Basil Essential Oil and its Nanoemulsion Mitigate Non Alcoholic Steatohepatitis in Rat Model with Special Reference to Gut Microbiota

Sahar Y. Al-Okbi1*, Magdy A. Amin2, Amal E. A. Mohamed2, Amr E. Edris3, Osama M. Sharaf4, Hoda B Mabrok1, and Asmaa A. Ramadan1

1 Nutrition and Food Sciences Department, National Research Centre, Cairo, EGYPT
2 Microbiology and Immunology department, Faculty of Pharmacy, Cairo University, Cairo, EGYPT
3 Aroma and Flavor Chemistry, Food Industries and Nutrition Division, National Research Centre, Dokki, Cairo, EGYPT
4 Dairy Sciences Department, National Research Centre, Cairo, EGYPT

Abstract: The present research evaluated the protective effect of basil essential oil nanoemulsion (BNO) and its parent basil essential oil (BO) towards steatohepatitis. Chemical composition of BO was assessed followed by formulation into different BNOs using the low energy spontaneous emulsification technique. An ideal formula of BON was selected among the others based on its ultra-fine particle size (15.42 nm) and physical stability at 25-37°C and tested in steatohepatitis rat model along with BO. Rats were divided into four groups, the first was fed on balanced diet (C), and the other groups were maintained on high fructose saturated fat diet deficient in choline to induce steatohepatitis, one of such groups served as control steatohepatitis (SC), the other groups received daily oral dose of BO and BNO, respectively. Microbiota (Fermicutes and Bacteroidetes) were counted in colon content and their ratio (F/B) was calculated. Liver fat, plasma lipid profile, plasma interleukin-6, plasma lipopolysaccharides and plasma and colon content of lipocaline were assessed with histopathological examination of liver and colon. Results showed that the major volatile components of BO were linalool (60.9 %), eugenol (5.1 %) and eucalyptol (9.5%). SC group exhibited significant increase in liver lipids, plasma triglycerides, total cholesterol (TC), low density lipoprotein cholesterol and significant reduction in high density lipoprotein-cholesterol (HDL-C) compared to C group. Significant increase in plasma TC/HDL-C, interleukin-6, and lipocaline and F/B ratio and lipocaline in colon content were demonstrated in SC group without changes in plasma lipopolysaccharides compared to C. Histopathology of SC group showed liver fatty degeneration and fibroblasts activation while the colon demonstrated erosion and mucosal epithelium detachment. Treatment with either BNO or BO showed improvement compared to SC group. BNO was superior in reducing F/B ratio, liver lipids and histopathological changes. BO was more efficient in reducing TC, triglycerides and low density lipoprotein cholesterol. It is concluded that BO and BNO reduced the progression of nonalcoholic steatohepatitis in rat model. Gut microbiota in relation to steatohepatitis and related new therapies needs further investigations.

Key words: basil essential oil, basil essential oil nanoemulsion, steatohepatitis, microbiota, biomarkers

1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common condition among population worldwide. Changes in lifestyle and eating habits of rapidly evolving societies have compromised the health of many by fostering the development of metabolic syndrome. NAFLD is a major consequence of metabolic syndrome along with type 2 diabetes and insulin resistance. The disease results from excessive accumulation of liver fat which leads to the incidence of high oxidative stress and inflammation. If not closely monitored, NAFLD could induce non-alcoholic steatohepatitis (NASH). NASH is a major risk factor for cardiovascular disease1 and could eventually progress to liver cirrhosis, hepatocellular carcinoma and liver failure2.

Several therapeutic approaches have been proposed to manage NAFLD. For instance, ursodeoxycholic acid has...
been reported to have hepatoprotective effects that may delay the complications of the disease\textsuperscript{5}. Insulin sensitizer drugs such as biguanides, and blood lipid lowering drugs (e.g. statins) might also improve the condition and reduce inflammation\textsuperscript{3}. However, the efficacy of those drugs has been controversial and so far no specific drug therapies have been reported to successfully cure fatty liver diseases\textsuperscript{5}.

In previous studies, some of the research team of the present research reported the impact of nutraceuticals or functional food ingredients in prevention of steatohepatitis and liver cirrhosis\textsuperscript{15–19}. Essential oils have long been investigated for their anti-inflammatory properties\textsuperscript{10}. Some essential oils were proven to have hepatoprotective effects and have been successfully used as nutraceuticals to manage liver tissue injury\textsuperscript{11,12}. Adopting the same line of using essential oils to mitigate NALFD, the current investigation selected basil essential oil (BO) for valuation against that disease. BO is commonly used in traditional medicine for its antiseptic, anti-inflammatory, and hypoglycemic functions\textsuperscript{13–16}. It has recently gained momentum as a novel nutraceutical that possesses several health benefits that might have a role in management of fatty liver diseases.

Pathogenesis of liver diseases, including steatohepatitis is speculated to be due to altered gut microbiota\textsuperscript{17–20}. Dysbiosis have been strongly related to different inflammatory diseases\textsuperscript{1,19}. Essential oils can improve gut dysbiosis and correct gut microbiota alteration\textsuperscript{20}. Therefore, it is hypothesized that administration of nutraceuticals like essential oils could prevent the progression of fatty liver to steatohepatitis. That is due to their potentials for improving the profile of gut microbiota along with their anti-inflammatory activity.

Formulation of essential oils in a water-based colloidal delivery system like nanoeulsion is a convenient dosage form for oral administration. In addition, the nano-size of essential oils in nanoeulsion can be more bioavailable and more biologically effective compared to the same essential oil in its neat state without formulation\textsuperscript{37}.

Based on the above mentioned, the aim of the present investigation was to study the impact of dietary intervention with basil essential oil on reducing the severity of NALFD. This was accomplished by studying modulation of gut microbiota and alleviation of hepatic tissue injury and inflammation in rats fed on high-fructose-saturated fat-choline deficient diet through assessing essential biomarkers and histopathological changes. The dosage forms of BO include two delivery systems; neat unformulated (BO) delivered in vegetable oil and a nanoeulsion (BNO) delivered in water.

2 Experimental

2.1 Materials

The essential oil of \textit{Ocimum basilicum} was provided from the farm of the Aromatic and Medicinal Plant Research Center, Kanater, Egypt. The essential oil was extracted by steam distillation of the fresh herb that was grown in the same farm under conventional agricultural practices. Surfactants represented by Tween 80 (polyoxyethylene sorbitan mono-oleate), Tween 20\textsuperscript{®} (polyoxyethylene sorbitan mono-oleate) and Cremophore RH40\textsuperscript{®} (hydrogenated polyoxyethylene castor oil) were supplied from Sigma-Aldrich Chemical Co. St. Louis, MO, U.S.A. Propylene glycol (99.0 %) as co-surfactant was supplied from Fischer Scientific (Leicestershire, U.K.).

2.2 Animals

Twenty four male Sprague-Dawley rats of body weight 150-160 g were purchased from the Animal Health Research Institute, Giza, Egypt. Rats were kept individually in stainless steel cages at ambient temperature (25 ºC ± 2), with 12 light/dark cycle. Food and water were supplied \textit{ad-libitum}. Handling and care of animals was carried out in accordance to Ethics Committee of National Research Centre, Cairo, Egypt (NIH publication No. 85-23, revised 1985) and according to the guidelines of the Research Ethics Committee for experimental and clinical studies of Faculty of Pharmacy, Cairo University, Cairo, Egypt.

2.3 GC-MS analysis of basil essential oil

The chemical composition of Basil essential oil was evaluated using GC-MS analysis. An appropriate amount of oil (20 μL) was diluted in 1 mL diethyl ether and 2 μL of that mixture was injected at split ratio 10 to 1 into a GC-MS Trace GC Ultra apparatus (Thermo Electron Corporation, Milan, Italy) with FID and MS DSQ II detector. A simultaneous GC-FID and MS analysis was performed using a MS-FID splitter (SGE Analytical Science). A capillary column Rtx-1ms (Restek), 60.0 m × 0.25 mm i.d., with film thickness of 0.25 μm was used to separate the essential oil components. The temperature was programmed from 50.0 ºC to 220.0 ºC at 2.0 ºC/min, and the injector and detector (FID) temperatures were adjusted at 280.0 ºC and 300.0 ºC, respectively. Helium at a regular pressure of 300.0 kPa was used as a carrier gas. Mass spectra were acquired over the mass range of 30.0-400.0 Da, ionization voltage 70.0 eV and ion source temperature of 200.0 ºC. Identification of the components was based on a comparison of their MS with computer libraries NIST 98.1 and MassFinder 4.1, along with the relative retention indices (RI, nonpolar column) associated with a series of alkanes with linear interpolation (C\textsubscript{19}-C\textsubscript{40}). Percentages were obtained from FID response without the use of correction factors. The volatile compounds were identified by comparing their mass fragmentation patterns with that of reference samples stored...
at an electronic mass spectroscopic library using X-caliber software.

2.4 Formulation of basil essential oil in vegetable oil-based delivery system

To facilitate dosing of essential oil to rats; the first dosage form of BO was prepared as an oil-based delivery system and formulated as follows. BO (3.0 wt\%) was dissolved in 97.0 wt\% corn oil followed by vigorous stirring via magnetic bar for 5 minutes until a homogenous clear oil mixture was obtained. This formula was freshly prepared before dosing rats. The applied dose via oral route was 100 mg basil essential oil/kg rat body weight/day as reported previously\(^{22}\).

2.5 Formulation of basil essential oil in water-based nanoemulsion delivery system

The second dosage form of BO was prepared as water-based delivery system and formulated as follows. Six different BO formulations were prepared using the low energy spontaneous emulsification method\(^{23}\) aiming to find out the appropriate formula which has the smallest particle size. The composition of the six formulations are quantitatively the same but qualitatively different in the type and ratio of the surfactant(s) (Table 1).

Preparation of the previously mentioned formulas in Table 1 took place as follows: the surfactant (or the surfactant mixture) and propylene glycol were intimately mixed using a vortex. Then BO was added and the whole system was vortexed again to give a homogenous concentrate. Each concentrate was titrated into a conical flask containing distilled water. The concentrate that instantly gave a translucent or transparent system just by swirling the conical flask -without using any shear equipment- was considered as nanoemulsion. On the other hand, milky white cloudy formulas were excluded. The essential oil nanoemulsions were left for another 24 h at room temperature to equilibrate before their particle size distribution was determined.

2.5.1 Particle size analysis of basil nanoemulsions

The average particle size diameter of BO nanoemulsions was measured using dynamic light scattering instrument Zetasizer (Nano-ZS model ZEN3600, Nanoseries, Malvern Instruments, U.K). Measurements were done at 25°C, with a fixed angle of 172° using 1.46 as a refractive index for the dispersed particles. Sizes quoted are the z-average mean (\(d_z\)) for the particles hydrodynamic diameter (nm). Each nanoemulsion preparation was measured at least 3 times; data was reported as the mean volume percent distribution ± S.D.

2.5.2 Evaluation of basil nanoemulsion stability

Basil nanoemulsion was evaluated for temperature stability. Different concentrations of the nanoemulsion (1, 1.5, 2, 2.5, and 3 wt. \%) were incubated at 25°C and 37°C for 24 h and watched for stability.

2.6 Antibacterial evaluation of BO and BNO

The antibacterial activity of BNO was evaluated in comparison to the BO. Both formulas were tested against 6 pathogenic bacterial strains: *E. coli* O157:H7, *Staphylococcus aureus* (ATCC 6538), *Salmonella typhimurium*, *Bacillus cereus* B-3711, *Listeria monocytogenes* 598, and *Yersinia enterocolitica*. A concentration of 1.0 wt. % of BO was prepared by dissolving 3.33 mL corn oil-based BO in 6.67 mL absolute isopropyl alcohol. Similarly; 1.0 wt. % of BNO (Formula 4, Table 1) was also prepared by dissolving 3.33 mL of that formula in 6.67 mL absolute isopropyl alcohol. Then, 20 \(\mu\)L of each strain suspension was inoculated on the surface of nutrient agar plates using the cup-plate diffusion method. Each cup was filled with 50 \(\mu\)L of either 1.0% BO or 1.0% BNO. The plates were then incubated for 24 hours and inhibition zone diameters were

### Table 1 Composition of the different formulas that were tested for production of basil essential oil nanoemulsion.

| Formula No. | Surfactants (wt\%) | Basil essential oil (wt\%) | Propylene glycol (wt\%) | Water (wt\%) | Appearance of the final obtained formula* | Particle size (nm) and polydispersibility index (PDI) |
|-------------|---------------------|---------------------------|-------------------------|-------------|------------------------------------------|--------------------------------------------------|
| F1          | T80 (5.0)           | 3.0                       | 2.0                     | 90.0        | Milky white                              | –                                                 |
| F2          | T20 (5.0)           | 3.0                       | 2.0                     | 90.0        | Milky white                              | –                                                 |
| F3          | Cr RH40 (5.0)       | 3.0                       | 2.0                     | 90.0        | Milky white                              | –                                                 |
| F4          | (Cr RH40/T80, ratio 1:1) (5.0) | 3.0                       | 2.0                     | 90.0        | Translucent                              | 15.42 (PDI, 0.18)                                 |
| F5          | (Cr RH40/T80, ratio 3:1) (5.0) | 3.0                       | 2.0                     | 90.0        | Translucent                              | 17.47 (PDI, 0.22)                                 |
| F6          | (Cr RH40/T80, ratio 1:3) (5.0) | 3.0                       | 2.0                     | 90.0        | Translucent                              | 17.36 (PDI, 0.35)                                 |

*After equilibration for 1 week at room temperature
T80: Tween 80
T20: Tween 20
Cr: Cremophor

J. Oleo Sci.
measured. Each plate contained 3 cups and duplicate plates were used for each tested strain. Absolute isopropyl alcohol, tween 80, chremophor RH40, and propylene glycol were used as negative controls.

2.7 Preparation of diets
Two types of diets were used during the animal experiment. A balanced diet was prepared as previously mentioned. A high fructose-saturated fat diet (HFFD) was used to induce steatohepatitis as previously described with some modifications where vitamin mixture free from choline was used for preparation of the diet. Table 2 shows the composition of the two types of diets.

2.8 Design of animal experiment
Twenty four rats were divided into 4 groups with similar average body weight; each of 6 rats. Rats of group 1 were fed on a balanced diet and designated as control healthy group (C). Rats of group 2 were fed on HFFD and served as steatohepatitis control group (SC). Rats of group 3 were fed on HFFD throughout the experiment along with a daily oral dose of 150 mg BNO/kg rat body weight. Rats of group 4 were fed on HFFD with a daily oral dose of 50 mg BNO/kg rat body weight. It is worth indicating that BNO used in the oral dosing experiment is formula F4 (Table 1). That is due to its promising feature as nanoemulsion compared to the other 5 formulas, as discussed in details in the result section. During the experiment, which was continued for 5 weeks, food intake was recorded and animals were weighed once a week. At the end of the experiment, body weight gain, total food intake, and food efficiency ratio (body weight gain/total food intake) were calculated. Blood was centrifuged at 3000 rpm for 15 minutes to separate plasma for biochemical analysis. Rats were dissected; samples were collected from the colon content and immediately stored at −80°C until analyzed. Livers and colons were excised from all rats; livers were weighed and a part was taken from each liver and stored at −20°C until analyzed for lipid content. The rest of the livers and colons were kept in 10% formalin for histopathological examination.

2.8.1 Determination of plasma lipid profile, interleukin-6 and lipopolysaccharides and lipocalin-2 in plasma and colon content.
Plasma total cholesterol (TC), high density lipoprotein (HDL-C) and triglycerides (TG) were determined as described previously; the tests were carried out using commercial kits purchased from Salucea, Netherlands. Low density lipoprotein-cholesterol was calculated according to the equation reported previously. Plasma Interleukin-6 (IL-6) was measured as an inflammatory biomarker using enzyme-linked immuno-sorbent assay (ELISA) for rat (Koma Biotech, South Korea). Plasma lipopolysaccharides (LPS), plasma lipocalcin-2 (LCN2) and LCN2 in colon content were determined using ELISA for rat (SinoGene-Clon Biotech, China).

2.8.2 Determination of liver total lipids and liver index
Liver lipids were extracted using dichloromethane/methanol (2/1, v/v) and determined as reported previously. Liver index was calculated according to the formula; liver index = liver weight (g) × 100/body weight (g).

2.8.3 Real-time polymerase chain reaction (real-time PCR) (qPCR)
Total genomic DNA was extracted from colon content according to the manufacturer’s protocol (Quick-DNasection colon content/soil microbe Miniprep kit, Zymo Research, U.S.A. Cat. No. D6010). The DNA concentration and purity (A260/A280 ratio) was measured by NanoDrop spectrophotometer. DNA samples were analyzed for quantitatively determination of *Firmicutes* and *Bacteroidetes* using real time PCR. Real-time PCR was performed using a Rotor-Gene® MDx instrument. The reaction was performed in a total volume of 25 μL contained 2 μL genomic DNA, 4 μL EvaGreen® qPCR master mix plus (Solis BioDyne,

| Table 2 Composition of the experimental diets (g/100 g). |
|-----------------------------------------------------------|
| Ingredients                                               | Balanced diet | High fructose-saturated fat diet deficient in choline |
| Casein                                                    | 12            | 12                                           |
| Starch                                                    | 68.5          | 10                                           |
| Fructose                                                  | –             | 58.5                                         |
| Salt mixture                                              | 3.5           | 3.5                                          |
| Vitamin mixture                                           | 1             | –                                            |
| Vitamin mixture without choline chloride                  | –             | 1                                            |
| Corn oil                                                  | 10            | –                                            |
| Coconut oil: sheep tallow (1:1)                           | –             | 15                                           |
| Cellulose                                                 | 5             | –                                            |
Estonia. Cat. No. 08-24-00001) and 0.3 μM of the forward and reverse primers (Invitrogen, U.K.). Specific oligonucleotide primers were adopted from previous studies \(^{34, 35}\) (Table 3). Primers sequences were confirmed for specificity using NCBI BLAST database. The PCR reaction conditions for the amplification of DNA were 50°C for 2 min, 95°C for 15 s, 50 cycles of 15 s at 95°C, 60 s at 60°C, 15 s at 72°C, melting curve program (55-95°C). No template control was measured using PCR water instead of DNA templates. The copy numbers were calculated from standard curve and normalized to gram of colon content.

Genomic DNA of Lactobacillus plantarum (L. plantarum) was used as standard. L. plantarum genomic DNA was extracted as previously described from L. plantarum activated culture. The genomic DNA was used as the standard for determining the bacterial copy number by real-time PCR. L. plantarum specific primer sequence was adopted from a previous study \(^{35}\) (Table 3). Bacterial genomic DNA from L. plantarum was used to prepare 10 fold dilution series (10\(^{-2}\) to 10\(^{6}\) copies). A standard curve was generated by plotting the Ct value (the cycle number when the fluorescence of a PCR product can be detected above the background signal) against bacterial copy number. The mass for L. plantarum was calculated by using the Avogadro constant and assuming the mean molecular weight of a base pair to be 660 g/mol.

### 2.8.4 Histopathology

Histopathological examination was carried out in Animal Health Research Institute, Giza, Egypt. Formalin fixed tissue specimens (liver and colon) of all groups were routinely processed for paraffin blocks. Specimens were dehydrated in graded series of ethyl alcohol, cleared in xylene and embedded in paraffin. Sections of 4-5 microns thickness were prepared and stained by hematoxylin and eosin dye (H&E) as described previously \(^{36}\).

### 2.9 Statistical analysis

Results of animal experiment were expressed as mean ± SE. Different groups were compared using Kruskal-Wallis test followed by Mann Whitney test. Means were considered significantly different at \(p \leq 0.05\). Pearson’s correlation was used to determine the correlation between colonic microbiota and different parameters; \(p \leq 0.05\) denotes significant correlation, while \(p \leq 0.01\) points to highly significant correlation (SPSS, version 22).

## 3 Results

### 3.1 GC-MS analysis of basil essential oil

Table 4 shows the composition of Basil essential oil using GC-MS. The analysis revealed that BO contained 23 volatile components; the major components were Linalool (60.96%), eucalyptol (9.51%), eugenol (5.15%) and α-bergamotene (4.37%). The presence of α-epi-cadinol, β-elemen, Germacrene-D and α-epi-cadinol ranged from 1.98% to 1.36%. The percentage of each of the components was less than 1%.

### 3.2 Particle size analysis of BNO and selection of the ideal formula of BNO for usage in the biological assay

Six formulas of BO were fabricated as indicated in Table 1 using the same quantitative ingredients which differ only in the type of surfactants. The aim of this part of the study is to select an ideal formula which has the smallest particle size with the lowest polydispersibility index to be used in the biological evaluation. From Table 1, it is clear that formula F1-F3 are milky white in appearance indicating failure in formation of nanoemulsion. On the other hand formula F4, F5 and F6 were translucent pointing to potentials of being nanoemulsions.

Particle size analysis of these nanoemulsions formulas indicates that the three formulas had a close particle size diameter ranging from 15.42 nm to 17.47 nm (Table 1 and Fig. 1). However, formula F4 had the smallest polydispersibility index (PDI, 0.18) compared to formula F5 (PDI 0.22) and F6 (PDI 0.35) as shown in Table 1. In addition the second population of larger particles (2.04 peak) of formula F4 had the lowest intensity (4.0%) compared to F5 (11.5%) and F6 (26.4%) indicating narrow particle size distribution in almost single population (Fig. 1). Based on these findings, formula F4 which was fabricated using cremophor

### Table 3 Oligonucleotide primers used in the real-time PCR reaction.

| Primer name | Primer sequence 5‘-3’ | Annealing temperature (°C) | Product size (bp) | Reference No. |
|-------------|-----------------------|-----------------------------|------------------|---------------|
| BactF       | CATGTGGTTTAATTGCAGAT  | 60                          | 126              | 35            |
| BactR       | AGCTGACGACAACCATGCAG  |                             |                  |               |
| FirmF       | ATGTGGTTAATTCGAAGCA   | 60                          | 126              | 35            |
| FirmR       | AGCTGACGACAACCATGCAC  |                             |                  |               |
| PlantarumF  | TGGATCACCTCCTTCTAAGGAAT| 58                          | 144              | 34            |
| PlantarumR  | TTTCCTCGGTTCATTATGAAAAATA|                         |                  |               |
S. Y. A.-Okbi, M. A. Amin, A. E. A. Mohamed et al.

3.3 Evaluation of basil nanoemulsion stability

Different concentrations of BNO (1, 1.5, 2, 2.5, and 3 wt. %) were stable at 25°C and 37°C for 24 h.

3.4 Antibacterial test of BO and BNO

The BNO did not have an effect against any of the tested strains while BO showed a moderate effect against *Yersinia enterocolitica* with an average inhibition zone of 13.83 ± 0.31 mm. It also had an activity against *Staphylococcus aureus* (ATCC 6538) and *Listeria monocytogenes* 508 with mean inhibition zones of 11.5 mm ± 0.34 and 8 mm ± 0.00, respectively.

3.5 Results of the animal experiment

3.5.1 Nutritional parameters, liver index and liver total lipid content

Nutritional parameters (initial body weight, final body weight, body weight gain, total food intake and food efficiency ratio), liver weight, liver index and liver total lipid content of different experimental groups are shown in Table 5. All nutritional parameters showed insignificant change among the different experimental groups except for the group that received BO that demonstrated significant reduction in final body weight compared to C group. Liver weight and % liver weight/body weight of SC group showed significant increase compared to C. Liver weight and % liver weight/body weight of the treated groups (by BO and BNO) demonstrated insignificant changes from SC group. Total liver lipids of SC group was significantly higher than C group while total liver lipids of the test groups (BO and BNO) was significantly lower than that of SC group. The nano-form of basil oil produced significant reduction of liver lipids compared to C group.

3.5.2 Lipid profile, interleukin-6, lipopolysaccharides and lipocalin-2 levels

Plasma lipid profile is present in Table 6. There was significant increase in plasma TC, TG, LDL-C and TC/HDL-C with concomitant significant reduction of HDL-C in SC group compared to C. Treatment with BO produced significant reduction in plasma TC, TG and LDL-C and insignificant change in HDL-C and TC/HDL-C compared to SC group; all lipids levels matched that of C group. BNO only significantly reduced plasma TG compared to SC while insignificant changes were noticed in the other lipid parameters.

Figure 2 A-D demonstrated the levels of plasma LPS, IL-6 and LCN2 and LCN2 in colon content of different experimental groups. No significant differences were observed in plasma LPS among all groups. Plasma IL-6 and LCN2 showed significant increase in SC group compared to C. Treating rats with either BO or BNO produced significant reduction of plasma LCN2 compared to SC group that reaches the normal levels. Plasma IL-6 showed reduction on administration of either oils compared to C group but the reduction was only significant in case of BO that
Table 5 Nutritional parameters (initial body weight, final body weight, body weight gain, total food intake and food efficiency ratio), liver weight, liver index and liver total lipid content of different experimental groups.

| Parameters                  | Groups | C     | SC    | BO    | BNO   |
|-----------------------------|--------|-------|-------|-------|-------|
| Initial body weight (g)     |        | 156.3 ± 3.88 | 156.2 ± 2.41 | 156.3 ± 3.57 | 156.3 ± 4.02 |
| Final body weight (g)       |        | 205.60 ± 4.38 | 200.67 ± 3.34 | 189.67 ± 5.97 | 199.00 ± 10.23 |
| Body weight gain (g)        |        | 47.80 ± 4.32  | 44.50 ± 3.82  | 33.33 ± 6.82  | 40.00 ± 6.82  |
| Total food intake (g)       |        | 474.40 ± 35.46 | 556.83 ± 27.21 | 514.83 ± 22.51 | 531.75 ± 35.18 |
| Food efficiency ratio       |        | 0.11 ± 0.02  | 0.08 ± 0.01  | 0.07 ± 0.01  | 0.07 ± 0.01  |
| Liver weight (g)            |        | 5.65 ± 0.20  | 6.92 ± 0.14  | 6.90 ± 0.34  | 6.83 ± 0.42  |
| Liver index                 |        | 2.82 ± 0.06  | 3.45 ± 0.07  | 3.68 ± 0.19  | 3.42 ± 0.04  |
| Liver total lipids (mg/g tissue) | | 120 ± 6.83  | 184 ± 17.63  | 116 ± 9.17  | 60 ± 10.1  |

Values are Mean ± SE, n=6. Within the same row, means with different letters are significantly different at p ≤ 0.05.
C: Normal healthy control, SC: control group with steatohepatitis, BO: Essential oil, BNO: Basil oil nano-form.
Liver index: Liver weight/body weight %.

Table 6 Lipid profile (Plasma TC, TG, HDL-C, LDL-C and TC/HDL-C) of different experimental groups.

| Parameters       | Groups | C      | SC     | BO     | BNO     |
|------------------|--------|--------|--------|--------|---------|
| TC (mg/dL)       |        | 98.52 ± 5.30 | 114.31 ± 3.79 | 95.93 ± 3.22 | 112.91 ± 1.66 |
| TG (mg/dL)       |        | 94.36 ± 4.09 | 131.26 ± 8.98 | 102.67 ± 7.21 | 114.36 ± 10.47 |
| HDL-C (mg/dL)    |        | 48.92 ± 1.74 | 37.85 ± 0.76  | 41.65 ± 3.92  | 39.63 ± 2.39  |
| LDL-C (mg/dL)    |        | 30.73 ± 6.59 | 50.21 ± 3.15  | 33.75 ± 4.97  | 50.41 ± 4.70  |
| TC/HDL-C         |        | 2.04 ± 0.17  | 3.02 ± 0.09  | 2.41 ± 0.24  | 2.88 ± 0.19  |

Values are Mean ± SE, n=6. Within the same row, means with different letters are significantly different at p ≤ 0.05.
C: Normal healthy control, SC: Control with steatohepatitis, BO: Essential oil, BNO: Basil oil nano-form.
TC: Total cholesterol, HDL-C: High density lipoprotein-cholesterol, LDL-C: Low density lipoprotein-cholesterol.

matched the normal level. However it could be noticed that the level of IL-6 of BNO group is intermediate between SC and BO groups and that there is no significant change from that of BO. LCN2 of colon content was significantly elevated in SC group compared to C. Receiving BO or BNO produced insignificant change in LCN2 compared to SC group while that of BO group did not demonstrate any significant change from C group.

3.5.3 Real-time PCR

Quantification of colon **Firmicutes** and **Bacteroidetes** phyla using real-time PCR revealed different levels of both phyla in different experimental groups (Fig. 3A). The count of **Bacteroidetes** was lower in SC group compared to the C group, while the count of **Firmicutes** was only slightly lower. These differences, however, were not statistically significant. Counts of **Firmicutes** were insignificantly higher in the BO group compared to C group while those of **Bacteroidetes** were insignificantly higher. BNO group showed **Bacteroidetes** counts significantly higher than the SC group. The counts were also higher than that of C group although the increase was not significant. In comparison to SC group, BO group had comparable **Firmicutes** and **Bacteroidetes** counts.

Looking at the **Firmicutes/Bacteroidetes** ratio of different groups (Fig. 3B), a significant increase can be observed in SC group compared to C group. Basil oil did not change the **Firmicutes/Bacteroidetes** ratio compared to the SC group while BNO, on the other hand, reduced the ratio significantly to a value similar to the healthy control.

Statistical correlation study of colon microbiota (**Firmicutes**, **Bacteroidetes** and **Firmicutes/Bacteroidetes**) with all other biochemical and nutritional parameters showed significant negative correlation between liver lipids and **Bacteroidetes** phylum count present in colon content (r = -0.541, p < 0.01). No other significant correlations were observed.
3.5.4 Histopathological examination of liver and colon

Histopathological examination of liver and colon of different groups (Figs. 4 and 5) revealed remarkable changes in treatment and control groups. Healthy control group (Fig. 4A) showed normal structure of hepatocytes. On the other hand, severe inflammation was observed in SC group (Fig. 4B) which was marked by diffuse fatty degeneration in the hepatocytes, congestion of liver blood vessels, and accumulation of fat vacuoles all over the tissue. Both BO and BNO groups showed noticeable improvement in liver tissues. Hepatocytes of the BO group had mild fatty degenerative changes compared to SC (Fig 4C). Similarly, BNO group (Fig. 4D) showed remarkable correction of fatty degeneration in the liver with much less focal areas. Table 7 demonstrated the scores of histopathological changes in livers of different groups which clarified that BNO adminis-
Alleviating Steatohepatitis by Basil Essential Oil and Its Nano-form

J. Oleo Sci.

4 Discussion

The pathophysiology of steatohepatitis is not fully understood; different multiple hits are proposed including intestinal dysbiosis, inflammatory cytokines, lipid metabolism alteration, insulin resistance, oxidative stress and endoplasmic reticulum stress. In a previous study, high fructose diet induced loss of the diversity of gut microbiota with lower proportion of Bacteriodetes and increased proportion of Proteobacteria which accompanied by inflammation that caused alteration in the tight junction protein leading to gut permeability. The aforementioned changes precede the development of dyslipidemia, liver inflammation, lipid accumulation and metabolic endotoximia which lead to steatohepatitis with normal weight obesity. Gut permeability induced by fructose results in translocation of bacteria and bacterial endotoxin into blood stream. So, keeping on a healthy balance of microbiota is highly important. Fructose produced reduction of beneficial Bifidobacterium and Lactobacillus species along with increase in clostridium. Also the level of the inflammatory biomarkers IL-6 and TNF-α were elevated in adipose tissue on fructose consumption. The SC group in the present study which is the control fed high fructose showed similar results to the above mentioned.
studies represented by elevated IL-6 and liver lipids, dyslipidemia, imbalance of colonic microflora, erosion and detachment of colon mucus epithelium and high proliferation of mucus secreting cells with moderate leukocytic infiltration (H&E ×200). C: Colon of the BO group with marked regeneration signs of the colon mucosa and very high proliferation of mucus secreting glands. (H&E ×200), D: Marked improvement in the colon of the BNO group with remarkable regeneration of colon mucosa (H&E ×200). SC: Control with steatohepatitis, BO: Essential oil, BNO: Basil oil nano-form.

Table 7  Scores of histopathological changes of the liver of different experimental groups.

| Pathological Changes          | C    | SC   | BO   | BNO |
|-------------------------------|------|------|------|-----|
| Hepatic degeneration          | _    | +++  | ++   | +   |
| Fat vacuoles                  | _    | +++  | ++   | +   |
| Necrosis of hepatocytes       | _    | +++  | ++   | +   |
| Activation of fibroblasts     | _    | ++   | +    | _   |

- : Negative, +: Slight changes, ++: Moderate changes, +++: Advanced changes.

C: Normal healthy control, SC: control group with steatohepatitis, BO: Essential oil, BNO: Basil oil nano-form.

Gradual persistent gut microbiota and dysbiosis from 2 to 4 weeks were reported in mice fed choline-methionine deficient diet with reduction in *Bifidobacterium* and promotion of *Bacteroides* abundance. That report support the colonic dysbiosis observed in the present study on feeding high fructose saturated fat choline-deficient diet. In healthy state, the crosstalk between immune system and intestinal microbiota enforce the symbiotic between liver. Choline deficient diet thereby promoted accumulation of TG in liver resulting in steatohepatitis and fibrosis. Gradual persistent gut microbiota and dysbiosis from 2 to 4 weeks were reported in mice fed choline-methionine deficient diet with reduction in *Bifidobacterium* and promotion of *Bacteroides* abundance. That report support the colonic dysbiosis observed in the present study on feeding high fructose saturated fat choline-deficient diet.
Alleviating Steatohepatitis by Basil Essential Oil and Its Nano-form

Table 8: Scores of histopathological changes of the colon of different experimental groups.

| Pathological Changes                  | C   | SC  | BO  | BNO |
|--------------------------------------|-----|-----|-----|-----|
| Erosion of outer mucosa layer        | +   | ++  | +   | +   |
| Proliferation of mucous cells        | +   | +++ | +++ | ++  |

+: Within normal limit, ++: Moderate increase, +++: Advanced increase.
C: Normal healthy control, SC: control group with steatohepatitis, BO: Essential oil, BNO: Basil oil nano-form.

the host and the commensal bacteria; therefore dysbiosis in certain chronic diseases could lead to adverse effect on the host. *Firmicutes* and *Bacteriodetes* phyla contribute about 90% of total bacterial population of intestinal microbiota. An increased *Firmicutes* and reduced *Bacteriodetes* as the case in the present study were reported during obesity and steatohepatitis. The role of such dysbiosis is based on the increased capacity of microbiota to harvest energy from diet. Not only the microbiome composition but also the microbial genome and its function determine the fatty liver prone state. Gut microbiota control pro-inflammatory signaling pathways like toll-like receptors (TLR) and Nod-like receptors (NLR) as part of their impact on the innate immune system. When the balance in gut microbiota is lost, it triggers one or more of these pathways which leads to low grade inflammation and metabolic endotoxemia. The elevated IL-6 an inflammatory cytokine in the present study demonstrated the incidence of such inflammation due to dysbiosis. The relationship between cytokines and the pathogenesis of NAFLD is a complex process that involves interactive endocrine and paracrine mechanisms. Elevated IL-6 has been linked to increased insulin resistance. The function of IL-6 in the inflammatory process is multifactorial. It is a cytokine that regulates innate immunity and homeostasis. In the liver, IL-6 stimulates some acute phase proteins like CRP and PTX3. It has been also associated with hepatic lipogenesis in insulin resistance and obesity. Some studies correlate high serum IL-6 levels with NAFLD.

The translocation of gut microflora or their metabolites reported in fatty liver might be indicated by the presence of LPS, the cell wall component of gram negative bacteria. Translocation might occur due to bacterial overgrowth, increased mucosa intestinal permeability and impaired immunity and which may lead to complication of steatohepatitis like liver cirrhosis, morbidity and mortality. The use of antibiotic like natural agent might lead to selective intestinal decontamination thereby modulating intestinal microbiota. In the present research although the histopathological results of colon group showed erosion and detachment of mucosal epithelium of SC group however yet no translocation has been observed represented by LPS which might explained on the basis that if the time of experiment was elongated resulting in severe injury in intestinal wall, then translocation might occur as reported previously after 17 week in animal experiment.

In previous studies feeding rat similar diets to that used in the present study produced elevation of transaminases, total bilirubin and direct bilirubin demonstrating liver dysfunction, which support the induction of steatohepatitis in the present study. Further; the elevated LCN2 and IL-6 in the present study indicated liver damage and inflammation in the SC group together with the significant deposition of liver fat and the histopathology changes in liver pointed to deposition of fat, inflammation and starting of fibrosis which are typical changes in steatohepatitis.

LCN2 has a great role in liver homeostasis. A growing evidence suggests a link between LCN2 upregulation and liver inflammation in NASH. The increase in LCN2 expression is a strong biomarker of fatty liver, hepatic inflammation and hepatic damage; so it is considered as a putative NASH marker. Some pro-inflammatory cytokines like IL-B can induce the release of LCN2, so it might be assumed that the hepatoprotective function of LCN2 accounts for its elevated levels in NAFLD. It is suggested that LCN2 is somehow connected to hepatic lipid uptake. LCN2 has the capacity to limit bacterial growth by sequestering iron, thereby may affect microflora balance. LCN2 prevents intestinal inflammation through enhancing phagocytic bacterial clearance in macrophages and it is considered as a master mediator of intestinal and metabolic inflammation. It was reported that LCN2 act in a lipid-independent manner to protect the liver from fructose induced damage; so the elevated LCN2 in SC group might reflect its use a biomarker of NASH and might be a compensatory mechanism to protect the liver. New therapies for NASH must target such biomarker.

Treatment with either basil oil forms produced reduction in plasma IL-6, LCN2, liver lipids and plasma TG and improved histopathological changes compared to SC group. BNO was superior in reducing F/B ratio, liver lipids and histopathological changes of liver and colon. BO was more
efficient in reducing TC, triglycerides and low density lipoprotein cholesterol. These results indicated anti-inflammatory and/or antioxidant activity, lipid lowering and liver protective effect of both basil oil forms which might be attributed to the synergistic effect of the volatile constituents present in BO specially linalool (60.9%), eugenol (5.15%), eucalyptol (9.5%) and α-bergamotene (4.37%). Improving F/B ratio and liver lipids on treatment with BNO with half the dose compared to BO might pointed to the beneficial effect of formulating BO into nano-form that might lead to increased bioavailability of BO even in a reduced dose which could be reflected in the enhanced reduction of liver lipids and F/B ratio and boosting the elevation of both Firmicutes and Bacteriodetes phyla induced by BNO compared to BO. The reduction of particle size of BO into nano level might also change some of the properties of the oil that related to its bioactivity as could be noticed from the abolishing of the hypocholesterolemic effect, the reduction of plasma LDL-C and the decrease in colon content of LCN2 along with the eradication of reduction in IL-6 induced by BO. However the level of IL-6 was insignificantly reduced compared to SC in case of BNO. Also it could be noticed that the level of IL-6 in BNO group is intermediate between SC and BO groups and that there is no significant change from that of BO which might suggest that if the experimental period extended a significant reduction of IL-6 might be demonstrated by BNO.

5 Conclusion
Administration of BO and BNO reduced the progression of nonalcoholic steatohepatitis in rat model. BNO was more promising in reducing F/B ratio, liver lipids and the histopathological changes in liver and colon compared to BO. On the other hand, BO was superior in reducing plasma TC, triglycerides and low density lipoprotein cholesterol. Changes in colon microbiota specially F/B ratio might be linked to nonalcoholic steatohepatitis and should be well investigated in prospective researches.

Acknowledgments
The research was carried out and financed by National Research Centre, Egypt.

Conflict of Interest Statement
The authors declare that they have no conflict of interest.

References
1) Chassaing, B.; Gewirtz, A.T. Gut microbiota, low-grade inflammation, and metabolic syndrome. Toxicol
Alleviating Steatohepatitis by Basil Essential Oil and Its Nano-form

J. Oleo Sci. 42, 49-53 (2014).

2) Chu, H.; Williams, B.; Schnabl, B. Gut microbiota, fatty liver disease, and hepatocellular carcinoma. Liver Res. 2, 43-51 (2018).

3) Kumar, D.; Tandon, R.K. Use of ursodeoxycholic acid in liver diseases. J. Gastroenterol. Hepatol. 16, 3-14 (2001).

4) Matafome, P.; Louro, T.; Rodrigues, L.; Crisostomo, J.; Nunes, E.; Amaral, C.; Monteiro, P.; Cipriano, A.; Seica, R. Metformin and atorvastatin combination further protect the liver in type 2 diabetes with hyperlipidemia. Diabetes Metab. Res. Rev. 27, 54-62 (2011).

5) Reardon, J.; Hussaini, T.; Alshahfi, M.; Azalghara, V.M.; Erb, S.R.; Partovi, N.; Yoshida, E.M. Ursodeoxycholic acid in treatment of non-alcoholic fatty liver diseases: A systematic review. J. Clin. Transit. Hepatol. 4, 192-205 (2016).

6) Al-Okbi, S.Y.; Mohamed, D.A.; Hamed, T.E.; Edris, A.E. Potential protective effect of Nigella sativa crude oils towards fatty liver in rats. Eur. J. Lipid Sci. Technol. 115, 774-782 (2013).

7) Al-Okbi, S.Y.; Mohamed, D.A.; Hamed, T.E.; Esmaiel, R.S.H. Rice bran oil and pumpkin seed oil alleviate oxidative injury and fatty liver in rats fed high fructose diet. Pol. J. of Food Nutr. Sci. 64, 127-133 (2014).

8) Al-Okbi, S.Y.; Mohamed, D.A.; Hamed, T.E.; Edris, A.E. 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. Grasas y Aceites 66, e103 (2015).

9) Al-Okbi, S.Y.; Mohamed, D.A.; Hamed, T.E.; Esmaiel, R.S.H.; Kareem Fouda. Hepatic regeneration and reno-protection by fish oil, Nigella sativa oil, and combined fish oil/Nigella sativa volatiles in CC14-treated rats. J. Oleo Sci. 67, 345-353 (2018).

10) Miguel, M.G. Antioxidant and anti-inflammatory activity of essential oils: A short review. Molecules 15, 9252-9287 (2010).

11) Ogaly, H.A.; Eltahlawy, N.A.; El-Behairy, A.M.; El-Hindi, H.; Abd-Elsalam, R.M. Hepatocyte growth factor mediates the antiﬁbrogenic action of Ocimum basilicum essential oil against CC14-induced liver ﬁbrosis in rats. Molecules 20, 13518-13535 (2015).

12) Samojlik, I.; Lækic, N.; Mimica-Dukic, N.; Dakovic-Svajic, K.; Bozin, B. Antioxidant and hepatoprotective potential of essential oils of coriander (Coriandrum sativum L.) and caraway (Carum carvi L.). J. Agric. Food Chem. 58, 8848-8853 (2010).

13) Issazadeh, K.; Pahlaviiani, M.R.; Massiha, A.; Bidarigh, S.; Ghai, M.; Muradov, P.Z. Analysis of the phytochemical contents and anti-microbial activity of Ocimum basilicum L. Int. J. Mol. Clin. Microbiol. 2, 141-147 (2012).

14) Benedec, D.; Parvu, A.E.; Oniga, I.; Toiu, A.; Tiperciuc, B. Effects of Ocimum basilicum L. extract on experimental acute inflammation. Rev. Med. Chir. Soc. Med. Nat. Iasi. 111, 1065-1069 (2007).

15) El-Beshbishy, H.; Bahashwan, S. Hypoglycemic effect of basil (Ocimum basilicum) aqueous extract is mediated through inhibition of α-glucosidase and α-amylase activities: An in vitro study. Toxicol. Ind. health. 28, 42-50 (2012).

16) Cani, P.D.; Osto, M.; Geurts, L.; Everard, A. Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. Gut Microbes 3, 279-282 (2012).

17) Miura, K.; Ohnishi, H. Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease. World J. Gastroenterol. 20, 7381-7391 (2014).

18) Porras, D.; Nistal, E.; Martinez-Florez, S.; Gonzalez-Gallego, J.; Garcia-Medivilla, M.V.; Sanchez-Campos, S. Intestinal microbiota modulation in obesity-related non-alcoholic fatty liver disease. Front. Physiol. 9, 1813 (2018).

19) Nathan, C. Epidemic inflammation; Pondering obesity. Mol. Med. 14, 485-492 (2008).

20) Hawrelak, J.A.; Cattley, T.; Myers, S.P. Essential oils in the treatment of intestinal dysbiosis: A preliminary in vitro study. Altern. Med. Rev. 14, 380-384 (2009).

21) Hamed, S.F.; Sadek, Z.; Edris, A.E. Antioxidant and antimicrobial activities of clove bud essential oil and eugenol nanoparticles in alcohol-free microemulsion. J. Oleo Sci. 61, 641-648 (2012).

22) Mondal, S.; Mirdha, B.R.; Mahapatra, S.C. The science behind sacredness of Tulsi (Ocimum sancum) Linn.. Indian J. Physiol. Pharmacol. 53, 291-306 (2009).

23) El-Sayed, H.S.; Chizzola, R.; Ramadán, A.A.; Edris, A.E. Chemical composition and antimicrobial activity of garlic essential oils evaluated in organic solvent, emulsifying, and self-microemulsifying water based delivery systems. Food Chem. 221, 196-204 (2017).

24) Al-Okbi, S.Y.; Abdel-Razek, A.G.; Mohamed, S.E.; Ottai, M.E. Roselle seed as a potential new source of healthy edible oil. J. Biol. Sci. 17, 267-277 (2017).

25) Kawasaki, T.; Igarashi, K.; Koeda, T.; Sugimoto, K.; Nakagawa, K.; Hayashi, S.; Yamaji, R.; Inui, H.; Fukusato, T.; Yamanoouchi, T. Rats fed fructose-enriched diets have characteristics of nonalcoholic hepatic Steatosis. J. Nutr. 139, 2067-2071 (2009).

26) Denimicz, R.; de Castro, G.S.F.; Francisco, L.V.; da Silva, L.E.C.M.; Cardoso, J. F.R.; Frajacomo, F. T.; Tedoro, B.G.; Silveira, L.R.; Jordao, A.A. Creatine supplementation prevents fatty liver in rats fed choline-deficient diet: A burden of one-carbon and fatty acid metabolism. J. Nutr. Biochem. 26, 391-397 (2015).

27) Watson, D. A simple method for the determination of serum cholesterol. Clinica Chim. Acta 5, 637-643 (1960).
28) Burstein, M.; Scholnick, H.R.; Morfin, R. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11**, 583-595 (1970).

29) Megraw, R.; Dunn, D.; Biggs, H. Manual and continuous flow colorimetry of triglycerols by a fully enzymatic method. *Clin. Chem.* **25**, 273-284 (1979).

30) Friedewald, W.T.; Levy, R.I.; Fredrickson, D.S. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18**, 499-502 (1972).

31) Odeh, M.; Sabo, E.; Srugo, I.; Oliven, A. Serum levels of tumor necrosis factor-alpha correlate with severity of hepatic encephalopathy due to chronic liver failure. *Liver International* **24**(2), 110-116 (2004).

32) Folch, J.; Lees, M.; Stanley, G. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226**, 479-500 (1957).

33) Cequier-Sánchez, E.; Rodriguez, C.; Ravelo, G.; Zarate, 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).

34) Haarmon, M.; Knol, J. Quantitative real-time PCR analysis of fecal *Lactobacillus* species in infants receiving prebiotic infant formula. *Appl. Environ. Microbiol.* **72**, 2359-2365 (2006).

35) Guo, X.; Xia, X.; Tang, R.; Zhou, J.; Zhao, H.; Wang, K. Development of a real-time PCR method for *Firmicutes* and *Bacteroidetes* in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett. Appl. Microbiol.* **47**, 367-373 (2008).

36) Bancroft, J.D.; Gamble, M. *Theory and practice of histological techniques*. Churchill Livingstone Elsevier, London (2008).

37) Buzzetti, E.; Pinzani, M.; Tschatzis, E.A. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *J. Metab.* **65**, 1038-1048 (2016).

38) Al-Okbi, S.Y. Role of nutraceuticals in prevention of non-alcoholic fatty liver in *Plant- and marine-based phytochemicals for human health: Attributes, potential, and use* (Goyal, M.R.; Chauhan, D.N. eds.). Apple Academic Press, Taylor and Francis, pp.131-149 (2019).

39) Do, M.H.; Lee, E.; Oh, M.J.; Kim, Y.; Park, H.Y. High-Glucose or -fructose diet cause changes of the gut microbiota and metabolic disorders in mice without body weight change. *Nutrients* **10**, 761 (2018).

40) Lambertz, J.; Berger, T.; Mak, T.W.; van Helden, J.; Weiskirchen, R. Lipopolacin-2 in fructose-induced fatty liver disease. *Front. Physiol.* **8**, 964 (2017).

41) Hsieh, F.C.; Lee, C.L.; Chai, C.Y.; Chen, W.T.; Lu, Y.C.; Wu, I., C.S. Oral administration of *Lactobacillus reuteri* GMNL-263 improves insulin resistance and ameliorates hepatic steatosis in high fructose-fed rats. *Nutr. Metab.** **10**, 38 (2013).

42) Ghoshal, A.K. New insight into the biochemical pathology of liver in choline deficiency. *Crit. Rev. Biochem. Mol.* **30**, 263-273 (1995).

43) Vetelainen, R.; Van Vliet, A.; Van Gulik, T.M. Essential pathogenic and metabolic differences in steatosis induced by choline or methionine-choline deficient diets in a rat model. *J. Gastroenterol. Hepatol.* **22**, 1526-1533 (2007).

44) Ye, J.Z.; Li, Y.T.; Wu, W.R.; Shi, D.; Fang, D.Q.; Yang, L.Y.; Bian, X.Y.; Wu, J.J.; Wang, Q.; Jiang, X.W.; Peng, C.G.; Ye, W.C.; Xia, P.C.; Li, L.J. Dynamic alterations in the gut microbiota and metabolome during the development of methionine-choline-deficient diet-induced nonalcoholic steatohepatitis. *World J. Gastroenterol.* **24**, 2468-2481 (2018).

45) Ley, R.E.; Turnbaugh, P.J.; Klein, S.; Gordon, J.I. Microbial ecology: Human gut microbes associated with obesity. *Nature* **444**, 1022-1023 (2006).

46) Zhu, L.; Baker, R.D.; Baker, S.S. Gut microbiome and nonalcoholic fatty liver diseases. *Pediatr. Res.* **77**, 245-251 (2015).

47) Rabot, S.; Membrez, M.; Bruneau, A.; Gérard, P.; Harbach, T.; Moser, M.; Raymond, F.; Mansourian, R.; Chou, C.J. Germ-free C57BL/6J mice are resistant to high-fat-diet-induced insulin resistance and have altered cholesterol metabolism. *FASEB J.* **24**, 4948-4959 (2010).

48) Geurts, L.; Lazarevic, V.; Derrien, M.; Everard, A.; Van Roye, M.; Knauf, C.; Valet, P.; Girard, M.; Muccioli, G.G.; François, P.; de Vos, W.M.; Schrenzel, J.; Delzenne, N.M.; Cani, P.D. Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue. *Front. Microbiol.* **2**, 149 (2011).

49) Polyzos, S.A.; Kountouras, J.; Zavos, C. Nonalcoholic fatty liver disease: The pathogenic roles of insulin resistance and adipocytokines. *Curr. Mol.* **9**, 299-314 (2009).

50) Ferreira, V.S.G.; Pernambuco, R.B.; Lopes, E.P.; Morais, C.N.; Rodrigues, M.C.; Arruda, M.J.; Silva, L.M.; Vilar, L. Frequency and risk factors associated with non-alcoholic fatty liver disease in patients with type 2 diabetes mellitus. *Arq. Bras. Endocrinol. Metab.* **54**, 362-368 (2010).

51) Jarrar, M.H.; Baranova, A.; Collantes, R.; Ranard, B.; Stepanova, M.; Bennett, C.; Fang, Y.; Elariny, H.; Goodman, Z.; Chandhoke, V.; Younossi, Z.M. Adipokines and cytokines in non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* **27**, 412-421 (2008).

52) Lin, R.S.; Lee, F.Y.; Lee, S.D.; Tsai, Y.T.; Lin, H.C.; Lu, R.H.; Hsu, W.C.; Huang, C.C.; Wang, S.S.; Lo, K.J. Endotoxemia in patients with chronic liver diseases: rela-
Alleviating Steatohepatitis by Basil Essential Oil and Its Nano-form

J. Oleo Sci.

53) Pinzone, M.R.; Celesia, B.M.; DiRosa, M.; Cacopardo, B.; Nunna, G. Microbial translocation in chronic liver diseases. Int. J. Microbiol. 2012, 694629 (2012).

54) Jin, X.; Zeng, D.; Wang, H.; Ni, X.; Yi, D.; Pan, K.; Jing, B. Preventing non-alcoholic fatty liver disease through Lactobacillus johnsonii BS15 by attenuating inflammation and mitochondrial injury and improving gut environment in obese mice. Appl. Microbiol. Biotechnol. 98, 6817-6829 (2014).

55) Borkham-Kamphorst, E.; Drews, F.; Weiskirchen, R. Induction of lipocalin-2 expression in acute and chronic experimental liver injury moderated by pro-inflammatory cytokines interleukin-1B through nuclear factor-kB activation. Liver Int. 31, 656-665 (2011).

56) Sembra, T.; Nishimura, M.; Nishimura, S.; Ohara, O.; Ishige, T.; Ohno, S.; Nonaka, K.; Sogawa, K.; Satoh, M.; Sawai, S.; Matsushita, K.; Imazeki, F.; Yokosuka, O.; Nomura, F. The FLS (Fatty liver Shionogi) model reveals local expressions of lipocalin-2, CXCL1 and CXCR2 in the liver with non-alcoholic steatohepatitis. BMC Gastroenterol. 13, 120 (2013).

57) Asimakopoulou, A.; Weiskirchen, S.; Weiskirchen, R. Lipocalin 2 (LCN2) Expression in Hepatic Malfunction and Therapy. Front. Physiol. 7, 430 (2016).

58) Labbus, K.; Henning, M.; Borkham-Kamphorst, E.; Geisler, C.; Berger, T.; Mak, T.W.; Knuchel, R.; Meyer, H.E.; Weiskirchen, R.; Henkel, C. Proteomic profiling in lipocalin 2 deficient mice under normal and inflammatory conditions. J. Proteomics 78, 188-196 (2013).

59) Berger, T.; Togawa, A.; Duncan, G.S.; Elia, A.J.; You-Ten, A.; Wakeham, A.; Fong, H.E.H.; Cheung, C.C.; Mak, T.W. Lipocalin 2-deficient mice exhibit increased sensitivity to Escherichia coli infection but not to ischemia-reperfusion injury. Proc. Natl. Acad. Sci. USA 103, 1834-1839 (2006).

60) Toyonaga, T.; Matsuura, M.; Mori, K.; Honzawa, Y.; Minami, N.; Yamada, S.; Kobayashi, T.; Hibi, T.; Nakase, H. Lipocalin 2 prevents intestinal inflammation by enhancing phagocytic bacterial clearance in macrophages. Sci. Rep. 6, 35014 (2016).

61) Moschen, A.R.; Adolph, T.E.; Gerner, R.R.; Wieser, V.; Tilg, H. Lipocalin-2: A master mediator of intestinal and metabolic inflammation. Trends Endocrinol. Metab. 28, 388-397 (2017).

62) Nagababu, E.; Rikkind, M.; Boindala, S.; Nakka, L. Assessment of antioxidant activity of eugenol in vitro and in vivo. Methods Mol. Biol. 610, 165-180 (2010).

63) Al-Okbi, S.Y.; Mohamed, D.A.; Hamed, T.E.; Edris, A.E. Protective effect of clove oil and eugenol microemulsions on fatty liver and dyslipidemia as components of metabolic syndrome. J. Med. Food 17, 764-771 (2014).

64) Pandey, A.K.; Singh, P.; Tripathi, N.N. Chemistry and bioactivities of essential oils of some Ocimum species: An overview. Asian Pac. J. Trop. Biomed. 4, 682-694 (2014).

65) Perera, I.; Severino, P.; Santos, A.C.; Silva, A.M.; Souto, E.B. Linalool bioactive properties and potential applicability in drug delivery systems. Colloids Surf. B. 171, 566-578 (2018).

66) Wang, L.; Zhang, Y.; Fan, G.; Ren, J.N.; Zhang, L.L.; Pan, S.Y. Effects of orange essential oil on intestinal microflora in mice. J. Sci. Food Agric. 99, 4019-4028 (2019).

67) Seol, G.H.; Kim, K.Y. Eucalyptol and its role in chronic diseases. Adv. Exp. Med. Biol. 929, 389-398 (2016).

68) Cable, E.E.; Fan, P.D.; Stebbins, J.W.; Hou, J.; Ito, B.R.; van Poelje, P.D.; Linemeyer, D.L.; Erion, M.D. Reduction of hepatic steatosis in rats and mice after treatment with a liver-targeted thyroid hormone receptor agonist. Hepatology 9, 407-4017 (2009).