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Role of Cymene in the attenuation of fatty liver and UCP2 gene expression

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ABSTRACT

Cymene is an organic aromatic and monoterpenic compound that has anti-inflammatory and antioxidant properties. This study investigates the effect of Cymene on biochemical and histological parameters and UCP2 gene expression in nonalcoholic steatosis

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model induced in male wistar rats. 40 male wistar rats randomized to 5 groups: control (normal diet with standard rat chow) HCD (high cholesterol diet for 12 weeks), sham (high cholesterol diet for 12 weeks and then received normal diet and p-cymene vehicle (sunflower oil) for 4 weeks) and two experimental groups (HCD for 12 weeks and then received normal diet and either 15 mg/kg or 50 mg/kg Cymene for 4 weeks). In the HCD group and sham group: body weight, and serum levels of triglycerides, total-cholesterol, glucose, insulin, liver enzymes (ALT، AST، ALP), total-bilirubin, direct-bilirubin, and low-density lipoprotein cholesterol had significantly increased. The serum levels of high-density lipoprotein cholesterol, adiponectin, superoxide dismutase and catalase were decreased. Histologic analyse of liver section revealed hepatic fibrosis and steatosis. The UCP2 gene expression significantly increased. Cymene treatment at both dose specifically the 50mg/kg dose ameliorated these changes and levels of UCP2 mRNA down-regulated. Administration of Cymene improved the liver fibrose via decreased ucp2 expression.

Keywords: Cymene, fatty liver, UCP2, gene expression

1. INTRODUCTION

As a result of metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) may be occurred (Hübscher, 2006). NAFLD could cause steatohepatitis, fibrosis, cirrhosis, and even cancer (Park et al., 2015). The increase between 5-10% of body weight as a result of fat amassing in the liver could be an indicator of NAFLD (Kotronen and Yki-Järvinen, 2008). The accumulation of free fatty acid is happened as a result of lipolysis. Immoderate triacylglycerol is precipitated in the liver which reduces fatty acid oxidation and also rises hepatic de novo lipogenesis (Li et al., 2014, Hijmans et al., 2015). One of the most important pathogenesis factors in NAFLD is free fatty acid (FFA)-inducedlipotoxicity. The plasma FFA levels is a crucial element in diagnosis ofdisease grade (Wu et al., 2008). These metabolic changes cause fatty liver. The histological researches have revealed that some changes can be detected in the preivenular regions of the liver parenchyma (Hübscher, 2006). Furthermore, it is realized that the progression of NAFLD is directly depends on obesity, diabetes, and the metabolic syndrome (Oosterveer et al., 2009, Wu et al., 2008). *p*-Cymene is a naturally occurring aromatic compound. This natural isomer could be found in the structures of some necessary oil including cumin and thyme. P-cymene is insoluble in aqueous solutions. It is consisted of alkylbenzene and it is categorized as monoterpenes (Bennett et al., 2007). The study shows that p-cymene is responsible for antinociceptive, anti-inflammatory and antioxidant activity of monoterpenes. It is verified that oral administration of nigella sativa crude oil, which contains significant amount of p-cymene, leads to improving lipid factors during high sucrose diet.(Al-Okbi et al., 2013)The reduction in lipid peroxidation and nitrite content, SOD and catalase enhancement in mice hippocampus and also neuroprotection of the brain are occurred as a p-cymene treatment(Souza, 2016). P-cymene is applied as an anti-microbial agent during pasteurizing process in order to increase the shelf life of raw juices(Fan et al., 2015). P-cymene and thymoquinone are two paramount components identified by gas chromatography-mass spectrometry in both raw and roasted black cumin seeds (Kiralan, 2012). Uncoupling protein 2 (UCP2) an inner mitochondrial membrane anion carrier uncouples respiration from ATP synthesis (Pi et al., 2010) and has high homology to the UCP1. Althoug UCP2 is expressed widely but its frequency is so low (Fülöp et al., 2006). Approximately 0.01% to 0.1% of the membrane protein, and when activated in particular has ability to transfer protons (Brand and Esteves, 2005). UCP2by reducing mitochondrial production of reactive oxygen species prevents oxidative damage (Echtay et al., 2002). UCP2 responds to oxidative stress situations (Pecqueur et al., 2001). In a normal liver, UCP2 expression located in Kupffer cells (Larrouy et al., 1997). But in fatty liver, its prevalence increases within hepatocytes conversely an effect of UCP2 amplifies by accumulation and peroxidation of lipids in fatty hepatocytes (Fülöp et al., 2006). UCP2 can cause type 2 diabetes as developed from obesity with important role in the pathogenesis of type-2 diabetes (Souza et al., 2011).

2. MATERIAL AND METHODS

P-cymene was purchased from Sigma-Aldrich, USA. Cholesterol (extra pure) was acquired from Scharlau,Spain. All evaluation kits including LDL-C, HDL-C, TC, total bilirubin, direct bilirubin, ALT, AST, and ALP were purchased from Pars Azmun Company, Iran. Serum adiponectin, insulin measurement kits (Rat adiponectin, ELISA kit; and Rat insulin, INS ELISA kit), Superoxide dismutase (SOD) and CAT enzymes kit were bought from Gmbh, Ulm Zellebio Germany.

Required materials for RNA isolation and cDNA synthesis

Buffer component, Tag DNA Polymerase Enzyme (TA8109C), dNTP (10 mM) (DN7604C), and $MgCl_2$ (50 mM) were obtained from CinnaGen Company, Iran. Ladder 50, 6X Loading Dye were purchased from Fermentas Company, USA. Water nuclease free, Random

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Hexamer primer, Ribolock RNAse inhibitor (RI), Reverse Transcriptuse (RT) were acquired from Thermo scientific Company, Germany. Power SYBER green master mix was purchased from Applied Biosystems Company, USA. Trizole obtained from Invitrogen Company, America. Forward Primer and Reverse Primer for the evaluation of UCP2 and B-actin gene expression were purchased from Pishgam Company, Iran (Table 1). cloroform and isopropanol were obtained from Merck Company, Germany.

Table 1 Sequences of probe and oligonucleotides used in real-time PCR analysis

Gene	Sequence
Ucp2(forward)	CTCCTGTGTTCTCCTGTG
Ucp2(reverse)	GTGTCCCGTTCTTCAAAG
β-ACTIN(forward)	AGCACA GAG CCTCGCCTT
β-ACTIN(reverse)	CAC GAT GGAGGGGAAGAC

Animals

Forty male wistar rats, 6 weeks old, with the approximate weight of 205-233 g were purchase from Pastour Institute, Karaj, Iran. All rats were kept under the same standard room conditions with 12 hours light/dark cycle. The rats have Ad libitum permission to standard pellet and water.

Experimental protocol

The rats were adopted by laboratory conditions for a period of one week and were received standard pellet food. Afterwards, the rats were weighted and categorized into five groups (n=8) as follows;

Animal groups

Control group: receiving a normal diet (ND)

HCD group: receiving a high cholesterol diet (HCD: normal dies which enriched with 2% additional cholesterol for 12 weeks)

Sham group (Following 12 weeks of high cholesterol diet, a normal diet supplemented with p-cymene vehicle was applied for a period of 4 weeks.

Group I: A HCD diet was utilized for 12 weeks and afterwards, a ND diet containing p-cymene (15mg/kg) was orally administrated for 4 weeks.

Group II: For a period of 12 weeks HCD diet was used. Then a ND diet containing p-cymene (50mg/kg) was taken for 4 weeks.

The supplemented HCD was consisted of 1% cholesterol mixed with standard pellet and 1 % cholesterol mixed with sunflower oil which was given by oral gavage.

Following 12 weeks of cholesterol treatment, the rats were randomly selected and their livers were detached under diethyl ether anesthesia condition. The outcomes of histopathological studies have revealed that fatty liver was formed. The rats were treated for a period of four weeks in accordance with international guidelines established in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and further approved by the University's Internal Ethics committee (approval code: 176947).

Ethical Clearance

The project was ethically certified by Anima Ethics Committee of the Science and Research Branch, Azad University, Tehran.

By completing of 12 weeks treatment, the rats were undergone 14 hours of fasting. Then they were weighted and anesthetized by inhalation of diethyl ether. The samples of blood were collected from cardiac ventricles with application of 5ml syringes. Subsequently, liver tissues were affixed in 10% formalin buffer solution for further histopathological evaluation. In accordance to conventional protocols, the tissue samples were embedded in paraffin and cross-sectioned into 5 mm segment. The segments were later stained with Masson's trichrome (MT) and Hematoxylin and Eosin (H&E) in 2 parts. The slides were studied using light microscopy.

The blood samples were clotted at room temperature for a period of 30 min and afterwards they were centrifuges at 2500 rpm, 37 °C for 10 min.

The serum levels of LDL-C, HDL-C, TG, TC, ALT, AST and ALP, total bilirubin, direct bilirubin, glucose, insulin, adiponectin; CAT and SOD were determined using commercially available kits.

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RNA isolation and cDNA synthesis

With respect to supplier's guideline, total RNA was isolated from rats' liver using TRIzol (Invitrogen). The RNA was treated with DNAse1 and spectrophotometrically determined at wavelength of 260 and 280 nm (Biophot- ometer, Eppendorf, Hamburg, Germany).

The integrity of RNA was confirmed using 1.5% gel agarose electrophoresis. Ethydium Bromide was used for staining of RNA. Finally, the pure RNA was frozen at -70 °C. Random hexamer (1 ml), 5 X Reaction buffer (4 ml), dNTP (2 ml), Ribolock RNase Inhibitor (1 ml), and Reverse Transcriptase (1 ml) were applied in order to synthesize cDNA. Final volume of reaction was 20 ml. The synthesis of cDNA protocol was as follows, 5min at 25 °C, 60 min at 42°C and 5 min at 70°C. The primer sequence of UCP2 and B-actin were acquired from NCBI website. The primer express program was utilized to design specific primers.

Quantitative real-time polymerase chain reaction with SYBR Green, and data analysis Real-time PCR relative quantification was performed using the ABI-step 1 system by measuring the increase in fluorescence emission resulting from SYBR Green. The final volume of the real- time PCR components was 20 ml and included SYBR TM (2X) Master Mix (10 ml), forward primer (0.5 ml), reverse primer (0.5 ml), reverse transcription reaction solution (2 ml cDNA) and dH2O (7 ml). The reactions were performed with the following settings: initial denaturation at 95 C for 10 s, 1 cycle; second denaturation at 95 C for 5 s, followed by 5 cycles of annealing and extension for 34 s at 60 C, 50 cycles. A reaction without cDNA was used as a negative control. Reactions were performed in triplicate.

The evaluation of gene expression was executed using the 2-DDCt method. The level of UCP2 gene expression was delineated on the basis of the RQ assay. Statistical analysis One-way ANOVA was used, and the results were expressed as the mean _ SEM (standard error of the mean) followed by Tukey's post hoc test. The level of statistical significance was set at $p < 0.05$.

3. RESULTS

Body weight

Animals Body weight at the beginning of the experiment (initial weight) had no significant difference between various groups. After 12 weeks Treatment with high-cholesterol food all groups except group 2 (cym50mg/kg) showed a significant increase in body weight compared with the control group. In the other words HCD group and sham group in comparison with the control group showed a significant increase in weight also Group 1 has a significant increase in body weight compared with control group. The results indicate that Animals treatment with p-cymene in both dose (15, 50 mg/kg) causes to weight reduction compared to the HCD group. But only treatment with higher dose (50 mg /kg) creates a significant reduction in weight compared to the HCD group. These results show a correlation between the doses of p-cymene and reduced body weight in HCD-fed animals (Table 2).

Table 2

	Control	HCD	Sham	HCD+Cym 1	HCD+Cym 2
Initial Weight	220.66±3.78	222±4.35	222.000±4.16	224.000±4.04	227.3333±2.60
Final Weight	274.33±8.45	361.66±11.53***	376.66±12.01***	360.33±9.26***	287.66±6.74###

Table 3

	Control	HCD	Sham	HCD+Cym 1	HCD+Cym 2
TG	135.7±8.090	260.7±21.46**	244.0±23.30*	230.0±19.70*	180.7±12.60
LDL	18.67±1.202	30.33±1.856*	29.00±2.30	22.33±2.40	19.67±3.38
Cholesterol	64.67±2.728	119.7±8.950**	110.7±6.06*	110.0±6.42*	71.67±11.67##
HDL	67.33±1.856	43.00±1.528**	47.67±2.84*	49.00±5.85*	54.33±3.18
Glucose	72.00±4.933	146.7±7.881***	131.0±5.68**	114.0±8.50*	104.7±8.66#
Insulin	0.3233±0.02186	0.8133±0.08212**	0.7400±0.06**	0.6033±0.07	0.5067±0.07
ALT	44.67±3.180	75.67±2.963**	73.00±5.13**	68.00±4.04*	70.67±3.48**
AST	155.7±6.692	176.3±12.41	185.7±11.61	119.7±7.79#	88.67±7.12**###
ALP	250.0±26.76	389.7±23.10*	373.7±15.32*	284.7±13.42	300.3±13.35
D.Bilirubin	0.0500±0.005774	0.1033±0.008**	0.0900±0.005*	0.0640±0.00321#	0.05333±0.008##

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T.Bilirubin	0.1367±0.01202	0.1967±0.0145	0.1667±0.02028	0.1567±0.01764	0.1200±0.01528#
CAT	78.87±4.518	62.57±6.973	65.90±6.621	96.33±4.265#	101.2±4.511##
SOD	143.3±11.88	87.60±8.640**	91.27±5.720*	89.37±6.821**	102.0±8.631*
Adiponectin	10.75±0.6064	5.623±0.5379**	7.203±0.480*	8.390±0.539	9.997±1.147##

Data are expressed as means _ SEM.
* p< 0.05 compared with the control group.
** p< 0.01 compared with the control group.
*** p< 0.001 compared with the control group.
# p< 0.05 compared with the HCD group.
p< 0.01 compared with the HCD group.
p < 0.001 compared with the HCD group (n = 7)

UCP2 gene expression was also assessed in liver and, as shown in Fig. 1, the UCP2 gene expression in HCD group showed a significant increase in comparison with the control group (p< 0.001). Cymene treatment in both doses (15, 50 mg/kg) caused to decrease expression but it is not significant (p< 0.05).

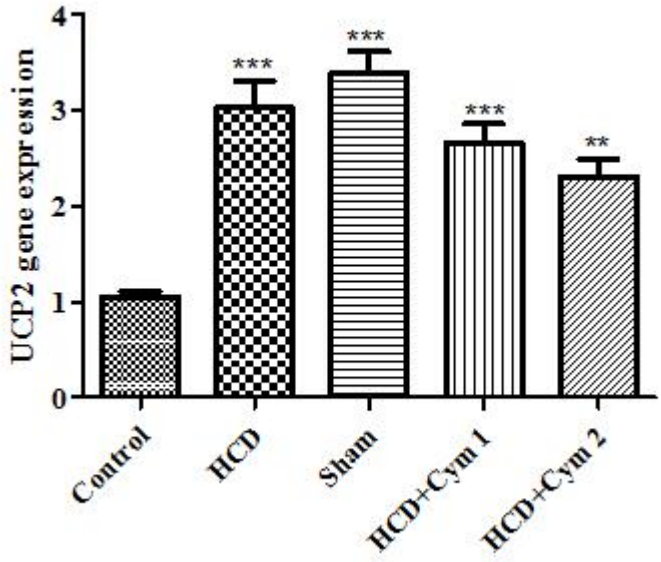

Figure 1 Effect of cymene on the ucp2 gene expression

Data are expressed as the means _ SEM. ***p < 0.001 and ** p < 0.01 compared with the control group.

Biochemical parameters

Biochemical parameters, including lipid profiles, glucose, insulin, and liver enzymes, are reported in Table 3. The serum levels of TC and TG (p< 0.01) and LDL-C (p< 0.05) in the HCD group showed a significant increase in comparison with the control group. The levels of TC (p < 0.01) and TG and LDL-C (p< 0.05) were significantly decreased in the treated groups compared with the HCD group. But this reduction is significant in the higher dose. The TG levels in group 1 were significantly increased compared with the control group (p< 0.05). The HDL-C levels were markedly reduced in the HCD and sham (p< 0.01), yet were increased in the treated groups. But this reduction is not significant (p< 0.05). The levels of the hepatic enzymes AST, ALP and ALT also direct and total bilirubin, were increased in the HCD group. Treatment with p-cymene resulted in decrease in the amount of liver enzymes compared with the HCD group (Table 3), the levels of AST (p < 0. 001), Total bilirubin (p< 0.05) and direct bilirubin (p < 0.01) showed a significant decrease. The levels of ALT and ALP were decreased but it is not significant (p< 0.05).Glucose and serum insulin levels were significantly increased in the HCD compared with the control group (p < 0.001). P-cymene caused reduction in comparison with the HCD. But this reduction is significant in higher dose (p< 0.05). The serum adiponectin levels were markedly reduced in the

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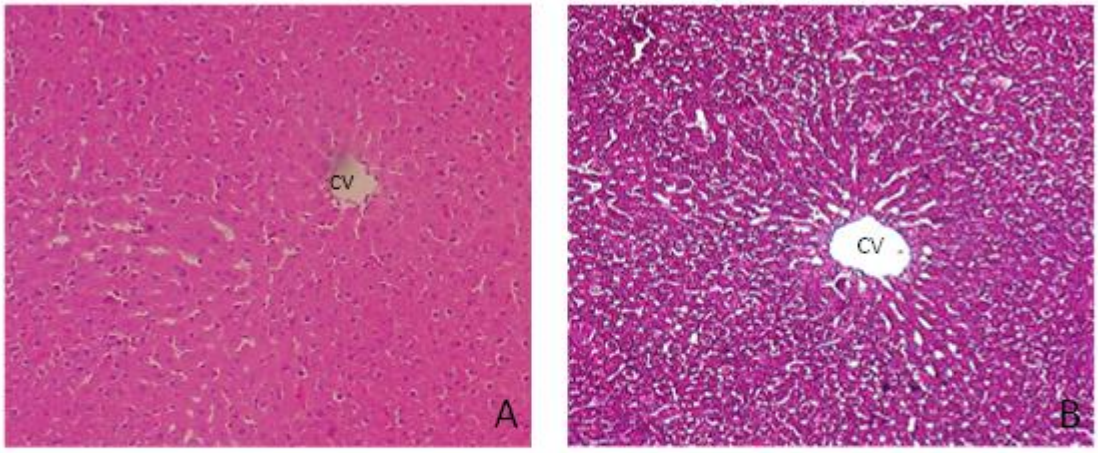
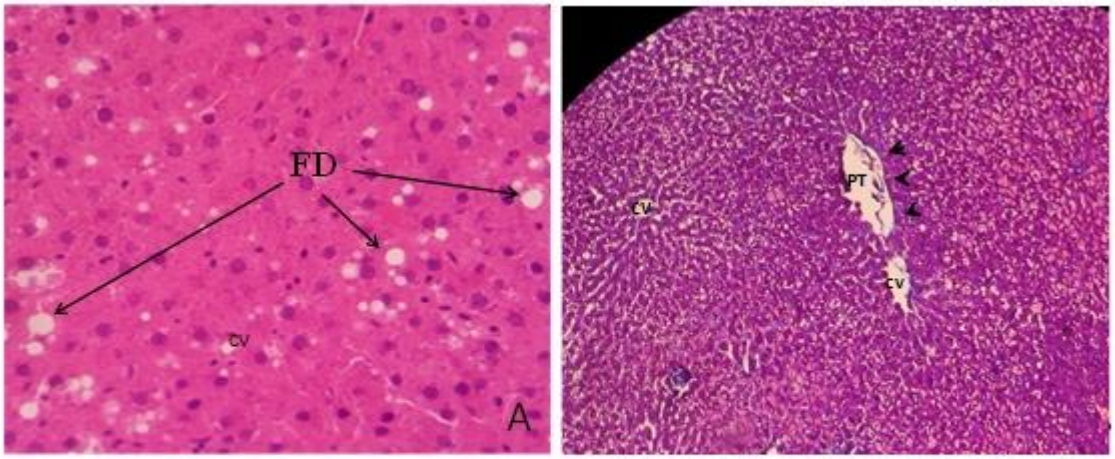
HCD group ($p < 0.01$) and p-cymene treatment were increased adiponectin levels compared with the HCD group. At higher dose was significant ($p < 0.01$). The SOD and CAT serum levels were reduced in HCD group in comparison with the control group. But SOD had a significant change ($p < 0.01$). P-cymene treatment increases SOD and CAT serum levels in both doses (15, 50 mg/kg) but this increase is significant in CAT ($p < 0.01$).

Histopathological evaluation

Liver sections of the control group showed unremarkable tissue with normal structure and hepatocytes and centralvein was observed. In the HCD group Collagen deposition in the form of thin strands detected among hepatocytes, fatty Macrovesicul and microvesicul were observed that represents the accumulation of fat and start steatosis. pericellular fibrosis was observed. The liver sections from group II (cym 50mg/kg) exhibited decrease in the number of fatty vesicles, especially Macrovesicul among hepatocytes (Fig. 2).



CONTROL



HCD

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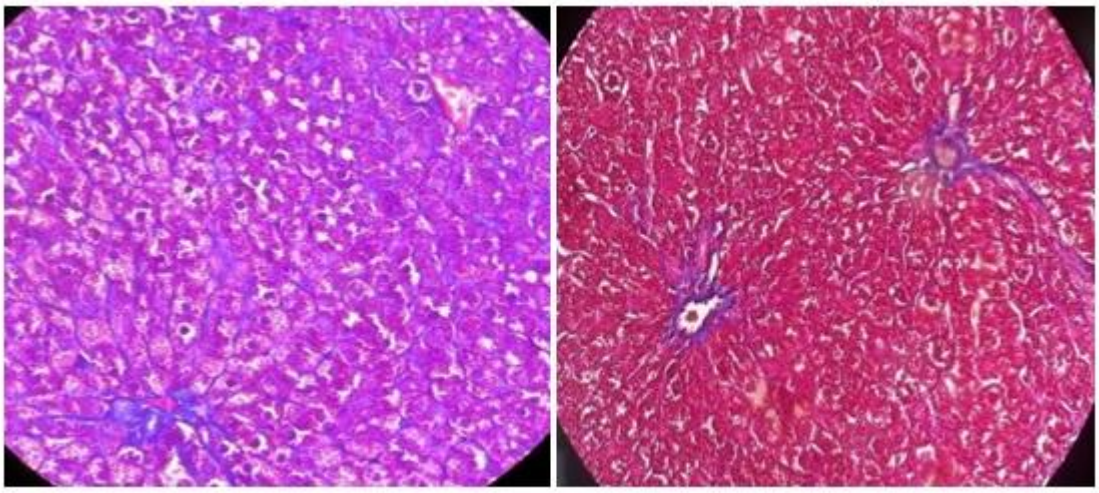
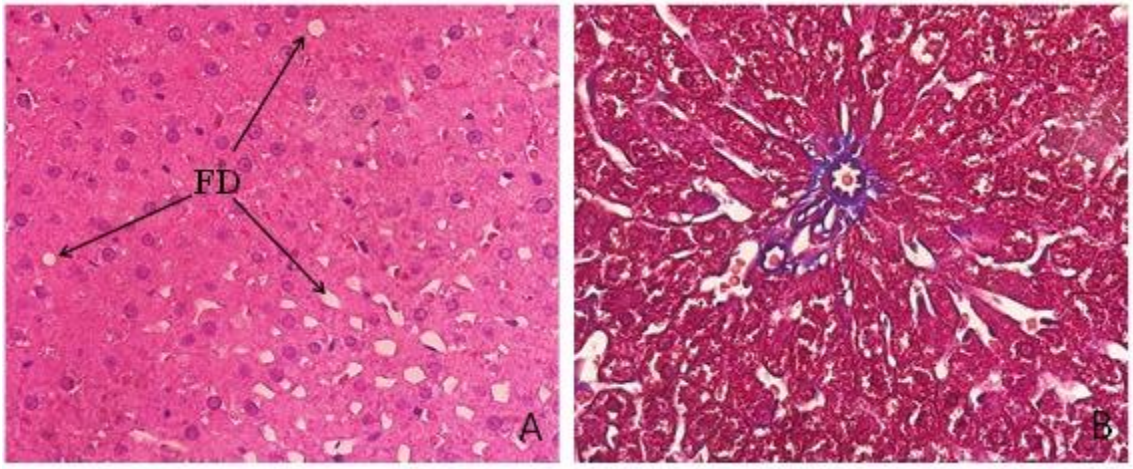
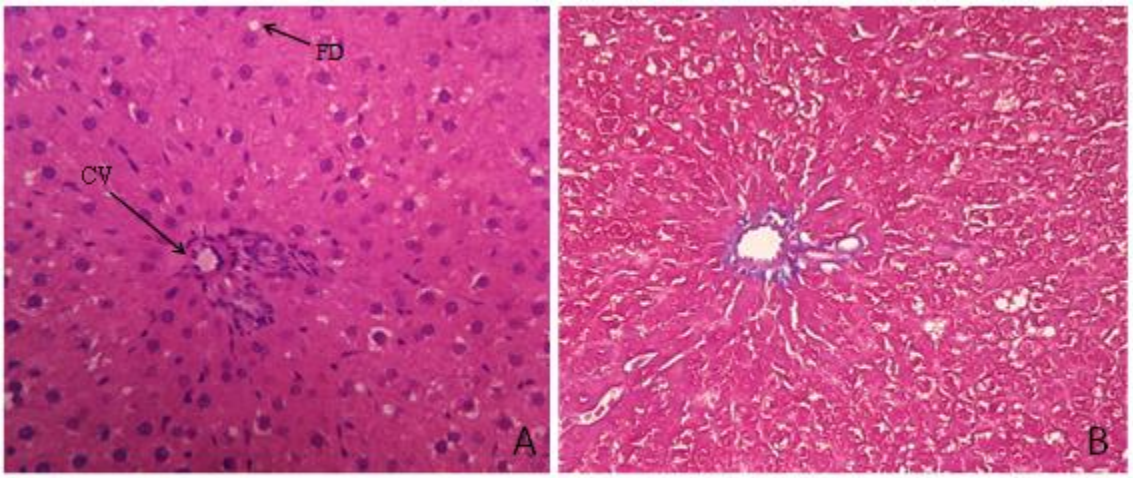


Sham



HCD+cym 1



HCD+cym 2

Figure 2 Histopathological findings of effects of cymene on liver tissues in HCD-fed rat. At the end of experimental period, liver sections were assessed by H & E & Masson's trichrome (MT) staining.

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4. DISCUSSION

In this project, the Wistar rats were treated with HCD diet for a period of 12 weeks in order to formation of fatty liver. At the end of treatment session, fatty liver was obviously distinguished in liver sections (Fig. 2). Then cymene was administrated for 4 weeks. The outcome of cymene was assessed through identifying several factors including body weight; lipid profiles; antioxidant enzyme levels; serum levels of adiponectin, glucose and insulin; liver tissue histology; and UCP2 gene expression in liver. The previous studies have revealed that as a result of 12 weeks treatment with HCD diet, the body weight will increase and obesity in rodent will induce (Hu et al., 2014).

The outcome of this research is also in good agreement with previous reports. It is clarified that 12-weeks HCD diet is significantly increase the body weight in compare to normal diet. Nevertheless, 4-weeks cymene diet is led to body weight reduction. It is also observed that administration of 50 mg/kg dose of cymene in group II notably decrease body weight. Moreover, HCD diet is raised the lipid profile indicators, TG, TC, and LDL-C, and is reduced the level of HDL-C which are all considerable signs of dyslipidemia. All these symptoms are noticed in obesity (Lohmann et al., 2009) due to activation of ROS and could be risk factors in progress of hepatic diseases (Saravanan et al., 2014, Blokhina et al., 2003).

The liver is responsible for lipid metabolism. The hepatic steatosis is occurred as a consequence of imbalance between construction and consuming of lipid (Stanton et al., 2011). It is demonstrated that application of cymene decrease body weight and enhance lipid profiles in rats (Lotfi et al., 2015, Al-Okbi et al., 2013). The results of this study are also confirmed advantageous effect of cymene on body weight and serum lipids. The liver function could be indirectly measured AST, ALT, ALP, total bilirubin and direct bilirubin levels of serum (Hall and Cash, 2012). The rats treated with HCD were shown growth in the plasma levels of these factors and following cymene treatment, the levels of factors were decreased. Furthermore, cymene could be a potential candidate in curing of hepatic damages through improving antioxidant defenses. The studies have demonstrated that obesity and HCD raise production of free radical and induce oxidative stress (Mohammadi et al., 2006).

Hypercholesterolemia is defined as peroxidation of lipids and depletion of antioxidant enzyme activity. Hypercholesterolemia leads to cell injury increases in free fatty acids cause an imbalance of oxidants and antioxidants in obese rats (Noeman et al., 2011). The SOD and CAT levels will be increased as a result of cymene treatment. The SOD and CAT, endogenous antioxidant enzymes, have detoxification effect and prevent cell damage (De Oliveira, 2012). The therapeutic effects on hepatic fibrosis and steatosis could be similarly related to these effects.

Enhanced adipose tissue lipolysis and improvement of hepatic synthesis of free fatty acid are related to insulin resistance (Yao et al., 2011). In this research, it is demonstrated that HCD diet raises the serum levels of glucose and insulin compared to normal diet. Administration of cymene leads to decreasing of glucose and insulin levels of serum. Furthermore, cymene treatment could postpone development of IR associated with tissue steatosis (Lotfi et al., 2015).

It is verified that high fat diets reduce adiponectin levels of animal models. Adiponectin plays a key role in preventing of obesity, insulin resistance and fatty liver disease (Kadowaki et al., 2006). Also, the beta-oxidation of free fatty acids in hepatocytes could be improved in presence of adiponectin (Ma et al., 2002). Therefore, alteration of adiponectin levels is resulted in improving obesity, insulin resistance and fatty liver (Ghantous et al., 2015). Other studies have shown that high fat diet decreases adiponectin levels in animal models (Sung et al., 2014, Barnea et al., 2006). In this study, the cymene enriched diet is significantly improved serum adiponectin levels.

The variance between up taking and consuming of energy substrate causes obesity. The ATP is mainly synthesized in mitochondria (Chavin et al., 1999). The inner mitochondrial membrane carrier, UCP2, which facilitate proton transfer in different mammalian cells, could cause some changes in ATP levels of mitochondria through competing with ATP synthesize (Fülöp et al., 2006).

During obesity, hepatocyte cells resist against excess substrate supply and induce UCP2 mRNA and protein expression (Chavin et al., 1999). The outcome of this study is also indicated that UCP2 gene expression enhanced succeeding HCD diet. Abundant substrate reservoir improves the possibility of ROS formation and accumulation of ROS will lead to oxidative stress.

The engendering of UCP2 impacts on uncoupling of oxidative phosphorylation and also limits mitochondrial ROS production (Chavin et al., 1999, Ruiz-Ramírez et al., 2011). Hence in the oxidative stress circumstances, the cells will show a defensive response by increasing UCP2 gene expression (Horimoto et al., 2004). Cymene wipes the cells from free radicals and therefore, reduces the obesity caused by oxidative stress (Quintans-Júnior et al., 2013). The expression of UCP2 gene is down regulated cell responses (Yang et al., 2011).

The results from this research have indicated that administration of cymene decreases UCP2 gene expression. Over expression of UCP2 could lead to endotoxin liver injury (Chavin et al., 1999, Rashid et al., 1999). Cymene could also reduce the UCP2 gene expression. Our histopathological assay is manifested that HCD significantly induced fatty liver. Steatosis and fibrosis were declined

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following cymene treatment. All these results indicate that cymene could restore hepatocyte cells through enhancing anti-oxidant activity and impeding free radical damage caused by lipid peroxidation.

5. CONCLUSION

This research is demonstrated that cymene has positive effect on improvement of inflammation and hepatic steatosis. The impact of cymene is caused due to down regulation of UCP2 modulation of lipid profiles, enhancing the level of anti-oxidant enzymes, removing free radicals, decreasing the oxidative stress and improving hepatic steatosis. It is suggested that application of cymene could be a promising strategies in treatment of hepatic steatosis.

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