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Antiplasmodial activity of *Artemisia annua* plant cell cultures

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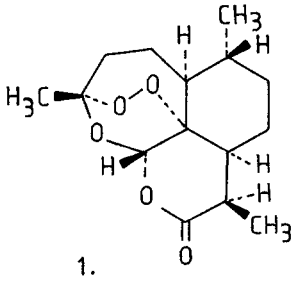
ABSTRACT

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.

INTRODUCTION

Malaria must be considered as one of the most common tropical diseases. It causes a greater economic loss than any other disease and it is a major cause of infant mortality in many developing countries, particularly in Africa (Wyler 1983). It is recognized that new drugs are urgently needed because of the resistance of Plasmodium species to current chemotherapy. Therefore, searching for new antimalarial compounds from plants is of increasing clinical interest. The Chinese antimalarial drug artemisinin (Qinghaosu, QHS) is one such new drug of current interest. Artemisinin has rapid action and is effective against chloroquine-resistant malarial parasites and has a different mode of action from existing blood schizonticides (Anon.1979; **Anon. 1981**; Nair et al.1986).

Qinghaosu (1), a novel sesquiterpene lactone endoperoxide is obtained from the traditional Chinese herbal remedy "Qing hao" (Artemisia annua, Asteraceae) (Anon. 1979) used for centuries as a febrifuge. The clinical usefulness of artemisinin and its derivatives has resulted in a great deal of scientific activity. The relatively low content of artemisinin in A. annua has been one of the limiting factors for clinical utility, and synthesis of the molecule is not an economic feasibility (Xu et al. 1986).



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The potential of plant cell and tissue culture as a means of producing pharmaceutically useful secondary metabolites has been the focus of intensive investigation (Fowler 1983 and 1986). It has been reported that low yields of artemisinin are produced from roots and rooted plantlets derived from leaf explants or callus cultures (Nair et al. 1986). In our search for natural products with antimalarial activities that could provide alternatives to chloroquine, we have established cell cultures of Artemisia annua and we have investigated the effect of the basal media, hormone levels and light on both growth and antiplasmodial activity of A. annua callus cultures.

EXPERIMENTAL

Plant material

Seeds of Artemisia annua were kindly supplied by Professor W. Peters, London School of Hygiene and Tropical Medicine.

Culture method

The seeds were surface sterilised in 2% sodium hypochlorite containing 1% triton X-100 for 15 minutes followed by washing with sterile distilled water. The seeds were germinated on damp filter paper in sterile petri dishes and callus culture was initiated by transferring aseptic seedlings onto solid (1.5% agar) Murashige and Skoog (1962) medium containing 2,4-dichlorophenoxy acetic acid (2,4-D) (1.0 mg l^{-1}), kinetin (kin) (0.1 mg l^{-1}) and sucrose (5%). The pH was adjusted to 5.8-5.9 before sterilization by autoclaving and cultures were maintained at 25°C under constant illumination and subcultured every 4 weeks. This medium serves as a control medium (A), see Tables 2 and 3 .

Cell suspension cultures were established by transferring third generation, friable callus cells to liquid medium (40 ml) in 250 ml conical flasks. The medium contained the same proportion of ingredients as above, without agar. The cultures were maintained on an orbital shaker (120 r.p.m.) at 25°C under continuous illumination and subcultured into fresh medium every 4 weeks.

Growth study in liquid suspension culture

Cell suspension cultures were filtered aseptically and distributed into flasks, containing 40 ml fresh medium to give an initial inoculum of 3 gm fresh weight per flask (0.4 gm total dried weight per flask)

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Table 1. In vitro activity of crude extracts obtained from callus suspension cells & medium of A. annua

Solvent extracts[2]	Approximate IC_{50} values (μgml^{-1})[1] Age in months												
	Callus cells						Suspension cells				Fluid medium		
	3	4	5	6	7	8	4	6	8	12	6	8	12
MeOH crude extract	500	50	67	56	500	500	50	167	NT[3]	NT	167	NT	NT
n-hexane fraction	50	50	18.5	56	56	167	NT	56	56	50	50	19	0.06
$CHCl_3$ fraction	50	50	18.5	18.5	18.5	18.5	NT	6.2-18.5	6	na[4]	NT	56	50
H_2O fraction	NT	NT	167	500	500	500	50	167	NT	na			

(1) Approximate IC_{50} values were determined in duplicate and calculated on the basis of either 10 fold or 3 fold dilutions. (2) See Experimental section. (3) NT: not tested. (4) na: not active. IC_{50} values of reference artemisinin = 0.0028 μgml^{-1}

and were harvested on alternate days over a 32 day period. The cells were filtered from the medium, air dried and weighed. All procedures were carried out in duplicate.

Establishment of callus cultures on different basal media, plant growth regulators

For the experiments, small pieces of callus cultures (15 ± 5 mg fresh weight) were transferred aseptically onto a fresh medium consisting either of Linsmaier and Skoog (1986) (LS), Shenk and Hildebrandt (1972) (SH) or Gamborg's B_5 (Gamborg et al. 1968) basal salts, with plant growth regulators and sucrose as in the control MS medium (A) (Murashige and Skoog 1962), see Table 2 and Fig. 2. In addition, callus cultures were established on MS medium using different concentrations of either indole acetic acid (IAA) (B, 0.1: C, 1.0; D, 3.5 and E 5.0 mg l^{-1}) or naphthalene acetic acid (NAA) (F, 1.0 and G, 5.0 mg l^{-1}) with a constant concentration of Kin. (0.1 mg l^{-1}) and sucrose (5%). A batch of MS cultures were kept in the dark for comparison purposes. The cultures were maintained under identical conditions (see culture method) and harvested after 2 months, air dried and weighed.

Extraction procedure

Callus cultures from third and subsequent generations and suspension cultures from fourth and subsequent generations were air dried and ground to fine powder prior to extraction. Medium from suspension cultures was extracted separately. Dried cells and freeze dried medium were extracted with methanol (x3) and the total methanolic extracts concentrated to dryness under reduced pressure. The dried methanolic extracts were partitioned between n-hexane and water and the aqueous fraction further partitioned with chloroform. The hexane and chloroform extracts were concentrated to dryness in vacuo and the remaining aqueous extract was freeze dried.

Thin layer chromatography (TLC)

Extracts were examined by TLC using silica gel GF_{254} together with authentic artemisinin. The following solvent systems were used:

S_1 : $CHCl_3$: EtOAc (8:2)
S_2 : $CHCl_3$: EtOAc (92.5:7.5)
S_3 : cyclohexane : Et_2O (9:1)
S_4 : cyclohexane : Et_2O (4:1)

Visualization was carried out by spraying with 60% concentrated sulphuric acid and heating. Reference artemisinin appeared as a yellow spot in daylight and a fluorescent blue spot when examined under UV light at 254 and 330 nm.

In vitro test for antimalarial activity

The cells and medium extracts were assessed for in vitro activity against P. falciparum (Thai K-1 strain multi-drug resistant) (Thaithong et al, 1983), utilising published procedures (Osisanya et al. 1981; Trager and Jensen, 1976; Fairlamb et al, 1985). The test measures inhibition of incorporation of [^{3}H]-hypoxanthine into the parasite, based on the method of Desjardins et al (1979) using the procedure previously described (O'Neill et al, 1985). Extracts were tested in duplicate, each at concentrations in ten fold dilution. For more accurate determination of IC_{50} values, three fold dilutions were prepared to give concentrations around the range of the IC_{50} value on the basis of ten fold dilution results. Two series of controls were used, one with infected red blood cells in the absence of drug and the other with uninfected red blood cells. Chloroquine diphosphate was tested simultaneously to monitor the sensitivity of the P. falciparum strain. The IC_{50} value of chloroquine diphosphate and of artemisinin were determined as 0.21 and 0.0028 $\mu g\ ml^{-1}$, respectively.

RESULTS AND DISCUSSION

The total methanolic extracts of the cell cultures were tested for in vitro antimalarial activity (see Experimental) at different stages in their growth and development. The results are based on tenfold and threefold dilutions and are summarised in Table 1.

The methanolic extract of callus and suspension cells were relatively inactive (IC_{50} values between 50-500 $\mu gm\ l^{-1}$). Fractionation into n-hexane, chloroform and water (see extraction procedure), yielded more active fractions, particularly in the chloroform fractions at months 5-8 for callus (IC_{50} value of 18.5 μgml^{-1}) and months 6 and 8 for suspension cells (IC_{50} value of 6-18.5 μgml^{-1}) (Table 1). The activity of suspension cells was subsequently lost. If the cultures had produced artemisinin, higher activities would have been expected from the n-hexane fractions (Artemisinin, IC_{50} value of 0.0028 μgml^{-1}). The chloroform fractions showed little activity after 8 - 12 months (see Table 1).

The results from the medium differ from the cells in that activity increased with age and the active fractions were obtained from n-hexane rather than from chloroform. Apparently the cultures contain at least two active ingredients, one 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 plant cell membranes.

TLC examination did not indicate the presence of artemisinin in extracts of either callus, suspension cells or medium. These results contrast with those reported previously (Nair et al. 1986) when artemisinin was obtained in low yields from fluid medium of Artemisia annua cultures (Nair et al. 1986). In the present investigation, the chloroform extracts of callus, suspension cells and medium indicated the presence of two major compounds which were isolated by preparative TLC, and identified as stigmasterol (co TLC, S_1; MS) and scopoletin (co TLC, S_1; MS). The IC_{50} values of both compounds were determined as 56 µg ml^{-1} based on 3 fold dilutions.

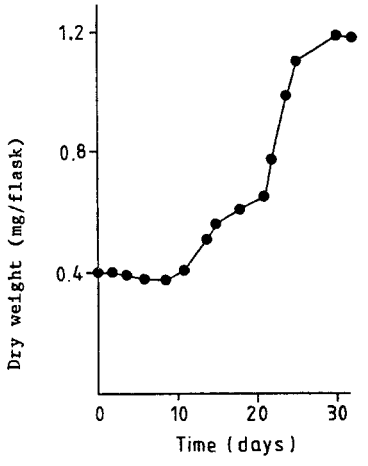


Figure 1 Growth curve of Artemisia annua cells in suspension cultures

Growth cycle

A time-course study of the growth of A. annua cells in suspension cultures has shown that the cells grow well, undergoing a 3-fold increase in total dry weight by the end of the growth period (Fig. 1). 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.

Effect of basal salts, plant growth regulators and absense of light on growth and in vitro antimalarial activity

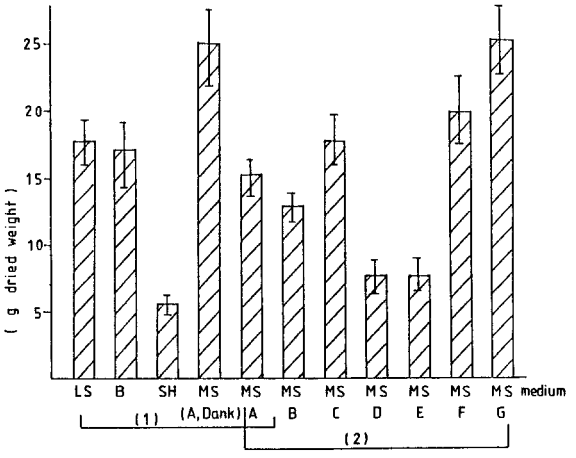
Time-course studies have been carried out to compare growth and possible production of artemisinin, utilising the inhibition of uptake of [^{3}H]-hypoxanthine into P. falciparum, to assess the activity of crude extracts of A. annua.

Callus cultures were produced on four commonly used basal media, MS, LS, SH and B_5. The results show that there was approximately a similar 3-fold increase in the dry weight for MS (A), LS and B_5 media over the initial inoculum (5 gm d/wt) (Fig. 2). Growth on LS and B_5 media was slightly enhanced with a 1.2 and 1.13 fold increase in the dry weights, respectively, compared to the control medium, MS (A). On the other hand, growth on SH medium was relatively poor over a two month period.

Growth of callus grown in the dark on MS medium was enhanced with a 1.7-fold increase in dry weight compared to the control medium MS (A). The change in the basal salts had a significant effect on the antiplasmodial IC_{50} values of prepared extracts (Table 2). The IC_{50} value of the n-hexane extract of the cells grown on B_5 medium was approximately similar to the n-hexane extract of the control medium cells MS (A) (16.5, 18.5 µm l^{-1}, respectively) while the $CHCl_3$ extract was inactive.

However, LS medium resulted in increased activity of both n-hexane and $CHCl_3$ extracts of cells (IC_{50} values of 5 µg ml^{-1} for each extract). In vitro antimalarial activity was inhibited, as was cell growth, by transferring calli to SH medium. Although there was a 1.7-fold increase in the dry weight of the calli grown on MS medium kept in the dark, there was no increase in antiplasmodial activity.

Figure 2. Effect of various basal media, absence of light and auxins on the growth of A. annua callus cultures after a period of 2 months.



(1) see Experimental section and Table 2
(2) see Experimental section and Table 3
MS: Murashige and Skoog basal medium
A-G represent different concentrations of auxins (see Table 3)

Table 2. Influence of basal media and absence of light on inhibition of uptake of [^{3}H]-hypoxanthine into P. falciparum in vitro by extracts of callus cells.

Media	Solvent extracts(1)	In vitro anti P. falciparum activity (IC_{50} value µgml $^{-1}$(2)
LS	n-hexane	5
	$CHCl_3$	5
	H_2O	50
B_5	n-hexane	16.5
	$CHCl_3$	na(3)
SH	n-hexane	na
MS (A) (light)	n-hexane	18.5
	$CHCl_3$	18.5
	MeOH + H_2O	500
MS (A) (dark)	n-hexane	18.5
	$CHCl_3$	18.5

(1) see experimental section
(2) based either on 10 or 3 fold dilutions in duplicate
(3) na: not active
MS (A): Murashige and Skoog control medium (see culture method)

The improvement in the IC_{50} values achieved by changing the basal salts from MS to LS is of particular interest because the formulations are very similar. In LS medium, pyridoxine, nicotinic acid and glycine are absent and the thiamine level is raised 4-fold in comparison to MS medium.

The deleterious effect of SH medium on growth as well as on antimalarial activity cannot easily be attributed to one component or group of components as the formulation differs significantly from that of the other media in macro and micro nutrients, as well as in 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 (Mantell and Smith 1983). The dry weight differences resulting from the change in the nature and concentration of auxins (Fig. 2, Table 3) are not very significant although NAA at a concentration of 5 mg l^{-1} (G) resulted in a 1.7-fold increase in dry weight when compared to MS medium (A), but activity was lost.

The greater activity was achieved from MS basal medium containing 1.0 mg l^{-1} of IAA (C) (IC_{50} value of 6 mg ml^{-1}). This finding is in agreement with the general acknowledgement that IAA increases production of secondary metabolites while NAA suppresses their production. The results presented in Table 3 indicate that antimalarial activity was lost when higher auxin levels were used.

Table 3. Influence of auxins on inhibition of uptake of [^{3}H]-hypoxanthine into *P. falciparum* *in vitro* by extracts of callus cells.

MS basal salt	Auxin (mgl^{-1})	Solvent extract(1)	*In vitro* anti *P. falciparum* activity (IC_{50} value µg ml^{-1})(2)
A	2,4-D (1.0)	n-hexane	18.5
		$CHCl_3$	18.5
		MeOH + H_2O	500
B	IAA (0.1)	n-hexane	50
C	IAA (1.0)	n-hexane	6
D	IAA (3.5)	n-hexane	na(3)
E	IAA (5.0)	n-hexane	na
F	NAA (1.0)	n-hexane	50
G	NAA (5.0)	n-hexane	na

(1) see experimental section

(2) based either on 10 or 3 fold dilution in duplicate

(3) na: not active

In this context, it was encouraging to note that the cultures have been highly active although artemisinin was not detected by less sensitive methods (TLC), but the results of the present study suggest that the technique of [^{3}H]-hypoxanthine incorporation can be used successfully to screen crude extracts of cell time cultures for antimalarial activity in order to monitor their fractionation and to select active principles.

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