CHAPTER II

EXPERIMENTAL

1. Materials and Technique Used in Microscopic Investigation.

Tuberous roots and nearly full grown leaves of Pueraria mirifica Airy Shaw et Suvatabandhu from Utaradit province in May, 1974, authenticated by Professor Kasin Suvatabandhu.

Tuberous roots and nearly full grown leaves of Butea superba Roxb. from Saraburi province in February, 1975, authenticated by Miss Ampai Yongboonkerd.

Chloral hydrate

Glycerin

Apparatus

Microscope (Olympus)

Drawing Apparatus (Carl Zeiss)

Microprojector Drawing Apparatus (RCA.)

Procedure

Examine and illustrate the external characters of leaves and roots of Pueraria mirifica Airy Shaw et Suvatabandhu, and Butea superba Roxb.

Method in Clearing Leaves

Transverse strips about 5 mm square of leaves other than extreme margin or midrib are cut and placed in a beaker of boiling chloral hydrate solution until they are clarified by simultaneous changes of the chloral hydrate solution. The strips are then washed with distilled water and kept in glycerin.

In case of Butea superba Roxb. the colour of the leaves are hardly removed, so chlorinated soda is used instead of chloral hydrate.

Procedure in Palisade Ratio Determination

Clarified leaf strips are placed under the microscope, four contiguous epidermal cells and the underlying palisade cells are traced. Count the total number of palisade cells lying beneath the four epidermal cells. In making this count, those palisade cells of which more than half the area lay under the epidermal cell are included, while those of which more than half the area lay outside the epidermal cells are excluded. The total numbers are divided by four.

Procedure in Stomatal Number and Stomatal Index Determination

By means of stage micrometer, measure the diameter of field of view. Calculate the area in square millimeters. Place the slide of lower surface of clarified leaf strips in

place of stage micrometer. Locate upper and lower epidermal cells together with stomata under the field of view in the same area.

Count the epidermal cells and stomatal apparatus in the field of view, part of the cells in one semicircle are inclusive, while those on another are exclusive.

Calculate the stomatal index of upper and lower surfaces by applying the formula shown.

Procedure in Vein-islet Number Determination

By means of stage micrometer, draw an enlarged square corresponding to 2 mm x 2 mm under the microprojector. Replace stage micrometer by clarified leaf strips slide, trace the veins of the lower epidermis within this four square millimeter area.

Count the vein-islet, including parts of those that are intersected by two adjacent sides but excluding those intersected by the other two adjacent sides, then divided by four.

Procedure in Veinlet Termination Number Determination

This procedure is the same as in the vein-islet number determination. Count the number of veinlets in four square millimeters, parts of those intersected by two adjacent sides included and parts of those intersected by the

other two adjacent sides excluded.

Procedure for Illustration of Histology

Examine the dried tuberous root powder of Pueraria mirifica Airy Shaw et Suvatabandhu and Butea superba Roxb. under the microscope. Illustrate the form of the starch grains under day light and under polarised light and determine the size of the starch granules microscopically.

Determination of Foreign Organic Matters of the Tuberous Roots

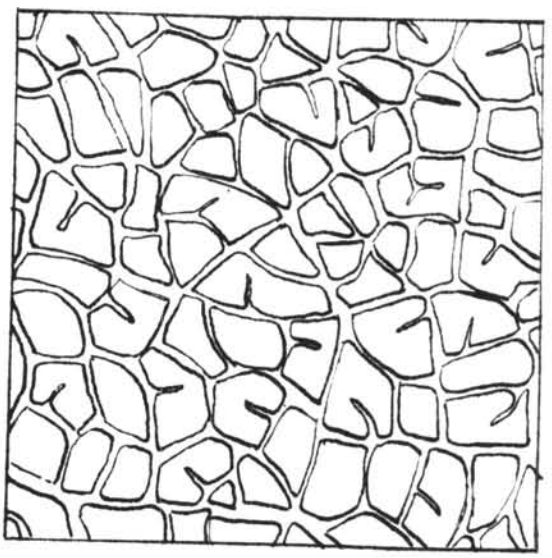
Accurately weigh 2-4 g of dried powdered root of Pueraria mirifica Airy Shaw et Suvatabandhu in a tared-crucible. Place them in the muffle, heat at about 525°C. until white ash is obtained. Weigh the ash, and reash until the weight is constant. The constant weight residue is the total ash[29].

Acid-insoluble Ash

Boil the total ash mentioned with 25 ml of dilute hydrochloric acid for 5 minutes. Filter with ashless filter paper, wash the residue with hot distilled water. Ignite the residue and weigh it until constant weight is obtained. Calculate the percentage of total ash and acid-insoluble ash.

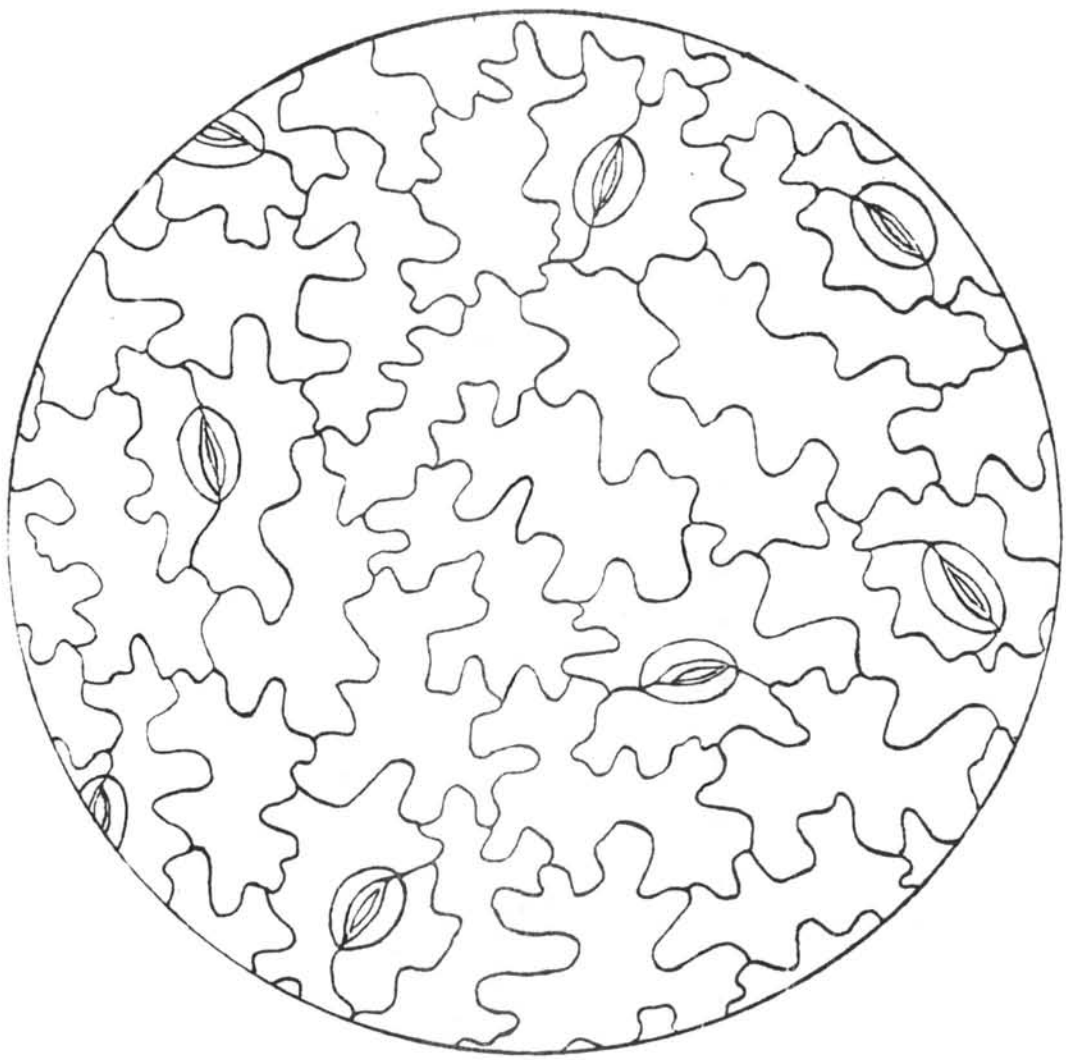
x 400

Fig. 1. Palisade ratio. Four epidermal cells with the underlying palisade cells of Pueraria mirifica leaf



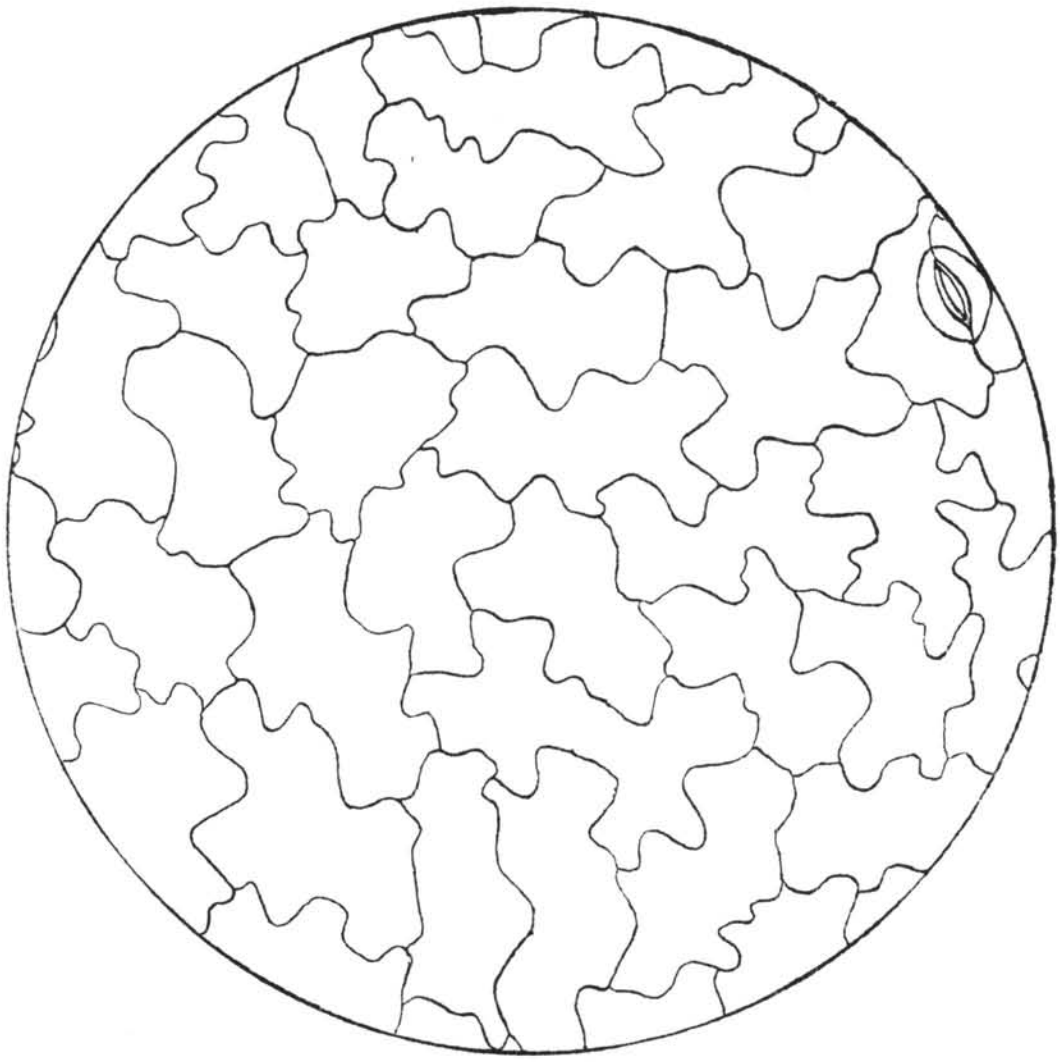
x 10

Fig. 2. Vein-islets of Pueraria mirifica leaf



x 375

Fig. 3. Stomatal cells and epidermal cells of lower surface of Pueraria mirifica leaf

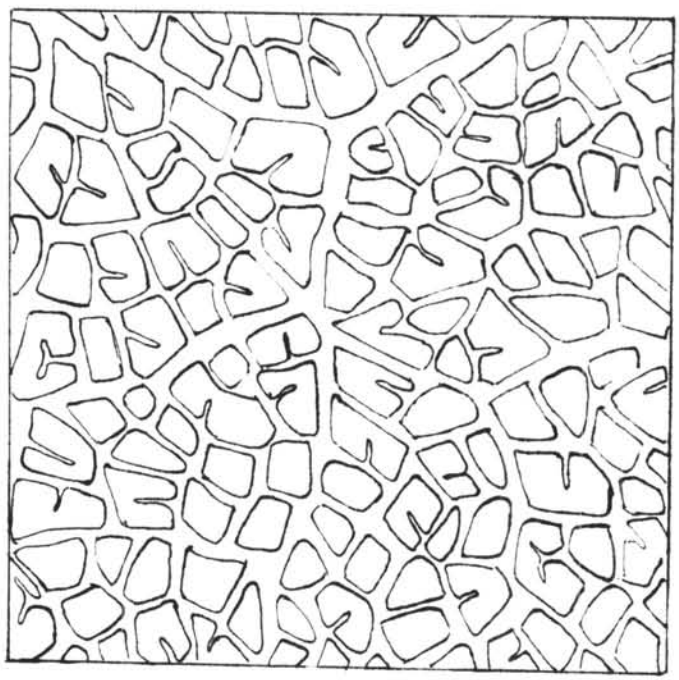


x 375

Fig. 4. Stomatal cells and epidermal cells of upper surface of Pueraria mirifica leaf

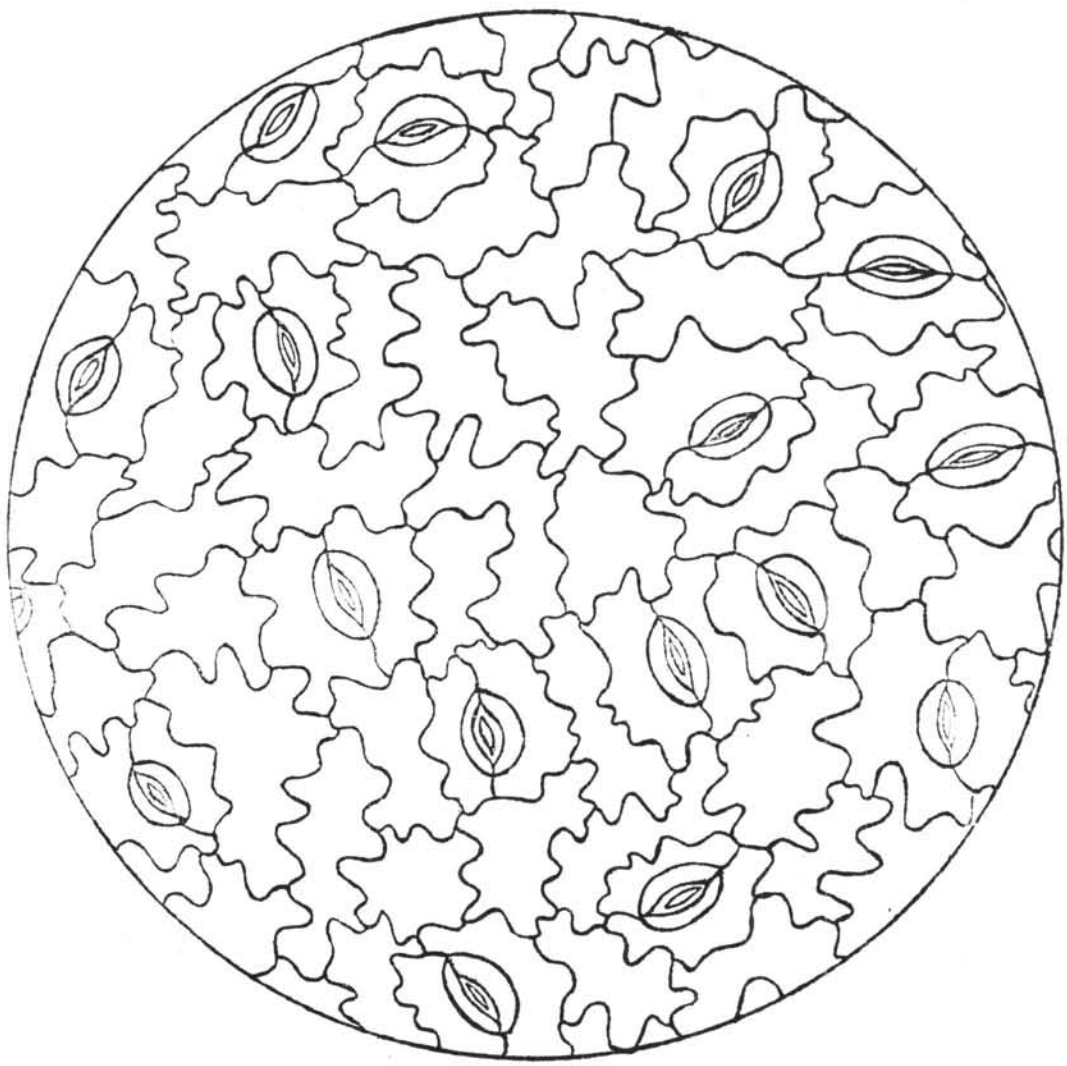
x 400

Fig. 5. Palisade ratio. Four epidermal cells with the underlying palisade cells of Butea superba leaf



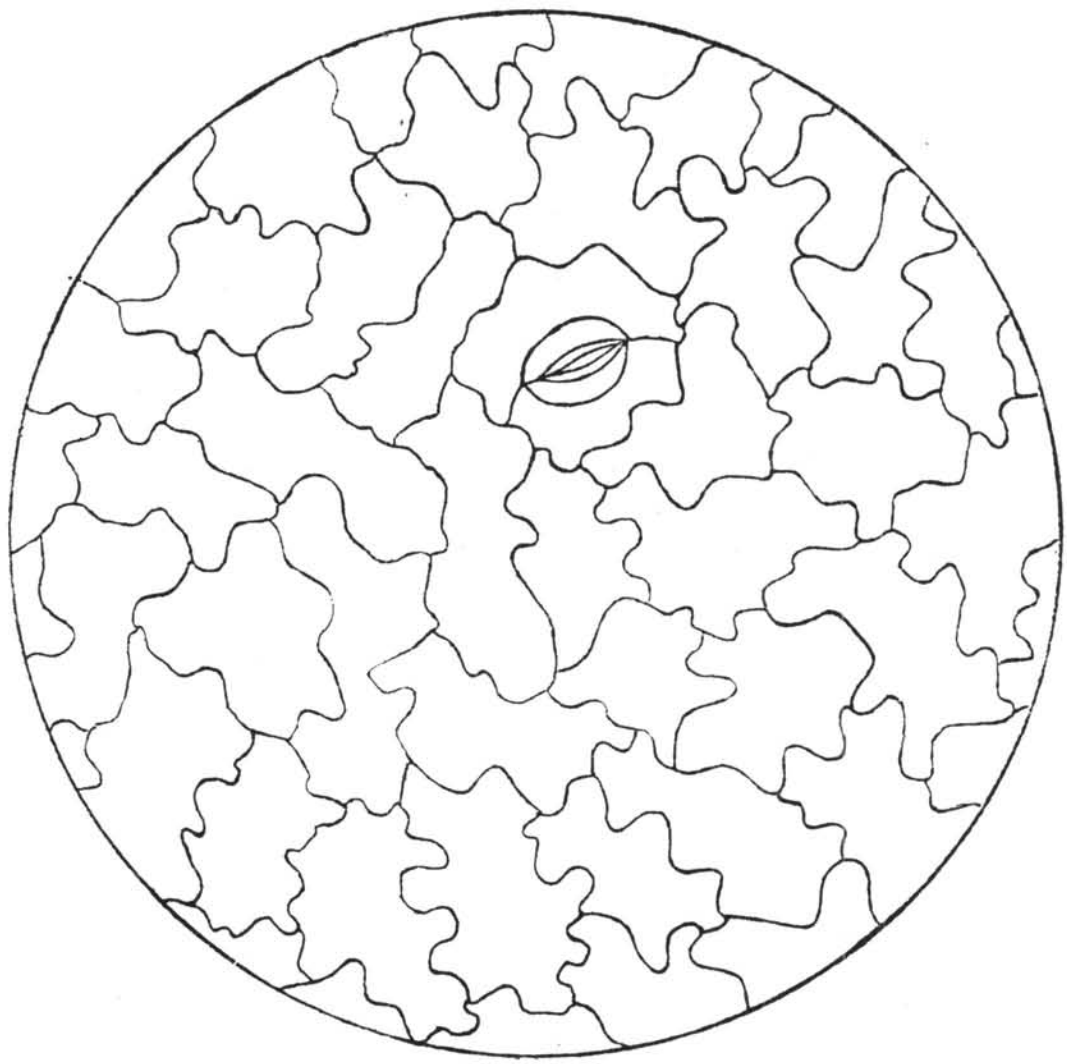
x 10

Fig. 6. Vein-islets and veinlet termination of Butea superba leaf



x 375

Fig. 7. Stomatal cells and epidermal cells of lower surface of Butea superba leaf.



x 375

Fig. 8. Stomatal cells and epidermal cells of upper surface of Butea superba leaf

2. Materials and Techniques Used on Chromatography

Leaves and tuberous root of *Pueraria mirifica* Airy Shaw et Suvatabandhu.

Tuberous root of *Butea superba* Roxb.

Solvents

Acetic acid, glacial	E. Merck, Darmstadt.
Butanol	E. Merck, Darmstadt.
Distilled water	Government Pharmaceutical Organisation, Bangkok.
Ethanol	Government Pharmaceutical Organisation, Bangkok.

Reagents

Ammonium Solution	May & Baker Lab., England.
Ferric chloride, A.R.	Riedel, Germany.
Ferric ammonium sulphate, A.R.	E. Merck, Darmstadt.
Iodine	
Silver nitrate, Lab. Chemicals	M & B Lab., England.
Sodium hydroxide	M & B Lab., England.
Potassium Iodide	
Sulphuric acid	M & B Lab., England.

Ultraviolet lamp — Ultraviolet Products, Inc. San Gabriel, California.

Whatman Chromatographic Paper No. 1, size 46 cm x 57 cm.

Chromatographic Chamber : A glass chamber of 58 cm long, 23 cm wide and 58 cm high (outside measurements) with glass cover.

Method of Extraction

Four grams of dried coarse powdered tuberous roots and leaves were macerated separately in 30 ml of 70% ethanol overnight. Filter through Whatman filter paper No. 1. The filtrates were evaporated to nearly dryness. Dissolved the extracts with 15 ml of 70% ethanol and filtered. Each of the alcoholic solutions was kept for chromatographic spotting.

Preparing of Paper Chromatograms

Whatman Chromatographic Paper No. 1 (46 cm x 46 cm) were used. Two pencil lines were drawn across the sheet at 6 cm from the rims of two adjacent sides. Spotting was made with 0.4 ml of the alcoholic extract by means of capillary tube, to form a circle of 2.5 cm diameter at the intersection point. The chromatogram is then ready for developing in chromatographic chamber.

Solvent System for Two-Dimensional Descending Chromatography

First dimension : normal Butanol - Glacial Acetic Acid - Water (4 + 1 + 1) was used. This solvent system provides an adequate separation of a great varieties of chemical compounds (sugars, glycosides, amino acids, polyphenols, alkaloids and others).

Second dimension : 3% of Acetic acid in water constituted this system. It causes a moderate distribution of the compacting compounds mentioned above, except the sugars.

Development of the Chromatograms

The chromatographic chamber was equilibrated with the particular solvent before Descending Chromatography was made. An excess of solvent was flooded at the bottom of the chamber as a pool for vapour saturation to assure greater constancy of the R_f values obtained. The room was air conditioned, the temperature remained fairly constant at about 22°-24°C.

First dimension : The spotted papers were developed in the normal Butanol - Acetic acid - Water (4 + 1 + 1) chamber in the machine direction of the paper until solvent front reached the end of the paper. Then the paper was removed and hung up to dry in the air.

Second dimension : Development was made in the perpendicular direction to that the first dimension in

3% Acetic acid in a similar manner.

After drying the chromatograms were kept separately until they were examined and dipped in the reagent.

Detection of Spots and Treatments of Chromatograms

Treatments and chromogenic reagents applied were as follow :

1. Colours in daylight : In order to provide a uniform light intensity over the whole chromatograms a 60 Watts (General Electric) lamp was used.
2. Fluorescence : The chromatograms were examined under the Ultraviolet lamp. A pair of U.V. light filter goggles were used during observation.
3. Fluorescence by treatment with Ammonia : During observation under U.V. light mentioned above, the chromatogram was exposed to ammonia vapour, the changes of colours due to the effect of ammonia were recorded.
4. Ferric ammonium alum solution : Dipping technique in 3% freshly prepared ferric ammonium sulphate solution was used. Phenols are detected.
5. Ferric ammonium alum solution and ammonia vapour : After treatment with reagent, dried, then the chromatogram was exposed to ammonia vapour. The changes of colours were recorded.

6. Tollen's reagent (Alkaline silver nitrate reagent) :

   10 ml of 0.1 N $AgNO_3$

   10 drops of Conc. NH_4OH

   5 ml of 10% NaOH

   The mixture must be freshly prepared. The chromatogram was dipped in this solution, after fully developed the paper was washed, firstly in 5% sodium thiosulphate solution and then in water to prevent development of background colours. After this treatment, the developed chromatogram can be kept permanently without changing of colours. Aldehydes and phenolic compounds were detected.

7. Iodine-potassium iodide solution : Chromatograms were treated with a solution consisting of 0.2 g iodine and 0.4 g of KI in 100 ml of water. Most of steroids were detected.

8. Liebermann-Burchardt's reaction : Sulphuric acid (1 ml) was mixed with acetic anhydride (20 ml) and chloroform (50 ml). After dipping the developed chromatogram was laid flat, dried, then heated at 85°-90°C for 15 minutes, the colours were recorded. This reaction can be used to detect all classes of steroids which will give dark green colour.

9. Ortho-phosphoric acid : Chromatogram was dipped briefly in 15% aqueous o-phosphoric acid and laid flat, dried, then heated for 20 minutes at exactly 90°C and detected spot under U.V. light. Steroids which turns brown after treatment were detected[30].

Recording of R_f Value and Colours

The locations and colours of the spots were recorded after each treatment. Concommitant use of more than one compatible chromatographic sprays, revealed at times more than one compound in some areas of the chromatogram and made it possible to mark each. A sample of chromatogram is given. An R_f grid was used as an aid in recording the values. The R_f values were coded as follows :

00 (Zero Zero)	corresponds to R_f	.000 to .075
08	" "	.075 to .125
10	" "	.125 to .175
18	" "	.175 to .225
20	" "	.225 to .275
28	" "	.275 to .325
30	" "	.325 to .375
and so on		
80	corresponds to R_f	.825 to .875
88	" "	.875 to .925
90	" "	.925 to 1.000

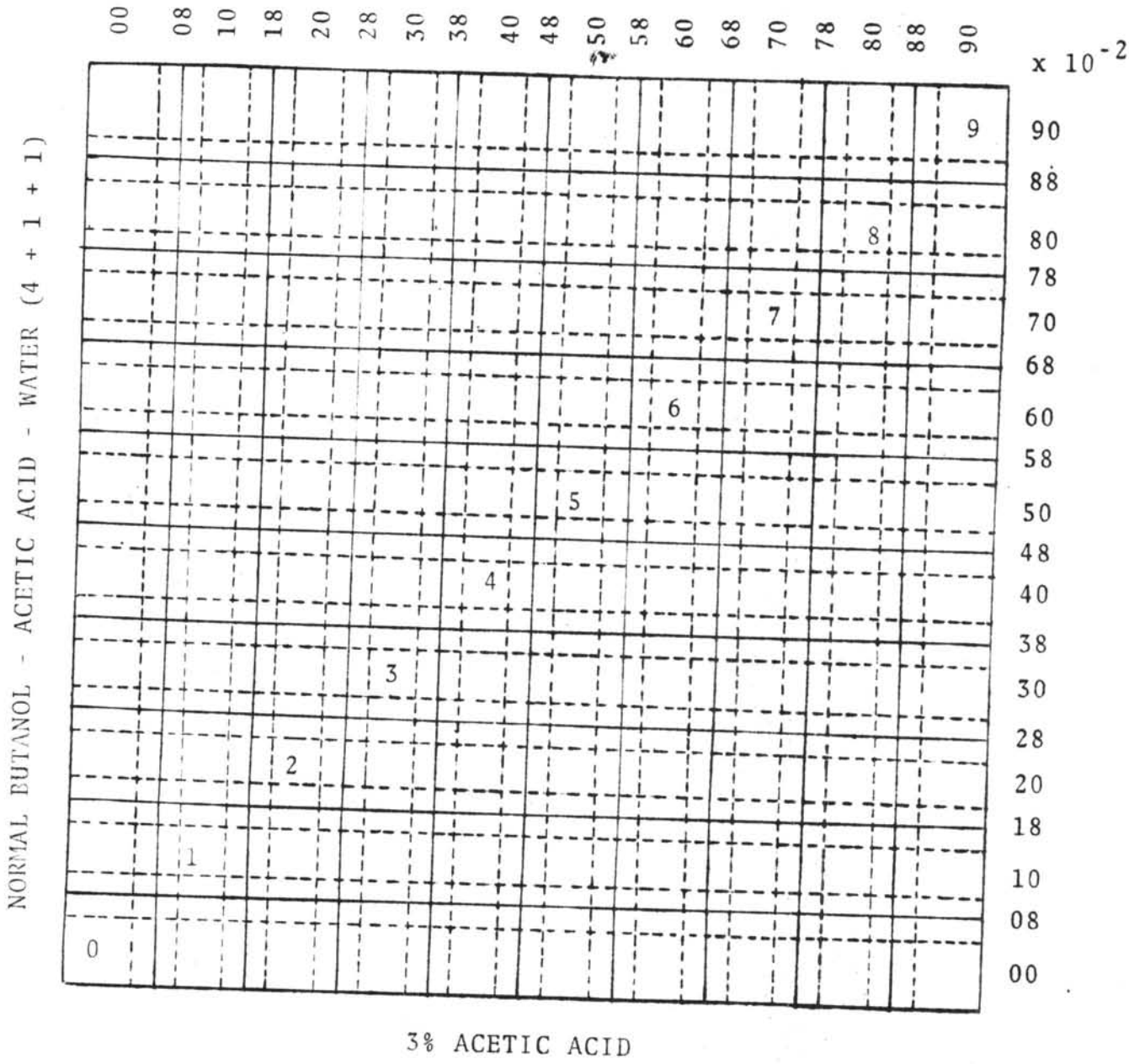


Fig. 9. R_f GRID

(Scale 1:2.66)

Coding of the Data

The chromatographic data were coded for making the table of basic plant data.

The following categories were considered for coding the name of plant species and characters of each chemical constituent.

1. Source (name of plant)

   Phylum or Division is under "PHY" 24 Anthophyta or Flowering Plant (Gray's, Manual of Botany, 1950).

   Class or Group is under "CL" 2 Dicotyledoneae (Gray's, Manual of Botany, 1950).

   Order is under "ORD" 32 Rosales.

   Family is under "FAM" 02 Papilionaceae

   Plant names are :

   242320201 Pueraria mirifica Airy Shaw et Suvatabandhu.

   242320202 Butea superba Roxb.

2. Treatment and/or Chromogenic Reagents

   The colours obtained with the various reagents were recorded in numerical system as described under "Colour Designation".

   The treatment and/or chromogenic reagents are under "TRT" (two columns) and they are numbered as follows :

Code number	Treatment, Chromogenic reagent
01	Colour in daylight
02	Fluorescence
03	Fluorescence by treatment with ammonia vapour
04	Ferric ammonium alum solution
05	Ferric ammonium alum solution and ammonia vapour
06	Tollen's reagent
07	Iodine-potassium iodide solution
08	Liebermann-Burchardt's reagent
09	O-phosphoric acid solution (15%).

Solvent systems

The first solvent system, normal Butanol - Acetic acid - Water (4 + 1 + 1), was run in the machine direction of the chromatogram. The second solvent system, 3% Acetic acid was run perpendicular to the first one.
Under X, the R_f values refer to the BAW solvent system.
Under Y, they refer to 3% Acetic acid solvent system.

R_f Values

By using the R_f grid, the R_f values were coded. In coding work, when informations become truely voluminous the

two digits of coding R_f values may easily be replaced by a one-digit system by shifting the 08, 18, ... etc. to the higher whole number, thus 1, 2, ... etc. respectively.

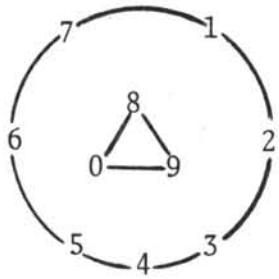
Colours of spots by treatment given (Coding of colours)[27]

Under "C" two columns, colours are arranged in a continuous disc system according to the Solar spectrum, colours No. 1 to 7 are used.

In the coding system used, the first digit stands for the basic colour itself, the second digit "0" (Zero) stands for light or pale shade, and "5" for darker. Thus :

10	pink	15	red
20	pale orange	25	orange
30	brown	35	dark brown
40	light yellow	45	yellow
50	green	55	dark green
60	blue	65	dark blue
70	heliotrope	75	violet
80	grey	85	black
90	quenching	95	strong quenching (under UV)
00	nil	-	

Colour Designation



It is difficult to indicate colour with precision (e.g. shade of pink and pale violet), but moderate errors will not invalidate the present system. It is possible to allow wide tolerance (with, of course, less precision) in the computor analysis by including preceding and following number so that moderate error in recording in colour will not discriminate against identifying spot.

Co-occurrence of constituents in the investigated samples based on occurrence of chemical constituents.

Any chemical in the investigated samples is identified by a coding system based on the decadic number system. Thus, there are 10 units in any one type of information used for its characterisation. This means that only 10 different R_f values are assignable to all compounds in any one solvent system, in two systems a possible total of 10^2. It would have been possible to use a system in which 100 different R_f values were assigned for one solvent system, but this would entail a doubling of the number of digits to be used and a doubling of the volume of data.

Using only 10 R_f values for any one direction, the number of places could be reduced by a factor of 10^2, from 10^4 to 10^2 locations over the whole chromatogram. The first digit in the coding system stands for R_f value of the second dimension of a given substance. In a two dimensional system more than one chemical (designed as spot) might sometimes be present in the same particular location. It is necessary in a two dimensional system, therefore, to differentiate unambiguously between compounds to use a number of chromogenic reagents, coded in this case by small letters.