Pharmacological interactions of essential oil constituents on the *in vitro* growth of *Plasmodium falciparum*

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Abstract

The pharmacological properties of essential oils have been widely studied. However the interaction and contribution of the individual constituents assayed singularly and in combination remains relatively unexplored. To investigate these possible interactions, various essential oil constituents (EOC's) were combined and the interactions on their antimalarial and toxicity properties determined using the tritiated hypoxanthine incorporation and the tetrazolium-based cellular viability assays, respectively. In combination, two inactive EOC's (IC50 values greater than 1 mM), *ρ*-cymene and carvacrol interacted in a synergistic manner ($\sum$FIC = 0.02) against a chloroquine-resistant strain of *Plasmodium falciparum*. This interaction was comparable to that between the potent *E*- and *Z*-(±)-nerolidol (IC50 value: 0.9 ± 0.3 μM) and the standard antimalarial, quinine (IC50 value: 0.13 ± 0.04 μM) ($\sum$FIC = 0.01). However, this latter combination also potentiated the toxicity ($\sum$FIC = 0.001) of each molecule. A similar increase in toxicity was noted between *ρ*-cymene and γ-terpinene, as well as *E*- and *Z*-(±)-nerolidol and (−)-pulegone. These results show that the pharmacological activities of individual EOC's can vary greatly when used in combination, and show potential as adjuvants in the elimination of malaria infections.

Keywords: Antagonism; Combination; Essential oil; Malaria; Synergy; Toxicity

1. Introduction

If medical science has to only rely on monotherapy alone to eradicate disease, it would implicate a serious catastrophe to human health as parasitic resistance is increasing at an unprecedented rate (Cassella et al., 2002; Cox et al., 2001). To counteract this increase in resistance, combination therapy has been implemented to ensure successful treatment of malaria infections. In South Africa, the current antimalarials used in combination therapy include lumefantrine with artemether and quinine with either doxycycline or clindamycin (South African Department of Health, 2009). However, another concern is the possibility of increased cytotoxicity following the addition of two agents which may interact with each other. Similarly, the cytotoxicity of an essential oil may be changed by the presence of other compounds, where the compounds may potentiate each other's activity or complex together to enhance overall toxicity or alter their respective bioavailability such that their therapeutic range is exceeded. The interaction between two or more molecules is said to be synergistic if the potency of the combination is higher than the expected activity of the individual molecule. However, if the potency of the combination is lower, then the end result is defined as an antagonistic interaction (Bell, 2005).

It has been claimed that the most dominating constituents in the essential oil are those eliciting the activity attributed to the whole oil (Nakatsu et al., 2000). Despite this fact, constituents in low concentrations are often found to be as effective as the principal constituent (Cassella et al., 2002; Kaur et al., 2009; Nakatsu et al., 2000). Moreover, synergistic and antagonistic effects could also be achieved by using the essential oils in combination, but also by combining the essential oils with other

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standard drugs or solvent extracts. However, there are few published studies investigating such interactions (Cassella et al., 2002; Kamatou et al., 2008a; Su et al., 2008; Van Vuuren and Viljoen, 2009b; Van Vuuren et al., 2009a). Studies on these interactions are vital, since essential oils have great potential as adjuvants in the symptomatic management or treatment of a wide range of health problems (Cowan, 1996; De Smet, 1997). Several studies have shown the antimalarial activity of essential oils isolated from plants such as *Cochlospermum* (Benoit-Vical et al., 1999), *Helichrysum* (Van Vuuren et al., 2006), *Lippia* (Valentin et al., 1995), *Salvia* (Kamatou et al., 2005), *Tetradenia* (Campbell et al., 1997) and *Virola* (Lopes et al., 1999).

Although these studies have determined the composition and biological activity of the essential oil, there has been little or no work done with regards to which of the major and/or minor constituents are responsible for the inhibitory activity of the malaria parasite. Since the essential oil is composed of numerous EOC's, it is likely that an interaction will exist between two or more of the EOC's. To examine this possibility, selected EOC's with varying activity profiles were combined and their antimalarial and toxicity properties recorded.

2. Materials and methods

2.1. Essential oil constituents

Selected essential oil constituents were purchased from Sigma-Aldrich and the purity and percentage composition verified to the supplier's specifications by gas chromatography (minimum purity ≥ 95%).

2.2. Antimalarial activity

A chloroquine-resistant strain of *Plasmodium falciparum* (FCR-3) was continuously maintained in vitro according to the method of Jensen and Trager (1976) and Van Zyl et al. (2006) and synchronised using the 5% (w/v) D-sorbitol methodology as described by Lambros and Vanderberg (1979). To determine the effects of the EOC's on parasite growth, the basic method of tritiated hypoxanthine uptake assay according to Desjardins et al. (1979) was modified and used to accommodate testing of select EOC's in combination. These combinations were repeated in triplicate to confirm consistency. Based on the antimalarial activity of the individual EOC's, eight combinations were selected as seen in Table 1, and one of the most active EOC was combined with quinine. In this assay a parasite suspension consisting predominantly of the ring stage, was adjusted to 0.5% parasitaemia and 1% haematocrit and plated out with various combined ratios and concentrations of the two test compounds for 24 h. Six fixed ratios of the two EOC's or EOC and quinine were prepared such that as the concentration of the EOC increased, the concentration of the other EOC/quinine decreased. The concentrations chosen for the ratios were based on the concentration of the individual EOC or quinine required to inhibit 50% parasite growth (IC50 value). Positive controls containing only one compound were also prepared. From each of the fixed ratios, a further six 1 in 10 dilutions were prepared such as to determine the IC50 value. All assays were carried out using untreated parasites and uninfected red blood cells as controls. After 24 h, labelled ^{3}H-hypoxanthine was added and 24 h thereafter the ^{3}H-DNA harvested on glass fibre filter paper with a Titertek® cell harvester. The IC50 value was determined for each ratio from the log sigmoid dose response curve generated by the Enzfitter® software.

Table 1
The combined interaction between two essential oil constituents or an essential oil constituent and control drug on the in vitro growth of *P. falciparum* and human kidney epithelial cells.

EOC_1	EOC_2/control drug	∑FIC value	Interaction
Antimalarial interactions			
p-Cymene	(−)-Pulegone	11.30	Antagonistic
E- and *Z*-(±)-Nerolidol	(−)-Pulegone	2.03	Antagonistic
Linalyl acetate	(+)-α-Pinene	0.91	Additive
p-Cymene	γ-Terpinene	0.37	Synergistic
(+)-α-Pinene	Carvacrol	0.22	Synergistic
Linalyl acetate	Carvacrol	0.14	Synergistic
E- and *Z*-(±)-Nerolidol	*p*-Cymene	0.09	Synergistic
Carvacrol	*p*-Cymene	0.02	Synergistic
E- and *Z*-(±)-Nerolidol	Quinine	0.01	Synergistic
Toxicity interactions			
p-Cymene	(−)-Pulegone	0.306	Synergistic
E- and *Z*-(±)-Nerolidol	(−)-Pulegone	0.076	Synergistic
p-Cymene	γ-Terpinene	0.002	Synergistic
E- and *Z*-(±)-Nerolidol	Quinine	0.001	Synergistic

2.3. MTT toxicity assay

The transformed human kidney epithelial (Graham) cells were continuously maintained in vitro at 37 °C in 5% CO_2 according to the method of Mosmann (1983) and Van Zyl et al. (2006). The MTT [3-[4,5-dimethyl-2-thiazol-2yl]-2,5-diphenyl-2H-tetrazolium bromide]-microculture cellular viability assay was used to determine the interaction of the EOC's/controls on the growth of human cells. The trypsinized cell suspension was adjusted and 0.5 million cells/mL plated out with the two EOC's/controls as described for the antimalarial combination assay. After 44 h of incubation, 20.1 mM MTT (USB) was added to the plates and incubated for a further 4 h. Dimethyl sulfoxide (DMSO) was added to stop the reaction and dissolve the formazan crystals. The absorbance was read at the test wavelength of 540 nm and reference wavelength of 690 nm and the percentage cellular viability calculated with the appropriate controls taken into account. The concentration that inhibited 50% of cellular growth (IC50 value) was determined for each ratio from the log sigmoid dose response curve generated by the Enzfitter® software.

2.4. Data analysis

The IC50 values of each ratio was determined and the type of interaction determined in two ways, namely an isobologram and determination of the sum fractional inhibitory concentration (ΣFIC) value for all ratios and individual EOC's/controls. The data points for construction of the isobolograms were obtained

for the various ratios by plotting the $IC50_{12}$ in combination/ $IC50_1$ alone (on the X-axis) versus the $IC50_{12}$ in combination/ $IC50_2$ alone (on the Y-axis). Values on the isobologram lying above the line were interpreted as being antagonistic, below as synergistic and those on or near the line as additive. It must be noted that there may be some points on the isobolograms overlying each other and sometimes cannot be clearly seen. Secondly, to verify the interaction read from the isobologram, the ∑FIC of the two selected EOC's (1 and 2) used in the combination was calculated for each combination according the following equation:

$$\sum \mathrm{FIC} = \frac{\mathrm{IC}_{50_{12}} \text{ in combination}}{\mathrm{IC}_{50_1} \text{ alone}} + \frac{\mathrm{IC}_{50_{12}} \text{ in combination}}{\mathrm{IC}_{50_2} \text{ alone}}$$

Values from these ∑FIC calculations were interpreted as follows: <0.5 as synergistic, 0.5–1.0 as additive, >1.0 as indifferent, while that >2.0 as antagonistic (Bell, 2005). Each combination was done in triplicate to confirm consistency.

3. Results and discussion

3.1. Antimalarial activity of the combined essential oil constituents

Essential oils have widely been reported to possess in vitro and in vivo antimalarial activity at relatively low concentrations (Kamatou et al., 2008b; Lopes et al., 1999; Tchoumbougnang et al., 2005; Van Zyl et al., 2006). However, the activity of the individual and combined effect of the EOC's has not been extensively investigated.

When two EOC's were combined and assessed for their antiplasmodial activity the overall interaction between the various combinations were found to be synergistic, although to varying degrees (Table 1; Fig. 1B). The combination between the least active and most active EOC's, namely, *p*-cymene (1528.8± 44.9 μM) and *E*- and *Z*-(±)-nerolidol (0.9±0.3 μM) (Van Zyl et al., 2006) displayed a pronounced synergistic interaction (Fig. 1B). *E*- and *Z*-(±)-Nerolidol with its reported mechanism of action of interfering with isoprenoid biosynthesis (Goulart et al., 2004) potentiated the antimalarial activity of *p*-cymene which had

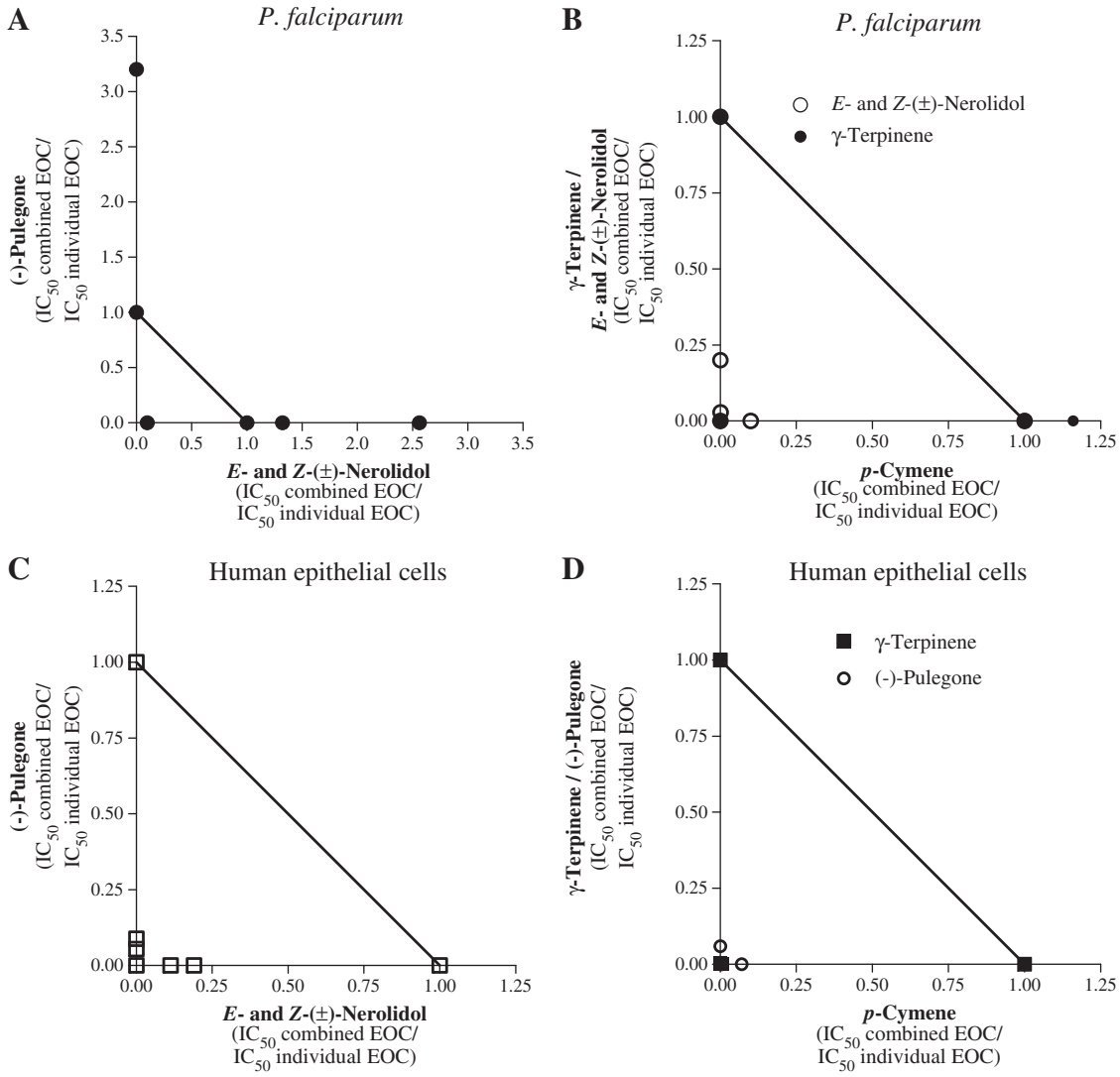

Fig. 1. The combined effect of essential oil constituents on the in vitro growth of malaria (A, B) and human kidney epithelial cells (C, D).

minimal activity when used individually. It is possible that *E*- and *Z*-(±)-nerolidol might assist *p*-cymene to reach the site of action in the parasites, or they may have acted on the complementary targets (Aqil et al., 2007).

This latter synergistic interaction could account for the good antimalarial activity of the volatile oils from *Virola surinamensis*, which predominantly consisted of *p*-cymene (42.0%), with smaller amounts of α-pinene (11.7%), β-pinene (5.2%), nerolidol (3.8%) and γ-terpinene (1.0%) (Lopes et al., 1999). It was reported that 100 μg/ml of this oil and nerolidol inhibited 100% development of the young trophozoites to schizont stage without haemozoin formation over 48 h of treatment. The promising antimalarial activity of this oil could also have been due to the potent synergistic interaction between *p*-cymene with nerolidol and/or γ-terpinene (1006.7±18.7 μM) (Table 1). Whereas nerolidol and α-pinene (1.2±0.2 μM) have shown to possess potent antimalarial activity, *p*-cymene, γ-terpinene and β-pinene (318.5±35.6 μM) probably did not significantly contribute to the overall effect when working individually (Van Zyl et al., 2006). This illustrates the importance of considering the combined effect of EOC's, even though the active constituents only represent 15.5% of the essential oil.

Two antagonistic interactions were observed, namely between (−)-pulegone with *E*- and *Z*-(±)-nerolidol or *p*-cymene (Table 1; Fig. 1A). In contrast, when two active EOC's [linalyl acetate (1.4±0.1 μM) and (+)-α-pinene] were combined, an additive interaction was observed (Table 1). It may be proposed that the antagonistic interactions are due to competition of the two EOC's when partitioning into the red blood cell or parasitic membranes (Turina et al., 2006) or accumulating at the same site and thereby interfering with each other's inhibitory effects. This has been described for mefloquine and chloroquine, where mefloquine inhibits the ability of chloroquine to cause an accumulation of undigested haemoglobin in the parasitic food vacuole (Famin and Ginsburg, 2002). Alternatively, the two EOC's could compete with each other and thereby neutralise their respective parasitocidal effect. The combination of artemisinin with iron chelators has been shown to interact antagonistically; presumably due to the chelators binding iron which is essential for the formation of C-centred radicals that are formed by the interaction of the endoperoxide bridge in artemisinin and iron (Krishna et al., 2006).

Moreover, this study shows that when two EOC's are combined, variable interactions exist depending on the concentration and ratio of the agents used. Boyom et al. (2003) stated that in the case of malaria, the physical properties of essential oil constituents namely its low density (~0.94 g/ml) and lipophilicity which facilitates rapid diffusion across cell membranes, might enhance targeting of the active components into the intracellular malaria parasite. However, the final interaction between the two EOC's depends on their respective cellular targets.

3.2. Cytotoxic profile of the essential oil constituents

The combined effects of various EOC's were assessed for their resultant toxic properties (Table 1; Fig. 1C, D). Essential oils consist of numerous constituents present in variable concentrations. Thus, to examine the interaction between some of these EOC's various combinations were assessed to determine how they may contribute to the overall toxicity profile of the essential oil. All tested EOC combinations potentiated each other's toxicity profile (Table 1). For example, the toxicity IC50 values of the individual EOC's, such as *E*- and *Z*-(±)-nerolidol and *p*-cymene were 5.5±1.2 μM and 673.6±35.4 μM respectively; but when combined the IC50 values decreased dramatically by more than 10 fold. Similarly, for the other combined EOC's, the combined toxic effect was more potent compared to the effects of the individual EOC's (Table 1).

Thus, although the linear alcohol, *E*- and *Z*-(±)-nerolidol and the monocyclic ketone, (−)-pulegone displayed very promising antimalarial activity when used individually (Van Zyl et al., 2006); their antagonistic interaction voided any potential of their combined administration (Table 1; Fig. 1A). These initial results indicate that they are not ideal candidates to be used in combination therapy due to the increased toxicity profile of the two EOC's when used in combinations (Fig. 1C; Table 1).

Thus when comparing the two latter combinations it would appear as if EOC's should not be combined for therapeutic application. However, when used individually they have proven to be effective in increasing the transdermal permeation of drugs such as cardiovascular (propranolol, nicorandil) and cancer drugs (tamoxifen, 5-fluoruracil) (Aqil et al., 2007; Krishnaiah et al., 2005). When tested in animal or human studies, the application of terpenes resulted in variable adverse effects depending on the route of administration. But if applied topically the reaction ranged from little to no skin irritation to eczema and dermatitis depending on the dose and duration of application (Aqil et al., 2007; Bakkali et al., 2008; Pisseri et al., 2008; RIFM, 2008). It has been reported that cytotoxicity is associated more often with phenols, aldehydes and alcohols (Bakkali et al., 2008).

The combination of two monoterpenes, namely γ-terpinene and *p*-cymene (Fig. 1D) also displayed increased toxicity towards the human kidney epithelial cells (∑FIC=0.002). Likewise when the ketone, (−)-pulegone was combined with *p*-cymene (Fig. 1D), the combination had a more toxic profile (∑FIC=0.306) (Table 1). Thus, based on these observations of the in vitro effect of the EOC's on human kidney epithelial cells, the route of administration and the concentration used should be carefully monitored to avoid detrimental and even fatal adverse effects. These cytotoxic effects in the kidney could be due to a membrane or a membrane–protein interaction, since monoterpenes are known to penetrate and partition in the monomolecular layers of dipalmitoyl-phosphatidylcholine in cellular membranes (Lawrence, 2000; Turina et al., 2006).

3.3. The combined antimalarial and cytotoxic effects of quinine and E- and Z-(±)-nerolidol

The combination of a classical antimalarial drug, namely quinine with *E*- and *Z*-(±)-nerolidol displayed a synergistic interaction (∑FIC value=0.01), but had a high toxicity profile (∑FIC=0.001) (Table 1). The ability of these two agents to target separate cellular components may have contributed to their enhanced interaction; with *E*- and *Z*-(±)-nerolidol

proposed to inhibit isoprenoid biosynthesis and to inhibit the isoprenylation of proteins, resulting in the interference with mitochondrial metabolic processes like pyrimidine biosynthesis (De Macedo et al., 2002; Goulart et al., 2004). In contrast, quinine is reported to inhibit haemozoin formation and to decrease plasmodial DNA strand separation and transcription, thereby inhibiting protein synthesis (Warhurst, 2001; Noedl et al., 2003). Thus, if a patient were to be admitted with a malaria infection and had been taking traditional aromatic phytomedicines for his fever and chills, the treatment of the patient would not be compromised if quinine were to be administered. In addition, nerolidol has been reported to increase the transdermal permeability of hydrophilic drugs (e.g. quinine) through biological membranes, providing an alternative route of administration (Aqil et al., 2007).

In the absence of an effective vaccine against malaria, people rely on chemotherapeutic agents to prevent and treat an infection (Craig et al., 2009). These results indicate that plant-derived or chemically synthesised EOC's have the potential to be used in adjunct therapy with antimalarial agents. In addition, they could be used as new templates for drug design.

In many chemotherapeutic regimens, drugs are used in combination to delay or overcome factors of resistance. If EOC's were to be combined with a standard antimalarial agent, such as quinine, the combined antimalarial effect would be potentiated ($\sum$FIC = 0.01). However, so would the likelihood of increased adverse effects ($\sum$FIC = 0.001; Table 1). Thus, the interaction in an in vivo system needs to be evaluated to determine if the adverse effects would outweigh the therapeutic efficacy of this combination.

It is clear that further research needs to be conducted to determine the toxic profiles of the whole essential oils, the individual EOC's and combinations with other essential oils or "western" drugs. In addition, it is known that the essential oil may exert a severe effect on an individual organism or cell type, but maybe inert to other cell types. Thus, it is essential that the in vivo effects be determined, taking into account the metabolism of the individual molecules and the resultant metabolites which could alter the inhibitory properties observed in this study.

From the results obtained, an inference can be drawn that some EOC's are not ideal candidates to form an integral part of the combination therapy, due to their increased toxicity profiles. Although the two more active compounds tested displayed potent antimalarial activity; in combination, *E*- and *Z*-(±)-nerolidol with (−)-pulegone or quinine displayed high toxicity to the test cell line. Although this does indicate some cytotoxicity to mammalian cells, the complete profile and metabolism of these terpenes need to be investigated. It can be concluded that in combination some EOC's potentiate each others' mechanisms of action which may contribute to the management and treatment of microorganisms resistant to standard drugs and warrants further investigation.

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