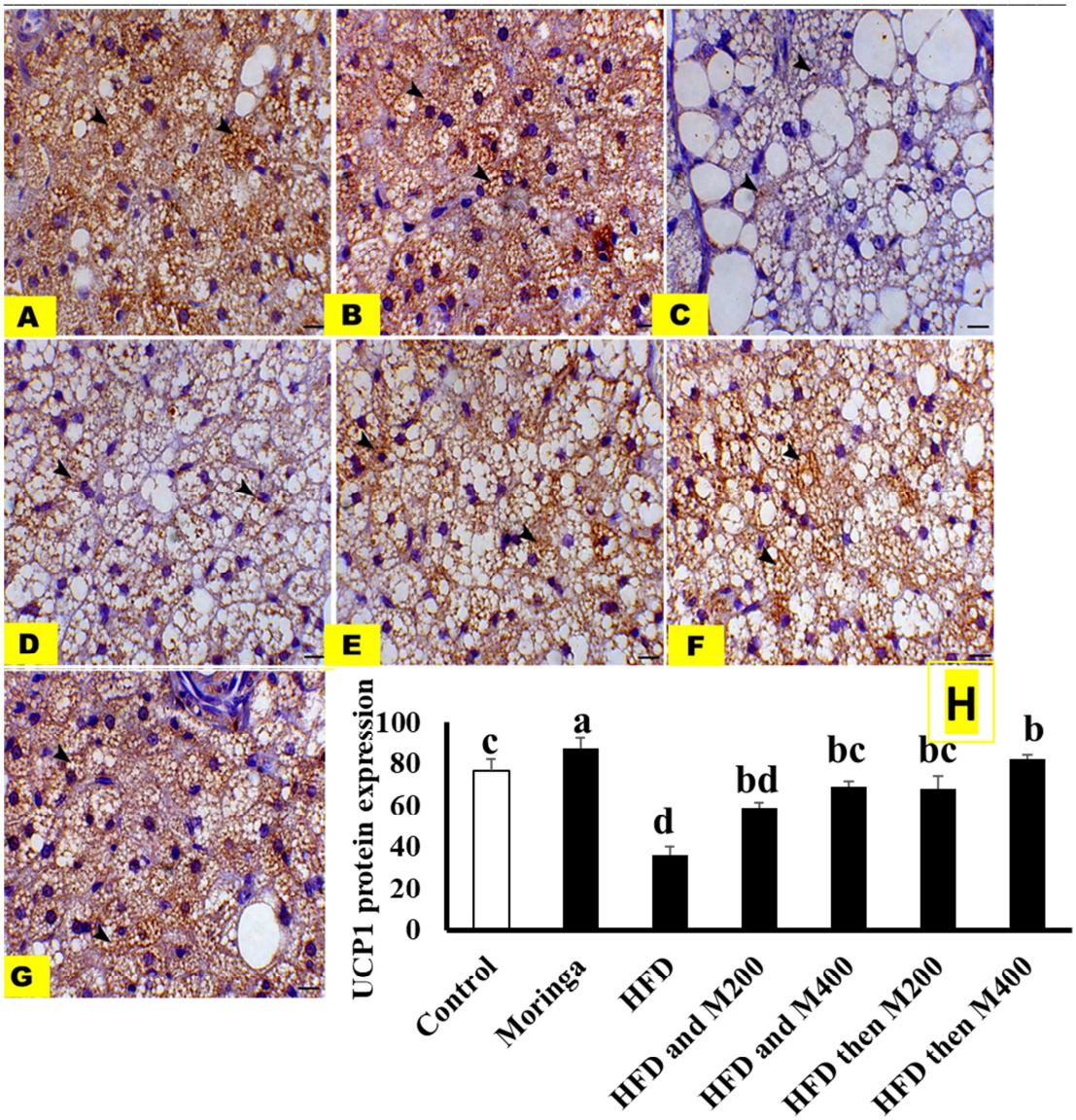

Figure 3: showed the effect of high fat diet and/ or *Moringa Oleifera*leaves alcoholic extract on uncoupling protein 1 in brown adipose tissues levels. **A**: Brown adipose tissue of control animal showing marked cytoplasmic expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**B**: Brown adipose tissue of normal animal treated with MOLAE showing marked increase the cytoplasmic expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**C**: Brown adipose tissue of HFD animal showing marked decrease the expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**D**: . Brown adipose tissue of HFD animal treated with MOLAE at a dose of 200 mg/kg showing increased expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**E**: Brown adipose tissue of HFD animal treated with MOLAE at a dose of 400 mg/kg showing marked increase the expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**F**: Brown adipose tissue of HFD animal pretreated with MOLAE at a dose of 200 mg/kg showing marked increase the expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**G**: Brown adipose tissue of HFD animal pretreated with MOLAE at a dose of 400 mg/kg showing marked expression of UCP1 within the adipocytes (arrowheads), UCP1 IHC, X200, bar= 50 µm.**H**: Un coupling protein expression (UCP1) of Brown Adipose Tissue.

3. Discussion

Diabetes mellitus (DM) is a complex metabolic disorder influenced by a myriad of genetic and environmental factors [21]. One well-established risk factor for the development of DM is obesity. In rodent models, obesity can be effectively induced through the consumption of highly palatable, energy-dense diets, such as a high-fat diet [22]. This approach closely mimics the development of obesity in humans [23].

In this study, rats fed HFD exhibited hyperglycemia, as evidenced by significantly increased levels of blood glucose and HbA1c. Additionally, there were elevated levels of serum adipokine levels, particularly an increase in RBP4 and a decrease in

CTRP15 and IGF-1 serum levels. Furthermore, there was a noticeable reduction in the expression of UCP1 protein in both white and brown adipose tissues, along with alterations in and GLUT4 gene expression in muscle and white adipose tissue. These findings agreed with previous studies by Felber and Golay in 2002 [24] who highlighted the pivotal role of elevated plasma free fatty acids (FFA) and their dominant utilization by muscles in contributing to insulin resistance in obesity. Similarly, Montgomery et al [25] proposed various mechanisms responsible for the insulin resistance associated with obesity, encompassing factors such as inflammation, lipid deposition in non-adipose tissues, mitochondrial dysfunction, and oxidative stress.

Interestingly, the oral administration of rats fed HFD MOLAE led to a significant decrease in fasting blood glucose and HbA1c levels, which is consistent with findings reported by Kamrul et al. [26] and Metwally et al [27]. These studies have previously documented notable reductions in fasting blood glucose and insulin levels following the oral administration of *Moringa Oleifera*leaves extract to HFD-feedrats. This aligns with the studyof Bamagous et al [28]and Handayati et al [29], who observed significant declines in blood glucose and glycosylated hemoglobin (HbA1c) levels in rats undergoing *Moringa Oleifera* treatment. Additionally, a 3-month treatment with *Moringa Oleifera*was found to substantially lower fasting glucose levels, as evidenced by Schaffer et al [30].

The mechanisms underpinning the hypoglycemic properties of *Moringa Oleifera*are multifaceted and encompass various factors. Potential mechanisms may involve the deceleration of gastric emptying, which could be linked to the presence of quercetin-3-glucoside and dietary fiber within *Moringa* leaves, as suggested by Abdelazim et al [31]. Another plausible mechanism may be the downregulation of pyruvate carboxylase in the liver, coupled with the regeneration of damaged β-cells in the pancreas and hepatocytes. These effects have been associated with the antioxidant properties inherent in *Moringa Oleifera*, as described by Abd El Latif et al [32]. *Moringa* leaves contain alkaloid and steroid/triterpenoid compounds, which can stimulate pancreatic cells to secrete insulin, as proposed by Handayati et al [33]. Furthermore, the presence of carotenoids , flavonoids and phenolic acids in *Moringa* leaves may slow down intestine's glucose absorption, inhibition of intestinal α-glucosidase, and pancreatic α-amylase activity, ultimately contributing to the regulation of glucose levels and a reduced risk of diabetes development, in accordance with the findings of Zarina et al [34]. Another plausible mechanism may be the presence of vitamin C in *Moringa oleifera* leaves (1,89 mg/g) [35],The accession of vitamin C dominates the glucose of blood, advances endothelium dependent vasodilation and increases the resistance of lipoprotein in the direction of oxidation in the affected person by both sort one or sort two diabetes mellitus [36].

Rats that were fed HFD exhibited hyperglycemia which can influence host responses to bacteria. For example, poor glucose control promotes risk and severity of enteric bacterial infections ,the composition of the gut microbiota differs between obese and/or T2D individuals and those who are lean and non-diabetic , The relationship between microbes and host metabolism is bidirectional [37], [38].On the other hand, the curdpolysaccharide extracted from *Moringa oleifera* Leaves could effectively prevent HFD-induced weight gain and lipid accumulation, ameliorate blood lipid levels and insulin resistance, andprevents obesity in association with modulating gut microbiota in HFD fed mice [39].The bacterial cell wall component muramyl dipeptide (MDP) is an insulin-sensitizing agent that promotes immune tolerance and lowers blood glucose during stressors such as obesity [40].

In this study, elevated levels of blood urea and creatinine were observed in the serum of HFD-fed rats, reflecting findings by Adugba et al [41], Sun et al [42], who highlighted oxidative stress and renal tissue damage associated with obesity induced by HFD. [43] also reported renal lipid accumulation, macrophage infiltration, oxidative stress, and impaired sodium excretion in the kidneys of HFD-treated mice.

Remarkably, the administration of MOLAE to HFD-fed rats led to a significant reduction in blood urea levels. These results were consistent with previous studies by Adeyemi et al [44], Nafiu et al [45], Akinrinde et al [46], Tang et al [47], and Soliman et al [48]. The rich antioxidant content and healing properties of *Moringa Oleifera*have been shown to protect against kidney damage caused by diabetes, preserving renal function and mitigating oxidative stress and inflammation in the kidneys of diabetic mice [49]. Patients with diabetes mellitus usually suffer from oxidative stress that is a major culprit of diabetic nephropathy. Diabetic nephropathy is a worrying problem of this disease that is the main factor for causing end-stage renal disease in the world, the relationship between oxidative stress and inflammation is described as symmetric [50].

Retinol binding protein 4 plays a pivotal role as the specific transporter of vitamin A in circulation, delivering this essential vitamin to target tissues. Therefore, assessing serum RBP4 levels is valuable in understanding metabolic disorders. In this study, we observed a significant increase in RBP4 levels in rats fed HFD compared with the control group. Interestingly, these elevated RBP4 levels were ameliorated in prophylactic and treated groups following the administration of MOLAE to HFD-fed rats. These results were in line with previous research.Zhao et al in 2024 [51] noted that RBP4 negatively regulates insulin sensitivity, Esteve et al in 2009 [52] noted that adipokines like RBP4 can induce insulin resistance by antagonizing insulin in peripheral tissues, particularly the liver and skeletal muscle. Fan et al in 2024 [53] found that increased serum RBP4 levels are associated with insulin resistance and glucose intolerance in obese mice on a high-fat diet. Additionally, KUČEROVÁ et al in 2024 [54]reported that RBP4 can impact insulin signaling in human adipocytes, blocking insulin-induced phosphorylation, and potentially contributing to insulin resistance. Broch et al [55] highlighted that elevated RBP4 levels can interfere with the stimulatory effects of transthyretin in pancreatic cell secretion. Jin et al [56] observed a correlation between plasma RBP4 during pregnancy and insulin resistance in the second trimester. Furthermore, Flock et al [57] emphasized that factors like RBP4 and free fatty acids (FFAs) could mediate obesity-induced insulin resistance. It's noteworthy that flavonoids present in *Moringa* leaves have been shown to inhibit RBP4 expression by reducing oxidative stress, subsequently leading to lower RBP4 levels [58].

In obesity and type II diabetes (T2D), the expression of GLUT4 is significantly reduced in adipocytes. GLUT4 plays a crucial role in the transport of glucose in muscle and adipose tissue. Our study revealed that the gene expression level of GLUT4 in

white adipose tissue increased in rats fed a diet containing MOLAE. This observation aligned with findings by Ahmed et al in 2021[59], which indicated that long-term obesity contributes to chronic low-grade systemic inflammation, impairs insulin sensitivity, and increases the risk of various chronic diseases. Consistent with these findings, Kahn et al in 2019 [60] discovered that consuming excess nutrients leads to fat accumulation in visceral and subcutaneous depots, resulting in hypertrophy and hyperplasia of these depots. Additionally, in2019 Balakrishnan et al [61] and Mohamed et al [62] postulated that the upregulation of GLUT4 gene expression and the downregulation of RBP4 expression may reduce insulin resistance, making them potential mechanisms underlying the antidiabetic properties of *Moringa Oleifera*.Reilly et al [63] suggested that a high-fat diet can selectively alter insulin-stimulated but uncontracted glucose transport by reducing GLUT4 translocation to the plasma membrane. In agreement, Yang et al [64] found that adipocyte specific GLUT4 deletion leads to a significant increase in RBP4, resulting in systemic insulin resistance. These results collectively suggest that alkaloids in *Moringa* can disrupt the action of the enzyme α-glucosidase and reduce glucose transport through intestinal epithelial cells [65]. This increase in GLUT4 gene expression was in line with the study conducted by Ahmed et al [59], which suggested that long-term obesity can lead to chronic low-grade inflammation, impair insulin sensitivity, and increase the risk of various chronic diseases. These findings were also consistent with studies by Balakrishnan et al [61] and Mohamed et al [62], supporting the idea that the modulation of GLUT4 and RBP4 expression could be a key mechanism underlying the antidiabetic potential of *Moringa Oleifera*.

Insulin-like Growth Factor-1 is essential in mediating the anabolic and mitogenic actions of growth hormone (GH). In this study, we observed a significant decrease in IGF-1 levels in the HFD-fed rats' group, but a significant increase in the group that received MOLAE. These findings corresponded with the work of Elabd et al [66], who found that *Moringa oleifera*leaves meal significantly upregulated the expression of the IGF-1 gene. Macvanin et al [67] highlighted those rats with the lowest GH secretion exhibited more severe metabolic consequences when fed a high-fat diet. Notably, *Moringa*'s high protein content is suggested to stimulate the release of ghrelin, a hormone that induces hunger, ultimately leading to GH and IGF-1 release [68].

The levels of CTRP15 have a significant association with obesity and type II diabetes mellitus (T2DM), and this connection with insulin resistance and inflammation may represent a compensatory mechanism in response to metabolic imbalances. In our study, we observed a notable increase in CTRP15 levels in the group of rats fed a high-fat diet (HFD), which subsequently decreased significantly following the administration of MOLAE. These findings were consistent with the research conducted by Shokoohi Nahrkhalaji et al [69], who reported elevated CTRP15 levels in individuals with metabolic syndrome, demonstrating a positive correlation with the number of metabolic syndromes. The elevated CTRP15 levels in these cases might be a consequence of altered signaling in target tissues among individuals with glucose tolerance and T2DM. Additionally, higher CTRP15 levels could result from dysregulation in CTRP15 synthesis or a response to factors such as hyperinsulinemia, hyperglycemia, or cytokines in insulin-resistant states, as suggested by Serazin et al [70]. Similar findings were reported by Shokoohi et al. (2022) [11] in patients with coronary artery disease, where increased CTRP15 levels were directly associated with various metabolic parameters.

Finally, our study investigated the effects of MOLAE on the thermogenic pathway of adipocytes, focusing on the expression of Uncoupling Protein 1 (UCP1). Brown adipose tissue (BAT), known for its mitochondria rich in UCP1, plays a critical role in heat generation. The results showed an increased expression of UCP1 in white adipose tissue (WAT) following MOLAE supplementation. This aligns with Nurhayati et al [71] and Sosa et al [72] and suggests that MOLAE may induce the browning of WAT and upregulate UCP1 in BAT. Flavonoids and saponins present in *Moringa* are believed to contribute to this effect, as they can stimulate the browning of white adipose tissue and reduce systemic inflammation caused by highfat diets [73].Fatty acids, as the substrate of UCP1, can activate UCP1 directly and overcome the inhibition of purine nucleotides on UCP1. This cascade of events initiates a diverse array of alterations in adipose tissue, ultimately resulting in increased heat production [74].

4. Experimental

Animal:

A total of fifty-six adult male albino Wistar rats, with weights ranging from 100 to 120 grams, were used in this study. These rats were accommodated in polypropylene cages maintained under hygienic conditions, with access to rat chow and clean water ad libitum. The rats were exposed to natural ventilation and a natural light/dark cycle, and they were acclimated to laboratory conditions for a period of two weeks prior to the beginning of the experiment. The rearing, handling of rats, and experimental procedures were approved by the Research Ethics Committee of the Faculty of Medicine, Menoufia University, Egypt, under approval number 1482019BIO.

Diet Preparation:

Two types of diets were used in the study:

Standard Diet:

The standard diet used in this study was procured from Al Wady Company in Cairo, Egypt, and consisted of a mixture of essential ingredients. These included yellow corn, soybean seeds, soybean oil, limestones, monocalcium phosphate, sodium chloride, sodium bicarbonate, lecithin, as well as a combination of various minerals and vitamins (Table 3).

Table 3: Chemical composition of basal diet

Basal Diet Chemical Composition	(g %)
Protein	17%
Fat	4.9%
Choline chloride	2%
Vitamin mixture	1%
Salt mixture	3.5%
Fiber	3.44%
Carbohydrates	68.16%

High Fat Diet (HFD):

The HFD was prepared following the method of Abd Eldaim et al [75] and Orabi et al [76]. It consisted of 7% beef tallow, 8.3% egg yolk, 18.7% sucrose, and 66% standard diet (Table 4).

Table 4: Chemical composition of high fat diet

High Fat Diet Chemical Composition	(g %)
Crude protein	16.68 %
Fat	31.59 %
Carbohydrates	51.73 %
Energy	4.66 kcal/g

Food intake was monitored in our diet study by weighing the food prior to serving and subtracting the amount left to get the total intake.

2.3. Preparation and Phytochemical Analysis of *Moringa Oleifera*Leaves Alcoholic Extract:

Fresh leaves of *Moringa Oleifera*were obtained from a farm in Sadat City, Menoufia, Egypt. These leaves underwent a thorough cleaning process with distilled water, followed by shade drying and finely chopping. The dried plant material was then homogenized using a grinder and stored in sealed brown glass containers at a temperature of 22°C until the extraction phase. The extraction was carried out using 70% ethanol, following a procedure previously detailed by Mousa et al. (2019)[77]. In brief, the plant material was soaked in 70% ethanol for 48 hours at room temperature (22°C) with intermittent agitation. The resulting extract was filtered through Whatman filter paper No. 1, and the filtrate was air-dried at room temperature. The extract was subsequently stored in a sealed container at 4°C until needed.), following the method described by Mousa et al [77].

Experimental Design:

A total of 56 male Wistar albino rats were randomly allocated into 7 groups, each comprising 8 rats:

Control group: Rats were given a standard basal diet and had access to distilled water throughout the entire experimental period.

*Moringa Oleifera*Leaves Alcoholic Extract (MOLAE) group: Rats were provided with a standard basal diet and received MOLAE orally at a dosage of 400 mg/kg of body weight [78].

High Fat Diet (HFD) group: Rats were fed a high-fat diet for a duration of 12 weeks.

HFD and MOLAE 200 group: Rats were fed HFD and concurrently administered MOLAE orally at a dose of 200 mg/kg of body weight [78] for 12 weeks.

HFD and MOLAE 400 group: Rats were fed HFD and concurrently administered MOLAE orally at a dose of 400 mg/kg of body weight [78] for 12 weeks.

HFD then MOLAE 200 group: Initially, rats fed HFD for 8 weeks, followed by a switch to a standard basal diet, and subsequently administered MOLAE at a dose of 200 mg/kg of body weight 23 for an additional 4 weeks.

HFD then MOLAE 400 group: Initially, rats fed HFD for 8 weeks, followed by a switch to a standard basal diet, and subsequently administered MOLAE at a dose of 400 mg/kg of body weight 23 for additional 4 weeks.

Blood sampling and determination of serum metabolites:

For the samples collection process, rats were anesthetized using Isoflurane following an overnight fasting period. Blood samples were drawn from the retro-orbital venous plexus and divided into two parts for distinct analyses. The first part of the blood was placed in an EDTA-containing tube for the quantitative turbidimetric assessment of blood HbA1c. This analysis utilized kits provided by BioMed Egy Chem for lab technology in Cairo, Egypt, following the method outlined by Weykamp [79].

The second part of the blood samples were collected into a plain tube, allowed to clot for 30 minutes at room temperature, and subsequently centrifuged at 3000 rpm for 15 minutes. The resulting sera was stored at -80°C for further analysis.

Determination of fasting blood glucose was done following the protocols described by Young [80]. Determination of blood urea nitrogen was carried out according to Kaplan [81] and the determination of serum creatinine level [82]. These analyses were carried out using kits from Diamond Company, Cairo, Egypt.

The quantitative assessment of rat Complement C1q tumor necrosis factor-related protein 15 (myonectin) (CTRP15) was performed using Enzyme-Linked Immunosorbent Assay (ELISA). In this procedure, the ELISA plate was pre-coated with rat CTRP15 antibody, and CTRP15 present in the sample was bound to these antibodies on the plate. Subsequently, biotinylated rat CTRP15 antibody was introduced, binding to CTRP15 in the sample. Streptavidin-HRP was added, binding to the Biotinylated CTRP15 antibody. Following incubation and washing steps, any unbound Streptavidin-HRP was removed, and a substrate solution was added, leading to color development proportional to the amount of rat CTRP15 levels. The reaction was terminated with an acidic stop solution, and the absorbance was measured at wavelength 450 nm.

ELISA was used to quantify the level of rat Retinol binding protein 4 (RBP4) in the samples. RBP4 was added to a monoclonal antibody enzyme well pre-coated with rat RBP4 monoclonal antibody, and after incubation and washing steps, biotin labeled antibodies and Streptavidin HRP were introduced. The addition of Chromogen solutions A and B led to a color change, and the concentration of RBP4 in the sample was correlated with the color intensity.

Serum levels of Insulin-Like Growth Factor 1 (IGF1) were determined using an ELISA assay. The microtiter plate provided in this kit was pre-coated with an antibody specific to IGF1. Standards or samples were added to the appropriate wells, along with a biotin-conjugated antibody specific to IGF1. Avidin conjugated to Horseradish Peroxidase (HRP) was added to each well and incubated. After adding TMB substrate solution, the change in color in wells containing IGF1 was measured spectrophotometrically at a wavelength of 450 nm.

The concentration of CTRP15, RBP4 and IGF1 in the samples were determined by comparing the optical density of the samples to a respective standard curve.

Determination of glucose transporter 4 (GLUT4) gene expression in muscles and white adipose tissues:

RNA isolation from muscle and white adipose tissue samples was carried out using the QIAamp RNA MiniKit (Qiagen, USA, 2013). RNA quality and purity were assessed using Genova UV spectrophotometry (JENWAY). Complementary DNA (cDNA) was synthesized using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., EU/Lithuania). For cDNA synthesis, 10 µL (400 ng) of RNA extract was combined with a mixture of reverse transcriptase enzyme, Trans Amp buffer, and RNase-free water. The reaction was carried out using a thermal cycler, following specific temperature cycles. The synthesized cDNA was stored at -20°C.

Quantification of GLUT4 and β-actin mRNA expression was performed using specific primer sequences: forward primer 5`-AGTTGGAAAGAGAGCGTCCACTGT-3`and reverse primers 5`-GCTGCAGCACCACTGCAATAATCA -3`for GLUT4, forward primer 5`-GACCTCTATGCCAACACAGT-3`and reverse 5`-CACCAATCCACACAGAGTAC-3`for actin. The gene amplification was carried out with SYBR Low ROX Master Mix. The amplification conditions included an initial phase at 95°C, followed by 40 cycles of denaturation, annealing, and extension. Fluorescence detection and data analysis were conducted, and the relative quantification of GLUT4 gene expression was calculated using the comparative ΔΔCt method [83]. The gene expression levels were normalized to the endogenous housekeeping gene β-actin and compared to the control group.

Immunohistochemical detection of uncoupling protein 1 (UCP1) expression in white and brown adipose tissues:

Paraffin sections were treated to remove the paraffin and then rehydrated. To block the endogenous peroxidase activity, the sections were exposed to a 10% hydrogen peroxide solution. Next, these sections were incubated with a primary monoclonal anti-UCP1 antibody obtained from Lab Vision Corporation, Westing House, Thurmont, CA, USA. Following a phosphate buffer wash, a secondary antibody (biotinylated goat anti-rabbit) was applied. Labeled avidin-biotin peroxidase, which binds to the biotin on the secondary antibody, was then introduced. The areas where the antibody had bound were made visible by adding diaminobenzidine chromogen, which was converted into a brown precipitate by peroxidase. The slides were gently rinsed in distilled water, immersed in hematoxylin for about half a minute, and washed in tap water until a blue color appeared. Subsequently, the slides were dehydrated using increasing concentrations of alcohol, cleared with xylene, mounted with Canada balsam, and covered with a cover slip [84].

Statistical analysis:

All data were presented as mean ± standard deviation (SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc test to determine the significant differences. $P < 0.05$ was considered significant.

5. Conclusions

The high fat diet induced hyperglycemia in rats via decreasing the production of IGF-1, GLUT4, and UCP1 proteins, while elevating the levels of RBP4 and CTRP15. However, *Moringa Oleifera*leaves mitigated the adverse effects of the high fat diet through decreasing CTRP15, RBP4, serum levels and normalizing the reduction in IGF-1, GLUT4, and UCP1 synthesis and production, indicating that MOLAE has potential hypoglycemic activity.

6. Conflicts of interest

"There are no conflicts to declare".

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