Materials & Methods
MATERIALS AND METHODS:

PHARMACOGNOSTICAL STUDIES:

Procurement and identification of crude drug:

The bark of *Ficus bengalensis* was collected during the month of January to March from the wildly growing plants and root sample of *Hemidesmus indicus* was purchased from local market (L. V. Gandhi, Ahmedabad). Their identity was confirmed by comparing with Herbarium specimens and were preserved for further use. These drugs were then chopped into small pieces and air-dried under the shade. The extraneous matter was removed by handpicking from an accurately weighed amount of crude drug and the percentage content of foreign organic matter was determined. The drugs were then powdered to 60-mesh size using an electric grinder.

Macroscopical evaluation:

Macroscopic evaluation of these air-dried crude drugs was carried out by subjecting them to various morphological examinations. The drug was subjected to macroscopic studies, which comprised of study of organoleptic characters of the drugs e.g. colour, odour, appearance, taste, smell, texture and fractures.

Microscopical evaluation:

Microscopic evaluation of these crude drugs both entire and powders form was carried out by histological examination of cleared powder mounts.

Proximate analysis:

Proximate analysis of these crude drug powders was carried by determining various ash and extractive values as follows (WHO, 2002):
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➢ **Determination of total ash:**

Accurately weighed 2g of plant powder was incinerated in crucible at a temperature not exceeding 450°C in a muffle furnace, until ash free from carbon was obtained. It was then cooled in a dessicator, weighed and percentage of ash was calculated with reference to the air-dried drug.

➢ **Determination of acid insoluble ash:**

The ash obtained in the above was boiled for 5 minutes with 25 ml of dilute hydrochloric acid and filtered using an ash less filter paper to collect insoluble matter. The ash obtained was washed with hot water and filter paper was burnt to a constant weight. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

➢ **Determination of water-soluble ash:**

Ash was boiled for 5 minutes with 25 ml of water, and insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C and weight of water insoluble matter gave the weight of water soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

➢ **Determination of alcohol soluble extractive:**

The air-dried powdered drug 5g was macerated with 100ml of alcohol in a closed flask for 24 hours, shaking frequently at an interval of six hours. It was then allowed to stand for 18 hours and filtered rapidly. To prevent any loss during evaporation, 25 ml of the filtrate was evaporated to dryness in a porcelain dish and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air-dried drug.

➢ **Determination of water-soluble extractive:**

A 5g of the air-dried powdered drug was macerated with 100ml of distilled water in a closed flask for 24 hours, shaking in a closed flask at an interval of six hours. It was then
allowed to stand for 18 hours and filter rapidly. To prevent any loss during evaporation, 25 ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105°C and weighed. The percentage of water-soluble extractive was calculated with reference to the air-dried drug.

➤ **Determination of ether-soluble extractive (Fixed oil content):**

A 5g of accurately weighed powdered drug was extracted with petroleum ether, (40°C-60°C) in a Soxhlet extractor for 6 hours and filtered. The solvent was evaporated off on a water bath and the residue was dried at 105°C weighed. The percentage of ether soluble extractive was calculated with reference to the air-dried drug.
Extraction, Fractionation and Phytochemical studies:

The air-dried coarsely powdered drug 5kg was extracted in Soxhlet extractor with petroleum ether (60°-80°C) for defatation. The defatted powdered drug was then extracted with methanol and water separately. The crude methanol extract was fractionated with different solvents of increasing polarity such as ether, toluene, chloroform, ethyl acetate and butanol. Each time before extracting with the next solvent the powdered drug or crude extract were dried in hot air oven below 50°C. All the extracts, after recovering the solvent by distillation, were dried in oven at 50°C. The percentage yields of each extract was calculated and were then subjected to qualitative chemical examination for various phytoconstituents as per method described by Harborne (1973).
Figure 4. Scheme for Extraction and Fractionation

Thin layer chromatographic studies of selective extracts:

The selective extracts of each crude drug were then subjected to thin layer chromatographic studies to detect the presence of various types of constituents using various solvent systems on silica gel G adsorbent layers with different detecting reagents. The $R_f$ values of different spots were recorded (Harborne, 1973).
PHARMACOLOGICAL EVALUATION:

*In vitro* Free radical scavenging activity:

**Diphenyl-picryl-hydrazyl (DPPH) assay:**

The free radical scavenging capacity of extract and fractions were tested by its ability to bleach the stable 2,2 diphenyl 2-picryl hydrazyl radical (DPPH) (Bonnia et al., 1998). A stock solution of DPPH (1.5 mg /ml of methanol) was prepared such that 75 μl of it in 3 ml methanol gave initial absorbance of 0.9. This stock solution was used to measure the antiradical activity. Decrease in absorbance in the presence of methanolic extract of bark of *F. bengalensis* at different concentration was noted after 15 minutes. IC\(_{50}\) was calculated from percentage inhibition. Ascorbic acid was used as reference standard.

**Scavenging effect on superoxide radical:**

Superoxide anion radicals were estimated by spectrophotometric measurement of the reduction products of nitroblue tetrazolium (NBT) generated in riboflavin-light system according the method of McCord and Fridovich (1971). The reaction mixture consisted of EDTA (6μM; with 3μg NaCN), riboflavin (2 μm), NBT (50μM), different concentrations of methanolic extract and phosphate buffer (67 mM; pH 7.8) added in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min, and then the optical density was measured at 530 nm before and after illumination (Robak Gryglewski, 1988). Ascorbic acid was used as a positive control.

**Nitric oxide scavenging activity:**

The interaction of extract or fractions with nitric oxide was assessed by the nitrite detection method. The chemical source of NO was sodium nitroprusside (10mM) in 0.5 M phosphate buffer, pH 7.4, which spontaneously produces nitric oxide in an aqueous solution. Nitric oxide interacts with oxygen to produce stable products, leading to the production of nitrites. After the incubation of 60 min at 37°C, the Greiss reagent (α-naphthyl-ethylenediamine 0.1% in water and sulfanilic acid 1% in H\(_3\)PO\(_4\) 5%) was added.
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The same reaction mixture without the methanolic extract of sample but with equivalent amount of methanol served as control. (Sreejayan and Rao, 1997; Green et al, 1992). Ascorbic acid was used as positive control.

Phenylhydrazine induced haemolysis of erythrocytes (membrane stabilization study):

20 % PCV (packed cell volume) of erythrocyte suspension (from human blood) was prepared according to the procedure described by Hill and Thornalley, (1983). The assay was carried out according to the procedure described by Cazana et al. (1990) with certain modifications. In brief, the incubation mixture comprising of 1 ml of phenylhydrazine hydrochloride (0.5 mM), different concentrations of sample extract and 0.1 ml of 20% erythrocyte suspension was made to a total volume of 3 ml with phosphate buffered saline (PBS) solution. The mixture was incubated at 37°C for 1 hour and centrifuged at 1000g for 10 min. The extent of haemolysis was measured by recording the absorbance of the supernatant at 540 nm. Suitable controls were kept to nullify the effect of solvents and inherent haemolysis.

Inhibition of Lipid Peroxide formation induced by Fe²⁺/ascorbate system:

The reaction mixture containing rat liver homogenate (0.1 ml, 25 % w/v) in Tris-HCl (30 mM), ferrous ammonium sulphate (0.16 mM), ascorbic acid (0.06 mM) and different concentrations of the extract (from 10 to 1000 µg/ml) in a final volume of 0.5 ml was incubated for 1 h at 37°C and the resulting thiobarbituric reacting substance (TBARS) was measured by the method of Ohkawa et al. (1979). A 0.4 ml aliquot of the reaction mixture was treated with sodium dodecyl sulfate (0.2 ml, 8.1%), thiobarbituric acid (1.5 ml, 0.8%), and acetic acid (1.5 ml, 20%, pH 3.5), made to a total volume of 4 ml by adding distilled water, and kept in a water bath at 95°C for 1 h. After cooling, distilled water (1 ml) and 5 ml of n-BuOH/py 15:1 (v/v) were added. After shaking and centrifugation, the organic layer was separated and the absorbance measured at 532 nm.
In-vitro Hepatoprotective activity using rat hepatocytes:

Fresh hepatocyte preparations and primary cultured hepatocytes were used to study antihepatotoxic activity of drugs. Hepatocytes were treated with hepatotoxins and the effect of the plant drug on the same was evaluated. The activities of the transaminases released into the medium were determined.

Isolation of rat hepatocytes (Seglon, 1976):

The abdomen of the rat was opened under ether anaesthesia and 0.2 ml of 0.2%w/v heparin in 0.9%w/v NaCl was injected into the tail vein to prevent blood from clotting.

A midline incision was made and the portal vein was cannulated with a needle fitted with a Teflon catheter. The Teflon catheter was tied in place and the needle was removed. The inferior vena cava was cut below the renal vein. The liver was perfused in-situ through the portal vein using Ca\(^{2+}\) free HBSS (pH 7.4) containing 1% bovine serum albumin Fr. V and 0.5 mM EGTA. The initial flow rate was 30 ml/min and aeration was carried out with 95% O\(_2\) /5% CO\(_2\) to pH 7.4 at 37\(^\circ\) C. After ten minutes of perfusion when liver was completely bleached and freed from the blood, the inferior vena cava was tied off above the renal vein and the thorax portion of the superior venacava was cannulated. The perfusion of the liver was then done for 10 min with the Ca\(^{2+}\) –free Hank's buffer (100 ml) (containing additionally 0.075% collagenase and 4 mM CaCl\(_2\)).

After 10-15 min perfusion, the liver was transferred to a beaker containing Ca\(^{2+}\) -free Hank’s buffer (50ml) and gently dispersed with two forceps. The crude cell suspensions were then rotated in a rotator for 10 min. The cell suspension was then cooled in ice and filtered gently through cotton gauze into centrifuge tubes. The preparation is centrifuged at 50 g for 1 min. the supernatant was removed and the loosely packed pellet of cells were gently resuspended in Ca\(^{2+}\) –free Hank’s buffer. The washing procedure was repeated 3-5 times.
Measurement of cell viability:

The viability of cells to exclude trypan blue was determined by incubating the cell suspension (0.1ml) with 0.4% trypan blue (0.9ml). Viability of the isolated cells was determined by Trypan blue exclusion assay by counting the number of stained and unstained cells (viable cells). The concentration of the viable cells was adjusted to $1 \times 10^6$ cells per ml. 100 mg of different extracts and fractions were dissolved in 100 ml of PBS (Phosphate buffer saline), pH 7.4 to give 1 mg/ml concentration. Further 1:10 and 1:100 dilution of this solution with PBS (Phosphate buffer saline), pH 7.4 was carried out to give 0.1mg/ml and 0.01mg/ml of extracts respectively. Carbon tetrachloride 0.25 ml was diluted to 257 ml with PBS (Phosphate buffer saline), pH 7.4. Methanolic extract of root of H. indicus and its various fractions and methanolic extract of bark of F. bengalensis and its different fractions were tested for their hepatocyte protecting activity at different concentrations ranging from 0.01mg/ml to 1.0mg/ml.

Group-I received only vehicle and group II received only CCl$_4$ (10nM). All the incubations were done in triplicate. The pre-incubated cells were exposed to the mixture of 10 mM carbon tetrachloride and different concentrations of drug solution in PBS.

Both aqueous and alcoholic extracts solutions at different dose range were treated in the same manner. Test tubes were incubated for 1 hr at 37 °C.

After incubation for 1 hour the cell suspension was centrifuged at low speed (50 g) and the supernatant solution was used for the estimation of cytosolic enzyme like GOT, GPT.

The pellet obtained after centrifugation was resuspended in the HBSS (1ml), pH 7.4 and used for Trypan blue exclusion assay.

Trypan Blue Exclusion Assay:

To 1 ml of above resuspended cells 10 µl of Trypan blue dye (0.5% in Phosphate buffer saline) was added and allowed for 2-3 min. Cells were then loaded onto a
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haemocytometer or on a glass slide. Covered with coverslip and scored for dye uptake (blue cells=dead cells).

A minimum of 100 cells were counted in three separate fields using inverted microscope,

\[
\text{% Of viable cells} = \frac{\text{Number of cells excluding dye}}{\text{Total no of cells counted}} \times 100
\]

Calculated the viability as a percentage of these separate determinations.

*In-vivo* hepatoprotective study:

**Experimental animal:**

Wistar albino rats (150-200gm) of either sex were selected for hepatoprotective studies. Animals were housed in polypropylene cages in room under standard condition like controlled light / dark cycle n temperature (22 ±2°C), 60 to 70% relative humidity. The study protocol was approved by the Institutional Animal Ethics Committee of our Institute as per the requirements of Committee for the Purpose of Control and Supervision on Animals (CPCSEA), New Delhi.

**CCl₄ induced hepatotoxicity** (Singh et al., 1999):

Rats were divided into five groups of five animals. Group I served as vehicle control and received normal saline (5 ml/kg). Group II was administered with CCl₄ / Olive oil (1:1, 0.7 ml/kg i.p.on alternate days). Group III and IV received methanolic extract (100 mg/kg and 250 mg/kg p.o respectively daily for seven days) simultaneously with toxicant CCl₄/Olive oil. Group V was administered with reference drug, silymarin (Majumdar et al., 1998) (100mg/kg p.o.) simultaneously with toxicant.

**Paracetamol induced hepatotoxicity** (Rege et al., 1984):

In a parallel study of five similar groups of rats were treated similarly to the study mentioned above except that paracetamol. A single dose of paracetamol on sixth day of
treatment was administered 48 hours before the animals were sacrificed to all the groups except vehicle control normal group I.

Assessment of hepatoprotective activity:

On the seventh day of the start of respective treatment the rats were anaesthetized by light ether anesthesia and the blood was withdrawn by making intracardiac puncture to the rats. It was allowed to coagulate for 30 minutes and serum was separated by centrifugation at 2500 rpm. The serum was used to estimate Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT), Alkaline Phosphatase (ALP), Total Bilirubin, Direct Bilirubin.

Curative effect study in CCl₄ and paracetamol induced hepatic damage:

Rats were divided into seven groups of five animals each. Group I served as normal control and received normal saline (5 ml/kg). Group II and Group III and Group IV was administered with CCl₄ / Olive oil (1:1, 0.7 ml/kg i.p.) on alternate days for 15 days. Group V, Group VI and Group VII received two doses of paracetamol 2 gm/kg each after 48 hrs. After 15 days of CCl₄ treatment to the Group II, III and IV; Group III was treated with methanolic extract of F. bengalensis 250 mg/kg p.o daily for fifteen days while Group IV was treated with methanolic extract of H. indicus 500 mg/kg p.o daily for twenty-one days and group II received vehicle only (named as CCl₄ untreated group). After 96hrs of paracetamol treatment to the Group V, VI and VII; Group VI was treated with methanolic extract of F. bengalensis 250 mg/kg p.o daily for fifteen days while Group VII was treated with methanolic extract of H. indicus 500 mg/kg p.o. daily for twenty-one days and Group V received vehicle only (named as paracetamol untreated group).

Statistical Analysis: Results are presented as mean ± S.E.M. Statistical differences between the mean of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test for preventive effect studies. Data were considered statistical significant at P value ≤ 0.05. Paired and unpaired t-test was applied.
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for curative effect studies and data were considered statistical significant at P value ≤ 0.05.

SGPT (ALT):

Principle:

SGPT (ALT) catalyses transfer of amino group from L - Alanine to α - Ketoglutarate with formation of glutamate and pyruvate. The pyruvate so formed, allowed to react with 2, 4-Dinitrophenyl hydrazine (2, 4-DNPH) derivative, which gives brown color in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGPT activity by plotting a calibration curve using pyruvate standard. L - Glutamate the following reaction:

\[
\begin{align*}
\text{L - Alanine} & \quad \text{SGPT} & \quad \text{Pyruvate} \\
+ & \quad & + \\
\alpha - \text{Ketoglutarate} & \quad \text{pH 7.4} & \quad \text{L - Glutamate}
\end{align*}
\]

\[
\begin{align*}
\text{Pyruvate} & \quad \text{Alkaline} & \quad 2,4\text{-donitrophenyl} \\
+ & \quad & \text{hydrazone} \\
\text{2,4DNPH} & \quad \text{medium} & \quad \text{(Brown coloured)}
\end{align*}
\]

Procedure

Calibration curve

In the five clean test tubes buffered substrate, pyruvate standard distilled water and DNPH colour reagents were added as per mentioned ion the leaflet supplied in with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. absorbance of tubes 2 to 5 was measured against tube 1 as reagent blank at 505nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).
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Assay

0.5ml buffered substrate was incubated at 37°C for 3 min. 0.1ml serum sample was added to buffered substrate and incubated at 37°C for 60 min. to this DNPH colour reagent was added and allowed to stand at room temperature for 20 min. Finally 5.0ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedure was followed except that instead to serum 0.1 ml distilled water was added. Absorbance of test samples was measured against reagent blank at 505nm and was read on calibration curve to find out enzyme activity.

SGOT (AST):

Principle:

SGOT (AST) catalyses transfer of amino group from L – Aspartate to α – Ketoglutarate with formation of glutamate. The oxaloacetate so formed, allowed to react with 2, 4-Dinitrophenyl hydrazine (2, 4-DNPH) derivative, which gives brown color in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGOT activity by plotting a calibration curve using pyruvate standard. L – Glutamate the following reaction:

\[
\begin{align*}
\text{L - aspartate} & \quad \text{ SGOT } \quad \text{ Pyruvate} \\
\alpha - \text{Ketoglutarate} & \quad \text{ pH 7.4 } \quad \text{ L - Glutamate} \\
\text{Oxaloacetate} & \quad \text{ Alkaline } \quad 2,4\text{-donitrophenyl} \\
2,4\text{DNPH} & \quad \text{ medium } \quad \text{hydrazone}
\end{align*}
\]
Materials and Methods

Procedure

Calibration curve

In the five clean test tubes buffered substrate, pyruvate standard distilled water and DNSH colour reagent were added as per mentioned in the leaflet supplied with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. Absorbance of tubes 2 to 5 was measured against tube 1 as reagent blank at 505 nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).

Assay

0.5 ml buffered substrate was incubated at 37°C for 3 min. 0.1 ml serum sample was added to buffered substrate and incubated at 37°C for 60 min. To this DNSH colour reagent was added and allowed to stand at room temperature for 20 min. Finally 5.0 ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedure was followed except that instead to serum 0.1 ml distilled water was added. Absorbance of test samples was measured against reagent blank at 505 nm and was read on calibration curve to find out enzyme activity.

Alkaline phosphatase (ALP):

Principle:

Alkaline phosphatase from serum converts Phenyl Phosphate to inorganic phosphate and Phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in presence of oxidising agent potassium ferricyanide and forms an orange-red coloured complex, which can be measured colorimetrically. The color intensity is proportional to enzyme activity.

The reaction can be represented as
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Aik Phoshatase

Phenyl Phosphate \[\xrightarrow{\text{Alk Phoshatase}}\] Phenol + Pi

\[\text{pH } 10.0\]

Pot. Ferencyanide

Phenol + 4-Aminoantpyrine \[\xrightarrow{}\] Orange-red coloured complex

Bilirubin:

Principle:

Direct: (Conjugated) Bilirubin couples with diazotised Sulfanilic acid, forming Azobilirubin, a red-purple coloured product in acidic medium.

Indirect: (Unconjugated) Bilirubin is diazotised only in the presence of its dissolving solvent (methanol). Thus the red-purple coloured Azobilirubin produced in the presence of methanol originates from both direct and indirect fractions and thus represents Total Bilirubin concentration. The difference of Total and Direct Bilirubin gives Indirect Bilirubin.

The intensity red-purple colour so developed above is measured colorimetrically and is proportional to concentration of the appropriate fraction of Bilirubin.

The reaction can be represented as

\[\text{Bilirubin + Diazotized Sulfanilic Acid} \xrightarrow{H^+} \text{Azobilirubin} \xrightarrow{H^+} \text{Red-purple color}\]
**Materials and Methods**

**Procedure**

Reagents were reconstituted as described in the leaflet supplied along with the kit. 50 μl of serum samples, distilled water serving as control and standard bilirubin (10 mg %) serving as standard were mixed well with distilled water, diazo reagent and methanol. Mix well and measure the optical density at 540nm against distilled water as blank.

**Histopathology:**

The method for histological studies was as described by (Garg et al., 1996). Briefly the procedure used included fixation of the tissue with formalin, embedding in paraffin blocks, sectioning with microtome (0.7 μ thickness) and finally staining by Haemotoxylin and Eosin stain technique.

Haemotoxylin stains nucleus light blue, which turns red in presence of acid. The cell differentiation is achieved by treating the tissue with acid solution the counter staining is performed by using Eosin, which imparts pink colour to cytoplasm

**Staining**

Tissues were stained using Haemotoxylin and Eosin (H & E) stain.
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**Anti-inflammatory activity**

Albino rats weighing between 160-200 g of either sex were used. They were kept in standard environmental condition and maintained on standard chow diet and water *ad libitum*. Acute inflammation was induced by 0.1 ml of 1% (w/v) carrageenin into the plantar aponeurosis of the right hind paw of rats (Winter et al., 1962). Suspension of methanolic extract (100,150 and 200 mg/kg) and different fractions prepared using Na-CMC was administered orally 45 minutes before carrageenin injection. Diclofenac sodium (10 mg/kg) was used as reference standard. Paw volume was measured with plethysmometer before and 3 hour after carrageenin injection. The percent inhibition of paw oedema was calculated.