CHAPTER II
EXPERIMENTAL

The present section has been devoted to the detailed descriptions of various methods and techniques employed for carrying out different experiments. The experiments were performed in triplicate and the calculated mean values ± standard error of mean are recorded unless otherwise mentioned. The results so obtained from each experiment are then presented and discussed in separate section. The determinations were performed for all the chosen drugs and described under separate headings:

2.1 PHARMACOGNOSTIC STUDIES ON SELECTED CRUDE DRUGS

2.1.1 Procurement and Identification of Crude Drugs

Rhizomes of C. orchioides; whole plants of F. indica; roots of I. racemosa; fruits, leaves, roots and stem bark of M. pterygosperma were purchased from the local market, while, whole plants of S. acuta, S. cordifolia and S. rhombifolia were collected during August to November from the campus of Faculty of Technology and Engineering, M.S.University of Baroda, in required amount. Their identity was confirmed by comparing them with the herbarium specimens preserved in the Herbarium-cum-Museum of Botany Department of M.S.University, Baroda and also with those of Central Drug Research Institute, Lucknow. These drugs were then chopped into small pieces and air dried under shade.

The extraneous matter was removed by hand picking from an accurately weighed amount of the crude drug and the percentage content of foreign organic matter was determined. The drugs were powdered to 60 mesh size, for phytochemical screening, and 120 mesh size for biological screening using an electric grinder.

2.1.2 Macroscopic Evaluation

Macroscopic evaluation of these air-dried crude drugs was carried out by subjecting them to various morphological examinations using reported methods.[161]

2.1.3 Microscopic Evaluation

Microscopic evaluation of these crude drug powders was carried out by histological examination of cleared powder mounts using reported methods.[162]

2.1.4 Proximate Analysis

Proximate analysis of these crude drug powders was carried out using reported methods by subjecting them to various determinations[163] such as :

(i) Total Ash

(ii) Acid-insoluble Ash

(iii) Water-soluble Ash

(iv) Sulphated Ash

(v) Moisture Content/ Loss on Drying

(vi) Alcohol Soluble Extractive

(vii)Water Soluble Extractive

2.1.5 Estimation of Different Inorganic Metal Ions

Acid-soluble ash of these crude powders was subjected for quantitative estimation of different inorganic metal ion content like calcium, cobalt, copper, lead, magnesium, nickel, potassium, sodium and zinc using Atomic Absorption Spectrophotometer (GBC-901).

i. Preparation of Standard Solutions

The stock solutions of different standard inorganic metal ions were prepared using reported methods[164] and were diluted to different strengths of optimum working range (Table 1) as and when required.

ii. Preparation of Test Sample Solutions

The test sample solutions were prepared by boiling 100 mg of an accurately weighed amount of total ash of these crude drugs for 5 min in 10 ml of 1N HCl. These were then filtered through Whatman 40 ashless filter paper and final volumes were adjusted to 50 ml with double distilled water.

iii. Procedure

The standard and test sample solutions were aspirated and their absorbances were recorded using an Atomic Absorption Sepctrophotometer (GBC-901) according to the working parameters (Table 1) and the corresponding concentrations of the inorganic metal ions present in these crude drugs were calculated.

Table 1 : Working Parameters Selected for Atomic Absorptions Spectrophotometric Studies.

Flame type: oxidising (Air/Acetylene) Slit width = 0.5 nm

Metal estimated	Wave length (nm)	Lamp current (mA)	Optimum working range (mcg/ml)	Sensitivity (mcg/ml)
Calcium	422.7	3.0	1.0 - 4.0	0.020
Cobalt	304.4	6.0	45.0 - 180.0	1.000
Copper	324.7	3.0	1.0 - 5.0	0.025
Lead	283.3	5.0	7.0 - 50.0	0.160
Magnesium	285.2	4.0	0.1 - 0.4	0.003
Nickel	352.4	3.5	6.0 - 28.0	0.140
Potassium	766.5	6.0	0.4 - 1.5	0.008
Sodium	589.0	5.0	0.18 - 0.7	0.004
Zinc	307.6	5.0	3000-12000	66.000

2.2 PHYTOCHEMICAL STUDIES

2.2.1 Preliminary Phytoprofiles of Different Crude Drugs

The air-dried powdered drugs, 50 g each, were successively extracted in a soxhlet extractor with solvents of increasing polarity such as petroleum ether (60°-80°C), benzene, chloroform, acetone, methanol and water. Each time, before extracting with the next solvent, the powdered drugs were dried in hot air oven below 50°C. All the extracts, after recovering the solvents by distillation, were dried in oven at 50°C. The percentage yield of each extract was calculated and were then subjected to qualitative chemical examination for various phytoconstituents using reported methods.[165]

2.2.2 Preparation of Selective Extracts

The crude drug powders were again subjected to successive solvent extraction in a selective manner using solvents of non-polar, semi-polar and polar nature like petroleum ether (60°-80°C), methanol and water. The total aqueous extracts of each drug, however, were also prepared separately using decoction method. These four extracts, after removing the solvents by distillation, were dried in oven at 50°C and their percentage yields were calculated. These were then subjected to preliminary qualitative

phytochemical screening and were used for further studies.

i. Thin Layer Chromatographic Studies of the 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 phytoconstituents using various solvent systems on silica gel G adsorbent layers with different detecting reagents. The Rf values of various spots were recorded.

2.3 BIOLOGICAL STUDIES

Animals: Wistar strain albino rats (150-200 g) of either sex, procured from Deep Biomed Animal Supply, Ahmedabad maintained under standard husbandary conditions (temperature: 23±2°C, relative humidity: 55±10% and 12 hr light-dark cycle) were used for all sets of experiments in groups of six animals unless otherwise mentioned. The animals were allowed to take standard laboratory feed and tap water ad libitum.

Vehicle: 5% w/v acacia mucilage was used as a vehicle in the dose of 1 ml/kg, p.o., unless otherwise mentioned.

Preparation of Test Samples: All the test suspensions (100 mg/ml) were prepared in the vehicle and were

administered in the dose of 100 mg/kg, p.o. except the powdered drug suspensions which were administered in the dose of 500 mg/kg,p.o. unless otherwise mentioned.

Preparation of Standard Solutions: Ibuprofen and Silymarin: 100 mg ml suspensions were prepared in the vehicle and were administered at a dose of 100 mg/kg, p.o.

Indomethacin and Ketoprofen: 20 mg/ml suspensions were prepared in the vehicle and were administered at a dose of 20 mg/kg,p.o.

Carrageenan: 1% w/v suspension was prepared in saline and 0.1 ml/rat was injected into the subplantar region of the right hind paw.

2.3.1 Acute Toxicity Studies[166]

The animals were divided into control and test groups. The control group received a single oral dose of the vehicle whereas the test groups received a single oral dose of various test suspensions ranging from 0.1 to 10 g/kg and were observed for any mortality uptil 48 hrs.

2.3.2 Studies on Anti-Inflammatory Activity[167]

Studies on AIA were carried out in male albino rats

against carrageenan induced artificial inflammation. In all sets of experiments, the animals were divided into negative control (received vehicle), positive control (received standard suspensions) and test (received test samples) groups. All the suspensions were administered orally 30 min before the injection of 0.1 ml of 1% w/v Carrageenan suspension into subplantar region of the right hind paws of the rats. The paw volumes were measured just before and after injection of carrageenan, every hour till five hours, using a plethysmometer. The differences in paw volumes at zero hour and at different time intervals were calculated and compared with those of standards. The mean differences in paw volumes ± standard error of means (SEM) were calculated. The percentage inhibition of oedema has been calculated by $(V_c-V_t)/V_c$ x100 where V_c = average differences in paw volumes of control.

V_t = average differences in paw volumes of test.

For determination of significant inter group differences at different time intervals, each value was analysed separately and one way analysis of variance[168] (ANOVA) was carried out. After that, individual comparisons of group mean values were done using Dunnet's test.[169]

2.3.3 Effects on Normal Liver Function

The crude drug powders (120 mesh), along with their extracts and marketed preparations, were evaluated for

their effects on liver functions by studying their effects on normal serum and urinary parameters.

i. Effect on Normal Serum Parameters

The test protocol with dose schedule is given in Table 2. After 36 hrs of first dose administration blood was collected by puncturing the retro-orbital plexus and was allowed to clot at room temperature for 30 min. Serum was separated by centrifuging at 2500 rpm and was analysed for various biochemical parameters such as serum transaminases[170] viz., serum glutamic oxalacetic transaminase (SGOT) or serum asparatate aminotransferase (AST), serum glutamic pyruvic transaminase (SGPT) or serum alanine amino transferase (ALT), serum alkaline phosphatase[171] (ALKP); total and direct bilirubin[172] (TBil and DBil), using reported methods.

Table 2: Protocol for Effects on Normal Serum Parameters :

Group	Time (Hrs)			
	0	(+)12	(+)24	(+)36
Control	Veh.	Veh.	Veh.	Biochemical
Standard	Std.	Std.	Std.	Estimations
Test	T.S	T.S	T.S	

Veh. : Vehicle; Std. = Standard; T.S. = Test samples

(+) : After treatment.

(a) Determination of Serum Glutamine Oxalacetic and Glutamic Pyruvic Transaminases[170] (Reitman and Frankel Method)

Reagents:

1. Phosphate buffer, 0.1N, pH 7.4:
   The buffer was prepared by mixing 420 ml of 0.1M Disodium Hydrogen Phosphate and 80 ml of 0.1M potassium dihydrogen phosphate.
2. Pyruvate, 2 mM/liter (for standard curve); 22 mg of sodium pyruvate was dissolved and made to 100 ml with phosphate buffer. (Stable for two weeks on refrigeration).
3. α- Ketoglutarate, 2mM/liter, dl-aspartate, 200 mM/liter (GOT substrate): 29.2 mg of α-Ketoglutaric acid and 2.66 g of dl-aspartic acid were dissolved in 1N NaOH and the pH was adjusted to 7.4. Then it was made to 100 ml with phosphate buffer. (Stable for 6 months on refrigeration)
4. α- Ketoglutarate, 2mM/liter, dl-alanine, 200mM/liter (GPT substrate): 29.2 mg of α-ketogluaric acid and 1.78g of dl-alanine were dissolved in 1N NaOH and volume was made to 100 ml with phosphate buffer after the pH was adjusted to 7.4 (stable for 3 months on refrigeration).
5. 2,4-Dinitrophenyl hydrazine (DNPH), 1mM/liter: 19.8 mg of 2,4-DNPH was dissolved in 100 ml of 1N HCl. (Stable

for 6 months at room temperature).

6. Sodium Hydroxide (NaOH) Solution, 0.4 N: A stock solution of 4N NaOH was prepared and it was diluted as when required with distilled water.

Calibration Curves for SGOT and SGPT:

Procedure : The calibration curves for SGOT and SGPT were prepared according to the test protocol (Table 3) and the test serum samples were then analysed accordingly.

Table 3: Protocol for Determination of SGOT and SGPT:

	Blank	1	2	3	4	Unknown
Buffered substrates GOT/GPT	0.50	0.45	0.40	0.35	0.30 (Incubated	0.50
Distilled water (ml)	0.10	0.10	0.10	0.10	0.10 at 37°C for 3 min.)	
Pyruvate standard (ml)	-	0.05	0.10	0.15	0.20	-
Serum (ml)	-	-	-	-	- mixed well and incubated at 37°C for60 min (GOT) & 30 min (GPT)	0.10
2,4-DNPH (ml)	0.50	0.50	0.50	0.50	0.50	0.50

Fig. 2a. CALIBRATION CURVE FOR SGOT

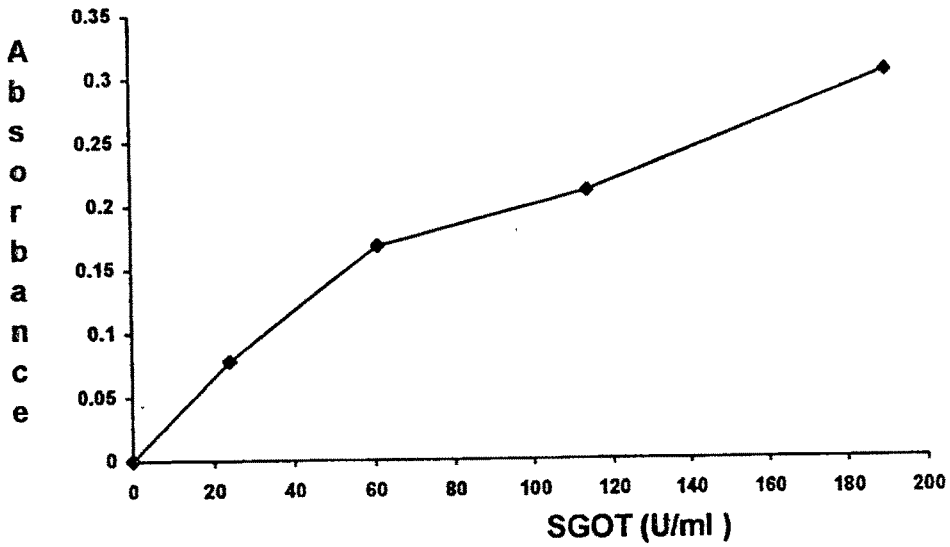
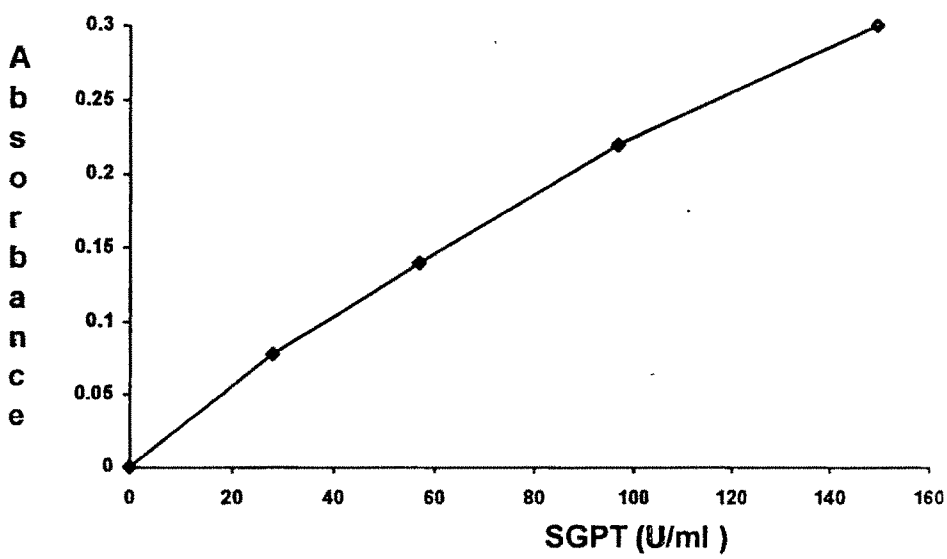


Fig. 2b. CALIBRATION CURVE FOR SGPT



Mixed well and allowed to stand at room temperature for 20 min.

0.4N NaOH (ml)	5.0	5.0	5.0	5.0	5.0	5.0

Mixed well and allowed to stand at room temperature for 10 min. and their absorbances were read against blank at 505 nm using Spectronic 20 (Bausch & Lomb) Spectrophotometer. Calibration curves were plotted by taking absorbances on the Y-axis and their corresponding enzyme activities on X-axis (Fig.2). The test samples were treated similarly and their corresponding enzyme activities were computed using the calibration curve. (Table 4)

Table 4: Calibration Curve for SGPT and SGOT:

Enzyme Activities GOT/GPT/(U/ml)	Absorbances
24/28	0.078/0.078
61/57	0.168/0.138
114/97	0.21 /0.218
190/150	0.302/0.281

(b) Determination of Alkaline Phosphatase[171] (ALKP):

(PNPP Method)

Reagents:

0.1M Diethanolanine, 0.526 g 1.25mM PNPP buffer of

diethanolamine and 23.2 mg of p-nitrophenylphosphate disodium salt (PNPP) were dissolved in 4 ml of 0.1N HCl and the volume was made to 50 ml with double distilled water after adjusting its pH to 9.8 with 0.1N HCl (stable for three days at 2-8°C; stored in amber coloured bottles).

Procedure: 1 ml of the buffered substrate and 30 ml of serum sample were mixed thoroughly and aspirated quickly using Ames, RA-50 Chemistry Analyser (Miles (I) Ltd, Baroda) and the values were recorded.

(c) Determination of total and direct bilirubin[172] (TBil and DBil) (Jendrassik and Grof Method):

Reagents

1. Sodium Nitrite ($NaNO_2$) Reagent 500 mg of $NaNO_2$ was dissolved and was made to 100 ml with distilled water (Stable for 2 weeks on refrigeration).
2. Sulphanilic acid Reagent (0.5% in HCl): 500 mg of sulphanilic acid was dissolved in 1.5 ml of HCl and the volume was made to 100 ml with distilled water (Stable indefinitely at room temperature).
3. Caffeine - Benzoate Reagent: 75 g of sodium benzoate, 125 g of sodium acetate (trihydrate) and 50 g of caffein were dissolved successively and the volume was adjusted to 1000 ml with warm distilled water (stable for 6 months).

Procedure: The determination of total and direct bilirubin were carried out according to the protocol (Table 5) using Ames, RA-50 Chemistry Analyser (Miles (I) Ltd, Baroda).

Table 5: Protocol for Estimation of TBil and DBil:

Reagents	Blank	Test	
		TBil	DBil
$NaNO_2$	-	50 ul	50 ul
Sulphanilic acid	-	50 ul	50 ul
Serum	-	50 ul	50 ul
Caffeine-Benzoate Reagent	-	0.5 ml	-
Distilled water	1.0 ml	0.5 ml	1.0 ml

Mixed well and incubated at room temperature for 5 min and read within 10 min.

ii. Effects on Urinary Parameters

These studies were carreid out in overnight fasted albino rats (4 animals/group) kept in separate metabolic cages. In all sets of experiments, the animals were divided into control and test groups. The control group received a single oral dose of the vehicle while the test groups received a single oral dose of the respective test suspensions. 25 ml of tap water was administered to each

rat 30 min after the respective doses of the suspensions. Total volume of urine collected till 3 hrs was measured and was then estimated for ascorbic acid[173] (Vit.C), cholesterol[174], glucose[175] and total proteins[176] using reported methods.

(a) Estimation of Ascorbic Acid[175] (Roe and Kuether Method):

Reagents:

1 2,4-DNPH: 2 g of 2,4-DNPH and 4 g of thiourea were dissolved in 100 ml of 9N H_2SO_4

2 85% v/v H_2SO_4

3 Ascorbic acid standard solution: 10 mg/ml solution of ascorbic acid was prepared in distilled water.

Procedure: Aliquots of ascorbic acid standard solution (0.1 to 1.0 ml) were taken and were adjusted to 1 ml with distilled water. To each of these, 1 ml of 2,4-DNPH solution was added and incubated at 37°C for 3 hrs. Then the final volumes were adjusted to 5 ml with 85% H_2SO_4 and were allowed to stand at room temperature for 30 min. The absorbances were read at 540 nm using Spectronic 20 (Bausch & Lomb) and the calibration curve was plotted (Fig.3). The unknown samples were treated in the same way and their concentrations were computed from the calibration curve. (Table 6)

Fig. 3. CALIBRATION CURVE FOR ASCORBIC ACID

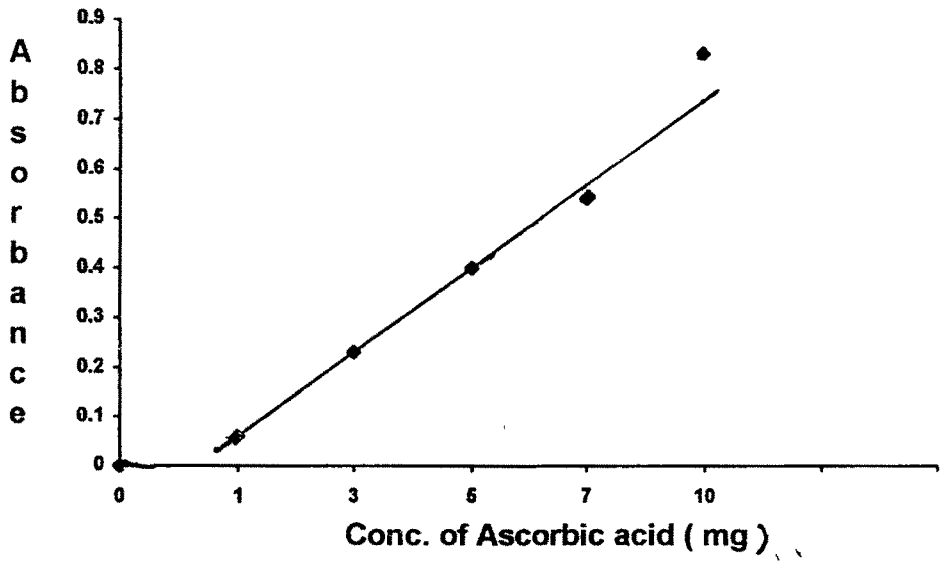


Table 6 : Calibration Curve for Ascorbic acid

Conc. of ascorbic acid (mg/5ml)	Absorbance
1	0.057
3	0.228
5	0.400
7	0.571
10	0.828

Regression analysis Y = mx+c, where m = 0.0858 c= -0.0293, correlation coefficient = 0.9987

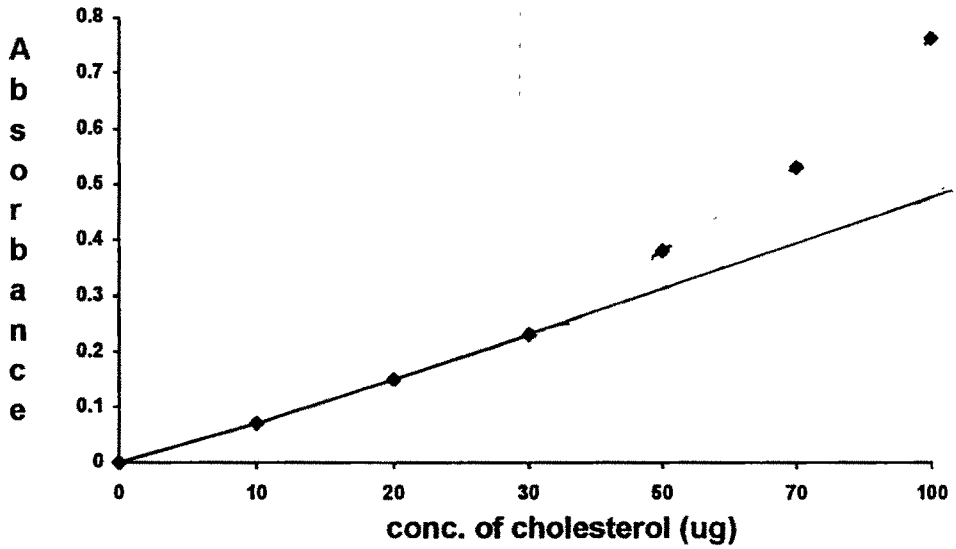
(b) Estimation of Cholesterol[174] (Zlatkis, Zak and Boyle Method):

Reagents:

1. Cholesterol standard: 100 ug/ml solution of cholesterol was prepared in glacial acetic acid.
2. Ferric chloride, ($FeCl_3$ $6H_2O$) solution of $FeCl_3$ 0.05% in glacial acetic acid was prepared.

Procedure: Aliquots of cholesterol standard solution (0.1 to 1.0 ml) were taken in a 5 ml volumetric flask. To these, 2 ml of ferric chloride solution and 2 ml of H_2SO_4 were added and were allowed to stand at room temperature for 30 min after the volumes were adjusted to 5 ml with glacial

Fig. 4. CALIBRATION CURVE FOR CHOLESTEROL



acetic acid. Then the absorbances were read against blank at 560 nm using Spectronic 20 (Bausch & Lomb) and the calibration curve was plotted (Fig.4). The test samples were also treated in the same way and their concentrations were calculated using the calibration curve (Table 7). (Note: Any precipitate formed after adding $FeCl_3$ was removed before adding H_2SO_4)

Table 7 : Calibration Curve for Cholesterol

Concentration of cholesterol (mcg/5 ml)	Absorbance
10	0.074
20	0.150
30	0.226
50	0.378
70	0.530
100	0.758

Regression analysis: y= mx+c, where m=0.0076, c=-o.0019
correlation coefficient = 0.9988

(c) Estimation of Glucose[175] (GOD/POD Method):

Reagents

1. Glucose Kit (International Biochemical and Equipment Co., Baroda)

a) Standard, 100 mg/dl, glucose solution

b) GOD-POD enzyme powder

2. Preparation of working solution: The GOD-POD enzyme powder supplied in the enzoset was dissolved in 50 ml of distilled water. It was refrigerated and protectd from light.

Procedure: 1 ml of GOD-POD working solution and 10 ul of sample were mixed thoroughly and was allowed to stand at RT for 30 min. The glucose content was read using Ames, RA-50 Chemistry Analyser after calibration.

(d) Estimation of Total Proteins[176] (Biuret Method)

The estimation of total proteins was carried out according to biuret method using Ames RA 50 Chemistry Analyser.

2.3.4 Evaluation of Hepatoprotective Activity (In vivo)

Hepatoprotective activity of these crude drug powders, extracts and their marketed preparations were studied against different toxicants such as chemical (CCl_4) and drug (paracetamol and rifampicin) induced hepatotoxicities.

i. Chemical (CCl_4) Induced Hepatotoxicity[177.178]

The test protocol mentioned in Table 8 was followed. The vehicle and test samples were administered orally and

the liver damage was induced by administration of a single oral dose of CCl_4 in olive oil (1:1 ratio) 2.5 ml/kg. In all the cases, CCl_4 was administered 30 min after the first dose of respective suspension and blood was collected by turning the retro-orbital plexus, 36 hrs after CCl_4 intoxication. Serum was separated and used for biochemical estimations to assess liver function.

Table 8: Protocol for CCl_4 induced Hepatotoxicity:

Group	Time (Hr)			
	0	(+)12	(+)24	(+)36
Control	Veh.+OL	Veh.	Veh.	Biochemical
Toxicant	Veh+CCl_4	Veh.	Veh.	Estimations
Test (T.S + Toxicant)	T.S.+CCl_4	T.S	T.S	

Veh. : 5% w/v acacia (1 ml/kg); Toxicant : CCl_4 [(CCl_4 in olive oil (1:1) = 2.5 ml/kg,p.o); T.S. = Test samples (100 mg/kg); (+) : After intoxication

ii. Drug Induced Hepatotoxicity

(a) Paracetamol induced hepatotoxicity:[179] The protocol mentioned in Table 9 was followed in all sets of experiments. On the 5th day of the treatment i.e. 48 hr after paracetamol (3g/kg,p.o) intoxication, blood was

collected by puncturing the retro-orbital plexus and serum was used for biochemical estimation to assess liver function.

Table 9: Protocol for Paracetamol induced Hepatotoxicity

Group	Time (Days)				
	1	2	3	4	5
Control	Veh.	Veh.	Veh.	-	Biochemical
Toxicant	Veh.	Veh.	Veh.+PCl	-	Estimations
Test (T.S.+Toxicant)	T.S	T.S	T.S +PCl	-	

Veh. : 5% w/v acacia (1 ml/kg,p.o.);
PCl : Paracetamol in vehicle (3g/kg, p.o., 30 min. after 3rd dose); T.S. : Test samples (100 mg/kg, p.o.)

(b) Rifampicin induced hepatotoxicity:[180] The hepatoprotective activity of all these drugs was studied according to test protocol described in Table 10. 48 hr after rifampicin (1 g/kg,p.o.) intoxication, blood was collected from retro orbital plexus and the serum separated was used for assessment of liver function.

Table 10 : Protocol for Rifampicin induced Hepatotoxicity

Group	Time (Hr)				
	0	(+)12	(+)24	(+)36	(+)48
Control	Veh.	Veh.	Veh.	Veh.	Biochemical
Toxicant	Veh+RMP	Veh.	Veh.	Veh.	Estimations
Test (T.S+Toxicant)	T.S+RMP	T.S	T.S	T.S	

Veh. : 5% w/v acacia, (1 ml/kg, p.o.); RMP : rifampicin in vehicle (1 g/kg,p.o., 30 min after 1st dose); T.S : Test samples, (100 mg/kg, p.o.)

iii. Assessment of Liver Function

Assessment of liver function was done by studying changes in biochemical parameters and histopathology.

(a) Biochemical parameters i.e. serum transaminases viz., serum glutamic oxalacetic transaminase (SGOT) or aspartate amino transferase (AST), serum glutamic pyruvic transaminase (SGPT) or alanine amino transferase (ALT), serum alkaline phosphatase (ALKP), serum total and direct bilirubin (TBil and DBil) were estimated.

Statistical Analysis: The mean values ± SEM were calculated for each parameter. Percentage reduction against

the hepatotoxin by the test samples were calculated by considering enzyme level difference between the hepatotoxin treated and the control group as 100% levels of reduction. For the determination of significant intergroup differences each parameter was analysed separately and one way analysis of variance[168] (ANOVA) was carried out. After that, individual comparisons of group mean values were done using Dunnet's test.[169]

(b) Histopathological Studies: The hepatoprotective activity of the various drug samples in powdered as well as extracted form was again confirmed by performing the histopathological examination of the treated livers. One animal, from the treated group showing maximal activity as indicated by improved biochemical parameters from each test and toxicant groups was utilised for this purpose. The animals were sacrificed and the abdomen was cut open and the liver was collected after clearing the adhering tissues. The liver was then cut into 5 mm thick pieces and blotted with a filter paper until free from blood. These liver pieces were then fixed in Bouin's solution (Mixture of 75 ml of saturated picric acid, 25 ml of 40% formaldehyde and 5 ml of glacial acetic acid) for 12 hr. These liver pieces were then thoroughly washed in running water until free from Bouin's fluid. These fixed tissues were then processed for paraffin embedding using conventional methods.[181] 5 um thick sections were taken using rocking microtome, stained with

haematoxylin eosin and finally mounted in diphenyl xylene (DPX). These stained sections were then examined under a light microscope for any histopathologilcal changes in liver architecture and their photomicrographs were taken, illustrating changes in cellular structure in those of test groups, control and toxicant groups.

2.4 ISOLATION OF COMPONENTS FROM BIOACTIVE EXTRACTS

The phytochemical and chromatographic studies revealed the presence of various constituents in the extracts of these drugs under test. Those extracts which showed promising biological activities either on artificially induced inflammation or hepatotoxicity were subjected for isolation and characterisation of components which would be responsible for their biological activity.

2.4.1 Isolation of C-1 and C-2 from C. orchioides

The residue of the dried petroleum ether (60-80°C) extract, after saponification, was dried and fractionated into different solvents of increasing polarity such as petroleum ether (60-80°C) benzene, acetone and methanol. The acetone insoluble portion of the benzene soluble fraction afforded a compound (C-1) which was purified and recrystallised from chloroform. The methanolic fraction afforded a compound (C-2) which was purified and

recrystallised from methanol after treatment with activated charcoal. The homogenity of C-i and C-2 was checked by TLC on silica gel G with benzene: dioxane (8:2) as mobile phase and Iodine vapours as detecting reagent.

2.4.2 Isolation of F-1 and F-2 from *F. indica*

The dried methanolic extract after treatment with petroleum ehter (60-80°C), chloroform, acetone and methanol successively afforded a colourless, crystalline, flaky residue, which on recrystallization after treatment with activated charcoal gave colourless needles (F-1). The homogenity of F-1 was checked by TLC on silica gel G with chloroform:methanol: (3:5:2) as a mobile phase and bromocresol green as a detecting reagent.

The methanol insoluble portion of the petroleum ether (60-80°C) extract afforded a colourless, free flowing powder (F-2), recrystallised from acetone after repeated washings with methanol. The homogenity of the compound F-2 was checked by TLC on silica gel G with toluene:dioxane (8:2) as a mobile phase and vanilline-sulphuric acid as a spraying reagent.

2.4.3 Isolation of I-1 from *I. racemosa*

The methanol soluble portion of the dried petroleum

ether (60-80°C) extract, on standing at room temperature afforded colorless blunt needles, which were separated from the mother liquor by repeated washing with methanol and were finally recrystallized from methanol. The homogenity was checked by TLC on silica gel G using toluene: dioxane (8:2) as a mobile phase and vanillin - sulphuric acid as a detecting reagent.

2.4.4 Isolation of M-1, M-2 and M-3 from M. pterygosperma

The residue of the dried petroleum ehter (60-80°C) extract after saponification was dried and fractionated successively with benzene. This fraction on repeated washing afforded the compound M-1 which was recrystallised from benzene and its homogenity was confirmed by TLC on silica gel G using bensene: methanol (8:2) as mobile phase and 5% sulphuric acid in ethanol as a detecting reagent.

The marc leftover after successive solvent extraction of the dried total aqueous extract of the Moringa stem bark with petroleum ether (60-80°C), chloroform and methanol, was dried and hydrolysed with 2N hydrochloric acid on a water bath for 1 hr and filtered and the filterate was extracted with diethyl ether. The diethyl ether extract on evaporation afforded colourless spheres (M-2), which were separated from the adhering mother liquor by treatment with Chloroform : Acetone (9:1) and were recrystallised from acetone. The

homogenity was checked by TLC on silica gel G using toluene: acetone (8:2) as mobile phase and 5% sulphuric acid in ethanol as a detecting reagent.

The residue of the dried petroleum ether extract after saponification was fractionated successively with different solvents of increasing polarity. The methanolic fraction on repeated washing afforded the compound M-3 which was recrystallised from methanol and its homogenity was confirmed by TLC on silica gel G using benzene methanol (8:2) as mobile phase and sulphuric acid as a detecting reagent.

2.4.5 Isolation of S-1 from S. cordifolia

The dried total aqueous extract was hydrolysed with 2N HCl on a boiling water bath for 1 hr and filtered. The filtrate, after cooling, was extracted with diethyl ether which on evaporation at room temprature afforded colorless clusters of sphere type crystals, S-1, which were separated from the adhering mother liquor by treatment with chloroform: methanol (9:1). S-1 was recrystallised from methanol after treatment with charcoal. The homogenity of S-1 was confirmed by TLC on silica gel G using toluene: ethyl acetate: diethylamine (7:2:1) as a mobile phase and Iodine vapour as a detecting reagent.

2.5 IDENTIFICATION, CHARACTERISATION AND BIOLOGICAL SCREENING OF THE ISOLATED COMPOUNDS

The compounds isolated from these extracts were then subjected to the following studies:

2.5.1 Physico-chemical Characterisation

The isolated components were subjected to physical characterisation by studying their state, melting point, solubility and max, etc. and chemical characterisation by qualitative elemental analysis, detection of different functional groups and quantitative elemental analysis. (Courtesy : UDCT, Bombay and Chemistry Dept, Faculty of Science, M.S.University, Baroda).

2.5.2 Characterisation by Spectral Analaysis

The components were then subjected for various spectral analysis such as Mass spectroscopy (Courtesy: IICT, Hyderabad), IR spectroscopy (Courtesy: MJIR, Baroda), ^{1}H NMR and ^{13}C NMR spectroscopy (Courtesy: RSIC, IIT Bombay), in order to characterise these components.

2.5.3 Hepatoprotective Activity of the Isolated Components

The hepatoprotective activity of the components isolated

from these drugs were also screened using *in vitro* as well as *in vivo* methods.

i. *In vitro* Studies

The compounds were subjected to *in vitro* hepatoprotective activity assessment using the following method. (These studies were carried out at CDRI, Lucknow).

Reagents and Chemicals:

Sodium chloride (NaCl) A.R.; Potassium Chloride (KCl) A.R.; Sodium Hydroxide (NaOH) A.R.; Thioacetamide (TAA) of Loba Chemie, Bombay

HEPES (N-2-Hydroxyethyl piperzine-N-2- Ethane Sulphonic acid)

Collagenase Type IV; D-Galactosamine (GalN) (Sigma Chemical Co. USA)

Typan blue (National Chemicals, Baroda)

Table 11: Composition of Calcium-free HEPES Buffer

Ingredients	Amounts (Conc.)	
	Buffer I	Buffer II
NaCl	4.15 g (0.142M)	1.95 g (0.0667M)
KCl	0.26 g (0.0067M)	0.25 g (0.0067M)
HEPES	1.20 g (0.01M)	12.00 g (0.1M)
Collagenase type IV 630 units/mg solid	-	1.6 $x10^{-9}$ g/ml
pH	7.4	7.6
Double Distilled water	500 ml	500 ml

Collagenase was added to buffer II (10 ul/50 ml) just before the isolation procedure. Buffer I was bubbled with O_2 for 10 min. pH was adjusted with 1N NaOH

Test solutions: 100, 1000 and 10000 ug/ml solutions of the compounds were prepared in double distilled water (DDW) D(+)Galactosamine HCl:(GalN) 4 mg/ml solution was prepared in DDW.

Thioacetamide:(TAA) 2 mg/ml solution was prepared in DDW

Liver cell suspension: The pellet of hepatocytes obtained after centrifugation was diluted in 1:3 ratio with HEPES buffer I.

Trypan blue solution : 0.2% w/v solution was prepared in buffer I.

(a) Isolation of hepatocytes: The hepatocytes were isolated by using a slightly modified method of Seglen et al.[182]

The rat was cleaned thoroughly with rectified spirit and anaesthetised with ether. Dissection of the animal was done under aseptic conditions using sterilized instruments. A midline incision was made on the abdomen of the anaesthetized animal and the liver, portal vein, right kidney and inferior vena cana were exposed by displacing intestine. The inferior vena cava near the kidney and the portal vein were ligated at the lower ends. The portal vein

was cannulated at its upper end and ligated. The other end of the cannula was attached to a plastic tubing connected to a buffer reservoir through a peristaltic pump and a constant temperature water bath maintained at 37°C. Just before starting perfusion with calcium-free buffer I (100 ml), a second cut was made in the upper vena cava near the heart for collection of perfusate. The buffer I was perfused at a flow rate of 2.5-3.0 ml/min./g of liver for 12-15 min with light massaging of the liver lobes until it became greyish brown. Then the liver was perfused with recirculating collagenase HEPES buffer II (50 ml) at the same flow rate for 7-8 min i.e. until it swelled up with the appearance of rashes. Then the collapsed liver was removed, washed and transferred to a ice cooled petri dish containing Buffer I. The cells were dispersed after disruption of Glissin's capsule with the help of blunt forcep. The cell suspension was filtered through muslin cloth and the tissue debris was removed. The cells were then washed throughly 3-4 times by slow centrifugation (200 rpm), suspended in Buffer I and subjected to viability test by the Trypan blue exclusion test. The viability of the cells in each of the experiment performed was found to be nearly 90%. The hepatocytes thus isolated were utilised for screening of the isolated components for their hepatoprotective activity.

The hepatocytes were induced with galactosamine (40 ug/ml) and thioacetamide (20 ug/ml) cytotoxitities

according to the test protocol mentioned in Table 12. After incubation in carbon dioxide incubator for 24 hrs they were subjected to viability and O_2 uptake tests.

Table 12: Protocol for in vitro Hepatoprotective Activity testing

Group	Liver cell suspension (ml)	HEPES buffer I (ml)	GalN/TAA (ml)	Vehicle (ml)	Test samples (ml)
Control	0.1	0.8	-	0.1	-
Toxicant	0.1	0.7	0.1	0.1	-
Test compounds	0.1	0.8	-	-	0.1
Test compounds + Toxicant	0.1	0.7	0.1	-	0.1

Vehicle: double distilled water

(b) Viability Tests: The viability of the cells was then determined by using trypan blue exclusion test and also by oxygen uptake tests.

1. Trypan blue exclusion test: The test was performed by mixing thoroughly 0.1 ml of 0.2% Trypan blue solution

to each 1 ml of liver cell suspension for 30 sec and by observing under light microscope. The ratio of viable cells i.e. nonstained or viable cells to stained or nonviable cells in different fields were counted and the percentage of viable cells were calculated. The results are represented as % mean viable cells ± SEM.

2. Oxygen uptake test: The uptake of oxygen in the isolated hepatocytes was determined by using Gilson's oxygraph according to Estabrook method.[183] The reaction mixture containing 1.7 ml of HEPES buffer I and 0.1 ml of liver cell suspension was placed into the probe and a polarising voltage (-0.8 volt) was applied across the probe through electrode. The downward movement of the styles on the chart was recorded until a straight line was obtained. The O_2 uptake by the cells was calculated after estimating the protein content of the cells.

(c) Estimation of Total Protein[184] (Folin & Ciocalteu Phenol reagent method):

Reagents:

1. Solution A : 8% w/v solution of sodium carbonate was prepared in distilled water.
2. Solution B: 600 mg of sodium potassium tartrate and 300 mg of copper sulphate were dissolved in 500 ml of distilled water.

Bovine Serum Albumin Standard: Bovine Serum Albumin 400 ug/ml solution was prepared in distilled water.

Trichloroacetic Acid (TCA) solution: A 1% TCA solution was prepared in distilled water.

Folin and Ciocalteu's Phenol reagent (2N): The readymade reagent was diluted with distilled water in 1:1 ratio just before use.

Preparation of Test Samples: The hepatocyte suspensions after subjecting to oxygen uptake test were centrifuged at 7000 rpm and to the sediment, 1 ml of 10% TCA solution was added and the precipitated proteins, after centrifugation, were dissolved in 1 ml of 1N NaOH.

Preparation of Calibration Curve: To different aliquots of bovine serum albumin (0.05 to 0.5 ml), 5 ml of solution A and solution B (1:1) mixture was added and allowed to stand for 10 min at 37°C. Then, to this, 0.5 ml Folin & Cioalteu's Phenol Reagent was added and incubated for 3 min at 37°C and their absorbances were read against blank prepared in the serum except standard solution at 625 nm and the calibration curve plotted (Fig.5). The test samples (0.1 ml) were treated in the same way and their concentrations were calculated using the calibration curve (Table 13).

Fig. 5. CALIBRATION CURVE FOR TOTAL PROTEINS

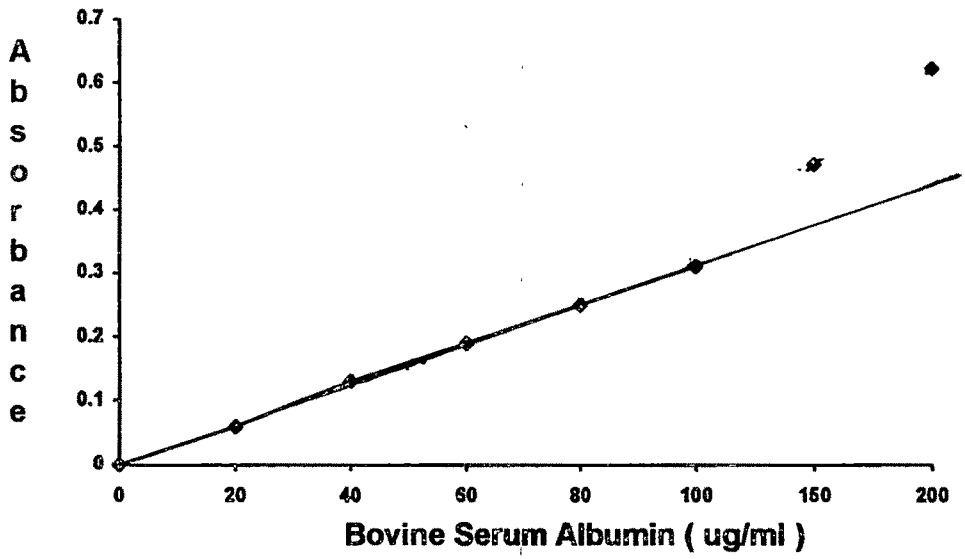


Table 13: Calibration Curve for Total Protein

Conc. of Bovine Serum Albumin mc g/ml	Absorbence
20	0.064
40	0.126
60	0.188
80	0.250
100	0.312
150	0.467
200	0.622

Regression Analysis: $y = mx+c$, when $m = 0.0031$, $c = 0.0023$

correlation coefficient = 0.9996

ii. In vivo Hepatoprotective Activity Studies

The compounds which were obtained in high yields and showed significant activity in in vitro hepatoprotective activity were only screened for in vivo hepatoprotective activity testing against CCl_4, paracetamol and rifampicin induced hepatotoxicities. The anti-inflammatory activity of these compounds was also confirmed by testing these compounds against carrageenan induced artificial inflammation in rat hind paw. The method adopted were similar to those described earlier.

2.6 EVALUATION OF SOME AVAILABLE MARKETED PREPARATIONS FOR ANTI-INFLAMMATORY AND HEPATOPROTECTIVE ACTIVITIES

Marketed preparations used:

2.6.1 Polyherbal Formulation Containing Kali Musli (C. orchioides)

1. Musli churna (Shree Bhuvaneshwari Aushadhashram, Gondal)
2. Muslipak (Shree Baidyanath Ayurved Bhavan Ltd., Nagpur)
3. Ashwabal (Raka Labs, Ahmedabad)
4. Virilex (TTK Pharma, Madras)

2.6.2 Polyherbal Formulations Containing Pitpapda (F. indica)

1. Livomyn (Charak Pharmaceuticals (I) Ltd., Umbergaon)
2. Stimuliv (Franco Indian Pharm. Ltd., Bombay)
3. Livpar (Gufic Ltd., Navsari)
4. Livfit (Dabur (I) Ltd., Sahibabad.
5. Liv52 (The Himalaya Drug Co., Bangalore)
6. Styplon (The Himalaya Drug Co., Bangalore)
7. Safi (Hamdard (WAKF) Labs, Ghaziabad)

2.6.3 Polyherbal Formulatiuons Containing Pushkarmool (I. racemosa)

1. Pushkar mool churna (S.B.A, Gondal & Mehta agencies, Baroda)

2. Kofostal (Nukem Remedies Ltd, Bombay)

2.6.4 Polyherbal Formulations Containing Sahajna (*M. pterygosperm*)

1. Rumalaya (The Himalaya Drug Co., Bangalore)

2.6.5 Polyherbal Formulations Containing Bala (*S. cordifolia*)

1. Alpitone (Zandu, Vapi)
2. Blumyn (Vasu Pharm. Pvt. Ltd, Vadodara)
3. Balantkadha No.3, (D.K.Dandu Bros. (Chembur), Bombay)
4. Tentex forte (The Himalaya Drug Co., Bangalore)

The above mentioned available marketed polyherbal formulations containing the selected drugs were also studied for their *in vivo* anti-inflammatory and hepatoprotective activities using the methods already described in previous sections. In case of solid dosage forms 100 mg/kg,p.o., liquid dosage forms 1 ml/kg,p.o., and powders 500 mg/kg,p.o. were administered.