CHAPTER II

EXPERIMENTAL

Materials and Techniques Used in Microscopic Investigation

Branches with full grown leaves of *Barleria lupulina* Lindl., *Barleria prionitis* Linn. and *Barleria strigosa* Willd. were collected from the garden of the Department of Pharmacognosy, Chulalongkorn University Faculty of Pharmaceutical Sciences, in July, November and December, 1979, respectively; and branches with full grown leaves of *Barleria cristata* Linn. were collected from Thonburi-rom Garden, Bangkok, in June, 1980. Flowers and fruits were also collected later. The authenticity of the mature plants were checked using the description given in standard taxonomic literatures, Flora of British India[13] and Flora of Java,[15] and also comparing with the herbariums preserved in the Botany Division of the Royal Forest Department, Bangkok.

Apparatus

Microscope (Olympus)

Drawing Apparatus (Carl Zeiss)

Microprojector Drawing Apparatus (RCA.)

Procedure

Examine and illustrate the external characters of the plants. For histological studies, free hand cut sections of fresh material are studied. The dried powder, sifted through a sieve no. 60, of the leaf of each plant is studied microscopically. For quantitative microscopy leaf fragments of certain regions are cleared by gently warming in concentrated solution of chloral hydrate and 30 readings are taken for each value. The data are analysed statistically and the standard deviation is given in parenthesis in each case.

Method of Palisade Ratio Determination

Pieces of leaf about 5 mm square are cleared by boiling with chloral hydrate solution, mounted and examined under the microscope. A drawing apparatus is arranged so that the epidermal cells and the palisade cells lying below them may be traced. First, four epidermal cells are traced and their outlines linked in to make them more conspicuous. The palisade cells lying beneath are then focused and traced. The palisade cells in each group are counted, those being included in the count which are more than half covered by the epidermal cells; the figure obtained divided by four gives the palisade ratio of that group.

Method of Stomatal Number and Stomatal Index Determination

Fragments of leaf other than extreme margin or midrib are cleared with chloral hygrate solution. The lower surface of the

clarified leaf strips are examined under the microscope. The epidermal cells together with stomata are traced with the aid of a drawing apparatus. The diameter of field of view is measured by means of a stage micrometer. Calculate the area in square millimetres.

Counts are made of the number of ordinary epidermal cells and of stomata. A trichome is also counted as an ordinary epidermal cell. The stoma, consisting of the two guard cells and osteole plus the adjacent subsidiary cells, is counted as one unit. Count the epidermal cells and stomatal apparatus in the field of view; incomplete part of the cells in one semicircle are inclusive, while those in the other are exclusive.

The average number of stomata per square millimetre, known as 'stomatal number', is calculated by dividing the number of stomata by the area of the field of view. The results should be recorded for each surface of the leaf and the ratio between the two surfaces.

Calculate the stomatal index by the formula of Salisbury.[42] If the leaf bears stomata on both surfaces, the stomatal index may be determined for each surface.

Method of Vein-Islet Number Determination

Pieces of mature leaf blade approximately 5 mm square are cut from the central part of the lamina, midway between the margin and midrib, cleared by gently warming in concentrated solution of chloral hydrate.

A microprojector drawing apparatus is set up and by means of a stage micrometer an enlarged square corresponding to 2 mm x 2 mm is drawn on the paper. The stage micrometer is then replaced by the cleared preparation and the veins are traced within this 4 mm square area.

When counting, it is convenient to number each vein-islet on the tracing. Each numbered area must be completely enclosed by veins, and those which are incomplete are included if cut by a two adjacent sides but excluded if cut by the other two sides. The total number of vein-islets divided by 4 gives the average number of vein-islets per square millimetre, or 'vein-islet number'.

Method of Veinlet Termination Number Determination

Count the number of free vein-tips, or veinlet terminations, in 4 mm square of the above tracing of vein-islets. The total number divided by 4 gives the average number of veinlet terminations per square millimetre of leaf surface.

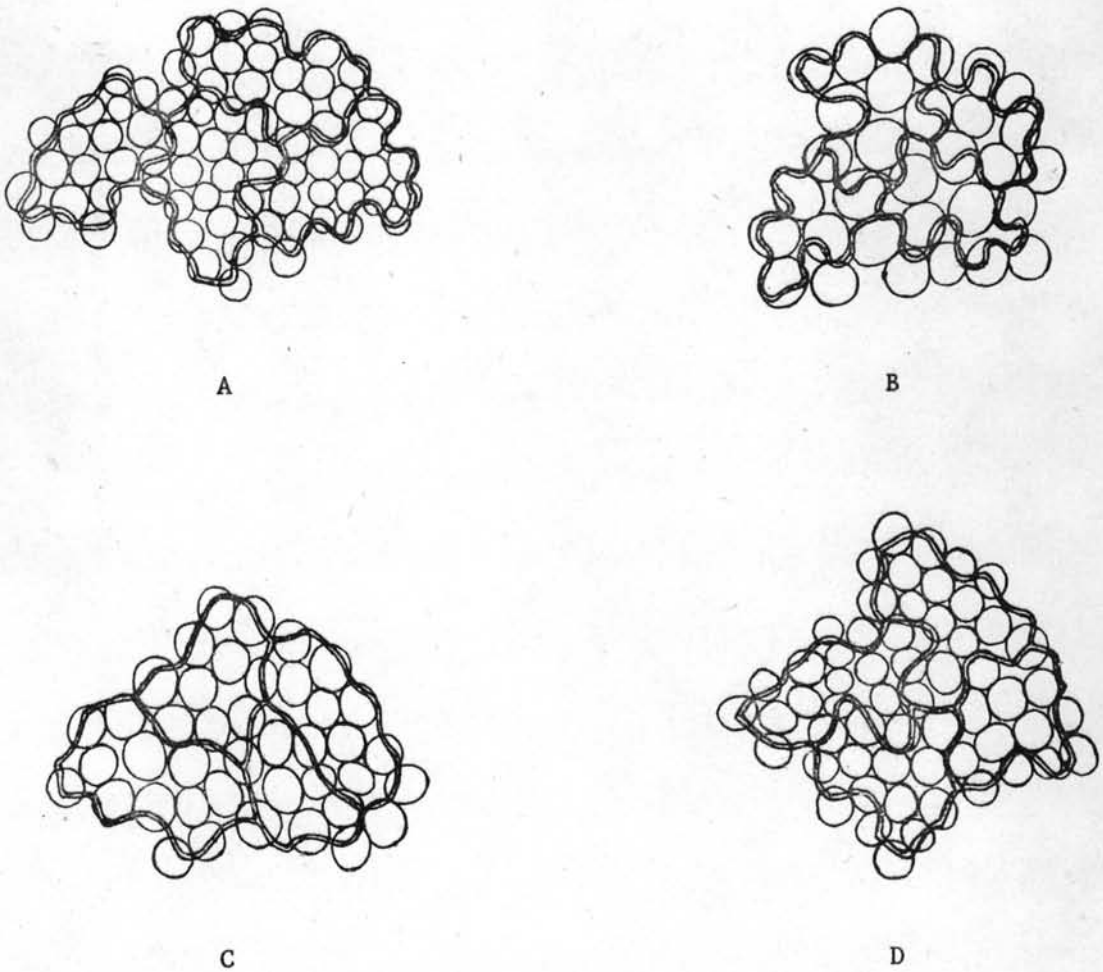


Fig. 1. Palisade Ratio. Four upper epidermal cells with the underlying palisade cells in surface view - x 400.
A, *Barleria cristata* Linn.; B, *Barleria lupulina* Lindl.;
C, *Barleria prionitis* Linn.; D, *Barleria strigosa* Willd.

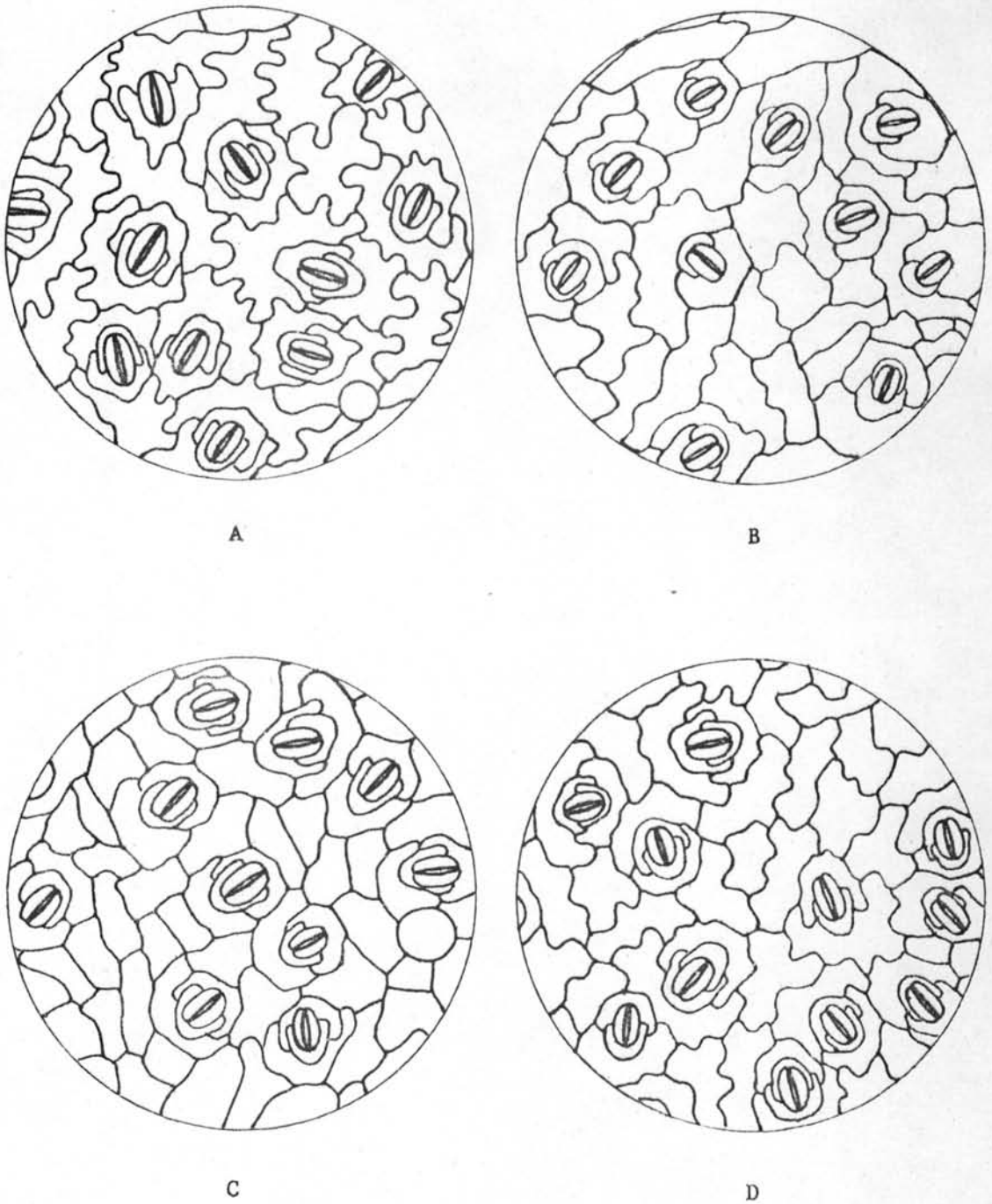


Fig. 2. Lower epidermis of leaves in surface view - x 375.
A, *Barleria cristata* Linn.; B, *Barleria lupulina* Lindl.;
C, *Barleria prionitis* Linn.; D, *Barleria strigosa* Willd.

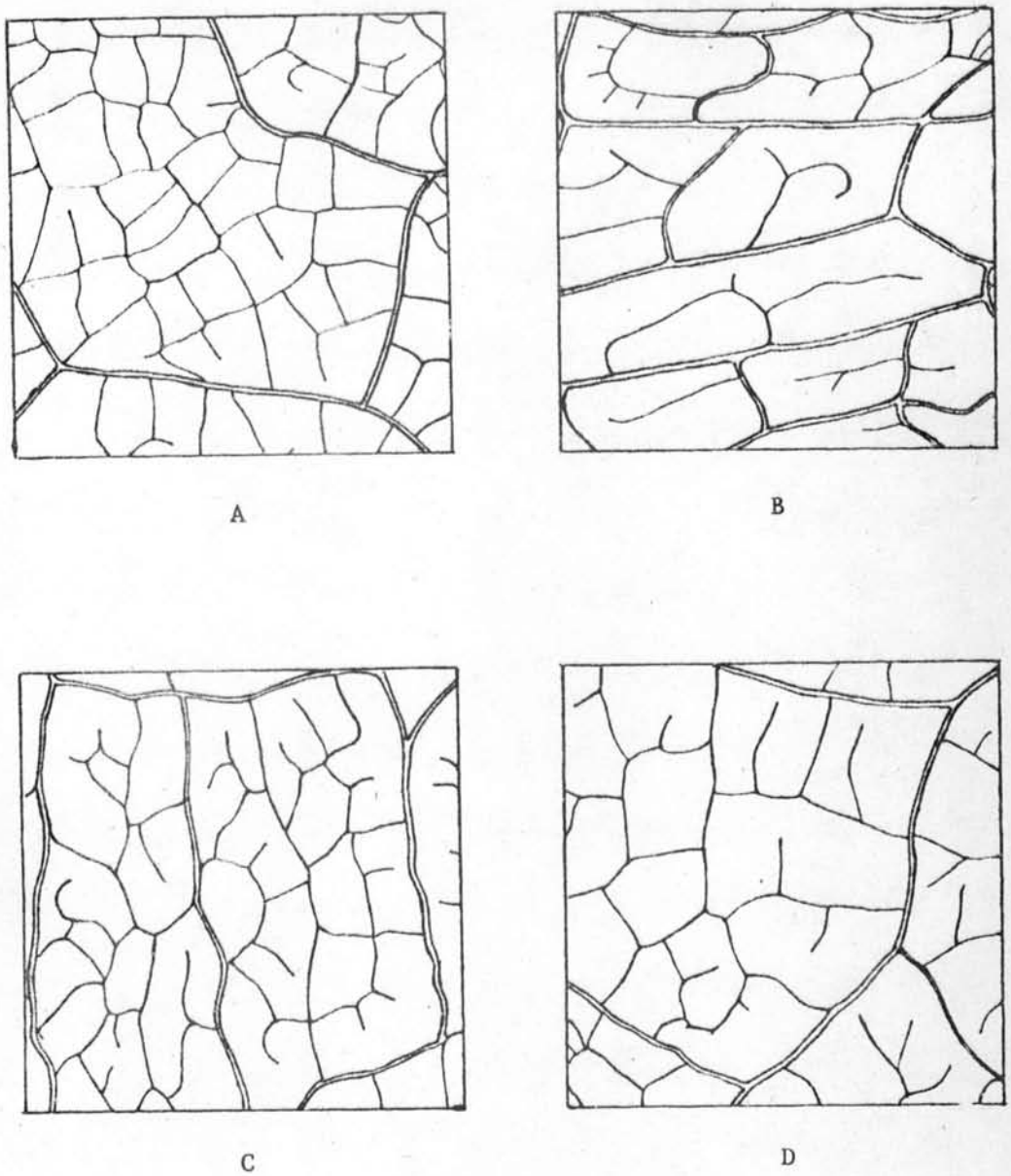


Fig. 3. Vein-islets and veinlet terminations of leaves in surface view - x 30.
A, *Barleria cristata* Linn.; B, *Barleria lupulina* Lindl.;
C, *Barleria prionitis* Linn.; D, *Barleria strigosa* Willd.

Materials and Techniques Used in Determining Thin-Layer Chromatographic Patterns of Chemical Constituents

Leaves of *Barleria cristata* Linn., *Barleria lupulina* Lindl., *Barleria prionitis* Linn. and *Barleria strigosa* Willd. were collected and dried in the oven at 60° C.

An adsorbent "Kieselgel G" (Type 60) for thin-layer chromatography, from E. Merck, A.G., Darmstadt, was used to prepare thin layers of 250 micron thick on 20 x 20 cm glass plates.

Solvents

Acetic Acid, glacial	BDH Chemicals, England.
Butanol-(1)	E. Merck, A.G., Darmstadt.
Distilled Water	-
Ethanol	Government Pharmaceutical Organisation, Bangkok.
Methanol	Government Pharmaceutical Organisation, Bangkok.

Reagents

Anisaldehyde	E. Merck, A.G., Darmstadt.
Hydrochloric Acid, concentrated	Riedel, A.G., Germany.
Sulphuric Acid, concentrated	Searle Co., England.
Copper (-ic) Sulphate, A.R.	Riedel, A.G., Germany.
Ninhydrin	May & Baker Co., England.
o-Phosphoric Acid	E. Merck, A.G., Darmstadt.
Vanillin, crystal 100%	TH. Muhlethaler, Switzerland.

Apparatus

Chromatography Chambers: rectangular TLC glass tanks with lids, for 20 x 20 cm plates

Ultraviolet Lamp (Ultraviolet Products, California)

Hot Air Oven

Desiccator

Stoppered Erlenmeyer Flask, 250 ml

Glass Plates, 20 x 20 cm, of uniform thickness (4 mm); and two 5 x 20 cm end plates

Plastic Aligning Tray, for coating five 20 x 20 cm plates

Thin-Layer Spreader, with layer thickness regulator (Desaga Co., Heidelberg, Germany)

Drying Rack: light metal drying rack to take ten 20 x 20 cm plates

Spotting Template, of transparent plastic, with scale

Capillary Tubes, for applying samples

Spraying Apparatus

Hot Plate and Hot Air Blower

Method of Extraction

Ten grams of dried coarse powdered leaves of each plant was macerated separately in 200 ml of 70% ethanol overnight. Filter through Whatman filter paper No. 1. The filtrates were evaporated under vacuum at 60-70°C to nearly dryness. Pastes of the crude extracts obtained were then kept in small well-closed containers and stored in a refrigerator.

Method of Layer Preparation

Five 20 x 20 cm glass plates of equal thickness, which are carefully cleaned and completely free of grease, are arranged in a continuous surface on a plastic aligning tray having a raised edge on two sides to hold the plates in position during the coating operation. Both ends of the row of plates are completed with two 5 x 20 cm plates, the end plates. Wipe the arranged plates with cotton soaked with ethanol.

Thirty grams of Kieselgel G (Type 60) for thin-layer chromatography is evenly mixed with 60 ml distilled water by vigorous shaking in a stoppered 250 ml Erlenmeyer flask for 1 minute. Transfer the mixture immediately to the open spreader, on which the layer thickness of 250 micron has previously been set, placing on the end-plate at one end of the tray. Turn the lever of the spreader through 180° to let the slurry coming out and draw the spreader towards the other end of the tray. Remove the spreader and leave the coated plates in position for 30 minutes so that the slurry can "set" and their surface becomes completely mat. Activate the layers at 100 to 110°C for 30 minutes by placing the coated plates in vertical position in the preheated hot air oven. Since active plates become deactivated in moist air, they are then stored over a desiccant in a desiccator until use.

Solvent Systems for Two-Dimensional Thin-Layer Chromatography

In the present work three thin-layer patterns of chemical constituents were illustrated for each of the plants. Three different

couples of solvent systems, as listed in Table 1, were used for developing the chromatograms.

For better separations of various components of the crude extracts, process of double development in the same solvent system was applied to the development in dimension 1. Fresh solvent was used in each development in order to improve the reproducibility of R_f values.

Table 1. Developing Systems for Two-Dimensional Thin-Layer Chromatography

Pattern	Dimension	Solvent Systems
A	1	n-Butanol-glacial acetic acid-water (4:1:1)
	2	3% Acetic acid in water
B	1	Chloroform-methanol (8:2)
	2	n-Butanol-ethanol-water (40:11:19)
C	1	Chloroform-methanol (6:4)
	2	n-Butanol saturated with water

Method of Chamber Saturation

Care should be taken to saturate the chromatographic chamber with solvent vapour as completely as possible before use since it strongly influences R_f values.[57,66]

In order to accomplish uniform saturation, the solvent system (100 ml) was introduced into the chamber, of which the walls were lined

as completely as possible with filter paper, 30 minutes before the introduction of the chromatogram. The vessel was then swirled round so that the filter paper was soaked with solvent.

Method of Sample Application

An amount of sample to be applied is chosen which is large enough to be effectively visualized to show trace substances, and small enough to give discrete spots with a minimum of tailing.

It should be noted that the lower limits of detection are higher for two-dimensional chromatograms, since diffusion effects cause greater dilution of the substance in the longer development time of the two-dimensional method.[66] Thus, higher concentrations of sample solution are required for the preparation of two-dimensional chromatograms.

The extracts (0.2 g) were dissolved in 70% ethanol (1 ml). Spotting was made by means of a filled capillary tube (1 mm in diameter) onto the starting point at a corner of the plate, 3 cm from the edges. Two to three applications with solvent evaporation after each were required. The spot was kept small (not exceeding 0.5 cm in diameter) by using an air blower to produce warm current air for rapid evaporation of the solvent. The solvent was removed as completely as possible before commencing chromatography.

(Concentration of the extract solution to be applied was about 0.2 mg/µl; and the volume of the extract solution applied onto each spot was about 2.85-3.00 µl.)

Method of Development of the Chromatograms

When the sample solution had already been applied to the starting point and allowed to dry, the plate was placed in a vertical position in a closed, saturated chromatography chamber such that the bottom of the layer dipped into the developing solvent. The chromatogram was then developed. The distance for development was 15 cm, marking by the finishing line which was drawn beforehand by means of a spotting template and a sharp object. Ambient temperature was about 25°C.

First dimension: The spotted TLC plate was developed firstly in direction 1 with solvent 1. After the solvent ascended 15 cm, the plate was removed from the tank and allowed to dry in the air. If solvent 1 was n-Butanol-acetic acid-water (4:1:1) the plate was allowed to dry overnight. If solvent 1 was either Chloroform-methanol (8:2) or Chloroform-methanol (6:4), only 2 hours were required for the drying. Complete removal of the solvent from the plate was then obtained.

Re-developed the air-dried plate in the same solvent system, using fresh solvent. The plate was allowed to dry in the air overnight before commencing the development in the second direction.

Second dimension: The plate from the first dimension development was now placed in a second solvent and developed in the direction perpendicular to the first dimension. After the solvent ascended 15 cm, the plate was removed and allowed to dry in the air overnight.

It is essential to be noted that in order to improve the reproducibility of R_f values the plate must be removed from the tank

as soon as the solvent reaches the finishing line. If several plates are being chromatographed simultaneously in a single container, all the plates have to be removed at the same time.

The process of double development in the first dimension provided considerable advantages in better separations of various components of the sample such that discrete spots with a minimum of tailing were obtained.

Methods of Detection

Special methods were used to detect compounds which could not be directly distinguishable by their own colours. Many compounds became visible when the chromatogram was viewed in short and long wave ultraviolet light. Some of them had to be visualized by spraying with special detection reagents.

Treatments and detection reagents applied were as follows:

1. Colours in Daylight.

   In order to provide a uniform light intensity over the whole chromatograms, a 20 Watts (Daylight) Fluorescence lamp was used.

2. Fluorescence.

   The chromatograms were examined in 365 nm ultraviolet light.

3. Fluorescence after Treatment with Ammonia.

   After observation in ultraviolet light, the chromatogram was exposed to ammonia vapour for 20 minutes, then inspected again in

ultraviolet light of the same wavelength as above. New visualized spots and/or the changes in colours due to the effect of ammonia were recorded.

4. Sulphuric Acid Reagent: for general detection.

Reagent: Equal volumes of concentrated sulphuric acid and glacial acetic acid.

Procedure: The chromatogram was sprayed with the reagent and allowed to dry in the air for 15 minutes, then heated by means of a hot air blower until the colour developed to its maximum.

Note: Sterols will turn red, then purplish-red, or red-violet. Most compounds may be subsequently charred, yielding brown spots.

5. Fluorescence due to Sulphuric Acid.

After treatment with the above sulphuric acid reagent, the the chromatogram was examined in 365 nm ultraviolet light.

6. Reagent for Iridoids.

Reagent: Glacial acetic acid (10 ml) was mixed with 2% copper sulphate solution (1 ml) and concentrated hydrochloric acid (0.5 ml).

Procedure: The chromatogram was sprayed with the reagent, then heated for 0.5-1 minute by means of a hot air blower. Iridoid compounds yield blue spots.

7. Ninhydrin Reagent: for aminoacids, amines and aminosugars.

   Reagent: Ninhydrin (0.2 g) was dissolved in ethanol (100 ml).

   Procedure: After spraying with the reagent, the chromatogram was heated at 110°C until the best colour development was reached. Violet, purple or pink spots were obtained.

8. Vanillin-phosphoric Acid Reagent: for sterols.

   Reagent: Vanillin (1 g) was dissolved in 50% aqueous solution of phosphoric acid (100 ml).

   Procedure: The chromatogram was heated to 120°C for 10-20 minutes after spraying with the reagent. Sterols will turn to purplish-red spots.

9. Modified Anisaldehyde-Sulphuric Acid Reagent: for the location of sugars.

   Reagent: Concentrated sulphuric acid (0.5 ml) was added to a solution of anisaldehyde (0.5 ml) in 95% ethanol (9 ml) and glacial acetic acid (0.1 ml). Freshly prepared before use.

   Procedure: After spraying with this solution, the chromatogram was heated to 90-100°C for 5-10 minutes. Sugars which turn grey after the treatment were detected.

Recording of R_f Values and Colours

The locations and colours of the spots were recorded after each treatment. R_f values were determined from the mean of a series of independent observations undertaken on three chromatograms of the same

solvent system development.

$$R_f = \frac{\text{distance of spot moving from start point}}{\text{distance of solvent front from start point}}$$

Concomitant 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. Samples of the chromatograms are given. An R_f grid was used for convenience 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
38	"	.375	to .425
40	"	.425	to .475
48	"	.475	to .525
50	"	.525	to .575
58	"	.575	to .625
60	"	.625	to .675
68	"	.675	to .725
70	"	.725	to .775
78	"	.775	to .825
80	"	.825	to .875
88	"	.875	to .925
90	"	.925	to .975
98	"	.975	to 1.000

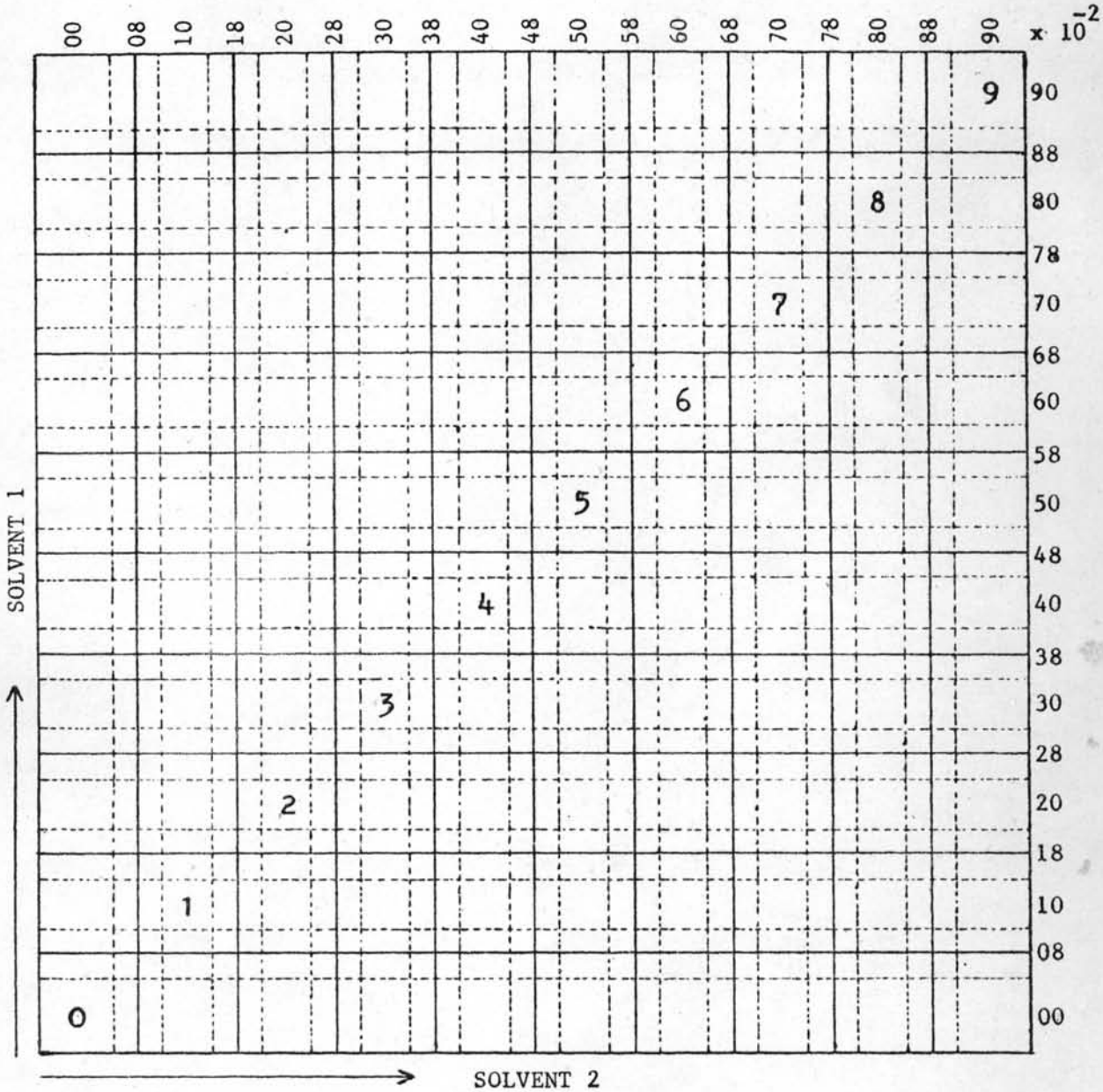


Fig. 4. R_f GRID

(Scale 1:1)

Coding of the Data

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

The following categories were considered for coding of each spot: 1. Source (plant identification, or name of plant), 2. Treatments and/or detection reagents, 3. Solvent systems, 4. R_f values, and 5. Colours of spot by treatment given (coding of colours).

1. Source (Plant identification) (11 columns).

Phylum or Division	is under "PHY"	24	Anthophyta or Flowering Plants(80)
Class or Group	" " "CL"	2	Dicotyledoneae(80)
Order	" " "ORD"	32	Tubiflorae(80)
Family	" " "FAM"	03	Acanthaceae
Genus	" " "GEN"	01	*Barleria* (listed in sequence of the investigation)
Species	" " "SP"	01	*B. cristata* Linn.
		02	*B. lupulina* Lindl.
		03	*B. prionitis* Linn.
		04	*B. strigosa* Willd. (listed in sequence of the investigation)

2. Treatments and/or Detection Reagents.

The treatments and/or detection reagents are under "TRT" (2 columns) and they are numbered as follows:

Code Number	Treatment, Detection Reagent
01	Colours in daylight
02	Fluorescence
03	Fluorexcence after treatment with ammonia
04	Sulphuric acid reagent
05	Fluorescence due to sulphuric acid
06	Reagent for iridoids
07	Ninhydrin reagent
08	Vanillin-phosphoric acid reagent
09	Modified anisaldehyde-sulphuric acid reagent

3. Solvent Systems.

Pattern A:	R_f values	in direction	1	is under	"A_1"
	"	"	2	"	"A_2"
Pattern B:	"	"	1	"	"B_1"
	"	"	2	"	"B_2"
Patern C:	"	"	1	"	"C_1"
	"	"	2	"	"C_2"

A_1	refers to	n-Butanol-glacial acetic acid-water (4:1:1)
A_2	"	3% Acetic acid in water
B_1	"	Chloroform-methanol (8:2)
B_2	"	n-Butanol-ethanol-water (40:11:19)
C_1	"	Chloroform-methanol (6:4)
C_2	"	n-Butanol saturated with water

4. R_f Values.

By using the R_f grid, the R_f values were coded. In coding work, when informations become truly voluminous the two digits of coding R_f values may easily be replaced by a one-digit system by shifting the 08, 18, 38, etc. to the higher whole number, thus 1, 2, 3, 4, etc.

5. Colours of Spot by Treatment Given (Coding of Colours).

The colours obtained with the various reagents were recorded in numerical system under "C" (2 columns). They are arranged in a continous disc system according to the natural solar spectrum colours. No. 1 to 7 are used.(81)

In the coding system used, the first digit stands for the basic colour itself, the second digit 0 stands for light or pale shade, and 5 for darker. For combination of colours, two first digits of the basic colours were used to indicate the various shades of colours. Thus:

10	pink	13	purplish-red	15	red
20	pale orange	21	pinkish-orange	25	orange
		24	yellowish-orange	28	reddish-brown
30	brown			35	dark brown
				38	yellowish-brown
40	pale yellow			45	yellow
50	pale green	53	brownish-green	55	green
				56	bluish-green
				58	greyish-green
60	prussian-blue			65	blue
70	purple			75	violet
				78	pinkish-grey

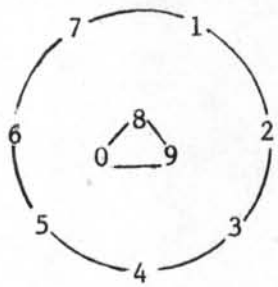
80 grey

90 quenching

00 nil

85 black

95 strong quenching (under UV)



Colour Designation

It is difficult to indicate colour with precision (e.g. shade of pink and pale purple), 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 of colour will not discriminate against identifying spot.