MATERIALS

AND

METHODS

4 MATERIALS AND METHODS

The *Pterocarpus marsupium* heartwood was purchased from Yucca Enterprises, Mumbai and was identified at the Department of Raw Materials Herbarium and Museum, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. It was assigned reference no. NISCAIR/RHMD/Consult/-2010-11/1469/67 and was deposited in the Departmental Herbarium. The heartwood was shade dried and was powdered in an electric grinder. The powder was passed through sieve no. 36 and was used for extraction. For the aqueous extractions purified water was used and for ethanol extractions absolute ethanol (Hayman Ltd., Batch No. 07/005 A2) was utilized. The filter papers used were of Whatman Cat no.1001125.

4.1 AQUEOUS AND ETHANOLIC CONVENTIONAL EXTRACTION METHODS

4.1.1 INFUSION

200 g of the powdered heartwood of *Pterocarpus marsupium* was taken in a 2 litre beaker and 1.6 litre purified water, at room temperature, was added. The contents were stirred occasionally and were left overnight. The lid of the container was closed. In the morning, a new beaker was taken and weighed. The contents were strained in the second beaker and the filtrate thus collected was dried to constant weight at 60 °C. The final weight of the second beaker was noted. The residue was scratched and

collected in a previously weighed 100 ml. beaker, carefully covered and stored at room temperature.

The same procedure was adopted for the ethanol extraction, instead of 1.6 litre purified water, 1.6 litre of absolute ethanol was used.

4.1.2 DECOCTION

200 g of the powdered heartwood of *Pterocarpus marsupium* was taken in a 2 litre beaker and 1.6 litre purified water, at room temperature, was added. The contents were boiled for 15 min. keeping the lid closed and stirring occasionally. After that they were cooled to room temperature. A new beaker was taken and weighed. The contents were strained in the second beaker and the filtrate thus collected was dried to constant weight at 60 °C. The final weight of the second beaker was noted. The residue was scratched and collected in a previously weighed 100 ml. beaker, carefully covered and stored at room temperature.

The same procedure was adopted for the ethanol extraction, instead of 1.6 litre purified water, 1.6 litre of absolute ethanol was used.

4.1.3 MACERATION

200 g of the powdered heartwood of *Pterocarpus marsupium* was mixed with 1.6 litres of purified water and kept in a sealed container for 7 days at room temperature. The contents were shaken several times in a day. A beaker was taken and weighed. The contents were filtered and the solution was expressed in the beaker. The contents were dried to constant weight at 60 °C. The final weight of the beaker was noted. The residue was

scratched and collected in a previously weighed 100 ml. beaker, carefully covered and stored at room temperature.

The same procedure was adopted for the ethanol extraction, instead of 1.6 litre purified water, 1.6 litre of absolute ethanol was used.

4.1.4 PERCOLATION

200 g of the powdered heartwood of *Pterocarpus marsupium* was mixed with the appropriate quantity, out of 1.6 litres, of the purified water to make it evenly and uniformly damp. It was allowed to stand for 15 min., then transferred to a percolator and packed. Sufficient purified water was added to saturate the drug. The top was placed on the percolator, and when the liquid was about to dip from the apparatus, the lower opening was closed. The drug was allowed to macerate for 24 hrs. The percolation was allowed to proceed slowly at a specified rate, gradually adding the remaining solvent. The percolate was collected in a previously weighed beaker. The contents were dried to constant weight at 60 °C. The final weight of the beaker was noted. The residue was scratched and collected in a previously weighed 100 ml. beaker, carefully covered and stored at room temperature.

The same procedure was adopted for the ethanol extraction, instead of 1.6 litre purified water, 1.6 litre of absolute ethanol was used.

The various methods were statistically compared using student t test and ANOVA, followed by Dunnet's t test.

4.2 AQUEOUS AND ETHANOLIC MICROWAVE-ASSISTED EXTRACTION

The MAE was carried out using LG Microwave, Model No. MG- 555 F, **Figure 19.** The various specifications were:

Power: 1350 Watts,

Operating frequency: 2450 MHz.

Input: 230 V- 50 Hz.

Adjustable power: 0-100 %

Adjustable time: 0- 99 min.

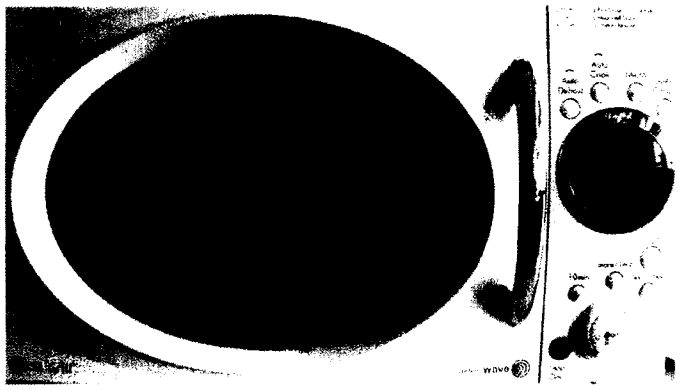

Fig. 19: Microwave MG- 555 F (LG).

50 g of the powdered heartwood of *Pterocarpus marsupium* was taken in 1 litre container and 400 ml. purified water, at room temperature, was added. It was kept in the microwave oven along with glass tube to prevent bumping. It was subjected to microwave irradiation for 30 min. at 100 % power. The container was removed from the oven and was kept aside for 4 hrs. to achieve the room temperature. The contents were filtered and the

solution expressed into another beaker which has been previously weighed. The contents were dried to constant weight at 60 °C. The final weight of the beaker was noted. The residue was scratched and collected in a previously weighed 100 ml. beaker, carefully covered and stored at room temperature.

The same procedure was adopted for the ethanol extraction, instead of using 400 ml. purified water, 400 ml. of absolute ethanol was used. The aqueous and ethanol methods were statistically compared using student t test.

4.2.1 OPTIMIZATION OF MAE

The constrained optimization was adopted by obtaining the experimental data using **factorial designed experiments.** It is a system of experimental design by which the factors involved in a reaction or a process can be identified and their relative importance assessed. It is thus a means of separating those factors which are important from those which are not. The factorial design of experiments was intended to avoid the problems of the interaction among the various parameters which were influencing the experiments.

4.2.1.1 TWO-LEVEL, TWO-FACTOR EXPERIMENTAL DESIGN

The MAE of powdered heartwood of *Pterocarpus marsupium* was performed and the effect of the two parameters *i.e.* microwave power (Factor-A) and irradiation time (Factor-B) was studied. The high (+) and the low (-) levels of the factors were predefined as given in **Table 7.** Any

experiment in which factor A/B was at high level was designated by the corresponding lower case letter, a/b. An experiment in which factors A and B were at higher level was thus designated ab and the experiment where all factors were at their lower level was denoted by (1). A simple factorial designed experiment as shown in **Table 8** would help assess the relative importance of these two factors. The effect of any factor was the change in the response produced by altering the level of that factor, averaged over the levels of all the other factors. The factors interaction was detected graphically. If a line was drawn joining the two results with factor B at high level and another line joining the two experiments in which factor B was at low level, and then if no interaction occurs, two parallel lines would result. For the quantitative estimation of the factor interaction another column of factor interaction was added, the signs of which were derived by the normal algebraic rules. The magnitude of the interaction term was calculated in the same way as that of the main factors, *i.e.* the mean of the results of all the experiments with a + in the interaction column minus the mean of all those with a – in that column.

The factor B was optimized by performing various experiments taking factor A constant at 100 % as shown in **Table 9.** The yield was calculated. The results were statistically arrived using ANOVA.

Table 7: Levels of the Factors of MAE.

Factors	Lower level (-)	Higher level (+)
Factor A (microwave power)	20 %	100 %
Factor B (irradiation time)	5 min.	25 min.

Table 8: ***Two-Level, Two-Factor Experimental Design in MAE.***

Experiment	Factor A (%)	Factor B (min.)
(1)	-	-
a	+	-
b	-	+
ab	+	+

Table 9: ***Optimization of Factor B (Time) in aqueous MAE.***

Expt. No.	Time (min.)	Expt. No.	Time (min.)
1	2	11	20
2	2	12	20
3	5	13	25
4	5	14	25
5	8	15	30
6	8	16	30
7	11	17	35
8	11	18	35
9	15	19	40
10	15	20	40

4.3 AQUEOUS AND ETHANOLIC ULTRASOUND-ASSISTED EXTRACTION

The UAE was carried out using Ultrasonic Bath (Model No. UCB- 5200 D), (Macro Scientific Works Pvt. Ltd.), **Figure 20**. The various specifications were:

Size of the interior trough: 300 X 240 X 150 mm.

Capacity: 10 Litres.

Adjustable temperature: Room temperature unto 60 °C.

Operating frequency: 40 KHz.

Ultrasonic power: 600Watts.

Heating power: 300 Watts.

Adjustable time: 1-99 min.

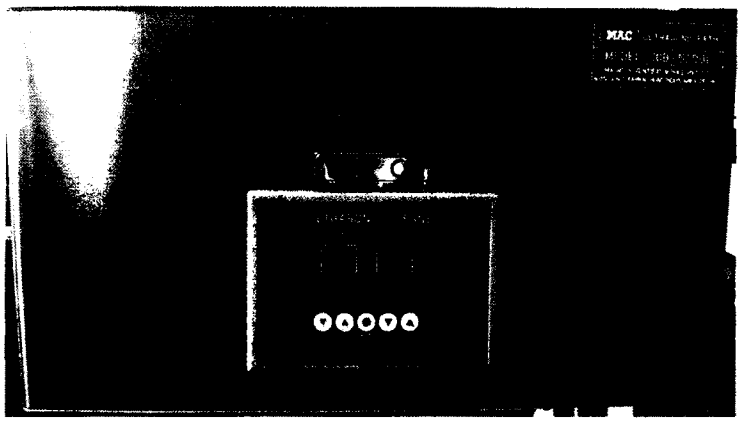

Fig. 20: ***Ultrasonic Bath UCB 5200 D.***

50 g of the powdered heartwood of *Pterocarpus marsupium* was taken in a 500 ml. container and 400 ml. purified water, at room temperature, was added. It was kept in the ultrasonic bath along with glass tube to prevent bumping. It was subjected to ultrasonic waves for 25 min. at 47 °C. The container was removed from the bath and was kept aside for 4 hrs. to achieve the room temperature. The contents were filtered and the solution expressed into another beaker which had been previously weighed. The contents were dried to constant weight at 60 °C. The final weight of the beaker was noted. The residue was scratched and collected in a previously weighed 100 ml. beaker, carefully covered and stored at room temperature.

The same procedure was adopted for the ethanol extraction, instead of using 400 ml. purified water, 400 ml. of absolute ethanol was used. The

aqueous and ethanol methods were statistically compared using student t test.

4.3.1 TWO-LEVEL, TWO-FACTOR EXPERIMENTAL DESIGN

The UAE of powdered heartwood of *Pterocarpus marsupium* was performed and the effect of the two parameters *i.e.* Temperature (Factor-A) and Time (Factor-B) was studied. The high (+) and the low (-) levels of the factors were predefined as given in **Table 10.** Any experiment in which factor A/B was at high level was designated by the corresponding lower case letter, a/b. An experiment in which factors A and B were at higher level was thus designated ab and the experiment where all factors were at their lower level was denoted by (1). A simple factorial designed experiment as shown in **Table 11** would help assess the relative importance of these two factors. The effect of any factor was the change in the response produced by altering the level of that factor, averaged over the levels of all the other factors. The factors interaction was detected graphically. If a line was drawn joining the two results with factor B at high level and another line joining the two experiments in which factor B was at low level, and then if no interaction occurs, two parallel lines would result. For the quantitative estimation of the factor interaction another column of factor interaction was added, the signs of which were derived by the normal algebraic rules. The magnitude of the interaction term was calculated in the same way as that of the main factors, *i.e.* the

mean of the results of all the experiments with a + in the interaction column minus the mean of all those with a – in that column.

Table 10: Levels of the Factors of UAE.

Factors	Lower level (-)	Higher level (+)
Factor A (temperature)	25 °C	60 °C
Factor B (time)	5 min.	60 min.

Table 11: Two-Level, Two-Factor Experimental Design in UAE.

Experiment	Factor A (°C)	Factor B (min.)
(1)	-	-
a	+	-
b	-	+
ab	+	+

4.3.2 OPTIMIZATION OF AQUEOUS UAE BY SIMPLEX SEARCH

The simplex search method is an optimization procedure which adopts an empirical approach rather than making assumptions about the response surface. The results of the previous experiments were used to define the experimental conditions of subsequent experiments in an attempt to find the optimal response. The optimum was approached by moving away from the low values of the response. The name simplex derives from the shape of the geometric figure which moves across the response surface. It is defined by a number of vertices equal to one more than the number of variables in the space. Thus a simplex of two variables is a triangle. All the variables must be put on the same unitary basis, and this was achieved

by Normalization. Normalization was carried out by using the following equation:

$$N = [\ \frac{(X - L)}{(H - L)}\ ] \times 100\ \%$$

Where: N is the normalized value,

X is the original uncorrected value of that variable,

L and H are the lowest and highest values of that factor which are likely to be of interest.

The simplex was constructed by selecting three combinations of these two variables, temperature and time. The three experiments were carried out and the response, *i.e.*, extraction yield (Ra, Rb and Rc) was measured in each case. The worst response was Rb and the values of the independent variables for the next experiment D were chosen by moving away from point B (reflection). This was achieved by reflecting the triangle ABC about AC axis. Hence BP = DP. The experiment at point D was performed and the response Rd was compared with the Ra, Rb and Rc. Now Rd > Rb and Rd < Ra, Rc. The response at D was lower than response at A and at C whereas it was greater than response at B. The procedure was to locate the next experiment along the DP axis at P + 0.5 P (contraction). Now, Re > Rd, Rb and Re < Ra, Rc. The response at point E was greater than that at D and B but it was less than that at A and C. Thus considering the triangle AEC, the values of the variables for the next experiment were chosen by reflecting the Δ AEC along the AC axis and the point F was chosen such

that EP = PF. Now, Rf < Rc, Ra and Rf > Re. The response at F was lower than that at A and C and was greater than that at E, the next point G was located along the EF axis at EP + 0.5 P (contraction). The yield at point G was maximum, *i.e.,* Rg > Ra, Rc and Rf. Now taking Δ AGC into consideration, the lowest yield was at point C, reflecting the point C moves the parameters out of the boundaries. Thus, moving to next lower point A, reflecting the point A also moves the parameters out of the boundaries. So, point G was the only point left. In order to compliment, another point H was chosen, the yield at H was nearly equal to Rg. The yields at last two points were virtually the same indicating that the maximum was nearby. Thus the process was optimized, **Figure 21.**

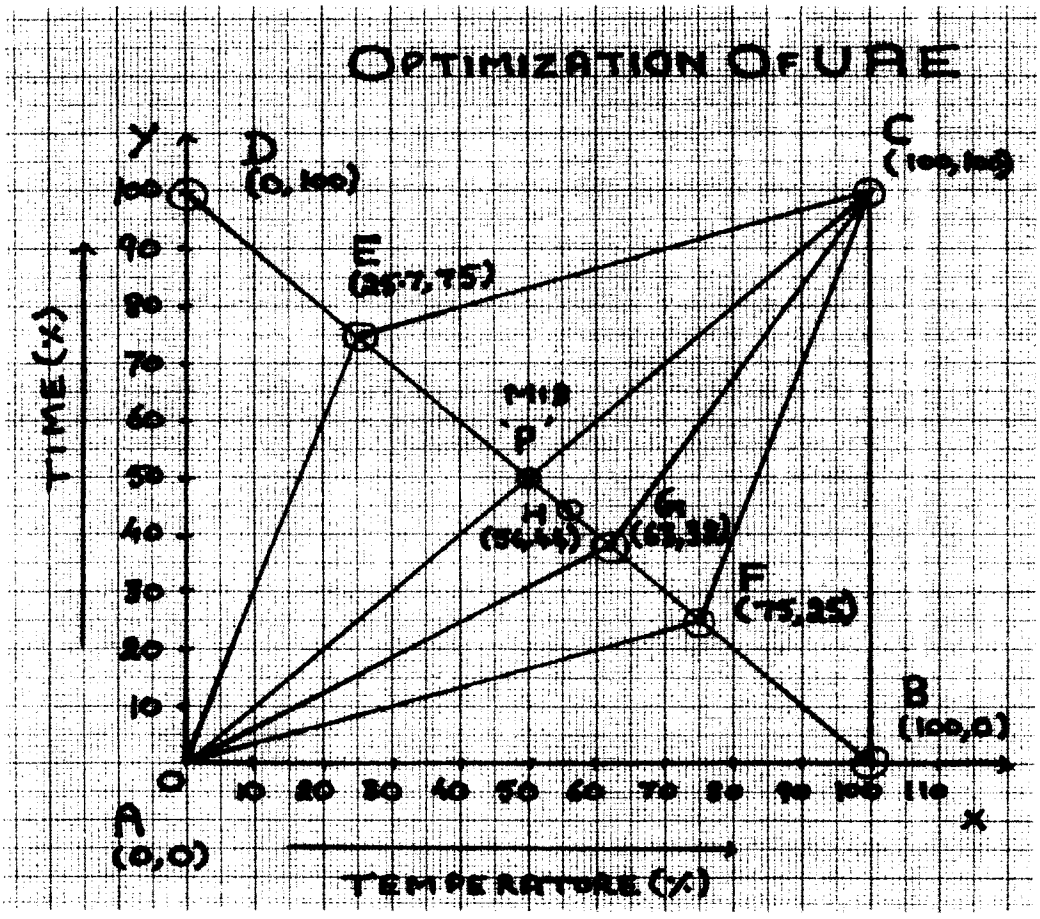

Fig. 21: Simplex Optimization of UAE.

4.4 QUALITATIVE CHEMICAL EXAMINATION

The compounds that are responsible for therapeutic effects are usually the secondary metabolites. The phytochemical screening involves testing of different plant extracts for their content of different classes of compounds. The methods used to detect various phytochemicals must be simple and rapid, but they should also be selective and easy to use.

Procedure:

4.4.1 DETECTION OF ALKALOIDS

A small portion of solvent free extract was stirred with a few drop of dilute hydrochloric acid and filtered. The filtrate was tested with various alkaloidal reagents such as Mayer's reagent (cream precipitate), Dragendorff's reagent (orange brown precipitate), Hager's reagent (yellow precipitate) and Wagner's reagent (reddish brown precipitate).

4.4.2 DETECTION OF CARBOHYDRATES AND GLYCOSIDES

i) Small quantities of extracts were dissolved separately in purified water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates.

ii) A Small portion of the extract was hydrolysed with dilute hydrochloric acid for a few hours in water bath and was subjected to Leibermann-Burchard's, Legal's and Borntrager's tests to detect the presence of different glycosides.

iii) A small portion of extract was dissolved in water and treated with Fehling's, Barfoed's and Benedict's reagents to detect the presence of different sugars.

4.4.3 DETECTION OF PHYTOSTEROLS

i) The extract was treated with few drops of acetic anhydride, boiled and cooled. Then concentrated sulphuric acid was added from the side of the test tube. Formation of brown ring at the junction of the two layers and the upper layer turning green showed the presence of sterols.

ii) Salkowski test: 2 ml of the concentrated sulphuric acid was added to the extract and observed for the formation of yellow ring at the junction, which would turn red after one minute.

4.4.4 DETECTION OF FIXED OILS AND FATS

i) Filter paper test: The extract was pressed between filter papers; production of oil stain indicated the presence.

ii) Saponification test: A few drops of 0.5 N alcoholic Potassium hydroxide was added to a small quantity of extract along with a drop of Phenolphthalein and heated on a water bath for 1-2 hours. The formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

4.4.5 DETECTION OF SAPONINS

i) 1 ml of extract was diluted with purified water to 20 ml and shaken in graduated cylinder for 15 minutes. A one centimetre layer of foam indicated the presence of saponins.

ii) Haemolysis test: 0.2 ml solution of saponin was added to 0.2 ml of blood in normal saline and mixed well. Centrifuged and the red colour supernatant was noted. It was compared with control tube containing 0.2 ml of 10 % blood in normal saline diluted with 0.2 ml of normal saline.

4.4.6 DETECTION OF PHENOLIC COMPOUNDS AND TANNINS

Small quantities of extracts were taken separately in water and tested for the presence of phenolic compounds and tannins with dilute ferric chloride

solution (5 %), 1 % solution of gelatin containing 10 % sodium chloride, 10 % lead acetate and aqueous bromine solution.

4.4.7 DETECTION OF PROTEINS AND FREE AMINO ACID

Small quantities of extract were dissolved in little ml water and the solution was subjected to Millon's, Biuret and Ninhydrin tests.

4.4.8 DETECTION OF GUMS AND MUCILAGES

About 10 ml of extract was added to 25 ml of absolute alcohol with constant stirring. The precipitates were filtered and dried in air. The precipitates were examined for its swelling properties.

4.4.9 DETECTION OF FLAVONOIDS

a) Ferric chloride test: The extract was taken in water, warmed and observed for the formation of green or blue colour on addition of 2 ml ferric chloride solution.

b) Lead acetate test: Lead acetate was added to 2 ml of the extract and observed for the formation of precipitate (Kokate, 1994; Kokate *et al.*, 2007 and Ansari, 2005).

4.5 THIN LAYER CHROMATOGRAPHY (TLC)

Thin Layer Chromatography is one of the widely used techniques for rapid identification of drugs and its formulation. There are several reasons for the practical use of this technique. It is simple to learn and perform, always available for use since precoated plates are available. The time required to demonstrates the presence of constituents of drug formulations by TLC is very short. The method is economical as the solvent

consumption is small. The method of detection does not place any restriction on the choice of the mobile phase. In addition to qualitative detection, TLC also provides visual semi-quantitative information on the chief active constituents of drugs. TLC provides a chromatographic drug finger print; it is, therefore, suitable, for monitoring the identity and purity of drugs and to detect adulteration and substitution. It allows possibility of separating wide classes of drugs. TLC chromatogram can be documented and kept permanently in the record.

4.5.1 OPTIMIZATION OF TLC

The TLC Silica Gel 60 F_{254} Aluminium sheets of Merck, product code 1.05554.0007 were used. All the reagents used were of AR/HPLC grade. The ultra pure water, of Organo Biotech Lab. Pvt. Ltd., batch no. 120 was used to make the solutions.

a) Neat solvents like chloroform, n-hexane, ethyl acetate etc. were tried.

b) Taking first n-hexane (Loba Chemie, Batch No. HB039807), the solvent strength was increased by adding solvents like ethyl acetate (Qualigens Fine Chemicals, Lot No. 89846807-3). Various combinations of strengths were tried taking pterostilbene (Chromadex, Lot: 16995-508) as reference standard.

Hence, the optimized mobile phase was observed as n-hexane and ethyl acetate (7.5:2.5).

4.5.2 PROCEDURE

The extracts obtained by aqueous Percolation, Optimized MAE and Optimized UAE were taken.

a) Sample Preparation: The samples were taken and ethyl acetate soluble fractions were prepared (Mallavadhani and Sahu, 2003).

b) Activation of precoated plates:

Freshly opened box of precoated TLC plates usually do not require activation. However, plates exposed to high humidity or kept on hand for long time may have to be activated by placing in oven at 110-120 °C for 30 minutes prior to sample spotting.

c) Application of sample/reference standard:

Sample application is the most critical step for obtaining good resolution. The sample was completely transferred to the layer without damaging the layer. The sample was applied through clean upper end of the capillary. Usually 1-10 µl volume of the sample was applied so that the size of the starting zone is 2-4 mm.

d) Mobile Phase:

The mobile phase has already been optimized.

e) Chamber saturation:

When the plate is introduced into an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvent is required for a given distance, hence resulting in increase in Rf values. If the tank is saturated (by lining with filter paper) prior to

development, solvent vapours soon get uniformly distributed throughout the chamber. As soon as the plate is placed in such a saturated chamber, it soon gets preloaded with solvent vapours, hence less solvent shall be required to travel a particular distance, resulting in lower Rf values.

f) Development and drying:

The TLC was developed using ascending chromatographic technique. After development the plate was removed from the chamber and mobile phase was removed. The plate was laid horizontally so that while mobile phase evaporates the separated substance will migrate evenly to the surface where they can be easily detected.

g) Detection and visualization:

As soon as the development process was complete, the plate was removed from the chamber and dried to remove the mobile phase completely. The coloured zones were located visually and also under the long wave length UV (365 nm) and short wave length (254 nm). After that the Iodine chamber was used to have the zone marked.

h) Evaluation:

A parameter often used for qualitative evaluation is the Rf value (retention factor). The Rf value is defined as follows:

Rf = Distance starting line-middle of the spot/Distance starting line-solvent front (Sethi, 1999).

4.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The HPLC was used to evaluate the samples. The pterostilbene (Chromadex, Lot: 16995-508) was used as reference standard marker compound (Mallavadhani and Sahu, 2003).

4.6.1 EQUIPMENTS

The Agilent HPLC system comprising of Agilent HPLC pump (JP 94174012), Agilent auto sampler (DE 62973678), column oven, Agilent UV-VIS detector (VWDDE 71366452), Zorbax C-18 column (5 µm, 150 x 4.60) and Agilent chemstation software was used for data analysis and data processing. The Trifluoroacetic acid (Merck, AE 9A 590224) in Milli-Q water was used and Acetonitrile (Merck, IK 91 F 90360) was of HPLC grade.

4.6.2 PREPARATION OF MOBILE PHASE

The mobile phase consisted of (A) 0.1 % Trifluoroacetic acid in Milli-Q water and (B) Acetonitrile. The 5 % B was isocratic for 5 min., then increasing to 95 % B over 20 min. Hold at 95 % B for 5 min. Then back to starting condition, **Table 12.**

4.6.3 PREPARATION OF STOCK SOLUTION

STANDARD: The pterostilbene was used as reference standard marker compound, initially a concentration of 0.9 mg/ml (in HPLC grade

methanol, Merck, SHOSF 60663) was prepared and then 0.18 mg/ml solution was prepared to get the stock solution.

SAMPLE: All the three samples, sample no. 1, sample no. 2 and sample no. 3 were weighed separately and about 500 mg of each was dissolved in 25 ml of HPLC grade methanol to get three stock solutions.

4.6.4 STANDARD CHROMATOGRAM

The 2.5 µL of the stock solution was injected at a flow rate of 0.5 ml/min. Various columns and number of combinations of mobile phase were tried at first and the technique was optimized. The optimized parameters are given in **Table 13.**

Table 12: Gradient Table:

S. No.	Time (min)	% A	% B
1	0	95	5
2	5	95	5
3	20	5	95
4	25	5	95
5	30	95	5

A: 0.1 % Trifluoroacetic acid.
B: Acetonitrile.

Table 13: Optimized Chromatographic Conditions.

Chromatograph	Agilent 1200, Agilent pump (JP 94174012), Agilent UV-VIS detector (DE 71366452) and Agilent Chemstation software.
Mobile phase	0.1 % Trifluoroacetic acid and Acetonitrile (gradient)
Column	Zorbax C-18 column (5 µm, 150 x 4.60)
Column Temp.	55 °C
Flow rate	0.5 ml/min
Detection	250 nm
Injection volume	2.5 µL
Retention time	
Pterostilbene	20.32

4.6.5 METHOD VALIDATION

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. In other words, the validation of an analytical procedure requires us to demonstrate scientifically that risks in decision by testing, caused by errors from analytical steps are acceptably small. Analytical methods need to be validated or revalidated before their introduction into the routine use or whenever the condition change for which the method has been validated or whenever the method is changed and the change is outside the original scope of the method. The International Conference on Harmonization (ICH) guidelines on Validation of Analytical Procedures (Q2A and Q2B) delineates the guidance and methodology for validation characteristics of an analytical procedure. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

4.6.5.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, *etc.* The specificity is a measure of discriminating ability. The specificity was assessed by comparing analytical results obtained from samples containing the analytes only with results obtained from samples containing excipients,

related substances or degradation products, and including or excluding the analyte.

4.6.5.2 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. For the establishment of linearity, five solutions of different concentrations were injected **(Table 14)** chromatograms obtained and the calibration plot was drawn between concentration and area. The linearity was evaluated by the visual inspection of the plot and also by calculating correlation coefficient.

Table 14: Concentration of the Solutions for Linearity Study.

S. No. of Solution	Concentration (ppm)
1	25
2	45
3	55
4	110
5	220

4.6.5.3 System Suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipments, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. The system suitability tests were applied to check the various parameters like column efficiency, resolution, precision and peak tailing. The system was injected with 6 replicate injections of pterostilbene in the working concentration of 45 ppm.

4.6.5.3.1 Calibration Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The specified range was derived from linearity studies and depends on the intended application of the procedure.

4.6.5.3.2 Plate Number or Number of Theoretical Plates

This is a measure of the sharpness of the peaks and therefore the efficiency of the column. This was calculated using the half width method, given by the formula:

$$n = 5.545 \times \left[\frac{t}{Wh}\right]^2$$

Where:

t: retention time of peak

Wh: peak width at half peak height.

4.6.5.3.3 Tailing Factor

The tailing factor is a measure of peak tailing. It is the distance from the front slope of the peak to the back slope divided by twice the distance from the centre line of the peak to the front slope, with all measurements made at 5 % of the maximum peak height.

4.6.5.3.4 Relative Standard Deviation or Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The **Repeatability** expresses the precision under the same operating conditions over a short period of time. The **Retention Time precision** is important, because not only is retention time the primary method for peak identification, but also variations can indicate problems within the HPLC system (*i.e.* with the piston seals, check valves, *etc.*). The Retention Time precision was affected by column temperature, pump flow and mobile phase composition. The most dominant factor controlling the **Peak Area precision** was the auto sampler, though the affect of noise and integration parameters will become more significant with small peaks. The precision is usually expressed in terms of Relative Standard Deviation (%), which was calculated by using the given formula:

$$RSD\ (\%) = \frac{S.D}{Mean} \times 100$$

Where:

S.D: Standard deviation.

Mean: Mean of the responses.

4.6.5.3.5 Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessarily quantitated as an exact value. According to ICH guidelines, in an

analytical procedure which exhibits baseline noise, the determination of limit of detection can be based on signal-to-noise ratio. The determination of the signal-to-noise ratio was done by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3:1 and 2:1 is generally considered acceptable for estimation of detection limit.

4.6.5.3.6 Limit of Quantification

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. According to ICH guidelines, in an analytical procedure which exhibits baseline noise, the determination of limit of quantification can be based on signal-to-noise ratio. The determination of the signal-to-noise ratio was done by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

4.6.6. SAMPLE PREPARATION AND STANDARDIZATION

The stock solutions of the three samples, sample no. 1, 2 and 3, were already prepared. Volume of 2.5 µl from each stock solution was taken

and injected in the column. The column was run for 30 min. The chromatograms were plotted between Peak height and Time. The samples were thus standardized. In order to quantify the pterostilbene in different samples, the following formula was applied:

$$Pterostilbene\ (\%) = \frac{Sample\ \ Area}{Avg.\ \ Std.\ \ Area} \times \frac{Std.\ \ Conc.}{Sample\ \ Conc.} \times \frac{Std.\ \ Potency}{100} \times 100$$

Where:

Avg. Std. Area: Average Standard Area.

Std. Conc.: Standard Concentration.

Sample Conc.: Sample Concentration.

Std. Potency: Standard Potency.

(Validation of analytical procedures: text and methodology Q2 (R1), 2005; Iterson, 2010; Walfish, 2006; Validation of analytical procedures, 2010 and Huber, 2010)

4.7 ANTIDIABETIC ACTIVITY

4.7.1 EXTRACTION OF AQUEOUS EXTRACT

The *Pterocarpus marsupium* heartwood has been extracted by conventional methods, *i.e.,* **infusion, decoction, maceration and percolation.** The maximum extraction efficiency was in case of **percolation.** The heartwood has also been extracted by the non conventional methods, microwave-assisted extraction and ultrasound-assisted extraction. Both of these methods have been optimized using

factorial design and simplex model independent method respectively. Thus on the whole, the aqueous extracts obtained from three different types of methods were used for the *in-vivo* studies. All the extracts were dissolved in sterile normal saline

4.7.2 TEST ANIMALS

Sprague-Dawley (220-250 g) male rats with average age of 10-12 weeks were used. They were procured from the in-house bred animals after approval under project BIO IAEC 216-10/4. These were housed in colony cages at an ambient temperature of 22 ± 3 °C and standard 30-70 % relative humidity and a 12 hours light- dark schedule, with 12-15 air changes per hour. The animals had free access to water (Aquaguard purified water). They were fed with standard laboratory diet (Vetcare/Nutrilab pellet feed). The animals were acclimatized to laboratory conditions for a period of 5 days.

4.7.3 EFFECT OF TEST DRUG ON BLOOD GLUCOSE LEVELS IN NORMAL FASTED RATS

The animals were fasted for 16 hours prior to the experiment, water *ad libitum.* The rats were arranged in 5 groups of 8 rats in each group. **Group 1** served as control and received only vehicle (sterile normal saline, 1 ml/100 g body weight). **Group 2** served as standard and received standard drug gliclazide (Panacea Biotec Ltd., Batch No. 2179501) at an oral dose of 25 mg/kg (in sterile normal saline). **Group 3-5** served as test and received the extracts at an oral dose of 250 mg/kg body weight (in sterile

normal saline). For extracts and the reference drug (gliclazide) the volume to be given to the rats orally was not greater than 1 ml/100g of body weight. The formulated doses of standard and plant extracts were administered through oral route by gavage to animals.

4.7.3.1 Estimation of blood glucose levels

Blood samples were collected through tail vein. The blood glucose levels were estimated by the Accuchek strip test immediately before receiving, *i.e.,* at 0 hr, 1 hr after and 3 hrs after receiving the vehicle/gliclazide/plant extracts.

All the 40 rats used in the above experiment were given a washing period of 7 days after the completion of the above experiment, so that the rats can be optimally utilized for the subsequent experiments.

4.7.3.2 Body weight estimation

The body weight measurements were done on day 1 before administration.

4.7.4 EFFECT OF TEST DRUG ON BLOOD GLUCOSE LEVEL IN ALLOXAN INDUCED DIABETIC RATS

4.7.4.1 Induction of experimental hyperglycaemia

Hyperglycaemia was induced by a single intra-peritoneal injection of 120 mg/kg alloxan monohydrate (Spectrochem Pvt. Ltd., Mumbai, India, and Batch No. 4244476) in sterile saline. The animals were allowed for 7 days to develop diabetes. On day 7, the blood glucose concentration of the animals was checked using Accuchek strip method. Animals showing blood glucose range of > 200 mg/dl were considered as diabetic. Animals

had free access to food and water till before 30 min of sampling and laboratory diet was returned 1 hr after Alloxan administration and remained freely available for the remainder of the study.

4.7.4.2 Acute treatment

The hyperglycaemic rats were divided into 6 groups of 8 animals each. **Group 1** was selected from the previously selected normal fasted rats and served as normal control and received only vehicle and not alloxan, food and water being freely available. **Group 2** served as diabetic control and was given only vehicle (sterile normal saline, 1 ml/100 g body weight). **Group 3** received standard antidiabetic drug gliclazide at an oral dose of 25 mg/Kg. **Group 4-6** were treated orally with extracts at a dose of 250 mg/kg.

4.7.4.2.1 Estimation of blood glucose levels

Blood samples were collected through tail vein. The blood glucose levels were estimated by the strip test immediately before receiving that is at 0 hr, 1 hr after, and 3 hrs after receiving the extract/reference drug/vehicle.

4.7.4.3 Sub-acute treatment

In sub-acute treatment the administration of extract/standard drug was continued for 10 days, once daily. Blood samples were collected through tail vein after day 1 and 1 hour and 3 hours on day 3^{rd}, 7^{th}, 10^{th} of the extract/standard drug administration and the blood glucose levels were determined using Accuchek strip method. After the completion of the

study the animals were humanely sacrificed. The carcasses were disposed through SembRamky Biowaste Management Pvt. Ltd.

4.7.4.4 Body weight estimation

The body weight measurements were done on day 1 before the test item administration and after the completion of 10^{th} day of administration.

4.7.5 Data and Statistical analysis

Statistical significance was determined by using Analysis of Variance (ANOVA) followed by Dunnet's t-test, $P < 0.05$ indicates significant difference between group means.