Hepatic Regeneration and Reno-Protection by Fish oil, *Nigella sativa* Oil and Combined Fish Oil/*Nigella sativa* Volatiles in CCl₄ Treated Rats

Sahar Y. Al-Okbi¹, Doha A. Mohamed¹, Thanaa E. Hamed¹, Amr E. Edris^{2*} and Karem Fouda¹

¹ Nutrition and Food Sciences Department, National Research Centre, Dokki, Cairo, EGYPT

² Aroma and Flavor Chemistry Department, National Research Centre, Dokki, Cairo, EGYPT

Abstract: The aim of the present research was to investigate the effect of fish oil, crude *Nigella sativa* oil and combined fish oil/*Nigella sativa* volatile oil as hepato-regenerative and renal protective supplements. The oils were administered as emulsions to rat model with liver injury induced by CCl₄. Plasma activities of transaminases (AST and ALT) were evaluated as liver function indicators, while plasma creatinine and urea and creatinine clearance were determined as markers of kidney function. Plasma malondialdehyde (MDA), nitrite (NO) and tumor necrosis factor- α (TNF- α) were estimated to assess the exposure to oxidative stress and subsequent inflammation. Liver fat was extracted and their fatty acids' methyl esters were determined using gas chromatography. Results showed that plasma activities of AST and ALT were significantly higher in CCl₄ control group compared to control healthy group. Plasma levels of creatinine and urea increased significantly in CCl₄ control, while creatinine clearance was reduced significantly in the same group. All rat treated groups given the three oil emulsions showed improvement in liver function pointing to the initiation of liver regeneration. The combination of fish oil/*Nigella sativa* volatiles showed the most promising regenerative activity. Oxidative stress and inflammation which were increased significantly in CCl₄ control group showed improvement on administration of the three different oil emulsions. Fatty acids methyl ester of liver fat revealed that rats treated with fish oil/*Nigella sativa* volatile oil presented the highest content of unsaturated fatty acids (45.52% $\pm$ 0.81) while fish oil showed the highest saturated fatty acids (53.28% $\pm$ 1.68). Conclusion; Oral administration of oil emulsions of native fish oil, *Nigella sativa* crude oil and combined fish oil/*Nigella sativa* volatile oil reduced liver and kidney injury in rat model of CCl₄ through exerting anti-inflammatory and antioxidant activity. Fish oil/*Nigella sativa* volatile oil emulsion was the most promising hepato-regenerative and reno-protective formula among the different groups.

Key words: liver regeneration, carbon tetrachloride, fish oil, *Nigella sativa* crude oil, *Nigella sativa* volatile oil, renal dysfunction

1 Introduction

The liver plays an important role in different vital processes including metabolism, detoxification and immunity. Liver damage could occur through exposure to chemicals, extreme fat deposition and/or viral infection which all lead ultimately to additional functional renal failure. The advancement of both liver and kidney dysfunction might be related to both high oxidative stress and inflammatory conditions¹. Fortunately, the liver has unmatched capacity for regeneration after exposure to hepatic damage factors^{2,3}. Regeneration is also a part of remodeling of the liver that

occurs in different other cases like cirrhosis, the replenishment of lost cells after hepatotoxic insults, and the transient hepatomegaly during pregnancy in response to the increased metabolic demand by the fetus⁴. The cells associated with liver regeneration are closely related to the degree of liver damage. Multiple genes and cytokines as well as the liver microenvironment play crucial roles in the dynamic regulation of regeneration via complex mechanisms that are not fully understood at present⁵.

Beside the inherent capacity of liver for self-regeneration, some bioactive plant constituents like silymarin and

* Correspondence to: Amr E. Edris, Aroma and Flavor Chemistry Department, National Research Centre, Dokki, Cairo, EGYPT
E-mail: amr_edris@hotmail.com

Accepted October 27, 2017 (received for review September 17, 2017)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/> <http://mc.manuscriptcentral.com/jjoc>

silibinin can stimulate the regeneration process⁶⁾. In addition dietary components like curcumine, vitamin K2 and vitamin E can also play a crucial role in enhancing liver regeneration⁷⁻⁹⁾. Omega-3-polyunsaturated fatty acids (ω -3 PUFA) have been shown to promote liver regeneration and functional recovery in living liver donors after resections for liver transplantation¹⁰⁾. They also showed effectiveness for treatment of patients after hepatectomy when administered in the form of lipid emulsion¹¹⁾.

Based on the advantages of ω -3 PUFA in the enhancement of liver regeneration, the authors of the current work planned to examine the possibility of further enhancement of liver regeneration ability of ω -3 PUFA by combination with a small amount of the volatile oil fraction of black cumin (*Nigella sativa*) seeds. This plant-derived volatile oil is co-extracted simultaneously with the crude oil of *N. sativa* during seeds' pressing. It was chosen due to its liver-related biological activity that includes protection against injury¹²⁾, reduction of formation of malondialdehyde, and enhancement of antioxidant enzyme activities which all lead to potential treatment of fibrosis¹³⁾. The volatile oil of *N. sativa* was also found to be responsible for the protecting activity of its parent bearing crude oil against fatty liver in rat model¹⁴⁾. The main components of the volatile oil fraction of *N. sativa* namely thymoquinone and p-cymene were deemed to be responsible for the anti-inflammatory, hepato and reno-protective effect of the whole crude oil of *N. sativa*^{14, 15)}.

For these reasons it is hypothesized that combination of *N. sativa* volatile oil with fish oil (as a rich source of ω -3 PUFA) could potentially elicit a recognized liver regeneration together with prevention of renal failure. Therefore, the current work was designed to evaluate the enhancement of liver regeneration and subsequent prevention of renal failure in rat model deliberately injured by carbon tetrachloride. The liver regenerating agents (oils) planned to be studied in the current work are the whole crude oil of *N. sativa*, fish oil and fish oil combined with the volatile oil fraction of *N. sativa* (at a percentage similar to its natural abundance in the whole crude oil of *N. sativa*).

2 Experimental

2.1 Chemicals, fish oil and plant material

CCl₄ was purchased from (Sigma-Aldrich St. Louis, MO USA). Fish oil was purchased from Natrol, Inc. (Chatsworth, CA 91311 USA). Mature dried seeds of black cumin (*Nigella sativa* L, family Ranunculaceae) were purchased from local herbal retail store specialized in medicinal and aromatic plants trading (Cairo, Egypt).

2.2 Animals

Male Sprague Dawley rats weighing 143–179 g (age: 6-8

weeks) were used in the present study. Animals were obtained from Animal house of National Research Centre, Cairo, Egypt. Animals were kept individually in metabolic stainless steel cages; water and food were given *ad-libitum*.

2.3 Preparation of carbon tetrachloride for dosing

A certain volume of carbon tetrachloride was added to equal volume of corn oil and the whole mixture was stirred till homogeneity.

2.4 Diet

A balanced diet composed of 12.5% casein, 10% corn oil, 23.3% sucrose, 46.7% maize starch, 3% fiber, 3.5% salt mixture and 1% vitamin mixture was prepared for feeding rats all over the experimental period according to the literature¹⁶⁾.

2.5 Extraction of *N. sativa* whole crude oil

The crude oil of *N. sativa* was extracted from the seeds according to the previous study¹⁷⁾. In detail, the seeds were exhaustively pressed for two successive cycles by using a commercial scale expeller screw pressing machine located at the same herbal retail store from which the seeds were purchased. The extracted crude oil of *N. sativa* was filtered and received in dark brown glass bottles equipped with screw caps and stored immediately under refrigeration conditions at -4°C until used.

2.6 Isolation of *N. sativa* volatile oil from its parent crude oil

Part of the whole crude oil of *N. sativa* which was obtained from the previous extraction step was subjected to a hydro-distillation process as described in the same previous study¹⁷⁾ to isolate its volatile oil fraction. In detail, 100 g of the crude oil (which is corresponding to ~ 500 g seeds) was mixed with distilled water (1:7 w/v, respectively) in a 5 liter round-bottom flask and distilled for three hours using the Clevenger-type apparatus. The cup that receives the pure volatile oil fraction in the side arm of the apparatus was wrapped with aluminum foil to protect the volatile oil from light. At the end of the distillation process the volatile oil was collected, dried over anhydrous sodium sulfate and its yield percent relative to the weight of the parent crude oil was assessed from three different extractions $\pm$ S.D. Finally the collected samples of the volatile oils were stored at -4°C until used.

2.7 Preparation of different sets of oil emulsions for rat dosing

Three different sets of oil-in-water emulsions were prepared having the same quantitative composition but differ only in the type of the oil phases. These oils included: 1- the whole crude oil *N. sativa*, 2- pure fish oil, 3- pure fish

oil combined with the isolated volatile oil fraction of *N. sativa* which was spiked at 2.6% of the fish oil's weight. That chosen percentage is typically similar to the natural abundance of that volatile oil fraction in the whole crude oil of *N. sativa* (as would be seen from the results section).

The three emulsions were formulated with 6% total oil phase, 1.2% surfactant (Tween 80) and 92.8% distilled water. First, the surfactant was dispersed in each oil phase separately using mechanical steering for 20 min. Then, each oil-surfactant mixture was added drop wise to the aqueous phase (distilled water) to form spontaneously a coarse milky white emulsion. The three different oil emulsions were further homogenized to reduce the particle size of the dispersed oil droplets by using high speed rotor-stator homogenizer (WiseTis HG-15D, Wise Laboratory instruments, Korea) at 3000 rpm for 3 min. The final emulsions were kept in refrigerator at 4°C and administered to rats at the next day of formulation. All oil emulsions were stored for only 10 days during the feeding experiment, after which the emulsions were discarded even if they did not manifest any signs of separation. Consequently fresh new batches of the three oil emulsions were re-formulated regularly every 10 days till the end of the experimental period. Beside the three oil emulsions, a vehicle composed of 1.2 wt% surfactant and 98.8wt% distilled water was also formulated without any kind of oil phase, for comparison.

2.8 Design of animal experiment

Thirty male rats were divided into five groups, each comprised of 6 rats. Group one served as normal control. Group two was that of control rats with induced liver injury. The other three groups (3-5) were the test groups. Hepatic injury was induced in rats of groups 2-5 according to literature¹⁸⁾, with some modifications, by intra-peritoneal injection of CCl₄/corn oil (at a dose of 2 ml CCl₄/kg rat body weight thrice during ten days). After induction of hepatic injury rats of groups 3-5 were treated by daily oral dose of the oil emulsions separately that provide 300 mg oil/kg rat body weight, for 20 days. Rats of the two control groups were given daily oral dose of the vehicle. After elapse of experimental period (a month); blood samples were withdrawn from eye vein orbital of anaesthetized fasted rats and received in heparinized tubes for separation of plasma. Twenty-four hours urine samples were collected during the last 24 h of the experiment for determination of creatinine¹⁹⁾ and for calculation of creatinine clearance. Rats were dissected and livers were immediately separated, weighed and stored at -20°C till being analyzed. Plasma activities of alanine transaminases and aspartate transaminase (ALT and AST) were estimated as indicator of liver function²⁰⁾. Plasma creatinine and urea were also determined^{19, 21)}, respectively to assess kidney function. Plasma tumor necrosis factor- α (TNF- α) was estimated²²⁾ as inflammatory marker. Plasma nitrite (NO) was assessed as both

oxidative stress and inflammatory biomarker²³⁾. Plasma malondialdehyde (MDA) as indicator of lipid peroxidation was determined²⁴⁾.

Animal procedure was performed in accordance with the Ethics Committee of the National Research Centre, Cairo, Egypt, and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.9 Extraction, preparation and gas chromatographic analysis of hepatic lipids

Total hepatic lipids were extracted according to published procedure²⁵⁾ which was modified by other authors²⁶⁾. Fatty acids methyl esters of hepatic lipids were prepared for GC analysis according to AOAC (2000)²⁷⁾. Assessment of the methyl ester was carried out by injecting 2 μ L into a Hewlett Packard HP-system 6890 gas chromatograph equipped with FID. HP-5 capillary column (30 m $\times$ 0.32 mm i.d.; 0.25 μ m film thickness) was used to separate the different methyl esters. The chromatographic analysis conditions were: initial temperature 70°C with a hold for 1 min, then raised to 120°C at a rate of 40°C /min with 2 min hold then the temperature was finally raised to 220°C at a rate of 4°C /min with another 20 min hold. The injector and detector temperatures were 250°C and 280°C respectively. Identification of the fatty acid methyl esters were carried out by direct comparison of retention times of each of the separated compounds with standards of the fatty acid methyl esters analyzed under the same conditions. Quantization was based on peak area integration.

2.10 Statistical analysis

The results of biological experiment were expressed as the mean $\pm$ SE and analyzed statistically using the one-way analysis of variance ANOVA followed by Duncan's test. In all cases $p < 0.05$ was used as the criterion of statistical significance.

3 Results

A whole crude oil was obtained from the seeds of *N. sativa* reaching ~21% of the seeds' weight after being subjected to a screw pressing process. This crude oil was found to contain a natural volatile oil fraction accounting for 2.6% of its weight, as revealed from the hydro-distillation process of that crude oil.

Table 1 showed that plasma AST and ALT activities were significantly higher in CCl₄ control group than normal control group, indicating liver dysfunction. Plasma levels of creatinine and urea as biomarkers of kidney function increased significantly together with significant reduction of creatinine clearance in CCl₄ control group compared to normal control. Oral administration of the three different

Table 1 Biochemical parameters of different experimental groups (mean $\pm$ SE).

	Normal control	CCl ₄ control	<i>N. sativa</i> crude oil emulsion	Fish oil emulsion	Fish oil / volatile oil emulsion
Plasma ALT (U/L)	9.83 ^a $\pm$ 0.75	21.00 ^b $\pm$ 1.46	8.67 ^a $\pm$ 0.71	10.66 ^a $\pm$ 1.63	8.33 ^a $\pm$ 0.42
Plasma AST (U/L)	33.33 ^a $\pm$ 1.38	83.67 ^c $\pm$ 2.93	50.16 ^b $\pm$ 1.25	48.17 ^b $\pm$ 2.95	48.00 ^b $\pm$ 1.91
Plasma urea (mg/dL)	25.74 ^a $\pm$ 1.10	34.56 ^c $\pm$ 0.92	25.40 ^a $\pm$ 1.10	29.07 ^b $\pm$ 0.57	28.55 ^b $\pm$ 0.87
Plasma creatinine (mg/dL)	0.69 ^a $\pm$ 0.02	1.32 ^d $\pm$ 0.03	1.00 ^c $\pm$ 0.07	0.96 ^{bc} $\pm$ 0.08	0.81 ^b $\pm$ 0.06
Plasma TNF- α (pg/mL)	18.17 ^a $\pm$ 1.25	49.33 ^d $\pm$ 2.27	34.00 ^{bc} $\pm$ 1.81	38.83 ^c $\pm$ 2.09	32.00 ^b $\pm$ 1.81
Plasma NO (μ mol/L)	20.65 ^a $\pm$ 0.71	35.13 ^b $\pm$ 1.71	19.25 ^a $\pm$ 0.90	22.09 ^a $\pm$ 1.44	21.04 ^a $\pm$ 1.22
Plasma MDA (nmol/mL)	8.26 ^a $\pm$ 0.58	17.28 ^b $\pm$ 0.44	8.72 ^a $\pm$ 0.84	8.89 ^a $\pm$ 0.87	9.22 ^a $\pm$ 0.62
Creatinine clearance	1.00 ^c $\pm$ 0.05	0.53 ^a $\pm$ 0.02	0.72 ^b $\pm$ 0.05	0.77 ^b $\pm$ 0.09	0.90 ^{bc} $\pm$ 0.08

In each column the same letters mean non-significant difference; and different letters mean significant difference at 0.05 probabilities.

Table 2 Fatty acids content of liver fat of different experimental groups as percentage of total fatty acids (mean $\pm$ SE).

	Normal control	CCl ₄ control	<i>N. sativa</i> crude oil emulsion	Fish oil emulsion	Fish oil / volatile oil emulsion
Palmitic (C16:0)	22.93 ^a $\pm$ 0.58	22.44 ^a $\pm$ 0.26	21.09 ^a $\pm$ 0.30	23.41 ^a $\pm$ 0.00	23.93 ^a $\pm$ 2.55
Stearic (C18:0)	14.64 ^d $\pm$ 0.84	11.67 ^{bc} $\pm$ 0.86	12.79 ^{cd} $\pm$ 0.42	10.05 ^{ab} $\pm$ 0.03	7.68 ^a $\pm$ 0.65
Oleic (C18:1)	15.34 ^a $\pm$ 0.92	19.16 ^b $\pm$ 0.29	18.35 ^b $\pm$ 0.55	23.80 ^c $\pm$ 0.27	24.07 ^c $\pm$ 0.71
Linoleic (C18:2)	20.28 ^c $\pm$ 0.11	19.44 ^c $\pm$ 0.10	21.16 ^d $\pm$ 0.25	18.57 ^a $\pm$ 0.10	21.45 ^d $\pm$ 0.11
Behenic (C22:0)	21.07 ^a $\pm$ 0.49	18.23 ^a $\pm$ 0.21	11.92 ^a $\pm$ 5.12	16.38 ^a $\pm$ 0.99	16.63 ^a $\pm$ 4.12
Lignoceric (C24:0)	1.18 ^{ab} $\pm$ 0.32	1.62 ^b $\pm$ 0.01	—	3.45 ^c $\pm$ 0.71	0.68 ^{ab} $\pm$ 0.32
Total saturated fatty acids	58.42 ^b $\pm$ 1.05	53.95 ^{ab} $\pm$ 0.92	45.79 ^a $\pm$ 5.84	53.28 ^{ab} $\pm$ 1.68	48.92 ^a $\pm$ 0.61
Total unsaturated fatty acids	35.58 ^a $\pm$ 0.81	38.60 ^b $\pm$ 0.18	39.51 ^b $\pm$ 0.80	42.37 ^c $\pm$ 0.38	45.52 ^d $\pm$ 0.81

In each column same letters mean non-significant difference; different letters mean significant difference at 0.05 probabilities.

sets of oil emulsions used in the present study showed significant improvement in liver and kidney function with different degrees, pointing to liver regeneration. It was also inferred from **Table 1** that the deliberate combination of fish oil/*N. sativa* volatile oil was the most effective treatment for improving AST and ALT. In addition, that oil combination was the most promising in improving creatinine and creatinine clearance.

Plasma levels of MDA and TNF- α were significantly high in CCl₄ control group compared with normal control referring to elevated oxidative stress and inflammation. Also plasma nitrite level was significantly elevated in CCl₄ control compared to normal control. Plasma levels of TNF- α as inflammatory biomarker reduced significantly in all oil emulsions treated groups with the lowest level (32.0 $\pm$ 1.81 pg/mL) observed for rats given the combination of fish oil/volatile oil. On the other hand, oral administration of *N. sativa* whole crude oil emulsion (which is naturally containing 2.6 wt% of the volatile oil) was the most promising in improving MDA and NO levels.

All treatments were able to normalize plasma ALT activity, NO and MDA. Plasma urea matched the control level

only on administration of *N. sativa* crude oil. Creatinine clearance showed insignificant change from control normal rat only on treatment with fish oil/ volatile oil combination.

Fatty acids' content of liver fat (**Table 2**) revealed no distinct variation between control normal rats and CCl₄ control group concerning saturated fatty acids; palmitic, lignoceric and behenic and the unsaturated linoleic acid. On the other hand, oleic acid was significantly higher in CCl₄ control group than control normal rats. Stearic was significantly high in control normal rats compared to CCl₄ control group. Total unsaturated fatty acids were significantly high in CCl₄ control group compared to normal group. Upon administration of the three oil emulsions, rats given oral administration of *N. sativa* crude oil emulsion showed the lowest content of liver saturated fatty acids (45.79%) followed by rats given oral administration of fish oil/volatile oil combination (48.92%), however no significant change was present among test groups and CCl₄ control. Regarding the effect of treatments on unsaturated fatty acids it was found that rats treated with fish oil/ volatile oil combination presented the highest content of unsaturated fatty acids (45.52%) followed by rats adminis-

Table 3 Nutritional parameters and liver/body weight % of different experimental groups (mean $\pm$ SE).

	Normal control	CCl ₄ control	<i>N. sativa</i> crude oil emulsion	Fish oil emulsion	Fish oil / volatile oil emulsion
Initial body weight (g)	159.00 ^a $\pm$ 5.23	158.83 ^a $\pm$ 3.75	158.50 ^a $\pm$ 3.05	158.67 ^a $\pm$ 3.72	158.67 ^a $\pm$ 4.62
Final body weight (g)	220.50 ^a $\pm$ 5.14	228.33 ^a $\pm$ 4.45	219.17 ^a $\pm$ 4.08	218.00 ^a $\pm$ 4.19	220.50 ^a $\pm$ 4.37
Body weight gain (g)	61.50 ^a $\pm$ 2.14	69.50 ^b $\pm$ 1.87	60.67 ^a $\pm$ 1.99	59.33 ^a $\pm$ 2.23	61.83 ^a $\pm$ 1.33
Total food intake (g)	529.83 ^b $\pm$ 5.29	492.00 ^a $\pm$ 8.08	512.17 ^{ab} $\pm$ 4.93	505.00 ^{ab} $\pm$ 13.35	523.00 ^b $\pm$ 4.16
Feed efficiency ratio	0.116 ^a $\pm$ 0.003	0.141 ^b $\pm$ 0.003	0.118 ^a $\pm$ 0.003	0.117 ^a $\pm$ 0.002	0.118 ^a $\pm$ 0.002
Liver weight (g)	7.31 ^a $\pm$ 0.21	7.99 ^a $\pm$ 0.16	7.34 ^a $\pm$ 0.10	7.67 ^a $\pm$ 0.34	7.63 ^a $\pm$ 0.13
Liver/body weight %	3.33 ^a $\pm$ 0.14	3.51 ^a $\pm$ 0.12	3.35 ^a $\pm$ 0.05	3.54 ^a $\pm$ 0.22	3.47 ^a $\pm$ 0.08

In each row same letters mean non-significant difference; different letters mean significant difference at 0.05 probabilities.

tered fish oil (42.37%). Oleic acid was the major unsaturated fatty acid in rats treated by fish oil /volatile oil combination (24.07%) and also fish oil emulsion (23.8%). Conversely, linoleic acid was the major unsaturated fatty acid in normal control group and those treated with *N. sativa* crude oil. Palmitic acid was the major saturated fatty acid in all the experimental groups.

The nutritional parameters and liver/body weight percentage of different experimental groups are shown in Table 3. Final body weight and liver/body weight percentage of the different groups showed non-significant changes. Body weight gain and feed efficiency ratio of CCl₄ control group were significantly high compared to different groups. Total food intake of CCl₄ control group was significantly low compared with normal control group and that treated by fish oil/ volatile oil emulsion. The value of total food intake of the CCl₄ was only below that of the groups given either fish oil or *Nigella* crude oil without significant difference.

4 Discussion

Hepatic diseases are the most common of all the pathologies worldwide and constitute up to 83% of all the cases. Among the causative factors of hepatic toxicity are food additives, alcohol, toxic industrial chemicals and air and water pollutant²⁸. The liver is a unique organ with ability to regenerate and recover its original function after extended resection or injury. This occurs due to the hyperplasia of the residual lobes and mitosis of the hepatocytes which are quiescent under normal conditions^{29, 30}. Some studies have speculated that slight genesis and/or formation of low level of free radicals are important for stimulating liver regeneration and necessary for their natural course³¹. These speculations seem to be only coincided on low concentration of reactive oxygen species because logically elevated oxidative stress could contribute to liver damage. It is mandatory that the continual hepatic exposure to oxidative stress and inflammation could hinder the

intrinsic liver regeneration and require external intervention with antioxidant and anti-inflammatory agent. In order to fulfill that requirement, the current study evaluated the liver regeneration potentials of some natural oils like fish oil, *N. sativa* whole crude seeds oil and fish oil / *Nigella* volatile oil combination.

Based on the above mentioned, the current study was initiated by inducing hepatic injury in rats using CCl₄ which is one of the most potent inducers of acute liver injury³². It is also used in experimental model to investigate the hepato-protective role of natural products and drugs³³. Liver injury was observed in the present study through significant elevation of ALT and AST as previously reported³⁴.

CCl₄ induced toxicity depends on the dose and duration of exposure. At low CCl₄ doses, transient effects occur, including the loss of Ca²⁺ sequestration, impaired lipid homeostasis and release of several cytokines. Longer CCl₄ exposure alters fatty acid metabolism, and induces fibrosis, cirrhosis and cancer³⁵. One distinctive feature of CCl₄ toxicity is rapid triglycerides accumulation in the liver, similar to observation in liver steatosis³⁶.

In CCl₄ treated control rats plasma levels of MDA, NO and TNF- α were significantly elevated pointing to the incidence of lipid peroxidation, oxidative stress and inflammation. All these changes are expected consequences of treatment with CCl₄ which induces elevation of reactive oxygen species (ROS) and MDA that lead to destruction and disruption of lipids of the membranous system, cellular proteins, intracellular organelles and causes DNA damage inducing several pathological changes^{34, 37}. CCl₄ is transformed in the liver to trichloromethyl free radical that disrupts Ca²⁺ homeostasis and induces lipid peroxidation that leads to hepatocellular injury³⁸.

In the present study significantly elevated plasma levels of creatinine and urea together with reduction of creatinine clearance indicated kidney dysfunction, in CCl₄ control. The results are in agreement with other studies³⁹ which reported that CCl₄ induced nephrotoxicity in rats through elevation in creatinine and blood urea nitrogen. This nephrotoxicity might be due to direct action of CCl₄ or could be

attributed to either elevated ROS and TNF- α or a consequence of severe liver injury.

Administration of different oil emulsions in the present study produced reduction in oxidative stress and inflammation reflected in inhibiting MDA, NO and TNF- α level in CCl₄ treated rats. This effect leads to improvement in both liver and kidney functions as noticed from the reduction in plasma ALT and AST activities, creatinine and urea with the simultaneous increase in creatinine clearance.

The hepato-renal improvement in rats treated with the whole crude oil of *N. sativa* emulsion might originates from its natural content of the volatile oil fraction¹²⁻¹⁴, which reached 2.6% of its weight in the current study. This volatile oil contains a major component namely thymoquinone (TQ) in addition to *p*-cymene which are considered to be responsible for the protective effect of that volatile oil fraction and its parent crude oil that bears that volatile fraction^{13, 15}. The content of TQ and *p*-cymene in the current investigation reached 68.13% and 20.11%, respectively of the total volatile oil composition, as determined by full GC analysis that was illustrated in our recent study¹⁷. That previous study was conducted concomitantly with the current study by using the very same seeds of *N. sativa* and its whole crude oil under the same extraction and storage conditions. Therefore it can be comfortably indicated that the chemical composition of the volatile oil and its content of TQ and *p*-cymene in the current study is exactly the same as that illustrated before¹⁷. One should also bear in mind that the other non-volatile components of the whole crude oil of *N. sativa* like the vitamin E, phytosterols and linolenic acid can also participate beside the volatile oil fraction in the hepato-renal improvement effect of that crude oil due to their anti-inflammatory and/or antioxidant activities¹⁴. It is also worth indicating that the whole crude oil of *N. sativa* previously showed hepato and reno-protective effect in rat model treated with galactosamine hydrochloride and fed on high fructose diet¹⁵.

The hepato-renal ameliorating effect of fish oil seen in the present study can be justified based on its content of the anti-inflammatory ω -3 fatty acids like eicosa-pentaenoic (EPA) and docosahexaenoic acid (DHA). Previous reports indicated that DHA has protective effect towards glomerulonephritis⁴⁰ and can improve liver MDA in CCl₄ induced liver dysfunction⁴¹. Despite the high unsaturation of fish oil fatty acids however improvement in MDA and NO was noticed in the present study which agreed with other studies⁴². In addition, ω -3 fatty acids were demonstrated to have remedial effect in experimental lupus nephritis⁴³. The anti-inflammatory activity of ω -3 fatty acids is related to amelioration of cytokine^{44, 45}. These fatty acids were also reported to reduce oxidative damage in various tissue^{46, 47} which agreed with the current study. However other authors demonstrated elevated thiobarbituric acid reactive substance in liver of mice treated by fish oil⁴⁸. It is worth

noting that fatty acids composition of fish oil was not determined in the present study. However in our previous work¹⁵, the fatty acids of this oil was assessed by GC-FID and GC-MS and the main fatty acids were EPA (20.15%), DHA (13.77%), palmitic (13.44%), oleic (10.85%) and other minor fatty acids including linoleic (1.57%).

The evaluation of the liver regeneration ability of fish oil/*N. sativa* volatile oil emulsion combination is considered to be the main target of the current study and the first report in this field. The data in the present study indicated that this oil combination was almost the best among the other sets of oils in its liver ameliorative effect which is an indication of regeneration. The mechanism by which this oils' combination exert its action is axiomatically inferred from the additive regenerative activity of fish oil as a source of ω -3 fatty acids along with *N. sativa* volatile oil as a rich source of thymoquinone. Although there were insignificant difference between the inflammation, oxidative stress, liver and kidney function biomarkers of rats treated by the whole crude oil of *N. sativa*, fish oil and fish oil/*N. sativa* volatile oil combination but the values of the latter indicates almost the highest regenerative effect.

The liver is a major metabolic organ involved in fatty acid metabolism. Lipid homeostasis and fatty acids metabolism are strongly affected by exposure to CCl₄³⁵. A decrease in saturated fatty acids and an increase in unsaturated fatty acids were noticed after treatment with CCl₄³⁴ which agreed with the data of the current study. In the present work; CCl₄ treatment induced significant increase in oleic without affecting linoleic with significant reduction in stearic acid. CCl₄ treatment provoked fatty metamorphosis in liver⁴⁹. It was also reported that oleic acid and linoleic acid were increased in liver tissue after partial hepatectomy⁵⁰. These authors suggested that some significant signals are transmitted during the regeneration process owing to alterations in the membrane structure by the high levels of fatty acid after partial hepatectomy.

In the present study; the three different oils treatment produced significant increase in both oleic and linoleic acid (except for linoleic acid in case of fish oil) compared to normal rats reflecting the possible increase in regeneration process as previously reported⁵⁰. The fatty acid profile in liver was rarely studied during intake of ω -3 or ω -6 rich oils or even after treatment with CCl₄; the scarce studies found could not explain the mechanism by which alteration in hepatic fatty acids profile occur. Fish oil was reported to produce hepatic increase in genes expression related to beta-oxidation of fatty acids and reduce expression of those induce lipogenesis⁵¹. The body is dependent on the exogenous supply of omega-6 (present in *Nigella sativa* crude oil) and omega-3 (present in fish oil). These essential fatty acids are key players in regulating metabolic signaling. Omega-3 fatty acids exert anti-inflammatory properties in addition to its beneficial effect on hepatic steatosis, re-

generation and inflammatory insults⁵²⁾. It was revealed that intake of omega-3 fatty acids by normal mice produced enrichment of ω -3 in liver and plasma phosphatidylcholine, lysophosphatidylcholine, cholesteryl ester and free fatty acids which are important bioactive lipids⁵³⁾. In the present results no ω -3 fatty acid was seen among the analyzed hepatic fatty acids during treatment with ω -3 fatty acids represented by either fish oil or fish oil/*N. sativa* volatile. This could be due to the complex metabolic effect of fish oil including the increased fatty acids beta-oxidation and the reduction in hepatic lipogenesis and liver fats along with increased export of lipids from liver that could alter the expected hepatic fatty acids profile. Fatty acids composition could be influenced by the rate of lipid peroxidation and the activity of delta-6 desaturase enzyme. PUFAs up regulate peroxisomal proliferator-activated receptors that lead to increased genes transcription that are responsible for fatty acids degradation. These fatty acids also down regulate steroid-responsive element binding protein that increases transcription of genes responsible for fatty acids synthesis^{54, 55)}. Linoleic acid (ω -6) is the major fatty acid in *Nigella sativa* seed oil accounting for 54.0–70.0%⁵⁶⁾. This fatty acid showed significant increase in the liver of rats treated by *N. sativa* oil compared to normal control group, CCl₄ group and the rats treated with fish oil which could be a reflection of its intake. However the non significant difference of liver linoleic acid between the rats given *N. sativa* oil and those treated by fish oil/ volatile is hardly to be explained. Full identification of the fatty acid composition of the crude oil of *N. sativa* was determined in our previous article¹⁵⁾ where the major fatty acids were linoleic (60.4%), oleic (21.9%), palmitic (9.5%), stearic (3.3%) and other minor fatty acids.

In the present study; the body weight gain of CCl₄ control group increased significantly compared with different groups also final body weight increased non-significantly. This result agreed with that of previous study⁵⁷⁾. The apparent increase in body weight gain is actually due to ascitis that results from drainage of fluid in the abdominal region that was noticed during rats' dissection in addition to the slight hepatomegaly but not to an increase in the lean or fat compartment of the body. The significant reduction of total food intake of CCl₄ control group supports this explanation. Oral administration of different oil emulsions attenuated elevation in body weight to reach normal level which reflects an improvement in the damaged liver and subsequent reduction of ascitis. Also, ω -3 fatty acids were reported previously to reduce body weight through decrease hepatic and total body fats⁵⁸⁾. Crude oil of *N. sativa* produced significant reduction in rat body weight in a previous study¹⁵⁾ which supports the present results.

Conclusion

Oral administration of *N. sativa* crude oil, fish oil and/or fish oil supplemented with *N. sativa* volatile oil in emulsion form could reduce liver injury and enhance liver regeneration with concomitant reno-protection in rat model of CCl₄. These activities were accomplished through the anti-inflammatory and antioxidant effects which were excreted by these natural oils. Fish oil/*N. sativa* volatile oil combination was superior to the other oils. This work can potentially be a new achievement in the dietary supplements filed that can help in protection and/or amelioration of liver dysfunction.

References

- 1) Al-Okbi, S.; Mohamed, D.; Hamed, Th.; Hussein, A.; Fouda, K. Evaluation of functional foods for prevention of hepatorenal syndrome in rat model. *Res. J. Pharm. Biol. Chem. Sci.* **8**, 1202-1211 (2017).
- 2) van de Laarschot, F.; Jansen, L.; Schaap, G.; Olde, W. The role of bile salts in liver regeneration. *Hepatol. Int.* **10**, 733-740 (2016).
- 3) Itoh, N.; Nakayama, Y.; Konishi, M. Roles of FGFs as paracrine or endocrine signals in liver development, health, and disease. *Front. Cell Dev. Biol.* **4**, 1-9 (2016).
- 4) Milona, A.; Owen, B.; Mil, S.; Dormann, D. Matak, C.; Boudjelal, M.; Cairns, W.; Schoonjans, K.; Milligan, S.; Parker, M.; White, R.; Williamson, C. The normal mechanisms of pregnancy-induced liver growth are not maintained in mice lacking the bile acid sensor Fxr. *Am. J. Physiol. Gastrointest. Liver Physiol.* **298**, 151-158 (2010).
- 5) Tan, Q.; Hu, J.; Yu, X.; Guan, W.; Lu, H.; Yu, Y.; Yu, Y.; Zang, G.; Tang, Z. The role of IL-1 family members and Kupffer Cells in liver regeneration. *Biomed. Res. Int.* **2016**, 1-5 (2016).
- 6) Wu, J.; Tsai, C.; Yeh, Y.; Lin, Y.; Lin, C.; Day, C.; Shen, C.; Padma, V.; Pan, L.; Huang, C. Silymarin accelerates liver regeneration after partial hepatectomy. *Evid. Based Complement. Alternat. Med.* **2015**, 1-14 (2015).
- 7) Toydemir, T.; Kanter, M.; Erboğa, M.; Oguz, S.; Erenoglu, C. Antioxidative, antiapoptotic, and proliferative effect of curcumin on liver regeneration after partial hepatectomy in rats. *Toxicol. Ind. Health* **31**, 162-72 (2015).
- 8) Lin, M.; Sun, P.; Zhang, G.; Xu, X.; Liu, G.; Miao, H.; Yang, Y.; Xu, H.; Zhang, L.; Wu, P.; Li, M. Vitamin K2-enhanced liver regeneration is associated with oval cell expansion and up-regulation of matrilin-2 expression in 2-AAF/PH rat model. *Currnt. Mol. Med.* **14**, 361-369 (2014).

- 9) Gavino, C.; Dillard, J.; Tappel, L. Effect of dietary vitamin E and Santoquin on regenerating rat liver. *Life Sci.* **36**, 1771-1777 (1985).
- 10) Ibrahim, E.; Saleh, S.; hoseeny, M.; shaarawy, A. Effect of omega-3 on hepatic regeneration in adult living donors undergoing hepatic resections for liver transplantation: A randomized controlled trial. *J. Critical Care* **31**, 157-162 (2016).
- 11) Gong, Y.; Liu Z.; Liao, Y.; Mai, C.; Chen, T.; Tang, H. Effectiveness of ω -3 polyunsaturated fatty acids based lipid emulsions for treatment of patients after peptectomy: A prospective clinical trial. *Nutrients* **8**, 357-364 (2016).
- 12) Mollazadeh, H.; Hosseinzadeh, H. The protective effect of *Nigella sativa* against liver injury: a Review. *Iran J. Basic Med. Sci.* **17**, 958-966 (2014).
- 13) Saricicek, E.; Tarakcioglu, M.; Saricicek, V.; Gulsen, M.; Karakoke, M.; Baltaci, Y.; Taysi, S. Effect of *Nigella sativa* on experimental liver fibrosis. *Biomed. Res.* **25**, 32-38 (2014).
- 14) Al-Okbi, Y.; Mohamed, D.; Hamed, Th.; Edris, A. Potential protective effect of *Nigella sativa* crude oils towards fatty liver in rats. *Eur. J. Lipid Sci. Technol.* **115**, 774-782 (2013).
- 15) Al-Okbi, Y.; Mohamed, D.; Hamed, Th.; Edris, A. Evaluation of the therapeutic effect of *Nigella sativa* crude oil and its blend with omega-3 fatty acids-rich oils in a modified hepatorenal syndrome model in rats. *Grasas y Aceites* **66**, e103-e114 (2015).
- 16) Mohamed, A.; Hamed, E.; Al-Okbi, Y. Reduction of hypercholesterolemia and risk of cardiovascular diseases by plant foods extracts mixtures: A study on plasma lipid profile, oxidative stress and testosterone in rats. *Grasas y Aceites* **61**, 378-389 (2010).
- 17) Hamed, S.; Shaaban, H.; Ramadan, A.; Edris, A. Potentials of enhancing the physicochemical and functional characteristics of *Nigella sativa* oil by using the screw pressing technique for extraction. *Grasas y Aceites* **68**, e188-e196 (2017).
- 18) Jayasekhar, P.; Mohanan, V.; Rathinam, K. Hepatoprotective activity of ethyl acetate extract of *Acacia catechu*. *Ind. J. Pharma.* **29**, 426-428 (1997).
- 19) Houot, O. in *Interpretation of Clinical Laboratory Tests* (Siest, G.; Henny, J.; Schiele, F.; Young, D. eds.), Biomedical Publications, p. 250 (1985).
- 20) Reitman, S.; Frankel, S. Colorimetric methods for aspartate and alanine aminotransferase. *Am. J. Clin. Pathol.* **28**, 55-60 (1957).
- 21) Fawcett, K.; Scott, E. A rapid and precise method for the determination of urea. *J. Clin. Pathol.* **13**, 156-159 (1961).
- 22) Stepaniak, A.; Gould, E.; Sun, D.; Swanborg, H. A comparative study of experimental autoimmune encephalomyelitis in Lewis and DA rats. *J. Immunol.* **155**, 2762-2769 (1995).
- 23) Montgomery A.; Dymock, F. The determination of nitrite in water. *Analyst* **86**, 414-416 (1961).
- 24) Satoh, K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clin. Chim. Acta* **90**, 37-43 (1978).
- 25) Folch, J.; Lees, M.; Sloane Stanley, H. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509 (1957).
- 26) Cequier-Sánchez, E.; Rodríguez, C.; Ravelo, G.; Zárata, R. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. *J. Agric. Food Chem.* **56**, 4297-4303 (2008).
- 27) AOAC. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists International, Arlington, Virginia, USA (2000).
- 28) Younis, T.; Khan, R.; Sajid, M. Protective effects of *Fraxinus xanthoxyloides* (Wall.) leaves against CCl₄ induced hepatic toxicity in rat. *BMC Complement. Altern. Med.* **16**, 407-418 (2016).
- 29) Michalopoulos, K.; DeFrances, C. Liver regeneration. *Science* **276**, 60-66 (1997).
- 30) Schibler, U. Circadian rhythms. Liver regeneration clocks on. *Science* **302**, 234-235 (2003).
- 31) Kurir, T.; Markotić, A.; Katalinić, V.; Bozanić, D.; Cikes, V.; Zemunik, T. Effect of hyperbaric oxygenation on the regeneration of the liver after partial hepatectomy in rats. *Braz. J. Med. Biol. Res.* **37**, 1231-1237 (2004).
- 32) Chen, M.; Huang, W.; Wang, C.; Nie, H.; Li, G.; Sun, T.; Yang, F.; Zhang, Y.; Shu, K.; Wang, C.; Gong, Q. High-mobility group box 1 exacerbates CCl₄-induced acute liver injury in mice. *Clin. Immunol.* **153**, 56-63 (2014).
- 33) Yang, J.; Li, Y.; Wang, F.; Wu, C. Hepatoprotective effects of apple polyphenols on CCl₄-induced acute liver damage in mice. *J. Agric. Food Chem.* **58**, 6525-6531 (2010).
- 34) Torres-Durán, V.; Paredes-Carbajal, C.; Mascher, D.; Zamora-González, J.; Díaz-Zagoya, C.; Juárez-Oropeza, A. Protective effect of *Arthrospira maxima* on fatty acid composition in fatty liver. *Arch. Med. Res.* **37**, 479-83 (2006).
- 35) Cui, Y.; Yang, X.; Lu, X.; Chen, J.; Zhao, Y. Protective effects of polyphenols-enriched extract from Huangshan Maofeng green tea against CCl₄-induced liver injury in mice. *Chem. Biol. Interact.* **220**, 75-83 (2014).
- 36) Lee, H.; Bhandary, B.; Lee, M.; Park, K.; Jeong, S.; Kim, K.; Kim, R.; Chae, J. The roles of ER stress and P450 2E1 in CCl₄-induced steatosis. *Int. J. Biochem. Cell Biol.* **43**, 1469-1482 (2011).
- 37) Shah, A.; Khan, R.; Naz, K.; Khan, A. Antioxidant potential, DNA protection, and HPLC-DAD analysis of

- neglected medicinal Jurineadolomiaea roots. *Biomed. Res. Inter.* **2014**, 1-10 (2014).
- 38) Hu, L.; Li, L.; Xu, D.; Xia, X.; Pi, R.; Xu, D.; Wang, W.; Du, H.; Song, E.; Song, Y. Protective effects of neohesperidin dihydrochalcone against carbon tetrachloride-induced oxidative damage *in vivo* and *in vitro*. *Chem. Biol. Interact.* **213**, 51-59 (2014).
- 39) Yoshioka, H.; Usuda, H.; Fukuishi, N.; Nonogaki, T.; Onosaka, S. Carbon Tetrachloride-induced nephrotoxicity in mice is prevented by pretreatment with zinc sulfate. *Biol. Pharm. Bull.* **39**, 1042-1046 (2016).
- 40) Zararsiz, I.; Sonmez, F.; Yilmaz, R.; Tas, U.; Kus, I.; Kavakli, A.; Sarsilmaz, M. Effects of omega-3 essential fatty acids against formaldehyde-induced nephropathy in rats. *Toxicol. Ind. Health* **22**, 223-229 (2006).
- 41) Saygili, O.; Paksoy, S.; Hismiogullari, S.; Kus, M.; Sunay, F.; Gulcen, B.; Ozcan, E. Prevention of carbontetrachloride-induced liver injury in rats by omega-3-fatty acids. *Acta Med. Mediterr.* **33**, 137-144 (2017).
- 42) Higdon, V.; Liu, J.; Du, H.; Morrow, D.; Ames, N.; Wander, C. Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater *in vivo* lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F2-isoprostanes 1-3. *Am. J. Clin. Nutr.* **72**, 714-722 (2000).
- 43) Clark, F.; Parbtani, A. Omega-3 fatty acid supplementation in clinical and experimental lupus nephritis. *Am. J. Kidney Dis.* **23**, 644-647 (1994).
- 44) Chen, Y.; Lin, Y.; Pan, Y.; Liao, L.; Chuang, H.; Yen, J.; Lin, Y.; Chen, J. Beneficial effect of docosahexaenoic acid on cholestatic liver injury in rats. *J. Nutr. Biochem.* **23**, 252-264 (2012).
- 45) Yilmaz, R.; Songur, A.; Ozyurt, B.; Zararsiz, I.; Sarsilmaz, M. The effects of n-3 polyunsaturated fatty acids by gavage on some metabolic enzymes of rat liver. *Prostaglan. Leuk. Essent. Fatty Acids* **71**, 131-135 (2004).
- 46) Zararsiz, I.; Meydan, S.; Sarsilmaz, M.; Songur, A.; Ozen, A.; Sogut, S. Protective effects of omega-3 essential fatty acids against formaldehyde-induced cerebellar damage in rats. *Toxicol. Ind. Health* **27**, 489-495 (2011).
- 47) Meydan, S.; Altas, M.; Nacar, A.; Ozturk, H.; Tas, U.; Zararsiz, I.; Sarsilmaz, M. The protective effects of omega-3 fatty acid against toluene-induced neurotoxicity in prefrontal cortex of rats. *Hum. Exp. Toxicol.* **31**, 1179-1185 (2012).
- 48) Yasuda, S.; Watanabe, S.; Kobayashi, T.; Okuyama, H. Effects of dietary unsaturated fatty acid and chronic carbon tetrachloride treatment on the accumulation of oxidation products, alpha-tocopherol and liver injury in mice. *Biol. Pharm. Bull.* **21**, 1050-1056 (1998).
- 49) Lertprasertsuke, N.; Shinoda, M.; Takekoshi, S.; Tsutsumi, Y.; Yamamoto, Y.; Niki, E.; Watanabe, K. Different effects of carbon tetrachloride on carcinogen-induced hepatocellular carcinoma and normal liver of the rat: lowered lipid peroxidation and accelerated necrosis in cancer. *Jpn. J. Cancer Res.* **82**, 503-510 (1991).
- 50) Kishino, T.; Tanno, M.; Yamada, H.; Saito, S.; Matsumoto, S. Changes in liver fatty acid unsaturation after partial hepatectomy in the rat. *Lipids* **35**, 445-452 (2000).
- 51) de Castro, G.; Deminice, R.; Cordeiro, Simões-Ambrosio, L.; Calder, P.; Jordão, A.; Vannucchi, H. Dietary docosahexaenoic acid and eicosapentaenoic acid influence liver triacylglycerol and insulin resistance in rats fed a high-fructose diet. *Mar. Drugs* **13**, 1864-1881 (2015).
- 52) Linecker, M.; Limani, P.; Botea, F.; Popescu, I.; Alikhanov, R.; Efanov, M.; Kim, P.; Khatkov, I.; Raptis, A.; Tschuor, C.; Beck-Schimmer, B.; Bonvini, J.; Wirsching, A.; Kron, P.; Slankamenac, K.; Humar, B.; Graf, R.; Petrowsky, H.; Clavien, A. A randomized, double-blind study of the effects of omega-3 fatty acids (Omegaven) on outcome after major liver resection. *BMC Gastroenterol.* **15**, 102-107 (2015).
- 53) Balogun, K.; Carolyn, J. Albert, David A. Ford, Robert J. Brown, Sukhinder K. Cheema. Dietary omega-3 polyunsaturated fatty acids alter the fatty acid composition of hepatic and plasma bioactive lipids in C57BL/6 mice: A lipidomic approach. *PLOS ONE* **8**, e82399 (2013).
- 54) Zhou, T.; Shimabukuro, M.; Wang, Y.; Lee, Y.; Higa, M.; Milburn, L.; Newgard, B.; Unger, H. Role of peroxisome proliferator-activated receptor alpha in disease of pancreatic beta cells. *Proc. Natl. Acad. Sci. USA* **95**, 8898-8903 (1998).
- 55) Osborne, F. Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *J. Biol. Chem.* **275**, 32379-32382 (2000).
- 56) Tulukcu, F. A comparative study on fatty acid composition of black cumin obtained from different regions of Turkey, Iran and Syria. *Afric. J. Agric. Res.* **6**, 892-895 (2011).
- 57) Lee, Y.; Kim, W.; Lee, H.; Choi, K.; Jung, W.; Kim, J.; Kwon, J.; Chae, J. Turmeric extract and its active compound, curcumin, protect against chronic CCl₄-induced liver damage by enhancing antioxidation. *BMC Complement. Altern. Med.* **16**, 316-324 (2016).
- 58) Levy, R.; Clore, N.; Stevens, W. Dietary n-3 polyunsaturated fatty acids decrease hepatic triglycerides in Fischer 344 Rats. *Hepatol.* **39**, 608-616 (2004).