TITLE PAGE

ANTHRACENE DERIVATIVES IN TISSUE CULTURE OF SOME NIGERIAN *CASSIA* SPECIES

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DOCTOR OF PHILOSOPHY

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DECLARATION

This is to certify that all the experimental work described in this thesis was carried out in the laboratories of Department of Pharmacognosy & Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The work of other investigators is acknowledged and referred to accordingly.

I solemnly declare that no part of this thesis has been accepted in substance or concurrently submitted in candidature for any other degree.

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ABSTRACT

This study was undertaken to determine the nature and quantity of anthraquinones produced in callus cultures of four Nigerian species of Cassia: C. nodosa Roxb., c. alata L., .C. occidentalis L. and C. podocarpa Guill. 4 Perr. A visual selection procedure for a high yielding anthraquinone cell line from callus cultures of C. podocarpa was attempted for the first time. The use of plant tissue culture for secondary product biosynthesis, particularly in plants of pharmaceutical importance, holds promise for the controlled production of plant constituents. In all of the Cassia cultures studied, only hydroxyanthraquinones, anthrones, dianthrones, and anthraquinone monoglycosides were produced and the most pharmacologically active dianthrone glycosides based on rhein were particularly absent.

Callus cultures from seedling hypocotyls and cotyledons of Cassia nodosa, C. alata, C. occidentalis and C. podocarpa were established. Cultures were grown at 28 C under constant illumination in an incubator, and these cultures maintained a satisfactory growth on Plurashige and Skoog (M/S) medium with 2,4-dichlorophenoxyacetic acid (2,4-D) (0.6 mg/l) and kinetin (0.35 mg/l). For pigment production and tissue growth, the optimal concentration of 2,4—D was 0.6 mg/l medium in the presence of kinetin (0.4 mg/l). The results indicated that the production of anthraquinones was usually correlated with the rate of tissue growth, and appeared to depend on the relative quantities of 2,4-D and kinetin in the culture media.

An approach to visual selection of high yeilding cell lines in C. podocarpa callus resulted in en increasing accumulation of anthraquinones from 1.3 to *1.8%* dry weight of the material. It is

suggested that anthraquinone synthesis in Cassia callus cultures is associated with brown pigmentation of tissues.

The composition of the mixture of anthracene derivatives found in callus cultures of Cassia spp. was investigated using a number of chromatographic separation techniques. Various methods of paper, thin-layer (including "Vario-Ks-Chamber" with controlled humidity), column, and high performance liquid chromatography for the separation and identification of anthraquinones were critically evaluated. Some new solvent mixtures were developed which provided superior resolution of hydroxyanthraquinones in paper and thin-layer chromatographic systems.

An improved system of high performance liquid chromatography (HPLC) utilising a reversed phase ion pairing technique was developed, which enabled the separation of a five component mixture containing principal anthraquinones: chrysophanol, physcion, emodin, rhein and aloe emodin. The resolution of rhein, one of the acidic components of many naturally occurring anthraquinone mixtures, had not been possible by HPLC methods reported previously.

The amount of free and combined anthraquinones, and the total anthracene derivatives content was estimated colorimetrically. The amount of some individual anthraquinone compounds was determined on thin-layer adsorbent material using a 'Vitatron' densitometer. A densitometric method reported earlier was modified to enable the estimation of individual anthraquinones as equivalent to 1,2-dihydroxyanthraquinone, used as an internal standard. This modified procedure was simpler and did not involve the use of large quantities of reference substances.

Whilst a number of anthraquinones, anthrones and dianthrones were produced in all the Cassia cultures in low concentrations; anthraquinone glycosides were present in "fair amount" in callus cultures of C. podocarpa only. The total yield of anthraquinone compounds in C. podocarpa cultures was nearly the same as obtained in the leaves of the whole plant. However, sennosides were not detected in any of the Cassia cultures.

It wae evident from the visual selection studies on C. podocarpa that biosynthetic capabilities of callus tissues could be improved progressively by selective subcultures. Qualitative and quantitative results, and the absence of sennosides particularly in cultures of C.Podocarpa, emphasize the variation in the anthracene derived constituents of the derived callus and the parent plant.

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CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

Anthraquinone glycosides are important cathartic drugs derived exclusively from plant sources such as *Senna* and *Rheum* species. "Sennosides" (rhein dianthrone glycosides) are the main cathartic principles of these plants. A preparation commercially known as 'Senokot' which is derived from sennosides is widely used for constipation and allied disorders. As with many plant drugs, the availability of *Senna* and *Rheum* plants has diminished over the years, and in view of a possible shortage (Fluck, 1976), phytochemical analysis of several other *Cassia* species: *C. podocarpa* Guill. & Perr. *C. alata* L., *C. nodosa* Roxb. and *C. occidentalis* L. has been carried out in order to find a possible substitute for the traditional purgative plants *Senna* and *Rheum*.

Plant tissue culture is amongst the new material sources sought as an alternative to whole plants. The possibility of using plant cells, isolated from the parent plant, for the production or modification of secondary plant products of pharmaceutical importance is an area of increasing experimental and industrial interest. For over two decades, callus and cell cultures from pharmaceutical plants have been initiated in order to establish suitable conditions for the possible production of pharmaceutically important organic metabolic products '*in vitro*'.

Biosynthesis of anthraquinones in cell cultures of *Cassia angustifolia* Vahl (Friedrich and Baier, 1973), *Cassia senna* L. (Rai *et al.*, 1974), *Cassia tora* (Tabata *et al.*, 1975), *Rheum palmatum* L. (Rai, 1978b), and *Morinda citrifolia* (Zenk *et al.*, 1975) have been investigated and it has shown that the cultures retain the capacity

for synthesis. However, it is usually difficult to obtain high yields of specific compounds in tissue cultures. Even where yields of compounds approach or exceed those found in whole plants (Tabata et al., 1975; Zenk et al., 1975), in many of these cultures only oxyanthraquinones and dianthrones are produced and the pharmacologically more potent dianthrone-glycosides are particularly absent. Incorporation of precursors in the culture medium, with a view to increasing the yield and forcing the synthesis of desired compounds, has met with limited success (Rai, 1977a).

The present work extends the investigation to callus cultures of four other Cassia species: C. podocarpa Guill. & Perr., C. alata L., C. nodosa Roxb. and C. occidentalis L. All of these species are commonly found in Nigeria. The main aim of this investigation was to find out ways to facilitate rapid culture growth and high yield of medicinal compounds, and to investigate constituents produced in these cultures.

Various chromatographic separation methods were employed in the determination of anthracene derivatives in callus cultures of Cassia species.

PART I

OCCURRENCE AND BIOSYNTHESIS OF ANTHRAQUINONES

(i) Anthraquinones and their occurrence in plants

The anthraquinone laxatives represent a group of natural products which have been used for medicinal purposes for thousands of years. Long before anything was known of their chemistry, rhubarb, aloes, senna and cascara were recognised as forming a natural group of purgative drugs.

Some forty different anthraquinone derivatives have now been found in nature, about thirty of which occur in the Rubiaceae. In the monocotyledons they are found only in the Liliaceae, in the form of C-glycosides e.g. barbaloin. Among dicotyledons, they occur in the Rubiaceae, Leguminosae, Lythraceae, Saxifragaceae, Scrophulariaceae and Verbenaceae. They are absent from Bryophyta, Pteridophyta and Gymnosperms but occur in certain fungi and lichens.

Chemical structures of various anthracene derivatives are shown in Figures 1 to 7.

(ii) Cassia species possessing anthracene derivatives

TABLE 1 gives different Cassia species found to contain anthracene derivatives. In all, 27 species of Cassia, from different countries, have been studied. Most of these contain anthracene derivatives, both in the free and in the combined form (glycosides). Of all the Cassia species, only C. angustifolia and C. acutifolia are reported

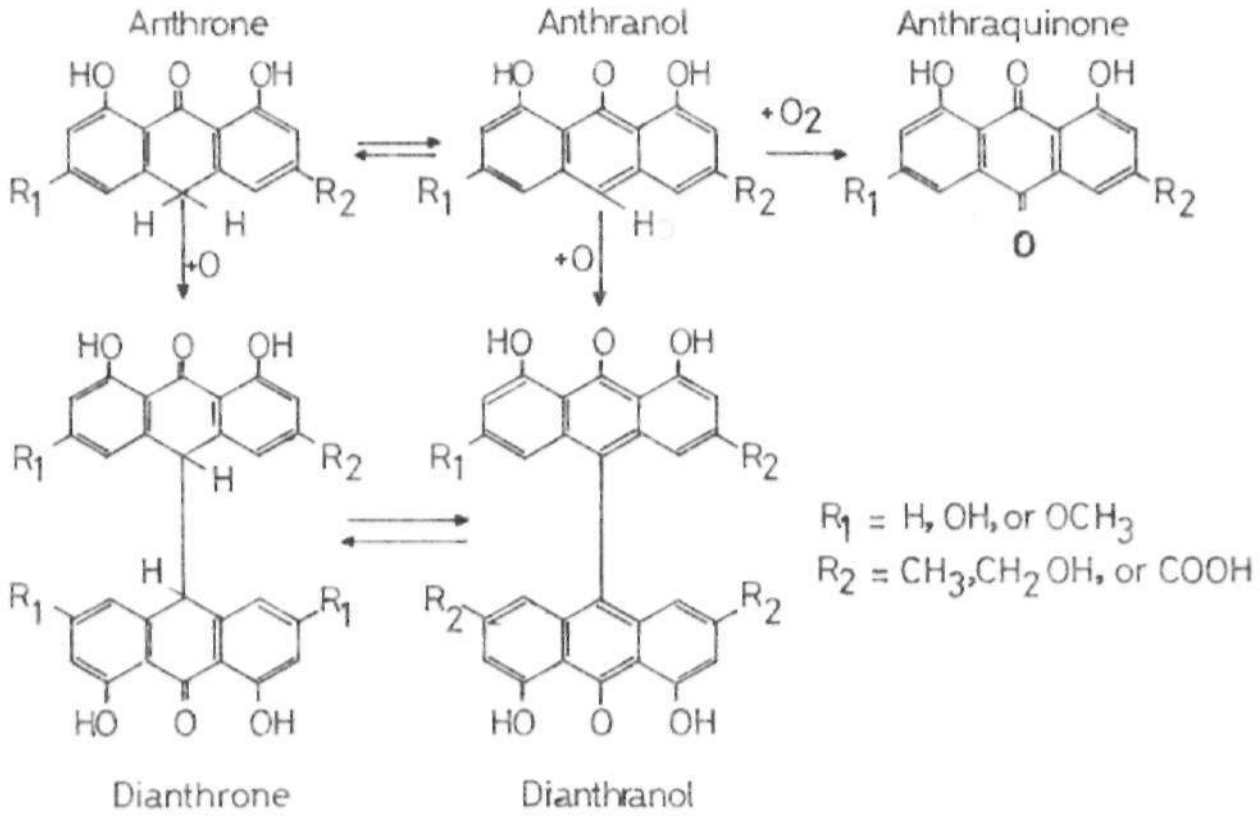


FIGURE 1. ANTHRAQUINONE AGLYCONES

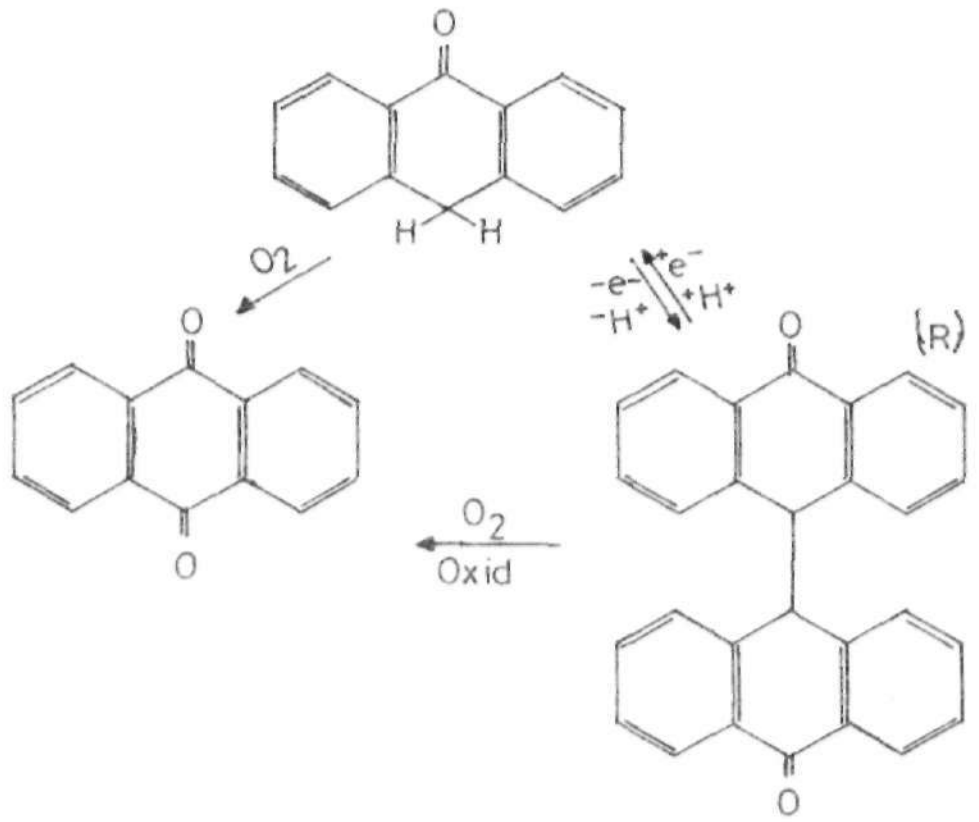
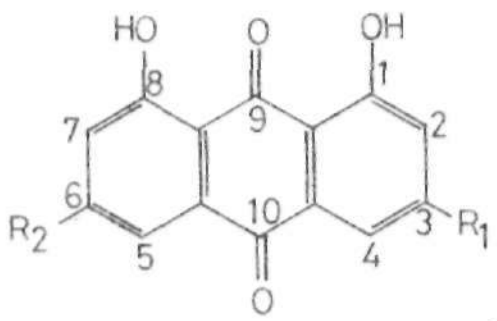
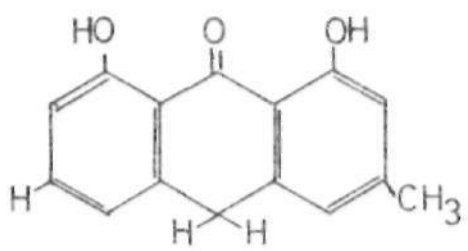


FIGURE 2. STRUCTURAL RELATIONSHIP OF ANTHRAQUINONE, ANTHRONES AND DIANTHRONES

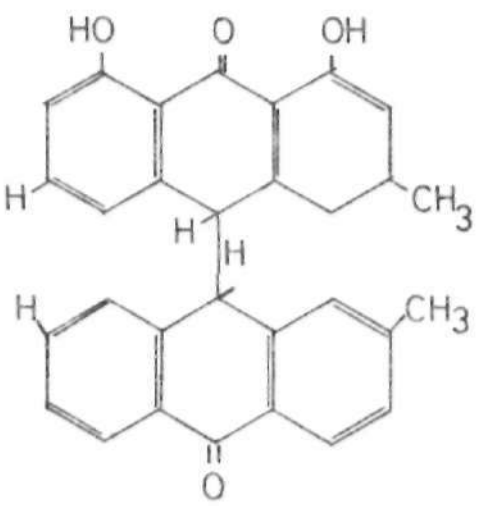


$R_1 = CH_3$	$R_2 = H$	**chrysophanol**
$R_1 = CH_3$	$R_2 = OCH_3$	**physcion**
$R_1 = CH_3$	$R_2 = OH$	**emodin**
$R_1 = CH_2OH$	$R_2 = H$	**aloe emodin**
$R_1 = COOH$	$R_2 = H$	**rhein**

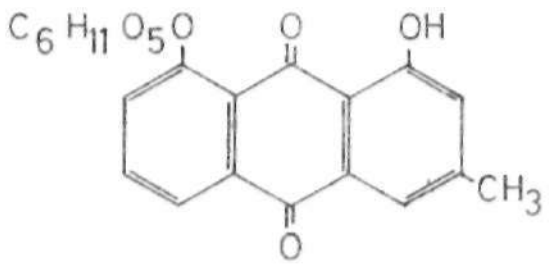
FIGURE **3** . THE PRINCIPAL ANTHRAQUINONE AGLYCONES



Chrysophanol anthrone

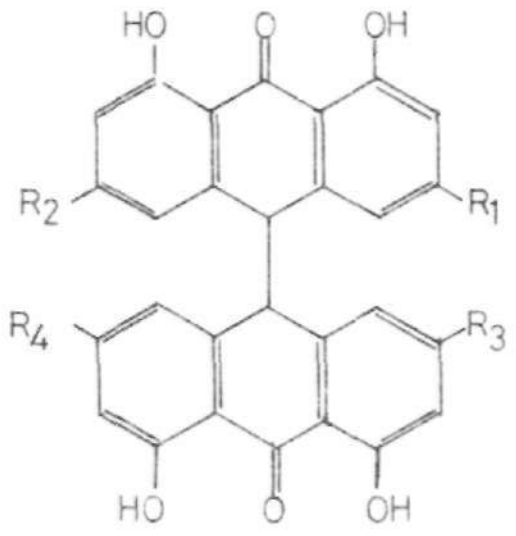


Chrysophanol dianthrone



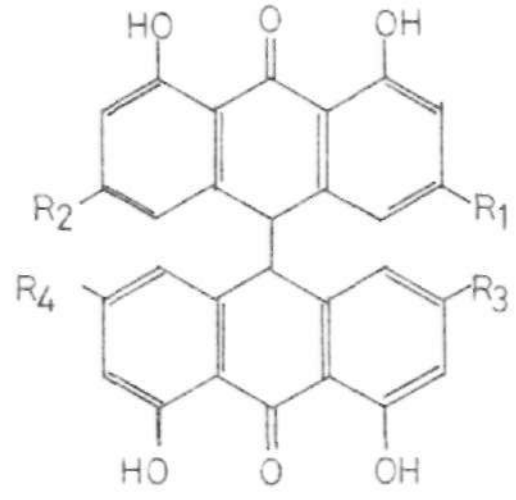
Chrysophanol monoglucoside

FIGURE 4. CHRYSOPHANOL DERIVATIVES



Homodianthrone

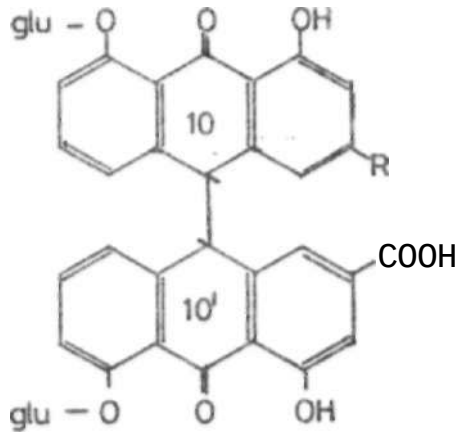
$R_1 = R_3 = -CH_3$	$R_2 = R_4 = H$	Chrysophanoldianthrone
$R_1 = R_3 = -CH_3$	$R_2 = R_4 = -OCH_3$	Physciondianthrone
$R_1 = R_3 = -CH_3$	$R_2 = R_4 = -OH$	emodindianthrone
$R_1 = R_3 = -CH_2OH$	$R_2 = R_4 = H$	aloe-emodindianthrone
$R_1 = R_3 = -COOH$	$R_2 = R_4 = H$	rhein dianthrone = sennidine A,B



Heterodianthrone

$R_1 = -CH_3$	$R_2 = H$	$R_3 = -CH_3$	$R_4 = -OCH_3$	= palmidin D
$R_1 = -CH_3$	$R_2 = H$	$R_3 = -CH_3$	$R_4 = -OH$	= palmidin C
$R_1 = -CH_3$	$R_2 = H$	$R_3 = -CH_2OH$	$R_4 = H$	= palmidin B
$R_1 = -CH_3$	$R_2 = H$	$R_3 = -COOH$	$R_4 = H$	= rheidin B
$R_1 = -CH_3$	$R_2 = -OCH_3$	$R_3 = -COOH$	$R_4 = H$	= rheidin C
$R_1 = -CH_3$	$R_2 = -OH$	$R_3 = -CH_2OH$	$R_4 = H$	= palmidin A
$R_1 = -CH_3$	$R_2 = -OH$	$R_3 = -COOH$	$R_4 = H$	= rheidin A
$R_1 = -CH_2OH$	$R_2 = H$	$R_3 = -COOH$	$R_4 = H$	= sennidine C,D

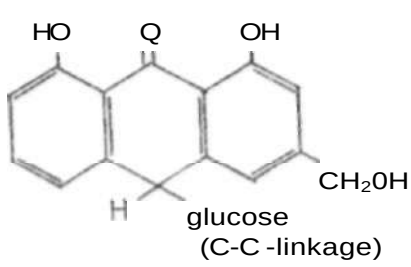
FIGURE 5. VARIOUS HOMODIANTHRONES AND HETERODIANTHRONES



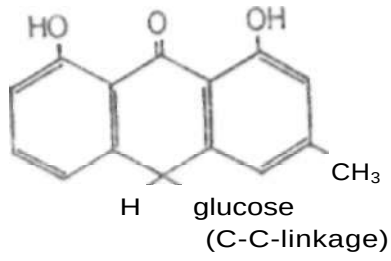
		10-10
Sennoside A	GOOH	trans
Sennoside B	COOH	meso
Sennoside C	*CSCOE*	trans
Sennoside D	$CH_?0H$	meso

FIGURE 6 STRUCTURES OF SENNOSIDES A, B, C AND D

C



Barbaloin



Chrysaloin

FIGURE 7 EXAMPLES OF C-GLYCOSIBES

TABLE 1 Anthracene derivatives from Cassia species

Species	Active principle isolated	Reference
C. alata	Rhein and other anthraquinones	Hauptmann and Nazario, 1950; Tiwari and Yadava 1971; Rai, 1978c
C. auriculata	Emodin	Varshney et al., 1973
C. bicapsularis	-	Matos, 1957 - 1958
C. chrysocarpa	-	Matos, 1957 - 1958
C. fistula	Rhein; sennosides A, B; sennidin like compounds	Kapadia and Khorana, 1962; Kaji et al., 1965, 1968; Kumar et al., 1966
C. hoffmansenggi	-	Matos, 1957 - 1958
C. jaegeri	Heterosides of chrysophanol, emodin and physcion	Jaeger et al., 1969
C. javanica	Chrysophanol, rhein, aloe emodin, glycoside of rhein	Bhutani et al., 1966
C. marilandica	(i) Chrysophanol, physcion and their glycosides (ii) Heterosides of the emodin of glucofrangulin type	Anton and Duquenois, 1968a
C. mimosoides	Emodin and emodin glycoside, physcion and emodic acid	Subramanian and Nagarajan, 1969, 1970
C. nigricans	Mainly non-Rhein derivatives	Duquenois and Anton, 1968a

TABLE 1 (Cont'd.)

Species	Active principle isolated	Reference
C. nodosa	Nodososide - an anthraquinone glycoside	Rizvi et al., 1971
	Rhein	Rai, 1979a
C. obovata	Rhein, aloe emodin, sennidins, reduced aloe emodin, chrysophanol, sennosides A & B	Saber et al., 1962
C. obtusifolia	Gluco-obtusifolin, gluco-aurantio-obtusifolin (Anthraquinone glycosides)	Takido and Takahashi, 1963; Takido, 1958
C. occidentalis	Physcion, chrysophanol, rhein, aloe emodin	Anton and Duquenois, 1968b; Shah and Shinde, 1969; Rai and Shok (1983)
C. obovata	Emodin-anthrone	Nazirov, 1960
C. podocarpa	Sennosides A & B, rhein anthrone glycoside, rhein and rhein glycoside	Paris and Chartier, 1948; Rai and Obayemi, 1978
C. reticulata	Rhein, aloe emodin, chrysophanol	Mesmer et al., 1968
C. sieberiana	Rhein and rhein glycoside	Duquenois and Anton, 1968b; Rai, 1979b
C. siamea	Siameanin dimer of chrysophanol, physcion and rhein	Chatterjee and Bhattacharjee, 1964; Rai, 1977b; Wagner et al., 1978

TABLE 1 (Cont'd.)

Species	Active principle isolated	Reference
C. singueana	Chrysophanol, physcion and two other anthraquinones	Margarida and Correia, 1968
C. splendida	-	Matos, 1957 - 1958
C. senna	Sennosides A & B. A number of both mono and polyglycosides of rhein, rhein anthrone, aloe emodin	Crellin et al., 1961; Lemli and Cuveele, 1967
C. tora	Chrysophanol, physcion, emodin, aloe emodin, rhein and glycosides of some of these	Shah and Shinde, 1969
	A new anthraquinone pigment	Tiwari and Behri, 1972

to be used as official drugs for cathartic activity.

(iii) Biosynthesis of anthraquinone derivatives

The formation of anthraquinones in plants other than those belonging to the genus Cassia has been studied in considerable detail. The biosynthesis of anthraquinone derivatives has been shown to take place by two different pathways.

(a) The acetate polymalonate pathway

In this pathway, anthraquinone derivatives are believed to be formed in the following manner (FIGURE 8).

Emodin, its dimer skyrin and islandicin are formed in this manner in Penicillium islandicum (Saul, 1974). The stages where the water molecules and CO_2 are lost from the polyketomethylene chain are not known. This type of process also occurs in non-fungal plants. Leistner and Zenk (1969) fed young leaves of Rumex alpinus through the cut ends with (±) 1, 2-^{14}C shikimic acid, (±) 2-^{14}C mevalonate, (±) 1-^{14}C acetate or 2-^{14}C acetate in complete darkness. After 24 hours, chrysophanol was isolated from leaves, purified and subjected to degradative studies, using alkaline H_2O_2. No incorporation of labelled shikimic acid and labelled mevalonate into the emodin type anthraquinones occurred. 1-^{14}C and 2-^{14}C acetates were, however, incorporated into chrysophanol. The distribution of radioactivity, in the oxidation and degradation products of labelled chrysophanol, is in complete agreement with the acetate-polymalonate pathway. The alternate labelling of the chrysophanol molecule (Leistner, 1971) after 1-^{14}C or 2-^{14}C acetate feeding, strongly supports the view

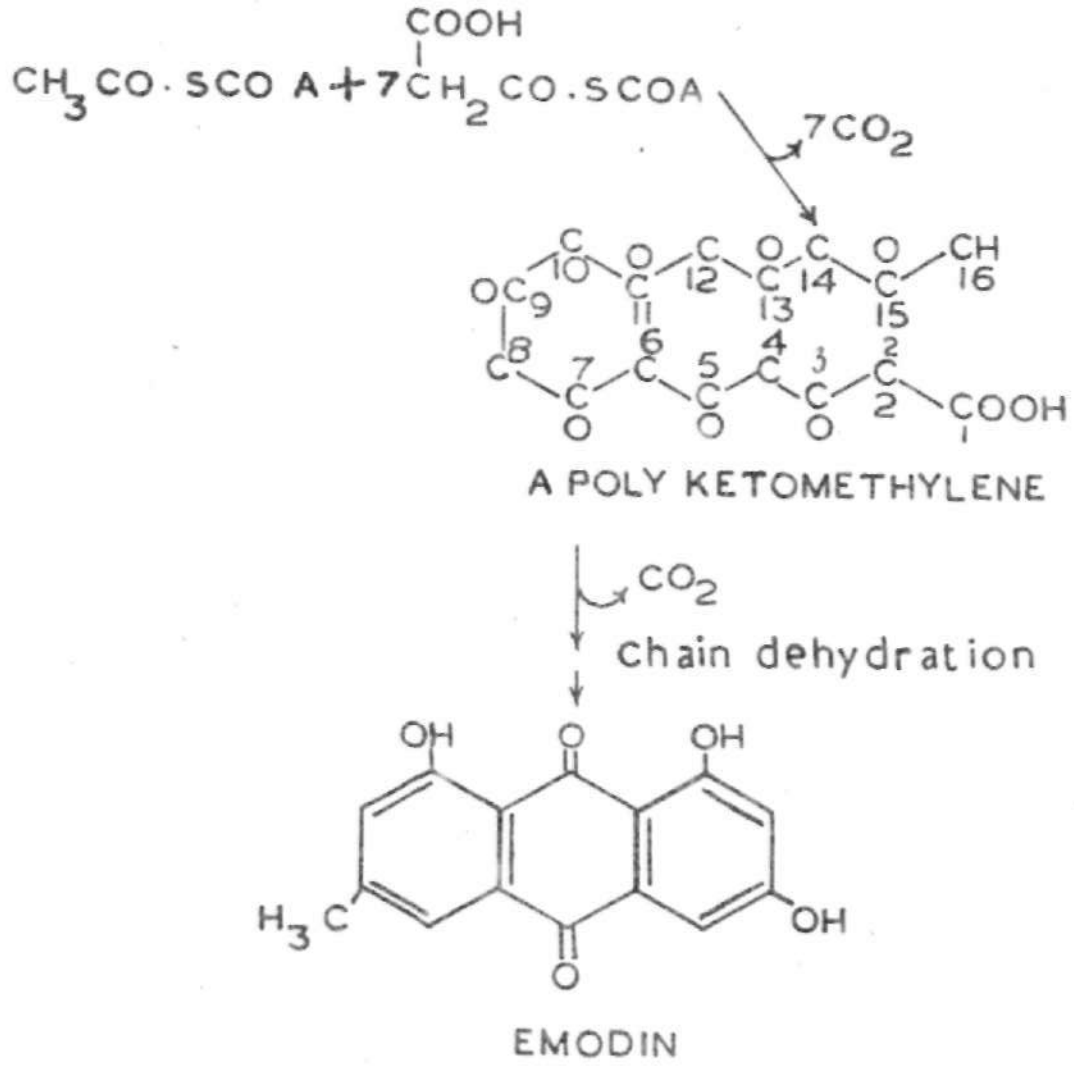


FIGURE 8 THE ACETATE POLYMALONATE PATHWAY

that acetate units are linked together by way of acetyl CoA and malonyl CoA. Cyclization of the polyketide unit most probably gives rise to an anthraquinone precursor which in several steps is transformed to chrysophanol. Similar conclusions were derived from studies on Rumex obtusifolius (Fairbairn and Muhtadi, 1972).

(b) Biosynthesis of anthraquinones starting from shikimic acid

Uniformly labelled shikimic acid (U-^{14}C) was specifically incorporated into alizarin and purpurin in Rubia tinctorum (Leistner and Zenk, 1967). Thus the existence of a biosynthetic pathway for the formation of anthraquinones entirely different from the acetate - polymalonate route was established. The carboxyl carbon of shikimic acid was incorporated into the anthraquinone nucleus. The same thing happens when naphthoquinones are formed from shikimic acid in bacterial and higher plants. 2-carboxy-1, 4-naphthoquinol was in fact formed from shikimic acid and 2-ketoglutarate in excised shoots of 6 to 8 weeks old Impatiens balsamina (Campbell, 1969). The ring junction of the naphthoquinone has been shown to originate from the ethylenic carbons of shikimic acid in Mycobacterium phlei (Le Duc et al., 1970). The carbon atoms 2, 3, 4 from 2-ketoglutarate are incorporated into 2, 3, 4 positions of the naphthoquinol (Campbell, 1969).

Since -COOH carbon of shikimic acid was incorporated into the anthraquinone nucleus (Leistner and Zenk, 1967) and is also incorporated into the naphthoquinone nucleus (Campbell, 1969), it was thought that alizarin is formed via naphthoquinone in the Rubiaceae (Leistner and Zenk, 1968). 1, 4-naphthoquinone (1, 4-^{14}C

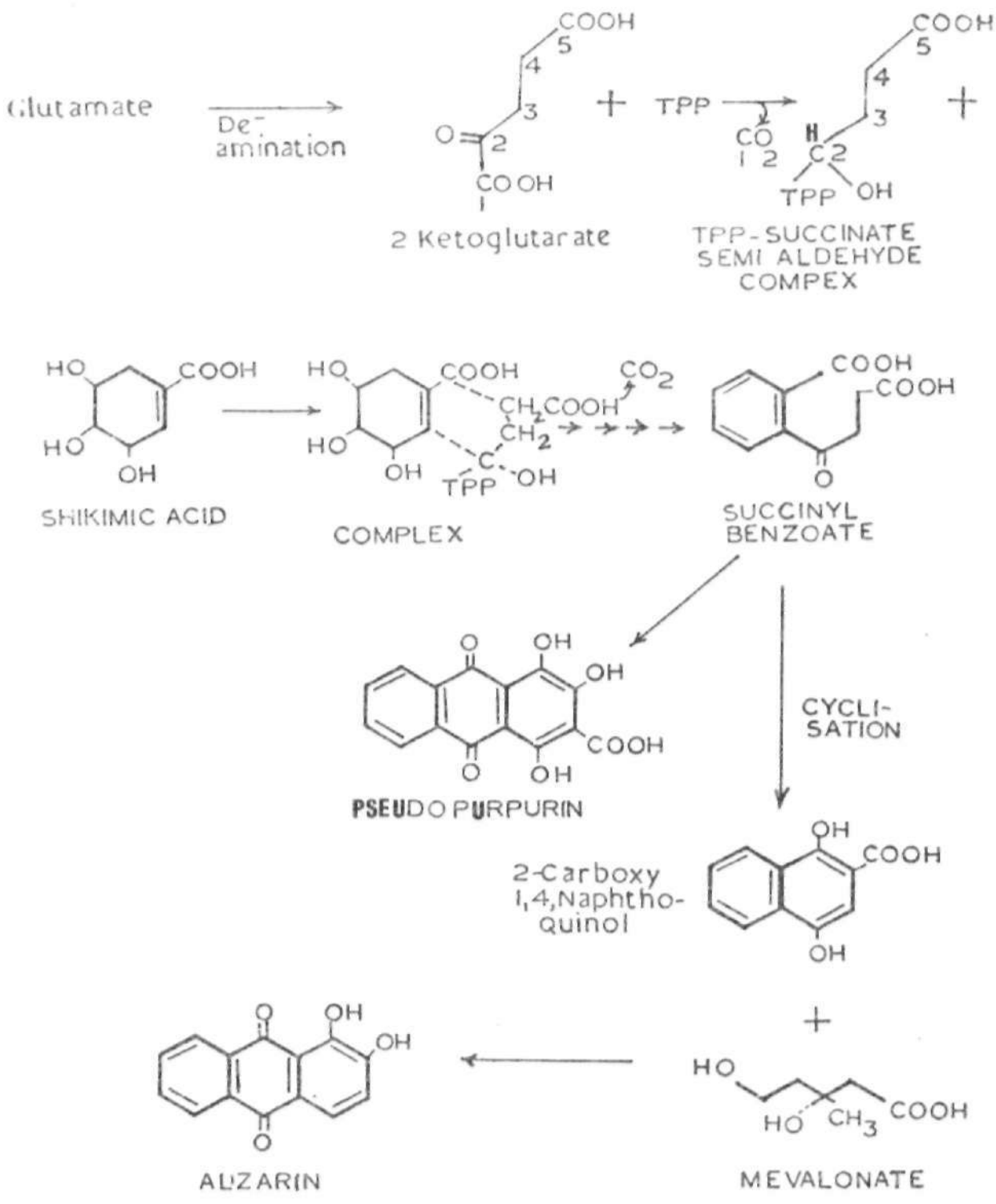


FIGURE 9 ANTHRAQUINONE BIOSYNTHESIS FROM SHIKIMIC ACID

and 2, 3, 9, 10-^{14}C) was synthesized and supplied to the root system of 1½ year old Rubia tinctorum plants. Radioactive alizarin was isolated and degradation studies of the same confirmed that 1, 4-naphthoquinone was incorporated into the anthraquinone nucleus.

One year old Rubia tinctorum plants fed with 2-^{14}C mevalonate were found to incorporate it into 4-anthraquinone pigments, the highest amount of incorporation being into rubiadin and pseudopurpurin. Decarboxylation of pseudopurpurin and degradation of purpurin and other anthraquinones showed that mevalonate had been incorporated into ring C of the anthraquinone nucleus. The C_2 of mevalonic acid provided the side chain carbon atom and also the ring carbon at C_4 (Burnett and Thompson, 1967; 1968). A new intermediate, synthetic ^{14}C- O- succinyl benzoic acid has also been found to be efficiently incorporated into naphthoquinones and anthraquinones of Rubia peregrina (Dansette and Azerad, 1970).

Anthraquinone biosynthesis by the shikimic acid pathway has been demonstrated in FIGURE 9.

(iv) Occurrence of anthraquinones in some selected Cassia species

Cassia alata L., C. nodosa Roxb., C. occidentalis L. and C. podocarpa Guill. & Perr. may be easily identified in the field using keys and descriptions given in Hutchinson and Dalziel (1958).

(a) Cassia alata L.

C. alata is a soft-wooded shrub up to 5 metres tall with large leaves up to 75cm long. Flowers are yellow, in stout dense erect racemes. Pods are 12-19cm long, with a wing along the middle of

each valve, containing 30-60 seeds when ripe.

The various medicinal uses of this plant have been described by Dalziel (1937).

Anthraquinone compounds are reported to be present in leaves and fruits (Hauptmann and Nazario, 1950; Rai, 1978c), and quinone pigments in roots (Tiwari and Yadava, 1971) of Cassia alata.

(b) Cassia occidentalis L.

C. occidentalis is a common weed and it grows as a small shrub (height up to one metre) on uncultivated dry land throughout Africa and the Indian Sub-continent. The flowers are yellow in few flowered racemes, the pods flattish up to 14cm long and 0.5cm wide, often slightly curved upwards, and abruptly beaked. Seeds are many, compressed, lying at right angles to long axis of pod.

In Nigeria, this plant is known as 'Rai dore' in Hausa, 'Rere' or 'Abo-rere' in Yoruba and 'Akede-agbara' in Igbo.

The various medicinal uses of this plant have been described by Dalziel (1937).

Chemical analysis of the root of this plant has revealed the presence of a phytosterol and emodin and the leaves have been found to contain flavonoids and some dianthrones (Anton and Duquenois, 1968b). Occurrence of free anthraquinones have been reported in the seeds of this plant (Shah and Shinde, 1969). Rai and Shok (1983) have detected a number of anthracene derivatives in the various parts of the plant.

(c) Cassia podocarpa Guill. & Perr.

C. podocarpa occurs in forest regions of West Africa, a common shrub up to 5 metres tall on old farm land. The yellow flowers are in dense erect spike-like racemes. The leaves are up to 30 cm long, the rachis has no wing; the fruits are thin flat pods (10-12cm x 1.5cm) with transverse ridges.

In Nigeria, it is mostly found in the southern part of the country. It is called 'Azuwon' or 'Asunwon' or 'Ajan-rere' in Yoruba; and 'Okpo' in Igbo.

C. podocarpa is closely related to official "senna" (Paris and Chartier, 1948). The leaves and fruits of this plant are mentioned as purgatives (Paris and Chartier, 1948). In view of C. podocarpa being considered a possible substitute to C. senna (Labri, 1976), Rai and Obayemi (1978) have re-examined the leaves of C. podocarpa for its anthraquinone compounds. Chrysophanol, emodin and rhein were detected as free anthraquinones but rhein alone was present in the glycosidic form in the leaves of this plant. The estimated content of free anthraquinones was 0.65% w/w, and of combined anthraquinones (rhein glycoside) 1% w/w, giving a total of 1.65% w/w in the dry powder.

(d) Cassia nodosa Roxb.

C. nodosa is a deciduous tree of up to 10 - 14 metres high. It is widely distributed in the tropics and is often grown in West African gardens. Racemes mostly borne singly (occasionally once or twice branched) are found at the end of short normally leafless shoots arising from older twigs. Stipules are 5mm long, narrow and not

leafy; the leaflets are usually acute. Flowers have pink corolla (Dalziel, 1937).

It is a fine decorative tree, easily propegated by seeds and transplanting. It does best in regions having good rainfall.

The leaves of this plant are mentioned to function as purgatives (Irvine, 1961). Rai (1979a) isolated and identified an active principle rhein from the leaves, and indicated that this compound was responsible for purgative action of the leaves.

PART II

PLANT TISSUE CULTURE

(i) History and state of plant tissue culture

Plant tissue culture is the technique of growing plant cells, tissues, and organs in a prepared nutrient medium in the absence of microorganisms.

The reasons for wishing to culture cells in isolation from the plant were first clearly stated by the German botanist Gottlieb Haberlandt in 1902. Although he was unsuccessful in his attempts to culture cells he foresaw that they could provide an elegant means of studying morphogenesis. The principal rationale for using cell cultures for this purpose was the belief that a knowledge of the properties of cells isolated from the plant would give important information regarding the properties and interrelationships of cells within the plant.

There was little progress towards culturing isolated cells or tissues until the early 1930's when technical developments introduced by a number of investigators led to the successful aseptic culture of isolated roots of several species. The next significant advances came in 1938 with the culture of wound callus of carrot and tumour tissues from a tobacco hybrid. These successes were followed by a period of development when culture media and technical methods were improved, permitting the culture of a wide range of organ and callus cultures from many different species. Since 1960, tissue culture methods have progressively become more advanced and have culminated

in highly specialized techniques for culturing single cells, cell suspensions and naked protoplasts.

During the last two decades it has been realized that some of the special properties of plant tissue cultures make them highly suitable as experimental material for many fields of study in addition to morphogenesis. This has led to a complete change in the status of tissue culture methods. Whereas, previously, tissue cultures were the prerogative of a few investigators primarily interested in growth and development, they are now used by scientists, including biochemists, phytochemists, geneticists, plant breeders and plant pathologists.

Aseptic plant cells, tissues, and organs are also used to better understand the biochemical processes of a plant. After nearly two decades of study, it has been demonstrated that serially propagated plant tissue culture systems will produce many useful secondary compounds, for example, cell strains can be selected for high production of some alkaloids and pigments. It is also possible now to grow plant cells in fermentors containing thousands of litres of medium.

The possibility of cultivating plant cells for the production of natural compounds by methods similar to those used to culture microorganisms has found increasing interest and opens new approaches in biology and biotechnology.

(ii) Potential problems with whole plants as sources of pharmaceuticals

Higher plants are the most important sources of pharmaceutically important natural products and occupy an important place in modern medicine. However, there are some potential problems associated with

the use of whole plants as sources of pharmaceuticals. These problems are mainly environmental, economic and political. Yields can be affected by environmental factors such as excessively cold seasons, drought or loss of crops due to natural disasters. Economic difficulties can arise from variation in yield of a desired compound and changing costs of growing or harvesting plants. As more land is diverted to food production to meet population needs, less land will be available for non-food i.e., drug crop usage. Uncontrolled exploitation and disappearance of the natural habitat continues and there may also be difficulties in bringing wild plants into cultivation. The availability of plant material may also be affected by transportation problems, difficulties of collecting plants of a species that grow at low population densities, and quarantine requirements. For those plants which are harvested from wild populations, continued use may exceed the replenishment rate of the natural populations leading to decreased availability and increased cost. Changes in currency exchange rates may also affect the availability of raw material. For plants from foreign countries, political problems such as warfare, internal strife or discriminatory governmental action may alter the supply of source plants. These aspects, together with the difficulty of adapting some native plants to new habitats, suggest that alternative sources need to be found.

Although a variety of substances used in the pharmaceutical industries originate from plants, the trend over the last fifty years has been towards chemical synthesis, as an alternative source for production of some of these substances. However, for a number of reasons, the chemical synthesis may not be viable and plants still remain an important source of many of these compounds.

(1) The compounds are difficult and/or costly to synthesize.

(2) Complex mixtures, e.g. rose oil, cannot be constituted successfully by man.

(3) Isolation from a natural source circumvents many of the regulations which must be satisfied before a chemically synthetic compound can be used commercially as a drug.

(4) Chemical synthesis may, depending on the compound, result in a mixture of isomers which cannot be separated, or separation is not viable on a commercial scale. This is of obvious importance when the major desirable property resides with one isomer.

Many of the problems associated with the availability of whole plants can be, and often are being, attacked by classical plant breeding and management approaches but the new technology evolving within the biological and pharmaceutical sciences offers the potential of providing solutions more rapidly in some cases, or of attaining specific ends which are impossible to achieve by classical means. The primary tool in these new approaches is plant tissue culture technology.

The quantity of a plant species available for extraction of a particular secondary metabolite may be limited by a variety of factors mentioned above. Plant cell cultures may therefore be an alternative to whole plants as sources of pharmaceuticals. This idea was expressed

in the patent issued to Routien and Nickell (1956). They proposed that cells of a species producing a desired pharmaceutical would be grown in fermentors as are bacteria or fungi, harvested and the desired pharmaceutical recovered. Plant cell culture has advanced dramatically since that time, so that a concept which was then a theoretical possibility, is now moving into the realm of reality.

Since the early days of their comparatively short history, plant cell cultures have been regarded as potential sources of useful secondary constituents and especially for medicinal substances. Many plant products which are indispensable as pharmaceuticals and materials for diagnostic purposes are in rapidly increasing demand (Farnsworth and Morris, 1976), so that plant cell cultures have been seriously considered as sources alternative to agricultural production.

(iii) Advantages of tissue cultures

The main advantages of cell culture over the conventional agricultural production of plant materials are:

(1) The production of desired metabolites would be possible independent of environmental factors.

(2) The quality of the raw products could reach a uniformity never observed with a field grown material.

(3) A newly discovered drug plant could immediately be put into culture and the

lengthy trials of agricultural propagation and cultivation could be shortened.

(4) The production of important drugs like morphine and codeine could be done under stringent state control to prevent abuse.

The advantages of using tissue cultures over whole plants for biosynthetic studies were recently summarised by Overton (1977) to be:

"(1) Undifferentiated cells, simple organisation: minimise problems of translocation, permeability, segregation of metabolic pools; hence good precursor incorporation.

(2) Can be grown under standardised conditions, often on synthetic media.

(3) Short growth cycles; no seasonal variation.

(4) Incorporation and turnover of labelled precursors can be studied over short periods.

(5) Sterile conditions ensure observed biosynthesis is mediated by plant tissue, not microorganism associated with it."

(iv) Accumulation of secondary metabolites

Plants are used as sources of many compounds and mixtures of compounds that are useful to man. These compounds, which are referred

to as secondary products include drugs, flavours, enzymes, essential oils and colourings.

The production of secondary metabolites, and of medicinal substances in particular, by plant cell cultures, has interested scientists since these tissues were found to show pigmentation, crystal formation, and extracellular enzyme activities. Attempts to exploit metabolite production were reported as early as 1960 by Tulecke and Nickell (1960). In general, yields were low and discouraged the implementation of plant cell culture in industry. Metabolite production in cell cultures, however, remained an area of academic research (Kurz and Constabel, 1979) for two reasons:

(1) Chemists recognised cell cultures as suitable systems for studying biosynthetic pathways.

(2) Physiologists were attracted to product formation because it equalled simple morphogenetic processes open to factorial analysis.

Lately, a rapidly growing literature on product formation in plant cell cultures clearly indicates that considerable and significant advances have been achieved (Staba, 1980).

Reviews on the biosynthetic potential of plant cell cultures include those by Steck and Constabel (1974); Butcher (1977); Staba (1977, 1980); Misawa (1977); Tabata et al. (1978); Dougall (1979) and Kurz and Constabel (1979). The biotechnical application and the production of useful compounds from plant cell cultures have also been discussed at symposia held in Munich

(Barz et al., 1977); Tubingen (Alfermann and Reinhard, 1978); Calgary (Thorpe, 1978) and Tokyo (Fujiwara, 1982).

When the idea of producing medicinal compounds through tissue cultures was initially conceived, more attention was given to the study of those compounds which are at present important pharmaceutical products of the plant sources.

The most common and essential drug compounds derived from higher plants are:

steroids (from diosgenin), codeine,
atropine, reserpine, hyoscyamine,
digoxin, scopolamine, digitoxin,
pilocarpine, quinidine, colchicine,
emetine, morphine, quinine,
sennosides, tubocurarine and
vincristine (Farnsworth and Morris, 1976).

These compounds would be of immediate interest from the point of view of attempting to obtain them from cultures. The list of types of compounds already shown to be produced from plant cell cultures is quite impressive. These range from alkaloids to allergens, antileukemic and antitumour agents, antimicrobial agents, benzo-compounds, cardiac glycosides and other active cardioactive substances, enzymes and inhibitors, commercial and volatile oils, phenolics, pigments, steroids, sterols, saponins and sapogenins, terpenes and terpenoids, etc.

When a cell culture is established for biosynthetic studies, there may be one of three possibilities:

(1) Production of the same compounds in culture that are produced by the parent plant in natural habitat from which the culture was derived, at the same or different concentrations.

(2) No production at all of the metabolite of interest under the given cultural conditions.

(3) Production of compounds in culture which are not present in the parent plant (i.e. the plant from which the culture originated).

Two main techniques are used in plant tissue culture. Tissues may be cultured in liquid nutrient media or on those solidified with agar. When grown on agar, the tissue forms a callus or mass of unorganised cells. This procedure is simple and a favoured method for starting and maintaining cell lines.

Liquid suspension cultures consist of mixtures of cell aggregates and single cells. This technique usually results in higher growth rates and better control of the environment in which the cells are growing because most of the cells remain in contact with the medium and problems such as nutrient gradients are eliminated. The choice of technique to be used depends on how easy it is to initiate the cultures and this varies with the type of plant from which the tissue originates.

The procedures for isolation of constituents from plant tissue

cultures are similar to those applied to whole plants. In the last decade numerous studies with callus and suspension cultures from various plants have been published describing the formation, and sometimes accumulation in rather high concentrations, of many different secondary metabolites. The accumulation of these compounds in cell cultures has often been compared to the concentration of these compounds in the normal plant (Zenk, 1978) and various cases have been reported in which the accumulation in cell cultures was considerably higher than in the normal plant. The reports dealing with accumulation in cell cultures clearly demonstrate the general potential of plant cell cultures for producing compounds.

The following TABLE 2 shows a list of the interesting plant products reported so far from tissue cultures.

(v) Factors affecting the nature and yield of product in plant tissue cultures

Like intact plants, plant cells, tissues, explants, organs and embryos in culture require a carbohydrate as an energy source (usually consisting of sucrose), a nitrogen source (such as nitrate or ammonium, or both) and elements such as phosphorus, sulphur, potassium, sodium, calcium, magnesium and chlorine. Micronutrients such as boron, cobalt, copper, iron, manganese, molybdenum and zinc are also necessary as are auxins, cytokinins and sometimes vitamins. In addition, amino acids or alternatively supplements such as coconut milk may also be required. In other words, while the majority of intact plants have an autotrophic nutrition, plant cultures are heterotrophic.

Efficient production of secondary metabolites by plant cell

TABLE 2 Interesting Plant Natural Products Isolated From Cell Cultures

(After Barz and Ellis, 1981)

Compound	Class of Compound	Plant
Ginsenosides	Saponin	*Panax ginseng*
Glycyrrhizin	Saponin	*Glycyrrihiza glabra*
Solasonine	Steroidal Alkaloid	*Solanum laciniatum*
Visnagin	Furochrome	*Ammi visnaga*
Quinones	Anthraquinones	*Cassia tora*
Shikonin	Naphthoquonone	*Lithospermum erythrorhizon*
Protopine	Protoberberine Alkaloid	*Macleaya microcarpa*
Scoulerine	Tetrahydroprotoberberine Alkaloid	*Macleaya microcarpa*
Sanguinarine	Berberine Alkaloid	*Macleaya microcarpa*
Scopolamine	Tropane Alkaloid	*Datura stramonium*
Hyoscyamine	Tropane Alkaloid	*D. tatula*
Caffeine	Purine	*Coffea arabica*
Thebaine	Morphinane	*Papaver bractcatum*
Codeine	Morphinane	*Papaver bracteatum*
Ephedrine	Phenylethylamine	*Ephedra gerardiana*
Berberine	Berberine Alkaloid	*Coptis japonica*
Cephalotaxine	Alkaloid	*Cephalotaxus harringtonia*
Harringtonine	Alkaloid	*Cephalotaxus harringtonia*

culture systems is largely dependent on environmental and biological factors. Some of the important factors will be discussed with a few examples for each case.

(a) Light

The effect of light on growth and metabolite production has been extensively studied. The problem, when comparing results, lies in the use of widely different sources of light, quality of light, and quantity of irradiation. Light may exert a significant effect on the accumulation of metabolites as demonstrated by Mohr (1969) when investigating anthocyanin formation in Sinapis seedling. A characteristic alteration in the pattern of chemical composition between light-grown and dark-grown callus has been reported for the volatile oil in Ruta graveolens cultures by Corduan and Reinhard (1972). It has been shown in a number of tissue cultures that light generally stimulates the formation of compounds including carotenoids, flavonoids, and polyphenols (Hahlbrock et al., 1976).

Several authors preferred to maintain cultures in the dark, so rigidly controlling the production of, for instance, anthraquinones in Cassia tora (Tabata et al., 1975), and alkaloids in Scopolia parviflora (Tabata et al., 1972b), Nicotiana tabacum (Furuya et al., 1971) and Catharanthus roseus (Doller et al., 1976).

In sharp contrast to the stimulatory effects of light, Tabata and co-workers (1974) found that either white or blue light almost completely inhibits the formation of shikonin derivatives in Lithospermum cultures at all stages of the cell growth cycle.

Similarly, Nagel and Reinhard (1975) have demonstrated that blue or intense white light inhibits the synthesis of terpenoids, whereas these metabolites are produced in the dark.

Saleh and Kamak (1982) have recently reported on the effect of light intensity and temperature on the growth and alkaloid accumulation in callus tissue of Datura stramonium. They found that the rate of callus tissue growth as well as alkaloids formation increased with increasing light intensity.

From a practical viewpoint, it would be more economical if metabolites could be produced in the dark, because no lighting facility would be needed.

(b) Temperature

Cell cultures generally are grown at 25° - 30°C. Higher temperatures have been shown to be stimulatory to product formation in the case of anthocyanin (Matsumoto et al., 1973). Temperature may influence the kind of substance synthesized, the degree of saturation of fatty acids, for example. Incubation of soybean cell suspensions at the low temperature of 15°C led to an increase in the level of linoleic and linolenic acids in the cell lipids (Wilson et al., 1978). Saleh and Kamak (1982) have shown that in the case of Datura stramonium var., 25°C proved to be the most favourable temperature for callus tissue growth, while 20°C was the best for alkaloid formation.

(c) Nutrients and plant growth regulators

The medium composition and culture conditions play a key role on the yields of desired compounds. Evidence for alteration of yield of secondary metabolites in cell cultures by the conditions of culture is shown in TABLE 3.

Historically, the highest priority for investigation has been given to growth substances because:

(1) These are required for growth of cultures.

(2) Altering the concentrations of growth substances in the culture medium initiates organised structures such as shoots or roots in some plant cell cultures.

(3) In some cases there appear to be associations between production of secondary compounds and formation of organised structures.

The types of growth substances required by plant cell cultures fall into two classes, auxins and cytokinins. Auxins are required for growth of cultures of normal plant cells. Examples of auxins are: indole-acetic acid (IAA), naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). Cells from some plant species require cytokinins in addition to auxins for growth. Examples of cytokinins are: 6-benzyladenine (kinetin), 6-furfuryladenine and 6-isopentyladenine. The specific auxin or cytokinin used, as well as the concentration of each, alters both growth of cells

TABLE 3 Effects of Medium Components on Secondary Products in Plant Tissue Culture

Compound(s)	Species	Range of Levels	Factors that Affect yield	Reference
Nicotine	*Nicotiana tabacum*	0 - 0.25% dw	Type and concentration of auxin	Furuya *et al.*, 1971
Nicotine	*Nicotiana tabacum*	0 - 0.25% dw	Concentration of kinetin	Tabata *et al.*, 1971b
Carboline alkaloids	*Peganum harmala*	0 - 850 nanomoles/gfw	Concentration of 2, 4-D Concentration of phosphate Type of nitrogen source Time of culture	Nettleship and Slaytor, 1974
Thebaine	*Papaver bracteatum*	0.1 - 130µg/gdw	Concentration of auxin Concentration of kinetin Coconut milk	Kamimura *et al.*, 1976
Serpentine	*Catharanthus roseus*	0.01 - 0.5% dw	Type and concentration of auxin Light or dark	Doller *et al.*, 1976
Serpentine	*C. roseus*	0 - 0.8% dw	Type and concentration of auxin	Zenk *et al.*, 1977b
Ajmalicine	*C. roseus*	0 - 1.0% dw	Presence of precursor (tryptophan)	Zenk *et al.*, 1977b
Berberine	*Coptis japonica*	432 - 774µg/gfw	Concentration of 2,4-D	Ikuta *et al.*, 1975
Jatrorrhizine	*Coptis japonica*	459 - 912µg/gfw	Concentration of kinetin	Furuya *et al.*, 1972c
Anthocyanin	*Haplopappus gracilis*	0 - 6mg/gdw	Light or dark Concentration of 2,4-D Time of exposure to light	Stickland and Sunderland, 1972

TABLE 3 (Cont'd.)

Compound(s)	Species	Range of Levels	Factors that Affect yield	Reference
Anthraquinones	Plorinda citrifolia	0 - 900M moles/ gdu	Type and concentration of auxin Type and concentration of carbohydrates Type and concentration of nitrogen source	Zenk et al., 1275
Anthraquinones	Cassia tcra	0.025 - 0.334^ of fu	Concentration of auxin Concentration of kinetin	Talata et al.f 1975
Polyphenols	Rosa sp.	150 - 300ju. moles/ 30iTd culture	Type and concentration of auxin Concentration of nitrate Light or dark	Dauies, 1972a Dswies, 1972b
Phenolic acids	Daucus carota	54 - 1070ug/gdu	Period of growth Concentration of auxin	Suganc et al., 1975
Phsnolics	Rosa sp.	0.9 - 13.4^ig/50rr,l	Concentration of sugar Concentration of nitrate	Amorim et al., 1977
Ubiquinone	Kicotiana tabacum	200 - 600^ug/gdu.'	Concentration of 2,4-D Concentration of cytokinin	Ikeda et al., 1977
Steroids	Solanum xanthocarpu-	0.32 - 0.55mg/gdu;	Types of auxin	Heble et al., 1971
Diosgenin	Dioscorea dcltoidea	0.77 - 2.6mg/gdw	Cholesterol concentration Yeast extract concentration Concentration of 2,4-D	Kaul j *i* al., 1969

TABLE 3 (Cont'd.)

Compound(s)	Species	Range of Levels	Factors that Affect yield	Reference
Plasmin inhibitor	Scopolia japonica	0.1 - 1.5mg/ml culture	Concentration of 2,4-D Concentration of kinetin Nitrogen source	Misawa et al., 1975
Podophyllotoxin	Podophyllum peltatum	-	Concentration of 2,4-D Concentration of kinetin	Kadkade, 1982

dw = dry weight

fw = fresh weight

gdw = gram dry weight

gfw = gram fresh weight

and yield of specific compounds. These compounds are normally used in the concentration range of 0.1 - 10mg/l.

Plant growth regulators affect not only growth and differentiation of cultured cells but also secondary metabolism. Their effects on secondary metabolism vary greatly depending upon the kinds of metabolites. Hormone type and product formation have been well studied. In general, IAA appears to be more favourable in permitting product synthesis and accumulation than NAA and 2,4-D. The synthesis and accumulation of indole alkaloids in cell cultures of Catharanthus roseus was suppressed as long as this material was grown in media with 2,4-D, while in IAA media yields were remarkably high (Zenk et al., 1977a). Also, callus of Datura innoxia, Hyoscyamus niger, Atropa belladonna and Papaver somniferum lacked alkaloids when fed with 2,4-D, while metabolite production was enhanced in the presence of IAA (Khanna, 1978).

Knobloch et al.. (1982) reported that the formation of secondary compounds in cell suspension cultures of Nicotiana tabacum and Catharanthus roseus was induced by adjusting the media to restrict growth, mainly by the lack of phosphate and auxins. The enhanced accumulation of products was preceded by the enhanced activity of key enzymes, diverting primary metabolites into secondary pathways. The results indicate that enhanced activities of key enzymes are a necessary but possibly not the only prerequisite for the increased accumulation of secondary products.

Higher concentrations of auxins suppress while lower concentrations permit the production of metabolites in cell cultures of, for instance, Digitalis lanata (Lui and Staba, 1978), Arachis hypogaea

(Mehta and Shailaja, 1978), and Haplopappus gracilis (Constabel et al., 1971).

The effects of nutritional factors on digitoxin formation by shoot-forming cultures of Digitalis purpurea in liquid media has been studied (Hagimori et al., 1982).

A remarkable difference between auxins in the control of secondary metabolism has also been demonstrated by Zenk et al. (1975). They found that the production of anthraquinones in Morinda cultures occurs in the presence of NAA, but not in the presence of 2,4-D. 2,4-D does not affect the production of emodin-type anthraquinones in Cassia tora cultures (Tabata et al., 1975), but it rather stimulates the production of certain compounds such as diosgenin (Kaul et al., 1969) and L-Dopa (Brain, 1976; Rai, 1978a).

Today most kinds of cells can be grown successfully on or in three to five different media (Gamborg et al., 1976). These media differ mainly in the concentration of mineral salts and the nitrogen source. Zenk et al. (1977a) compared the growth of cell cultures derived from Catharanthus roseus and cultured in media after Gamborg et al., (1968); Heller (1953); Linsmaier and Skoog (1965); Murashige and Skoog (1962); Veliky and Martin (1970), and White (1963). While the cells grew well in all those media, the alkaloid content fluctuated widely. Gamborg's, Linsmaier's, Veliky's and White's media did not yield detectable alkaloids. The medium of Murashige and Skoog, however, enhanced product formation.

Some plant tissue cultures will grow on nitrate as the sole nitrogen source, others require a mixture of nitrate and ammonium ions, and some require glutamine. Other variations include the

presence or absence of casein hydrolysate. In some cases the nature of the nitrogen source, and its concentration, affects the yields of specific compounds.

Sucrose is the carbon and energy source for heterotrophic growth of plant cell cultures that generally gives the highest growth rate. However, cultures from different species may achieve their highest growth rate on other carbon sources such as mannose, galactose, or glucose. Both the sugar and its concentration affect the yield of secondary products in some cases (Dougall, 1979).

In a limited number of cases, medium pH, phosphate concentration, gas type and concentration (O_2, CO_2, or ethylene), and the addition of biologicals (e.g., yeast extract or coconut water), have led to alterations in yield of secondary products (Dougall, 1979).

(d) Precursors

An exogenous supply of a biosynthetic precursor in a culture medium may increase the yield of the final product when productivity is limited by the lack of precursor. Optimization of conditions for product formation in cell cultures is not, therefore, only limited to modifications of the nutrient media and physical environment of the cells, but includes the regulation of secondary metabolism. From experiments on the regulation of phenylpropanoid metabolism, Margna (1977) concludes that the availability of intermediates (phenylalanine, for instance,) exerts a greater influence on the rate of synthesis than the level of the enzyme (phenylalanine-ammonia lyase). Supplying precursors, therefore, would increase yields of metabolites.

In both Scopolia and Datura cultures, experiments showed that production of tropane alkaloids can be markedly increased by the addition of tropic acid, a direct precursor (Tabata et al., 1971a). On the other hand, it was found that tropine, another direct precursor, administered to Datura cells, yields only acetyltropine which is of no medicinal value (Hiraoka et al., 1973).

The administration of a direct precursor is not necessarily effective in increasing the final yield. For example, in Lithospermum cultures, the addition of L-phenylalanine to the culture medium increased the shikonin content more than three-fold, whereas the addition of more direct precursors including p-hydroxybenzoic acid failed to produce any increase (Tabata et al., 1976).

Zenk et al., (1975)reported that production of anthraquinones could not be increased by the supply of indirect precursors but was increased two-fold by the supply of a more direct precursor, O-succinylbenzoic acid. Rai (1977a) studied the effect of additives on the constituents of callus cultures of Cassia senna and found that magnesium acetate and shikimic acid when added together to the basal medium, increased the production of anthraquinones; magnesium acetate alone did not influence the total yield of anthraquinone content and 1,4-naphthoquinone lowered the anthraquinone content.

Adding a precursor to cell cultures may lead to the formation of a metabolite not normally present in cell cultures, nor in the plant from which the cell culture has been derived. The furoquinoline alkaloids dictamine and 8-methoxydictamine found in Ruta plants are synthesized by cell cultures of this plant only after feeding the precursor 4-hydroxy-2-quinolone (Steck and Constabel, 1974;

Steck et al., 1973). After feeding them tryptophan, cell cultures of Phaseolus vulgaris were able to produce the indole alkaloids harmane and norharmane, which are otherwise unknown in this species (Veliky, 1972).

Kurz et al.. (1982) tested serially cultured cell suspensions derived from three cultivars of Catharanthus roseus for their alkaloid production capabilities. They established several cell lines and found that many subcultures, although they had similar growth pattern, differed in their capabilities to synthesize or biotransform specific alkaloids.

Precursor feeding aimed at increasing yields may easily attract attention, because it appears a logical procedure to follow, but, as all the above examples show, it is not simple to find the most effective precursor for increasing the content of the final product. Moreover, from the practical viewpoint, it is important that the precursor be of low cost. In this respect, the experimental demonstration of Ogutuga and Northcote (1970) is of interest in showing that the caffeine content in tea suspension cultures could be increased nearly four times by the supply of inexpensive ammonium, which is presumably the nitrogen source for the purine ring of caffeine.

(e) Mutations

Attempts to induce mutations for the improvement of secondary metabolites production have been reported for a few plant cell cultures. Induced variation in cell cultures has recently been reviewed by Skirvin (1978). Mutagenic agents employed are UV-, X-, and gamma-irradiation, and chemical mutagens.

Eriksson (1965, 1967) tested many mutagens on suspension cultures of Haplopappus gracilis. He was able to observe the effects on cell division and chromosomes. As a result of UV-irradiation, cell cultures varied in pigmentation. More recently Deus (1978) irradiated cells of Catharanthus roseus. Out of 700 clones developed from irradiated cells, 14 showed higher yields of serpentine and ajmalicine (0.69 - 2.0% of dry weight) than controls.

Nishi et al. (1974) treated cultured cells of carrot with the chemical mutagen N-methyl-N'- nitro-N- nitro-soguanidine and obtained many variant clones, which differed in their capabilities to synthesize β-carotene and lycopene. The carotenoid content of these clones was increased nearly three-fold compared with the original strain and it was four times higher than the carotenoid content in the intact roots.

Biosynthetic capability of cultured cells may be improved by the artificial induction of genetic mutation (Berlin and Widholm, 1978), but available information suggests that there is need for more work on the artificial induction of mutant cells useful for medicinal compound production.

(vi) Selection of plant cell lines

This approach involves the exploitation of natural or induced variability among cells by selecting variant cell lines which produce the desired compounds. These lines may be selected at a low frequency from large populations. This is in contrast to the usual approach where attempts are made to induce the entire "wild-type" culture to produce the compound. Selection of cell lines with a capacity for maximum product formation requires the following conditions

(after Tabata et al., 1978):

"(1) The variability in the original cell population must be high enough to offer good starting material for selection.

(2) The preparation of single cell-clones must be possible by protoplast or cell plating techniques.

(3) A simple but sensitive assay method for specific secondary metabolites must be available for the screening of numerous clones, and selected cell lines must be stable."

There are two main methods for selecting the variant cell lines.

(a) Visual selection

Many cell lines which accumulate high levels of various coloured compounds have been selected over the years by visual selection. Cells which show the desired colouration are picked out and subcultured until a pure line is established. Cell cultures which produce pigments would provide a model system to test the feasibility of selecting higher yielding clones, because, for obvious reasons, an assay method is not required.

The isolation of variant cell lines producing high amounts of anthocyanins and carotenoids from respective cell cultures has been reported repeatedly (Zenk, 1978). Eichenberger (1951) reported the isolation of a pure Daucus carota callus line high in β-carotene. This high level was maintained in the line for more than 10 years

(Naef and Turian, 1963). Strains of Macleaya microcarpa with both high and low levels of protopine alkaloids were selected using pigmentation as a marker (Koblitz et al., 1975). Cell clones of Atropa belladonna were also shown to give different pigmentation patterns (Davey et al., 1971).

Mok et al. (1976) initiated cultures from several carrot-root-pigment phenotypes (red, dark orange, light orange, yellow and white). Variations in colourations were noted in lines derived from different sources and also in lines from the same source. However, some relationship to the source of the culture was noted as carotenes predominated in cultures from red and orange roots, while xanthophylls predominated in cultures from yellow and white roots.

(b) Chemical analysis selection

Another method of selection similar to visual selection is one in which compounds are measured chemically in cell colonies in order to identify high producing lines. This method was used by Zenk et al. (1977a) to select Catharanthus roseus strains which produce much higher than normal levels of the indole alkaloids, serpentine and ajmalicine.

Zenk et al. (1977a) developed a highly sensitive and specific radioimmunoassay (RIA) for both serpentine and ajmalicine. Besides specificity and sensitivity, the successful application of chemical analysis methods to selection also requires rapidity so that large numbers of samples can be analysed. Zenk's method was applied to crude extracts where more than 200 samples per day could be processed. This RIA was capable of detecting as low as 0.1μg serpentine in

0.1ml samples.

With this analytical method in hand, Zenk et al. (1977a) used the following strategy to select high serpentine - and ajmalicine - producing strains of C. roseus:

(1) Use of callus derived from high-producing plants.

(2) Selection of variant strains capable of high production.

(3) Development of the optimal production medium.

Ohta and Yatazawa (1982) have isolated and established stable and high nicotine producing tobacco cell lines by selection based on genetic variation and phenotypic expression.

Endo et al. (1982) have made an interesting comparison between two approaches used to produce useful metabolites in cultured plant cells. These two approaches are: metabolic regulation and selection of lines that produce large amounts of metabolites. They compared these two ways to enhance berberine production in cultured Coptis japonica cells. Improvement and optimization of culture conditions gave a berberine production of up to 5% dry weight using non-selected cell lines, but repeated cloning of selected cell lines produced berberine up to 10.1% dry weight.

Watanabe and Yamada (1982) have succeeded in establishing Lavandula vera cell lines which had high biotin contents by a repeated selection method.

(vii) Products with high yields

The yield of a secondary product, expressed per unit weight of tissue, is used as a crude comparison of the productivity of cell cultures with that of whole plants. If the yield approaches or exceeds that of the whole plant, then there is some basis for hope that cell cultures can become a realistic alternative to whole plants.

The yields obtained in early measurements of substances in plant cell cultures were lower than those found in the plant, generally by several orders of magnitude. This line of work has been reviewed by Krikorian and Steward (1969); Puhan and Martin (1971); Constabel et al. (1974); Butcher (1977) and Staba (1977).

As a result of the improvement of media for the growth of plant cells and some major developments in other aspects of plant cell culture, tissue cultures from a number of plants have been established which produce a variety of specific secondary products at levels approximating or exceeding those found in the whole plant. These examples are shown in TABLE 4. In particular, Kaul et al. (1969) obtained good yields of the steroid diosgenin from cell cultures of Dioscorea deltoidea. Since 1973, there has been a substantial increase in the number of reports showing yields of compounds approaching those found in the plant. In the cases of tissue cultures from Catharanthus roseus, Morinda citrifolia, Symphytum officinale, Daucus carota, Nicotiana tabacum (producing ubiquinone) and Coleus blumei, the yields are from five to a hundred-fold higher than recorded for the whole plant or its parts. It is to be noted also that in the cases of the tissue cultures of Ruta graveolens

TABLE 4 Compounds Isolated from Plant Tissue Cultures in Yields Approaching or Exceeding those found in Whole Plants

Compound	Species	Yield from Tissue Culture	Yield from Plant	Reference
Diosgenin	Dioscorea deltoidea	26mg/gdw	-	Kaul et al., 1969
Diosgenin	Trigonella occulta	0.37% dw	Seeds 0.04% dw	Jain et al., 1977
Alkaloids	Ruta graveolens	Kokusaginine, edulinine	Not present	Steck et al., 1971
Sesquiterpene lactones	Andrographis paniculata	Paniculides A, B, C	Not found	Butcher and Connolly, 1971
Glutamine	Symphytum officinale	924µ moles/ gdw	Leaf: 3.5µ moles/ gdw	Tanaka et al., 1974
Biscoclaurine alkaloids	Stephania cepharantha	10 - 22.9mg/ gdw	Tuber: 8.2mg/gdw Aerial part: 1.0mg/gdw	Akasu et al., 1976
Nicotine	Nicotiana rustica	0.291% dw	Root: 0.25% of dw Leaf: 0.35% of dw	Tabata and Hiraoka, 1976
Serpentine	Catharanthus roseus	1.3 dw	Stem: 0.6% dw	Zenk et al., 1977a
Anthraquinones	Morinda citrifolia	900µ moles/ gdw	Root: 110µ moles/gdw	Zenk et al., 1975
Anthraquinones	Cassia tora	0.334% of fw	0.209% of dw of seeds	Tabata et al., 1975
Ubiquinone	Nicotiana tabacum	0.5mg/gdw	Leaf: 16µg/gdw	Ikeda et al., 1977
Proteinase inhibitors	Scopolia japonica	41.0mg/gdw	Stem: 12.5mg/gdw Leaf: 24.1mg/gdw Root: 37.1mg/gdw	Misawa et al., 1975

TABLE 4 (Cont'd.)

Compound	Species	Yield from Tissue Culture	Yield from Plant	Reference
Ginseng saponins	Panax ginseng	0.38% fw	0.3 - 3.3% fw	Zhang et al., 1974
	Panax quinquefolium	0.41% fw		
Serpentine + Ajmalicine	Catharanthus roseus	1.3% dw	0.26% dw	Zenk et al., 1977a
Cyanidin	Daucus carota	10% dw	trace	Alfermann et al., 1975
Phenolics	Acer pseudoplatanus	132mg/gdw	Buds 72mg/gdw Bark 32mg/gdw Root 14mg/gdw	Westcott and Henshaw, 1976
Pseudoephedrine	Ephedra foliata	2.25% dw	Stem 0.6% dw	Khanna et al., 1976
Rosemarinic Acid	Coleus blumei	11.0% dw	2.1% dw in Stems 4.3% dw in Leaves	Razzaque and Ellis, 1977
1,4-Naphthoquinone	Lithospermum erythrorhizon	0.57% dw	root 1.16% dw	Mizukami et al., 1978

and Andrographis paniculata, a series of novel compounds were identified. These compounds were not found in the whole plant.

(viii) Novel compounds from cell cultures

If the spectrum of secondary constituents in plant cell cultures is compared with that in the intact plant and its various organs, it would be found that the plant contains in most cases a wider spectrum than do the cells derived from that plant. TABLE 5 presents examples of secondary products produced predominantly in cell cultures rather than in the plant.

(ix) Possible commercial use of plant cell cultures

The range of products of cell culture origin suggests the feasibility of commercial exploitation, and already a variety of processes which could lead to the production of physiologically active compounds using this technique are covered by patents (Misawa, 1977). Although in some instances cell suspensions are grown in large scale operations (Kato et al., 1972), or produce metabolites in extremely high amounts (Zenk, 1978), their industrial use has not yet been fully realised.

One chemical that has been produced commercially from tobacco cell cultures is acid pyrophosphatase, an enzyme used as a reagent in the study of RNA structure (Efstratiadis et al., 1977). Commercial use of plant cell cultures for large scale production of metabolites appears not to have been achieved so far, although several possibilities exist. The examples are ajamalicine from Catharanthus roseus, and β-methyl digoxin from Digitalis lanata (Reinhard et al., 1978).

TABLE 5 Examples of Secondary Products Predominantly Present in Cell Cultures Rather Than in the Parent Plant

Compound	Class	Plant	Reference
Eduline	Quinoline Alkaloid	*Ruta graveolens*	Steck *et al.*, 1971
Isopimpinellin	Furocoumarin	*Ruta graveolens*	Steck *et al.*, 1971
Rutacultin	Furocoumarin	*Ruta graveolens*	Steck *et al.*, 1971
Armorine	Biscoclaurine Alkaloid	*Stephania cepharantha*	Akasu, 1976
Norsanguinarine	Benzophenantheridine Alkaloid	*Papaver somniferum*	Furuya *et al.*, 1972a
3-Methylpurpurin	Anthraquinone	*Digitalis lanata*	Furuya *et al.*, 1972b
5-Hydroxydigitolutein	Anthraquinone	*Tectona grandis*	Dhruva *et al.*, 1972
Lucidine	Anthraquinone	*Morinda citrifolia*	Leistner, 1975
Paniculides	Sesquiterpene	*Andrographis paniculata*	Overton, 1977

(x) Anthracene derivatives in plant tissue culture

Although small in number, the reports on the presence of anthraquinones in plant tissue cultures have been encouraging. The production of anthraquinones in tissue cultures of Cassia senna L. (Rai et al., 1974), Cassia angustifolia Vahl. (Friedrich and Baier, 1973), Cassia tora (Tabata et al., 1975), Rheum palmatum (Rai, 1978b), Digitalis lanata (Furuya et al., 1972b), Morinda citrifolia (Zenk et al., 1975), Galium mollugo L. (Wilson and Marron, 1978; Bauch and Leistner, 1978) and Rubia cordifolia (Suzuki et al., 1982) has been reported.

Furuya et al. (1972b) demonstrated for the first time the presence of anthracene derivatives in plant tissue culture by isolating anthraquinones from callus cultures of Digitalis lanata. Six anthraquinones were isolated from D. lanata cells, and it was suggested that the anthraquinones were biosynthesized from mevalonate.

In the subsequent years, Baier and Friedrich (1970) and Friedrich and Baier (1973) reported on the isolation and identification of a number of anthraquinone compounds including a trace of sennosides from callus cultures derived from the cotyledons of Cassia angustifolia. The cultures were shown to contain dianthrones. The basic components were chrysophanol, physcion, emodin, rhein and aloe emodin.

In view of the existing biochemical similarities between the two species Cassia senna and C. angustifolia, Rai et al. (1974) have investigated the constituents produced by callus cultures of C. senna and identified chrysophanol, physcion, emodin, rhein and aloe emodin in the oxidative-hydrolysate of the callus. With the exception of physcion, all of these compounds were found in the glycosidic as

well as in the free form. On the basis of these results, Rai et al. (1974) concluded that the callus of C. senna performed in a similar manner to that of C. angustifolia but differed particularly in the absence of sennosides; it had a concentration of free anthraquinones approximately four times greater than the plant and a concentration of glycosides only one sixth of that found in the mature plant. These observations demonstrate not only the expected variations between the constituents of a callus and its parent plant, but also indicate the difference between callus from the two Cassia species (Rai et al., 1974).

In another study, morindone, alizarin and damnacanthol were isolated from suspension cultures of Morinda citrifolia (Leistner, 1973). Again the patterns of compounds produced differed from those found in the intact plants. While damnacanthol was the major compound in the cultures, morindone was dominant in one year old plants. In addition, alizarin was absent in the latter.

Morinda citrifolia cultures produce anthraquinones of the morindone type in amounts up to 10% of dry weight, which is more than nine times the amount produced by the plant itself (Zenk et al., 1975).

A phytochemical investigation of callus culture of Cassia tora (Tabata et al., 1975) demonstrated the presence of chrysophanol, emodin, physcion and an unidentified pigment, all of which are contained in the seeds of the original plant. The content of total anthraquinones in the callus was higher than in dry seeds. Furthermore, it was shown that the concentrations of auxins and cytokinins in the medium influenced the production of total anthraquinones.

In Rheum palmatum callus cultures, Rai (1978b) detected

chrysophanol, emodin and a crystalline compound which appeared to be absent from the mother plant. The total anthraquinone content was 0.3% of the dry weight of callus.

Recently, in another study, Suzuki et al. (1982) have identified a number of colouring compounds such as pseudopurpurin, purpurin, lucidin and their glycosides in callus cultures of Rubia cordifolia, a plant of the Rubiaceae family used as dyestuffs in Japan.

CHAPTER TWO

MATERIALS, METHODS AND RESULTS

PART I

TISSUE CULTURE OF CASSIA SPECIES

(i) Materials

(a) Seeds

The seeds of Cassia occidentalis, C. nodosa and C. alata were obtained from locally growing plants. C. podocarpa seeds were received as a gift from the Forest Research Institute, Ibadan.

(b) Chemicals

The chemicals used throughout the investigation were of 'Analar' grade except where otherwise stated.

(c) Glassware

Petri dishes, and Erlenmeyer (Pyrex) flasks of different capacities were the main glassware used.

(d) Equipment

(d.1) pH meter 7010 (Electronic Instruments Ltd., U.K.)

(d.2) Table-Top-Model Horizontal Flow Air Cabinet (Flow Laboratories Ltd., U.K.)

(d.3) Air-cooled incubator (Gallenkamp, U.K.)

(ii) Methods

(a) Preparation of culture media

The tissues from all the four species of Cassia: C. nodosa, C. occidentalis, C. alata and C. podocarpa were first cultured on laboratory formulated Murashige & Skoog's (1962) revised tobacco medium (TABLE 6) and later, on ready-to-use

Murashige & Skoog (M/S) powdered medium (TABLE 7) from Flow Laboratories Ltd. (U.K.). In both cases the medium was supplemented with appropriate quantities of the growth regulators 2,4-dichlorophenoxy acetic acid and kinetin as determined in section (ii) (e). These were the standard media used throughout the work for the growth of cultures.

(a.1) Preparation of Murashige & Skoog laboratory media (M/S-L)

The basic media were prepared from concentrated stock solutions stored at $4^{o}C$. When supplements were to be incorporated, they were added to the basic media before the adjustment of pH and volume, and before sterilization. After the final adjustment of the volume, the pH of the media were adjusted by addition of 3% KOH or 1% HC1. Media were solidified by the addition of 'Difco' Noble agar, which was dissolved in the boiling media before sterilization. This agar contains a very low content of copper and hence was selected for use. The concentration of agar used was 0.8% w/v.

TABLE 6 Laboratory formulation of Murashige & Skoog's (1962) (revised tobacco) medium (M/S-L)

Stock solution A	
$MgSO_4.7H_2O$	37g
$MnSO_4.4H_2O$	2.23g
$ZnSO_4.7H_2O$	0.86g
distilled water to 1000 ml.	

TABLE 6 (Cont'd.)

Stock solution B	
KNO_3	190g
NH_4NO_3	165g
distilled water to 1000 ml.	
Stock solution C	
$CaCl_2.2H_2O$	44g
KH_2PO_4	17g
H_3BO_3	0.62g
KI	0.083g
distilled water to 1000 ml.	
Stock solution D	
$CuSO_4.5H_2O$	2.5mg
distilled water to 1000 ml.	
Stock solution E	
$Na_2MoO_4.2H_2O$	25.0mg
distilled water to 1000 ml.	
Stock solution F	
$CoCl_2.6H_2O$	2.5mg
distilled water to 1000 ml.	
Stock solution G	
$FeSO_4.7H_2O$	2.78g
Na_2EDTA	3.73g
distilled water to 1000 ml.	

TABLE 6 (Cont'd.)

Stock solution H	
Inositol	10g
distilled water to 1000 ml.	
Stock solution I	
Cyanocobalamin	0.15mg
Folic acid	50mg
Riboflavin	50mg
PABA (para amino benzoic acid)	50mg
Biotin	100mg
Choline chloride	100mg
Ca pantothenate	100mg
Pyridoxine PO_4	100mg
Thiamine HC1	100mg
Nicotinamide	200mg
distilled water to 1000 ml.	
Stock solution J	
2,4-D	100mg
distilled water to 1000 ml.	
(1 ml = 0.1 mg, 10 ml = 1.0 mg)	
Stock solution K	
Kinetin	100mg
distilled water to 1000 ml.	
(1 ml = 0.1 mg, 10 ml = 1.0 mg)	

TABLE 6 (Cont'd.)

Stock solution L	
NAA	400mg
distilled water to 1000 ml.	
(1 ml = 0.4 mg, 10 ml = 4.0 mg)	
Stock solution M	
IAA	200mg
distilled water to 1000 ml	
(1 ml = 0.2 mg, 10 ml = 2.0 mg)	

Preparation instructions

1) Add 10 ml of each of the stock solutions A to I to 300 ml distilled water.

2) Add 35g sucrose and dissolve this. Make up to 500 ml with distilled water.

3) Divide into portions as necessary for addition of appropriate growth regulators.

4) Add the further supplements (yeast extract, coconut water, etc.) where appropriate.

5) Make the total volume of all portions almost 1000 ml with distilled water.

6) Adjust pH to 6.0 with 3% KOH or 1% HCl.

7) Make total volume of all portions up to 1000 ml.

Preparation instructions (Cont'd.)

8) For solid media, add 8g noble agar, bring to the boil on a water bath and pour into flasks.

9) Cap flasks with aluminium foil.

10) Sterilize by autoclaving at 121°C (15 lb/in^2 or 1kg/Cm^2) for 15 minutes. Care should be taken to avoid autoclaving at higher temperatures and/or longer or shorter times.

The following differences exist between Murashige & Skoog's (M/S) media and the laboratory formulated Murashige & Skoog's (M/S-L) media.

M/S medium pH 5.7 - 5.8;

M/S Laboratory media pH 6.

Vitamins and organics in M/S are:

glycine	2.0 mg/l
nicotinic acid	0.5 mg/l
pyridoxine HCl	0.5 mg/l
thiamine HCl	0.1 mg/l

Vitamin complex in laboratory media are according to Khanna & Staba (1968).

(a.2) Ready to use Murashige & Skoog powdered media (M/S)

Flow Laboratories have produced ready-to-use powdered media to published formulations, using chemicals of the highest purity and tested against exacting quality control specifications.

Because of time, cost and space saving and increased standardization, Murashige & Skoog medium as formulated by Flow Laboratories (TABLE 7) was used in the latter part of the work.

TABLE 7 Description of Flow Laboratories powder media: Murashige and Skoog Medium

Formulation:	
Microelements	**mg/l**
$CaCl_2.2H_2O$	440.00
KH_2PO_4	170.00
KNO_3	1900.00
FeNa.EDTA	1650.00
$MgSO_4.7H_2O$	370.00
Microelements	**mg/l**
$CoCl_2.6H_2O$	0.025
$NaMoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
KI	0.83
H_3BO_3	6.20
$MnSO_4.7H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
Amino Acids & Vitamins	
Inositol	100.00
Nicotinic acid	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine	2.00

TABLE 7 (Cont'd.)

Sterile Liquid Supplements (used as appropriate)	
2,4-Dichlorophenoxyacetic acid	5mM
Naphthaleneacetic acid	1mM
Kinetin	1mM
Indoleacetic acid	1mM

Preparation instructions

1) Measure out approximately 25% less water (distilled or deionized) than the required final volume of medium.

2) Add the contents of the package slowly to the water, gently stirring until dissolved. Rinse out the inside of the container to remove all traces of powder.

3) Add the required supplements.

   Note: Heat labile substances should be added aseptically after autoclaving.

4) Adjust the pH of the medium to the desired level (normally 5.5 $\pm$ 0.1) using NaOH or HCl. Mix thoroughly during addition.

5) Add sufficient water to make up to volume.

6) If the medium includes agar, the mixture should be heated, mixing continuously until the solution is clear. Care should be taken to avoid boiling. The temperature of the medium should not fall below 50^{o}C before dispensing.

Preparation instructions (Cont'd.)

7) Dispense into containers, cap or plug and autoclave at 121°C (15 lb/in^2 or 1 kg/cm^2) for 15 minutes.

8) Allow the medium to cool, label and store at 4°C.

(b) Aseptic techniques

All manipulations involving sterile tissues and media were carried out in a Laminar Flow air cabinet. Contamination in the cabinet was reduced by swabbing down the interior area with 70% ethanol 30 minutes before use. Contamination taken into the cabinet was reduced by spraying all culture vessels with either hibitane (5% chlorohexidine gluconate) or cetrimide solution (cetyltrimethylammonium bromide, 0.5% w/v in methylated spirit).

(c) Culture vessels and culture growth chamber

Callus cultures were grown on agar medium in 100, 250 and 500 ml Erlenmeyer flasks. The mouth of each flask was capped with a layer of thick aluminium foil, which had been sterilized in a flame. This was a fine quality foil which had no pores and proved useful in preventing contamination occurring through the mouth of the flasks.

All culture vessels used in the preparation of media including culture flasks were cleaned by a special detergent "7X" marketed by Flow Laboratories Ltd.

Glassware were normally submerged in 5% solution of

"7X" detergent for 30 minutes, removed and rinsed thoroughly in tap water, and finally in distilled water.

After adjustment of pH and volume, media were distributed in flasks as follows: 25 ml in 100 ml flasks; 50 ml in 250 ml flasks; and 100 ml in 500 ml flasks.

The culture vessels were then autoclaved for 15 minutes at a pressure of 15 lbs/sq inch. Instruments (spatula, knife, forceps, etc.) were sterilized by dipping in 70% ethanol and flaming.

All cultures were grown in an incubator at 28°C under constant illumination of approximately 2000 lux intensity, obtained from cool-white fluorescent tubes.

(d) Initiation of callus cultures

The cultures derived from the different plant materials were grown and maintained as callus cultures on solid media.

(d.1) Seed germination

The initial attempts to germinate the seeds of *Cassia occidentalis*, *C. nodosa*, *C. podocarpa* and *C. alata* on sterile nutrient agar were unsuccessful. However, the seeds germinated within 5-7 days on ordinary agar (Agar-Agar; Kobe), and the average rate of germination was higher in the dark than in the light. A reasonably good growth of hypocotyl and cotyledon was obtained for the seeds of all *Cassia* species within 10 days. The final procedure adopted for the germination of seeds was as follows:

1) Dry seeds of Cassia occidentalis, C. nodosa, C. podocarpa and C. alata were transferred to 70% ethanol in separate flasks and put on a shaker for 2-5 minutes.

2) The alcohol was discarded and 20% commercial bleach containing 5% hypochlorite or 1-2% sodium hypochlorite was added. The flasks were left on a shaker for 15-20 minutes. Floating seeds were discarded. In cases where seeds were suspected to have been heavily contaminated with microbial organisms, a second treatment with hypochlorite was necessary.

3) The seeds were rinsed in sterile distilled water.

4) The seeds were then placed on double layers of pre-sterilized filter paper in petri dishes. A small quantity of sterile distilled water was added and petri dishes sealed with parafilm or aluminium foil. The seeds germinated well in the dark at 25-28^{o}C. Relatively few seeds per dish were used, to reduce the possibility of contamination.

5) When the seeds germinated, both hopocotyl and cotyledon were used together as explants for callus formation.

Germination percentages of 60-70% were obtained in C. nodosa, C. alata and C. occidentalis, and 45-55% in C. podocarpa.

(d.2) Initiation, maintenance and harvesting of cultures

Ten-day-old seedlings were chosen for the initiation of cultures because, after such a period, suitable sizes of explants could be obtained. Pieces of cotyledon approximately $6mm^2$ and segments of hypocotyl approximately 10 mm in length were aseptically transferred to M/S medium supplemented with 2,4-dichlorophenoxyacetic acid (0.5 ~ 0.6 mg/l), kinetin (0.3 - 0.4 mg/l) and sucrose (35 g/l). These values of 2,4-D and kinetin chosen were based on an earlier experience (Rai et al., 1974). The pH of the medium was adjusted to 5.5 $\pm$ 0.1. Proliferation on the cut pieces of hopocotyl and cotyledon was normally observed after 8-10 days; after a period of 3 to 4 weeks the growth was about 5 times the size of the explant and turned into a true callus.

Callus cultures were routinely subcultured every six to eight weeks. Each callus mass was divided by a sterile spatula into 3-4 pieces, which were then transferred aseptically to flasks containing fresh medium. Calli were maintained on the same medium from February 1980 to December 1982 and were subcultured at intervals of 6 to 8 weeks. It was observed that by this time cultures would have grown to cover the available surface area of the culture flasks, and that most of the medium had been depleted.

During this period it was observed that the callus cultures of C. nodosa, C. alata and C. podocarpa varied from grey to light brown in colour, but that of C. occidentalis varied from pale green to dark brown.

Callus cultures were routinely harvested and examined chemically for their constituents in the fresh state, or they were dried at 60°C for 24 hours and stored at room temperature.

(e) Influence of hormonal supplementation on callus growth

Initially cultures had been grown on 2,4-D (0.5 to 0.6 mg/l) and kinetin (0.3 to 0.4 mg/l) media. The callus grew successfully, but in order to achieve satisfactory growth rate it was decided to evaluate the need for indoleacetic acid (IAA) and naphthaleneacetic acid (NAA) (either alone or with either 2,4-D or kinetin). It was also intended to find a suitable 2,4-D and kinetin concentration combination. Therefore, preliminary experiments were conducted as outlined below (see also TABLE 8).

A known quantity (5g) of callus was transferred to 100 ml flasks containing different growth supplements and concentration levels (TABLE 8). The growth rate of callus tissue of all Cassia species was assessed at different concentrations of the growth regulators by visual comparisons of the culture flasks. The value of callus prohibited sacrificial measurements.

Ten flasks (100 ml) were used at each concentration of the growth regulators for each of the four Cassia cultures.

TABLE 8 Growth supplements and their concentration levels

Combination number	2,4-D (mg/l)	Kinetin (mg/l)	IAA (mg/l)	NAA (mg/l)
1			0.40	0.40
2		0.30	0.30	
3	0.30			0.30
4	0.50		0.30	
5	0.50			0.30
6		0.30		0.30
7	0.30	0.40		
8	0.40	0.40		
9	0.40	0.20		
10	0.50	0.40		
11	0.60	0.35		
12	0.80	0.50		
13			0.40	
14				0.40
15		0.40		
16	0.40			

(f) The effect of auxin and kinetin on the growth of and anthraquinone formation in callus cultures of C. podocarpa

Callus tissues used in this study were derived from the seedlings of C. podocarpa which had been placed on M/S agar medium supplemented with 2,4-D (0.6 mg/l) and kinetin (0.35 mg/l). The cultures had been maintained on the same medium for 12 months, and had been subcultured at intervals of 5 to 6 weeks.

To examine the influence of plant growth regulators on the growth and pigment production in cultured tissues, 0.5 g of callus tissues (culture age: 12 months) were transferred to 100 ml flasks, each containing 25 ml culture medium supplied with various concentrations of 2,4-D (0.3, 0.4, 0.5, 0.6 and 0.7 mg/l) and kinetin (0.0, 0.2 and 0.4 mg/l). 0.5 g of callus was assessed by weighing each flask before and after inoculation with callus. The callus was allowed to grow for ten weeks in continuous light at 28°C. After ten weeks the weight of callus in each flask was obtained by difference (weight of flask with and without harvested callus). Five flasks were used at each concentration of the growth regulators, to estimate growth and yield of anthraquinones. The combined tissues from identical treatments (i.e. groups of five flasks) were dried in an oven at 60°C for 24 hours prior to estimation of anthraquinones. The combination of calli from five flasks was necessary because preliminary observations showed that the amount of dried tissue left in one flask after drying was too little for the purpose of estimation of anthraquinones. Stale media on which these tissues were grown were also collected

separately and examined for anthraquinones.

The detection of anthraquinone glycosides and their aglycones was carried out by thin-layer chromatographic (TLC) procedures. Colorimetric methods (Rai et al., 1974; Rai, 1978b) were employed for the estimation of these compounds in callus tissue extracts.

Growth indices (GI) of these tissue cultures were calculated for each flask using the following formula (Khanna et al., 1976).

$$GI = \frac{\text{Final wet weight} - \text{Initial wet weight}}{\text{Initial wet weight}}$$

(g) The visual selection of a high yielding cell line in C. podocarpa

Callus tissues used in this experiment were derived from the seedlings of C. podocarpa which had been grown on M/S agar medium supplemented with 2,4-D (0.6 mg/l) and kinetin (0.35 mg/l). The cultures had been maintained on the same medium for 12 months with subculturing at intervals of 5 to 6 weeks.

The grey and brown tissues of C. podocarpa callus were, during subculture, manually separated using sterile forceps and spatula and grown separately in different flasks on fresh medium. It was not possible to separate completely all the brown tissues and grey tissues from one another. Therefore, great care was taken in removing these lumps of brown and grey tissues. It was possible to remove approximately 90% of all the tissues of each colour type from the source callus.

This exercise of separating grey and brown tissues was repeated thrice for each flask at subsequent subculture stages, at six week intervals. During the initial two passages, it was noted that both grey and brown tissues re-acquired their original mixed colour appearance, but towards the end of the third passage it was observed that grey and brown pigments had become dominant in their respective flasks (FIGURE 10).

Qualitative and quantitative analyses were carried out on callus tissues at every subculture stage. The qualitative pattern of anthraquinones was mainly examined by paper and thin-layer chromatographic techniques, and quantitative determination by colorimetric procedures described by Rai et al. (1974) and Rai (1978b).

(iii) Results

(a) Appearance of callus cultures

Callus cultures of Cassia species consist of a soft friable mass. Calli showing no gross differences in the cell characters, were successfully initiated from cotyledon and hypocotyl of all the four Cassia species studied (see Plates 1, 2, 3 & 4).

(b) Influence of hormonal supplementation on callus growth

The M/S medium with 2,4-D (0.6 mg/l) and kinetin (0.35 mg/l) sustained a satisfactory growth of all callus cultures for over two years with subculturing at intervals of 5 to 6 weeks.

The combinations of growth regulators and their respective

FIGURE IQ SCHEME FOR THE VISUAL SELECTION OF A HIGH YIELDING CELL LINE IN C.PODOCARPA CALLUS CULTURES (GROWN ON MS MEDIUM WITH ·6mg/l 2,4-D AND ·35mg/l KINETIN

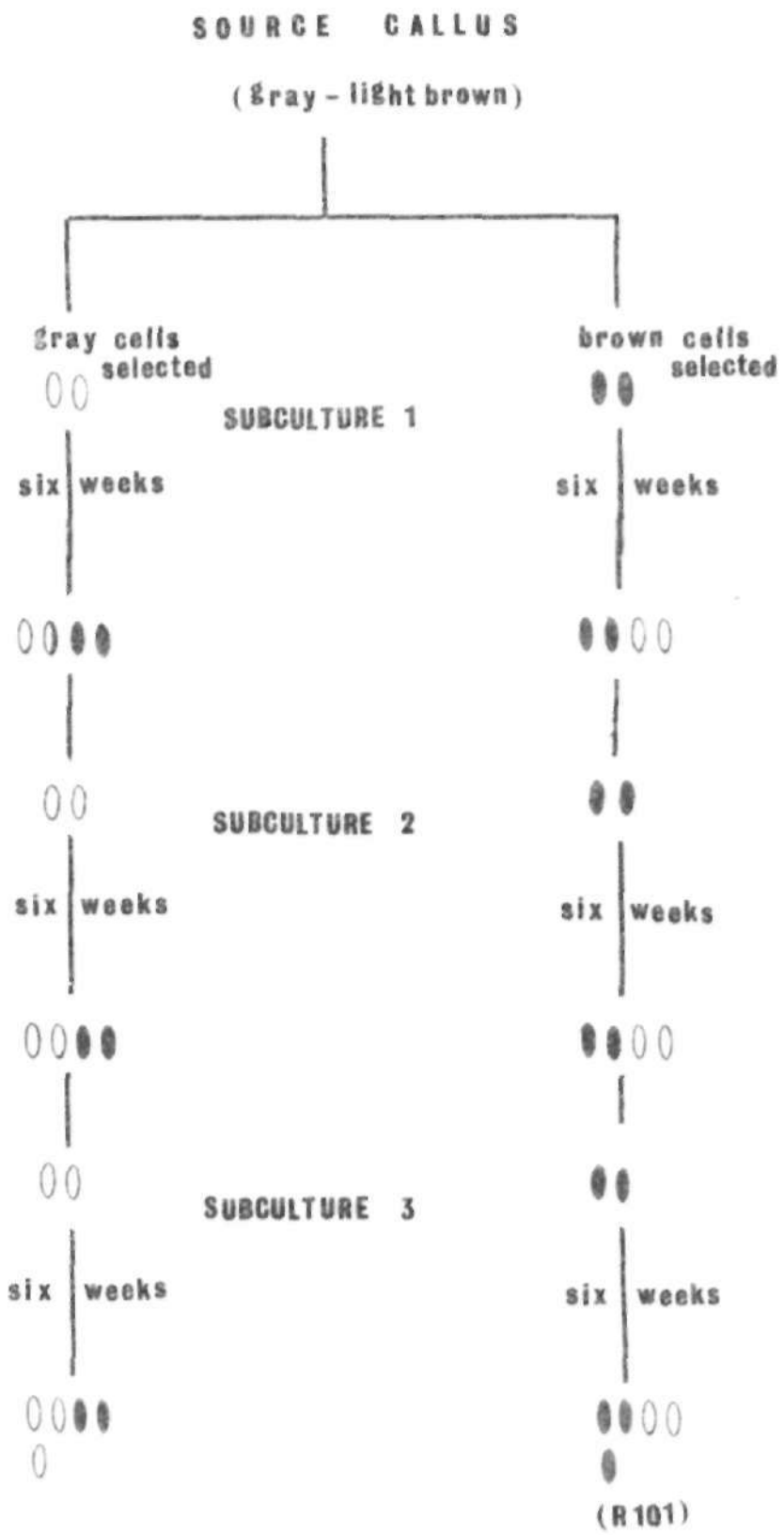




PLATE 1 CALLUS CULTURE OF CASSIA NODOSA GROWN ON MURASHIGE & SKOOG (M/S) MEDIUM

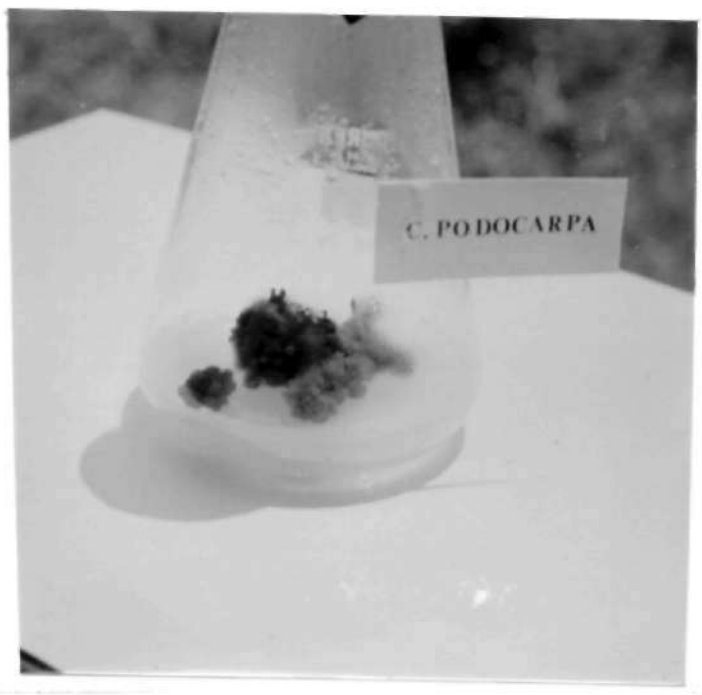


PLATE 2 CALLUS CULTURE OF CASSIA PODOCARPA GROWN ON M/S MEDIUM (Note the larger 'brown' tissue on the left and the smaller mass of 'gray' tissue on the right)



PLATE 3 CALLUS CULTURE OF CASSIA ALATA GROWN ON M/S MEDIUM



PLATE 4 CALLUS CULTURE OF CASSIA OCCIDENTALIS GROWN ON M/S MEDIUM

concentration levels have already been listed in Table 8. Combinations 1 - 6 were rejected because the growth of Cassia cultures was not possible with them; it was observed that the presence of IAA or NAA either with 2,4-D or kinetin tended to induce differentiation in the callus cultures. Combinations 13, 14, 15 and 16 were also rejected because medium with NAA and IAA alone or with kinetin or 2,4-D did not support tissue growth.

The undifferentiated, compact callus tissues of Cassia showed little growth on the culture medium containing a high concentration of kinetin and low concentration of 2,4-D, or a similar concentration of the two (combinations 7, 8). The tissue growth was stimulated by increasing the concentration of 2,4-D up to a level of 0.6 mg/l and kinetin up to a level of 0.35 mg/l medium (combinations 9, 10, 11). The 2,4-D concentration higher than 0.6 mg/l medium appeared to depress the culture growth (combination 12).

On the basis of these preliminary observations, combination 11 (2,4-D, 0.6 mg/l; kinetin, 0.35 mg/l) was chosen for subsequent investigation.

(c) The effect of auxin and kinetin on the growth of and anthraquinone formation in callus cultures of C. podocarpa

The undifferentiated, compact callus tissues of C. podocarpa grew relatively slowly on the culture medium containing 7 mg/l 2,4-D and 4 mg/l kinetin and turned dark brown with the accumulation of pigments during the culture period.

Three anthraquinones, i.e., chrysophanol, emodin and rhein

FIGURE 11 - THE EFFECT OF 2,4-D and KINETIN ON ANTHRAQUINONE YIELD IN CALLUS CULTURES OF C. PODOCARPA

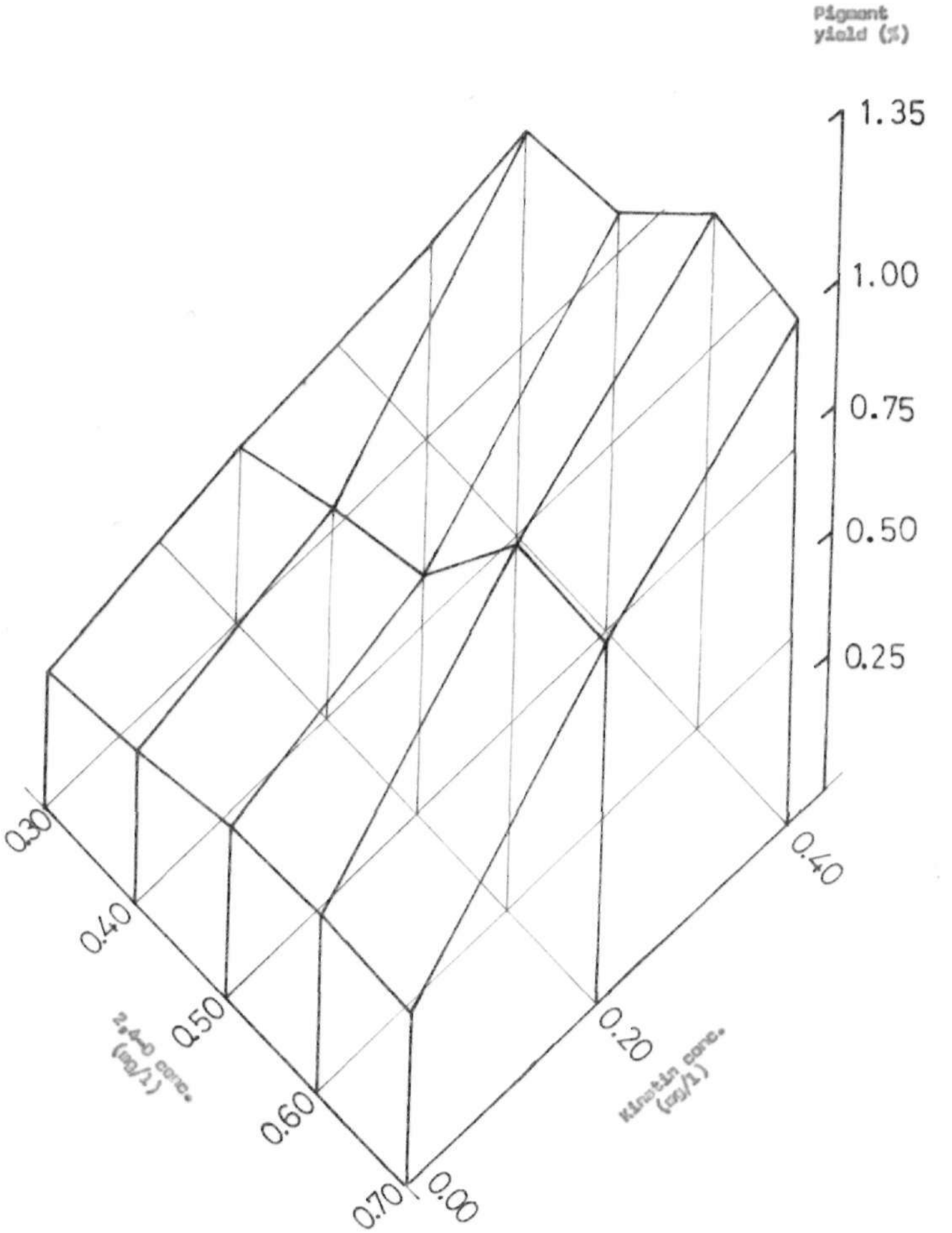
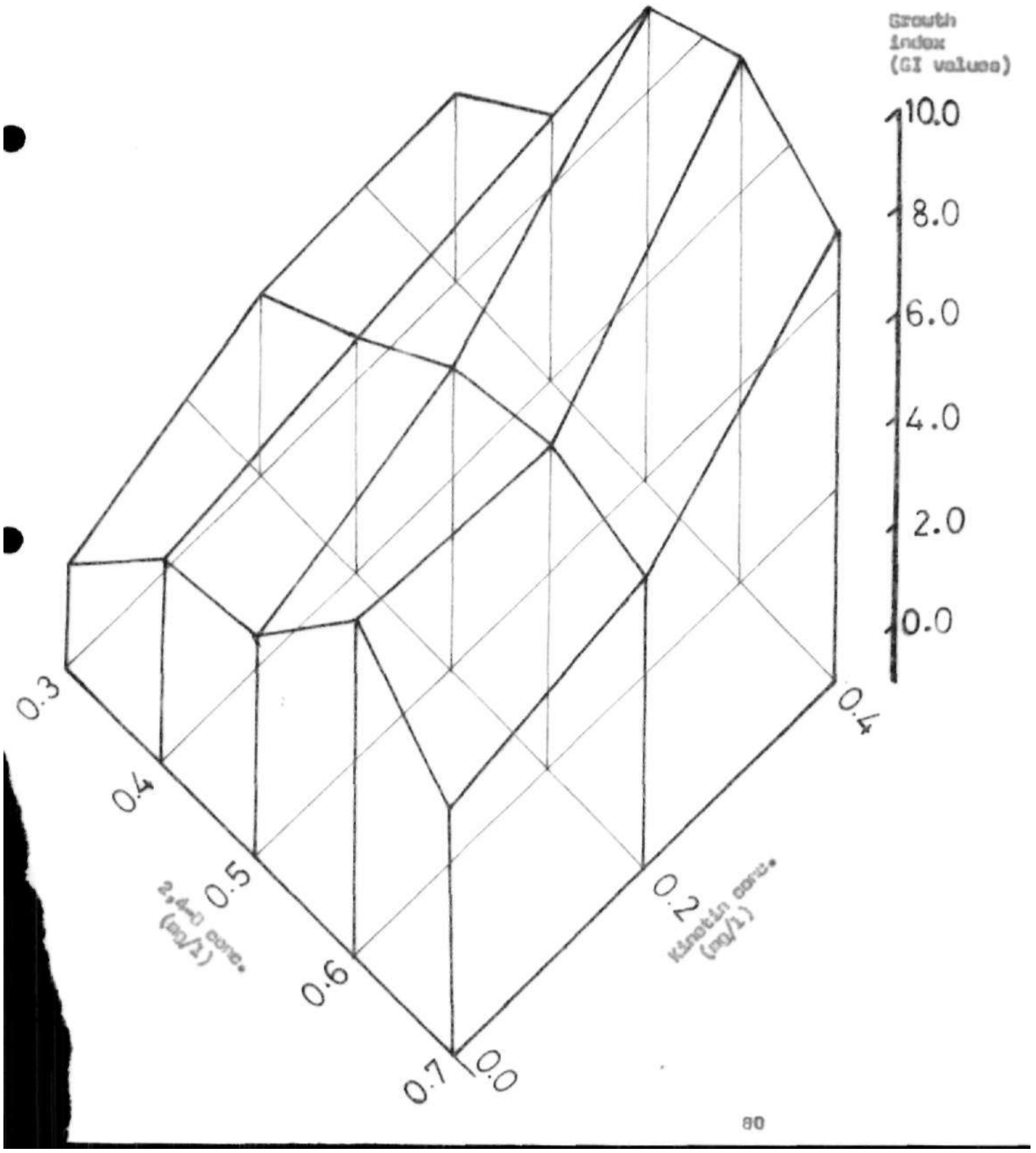


FIGURE 12 - THE EFFECT OF 2,4-D AND KINETIN ON GROWTH OF CALLUS TISSUES OF C. PODOCARPA



five concentrations of 2,4-D (F = 14.76, p = <.01). Because of the design of the experiment (no replication, due to bulking of callus from groups of five flasks) it was not possible to test for interactions.

TABLE 9 The effect of auxin and kinetin on the growth and anthraquinone formation in callus cultures of Cassia podocarpa

Concentration of 2,4-D (mg/l)	Concentration of Kinetin (mg/l)	Growth Index (GI)	Total Yield of Anthraquinones (on % dry weight basis)
		SE	
0.30	0.00	2.00 ± 0.042	0.27
	0.20	3.40 ± 0.022	0.35
	0.40	3.60 ± 0.008	0.39
0.40	0.00	3.80 ± 0.012	0.29
	0.20	4.40 ± 0.019	0.41
	0.40	5.00 ± 0.033	0.80
0.50	0.00	4.20 ± 0.085	0.33
	0.20	5.80 ± 0.032	0.47
	0.40	9.00 ± 0.149	0.83
0.60	0.00	6.40 ± 0.014	0.35
	0.20	6.20 ± 0.049	0.73
	0.40	10.00 ± 0.176	1.31
0.70	0.00	4.60 ± 0.054	0.34
	0.20	5.80 ± 0.029	0.72
	0.40	8.60 ± 0.111	1.00

SE = Standard Error

(d) The visual selection of a high yielding cell line in C. podocarpa

Little change in qualitative and quantitative spectra with respect to anthraquinone constituents was observed in grey cells and brown cells examined six weeks after first and second subculture stages. The chemical analysis carried out six weeks after the third transfer also revealed that there was no change in the qualitative spectrum, but the total accumulation of anthraquinones was higher in the brown cell line, by nearly 40 percent, than in the source callus (TABLE 10). By contrast, the total concentration of anthraquinone compounds was found to be lower in grey callus tissues in comparison to the source callus. In three separate analyses of variance, the differences in free anthraquinones ($F = 166.9$), bound anthraquinones ($F = 4281.3$) and total anthraquinones ($F = 1057.5$) between the original cells, brown cell line and grey cells (see Table 10), are all highly significant ($p = <.01$).

The brown cell line, thus obtained, after three successive selective transfers was coded R101. The determinations carried out on this cell line 6, 12 and 18 weeks after the initial determinations, suggested that the anthraquinone level remained virtually unchanged.

The chemical tests of the media on which the tissues grew gave a negative test for anthraquinones, indicating that anthraquinones are retained in the tissues and not released into the medium.

TABLE 10 Anthraquinone content of cell line R101 and original callus of Cassia podocarpa, expressed as % w/w on a dry weight basis (mean of ten determinations)

	Original cells	Brown cell line R101	Grey cells
	SE		
Free anthraquinones	0.80 ± 0.003	1.00 ± 0.016	0.80 ± 0.003
Bound anthraquinones	0.50 ± 0.003	0.80 ± 0.002	0.40 ± 0.005
Total content	1.30 ± 0.004	1.80 ± 0.016	1.20 ± 0.005

SE = Standard Error

PART II

DETERMINATION OF ANTHRAQUINONES

(i) Materials

The chemicals used were of 'Analar' grade. Also, a number of reference compounds were used, all of which were received as gifts from different sources.

	Reference compounds	Source
1.	Chrysophanol	Koch-light Laboratories Ltd., Bucks,, U.K.
2.	Physcion	Dr. Haruj Oshio, Takeda Chemical Industries Ltd., Opaka, Japan.
		Dr. C.W. Van Eijk, Central bureau voor Schimmal-cultures, Baarn, The Netherlands.
		Reckitt & Colman, Hull, U.K.
3.	Emodin	Ralph N. Emmanuel Ltd., Middx., U.K.
4.	Rhein	Prof. J.A. Lemli, University of Leuven, Belgium.
5.	Aloe emodin	Prof. J.A. Lemli, Roth Laboratories, W. Germany.
6.	Rhein glucoside	Dr. H. Oshio.
7.	Aloe-emodin glucoside	Dr. H. Oshio.
8.	Mixture of 1- and 8-glucosides-chrysophanol	Dr. H. Oshio.
9.	Rhein monoglucoside	Dr. J.K. Zwaving, Laboratorium vbor Farmacognosie, Groningen, The Netherlands.
10.	Physcion monoglucoside	Prof. Lemli.
11.	Aloe-emodin monoglucoside	Prof. Lemli.
12.	Emodin monoglucoside	Prof. Lemli.
13.	Chrysophanol-anthrone	Pharmacognosy Laboratories, Welsh School of Pharmacy, UWIST, Cardiff, U.K.

	Reference compounds	Source
14.	Chrysophanol-dianthrone	Pharmacognosy Laboratories, Welsh School of Pharmacy, UWIST, Cardiff, U.K.
15.	1,8-dihydroxy-anthraquinone	Prof. Lemli.
16.	1,2-dihydroxy-anthraquinone	Prof. Lemli.
17.	Sennoside A	Reckitt & Colman, Hull, U.K.
18.	Sennoside B	Reckitt & Colman, Hull, U.K.
19.	Sennoside C	Dr. H. Oshio.
20.	Sennoside D	Dr. H. Oshio.
21.	Sennidine A	Sandoz Products Ltd., London.
22.	Sennidine B	Sandoz Products Ltd., London.
23.	Sennidine A + B	Dr. H. Oshio.
24.	Physcionanthrone	Dr. H. Oshio.
25.	Emodinanthrone	Dr. H. Oshio.
26.	Aloe emodinanthrone	Dr. H. Oshio.
27.	Rheinanthrone	Dr. H. Oshio.

Equipment

1. 'Shandon' chromatographic tank.
2. 'Vario-Ks-Chamber' (Camag, Switzerland).
3. 'UVISPEK D275' spectrophotometer.
4. Vitatron TLD 100 Universal Densitometer. (Dieren, The Netherlands.)
5. High performance liquid chromatography unit. (Waters Associates, Milford, Massachusetts.)
6. Infra-red spectrophotometer (Pye-unicam.)
7. U.V. spectrophotometer (Pye-unicam.)

(ii) Methods

(a) Qualitative analysis

The callus tissue of Cassia spp. was subjected to different extraction procedures and the extracts were examined for the presence of anthracene derivatives by chemical methods as well as by paper, thin-layer, column and high performance liquid chromatographic methods.

(a.1.) Chemical tests

The dried as well as the fresh callus material was tested for the presence of anthracene derivatives applying the Borntrager reaction. There are several published modifications of the Borntrager test (Christensen and Abdel-Latif, 1949). However, Fairbairn's modification (1942) was found to give superior reproducibility and was adopted for the routine test.

(a.1.1) Borntrager test

0.1g of the powdered material was boiled with 5ml of 100% sulphuric acid for at least two minutes, filtered whilst hot and the filtrate was cooled and shaken gently for a minute with an equal volume of benzene. The benzene layer was allowed to separate completely from the aqueous layer and by means of a teat-pipette, the benzene layer was transferred to a test tube. About half of its volume of ammonia (10%) was added, the test tube was then shaken gently and the layers allowed to separate. If anthraquinones were originally present in the tissue material, the lower ammoniacal layer would acquire a delicate rose-pink colour.

(a.2.) Paper chromatography

(a.2.1) Chromatographic paper

Whatman No.1 paper gave satisfactory results for qualitative work with anthraquinone derivatives, and was used for this purpose.

(a.2.2) Solvent system

The following solvent systems were used:

I - toluene (Betts et al., 1958)

II - acetone - water (7 : 3) (Kaji et al., 1965)

III - water - acetone - benzene (2 : 1 : 4) lower phase (Fairbairn & Shrestha, 1966)

IV - petroleum ether (40 - 60°C) - toluene - xylene - methanol (4 : 1 : 1 : 1)

V - petroleum ether (40 - 60°C) - toluene - xylene - methanol (4 : 1 : 1 : 2)

VI - n-butanol saturated with water (Paris and Durand, 1956)

VII - toluene - cyclohexane - n-propanol - n-butanol (40 : 50 : 7.5 : 2.5) (Kinget, 1966)

VIII - n-propanol - ethyl acetate - water (4 : 3 : 3) (Horhammer et al., 1959)

(a.2.3) Preparation of extracts

Method 1

0.5g of the dry callus material was macerated in chloroform at room temperature for 24 hrs. and filtered. The filtrate was concentrated to about 2ml under reduced pressure in vacuum. Approximately 5 μl of chloroform solution were spotted on the paper.

Method 2

0.5g of the dry callus material was mixed with 20ml dil. HC1. The mixture was heated, boiled for 1 min., cooled and shaken with 5ml benzene. Approximately 10 μl benzene solution were spotted on the paper.

Method 3

0.5g of the dry callus material was added to aqueous $FeCl_3$ (15ml, 25%) + HC1 (15ml, 4N) and refluxed on a boiling water bath for 30 min. The mixture was extracted with chloroform (30ml) and the extract evaporated to dryness. The residue was taken up in methanol (2ml). Approximately 5 μl methanol solutions were spotted on the paper.

(a.2.4) Preparation of reference solutions

Solutions of anthraquinones and anthrones were prepared by dissolving small quantities of these in chloroform, and of their glycosides in methanol.

(a.2.5) Application of sample

The extracts and reference solutions were applied on chromatographic paper by means of a capillary pipette, using an air-blower to prevent the spots from spreading further than about 6mm diameter. In two phase solvent systems, the mobile phase was placed at the bottom of the chromatographic tank while the stationary phase was kept separately in a beaker placed at the bottom of the tank. The chromatograms were developed

at room temperature between 15 - 22°C.

(a.2.6) Detection of spots

After development was complete, the chromatograms were removed and dried in an electrically heated oven at 60 - 90°C. These were then viewed in daylight and ultraviolet light before and after applying a suitable detection reagent.

When a quick observation or intensification of the colours of substances of the treated chromatograms was required, ammonia vapour was applied. Some other chemical reagents used for the detection of various anthraquinones were: 0.5% (w/v) magnesium acetate in methanol (Shibata et al., 1950), 0.5% (w/v) aluminium acetate in methanol, 1% (w/v) aluminium chloride in methanol, and 2.5% (w/v) aniline phthalate in butanol.

(a.2.7) R_f values

The R_f values quoted are the average of five determinations, and were obtained using pure compounds.

(a.3.) Thin-layer chromatography

(a.3.1) Purification of kieselguhr G (Merck)

A slurry was prepared by mixing 200g of kieselguhr G (Merck) and one litre of 36 per cent HCl. The mixture was left overnight. Then the adsorbent was filtered off and washed thoroughly with

water to remove the acid. After drying for 3 hrs. at 110 - 120°C the adsorbent was allowed to cool and used for the preparation of thin layer plates.

(a.3.2) Preparation of thin layer plates

Method 1

A slurry of silica gel was prepared from 35g of a mixture of Kieselgel G (Merck) and purified Kieselguhr G (Merck) (1 + 6) and 70 ml of water. This was applied to glass plates 20 x 20 cm by means of a leveller (Shandon & Co.), with the spreader set at 0.25mm. The plates were activated at 100°C for one hour, cooled and stored in a desiccator over anhydrous silica gel till they were required for use.

Method 2

Kieselgel G (Merck) or Kieselgel 60 (Merck) 35g was shaken vigorously with 70 ml distilled water and applied to glass plates 20 x 20 cm. The plates were allowed to stand for 15 min. at room temperature and then activated by drying in an oven at 100°C for one hour. The coated plates were stored in a desiccator over anhydrous silica gel till they were required for use. The thickness of the thin layer plates was 0.25mm.

(a.3.3) Solvent Systems

The following solvent systems were used for the separation of hydroxyanthraquinones, anthrones, dianthrones, dianthrone glycosides and anthraquinone glycosides. Some of the solvent

mixtures were newly developed while some have been used before by other workers.

I. toluene-diethylether-ethanol (14 : 5 : 1)

II. light petroleum (40 - 60°C) - ethyl formate - formic acid (90 : 4 : 1)

III. benzene-methanol (1 : 4)

IV. benzene-ethyl formate-formic acid (15 : 5 : 1) (Stage I); light petroleum (40 - 60°C) - ethyl acetate - acetic acid (90 : 5 : 5) (Stage II)

V. hexane-glacial acetic acid (4 : 1) (Anton and Duquenois, 1967)

VI. benzene-glacial acetic acid (4 : 1) (Moes, 1964)

VII. light petroleum (40 - 60°C) - ethyl acetate - formic acid (90 : 4 : 1)

VIII. toluene - amyl alcohol - ethanol (14 : 5 : 1)

IX. acetone - ethyl acetate - water (3 : 4 : 3)

X. iso-propyl alcohol- ethyl acetate - water (4 : 4 : 3)

XI. n-propanol - ethyl acetate - water (Horhammer et al., 1963) (4 : 4 : 3)

XII. formic acid - water - tertiary butanol - ethyl methylketone (15 : 15 : 40 : 30)

(a.3.4) Development

The development (ascending) was carried out at room temperature between 15 - 22°C in a 'Shandon' chromatographic tank. Prior to the development, the atmosphere within the tank was saturated with the solvent system used by means of a solvent saturation pad.

The separation of dianthrone glycosides and anthraquinone glycosides was also performed in a "Vario-Ks-Chamber". The

plate was equilibrated for 30 mins. at 32% relative humidity (RH) prior to development. The desired humidity was obtained with an appropriate sulphuric acid + water mixture; it was found that in this case the RH which produced optimum separation was 32%.

(a.3.5) Detection and visualization

First, the spots were studied directly on the chromatogram in daylight and UV light, then the following detection reagents were used, where necessary.

1. 5% KOH (w/v)
2. 5% KOH in methanol
3. 5% KOH in 50% methanol
4. pyridine-methanol (1 : 1)
5. 25% nitric acid
6. aniline - phosporic acid in butanol (1 : 2)

Chromatograms sprayed with reagent 1, 2 or 3 were heated at 90°C for 5 minutes, while those treated with reagents 4 and 5 were heated at 120°C for 20 min. All were examined in daylight and under UV light. The spray reagent 6 was used only for detection of sugar molecules.

(a.3.6) Preparation of extracts

The qualitative analysis of anthraquinone derivatives present in callus cultures of *Cassia podocarpa*, *C. alata*, *C. nodosa* and *C. occidentalis* was carried out by isolating

the free and combined anthraquinone derivatives separately and identifying these on TLC plates, using authentic reference substances.

In addition, extracts containing anthraquinone aglycones of the oxidative-hydrolysate, and dianthrone as well as anthraquinone glycosides were prepared and examined by TLC procedures.

Method 1 Free anthraquinones

I. About 1g of the dry callus powder was completely extracted with hot benzene and concentrated under reduced pressure at 40^{o} to about 2 ml.

II. About 1g of the dry callus powder was completely extracted with diethyl ether. The ether extract was concentrated by atmospheric evaporation of diethyl ether.

Extracts I and II were used for TLC studies using solvent systems no's. V, VI and VII.

Method 2 Combined anthraquinones

I. O-glycosidic anthraquinones

The marc, after extraction of free anthraquinones (from II, Method 1), was heated with dilute hydrochloric acid (25 ml) for fifteen minutes on a water bath; the solution was cooled and the liberated aglycones were extracted with diethyl ether. The diethyl ether solution was concentrated by atmospheric evaporation.

This extract was used for TLC studies involving solvent systems no's. I, II, III, IV, V, VI and VII.

The aqueous phase was retained for testing the presence of C-C-glycosides and for the TLC identification of sugar molecule.

II. C-C-glycosides

The aqueous extract, after separating the ether extract as above, was used for isolating the C-C-glycosides. A part of the aqueous portion was boiled with ferric chloride and HCl for 15 minutes (Fairbairn and Simic, 1963). After cooling, the solution was extracted with ether. The ether extract was tested with ammonia for the presence of C-C-glycosides. The test for C-C-glycosides was negative in all the four Cassia callus tissues.

Method 3 Anthraquinone aglycones of the oxidative-hydrolysate

About 1g of the powdered callus material was added to aqueous $FeCl_3$ (15 ml, 25%) + HCl (15 ml, 4N) and refluxed on a boiling water bath for 30 min. The mixture was extracted with chloroform (30 ml) and the extract evaporated to dryness. The residue was taken up in methanol (2 ml). 5 μl methanol solutions were spotted on the TLC plate.

This extract was used for TLC studies using solvent systems no's. I, II, III and IV.

Method 4 Solutions containing dianthrone and anthraquinone glycosides

About 1g of the powdered callus material was extracted with 15 ml 70% methanol for 10 min. by vigorously shaking in a glass-stoppered centrifuge tube. The extract was centrifuged and the supernatant liquid collected. This was reduced to 1 ml at 40°C. 10 μl of this solution were spotted on the TLC plate.

This extract was used for TLC studies using solvent systems no's. VIII, IX, X and XI.

Method 5 Extract containing the sugar molecule

The aqueous phase (see combined anthraquinones) obtained after the hydrolysis of the marc and subsequent extraction with ether, was chromatographed on precoated cellulose (Polygram MN 300), 0.1mm, thin layer plates and developed with solvent system XII. Only one spot was observed on the chromatogram and it produced an identical R_f value and colour response as that of standard glucose.

(a.3.7) Preparative thin-layer chromatography

The plates (adsorbent, kieselgel G (Merck), for preparative TLC were prepared by means of a leveller (Shandon & Co.), with the spreader set at 1.00mm. The extracts were applied in the form of a band (approx. 5mm wide). The plates were developed in the solvent system: toluene - diethyl ether - ethanol (70 : 25 : 5). The compounds were recovered by scraping off the separated zones separately and extracting them with chloroform.

The identity of these isolated compounds was further confirmed by melting point, mixed melting point, ultraviolet, visible and infrared spectra.

(a.4.) Separation of anthraquinone glycosides on a column of Sephadex LH-20

Anthraquinone glycosides in callus cultures of different Cassia species were extensively investigated by various thin layer chromatographic systems described earlier. In order to ascertain if some of these glycosides could be separated and isolated in their genuine form without destroying their molecular composition, the Zwaving procedure (1968) recommended for the separation of anthraquinone glycosides in senna was followed.

Because of the greater concentration level of glycosides in callus cultures of Cassia podocarpa, the column chromatographic analysis was limited to C. podocarpa cultures.

An extract was prepared by boiling about 500mg of the callus tissue with 50ml 70% methanol and drying 25ml of this solution in vacuo below 30°C.

Sephadex LH-20 (Pharmacia) was allowed to swell in 70% methanol for 30 mins. before packing into a glass column (20 x 1cm). The extract was dissolved in 70% methanol and applied to the top of the column. Of the different solvents tested, 70% methanol was found superior and used as an eluent, at a column flow rate of 0.1 ml/min. approximately. Zones eluted from the column were evaporated to dryness in vacuo and investigated by thin layer chromatography on Kieselgel G (Merck), using n-propanol -

ethyl acetate - water (4 : 3 : 3), as the solvent.

(a.5.) High performance liquid chromatography

High performance liquid chromatography (HPLC) has been used in the study of the anthraquinone components of rhubarb species by Castagnola et al. (1975); Rai et al. (1975), and Rai (1978b). These investigators used reverse phase partition and adsorption HPLC systems for the separation of anthraquinone mixtures, but the wide range of polarities of anthraquinones resulted in unsatisfactory, or incomplete separations.

Rhein could not be satisfactorily resolved from the other components of a mixture of rhubarb anthraquinone constituents by the HPLC methods previously reported. This necessitated the development of an improved HPLC procedure utilising ion-pairing and gradient elution which provides separation of a mixture of the most commonly occurring anthraquinone aglycones.

The HPLC instruments used for the separation of anthraquinones were "Waters 600CA and 6000 solvent delivery systems" supplying a gradient through the "Waters 600 gradient eluter", fitted with a "Cecil CE 272" UV detector set at a fixed wave length of 260nm.

(a.5.1) Preparation of paired ion chromatography reagent (PIC A) (tetrabutyl ammonium phosphate)

One bottle of PIC Reagent A was added to 1000ml of the methanol/water (60 : 40) mixture and stirred for about five minutes. The mixture was filtered through a 0.45 micron vacuum

filter (Millipore Corporation, Massachusetts).

(a.5.2) Conditions of separation

Column	:	Apex 5µm. octadecyl silane-silica (ODS), 30cm x 4mm I.D.
Solvent	:	Initial conditions - Methanol - water (60 : 40) with the addition of 1.75 ml/l of PIC A (Waters). Final conditions - Methanol - water (86 : 14) Gradient - Linear gradient (curve 6) for 20 mins.
Flow rate	:	2 mls/min.
Preparation of standard solutions	:	0.05% w/v solutions of pure chrysophanol, physcion, emodin, aloe-emodin and rhein in dichloromethane.

(a.5.3) Preparation of callus tissue extracts for HPLC analysis

1g each of the dried, finely powdered callus material of C. podocarpa, C. alata, C. nodosa and C. occidentalis was added separately to each 50ml HCl (4N) and refluxed on a water bath for 25 min. The solution was cooled, and then repeatedly extracted with diethylether until all the pigments were extracted from the acid solution (as was seen by colourless ether). The combined ether solution was evaporated to dryness under reduced pressure and the residue collected in 2ml dichloromethane.

(a.5.4) Identification

Identification of compounds was based on comparison of

peak retention times with those of standard substances.

(b) Quantitative analysis

Most of the published methods for estimating free and combined anthraquinones in crude drugs do not allow the compounds to be determined separately (Lemli, 1964; Shah et al., 1972). In the present method, the anthracene derivatives in callus cultures of Cassia species have been estimated as free anthraquinones and combined anthraquinones separately by colorimetric procedures. In the case of C. podocarpa cultures, the amount of the individual components was also estimated by densitometry.

(b.1) Colorimetric estimation of anthraquinones in callus cultures of Cassia spp.

The quantitative estimation of anthraquinone derivatives in callus cultures was based on the method described by Rai et al. (1974) and Rai (1978b). The quantitative determination of aglycones is subject to error due to the presence of plant pigments in the extract, which may be partitioned into the organic layer assayed. Therefore, the organic liquid used to partition aglycones should be highly selective and should give good quantitation. Chloroform was found to be the organic solvent of choice for the extraction of free anthraquinones from the callus tissue, but because ether has greater volatility, it was preferred for the extraction of the aglycones liberated by hydrolysis.

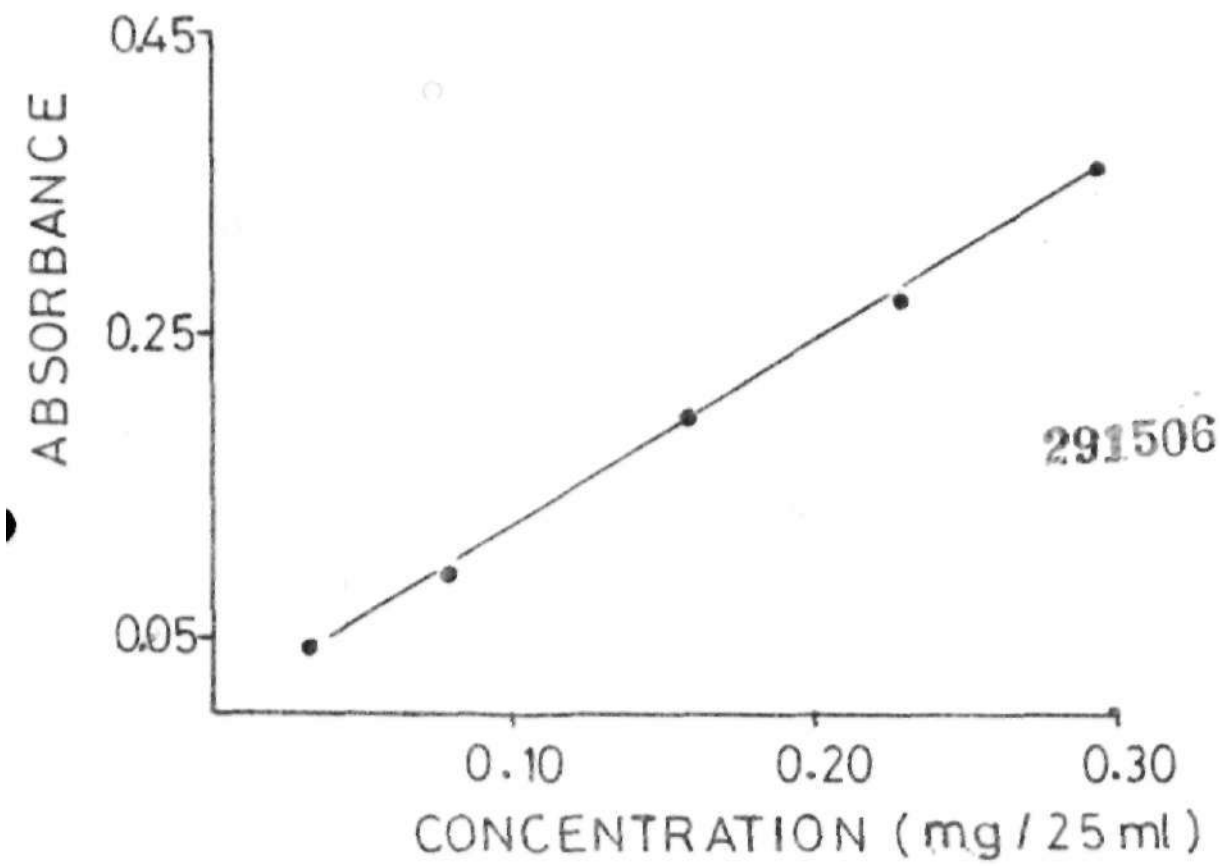


FIGURE 13 CALIBRATION CURVE FOR 1,8-DIHYDROXYANTHRAQUINONE

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(b.1.2) was heated with water (50 ml) for 20 minutes to extract the water soluble glycosides, the suspension was then cooled and centrifuged. To 10 ml of the supernatant HCl (2 ml, 10N) was added, and the mixture was refluxed for 20 min. The solution was cooled, extracted with ether (3 x 25 ml) and the extracts combined and washed with water (25 ml) to remove excess acid. The volume of this extract was made up to 100 ml with ether. 10 ml of this solution was evaporated and the residue taken up with 10 ml of 5% NaOH + 2% ammonium hydroxide solution and the extinction measured at 510 nm.

(b.1.4) Estimation of total anthraquinones

0.5g of accurately weighed powdered callus tissue was extracted to exhaustion with methanol (100 ml) at room temperature, filtered, and the methanol was evaporated *in vacuo*. The residue was dissolved in $FeCl_3$ (20 ml, 10%) and HCl (2 ml, 10N) and the mixture refluxed. The solution was cooled, extracted with ether and washed with water, and the volume was made up to 100 ml. 10 ml of this solution was evaporated and the residue taken up with 10 ml of 5% NaOH + 2% ammonium hydroxide solution. The extinction was measured at 510 nm.

(b.2.) Thin-layer densitometric determination of anthraquinones in callus cultures of C. podocarpa

Lemli (1963) had devised a method for the paper densitometric estimation of the dianthrone aglycones of some glycosides in senna leaf and pods, and later Dequeker *et al.* (1964) estimated dianthrone aglycones in *Rheum palmatum* by densitometry on paper

chromatograms. Direct densitometric determination of anthraquinones (Rai and Turner, 1975) and anthrone glycosides (Lemli and Cuveele, 1975) on thin layer chromatograms has now also been reported.

The method used here, involved an internal standard to obviate both problems associated with accurate sample application on thin layer chromatograms (Brain and Turner, 1971), and the need for relatively complex apparatus.

The thin-layer densitometric method described earlier by Rai and Turner (1975) enabled the estimation of individual anthraquinones with reference to their corresponding pure compounds. It, however, required the preparation of separate calibration curves for each reference compound, and for each chromatogram. In the routine analysis of tissue cultures of Cassia species, it was found that this method could not be applied on a regular basis because of its tedious nature and also the involvement of reasonably high quantities of reference compounds. This necessitated the development of a simpler method for the estimation of individual dihydroxyanthraquinone derivatives, by which anthraquinone compounds in callus cultures are estimated as equivalent of 1,2-dihydroxyanthraquinone (alizarin), used as an internal standard.

(b.2.1) Procedure

The extraction procedures were the same as described for the qualitative processes; 5% KOH in methanol w/v was used as the spray reagent. The extracts were evaporated to dryness and

collected separately in 2 ml chloroform containing alizarin (0.02% w/v). Alizarin was adequately resolved from other anthraquinones and was therefore used as an internal standard. The callus extracts and reference solutions (5 μl) of chrysophanol, physcion, emodin, rhein and aloe emodin were applied on precoated silica gel N-HR (Camblab Cambridge), 250 μm plates.

After development with a benzene - ethyl formate - formic acid mixture (75 : 25 : 2) visualization was effected by spraying the plate with 5% KOH in methanol (w/v) and heating at 90^{o}C for five minutes. All the compounds produced an orange-pink colour except alizarin which gave a purple colour. The R_f values were chrysophanol, 0.74; physcion, 0.65; alizarin, 0.44; emodin, 0.35; rhein, 0.29; and aloe emodin, 0.25.

Scanning of the coloured spots on the chromatogram was carried out with a "Vitatron TLC 100 Universal Densitometer" employing a 'flying spot' scanning system with transmitted light in a logarithmic mode. An interference filter of 525 nm was used at which the compounds produced optimal light absorptions (FIGURE 14). A typical densitometric trace is shown in FIGURE 15. As a spot is scanned, a peak is produced on the recorder chart by the sinusoidal scanning of the densitometer. The integrated area under a peak is related directly to the amount of material in the spot.

A calibration curve was prepared for alizarin which showed a linear response between concentration and integrated peak area over a range of 0.40 to 6.00 μg. (FIGURE 16). The

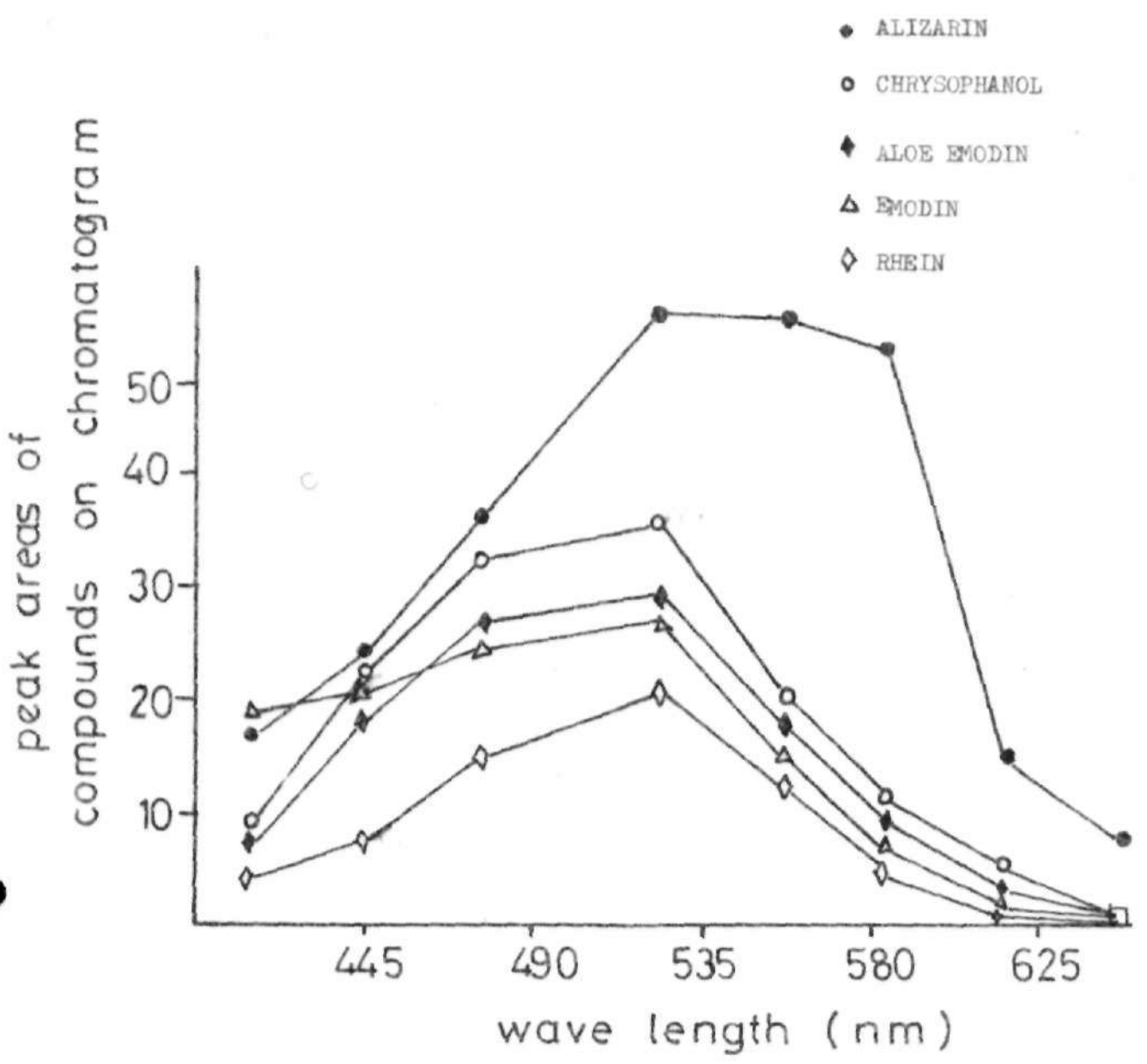
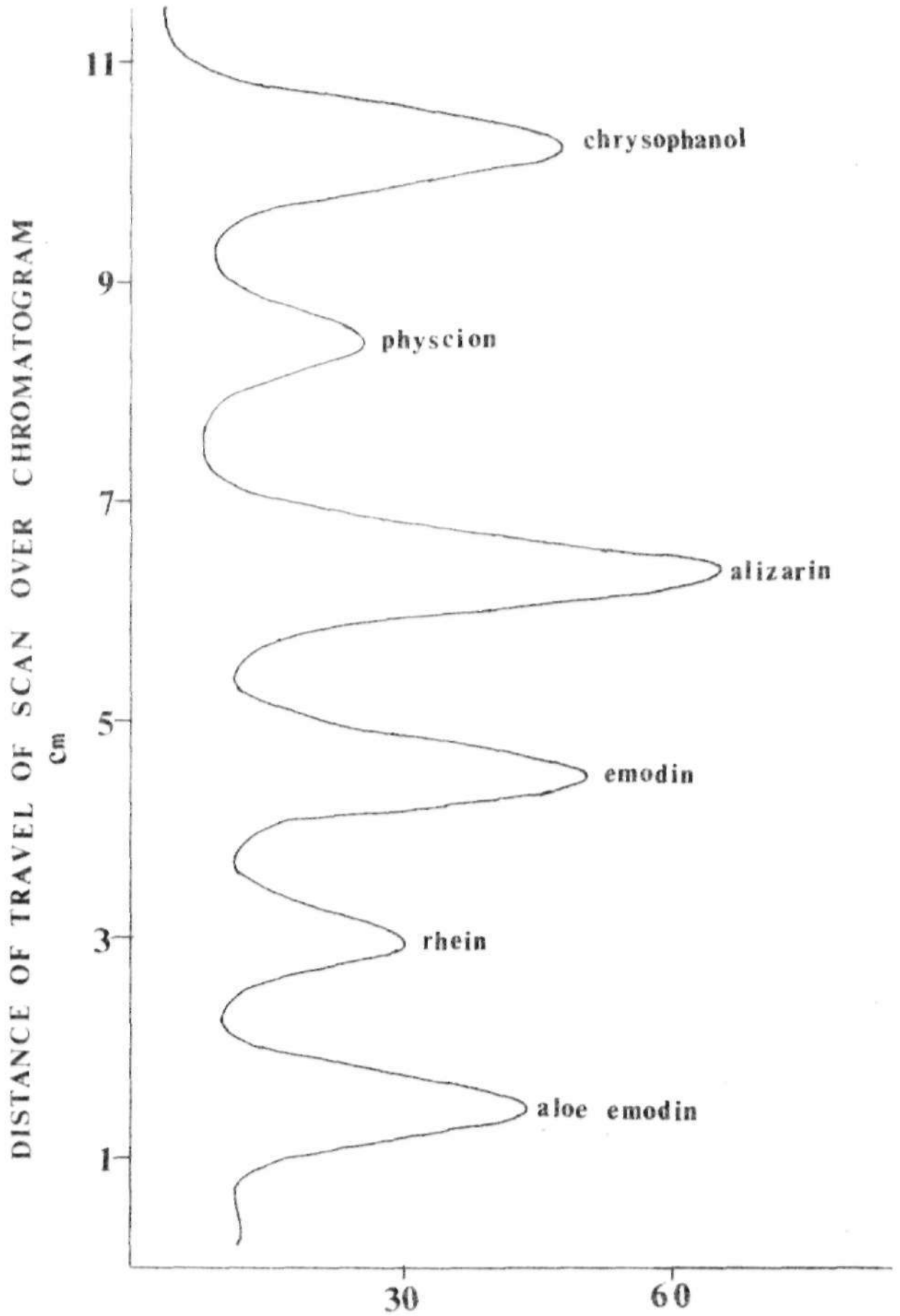


FIGURE 14 THE EFFECT OF WAVE LENGTH ON LIGHT ABSORPTION BY ANTHRAQUINONES


DISTANCE OF TRAVEL OF SCAN OVER CHROMATOGRAM
cm
11
9
7
5
3
1
chrysophanol
physcion
alizarin
emodin
rhein
aloe emodin
30
60
RECORDER RESPONSE

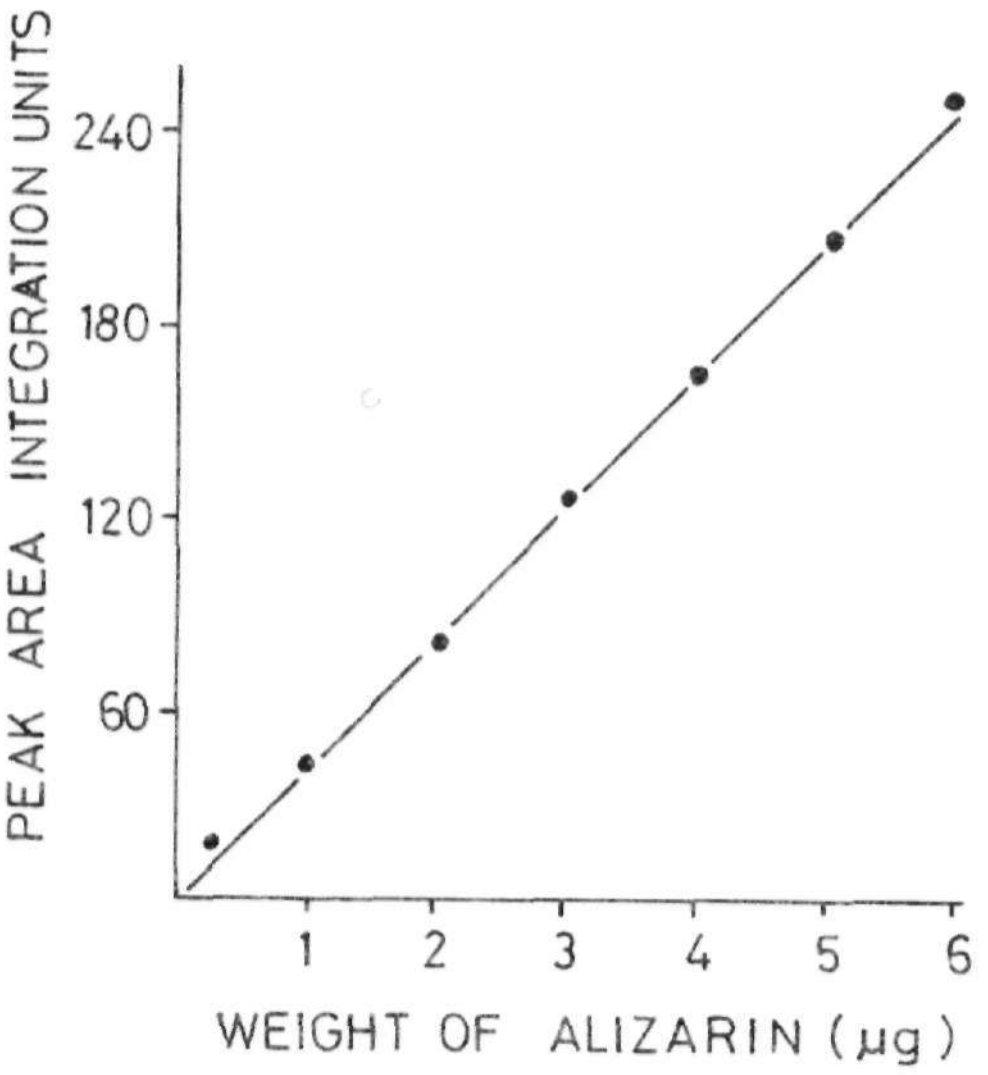


FIGURE 16 ALIZARIN CONCENTRATION VS PEAK AREA INTEGRATION UNITS

concentrations of anthraquinones in the extracts were determined by reference to the calibration curve and expressed as the equivalent of alizarin and as a percentage dry weight of the callus material (see TABLE 20; each figure represents a mean of three determinations).

(iii) Results

(a) Chemical tests

Callus cultures of all the four Cassia species: C. occidentalis, C. nodosa, C. alata and C. podocarpa gave a positive Borntrager test, indicating the presence of anthraquinones.

(b) Paper chromatographic separation of anthraquinones

TABLE 11 shows the R_f values obtained for anthraquinone compounds in different solvent systems.

Although the separation of many anthraquinones, their reduced derivatives and glycosides, was possible using solvent systems I - VIII, chrysophanol and physcion were not resolved from one another by any of the solvent mixtures used. Solvent systems IV and V had not been previously used for the separation of anthraquinones by other workers.

Anthracene derivatives identified by paper chromatographic analysis of callus cultures of Cassia species are given in TABLE 16, together with the results obtained by thin-layer chromatographic analyses of these cultures.

TABLE 11 R_f values for anthraquinones obtained on paper chromatogram in solvent systems I - VIII

Compound	Solvent System							
	I	II	III	IV	V	VI	VII	VIII
chrysophanol	0.98	S	0.00	0.88	0.91	0.78	0.83	0.97
physcion	0.98	S	0.00	0.88	0.91	0.78	0.83	0.97
emodin	0.42	S	0.26	0.50	0.61	0.89	0.62	0.97
rhein	0.00	0.84	0.77	0.02	0.10	0.30	0.00	0.67
aloe emodin	0.70	S	0.20	0.28	0.50	0.84	0.37	0.97
chrysophanol anthrone				0.00	0.00	0.00	0.97	
physcionanthrone				0.00	0.00	0.00	0.96	
emodinanthrone				0.00	0.00	0.00	0.31	
aloe emodin anthrone				0.30	0.53	0.78	0.15	
rhein anthrone				0.05	0.14	0.21	0.00	
physcion monoglucoside								0.95
chrysophanol monoglucoside								0.95
emodin monoglucoside								0.93
aloe emodin monoglucoside								0.82
sennoside C								0.39
rhein monoglucoside								0.34
sennoside D								0.25
sennoside A								0.23
sennoside B								0.12

S = solvent front
I = toluene (Betts et al., 1958),
II = acetone - water (7 : 3) (Kaji et al., 1965),
III = water - acetone - benzene (2 : 1 : 4), lower phase (Fairbairn & Shrestha, 1966),
IV = petroleum ether (40 - 60°C) - toluene - xylene - methanol (4 : 1 : 1 :1),
V = petroleum ether (40-60°C) - toluene - xylene - methanol (4 : 1 : 1 : 2),
VI = n-butanol saturated with water (Paris & Durand, 1956),
VII = toluene - cyclohexane - n-propanol - n-butanol (40 : 50 : 7.5 : 2.5) (Kinget, 1966),
VIII = n-propanol - ethyl acetate - water (4:3:3) (Horhammer et al., 1959).

(b.1.) Detection and visualization

Anthraquinones are coloured substances, and were visible as yellow spots in daylight on a paper chromatogram. Intensification of spots was possible by exposing the chromatogram to ammonia vapour, though the colour faded quickly. The reagent, magnesium acetate in methanol (0.5% w/v), recommended by Shibata et al. (1950), was found very satisfactory for the detection of free anthraquinones; the free anthraquinones became pink in daylight and red in ultraviolet light (366 nm). The colour response of anthraquinone glycosides and dianthraquinone glycosides to various detection reagents are shown in TABLE 12.

TABLE 12 Colour characteristics of anthraquinone glycosides and dianthrone glycosides on paper chromatogram after treatment with detection reagents

Detection reagent	anthraquinone glycosides		dianthrone glycosides	
	daylight	UV(360nm)	daylight	UV(360nm)
magnesium acetate 0.5% in methanol	orange	orange-red	light orange	orange-brown
aluminium acetate 0.5% in methanol	orange	orange-red	orange	dark-brown
aluminium chloride 1.0% in ethanol	orange	orange	orange-brown	orange-brown
aniline phthalate 2.5% in n-butanol	yellow	orange	brown-yellow	red-brown

(c) Thin-layer chromatographic separation of anthraquinones

R_f values of anthraquinone compounds obtained in various solvent systems are given in TABLES 13, 14 and 15.

TABLE 13 R_f values of anthraquinones on thin layer (Kieselgel G) in various solvent systems (I - IV)

Compound	Solvent system			
	I	II	III	IV*
chrysophanol	0.66	0.49	0.72	0.71
physcion	0.64	0.32	0.62	0.68
emodin	0.52	0.13	0.33	0.40
rhein	0.08	0.04	0.28	0.33
aloe emodin	0.42	0.47	0.21	0.25

I = toluene - diethyl ether - ethanol (70 : 25 : 5),
II = light petroleum (40 - 60°C) - ethyl formate - formic acid (15 : 5 : 1),
III = benzene - methanol (1 : 4),
IV* = benzene - ethyl formate - formic acid (15 : 5 : 1) : stage I,
light petroleum (40 - 60°C) - ethyl acetate - acetic acid (90 : 5 : 5) : stage II.

*The plate was developed first to 7.5 cm with benzene - ethyl formate - formic acid, removed, dried and then developed to 15 cm with light petroleum - ethyl acetate - acetic acid.

TABLE 14 R_f values of anthrones, dianthrones and anthraquinone aglycones on thin layer in various solvent systems (V - VII)

Compound	Solvent system		
	V	VI	VII
chrysophanol	0.83	0.75	0.80
chrysophanolanthrone	0.85	0.74	0.75
physcion	0.71	0.72	0.61
physcionanthrone	0.52	0.71	-
chrysophanoldianthrone	-	-	0.50
emodindianthrone	-	0.53	-
rheinanthrone	0.33	0.63	-
rhein	0.35	0.60	-
emodinanthrone	0.20	0.53	-
emodin	0.22	0.51	-
rheindianthrone (sennidin A/B)	-	0.43	-
aloe emodin	0.08	0.41	-
aloe emodinanthrone	0.07	0.39	-
aloe emodindianthrone	-	0.15	-

V = hexane - glacial acetic acid (4 : 1) (Anton and Duquenois, 1967), adsorbent Kieselgel G,
VI = benzene - glacial acetic acid (4 : 1) (Moes, 1964), adsorbent Kieselgel G,
VII = light petroleum (40 - 60 °C) - ethyl acetate - formic acid (90 : 4 : 1), adsorbent Kieselgel + Kieselguhr (1 : 6).
- = compound not applied to plate.

TABLE 15 R_f values of dianthrone glycosides (sennosides) and anthraquinone glycosides on thin layer in various solvent systems (VIII - XI)

Compound	Solvent system				
	VIII	IX	X_1	X_2	XI
sennoside A	0.00	0.00	0.44	0.41	0.22
sennoside B	0.00	0.00	0.34	0.28	0.15
sennoside C	0.00	0.00	0.77	0.56	0.42
sennoside D	0.00	0.00	0.85	0.72	0.27
rheinmonoglucoside	0.30	0.38	0.53	0.64	0.38
aloe emodinmonoglucoside	0.11	0.32	0.70	0.86	0.71
chrysophanolmonoglucoside	0.22	0.42	0.73	0.92	0.79
physcionmonoglucoside	S	S	S	S	0.79
emodinmonoglucoside	S	S	S	S	0.79

S = solvent front, Adsorbent - Kieselgel 60 (Merck),
VIII = toluene - amyl alcohol - ethanol (14 : 5 : 1),
IX = acetone - ethyl acetate - water (3 : 4 : 3),
X_1 = iso - propyl alcohol - ethyl acetate - water (4 : 4 : 3),
X_2 = iso - propyl alcohol - ethyl acetate - water (4 : 4 : 3), in Vario-Ks Chamber,
XI = propanol - ethyl acetate - water (4 : 4 : 3)
(Horhammer et al., 1963)

(c.1.) Detection and visualization

On chromatograms, which were completely free from formic acid, the hydroxyanthrones and dianthrones (e.g. chrysophanolanthrone, chrysophanoldianthrone) were seen as yellow spots, whereas the anthraquinones (chrysophanol, physcion, etc.) formed bright

yellowish to orange spots. In UV light (366 nm) the hydroxyanthrone spots were light orange-red, the dianthrone spots were dark red and the anthraquinone spots bright orange. With the KOH reagent, the hydroxyanthrone spots turned to yellow, then changed immediately to a purple colour. The hydroxydianthrone spots also turned to yellow; after some hours, however, the colour changed to light brown. The anthraquinone compounds turned to orange-pink after spraying with alkali solution.

The anthrones could not be detected by the KOH reagent unless they were completely separated from the anthraquinones. The pyridine-methanol reagent, however, distinguished the reduced anthraquinones (violet colour) from anthraquinones (orange-yellow).

In daylight, both dianthrone glycosides and anthraquinone glycosides gave yellow colours which intensified with ammonia vapour; under UV light (366 nm) dianthrone glycosides gave dull brownish colours, whereas anthraquinone glycosides produced orange colours. After treatment with 5% KOH in 50% methanol, the dianthrone glycosides changed to red colour and anthraquinone glycosides became deep yellow.

(c.2.) Identification of anthraquinones in callus cultures of Cassia species

A number of anthraquinone compounds, both in free and bound forms, were identified in callus tissue extracts of Cassia species (TABLE 16) by various paper chromatography (a.2.2) and thin-layer chromatography systems described earlier.

TABLE 16 Anthraquinones of callus cultures of Cassia podocarpa, C. alata, C. occidentalis, and C. nodosa identified by by various TLC and PC systems

	C. podocarpa	C. alata	C. occidentalis	C. nodosa
chrysophanol	+	+	+	+
emodin	+	-	+	-
rhein	+	+	-	-
aloe emodin	-	+	+	-
chrysophanolanthrone	+	+	+	+
chrysophanoldianthrone	+	-	+	-
emodinanthrone	+	-	-	-
rheinanthrone	+	-	-	+
chrysophanol-monoglucoside	+	+	+	+
emodin-monoglucoside	-	-	+	-
rhein-monoglucoside	+	+	-	+
aloe emodin-monoglucoside	-	+	+	-

+ = present

- = not present

(c.3.) Preparative thin-layer chromatography

About 5 - 10mg of each of the hydroxyanthraquinones detected on the chromatograms from the four Cassia species were isolated. TABLE 17 lists the physical properties of these anthraquinones. The data (M.P., mixed M.P., UV and IR absorptions) compared

favourably with the values reported by Fairbairn and Muhtadi (1972).

TABLE 17 Physical properties of principal anthraquinone aglycones

Compound	M.P. (^{o}C)	Mixed M.P. (^{o}C)	UV max. (nm) in MeOH	IR absorptions (cm^{-1}) in Nujol
chrysophanol	193 - 195	193 - 195	226	1630
			256	
			277	1670
			288	1570
emodin	265	263 - 265	223	1630
			254	1670
			265	1570
			290	2990
			435	
rhein	321	320 - 321	228	1630
			260	1670
			432	1705
aloe emodin	223 - 224	223	256	1630
			279	1670
			288	1570
			430	2890

(d) Column chromatographic separation of anthraquinones in a callus tissue extract of C. podocarpa

The callus tissue extract of C. podocarpa developed four distinct well separated yellow coloured zones on the column

during elution, anthraquinone monoglycosides were eluted first. Anthraquinone aglycones which have the smallest molecular dimension, were retained on the column, and could only be eluted with 70% acetone. TABLE 18 shows the results of the fractionation of the callus extract investigated.

Each fraction was further examined using thin layer chromatography. The chromatogram was observed under UV light (366 nm). Anthraquinone glycosides and aglycones both gave an orange colour. With spray reagent 5% KOH in 50% methanol anthraquinone glycosides produced a faint yellow colour which intensified with ammonia vapour or alcoholic KOH.

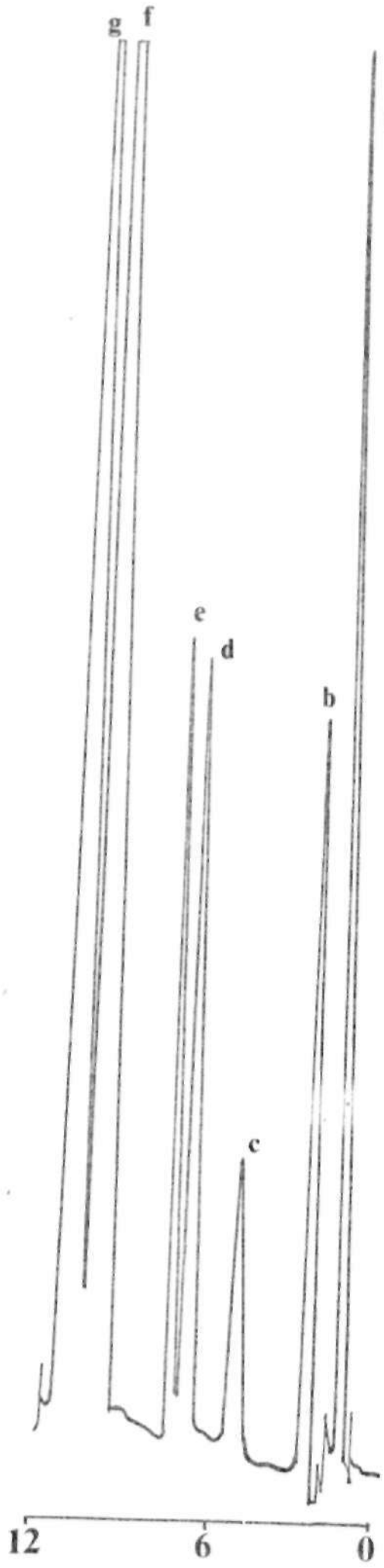
TABLE 18 *Cassia podocarpa* callus tissue fractionation on a Sephadex LH-20 column

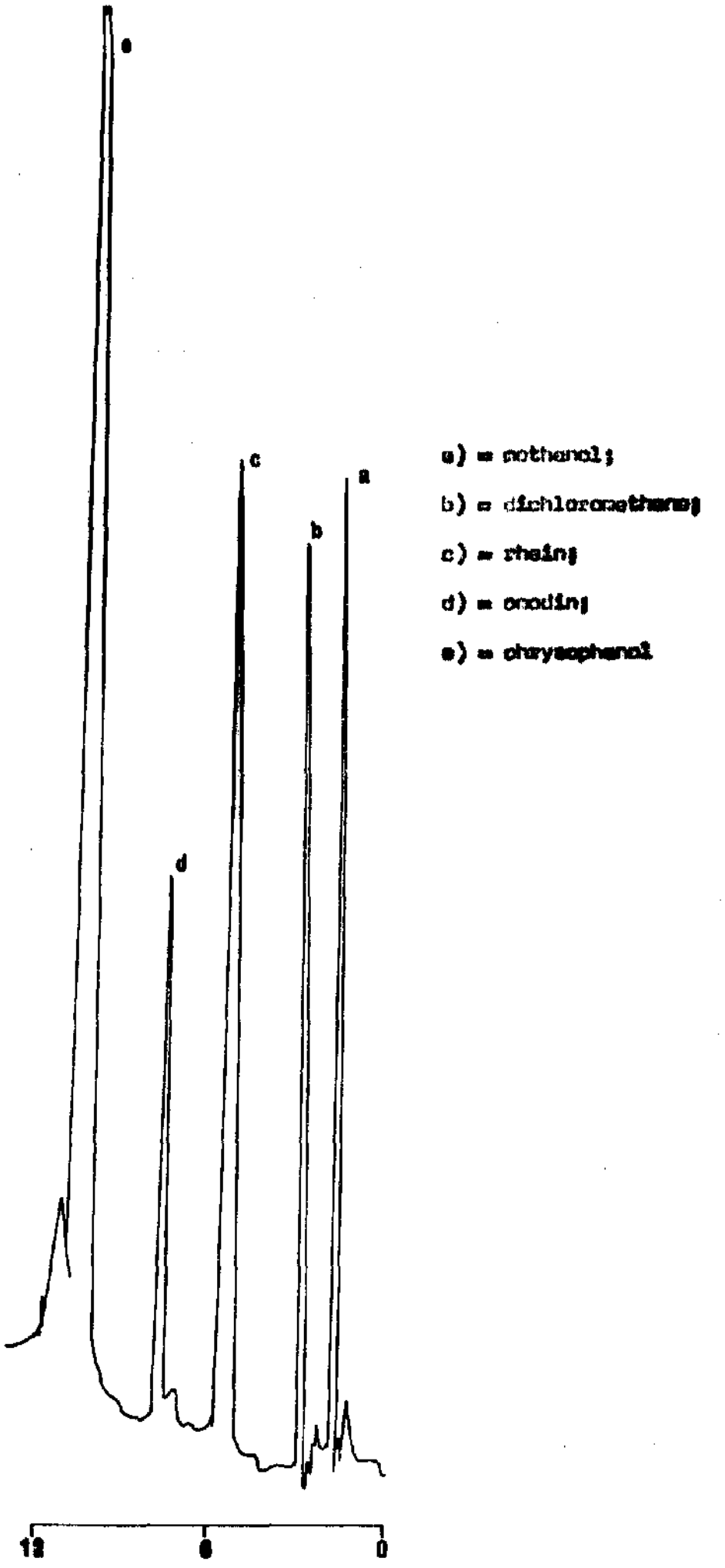
Zone colour	Components	R_f value (TLC)*
1. Faint yellow	Unknown	0.00
2. Yellow	Rhein monoglycoside	0.23
3. Bright yellow	Chrysophanol monoglycoside	0.75
4. Light yellow	Chrysophanol, Rhein	0.85

*Adsorbent - Kieselgel G (Merck); solvent system - n-propanol - ethyl acetate - water (4 : 3 : 3)

(e) HPLC separation of anthraquinones

The use of a reversed-phase water/methanol gradient elution system was found to give a satisfactory separation of the anthraquinone aglycones aloe-emodin, emodin, chrysophanol and


g
f
a
e
d
b
c
12
6
0
TIME - min



CURE 10 - HPLC chromatogram of anthraquinones from callus tissue hydrolysate of *S.* [redacted]

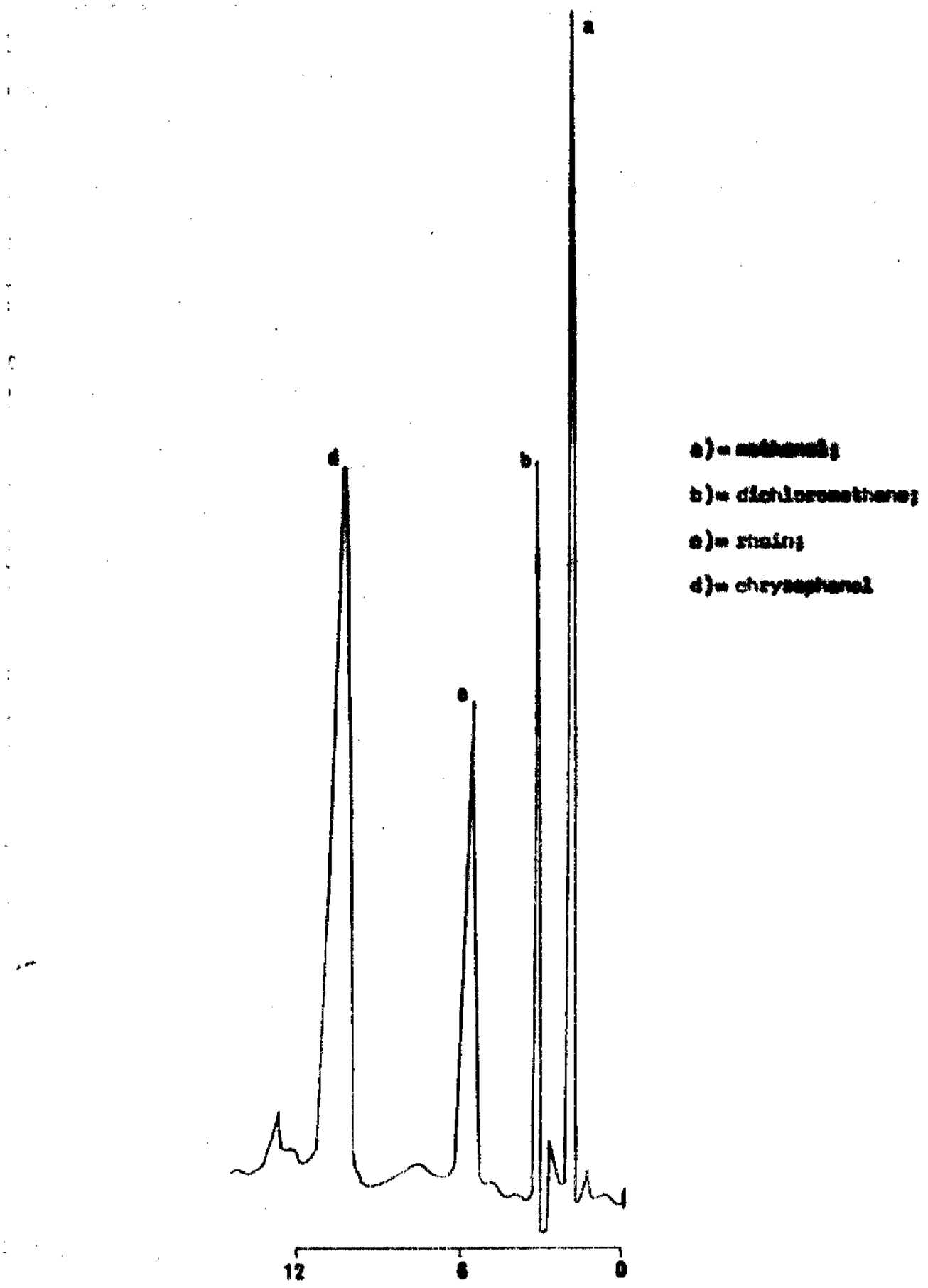


FIGURE 19 - HPLC chromatogram of anthraquinones from callus tissue hydrolysate of *C. racemosa*

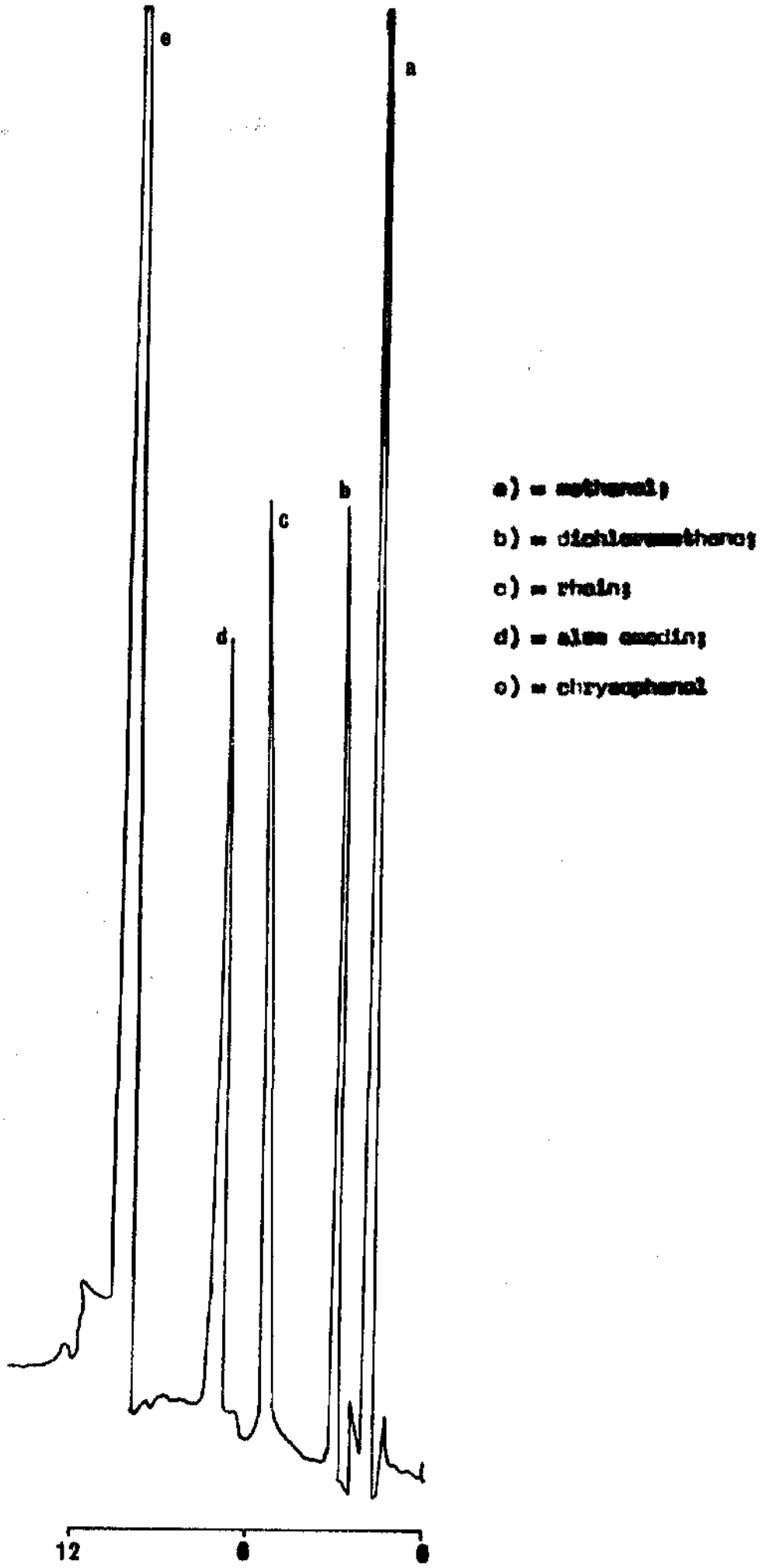


FIGURE 22. HPLC chromatogram of anthraquinones from callus tissue hydrolysate of C. alata

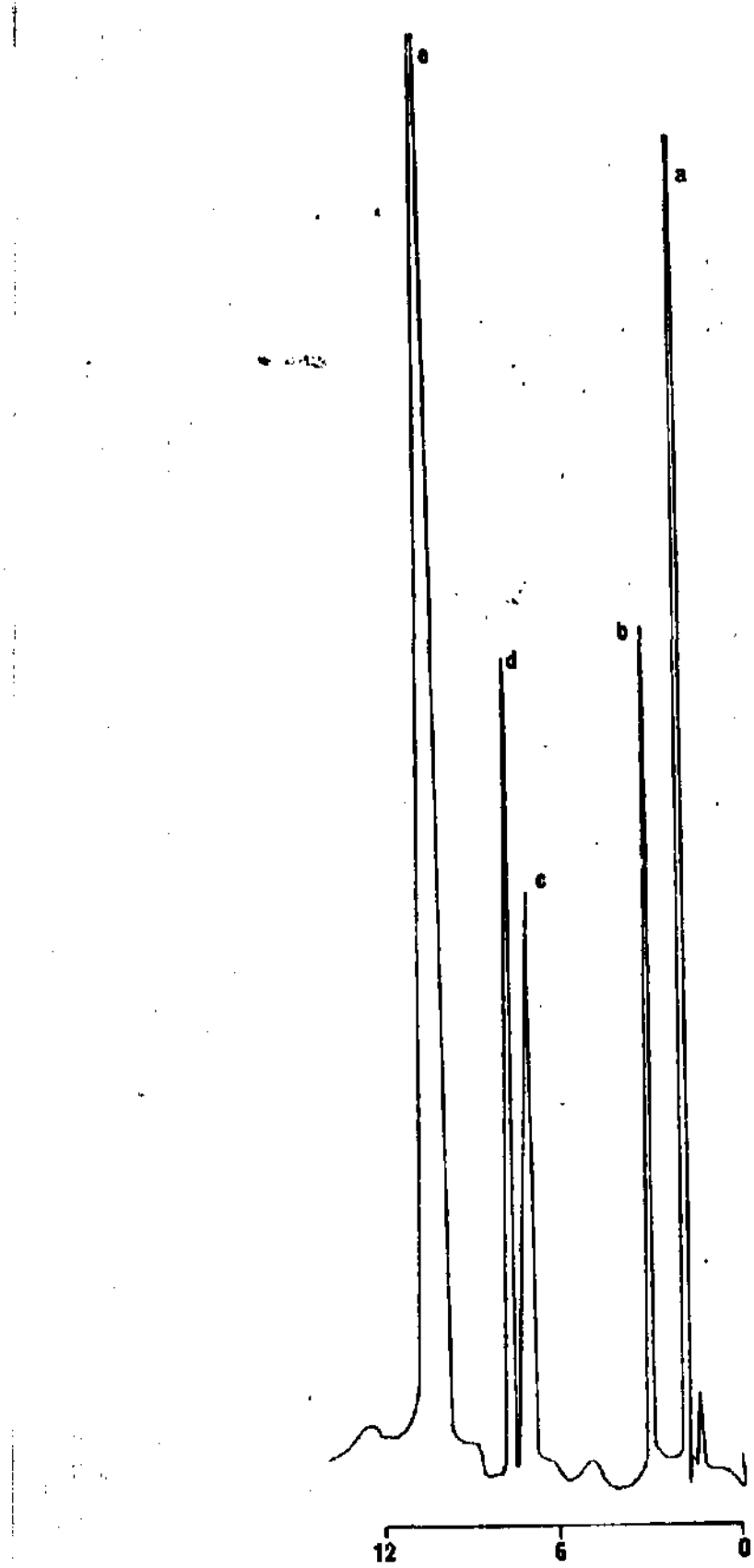


FIGURE 21 HPLC CHROMATOGRAM OF ANTHRAQUINONES FROM CALLUS TISSUE HYDROLYSATE OF CASSIA OCCIDENTALIS

a) methanol; b) dichloromethane; c) aloe emodin; d) emodin; e) chrysophanol

0.300mg/25 ml of 1,8-dihydroxyanthraquinone. Results of colorimetric estimation of anthraquinones in callus cultures of Cassia spp. are summarized in TABLE 19.

TABLE 19 Concentration of anthraquinones detected in callus cultures of Cassia species

	Free form % d.wt.	Bound form % d.wt.	*Total content % d.wt.	**Total anthraquinones % d.wt.
	SE			
C. nodosa	0.10 ± 0.002	0.40 ± 0.001	0.50 ± 0.003	0.53 ± 0.002
C. occidentalis	0.23 ± 0.001	0.30 ± 0.002	0.62 ± 0.003	0.68 ± 0.001
C. alata	0.30 ± 0.002	0.45 ± 0.001	0.75 ± 0.002	0.75 ± 0.001
C. podocarpa	0.80 ± 0.003	0.50 ± 0.003	1.30 ± 0.004	1.31 ± 0.002

d.wt. = dry weight,
SE = Standard error

*Average of ten determinations, calculated as equivalent to 1,8-dihydroxyanthraquinone.

**Estimation of anthracene derivatives in a total extract obtained after oxidative-hydrolysis.

(g) Thin-layer densitometric determination of anthraquinones in callus cultures of Cassia podocarpa

Applying the thin-layer densitometric procedure, callus cultures of C. podocarpa were quantitatively examined and the concentration of individual anthraquinone compounds determined (TABLE 20).

TABLE 20 *Concentration of anthraquinone compounds in callus cultures of C. podocarpa estimated by thin-layer densitometry

	Aglycones % w/w		O-glycosides % w/w
Chrysophanol	0.14		0.22
emodin	0.15		-
rhein	0.22		0.26
Total	0.51	Total	0.48

*Expressed as equivalent of 1,2-dihydroxyanthraquinone; each figure represents a mean of three determination.

However, the individual estimation of some of the anthrones and dianthrones earlier detected in callus cultures of C. podocarpa was not possible in the solvent system benzene - ethyl formate - formic acid (75 : 25 : 2), because alizarin could not be completely resolved from anthrones and dianthrones.

Using this method, determinations on standard solutions differed from the known value by $\pm$ 6%. The variation was assessed as $\frac{\text{range}}{\text{mean}} \times 100\%$ as in Rai and Turner (1975).

CHAPTER THREE

DISCUSSION

PART I

DETERMINATION OF ANTHRAQUINONES

The aim of this project, as has already been stated, was to establish optimum conditions for the growth of and anthraquinone formation in tissue culture of *Cassia* spp. This project is, therefore, of two parts: growth of callus cultures and determination of anthraquinones. To ensure that reliable methods were obtained for the determination of anthraquinones in callus cultures, various experiments were carried out using established chromatographic processes e.g., PC, TLC, HPLC, colorimetry, and densitometry. Having done this on pure anthraquinone samples, the methods were then applied to extracts from callus cultures for determination of these compounds.

(i) Characterization of anthraquinones in callus cultures of Cassia species

(a) Separation of anthraquinones by paper chromatography

In this work, eight solvent systems were selected for the paper chromatography of anthraquinones. The selection of the first three solvent systems (I) toluene (Betts *et al.*, 1958), (II) acetone - water (7 : 3) (Kaji *et al.*, 1965), and (III) water - acetone - benzene (2 : 1 : 4) (Fairbairn and Shrestha, 1966) was based on the fact that these systems allowed the separation of chrysophanol, emodin, rhein and aloe emodin on Whatman paper No.1, using the ascending development technique. None of the three solvent systems however separated more than three anthraquinone compounds. A related aglycone physcion, was not separated by any of the three solvent mixtures. The solvent

system II afforded the separation of only a single compound, rhein.

The use of solvent mixtures (IV) pet. ether - toluene - xylene - methanol (4 : 1 : 1 : 1), (V) pet. ether - toluene - xylene - methanol (4 : 1 : 1 : 2), (VI) n-butanol saturated with water (Paris and Durand, 1956) and (VII) toluene - cyclohexane - n-propanol - n-butanol (40 : 50 : 7.5 : 2.5) (Kinget, 1966), enabled the separation of all the principal anthraquinone aglycones and their reduced anthrone forms, except the separation of chrysophanol and physcion. Solvent mixtures IV and V were used for the first time for separation of anthraquinones, they varied only in their methanol content. Higher R_f values and a better separation of compounds were recorded with the solvent mixture V. Solvent mixture VII produced the separation of the maximum number of compounds.

Solvent system VIII: n-propanol - ethyl acetate - water (4 : 3 : 3) developed by Horhammer et al. (1959) is a classical solvent system used for the separation of anthraquinone glycosides. By this solvent mixture it was possible to achieve the separation of monoglycosides of emodin, aloe emodin and rhein and of the dianthrone glycosides: sennoside A, sennoside B, sennoside C and sennoside D, but the monoglycosides of chrysophanol and physcion gave a similar R_f value (0.95) and were not separable. As anticipated, the separation of anthraquinone aglycones, except rhein, was not possible in this solvent system which is basically hydrophilic.

Having obtained the above results, it was considered useful to employ the methods of TLC to further substantiate them.

(b) Separation of anthraquinones by thin-layer chromatography

Silica gel has always been the adsorbent of choice for the separation of anthraquinones. However, for the separation of anthrones and dianthrones, thin layers of lesser activity are normally used. These were prepared by coating the plates with a slurry consisting of one part Kieselgel G (Merck) and six parts Kieselguhr G (Merck).

(b.1.) Solvent system

In the routine examination of extracts and hydrolysates from callus tissue for the presence of hydroxyanthraquinones, a method is required which is rapid and which will achieve a satisfactory separation of the principal constituents preferably using a single solvent system. The existing methods for the separation of plant anthracene derivatives by thin-layer chromatography (Horhammer et al., 1963; Gyanchandani et al., 1969; Labadie, 1969) either require the plate to be developed successively in two different solvent systems, or, where a single solvent system is used, not all the hydroxyanthraquinones are resolved satisfactorily.

Systematic chromatographic structural analysis of a great number of naturally occurring anthracene derivatives has not, up till now, been carried out. However, it seems certain that the polarity of an anthracene derivative depends on the position of the hydroxyl group in the anthracene ring. This was taken into consideration in formulating the composition of the solvent mixtures.

The classical solvent system: benzene - ethyl formate - formic acid (15 : 5 : 1) was used by Gyanchandani et al. (1969). They claimed to be able to separate all the five principal anthraquinones (chrysophanol, physcion, emodin, rhein and aloe emodin) on silica gel. However, it was found that with this solvent mixture in its originally stated composition, chrysophanol and physcion were not completely separated from each other. The difference in the R_f values of these two substances was so small that it would not have permitted the densitometric determination of individual anthraquinones efficiently on the chromatogram.

In this investigation, eleven solvent mixtures were used for the separation of different groups of anthracene derivatives. The separation of the principal hydroxyanthraquinones: chrysophanol, physcion, emodin, rhein and aloe emodin was achieved by four newly developed solvent mixtures: I to IV (see TABLE 13). The noteworthy aspect of this was a distinct separation of chrysophanol and physcion in all the four solvent systems, the separation of which had proved unsuccessful on paper.

A satisfactory separation of anthrones and anthraquinone aglycones was achieved on TLC using silica gel in solvent mixture V hexane-glacial acetic acid (4 : 1). This system was found to be superior to TLC on Kieselgel + Kieselguhr (1 : 6) in solvent mixture VII light petroleum (40 - 60°C) - ethyl acetate - formic acid (90 : 4 : 1) for separation of the same compounds (see TABLE 14). The solvent mixture VI benzene - glacial acetic acid (4 : 1), first used by Moes (1964) was even better and provided the separation of thirteen compounds on a single chromatogram

in a 15cm run (TABLE 14). In addition to anthraquinones and anthrones, dianthrones were also distinctly separated by this solvent mixture.

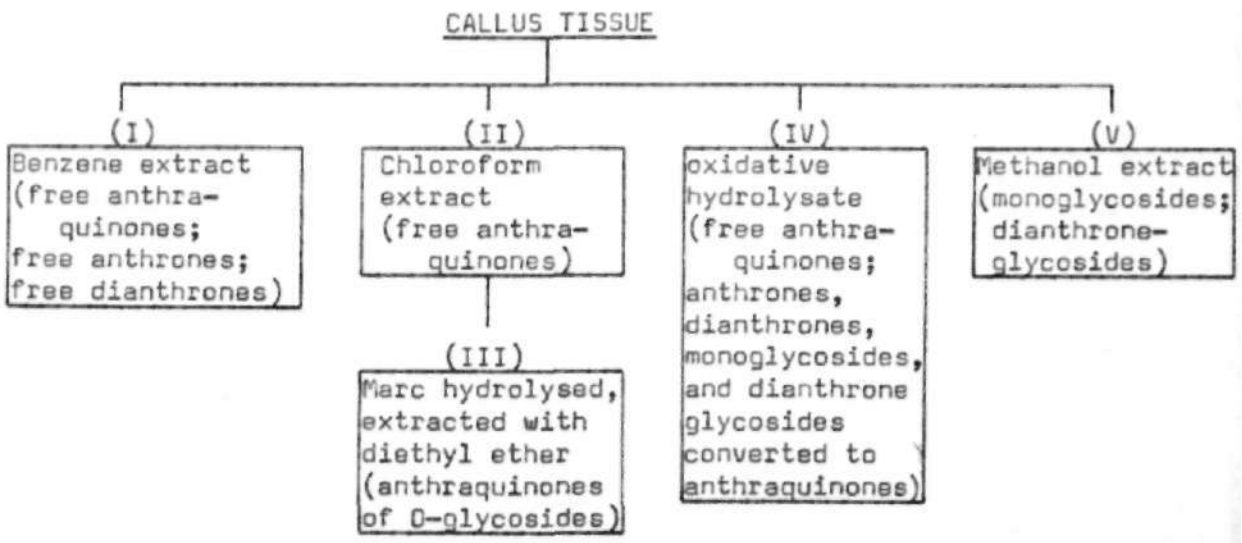
The analytical problems involved in separating dianthrone glycosides (sennosides) and anthraquinone glycosides are considerable. These two groups of glycosides are active purgative components of some important Cassia species, including Cassia podocarpa (Paris and Chartier, 1948), and these glycosides possess different pharmacological activity (Fairbairn, 1965). The separation of dianthrone and anthraquinone glycosides is necessary if the determination of individual glycosides is intended. Amongst the known thin-layer chromatographic procedures (Anton and Duquenois, 1967; Horhammer et al, 1963; Khafagy et al., 1972) employed for the separation of dianthrone and anthraquinone glycosides, none was found satisfactory; either the resulting separation was incomplete or such separation as was achieved was inconclusive because of the very close R_f values. In this investigation three new solvent mixtures: toluene - amyl alcohol - ethanol (14 : 5 : 1) no.VIII, acetone - ethyl acetate - water (3 : 4 : 3) no.IX, and isopropyl alcohol - ethyl acetate - water (4 : 4 : 3) no.X, were used, in addition to the more commonly used solvent mixture n-propanol - ethyl acetate - water (4 : 4 : 3) no.XI, first proposed by Horhammer et al. (1963). It was found that most of the anthraquinone monoglycosides were separable with all the above solvent mixtures but that dianthrone glycosides were only separated with the latter two: isopropyl alcohol - ethyl acetate - water (4 : 4 : 3) no.X and n-propanol - ethyl acetate - water (4 : 4 : 3)

no.XI. A distinct separation of these compounds due to differing R_f values (TABLE 15), may facilitate removal of the respective spot areas from the chromatogram and if necessary, permit the densitometric determination of individual components.

It was also observed that dianthrone and anthraquinone glycosides were better resolved when separation of extracts containing these compounds was performed in a "Vario-Ks-Chamber" under controlled humidity conditions (32% RH) (see TABLE 15). The TLC separation of anthraquinone glycosides under a set of programmed humidity conditions had not been attempted earlier; and it would seem that chromatography at a low humidity favours a better resolution of these two groups of glycosides.

(b.2.) Preparation of extracts for qualitative analysis by TLC

Paper chromatographic examination of various extracts had earlier indicated the presence of a number of different forms of anthraquinone compounds in callus tissues of Cassia species. In order to make effective use of TLC for qualitative analysis, five different extracts, each to contain one or more groups of anthraquinones were prepared as shown below.



It can be seen that different groups of compounds were present in the different extracts so that to obtain a complete analysis, all five extracts were used. Thin-layer chromatographic analysis of the various extracts prepared from the callus tissue of Cassia species demonstrated a more complete qualitative spectrum than had been possible by paper chromatography, with respect to the nature and type of anthracene derivatives present.

(c) Separation of anthraquinone glycosides on a Sephadex LH-20 column

Thin-layer chromatographic studies of callus tissue of Cassia podocarpa indicated the presence of rhein monoglycoside and chrysophanol monoglycoside, and some free anthraquinones and anthrones, but no dianthrone glycosides were detected by any of the TLC systems used. In order to ascertain if the same anthraquinone monoglycosides could be separated on a column of Sephadex LH-20, and also to further confirm if the dianthrone glycosides were actually absent in the callus tissue, the Zuaving procedure (1968) recommended for the separation of dianthrone and anthraquinone glycosides was followed.

The TLC results of the column fractions obtained from callus tissue indicated the presence of monoglycosides of rhein and chrysophanol but did not yield any dianthrone glycosides. This further strengthened the earlier observation, based on TLC investigation, that callus cultures of C. podocarpa probably did not synthesize these compounds.

(d) HPLC separation of hydroxyanthraquinones

Of several solvent mixtures investigated, a linear gradient from 60% to 86% methanol in water utilising reversed phase technique (by using a chemically bonded column: Apex octadecylsilane) provided a superior resolution of anthraquinones (see FIGURE 17). The initial solvent mixture (60 : 40) contained reagent A (PIC A Waters), but this was omitted from the final solvent mixture (86 : 14). The addition of PIC A to the initial solvent proved essential to facilitate elution of the acidic component, rhein, of many naturally occurring anthraquinones.

In an HPLC system reported earlier (Rai et al., 1975), it was not possible to separate rhein from the solvent peak because it was strongly dissociated in the system used and would not stay on the column. The addition of PIC A to the initial solvent (solvent A) helped form an ion-pair and enabled the rhein to be separated from the solvent peak. PIC A was not added to the solvent B, and subsequent elution of other anthraquinones occurred by normal reversed phase separation as the gradient proceeded and PIC A was eluted from the column.

It was noted that a careful preparation of solvent A and the addition of an accurate volume of PIC A was essential for the reproducibility of separation. More importantly, a careful equilibration of the column over a period of 15 minutes with the initial solvent containing PIC A was found to be critical for reproducibility.

The emergence of compounds from the column was in order of their reducing polarities in the following sequence: rhein,

aloe emodin, emodin, physcion and chrysophanol.

The addition of an increasing volume of PIC A (1.8 ml to 2 ml/l) to the initial solvent not only progressively increased retention times of compounds (see FIGURE 22 & 23) but also produced unsatisfactory peaks, primarily of rhein. Addition of PIC A at less than 1.75 ml/l to the initial solvent could not bring about the separation of rhein from the solvent peak.

FIGURE 24 shows separation peaks of some pure anthraquinones introduced on the HPLC column either singly or in a mixture.

The most satisfactory HPLC separation of Cassia species callus tissue extracts was achieved using callus tissue hydrolysates (see FIGURES 18, 19, 20, 21) as described in the experimental. No conclusive identification and separation of anthraquinones was possible when callus tissue extracts containing only free anthraquinones were introduced on the HPLC column. However, a number of peaks emerged for each callus tissue extract (e.g. FIGURE 25). This could primarily be due to the presence, in addition to anthraquinones, of anthrones and dianthrones in the extracts. In general, very complicated HPLC chromatograms were obtained, and interpretation of these was rather difficult. In addition, it should also be noted that an extraction procedure designed to contain only free anthraquinones (in this case a simple chloroform extract of callus material) would normally also contain other pigments, which might also interfere with the programmed separation.

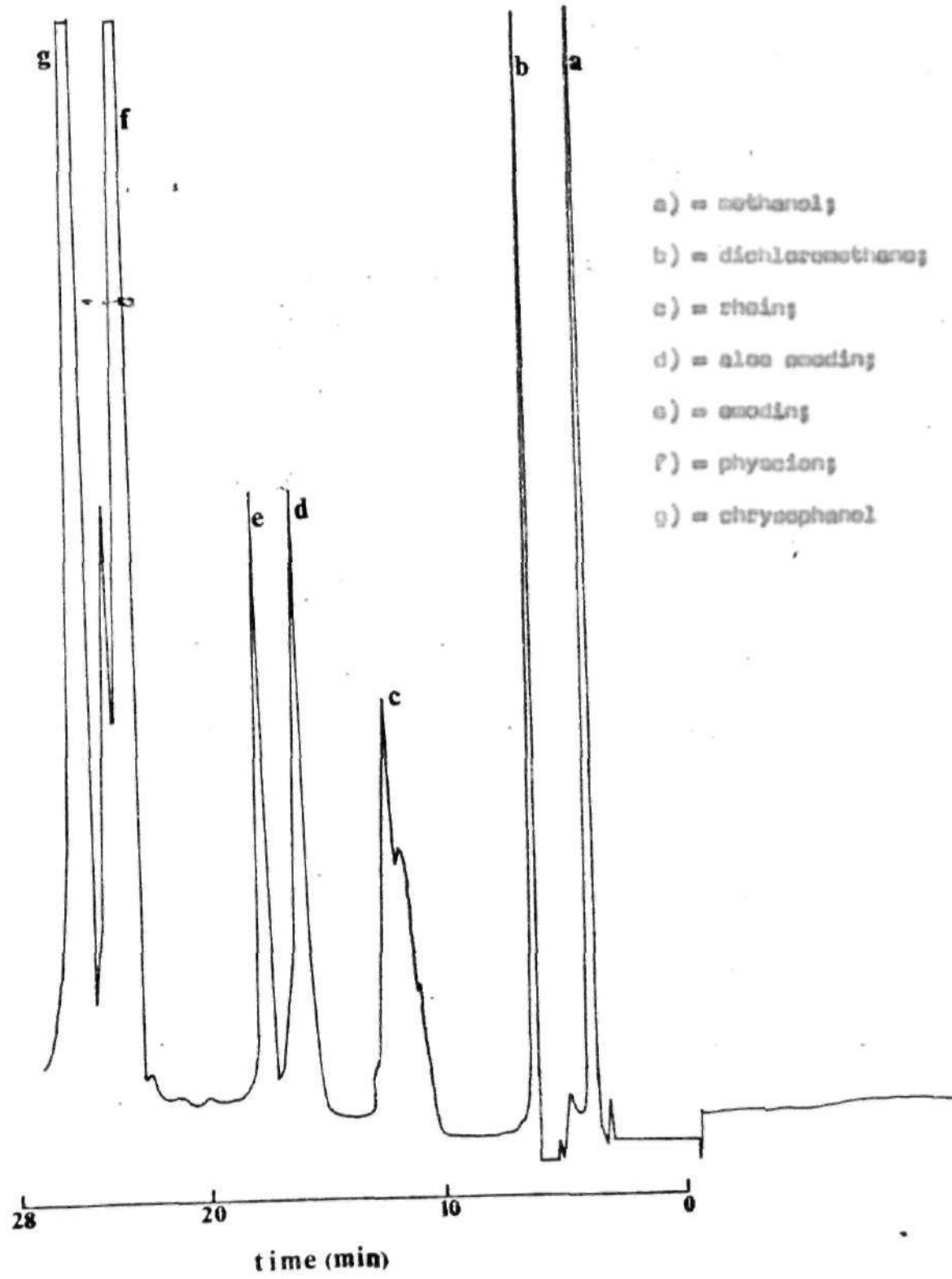
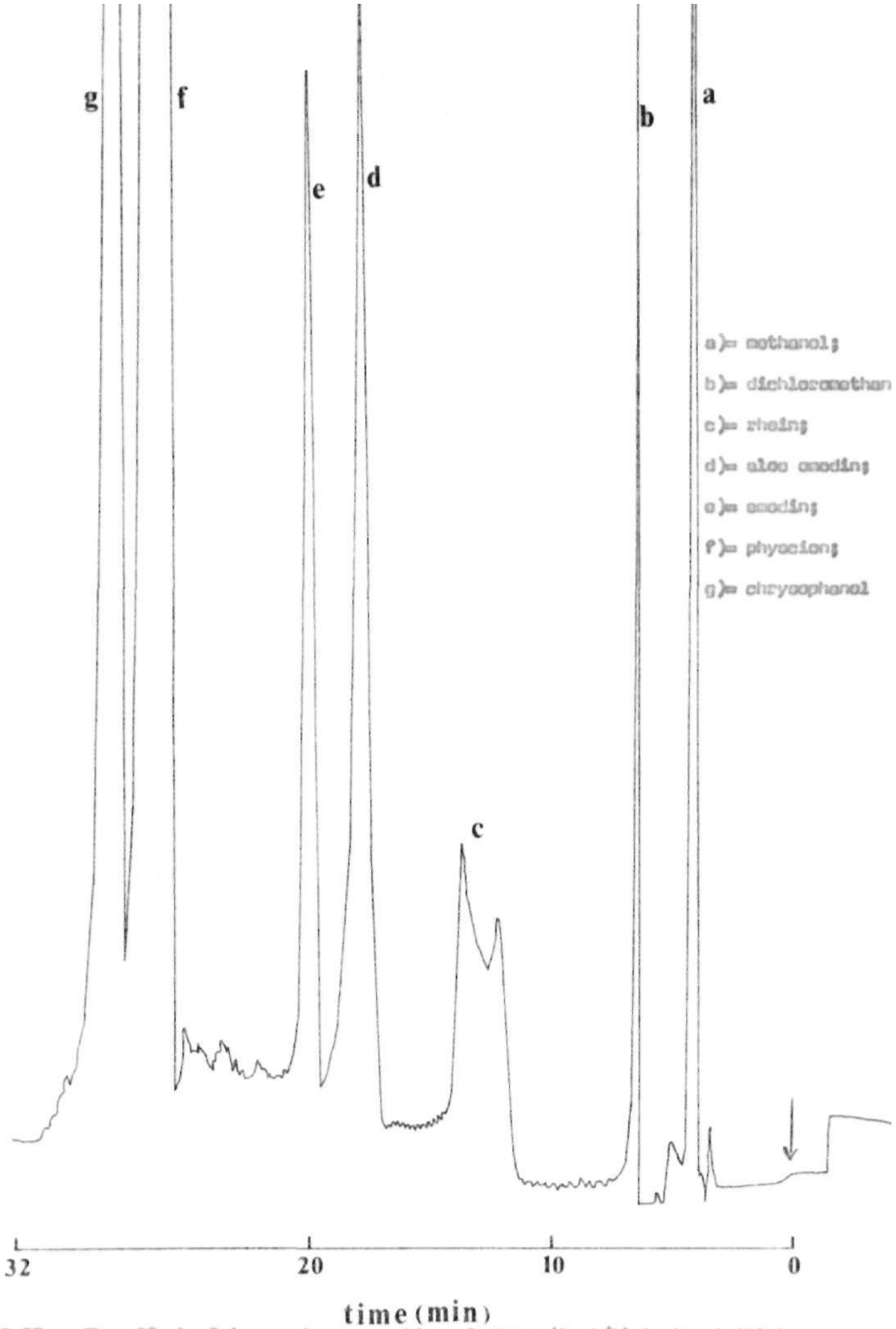


FIGURE 22 - The effect of increasing proportion of PIC A (1.6 ml/l) in the initial solvent (methanol : water, 60 : 40) on separation of anthraquinones



GURE 23 - The effect of increasing proportion of PIC A (2 ml/l) in the initial solvent (methanol : water, 60 : 40) on separation of anthraquinones

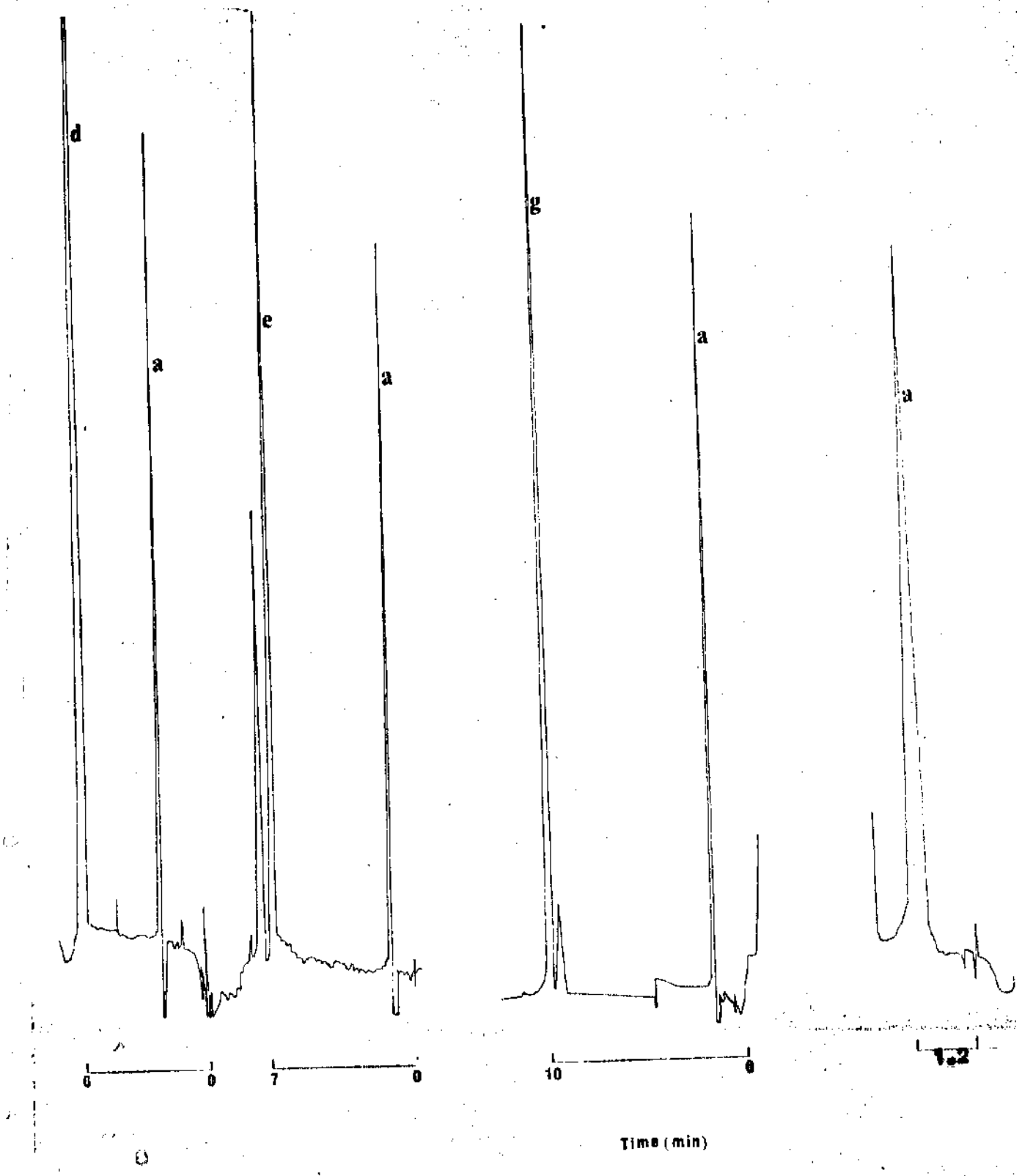
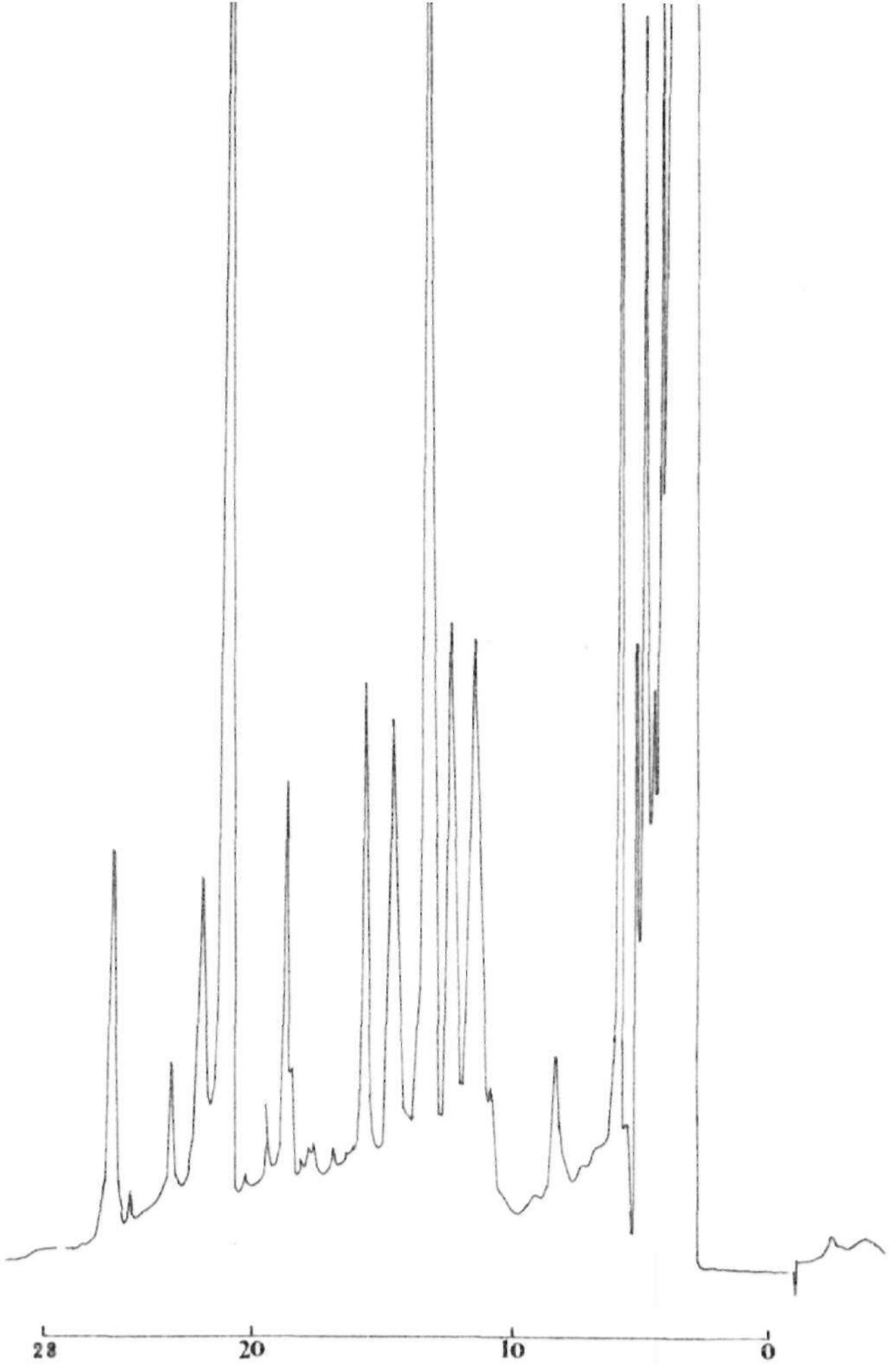


FIGURE 24 - HPLC SEPARATION PEAKS OF SOME PURE ANTHRAQUINONE COMPOUNDS
a) chains d) aloe emodins e) emodins g) physcion



URE 25 -- HPLC chromatogram of a chloroform extract from callus tissue of C. roseocarpa

(ii) Quantitative analysis

(a) Colorimetric estimation

(a.1.) Determination of free forms

The colorimetric procedure used in this investigation only allowed the determination of free hydroxyanthraquinones and did not fully include free anthrones and dianthrones.

Anthrones and dianthrones are oxidised in the alkaline medium partly to anthraquinones and partly to a mixture of other non-defined oxidation products. A quantitative transformation of free anthrones and dianthrones into anthraquinones has so far not been possible.

The determination of the anthraquinone form as such was no longer a problem since the reaction of Borntrager was used, giving excellent results if the anthraquinones are in a relatively pure state. Many substances occurring in the callus tissue may not indeed be anthracene derivatives but may absorb in the same region, in the alkaline medium of the Borntrager reaction. Thus the callus tissue extracts were prepared in such a manner that very little interfering substances were present in them. This was possible by a judicious choice of the solvents used for extraction. The lipophilic character of the anthraquinones gives the opportunity to use lipophilic and selective solvents such as chloroform and ether. Also, in order to obtain higher extinction values, as suggested by some workers (Muller et al., 1962), ammonium hydroxide was added to the alkaline solution. This not only gave higher

extinction values but also shifted the absorption maxima to longer wavelengths, where absorption of the interfering substances of non-anthracene type was little.

The chemical assay based on the Borntrager reaction is a classical one, and was used in the determination of free forms of anthraquinones in callus tissues of Cassia spp.

(a.2.) Determination of combined forms

In contrast to the free forms, where a separete estimation of dianthrones and anthraquinones is not possible, glycosides can be estimated as groups.

In this work, since the presence of O-glycosidic anthraquinones, and the absence of dianthrone and C-glycosides had earlier been established, the extraction procedure was designed to yield only anthraquinones of the O-glycosides. This was accomplished by a simple hydrolysis of the water extract of the marc which had been earlier treated with an organic solvent to remove from it free forms of dihydroxyanthraquinones.

(a.3.) Determination of the total anthracene derivatives

The estimation of total anthracene derivatives in callus tissue of Cassia species was carried out by a colorimetric procedure based on oxidative-hydrolysis. The resultant mixture contained only anthraquinones whose extinction was measured in the usual manner.

The total anthracene derivatives estimated by this procedure recorded a slightly higher value in all the callus tissues

compared to a combined estimated value of free forms and bound forms (TABLE 19). The reason for obtaining a higher value was because this method included the estimation of free anthrones and dianthrones (by converting these into anthraquinones), the estimation of which had not been possible in the method used for the determination of free forms.

(b) Thin-layer densitometric estimation of anthraquinones in callus tissue of C. podocarpa

Densitometry is a method whereby the intensity of colour of a substance is measured directly on the chromatogram.

By thin-layer densitometric procedure, it was estimated that rhein was the major anthraquinone component of C. podocarpa callus cultures, both in the free and combined forms. Chrysophanol was present in relatively low concentration. Taking chrysophanol as unity, the quantitative ratio for free oxidized anthraquinones can be expressed as emodin 1: 1.07, rhein 1: 1.57, and for O-glycosidic compounds the ratio was rhein 1: 1.8. The concentration of oxidized algycones was 0.51% w/w and combined anthraquinones 0.48% w/w, giving a total anthraquinone content of 0.99% w/w.

The total concentration of free and combined anthraquinones in C. podocarpa cultures estimated by the colorimetric method was 1.31% w/w, a value higher than that obtained by the densitometric method (0.99%). This difference may be attributed to anthrones and dianthrones not being included in the densitometric estimation.

Using this densitometric method, determinations on standard solutions differed from the known value by ± 6%, whereas in the previous method (Rai and Turner, 1975) the deviation factor was slightly lower (± 4%). However, the greater ease and simplicity of this method made it more suitable for the routine analysis of individual dihydroxyanthraquinones directly on the chromatogram.

Using the same criterion (range as % of mean) for assessing variation in colorimetry that was used in densitometry, the colorimetric procedure used shows a variation of 4% (TABLE 19), compared with the 6% for densitometry (TABLE 20). It can be concluded that the colorimetric method is slightly more consistent.

PART II

TISSUE CULTURE OF CASSIA SPECIES

(i) Callus initiation, pigmentation and medium

In this work, no problems were experienced in initiating callus cultures from all four species of *Cassia* investigated. There was no sign of microbial contaminants, which was an indication that sterilization of the explants had been adequate. A double treatment (firstly 70% ethanol, followed by sodium hypochlorite solution) was used in the present investigations because the seeds had proved initially to be heavily contaminated, a single treatment was therefore inadequate. The reason for this heavy contamination was possibly the source of seed: from wild growing plants.

An earlier experience (Rai *et al.*, 1974) had shown that explants of the size utilized (6mm^2 of cotyledon and 8 - 10mm of hypocotyl) were large enough to overcome any aspects of minimal size which might affect callus formation or response to cultural conditions.

Callus cultures of all the four *Cassia* species consisted mostly of a friable mass in which the tissue colour varied from light brown to grey colour. Brown pigments are usually associated with dead or dying callus, but in this case, when the brown tissue was separated from the rest of the callus mass and cultured, it gave rise to rapid growth of the same brown-grey callus. It appears that this colour pattern is an integral part of the callus character of these *Cassia* species.

In contrast, callus cultures of *C. senna* (Rai *et al.*, 1974) and *C. angustifolia* (Friedrich and Baier, 1973) were of pale green

to light brown colour. The green pigmentation in these cultures may have been due to the presence of coconut milk in the growth medium which provided a faster tissue multiplication rate. The growth medium used in the present work was devoid of coconut milk.

In this work, the media were heat sterilized, since degradation of media components by heat was not suspected. Also, by this method, sterilization of culture vessels as well as the media was achieved at the same time.

(ii) Temperature, pH and light

Studies on the in vitro growth of plant cells have indicated that the optimum temperature is generally within the range 25 to 30°C, but that species could differ considerably (Carew and Staba, 1965; Puhan and Martin, 1971). Riker and Hilderbrandt (1958) have concluded that the temperature range for tissue culture is approximately the same as it is for the whole plant. Little if anything seems to have been reported on temperature optima for growth versus those for metabolite production.

Although the effect of temperature on growth and anthraquinone formation in tissue cultures of Cassia species has not been studied yet, the use of a temperature range 25 to 30°C has been reported: Cassia angustifolia, 30°C (Friedrich and Baier, 1973); Cassia senna, 28°C (Rai et al., 1974); Cassia tora, 25°C (Tabata et al., 1975). It would appear that the temperature (28°C) at which cultures of C. podocarpa, C. nodosa, C. occidentalis and C. alata were grown and maintained was quite suitable for growth and for metabolite production.

Media used for the growth of callus cultures of Cassia species were adjusted to 5.5 $\pm$ 0.1. In general, the most favourable pH for the growth of plant cell cultures is in the range pH 5 to 6, and most media are so adjusted prior to autoclaving. Murashige and Skoog (1962) medium used in this study is fairly well buffered, and very little pH shift would be expected in the course of culture growth.

Light, in addition to its effect on in vitro plant growth and development, also influences the production of plant metabolites (Seibert and Kadkade, 1980). Callus cultures of Cassia angustifolia Vahl have been grown and propagated in light and darkness (12 hr. rhythm) (Baier and Friedrich, 1970). In contrast, callus cultures of C. tora have been grown in constant darkness (Tabata et al., 1975), and Cassia senna L. cultures have been grown under constant illumination (Rai et al., 1974). In all these cases, a number of anthracene derivatives were isolated and identified. In this investigation, tissue cultures of all the Cassia species were grown under the constant illumination of 2000 lux (one foot candle = 10.76 lux). The influence of the light intensity on the growth and pigment production in the cultures from anthraquinone producing plants has not been examined in the present work. Under the conditions utilized, in the present work, moderate amounts of metabolites have been produced. Further work will be needed to see whether a change in light intensity and periodicity of illumination can affect yeild.

(iii) Nutritional requirements and growth regulators

Cultures of plant tissues can be initiated in nutrient media containing a range of formulations of minerals, vitamins, and sugar (Gamborg et al., 1976). The major difference between the nutrient

media used to initiate cultures of different species lies in the concentrations and nature of the growth stimulating compounds of the classes auxin and cytokinin.

The presence of an auxin at a suitable concentration in the medium is required for the initiation of tissue cultures from primary explants. The presence of a cytokinin at a suitable concentration may also be required. In addition to their effects on initiation and maintenance of growth in tissue cultures, auxins and cytokinins have substantial effects on yields of biologicals. This may be illustrated by considering the effects of the three widely used auxins: indoleacetic acid (IAA), naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D).

The results of experiments on the influence of plant growth regulators showed that there are considerable differences not only in growth but also in pigment production among callus cultures of Cassia tora, depending upon the concentration of hormones in the medium (Tabata et al., 1975). They showed that in the absence of kinetin, the tissue growth was stimulated by increasing the concentration of 2,4-D. The maximum yield of anthraquinones was achieved with the combination of growth hormones 2,4-D and kinetin in concentration of 10^{-8}M and 10^{-5}M respectively. Friedrich and Baier (1973) demonstrated that in callus cultures of C. angustifolia, modifications of the growth regulator composition of the culture medium gave no quantitative changes in the content spectrum of the cultures. Rai et al (1974) have shown that in callus cultures of Cassia senna, modifications of the medium with naphthalene acetic acid (NAA), indol-3yl-acetic acid (IAA) alone or with kinetin or 2,4-dichlorophenoxyacetic acid (2,4-D), did not improve callus growth,

and the Murashige and Skoog medium containing 2,4-D (0.55 mg/l), kinetin (0.3 mg/l) and coconut milk provided a satisfactory growth of cultures.

In this investigation, the effects of different concentrations of auxin and kinetin on the growth and pigment formation in callus cultures of C. alata, C. nodosa, C. occidentalis and C. podocarpa were not quantitatively determined. However, the growth rate of these tissues was observed at different concentrations of the growth regulators by visual comparisons of the culture flasks. The combinations of growth regulators used were: 2,4-D and NAA; 2,4-D and IAA; 2,4-D and kinetin; kinetin and IAA; kinetin and NAA; and IAA and NAA. Six different concentration combinations of 2,4-D and kinetin were used. It was observed that the presence of IAA or NAA either alone or with 2,4-D or kinetin tended to induce organ regeneration in all the callus cultures and, generally, the growth of cultures was very slow. The organ differentiation was more pronounced in the medium completely devoid of 2,4-D, and also in the medium containing IAA. This is in contradiction to the observations of Tabata et al. (1975) on callus cultures of C. tora where none of the cultures showed any organ formation whatever the hormone supply.

The callus cultures of Cassia species grew more slowly on the media containing higher concentrations of kinetin than 2,4-D (see TABLE 8). For achieving a reasonable culture growth it was necessary that 2,4-D and kinetin were present in the medium with 2,4-D in higher concentration than kinetin. It was apparent that the tissue growth was stimulated by increasing the concentration of 2,4-D up to a level of 0.6 mg/l, and kinetin up to 0.35 mg/l medium. The

2,4-D concentration higher than 0.6 mg/l medium appeared to inhibit the culture growth.

With respect to anthraquinone accumulation, results obtained from cultures of C. podocarpa, C. alata, C. nodosa and C. occidentalis present a marked contrast to Furuya's finding that IAA was much more favourable than 2,4-D to the synthesis of anthraquinones in Digitalis lanata cultures (Furuya and Kojima, 1971). It is in agreement however, with the observations of Tabata et al. (1975) that 2,4-D was better than IAA for the synthesis of anthraquinones in cultures of C. tora; they suggested that this difference might be partly due to the emodin-type anthraquinones in Cassia and the alizarin type pigments in Digitalis. The emodin-type compounds are believed to be derived from acetate units (Leistner, 1971), and the alizarin type compounds are derived from shikimic acid, α-ketoglutaric acid and mevalonic acid (Leistner and Zenk, 1968). In this investigation, it was also observed that growth regulators did not apparently affect the qualitative aspect of pigment synthesis. This result is in agreement with the observations of Tabata et al. (1975) who have demonstrated that growth regulators did not affect the qualitative pattern of anthraquinone synthesis in cultures of C. tora but the growth regulators did influence the quantitative aspect of pigment synthesis.

Besides 2,4-D and kinetin, an addition of coconut milk (up to 18% v/v medium) into the culture media was necessary to induce vigorous growth of callus tissues of Cassia senna (Rai et al., 1974). Similar observation has been made by Friedrich and Baier (1973) in cultures of C. angustifolia where incorporation of coconut milk into the media was an important factor for good culture growth. In contrast,

cultures of C. tora were grown on media devoid of coconut milk (Tabata et al., 1975). It is evident that an accelerated and much faster growth of tissue could be possible by the addition of coconut milk into the medium. However, in this work it was preferred not to use coconut milk for the following reasons:

1. Recent literature on product synthesis in tissue cultures suggest that rapidly growing cultures accumulate cells and not secondary compounds.

2. Coconut milk is as yet an undefined mixture of substances, therefore, culture medium containing coconut milk remains a chemically undefined medium.

The reasons for a lower concentration of anthraquinone content in C. senna (Rai et al., 1974) and C. angustifolia (Friedrich and Baier, 1973) compared with a higher concentration in C. tora (Tabata et al., 1975) and C. podocarpa in this work, may perhaps be attributed to the fact that a faster growth was obtained due to coconut milk and hence lower pigment accumulation as is the case in C. senna and C. angustifolia.

(iv) Hormonal supplementation in C. podocarpa cultures

Having ascertained that 2,4-D was the best choice as an auxin and that a combination of 2,4-D and kinetin was essential for growth as well as pigment accumulation in cultures of Cassia species, it was decided to study quantitatively the effect of varying concentrations of 2,4-D and kinetin on C. podocarpa cultures with a view to determining an optimum level of 2,4-D and kinetin for both tissue growth and anthraquinone production. C. podocarpa cultures

were selected for this experiment because the initial studies had shown that C. podocarpa cultures accumulated a higher amount of anthraquinone than other Cassia cultures.

The ideal situation would be to find a system in which a very high pigment accumulation as well as equally fast growing tissues could be obtained for a given culture. But in practice it is often difficult to realize this goal. It has been suggested (Yeoman et al., 1980) that actively growing tissues normally produce a lower concentration of secondary products; and similarly, extremely slow growing cultures would produce less cell mass over a given period and therefore, in effect, a low gross yield of secondary products, though the yield would be higher on the basis of per unit cell mass. The emphasis should be on the yield of desired secondary compounds and a compromise situation between the tissue growth and pigment accumulation will need to be worked out for each species.

In this investigation, callus cultures were allowed to grow for ten weeks, a period during which it was assumed that all likely anthraquinone synthesis and accumulation would have been completed. In fact, it was observed that cultures tended to turn dark brown with accumulation of pigments towards the latter stage of this growth period. As mentioned earlier, there is a close parallel in TABLE 9 between the effects of the growth regulators (2,4-D and kinetin) on growth index and anthraquinone production. The depression effect of 2,4-D on callus tissue growth at 0.7 mg/l is similar to that found by Tabata et al. (1975), although they were using 2,4-D level at 1 mg/l. The depression effect of 2,4-D at this concentration on anthraquinone production does not seem to have been recorded previously.

(v) C. podocarpa high yielding cell line

Since anthraquinones are coloured pigments, the 'visual selection' method was used for isolating a high yielding cell line from callus cultures of C. podocarpa. Like many other callus cultures originating from Cassia species, C. podocarpa callus tissue was also composed of primarily brown and grey tissues. The brown and grey colouration is apparently an indication of comparatively high and low levels of pigment formation in respective tissues, and hence, of the differential capacity of these tissues for metabolite production. It was hoped that by selective separation of these brown and grey tissues, it would be possible to establish, eventually, a cell line which would give a higher yeild of anthraquinone metabolites.

The 'visual selection' procedure (see FIGURE 10) applied in this study has not been reported in literature for any other plant tissue culture, and it was particularly developed to suit the problem at hand. This procedure is simple to operate and reproduce.

The method was based on a repeated selective differential separation of brown and grey tissues, and successive culturing in different culture flasks. After three such successive transfers at six weeks interval each, a clear difference in anthraquinone accumulation pattern emerged in brown and grey tissues towards the end of 18 weeks since the initial exercise began (see TABLE 10).

The repeated selection procedure has helped in segregating and/or isolating certain cell types which may be called 'homogenous' with respect to, at least, chemical-variability. However, the possibility of individual cells within this 'homogenous mass' to re-exert their inherent chemical variability over a long period

cannot be ruled out.

An important aspect of the callus cultures of C. podocarpa was their ability to synthesize a "fair amount" of anthraquinone glycosides. These glycosidic compounds are primarily of the 'O-type' and are retained as such in cultures without being transformed to their aglycone forms which are relatively more stable.

It may be noted that though rhein dianthrone glycosides (sennosides) occur in whole plants of C. podocarpa, it was found absent from the callus tissue cultures; this parallels the occurrence of these in plants and its absence in callus cultures of C. senna (Rai et al., 1974), and Rheum palmatum (Rai, 1978b). However, Friedrich and Baier (1973) were able to recover small quantities of sennosides from callus cultures of C. angustifolia. It seems possible that in the future, by probing into cultural conditions, some cell lines of C. podocarpa may be able to form sennosides.

(vi) Product synthesis

Qualitative and quantitative analyses of callus cultures of Cassia spp. investigated have been discussed in Part I of this chapter. A comparative study (TABLE 21), of the anthraquinone contents of Cassia species: C. podocarpa, C. alata, C. nodosa and C. occidentalis and derived callus cultures, shows that accumulation of anthraquinones on the basis of total content is generally lower in cultures than in the plant, except in C. podocarpa cultures where a visual selection method for brown pigmented tissues yielded an appreciably higher anthraquinone content than originally present in the entire plant. It seems probable, that biosynthetic capabilities of callus tissues could be improved progressively by selective subcultures.

TABLE 21. A comparison of the anthraquinone content* in Cassia species and their derived cultures

Anthraquinone content	C. alata		C. occidentalis		C. nodosa		C. podocarpa	
	Plant 1	Callus	Plant 2	Callus	Plant 3	Callus	Plant 4	Callus
Free form	0.30	0.30	0.35	0.32	0.50	0.10	0.65	1.00
Bound form	1.00	0.45	0.35	0.30	0.30	0.40	1.00	0.80
Total	1.30	0.75	0.70	0.62	0.80	0.50	1.65	1.80

*% w/w in dry weight

1 - Rai, 1978c; 2 - Rai and Shok (1983); 3 - Rai (1979a); 4 - Rai and Obayemi (1978)

Investigations in the area of plant tissue culture as a possible source of pharmaceuticals have been directed almost exclusively towards the search for the same isolates as would have been found in the entire plant. It was of course recognised that tissue cultures do not possess either the complete biochemical ingredients or the facility for translocation found in the parent plant. It is now known that there are certain tissue cultures which synthesize none of the secondary products found in the organ from which they were derived (Bohm, 1982). It would seem that the change from the differentiated tissues to undifferentiated callus may involve the loss of certain synthetic abilities, possibly not necessarily an irreversible loss, but one which may have to be revitalized by a change in the growth environment and cultural conditions.

On the other hand, the production in tissue culture, of constituents

not normally found in the parent plants, might provide the means of manipulating the culture system to produce novel compounds that might be of pharmaceutical interest. However, having said that, it should be recognised that tissue culture should normally be viewed as a system that allows for the enhancement of a process which can be observed in nature, i.e., as a means to enhance an ability already present. Tissue culture is not the miracle which allows for the expression of genetically non-existent characters.

CHAPTER FOUR

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