Evaluation of the therapeutic effect of *Nigella sativa* crude oil and its blend with omega-3 fatty acid-rich oils in a modified hepatorenal syndrome model in rats

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SUMMARY: In the present study, the hepato and reno-protective effect of *Nigella sativa* crude oil and its binary blend with omega-3 fatty acid-rich oils (fish and flaxseed oils) was studied in a modified hepatorenal syndrome model (MHRS) in rats. MHRS was induced through feeding a high fructose diet followed by an intraperitoneal injection of galactosamine hydrochloride. *Nigella* oil and its different blends were given as a daily oral dose to MHRS rats. Two control groups of MHRS and normal healthy rats were run. Different biochemical and nutritional parameters were assessed. The induction of MHRS produced liver and kidney dysfunction, and elevated oxidative stress, an inflammatory biomarker, endothelin 1, and plasma cholesterol. Reduced plasma high density lipoprotein cholesterol, albumin and Ca and elevated urinary N-acetyl-β-D-Glucosaminidase and liver fats were noticed. The administration of *Nigella* crude oil that originally had 0.2% total omega-3 fatty acids or its blend with fish oil (17.9% omega-3) or flaxseed oil (42.1% omega-3) significantly improved all biochemical parameters of MHRS. There was no significant difference in the biochemical parameters among the different oil treated groups regardless of the omega-3 fatty acid content. This may point out to the potential profound effect of the volatile oil fraction of *Nigella* crude oil which may compensates for its low omega-3 content.

KEYWORDS: Hepatorenal syndrome; Nigella *crude oil; Oil blends; Omega-3 fatty acids*

RESUMEN: ***Evaluación del efecto terapéutico del aceite crudo de* Nigella sativa *y su mezcla con aceites ricos en ácidos grasos omega-3 en un modelo de síndrome hepatorenal modificado en ratas***. En el presente estudio, el efecto hepato- y reno-protector de aceites crudos de *Nigella sativa* y su mezcla binaria con aceites ricos en ácidos grasos omega-3 (pescado y aceites de linaza) fue estudiado en un modelo modificado de síndrome hepatorenal (MHRS) en ratas. MHRS fue inducido a través de la alimentación de una dieta alta en fructosa seguido de la inyección intraperitoneal de clorhidrato de galactosamina. Diferentes aceites fueron suministrados como dosis oral diaria a ratas con MHRS. Se realizaron dos grupos de control de MHRS y ratas sanas normales. Se evaluaron diferentes parámetros bioquímicos y nutricionales. La inducción de la MHRS produce disfunción hepática y renal, elevado estrés oxidativo y biomarcadores inflamatorios, endotelina 1, y colesterol en plasma. Se observó una reducción del colesterol plasmatico de lipoproteínas de alta densidad, albúmina y Ca, elevación en orina de N-acetil-β-D-glucosaminidasa y de la grasa del hígado. La administración de aceite crudo de *Nigella* que tiene un 0,2% de ácidos grasos omega-3 totales o su mezcla con aceite de pescado (17,9% de omega-3) o aceite de linaza (42,1% de omega-3) mejoró significativamente todos los parámetros bioquímicos de MHRS. No hubo diferencia significativa en los parámetros bioquímicos entre los diferentes grupos tratados con los aceites ensayados independientemente del contenido de ácidos grasos omega-3. Con esto se puede resaltar el potencial efecto de la fracción volátil del aceite crudo de *Nigella*.

1. INTRODUCTION

Chronic liver disease causes significant morbidity and mortality because of the number of complications associated with this disease. This can comprise hepatic encephalopathy, ascites and hepatorenal syndrome. Hepatorenal syndrome (HRS) is defined as renal failure caused by acute or chronic liver failure without any histological reasons (Bataller *et al.*, 1998). Two categories of HRS are described: Type 1 (HRS) is considered to be a specific complication of advanced cirrhosis characterized by a rapidly progressive decrease in kidney function. It is defined as a 100% increase in serum creatinine to a final value >2.5 mg·dL^{-1} in less than two weeks in humans. Type 2 HRS occurs in the setting of refractory ascites with a steady, moderate degree of functional renal failure (Arroyo *et al.*, 1996). Considering the management of HRS, patients with type 1 HRS usually require hospitalization, whereas those with type 2 HRS can be treated as outpatients.

HRS is still one of a few great secrets of today's medicine. The Pathomechanism of this syndrome is still poorly understood. Therefore, the pathogenesis of renal function alteration associated with liver disease remains to be elucidated. The exact etiology of HRS is unknown, although vasodilatation in the splanchnic area is suggested to be the basic pathophysiological reason for this disease (Demirbas *et al.*, 2012). Vasodilatation can develop as a result of cirrhosis, portal hypertension, reflex systemic and splanchnic vasoconstriction. On the other hand, some factors were suggested to be the causes of type 1 HRS including sepsis, variceal hemorrhage, shock, severe acute alcoholic hepatitis, or use of nephrotoxics (Muñoz, 2008). The decrease in renal perfusion and also in the glomerular filtration rate, sodium retention and deterioration of excretion of free water are the major renal problems which remain progressive according to the stage of liver disease (Demirbas *et al.*, 2012). Therefore, a new approach for future studies of the pathology in cirrhotic patients with renal dysfunction is required. Based on the ignorance of the exact mechanism of HRS, therapy for such a syndrome has not yet been established, where symptomatic treatment is only adopted which is not a radical remedy. Symptomatic treatments include vasoconstrictor therapy, in order to reverse splanchnic vasodilation, together with albumin. This treatment was found to be effective in about 50% of patients with type 1 HRS and improves survival (Fagundes and Ginè, 2012). These symptomatic treatments could reduce the risk of developing type 2 HRS (Planas *et al.*, 2006). Another treatment like renal vasodilators (Snowdon *et al.*, 2013) is also an option for managing HRS. However, liver transplantation is considered the preferred treatment because it offers a cure for both liver and renal dysfunction (Wadei *et al.*, 2006). Different reviews are available disclosing different aspects of this disease and more detailed strategies for treatment (Mohindra and Kumar, 2013; Davenport *et al.*, 2012).

In Egypt and in many other developing countries there is a continuous increase in the percentage of patients with liver and kidney dysfunction. This motivated the research team of the current investigation to study the interrelation between both diseases represented by HRS and how to protect, overcome or manage through the oral administration of some natural nutraceutical oils from plant and marine sources. Experimental animal models could be utilized in order to explain the patho-physiological state, and the mechanisms involved in the etiology of HRS as well as studying new efficient therapy. So, in the present research galactosamine hydrochloride was used to induce HRS in Sprague dawley rats according to Saracyn *et al.* (2012). In our previous work, (Al-Okbi *et al.*, 2013) it was revealed that the crude oil of *Nigella sativa* had a promising protection from steatohepatitis, which is a fatty liver with inflammation in rats.

In a continuation of that trend, the present study was dedicated to study the protective effect of *Nigella sativa* crude oil and its mixture with other omega-3 fatty acid-rich oils like flaxseed or fish oil toward the induced modified hepatorenal syndrome (MHRS). MHRS was induced in rats via feeding a high fructose diet with a simultaneous intraperitoneal injection of galactosamine hydrochloride to induce steatohepatitis and HRS. Another objective of the current study was to shed light on the biochemical changes and biomarkers that may be related to MHRS.

2. MATERIALS AND METHODS

2.1. Materials

Mature dried seeds of black cumin (*Nigella sativa* L, family Ranunculaceae) and flaxseeds (*Linum usitatissimum* L, family Linaceae) were purchased from a specialized local herbal store located in Cairo, Egypt.

The seeds were authenticated by Dr. Tereez Labib, a consultant of Plant Taxonomy, Ministry of Agriculture, Egypt. A voucher specimen from each plant seed was deposited in the National Research Centre Herbarium, Egypt. Fish oil (a mixture of anchovy cod, mackerel and sardine oils) was obtained from an Egyptian local market in soft gelatin capsules under the trade name "Natrol Omega-3 Fish oil". This dietary supplement is manufactured by Natrol. Inc. Chatsworth, CA 91311 USA. D-(+)-galactosamine hydrochloride was obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals used in the experiment were of analytical grade and used as is without further purification.

2.2. Animals

Male Sprague Dawley rats weighing 120.0–165.0 g (mean ± SD of 141.2±12.2) were used in the present study. The animals were obtained from the Animal House of National Research Centre, Cairo, Egypt. The animals were kept individually in stainless steel metabolic cages; water and food were given *ad-libtium*.

2.3. Diets

Experimental diets were prepared as in Table 1. A salt mixture and vitamin mixtures were prepared according to previous methods (Briggs and Williams, 1963; Morcos, 1976). Oil soluble vitamins were given orally in a dose of 0.1 mL/rat/week. Two types of diets were prepared; a balanced and a high fructose diet as described by Kawasaki *et al.* (2009) and Al-Okbi *et al.* (2013).

2.4. Extraction of *Nigella* and flaxseed crude oils.

Nigella sativa seeds and flaxseeds were crushed twice using a grinder model (MF10 microfine grinder drives). The crushed samples were pressed separately with a laboratory type Carver hydraulic press under 10.000 lb/inch pressure at room temperature

TABLE 1.   Composition of different diets (g per 100 g).

Ingredients	High fructose diet (HFD)	Balanced diet
Casein	17	12
Corn oil	–	10
Butter fat	5.5	–
Fructose	70	–
Starch	–	47
Sucrose	–	23.5
Salt mix.	3.5	3.5
Vitamin mix.	1	1
Cellulose	3	3

according to Üstun *et al.*, 1990. The produced oil was filtered and kept in dark bottles under deep freezing conditions until use.

2.5. Blending of *Nigella* crude oil with flaxseed and fish oils

A certain weight of *Nigella* crude oil was mixed separately with an equal weight of either flaxseed oil or fish oil with continuous stirring for 5 minutes to ensure homogeneity. These binary oil blends (*Nigella*/flaxseed and *Nigella*/fish at 1:1 weight ratios) were left for 1 h to equilibrate at room temperature before formulation into water-based emulsion for oral administration.

2.6. Formulation of *Nigella* crude oil and its binary blends in water-based emulsion for oral administration

Nigella crude oil and its binary blends with flaxseed and fish oils were mixed separately with Tween 80 as a non-ionic surfactant at 10.0% of the oils' weight (e.g. 1.0 g surfactant for each 10.0 g oil or oil blend). The oil-surfactant mixtures were vortexed for 1 min then left for 1h to equilibrate before a drop-wise addition of the oils to a calculated amount of distilled water. This oral administration system is called a self-emulsifying delivery system which forms oil-in-water emulsion spontaneously upon titration in water.

2.7. Preparation and analysis of the fatty acid composition of *Nigella* crude oil and its binary blends

The oils were transformed into their corresponding volatile fatty acid methyl esters via a trans-esterification process using 3.0% methanol/sulfuric acid according to A.O.A.C. (2000). Two microliters of the prepared fatty acid methyl esters were injected (at a split ratio 10:1) into a Hewlett Packard HP 6890 gas chromatograph equipped with a flame ionization detector. A 30 m×0.32 mm i.d. fused silica capillary column coated with DB-5 was used to separate the different methyl esters. The oven temperature was programmed from 150 °C to 240 °C at a rate of 2.5 °C·min^{-1} with a 30 min hold at the final temperature. The injector and detector temperatures were 230 °C and 250 °C, respectively. Helium was used as a carrier gas at a flow rate of 1.0 mL·min^{-1}. Methyl esters were identified by comparing their retention times with the available authentic standards (Sigma-Aldrich, St. Louis USA). Values of area% were the means of two injections from two different extractions±SD.

A gas chromatographic-mass spectroscopic (GC-MS) analysis was also conducted for the full identification of fatty acid methyl esters. A Hewlett-Packard 5972 GC-MS system was equipped with the

same column and conditions used as in GC analysis. The ionization voltage was 70 eV and the ion source temperature was 170 °C.

The full identities of the fatty acid methyl esters were revealed and verified by automatic matching of their mass fragmentation patterns (m/z) with that of standard components stored in the electronic mass spectral library (NIST: National Institute of Standards and Technology) that was built in to the GC-MS software.

2.8. Extraction and analysis of *Nigella* volatile oil

Nigella volatile oil was extracted from the crude oil via the hydro-distillation method using a Clevenger-type apparatus (European Pharmacopoeia, 1996). A certain weight of the crude oil was mixed with distilled water at 1:5 weight/volume ratios and distilled for 3h. The weight of the obtained volatile oil was determined and correlated to the weight of the crude oil to give the yield percent. The value of the yield percent was an average of two extractions±SD. A chemical analysis of the volatile oil was conducted using gas chromatography in order to estimate the percentage of thymoquinone and p-cymene which are the most prominent and biologically active components of the volatile oil. Twenty microliters of the volatile oil were diluted in 1.0 mL diethyl ether and 2.0 µL of this mixture were injected at a 10:1 split ratio into a Hewlett Packard HP 6890 gas chromatograph equipped with a flame ionization detector. A 30 m×0.32 mm i.d. fused silica capillary column coated with DB-5 was used to separate the volatile oil components. The oven temperature was programmed from 50 °C to 220 °C at a rate of 3 °C·min^{-1}. The injector and detector temperatures were 230 °C and 250 °C, respectively. Helium was used as a carrier gas at a flow rate of 1.0 mL·min^{-1}. Thymoquinone and p-cymene were identified by comparing their retention times with authentic standards (Sigma-Aldrich, St. Louis USA). Values of the area% of these two components were the means of two injections from two different extractions±SD.

2.9. Biological experiment

Thirty rats were divided into five groups, each comprised of six rats. The first was the normal control group where rats received a balanced diet without any treatments. The rats of the second group were fed a high fructose diet for the induction of steatohepatitis (Kawasaki *et al.*, 2009). The rats of groups 3, 4 and 5 were fed a high fructose diet and given a daily oral dose of 265mg·kg^{-1} rat body weight of *N. sativa* crude oil, a mixture of *N. sativa* crude oil and fish oil (1:1) and a mixture of *N. sativa* crude oil and flaxseed oil (1:1), respectively by stomach tube. The dose of 265 mg·kg^{-1} was used because *N. sativa* crude oil was reported previously to improve fatty liver at this dose level (Al-Okbi *et al.*, 2013). On the 34th day all rats except the normal healthy control (group: 1) were given 1.1 g·kg^{-1} body weight of galactosamine hydrochloride via intraperitoneal injection as a 200 mg·mL^{-1} solution in saline to induce hepatorenal syndrome (Saracyn *et al.*, 2012). Therefore, according to the last treatment, group 2 served as a control with both steatohepatitis and hepatorenal syndrome (MHRS) while groups 3-5 are test groups with MHRS and treated with different oils. During the experiment, body weight and food intake were recorded weekly. At the end of the study, the total food intake, body weight gain and food efficiency ratio (Body weight gain/total food intake) were calculated. The whole experimental period was thirty-five days.

Urine samples were collected twenty-four hours after the galactosamine hydrochloride injection for the determination of N-acetyl-β-D-Glucosaminidase (NAG) (Price and Whiting, 1992). Creatinine was determined in the collected 24h urine (Houot, 1985) for the calculation of creatinine clearance. Blood samples were collected from the fasting rats 24 hours after the galactosamine injection. An aliquot of blood was used for the determination of hemoglobin (Hb) (VanKampen and Zijlstra, 1965). Another aliquot was placed in heparinized tubes and centrifuged for the separation of plasma. Plasma total cholesterol (T-Ch) (Watson, 1960), high density lipoprotein cholesterol (HDL-Ch) (Burstein *et al.*, 1970), low density lipoprotein cholesterol (LDL-Ch) (Schriewer *et al.*, 1984) and triglycerides (TG) (Megraw *et al.*, 1979) were determined. The HDL-Ch/T-Ch ratio was calculated. Plasma malondialdehyde (MDA) (Satoh, 1978) and total antioxidant capacity (TAC) (Koracevic *et al.*, 2001) were determined as indicators of lipid peroxidation and antioxidant state, respectively. The plasma tumor necrosis factor-α (TNF-α) (an inflammatory biomarker) (Stepaniak *et al.*, 1995) was assessed. The activity of aspartate transaminase (AST) and alanine transaminase (ALT) (Reitman and Frankel, 1957), plasma albumin (Doumas *et al.*, 1972) and total and direct bilirubin (Gambino, 1965) were determined as indicators of liver function. Plasma creatinine (Houot, 1985) and urea (Fawcett and Scott, 1960) were assessed as indicators of kidney function. Plasma endothelin 1 (ET-1) (Zhou *et al.*, 2002) was determined as an expected biomarker of the hepatorenal syndrome. Livers and kidneys were immediately removed and weighed. The livers were stored at −20 °C until analysis. Hepatic lipids were extracted and weighed as described by Folch *et al.* (1957) and Cequier-Sánchez *et al.* (2008). Triglyceride and cholesterol contents in the liver were measured according to Megraw *et al.* (1979) and Watson, (1960), respectively. The animal experiments were carried out according to the Medical Research Ethics Committee, National Research Centre; Cairo, Egypt.

2.10. Statistical analysis

The results of animal experiments were expressed as the mean±SE and they were 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 for statistical significance.

3. RESULTS

The fatty acid composition of *Nigella* crude oil and its binary blend with flaxseed and fish oils is shown in Table 2. From the table it is evident that *Nigella* crude oil is poor in omega-3 fatty acids (0.2%) however, it is rich in omega-6 (60.4%) and omega-9 (25.2%) fatty acids. Blending *Nigella* crude oil with either fish or flaxseed oil improved the total omega-3 content of the mixture to reach 17.9% and 42.1% of the total fatty acids, respectively. This provides omega-6 to omega-3 ratios of 1.88 and 0.73 for *Nigella*/fish and *Nigella*/flaxseed oil blends, respectively.

Table 3 shows the fatty acid composition of the pure individual flaxseed and fish oils that were used to blend with *Nigella* crude oil. The table confirmed the fact that these oils are rich sources of omega-3 fatty acids which reached 42.7% and 57.9% for both fish and flaxseed oils, respectively.

The extraction of the volatile oil from *Nigella* crude oil revealed that this fraction constitutes 2.6%±0.3 of the crude oil's weight. The gas chromatographic analysis revealed that thymoquinone (68.1±0.8%) and p-cymene (20.1±0.4%) are the major constituents of the volatile oil fraction.

Table 4 shows the liver and kidney functions of the different experimental groups. The plasma levels of the total and direct bilirubin and the plasma activities of AST and ALT were increased significantly in the control rats with MHRS indicating liver dysfunction. Also plasma albumin was reduced significantly in the MHRS rats compared with the normal control rats. The plasma levels of creatinine, urea and endotheline-1 (ET-1) increased significantly in the MHRS control rats compared with the normal control rats, indicating kidney dysfunction. The oral administration of *Nigella* crude oil, *Nigella*/fish or *Nigella*/flaxseed oils (which encompass the three test groups) showed significant improvement in liver and kidney functions with different degrees compared with the MHRS control group. On the other hand, plasma urea and creatinine were completely normalized on oral administration of different oils where they matched that of the normal control rats. When comparing the test groups with each other, there were no significant changes in the parameters indicating liver and kidney functions

TABLE 2. Fatty acids composition of *Nigella*, *Nigella*/fish and *Nigella*/flaxseed oils blended at 1:1 weight ratio

Fatty acid carbon No.	Common name	CAS Name	Average area (%)		
			Nigella	*Nigella*/fish	*Nigella*/flaxseed
C14:0	Myristic	Tetradecanoic	0.1±0.07	2.0±0.4	<0.1
C16:4	–	6,9,12,15-Hexadecatetraenoic	nd	0.7±0.01	nd
C16:3 (n-3)	Roughanic	7,10,13-Hexadecatrienoic	nd	0.6±0.01	nd
C16:1 (n-7)	Palmitoleic	9-Hexadecenoic	0.1±0.07	4.2±0.2	<0.1
C16:0	Palmitic	Hexadecanoic	9.5±0.5	11.5±0.5	11.4±0.07
C18:2 (n-6)	Linoleic	9,12-Octadienoic	60.4±3.6	32.8±1.6	30.9±0.7
C18:3 (n3)	α-linolenic	9,12,15 -Octadecatrienoic	0.2±0.07	0.2±0.05	42.1±0.6
C18:1 (n-9)	Oleic	9-Octadecenoic	21.9±2.6	21.7±1.0	11.7±0.1
C18:1 (n-7)	vaccenic	11-Octadecenoic	1.0±0.07	2.8±0.1	<0.1
C18:0	Stearic	Octadecanoic	3.3±0.2	3.5±0.3	3.1±0.1
C20:4 (n-6)	Arachidonic	Eicosa-5,8,11,14-tetraenoic	nd	0.9±0.01	nd
C20:5 (n-3)	Timnodonic	Eicosa-5,8,11,14,17-pentaenoic	nd	10.2±0.6	nd
C20:4 (n-3)	Eicosatetraenoic	Eicosa-8,11,14,17-tetraenoic	nd	0.7±0.01	nd
C20:1 (n-9)	Eicosenoic	11-Eicosenoic	3.3±0.2	3.1±0.04	1.2±0.1
C22:6 (n-3)	Cervonic acid	Docosa-4,7,10,13,16,19-hexaenoic	nd	5.2±0.5	nd
C22:5 (n-3)	Clupanodonic	Docosa-7,10,13,16,19-pentaenoic	nd	1.0±0.2	nd
Total n-9			25.2%	24.8%	12.9%
Total n-6			60.4%	33.7%	30.9%
Total n-3			0.2%	17.9%	42.1%
Ratio n-6/n-3			302	1.88	0.73

nd: not detected.

TABLE 3.   Fatty acid composition of the pure fish and flaxseed oils.

Fatty acid carbon No.	Common name	CAS Name	Average area (%)	
			Fish oil	Flaxseed oil
C14:0	Myristic	Tetradecanoic	4.82±0.2	nd
C16:4	–	6,9,12,15-Hexadecatetraenoic	1.72±0.01	nd
C16:3 (n-3)	Roughanic	7,10,13-Hexadecatrienoic	1.28±0.01	nd
C16:1 (n-7)	Palmitoleic	9-Hexadecenoic	8.45±0.1	nd
C16:0	Palmitic	Hexadecanoic	13.44±0.3	7.0±0.1
C18:4 (n3)	Stearidonic	6,9,12,15-Octadecatetraenoic	3.20±0.1	nd
C18:2 (n-6)	Linoleic	9,12-Octadienoic	1.57±0.01	12.4±0.2
C18:1 (n-9)	Oleic	9-Octadecenoic	10.85±0.2	16.8±1.5
C18:3 (n-3)	α -linolenic	9,12,15 -Octadecatrienoic	0.44±0.01	57.9±2.4
C18:1 (n-7)	vaccenic	11-Octadecenoic	3.90±0.1	nd
C18:0	Stearic	Octadecanoic	3.64±0.1	4.5±0.2
C20:4 (n-6)	Arachidonic	Eicosa-5,8,11,14-tetraenoic	1.17±0.01	nd
C20:5 (n-3)	Timnodonic	Eicosa-5,8,11,14,17-pentaenoic	20.15±1.8	nd
C20:4 (n-3)	Eicosatetraenoic	Eicosa-8,11,14,17-tetraenoic	1.37±0.01	nd
C20:1 (n-9)	Eicosenoic	11-Eicosenoic	1.73±1.03	nd
C22:6 (n-3)	Cervonic acid	Docosa-4,7,10,13,16,19-hexaenoic	13.77±0.4	nd
C22:5 (n-3)	Clupanodonic	Docosa-7,10,13,16,19-pentaenoic	2.51±0.1	nd
Total n-9			12.5%	16.8%
Total n-6			2.7%	12.4%
Total n-3			42.7%	57.9%

nd: not detected.

TABLE 4.   Liver and kidney functions of different experimental groups*

Plasma Parameters	Normal control	MHRS control	*Nigella* oil	*Nigella* oil & Fish oil (1:1)	*Nigella* oil & Flaxseed oil (1:1)
Creatinine (mg·dL^{-1})	0.686[a]±0.04	1.04[b]±0.1	0.761[a]±0.03	0.744[a]±0.04	0.806[a]±0.03
Urea (mg·dL^{-1})	30.3[a]±1.1	44.3[b]±2.8	30.3[a]±0.5	30.2[a]±0.2	30.1[a]±0.7
Albumin (g·dL^{-1})	4.2[a]±0.2	2.65[b]±0.1	3.1[c]±0.1	3.2[c]±0.1	3.2[c]±0.1
ALT (U/l)	55.8[a]±1.2	86.2[b]±1.9	76.7[c]±2.2	74[c]±1.8	72.7[c]±1.4
AST (U/l)	40.8[a]±0.8	80.5[b]±1.4	70.2[c]±2.6	67.5[c]±2.1	66.7[c]±1.7
T. Bilirubin (mg·dL^{-1})	0.356[a]±0.01	0.497[b]±0.01	0.411[c]±0.01	0.395[c]±0.01	0.383[c]±0.02
D. Bilirubin (mg·dL^{-1})	0.157[a]±0.004	0.242[b]±0.01	0.18[c]±0.01	0.167[c]±0.01	0.162[c]±0.01
ET-1 (ng·mL^{-1})	22.0[a]±1.3	39.7[b]±2.8	27.2[c]±1.7	26.3[c]±2.4	27.7[c]±1.1

*Mean±S.E.
In the same row, values having the same letter are insignificantly different and values having different letters are significantly different at the level p<0.05.

reflecting an equal effect of the different oils in improving such parameters.

Table 5 shows the plasma and liver lipid profile of the different experimental groups. MHRS control rats exhibited a significant increase in plasma total cholesterol, triglycerides, LDL-Ch and the ratio of T-Ch/HDL-Ch compared with the normal control rats. In addition, significant increases were observed in total fat, T-Ch and TG in the liver tissue of the MHRS control rats compared to the normal control group.

The rats of the test groups showed a significant improvement in plasma lipid profile, T-Ch/HDL-Ch and the contents of total liver fat, T-Ch and TG with different degrees.

Table 6 shows the blood hemoglobin, total antioxidant capacity, malondialdehyde and tumor necrosis factor-alpha of the different experimental groups. Blood hemoglobin and total plasma antioxidants were reduced significantly in the MHRS control rats compared with the normal control group. The oral administrations of different oils elevated

TABLE 5. Plasma and liver lipid profile of different experimental groups*

Parameters	Normal control	MHRS control	*Nigella* oil	*Nigella* oil & fish oil	*Nigella* oil & flaxseed oil
Plasma					
T-Cholesterol (mg·dL^{-1})	82.8[a]±3.0	112.1[b]±2.8	92.4[c]±2.4	91.5[c]±1.8	92.5[c]±1.1
Triglycerides (mg·dL^{-1})	83.1[a]±2.7	162.4[b]±2.4	130.3[c]±4.0	128.7[c]±2.5	129.5[c]±1.6
HDL-Ch (mg·dL^{-1})	31.8[a]±1.2	21.8[b]±0.6	28.7[c]±0.7	30.5[c]±1.1	30.7[c]±0.9
LDL-Ch (mg·dL^{-1})	22.3[a]±0.4	81.5[b]±2.1	55.5[c]±2.9	52.3[c]±2.4	53.3[c]±2.3
T-Ch/ HDL-Ch	2.6[a]±0.1	5.2[b]±0.2	3.2[c]±0.1	3.0[c]±0.1	3.0[c]±0.1
Tissue					
Liver total fat (mg·g^{-1} tissue)	23.2[a]±0.3	44.2[b]±1.2	32[c]±0.7	30.5[c]±0.7	30.8[c]±0.4
Liver T-Cholesterol (mg/g tissue)	2.2[a]±0.2	7.1[b]±0.2	5.2[c]±0.1	4.9[c]±0.05	5.1[c]±0.1
Liver Triglycerides (mg·g^{-1} tissue)	5.3[a]±0.1	13.95[b]±0.5	8.4[c]±0.2	8[c]±0.2	8.2[c]±0.2

*Mean±S.E.
In the same row, values having the same letter are insignificantly different and values having different letters are significantly different at level p<0.05.

TABLE 6. Hemoglobin, total antioxidant capacity, malondialdehyde and tumor necrosis factor-alpha of different experimental groups*

Parameters	Normal control	MHRS control	*Nigella* oil	*Nigella* oil & Fish oil	*Nigella* oil & Flaxseed oil
Hb (g·dL^{-1})	13.3[a]±0.543	9.9[b]±0.467	11.8[c]±0.718	12.1[c]±0.774	12.6[c]±0.581
TAC (mm·L^{-1})	1.57[a]±0.038	0.872[b]±0.036	1.21[c]±0.058	1.28[c]±0.070	1.3[c]±0.058
MDA (nmol·mL^{-1})	5.5[a]±0.217	23.1[b]±0.634	15.7[c]±0.685	14.2[c]±0.958	14.8[c]±0.777
TNF-α (pg·mL^{-1})	19.7[a]±0.604	31.7[b]±0.735	15.1[c]±0.506	14.1[c]±0.774	14.7[c]±0.882

*Mean±S.E.
In the same row, values having the same letter are insignificantly different and values having different letters are significantly different at the level p<0.05.

blood hemoglobin and plasma TAC levels to different degrees. The plasma levels of MDA and TNF-α increased significantly in the MHRS control rats compared with the normal control group. The MHRS rats administered different oils showed a significant reduction in MDA levels and TNF-α. Blood hemoglobin, plasma total antioxidant capacity and plasma malondialdehyde levels of the test groups were still not matching that of the normal control group.

Table 7 shows that creatinine clearance was reduced significantly in the MHRS control group compared to normal control rats. The administration of different oils showed a significant increase in creatinine clearance compared with the MHRS control group.

The urine level of NAG (Table 5) was elevated significantly in the MHRS group compared to the normal control rats. The administration of different oils showed a significant reduction in NAG compared with the MHRS control group.

Table 8 shows the non-significant changes when all nutritional parameters, liver weight/body weight % and kidney weight/body weight % of the MHRS control rats were compared with the normal control

TABLE 7. Creatinine clearance and NAG of different experimental groups*

Groups	Creatinine clearance (ml/min)	NAG (IU/l)
Normal control	0.945[a]±0.05	38.1[a]±0.66
MHRS	0.501[b]±0.07	46.1[b]±1.8
Nigella sativa oil	0.636[c]±0.05	36.9[c]±2.5
Nigella sativa oil & fish oil	0.686[c]±0.06	35.2[c]±1.7
Nigella sativa oil & Flaxseed oil	0.705[c]±0.04	37.0[c]±1.3

*Mean±SE.
In the same column, values having the same letter are insignificantly different and values having different letters are significantly different at level p<0.05.

rats. The oral administration of different oils showed non-significant changes in all the aforementioned parameters except for the rats given the oral administration of *Nigella sativa* crude oil which showed a significant reduction in final body weight, body weight gain and food efficiency ratio in comparison to all the conducted groups.

TABLE 8. Nutritional parameters of different experimental groups*

Parameters	Normal control	MHRS control	*Nigella* oil	*Nigella* oil & fish oil	*Nigella* oil & flaxseed oil
Initial BW(g)	141.3[a]±6.2	141[a]±6.8	141.2[a]±6.69	141.3[a]±2.4	141[a]±3.2
Final BW (g)	225[a]±6.8	228.2[a]±4.7	207.3[b]±7.1	227.8[a]±7.6	228.2[a]±4.2
Body weight gain (g)	83.7[a]±2.01	87.2[a]±3.7	66.2[b]±2.8	86.5[a]±5.8	87.2[a]±5.5
Total Food intake (g)	512.8[a]±8.1	518.8[a]±4.2	508.5[a]±2.3	510.3[a]±6.9	514.8[a]±7.8
Food efficiency ratio	0.163[a]±0.01	0.168[a]±0.07	0.130[b]±0.05	0.169[a]±0.09	0.169[a]±0.01
Liver weight/body weight %	2.6[a]±0.1	2.7[a]±0.2	2.9[a]±0.3	2.8[a]±0.2	2.8[a]±0.2
Kidney weight/body weight %	0.699[a]±0.02	0.74[a]±0.01	0.896[a]±0.05	0.805[a]±0.05	0.843[a]±0.04

*Mean±S.E.
In the same row, values having the same letter are insignificantly different and values having different letters are significantly different at level p<0.05.

4. DISCUSSION

In the present study it was decided to induce steatohepatitis before the induction of HRS with the aim of enhancing the induction of HRS on intraperitoneal injection with galactosamine hydrochloride. MHRS in the current study referred to the blend of steatohepatitis and HRS. It was also the interest of the authors in the present study to assess the biochemical and nutritional changes that occur during MHRS in a trial to study the mechanism of action and patho-physiology of the hepatorenal syndrome.

From the obtained results, this model was found to be satisfactory for studying the therapeutic potential of the investigated oils against HRS, because both renal and hepatic dysfunction occurred on intraperitoneal injection of galactosamine hydrochloride.

The increased plasma creatinine and reduced creatinine clearance in MHRS in the present study reflected that glomerular function, proximal tubules and glomerular filtration rate are affected in HRS as reported previously (Van Lente and Sult, 1989; Skálová, 2005). Renal dysfunction in patients with cirrhosis with superimposed inflammation was reported previously to be associated with significant tubular injury (Shah et al., 2013). This model also showed elevated oxidative stress in the present study, represented by the increased MDA and the reduced total antioxidant which might be a biomarker of tissue destruction and inflammation in both kidney and liver. In the MHRS model, an elevated inflammatory biomarker (tumor necrosis factor-alpha) was also noticed in the current results. The inhibition of oxidative stress and simultaneous inflammatory biomarker, were reported to prevent the development of renal failure in an animal model of HRS (Saracyn, et al., 2008). Therefore, a natural anti-inflammatory and antioxidant might have therapeutic potential towards HRS.

ET-1 showed a significant elevation in the MHRS model in the present study, which supports the previous proposal of being a biomarker of HRS (Anand et al., 2002) due to the increased endothelin (A) of the renal cortex which is important in the pathogenesis of renal failure that occurs in patients with acute liver failure or HRS. Urinary NAG was significantly elevated in the MHRS model in the present study reflecting tubular dysfunction and kidney parenchymal damage as reported by Skálová (2005). In agreement with this result, high urinary NAG was reported in infants with liver cirrhosis (Aydogdu et al., 2004) pointing to an occurrence of renal dysfunction and HRS. The plasma albumin level was reduced significantly in MHRS rats in the present study which confirms the work of García-Martínez et al. (2013), who showed that not only albumin concentration but also albumin function is reduced in liver failure.

Hemoglobin reduction was noticed in MHRS in rats, pointing to some sorts of anemia. Anemia is prevalent in chronic kidney disease which could be due to erythropoietin deficiency, inhibition of erythropoiesis by uremic solutes, and reduction in red blood cell life span. Other possible causes include iron, B12 or folic acid deficiency or blood loss. Dysfunction of the endogenous erythropoietin is usually clinically evident once the glomerular filtration rate falls below 20.0–25.0 mL·min^{-1} in humans (Ansari et al., 2014).

Rats with MHRS showed a significant elevation in all liver fat with dyslipidemia which may be due to feeding high fructose as described by Kawasaki et al. (2009) and Al-Okbi et al. (2013). *Nigella sativa* oil showed previously to produce a hepatoprotective effect in rats (Al-Suhaimi, 2012) which was confirmed by the work of Al-Okbi et al. (2013), who reported its protective effect against steatohepatitis. This effect was ascribed to the volatile oil fraction, particularly thymoquinone and p-cymene and to a lesser extent to the fatty acid profile of *Nigella* crude oil. So, it was important to extend the study on the protective effect of *Nigella sativa* crude oil in rats in which MHRS was induced. The quantity and quality of the volatile oil fraction of *Nigella sativa*

crude oil used in the present study differs from that used to evaluate the protective effect against steatohepatitis in our previous work (Al-Okbi *et al.* 2013). The crude oil in the current study contains a higher volatile oil fraction (2.6 wt%) and different contents of the volatile constituents thymoquinone (68.1%, GC area) and p-cymene (20.1%, GC area) than in the previous work in which these parameters were 0.1 wt%, 39.1%, and 40.2%, respectively.

Concerning the fatty acid composition of *Nigella* crude oil, the current study showed that the oil is rich in omega-6 (60.4%) and omega-9 (25.2%) but scarce in omega 3- fatty acids (0.2%). Therefore, due to the role of omega-3 fatty acids as hepatoprotective and anti-inflamatory agents (Raptis *et al.*, 2014; Al-Okbi and Mohamed, 2012), it was decided in the present study to blend *Nigella* crude oil with other omega-3 fatty acid rich oils from marine as well as plant origins such as fish and flaxseed oils, respectively.

In the current study, treatment with *Nigella* crude oil or its binary blend with either fish or flaxseed oils produced a significant improvement in all biochemical parameters. This improvement only reaches the normal levels in the case of plasma creatinine and urea and even showed a more significant improvement than normal concerning plasma TNF-α. All other biochemical parameters, although significantly improved, did not reach the level of the normal group. This means that the different treatments did not provide complete protection, but reduced the progression of HRS. There were no significant changes in the biochemical parameters among the different test groups (kept on different oil treatments). This result showed that blending half the dose of *Nigella* crude oil with either flaxseed oil or fish oil produced no further improvement in the biochemical parameters compared with *Nigella* crude oil alone. This means that increasing omega-3 fatty acids at the expense of crude oil constituents including fatty acids and volatile oils did not add to the hepato-protective effect of *Nigella* crude oil. It is well documented that decreasing the ratio of n-6/n-3 fatty acids renders the oil anti-inflammatory and beneficial health effect. Although the present oil blends showed lower n-6/n-3 fatty acids than *Nigella* crude oil, they did not afford extra beneficial effects towards MHRS. This may be explained on the basis of the presence of volatile oils in *Nigella* crude oil (present at 2.6%) that possess antioxidant and anti-inflammatory activity. Some other constituents not determined in the present study could also be responsible for the biological activity of *Nigella sativa* crude oil; these are phytosterols and alpha and gamma- tocopherols (Cheikh-Rouhou *et al.*, 2008; Ramadan *et al.*, 2012) that possess antioxidant and anti-inflammatory effects. Although fish oil and flaxseed oil contain high percentages of omega-3 fatty acids, they lack the presence of antioxidants compared to *Nigella* crude oil.

Hyperbilirubinemia as shown in MHRS models in the present study might be detrimental to liver and renal functions. The systemic reduction in bilirubin concentration by the different oil treatments might contribute to the normalization of the urinary levels of prostaglandins and thromboxane B2, to decrease in serum bile acid levels, and to the improvement of the hepatic energy charge that may reflect improvement in both liver and kidney dysfunctions as reported by Kamisako *et al.* (1998).

It could be noticed from the present study that although polyunsaturated omega-3 fatty acids were higher in rats given the mixture of *Nigella* crude oil with either fish oil or flaxseed oil, MDA was not elevated and total antioxidant was not reduced, but improved. This could be due to the presence of antioxidant constituents supplemented from *N. sativa* crude oil. The results of studies examining the effects of increased proportions of polyunsaturated fatty acids in the diet on indexes of lipid peroxidation in vivo are contradictory (Wander *et al.*, 1996; Harats *et al.*, 1991; Meydani *et al.*, 1991). With fish-oil supplementation, there was no evidence of increased lipid peroxidation when assessed by plasma F2-isoprostanes and MDA (Higdon *et al.*, 2000), which agreed with the present study.

Diets relatively high in unsaturated fat have favorable effects on lipoprotein-cholesterol profiles (Mensink and Katan, 1992; Mattson and Grundy, 1985) which agreed with the present results. It was also shown previously that chronic supplementation of fish oil demonstrated a reno-protective effect (Fernandez *et al.*, 2004) which confirms the present results.

Flaxseeds is a leading source of α –linolenic acid (ALA) which account for 57.9% of total fatty acids. In the present study, this amount was higher than that reported previously (52%) by Oomah and Mazza (1995). Mohamed *et al.* (2005) showed that flaxseed produced a reduction in plasma T-ch and LDL-ch in hyper-cholesterolemic rats. The same effect was reported for the consumption of flaxseed in humans (Clark *et al.*, 1995; Jenkins *et al.*, 1999). ALA, which is an omega-3-fatty acid, has been reported to be useful in the prevention and treatment of coronary artery disease and hypertension (Simopoulos, 1999). This is because ALA is the precursor for the synthesis of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) which are associated with the control of cardiovascular diseases (Bibus *et al.*, 1998). ALA also reduces serum triglycerides and the development of thrombosis and arteriosclerosis (Pszczola, 1998). Also, flaxseed was shown to have an impact on the treatment of lupus nephritis (Clark *et al.*, 1995) in humans and improvement of renal dysfunction during cisplatin treatment in rats (Al-Okbi *et al.*, 2014), pointing to its reno-protective effect. Flaxseed oil attenuated the decline in renal function and reduced glomerular

injury (Caligiuri *et al.*, 2013). It has also been shown that treatment with flaxseed oil caused a significant improvement in the histopathological picture of the kidney as well as the kidney function and antioxidant status, against lead acetate-induced renal toxicity (Abdel-Moneima *et al.*, 2011). It was reported that rats fed a flaxseed-supplemented diet had significantly lower blood pressure and a significantly improved lipid profile, liver and kidney functions in the hypertensive condition. These effects are likely to be mediated by the ALA and linoleic acid contents of flaxseed oil (Al-Bishri, 2013).

The treatment of MHRS rats with mixtures of oils containing omega-3 fatty acids produced an improvement in renal function in the present study. Over the past 25 years, several studies have suggested the efficacy and potential clinical utility of dietary supplementation with omega-3 fatty acids in human renal diseases (Donadio, 2001; De Caterina *et al.*, 1994; Baggio *et al.*, 2002; Azar *et al.*, 1989).

It is accepted that the effect of omega-3 fatty acids on kidney diseases related to the activities of metabolites derived from EPA opposed those derived from arachidonic acid (AA). Alterations in eicosanoid synthesis and metabolism are produced when concentrations of EPA and DHA increase in relation to AA. For example, the renal production of thromboxane A2 is elevated in several renal diseases and treatment with n-3 fatty acids reduced it (Fernandez *et al.*, 2004).

The non significant changes in different nutritional parameters in the present study are similar to the results reported by Kawasaki *et al.* (2009) and Al-Okbi *et al.* (2013) when rats fed similar high fructose diets without a glucosamine injection were compared with balanced diets. The significant reduction in final body weight, body weight gain and food efficiency ratio on administration of *Nigella* crude oil agreed with previous results (Al-Okbi *et al.*, 1997; Houcher *et al.*, 2007).

5. CONCLUSION

MHRS induced in rats through feeding a high fructose diet followed by an intraperitoneal injection of galactosamine hydrochloride produced liver and kidney dysfunction. The same model showed reduced hemoglobin, elevated oxidative stress, increased inflammatory biomarker, elevated ET-1, elevated plasma cholesterol, reduced plasma HDL-Ch, reduced plasma albumin, reduced plasma Ca and elevated urinary NAG and liver fats. These changes confirm the induction of HRS. The administration of *Nigella* crude oil or its blend with fish or flaxseed oils significantly improved all biochemical parameters of MHRS but without complete protection. Changing the fatty acid profile of *Nigella* crude oil by blending with those omega-3 fatty acid-rich oils did not make any difference in the therapeutic efficiency of *Nigella*

crude oil alone. That can reflect the superiority of this oil and potentially its volatile oil fraction as hepatoprotective agents.

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