Results
3.1 Average Yield of Nonsaponifiable (NSF) and Volatile Oil (VO) Extracts from Nigella sativa (NS) Seed Oil, their Total Phenolic Contents (TPC) and Fe$^{+2}$-Chelating Activities

Results in Table 1 show an average NSF yield of 4.076±0.005 % from 40 extractions of NS seed oil with methanol. The average yield of volatile or essential oil from 23 extractions of NS seed oil with hydrodistillation was 1.024±0.007 %. Because phenolic compounds are known to contribute to overall antioxidant activities, TPC was measured in NS seed oil, NSF and VO fractions. The average TPC was 320.00±3.00 mg gallic acid equivalents (GE) in 100 g of NS seed oil. While the average yield of TPC from 3 of NSF or 4 VO extracts was 300.12±0.04 and 288.41±0.01 mg GE/4.298 g NSF or per 1.170 g of VO, respectively. These results indicate that 90-94 % TPC associated with NS seed oil was detected in NSF and VO extracts, with some loss (6-10 %) during their isolation.

Chelating agents such as EDTA may reduce the availability of transition metals and inhibit the radical-mediated oxidation chain reactions in biological or food systems, and consequently improve human health, and food quality, stability and safety. The results presented in Table 1 demonstrate a significant chelating activity against Fe$^{+2}$ in NS seed oil and it’s NSF and VO extracts. Oil from NS seeds exhibited a value of 870 mg EDTA equivalent per 100 g oil, whereas in NSF or VO the chelating capacity was 222 or 39 mg EDTA equivalent per 4.298 g of NSF or per 1.170 g VO isolated from 100 g of NS seed oil. These results suggest the potential of NS seed oil or it’s VO fraction to prevent oxidative damage from free radical-mediated oxidation.

3.2 Determination of Antioxygenic Activities of NS Seed Oil and its NSF and VO Extracts by the 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH) Radical Scavenging Method

Since 90-94 % of TPC/antioxidants present in NS seed oil were recovered in NSF or VO (Table 1), their antioxygenic activities were measured in terms of hydrogen donating or radical scavenging capacities using the DPPH radicals. All test fractions directly reacted with and quenched DPPH radicals. The NS seed oil at 9.28 mg per ml of reaction mixture quenched 95 % of the DPPH$^\cdot$ (Fig. 3.1). On the other hand, scavenging capacities for 95 % DPPH radicals by NSF and VO extracts of NS
### TABLE 1
PERCENT YIELD OF NONSAPONIFIABLE (NSF) AND VOLATILE OIL (VO) EXTRACTS FROM *Nigella sativa* (NS) SEED OIL, THEIR TOTAL PHENOLIC CONTENTS (TPC) AND Fe²⁺-CHELATING ACTIVITIES

<table>
<thead>
<tr>
<th>NS oil/fractions</th>
<th>Percent yield (g/100 g oil)†</th>
<th>Total phenolic contents (mg gallic acid equivalent)#</th>
<th>Chelating capacity (mg EDTA equivalent)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS oil</td>
<td>-</td>
<td>320.00±3.00*</td>
<td>870.00±2.00*</td>
</tr>
<tr>
<td>NSF</td>
<td>4.076±0.005</td>
<td>300.12±0.04</td>
<td>222.31±5.80a</td>
</tr>
<tr>
<td>VO</td>
<td>1.024±0.007</td>
<td>288.41±0.01</td>
<td>38.59±1.43a</td>
</tr>
</tbody>
</table>

†Values are mean ± SD from 40 NSF or 23 VO extracts.  
#TPC or chelating capacity of NS oil and its fractions are expressed as mg gallic acid or mg EDTA equivalent per 100 g of NS oil; or per 4.298 g of NSF (average yield per 100 g of NS oil from 3 extractions); or per 1.170 g VO (average percent yield from 4 preparations).  
*Values are mean ± SD from 3 to 8 different experiments done in triplicate.  
Significantly different from NS oil at *p< 0.001.
**Results**

**Fig. 3.1.** Comparison of DPPH$^*$ scavenging activities of *Nigella sativa* (NS) seed oil, nonsaponifiable (NSF) and volatile oil (VO) extracts of NS seed oil. The radical DPPH$^*$ scavenging capacities of the above fractions at the indicated concentrations were determined by measuring DPPH$^*$ remaining as compared to control samples lacking above fractions. All tests were conducted in triplicate and means were used.
Results

seed oil were achieved at 0.142 mg/ml and 0.342 mg/ml, respectively. Consistent with these results, IC₅₀ values for NS seed oil, NSF and VO extracts were 5.12 mg/ml, 42.14 and 88.39 µg/ml, respectively. In comparison to VO fraction, a 2-fold stronger DPPH• scavenging capacity of NSF extract suggested that extraction of NS seed oil with methanol not only removed all the nonsaponifiable antioxidant agents, including TQ, from VO fraction, but also phytosterols associated with fixed oil, in the resultant NSF extract (Fig. 3.1).

3.3 Comparison of Scavenging Activities of NS Seed Oil and it’s NSF and VO Extracts; Pure Thymoquinone (TQ) and Limonene (LMN) against 2,2-Diphenyl-1- Picryl Hydrazyl (DPPH•), Hydroxyl (•OH), 2,2-Azinobis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS•+) and Nitric Oxide (NO•) Free Radicals

The results depicted in Fig. 3.1, demonstrated that the DPPH• scavenging capacity of NS seed oil is associated with the VO extract, which is also fractionated in NSF fraction of NS seed oil extracted with methanol. Data in Table 2 represent the comparison of scavenging activities of NS seed oil, NSF and VO with pure TQ (99 %, Sigma-Aldrich) and LMN (98 %, Sigma-Aldrich), two constituents of VO against DPPH•, •OH, ABTS•+ and NO•. The NSF extract had the strongest DPPH• scavenging capacity, with an IC₅₀ of 42 µg/ml, followed by the pure TQ (57 µg/ml) and VO (88 µg/ml), respectively, while NS seed oil and LMN had an IC₅₀ value of 5 mg/ml and 8 mg/ml. Similar to DPPH• scavenging capacities among the five test fractions/compounds, the order of quenching efficiencies of OH radicals was NSF extract > pure TQ > VO extract > NS oil > pure LMN. The IC₅₀ values for these antioxidants were 140, 186, 290 µg/ml, 15 and 49 mg/ml, respectively. Radical ABTS•+ scavenging activity is widely used measurement of radical scavenging capacity. In the present study, the NS seed oil and it’s two extracts, pure TQ and LMN were evaluated for their scavenging activity against the ABTS•+ generated through potassium persulfate mediated oxidation reaction (Table 2). The NS seed oil, NSF and VO extracts as well as pure TQ and LMN directly reacted with and quenched ABTS•+, with an IC₅₀ of 200, 8, 17, 11 µg/ml and 5 mg/ml, respectively. Consistent with these results, incubation of above five test fractions/compounds...
### TABLE 2

**COMPARISON OF SCAVENGING ACTIVITIES OF Nigella sativa (NS) OIL AND IT’S NONSAPONIFIABLE (NSF) AND VOLATILE OIL (VO) FRACTIONS, PURE THYMOQUINONE (TQ) AND PURE LIMONENE (LMN) AGAINST DPPH*, OH, NO* AND ABTS** FREE RADICALS**

<table>
<thead>
<tr>
<th>Type of free radical</th>
<th>Test fractions or compounds</th>
<th>NS oil</th>
<th>NSF</th>
<th>VO</th>
<th>TQ</th>
<th>LMN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC₅₀ # (µg or mg***/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH*)</td>
<td></td>
<td>5.12±0.03* (1.42-10.0)</td>
<td>42.14±0.0 (7.14-142.85)</td>
<td>88.39±0.04 (14.28-342.85)</td>
<td>57.15±0.05 (8.57-171.42)</td>
<td>8.40±0.01* (1.42-22.85)</td>
</tr>
<tr>
<td>B. Hydroxyl (OH)</td>
<td></td>
<td>14.91±0.01 (5.0-35.0)</td>
<td>140.0±0.53 (25.0-500.0)</td>
<td>290.0±0.27 (50.0-1000.0)</td>
<td>186.0±0.5 (25.0-500.0)</td>
<td>48.75±0.05 (10.0-100.0)</td>
</tr>
<tr>
<td>C. 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS**+)</td>
<td></td>
<td>200.0±0.83 (45.45-545.45)</td>
<td>7.81±0.11 (1.81-27.27)</td>
<td>17.36±0.17 (4.54-54.54)</td>
<td>10.68±0.07 (2.72-22.72)</td>
<td>4.86±0.15 (0.90-10.90)</td>
</tr>
<tr>
<td>D. Nitric oxide (NO*)</td>
<td></td>
<td>7.60±0.01 (1.0-20.0)</td>
<td>68.75±0.24 (5.0-250.0)</td>
<td>140.0±0.22 (25.0-500.0)</td>
<td>89.75±0.25 (5.0-250.0)</td>
<td>23.65±0.025 (5.0-50.0)</td>
</tr>
</tbody>
</table>

*IC₅₀ is defined as the concentration (µg or mg***/ml; with range in parenthesis) of test fractions or compounds sufficient to quench or inhibit 50% of the above free radicals (A-D) under standard experimental conditions.

*Values are mean ± SD, of triplicate determinations, were calculated from a concentration dependent plot of each test fraction or compound versus the percent of antioxidant activity.
caused a concentration dependent inhibition of NO\textsuperscript{•} generation during \textit{in vitro} incubation. The 50% inhibition of NO\textsuperscript{•} generation for NS seed oil and LMN was 8 and 24 mg/ml, while for NSF and VO fractions of NS seed oil as well as pure TQ the values were 69, 140 and 90 µg/ml. These data suggest that NS seed oil may serve as excellent dietary source of natural antioxidants in the protection of biologically important components, such as membrane lipids, DNA and proteins, from variety of free radical attacks and radical-mediated oxidative damage.

### 3.4 Inhibition of Nonenzymatic Lipid Peroxidation in Phospholipid Liposomes by NSF and VO Fractions of NS Seed Oil and Pure TQ

Ox brain phospholipid liposomes undergo rapid nonenzymatic peroxidation when incubated with FeCl\textsubscript{3} and ascorbic acid. The use of Fe\textsuperscript{3+} in the presence of reducing agent such as ascorbic acid produces \textsuperscript{•}OH. As shown in Fig. 3.2, the TQ rich NSF and VO fractions of NS seed oil as well as pure TQ exhibited a very strong antioxidant effect. The IC\textsubscript{50} for pure TQ was 1.72 µg/ml, whereas for NSF and VO fractions containing TQ as a major antioxidant, the IC\textsubscript{50} values were 1.62 and 3.15 µg/ml, respectively. These results indicate that NSF and pure TQ are equally potent in the inhibition of lipid peroxidation as shown by reduced formation of malondialdehyde.

### 3.5 Average Body Weight, Food Intake and Liver Weight

The average body weight of rats in each group at the beginning of the experiment period averaged 221 g (Table 3). After 30 days of treatment the average body weight of rats in NLP-C group was significantly increased from 223 to 279 g (+25 %), while in HLP-C the increase was 52 % (from 220 to 335 g). The final body weights in HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN treated rats were increased by 9 %, 14 %, 13 % and 13 %, respectively, when compared to their respective initial body weights. There was no significant differences in the average diet consumption /rat/day during the course of 30-day treatment among six groups. However, in comparison to final body weight of NLP-C rats, a significant increase of 20 % was seen in HLP-C group, while a significant decrease of 14 %, 10 %, 12 % and 10 % in the body weights of HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN groups had occurred after 30 days of treatment. A maximum average food intake of 22.8 g /rat/day in NLP-C group was seen, while a minimum of 19.7 g was consumed
Fig. 3.2. Inhibition of nonenzymatic lipid peroxidation in phospholipid liposomes by NSF and VO fractions and pure TQ. Lipid peroxidation in phospholipid liposomes, prepared from type VII Folch bovine brain extract (Sigma), was induced by Fe$^{3+}$-ascorbate. Lipid peroxidation was monitored by measuring the formation of malondialdehyde. MDA value in control samples incubated without NSF, VO or TQ is represented as 100 %, whereas inhibition of lipid peroxidation at indicated concentrations of the above antioxidants are represented as a percentage of the control value. MDA values represent mean of triplicate determinations and at each concentration of NSF, VO and TQ are significantly (p<0.001) different from control value.
### TABLE 3

AVERAGE BODY, LIVER WEIGHT AND DIET CONSUMPTION IN EACH GROUP OF RATS DURING 30 DAYS OF TREATMENT

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight/rat (g) Before treatment</th>
<th>Body weight/rat (g) After treatment</th>
<th>Liver weight/rat (g)</th>
<th>Diet consumption /rat/day (g) During treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLP-C</td>
<td>223.30±20.8*</td>
<td>278.60±16.72*</td>
<td>5.44±0.22*</td>
<td>22.76±0.82*</td>
</tr>
<tr>
<td>HLP-C</td>
<td>220.00 ±14.71</td>
<td>334.50±14.50 (+20.06%)a</td>
<td>6.51±0.01 (+19.65%)d</td>
<td>21.40±0.70</td>
</tr>
<tr>
<td>HLP-NSF</td>
<td>221.25±13.14</td>
<td>240.25±9.74 (-13.76%)a</td>
<td>5.48±0.03</td>
<td>21.31±0.50</td>
</tr>
<tr>
<td>HLP-VO</td>
<td>220.00±10.80</td>
<td>250.75±4.85 (-10.00%)b</td>
<td>5.43±0.07</td>
<td>21.50±0.40</td>
</tr>
<tr>
<td>HLP-TQ</td>
<td>218.75±15.47</td>
<td>246.00±16.80 (-11.70%)c</td>
<td>6.37±1.39 (+17.10%)c</td>
<td>19.71±0.64</td>
</tr>
<tr>
<td>HLP-LMN</td>
<td>222.50±14.43</td>
<td>251.00±10.81 (-9.90%)c</td>
<td>6.21±1.21 (+14.15%)c</td>
<td>21.15±0.77</td>
</tr>
</tbody>
</table>

*Values are mean ± SD from 4 rats in each group except NLP (n=5).

NLP-C, normolipidemic control fed 1 ml saline/rat/day; HLP-C, hyperlipidemic control given 1 ml of saline 30 min before the feeding of 1 ml suspension containing 5 mg cholesterol, 30 mg coconut oil and 2.5 mg cholic acid /rat/day; whereas rats in HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN were fed 1 ml of 100 mg NSF, 20 mg VO, 10 mg TQ or 400 mg LMN 30 min prior to administration of 1 ml of the above atherogenic suspension /rat/day for 30 days.

Significantly different from NLP-C at a p< 0.01, b p< 0.02, c p<0.05, d p< 0.001 and e not significant.
Results by HLP-TQ rats, while rest of the four treated groups consumed an average of 21.3 g /rat/day. After 30 days of treatment the average liver weight /rat in HLP-C was increased by 20 % in comparison with NLP-C, while no increase was seen in HLP-NSF and HLP-VO rats. The increase in the average liver weight in HLP-TQ and HLP-LMN was 17 % and 14 %, respectively.

3.6 Impact of NSF and VO Fractions from NS Seed Oil; Pure TQ and LMN on Antioxidant and Lipid Peroxidation Status in Plasma, Erythrocyte, Liver and Erythrocyte Membrane Bound ATPase Activities in Hyperlipidemic Rats Treated for 30 Days

3.6.1 Impact on plasma ABTS$^{•+}$ scavenging activity, FRAP arylesterase antioxidant enzyme activity and lipid peroxidation products

Data in Fig. 3.3, show the antioxidant impact of NS seed oil fractions, NSF and VO; pure TQ and LMN on ex vivo plasma ABTS$^{•+}$ scavenging activity, antioxidants, measured as ferric reducing ability (FRAP), HDL-associated arylesterase antioxidant enzyme activity, conjugated diene (CD), hydroperoxide (LOOH) and malondialdehyde (MDA) in atherogenic suspension fed rats. After 30 days of treatment, plasma antioxidant capacity of experimental rats against radical cation ABTS$^{•+}$ was measured. The plasma from hyperlipidemic control (HLP-C) rats exhibited a significantly (p< 0.001) reduced ABTS$^{•+}$ scavenging capacity with an increase of 36 % in IC$_{50}$ value, when compared to an IC$_{50}$ value of 1.81 µl/ml from plasma of NLP-C rats. In HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN treated groups, the IC$_{50}$ values were significantly reduced to 1.90, 2.09, 2.00, and 2.18 µl/ml, respectively, from an increased IC$_{50}$ value of 2.46 µl/ml in HLP-C. This implies that oral administration of NS seed oil extracts, NSF, VO, pure TQ or LMN to rats fed an atherogenic suspension, is capable of significantly improving the plasma antioxidant capacity, as evidenced by their increased ABTS$^{•+}$ scavenging activities. Due to increase in oxidative stress in HLP-C rats, plasma ferric reducing ability and arylesterase antioxidant enzyme activity was reduced by 32 % and 21 %, while CD, LOOH and MDA levels were increased by 43 %, 60 % and 110 %, respectively. In comparison to corresponding normal values in NLP-C, simultaneous administration of NSF, VO, pure TQ or LMN to HLP rats, significantly increased the FRAP level by 96 %, 80 %, 86 % and 77 %, while arylesterase activity by,
Fig. 3.3. Protective effect of NSF and VO extracts, pure TQ and LMN on plasma *ex vivo* ABTS*-•* scavenging activity (ABTS*-•*-SA), ferric reducing ability (FRAP), arylesterase enzyme activity (AeA), conjugated diene (CD), lipid hydroperoxide (LOOH) and malondialdehyde (MDA) contents in hyperlipidemic rats after 30 days of treatment.

IC$_{50}$ is defined as the concentration of plasma antioxidants (µl/ml incubation; range, 0.45 to 4.54 µl of plasma) sufficient to quench or inhibit 50 % of the radical cation ABTS*-•* under standard experimental conditions.

Values are mean ± SD, of triplicate determinations, were obtained by plotting the percent of remaining radical ABTS*-•* at steady state of reaction against the corresponding levels of pooled plasma samples in each group.

Values are mean (µmole/min/dl for FRAP and AeA or µmole/dl for LOOH and MDA; conjugated diene values are expressed as n mole MDA equivalents) ± SD from pooled plasma samples in each group.

Significantly different from NLP-C at p< 0.001.

Significantly different from HLP-C at p< 0.001.
Results

IC₅₀ (µM plasma/ml) or µmole/min/dl or nmole MDA equivalents or µmole/dl

- ABTS⁺-SA
- FRAP
- AeA
- CD
- LOOH
- MDA

Legend:
- NLP-C
- HLP-C
- HLP-NSF
- HLP-VO
- HLP-TQ
- HLP-LMN
107 %, 102 %, 104 % and 89 % respectively. On the other hand, CD, LOOH and MDA levels were significantly decreased by 88 %, 89 % and 95 % in HLP-NSF, 84 %, 79 % and 86 % in HLP-VO, 86 %, 83 % and 90 % in HLP-TQ, 81 %, 73 % and 56 % in HLP-LMN fed rats, when compared to corresponding values in NLP-C. These results demonstrate that in HLP rats, due to increase in oxidative stress, plasma ABTS$^{•+}$ scavenging capacity, FRAP and arylesterase activity levels were decreased, while the above lipid peroxidation products in plasma were significantly increased. In four treated groups, decreases in plasma ABTS$^{•+}$ scavenging capacity, FRAP and arylesterase activity, while increases in plasma CD, LOOH and MDA levels were restored to a level close to corresponding normal values in NLP-C group. These results indicate a potent in vivo antioxidant effect of above test fractions/compounds in the order of NSF extract > pure TQ > VO extract > pure LMN.

3.6.2 Effect on membrane lipid peroxidation in erythrocytes

Since hypercholesterolemia is characterized by excess generation of oxygen free radicals, which exhibit erythrocyte membrane lipid peroxidative damage in vivo, we have examined the cause of this membrane damage by quantifying the end product of fatty acid peroxidation, i.e. MDA. As shown in Table 4, ex vivo MDA content of erythrocyte hemolysate was increased (94 %) from 6.56 in NLP-C to 12.75 nmol/gHb in HLP-C. In rats treated with NSF, VO or pure TQ, 94 % increase in MDA was significantly blocked and reduced to 96 %, 84 % and 92 %, respectively, of NLP-C value, while in HLP-LMN this reduction in MDA was 62 %. Consistent with above results, intact erythrocytes from HLP-C stressed rats showed a greater susceptibility to H$_2$O$_2$-induced lipid peroxidation than those from NLP-C group. An increase of 88 % (p< 0.001) in release of MDA in HLP-C was seen in comparison to NLP-C value. This increase in the formation of MDA was significantly reduced to a level which was 89 %, 80 %, 82 % and 58 % of control value in NLP-C, after 30-day treatment of atherogenic suspension fed rats with NSF, VO, pure TQ or LMN, respectively. Based on these results one can conclude that hyperlipidemia induced in rats by feeding an atherogenic suspension is associated with a significant increase in in vivo and H$_2$O$_2$-induced in vitro erythrocyte membrane lipid peroxidation, which was significantly prevented by the simultaneous administration of NSF, VO, pure TQ or LMN to these rats.
## TABLE 4

BASAL MALONDIALDEHYDE (MDA) CONTENT AND IT’S 
H$_2$O$_2$-INDUCED RELEASE IN INTACT ERYTHROCYTES FROM 
HYPERLIPIDEMIC RATS TREATED WITH NSF AND VO FRACTIONS 
OF NS OIL, PURE TQ AND LMN FOR 30 DAYS

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA Content (nmole/g Hb)</th>
<th>MDA release (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLP-C</td>
<td>6.56±0.04$^a$</td>
<td>22.54±0.05$^a$</td>
</tr>
<tr>
<td>HLP-C</td>
<td>12.75±0.08</td>
<td>42.39±0.10</td>
</tr>
<tr>
<td></td>
<td>(+94.35%)$^a$</td>
<td>(+88.06%)$^a$</td>
</tr>
<tr>
<td>HLP- NSF</td>
<td>6.83±0.04</td>
<td>25.36±0.07</td>
</tr>
<tr>
<td></td>
<td>(-46.43%)$^a$</td>
<td>(-40.17%)$^a$</td>
</tr>
<tr>
<td>HLP-VO</td>
<td>7.84±0.06</td>
<td>28.36±0.07</td>
</tr>
<tr>
<td></td>
<td>(-38.50%)$^a$</td>
<td>(-33.09%)$^a$</td>
</tr>
<tr>
<td>HLP-TQ</td>
<td>7.14±0.05</td>
<td>27.34±0.08</td>
</tr>
<tr>
<td></td>
<td>(-44.00%)$^a$</td>
<td>(-35.50%)$^a$</td>
</tr>
<tr>
<td>HLP-LMN</td>
<td>10.55±0.06</td>
<td>39.06±0.09</td>
</tr>
<tr>
<td></td>
<td>(-17.25%)$^a$</td>
<td>(-7.89%)$^a$</td>
</tr>
</tbody>
</table>

$^a$Values are mean ± SD either from hemolysates or intact erythrocytes of pooled samples in each group. 
Significantly different from NLP-C at $^a$p< 0.001. 
Significantly different from HLP-C at $^a$p< 0.001.
3.6.3 Impact on liver lipid peroxidation products

As depicted in Table 5, CD, LOOH and MDA formation in liver were increased from 5.26, 0.88 and 2.40 in NLP-C to 7.28 (38 %), 1.27 (44 %) and 3.31 (38 %) nmol/mg protein, respectively, in HLP-C rats. Treatment of HLP rats with four test fractions/compounds blocked the increases in CD, LOOH and MDA formation and restored them to a value which was 93 %-98 % in HLP-NSF, 85 %-91 % in HLP-VO, 90 %-95 % in HLP-TQ and 81 %-86 % in HLP-LMN of corresponding normal values in NLP-C.

3.6.4 Effect on erythrocyte membrane bound total, Mg++- and Na+, K+-dependent ATPase activities

Data presented in Table 6 revealed that due to increase in oxidative stress in HLP-C rats, a significant decline of 33 % and 66 % in erythrocyte total and Na+, K+-ATPase activities had occurred, while an increase of 50 % in Mg++-dependent ATPase was seen, when compared to respective values in NLP-C. Following simultaneous treatment of atherogenic suspension fed rats with NSF, VO, pure TQ or LMN, these ATPase activities were restored close to corresponding values in NLP-C, indicating a significant improvement in the damaged membranes of erythrocytes from HLP-stressed rats.

3.7 Modulatory Effects of NSF, VO, pure TQ and LMN on Antioxidant Defense System in Hyperlipidemic Stressed Rats

Cholesterol rich diets are associated with free radical production, followed by oxidative stress and hypercholesterolemia. Oxidative stress is, on the other hand, one of the factors that links hypercholesterolemia with atherogenesis. In addition, oxidative stress is known to contribute to the development of atherosclerosis in the vascular wall through the formation of reactive oxygen species (ROS). Protection against ROS and the breakdown products of oxidized lipids and proteins is provided by enzymatic and nonenzymatic antioxidants. Therefore, the status of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx), glutathione reductase (Gred) and glutathione-S-transferase (GST) including reduced and oxidized glutathione concentrations in erythrocytes were examined.
## TABLE 5

Effect of NSF and VO extracts of NS oil, pure TQ and LMN on liver conjugated diene (CD), lipid hydroperoxide (LOOH) and malondialdehyde (MDA) contents in hyperlipidemic rats after 30 days of treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>CD</th>
<th>LOOH</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLP-C</td>
<td>5.26±0.014*</td>
<td>0.880±0.016*</td>
<td>2.40±0.020*</td>
</tr>
<tr>
<td>HLP-C</td>
<td>7.28±0.034 (+38.40%)a</td>
<td>1.270±0.017 (+44.31%)a</td>
<td>3.31±0.020 (+37.91%)a</td>
</tr>
<tr>
<td>HLP-NSF</td>
<td>5.45±0.020 (-25.13%)a</td>
<td>0.899±0.012 (-29.21%)a</td>
<td>2.59±0.015 (-21.75%)a</td>
</tr>
<tr>
<td>HLP-VO</td>
<td>5.87±0.018 (-19.36%)a</td>
<td>1.039±0.016 (-18.18%)a</td>
<td>2.64±0.010 (-20.24%)a</td>
</tr>
<tr>
<td>HLP-TQ</td>
<td>5.54±0.017 (-23.90%)a</td>
<td>0.980±0.017 (-22.83%)a</td>
<td>2.61±0.020 (-21.14%)a</td>
</tr>
<tr>
<td>HLP-LMN</td>
<td>6.14±0.019 (-15.65%)a</td>
<td>1.081±0.019 (-14.88%)a</td>
<td>2.79±0.015 (-15.70%)a</td>
</tr>
</tbody>
</table>

*Values are mean (nmole/mg protein; CD values are expressed as nmole MDA equivalents) ± SD from homogenates of pooled liver samples in each group.
Significantly different from NLP-C at a p< 0.001.
Significantly different from HLP-C at a p< 0.001.
### TABLE 6

**REGULATORY EFFECT OF NSF AND VO FRACTIONS, PURE TQ AND LMN ON ERYTHROCYTE MEMBRANE BOUND TOTAL, Mg\(^{++}\)-DEPENDENT AND Na\(^+\), K\(^+\)-DEPENDENT ATPase ACTIVITIES IN HYPERLIPIDEMIC RATS AFTER 30 DAYS OF TREATMENT**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total ATPase (µmole Pi/hr/mg protein)</th>
<th>Mg(^{++})-ATPase (µmole Pi/hr/mg protein)</th>
<th>Na(^+), K(^+)-ATPase (µmole Pi/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLP-C</td>
<td>0.8542±0.002*</td>
<td>0.2435±0.001*</td>
<td>0.6107±0.002*</td>
</tr>
<tr>
<td>HLP-C</td>
<td>0.5701±0.001</td>
<td>0.3652±0.002*</td>
<td>0.2049±0.001</td>
</tr>
<tr>
<td>HLP-NSF</td>
<td>0.7549±0.002</td>
<td>0.2683±0.003*</td>
<td>0.4866±0.001</td>
</tr>
<tr>
<td>HLP-VO</td>
<td>0.7103±0.004</td>
<td>0.2792±0.002*</td>
<td>0.4311±0.002</td>
</tr>
<tr>
<td>HLP-TQ</td>
<td>0.7326±0.010</td>
<td>0.2690±0.003*</td>
<td>0.4636±0.007</td>
</tr>
<tr>
<td>HLP-LMN</td>
<td>0.6672±0.002</td>
<td>0.2986±0.002*</td>
<td>0.3686±0.0008</td>
</tr>
</tbody>
</table>

*aValues are mean (µmole Pi/hr/mg protein) ± SD in membranes isolated from pooled packed erythrocytes in each group.

Significantly different from NLP-C at *p < 0.001.

Significantly different from HLP-C at *p < 0.001.
3.7.1 Effect on erythrocyte catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx) and glutathione reductase (Gred) activities

Fig. 3.4 shows that the erythrocyte CAT, SOD and Gred enzyme activities were significantly decreased by 29 %, 20 % and 68 %, while Gpx activity was increased (25 %) in the HLP-C group compared to the NLP-C rats after 30-day feeding of cholesterol-coconut oil-rich suspension. However, NSF, VO, pure TQ or LMN treatments effectively blocked the decreases in CAT, SOD and Gred activities and elevated them to 90 %, 84 %, 88 % and 77 %; 127 %, 115 %, 122 % and 90 % or 94 %, 91 %, 92 % and 72 % of respective NLP-C values of CAT, SOD and Gred, respectively. On the other hand, increased (25 %) Gpx activity in HLP-C rats was reduced to near normal activity levels in NSF, VO, pure TQ or LMN treated rats.

Reduced glutathione, a major nonprotein thiol plays a crucial role in coordinating the body’s antioxidant defense processes. Perturbation of GSH status of a biological system can lead to serious consequences. Consistent with an increase (25 %) in Gpx and a decrease (68 %) in Gred activities in erythrocytes of HLP stressed rats (Fig. 3.4), a significant decrease of 39 % in free SH and 23 % in membrane bound SH with a concomitant increase of 42 % in oxidized glutathione (GSSG), which resulted in a substantial increase of 134 % in GSSG:GSH ratio (Fig. 3.5). As expected, supplementation with natural antioxidant rich factions of NS seed oil or pure antioxidants attenuated the above alterations in free and membrane-SH, GSSG and GSSG:GSH ratio close to corresponding values in NLP-C, indicating a strong antioxidant property of phytosterols and TQ rich NSF, TQ rich VO, pure TQ or LMN in the order NSF extract > pure TQ > VO extract > pure LMN.

3.7.2 Impact on the in vivo modulation of liver catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx), glutathione reductase (Gred) and glutathione-S-transferase (GST) activities

Fig. 3.6 depicts the results of CAT, SOD, Gpx, Gred and GST activities in liver of NLP-C, HLP-C, HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN rats. Due to increase in oxidative stress in HLP rats, the enzymatic activities of CAT, SOD, Gpx, Gred and GST in liver were significantly decreased from the NLP-C values of 4.30, 0.732, 52.78, 8.81 and 141.41 U/mg protein to 2.82 (35 %), 0.515 (30 %), 26.36 % (50 %), 5.17 % (41 %) and 95.66 (32 %) U/mg protein, respectively. Simultaneous
Fig. 3.4. Regulatory effect of NSF and VO fractions, pure TQ and LMN on erythrocyte catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx) and glutathione reductase (Gred) activities in hyperlipidemic rats after 30 days of treatment.

One unit of CAT activity (U/g Hb) is defined as the µmoles of H₂O₂ decomposed /min/g Hb.

One kilounit of SOD activity (KU/10 g Hb) is defined as the amount of enzyme required to inhibit O.D. at 560 nm of chromogen production by 50 % in one minute.

One unit of Gpx activity (U/10 g Hb) is defined as the µmoles of oxidized glutathione formed /min/g Hb.

One unit of Gred activity (U/g Hb) is defined as the µmoles of NADPH oxidized /min/g Hb.

Values are mean ± SD from hemolysates of pooled erythrocytes in each group. Significantly different from NLP-C at p< 0.001. Significantly different from HLP-C at p< 0.001.
Results

The graph shows the activity of various enzymes (CAT, SOD, Gpx, and Gred) expressed as U/g Hb or KU/10 g Hb or U/10 g Hb. The enzymes are grouped into different categories:

- **NLP-C**
- **HLP-C**
- **HLP-NSF**
- **HLP-VO**
- **HLP-TQ**
- **HLP-LMN**

The data indicates that the enzyme activities vary significantly across different categories and conditions, with CAT showing the highest activity, followed by SOD, Gpx, and Gred.
Fig. 3.5. Protective effects of NSF and VO extracts of NS oil, pure TQ and LMN on erythrocyte free, membrane bound sulfhydryl groups of GSH (Mem-SH) and oxidized glutathione (GSSG) in hyperlipidemic rats after 30 days of treatment.
Values of GSH and GSSG (µmole/g Hb) are mean ± SD from pooled erythrocyte hemolysates in each group.
Values of Mem-SH (µmole/mg protein) are mean ± SD from pooled erythrocyte membranes in each group.
Significantly different from NLP-C at p< 0.001.
Significantly different from HLP-C at p< 0.001.
Results

GSH Mem-SH GSSG GSSG:GSH

µmole/g Hb or µmole/mg protein or ratio

NLP-C
HLP-C
HLP-NSF
HLP-VO
HLP-TQ
HLP-LMN

GSH Mem-SH GSSG GSSG:GSH

µmole/g Hb or µmole/mg protein or ratio

NLP-C
HLP-C
HLP-NSF
HLP-VO
HLP-TQ
HLP-LMN
Fig. 3.6. Impact of NSF and VO fractions, pure TQ and LMN on liver catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx), glutathione reductase (Gred) and glutathione-S-transferase (GST) activities in hyperlipidemic rats after 30 days of treatment.

One unit (U/mg protein) of CAT activity is defined as the μmoles of H₂O₂ decomposed /min/mg protein.

One unit (U/10 mg protein) of SOD activity is defined as the amount of enzyme required to inhibit O.D. at 560 nm of chromogen production by 50% in one minute.

One unit (U/mg protein) of Gpx activity is defined as nmole oxidized glutathione formed /min/mg homogenate protein.

One unit (U/mg protein) of Gred activity is defined as nmole NADPH oxidized /min/mg PMS protein.

One unit (U/mg protein) of GST activity is defined as the nmole of 1-chloro 2,4-dinitrobenzene (CDNB) conjugate formed /min/mg PMS protein.

Values are mean ± SD from pooled liver samples in each group.

Significantly different from NLP-C at p< 0.001.

Significantly different from HLP-C at p< 0.001.
Results

U/mg protein or U/10 mg protein
feeding of TQ rich NSF or VO extracts of NS seed oil to rats along with atherogenic suspension significantly blocked the decreases in the activities of above five antioxidant enzymes and in NSF treated rats restored them to 94 %, 87 %, 90 %, 88 % and 93 % of respective control values in NLP-C, while in HLP-VO, this restoration was 86 %, 86 %, 77 %, 81 % and 89 % of corresponding CAT, SOD, Gpx, Gred and GST activities in NLP-C. Similarly, the restoration in the above enzymatic activities in pure TQ treated HLP rats was 91 %, 89 %, 86 %, 83 % and 91 % of respective normal control values. In HLP-LMN group, in comparison to corresponding values of these enzymes in NLP-C, the percent increase was 77, 83, 68, 73 and 85, respectively.

3.8 Effect on Hepatic Total, Free and Protein-Bound Sulfhydryl Contents, Oxidized Glutathione (GSSG) and GSSG:GSH ratio

As evident from Fig. 3.7, hepatic total, free and protein-bound-SH contents of glutathione in HLP-C were reduced by 50 %, 24 % and 56 %, respectively, when compared to respective NLP-C values. Treatment of HLP rats with NSF, VO, pure TQ or pure LMN prevented these decreases and exhibited significant increases in hepatic total (95 %), free (101 %) and protein-bound-SH (94 %) levels of glutathione in comparison to corresponding values in NLP-C group. In HLP-VO, these restorations were 90 %, 93 % and 89 % of control values, while in HLP-TQ the increases were 92 %, 94 % and 91 % of respective NLP-C values. LMN was least effective antioxidant and was able to increase total, free and protein-SH by 62 %, 82 % and 58 %, respectively, when compared to corresponding NLP-C values. In contrast, hepatic GSSG and GSSG:GSH ratio values were increased by 30 % and 71 % in untreated HLP rats, which were reduced to 106 % and 107 % in HLP-NSF, 96 % and 89 % in HLP-VO, 101 % and 94 % in HLP-TQ and 91 % and 75 % in HLP-LMN, when compared to respective GSSG and GSSG:GSH ratio values in NLP-C rats. These results indicate that similar to erythrocytes, a significant reduction in the hepatic enzymatic and nonenzymatic (total, free and protein-bound-SH contents of glutathione) antioxidant defense had occurred during experimental hypercholesterolemia, which may be due to enhanced production of free radicals. Supplementation of HLP rats with NSF or VO fractions of NS seed oil, pure TQ or LMN resulted in the upregulation of liver enzymatic activities of CAT, SOD, Gpx,
Fig. 3.7. Protective effects of NSF and VO extracts of NS oil, pure TQ and LMN on hepatic total, free and protein bound sulfhydryl groups of GSH (Pro-SH) and oxidized glutathione (GSSG) in hyperlipidemic rats after 30 days of treatment. Values are mean (µmole SH group/mg protein or nmole GSSG/mg protein) ± SD from homogenates of pooled liver samples in each group. Significantly different from NLP-C at p< 0.001. Significantly different from HLP-C at p< 0.001.
**Results**

<table>
<thead>
<tr>
<th>NLP-C</th>
<th>HLP-C</th>
<th>HLP-NSF</th>
<th>HLP-VO</th>
<th>HLP-TQ</th>
<th>HLP-LMN</th>
</tr>
</thead>
</table>

**NLP-C**

- Total-SH: 0.06
- Free-SH: 0.04
- Pro-SH: 0.02
- GSSG: 0.18
- GSSG:GSH: 0.14

**HLP-C**

- Total-SH: 0.12
- Free-SH: 0.10
- Pro-SH: 0.08
- GSSG: 0.16
- GSSG:GSH: 0.14

**HLP-NSF**

- Total-SH: 0.06
- Free-SH: 0.04
- Pro-SH: 0.02
- GSSG: 0.18
- GSSG:GSH: 0.14

**HLP-VO**

- Total-SH: 0.12
- Free-SH: 0.10
- Pro-SH: 0.08
- GSSG: 0.16
- GSSG:GSH: 0.14

**HLP-TQ**

- Total-SH: 0.06
- Free-SH: 0.04
- Pro-SH: 0.02
- GSSG: 0.18
- GSSG:GSH: 0.14

**HLP-LMN**

- Total-SH: 0.12
- Free-SH: 0.10
- Pro-SH: 0.08
- GSSG: 0.16
- GSSG:GSH: 0.14
Results

Gred and GST, which were responsible for the increase in total GSH with a reduction in GSSG and GSSG:GSH ratio to near normal levels, indicating a strong antioxidant effect of test fractions/compounds in the order NSF extract > pure TQ > VO extract > pure LMN. These results also demonstrate that TQ rich fractions of NS seed oil, pure TQ and LMN effectively improved the erythrocyte and liver enzymatic as well as nonenzymatic antioxidant defense capacities during cholesterol-induced hypercholesterolemia and restored them to near normal levels of NLP-C rats.

3.9 Effect of NSF and VO Fractions from NS Seed Oil; Pure TQ and LMN on Plasma Lipids, Plasma Lipoprotein Lipids, Hepatic HMG-CoA Reductase Activity in Hyperlipidemic Stressed Rats

3.9.1 Effect on plasma and lipoprotein lipids

As seen in Fig. 3.8, Panel (a) feeding rats one ml suspension supplemented with 5 mg cholesterol, 30 mg coconut oil and 2.5 mg cholic acid for 30 days resulted in a significant elevation of plasma TG (+105 %), TC (+168 %), VLDL-C (+105 %), LDL-C (+207 %) and non-HDL-C (TC minus HDL-C) (+222 %) levels in the HLP-C group compared to the corresponding values in NLP-C rats. On the other hand, a significant decline in plasma HDL-C (-6 %) and HDL$_2$-C (-13 %) with no significant change (-1.90 %) in HDL$_3$-C was seen. After 30-day treatment of HLP rats with 100 mg NSF, 20 mg VO, 10 mg TQ or 400 mg LMN per rat/day, the increase in the above lipid parameters were significantly blocked and reduced when compared to corresponding values in HLP-C group. NSF extract of NS seed oil was most effective in reducing plasma TG, TC, VLDL-C, LDL-C and non-HDL-C to a level which was 97 %, 96 %, 95 %, 103 % and 99 % of the corresponding control values in NLP-C. In VO and TQ treated HLP rats the level of decline in TC, LDL-C and non-HDL-C was similar to HLP-NSF rats, while TG and VLDL-C levels in HLP-VO and HLP-TQ groups were reduced by 72 % and 85 % of the respective value in NLP-C. The lipid lowering efficacy of LMN was lowest among four treated groups; plasma TG, TC, VLDL-C, LDL-C and non-HDL-C levels were restored by 53 %, 89 %, 53 %, 87 % and 86 %, respectively, of corresponding values in NLP-C rats. In contrast, nonatherogenic HDL-C, from a 6 % decline in HLP-C, was significantly (p<0.001) increased by 23 % in HLP-NSF, 12 % in HLP-VO, 15 % in HLP-TQ and
Fig. 3.8. Panel (a): Impact of NSF and VO extracts of NS oil, pure TQ and pure LMN on plasma TG, TC, VLD-C, LDL-C, HDL-C, HDL_{2-C} and HDL_{3-C} subfractions in hyperlipidemic rats after 30 days of treatment. Values are mean (mg/dl) ± SD from pooled plasma samples in each group. NLP-C, normolipidemic control fed 1 ml saline/rat/day; HLP-C, hyperlipidemic control given 1 ml of saline 30 min before the feeding of 1 ml suspension containing 5 mg cholesterol, 30 mg coconut oil and 2.5 mg cholic acid /rat/day; whereas rats in HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN were fed 1 ml of 100 mg NSF, 20 mg VO, 10 mg TQ or 400 mg LMN 30 min prior to administration of 1 ml of the above atherogenic suspension /rat/day for 30 days. Significantly different from NLP-C at p< 0.001, except in the case of HDL-C in HLP-C group which is p< 0.01. Significantly different from HLP-C at p< 0.001. Panel (b): Effect of NSF and VO fractions, pure TQ and LMN on the ratios of HDL-C/LDL-C, HDL-C/TC and HDL-C/TG in hyperlipidemic rats after 30 days of treatment. For the calculation of ratios, data is taken from Fig. 3.8. Panel (a). Values are mean ± SD from pooled plasma samples in each group. Significantly different from NLP-C at p< 0.001. Significantly different from HLP-C at p< 0.001.
Results

Panel (a)

Panel (b)
Results

2 % in HLP-LMN treated rats. Among HDL subfractions, HDL\textsubscript{2C} was differentially increased from a value of 6.09 mg/dl in HLP-C (-13 % vs. NLP-C value of 7.01 mg/dl) to 9.19 (+51 %), 7.39 (+21 %), 7.59 (+25 %) and 6.49 mg/dl (+7 %) in NSF, VO, pure TQ and LMN treated HLP rats, respectively. These results demonstrate that feeding of the indicated concentrations of NSF or VO extracts of NS seed oil, pure TQ or LMN to rats, 30 min prior to administration of an atherogenic suspension, mediated a significant decrease in plasma TG and atherogenic non-HDL-C with a concomitant increase in antiatherogenic HDL-C in the order HLP-NSF > HLP-TQ > HLP-VO > HLP-LMN (Fig. 3.8. Panel (a).

3.9.2 Effect on enzymatic activity of hepatic HMG-CoA reductase

As seen in Table 7, liver HMG-CoA reductase activity in hyperlipidemic stressed rats (HLP-C) was significantly reduced by 32 % in comparison to NLP-C value. Feeding of NSF, VO, pure TQ or LMN to these rats along with atherogenic suspension resulted in a further decline in HMG-CoA reductase activity by 44 %, 42 %, 43 % and 42 %, respectively, when compared to the activity in NLP-C. These results show that test fractions of NS seed oil/compounds mediated a reduction in total cholesterol levels of HLP rats apparently by inhibiting the enzyme activity of HMG-CoA reductase, the rate controlling enzyme in the biosynthetic pathway of cholesterol.

3.9.3 Effect on plasma LDL, small dense LDL and large buoyant LDL subfractions

Fig. 3.9 shows cholesterol and apoB concentrations of plasma LDL, small dense (sd-) LDL and large buoyant (lb-) LDL. In normal rats, cholesterol and apoB content of LDL were 61 and 117 mg/dl. The cholesterol and apoB concentrations of sd-LDL subfractions, isolated from LDL, were 18 and 36 mg/dl, while lb-LDL-C and lb-LDL-apoB levels were 43 and 81 mg/dl, respectively. In comparison to these values in NLP-C, hyperlipidemic stressed rats (HLP-C) showed a significant increase in plasma LDL-C from 61 to 188 mg/dl (207 %) and LDL-apoB content from 117 to 136 mg/dl (17 %). In contrast, sd-LDL-C level was substantially (p<0.001) increased from 18 mg/dl in NLP-C to 105 mg/dl (498 %) in HLP-C rats, while it’s apoB content was significantly increased by only 127 %. On the other hand, in HLP-C rats, lb-LDL-C level was increased by only 89 % whereas lb-LDL-
### Results

**TABLE 7**

*IN VIVO MODULATION OF HEPATIC HMG-CoA REDUCTASE ACTIVITY IN HYPERLIPIDEMIC RATS TREATED WITH NSF AND VO FRACTIONS, PURE TQ AND LMN AFTER 30 DAYS OF TREATMENT*

<table>
<thead>
<tr>
<th>Group</th>
<th>HMG-CoA reductase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLP-C</td>
<td>28.90± 0.04*</td>
</tr>
<tr>
<td>HLP-C</td>
<td>19.72 ± 0.07 (-31.76%)a</td>
</tr>
<tr>
<td>HLP-NSF</td>
<td>16.33±0.06 (-43.49%)a</td>
</tr>
<tr>
<td>HLP-VO</td>
<td>16.74±0.05 (-42.07%)a</td>
</tr>
<tr>
<td>HLP-TQ</td>
<td>16.52±0.06 (-42.83%)a</td>
</tr>
<tr>
<td>HLP-LMN</td>
<td>16.89±0.04 (-41.55%)a</td>
</tr>
</tbody>
</table>

†Expressed as percent of nmoles of HMG-CoA to mevalonate formed per mg liver protein.

*Values are mean ± SD from pooled liver samples in each group.

NLP-C, normolipidemic control fed 1 ml saline/rat/day; HLP-C, hyperlipidemic control given 1 ml of saline 30 min before the feeding of 1 ml suspension containing 5 mg cholesterol, 30 mg coconut oil and 2.5 mg cholic acid /rat/day; whereas rats in HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN were fed 1 ml of 100 mg NSF, 20 mg VO, 10 mg TQ or 400 mg LMN 30 min prior to administration of 1 ml of the above atherogenic suspension /rat/day for 30 days.

Significantly different from NLP-C at *p< 0.001.
Fig. 3.9. Impact of NSF and VO extracts of NS oil, pure TQ and LMN on plasma LDL, small dense and large buoyant LDL subfractions in hyperlipidemic rats after 30 days of treatment. Values are mean (mg/dl) ± SD from pooled plasma samples in each group. Significantly different from NLP-C at p< 0.001. Significantly different from HLP-C at p< 0.001.
apoB was reduced by 32 %, when compared to respective values in NLP-C. Treatment of hyperlipidemic rats with NSF, VO, pure TQ or LMN for 30 days significantly reduced both the cholesterol and apoB concentrations of LDL to a level similar to the values shown in NLP-C, except in HLP-LMN rats, where the decline was 87 % - 89 % of control values. A substantial and significant decreases in sd-LDL-C from a value of 105 mg/dl in HLP-C to 17, 20, 19 and 24 mg/dl in NSF, VO, pure TQ and LMN treated rats, respectively, were observed, which are similar to sd-LDL-C value of 18 mg/dl in NLP-C. Similarly, an enhanced value of 81 mg/dl in sd-LDL-apoB from HLP-C rats was reduced to 29, 46, 35 and 54 mg/dl in the four treated groups. These data represent a reduction of 125 %, 78 %, 101 % and 66 % in sd-LDL-apoB of four treated groups, when compared to control value of 36 mg/dl in NLP-C rats. On the other hand, lb-LDL-C registered an increase of only 89 % from a control value of 43 mg/dl in NLP-C to 81 mg/dl in HLP-C, while lb-LDL-apoB in HLP-C was reduced by 32 % in comparison to NLP-C value. The 89 % increase in lb-LDL-C with 32 % decline in lb-LDL-apoB in HLP-C group was significantly restored to near respective NLP-C values (41-46 mg/dl for lb-LDL-C and 77-83 mg/dl for lb-LDL-apoB) of 43 and 81 mg/dl, respectively, in NLP-C following 30-day administration of test fractions/compounds to atherogenic suspension fed rats.

In normolipidemic rats, the percent wise distributions of cholesterol and apoB from LDL particle to sd-LDL and lb-LDL subfractions were 29 %, 31 %, 70 % and 69 %, respectively. In HLP-C group, the percent sd-LDL-C and sd-LDL-apoB were significantly increased by a common value of 95 %, while the percent lb-LDL-C and lb-LDL-apoB were significantly decreased by 39 % and 42 %, when compared to corresponding percent values in NLP-C rats. In the four treated groups, percent sd-LDL-C was significantly reduced to a level which is 99 %, 88 %, 92 % and 84 % of control value of 29 mg/dl in NLP-C, while decline in percent sd-LDL-apoB was 120 %, 83 %, 101 % and 74 % of control value (31 mg/dl) in NLP-C rats. On the other hand, both cholesterol and apoB concentrations in lb-LDL were significantly increased to near control percent values of 70 and 69 mg/dl in NLP-C rats after 30-day treatment of HLP rats with NSF, VO, pure TQ or LMN. These results demonstrate that the prevalence and concentration of sd-LDL subspecies, which is considered more atherogenic than buoyant LDL or LDL, was significantly increased, while lb-LDL was proportionally decreased in hyperlipidemic rats. The
Results

percent share of LDL in sd-LDL subpopulation was increased from 30 % in NLP-C to 58 % in HLP-C, whereas in lb-LDL it was decreased from 70 % to 42 %. Oral feeding of test fractions/compounds to HLP rats almost completely blocked this shift in buoyancy and restored the percent distribution of LDL cholesterol and apoB into sd-LDL and lb-LDL subfractions by an average value of 33 % and 67 %, which are close to NLP-C values of 30 % and 70 %, respectively.

3.9.4 Effect on HDL-C/LDL-C, HDL-C/TC and HDL-C/TG ratios

As shown in Fig. 3.8. Panel (b), HDL-C/LDL-C, HDL-C/TC and HDL-C/TG ratios were calculated from the data presented in Fig. 3.8. Panel (a). As expected, HDL-C/LDL-C ratio was substantially decreased from 0.345 in NLP-C to 0.106 (69 %) in HLP-C rats. After 30 days of treatment, this decrease in HDL-C/LDL-C ratio was significantly prevented and substantially increased to 0.415 (292 %) in HLP-NSF, 0.365 (244 %) in HLP-VO, 0.381 (259 %) in HLP-TQ and 0.288 (172 %) in HLP-LMN groups. The results also show that HDL-C/LDL-C ratio in NSF, VO, TQ and LMN treated HLP rats were restored by 120 %, 106 %, 110 % and 83 % of control ratio value in NLP-C. On the other hand, the HDL-C/TC ratio in HLP-C was decreased by 65 % in comparison to a ratio of 0.235 in NLP-C. After 30-day treatment, HDL-C/TC ratios in HLP-NSF, HLP-VO, HLP-TQ and HLP-LMN groups were increased and restored by 112 %, 101 %, 103 % and 86 % of control ratio value. In NLP-C, an HDL-C/TG ratio of 0.372 was obtained, which is significantly higher than HDL-C/LDL-C and HDL-C/TC ratios of 0.345 and 0.235, respectively. In HLP-C rats, this ratio was decreased to 0.171 (54 %). NSF, VO, pure TQ or LMN treatment significantly prevented this decrease in HDL-C/TG ratio and markedly increased to a level, which is 113 %, 76 %, 93 % and 51 %, respectively, of control ratio value. The ratios related to TG, TC, LDL-C and HDL-C in four treated groups except HLP-LMN were positively modulated and restored similar or better to corresponding ratio values in NLP-C.
Results

3.10 *Ex Vivo* and *In Vitro* Cu\(^{++}\)-Mediated Oxidation of LDL, Sd-LDL and Lb-LDL in Hyperlipidemic Stressed Rats Treated with NSF, VO, pure TQ or LMN

3.10.1 Antioxidant effect on basal and maximal levels of CD formation and lag time in LDL, sd-LDL and lb-LDL

As seen in Fig. 3.10, the *ex vivo* basal CD levels of LDL, sd-LDL and lb-LDL of HLP-C stressed rats were significantly increased by 52 %, 55 % and 20 %, respectively, in comparison to the corresponding NLP-C values. Treatment of these stressed rats with NSF, VO or pure TQ significantly blocked their *in vivo* oxidation and reduced the basal CD values of LDL, sd-LDL and lb-LDL to 80-84 %, 87-95 % and 91-96 % of respective control values, while in HLP-LMN, restoration of 75 %, 76 % and 88 % was seen. The maximal *in vitro* Cu\(^{++}\)-mediated oxidation of LDL and lb-LDL was achieved after 2 h of incubation, while for sd-LDL, maximal CD value was obtained after 30 min. The maximal CD values of LDL, sd-LDL and lb-LDL in NLP-C rats were substantially increased by 299 %, 305 % and 290 %, respectively, when compared to corresponding *ex vivo* basal CD values in NLP-C. In comparison to corresponding maximal CD values in normolipidemic rats, LDL, sd-LDL and lb-LDL associated CD formation of HLP-C rats were increased by 61 %, 80 % and 37 %, respectively. Similar to *ex vivo* basal CD values, maximal CD values of LDL, sd-LDL and lb-LDL were significantly reduced close to corresponding normal values following 30-day feeding of NSF, VO, pure TQ or LMN to atherogenic suspension fed rats. As expected the lag time of LDL and lb-LDL oxidation was reduced from 90 min and 51 min in NLP-C to 53 min and 36 min in HLP-C, respectively. In contrast, sd-LDL oxidation resulted in a strikingly lower lag time of 17 min in NLP-C, which was further reduced to 11 min in HLP-C group. Treatment with NSF, VO or pure TQ restored the lag time of LDL oxidation to 88 min, 85 min and 87 min, while for lb-LDL, it was increased to 49 min, 46 min and 48 min, respectively. In LMN treated rats, the restoration of lag time was 62 min and 40 min for LDL and lb-LDL oxidation, respectively. The lag time of sd-LDL oxidation was increased from 11 min in HLP-C to 15 min in HLP-NSF, 13 min in HLP-VO, 14 min in HLP-TQ and 12 min in HLP-LMN. These results demonstrate a significantly enhanced *in vivo* and Cu\(^{++}\)-induced *in vitro* susceptibility of sd-LDL to
Fig. 3.10. Copper-catalyzed *in vitro* oxidation of LDL and its Sd-LDL and Lb-LDL subfractions from plasma of hyperlipidemic rats after 30 days of NSF and VO extracts, pure TQ and LMN treatment.

The CD values are expressed as nmole MDA equivalents/mg protein. Basal CD values represent the status of oxidized LDL, sd-LDL and lb-LDL *in vivo*. Maximal CD and MDA values of LDL and lb-LDL oxidation were achieved after 2 h of incubation with CuSO4, whereas for sd-LDL these values were obtained after 30 min.

The lag time is defined as the interval between the intercept of the tangent of the slope of the curve with the time expressed in minutes. Values are obtained from LDL, sd-LDL and lb-LDL subpopulations, isolated from pooled plasma samples in each group.
oxidative modification than lb-LDL, which may contribute to the increased risk associated with atherogenesis. In addition, simultaneous feeding of NSF or VO extracts of NS seed oil, pure TQ or LMN along with an atherogenic suspension to rats exhibited a potent antioxidant effect by preferentially blocking the *ex vivo* and *in vitro* formation of CD and increasing the lag time of more atherogenic sd-LDL subpopulation than less atherogenic lb-LDL.

### 3.10.2 Antioxidant impact on basal and maximal levels of MDA in LDL, sd-LDL and lb-LDL

As evident from the data in Fig. 3.11, the *ex vivo* basal levels of MDA in LDL and its subfractions, sd-LDL and lb-LDL were significantly increased by 106 %, 71 % and 61 % in HLP-C compared to NLP-C group. Treatment of these stressed rats with NSF, VO or pure TQ significantly blocked their *in vivo* oxidation and reduced the basal MDA values of LDL, sd-LDL and lb-LDL to 65-78 %, 68-81 % and 72-80 % of respective control values, while in HLP-LMN, restoration of 59 %, 65 % and 68 % was seen. The maximal MDA values *in vitro* Cu$^{++}$-mediated oxidation of LDL and lb-LDL were achieved after 2 h of incubation, while for sd-LDL, maximal MDA value was obtained after 30 min. The maximal MDA values of LDL, sd-LDL and lb-LDL in NLP-C rats were substantially increased by 750 %, 601 % and 550 %, respectively, when compared to corresponding *ex vivo* basal MDA values in NLP-C. In comparison to corresponding maximal MDA values in normolipidemic rats, LDL, sd-LDL and lb-LDL associated MDA formation of HLP-C rats were increased by 81 %, 66 % and 32 %, respectively. The increase in maximal MDA formation in LDL, sd-LDL and lb-LDL from HLP rats treated with NSF, VO, pure TQ or LMN was significantly restored by 83 %, 72 %, 74 % and 64 %; 84 %, 70 %, 71 % and 68 %; 93 %, 87 %, 89 % and 83 % of respective NLP-C values in LDL, sd-LDL and lb-LDL. These results indicated that similar to increase in *in vivo* and *in vitro* CD formation in LDL, more atherogenic sd-LDL and less atherogenic lb-LDL from HLP-stressed rats, the *ex vivo* basal and Cu$^{++}$-catalyzed maximal MDA levels were also increased, which were significantly inhibited in rats treated with natural antioxidant rich fractions or pure antioxidants.
Fig. 3.11. *In vitro* Cu^{++}-induced generation of malondialdehyde in LDL and its Sd-LDL and Lb-LDL subfractions from plasma of hyperlipidemic rats after 30 days of NSF and VO fractions, pure TQ and LMN treatment. The values are expressed as nmole/mg protein. Basal MDA values represent the *in vivo* status of oxidized LDL, sd-LDL or lb-LDL, maximal MDA values of LDL and lb-LDL Cu^{++}-induced oxidation were achieved after 2 h, while for sd-LDL these values were obtained after 30 min. Values are mean ± SD from LDL, sd-LDL and lb-LDL subfractions, isolated from pooled plasma samples in each group. Significantly different from NLP-C at p< 0.001. Significantly different from HLP-C at p< 0.001.
Results

Basal Maximal Basal Maximal Basal Maximal
nmole/mg protein

NLP-C HLP-C HLP- NSF HLP-VO HLP-TQ HLP-LMN

LDL Sd-LDL Lb-LDL