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Phytochemical and biological investigation of the extracts of *Nigella sativa* L. seed waste

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Different extracts of *Nigella sativa* L. seed waste; aqueous (AE) 200 mg/kg, ethanol 70% (EE) 250 mg/kg and hexane (HE) 10 mg/kg, were evaluated for their hepatoprotective activities. They were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of carbon tetrachloride (10 mg/kg b. w. of 0.25% (*v*/*v*). Hepatoxicity produced, was evaluated by both biochemical and histopathological investigations. The aqueous extract attenuated the CCl_4-induced liver damage likely due to the decrease of proinflammatory cytokines and T-cell proliferation. This was noticed by a significant decrease in both serum and tissue cytokines; tumor necrosis factor-alpha (TNF-α), interferon-gamma (INF-γ) and interlukin-beta (IL-1β), in the markers of liver functions; bilirubin and glutamic pyruvic transaminase (GPT) and in the oxidative stress markers; malondialdehyde (MDA) and glutathione content (GSH). Fractionation of this extract was performed and its component, protein, saponin, and polyphenol fractions were evaluated by appropriate analytical procedures. The crude protein of the seed waste reached 36.85% while protein fingerprint showed four bands ranging from 91.97 KD and 29.00 KD. The saponin content was evaluated through the determination of the haemolytic index and reached 15.56 mg/g dry powder. Finally, Folin Ciocalteu method was used for the determination of the total polyphenols. The same biochemical and histopathological studies were again performed on the different fractions of the aqueous extract; protein fraction (PF) 10 mg/kg, saponin fraction (SF) 5 mg/kg and polyphenol fraction (FF) 10 mg/kg. The biochemical changes were improved only by the protein fraction (PF) of the seed waste of *Nigella sativa*. This was manifested by a significant reduction in both serum and tissue cytokines in the liver markers and in the oxidative stress markers. Moreover, liver histopathology showed that (PF) reduced the incidence of liver lesions including hepatic cells cloudy swelling, lymphocytes infiltration, hepatic necrosis and fibrous connective tissue proliferation induced by CCl_4 in mice. From this study, it is concluded that the protein fraction of the aqueous extract of *Nigella sativa* seed waste exhibited a promising hepatoprotective effect in the management of different liver disorders.

Keywords: *Nigella sativa* seed waste; immunostimulant aqueous extract

Introduction

Nigella sativa L. seeds known as black seed or black cumin, Family Ranunculaceae, have been used for medicinal purposes for centuries in Asia, the Middle East, and Africa.[1] More than 150 studies conducted since 1959 confirmed the effectiveness of *Nigella sativa* seed constituents.[2] It has been traditionally used as a natural remedy for a number of ailments that include asthma, chest congestion, hypertension, diabetes, inflammation, cough, bronchitis, headache, fever, dizziness, and influenza and for general well-being.[1,3–5] The plant has been extensively studied pharmacologically to justify its broad traditional therapeutic value, from which, it was found to have hepatoprotective[6] and immunopotentiating properties.[2,7–8] *Nigella sativa* seeds contain a complex mixture of more than 100 compounds, some of which have not yet been identified or studied. A combination of volatile oils, fatty acids, flavonoids, saponins, proteins, and trace elements are believed to contribute to its effectiveness.[3] It was found that both the oil and their active ingredients of the seeds, in particular thymoquinone (TQ), possess reproducible anti-oxidant effects through enhancing the oxidant scavenger system, which as a consequence lead to antitoxic effects induced by several insults. The oil and TQ have shown also potent anti-asthmatic[5] and anti-inflammatory effects on several inflammation-based models including experimental encephalomyelitis, colitis, peritonitis, oedema, and arthritis through suppression of the inflammatory mediators' prostaglandins and leukotriens.[9,10] The oil and certain active ingredients showed beneficial immunomodulatory properties, augmenting the T cell- and natural killer cell-mediated immune responses.[2] The aim of this work is to determine and investigate bioactive seed waste extracts focusing on the constituents responsible for activity. Moreover, the potential beneficial hepatoprotective and immunopotentiating effects of the chosen extract were evaluated.

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Material and Methods

Plant material

Nigella sativa L. seed waste were collected from local mills in Cairo, Egypt (2008).

Animals

Male Albino mice were obtained from the National Scientific Research Centre (Giza, Egypt), fed a standard pellet chow (El-Nasr Chemical Company, Cairo, Egypt) and had free access to water. All animals were maintained on a 12-h light, 12-h dark cycle and housed for 1 week before experimentation. Mice weighing between 26 and 30 g on the day of the experiment were used. This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy Cairo University, Cairo, Egypt.

Apparatus and solvents

Heating mantle 5 litres capacity, electric balance four digits and hot plate with magnetic stirrer. ELISA was used for estimation of TNF-α, IL-1β and IFN-γ. All chemicals were of the analytical grade.

Immunostimulant kits

Tumor necrosis factor-alpha (TNF- α), interlukin-beta (IL-1β) and interferon-gamma (IFN- γ) (R&D Systems), Minnesota, USA.

Preparation of the different extracts for biological screening

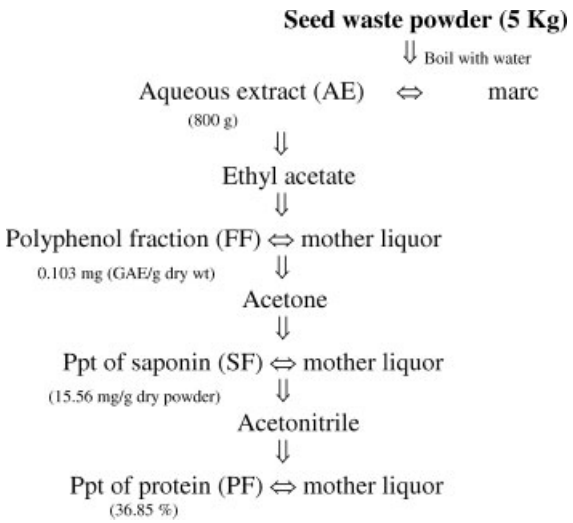
Extraction was performed by refluxing 100 g of seed waste first, with n-hexane (40–60 °C) and then with ethanol 70% till exhaustion (2 L., 24 h). The extracts were filtered and evaporated to dryness at reduced temperature (40 °C) and pressure to afford the hexane extract (HE) and the ethanol 70% extract (EE) respectively. Another aliquot of seed wastes (100 grams) were boiled with distilled water (500 ml X 4,4h, with continuous stirring), filtered and concentrated to afford aqueous extract (AE).

Fractionation of the bioactive aqueous extract

Based on biological findings, aqueous extraction was performed using 5 kg of seed waste adopting the aforementioned procedure. Percentage yield of the aqueous extract (AE) was calculated and found to be 16% (as mean of three determinations) by keeping in desiccators for at least one week till constant weight. The aqueous extract was resuspended in water and fractionated with ethyl acetate (5L. × 5). The ethyl acetate extract was separated and concentrated to dryness under reduced temperature and pressure to afford the polyphenols fraction (FF). The saponin fraction (SF) was separated by precipitation with acetone from the aqueous layer then dried. Proteins fraction (PF) was separated from the mother liquor by precipitation with acetonitrile, dried and kept for further investigation (scheme 1).

Material and method for protein electrophoresis

Protein pattern of the whole seed waste was detected using continuous polyacrylamide gel electrophoresis (SDS-PAGE) of 12% and 0.75 mm thick by means of Hoefer vertical minigel. According to Gallagher and Smith,[11] seed waste (0.5g and 0.25g) have been extracted with 1 ml buffer (1.21g Tris HCl, 1 ml 10% SDS, 0.5 ml β- mercaptoethanol and 5g sucrose completed to 50 ml distilled water, pH 8.0); precipitated at −4 °C with 4000 rpm for 15 min. Pellets were dissolved with sample buffer (1.21 ml Tris HCl, 2 ml 10% SDS, 1 ml glycerol, 0.5 ml 0.4% bromophenol, 0.5 ml β- mercaptoethanol, 4.8 ml distilled water), marker from Jena Bioscience Germany was used. The loaded samples were allowed to run at 70 volt, 40 mA. The gel was stained with comassie blue and distained according to Wilson,[12] photographed to be analyzed by BioRad documentation system St. Louis, MO, USA.



Scheme 1. Preparation and fractionation of the aqueous extract of *Nigella sativa* seed waste.

Material and method of determination of saponin content of the *Nigella* waste

The haemolytic activity of saponin was used for the determination of total saponins in *Nigella sativa* seed waste. The determination was based on the absorbance of erythrocyte suspensions after it had been haemolyzed by increased volumes of saponin.[13,14]

Biochemical experiments

Experimental design

The doses of the AE, EE, and HE, as well as, for the different aqueous extract fractions. PF, SF and FF were selected on the basis of acute toxicity study and the LD_{50} of the extract was found to be 5g/kg b.w. The LD_{50} values were determined by Miller and Tainter method.[15] The extracts and fractions administration did not produce any abnormalities such as atoxic, circling, lacrimation, labowed breathing, *etc*., in the animals throughout the experimental period. The dose level selected for the present study was non-toxic and safe.

Preparation of the blood samples

The animals were divided into five groups of 6–8 mice each: Group I: normal control tween 80 (1%) treated; Group II: CCl_4 positive control treated; Group III: hepatotoxic treated with 200 mg/kg AE; Group IV: hepatotoxic treated with 250 mg/kg EE and Group V: hepatotoxic treated with 10 mg/kg HE. Different extracts were dissolved in 10% tween 80 and administered orally, once daily, for

5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25% (*v/v*) solution in corn oil. One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation and used for estimation of TNF-α, IL-1β and IFN-γ levels in the serum of mice and estimation of GPT activity and bilirubin level in plasma.

Preparation of the liver samples

Different extracts AE, HE, and EE were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25% (*v/v*). One day thereafter, mice were sacrificed by cervical dislocation and livers were rapidly excised and homogenized in chilled 1.15 KCL (PH 7.4) to yield 10% homogenates then used for estimation of TNF-α, IL-1β, IFN-γ levels; LP level and GSH content in liver homogenates of mice.

Determination of tumour necrosis factor-α, interlukin-1 β and IFN-γ assays

TNF-α, IL-1β and IFN-γ levels were measured in serum and tissue homogenates by using a Quantikine rat TNF-α, IL-1β and IFN-γ ELISA kits (R&D Systems), Minnesota, USA.

Determination of MDA, GSH

Lipid peroxidation products were estimated by the determination of the content of the thiobarbituric acid-reactive substances (TBARS) that was measured as malondialdehyde[16] and expressed as nmol/g wet tissue. Estimation of GSH content was performed spectrophotometrically at 412 nm, using Ellman's reagent[17,18] and expressed as μmol/g wet tissue.

Determination of GPT and bilirubin

Serum activities of ALT enzyme were measured using test reagent kits based on the method of Reitman and Frankel.[19] Serum bilirubin levels were estimated according to Jendrassik and Grof.[20]

Histopathological experiments

The representative sections of livers ($n = 6$) were prepared by formalin fixation, routinely processed and embedded in paraffin. Three-micrometer-thick sections were placed on slides and stained with hematoxylin and eosin (H&E). The slides were then investigated under light microscope (Nikon XDS-1B). Sections were examined to investigate the toxic effect of CCl_4; the congested central veins, massive inflammatory infiltration, cytoplasmic vacuolations and loss of normal hepatic architecture.

Statistical analysis

Comparisons between different groups were carried out by one way analysis of variance (*ANOVA*) followed by Tukey-Kramer multiple comparisons test. The level of significance was set at $p < 0.05$. GraphPad Software InStat (version 2) was used to carry out these statistical tests.

Chemical screening of the bioactive extract

Chemical screening of the bioactive aqueous extract was performed according to published procedures[21,22].

Proteins and amino acids determination

Quantitative determination of the amino acids

This was performed according to the method of the *Official Journal of the European Communities* (19-9-98) using Keldahl method. Amino acid determination was performed by oxidation with performic acid to protect methionine and cystine from destruction followed by acid hydrolysis. The filtered buffered hydrolysate was determined by subjecting to ion exchange chromatography using strong acid cation exchanger and a colorimetric detection for amino acids after reacting with ninhydrin.

Preparation of isotonic buffer solution (pH 7.4)

Isotonic buffer solution used for the extraction of saponins from the *Nigella sativa* seed waste and for the dilution of blood saponin mixture was prepared by dissolving 19.00 g dibasic sodium hydrogen phosphate, 1.815 g potassium hydrogen phosphate and 1.283 g sodium chloride in a sufficient amount of water and the volume was finally adjusted to 1000 ml.

Defibrinated blood

(A) Blood stock was prepared by mixing the blood of freshly slaughtered buffaloes, *Bos bubolis* Family Bovidae, with broken glass in a suitable container and stirred. The precipitated fibrin was collected around the glass particles in the form of soft clot which was then removed. The remainder of blood was placed in a sterile well-closed bottle and preserved in a refrigerator until required for use.

(B) Blood reagent prepared by mixing 5 ml of defibrinated blood stock with sufficient isotonic buffer solution to produce 10 ml., the blood stock should be thoroughly agitated before measuring the 5 ml to resuspend any ppted RBCs. This solution should be freshly prepared just before the estimation of saponins.

Standard saponin solution

The 'white saponin of Merck' was used as a standard. The standard solution was prepared by dissolving accurately weighed 0.5 g of the saponin in a suitable volume of isotonic buffer solution in a volumetric flask and the volume was completed to 1000 ml with isotonic buffer. This solution should be freshly prepared just before the estimation of saponins.

Colourimetric determination of total polyphenol content of the Nigella seed waste

Total polyphenol (TP) content of seed waste was assayed using Folin-Ciocalteu colorimetric method according to the European Pharmacopoeia, 2002.[23] TP content was calculated as mg of gallic acid equivalents of the dry plant material (mg/GAE/g)

Table 1. Effect of AE, EE and HE of *Nigella sativa* seed waste on the TNF-α, IL-1β and IFN-γ levels in the serum of mice

Groups Parameters	Normal Control	CCl_4-treated	Aqueous extract (AE) 200 mg/kg	Ethanol 70% extract (EE) 250 mg/kg	Hexane extract (HE) 10 mg/kg
TNF-α (pg/ml)	85.25 ± 3.56	256.75 ± 20.65*	94.54 ± 12.67@	109.44 ± 32.68	220.24 ± 33.5*
IL-1β (pg/ml)	46.66 ± 9.42	180.34 ± 34.66*	67.92 ± 11.22@	98.74 ± 19.86*	147.46 ± 24.6*
IFN-γ (pg/ml)	517.65 ± 51.78	1543 ± 121.23*	851.23 ± 57.67@	1304.76 ± 110.44*	1378.56 ± 203.43*

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (v/v) solution in corn oil). One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from the CCl4 group at $p<0.05$

Table 2. Effect of AE, EE, and HE of *Nigella sativa* seed waste on the TNF-α, IL-1β and IFN-γ levels in the liver homogenates of mice

Groups Parameters	Normal control	CCl_4-treated	Aqueous extract (AE) 200 mg/kg	Ethanol 70% extract (EE) 250 mg/kg	Hexane extract (HE) 10 mg/kg
TNF-α (pg/ml)	103.25 ± 3.56	326.75 ± 20.65*	115.54 ± 12.67@	258.44 ± 32.68*	290.24 ± 33.5*
IL-1β (pg/ml)	56.66 ± 9.42	211.34 ± 34.66*	83.92 ± 11.22@	158.74 ± 19.86*	187.46 ± 24.6*
IFN-γ (pg/ml)	723.65 ± 51.78	1776 ± 121.23*	851.23 ± 57.67@	1204.76 ± 110.44*	1378.56 ± 203.43*

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (*v/v*) solution in corn oil). One day thereafter, rats were sacrificed by cervical dislocation and livers were rapidly excised and homogenized in chilled 1.15 KCL (PH 7.4) to yield 10% homogenates. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from the CCl_4 group at $p<0.05$.

Table 3. Effect of AE, EE and HE of *Nigella sativa* seed waste on CCl4-induced biochemical changes [glutamic pyruvic transaminase (GPT) activity and bilirubin level in plasma]

Groups Parameters	Normal control	CCl_4-treated	Aqueous extract (AE) 200 mg/kg	Ethanol 70% extract (EE) 250 mg/kg	Hexane extract (HE) 10 mg/kg
GPT (units/ml)	184 ± 38.56	1454 ± 134.76*	255 ± 33.54@	320 ± 40.61@	781.98 ± 87.78*
Bilirubin (μmol/l)	2.4 ± 0.12	6.67 ± 0.56*	3.43 ± 0.34@	5.5 ± 0.42*	5.96 ± 0.65*

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (*v/v*) solution in corn oil).). One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from the CCl4 group at $p<0.05$.

Table 4. Effect of AE, EE and HE of *Nigella sativa* seed waste on CCl4-induced biochemical changes in lipid peroxides (LP) level and reduced glutathione (GSH) content in liver homogenates of mice

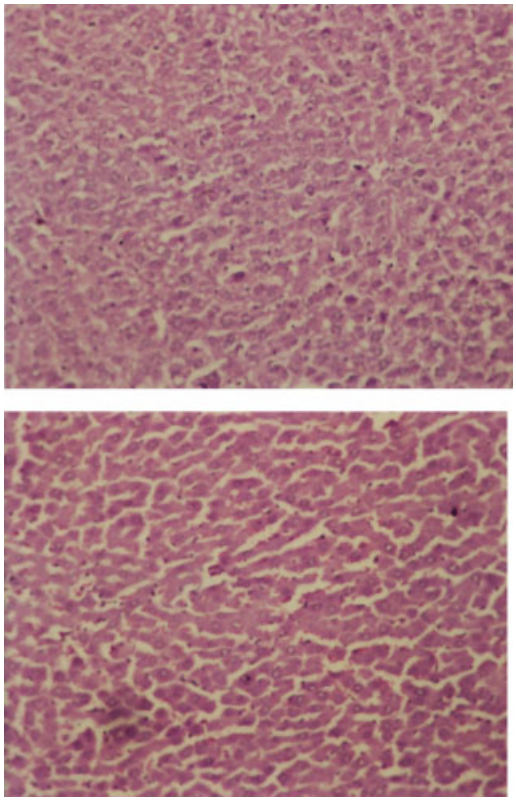
Groups Parameters	Normal control	CCl_4-treated	Aqueous extract (AE) 200 mg/kg	Ethanol 70% extract (EE) 250 mg/kg	Hexane extract (HE) 10 mg/kg
LP (nmol MDA/g)	405.23 ± 23.65	978.56 ± 56.54*	516.90 ± 62.46@	750.12 ± 65.35*	789.46 ± 87.43*
GSH (μmol/g)	8.22 ± 0.42	15.32 ± 1.67*	6.06 ± 0.24@	9.26 ± 0.46	11.29 ± 0.44

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (v/v) solution in corn oil). One day thereafter, rats were sacrificed by cervical dislocation and livers were rapidly excised and homogenized in chilled 1.15 KCL (PH 7.4) to yield 10% homogenates. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from the CCl_4 group at $p<0.05$

Results

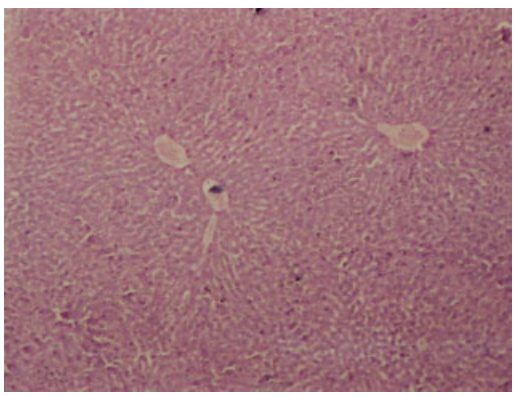
Bioactivity-guided screening of the different extracts

The different extracts, AE, EE, and HE were administered orally for five consecutive days to mice before treating them intraperitoneally with CCl_4 at a dose of 10 mg/kg b wt of 0.25 (*v*/*v*) solution in corn oil. Liver injury produced was evaluated by biochemical parameters and histopathological examination. These changes were improved only by AE and EE of the seed waste of *Nigella sativa*. Notably, administration of CCl_4 caused liver injury manifested by a twofold increase of TNF-α and INF-γ and a threefold rise of IL-1β which are all important mediators of the progress of liver injury, as compared to the normal control group. Later, the acute liver injury was evidenced in the plasma by about sevenfold rise in glutamic pyruvic transaminase (GPT) activity and twofold increase in bilirubin level. Hepatotoxicity was further manifested by twofold increase in the hepatic level of MDA and an increase by 86% of the GSH. Moreover, liver histopathology showed that both extracts reduced the incidence of liver lesions including hepatic cells cloudy swelling, lymphocytes infiltration, hepatic necrosis and fibrous connective tissue proliferation induced by carbon tetrachloride in mice. Table 1 shows that AE possessed a potent effect by a significant reduction of TNF-α, IL-1β and IFN-γ levels by 63%, 62%, and 50% respectively compared to CCl_4-treated animals. The EE showed less potent effect while the HE was almost ineffective. Similar results were concluded from the effect of extracts on TNF-α, IL-1β and IFN-γ in liver homogenates of mice with a significant reduction of TNF-α, IL-1β and IFN-γ levels by 65%, 90%, and 44% respectively compared to CCl_4-treated animals (Table 2). The AE causes a significant reduction in the GPT and bilirubin levels by 82% and 48%, respectively, as compared to CCl_4-treated animals. The EE showed only a significant reduction in bilirubin level by 48% as compared to CCl_- treated mice (Table 3). Concerning the antioxidant parameters, the AE showed significant reduction of both LP and GSH by 47% and 60% respectively as compared to CCl_{4-} treated mice (Table 4).

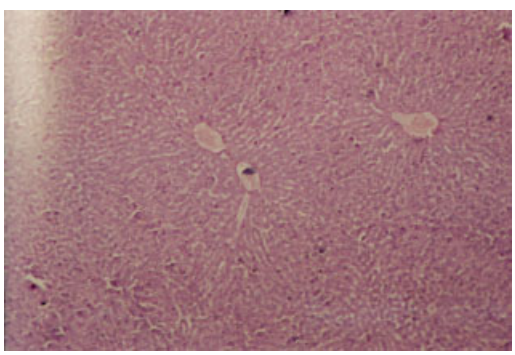


A photomicrograph of liver sections obtained from the median lobes of adult male albino mice subjected to CCl4 (H & E stain $\times$ 100). The section shows congested central veins, massive inflammatory infiltration, cytoplasmic vacuolations and loss of normal hepatic architecture.

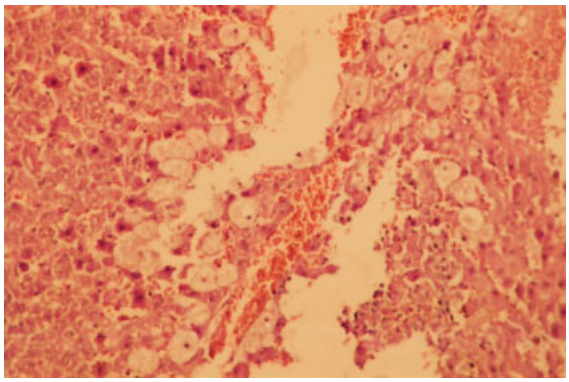
Histopathology



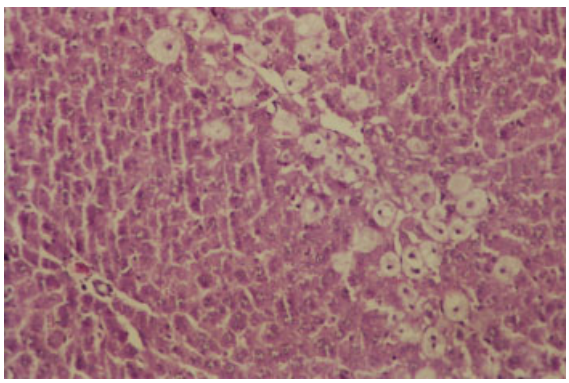
A photomicrograph of liver section obtained from the median lobe of an adult male albino mouse (H & E stain $\times$ 100). The section shows normal hepatocytes and normal hepatic architecture.



A photomicrograph of liver section obtained from the median lobe of adult male albino mice subjected to CCl_4 and pretreated with the aqueous extract (AE) of *Nigella sativa* seed waste (H & E stain $\times$ 100). The section shows normal hepatic architecture with slight narrowing of blood sinusoids and congestion of central vein.



A photomicrograph of liver section obtained from the median lobe of adult male albino mice subjected to $CCl_4$and pre-treated with ethanol 70% extract (EE) of *Nigella sativa* seed waste (H & E stain × 100). The section shows slight dilation of central vein with blood congestion, restoration of normal hepatic architecture and some heterochromatic nuclei.



A photomicrograph of liver section obtained from the median lobe of adult male albino mice subjected to CCl4 and pre-treated with the hexane extract (HE) of *Nigella sativa* seed waste (H & E stain × 100). The section shows congested central vein and low inflammatory infiltration

Table 5. Amino acid profile of *Nigella sativa* seed waste

Amino acids	%	Amino acids	%
Aspartic (ASP)	3.07	Methionine (MET)	0.53
Therionine (THR)	1.21	Isoleucine (ILE)	1.06
Serine (SER)	1.31	Leucine (LEU)	2.04
Glutamic (GLU)	7.78	Tyrosine (TYR)	–
Proline (PRO)	1.58	Phenylalanine (PHE)	1.16
Glycine (GLY)	1.89	Histidine (HIS)	1.05
Alanine (ALA)	1.47	Lysine (LYS)	1.10
Cystein (CYS)	0.75	Arginine (ARG)	2.86
Valine (VAL)	1.34		–

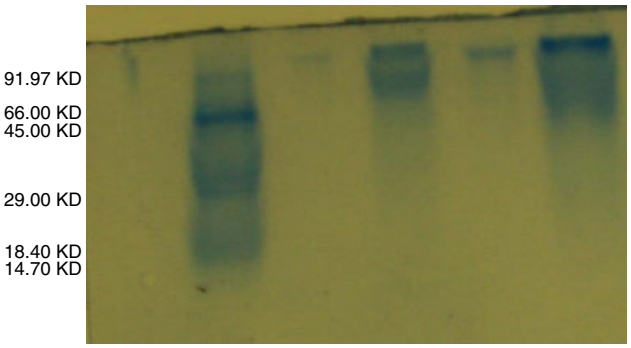


Figure 1. Polyacrylamide gel illustrates electrophoretic band profile of *Nigella sativa* : M: Jena Bioscience marker; 1: 0.5g; 2: seed residue: 0.25g seed residue.

The use of protein profile of the seeds storage protein has extensively been used for differentiation between varieties of the same species as well as for identification. There are no prospective changes in proteins of the dry mature seeds which have high stability under all environmental conditions as previously reported by Stegeman,[24] Harborn and Turner[25] and Gallagher and Smith.[11] Protein finger print was previously used by Chauhan *et al*.[26] to discriminate between 12 varieties of sorghum. However, Rashed *et al*.[27] used the SDS-PAGE to differentiate between 17 different sorghum cultivars. On the other hand, protein profile has provided valid evidence for addressing biodiversity problems within many species of genus *Lathyrus* as mentioned by Badr *et al*.[28] Seed protein banding profile observed in SDS-PAGE of *Nigella sativa* L. is represented in Figure 1. The molecular weight of the revealed bands is dictated in Table 6 and the total number of bands is 4 ranging from 91.97 KD and 29.00 KD.

Chemical investigation on the bioactive extract

The chemical screening of the bioactive aqueous extract revealed the presence of sterols and/or triterpenes, flavonoids, carbohydrates and/or glycosides, tannins, alkaloids and/or nitrogenous compound and traces of saponins. Proteins were also detected in the aqueous extract. Quantitative determination of proteins revealed that crude protein reached 36.85% while the amino acids percentages were highest for GLU followed by ASP and LEU as reported in Table 5.

Immunostimulant effect of the different fractions of the aqueous extract

Experimental design

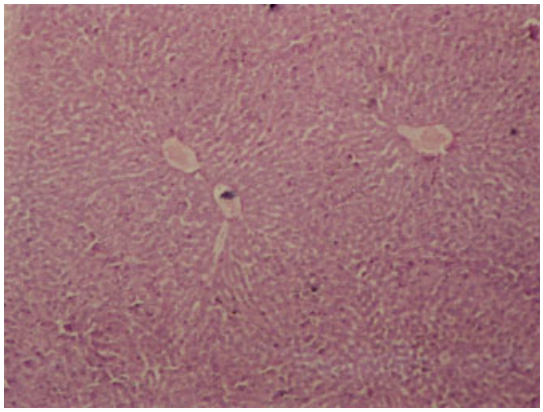
Based on the aforementioned positive hepatoprotective effect of the bioactive aqueous extract of *Nigella sativa* seed waste, the same biochemical and histopathological studies were again performed on the different fractions of the aqueous extract. The doses of these fractions were identified as the most effective doses, and therefore selected for the present study.

Table 6. Electrophoresis banding pattern of *Nigella sativa* L. seed waste. Lane1: 500 mg; lane 2 : 250 mg total protein

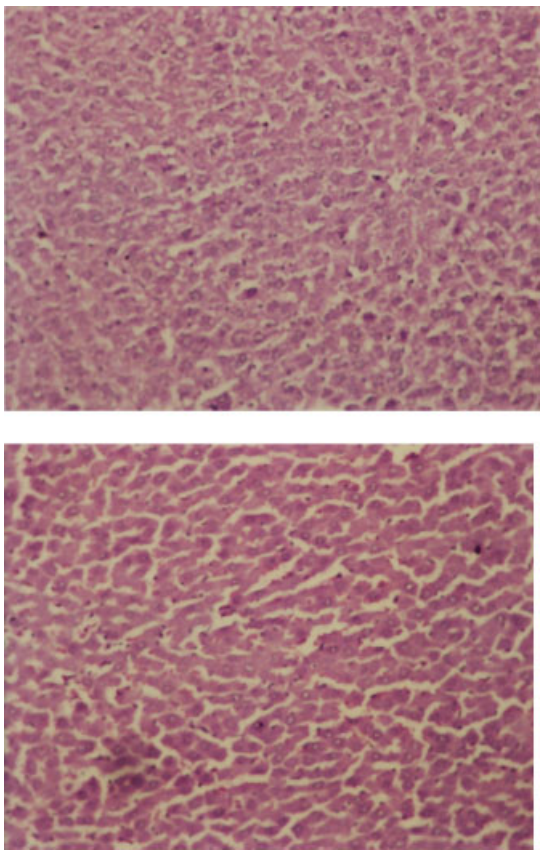
Mol. Wt. KD	Marker	Lane 1	Lane2	Relative Front Value
91.971	+	+	+	**0.079**
66.00	+	–	–	**0.159**
64.861	–	+	+	**0.164**
45.000	+	+	+	**0.246**
29.000	+	+	+	**0.319**
18.400	+	–	–	**0.512**
14.700	+	–	–	**0.729**
Sum	**6**	**4**	**4**	

The animals were divided into five groups of 6–8 mice each: Group I: normal control tween 80 (1%) treated; Group II: CCl_4 positive control treated; Group III: hepatotoxic treated with 5 mg/kg of PF; Group IV: hepatotoxic treated with 10 mg/kg of SF and Group V: hepatotoxic treated with 5 mg/kg of FF. and PF (5 mg/kg), SF (10 mg/kg) and FF (5 mg/kg) of AE were dissolved in 10% tween 80 and administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b. w.) of 0.25% (*v/v*) solution in corn oil. One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation and used for estimation of TNF- α, IL-1β and IFN-γ levels in the serum of mice and estimation of GPT activity and bilirubin level in plasma. The biochemical changes were improved only by the protein fraction (PF) and to a very less extent by the flavonoid fraction of the seed waste of *Nigella sativa*. Notably, administration of CCl_4 caused liver injury manifested by a onefold increase of TNF- α, a threefold rise of IL-1β and twofold increase in IFN-γ levels in the serum of mice. Similarly, CCl_4 administration resulted in twofold increase in these cytokines in liver homogenates, which are all important mediators of the progress of liver injury, as compared to the normal control group. Later, the acute liver injury was evidenced in the plasma by about sevenfold rise in GPT activity and twofold increase in bilirubin level. Hepatotoxicity was further manifested by one-fold increase in the hepatic level of MDA and an increase by 86% of the GSH. Moreover, liver histopathology showed that protein fraction reduced the incidence of liver lesions including hepatic cells cloudy swelling, lymphocytes infiltration, hepatic necrosis and fibrous connective tissue proliferation induced by CCl_4 in mice. Table 7 shows that PF possesses a potent effect by a significant reduction of TNF-α, IL-1β and IFN-γ levels by 58%, 43%, and 42%, respectively compared to CCl_4-treated animals. The FF showed less potent effect while the SF was almost ineffective. Similar results were concluded from the effect of different fractions on TNF-α, IL-1β and IFN-γ in liver homogenates of mice. Only the PF exerted a significant reduction of TNF-α, IL-1β and IFN-γ levels by 63%, 63%, and 47% respectively compared to CCl_4-treated animals (Table 8). The PF caused a significant reduction in the GPT and bilirubin levels by 67% and 60% respectively as compared to CCl_4-treated animals (Table 3). Concerning the antioxidant parameters, the PF showed significant reduction of both LP and GSH by 43% and 70% respectively and the SF decreased the GSH by 46% as compared to CCl_4– treated mice (Table 8).

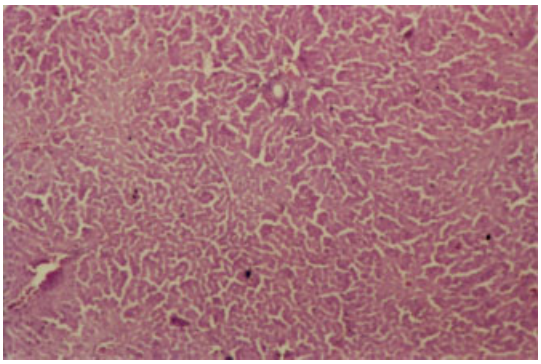
Histopathology



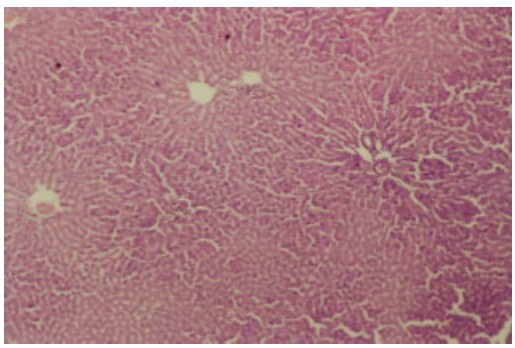
A photomicrograph of liver section obtained from the median lobe of an adult male albino mouse (H & E stain × 100). The section shows normal hepatocytes and normal hepatic architecture.



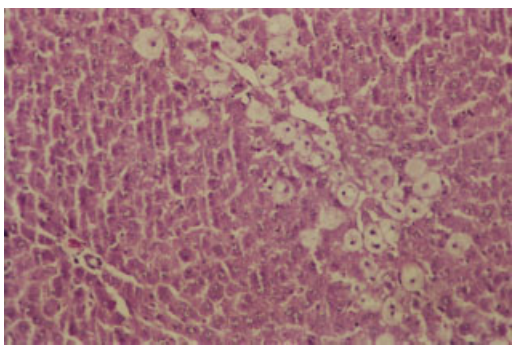
A Photomicrograph of liver sections obtained from the median lobes of adult male albino mice subjected to Ccl4 (H & E stain × 100). The section shows congested central veins, massive inflammatory infiltration, cytoplasmic vacuolations and loss of normal hepatic architecture.



A photomicrograph of liver section obtained from the median lobe of adult male albino mice subjected to CCl4 and pre-treated with protein fraction (PF) of *Nigella sativa* seed waste (H & E stain × 100). The section shows normal hepatic architecture with slight narrowing of blood sinusoids and congestion of central vein.



A photomicrograph of liver section obtained from the median lobe of adult male albino mice subjected to CCl4 and pre-treated with saponin fraction (SF) of *Nigella sativa* seed waste (H & E stain × 100). The section shows slight dilation of central vein with blood congestion, restoration of normal hepatic architecture and some heterochromatic nuclei.



A photomicrograph of liver section obtained from the median lobe of adult male albino mice subjected to CCl4 and pre-treated with polyphenol fraction (FF) of *Nigella sativa* seed waste (H & E stain × 100). The section shows congested central vein and low inflammatory infiltration.

Discussion

In the current study, CCl_4 produced an acute liver inflammation with significant increase in TNF-α, IL-1β and IFN-γ levels in both the serum and liver homogenates of mice as compared to the respective normal values (Tables 1, 2, 7, and 8). CCl_4 administration significantly increased bilirubin and GPT, as well as, GSH and LP compared to the normal control group (Tables 3, 4, 9, and 10).

Treatments with AE and PF significantly prevented CCL_4-induced increase in the cytokines production (Tables 1, 2, 7, and 8). Similarly, treatment with AE and PF significantly reduced CCl_4-induced increase in bilirubin and GPT as compared to CCL_4- treated group (Tables 3 and 9). The FF resulted in a significant reduction in only GPT as compared to CCl_4-treated group (Table 9). Treatments with AE and the PF significantly prevented CCl_4-induced increase in LP and GSH levels (Tables 4 and 10).

Serum TNF-α level in CCl_4 model groups was significantly higher than that in control groups. IL-1β is another critical inflammatory mediator and cytokine in liver injury. Although IL-1 β itself has no damage on liver, its elevation could stimulate inflammatory cells to excrete many other cytokines including TNF- α, IL-6 and IL-8, which contribute to acute liver injury (ALI).[29] Our data provided further evidence for the role of cytokines including TNF- α and IL-1 β during CCl_4- induced liver injury. Serum level of TNF- α and IL-1 β elevated significantly in CCl_4 model group. AE and PF significantly reduced the elevated level of TNF- α and IL-1 β in serum. Therefore, inhibition of pro-inflammatory mediator and cytokines is partly the mechanisms of the AE and especially the PF's protective effect.

This study showed that the level of IFN-γ was positively correlated with serologic markers of hepatic injury. Prior clinical reports also indicated that IFN-γ was positively associated with elevated levels of ALT in chronic hepatitis B patients.[30,31] The role of the elevated level of IFN-γ in the development of hepatic injury and fibrogenesis remains unclear. It was postulated that the elevation in the level of IFN-γ might be advantageous in the control of hepatic viral infection.[32] Therefore, it is plausible to assume that in addition to its role in the induction of inflammation, the elevation of the level of IFN-γ in the mouse model might be an advantageous response for the animals to protect the liver from CCl_4-induced injury.

CCl_4 causes acute hepatotoxicity with necrotic and apoptotic hepatocellular injury and impairment of liver function.[33,34] In this study, a significant increase in lipid peroxidation was observed after CCl_4 injection and treatment with AE and PF attenuated this increase. The mechanism of CCL_4 injury involves oxidative damage by metabolism of CCl_4 to $CCl_3^{\bullet}$ in hepatocytes; $CCl_3^{\bullet}$ can react with oxygen to form another reactive oxygen species (ROS), trichloromethylperoxy radical ($CCl_3OO^{\bullet}$), which triggers lipid peroxidation[35,36], this causes cell death with accumulation of lipid peroxides, intracellular calcium ions and triggers secondary damage from the inflammatory process.[37]

Anti-oxidative defence systems such as uncoupling protein have been reported to prevent CCl_4 damage and GSH has beneficial effects through its restoration of the Ca^{2+} -pump disorder caused by CCl_4.[38] In the current investigation, CCL_4 administration strongly increased GSH, as compared to the respective normal values. GSH is the most abundant redox system and the GSH/GSSG ratio represents the cellular ability to prevent oxidative damage caused by most hepatotoxins. Treatments with aqueous and proteins fractions significantly decrease GSH levels.

In the present study, the GPT and bilirubin activities were dramatically increased in the CCl_4-treated group compared with the normal control group, indicating severe hepatocellular damage. In contrast, treatments with PF significantly reduced CCl_4-induced increase in bilirubin and GPT as compared to the control CCl_4 group. These results were also strongly supported by

Table 7. Effect of PF, SF and FF of *Nigella sativa* seed waste on the TNF-α, IL-1β and IFN-γ levels in the serum of mice

Groups Parameters	Normal control	CCl_4-treated	Protein fraction (PF) 5 mg/kg	Saponin fraction (SF) 10 mg/kg	Polyphenol fraction (FF) 5 mg/kg
TNF-α (pg/ml)	95.25 ± 3.56	216.75 ± 12.65*	90.24 ± 17.5*@	220.24 ± 23.5*	109.44 ± 22.68@
IL-1β (pg/ml)	41.66 ± 9.42	170.34 ± 24.66*	97.46 ± 24.6*@	147.46 ± 24.6*	98.74 ± 19.86*
IFN-γ (pg/ml)	457.65 ± 51.78	1347 ± 101.23*	780.56 ± 203.43*	1078.56 ± 203.43*	1104.76 ± 110.44*

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (*v/v*) solution in corn oil). One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from CCl4 group at $p<0.05$.

Table 8. Effect of PF, SF and FF of *Nigella sativa* seed waste on the TNF-α, IL-1β and IFN-γ levels in the liver homogenates of rats

Group Parameters	Normal control	CCl_4-treated	Protein fraction (PF) 5 mg/kg	Saponin fraction (SF) 10 mg/kg	Polyphenol fraction (FF) 5 mg/kg
TNF-α (pg/ml)	103.25 ± 3.56	326.75 ± 20.65*	121.23 ± 12.90@	290.24 ± 33.5*	258.44 ± 32.68*
IL-1β (pg/ml)	56.66 ± 9.42	211.34 ± 34.66*	78.98 ± 6.55@	187.46 ± 24.6*	158.74 ± 19.86*
IFN-γ (pg/ml)	723.65 ± 51.78	1776 ± 121.23*	945.60 ± 34.98@	1378.56 ± 203.43*	1204.76 ± 110.44*

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (*v/v*) solution in corn oil). One day thereafter, rats were sacrificed by cervical dislocation and livers were rapidly excised and homogenized in chilled 1.15 KCL (PH 7.4) to yield 10% homogenates. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from the CCl_4 group at $p<0.05$.

Table 9. Effect of Effect of PF, SF and FF of *Nigella sativa* seed waste on CCl_4-induced biochemical changes (glutamic pyruvic transaminase (GPT) activity and bilirubin level in plasma

Group Parameters	Normal control	CCl_4-treated	Protein fraction (PF) 5 mg/kg	Saponin fraction (SF) (10 mg/kg	Polyphenol fraction (FF) 5 mg/kg
GPT (units/ml)	184 ± 38.56	1454 ± 134.76*	**470 ± 15.09**@	781.98 ± 87.78*	320 ± 40.61@
Bilirubin (μmol/l)	2.4 ± 0.12	6.67 ± 0.56*	**2.7** ± 0.2@	5.96 ± 0.65*	5.5 ± 0.42*

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (*v/v*) solution in corn oil). One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from the CCl4 group at $p<0.05$.

Table 10. Effect of PF, SF and FF of *Nigella sativa* seed waste on CCl_4-induced biochemical changes (lipid peroxides (LP) level and reduced glutathione (GSH) content in liver homogenates of mice

Groups Parameters	Normal control	CCl_4-treated	Protein fraction (PF) 5 mg/kg	Saponin fraction (SF) 10 mg/kg	Polyphenol fraction (FF) 5 mg/kg
LP (nmol MDA/g)	405.23 ± 23.65	878.56 ± 46.54*	501.90 ± 62.46@	750.12 ± 65.35*	789.46 ± 87.43*
GSH (μmol/g)	8.22 ± 0.42	17.23 ± 1.54*	5.08 ± 0.21@	9.26 ± 0.46@	11.29 ± 0.44

Different extracts were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl_4 (10 mg/kg b wt of 0.25 (*v/v*) solution in corn oil). One day thereafter, rats were sacrificed by cervical dislocation and livers were rapidly excised and homogenized in chilled 1.15 KCL (PH 7.4) to yield 10% homogenates. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. * Significant difference from the control group at $p<0.05$. @ Significant difference from the CCl_4 group at $p<0.05$

the histological observations. The hematoxylin-eosin stained liver sections showed significantly fewer histological changes in the extracts and fractions-treated CCl_4 group than the vehicle-treated CCl_4 group. These results suggest that the test extracts may be clinically applied to treat liver diseases.

Concerning the histopathological findings, CCL_4 produced congested central veins, massive inflammatory infiltration, cytoplasmic vacuolations, and loss of normal hepatic architecture as compared with the normal control group. Treatment with the AE and PF showed normal hepatic architecture with slight narrowing of blood sinusoids and congestion of central vein and low inflammatory infiltration.

In summary, this study demonstrates that the aqueous extract and specially the protein fraction of this extract can protect against CCl_4-induced acute hepatotoxicity through restoration of the anti-oxidative defence system and down-regulation of the pro-inflammatory pathway. This study provides evidence that this extract may be an alternative treatment for liver diseases caused by xenobiotics.

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