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Moringa oleifera Lam. improves lipid metabolism during adipogenic differentiation of human stem cells

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Abstract. – OBJECTIVE: ***Moringa oleifera*** **Lam., a multipurpose tree, is used traditionally for its nutritional and medicinal properties. It has been used for the treatment of a variety of conditions, including inflammation, cancer and metabolic disorders.**

MATERIALS AND METHODS: We investigated the effect of ***Moringa oleifera*** **Lam. on adipogenic differentiation of human adipose-derived mesenchymal stem cells and its impact on lipid metabolism and cellular antioxidant systems.**

RESULTS: We showed that ***Moringa oleifera*** **Lam. treatment during adipogenic differentiation reduces inflammation, lipid accumulation and induces thermogenesis by activation of uncoupling protein 1 (UCP1), sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor alpha (PPARα), and coactivator 1 alpha (PGC1α). In addition,** ***Moringa oleifera*** **Lam. induces heme oxygenase-1 (HO-1), a well established protective and antioxidant enzyme. Finally** ***Moringa oleifera*** **Lam. significantly decreases the expression of molecules involved in adipogenesis and upregulates the expression of mediators involved in thermogenesis and lipid metabolism.**

CONCLUSIONS: Our results suggest that ***Moringa oleifera*** **Lam. may promote the brown remodeling of white adipose tissue inducing thermogenesis and improving metabolic homeostasis.**

Key Words:
Moringa Oleifera Lam., lipid metabolism, adipocytes, stem cells, differentiation.

Introduction

Moringa oleifera Lam. (*M. oleifera* Lam.) is cultivated in many tropical and subtropical countries of Asia and Africa. It is a common herb and has been documented to contain various phytochemicals, macronutrients, and micronutrients that contribute to its great medicinal value including the management of diseases such as asthma, bronchitis, diabetes, mastitis, skin conditions, worm infestations, and HIV/AIDS symptoms among others[1,2]. Its ability to treat most of these conditions has been attributed to the nutritional and immunomodulatory properties as well as to its antioxidant, hypoglycemic, hypotensive, antidyslipidemic, anticancer, and anti-inflammatory properties[1]. Most of the reviewed literature reported changes in the biochemical parameters of diabetes mellitus after short-term treatment with an extract of *M. oleifera* Lam. leaves[3,4] though only relatively few studies have described the related histological pancreatic changes[5]. Furthermore, it has been reported that *M. oleifera* Lam. administration to diabetic patients can induce better glucose tolerance by extending the treatment period[6]. Recently, a study demonstrated that administration of benzylamine, one of the constituents found in *M. oleifera* Lam., reduces body weight gain, circulating cholesterol, improves glucose tolerance and lipid metabolism in rats[7,8]. Among these characteristics representing some of the hallmarks of metabolic syndrome, adipocytes also play an important role in this process through their metabolism[9]. Adipose tissue growth involves the formation of new adipocytes from precursor cells, further leading to an increase in adipocyte size. Moreover, in patients affected by metabolic syndrome, adipose tissue expansion is associated with adipocyte

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dysfunction and increased inflammatory processes[9-11]. The transition from undifferentiated fibroblast-like pre-adipocytes into mature adipocytes constitutes the adipocyte life cycle, and treatments that regulate both size and number of adipocytes may provide a better therapeutic approach for treating obesity.

Recently, some studies reported a novel perspective on functional adipogenesis that appears to play a central role in providing adipose tissue insulin sensitivity and functionality and in preventing the development of insulin resistance and type 2 diabetes.

The aim of this study was to investigate the *in vitro* effect of administration of *M. oleifera* Lam. during adipogenic differentiation of human adipose tissue mesenchymal stem cells.

Material and Methods

Quenching of DPPH

The free radical-scavenging capacity of *M. oleifera* Lam. was tested by their ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH). The reaction mixture contained 86 μM DPPH, different concentrations of extracts (500-250-100-50-10-5 μg/ml) in 1 mL of ethanol. After 10 min at room temperature the absorbance at λ = 517 nm was recorded. Trolox (30 μM) was used as a standard. Each result represents the mean ± S.D. of five experimental determinations.

Adipose Stem Cells Isolation and Culture

The subcutaneous tissue was collected from a healthy subject of 23 years underwent surgery for umbilical hernia[12] following written consent. The adipose tissue was minced by means of the use of scissors into pieces of a size less than 3 mm[12]. Briefly, after gentle shaking with an equal volume of phosphate buffered saline (PBS), the mixture separated into two phases. The upper phase (containing stem cells, adipocytes and blood) after three washes with PBS was enzymatically dissociated with 0.075% collagenase (type I)/PBS for 1 h at 37°C with gentle shaking. The dissociated tissue was then mixed with an equal volume of Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% FBS and incubated 10 min at room temperature. The solution was separated into two phases. The lower phase was centrifuged at 1500 rpm for 5 min at 20°C. The cellular pellet was resuspended in 160 mM NH_4Cl to eliminate erythrocytes and passed through a 40 μm mesh filter into a new tube. Cells were resuspended in an equal volume of DMEM/10% FBS (fetal bovine serum) and then centrifuged.

Detection of ASCs Cell Markers by FACS Analysis

ASCs phenotype was evaluated by flow-cytometry analysis (FC500 Beckman Coulter, Cassina De' Pecchi, Milan, Italy). The ASCs presented as a homogeneous fibroblastic cell population. Flow cytometric analysis of passage 4 cells revealed that they were negative for CD34 and CD45, and positive for CD105 and CD90.

Differentiation of Human ASCs Into Adipocytes

ASCs were plated in a 75-cm^2 flask at a density of 1 to 2 × 10^4 cells and cultured in DMEM with 10% FBS for 7 days. The medium was replaced with an adipogenic medium, and the cells were cultured for an additional 14 days. The adipogenic media were prepared as follows: complete culture medium supplemented with DMEM-F12 high glucose, 3% (v/v) FBS, 100 nM insulin, 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO, USA), 60 μM indomethacin (Sigma-Aldrich, St. Louis, MO, USA) and transferrin 10 μg/ml. Human ASCs were cultured in the presence of *M. oleifera* Lam. (100 μg/ml) which was added every 3 days.

RNA Extraction and qRT-PCR

Trizol (Invitrogen, Carlsbad, CA, USA) reagent was used to extract RNA[13]. First strand cDNA was then synthesized with Applied Biosystem (Foster City, CA, USA) reverse transcription reagent[14]. Quantitative real-time PCR was performed in *Roche* Light Cycler *Nano* (*Roche* Diagnostics) using the SYBR Green PCR MasterMix (Life Technologies)[15]. The primer sequences used for these set of experiments are reported in Table I. The specific PCR products were detected by the fluorescence of SYBR Green, the double-stranded DNA binding dye. The reaction was followed by a melting curve protocol (Figure 1) according to the specifications of the *Roche* Light Cycler *Nano* (*Roche* Diagnostics, Basel, Switzerland). The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product

Table I. PCR primers used in this study.

Gene	Primer forward	Primer reverse	Size
Adiponectin	CCCTCTCTTACAAGCCCATCA	GAGCCAGTCTGGTAGTACATCA	109
CEBPα	TAACTCCCCCATGGAGTCGG	ATGTCGATGGACGTCTCGTG	207
DGAT1	GGTCCCCAATCACCTCATCTG	TGCACAGGGATGTTCCAGTTC	165
FABP4	AAACTGGTGGTGGAATGCGT	GCGAACTTCAGTCCAGGTCA	95
FAS	AAGGACCTGTCTAGGTTTGATGC	TGGCTTCATAGGTGACTTCCA	106
GAPDH	AGACACCATGGGGAAGGTGA	TGGAATTTGCCATGGGTGGA	56
HO-1	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCG	247
IL6	GAAAGCAGCAAAGAGGCACT	TTTCACCAGGCAAGTCTCCT	108
IRS1	CCCAGGACCCGCATTCAAA	GGCGGTAGATACCAATCAGGT	89
PGC1α	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTCACATCTAGTTCA	112
PPARα	TTCCGAATCCTCTTTACTCCAGA	CCCTCTAGCTTTGATGGGTACA	102
PPARγ	ACCAAAGTGCAATCAAAGTGGA	ATGAGGGAGTTGGAAGGCTCT	100
SIRT1	TGTGTCATAGGTTAGGTGGTGA	AGCCAATTCTTTTTGTGTTCGTG	101
SREBP-1c	GCCCCTGTAACGACCACTG	CAGCGAGTCTGCCTTGATG	84
UCP1	AGGTCCAAGGTGAATGCCC	TTACCACAGCGGTGATTGTTC	75

and normalized with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by using comparative $2^{-\Delta\Delta Ct}$ method[14,16].

Statistical Analysis

Statistical significance ($p < 0.05$) of differences between experimental groups was determined by the Fisher method for analysis of multiple comparisons[17]. For comparison between treatment groups, the null hypothesis was tested by either single-factor analysis of variance (ANOVA) for multiple groups, or the unpaired *t*-test for two groups, and the data are presented as mean ± standard error mean (SEM).

Results

Free Radical Scavenging Activity of *M. oleifera* Lam.

The free radical scavenging activity of *M. oleifera* Lam. was tested by their ability to bleach the stable DPPH radical. This assay provides information on the reactivity of test sample with a stable free radical. Because of its odd electron, DPPH gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting destaining is stoichiometric with respect to the number of electrons taken up. In this assay, the *M. oleifera* Lam. showed a radical scavenger of DPPH in a dose-dependent manner. In particular, the percentage of inhibition of DPPH resulted were about 81%, 63%, 42%, 33%, 13% 18% respectively at concentration of *M. oleifera* Lam. of 500-250-100-50-10-5 µg/ml (Figure 2).

Analysis of Adipogenic Differentiation

To investigate signals that might regulate the differentiation of ASCs, we analyzed the mRNA levels of peroxisome proliferator-activated receptor gamma (PPAR), CCAAT/enhancer-binding protein alpha (CEBPα), diacylglycerol O-acyltransferase 1 (DGAT1), fatty acid binding protein 4 (FABP4), fatty acid synthase (FAS), and sterol regulatory element-binding protein 1c (SREBP-1c).

We showed that all of these markers resulted in a significant increase after 14 days of adipogenic differentiation (Figure 3).

The effect of M. oleifera Lam. on Adipogenesis

As seen in Figure 4 the administration of *M. oleifera* Lam. during the adipogenic differentiation was able to reduce significantly the mRNA levels of FABP4, FAS and SREBP-1c (Figures 4A-C).

Analysis of Inflammatory Cytokines Gene Expression

To study the eventual effect of OLE on inflammation, we investigated Il-6 expression during differentiation. We showed a significant increase of mRNA level of IL-6 in differentiated adipocytes (Figure 5). The *M. oleifera* Lam. treatment was able to significantly decrease the Il-6 mRNA levels in differentiated cells.

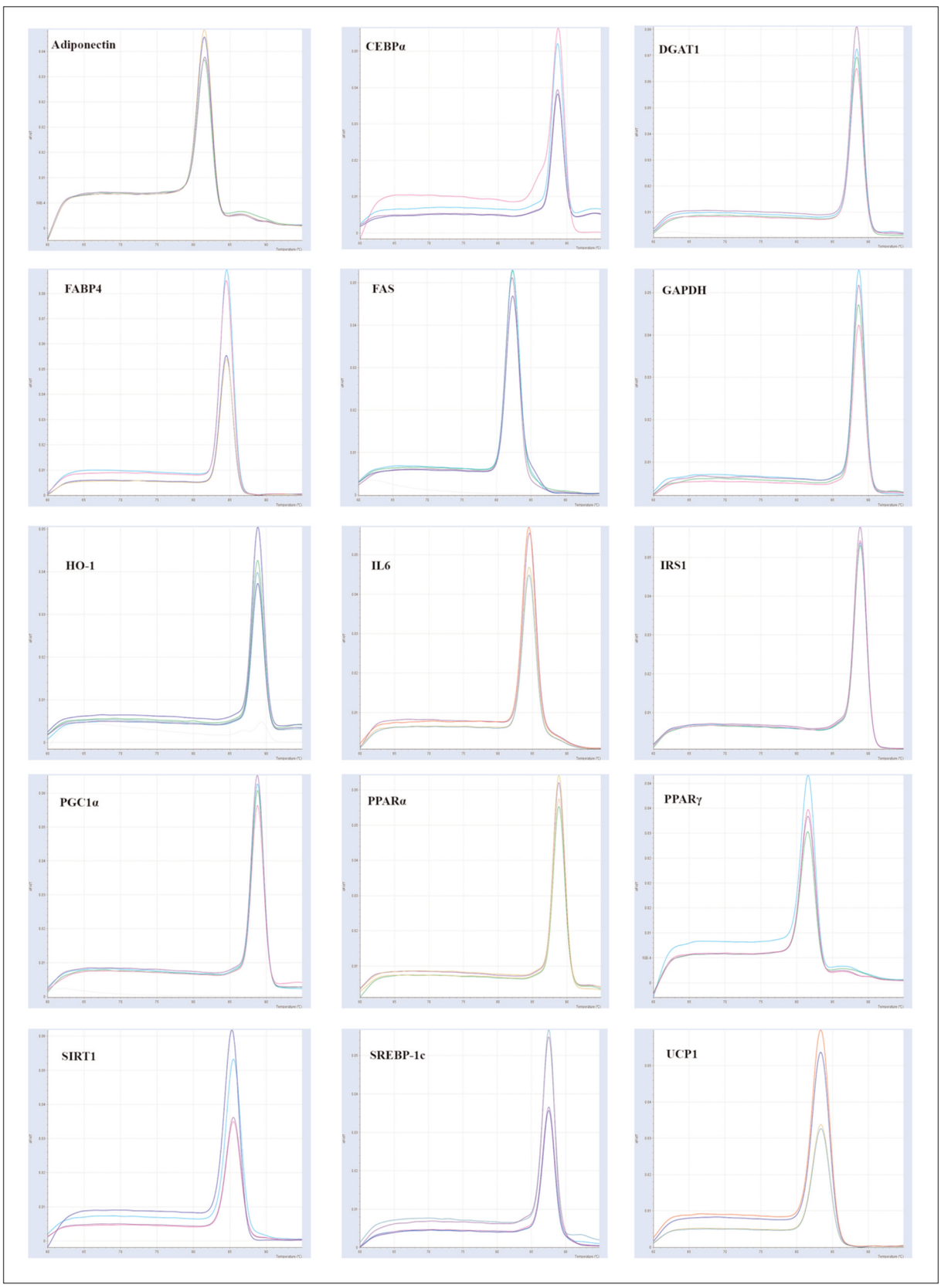

Figure 1. Melting curve analysis.

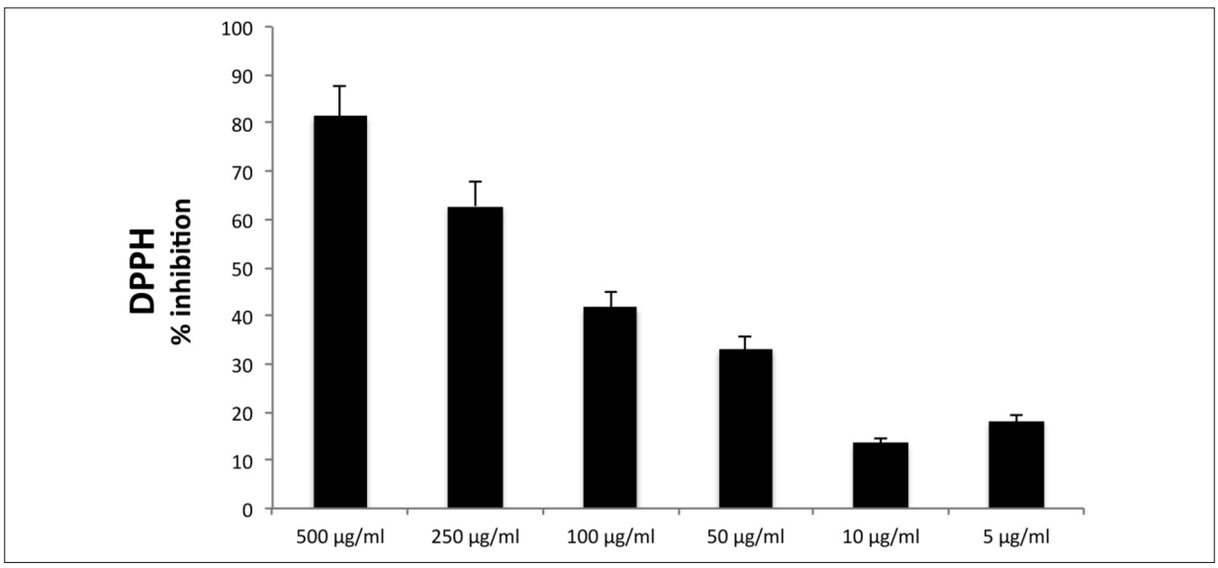

Figure 2. Scavenger effect of *M. oleifera* Lam. Extract expressed as the capacity to bleach the stable DPPH. Results are expressed as the percentage decrease in absorbance at = 517 nm. Each value represents the mean ± SEM of 4 experiments. Significance *vs*. control $p < 0.005$.

Effect of M. oleifera on IRS-1, HO-1 and Adiponectin Expression

Interesting, *M. oleifera* Lam. effects show a significant increase of insulin receptor substrate 1 (IRS-1) genes expression (Figure 6A). To investigate the possible role of *M. oleifera* Lam. on the adiponectin expression we performed a Real-time PCR assay. The *M. oleifera* Lam. treatment resulted in a significant increase of adiponectin expression during adipose differentiation (Figure 6C). Heme oxygenase-1 (HO-1) gene expression, an antioxidant protein, resulted in a significant increase in *M. oleifera* Lam. treated cell cultures after 14 days of adipogenic differentiation respect the differentiated untreated cells (Figure 6B).

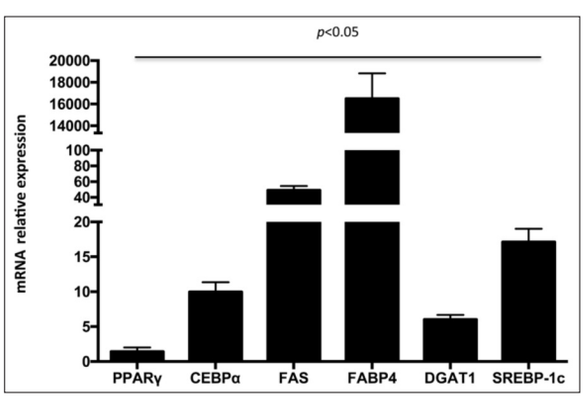

Figure 3. Analysis of gene expression by Real-time PCR of PPARγ, CEBPα, FAS, FABP4, DGAT1 and SREBP-1c after 14 days of adipogenic differentiation. All values are expressed as mean ± SEM of four experiments (n = 4) in duplicate. ($p < 0.05$ *vs*. undifferentiated).

M. oleifera Lam. Induces Thermogenic Pathway

To investigate the effect of *M. oleifera* Lam. on the lipid metabolism, we analyzed the expression of the thermogenic pathway markers, which are characterized in the brown adipocyte.

The administration of *M. oleifera* Lam. during adipogenic differentiation was able to significantly increase mRNA levels of sirtuin 1 (SIRT-1), peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (Pgc-1α) (Figures 7A-C). Moreover, to study the activation of the heat-generating pathway, which is the futile cycle of proton pumping through the actions of uncoupling proteins (UCPs), we analyzed the expression of UCP1 (Figure 7D).

Discussion

The important effects of natural antioxidants on metabolic diseases are rewiring the approaches on the therapeutic strategy versus this kind of condition. It was demonstrated that natural antioxidants lead their effect through the expression

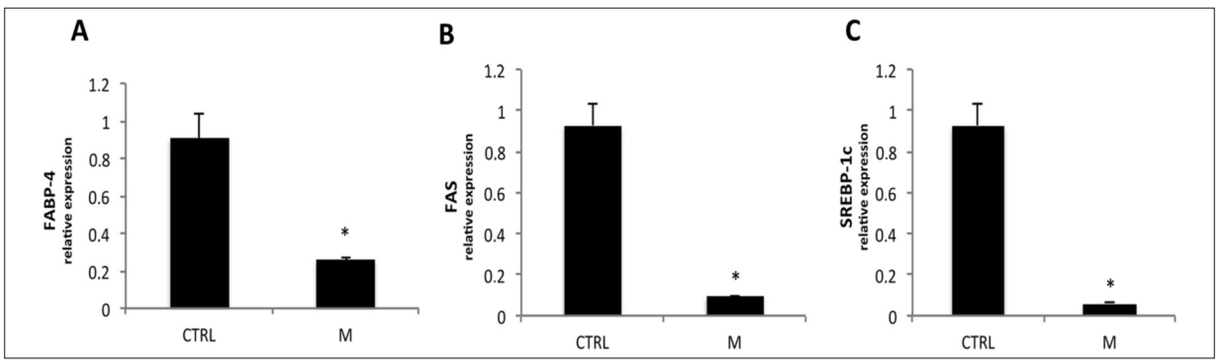

Figure 4. Analysis of gene expression of FABP4, FAS and SREBP-1c by Real-time PCR of cells treated with *M. oleifera* Lam. during adipogenic differentiation (M). All values are expressed as mean ± SEM of four experiments (n = 4) in duplicate. (*$p < 0.05$ *vs*. control cells).

of antioxidant enzymes and activation or inhibition of specific pathways[18-21]. In particular, different studies on natural antioxidants effects in the metabolic suitability of adipose tissue or adipocytes showed that modulation on the expression of specific protein/enzymes is responsible for the improving of the metabolic processes[22-25]. Maintenance of metabolic homeostasis of adipocytes and adipose tissue is an important factor for the onset of metabolic syndrome. The main function of adipose tissue is the storage of fat under conditions of calories excess and the release of free fatty acids during fasting. Also, adipocytes play a role in thermoregulation, mechanical organ protection and as an endocrine organ by adipokines secretion[26,27]. Adipokines regulate important biological processes in target organs including the brain, liver, skeletal muscle, vessels, heart, immune system and pancreatic islets. Altered adipokines secretion may contribute to impaired regulation of fat distribution, insulin secretion and sensitivity, energy expenditure, endothelial function, inflammation, blood pressure and hemostasis. Therefore, alteration in adipokine secretion may link obesity to its inflammatory, metabolic and cardiovascular comorbidities. Because adipokines also act in an autocrine and paracrine manner they could contribute to the development of adipose tissue dysfunction initiated from disturbances of individual adipocytes. In addition, to changes in adipokine secretion, impaired expandability of subcutaneous adipose tissue, ectopic fat deposition and hypoxia may contribute to adipose tissue inflammation or dysfunction. As a consequence, adipocyte dysfunction contributes to a proinflammatory, atherogenic, and diabetogenic state and may be mechanistically linked to the development of chronic inflammation, insulin resistance, metabolic syndrome and other obesity-associated disorders. Targeting adipocyte dysfunction may be a promising approach to prevent or treat obesity-related diseases in the future.

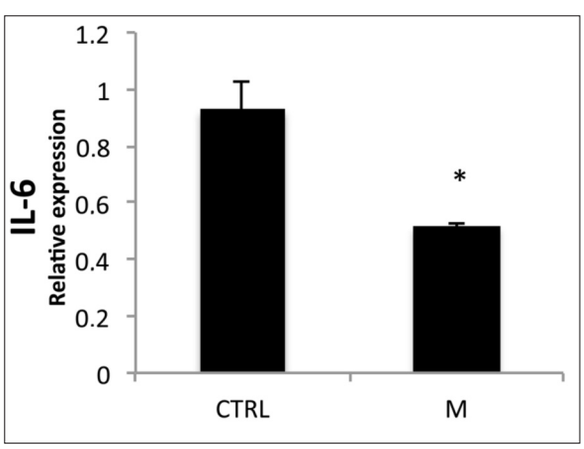

Figure 5. Analysis of gene expression by Real-time PCR of cytokine IL-6 of cells treated with *M. oleifera* Lam. during adipogenic differentiation (M). All values are expressed as mean ± SEM of four experiments (n = 4) in duplicate. (*$p < 0.05$ *vs*. control cells).

Many *in vitro* and *in vivo* studies have shown antioxidant properties of *M. oleifera* Lam. by its abundant amounts of antioxidant compounds. Further, scientific evidences suggest a positive effect of *M. oleifera* Lam. on lipid homeostasis[28]. Previous studies[29-31] have shown that supplementation of *M. oleifera* Lam. leaves extract exhibited anti-obesity effects in high fat fed mice. Furthermore, other study showed that *M. oleifera* Lam. leaves supplementation in diabetes-induced rats leads a hypolipidemic effects, presented low-

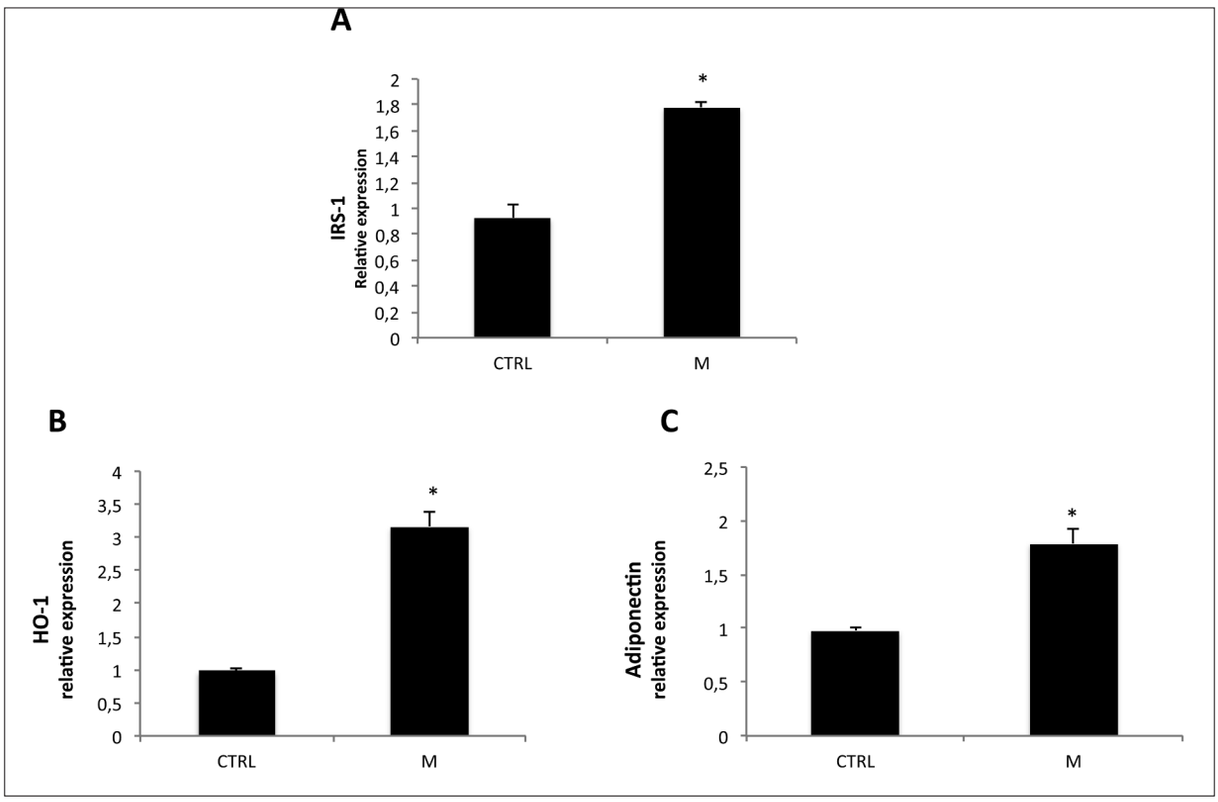

Figure 6. Analysis of gene expression by Real-time PCR of IRS-1 ***(A)***, HO-1 ***(B)*** and Adiponectin ***(C)*** of cells treated with *M. oleifera* Lam. during adipogenic differentiation (M). All values are expressed as mean ± SEM of four experiments (n = 4) in duplicate. (*$p < 0.05$ *vs*. CTRL).

er plasma total cholesterol, triglycerides and LDL, than diabetic rats untreated[3]. In this study, we report that *M. oleifera* Lam. has higher free radical-scavenging capacity measured by DPPH assay. In particular, our data showed that *M. oleifera* Lam. administration during adipogenic differentiation of adipose-derived human mesenchymal stem cell resulted in a decrease of FABP4, FAS, and SREBP-1c gene expression during the differentiation, confirming that its administration is able to reduce fatty acid accumulation. Moreover, a decrease in IL-6 expression in adipocytes differentiated whit *M. oleifera* Lam. respect to the normal adipocyte confirms a reduction of inflammation. We reported also that in adipocyte treated with *M. oleifera* Lam. we found an increase of IRS1 gene expression, which has been previously reported that low-IRS1 expression and protein levels are predictive of insulin resistance and type 2 diabetes[32]. In order to understand the mechanism by which *M. oleifera* Lam. may act as a protective modulator in adipocytes, we evaluated the gene expression of HO-1 and adiponectin. Adiponectin is an adipokine secreted by adipocytes and increased its expression was correlated to an improvement in glucose tolerance and an increase in insulin sensitivity[33]. Furthermore, the induction of HO-1, an antioxidant and cytoprotective enzyme, regulates adiponectin expression by reducing inflammation and insulin resistance both in *in vivo* and *in vitro*[34-39]. Our data show that *M. oleifera* Lam. treatment during differentiation leads to an overexpression of HO-1 and Adiponectin confirming a protective effect of this extract. Finally, we investigated the eventually effect of this plant on the thermogenic pathway of adipocytes analyzing the expression of uncoupling protein UCP1, SIRT-1, PPARα and Pgc-1α. Thermogenesis contributes to ameliorate the metabolic rate as well as glucose and lipid metabolism in humans[37,40] and it represents the distinctive feature of adipocytes contained in the brown adipose tissue. Brown adipose tissue (BAT) is densely populated with mitochondria containing the inner mitochondrial proton carrier UCP1 that uncouples oxidative phosphorylation and allowing the mitochondrial membrane potential to be dissipat-

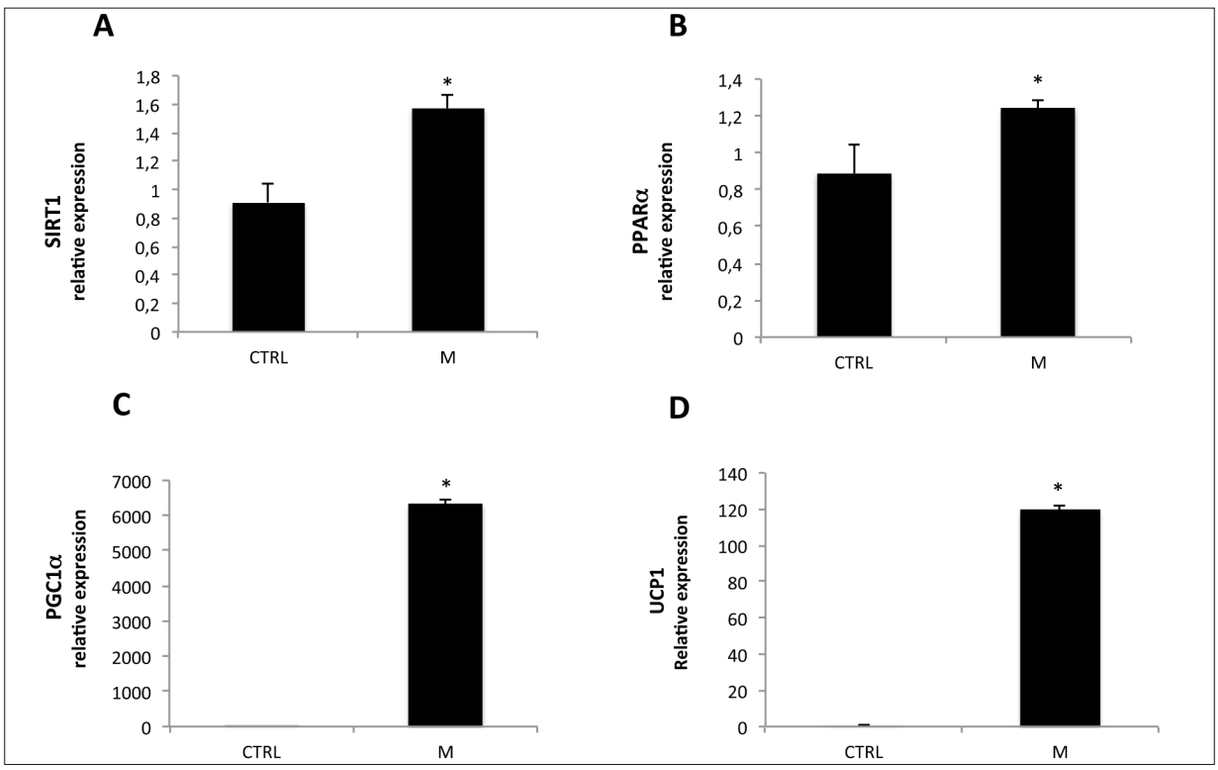

Figure 7. Analysis of gene expression by Real-time PCR of thermogenic pathway of cells treated with *M. oleifera* Lam. during adipogenic differentiation (M). SIRT1 ***(A)***, PPARα ***(B)***, PGC1α ***(C)*** and UCP1 ***(D)***. All values are expressed as mean ± SEM of four experiments (n = 4) in duplicate. (*$p < 0.05$ *vs*. control).

ed as heat[41]. In this study, we report, for the first time, that *M. oleifera* Lam. treatment during adipose tissue differentiation induce thermogenesis through overexpression of UCP1, SIRT-1, PPARα and Pgc-1α. Our data suggest that *M. oleifera* Lam. during adipogenesis improves adipocyte functionality and upregulates the expression of molecules involved in thermogenesis modulating lipid metabolism.

Conclusions

Our findings show that *M. oleifera* Lam. should be taken in to account for future nutritional researches focused on using *M. oleifera* Lam. as minerals supplementation.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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