

Figure Culture of Antimalarial Plants

Thesis

Presented by

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for the degree of

Master of Philosophy

of the

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سورة الرحمن الرحيم

To my parents and
beloved children
Abote, Saaf and Siba

ABSTRACT

Investigations utilising in vitro cultures of two species, Cinchona ledgeriana (Rubiaceae) and Artemisia annua (Asteraceae) were carried out. Root organ suspension cultures of C. ledgeriana produce a considerable amount of anthraquinones. In the present investigation scopoletin and five new anthraquinones have been isolated and identified as: 1-hydroxy-2-methyl-(3,4,6) or (3,4,7)-trimethoxy anthraquinone; 1-hydroxy 2,3,5-(or 2,3,8)-trimethoxy anthraquinone; anthragallol 1,2,3-tri-methyl ether; 1,6-dihydroxy-2-methyl-7-methoxy or 1,7-dihydroxy-2-methyl-6-methoxy anthraquinone; 2-methyl-6-hydroxy-7-methoxy or 2-methyl-7-hydroxy-6-methoxy anthraquinone.

Thin-layer, column and high performance liquid chromatography were utilised for preparative and analytical separations. The isolated compounds were characterised by the use of ultraviolet, infrared and proton magnetic resonance spectroscopy together with mass spectrometric techniques. Substitution pattern of the A and C rings were established by nuclear Overhauser techniques.

Extracts of Artemisia annua cultures have been assessed for in vitro activity against the malarial parasite Plasmodium falciparum. Callus and suspension cells and medium were analysed and examined for their activity at different stages of growth and development.

Time-course experiments were carried out to investigate the influence of various basal media, plant growth regulators and light on both growth and possible artemisinin production. Two active fractions were obtained but artemisinin was not detected using thin-layer chromatography only.

ACKNOWLEDGEMENTS

I am deeply grateful to Professor J.D. Phillipson for his help and guidance throughout the course of this project.

I would like to thank Dr. M.F. Roberts for her supervision during this work.

I am extremely grateful to Dr. L.A. Anderson for developing the plant tissue cultures and especially for all her support and continual encouragement over the years.

I wish to thank Dr. D.H. Bray and Dr. W.C. Warhurst of The London School of Hygiene and Tropical Medicine for the antimalarial testing.

I am grateful to the staff of the mass spectrometry unit and Mr. W. Baldeo of the NMR department at The School of Pharmacy for running the mass spectra and the 60 MHz spectra and also to the staff of the NMR department, King's College, London for the 250 MHz spectra.

My thanks also go to the technical staff and fellow postgraduate students of the Department of Pharmacognosy and the staff of the Department of Microbiology for their assistance over the two year period.

I am grateful to Mrs E. Baldeo for her help, care, patience and kindness during typing this thesis.

Last, but not least, I would like to express my sincere thanks to my parents, husband and family for all their patience, financial support and continual encouragement.

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ABBREVIATIONS

NAA :naphthalene acetic acid
IAA :indole acetic acid
IBA :indole butyric acid

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Section 1.1 Plant cell and tissue cultures as alternative methods for the production of secondary metabolites.

Within the last decade there has been a rapid development of plant tissue culture techniques (1). These techniques have become recognized as a possible means of producing valuable chemical substances, e.g. pharmaceuticals. The potential of plant cells for the synthesis and accumulation of secondary products such as alkaloids and glycosides has led to the suggestion that cell cultures could provide an alternative to whole plants as a source of these products (2 - 3). A series of investigations on the production of secondary metabolites by plant tissue cultures are summarized in the reviews (4 - 6) and these techniques have advantages which might be summarized as follows: independent of climatological or political circumstances a constant flow of products of constant quality is assured without the risk that species may become extinct due to too extensive harvesting. Plant cell and tissue cultures are also considered to be ideal systems for studying the biosynthesis of secondary metabolites. Plant cell and tissue cultures can be employed as a source for enzymes, that can be employed either as a crude enzyme preparation for cell free secondary product synthesis (7 - 9), or purified to catalyze certain specific reactions (10 - 12).

Many of the substances used in the pharmaceutical food, flavour, and perfume industries originate from plants (13) and many such compounds are in rapidly

increasing demand. Their availability and cost is frequently affected by political and economic reasons; an alternative source is clearly desirable so that an adequate supply of these materials is available (13). In order to be useful as an alternative source of secondary compounds, a cell culture must produce a good yield of the final product, greater than that of the parent plant. Examples can be given of plant cell cultures that produce the desired secondary metabolites in relatively large amounts, e.g. anthraquinones in Morinda citrifolia cell cultures (14 - 15), and in cell cultures of many other Rubiaceae species (15), berberin and Thalictrum minus (16) and Coptis japonica cell cultures (17), shikonin in Lithospermum erythrorhizon cell cultures (18) and rosmarinic acid in Anchusa officinalis (19 - 20) and Coleus blumei cell cultures (21). However, the cases in which low yields or none at all are far more numerous.

In this particular study, plant tissue culture was used, with the aim of producing larger yields of secondary metabolites particularly with Artemisia annua where the percentage of artemisinin in the whole plant is usually low. It is essential to provide sufficient amounts of artemisinin for antimalarial studies. Biotechnological uses of plant cell cultures require cell suspension cultures because only this type of cell culture possesses the sufficient growth rates and the necessary cellular homogeneity with respect to cellular differentiation. Newly established cell suspensions are very heterogenous populations with respect to synthetic capacity and accumulation behaviour of secondary compounds. Individual cells

with a greatly enhanced rate of biosynthesis have therefore to be selected (1).

The method for selection has been described (22 - 23), the main aim being to produce genetically stable, high-producing strains (1). These techniques have been successfully employed in the production of ajmalicine (1.02% d.wt) from Catharanthus roseus cultures (24), and jatrorrhizine (10% d.wt) from Berberis cultures (25).

In cell suspensions the cells are thought to be devoid of the regulatory pattern which exists in the intact plant tissue, secondary products often differing qualitatively and quantitatively in the cell cultures and parent plant. The extreme variability of secondary constituents being exemplified by studies on the formation of indole alkaloids in Catharanthus roseus cell cultures (1). Furthermore, many cell culture strains were found to contain complex strain specific mixtures of alkaloids, several being biosynthetic intermediates which do not occur in the intact plant.

In addition, several other methods of producing high yielding cell strains have been developed. These may involve treating the cells with mutagenic agents (chemicals, high energy irradiation), or by growing cells in the presence of various chemicals which kill wild-type cells and favour regulatory mutants (1).

Plant suspension cultures have also been used for biotransformation of organic substrates e.g. the 12 - hydroxylation of digitoxin (not used pharmaceutically) to clinically useful digoxin. The process could be economically feasible if not competing with one that can be carried out by micro-organisms (25).

Section 1.2 Anthraquinones

1.2.1 Anthraquinones in the Rubiaceae

In 1971 about 50 new anthraquinones had been isolated from members of the Rubiaceae (26). Many species in the family Rubiaceae, have long been known to contain substantial amounts of anthraquinones, well known are the genera Rubia, Galium and Morinda (26), with the roots being especially rich sources of anthraquinones. Tissue cultures of Rubiaceae also produce large amounts of anthraquinones (14 - 15) and in some cases the levels of anthraquinones found in cultured cells exceed those in the intact plant (15). The anthraquinones are most often present as aglycones and sometimes in the form of glycosides; in the latter case the sugar moiety is most often (glucose-xylose). Because of their anthraquinone content many plants of the Rubiaceae have been used, all over the world for the preparation of natural dyes. The best known for this purpose is madder, i.e. the ground root of Rubia tinctorum L., which produces alizarin (27). The colours most frequently observed when alizarin is used with aluminium mordant are various shades of red. As alizarin forms lakes with many metal oxides many other colours can be obtained as well. The colours for which madder has been used most frequently are red, pink, violet, lilac, dark-brown and black (28).

The cultivation of madder for dyeing purposes was once of great economic importance as can be seen from the value of the traditional madder industry in France, which around 1865 was worth some 16 million US dollars per year. The synthesis of alizarin in 1869 by Graebe and Liebermann wiped out the madder industry in about four years, with the

revenues being transferred completely from France to Germany (28).

Tissue culture of some species in particular have been studied extensively for their anthraquinone production Morinda citrifolia (14, 29, 30, 31), Galium mollugo (32, 33), and Rubia cordifolia (34 - 36). Schulte et al. (35) reported on the optimization of the anthraquinone production in cell suspension culture of 19 Rubiaceae species belonging to the genera Asperula Galium, Rubia and Sherardia. In all cases it proved possible to improve the production of anthraquinones substantially by optimization of the culture medium. Anthraquinone concentrations in cultural cells are among the highest known today for any product (37).

1.2.2 Biological activity

The naturally occurring anthraquinones can be divided into two groups on the basis of their biosynthetic pathways, a division which parallels therapeutic use. The first of the two groups is the acetate derived class of anthraquinones (38). This group of anthraquinones which occurs mostly in the form of glycosides is found in the Leguminosae (Cassia Sp.), Rhamnaceae (Rhamnus sp.) and Polygonaceae (Rheum sp.) and is widely used in medicine because of their laxative action (43, 44) (see Figure 1a, P.7).

The second group of anthraquinones found in the Rubiaceae and some related families, e.g. the Bignoniaceae, Scrophulariaceae and Verbenaceae, has been used in traditional medicine (41), for example in Japan, for the coagulation of blood (29). In West and East Germany, extracts of the roots of Rubia tinctorum L. have been used for the treatment of kidney stones (42). To our knowledge

no other therapeutic uses have been reported for Rubiaceous anthraquinones in western medicine, certainly they do not have any laxative action.

Some of the Rubiaceous anthraquinones, however, exhibit very interesting biological in vitro activities: antimicrobial (41), hypotensive (43) and antileukemic (44, 45) properties have been reported.

1.2.3 Biosynthesis of Rubiaceous anthraquinones

During the past twenty years, much work on the biosynthesis of anthraquinones in the Rubiaceae has been performed by Leistner and co-workers. Their work has showed that the rubiaceous anthraquinones are formed through the shikimate-mevalonate pathway (46 - 48) and that they are biosynthesised from *o*-succinyl benzoic acid, derived from shikimic acid, α -ketoglutaric acid, and mevalonic acid (Fig. 1 p 8) (46 - 50). In a more recent study, Inoue and co-workers (51) have also shown that shikimic acid and *o*-succinylbenzoic acid are involved in anthraquinone biosynthesis in Galium mollugo L. cell suspension cultures and that 1,4-dihydroxy naphthoic acid and 1,4-dihydroxy-3-prenyl-2-naphthoic acid are important intermediates in this biosynthetic pathway.

This biosynthetic route clearly indicates that a methyl group or an oxidized methyl group is always found in the C-2- or in the C-6-position which are not equivalent biosynthetically. It is therefore proposed to use a biogenetic numbering for the anthraquinones found in the Rubiaceae (see Figure 9 , p. 79). Comparing the biosynthetic pathway leading to the Cinchona anthraquinones (Fig. 1b) and the alkaloids, it is obvious that

for both biosynthetic pathway mutual precursors are being involved. Chorismic acid is a precursor of L-tryptophan, a precursor for the terpenoid indole and quinoline alkaloids, as well as a precursor for iso-chorismic acid, which is the key precursor for the biosynthesis of anthraquinone. In addition, mevalonic acid which is a product of primary metabolism, is a precursor of the iridoid moiety of Cinchona indole and quinoline alkaloids as well as ring C of the anthraquinones.

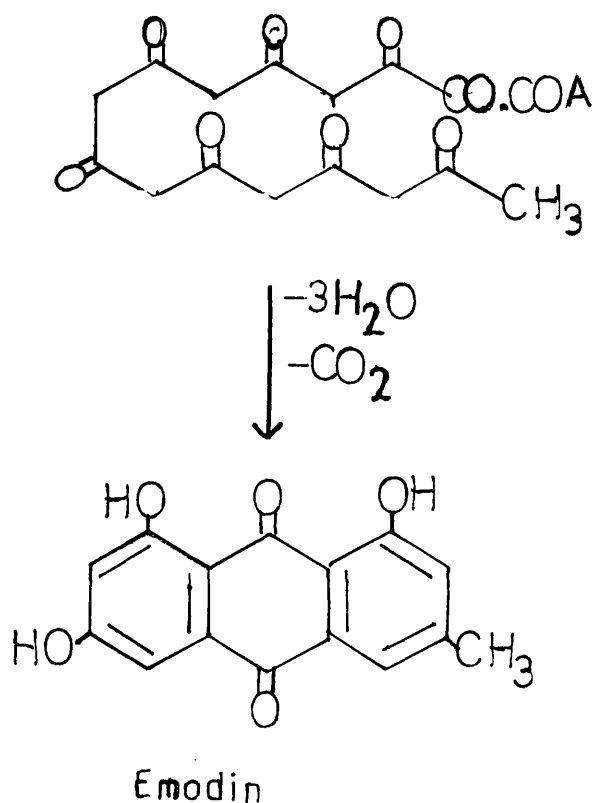


Figure 1a Biosynthesis of anthraquinones via acetate pathway.

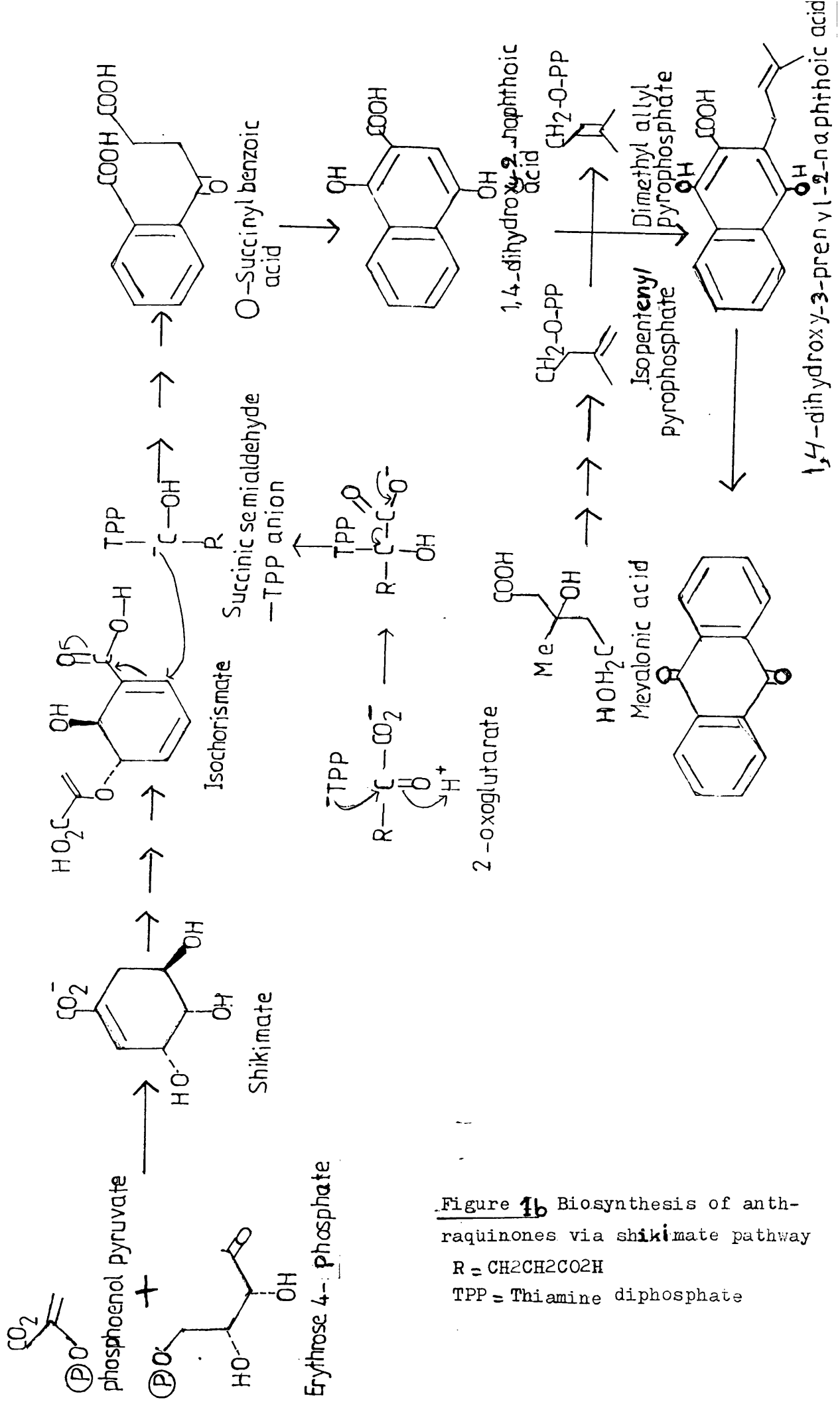


Figure 1b Biosynthesis of anthraquinones via shikimate pathway

R = CH₂CH₂CO₂H

TPP = Thiamine diphosphate

Section 1.3 Spectroscopy of anthraquinones

The anthraquinone pigments are common metabolite products of fungi and may be found with a multiplicity of hydroxyl functions. The anthraquinones found in the Rubiaceae constitute a homogeneous group of compounds, all being derivatives from the basic tricyclic structure given in (Figure 9, p. 79). They differ only in the nature of substituents and the substitution pattern of rings A and C. In addition to the already mentioned (oxidized) methyl group in the 2-position, hydroxyl and methoxyl groups are found most frequently as substituents. These substituents are mostly found in ring C at the C-1, C-2, C-3 and C-4 positions and in ring A at the C-5 and C-6 positions. To establish the nature and number of substituents as well as the substitution pattern spectroscopic methods have proved extremely valuable.

1.3.1 UV Spectroscopy

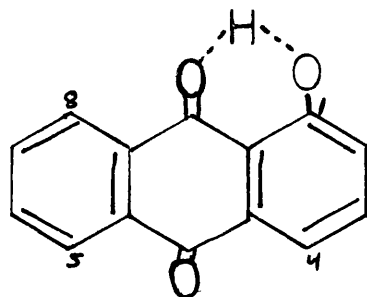
This large group of pigments consists mostly of polyhydroxy or alkoxy derivatives, and the influence of these substituents dominates the spectra (26). The UV spectrum of anthraquinone indicates a link between the spectra of natural quinones with the spectra of hydroxy-aromatic ketones. Therefore, the spectrum of anthraquinone may then be regarded as a conjugated acetophenone-benzoquinone system (52). Anthraquinone shows intense benzenoid absorption at ca. 250 nm and medium absorption at 322 nm, strong quinonoid bands are seen at 260 - 290 nm. A typical UV spectrum of naturally occurring anthraquinones can be seen in (Fig. 2 and 3, p. 11) (53). The UV spectrum recorded in alkaline methanol or ethanol gives information on the

location of hydroxyl groups (54). The values for UV maxima in alkaline ethanol are listed in Table 1, p. 12) recorded for pure hydroxy-anthraquinones not bearing any other substituents. However, naturally occurring anthraquinones which contain other substituents may show either positive or negative effects in their UV spectra maxima, due to these substituents. The extent of chromic shifts may also vary.

1.3.2. IR Spectroscopy

The carboxyl frequencies of quinones are useful diagnostic aids in structure determination and have been studied extensively. The IR spectrum of an anthraquinone gives information on the environment of the carboxyl group in the C-9 and C-10 positions. Most anthraquinones have an intense absorption band in the region $1600-1575\text{ cm}^{-1}$ but this overlaps the carbonyl peak in highly chelated derivatives (55).

If a perihydroxyl group is present, the carbonyl absorption band will show a shift to smaller wave numbers due to hydrogen bonding between the perihydroxyl and the adjacent carbonyl group (26). The presence of a free (or C-2, C-3, C-6, C-7 positioned) hydroxyl group can also be readily deduced from the IR spectrum (see Table 2, p. 12). The peri position refers to C-1, C-4, C-5 and C-8.



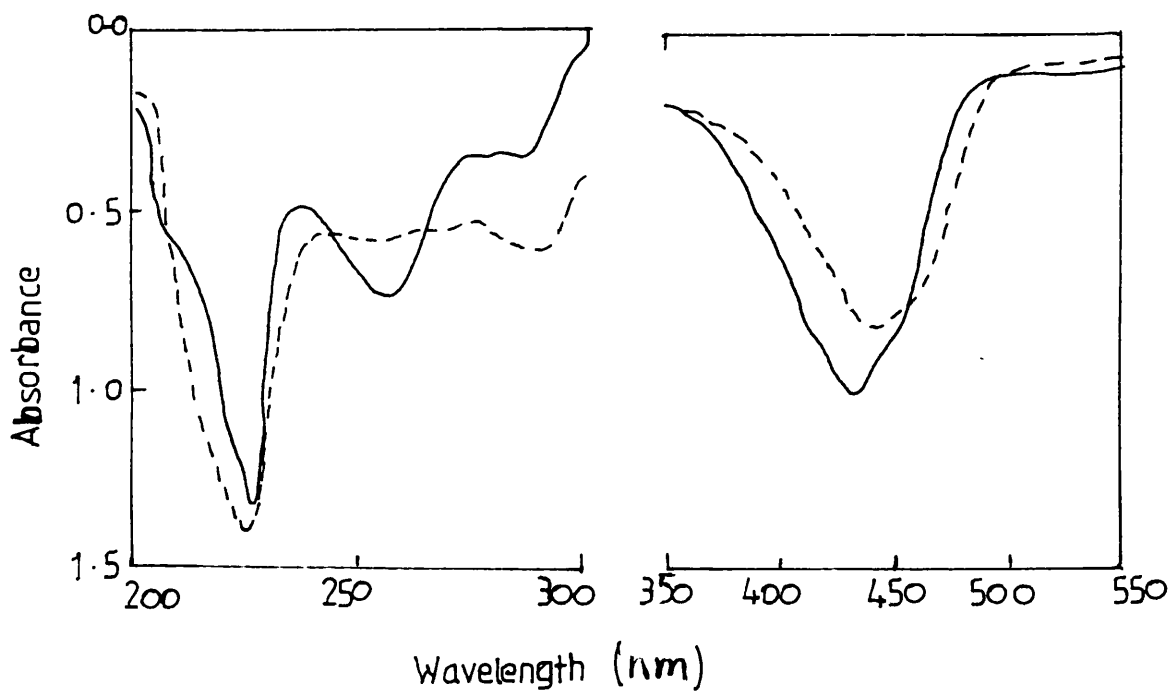


Figure 2 Typical U.V. spectra of anthraquinones with one chelated CO and the other is free

— Aloe-emodin (position 6 is free)
 - - - Emodin (position 6 is substituted).

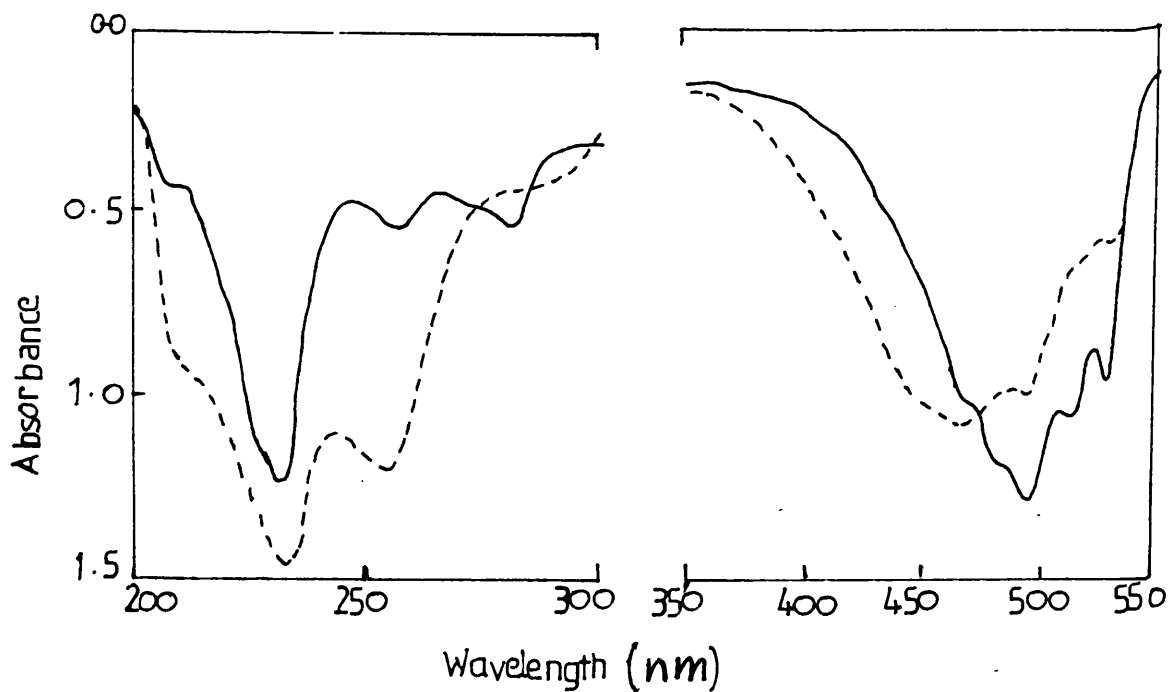


Figure 3 Typical U.V. spectra of anthraquinones with both CO chelated.

— Catenarin
 - - - Catenarin acetate

	λ_{\max} (nm)
1-hydroxy anthraquinone	500
2-hydroxy anthraquinone	478
1,2-dihydroxy anthraquinone	576
1,3-dihydroxy anthraquinone	485
1,4-dihydroxy anthraquinone	560
1,5-dihydroxy anthraquinone	510
1,8-dihydroxy anthraquinone	515
1,4,5,8-tetrahydroxy anthraquinone	630

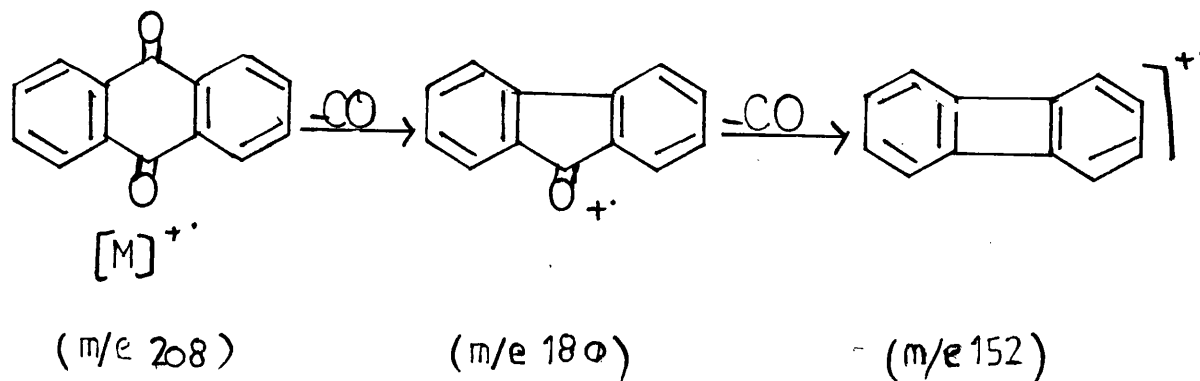
Table 1 Longest wavelength maxima in the UV spectra of hydroxy anthraquinones recorded in alkaline ethanol, useful for the determination of hydroxyl group substitution patterns. (54)

	ν_{\max} (cm ⁻¹)
Free hydroxyl group	3400
Non-chelated carbonyl group	1675
Chelated carbonyl group	1630

Table 2 Absorption bands of primary diagnostic value in the IR spectra of anthraquinones. (26,55)

1.3.3 Mass Spectrometry

Mass spectrometry gives information on the molecular weight of a compound, the molecular ion almost invariably forms the base peak in the EI mass spectra of the anthraquinone aglycones. Anthraquinone itself (56) |see below, undergoes successive elimination of two molecules of carbon monoxide to give strong peaks at m/e 180 ($M-CO$) and 152 ($M-2CO$) (and strong doubly charged ions at m/e 90 and 76). Spectra of derivatives follow the same pattern with additional peaks appropriate to the substituents and whether they are α - or β - to the keto group (57,58). A mass spectrum may also provide information on the location of methoxyl groups. In the case of a peri methoxyl group fragmentation peaks corresponding to $M^+ - H_2O$ and $M^+ - H_2O$ are observed and such ion peaks are not observed in the corresponding β -methoxyl derivatives, (59). 1,8-Dihydroxy anthraquinone can be distinguished from its 1,5-isomer by virtue of the abundant $M^+ - Me$ ion peak (60). Both α - and β -methoxyl compounds eliminate a formyl radical ($M^+ - CHO$) but again this fragmentation is only significant for peri-isomers (57). However, this information should be viewed with caution if in the same molecule a hydroxyl group is present. The loss of water from the molecular ion will also occur when a hydroxyl group is positioned ortho to a methoxyl group (57).



1.3.4 ^1H -NMR Spectroscopy

As is generally the case in natural products chemistry ^1H -NMR spectroscopy is an indispensable tool for structure elucidation of anthraquinones. In many instances, the ^1H -NMR spectrum allows deduction of the nature and the number of substituents, because generally the substituents will give rise to signals with characteristic chemical shifts (see Table 3, p. 16). However, hydroxyl groups in C-2, C-3, C-6 or C-7 position are not always readily observable. By means of high resolution ^1H -NMR spectrometry the substitution pattern for both ring A and C (see p 79) can also be deduced from the splitting patterns observed in the aromatic region between δ 7.0 and 8.5 ppm (26). The usual coupling constants for the aromatic protons are 7-8 Hz for ortho coupling and ca. 1.5 Hz for meta coupling. Also from the chemical shifts observed for the aromatic protons information on their position can be obtained. In the case of a proton at C-1, C-4, C-5 or C-8 a signal greater than 7.5 ppm will be seen whereas a proton at C-2, C-3, C-6, C-7 will give rise to a signal less than 7.5 ppm, because the two carbonyl groups in the molecule exert a relatively strong deshielding effect on substituents in the peri position (61). A peri positioned methoxyl group will give rise to a signal at ca. δ 4.05 ppm but when a methoxyl group is in the C-2, C-3, C-6, or C-7 position, the signal will be found at ca. δ 3.9 ppm. The deshielding effect of and the hydrogen bonding with carbonyl groups helps in discriminating between other hydroxyl group and a hydroxyl group in the peri position. The signal due to a peri positioned hydroxyl group will be found

between δ 12 and 13 ppm's the signal due to a free hydroxyl group - if it is seen at all - will be found at ca. 10 ppm. All chemical shift values given in (Table 3, p. 16) are derived from spectra recorded in CDCl_3 , and the values observed for the chemical shifts are lower when recorded in $\text{DMSO } d_6$ or CD_3OD , usually by ca. 0.10-0.20 ppm.

1.4.6.5 ^{13}C NMR Spectroscopy

^{13}C NMR data for both synthetic and naturally occurring anthraquinones have been reported recently and attempts have been made to determine substituent induced chemical shift parameters. Hydroxyl groups in the peri position cause a considerable paramagnetic shift in the C-9 (C-10) signal, ca. 5 ppm due to hydrogen bonding. Typical shifts for C-9 and C-10 are 183 ppm; in the case of hydrogen bonding to one hydroxyl group this is shifted to 188 ppm, and if there is hydrogen bonding to two hydroxyl groups to 192 ppm (61). Methoxyl signals are usually observed at ca. 55 ppm; however, in case of a 1-methoxyl group in combination with a substituent at C-2 the methoxyl signal is shifted downfield to ca. 61-64 ppm, due to steric hindrance (62). Moreover, it should be noted that the signals due to the carbon 11, 12, 13, 14 are difficult to observe due to their very long relaxation times, and by using a pulse delay of 10 sec. (63) or adding of a relaxation agent such as chromium tris (acetyl acetonate) (62,64) the signals of these carbons can be made visible, the shift differences of the spectra recorded in CDCl_3 and $\text{DMSO } d_6$, due to the solvent being in range of 0-1 ppm (62).

Hoffle (65) found assignment of the various signals in the anthraquinone spectra quite difficult due

Substituents	Chemical Shift (ppm)
CH ₃	2.1 - 1.4
OCH ₃ (in 2,3,6,7-position)	3.8 - 3.95
OCH ₃ (in peri-position)	3.9 - 4.15
CH ₂ O	4.4 - 4.8
O-CH ₂ -O	6.2
H-6, H-7 (of an unsubstituted A-ring)	7.7 - 7.8
H-5, H-8 (of an unsubstituted A-ring)	8.1 - 8.3
Aldehydic H	ca. 10.5
Chelated OH	ca. 13

A signal due to an unchelated hydroxyl group is usually not observed in the ¹H-NMR spectra, but according to some authors it is seen at ca. 10 ppm.

Table 3 Characteristic features in the ¹H-NMR spectra of anthraquinones (CDCl₃). (26,61)

to the small chemical shift differences. The anthraquinones were converted into the corresponding acetoxy derivatives to improve their solubility in organic solvents. He concluded that the use of long-range C-H couplings is useful in some cases for making assignments and thus to establish the sites of substitution. Itakawa et al. (66) reported the ^{13}C NMR spectra of some anthraquinones isolated from Rubia species.

In conclusion it can be said that for structure elucidation of anthraquinones ^{13}C NMR spectroscopy does not yield new information that cannot also be obtained from other spectral data such as UV, IR, MS and ^1H NMR; however, for multi-substituted anthraquinones ^{13}C NMR data might conceivably lead to more definitive structure assignments than are now possible using the other spectroscopic methods.

Section 1.4 Plant tissue culture of Cinchona species.

The genus Cinchona (Rubiaceae) has been of great therapeutic value for many years. The antimalarial activity of extracts of Cinchona bark has been known for centuries and this has led to its widespread use. The compound found to be responsible for this activity is quinine. Quinidine, another major Cinchona alkaloid, is used for the treatment of cardiac arrhythmias and it has recently been shown also to be an effective antimalarial agent (67). Besides their pharmaceutical uses, Cinchona alkaloids are used extensively as a bitter flavouring in the soft drink industry. Because of the demand and the length of time required for alkaloid production to reach any significant levels in the bark and the problems associated with the mass cultivation, attempts have been made to utilise in vitro methods of cultivation. The initial reports of the plant tissue culture of Cinchona were concerned with the in vitro propagation of Cinchona plants with particular interest in the propagation of high yielding clones (68). Over the past 6 years there have been a number of reports concerning the production of secondary metabolites by Cinchona cultures (69-80).

The main focus of research has been the production of quinoline alkaloids particularly quinine and quinidine (86). The fact that, Cinchona cell cultures produce another kind of secondary metabolite, the anthraquinones, has also received considerable attention (81-85).

1.4.1 Anthraquinones produced by Cinchona cultures.

In addition to the quinoline and indole alkaloids, a further category of secondary metabolites, the anthraquinones, has been reported from Cinchona cultures. The production of these compounds is of particular interest as they have only been tentatively reported in the parent plant (87).

The first report on the occurrence of anthraquinones in the genus Cinchona was by Mulder-Krieger et al. (69) who identified five anthraquinones in callus cultures of C. ledgeriana. Prior to this, only one report had appeared in the literature indicating that the genus Cinchona might possibly contain anthraquinones (87). In that report it was concluded on the basis of TLC that anthraquinones were present in Cinchona bark; more exact data, however, were not given. In a study on the secondary metabolites in callus cultures of C. pubescens, Mulder-Krieger et al. (74) identified the same five anthraquinones as in C. ledgeriana by means of Co-TLC with reference compounds: alizarin, alizarin-1-methyl ether, 1-hydroxy-2-hydroxymethylanthraquinone, rubiadin and 1,8-dihydroxyanthraquinone. Fifteen anthraquinones were isolated and identified from callus cultures of C. ledgeriana (82). All anthraquinones were of the structural type characteristic for the Rubiaceae anthraquinones (26,27). Of these anthraquinones seven were known from other members of the Rubiaceae, eight of them had not been found previously. From callus cultures of C. pubescens twelve anthraquinones were isolated and identified (83), seven of them were known compounds, six of which had also been found in C. ledgeriana callus cultures, five

of them were reported for the first time. Recently a report on the isolation and identification of fifteen anthraquinones from suspension cultures of C. ledgeriana appeared (88), of these, five were isolated from Cinchona for the first time. In Table 4, p. 27, all anthraquinones identified in tissue cultures of Cinchona species are summarized. The majority of them are hydroxylated, methoxylated derivatives of the basic anthraquinone skeleton (see fig. 4, p. 24). To date, no glycosides have been reported.

1.4.2 Factors affecting anthraquinone production in Cinchona cultures.

There have been several studies investigating the effect of various factors on anthraquinone production by Cinchona cultures.

Robins et al. (89) reported the influence of medium factors such as plant growth regulators, sucrose and phosphate concentrations, and the addition of organic nitrogen sources on the production of anthraquinones by suspension cultures of C. ledgeriana. It was found that the nature of the plant growth regulator used had a profound influence on the anthraquinone biosynthesis, with 2,4-D giving lower anthraquinone yields than the non-phenoxy acetic acid and auxins such as NAA, IAA and IBA. This finding is in full agreement with the results reported by Zenk et al. (14) for anthraquinone production by Morinda citrifolia cell cultures. However sucrose and phosphate concentrations did not influence significantly anthraquinone production (89). Addition of N.Z-amine (amino acid) (0.1% w/v) proved to stimulate the anthraquinone production.

Hawkes et al. (75) investigated the influence of a number of medium components on the alkaloid and anthraquinone production by C. ledgeriana tissue cultures. They found that the concentration of anthraquinones in the tissue cultures (expressed as $\mu\text{g/g}$ fresh weight) were influenced considerably by these medium variations, although the yield of anthraquinones (expressed as $\mu\text{g/petri-dish}$) was not influenced significantly. It was also found that the effect of illumination being inhibitory on anthraquinone yield.

Results reported by Wijnsma et al. (76) on the effect of varying sucrose concentration in the medium of C. ledgeriana cell suspension cultures, showed that raising sucrose concentration in the medium from 0.5% to 8.0% resulted in a 15-fold increase of the anthraquinone yield, this finding did not agree with the aforementioned report, while the initial nitrate concentration showed no significant influence on the anthraquinone yield (76). Robins et al. (89) also reported on inhibition of the anthraquinone biosynthesis by addition of L-tryptophan to the cultures (0.1 - 5.0 mM, 20-60% inhibition). This inhibitory effect was also reported by Wijnsma et al. (80) for C. ledgeriana cell suspension cultures and by Heide and Leistner (90) for Galium molluga cell cultures and by Schulze et al. (15) for Morinda citrifolia cell cultures. Inhibition of anthraquinone biosynthesis in C. ledgeriana suspension cultures was also caused by glyphosate (N-(phosphomethyl) glycine) (1.0 mM) in the culture medium (89). Glyphosate, an inhibitor of 5-enolpyruvylshikimate 3-phosphate synthase, has previously been reported to inhibit anthraquinone biosynthesis in Galium mollugo cell

cultures (90). Robins et al. (89) reported on the inhibitory effect of high concentrations of anthraquinones on the growth of C. ledgeriana suspension cultures. It was found that 0.1 ng/ml of a crude anthraquinone extract caused 30% growth inhibition.

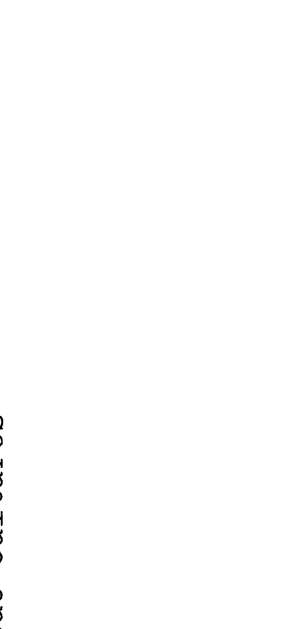
In another study, Robins and Rhodes (91) reported that, the addition of polymeric adsorbents to the suspension cultures of C. ledgeriana stimulates anthraquinone production, which has also been reported by Becker and Herold (92) who used Lichroprep RD8 reversed phase material as an adsorbent for valepotriates in suspension cultures of Valeriana wallichii. Similar experiments were reported by Bisson et al. (93) who used Migbyol 812^(R), a mixture of triglycerides, in a two-phase system with Matricaria chamomilla cell suspension cultures and by Berlin et al. (94) who used the same material in a two-phase culture system with Thuja occidentalis cells.

Robins and Rhodes (91) tested a range of polymeric adsorbents and concluded that Amberlite XAD-7 was the most effective in stimulating the anthraquinone production. A 15-fold improvement of anthraquinone yield, expressed as ml/L/day, was achieved compared to the control culture. Total yield of anthraquinones obtained, using XAD-7 as an adsorbent, was 20 mg/L/day.

Wijnsma et al. (84) found stimulation of the anthraquinone production in cell suspension cultures of C. ledgeriana by addition of sterilized fungal mycelia to the cultures. It was also demonstrated that anthraquinone aglycones exhibit antimicrobial properties and by using the agar-diffusion technique, growth inhibition of a number

of bacteria and a yeast was shown for anthraquinones isolated from callus cultures of C. pubescens. These findings - antimicrobial properties of anthraquinones and the elicitation of the production of anthraquinones - led to the conclusion that anthraquinones in Cinchona species act as phytoalexins (84). Further proof for the phytoalexin nature of the anthraquinones was given in a recent paper (85) in which it was demonstrated that bark of a C. ledgeriana tree infected with Phytophthora cinnamomi, a known phytopathogenic fungus, contained 0.8% anthraquinones, whereas anthraquinones could not be detected in healthy bark pieces, from the same tree.

Figure 4. Structures of anthraquinones produced by Cinchona tissue cultures



Anthraquinone (AQ)	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	Ref.
Alizarin	OH	OH	H	H	H	H	H	H	69,74
Alizarin-1-methylether	OCH ₃	OH	H	H	H	H	H	H	74
Alizarin-2-methylether	OH	OCH ₃	H	H	H	H	H	H	83
Anthragallol-1,2-dimethyl-ether	OCH ₃	OCH ₃	OH	H	H	H	H	H	82,165
Anthragallol-1,3-dimethyl-ether	OCH ₃	OH	OCH ₃	H	H	H	H	H	165
Anthragallol-1,2,3-trimethylether	OCH ₃	OCH ₃	OCH ₃	H	H	H	H	H	165
1,8-DihydroxyAQ	OH	H	H	H	H	H	H	OH	69,74
1,3-Dihydroxy-2,5-dimethoxyAQ	OH	OCH ₃	OH	H	OCH ₃	H	H	H	82,165
4,6(or 4,7)-Dihydroxy-2,7(or 2,6)-dimethoxyAQ	H	OCH ₃	H	OH	H	OH or OCH ₃	OCH ₃ or OH	H	83
6,7-Dihydroxy-1-methoxy-2-methylAQ	OCH ₃	CH ₃	H	Y	H	OH	OH	H	83

Figure 4. Continued

Anthraquinone (AQ)	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	Ref.
1,6-Dihydroxy-5-methoxy-1-methylAQ	OH	CH ₃	H	H	OCH ₃	OH	H	H	165
1,6(or 1,7)-Dihydroxy-2-methylAQ	OH	CH ₃	H	H	H	OH or H	H or OH	H	83
2,5(or 3,5)-Dihydroxy-1,3,4(or 1,2,4)-trimethoxyAQ	OCH ₃	OH or OCH ₃	OCH ₃ or OH	OCH ₃	OH	H	H	H	82,83
5,6-Dimethoxy-1(or 4)-hydroxy-2(or 3)-hydroxymethylAQ	OH or H	CH ₂ OH or H	H or CH ₂ OH	H or OH	OCH ₃	OCH ₃	H	H	82
1,4-Dimethoxy-2,3-methylenedioxyAQ	OCH ₃	O-CH ₂ -O	O-CH ₂ -O	OCH ₃	H	H	H	H	82,165
1,5-Dimethoxy-2,3-methylenedioxyAQ	OCH ₃	O-CH ₂ O	O-CH ₂ O	H	OCH ₃	H	H	H	165
1,3-Dimethoxy-2,4,6-trihydroxyAQ	OCH ₃	OH	OCH ₃	OH	H	OH	H	H	165
1-Hydroxy-2-hydroxy-methylAQ	OH	CH ₂ OH	H	H	H	H	H	H	69,74,82,83,165
1-Hydroxy-2-methylAQ	OH	CH ₃	H	H	H	H	H	H	82,165
5-Hydroxy-2-methylAQ	H	CH ₃	H	H	OH	H	H	H	165
5-Hydroxypurpurin-1-methylether	OCH ₃	OH	H	OH	OH	H	H	H	83

Figure 4. Continued

Anthraquinone (AQ)	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	Ref.
2-Hydroxy-1,3,4,6(or 1,3,4,7)-tetramethoxyAQ	OCH ₃	OH	OCH ₃	OCH ₃	H	OCH ₃ or H	H or OCH ₃	H	83
2-Hydroxy-1,3,4-trimethoxyAQ	OCH ₃	OH	OCH ₃	OCH ₃	H	H	H	H	82,83,165
5-Methoxy-2(or 3)-methyl- 1,4,6-trihydroxyAQ	OH	CH ₃ or H	H or CH ₃	OH	OCH ₃	OH	H	H	82
4-Methoxy-1,3,5-trihydroxy AQ	OH	H	OH	OCH ₃	OH	H	H	H	82
2-Methyl-1,4,5-trihydroxyAQ	OH	CH ₃	H	OH	OH	H	H	H	165
Morindone-5-methylether (or 1,7-dihydroxy-8-methyl- 2-methylAQ)	OH	CH ₃	H	H	OCH ₃ or H	OH or H	H or OH	H or OCH ₃	82
Purpurin	OH	OH	H	OH	H	H	H	H	82,165
Purpurin-1-methylether	OCH ₃	OH	H	OH	H	H	H	H	82,83
Rubiadin	OH	CH ₃	OH	H	H	H	H	H	82
1,2,5,6-TetramethoxyAQ	OCH ₃	OCH ₃	H	H	OCH ₃	OCH ₃	H	H	165

Table 4. Anthraquinones produced by Cinchona tissue cultures

	<u>Species</u>	<u>Culture System (Ref.)</u>
1-Hydroxy-2-hydroxymethyl AQ	<u>C. ledgeriana</u>	C(69,82), S(81, 165)
	<u>C. pubescens</u>	C(83,74)
Rubiadin	<u>C. ledgeriana</u>	C(69,82, 81)
	<u>C. pubescens</u>	C(74)
Alizarin; Alizarin-1-methylether; 1,8-Dihydroxy AQ	<u>C. ledgeriana</u>	C(69)
	<u>C. pubescens</u>	C(74)
2-Hydroxy-1,3,4-trimethoxy AQ; Purpurin	<u>C. ledgeriana</u>	C(82)
	<u>C. pubescens</u>	S(.81)
	<u>C. pubescens</u>	C(83)
Anthragalloyl-1,3-dimethylether; 1-Hydroxy-2-methyl AQ; 1,4-Dimethoxy-2,3-methylenedioxy AQ; 1,3-Dihydroxy-4- methoxy AQ; 1,3-Dihydroxy-2,5-dimethoxy AQ	<u>C. ledgeriana</u>	C(82)
	<u>C. ledgeriana</u>	S(.81, 165)
2,5-(or 3,5)-Dihydroxy-1,3,4-(or 1,2,4)-trimethoxy AQ	<u>C. ledgeriana</u>	C(82)
	<u>C. pubescens</u>	C(83)

Table 4. Continued

	<u>Species</u>	<u>Culture System (Ref.)</u>
Morindone-5-methylether (or 1,7-Dihydroxy-8-methoxy-2-Methyl AQ); 5,6-Dimethoxy-1-(or 4)-hydroxy-2-(or 3)-hydroxymethyl AQ; 4-Methoxy-1,3,5-trihydroxy AQ	<u>C. ledgeriana</u>	C(82)
Anthragalloyl-1,2,3-trimethylether; 1,4,5-Trihydroxy-2-Methyl AQ; 5-Hydroxy-2-Methyl AQ; 1,2,5,6-Tetramethoxy AQ; 1,5-Dimethoxy-2,3-methylenedioxy AQ; 2,4,6-Trihydroxy-1,3-dimethoxy AQ; 1,6-Dihydroxy-5-methoxy-2-methyl AQ	<u>C. ledgeriana</u>	S(81 , 16S)
Alizarin-2-methylether; Purpurin-1-methylether; 2-Hydroxy-1,3,4,6- (or 1,3,4,7)-tetramethoxy AQ; 1,6-(or 1,7)-Dihydroxy-2-methyl AQ; 5-Hydroxypurpurin-1-methylether; 4,6-(or 4,7)-Dihydroxy-2,7-(or 2,6)-dimethoxy AQ; 6,7-Dihydroxy-1-methoxy-2-methyl AQ	<u>C. pubescens</u>	C(83)

Abbreviations:

AQ - anthraquinone

C - callus

S - suspension

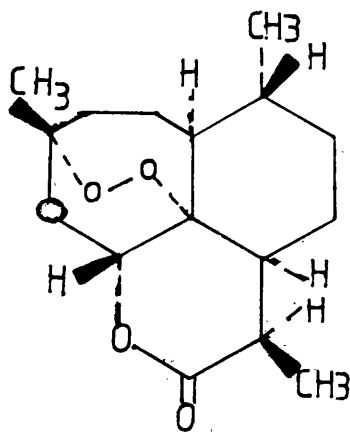
Section 1.5 Medicinal uses of *Artemisin annua* L.

Synonyms. Qing hao, sweet worm wood,
Annual worm wood.

The genus Artemisia has always been of great botanical and pharmaceutical interest, as well as in the liqueur-making industry. For many years members of the Compositae have been used in folk medicine throughout the world and the use may, at least partly, be ascribed to the composition of their main constituents in particular, the sesquiterpene lactones and essential oils. Extracts of the leaves, flowers, or the whole plant have been employed in treatment of gastric disturbances (95), malaria (96,97), and used as anthelmintics (98). Traditionally these plants have been used as bitter tonics to increase the appetite.

Interest has focussed recently on one particular species of Artemisia, namely Qing hao (99) (A. annua L.). Its earliest reported use was for the treatment of haemorrhoids. Ge Hong, used the herb to reduce fevers by soaking one handful of Qing hao in 1 litre of water, straining the liquor and drinking it (100). However, although the herb has been used for many centuries in Chinese traditional medicine as a treatment for fever and malaria, the active principle of the herb was not isolated and characterised until 1972, when Chinese scientists isolated from the leafy parts of the plant the substance responsible for its reputed medicinal action. This compound, called Qinghao Su (QHS, artemisinin) (see the structure below) (101), is a sesquiterpene lactone that bears a peroxide grouping and, unlike most other antimalarials, lacks a nitrogen-containing heterocyclic ring system. The compound has been used successfully

in several thousand malaria patients in China, including those with both chloroquine-sensitive and chloroquine-resistant strains of P. falciparum. Thus QHS and its derivatives offer promise as a totally new class of anti-malarial. Subsequently, from that date to the present, this drug and several derivatives have been studied by Chinese scientists with regard to their efficacy in laboratory malarial models, their pharmacology and pharmacokinetics and their toxicology. Also Qing hao su and its derivatives have been used in clinical trials under the auspices of the Ministry of Public Health, People's Republic of China, to treat cases of uncomplicated falciparum malaria, cerebral and vivax malaria.



Qing hao Su (QHS, artemisinin)

Section 1.6 The development of artemisinin and its derivatives as antimalarial drugs.

The spread of resistance of Plasmodium falciparum to 4-aminoquinolines, particularly chloroquine, through South East Asia, the West and South America, and its recent appearance in East Africa, is a major impediment to the control of malaria in these areas. Malaria causes a greater economic loss than any other disease and it is a major cause of infant mortality in many less developed countries, particularly in Africa (102). Therefore, it should be realised that malaria must be considered as the world's most common tropical diseases. The World Health Organisation, through its expert committee on malaria, has instituted extensive programmes for the drug control and eradication of malaria (103-104). This situation has been made more desperate with reports, during the last two years, of resistance to pyrimethamine/sulphonamide combinations which were until recently, the only operationally useful formulations against such chloroquine-resistant strains. Although it is hoped that the quinoline-methanol drug, mefloquine, will become available in the near future for the treatment of multi-resistant strains of P. falciparum, it must be recognized that other alternative drugs should be made available. This is especially true in view of the possibility of mefloquine resistance appearing, following its widespread and possibly uncontrolled use. Ideally, any new drug should have a novel structure and mode of action, since this may lead to a longer operational life, as it may take more time for resistance to the drug to arise. In addition, there is still a need for a well-tolerated, safe

formulation which shows a rapid action for use against severe forms of falciparum malaria, especially cerebral malaria.

The Chinese antimalarial drug, artemisinin, apparently fulfills most of these criteria, since it has novel structure, appears to have a mode of action different from existing blood schizonticides, and has a rapid action.

1.6.1 Artemisinin produced by Artemisia annua herb

Attempts to confirm the antipyretic and antimalarial activity of a hot-water extract of A. annua were disappointing. In 1971, in vivo tests using P. berghei in mice indicated that the antimalarial activity of ethereal extracts of A. annua resided in the neutral portion (105). Subsequently, seven sesquiterpenes were isolated from this fraction of which artemisinin (106), was shown to have antimalarial properties.

Although, antimalarial peroxides can be obtained from another Chinese herb, Artemisia annua is the only one which is readily available and gives good yields of the active compounds (103).

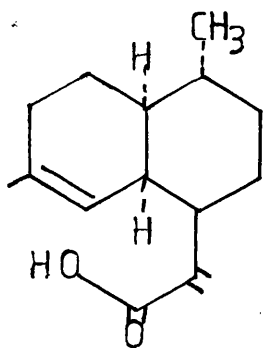
In general artemisinin is obtained only from the aerial parts of the plant, and the yield varies with the agronomical conditions. The relatively low content of artemisinin in cultivated European and New World types of A. annua L. has been up to now the limiting factor for an economic isolation on a technical scale. In Chinese variety of the plant, the maximum yields obtained are between 0.3 - 0.5% (w/w), whereas poor yields may be as low as 0.01% (w/w) (103). However, artemisinin was obtained in 1983 from the dried leaves or flowers in 0.06% yield

from plants growing in the Washington, D.C., vicinity (101).

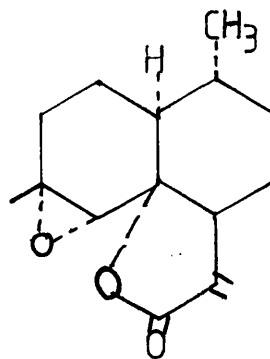
By comparison, yields in China (103) with the varieties growing in Sinchuan province | it was found to be the richest in artemisinin (107). More recent work has been undertaken, when the plant of A. annua were cultivated and analysed at different stages of development, in order to investigate the possibility of isolation a greater amounts of artemisinin during one vegetation period. It was found that the highest content occur just before flowering (108). In same study, the analytical results of artemisinin content in various Artemisia plants showed a range from 0 to a maximum of 0.1%. Also they found that the application of growth regulators like chloromequat during the growth period of the plant, increases artemisinin by 30% over the untreated plants (108).

In addition, thirty other species of Artemisia have been examined by Chinese researchers but none yield extracts with antimalarial activity (103). American workers (109) have extracted A. ludoviciana, A. vulgaris, A. schmidtiana, A. portica, A. arbuscula, and A. dracunculus, and none of these show the presence of artemisinin. More interest, artemisinin has been reported from A. apiacea (0.08%) (108), therefore, this is the only other variety up to date containing artemisinin besides A. annua.

Structures of related sesquiterpenes in A. annua are listed below.



Qinghao acid



Arteannin B

1.6.2 Artemisinin production by *Artemisia annua* tissue cutlures.

To date, only one report has appeared concerning the production of artemisinin by tissue culture techniques. Nair et al. (110) have been able to induce callus, root, shoot, or whole plant regeneration using explants from stems, leaves and flower buds, cultured on either Murashige - Skoog (111) or B₅ inorganic Salts (112) modified by supplementing with the following nutrients (mg/L): thiamine HCl (1.0), inositol (100), nicotinic acid (0.5), pyridoxine HCl (0.5) and sucrose (20,000). Preliminary results with various culture systems indicated the presence of artemisinin in roots and rooted plantlets derived from leaf explants or callus cultures therefrom. It was found that callus and unrooted shoots did not produce the compound in amounts detectable in highly sensitive assay system using HPLC with an electrochemical (EC) detector. Even though the preliminary studies by Nair et al. showed that the suspension cultures produced artemisinin in the medium, the amounts obtained were low (ca. 8 µg/ml).

1.6.3 Testing for antimalarial activity.

Assay of artemisinin against *P. falciparum* in vitro revealed that its potency is comparable to that of chloroquine in two Haihan strains (113,114) and of mefloquine in Camp (chloroquine-susceptible) and Smith (chloroquine-resistant) strains (109). Milhous et al. (115) compared the activity of QHS in vitro with that of other well-known antimalarials and reported that the compound is both potent and rapidly acting. The efficacy of QHS relative to some of its derivatives has been evaluated (116,117). The Qing hao su anti-

malarial co-ordinating research group (96), suggested that QHS has no practical effect on either the early or persistent exo erythrocytic tissue stage at daily doses of 50 mg/kg and 200 mg/kg for 3 days in mice infected with P. berghei or in monkeys infected with P. cynamolgi respectively. Also in the same study it was found that QHS had no detectable prophylactic effects on the pre-erythrocytic stages in chickens infected with sporozoites of P. gallinaceum and treated with QHS, 200 mg/kg daily for 6 days.

1.6.4 Cerebral malaria and other therapeutic applications of artemisinin.

The most striking results received with QHS and its derivatives are seen in the treatment of cerebral malaria. This potentially fatal disease is an advanced form of P. falciparum malaria that can occur when more than 5 per cent of erythrocytes are infected with parasites. However, the cure rate average of QHS was about 90% (118), and the time for recovery from the coma was about 21 hours when oil suspensions was used. Chloroquine and quinine are considerably slower acting. Because of its unusual structure, QHS is being examined for further therapeutic applications. Investigators have studied QHS in the treatment of systemic Lupus erythematosus (119) and have reported that the compound has antiviral activity effect on influenza virus in chick embryo (120). It has an adjuvant effect on cell-mediated immunity, as well as a marked immuno-suppressive effect on antigen-binding and antibody-forming spleen cells in mice (120), but it is less potent than cyclophosphamide. Moreover, when QHS was tested against clinical isolates of Gram-positive and Gram-negative organisms, it exhibited no

antibacterial activity (121). Furthermore, the compound was also inactive against P 388 leukemia (122).

Section 1.7 Factors affecting secondary metabolite production in plant cell and tissue cultures.

Numerous factors in the cultural environment have been shown to affect secondary metabolite production, particularly of alkaloids, in plant cell cultures. It has long been acknowledged that factors such as the degree of differentiation and components of the culture medium, in particular, carbon source, macro- and micro-nutrients and plant growth regulators, can have a profound effect on secondary metabolite yield (123-125).

The degree of differentiation or organogenesis shown by the cultures may be an important factor as to whether they are capable of accumulating the desired secondary metabolites. It has been suggested that the lack of the necessary enzymes for secondary metabolism is the main reason that plant cells may be unproductive; however, this would not appear to be the case with cultures of Lupinus species. Although the enzymes required for biosynthesis are present, the alkaloids do not accumulate in large quantities, which suggests that inadequate storage facilities result in further metabolism of the alkaloids (126). Cell selection techniques have proved to be some of the major tools in improving the productivity of cell cultures. Simple methods of selection are based on visual characteristics, in particular, fluorescence microscopy for compounds such as serpentine and berberine (96, 127). Sensitive radio- and enzyme-immunoassay methods have

been developed for some alkaloids eg. quinine (128), serpentine and ajmalicine (129). Flow cytometry techniques developed for plant cells have proved to be a major advance in cell selection procedures and they have already been used successfully to sort protoplasts derived from C. roseus cultures (130, 131).

There are many different combinations of culture medium used for initiating and maintaining in vitro cultures, they generally differ in their combination of mineral salts, organic growth factors and plant growth regulators. In order to study the effect of medium constituents on secondary metabolite formation, one approach has been to first obtain a culture medium in which cell growth is maximal and subsequently investigate the influence of various constituents on product formation.

The following sections highlight the influence of medium composition on secondary metabolite production.

1. Carbon source: The most common sources of carbon are sucrose and glucose. In general raising the initial sucrose levels leads to an increase in secondary metabolite production (123).
2. Macro- and micro-nutrients: In general, increased levels of nitrate, potassium, ammonium and phosphate support rapid cell growth whereas decreasing the levels often results in growth being limited and secondary metabolism being enhanced (123). Product formation has been found to be stimulated by low initial phosphate concentrations and this has led to the observation that lack of phosphate increases secondary metabolism more than any other nutrient.

3. Plant growth regulators: Plant growth regulators are required by most in vitro cultures for growth and the degree of differentiation exhibited by a culture can be controlled by the concentrations of the auxins and cytokinins present. It is generally acknowledged that 2,4-dichlorophenoxy acetic acid (2,4-D) inhibits secondary metabolism and this has been illustrated with many cultures, including Morinda citrifolia where anthraquinone production was totally inhibited in the presence of 2,4-D (14).
4. Precursors: There have been a number of attempts to increase the productivity of a cell culture by adding the appropriate biosynthetic precursors to the culture medium.

The physical conditions of cultivation such as light, temperature and pH have also been shown to affect secondary metabolite production (123,125, 132-134).

The immobilisation of plant cell cultures within matrices such as calcium alginate beads or polyurethane reticulate foam can result in increased productivity by the cells (135-137) eg. cells of Capsicum frutescens immobilised in polyurethane foam produced levels of capsaicin almost 1000-fold greater than freely suspended cells (138) and Morinda citrifolia cultures immobilised in alginate produced a 10-fold greater level of anthraquinones (139). In most immobilised systems the desired secondary metabolites are released spontaneously into the medium, but in some cases this does not occur readily and has led to the release being induced by permeabilisation of the cells eg. dimethylsulphoxide was used to induce the release of

quinoline alkaloids from immobilised cultures of Cinchona (140). An area of increasing interest is the use of elicitors to induce and enhance alkaloidal production (125,141). The elicitors can be either crude preparations eg. the addition of autoclaved Verticillium dahline conidia or Fusarium moniliforme to Papaver somniferum cell suspension cultures caused a marked increase in morphine and codeine levels (142), or isolated preparations eg. chitosan, a fungal cell wall component, has been reported to enhance yields of acridone alkaloids epoxide in cell suspension cultures of Ruta graveolens (143).

Section 2.1 Plant tissue culture of *Cinchona ledgeriana* and *Artemisia annua*.

2.1.1 Seed source.

The *Cinchona ledgeriana* Moens (Zapote variety) seeds were obtained from Kenya by R. Lennox of Brook-Bond-Liebig.

Artemisia annua seeds were kindly supplied by Prof. W. Peters of the Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, University of London.

2.1.2 Initiation of callus cultures.

The cultures of *Cinchona ledgeriana* (144) and *Artemisia annua* (145) were initiated and maintained using the same procedure. The seeds were surface sterilised in 2% sodium hypochlorite containing Triton-X-100 (BDH) for 15 minutes followed by washing with sterile, distilled water. The seeds were germinated on wetted filter paper in petri dishes. Seedlings were then transferred aseptically onto solid medium composed of Murashige and Skoog basal salts (111; Flow Laboratories), 1.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (Flow Laboratories), 0.1 mg L^{-1} kinetin (Flow Laboratories), 5% sucrose (BDH) and 1.5 Lab.M. agar (London Analytical And Biological Media Inc.) The pH was adjusted to 5.8-5.9 before sterilisation by autoclaving.

2.1.3 Culture conditions.

The callus were maintained at 25°C under continuous illumination and subcultured every four weeks-onto fresh medium.

2.1.4 Suspension cultures.

These cultures were initiated by transferring callus tissue to sterile 250 ml conical flasks containing 40 ml of the above medium (without agar). The cultures were maintained as before in addition to being continually agitated (120 r.p.m., LH Fermentation) on an orbital shaker.

2.1.5 Harvesting.

Fourth generation suspension cultures of Cinchona ledgeriana and callus cultures from third and subsequent generations and suspension cultures from fourth and subsequent generations of Artemisia annua were harvested.

Section 2.2 Phytochemical studies.

2.2.1 General techniques.

2.2.1.1 Organic solvents.

All the organic solvents used were obtained from May and Baker or BDH and redistilled prior to use.

2.2.1.2 Thin-layer chromatography (TLC).

Analytical TLC was carried out using pre-coated silica gel GF₂₅₄ plates (Merck). For identification of anthraquinones, coumarin and some pure compounds obtained from A. annua culture extracts, glass plates measuring 20 x 20 cm were prepared using a slurry of the acetone washed gel suspended in distilled water, and were spread to a thickness of 0.5 mm for preparative work. Si 60 silica gel GF₂₅₄ (Merck) plates were prepared and then run in methanol, dried and activated for one hour at 120°C, these were allowed to cool before use. After development

and visualisation, the component bands were then scraped off the silica gel, transferred into glass columns and eluted with methanol. The solution was evaporated to dryness under reduced pressure.

2.2.1.3 Column chromatography.

Column chromatography was carried out using 70-325 mesh silica gel (E. Merck, Darmstadt, F.R. Germany). Prior to use, silica gel was washed with methanol, air dried and packed into the column (2.5 cm x 30 cm).

2.2.1.4 High performance liquid chromatography (HPLC).

The HPLC separation were obtained using an Altex Isocratic Liquid Chromatography (Model 330) equipped with a variable Hitachi UV spectrophotometer (Model 100-10).

All the solvents used were HPLC grade. The compounds of interest were identified using UV, PMR, IR and MS.

2.2.1.5 Ultraviolet spectroscopy (UV).

The ultraviolet spectra of all anthraquinones were obtained in solution using spectroscopic grade methanol. The instrument used was a double beam Perkin-Elmer 402, Ultraviolet-Visible Spectrometer. The bathochromic shifts in the absorption maxima of anthraquinones possessing a hydroxyl moiety were observed on the addition of one drop of 1N sodium hydroxide.

2.2.1.6 Infrared spectroscopy (IR).

Infrared spectra were obtained using a Perkin-Elmer 298 Infrared-Spectrophotometer. The spectra were recorded in spectroscopic grade chloroform.

2.2.1.7 Mass spectrometry (MS)

Mass spectra were obtained by direct insertion at 70 eV using a ZAB-IF (VG-Micromass Ltd.) mass spectrometer, at inlet temperatures of 170°C to 240°C.

2.2.1.8 Proton nuclear magnetic resonance (¹H-NMR).

Spectra were run by Mrs J.E. Hawkes and Mrs F. Galloway of the NMR Department of King's College, University of London, using a Bruker WM-250 spectrometer and by Mr W. Baldeo at the School of Pharmacy, University of London, using a Bruker WP-80 NMR spectrometer. The samples were dissolved in CDCl₃ and contained TMS as an internal standard.

2.2.2 Extraction of *C. ledgeriana* root organs.

2.2.2.1 Extraction of quinoline alkaloids.

The *C. ledgeriana* root organs were air dried before being exhaustively extracted with methanol. The methanol fraction was dried under reduced pressure and reconstituted in a small volume of 0.9 M sodium hydroxide. The basic extract was applied to Extrelut columns (Merck) and allowed to stand for 30 minutes. The alkaloids were eluted with chloroform.

2.2.2.2 Extraction of anthraquinones.

Ground root organ suspension cultures of *C. ledgeriana* were sonicated with 80% ethanol (aq.) until the solution was colourless. Concentration of the ethanol extract in vacuo at 40°C gave a residue which was suspended in 2% H₂SO₄ (aq.) and extracted with chloroform (x3). The acidic aqueous phase was separated and purified by acid-base extraction to yield the alkaloids. The combined chloroform phase was concentrated in vacuo and a crude extract

containing anthraquinones was obtained.

2.2.2.3 Thin-layer chromatography.

Analytical and preparative procedures utilised adsorption chromatography were carried out (see Section 2.2.1.2, p. 41).

2.2.2.3.1 Authentication.

Quinoline alkaloids quinine, quinidine, cinchonine and cinchonidine were obtained from Sigma. Scopoletin was obtained from Prof. S.A. Brown, University Peterborough, Ontario, Canada and the samples of anthraquinones were provided from Prof. R. Verpoorte, University of Leiden.

2.2.2.3.2 Solvent systems used in TLC.

The following systems were used according to the particular group of compounds under study.

- A Chloroform : diethylamine (9:1)
- B Toluene : ethyl acetate : glacial acetic acid
(74:25:1)
- C Toluene : ethyl acetate : glacial acetic acid
(97:30:2)
- D Toluene : ethyl acetate (74:25)
- E Chloroform : methanol : ammonia (0.88) (85:14:1)
- F Toluene : ether (1:1) saturated with 10% acetic acid
- G Chloroform : methanol : water (lower layer) (65:35:10)

System A was used exclusively for the separation of alkaloids and the following R_f values for reference alkaloids were obtained: quinine, 0.15 and quinidine, 0.26.

The anthraquinones were studied using system B to E. The coumarin being separated using system B and F. The solvent system G was used to separate the main proportion

of the anthraquinones extract which are extremely polar and stayed on the base-line when using the solvent systems B to E.

Wherever possible, compounds were co-chromatographed with authentic standards.

2.2.2.3.3 Visualisation.

For both alkaloids and coumarin chromatograms, the visualisation was carried out under UV light (254 and 366 nm). In addition, the alkaloid-chromatograms were sprayed with methanolic sulphuric acid (10%) and viewed under UV light (366 nm). Anthraquinones were detected by their colour in day light, their fluorescence in UV light 366 nm and by spraying with 5% KOH (aq.) (see Table 5, p. 70).

2.2.2.4 Isolation and purification of the anthraquinones.

Anthraquinones crude extract was separated into three major bands by preparative TLC using system B (see Section 2.2.2.3.2, p. 44). Analytical TLC of the separated bands indicated the presence of compounds of a similar R_f value to the separated anthraquinones which gave rose pink colourations on spraying with 60% sulphuric acid. At this stage a bright blue fluorescent compound was located when viewed under ultraviolet light. Each band was further purified using acid-base partition. The crude extract contained minor anthraquinones, but because of their low amounts, no further work was undertaken to separate them.

2.2.2.5 Acid-base partition.

The anthraquinone-rich bands were dissolved in 0.9 M NaOH giving red colouration, partitioned with chloroform (x4), followed by acidifying the basic fraction with

2% sulphuric acid until the colour changed to yellow-orange. The aqueous acidic portion was partitioned with chloroform (x4), washed with distilled water to remove any salt present and dried over (5 gm) anhydrous sodium sulphate. The chloroform extracted was taken to dryness in vacuo for the analysis.

2.2.2.6 Column chromatography.

Analytical TLC of the pure anthraquinones, indicated that several bands were enriched in these particular compounds. In an attempt to isolate a number of closely related anthraquinones from each other, column chromatography was carried out. The initial separation was performed using the method described in Section 2.2.1.3, p. 42. The column was eluted with hexane, followed by 2.5% ethyl acetate in hexane and finally 5% ethyl acetate in hexane. 25 ml fractions were collected and monitored using (TLC). Fractions containing similar TLC profiles were pooled before subjected to preparative HPLC (see Figure 7,P.75). After the addition of 2.5% of the above mixture a yellow band immediately moved down the column leaving an orange band in the top, which eluted by using 5% ethyl acetate in hexane mixture. Ultimately, 10 fractions were obtained and further analysed by HPLC.

2.2.2.7 High performance liquid chromatography.

Before preparative HPLC was achieved, the 10 column fractions were analysed by analytical HPLC (see Section 2.2.1.4, p. 42) and the following conditions were used.

Analytical HPLC equipment and conditions.

Column: Spherisorb-S5W (25 cm x 4.9 mm I.D.,
particle size 5 um), analytical column

Loop volume: 20 μ L

Instrumental conditions

Flow rate: 2 ml/min, pressure 1000 p.s.i.,

Temperature: ambient, UV detector: 280 nm,

Chart: 2 mm/min.

Chromatographic conditions

The following mobile phases were used:

A n-hexane : ethyl acetate (80:20)

B n-hexane : ethyl acetate (98:20)

C n-hexane : ethyl acetate (95:5)

D n-hexane : ethyl acetate (93:7)

E Toluene : ethyl acetate : glacial acetic acid
(100:1:1)

F Toluene : ethyl acetate : glacial acetic acid
(100:5:1)

G n-hexane : ethyl acetate : glacial acetic
acid (98:2:0.05)

H n-hexane : ethyl acetate : glacial acetic
acid (80:20:0.5)

I n-hexane : ethyl acetate : glacial acetic
acid (95:5:0.2)

Mobile phase (I) was used for the separation
of the anthraquinones (see Figure 7, p. 75).

Retention times.

1-Hydroxy-2-methyl-3,4,6-(or -3,4,7)-trimethoxy
anthraquinone (1) R_t 9 mins.; 1-hydroxy-

2,3,5-(or 2,3,8)-trimethoxy anthraquinone (2), R_t 10.6 mins.; anthragallol 1,2,3-trimethyl ether (3), R_t 12.8 mins.; 1,6-dihydroxy-methyl-7-methoxy anthraquinone or 1,7-dihydroxy-2-methyl-6-methoxy anthraquinone (4), R_t 20.6 mins.; 2-methyl-6-hydroxy-7-methoxy anthraquinone or 2-methyl-7-hydroxy-6-methoxy anthraquinone (5), R_t 25 mins.

Preparative HPLC equipment and conditions.

Column: Spherisorb-S5W (25 cm x 8 mm I.D., particle size 5 μ m), preparative column.

Loop volume: 200 μ L.

Instrumental conditions

Flow rate: 3 ml/ min., pressure: 2000 p.s.i., temperature: ambient, UV detector: 280 nm, chart: 2 mm/min.

Chromatographic conditions

Mobile phase: n-hexane : ethyl acetate : glacial acetic acid (95:5:0.2)

2.2.3 Extraction of *Artemisia annua* cell cultures.

2.2.3.1 Extraction of callus cultures.

The cells were air dried yielding 12.47 g and ground to a fine powder prior to extraction. They were then macerated and extracted with methanol (x3) and the total methanolic extracts concentrated to dryness, using a rotary evaporator (Rota Vapor, R. Buchi, Switzerland).

2.2.3.2 Extraction of suspension cultures.

The suspension cells were separated from the medium by filtration and were air dried yielding 4.6 gm and ground to fine powder prior to extraction. They were then macerated and extracted with methanol (x3), this extract was then taken to dryness, using a rotary evaporator.

2.2.3.3 Extraction of medium.

The filtered medium of cell suspensions were collected and freeze dried, yielding 9.6 g. The dried medium were extracted with methanol (x3), and the total methanolic extract concentrated to dryness, using a rotary evaporator, yielding 5.86 g.

2.2.3.4 Solvent fractionation.

The dried methanolic extracts of callus, suspension cells and medium were subjected further to solvent fractionation as outlined in Figure 5, p. 51. Methanol extract (Fraction 1) was partitioned between n-hexane and water yielding hexane extract (Fraction 2) and water. The total hexane extracts from callus, suspension cells and medium were weighed, yielding (182.31, 90.87 and 2.12 mg) respectively. The aqueous fraction was further partitioned with chloroform to give a chloroform extract (Fraction 3) and water extract (Fraction 4). The total chloroform extracts for callus, suspension cells and medium were weighed yielding (69.78, 14.45 and 3.75 mg) respectively. The remaining aqueous extract (Fraction 4) was freeze dried using an Edwards Modulyo freeze dryer. The obtained fractions were subjected to antimalarial testing in vitro.



2.2.3.5 Thin-layer chromatography.

Analytical and preparative procedures utilising adsorption TLC were carried out on silica gel G (see Section 2.2.1.2, p. 41).

2.2.3.5.1 Authentication.

Stigmasterol was obtained from Sigma and artemisinin (QHS) was provided kindly by Prof. W. Peters.

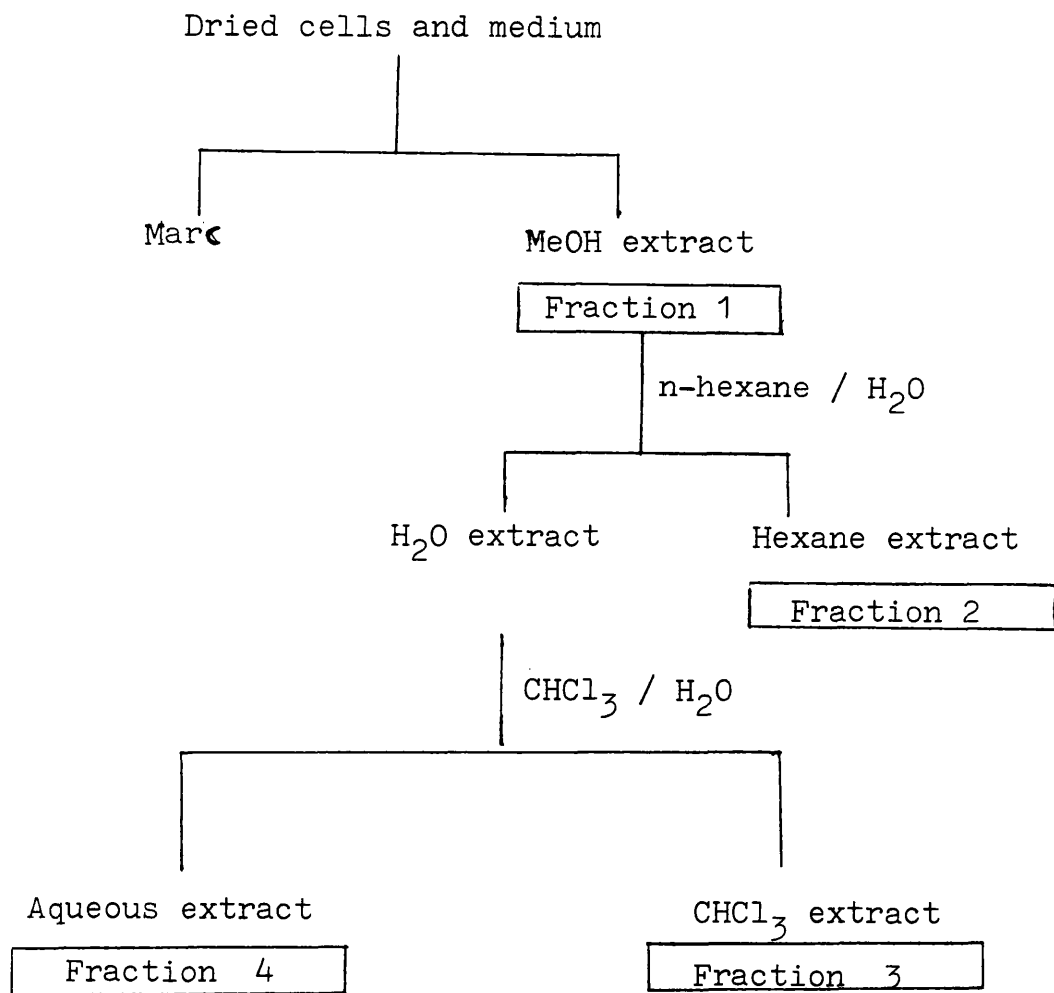
2.2.3.5.2 Solvent systems used in TLC.

The fractions were chromatographed using the following solvent systems:

- A Chloroform : methanol (99:1)
- B Benzene : ethyl acetate (85:15)
- C Chloroform : ethyl acetate (92.5:7.5)
- D Chloroform : benzene (75:25)
- E Chloroform : ethyl acetate (80:20)
- F n-hexane : diethyl ether (1:1)
- G n-hexane : diethyl ether (1:4)
- H n-hexane : diethyl ether (2:3)
- I 7.5% ethyl acetate in chloroform
- J Cyclohexane : diethyl ether (9:1)
- K Cyclohexane : diethyl ether (4:1)
- L Toluene : diether (1:1) saturated with 10% acetic acid

All the fractions were co-chromatographed with authentic sample of artemisinin. System E was used for the separation of stigmasterol and the systems E and L were used for the separation of scopoletin. The R_f value for reference artemisinin was 0.51, using solvent system E.

Figure 5. Fractionation of cell and medium extracts for in vitro antimalarial testing.



2.2.3.5.3 Visualisation.

Several different reagents were used to detect authentic artemisinin. Anisaldehyde and vanillin-sulphuric acid was used for the detection of artemisinin and its metabolites (146). However, visualisation being achieved by means of p-dimethylaminobenzaldehyde (147) and I₂ vapour (109). In this study 60% sulphuric acid was used for the detection of artemisinin.

2.2.3.6 Isolation and purification of stigmasterol and scopoletin

The chloroform extract contained compounds which gave pink and purple colours on spraying with sulphuric acid in particular sterols. Stigmasterol was found as a major compound in this extract when co-chromatographed with authentic stigmasterol. This compound was separated by preparative TLC system E (see Section 2.2.3.5.2, p. 50) and analysed (TLC, MS). The chloroform extract contained the UV fluorescent scopoletin as detected in the callus, suspension cells and medium when co-chromatographed with authentic scopoletin. This compound was separated by preparative TLC systems E and L (TLC, MS).

2.2.3.7 Isolation and purification of the active anti-malarial compound.

Column chromatography was carried out using the method described in Section 2.2.1.3. 45 mg of the active hexane crude extract of the medium (fraction 2) was dissolved in a minimum volume of chloroform and was eluted with n-hexane followed by ethyl acetate in chloroform gradient, ethyl acetate-chloroform and methanol gradient,

ethyl acetate-methanol gradient and finally methanol. 25 ml fractions were collected and examined by TLC using the solvent system I (see Section 2.2.3.5.2, p. 50). The first two fractions showed the presence of several pink-grey spots with 60% sulphuric acid. Fractions containing similar TLC profiles were pooled before being subjected to antimalarial testing in vitro.

Section 2.3 Characterisation and identification of anthraquinones and scopoletin isolated from C. ledgeriana root organ.

2.3.1 Anthraquinones.

2.3.1.1 1-hydroxy-2-methyl-3,4,6-(or 3,4,7)-tri-methoxy anthraquinone (1).

TLC: R_f value 0.48, System B (Section 2.2.2.3.2, p. 44) appearance in day light, dark yellow, in UV light 366 nm, red-orange absorbance and with 5% KOH gives yellow colour.

HPLC: R_t value 9 mins., (Section 2.2.2.7, p. 46).

UV spectrum: $\lambda_{\text{max}}^{\text{MeOH}}$ 250, 260-290 nm.

IR spectrum: CHCl_3 max 1665 cm^{-1} .

EIMS: m/z (%) 328.0939 (M^+ ; 100) (Calc. for $\text{C}_{18}\text{H}_{16}\text{O}_6$: 328.0947), 313 (47), 298 (52), 285 (23), 267 (22), 255 (21), 227 (30).

PMR: CDCl_3 , 250 MHz. TMS as internal standard.
 \int 2.37 (3H, s, CH_3), 4.00 (6H, s, 2 OCH_3), 4.05 (3H, s, OCH_3), 7.47 (H, d, $J = 7.8$ Hz, H-7 or H-6), 7.67 (H, d, $J = 7.8$ Hz, H-5 or H-8),

7.69 (H, s, J = 2.8 Hz, H-5 or H-8), 13.3
(H, s, OH).

Nuclear Overhauser effects (NOE)

On irradiation of the methyl signal at δ 2.37 no effects were observed on either the signal which appeared at δ 13.3 or δ 4.00 which was attributed to the C-1 OH and C-3 OCH₃ groups respectively. Also irradiation of the two methoxyl signals at δ 4.00 gave no effect on the signal at δ 2.37, which was attributed to the C-2 CH₃. However, on irradiation of the methoxyl signal at δ 4.05 the signal at δ 7.69 which was attributed to either C-5 or C-8 H increased by 18%, while at the same time no effects were observed on the signal which appeared at δ 7.47, which attributed to either C-6 or C-7 H.

2.3.1.2 1-hydroxy-2,3,5-(or 2,3,8)-tri-methoxy anthraquinone (2)

TLC: R_f value 0.46, System B (Section 2.2.2.3.2, p.44)
appearance in day light, dark yellow, in UV
light 366 nm, red-orange absorbance and with
5% KOH gives orange colour.

HPLC: R_t value 10.6 mins. (Section 2.2.2.7, p. 46).

UV spectrum: $\lambda_{\text{max}}^{\text{MeOH}}$ 250, 260-290 nm.

IR spectrum: CHCl₃ max 1665 cm⁻¹

EIMS: m/z (%) 314.0799 (M⁺, 100)
(Calc. for C₁₇H₁₄O₆: 314.0790), 299 (67),
296 (26), 281 (9), 271 (13), 268 (13), 253
(12), 241 (19), 213 (26).

PMR CDCl₃, 250 MHz. TMS as internal standard.
 δ 4.00 (6H, s, 2 OCH₃), 4.06 (3H, s, OCH₃),

7.39 (H, d, J = 7.9 Hz, H-6 or H-7), 7.61
(H, t, J = 7.9 Hz, H-6 or H-7), 7.70 (H, s,
H-4), 7.77 (H, d, J = 1.0 Hz and J = 6.5 Hz,
H-5 or H-8), 13.00 (H, s, OH).

Nuclear Overhauser effects (NOE)

On irradiation of the hydroxyl signal at δ 13.00
no effects were observed on the signal which appeared at
 δ 4.00 which was attributed to the C-2 OCH₃. However,
irradiation of the two methoxyl signals at δ 4.06 the signal
at δ 7.70 which was attributed to C-4 H increased by 23%.

2.3.1.3 Anthragallol 1,2,3-trimethyl ether (3)

TLC: R_f value 0.4, System B (Section 2.2.2.3.2, p.44)
appearance in day light faint yellow, in UV
light 366 nm, orange absorbance and with 5%
KOH gives faint yellow colour.

HPLC: R_t value 12.8 mins., (Section 2.2.2.7, p.46)

UV spectrum: $\lambda_{\text{max}}^{\text{MeOH}}$ 250 260-290 nm.

IR spectrum: CHCl₃ max 1665 cm⁻¹

EIMS: m/z (%) 298.0835 (M⁺, 100)
(Calc. for C₁₇H₁₄O₅: 298.0841), 283 (70),
269 (17), 255 (14), 240 (12), 225 (13),
212 (8), 197 (14).

PMR CDCl₃, 250 MHz. TMS as internal standard.
 δ 4.05 (3H, s, OCH₃), 4.01 (3H, s, OCH₃),
3.97 (3H, s, OCH₃), 7.70 (H, s, H-4), 7.75
2H, m, H-6, H-7), 8.28 (2H, m, H-5, H-8).

2.3.1.4 1,6-dihydroxy-2-methyl-7-methoxy anthraquinone or
1,7-dihydroxy-2-methyl-6-methoxy anthraquinone (4)

TLC: R_f value 0.3, System B (Section 2.2.2.3.2, p.44),
appearance in day light, yellowish-orange,
in UV light 366 nm, orange absorbance and
with 5% KOH gives orange colour.

HPLC: R_t 20.6 mins., (Section 2.2.2.7, p.46)

UV spectrum: $\lambda_{\max}^{\text{MeOH}}$ 250, 260-290 nm.

IR spectrum: CHCl_3 \max 1665 cm^{-1}

EIMS: m/z (%) 284.0674 (M^+ , 100)
(Calc. for $C_{16}H_{12}O_5$: 284.0685), 269 (14),
255 (13.5), 241 (25), 213 (16).

PMR CDCl_3 , 250 MHz. TMS as internal standard.
 \int 2.37 (H, s, CH_3), 4.09 (H, s, OCH_3),
7.50 (H, d, $J = 7.9$ Hz, H-3), 7.72 (H, d,
 $J = 6.4$ Hz, H-4), 7.74 (H, s, H-5 or H-8),
7.77 (H, s, H-5 or H-8), 12.99 (H, s, OH).

Nuclear Overhauser effect (NOE)

On irradiation of the hydroxyl signal at \int 12.99
no effects were observed on the signal which appeared at
 \int 2.37 which was attributed to the C-2 CH_3 . However,
irradiation of the methyl signal at \int 2.37 the signal at
 \int 7.50 which was attributed to C-3 H increased by 25%,
while at the same time no effects were observed on the
signal which appeared at \int 12.99, which was attributed to
the C-1 OH. In addition to, irradiation of the methoxyl
signal at \int 4.09 the signal at \int 7.74 which was attributed

to either C-5, or C-8 H increased by 42%, while at the same time no effects were observed on the signal which was attributed to either C-6 or C-7 OH.

2.3.1.5 2-methyl-6-hydroxy-7-methoxy anthraquinone or
2-methyl-7-hydroxy-6-methoxy anthraquinone (5)

TLC: R_f value 0.28, System B (Section 2.2.2.3.2, p.44), appearance in day light, yellowish-orange, in UV light 366 nm, orange absorbance and with 5% KOH gives pink colour.

HPLC: R_t 25 mins., (Section 2.2.2.7, p.46)

UV spectrum: $\lambda_{\max}^{\text{MeOH}}$ 250, 260-290 nm.

IR spectrum: CHCl_3 max 1665 cm^{-1}

EIMS: m/z (%) 268.0735 (M^+ , 100)
(Calc. for $\text{C}_{16}\text{H}_{12}\text{O}_4$: 268.0736), 253 (10),
239 (26), 225 (41), 197 (24).

PMR: CDCl_3 . 250 MHz. TMS as internal standard.
 δ 2.52 (H, s, CH_3), 4.09 (H, s, OCH_3),
7.57 (H, d, $J = 5.6$ Hz, H-3), 7.74 (H, s,
H-5 or H-8), 7.76 (H, s, H-5 or H-8), 8.07
(H, s, H-1), 8.17 (H, d, $J = 7.9$ Hz, H-4).

Nuclear Overhauser effects (NOE)

On irradiation of the methyl signal at δ 2.52 the signal at δ 8.07 which was attributed to C-1 H increased by 30%, while at the same time no effects were observed on the signal which appeared at δ 7.57, which was attributed to the C-3 H. In addition, irradiation of the methoxyl signal at δ 4.09, the signal at δ 7.74 which was attributed

to either C-5 or C-8 H, increased by 20%, while at the same time no effects were observed on the signal which was attributed to either C-6 or C-7 OH.

2.3.2 Scopoletin.

TLC: R_f value 0.18, System B (Section 2.2.2.3.2), appearance in day light, faint yellow and in UV light 366 nm, blue fluorescence.

EIMS: m/z (%) 192 (M^+ , 100), 177 (60), 164 (30, 149 (69), 137 (23), 121 (30).

This sample was identical with authentic scopoletin (TLC, MS).

Section 2.4 Investigation into the growth cycle of C. ledgeriana root organ and anthraquinone production during the growth cycle.

Root organ cultures were filtered aseptically and distributed into 250 ml conical flasks containing 40 ml fresh medium (Section 2.1.3, p. 40) to give an initial inoculum of 5 g fresh weight per flask. The flasks were maintained at 25°C under continuous illumination. Flasks were harvested on alternate days over a 32 day period. On harvesting, the root organs were filtered from the medium and the fresh weight determined, air dried and the total dry weight calculated. All the measurements were carried out in duplicate. The anthraquinones were extracted from the roots (Section 2.2.2.2) on alternate days over the growth period and the amount of the crude anthraquinone extracts were determined.

Section 2.5 Testing plant extracts and pure compounds
for antimalarial activity *in vitro*.

2.5.1 Antimalarial testing of crude extracts and pure
compounds, using [$G-^3H$] hypoxanthine incorporation
by *P. falciparum*.

The *in vitro* activity of crude extracts of *Artemisia annua* cultures were tested against chloroquine-resistant strains of *Plasmodium falciparum* by Dr. D.H. Bray in collaboration with Dr. D. Warhurst at the School of Hygiene and Tropical Medicine.

The cultures of *P. falciparum* (K-1, chloroquine-resistant strain) (148) were maintained *in vitro* in human blood utilising published methodology (149-151). Dried extracts of plant material were dissolved or micronised in ethanol and a series of 6 concentrations prepared by 10-fold dilutions in RPMI 1640 medium (151). The ethanol concentration for tested dilutions was not more than 0.1%. 50 μ L Aliquots of diluted crude extracts were dispensed into 96-well-microtiter plates so as to yield final concentrations at test of 500 μ g ml⁻¹, 50, 5, 0.5, 0.05 and 0.005 μ g ml⁻¹. For the more accurate determination of IC₅₀ values of extracts and of pure compounds two-fold dilutions were prepared to give concentrations around the range of the IC₅₀ on the basis of 10-fold dilution results. All tests were performed in duplicate. To each well was added 50 μ L of human red blood cells (0⁺ ve, 5% haematocrit) containing 1% parasitaemia. Dilutions to produce 1% parasitaemia were made with uninfected washed red blood cells. Two series of controls were performed, one with parasitised blood without addition of plant extract and a second with

uninfected red blood cells. After incubation in a 3% O₂, 4% CO₂ and 93% N₂ gas phase for 18 hours at 37°C, 5 μL (G-³H)-hypoxanthine (40 μ Ci ml⁻¹, Amersham) was added to each well and incubation continued at 37°C for a further 18-24 hours.

2.5.2 Harvesting of parasites and scintillation counting.

Red blood cells were washed out from the wells with normal saline using a Titertek cell harvester (Flow Laboratories) through a glass fibre membrane pre-damped with saline. The glass fibre membrane was then flushed with distilled water for 20 seconds to remove remaining traces of haemoglobin. After further washing with distilled water and saline (20 secs each) the membrane was dried and the glass fibre disc for each well was pushed out into polyethylene scintillation vials (Packard). 4 ml Betafluor scintillation liquid (National Diagnostics) was added and the vials were counted for 10 minutes in liquid scintillation spectrometer (Packard TRI-CARB) after cooling to 2°C to 4°C. The counting efficiency was around 30%.

2.5.3 Analysis of the results.

Cpm were converted to dpm using an external standard and the percentage inhibition was calculated from the equation:

$$\% \text{ inhibition} = 100 - \frac{\text{dpm infected blood and plant extract} - \text{dpm uninfected blood}}{\text{dpm infected blood} - \text{dpm uninfected blood}} \times 100$$

Section 2.6 Investigation into the growth cycle of
A. annua cell suspension cultures.

Cell suspension cultures are filtered aseptically and distributed into flasks, containing 40 ml fresh medium (Section 2.1.3, p. 40) to give an initial inoculum of 3 g fresh weight per flask (0.4 gm total dried weight per flask) and were harvested on alternative days over a 32 day period. The cells were filtered from the medium, air dried and weighed. All the measurements were carried out in duplicate.

Section 2.7 Investigation into the effect of the medium
composition on A. annua callus cultures.

2.7.1 Control medium.

The medium that was used as a control in all the investigations was composed of 1.5% Lab.M. agar (London Analytical and Biological Media Inc.), Murashige and Skoog based salts (111), 1 mg L^{-1} 2,4-D, 0.1 mg L^{-1} Kin. and 5% sucrose (BDH).

2.7.2 Analysis of samples.

On harvesting, the callus cultures were collected and the mean fresh weight determined, air dried and the 95% confidence limits of the weights were calculated. The cells were extracted and subjected further to solvent fractionation (Section 2.2.3.4, analysed by TLC (see Section 2.2.3.5 and tested in vitro for antimalarial activity (Section 2.5, p. 59).

2.7.3 Nature of basal salts.

In these investigations, three basal salts were studied viz. Linsmaier and Skoog (LS) (152), Shenk and Hildbrandt (SH) (153) and Gamborg's B₅ (B₅) (112), 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4 D, Flow Laboratories), 0.1 mg L⁻¹ kinetin (Flow Lab.) and 5% sucrose (BDH) with 1.5% Lab.M. agar (London Analytical and Biological Media Inc.).

Little pieces of callus (15 ± 5 mg fresh weight) were transferred aseptically to one of the three media and all the petri-dishes were harvested at the end of two months (see Section 2.7.2).

2.7.4 Nature and concentration of plant growth hormones (auxin).

In these investigations, two auxins were studied viz. indole acetic acid (IAA) and naphthalene acetic acid (NAA), at concentrations of 0.1, 1.0, 3.5, 5.0 mg L⁻¹ and 1.0, 5.0 mg L⁻¹ respectively, keeping kinetin concentration constant as in the control medium (Section 2.7.1). All the auxins were obtained from Flow Laboratories. Little pieces of callus (15 ± 5 mg fresh weight) were transferred aseptically to one of the six media combinations and harvested after two months (see Section 2.7.2).

2.7.5 Effect of light.

For this experiment, a number of petri-dishes containing the same composition of the control medium (Section 2.7.1), were kept in the dark, whereas the control medium kept under continuous illumination and harvested after two months (see Section 2.7.2, p. 61).

Section 3.1 Plant tissue cultures.3.1.1 Cinchona ledgeriana

Root organ cultures of C. ledgeriana Moens (Rubiaceae) were developed in our laboratory, from sterilised seeds and once germinated, portions of the hypocotyl were aseptically transferred to medium composed of Murashige and Skoog salts (111), 1 mg L^{-1} 2,4-D, 0.1 mg L^{-1} kinetin and 5% sucrose. The cultures are maintained at 25°C under constant illumination (144). The developed callus cultures were transferred into suspension culture (plate 2, p. 65) and maintained under the same conditions with continuous agitation (120 r.p.m.). The cells have been in cultures for many years and require subculturing every 28-30 days (144). The optimum inoculum density has been found to be 3-5 g fresh weight per 40 ml medium.

During the study of these cultures, a red pigmentation of the sterile seedlings, both the stem and leaf parts, was noticed after 7 days of transferring the seedlings on to the solid medium (plate 1, p. 64). In addition to the seedlings, orange colouration of the callus and suspension cells were often observed.

The tissues which developed were highly differentiated and were named root organs (86).

It can be seen that they are pigmented. The orange/brown appearance has been shown to be due to the presence of phenolic compounds, mainly anthraquinones, a class of compound not normally produced by whole plants

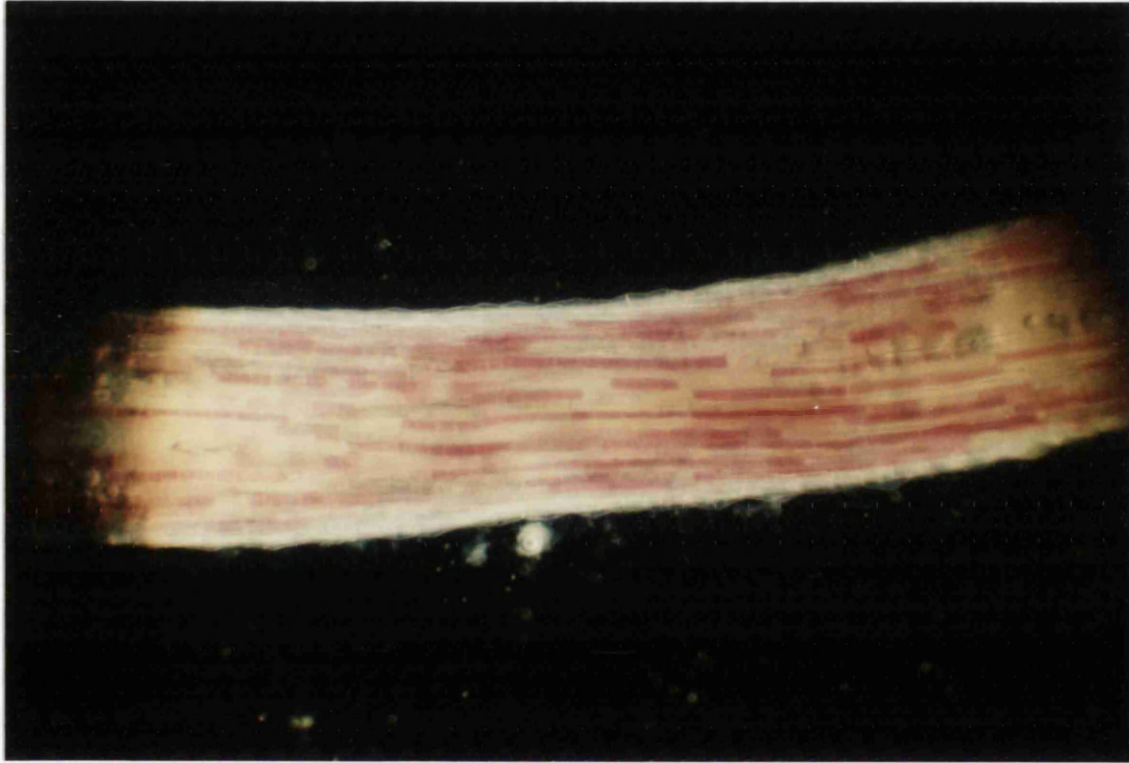
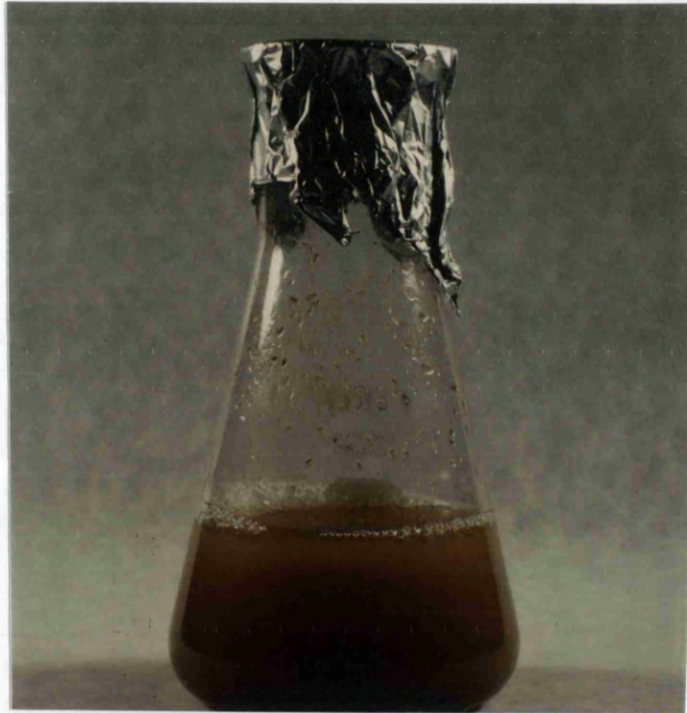


Plate 1:- Cinchona ledgeriana seedlings.

Plate 2:- Cinchona ledgeriana suspension culture.



of Cinchona. However, anthraquinones are typical of the Rubiaceae (Section 1.2.1, p. 4).

3.1.2 Artemisia annua

In vitro cultures of A. annua (Compositae, Asteraceae) were initiated by germinating sterilised seeds and aseptically transferring seedlings to medium composed of Murashige and Skoog basal salts (111), 5% sucrose, 1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin. The cultures were maintained at 25°C under constant illumination (145). Plate 3, p. 67 shows callus tissue typical of A. annua. Biomass was increased until sufficient had been obtained to enable suspension cultures to be produced by transferring calli to liquid medium. The suspension cultures (plate 4, p. 67) are maintained under the same conditions as described above along with continuous agitation (120 r.p.m.) (145). The suspensions are subcultured every 28-30 days and have been in cultures for 3 years. The optimum inoculum density range has been found to be 3 g fresh weight per 40 ml medium (145).

When the cultures are examined macroscopically, they were found to consist of spherical clusters and were distinctly different from the Cinchona root organs.

Plate3 :- Artemisia annua callus.



Plate4 :- Artemisia annua suspension culture.



Section 3.2 Separation and isolation of anthraquinones from *C. ledgeriana* root organ cultures.

Root organ cultures of *C. ledgeriana* were initiated by Dr L.A. Anderson and have been found to produce four quinoline alkaloids. Quinine and quinidine are the major alkaloids present along with cinchonine and cinchonidine as minor compounds (Figure 6) (144).

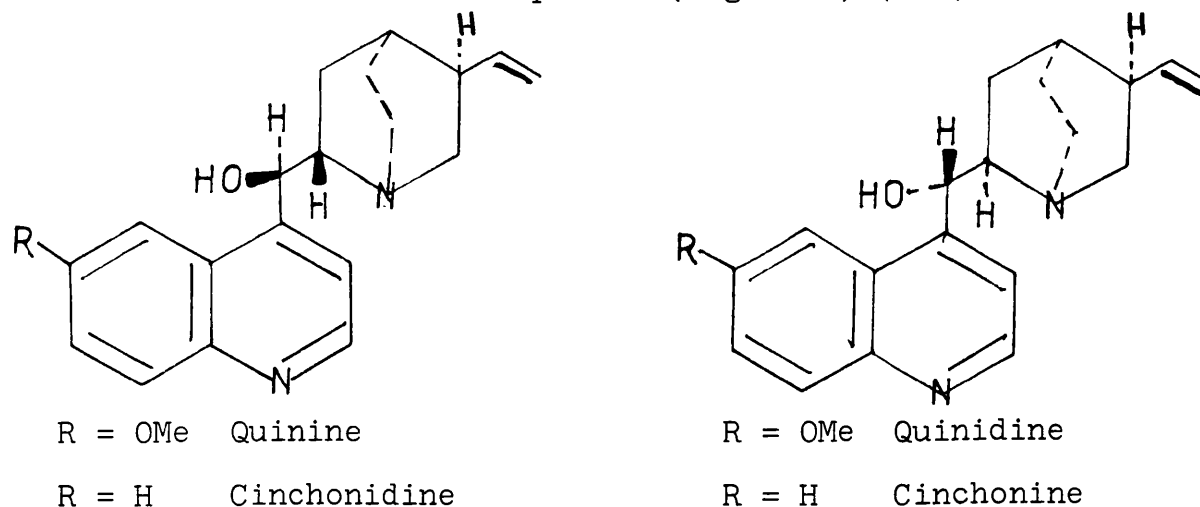


Figure 6 Quinine, quinidine, cinchonine and cinchonidine.

In addition to the quinoline alkaloids, the *C. ledgeriana* root organ cultures produce high levels of anthraquinones as seen by the orange/brown pigmentation. In this study, five new anthraquinones were isolated and identified as methoxylated aglycones (154).

To date, 25 anthraquinones have been isolated from cell cultures by other research groups, a number of them are new for *Cinchona* species but known from other species of Rubiaceae (see Section 1.4.1, p. 19). The anthraquinones were extracted from the root organs using the traditional acid/base method of extraction. Crude extract of the anthraquinones were analysed by TLC and a number of solvent systems were tried in order to obtain the best separation and this was achieved using toluene :

ethyl acetate : glacial acetic acid (74:25:1) as a solvent system. This system was used in all investigations, mainly for preparative TLC. Since anthraquinones are naturally coloured compounds, visualisation of the thin layer chromatograms was carried out in day light and UV light (366 nm). Phenolic anthraquinones were detected using 5% KOH (aq.) which gives orange or pink colours depending on the number of hydroxyl groups present in the molecules (see Table 5, p. 70).

The preliminary separation of the anthraquinones on a large scale was carried out using preparative TLC. This was essential for the removal of

major anthraquinones, since there are minor anthraquinones present in the crude extract. A band of crude anthraquinones extract was applied to the plates and run in the above solvent system. When the plates were visualised under UV light, there were three major bands present and it was decided to concentrate on these. In addition to the three bands, a number of minor bands were observed but their amounts were low and hence they were not further investigated. At this stage a blue fluorescent band was noticed and isolated by preparative TLC and identified as the coumarin, scopoletin. The isolated scopoletin was co-chromatographed on TLC with reference scopoletin and characterised by (MS,). Scopoletin has not previously been reported from plant cell cultures. However, the bulk of the anthraquinones crude extract are extremely polar and stay on the base-line when the above solvent system was used for the isolation. Hence a more polar solvent system was needed for their separation.

Table 5 Visualisation of the isolated anthraquinones and scopoletin.

Anthraquinones	Colour		
	day light	UV 366 nm	5% KOH
1-hydroxy-2-methyl-3,4,6 (or 3,4,7)-trimethoxy	dark- yellow	red-orange	yellow
1-hydroxy-2,3,5-(or 2,3,8)-trimethoxy	dark- yellow	red-orange	orange
Anthragallol 1,2,3- trimethyl ether	faint- yellow	orange	faint- yellow
1,6-dihydroxy-2-methyl- 6-methoxy or 1,7- dihydroxy-2-methyl-7- methoxy	yellowish -orange	orange	orange
2-methyl-6-hydroxy-7- methoxy or 2-methyl-7- hydroxy-6-methoxy	yellowish -orange	orange	pink
Scopoletin	faint- yellow	blue- fluorescent	-

Therefore, the base-line from the above solvent system was extracted and further separated by TLC using chloroform : methanol : water (65:35:10) as solvent system. It was found that 4 to 5 major spots were separated and a number of minor spots were detected. But unfortunately there was not enough time to isolate and identify these more polar anthraquinone aglycones. When the isolated anthraquinones was subjected to spectroscopic identifications, it was found that they were mixtures as indicated by their EIMS spectra. In an attempt to obtain the isolated anthraquinones as pure compounds, acid-base partition was carried out as a general method to remove any lipophilic materials, particularly sterols.

Despite the different purification steps the isolated compounds still showed the presence of impurities in the 250 MHz ^1H -NMR spectra and each isolated band was seen to be a mixture of two very closely related anthraquinones. Therefore, in order to isolate these anthraquinones from the mixture, column chromatography was utilised and the initial separation was performed using a silica gel column. Each isolated band by preparative TLC was added separately to a chromatographic column. The column was eluted using 100% n-heptane followed by ethyl acetate in a hexane gradient and resulted in the separation of two (yellow and orange) bands. Ten fractions were collected and checked by TLC. Again it was found that some of these fractions were not totally pure and comprised a mixture of two anthraquinones. Even so this method of isolation is preferable to preparative TLC as less impurities are introduced.

An HPLC system has been reported for the separation of anthraquinones using normal phase (88) and column partisil-5 (4.6 x 25 mm) eluted at 25°C with toluene-EtOAc-HOAc (100:5:1), flowing at 1.5 ml/min with a detector at 280 or 410 nm.

The above solvent system proved to be inadequate and separation of the anthraquinones was not achieved. Various HPLC mobile phases were tried in order to separate the very closely related anthraquinones and it was found that hexane : ethyl acetate : glacial acetic acid (95:5:0.2) mobile phase proved to be ideal for the selective retention of the various anthraquinones and produced sharp discrete peaks (see figure 7, p. 75) (154). Other mobile phases were tried and found to give broad unresolved peaks. The incorporation of a small percentage of acid in the system used to separate the anthraquinones was needed in order to suppress the ionization of the phenolic groups. Finding a suitable mobile phase was a necessary requirement for the analysis of the compounds obtained from the column chromatography. Anthraquinones were readily separated by HPLC and this proved to be a sensitive technique for analytical purposes and was also used for preparative work. As a result, column fractions were monitored using HPLC and fractions containing similar HPLC profiles were combined before being subjected to spectroscopic analysis. Fractions with different HPLC profiles were further purified using preparative HPLC and checked further using analytical HPLC. The five isolated anthraquinones (Figure 7, p. 75) were fully characterised by UV, IR, MS and ¹H-NMR and the substitution pattern of the A and C rings was established by nuclear Overhauser

effects (NOE) and found to be new anthraquinones from C. ledgeriana cultures (Section 3.3, p. 77) (154).

In addition to the anthraquinone aglycones, some anthraquinones occur as glycosides but to date no anthraquinone glycoside has been reported from C. ledgeriana. These are currently investigated in our laboratory and analysed by TLC using (chloroform : methanol : water) (65:35:10) as a solvent system and 4-5 glycosidic anthraquinones was detected, but unfortunately there was not enough time to continue the work.

Having isolated and characterised the anthraquinones, it was necessary to obtain data with regard to the growth cycle of the root organs and anthraquinone production during the growth period. In order to do this, detailed time course studies were carried out (154). C. ledgeriana root organs were transferred to fresh medium to give an initial inoculum of 5 g fresh weight per 40 ml medium and flasks were harvested on alternate days over a 32 day period. On harvesting, these roots were filtered from the medium and the fresh weight determined, air dried and weighed.

Figure 8, p. 76 shows that the typical growth cycle consists of a long lag phase of up to 15 days, followed by a period of growth until day 30 when the root organs enter the stationary phase. By the end of the growth cycle, the root organs have undergone a 3-fold increase in the total dry weight over the initial inoculum. In addition, the anthraquinone content was studied during the growth cycle and the same figure shows that, there was a decrease in the dry weight of the crude anthraquinone

extract during the lag phase up to 15 days, followed by an increase in the anthraquinone production with a 3-fold increase by the end of the growth period until day 28, when the root organs enter the stationary phase.

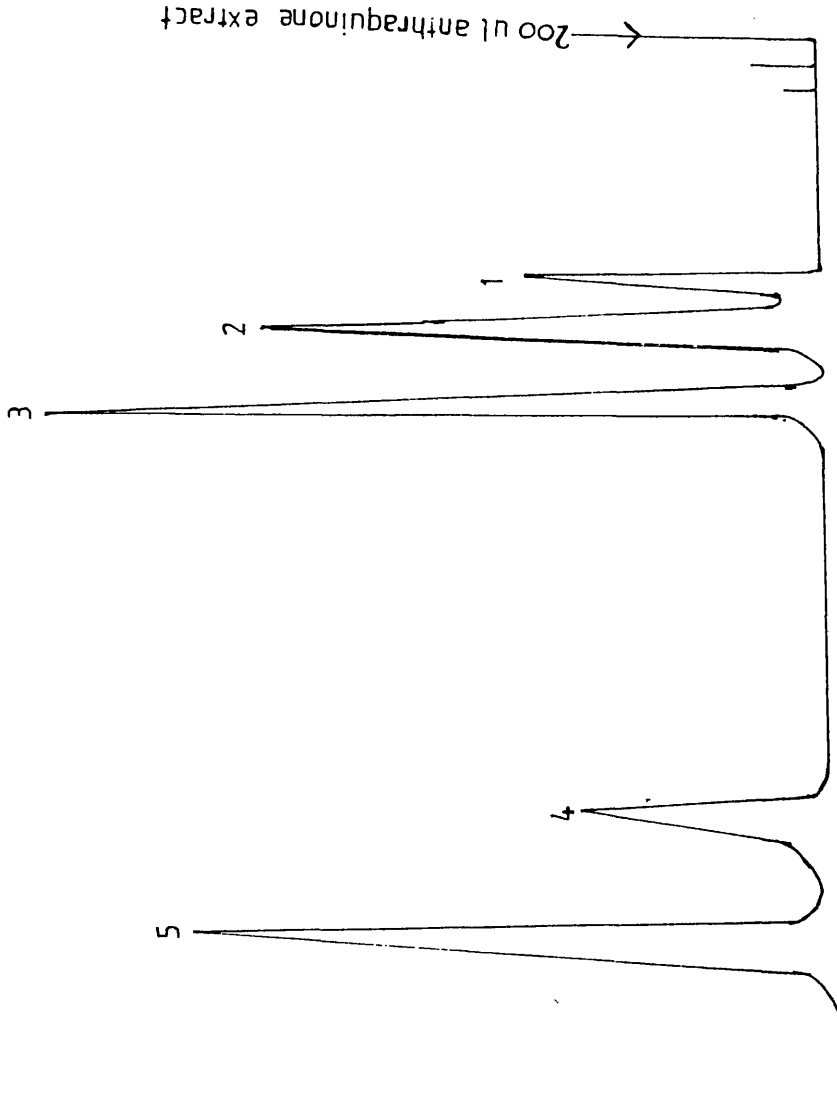


Figure 7 Preparative HPLC profile for the separated anthraquinones,
(See Section 2.5, p. 55)

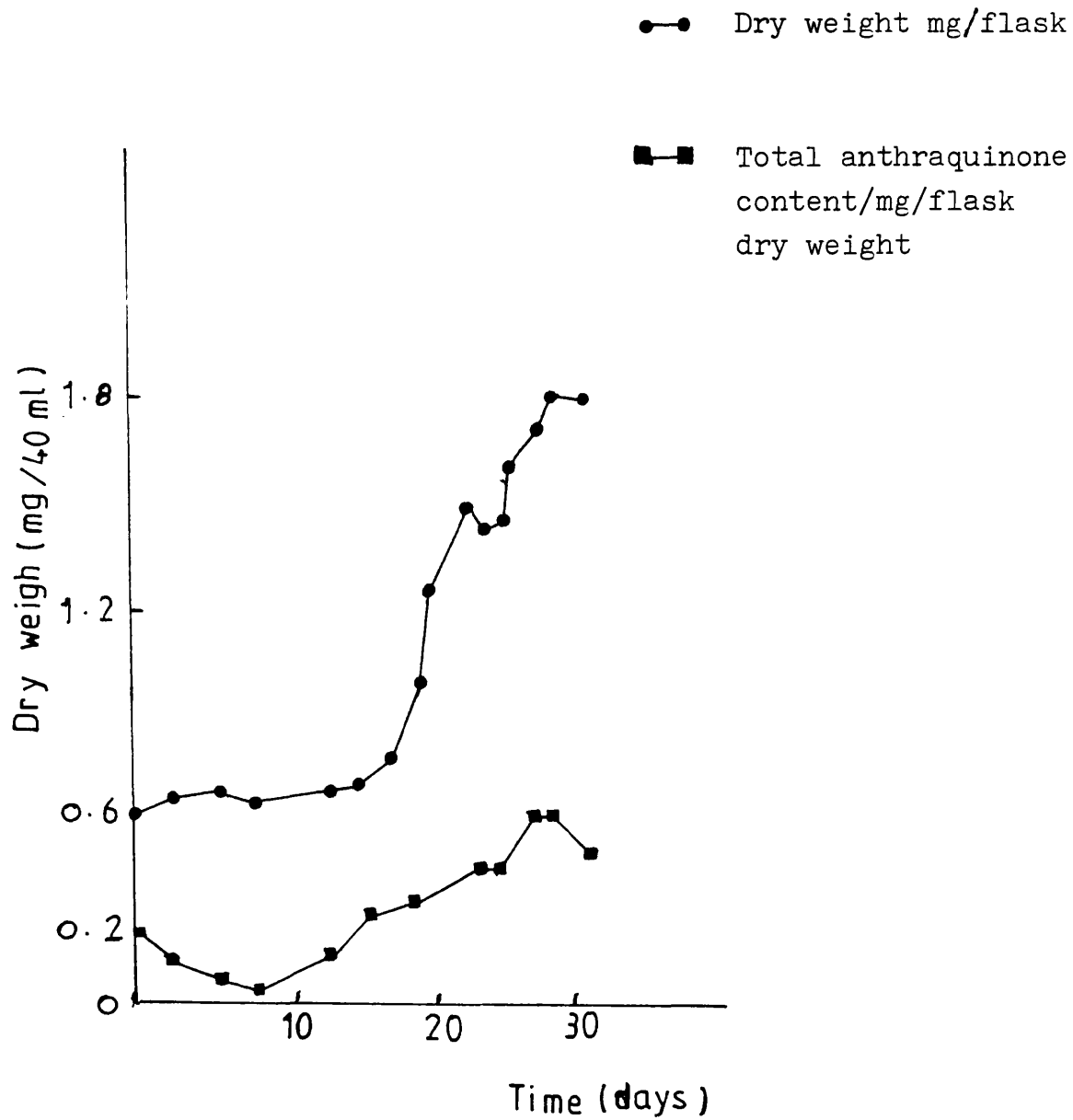
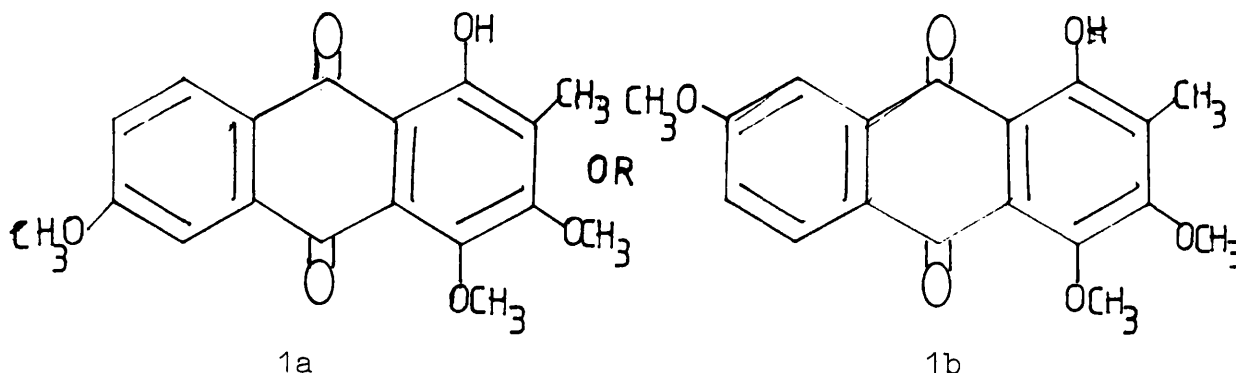


Figure 8 Growth cycle and anthraquinone production in C. ledgeriana root suspension culture.

Section 3.3 Identification of the isolated anthraquinones and coumarin.

3.3.1 Anthraquinones.

3.3.1.1 1-hydroxy-2-methyl-3,4,6 (or 3,4,7)-trimethoxy anthraquinone. $C_{18}H_{16}O_6$



This dark yellow anthraquinone has a molecular ion base peak at m/z measured 328.0939 and calculated for $C_{18}H_{16}O_6$; 328.0947. The high intensity of this ion being due to the molecule's highly aromatic and therefore fairly stable structure. Other significant ions were found at m/z 313 corresponding to the loss of a methyl group from the parent ion. Loss of a water moiety from the molecular ion leads to the peak at m/z 310 and loss of two methyl groups from the parent ion leads to the peak at m/z 298. The m/z 313 and 298 are the major fragment of this anthraquinone. However, anthraquinone itself undergoes successive elimination of two molecules of carbon monoxide to give strong peaks at m/z 180 (M-CO) and 152 (M-2CO) (and strong doubly charged ions at m/z 90 and 76) which correspond to the molecular ions of fluorenone and biphenylene, respectively (56). This pattern of fragmentation indicates that the molecule is heavily substituted

with either a methyl or methoxyl group. Its UV spectrum shows intensive benzenoid absorption at 250 nm and quinonoid band(s) at 260-290 nm, indicated a compound with a quinone chromophore. Moreover, addition of one drop of methanolic solution of 1 N sodium hydroxide resulted in a colour change of the solution and a bathochromic shift in the UV spectrum, indicative of the presence of a phenolic hydroxyl group in the molecule. The IR absorption in CHCl_3 at 1665 cm^{-1} was consistent with the presence of carbonyl moieties. The $^1\text{H-NMR}$ spectrum obtained at 250 MHz revealed the presence of five substituents, a hydroxy (δ 13.3), one methyl (δ 2.37) and three methoxyl (δ 4.00, 6H; 4.05, 3H) groups. Signals for three aromatic protons indicated that two were ortho coupled (δ 7.47, 7.67) and one was meta coupled (7.69). Hence, one aromatic ring contains a single substituent. The chemical shift of the hydroxyl substituent was indicative of peri substitution and in all of the known anthraquinones a methyl substituent is only found at C-2 (Figure 9, p. 79) on the basis of the biosynthetic pathway leading to the anthraquinone in the Rubiaceae (Section 1.2.3, p. 6) (46,47,155).

In a NOE experiment, irradiation of the methoxyl signal which occurred at δ 4.05 gave an 18% increase of the aromatic proton signal which occurred at δ 7.69. Hence, one of the methoxyl groups must be present as the single substituent in one aromatic ring. If C-2 carries a methyl substituent, it follows that one methoxyl group is located at either C-6 or C-7 and that ring C if fully substituted with one hydroxyl (either at C-1 or C-4)

one methyl and two methoxyl groups and hence the anthraquinone was 1-hydroxy-2-methyl-3,4,6(or 3,4,7)-trimethoxy. It has not proved possible to distinguish between these two alternative structures (83).

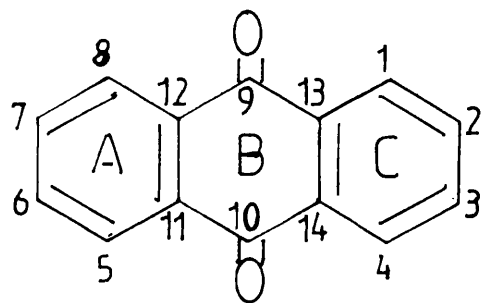
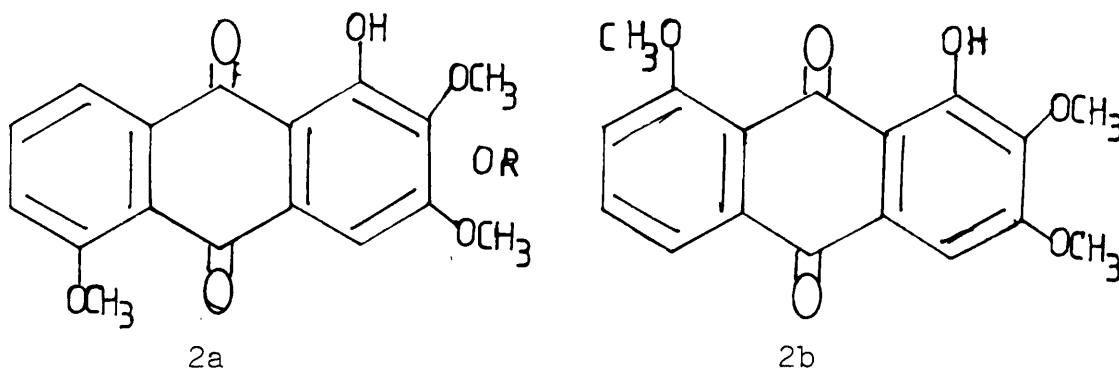


Figure 9 Numbering of the anthraquinone skeleton.

3.3.1.2 1-hydroxy-2,3,5-(or 2,3,8)-trimethoxy
anthraquinone. C₁₇H₁₄O₆

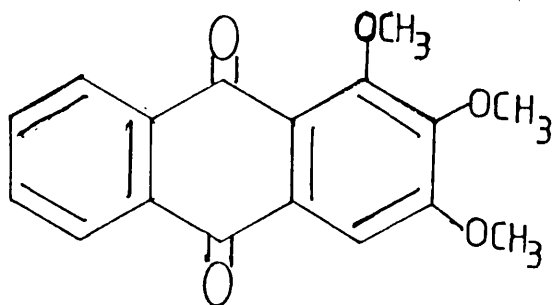


This yellow anthraquinone has a molecular ion base peak at m/z measured 314.0799 and calculated for C₁₇H₁₄O₆; 314.0790. The high intensity of this ion being due to the molecule's highly aromatic and therefore fairly stable structure. The fragment at m/z 299 would correspond to the loss of the methyl group from the parent ion. The loss of a water molecule from the parent ion would give the fragment m/z 296. The peak at m/z 281 could correspond to the loss of one molecule of carbon monoxide and a methyl group from the parent ion. Other significant ions can be seen and it can be noticed that we have loss of methyl groups rather than carbon monoxide loss (Section 1.3.3, p. 13). The number of the substituents fits with the calculated molecular formula that it composed from three methoxy and one hydroxyl groups. Its UV spectrum shows intensive benzenoid absorption at 250 nm and quinonoid band(s) at 260-290 nm, indicated a compound with methanolic solution of 1N sodium hydroxide resulted in a colour change of the solution and a bathochromic shift in the UV spectrum, indicative of the presence of a phenolic hydroxyl group in the molecule. However, its IR

absorption in CHCl_3 at 1665 cm^{-1} is consistent with the presence of carbonyl moieties. The $^1\text{H-NMR}$ spectrum obtained at 250 MHz revealed the presence of four aromatic substituents, one hydroxyl ($\delta 13.00$, peri), and three methoxyl substituents ($\delta 4.00$, 6H; 4.00 , 3H). An aromatic proton singlet at ($\delta 7.70$) is indicative of a trisubstituted aromatic ring with a free peri-position. The remaining three aromatic proton signals ($\delta 7.61$, t; 7.77 , d; 7.39 , d) indicated that the other aromatic ring contained a single substituent and that there were three adjacent aromatic protons. Irradiation of the methoxyl proton at $\delta 4.00$ increased the signal at $\delta 7.70$ by 23% and hence there must be a single methoxyl substituent in one ring. The presence of a relatively large $(\text{M-H } 20)^+$ peak in the E.I.MS spectrum at m/z 296 (28% rel.int.), due to the loss of H_2O molecule from the parent ion caused by ortho positioning of a methoxy and a hydroxyl groups (156), confirms the presence of the hydroxyl group at C-1 rather than C-5 or C-8.

Thus the two possible alternative structures are 1-hydroxy-2,3,5-(or 2,3,8)-trimethoxy anthraquinone. The spectral data and the amount isolated did not allow for discrimination between these two possible structures.

3.3.1.3 Anthragallol 1,2,3-trimethyl ether. C₁₇H₁₄O₅

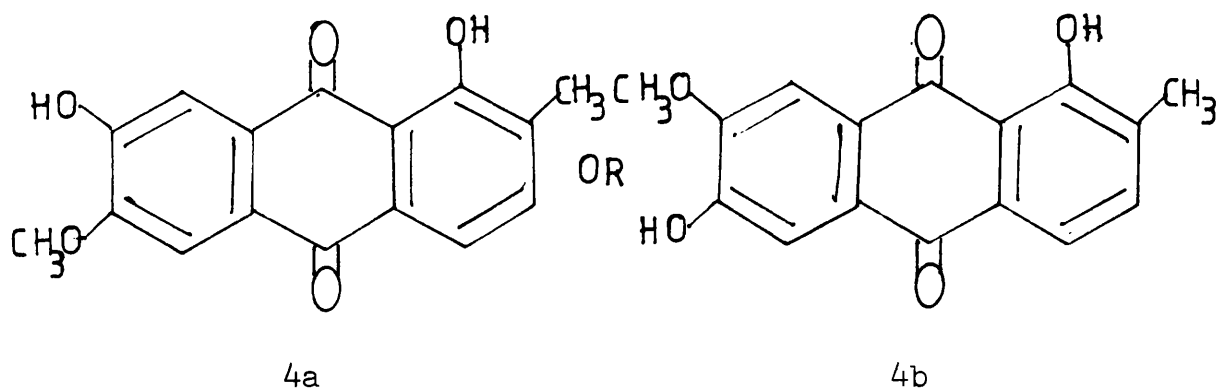
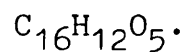


(3)

This pale yellow anthraquinone has a molecular ion base peak at m/z measured 298.0835 and calculated for C₁₇H₁₄O₅; 298.0841. The high intensity of this ion being due to the molecule's highly aromatic and therefore fairly stable structure. The major fragments of m/z at 283 and 269 corresponds to the loss of a methyl and CHO groups, respectively. Loss of one carbon monoxide and a methyl moieties from the parent ion leads to the peak at m/z 255. Its UV spectrum shows intensive benzenoid absorption at 250 nm and quinonoid band(s) at 260-290 nm indicated a compound with a quinone chromophore. Interestingly, upon the addition of one drop of methanolic solution of 1N sodium hydroxide no change in the colour of the solution and no bathochromic shift in the UV spectrum was observed, an indication that the compound does not possess a hydroxyl group. However, its IR absorption in CHCl₃ at 1665 cm⁻¹ was consistent with the presence of carbonyl moieties. The ¹H-NMR spectrum obtained at 250 MHz displayed three signals each of a three proton singlet at δ 3.97, δ 4.01 and δ 4.05 which corresponds to a three methoxyl signals. A proton signal appeared as a singlet at δ 7.70

which corresponds to the C-4 proton. The C-5 and C-8 proton signals appeared as a multiplet at δ 8.2. The C-6 and C-7 proton signals appeared as a multiplet at δ 7.73. The presence of a singlet and two multiplets, characteristic of an unsubstituted A-ring (Figure 9, p. 79). On the basis of this spectral data it is concluded that this compound is anthragallof, 1,2,3-trimethyl ether. This anthraquinone has been isolated in Rubiaceae from Oldenlandia umbellata (157).

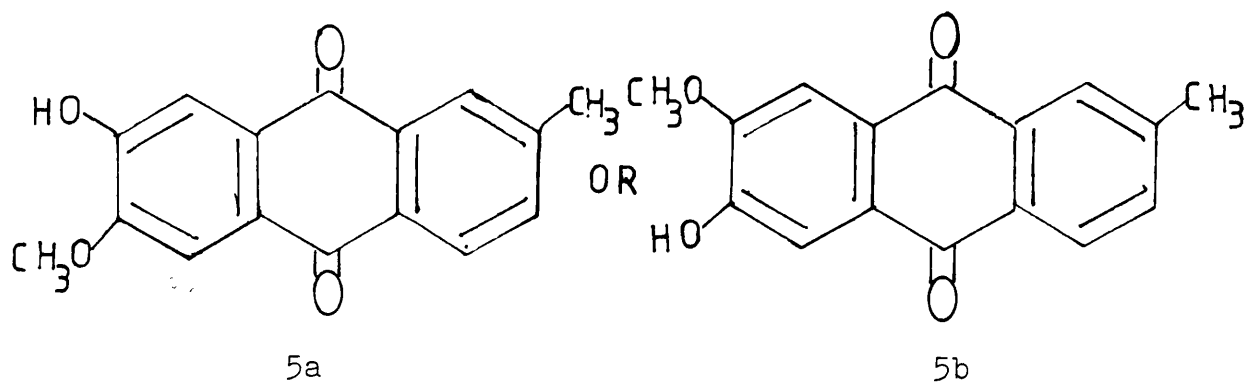
3.3.1.4 1,6-dihydroxy-2-methyl-7-methoxy or 1,7-dihydroxy-2-methyl-6-methoxy anthraquinone.



This yellowish-orange anthraquinone has a molecular ion base peak at m/z measured 284.0674 and calculated for $C_{16}H_{12}O_5$; 284.0685. The peak at m/z 269 corresponds to the loss of a methyl group from the parent ion and loss of a CHO group from the parent ions leads to a peak at m/z 255. The major fragment at m/z 241 corresponds to the loss of a methyl and CHO groups from the parent ion. Its UV spectrum shows intensive benzenoid absorption at 250 nm and quinonoid band(s) at 260-290 nm, indicated a compound with a quinone chromophore. Moreover, addition of one drop of methanolic solution of 1N sodium

hydroxide resulted in a colour change of the solution and a bathochromic shift in the UV spectrum, indicative of the presence of a phenolic hydroxyl group in the molecule. Its IR absorption in CHCl_3 at 1665 cm^{-1} was consistent with the presence of carbonyl moieties. The $^1\text{H-NMR}$ spectrum obtained at 250 MHz showed the presence of a methyl substituent (δ 2.37), a methoxyl group (δ 4.09), a hydrogen bonded hydroxyl (δ 12.99) and a free phenolic hydroxyl (δ 6.17). Signals for the four aromatic protons appeared as two, one proton singlets (δ 7.74 and 7.77) and two ortho coupled signals (δ 7.50 and 7.72). NOE experiments showed that irradiation at δ 2.37 resulted in a 25% increase in the signal at δ 7.50. Thus if C-2 is assigned the methyl substituent on biosynthetic grounds (46,46,155), positions C-3 and C-4 are unsubstituted and C-1 is substituted. Irradiation of the methoxyl signal at δ 4.09 resulted in a 42% increase of the singlet at δ 7.74. Thus C-6 and C-7 are substituted and since one hydroxyl is strongly hydrogen bonded it can only be located at C-1 whilst one methoxyl and one hydroxyl can be assigned to either the C-6 or C-7 positions. Hence the compound was 1,6-dihydroxy-2-methyl-7-methoxy or 1,7-dihydroxy-2-methyl-6-methoxy anthraquinone. It has not proved possible to distinguish between these two alternative structures (83).

3.3.1.5 2-methyl-6-hydroxy-7-methoxy or 2-methyl-7-hydroxy-6-methoxy anthraquinone. $C_{16}H_{12}O_4$



This yellowish-orange anthraquinone has a molecular ion base peak at m/z measured 268.0735 and calculated for $C_{16}H_{12}O_4$; 268.0736. The high intensity of this ion being due to the molecule's highly aromatic and therefore fairly stable structure. A number of significant ions were found at m/z 253, 239, 225, 197. Loss of the methyl group from the parent ion leads to the peak at m/z 253 and loss of the CHO moiety from the parent ion leads to the peak at m/z 239. The major fragment at m/z 225 corresponds to the loss of both methyl and a carbon monoxide moieties from the parent ion. Its UV spectrum shows intensive benzenoid absorption at 250 nm and quinonoid band(s) at 260-290 nm, indicated a compound with a quinone chromophore. Moreover, addition of one drop of methanolic solution of 1N sodium hydroxide resulted in a colour change of the solution and a bathochromic shift in the UV spectrum, indication of the presence of a phenolic hydroxyl group in the compound. However, its IR absorption in $CHCl_3$ at 1665 cm^{-1} is consistent with the presence of carbonyl moieties. The 1H -NMR spectrum obtained at 250

MHz showed the presence of one methyl (δ 2.52), and one methoxyl (δ 4.09) substituent. The MS indicated that there was further substitution with a hydroxyl group and since this did not appear as a sharp signal in the $^1\text{H-NMR}$ spectrum, it is unlikely to be at one of the peri positions. The signals of the five aromatic protons were seen as three singlets (δ 7.74, 7.76 and 8.07) and as two ortho coupled doublets (δ 7.57 and 8.17). Irradiation at δ 2.52 resulted in a 30% increase in the singlet at δ 8.07 and hence if the C-2 methyl groups can be assigned on biosynthetic grounds (46,47,155), the C-1H signal is at δ 8.07 and C-3 and C-4 must be unsubstituted. Irradiation of the methoxyl signal at δ 4.09 resulted in 20% increase in the intensity of the one proton singlet at δ 7.74, thus C-5 and C-8 are unsubstituted, the methoxyl and hydroxyl substituents may be at either C-6 or C-7. Hence the compound was either 2-methyl-6-hydroxy-7-methoxy or 2-methyl-7-hydroxy-6-methoxy anthraquinone. It has not proved possible to distinguish between these two alternative structures (83).

3.3.2 Scopoletin. $\text{C}_{10}\text{H}_8\text{O}_4$

Scopoletin (Figure 10, p. 91) has been isolated for the first time from C. ledgeriana plant tissue culture (154) by means of prep. TLC. The sample of scopoletin was identical with an authentic standard (TLC,MS) see Section 2.2.2.3 and 2.2.2.4, p. 44 and 45. Its mass spectrum showed a molecular ion base peak at m/z 192 and other significant ions were found at 177, 164, 149, 137, 121.

Section 3.4. Separation and isolation of natural product constituents from *A. annua* cultures.

Since there is a demand for high yielding sources of new antimalarial drugs, it was decided to establish plant tissue cultures of *A. annua* from seed material (see Section 2.1.2, p. 40). Standard techniques were used to initiate callus from sterile seedlings by the author (145).

The cultures were extracted and examined by TLC for the presence of artemisinin. Preliminary work with different cultures, did not reveal the presence of artemisinin in amounts detectable by means of TLC techniques only, but in vitro antimalarial testing, clearly showed the presence of active fractions when tested against multi-drug resistant strains of *P. falciparum* (see Section 3.5, p.93). To date, there is only one report of artemisinin production by tissue culture of *A. annua* (110). The method that was employed for all of the present investigations consisted of first extracting the cells with methanol. Since the methanolic extracts of callus and suspension cells were relatively inactive against *P. falciparum* in vitro, a solvent fractionation procedure was adopted (Figure 5, p. 51), which was aimed at obtaining three major fractions by extracting initially with methanol (fraction 1) followed by partition with n-hexane (fraction 2), anticipating that any artemisinin would be present in this fraction. The aqueous extract was further partitioned with chloroform to obtain the chloroform extract (fraction 3) and finally the remaining aqueous extract (fraction 4) freeze dried before being subjected to in vitro antimalarial

testing. The culture medium was also investigated for the presence of artemisinin, but it was not detected and therefore the medium was not screened routinely.

The extracted crude fractions were analysed by TLC, and the most satisfactory solvent system was chloroform : ethyl acetate (80:20). This solvent system resulted in reference artemisinin (R_f value 0.51) being well resolved and thus was useful for the present investigations. Since there is no specific colour reagent that will react with artemisinin, therefore various sprays were tried. Anisaldehyde and vanillin-sulphuric acid (146) was used as a general spray for the detection of essential oils, pungent and bitter principles, but the most useful spray proved to be 60% sulphuric acid followed by heating. Artemisinin gave (a) a yellow spot in day light and (b) a blue fluorescent spot when examined under UV light at 254 and 366 nm. The spray reagent has not previously been reported for the detection of artemisinin and it is the first report of such method of detection (145). The spray has been used as a general method for the detection of terpenes and it proved useful in this study for the detection of artemisinin. Scopoletin is detected by its blue fluorescent absorbance under UV light at 366 nm.

A hexane extract of the culture of cells grown for more than 12 months showed antiplasmodium activity when tested in vitro (Section 2.5, p. 59). Analytical TLC of this fraction did not reveal the presence of artemisinin and if it was present then it could only have been in minute quantities. For the analysis of these active extracts, a rapid analytical technique was required.

Column chromatography was carried out (see Section 2.2.3.7, p. 52). As a result, six column fractions were collected and subjected to antimalarial testing in vitro (see Table 9, p. 97).

Stigmasterol and scopoletin have been isolated from the cultures (Figure 10, p. 91) by means of preparative TLC using chloroform : ethyl acetate (80:20) as a solvent system. The isolated stigmasterol and scopoletin co-chromatographed on TLC with reference standard and were characterised by MS and ¹H-NMR. These two compounds have been isolated from A. annua whole plant (101).

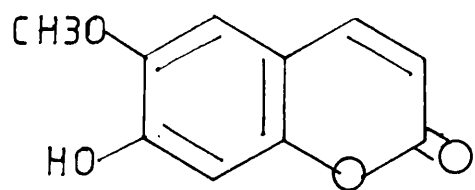
Interestingly the cells were found to release the active compounds into the medium. This feature is essential when immobilised cell techniques were employed for the production of secondary metabolites. No evidence was obtained for the presence of artemisinin in either callus, suspension cells or medium.

Plant cell cultures are potentially a viable source of secondary plant products (2,3). However, some cultures have been shown to synthesize secondary metabolites in concentrations comparable to their parent plants (13). In particular, the yields of alkaloids by plant cell cultures have generally been low. Very little is known about artemisinin production in A. annua cultures and the yield in the whole plant is low.

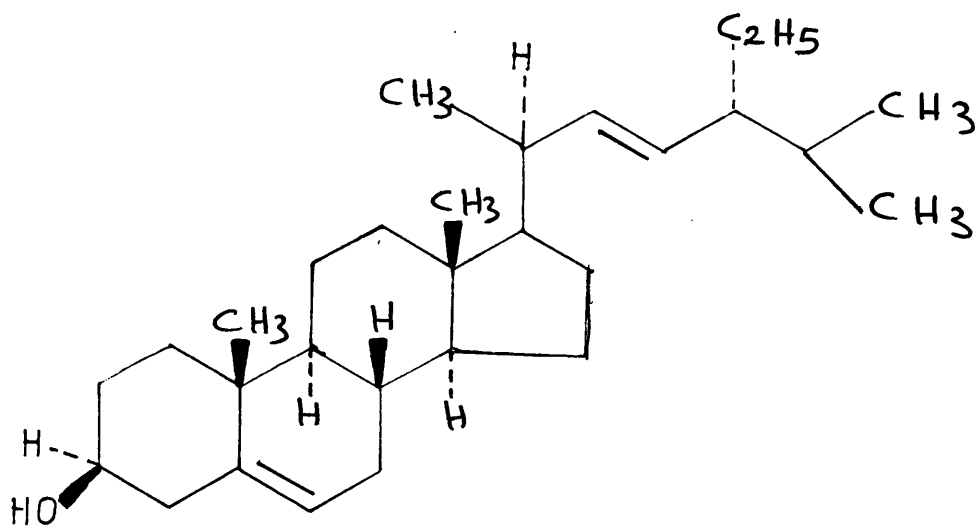
Since the cultures have been found to be active against P. falciparum it is hoped that artemisinin production can be encouraged by the use of precursor feeding experiments.

In order to ascertain the growth characteristics, detailed time course studies were carried out (145). Artemisia annua cells were transferred into fresh medium to give an initial inoculum of 3 g fresh weight 40 ml^{-1} and the cultures were then harvested on alternate days throughout the growth cycle until day 32. On harvesting, the fresh weight of the cells was determined, air dried and weighed.

The growth cycle shown in (Figure 11, p. 92) shows that the cells grow well, undergoing a 3-fold increase in the dry weight by the end of the growth period. The growth cycle is characterised by a relatively long lag phase, up to 12 days, followed by a period of growth until day 30 when the cells enter the stationary phase (145).



Scopoletin



Stigmasterol

Figure 10 Stigmasterol and Scopoletin

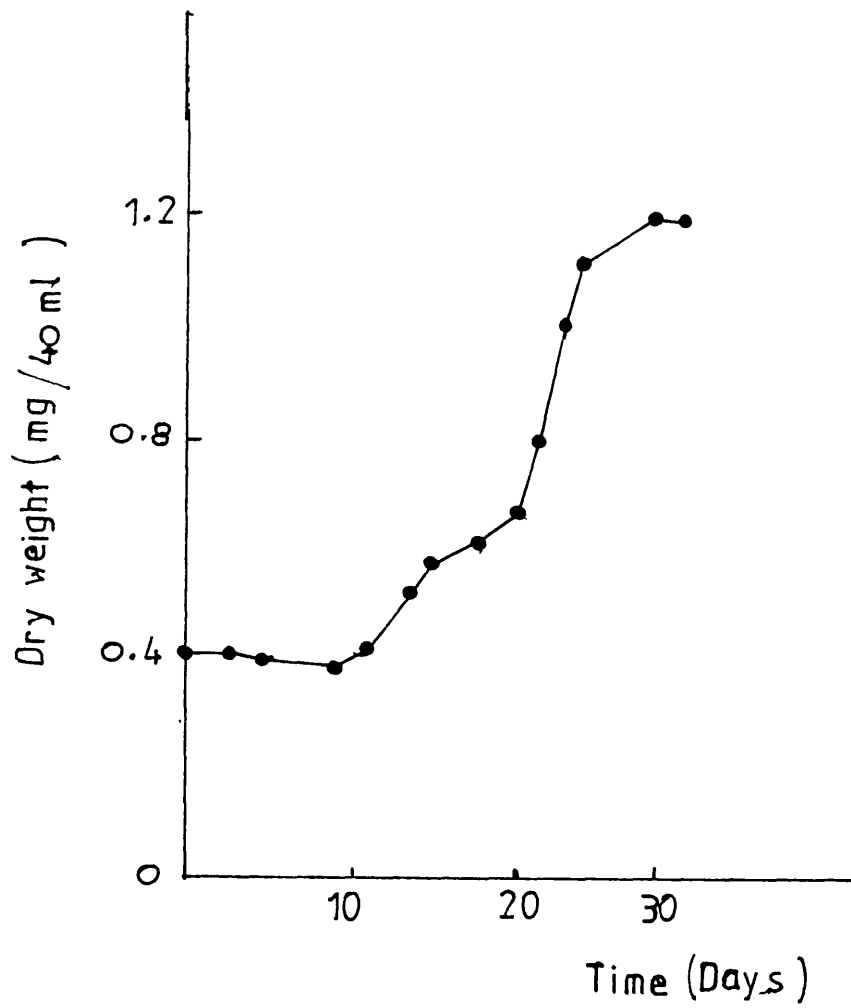


Figure 11 Growth cycle of Artemisia annua cell suspension cultures.

Section 3.5. In vitro antimalarial activity of crude extracts of A. annua cell cultures.

The micro dilution technique of Desjardins et al. (158), based on the inhibition of incorporation of the radio-labelled nucleic acid [^3H]-hypoxanthine precursor, provides quantitative measurements of the anti-malarial activity of large numbers of compounds. For the purpose of the present study the Desjardins technique has been used for preliminary evaluation of antimalarial activities of crude plant extracts and pure compounds.

3.5.1. Activities of callus and suspension cells.

Dried, powdered, callus and suspension cells were extracted using cold maceration techniques as outlined in Figure 5, p. 51.

All the extracts were assessed at different stages in their growth and development against Plasmodium falciparum. The in vitro antimalarial activities of these extracts are shown in Table 6 for callus cultures and Table 7 for cell suspension cultures and are based on 10-fold and 3-fold dilutions. The methanolic extracts (fraction 1) of callus and suspension cells were relatively inactive (IC_{50} values ranging between 50-500 $\mu\text{g ml}^{-1}$). Fractionation of the methanolic extract into n-hexane (fraction 2), chloroform (fraction 3) and water (fraction 4) as outlined in Figure 5, p. 51, yielded more active fractions particularly in chloroform at month 5 for callus (IC_{50} value 18.5 $\mu\text{g ml}^{-1}$) and months 6 and 8 for suspension cells (IC_{50} value 6-18.5 $\mu\text{g ml}^{-1}$) (Tables 6 and 7). The activity of callus, suspension cells were subsequently lost.

If the cultures had produced artemisinin then higher activities would have been observed in the n-hexane fractions. Moreover, the crude extracts of the culture medium in which the suspension cells had been grown were also tested for in vitro antimalarial activity and the results expressed in Table 8, p.95. The hexane extract (fraction 2) showed activity after 8 months (IC_{50} value $19 \mu\text{g ml}^{-1}$) and after 12 months there was a dramatic increase in activity (IC_{50} value $0.06 \mu\text{g ml}^{-1}$). The chloroform extracts (fraction 3) showed little activity after (8-12) months. The results from the medium differ from the cells in that activity increased with age and the active fractions were obtained from hexane rather than chloroform.

Table 6 In vitro antimalarial activities (approximate IC_{50} values in $\mu\text{g ml}^{-1}$) of crude extracts (fractions 1-4) obtained from callus cells of Artemisia annua.

Fraction Number	Age of callus cells in months					
	3	4	5	6	7	8
1 methanol	500	50	67	56	500	500
2 n-hexane	50	50	18.5	56	56	167
3 chloroform	50	50	18.5	18.5	18.5	18.5
4 water	N.T.	N.T.	167	500	500	500

N.T. = not tested

Table 7 In vitro antimalarial activities (approximate IC_{50} values in $\mu\text{g ml}^{-1}$) of crude extracts (fractions 1-4) obtained from cell suspensions of Artemisia annua.

Fraction Number	Age of suspension cells in months			
	4	6	8	12
1 methanol	50	167	N.T.	N.T.
2 n-hexane	N.T.	56	56	50
3 chloroform	N.T.	6.2 - 18.5	6	n.a.
4 water	50	167	N.T.	n.a.

N.T. = not tested

n.a. = not active

Table 8 In vitro antimalarial activities (approximate IC_{50} values in $\mu\text{g ml}^{-1}$) of crude extracts (fractions 1-3) obtained from the medium of Artemisia annua cell suspensions.

Fraction Number	Age of the medium in months		
	6	8	12
1 methanol	167	N.T.	N.T.
2 n-hexane	50	19	0.06
3 chloroform	N.T.	56	50

N.T. = not tested

Apparently the cultures contain at least two active constituents one, being extracted from the cells by chloroform and not by hexane whilst the other is extracted by hexane from the medium. This latter activity is associated with a compound(s) which is able to pass across cell membranes. TLC examination did not indicate the presence of artemisinin in either callus, suspension cells or medium. It has been reported previously by Nair et al. (110) that artemisinin is produced in low yields in the medium of A. annua cultures.

However, it should be pointed out that, the medium of the cell suspensions was not tested regularly each month as in case of the cell suspensions (Tables 7 and 8). The reason for this is because of the problems associated with the freeze drying of a large volume of the filtered medium. In addition, extraction of the medium is difficult and this is due to the fact that, the concentrated medium was syrupy (containing 5% sucrose), which made it difficult to obtain efficient extraction..

The aqueous extracts (fraction 4), were examined for in vitro antiplasmodial activity and they were inactive with IC₅₀ values of 50 $\mu\text{g ml}^{-1}$ or more.

3.5.2. Activities of artemisinin and pure compounds.

The isolated and identified scopoletin and stigmasterol (Section 2.2.3.6, p. 52) were tested against Plasmodium falciparum and the IC₅₀ values of both compounds were determined as 56 $\mu\text{g ml}^{-1}$ based on 3-fold dilutions. Reference artemisinin was tested and its IC₅₀ value was determined as 0.0028 $\mu\text{g ml}^{-1}$ (159).

3.5.3. Activities of column fractions obtained from the hexane extract of the medium which showed high antimalarial activity in preliminary tests.

The hexane extract of the medium upon column chromatography yielded six fractions (Section 2.2.3.7, p. 52). Each fraction was tested for in vitro antimalarial activity and the results based on three fold dilution are given in Table 9).

Fractions 1 and 2 were relatively inactive (IC_{50} values of $167 \mu\text{g ml}^{-1}$), while fractions 3 and 6 showed little activity (IC_{50} values of $50 \mu\text{g ml}^{-1}$). However, fractions 4 and 5 were totally inactive.

The antimalarial activity of the crude hexane extract of the medium was lost by using a silica gel column and this could be explained either by the method not being suitable with the activity being lost during the process or because the active compound is highly unstable and needs a more rapid method of separation.

Table 9 In vitro antimalarial activity (approximate IC_{50} values in $\mu\text{g ml}^{-1}$) of fraction from a silica gel column chromatogram obtained from the hexane extract of A. annua medium.

Column Fraction	approximate IC_{50}
1	167
2	167
3	50
4	n.a.
5	n.a.
6	50

n.a. = not active

Section 3.6. Influence of medium composition on growth and *in vitro* antimalarial activity of *A. annua* callus cultures.

3.6.1. Nature of the basal salts.

A wide range of media components are known to affect secondary product synthesis (123) and in this investigation we have compared growth and possible production of artemisinin, utilising the inhibition of uptake of [^3H]-hypoxanthine into Plasmodium falciparum to assess the activity of crude extracts of *A. annua* callus cultures. Four commonly used basal media were investigated namely, Murashige and Skoog (MS), Linsmaier and Skoog (LS), Schenk and Hildbrandt (SH) and Gamborg's B₅ (B₅) (Section 2.7.3) (345). The standard MS medium was used as a control. Small pieces of callus cells (15 \pm 5 mg fresh weight) were transferred from stock cultures into one of the four media and the cultures were harvested after 2 months.

On harvesting, both fresh and dried weights of the callus cells were determined. The results (Figure 12, p. 102) show that, there was approximately a similar 3-fold increase in the dry weight for MS, LS and B₅ media over the initial inoculum (5 g d.wt). Growth on LS and B₅ media were extracted with a 1.2 and 1.13, fold increase in the dry weight respectively compared to the control medium (MS).

On the other hand, growth on SH medium was relatively poor with about 20% increase in the dry weight over the initial inoculum, being achieved over a two months period.

In addition to growth, there was a difference in

the morphological appearance of the calli. Calli growing on MS medium consisted of fast growing, cream-brown undifferentiated cells. Many roots were formed by calli grown on SH medium.

Moreover, the change in the basal salts did have a significant effect on the IC_{50} values obtained. Results in Table 10 illustrate that the IC_{50} value of the n-hexane extract of the B_5 medium was approximately similar to the n-hexane extract of the control medium (16.5, 18.5 $\mu\text{g ml}^{-1}$ respectively), while the chloroform extract was not active. However, LS basal salts resulted in increased activity of both n-hexane and chloroform extracts (IC_{50} values 5 $\mu\text{g ml}^{-1}$). In vitro antimalarial activity was inhibited, as was cell growth by transferring calli to SH medium.

TLC analysis of these fractions did not show the presence of artemisinin when co-chromatographed with authentic artemisinin.

The improvement in the IC_{50} values achieved by changing the basal salts from MS to LS is of particular interest because the formulation of LS is very similar to that of MS, they differ only in their vitamin/organic components, with the pyridoxine nicotinic acid and glycine deleted, whereas the thiamine level is raised 4-fold. The author concluded that the best medium for sustaining growth with high antimalarial activity in stock callus culture of A. annua is LS medium (145).

The deleterious effect of SH medium on growth as well as antimalarial activity can not easily be attributed to one component or group of components as the formulation differs significantly from the others in macro and micro

nutrients, as well as vitamins. Relatively low levels of secondary metabolites have been reported from cultures using SH medium, and this has been attributed to the high phosphate levels (123). However, from other experiments with A. altissima cultures, transfer of cell suspensions from MS to SH medium has resulted not only in reduced growth and alkaloidal accumulation but also in a major change in the ratio of 1-methoxy-canthin-6-one to canthin-6-one (160). The adverse effect of SH medium on growth is not solely due to the raised phosphate concentration (161).

3.6.2. Nature and concentration of plant growth regulator (auxin)

A great deal of research has been carried out in order to study the effects exerted by the plant growth regulators present in the culture medium on growth and secondary metabolite production by plant cell and tissue cultures (Section 1.7, p. 36). In general, 2,4-dichlorophenoxy acetic acid (2,4-D) is thought to suppress secondary metabolism eg. the presence of 2,4-D totally inhibited the production of serpentine by C. roseus cultures (5). There does not appear to be any general trends with regard to the influence of other auxins such as indole acetic acid (IAA) and naphthalene acetic acid (NAA).

Continuing the studies investigating the effects of the composition of the culture medium on A. annua callus cultures, experiments were designed to determine the influence of three auxins at different concentrations 2,4-D (1.0), IAA (0.1, 1.0, 3.5, 5.0) and NAA (1.0, 5.0) mg L⁻¹, respectively. The cytokinin, kinetin, remained

constant at 0.1 mg L^{-1} (Section 2.7.4, p. 62) (145). Small pieces of calli ($15 \pm 5 \text{ mg}$ fresh weight) were transferred from stock medium containing 1.0 mg L^{-1} 2,4-D to the test medium. The cultures were harvested 2 months after subculturing and the fresh and dried weight of the callus cells determined. The dry weight differences resulted from the change in the nature and concentration of auxins (Figure 13, Table 11) are not very significant although NAA at a concentration of 5 mg L^{-1} resulted in 1.7-fold increase in dry weight when compared to MS medium, but activity was lost. The greater activity was achieved from MS basal salts containing 1.0 mg L^{-1} of IAA (IC_{50} value $6 \mu\text{g ml}^{-1}$). This finding is in agreement with the general acknowledgement that IAA increases secondary metabolite production while NAA suppresses their production. Also Table 11 indicates that antimalarial activity was lost with higher levels of auxins. However, TLC examination of these fractions did not show the presence of artemisinin when co-chromatographed with authentic artemisinin. In addition to growth and antimalarial activity, there were differences in appearance of the calli. The growth of calli on IAA media with higher auxin level was less, but the cultures had better growth characteristics and were green-brown in the light. Many roots were formed by calli grown on media with high auxin level. Only a few explants on media containing 1.0 mg L^{-1} of 2,4-D, IAA or NAA, showed rooting. In other cases no organogenesis was observed. The nature and level of auxin has a profound effect on in vitro antimalarial activity exerted by A. annua callus cultures however, there does not appear

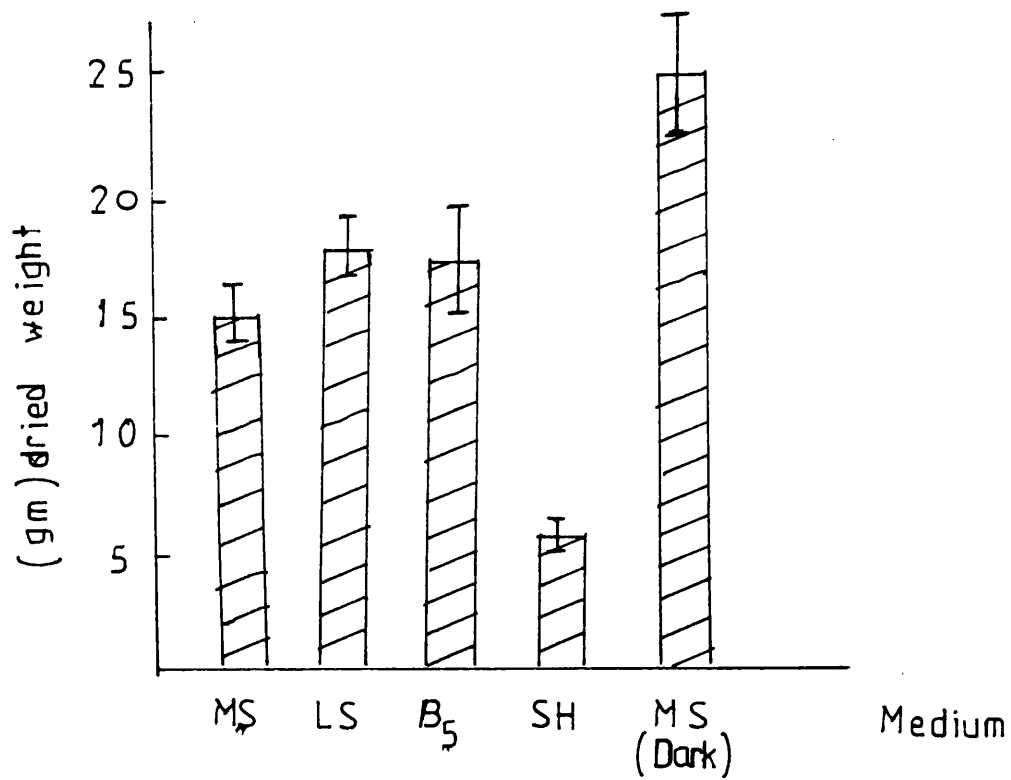


Figure 12 Effect of various basal media and dark growth of A. annua callus cultures.

Table 10. Influence of basal media and dark on inhibition of uptake of [^3H]-hypoxanthine into P. falciparum in vitro by extract fractions from callus cells.

Media	Solvent extracts	<u>In vitro</u> anti <u>P. falciparum</u> activity (IC ₅₀ value $\mu\text{g ml}^{-1}$)
LS	n-hexane	5
	CHCl ₃	5
	H ₂ O	50
B ₅	n-hexane	16.5
	CHCl ₃	n.a.
SH	n-hexane	n.a.
MS (control)	n-hexane	18.5
	CHCl ₃	18.5
	MeOH and H ₂ O	500
MS (dark)	n-hexane	18.5
	CHCl ₃	18.5

n.a. = not active

Table 11 Influence of auxins on inhibition of uptake of [³H]-hypoxanthin into P. falciparum in vitro by extract fractions from callus cells.

Auxin	Concentration ug L ¹	Solvent extracts	<u>In vitro</u> anti <u>P. falciparum</u> activity (IC ₅₀ value μg ml ⁻¹)
2,4-D	1.0	n-hexane	18.5
		CHCl ₃	18.5
		MeOH and H ₂ O	500
IAA	0.1	n-hexane	50
	1.0	n-hexane	6
	3.5	n-hexane	n.a.
	5.0	n-hexane	n.a.
NAA	1.0	n-hexane	50
	5.0	n-hexane	n.a.

n.a. = not active

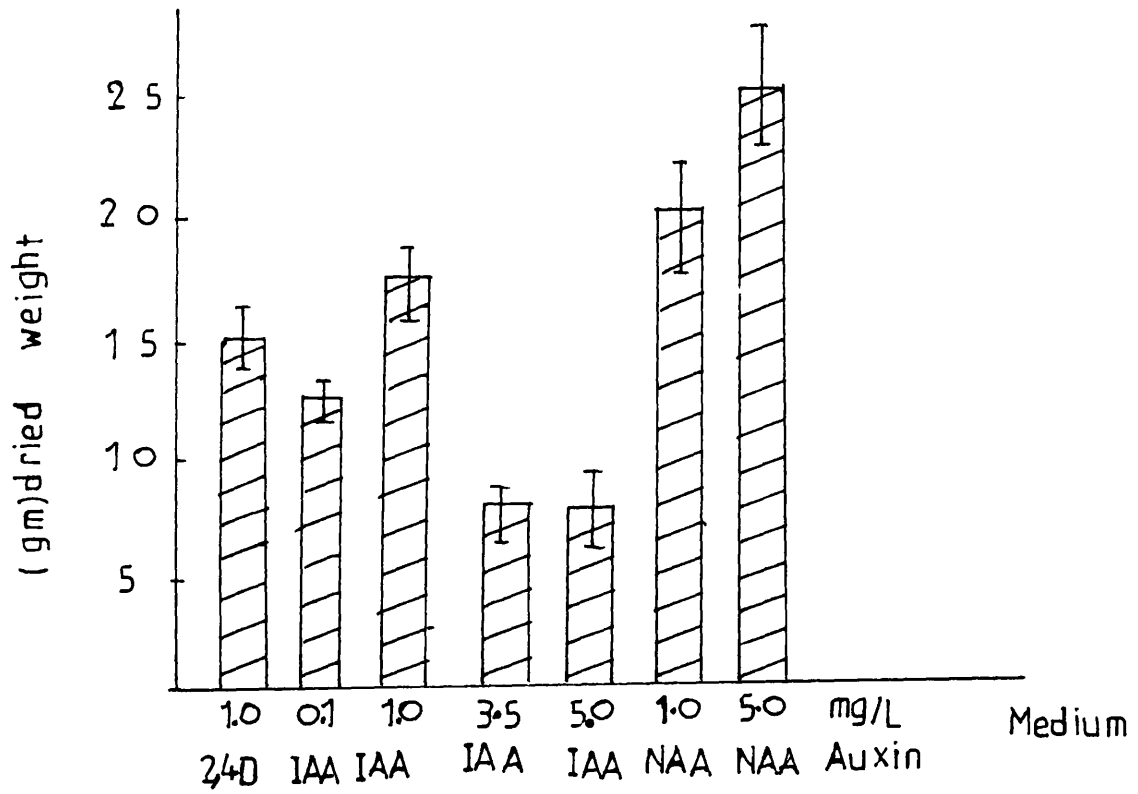


Figure 13 Effect of various auxin concentrations on growth of A. annua callus cultures.

to be a standard combination of auxin and cytokinin that would result in optimal growth and increased antimalarial activity. Therefore, for such culture systems investigations should be carried out in order to achieve the ideal composition of plant growth regulators for both growth and secondary metabolite production.

3.6.3. Effect of the dark on growth and in vitro antimalarial activity.

In addition to the components of the internal and external environment such as basal medium, plant growth regulators, sucrose levels, precursor feeding, it has been found that light has a great effect on the production of secondary metabolites. It has been reported (162) that the concentrations of quinoline alkaloids are low from C. ledgeriana cultures which grow in the light than from those grown in the dark. Mulder-Krieger et al. (74) found that light stimulates alkaloid production by callus cultures of C. pubescens. Nothing has been reported about the effect of various factors in the cultural environment on artemisinin production. Therefore, studies investigating the effect of the dark on growth and possible production of artemisinin, utilising the inhibition of uptake of [^3H]-hypoxanthine into P. falciparum to assess the activity of crude extracts of A. annua callus cultures was carried out (145). The stock medium composed of MS basal salts kept under continuous illumination was used as the control. Small pieces of the callus cells were transferred also to a medium based on MS basal salts and kept in the dark (Section 2.7.5). After 2 months the

cultures were harvested. Growth was enhanced with a 1.7-fold increase in the dry weight compared to the control medium (Figure 12, p. 102). However, the increase in the growth does not show any increase in the antiplasmodial activity (IC_{50} values of both n-hexane and $CHCl_3$ extracts was $18.5 \mu g ml^{-1}$) (Table 10, p. 103). In addition differences in the morphological appearance of the callus was observed. The dark-brown colour of the tissues was replaced by creamy coloured cells compared to those with illumination, also there was a change in the colour of the medium to deep brown. Many deep brown roots with white shoots of two leaves were formed when the calli were left for more than 50 days without subculturing. Moreover, more fluorescent bands have been noticed in a TLC chromatogram compared to those under illumination. TLC analysis of these fractions does not show the presence of artemisinin when co-chromatographed with authentic artemisinin.

Plant cell cultures offer possible alternative as commercial sources of secondary products, providing that the processes are economically viable. However, much has yet to be accomplished in our understanding of the regulation of secondary metabolite production before commercially feasible processes are routinely available.

Anthraquinones are an important category of secondary metabolites produced by Cinchona species. Some 1,8-dihydroxy anthraquinone glycosides are used in medicine because of their laxative action. However, some of the anthraquinones from the Rubiaceae exhibit other very interesting biological in vitro activities (Section 1.2.2) and this investigation was undertaken to obtain information with regard to their production in vitro. Highly differentiated structures termed root organs of Cinchona ledgeriana were developed in our laboratories and found to produce a substantial amount of anthraquinones in addition to quinoline alkaloids, quinine and quinidine being the major constituents.

In this particular study, five hitherto unreported anthraquinones were isolated from Cinchona ledgeriana root organ cultures and identified on the basis of spectroscopic data (Section 2.3, p. 53). All the anthraquinones found are of the type that is characteristic for the Rubiaceae. The first report on the occurrence of anthraquinones in the genus Cinchona was by Mulder-Krieger et al. (69) who identified five anthraquinones in callus cultures of C. ledgeriana. Subsequently Wijnsma et al.

(61) isolated and identified 15 anthraquinones, whilst Robins et al. (88) reported the isolation of 15 anthraquinones from suspension cultures of C. ledgeriana, five of which were reported for the first time. Wijnsma and co-workers have also reported the isolation of 12 anthraquinones from callus cultures of C. pubescens, seven of which corresponded to those previously isolated from C. ledgeriana callus cultures, whilst five were reported for the first time from Cinchona species (163).

Many factors are known to affect anthraquinone production in vitro, for example, the composition of the medium, light, temperature and pH (Section 1.4.2), have been studied and reported. Wijnsma et al. (84) have demonstrated that anthraquinones exhibited antimicrobial activity and acts as phytoalexins in cell and tissue cultures of Cinchona species and suggested that these compounds might be correlated with the defence mechanisms of plants against microbial infections.

Since the level of anthraquinones in cultured cells is among the highest known today for any product (37) and because of their antimicrobial activity, it is possible these compounds may be targets for commercial production.

In view of the urgent need to replace the existing antimalarials, artemisinin extracted from the Chinese herb Artemisia annua provides an encouraging example of a novel antimalarial drug from the plant source. The plant contains low yield of artemisinin and synthesis is not economic (164), hence cell cultures meant further investigations.

In vitro cultures of Artemisia annua developed in

our laboratories, produced no artemisinin. Nair et al. (110) reported the presence of artemisinin in a very low amount in the medium and in rooted callus cultures by means of HPLC with Electro Chemical Detector.

In contrast to the highly organised Cinchona cultures our Artemisia cultures are composed of indifferentiated cells, either single or in small aggregates. The degree of differentiation or organogenesis shown by the cultures may be an important factor as to whether the cultures are capable of accumulating the desired secondary metabolites. However, the lack of artemisinin production in our cultures could be due to inadequate storage facilities resulting in further metabolism (126). Other reasons for non-production of artemisinin may include the lack of biosynthetic precursors or of the necessary enzymes for secondary metabolism.

In this study, an in vitro antimalarial test with Plasmodium falciparum was used to assess the activity of the crude extracts obtained from Artemisia annua cultures at different ages of growth and development. Results in Tables 6, 7 and 8 show that although the cultures were highly active, artemisinin was not detected by means of TLC. Nonetheless, these initial results suggest that the technique of $[G-^3H]$ -hypoxanthine incorporation is clearly applicable to screening crude extracts of plant culture for antimalarial activity in order to monitor their fractionation and to select their active principles. The increase in the antimalarial activity as the cultures aged confirms that the cells are genetically stable over a two year period. However, the IC_{50} values of the callus

suspension cells and medium were determined at different ages of the cultures. Scopoletin and stigmasterol were isolated and identified from these cultures, their IC_{50} values were determined and found to be relatively inactive. The research on A. annua plant tissue culture is relatively recent and very little has been reported about artemisinin production from tissue culture. No papers have been published on the effects of altering the nature and levels of auxins and cytokinins present in the medium on artemisinin production.

In this work a series of investigations were carried out to study the effect of basal salts, light and auxin levels on growth, and possible production of artemisinin by A. annua callus cultures. The activity of the crude extracts was also assessed using Desjardins et al. technique (158). The results of our studies (Section 3.6) would indicate that the optimal production medium with a higher antimalarial activity of those investigated, is one composed of Linsmaier and Skoog (LS) basal salts with 1.0 mg L^{-1} (IAA), 0.1 mg L^{-1} kinetin and 5% sucrose. However, due to the diversity of the cultural systems that can exist for any given species, the optimal medium composition should be determined for each individual culture.

It has not proved possible to detect artemisinin in cell cultures or media during the present investigation. Nevertheless, the finding of antiplasmodium activity warrants further investigations. It is possible that artemisinin may be present in low quantities in hexane fractions but the presence of antiplasmodial activity in

other fractions indicates additional active constituents must be produced by a cell culture. Therefore, it is interesting and essential as a first step to isolate and identify the active compound(s) from the cultures using more sensitive methods (eg. HPLC with EC Detector). Quantitative analysis of the active compounds should be carried out followed by their in vitro and in vivo evaluation. It has been shown that the in vitro anti-plasmodial activity of artemisinin is potentiated by the presence of certain methoxylated flavanoids (eg. casticin) (166) and at higher concentrations some of these flavanoids are cytotoxic (167).

The possibility that low concentrations of artemisinin may be present in our tissue cultures being potentiated by flavanoids present in the cultures.

Since the cells started to release active constituents into the medium after a 12 month period, it is proposed that this experiment should be repeated and that cell immobilisation techniques be completed.

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