Materials and Methods
MATERIALS AND METHODS

Materials: Acrylamide (99.9%), Glutathione, (GSH), Glutathione-CL-Agarose affinity column, N,N,N',N-tetramethylethylenediamine (TEMED) 2-mercaptoethanol, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitroblue tetrazolium (NBT), comassie brilliant blue R-250, bromophenol blue, Ponceau-S stain, Bromosulfophthalein (BSP), 1-chloro 2,4-dinitro benzene(CDNB), 1,2-Epoxy-3(p-nitro phenoxy propane (EPNP), p-Nitrobenzyl chloride, and p-Nitrophenyl acetate (PNPA) were purchased from Bio-Rad laboratories (Richmond, USA) and Genei, Bangalore, India. Cellulose membrane dialysis tubings were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

Ethylene diaminetetraacetic acid (EDTA), ammonium persulphate (APS), Acetic acid-glacial, Agarose, Cumene Hydroperoxide (CHP), Hydrochloric acid (HCL), Hydrogen Peroxide (H2O2), sucrose, hydroxymethyl aminomethane (Tris), silver nitrate, glycine, glycerol, sodium carbonate, sodium thiosulfate, Tween-20, guanidine hydrochloride, dithiothreitol (DTT), formaldehyde, phenylmethanesulphonyl fluoride (PMSF), sodium azide, Sucrose, Sodium Potassium tartrate, Sodium dodecyl sulphate (SDS), Sodium Chloride (NaCl), Skim Milk Powder, Potassium chloride(KCl) and All other chemicals procured from the local companies and were pure of high quality. β-methylcholanthrene procured from SD fine chemical LTD, India, The formalin, Haematoxylin, and Eosin were purchased from BDH, chemical company, Bombay.

Freunds complete and incomplete adjuvants were purchased from Genei, Bangalore, India. Goat anti rabbit IgG antibodies (Bio-Rad laboratories, Richmond, USA) and Nitrocellulose membranes for immunoblotting were from Amersham (England, UK). were procured.
METHODOLOGY

Acrylamide treatment:

Male Wistar rats procured from Venkateswara Enterprises, Bangalore, weighing about 100-150 gms were selected for treatment studies. The rats were treated with intra peritoneal administration of acrylamide 16mg/100gm body weight for 6 days with an interval of 24 hours to a total of 96mg. After injection the rats were maintained by feeding ad libitum with water and sacrificed after each dose and after interval of 24 hrs. Control animals maintained by giving water alone.

Tissue collection and sample preparation

Normal and induced rat liver tissues were collected by after decapitation of animals and the fishes were washed with cold 50 mM Tris HCl buffer, (pH 8.0), containing 1mM ethylenediaminetetraacetic acid (EDTA) in order to remove excess blood and body fluids instantly collected tissues were preserved at -20°C for further experimentation. At the time of experimentation the collected normal and treated rat liver tissues were thawed, minced with scissors and 20% of liver tissue homogenate was prepared in 50 mM Tris-HCl buffer, pH 8.0, containing 0.25M sucrose and 1mM phenylmethanesulphonyl fluoride (PMSF) using a Potter Elvijhem homogenizer. Homogenization was done by keeping the glass homogenizer in an ice jacket and care was taken to minimize the froth formation. The homogenate was filtered through two layers of cheese cloth to remove floating lipid materials and the resulted supernatant was centrifuged at 10,000 rpm on high speed refrigerated centrifuge (Remi) two times for 45 min at 4°C. The collected supernatant was known as cytosolic GSTs fraction of rat liver and it was used as the enzyme source for analysis and protein purification for the preparation antibodies.

The protein determination, enzyme activity measurement and purification process were started immediately after the preparation of the cytosolic fraction.
During this process the cytosolic fraction in small aliquots of 0.5 ml were stored at -80°C for the use of SDS-PAGE and Western blotting.

**Purification of GSTs**

Cytosolic fraction of 10,000 rpm after dialysis was loaded onto Glutathione-CL-Agarose affinity column previously equilibrated with 50mM Tris-HCl, pH 8.0 and then washed with the same buffer till the optical density (O.D) reached to 0.005 at 280 nm. To remove non specific bound proteins (by spectrophotometric detection) 50mM Tris-HCl containing 0.2M KCl buffer was used. The affinity bound GST proteins were eluted with 50mM Tris-HCl, pH 8.0 containing 5mM reduced glutathione (GSH), till the optical density (O.D) was reached to 0.005 at 280 nm and the fractions were collected in 5 ml aliquots (Frangioni *et al.*, 1993; Simons *et al.*, 1977). All the active fractions after elution of protein from the column were pooled and dialysed against 25 mM of Tris-HCl buffer, pH 8.0 and centrifused to remove dead proteins, if any present.

**Protein Determination:**

The protein concentration in the prepared cytosol and also in purified GSTs was determined by the method of Lowry *et al* (Lowry *et al.*, 1951) with crystalline bovine serum albumin (BSA) using as a standard and prepared standard calibration curve of 20μg per 200μl for experiments. The collected cytosolic GST aliquot samples were taken into test tubes and were made to a final volume of 1ml with distilled water. Then, alkaline copper reagent was prepared by mixing 2% of copper sulfate, 2% of sodium potassium tartarate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 2.5 ml of the alkaline copper reagent was added to each tube, mixed by vortex and allowed to stand for 10 minutes at room temperature. Finally, 0.5 ml of 1 N Folin Phenol reagent (1:1) was added to each test tube, mixed immediately by vortex and incubated for 10 minutes at 50°C in water bath. The intensity of color developed in each tube was measured at 660 nm. The protein concentrations in the crude extracts were calculated from a standard calibration curve that was
constructed from the corresponding O.D, at 660nm, values of BSA standards (0 to 120 μg).

**GST Assay**

The rat liver tissue GST activity was assayed by the conventional method of Habig *et al.* (1974). The increase in the measurement of absorbance is due to the conjugation of CDNB with GST, which is catalyzed by GSH, to produce a thioether which can be read on the spectrophotometer at the wavelength of 340 nm (Nunes *et al.*, 2006). The typical reaction mixture in a total volume of 3 ml consists of 1 ml of 0.3M PO₄ buffer, pH 6.5, 100 μl of each of enzyme, 30mM CDNB and 30mM GSH and 1.7 ml of distilled water and the increase in absorbance was read at 340 nm and quantification was done using 9.6x10⁻³ -mm⁻¹cm⁻¹ as the molar extinction coefficient. One unit of enzyme activity was defined as the formation of one micromole of 2, 4-dinitrophenol GSH product per minute or one micromole of substrate consumed per minute. Specific activity was expressed as micromoles of GSH conjugate formed per milligram of protein in a minute of time.

**Substrate Specificities**

In order to screen different isozymes for substrate specificities they were assayed for activity with BSP, EPNP, pNPA, pNBC, in addition to CDNB, the classical substrates for GSTs by the method of Habig and Jakoby (1981, Table-8).
Table 3: A summary of procedures for GST substrate specificity measurement

<table>
<thead>
<tr>
<th>Name of substrate</th>
<th>Contents of reagent mixture</th>
<th>Molar extinction coefficient</th>
<th>Absorbance</th>
<th>Enzyme protein</th>
<th>Initiation of reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPA</td>
<td>125 mM KH₂PO₄ - pH 7.0, 0.3 mM pNPA, 0.5 mM GSH, Total Vol. 3 ml</td>
<td>8.79x10⁴ cm⁻¹</td>
<td>400 λ</td>
<td>150-300μg</td>
<td>GSH</td>
</tr>
<tr>
<td>pNBC</td>
<td>1005 mM KH₂PO₄ - pH 6.5, 1mM substrate, 5 mM GSH, Total Vol. 3ml</td>
<td>1.9x10⁴ cm⁻¹</td>
<td>310 λ</td>
<td>50-100μg</td>
<td>GSH</td>
</tr>
<tr>
<td>EPNP (1,2-epoxy p-nitrophenox y) propen</td>
<td>125 mM KH₂PO₄ - pH 6.5, 1mM EPNP, 5 mM GSH, Total Vol. 3ml</td>
<td>0.5x10⁴ cm⁻¹</td>
<td>360 λ</td>
<td>150-300μg</td>
<td>GSH</td>
</tr>
<tr>
<td>Bromosulfaphthalein</td>
<td>125 mM KH₂PO₄ - pH 7.5, 1mM BSP, 5 mM GSH, Total Vol. 3ml</td>
<td>4.5x10⁴ cm⁻¹</td>
<td>330 λ</td>
<td>150-300μg</td>
<td>GSH</td>
</tr>
</tbody>
</table>

Assay of Glutathione Peroxidase

Glutathione peroxidase assay was carried out by monitoring the oxidation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) in a recycling assay as described by Wendel, 1981. Total GPx activity was determined by using CHP (ROOH) as substrate. Selenium dependent GPx activity was measured using H₂O₂ as substrate.

Buffers and Substrates required for GPX assay

Potassium phosphate buffer, 0.25M, pH 7.0, containing 2.5mM tetra sodium ethylene diamine tetra acetic acid (EDTA) and 2.5mM sodium azide.

Glutathione reductase (GR) in phosphate buffer (0.34 mg/ml at 25⁰C)

GSH – 10mM in distilled water.
NADPH – 2.5 mM in 0.1% sodium bicarbonate.

ROOH – 12 mM in GPx buffer (Phosphate buffer 0.25 M pH 7.0 containing EDTA and sodium azide. (R=H or CHP).

The Cocktail mixture, which contained 1.8 ml of assay buffer, 100 µl of each of glutathione reductase (GR), glutathione reduced (GSH) and NADPH, was transferred to 3 ml cuvette. Enzyme (250 µg of protein) was added and the reaction was initiated by the addition of 100 µl of CHP/H2O2. The linear decrease in NADPH absorption at 340 nm was recorded. The blank reaction was carried out without enzyme and was subtracted. Amount of NADPH oxidized was calculated using molar extinction coefficient 6.32 x 10^3. Specific activity of GPx was expressed as nano moles (n moles) of NADPH oxidized / min/ mg protein at room temperature.

SDS-Polyacrylamide Gel Electrophoresis:

Polyacrylamide slab gel electrophoresis, in the presence of the anionic detergent sodium dodecyl sulfate (SDS), was performed on 4 % stacking gel and 12 % separating gel in a discontinuous buffer system as described by Laemmli (1970).

Preparation of Reagents:

Separating Gel Buffer: (1.5 M Tris-HCl, pH 8.8): 36.3 gm of Tris base was dissolved in about 100 ml distilled water and pH 8.8 was adjusted with 1 M HCl and then made upto 200 ml.

Stacking Gel Buffer: (0.5 M Tris-HCl, pH 6.8): 12.1 gm of Tris base was dissolved in about 100 ml distilled water and pH 6.8 was adjusted with 1 M HCl and then made upto 200 ml.

Acrylamide -Bis Solution (30 %): 60.0 gm acrylamide were dissolved in about 175 ml distilled water and then 1.6 gm Bis (Bis-acrylamide) were added in 37.5:1 ratio and solution was completed to 200 ml with distilled water. Finally, the
solution was filtered through ordinary filter paper to removed floating acrylamide particles.

A: 10% SDS Solution:

10 gm SDS was dissolved in water with gentle stirring and made to a final volume of 100 ml.

B: Catalyst: 10% Ammonium Persulfate "APS" was prepared by dissolving 10 gms in 100ml of distilled water.

C: Tracking Dye: 0.05% Bromophenol Blue

Tracking dye solution was prepared by dissolving 5 mg of solid bromophenol blue in a final volume of 10 ml.

G: 5 X Electrode (Running) Buffer: (25 mM Tris, 192 mM Glycine, pH 8.3)

Stock running buffer: This solution was prepared by dissolving and completing 15 gm of Tris base and 72 gm of glycine in one liter distilled water. The pH of the buffer was not adjusted with acid or with base, since the mixture directly reaches to pH 8.3. This buffer was diluted in 1:5 ratio and 1 gm of solid SDS was added to 1 liter of buffer before use.

H: 4 X Sample Dilution Buffer: (SDS Reducing Buffer)

It was prepared freshly by mixing 1ml of 100 mg of ammonium persulfate (APS) dissolved in a final volume of 10ml of distilled water, 2.5ml of 1 M Tris-HCl, pH 6.8 40%, Glycerol 20%, 2-mercaptoethanol 0.004%, Tracking Dye (BPS) 8%, SDS (0.8 gm) and distilled water to make upto 10ml. This mixture was stored in refrigerator for necessary use.

Electrophoresis Procedure:

Vertical slab gel electrophoresis was carried out using the EC120 Big Vertical Gel System (Yarcaud) that can be used to run two gels simultaneously. Aliquots of the protein samples to be analyzed and the standards were diluted in
3:1 with the 4X tracking dye (3 parts sample and 1 part sample buffer), and there were boiled and centrifuged. The supernatant (50μl) of each vial was loaded into different using a capillaries.

Table-4: Formulations for SDS-PAGE separating and stacking gels

<table>
<thead>
<tr>
<th></th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer concentration</td>
<td>12%</td>
<td>10%</td>
</tr>
<tr>
<td>Acrylamide-Bis (30%)</td>
<td>8 ml</td>
<td>6.7 ml</td>
</tr>
<tr>
<td>solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>8 ml</td>
<td>8.3 ml</td>
</tr>
<tr>
<td>1.5 M Tris-Hcl ,pH 8.8</td>
<td>4 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>0.5 M Tris-Hcl ,pH 6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% ( w/v ) SDS</td>
<td>200 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>200 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>Total Monomer</td>
<td>20 ml</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

The power supply was adjusted to give a constant current of 50 mA when the samples were in the stacking gel and 75 mA when the samples passed to the separating gel. Under these conditions the voltage was about 75 V at the beginning and elevated up to 100V at the end of the run that took a total of about 3.5 hour. The power supply was switched off, when the dye front is just 0.5 cm from the lower end of the glass plates, the running frame was taken out and the buffer was removed from the upper buffer compartment. Afterwards, the clamps were detached and the cassettes were removed from the running frame. To gain access to the gels in the cassette, the glass plates were removed gently using a spatula taking care not to chip the edges of the glass plates. The gel was stained.
with appropriate strain and stored. The same gel or another gel was used for transblot analysis.

**Silver Staining of the SDS-PAGE Gel:**

The silver staining of the SDS-PAGE gels was carried out with a revised method of Blum and co-workers (Blum et al., 1987) as explained in **Table-5**.

**Table-5: Procedure for rapid method of Silver Staining of Proteins in Polyacrylamide Gels**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Solutions</th>
<th>Time of Incubation of gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>In the mixture of 50% methanol, 7.5% acetic acid 50μl of formaldehyde</td>
<td>One hour</td>
</tr>
<tr>
<td>Washing</td>
<td>With 10% &amp; 5% HAC ethanol</td>
<td>Three times, Each 30 minutes</td>
</tr>
<tr>
<td>Pre treat</td>
<td>In 0.2% solution of sodium thiosulphate (Hypo)</td>
<td>One minute</td>
</tr>
<tr>
<td>Washing</td>
<td>With double distilled water</td>
<td>Three times each 5 minutes</td>
</tr>
<tr>
<td>Impregnate</td>
<td>In 0.2% solution of silver nitrate and 75μl formaldehyde</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Washing</td>
<td>With double distilled water</td>
<td>Three times each 5 minutes</td>
</tr>
<tr>
<td>Development</td>
<td>With mixture of 6% sodium carbonate, 50μl Formaldehyde and Hypo</td>
<td>As quick as possible</td>
</tr>
<tr>
<td>Stop</td>
<td>10% Acetic acid</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Washing</td>
<td>With double distilled water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Store</td>
<td>In double distilled water (If necessary dry and store)</td>
<td></td>
</tr>
</tbody>
</table>
The electrophoresed Gels were treated with a solution of 50% methanol, 7.5% acetic acid and 50µl of HCHO for five minutes to fix the proteins and followed the washings thrice (5 min each) with 10% ethanol and 5% acetic acid. The proteins fixed gels were treated with sodium thiosulphate (Hypo 200 mg /100ml) for one min and washed thoroughly with distilled water to remove excess Hypo. The gels were further treated with silver nitrate solution (200 mg /100ml) for 20 min, rinsed with distilled water thrice and color was developed using 6% sodium carbonate solution containing formaldehyde and Hypo. The color development of the proteins was stopped by the addition of 1% acetic acid and gels were preserved in double distilled water.

Production of Antisera

The affinity purified GST protein after electrophoretic analysis was used for immunization of Rabbits (Newzealand White Male, 3 months old) to produce antibodies. Rabbits obtained from Poultry Department of Sri Venkateswara Veterinary University, Tirupati, were used for this experiment. Freund's complete and incomplete adjuvants were purchased from Genie, Bangalore, India. Prior to the injection of the sample to rabbit the blood was collected from it and serum was tested for immunocross reactivity and stored the non cross reacted serum as control for further experimental analysis. After observation no immunocross reactivity with collected serum and purified GST protein the rabbits were prepared for immunization after removal of hair from the body. 200 µg of affinity purified chick rat liver GST protein per ml was emulsified with an equal volume of Freund's complete adjuvant. The equally emulsified mixture was injected subcutaneously to the rabbit at 4 to 6 sites. The booster doses were given with an interval of a week for about four to five times. The titre of antibodies was tested before the fifth dose of immunization. The last dose was given with an incomplete adjuvant. One week after the last injection, the rabbits were bled and the serum was prepared by centrifuging at 6,000 rpm in Remi refrigerated centrifuge for half an hour at 4°C and the supernatant obtained thus was considered as antisera of chick rat liver GSTs.
**Immunodiffusion**

The Ouchterlony, (1968) double immunodiffusion method was followed for the cross reactivity determination between antibody and antigen. The slides were prepared with 0.8% agarose in normal saline and kept in a humid chamber. The wells were punched on gels. The central well was loaded with rat liver cytosolic GSTs antisera and the four encircling wells were loaded with the specific affinity purified GST proteins as antigens. The precipitin bands were visualized by staining with 0.1% commassie brilliant blue R. 250 and upon destaining with methanol and acetic acid. The titre value of the antisera was determined by the same procedure.

**Electroblotting of the Gels from SDS-PAGE:**

Electroblotting was carried out using EC140 Mini Blot Module of the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A.), and Nitro cellulose membrane (NC) was used as a blotting membrane. The gels obtained from the SDS-PAGE were electroblotted directly. Prior to electroblotting, the gels run from SDS-PAGE were placed for 30 min, with shaking, in the Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol; Towbin *et al.*, 1979). While the gels were incubated in the transfer buffer, the required NC membrane was prepared.

Every precaution was taken to see that the membrane should not become to dry, so that proteins must bind to it properly. Afterwards, two pieces of filter paper, the Scotch Brite sponge pads, and the transfer membrane were soaked in the transfer buffer for 15 min with continuous shaking. The blotting stack was assembled on the top of stainless steel grid cathode located in the trough of the frame stand of the Mini Blot Module, to which a small amount of transfer buffer was added.

After the assembly of the transfer module the lid of the electroblotting module was pressed onto the blotting stack and fixed with the clamps after turning assembled blotting unit upright and then filled with the transfer buffer (about 2.5
Thereafter, the fully assembled module was inserted into the outer tank and the safety cover with leads was replaced. The red lead was connected to the anode and the black lead to the cathode, and the proteins transfer was allowed to move anions to the direction of anode.

The transfer process was performed at 4°C for over night using a constant voltage of 35V. After the completion of the blotting, the NC membrane was immediately removed and placed in the Ponceau-S stain and checked for transferred of protein bands from SDS-PAGE onto NC membrane.

**Immunostaining of the NC Membranes**

Immunostaining was carried out according to the instruction manual provided with the Alkaline Phosphatase (AP), Western Blotting Kit that was used in the immunostaining of the electroblotted NC membranes. All of the incubations were performed in a minimum of 5 ml of solutions in each step with continuous shaking at room temperature.

The electroblotted NC membrane was incubated in the blocking solution (5 % non-fat dry milk in TBS buffer) for 30 minutes. Afterwards, the membrane was incubated with the anti GST polyclonal primary antibodies raised in rabbit diluted (1/5,000 diluted) in the blocking solution for 30 minutes. The membrane was then washed five times, each for 5 min with TBS and incubated with the secondary antibody (goat anti-rabbit IgG-ALP conjugate) with the 1/5000 dilution in TBS for 5 hours. Afterwards, the membrane was washed three times, 5 min each, again with TBS and the AP color developing solution (BCIP/NBT) was added. The specific protein bands started to appear after 10 - 30 min. Finally, the membranes were carefully dried and the images were obtained using a scanner connected to the computer.

**Isolation of genomic DNA :**

Genomic DNA was isolated by the method of Herrmann and Kalden (1994). The methodology is as follows:
1. **Preparation of lysis buffer:** 1% nonidet p-40 in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM EDTA

2. 1% SDS

3. **RNase A stock:** 500μg / ml of sterilized water

4. **Proteinase K stock:** 25mg / ml of sterilized water

5. **10M Ammonium acetate**

Both control and AC treated fresh rat liver tissues were washed in phosphate buffer saline (PBS) to remove fat debris and excessive blood and then homogenized in above said lysis buffer (this is to lyse the cell to release genomic DNA) and centrifuged for five minutes at 1600 x g. With the resultant supernatant the procedure was repeated again.

The resultant supernatant (the resuspended nuclei as control for the complete recovery of apoptic DNA fragments) were brought to 1% SDS and treated for two hours with RNase A (final concentration 5μg / ml) at 56°C followed by digestion with proteinase K (final concentration 2.5μg / μl) for at least two hours at 37°C. After addition half volume of Ammonium acetate the DNA was precipitated with 2.5 ml of ethanol and dissolved in gel loading buffer and then separated by 0.8% agarose gel electrophoresis.

**Histopathological Analysis**

Fixation and staining of control and treated samples: The liver tissues were isolated from control and treated rats were gently rinsed with physiological saline to remove blood and debris adhering to them. They were fixed in Bouin’s solution until processing. The tissues were washed through running tap water, overnight to remove Bouin’s solution. After dehydrating through a graded series of alcohols, the tissues were cleaned in methylbenzoate and embedded in paraffin wax. Sections were cut at 6 μ thickness and stained with haematoxylin (Harris, 1900) and counter stained with Eosin dissolved in 95% alcohol. After
dehydration and clearing, sections were mounted using Canada balsam. Histological examinations of the tissues were followed according to Humason, 1972 and the specimens were observed under the light microscope.

Statistical Analysis

All the data related to rat liver and their results of treatments presented as the mean ± standard deviation (SD) for the calculation of three experiments. Student’s “t” tests were performed to identify the time points at which the mean for acrylamide treated rat liver differed from the mean for the respective vehicle controls.