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Nigella sativa L. Attenuates Oxidative Stress, Inflammation and Apoptosis in Concanavalin A-induced Acute Immunological Liver Damage in Mice

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HIGHLIGHTS

- *Nigella sativa* seed extract (NSE) possess significant polyphenol contents and antioxidant potential.

- NSE attenuated concanavalin A-induced liver injury and oxidative stress.

- NSE suppressed liver inflammation through modulating PPARγ/JAK2/STAT3 pathway.

- NSE inhibited hepatocyte apoptosis *via* downregulating Bcl-2/Bax/Caspase-9 pathway.

Abstract: Liver's contribution to innate immunity is eminent. However, uncontrolled inflammatory conditions predispose the liver to immune-mediated injury. *Nigella sativa* L. is traditionally implicated in infectious, inflammatory, metabolic and hepatorenal complications. This study aimed to evaluate the protective role of *N. sativa* seed extract (NSE) against concanavalin A (ConA)-induced acute immunological liver injury in mice. *In vitro*, NSE was subjected to quantitative phytochemical characterization and 1,1-diphenyl-2-picryhydrazyl (DPPH) analysis. *In vivo*, male Balb/c mice were pretreated with NSE (100, 200 and 400 mg/kg/day, p.o.) and pioglitazone (5 mg/kg/day, p.o.) for seven consecutive days. A single dose of ConA (12 mg/kg, i.v.) was injected and samples were collected for biochemical, histopathological and qRT-PCR analyses after 8 h of ConA injection. *In vitro* analysis showed considerable quantities of polyphenols and significant DPPH scavenging ability of NSE. In mice, ConA resulted in a significant ($p<0.05$) increase in liver injury markers (ALT, AST, ALP and TBil) and hepatic oxidative stress (SOD, CAT and MDA). Also, a substantial elevation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in liver tissues was noticed. Furthermore, ConA

markedly downregulated PPARγ and upregulated JAK2 and STAT3 expressions. In addition, considerably decreased expressions of Bcl-2 and increased Bax and Caspase-9 were observed. NSE demonstrated hepatoprotective effect in a dose-dependent manner through attenuating liver injury markers, oxidative stress parameters, pro-inflammatory cytokines levels as well as liver inflammation and hepatocyte apoptosis *via* modulating PPARγ/JAK2/STAT3 and Bcl-2/Bax/Caspase-9 pathways. Conclusively, the antioxidative, anti-inflammatory and anti-apoptotic actions of NSE could protect against acute immunological liver injury.

INTRODUCTION

Liver is a vital organ that not only plays key metabolic and detoxification functions but also involves in the regulation of crucial immunological actions as various immune cells reside in it [1]. Autoimmune hepatitis (AIH) is an inflammatory disease manifested by hepatic parenchymal damage which ultimately leads to fibrosis, cirrhosis and cancer of liver, if left untreated or treatment delayed. AIH can affect individuals of any age, however, it is more frequent in women. The pathogenesis of AIH involves an aberrant immune response that targets liver autoantigens and causes self-perpetuating and chronic liver inflammation. Various factors including drugs, chronic alcohol abuse, infections and autoimmunity may induce abnormal stimulation of liver immunity [2]. Currently, the standard therapeutic choice for AIH includes corticosteroids and other immunosuppressive medicines to induce and maintain remission of liver inflammation and fibrosis for long term. However, the non-specific action and associated adverse effects of these medicines led several patients to discontinue their use. While liver transplantation is not always considered an ideal approach. Therefore, therapeutic strategies for liver diseases are primarily focused to prevent liver injury [2,3].

Concanavalin A (ConA), a lectin possessing mitogenic and agglutination properties, is extracted from Jack beans (*Canavalia ensiformis*) [4]. ConA is commonly preferred to develop acute liver injury animal model as it selectively induces liver inflammation and extensive hepatocyte damage in experimental animals through activating an immunological response. Its pathological features are clinically similar to viral hepatitis and human AIH [5]. The administration of ConA activates abnormal hepatocyte immunity mainly through stimulating immune cells such as neutrophils, T cells and NK cells. Also, inflammation and immune stimulation are maintained by various endogenous inflammatory mediators such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukins (IL-1β, IL-6, IL-10 and IL-17) [6,7].

Peroxisome proliferator-activated receptors (PPARs) are transcriptional factors that control lipid metabolism and regulate cell growth and differentiation. Three subtypes of PPARs (PPARα, β, γ) are mainly involved in the pathogenesis and regulation of inflammatory responses [8]. Among all subtypes of PPARs, PPARγ gained more attention due to its particular relationship with inflammatory signaling pathways such as Nuclear factor-kappaB (NF-κB) pathway [9] and Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling [10]. While apoptosis is mainly regulated by genes of B cell leukemia/lymphoma 2 (Bcl-2) and Caspase families [11,12]. Studies have emphasized the involvement of nuclear receptors (PPARs) in liver injury. PPAR agonists suppress hepatic inflammation and delay the progression toward cirrhosis [13]. Pioglitazone, used in this study as a reference hepatoprotective treatment, is a PPARγ agonist that suppresses pro-inflammatory cytokines through downregulating nuclear transcriptional factors [14].

Medicinal plants provide an alternative safe and effective approach for the treatment of inflammatory and immunological liver diseases. The immunomodulatory effect of herbal therapies might involve the regulation of lymphocyte proliferation and production of cytokines and immunoglobulins [15]. *Nigella sativa* L., known as 'Black seeds' in English and 'Kalonji' in Urdu, belongs to the *Ranunculaceae* family. The plant is widely cultivated in Pakistan, India, Saudi Arabia, Iran, Egypt, Syria, Turkey, Greece and Albania [16]. Traditionally, it is used to cure inflammation, rheumatism, bronchitis, eczema and hypertension [17]. Studies reported that *N. sativa* seed extract and/or oil could prevent chemical [18] and infection [19] induced liver injury in experimental animal models. An *in vitro* study conducted by Ciesielska-Figlon and coauthors [20] reported the immunomodulatory effect of *N. sativa* seed oil by inhibiting proliferation and inducing apoptosis of human T lymphocytes. In another study, *N. sativa* ethanol extract showed renoprotective and immunomodulatory effects in mice by suppressing the pro-inflammatory cytokines [21]. Furthermore, antioxidant activity of *N. sativa* promisingly inhibited the oxidative stress-mediated hepatic damage [22]. However, studies of hepatoprotective activity of *N. sativa* against immune-mediated liver injury are limited. Therefore, we aimed to assess the protective effect of oral administration of methanolic extract of *N. sativa* seeds (NSE) against oxidative stress, inflammation and apoptosis in ConA-induced acute liver injury in Balb/c mice.

MATERIAL AND METHODS

Preparation of extract

Seeds of *N. sativa* were collected from Punjab Forestry Research Institute Gatwala (31°28′12″ N, 73°12′39″ E), Faisalabad, Pakistan. A taxonomist of Department of Botany, University of Agriculture Faisalabad (UAF), Pakistan, identified the specimen (voucher no. 245119). Seeds were thoroughly washed, dried and coarsely powdered. To prepare *N. sativa* seed extract (NSE), 200 g of powdered seed material was macerated in 1.5 L of 70% (v/v) methanol at room temperature for 48 h, filtered twice through Whatman filter paper and the excess solvent was removed using a rotatory evaporator (Heidolph®, Germany). The resultant concentrated extract was stored at 4°C until further characterized.

Estimation of total phenolic and flavonoid contents

The Folin-Ciocalteu method was adopted to quantify the total phenolic content (TPC) of NSE [23]. Briefly, 50 µL of extract was combined with 500 µL of Folin-Ciocalteu reagent and 1.5 mL of 20% Na_2CO_3 solution. Absorbance of mixture was measured spectrophotometrically at 765 nm after 60 min of incubation. A calibration curve (R^2 = 0.9955; y = 0.0116x+0.0927) of gallic acid (reference antioxidant compound) was used for quantification and TPC was presented as mg GAE/g of dry extract. Total flavonoid content (TFC) of NSE was estimated following the $AlCl_3$ colorimetric method [24]. For the preparation of sample, 500 µL NSE mixed with 150 µL of 5% $NaNO_2$ and 150 µL of 10% $AlCl_3$ solutions was further added to 1 mL of 1 M NaOH solution. After incubating for 15 min, absorbance was measured at 510 nm. The TFC was determined using a calibration curve of catechin (R^2 = 0.9938; y = 0.0027x+0.1609) and the content was mentioned as mg CE/g of extract.

HPLC characterization of extract

HPLC analysis was performed to elucidate the polyphenols of NSE according to a modified method [25]. An HPLC instrument (Shimadzu® (Japan) comprised of a C_{18} column (Shim-Pack CDC-ODS) and UV-visible detector was used. Briefly, extract (50 mg) diluted in methanol was acidified by HCl and boiled at 90°C for 2 h. A gradient mode was set for gradient elution using two mobile phases, i.e., A: acetonitrile (100% v/v) and B: acetic acid (6% v/v; pH = 2.27). A 20 µL sample was manually injected maintaining a 1 mL/min flow rate of mobile phase and 27°C of column temperature. Absorbance was measured at 280 nm. Amounts of phytocompounds were quantified by comparing retention times and concentrations [26].

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

A modified DPPH assay was conducted to determine the *in vitro* antioxidant potential of NSE [27,28]. In short, different concentrations (0.312 to 20 mg/mL) of NSE and ascorbic acid (standard antioxidant) were prepared. Then, 1 mL of each sample was diluted in ethanol and mixed with 2 mL of DPPH ethanolic solution of 0.2 mg/mL concentration. Reaction mixtures were incubated for 30 min and absorbance was taken at 517 nm using a spectrophotometer (Shimadzu®, Japan). Percentage inhibitions were calculated according to the given formula and the obtained values were used to calculate IC_{50} values.

$$\% \text{ Inhibition} = [(A_O - A_S)/A_O] \times 100 \tag{1}$$

A_O: absorbance of blank solution, A_S: absorbance of extract/standard.

Animals

Male Balb/c mice weighing 21±2 g were bred and housed at animal housing facility of Institute of Microbiology, University of Agriculture Faisalabad, Pakistan. Mice were acclimatized for one week prior to conduct experimental work. Standard conditions such as room temperature (25±1°C), humidity (50±5%) and 12 h light/dark cycles were maintained during the experiment. Standard pellet diet and water *ad-libitum* were provided.

Ethical approval

The study protocols were approved by Institutional Bioethics Committee (IBC), University of Agriculture Faisalabad, Pakistan (letter no.: D. No. 503/ORIC). In addition, guidelines for the care and use of laboratory animals were followed [29].

Experimental design

Thirty-six male Balb/c mice were allocated to six groups, with six mice in each group (n=6), and pretreated orally for seven successive days. Vehicle control and ConA control groups were given 3 mL/kg/day of normal saline. Three groups were administered with NSE at 100, 200 and 400 mg/kg/day, respectively [30]. Pioglitazone was given at 5 mg/kg as a reference hepatoprotective treatment. After 1 h of last treatment administered, 12 mg/kg of ConA dissolved in normal saline was injected into mice *via* tail vein [31], except those in vehicle control group. After 8 h of ConA injection, blood samples were withdrawn through a cardiac puncture under the influence of anesthesia (a combination of 2% xylazine and 10% ketamine) and stored in gel clot activator tubes. Then mice were decapitated and livers were immediately collected and preserved.

Measurement of liver function markers

Blood samples were centrifuged at 3,500 rpm for 15 min using a cooling centrifuge and separated sera were used to assess the liver injury markers. The calorimetric kits used to determine serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TBil) were purchased from SBio®, Singapore and Analyticon®, Germany. Analysis was done with the help of a spectrophotometer (Multiskan GO™, Thermo-Scientific, UK). The manufacturer's instructions were followed.

Histopathological examination

Small portions of liver tissues fixed in 10% buffered formalin were embedded in paraffin and sliced into thin sections of 5 µm thickness. Hematoxylin and eosin (H&E) staining was performed and slides were assessed for conventional histopathological changes under a light microscope (IRMECO®, Germany). Images were captured using a camera (TOUPCAM®, China) at 10 x 40 magnification.

Preparation of liver tissue homogenate (10% w/v)

Liver tissue of each experimental animal was homogenized in ice-cold sodium phosphate buffer (pH 7.4) and centrifuged at 3500 rpm for 20 min at 4°C. The collected supernatants were analyzed to measure oxidative stress parameters and pro-inflammatory cytokines levels.

Assessment of oxidative stress markers

Liver tissue homogenate was used to assess the superoxide dismutase (SOD) and catalase (CAT) activities. Lipid peroxidation in terms of malondialdehyde (MDA) was measured. The SOD activity was determined by its potential to inhibit pyrogallol autoxidation and absorbance of reaction mixture was taken at 412 nm [32]. Results were mentioned as U/mg protein. The CAT activity was measured [33] as a decrease in absorbance of reaction mixture due to hydrolysis of H_2O_2 when measured at 240 nm and values were expressed as nmol H_2O_2/min/mg protein. Malondialdehyde (MDA) level was estimated through the thiobarbituric acid reacting substances (TBARS) method [34]. Absorbance was measured at 532 nm and values were presented as nmol/mg protein.

Estimation of pro-inflammatory cytokine levels

The concentrations of tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β) and interleukin-6 (IL-6) in liver homogenate were measured spectrophotometrically using ELISA kits acquired from BT Lab®, Shanghai, China.

Gene expression (qRT-PCR) analysis

A small portion of each liver was preserved in RNALater (Thermo-Fisher-Scientific®, USA) for qRT-PCR analysis. The TRIzol reagent (Thermo-Scientific®, UK) was implicated in the extraction of total RNA from liver tissues and quantified using a Nanodrop spectrophotometer. The protoScript first-strand cDNA synthesis kit (Thermo-Scientific®, UK) with equally distributed mRNA concentrations in each sample was used to synthesize cDNA (Thermo-Scientific®, UK), according to the kits manufacturer's protocol. Oligo-primers (Macrogen®, USA) including TNF-α: Forward–5′CCCCGCTTACAGTTCCTCTT3′, Reverse–5′GAGATCAAGCACAGAGGGCA3′; IL-1β: Forward–5′CTCTAGTTCAGGGCAGGCAT3′, Reverse–5′GACCTATACAACGGCTCCTCC3′; IL-6: Forward–5′GTGTGGGAAAGAGGACACCC3′, Reverse–5′CAGACTCTCTCCCTTCTGAGC3′; PPARγ: Forward–5′GCCCTTTGGTGACTTTATGGA3′, Reverse–

5′GCAGCAGGTTGTCTTGGATG3′;   JAK2:   Forward–5′CCACGGCCCAATATCAATG3′,   Reverse–
5′CCCGCCTTCTTTAGTTTGCTA3′;   STAT3:   Forward–5′ACCCAACAGCCGCCGTAG3′,   Reverse–
5′CAGACTGGTTGTTTCCATTCAGAT3′; Bcl-2: Forward–5′GCTACCGTCGTCGTGACTTCGC3′, Reverse–
5′CCCCACCGAACTCAAAGAAGG3′;   Bax: Forward–5′CATCGCACTCACCCCTGTAG3′,   Reverse–
5′ATCCATCCCCCTGACTCTCC3′; Caspase-9: Forward–5′GGCTGTTAAACCCCTAGACCA3′, Reverse–
5′TGACGGGTCCAGCTTCACTA3′;   β-actin: Forward–5′GGCTATAGTCACCTCGGGGC3′,   Reverse–
5′GTAATAATGCGGCCGGTCTG3′ were purchased. The qRT-PCR was conducted using Maxima SYBR
Green/ROX Master Mix (Thermo-Scientific®, UK) on an iQ5 Bio-Rad machine, adjusting the denaturation at
95ºC, annealing at 60ºC and extension at 72ºC for 39 cycles. Subsequently, the $2^{-\Delta\Delta Ct}$ method was applied
to calculate the gene expression, using β-actin as a housekeeping gene.

Statistical analysis

The GraphPad Prism® software v6 was used to statistically analyze the experimental data by applying
one-way and two-way ANOVA and Tukey's test and data were presented as mean±standard deviation (SD)
values. A difference of $p<0.05$ was considered significant.

RESULTS

Phytochemical characterization of NSE

The TPC of NSE was 81.91±1.13 mg GAE/g as determined by the Folin-Ciocalteu method, whereas TFC
of examined plant extract was 136.01±1.24 mg CE/g. HPLC analysis was used to identify and quantify the
phytocompounds in NSE. The analysis confirmed the presence of seven polyphenols including quercetin,
gallic acid, chlorogenic acid, ferulic acid, sinapic acid and *m*-coumaric acid, as detailed in Table 1.

Table 1. Phytocompounds of NSE identified by HPLC analysis.

Retention time (min)	Phytocompounds	Area (mV.s)	Concentration (ppm)
3.32	Quercetin	3237.28	171.56
5.14	Gallic acid	551.16	19.83
15.15	Chlorogenic acid	2417.13	188.52
19.82	*m*-Coumeric acid	767.70	9.24
22.56	Ferulic acid	1502.81	108.14
26.06	Sinapic acid	1864.31	24.27

In vitro antioxidant activity of NSE

Antioxidant activity of NSE was assessed using its ability to scavenge DPPH radicals. Based on the
concentrations employed, results showed that NSE inhibited free radical formation, as presented in Figure 1.
In comparison to IC50 value of ascorbic acid (1.57 mg/mL), NSE inhibited DPPH by 68.30% at maximal
concentrations of 20 mg/mL with an IC50 of 5.34 mg/mL. (Original source data underlying Table 1 and Figure
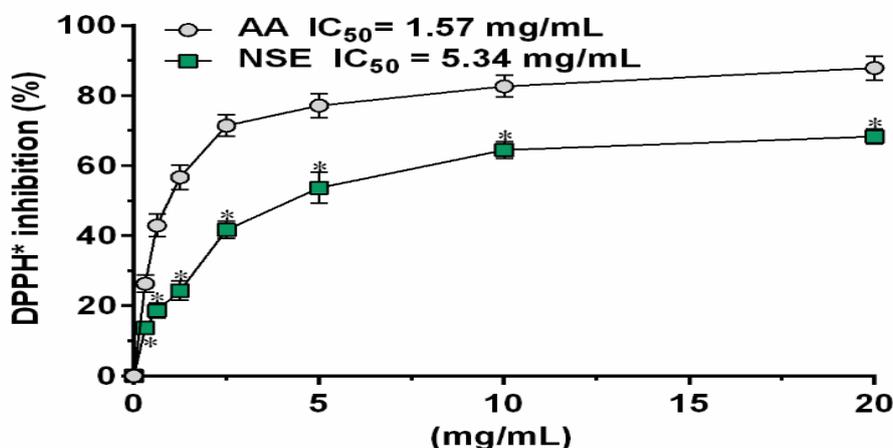1 were provided as supplementary material file 1).

Figure 1. *In vitro* antioxidant activity of NSE at various concentrations in DPPH assay. Triplicate values are presented
as mean±SD. *$p<0.05$ significance from AA. AA: ascorbic acid; NSE: *Nigella sativa* seed extract.

Effect of NSE on biochemical markers and liver histology

Serum levels of ALT, AST, ALP and TBil were measured to evaluate changes in liver function markers (Table 2). The ConA administration resulted in substantial ($p<0.05$) elevation in ALT, AST, ALP and TBil levels as compared to vehicle control. However, it was observed that pretreatment with NSE and pioglitazone protected liver from damaging effect of ConA and significantly ($p<0.05$) lowered these markers when compared to ConA control. NSE dose-dependently lowered liver injury markers and highest effect was observed at 400 mg/kg dose. Additionally, histopathological findings (Figure 2) demonstrated that ConA caused extensive infiltration of inflammatory cells, condensed nuclei and hepatocyte damage in ConA control group in contrast to vehicle control. Whereas mice pretreated with NSE at graded doses revealed improvement in liver histological changes in a dose-dependent manner after 8 h of ConA injection. Furthermore, the attenuated effect of NSE at 400 mg/kg on liver function markers and liver histology was comparable to pioglitazone.

Table 2. NSE attenuated serum levels of liver injury indices in ConA-induced acute liver injury in mice.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	TBil (µmol/L)
Vehicle control	39.91±3.58	32.94±3.77	26.21±4.65	4.93±0.23
ConA control	1243.31±104.47*	1461.91±82.17*	607.13±53.01*	47.37±5.25*
NSE (100 mg/kg)	932.52±44.54*#+	992.23±87.85*#+	442.12±32.11*#+	36.97±3.59*#+
NSE (200 mg/kg)	587.25±49.79*#+	730.62±61.97*#+	291.07±29.29*#+	28.38±1.98*#+
NSE (400 mg/kg)	441.46±59.61*#	513.21±60.09*#	222.91±33.38*#	21.49±3.41*#
Pioglitazone	394.80±44.72*#	474.26±56.28*#	203.01±35.61*#	19.42±2.46*#

Data are presented as mean±SD, n=6. *$p<0.05$, Vehicle control *vs* other groups; #$p<0.05$, ConA control *vs* treated groups; +$p<0.05$, Pioglitazone *vs* NSE pretreated groups. ConA: Concanavalin A, NSE: *Nigella sativa* seed extract.
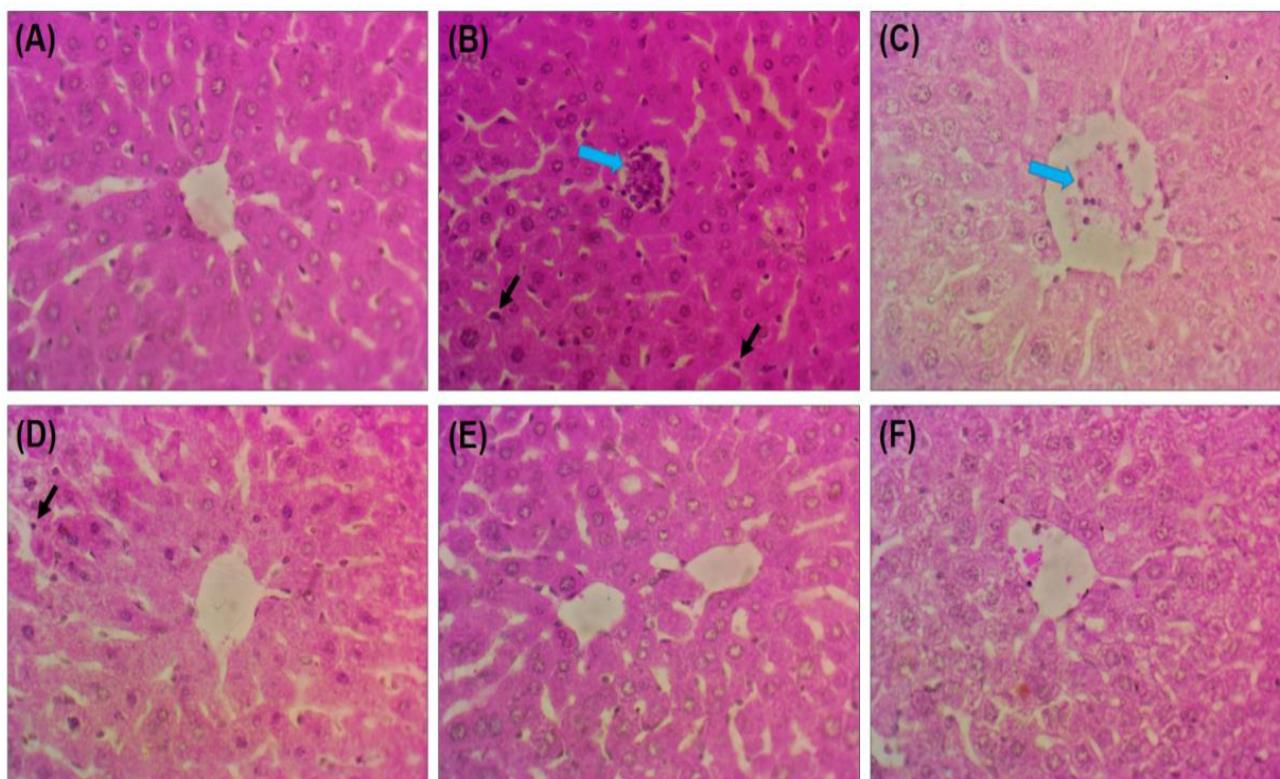

Figure 2. Effect of NSE pretreatment on liver histology of ConA-induced acute liver damage in mice (H&E stain, x400 magnification). (A) Vehicle control showed the intact histological structure of liver as well as normal hepatocytes. (B) ConA control exhibited intense inflammatory cell infiltration, nuclei condensation and damaged hepatocytes. (C-D) Mice pretreated with NSE (100 and 200 mg/kg) indicated moderate degenerative alterations in liver tissue. (E-F) Meanwhile, NSE (400 mg/kg) demonstrated mild histopathological changes in liver which were comparable to pioglitazone. NSE: *Nigella sativa* seed extract.

Effect of NSE on hepatic oxidative stress markers

Results presented in Table 3 indicate the changes in antioxidant/oxidant markers in liver tissues. The ConA caused a significant ($p<0.05$) decrease in activities of antioxidant enzymes (SOD and CAT) and raised MDA level in comparison to vehicle control. However, pretreatment with NSE dose-dependently boosted antioxidant enzyme activities and lowered MDA level in liver tissue. As a result, NSE was shown to minimize the oxidative stress induced by ConA with promising effects observed at 200 and 400 mg/kg in comparison to pioglitazone.

Table 3. NSE alleviated hepatic oxidative stress parameters in ConA-induced acute liver damage in mice.

Groups	SOD (U/mg protein)	CAT (nmol H_2O_2/min/mg protein)	MDA (nmol/mg protein)
Vehicle control	109.24±9.49	34.26±2.50	0.27±0.07
ConA control	51.28±8.26*	16.51±2.44*	1.83±0.10*
NSE (100 mg/kg)	65.74±14.86*	21.68±3.76*#+	1.27±0.10*#+
NSE (200 mg/kg)	81.19±12.25*#	25.17±1.87*#	0.88±0.12*#
NSE (400 mg/kg)	87.56±11.92*#	29.13±2.15*#	0.71±0.11*#
Pioglitazone	82.59±12.06*#	28.28±2.53*#	0.78±0.12*#

Data are presented as mean±SD, n=6. *$p<0.05$, Vehicle control vs other groups; #$p<0.05$, ConA control vs treated groups; +$p<0.05$, Pioglitazone vs NSE pretreated groups. ConA: Concanavalin A, NSE: *Nigella sativa* seed extract.

Effect of NSE on pro-inflammatory cytokine levels

The levels of pro-inflammatory cytokines were assessed to determine the effect of NSE and pioglitazone on inflammatory state in liver tissues (Table 4). Administration of ConA caused a significant ($p<0.05$) increase in TNF-α, IL-1β and IL-6 levels in liver tissues in comparison to vehicle control. It was noticed that NSE pretreatment dose-dependently reduced the TNF-α, IL-1β and IL-6 levels. However, a non-significant ($p>0.05$) difference was observed at 200 and 400 mg/kg doses of NSE as compared to pioglitazone.

Table 4. NSE suppressed pro-inflammatory cytokine levels in liver tissues of ConA-induced acute liver injury in mice.

Groups	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
Vehicle control	26.14±4.13	33.04±6.21	42.21±4.60
ConA control	371.29±19.76*	227.38±26.43*	358.36±29.78*
NSE (100 mg/kg)	242.80±15.35*	171.59±18.86*#+	316.89±30.06*#+
NSE (200 mg/kg)	159.61±18.62*#	134.92±26.94*#	173.81±24.28*#
NSE (400 mg/kg)	134.68±17.71*#	103.34±18.80*#	146.90±20.44*#
Pioglitazone	148.94±15.01*#	110.81±18.21*#	194.14±28.69*#

Data are presented as mean±SD, n=6. *$p<0.05$, Vehicle control vs other groups; #$p<0.05$, ConA control vs treated groups; +$p<0.05$, Pioglitazone vs NSE pretreated groups. ConA: Concanavalin A, NSE: *Nigella sativa* seed extract.

Effect of NSE on TNF-α, IL-1β and IL-6 expressions

Inflammation plays a crucial role in the maintenance of liver homeostasis. Therefore, the mRNA expression levels of pro-inflammatory cytokines in liver tissues of ConA-induced immune-mediated liver injury in mice were estimated by qRT-PCR analysis. Results presented in Figure 3 indicate that ConA injection induced inflammation by causing a significant ($p<0.05$) increase in TNF-α, IL-1β and IL-6 expressions in liver. NSE pretreatment exhibited a significant ($p<0.05$) reduction in expression levels of TNF-α, IL-1β and IL-6 in a dose-dependent manner (Figure 3A-C). Moreover, the high attenuating effect of NSE on pro-inflammatory cytokine levels was observed at 200 and 400 mg/kg doses when compared to pioglitazone pretreated group.
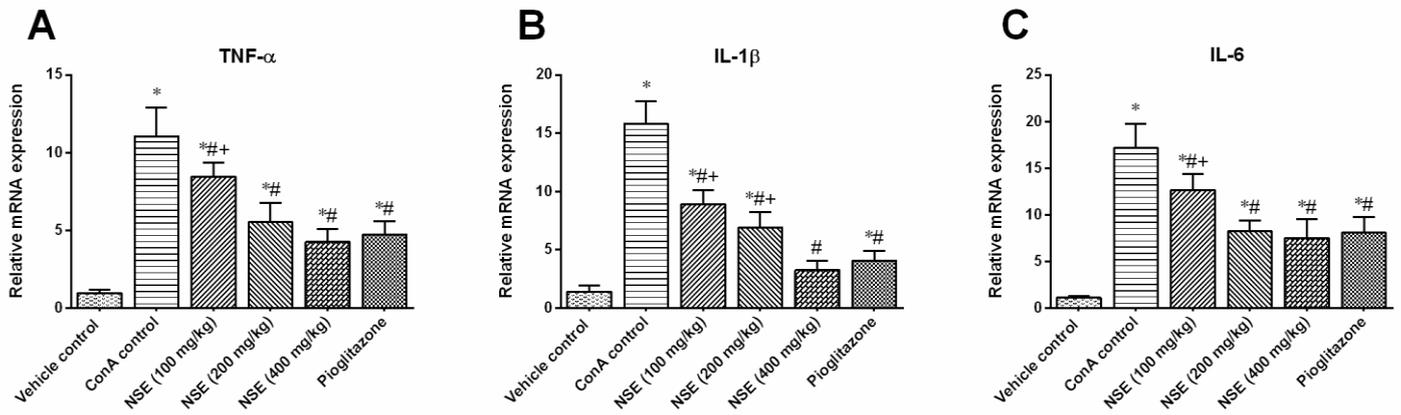
Figure 3. NSE pretreatment downregulated the expression levels of pro-inflammatory cytokines in ConA-induced immunological liver injury in mice. Relative mRNA expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 in liver tissues of mice after 8 h of ConA injection. Data are presented as mean±SD, n=6. *$p<0.05$, Vehicle control vs other groups; #$p<0.05$, ConA control vs treated groups; +$p<0.05$, Pioglitazone vs NSE pretreated groups. ConA: Concanavalin A, NSE: *Nigella sativa* seed extract.

Effect of NSE on PPARγ, JAK2 and STAT3 expressions

In this study, after ConA was injected into NSE and pioglitazone pretreated mice, the influence of PPARγ on JAK2/STAT3 pathway was assessed by qRT-PCR analysis (Figure 4). According to the obtained results, it was noticed that ConA substantially ($p<0.05$) upregulated JAK2 and STAT3 expressions by decreasing PPARγ expression. However, it was seen that NSE dose-dependently increased PPARγ expression and downregulated JAK2 and STAT3 expressions (Figure 4A-C). In particular, NSE at 400 mg/kg demonstrated a better modulatory effect on PPARγ, JAK2 and STAT3 expressions which was comparable to pioglitazone.
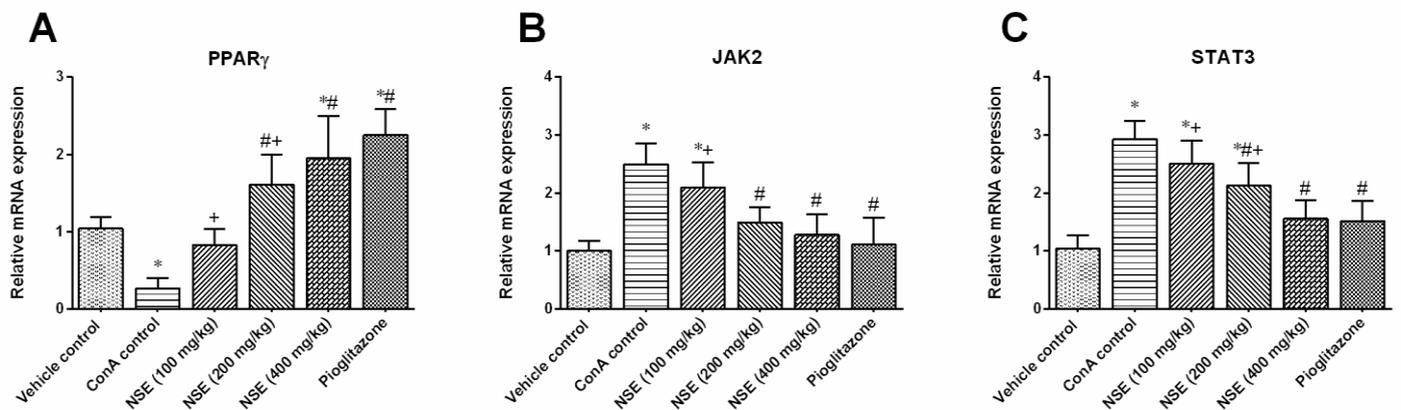

Figure 4. NSE pretreatment modulated PPARγ influenced JAK2/STAT3 expressions in ConA-induced immunological liver injury in mice. Relative mRNA expression levels of (A) PPARγ, (B) JAK2 and (C) STAT3 in liver tissues of mice after 8 h of ConA injection. Data are presented as mean±SD, n=6. *$p<0.05$, Vehicle control vs other groups; #$p<0.05$, ConA control vs treated groups; +$p<0.05$, Pioglitazone vs NSE pretreated groups. ConA: Concanavalin A, NSE: *Nigella sativa* seed extract.

Effect of NSE on Bcl-2, Bax and Caspase-9 expressions

The mRNA expression levels of Bcl-2, Bax and caspase-9 in liver tissues of mice are displayed in Figure 5. The qRT-PCR analysis revealed that ConA significantly ($p<0.05$) suppressed Bcl-2 expression and triggered the overexpression of Bax and caspase-9, causing apoptosis of hepatocytes (Figure 5A-C). Pretreating mice with NSE (100, 200 and 400 mg/kg) and pioglitazone provided liver protection through significantly ($p<0.05$) increasing Bcl-2 expression and decreasing Bax and caspase-9 expressions. Additionally, there was a non-significant ($p<0.05$) difference among NSE (400 mg/kg) and pioglitazone pretreated groups. (Original source data underlying Table 2-4 and Figures 3-5 were provided as supplementary material file 2).
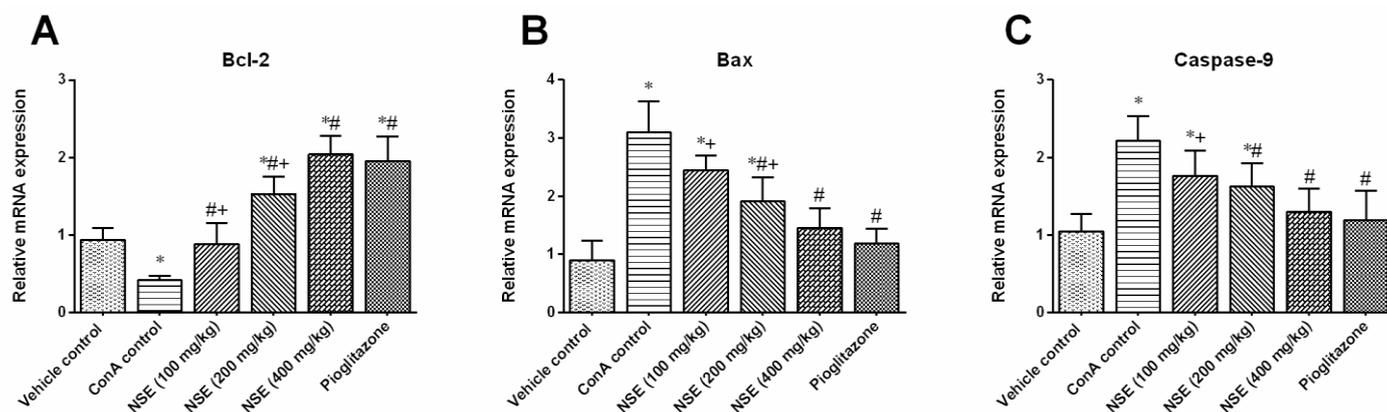
Figure 5. NSE pretreatment upregulated Bcl-2 expression and downregulated Bax and Caspase-9 expressions in ConA-induced immunological liver injury in mice. Relative mRNA expression levels of (A) Bcl-2, (B) Bax and (C) Caspase-9 in liver tissues of mice after 8 h of ConA injection. Data are presented as mean±SD, n=6. *$p<0.05$, Vehicle control vs other groups; #$p<0.05$, ConA control vs treated groups; +$p<0.05$, Pioglitazone vs NSE pretreated groups. ConA: Concanavalin A, NSE: *Nigella sativa* seed extract.

DISCUSSION

Although the diagnosis rate of autoimmune hepatitis (AIH) cases has grown year after year, however, the etiology of AIH remains uncertain with genetic and environmental variables both likely to be involved [2,3]. The failure of self-tolerance mechanisms promotes the development of autoimmunity-induced diseases and AIH is no exception. Due to immune regulatory dysfunction, the immune cells target liver autoantigens, resulting in an uncontrolled immunological response and permanent liver damage [35,36]. Our study aimed to elucidate the protective potential of NSE on acute immune-mediated liver damage induced by ConA. Our results showed that NSE reduced liver injury indices, attenuated histopathological changes and reduced hepatic oxidative stress. Moreover, NSE significantly suppressed levels as well as gene expressions of pro-inflammatory cytokines in liver. Furthermore, NSE markedly inhibited inflammation and hepatocyte apoptosis *via* PPARγ influenced modulation of JAK2/STAT3 and Bcl-2/Bax/Caspase-9 signaling pathways, suggesting a possible hepatoprotective role of NSE.

NSE, rich in polyphenols such as quercetin, gallic acid, chlorogenic acid, *m*-coumeric acid, ferulic acid and sinapic acid, has been recognized for antioxidative [37,38], analgesic [39], anti-inflammatory, immunomodulatory [16,19] and anticancer [40] activities. Therefore, NSE was tested for its hepatoprotective effectiveness in this study. A significant ($p<0.05$) reduction in liver damage indicators (ALT, AST, ALP and TBil) and markedly prevented histopathological changes in liver tissue. Moreover, levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in NSE pretreated mice revealed that NSE also demonstrated a potent anti-inflammatory effect in acute liver injury induced by ConA, as similar findings were reported in earlier studies [41,42]. As previously stated [43], the ConA-induced liver damage is mostly mediated by oxidative stress, as evidenced by a significant ($p<0.05$) increase in SOD and CAT activities and a decrease in MDA level in liver tissues of NSE pretreated groups. Our findings showed that NSE has anti-inflammatory and antioxidative actions on the development of ConA-induced acute liver injury in mice.

The effect of oxidative stress on liver injury has been established several times and various studies reported that "endoplasmic reticulum stress" may potentially be a key factor in the development of reactive oxygen species (ROS)-induced liver injury [44]. The excessive ROS production in mitochondria and generation of many important cytokines during the oxidative damage process result in permanently damaged hepatocytes. Additionally, studies have indicated that various signal transduction pathways (MAPK families) activated by ROS led to an impaired cellular redox state [45]. In this study, we found a significant ($p<0.05$) decrease in TNF-α, IL-1β and IL-6 expressions, suggesting that NSE exhibited a protective role by lowering ROS-induced liver damage.

PPARγ is a member of nuclear transcription factor superfamily which contributes to cellular differentiation and apoptosis. It is well established that PPARγ activation is linked to JAK2/STAT3 pathway. JAK could stimulate hepatocyte apoptosis through stimulating STAT proteins and downstream pathways. Thus, JAK2/STAT3 pathway is considered important in apoptosis onset [46,47]. The JAK2/STAT3 pathway is also modulated by IL-6 which is one of the routes that promote inflammation. It has been shown that blocking the IL-6/JAK2/STAT3 pathway prevents inflammation and thereby protects against possible tissue injury [48]. The current study depicted a significant ($p<0.05$) decrease in PPARγ expression and an increase in JAK2

and STAT3 expressions in liver tissues after ConA administration. Nevertheless, NSE showed anti-inflammatory and anti-apoptotic effects on liver by significantly ($p<0.05$) increasing PPARγ expression and lowering JAK2 and STAT3 expressions.

The Bcl-2 family of proteins includes both pro-apoptotic and anti-apoptotic proteins that regulate cell survival. Bax activation causes apoptosis while stimulation of Bcl-2 inhibits apoptosis. Therefore, Bcl-2/Bax ratio determines the fate of cell survival [49]. A decrease in the Bcl-2/Bax ratio triggers caspase-9 [50]. Studies have revealed that a variety of chemicals induces apoptosis in liver by reducing Bcl-2 and increasing Bax and Caspase-9 expressions, subsequently leading to severe tissue damage [51]. In present study, ConA injection caused a significant ($p<0.05$) reduction in Bcl-2 expression while promoted the expressions of Bax and Caspase-9. It indicated that ConA could alter organ function by triggering apoptosis in liver tissue. However, NSE administration dose-dependently reduced hepatocyte apoptosis by upregulating the Bcl-2 and lowering Bax and caspase-9 expressions, as seen in an earlier study [48].

Overall, hepatocytes can easily persist in an oxidative stress microenvironment due to their distinctive functional characteristics and inflammation stimulation worsens liver dysfunction and induces organ damage. Therefore, natural antioxidants are considered essential in the treatment of liver diseases [52]. In our study, phytochemical characterization showed the presence of considerable polyphenol contents and potent antioxidant activity of plant extract was confirmed. In mice, NSE has shown antioxidative, anti-inflammatory and anti-apoptotic activities and protected the development of ConA-induced immunological liver damage. Furthermore, the focus of our future research will be on the further isolation and characterization of bioactive compounds of *N. sativa* and their effects on specific cellular mechanisms in an immune-mediated liver injury model.

CONCLUSION

This study demonstrated that pretreatment of NSE dose-dependently attenuated ConA-induced liver inflammation and hepatocyte apoptosis in mice by inhibiting pro-inflammatory cytokines and modulating PPARγ/JAK2/STAT3 and Bcl-2/Bax/Caspase-9 pathways. Also, NSE at 400 mg/kg provided hepatoprotection which was comparable to pioglitazone. Thus, our findings suggest that NSE might be used as an alternative therapeutic approach to treat immune-mediated liver diseases.

Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Material: Data were provided as supplementary material files. Available in:
File 1: https://www.documentador.pr.gov.br/documentador/pub.do?action=d&uuid=@gtf-escriba-tecpar@d48932ac-75fe-4da1-b273-6bec803ae46a
File 2: https://www.documentador.pr.gov.br/documentador/pub.do?action=d&uuid=@gtf-escriba-tecpar@3eda25ab-9f67-4594-b888-b930c6a499e0

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