CHAPTER 5

STUDIES ON FUNCTIONAL CHARACTERIZATION OF SEABUCKTHORN WINE (FUNCTIONAL BEVERAGE I)
ABSTRACT

We evaluated the protective effects of seabuckthorn wine against phorone-induced oxidative stress and high-cholesterol diet induced hypercholesterolemia in male LACA mice. Oral administration of seabuckthorn wine increased the redox ratio (1.24) accompanied by reduction of oxidized glutathione levels (21.07 nmoles/mg protein) leading to attenuation of phorone-induced oxidative stress. Furthermore, the seabuckthorn wine supplementation reduced hepatic lipid peroxidation and increased the superoxide dismutase activity (1.59 Units/mg protein) indicating improved resistance to oxidative stress. The seabuckthorn wine performed better with respect to reduced glutathione (GSH), catalase, glutathione peroxidase (Gpx) & glutathione-S-transferase (GST) as compared to Cabernet Shiraz. This study also provided important evidence that seabuckthorn wine exerts protective effects against hypercholesterolemia. In addition, high-cholesterol-fed mice administered with seabuckthorn wine exhibited a 197% increase in the HDL-C/LDL-C ratio compared to high-cholesterol diet treated mice. Interestingly both wine (seabuckthorn wine and Cabernet Shiraz) showed similar in vivo protective effects inspite of the fact that resveratrol (which is one of the most important health improving factor of red wine) is absent in seabuckthorn wine. These studies provide experimental proof that seabuckthorn wine exerts protective effects against oxidative stress and hypercholesterolemia.
5.1 INTRODUCTION

Today, consumer beliefs in the health benefits of functional foods appear to be increasing at an unexpected pace [Meyer, 1998]. Moreover, any statements about the benefits of a functional food or its component(s) must be based on sound scientific information [Clydesdale, 1997]. Hence, it is essential that consumers receive evidence based and consistent information to make well-versed decisions about the benefits associated with functional foods. The positive effects of a functional food can be either maintenance of a state of well-being and health or reduction of the risk of disease.

The design and development of functional foods is a scientific challenge that should rely on the stepwise process. The initial step in design and development of a functional food is the identification of a specific function (i.e., cellular, biochemical, or physiologic) that is potentially beneficial to maintenance of well-being and health, and may be reduce the risk of a disease. This step is fundamental research and should lead to the development and validation of relevant biomarkers. Next is a new generation of hypothesis-driven in vivo studies that will include the use of these validated, relevant markers and allow the establishment of effective and safe intakes.

5.1.1 Biomarkers

Biomarkers are needed not only to assess the value of functional foods and their biological components as modifiers of disease, but also to evaluate their ability to promote health, growth and well-being. Thus biomarkers capable of assessing the following will be required [Milner, 1999]:

1. Active agents capable of modifying target tissues (Intake Biomarkers)
2. Specific biological responses that relate directly to either disease risk or health maintenance (Effect Biomarkers)
3. Modifiers of the response by genetic and other environmental factors (Susceptibility Biomarkers)
To assess whether a food or its constituent is having a physiologic effect and hence is ‘functional’, it is imperative that valid intakes and exposures to the active site(s) be determined. This assessment is by itself not an easy task due to the questionable reliability of food disappearance data and the dearth of information available about many, if not most of the functional food constituents of the diet [Ervin and Smiciklas-Wright 1998; de Vries et al. 1998]. Effect biomarkers refer to the consequences of interactions between a food component and a specific genomic, biochemical, cellular or physiologic event. Generally, the effect biomarkers are aimed at predicting a long-term consequence such as general health or disease risk. However, some may be more immediate, including those associated with physical or mental performance. Some effect biomarkers may actually not be within the body proper such as the effect seen with the biofermogenic stimulation caused by enhanced intake of inulin and oligofructose [Roberfroid, 1993]. Clearly, the sensitivity of the effect biomarker is highly dependent on a number of factors, including genetics and environmental factors. Collectively, these factors constitute susceptibility biomarkers that can affect the effect biomarker being examined.

Identifying specific cause-effect relationships between functional food and health is challenging and, in some cases, controversial because of the complexity of human biology and physiology. Biomarkers and their relationship to health status are often identified through observational studies and correlations. At best, correlated factors may suggest a complex, multi-factorial relationship among functional food and health and may be supported by scientific theory that appears reliable; at worst, the correlations are the result of another unrelated factor and have no basis in fact. Hence it is essential to identify biomarkers that signal changes in health status and then determine the meaning of changes in those biomarkers relative to a defined health condition. The effects of functional food on biomarkers must be validated through prospective in vivo studies.
Studies exploring the protective and disease preventing potential of foods have shown that in addition to red wine, non-grape wine such as cherries, blueberries, blackcurrant, and cranberries have comparable or even higher flavonoid and phenolic content [Heinonen et al., 1998; Schmitzer et al., 2010; Lim et al., 2012; Mudnic et al., 2012; Johnson and Mejia et al., 2012]. Consequently, these have given a new dimension to the non-grape wines. Epidemiological studies also suggest that moderate consumption of red wine is associated with many health benefits including a reduced risk of cardiovascular disease and with reduced mortality for all causes.

The current study undertaken to evaluate the functional effects of seabuckthorn wine on phorone-induced oxidative stress is important for two reasons. Firstly, it assesses the effect of the wine on the liver as excessive alcohol consumption may lead to liver damage (hepatotoxicity). Secondly, it also evaluates the seabuckthorn wine, an external source of the antioxidant protection system because oxidative stress is considered to contribute to the pathogenesis of various other diseases. Moreover, a significant degree of cardioprotection has been attributed to ingestion of wines rich in flavonoids. Although a few studies have reported the effects of seabuckthorn berry fractions on cardiovascular risk factors [Eccleston et al., 2002; Basu et al., 2007], this is the first study on the impact of seabuckthorn wine on cardiovascular risk factors. The sub-objectives of this study were:

a. Studies on protective effects of seabuckthorn wine (Functional beverage I) on phorone-induced oxidative stress in male LACA mice
b. Studies on protective effects of seabuckthorn wine (Functional beverage I) on diet-induced hypercholesterolemia in serum of male LACA mice
5.2 MATERIALS AND METHODS

5.2.1 Chemicals and reagents

Bovine serum albumin (BSA), EDTA, trichloroacetic acid, N-ethylmaleimide, glutathione reductase, dithiothreitol, o-phenaldehyde, nitroblue tetrazolium, thiobarbituric acid, oxidized glutathione, reduced glutathione and 1-chloro-2,4-dinitrobenzene were purchased from Sigma-Aldrich, St Louis (USA). All other chemicals and reagents used in the present study were of analytical grade.

5.2.2 Animals

Male LACA mice (25-30 g body weight, 8-10 weeks of age) were obtained from the Central Animal House, Panjab University, Chandigarh, India. The animals were kept in plastic cages under hygienic conditions and were provided standard animal feed. Mice were acclimatized to the laboratory conditions for a week before initiating the study. Necessary approvals (CAH/131) for animal studies were obtained from the Institutional Ethics Committee, Panjab University, Chandigarh, India. The animals care and handling were done according to the guidelines set by the World Health Organization (WHO), Geneva, Switzerland, and the Indian National Science Academy (INSA), New Delhi, India. The treatment protocol was for 15 days.

5.2.3 Treatment protocol for phorone induced oxidative stress

Twenty four male LACA mice were randomly divided into following four groups of six mice in each group: group 1- control, group 2- phorone treated, group 3- Cabernet Shiraz and phorone treated, group 4- seabuckthorn wine and phorone treated. Phorone (450 mg/Kg of body weight) [Geneve et al., 1987] was administered daily intraperitonially for 15 days to all groups except control group (Table 5.1). This dose was chosen since it is not hepatoxic and it is known to markedly diminish hepatic GSH content. An aliquot of 500 µL of Cabernet Shiraz and seabuckthorn wine were given daily through oral administration. Control group animals received only 0.9% saline.
Body weights of mice and their daily food and fluid intake were recorded on alternate days.

**Table 5.1 Treatment protocol for oxidative stress**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Animals (male LACA mice)</th>
<th>Dose</th>
<th>Delivery method</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 Control</td>
<td>6</td>
<td>Normal chow diet</td>
<td>Free access</td>
<td>Daily for 15 days</td>
</tr>
<tr>
<td>Group 2 Phorone treated</td>
<td>6</td>
<td>450 mg/Kg (BW)</td>
<td>Intraperitonially</td>
<td>Daily for 15 days</td>
</tr>
<tr>
<td>Group 3 Phorone + Cabernet Shiraz treated</td>
<td>6</td>
<td>Phorone: 11.25 mg/25 g (BW)</td>
<td>Phorone: Intraperitonially</td>
<td>Daily for 15 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wine: 500 μL/25 g (BW)</td>
<td>Wine: oral administration</td>
<td></td>
</tr>
<tr>
<td>Group 4 Phorone + seabuckthorn wine treated</td>
<td>6</td>
<td>Phorone: 11.25 mg/25 g (BW)</td>
<td>Phorone: Intraperitonially</td>
<td>Daily for 15 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wine: 500 μL/25 g (BW)</td>
<td>Wine: oral administration</td>
<td></td>
</tr>
</tbody>
</table>

After completion of the treatment, mice were euthanized by cervical dislocation following etherization. Livers were rapidly harvested. To obtain post-mitochondrial fraction (PMF) 10% homogenate was prepared in ice cold 20 mM Tris- HCl buffer (pH 7.4) and centrifuged at 10,000 g for 30 min at 4°C. Further assays were carried out in PMF.
5.2.3.1 Estimation of glutathione levels

The levels of total and oxidized glutathione were measured by the fluorimetric method of Hissin and Hilf [1976]. This method is based on the principle that glutathione reacts specifically with o-pthalaldehyde (OPT) at pH 8 and with GSSG at pH 12 resulting in the formation of a highly fluorescent product which is activated at 350 nm with an emission at 420 nm. To the PMF, trichloroacetic acid (TCA; final concentration 5%) was added to precipitate the protein and supernatant was obtained after centrifugation at 10,000 g for 10 min at 4°C. The samples were then processed differently for measuring total and oxidized glutathione.

Total glutathione

The reaction mixture consisted of:

0.1 M sodium phosphate-5mM EDTA buffer (pH 8) 9.4 mL

Tissue supernatant (after TCA precipitation) 100 μL

OPT (100mg% in methanol) 500 μL

The samples were mixed by vortex and incubated at room temperature for 15 min. Reduced glutathione (GSH) in the range of 5-40 μg was used as standard.

Oxidized glutathione (GSSG)

For the estimation of GSSG, 0.5 mL of the supernatant after TCA precipitation was mixed with 200 μL of 0.04M N-ethylmaliemide (NEM) at room temperature for 30 min. This causes inhibition of GSH in alkaline pH 12.0 and only OPT-GSSG complex is formed.

The reaction mixture contained:

0.1N sodium hydroxide 9.4 mL
Supernatant containing NEM 100 µL

OPT (100 mg% in methanol) 500 µL

The samples were mixed by vortex and incubated at room temperature for 15 min. Oxidized glutathione (GSSG) in the range of 5-40 µg was used as standard. The fluorescence intensity was recorded in a fluorescence spectrophotometer (Perkin Elmer, USA) with an excitation filter of 350 nm and an emission filter of 420 nm. Results were expressed as nmol of GSSG/mg protein.

Reduced Glutathione (GSH)

GSH levels were quantitated by subtracting the value of GSSG from the total glutathione levels. Redox ratio was determined by taking the ratio of reduced GSH to oxidized GSSG.

5.2.3.2 Superoxide dismutase

Activity of superoxide dismutase (SOD) was estimated by the method of Kono and Fridovich [1982]. The reaction is designed to observe the inhibition of the rate of oxidation of nitroblue tetrazolium (NBT), which was recorded using hydroxylamine hydrochloride as the electron donor.

The reaction mixture consisted of:

Solution A (3.8 mg EDTA and 0.53 g 1.3 mL
Na₂CO₃ in 100 mL distilled water, pH 10)

NBT (8 mg/100 mL of Sol A) 0.5 mL

0.6% Triton X in Solution A 0.1 mL

2mM hydroxylamine hydrochloride (pH 6) 0.1 mL
After the addition of hydroxylamine hydrochloride, the change in absorbance \( A_x \) was measured at 560nm. To this reaction mixture, enzyme source (PMF) was added and again change in absorbance \( A_y \) was recorded at 560nm. Difference in the rate was compared and expressed as percent inhibition.

\[
\text{% inhibition} = \left( \frac{A_x - A_y}{A_x} \right) \times 100
\]

One enzyme unit is defined as the enzyme required resulting in 50% inhibition of \( A_x \). The enzyme was expressed as units/mg protein.

**5.2.3.3 Lipid peroxidation**

The levels of malondialdehyde (MDA) were used as an index for measuring the level of lipid peroxidation according to the method of Wills et al. [1966].

To 250 µL of PMF, 500 µL of ice-cold TCA (10%) was added and centrifuged at 800 g for 10 min. The supernatant was collected and to 500 µL of supernatant an equal volume of thiobarbituric acid, TBA (0.67% in distilled water) was added. The tubes were kept in boiling water bath for 10 min. Since malondialdehyde is a degradation product of peroxidized lipids, the development of pink colour with the absorption characteristics (maxima 532 nm) as TBA-MDA chromophore was taken as an index of lipid peroxidation. For standard, 2-10 nM range of 1,1',3,3'-tetraethoxypropane (TEP) in 250 µL volume was used and for control, distilled water was used instead of the sample. Results were expressed as nmoles MDA/mg protein.

**5.2.3.4 Catalase**

Catalase activity was estimated according to the method of Luck [1963] using hydrogen peroxide as the substrate.
The reaction mixture consisted of:

50 mM phosphate buffer (pH 7) 2.5 mL
0.75 M $H_2O_2$ 50 μL

The reaction was started by adding 100 μL of enzyme (PMF) to the above reaction mixture and the rate of change in absorbance was recorded at 240 nm for 2 min. The enzyme activity was expressed as the rate of decrease in absorbance using the molar extinction coefficient of $H_2O_2$ (0.0394 mM/cm).

5.2.3.5 Glutathione reductase

Glutathione reductase activity was estimated according to the method of Massey and Williams [1965]. The activity was monitored by following the oxidation of co-factor NADPH by oxidized glutathione (GSSG) at 340 nm. The utilization of NADPH was directly related to the activity of glutathione reductase.

The reaction mixture consisted of:

3 mM EDTA 0.1 mL
2% BSA 0.1 mL
0.1 mM NADPH 0.1 mL
3 mM GSSG 0.1 mL
0.1 M potassium phosphate buffer (pH 7.5) 0.6 mL

The reaction was started by the addition of 50 μL of the PMF and the oxidation of NADPH was recorded at 340 nm for 3 min. Enzyme activity was expressed as μmoles of NADPH oxidized/min/mg protein.
5.2.3.6 Glutathione peroxidase

Total glutathione peroxidase (GSH-Px) activity was estimated in the liver by the method of Lawrence and Burk [1976] using $H_2O_2$ as substrate.

The reaction mixture consisted of:

- 50 mM Potassium phosphate buffer, pH 7.0: 566 µL
- 1 mM EDTA: 100 µL
- 1 mM Sodium azide: 10 µL
- 0.2 mM NADPH: 100 µL
- Glutathione reductase (1E.U/mL): 4 µL
- 1mM GSH: 100 µL
- 0.25 mM $H_2O_2$: 100 µL

The reaction was started by adding 20 µL of the PMF (20 µL of sample diluted 1:20 with 50 mM potassium phosphate buffer) to 880 µL of the above mixture (except $H_2O_2$) followed by incubation for 5 min at room temperature. Then 100 µL of the $H_2O_2$ was added. Absorbance was recorded at 340 nm (UV-160A, Shimadzu) for 3 min. Enzyme activity was calculated from the slope of these lines and expressed as µmoles NADPH oxidized/min/mg protein.
5.2.3.7 Glutathione-S-Transferase

The activity of Glutathione-S-transferase (GST) was measured by using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to the method of Habig et al. [1974].

The reaction mixture consisted of:

- 0.1 M potassium phosphate buffer (pH 6.5) 1 mL
- 1 mM reduced glutathione (GSH) 35 μL
- 1 mM CDNB (in absolute alcohol) 35 μL

To the above mixture, 50 μL of the sample was added and the formation of conjugated complex of CDNB was monitored at 340 nm. The activity was expressed as nmol CDNB conjugated/min/mg protein using the molar extinction coefficient of CDNB (9.6 mM/cm).

5.2.3.8 Protein estimation

Protein estimation was done by the method of Lowry et al., [1951]. To 10 μL of samples from each treatment group was added 3 mL of 50:1 mixture of 2% sodium carbonate in 0.1 N NaOH and 0.5% CuSO₄/1% Na-K tartrate in distilled water. The tubes were incubated for 10 min at room temperature. Then added 300 μL 1N Folin's phenol reagent to each tube, mixed it and again incubated for 30 min at room temperature. The optical density was then measured at 620 nm on spectrophotometer (UV-160A, Shimadzu). Bovine serum albumin (BSA) was used as standard (10-100 μg).
5.2.3.9 Reactive oxygen species (ROS) levels

Determination of ROS was based on the modified method of Driver et al. [2000]. Liver homogenates were prepared in ice-cold Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2 mM CaCl₂, 10 mM d-glucose and 5 mM HEPES pH 7.4). The homogenates were allowed to warm at 21°C for 5 min. The reaction mixture containing 10 μM DCFH-DA and 5 mg tissue/ml was incubated for 15 min at room temperature (21°C). After another 30 min of incubation, the conversion of DCFH to the fluorescent product 2, 7 dichlotoflourescein (DCF) was measured using fluorescence spectrophotometer with the excitation at 485 nm and emission at 530 nm. Background fluorescence (conversion of DCFH-DH to DCF in the absence of homogenate) was corrected by inclusion of parallel blanks. The relative fluorescence intensity was taken as the measure of amount of ROS in different groups.

5.2.4 Treatment protocol for hypercholesterolemia induced by feeding a high cholesterol diet

Male I.ACA mice (75-30 g body weight, 8-10 weeks of age) were obtained from the Central Animal House, Panjab University, Chandigarh, India. Animals were divided into four groups (six animals in each group) for the study (Table 5.2). Group 1 animals served as control. These animals were fed on normal chow diet. Group 2 animals were fed on high-fat diet (HFD) (2% cholesterol with control diet) [Dhingra et al., 2003]. Group 3 animals were fed on HFD with Cabernet Shiraz (Sula Vincyards). Group 4 animals were fed on HFD with seabuckthorn wine. 500 μL (per 25 gm body weight of animal) of seabuckthorn wine and Cabernet Shiraz were given daily through oral administration for 15 days. Body weights of mice and their daily food and fluid intake were recorded on alternate days. After completion of treatment period, blood was drawn from the retro-orbital sinus of the ether anaesthetized overnight (12 h) fasting mice. Blood was collected into a clean, sterile, and labelled centrifuge tubes without an anticoagulant. After clotting, the serum was separated by centrifugation at 1500 g for 10 min.
Table 5.2  Treatment protocol for hypercholesterolemia induced by feeding a high cholesterol diet

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Animals (male I.A.C.A mice)</th>
<th>Dose</th>
<th>Delivery method</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1 Control</strong></td>
<td></td>
<td>Normal chow diet</td>
<td>Free access</td>
<td>Daily for 15 days</td>
</tr>
<tr>
<td><strong>Group 2 High-cholesterol diet treated</strong></td>
<td>6</td>
<td>2% cholesterol with a normal chow diet</td>
<td>Free access</td>
<td>Daily for 15 days</td>
</tr>
<tr>
<td><strong>Group 3 High-cholesterol diet +Cabernet Shiraz treated</strong></td>
<td>6</td>
<td>2% cholesterol with a normal chow diet</td>
<td>High cholesterol diet= Free access Wine=oral administration</td>
<td>Daily for 15 days</td>
</tr>
<tr>
<td><strong>Group 4 High-cholesterol diet +seabuckthorn wine treated</strong></td>
<td>6</td>
<td>2% cholesterol with a normal chow diet</td>
<td>High cholesterol diet= Free access Wine=oral administration</td>
<td>Daily for 15 days</td>
</tr>
</tbody>
</table>

5.2.4.1  Lipid profile analysis

5.2.4.1.1  Determination of serum total cholesterol

Total cholesterol level was estimated using CHOD-POD (cholesterol oxidase/peroxidase phosphotungstate method) based kit obtained from Human Diagnostics, Germany. To find out total cholesterol and fatty acid by the action of cholesterol esterase, free cholesterol in the sample is oxidized to 4 Cholestene-3-one and hydrogen peroxide. The amount of hydrogen peroxide formed is directly proportional to the amount of cholesterol in the blood. Hydrogen per oxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase and produces a red colored quinine compound, which is measured at 500 nm.
Following is the kit composition of Total Cholesterol PAP, Enzymatic Colorimetric Method of Human Diagnostics, Germany.

**Reagent 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipes buffer, pH 6.90</td>
<td>50 mmol/L</td>
</tr>
<tr>
<td>Sodium Chlorate</td>
<td>0.5 mmol/L</td>
</tr>
<tr>
<td>Phenol</td>
<td>24 mmol/L</td>
</tr>
</tbody>
</table>

**Reagent 2**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol esterase</td>
<td>≥ 200 U/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥1000 U/L</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>≥250 U/L</td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.5 mmol/L</td>
</tr>
</tbody>
</table>

**Standard**

Cholesterol 200 mg/dL

Working reagent was prepared by dissolving reagent 2 in reagent 1. 10 μL of standard or sample and 1 mL of working reagent were added to test tube. The reagents were mixed and after 5 min incubation optical density (OD) was measured at 500 nm.

**5.2.4.1.2 Determination of high density lipoprotein cholesterol**

The serum is centrifuged with magnesium chloride and tungsten phosphoric acid, so that β-apoprotein is precipitated (LDL-C and VLDL-C). The supernatant thus obtained contains α-apoproteins (HDL cholesterol). HDL-C then reacts with cholesterol reagent and oxidized to cholestenone and hydrogen peroxide by the action of cholesterol oxidase. Hydrogen peroxide thus produced is directly proportional to the HDL-C in serum. Hydrogen peroxide, after reacting with 4-aminoantipyrine, gives 4-p-(benzoquinone-monoamino)-phenazine, which is a red colored compound.
0.2 mL of serum and 0.5 mL of reagent were added in a tube and incubated for 10 min at room temperature. Afterward the contents were centrifuged for 15 min at 1500. 0.1 mL of supernatant was then taken, mixed with 1 mL of cholesterol reagent and incubated for 15 min at room temperature. The absorbance was measured at 546 nm.

5.2.4.1.3 Determination of Triglycerides (TG)

The reagents used for Triglyceride enzymatic liquid colorimetric test, GPO-PAP method were obtained from Sigma–Aldrich Co., USA.

In blood triglyceride level was determined enzymatically by the oxidation of triglycerides into glycerol and fatty acids using lipoprotein lipase. Glycerol is phosphorylated into glycerol-3 phosphate using glycerol kinase in the presence of ATP. Glycerol-3 phosphate is oxidized under the action of glycerol-3 phosphate oxidase, with immediate production of hydrogen peroxide. Hydrogen peroxide then reacts with 4-aminoantipyrine and produce a red quinine compound under the action of peroxidase. The absorbance of quinine is then measured at 546 nm

**Reagents:**

Reagent 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-amino-antipyrine</td>
<td>0.7 mmol/L</td>
</tr>
<tr>
<td>ATP</td>
<td>0.3 mmol/L</td>
</tr>
<tr>
<td>Enzymes lipoprotein lipase</td>
<td>≥1100 U/L</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>≥800 U/L</td>
</tr>
<tr>
<td>Glycerol -3-phosphate oxidase</td>
<td>≥3000 U/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥350 U/L</td>
</tr>
</tbody>
</table>
Reagent 2

Buffer solution of pH 7.50 50 mmol/L.

10 μL of standard or sample and 1 mL of working reagent was added to each test tube. The solution was mixed and incubated for 5 min at 37°C. The absorbance of the solution was measured at 546 nm.

5.2.4.1.4 Determination of low density lipoprotein cholesterol

LDL-C was calculated by the Friedewald formula [Friedewald et al., 1972].

LDL cholesterol (mg/dl) = TC – HDL-cholesterol- TG/5.

5.2.5 Statistical Analysis

All data were presented as the average of triplicate experiments with standard deviation. Results were statistically interpreted with one-way and two way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis to locate the significant differences indicated with ANOVA. The data for ANOVA were analyzed at different significance level using statistical package MSTAT/Minitab (Minitab Inc. USA, Version 13, 2004 for Windows®). Correlation between total phenolic content and antioxidant activities was established using the MS Excel correlation coefficient statistical option.
5.3 RESULTS AND DISCUSSION

Fruit wines and wine flavonoids have emerged as potential sources of antioxidants in reducing free radical induced tissue injury. Consequently, the health benefits of the antioxidant potential of wines have received considerable attention because many synthetic antioxidants have been shown to have one or the other side effects [Nocentini et al., 2001]. Our aim was to investigate the protective role of novel seabuckthorn wine that was developed in our laboratory and there by ascribe potential health benefits to the developed functional beverage.

5.3.1 Standardization of wine dose

Wine dose was standardized based on the high concentration of blood plasma phenolic content in LACA mice (Table 5.3). To standardize the wine dose animals were divided to 7 groups. Each group animals were given a free access to normal chow diet and water. Group 1 animal treated as control group. Wine dose of 50, 100, 250, 500, 750 and 1000 μL/25 gm BW (Body Weight of mice) were given daily through oral administration to group 2, 3, 4, 5, 6 & 7, respectively. The treatment protocol was for 4 weeks.
### Table 5.3  
**Standardization of wine dose in blood plasma of mice**

<table>
<thead>
<tr>
<th>Oral administration of seabuckthorn wine</th>
<th>Total Phenolic Content (µg GAE/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 week</td>
</tr>
<tr>
<td>Group 1 (no treatment)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>49 ± 1.23</td>
</tr>
<tr>
<td>Group 2 (BW) 50µL/25gm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 ± 0.59</td>
</tr>
<tr>
<td>Group 3 (BW) 100µL/25gm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52 ± 0.83</td>
</tr>
<tr>
<td>Group 4 (BW) 250µL/25gm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51 ± 0.92</td>
</tr>
<tr>
<td>Group 5 (BW) 500µL/25gm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>49 ± 1.39</td>
</tr>
<tr>
<td>Group 6 (BW) 750µL/25gm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52 ± 1.39</td>
</tr>
<tr>
<td>Group 7 (BW) 1000µL/25gm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51 ± 2.60</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation (n = 6). Each assay was run using triplicate samples. The mean values represent statistically significant differences (P ≤ 0.001) according to the one-way ANOVA analysis.
On test day, animals were fasted overnight and blood sample was drawn. Plasma total phenolic content of all groups is provided in Table 5.3. Wine dose of 500 µL/25 gm BW (Body Weight of mice) showed a significant increase in plasma phenolic concentration followed by 750 and 1000 µL/25 gm (BW). Whereas, dose 50, 100 & 250 µL/25 gm (BW) showed no change in plasma phenolic content. Based on these results wine dose of 500 µL/25 gm (BW) was used for further studies.

5.3.2 Protective effects of seabuckthorn wine (Functional beverage I) on phorone-induced oxidative stress in male LACA mice

Several reports have been cited to support the theory a light to moderate wine intake showed several health benefits (Section 1.5.2, Chapter 1). However, chronic or excessive alcohol consumption produces deleterious effects directly or indirectly by accelerating oxidative mechanisms via increased production of reactive oxygen species (ROS) and by impairing protective mechanisms against them [Mari et al., 2001]. Liver provides the primary site for alcohol metabolism, therefore the effects of alcohol are more pronounced in liver than in any other organ. The oxidative damage is further potentiated by an excessive alcohol-induced decrease particularly in the glutathione (GSH) level, superoxide dismutase (SOD) and redox state changes [Kaur et al., 2010]. Similar conditions of oxidative stress and decreased glutathione (GSH) may be induced chemically by phorone. Phorone is an α, β-unsaturated compound which, by combining with GSH through the action of glutathione-S-transferase, leads to a 70–90% depletion of hepatic intracellular GSH concentrations [Autrup et al., 1996] leading to hepatotoxicity [Gao et al., 2010].

For this study, animals were divided into following four groups: group 1-control, group 2-phorone treated, group 3-Cabernet Shiraz and phorone treated and group 4-seabuckthorn wine and phorone treated. None of the groups showed any change in body weight of mice during treatment period. We analyzed the redox status and degree of lipid peroxidation, activity of antioxidant enzymes in mice liver. The enzymatic antioxidants include superoxide dismutasc (SOD), glutathione peroxidasc (GPx),
glutathione reductase, glutathione-S-transferase (GST) and catalase. As shown in Table 5.4, phorone treated mice (Group 2) caused a definite increase ($P \leq 0.001$) in hepatic oxidized glutathione (GSSG) (38.20 nmoles/mg protein) as compared to control group (15.85 nmoles/mg protein). The phorone treatment also resulted in decreased levels of hepatic reduced glutathione (GSH) (12.03 nmoles/mg protein) in comparison to control mice (33.78 nmoles/mg protein). Oral administration of seabuckthorn wine reversed the above observed changes (GSH 26.21 nmoles/mg protein; GSSG 21.07 nmoles/mg protein). A significant decrease in redox ratio (GSH/GSSG 0.31) was observed in group 2. The hepatic GSH/GSSG ratio (1.24) increased after oral administration of seabuckthorn wine.

The phorone-induced oxidative stress (group 2) resulted in 64.3% decrease in hepatic GSH (reduced glutathione) concentration as compared to control group mice. To cope with oxidative stress, it caused a significant increase in hepatic GSSG (oxidized glutathione) levels by 141% compared with the control group. The total glutathione concentration was not affected by any of treatments. The redox ratio (GSH/GSSG) in mice liver decreased by 85.4% in phorone treated mice (group 2) as compared to control mice (group 1).
Table 5.4. Effect of Seabuckthorn wine and Cabernet Shiraz administration on glutathione levels in phorone-treated mice liver

<table>
<thead>
<tr>
<th></th>
<th>Group 1 control</th>
<th>Group 2 Phorone treated</th>
<th>Group 3 Phorone + Cabernet Shiraz treated</th>
<th>Group 4 Phorone + seabuckthorn wine treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Glutathione</td>
<td>49.63 ± 1.65</td>
<td>50.23 ± 2.25</td>
<td>47.37 ± 2.06</td>
<td>47.28 ± 1.99</td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td>33.78 ± 1.25</td>
<td>12.03 ± 0.75a</td>
<td>22.48 ± 1.17ab</td>
<td>26.21 ± 1.05ab</td>
</tr>
<tr>
<td>Oxidized Glutathione (GSSG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td>15.85 ± 0.97</td>
<td>38.20 ± 1.87a</td>
<td>24.89 ± 1.09ab</td>
<td>21.07 ± 1.01b</td>
</tr>
<tr>
<td>Redox ratio (GSH/GSSG)</td>
<td>2.13</td>
<td>0.31</td>
<td>0.90</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation (n = 6). Each assay was run using triplicate samples. The mean values represent statistically significant differences (P ≤ 0.001) according to the post hoc comparisons (Tukey’s test) of the one-way ANOVA analysis.

a significantly different (P ≤ 0.001) than group 1; b significantly different (P ≤ 0.001) than group 2

Mechanism that contributes to the phorone-induced oxidative stress includes depletion of the hepatic GSH concentration [Gao et al., 2010]. The administration of seabuckthorn wine along with phorone treatment (group 4) increased hepatic GSH by
118% and decreased GSSG by 45% as compared to phorone treatment (group 2). One of the most significant results of the study is that the seabuckthorn wine administration restored the glutathione levels and the redox ratio.

Phorone treatment (group 2) decreased the activity of hepatic SOD (0.33 Units/mg protein) compared with the control group (1.95 Units/mg protein), (Table 5.5). SOD is one of the potent antioxidant enzymes [Chen et al., 2005] and mice liver contains two types of superoxide dismutases (Cu-Zn or Mn-containing dismutase) [William et al., 1998], which catalyzes the conversion of superoxide anions into oxygen and hydrogen peroxide in cell cytosol and mitochondria to protect the cell from superoxide radical damage [Salin and McCord, 1974]. Decreased SOD activity may be responsible for increased levels of toxic oxygen radicals in mice liver homogenate and may contribute to the progression of liver injury [Cederbaum, 2001]. Under these conditions when there is excessive production of superoxide anions and hydrogen peroxide or loss of antioxidant enzyme activities, these reactive oxygen species can interact with transition metals and produce highly reactive hydroxyl radicals [Caro and Cederbaum, 2004]. Under these intracellular condition when seabuckthorn wine was administered along with phorone (group 4), SOD activity was enhanced from 0.33 to 1.59 Units/mg protein (Table 5.5). Therefore, oral administration of seabuckthorn wine along with phorone treatment restored the hepatic SOD activity in phorone treated mice (group 2) as that of control level.

Phorone administration (group 2) also caused a significant increase (P ≤ 0.001) in hepatic MDA (malondialdehyde) levels (0.32 µmoles MDA/mg protein) when compared to control group (0.20 µmoles MDA/mg protein) indicating an increase of 60%. Interestingly, administration of seabuckthorn wine (group 4) significantly decreased (P ≤ 0.001) and reverted the MDA levels (0.22 µmoles MDA/mg protein) to that of control values (0.20 µmoles MDA/mg protein). Thus, seabuckthorn wine administration resulted in a protection against lipid peroxidation.
Table 5.5. Effect of Seabuckthorn wine and Cabernet Shiraz administration on biochemical parameters in phorone-treated mice liver

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1 Control</th>
<th>Group 2 Phorone treated</th>
<th>Group 3 Phorone + Cabernet Shiraz treated</th>
<th>Group 4 Phorone + seabuckthorn wine treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (Units/mg protein)</td>
<td>1.95 ± 0.03</td>
<td>0.33 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.31 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.59 ± 0.06&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipid peroxidation (μmoles MDA/mg protein)</td>
<td>0.20 ± 0.003</td>
<td>0.32 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase (μmoles of H₂O₂ decomposed/min/mg protein)</td>
<td>49.33 ± 1.02</td>
<td>57.12 ± 0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.25 ± 1.92</td>
<td>49.09 ± 0.76&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione reductase (μmoles NADPH oxidized/min/mg protein)</td>
<td>20.22 ± 1.39</td>
<td>28.97 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.89 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.96 ± 0.94&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione peroxidase (Gpx) (μmoles NADPH oxidized/min/mg protein)</td>
<td>38.09 ± 1.70</td>
<td>53.57 ± 2.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.6 ± 1.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.01 ± 1.15&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione-S-Transferase (μmoles DNB conjugated/min/mg protein)</td>
<td>42.60 ± 0.96</td>
<td>60.16 ± 1.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.82 ± 1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.30 ± 0.87&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation (n = 6). Each assay was run using triplicate samples. The mean values represent statistically significant differences (P ≤ 0.001) according to the post hoc comparisons (Tukey’s test) of the one-way ANOVA analysis.

<sup>a</sup> significantly different (P ≤ 0.001) than group 1
<sup>b</sup> significantly different (P ≤ 0.001) than group 2
<sup>c</sup> significantly different (P ≤ 0.001) than group 3
Among the enzymes important for cellular antioxidant defense, catalase forms an integral component of the defense mechanism [Caro and Cederbaum, 2004]. GST is a detoxifying enzyme induced by a variety of electrophilic drugs and toxins. It is mainly involved in the free radical scavenging, peroxide reduction and detoxification of GSH-S-Conjugates [Sies, 1988; Dolphin et al., 1989]. The GPx is capable of reducing organic and inorganic hydroperoxides by converting reduced glutathione into oxidized glutathione. Glutathione reductase recycles oxidized glutathione (GSSG) by converting it to the reduced form (GSH) using NADPH as the electron donor [Kaneko et al., 2001]. In the present study, phorone-induced ROS generation resulted in increased hepatic catalase, glutathione reductase, GPx and GST activities in group 2, which in turn caused a marked reduction in the hepatic SOD activity and GSH levels. Thus, increased activities of catalase, glutathione reductase, GPx and GST as observed in this study may be explained as an adaptive response against ROS over production. Administration of seabuckthorn wine with phorone (group 4) resulted in restoration of the catalase and glutathione reductase enzyme activities (Table 5.5) compared to the control level (group 1). Thus, it may infer that the adverse effects of phorone were significantly curtailed by seabuckthorn wine administration.

To confirm the above results the reactive oxygen species (ROS) levels in liver homogenates were estimated using a fluorescence probe Dichlorofluorescein diacetate (DCFH-DA). Oxidation of DCFH-DA to DCF was measured as an index of total ROS. As shown in Figure 5.1, there was a significant increase ($P \leq 0.001$) in the hepatic ROS levels by 159% as shown by increased fluorescence intensity in phorone treated mice (group 2). Interestingly, oral administration of seabuckthorn wine diminished the phorone induced ROS generation in mice liver by 51%.

Overall results of the present study confirmed that the adverse effect of phorone could be significantly curtailed by administering seabuckthorn wine to the mice. The decreased hepatic MDA levels, restoration of catalase and glutathione reductase activities, an enhanced hepatic redox ratio and increased SOD activity are all
indicative of the protective effect of seabuckthorn wine on phorone-induced oxidative stress. These results are comparable with in vivo study conducted earlier which reported that the fruit extract of seabuckthorn restored the MDA level in rat liver in the nicotine-induced oxidative stress in 21 days; whereas GST activity was not affected [Taysi et al., 2010]. Another study conducted by Geetha et al. [2003] reported that the leaf extract of seabuckthorn protected the animals from the chromium-induced oxidative injury but only at high concentration of 100 and 250 mg/Kg body weight in 30 days.

Figure 5.1 Effect of Seabuckthorn wine and Cabernet Shiraz administration on reactive oxygen species (ROS) levels in phorone treated mice.

Values are means ± standard deviation (n = 6). Each assay was run using triplicate samples. The mean values represent statistically significant differences (P ≤ 0.001) according to the post hoc comparisons (Tukey’s test) of the one-way ANOVA analysis. *significantly different (P ≤ 0.001) than group 1; †significantly different (P ≤ 0.001) than group 2; Group 1, Control (untreated); Group 2, Phorone treated; Group 3, Cabernet Shiraz and Phorone treated; Group 4, Seabuckthorn wine and Phorone treated.
As it has been mentioned earlier the seabuckthorn wine had high proportion of rutin, myricetin and quercetin which is not very common in red wines. In addition to flavonoids, the berries are also rich in carotenoids, unsaturated fatty acids, Vitamins, C, and E [Zeb, 2004]. Thus, seabuckthorn berries have a unique composition, combining a cocktail of components usually only found separately. These antioxidants are involved in various signaling pathways of metabolism either directly or indirectly to alter the expression of genes [Kaput and Rodriguez, 2004].

It may be inferred that the observed protective action against oxidative stress may be due to the synergistic effects of bioactive compounds present in the seabuckthorn wine which also contributes to the uniqueness of the product.

From the in vivo studies, it is indicated that seabuckthorn wine has performed better with respect to GSH, catalase, Gpx & GST as compared to Cabernet Shiraz. It is interesting to observe similar in vivo protective effects both in the case of seabuckthorn wine and Cabernet Shiraz inspite of the fact that resveratrol (which is one of the most important health improving factor of red wine) is absent in seabuckthorn wine.

5.3.3 Protective effects of seabuckthorn wine (Functional beverage I) on diet induced hypercholesterolemia in serum of male LACA mice

A significant degree of cardioprotection has been attributed to ingestion of wines rich in flavonoids. Although a few studies have reported the effects of seabuckthorn berry fractions on cardiovascular risk factors [Eccleston et al., 2002; Basu et al., 2007], this is the first study on the impact of seabuckthorn wine on cardiovascular risk factors.

For this study animals were divided into following four groups: group 1 animals served as control, group 2 animals were fed on high-cholesterol diet (HCD), group 3 animals were fed on HCD with Cabernet Shiraz (Sula Vineyards) and group 4 animals were fed on HCD with seabuckthorn wine. The final body weight of mice in group 2 (35.17 g) were significantly higher (P ≤ 0.001) than that of the group 1 (27.14 g), group 3 (27.91 g) and group 4 (27.78 g). The oral administration of Cabernet Shiraz and
seabuckthorn wine along with high-cholesterol diet did not lead to any change in body weights of mice.

To evaluate the extent of hypercholesterolemia, lipid profile was analyzed in all the four groups (Figure 5.2). Mice were fasted overnight (12h) before blood collection. Administration of high-cholesterol diet (HCD) resulted in increase in total cholesterol, triglycerides, HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) significantly ($P \leq 0.001$) in group 2 as compared to group 1 (control). On administration of seabuckthorn wine, total cholesterol content decreased significantly ($P \leq 0.001$) in group 4 as compared to group 2. Also, the LDL-cholesterol showed a significant decrease ($P \leq 0.001$) to 15.16 mg/dL in group 4 from 44.18 mg/dL in group 2. On the other hand, the triglycerides and HDL-cholesterol did not show any remarkable change. The ratio of HDL-cholesterol to LDL-cholesterol depicted a noteworthy decrease in group 2 (HDL-C/LDL-C: 1.50) as compared to group 1 (HDL-C/LDL-C: 3.82), while on the oral administration of seabuckthorn wine along with HCD (group 4), this ratio was 4.46.
Figure 5.2  Effect of Seabuckthorn wine and Cabernet Shiraz on diet induced hypercholesterolemia in blood serum

Group 1, Control mice; Group 2, High-cholesterol diet treated mice; Group 3, High-cholesterol diet treated mice along with oral administration of Cabernet Shiraz (Sula Vineyards); Group 4, High-cholesterol diet treated mice along with oral administration of seabuckthorn wine.

Values are means ± standard deviation (n = 6). Each assay was run using replicates samples. The mean values represent statistically significant differences (P ≤ 0.001) according to the post hoc comparisons (Tukey’s test) of the one-way ANOVA analysis. \(^a\)significantly different (P ≤ 0.001) than group 1; \(^b\)significantly different (P ≤ 0.001) than group 2;

HDL-C/LDL-C ratio: Group 1 (3.82); Group 2 (1.50); Group 3 (4.44); Group 4 (4.46).
The triglycerides in serum, increased by 24% in the HCD group as compared to the control group. Supplementation of Cabernet Shiraz or seabuckthorn wine did not lower the serum triglyceride levels significantly. However, a more interesting observation was the decrease of serum total cholesterol by 21% as a result of seabuckthorn wine ingestion, and 18% as a result of Cabernet Shiraz consumption, as compared to the HCD treated group. A reduction of 66% of LDL-cholesterol was observed in group 4 (HCD + seabuckthorn wine) in comparison to high-cholesterol diet treated mice. The HDL-C/LDL-C ratio increased significantly by 197% in the mice fed with seabuckthorn wine in comparison to HCD group. It may be concluded that the increase in HDL/LDL-cholesterol ratio is more significantly due to the decrease in LDL-cholesterol concentration. Considering that high LDL-cholesterol levels is correlated to high atherogenic process [Ho et al., 2003], the effect of seabuckthorn wine on reduction of LDL-cholesterol levels is an important finding of this study. Since seabuckthorn wine reduced the LDL-cholesterol levels, the dietary interventions with this wine are likely to prove beneficial.

The various in vivo studies reported in past by other workers on the effects of different non-fermented seabuckthorn products on the lipid profile are not conclusive. These results obtained in this study is similar to that of a recent report where the administration of seabuckthorn tea showed a reduction in the plasma total cholesterol in high-fat diet-induced obese mice in 42 days, but did not affect the plasma HDL-cholesterol concentration [Lee et al., 2011b]. On the other hand, I armo et al. [2009] did not detect any changes in serum total, LDL and HDL-cholesterol in healthy adults after consumption of seabuckthorn berries.

To the best of our knowledge the impact of seabuckthorn wine on lipids and lipoproteins in diet-induced hypercholesterolemic has not been reported so far. The studies clearly established that the seabuckthorn wine exerts positive effects by reducing the total cholesterol and LDL-cholesterol in hyperlipidemic conditions.
5.4 CONCLUSIONS

The present study provided important evidence into the potential protective effects of seabuckthorn wine against oxidative stress. The seabuckthorn wine performed better with respect to GSH, catalase, Gpx & GST as compared to Cabernet Shiraz. This study also provided important evidence that seabuckthorn wine exerts protective effects against hypercholesterolemia. In addition, high-cholesterol-fed mice administered with seabuckthorn wine exhibited a 197% increase in the HDL-C/LDL-C ratio compared to high cholesterol diet treated mice. Since the administration of seabuckthorn wine positively, influenced the biomarkers of oxidative stress and hypercholesterolemia, it can be concluded that inclusion of seabuckthorn wine in a diet may be an effective way of reducing the total cholesterol and LDL cholesterol under hyperlipidemic conditions.