CHAPTER - II

MATERIALS AND METHODS

HOUSING AND CARE OF ANIMALS

All the experimental protocols of animal studies were sanctioned by Institutional Animal Ethics Committee of Gujarat University, Ahmedabad and approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg – 167/1999/CPCSEA), New Delhi, India. Healthy adult male albino mice of Swiss strain (*Mus musculus*) weighing 30-35 gm were obtained from Cadila Pharmaceuticals, Dholka, Ahmedabad, India. Animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (temperature 25±2°C, relative humidity 50-55% and 12 h light/dark cycle). They were fed with certified pelleted rodent feed supplied by Amrut feeds, Pranav Agro Industries Ltd., Pune, India and potable water *ad libitum*. Animals were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India (1991).

CHEMICALS

Bisphenol A was purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India and was of analytical grade. All the other chemicals used in the study were of AR grade and procured from Hi Media Laboratories Pvt. Ltd., Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Sigma – Aldrich St. Louis, MO, USA. Olive oil was obtained from Figaro, Madrid, Spain.
INSTRUMENTATION

The optical density measurements were carried out on Systronics-Visiscan 167 spectrophotometer and Systronics 118 UV-Vis spectrophotometer (Ahmedabad, India).

Based on our literature survey and preliminary study the present work was divided into four parts:

PART I: Evaluation of bisphenol A toxicity in liver of mice

PART II: Phytochemical screening and analysis of antioxidative potential of green tea extract

PART III: Evaluation of bisphenol A toxicity on some \textit{in vitro} and \textit{in silico} models and its amelioration by green tea extract, and

PART IV: Ameliorative effect of green tea extracts against bisphenol A – induced toxicity in mice.
PART I
EVALUATION OF BISPHENOL A TOXICITY IN LIVER OF MICE

STUDY DESIGN

The initial study was focused to determine the best possible dose of bisphenol A to induce liver damage in mice. Three different doses (1/10\textsuperscript{th} – 240 mg/kg bw/day, 1/20\textsuperscript{th} – 120 mg/kg bw/day and 1/30\textsuperscript{th} – 80 mg/kg bw/day) of bisphenol A were selected based upon LD\textsubscript{50} (2400 mg/kg bw) and standardized (MSDS, 2004; Hussein and Eid, 2013). Fifty animals were randomized in five different groups and caged separately. Animals of group I (untreated control) were maintained without any treatment and given free access to feed and drinking water. Animals of group II (vehicle control) received olive oil (0.2 ml/animal/day) for 30 days as olive oil was used as vehicle to dissolve bisphenol A. Animals of group III, IV and V were treated with low dose (80 mg/kg bw/day), mid dose (120 mg/kg bw/day) and high dose (240 mg/kg bw/day) of bisphenol A respectively (Table 2.1). All treatments were given orally using a feeding tube attached to a hypodermal syringe for 30 days.

Mortality rate, behavioral and clinical changes were noted throughout the experiments. The body weight of control and all treated groups of mice were recorded to the nearest gm on a weighing balance. At the end of the treatment, the animals were weighed and humanly sacrificed by cervical dislocation. Fresh flowing blood of animals were collected by cardiac puncture in non–anticoagulant added tubes, allowed to clot and centrifuged at 1000 x g for 10 min at 4°C. The non–haemolysed serum samples were obtained, then stored under cold conditions and used for biochemical analysis. The liver was dissected out, blotted free of blood and weighed to the nearest mg on a balance and used for histopathological and biochemical analysis.
Table 2.1: Experimental protocol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Duration of treatment</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Untreated control</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>II</td>
<td>Olive oil control (0.2 ml/animal/day)</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>III</td>
<td>BPA Low dose (80 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>IV</td>
<td>BPA Mid dose (120 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>V</td>
<td>BPA High dose (240 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
</tbody>
</table>
HISTOPATHOLOGICAL EXAMINATION

Histopathological studies were carried out using the standard technique of hematoxylin and eosin (H & E) staining. Liver tissues of all control and treated animals were preserved in 10% neutral buffered formalin immediately after the autopsy. The tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58 to 60°C mp). 5 μm thick sections were cut on a rotary microtome and stained with H & E, dehydrated in alcohol, cleared in xylene, mounted in DPX and examined under a light microscope.

BIOCHEMICAL ANALYSIS

Estimation of protein, lipid, carbohydrate and nucleic acid contents

Protein content:

Protein content in liver and serum samples were estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Protein reacts with phenol reagent of Folin Ciocalteu, resulting in a deep blue coloration. The color development is due to two reactions occurring simultaneously i.e., the reaction of alkaline copper sulfate solution with peptide bonds and reaction of phosphomolybdic and phosphotungstic acids by aromatic amino acids present in the protein. The blue color that develops is quantitatively proportional to the total protein, which was measured at 540 nm. The protein contents were expressed as mg/100 mg tissue weight in case of liver and as gm/dl for serum.

Glycogen content:

The glycogen content of the liver was estimated by the method of Seifter et al. (1950). The glycogen present in tissue is converted to glucose, which reacts with anthrone reagent to give a green colored product. The color intensity was read at 620 nm which was directly proportional to glycogen content in liver. The glycogen content was expressed as mg/100 mg tissue weight.
**Total lipid content:**

Total lipid content of the liver was estimated according to the method of Fringes *et al.* (1972). Olive oil was used as a standard. Lipid on being heated with sulfuric acid followed by addition of vanillin and phosphoric acid produces a pink color whose optical density is measured at 530 nm. The total lipid content was expressed as mg/100 mg tissue weight.

**Cholesterol content:**

The concentration of cholesterol was estimated in the liver by the method of Zlatki *et al.* (1953). Cholesterol forms a colored complex with ferric chloride (FeCl$_3$) in the presence of concentrated sulphuric acid and glacial acetic acid which can be measured at 540 nm. The cholesterol content was expressed as mg/100 mg tissue weight in liver.

**Estimation of nucleic acid:**

**Extraction:**

A known weight of fresh liver tissue was homogenized in 5 ml of 5% cold TCA and the homogenate was kept at 0-4°C for 30 min. The precipitate obtained after centrifugation (10 min at 1000 x g) was dissolved in 5 ml of 5% cold TCA and left for 30 min at 0-4°C. Thereafter, centrifugation (10 min at 1000 x g) was carried out and the precipitate obtained was dissolved in alcohol: ether (1:3, v/v) mixture and left for 30 min at 50°C. This process was repeated once again. The contents were centrifuged at 1000 x g for 10 min and the supernatant was discarded. The lipid free pellet obtained was dissolved in 5 ml of 0.1 N KOH and incubated at 37°C for 16-18 h. Then 0.17 ml of 6 N HCL and 5 ml of 10% TCA were added to the incubated suspension and precipitate was allowed to be formed at 4°C for 30 min. After centrifugation at 1000 x g for 10 min the supernatant was separated and used for estimation of RNA. The pellet containing DNA and protein was heated at 90°C for 15 min after adding 5 ml of 5% TCA. The supernatant
was then separated by centrifugation (10 min at 1000 x g) after cooling at 4°C for 30 min and used for estimation of DNA.

**Deoxyribonucleic acid (DNA):**

The estimation of DNA in the liver was carried out by the method of Giles and Meyer (1965). The DNA in the supernatant reacts with diphenylamine to give a blue colored complex whose optical density was read at 620 nm. The DNA content was expressed as μ moles/100 mg tissue weight.

**Ribonucleic acid (RNA):**

The estimation of RNA in liver was carried out by the method of Schneider (1945). The RNA in the supernatant reacts with the orcinol reagent to give a greenish color, whose absorbance was read at 670 nm. The concentration of RNA was expressed as μ moles/100 mg tissue weight.

**ESTIMATION OF LIVER MARKER ENZYMES**

**Alanine transaminase (E.C.2.6.1.2) activity:**

The alanine transaminase (ALT) activity in liver and serum was assayed by the method of Reitman and Frankel (1957). A buffered solution of α–ketoglutarate and L-alanine was made to react with the liver homogenate for 30 min. The pyruvate formed from L-alanine by the enzymatic reaction reacts with 2, 4-dinitrophenyl hydrazine (DNPH) in alkaline medium was measured at 540 nm. The enzymatic activity was expressed as mU/mg protein/30 min in case of liver and mU/mL in case of serum.

**Aspartate transaminase (E.C.2.6.1.1) activity:**

The aspartate transaminase (AST) activity in liver and serum was assayed by the method of Reitman and Frankel (1957). Assay method is similar as described in ALT activity assay, except buffered solution contained L-aspartate instead of L-alanine and allowed to react for 1 h.
The enzymatic activity was expressed as mU/mg protein/60 min in case of liver and mU/mL in case of serum.

**Alkaline phosphatase (E.C.3.1.3.1) activity:**

The alkaline phosphatase (ALP) activity in liver and serum was determined by the method of Bessey *et al.* (1946). Alkaline phosphatase at optimum pH 10.5 catalyzes the hydrolysis of p-nitrophenyl phosphate (disodium salt) to p-nitrophenol and inorganic phosphate. The liberated p-nitrophenol reacts with sodium hydroxide to form yellow colored complex which was measured at 410 nm. The ALP activity was expressed as μmoles p-nitrophenol released/mg protein/30 min in liver and IU/mL in serum.

**Acid phosphatase (E.C.3.1.3.2) activity:**

The acid phosphatase (ACP) activity was assayed in the liver and serum by the method as described in Sigma Technical Bulletin (Sigma Technical Bulletin, MO, USA). Acid phosphatase at optimum pH 4.8 catalyzes the hydrolysis of p-nitrophenyl phosphate (disodium salt) to p-nitrophenol and inorganic phosphate. The liberated p-nitrophenol reacts with sodium hydroxide to form a yellow colored complex which was measured at 420 nm. The enzyme activity was expressed as μmoles p-nitrophenol released/mg protein/30 min in liver and IU/mL in serum.
EFFECTS ON MITOCHONDRIAL FUNCTION

Succinic dehydrogenase (E.C.1.3.99.1) activity:

The succinic dehydrogenase (SDH) activity in the liver was measured by the method of Beatty et al. (1966) using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tertazolium chloride (INT) as an electron acceptor. The electrons released as a result of enzyme activity from the substrate are taken up by INT, which was reduced to a red colored formazan. This was extracted in ethyl acetate and the absorbance was read at 420 nm. The enzyme activity was expressed as μg formazan formed/mg protein/15 min.

Adenosine triphosphatase (E.C.3.6.1.3) activity:

The liver adenosine triphosphatase (ATPase) activity was assayed by using the method of Quinn and White (1968). Adenosine triphosphatase causes hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate (i.p.). The liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (1925). The optical density was read at 660 nm. The enzyme activity was expressed as μmoles inorganic phosphate released/mg protein/30 min.

EFFECTS ON LIPID PEROXIDATION AND ANTIOXIDATIVE DEFENCE MECHANISM

Lipid peroxidation:

The level of lipid peroxidation (LPO) in the liver was measured by the method as described by Ohkawa et al. (1979). This method is based on the formation of a red chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with products of lipid peroxidation like malondialdehyde (MDA) and others collectively called as thiobarbituric
acid reactive substances (TBARS). The results were expressed as nmoles MDA formed/mg protein/60 min.

**Non-enzymatic antioxidants:**

*Glutathione content:*

The glutathione (GSH) content in the liver was measured by the method of Grunert and Philips (1951). In saturated alkaline medium, the GSH present in the tissues reacts with sodium nitroprusside to give a red colored complex which was measured at 520 nm. The glutathione content was expressed as μg/100 mg tissue weight.

*Total ascorbic acid content:*

Total ascorbic acid (TAA) content in the liver was estimated by the method of Roe and Kuether (1943). Total ascorbic acid is oxidized to dehydroascorbic acid (DHA) by Norit reagent in the presence of TCA. This couples with 2, 4-dinitrophenyl hydrazine in the presence of thiourea and sulphuric acid to yield a red colored complex which was read at 540 nm. Total ascorbic acid content was expressed as mg/gm tissue weight.

**Enzymatic antioxidants:**

*Catalase (E.C.1.11.1.6) activity:*

The catalase (CAT) activity was measured in the liver by the method of Sinha *et al.* (1972) using hydrogen peroxide as standard substrate. Potassium dichromate-acetic acid reagent was added at the interval of 0, 15, 30 and 60s to terminate the reaction. Resulting orange-yellow color was read at 590 nm. Catalase activity was expressed as μ mole H₂O₂/mg protein/min.
**Superoxide dismutase (E.C.1.15.1.1):**

The superoxide dismutase (SOD) activity in the liver was measured by the method of Kakkar et al. (1984) with slight modification. This method is based on the NADH-phenazine methosulfate (PMS) - nitroblue tetrazolium (NBT) formazan inhibition. The formazan formed at the end of the reaction was extracted into butanol layer, upon inactivation of the reaction with acetic acid. The enzyme activity was expressed as units/mg protein. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the optical density of chromogen production at 560 nm by 50% in 1 min under the assay condition.

**Glutathione peroxidase (E.C.1.11.1.9) activity:**

The glutathione peroxidase (GSH-Px) activity in the liver was assayed by the modified method of Pagila and Valentine (1976). The enzyme activity was expressed as units/mg protein/min, where 1 unit of GSH-Px equals to nmoles of NADPH consumed/mg protein/min.

**Glutathione reductase (E.C.1.6.4.2) activity:**

The glutathione reductase (GR) activity in the liver was assayed by the method of Mavis and Stellwagen (1968). The enzyme catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The decrease in absorbance was recorded for 5 min at 340 nm. The enzyme activity was calculated as nmoles NADPH consumed/mg protein/min.

**Glutathione-S-transferase (E.C.2.5.1.18) activity:**

The glutathione-S-transferase (GST) activity was measured by the method of Habig et al. (1974). The increase in absorbance was noted at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB). The enzyme activity was calculated as μmoles CDNB conjugates formed/mg protein/min.
PART II
PHYTOCHEMICAL SCREENING AND ANALYSIS OF ANTIOXIDATIVE POTENTIAL
OF GREEN TEA EXTRACT

Extract preparation:

Green tea leaves was purchased from Brook-Bond Company, Darjeeling, India. Green tea extract was prepared according to the method of Bhargava and Singh (1981) with slight modification. The finely ground 5 gm of green tea powder was mixed with 100 ml 50% ethanol in water and allowed to stand overnight for maximum extraction of polyphenols. Percolation of the extract was performed at room temperature. Collected filtrate was evaporated below 50°C to obtain a residue which was stored under refrigerated conditions. Percent yield of the extract was calculated. Polyphenolic contents of the extract were qualitatively and quantitatively analyzed using standardized methods. The antioxidative potency of both the extracts was estimated using various chemical assay systems as described below:

Qualitative analysis of polyphenolic content:

Qualitative analysis for the presence of tannins, saponins, flavonoids and alkaloids in the plant extracts were carried out using standard methods as described by Harborne (1973), Trease and Evans (1983) and Sofowara (1993).

Test for tannins:

0.5 gm of extract was dissolved in 20 ml distilled water in a test tube and then filtered. A few drops of 0.1% FeCl₃ was added and observed for brownish green or blue black color.
**Test for saponins:**

2 gm of the extract was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously. The froth was mixed with 3 drops of olive oil and shaken vigorously and then observed for formation of emulsion.

**Test for flavonoids:**

5 mL of dilute ammonia solution was added to plant extract, followed by addition of concentrated H₂SO₄. A yellow coloration indicated presence of flavonoids.

**Test for alkaloids:**

The test was performed with Mayer’s, Wagner’s and Dragendorff’s reagents. Observation of white, brown, orange coloration indicated the presence of alkaloids.

**Quantitative analysis of polyphenolic content:**

The quantitative analysis of the plant extract was performed using standard protocols as mentioned below:

**Total phenolic content (TPC):**

Total phenolic content of hydro-alcoholic extract was estimated by the method as described by Singleton *et al.* (1999). Briefly, the extract was made to react with Folin-Ciocalteu reagent in the presence of sodium carbonate to form a blue colored complex which was read at 760 nm. Total phenolic content of the extract was expressed as mg gallic acid equivalents/gm dry weight of extract.

**Flavonoid content:**

The flavonoid content in the extract of green tea was estimated by the method of Lamaison and Carnat (1990). Briefly 1 ml of plant extract was mixed with 1 ml of aluminium chloride
reagent and resultant color was read at 430 nm. The flavonoid content of the extract was expressed as mg quercetin equivalents/gm dry weight of extract.

**Tannin content:**

The tannin content in the green tea extract was estimated by the method of Prince and Butler (1977). Plant extract was allowed to react with (Potassium hexacyanoferrate) K$_3$Fe(CN)$_6$-FeCl$_3$ (Ferric chloride) reagent for five min and the intensity of color develop was measured spectrophotometrically at 720 nm. The tannin content of the extract was expressed as mg rutin equivalents/gm dry wt. of extract.

**Ascorbic acid content:**

Ascorbic acid, also known as vitamin C, is one of the most abundant antioxidant present in plant, was quantified by the method of Jagota and Dani (1982). The ascorbic acid content of plant extract was expressed as μg/gm dry weight of extract.

**Antioxidative potency:**

**Superoxide radical scavenging assay:**

Superoxide radical scavenging activity was assessed by the method of Liu et al. (1997). In the PMS/NADH-NBT system, superoxide anion derived from dissolved O$_2$ by PMS/NADH coupling reaction reduces NBT. The addition of various concentrations of hydro-alcoholic extract resulted in decreased color intensity which was read at 560 nm against blank to determine the quantity of the formazon generated. Inhibitory constant (IC$_{50}$) value of the extract (concentration required to scavenge 50% of the radicals) was calculated.
**Hydroxyl radical scavenging assay:**

The hydroxyl radical scavenging activity of the extract was estimated by the method of Halliwell *et al.* (1987), where radicals were generated from Fe$^{3+}$/ascorbate/EDTA/H$_2$O$_2$ from Fenton’s reaction. Briefly different concentrations of plant extract were made to react with 2-deoxy-2-ribose, H$_2$O$_2$, FeCl$_3$ and EDTA. The reaction was initiated by the addition of ascorbic acid. After incubation for 90 min the reaction was terminated by addition of thiobarbituric acid (TBA) and resulting color was read at 590 nm. Percent inhibition by various concentrations of plant extract and IC$_{50}$ was calculated.

**Nitrous oxide radical scavenging assay:**

Nitrous oxide radical scavenging activity was measured using method of Sreejayan and Rao (1997). Various concentrations of plant extract was incubated with 10 mM sodium nitroprusside and incubated for 150 min. After incubation, Griess reagent was added to the tubes and absorbance of chromophore formed was read at 590 nm. IC$_{50}$ value and percent inhibition by various concentrations of plant extract was calculated comparing the absorbance of control and test compounds against blank.

**Reducing ability:**

Reducing power of hydro-alcoholic green tea extract was evaluated using method of Yildirim (2000). Briefly, various concentrations of hydro-alcoholic green tea extract was mixed with potassium phosphate buffer and potassium hexacyanoferrate [K$_3$Fe(CN)$_6$] and incubated for 30 min. Reaction was terminated by addition of trichloroacetic acid (TCA) followed by addition of FeCl$_3$. Reductant (antioxidant) present in the tested plant samples reduces Fe$^{3+}$/ferricyanide complex to the ferrous form (Fe$^{2+}$) resulting in formation of perl’s Prussian blue color which was read at 700 nm.
PART III

EVALUATION OF BISPHENOL A TOXICITY IN SOME ON VITRO AND IN SILICO MODELS AND ITS AMELIORATION BY GREEN TEA EXTRACT

IN VITRO

A. Effect of bisphenol A and green tea extract in mice liver homogenate:

Healthy adult Swiss strains of male albino mice were used in the study. Animals were humanely sacrificed by cervical dislocation, liver was dissected out and blotted free of blood. Liver homogenates (10%) were prepared in 0.1 M phosphate buffered saline (pH 7.4) and treated with five different concentrations of bisphenol A (50, 100, 150, 200 and 250 μg/ml). Highest concentration of bisphenol A (250 μg/ml) was taken further to evaluate protective effect of various concentrations (10, 20, 30, 40 and 50 μg/ml) of green tea extract and following parameters were studied.

Methodology used for measurement of LPO, CAT, SOD, SDH and ATPase activities were as described in part I in vivo study section.

B. Effect of bisphenol A and green tea extract on red blood cells:

Preparation of RBC suspension:

Red blood cell (RBC) suspension was prepared according to the method as described by Verma and Raval (1991). Participants were recruited to participate in the study after approval of Institutional Ethics Committee of Zoology Department of Gujarat University, Ahmedabad. Briefly, venous blood samples from well fed healthy adult human beings of age group 25-30 years were collected in EDTA vials for preparation of RBC suspension. Collected blood samples were diluted with normal saline (0.9% NaCl w/v) and centrifuged at 1,000 × g for 10 min. RBC pellets
were collected, washed twice with normal saline and diluted to obtain a cell density of $2 \times 10^4$ RBC/ml which was used for studying hemolysis.

* Treatments of erythrocytes suspension for hemolysis:*

Effect of different concentrations of BPA (50-250 μg/ml) on erythrocytes cell suspension was studied. Highest concentration of BPA (250 μg/ml) was selected to evaluate protective effect of green tea extract. Hydrogen peroxide ($H_2O_2$) – a well known potent oxidant, was used as standard to investigate mechanism of bisphenol A – induced oxidative damage to human RBCs.

*Following sets of treatments were prepared:*

1. Control tubes having 2 ml of erythrocytes suspension.
2. Antidote control tubes containing 50 μg/ml of green tea extract and 2 ml of erythrocytes suspension.
3. Hydrogen peroxide - treated tubes having different concentrations of $H_2O_2$ (0-0.25 mM) and 2 ml of erythrocytes suspension.
4. Bisphenol A- treated tubes having different concentrations of bisphenol A (0-250 μg/ml) and 2 ml of erythrocytes suspension.
5. Tubes containing bisphenol A (50 μg/ml) along with different concentrations of $H_2O_2$ (0-0.25 mM/ml) and 2 ml erythrocytes suspension.
6. Tubes having different concentrations of bisphenol A (0-250 μg/ml) along with $H_2O_2$ (0.05 mM/ml) and 2 ml of erythrocytes suspension.
7. Tubes having 250 μg/ml bisphenol A along with varying concentrations of green tea extract (0-50 μg/ml) and 2 ml erythrocytes suspension.
8. To achieve, 100% hemolysis, 2 ml distilled water was added to 2 ml of erythrocytes suspension.
Total volume of each tube was made up to 4 ml with addition of normal saline. All sets of tubes were incubated with intermittent shaking for 4 h at 37°C. After incubation, tubes were centrifuged at 1,000×g for 10 min and collected supernatant was read spectrophotometrically at 540 nm. Percent hemolysis was calculated by using the formula.

\[
\text{% hemolysis} = \left( \frac{\text{Absorbance of individual tubes}}{\text{Absorbance with 100% hemolysis}} \right) \times 100
\]

C. Interaction of green tea extract with bisphenol A

UV analysis:

Green tea extract (0.25 mg/ml) and bisphenol A (4 mg/ml) solutions were prepared in ethanol and water (1:1) mixture and scanned (200-400 nm) individually as well as in a mixture (v/v) on UV spectrophotometer.

In Silico

Docking Studies

Binding capacity of bisphenol A with erythrocyte proteins (hemoglobin, catalase and glutathione peroxidase) were inspected using molecular docking tool, which showed presence of various hydrogen bonds of the compound with the proteins.

Preparation of Protein Target Structure and Ligands:

The X-ray crystal structure of hemoglobin (PDB id-2HHB), catalase (PDB id-1QQW) and glutathione peroxidase (PDB id-2f8A) retrieved from the Protein Data Bank (PDB ID- 2YK0) were subjected to energy minimization using GROMOS96 utility (without reaction field) implemented in Swiss-Pdb Viewer 4.0.1.

Ligand structure of bisphenol A was retrieved in Structure Data Format (SDF) from NCBI PubChem and wherever applicable, drawn using ChemSketch v. 10 with reference to 2D
structures available in the literature. The structure of bisphenol A was energy minimized using molecular mechanics geometry optimization module implemented in HyperChem. AMBER force field with distant dependent dielectric constant, scale factor for electrostatic and van der Waals forces set to 0.5 and without any cutoffs to bond types and its lengths were chosen to determine global minimum energy conformation. Subsequently, all the structures were minimized and exported to hard disk.

**Active site prediction:**

This prediction wasn’t carried out for protein structures co-crystallized with ligand as the ligand binding site was implicated as active site for docking with the ligand dataset. Structures with unbound ligands were computationally analyzed for active site using Q-Site Finder. It detects pockets on the protein surface through calculation of van der Waals interaction energies using a methyl probe and probes with favorable interaction energies were clustered and ranked.

**Virtual screening:**

Bisphenol A under study were virtually screened (docked) into the binding site of the target proteins as hemoglobin (PDB id-2HHB), catalase (PDB id-1QQW) and glutathione peroxidase (PDB id-2f8A) using Argus Lab 4.0.1 from Planaria Software LLC. To enable fast sampling, the binding site was constructed which consist of all residues that have at least one atom within 3.5 Å from any atom in the co-crystallized ligand. This procedure of constructing binding site was applied for protein structures with co-crystallized ligand. A different approach was performed for proteins with unbound ligand whose active site was predicted using Q-Site Finder. Best 3 scored pockets was computationally analyzed for each protein target using Jmol Java plugin implemented in Q-Site Finder and the amino acids embedded in the predicted cavity volume was utilized as active site residues.
These two approaches generally gave a good representation of the important residues in the binding pocket for a protein target. A grid box of size 22 X 15 X 16 with atom scaling of 0.40 Å was generated and high precision Argus Dock engine with a Score as scoring function were selected. After grid generation, the ligands were flexibly docked with the protein and 1000 poses were generated, among which best 10 poses of low-energy which were clustered in rank 1, were examined. Argus Dock engine makes use of ligand torsionality as a hierarchical tree in which the root’s node (group of bonded atoms that do not have rotatable bonds) is placed in a search point inside a grid comprised of residues of the active site. A set of diverse and energetically favorable translations are generated and poses that survives in torsional search through an approximate exhaustive search are retained and finally clust.

PART IV

AMELIORATIVE EFFECT OF GREEN TEA EXTRACTS AGAINST BISPHENOL A – INDUCED TOXICITY IN MICE

STUDY DESIGN:

Based on the results of part – I, the high dose (HD) of BPA was chosen to evaluate the hepatoprotective effect of green tea extract. Sixty animals were divided into six groups. Animals of group I received 0.2 ml olive oil/animal/day for 30 days and marked as vehicle control. Antidote control groups (group II and III) animals were orally administered with green tea extract (100 mg/kg body weight/day) and Liv. 52 (100 mg/kg bw/day) respectively, for 30 days. Animals of group IV received HD (240 mg/kg body weight/day) of BPA for 30 days. Animals of group V, VI and VII were treated with HD
<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Duration of treatment</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Olive oil Control (0.2ml/animal/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>Green tea control (100 mg/ kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>III</td>
<td>Liv. 52 control (100 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>BPA - HD (270 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>BPA - HD + GT (25 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>VI</td>
<td>BPA - HD + GT (50 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>VII</td>
<td>BPA - HD + GT (100 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>VIII</td>
<td>BPA – HD + Liv. 52 (100 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
of BPA along with 25, 50 and 100 mg/kg bw/day of green tea extract for 30 days. Animals of **group VIII** received HD (240 mg/kg bw/day) of BPA along with 100 mg/kg bw/day of Liv. 52 for 30 days (Table 2.2).

Behavioral and clinical changes throughout the treatment were observed in the animals of all groups. On completion of the treatment of 30 days, animals were humanely sacrificed on 31st day by cervical dislocation. The liver was dissected out carefully, blotted free of blood, weighed to the nearest mg and utilized for the study. For studying serum parameters, the blood collected by cardiac puncture was allowed to clot and centrifuged at 1000 × g for 10 min to obtain serum. The obtained serum samples were stored under refrigerated conditions and used within 24 h.

**HISTOPATHOLOGICAL STUDIES:**

Tissues of all control and treated animals were preserved in 10% neutral buffered formalin for histopathological examination after autopsy. The standard technique of H & E was followed as described in part I materials and methods section.

**BIOCHEMICAL ANALYSIS**

All biochemical analysis (protein, lipid, carbohydrate, nucleic acid contents, liver marker enzymes, SDH, ATPase, lipid peroxidation, enzymatic and non-enzymatic antioxidants) were carried out using standard protocols as described in part I materials and methods section.

**Hepatoprotective index:**

The liver protecting activity of green tea extract was expressed as hepatoprotective percentage (H) (Chandan et al., 2007), which was calculated using the formula:

\[ H = \left(1 - \frac{T - V}{C - V}\right) \times 100 \]
Where $T$ is the mean value of plant extracts along with the BPA, $C$ is the mean value of BPA alone and $V$ is the mean value of vehicle control animals.

**Statistical analysis:**

The results were expressed as the mean ± S.E.M. The data were statistically analyzed using one way analysis of variance (ANOVA) followed by Turkey’s test in Graph pad prism 5 (graph pad, software, USA). Statistical significance was accepted with $p< 0.05$. Correlation Coefficient was measured to estimate the strength of linear association between two variables. Pearson’s correlation analysis was used to find the correlation between dose administration and alteration in biochemical parameters in liver and serum of mice.