

Plasmodium falciparum growth is arrested by monoterpenes from eucalyptus oil

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ABSTRACT: Cerebral malaria is a major health problem in the developing world. Widespread resistance to existing drugs by the parasite *Plasmodium falciparum* has coincided with an increase in mortality, particularly in children. One potential source of new drugs comes from plant natural products. We found that commercially available, pharmaceutical grade eucalyptus oil and its principal component 1,8-cineole inhibited the growth and development of chloroquine-sensitive and chloroquine-resistant *P. falciparum*. This was true both when the oil was added directly to the parasite cultures and when cultures were exposed to the vapours. The development of the parasite was arrested at the early trophozoite stage, irrespective of when the oil was introduced. We used a new approach where the concentration of monoterpenes actually taken up by the cultures was measured directly using HS–GC. We found that the critical concentration required to inhibit and kill the parasite did not adversely affect the host erythrocytes, placing it in the range suitable for drug development. Given the ready availability and existing quality control of eucalyptus oils, this may represent an economically viable adjunct to current antimalarial therapies. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: malaria; plant defence; cineole; ethnobotany; HS–GC; antiplasmodial

Introduction

With the widespread resistance by the malarial parasite *Plasmodium falciparum* to existing drugs, there is an urgent need for new, cheap drugs to replace or supplement existing treatments.^{1,2} One potential source of new drugs comes from plant natural products, many of which have antimicrobial and antiherbivore properties.³ One approach to finding new drugs is to investigate traditional medicines. Essential oils, particularly those rich in terpenes, have been used in traditional treatments for malaria with varying degrees of success.^{4,5} One problem associated with natural plant products is that they are highly variable in concentration and quality, depending on genotype and environmental conditions.^{6,7} Furthermore, economic production can be limited by relatively low endogenous concentrations.⁸

Our research into plant–animal interactions in *Eucalyptus* led us to an analysis of the antimicrobial properties of eucalyptus oil.⁹ Pharmaceutical grade eucalyptus oil is readily available, inexpensive and has a consistent composition and might, therefore, provide a cheap alternative,

or complement to, existing drug treatments. We tested the inhibitory effect by exposing the parasite to eucalyptus oil and its principle component, 1,8-cineole, at different points in the life cycle and for different lengths of time.

Materials and Methods

Eucalyptus Oil and Monoterpenes

Commercially available pharmaceutical grade eucalyptus oil must contain >80% 1,8-cineole and <15% terpene hydrocarbons (International Organization for Standardization; ISO 3065:1974). All trials here used Bosisto's 'Parrot Brand' eucalyptus oil or pure 1,8-cineole (ca. 99%; Felton Grimwade Bickford, Melbourne, Australia). The concentration of 1,8-cineole actually taken up by the cultures was measured using headspace–gas chromatography (HS–GC)¹⁰ and quantified using known standards of pure 1,8-cineole. Aliquots (2 ml of culture) were added to a 20 ml headspace vial, sealed and analysed using a Turbomatrix 40 headspace sampler (Perkin-Elmer, Melbourne, Australia). Vials were heated to 97°C for 15 min, then pressurized (He) to 125 kPa for 3 min before transfer of the sample through a heated (110°C) transfer line to a GC–FID (injector temperature, 150°C; split, 1:10; detector temperature, 200°C). Further GC–FID conditions are described in King *et al.*⁷

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Parasite Culture

Plasmodium falciparum was grown in RPMI–HEPES medium [RPMI1640 (Sigma), 25 mM HEPES, pH 7.4, 0.5% Albumax II (Invitrogen)].¹¹ Eucalyptus oil was serially diluted in growth medium and added directly to mixed-stage cultures of chloroquine-sensitive (D10, 3D7) and -resistant (W2-mef) strains of *P. falciparum*^{12,13} and incubated at 37°C with gentle agitation at 80 r.p.m. for 48 h.¹⁴ Chloroquine diphosphate (Sigma) was used as the positive control. A modified SYBRgreen I fluorescence assay was used to quantify parasite growth for all assays¹⁵ and confirmed using the [³H] hypoxanthine uptake assay.^{16–18} The effect on the host cells was tested by exposing uninfected erythrocytes to eucalyptus oil (IC₉₀) for 48 h, after which they were washed in RPMI–HEPES before introducing *P. falciparum*.

Cultures were also exposed to volatilized oil. *Plasmodium falciparum* (2 ml) was cultured in 12-well flat-bottomed multi-well plates. Aliquots of eucalyptus oil or 1,8-cineole (0–100 µl) were added to a dish with 10 ml water in sealed boxes and incubated as above. All experiments were repeated with 99% pure 1,8-cineole.

Plasmodium falciparum has a 48 h asexual life cycle with distinct life stages: rings (0–24 h), trophozoites (24–36 h) and late trophozoites/schizonts, followed by reinvasion. In order to determine whether the sensitivity of the parasites was associated with a particular stage, cultures were synchronized by treatment with 5% w/v sorbitol (Sigma).¹⁹ Oils were introduced at different points in the life cycle and incubated for 48 h (one life cycle) or 96 h (two life cycles). The water–oil mix was replenished after 48 h in order to sustain the monoterpene concentration in the atmosphere. When exposure was limited to 48 h, the mix was replaced with water. The life stages of live *P. falciparum* were analysed with Giemsa staining every 24 h to assess their development and characterized by the different morphology of the organelles.¹⁷ Nuclei were labelled with Hoechst 33258.¹⁷ A Leica confocal microscope was used to analyse images of live *P. falciparum* D10 parasites containing fluorescent mitochondrial and apicoplast reporter genes.¹⁸

Results

Both eucalyptus oil and its principle component, 1,8-cineole, inhibited the growth and development of chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum*. Based on the concentration of eucalyptus oil added directly to the cultures, the IC₅₀ (i.e. the concentration at which 50% of parasite growth was inhibited) = 0.12 mg/ml and the IC₉₀ = 0.24 mg/ml. Experiments using the monoterpene 1,8-cineole gave similar values.

As a control, erythrocytes that had previously been exposed to eucalyptus oil were infected with the parasite.

There was no visible difference in the ability of the parasite to invade and grow in erythrocytes that had been exposed to the IC₉₀ (0.24 mg/ml) and the control erythrocytes.

Although we knew how much oil was added, it was not clear how much 1,8-cineole was actually taken up into solution. In order to determine this, we took an aliquot of the culture medium and measured the 1,8-cineole concentration directly using HS–GC. Using this method we found that the IC₅₀ was only 0.02 mg/ml, over 80% less than the calculated value.

Eucalyptus oil is highly volatile and simply exposing cultures of *P. falciparum* to the vapours alone also actively inhibited growth. This meant that the volatile oil must have been taken up into the culture solution. The concentration of 1,8-cineole that had passed into the culture medium from the open dishes was also measured directly, using HS–GC. The IC₅₀ was again found to be 0.02 mg/ml.

In order to determine whether the sensitivity of the parasites was associated with a particular stage, cultures were synchronized and oils introduced at different points in the life cycle and for different lengths of time. Confocal and brightfield microscopy confirmed that when the parasites were exposed to a concentration of vapour equivalent to the IC₉₀ at the ring stage (0–24 h), parasite growth was stalled at the early trophozoite stage (24–36 h; Figures 1a, 2). When other life stages of the parasite were exposed to the vapours, parasite development proceeded normally until the early trophozoite stage. No late trophozoites or schizonts (36–48 h) were ever formed or observed (Figures 1b, 2). The parasites were able to recover if the eucalyptus oil was removed after 48 h (Figure 1c) but were unable to resume growth if exposed for more than 96 h (Figure 1b).

Discussion

Three important conclusions can be drawn from the data presented here: (a) eucalyptus oil can inhibit chloroquine-resistant and -sensitive strains of *P. falciparum*; (b) growth is always arrested at the early trophozoite stage; and (c) the IC₅₀ of 1,8-cineole actually taken up into the culture medium is much lower than predicted and therefore is suitable for drug development.

The concentration of active components in *in vitro* studies is typically calculated using the concentration and volume of the aliquot added to the cultures.^{4,5,15,17,20} Using this approach, the IC₅₀ in this study would be considered to be fairly high, at 0.12 mg/ml. However, when we measured the concentration of 1,8-cineole in the culture medium itself, the IC₅₀ was only 0.02 mg/ml. This is important, because it appears that 1,8-cineole can effectively inhibit the parasite at concentrations that are unlikely to be toxic to the erythrocytes themselves.^{5,21}

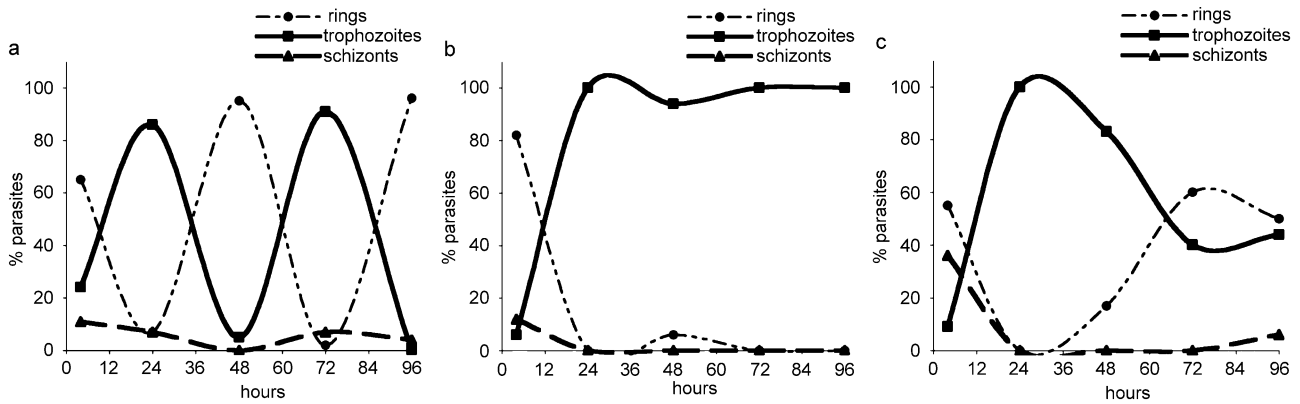


Figure 1. Changes in *P. falciparum* after exposure to eucalyptus oil vapour (IC_{90}). Cultures were synchronized at the ring stage and observed over 96 h (two life cycles). (a) Control, without eucalyptus oil. (b) Culture exposed to eucalyptus oil for 96 h. (c) Culture exposed to eucalyptus oil for 48 h followed by 48 h recovery. Graphs represent the percentage of parasites at a certain life stage as a proportion of the total number of parasites, measured every 24 h

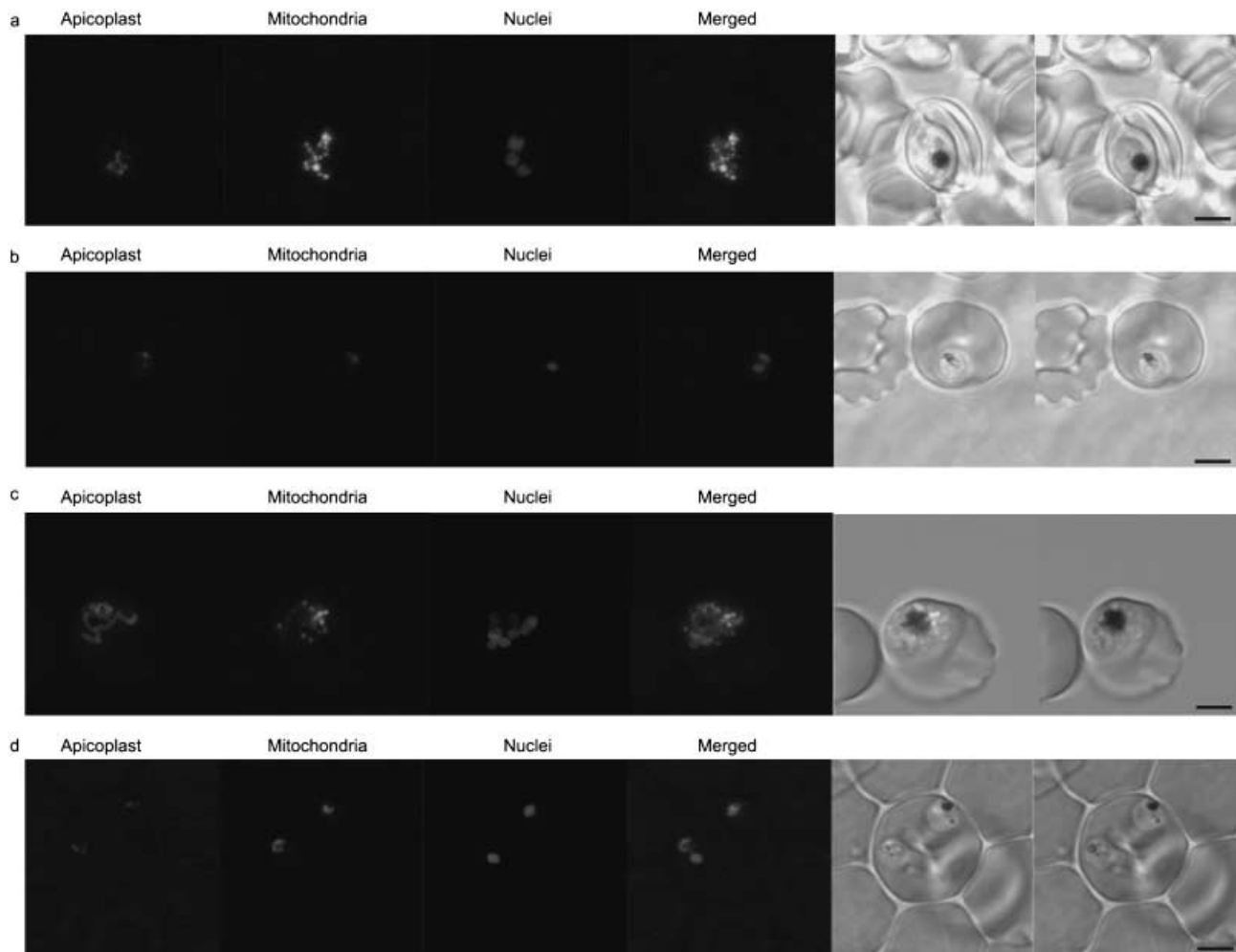


Figure 2. Confocal microscopy images of live *P. falciparum* cells exposed to eucalyptus oil vapour (IC_{90}). Cells were synchronized at the ring stage and followed over 96 h (two life cycles). Cells were CS(l)YFP/ACP(l)DsRed double transfectants, co-labelled with the nuclear dye Hoechst 33 258.¹⁶ Scale bar = 2 μ m. (a) Control culture not exposed to eucalyptus oil, at 30 h: late trophozoite stage. (b) Culture exposed to eucalyptus oil at 30 h: stalled at the early trophozoite stage. (c) Control culture, now at 72 h: mid-schizont stage. (d) Exposed culture, now at 72 h: still stalled at the early trophozoite stage

The *in vitro* toxicity of several monoterpenes has been tested on different human cell lines, with cineole reported to be, overall, the least toxic to erythrocytes.²¹ Boyom *et al.*⁵ reported that terpene-rich essential oils were toxic to erythrocytes at concentrations above 0.6 mg/ml, while Hayes *et al.*²¹ calculated that the IC₅₀ for erythrocytes was in the range 0.14–4.2 mg/ml. Both of these values are much higher than the concentrations of vapour reported to be antiplasmodial in our study.

Other monoterpenes have been shown to affect particular stages of *Plasmodium* species. Limonene, for example, arrests development at the ring stage.²⁰ Nerolidol, by contrast, prevents parasites from developing past the trophozoite stage.⁴ Little is known about how these terpenes act on the parasite but both limonene and nerolidol are thought to affect the isoprenoid pathway;^{20,22} nerolidol appears to inhibit the biosynthesis of the isoprenic chain attached to the benzoquinone ring, while limonene appears to act by inhibiting the isoprenylation of protein. The fact that development is arrested at different stages of the life cycle by different monoterpenes suggests that the underlying mechanisms are likely to be different. The mechanism of action of 1,8-cineole is not known either, but its rapid absorption by cell membranes²³ indicates some kind of membrane disruption, perhaps inhibiting transportation and uptake of compounds essential for parasite growth and development.

Pharmacokinetic studies have shown that 1,8-cineole is rapidly absorbed via inhalation and oral administration.^{16,24} After 60 min of exposure to the vapour, 1,8-cineole has been observed to accumulate in the blood to a concentration of 0.015 mg/ml.²⁴ Moreover, the oral acute toxicity value (LD₅₀) is around 2.5 g/kg, or an equivalent of 25 mg/ml in the bloodstream.²⁵ The active antiplasmodial concentration of 1,8-cineole detected *in vitro* here is well below the LD₅₀, thus the required concentrations could be readily achieved *in vivo* through inhalation alone.

Cerebral malaria is widespread in developing countries with poor infrastructure. Many people infected with the parasite must travel long distances to receive medical treatment. Eucalyptus oil is readily available, cheap and its composition is internationally regulated. The finding that the actual concentration required to inhibit *P. falciparum* is much lower than expected has led us to re-evaluate what was initially considered an unlikely option. The fact that it is also an effective insect repellent is an added bonus.²⁵ It may ultimately prove useful as part of a multi-drug program. In the meantime, it

could be a simple and effective way to inhibit the progression of the disease until other treatments can be accessed.

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