MicroRNA-33a and MiR-34a as a Molecular Targets for Pomegranate Peel Extract During Treatment of Non-Alcoholic Fatty Liver Disease in Rats.

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD), hepatic manifestation of metabolic syndrome, has been considered the most common liver disease nowadays. The aim of this study is to evaluate the potential therapeutic effect of pomegranate peel extract on the experimental rat model of non-alcoholic fatty liver diseases (NAFLD) and to explore the role of mir-33a and mir-34a as the molecular targets for these therapeutic effects.Fifty male albino Wistarrats were used in the present study. The NAFLD was induces in rats using high fat diet (HFD). The pomegranate peel extract (PPE) was used in different doses (50,100 and 150) for 30 days.The present study documents the ameliorating effects of PPE on hyperglycemia, insulin resistance, lipid profile and liver functions in NAFL rats in a dose-dependent manner compared to untreated rats.The ameliorating effects on the metabolic parameter are associated with significant correction in the hepatic expression of MiR-33a and MiR-34ain a dose-dependent manner. From the present study we can concluded that the PPE have efficient anti-steatotic effect which may be mediated through modulation of MiR-33a and MiR-34.

***Key words**: NAFL, MiR-33a, MiR-34a, PPE.*

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease, affecting approximately 30% of global populations. Fatty liver has been documented in up to 10 to 15 percent of normal individuals and 70 to 80 percent of obese individuals (**Younossi*et al.*, 2002**). Fatty liver includes a wide spectrum of histological alterations ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), characterized by inflammation and fibrosis (**Pais*et al.*, 2013**). NAFLD is regarded as the liver manifestation of the metabolic syndrome, as it is strongly associated with obesity, insulin resistance, hypertension, and dyslipidemia (**Souza and Diniz, 2012**), conditions associated with high cardiovascular risk. The development of NAFLD and its progression are multifactorial processes due to the involvement of many different pathways remain unclear and require further investigations (**Pastori*et al.*, 2015**).

Mir-33a is one of the most important microRNAs in liver, miR-33 contributes to the modulation of fatty acid metabolism and insulin signaling pathways, as well as the dysregulation of cholesterol synthesis and high-density lipoprotein (HDL) levels (**Zhen and Cheng, 2016**). miR-33 reduced the expression of genes associated with fatty acid synthesis (SREBP, fatty acid synthase, ATP citrate lyase, and acetyl-CoA carboxylase α) and concomitantly elevated plasma HDL levels(**Rayner*et al*., 2011**).

MicroRNA-34a is a family of microRNA, it found in several animal species, including humans. MicroRNA-34a was found to be up-regulated in patients with nonalcoholic fatty liver disease and hepatitis C virus infection (**Cermelli*et al*., 2011**) and down-regulated in multiple tumors including hepatocellular carcinoma (**Budhu*et al*., 2008**). MiR-34a plays an important role in lipid metabolism (**van der Meer *et al*., 2012**).It is highly expressed in patients with non-alcoholic fatty liver disease, non-alcoholic steatohepatitis (NASH) (**Cheung *et al*., 2008)** and type 2 diabetes (**Kong *et al*.,2011**).

The Pomegranate fruit could be considered a functional food because it has valuable compounds in different parts of the fruit that display functional and medicinal effects. These compounds can act as antioxidant (**Cam *et al*., 2009**), as antitumor (**Hamad and Al-Momene, 2009**) as antihepatotoxic agents (**Celik*et al*., 2009**), and improve cardiovascular health (**Davidson *et al*., 2009**). They have been reported to have antidiabetic properties and help to prevent Alzheimer's disease (**Singh *et al*., 2008**).Pomegranate peel extract (PPE) also have an effect on lipid metabolism, (**Hossin, 2009**).

The present study was designated to investigate the therapeutic effects of pomegranate peel extract (PPE) on HFD-induced NAFLD in rats at different levels; hepatic lipid content, liver function test, glucose homeostasis, lipid abnormalities and hepatic expression of microRNAs (miR-33a and miR-34a).

2. Material and Method

2.1 Animals and diet

In the present study, we used 50 local Wistar male 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 Fiorese, et al. 2008 (**Sene-Fiorese*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.

2.2 Pomegranate Peel Extract:

For preparation of pomegranate peel extract, a peel was removed and dried. The dried plant materials were 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 Arotary evaporator at 45 °C under reduced pressure (**Laporni*et al*., 2005**).

2.3 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-Baroni*et al*., 2006**). At the end of treatment period, overnight fasting rats were weighed and sacrificed by cervical dislocation and blood samples were collected to obtain serum for assessment of glucose, insulin, bilirubin, lipid profile, alanine aminotransferase (ALT),asparatate aminotransferase (AST) and gamma-glutamyltransferase (GGT).

The liver of animals were quickly removed and washed with ice-cold saline. Livers were then divided into small parts for total RNA extraction.

2.4 Methods:

2.4.1 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)**(Burtis*et al*., 2006)**, insulin was determined by ELISA kit (Biospes, China) **(Weyer*et al*., 2000)** and fasting serum glucose was assessed using SPINREACT colormetric Kit (SPINREACT, SPAIN) **(Sacks, 2008)**. The homeostasis model assessment index for insulin resistance (HOMA-IR) was determined using the following formula:

HOMA-IR = [fasting glucose (mg/dl) × fasting insulin (μU/ml)]/ (22.5 × 18) **(Feng*et 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: **(Friedewald*et al*., 1972).**

LDL-C = total cholesterol - (HDL-C) – (TG/5)

2.4.2 Liver parameters:

Immediately after blood collection, livers 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.

2.4.2.1 Assessment of tissue lipid contents

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

2.4.2.2 Reverse transcription of total RNA

Reverse transcription was done using miScript II RT Kit (Qiagen,Germany) according to the manufacturer instructions. The miScript II RT Kit is used to perform a one-step, single-tube reverse transcription reaction. miScriptHiFlex Buffer was used to promote conversion of all RNA species (mature miRNA, precursor miRNA, noncoding RNA, and mRNA) into cDNA. This enables flexibility to study miRNA biogenesis and mRNA regulation in a single cDNA sample.

2.4.2.2.1 Assessment of hepatic expression of microsRNAs:

The obtained cDNAs were used for real-time PCR quantification of mature miRNAs (mir-34a and mir-33a) using Primer Assays (forward primers) and the miScriptSYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTectSYBR Green PCR Master Mix (Qiagen,Germany). The kit was used with miScriptPCR Control U6(Qiagen,Germany). Data were collected using Rotor-Gene Q-Pure Detection version 2.1.0 (build 9) (Qiagen, USA). The relative expression of miRNAs was quantified relative to the expression of the reference gene (U6) in the same sample by calculating and normalizing the threshold cycles (Ct) values of target miRNAs to that of U6 using ΔΔCt method.

3. 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 and Feeney, 2013**).The Kolmogorov-Smirnov test was used to verify the normality of distribution Quantitative data was described using mean and standard deviation. Significance of the obtained results was judged at the 5% level.

4. Results:

4.1 Liver parameters

The results show the untreated NAFL rats showed significantly higher serum activities of ALT, AST and GGT by about 95.2, 53.1 and 292.6 % respectively compared to control rats. Also, the results showed an elevation in serum bilirubin by about 253.9%. The rats which treated with Pomegranate peel extract showed significantly lower level of liver enzymes (ALT, AST and GGT) and bilirubin compared to untreated rats in a dose-dependent manner, **Table (1)**.

Table 1.Liver enzymes and bilirubin

	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	33.0±5.03	64.4[a]±15.46	40.30[b]±6.62	36.90[b]±6.64	34.40[b]±9.57
% of change 1		↑95.2	↑22.1	↑11.8	↑4.2
% of change 2			↓37.4	↓42.7	↓46.6
AST	110.20±22.26	168.70[a]±22.10	147.10[a]±12.47	130.50[b]±21.42	115.30[bc]±14.26
% of change 1		↑53.1	↑33.5	↑18.4	↑4.6
% of change 2			↓12.8	↓22.6	↓31.7
Total Bilirubin	0.36 ±0.05	1.27[a]±0.15	0.59[ab]±0.27	0.45[b]±0.14	0.33[bc]±0.13
% of change 1		↑253.9	↑63.9	↑25.0	↓8.3
% of change 2			↓53.5	↓64.6	↓74.0
GGT	12.10 ± 2.65	47.50[a] ± 5.93	25.06[ab] ± 5.32	17.15[abc] ± 2.19	14.78[bc] ± 1.64
% of change 1		↑292.6	↑107.1	↑41.7	↑22.1
% of change 2			↓47.2	↓63.9	↓68.9

Data was expressed as mean ± 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** group at $p \leq 0.05$
% of change 1: from **Control** group
% of change 2: from **Untreated** group
*: Statistically significant

4.2 Glucose homeostasis parameters:

The results indicated that the untreated NAFL rats showed significantly higher serum levels of fasting blood glucose and insulin compared to control rats by about 74.2% and 118.8%, respectively. HOMA-IRI also increased by 3.7- folds compared to control rats. The NAFL rats treated withPomegranate peel extract showed significantly lower values of fasting blood glucose, insulin and HOMA-IRI compared to untreated in a dose dependent manner, **Table (2)**.

Table 2. Glucose homeostasis parameters

	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)
Insulin (uIU/ml)	3.82±0.85	8.36[a]±0.94	4.99[ab]±0.78	3.90[bc]±0.63	3.58[bc]±0.54
% of change 1		↑118.8	↑30.6	↑2.1	↓6.3
% of change 2			↓40.3	↓53.3	↓57.2
FBS	99.20±10.25	172.8[a]±44.3	144.0[a]±17.33	126.6[b]±23.85	119.2[b]±23.44
% of change 1		↑74.2	↑45.2	↑27.6	↑20.2
% of change 2			↓16.7	↓26.7	↓31.0
HOMA	0.95±0.27	3.55[a]±0.93	1.78[ab]±0.38	1.23[b]±0.33	1.05[bc]±0.24
% of change 1		↑274.1	↑87.8	↑29.7	↑10.8
% of change 2			↓49.9	↓65.4	↓70.4

Data was expressed as mean ± 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 $p \leq 0.05$
% of change 1: from **Control** group
% of change 2: from **Untreated** group

4.3 Lipid parameters:

4.3.1 Serum lipid profile:

The results indicated that the untreated NAFL rats showed significant higher levels of TG, TC and LDL-C (by about 37.9, 58.9 and 142.9%; respectively), while showed significant lower level of HDL-C (by about 29.7%) compared to control rats, **Table (3)**. The treatment of NAFL rats with Pomegranate peel extract result in significantly dose-dependent decline in the level of TG and TC compared to the untreated rats, Table (3). On the other hand the level of HDL-C showed dose–dependent increase in the NAFL rats treated with Pomegranate peel extract,**Table (3)**.

Table 3. Lipid parameter

	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)
Triglycerides	80.2±9.34	110.7[a]±12.2	92.10±17.55	86.60[b]±22.91	83.40[b]±20.59
% of change 1		↑38.0	↑14.8	↑8.0	↑4.0
% of change 2			↓16.8	↓21.8	↓24.7
T. Cholesterol	107.6±14.0	171.0[a]±20.1	146.2[ab]±7.28	132.8[ab]±5.27	127.3[abc]±5.03
% of change 1		↑58.9	↑35.9	↑23.4	↑18.3
% of change 2			↓14.5	↓22.3	↓25.6
HDL-C	42.6±2.27	29.96[a]±3.26	32.90[a]±3.03	37.8[abc]±3.49	41.70[bcd]±2.83
% of change 1		↓29.7	↓22.8	↓11.3	↓2.1
% of change 2			↑9.8	↑26.2	↑39.2
LDL-C	48.96±15.1	118.9[a]±19.0	94.88[ab]±7.9	77.68[abc]±7.84	68.92[abc]±7.07
% of change 1		↑142.9	↑93.8	↑58.7	↑40.8
% of change 2			↓20.2	↓34.7	↓42.0

Data was expressed as mean ± 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 $p \leq 0.05$
d: significant with **100 mg/Kg** treatedgroup at $p \leq 0.05$
% of change 1: from **Control** group
% of change 2: from **Untreated** group

4.3.2 Hepatic lipid contents:

The hepatic tissues of untreated NAFL rats have greatly elevated contents of triglycerides (by about 283.2 %) and cholesterol (by about 252.2 %) compared to the hepatic tissues of control rats Table (4).The NAFL rats treated with pomegranate peel extract showed significant down regulation of hepatic contents of triglycerides and cholesterol in a dose-dependent manner,**Table (4)**.

Table 4. Hepatic lipid contents

Hepatic	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)
Cholesterol	24.80±4.59	87.35[a]±9.43	50.90[ab]±19.13	33.10[bc]±12.27	25.90[bc]±13.35
% of change 1		↑252.2	↑105.2	↑33.5	↑4.4
% of change 2			↓41.7	↓62.1	↓70.3
Triglycerides	33.40±10.80	128.0[a]±15.64	71.20[ab]±19.10	52.20[abc]±3.36	37.90[bc]±8.91
% of change 1		↑283.2	↑113.2	↑56.3	↑13.5
% of change 2			↓44.4	↓59.2	↓70.4

Data was expressed as mean ± 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 $p \leq 0.05$
% of change 1: from **Control** group
% of change 2: from **Untreated** group

4.4 Hepatic expression of MicroRNA-33a and MicroRNA-34a:

The hepatic level miR-34a was greatly increased in the untreated NAFL rats by about 909.7% compared to control rats,**(figure 1)**. The NAFL rats treated with pomegranate peel extract showed a dose-dependent reduction of miR-34a compared to the untreated rats.

The hepatic level miR-33a was decreased in the untreated NAFL rats by about 42.4% compared to control rats, **(figure 1)**. The NAFL rats treated with pomegranate peel extract showed a dose-dependent elevation of miR-33a compared to the untreated rats **(figure 1)**.

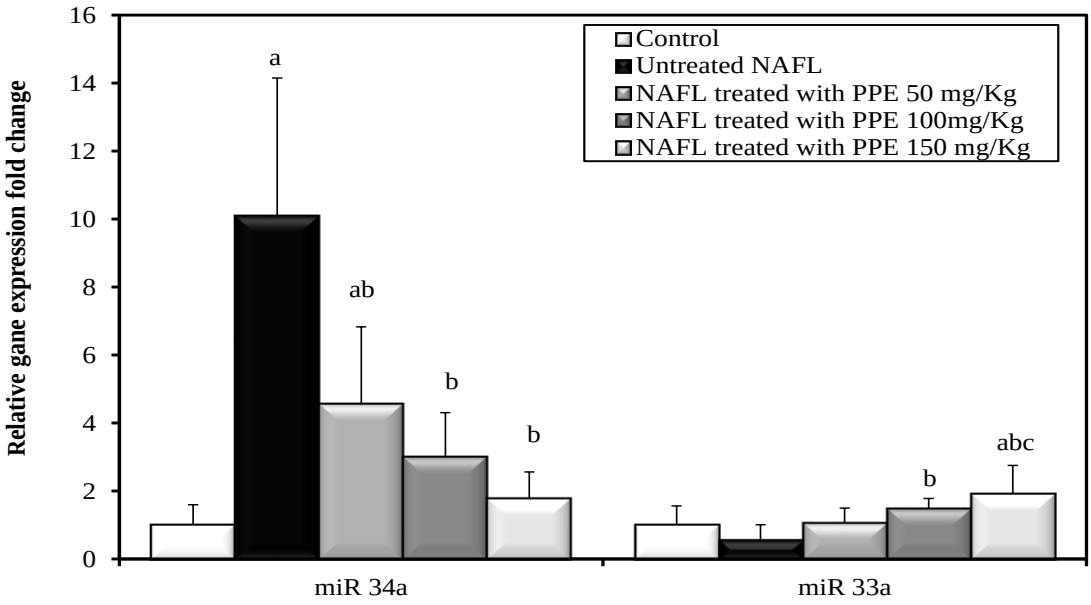

Fig. 1. MicroRNA-33a and MicroRNA-34a

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 $p \leq 0.05$

5. DISCUSSION:

In the present study, feeding male Wistar rats HFD for 14 weeks resulted in manifestations of fatty liver with signs of steatohepatitis. In accordance with our results,using the same type of high-fat diet in rats (**Lieber *et al.*, 2004**) and mice (**Van Rooyen *et al.*, 2011**), produced similar hepatic lesions of human NASH. These histological changes in hepatic tissue were associated with significant alterations in many biochemical pathways that may be involved in the pathogenesis of the NAFL or may be consequences of the disease condition.

NAFL rats demonstrated significant hepatic accumulation of triglycerides and cholesterol which were higher by about 283.2 and 252.2 % respectively compared to control values. Also, the activities of serum transaminases; AST, ALT and GGT were significantly elevated by about 53.1%, 95.2% and 292.6%; respectively

compared to control rats. Also, NAFL rats showed higher bilirubin value by about 252.8%. These abnormalities of liver function tests indicated significant hepatocytes inflammation, damage and necrosis.

At the glucose homeostasis level, NAFL rats showed a significant elevation in fasting blood glucose, insulin and HOMA-insulin resistance index (by about 74.2, 118.8 and 274.1%, respectively) that indicated a state of insulin resistance which is considered as the first hit for hepatic fat accumulation and steatosis. The increase in fasting plasma glucose concentration is due to the impaired ability of insulin to inhibit hepatic glucose production (by glycogenolysis and gluconeogenesis) (**Ryysy L *et al*., 2000**).

In the present study, NAFL rats demonstrated significant hepatic accumulation of lipids where the hepatic contents of triglycerides and cholesterol were increased. The results of lipid content of the liver of NAFL rats showed significantly higher level.

The present study indicated that the metabolic abnormalities in NAFL rats are associated with deranged hepatic expression of microRNAs as indicated by significant suppression of miR-33a by about half-control value and significant induction of miR-34a expression by about 9-fold control value.

The Pomegranate considered one of the most important plants used in alternative medicine because it contains phenolic and flavonoid substances are very important plant constituents because of their antioxidant activity (**Abdel Moneim, 2012**).

The present study was undertaken to evaluate the anti-steatotic effects of pomegranate peel extract (PPE) and to explore microRNAs (miR-33a and miR-34a) as a molecular target of its effect.

The present study documents the ameliorating effects of PPE on hyperglycemia, insulin resistance and liver functions in NAFL rats in a dose-dependent manner compared to untreated rats. The results clearly indicated the strong glucose-lowering effect of PPE as it significantly corrects the fasting blood glucose, insulin and normalizes insulin sensitivity that may mean PPE enhance insulin sensitivity in peripheral tissues which manifested as decreased HOMA-IRI. PPE may thus have a potential clinical utility in combating NAFLD. The exact molecular mechanism involved in the influence of PPE on glucose and lipid homeostasis is unclear, so in this study, the probable involvement of miR-33a and miR-34a in this mechanism was explored.

The results of the present study showed that there were significant decreases in serum activities of AST, ALT and GGT in NAFL rats treated with PPE in a dose-dependent manner. This effect is due to antioxidant content of pomegranate peel. Also, treatment by PPE completely normalizes the level of serum bilirubin.

The PPE supplementation causes a significant dose-dependent decline in the hepatic expression of miR-34a while significantly enhance the hepatic expression of miR-33a in a dose-dependent manner. The modulating

effects of PPE on gene expression are in line with the previous study of Taha et 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 (**Taha *et al*., 2016**).

The correlation pattern obtained in the present study may suggested that, the therapeutic effects of PPE may be mediated through down-regulation of miR-34a and up-regulating miR-33a; as hepatic miR-34a in treated rats is positively correlated with the activity of the liver function parameters (ALT, AST, and bilirubin), glucose homeostasis parameters (FBS and HOMA), and lipid profile (serum TG, serum TC(r=0.659), LDL-C, hepatic cholesterol and hepatic TG) and negatively correlated with serum HDL-C. On the other hand miR-33a showed opposite pattern of correlation.

The suggested role of miR-34a and miR-33a in mediating the anti-steatotic effect of PPE may be supported by the study of **Ding *et al*., 2015** who showed that silencing miR-34a led to an initially increased expression of PPARα, SIRT1 and PPARα's downstream genes and activation of the AMP-dependent kinase (AMPK) the central metabolic sensor. The miR-34a inhibitor suppressed lipid accumulation and improved the degree of steatosis.

Taken together, our data indicated the anti-steatotic effects of PPE in HFD-induced NAFLD in rats and these effects may be mediated through modulation of hepatic expression of expression of miR-34a and miR-33a which potentially contribute to altered lipid and glucose metabolism in NAFLD.

6. CONCLUSION

From the results of the present study we can conclude thatNAFLD is easy to be established by high-fat diet and In NAFLD state the liver function (SGPT, SGOT, GGT and bilirubin) and glucose hemostasis (HOMA-IR, insulin and FBG) are increased. Treatment by PPE reduces accumulation of fat in the liver and decline the hepatic lipid content and serum TG, TC, and LDL-cholesterol. Treatment by PPE improves liver enzymes. From this study we observed that there is a relation between MiR-34a and MiR-33a expression with NAFLD and its expression changed by treatment of PPE.

Recommended, the medical company to apply this research on human to using PPE as medical drug for treatment of NAFLD.

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