Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2017; 9(2): 156-168
DOI number: 10.25258/ijpcr.v9i1.8299
ISSN- 0975 1556

Research Article

The Potential of *Moringa oleifera* to Induce Cerebral Leptin mRNA Expression and to Attenuate Oxidative Stress, Cognitive and Motor Deficits, Depression- and Anxiety- Like Behavior in Experimental Obese Model

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Available Online: 25th February, 2017

ABSTRACT
Moringa oleifera showed an evident role against obesity and leptin resistance. However, *Moringa oleifera* potential effects on alteration of moods, cognitive as well as motor deficits in obese animal model have not been evaluated in a mechanistic way. So, the aim of the current study is to examine the potential of *Moringa oleifera* to induce cerebral leptin mRNA expression, and consequently its effects on amelioration of behavioral and biochemical alterations in obese female rats. Ethanolic extract of *Moringa oleifera* was orally administered (600 mg/kg b.wt) for 12 weeks to those obese rats. Memory behavior, depression- and anxiety-like behavior, as well as motor activity were examined by object recognition test, forced swim test, light and dark test, and open field test, respectively. Leptin mRNA gene expression and its concentration were determined in cerebral cortex using quantitative real time-PCR and ELISA, respectively. Oxidative stress markers malondialdehyde and glutathione were also evaluated in the cerebral cortex. *Moringa oleifera* significantly up-regulated cerebral leptin mRNA expression and its level, as well compared to control obese rats. Moreover, *Moringa oleifera* decreased lipid peroxidation significantly, whereas they improved glutathione significantly in comparison with those untreated rats. Regarding behavioral deficits, *Moringa oleifera* attenuated the declined memory, depression and anxiety-like behavior, as well as the motor deficit that observed in obese untreated rats**.** This study indicated the potential of *Moringa oleifera* in triggering cerebral leptin mRNA expression, hence protection of brain from oxidative damage as well as improvement of cognition, moods and motor deficits in obese rats.

Keywords: *Moringa oleifera*, Leptin mRNA expression, memory, depression, anxiety, oxidative stress.

INTRODUCTION

The comorbid obesity and high-cholesterol diet (HCD), as well as their implications in the pathogenesis of metabolic syndrome[1], alterations of moods, impairments in cognitive statuse, and motor activities among obese subjects, were always a matter of investigations. Long-term utilization of high-caloric diet rich in cholesterol has been contributed to the cognitive decline, due to the impairment of lipid homeostasis, that causes reduction of entry of essential fatty acids into the CNS, which results in dysfunction of membrane proteins and neuronal damage[2]. Further, it has been recorded that HCD induces memory and learning deficits in rats[3,4]. This might be attributed to the positive correlation between the hypercholesterolemia[1], and visceral adiposity. Using HCD has been noticed to trigger the expansion of visceral adiposity and it dysfunction which results in alterations in adipokines releasing levels particularly leptin[1], which in turn would disturb all the body metabolism and interrupt the feedback of leptin about energy homeostasis to the brain[5].

Leptin is the key regulator of food intake and in turn body energy expenditure. It is biosynthesized in adipose tissue as a major adipocytokine, circulated and passed through the blood-brain barrier (BBB), to be taken by leptin receptors. Leptin receptors are widely distributed in the hypothalamus, hippocampus and cerebral cortex[5]. Thus, the dysregulation of leptin actions could influence obesity and other metabolic disorders development, such as insulin resistance and type II diabetes[1,6]. Previous studies suggested that the main factors enhancing the cognitive impairment and depression and various behavioral deficits in obesity were leptin resistance[7,8], and its association with dysfunction of leptin receptors in the brain as well as the disturbance in the leptin transport from the peripheral circulation across the blood-brain barrier[9]. Obesity has been noticed to impair leptin transport across BBB as result of leptin resistance[10]. Insulin resistance and leptin

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resistance (hyperleptinemia)[11] are implicated in an incidence of memory deficit, dementia or Alzheimer's disease in advanced stage[12].However, in rodent, leptin administration improved cognitive deficits[13].

Orientation toward herbal alternatives beyond or instead of the drugs has been focused and paid attention worldwide. *Moringa oleifera* Lam. (*M. oleifera*) is a plant of family Moringaceae. It is commonly called drum Stick tree, which is a widespread growing plant in tropical and subtropical areas[14], as well as in Egypt. It is a traditional indigenous material due to the high nutritional contents of its various parts, hence the name of the miracle tree[15]. Interestingly, the usage of various successful preparations of *M. oleifera* leaves in folk medicine positively influenced the researchers to evident its active constituents and their functions as antibiotic, hypotensive, anti-ulcer, anti-inflammatory, as well as anti-cancer properties without adverse effects on liver and kidney[1,15,16]. Recently, the ethanolic extract of *M. oleifera* leaves demonstrated potent antioxidant[11], and anti-diabetic activities[1]. Additionally, ethanolic extracts of *M. oleifera* leaves restored body weight towards normal status and mechanistically declined dyslipidemia, and insulin resistance through its action on the gene expression of leptin, resistin as well as adiponectin of the visceral adipose mass. Therefore, the ethanolic extract of *M. oleifera* leaves ameliorated hyperleptinemia, hyperresistenemia as well as hypoadiponectinemia in obese hypercholesterolemic rats[1,11].

Consequent to our previous works[1,13], which documented the leptin resistance in obese female rats, and showed the ameliorating activity of *M. oleifera* in reducing the leptin resistance as well as the risk obesity and metabolic syndrome. the present study was conducted to investigate whether brain leptin level in those obese female rats is attributed to leptin that passes across blood brain barrier from circulation as well known or could be attributed to expression of leptin endogenously in neuronal cells, as well. Also, to study the effect of leptin on oxidative stress markers, as well as cognitive, anxiety, depression, and, locomotor activities as assessed by behavioral tests. Also, the present study aimed to clarify the potential role of ethanolic extracts *M. oleifera* in reducing the risk of leptin resistance, behavioral deficits and oxidative stress induced by obesity in those rats.

MATERIALS AND METHODS

Plant materials and extracts

Moringa oleifera Lam. (Family: Moringaceae) was provided by the Department of Horticulture Crops Technology, National Research Centre, Giza, Egypt. Meanwhile, identification of *Moringa oleifera* Lam. was accomplished at Botany Department, Faculty of Science, Cairo University, Giza, Egypt. A voucher herbarium specimen (MO-2014-13) was processed and stored for long-term maintenance at the herbarium museum of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt. Preparation of *Moringa oleifera* ethanolic extract, and determination of phenolic and flavonoids contents of the extract were described previously in our former study Metwally et al. 2016[1].

Animals

Thirty-two adult female Wistar rats at 90 days of age (130±10 g) obtained from a breeding stock maintained in the Animal House of the National Research Centre, Giza, Egypt. Animals were maintained in polypropylene cages under environmentally controlled conditions with a temperature of 24±1°C, a 12 h light/12 h dark cycle and a relative humidity of 60±5%. They were fed with standard rat diet and water provided ad libitum. Rats were allowed two weeks to acclimatize to animal room conditions before the commencement of the experiment. The experimental protocol was approved by Institutional Ethics Committee of the National Research Centre, Giza, Egypt, and experiments were performed as per guidelines of National Research Centre Ethical Committee for Medical Research "Animal Experimentation Sector".

Experimental groups

After the acclimatization period, eight rats were chosen as the normal control group (NC). They received water ad libitum and standard laboratory rodent diet (26.5% protein, 3.8% fat, 40% carbohydrate, 4.5% crude fiber) throughout the experimental period. The rest of the rats (thirty-two) received water ad libitum and high-cholesterol diet (HCD) (19.93% protein, 15% cholesterol (Sigma Chemical Co., USA), 57.50% carbohydrate, and 2.81% dietary fiber) for 12 consecutive weeks, it was the same as in our previous work[1]. The dietary constituents were homogenized in distilled water at 60°C and the resulting homogenate was used to prepare the pellets. Diets were introduced fresh each day as dry pellets.

All rats were allocated into 4 groups (8 rats each) to undergo treatments as follows for 12 weeks: Group 1 (NC): normal control group administered with vehicle (Dist. water) only. The obese rats were deprived of HCD, fed with standard laboratory rodent diet and further assigned into the following 4 groups; Group 2(ob): untreated obese group orally administered with vehicle (dist. water) only, Group 3(Ob+Sim): obese group orally administered with cholesterol-lowering drug (simvastatin), purchased from MSD B.V Co., UAE, (5 mg/ kg b.wt) [15]. Group 4 (Ob + *M. oleifera*): obese group orally administered with ethanolic extract of *M. oleifera* (600 mg/ kg b.wt)[17].

Behavioral assessment

Animals were trained in all behavioral assessments at last week of the treatment period.

Object recognition test (ORT)

The object recognition test is used to assess the cognition, especially the recognition memory or short-term memory. The object recognition test was carried out in the open field arena (as described before). The rats were placed in the arena for 5 min to explore freely to be habituated on the empty place without objects, prior the test sessions. The object recognition test is composed of two trials: a training trial at which, each rat was allowed to explore the objects (2 identical plastic blue square cubes), that were placed in different corners of the arena for 5 min. The training was followed by a retention trial 30 min later. In the retention

trial, one copy of the square cube was replaced by a novel black cylinder (identical in height and volume with the original object). Both objects were placed in the same corners, then the rats were allowed individually to explore the familiar and novel objects in the open field for another 5 min. The animal was defined to be exploring the object when the head of the animal was directed to the object or the animal was touching, licking or sniffing the object. The exploration times were recorded with video and analyzed later in order to calculate the discrimination index as follows:

Discrimination index (OR) = [time spent with familiar object - time spent with novel object] / [time spent with familiar object + time spent with novel object] for the test session.

Increased time spent exploring the novel object rather than the familiar one and higher OR was interpreted as an excellent short-memory ability and retention for the familiar object [18].

Forced swim test (FST)

Depression-like behavior was assessed using the forced swim test. Each rat was placed individually in a transparent plastic cylinder (diameter: 40 cm, height: 60 cm) filled with water to a height of 40 cm. The rats were forced to swim for 5 min, the duration of immobility was recorded during the last 3 min. Stopping, struggling and floating on the surface of water indicated immobility. The test was video-recorded and analyzed later. Water was renewed each time and temperature was kept at 26 ºC[19].

Light and dark test (LDT)

The light and dark test was performed to assess the anxiolytic effect of both herbal extracts in obese rats. The test depends on the innate aversion behavior of rats to the brightly lighted areas and on the spontaneous exploratory behavior of rats due to mild stressors (novel environment and light). The light/dark box consisted of 2 compartments (40 cm x 30 cm x 24 cm) joined together. Between the 2 partitions, there is a central hole (12 cm x 14 cm), that permits the rats to freely move between partitions. One box was turned black with cardboard (black box), while the other box was left transparent and uncovered, but illuminated with white light source (white box). At the beginning of the experiment, an animal was introduced in the center of the light box, facing away from the hole, and allowed to freely access between the 2 compartments for 5 min. Each rat was observed and tracked with video recorded and analyzed later for the time spent in the light and dark boxes, also, for the number of crossings between the boxes[19]**.** The boxes were cleaned after each session using a 70% v/v ethanol.

Open field test

The locomotor activity was assessed in an open field apparatus (40×40×15 cm, divided into 25 squares). The rats were individually placed at the center of a clean open field apparatus, and the activity of each was video-recorded for 3 minutes and analyzed later. The latency (time in the center square), ambulation (number of squares crossed indicating spontaneous locomotor activity) and mobility duration (time of paw movement), rearings on hind limbs (exploratory activity), freezing time and other behavioral disturbances for individual rat were scored as described by[20,21]. The arena was cleaned after each test using a 70% v/v ethanol.

Sample collection

At the end of the experimental period, the final body weight of rats was recorded. Rats were euthanized after withholding food for 14 h. The brain was immediately excised, blotted, then stored immediately in RNAlater and frozen at -80 ◦C until used for gene expression analysis of leptin using quantitative real-time polymerase chain reaction (qRT-PCR). The other brain Brain samples were then processed for biochemical assessments.

Brain homogenates preparation

Sagittal half of the brain of each animal was weighed and washed using chilled saline solution. Tissues such as cerebral cortex tissue were then minced and homogenized in ice-cold phosphate buffer containing 50 mM Tris/HCl and 300 mM sucrose at pH 7.4 to give a 10% (w/v) homogenate[22]. The homogenate was centrifuged at 1400 g for 10 min at 4°C. The resultant supernatant was stored at–80°C until use.

Oxidative stress markers

The protein content oxidative stress markers activities in cerebral tissue were estimated using commercially available kits (Biodiagnostics Co., Dokki, Egypt) according to the manufacturer's instructions as follows:

Determination of lipid peroxidation level

Lipid peroxidation was determined by estimating the level of malondialdehyde (MDA) as described by Mihara and Uchiyama[23]**.** In brief, 100 ul aliquots of the supernatants were added to 1 mL 10 % trichloroacetic acid and 1 mL 0.67 % thiobarbituric acid, mixed well, the test tubes was covered with glass beads. They were then heated in a boiling water bath for 30 min. After cooling, butanol (2:1, v/v) was added to the solution. The mixture was centrifuged for 5 min at 5000 g. Finally, the absorbance of samples was read against blank by spectrophotometry at 534 nm. Results were expressed as MDA nmole/mg protein.

Determination of reduced glutathione level

Reduced glutathione (GSH) was determined according to Beutler et al. 1963[24]. Supernatants of brain homogenates were collected and each sample was mixed well with 0.5 M trichloroacetic acid. The samples were allowed to stand for 5 min 25 ºC and centrifuged at 9000 g for 15 min. The supernatants were then mixed with 0.4 M TRIS buffer and 0.01 M 5,5 dithiobis (2-nitrobenzoic acid) (DTNB). The method based on the reduction of DTNB with GSH, and measuring the absorbance of the produced yellow compound by spectrophotometry at 405 nm. The reduced compound is directly proportional to GSH concentration which is expressed as nmole/mg protein.

Determination of leptin concentration

The leptin concentration in cerebral tissue was assayed by enzyme linked immunosorbent assay (ELIZA) technique using Gscience kits purchased from Glory Science Co., Ltd, USA, according to manufacturer's instruction.

Determination of protein content

The protein content in cerebral tissue was determined according to the method described by Bradford (1976)[25], using bovine serum albumin as standard.

RNA extraction and cDNA synthesis

Rat cerebral tissues (100 mg each) were collected from 8 rats per group and stored for a maximum of 4 days in the RNAlater (RNA stabilizer reagent) solution (Qiagen, Germany). About 30 mg of stored samples were ground and homogenized using a mortar and pestle in liquid nitrogen. Total RNA isolation from tissues was performed using GeneJet RNA Purification Kit, Thermo Scientific. RNA was quantified spectrophotometrically using (ND-1000 Spectrophotometer, NanoDrop). C-DNA was obtained from 1 µg samples of tissue-derived RNA (High capacity cDNa) with RevertAid First Strand cDNA synthesis Kit (Thermo Scientific) and the incubation was performed on the gradient thermal cycler (Bio-Rad).

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was carried out in Stratagene Mx3000p, real-time PCR system, Agilent Technologies. The qRT-PCR was performed in a duplicate for each sample using Maxima SYBR Green qPCR Master Mix, Thermo Scientific. The reaction mixture was performed in a total volume of 20 µl containing 4 ul of cDNA (100ng/ul), 300 nM of each primer set for leptin gene, and 10 µl of SYBR Green Master Mix and completed to 20ul with nuclease-free water. Leptin gene expression was normalized with the housekeeping gene beta-actin (β-actin). The primer sequences for different genes (synthesized by Biosearch Technologies, USA) are listed in Table 1[26]. The thermal cycler program was 95 ºC for 5 min and 40 cycles of 94 ºC for 15 sec, annealing for 60 sec according to melting temperature suitable for each primer set, extension at 72 ºC for 10 sec. The $2^{-\Delta\Delta CT}$ formula, the method of relative quantification of mRNA was used to determine the fold difference in gene expression.

Statistical analysis

Data obtained were expressed as mean ± standard error of mean (SEM). The significance of the interrelation of different groups was tested using One-Way Analysis of Variance, ANOVA and Tukey's multiple comparison tests using GraphPad Prism™ (version 4.03, GraphPad software, San Diego, CA, USA). Difference values were considered significant at $P < 0.05$. Bivariate comparisons were examined using Pearson's correlation coefficient (r) to analyze the correlation between the behavioral variables and leptin gene expression.

RESULTS

Behavioral assesment

Novel object recognition test

Analysis of the Performance of all examined groups as shown in Fig. 1 revealed decreased significant discrimination between the novel and familiar objects in obese control rats relative to normal control ones (P<0.05), which indicated the impairment in working short memory as a result of HFD. Conversely, administration of simvastatin to obese rats increased statistically (P<0.05) the time that they spent with the novel object relative to untreated obese control group. similarly, M.oleifera extract treatment demonstrated higher discrimination rate in the short-term memory for the animals as compared to obese control females (P<0.05). Moreover, *M. oleifera* significantly improved the discrimination index rather than simvastatin (P<0.05).

Forced swim test

Fig. 1 illustrated that the high-fat diet induced depressive-like behavior in female rats in the forced swim test which evidenced by the significant increase in immobility duration regarding the control rats (P<0.05). On the contrary, the obese rats that received the cholesterol-lowering agent as well as *M.oleifera* exhibited a significant reduction in immobility duration with respect to the obese rats (P<0.05), suggesting that they might function as antidepressant- like agents.

Dark and light box

As shown in Fig. 2(a,b) High-fat diet showed a notable decrease the time spent in the light zone (P<0.05), and the number of entries in the light compartment in female obese rats as compared to females fed on normal chow diet (P<0.05). On the other hand, treatment of obese rats with simvastatin and ethanolic extracts of *M. oleifera* increased the time spent in the light zone with respect to the obese rats (P<0.05) (Fig. 2a), however, all treatments did not affect number of entries in the light zone (2b**).** Interestingly, the anxiogenic effect exhibited by obesity was ameliorated by M. oleifera treatment, which reflects its anxiolytic-like effects.

Open field test

As shown in Fig. 3 (a-e) the locomotor activity was evaluated in an open field apparatus. Different activities (latency, ambulation, rearings, grooming, as well as freezing time) were analyzed in all the experimental groups. The obese group showed statistically significant elevation of latency period (P<0.05), reduction in number of crossed squares (P<0.05), number of rearings (P<0.05), accompanied by higher freezing time (P<0.05) as well as grooming time (P<0.05) more than monitored in the control group, which in turn indicated the role of HFD in reduction of locomotor activity.

The obese rats treated with simvastatin exhibited a significant improvement in spontaneous locomotor activity represented by lower latency period (P<0.05), higher number of crossings, and freezing time as well as in exploratory activity associated with reduced grooming time, compared to the control obese rats(P<0.05) (Fig. 3a-3e).

Treatment of rats with the ethanolic extracts of *M.oleifera* showed a significant triggering effect on the spontaneous locomotor activity of rats, as the latency period and freezing time decreased, and ambulation increased compared to obese control group (P<0.05). Regarding the exploratory activity, *M.oleifera* increased the number of rearings significantly (P<0.05). Grooming events also consumed lesser time than observed in obese control rats (P<0.05).

Body weight gain

As shown in Table 2 the high-cholesterol diet administration to rats for 12 weeks increased body weight gain significantly as compared to the normal control rats

Table 1: Primer sequences of the studied genes.

Gene	Primer sequence (5′ –3′)	AT (ºC)	GenBank accession no. & references
Leptin	Forward CTCAGCATTCAGGGCTAAGG	62	NM_013076 [26]
	Reverse AAGCCTCGCTCTACTCCACA		NM_031144 [26]
β-actin	Forward GTGGGGCGCCCCAGGCACCA	60	
	Reverse CTCCTTAATGTCACGCACGATTTC		

Table 2: Effect of ethanolic extract of *M. oleifera* on body weight gain, oxidative stress markers (MDA & GSH) and leptin levels in female obese rats.

Parameter	NC	Ob	Ob+Sim	Ob+ *M.oliefera*
Body weight gain (%)	87.69±4.37	164.85±4.21^{a}	-42.37±3.27^{b}	-40.22±3.57^{b}
MDA (nmole/mg protein)	1.46±0.09	2.39±0.11^{a}	1.61±0.04^{b}	1.63±0. 05^{b}
GSH (nmole/mg protein)	25.40±2.14	13.26±1.82^{a}	20.19±1.22^{b}	19.87±0.71^{b}
Leptin (Pg/ mg protein)	189.76±7.63	99.27±5.32^{a}	128.41±5.52^{b}	130.34±8.17^{b}

that fed on normal chow diet, the increase in body weight gain was amounted to 164.85% (P < 0.05) and indicated frank obesity. On the other hand, posttreatment of the obese group with simvastatin and ethanolic extracts of *M. oleifera* induced body weight loss and decreased obesity significantly as compared to the control obese group, the body weight loss was amounted to -40% to- 42% (P < 0.05).

Oxidative stress biomarkers

High- cholesterol diet resulted in increased lipid peroxidation level (MDA) in the cerebral cortex of the obese female rats as compared to the control female ones (P < 0.05). Interestingly, the MDA levels were restored to normal levels by simvastatin and, *M. oleifera* treatments (Table 2). Further, GSH decreased significantly in the cerebral tissue of the obese rats relative to normals (P < 0.05). However, oral administration of simvastatin and herbal extract enhanced the levels of GSH in the brain of treated rats significantly as compared to control obese group (P < 0.05).

Effect of simvastatin, M. oleifera extract on concentration and gene expression of leptin in brain (cerebral tissue)

Serum leptin level was decreased significantly in the cerebral cortex of the obese control group, compared with the control group (P < 0.05) (Table 2). Conversely, treatment of the obese rats with simvastatin improved the level of leptin significanty (P < 0.05). Additionally, ethanolic extract of *M. oleifera* increased the level of leptin in cerebral tissue significantly with respect to the obese rats (*P*<0.05).

Comparative gene expression analysis by RT-PCR showed that leptin gene expression in obese group was down-regulated to 0.52±0.03fold as that of the normal control group (Fig. 4). Simvastatin and ethanolic extract of *M. oleifera* treatment in obese rats showed significant up-regulation of the leptin gene expression reaching 1.58±0.07-fold, and 2.39±0.04-fold that of the untreated obese group, respectively, *P*<0.05 (Fig. 4). Moreover, the up-regulation of leptin expression was significantly in favor of *M. oleifera* treatment.

The correlations between behavioral variables, the leptin levels and mRNA expression in the cerebral cortex

The current data showed negative correlation between leptin mRNA and body weight gain (r= -0.646, p= 0.000). The results showed significant correlation between leptin mRNA and the levels of leptin (r= 0.880, p=0.006) in the cerebral cortex. Further, the data showed significant correlation between behavioral variables and the levels of leptin mRNA [object recognition test: r = 0.938, p = 0.000; light and dark test: (no of entries into light compartment r = 0. 980, p = 0.000 and time spent in light r = 0. 931, p = 0.000); forced swim test (negative correlation with immobility duration): r = -0. 863, p = 0.000 and open field test (ambulation r = 0.959 , p = 0.000; rearing r=0.851, p= 0.000 ; negative correlation with freezing time r= -0.946, p= 0.000 as well as with grooming time r = -0.707, p= 0.000)].

DISCUSSION

The current study reported the endogenous expression of leptin in the cerebral brain tissue, and the casual link between the high intakes of HCD, (which was confirmed by increased weight gain and induction of obesity) and the down-regulation of leptin expression, and its level in brain tissue. The possibility of expression of leptin in the brain is a paradoxical issue. Although there is some recent data did not report leptin expression in mice brain astrocytes or hippocampal neurons[27], our results evidenced the idea that, the leptin which was detected in the brains of obese female rats is due to the leptin that was expressed in cerebral cortex of the brains, and did not come from the peripheral circulation only, since brain leptin levels that were recorded in the current study, were reversely proportional to the serum leptin levels that were reported formerly[11], which support previous studies of Morash et al. 1999[28], and Marwarha et al. 2016[29]. Moreover, Marwarha et al. 2011[30] reported the endogenous expression of leptin in the cortex and hippocampus of the rabbit's brain. *M. oleifera* and simvastatin treatments significantly up-regulated the leptin expression, and indicated the effect significantly by raising leptin levels in brains of obese female rats. This was in agreement with our previous reports[1], which indicated the role of those treatments in attenuation of obesity, hypercholesterolemia, insulin resistance, and leptin resistance conditions.

Our study delineates that down-regulation of leptin expression was significantly and positively correlated to memory deficit, anxiogenic -and –depressive-like

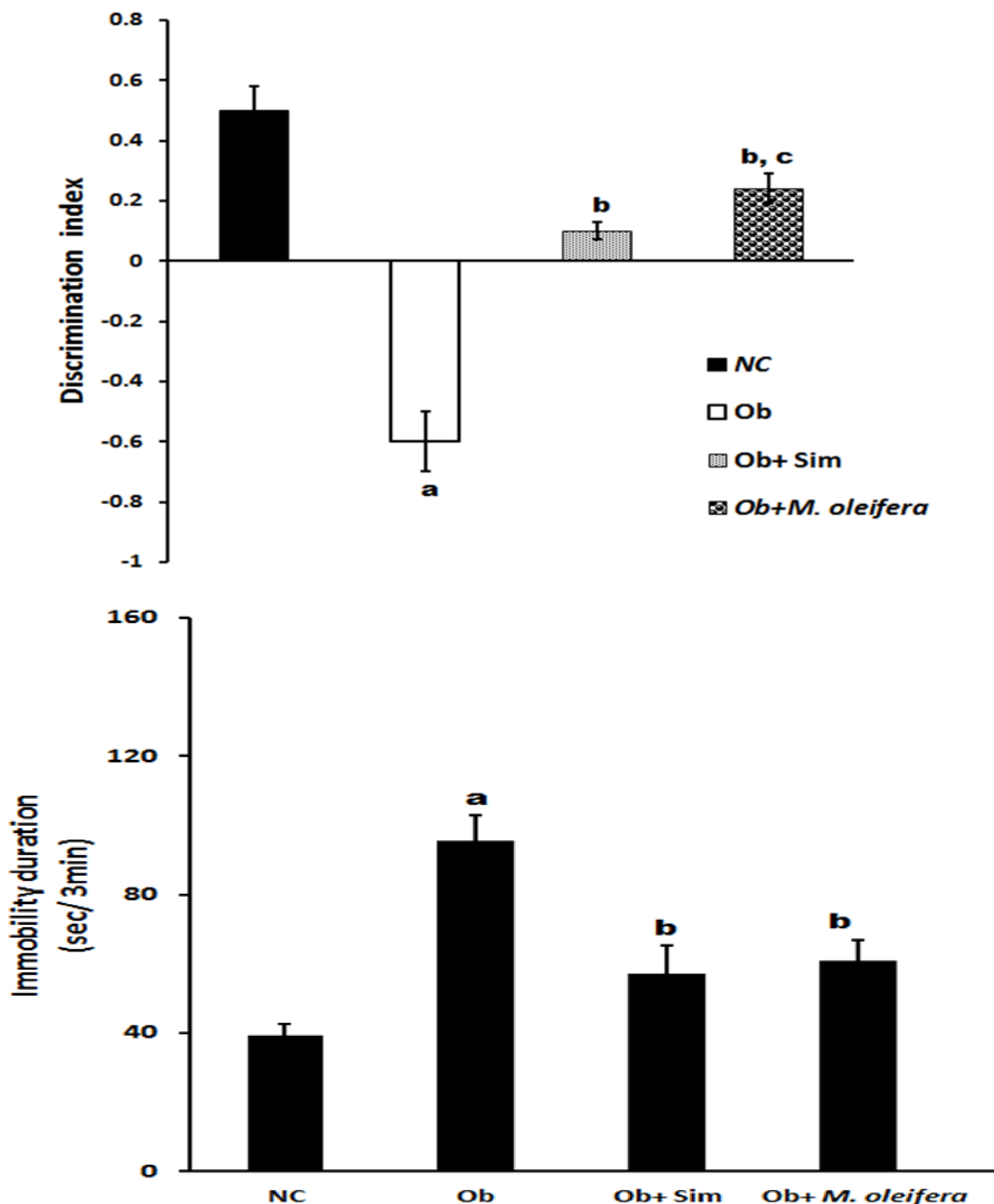


Figure 1: Effect of ethanolic extract of *M. oleifera* on discrimination index in the novel object recognition test, and on immobility duration in forced swim test in female obese rats. Data are represented as mean ± S.E.M (n=8). a: Significant change at $P < 0.05$ in comparison with NC group; b: Significant change at $P < 0.05$ in comparison with Ob group; c: Significant change at $P < 0.05$ in comparison with Ob+ sim group.

behaviors as well as impairment in locomotor activity, as assessed in object recognition test, forced swim test, light and dark box test, and open field test, respectively. Interestingly, to our knowledge, endogenous leptin expression in rats' brain tissues and its association with cognition and other behavioral changes have not been investigated.

Obesity, particularly as a life style-related disease, and as a feature of depending on HCD is directly linked to brain structure alterations, cognitive and memory decline, as well as Alzheimer disease[31]. In compliance with this, obesity resulted in cognitive and memory deficits in experimental animal models[32,33]. This was in agreement with our finding that showed that hypercholesterolemia in obese rats showed a decline in their memory function as

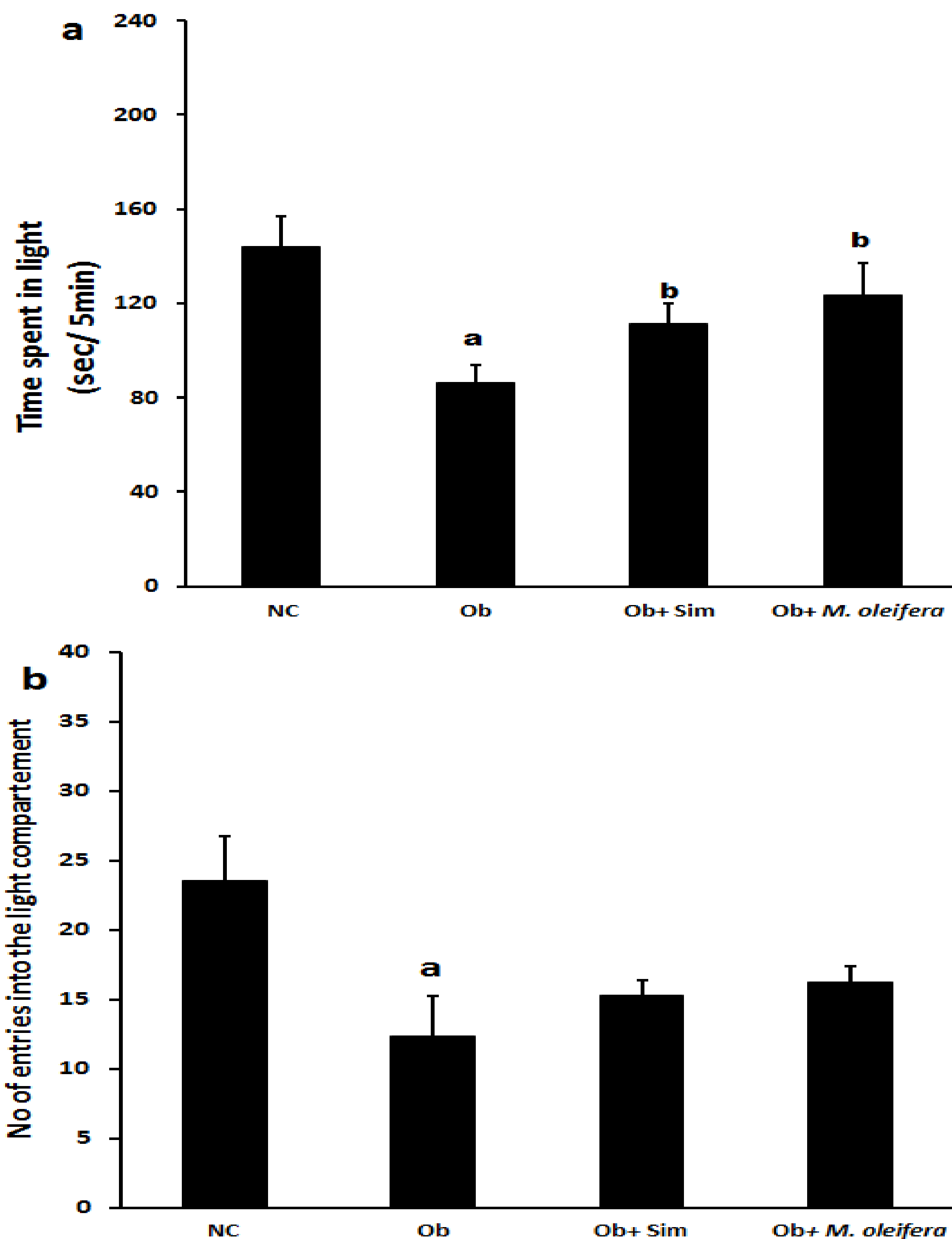
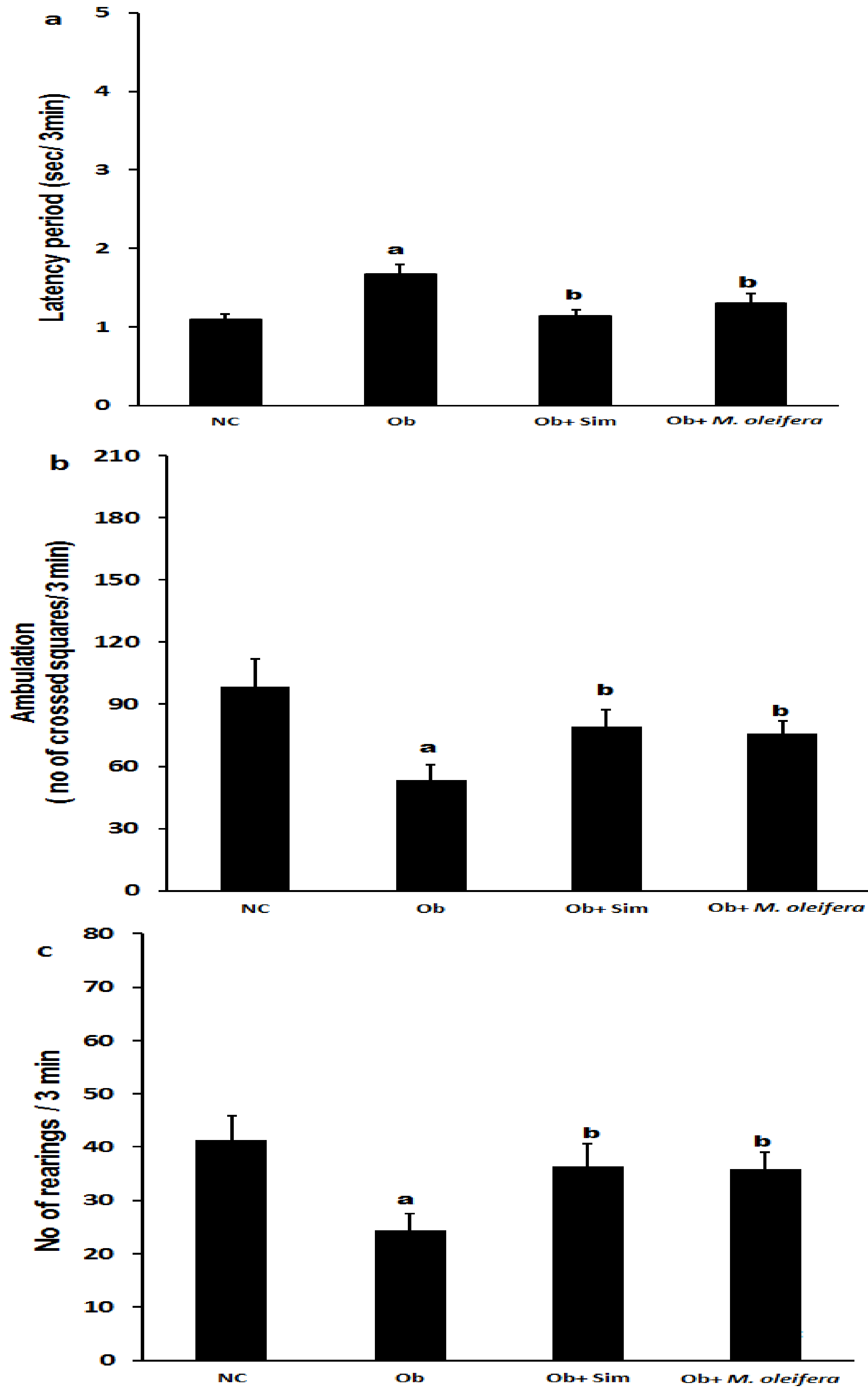


Figure 2: Effect of ethanolic extract of *M. oleifera* on time spent in light (a), and on no of entries into the light compartment (b) in light and dark test in female obese rats. Data are represented as mean ± S.E.M (n=8). a : Significant change at $P < 0.05$ in comparison with NC group; b: Significant change at $P < 0.05$ in comparison with Ob group.

displayed in the ORT (the assessment is based on the higher time that rat spent exploring in the favor of the novel object). However, whose received therapeutic doses of simvastatin or *M. oleifera* significantly exhibited recovered cognitive and memory functions due to up-regulation of leptin expression. Further, the cholesterol-lowering drugs (simvastatin) as well as *M.oleifera* exhibited reduction in the lipid level, ameliorated leptin resistance[1], which could be the cause of restoring the cognitive function in obese rats of the present study. Thus, increasing expression of brain leptin as a neurotrophic cytokine[34] would confirm its role as a neuroprotector, cognitive enhancer as well as in improving memory functions[35].

The forced swim test is the most predictive and validated assessment to describe depression in animal models,


a
Latency period (sec/ 3min)
5
4
3
2
1
0
a
b
b
NC
Ob
Ob+ Sim
Ob+ M. oleifera
b
Ambulation
(no of crossed squares/3 min)
210
180
150
120
90
60
30
0
a
b
b
NC
Ob
Ob+ Sim
Ob+ M. oleifera
c
No of rearings / 3 min
80
70
60
50
40
30
20
10
0
a
b
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NC
Ob
Ob+ Sim
Ob+ M. oleifera

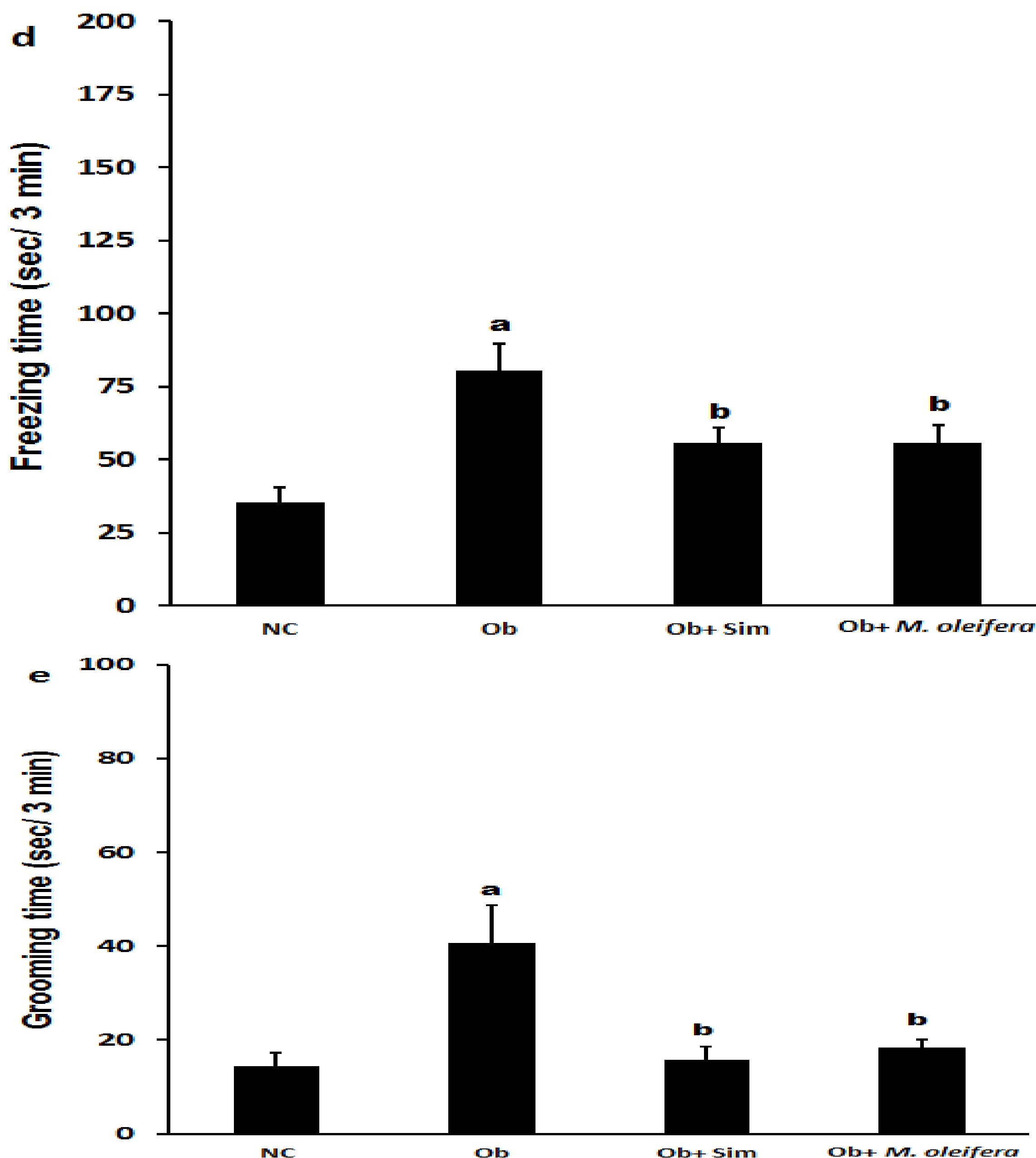

Figure 3: Effect of ethanolic extract of *M. oleifera* on the latency period (A), ambulation (B), no of rearings (C), freezing time (D) and grooming (E) in the open field test in female obese rats. Data are represented as mean ± S.E.M (n=8). a : Significant change at $P < 0.05$ in comparison with NC group; b : Significant change at $P < 0.05$ in comparison with Ob group.

particularly upon examination new chemical agents in the pharmaceutical companies, since it monitors the changes between immobility while keep floating and swimming activity in depressed patient and rodents[36,37]. Recently, leptin has been suggested to be involved in mood alteration. This was evidenced by the prevalence of depression among obese subjects, which could be attributed to leptin resistance[38]. Increasing immobility duration, and reduction of the swimming time in obese rats in the current study, As well as, reduction in explorative behavior that represented by decreased rearing, and ambulation during the open field test, would reveal some kinds of anhedonia and reflect the prevalence of depression among them, that could be attributed to the peripheral leptin resistance[11], and endogenous down-regulation of leptin expression, as well as its levels in the brain, that was in compliance with the previous study of Kurhe et al. 2015a[39].

Our results showed the antidepressant-like effects of simvastatin and *M. oleifera* that were clearly observed by reduction of immobility duration, increased explorative behavior during the open field test, which was positively correlated to up-regulation of leptin expression and levels in the brain, upon administration of those treatments. This was in agreement with the previous report that suggested that the hyperleptinemia was associated with depression-

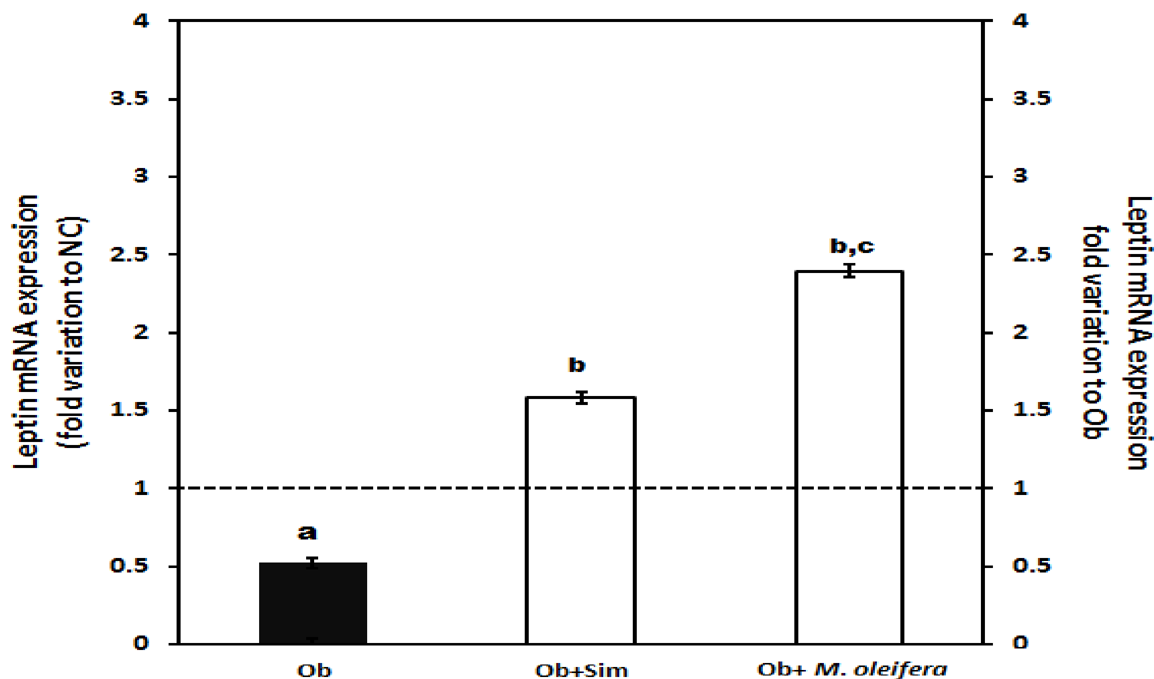

Figure 4: Effect of ethanolic *M. oleifera* extract on leptin mRNA expression in brain cerebral tissue of female obese rats. The fold variation of mRNA in Ob group is compared with the NC condition (dashed line), while the fold variation of mRNA in Ob + sim and Ob + *M. oleifera* is compared with the Ob condition (dashed line). Data are represented as mean ± SEM (n = 8). a : Significant change at $P < 0.05$ in comparison with NC group; b : Significant change at $P < 0.05$ in comparison with Ob group; c: Significant change at $P < 0.05$ in comparison with Ob+ sim group.

like behavior that was recovered upon injection of leptin into the brain of rats[7]. Moreover, administration of leptin to stressed rats reversed their depressive status[40]. In this respect, *Moringa oleifera* ethanolic extract showed antidepressant effect approachable to fluoxetine (well-known antidepressant drug) as examined in FST and OPT[41].

The light and dark test was a behavioral paradigm used in the assessment of anxiety, and the anxiolytic effect of both herbal extracts in obese rats. The test is based on the innate aversion behavior of rats to the novel environment and light areas as mild stressors and on the spontaneous exploratory behavior of rats. Previously, anxiety-like behaviors that were monitored in diabetic rats was inversely proportional to leptin receptors expression in brain cortex[42]. In another study, leptin was found to be lower in depressed and anxious patients[43]. The current data confirmed the role of leptin in the earlier studies and suggested the anxiety-like behavior would be due to inhibition of endogenous neuronal leptin expression as a result of hypercholesterolemia and obesity. The natural dislike of rats to be in lighted- zones is ameliorated by the impact of simvastatin and *M. oleifera* in such a way that they showed marked increases in the time spent in the lighted box and displayed anxiolytic-like effect, compared with those obese rats. Also, increasing rearing as well as number of crossed squares (signs of explorative interest) observed in the open field test[44], confirmed the anxiolytic-like activity of all treatments.

Interestingly, ambulation and mobility duration test motor activity. However, rearing is an exploratory behavior that demonstrates the visual assessment and alertness of the animal as well as degree of anxiety[45]**.** Thus, this paradigm clearly showed that leptin resistance and down-regulation of leptin expression in the brain is responsible for the motor and exploratory- behavioral deficits monitored in the obese rats. Skeletal muscle activity is implicated in displaying the body leptin and insulin resistance and metabolic status, as well. Since increasing motor activity in response to increased leptin sensitivity could be related to its role in induction of muscle-derived myokines[46]. Moreover, grooming behavior in rats is a kind of innate self-care behavior, it takes places under normal conditions and consumes approximately 40% of their locomotor activity[47]**.** Self-paced grooming is triggered under stress, and its increase would be a clear sign of anxiety, particularly if the rats we examined in open field test[48]. Previous reports illustrated that the increase in grooming level is directly correlated to the degree of anxiety[49], as well as depression[50]. Conversely, long-term treatments with ethanolic extracts of *M. oleifera* or simvastatin showed beneficial impact in attenuation of motor deficits. These results indicated the significant correlation between, attenuation of obesity-induced leptin resistance by *M. oleifera* in our former work[1], their role in up-regulation of leptin expression in the brain of obese rats and restoring of motor and exploratory interests of those rats, which were evidenced by enhancing rearing activity and decreasing freezing time.

Rats fed the HCD for a long period, would exhibit higher production of ROS in their brain tissues rather than in the controls. Therefore, the brain vulnerability to oxidative stress is associated with GSH depletion and lipid peroxidation rise[51]. Brain dysfunction is considered a potential results of obesity as a stressful condition implicated in induction of ROS and oxidative stress[56]. Various studies were in agreement with the present work, and reported the direct correlation between depression and raising of brain MDA, and reduction in brain GSH in mice[39,53]. Oxidative stress shows deleterious effects that would lead to neuronal cell death, which is accompanied by memory impairment in the rats[54,55]. The present data showed potent antioxidant activities of simvastatin, *and M. oleifera*, since they declined the observed rise in MDA level, and improved the reduction in GSH content in the brain tissue of hypercholesterolemic rats. This was a confirmation of our previous investigation[1], that recorded the pronounced antioxidant effects of *M. oleifera* since they exhibited significant inhibition of lipid peroxidation induced by leptin resistance in obese rats' liver homogenate. The previous data reported that that simvastatin improved the antioxidant status via prevention of oxidative stress[56]. Also, Zhang et al. 2016[57] indicated statins reversed the increase in lipid peroxidation the decrease in GSH-Px expression in the hippocampus hypercholesterolemic rabbits compared to the control group. *Moreinga oleifera* extract has been shown to decrease immobility duration, as well as a reduction in blood MDA[58]. This would give a good evidence and support of the idea of the association of improvement of antioxidant capacity with reduction of despair and depression.

CONCLUSION

The current study suggested that the long-term treatment of obese rats with *M. oleifera* reported its role in up-regulation of brain leptin expression and induction of endogenous leptin production in cerebral cortex of those rats. Further, *M. oleifera* administration suggested the direct correlation of its antidepressant and anxiolytic effects, correction of the motor and the cognitive behaviors, as well as improvement of the antioxidant status to its role in the induction of cerebral leptin production.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by Science & Technology Development Fund in Egypt (STDF) project entitled "Recent approaches in the utilization of *M. oleifera* and *Moringa peregrina* as a good nutritional, medicinal and industrial plant in Egypt" (Grant No. 5979).

ABBREVIATIONS

BBB, blood-brain barrier; FST, Forced swim test; GSH, reduced glutathione; HCD, high-cholesterol diet; LDT, Light and dark test; MDA, malondialdehyde; *M. oleifera Moringa oleifera* Lam.; NC, normal control group; ob, untreated obese group; ORT, Object recognition test; OPT, Open field test; qRT-PCR, quantitative real-time polymerase chain reaction; *r,* Pearson's correlation coefficient; RNA stabilizer reagent, RNAlater; SEM, standard error of mean; sim, simvastatin.

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