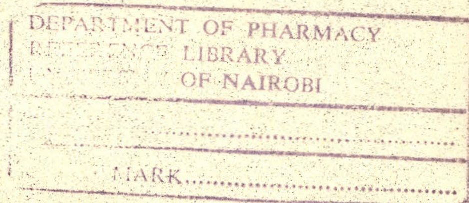
PHARMACOGNOSTICAL INVESTIGATION
OF *Cassia didymobotrya* Fres.
OCCURRING NATURALLY IN KENYA



by

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DEDICATION

This Research Project is dedicated to my mother and my father who are my ever unfailing source of inspiration.

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CONTENTS

PAGE

1. Abstract 1
2. Introduction 2
3. Experimental work 6
   - 3.1 Collection and Preparation of plant material 6
   - 3.2 Macroscopic and Microscopic Examination of plant material 6
   - 3.3 Qualitative tests for Anthraquinones 7
   - 3.4 Thin Layer Chromatographic Examination of plant material 7
   - 3.5 Determination to total anthraquinone Content 9
4. Results 11
5. Discussion 13
6. Conclusion 15
7. Figures and tables 16
8. References 27

1. ABSTRACT

The aim of this work was to determine the total anthraquinone glycosides present in the leaves of the naturally occurring *Cassia didymobotrya* Fres., and to study the basic pharmacognostic features (microscopic and chromatographic). Senna, a drug from the same genus, was used as a basis for reference.

The results obtained indicate that the plant contains anthraquinone glycosides, accounting for the reported purgative action.

The total anthraquinone content calculated as sennoside B in the dried plant material (leaf) was found to be 2.1%.

Thin layer chromatography revealed that three of the anthraquinones present in *Cassia didymobotrya* Fres. did not occur in detectable quantities in prepared senna, and that the two main anthraquinones detected in senna,
(*Cassia angustifolia*, *Cassia aculifolia*), aloe-amodin and rhein, were also present in *Cassia didymobotrya* although in lower quantities.

The plant is a possible source of purgative drug.

2 INTRODUCTION

Cassia didymobotrya Fres. belongs to the order Rosales and the family Leguminosae. The family is the second largest among the flowering plants and contains 600 genera and over 13,000 species, including more important drugs than any other. It is divided into the subfamilies Papilionaceae, Mimosoidae and Caesalpiniodae, containing about 377, 40 and 133 genera respectively.
Cassia didymobotrya belongs to the subfamily caesalpiniodae (3).

The plant is a shrub which is usually 0.6 to 4.5 metres high with compound leaves mostly 10 to 30 cm long which contain 8 to 18 pairs of oblong elliptic leaflets 2 to 6.5 cm long, 0.6 to 2.5 cm wide mostly rounded to obtuse at apex.
The petals are bright yellow 1.8 to 2.7 cm long, 1 to 1.6 cm wide.
There are 10 stamens with straight or nearly straight filaments, two large anthers, five medium ones and three small ones. The pods are oblong and flattened, about 8 to 12 cm long and 1.5 to 2.5 cm wide, dehiscent and not winged. The seeds are compressed in the same plane as pod, oblong, apiculate near the hilum, 8 to 9 mm. long, 4 to 5 mm wide, and 2.5 mm thick (16).
The plant usually occurs by lake shores, rivers and other damp places in upland rainforest, grassland, and woodland. It is also common in old plantations and in hedges near buildings. It is widely distributed throughout Eastern, Central and Southern Africa (16).
In Kenya it has been found growing naturally in Central, Eastern, Nairobi, Nyanza, Rift Valley and Western Provinces (12).

The plant is reported to be poisonous.
The poisonous nature of the leaves of this plant has been mentioned by KOKWARO J.O. (1976) (2) and by WALKER J. (1929) (4). Kokwaro indicates that an overdose in the use of leaf decoction could be fatal, while Walker states that the foliage is poisonous to cattle.
METTAM (1) demonstrated that doses of 72 to 185 g (10 to 30 fresh leaves) of the plant are lethal to sheep causing intense inflammation of the intestinal canal (gastroenteritis).
The use of the leaf as fish poison (15) has been reported by BRENAM J.P.M and GREENWAY P.J (1949) (7). The rabbit reacts to the material by softish stools after ingestion of a couple of leaves and by watery diarrhoea after ingestion of a greater quantity (1). WATT (1) has referred to the plant as wild senna. Observations that stock do not browse the plant have been made locally (15) and also in Queensland (1).

In spite of its reported poisonous effects, the plant has been put into medicinal use for various purposes among some indigenous communities in Kenya. The Luhya have used the burnt leaf ashes to treat skin discoloration topically (2). The Maasai use this "ol senetoi" as a purgative (about a dozen leaves to a dose) and as an antimalarial (1). A Kisii medicinewoman (15) claimed that burnt leaf ashes of "omobeno" could resolve enlargement of the spleen in children if the ashes were drunk with water daily for two weeks. Several other claims regarding the medicinal value of cassia didymobotrya have been made by the Luo and the Luhya:-

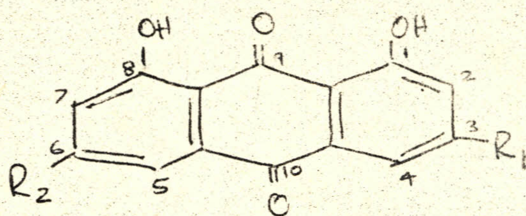
> The leaves are cooked and the decoction drunk in cases of gonorrhoea and for backache in women. A similar decoction can also be given to weaned childred in their food to act as an appetizer. The bark and roots are soaked in water and the resulting solution drunk as a purgative and emetic. Leaves are boiled with water and the decoction drunk as a cure of stomach troubles. Doses vary from two to five glasses (2).

Large amounts of decoction have been reported to cause miscarriage in women (2).
The leaf and root are reported to contain anthraquinones, thus accounting for the purgative effects (1). The bark contains tannin and is used by the African in Kenya for tanning (13). A trisaccharide, raffinose and choline have been isolated from the seed. A small amount of an alkaloid casseine $C_{33}H_{47}O_{14}N_3$ has been isolated from the pod (1). GITHENS (1949) (10) suggests that the plant is of probable value as a purgative drug.

Anthraquinones occur in the monocotyledonous family Liliaceae and in several dicotyledonous families, namely, Rubiaceae, Leguminoseae, Polygonaceae, Rhamnaceae, Ericaceae, Euphorbiaceae, Lythraceae, Saxifragaceae, Scrophulariaceae, and Verbenaceae.

The derivatives of anthraquinone present in purgative drugs may be dihydroxy-phenols such as chrysophanol, trihydroxyphenols such as emodin or tetrahydroxyphenols, carminic acid. Other groups also occur, such as methyl ($-CH_3$) chrysophanol, hydroxymethyl ($-CH_2OH$) in aloe-emodin, and carboxyl ($-COOH$) in rhein and carminic acid (3).

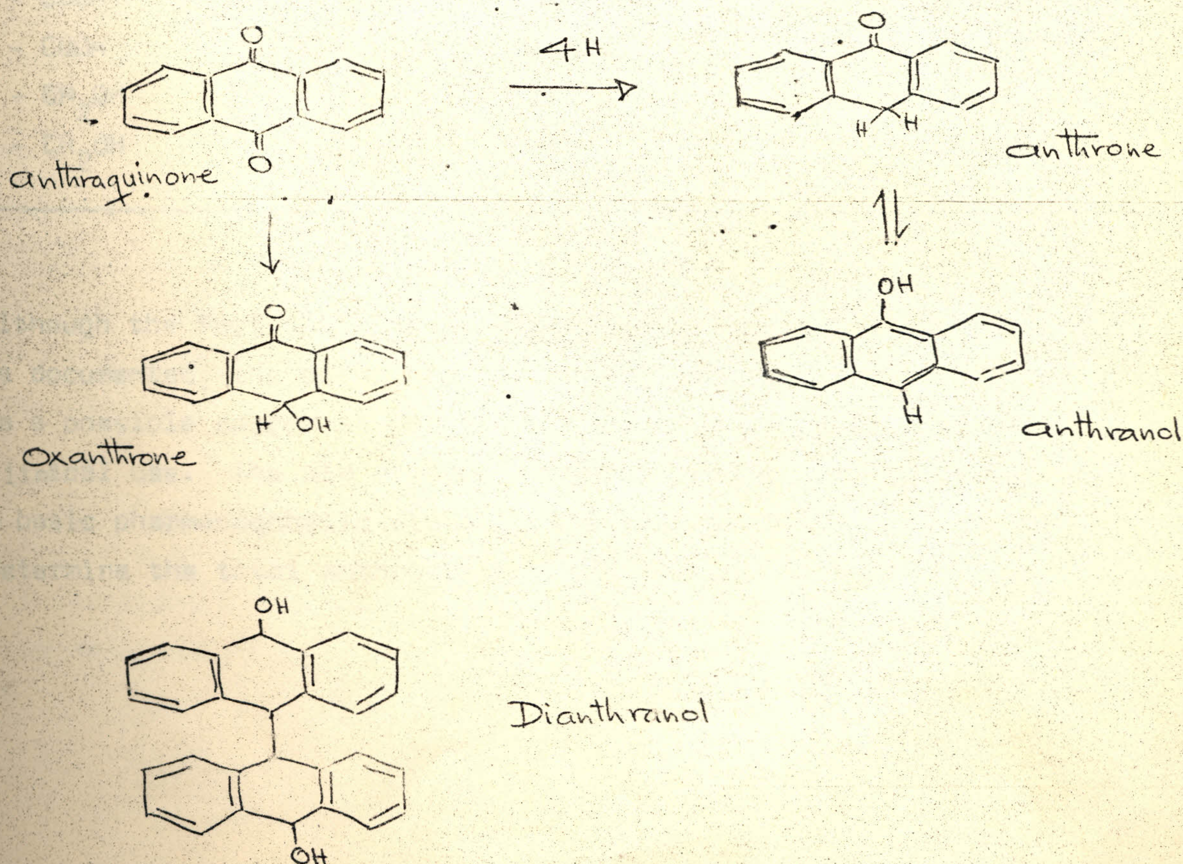
The anthraquinone glycosides which occur in cassia species include glucorhein and gluco aloe-emodin. The structures of some of the aglycones found in cassia species are given below:-



R_1	R_2	Aglycone
- CH_2OH	- H	Aloe-emodin
- COOH	- H	Rhein
- CH_3	- H	Chrysophanol
- CH_3	- OCH_3	Physcion

The glucose moiety is attached via the hydroxyl group at position 1 or 8.

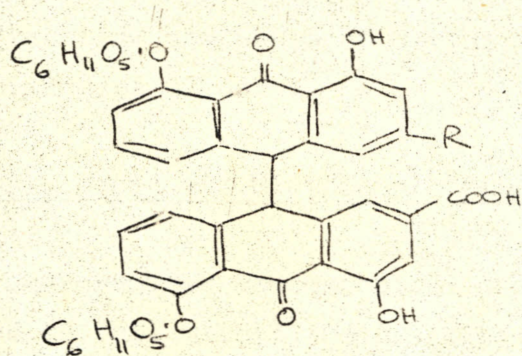
The aglycones may occur in various oxidation forms:
as anthraquinone, anthrone, anthranol, oxanthrone or dianthranol:-



The two anthrone molecules which form the dianthrone may be identical

or different. Dianthrones are important aglycones in the species of Cassia, Rheum and Rhamnus; the sennosides (structures of which are given below) are the best examples. Rheidin A, B and C which occur in senna and rhubarb are heterodianthrones and involve aloe-emodin, rhein, chrysophanol and physcion (3).

The sennosides have the following structures:-



R	10-10'	Sennoside
- COOH	trans	Sennoside A
- COOH	meso	sennoside B
- CH_2OH	trans	sennoside C
- CH_2OH	meso	sennoside D

Although the fact that Cassia didymobotrya contains anthraquinones is documented, the plant has not been given adequate consideration as a possible source of purgative drug that could be put into clinical use. The aim of this research project was to carry out a basic pharmacognostic study of the leaf of this plant and to determine the total anthraquinone content in the leaf.

3. EXPERIMENTAL WORK

3.1 Collection of Plant Material

The initial plant material for identification was collected in Riosiri area of South Mugirango Chache Location in Kisii district, in October 1984, and identified at the East African Herbarium, Nairobi. The rest of the plant material used for the experiments was obtained from the banks of Nairobi river next to the University of Nairobi Sports grounds.

3.2 Macroscopic and Microscopic Examination of Plant Material

The leaf was examined macroscopically and the structural featured were found to fall within the description given earlier (page 2). A diagramatic presentation of the macroscopic features of the leaf and leaflet is given in figures 1 and 2.

A transverse section of a fresh leaflet was made on a microscopic slide, cleared with chloral hydrate solution and observed under low (X100) and high (X400) powers of the light microscope. Fig 3 gives a diagramatic view of the features noted in the transverse section and described (See Results P. 11).

To test for lignification, the Phloroglucinol Test was done on a cleared transverse section on a microscopic slide. Two drops of phloroglucinol reagent were added and the preparation dried in air. Concentrated hydrochloric acid was added, cover-slip put in place and the preparation observed and low and high power of the light microscope.

A section of the lower epidermis of the leaflet was peeled off, cleared with chloral hydrate and observed under low and high power of the light microscope. The stomatal and epidermal cell arrangement was observed (Fig. 4).

3.3 Qualitative Tests for Anthraquinones

The plant material was divided into three parts. The first portion was used for the tests when fresh, the second portion was dried in an oven at 50°C for 24 hours, the third portion being dried at room temperature for one month. The dried leaves were powdered and stored in a well corked sample container in a dark locker until used.

a) The potassium hydroxide test

Some powdered plant material was placed on a clean white tile and two drops of alcoholic solution of potassium hydroxide added. The colour was observed for anthraquinone reaction.

b) Borntrager's Test

About 1g. of plant material (crushed if fresh, and pardered if dry) was boiled in about 10 mls of 2M hydrochloric acid for five minutes and filtered through Whatman paper No. 1. The filtrate was cooled to room temperature and extracted with an equal volume of carbontetrachloride by gentle swirling to avoid the formation of emulsion. The carbon tetrachloride was then shaken with half its volume of dilute ammonia solution. The color change in ammonia layer was observed (11).

c) Modified Borntrager's Test

The Borntrager's test was repeated with the inclusion of 5mls of 5% ferric chloride in the boiling mixture.

3.4 Thin Layer Chromatographic (TLC) Separation of the Plant Material Extract.

Anthraquinone derivatives are usually soluble in hot water or dilute alcohol (3).

The method of extraction used was basically the same as that used in the Borntrager's test, but concentration of glycosides by evaporation of solvent was found to be necessary before spotting in order to ease visualisation.

The method described in the Pharmacognosy laboratory manual for Chelsea College (University of London) (9) was found to be suitable for separation of the anthraquinone derivatives.

2M hydrochloric acid was used for hydrolysis. The extract was obtained in two ways

The first method was carried out as follows:
3g. of powdered plant material was boiled in 30 mls of dilute hydrochloric acid for five minutes to hydrolyse the glycosides into sugar and aglycone, and then centrifuged while hot (see discussion note 1). The supernatant was filtered off, cooked and shaken with an equal volume of carbon tetrachloride (discussion note 2) in separatory funnel, the carbon tetrachloride layer retained and concentrated to 2mls using the rotary vacuum evaporator.
This was used for spotting.

In the second method, 5 mls of five percent ferric chloride solution was included in the boiling mixture during the extraction process described in the first method.

Powdered senna tablets were treated in a similar manner as plant material.

The TLC Process

Two methods were found to work well in the TLC process; that described by HARBONE J.B. (5) and the one described in the Chelsea College Pharmacognosy manual (9).

Using the method described by Harbone, the slurry was prepared by using silica gel GF 254 m 0.01M Sodium hydroxide solution in the ratio 1:2. The mobile solvent system used was a mixture of benzene, ethylacetate and acetic acid in the rations 75:24:1. After running the plates, detection was achieved by using ten percent methanolic potassium hydroxide solution as a spray reagent.

In the Chelsea College method, the slurry was prepared using silica gel GF 254 in water (1:2). Two mobile solvent systems were used:-
Solvent System 1; ethylacetate: n-propanol: water (40:40:30)
(Fig. 6)

Solvent system 2: ethylacetate: methanol: water (77:13:10)
(Fig. 7).

The examination of the TLC plates was done in ultraviolet light before spraying and in daylight after spraying. The spraying reagent used was twenty five percent nitric acid, followed by heating at 120°C for ten minutes, cooling and then spraying with five percent potassium hydroxide solution in fifty percent methanol.

Certain aspects of the TLC process were similar in both methods adopted.

A size of 20 cm by 20 cm plates were used. The layers were prepared using the "Desaga" spreader with a 0.250 mm applicator to give a layer thickness of 250 mm. The development was done in a 21 cm by 21 cm by 9 cm chromatographic tank.

The technique used was one-way ascending (OWA). Prior to development the chamber was saturated and the plate pre-equilibrated with mobile phase. Sufficient time was allowed for the mobile solvent to run about 10 cm.

3.5 Determination of Total Anthraquinone Content

The total content of anthraquinones was calculated as sennoside B in the dry plant material. The method which was adopted is that described for senna tablets by the British Pharmacopoiea (1973) (8)

0.3g of powdered plant material was placed in around bottom flask, 30 ml water added, weighed and heated on a water bath under reflux for fifteen minutes, allowed to cool, weighed and adjusted to the original weight with water. This was transfered into a centriguge tube and contrifuged for two minutes. 20 ml of the supernatant liquid was transfered to a separator, and one drop of hydrochloric acid added. This was shaken with two quantities, each 15 ml, of chloroform, allowed to separate and the chloroform layer discarded. The aqueous layer was centrifuged and 10 ml of the liquid transfered to a round-bottom flask fitted with a ground-glass stopper.

The PH of the solution was adjusted to between seven and eight with a five per cent solution of sodium carbonate, 20 ml of a mixture of 8 ml ferric chloride solution and 12 ml water was added, mixed and heated on a water bath under reflux condenser for twenty minutes, then adding one millilitre of hydrochloric acid and continuing the heating for a further twenty minutes with frequent shaking until precipitate dissolved. The mixture was allowed to cool, transfered to a separator and extracted with three quantities, each of 25 ml, of solvent ether previously used to rinse the flask. The combined extract was washed with two quantities, each of 15 ml, of water and sufficient solvent ether added to produce 100 ml. 10 ml of this was evaporated just to dryness on a water bath and the residue dissolved in 10 ml of N potassium hydroxide, and then filtered through a sintered glass filter.

The extinction of the resulting solution was measured immediately without delay at a maximum of 500 nm. The total anthraquinone content was calculated as sennoside B taking 170 as the value of the extinction coefficient, E (1%, 1cm) at about 500 nm.

Senna tablets were treated similarly.

4. RESULTS

4.1 Macroscopic and microscopic Examination of the Leaflet

The macroscopic features of the leaf and leaflet are given in figures 1 and 2. The microscopic features of the leaflet transverse Section and lower epidermis are given in figures 3 and 4.

Regarding the macroscopic and microscopic features of the plant material, the following description has been drawn from the macroscopic and microscopic examination:-

Cassia didymobotiya Fres leaflet bears stout petiolules, entire margin and assymmetric base. The surfaces appear smooth but with some trichomes on either side. It has an unpleasant odour and a bitterish mucilagenous taste. The transverse section reveals a dorsiventral structure. The epidermal cells have straight walls, both surfaces bearing unicellular non-lignified smooth covering trichomes. The stomata are of paracytic type. The mesophyll, consisting of the palisade layer toward the lower epidermis, contains calcium oxalate cluster crystals. Below and above the midrib bundle are zones of lignified fibres and a sheath of parenchymatous cells containing prisms of calcium oxalate.

4.2 Qualitative Tests for Anthraquinones

All the tests for anthraquinones which were carried out gave positive results: potassium hydroxide tests gave red color, Borntrager's test red in the ammonia layer. There was no notable difference in color intensity between the Borntrager's test and the modifie Borntrager's test.

4.3 Thin Layer Chromatography

The chromatographic patterns obtained using various solvent systems are given in figures 5, 6 and 7, and the Rf values drawn therefrom are given in tables 1, 2 and 3 respectively.

4.4 Total Anthraquinone Content

The total anthraquinone content, calculated on dry plant material using the B.P. (1973) method, as for Senna, was found to be 2.1 per cent.

5. DISCUSSION

In this study, fresh plant material was found to be inappropriate for use in TLC analysis since there was much interference from the colored material and the gelly-like mass when crushed. Dried plant material was therefore used. No significant difference was seen between the results obtained from the materials dried by the two methods,- drying in an oven at fifty degrees celcius for twenty-four hours and drying ar room temperature for one month - , the less time-consuming oven-drying being preferred.

During the process of extraction of anthraquinones for TLC. the following facts were considered:-

Note 1: Anthracene aglycones are soluble in hot water but insoluble in cold water; hence the necessity of filtering the aqueous extract while hot.

Note 2: Aglycones dissolve preferentially in carbontetrachloride and are therefore easily extractible from cold water using this solvent.

Note 3: Glycosides are often easily hydrolysed to give products of hydrolysis, the aglycone and sugar moiety (3);

Note 4: Some plants contain anthracene aglycones in reduced form and if ferric chloride is used during the extraction, oxidation to anthraquinones takes place.

The TLC patterns obtained (figures 5, 6 and 7) and the Rf values calculated therefrom compare well with the values obtained by other authors. According to HARBORNE J.B. (5), when the mobile phase used is a mixture of benzene, ethyl acetate and acetic acid in the ratios 75:24:1 at room temperature (25°C) the Rf values obtained for the various anthraquinones are as given in table 4. This method (of Harborne) was found to be useful in the identification of the anthraquinones.

The TLC results obtained show *Cassia didymobotrya* to contain five anthraquinones (Fig. 5) two of which were identified as *aloe-amodin* (spot 3) and *rhein* (spot 2) using senna - known to contain aloe-emodin and rhein - as a basis for reference.

6. CONCLUSION

The anthraquinone content of naturally occurring and locally available *Cassia didymobotrya* Fres. has been determined and found to be sufficiently high to necesitate consideration as a purgative drug. The investigation of the reported poisonous nature was, however, beyond the scope of this project. This plant is a possible source of purgative drug, its chemical, pharmacological and toxicological evaluation being necessary before use.

7. FIGURES AND TABLES

7.1 Figures

Fig No.

1: Macroscopical features of Cassia didymobotrya leaf.

2: Macroscopical features of Cassia didymobot.rya leaflet.

3: Transverse Section through Cassia didymobot.rya leaflet.

4: Microscopical Featured of Lower Epidermis Cassia didymobot.rya leaflet.

5: TLC separation; system 1

6: TLC separation; system 2

7: TLC separation; system 3

7.2 Tables

Table No.

1: Rf values obtained from system 1

2: Rf values obtained from system 2

3: Rf values obtained from system 3

4: Rf values for some anthraquinones as given by Harborne J.B. (1973) (5).

It was not possible to identify the other three anthraquinones as reference standards were not available. By correlation with the Rf values given by HARBORNE (5) it was proposed that the unidentified anthraquinones were likely to be emodic acid (spot 1), emodin (spot 4) and chrysophanol or physcion (spot 5).

Due to the effects of some factors such as temperature of running the chromatogram, the Rf values obtained experimentally were not directly superposable with the literature values.

The methods in the Chelsea College pharmacognosy laboratory manual gave good separation but had no basis for identification of the spots.

The total anthraquinone content, determined using the B.P method for the assay of anthraquinones in senna, was found to be 2.1 per cent of the dried plant material, a value which compares well with that obtained for prepared senna (2.5 per cent) by the same method.

ig. 1: Macroscopical features of Cassia didymobotrya leaf

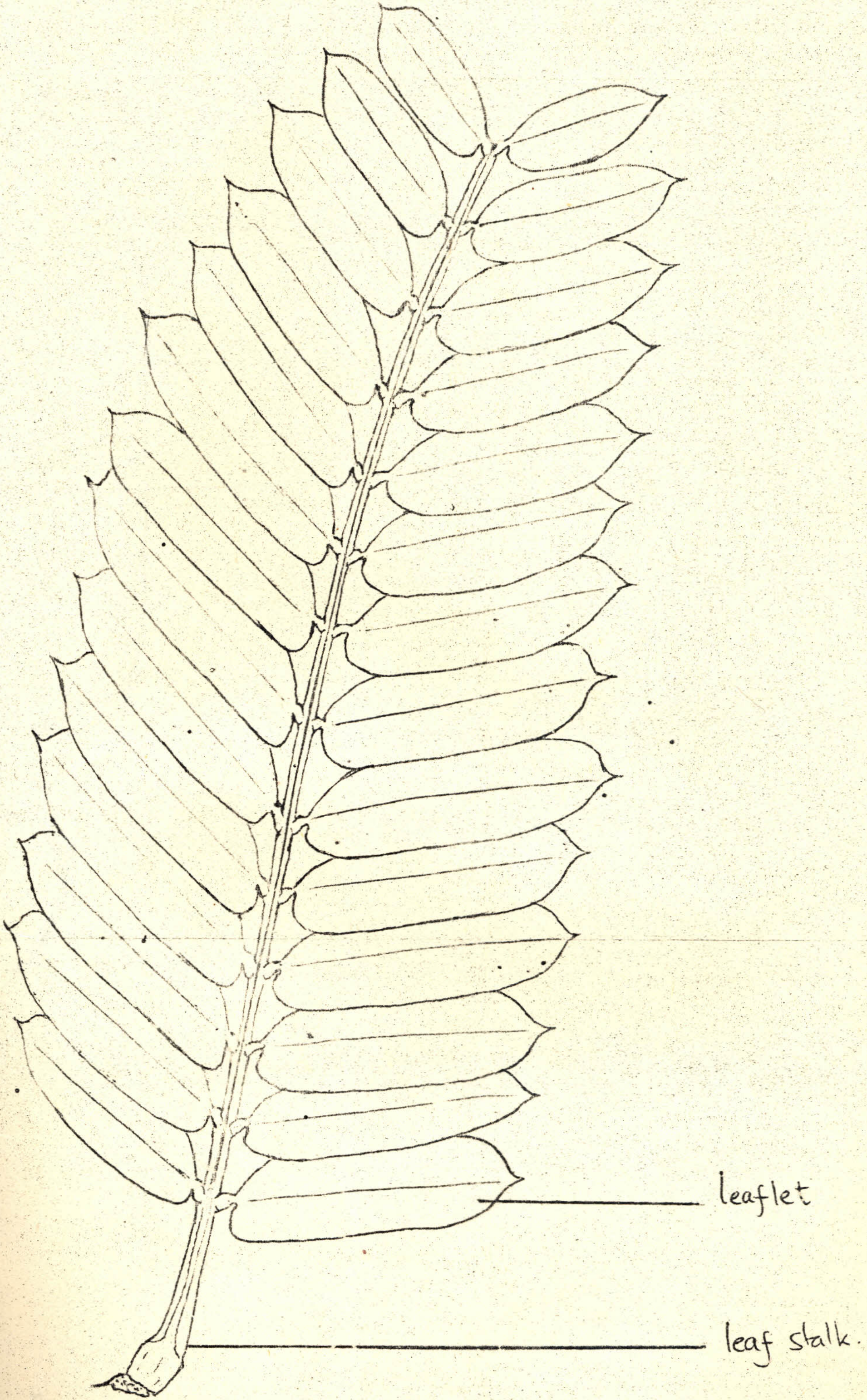


Fig 2: Macroscopical Features of *Cassia didymobotrya* Leaflet (X 2)

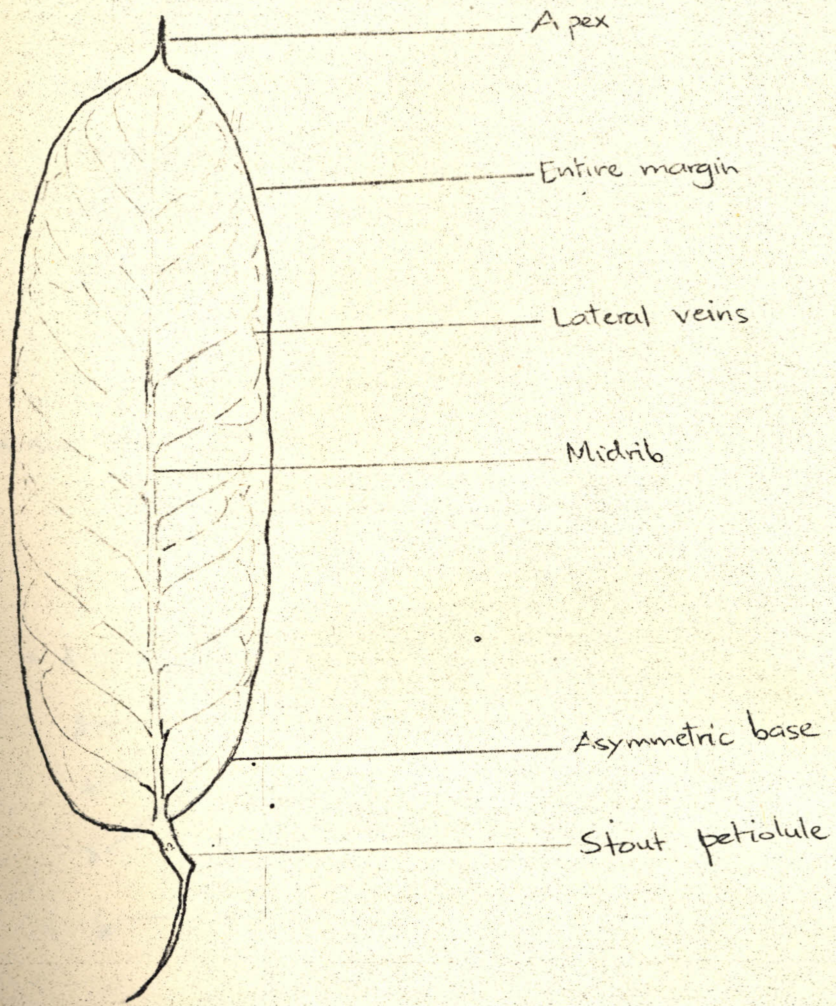
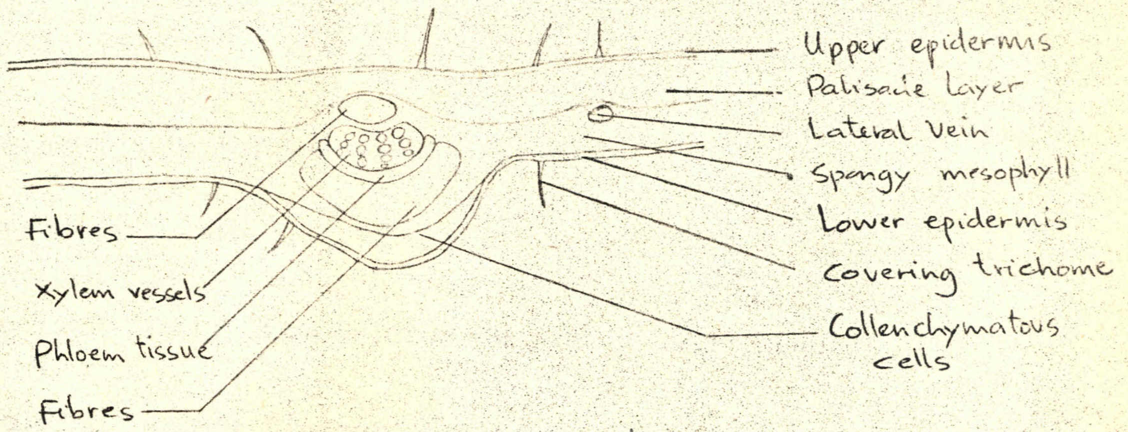


Fig 3: Transverse Section through *Cassia didymobotrya* Leaflet

x 100 (Not to scale)



x 400 (Not to scale)

1
2
3
4
5
6
7
8
6
5
11
12
9
10

Key to Fig. 3

1. Smooth unicellular covering trichome

2. Upper epidermis

3. Palisade layer with calcium oxalate cluster crystals (4)

4. Calcium oxalate Cluster crystals

5. Parenchymatous cells with calcium oxalate prisms

6. Lignified fibres

7. Lignified xylem vessels

8. Phloem tissue

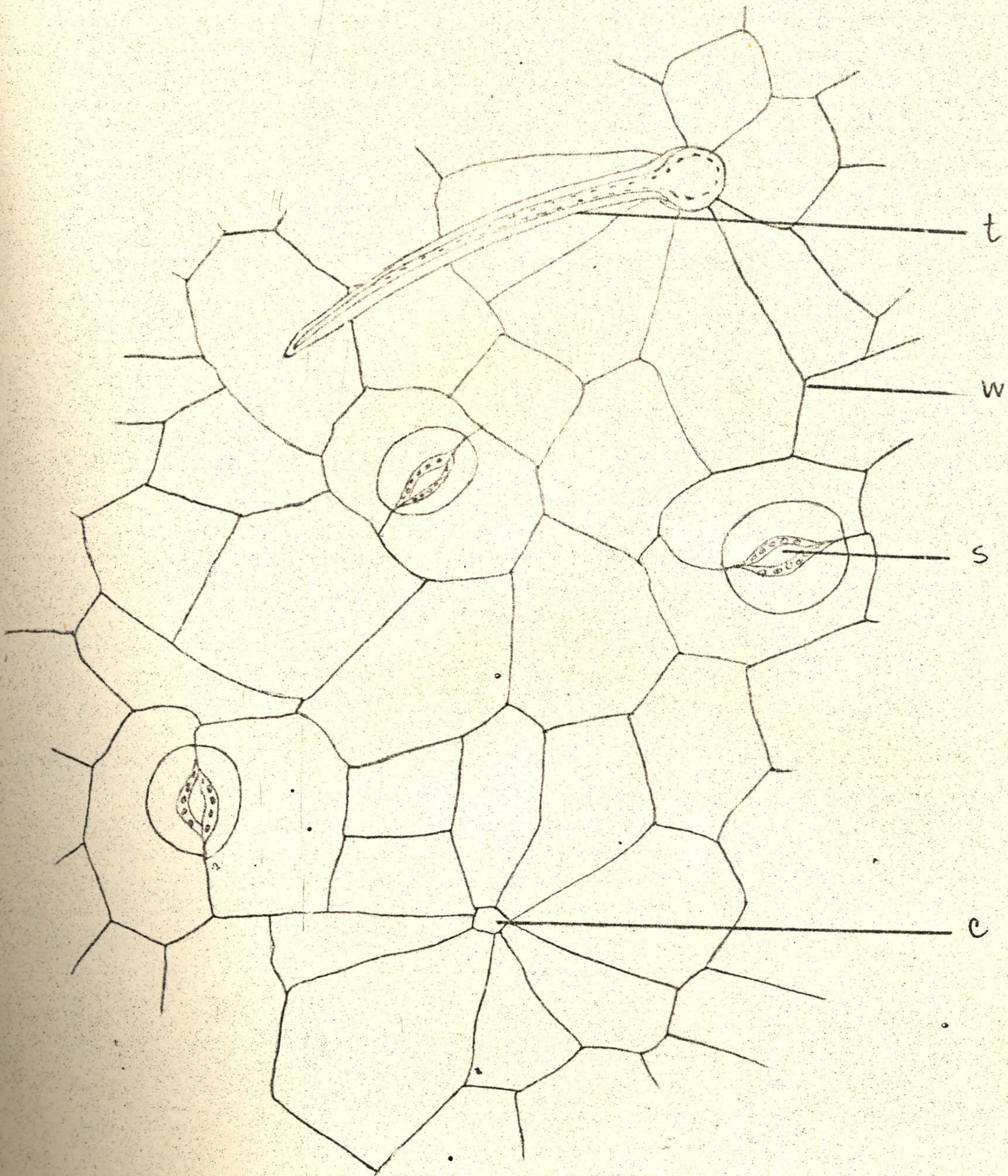
9. Lateral veins surrounded with a sheath of parenchyma cells containing prisms of calcium oxalate

10. Spongy mesophyll layer

11. Collenchyma cells

12. Lower epidermis.

Fig 4 : Microscopical Features of Lower Epidermis of Cassia didymobotrya Leaflet x 400 (Not to scale)



Key to figure 3 :

c, cicatrix ; s, stoma ; t. smooth unicellular covering trichome; w, straight epidermal cell walls.

Fig 5: Thin Layer Chromatographic Separation of Anthraquinones in Cassia didymobotrya Extract; System I

- Technique : OW Ascending. Temperature 27°C
- Mobile Solvent System : Benzene : Ethyl acetate : Acetic acid
  75 : 24 : 1
- Support System : Silica Gel GF254 (with Sodium hydroxide)

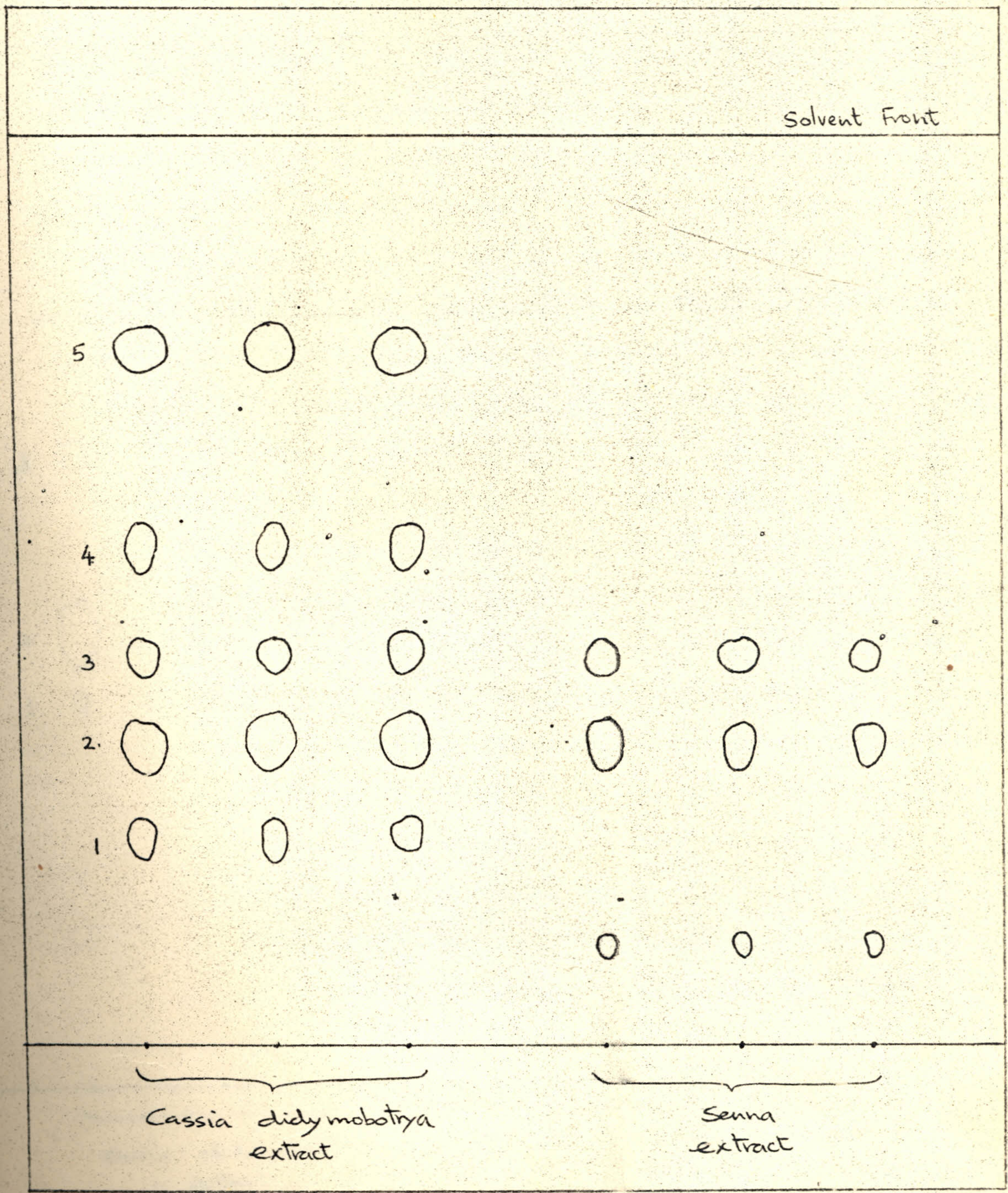


Fig 6: Thin layer chromatographic separation of anthraquinones in Cassia didymobotrya Extract, System 2

- Technique: O.W. Ascending ; Temperature: 27°C
- Mobil Solvent System: Ethyl acetate : n-propanol : water
  40 : 40 : 30
- Support System : Silica Gel GF_{254}

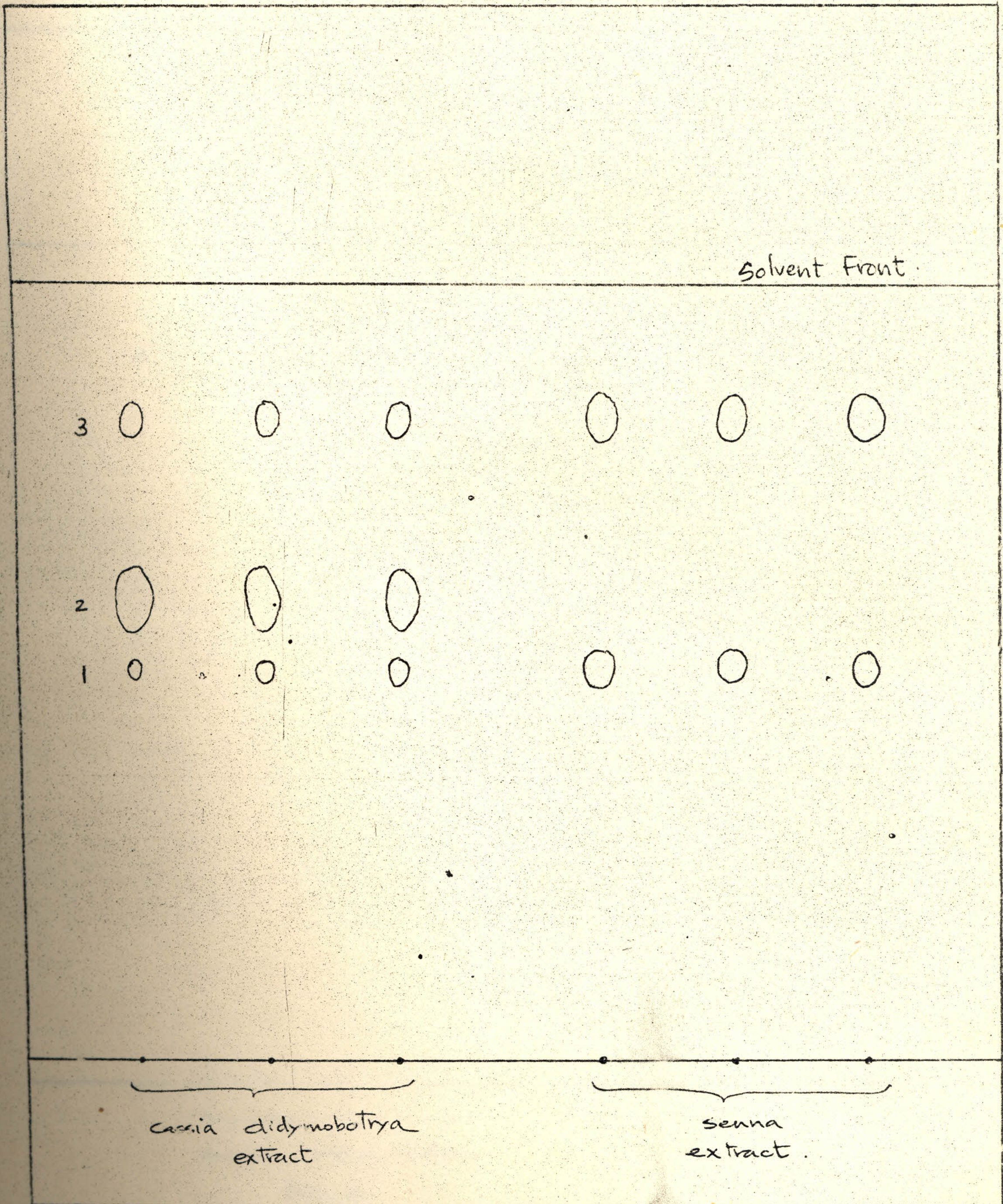


Fig. 7 Thin Layer Chromatographic Separation of Anthraquinones in Cassia didymobotrya Extract ; System 3.

- Technique : OW. Ascending. Temperature 27°C
- Mobile solvent system: Ethylacetate : Methanol : water
  77 : 13 : 10
- Solid support system: Silica Gel GF 254

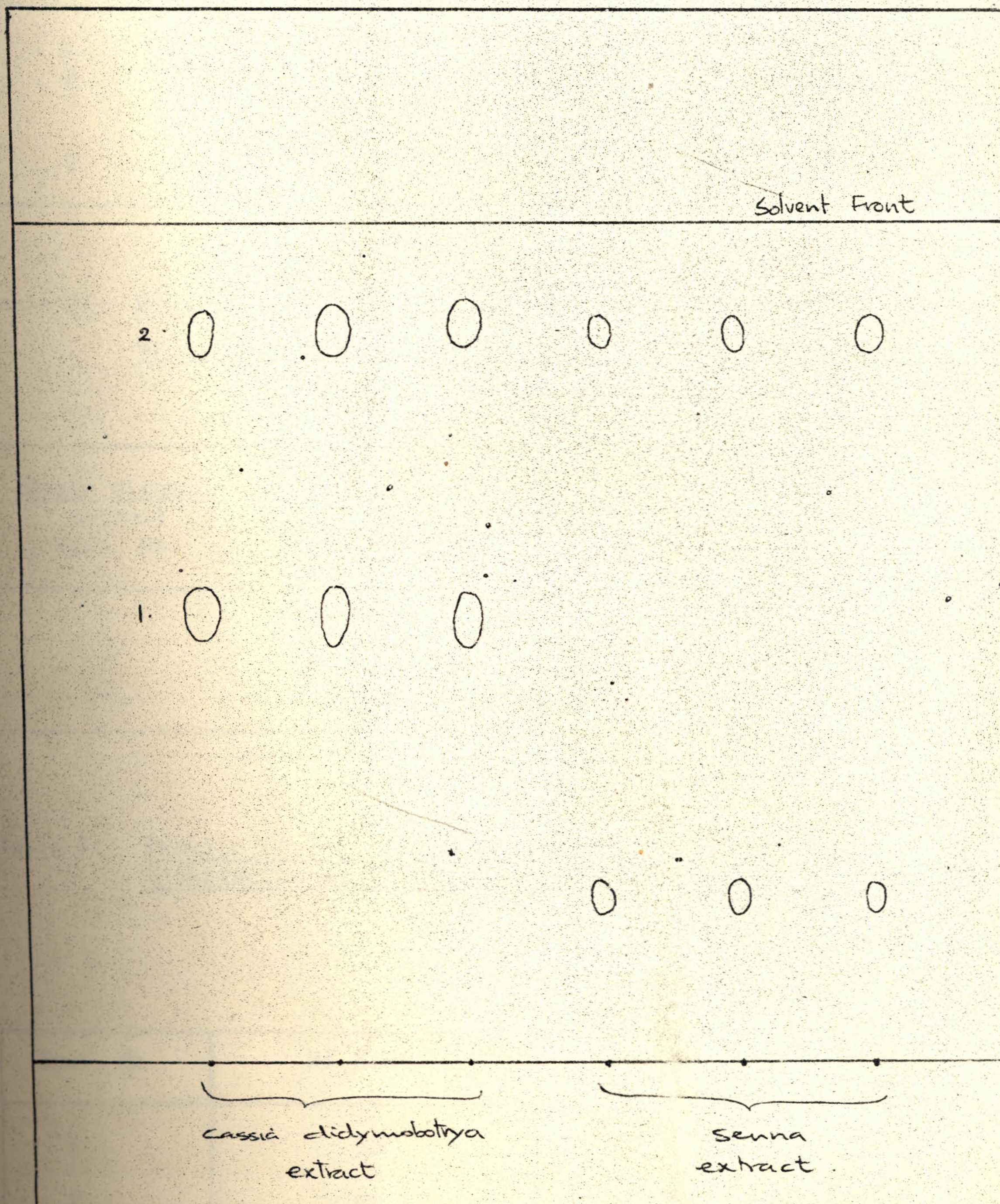


Table 1: Results of Thin Layer Chromatographic Separation of Anthraquinones in Cassia didymobotrya Fres., System 1 (Fig 5)

Cassia didymobotrya extract Spot No.	C. didymobotrya extract hRf	hRf of anthraquinone of senna extract	Name of identified anthraquinone
1	22	-	-
2	33	33	Rhein
3	43	43	Aloe-emodin
4	55	-	-
5	77	-	-

Table 2: Results of Thin Layer Chromatographic Separation of Anthraquinones in Cassia didymobotrya Fres., System 2 (Fig 6)

Cassia didymobotrya extract Spot No.	Rf X100
1	50
2	59
3	83

Table 3: Results of Thin Layer Chromatographic Separation of Anthraquinones in Cassia didymobotrya Fres., System 3 (Fig 7)

Spot No	Rf X100
1	53
2	80

'abl 4: Rf values for some Anthraquinones as given by HARBORNE J.B. (1973) (5)

Anthraquinone	Rf (X100)
Aloe-emodin	36
Chrysophanol	76
Emodic acid	18
Emodin	52
Physcion	75
Rhein	24

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