IN VITRO AND *IN VIVO* ANTI-PLASMODIAL ACTIVITY OF ESSENTIAL OILS, INCLUDING HINOKITIOL

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Abstract. The anti-plasmodial activity of 47 essential oils and 10 of their constituents were screened for *in vitro* activity against *Plasmodium falciparum*. Five of these essential oils (sandalwood, caraway, monarda, nutmeg, and *Thujopsis dolabrata* var. *hondai*) and 2 constituents (thymoquinone and hinokitiol) were found to be active against *P. falciparum in vitro*, with 50% inhibitory concentration (IC_{50}) values equal to or less than 1.0 μg/ml. Furthermore, *in vivo* analysis using a rodent model confirmed the anti-plasmodial potential of subcutaneously administered sandalwood oil, and percutaneously administered hinokitiol and caraway oil against rodent *P. berghei*. Notably, these oils showed no efficacy when administered orally, intraperitoneally or intravenously. Caraway oil and hinokitiol dissolved in carrier oil, applied to the skin of hairless mice caused high levels in the blood, with concentrations exceeding their IC_{50} values.

Keywords: anitplasmodial activity, essential oils, *in vitro*, *in vivo*

INTRODUCTION

Malaria is endemic in many tropical and subtropical countries. Global warming accompanied by increasing global traffic may promote the expansion of malaria to regions with moderate climates, where malaria is not currently present. This disease poses an increasing threat to millions of people because the parasite strains are less susceptible or more resistant to previously effective anti-malarial agents. The increasing prevalence of *Plasmodium falciparum* resistant to standard treatment has initiated numerous studies to search for new anti-malarial agents.

The discovery of artemisinin, a constituent of *Artemisia annua* (Abdin *et al*, 2003) has prompted the evaluation of essential oils as a novel class of anti-malarial candidates. So far, essential oils of *Lippia multiflora* (Valentin *et al*, 1995), *Lavandula angustifolia,* other oils (Milhau *et al*, 1997), *Cochlospermum tinctorium* (Benoit-Vical *et al,* 1999), *Virola surinamensis* (Lopes *et al*, 1999), *Hexalobus crispiflorus* (Boyom *et al,* 2003), *Lippia javanica* (Manenzhe *et al*, 2004), and *Uvariastrum pierreanum* (Boyom *et al,* 2011) have been shown to be effective against *P. falciparum in vitro.* Furthermore, *in vivo* activity of essential oils of *Cymbopogon citratus* and *Ocimum gratissimum* was shown to be effective

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against *Plasmodium* species (Tschoumbougnang *et al*, 2005).

In this study, we evaluated the anti-plasmodial activity of 47 essential oils and 10 constituents against *P. falciparum*.

MATERIALS AND METHODS

Essential oils and chemicals

The majority of essential oils tested were obtained from Sanoflore Laboratoire, France, via Hyperplants (Tokyo) and Pranarom International SA, Belgium, via Kenso-Igakusha (Yamanashi) and in part from Aroma Life, Germany, via Tennokaori (Tokyo). East Indian sandalwood (*Santalum album*) Lot. 22269 10/2003 (Sanoflore), used for *in vivo* experiments, was produced in India and distributed via Hyper Plant. Caraway (*Carum carvi*) oil used for *in vivo* experiments was supplied by Pranarom (P-26) via Kenso Igakusha. Essential oil of Aomori Hiba (*Thujopsis dolabrata* var. *hondae*) and hinokitiol were provided by Kiseitec (Wakayama, Japan). Essential oils of *Houttuynia cordata, Lindera obtusiloba, Lindera umbellata,* and *Parabenzoin praecox* grown in Japan were prepared by steam distillation at our institute. Pure chemical compounds were primarily obtained from Tokyo Kasei Industries (Tokyo, Japan) and in part from Wako Pure Chemical (Osaka, Japan). α-Santalol was a gift from Dr Hadji-Minaglou, Montauroux, France. Artemisinin was obtained from Sigma (St Louis, MO). Vaseline and olive oil were obtained from Wako Pure Chemical. Faneous oil, a carrier oil developed for essential oils was obtained from Kenso-Igakusha.

Essential oils and constituents were dissolved in DMSO at a concentration of 10 % (w/v) and stored in a dark, cold room until *in vitro* evaluation.

Gas chromatographic analysis

The major constituents of the essential oils used were identified by gas chromatography (GC)/mass spectrometry (MS) using a Shimadzu QO-2010 instrument (Shimadzu, Kyoto, Japan). These constituents are listed in Table 1. The quantitative analyses of essential oil composition and blood concentrations were performed by GC using a Model 353B apparatus (GL Sciences, Tokyo, Japan) coupled with a TC-5 column (0.5 mm x 30 m) and hydrogen flame detector. The detailed analytical conditions were reported earlier (Inouye *et al*, 2010).

Parasites and animals

The parasite used for *in vitro* screening was the knob-bearing, wild-type isolate of the strain *P. falciparum* FCR-3/Gambia, which is sensitive to chloroquine and artemisinin, and was maintained at the Department of Parasitology, Teikyo University. Parasites were stored at -80ºC, and passaged once in culture before the *in vitro* assays were conducted, as previously described (Jensen and Trager, 1978). A rodent malaria parasite, *P. berghei* strain NK-5, also maintained at Teikyo University, was used for *in vivo* experiments.

Three-week-old male ICR mice or male ddY mice (Sankyo Lab Service, Shizuoka, Japan) were bred in our laboratory until their body weight exceeded 30 g (>1 month). Four- to 5- week old hairless male mice (Hos-HR-1) were also purchased from Sankyo Lab Service. These mice were housed for 2 weeks at 24.5ºC ± 0.5ºC under conditions of light and dark cycles of 10 hours and 14 hours, respectively, before the *in vivo* experiments were conducted. All mice were fed a standard diet (MF, Orientaru Kobo, Tokyo) and provided water *ad libitum*. The hair of the ICR and ddY mice was removed using scissors and

cream immediately before a test oil was percutaneously administered.

In vitro screening for anti-plasmodial activity

The *in vitro* activity of essential oils against the human malarial parasite *P. falciparum* FCR-3/Gambia, was performed according to the method described in our previous paper (Kamei *et al*, 2000) by using 96 flat-bottom micro-titer plates. The positive control was artemisinin dissolved in DMSO. The oil stock solutions were diluted in 10-fold series in RPMI-1640 medium containing human serum and erythrocytes, such that the maximum final concentration of DMSO was 1.0%. The plates were sealed (Plate Seal, Sumitomo Bakelite, Tokyo, Japan) to prevent vapor contamination to neighboring wells. The IC_{50} values of the essential oils were determined after 72-hour culture by comparison to non-treated negative controls.

Anti-plasmodial activity _in vivo_

In vivo experiments for anti-malarial effect were carried out according to the method used in our previous study (Kamei *et al*, 2000), with the following modifications. Mice were inoculated intraperitoneally (i.p.), using a 26-gauge needle, with red blood cells infected with 100 *P. berghei* in 200 µl blood. Experimental groups consisted of 3~5 mice each. One hour later, an essential oil or a constituent with or without dilution with a vehicle (olive oil, essential oil, Faneous oil, 7% $NaHCO_3$ aqueous solution or DMSO) was applied either i.p, intravenously (i.v.), orally, subcutaneously (s.c.) or percutaneously. Treatment schedules ranged from 2-6 times a day for 3 - 9 days. Control groups received only the vehicle. The maximum dose was determined by physically permissible maximum loading below LD_0, and then diluted two-fold successively.

Besides monitoring for survival of the mice, parasitemia was measured from tail vein bloods, up to 9 days after inoculation. Values presented in the tables are mean ± standard deviation. The positive control, artemisinin was consistently given after infection, in 3 doses at 40 mg/kg i.v., resulting in complete suppression of parasitemia in this mouse model.

Determination of blood concentration of an oil constituent after percutaneous application in hairless mouse

Four-week-old hairless male mice, divided into groups of 3, received essential oil or a constituent applied percutaneously on the dorsal side as follows: 20 µl of caraway essential oil (0.8 ml/kg) or 5 mg hinokitiol dissolved in 50 µl Faneous carrier oil (307 mg/kg). The test solution was evenly spread on the skin of the back of the mice with a glass spreader; the mice were maintained at room temperature. At a predetermined time, 3 mice were immobilized using carbon dioxide, and blood samples (ca 0.5 ml) were obtained 10, 20, 30, and 60 minutes after exposure via cardiac puncture using a syringe containing a minute amount of heparin. The blood samples were extracted with an equal volume of ethyl acetate. The extract was dried over anhydrous sodium sulfate and subjected to GC analysis using a TC-5 column, as described already. Blood concentrations of the constituents were determined from their respective peak area by using a concentration calibration curve of the authentic compound run in series with the sample. The recovery of hinokitiol and (+)-carvone from mouse blood with ethyl acetate was determined to be over 95%.

Statistical analysis

Two-tail unpaired *t*-test was used for comparison between values of test

Table 1
In vitro evaluation of anti-plasmodial activity of essential oils and their constituents against *P. falciparum* FCR-3.

IC_{50} (μg/ml)	Essential oil (major constituent, content)
10,000	Rosewood (linalool, 83%), Houttuynia cordata (2-undecanone, 26%)
1,000	Black pepper (caryophyllene, 29%), coriander (linalool, 71%), Eucalyptus citriodora (citronellal, 73%), Eucalyptus radiata (1,8-cineole, 65%), German chamomile (bisabolol oxide A, 34%), geranium Bourbon (citronellol, 26%), helichrysum (neryl acetate, 52%), lemongrass (citral, 69%), Lindera obtsusiloba (caryophyllene oxide, 28%), Lindera umbellata (linalool, 42%), sweet marjoram (terpinen-4-ol, 25%), Parabenzoin praecox (camphor, 12%), patchouli (patchoulool, 42%), peppermint (menthol, 33%), rosemary camphor (1,8-cineole, 32%), rosemary verbenone (α-pinene, 31%), sage (α-thujone, 26%), speamint [(-)-carvone, 55%], tansy (chrysanthenyl acetate, 69%), vetiver (bicyclovetivenol, 11%), ylangylang (germacrene D, 20%)
100	Bergamot (limonene, 35%), cedarwood Himalaya (β-himachalen, 36%), citronella (geraniol, 18%), clove (eugenol, 86%), galbanum (3-carene, 68%), hyssop (isopinocamphone, 30%), true lavender (linalyl acetate, 37%), myrrh (lindestrene, 38%), ravensara (1,8-cineole, 50%), tea tree (terpinen-4-ol, 43%), thyme thujanol (thujanol, 45%), yuzu (limonene, 93%), ginger (zingiberene, 27%)
10	Frankincense (α-pinene, 31%), oregano (carvacrol, 26%), palmarosa (geraniol, 84%), perilla (perillaldehyde, 66%), thyme geraniol (geraniol, 30%), thyme thymol (thymol, 25%)
1	Aomori Hiba (thujopsene, 64%), caraway [(+)-carvone, 57%], monarda (thymoquinone, 30%), nutmeg (α-pinene, 23%), sandalwood (α-santalol, 50%)
IC_{50} (μg/ml)	Constituent
1,000	Myristicin, (-)-carvone
100	Plaunotol
10	Carvacrol, thymol, geraniol, perillaldehyde, (+)-carvone
1	Hinokitiol, thymoquinone

group and control in parasitemia rate and mean survival days. Sample number of each group was described in the table footnotes. *P*-value <0.05 or <0.01 was considered statistically significant.

RESULTS

In vitro screening for anti-plasmodial activity

Table 1 shows IC_{50} values of 47 essential oils and 10 oil constituents effective against *P. falciparum*. Among the essential oils examined, the most active were monarda, Aomori Hiba, caraway, nutmeg and sandalwood oils. Parasite counts in wells containing 1 μg/ml of these oils increased slightly at 24 hours, but decreased at 48 hours and the parasites were undetectable by 72 hours. Monarda oil was also active at 0.2 μg/ml. With regard to the oil constituents, hinokitiol and thymoquinone, active ingredients of Aomori Hiba and monarda, respectively,

Table 2
Effect of sandalwood oil on the *in vivo* efficacy of *P. berghei*-infected mice by subcutaneous administration.

ICR male	Dose	Vehicle	Parasitemia (%)			Mean survival days
			Day 6	Day 7	Day 8	
Experiment 1[a]						
34.7±2.4g	50 µl	Olive oil 150 µl	43.3±7.5	62.4±15.9	67.2±15.4	9.4±1.5
35.3±0.9g	100 µl	Olive oil 100 µl	33.5±12.7	59.9±10.0	73.0±7.2	10.2±1.5
34.4±1.4g	200 µl	None	10.2±3.2[d]	21.1±11.4[c]	22.7±6.6[d]	19.8±2.7[d]
34.6±2.1g	Control	Olive oil 200 µl	29.4±.8.1	58.1±20.4	56.0±8.4	10.2±2.5
Experiment 2[b]						
36.3±0.8g	200 µl	None	13.4±4.5[d]	20.0±2.5[d]	28.2±5.1[c]	20.0±4.0[d]
36.3±2.0g	Control	Olive	35.2±3.9	67.2±17.7	54.9±12.9	8.4±0.9

[a]Dosing: subcutaneous administration per mouse, once a day for 9 days using 5 mice (n=5). The figure presented is mean± standard deviation.
[b]Dosing: subcutaneous administration per mouse, once a day for 7 days (n=3).
[c] $p<0.05$; [d]$p<0.01$

were most potent with IC_{50} values of 1 µg/ml or less. Thymoquinone was active at 0.2 µg/ml. (+)-Carvone (Fig 1) exhibited an IC_{50} value of 10 µg/ml in contrast to (-)-carvone with an IC_{50} value of 1,000 µg/ml, indicating different bioactivity by stereoisomers. As a positive reference, artemisinin exhibited an IC_{50} value of 0.5 ng/ml under these conditions.

***In vivo* anti-plasmodial activity**

Table 2 shows the results of *in vivo* anti-plasmodial activity of sandalwood oil administered s.c. The effective dose was 200 µl oil (n=5), as indicated by the significant reduction in parasitemia on days 6, 7, and 8, and longer survival times than those of the control mice. However, lower doses of sandalwood oil, *ie*, 50 µl and 100 µl, did not suppress the growth of parasites, or extend survival.

The same results were obtained in a second experiment, confirming the anti-plasmodial effect of sandalwood *in vivo*. Sandalwood administered s.c. at 200 µl/mouse, again significantly reduced parasitemia on days 6, 7, and 8. The mean survival times of 20.0 ± 4.0 days was comparable to that of the first experiment (19.8 ± 2.7 days), and significantly longer than that of the control group (8.4 ± 0.9 days).

Table 3 shows the *in vivo* effect of α-santalol (Fig 1), a major component of sandalwood and hinokitiol (Fig1), an active component of Aomori Hiba. a-Santalol administered s.c. at a dose of 100 µl/mouse thrice daily for 3 days, showed positive effect against rodent parasite growth, and the efficacy of this regimen was comparable to that of sandalwood oil at 200 µl/mouse. Intravenous or subcutaneous administration of hinokitiol dissolved in 7% sodium bicarbonate aqueous solution showed no significant protective effect.

Furthermore, i.p. and orally administered hinokitiol were ineffective (Table 4).

Table 3
Effect of α-santalol by subcutaneous dosing and hinokitiol by intravenous and subcutaneous dosing against parasite-infected mice.

ddY male	Dose	Vehicle	Parasitemia (%) Day 5	Day6	Day7	Mean survival days
31.3±1.2g	α-santalol 100 µl[a]	None	8.5±3.2	17.1±4.8	29.4±1.7[d]	20.3±4.1[f]
31.4±2.2g	Hinokitiol 2 mg	H_2O 100 µl[b]	10.3±1.3	31.7±4.3	48.6±4.5	11.3±1.5
30.7±0.3g	Hinokitiol 2 mg	H_2O 100 µl[c]	8.3±1.3	19.7±10.3	36.2±11.4	17.0±7.0
32.3±1.5g	Control	-	18.0±10.4	36.6±11.7	48.5±5.9	10.0±1.0

[a]Subcutaneous injection, thrice daily for 3 days (n=3).
[b]Intravenous injection after dissolving in 7% $NaHCO_3$ solution, thrice daily for 3 days (n=3).
[c]Subcutaneous injection after dissolving in 7% $NaHCO_3$ solution, thrice daily for 3 days (n=3).
All figures are presented as means ± standard deviation.
[d] $p<0.05$; [f] $p<0.01$

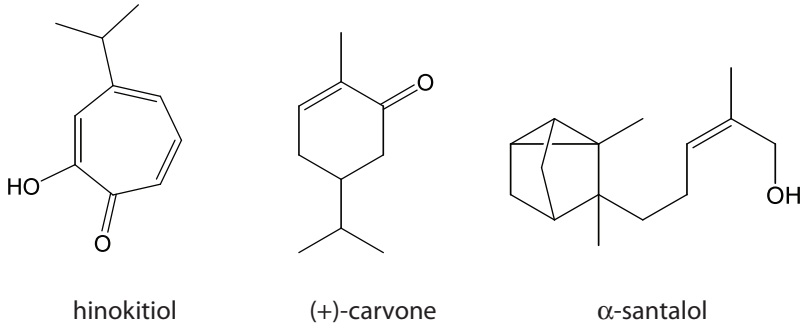

Fig 1–Structures of hinokitiol, (+)-carvone and α-santalol.

However, percutaneous administration of 10 mg/mouse of hinokitiol dissolved in DMSO showed a positive effect, as indicated by a significantly longer survival of the treated mice than that of the control mice. Unfortunately, repeated applications of DMSO damaged the surface of the skin. Therefore, vehicles other than DMSO were sought.

In the second experiment (Table 4), we examined the efficacy of percutaneously administered hinokitiol prepared in either vaseline, joboba oil, or essential oil of Aomori Hiba. The anti-plasmodial activity of hinokitiol *in vivo* depended on the vehicles used. Parasitemia on days 4 and 9 was significantly suppressed by all 3 oil preparations. However, evaluation of mean survival indicated only hinokitiol dissolved in essential oil of Aomori Hiba significantly prolonged survival of the treated mice compared to control mice.

When Faneous oil was used as a vehicle, an even more potent *in vivo* anti-plasmodial effect was observed (Experiment 3, Table 4). In this experiment, hairless mice were used instead of ICR mice, because of the ease of topical application. In the group treated with 10% hinokitiol in Faneous oil at a dose of 5 mg/mouse (385 mg/kg),

Table 4
In vivo effect of hinokitiol against *P. berghei*-infected mice.

ICR male mouse	Dose	Vehicle	Route[a]	Parasitemia (%) Day 4	Parasitemia (%) Day 5	Mean survival days
Experiment 1. Effect of administration route						
35.2±1.7g	1 mg	Olive oil 100 µl	Intraperitoneal	-	-	1.7±2.4
32.9±2.5g	1 mg	Olive oil 100 µl	Oral	1.7±0.3	10.0±1.7	10.3±7.8
36.0±1.9g	10 mg	DMSO 100 µl	Percutaneous	0.6±0.4	3.3±3.2	18.5±3.0[d]
33.2±1.0g	Control	-	-	2.3±1.8	8.1±4.4	6.5±1.7

[a]Dosing: thrice daily for 3 days (intraperitoneally) or 8 days (orally, percutaneously) (n=3).

ICR male mice	Dose	Vehicle	Parasitemia (%) Day 4	Parasitemia (%) Day 9	Mean survival days
Experiment 2. Effect of vehicle by percutaneous administration[a]					
34.4±0.9g	20 mg	Vaseline 200 µl	0.8±0.3[c]	22.8±12.6[c]	17.0±4.6
32.9±2.5g	20 mg	Joboba oil 200 µl	1.0±0.6[c]	22.9±2.9[d]	19.3±5.5
34.7±3.0g	4 mg	Essential oil[b] 200 µl	0.8±0.4[c]	28.9±9.3[c]	25.0±3.6[d]
37.0±2.1g	Control	-	2.8±1.2	48.4±1.3	11.2±4.3

[a]Dosing: twice daily for 8 days (n=3).
[b]Aomori Hiba (*Thujopsis dolabrata* var. *hondae*) oil which contained 2.7% hinokitiol.

Hairless male mice	Dose	Vehicle	Parasitemia (%) Day 9	Mean survival days
Experiment 3. Effect of vehicle by percutaneous administration[b]				
17.2±0.4g	5 mg	Faneous oil 50 µl	5.6±8.9[d]	>35.8±22.3[a,c]
17.6±0.7g	Control	Faneous oil 50 µl	28.7±3.4	10.3±4.6

[a]Dosing: thrice daily for 3 days (n=5).
[b]Two mice were free of parasites in blood.
Figures are presented as means ± standard deviation.
[c]p<0.05; [d]p<0.01

parasitemia was 5.6% on day 9, which was significantly lower than that in the control group (28.7%). The mean survival for this group was >35.8 ± 22.3 days; 2 mice in this group were alive for more than 2 months with no evidence of parasitic infection. This was in sharp contrast to the control group with a mean survival of only 10.3 ± 4.6 days.

Table 5 shows the anti-plasmodial efficacy of caraway in the parasite-infected hairless mice. When 50 µl of caraway oil without vehicle was administered percutaneously on the hairless mice thrice daily for 3 days, a significant increase in mean survival was observed, compared to control mice. However, parasitemia was not significantly lower by day 8.

Table 5
Effect of caraway oil against *P. berghei*-infected mice by percutaneous administration.

Hairless male mice	Dose	Vehicle	Dosing	Parasitemia (%) Day 8	Mean survival days
21.1±1.5g	50 µl	None	Thrice daily for 3 days[a]	10.3±6.8	19.3±4.5[b]
21.2±2.3g	Control	-	-	18.5±2.7	9.0±1.0

[a] n=3. Values are presented as means ± standard deviation.
[b] p<0.05

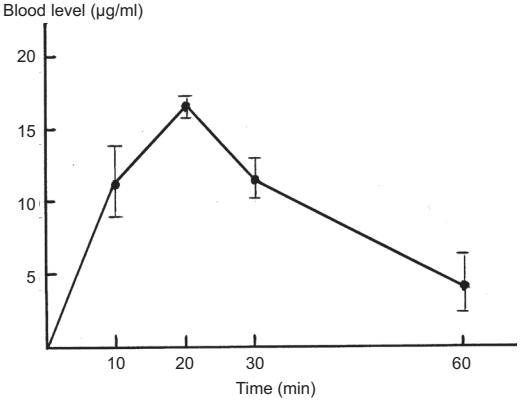

Fig 2–Blood concentration of hinokitiol at a dose of 5 mg/mouse (n=3) over time.

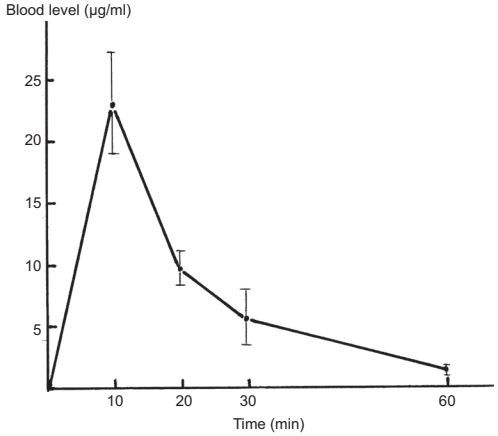

Fig 3–Blood concentration of (+)-carvone at a dose of 20 µl/mouse of caraway oil (n=3) over time.

Time course of blood concentration of hinokitiol and (+)-carvone in hairless mice after percutaneous administration

Fig 2 shows the concentration of hinokitiol in the blood over time. Hinokitiol dissolved in Faneous oil (50 µl) was applied percutaneously at a dose of 5 mg/mouse (307 mg/kg). Hinokitiol was detected in circulation 10 minutes after administration, showing a peak around 20 minutes (16.4 ± 0.1 µg/ml); hinokitiol concentration gradually decreased until 60 minutes, with a half-life of 43 minutes. The blood level at 60 minutes (4.3 ±2.5 µg/ml) still exceeded the IC_{50} value (1 µg/ml) of hinokitiol.

Fig 3 shows the concentration of (+)-carvone, a major constituent of caraway oil, in the blood over time. Mice were percutaneously administered 20 µl caraway (0.8 ml/kg) dissolved in 50 ml of Faneous oil. Carvone levels in blood reached a maximum (23.2 ± 4.3 µg/ml) 10 minutes after topical application, and rapidly decreased until 60 minutes, with a half-life of 15 minutes. At 20 minutes after treatment, the circulating level of carvone was 9.8 ± 1.4 µg/ml, which is equivalent to its IC_{50} value determined *in vitro*. When the dose was increased to 100 µl without a vehicle, the level of carvone detected in the blood

was increased in a dose-dependent manner, and was found to be 109 μg/ml at 10 minutes (data not shown).

DISCUSSION

Thus far, few studies have investigated the potential anti-malarial activity of essential oils, as compared to the antimicrobial activity of these oils. The IC_{50} values reported in the literatures were 50~100 μg/ml for *Lippia multiflora* oil (Valentin *et al,* 1995), 100 μg/ml for nerolidol of *Virola surinamensis* (Lopes *et al,* 1999), 22~35 μg/ml for *Cochlospermum planchonii* oil (Benoit-Vical *et al,* 1999), 6.08 μg/ml for *Uvariastrum pierreanum* oil (Boyom *et al,* 2011) and 2 μg/ml for *Hexalobus crispiflorus* oil (Milhau *et al*, 1997). In addition to essential oils, potent *in vitro* activity of several diterpens has been reported. Abietane and totarane-like diterpenes from *Harpagophytum procumbens* exhibited an IC_{50} value <1 μg/ml (Clarkson *et al,* 2003). The IC_{50} value of abietane-like diterpene from *Hyptis suaveolens* was 0.1 μg/ml (Chukwujekwu *et al,* 2005).

Ours is the first study screening a large number of essential oils for their anti-malarial activity. Five essential oils and 2 oil constituents exhibited IC_{50} values ≤1 μg/ml, which indicates the activities of these oils and constituents are comparable to or more potent than those of oils and constituents previously identified.

Further, the oil constituents, such as hinokitiol, thymoquinone, carvacrol, thymol, geraniol, and perillaldehyde, with potent anti-plasmodial activity also had potent anti-*Trichophyton* activity (Inouye *et al*, 2006). A close correlation between anti-plasmodial and antifungal activities was reported for quinone antibiotics (Tanaka *et al*, 1999) and antifungal azoles (Nuy *et al*, 2002).

Although sandalwood oil and its constituent, α-santalol, showed comparable efficacy *in vivo*, this finding does not confirm α-santalol itself is an active ingredient *in vivo*, because sandalwood oil is metabolically unstable in mouse blood and results in the generation of a variety of metabolites (data not shown). Furthermore, the *in vivo* activity of sandalwood oil varied according to the oil supplier, and sandalwood oil purchased from another company was ineffective (data not shown). The exact reason for this inconsistency remains unclear.

Hinokitiol showed not only potent anti-malarial activity *in vitro* but also metabolic stability *in vivo*, unlike other constituents including thymoquinone. The high blood concentration and relatively long half-life of hinokitiol after percutaneous administration possibly supports its *in vivo* efficacy, because the anti-plasmodial activity of hinokitiol was time-dependent, and complete suppression of parasites was achieved after 72 hours exposure *in vitro*. The *in vivo* activity of percutaneously administered caraway was also in agreement with the high blood concentration of (+)-carvone, which was metabolically stable.

Currently, anti-malarial agents are clinically used orally or i.v. Percutaneous administration may be advantageous, because it could help in avoiding gastrointestinal disorders, frequently caused by oral and i.v. administration of anti-malarial drugs. Additionally, this formulation can avoid the first-pass effect of the liver which may metabolize a drug before the drug reaches systemic circulation. A patient could easily apply the treatment at home by topical application. Thus, the results obtained in our study may pave a way to topical application of new anti-malarial drugs.

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