5. Methods

5.1 In vitro studies

5.1.1 In vitro antioxidant activity

5.1.1.A) ABTS (2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Method: (Sithisarn et al., 2005)

Principle

The pre-formed radical monocation of 2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) is generated by oxidation of ABTS with potassium persulfate (a blue chromogen) and is reduced in the presence of such hydrogen donating antioxidants.

Chemicals and Reagents Used

Preparation of ABTS solution

Solution I: ABTS (2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (2 mM solution is prepared using distilled water).

Solution II: Potassium persulfate (17 mM solution is prepared using distilled water)

0.3 mL of solution II was added to 50 mL of solution. The reaction mixture was left to stand at room temperature overnight in dark before use.

Preparation of Test Solution

10 mg of each of the drug samples and the standard (ascorbic acid) were accurately weighed separately and dissolved in 1 mL of DMSO. These solutions were serially diluted with DMSO to obtain the lower dilutions.
Method

1 mL of distilled DMSO was added to 0.2 mL of various concentrations of the drug samples or standard, and 0.16 mL of ABTS solution was added to make a final volume of 1.36 mL. Absorbance was measured spectrophotometrically, after 20 min at 734 nm using ELISA reader. Blank was maintained without ABTS. IC\textsubscript{50} value obtained is the concentration of the sample required to inhibit 50 % ABTS radical mono cation.

5.1.1.B) Scavenging of Superoxide radical by Alkaline DMSO Method: (Rao and Kunchandy., 1990)

Principle

Superoxide is generated according to the alkaline DMSO method. The reduction of Nitro Blue Tetrazolium (NBT) by superoxide was determined in the presence and absence of the extracts.

Chemicals and Reagents used

NBT: 10 mg of NBT in 10 mL of Distilled Water.

Alkaline DMSO: 20 mg of Sodium Hydroxide pellet is dissolved in 1 mL DMSO and then final volume is made up with 99 mL of DMSO. This solution should be prepared before starting an experiment.

Preparation of Test and Standard solutions

10 mg of the drug samples and the standard (ascorbic acid) were weighed accurately and separately dissolved in 1 mL of DMSO. These solutions were serially diluted with DMSO to obtain the lower dilutions.

Method

To the reaction mixture containing 1 mL of alkaline DMSO, 0.3 mL of the drug samples and standard was added in DMSO at various concentrations followed by 0.1 mL of NBT (0.1 mg) to give a final volume of 1.4 mL. The absorbance was measured at 560 nm.
5.1.2 In vitro cytotoxicity studies for selected drug samples

5.1.2.A) Method for passaging the cells (Freshney., 2000a)

All the reagents were brought to 37°C before use.

a. Sufficient amount of TPVG solution was added to cover the monolayer, rinsed and discarded.

b. Fresh TPVG solution was added and allowed to stand at room temperature for 2-3 minutes.

c. TPVG solution was discarded and the flask containing the monolayer was incubated at 37°C for 3-5 minutes and slightly tapped to free the cells from the surface.

d. 10ml of Minimum Essential Medium (MEM) containing 10% serum was added to the flask and pipetted to breakdown the clumps of cells.

e. Total cell count was taken using a haemocytometer.

f. The medium was added according to the cell population needed. Required amount of medium containing the required number of cells (0.5-1.0x10^5 cells/ml) was transferred into bottles according to the cell count and the volume was made up with medium and required amount of serum (10% growth medium and 2% maintenance medium) was added.

g. The flasks were incubated at 37°C and the cells were periodically checked for any morphological changes and contamination. After the formation of monolayer, the cells were further utilized.

5.1.2.B) Determination of Mitochondrial Synthesis by Microculture Tetrazolium (MTT) Assay: (Eisenbrand et al., 2002)

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell.
The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based.

The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2 yl) - 2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

**Preparation of Test solutions**

10 mg of the drug samples were weighed accurately and separately dissolved in 1 mL of DMSO and made up the volume to 10 ml with maintenance medium. These solutions were serially diluted with maintenance medium to obtain the lower dilutions.

**Requirements**

1. Confluent monolayer of chang liver cells
2. TPVG Solution
3. Dulbecco’s Modified Eagle’s Medium (DMEM) with antibiotics
4. New born calf serum / sheep serum
5. Eppendorf tubes
6. Microtitre plate (96 well)
7. Drug dilutions
8. MTT (prepared in Hank’s Balanced Salt Solution (HBSS) without phenol red, 2mg/mL) (Sigma Chemicals)
9. Isopropanol
10. Microplate reader (ELISA Reader, Bio-Tek)
11. Inverted Microscope (Olympus)

**Procedure**

a. The monolayer cell culture of Chang liver cells was trypsinized and the cell count was adjusted to $1.0 \times 10^5$ cells/mL using medium containing 10% new born calf serum.
b. To each well of the 96 well microtitre plate, 0.1mL of the diluted cell suspension (approximately 10,000 cells) was added.

c. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations was added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations recorded every 24 hours.

d. After 72 hours, the drug solutions in the wells were discarded and 50 µL of MTT in MEM- PR was added to each well.

e. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO₂ atmosphere.

f. The supernatant was removed and 50 µL of propanol was added and the plates were gently shaken to solubilize the formed formazan.

g. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage growth inhibition was calculated using the formula below:

\[
\text{% Growth Inhibition} = 100 - \left( \frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \right) \times 100
\]

5.1.2.C) Determination of Total Cell Protein Content by Sulphorhodamine B (SRB) Assay:
(Eisenbrand et al., 2002)

SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under mild acidic conditions, SRB binds to protein basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two orders of magnitude.

Colour development in SRB assay is rapid, stable and visible. The developed colour can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96 well plate reader. When TCA-fixed and SRB stained samples are air-dried, they can be stored indefinitely without deterioration.
**Preparation of Test solutions**

10 mg of the drug samples were weighed accurately and separately dissolved in 1 mL of DMSO and made up the volume to 10 ml with maintenance medium. These solutions were serially diluted with maintenance medium to obtain the lower dilutions.

**Requirements**

1. The same as that needed for MTT assays.
2. SRB dye (0.4% prepared in 1% acetic acid) (Sigma Chemicals)
3. 10mM Tris base
4. 50% trichloro acetic acid
5. Microplate reader (ELISA Reader, Bio-Tek)

**Procedure:**

Same as that of MTT assay (Sl. No. a. to c.)

d. After 72 hours, 25 μl of 50% trichloro acetic acid was added to the wells gently such that it forms a thin layer over the drug dilutions to form a overall concentration of 10%.

e. The plates were incubated at 4°C for one hour.
f. The plates were flicked and washed five times with tap water to remove traces of medium, drug and serum, and were then air-dried.
g. The air-dried plates were stained with SRB for 30 minutes. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried.
h. 100 μl of 10mM tris base was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 minutes.
i. The absorbance was measured using microplate reader at a wavelength of 540nm.

The percentage growth inhibition was calculated using the formula below:
Mean OD of Individual Test Group
Mean OD of Control Group
\[
\text{% Growth Inhibition} = 100 - \left( \frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \right) \times 100
\]

5.1.3 In vitro cytotoxicity studies for selected liver toxicants

In vitro cytotoxicity studies of the selected liver toxicants were performed against chang liver cells using MTT and SRB assay methods. Procedure followed was same as explained above.

5.1.4 Hepatoprotective activity of drug samples against selected liver toxicants (Vijayan et al., 2003)

5.1.4.1 In vitro hepatoprotective activity against D- galactosamine induced toxicity

Below the CTC_{50} value three dose levels were selected for each drug sample and used for further studies.

a. The monolayer cell culture of Chang liver cells was trypsinized and the cell count was adjusted to 1.0x10^5 cells/mL using medium containing 10% new born calf serum.

b. To each well of the 96 well microtitre plate, 0.1mL of the diluted cell suspension (approximately 10,000 cells) was added.

c. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once and treated with 100\μl of different drug concentrations for 24 hrs.

d. After 24 hrs of pretreatment, the cells were challenged with D-Galactosamine (30 mM) where 100\μl of different drug concentration and 100\μl of D-galactosamine was added. The plates were then incubated at 37°C for further 24 hours in 5% CO_2 atmosphere. Microscopic examination was carried out and observations were recorded every 24 hours.

e. After 72 hours, the drug solutions in the wells were discarded and 50\μl of MTT in MEM - PR was added to each well.

f. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO_2 atmosphere.

g. The supernatant was removed and 50\μl of propanol was added and the plates were gently shaken to solubilize the formed formazan.
h. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage growth inhibition was calculated using the formula below:

\[ \text{% Growth Inhibition} = 100 - \left( \frac{\text{Mean OD of Individual Test group}}{\text{Mean OD of Control Group}} \right) \times 100 \]

5.1.4.2 *In vitro* hepatoprotective activity against alcohol induced toxicity

Procedure same as section 5.1.4 except step d, which is explained below.

d. After 24 hrs of pretreatment, the cells were challenged with alcohol (60 mM) in which 100μl of different drug concentration and 100μl of alcohol was added. The plates were then incubated at 37°C for further 24 hours in 5% CO\(_2\) atmosphere. Microscopic examination was carried out and observations recorded every 24 hours.

Remaining steps (e), (f), (g) and (h) remain the same as section 5.1.4

5.1.4.3 *In vitro* hepatoprotective activity against CCl\(_4\) induced toxicity

Procedure same as section 5.1.4 except step d, which is explained below.

d. After 24 hrs of pretreatment, the cells were challenged with CCl\(_4\) (60 mM) (100μl of different drug concentration and 100μl of CCl\(_4\)) was added. The plates were then incubated at 37°C for further 24 hours in 5% CO\(_2\) atmosphere. Microscopic examination was carried out and observations were recorded every 24 hours.

Remaining steps (e), (f), (g) and (h) remain same as section 5.1.4

5.1.4.4 *In vitro* hepatoprotective activity against paracetamol induced toxicity

Procedure same as section 5.1.4 except step d, which is explained below.

d. After 24 hrs of pretreatment, the cells were challenged with paracetamol (50 mM) (100μl of different drug concentration and 100μl of paracetamol) was added. The plates were then incubated at 37°C for further 24 hours in 5% CO\(_2\) atmosphere. Microscopic examination was carried out and observations recorded every 24 hours.

Remaining steps (e), (f), (g) and (h) remain same as section 5.1.4
Methods

5.1.4.5 In vitro hepatoprotective activity against INH: RIF: PYZ induced toxicity

Procedure same as section 5.1.4 except step d, which is explained below.

d. After 24 hrs of pretreatment, the cells were challenged with INH: RIF: PYZ {0.5:1:7} (100μl of different drug concentration and 100μl of INH: RIF: PYZ) was added. The plates were then incubated at 37°C for further 24 hours in 5% CO₂ atmosphere.

Microscopic examination was carried out and observations recorded every 24 hours.

Remaining steps (e), (f), (g) and (h) remains same as section 5.1.4

5.1.5 Preparation of Freshly isolated rat hepatocytes: (Seglen, 1994) (Freshney, 2000b)

The availability of methods for isolation of large quantities of intact cells had made isolated hepatocytes culture a favorite experiment system for pharmacological, toxicological and biochemical research. The pioneering studies have established the superiority of collagenase treatment over the older mechanical and chemical methods of liver cell preparation and the introduction of enzymatic liver perfusion techniques increased the efficiency of tissue dissociation to such an extent to allow most of the liver tissue to be converted to a suspension of intact cells. In later studies, a quantitative liver dissociation assay to study the methodological parameters of collagenase perfusion established that the most optimal and reproducible results are obtained by a two-step procedure.

In the first step the liver is subjected to non-recirculating perfusion with calcium free buffer or with a calcium chelator like EDTA, causing irreversible separation of desmosomal cell contacts.

In the second step liver is perfused with collagenase to dissolve the extra cellular matrix, calcium being added back to ensure maximal enzyme activity. This optimal treatment dissociates the liver completely within 10 –15 mins, that is, sufficiently rapid to obviate the need for continuous oxygenation during perfusion. (Tanaka et al., 2006)
Requirements

Sterile:

1. L-15 Leibovitz medium
2. Tygon tube (ID 3.0mm; OD 5.0mm)
3. Disposable scalp vein infusion needles (24 gauge)
4. Sewing thread for cannulation
5. Sterile surgical instruments
   a. 4” scissors
   b. Toothed forceps
   c. Small forceps
   d. Blade holder
   e. Pithing needle
6. Graduated bottles and petridishes
7. Iodine solution
8. 2 x 1ml disposable syringes
9. Heparin
10. Thiopental sodium
11. Calcium free HEPES buffer (pH 7.65)
12. Collagenase solution (Sigma; Type IV)
13. Trypsin – Versene – Glucose (TPVG) solution
14. F12 Coon’s modified medium
15. Bovine Insulin
16. Bovine Albumin
17. Dexamethasone
18. Standard drug (silymarin 70mg)

Non-Sterile:

1. Peristatic pump (10 to 200rpm)
2. Water Bath maintained at 40°C
Procedure

a. The HEPES buffer and collagenase solution were warmed in a water bath usually (38°C-39°C to achieve 37°C in the liver)
b. The pump flow rate was adjusted to 30ml/min.
c. The rat (180-200gms) was anaesthetized by intra peritoneal administration of Thiopental sodium 45mg/kg b.w.
d. The abdomen was opened and a loosely tied ligature was placed around the portal vein approximately 5mm from the liver, and the cannula was inserted up to the liver and then the ligature was tightened, and heparin (1000 IU) was injected into the femoral vein.
e. Sub hepatic vessels were rapidly incised to avoid excess pressure and 600ml of calcium free HEPES buffer was perfused at a low rate of 30ml/min for 20 minutes. The liver swells during this time slowly changing color from dark red to greyish white.
f. 300ml of collagenase solution were perfused at a flow rate of 15ml/min for 20 minutes during which the lobes swell.
g. The lobes were removed and washed with HEPES buffer, after disrupting the Glison capsule.
h. The cell suspension was centrifuged at 1000 RPM to remove the collagenase, damaged cells and non-parenchymal cells.
i. The hepatocytes were collected in Ham’s F12 medium enriched with 0.2% bovine albumin, 10 μg/ml bovine insulin and 0.2% of dexamethasone.

5.1.5.1 In vitro estimation of biochemical parameters against D-galactosamine intoxicated rat hepatocytes (Kucera et al., 2006)

a. The hepatocytes isolated were incubated for 30 minutes at 37°C for stabilization.
b. The cells were then diluted in F12 coons modified medium to obtain a cell count 5x10⁵ cells/ml.
c. 100 ml of this cell suspension was seeded in 96 well plates in each well.
d. After 2 hours of pre-incubation, the medium was replaced with fresh medium.
e. Then the hepatocytes were pretreated with extracts for one hour before Galactosamine (30 mM) - induced treatment (100μl of different extract concentration and 100μl of D-galactosamine into each well).

f. Hepatocytes were further incubated for 24 hours at 37°C and 5% CO₂.

g. After incubation, the toxicant and drug treated cell suspensions were pooled into eppendorff tubes and centrifuged at 4000 rpm for 10 -15 min.

h. Supernatant was collected and the following enzyme levels were determined

- ASAT (Asparate Aminotransferase) (Bergmeyer et al., 1986)
- ALAT (Alanine Aminotransferase) (Lustig et al., 1988)
- ALP (Alkaline Phosphatase) (Tietz et al., 1983)
- LDH (Lactate dehydrogenase) (Bakker et al., 2006)

5.1.5.2 *In vitro* estimation of biochemical parameters against alcohol intoxicated rat hepatocytes (Adachi et al., 2004)

Procedure same as section 5.1.5.1 except step e, which is explained below.

- The hepatocytes were pretreated with drug samples for one hour before alcohol (60 mM) - induced treatment (100μl of different drug sample and 100μl of alcohol into each well).

Remaining steps (f), (g) and (h) remain same as section 5.1.5.1

5.1.5.3 *In vitro* estimation of biochemical parameters against CCl₄ intoxicated rat hepatocytes (Raj et al., 2010b)

Procedure same as section 5.1.5.1 except step e, which is explained below.

- The hepatocytes were pretreated with drug samples for one hour before CCl₄ (15 mM) - induced treatment (100μl of different drug sample and 100μl of CCl₄ into each well).

Remaining steps (f), (g) and (h) remain same as section 5.1.5.1
5.1.5.4 *In vitro estimation of biochemical parameters against paracetamol intoxicated rat hepatocytes* (Burcham and Harman 1991)

Procedure same as section 5.1.5.1 except step e, which is explained below.

   e. The hepatocytes were pretreated with different drug samples for one hour before paracetamol (50 mM) - induced treatment (100µl of different drug sample and 100µl of paracetamol into each well).

Remaining steps (f), (g) and (h) remain same as section 5.1.5.1

5.1.5.5 *In vitro estimation of biochemical parameters against INH: RIF: PYZ intoxicated rat hepatocytes* (Schwab and Tuschl 2003)

Procedure same as section 5.1.5.1 except step e, which is explained below.

   e. The hepatocytes were pretreated with different drug sample for one hour before INH: RIF: PYZ (90 µg/ml) - induced treatment (100µl of different drug sample and 100µl of INH: RIF: PYZ into each well).

Remaining steps (f), (g) and (h) remain same as section 5.1.5.1.

5.1.6 *Nuclear morphological studies* (Matzinger et al., 1991)

In order to observe the alteration or morphological changes in the nucleus specific fluorescent dyes which will reemits visible light upon absorbing ultraviolet light are used. Ethidium bromide a photoactive stains which will covalently bind with the nucleic acids in the fixed cells and stains DNA in red colour. Acridine orange is another dye which stains nucleus as green and cytoplasm red in colour. Common fluorochromes used to stain the genomic DNA of viable and/or non-viable cells.
Table No-5.1: Commonly used fluorochromes

<table>
<thead>
<tr>
<th>DNA-binding dyes (Fluorochromes)</th>
<th>Dye enters</th>
<th>Dye stains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable cells</td>
<td>Nonviable cells</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>DAPI</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

5.1.6.1 Nuclear staining of Chang liver cells using acridine orange against DGaLN induced toxicity

a. 24 h before the drug treatment, 50,000 cells were seeded in each well of 24 well plates with culture medium containing 10 % FBS.

b. After 24 h, cells were treated with different drug samples and further incubated overnight at 37°C in 5% CO₂ atmosphere.

c. After overnight incubation, the pre treated cells were challenged with DGaLN (30 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

d. After overnight incubation with the toxicant, medium from wells were discarded and cells were washed with PBS. The cells were fixed with 1 ml of methanol (90%) at -20°C for 20min. The methanol was removed and air-dried. Fixed cells were washed with ice cold PBS 2-3 times.
e. The cells were incubated with PBS containing 1% BSA and 0.1% triton X-100 at 37°C for 30 min.
f. Plate was washed with PBS 2-3 times and 200 μl of acridine orange (0.01% in PBS, pH-7.4) and was added and incubated at 37°C for 20 min.
g. The plate was washed thrice with PBS and observed under fluorescent microscope for any nuclear changes and photographs were taken.

5.1.6.2 Nuclear staining of Chang liver cells using acridine orange against alcohol induced toxicity

Procedure same as section 5.1.6.1, except step c, which is explained below.

c. After overnight incubation, the pre treated cells were challenged with alcohol (60 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

Remaining steps (d), (e), (f) and (g) remain same as section 5.1.6.1

5.1.6.3 Nuclear staining of Chang liver cells using acridine orange against CCl₄ induced toxicity

Procedure same as section 5.1.6.1, except step c, which is explained below.

c. After overnight incubation, the pre treated cells were challenged with CCl₄ (15 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

Remaining steps (d), (e), (f) and (g) remain same as section 5.1.6.1

5.1.6.4 Nuclear staining of Chang liver cells using acridine orange against paracetamol induced toxicity

Procedure same as section 5.1.6.1, except step c, which is explained below.

c. After overnight incubation, the pre treated cells were challenged with paracetamol (50 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

Remaining steps (d), (e), (f) and (g) remains same as section 5.1.6.1
5.1.6.5 Nuclear staining of Chang liver cells using acridine orange against INH: RIF: PYZ induced toxicity

Procedure same as section 5.1.6.1, except step c, which is explained below.

c. After overnight incubation, the pre treated cells were challenged with INH: RIF: PYZ (90µg/ml) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

Remaining steps (d), (e), (f) and (g) remain same as section 5.1.6.1

5.1.6.6 Nuclear staining of Chang liver cells using Hoechst 33342 against selected toxicants

Procedure same as section 5.1.6.1, except step (c) and (f)

c. After overnight incubation, the pre treated cells were challenged with selected toxicants individually and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

Next steps (d) and (e) remain same as section 5.1.6.1

f. Plate was washed with PBS 2-3 times and 200 µl of Hoechst 33342 (10 µg/ml in PBS pH-7.4) and was added and incubated at 37°C for 20 min.

Remaining step (g) remains same as section 5.1.6.1

5.1.7 Isolated mitochondrial staining using JC-1 dye against selected toxicants

5.1.7. A) Mitochondrial isolation (Guthrie and Welch 2008)

Mitochondria were isolated from Chang liver cells using mitochondrial isolation kit from Sigma company, St Louis, USA.

a. 50,000 cells were seeded in each well of 24 well plates with MEM medium containing 10% FBS.
Methods

b. After 24 h, cells were treated with different drug samples and further incubated overnight at 37°C in 5% CO₂ atmosphere.

c. After overnight incubation, the pre treated cells were challenged with different toxicants individually and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

d. After overnight incubation with the toxicant, the cells were trypnised, growth medium was added and centrifuged at 600 x g for 5 min. Supernatant was discarded and again the samples were centrifuged with growth medium at 600 x g for 5 min.

e. The cell pellet was re suspended in ice cold phosphate buffer saline and cell count was determined. Centrifuge the samples at 600 x g for 5 min and cell pellet was collected.

f. Cell pellet was re suspended in lysis buffer (2 ml per 1 X 10⁵ cells). The samples were incubated for 5 minutes on ice and two volume of extraction buffer was added.

g. The homogenate was centrifuged at 600 x g for 10 minutes at 4°C.

h. The supernatant was carefully transferred to a fresh tube, centrifuged at 11,000 x g for 10 minutes at 4°C.

i. The supernatant was removed, and the cell pellet was suspended in storage buffer and kept at ice cold conditions until mitochondrial staining procedure.

5.1.7. B) Isolated mitochondrial staining

Isolated mitochondrial preparation was stained with help of JC-1 (5, 5, 6, 6′-tetrachloro-1, 1′-3, 3′-tetraethyl benzimidazolocarbocyanine iodide) dye. The concentration of mitochondrial preparation for staining was 40 µg/ml. Final concentration of JC-1 staining solution was 0.2 µg/ml. 90 µl of JC-1 staining solution was added to 10 µl of isolated mitochondrial sample and excitation wave length of 490 nm and emission wave length of 590 nm was used to visualize the samples with the help of Olympus inverted microscope with fluorescence attachment.

5.1.8 Effect of toxicants on the expression of Bax with respect to time using RT PCR

5.1.8.A) Extraction of total RNA from Chang liver cells (Liu et al., 1978)

a. The cells were lysed directly with 1 ml Trizol reagent. The lysate was passed through a pipette several times.
b. The homogenized samples were transferred into eppendorf tubes and incubated for 5 min at 25°C.

c. 0.2 ml of chloroform per ml of Trizol was added to each sample.

d. The tubes were shaken vigorously for 15 seconds and incubated at 25°C for 2 to 3 min.

e. The samples were centrifuged at 12,000 × g for 15 min at 2 to 8°C.

f. The colourless upper aqueous phase was transferred to a fresh tube for RNA isolation.

g. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. Samples were incubated at 25°C for 10 min and centrifuged at 12,000 × g for 10 min at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

h. The supernatant was removed and the RNA pellet was washed once with at least 1 ml of 75% ethanol (prepared using RNase-free water). The sample was mixed by vortexing and centrifuged at no more than 7,500 × g for 5 min at 2 to 8°C.

i. The RNA pellet was briefly air-dried for 5 to 10 min. RNA was dissolved in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubated for 10 min at 55 to 60°C.

j. This extracted RNA was used for subsequent reverse transcriptase - polymerase chain reaction RT-PCR.

5.1.8. B) Reverse transcriptase PCR

RT-PCR can be done by two methods:
One step RT-PCR
Two step RT-PCR

In one-step RT-PCR, the components of RT and PCR are mixed in a single tube at the same time. The one-step protocol generally works well for amplifying targets that are reasonably abundant.
Alternatively, RT-PCR can be done in two steps, first with the reverse transcription and then the PCR. The two-step protocol is usually more sensitive than the one-step method; yields of rare targets may be improved by using the two-step procedure.

**One-step RT-PCR:**
- Convenient

\[
\text{RT + PCR} \\ 
\text{RNA} \rightarrow \text{RT-PCR product of gene of interest}
\]

**Two-step RT-PCR:**
- Saves RT reagents. One RT reaction will provide templates for multiple PCR’s
- Can be more sensitive than one-step RT-PCR

\[
\text{PCR for gene X} \\ 
\text{RNA} \rightarrow \text{cDNA pool} \rightarrow \text{RT-PCR product of Gene X} \\
\text{PCR for gene Y} \\
\text{RT-PCR product of Gene Y} \\
\text{PCR for gene Z} \\
\text{RT-PCR product of Gene Z}
\]

We used two step RT-PCR protocol for studying the extent of Bcl-2, Bax and P53 mRNA expression with the use of specific primers.

**Two step RT-PCR**

**Step One: Reverse Transcription**

Reagents:
- AMV reverse transcriptase (100 units/10 µl)
- 5X RT buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30mM MgCl\(_2\) 50 mM DTT)
- Random decamers (50µM)
- dNTPs (10 mM each dNTP)
- RNase Inhibitor (10 units/µl)
• All the reagents were added to small 0.5 ml PCR tubes sequence wise and mixed well.

**Table No 5.2:** Reaction mix for reverse transcription

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock</th>
<th>Final amount</th>
<th>Experiment (+RT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>-</td>
<td>2 μg</td>
<td>2 μl</td>
</tr>
<tr>
<td>Random Decamers</td>
<td>5 μM</td>
<td>5 μM</td>
<td>2 μl</td>
</tr>
<tr>
<td>5X RT Buffer</td>
<td>5X</td>
<td>1X</td>
<td>4 μl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>10 mM</td>
<td>0.25 mM</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>10 U/μl</td>
<td>10U</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>10 U/μl</td>
<td>100U</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>x μl (to total of 20 μl)</td>
<td>x μl (to total of 20 μl)</td>
<td>8.5 μl</td>
</tr>
</tbody>
</table>

• All the reagents were mixed well and vortexed in PCR tubes.

• All the tubes were incubated in the thermocycler at:
  a. 44°C for 1 h
  b. 92°C for 10 min to inactivate the reverse transcriptase

• RT products were stored at -20°C until the next PCR step.

**Step Two: PCR**

Reagents:
  • JumpStart® Taq ready mix
  • All the reagents were added to small 0.5 ml PCR tubes sequence wise and mixed well.
Table No 5.3: Reaction mix for bulk PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>JumpStart® Taq ready mix</td>
<td>25 µl</td>
</tr>
<tr>
<td>50 mM Mn(OAc)$_2$</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2 µl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2 µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>2 µl</td>
</tr>
<tr>
<td>RNase free water</td>
<td>16.5 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

- All the tubes were incubated in the thermocycler with following programme:

Table No 5.4: Program for PCR reaction

<table>
<thead>
<tr>
<th>Cycling step</th>
<th>Time and temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>2 min at 94°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>30 s at 51°C for Bax, 30 s at 60°C GADPH, 30 s at 51°C Bcl2 and 30 s at 46°C P53.</td>
</tr>
<tr>
<td>Extension</td>
<td>1.30 min at 72°C</td>
</tr>
<tr>
<td>Final extension</td>
<td>6 min at 72°C</td>
</tr>
</tbody>
</table>

Note: Annealing temperature was fixed about 5°C below the $T_m$.

- Result were analysed on a 1% agarose gel containing 0.5 μg/ml ethidium bromide.
- Electrophoresis was then carried out with 1× TAE buffer at a constant voltage of 50 V for 1 h.
- Bands were visualised under a UV transilluminator at the wavelength of 365 nm.
5.1.8. C) Agarose gel electrophoresis

Agarose is a linear polymer extracted from a sea weeds. Purified agarose is a powder insoluble in water or buffer at room temperature but dissolves on boiling. Molten solution is then poured into a mould and allowed to solidify. As it cools, agarose undergoes polymerization i.e., sugar polymers cross-link with each other and cause the solution to gel, the density or pore size of which is determined by concentration of agarose.

DNA is negatively charged at neutral pH and when electric field is applied across the gel, DNA migrates towards the anode. Migration of DNA through the gel is dependent upon:

1. Molecular size of DNA
2. Agarose concentration
3. Conformation of DNA
4. Applied current

Matrix of agarose gel acts as a molecular sieve through which DNA fragments move on application of electric current. Higher concentration of agarose gives firmer gels, i.e., spaces between cross-linked molecules is less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases, it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps in movements of larger DNA fragments as the spaces between the cross-linked molecules is more.

The progress of gel electrophoresis is monitored by observing the migration of a visible dye (tracking dye) through the gel. We used two dyes namely xylene cyanol and bromophenol blue that migrate at the same speed as double stranded DNA of size 5000 bp and 300 bp respectively. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of run, when the tracking dye reaches towards the anode, run is terminated.
Visualization of DNA fragments

Since DNA is not naturally colored, it will not be visible on the gel. Hence the gel, after electrophoresis, is stained with a dye specific to the DNA. Discrete bands are observed when there is enough DNA material present to bind the dye to make it visible, otherwise the band is not detected. The gel is observed against a light background wherein DNA appears as dark colored bands.

Alternatively, an intercalating dye like Ethidium bromide is added to agarose gel and location of bands determined by examining the gel under UV light, wherein DNA fluoresces. We used Ethidium bromide to visualize the DNA fragments.

Procedure for preparation of agarose and electrophoresis

a. 1X TAE was prepared by diluting appropriate amount of 50X TAE buffer (for one experiment, approximately 200 ml) and make up volume was made up by adding 4 ml of 50X TAE to 200 ml with Distilled Water.

b. 1 g agarose was weighed and to 100 ml of 1X TAE this gives 1% agarose gel.

c. Agarose was boiled it till dissolves completely and a clear solution results, a pinch of Ethidium Bromide was added to it and mix well.

d. The Agarose solution was poured in central part of tank when temperature reached approximately 60°C before pouring the gel and the comb of electrophoresis was placed so that it was set so that it is 2 cm away from cathode.

e. Air bubbles should not be generated during and after pouring the gel, thickness of the gel should be around 0.5 to 0.9 cm. The gel was kept undisturbed at room temperature for the agarose to solidify.

f. 1X TAE buffer was poured into the gel tank till buffer level stands at 0.5 to 0.8 cm above the gel.

g. The comb was lifted gently, ensuring that wells remained intact.

h. Power cords were connected to the electrophoresis power supply. Red-Positive. Black- Negative.

i. Samples were numbered according to the well in which they were loaded.
j. 2.5 µl of gel loading buffer was added to 25 µl of sample and minimum of 10 µl samples were loaded in the respective wells.

k. Voltage was set between 50-100V and power was switch on.

l. When the dye bands reached ¾ th length of the gel, the running was stopped.

m. The bands were observed in U.V. light and gel picture captured, saved and analysed using alpha imager software.

5.1.8.1 Expression of Bax after D galactosamine induced toxicity at different time periods

a. 50,000 cells were seeded in each well of 24 well plates with MEM medium containing 10 % FBS.

b. After 24 h, cells were treated with DGalN (30 mM) and incubated at 37⁰C in 5% CO₂ atmosphere.

c. Toxicant treatment was performed in five replicates and each replicate is incubated for different time periods of 6, 12, 24, 48 and 72 h respectively.

d. After different time periods of 6, 12, 24, 48 and 72 h respectively after toxicant treatment the cells were trypnised, growth medium was added and centrifuged at 600 x g for 5 min. Supernatant was discarded and again the samples were centrifuged with growth medium at 600 x g for 5 min.

e. The cell pellet was re suspended in ice cold phosphate buffer saline and cell count was determined. Centrifuge the samples at 600 x g for 5 min and cell pellet was collected.

f. Total RNA was extracted from the cell pellet as per section 5.1.8.A.

g. Reverse transcriptase PCR was performed using the extracted RNA sample as per section 5.1.8. B.

5.1.8.2 Expression of Bax after alcohol induced toxicity at different time periods

Procedure followed as per section 5.1.8.1 except step b, which is explained below.
b. After 24 h of seeding, chang liver cells were treated with alcohol (60 mM) and incubated at 37°C in 5% CO₂ atmosphere.

Remaining steps (c) to (g) remains the same as per section 5.1.8.1.

**5.1.8.3 Expression of Bax after CCl₄ induced toxicity at different time periods**

Procedure followed as per section 5.1.8.1 except step b, which is explained below.

b. After 24 h of seeding, chang liver cells were treated with CCl₄ (15 mM) and incubated at 37°C in 5% CO₂ atmosphere.

Remaining steps (c) to (g) remains the same as per section 5.1.8.1.

**5.1.8.4 Expression of Bax after paracetamol induced toxicity at different time periods**

Procedure followed as per section 5.1.8.1 except step b, which is explained below.

b. After 24 h of seeding, chang liver cells were treated with paracetamol (50 mM) and incubated at 37°C in 5% CO₂ atmosphere.

Remaining steps (c) to (g) remains the same as per section 5.1.8.1.

**5.1.8.5 Expression of Bax after INH: RIF: PYZ induced toxicity at different time periods**

Procedure followed as per section 5.1.8.1 except step b, which is explained below.

b. After 24 h of seeding, chang liver cells were treated with INH: RIF: PYZ (90 µg/ml) and incubated at 37°C in 5% CO₂ atmosphere.

Remaining steps (c) to (g) remains the same as per section 5.1.8.1.

**5.1.9 Effect of selected drugs on the expression of genes related to mitochondrial pathway**

After fixing the time of exposure of toxicant to 12 hrs, we tested the expression of three genes Bax, Bel-2 and P53 which are related to the mitochondrial pathway. Chang liver cells were pre
treated with drug samples and then challenged with individual toxicants. After 12 hrs of challenge with toxicant, the cells were trypnised, RNA was extracted and RT PCR was performed using primers specific for Bax, Bcl-2 and P53 respectively.

5.1.9.1 Effect of selected drugs on Bax expression against DGalN induced toxicity

a. 50,000 cells were seeded in each well of 24 well plates with MEM medium containing 10 % FBS.
b. After 24 h, cells were treated with selected drug samples and further incubated overnight at 37°C in 5% CO₂ atmosphere.
c. After overnight incubation, the pre treated cells were challenged with DGalN (30 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.
d. After 12 hrs of incubation with the toxicant, the cells were trypnised, growth medium was added and centrifuged at 600 x g for 5 min. Supernatant was discarded and again the samples were centrifuged with growth medium at 600 x g for 5 min.
e. The cell pellet was re suspended in ice cold phosphate buffer saline and cell count was determined. Centrifuge the samples at 600 x g for 5 min and cell pellet was collected.
f. Total RNA was extracted from the cell pellet as per section 5.1.8.A.
g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bax. Procedure followed for RT PCR as per section 5.1.8. B.

5.1.9.2 Effect of selected drugs on Bcl2 expression against DGalN induced toxicity

Procedure followed as per section 5.1.9.1 except step g, which is explained below.

   g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bcl2. Procedure followed for RT PCR as per section 5.1.8.B.

5.1.9.3 Effect of selected drugs on P53 expression against DGalN induced toxicity

Procedure followed as per section 5.1.9.1 except step g, which is explained below.
g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for P53. Procedure followed for RT PCR as per section 5.1.8.B.

5.1.9.4 Effect of selected drugs on Bax expression against alcohol induced toxicity

a. 50,000 cells were seeded in each well of 24 well plates with MEM medium containing 10% FBS.

b. After 24 h, cells were treated with selected drug samples and further incubated overnight at 37°C in 5% CO₂ atmosphere.

c. After overnight incubation, the pretreated cells were challenged with alcohol (60 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

d. After 12 hrs of incubation with the toxicant, the cells were trypnised, growth medium was added and centrifuged at 600 x g for 5 min. Supernatant was discarded and again the samples were centrifuged with growth medium at 600 x g for 5 min.

e. The cell pellet was resuspended in ice cold phosphate buffer saline and cell count was determined. Samples were centrifuged at 600 x g for 5 min and cell pellet was collected.

f. Total RNA was extracted from the cell pellet as per section 5.1.8.A.

g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bax. Procedure followed for RT PCR as per section 5.1.8. B.

5.1.9.5 Effect of selected drugs on Bcl2 expression against alcohol induced toxicity

Procedure followed as per section 5.1.9.4 except step g, which is explained below.

g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bcl2. Procedure followed for RT PCR as per section 5.1.8.B.

5.1.9.6 Effect of selected drugs on P53 expression against alcohol induced toxicity

Procedure followed as per section 5.1.9.4 except step g, which is explained below.

g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for P53. Procedure followed for RT PCR as per section 5.1.8.B.
5.1.9.7 Effect of selected drugs on Bax expression against CCl₄ induced toxicity

a. 50,000 cells were seeded in each well of 24 well plates with MEM medium containing 10% FBS.

b. After 24 h, cells were treated with selected drug samples and further incubated overnight at 37°C in 5% CO₂ atmosphere.

c. After overnight incubation, the pre treated cells were challenged with CCl₄ (15 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

d. After 12 hrs of incubation with the toxicant, the cells were trypnised, growth medium was added and centrifuged at 600 x g for 5 min. Supernatant was discarded and again the samples were centrifuged with growth medium at 600 x g for 5 min.

e. The cell pellet was resuspended in ice cold phosphate buffer saline and cell count was determined. Centrifuge the samples at 600 x g for 5 min and cell pellet was collected.

f. Total RNA was extracted from the cell pellet as per section 5.1.8.A.

g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bax. Procedure followed for RT PCR as per section 5.1.8. B.

5.1.9.8 Effect of selected drugs on Bcl2 expression against CCl₄ induced toxicity

Procedure followed as per section 5.1.9.7 except step g, which is explained below.

  g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bcl2. Procedure followed for RT PCR as per section 5.1.8.B.

5.1.9.9 Effect of selected drugs on P53 expression against CCl₄ induced toxicity

Procedure followed as per section 5.1.9.7 except step g, which is explained below.

  g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for P53. Procedure followed for RT PCR as per section 5.1.8.B.
5.1.9.10 Effect of selected drugs on Bax expression against paracetamol induced toxicity

a. 50,000 cells were seeded in each well of 24 well plates with MEM medium containing 10% FBS.
b. After 24 h, cells were treated with selected drug samples and further incubated overnight at 37°C in 5% CO₂ atmosphere.
c. After overnight incubation, the pre treated cells were challenged with paracetamol (50 mM) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.
d. After 12 hrs of incubation with the toxicant, the cells were trypnised, growth medium was added and centrifuged at 600 x g for 5 min. Supernatant was discarded and again the samples were centrifuged with growth medium at 600 x g for 5 min.
e. The cell pellet was re suspended in ice cold phosphate buffer saline and cell count was determined. Centrifuge the samples at 600 x g for 5 min and cell pellet was collected.
f. Total RNA was extracted from the cell pellet as per section 5.1.8.A.
g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bax. Procedure followed for RT PCR as per section 5.1.8. B.

5.1.9.11 Effect of selected drugs on Bcl2 expression against paracetamol induced toxicity

Procedure followed as per section 5.1.9.10 except step g, which is explained below.

g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bcl2. Procedure followed for RT PCR as per section 5.1.8.B.

5.1.9.12 Effect of selected drugs on P53 expression against paracetamol induced toxicity

Procedure followed as per section 5.1.9.10 except step g, which is explained below.

g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for P53. Procedure followed for RT PCR as per section 5.1.8.B.
5.1.9.13 Effect of selected drugs on Bax expression against INH: RIF: PYZ induced toxicity

a. 50,000 cells were seeded in each well of 24 well plates with MEM medium containing 10% FBS.

b. After 24 h, cells were treated with selected drug samples and further incubated overnight at 37°C in 5% CO₂ atmosphere.

c. After overnight incubation, the pre-treated cells were challenged with INH: RIF: PYZ (90µg/ml) and the plates were further incubated overnight at 37°C in 5% CO₂ atmosphere.

d. After 12 hrs of incubation with the toxicant, the cells were trypnised, growth medium was added and centrifuged at 600 x g for 5 min. Supernatant was discarded and again the samples were centrifuged with growth medium at 600 x g for 5 min.

e. The cell pellet was re suspended in ice cold phosphate buffer saline and cell count was determined. Centrifuge the samples at 600 x g for 5 min and cell pellet was collected.

f. Total RNA was extracted from the cell pellet as per section 5.1.8.A.

g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bax. Procedure followed for RT PCR as per section 5.1.8.B.

5.1.9.14 Effect of selected drugs on Bcl2 expression against INH: RIF: PYZ induced toxicity

Procedure followed as per section 5.1.9.13 except step g, which is explained below.

 g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bcl2. Procedure followed for RT PCR as per section 5.1.8.B.

5.1.9.15 Effect of selected drugs on P53 expression against INH: RIF: PYZ induced toxicity

Procedure followed as per section 5.1.9.13 except step g, which is explained below.

 g. Reverse transcriptase PCR was performed using the extracted RNA sample and primers specific for Bcl2. Procedure followed for RT PCR as per section 5.1.8.B.
5.1.10 Flow cytometric analysis (Kumar et al., 2009)

Flow cytometric analysis was performed using propidium iodide.

5.1.10.1 Flow cytometry analysis of control untreated cells

a. Cells were plated at 1 X 10^6 cells/ml in T-25 cm² flasks and incubated at 37³C for attachment and growth.

b. After 3 days of incubation, cells were collected, washed twice in ice-cold PBS, and then re suspended in binding buffer at a density of 1 X 10^6 cells/ml.

c. Propidium iodide (PI, 10 µl) was added to the cells and analysed with FACS Calibur TM flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

5.1.10.2 Flow cytometry analysis of DGalN intoxicated cells

a. Cells were plated at 1 X 10^6 cells/ml in T-25 cm² flasks and incubated at 37³C for attachment and growth.

b. After 48 hrs of initial plating, the cells were challenged with DGalN (30 mM).

c. After 12 hrs of toxicant challenge, cells were collected, washed twice in ice-cold PBS, and then re suspended in binding buffer at a density of 1 X 10^6 cells/ml.

d. Propidium iodide (PI, 10 µl) was added to the incubated cells and analysed with FACS Calibur TM flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

5.1.10.3 Flow cytometry analysis of silymarin pretreated cells challenged with DGalN

a. Cells were plated at 1 X 10^6 cells/ml in T-25 cm² flasks and incubated at 37³C for attachment and growth.

b. Twenty four hours later, cells were treated with silymarin (40µg/ml) and incubated for further 24 hrs.
Methods

c. After 48 hrs of initial plating, the cells were challenged with DGalN (30 mM) for further 12 hours.

d. After 12 hrs of toxicant challenge, cells were collected, washed twice in ice-cold PBS, and then re suspended in binding buffer at a density of $1 \times 10^6$ cells/ml.

e. Propidium iodide (PI, 10 µl) was added to the incubated cells and analysed with FACS Calibur TM flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

5.1.10.4 Flow cytometry analysis of catechin pretreated cells challenged with DGalN

Procedure followed as per section 5.1.10.3 except step b, which is explained below.

b. Twenty four hours later, cells were treated with catechin (40µg/ml) and incubated for further 24 hrs.

Remaining steps (c) to (e) remains the same as per section 5.1.10.3.

5.1.10.5 Flow cytometry analysis of LOLA pretreated cells challenged with DGalN

Procedure followed as per section 5.1.10.3 except step b, which is explained below.

b. Twenty four hours later, cells were treated with LOLA (75µg/ml) and incubated for further 24 hrs.

Remaining steps (c) to (e) remains the same as per section 5.1.10.3.

5.1.10.6 Flow cytometry analysis of alcohol intoxicated cells

Procedure followed as per section 5.1.10.2 except step b, which is explained below.

b. After 48 hrs of initial plating, the cells were challenged with alcohol (60 mM).

Remaining steps (c) and (d) remains the same as per section 5.1.10.2.
5.1.10.7 Flow cytometry analysis of silymarin pretreated cells challenged with alcohol

a. Cells were plated at $1 \times 10^6$ cells/ml in T-25 cm$^2$ flasks and incubated at 37°C for attachment and growth.

b. Twenty four hours later, cells were treated with silymarin (40µg/ml) and incubated for further 24 hrs.

c. After 48 hrs of initial plating, the cells were challenged with alcohol (60 mM) for further 12 hours.

d. After 12 hrs of toxicant challenge, cells were collected, washed twice in ice-cold PBS, and then re suspended in binding buffer at a density of $1 \times 10^6$ cells/ml.

e. Propidium iodide (PI, 10 µl) was added to the incubated cells and analysed with FACS Calibur TM flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

5.1.10.8 Flow cytometry analysis of catechin pretreated cells challenged with alcohol

Procedure followed as per section 5.1.10.7 except step b, which is explained below.

b. Twenty four hours later, cells were treated with catechin (40µg/ml) and incubated for further 24 hrs.

Remaining steps (c) to (e) remains the same as per section 5.1.10.7.

5.1.10.9 Flow cytometry analysis of LOLA pretreated cells challenged with alcohol

Procedure followed as per section 5.1.10.7 except step b, which is explained below.

b. Twenty four hours later, cells were treated with LOLA (75µg/ml) and incubated for further 24 hrs.

Remaining steps (c) to (e) remains the same as per section 5.1.10.7.
5.10.10 Flow cytometry analysis of CCl<sub>4</sub> intoxicated cells

Procedure followed as per section 5.10.2 except step b, which is explained below.

b. After 48 hrs of initial plating, the cells were challenged with CCl<sub>4</sub> (15 mM).

Remaining steps (c) and (d) remains the same as per section 5.10.2.

5.10.11 Flow cytometry analysis of paracetamol intoxicated cells

Procedure followed as per section 5.10.2 except step b, which is explained below.

b. After 48 hrs of initial plating, the cells were challenged with paracetamol (50 mM).

Remaining steps (c) and (d) remains the same as per section 5.10.2.

5.10.12 Flow cytometry analysis of silymarin pretreated cells challenged with paracetamol

a. Cells were plated at 1 X 10<sup>6</sup> cells/ml in T-25 cm<sup>2</sup> flasks and incubated at 37ºC for attachment and growth.

b. Twenty four hours later, cells were treated with silymarin (40µg/ml) and incubated for further 24 hrs.

c. After 48 hrs of initial plating, the cells were challenged with paracetamol (50 mM) for further 12 hours.

d. After 12 hrs of toxicant challenge, cells were collected, washed twice in ice-cold PBS, and then re suspended in binding buffer at a density of 1 X 10<sup>6</sup> cells/ml.

e. Propidium iodide (PI, 10 µl) was added to the incubated cells and analysed with FACS Calibur TM flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA, USA).
5.1.10.13 Flow cytometry analysis of catechin pretreated cells challenged with paracetamol
Procedure followed as per section 5.1.10.12 except step b, which is explained below.

b. Twenty four hours later, cells were treated with catechin (40µg/ml) and incubated for further 24 hrs.

Remaining steps (c) to (e) remains the same as per section 5.1.10.12.

5.1.10.14 Flow cytometry analysis of LOLA pretreated cells challenged with paracetamol
Procedure followed as per section 5.1.10.12 except step b, which is explained below.

b. Twenty four hours later, cells were treated with LOLA (75µg/ml) and incubated for further 24 hrs.

Remaining steps (c) to (e) remains the same as per section 5.1.10.12.

5.1.10.15 Flow cytometry analysis of INH: RIF: PYZ intoxicated cells
Procedure followed as per section 5.1.10.2 except step b, which is explained below.

b. After 48 hrs of initial plating, the cells were challenged with INH: RIF: PYZ (90µg/ml).

Remaining steps (c) and (d) remains the same as per section 5.1.10.2.
5.2 In vivo studies

5.2.1 Determination of hepatic SOD and CAT levels

5.2.1.A) SOD Estimation

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD). Superoxide dismutase is the antioxidant enzyme that catalyzes the dismutation of $O_2^\cdot^-$ to $O_2$ and to the less reactive species $H_2O_2$. While this enzyme was isolated as early as 1939, it was only in 1969 that McCord and Fridovich proved the antioxidant activity of SOD (McCord and Fridovich, 1969).

Superoxide dismutase exists in several isoforms, differing in the nature of active metal centre and amino acid constituency, as well as their number of subunits, cofactors and other features.

Under physiological conditions, a balance exists between the level of reactive oxygen species (ROS) produced during normal cellular metabolism and the level of endogenous antioxidants, which serve to protect tissues from oxidative damage. Disruption of this balances either through increased production of ROS or decreased levels of antioxidants; produce a condition known as oxidative stress and leads to variety of pathological conditions. To protect against oxidative damage, organisms have developed a variety of antioxidant defenses that include metal sequestering proteins, use of compounds such as vitamin C, E and specialized antioxidant enzymes. One family of antioxidant enzymes, the superoxide dismutase (SOD) function to remove damaging ROS from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Tortora et al., 2004).

$$O_2^\cdot^- + O_2^\cdot^- \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

SOD measurement was carried out on the ability of SOD to inhibit spontaneous oxidation of epinephrine to adrenochrome.
5.2.1.B) Estimation of Catalase (CAT):
Catalase is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria (Tortora et al., 2004). Catalase is located in a cell organelle called the peroxisome. In animals, catalase is present in all major body organs. The role of catalase is to scavenge hydrogen peroxide and prevent oxidative damage in the cell. Catalase is a heme containing protein that can convert hydrogen peroxide to water and oxygen in two-step reaction cycle. In the first step, one molecule of hydrogen peroxide is converted to water. The catalytic cycle begins with the oxidation of the ferric heme by two electrons by hydrogen peroxide to form the ferryl-oxo porphyrin/protein radical intermediate known as compound 1. The catalase cycle is completed by the reduction of compound 1 to the ferric enzyme by hydrogen peroxide, resulting in production of molecular oxygen (Murray et al., 2007).

\[
\begin{align*}
\text{Fe}^{3+} & \quad \text{Ferric Heme} \\
\text{H}_2\text{O}_2 & \quad \text{H}_2\text{O} \\
\text{H}_2\text{O} + \text{O}_2 & \quad \text{Fe}^{4+}=\text{O} \\
\text{H}_2\text{O}_2 & \quad \text{Ferryl-oxoporphyrin / protein} \\
\end{align*}
\]

5.2.1.C) Preparation of the formulation:
Catechin, lecithin, LOLA, L-ornithin and silymarin drug samples were dissolved or suspended in 0.5% sodium carboxy methylcellulose and administered.

5.2.1.D) Test concentrations selected for study:
Acute toxicity studies were done according to OECD guidelines 425. Dose of 2000 mg/kg.bt.wt was found to be safe in rat models. After extensive literature survey and considering the efficiency of the drug samples we selected one by twenty concentration (100 mg/kg.bt.wt) and one by forty concentration (50 mg/kg.bt.wt) for in vivo studies.

5.2.1.E) Selection of Animals:
Species: Wistar Rats
Sex: Male and female
Weight: 150-200g
5.2.1.1 Effect of drugs on hepatic SOD and CAT levels in rats with D-GalN induced toxicity

5.2.1.1.A) Randomization Numbering and Grouping of Animals:

The experimental design of the investigation was carried out twelve groups with six animals in each group and given the regiments described below.

GROUP I Served as normal control which received 1mL 0.5% sodium carboxy methyl cellulose (CMC) orally once a day.

GROUP II Served as toxicant treated control which received 1mL of 0.5% CMC orally once a day for 7 days. On the last day single dose of D-GalN (400mg/kg) was administered i.p route.

GROUP III Received a single dose of 50 mg/kg bt.wt of catechin for 7 days followed by treatment with the toxicant on the last day.

GROUP IV Received a single dose of 100 mg/kg bt.wt of catechin for 7 days followed by treatment with the toxicant on the last day.

GROUP V Received a single dose of 50 mg/kg bt.wt of lecithin for 7 days followed by treatment with the toxicant on the last day.

GROUP VI Received a single dose of 100 mg/kg bt.wt of lecithin for 7 days followed by treatment with the toxicant on the last day.

GROUP VII Received a single dose of 50 mg/kg bt.wt of LOLA for 7 days followed by treatment with the toxicant on the last day.

GROUP VIII Received a single dose of 100 mg/kg bt.wt of LOLA for 7 days followed by treatment with the toxicant on the last day.

GROUP IX Received a single dose of 50 mg/kg bt.wt of L-ornithin for 7 days followed by treatment with the toxicant on the last day.

GROUP X Received a single dose of 100 mg/kg bt.wt of L-ornithin for 7 days followed by treatment with the toxicant on the last day.
GROUP XI  Received a single dose of 50 mg/kg bt.wt of silymarin for 7 days followed by treatment with the toxicant on the last day.

GROUP XII  Received a single dose of 100 mg/kg bt.wt of silymarin for 7 days followed by treatment with the toxicant on the last day.

After 24 hrs of toxicant treatment the rats were anesthetized using ether and the blood was collected from retro-orbital plexus. Serum was separated and used for various biochemical parameter estimations.

After removing blood the rats were sacrificed by spinal cord dislocation. Liver tissue was perfused with normal saline solution and dissected out. One lobe of the liver sample from each group was used for histopathology and DNA fragmentation studies. Another lobe of the liver tissue was used for preparing tissue homogenate. The tissue homogenate was prepared by using homogenizer in ice cold 10% calcium chloride solution. The tissue homogenate was centrifuged at 10,000 rpm for 10 min and supernatant was taken out and transferred into fresh tubes and used for estimating hepatic SOD and CAT levels.

5.2.1.1.B) Estimation of Catalase (CAT)

Chemicals and reagents

1. Hydrogen peroxide (7.5 mM): 1.043 mL of 30% w/w H₂O₂ was made up to 100 mL with sodium chloride and EDTA solution (9 g of NaCl and 29.22 mg of EDTA dissolved in 1 L distilled water).

2. Potassium phosphate buffer (65 mM, pH 7.8): 2.2 g of potassium dihydrogen phosphate and 11.32 g of dipotassium hydrogen phosphate were dissolved in 250 mL and 1 L distilled water, respectively and mixed together. The pH was adjusted to 7.8 with KH₂PO₄.

3. Sucrose Solution: 10.95 g of sucrose was dissolved in 100 mL of distilled water.

Procedure

2.25 mL of potassium phosphate buffer (65 mM, pH 7.8) and 100 μL of the tissue homogenate or sucrose (0.32 M) were incubated at 25°C for 30 min. 0.65 mL of H₂O₂ (75 mM) was added to initiate the reaction. The change in absorption at 240 nm was measured for 2-3 min, and dy/dx
for 1 min for each assay was calculated and the results are expressed as CAT units / mg of tissue (Beers and Seizer, 1952).

\[
\frac{\text{Cat (U)}}{100 \ \mu l \ of \ Sample} = \frac{[(dy/dx) \times 0.003]}{[38.3956 \times 10^{-6}]}
\]

The \( dy/dx \) (change in absorbance / min) was calculated for each assay divided by \( 38.3956 \times 10^{-6} \) (molar extinction coefficient of \( \text{H}_2\text{O}_2 \) at 240 nm) to obtain \( \mu \text{M/L} \) of \( \text{H}_2\text{O}_2 \) converted to \( \text{H}_2\text{O} \) per min, multiplied by 0.003 to obtain micromoles. \( \text{H}_2\text{O}_2 \) converted to \( \text{H}_2\text{O} \) per min in 3 mL by 0.1 mL sample.

5.2.1.1.C) SOD Estimation

**Chemicals and reagents**

1. **Sodium carbonate buffer (0.05 M, pH 10.2):** 5.3 g of sodium carbonate and 1.2 g of sodium bicarbonate were dissolved separately in 1 L of distilled water, which served as a stock solution. Buffer was prepared by mixing 64 mL of sodium carbonate and 70 mL of sodium bicarbonate solutions. The pH of the buffer was adjusted to 10.2 using the above stock solution accordingly.

2. **Adrenaline (9 mM):** 0.03 g of adrenaline was dissolved in distilled water and the final volume was made up to 10 mL with distilled water containing a drop of concentrated HCl (to bring pH down to 2). Adrenaline being sensitive, the vial was kept covered with aluminum foil at all times.

3. **Sucrose (0.3199 M) solution:** 10.96 g of sucrose was dissolved in distilled water and the volume was made up to 100 mL.

**Procedure**

2.8 mL of sodium carbonate buffer (0.05 mM) and 0.1 mL of tissue homogenate or sucrose (blank) was incubated at 30°C for 45 min. Then, the absorbance was adjusted to zero to sample. Thereafter, the reaction was initiated by adding 10 \( \mu \)L of adrenaline solution (9 mM). The change in absorbance was recorded at 480 nm for 8-12 min. Throughout the assay, the temperature was maintained at 30 °C. Similarly, SOD calibration curve was prepared by taking 10 unit/mL as standard solution. One unit of SOD produced approximately 50% inhibition of auto-oxidation of adrenaline. The results are expressed as unit (U) of SOD activity/mg of tissue.
5.2.1.2 Effect of drugs on hepatic SOD and CAT levels in rats with alcohol induced toxicity

**GROUP I**  
Served as normal control which received 1mL 0.5% sodium carboxymethyl cellulose (CMC) orally once a day.

**GROUP II**  
Served as toxicant treated control which received alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP III**  
Received a daily dose of 50 mg/kg bt.wt of catechin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP IV**  
Received a daily dose of 100 mg/kg bt.wt of catechin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP V**  
Received a daily dose of 50 mg/kg bt.wt of lecithin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP VI**  
Received a daily dose of 100 mg/kg bt.wt of lecithin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP VII**  
Received a daily dose of 50 mg/kg bt.wt of LOLA, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP VIII**  
Received a daily dose of 100 mg/kg bt.wt of LOLA, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP IX**  
Received a daily dose of 50 mg/kg bt.wt of L-ornithin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP X**  
Received a daily dose of 100 mg/kg bt.wt of L-ornithin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP XI**  
Received a daily dose of 50 mg/kg bt.wt of silymarin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.

**GROUP XII**  
Received a daily dose of 100 mg/kg bt.wt of silymarin, along with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days.
At the end of 45 days, animals were sacrificed and part of liver tissue is used for CAT and SOD levels. Procedure followed for estimation of CAT and SOD remains the same as section 5.2.1.1.B and 5.2.1.1.C respectively.

### 5.2.1.3 Effect of drugs on hepatic SOD and CAT levels in rats with CCl$_4$ induced toxicity

Procedure followed same as section 5.2.1.1.A except group II

**GROUP II**  
Served as toxicant treated control which received 1mL of 0.5% CMC orally once a day for 7 days. On the last day single dose of CCl$_4$ (0.5 ml/kg.bt.wt, 1:1 with olive oil) was administered i.p route.

Remaining groups remains same as section 5.2.1.1.A.

Procedure followed for estimation of CAT and SOD remains the same as section 5.2.1.1.B and 5.2.1.1.C respectively.

### 5.2.1.4 Effect of drugs on hepatic SOD and CAT levels in rats with paracetamol induced toxicity

Procedure followed same as section 5.2.1.1.A except group II

**GROUP II**  
Served as toxicant treated control which received 1mL of 0.5% CMC orally once a day for 7 days. On the last day single dose of paracetamol (3.5 g/kg.bt.wt, 0.25% CMC) was administered oral route.

Remaining groups same as section 5.2.1.1.A.

Procedure followed for estimation of CAT and SOD remains the same as section 5.2.1.1.B and 5.2.1.1.C respectively.

### 5.2.1.5 Effect of drugs on hepatic SOD and CAT levels in rats with INH: RIF: PYZ induced toxicity

Procedure followed same as section 5.2.1.1.A except group II

**GROUP II**  
Served as toxicant treated control which received 1mL of 0.5% CMC orally once a day for 7 days. On the last day single dose of INH (50 mg/kg.bt.wt, distilled water) single dose (i.p route), RIF (100mg/kg.bt.wt,
0.25% CMC) (i.p route) and PYZ (350 mg/kg.bt.wt, 0.25% CMC) (i.p route) was administered.

Remaining groups same as section 5.2.1.1.A.

Procedure followed for estimation of CAT and SOD remains the same as section 5.2.1.1.B and 5.2.1.1.C respectively.

5.2.2 Studies on the biochemical parameters of liver (Fujii, 1997)

Important liver biochemical parameters such as ASAT, ALAT, ALP and LDH were estimated to confirm the effect of toxicants on the liver as well as to check the hepatoprotective potential of the drug samples against each toxicant individually.

5.2.2.A) Assay of aspartate amino transferase (ASAT or SGOT) (Bergmeyer et al., 1986)

Aspartate amino transferase in serum and liver homogenate was assayed by using Cobas diagnostic kit. ASAT catalyzes by the following reactions.

\[
\text{2-Oxoglutarate + L-Aspartate} \rightleftharpoons \text{Glutamate + Oxaloacetate}
\]

\[
\text{Oxaloacetate + NaDH + H}^+ \rightleftharpoons \text{Malate + NAD}^+ 
\]

Aspartate amino transferase level in kidney and liver tissue homogenate was expressed as U/L.

5.2.2.B) Assay of alanine amino transferase (ALAT or SGPT) (Lustig et al., 1988)

Alanine amino transferase in serum and liver homogenate was assayed by using Cobas diagnostic kit. ALAT catalyzes by the following reactions.

\[
\text{2-Oxoglutarate + L-Alanine} \rightleftharpoons \text{Glutamate + Pyruvate}
\]

\[
\text{Pyruvate + NaDH + H}^+ \rightleftharpoons \text{Lactate + NAD}^+ 
\]

Alanine amino transferase level in kidney and liver tissue homogenate was expressed as U/L.

5.2.2.C) Assay of Alkaline Phosphatase (ALP) (Tietz et al., 1983)

Alkaline phosphatase in kidney and liver homogenate was assayed by using Cobas diagnostic kit. ALP catalyzes by the following reaction

\[
\text{4-Nitrophenyl phosphate + H}_2\text{O} \rightleftharpoons \text{Phosphate + 4-Nitrophenolate}
\]
Alkaline phosphatase level in kidney and liver tissue homogenate was expressed as U/L.

5.2.2.D) Lactate dehydrogenase (LDH) (Bakker et al., 2006)

Lactate dehydrogenase (LDH) an enzyme that catalyzes the conversion of lactate to pyruvate. This is an important step in energy production in cells. Some of the organs relatively rich in LDH are the heart, kidney, liver and muscle. As cells die, their LDH is released and finds its way into the blood. LDH levels were determined by a decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit causes the oxidation of one micromole of NADH per minute at 25°C and pH 7.3.

5.2.2.1 Study on the biochemical parameters of rats pretreated with drugs and challenged with D-GalN (Jaishree and Badami 2010)

Procedure followed same as section 5.2.1.1.A

At the end of the study the rats were anesthetized using ether and the blood was collected from retro-orbital plexus. Serum was separated and used for estimating important biochemical parameters such as ASAT, ALAT, ALP and LDH.

5.2.2.2 Study on the biochemical parameters of rats pretreated with drugs and challenged with alcohol (Lin et al., 2002)

Procedure followed same as section 5.2.1.2

At the end of the study the rats were anesthetized using ether and the blood was collected from retro-orbital plexus. Serum was separated and used for estimating important biochemical parameters such as ASAT, ALAT, ALP and LDH.

5.2.2.3 Study on the biochemical parameters of rats pretreated with drugs and challenged with CCl₄ (Shahjahan et al., 2004)

Procedure followed same as section 5.2.1.1.A except group II

GROUP II Served as toxicant treated control which received 1mL of 0.5% CMC orally once a day for 7 days. On the last day single dose of CCl₄ (0.5 ml/kg.bt wt, 1:1 with olive oil) was administered i.p route.

Remaining groups same as section 5.2.1.1.A.
At the end of the study the rats were anesthetized using ether and the blood was collected from retro-orbital plexus. Serum was separated and used for estimating important biochemical parameters such as ASAT, ALAT, ALP and LDH.

5.2.2.4 Study on the biochemical parameters of rats pretreated with drugs and challenged with paracetamol (Sabir and Rocha., 2008)

Procedure followed same as section 5.2.1.1.A except group II

GROUP II Served as toxicant treated control which received 1mL of 0.5% CMC orally once a day for 7 days. On the last day single dose of paracetamol (3.5 g/kg.bt.wt, 0.25% CMC) was administered oral route.

Remaining groups same as section 5.2.1.1.A.

At the end of the study the rats were anesthetized using ether and the blood was collected from retro-orbital plexus. Serum was separated and used for estimating important biochemical parameters such as ASAT, ALAT, ALP and LDH.

5.2.2.5 Study on the biochemical parameters of rats pretreated with drugs and challenged with INH: RIF: PYZ (Sodhi et al., 1997)

Procedure followed same as section 5.2.1.1.A except group II

GROUP II Served as toxicant treated control which received 1mL of 0.5% CMC orally once a day for 7 days. On the last day single dose of INH (50 mg/kg.bt.wt, distilled water) single dose (i.p route), RIF (100mg/kg.bt.wt, 0.25% CMC) (i.p route) and PYZ (350 mg/kg.bt.wt, 0.25% CMC) (i.p route) was administered.

Remaining groups same as section 5.2.1.1.A.

At the end of the study, rats were anesthetized using ether and the blood was collected from retro-orbital plexus. Serum was separated and used for estimating important biochemical parameters such as ASAT, ALAT, ALP and LDH.
5.2.3 Histopathology studies of liver (Fijii, 1997)

At the end of the study, rats were sacrificed by spinal cord dislocation. Liver tissue was perfused with normal saline solution and dissected out. One lobe of the liver sample from each group was used for histopathology studies.

Procedure

Part of the liver sample is fixed overnight in 10% buffered formalin and paraffin-embedded. The sections were stained with hematoxylin and eosin (H&E) for histological evaluation and examined under light microscope. In brief, 4 μm thick sections of paraffin-embedded rat liver were dewaxed in xylene, rehydrated in graded alcohol series, and washed with distilled water for 2 min. Subsequently, the sections were stained with hematoxylin for 5 min at room temperature. After 15 min, the sections were counter-stained with eosin for 2 min, dehydrated in graded alcohol series, washed with xylene, and blocked by rosin. H&E stained slides were observed under microscope at 40 × magnification.

5.2.3.1 Histopathology studies on the biochemical parameters of rats pretreated with drugs and challenged with DGalN.

Animals were pretreated with drug samples and toxicant challenge as per section 5.2.1.1.A.

5.2.3.2 Histopathology studies on the biochemical parameters of rats pretreated with drugs and challenged with alcohol.

Procedure followed for drug pretreatment and toxicant challenge is same as section 5.2.1.2. Animals were challenged with alcohol (45 %, 10ml/kg.bt.wt, twice daily) for 45 days along with/without drug samples before sacrifice on the last day.

Procedure followed for histopathology is same as section 5.2.3.

5.2.3.3 Histopathology studies on the biochemical parameters of rats pretreated with drugs and challenged with CCl₄.

Procedure followed for drug pretreatment is same as section 5.2.1.1.A except group II
All the animals were challenged with CCl₄ (0.5 ml/kg.bt.wt, 1:1 with olive oil) single dose (i.p route) on 7th day. After 24 hrs of toxicant challenge the animals were sacrificed by spinal cord dislocation.

Procedure followed for histopathology is same as section 5.2.3.

5.2.3.4 Histopathology studies on the biochemical parameters of rats pretreated with drugs and challenged with paracetamol.

Procedure followed for drug pretreatment is same as section 5.2.1.1.A except group II

All the animals were challenged with paracetamol (3.5 g/kg.bt.wt, 0.25% CMC) single dose (oral route) on 7th day. After 24 hrs of paracetamol challenge the animals were sacrificed by spinal cord dislocation.

Procedure followed for histopathology is same as section 5.2.3.

5.2.3.5 Histopathology studies on the biochemical parameters of rats pretreated with drugs and challenged with INH: RIF: PYZ.

Procedure followed for drug pretreatment is same as section 5.2.1.1.A except group II

All the animals were challenged with INH (50 mg/kg.bt.wt, D.W) single dose (i.p route), RIF (100mg/kg.bt.wt, 0.25% CMC) (i.p route) and PYZ (350 mg/kg.bt.wt, 0.25% CMC) (i.p route) on 7th day. After 24 hrs of INH: RIF: PYZ challenge the animals were sacrificed by spinal cord dislocation.

Procedure followed for histopathology is same as section 5.2.3.

5.2.4 DNA Fragmentation studies (Raj et al., 2010a)

At the end of the study, rats were sacrificed by spinal cord dislocation. Liver tissue was perfused with normal saline solution and dissected out. One lobe of the liver sample from each group was used for DNA fragmentation studies.

5.2.4.A) DNA extraction (Ribeiro et al., 2004)

DNA was extracted from excised liver of animals from every group. The liver tissues were lysed by exposing them at -70°C and subsequent trituration with liquid nitrogen before extraction. The tissues were lysed with digestion buffer (pH 7.5) containing 0.5% SDS, 25 mM
tris-HCl, 0.5% mg/ml proteinase K and 5 mM EDTA at 55°C overnight. After extracting the cell lysates with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), DNA was precipitated with 3M sodium acetate (pH 5.2) and absolute ethanol, and was washed, dried and resuspended with tris-EDTA buffer containing RNase A (100 µg/ml) at 37°C for 30 min.

5.2.4.B) Agarose gel electrophoresis

Procedure followed for agarose gel electrophoresis is already explained at section 5.1.8.C

5.2.4.1 DNA fragmentation studies of DGalN induced toxicity

Animals were pretreated with drug samples and toxicant challenge as per section 5.2.1.1.A.

DNA isolated from liver samples from different treated groups were analysed using 1% agaorse gel electrophoresis and DNA fragments were visualized using ethidium bromide staining under UV light. DNA bands were analysed and comparison between groups was done with help of alpha imager software.

5.2.4.2 DNA fragmentation studies of alcohol induced toxicity

Procedure followed for drug pretreatment and toxicant challenge is same as section 5.2.1.2.

DNA isolated from liver samples from different treated groups were analysed using 1% agaorse gel electrophoresis and DNA fragments were visualized using ethidium bromide staining under UV light. DNA bands were analysed and comparison between groups was done with help of alpha imager software.

5.2.4.3 DNA fragmentation studies of CCl₄ induced toxicity

Procedure followed for drug pretreatment is same as section 5.2.1.1.A except group II

All the animals were challenged with CCl₄ (0.5 ml/kg.bt.wt, 1:1 with olive oil) single dose (i.p route) on 7th day. After 24 hrs of toxicant challenge the animals were sacrificed by spinal cord dislocation. The isolated liver lobes were used for DNA extraction and fragmentation studies.

DNA fragmentation studies were performed in same way as explained in section 5.2.4.1.
5.2.4.4 DNA fragmentation studies of paracetamol induced toxicity

Procedure followed for drug pretreatment is same as section 5.2.1.1. A except group II

All the animals were challenged with paracetamol (3.5 g/kg.bt.wt, 0.25% CMC) single dose (oral route) on 7\(^{th}\) day. After 24 hrs of paracetamol challenge the animals were sacrificed by spinal cord dislocation. The isolated liver lobes were used for DNA extraction and fragmentation studies.

DNA fragmentation studies were performed in same way as explained in section 5.2.4.1.

5.2.4.5 DNA fragmentation studies of INH: RIF: PYZ induced toxicity

Procedure followed for drug pretreatment is same as section 5.2.1.1. A except group II

All the animals were challenged with INH (50 mg/kg.bt.wt, D.W) single dose (i.p route), RIF (100mg/kg.bt.wt, 0.25% CMC) (i.p route) and PYZ (350 mg/kg.bt.wt, 0.25% CMC) (i.p route) on 7\(^{th}\) day. After 24 hrs of INH: RIF: PYZ challenge the animals were sacrificed by spinal cord dislocation. The isolated liver lobes were used for DNA extraction and fragmentation studies.

DNA fragmentation studies were performed in same way as explained in section 5.2.4.1.

5.2.5 Statistical analysis

The statistical analysis was carried out by one way analysis of variance (ANOVA). The values are represented as mean ± S.E.M. Comparison of mean values of different groups treated with different dose levels of selected drug samples with normal was performed by Turkey’s Multiple Comparison Test. P< 0.01 was considered significant.