CHAPTER – II

MATERIALS AND METHODS
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CHEMICALS

Butyl p-hydroxybenzoic acid 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.

ANIMALS

Inbred adult healthy Swiss strain female albino mice (*Mus musculus*) weighing 30-35 gm were obtained from Torrent Research Centre, Bhat, Gandhinagar-382 428, India. Animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (temperature 25±2 °C, 12 h light/dark cycle and relative humidity 50-55%). They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and water *ad libitum*. All the experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experiment on Animals (Reg-167/1999/CPCSEA), New Delhi, India. Animals were handled according to the guidelines published by Indian National Science Academy, New Delhi, India (1991).
Objectives of the study planned:

- To study the toxic effects of butylparaben using *in vitro* and *in vivo* models, and
- To study the ameliorative role of *O. sanctum* (tulsi) on toxic effects induced by butylparaben.

Based on our literature survey and preliminary study the present work was divided into five parts.

(I) Phytochemical screening and antioxidative potency analysis of two different extracts (aqueous and alcoholic) of *O. sanctum*

(II) Evaluation of butylparaben - toxicity on some *in vitro* models and its amelioration by *O. sanctum* extract

(III) Toxicological evaluation of butylparaben in liver of mice

(IV) Protective effect of *O. sanctum* extract in liver of butylparaben - treated mice

(V) Quantification of one of the active component (luteolin) of *O. sanctum* (aqueous extract) using HPLC as well as to study its chemical interaction with butylparaben
PART-I

PHYTOCHEMICAL SCREENING AND ANTIOXIDATIVE POTENCY ANALYSIS OF TWO DIFFERENT EXTRACTS (AQUEOUS AND ALCOHOLIC) OF *O. SANCTUM*

*Extract preparation:*

Aqueous and alcoholic extracts of *O. sanctum* (tulsi) were prepared according to the method of Bhargava and Singh (1981) with slight modification. Fresh leaves of *O. sanctum* were collected from botanical garden of Botany Department of Gujarat University, Ahmedabad in the months of August-September, 2008. Leaves were washed, shade-dried and finely powdered for extraction. Aqueous polyphenols were extracted in distilled water and stored at 4°C. Absolute ethanol was used to extract the alcoholic polyphenols of *O. sanctum* and percent yield of both the extracts were calculated. Herbarium specimen was prepared and authenticated by Dr. Hitesh Solanki, Reader, Department of Botany, Gujarat University, Ahmedabad. Polyphenolic contents of both the extracts were qualitatively and quantitatively analyzed using standardized methods. Antioxidative potency of both the extracts was estimated using various chemical assay systems as described below:

*Polyphenolic content:*

**Total phenolic content (TPC):**

Total phenolic content of aqueous and alcoholic extracts were estimated by the method as described by Singleton *et al.* (1999). Briefly extract phenols react with Folin-ciocalteu reagent in presence of sodium carbonate to form blue colored complex
which was read at 760 nm. Various concentrations of gallic acid were used to plot standard curve. Total phenolic content of the extracts were expressed as mg gallic acid equivalents/gm dry wt. of extract.

**Flavonoid content:**

The flavonoid content in the extract 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 both the extracts was expressed as mg quercetin equivalents/gm dry wt. of extract.

**Tannin content:**

Tannin content of both the extracts were estimated by the method as described by Price and Butler (1977). Plant extracts were allowed to react with $K_3Fe(CN)_6 - FeCl_3$ reagent for five min and the intensity of color developed 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 content of plant extracts were expressed as µg/gm dry wt. 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
O₂ by PMS/NADH coupling reaction reduces NBT. Addition of various concentrations of aqueous and alcoholic extracts resulted in decreased color intensity which was read at 560 nm against blank to determine the quantity of the formazon generated. IC₅₀ values of the extracts (concentration required to scavenge 50% of the radicals) were calculated.

**Hydroxyl radical scavenging assay:**

Hydroxyl radical scavenging activity of the extracts was estimated by the method of Halliwell *et al.* (1987), where radicals were generated from Fe³⁺/ascorbate/EDTA/H₂O₂ from Fenton’s reaction. Briefly different concentrations of plant extracts were made to react with 2-deoxy-2-ribose, H₂O₂, FeCl₃ and EDTA. The reaction was initiated by the addition of ascorbic acid. After incubation of 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 extracts and IC₅₀ were calculated.

**Nitrous oxide radical scavenging assay:**

Nitrous oxide radical scavenging activity was measured using method of Sreejayan and Rao (1997). Various concentrations of plant extracts were 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. IC₅₀ values and percent inhibition by various concentrations of plant extracts were calculated comparing the absorbance values of control and test compounds against blank.
DPPH radical scavenging assay:

Ability of plant extracts to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured by the method of Gyamfi et al. (1999). DPPH is a purple color radical compound which changes to stable compound having yellow color by reacting with antioxidant compounds. Addition of 0.1 mM DPPH solution in various concentrations of extracts in presence of tris-HCl buffer (50mM, pH-7.4) resulted in decreased absorbance which was measured at 517 nm. Percent inhibition was calculated by measuring the absorbance of plant extract treated samples against blank. IC$_{50}$ values for both the extracts were calculated.

Ferric reducing antioxidant power (FRAP):

Ferric reducing antioxidant power (FRAP) reagent was prepared as described by Benzie and Strain (1996) and was used to measure total antioxidative potency of both the extracts. Plant extracts were allowed to react with FRAP reagent containing acetate buffer, 2,4,6-tripyridyl-S-triazine (TPTZ) and FeCl$_3$ solutions. Resulted color was read against blank at 593 nm. Aqueous solution of Fe (II) was used to make calibration curve. FRAP value was expressed as $\mu$M Fe$^{2+}$/gm dry wt. of extract.

Reducing ability:

Reducing power of aqueous and alcoholic plant extracts was evaluated using method of Yildirim (2000). Briefly various concentrations of aqueous and alcoholic extracts were 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$^{2+}$/ferricyanide complex to the ferrous
form (Fe$^{2+}$) resulting in formation of perl's Prussian blue color which was read at 700 nm.

**Fe$^{2+}$ chelating ability:**

The Fe$^{2+}$ chelating activity of both the extracts was estimated using the method of Dinis et al. (1994). Plant extracts were allowed to react with ferrozine (5 mM) in presence of FeCl$_3$ (2 mM). Blue colored Fe$^{2+}$–ferrozine complex formed was read at 562 nm. Chelating ability of the extracts was compared with EDTA (0.01 mM). Percent inhibition and IC$_{50}$ value for both the extracts were calculated by comparing test samples with the control.

**ACUTE ORAL TOXICITY STUDY OF OCIMUM SANCTUM:**

Acute oral toxicity of aqueous and alcoholic extract of *O. sanctum* was studied using up and down procedure as per the guideline provided by Organization for Economic Co-operation and Development (OECD – 425, 2001). Female Swiss albino mice were fasted for 3 h prior to the dosing period and orally administered with single dose of the extracts and observed for mortality up to 48 h. Dosage for the next animal was determined based on the results of the earlier dose. Animals were kept under observation for 14 days.
PART—II

EVALUATION OF BUTYLPARABEN TOXICITY ON SOME IN VITRO MODELS AND ITS AMELIORATION BY O. SANCTUM EXTRACT

1. Effect of butylparaben and O. sanctum extracts in mice liver homogenate:

Adult healthy Swiss strain of female albino mice was used for the study. Animals were sacrificed by cervical dislocation and liver was dissected out and blotted free of blood. 10% tissue homogenate was prepared in tris-HCl buffer (0.1 M, pH-7.4) and treated with five different concentrations of butylparaben (50, 100, 150, 200 and 250 µg/ml). Highest concentration of butylparaben (250 µg/ml) was taken further to evaluate protective effect of various concentrations (25, 50, 75, 100 and 125 µg/ml) of O. sanctum extracts (aqueous and alcoholic). Tissue homogenates were incubated for 1 hr with butylparaben and/or O. sanctum extracts (aqueous and alcoholic) and following parameters were studied.

Lipid peroxidation:

Lipid peroxidation in liver homogenate was measured by estimating malonyldialdehyde (MDA) – intermediary product of lipid peroxidation by TBARS method as described by Devasagayam and Tarachand (1987). The formed MDA was measured spectrophotometrically at 530 nm. The levels of lipid peroxidation was expressed as nmole of MDA formed/mg protein/60 min.
Superoxide dismutase:

Activity of superoxide dismutase (SOD) was measured by the method of Marklund and Marklund (1974). Superoxide radical formed reacts with pyrogallol dye causing its autooxidation resulting in blue color which is read at 470 nm. Superoxide dismutase activity in liver tissue was expressed as U/mg protein.

Catalase:

Catalase (CAT) activity in tissue was measured 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 \( \mu \text{m H}_2\text{O}_2/\text{mg protein/min} \).

Glutathione:

Glutathione content (GSH) was determined by Ellman’s reaction using 5’5’-dithio-bis-2-nitrobenzoic acid (DTNB) as described by Moron et al. (1979). The amount of reduced glutathione was measured at 412 nm on spectrophotometer and was expressed as \( \mu \text{g of GSH/mg protein} \).

Protein:

Protein content was measured in liver by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Reaction of protein with Folin Ciocalteau reagent results in blue color which is due to two reactions occurring simultaneously i.e., the reaction of alkaline copper sulphate solution with peptide bonds and reduction of phosphomolybdic and phosphotungstic acids by aromatic amino acids present in the protein. Resulting blue color was measured at 540 nm. The protein contents were expressed as mg/100 mg tissue weight.
2. Effect of butylparaben and *Ocimum sanctum* on human blood:

In another *in vitro* model - human blood was used to study toxicity of butylparaben and its amelioration by *O. sanctum* extract. Venous blood was collected from well-nourished healthy volunteers (25-30 years age group) in EDTA vials.

*Measurement of Plasma MDA levels:*

In first part of experiment whole blood was treated with various concentrations of butylparaben (50, 100, 150, 200 and 250 µg/ml) and incubated for 1 h. After incubation period tubes were centrifuged at 1,000 × g for 10 min and plasma samples were collected. Plasma MDA level was measured according to the method described by Stocks and Dormancy (1972).

Second part of experiment was designed to evaluate protective effect of *O. sanctum* extract on butylparaben-induced toxicity. Whole blood was treated with butylparaben (250 µg/ml) along with five different concentration of aqueous extract of *O. sanctum* (25, 50, 75, 100 and 125 µg/ml). Tubes were centrifuged at 1,000 × g for 10 min after the incubation period. Collected plasma samples were used for TBARS assay and estimated MDA levels were expressed as nmole of MDA formed/mg protein/60 min. Butylparaben-induced change in lipid peroxidation was calculated (percent) by comparing it with control tubes. Extent of protection denoted by *O. sanctum* was also calculated.
Effect of butylparaben and O. sanctum 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). Briefly venous blood was collected in the EDTA vial from healthy adult well nourished human beings of 25-30 years age group. Collected blood samples were diluted with normal saline (0.9% NaCl) 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 2×10⁴ RBC/ml which was used for studying the morphology of RBCs and hemolysis.

Treatment of erythrocyte suspension for hemolysis and morphological studies:

Effect of various concentrations of butylparaben (50, 100, 150, 200 and 250 µg/ml) on erythrocyte cell suspension was studied. Highest concentration of butylparaben (250 µg/ml) was selected to evaluate protective effect of O. sanctum extract. Hydrogen peroxide – a known oxidant was used as standard to investigate mechanism of butylparaben - induced oxidative damage to human RBCs. Seven different sets of RBC suspension tubes were prepared as follow:

1) Control tubes having 2 ml RBC suspension.

2) Antioxidant control tubes containing 125 µg/ml of O. sanctum extract and 2 ml of RBC suspension.

3) H₂O₂ – treated tubes having 100 µl (0.1 M) of H₂O₂ in 2 ml RBC suspension.

4) Toxin - treated tubes containing varying concentration of butylparaben (50-300 µg/ml) in 2 ml RBC suspension.
5) Tubes having different butylparaben concentrations (50-300 µg/ml) along with 100 µl (0.1 M) of H₂O₂ and 2 ml RBC suspension.

6) Tubes containing butylparaben (250 µg/ml) along with varying concentrations of *O. sanctum* extract (10-125 µg/ml) and 2 ml of RBC suspension.

7) Tubes having 250 µg/ml butylparaben and 100 µl H₂O₂ (0.1 M) along with varying concentrations of *O. sanctum* extract (10-125 µg/ml) and 2 ml of RBC suspension.

Total volume of each tube was made up to 4 ml with addition of normal saline and was incubated for 4 h at 37°C with intermittent shaking. Samples were taken to study the morphological alterations microscopically. After incubation, tubes were centrifuged at 300 × g for 10 min and the collected supernatant was read spectrophotometrically at 540 nm and percent hemolysis was calculated.

**PART-III**

**TOXICOLOGICAL EVALUATION OF BUTYLPARABEN IN LIVER OF MICE**

**STUDY DESIGN**

Based upon LD₅₀ (13,200 mg/kg bw) three different doses (1/10th-1320 mg/kg bw/day, 1/20th-660 mg/kg bw/day and 1/30th-439.89 mg/kg bw/day) of butylparaben were selected and standardized. Fifty animals were randomized in five different groups and caged separately. Animals of group 1 (untreated control) were maintained without any treatment and given free access to feed and drinking water. Animals of group 2 (vehicle control) received olive oil (0.2 ml/animal/day) for 30 days as olive oil was used as vehicle to dissolve butylparaben. Group 3 animals
Table 2.1: Experimental protocol (Part III).

<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>BP Low dose (13.33 mg/0.2 ml/animal/day, 439.89 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>IV</td>
<td>BP Mid Dose (20 mg/0.2 ml/animal/day, 660 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>V</td>
<td>BP High Dose (40 mg/0.2 ml/animal/day, 1320 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31st</td>
</tr>
</tbody>
</table>
received low dose – LD of butylparaben (1/30th - 13.33 mg/animal/day). Animals of
groups 4 and 5 were given mid dose - MD (20 mg/animal/day) and high dose – HD
(40 mg/animal/day) of butylparaben for 30 days using a feeding tube attached to a
hypodermal syringe (Table 2.1). The above doses were calculated based on 33 gm
(mean) body weight of animal.

Mortality rate, behavioral and clinical changes were noted in the animals of all
groups. After 30 days treatment, animals were sacrificed on 31st day by cervical
dislocation. Fresh flowing blood of the animals was collected by cardiac puncture in a
vial and allowed to clot. Serum was separated by centrifuging the blood at 1,000 × g
for 10 min. Collected serum samples were stored under cold conditions and used
within 24 h. Liver was dissected out quickly, blotted free of blood, weighed and used
for biochemical analysis and histopathological studies. Relative organ weight was
calculated.

HISTOPATHOLOGICAL EXAMINATION

Liver tissues of all control and treated animals were preserved in 10% neutral
buffered formalin immediately after autopsy for histopathological examination.
Hematoxylin and eosin (H & E) staining technique was used to study histopathology
of liver tissue. 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 in H & E, dehydrated in alcohol,
cleared in xylene and mounted in DPX and examined under a light microscope.
BIOCHEMICAL ANALYSIS

EFFECTS ON PROTEIN, LIPID, CARBOHYDRATE AND NUCLEIC ACID CONTENTS

Protein content:
Methodology used for protein estimation was as described in Part II section.

Glycogen content:
Method described by Seifter et al. (1950) was used to estimate tissue glycogen content. Anthrone reagent reacts with tissue glucose coming from glycogen breakdown to give green color. The color was read at 620 nm and was directly proportionate to glycogen content of liver. The glycogen concentration 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) using olive oil as a standard. Reaction of tissue lipid with vanillin-phosphoric acid reagent resulted in pink color which was read at 530 nm. The total lipid content was expressed as mg/100 mg of tissue weight.

Cholesterol content:
Cholesterol content was estimated in the liver by the method of Zlatkis et al. (1953). Cholesterol forms a coloured complex with ferric chloride (FeCl₃) 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 of tissue weight.
Extraction of nucleic acids:

A known weight of liver was homogenized in 5 mL of cold 5% TCA and homogenate was kept at 0 - 4°C for 30 min. The precipitates obtained after centrifugation (10 min at 1,000 x g) were dissolved again in 5 mL of cold 5% TCA and left for 30 min at 0 - 4°C. Thereafter, centrifugation (10 min at 1,000 x g) was carried out and precipitates obtained were dissolved in alcohol: ether (1:3, v/v) mixture and left for 30 min at 50°C. This process was repeated once again. The tubes were centrifuged at 1,000 x g for 10 min and the supernatant was discarded. The pellet obtained finally which was lipid free was dissolved in 5 mL of 0.1N 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 precipitates were allowed to be formed at 4°C for 30 min. After centrifugation at 1,000 x g for 10 min the supernatant and the pellet were separated. The supernatant was used for RNA estimation. 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 1,000 x g) and used for DNA estimation.

Ribonucleic acid:

The estimation of ribonucleic acid (RNA) was carried out by the method of Schneider (1945). The RNA content in the supernatant reacts with orcinol reagent to give a greenish colour, which was read at 670 nm. The concentration of RNA was expressed as µmoles/100 mg tissue weight.

Deoxyribonucleic acid:

The estimation of deoxyribonucleic acid (DNA) was carried out by the method of Giles and Meyer (1965). The DNA in the supernatant reacts with diphenylamine to
give blue coloured complex whose optical density was read at 620 nm. The concentration of DNA was expressed as μmoles/100 mg tissue weight.

Assessment of liver function

ESTIMATION OF LIVER MARKER ENZYMES

Alanine transaminase (EC 2.6.1.2) activity:

The alanine transaminase (ALT) activity in liver was assayed by the method of Reitman and Frankel (1957). A buffered solution of α-ketoglutarate and L-alanine were 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 and formazone formed was measured at 540 nm. The enzyme activity was expressed as mU/mg protein/30 min in case of liver and mU/ml in case of serum.

Aspartate transaminase (EC 2.6.1.1) activity

The aspartate transaminase (AST) activity was assayed by the method of Reitman and Frankel (1957). As described in ALT activity estimation, here also buffered solution (phosphate buffer - 0.05 M of K$_2$HPO$_4$, 0.05 M of KH$_2$PO$_4$, pH 7.4) was made which contained α-ketoglutarate and L-aspartate and allowed to react with liver homogenate for 1 hr. The enzyme activity was expressed as mU/mg protein/60 min in case of liver and mU/ml in case of serum.

Alkaline phosphatase (EC 3.1.3.1) activity

Estimation of alkaline phosphatase (ALP) activity was done 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 combines with sodium hydroxide to form a yellow coloured complex which was measured at 410 nm. The alkaline phosphatase activity in liver and serum was expressed as μmoles p-nitrophenol released/mg protein/30 min.

**Acid phosphatase (EC 3.1.3.2) activity**

The acid phosphatase (ACP) activity was assayed in the liver and serum of mice 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 combines with sodium hydroxide to form a yellow coloured complex which was measured at 420 nm. The acid phosphatase activity in liver and serum was expressed as μmoles p-nitrophenol released/mg protein/30 min.

**Lactate dehydrogenase (EC 1.1.1.27) activity**

Lactate dehydrogenase (LDH) activity was measured by the method of King (1965). The method is based on the ability of LDH to convert lactate to pyruvate with the help of coenzyme nicotinamide adenine dinucleotide (NAD\(^+\)). The pyruvate formed is made to react with 2, 4-dinitrophenyl hydrazine in hydrochloric acid and the hydrazone formed was read at 540 nm. The LDH activity in liver and serum was expressed as μmoles pyruvate liberated/mg protein/min.
\textbf{\textit{\textgamma-\textit{G}lutamyl transpeptidase (EC 2.3.2.2) activity}}

The \gamma-glutamyl transpeptidase (\gamma-GT) activity in liver and serum was analysed following the method of Orlowski and Meister (1965). This enzyme catalyzes transfer of gamma glutamyl groups from gamma glutamyl peptides to suitable acceptor. The enzymatic reaction in the presence of substrate \gamma-glutamyl-p-nitroaniline results in the formation of p-nitroaniline whose release was monitored by noting increase in absorbance at 410 nm. The activity of the enzyme was expressed as \(\mu\)moles p-nitroaniline liberated/mg protein/min.

\textbf{EFFECTS ON MITOCHONDRIAL FUNCTION}

\textbf{Succinate dehydrogenase (EC 1.3.99.1) activity}

The succinate dehydrogenase (SDH) activity was measured by the method of Beatty \textit{et al.} (1966). The electrons released by the enzyme from the substrate are taken up by 2-(4-iodophenyl)-3(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) which acts as electron acceptor. Resulted red colored formazan was extracted in ethyl acetate and measured at 420 nm. The enzyme activity was expressed as \(\mu\)g formazan formed/mg protein/15 min.

\textbf{Adenosine triphosphatase (EC 3.6.1.3) activity}

The liver adenosine triphosphatase (ATPase) activity was assayed by using the method of Quinn and White (1968). Liberated inorganic phosphorus (i.p.) by ATPase activity was estimated by the method of Fiske and Subbarow (1925). The optical density was read at 660 nm. The enzyme activity was expressed as \(\mu\)moles inorganic phosphate released/mg protein/30 min.
EFFECTS ON LIPID PEROXIDATION AND ANTIOXIDATIVE DEFENSE MECHANISM

Method used for measurement of lipid peroxidation, glutathione content, superoxide dismutase and catalase activities were as described in Part II section study.

Total ascorbic acid content:

Total ascorbic acid (TAA) content in the liver was estimated by the method of Roe and Kuether (1943). TAA 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 coloured complex which was read at 540 nm against blank. The TAA content was expressed as mg/gm of tissue weight.

Glutathione peroxidase (EC 1.11.1.9) activity:

The glutathione peroxidase (GPx) activity in the liver was assayed by modified method of Pagila and Valentine (1967). The enzyme activity was expressed as units/mg protein/min, where 1 unit of GPx equals to nmoles NADPH consumed/mg protein/min.

Glutathione reductase (EC 1.11.1.9) activity:

The glutathione reductase (GR) activity in 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 (EC 2.5.1.18) activity:

The liver glutathione–S–transferase (GST) activity was assayed 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 - IV

PROTECTIVE EFFECT OF O. SANCTUM EXTRACT IN LIVER OF BUTYLPARABEN - TREATED MICE

EXPERIMENTAL PROTOCOL

Based on the results of part-III, the HD of butylparaben was chosen further to evaluate hepatoprotective effect of O. sanctum aqueous extract. Sixty animals were divided in six groups. Animals of group 1 received 0.2 ml olive oil/animal/day for 30 days and marked as vehicle control. Antidote control group (group 2) animals were given oral treatment of O. sanctum (300 mg /kg bw/day). Animals of group 3 received HD (40 mg/0.2 mL olive oil/animal/day) of butylparaben for 30 days. Animals of group 4, 5 and 6 were treated with HD of butylparaben along with 100, 200 and 300 mg /kg bw/day of aqueous O. sanctum extract (Table 2.2).

Animals were given treatment for 30 days and autopsied on 31st day. Behavioral and clinical changes throughout the experiment were also recorded. Liver were quickly isolated, blotted free of blood and used for biochemical parameters and histopathological studies. Serum parameters were performed by centrifuging blood samples at 1000 × g for 10 min. Obtained serum sample was stored under refrigerated conditions and used within 24 h.
Table 2.2: Experimental protocol (Part IV):

<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 (0.2 ml/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>Antidote control (300 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>BP High Dose (40 mg/0.2 ml/animal/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>BP Low dose (40 mg/0.2 ml/animal/day)+OS(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>V</td>
<td>BP Low dose (40 mg/0.2 ml/animal/day)+OS(200 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>BP Low dose (40 mg/0.2 ml/animal/day)+OS(300 mg/kg bw/day)</td>
<td>10</td>
<td>30</td>
<td>31&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
HISTOPATHOLOGICAL STUDIES:

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

Assessment of liver function

ESTIMATION OF LIVER MARKER ENZYMES

Measurements of ALT, AST, ALP, ACP, LDH and GT in liver were done using standard methods as described in part-III materials and methods section.

EFFECTS ON PROTEIN, LIPID, CARBOHYDRATE AND NUCLEIC ACID CONTENTS

The protein, glycogen, total lipid, cholesterol, DNA and RNA contents were estimated using standard protocols as described in part-III materials and methods section.

EFFECTS ON MITOCHONDRIAL FUNCTION

The SDH and ATPase activities were estimated as described in part-III materials and methods section.

EFFECTS ON LIPID PEROXIDATION AND ANTIOXIDATIVE DEFENSE MECHANISM

Lipid peroxidation:

Method used for measurement of lipid peroxidation was as described in Part II materials and method section.
Non - enzymatic antioxidants:

Methods used for measurement of glutathione and total ascorbic acid content were as described in Part II and III materials and method section.

Enzymatic antioxidants:

Methods used for assessment of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione transferase were as described in Part II and III materials and method section.

Hepatoprotective index:

The liver protecting activity of the O. sanctum extract was expressed as hepatoprotective percentage (H) (Prakash et al., 2008) which was calculated using the formula:

\[ H = \left(1 - \frac{T-V}{C-V}\right) \times 100 \]

where T is mean value of plant extracts along with the butylparaben, C is the mean value of butylparaben alone, and V is the mean value of vehicle control animals.

Statistical analysis

The data were statistically analyzed using SAS statistical software, version 16. The results were expressed as mean ± S.E.M. Hypothesis testing methods included one-way Analysis of Variance (ANOVA) followed by least significant difference (LSD) multiple comparison test. The level of significance was accepted with *p< 0.05.
PART V:

QUANTIFICATION OF ONE OF THE ACTIVE COMPONENT (LUTEOLIN) OF O. SANCTUM (AQUEOUS EXTRACT) USING HPLC AS WELL AS TO STUDY ITS CHEMICAL INTERACTION WITH BUTYLPARABEN

Hi-Performance Liquid Chromatography (HPLC):

Reagents:
Acetonitrile, methanol, double distill were of HPLC grade.

Standard stock (Luteolin) preparation:
Luteolin solution was prepared by dissolving standard luteolin to achieve concentration of 2 μg/ml acetonitrile.

Butylparaben:
Butylparaben solution was also prepared by dissolving 1 mg of butylparaben powder in 10 ml acetonitrile.

Plant extract (O. sanctum) preparation:
Leaf extract of O. sanctum was prepared was prepared by dissolving 10 mg of dry extract in 10 ml milique water.

Chromatographic conditions:
A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-10ADVP pump, SIL-HTc autosampler, CTO 10 ASvp column oven and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions.

Column: Inertsil ODS-C18 (150 mm length × 4.6 mm inner diameter, 5μ particle diameter) analytical column from Phenomenex Inc. (Torrance, CA, USA)
Column oven temperature: 30 °C

Detector: UV (276 nm)

Separation mode: Isocratic

Mobile phase: acetonitrile-methanol (70:30, v/v)

Flow rate: 0.6 mL/min

Total chromatographic run time: 20 min.

Auto sampler temperature: 10 °C

Injection volume: 20 μL

Identification and quantification of luteolin from *O. sanctum* aqueous extract:

Plant extract and luteolin standard were separately run on chromatographic column. Based on the retention period of luteolin standard and its corresponding peak in the crude extract chromatogram identification and quantification of the same was achieved. Spiking of the standard luteolin solution with plant extract was done to confirm presence of luteolin in the extract.

Interaction of aqueous *O. sanctum* extract/luteolin with butylparaben:

HPLC analysis:

Crude sample was incubated for 90 min with butylparaben the mixture was allowed to run under standardized chromatographic conditions. Any shift of decrease in the peak corresponding to luteolin and/or butylparaben was monitored.

UV analysis:

Standard luteolin (0.3 mg/10 ml) and butylparaben (0.5 mg/10 ml) solutions were prepared in acetonitrile and scanned (200 – 400 nm) individually as well as in a mixture (v/v) on UV spectrophotometer. Time – dependent variations were noted for 90 min in case of luteolin and butylparaben mixture.