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RESEARCH ARTICLE

Morus alba and *Morus rubra* Alleviate Hepatic Disorder, Oxidative DNA Damage and Gene Expression Profile Change in Obese Rats

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Abstract

Objective: To examine the impact of *Morus rubra* and *Morus alba* extracts on physiological and metabolic functions of obese male rats induced by high fat diet. Methods: Orlistat was used as standard drug. Seventy Wistar male rats (weighing 150-160 g) were fed for 12 weeks with high fat diet to induce obesity. The obese rats were randomly assigned to three groups (10 rats each) and treated with *Morus rubra* and *Morus alba* extracts or orlistat for 6 consecutive weeks. The level of visfatin was measured by enzyme-linked-immunosorbent. The suppression impact of extracts against DNA adducts 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG) generation was measured by high-performance liquid chromatography. Expression of genes including ATP- citrate-lyase, peroxisome-proliferator-activated receptor-γ and HMG CoA reductase in liver tissues were also measured by quantitative real time-polymerase chain reaction. Results: Treatment with the *Morus rubra* and *Morus alba* extracts and orlistat significantly decreased visfatin level. *Morus rubra* and *Morus alba* extracts significantly decreased the levels of mRNA expression of ATP-citrate-lyase, HMG CoA reductase genes, and peroxisome-proliferator-activated receptor-γ compared with high fat diet rats. In addition, *Morus rubra* and *Morus alba* extracts decreased the level of 8-OHdG/2-dG ratio. Conclusions: The ameliorating effect of *Morus rubra* and *Morus alba* extracts on visfatin levels reducing fat may be attributed to the rich content of polyphenolic which suppresses lipid synthesis in liver tissues. Moreover, anti-DNA damage effect of *Morus rubra* and *Morus alba* extracts suggests their role as natural antioxidants against diseases such as obesity associated with genetic damage.

Keywords: *Morus, Visfatin, MRNA expression, DNA adducts, Hepatic disorder.*

Introduction

Obesity coincides with metabolic alterations including inflammation, diabetes, nonalcoholic fatty liver disease (NAFLD) and insulin resistance [1, 2]. NAFLD is a progressive disease caused by imbalanced diets such as an excessive high-fat diet (HFD) intake. HFD has been shown to induce dysregulated gene expression of hepatic tissues which affect inflammatory pathways, oxidative, regulation of lipolysis and lipogenesis pathways [3].

Visfatin is one of the prominent adipokines secreted by adipose tissue and has an insulin-similar role in the tissues of liver [4]. Numerous genes coincided with lipid metabolism play important roles in the lipogenesis regulation and serum adipocytokine concentration increases in patients with NAFLD [5]. Many plant extracts were reported to contain polyphenolics which are considered as an important radical scavenger against DNA damage and gene expression alterations [3].

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These compounds can alter signaling pathways and gene expression because of their metal chelating, lipid peroxidation inhibition properties and may be associated with reduced hepatic and adipose tissue expression of inflammation-related genes [6,7]. Mulberries are possessing several potential pharmacological benefits including hepatoprotective, anti-obesity and anti-cholesterol effects. These activities might be associated with the presence of combinations of polyphenolics which show optimal preventive capacities such as flavonoids, anthocyanins, stilbenoids, lignans, tannins, and sterols [7].

Traditionally, mulberries are used for the prevention of liver and kidney diseases, joint damage, and anti-aging. It has been reported in the previous study [6] that addition of bilberries and lingonberries to diet with high fat can prevent the development of fatty liver and obesity in mice.

In conventional obesity, the profile of adipose gene expression reflects both the newly recruited adipocytes in various stages of maturity and the preexisting hypertrophic fat cells [7]. No data are published on pathways associated with gene expression in the liver affecting by *Morus* species *in vivo*. The effect of treatment of *Morus* species on the level of visfatin has not been thoroughly studied.

Therefore, this study aims to investigate the potency of both *Morus alba* (*M. alba*) and *Morus rubra* (*M. rubra*) on obesity induced by HFD by measuring different metabolic disorders-related physiological biomarkers. Further, the lipid metabolism-related gene expression levels in liver tissues of obese rats were studied. Additionally, the suppression impact of *Morus* species against DNA adducts [2-deoxyguanosine (2-dG) and 8-hydroxy-2-deoxyguanosine (8-OHdG)] generation due to HFD was also measured. Orlistat, which is a pharmacological agent promoting weight loss in obese subjects *via* pancreatic and gastric lipase inhibition was used as the standard drug.

Materials and Methods

Plant Material Collection

White *M. alba* L. and red *M. rubra* L. fresh fruits were collected from Delta region, Egypt.

The identification of the plant was varified by Therese Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and El-Orman Botanical Garden, Giza, Egypt, and by comparing with the voucher specimen located at the herbarium of the garden. The fruits were selected according to uniformity of shape and color. The fresh fruit samples were cleaned, stored in polyethylene bags and frozen at −20ºC, until needed.

The Extracts Preparation for Bioassays

The fresh fruits of each species (2 kg) were extracted separately with 700 mL for 3 times of 70% aqueous ethanol (EtOH) for 3 h on an orbital shaker at room temperature in the dark. The extracts were separated by centrifugation (13 000×*g*, 10 min), then the supernatant was taken. In the same solvent (50 mL), the residue was resuspended for 3 times. By centrifugation, the mixture was again separated. The two resulting supernatants were then combined and concentrated under reduced pressure at 40ºC till dryness to get 26.3% and 25.0% of crude EtOH extract of *M. alba* and *M. rubra*, respectively.

Experimental Animals

Seventy adult Wistar male rats (150-160 g), were obtained from the animal house colony of the National Research Centre, Dokki, Giza, Egypt. Animals were quarantined and allowed to acclimate for 10 d before experimentation. They were keept under a controlled -temperature (26-29ºC) with a fixed cycle of light/dark and with free access of food and *ad libitum*. Study protocol approval (approval number: 432117) and guidelines were in accordance with the moral rules of the Medical Ethical Committee of NRC, Egypt, assuring that the animals will not suffer at any stage of the experiment.

Induction of Obesity in Rats

Obesity was induced according to the method of Adaramoye *et al.* [8] by feeding HFD of cholesterol. Cholesterol was orally administrated at 30 mg/0.3 mL olive oil/1 kg animal 5 times a week for 12 consecutive weeks. Lard fat was mixed with normal diet. One kilogram of animal lard was added to 5 kg of normal diet. The occurrence of obesity was determined by measuring body weight gain percentages, visceral and fecal fat percentages [9].

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Experimental Design

After adaptation period in the animal facilities before beginning the experiment, the animals were divided into 7 groups (n=10 rats per group). Rats in group 1 received normal diet. Rats in groups 2 and 3 were given normal diet and 300 mg/kg b.wt. of *M. rubra* and *M. alba* extract, respectively [10] for six consecutive weeks. Rats in group 4 were treated with HFD for 6 weeks. Groups 5 and 6 were treated with 300 mg /kg b.wt. of ethanolic extract of *M. rubra* and *M. alba*, respectively. Rats in group 7 received with orlistat (12 mg/kg b.wt.) [11]. Health conditions were monitored daily and no adverse events occurred throughout the study. All experiments were conducted with triplicate measurements.

Blood Sample and Collection of Serum and Liver Tissue

At the end of experiment (week 12), the blood samples were obtained by rupture of subtongual vein post overnight fasting. Centrifugation of the blood (3 000 rpm, for 10 min) was carried out. The serum was separated and stored at -80 ºC for further analysis, and all the rats were sacrificed by decapitation and livers were obtained [12].

Biochemical Measurements

Determination of Visfatin

Determination of serum visfatin was carried out using enzyme-linked-immunosorbent assay kit (R&D Company, USA). Percentages change is calculated according to the following formula: [Mean of control–Mean of tested/Mean of control] ×100. Percentage of improvement is calculated according to the following formula: [Mean obese rats–Mean of obese –treated rats /Mean of control rats] ×100.

Assessment of the DNA Adducts (8-OHdG and 2-dG) by High-Performance Liquid Chromatography (HPLC)

Genomic DNA was isolated from liver tissues of rats by mixing the tissues with lysing buffer (pH 7.4), and incubation them for 16 h at 55°C with proteinase K (0.5 mg/mL). Afterwards, the lysed tissues were incubated with RNase enzyme for 10 min at 50°C and then the DNA was isolated using chloroform/isoamyl alcohol method. The DNA was precipitated with sodium acetate and absolute ethanol and kept at -20°C.

The isolated DNA materials were washed with seventy percent ethanol, air-dried and dissolved in Tris/EDTA solution (pH 7.4). The DNA was digested according to the method described previously [13]. HPLC (CoulArray system, Model 5600) and coulometric array containing four electrochemical sensors were used to measure the DNA adducts [13].

Expression of Obese Related Genes

Total RNA Isolation

Total RNA was isolated from lliver tissues of male rats using TRIzol® Reagent (Invitrogen, Germany) according to the manufacturer's instructions. One unit of RQ1 RNAse-free DNAse enzyme (Invitrogen, Germany) was used to digest DNA residues from isolated RNA and re-suspended in DEPC-treated water. Aliquots were used directly for reverse transcription (RT); otherwise, they were kept at -80°C.

RT Reaction

Total RNA isolated from liver tissues of male rats was reverse transcribed into complementary DNA (cDNA) in 20 µL total volume using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). The RT reaction tubes were transferred to the thermocycler (Applied Biosystem, USA) and the reaction program was carried out at 25°C for 10 min, followed at 42°C for 1 h, and finished with a denaturation step at 99°C for 5 min. The obtained cDNA was stored at -20°C or was used immediately for amplification through quantitative Real Time- polymerase chain reaction (qRT-PCR) [14].

Quantitative Real Time- PCR (qRT-PCR)

To determine the liver cDNA copy number of rat's genome quatitativelly a StepOne Real-Time PCR System (Applied Biosystem, USA) was used. The PCR reaction was performed in a reaction mixture (25 µL) according to the manufacturer's instructions of SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.).

The program of the reaction was consisted from 3 steps. In the first step the reaction was set at 95.0°C for 3 min. In the second step the reaction was set for 40 cycles for the following substeps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. In the third step the reaction was (carried out to obtain the melting curve)

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consisted of 71 cycle at 60.0°C up to 95.0°C (10 sec distance). The primer sequences of the obese related genes are listed in Table (1).

The quantitative values of qRT-PCR of the genes used were normalized on the bases of ß-actin expression using the $2^{-\Delta\Delta CT}$ method.

Table 1: Primers used for qPCR

Genes	Primer sequence [a]	Reference
ACLY	F: 5'-ATG GGA GGA GAG AAA AGT T-3`	[6]
	R: 5'-ATT TTT ATA AAA CTC TTT TTT CCT TTC TAC-3'	
PPARγ	F: 5'-CTG TTT TAT GCT GTT ATG GGT GAA-3'	[6]
	R: 5`-GCA CCA TGC TCT GGG TCA A-3`	
HMGCR	F: 5'-GCA GTC AGT GGG AAC-3'	[6]
	R- 5'-CGG CTT CAC AAA CCA-3'	
β-actin	F: 5`-GTG GGC CGC TCT AGG CAC CAA-3`	[14]
	R: 5`-CTC TTT GAT GTC ACG CAC GAT TT-3`	

F: forward primer; R: reverse primer. *ACLY*: ATP citrate lyase; *PPARγ*: peroxisome proliferator-activated receptor gamma; *HMGCR*: 3-hydroxy-3-methylglutaryl-CoA reductase.

Statistical Analysis

Statistical analysis was carried out using SPSS coupled with *co-state* computer programs, where different letters were considered significant at $P<0.05$. General Liner Models procedure of SAS (statistical analysis system, version 9.1, Statsoft Inc., Tulsa, USA) was used for statistically analysis of the genetic data. Afterwards, Scheffé-test was used to determine the significant differences between groups based on the probability of $P < 0.05$.

Results

Effect of *M. rubra* and *M. alba* on Serum Visfatin Level

At the beginning of the experiments, the weights of all rats were recorded at (155.00 ± 10.00) g (weight of rats after 10 days of acclimatization). Table 2 showed significant reduction (15.00%) in serum visfatin level in normal rats treated with *M. rubra* compared with normal control rats, while no significant change was detected in normal rats treated with *M. alba*. HFD administered rats showed a significant increase in visfatin level reached to 72.32%. On the other hand, rats treated with *M. rubra* (66.73%) and *M. alba* (59.83%) exhibited marked improvement compared to HFD rats, which was similar to orlistat (62.60%).

Table 2: Effect of *Morus alba* and *Moru rubra* on serum visfatin level (Data is expressed as mean±D)

Markers	Groups						
	Group1	Group2	Group3	Group4	Group5	Group6	Group7
Vastatin (ng/L)	[a]288.00 ±0.0	[b]245.70±30.25	[a]265.70±30.25	[c]496.30±16.25	[a]304.12±19.00	[a]324.00±16.32	[a]316.00±20.28
% Change	-	-15.00	-7.74	+72.32	+5.56	+12.50	+9.72
% Improvement	-	-	-	-	66.73	59.83	62.60

Group 1: normal diet, Group 2: rats fed with normal diet and treated orally with red *Morus alba* extract for 6 weeks. Group 3: rats fed with normal diet and treated orally with *Morus alba* extract for 6 weeks Group 4: rats fed with high fat diet for 12 weeks. Group 5: high fat diet fed rats treated orally with red *Morus rubra* for 6 weeks post-induction. Group 6: high fat diet fed rats treated orally with *Morus alba* for 6 weeks post-induction. Group 7: rats feed high fat diet and treated orally for 6 weeks with standard drug orlistat. Different letters mean significant differences at $P\leq0.05$.

Effect of *M. rubra* and *M. alba* on 8-OHdG Generation

The results found that 8-OHdG/2-dG generation ratio of normal control group was 5.2±0.2. However, the ratio was increased with a significant difference ($P<0.01$) to 17.9±0.4 in male rats fed with HFD. On the other hand, the 8-OHdG/2-dG ratios in

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normal rats treated with *M. rubra* and *M. alba* extracts were similar to the control rats. Moreover, the results exhibited that *M. rubra* (7.4±0.2) and *M. alba* (10.4±0.3) extracts significantly decreased the 8-OHdG/2-dG generation rates induced by HFD treatment (P<0.05). Furthermore, suppression impact of *M. rubra* extract against OHdG/2-dG generation was more effective than that of orlistat drug (Figure 1).

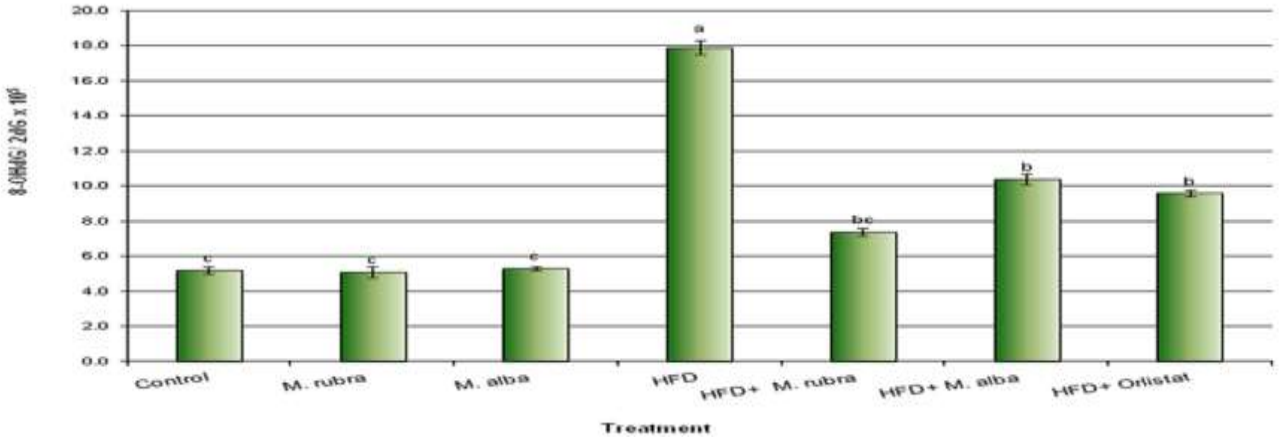

Figure 1: Generation of 8-OHdG/2dG in liver tissues. The DNA damage was expressed as the ratio of oxidized DNA base (8-OHdG) to non-oxidized base (2-dG) in liver DNA. Results are expressed as mean±SD of data from at least ten samples. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different (P < 0.05, Scheffé-Test)

Effect of *M. rubra* and *M. alba* on Expression Of Lipid Metabolisms-Related Genes

Expression of lipid metabolism-related gene ATP- citrate-lyase (*ACLY*), peroxisome-proliferator-activated receptor-γ (*PPARγ*) and, and cholesterol metabolism-related gene HMG CoA reductase (*HMGCR*) in liver tissues were determined. The results revealed that the mRNA expressions of *ACLY*, *PPARγ* and *HMGCR* were increased significantly in obesity rats (P<0.01) in comparison to healthy control rats. Normal rats treated with *M. rubra* and *M. alba* extracts revealed similar expression to normal control rats (Figure 2A-C).

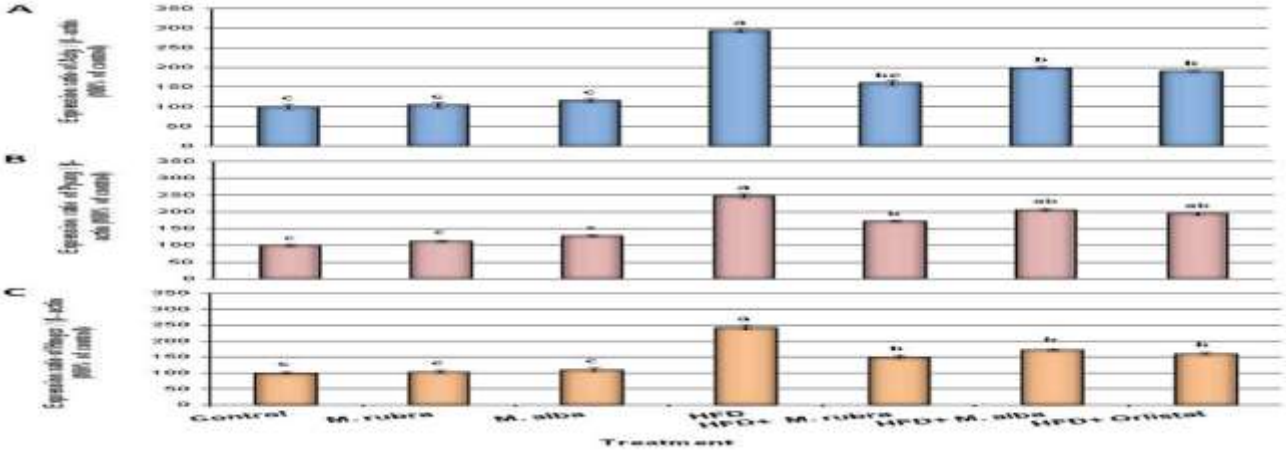

Figure 2: Alterations of *ACLY* (A), *PPARγ* (B) and *HMGCR* (C) gene in liver tissues. Data are presented as mean±SD. [a,b,c] Mean values within tissue with different superscript letters were significantly different ([a:] P<0.01, [b, c,d] P<0.05)

M. rubra extract significantly (P<0.01) decreased the levels of mRNA expression of *ACLY*, *PPARγ* and *HMGCR* genes in comparison to HFD rats; while *M. alba* extract significantly decreased (P<0.05) the mRNA abundance of *ACLY* and *HMGCR* genes. But the expression levels of *PPARγ* gene in obesity rats treated with *M. alba* extract and orlistat drug were decreased without significant differences compared with HFD rats.

Discussion

Morus species and their medicinally active phytochemicals display a wide range of biomedical functions such as anti-inflammatory, anti-diabetic, and hepatoprotective [2].

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Several studies have been reported that no toxic effects of supplementation with *Morus* extracts [18,21]. Visfatin has a principle role in controlling of insulin.

Visfatin is newly considered as an adipocytokine with great effect on metabolism of fat [15]. The role of visfatin in fat pathway eflected in body mass index and obesity [15]. Up till now, the role of mulberries in regulating the level of visfatin has not been thoroughly determined. In the current study, serum visfatin level was reduced significantly in normal rats treated with *M. rubra*, while no significant change was detected in normal rats treated with *M. alba*. However, obese rats showed a significant increase in visfatin levels compered with control rats. On the other hand, obese rats treated with *M. rubra* and *M. alba* exhibited marked improvement compared to obese rats without treatment. One of the mechanisms of *Morus* species therapy on the hyperglycemia may be the presence of potent α-glucosidase inhibitors such as 1-deoxynojirimycin and resveratrol [16,17].

The results are in accordance with the study of He and Lu [16], who showed that the combined treatment of polyphenolic-rich extract was effective on improving obesity-related phenotypes by lowering body weight gain and epididymal adipocyte size. The supplement has been reported to give a positive effect and to reduce the post-prandial hyperglycemia type-2 diabetes rats [18]. Polyphenolics such as flavonoids, anthocyanins, stilbenoids, lignans, and tannins have been reported in *Morus* genus [2]. Our previous study showed a higher level of total polyphenolics in *M. alba* [(622.4 ± 24.2) mg/g of ethanol extract] and *M. rubra* [(308.4 ± 17.6) mg/g of ethanol extract], respectively [19].

The present study showed that DNA damage (in the form of 8-OHdG/2-dG generation ratio) increased significantly in male rats fed with HFD. However, the damage of DNA in normal rats medicated with *M. rubra* and *M. alba* extracts was relatively similar to the normal rats. Moreover, the results revealed that *M. rubra* and *M. alba* extracts decreased significantly the DNA damage in obese rats. The polyphenolics content could in natural products prevent reactive oxygen species induced DNA damage and protect cells from free radicals to ameliorate oxidative stress [18, 19, 2, 22]. Quercetin 3-(6-malonylglucoside), present in various species of Asteraceae has been shown to prevent steatosis and obesity [20,21].

This compound could improve glucose metabolism through relaxation of oxidative stress in the liver cell. *Morus* species could effectively alleviate oxidative damage in DNA and attenuate the carbonylation level of proteins, which are related to the enhancement of peroxisome proliferator-activated receptor alpha (*PPARα*), *PPARγ* and its target genes. Obese rats with *M. rubra* showed better improvement than *M. alba* and standard drug [2,17,20]. The crude extract of *Morus* species may improve oxidative stress and transcriptional expression of lipid-related gene due to the presence of resveratrol, a stilbene compound that recently has shown therapeutic effect on hepatic steatosis in HFD-induced obese mice [22]. The treatment with *Morus* species extract may be able to attenuate insulin resistance by modulating gene and protein expression involved in glucose homeostasis in the liver cells [10].

The effects of *Morus* extract on the expression of related genes including *ACLY*, *PPARγ* (regulation of lipid metabolism), and *HMGCR* (cholesterol synthesis) in liver tissue were also studied in the present work. *PPARγ* is an essential modulator of fat cell function. ATP-citrate lyase (Acly) is one of the important cytosolic enzymes that synthesize acetyl-coenzyme A (CoA). Since that acetyl-CoA is an essential building block for triglycerides and cholesterol, Acly has been re-emerged as a therapeutic target for obesity and elevated plasma lipid levels. *PPARs* are a unique set of fatty acid-regulated transcription factors controlling lipid metabolism. Caballero et al [23].

Reported increased *HMG* CoA reductase (*HMGCR*) expression in nonalcoholic fatty liver disease but the reseason if this due to the liver disease or underlying obesity was not explained. The current study indicated that expression levels of lipid and cholesterol metabolism genes in liver tissues of male rats treated with *M. rubra* revealed similar expression to normal control rats, but the expression of these genes increase slightly in normal rats treated with and *M. alba*.

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The extract of *M. rubra* was more effective on the expression status than *M. alba* extract in this study. Moreover, *M. rubra* and *M. alba* extracts showed different gene expression in liver tissues and regulatory pathways, suggesting that these two extracts exert metabolic effects *via* different mechanisms [2,17]. Development of obesity coincides with increased fat in liver tissues, which could induce liver steatosis disease. HFD is able to induce fatty liver and to increase expression of several genes associated with steatosis [24]. *Morus* extracts could lower expression of genes involved in lipid biosynthesis pathway and insulin resistance to alleviate the steatosis[25]. These results suggested that mulberries extract may protect the liver tissues against HFD induced steatosis in part by down-regulation of lipid metabolism-related genes [6].

The reduction of fat and weight is due to polyphenolic products, which are suggested to have a range of health benefits and may alter the hepatic expression of lipid-related genes [7, 26].Delphinidin chloride, an anthocyanin commonly found in berries was reported to reduce dibenzo-[α,l]-pyrene-DNA adduct level in the cell line of human oral leukoplakia [27]. This compound enhances repair of bulky DNA adducts. The present study indicated that red *M. rubra* extract decreased the level of DNA adduct (8-OHdG/2-dG)-induced more significantly than the white *M. alba* extract. Anthocyanins that act as important antioxidants are rich in colored *Morus* species (red), while the white mulberry (*M. alba*) has low concentrations of flavonoids and anthocyanins pigment [27]. Two main anthocyanins of cyanidin (Cya) glycosides have been found in *M. rubra*; Cya-3-*O*-ß-rutinoside and Cya-3-*O*-ß-glucoside [22]. The anthocyanins-riched *M. rubra* extract could enhance the repair of HFD-induced DNA adduct.

In the current study, the biological action of red mulberry extract (*M. rubra* L.) on DNA protection may be attributed to the antioxidant properties and could be used against several diseases. As a protector against lipid peroxidation, *M. rubra* may cause an increase in the activity of antioxidant enzymes and a decrease in hepatic malonaldehyde [6,7]. In conclusion, the extracts of *Morus* species, which are rich with polyphenolics, have anti-accumulation action of lipids and consequently anti-obesity properties. They have therapeutic effects against hepatic disorder, oxidative DNA damage and change of gene expression due to obese. Thus, *Morus* species, especailly *M. rubra* are promising medicinal alternatives.

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