CHAPTER 6

In vitro hepatoprotective activities of B. oleracea var. gongylodes fruit pulp, L. siceraria leaf and V. faba fruit in isolated primary rat hepatocytes
Liver diseases are a worldwide problem. The management of liver diseases has become a critical concern in medical science. Drugs available in the present system of medicine are associated with toxic effects and injure the organ (Sallie, 1999), when taken in overdoses. However, there are not any satisfactory liver protective drugs in allopathic medical practice for serious liver disorders. Whereas, herbal drugs play a role in the management of various liver disorders most of which speed up the natural healing processes of the liver. Numerous medicinal plants and their formulations are used for liver disorders in ethno-medical practice as well as traditional system of medicine in India. More than fifteen of these plants are evaluated for their hepatoprotective action in the light of modern medicine (Subramoniam et al., 1998). Clinical trials have shown that silymarin exerts hepatoprotective effects in acute viral hepatitis and poisoning by ethanol, paracetamol and carbon tetrachloride (Fraschini et al., 2002; Kumar et al., 2010). In the past, several herbal compounds have also been screened to test their ability to reduce and/or nullify acetaminophen induced hepatotoxicity (Sharma et al., 2008).

The present study has been undertaken to investigate hepatoprotective activity and antioxidant role of the hydro-ethanolic extract (1:1) of B. oleracea var. gongylodes pulp extract (BOGPE), L. siceraria leaf extract (LSLE) and V. faba fruit extract (VFFE) on paracetamol induced cytotoxicity in rat liver.

6.1 Materials and Methods

6.1.1 Reagents: 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was procured from Calbiochem (USA). Rest of the chemicals used throughout the study was commercial products of the highest purity grade and purchased from
Sigma Chemicals Co. (St. Louis, MO, USA). Solutions were prepared in de-ionized ultra-pure water (Direct Q5, Millipore, Bangalore, India).

6.1.2 Collection of plant materials: As described in chapter 3.

6.1.3 Extraction and yield: As described in chapter 4.

6.1.4 Isolation of hepatocytes: Rat liver perfusion was performed according to the method of Seglen (1976). 400 ml Hepes buffer contains 130 μM NaCl, 3 mM KCl, 0.8 mM NaH₂PO₄ and 10 mM Glucose, 10 mM Hepes (pH 7.4; 37°C). After anaesthetization, 200 ml pre-wash solution (Hepes buffer + 0.5 mM EDTA) was perfused through portal vein for 15 min, followed by 100 ml collagenase solution (Hepes buffer + 5 mM CaCl₂ + 30 mg collagenase (Type IV) for 10 min. This was followed by washing with 100 ml post wash solution (Hepes buffer + 5 mM CaCl₂). The liver was mechanically disintegrated and filtered through a 100 mesh filter and resuspended in RPMI medium with 10% FBS, which was maintained during the experiments. The viability of hepatocytes was at least 95%. Cell viability was confirmed by trypan blue dye exclusion test within 1 h of cell isolation.

6.1.5 Primary cell culture: Hepatocytes were maintained in RPMI-1640 media supplemented with heat inactivated 10% fetal bovine serum and 1% of 10,000 units Penicillin, 10 mg Streptomycin, 25 μg Amphotericin B, 1 mM sodium pyruvate, 2 mM glutamine under an atmosphere of 5% CO₂, 95% air in an incubator (Thermoforma) with controlled humidity at 37°C. The cells were seeded at a density of 1.0X10⁴ cells/well (counted on hemocytometer) in 0.1% collagen pre-coated 96 well plate and used for drug exposure experiments after being cultured for 24 h. The viability of cells was checked by MTT reduction test.
6.1.6 Treatment of cells: Acetaminophen (APAP) was dissolved in 1% DMSO, filtered through 0.22 μM filter and used for subsequent treatment as described in linear diagram given below. The cells were pre-incubated with extracts as well as with standard (silymarin, 5μg/10⁴ cells). Cells were exposed to extract (45 min) prior to treatment with APAP (30 min). Hepatocytes were also treated (control vehicle) with 0.1% dimethyl sulphoxide (DMSO) but no changes were observed (data not shown). For each set of experiment silymarin (5μg/10⁴ cells) was used as control.

Pre treatment Schedule
Start  45 min (Extract)  30 min (675 μM acetaminophen)  End
Medium Change

6.1.7 Quantitative analysis of viable cells: Cell viability was determined by a colorimetric MTT assay as described by Mosmann (1983). MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase, which demonstrates functional mitochondrial dehydrogenase, i.e. functional mitochondria. Twenty four hours old hepatocytes were exposed to various concentrations of acetaminophen and extracts. At the end of the incubation period, the culture medium was removed and MTT (10 μl from a stock of 5 mg/ml) was added to each well. After 4 h incubation the medium was removed and in each well, 0.2 ml DMSO was added. Optical density (OD) was measured at 530 nm using a Spectramax PLUS 384 microplate reader (Soft max proversion 5.1; Molecular Devices, USA). The linear relationship between OD and cell density was taken into
account. The data are expressed as a percentage of viability measurement with reference to control (untreated cells).

6.1.8 SOD activity: SOD activity was determined spectrophotometrically by measuring inhibition of nicotinamide adenine dinucleotide (reduced)–phenazine methosulfate–nitroblue tetrazolium reaction system by the method of Kakkar et al. (1984; as described in chapter 4), after adoption on microplate. Superoxide radical is produced in situ, which is involved in the NBT reduction leading to the formation of blue formazan, which is read at 560 nm. Fifty percent inhibition of formazan formation in 1 min is taken as 1 unit activity/min.

6.1.9 MDA determination: Thiobarbituric acid reactive substance (TBARS) formation as a product of lipid peroxidation was estimated in pre-treated hepatocytes by using the method of Wallin et al. (1993). In this method, oxidation of phospholipids and evaluation of TBARS is achieved in single 96-well microplate. Major oxidative product of phospholipids, i.e. malondialdehyde (MDA) was estimated by measuring the amount of MDA formed as a breakdown product at 530 nm. The lipids were isolated by precipitating the cell lysate with TCA and then directly the lipid peroxide concentration was measured with TBA reaction. The amount of MDA formed as a breakdown product was measured at 530 and 600 nm.

6.1.10 Determination of nitric oxide (NO\(^-\)): Nitric oxide radical formation was measured in all treated and control hepatocytes by assaying nitrite, one of the stable end products of NO oxidation. In hepatocytes nitrite concentration was measured spectrophotometrically using Griess reagent [1% sulfanilamide in 5% phosphoric acid (sulfanilamide solution) and 0.1% N-1-naphthylethylenediamine dihydrochloride in distilled water (NED solution)] as described by Feelisch et al. (1987).
6.1.11 Statistical analysis: Data are expressed as mean ± SD of three replicates. Data were analysed on PRISM software version 3.0 using student’s t-test and one way ANOVA followed by the Tukey-Kramer multiple comparisons test. A p value less than 0.05 was considered as statistically significant.* p≤0.05; ** p≤0.01 and *** p≤0.001 were used as the criterion for significance.

6.2 Results

6.2.1 Extracts increased survival of acetaminophen stressed primary hepatocytes: Acetaminophen (Paracetamol) is a commonly and widely used analgesic and antipyretic agent. Hepatotoxic doses of acetaminophen deplete the normal levels of hepatic glutathione, when NAPQI covalently binds to cysteine groups on proteins to form 3-(cystein-S-yl) acetaminophen adducts (Tirmenstein et al., 1990). It was observed that acetaminophen (APAP) at 650 μM reduced the cell survival to 51.90% ± 1.22, i.e. the inhibitory concentration (IC_{50}) of APAP was found to be 675 μM (Fig. 6.1). This concentration of APAP was used for further experiments.

![Graph](image)

**Fig. 6.1** Concentration dependent effect of APAP toxicity on cell survival rate.

Cell viability was assayed using MTT and measurement of the solubilized formazan dye at Abs 530 nm. Isolated primary rat hepatocytes were treated with
varying concentration of all three extracts (1-10 μg/ml/10^4 cells) as shown in Fig. 6.2 (A, B and C) to study its effect on cell viability. All the tested concentrations of extracts were found to be non-toxic since no cytotoxicity was observed on treatment of hepatocytes with any of the individual concentrations of extracts with respect to control (Fig. 6.2 A, B and C). Hence, in order to ascertain their cyto-protective effects against APAP induced hepto-cellular toxicity, 2.5, 5.0 and 7.5 μg/10^4 cells concentrations of each of the extracts were selected for further experiments. Same concentrations (2.5, 5.0 and 7.5 μg/10^4 cells) of silymarin (Sil) were used as a positive control. The treatment of silymarin at 5.0 μg/10^4 cells concentration increased the cell survival rate of hepatocytes significantly (p≤0.001) up to 44.45%±4.33. During pretreatment schedule, 24 h cultivated hepatocytes were incubated with selected concentrations of extracts for 45 min before subjecting them to oxidative stress of APAP for 30 min. A positive correlation between dose-response in terms of viability was seen. The cells which were pre-incubated with extracts showed significant increase in cell survival rate up to 42.58%±2.83 (p≤0.001), in comparison to APAP treated cells. The VFFE showed significantly higher survival rate 42.58%±2.83 (p≤0.001) in comparison to LSLE (19.07%±2.28, p≤0.001) and BOGPE (30.97%±2.08, p≤0.001) (Fig. 6.3 A, B and C).

6.2.2 Restoration of antioxidant status in stressed rat hepatocytes by extracts treatment

6.2.2.1 SOD activity: Cultured hepatocytes subjected to APAP stress showed SOD activity 4.80±0.45 units/min/10^4 cells which were 2.3 fold lower than the untreated cells (Table 6.1). Cells that were treated with extracts showed 1.3 to 2.1 fold increase, whereas the cells treated with silymarin (5.0 μg/10^4 cells) showed 2.6 fold increases in superoxide dismutase activity 12.82±0.52 Units/min/10^4 cells (p≤0.001) with
Fig. 6.2 Survival rate of primary hepatocytes at different concentrations of BOGPE (A), LSLE (B) and VFFE (C). Data shown are mean ± SD evaluated from three different sets of experiments. Significant difference compared with control values, *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001.
Fig. 6.3 Survival rate of acetaminophen stressed primary hepatocytes at selected concentrations of silymarin and extracts (A) BOGPE, (B) LSLE and (C) VFFE, respectively. Data shown are mean ± SD evaluated from three different sets of experiments. APAP was compared with control and treatments were compared with APAP. Results with *p≤0.05, **p≤ 0.01 and ***p≤0.001 were considered significant.
Table 6.1 Dose dependent antioxidant activity in cells treated with extract and Acetaminophen

<table>
<thead>
<tr>
<th>Pre-treatments</th>
<th>Concentration (µg/10⁶ cells)</th>
<th>SOD activity (Units/min/10⁴ cells)</th>
<th>LPO (nM MDA formation/10⁴ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>11.31±0.53</td>
<td>0.38±0.017</td>
</tr>
<tr>
<td>APAP</td>
<td>675 µM</td>
<td>4.80±0.45***</td>
<td>0.63±0.015***</td>
</tr>
<tr>
<td><em>B. oleracea var. gongylodes</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(FP)</td>
<td>2.5</td>
<td>6.32±0.38*</td>
<td>0.54±0.014***</td>
</tr>
<tr>
<td><em>B. oleracea var. gongylodes</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(FP)</td>
<td>7.5</td>
<td>8.82±0.66***</td>
<td>0.44±0.012***</td>
</tr>
<tr>
<td><em>L. siceraria</em> (L)</td>
<td></td>
<td>6.81±0.30**</td>
<td>0.57±0.024**</td>
</tr>
<tr>
<td><em>L. siceraria</em> (L)</td>
<td></td>
<td>8.93±0.24***</td>
<td>0.48±0.018***</td>
</tr>
<tr>
<td><em>V. faba</em> (F)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>8.81±0.65***</td>
<td>0.43±0.018***</td>
</tr>
<tr>
<td><em>V. faba</em> (F)</td>
<td></td>
<td>10.06±0.24***</td>
<td>0.31±0.027***</td>
</tr>
<tr>
<td>Silymarin</td>
<td>5.0</td>
<td>12.82±0.52***</td>
<td>0.24±0.014***</td>
</tr>
</tbody>
</table>

SOD activity and LPO were quantified by spectrophotometric. Pretreatment of extracts was found to be effective over APAP induced oxidative injury. Values represent the mean of ± SD of three independent experiments. APAP was compared to control while extracts and silymarin were compared to APAP. Results with *p≤ 0.05, **p≤ 0.01 and ***p≤0.001 were considered significant.

respect to APAP treated hepatocytes. The cells pre-treated with VFFE (7.5 µg/10⁴ cells) showed 2.1 fold increase SOD activity 10.06±0.24 Units/min/10⁴ cells (p≤0.001) with respect to APAP treated hepatocytes and indicate that VFFE is comparable to silymarin and act as a strong antioxidant.

6.2.2.2 MDA determination: Oxidative stress induced in the hepatocytes by free radical generation due to APAP caused 0.63±0.015 nM MDA formation /10⁴ cells. In hepatocytes pre-treated with extracts, the peroxidative decomposition of the lipids was reduced from 0.57±0.024 to 0.31±0.027 nM MDA formation /10⁴ cells with respect to APAP treated cells (Table 6.1).
The results were dose dependent as MDA revel decreased with BOGPE, LSLE and VFFE (0.54±0.014; 0.57±0.024 and 0.43±0.018 nM MDA formation /10^4 cells, respectively) for 2.5/10^4 cells and (0.44±0.012; 0.48±0.018 & 0.31±0.027 nM MDA formation /10^4 cells, respectively) for 7.5 μg/10^4 cells concentration. The results showed by VFFE were highly significant (p≤0.001) and compared well with standard antioxidant silymarin (Table 6.1).

6.2.2.3 NO⁻ quenching activity: Generation of NO⁻ in APAP stressed cells was 25.74% more in comparison to untreated cells. The treatment of extracts showed dose dependent quenching of NO⁻ which ranged between 18.51%±1.67 to 53.61%±4.67 (Fig. 6.4). The cells pre-treated with silymarin (5 μg/10^4 cells) showed 39.55%±2.06 quenching of NO⁻. Maximum quenching was shown by BOGPE extract (53.61%±4.67 for 7.5 μg/10^4 cells) which was significant (p≤0.001) increase in comparison to APAP treated cells.

![Graph showing inhibition of NO⁻ production](image)

**Fig. 6.4** Effect of extracts on APAP induced NO⁻ production in primary rat hepatocytes. NO⁻ generated by APAP was taken as 100% from which % inhibition was calculated for each extract. APAP was compared by control. Data shown are mean ± SD evaluated from three different sets of experiments. Results with ***p≤0.001 were considered significant.
6.3 Discussion

Acetaminophen (Paracetamol) is a widely used antipyretic, analgesic drug which produces acute hepatic damage on accidental over dosage. It is established that, a fraction of acetaminophen is converted via the cytochrome P450 pathway to a highly toxic metabolite N-acetyl-p-benzo quinamine (NAPQI) that damage liver cells (Dahlin et al., 1984; Jos et al., 2001). This metabolite is normally conjugated with glutathione and excreted in urine (Parmar and Kandakar, 1995). Several Cyt P450 enzymes are known to play an important role in APAP bioactivation to NAPQI. Cyt P450 2E1 have been suggested to be primary enzymes for acetaminophen bioactivation in liver microsomes (Mitchell et al., 1973; Raucy et al., 1989).

In the present study, the results indicated that all the three extracts showed dose dependent antioxidant activity in pre-treated primary hepatocytes. Among the three extracts the *V. faba* fruit extract showed more effective protection at the concentrations of 2.5 μg/10^4 cells and 5.0 μg/10^4 cells which were well comparable with known hepatoprotective antioxidant silymarin (5 μg). Administration of APAP decreased the hepatic SOD activity compared to normal cells; whereas the administration of all three extracts significantly increased the hepatic SOD activity as compared to acetaminophen (APAP) damaged primary hepatocytes of rat. This indicates that the extract can reduce reactive free radicals that might diminish oxidative damage to the tissues and improve the activities of the hepatic antioxidant enzyme.

Lipid peroxidation has been postulated as a major destructive process of liver injury due to acetaminophen administration. The activated free radical binds covalently to the macromolecules and induces peroxidative degradation of membrane
lipids of endoplasmic reticulum which are rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides which in turn generate products such as malondialdehyde (MDA) that cause damage to the membranes. The increased MDA contents in primary hepatocytes of rat treated with hepatotoxins only suggest that the natural antioxidant defence mechanism to scavenge excessive free radicals has been compromised (Iniaghe et al., 2008). In the present study, treatment of extracts and silymarin showed significant effect in the reduction levels of end products of lipid peroxidation in the rat primary hepatocytes treated with paracetamol. This similar trend was observed in earlier reports (Muriel and Garciplana, 1992; Tseng et al., 1997). Our findings were also supported by the previous reports on antioxidant effects of Vismang, M. indica L. aqueous extract on in vitro and in vivo models (Rodeiro et al., 2007).

Nitric oxide (NO) is lipophilic and greatly diffusible solute that forms within the cell. It is important in the regulation of many physiological processes. Its functions are dependent upon concentration. It is involved in the mediation of neuronal signaling, hepatic metabolism, blood pressure, vasodilation, relaxation of smooth muscles and prevention of platelet aggregation. Excess production of nitric oxide leads to a number of diseases for instance neurodegenerative and muscle diseases (Ebrahimzadeh et al., 2010; Aslam et al., 2012).

Nitric oxide is formed from L-arginine by NO synthase (Packer, 2002). Free radicals liberated from phagocyte cells are important in inflammatory processes because they are implicated in the activation of nuclear factor κB (NF-κB), which induces the transcription of inflammatory cytokines and COX-2. Furthermore, antioxidants have been shown to be able to effectively block the activation of NF-κB through the stabilization of NF-κB/IκB-a complex (Halliwell, 1996; Huang et al.,
2001) and prevent NO production. In the present study, administration of extract reduced the production of NO which is in agreement with earlier report of Bor et al., (2006) which showed the scavenging effects of vegetable extracts on NO.

A number of plants have been shown to possess hepatoprotective properties by improving the antioxidant status (Kongcharoensuntorn et al., 2007; Ita et al., 2009; Malar and Bai, 2009; Patil and Ageely, 2011). Chirata is one of the oldest medicinal herbs of Indian Ayurvedic medicine used for the treatment of liver disorders, screened for their anti-hepatotoxic activity and was found to be a good protector against against carbon tetrachloride (CCl₄) and paracetamol (acetaminophen) toxicity in primary monolayer cultures of rat hepatocytes (Singh and Reen, 1999; Reen et al., 2001). These results indicate that plants posses their own antioxidant activity which suppresses the damage induced by free radicals. Thus, the herbal medicine with their natural antioxidants witnessed a credible role in health, food and cosmetic industry.

Our study indicated that hepatocytes treated with APAP showed reduced cell viability, increased LPO, NO and decreased SOD values. However, supplementation of BOGPE, LSLE and VFFE conferred significant protection against APAP induced injury to primarily cultured rat hepatocytes which was comparable to standard hepatoprotectant silymarin. Based on the above results, it could be concluded that all three extracts (BOGPE, LSLE and VFFE) have significant hepatoprotective (as an antioxidant) and DNA damage protective activities. In view of the above studies, VFFE, BOGPE and LSLE plant may be used as source of natural antioxidants.