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Effect of dietary fortification by dried *Moringa oleifera* leaves on the expression of some lipids and immune related genes in albino rats fed purified diet

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Abstract

An experimental feeding trial was conducted to visualize the effect of dietary fortification of graded levels of dried *Moringa oleifera* leaves (MLS) on the expression of some important genes including, resistin, adiponectin and leptin as adipokines, IL-1 and IL-6 as pro-inflammatory cytokines of albino rat fed on modified AIN-93G purified diet. A total of 30 male albino rats with an average weight of (80-90 g)were assigned into four equal experimental groups as follow: the first group(G1) was fed on modified AIN-93G purified diet supplemented with 1.25% $CaCO_3$ and served as control, the Second (G2)was fed on the same diet supplemented with only 0.06% $CaCO_3$ (low Ca diet), the third (G3)was fortified with 2.5% MLS and the forth (G4) was supplemented with $CaCO_3$ (1.25%) and the diet was fortified by MLS (2.5%) respectively. The experimental trial lasted for 11 weeks of which one week was considered as acclimatization period. At the end of the trial representative samples of abdominal fat and intestine were collected to estimate mRNA as indicator of adipokines and cytokinesgenes expression, respectively. Results showed that, low calcium diet resulted in over expression of Resistin, Leptin, IL-1α and IL-6 genes, down regulation of adiponectin gene and recorded the highest levels of serum cholesterol and triglycerides in comparison to the control group. It was clear also that, dietary fortification of MLS caused regulation of lipid metabolism manifested by down regulation of resistin, leptin, IL-1α and IL-6 genes, up regulation of adiponectin together with lower serum cholesterol and triglycerides. In addition, the obtained data showed positive relationship between the level of dietary Calcium irrespective to its source with Calcium levels in serum. It could be concluded that dietary fortification of albino rat purified diet with dried *Moringa oleifera* leaves powder (MLS) at aforementioned levels might be used as promising clinico-nutritional management tool for prevention and control of lipid dysfunction, insulin resistance and obesity problems.

Keywords: *Moringa oleifera*, obesity, white adipose tissue, insulin resistance, Calcium deficiency, resistin, leptin, adiponectin, RT-PCR, gene expression.

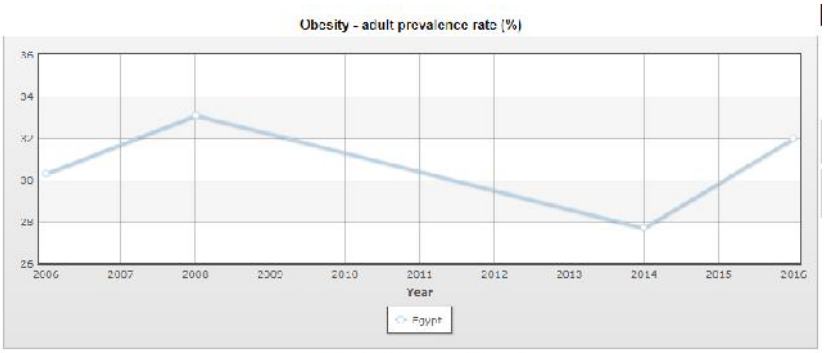
Introduction

Overweight problem is the most common nutrition-related health condition. It is characterized by the excessive accumulation of fat in subcutaneous and peri-visceral tissue. Research activities indicated that many conditions can contribute to obesity including food type, availability and palatability, activity level, age, physical environment and medications *(***WHO, 2019***)*. In addition, the more common consequences of overweight/obesity are serious health risks including, diabetes mellitus (sugar diabetes), damage to joints, bones, and ligaments, heart disease and increased blood pressure, difficulty breathing, decreased stamina, heat intolerance, decreased liver function, increased surgical and anesthetic risk, reproductive

problems, digestive disorders, decreased immune function, skin and hair coat problems, increased risk of cancer and decreased quality of life **(Linder and Parker, 2016).**These metabolic disorders are dramatically increasing among adults in the Eastern Mediterranean Region. Data for adults aged 15 years and older from 16 countries in the region showed the highest levels of overweight and obesity in Egypt, Bahrain, Jordan, Kuwait, Saudi Arabia and United Arab Emirates. The prevalence of overweight and obesity in these countries ranges from 74% to 86% in women and 69% to 77% in men. These data indicate a much higher prevalence of obesity among adult women, while overweight is more marked among adult men. Escalating levels of overweight and obesity among children and adolescents is of particular concern given recent evidence linking childhood and adolescent obesity to increased risk of obesity and morbidity in adulthood **(WHO, 2019).**

More than two billion children and adults in the world are suffering health conditions resulting from obesity and the death rates due to such cases are on the rise. About 19 million Egyptians are suffering from obesity, representing 35 percent of all adults, which is the highest rate in the world. Moreover, 3.6 million children (10.2% of Egyptian children) suffer from obesity. Among the most populous countries in the world, the U.S. came on top with the highest rate of obesity among children and youth (13%); while Egypt topped the list of obesity among adults (35%) (**David *et al.*, 2017).**

Many data sources have illustrated the increase of obesity rate in Egypt from 2006 to 2016 (**CIA, 2018)** in (Fig 1) and from 2007 to 2016 **(Statista, 2019**) in (Fig 2) which clarified that there is a continuous increase in this metabolic disorder reaching about 32% of the population in 2016.



Country	2006	2008	2014	2016
Egypt	30.3	33.1	27.7	32

Fig (1) Prevalence of obesity rate in adults from 2006 till 2016.

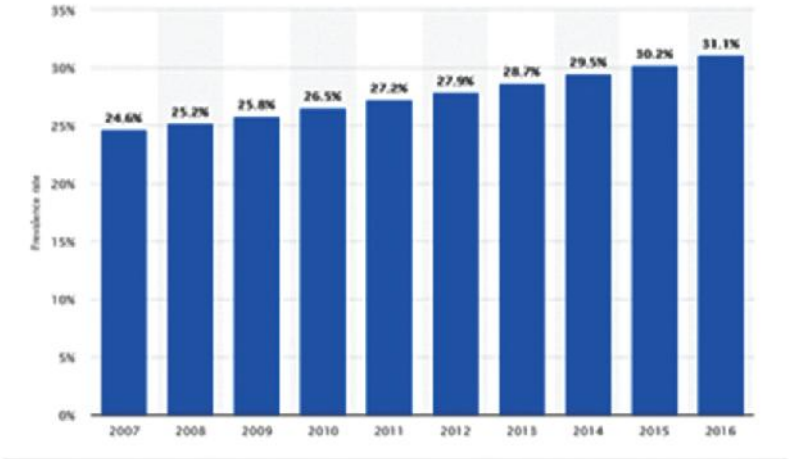


Fig (2) Prevalence of obesity in the adult population aged 18 years and older in Egypt from 2007 to 2016

The visceral adipose tissue was chosen to be studied at the gene expression level due to its anatomical position and pattern of secretion. The deep visceral or mesenteric adipose tissue in rats resembles the omental tissue in human as they act as active endocrine glands. Their secretions (adipokines) are collected by the portal vein, reaching the liver and contribute to the increased chronic inflammation, hepatic injury and impairment of liver enzymes, insulin resistance and cardiovascular risk associated with obesity. Thus, the gene expression analysis of the leptin, resistin and adiponectin would give a causal correlation between visceral obesity and symptoms of metabolic syndrome (**Fateheya M. Metwally *et al.*, 2017)**.

Abdominal fat, also called White Adipose Tissue (WAT) was considered for several decades as a storage place for energy before several research work studied its role as an endocrine organ producing number of biologically active compounds that regulate metabolic homeostasis. This dynamic tissue is composed not only from adipocytes but also of other cell types called the stroma-vascular fraction comprising blood cells, endothelial cells, pericytes and adipose precursor cells among others (**Guerre Millo, 2002** and **Marisa *et al.*, 2013**). It is now generally recognized that adipose tissue is an important organ of a complex network that participates in the regulation of a variety of quite diverse biological functions (Fig 3) **(Coelho *et al.*, 2013).**

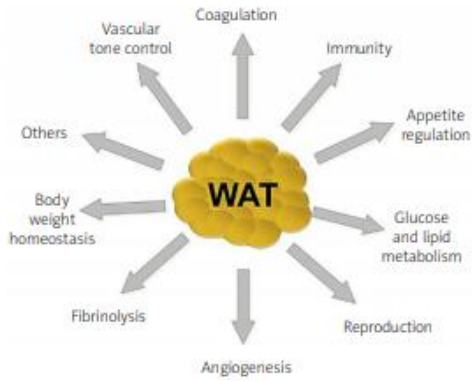


Fig (3) The most significant physiological functions of white adipose tissue such as coagulation, appetite regulation, immunity, glucose and lipid metabolism, reproduction, angiogenesis, fibrinolysis, body weight homeostasis and vascular tone control

Resistin, is one of the WAT hormones which is well known to have a very important role in insulin resistance as it causes impairment of the insulin receptors preventing insulin from reaching inside the cells and subsequently prevents proper metabolism of glucose helping in the elaboration of Diabetes Type 2 (**Berger, 2001**).

Adiponectin, which is another product of WAT, is responsible for fatty acid oxidation, decrease of plasma Triglycerides and improves glucose metabolism by increasing insulin sensitivity through activation of cellular AMP protein kinase and reduction of non-esterified fatty acids which are the main component of Triglycerides (**Khan and Joseph, 2014**). In addition, Adiponectin inhibits the inflammatory process which is an important predisposing factor for atherogenesis by suppressing the migration of monocytes/macrophages and their transformation into foam cells (**Beltowski, 2003**).

Leptin is considered as a hormone secreted by mature adipocytes. It is a 16 kDa protein, encoded by the obese (OB) gene. Leptin is secreted in plasma and its levels are strongly correlated with adipose mass in rodents as well as in humans. Its main effect was to act as a signal from adipose tissue to the brain regarding the quantity of fat tissue stored. It acts via hypothalamic receptors to inhibit feeding and increase thermogenesis (**Kouidhi *et al.*, 2010**).

Resistin, Adiponectin and Leptin are called adipokines which are considered potential targets for the management of obesity and cardiovascular disease. Therefore, the use of cholesterol-lowering agents is considered as the approach of choice (**Han *et al.*, 1995; Klein *et al.*, 2007**).

One of the most recent approaches is using plant sources, namely *Moringa oleifera*, and studying its effect on different parameters concerning fat metabolism and deposition in human body and/or experimental animal body (**Barbagallo *et al.*, 2016).**

Medicinal plants have been used to prevent and/or control of some physiological, nutritional and health related problems of both human and animals. They hold a great promise in clinical therapy as they possess minimal side effects that are usually associated with chemotherapy or radiotherapy; they are also comparatively cheap and thus significantly reduce health care cost.

Moringa oleifera is one of medicinal plants exerting various nutritional and pharmacological actions. *Moringa oleifera* leaves contains many biologically active compounds (Table 2a and 2b) that contribute to its great medicinal value including the management of diseases such as Diabetes.

Table (1) Taxonomic classification of *Moringa oleifera:* **(Bhattacharya *et al.*, 2018)**

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Capparales
Family	Moringaceae
Genus	*Moringa*
Species	*Oleifera*

Its ability has been attributed to the nutritional and immunomodulatory properties as well as to its antioxidant, hypoglycemic, hypotensive, anti-dyslipidemic, and anti-inflammatory properties.

Table (2a) Structures of some important phytoconstituents of *Moringa oleifera* **(Udechukwu *et al.*, 2018)**:

Plant part	Compound	Content [g·kg⁻¹]	Reference
Ethanolic leaves extract	Niaziridin	0.15	Shanker et al. 2007
Aqueous extract of defatted seeds	4-(α-L-Rhamnosyloxy)-benzyl isothiocyanate	3.6	Eilert et al. 1981
Aqueous extract of defatted seeds with 0.1% ascorbic acid at pH 6.8		6.9	
Ethanolic leaves extract	Niazirin	36	Faizi et al. 1994
	Niazirinin	22	
	Niaziminin A	23	
	Niaziminin B	6.2	
	Niazinin A	19	
	Niazinin B	21	
	4-(4-*O*-Acetyl-α-L-rhamnosyloxy)-benzyl isothiocyanate	10	
Ethanolic leaves extract	*O*-Ethyl-4-(α-L-rhamnosyloxy)-benzyl carbamate	1.25	Guevara et al. 1999
	Niazimicin	0.42	
	Niazirin	1.67	
	Glycerol-1-(9-octadecanoate)-β-sitosterol	8.9	
	3-*O*-(6'-*O*-Oleoyl-β-D-glucopyranosyl)-β-sitosterol	5.42	
	β-Sitosterol-3-*O*-β-D-glucopyranoside	2.5	
Methanolic seeds and leaves extracts	4-(α-L-Rhamnosyloxy)-benzyl glucosinolate	202[a]; 59.4[b]	Bennett et al. 2003
	Quercetin-3-*O*-glucoside	0.21[b]	
	Quercetin-3-*O*-(6''-malonyl) glucoside	0.25[b]	
	Kaempferol-3-*O*-glucoside	0.08[b]	
	Kaempferol-3-*O*-(6''-malonyl) glucoside	0.08[b]	
	3-Caffeolyquinic acid	8.9[b]	
	5-Caffeolyquinic acid	1.1[b]	
Aqueous seeds and leaves extracts	Ascorbic acid	0.62[a]; 0.91[b]	Singh et al. 2009
	Gallic acid	0.11[a]; 1.03[b]	
	Quercetin	0.81[b]	
	3-Caffeolyquinic acid	0.49[b]	
	Ferulic acid	0.13[b]	
	Vanillin	0.14[b]	
	Ellagic acid	0.19[b]	
	Kaempferol	0.1[a]; 0.2[b]	

Lowercase letters in superscript denote the plant part where the compound was identified (a – seeds, b – leaves).

Table 2b: Bioactive Components in *Moringa oleifera* and their Positive Effects on Chronic Disease **(Vergara-Jimenez *et al.*, 2017)**.

Compounds	Postulated Function	Model Used	Disease Protection
Flavonoids: Quercitin	Hypolipidemic and anti-diabetic properties	Zucker rat	Diabetes
	Lower hyperlipidemia	Rabbits	Atherosclerosis
	Decrease expression of DGAT	Guinea Pigs	NAFLD
	Inhibition of cholesterol esterase and α-glucosidase	In vitro study	Cardiovascular disease and Diabetes
	Inhibits activation of NF-kB	High fat fed Mice	Cardiovascular disease
Chlorogenic Acid	Glucose lowering effect	Diabetic rats	Diabetes
	Cholesterol lowering in plasma and liver	Zucker rat	Cardiovascular disease
	Decrease expression of CD68, SERBP1c	Guinea pigs	NAFLD
	Anti-obesity properties	High-fat induced obesity rats	Obesity
	Inhibit enzymes linked to T2D		Diabetes
Alkaloids	Cardioprotection	Cardiotoxic-induced rats	Cardiovascular disease
Tannins	Anti-inflammatory	Rats	Cardiovascular/Cancer
Isothiocyanates	Decreased expression of inflammatory markers	RAW Macrophages	Cardiovascular disease
	Reduction in insulin resistance	Mice	Diabetes
	Inhibition of NF-kB signaling	Cancer breast cells	Cancer
B-Sitosterol	Decrease cholesterol absorption	High-fat fed rats	Cardiovascular disease

Most of the reviewed literature reported changes in the biochemical parameters of diabetes mellitus after short-term administration of an extract of *M. oleifera* leaves. Furthermore, it has been reported that *M. oleifera* administration can induce better glucose tolerance, reduces body weight gain, circulating cholesterol, improves glucose tolerance and lipid metabolism in rats (**Adisakwattana and Chanathong. 2011; Jaiswal *et al.*, 2013 and Yassa and Tohamy, 2014**).

On the other hand, Calcium has been involved in dietary substances that have a significant positive effect on Insulin resistance and fat metabolism as it interferes with the expression of some Insulin-resistant gene (Resistin) and fat metabolism genes (Leptin and Adiponectin) (**Nobre *et al.*, 2011**).

Since the overweight problem is the most common nutrition-related health condition. Many attempts have been done to prevent and control such problem and minimize its serious health risks. In the current trial the impact of dietary fortifications of graded levels of dried *Moringa oleifera* leaves (MLS) on the expression of some important genes including, resistin, adiponectin and leptin as adipokines, IL-1 and IL-6 as pro-inflammatory cytokines of albino rat fed on modified AIN-93G purified diet as well as the possibility of its use as promising Clinico-Nutritional management tool to overcome the over-weight problem.

Materials and Methods

Preparation of *Moringa oleifera* leaves powder

Fresh MLS were collected from medicinal plants farm located at El-Fayoum- Egypt. The leaves were washed with distilled water to remove the residual soil, completely dried firstly under shed then oven dried at 60º C overnight, until constant weight, ground to fine powder, and stored at -20°C in tightly closed plastic containers until use.

Animals and husbandry

Thirty male rats (80-90 gm) were purchased from National Research Center (NRC) animal housing department, Giza, Egypt were utilized throughout the current study. Rats were housed in stainless steel cages in animal house at Regional Center for Food and Feed, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt, under controlled light and temperature conditions (12/12 h light/dark cycles at 23±3 ºC). During the acclimation period (1 week) and experimental period (10 weeks).

Experimental design

Thirty male rats (80-90 gm) were purchased from National Research Center (NRC) animal housing department, Giza, Egypt. Rats were housed in stainless steel cages in animal house at Regional Center for Food and Feed, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt under controlled light and temperature conditions (12/12 h light/dark cycles at 23±3 ºC). During the acclimation period (1 week) and experimental period (10 weeks), the rats were fed on standard diet (Table 3) based on AIN-93G purified diet (**Reeves *et al.* 1993**), mineral mix AIN-93G and vitamin mix AIN-93-VX. The diets were isocaloric in nature and differed only in calcium content and source(s). Purified tap water was supplied ad libitum. Rats were divided into five groups (six rats each) and were treated for 10 weeks as follow: G1-control: supplemented with 1.25% calcium carbonate, G2-low calcium: supplemented with 0.06% $CaCo_3$, G3: supplemented with 2.5% MLS and G4: supplemented with 1.25% and 2.5% Calcium Carbonate and MLS, respectively. Ca concentrations in diet expressed in (mg Ca/g dry mass). At the end of treatment period, all the animals were fasted overnight and were sacrificed on the next day. Abdominal fat layer and intestine were collected under hygienic conditions and were kept at -20^{o}C for further analysis. Blood samples were collected from the retro-orbital venous plexus under carbon dioxide anesthesia into no-anticoagulant coated tubes and were left to coagulate at 4°C and then centrifuged at 3000 x g for 15 minutes. The clear serum yields were collected and stored at −20°C for further biochemical analysis.

Table 3 Composition of the experimental diets (g/100 g dry matter) formulated according to AIN-93G:

ingredients	G1	G2	G3	G4
Diet Ingredients:				
Casein	20.00	20.00	19.36	19.36
Maltodextrin	13.20	13.20	13.20	13.20
Sucrose	10.00	10.00	10.00	10.00
Corn Starch	38.5	39.69	38.25	37.07
Cellulose	5.00	5.00	4.79	4.79
Corn Oil	7.00	7.00	6.83	6.80
***AIN-93G Mineral Mix**	3.50	3.50	3.50	3.50
L-Cystine	0.30	0.30	0.30	0.30
****AIN-93-VX Vitamin Mix**	1.00	1.00	1.00	1.00
Choline bitartrate	0.25	0.25	0.25	0.25
Calcium sources:				
Calcium carbonate	1.25	0.0625	-	1.25
Moringa	-	-	2.525	2.53

***Mineral Mix according to AIN-93G: **Monopotassium phosphate** (19.6%), **Potassium Citrate monohydrate** (7.078%), **Sodium Chloride (**7.4%), **Potassium Sulfate(** 4.66%), **Magnesium Oxide (**2.4%), **Ferric Citrate (**0.606%), **Zinc Carbonate (**0.165%), **Manganese Carbonate** (0.063%), **Copper Carbonate** (0.03%), **Potassium Iodate** (0.001%), **Sodium Selenate, Anhydrous (**0.00103%), **Ammonium Molybdate 4H_2O** (0.000795%), **Sodium Metasilicate 9 H_2O(** 0.145%), **Chromium Potassium Sulfate 12 H_2O** (0.0275%), **Boric acid** (0.00815%), **Sodium Floride (**0.00635%), **Ammonium Vanadate** (0.00066%), **Powdered Sugar** (57.8%).

****Vitamin Mix according to AIN-93 VX: **Nicotinic Acid** (3.0 **gm/kg)**, **D- Calcium Pantothenate(** 1.6 **gm/kg)**, **Pyridoxine HCl(** 0.7 **gm/kg**), **Thiamine HCl(** 0.6 **gm/kg)**, **Riboflavin(** 0.6 **gm/kg**), **Folic Acid(** 0.2 **gm/kg)**, **D-Biotin(** 0.02 **gm/kg**), **Vitamin B_{12}** (2.5 **gm/kg)**, **α-Tocopherol Powder (250 U/gm)(** 30 **gm/kg)**, **Vitamin A Palmitate (250,000 U/gm)** (1.6 **gm/kg)**, **Vitamin D3 (400,000 U/gm)(** 0.25 **gm/kg)**,**Pylloquinone (**0.075 **gm/kg)**, **Powdered Sucrose** (959.655 **gm/kg).**

Serum biochemical analysis:

Biochemical Blood Analyzer (Alfa Wassermann Diagnostic Technologies, Llc, Ace, Alera, USA) was used to measure the following parameters: total cholesterol (TC), triglycerides (TG) and total calcium using standard commercial kits Diagnostic Systems GmbH (DiaSys) Holzheim, Germany.

RNA extraction and cDNA synthesis:

Visceral adipose tissues and intestine (100 mg each) were collected from 6 rats per group. Samples were lysedand total RNA isolation from tissues was performed using Thermo Scientific TRIzol™ Reagent-RNA extraction Kit. RNA was quantified spectrophotometrically using ND-1000 Spectrophotometer (NanoDrop, USA). cDNA was obtained from using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and the incubation was performed on the gradient thermal cycler (Bio-Rad T100, Michan, USA) according to the manufacturer manual.

qRT-PCR

qRT-PCR was carried out in (7000 ABI, Biosystems, USA) Real-Time PCR. The qRT-PCR was performed using Maxima SYBR Green qPCR Master Mix, Thermo Scientific. The reaction mixture was performed in a total volume of 20 mL containing 4 µl of cDNA (100 ng/µL), 2.5 µl (20 nmol/µl) of each primer set for each gene, and 10 µL of SYBR Green Master Mix and completed to 20 µl with nuclease-free water. Each gene expression was normalized with the housekeeping gene β-actin. The primer sequences for different genes are listed in Table (4). The thermal cycler program was as follows: 95 ^{O}C for 5 min and 40 cycles of 94 ^{O}C for 15 s, annealing for 60 s according to melting temperature suitable for each primer set, extension at 72 ^{O}C for 10 s. The $2^{-\Delta\Delta}$CT formula, the method of relative quantification of mRNA was used to determine the fold difference in gene expression.

Table (4) Primer sequences of the studied genes:

Gene	Sequence	Annealing Temperature
Leptin	Forward: CTCAGCATTCAGGGCTAAGG Reverse: AAGCCTCGCTCTACTCCACA	62 °C
Adiponectin	Forward: AATCCTGCCCAGTCATGAAG Reverse: CATCTCCTGGGTCACCCTTA	65 °C
Resistin	Forward: AGTTGTGCCCTGCTGAGCTCTCTGCC Reverse: CCCATTGTGTATTTCCAGACCCTC	62 °C
B-actin	Forward: GTGGGGCGCCCCAGGCACCA Reverse: CTCCTTAATGTCACGCACGATTTC	60 °C
interleukin 1 β	Forward: GCACGATGCACCTGTACGAT Reverse: CACCAAGCTTTTTTGCTGTGAGT	60 °C
Interleukin 6	Forward: GAGGATACCACTCCCAACAGACC Reverse: AAGTGCATCATCGTTGTTCATACA	60 °C

Statistical analysis

Analyses were performed according to (**SAS, 2013)**. Data were expressed as mean ± SE. Differences between groups were tested for statistical significance using One-way ANOVA, followed by Duncan's test (**Duncan, 1955**). $P < 0.05$ was considered significant for all data analyses.

Results and Discussion

Data obtained from Table (5) illustrated that, groups fed on diet fortified with *M. oleifera* (G3 and G4) had the lowest Resistin and Leptin mRNA values followed by the control group while the highest level was recorded in G_2, which represented the group fed on lower Ca levels if compared to the dietary requirements.

The data are also clear from fig. 4 and 6 contents, which showed the amplification curves of the analyzed resistin and Leptin genes by RT-PCR, the tabulated values and the mathematical processes that were followed to calculate the amount of expressed mRNA depending on the ΔCT values, the calculated values and the graphs that showed the different values of resistin and Leptin mRNA expressed during the experiment.

From the same table it was clear that Adiponectin mRNA had almost the opposite behavior during the experiment as it showed the lowest value in G_2, followed by G_1, then no statistical differences were recorded for both mRNA in the G3 and G4 which had both the highest values. It is clear that, as reported by **Shi *et al.*, 2001**, the lower the dietary Ca level, the higher the adipocytes count, the higher of its secretions and the higher the incidence of its dysfunction.The obtained result in case of offering Ca-deficient diet in G_2 is the increase of the number of the adipocytes and the increase of resistin and leptin, which recorded the highest levels in G_2.Lower values of both mRNA were recorded in G_1 (the control group) which offered the exact requirement of Ca (0.5%) resulting in proper function of the adipocytes hence lower values of resistin and leptin expression if compared to G2. Individuals in G3 and G4showed lower resistin and Leptin values demonstrating that, the presence of MLS caused decrease in the expression of these genes. This result was attributed to the effect of *M. oleifera* as it has anti-cholesterogenic and antilipolytic effect which was clear from data obtained in Table (6) which showed reduced amount of total cholesterol and triglycerides in groups fed purified diets fortified with *M. oleifera leaves.* This anti-cholesterogenic and antilipolytic effect of *M. oleifera* was attributed to the effect of the active ingredients among the composition of this natural plant source which contains Quercetin, chlorogenic acid and niaziminin (as illustrated in Table 2a) which have antihypertensive & antihyperlipidemic effect (**Udechukwu *et al.*, 2018**).

These data are also clear from fig. 6 content, which showed the amplification curves of the analyzed genes, the tabulated values and the mathematical processes that were followed to calculate the amount of expressed mRNA depending on the ΔCT values, the calculated figures and the graphsthat showed the different values of ADI and Leptin mRNA expressed during the experiment, respectively. The obtained data was supported by that reported by **Ahmed *at al.*, 2014**, **Waterman *et. al.*, 2015** and **Fateheya M. Metwally *et al.*, 2017** whose results revealed that, MLS has a hypoleptinemic, hyperadiponectinemic and hyporesistenemic effect in female rat as affected by dietary supplementation of MLS which is attributed to antioxidants, vitamins, minerals, amino acids, carotenoids, alkaloids, flavonoids and the contained phenolic compounds, such as zeatin, quercetin, isoquercetin, kaempferol,apigenin and rutin.

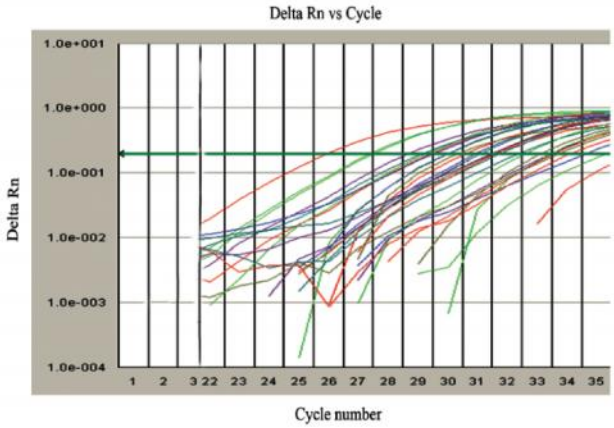
Table (5) Effect of M. oleifera on leptin, adiponectin, interlukin 1β and interlukin 6 mRNA expression in tissue of rats:

Treatment	Resistin	Adiponectin	Leptin	Interlukin1	Interlukin6
G1	1.45^{b}	1.36^{b}	1.85^{b}	4.59^{b}	1.14^{a}
G2	2.98^{a}	0.73^{c}	2.54^{a}	10.08^{a}	1.29^{a}
G3	0.74^{c}	2.31^{a}	0.16^{c}	1.15^{c}	0.91^{b}
G4	0.85^{c}	2.31^{a}	0.16^{c}	1.20^{c}	0.53^{c}
SE	±0.73	±0.45	±0.58	±2.12	±0.23

Data are represented as mean ± SEM (n = 6).

$^{a-c}$ Means with the same letters within each column of trait are not-significantly different (P≤0.05).

a



b

S	RP	CT	S	RP	CT	ΔCT	ΔΔCT	2^-ΔΔCT
C1	1	33.02	C1	1	29.42	-3.6	-1.515	2.86
C2	2	29.89	C2	2	27.38	-2.51	-0.425	1.34
C3	3	34.8	C3	3	34.65	-0.15	1.935	0.26
C4	4	34.99	C4	4	33.48	-1.51	0.575	0.67
C5	5	34.95	C5	5	31.57	-3.38	-1.295	2.45
C6	6	34.81	C6	6	33.45	-1.36	0.725	0.60
T1	1	33.06	T1	1	30.59	-2.47	-0.385	1.31
T1	2	33.01	T1	2	30	-3.01	-0.925	1.90
T1	3	33.47	T1	3	30.99	-2.48	-0.395	1.31
T1	4	33.73	T1	4	31.12	-2.61	-0.525	1.44
T1	5	33.76	T1	5	31.14	-2.62	-0.535	1.45
T1	6	36.25	T1	6	31.48	-4.77	-2.685	6.43
T2	1	27.75	T2	1	25.99	-1.76	0.325	0.80
T2	2	29.43	T2	2	27.49	-1.94	0.145	0.90
T2	3	31.88	T2	3	30.01	-1.87	0.215	0.86
T2	4	30.61	T2	4	28.66	-1.95	0.135	0.91
T2	5	33.86	T2	5	32.37	-1.49	0.595	0.66
T2	6	34.03	T2	6	33.14	-0.89	1.195	0.44
T3	1	35.87	T3	1	34.16	-1.71	0.375	0.77
T3	2	35.15	T3	2	35.37	0.22	2.305	0.20
T3	3	35.35	T3	3	33.54	-1.81	0.275	0.83
T3	4	33.44	T3	4	32.58	-0.86	1.225	0.43
T3	5	33.31	T3	5	31.75	-1.56	0.525	0.69
T3	6	36.38	T3	6	33.73	-2.65	-0.565	1.48

c

G1	1.45
G2	2.98
G3	0.74
G4	0.85

d

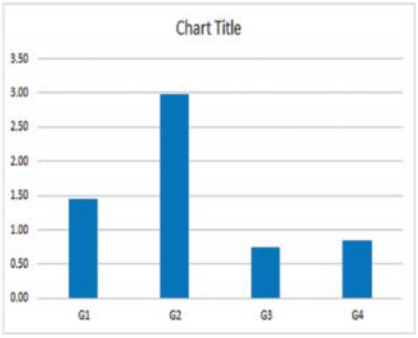
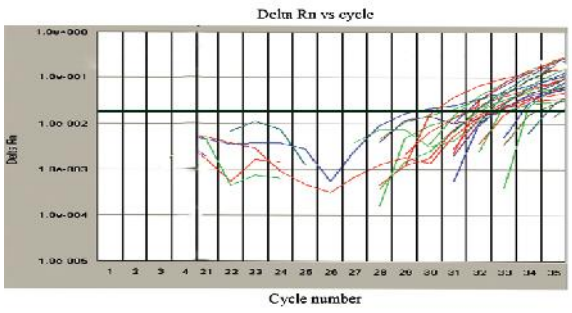
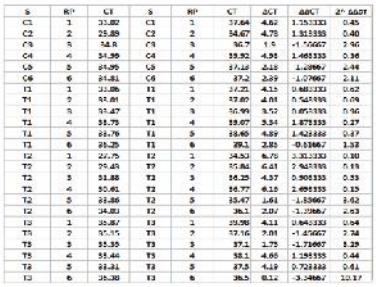


Fig (4) Results of Resistin Gene analysis: a) Amplification blot illustrating the amplification of signals related to Resistin gene reaching the plateau; b) Tabulated data used to calculate 2^-ΔΔCT; c) Calculated 2^-ΔΔCT for all treatments and d) Obtained graph illustrating the values of 2^-ΔΔCT in all groups.

a



b



c

G1	1.36
G2	0.70
G3	2.31
G4	2.31

d

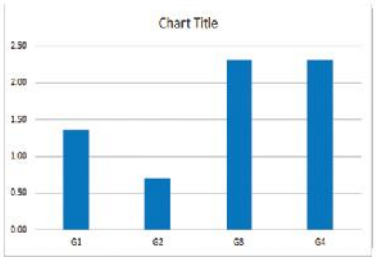
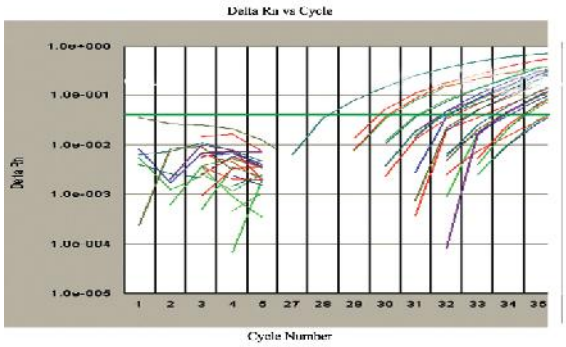


Fig (5) Results of ADI Gene analysis: a) Amplification blot illustrating the amplification of signals related to ADI gene reaching the plateau; b) Tabulated data used to calculate 2^-ΔΔCT; c) Calculated 2^-ΔΔCT for all treatments and d) Obtained graph illustrating the values of 2^-ΔΔCT in all groups.

a



b

S	RP	CT	ΔCT	ΔΔCT	2^-ΔΔCT
C1	1	34.57	1.55	1.868333	0.27
C2	2	30.98	1.09	1.09	0.47
C3	3	34.23	-0.57	-0.57	1.48
C4	4	34.95	-0.08	-0.08	1.04
C5	5	33.14	-1.81	-1.81	3.51
C6	6	32.7	-2.11	-2.11	4.32
T1	1	30.3	2.76	2.76	6.77
T1	2	32.02	-0.59	-0.59	1.50
T1	3	33.77	0.3	0.3	0.81
T1	4	32.31	-1.42	-1.42	2.68
T1	5	32.35	-1.41	-1.41	2.66
T1	6	37.82	1.57	1.57	0.34
T2	1	33.01	5.26	5.26	0.05
T2	2	44.02	4.59	4.59	0.04
T2	3	33.4	1.52	1.52	0.35
T2	4	33.72	3.11	3.11	0.12
T2	5	33.71	-0.15	-0.15	1.11
T2	6	35.65	1.62	1.62	0.33
T3	1	34.2	1.67	1.67	0.31
T3	2	34.4	-0.75	-0.75	1.68
T3	3	34.4	-0.95	-0.95	1.93
T3	4	36.61	3.17	3.17	0.11
T3	5	33.97	0.66	0.66	0.63
T3	6	35.61	0.43	0.43	0.74

c

G1	1.85
G2	2.54
G3	0.16
G4	0.16

d

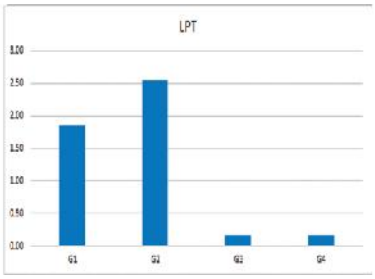
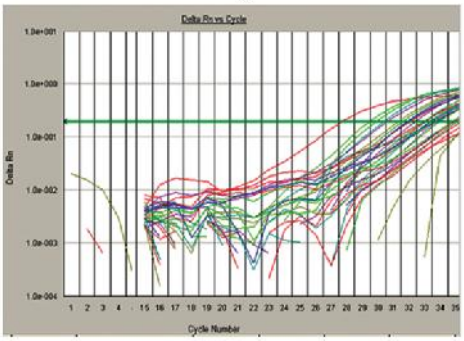
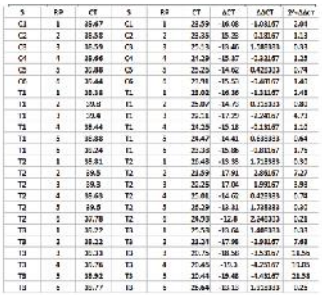


Fig (6) Results of Leptin Gene analysis: a) Amplification blot illustrating the amplification of signals related to Leptin gene reaching the plateau; b) Tabulated data used to calculate 2^-ΔΔCT; c) Calculated 2^-ΔΔCT for all treatments and d) Obtained graph illustrating the values of 2^-ΔΔCT in all groups.

a



b



c

G1	4.59
G2	10.08
G3	1.15
G4	1.20

d

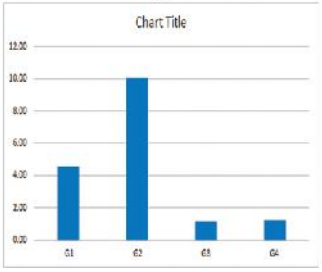
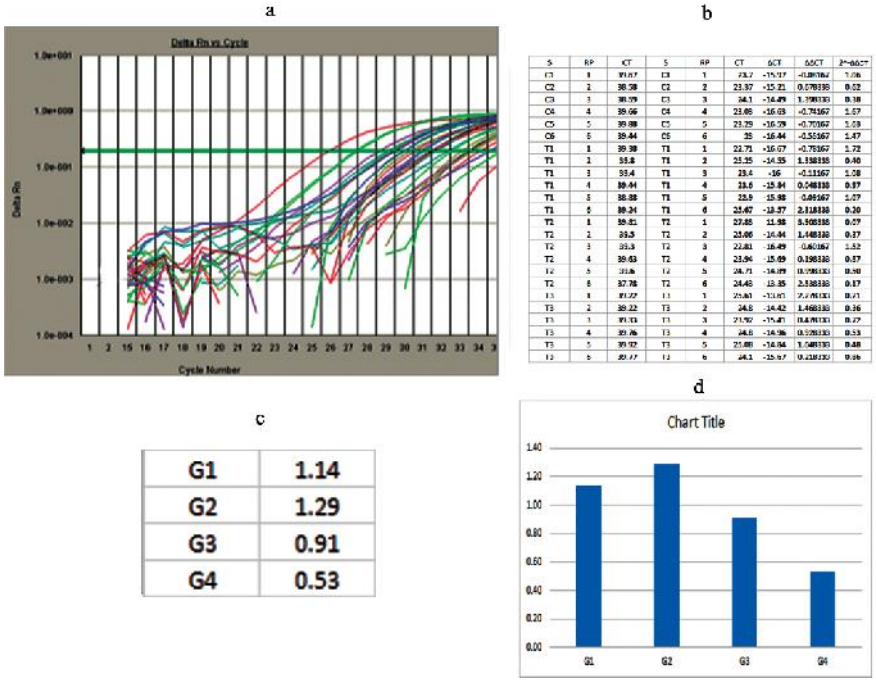


Fig (7) Results of IL-1α gene analysis: a) Amplification blot illustrating the amplification of signals related to IL-1 gene reaching the plateau; b) Tabulated data used to calculate 2^-ΔΔCT; c) Calculated 2^-ΔΔCT for all treatments and d) Obtained graph illustrating the values of 2^-ΔΔCT in all groups.

From the same table and from data illustrated in figures (7 and 8), it was found that, offering Calcium deficient diet to G2 caused significant increase in the expression of both IL-1α and IL-6 which are responsible for the initiation of inflammatory response due to undergoing stress condition in this case, while offering Calcium sufficient diet caused significant reduction in IL-1α in G1 but had no significant effect in the mRNA content of IL-6. The expression of IL-1α was significantly reduced in individuals belonged to G3 and G4 which were supplied with MLS in their diet and the same trend was obtained in G3 and G4 concerning IL-6 expression. These results were supported by that obtained by **Minaiyan *et al.*, 2014; Fard *et al.*, 2015 and Praengam *et al.*, 2015** who reported that MLS decreased the pro-inflammatory cytokines production including IL-1α and IL-6 by macrophages. Also, they added that, Moringa was effective in blocking production of several pro-inflammatory cytokines.



G1	1.14
G2	1.29
G3	0.91
G4	0.53

Fig (8) Results of IL-6 gene analysis: a) Amplification blot illustrating the amplification of signals related to IL-6 gene reaching the plateau; b) Tabulated data used to calculate 2^-ΔΔCT; c) Calculated 2^-ΔΔCT for all treatments and d) Obtained graph illustrating the values of 2^-ΔΔCT in all groups.

Data in Table (6) illustrated that, decrease of dietary Ca content (G_2) caused significant increase of serum cholesterol and triglycerides, which recorded the highest values among all treatments. This result was supported by the results of (**Shi *et al.*, 2001**) who demonstrated the inverse relationship between dietary Ca content & serum lipids due to not only the increase in the number adipocytes but also its dysregulation. From the same table it was clear that, supplementation of *M. oleifera* in the diet caused significant decrease of total cholesterol and triglycerides which showed significant decrease with the supplementation of *M. oleifera* as it became lower than the control group in G3 and recorded the lowest levels with the inclusion of *M. oleifera* together with Ca carbonate in G4.This result was supported by the finding of **Hsu and Culley, 2006** who stated that Ca supplementation caused a significant decrease in serum lipids while its deficiency causes increase of serum cholesterol and triglycerides. Also, it was reported by **Vergara-Jimenez *et al.*, 2017** that, many bioactive compounds found in MO leaves may influence lipid homeostasis. Phenolic compounds, as well as flavonoids, have important roles in lipid regulation as it has a strong effect on lipid profile through cholesterol reducing effects. *Moringa oleifera* (MO) leaves also contain the bioactive β-sitosterol, with documented cholesterol lowering effects, which might have been responsible for the cholesterol lowering action in plasma of experimental rats. Saponins, found in MO leaves, prevented the absorption of cholesterol, by binding to this molecule and to bile acids, causing a reduction in the enterohepatic circulation of bile acids and increasing their fecal excretion. The increased bile acid excretion is offset by enhanced bile acid synthesis from cholesterol in the liver, leading to the lowering of plasma cholesterol.

Table (6) Levels of Cholesterol, Triglycerides and Calcium as affected by the used treatments:

	Cholesterol* mg/dl	Triglyceride* mg/dl	Calcium* mg/dl
G1	99.35±8.66[b]	89.15±3.50[b]	13.43±0.83[b]
G2	124.18±6.31[a]	102.83±4.54[a]	11.77±0.45[c]
G3	77.27±4.26[c]	74.90±3.83[c]	13.33±0.65[b]
G4	65.85±6.18[d]	68.58±4.20[d]	14.37±0.95[a]
LSD	7.29	4.60	0.84

*Level in serum **Percent in bones

From the same table, results concerning the concentration of Ca in serum as affected by the inclusion rate of Ca source(s) were obtained as the highest concentration of calcium was recorded in G_4.No statistical differences were recorded in the other groups (except G_2) regarding the concentration of Ca as the inclusion rate of Ca was the same despite of its source. The lowest concentration of Ca in serum was recorded in G_2, which was fed on dietary Ca lower than its requirements. These results agreed with that mentioned by (**Hsu and Culley, 2006**) who illustrated that dietary Ca has direct correlation with both serum and tissue Ca levels. Also, **Parikh *et al.*, 2015** reported that, the phytochemicals present in MLS caused decrease in Calcium excretory rate suggesting that the components were helpful in keeping Calcium load in the body. From the overall data it could be concluded that, MLS can be considered as a reliable additive which can be used to control lipid dysfunction, insulin resistance and obesity. Moreover, from our data it is clear that, dietary Calcium deficiency causes higher Cholesterol, Triglycerides, while increased dietary supplementation with Calcium not only controlled the Cholesterol and Triglycerides levels but also caused higher rates of Calcium deposition in the examined femur bone.

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