CHAPTER 2

EXPERIMENTAL

2. Experimental

The present section deals with the detailed description of various methods and techniques adopted for carrying out different studies on the selected plants viz., aerial parts of *Pergularia daemia* and roots of *Baliospermum montanum.*

2.1 Pharmacognostic studies

Pharmacognostic evaluation is the initial step to confirm the identity and to assess the quality and purity of the crude drug. The selected plant drugs therefore were first subjected to pharmacognostic evaluation.

Aerial parts of *P. daemia* were collected from foot hills of Tirumala, Andhra Pradesh state and their identity was confirmed by Dr. R. Chandrasekhar, scientist, at The Botanical Survey of India, Southern circle, Coimbatore, India. The voucher specimen (BSI/SC/5/21/05-06/Tech 1512) was also deposited at The Madras herbarium, The Botanical Survey of India, Coimbatore.

The roots of the *B. montanum* were procured from Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal, Kerala state, India and authentified at the same centre by Dr. Udayan, scientist. The voucher specimen (HDT/CMPR/04/SVSK/2005) has been deposited in the Herbarium of the Institute.

2.1.1 Macroscopic evaluation

The aerial parts of the *P. daemia* and roots of the *B. montanum* were subjected to morphological examinations using reported methods (Kokate et al. 2001; Brain and Turner, 1975) and the results were then compared with reported monographs (Kirtikar and Basu, 1983; The Wealth of India, 1966; The Ayurvedic Pharmacopoeia India, 2001)

2.1.2 Microscopic evaluation

Microscopic evaluation of the different organs of *P. daemia* and roots of *B. montanum* were carried out by taking the transverse sections using standard procedures (Brain and Turner, 1975) and then subjecting them to microscopic examination. Further the powdered samples were also subjected to histological examinations using standard procedures (Lala, 1981)

mentioned in the text and their diagnostic features were identified and recorded.

2.2 Proximate analysis

Physical and physico chemical standards are to be determined for the crude drugs, wherever possible. These were determined using standard procedures.

2.2.1 Determination of foreign organic matter

Foreign organic matter is the material consisting of any or all of the following.

1. Parts of the organ or organs from which the drug is derived other than the parts named in the definition and description or for which the limit is prescribed in the individual monograph.
2. Any organs other than those named in the definition and description.
3. Matter not coming from the source plant and
4. Moulds insects or other animal contamination.

Method: 500 g of the sample was weighed and spread out in a thin layer. The sample was inspected with the eye and separated the foreign organic matter by hand. Then it was weighed and percentage was calculated from the amount taken for the experiment (WHO, 1998).

2.2.2 Loss on Drying

About 5 g of the air-dried material was weighed into a previously dried and tared flat weighing bottle and dried in an oven at 105°C. Drying was continued until two consecutive weighings do not differ by more than 5 mg. The difference in the weight of drug before and after drying was noted and loss on drying was calculated with reference to the air-dried material (WHO, 1998).

2.2.3 Determination of extractive values

This method determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material.

Water soluble extractive value: 4 g of coarsely powered air-dried material was macerated with 100 ml of chloroform water in a glass stoppered conical flask for 24 h shaking frequently during first 6 h and the allowing to stand for 18 h. The solution was filtered rapidly taking care not to lose any solvent and

evaporated 25 ml of the solvent in a tared flat bottomed dish on water bath. The residue was dried at 105°C for 6 h, cooled in a desiccator for 30 min and weighed. The content of extractable matter was calculated on the basis of air dried material (WHO, 1998).

Alcohol soluble extractive value: 4 g of coarsely powered air-dried material was macerated with 100 ml of 95% ethanol in a glass stoppered conical flask for 24 h shaking frequently during first 6 h and the allowing to stand for 18 h. The solution was filtered rapidly taking care not to lose any solvent and evaporated 25 ml of the solvent in a tared flat bottomed dish on water bath. The residue was dried at 105°C for 6 h, cooled in a desiccator for 30 min and weighed. The content of extractable matter was calculated on the basis of air dried material (WHO, 1998).

2.2.4 Ash values

The weighed amount (2-3 g) of dried and powdered plant materials were incinerated in a silica crucible in muffle furnace at a temperature not exceeding 600°C until the drug was free from carbon. Cooled and weighed. The total ash values were calculated with reference to the air dried drugs. The total ash was then subjected to the determination of acid-insoluble ash and water-soluble ash using official procedure (WHO, 1998).

Water-soluble ash: The total ash of the powdered drugs, obtained above was subjected separately for the estimation of water-soluble ash using the following procedure.

The total ash obtained was boiled with 25 ml of water for 5 min. Then the insoluble matter was collected in a Gooch crucible, and then the insoluble matter was ignited for 15 min at a temperature not exceeding 450° C. The weight of the insoluble matter was subtracted from the weight of the ash; the difference in weight represents the water-soluble ash. The percentage of the water-soluble ash was calculated with reference to air dried drug.

Acid-insoluble ash: The total ash of the powdered drugs obtained by the above procedure was subjected separately for the estimation of acid-insoluble ash using the following procedure.

The total ash was boiled with 25 ml of 2N hydrochloric acid for 5 min. The insoluble matter was then collected in a Gooch crucible, then washed with hot water, ignited and weighed. The percentage of the acid insoluble ash was then calculated with reference to air dried drug.

2.2.5 Determination of foaming index

About 2 g of the plant material was reduced to coarse powder. Weighed accurately 1 g and transferred to a 500 ml conical flask containing 100 ml of boiling water. The mixture was maintained at moderate boiling for 3 min. the mixture was cooled and filtered into a 100 ml volumetric flask and added sufficient amount of water to produce 100 ml. Decoction was poured into 10 stoppered test tubes in successive proportions of 1 ml, 2 ml, 3 ml, etc. up to 10 ml and adjusted the volume of the liquid in each tube with water to 10 ml. stoppered the tubes and shaken them in a lengthwise motion for 15 sec, two shakes per second. Allowed to stand for 15 min and measured the height of the foam. The foaming index was calculated by using the formula 1000/a where "a" is the volume in ml of the decoction used for preparing the dilution in tube where foaming to a height of 1 cm was observed (WHO, 1998).

2.2.6 Estimation of heavy metals

About 5 g of the powdered drug material was ignited in muffle furnace to obtain total ash. 100 mg of the ash was dissolved in 10 ml of 1 N HCl and then the solution was filtered and diluted to 50 ml with distilled water and used for quantitative determination of heavy metals by absorption spectroscopy (WHO, 1998).

2.2.7 Estimation of total phenolic content

The total phenolic content of the ethanol extract of *P. daemia* and methanolic extract of *B. montanum* were estimated by the method of Folin ciocalteu (Singleton and Rossi, 1965). The detailed procedure was as follows.

Stock solution (10mg/10ml) of the extract was prepared in respective solvents. From the stock solution 1ml of the extract was taken into a 25 ml volumetric flask. To this added 10 ml of water and 1.5 ml of Folin ciocalteu reagent. The mixture was kept aside for 5 min and then 4 ml of 20% sodium carbonate solution was added and volume was made up to 25 ml with double

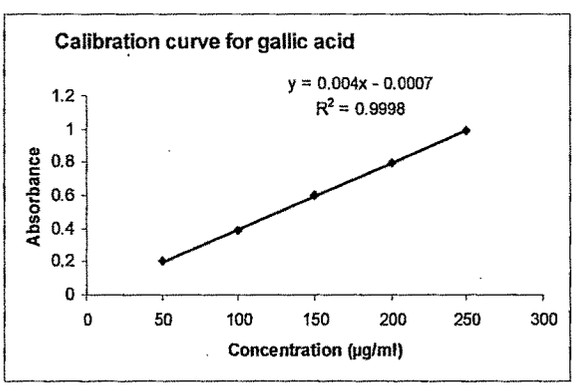
distilled water. The mixture was kept aside for 30 min and absorbance of blue color developed was recorded at 765 nm. For the preparation of calibration curve the solutions of standard gallic acid was prepared in concentration range of 50 to 250 µg/ml. Calibration curve for gallic acid was obtained by plotting absorbance on Y axis and their corresponding concentration on X axis (Graph 1).

Table 1: Concentration and absorbance data for gallic acid and extracts

Concentration (µg/ml)	Absorbance
50	0.199
100	0.391
150	0.601
200	0.792
250	0.991
P. daemia (1 mg/ml)	0.435
B. montanum (1 mg/ml)	0.484

The data representing the concentration and absorbances of gallic acid, ethanol extract of *P. daemia* and methanol extract of *B. montanum* is represented in Table 1.

Graph 1: Calibration curve for gallic acid



Total phenolic content of the test samples was computed from calibration curve of gallic acid and expressed as percentage gallic acid.

2.2.8 Estimation of total flavonoid content

The total flavonoid content of the ethanol extract of *P. daemia* and methanolic extract of *B. montanum* was estimated by reported methods (Chang et al., 2002).

I. Aluminium chloride method:

Standard solution: 1 mg of quercetin was dissolved in 10 ml of 80 % ethanol.

Reagents:

a.10% aluminium chloride solution: 10 g of aluminium chloride solution was dissolved in 100 ml of distilled water.

b.1 M potassium acetate: 9.814 g of potassium acetate was dissolved in 100 ml of water.

Procedure:

The aluminium chloride colorimetric method was carried out using the procedure reported by Chang et al. Quercetin was used to make the calibration curve. From the stock solution of standard 0.1, 0.2, 0.3, 0.4 and 0.5 ml were taken which gave 10, 20, 30, 40 and 50 µg concentrations respectively. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The absorbance of the reaction mixture was measured at 415 nm, after incubation at room temperature for 30 min. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in blank. Similarly 0.5 ml of ethanol extract of *P. daemia* and methanol extract of *B. montanum* (2 mg/ml) were reacted with aluminium chloride for determination of total flavonoid content as described above.

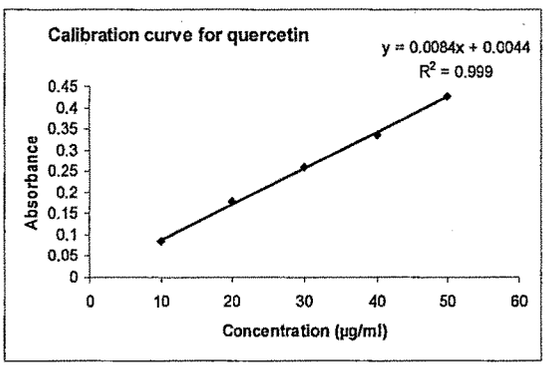
The principle of aluminium chloride colorimetric method is that aluminium chloride forms stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with ortho-dihydroxyl groups in the A or B-ring of flavonoids. Though the apigenin, kaempferol and quercetin can be used for the calibration curve, quercetin was used for this

purpose in the experiment because of its maximum absorbance after reaction with aluminium chloride in the selected concentration range.

Table 2: Concentration and absorbance data for quercetin and extracts

Concentration (µg/ml)	Absorbance
10	0.085
20	0.181
30	0.248
40	0.336
50	0.425
P. daemia (2 mg/ml)	0.301
B. montanum (2 mg/ml)	0.241

Graph 2: Calibration curve for quercetin



The data representing the concentration and absorbances of quercetin, ethanol extract of *P. daemia* and methanol extract of *B. montanum* is represented in Table 2. The calibration curve for the quercetin is represented in Graph 2.

II. 2, 4-dinitro phenyl hydrazine method:

Standard solution: 10 mg of Naringenin was dissolved in 10 ml of methanol.

Reagents:

a.1% 2, 4-DNPH: 1 g of 2, 4-dinitro phenyl hydrazine was dissolved in 2 ml of 96% sulphuric acid and then diluted to 100 ml with methanol.

b.1% KOH: 1 g of potassium hydroxide was dissolved in 100 ml of 70% methanol.

Table 3: Concentration and absorbance data for naringenin and extracts

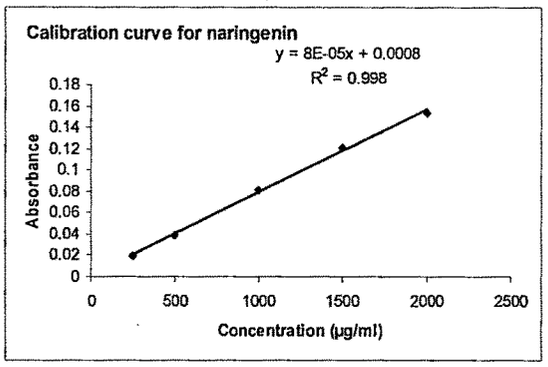
Concentration (µg/ml)	Absorbance
250	0.019
500	0.039
1000	0.081
1500	0.121
2000	0.154
P. daemia (5 mg/ml)	0.027
B. montanum (5 mg/ml)	0.031

Procedure: The method reported by Chang et al was used for this estimation. Naringenin was used as a reference standard to prepare calibration curve. 20 mg of naringenin was dissolved in methanol and then diluted to give concentrations of 250, 500, 1000, 1500 and 2000 µg/ml. one millilitre of each of the diluted standard solutions was separately mixed with 2 ml of 1% 2, 4-dinitro phenyl hydrazine reagent and 2 ml of methanol at 50°C for 50 min. After cooling to room temperature the reaction mixture was mixed with 5 ml of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then 1 ml of the mixture was taken, mixed with 5 ml of methanol and centrifuged at 1000 rpm/min to remove the precipitate formed. The supernatant was collected and adjusted to 25 ml. the absorbance of the supernatant was measured at 495 nm. Similarly 5 ml each of ethanol extract of *P. daemia* and methanol extract of *B. montanum* (5 mg/ml) were similarly treated with 2, 4-dinitro phenyl hydrazine reagent for determination of flavonoid content as described above. For the blank the amount of 2, 4-dinitro phenyl hydrazine reagent was replaced by methanol.

The principle of 2, 4-dinitro phenyl hydrazine method is that 2, 4-dinitro-phenylhydrazine reacts with ketones and aldehydes to form 2, 4-dinitrophenyl hydrazones. Flavones, flavonols and isoflavones with the C2-C3 double bond

could not react with 2,4-dinitro- phenylhydrazine, while the hydrazones of all flavanones show maximum absorbance at 495 nm.

Graph 3: Calibration curve for naringenin



The data representing the concentration and absorbances of naringenin, ethanol extract of *P. daemia* and methanol extract of *B. montanum* was represented in Table3. The calibration curve for the quercetin is represented in Graph 3.

The sum of the values obtained from these two methods was considered as total flavonoid content.

2.3 Phytochemical studies

2.3.1 Successive solvent extraction

The presence of different chemical constituents in crude drugs can be detected by subjecting them to successive extraction using solvents in the order of increasing polarity and subjecting the extracts so obtained to qualitative tests for various chemical constituents. The selected drug samples in the present study were therefore, subjected to successive extraction followed by qualitative chemical tests in order to know the phyto profiles on a preliminary basis.

The air dried powdered drugs weighing about 50 g each were taken and extracted successively in soxhlet apparatus using solvents in he order of increasing polarity, as follows (Kokate, 1999).

1. Petroleum ether
2. Benzene.

3. Chloroform.
4. Acetone.
5. Ethanol.

Each time before extracting with the next solvent, the material was dried in hot air oven at a temperature not exceeding 50°C. Finally the marc was macerated with chloroform water for 24 h to obtain the aqueous extract. All the extracts were concentrated by distilling off the solvents and evaporating to dryness on the water bath. Then physical characters and percentage yield of extracts were recorded.

2.3.2 Qualitative evaluation of successive extracts

The successive extracts were subjected to various qualitative tests to determine the presence of various phytoconstituents using reported methods (Kokate, 1999). Hydro distillation process was used to detect the presence of volatile oil in the drugs.

Alkaloids were tested by Dragendorff, Wagner and Mayer tests; cardenolides were tested by Kedde, Legal and Raymond tests; while phenolics and flavonoids were tested by Ferric chloride, Shinoda, Alkali and Lead acetate tests. Chlorosulphonic acid and Salkowski tests were used for the detection of tri terpenoidal saponins. Terpenoids and steroids were detected by vanillin sulphuric acid and Liebermann-Burchard tests. Phytosterols in unsaponifiable matter of petroleum ether extract were detected by Liebermann-Burchard tests. Fixed oils and fats were detected by spot and saponification tests. Carbohydrates were detected by Molisch and Fehling tests; while amino acids by Ninhydrin test.

2.3.3 Thin layer chromatographic studies on the extracts

The various extracts obtained in the successive extraction process were subjected to thin layer chromatographic studies using Silica gel $60F_{254}$ pre coated plates (Wagner and Blatt, 1996), to confirm the presence of various constituents as described above.

Cardenolides were developed using chloroform: methanol (90:10) as mobile phase and detected using Kedde's reagent as visualisation agent. Flavonoids were developed using ethyl acetate: formic acid: acetic acid: water

(100:11:11:27) as mobile phase and detected using Natural product-polyethylene glycol reagent (NP-PEG) as visualisation agent. Terpenoids were developed using toluene: ethyl acetate (93:7) as mobile phase and Vanillin-sulphuric acid as detecting agent. Alkaloids were detected by Wagner's reagent using toluene: ethyl acetate: diethyl amine (70:20:10) as mobile phase. Sterols were detected by Liebermann-burchard reagent after developing in ethyl methyl ketone: acetonitrile (70:30) as mobile phase. Steroids were detected by antimony trichloride reagent using chloroform: methanol: water (100:13.5:10) as mobile phase. Amino acids were developed using n-butanol: acetic acid: water (4:1:5) as mobile phase and detected using Ninhydrin reagent. Carbohydrates were detected by developing in n-butanol: acetic acid: water (4:1:5) and spraying with 5% ethanolic sulphuric acid. These results were compared with the results obtained in qualitative tests.

2.3.4 Preparation of selective extracts

Aerial parts of *P. daemia*

The shade dried aerial parts of about 500 g were subjected for size reduction to coarse powder. The powder was first defatted with petroleum ether (60-80°C) and then extracted using 5 l of 95% ethyl alcohol using soxhlet extractor to prepare the ethanol extract (EE). The aqueous extract (AE) was prepared by taking 200 g of the powdered drug using chloroform water. Both the ethanol and aqueous extracts were concentrated under vacuum. These extracts were then stored in desiccator.

Roots of *B. montanum:*

The shade dried roots of about 450 g were subjected for size reduction to coarse powder. The powdered drug was extracted using methanol in a soxhlet extractor to obtain methanol extract (ME) which was concentrated under vacuum and stored. The aqueous extract (AE) was prepared by taking 200 g of the powdered drug using chloroform water. The extract was concentrated under vacuum and stored in desiccator.

2.3.5 Fractionation of bio-active extracts

The selective extracts were subjected for preliminary screening for hepatoprotective activity and the extracts with positive activity were subjected to fractionation using different solvents in the order of increasing polarity.

Aerial parts of *P. daemia:*

60 g of activity guided total ethanol extract was adsorbed on to the 250 g of silica gel (60-120 mesh) and eluted (Figure 1) using chloroform and 95% ethyl alcohol by column chromatography. The fractions so obtained were then concentrated under vacuum. The yields of chloroform and 95% ethyl alcohol fractions obtained are found to be 2.46% and 71.78% respectively. Then 43 g of ethanol soluble fraction was again fractionated into benzene, chloroform, acetone and 95% ethyl alcohol soluble sub-fractions. All these fractions and sub-fractions were concentrated in rotary vacuum evaporator.

Figure 1: Fractionation of total ethanol extract from *P. daemia* (Flow diagram)

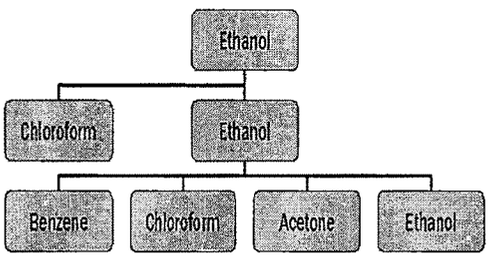
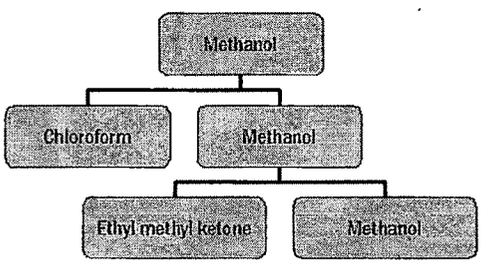


Figure 2: Fractionation of methanol extract from *B. montanum* (Flow diagram)



Roots of *B. montanum*:

The active methanolic extract (25 g) was then subjected to fractionation (Figure 2) into chloroform and methanol soluble fractions by column chromatography using silica gel (60-120 #). The fractions collected were concentrated under vacuum. The yields of chloroform and methanol soluble fractions are found to be 12.06% and 68.86% respectively. Then 40 g of methanol soluble fraction was sub-fractionated into ethyl methyl ketone and methanol soluble fractions. All the fractions and sub-fractions were concentrated in rotary vacuum evaporator.

2.3.6 Thin layer chromatographic studies on the extracts and their different fractions

The active extracts and their fractions were then subjected to TLC studies in order to detect separation of various types of phytoconstituents in different solvents using specific detecting reagents (Wagner and Blatt, 1996) The R_f values of various spots detected were recorded.

Cardenolides were developed using chloroform: methanol (90:10) as mobile phase and detected using Kedde's reagent as visualisation agent. Flavonoids were developed using ethyl acetate: formic acid: acetic acid: water (100:11:11:27) as mobile phase and detected by spraying with Natural product-polyethylene glycol reagent as visualisation agent and observing under UV 366 nm. Terpenoids were developed using toluene: ethyl acetate (93:7) as mobile phase and Vanillin-sulphuric acid as detecting agent. Steroids were detected by Antimony trichloride reagent using chloroform: methanol: water (100:13.5:10) as mobile phase. Amino acids were developed using n-butanol: acetic acid: water (4:1:5) as mobile phase and detected using Ninhydrin reagent. Carbohydrates were detected by developing the plate in n-butanol: acetic acid: water (4:1:5) and spraying with 5% ethanolic sulphuric acid.

2.4 Biological screening of extracts and fractions

2.4.1 Acute toxicity studies

Acute toxicity studies were performed for selective extracts and their fractions according to the acute toxic classic method as per guidelines 423

prescribed by OECD (OECD, 1996). Female albino rats were used for acute toxicity study. The animals were kept fasting for overnight providing only water. These were divided into groups of each containing three animals. Each of these groups was then administered separately with ethanol extract (EE), ethanol fraction of ethanol extract (EFEE), acetone sub-fraction of ethanol extract (AFEFEE) and ethanol sub-fraction of ethanol extract (EFEFEE) of aerial parts of *P. daemia* and methanol extract (ME), methanol fraction of methanol extract (MFME), ethyl methyl ketone sub-fraction of methanol extract (EMKMFME) and methanol sub-fraction of methanol extract (MFMFME) of roots of *B. montanum* at a dose of 300 mg/kg p.o. The animals were observed continuously after dosing during first 30 min and then periodically for first 24 h with special attention during first 4 h and thereafter daily, for a total of 14 days. The observations like sedation, convulsions, tremors, salivation, lethargy, death etc are systematically recorded with individual records for each animal. Since no mortality was seen at the dose level of 300 mg/kg, the procedure was repeated with higher dose of 2000 mg/kg p.o. in fresh animals.

2.4.2 Hepatoprotective activity

The plant *P. daemia* is used particularly used to treat jaundice by the folklore people in Chittoor district region of Andhra Pradesh state. The usage of the plant *B. montanum* to treat jaundice was mentioned in Ayurveda. It has become therefore, an important task to evaluate these drugs in a scientific manner. The hepatoprotective screening was, therefore, planned on the selective extracts as well as their fractions in the following manner.

2.4.2.1 Hepatoprotective activity in vivo

The extracts and their fractions were subjected to hepatoprotective screening after inducing acute hepatotoxicity using hepatotoxins like, carbon tetrachloride, paracetamol and thioacetamide using the following procedural details.

A. Animals:

Wistar albino rats weighing 175-225 g of either sex, maintained under standard husbandry conditions (Temp 23 ± 2°C, relative humidity 55 ± 10%

and 12 h light dark cycle) were used for all studies. Animals were allowed to take standard laboratory feed and tap water. The experiments were performed after the experimental protocols approved by the institutional animal ethics committee, The M.S.University of Baroda, Vadodara, Gujarat. The animals were divided into groups consisted of 6 rats.

B. Preparation of solutions for administration:

I. Carbon tetrachloride (CCl_4): 50% v/v solution of carbon tetrachloride was prepared in liquid paraffin. The solution was administered at the dose of 2.5 ml/kg b.w. i.p.

II. Paracetamol suspension (PCML): Known amount of paracetamol powder was suspended in 5% acacia mucilage and administered at the dose of 3g/kg b.w. p.o.

III. Thioacetamide (TAA): Known amount of thioacetamide was dissolved in distilled water and administered at the dose of 100 mg/kg b.w. i.p.

IV. Suspensions of test substances: All the selective extracts were suspended in 5% acacia mucilage and administered at the dose levels of 100, 200 and 300 mg/kg where as their fractions were administered at the dose levels of 50, 150 and 250 mg/kg; while the sub-fractions were administered at the dose levels of 50, 100, 150 mg/kg. Silymarin being positive control was suspended in 5% acacia mucilage and administered at a dose level of 100 mg/kg.

C. CCl_4 induced-hepatotoxicity:

Ethanol extract (EE) and aqueous extract (AE) obtained from aerial parts of *P. daemia* and methanol extract (ME) and aqueous extract (AE) obtained from roots of *B. montanum* were subjected to the evaluation of hepatoprotective activity in vivo on preliminary basis, against CCl_4-induced toxicity by assessing them through biochemical parameters and histopathological observations. Each set of experiment was divided into groups consisting of control, toxicant, standard, and test. Groups consisted of 6 rats each unless otherwise mentioned. The protocol followed for CCl_4-induced hepatotoxicity on preliminary basis (Sureshkumar and Mishra, 2005) was given in Table 4. Where as the further assessment of the activity using

selective extracts and their fractions the protocol mentioned in the Table 5 (Rao and Mishra, 1998a) was followed.

Table 4: The protocol for CCl_4-induced hepatotoxicity on preliminary basis

Group	0 h	24 h	48 h	72 h
Control	Vehicle	Vehicle	Vehicle	Withdrawal of blood
CCl_4	Vehicle	Vehicle+CCl_4	Vehicle	
Silymarin	Silymarin	Silymarin+ CCl_4	Silymarin	
Test	Extract	Extract+ CCl_4	Extract	

Vehicle: 5% acacia mucilage, Test: Extracts prepared in 5% acacia mucilage.

The rats of control group received three doses of 5% acacia mucilage (1 ml/kg, p.o.) at 24 h intervals (0 h, 24 h and 48 h). The animals in CCl_4 treated group received vehicle at 0 h and at 24 h vehicle followed by followed by CCl_4 diluted in liquid paraffin (1:1 i. p.) at a dose of 1.25 ml/kg, while at 48 h these animals received only vehicle. The test groups received the first dose of extracts at 0 h, second dose of extracts at 24 h, which was followed by a dose of CCl_4 and at 48 h the third dose of extracts. The positive control group received the first dose of silymarin (200 mg/kg) (Sureshkumar and Mishra, 2005) at 0 h, second dose of silymarin at 24 h followed by a dose of CCl_4 and at 48 h the third dose of silymarin. After 72 h blood was collected from all the groups, allowed to clot for the separation of serum. The serum was used for estimation of biochemical parameters.

Table 5: The protocol for CCl_4-induced hepatotoxicity

Group	0 h	12 h	24 h	36 h
Control	Vehicle	Vehicle	Vehicle	Withdrawal of blood
CCl_4	Vehicle+CCl_4	Vehicle	Vehicle	
Silymarin	Silymarin+ CCl_4	Silymarin	Silymarin	
Test	Extract+ CCl_4	Extract	Extract	

Vehicle: 5% acacia mucilage, Test: Extracts prepared in 5% acacia mucilage.

The rats of control group received three doses of 5% acacia mucilage (1 ml/kg, p.o.) at 12 h intervals (0 h, 12 h and 24 h). The rats of carbon tetrachloride group received three doses of vehicle at 12 h intervals and a single dose of carbon tetrachloride (1.25 ml/kg i.p.) diluted in liquid paraffin (1:1) 30 min after the administration of first dose of vehicle.

The animals in silymarin group received three doses of silymarin (100 mg/kg) at 0 h, 12 h and 24 h. CCl_4 (1.25 ml/kg i.p.) was administered 30 min after the first dose of silymarin while the test groups were given first dose of extract in acacia mucilage at 0 h which was followed by a dose of CCl_4 (1.25 ml/kg i.p.) after 30 min, while at 12 h, and 24 h the second and third dose of respective extracts. After 36 h of administration of CCl_4, blood was collected and serum was separated and used for determination of biochemical parameters.

D. Paracetamol-induced hepatotoxicity:

Each set of experiment was divided into groups consisting of control, toxicant, standard, and test. The protocol followed for paracetamol-induced hepatotoxicity (Rao and Mishra, 1998a) is given in Table 6.

Table 6: The protocol for paracetamol-induced hepatotoxicity

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Control	Vehicle	Vehicle	Vehicle	Vehicle	Withdrawal of blood
PCML	Vehicle	Vehicle	Vehicle+ PCML	Vehicle	
Standard	Silymarin	Silymarin	Silymarin+ PCML	Vehicle	
Test	Extract	Extract	Extract+ PCML	Vehicle	

Vehicle: 5% acacia mucilage, Test: Extracts prepared in 5% acacia mucilage.
PCML: Paracetamol.

The rats of control group received a single daily dose of 5% acacia mucilage (1 ml/kg p.o.). The rats of paracetamol group received a single daily dose of vehicle for three days and a single dose of paracetamol (3 g/kg) 30 min after the administration of the vehicle, on the third day of experiment.

The animals in silymarin group received a single daily dose of silymarin (100 mg/kg p.o.) for three days. Paracetamol ((3 g/kg p.o.) was administered 30 min after the third dose of silymarin while test groups were given orally a single daily dose of extracts in acacia mucilage for three days and a single dose of paracetamol (3 g/kg p.o.) on the third day 30 min after the administration of respective test suspensions (Rao and Mishra, 1998a). After 48 h of paracetamol intoxication blood was collected and serum was analysed for the biochemical parameters.

D. Thioacetamide induced hepatotoxicity:

Each set of experiment was divided into groups consisting of control, toxicant, standard, and test. The protocols followed for thioacetamide-induced hepatotoxicity (Handa and Singh, 1995) was given in Table 7.

Table 7: The protocol for thioacetamide induced hepatotoxicity

Group	Day 1	Day 2	Day 3	Day 4
Control	Vehicle	Vehicle	Vehicle	Withdrawal of blood
TAA	Vehicle	Vehicle + TAA	Vehicle	
Silymarin	Silymarin	Silymarin + TAA	Silymarin	
Test	Extract	Extract + TAA	Extract	

Vehicle: 5% acacia mucilage, Test: Extracts prepared in 5% acacia mucilage.
TAA: Thioacetamide.

The rats of control group received a single daily dose of 5% acacia mucilage (1 ml/kg, p.o.). The rats of toxicant group received a single daily dose of vehicle (1 ml/kg, p.o.) for three days and a single intraperitoneal injection of thioacetamide (100 mg/kg) in water for injection, 30 min after the administration of the vehicle on the second day of treatment. The rats of silymarin group received silymarin (100 mg/kg p.o.) three times at 24 h intervals. Thioacetamide was administered 30 min after the second dose of silymarin while test groups were given orally a single daily dose of extracts in vehicle for three days and a single dose of thioacetamide (100 mg/kg i.p.) on the second day 30 min after the administration of respective test suspensions. After 48 h of thioacetamide administration i.e 4^{th} day of the experiment the

blood was collected and serum was used for determination of biochemical parameters.

E. Assessment of liver function:

Blood was collected from all the groups by puncturing the retro-orbital plexus and was allowed to clot at room temperature and serum was separated by centrifuging at 2500 rpm for 10 min. the serum was used for estimation of biochemical parameters to determine the functional of the liver. Glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) were estimated by a UV-Kinetic method based on the reference method of International Federation of Clinical Chemistry (Bergmeyer et al., 1985). Alkaline phosphatase (ALKP) was estimated method by PNPP method (Mac Comb and Bowers, 1972), while total bilirubin (TBL) by Jendrassik and Grof method (Chhaya & Mishra, 1999), total cholesterol (CHL) by CHOD-PAP method (Richmond, 1973), total protein (TPTN) by color complexation with copper ions in an alkali solution (Peters, 1968). Albumin was estimated by bromo cresol green method (Webster, 1974). All the estimations were carried out using standard kits on auto analyser of Merck make (300 TX, E.Merck-Micro Labs, Mumbai).

Estimation of GOT and GPT:

The estimation of GOT and GPT were carried out by enzyme-coupled system based on reference method of International Federation of Clinical chemistry (IFCC) using standard kits.

Principle: The ability of the GOT (aminotransferase) to react with 2-oxoglutarate and L-aspartate to yield glutamate and a keto acid oxaloacetic acid forms the basis of the reaction. The resultant keto acid reacts in a system using NADH. The coenzyme is oxidized to NAD yielding malate and the reaction is catalysed by malate dehydrogenase (MDH). The rate of NADH consumption is measured photometrically and is directly proportional to the GOT activity in the sample. Similarly GPT reacts with 2-oxoglutarate and L-alanine to yield glutamate and pyruvate. The resulting pyruvate consumes NADH to yield lactate in presence of lactate dehydrogenase and the rate of

NADH consumption is measured photometrically and is directly proportional to the GPT activity in the sample.

Test reagents: The reagents for GOT determination includes Tris buffer, pH 7.8 (80 mmol/l), L-aspartate (240 mmol/l), 2-oxoglutarate (12 mmol/l), NADH (0.18 mmol/l) and MDH (≥ 420 U/l). The reagents for GPT determination includes Tris buffer, pH 7.8 (100 mmol/l), L-alanine (500 mmol/l), 2-oxo glutarate (15 mmol/l), NADH (0.18 mmol/l) and LDH (≥ 1.2 kU/l).

Procedure: For both GOT and GPT determinations 50 µl of serum was first mixed with 500 µl of reagent solution 1 and mix well. After 1 minute, 125 µl of reagent solution 2 was added and mixed well. Approximately after one minute the absorbance was measured at 340 nm.

Estimation of Alkaline phosphatase:

The estimation of alkaline phosphatase was carried out by the method of Bessey-Lowry-Brock modified by Bowers and Mc Comb using standard kits.

Principle: p-nitrophenylphosphate undergoes hydrolysis in presence of alkaline phosphatase to yield phosphate and p-nitrophenol which gives a strong yellow color in alkaline solution. Therefore the formation of this product can be monitored directly by measuring the change in absorbance at 405 nm.

Test reagents: The reagents and solutions includes Diethanolamine HCl buffer pH 9.8 (1 mol/l), Magnesium chloride (0.5 mmol/l) (Reagent 1) and p-nitrophenylphosphate (10 mmol/l) (Reagent 2).

Procedure: About 20 µl of the serum was mixed with 1000 µl of reagent solution 1 and 250 µl of reagent solution 2. Mixed well and after one minute the increase in absorbance was measured.

Estimation of Total bilirubin:

The method of Jendrassik and Grof was used for the estimation of total bilirubin.

Principle: Bilirubin reacts with diazotized sulphanilic acid to form an azocompound the color of which is measured at 546 nm and is proportional to the concentration of bilirubin. The reaction is accelerated by caffeine reagent.

Test reagents: Reagent 1 consisted of sodium nitrate 10 mmol/l, reagent 2 consisted of sulphanilic acid 23 mmol/l and reagent 3 consisted of sodium acetate 0.9 mol/l, sodium benzoate 0.5 mol/l and caffeine 0.25 mol/l.

Procedure: Reagent 1 and 2 are mixed in equal volumes to form working solution 1 and working solution 2 consisted of one volume of saline and two volumes of reagent 2, where as working solution 3 consisted of equal volumes of reagent 3 and distilled water. To the 50 µl of serum added 100 µl of solution 2 and 1000 µl and solution 3 and incubated for 5 min at room temperature and read before 10 min.

Estimation of cholesterol:

The method of CHOD-PAP described by Richmond was used for the determination of cholesterol.

Principle: Free cholesterol is hydrolysed by cholesterol oxidase to cholestenone-4-en-3-one and hydrogen peroxide. Hydrogen peroxide by the action of peroxidase liberates oxygen which reacts with 4-amino antipyrine and phenol to form red coloured compound which is measured at 500 nm.

Test reagents: Reagent solution containing Pipes buffer (pH 7.5) 99 mmol/l, Salicylic alcohol 3.96 mmol/l, 4-Amino antipyrine 0.5 mmol/l, Peroxidase, ≥ 1000 U/l, Cholesterol oxidase ≥ 100 U/l and Cholesterol esterase ≥ 100 U/l. and Standard solution containing cholesterol.

Procedure: 10 µl of the serum was mixed with 1000 µl of reagent solution and 10 µl of the standard solution was mixed with 1000 µl reagent solution. Both are mixed well and incubated for 5 min at 37°C and absorbance was measured.

Estimation of Total protein:

Total protein content was estimated by Biuret method.

Principle: Proteins produce a violet colour complex with copper ions in an alkaline solution. The absorbance of the color complex is directly proportional to the protein concentration in the sample.

Test reagents: Reagent 1 consists of sodium hydroxide 100 mmol/l, potassium-sodium-tartrate 16 mmol/l, reagent 2 consists of copper sulphate 6 mmol/l, potassium-sodium-tartrate 16 mmol/l, sodium hydroxide 100 mmol/l,

and potassium iodide 15 mmol/l where as reagent 3 consists of protein standard 5 g/dl.

Procedure: For sample 20 µl of serum was mixed with 1000 µl of reagent 1, for standard 20 µl of reagent 3 and for blank 1000 µl of reagent 1 was added. Then all the solutions were mixed well and incubated at 37°C and then added 250 µl of reagent 2 to all the solutions. Mixed well and incubated at 37°C for 5 min and absorbance was measured at 540 nm.

Estimation of Albumin:

The method of BCG was used for the estimation of albumin content in the serum.

Principle: albumin forms blue-green complex with bromocresol green at slightly acidic pH which is measured photometrically at 546 nm.

Test reagents: Reagent 1 consists of citrate buffer 30 mmol/l, bromocresol green 0.26 mmol/l and reagent 2 consists of albumin standard 5 g/dl.

Procedure: For sample 10 µl of serum was mixed with 1000 µl of reagent 1, for standard 10 µl of reagent 2 and for blank 1000 µl of reagent 1 was added. Then all the solutions were mixed well and incubated at 37°C for 10 min and absorbance was measured at 546 nm.

F. Statistical Analysis

The mean values±SEM were calculated for each parameter. Percentage restoration against the hepatotoxin by the test samples was calculated by considering the difference between the hepatotoxin treated group and the control group as 100% restoration. For the determination of significant inter group difference each parameter was analysed separately and one way analysis of variance (ANOVA) (Gennaro, 1995) was carried out. After that individual comparisons of group mean values were done using Dunnet's test (Dunnet, 1964).

G. Histopathological Studies

The hepatoprotective activity of the various extracts was again confirmed by performing the histopathological examination of the treated livers. The animals were sacrificed and the abdomen was cut open and 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 75ml of saturated picric acid, 25ml of 40 % formaldehyde and 5ml of glacial acetic acid) for 12 h. These liver pieces were then washed with water until free from Bouin's fluid. These tissues were then processed for paraffin embedding using conventional methods (Galighor and Kozloff, 1976). 5 μm 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 histopathological changes in liver architecture and their photomicrographs were taken, illustrating changes in cellular structure in those of test groups, control and toxicant groups.

2.4.2.2 Hepatoprotective activity in vitro

The studies were also performed for determination of in vitro hepatoprotective activity of the sub-fractions and isolated compounds against various hepatotoxins like, carbon tetrachloride, paracetamol and thioacetamide.

Isolated hepatocytes have become a useful model for pharmacological, toxicological, metabolic and transport studies of xenobiotics since the development of techniques for high yield isolation of rat hepatocytes (Skett, 1994). Various hepatotoxins viz. carbon tetrachloride, thioacetamide and paracetamol have shown reduction of viability of hepatocytes, and leakage of enzymes which are considered to be the markers of cellular injury (Belinsky et al., 1984; Zimmerman and Mao, 1965).

The in vivo studies required a large number of animals (six per group), and needed up to 3-5 days of drug administration for a significant effect to be produced and thus required large quantities of drugs, where as in vitro models on the other hand are more rapid and requires small quantities of test substances and fewer animals, generally useful in studies on natural products.

A. Isolation of rat hepatocytes:

Hepatocytes were isolated from rat liver as per the reported method (Sarkar and Sil, 2006) with some modifications. The liver was isolated under

aseptic condition and placed in chilled HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffer I containing HEPES (0.01 M), NaCl (0.142 M), and KCl (0.0067 M), pH 7.4. The liver pieces were then incubated in a second buffer containing HEPES (0.1 M), NaCl (0.0667 M), KCl (0.0067 M), and 0.5% Collagenase type IV, at pH 7.6 for about 45 min at 37°C. Hepatocytes were obtained after filtration through sterile muslin cloth and cold centrifugation (4°C, 200 rpm/min for 2 min, three times) and resuspended in 4-5 ml of HEPES buffer I. The viability of the hepatocytes was assessed by trypan blue (0.2%) exclusion method (Kiso et al., 1983).

Trypan blue dye exclusion method: This is the most commonly employed criterion of cellular integrity or viability. Cells with an intact plasma membrane exclude this dye whereas damaged cells become stained particularly in the nucleus. Under ordinary conditions the dye concentration of 0.2% is sufficient but staining intensity may show a considerable variation depending upon components in the cell suspension/solution or in a particular batch of trypan blue. The stained hepatocytes were observed under microscope and four different fields were scanned to calculate the viability of cells.

B. Primary cultures of rat hepatocytes:

The method of Tingstrom and Obrink (Tingstrom and Obrink, 1989) with slight modifications was used for this purpose. The freshly isolated viable hepatocytes were suspended in culture medium RPMI-1640 supplemented with calf serum (10%), HEPES and gentamicin (1 µg/ml). These cells (approximately 1-1.2 X 10^6/ml) were then seeded into culture bottles and incubated at 37°C in atmosphere of 5% CO_2. The hepatocytes formed a mono layer upon incubation for 24 h. The newly formed cells were round and mostly appeared as individual cells. These cells were 96-97% viable as confirmed by trypan blue exclusion test.

C. Hepatic cytotoxicity testing of sub-fractions:

The acetone and ethanol sub-fractions (AFEFEE and EFEFEE) of the EE from *P. daemia*; ethyl methyl ketone and methanol sub-fractions (EMKMFME and MFMFME) of ME from *B. montanum* were tested for their hepatic cytotoxicity at dose levels of 500, 1000 and 1500 µg/ml on isolated rat

primary cultured hepatocytes. The hepatocytes suspensions were incubated with respective sub-fractions for 24 h in CO_2 incubator at a temperature of 37°C. The hepatic cytotoxicity was assessed after 24 h of incubation with these sub-fractions, by calculating the percentage viability (Kiso et al., 1983) and also by estimating total protein content (Peters, 1968) in the surrounding medium. The test substances were dissolved in 30 % DMSO (Tasaduq et al., 2003) and used for activity. The protocol (Rao and Mishra, 1998b) used for hepatic cytotoxicity testing of extracts and compounds is as given in Table 8.

Table 8: Protocol for the in vitro assessment of hepatic cytotoxicity of the sub-fractions and isolated compounds

Group	Contents	24 h
Control	0.1 ml HS + 0.1 ml Vehicle + 0.8 ml HEPES buffer 1	Estimation of hepatocytes viability and TPTN
AFEFEE	0.1 ml HS + 0.1 ml AFEFEE + 0.8 ml HEPES buffer 1	
EFEFEE	0.1 ml HS + 0.1 ml EFEFEE + 0.8 ml HEPES buffer 1	
EMKMFME	0.1 ml HS + 0.1 ml EMKMFME + 0.8 ml HEPES buffer 1	
MFMFME	0.1 ml HS + 0.1 ml MFMFME + 0.8 ml HEPES buffer 1	
PD1	0.1 ml HS + 0.1 ml PD1 + 0.8 ml HEPES buffer 1	
BM1	0.1 ml HS + 0.1 ml BM1 + 0.8 ml HEPES buffer 1	

Vehicle: 30 % DMSO; HS: Hepatocyte suspension; PD1:100 and 500 µg/ml; BM1:100 and 500 µg/ml; AFEFEE: 500, 1000 and 1500 µg/ml; EFEFEE: 500, 1000 and 1500 µg/ml; EMKMFME: 500, 1000 and 1500 µg/ml; MFMFME: 500, 1000 and 1500 µg/ml;

D. Hepatoprotective activity of sub-fractions:

Table 9: Protocol for the in vitro hepatoprotective activity of the sub-fractions and isolated compounds

Group	Contents	24 h
Control	0.1 ml HS + 0.1 ml Vehicle + 0.8 ml HEPES buffer 1	Estimation of hepatocytes viability, GOT, GPT and TPTN
Toxicant	0.1 ml HS + 0.1 ml Toxicant + 0.8 ml HEPES buffer 1	
Silymarin	0.1 ml HS + 0.1 ml Silymarin + 0.1 ml Toxicant + 0.7 ml HEPES buffer 1	
AFEFEE	0.1 ml HS + 0.1 ml AFEFEE + 0.1 ml Toxicant + 0.7 ml HEPES buffer 1	
EFEFEE	0.1 ml HS + 0.1 ml EFEFEE + 0.1 ml Toxicant + 0.7 ml HEPES buffer 1	
EMKMFME	0.1 ml HS + 0.1 ml EFEFEE + 0.1 ml Toxicant + 0.7 ml HEPES buffer 1	
MFMFME	0.1 ml HS + 0.1 ml EFEFEE + 0.1 ml Toxicant + 0.7 ml HEPES buffer 1	
PD1	0.1 ml HS + 0.1 ml PD1 + 0.1 ml Toxicant + 0.7 ml HEPES buffer 1	
BM1	0.1 ml HS + 0.1 ml BM1 + 0.1 ml Toxicant + 0.7 ml HEPES buffer 1	

Vehicle: 30 % DMSO; Silymarin: 100 µg/ml; Toxicants: CCl_4 10 µl/ml, paracetamol 300 µg/ml and thioacetamide 40 µg/ml; HS: Hepatocyte suspension; AFEFEE: 100, 500 and 1000 µg/ml; EFEFEE: 100,500 and 1000 µg/ml; EMKMFME: 100, 500 and 1000 µg/ml; MFMFME: 100, 500 and 1000 µg/ml ; PD1: 10, 100 and 500 µg/ml; BM1: 10, 100 and 500 µg/ml.

The protocol (Rao and Mishra, 1998b) followed for the in vitro hepatoprotective activity of various fractions against different toxicants is as given in Table 9. Twenty four h after the establishment of the monolayers of hepatocytes, the medium was decanted and the culture was washed with HEPES buffer I and finally the hepatocytes were suspended in 5 ml of HEPES

buffer I. The hepatic cytotoxcities were induced with carbon tetrachloride (10 µl/ml), paracetamol (300 µg/ml) and thioacetamide (40 µg/ml).Test substances including silymarin were dissolved in 30 % DMSO. Hepatocyte suspensions (0.1 ml) in triplicate were distributed into various culture tubes labelled as control, toxicant, standard (silymarin + toxicant) and test (test sample + toxicant). The control group received 0.1 ml of vehicle (30% DMSO) and toxicant groups received 0.1 ml of appropriate hepatotoxins dissolved in 30% DMSO, while the test groups received 0.1 ml of respective test solutions (100, 500 and 1000 µg/ml of extracts dissolved in 30% DMSO) followed by 0.1 ml of appropriate hepatotoxins. The standard groups received 0.1 ml of silymarin solution (100 µg/ml) followed by respective hepatotoxins. The content of the all culture tubes were made up to 1 ml with HEPES buffer I. The contents of all the tubes were mixed well and incubated in a CO_2 incubator for 24 h at 37°C. In test and standard groups the hepatocytes were incubated with respective solutions for 30 min and then exposed to hepatotoxins. After incubation hepatocyte suspensions were collected to assess cell damage. Cell viability was evaluated by trypan blue exclusion method (Kurma and Mishra, 1998b). Hepatocytes suspensions were centrifuged at 200 rpm. The leakage of the enzymes GOT, GPT and total proteins secreted outside the cells were determined from the supernatant.

2.5 Isolation and characterisation of active principles from active extracts

Sub-fractions with proven activity from aerial parts of *P. daemia* and roots of *B. montanum* were further subjected to the isolation of active principles.

2.5.1 Isolation of active principles

2.5.1.1 Isolation of active principles from *P. daemia*

Acetone sub-fraction (AFEFEE) of ethanol extract when subjected for thin layer chromatographic studies using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase, on silica gel 60 F_{254} pre-coated plates and NP-PEG as a detecting agent, revealed nine spots under UV 366 nm, indicating presence of flavonoids. Among these three were found to be

major at R_f 0.41, 0.53 and 0.64 respectively. An attempt was made to isolate these compounds using column chromatography.

About 10 g of AFEFEE was dissolved in sufficient quantity of acetone and the resulting solution is adsorbed on to the minimum amount of silica gel 200 mesh. The glass column was (30 mm X 55 cm) prepared by dry packing method using ethyl acetate as solvent and 250 g of silica gel. The sub-fraction (AFEFEE) was loaded on to the silica gel. Then the column was eluted initially with ethyl acetate then by a mixture of ethyl acetate and acetone of different concentrations followed by acetone and mixture of acetone with methanol of different concentrations. In all about 56 fractions were collected, 50 ml each. All the fractions were then subjected for thin layer chromatography using ethyl acetate: formic acid: acetic acid: water (100:11: 11:27) as mobile phase and NP-PEG as a visualising agent to detect the pattern of elution of the components. The fractions which showed similar components were combined. The combined fractions were subjected to concentration in rotary evaporator.

The fractions 49-56 elute in acetone: methanol (80:20) showed three compounds of R_f of 0.30, 0.41 and 0.53 on TLC. These fractions were combined and evaporated. The residue was dissolved in sufficient amount of acetone and methanol mixture (80:20) and subjected for preparative TLC on silica gel 60 and ethyl acetate: formic acid: acetic acid: water (100:11:11:27) as mobile phase to afford individual components.

The individual components were obtained from the chromatogram by marking respective bands (under UV 366 nm) and scrapping the respective areas of the plate. Then the individual components were eluted in methanol and solvent was evaporated in vacuum. Although two compounds PD1 and PD2 (R_f 0.41 and 0.53) were obtained in sufficient quantities, PD2 was unstable at room temperature and it acquired brownish black colour from its original greenish yellow colour and lost its solubility in the solvents tried. The compound was identified after co-TLC using authentic sample as quercetin-3-glucoside (Samia et al., 2006). Hence only PD1 was used for further studies.

Purification of PD1: The compound was further purified by dissolving it in acetone and decolouring with charcoal.

2.5.1.2 Isolation of active principles from *B. montanum*

Methanol sub-fraction (MFMFME) of methanol extract was subjected for thin layer chromatography on silica gel 60 F_{254}, using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase and NP-PEG reagent as a visualising agent. This revealed ten bands under UV 366 nm with five as major bands of R_f values 0.13, 0.23, 0.32, 0.43 and 0.51 indicating the presence of flavonoids. An attempt was made to separate these compounds by preparative TLC using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27). The respective bands are marked after development under UV 366 nm and these bands are scrapped and components were eluted in methanol. This afforded sufficient amount of compound of R_f 0.43 (BM1) which was used for further studies. The yield of other compounds was too poor to be used for further studies.

Purification of BM1: The isolated compound when subjected for thin layer chromatography using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase and NP-PEG reagent as a visualising agent revealed under UV 366 nm, two bands with R_f values of 0.43(major) and 0.41(minor) respectively. This mixture was subjected for separation on column chromatography using silica gel and eluted with methanol. The compound with R_f of 0.43 was sufficient in quantity to be used for further studies and the remaining minor compound was quite in sufficient to be used for further studies.

2.5.2 Hepatoprotective activity of isolated compounds in vitro

2.5.2.1 Hepatic cytotoxicity testing of PD1 and BM1

PD1 isolated from AFEFEE of *P. daemia* and BM1 isolated from MFMFME of *B. montanum* were also tested for their toxicity on hepatocytes at dose levels of 500 and 1000 µg/ml.

For this purpose the hepatocytes suspensions were incubated with respective compounds for 24 h in CO_2 incubator at a temperature of 37°C. After 24 h of incubation with these isolated compounds, the hepatic

cytotoxicity was assessed by calculating the percentage viability of hepatocytes as is determined by trypan blue exclusion method (Kiso et al., 1983) and by estimating total protein content in the surrounding medium. The test substances are dissolved in 30 % DMSO (Tasaduq et al., 2003) and used for activity. The protocol (Rao and Mishra, 1998b) used for hepatic cytotoxicity testing of extracts and compounds was as given in Table 8.

2.5.2.2 Hepatoprotective activity of PD1 and BM1

The protocol followed for in vitro hepatoprotective activity of isolated compounds against different toxicants was as mentioned in Table 9. The hepatotoxicities were induced with carbon tetrachloride (10µl/ml), paracetamol (300 µg/ml) and thioacetamide (40 µg/ml). The compounds used as test substances including silymarin were dissolved in 30 % DMSO.

Hepatocyte suspensions (0.1 ml) in triplicate were distributed into various culture tubes labelled as control, toxicant, standard (silymarin + toxicant) and test (test sample + toxicant). The control group received 0.1 ml of vehicle (30% DMSO) and toxicant groups received 0.1 ml of appropriate hepatotoxins dissolved in 30% DMSO, while the test groups received 0.1 ml of respective test solutions (10, 100 and 500 µg/ml of compounds dissolved in 30% DMSO) followed by 0.1 ml of appropriate hepatotoxins. The standard groups received 0.1 ml of silymarin solution (100 µg/ml) followed by respective hepatotoxins. The content of the all culture tubes were made up to 1 ml with HEPES buffer I. The contents of all the tubes were mixed well and incubated in a CO_2 incubator for 24 h at 37°C. In test and standard groups the hepatocytes were incubated with respective solutions for 30 min and then exposed to hepatotoxins. After incubation hepatocyte suspensions were collected to assess cell damage as per the method described under sub tittle 'hepatoprotective activity of sub-fractions' (2.4.2.2.D).

2.5.2.3 Assessment of hepatoprotective activity:

The protective effect of isolated compounds was determined by measuring an increase in the percentage of viable cells in that group of cells incubated with compounds, compared with the control and toxicant groups. Reversal of toxin-induced alterations in the levels of enzymes and proteins

were also considered to be an important criterion of protective activity. The UV-Kinetic method based on the reference method of International Federation of Clinical Chemistry (Bergmeyer et al., 1985) was followed for the assessment of activity of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Total proteins were estimated by biuret method (Peters, 1968).

2.5.2.4 Statistical analysis

The mean values±SEM are calculated for each parameter. Percentage restoration against the hepatotoxin by the test samples was calculated by considering the difference between the hepatotoxin treated group and the control group as 100% restoration. For determining the significant inter group difference each parameter was analysed separately and one-way analysis of variance (Gennaro, 1995) was carried out and the individual comparisons of the group mean values were done using Dunnett's test (Dunnett, 1964).

2.5.3 Characterisation of isolated compounds

The isolated compounds PD1 and BM1 were then subjected to determinations of various physical characteristics like melting point, solubility index and absorption maxima in UV light etc. to elucidate the structures. The FT-IR, ^{1}H NMR, ^{13}C NMR, ^{13}C DEPT NMR (Distortion less Enhancement through Polarization Transfer), DQF COSY (Double Quantum Filtered Correlation Spectroscopy), NOESY (Nuclear Overhauser Effect Spectroscopy), HSQC (Heteronuclear Single Quantum coherence) and HMBC (Hetero Nuclear Multiple Bond Connectivity) and Mass spectra of individual compounds were performed. The various observations were recorded to elucidate the structure of the isolated compounds.

2.6 High performance thin layer chromatographic analysis

It is an accepted fact that qualitative and quantitative analysis of major chemical components (marker components) of plant material constitutes an important and reliable part of a quality control protocol because the change in quality of the plant material directly affects the constituents. If marker itself is a biologically active principle of the plant, qualitative and quantitative analysis of

these biomarkers not only helps to control the quality of the herbal material used, but also to estimate the quantity of the biologically active chemical entities required to produce specific pharmacological activity (Mukherjee at al., 2006).

2.6.1 Aerial parts of *P. daemia*

The biologically active ethanol extract (EE), ethanolic fraction of ethanol extract (EFEE) and acetone sub-fraction of ethanol extract (AFEFEE) were subjected to high performance thin-layer chromatographic studies (HPTLC). The isolated compound PD1 and quercetin-3-glucoside (Samia et al., 2006) were also run simultaneously to detect their presence in these extracts. An attempt was also made to estimate the amount of quercetin-3-glucoside and PD1 in the ethanol extract.

2.6.1.1 Fingerprint profile of EE, EFEE and AFEFEE

2 µg/µl solutions of each of EE, EFEE, and AFEFEE; 4 ng/µl solution of quercetin-3-glucoside and 8ng/µl of chlorogenic acid were subjected to HPTLC studies on silica gel 60 F_{254} thin layer chromatographic plate using ethyl acetate: formic acid: acetic acid: water (100:11:11:27) as mobile phase. NP-PEG reagent was used as detecting reagent. The plate was then scanned at 366 nm. The color, R_f value and spectral comparison was taken into consideration to detect the presence of quercetin-3-glucoside and PD1 in the extracts. The complete analysis was performed with Camag HPTLC instrument.

2.6.1.2 Estimation of PD1 in ethanol extract

The estimation of PD1 in the ethanol extract (EE) was carried out by linear calibration curve constructed for the compound.

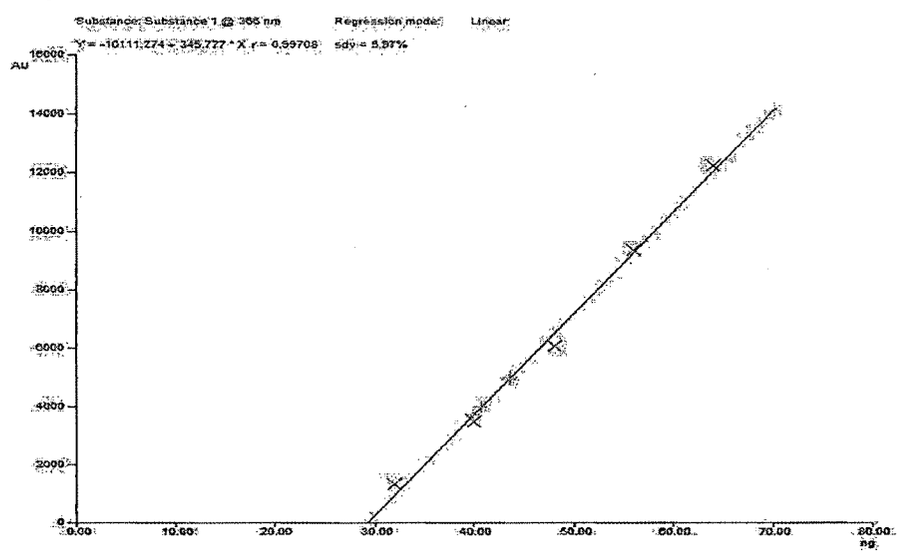
Stock solution of PD1 was prepared by dissolving 2 mg in 5 ml of methanol. Further 0.1 ml of the stock solution was diluted with methanol to 5 ml to give concentration of 8 ng/µl. The stock solution of ethanol extract was prepared by dissolving 20 mg of extract in 10 ml of ethanol. 4, 5, 6, 7 and 8 µl of PD1 (32, 40, 48, 56 and 64 ng) and 14 µl of EE (2 µg/µl) in ethanol were spotted on silica gel 60 F_{254} thin layer chromatographic plate. Then the plate was developed using ethyl acetate: formic acid: acetic acid: water

(100:11:11:27) as mobile phase. The post chromatographic derivatization was carried out with NP-PEG reagent. The chromatogram was then scanned at 366 nm. The concentration of PD1 in EE was calculated form calibration curve plotted by taking concentration on X-axis and area under peak on Y-axis. The calibration curve of PD1 is as shown in Graph 4. The Table 10 indicates the concentration and peak area of standard and sample (n=4).

Table 10: Concentration and peak area data for PD1 and EE

Concentration (ng/µl)	Peak area (AU)
32	1337.29
40	3464.59
48	6056.85
56	9341.41
64	12229.96
EE 1 (2 µg/µl)	4947.78
EE 2 (2 µg/µl)	4988.00
EE 3 (2 µg/µl)	3964.21
EE 4 (2 µg/µl)	3695.97

Graph 4: Calibration curve for PD1



2.6.1.3 Estimation of quercetin-3-glucoside in ethanol extract:

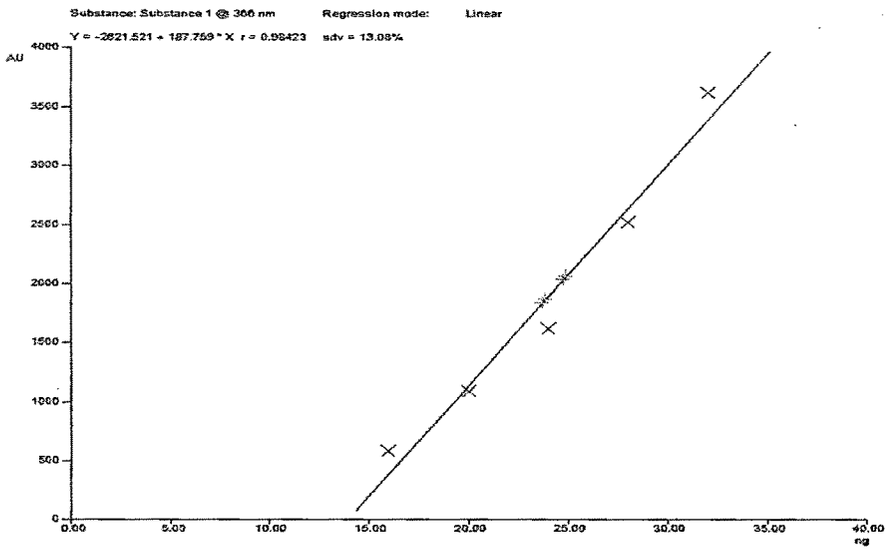
The estimation of quercetin-3-glucoside in the ethanol extract (EE) was carried out by linear calibration curve constructed for the compound.

Stock solution of quercetin-3-glucoside was prepared by dissolving 1 mg in 5 ml of methanol. Further 0.1 ml of the stock solution was diluted to 5 ml with methanol to give concentration of 4 ng/µl. The stock solution of total ethanol extract was prepared by dissolving 20 mg of extract in 10 ml of ethanol. 2, 4, 6, 8 and 10 µl of standard quercetin-3-glucoside (16, 20, 24, 28 and 32 ng respectively) and 14 µl of EE (2 µg/µl) in ethanol were spotted on silica gel 60 F254 thin layer chromatographic plate. Then the plate was developed using ethyl acetate: formic acid: acetic acid: water (100:11:11:27) as mobile phase. Then the post chromatographic derivatization was carried out with NP-PEG reagent. The chromatogram was then scanned at 366 nm. The concentration of quercetin-3-glucoside in EE was calculated form calibration curve plotted by taking concentration on X-axis and area under peak on Y-axis. The calibration curve of quercetin-3-glucoside was as shown in Graph 5. The Table 11 indicates the concentration and peak area of standard and sample.

Table 11: Concentration and peak area data for quercetin-3-glucoside and EE

Concentration (ng/µl)	Peak area (AU)
16	581.87
20	1085.29
24	1618.20
28	2516.79
32	3621.30
EE 1 (2 µg/µl)	1860.87
EE 2 (2 µg/µl)	1835.90
EE 3 (2 µg/µl)	2033.41
EE 4 (2 µg/µl)	2059.20

Graph 5: Calibration curve for quercetin-3-glucoside



2.6.2 Roots of *B. montanum*

The bioactive ME, MFME, EMKMFME and MFMFME exhibiting flavonoids were subjected for high performance thin layer chromatographic studies. The isolated compound BM1 was also run simultaneously to detect its presence in these extracts. An attempt was also made to estimate the amount of BM1 in the methanol extract. An attempt was also made to quantify the BM1 in the methanolic extract (ME).

2.6.2.1 Fingerprint profile of ME, MFME, EMKMFME and MFMFME

2 µg/µl solutions of each ME, MFME, MFMFME and EMKMFME; 200 ng/µl solution of BM1 were spotted on silica gel 60 F_{254} thin layer chromatographic plate and developed using mobile phase, ethyl acetate: formic acid: acetic acid: water (100:11:11:27). NP-PEG reagent was used as detecting reagent and the resulting chromatogram was scanned at 366 nm. The color, R_f value and spectral comparison was taken into consideration to detect the presence of BM1 in the extracts. The complete analysis was performed with Camag HPTLC instrument.

2.6.2.2 Estimation of BM1 in methanol extract

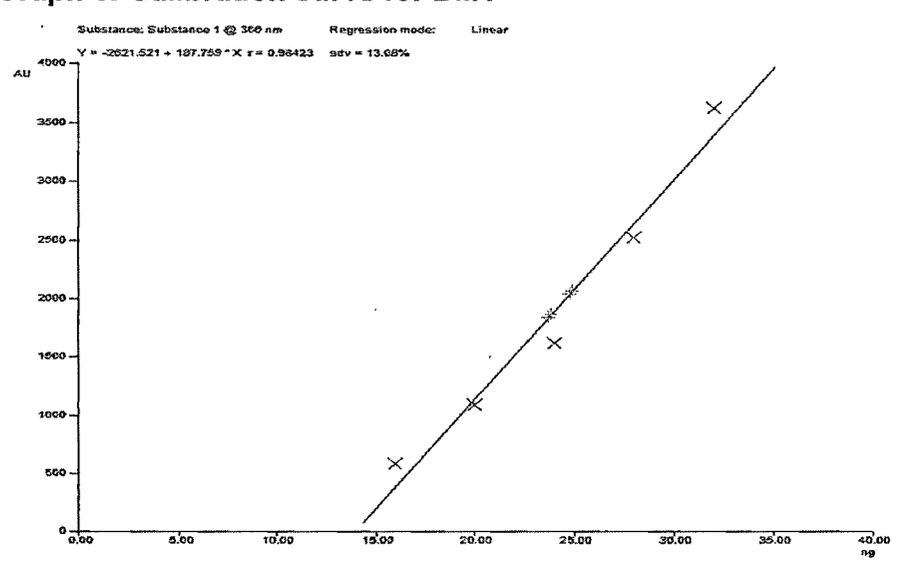
The estimation of BM1 in the methanol extract (ME) was carried out by linear calibration curve constructed for the compound.

Table 12: Concentration and peak area data for BM1 and ME

Concentration (ng)	Peak area (AU)
400	738.20
600	1589.20
1000	4714.03
1400	8637.36
ME 1 (2 µg/µl)	1274.12
ME 2 (2 µg/µl)	1337.11
ME 3 (2 µg/µl)	1289.59

Stock solution of BM1 was prepared by dissolving 4 mg in 10 ml of methanol. Further 5 ml of the stock solution was diluted to 10 ml with methanol to give concentration of 200 ng/µl. The stock solution of methanol extract was prepared by dissolving 20 mg of extract in 10 ml of methanol. 1, 2, 3, 5 and 7 µl of BM1 and 20 µl of ME (2 µg/µl) in methanol were spotted on silica gel 60 F254 thin layer chromatographic plate. The plate was developed using ethyl acetate: formic acid: acetic acid: water (100:11:11:27) as mobile phase. Then the resulting chromatogram was subjected for derivatization with NP-PEG. The chromatogram was scanned at 366 nm.

Graph 6: Calibration curve for BM1



The concentration of BM1 in ME (n=3) was calculated form calibration curve plotted by taking concentration on X-axis and area under peak on Y-axis. The calibration curve of BM1 was as shown in Graph 6. The Table 12 indicates the concentration and peak area of standard and sample.

2.7 References

Belinsky SA, Popp JA, Kauffman FC and Thurman RG: Trypan blue uptake as a new method to investigate hepatotoxicity in periportal and pericentral regions of the liver lobule: studies with allyl alcohol in the perfused liver. **Journal of Pharmacological experiments and Therapeutics** 1984, 20, 755-760.

Bergmeyer HU, Horder MR and Rej R: IFCC methods for the measurement of catalytic concentration of enzymes. **Journal of Clinical Chemistry and Biochemistry** 1985, 23, 899-901.

Brain KR and Turner TD: The Practical Evaluation of Phyto pharmaceuticals, Wright-Scientechnica, Bristol, 1975, pp. 4-35.

Chang CC, Yang M, Wen H and Chern J: Estimation of flavonoid content in propolis by two complementary colorimetric methods. **Journal of Food and Drug Analysis** 2002, 10 (3), 178-182.

Chhaya G and Mishra SH: Antihepatotoxic activity of *P*-methoxy benzoic acid from *Capparis spinosa*. **Journal of Ethnopharmacology** 1999, 66, 187-192.

Dunnet CW: New tables for multiple comparisons with a control. **Biometrics** 1964, 20, 482-491.

Galighor AE and Kozloff EN: Essentials of practical micro technique. Second ed. Lea and Febiger, New York, 1976, p. 210.

Gennaro AR: In. Remington: The Science and Practice of Pharmacy. Nineteenth ed. Vol I, Mack publishing company, Easton PA, 1995, p.111.

Handa SS and Singh S: Hepatoprotective activity of *Apium graveolens* and *Hygrophila auriculata* against paracetamol and thioacetamide intoxication in rats. **Journal of Ethnopharmacology** 1995, 49, 119-126.

Kirtikar KR and Basu BD: Indian Medicinal Plants. Second ed. Vol II, International book distributors, Dehradun, 1983, pp. 1616-1617.

Kiso Y, Tohkin M and Hikino H: Assay method for anti-hepatotoxic activity using galactosamine induced cytotoxicity in primary cultured hepatocytes. **Journal of Natural Products** 1983, 46, 841-847.

Kokate CK, Purohit AP and Gokhale SB: Text book of Pharmacognosy, 17th edition, Nirali Prakashan, Pune, 2001, pp.99-100.

Kokate CK: Practical Pharmacognosy. Vallab Prakashan, New Delhi, 1999, pp.107-111.

Lala PK: Practical Pharmacognosy, First ed. Lina publishers, Calcutta, 1981, pp.136-139.

Mac comb RB and Bowers GN: Alkaline phosphatase activity in serum. **Clinical Chemistry** 1972, 18, 97.

Mukherjee PK, Atul W, Kumar V, Rai S and Saha BP: Marker profiling of botanicals used for hepatoprotection in Indian system of medicine. **Drug Information Journal** 2006, 40, 131-139.

OECD: OECD guidelines for the Testing of Chemicals test no. 423: Acute oral Toxicity- Acute Toxic class method 1996.

Peters T: Proposals for standardisation of total protein assays. **Clinical Chemistry** 1968, 14, 1147-1159.

Rao KS and Mishra SH: Screening of anti-inflammatory and hepatoprotective activities of alantolactone isolated from the roots of *Inula racemosa*. **Indian Drugs** 1997, 34, 571-575

Rao KS and Mishra SH: Antihepatotoxic activity of monomethyl fumarate isolated from *Fumaria indica*. **Journal of Ethnopharmacology** 1998a, 60, 207-213.

Rao KS and Mishra SH: Hepatoprotective principles from the stem bark of *Moringa pterygosperma*. **Pharmaceutical Biology** 1998b,36 (4), 295-300.

Richmond W: Preparation and properties of a cholesterol oxidase nocardia species and its application to the enzymatic assay of total cholesterol in serum. **Clinical Chemistry** 1973,19, 1350-1356.

Samia H, Renee JG and Geoffrey CK: Flavonoidal glycosides from Egyptian species of the tribe Asclepiadeae. **Biochemical Systematics and Ecology** 2006, 34,575-584.

Sarkar K and Sil PC: A 43K Da protein from the herb *Cajanus indicus* L. protects thioacetamide induced toxicity in hepatocytes. **Toxicology in Vitro** 2006, 20, 634-640.

Singleton VL and Rossi JA: Colorimetry of total phenolics with phospho-molybdic-phosphotungstic acid reagent **American Journal of Ecology and Viticulture** 1965,16, 144-148.

Skett P: Problems in using isolated and cultured hepatocytes for xenobiotic metabolism based toxicity testing solutions. **Toxicology in Vitro** 1994, 3, 491-504.

Tasaduq SA, Singh K, Sethi S, Sharma SH, Bedi K, Singh J and Johri RK: Hepatocurative and antioxidant profile of HP-1, a polyherbal Phytomedicine. **Human & Experimental Toxicology** 2003, 22, 639-645.

Tingstrom A and Obrink B: Distribution and dynamics of cell surface associated cell Cam 105 in cultured rat hepatocytes. **Experiments in Cellular Research** 1989, 186, 132-142.

The Ayurvedic Pharmacopoeia of India, Part I, Vol III, Appendix 2, Ministry of Health and Family Welfare, The Controller of Publications, New Delhi. 2003, p. 227-240.

The Ayurvedic Pharmacopoeia of India, Part I, Vol III, Ministry of Health and Family Welfare, The Controller of Publications, New Delhi. 2001, p. 41

The Wealth of India, A Dictionary of Indian Raw Materials and Industrial Poducts.Vol VII, Publication and Information Directorate, CSIR, New Delhi. 1966, pp. 309-311.

Wagner G and Blatt S: Plant Drug Analysis. Second ed. Springer Verlag, Berlin, 1996, pp. 352-355.

Webster D: Interaction of bromocresol green with isolated serum globulin fractions. **Clinica Chimica Acta** 1974, 53, 109-115.

WHO: Quality control methods for medicinal plant materials, World Health Organisation, Geneva 1998.

Zimmermann HJ and Mao R: Cytotoxicity of carbon tetrachloride as measured by loss of cellular enzymes to surrounding medium. **American Journal of medical Sciences** 1965, 110, 688-692.