PROTECTIVE EFFECT OF *NIGELLA SATIVA* L. ON D-GALN/LPS INDUCED TOXICITY AND ITS CONSTITUENTS ANALYSIS BY HPLC TECHNIQUE

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ABSTRACT
The present work has been carried out to study the protective effect of alcoholic extract of Nigella sativa L. seeds (NSE) on D-Galactosamine (D-GalN) / Lipo polysaccharide (LPS) induced toxicity in rats and to analyze the constituents of the extract using HPLC technique. Twenty four adult wistar albino rats were divided into four groups viz Control, D-GalN/LPS induced toxicity, *Nigella sativa* alcoholic extract (NSE) treated and *Nigella Sativa* alcoholic extract pretreatment prior to the administration of D-GalN/LPS. After treatment, the rats were sacrificed, blood samples were collected for determination of serum high density lipo protein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL). Levels of total cholesterol, triglycerides (TG) and phospholipids were analyzed from serum and liver samples. Level of total lipid analyzed from liver sample. Mean LDL, VLDL, TG, total cholesterol and total lipid were found to be significantly higher in D-GalN/LPS induced hepatotoxic rats group (P<0.05), mean HDL and phospholipid levels were found to be significantly decreased in D-GalN/LPS induced hepatotoxic rats group (P<0.05) than in the control group. The HPLC analysis confirmed the presence of various groups of secondary metabolites such as sterols, alkaloids and quinones. Levels of various parameters in the NSE pretreated group were found to be nearer to control and the constituents of the seed were found to be antioxidants which protected rats from D-GalN/LPS induced toxicity. Results of the present study strongly indicate that NSE having protective efficiency against D-GalN/LPS induced toxicity in rats.
KEYWORDS: Nigella sativa L., D-Galactosamine, Lipopolysaccharide, HPLC, Lipid profile.

1. INTRODUCTION

*Nigella sativa L.* (NS) is a well-known medicinal plant, widely used in various traditional medicine systems, including Ayurveda, Siddha and Unani. The NS seed contains various active phytochemical constituents, including thymoquinone, thymohydroquinone, q-cymene, carvacrol, t-anethole, 4-terpineol, longifoline, nigelicine, nigellidine, nigellimine and isoquinolines.[1] NS is used to treat various chronic diseases, such as diabetes, hypertension, asthma, cancer and cardiovascular disease.[2] The NS seed constituents exhibited therapeutic properties viz., antioxidant, antiparasitic, anticancer, antimicrobial, anti-inflammatory, analgesic and antipyretic properties.[3–5] Several studies suggested that NS seed extract can be used to suppress cough, retard carcinogenesis, disintegrate renal calculi, and treat polio, diarrhea, abdominal pain and flatulence.[6,7] The active ingredients in NS seeds play a major role in inhibiting carcinogenesis and induces cell death in various cancer cells, including cervical cancer, hepatic cancer, colon cancer, blood cancer, pancreatic cancer, skin cancer, renal cancer, fibrosarcoma, lung cancer, prostate cancer and breast cancer.[8,9]

D-GalN is an amino sugar normally found *in vivo* only in acetylated form in certain structural polysaccharides. Administration of single dose of this compound results in dose dependent hepatic damage resembling viral hepatitis, with focal necrosis and periportal inflammation. It induces hepatotoxicity by inhibiting the synthesis of RNA and protein through a decrease in cellular UTP concentration which finally leads to the necrosis of liver cells. Rats intoxicated with D-GalN revealed morphologic features closely resembling those seen in viral hepatitis. Acidophilic degeneration, appearance of councilman bodies, single cell necrosis, foci of hepato cellular necrosis, enlarged liver macrophages and periportal inflammatory infiltrations were also found in the rats.[10]

Lipopolysaccharide (LPS; endotoxin) is a component of gram-negative bacteria that elicits a potent inflammatory response in mammals. At smaller doses, LPS increases liver sensitivity to galactosamine, ethanol, carbon tetra chloride, aflotoxin B1, monocrotaline and allyl alcohol.[11] Fulminant hepatitis can be induced in experimental animals by the synergistic action of a small dose of lipopolysaccharide and D-Galactosamine induced an inhibitor of hepatocellular RNA synthesis.
The aim of this work was to evaluate the protective effect of alcoholic extract of *Nigella sativa* L. seeds (NSE) against D-Galactosamine and lipo polysaccharide induced lipid metabolic changes in adult wistar albino rats and to carry out the HPLC analysis of alcoholic extract of *Nigella sativa* L. seeds.

2. MATERIALS AND METHODS
The powdered *Nigella sativa* seed was subjected to soxhlet extraction using 95% ethanol. The solvent was removed *in vacuo* to the extent that there is no smell of ethanol in the oily residue to give an appropriate yield 22%. The extract was named as NSE. NSE administration did not produce any abnormalities such as atoxic, circling, lacrimation, laboured breathing etc., in the animals throughout the experimental period. The dose level fixed for the present study was non-toxic and safe. Twenty four male wistar albino rats (180-230 gm), were fed standard rat pellets and drinking tap water *ad libitum*. Experimental protocol was approved by the Institutional Animals Ethics Committee (IAEC) of Periyar Maniyammai University, Vallam, Tamil Nadu, India. Rats were divided into 4 groups of 6 animals in each group. Control, D-GalN/LPS administered, NSE administered and NSE pretreatment prior to D-GalN/LPS administration. The control Group (Group I) received only tap water. Group II comprised rats were administrated (i.p) D-Galactosamine (D-GalN) (300 mg/kg B.Wt.) and Lipopolysaccharide (LPS) (Sero type 0111.B4 extracted by phenol water method from E.Coli 30μg/kg B.Wt.) 18 hrs before the experiment[12], Group III rats received NSE 500 mg/kg orally for 15 days. Group IV rats were given NSE pretreatment for 15 days prior to the administration of D-GalN/LPS. After the scheduled treatments the blood sample was taken from the tail vein and serum was trapped and then used for the analysis of HDL, LDL, VLDL[13] total cholesterol,[14] triglycerides,[15] and phospholipids.[16] Liver was immediately excised after perfusing with physiologic saline, the organs were blotted dried, weighed and then homogenized to get a 1% solution of tissue homogenate in Tris – Hcl buffer (10 mM, pH – 8.0) and were used for the determination of total lipids,[17] total cholesterol,[14] triglycerides,[15] and phospholipids.[16]

2.1. HPLC Analysis
The various fractions present in the alcoholic extract of *Nigella sativa* L. seed were analyzed using HPLC with the following specification.

Model : Schimadzu – Japan
Stationary Phase : Silica Gel (Reversed Phase)
Mobile Phase: Water:methanol:2-Propanol(50:45:5% v/v)
Main Column: Analytical - Shim – Pack CLC – OCTA DECYL SILANE (CDS – C18) (4.6mm ID* 25Cm)
Guard Column: Shim – pack G – ODS (4mm ID* 1Cm)
Detector: UV – Spectrophotometric
Flow Rate: 0.5 ml per minute
Column head Pressure: 125Kgf/Cm²
Injection Volume: 20µl
Wavelength: 254, nm

2.2. Statistical Analysis

Statistical significance of differences between the control and treatment groups was determined by ANOVA (Analysis of Variance) followed by Dunnet’s t-test using the SPSS 11 version. Data are expressed as mean ± standard deviation (mean ± S.D). The level of significance chosen was (P<0.05)

3. RESULTS AND DISCUSSION

Table 1: Levels of total cholesterol, triglycerides and phospholipids in the serum of control and experimental groups of rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>77.58 ± 1.28</td>
<td>105.29 ± 1.58a*</td>
<td>78.97 ± 1.69</td>
<td>81.59 ± 1.82b*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>44.11 ± 1.46</td>
<td>110.12 ± 1.51a*</td>
<td>44.25 ± 1.54</td>
<td>63.11 ± 1.60b*</td>
</tr>
<tr>
<td>Phospholipids (mg/dl)</td>
<td>107.40 ± 1.54</td>
<td>74.10 ± 2.02a*</td>
<td>109.18 ± 1.23</td>
<td>95.41 ± 2.03b*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group

a As compared with Group I. b As compared with Group II. *P<0.001, **P<0.01.

Table 2: Levels of total lipids, total cholesterol, triglycerides and phospholipids in the liver of control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Lipids (mg/g)</td>
<td>57.11 ± 1.53</td>
<td>80.15 ± 1.74a*</td>
<td>58.21 ± 1.54</td>
<td>61.13 ± 1.65b*</td>
</tr>
<tr>
<td>Total cholesterol (mg/g)</td>
<td>12.11 ± 0.17</td>
<td>20.10 ± 0.92a*</td>
<td>11.97 ± 0.50</td>
<td>14.15 ± 0.30b*</td>
</tr>
<tr>
<td>Triglycerides (mg/g)</td>
<td>23.12 ± 1.03</td>
<td>43.11 ± 1.54a*</td>
<td>24.11 ± 1.70</td>
<td>32.51 ± 0.58b*</td>
</tr>
<tr>
<td>Phospholipids (mg/g)</td>
<td>26.12 ± 1.31</td>
<td>16.16 ± 1.15a*</td>
<td>25.20 ± 1.36</td>
<td>21.16 ± 1.05b*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group

a As compared with Group I. b As compared with Group II. *P<0.001, **P<0.01.
Tables 1 and 2 show the levels of total lipids in the liver, total cholesterol, triglycerides and phospholipids in the liver and serum of control and experimental groups of rats. The results show that the levels of all the parameters mentioned above manifested a significant increase (P<0.001) in the Group II rats except the levels of phospholipids, which showed a significant decrease (P<0.001) compared to control rats. Rats pretreated with Nigella sativa extract prior to the administration of D-GalN/LPS showed a restoration of the altered lipid levels induced by D-GalN/LPS towards near normally thereby showing the modulating effect of the plant extract against D-GalN/LPS induced changes in the lipid levels in rats.

A number of agents that produce liver injury also cause the accumulation of abnormal amounts of fat, predominantly triglycerides in the hepatic parenchymal cells. In general, accumulation of triglycerides can be thought of as resulting from an imbalance between the rate of synthesis and the rate of release of triglycerides by the parenchymal cells into the systemic circulation[18]. Non esterified fatty acids (NEFAs) removed from the circulation or synthesized endogenously are processed through two main pathways in liver, (a) mitochondrial β-oxidation for production of metabolic energy and (b) incorporation into complex lipids, especially triglycerides, phospholipids, cholesterol esters and glycolipids[19]. Once synthesized, the complex lipids may be used for production of cellular membranes (Structural lipids) or continuously secreted from the liver into the blood. The latter pathway appears to be of the greatest interest in the triglyceride accumulation observed in the fatty liver caused by the administration of hepatotoxin.[20]

The hyper triglyceridemia observed in D-GalN/LPS induced toxicity may be due to the clearance defects associated with deficient LPL activity. Increased fatty acid mobilization from peripheral adipose tissues and decreased triglycerides clearance form blood circulation are considered causes for the hepatoma induced hyper triglyceridemia. Hypertriglyceridemia which is frequently observed in various degrees in tumour bearing animals in combination with increased VLDL and decreased HDL, which is very suggestive of a defective catabolism rather than elevated hepatic synthesis of triglycerols rich lipoproteins. Several earlier studies report increased accumulation of triglycerides during D-GalN/LPS toxication in animals[21]. The decreased content of triglycerides in Group IV animals may be due to the hypolipidemic activity of the NSE.
Liver plays a major role in cholesterol metabolism in mammals. The administration of D-GalN in rats caused a significant increase in total cholesterol levels.[22] In accordance with these reports, the present study elicited a significant increase in total cholesterol levels after D-GalN/LPS administration. The increased total cholesterol level in Group II rats may be due to decreased uptake of cholesterol from blood. Blood supply to hepatomas is decreased and hence 80% decrease in uptake of blood born substances occurred in hepatoma conditions[23]. The decreased cholesterol content to near normal in the pretreated groups (IV) can be due to strong hypocholesterolemic activity of NSE. This may inhibit cholesterol synthesis and accumulation.

Phospholipids are known to play a significant role in the molecular organization and in the activity of membrane bound enzymes. They also play an important role in maintaining the structural integrity of the hepatocellular membrane[24]. The decreased levels of phospholipids in Group II rats implicate the alteration and disturbance in the phospholipids metabolism after injection of D-GalN/LPS. The Ca\(^{2+}\) dependent enzyme phospholipase A\(_2\) is a key enzyme in the arachidonic cascade, and this enzyme may play an important role in the increased lipid peroxide formation in the animals injected with D-GalN/LPS. The increased concentration of intracellular calcium caused by the administration of D-GalN/LPS increases the activity of phospholipids A\(_2\) which in turn causes the hydrolysis of liver membrane phospholipids to release arachidonic acid[25]. This might account for the decreased levels of phospholipids observed in the liver and serum of the D-GalN/LPS challenged rats.

The considerable increase in the levels of phospholipids in the Group IV as compared with Group II rats suggest that Nigella sativa extract might decrease the activity of the enzyme phospholipase A\(_2\) probably by maintaining the levels of intracellular calcium within normal levels. This in turn decreases the degree of hydrolysis of membrane phospholipids to release arachidonic acid. This might also be one of the probable mechanisms, which is responsible for the anti-inflammatory activity of the plant extract.

**Table 3: Levels of HDL, LDL and VLDL in the serum of control and experimental groups of rats**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL (mg/dl)</td>
<td>31.17 ± 1.24</td>
<td>20.15 ± 1.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.98 ± 1.01</td>
<td>27.11 ± 1.54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>39.15 ± 1.10</td>
<td>80.11 ± 1.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.67 ± 1.75</td>
<td>48.22 ± 1.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VLDL (mg/dl)</td>
<td>7.26 ± 0.17</td>
<td>10.03 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.32 ± 0.13</td>
<td>6.26 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Values are expressed as mean ± SD for six animals in each group

a As compared with Group I. b As compared with Group II. *P<0.001, **P<0.01.

Table 3 shows the levels of HDL, LDL and VLDL in the serum of control and experimental groups of rats. The present study elicited a significant increase (P<0.001) in serum VLDL and LDL in the Group II rats compared to control rats. The abnormalities in the lipid and lipoprotein composition produced by D-GalN/LPS have been attributed to the decreased LCAT activity and concomitant defects in lipoprotein metabolism. The decreased levels of LDL and VLDL in the Group IV rats compared to Group II rats may be due to the optimal activity of serum lipoprotein lipase and due to the antioxidant effect of the extract.

HDL is considered to be a beneficial lipoprotein. It helps in the scavenging of cholesterol from the extra hepatic tissues in the presence of lecithin cholesterol acyl transferase and brings it to the liver. Decreased levels of serum HDL were seen in D-GalN/LPS intoxicated rats (Group II) compared to the control rats (Group I) (P<0.001). The lowered HDL levels can be attributed to the decreased serum lipoprotein lipase and lecithin cholesterol acyl transferase activity. The considerable increase in the levels of HDL in the Group IV compared to Group II rats (P<0.01) may be due to the delayed clearance and increased synthesis of HDL constituents. Therefore the increased HDL levels in the Group IV rats might be due to the increased activities of lipoprotein lipase and lecithin cholesterol acyl transferase.

![HPLC Analysis](image)

Figure 1

3.1. HPLC Analysis

The HPLC analysis of the alcoholic fraction at the specific conditions revealed 15 peaks with retention time and peak area (Table 4 & Fig.1).
The peaks at 6.720, 8.953 and 10.753 retention time revealed the presence of thymoquinone (TQ), dithymoquinone (DTQ) and thymol (THY) respectively in the extract. This is in agreement with the observation noted in previous studies. The HPLC fingerprint profile at 254 nm in the methanolic mobile system reveals the presence of steroids and terpenoids. β-sistosterol, cycloeucalenol, cycloartenol and α-hedrin has also been reported to occur along with thymoquinone in Nigella sativa. Nigelicine, nigellidine, nigellimine-N-oxide are the various alkaloids reported in Nigella sativa. Various polyphenolic compounds such as alkaloids, flavonoids, tannins and steroids responsible for the anti-inflammatory potential of the plant L.aspera have been documented.

Table 4: HPLC analysis of the Nigella sativa alcoholic fraction showing different retention times and peak areas.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention Time</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.880</td>
<td>155.32</td>
</tr>
<tr>
<td>2</td>
<td>4.880</td>
<td>891.28</td>
</tr>
<tr>
<td>3</td>
<td>6.720</td>
<td>1419.95</td>
</tr>
<tr>
<td>4</td>
<td>8.953</td>
<td>303.53</td>
</tr>
<tr>
<td>5</td>
<td>9.287</td>
<td>209.17</td>
</tr>
<tr>
<td>6</td>
<td>9.887</td>
<td>186.73</td>
</tr>
<tr>
<td>7</td>
<td>10.753</td>
<td>306.87</td>
</tr>
<tr>
<td>8</td>
<td>11.347</td>
<td>175.79</td>
</tr>
<tr>
<td>9</td>
<td>12.087</td>
<td>634.71</td>
</tr>
<tr>
<td>10</td>
<td>14.087</td>
<td>534.47</td>
</tr>
<tr>
<td>11</td>
<td>18.207</td>
<td>134.04</td>
</tr>
<tr>
<td>12</td>
<td>19.393</td>
<td>1694.41</td>
</tr>
<tr>
<td>13</td>
<td>35.687</td>
<td>590.79</td>
</tr>
<tr>
<td>14</td>
<td>41.913</td>
<td>1920.24</td>
</tr>
<tr>
<td>15</td>
<td>62.727</td>
<td>725.02</td>
</tr>
</tbody>
</table>

4. CONCLUSION
In the present study, it is concluded that Nigella sativa contains various groups of secondary metabolites such as sterols, alkaloids and quinones all of which have been reported to be antioxidants. Increase in LDL, VLDL, lipids (cholesterol, triglycerides) and a considerable decrease in HDL and phospholipids level were noted in D-GalN/LPS toxication. NSE pretreatment showed a tendency for the restoration of altered levels thereby showing the protecting effect of the NSE. Therefore N sativa seeds may be used in the regular food to protect health from harmful chemicals.
5. REFERENCES


