Experimental
## MATERIALS

### 2.1.1 Chemicals

The chemicals and reagents were obtained from various sources listed below.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Chloro 2, 4-Dinitrobenzene</td>
<td>Central drug house, Pvt. Ltd., India</td>
</tr>
<tr>
<td>1,1,3,3-Tetra Methoxy Propane</td>
<td>Sigma-Aldrich Inc., USA</td>
</tr>
<tr>
<td>2,2-Diphenyl-1-Picryl Hydrazyl</td>
<td>Sigma-Aldrich Inc., USA</td>
</tr>
<tr>
<td>2,4,6-Tripyridyl-s-Triazine</td>
<td>HiMedia Laboratories Pvt. Ltd., India</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>Miles Ltd. Inc., USA</td>
</tr>
<tr>
<td>Butylated Hydroxyl Toluene</td>
<td>HiMedia Laboratories Pvt. Ltd., India</td>
</tr>
<tr>
<td>Cholesterol (Extra pure)</td>
<td>Sisco Research Laboratories Pvt. Ltd., India</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue G 250</td>
<td>Ployscience Inc., USA</td>
</tr>
<tr>
<td>Dextran Sulfate</td>
<td>Sigma-Aldrich Inc., USA</td>
</tr>
<tr>
<td>5, 5’-Dithiobis (2-Nitrobezoic Acid)</td>
<td>Sisco Research Laboratories Pvt. Ltd., India</td>
</tr>
<tr>
<td>Glutathione Oxidized</td>
<td>Sisco Research Laboratories Pvt. Ltd., India</td>
</tr>
<tr>
<td>Glutathione Reduced</td>
<td>HiMedia Laboratories Pvt. Ltd., India</td>
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<td>Hemoglobin Assay Kit</td>
<td>Ranbaxy Diagnostics, India</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>Pharmacia, USA</td>
</tr>
<tr>
<td>Hydroxylamine Hydrochloride</td>
<td>HiMedia Laboratories Pvt. Ltd., India</td>
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<td>Limonene</td>
<td>Sigma-Aldrich Inc., USA</td>
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<tr>
<td>Mevalonolactone</td>
<td>United States Bioch.Corp., USA</td>
</tr>
<tr>
<td>NADP</td>
<td>HiMedia Laboratories Pvt. Ltd., India</td>
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</table>
Experimental

NADPH  HiMedia Laboratories Pvt. Ltd., India
N-Ethylmaleimide  HiMedia Laboratories Pvt. Ltd., India
Nitroblue Tetrazolium Salt  HiMedia Laboratories Pvt. Ltd., India
Ouabain  Sigma-Aldrich Inc., USA
Phenazine Methosulfate  HiMedia Laboratories Pvt. Ltd., India
Phenyl Acetate  Sigma-Aldrich Inc., USA
Phosphotungstic Acid  Loba Chemie, India
Rat Chow  Ashirwad Industries, Chandigarh, India
Sodium Dodecyl Sulfate  Bio-Rad Laboratories, USA
Sodium pyrophosphate  HiMedia Laboratories Pvt. Ltd., India
Thiobarbituric Acid  Sigma-Aldrich Inc., USA
Thymoquinone  Sigma-Aldrich Inc., USA
Triglyceride Assay Kit  Autospan kit SPAN Diagnostics, India
Triphenyl Phosphine  HiMedia Laboratories Pvt. Ltd., India
Xylenol Orange  HiMedia Laboratories Pvt. Ltd., India

All other chemicals and reagents used in this study were of analytical grade.
2.1.2 Animals/Exposure to Cigarette Smoke and Treatments

Healthy male albino rats, weighing 180-200 g were purchased from Indian Veterinary Research Institute, Barielly, were acclimatized to J. N. Medical College animal house environment for 3 weeks prior to the experiment. The rats had free access to standard rat chow and water. Thirty rats in experimental groups were exposed to cigarette smoke for 30 min, daily for 30 days with interval of 10 min between each 10 min exposure, using 3 cigarettes/day/2 rats in each group. Exposure to cigarette smoke was done each morning between 7 AM and 11 AM, by keeping two rats in bottomless metallic container (10 x 11 x 16 inch), having two holes of 3 and 1.5 cm diameter, one on the either side. A burning cigarette was introduced through one hole (3 cm) and the other hole (1.5 cm) was used for ventilation. Ten percent methanol extract (ME) and 2 % essential oil (EO) extracted from *Nigella sativa* seeds, one percent thymoquinone (TQ) and 15 % limonene (LMN) suspensions were prepared by dissolving in DMSO (12.5 %) and then homogenizing with saline. Thirty minutes prior to cigarette smoke exposure, five rats in CS-ME and CS-EO groups, six rats in CS-TQ and CS-LMN groups received one ml of above saline suspension containing 100 mg of ME, 20 mg of EO, 10 mg of TQ or 150 mg of LMN by using intragastric intubation in two equal doses (morning and evening) of 0.5 ml each for 30 days. Eight rats in cigarette smoke exposed control (CS-C) group, with no drug intervention, received 0.5 ml of saline containing 12.5 % DMSO, 30 minutes before exposure to cigarette smoke, twice daily for 30 days. Eight normolipidemic rats in control group (N-C) were administered 0.5 ml of saline twice daily.
METHODS

2.2.1 Preparation of Methanol and Essential Oil Extracts from Nigella sativa (NS) Seeds

*Nigella sativa* seeds were purchased from a local store, washed and dried at 50°C for 8 hr. The seeds were powdered in a grinder and used for the preparation of methanol extract (ME) and essential oil (EO).

**2.2.1.1 Extraction of ME**

The ME, used in the present study, was extracted by stirring 48 g of the NS seed powder with 180 ml of methanol for 90 min at ambient temperature. The methanol layer was removed, evaporated at 45°C and the resulting ME residue was flushed with nitrogen and kept in dark coloured bottle at 4°C. The ME from 41 extractions were pooled together giving an average yield of 1.252±0.002%.

**2.2.1.2 Extraction of EO**

The EO, used in the experiments, was extracted from NS seeds by steam distillation. The procedure is essentially the same as described by (Kanter *et. al.*, 2006). Briefly, 250 ml of distilled water was added to a distillation flask containing 70 g of NS seeds powder, and the temperature was set to boiling point. The process of distillation was continued until about 100 ml of the distillate containing essential oil was collected in a dark glass bottle. The distillate was extracted with 60 ml of diethyl ether, moisture was removed by the addition of anhydrous sodium sulphate and the resultant ether extract was evaporated at 40°C. The essential oil thus obtained was stored under nitrogen in dark coloured bottle at 4°C. The EO fractions from 34 extractions were pooled together resulting in an average yield of 0.166±0.008%.

**2.2.2 Analytical Procedures**

**2.2.2.1 Collection of blood and preparation of erythrocytes/membranes**

At the conclusion of the study, overnight fasted rats in each group were anaesthetized and blood was drawn from cardiac puncture, collected in heparinised tubes, mixed gently by inversion 2-3 times and incubated at 4°C for 2-3 h. Plasma was separated from blood by centrifugation at 2,500 rpm for 30 min, aliquoted and stored at 4°C. After the removal of plasma and buffy coat, the packed erythrocytes thus, obtained were resuspended gently with two parts of physiological saline and
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centrifuged again 1,500 rpm for 10 min at 4°C. The washing procedure was repeated two more times. A portion of the washed erythrocytes was used for the isolation of membranes according to the procedure of Hanahen and Ekholm, 1974. Hemolysates from a portion of packed erythrocytes were prepared as described by Lakshmi and Rajagopal (1998).

2.2.2.2 Preparation of liver homogenate and post-mitochondrial supernatant

At the end of the experiment, liver from each rat was promptly excised and chilled in ice cold saline. After washing with saline, each liver was blotted and weighed. Livers in each group were pooled, cut into small pieces, mixed and 10 g of wet tissue was homogenized with 90 ml of chilled 0.1 M sodium phosphate buffer, pH 7.4, containing 1.17% KCl in a waring blender. The volume of homogenate was recorded and centrifuged at 1,000 rpm for 10 min at 4°C. After centrifugation, a portion of the homogenate obtained from pooled liver samples in each group was aliquoted and stored at –20°C. The remaining portion of the homogenates was centrifuged at 12,000 rpm for 20 min at 4°C. The post-mitochondrial supernatant thus obtained was aliquoted and stored at –20°C for future use.

2.2.2.3 Determination of 2, 2-Diphenyl-1-Picryl Hydrazyl radical (DPPH·) scavenging activity (antioxidant capacity) of NS seed and it’s ME, EO fraction; pure Thymoquinone (TQ) and pure Limonene (LMN)

DPPH· scavenging activities of ME, EO extract of NS seed, pure TQ and pure LMN were determined in triplicate by the method of Mensor et al. (2001). The reaction mixture contained several concentrations of ME, EO extract or pure TQ in methanol, whereas pure LMN was dissolved in Tween 80-PBS. The reaction was started by the addition of freshly prepared methanolic solution of DPPH· (86 μM) in a total volume of 3.5 ml, mixed thoroughly and allowed to react in dark at ambient temperature. After 30 min the absorbance at 518 nm was read against a blank of pure methanol. The above fractions in methanol (3.5 ml) incubated without DPPH· were used as control. The concentration dependency of the above antioxidant fractions/compounds and DPPH· reactions were demonstrated by plotting the percent of DPPH· remaining against each level of antioxidant by using standard DPPH·.
2.2.2.4 Determination of plasma triglycerides

Triglycerides were determined by using enzymatic kit. The method uses a modified Trinder color reaction to produce a fast, linear, end point reaction (Trinder, 1969). The intensity of the color produced after incubation is directly proportional to the concentration of the triglycerides in the plasma samples when measured at 500 nm in a Beckman DU 640 spectrophotometer. Triglycerides in plasma samples were calculated by using a triolein standard.

2.2.2.5 Fractionation of plasma lipoproteins

The precipitation method described by Wieland and Seidel (1989) was used for the isolation of plasma low density lipoprotein (LDL). The precipitation buffer consisted of 64 mM trisodium citrate adjusted to pH 5.05 with 5 N HCl, containing 50,000 IU/L heparin. Before precipitation of LDL, plasma samples and precipitation reagent were allowed to equilibrate to room temperature. One ml of plasma sample was added to 7.0 ml of heparin-citrate buffer. After mixing with a vortex mixer, the suspension was allowed to stand for 10 min at 22°C. The insoluble LDLs were then sedimented by centrifugation at 1,500 rpm for 10 min at 22°C. The pellet was resuspended in 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9 % NaCl.

The method for the isolation of small dense (sd)-LDL and large buoyant (lb)-LDL from isolated LDL is based on the two-step procedure of Hirano et al. (2004), which employs quantification of sd-LDL from serum by heparin-Mg\(^{++}\) precipitation. In our modified method of sd-LDL and lb-LDL isolation, 0.1 ml of precipitation reagent containing 15 IU of heparin and 90 mM MgCl\(_2\) was added to 0.1 ml of isolated LDL sample as described above. After mixing, the sample was incubated at 37°C for 10 min. Then each sample was incubated in an ice-bath for 15 min and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant containing sd-LDL was carefully removed and saved. The pellet containing lb-LDL was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9 % NaCl. Appropriate aliquots of LDL, sd-LDL and lb-LDL fractions were used for the analysis of their cholesterol and apoB content.

The method of Kostner (1976) was used for the isolation of high density lipoprotein (HDL). The following solutions were used:
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- 9.7 mM Phosphotungstic acid
- 0.4 M magnesium chloride

0.2 volume of 1:1 mixture of Phosphotungstic acid and magnesium chloride was added to 1 volume of plasma. The samples were mixed immediately, allowed to stand for 5 min at room temperature and then centrifuged for 10 min at 12,000 rpm at room temperature. The clear supernatant was used for the analysis of HDL-cholesterol as well as for the fractionation of HDL₃-cholesterol and HDL₂-cholesterol.

Fractionation of HDL-cholesterol subfraction and VLDL-cholesterol was done by precipitation method described by Bachorik and Albers (1986). The following solutions were used for the fractionation of HDL-cholesterol:

- Dextran sulfate (Mr 50,000) 40 gm/L, pH 7.0.
- MgCl₂·6H₂O, 2 M, pH 7.0.
- Reagent ‘X’ was prepared by mixing 1 volume of 40 gm/L dextran sulfate with 3 volumes of 2 M MgCl₂·6H₂O.

For HDL-cholesterol fractionation, 0.1 volume of reagent ‘X’ was added to 1 volume of HDL-cholesterol sample and mixed immediately. The samples were allowed to stand at room temperature for 15 min and then centrifuged at 5,000 rpm for 30 min at 4°C. Aliquots of clear supernatant were used for HDL₃-cholesterol analysis, whereas the HDL₂ precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9 % NaCl.

For the isolation of VLDL, 0.075 volume of 10 % SDS solution was added to 1 volume of plasma. After mixing them well, the samples were incubated for 2 h at 37°C. These were then centrifuged at 10,000 rpm for 10 minutes at room temperature. The VLDL pellicle floats at the top of the tube. The supernatant was carefully removed with a hypodermic syringe and discarded. The pellicle residue was dissolved in 1 volume of 1 % SDS by warming at 37°C for 15 minutes.

**2.2.2.6 Determination of plasma cholesterol**

Total cholesterol in plasma, LDL, sd-LDL, lb-LDL, HDL, HDL₂, HDL₃ and VLDL subfractions was determined as described by Annino and Giese (1976), with
a minor modification. For the determination of cholesterol in plasma and lipoproteins, 0.1 volume of plasma was mixed with 1 volume of isopropanol, allowed to stand for 5 min and centrifuged at 3,000 rpm for 10 min. A suitable aliquot of isopropanol extract was used for cholesterol determination in a total volume of 0.75 ml. To each tube 0.25 ml of 7.02 mM ferric chloride dissolved in glacial acetic acid, was added, mixed instantly, followed by the addition of 0.8 ml of sulphuric acid with thorough mixing. After 5 min, the absorbance was read at 550 nm in a Beckman DU 640 spectrophotometer. The cholesterol content in the samples was determined by using a cholesterol standard.

2.2.2.7 Measurement of ex vivo and in vitro Cu\(^{++}\)-mediated susceptibility of LDL, sd-LDL and lb-LDL to oxidation

The ex vivo and in vitro Cu\(^{++}\)-mediated susceptibility of isolated LDL, sd-LDL and lb-LDL to oxidation was assessed by determining the lag time of conjugated diene formation using the method of Esterbauer et al. (1989; 1992). Prior to oxidation studies LDL, sd-LDL and lb-LDL samples were dialyzed against 10 mM phosphate buffer saline (PBS), pH 7.4, for 12 h. The incubation mixture contained 100 μg of LDL, sd-LDL or lb-LDL in 1.0 ml of 10 mM PBS, pH 7.0. At time zero, before the initiation of Cu\(^{++}\)-mediated oxidation, 1.0 ml aliquot of each sample was added to a tube containing 0.5 mM EDTA, pH 7.4, for the assessment of ex vivo levels of conjugated diene. Then, lipoprotein samples were mixed with CuSO\(_4\) to a final concentration of 5 μM and incubated at 37°C. In one series, at different time intervals of oxidation, 1.0 ml aliquots from LDL, sd-LDL and lb-LDL incubation mixture were taken out, mixed with 0.5 mM EDTA, pH 7.4, stored at 4°C and used for the assessment of conjugated diene. The oxidation for LDL and lb-LDL was carried out for 2 h, whereas for sd-LDL it was 30 min. The formation of conjugated diene in each aliquot was measured by monitoring absorbance at 234 nm in a Beckman DU 640 spectrophotometer. Conjugated diene was calculated by using an extinction coefficient of 2.52 x 10\(^4\) M\(^{-1}\) cm\(^{-1}\) and expressed as nmole malondialdehyde (MDA) equivalent per mg LDL, sd-LDL or lb-LDL protein.

The MDA content in LDL, sd-LDL and lb-LDL were assayed by the method of Niehaus and Samuelsson (1968). Briefly, 0.3 ml of LDL, sd-LDL and lb-LDL mixture in triplicate was thoroughly mixed with 2 ml of trichloroacetic acid (TCA)-
thiobarbituric acid (TBA)-hydrochloric acid (HCL) reagent containing 15 % TCA, 0.375 % TBA and 0.25 N HCL. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the samples was determined at 535 nm against a blank that contained all the reagents except the LDL, sd-LDL or lb-LDL. The MDA concentration of the samples was calculated by using an extinction coefficient (1.56 x 10^5 M^-1 cm^-1).

2.2.2.8 Ferric reducing activity (FRAP assay)

The method of Benzie and Strain (1996) was used for measuring the ferric reducing ability of plasma, the FRAP assay, which estimate the “total antioxidant power”, with minor modification. Ferric to ferrous ion reduction at low pH results in the formation of a colored ferrous-tripyridyl triazine complex. The assay was carried out in a total volume of 1.0 ml containing a suitable aliquot of plasma in 0.1 ml and 900 μl of freshly prepared FRAP reagent, prepared by mixing 10.0 ml of 22.78 mM sodium acetate buffer, pH 3.6, 1.0 ml of 20 mM ferric chloride and 1.0 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine solution prepared in 40 mM HCl. Before starting the reaction, both FRAP reagent and plasma samples were preincubated for 5 min at 30°C. Incubation was done for 5 min at 30°C and absorbance was recorded at 593 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Ferrous sulphate was used as a standard for calculating the “total antioxidant power”.

2.2.2.9 Assay of plasma arylesterase antioxidant enzyme activity

Plasma arylesterase activity was determined by the method of Ayub et al. (1999) by using phenyl acetate as the substrate. The reaction mixture included 100 mM tris buffer, pH 8.0, 1 mM CaCl2, suitable aliquots of plasma and 1 mM phenyl acetate. After 5 min preincubation at 25°C, the reaction was started by the addition of phenyl acetate and the samples were incubated for different time intervals at 25°C. The initial rates of hydrolysis (within linear range) were determined spectrophotometrically at 270 nm against a reagent blank. Nonenzymatic hydrolysis of phenyl acetate was subtracted from the total rate of hydrolysis. Arylesterase activity in the above samples was calculated by using the molar extinction coefficient of 1.31 x 10^3 M^-1 cm^-1 (La Du and Eckerson, 1984).
2.2.2.10 Measurement of malondialdehyde release from intact erythrocytes

The procedure of Cynamon et al. (1985) was employed for the determination of MDA release from erythrocytes. Two aliquots of 0.22 ml of washed packed erythrocytes in duplicate were taken in two separate tubes. One series of aliquots were suspended in 4.18 ml of phosphate buffered saline, pH 7.4, and the second series of aliquots of erythrocytes were suspended in 4.18 ml of phosphate buffered saline, pH 7.4, containing 4 mM sodium azide. Both the suspensions were vortexed for 15 seconds. One ml of erythrocytes suspension of series one was taken in triplicate and mixed with 1.0 ml of freshly prepared 3 % hydrogen peroxide, whereas, 1.0 ml of 0.75 % hydrogen peroxide was added to the second series of erythrocytes suspension. The samples were mixed for 10 seconds and incubated for 1 h at 37°C. At the end of incubation, 1.0 ml of 28 % TCA containing 100 mM sodium arsenite was added to each tube and centrifuged at 3,000 rpm for 10 min. Two ml of the supernatant from each tube was taken in triplicate and mixed with 0.5 ml of 1 % thiobarbituric acid prepared in 50 mM NaOH. The samples were then boiled for 15 min at 95°C, cooled to room temperature and the absorbance was recorded at 535 nm against a reagent blank in a Beckman DU 640 spectrophotometer. The concentration of MDA was calculated by using a standard malondialdehyde (Liu et al., 1982).

2.2.2.11 Determination of malondialdehyde in erythrocytes

The determination of MDA in erythrocytes was carried out according to the method of Stocks and Dormandy (1971). Briefly, 0.2 ml of packed erythrocytes in triplicate was made up to 1.0 ml in phosphate buffered saline, pH 7.4. To each tube, BHT in ethanol (0.75 mM) was added, followed by the addition of 0.5 ml of 30 % TCA. Tubes were vortexed and allowed to stand in ice-bath for 2 h. Tubes were then centrifuged at 2,000 rpm for 15 min and 1.0 ml of the supernatant of each tube was transferred to another tube. To each tube, 75 μl of 100 mM EDTA and 250 μl of 1 % thiobarbituric acid in 50 mM NaOH was added, mixed and kept in a boiling water bath for 15 min. After cooling the tubes to room temperature the absorbance of each sample was read against a reagent blank at 532 nm in a Beckman DU 640 spectrophotometer. Malondialdehyde was used as a standard for the calculation of MDA concentration (Liu et al., 1982).
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2.2.2.12 Assay of total and magnesium-dependent ATPase activities in erythrocyte membrane

Total and Mg\(^{++}\)-dependent ATPase enzyme activities in freshly isolated erythrocytes membrane was done as described by Quigley and Gotterer (1969), where as inorganic phosphate released in the medium by the action of ATPase enzymes was quantified by the method of Chen et al. (1956). For the assay of total ATPase activity, the incubation mixture consisted of 50 mM imidazole buffer, pH 7.4, 3.0 mM MgCl\(_2\), 120 mM NaCl, 20 mM KCl and 5 mM ATP. Whereas, for the assay of Mg\(^{++}\)-dependent ATPase activity, the reaction mixture in addition to the above ingredients also contained 1 mM ouabain. The reaction was started by the addition of 100 µg erythrocytes membrane protein suspension and the samples were incubated for 30 min at 37\(^\circ\)C. At the end of incubation, reaction was stopped by the addition of 1.0 ml of 10 % TCA. The tubes were then centrifuged at 3,000 rpm for 3 min and 0.5 ml of the supernatant from each tube was taken out for the assay of inorganic phosphate. The supernatant volume was made up to 4.0 ml with water followed by the addition of 4.0 ml of a reagent containing 1 volume of 20 mM ammonium molybdate, 1 volume of 567 mM ascorbic acid, 1 volume of 6 N H\(_2\)SO\(_4\) and 2 volumes of water. The tubes were incubated for 2 h at 37\(^\circ\)C and the absorbance was recorded at 820 nm against a suitable reagent blank in a Beckman DU 640 spectrophotometer. Total and Mg\(^{++}\)-dependent ATPase activities were calculated by using standard phosphorous calibration curve. The enzymatic activity of Na\(^+\)/K\(^+\)-dependent ATPase was then calculated as the difference between total and Mg\(^{++}\)-dependent ATPase activities.

Oxidative stress was induced in vitro by incubating 250 µl of washed erythrocytes suspension in PBS (Ph 7.4, 0.5 ml) with or without 100 µM tert-Butylhydroperoxide (Di Simplicio et al., 1998) in 0.9 % saline. In another series antioxidant effect of thymoquinone was investigated by the addition of 4.105 ng/ml of thymoquinone (12.5 µl in methanol) in the above t-BHP-stressed erythrocytes. Control samples contained 12.5 µl methanol. Samples were incubated for 1 hr at 37 \(^\circ\)C in shaking waterbath. At the end of incubation, erythrocytes were washed three times with PBS buffer Ph 7.4, and their total and magnesium-dependent ATPase enzyme activities in freshly isolated erythrocytes membrane was done as described.
by Quigley and Gotterer (1969), where as inorganic phosphate released in the medium by the action of ATPase enzymes was quantified by the method of Chen et al. (1956) as above.

2.2.2.13 Estimation of lipid peroxides in plasma and liver homogenate

For the extraction of lipid contents from plasma and tissues, the method of Folch et al. (1957) was employed. One volume of plasma or liver homogenate was mixed with 5.0 volume of chloroform: methanol (2:1), followed by centrifugation at 1,000 rpm for 5 min to separate the phases. Most of the upper layer was removed; and 3.0 ml of the lower chloroform layer was recovered. The chloroform layer was placed in a test tube and incubated at 45°C till dryness. For the determination of conjugated diene in plasma and liver, corresponding lipid residues were dissolved in 1.5 ml of cyclohexane and the absorbance was recorded at 234 nm against a cyclohexane blank in a Beckman DU 640 spectrophotometer. The concentration of conjugated diene formation was calculated by using a molar extinction coefficient of 2.52 x 10^4 M⁻¹ cm⁻¹.

In another experiment, quantification of lipid hydroperoxide was done according to the method Nourooz-Zadeh et al. (1996). The dried residues extracted from liver homogenate was dissolved in methanol and 90 μl of the samples or plasma were transferred into 1.5 ml microcentrifuge tubes together with 10 μl of methanol (in triplicate) or 10 μl of 10 mM triphenyl phosphine (TPP) in methanol (in triplicate). The samples were then vortexed and incubated for 30 min at 25°C, followed by addition of 900 μl of a reagent containing 250 mM ammonium sulphate, 100 mM xylene orange, 25 mM sulphuric acid and 4 mM BHT in 90% methanol. The samples were incubated for additional 30 min at 25°C, and centrifuged at 12,000 rpm for 10 min prior to the determination of the absorbance of the supernatants at 560 nm in a Beckman DU 640 spectrophotometer. The levels of lipid hydroperoxide in liver homogenate and plasma samples was then determined using the difference between the mean absorbance of samples with and without TPP pretreatment. Hydroperoxide contents were determined using a molar absorption coefficient of 4.3 x 10^4 M⁻¹ cm⁻¹.

Lipid peroxide contents in plasma were assayed by the method of Yagi (1987). Plasma (25 μl) was mixed with 4.0 ml of 0.083 N H₂SO₄ followed by the
addition of 0.5 ml of 10% phosphotungstic acid. The samples were mixed and incubated for 5 min at room temperature and then centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and the sediment was mixed with 2.0 ml of 0.083 N H$_2$SO$_4$ and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged at 3,000 rpm for 10 min, the sediment was suspended in 4.0 ml of water and 1.0 ml of TBA reagent (a mixture containing equal volumes of 0.67% aqueous TBA solution and glacial acetic acid) was added. The reaction mixture was heated for 60 min at 95°C, cooled and the tubes were centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant was determined at 532 nm against a reagent blank in a Beckman DU 640 spectrophotometer. The concentration of MDA was calculated by using a standard malondialdehyde (Liu et al., 1982).

The method of Ohkawa et al. (1979) was used for the determination of Lipid peroxides in liver homogenate. To 100 μl of 10% liver homogenate sample, 200 μl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of TBA were added and mixed. The mixture was made up to 4.0 ml with water and heated in a water bath for 60 min at 95°C. After cooling, the samples were centrifuged at 3,000 rpm for 5 min and the supernatant of each sample was read at 532 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Malondialdehyde was used as a standard for the calculation of MDA concentration (Liu et al., 1982).

2.2.2.14 Assay of HMG-CoA reductase activity in liver homogenate

HMG-CoA reductase enzyme activity in liver homogenate was estimated indirectly as described by Rao and Ramakrishnan (1975). Fresh 10 g liver was homogenized in 90 ml of saline arsenate (0.1% sodium arsenate in physiological saline). Then 5 ml of 10% liver homogenate was mixed with 5 ml of 5% perchloric acid, kept for 5 min at room temperature and centrifuged at 2,000 rpm for 10 min. One ml of the supernatant from each tube was taken out and mixed with 0.5 ml of freshly prepared 1 M aqueous hydroxylamine hydrochloride, whereas for the assay of HMG-CoA, 0.5 ml of alkaline hydroxylamine hydrochloride were added and mixed. After an incubation of 5 min at room temperature, 1.5 ml of a 0.616 M ferric chloride reagent containing 5.2% TCA, prepared in 0.65 N HCl was added, mixed
and absorbance was read at 540 nm against a reagent blank in a Beckman DU 640 spectrophotometer after 10 min of incubation at room temperature.

### 2.2.2.15 Activities of antioxidant enzymes

#### 2.2.2.15.1 Determination of catalase activity in erythrocytes and liver

The enzymatic activity of catalase in erythrocytes hemolysate and post-mitochondrial supernatant (PMS) of liver was measured by the procedure described by Sinha (1972). The reaction was carried out in a total volume of 1.0 ml containing 10 mM phosphate buffer, pH 7.0 and erythrocytes hemolysate equivalent to 27.7-31.67 μg hemoglobin, 1088-1295 μg liver PMS protein. After a preincubation of 10 min at 30°C, the reaction was started by the addition of hydrogen peroxide resulting in a final concentration of 100 mM and the tubes were incubated for 5 or 10 min at 30°C. At the end of incubation, the reaction was terminated by the addition of 1.0 ml of potassium dichromate-acetic acid reagent, prepared by mixing 1 volume of 5 % aqueous solution of potassium dichromate with 3 volumes of glacial acetic acid. For zero time control, hydrogen peroxide was added after stopping the reaction. The tubes were heated in a boiling water bath for 15 min, cooled and the optical density was measured at 590 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Liver and erythrocytes catalase activity was calculated by using a standard hydrogen peroxide calibration curve.

#### 2.2.2.15.2 Determination of superoxide dismutase activity in erythrocytes and liver

Enzymatic activity of superoxide dismutase (SOD) in erythrocytes hemolysate and PMS fraction of liver was determined by the method as described by Kakkar et al. (1984). The assay mixture containing 52 mM sodium pyrophosphate buffer, pH 8.3, 300 μM nitroblue tetrazolium, 186 μM phenazine methosulphate and erythrocytes hemolysate equivalent to 55.4-63.3 μg hemoglobin, 1088-1295 μg liver PMS protein was preincubated for 5 min at 30°C and the reaction was initiated by the addition of NADH (780 μM), by incubating the samples for different time intervals at 30°C. The reaction was stopped by the addition of 0.5 ml of glacial acetic acid at 1 min time interval up to 4 min. For zero time control, to the above incubation mixture, 0.5 ml of glacial acetic acid was added prior to the addition of NADH. To each tube including reagent blank 2.0 ml of n-butanol was added, rigorously extracted and centrifuged at 3,000 rpm for 5 min, color intensity of the chromogen in
the butanol extract was measured at 560 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Superoxide dismutase activity was calculated in terms of an arbitrary unit, which is defined as the enzyme concentration required to inhibit the chromogen formation by 50% in one min under the above assay conditions.

2.2.2.15.3 Determination of glutathione peroxidase activity in erythrocytes and liver

Glutathione peroxidase (Gpx) activity in erythrocytes hemolysate and liver homogenate was assayed by a modification of Mill's procedure 2 (1959) as reported by Hafeman et al. (1974). The reaction mixture contained 80 mM sodium phosphate buffer, 80 μM EDTA, pH 7.0, 1 mM sodium azide, 400 μM GSH and erythrocytes hemolysate equivalent to 27.7-31.67 μg hemoglobin, 711.5-846.0 μg liver homogenate protein in a total volume of 0.5 ml reaction mixture. After a 5 min preincubation at 37°C, the reaction was started by the addition of hydrogen peroxide (250 μM) and the tubes were incubated for 2, 4, 6 and 8 mins at 37°C. At the indicated time intervals, the reaction was terminated by the addition of 0.5 ml 10% TCA. The tubes were centrifuged at 3,000 rpm for 10 min and 0.5 ml supernatant from each tube was added to tubes containing 2.0 ml of 0.4 M sodium phosphate buffer, 0.4 mM EDTA, pH 7.0 and 0.5 ml of 1 mM 5, 5'-dithiobis (2-nitrobezoic acid, DTNB). The samples were mixed and optical density was recorded at 412 nm against a reagent blank within 2 min of DTNB addition in a Beckman DU 640 spectrophotometer. The erythrocytes and liver glutathione peroxidase activity was calculated after deducting the OD value of samples from the zero time control values by utilizing a standard glutathione.

2.2.2.15.4 Assay of glutathione reductase activity in erythrocytes and liver

The method of Carlberg and Mannervik (1975) was employed for the determination of Glutathione reductase (Gred) activity. The incubation mixture consisted of 82.5 mM sodium phosphate buffer containing 25 μM EDTA, pH 7.6, 25 μM oxidized glutathione and erythrocytes hemolysate equivalent to 27.7-31.67 μg hemoglobin, 544-647.5 μg liver PMS protein. After a 5 min preincubation at 25°C, the reaction was started by the addition of NADPH (5 μM) and the tubes were incubated for 1-4 min at 25°C. The reaction was terminated at 1 min time interval by incubating the samples in an ice-bath. Zero time control tubes were prepared as
above except 5 μM NADPH was added after stopping the reaction. The absorbance of each sample was recorded at 340 nm against a reagent blank in a Beckman DU 640 spectrophotometer. By using the zero time control value the enzyme activity was calculated on the basis of a molar extinction coefficient of NADPH (6.22 x 10^3 M^{-1} cm^{-1}).

2.2.2.15.5 Determination of glutathione-S-transferase activity in liver

Glutathione-S-transferase (GST) activity was measured in PMS fraction of liver by the method of Habig et al. (1974). The assay was carried out in a total volume of 1.0 ml containing 100 mM potassium phosphate buffer, pH 6.5, 1 mM GSH and 272.00-323.75 μg liver PMS protein. The samples were preincubated for 10 min at 30°C, the reaction was started by the addition of 1-chloro 2, 4-dinitrobenzene (CDNB) prepared in absolute ethanol (1 mM) followed by an incubation for 2 and 4 min at 30°C. At the end of incubation the tubes were placed in an ice-bath for 15 min, after which the optical density was recorded at 340 nm against a reagent blank in a Beckman DU 640 spectrophotometer. The GST activity was calculated on the basis of molar extinction coefficient of CDNB (9.6 x10^3 M^{-1} cm^{-1}) after an appropriate deduction of zero time control values.

2.2.2.16 Determination of GSH, GSSG and membrane-bound-SH contents in erythrocyte

Free sulfhydryl group of glutathione content in packed erythrocytes was determined essentially by the method of Ellman (1959) as modified by Sedlack and Lindsay (1968). Briefly, to 100 μl of washed packed erythrocytes, 1.0 ml of 10 % TCA was added, mixed and incubated for 1 h at 4°C. At the end of incubation, the tubes were centrifuged at 1,200 rpm for 20 min at 4°C and 0.5 ml of the supernatant was used for the assay of free sulfhydryl group. The reaction mixture contained 267 mM tris buffer, 13 mM EDTA, pH 8.9 and 1.7 mM DTNB (prepared in absolute methanol). The absorbance of the sample was read against a reagent blank at 412 nm within 2 minutes of DTNB addition. Free sulfhydryl content in the samples were calculated using a standard calibration curve of reduced glutathione.

The method of Sacchetta et al. (1986) was employed for the determination of glutathione disulfide. In order to assay glutathione disulfide content, 3.0 ml of
washed packed erythrocytes was hemolysed with 3.0 ml of chilled distilled water in the presence of 10 mM of NEM. After 15 min at room temperature, it was deproteinized with chilled perchloric acid to a final concentration of 1M in a total volume of 6.6 ml. Then, after 15 min the tubes were centrifuged at 23,793 rpm for 30 min at 4°C to remove the precipitate. The supernatants from each tubes were taken out and for hydrolysis of NEM pH 11.0 was adjusted with 10 mM potassium hydroxide followed by neutralization with 5 N HCl. Further, after freezing and thawing it was centrifuged at 10,000 rpm for 10 min at 4°C to remove potassium perchlorate. It was noteworthy that to prevent local extreme alkalinization pH adjustments were done with vigorous mixing in a pH meter. Thus the obtained supernatant was used for the quantification of glutathione disulfide. The incubation mixture consisted of 0.8 ml of the supernatant, 0.1 M sodium phosphate buffer containing 1 mM EDTA pH 7.0 and 180 μM NADPH. After a 3 min preincubation at 25°C, the reaction was started by the addition of 1.2 units of glutathione reductase and the tubes were incubated for 0.5-4.0 min at 0.5 min interval. The absorbance of each sample was recorded at 340 nm against a reagent blank in a Beckman DU 640 spectrophotometer and used to calculate GSSG content on the basis of a molar extinction coefficient of NADPH (6.22 x 10^3 M^-1 cm^-1).

Isolation of erythrocyte membranes was done as described by Quigley and Gotterer (1969), where membrane-SH group contents were quantified by the method of Kitajima et al. (1990). Freshly isolated erythrocyte membranes (100 μg protein) were solubilized in 0.3 ml of 20 % SDS and 3.0 ml of 100 mM sodium phosphate buffer containing 0.1 mM EDTA pH 8.0 then incubated at 37°C for 20 min. Finally, it was allowed to react with 100 μl of 10 mM DTNB dissolved in 100 mM sodium phosphate buffer pH 8.0 and incubated at 37°C for 15 min. The absorbance of each sample was recorded at 412 nm against a reagent blank in a Beckman DU 640 spectrophotometer and used to calculate membrane-SH group contents by using a standard curve of reduced glutathione.

Oxidative stress was induced in vitro by incubating 400μl (free and membrane bound-SH) and 1.5 ml (glutathione disulfide) of washed erythrocytes suspension in PBS (Ph 7.4, 4.0 and 15.0 ml) with or without 100 μM tert-Butylhydroperoxide (Di Simplicio et al., 1998) in 0.9 % saline. In another series
antioxidant impact of thymoquinone was investigated by the addition of 4.105 ng/ml of thymoquinone (20.0 and 75.0 µl in methanol) in the above t-BHP-stressed erythrocytes. Control samples contained 20.0 and 75.0 µl methanol. Samples were incubated for 1 hr at 37 ºC in shaking waterbath. At the end of incubation, erythrocytes were washed three times with PBS buffer Ph 7.4, and their glutathione content was assayed by the method of Ellman (1959) as modified by Sedlack and Lindsay (1968), where glutathione disulfide content quantified by the method of Sacchetta et al. (1986). Isolation of erythrocyte membranes was done as described by Quigley and Gotterer (1969), where membrane-SH group contents were quantified by the method of Kitajima et al. (1990) and except 100 µg erythrocytes membrane protein suspension was used. All methods described above.

2.2.2.17 Determination of total, free, membrane-bound-sulfhydryl and oxidized Contents of glutathione in liver

The total sulfhydryl content of glutathione in liver homogenate was determined by the method of Ellman (1959) as modified by Sedlack and Lindsay (1968). The incubation mixture contained 60 mM tris buffer and 6 mM EDTA, pH 8.2, 1.42-1.69 mg liver protein and 0.2 mM DTNB. The reaction mixture was mixed and made up to 5.0 ml by the addition of 3.2 ml absolute methanol. The tubes were centrifuged at 6,000 rpm for 5 min at 4°C and the absorbance of the clear supernatants were read at 412 nm against a reagent blank in a Beckman DU 640 spectrophotometer. The total glutathione content was calculated by using a standard curve of reduced glutathione.

For the determination of free sulfhydryl group of glutathione content in liver homogenate, the method of Ellman (1959) as modified by Sedlack and Lindsay (1968) was employed. In order to assay free sulfhydryl content of glutathione, 0.5 ml of 10 % homogenate was precipitated by the addition of 0.5 ml of 4 % sulphosalicylic acid (Jollow et al., 1974). The samples were incubated for 1 h at 4°C, centrifuged at 6,000 rpm for 5 min and 0.5 ml aliquots of the supernatant were used for quantification of free sulfhydryl group. The free sulfhydryl group of liver was determined according to the procedures described above for erythrocyte, except 0.33 mM DTNB was used. The membrane-bound sulfhydryl content of glutathione was calculated as the difference between total and free sulfhydryl content of glutathione.
The procedure of Sacchetta et al. (1986) was employed for the determination of glutathione disulfide in liver as described for erythrocyte (page No. 48) except that rat liver tissue (1/5, w/v) was homogenized in 0.15 M potassium phosphate buffer containing 1 mM EDTA pH 7.0 and 12.5 mM NEM.

2.2.2.18 *In vitro* Quantification of reduced glutathione: Effect of methanol extract, essential oil, pure thymoquinone and pure limonene on tert-Butylhydroperoxide-induced oxidative stress in intact erythrocytes

Oxidative stress was induced *in vitro* by incubating 100 µl of washed erythrocytes suspension in PBS (Ph 7.4, 1.0 ml) with or without 100 µM tert-Butylhydroperoxide (Di Simplicio et al., 1998) in 0.9 % saline. In another series, antioxidant effect of methanol extract, essential oil, pure thymoquinone and pure limonene was investigated by the addition of 0.4105 to 8.210 ng/ml of methanol extract, essential oil, pure thymoquinone and 102.6 to 4105 ng/ml of pure limonene (5.0 µl in methanol) in the above t-BHP-stressed erythrocytes. Control samples contained 5 µl methanol. Samples were incubated for 1 hr at 37°C in shaking waterbath. At the end of incubation, erythrocytes were washed three times with PBS buffer Ph 7.4, and their glutathione content was assayed by the method of Ellman (1959) as modified by Sedlack and Lindsay (1968), the procedures described above for erythrocytes.

2.2.2.19 Protein estimation

The protein was determined by the method of Bradford (1976), using bovine serum albumin as standard. Aliquots of liver homogenate, PMS, LDL, sd-LDL, lb-LDL HDL, HDL₂ and HDL₃ were first precipitated with 10 % TCA. The protein pellets were dissolved in 0.5 N NaOH and suitable aliquots were used for protein determination.

2.2.2.20 Statistical evaluation

Statistical analysis of data was done by employing two-tailed Student t- test as described by Bennet and Franklin (1967). P values less than 0.02 were considered significant.