# Experimental

# Chapter II - Experimental

The section deals with in detailed the methodologies adopted along with the modifications made whenever necessary, to perform various experiments.

## 2.1 Collection and Identification of the plants:

The whole plants of *Leptadenia reticulata* Wight and Arn, , *Dregea volubilis* Benth. Ex Hook. and *Pentatropis microphylla* Wight. and Arn. were collected from near by tribal areas and were compared with the authentic samples preserved in the Botany Department, The M.S. University of Baroda, Vadodara. The voucher specimens were preserved in Herbal Drugs Technology Laboratory, Pharmacy Department, The M. S. University of Baroda, Vadodara bearing specimen no. HDT/MR/20041801 for *L..reticulata*, HDT/MR/20042503 for *D.volubilis* and HDT/MR/20041101 for *P .microphylla*. The collected plant materials were first dried in sun and then under the shade. The aerial parts were then powdered using pulverizer and used for further studies.

## 2.2 Pharmacognostic Studies:

### 2.2.1 Morphological Evaluation:

The plants selected were subjected to the morphological evaluation. Various parts of the plants were observed carefully and the preliminary observations were noted.[64]

### 2.2.2 Microscopic studies:

The powdered aerial parts of the selected plants were subjected to the microscopic examination to detect different identifying characteristics. The slides were stained with various staining reagents to ascertain the presence of particular type of microstructures.[65]

## 2.3 Phytochemical studies

### 2.3.1 Preliminary Phytochemical screening of the plant extracts:

50 g. of the air dried powdered drug was extracted in a soxhlet apparatus with the solvents of increasing polarity as follows[66].

1)Petroleum ether ($60^{o}$-$80^{o}$), 2) Benzene, 2)Chloroform, 3)Ethyl acetate , 4)Methanol, 5) Water.

The material was dried in hot air oven below $50^{o}$ C after each extraction. The marc was finally macerated with chloroform water for 24 hours to obtain the aqueous extract. The extracts were concentrated by vacuum distillation and the concentrated extracts were allowed to dry at room temperature. The consistency, color and physical appearance of the extracts was noted. The extractive values for each extract was determined in terms of %w/w extract obtained on the air-dried weight basis of the plant material.

The extracts, thus, obtained from the successive solvent extraction were subjected to various qualitative chemical tests to ascertain the presence of various phytoconstituents such as Sterols, Triterpenoids, Alkaloids, Glycosides, Flavonoids, Phenolics, Carbohydrates and Amino acids etc.[67]

### 2.3.2 Development of Comparative Phytochemical profiles of the plant extracts using HPTLC:

The Pet.ether and Chloroform extract of all the three selected plants were subjected to the process of saponification by refluxing them indicvidually with 10% methanolic potassium hydroxide solution for 30 minutes. The unsaponifiable matter was collected by partitioning the mixture with water and diethyl ether. The diethyl ether fraction was evaporated to dryness at room temperature. The unsaponifiable matter, thus, collected was preserved in desiccator.

Methanolic and aqueous extracts of all the selected plants, were refluxed with 2N Hydrochloric acid and the mixture was partitioned with ethyl acetate. Ethyl acetate fraction, thus, obtained was dried and preserved in desiccator.

The extracts obtained from successive solvent extraction in the previous experiments, were subjected to the comparative thin layer chromatographic studies using High Performance Thin Layer Chromatography (HPTLC) to assess the similarity of the phytoconstituents present in the extracts.

The extracts were spotted on the pre coated TLC plates and the plates were developed using the optimized solvent system. The method of post chromatographic derivatization was adopted to detect the presence of various phytoconstituents on the TLC plates. The results are displayed in the form of chromatograms as comparative account of the phytoconstituents present in the extracts studied[68].

## 2.4 Development of standardization parameters for different plant drugs

### 2.4.1Determination of various physico chemical constant as per WHO guidelines[69]:

As two of the selected plants D. *volubilis* and *P.microphylla* are being used as the substitutes of *L. reticulata,* comparative studies were planned to evolve few of the preliminary standardization parameters as per WHO guidelines.

The dried aerial parts of the plants were subjected to the determinations as proposed in guideline. The methodology adopted to perform the experiments is as under:

#### 2.4.1.1 Determination of foreign matter:

250 g of plant material was weighed and spread in form of a thin layer. The foreign matter was sorted out by visual inspection and with the help of a magnifying lens (6x or 10x). The amount of this sorted foreign matter was weighed and the value in the bulk was determined on the percentage dried weight basis of plant material.

**2.4.1.2 Determination of Ash value:**

Accurately about 4g of the air-dried powdered material was taken in a previously ignited and tarred silica crucible. The material was spreaded in the form of an even layer and ignited by gradually increasing the heat to 500-600°C until the content was turned to white in color, indicating the absence of carbon. It was then allowed to cool in a desiccator and weighed. Content of total ash is reported in the form of percentage w/w ash obtained from the air-dried material.

**2.4.1.3 Determination of Acid insoluble Ash:**

About 25ml of 2N hydrochloric acid was added to crucible containing accurately about 2g of ash. The crucible was covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5 ml of hot distilled water and this liquid was added to the crucible. The suspension was then filtered through ash less filter paper. The insoluble matter on filter-paper was washed with hot water until the filtrate was neutral. The material retained on the filter paper was then allowed to dry on a hotplate and ignited to constant weight. The residues were allowed to cool in a desiccator for 30 minutes, and then weighed.

**2.4.1.4 Determination of Water soluble ash:**

About 25 ml of distilled water was added to the crucible containing the accurately weighted quantity of total ash and boiled for 5 minutes. The insoluble matter was collected on an ashless filter-paper, washed and ignited in a crucible for 15 minutes at a temperature 430°C. The weight of the amount obtained after ignition was subtracted from the weight of the total ash taken that is the water soluble portion of the ash.

**2.4.1.5 Determination of Alcohol soluble extractives:**

About 5.0 g of coarsely powdered air-dried material was macerated with 100 ml of the methanol for 6 hours, shaking frequently, and then allowed to stand for 18 hours.

Exactly about 25 ml of the filtrate was transferred to a tared flat-bottomed dish and evaporated to dryness on a water-bath. The extract obtained was dried at 105°C for 6 hours and weighed without delay. The amount of methanol soluble content, thus, obtained from the plant material was reported in the form of percentage w/w of the methanol soluble extractives on the dried weight basis of the plant materials.

**2.4.1.6 Determination of Water soluble extractives:**

About 5 g of the air-dried drug was macerated with 100 ml of chloroform water in a closed flask for 24 hours with frequent shaking. Exactly about 25 ml of the filtrate was evaporated to dry ness in a tarred flat-bottom dish. The weight of the collected extract was noted after drying. The percentage of the water-soluble extractive was calculated with reference to the air-dried drug.

**2.4.1.7 Determination of water and volatile matter:**

Loss on drying method was used for the determination of the water and volatile matter in the sample of air dried powdered plants.

Accurately about 5 gm of the sample was taken in a tarred evaporating dish and kept it in an oven at 105 $^{o}$C for 5 hrs, cooled and weighed repeatedly at intervals of an hour until the difference between two successive weighing did not alter more than 5 mg.

**2.4.1.8 Determination of foaming index:**

Many medicinal plants consist of phytoconstituents that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of foaming index.

Accurately about 1 g of the powdered plant material was boiled moderately for 30 minutes with 50 ml of water. The extract obtained was cooled and filtered. The aliquots of the solution were taken and diluted with water to produce serial dilutions. The solution of the tests material was shaken in a lengthwise motion for 15 seconds.

The solutions were allowed to stand for 15 minutes and the height of the foam was measured. Foaming index was measured from the formula mentioned below.

**Foaming index: 1000 / a**

Where a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

**2.4.1.9 Determination of hemolytic activity:**

The haemolytic activity of plant materials was determined by comparing the hemolytic potential of the aqueous extract prepared with that of a reference material, Saponin R, which has a haemolytic activity of 1000 units per g. The suspension of erythrocytes was mixed with equal volumes of a serial dilution of the aqueous extract of the plants. The lowest concentration exhibited complete haemolysis was determined. A similar test was performed simultaneously using Saponin R too. The tubes were examined and the minimum dilution exhibited total haemolysis was recorded.

**2.4.1.10. Determination of microbial count:**

The experiments were performed for determination of *total bacterial* and *total fungal* count as well as for determination of specific count of Escherichia *coli* too.

**2.4.2. Quantitative determination of various Inorganic ions in plant materials [70]:**

The plant materials were subjected to detailed qualitative as well as quantitative assessment of heavy metal and other inorganic ions detected, using Atomic Absorption Spectroscopy. The experimental method is mentioned as below:

About 5 g of dried and powdered aerial parts of the plant was ignited in muffle furnace to obtain total ash. Exactly about 250 mg of ash was dissolved in 25 ml of 5 % v/v Hydrochloric acid to produce the stock solution. Various aliquots of the solution were taken and different dilutions were prepared. The concentration range

selected for preparation of calibration curve along with the wavelength mentioned in the professional literature for estimation of different ions is mentioned in Table 2.1.

**Table: 2.1 Details of the preparation of calibration curve of selected elements using Atomic Absorption Spectroscopy:**

| Element | Standard used | Calibration range (µg/ml) | Wavelength used (nm) | Sensitivity (µg/ml) |
|---|---|---|---|---|
| Sodium | NaCl | 0.4 – 1.5 | 589.6 | 0.008 |
| Potassium | KCl | 0.4 – 1.5 | 766.5 | 0.008 |
| Magnesium | Mg Powder | 0.1 – 0.4 | 285.2 | 0.02 |
| Manganese | $MnSO_4$ | 1.0 – 1.4 | 279.8 | 0.02 |
| Cobalt | $CoCl_2$ | 2.5 – 9.0 | 240.7 | 0.05 |
| Copper | Cu metal | 1.0 – 5.0 | 324.7 | 0.025 |
| Zinc | $ZnSO_4$ | 0.4 – 1.5 | 213.9 | 0.008 |
| Lead | $Pb(NO_3)_2$ | 2.5 – 20.0 | 217 | 0.06 |
| Mercury | $HgSO_4$ | 73 - 290 | 253.7 | 1.6 |

## 2.4.3. Development of HPTLC finger prints of plant extracts:

The preliminary phytochemical evaluation revealed the presence of various phytoconstituents, believed to be active physiologically, in methanolic extract of all the selected plants.

The chromatogram of such extracts was developed to identify and differentiate *L.reticulata* from other two plants.

The methodology developed to establish HPTLC fingerprints of the methanolic extracts is as under:

The dried powder (50 g) of the aerial parts of the selected plants was refluxed with methanol (250 X 2 ml, 30 min). The extract was concentrated under vacuum and kept at room temperature for drying.

The extracts were dissolved in methanol and about 100 µg extract was spotted on Pre coated TLC plate using Linomet V (Applicator).

The TLC plates contained spots of markers and the spots of the Methanolic extracts of the selected plants were developed using optimized mobile phase.

The samples were spotted in the form of 8.0 × 0.45 mm bands using Camag micro liter syringe and Camag Linomet V. The application rate was set at 150 nl/ sec. The monochromator band width was set 20 nm and each track was scanned thrice. The plate was developed with 20 ml mobile phase comprised of Toluene: Ethyl Acetate: Formic Acid (9:4:0.05, v/v). The mobile phase was allowed to reach up to the distance of 80 mm. in a 200x 100 mm twin tough glass chamber using linear ascending technique. The plates were kept at room temperature (25 ± 2.0$^0$ C) for drying. The optimized chamber saturation time was 30 min at room temperature.

The plates were then scanned at 366 nm using the mercury lamp in fluorescent mode. The chromatograms, thus, developed were utilized to distinguish the extracts from each other. The plates were then derivatized using 10% w/w methanolic sulphuric acid reagent [71]and scanned at 366 nm after heating at 110$^0$ C for 10 minutes, using mercury lamp.

**2.4.4. Development of methods for detection and estimation of some markers in selected plant materials.**

**2.4.4.1 Detection and estimation of Salicylic acid and Stigmasterol in the plant extracts using HPTLC**

**2.4.4.1.1 Detection of Salicylic acid:**

Salicylic acid was isolated from the ethyl acetate extract of *P.microphylla*[63].As the plant *P.microphylla* is traditionally recommended as a substitute of *L.reticulata (Jivanti)* in treatment of cut and burns topically, the experiments were designed to detect Salicylic acid in *L.reticulata* and *D.volubilis.*

The dried powder of aerial parts of *L.reticulata* and *D.volubilis were* refluxed with Methanol for 1 hr at 65$^0$ C separately. The extract obtained was then concentrated under vacuum using rotary vacuum evaporator at 45$^0$ C. The extract, thus, obtained

was subjected to co-TLC along with Salicylic acid as standard. The experiments were performed using HPTLC and the $R_f$ values of corresponding spots were noted.

UV-spectra of the corresponding spots were taken and compared at three positions 1. Peak start, 2. Peak maxima 3. Peak end for their superimposability using the software. The comparative spectra of the spots are shown in Fig3. 14.

Chromatographic conditions for the set of experiments were as under:

Stationary Phase: Silica gel G 60 F 254.

Mobile Phase:Toluene : Ethyl acetate : Formic acid (1:2.5:0.05, v/v/v)

Saturation time: 30 minutes

Spotting amount: 100 µg/ spot
Of extract

Spotting amount: 400 ng/spot
Of standard

Scanning wavelength: 300 nm, fluorescent analysis using Mercury lamp, K400filter.

**2.4.4.1.2 Detection of Stigmasterol:**

The extracts thus obtained in previous case were subjected to co-TLC experiments along with Stigma sterol. The protocol of the experiment was as described below:

Stationary Phase: Silica gel G 60 F 254.

Mobile Phase: Toluene: Ethyl acetate: Formic acid (9.0:4.0:0.05, v/v/v)

Saturation time: 30 minutes

Spotting amount: 400 µg/ spot

of extract

Spotting amount: 20 ng/spot

Of standard

Postchromatographic derivatization:

Plates were dip in the solution of 10w/w methanolic sulphuric acid. The plates are heated for 10 min at $100^0$ C and then scanned at 366 nm using mercury lamp with K400 filter. The UV spectra of corresponding spots are recorded as in Fig 3.14

## 2.4.4.2. Estimation of Salicylic acid and Stigmasterol in the Plant extracts:

### 2.4.4.2.1. Preparation of standard solutions:

The stock solutions were prepared by dissolving both the compounds in ethyl acetate separately (20 mg in 10 ml methanol). The aliquot of both the solutions were diluted to produce combined standard solution. (20 µg/ml and 200 µg/ml for stigmasterol and salicylic acid respectively).

### 2.4.4.2.2 Preparation of sample solution:

Air dried powdered material was refluxed with methanol (25 g, 3 × 200 ml, each for 30 minutes). The collected extracts were mixed together and evaporated to dryness using vacuum evaporator at $50^0$ C. The extract was dissolved in ethyl acetate to produce the sample solution. The solution was directly applied on the TLC plates.

### 2.4.4.2.3 Chromatographic Conditions:

Stationary phase was precoated TLC plates. (TLC plates Silica gel G, 60 F254, Aluminium sheet support, 200 × 100 $(mm)^2$). The band size was 8 × 0.45 mm and 12 bands were spotted on each plate. The mobile phase was Toluene: Ethyl acetate: Formic acid (9.0:4.0:0.05, v/v/v). Chamber saturation time employed was 30 min and the developing distance was 70 mm. The scanning wavelength was 300 nm in fluorescence mode before postchrmoatographic derivatization to estimate amount of salicylic acid. The plates were derivatized by dipping in Methanolic Sulphuric acid (10 % w/w) and heated ($100^0$ C, 10 min). The scanning was performed at 366 nm in fluorescence mode to estimate the amount of stigmasterol. The slit dimensions were 6.0× 0.45 mm and scanning speed was 10 mm/s using K400 optical filter.

**2.4.4.2.4 Calibration Curve of Salicylic acid and Stigmasterol:**

Various amount of standard solution was applied on the TLC plates which correspond to 20-120 ng/spot for stigmasterol and 200-1200 ng /spot for salicylic acid. Each concentration was spotted three times on TLC plate.

**2.4.4.2.5 Estimation of stigmasterol and salicylic acid in test extracts:**

The samples were prepared by dissolving the Methanolic extract in ethyl acetate and applied in the form of sharp bands to estimate the amount of the phytoconstituents. (1μg/spot and 100 μg/spot for stigmasterol and salicylic acid respectively). The plates were developed and scanned as mentioned under the title chromatographic conditions. The results are expressed as average of three experiments along with standard deviation in Table 3.17.

**2.4.4.2.6 Method Validation[72]:**

The method was validated in terms of accuracy, inter-day and intra-day precision, specificity, limit of detection (LOD), limit of quantification (LOQ) as described in ICH guidelines [ICH, Q2(B)].

**2.4.4.2.6.1 Accuracy:**

Accuracy of the analysis was evaluated by performing recovery studies. The sample solution consisted of methanolic extract of aerial parts of *L.reticulata* was spiked with three different amounts of the standards separately for salicylic acid and stigmasterol. The average recoveries after the analysis were calculated. The results were recorded in along with Relative standard deviations (RSD) (Table; 3.16).

**2.4.4.2.6.2 Precision:**

Intraday precision was determined by analyzing standard solutions in the concentration range of 40, 80, 100 ng/spot and 400, 800, 1000 ng/spot for stigmasterol and salicylic acid respectively for 6 times in a day. The inter day

precision was measure in the similar manner at the interval of 7 days. The response was evaluated by measuring the area of the respective peaks. The results of inter day and intraday precision are tabulated as in Table 3.15.

**2.4.4.2.6.3 Limit of Detection and Limit of quantification:**

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank of the standard and various concentration of the standard solution were spotted on the plate. The plate was developed and detected by following the method as explained in chromatographic conditions. The signal to noise ratio was determined, for LOD it was 3:1 and for LOQ determinations it was 10:1.

**2.4.4.2.6.4 Specificity:**

Specificity of the method was ascertained by analyzing standard drug and sample. The purity of the spot for salicylic acid and stigmasterol were ascertained by comparing the $R_f$ and spectra of the spot with standard. The peak purity of both of the components were assessed by comparing the spectra at three different levels- peak start, peak apex and peak end positions of the spot.

**2.4.4.2.6.5 Robustness of method:**

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phase having different composition like Toluene:Ethyl acetate: Formic acid A) (9.0:3.0:0.05, v/v),B) (9:5.0:0.05, v/v),C) (9.0:2.0:0.1,v/v) , D) (9, 4.0, 0.1,v/v), E) (9, 4.0, 0.2,v/v) were utilized and chromatograms were developed. The amount of mobile phase was altered in the range of 20 ml ± 5.0%. Effect of various level of saturation in the tank on resolution was studied by altering the saturation time. Three various intervals 15, 20, 30, 40 min of were selected for this purpose. The plates were pre washed with methanol, dried at 60 $^0$C prior to chromatography. Time for spotting the plate and developing were altered at three time

intervals- 5, 10, 15 minutes. Time between heating and scanning were varied from 5, 10, 15 minutes and effects on the spot intensity were studied by scanning the plates at the end of mentioned periods.

**2.4.4.2.6 .6 Statistical Analysis:**

The results are expressed as Average ± SD. %RSD and SEM were also indicated where necessary. The datasets of the results were analyzed using ANOVA to assess the statistical significance of the result. $p<0.05$ was selected as the level of significance.

## 2.4.5 Estimation of β Carotene from Plant materials:

*L.reticulata* is traditionally recommended for diseases related to vision[25]. As there was no other markers reported for *L.reticulata* and other selected plants for the purpose, a method were developed to estimate β carotene from the plants using HPTLC.

**2.4.5.1. Detection of β-carotene:**

10 g of dried powdered plant material was refluxed with 3 × 150 ml acetone containing 5% w/w pyrogallol for 30 min in dark. The extract was filtered and concentrated in vacuum evaporator at $40^0$ C. The concentrated extract was then evaporated to dryness at room temperature by keeping it in desiccator while protected from the direct exposure to light.

The dried extract was dissolved in methanol and centrifuged. The supernatant collected was utilized to perform the analysis.

Stationary Phase: Silica gel G 60 F 254.

Mobile Phase: Hexane: Benzene (9.0:2.5, v/v)

Saturation time: 10 minutes

Spotting amount : 200 µg/ spot

of extract

Spotting amount : 40 µg/spot

Of standard

Scanning wavelength: 444 nm using tungsten lamp.

The UV spectra of corresponding spots recorded were as in Fig3. 19

### 2.4.5.2 HPTLC instrumentation:

The samples were spotted in the form of bands with 8.0 × 0.45 mm size on Precoated Silica gel plates. Camag micro liter syringe and Camag Linomet V (applicator) were utilized for this purpose. The plates were washed with methanol and activated at $60^0$ C for 10 min prior to application. The monochromator band width was set 20 nm, each track was scanned thrice and base line correction was used.

The plate was developed with 20 ml mobile phase comprised of Hexane: Benzene (10: 2.5, v/v), the mobile system was allowed to reach up to the distance of 50 mm. in a 200x 100 mm twin tough glass chamber using linear ascending technique. The plates were allowed to dry at room temperature (25 ± $20^0$ C) at relative humidity of 60% ±5. The optimized chamber saturation time was 5 min at room temperature (25 ± $20^0$ C).

The dried plates were scanned and quantified in reflectance- absorbance mode at 444 nm using the CAMAG TLC SCANNER-3 where tungsten lamp emits continuous radiation. the slit dimension was kept at 6.0x 0.45 mm with scanning speed of 2mm/sec. Data of peak areas of each band were recorded.

### 2.4.5.3 Calibration curve of β-carotene:

Stock solution of standard β-carotene (2000µg/ml) was prepared in methanol. The final standard solution consisted of 0.2 µg/ml β-carotene. Different volumes of this solution were spotted on the plates which covered the range of 400-1400 ng /spot.

### 2.4.5.4 Preparation of sample solution:

5 g of dried powdered plant material was refluxed with 100 ml acetone containing 5% w/w pyrogallol for 30 min in dark. The extract was filtered and concentrated in rotary vacuum evaporator at $40^0$ C. The concentrated extract was then evaporated to dryness at room temperature by keeping it in desiccator while protected from the direct exposure to light.

The dried extract was dissolved in methanol and centrifuged. The supernatant was collected to perform the analysis.

### 2.4.5.5 Method Validation[72]:

#### 2.4.5.5.1 Precision:

Intra day precision was determined for standard β carotene (400-1200 ng/spot) 3 times on the same day. The inter-day precision was calculated by spotting standard β carotene (400-1200 ng/spot) for 3 times over a period of 15 days. The values of the slopes of the calibration curve were subjected to the statistical analysis (ANOVA).

#### 2.4.5.5.2 Limit of detection and Limit of quantification:

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank of the standard was spotted six times following the same method as explained under the title 'HPTLC instrumentation'. The signal to noise ratio was determined, for LOD it was 3:1 and for LOQ determinations it was 10:1.

#### 2.4.5.5.3 Specificity:

The spot for β carotene was ascertained by comparing the $R_f$ and spectra of the spot with standard. The peak purity

was assessed by comparing the spectra at three different levels- peak start, peak apex and peak end positions of the spot.

**2.4.5.5.4 Recovery studies:**

The recovery studies were performed by applying the samples spiked with the known amount of the β carotene and the percentage recovery was calculated against the theoretical values. Pre-analyzed samples were spiked at three different concentration levels of β carotene and analyzed. The instrument set up was as same in case of the estimation of the sample. This was done to check the recovery of the drug at different levels in the extract. The experiments were performed in triplicate. The results of the experiments are summarized in Table 3.23.

**2.4.5.5.5 Robustness of the method:**

Small changes were introduced in the mobile phase composition and the effects of such alterations were examined. The studies were performed by altering the composition of benzene by 10% each time, up to 30% of the original value. The alterations in the time period allotted for saturation of tank were studied by altering the saturation time, ranging from 0-30 min. The time between spotting and dipping the plate in mobile phase were varied from 0, 10, 15 min to observe the stability of the compounds of the interest on TLC plates. Similar time durations were selected to observe the effect of delaying in scanning of the TLC plates after development of the chromatogram.

**2.4.5.5.6 Statistical Analysis:**

The results are expresses as Average ± SD. The datasets of the results were analyzed using ANOVA to assess the statistical significance of the results. $p<0.05$ was selected as the level of significance.

## 2.4.6 Determination of Total Phenolics and Flavonoidal content of plant extracts:

The plant extracts possess Flavonoids and Phenolic constituents. The quantitative estimation of such compounds serves a good parameter to develop the analytical profile of the plant materials.

### 2.4.6.1 Estimation of Total Phenolic components[73]:

The method adopted for the estimation of phenolic content of methanolic extract is as under:

**2.4.6.1.1Preparation of test sample:**

The aerial parts and stem of the selected plants were refluxed successively with methanol and water. The extracts were allowed to dry and the dried extracts were stored in desiccator. Stock solution of sample extract was prepared by dissolving 10 mg of methanolic and aqueous extract of the aerial parts and of stem in 10 ml of methanol individually.

**2.4.6.1.2 Preparation of standard:**

Gallic Acid (AR) was used as standard to plot the calibration curve. 10 mg of Gallic acid was dissolved in 100 ml of methanol to produce the stock solution. Various aliquots of this stock solution were taken to produce serial dilutions in the range of 50-200 µg/ml.

**2.4.6.1.3 Preparation of reagent:**

Folin ciocalteu reagent: The reagent was procured from Ms. Qualigen India LTD. 1: 2 dilution of the reagent with distilled water was prepared and utilized.

20 % sodium carbonate solution:

20 gm of anhydrous sodium carbonate was dissolved in 100 ml of distilled water.

Protocol for estimation of Total Phenolic Content:

1. 1 ml of the sample/standard solution was taken in 25 ml volumetric flask. 10 ml of water and 1.5 ml of Folin Ciocalteu reagent was added to each sample solution.
2. The above mixture was allowed to stand at room temperature for 5 min and then 4 ml of 20 % sodium carbonate solution was added. The solution was diluted up to 25 ml with distilled water.
3. The absorbance of the blue color was measured after 30 min at 765 nm.
4. Percentage of total phenolics was calculated from calibration curve of gallic acid as per the procedure described above. The amount of total phenolics was expressed as %w/w of total phenolics on dried wt basis of the extract.

## 2.4.6.2 Determination of Total flavonoidal content[74]

The total flavonoidal content of methanolic extract was determined by two complimentary methods and the sum of the results obtained provides the amount of total flavonoids presents in extracts assessed.

**2.4.6.2.1Preparation of Sample solution:**

The aerial parts and stem of the selected plants were refluxed successively with methanol and water. The extracts were allowed to dry and the dried extracts were stored in desiccator. Stock solution of sample extract was prepared by dissolving 10 mg of methanolic and aqueous extract of the aerial parts and of stem in 10 ml of methanol individually.

## 2.4.6.2.2 Aluminum chloride colorimetric method:

**2.4.6.2.2.1 Preparation of standard solution:**

A stock solution of Quercetin (1 mg/ml) was prepared in ethanol.

**2.4.6.2.2.2Reagents:**

10 % w/w Aluminium chloride:

10 g of Aluminium chloride was dissolved in 100 ml of distilled water.

1 M Potassium acetate:

9.814 gm of potassium acetate was dissolved in 100 ml distilled water.

**2.4.6.2.2.3 Protocol for estimation of total flavonoidal content:**

1. Quercetin was used to plot the calibration curve.
2. Various aliquots of the standard stock solution e.g. 0.1, 0.2, 0.3, 0.4 and 0.5 ml, were taken and diluted up to 10 ml, which represented 10, 20, 30, 40 and 50 µg/ml concentration respectively.
3. The standard solutions were separately mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10 % aluminium chloride, 0.1 ml of 1 M potassium acetate and diluted up to 20 ml with water.
4. After incubation at room temperature for 30 min. the absorbance of reaction mixture was measured at 415 nm using spectrophotometer against the reagent blank.
5. Similarly 1 ml of the sample solution was allowed to react with Aluminum chloride for determination of flavonoidal content as described in above procedure.

### 2.4.6.2.3 2, 4- Dinitrophenylhydrazine (2,4-DNPH) colorimetric method:

**2.4.6.2.3. 1. Reagents:**

1 % 2, 4-dinitrophenylhydrazine reagent: 1 gm 2, 4- DNPH was dissolved into the 100 ml of distilled water.

1 % potassium hydroxide:

1 gm of potassium hydroxide was dissolved in 100 ml of 70 % methanol.

**2.4.6.2.3.2. Preparation of standard solution for control:**

A stock solution, 1mg/ ml of Naringin was prepared in distilled water.

**2.4.6.2.3.3. Protocol for estimation of total flavonoidal content:**

1. Naringin was used to plot the calibration curve. Required quantities of stock solution were diluted to get 250, 500, 1000, 1500, 2000µg/ml concentration respectively.
2. 1 ml of the each standard solution was separately mixed with 2 ml of 1 % 2, 4 –DNPH reagent and 2 ml of methanol, then kept at $50^0$ C for 50 min.
3. After cooling to room temperature, the reaction mixtures were mixed with 5 ml of 1 % potassium hydroxide and incubated at room temperature for 2 min.
4. 1 ml of the mixture was diluted up to 5 ml of methanol and Centrifuged at 1000 rpm for 10 min
5. The supernatant was collected and final volume was adjusted to 25 ml. The absorbance of the supernatant was measured at 495 nm against the reagent blank.
6. Similarly, 5 mg /ml sample solution were reacted with 2, 4-dintophenyl hydrazine for determination of flavonoidal content as described in the above procedure.

## 2.5 Stability Studies of Methanolic extract of the plants[75]:

The stability studies of the methanolic extract of the selected plants, obtained in the previous study, were performed as per the EMEA guideline. (Acceptance criterions for Herbal Drugs and Products). The extracts were stored at 40 ± $2^0$ C temperature and 65 ± 5 % relative humidity in stability chamber. The samples were withdrawn on 0,7,14,28,56,92,128,180 days.

The samples collected were stored in refrigerator for further analysis. The collected samples were subjected to morphological and chemical evaluation. The extracts were evaluated for color, consistency and odor, estimation of total phenolic content and estimation of total flavonoidal content.

The experiments were performed in triplicate and average of the three results is tabulated along with standard deviation (Table 3.32).

### 2.5.1 Statistical analysis:

The samples were subjected to Analysis of Variance to ascertain the data sets are statistically different to each other. ($p < 0.05$, confidance interval = 95%).

## 2.6 Evaluation of market samples of *L. reticulata*:

The powders named *Jivanti* were collected from the local market. Total 5 samples were collected from five different places each containing 50 g fine powders. 5 samples of authentic materials were included as positive control and subjected to the analysis as the methods explained below. The samples were coded randomly at beginning to avoid biases. The comparative results were tabulated as in Table 3.33 and 3.34 after decoding.

### 2.6.1 Selection of Parameters to distinguish the powdered plant materials:

The methodology adopted to differentiate the powdered plant material from traditionally utilized substitutes is mentioned as shown in the Chart2.1

#### 2.6.1.1Microscopic Examination of the Powdered Materials:

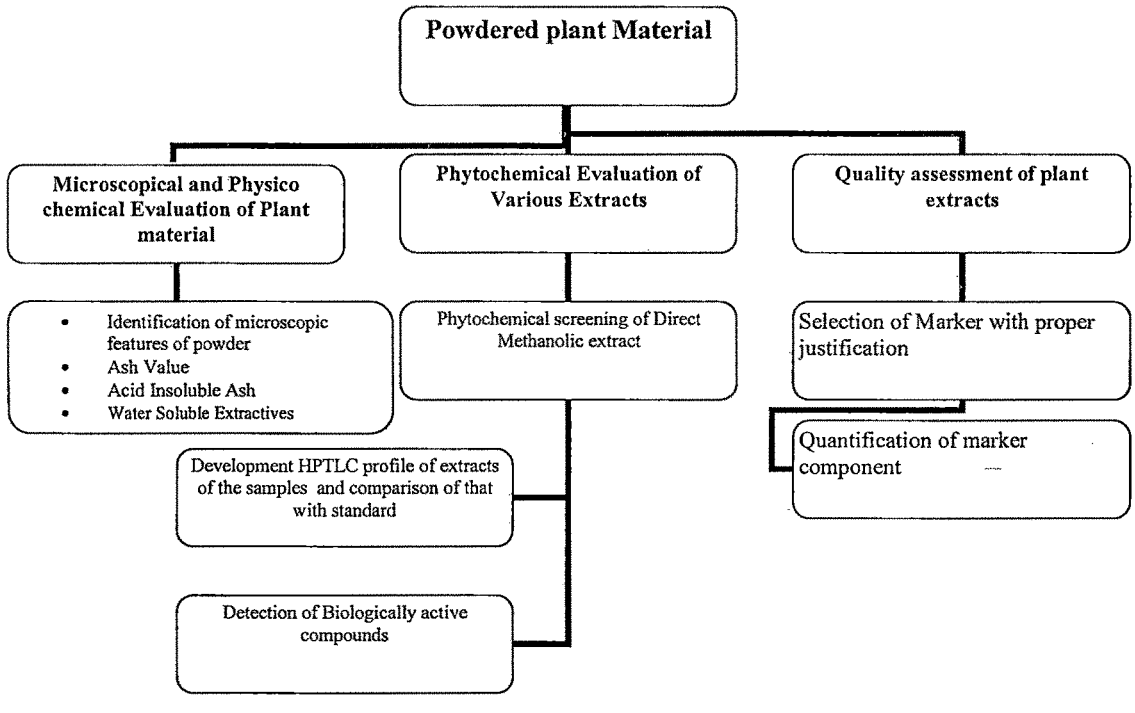
The powdered materials were subjected to the microscopic examinations as described in section 2.2.2

#### 2.6.1.2 Physical Evaluation of powdered plants:

Ash value, Acid insoluble ash value and amount of methanol soluble extractives were the physical parameters selected. The evaluation was performed by adopting the

methodologies proposed in WHO Standards for Evaluation of Herbal Drugs[69]. The results of the studies are tabulated as in Table 3.33

**Chart 2.1: Proposed methodology to differentiate *L.reticulata (Jivanti)* and traditional substitutes:**




### 2.6.1.3 Phytochemical Evaluation:

#### 2.6.1.3.1 Preparation of Extracts:

50 g of the powdered raw material of the drug was refluxed with 250 ml of Methanol (AR) for 4 hours. The Methanolic extract was then filtered and concentrated using rotary vacuum evaporator at temperature 45 $^{0}$ C. The concentrated mass was then dried at room temperature. The extract thus obtained was stored in vacuum desiccator

in dark. The extracts were subjected to phytochemical studies and development of HPTLC fingerprints.

**2.6.1.3.2 Phytochemical Analysis and Detection of Markers:**

Methanolic extracts of the plants were subjected to various phytochemical tests specified for different types of phytoconstituents[67]. Presence or absence of the phytoconstituents was then ascertained by performing Thin Layer Chromatographic studies[68].

**2.6.1.3.4 Development of HPTLC finger print:**

HPTLC fingerprints were developed by following the methodology proposed in section 2.4.3.

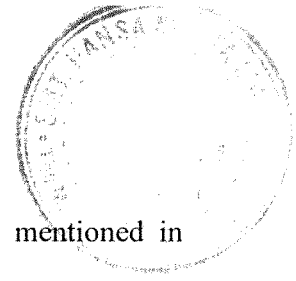
**2.6.1.3.5 Estimation of β-carotene from plant materials:**

Estimation of β-carotene was performed from the authentic plant material and from the sample as per the method proposed in section 2.4.5.

## 2.7 Evaluation of *Ayurvedic* formulation- *Jivantyadi Ghrita* and comparison of market samples.

### 2.7.1 Evaluation of *Jivantyadi Ghrita*:

*Jivantyadi Ghrita* is multi herbal formulation mentioned in Ayurvedic formulary of India, used in the treatment of diseases related to vision and eyes[76]. An attempt was performed to compare laboratory and marketed formulations in order to study the influence of variation of manufacturing parameters on chemical nature of the product. Apart from the regular parameters prescribed to evaluate *Ghrita* in official publications[77], Peroxide value[78, 79] and total Vitamin A content were additional parameters adopted in present investigation[80].



### 2.7.2 Formulation of *Jivantyadi Ghrita*[76]:

*Jivantaydi Ghrita* was prepared in laboratory using official method mentioned in Ayurvedic formulary of India. The procedure adopted for the preparation of the formulation is mentioned below.

The amount of cow ghee was taken accurately about 100 g and the contents of the other ingredients mentioned were added according to the formulae. Phase I: The contents were heated for 70 minutes in a stainless steel container of 14 cm diameter on a gas stove with adequate stirring to prevent the charring of the powdered drugs. The energy provided was 2064 kcal/hr. The previous experiments showed the moisture content was decreased up to20-25% w/w by this time. Phase II: The mixture was, after this phase, heated at the same temperature till the moisture content of the formulation was about 0.5% w/w, with vigorous stirring (This stage required about 15-20 minutes depending on temperature selected). The preservation of the characteristic aroma of the *ghrita* was ascertained and the content was kept at $40^0$ C for 2-3 minutes, for sedimentation of the other insoluble contents. The golden yellow/greenish colored upper part consisting of medicated *ghee* was collected by passing it through muslin cloth. The sample was used as control during the entire study.

### 2.7.3 Procurement of the market samples:

Three market samples of the *Jivantyadi Ghrita* of different make were purchased from retail shops of the city.

### 2.7.4 Evaluation of *Jivantyadi Ghrita*:

Different determinations like Acid value, Saponification value and Iodine value were determined using official methods[77]. Peroxide value[78, 79] and total retinol content[80] was determined as additional parameters to assess the quality of the formulations. The

samples were also evaluated to detect the presence of *Vanaspati Ghee* (Hydrogenated fat) as an adulterant following the methods described under standards for *Ayurvedic* formulation[76]. The results are recorded as in Table 3.34.

**2.7.5 Estimation of Vitamin A[80]:**

The amount of total retinol was determined by using the reported spectrophotoflurimetric method of analysis. The methodology adopted was as under:

**2.7.5.1 Sample Preparation[81]:**

About 5 g of the sample of *ghrit* was accurately weighed and boiled with 150 ml freshly prepared 3 M, methanolic Potassium hydroxide solution for 30 min under nitrogen atmosphere.

The solution was allowed to cool at room temperature by keeping it in ice and about 100 ml of water was added to the solution. The mixture was then extracted with 4 × 50 ml peroxide free diethyl ether and the combined fractions were washed with water. The fractions thus obtained were then evaporated dryness in vacuum evaporated at room temperature. The semisolid yellowish mass obtained was dissolved in minimum amount of methanol and the sample is diluted up to 25 ml with methanol. The solution obtained was centrifuged if any particulate matters are seen at room temperature.

**2.7.5.2 Preparation of Standard Solution[80]:**

Accurately weighed quantity of Retinol (Purity 90%) was dissolved in methanol to produce stock solution (1mg/ml). Various aliquots were collected from this stock solution and a series of dilutions were prepared in the range of 0.8-1.6 µg/ml.

**2.7.5.3 Estimation of the Retinol content:**

The fluorescent intensity was measured by means of spectrophotoflurometer, using the excitation wave length 353 and emission wave length 482 nm.

The calibration curve of relative intensity and concentration of the solution was plotted and from the curve the total vitamin A content of the sample of ghee was estimated.

**2.7.6 Studies on effect of Heating time and temperature on product quality:**

A prototype study was designed to observe and evaluate degradative effect of heating on *Jivantyadi Ghrita* in presence of water. The studies were performed on the batches comprised of 100 g of Ghee. The effects of variation of formulating techniques on the quality of the product were assessed by taking heating time and temperature as two variables. While preparing three formulations in the laboratory the ingredients were heated at three different temperatures (105 $\pm 2^0$C, 110$\pm 2^0$C and 115$\pm 2^0$C) and test samples from each batch were withdrawn at three different time intervals (10, 15, 20 min). These collected samples were immediately transferred to air tight container at 2-4 $^0$C and were then subjected to determinations of acid number[77], peroxide value[78], total retinol content[80] and moisture content[77] as indicator of thermal oxidative degradation. The experiments were repeated thrice to obtain a constant value. The results are recorded in Table 3.36.

**2.7.7 Statistical Analysis:**

The datasets obtained were subjected to the studies of Analysis of Covariance to ascertain that the datasets are significantly different to each other.

## 2.8 Biological assessment of different extracts from the selected plants.

**2.8.1 *In vitro* antioxidant activity of plant extracts:**

Methanolic extracts of the aerial parts of the selected plant material was evaluated to assess the antioxidant potential using four various methodologies.

The models selected were:

1. DPPH radical scavenging activity [82]
2. NBT super radical scavenging activity [83]
3. Nitrous oxide free radical scavenging activity [84]
4. Reducing power of the extracts [85]

The methodology adopted to perform the activities was as under:

**2.8.1.1 DPPH radical scavenging activity[82]:**

**2.8.1.1.1. Preparation of standard solution:**

Required quantity of Ascorbic acid was dissolved in methanol to produce the concentration of 10, 15, 20, 25, 30 and 35 µg/ml.

Preparation of sample stock solution

Test extracts were dissolved in methanol to produce stock solutions of 10 mg/ml.

**2.8.1.1.2. Preparation of test samples:**

100, 200, 400, 600, 800 and 1000 µg/ml concentration of the test samples were prepared by proper dilution of the stock solution with methanol.

Preparation of DPPH solution

1.3 mg of DPPH was dissolved in 1 ml of methanol. It was protected from the light by covering the test tube with aluminium foil.

**2.8.1.1.3. Methodology:**

1. 75 µl DPPH solution was added to 3 ml methanol and the absorbance was taken immediately at 516 nm and considered as control reading.
2. Different dose levels were screened by adding 75 µl DPPH to each vial containing 3 ml solution of test extract.
3. The absorbance was taken immediately after addition of DPPH solution at 516 nm using methanol as a blank at zero minute.
4. Decrease in absorbance in presence of test samples at different concentration was noted after 10 min, 20 min, and 30 min.

The effective concentration required to inhibit 50% of the generated radicals was calculated by plotting the graph of log concentration v/s percentage inhibition.

**2.8.1.1.4. Assessment of DPPH radical scavenging activity on TLC plates[86]:**

Different quantity the methanolic extract of the stem of all the selected plants was spotted on TLC plates (25, 50 µb/spot). The plates were run with reported solvent system for flavonoids[68]. The plates were derivatized with 0.04 M DPPH solution in

methanol and kept at room temperature for 30 minutes and the photograph of the plate was recorded.

**2.8.1.2 NBT Super Oxide Scavenging Activity[83]:**

**2.8.1.2.1 Reagents:**

1. Phosphate buffer: 200 ml of phosphate buffer of pH 7.6 was prepared according to I.P.
2. Riboflavin solution: 5 mg riboflavin was dissolved in 25 ml Phosphate buffer.
3. EDTA solution: 402 mg EDTA was dissolved in 10 ml phosphate buffer.
4. NBT reagent: 5 mg NBT was dissolved in 5 ml Phosphate buffer.

**2.8.1.2.2 Methodology:**

1. 100 µl riboflavin solution (20 µg), 200 µl EDTA solution (12 mM), 200 µl methanol and 100 µl NBT solution (0.1 mg) were mixed in a test tube and the reaction mixture was diluted up to 3 ml with phosphate buffer (50 ml).
2. The absorbance of the solution was measured at 590 nm using phosphate buffer as blank after illumination for 5 min. This was taken as control reading.
3. Different concentrations of all the extracts were screened. 100 µl riboflavin, 200 µl EDTA, 100 µl test sample, 200 µl EDTA, 100 µl NBT were mixed in the test tube and the reaction mixture was diluted up to 3 ml with phosphate buffer.
4. The absorbance of the solution was measured at 590 nm after illuminating the content for 5 min

The effective concentration required to inhibit 50% of the generated radicals was calculated by plotting the graph of log concentration v/s percentage inhibition.

**2.8.1.3 Nitrous oxide free radical scavenging activity[84]**

**2.8.1.3.1. Preparation of standard solutions:**

Required quantity of Cur cumin was dissolved in 10 ml of distilled water to produce the concentration of 5, 10, 15, 20, 25, and 30 µg/ml.

**2.8.1.3.2. Preparation of sample stock solution:**

Individual test samples were dissolved in methanol to produce the stock solutions of 10 mg/ml.

**2.8.1.3.3. Preparation of test samples:**

1, 2, 4, 6, 8 and 10 mg/ml concentration of the test samples were prepared by proper dilution of the stock solution with methanol.

Preparation of reagents used :

Phosphate buffer saline: 500 ml phosphate buffer saline was prepared according to I.P.'1996.

10 mM Sodium nitroprusside: 1.788 gm Sodium Nitroprusside was dissolved in 600 ml phosphate buffer saline.

Griess reagent: 100 ml of 1 % solution of Sulphanilamide in 2 % phosphoric acid was prepared. Separately 100 mg of N-(1- napthyl) ethylene diamine dihydrochloride was dissolved in 100 ml of distilled water. Both the solutions were mixed in equal proportion and were kept overnight.

**2.8.1.3.4. Methodology:**

1. 5 ml of 10 mM Sodium nitroprusside in phosphate buffer saline was mixed with different concentrations of sample and standard solutions, and kept for incubation for 15 min at room temperature.
2. The same contents with 1 ml of methanol without the standard or sample served as control.
3. After incubation, 0.5 ml of Griess reagent was added.
4. The absorbance of the chromophore formed was measured at 546 nm.

The effective concentration required to inhibit 50% of the generated radicals was calculated by plotting the graph of log concentration v/s percentage inhibition.

**2.8.1.4. Reducing power Assay[85]:**

Preparation of standard solution

10 mg of ascorbic acid dissolved in 10 ml of distilled water. Make dilutions of this solution with distilled water to give 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, and 1000 µg/ml solutions.

**2.8.1.4.1. Preparation of test sample:**

A required quantity of the test sample was dissolved in minimum quantity of methanol and volume was made up to 10 ml with phosphate buffer. Separately all the samples were diluted in 10 ml volumetric flask with phosphate buffer to produce 10, 20, 40, 60, 80, 100, 200, 400, 600, 800 and 1000 µg/ml solutions.

**2.8.1.4.2. Preparations of reagents:**

Phosphate buffer: 0.2 M phosphate buffer of pH 6.6 was prepared according to I.P. '96

1 % potassium ferricyanide solution: 2 g of potassium ferricyanide was dissolved in 200 ml of distilled water.

10 % Trichloro acetic acid: 40 g of Trichloro acetic acid was dissolved in 400 ml of distilled water.

0.1 % ferric chloride solution: 0.1 g of ferric chloride was dissolved in 100 ml of distilled water.

**2.8.1.4.3: Methodology:**

1. 2 ml of each, sample and standard solutions were spiked with 2.5 ml of 1 % potassium ferricyanide solution.
2. This mixture was kept at $50^0$C in water bath for 20 min.
3. After cooling, 2.5 ml of 10 % Trichloro acetic acid was added and the content was centrifuged at 3000 rpm for 10 min.
4. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % ferric chloride. The solution was kept at room temperature for 5 min.
5. The absorbance of resulting solution was measured at 700 nm and those of samples were compared with the standard solution.

**2.8.2 Animals:**

Wistar female rats weighing about 200-250 g were utilized as experimental animals. The animals were housed in standard conditions of temperature, humidity and light. They were fed with standard rodent diet and water *ad libitum*. Animal experiments were performed after obtaining necessary approval of Institutional Animal Ethics Committee.

**2.8.3 Statistical Analysis:**

Results were subjected to statistical analysis using one way test for analysis of variance (Anova) and student's *t-test*. $p < 0.05$ was selected as the level of significance.

### 2.8.4 Acute toxicity studies[87]:

Acute toxicity study was performed for selective extracts according to the acute toxic classic method (OECD, 1996). Female albino rats were used for acute toxicity study. The animals were kept fasting for overnight providing only water and the extracts were administered orally at the dose of 300 mg/kg and observed for 14 days. If mortality was observed in 2 out of 3 animals, then the dose administered was assigned as toxic dose. If the mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose i.e., 2000 mg/kg. The results of the acute toxicity tests are reported as in Table 3.41 including the extract evaluated and the minimum dose found to be toxic.

### 2.8.5Assessment of Hepatoprotective activity of plant extract *in vivo* and *in vitro*.

#### 2.8.5.1 *In-Vivo* Evaluation[88]:

The extracts were subjected to *in-vivo* evaluation of hepatoprotective activity after inducing acute hepatotoxicity in experimental animals using Paracetamol.

#### 2.8.5.1.2 Selection of extracts and Preparation of Dose:

As methanolic extract of aerial parts of the plants revealed the presence of flavonoidal moieties they were selected to assess the hepatoprotective potential against paracetamol induced toxicity.

Methanolic extract was prepared by extracting the powdered aerial part of the selected plants in soxhlet extractor. The extracts were concentrated in rotary vacuum evaporator at 50$^{o}$ C and then dried at room temperature in vacuum desiccator. The extract were administered to the animals by suspending appropriate quantity of the extract in 0.2ml of 0.1% sodium carboxy methylcellulose prepared in distilled water through oral route (p.o.).

Chapter II Experimental

Albino rats (150-250 g.) of either sex were divided into following groups of six each. All the animals were maintained on normal diet and tap water *ad libitum.*

1. Unstressed rats served as control.
2. Stressed rats were administered Paracetamol (3000mg/kg), suspended in 0.1% sodium carboxy methylcellulose solution.
3. The rats administered with 200 mg/kg silymarin served positive control
4. Treatment group animals received methanolic extract of the plants (200 mg/kg., 400 mg/kg. p.o) suspended in 0.1% sodium carboxy methylcellulose solution.

The animals were administered with the test extracts/blank/silymarin for 3 days. On the third day Paracetamol was administered through oral route in the form of suspension in water. After 72 hrs of administration of Paracetamol, 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 Viz, Serum glutamic oxaloacetic transaminase (SGOT) and Serum glutamic pyruvic transaminase (SGPT), Serum alkaline phosphatase(Alk P), Total bilirubin (T Bil), Total serum protein using reported methods.

The animals were dissected to isolate the liver. The histology of liver sections was studied to observe the alterations in integrity of the microstructures.

**2.8.5.1.3. Estimation of selected biochemical parameters:**

**2.8.5.1.3.1 Determination of Serum Glutamic Oxaloacetic and Serum Glutamic Pyruvic Transaminases (SGOT & SGPT)[89]:**

The level of Glutamic Oxaloacetic Transaminases and Glutamic PyruvicTransaminases was determined by the commercially available kit from Span Diangnostics LTD, India using Reitman and Frankel method

**2.8.5.1.3.2 Estimation of Alkaline Phosphatase[90] (Mod. Kind & King 's Method):**

The level of alkaline phosphatase in serum was estimated using the commercially available kit.

**2.8.5.1.3.3 Estimation of Total Bilirubin[91]:**

Total serum bilirubin (T.Bil) values were determined by Malloy and Evelyn Method using commercially available kit.

**2.8.5.1.3.4. Estimation of Total Protein[92]:**

The level of total protein was estimated using modified biuret method by adopting the methodology proposed in commercial literature obtained with protein estimation kit.

**2.8.5.1.4. Histopathological Studies[93]:**

The hepatoprotective activity of the various extracts was assessed by performing the histopathological examination of the liver section. One animal from the treated group showing maximal activity as indicated by improved biochemical parameters from each test and toxicant group was utilized for this purpose. The animals were sacrificed and the abdomen was incised to isolate liver. 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 hours. 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. 5µm 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.

**2.8.5.2 *In Vitro* Hepato protective Activity**

**2.8.5.2.1 Isolation of Hepatocytes[94]:**

The liver was irrigated with PBS (Phosphate buffer solution PH 7.4). Hepatocytes were isolated by following the methodology mentioned below:

1. Liver was dissected out under aseptic condition.
2. The liver was then minced into small pieces with blunt knives.
3. Minced pieces of liver were added to the PBS containing HEPES (0.01m – 1.2 g) collagenase 0.05 % and incubated at 37 °c for 45 – 60 minutes with constant shaking.
4. After incubation, to the mixture 10 % bovine calf serum was added, and then the suspension was subjected to the process of filtration through sterile muslin cloth.
5. The filtrate obtained was then centrifuged at 200 rpm for 2-3 minutes while temperature was maintained at $4^0$ C
6. The supernatant was discarded and the cells were washed with PBS till the clear supernatant was obtained
7. The hepatocytes were finally suspended in PBS and the viability was couned by trypan blue exclusion method.

**2.8.5.2.2 Culturing of isolated hepatocytes[95]:**

1. The hepatocytes were added to RPMI 1640 media, supplemented with 10 % calf serum. 1 μg/ml of gentamycineand 0.1 M HEPE'S buffer was added to

this suspension and fianally the suspension was distributed into various culture bottles and incubated in $CO_2$ incubator for 24 hours.

2. After 24 hours the culture was washed with RPMI and re suspended in fresh RPMI solution.
3. The viability was assessed again. The culture must possess viability more than 85 %.
4. The hepatocyte suspension was divided in various groups eg. control, toxicant, standard and test .

**2.8.5.2.3 Protocol for Hepatoprotective Activity[88], [96, 97]**

Control:

0.1 ml hepatocytes solution + 0.1 ml vehicle (30 % DMSO solution ) +0.8ml of PBS.

Toxicant

0.1 ml hepatocytes solution + 0.1 ml Paracetamol + 0.8 ml PBS.

Test:

0.1 ml hepatocytes solution + 0.1 ml of extract + 0.7 ml PBS ( keep a side for 30 minutes at 37 °c ) + 0.1 ml of toxicant ( 100µl Paracetamol).

The experiments were performed in triplicate. The hepatocytes were cultured for 24 hr in presence of 5% $CO_2$ at 37 $^0$ C. The viability of hepatocytes were determined at the end of 24 hr by trypan blue exclusion assay and the results are expressed as in the Table 3.43 in the form of average of % viability along with Standard Error of Mean value (SEM).

**2.8.6 Assessment of comparative adaptogenic potential shown by methanolic extracts in swim endurance test[98]. :**

The adaptogen should protect the body under various stress conditions (Vernikos-Danellis *et al*, 1980*)*. In present studies the stress conditions were generated by

enforcing the animals to swim in water for specified hours on suggested number of days. The effect of the drug/extract on the altered level of biochemical parameters was measured and compared with that of control animals. The Wistar rats were selected as the experimental animals.

The biochemical markers estimated to assess the performance of the drug during stressed condition were Serum GPT, Serum glucose, Serum Cholesterol, Serum trilglycerides and Blood Urea Nitrogen.

Control group was set and used parallel to the test groups. The experiments were performed at two arbitrary selected doses of total methanolic extract (200, 400 mg/kg body wt). The extracts were administered as suspension in 0.1% Sodium Carboxyl Methyl Cellulose (SCMC) and administered orally. (p.o.)

The methodology of the experiments along with the protocol employed for estimation of the biochemical markers is explained as under:

**2.8.6.1 Preparation of Extracts:**

Methanolic extract was prepared by extracting the powdered aerial part of the selected plants in soxhlet extractor. The extracts were concentrated in rotary vacuum evaporator at 50$^{o}$ C and then dried at room temperature in vacuum desiccator. Appropriate quantities of the extract were suspended in 0.2ml of 0.1% sodium carboxy methylcellulose, in distilled water and administered p.o. to the animals.

**2.8.6.2 Animals:**

Albino rats (180-230 g.) of either sex were divided into following groups of six each. All the animals were maintained on normal diet and tap water *ad libitum.*

1. Unstressed rats served as blank
2. Stressed rats were administered vehicle alone which served as control.

3. Stressed rats were administered methanolic extract of the plants (400 mg/kg., 200 mg/kg. p.o) at 11 am (1 hr before the application of stress) and 3 pm (1 hr after the application of stress). Extract was administered for a period of 15 days prior to application of stress.

4. Positive Control, the animals of the group was administered with aqueous extract of roots of *Borhaevia diffusa.* (200 mg/kg body wt/daily)

**2.8.6.3 Forced Swimming stress:**

Stress was exerted by enforcing the rats to swim in cylindrical vessels of 48 cm in length and 30 cm in diameter, filled with water to a height of 25 cm over the period for two hours (12:00 am to 2.00 pm) daily for 1$^{st}$ day to 7$^{th}$ day. Blood was collected and required serum was separated on 1$^{st}$ day and on 7$^{th}$ day of stress.

**2.8.6.4 Biochemical Tests:**

Serum Glucose, Cholesterol, Triglycerides, were estimated by using prepared reagents supplied by Monozyme India Limited Secunderabad India, while SGPT (serum Glutamate Pyruvate Transaminase), and BUN (Blood Urea Nirtrogen) were estimated using ready to use reagents supplied by Span Diagnostics Ltd. Surat India. All the samples were analyzed on UV-Spectrophotometer (1601) Shimadzu. The methodology adopted is mention below.

**2.8.6.4.1 Estimation of SGPT[99]:**

The SGPT was determined by 2,4-DNPH method.

**2.8.6.4.2 Estimation serum glucose[100]:**

Glucose was estimated by Enzymatic, Glucose oxidase (GOD) and Peroxidase (POD) kit.

**2.8.6.4.3 Estimation of Serum Total Cholesterol[101]:**

Total Cholesterol in serum was enzymatically estimated using proposed method.

**2.8.6.4.4 Estimation of Triglycerides from serum[102]:**

Glycerol Phosphate Oxidase (GPO) Peroxydase (POD) Enzymatic Method was used for estimation of triglycerides.

**2.8.6.4.5 Estimation of Blood Urea Nitrogen[103]:**

Blood Urea Nitrogen was estimated by DAM method (Diacetylmonoxime method)

**2.8.6.5 Statistical analysis:**

Results were subjected to statistical analysis using one way test for analysis of variance (Anova) and student's *t-test.* $p < 0.05$ was selected as the level of significance. The results obtained after the application of the stress in all the test groups were compared with the results obtained for the control in same conditions. The results of the test groups obtained before the application of the stress were also compared with the respective values of the unstressed rats.

**2.8.7 Assessment of lactogenic potential of extracts of the selected plants**

**2.8.7.1. Assessment of lactogenic potential of extracts of *D.volubilis* and *P.microphylla* in lactating rats and comparison with extracts of *L.reticulata:***

Pet. ether and methanol extracts of all the three plants were selected for present studies. The basis of selection was the probable presence mainly non polar components in Pet. ether extracts, polar components in Methanol extract and their structural similarities with Stigmasterol; a known lactogenic compound[44]

**2.8.7.1.1 Preparations of extracts:**

The dried aerial parts of all three drugs were converted to coarse powder and then successively extracted with Petroleum ether ($60^0$-$80^0$) and Methanol using Soxhlet

extractor. The extracts were evaporated in vacuum evaporator at $40^0$ C and then stored in vacuum desiccators. Stigmasterol and Domperidone were used as positive control.

**2.8.7.1.2 Preparation of dose:**

The extracts were suspended in Arachis oil and used in the treatment. The quantity of extracts was incorporated in such a manner so that 0.2 ml of the suspension represents the daily dose in mg/kg, calculated on the basis of weight of each animal. Pet ether extract of *L.reticulata* was administered by dissolving it in 0.2 ml butter oil too, to mimic the traditional therapy. The same extract was formulated in the form of o/w emulsions and administered. The dose of the extract selected was 100 mg/kg body wt of the animals. Respective control group were run parallel to the test groups. The doses were administered through oral route (p.o.)

**2.8.7.1.3 Methodology[44]:**

The colony of Wistar female rats was set up for breeding. Six female pregnant rats were randomly allotted to each group. The litter size was reduced to 6 pups with each mother rat on the second day of the delivery of pups. Treatments were administered from the 3rd to 23rd day, where the day of the delivery was counted as day 1. Weight of mother rat and pups were recorded daily from 1st day to 23rd day. Mamactomy of two pectoral and two abdominal mammary glands of mother rats was performed 23rd day. One abdominal mammary gland was preserved in 10% w/v formosaline solution and utilized for histological studies. The slides were stained with hematoxylline stain and the histology slides were observed for the alterations in the microstructure of the mammary glands[93]. The other set of mammary glands tissues were preserved in deep freeze at $-20^0$C for the estimation of total glycogen content, while pectoral glands isolated and preserved in the same manner were used to estimate total protein content. The observations on the following parameters were recorded and compared with the

results of control obtained in similar manner to evaluate the lactogenic potential of selected extracts during lactation.

The blood was collected from the retroorbital plexus of the mother rat on 3rd, 8th and 13th day of the pup birth and serum was separated. The separated serum was subjected to estimation of prolactin.

**2.8.7.1.3.1 Histological studies:**

The studies include observation of intensity of lactation changes in cellular architect of mammary glands quantified by the secretary ratings and percentage of parenchyma tissues was determined.

Percentage of parenchyma tissue is hundred times the ratio of the area covered by parenchyma cells to the stroma in unit area. The determinations were performed using camera lucida.

Secretory rating is the empirically set scale to assess the potential of mammary gland in secreting milk. The observations included size and shape of the epithelial cells, flattening of myelo epithelial cells and the size of the alvoelis. The rating was awarded in between 0-5.

**2.8.7.1.3.2. Weight of pups and mother rats:**

The weight of both mother rats and pups on 3rd day and 13th day was determined and the difference was recorded.

**2.8.7.1.3.3. Biochemical parameters:**

The stored mammary glands tissues were subjected to the estimation of total protein and glycogen content. The total protein content of mammary glands was determined using Lowry-Hartree method[105] and glycogen content was determined using Phenol – Sulphuric acid method for estimation of glycogen content in small amount of tissues[104].

Methodologies adopted for estimation of total Protein and Glycogen from mammary glands tissues:

**2.8.7.1.3.4. Estimation of Glycogen[104]:**

Reagents:-

Reagent 1: 30% Potassium hydroxide solution saturated with sodium Sulfate.

Potassium hydroxide pellets 30 g (AR) were dissolved in minimum quantity of distilled water and volume was made up to 100 ml. The solution was saturated with sodium sulfate (AR).Reagent 2: 95% v/v ethanol. Reagent 3: 5% Phenol solution: 5 g of phenol crystal (AR) was dissolved in 100 ml distilled water. Reagent 4: Sulfuric acid 96-98% (AR)

**2.8.7.1.3.4.1 Methodology for estimation of Mammary Glands Glycogen Content:**

Preparation of the sample solution:-

Rat mammary glands were isolated by dissecting the rat on 23rd day after the pup birth. All the visible fats, connective tissues and blood were separated with the help of a forceps. The samples were then immediately transferred to a weighing pan and weighed. Finally the weighed amount of mammary glands tissues were transferred to tightly closed type I glass vial (2 ml capacity).

The Vials containing the weighed amount of sample tissues were transferred to deep freezer for storage. The vials were kept on ice after removal from deep freezer storage on the day of the estimation. 1.5 ml of 30% potassium hydroxide solution, saturated with Sodium Sulphate was added to the samples, making sure that the tissue was completely immersed in the in the solution and then kept it in a boiling water bath for 20-30 min to produce a homogeneous solution after placing a tight seal. The vials were removed from the boiling water bath and allowed to cool on ice. The sample from the vials were transferred to centrifuge tubes (2ml each), rinsed previously with

0.5 ml 95% ethanol and finally 0.5 ml of 95% Ethanol was added in the centrifuge tubes to precipitate the glycogen from the alkaline digest.

The test tubes containing the alkaline digest were kept on ice for 30 minutes and then centrifuged at 840 X g for 20-30 min. The supernatant was carefully separated. The glycogen precipitates were present in the form of pallet at the bottom of the tube were dissolved in 2.0 ml distilled water. An aliquot of above glycogen solution was pipetted out into 150 X 20 mm size test tubes and was diluted up to 1 ml by addition of distilled water. 1 ml of 5% phenol solution was added to the above test solution followed by the addition of 5 ml of 96-98% sulfuric acid was added rapidly (within 10-20 sec.). The test tubes were then allowed to stand for 10 min, shaken carefully and then placed for 10-20 minutes in a water bath at 25-35 $^{o}$ C.

**2.8.7.1.3.4.2. Preparation of calibration curve:**

Standards were prepared by dissolving 3.5 mg of glycogen (S.D.Fine-chem Mumbai-India) in 2 ml water and the solution was transferred to tightly capped type I glass vial. The procedure remains same as mentioned in under the title Sample Preparation up to step 6,as in previous section. Glycogen precipitates obtained was then dissolved in 2 ml. of distilled water. Aliquots were pipette out from the above solution to produce the serial dilutions.1 ml. solution of the each standard was pippeted out into 150 X 20 mm test tubes. The solutions were in the range of 0.62μg/ml, to 4.37 μg/ml. Step No. 8 to 10 as mentioned in previous section were then followed. The absorbance was measured at 490nm using UV-Spectrophotometer against reagent blank.

**2.8.7.1.3.5. Estimation of Total Protein content of mammary glands tissues[105]:**

Protein content of the pectoral mammary gland of rat was determined by Lowry's method modified by Hartree. The method was adopted with minor modification for estimation of total protein contents of mammary glands tissues.

The methodology adopted is described as under:

**2.8.7.1.3.5.1Preparation of stock solution of standard:**

Serum albumin was procured from the commercial source, available in the form of Serum Albumin fraction V, was then employed as the reference standard. Stock solution of serum albumin was prepared by dissolving 25 mg of serum albumin in 25 ml of distilled water (1 mg/ml).

**2.8.7.1.3.5.2 Preparation of Reagents:**

Reagent 1: 0.2 g of Sodium Potassium Tartarate and 10 g of Sodium Carbonate were dissolved in 100 ml, 1N Sodium hydroxide.

Reagent 2: 2 g of Sodium Potassium Tartarate and 1 g of $CuSO_4$ 5 $H_20$ were dissolved in 90 ml distilled water volume was make up to 100 ml with 1N Sodium hydroxide solution.

**2.8.7.1.3.5.3 Preparation of Calibration Curve:**

0.1, 2.5, 0.5, 1.0, 2.0, and 3.0 ml from the stock of the standard solution was taken in the 10 ml volumetric flask individually and diluted up to 10 ml with distilled water. 1.0 ml from the each solution was pipette out in separate test tube labeled as $S_1$, $S_2$, $S_3$, $S_4$, $S_5$ and $S_6$. 0.9 ml of Reagent 1 was added in all six test tubes and kept the test tubes at room temperature for 10 minutes. 0.1 ml of Reagent 2 was added in all the test tubes and kept for 10 minutes at room temperature. 3.0 ml of Folin Ciocalteu's reagent was added in each test tube. The test tubes were kept for 30 minutes at room temperature.

Blank was prepared according to above the procedure by skipping the addition of standard solution. (Reagent Blank). Absorbance of standard and test samples were measured against reagent blank at 650nm on UV- Spectrophotometer.

**2.8.7.1.3.5.4 Preparation of sample solution:**

Dissected mammary glands tissues were weighed accurately about 100 mg. All visible fats, connective tissue, and blood were removed previously. The tissues were then transferred to the tissue homogenizer and homogenate obtained was dissolved in 1.5 ml of phosphate buffer and homogenate, thus, obtained was centrifuged at 1000 rpm for 20 minutes. The aliquots (50, 100, 300 µl) were taken from the solution and diluted up to 10 ml with distilled water. 1 ml of the above solution was transferred to the three test tubes and proceed according to the procedure mentioned in preparation of calibration curve. (Step 3 onwards)

**2.8.7.1.3.6 Estimation of serum prolactin[106]:**

The blood was collected from the mother rats on the 3$^{rd}$, 8$^{th}$ and 13$^{th}$ day following the pup birth. The serum was collected and sent to pathology laboratory to estimate serum prolactin content. The explanation of the adopted methodology is described below.

The assay was performed on direct chemoluminometric technology, which uses constant amounts of two antibodies. The first antibody is polyclonal goat anti-prolactin antibody labeled with acridinium ester. The second antibody is a monoclonal mouse anti-prolactin antibody, which is covalently coupled to paramagnetic particles.

25 µl of sample was incubated with 100 µl of goat anti prolactin antibody for 5.0 minutes at 37$^0$ C. 450 µl of mouse anti prolactin antibody was added to mixture and it was kept at 37$^0$ C for 2.5 minutes. 300 µl of acid reagent and base reagent were added and chemiluminescent reaction was initiated. The relative light units were measured and the content of prolactin was obtained from the standard curve.

**2.8.7.1.3.7. *In vitro* Permeation Studies[107]:**

The studies were performed to identify the type of the compounds which passed through the intestinal membrane and thus reached to the blood from the extract administered orally.

Franz Diffusion cell was used to perform the experiments and a part of rat intestine was used as the membrane. 2 g of the extract was first treated with 200 ml simulated gastric juice by stirring for 30 minutes at $37^0$ C. The resultant mixture was, then, partitioned with chloroform after cooling and chloroform from the collected fraction was evaporated using rotary vacuum evaporator at $50^0$ C. The residues obtained after drying was stored in desiccator in cool place.

The extract (100 mg) thus obtained, was mixed with phosphate buffer ph 7.4 (3 ml) and the mixture was added to the upper compartment of the Franz diffusion apparatus. The sink was maintained at $37^0$ C with continuous rotation. The solution from the lower compartment (1.0 ml) was collected after 30 minutes and partitioned with chloroform (10 ml × 3). The combined collected Chloroform layers were then subjected to the drying at room temperature and the dry mass obtained was then subjected to the TLC studies[74]. The chromatograms are displayed as in Fig .3.26

The chromatographic conditions are mentioned as below:

Stationary Phase: Silica Gel G 60 F 254

Mobile Phase:Toluene: Ethyl Acetate: Formic Acid (9.0:1.0:0.1)

Saturation Time: 20 minutes

Postchromatographic derivatization: Libermann Burchard Reagent and heating at $100^0$ C for 10 minutes.

Scanning Wavelength: 366 nm

**2.8.7.2. Assessment of lactogenic potential of the formulations prepared from Pet.ether extract of *L.reticulata*:**

Traditionally, *Jivanti* that is the plant of *L.reticulata* is used for lactogenic potential in the form of either its water extracts or boiled with butter oil and cooked. The assessment of efficacy of laboratory formulation and comparison with those available traditionally was undertaken. A formulation prepared by O/W technique by incorporating Pet.ether extract in oil phase of the emulsion of *L.reticulata* and compared with that of vehicle used and formulation prepared from butter oil.

**2.8.7.2.1 Preparation of Formulations:**

The extracts were dissolved by heating the butter oil in such a manner so that 0.2 ml of the formulation represents the 100 mg/kg dose. The o/w emulsion was prepared using acacia and olive oil[108]. The amount of the extract incorporated in such a way that 0.4 ml formulation consisted of 100 mg/kg body wt dose.

**2.8.7.2.2 Assessment of lactogenic potential:**

The lactogenic potential of the formulations were assessed in the manner mentioned in 2.9.1.The comparative results are shown in Table 3.46.

## 2.9 Development of software facilitating Quality Assessment of Herbal Raw Materials

The magnitude of information regarding herbal drugs increases day by day and at time it becomes rather impossible to retrieve any data while differentiating varieties, species and more so the controversial sources due to geographical variation. Software therefore was developed as a Microsoft Visual Basic Application having Windows XP interface to compile the data suggesting the probable interface and designing the report pattern as per administrator need. Market samples of *L.reticulata* were subjected to evaluation and data generated were compared with stored data for authentic *L.reticulata* using software.