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Standardized *Nigella sativa* seed oil ameliorates hepatic steatosis, aminotransferase and lipid levels in non-alcoholic fatty liver disease: A randomized, double-blind and placebo-controlled clinical trial

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ABSTRACT

Ethnopharmacological evidence: *Nigella sativa* (*N. sativa*) seeds are used in the Iranian traditional medicine for the treatment of liver diseases.
Aim of study: To study the efficacy and safety of *N. sativa* seed oil in the treatment of patients with non-alcoholic fatty liver disease (NAFLD).
Materials and methods: Sixty patients received 2.5 mL fully standardized *N. sativa* seed oil every 12 h and 60 other patients received placebo for 3 months. At the baseline and endpoint, hepatic steatosis ultrasound grade and blood levels of triglycerides, LDL-C (low-density lipoprotein cholesterol), HDL-C (high-density lipoprotein cholesterol), ALT (alanine aminotransferase), AST (aspartate aminotransferase), blood urea nitrogen, creatinine and complete blood cell count as well as body mass index were determined in the oil and placebo groups and compared.
Results: Grade of hepatic steatosis was significantly reduced in the oil group compared to the placebo group ($P = 0.004$). Mean ± standard deviation of changes of variables in the oil and placebo groups were respectively 32.6 ± 16.6 and 14.2 ± 19.7% for ALT ($P < 0/001$), 29.4 ± 16.3 and 12.3 ± 16.8% for AST ($P < 0.001$), 10 ± 13.9 and 0.22 ± 18.2% for triglycerides ($P = 0.001$), 14.1 ± 9.8 and 9.2 ± 11.1% for LDL-C ($P = 0.01$), 9.5 ± 7.7 and 4.8 ± 6.5% for HDL-C ($P = 0.001$). However, the oil did not significantly affect the other outcome variables compared to the placebo (all $P > 0.05$). No adverse effect was observed.
Conclusions: The *N. sativa* seed oil seems to be safe and improve liver steatosis and injury and blood levels of triglycerides, LDL-C and HDL-C in the NAFLD patients.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a prevalent disease affecting around 25% of the world population. NAFLD begins with simple steatosis (accumulation of triglycerides in the liver) which may progress to steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. NAFLD may lead to hepatic and cardiovascular morbidities and mortalities. NAFLD is highly associated with the components of metabolic syndrome especially insulin resistance (Friedman et al., 2018). Vitamin E and pioglitazone have the most evidence of efficacy in the treatment of NAFLD but have adverse effects and limitations. There is no approved pharmacologic therapy for the treatment of NAFLD. Body weight loss via dieting and physical activity is the only recommended modality for the treatment of NAFLD. Weight loss can be effective but it is hard to achieve and sustain. The need to develop new therapies with proven efficacy in the NAFLD patients is clear (Hung and Bodenheimer, 2018). The seeds of *Nigella sativa* L. (*N. sativa*) (black seed or black cumin) (family Ranunculaceae) are traditionally used for the treatment of liver diseases in Iran (Tavakkoli et al., 2017). Moreover, pharmacologic studies suggest that the *N. sativa* seeds could be effective in the treatment of NAFLD. The *N. sativa* seed ethanol extract demonstrated peroxisome proliferator-activated receptor gamma (PPARγ) agonistic action in vitro (Benhaddou-Andaloussi et al., 2010). Notably, agonists of PPARγ reduce insulin resistance. Oral administration of the *N. sativa*

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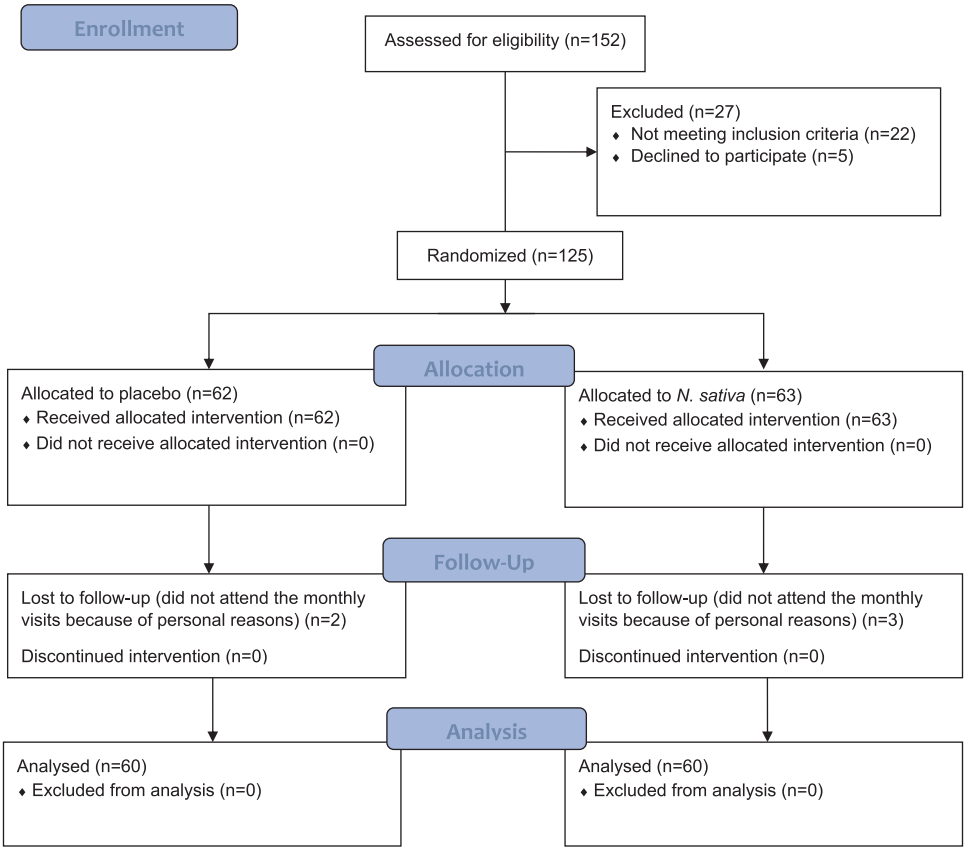

Fig. 1. CONSORT flow chart of the clinical trial.

seed oil significantly improved dyslipidemia, high blood levels of tumor necrosis factor alpha and malondialdehyde and liver steatosis and function tests in the rat model of high fructose diet-induced steatohepatitis (Al-Okbi et al., 2013). Additionally, the *N. sativa* seeds have prevented liver injury in the various experimental and clinical studies (Mollazadeh and Hosseinzadeh, 2014). Hepatoprotective supplements or nutraceuticals may improve hepatic steatosis and related biochemical, anthropometric and hemodynamic parameters in the NAFLD (Cicero et al., 2018). There has been no high-quality clinical trial investigating the efficacy of a standardized product of *N. sativa* seeds in the treatment of NAFLD. Therefore, in the present study, the efficacy of a fully standardized *N. sativa* seed oil in the treatment of NAFLD patients was evaluated.

2. Materials and methods

2.1. Drugs

The *N. sativa* seed oil with the batch number 402296 prepared by cold press method was purchased from the Barij Essence Pharmaceutical Company (Iran, Kashan). *N. sativa* seed oil mixed with honey and water with the volumes 2.5 mL, 1.25 mL and 1.25 mL respectively in each 5 mL of the mixture constituted the active treatment. The placebo contained 2.5 mL mineral oil, 1.25 mL honey and 1.25 mL water in each 5 mL of the mixture. The active treatment and placebo were similar in appearance and smell and were packaged in similar dark colored glass bottles.

2.2. Phytochemical analyses

The *N. sativa* seed oil was standardized by determination of the thymoquinone and fatty acid contents.

2.3. Determination of thymoquinone in the N. sativa seed oil

A Knauer HPLC (Germany) was used with pump K1001 and UV detector K2501 (Germany) and a C18 reversed-phase Phenomenex analytical column (250 × 4.6 mm). The isocratic mobile phase utilized was composed of water: methanol: 2-propanol (50:45:5% v/v) and was filtered through a 0.45 µm Millipore filter. Analysis was performed at room temperature. UV monitoring of the eluted solutes was carried out at 254 nm for thymoquinone. A flow rate of 2.0 mL/min was used. Calibration curve of peak area ratios was constructed by injecting different quantities of thymoquinone (Ghosheh et al., 1999).

2.4. Determination of fatty acids in the N. sativa seed oil

To determine the fatty acid content of the *N. sativa* seed fixed oil, fatty acid esters in fixed oil were trans-esterified, converting them to more volatile fatty acid methyl esters (FAMEs). These FAMEs were separated using gas chromatography (GC), detected using electron ionization mass spectrometry (MS), and identified using a mass spectral library. After integrating the GC peaks, the relative amounts of FAMEs in their samples were identified.

200 mg of sample was dissolved in 2 mL iso-octane and added 0.1 mL of potassium hydroxide 2 M, then 2 mL of 40% sodium chloride solution was added and mixed. After allowing the layers to settle, the upper (organic) layer was carefully transferred to a clean vial. The organic layer was dried by adding anhydrous sodium sulfate and thereupon the sample was ready to inject into the GC-MS instrument.

Gas chromatography–mass spectrometry was carried out on an Agilent 890 GC gas chromatograph coupled to a 5973N mass selective detector under electron impact ionization (EI) mode at 70 eV. The mass scan range was 50–500 at. mass units (AMU). BPX5 (30 m, 0.25 mm internal diameter) was employed with helium as carrier gas at a flow

rate of 0.5 mL/min. Injector temperature was 290 ºC. Sample was analyzed with the column held initially at 70 ºC for 5 min, increased to 300 ºC at 10 ºC/min and held for 3 min. Response time was 75 min. Peaks were identified by computer searches in commercial reference libraries NIST/MS and user-generated reference library (Christie, 1998).

2.5. Protocol of the clinical trial

A 2-arm, randomized, double-blind, placebo controlled parallel-group trial was performed in the Baqiyatallah hospital (Tehran, Iran). The trial was conducted from April 4, 2017 to April 20, 2018. Inclusion criteria: Iranian patients whose hepatic ultrasonography shows they have fatty liver disease; age of 20–70 years. Exclusion criteria: Child-Pugh score above 7; hepatic disease other than non-alcoholic fatty liver disease; intake of any drug affecting NAFLD; pregnant women; women planning pregnancy; lactating women.

One hundred and fifty two patients were screened. The enrolled patients were randomized to the *N. sativa* and placebo groups (Fig. 1). Block randomization with computer generated random number table and sequentially numbered containers each representing a block consisting of ten patients was used for the treatment allocation. The patients were instructed to take 5 mL of the *N. sativa* syrup or placebo every 12 h for 3 months. The patients were also asked to make no changes to their diet and physical activity compared to before trial. Further, the groups' blood levels of ALT (alanine aminotransferase), AST (aspartate aminotransferase), LDL-C (low-density lipoprotein cholesterol), HDL-C (high-density lipoprotein cholesterol) and triglyceride determined by an autoanalyzer (Hitachi 917, Japan), ultrasound grading of hepatic steatosis (grades 0, 1, 2 and 3 representing normal, mild, moderate and severe steatosis, respectively) (Gerstenmaier and Gibson, 2014) and body mass index were compared. Primary outcome variables: steatosis grade and blood levels of ALT, AST, LDL-C and triglyceride. Secondary outcome variables: body mass index, complete blood cell count and blood levels of HDL-C, blood urea nitrogen and creatinine. Three different persons generated the random allocation sequence, enrolled the patients and assigned them to interventions. These persons, care-providers and patients were blinded to interventions. Patient adherence to the treatments was measured by counting returned bottles and asking how many doses of the drugs were (or were not) taken. Sixty patients in each group was the sample size calculated to detect 3.5 U/L difference of the ALT levels between the groups, considering type I error = 0.05% and 80% power. The chi-squared and independent samples *t*-tests were used for data analyses and $P < 0.05$ was considered statistically significant. The data were analyzed by the per protocol approach. This study was approved by the Ethics Committee of the Baqiyatallah University of Medical Sciences (approval number: IR.BMSU.REC.1394.149). The clinical trial was performed according to the revised Declaration of Helsinki 2013. The participants gave written informed consent before enrolment. This trial was registered at the Iranian Registry of Clinical Trials (www.irct.ir) as IRCT20090804002288N13.

3. Results

3.1. Determination of thymoquinone in the N. sativa seed oil

The chromatogram of the *N. sativa* seed oil showed well resolved peaks with no interference. The run time for total analysis required less than 14 min. The retention time of thymoquinone was 9.5 min (Fig. 2). The sample and standard were analyzed three times (Figs. 2 and 3) and the amount of thymoquinone in the *N. sativa* seed fixed oil was calculated as 0.987 ± 0.07 mg/mL (mean ± standard deviation) using the calibration curve (Fig. 4).

3.2. Determination of fatty acids in the N. sativa seed oil

Myristic acid (0.63%), palmitic acid (20.27%), palmitoleic acid (0.94%), linoleic acid (49.37%), oleic acid (5.76%), stearic acid (5.48%) and arachidic acid (0.90%) were the main fatty acids of the *N. sativa* seed oil (Table 1).

3.3. Clinical trial

Sixty patients in each of the *N. sativa* seed oil and placebo groups completed the trial (Fig. 1). The patients took more than 80% of the prescribed *N. sativa* seed oil and placebo. Mean ± standard deviation of age was 46.64 ± 12.18 years. Sixty two (53.3%) patients were male. The mean ± standard deviation of body mass index was 27 ± 2.1 kg/m^2. The groups did not differ significantly in age, gender and body mass index. Table 2 shows that the changes in ALT, AST, LDL-C, HDL-C and triglycerides in the *N. sativa* seed oil group were significantly higher than in the placebo group. The *N. sativa* seed oil significantly decreased the steatosis grade ($P = 0.004$) and the levels of ALT ($P < 0.001$), AST ($P < 0.001$), LDL-C ($P = 0.01$) and triglycerides ($P = 0.001$) and increased the HDL-C level ($P = 0.001$) compared with the placebo (Table 2). However, the *N. sativa* seed oil did not significantly affect the other outcome variables compared with the placebo (all $P > 0.05$). The patients did not report any adverse drug effect.

4. Discussion

The results indicate that *N. sativa* seed oil reduces degree of steatosis and levels of aminotransferases, LDL-C and triglycerides, while it increases HDL-C levels and has no effect on body weight in the NAFLD patients. Imaging tests (ultrasound, CT and MRI) often report the severity of steatosis. The degree of steatosis quantified by imaging correlates significantly with histologic grading of steatosis (Rinella et al., 2003; Vuppalanchi et al., 2007; Mazhar et al., 2009; Gerstenmaier and Gibson, 2014). Some studies suggest that degree of steatosis may predict the severity of liver histologic alterations (e.g. ballooning, inflammation, fibrosis and steatohepatitis) (Chalasani et al., 2008). Reduction of hepatic steatosis and aminotransferases demonstrates that *N. sativa* seed oil may reverse hepatic injury and protect the liver in the NAFLD. Moreover, according to the results, *N. sativa* seed oil seems to affect lipid metabolism and specifically liver triglyceride metabolism. Lack of effect on the body weight suggests that the effects of the *N. sativa* seed oil are not due to weight loss. Since NAFLD is commonly associated with dyslipidemia (Friedman et al., 2018), the LDL-C and triglyceride lowering and HDL-C raising effects of the *N. sativa* seed oil can be useful in the NAFLD patients. Lack of adverse effects in the patients illustrates safety of *N. sativa* seed oil, as also reported previously (Zaoui et al., 2002).

Although liver biopsy is the gold standard for diagnosis of NAFLD and assessment of hepatic steatosis, fibrosis and inflammation in the NAFLD, it is limited by sampling error, intra- and inter-observer variability, patient anxiety and procedure-related morbidity and mortality (Chartampilas, 2018; Li et al., 2018). Magnetic resonance is the most accurate method and superior to liver biopsy and ultrasound for quantification of hepatic steatosis and fibrosis (Chartampilas, 2018; Li et al., 2018). Inflammation is more important than steatosis in the development of fibrosis, cirrhosis and hepatocellular carcinoma (Li et al., 2018). Therefore, the shortcomings of this study are short treatment duration and lack of investigation into the action mechanisms of the *N. sativa* seed oil, liver biopsy, magnetic resonance techniques and evaluation of fibrosis and inflammation.

The pathogenesis of NAFLD is not fully understood and multiple mechanisms have been implicated in it (Friedman et al., 2018). Bile acids affect lipid metabolism in the liver and extra-hepatic tissues and dysregulation of bile acid metabolism may play a role in the pathogenesis of NAFLD (Arab et al., 2017; Chow et al., 2017). *N. sativa* seed

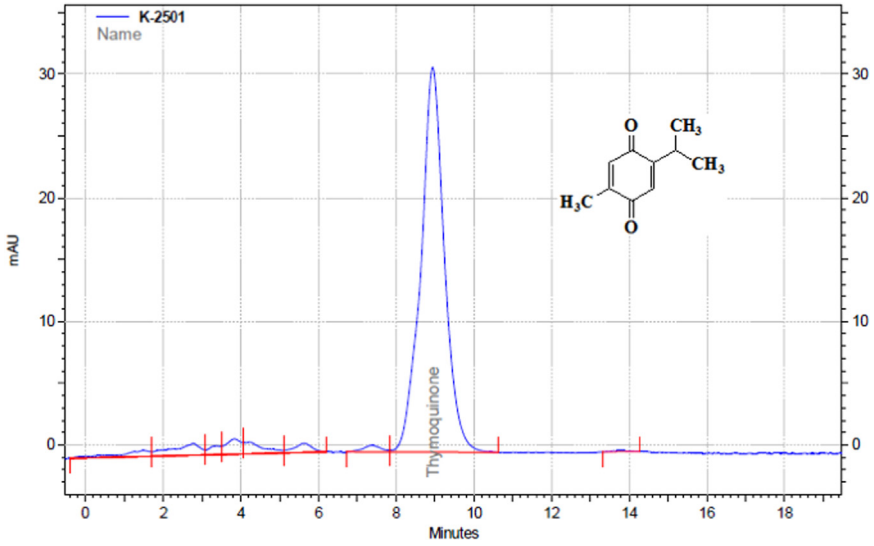

Fig. 2. HPLC chromatogram showing thymoquinone in the *Nigella sativa* seed oil.

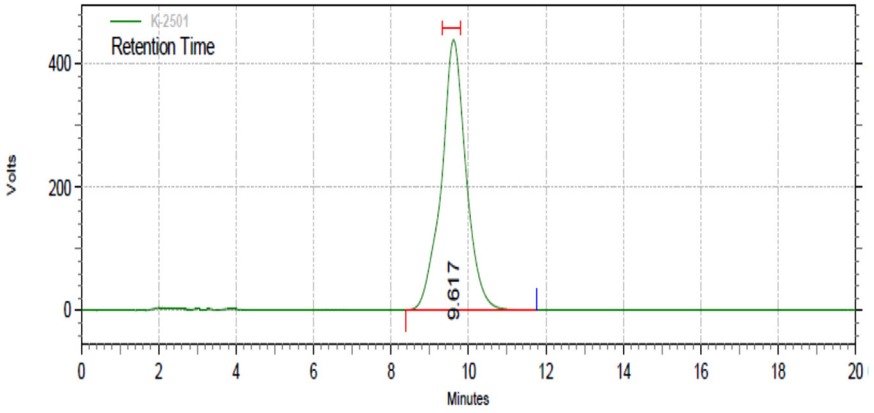

Fig. 3. HPLC chromatogram of the standard thymoquinone.

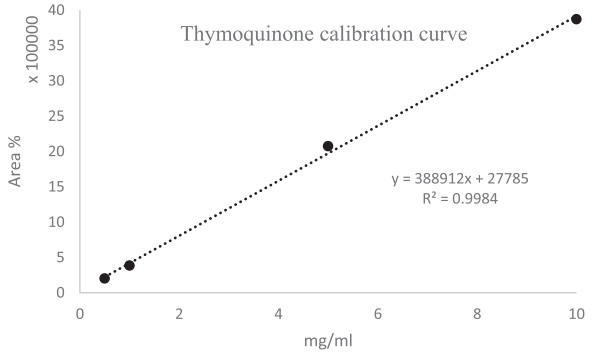

Fig. 4. Calibration curve used for determination of the thymoquinone content of the *Nigella sativa* seed oil.

oil has choleretic effect (Mollazadeh et al., 2017). Choleretic action is one of the mechanisms of hepatoprotection (Cicero et al., 2018). Thus, the choleretic effect of *N. sativa* seed oil may also be involved in its effects in this study. The active compounds identified in the *N. sativa* seed oil including thymoquinone (Awad et al., 2016), monounsaturated fatty acids (MUFA) (palmitoleic acid and oleic acid) and polyunsaturated fatty acids (PUFA) [linoleic acid (omega 6), linoleic acid (omega 3) and 11,13-eicosadecanoic acid] (Silva Figueiredo et al., 2018) may be involved in the effects observed in this study. Moreover, other components of the *N. sativa* seed oil alongside thymoquinone and fatty acids may also have some role in the effects. Thymoquinone is the major component of the *N. sativa* seed essential oil and responsible for most therapeutic effects of the *N. sativa* seed oil (Ghosheh et al., 1999; Mollazadeh and Hosseinzadeh, 2014; Tavakkoli et al., 2017; Tekbas et al., 2018). Thymoquinone is transferred in the blood as bound to the human serum albumin (HAS) and α1-acid glycoprotein (AGP) after intestinal absorption (Lupidi et al., 2012). Under normal physiological conditions, thymoquinone preferentially binds to HAS. However, because blood levels of AGP increases considerably in inflammatory

Table 1
Fatty acid content of the *Nigella sativa* seed oil.

Compound number	Fatty acid	%
1	Linoleic acid (omega-6)	48.29
2	Palmitic acid	20.27
3	11,13-Eicosadienoic acid	9.22
4	Oleic acid	5.76
5	Stearic acid	5.48
6	Linoleic acid (omega-3)	1.08
7	Palmitoleic acid	0.94
8	Arachidic acid	0.90
9	Myristic acid	0.63
10	Heptadecanoic acid	0.28
11	Pentadecanoic acid	0.18
12	Behenic acid	0.16
13	Lignoceric acid	0.10
14	Tricosylic acid	0.04

Table 2
Clinical characteristics at baseline and after intervention in each group.

Variables	Group	Baseline (mean ± standard deviation)	*P* value	After intervention (mean ± standard deviation)	*P* value	Changes (%)	*P* value
Fatty liver grade 0 (n)	1	0	0.194*	18	0.004*		
	2	0		4			
Fatty liver grade 1 (n)	1	24		18			
	2	22		24			
Fatty liver grade 2 (n)	1	31		19			
	2	37		30			
Fatty liver grade 3 (n)	1	5		5			
	2	1		2			
AST (U/L)	1	66.4 ± 8.9	0.134	46.6 ± 11.9	0.001	29.4 ± 16.3	< 0.001
	2	68.4 ± 4.9		59.9 ± 12.4		12.3 ± 16.8	
ALT (U/L)	1	72.3 ± 8	0.128	48.5 ± 12.6	0.001	32.6 ± 16.6	< 0.001
	2	74.3 ± 5.7		63.6 ± 15.2		14.2 ± 19.7	
LDL-C (mg/dL)	1	118.8 ± 23.4	0.491	101.2 ± 20.7	0.452	14.1 ± 9.8	0.01
	2	115.8 ± 23.6		103.9 ± 19.1		9.2 ± 11.1	
HDL-C (mg/dL)	1	39.8 ± 5.1	0.127	43.5 ± 5.7	0.001	9.5 ± 7.7	0.001
	2	38.5 ± 3.1		40.4 ± 3.5		4.8 ± 6.5	
Triglycerides (mg/dL)	1	122 ± 38.6	0.311	105.6 ± 31.4	0.001	10 ± 13.9	0.001
	2	128.7 ± 33.8		126.8 ± 37.2		0.22 ± 18.2	

1 = *Nigella sativa* seed oil group (n = 60), 2 = placebo group (n = 60).
* Chi-square test, other *P* values resulted from independent t-test.

conditions, the interaction of thymoquinone with AGP may substantially affect the pharmacokinetics and pharmacodynamics of thymoquinone in the NAFLD (Lupidi et al., 2012). Besides hepatic steatosis and insulin resistance, NAFLD encompasses hepatic inflammation, apoptosis, fibrosis and oxidative stress (Friedman et al., 2018). Thymoquinone may have hepatoprotective effect through up-regulation of PPARγ (Prabhakar et al., 2015; Awad et al., 2016; Yang et al., 2016) and anti-inflammatory, anti-apoptotic, antifibrotic and antioxidant actions (Tekbas et al., 2018). Thymoquinone improved hepatic steatosis and fibrosis, ALT and AST levels and dyslipidemia in a rat model of high fat diet-induced NAFLD (Awad et al., 2016). The pivotal role of interleukin-1 receptor-associated kinase 1 (IRAK1) in inflammation has been demonstrated in several inflammatory disease models including high-fat-diet-induced nonalcoholic steatohepatitis (Jiang et al., 2015). In a mouse model of hepatitis, thymoquinone alleviated hepatic inflammation by inhibiting IRAK1, and consequently nuclear factor-κB and activator protein-1, and reduced the ALT and AST levels (Hossen et al., 2017). Decrease in caspase 3 expression, enhancement of sirtuin 1 expression, normalization of proapoptotic Bax expression in hepatocytes and increase of antiapoptotic B-cell lymphoma-2 level have been implicated in the antiapoptotic effects of thymoquinone (Tekbas et al., 2018). Prevention of hepatic fibrosis by thymoquinone has been mainly attributed to inhibition of transforming growth factor-β induced hepatic stellate cell activation, downregulation of α-smooth muscle actin expression and activation of adenosine monophosphate-activated protein kinase phosphorylation (Tekbas et al., 2018). Thymoquinone may enhance the liver antioxidant system by increasing glutathione level and activities of catalase, superoxide dismutase and quinone reductase, inhibition of lipid peroxidase and scavenging $O._2$ and OH free radicals (Mollazadeh and Hosseinzadeh, 2014; Tekbas et al., 2018). There has been no clinical trial evaluating the effects of thymoquinone in the NAFLD. The MUFA and PUFA may reduce hepatic steatosis by suppressing triglyceride synthesis and increasing fatty acid oxidation in the liver (Silva Figueiredo et al., 2018). A systematic review found that PUFA reduced liver fat and AST levels in the NAFLD patients (Parker et al., 2012). A high-MUFA diet reduced liver fat in a controlled randomized clinical trial of type 2 diabetic patients (Bozzetto et al., 2012). However, the *N. sativa* seed oil used in the present study contained low MUFA and relatively high PUFA content. Therefore, the possible role of PUFA versus MUFA in the effects of *N. sativa* seed oil may be stronger. Finally, long-term clinical trials investigating the efficacy of *N. sativa* seed oil in the treatment of nonalcoholic steatohepatitis, hepatic fibrosis and cirrhosis seem warranted.

5. Conclusions

The *N. sativa* seed oil seems to be safe and improve liver steatosis and injury and blood LDL-C, triglyceride and HDL-C levels in the patients with nonalcoholic fatty liver disease.

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Conflict of interest

None.

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