

## Molecular Study of The Therapeutic Effect of Pomegranate Peel Extract Against Non-Alcoholic Fatty Liver Disease in Rats .

Wael S. Darwish,<sup>1\*</sup> Maher A. Kamel,<sup>2</sup> Nasser H. Abbas,<sup>1</sup> Mohamed E. Ebeid<sup>1</sup> Abada M. Khedr,<sup>1</sup>

1-Molecular Biology and Biochemistry department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt

2-Medical Research Institute, University of Alexandria

\*Corresponding author: Email: wael\_science@yahoo.com

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### ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a major health problem and is considered as the most common worldwide liver disease. High fat diet (HFD) leads to NAFLD through the dysregulation of genes involved in lipid metabolism. Pomegranate Peel Extract (PPE) has many biological activities and could modify the risk of hyperlipidemia. This study was performed to investigate whether and how the pomegranate peel extract has a hepatosteatotic effect in NAFLD rats, and its role in the modulation of sterol regulatory element binding protein-1C (SREBP-1C) and sterol regulatory element binding protein-2 (SREBP-2) as a molecular target for this therapeutic effects in the NAFLD rats. Fifty male albino Wistar rats will be used in the present study, The NAFLD was induced in rats using HFD. Treatment the NAFLD rats with PPE at a dose dependent manner was significantly ameliorate hyperlipidemia, liver profile, correct glycemic index, IR-HOMA and normalize the hepatic expression of SREBP-1C and SREBP-2. From the present study we can concluded that the PPE have efficient therapeutic effect against NAFLD through the modulation of SREBP-1C and SREBP-2.

*Key words: NAFLD, HFD, IR, SREBP-1C, SREBP-2, PPE.*

### 1.INTRODUCTION

Liver is the largest metabolizing organ in the body which regulates homeostasis of different body systems. The main important functions of the liver include protein synthesis, storage and metabolism of fats and carbohydrates, detoxification of drugs and other toxins, excretion of bilirubin and metabolism of hormones (Hassan *et al.*, 2017).It regulates several key aspects of lipid metabolism in

response to nutritional and hormonal signals. It is essential for the maintenance of systemic energy homeostasis in the body (Purushothamet *al.*,2009).Dysregulation of lipid metabolic pathways results in the development of insulin resistance, hepatic inflammation and NAFLD (Browning and Horton, 2004; Sanyal, 2005). Nonalcoholic fatty liver disease (NAFLD) is one of the most important chronic liver disorders worldwide

(**Ratziuet et al., 2015**). It covers a wide spectrum of hepatic damage in which steatosis with inflammation progresses to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and ultimately hepatocellular carcinoma (**Yatsujiet al., 2009; Ahmedet al., 2015**). NAFLD is considered to be the hepatic component of metabolic syndrome as its features are similar to those of metabolic disorders such as obesity, inflammation, insulin resistance, and type 2 diabetes (**Haaset al., 2016**). Thus, it is important to treat NAFLD as well as its associated metabolic diseases (**Abenavoliet al., 2016; Machado et al., 2016**). Fat accumulation within the liver during NAFLD is an indicator of disrupted lipid homeostasis that is usually controlled by sterol regulatory element-binding proteins (SREBPs) (**Eberleet al., 2004**).

The SREBP family members (SREBP-1a, -1c and -2) are master regulators of lipid metabolism. Both SREBP-1a and SREBP-1c are encoded by a single gene and have different N-termini; SREBP-2 is encoded by a separate gene. Both SREBP-1c and SREBP-2 are abundantly expressed in the liver. SREBP-1c activates the genes that control fatty acid and TAG synthesis, and SREBP-2 activates the genes that control cholesterol biosynthesis. SREBP-1b promotes both fatty acid and cholesterol synthesis (**Horton et al., 2002**). Abnormal expression of SREBPs may have a role in the pathogenesis of NAFLD. Indeed, SREBPs are potential therapeutic targets for NAFLD (**Eberleet al., 2004; Xiao and Song, 2013**). Pharmacotherapy of

NAFLD is an unmet clinical need. To date, no drug has received FDA approval for NAFLD (**Ratziuet al., 2015**). An increasing number of studies have focused on herbal extracts or natural products, and many of these studies have discovered herbal products with potent effects against NAFLD (**Yang et al., 2014; Xu Xet al., 2015**). Thus, herbal medicines are promising candidate drugs for the treatment of NAFLD.

Pomegranate peels are characterized by an interior network of membranes comprising almost 26-30% of total fruit weight and are characterized by substantial amounts of phenolic compounds, including flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolysable tannins (punicalin, pedunculagin, punicalagin, gallic and ellagic acid) (**El-Sherbini and Shoukry, 2012; Gianottiet al., 2013**). In vivo, in vitro, and epidemiological studies have shown the medicinal properties of PPE as an antioxidant, against colon cancer, T2DM, and inflammatory-mediated diseases (**Ben-Ali et al., 2014; Bostock-Cox, 2014**). Antioxidant activity of pomegranate peel has been proposed to play vital role in various pharmacological activities such as anti-aging, anti-inflammatory and anti-atherosclerotic activities. Inhibition of free radical induced damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of diseases (**Khan et al., 2017**). The present study was aimed to evaluate the therapeutic effect of PPE on the

experimental rat model of non-alcoholic fatty liver diseases through modulation of hepatic expression of sterol regulatory element binding protein-1c (SREBP-1c and SREBP-2).

## **2. Materials and Methods**

### **Animals and diet**

In the present study, we used 50 male Wistar rats weighing approximately (150-170 grams) and aged 2-3 months, obtained from animal house of Medical Research Institute. Rats were divided into two groups: control group (group I;  $n=10$ ) and non-alcoholic fatty liver (NAFL) group, (group II;  $n=40$ ). The control group was fed a standard diet. The NAFL group was fed a high fat diet (HFD) for 14 weeks. The model was based on the model reported by (Sene-Fiores *et al.*, 2008). The HFD consisted of commercial rat chow plus peanuts, milk chocolate, and sweet biscuit in a proportion of 3:2:2:1. All components of the high-fat diet were ground and blended.

### **Pomegranate Peel Extract:**

For preparation of pomegranate peel extract, a peel was removed and dried. The dried plant materials was powdered using a grinder. About 100 mg of dried, ground plant materials was soaked in methanol (1 L) for 24 hours at room temperature, followed by filtration through Whatman filter paper. The filtrate was centrifuged at 8000 rpm for 15 min, the clear supernatant will be collected, and then the methanol was evaporated in a rotary evaporator at 45 °C

under reduced pressure (Laporniket *et al.*, 2005).

### **Experimental procedures**

After establishing NASH the rats were divided into 2 groups; NAFLD group: 10 NAFLD rats without treatment and NAFLD treated with pomegranate peel extract (30 rats): after establishing NAFLD, the rats were treated daily with pomegranate peel extract using different doses (50, 100 and 150 mg/kg body weight) were administered to rats orally for 30 days by using 10 rats for each dose (Svegliati-Baroniet *et al.*, 2006). At the end of treatment period, overnight fasting rats were sacrificed by cervical dislocation and blood samples were collected to obtain serum for assessment of glucose, insulin, lipid profile, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and gamma glutamyltransferase (GGT). The liver of animals were quickly removed and washed with ice-cold saline and blotted dry. Livers were then divided into small parts for total RNA extraction and analysis of gene expression.

### **Methods:**

#### **Serum parameters:**

The AST, ALT and GGT were assessed using Biosystem kinetic kits (Biosystem, Spain) (Panteghini and Bais, 2008), Total Bilirubin was determined using BioMed colorimetric kit (BioMed, Germany) (Burtiset *et al.*, 2006), insulin was determined by ELISA kit (Biospes, China) (Weyeret *et al.*, 2000) and fasting serum glucose was assessed using SPINREACT colorimetric Kit

(SPINREACT, SPAIN) (Sacks, 2008). The homeostasis model assessment index for insulin resistance (HOMA-IR) was determined using the following formula:  $HOMA-IR = [\text{fasting glucose (mg/dl)} \times \text{fasting insulin } (\mu\text{U/ml})] / (22.5 \times 18)$  (Fenget *al.*, 2017). For lipid profile, the serum levels of triglycerides (TGs) and total cholesterol (TC) were determined using Boehringer Mannheim colorimetric kits (Mannheim, Germany), while HDL-C was determined according to the method described by Lopes-Virella et al (Rifai and Warnick, 2008).; one aliquot of the serum was mixed with the precipitating reagent phosphotungstic acid and magnesium chloride then the cholesterol content was evaluated in the clear supernatant using the Boehringer Mannheim kit (Mannheim, Germany). Finally, LDL-C was calculated according to the Friedewald equation:  $TC - (HDL-C + 1/5 TGs)$  (Friedewald *et al.*, 1972).

#### **Liver parameters**

Immediately after blood collection animals liver were excised and divided into aliquots; one aliquot was used for determination of hepatic triglycerides and cholesterol contents and 50 mg was used for total RNA extraction using miRNeasy kit (Qiagen, Germany) according to the manufacturer's instructions.

#### **Assessment of tissue lipid contents**

Hepatic lipids were extracted according to the method modified by Folich method (Folichet *al.*, 1975)., where the chloroform layer, containing all lipids, was utilized to assay TGs and TC, as mentioned before.

#### **Gene expression analysis using reverse transcriptase-polymerase chain reaction (RT-PCR)**

Quantitative analysis of SREBP-1c and SREBP-2 expression in hepatic tissues was performed using quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR). First, the total RNA was isolated from the tissues, then the isolated RNA was reverse transcribed using reverse transcriptase enzyme into complementary DNA (cDNA) then amplified and detected using specific primers by real-time PCR.

#### **RNA extraction**

mRNA were isolated from the liver tissue using miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer instructions.

#### **Relative quantification of SREBP-1c and SREBP-2 gene expression using PCR:**

The cDNA were used to quantify the hepatic gene expression of SREBP-1c and SREBP-2 by Rotor-Gene Q qPCR (Qiagen, USA) using QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany). Quantitative PCR amplification conditions started with initial denaturation for 10 minutes at 55°C and the then amplification by 40 cycles of PCR as follows: Denaturation at 95°C for 5 seconds, annealing at 55°C for 15 seconds and extension at 60°C for 15 seconds. The house keeping gene GAPDH was used as a reference gene for normalization. Primers used for rat genes were as follows:

**Table (1)** Primers used for rat genes were as follows:

Gene	primer sequence	
SREBP-1c	F:	5'-GACGACGGAGCCATGGATT-3'(Tianet <i>et al.</i> , 2016)
	R:	5'-GGGAAGTCACTGTCTTGGTTGTT-3'(Tianet <i>et al.</i> , 2016)
SREBP-2	F:	5'-CCCTGGGAGACATCGACGA-3' (Dong <i>et al.</i> , 2010)
	R:	5' -CGTTGCACTGAAGGGTCCA-3' (Dong <i>et al.</i> , 2010)
GAPDH	F:	5'-GGGTGTGAACCACGAGAAATA-3'(Sautinet <i>et al.</i> , 2007)
	R:	5'-AGTTGTCATGGATGACCTTGG-3'(Sautinet <i>et al.</i> , 2007)

The values of threshold cycle (Ct) were determined by Rotor-Gene Q-Pure Detection version 2.1.0 (build 9) (Qiagen®, Valencia, CA, USA). For each gene, the relative change in mRNA in samples was determined using the  $2^{-\Delta\Delta Ct}$  method and normalized to the housekeeping gene (GAPDH) (Livak and Schmittgen, 2001).

**Statistical analysis of the data** (Kotz *et al.*, 2006)

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) (Kirkpatrick L.A. and Feeney, 2013). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using mean and standard deviation. Significance of the obtained results was judged at the 5% level. The used tests were F-test (ANOVA) For normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons.

### 3. RESULTS

#### The effects of HFD on serum biochemical parameters

The HFD-induced NAFLD model is usually accompanied by hyperlipidemia, hyperglycemia, impaired insulin sensitivity. In the present study, there is a significantly high level of fasting blood glucose, insulin and insulin resistance (HOMA-IR) induced by HFD table (2). In addition, the serum level of triglyceride, cholesterol, low-density lipoprotein cholesterol (LDL-C) was elevated by HFD. While the serum level of high-density lipoprotein cholesterol (HDL-C), was decreased by HFD, and there is an elevation of liver enzymes (ALT, AST, GGT) and serum total bilirubin.

#### The effect of PPE on serum biochemical parameters of NAFLD rats

The NAFLD rats treated with PPE showed significantly decreased activity of ALT, AST, GGT in a dose-dependent manner (50, 100 and 150 mg/Kg) when compared to untreated rats, there is no significance in the activities of serum ALT, AST, GGT and total bilirubin at the

high dose (150 mg/Kg) of the PPE treated rats when compared to control rats table (2).

There is a significant decreased effect of NAFLD rats treated with PPE on the fasting blood glucose, insulin and insulin resistance (HOMA-IR) in a dose dependent manner when compared to untreated rats, and at the high dose (150 mg/Kg) of PPE in fasting blood glucose, insulin and insulin resistance (HOMA-IR) were normalized compared to control groups table (2).

The treatment of NAFLD rats with PPE result in significantly dose-dependent

decline in the level of TG, TC and LDL-C compared to untreated NAFLD rats table (2), On the other hand the level of HDL-C showed dose-dependent increased in the NAFLD rats treated PPE when compared to untreated rats table (2). The results clearly indicated that, PPE treatment of NAFLD group were completely normalized the serum levels of TG and HDL-C at the highest dose (150 mg/kg), however, TC and LDL-C still significantly higher than control groups table (2).

**Table (2)** Serum levels of hepatic profile parameters, Glucose homeostasis parameters and lipid profile parameters in the control rats and non-alcoholic fatty liver rats untreated and treated with different doses of PPE.

	Control (n=10)	NAFAL rats Untreated (n=10)	NAFAL rats treated		
			50 mg/Kg (n=10)	100mg/Kg (n=10)	150 mg/Kg (n=10)
ALT (U/L)	33.0±5.03	64.4 <sup>a</sup> ±15.46	40.30 <sup>b</sup> ±6.62	36.90 <sup>b</sup> ±6.64	34.40 <sup>b</sup> ±9.57
AST (U/L)	110.20±22.26	168.70 <sup>a</sup> ±22.10	147.10 <sup>a</sup> ±12.47	130.50 <sup>b</sup> ±21.42	115.30 <sup>bc</sup> ±14.26
Total Bilirubin(mg/dl)	0.36 ± 0.05	1.27 <sup>a</sup> ± 0.15	0.59 <sup>ab</sup> ± 0.27	0.45 <sup>b</sup> ± 0.14	0.33 <sup>bc</sup> ± 0.13
GGT(U/L)	12.10 ± 2.65	47.50 <sup>a</sup> ± 5.93	25.06 <sup>ab</sup> ± 5.32	17.15 <sup>abc</sup> ± 2.19	14.78 <sup>bc</sup> ± 1.64
Insulin (uIU/ml)	3.82±0.85	8.36 <sup>a</sup> ±0.94	4.99 <sup>ab</sup> ±0.78	3.90 <sup>bc</sup> ±0.63	3.58 <sup>bc</sup> ±0.54
FBS (mg/dl)	99.20±10.25	172.8 <sup>a</sup> ±44.3	144.0 <sup>a</sup> ±17.33	126.6 <sup>b</sup> ±23.85	119.2 <sup>b</sup> ±23.44
HOMA	0.95±0.27	3.55 <sup>a</sup> ±0.93	1.78 <sup>ab</sup> ±0.38	1.23 <sup>b</sup> ±0.33	1.05 <sup>bc</sup> ±0.24
Triglycerides(mg/dl)	80.2±9.34	110.7 <sup>a</sup> ±12.2	92.10±17.55	86.60 <sup>b</sup> ±22.91	83.40 <sup>b</sup> ±20.59
T.Cholesterol(mg/dl)	107.6±14.0	171.0 <sup>a</sup> ±20.1	146.2 <sup>ab</sup> ±7.28	132.8 <sup>ab</sup> ±5.27	127.3 <sup>abc</sup> ±5.03
HDL-C(mg/dl)	42.6±2.27	29.96 <sup>a</sup> ±3.26	32.90 <sup>a</sup> ±3.03	37.8 <sup>abc</sup> ±3.49	41.70 <sup>bcd</sup> ±2.83
LDL-C(mg/dl)	48.96±15.1	118.9 <sup>a</sup> ±19.0	94.88 <sup>ab</sup> ±7.9	77.68 <sup>abc</sup> ±7.84	68.92 <sup>abc</sup> ±7.07

Data was expressed as mean ± SD.

a: significant with Control group at p ≤ 0.05

b: significant with Untreated group at p ≤ 0.05

c: significant with 50 mg/Kg treated group at p ≤ 0.05

d: significant with 100 mg/Kg treated group at p ≤ 0.05

### The effect of HFD and PPE on Hepatic lipid content of NAFL rats

The hepatic tissues of untreated NAFLD rats have greatly elevated contents of triglycerides and cholesterol. The treatment of NAFLD rats with PPE showed significant decline of hepatic

contents of triglycerides and cholesterol in a dose-dependent manner. At the highest dose of PPE 150mg/Kg the hepatic TG and hepatic cholesterol content were completely normalized compared control rats figure (1).

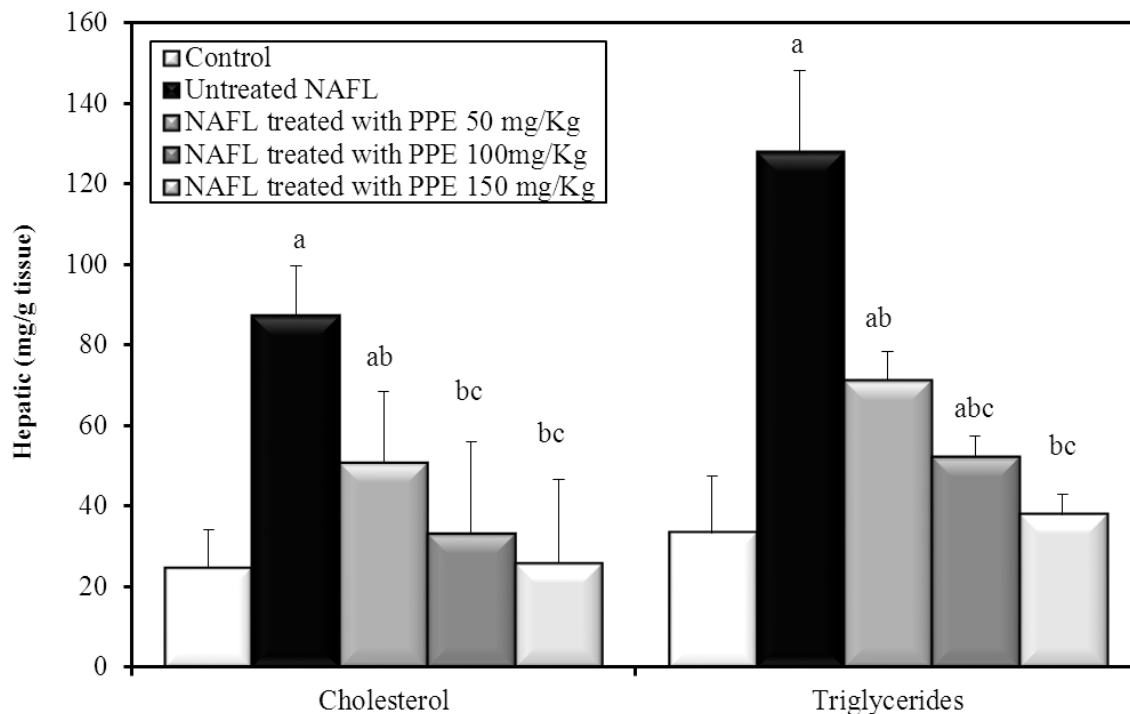


Figure (1): The hepatic triglycerides and cholesterol contents in the control rats, untreated NAFL rats and treated NAFL rats with different doses of pomegranate peel extract.

Data was expressed as mean  $\pm$  SD.

a: significant with **Control** group at  $p \leq 0.05$

b: significant with **Untreated** group at  $p \leq 0.05$

c: significant with **50 mg/Kg** treatedgroup at  $p \leq 0.05$

### The effect of HFD and PPE on Hepatic expression of (SREBP-1C, SREBP-2)

Previous reports have indicated that SREBP-1c and SREBP-2 pathway activation may be involved in the progression of NAFLD. We, therefore, examined the involvement of the SREBP-1c and SREBP-2 pathway in HFD-induced NAFLD and the effect of PPE on

their expression. The hepatic gene expression levels of SREBP-1C was greatly increased in the untreated NAFLD rats compared to control rats, on the other hand the level of SREBP-1C was markedly decreased in the treated rats compared to untreated rats. But their levels still higher than control value figure (2). The hepatic gene expression levels of SREBP-2 was significantly

increased compared to control rats. on the other hand the level of SREBP-2 was significantly decreased in the treated rats

compared to untreated rats. But their levels still higher than control value figure (2).

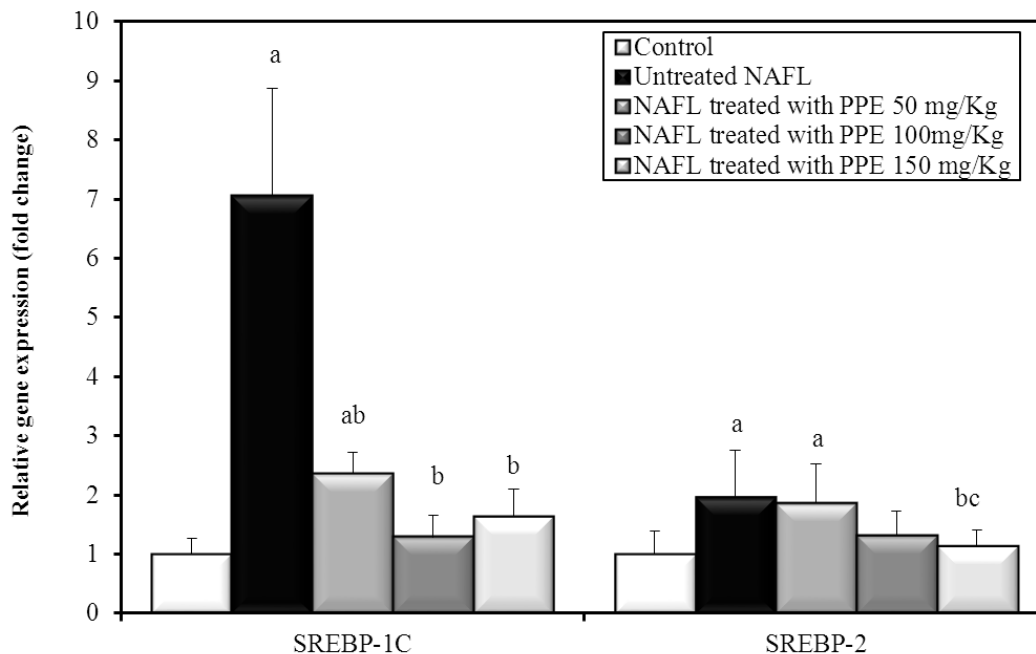


Figure (2): Gene expression of SREBP-1C and SREBP-2 in the control rats, untreated NAFL rats and treated NAFL rats with different doses of pomegranate peel extract.

Data was expressed as mean  $\pm$  SD.

a: significant with **Control** group at  $p \leq 0.05$

b: significant with **Untreated** group at  $p \leq 0.05$

c: significant with **50 mg/Kg** treatedgroup at  $p \leq 0.05$

#### 4. Discussion

In the present study HFD used to create a model of NAFLD in rats. The using of HFD model because it is easy to be established and mimics the pathophysiology of NAFLD in human (Liu *et al.*, 2012). From the results, The NAFLD rats showed significant hepatic accumulation of triglycerides and cholesterol which were higher compared to control values. Also, the activity of liver enzymes; AST, ALT,  $\gamma$ GT and the level of serum bilirubin were significantly elevated compared to control rats. The elevated level of liver

enzyme was an indication of fatty liver pathogenesis (Mukherjee *et al.*, 2013). At the glucose homeostasis level the NAFLD rats showed a significant elevation in fasting blood glucose, insulin and HOMA-insulin resistance index compared to control rats. The occurrence of insulin resistance considered as the first hit for hepatic fat accumulation and steatosis (Gaggini *et al.*, 2013). Also, There are significant abnormalities in the lipid profile; such as elevated serum levels of triglycerides, total cholesterol and LDL-cholesterol and lower HDL-cholesterol compared to control rats. This



condition commonly referred to as dyslipidemia (Tangvarasittichai, 2015).

Our results indicated up-regulation of the hepatic gene expression of SREBP-1c by about 6-fold and SREBP-2 by about 2-fold compared to control rats. Which are the main lipogenic transcription factors that activate the hepatic DNL (George and Liddle, 2007; Pettinelliet al., 2011). it is suggested that pharmacological manipulation of SREBPs may prove beneficial effect in the management of NAFLD and we used PPE for this purpose.

From the results PPE treatment had dose-dependent ameliorating effects on the different pathogenic pathways that participated in the induction of NAFLD in rats. It acted as a lipotropic factor that prevents or reduce accumulation of fat in the liver as indicated by the decline in the hepatic lipid content and serum triglycerides, total cholesterol, and LDL-cholesterol. The lipotropic effects of PPE are in agree with findings of other researchers who found significant changes in levels of blood glucose and lipids in metabolic syndrome subjects (Basuet al., 2013; Al-Shaibiet al., 2016) that consume pomegranate juice. Another report has indicated that pomegranate juice can protect against developing changes caused by high-fat diet or reduce the risk for development of NAFLD (Hassan et al., 2017). These findings agree with the present study and many other studies that demonstrated a correlation between the role of PPE and juice in regulating vital cellular functions, including cell proliferation and

differentiation and its potent antioxidant activity and free radical scavenging capability (Ahmed et al., 2014).

Also PPE treatment had an ameliorating effect on glycemic parameters, The anti-diabetic effect of the PPE may be partly, due to their positive effect on glycogen synthesis in the liver, skeletal muscle, and heart muscle, and partly, due to the insulin-like or insulin-releasing action of the ingredients present in the peel of the plant (Halawaet al., 2016). The compounds that present in pomegranate peel, such as ellagic, gallic and ursolic acid, have been identified as having antidiabetic actions (Hidayatet al., 2014). Also, the presence of one or more bioactive antihyperglycemic principles, such as flavonoids, isoflavones, and their synergistic effects (Ismail et al., 2012) are known to be natural antioxidants and thus protecting the existing  $\beta$ -cells from dying by their free radical scavenging action (Allen et al., 2005). It has been suggested that polyphenols, as the main component of pomegranate, might have hypoglycemic effects on different mechanisms, including increased glucose uptake by peripheral tissues, inhibition of glucose absorption in the gut, increased insulin release, or inhibition of gluconeogenesis (Viuda-Martoset al., 2010). In line with our data, reports have found that FBG levels were decreased significantly by punicic acid, and PPE (Banihaniet al., 2013). It was reported for both in vivo and in vitro studies that pomegranate exerts hypoglycaemic effects, including increased insulin sensitivity, inhibition of  $\alpha$ -glucosidase,

and has an impact on glucose transporter type 4 function (**Medjakovic and Jungbauer, 2013**).

The activities of ALT, AST,  $\gamma$ GT and the level of serum bilirubin were significantly decreased after the treatment of NAFLD rats with PPE. This results agree with (**Tokluet al., 2007**) which studied the effect of chronic administration of PPE on liver fibrosis induced by bile duct ligation. They showed that the elevated levels of AST and ALT were significantly decreased after treatment. Thus the hepatoprotective effect of PPE is due to its antioxidant and antifibrotic properties which may protect the liver from fibrosis and oxidative injury.

From the previous results, PPE have a potential clinical utility in combating NAFLD. The exact molecular mechanism involved is unclear, so in this study, the probable involvement of SREBP-1C and SREBP-2 in this mechanism was explored.

In the present study it is clear that the PPE treatment causes a significant dose-dependent decline in the hepatic expression of SREBP-1c and SREBP-2. The modulating effects of PPE on gene expression are in line with the previous study of (**Tahaet al, 2016**), who reported significant down-regulation of the gene expression of SREBP-1c, SREBP-2, fatty acid synthase (FAS) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) upon PPE treatment in HepG2 cell line. In accordance with the present work, a new report indicates that pomegranate exerts hepatoprotective effects against

CCl4-induced oxidative stress through anti-inflammatory effects by suppressing CCl4-induced IL-6 expression and suppressing CCl4-induced SREBP-1c expression (**Ibrahim et al., 2016**).

## 5. Conclusion

The results of the present study clearly indicate the ameliorating effect of PPE treatment against HFD-induced NAFLD in rats and the correction of glycemic and lipid homeostasis through modulation of hepatic gene expression of SREBP-1c and SREBP-2 especially SREBP-1c. However, further investigation is required to elucidate which of the major components in PPE are responsible for the antisteatotic effect seen with treatment.

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