Trends Phytochem. Res. 7(4) 2023 279-288

Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

Original Research Article

Anti-plasmodial diterpenes from the roots of *Hypoestes forsskaolii* (Vahl) R.Br. (Acanthaceae)

Fred Wanyonyi Sawenja[1,2], Ruth Anyango Omole[1,2], Peter Gakio Kirira[3], Hamisi Masanja Malebo[1,4], Alex Kingóri Machocho[1], Richard Maveke Musau[1] and Isaiah Omolo Ndiege[1]*

[1]Department of Chemistry, Kenyatta University, P.O. Box 43844, Nairobi 00100, Kenya
[2]School of Chemistry and Material Science, Technical University of Kenya, P.O. Box 52428, Nairobi 00200, Kenya
[3]Centre for Traditional Medicine & Drug Research, Kenya Medical Research Institute (KEMRI), P.O. Box 54840 Nairobi 00200, Kenya
[4]Department of Traditional Medicine Research, National Institute for Medical Research (NIMR), P.O. Box 9653, Dar es Salaam, Tanzania

ABSTRACT

Ethnopharmacological surveys previously revealed that *Hypoestes forsskaolii* (Vahl) R.Br. (Acanthaceae), a local medicinal plant, is one of the most widely used herbs in traditional malaria therapy by the Marakwet and Kisii communities in Kenya. This study aimed to validate the indigenous medicinal knowledge claims regarding the efficacy and safety of *H. forsskaolii* (Vahl) R.Br. as a traditional anti-malarial drug. It involved the sequential solvent extraction of plant material, bioassay-guided separation and isolation of components, and identification of anti-plasmodial principles of *H. forsskaolii* (Vahl) R.Br. and determination of their cytotoxicity and selectivity. From the roots of *H. forsskaolii* (Vahl) R.Br., 3,4a,7,7,10a-pentamethyl-1,3-vinyl-dodecahydro-benzo[*f*]chromene (**1**) and 3,4a,7,7,10a-pentamethyl-3-vinyl-dodecahydrobenzo[*f*]chromen-8-ol (**2**) with moderate and mild anti-plasmodial activity at IC_{50} 7.81 µM and 15.0 µM, respectively, were isolated plus the inactive kaur-16-en-19-oic acid (IC_{50} 65.40 µM) against *Plasmodium falciparum* D6 (CQ-sensitive) strain.

ARTICLE HISTORY

Received: 29 May 2023
Revised: 23 October 2023
Accepted: 29 October 2023
ePublished: 12 November 2023

KEYWORDS

Acanthaceae
Anti-plasmodial
Diterpenes
Hypoestes forsskaolii (Vahl) R.Br.
Malaria
*Plasmodium falciparum D6 (*CQ-sensitive)
Roots

1. Introduction

Medicinal plants have been vital to human health in various parts of the world and are of great importance in numerous scientific fields. A literature survey demonstrates that a large number of medicinal natural products, such as alkaloids, flavonoids, and coumarins, among others, have been characterized in herbal medicines (Nahar et al., 2021; Mohammadhosseini et al., 2022).

Local indigenous communities in Kenya have been utilizing plant-based herbal formulations to treat malaria, a life-threatening disease predominantly found in tropical countries. However, they have been doing so without any knowledge regarding the safety, efficacy, and bioactive components of these formulations (Okaiyeto and Oguntibeju, 2021). Previous ethno-pharmacological surveys revealed that *H. forsskaolii* (Vahl) R.Br. (Acanthaceae), a local medicinal plant, is one of the most widely used herbs in traditional malaria therapy by the Marakwet and Kisii communities in Kenya (Muregi et al., 2003; Kipkore et al., 2014). *H. forsskaolii (Vahl) R.Br.* is an perennial herb that can grow up to 1m tall. The stem and leaves of the plant are almost glabrous, and it produces pale pink or white flowers (Iain, 2015). Earlier studies demonstrated that the plant exhibits *in vitro* anti-plasmodial activity (Muregi et al., 2003). Due to the development of resistance by malaria parasites to the currently available anti-malarial drugs, the associated toxicity, and the widespread reports of vector resistance to insecticides, there is a pressing need for new and more effective yet less toxic alternatives (Kunasol et al., 2017; Deletre et al., 2019; Nsanzabana, 2019; Richards et al., 2020). The plant kingdom is an obvious source of anti-malarial drugs and/or templates due to the previously documented successes (Mojab, 2012; Atanasov et al., 2015; Harvey et al., 2015).

We conducted chemo-pharmacological investigations

✉ Corresponding authors: Fred Wanyonyi Sawenja and Isaiah Omolo Ndiege
Tel: ; Fax:
E-mail address: wanyonyi.sf@gmail.com; indiege@yahoo.com, **doi: 10.71596/tpr.2024.3051345**

to verify the validity of the traditional medicinal knowledge claims regarding the effectiveness of *H. forsskaolii* (Vahl) R.Br. through extraction, bioassay-guided isolation, and identification of anti-plasmodial compounds. Therefore, we hereby present the isolation of three compounds and their corresponding anti-plasmodial activity.

2. Experimental

2.1. Instruments, reagents and chemicals

Analytical grade or double distilled solvents (such as *n*-hexane, DCM, ethyl acetate, and methanol) were used for extraction, chromatography, and recrystallization. Column chromatography was performed using 200 g of silica gel (60-200 mesh), while gel filtration chromatography utilized sephadex LH-20. Analytical thin-layer chromatography (TLC) was performed using aluminum plates pre-coated with silica gel G/UV254. While performing preparative thin-layer chromatography (PTLC), glass plates (20 x 20 cm) coated with silica gel were used. The compounds isolated by PTLC were subjected to recrystallization. Melting points (mp) were determined using the Gallenkamp apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Hyper Infra Red spectrometer using potassium bromide pellets or chloroform as solvents. The maximum absorption frequencies (νmax) are reported in wave numbers (cm^{-1}). ^{1}H NMR spectral data were recorded in deuterated chloroform ($CDCl_3$) at 292.9 K using Bruker DRX-500 or Bruker DRX-400 Nuclear Magnetic Resonance spectrometers with gradient units. The resonance frequency was 500 or 400 MHz, respectively, and the results are reported in parts per million (ppm) or (δ). Tetramethylsilane (TMS) was used as the internal standard to calibrate the chemical shifts. ^{13}C NMR spectral data were recorded in (CD_3OD) at 292.9 K on Bruker DRX-500 or Bruker DRX-400 Nuclear Magnetic Resonance spectrometers with gradient units at a resonance frequency of 125 or 100 MHz, respectively. The data is reported in ppm (δ). EIMS data was obtained from the VG-12-250 Mass Spectrometer, and the *m/z* values are reported as atomic mass units (a.m.u.) with corresponding percentage intensities in parentheses. The *in vitro* anti-plasmodial tests were carried out using tritium radiolabeled hypoxanthine, [3H[-hypoxanthine]] from PerkinElmer (Lot. 1804926).

2.2. Plant material and extraction

The plant material was collected from Wire Hill, off Kisumu-Oyugis Road, near Ober Market in Rachuonyo District, Homa Bay County, Kenya, in August 2004. The plant specimen was authenticated by Mr. Simon Mathenge of the Botany Department Herbarium at the University of Nairobi, where the voucher specimen was deposited under Ref. No. SWF/01/2005. The plant material was air-dried in the shade for 7 days, pulverized using a laboratory mill, and then soaked in cold solvents of increasing polarity with occasional swirling. Briefly, 0.32 kg of the plant chaff was sequentially soaked in 3 × 250 mL of *n*-hexane (*n*-C_6H_{14}), 3 × 250 mL of dichloromethane (DCM), 3 × 250 mL of ethyl acetate (EtOAc), and 3 × 250 mL of methanol (MeOH) for 48 h each. The three extracts from the same solvent were combined, concentrated *in vacuo* at a rotary evaporator bath temperature of 30-50 °C. They were then weighed, appropriately labeled, and stored in a refrigerator at 4 °C until they were needed for chromatography. The plant material yielded 5.8 g of *n*-hexane (1.8%), 5.0 g of DCM (1.6%), 4.0 g of EtOAc (4.0%), and 11.2 g of MeOH (3.5%) extract by weight.

2.3. Isolation and purification

The DCM extract of *H. forsskaolii* (Vahl) R.Br. roots was subjected to column chromatography on silica gel, followed by gel filtration chromatography on sephadex LH-20, and PTLC to afford three pure compounds. Briefly, 12.0 g of the DCM extract was subjected to vacuum chromatography on silica gel (60-200 mesh, 120 g) using a mixture of *n*-C_6H_{14}, DCM and EtOAc with with a gradual increase in polarity from 100:0:0 to 0:100:0 and finally to 0:0:100. The 54 x 15 mL fractions were subjected to analytical TLC and combined into 10 mega-fractions (F_1-F_{10}) based on the R_f values of the components. Five of the mega-fractions (F_1, F_3, F_5, F_6, and F_{10}), which were available in sufficient amounts (325 mg) with a yield of 0.102%, were subjected to further purification using PTLC, resulting in six portions (CN 01-06). F_1 gave CN 01 (24 mg) after elution with DCM-EtOAc (30:70; v/v). F_6 yielded CN 02 (19 mg) after elution with DCM-EtOAc (50:50; v/v). F_{10} yielded CN 03 (21 mg) after elution with *n*-hexane-DCM-EtOAc (1:2:1; v/v/v), and CN 04 (26 mg) after elution with DCM-EtOAc (60:40; v/v). F3 yielded CN 05 (14 mg) after elution with DCM-EtOAc (80:20; v/v), and CN 06 (10 mg) after elution with DCM-EtOAc (9:1; v/v). These compounds were then subjected to TLC analysis. The relatively pure fraction, CN 04 (26 mg), was further fractionated by gel filtration chromatography on sephadex LH-20 (260 g), eluting with a mixture of DCM-MeOH (1:1), to give 36 x 10 mL sub-fractions (f_1-f_{36}). These sub-fractions were combined based on their Rf values in analytical TLC profiles and purified further by PTLC using 2% EtOAc in DCM as the mobile phase. This process yielded compounds **1** (1.2 mg), **2** (5 mg), and **3** (3.5 mg).

2.4. Spectroscopic data of isolated compounds

Compounds **1** was isolated as white crystalline solid: m.p. 119-121 °C (lit. 120 °C); IR (ν_{max}) 3080, 2971, 2869 1636, 1553, 1541, 1508, 1458, 1416, 1387, 1375, 1364, 1125, 1096, 1076, 1062 1011, 961, 943 925, and 841 cm^{-1}; EIMS *m/z* 290 $[M]^+$, ^{1}H NMR (Table 1); ^{13}C NMR (Table 1).

Compound **2** was isolated as white crystalline solid; m.p. of 123-124 °C (lit. 124-126 °C) (Konishi et al., 1996); IR (ν_{max}); 3424, 3083, 1648, 1558, 1541, 1508, 1456, 1410, 1387, 985, 960, 943, 910, 839, and 733 cm^{-1}; EIMS *m/z* 306 $[M]^+$, ^{1}H NMR (Table 2); ^{13}C NMR (Table 2).

Compound **3** was also isolated as white crystalline solid: m.p.170-172 °C (lit.178-180 °C); IR (ν_{max}); 2500-3500, 3300, 3100, 2924, 2853, 1734 (CO_2), 1619, 1581, 1339, 1140, 835, 763, 740, and 721 cm^{-1}; ^{1}H NMR (Table 3); ^{13}C

Table 1
NMR (500 MHz, $CDCl_3$) data of compound **1**.

Position	Compound 1			
No.	**δ_C**	**δ_H, m, J in Hz**	**HMBC (H to C)**	**^{1}H-^{1}H COSY**
1	39.4	0.82-0.88 (m)	5, 8, 10	2
		1.55-1.65 (m)		
2	18.7	1.39-1.51 (m);	4, 10	1, 3
		1.55-1.65 (m)		
3	42.2	1.14-1.21 (m)	1, 5, 18,19	2
		1.34-1.37 (m)		
4	33.4			
5	56.5	0.92 (dd, 12.2, 2.4)	7, 18, 19	
6	19.9	1.23-1.29 (m)	4, 8, 10	7
		1.55-1.65 (m)		
7	43.2	1.39-1.51 (m)	5, 9	6
		1.75 (dt, 12.0, 2.2)		
8	76			
9	58.5	1.17-1.21 (m)	1, 5, 7, 12	
10	36.9			
11	15.9	1.39-1.51 (m)	8, 10,13	12
12	34.9	1.39-1.51 (m)	14, 13	11
		2.18-2.20 (m)		
13	73.3			
14	147.7	6.00 (dd, 18.0, 11.0)	16, 12	15
15	109.4	4.95 (dd, 18.0, 1.2)	13, 14	14
		4.89 (dd, 11.0, 1.2)		
16	32.8	1.12 (s)	14, 13, 12	
17	24	1.21 (s)	8, 9, 7	
18	21.3	0.77 (s)	5, 3	
19	33.3	0.84 (s)	5, 3	
20	15.9	0.71 (s)	1, 5, 9	

NMR (Table 3).

2.5. Structural elucidation of isolated compounds

The chemical structures of the three diterpenes were established based on IR; ^{1}H and ^{13}C NMR, spectroscopic data; 2D-NMR correlation experiments including COSY, HMQC, HMBC; and mass spectroscopy (MS) data. ^{1}H and ^{13}C NMR data were assigned based on literature chemical shifts for hydrogen and carbon atoms. The assignments were further confirmed using correlation spectroscopy (COSY), homonuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC) experimental data.

2.6. *In vitro* anti-plasmodial assay

The *in vitro* anti-plasmodial tests were conducted to determine the ability of the isolated compound/ samples to inhibit the uptake of tritium radiolabeled hypoxanthine, [3H[-hypoxanthine]] by *P. falciparum* D6 strain. The tests were performed in RPMI 1640 medium

Table 2
NMR (500 MHz, $CDCl_3$) spectral data of compound **2**.

Position No.	Compound 2			
	δ_C	δ_H, m, *J* in Hz	¶HMBC (H to C)	^1H-^1H COSY
1	37.7	1.00-1.03 (m)	3, 5, 9	2
		1.61-1.67 (m)		
2	27.4	1.55-1.58 (m)	4, 10	1, 3
		1.61-1.67 (m)		
3	78.9	3.21 (dd, ,11.5, 4.5)	1, 5, 18, 19	2
4	38.9			
5	55.3	0.91 (dd, ,11.7, 2.3)	7, 18, 19	
6	19.6	1.33-1.37 (m)	4, 8, 10	7
		1.61-1.67 (m)		
7	43.1	1.33-1.37 (m	5, 9	6
		1.77 (dd, 11.7, 3.0)		
8	75.8			
9	58.3	1.15 (dd, 10.1, 3.8)	1, 5, 7, 12	
10	36.7			
11	16.1	1.41-1.48 (m)	8, 10, 13	12
12	34.9	1.41-1.48 (m)	13, 14	11
		2.20 (ddd, 7.0,7.0,3.0)		
13	73.4			
14	147.5	5.99 (dd, 18.0, 11.0)	12, 16	15
15	109.6	4.96 (dd, 18.0, 1.2)	13, 14	14
		4.90 (dd, 11.0, 1.2)		
16	32.8	1.12 (s)	12, 13, 14	
17	23.9	1.21 (s)	7, 8, 9	
18	28.1	0.96 (s)	3, 5	
19	15.3	0.74 (s)	3, 5	
20	16	0.72 (s)	1, 5, 9	

supplemented with human serum, using O + RBC as the host cells, at various concentrations, as previously described (Desjardins et al., 1979). Briefly, parasite cultivation was conducted aseptically in a laminar flow hood at the Malaria Research Laboratory in the Centre for Biotechnology Research and Development (CBRD) at KEMRI, Nairobi. *P. falciparum* D6 strain was obtained from the cultures preserved in the Malaria Parasite Bank at CBRD in KEMRI, Nairobi.

Samples of the compounds isolated from *H. forsskaolii* (Vahl) R.Br. The solutions were prepared by dissolving 0.45 mg of the sample in a final volume of 20 mL, resulting in a stock solution concentration of 22.50 µg/mL. Since the final volume in each well was 225 µL, this stock solution was intended to provide a concentration of 250 µg/mL for the first row.

Human serum was prepared from aseptically collected blood from donors. The blood was collected into anticoagulant-free blood bags and allowed to clot at room temperature for 90 minutes. After clotting, the blood bags were stored at 4 °C overnight. The following day, initially 2 mL of the serum were carefully dispensed into sterile 50 mL centrifuge tubes. The tubes were then centrifuged at 3000 rpm for 10 minutes at 4 °C. After centrifugation, the serum was transferred (aliquoted) aseptically into sterile 10 mL snap-top tubes. The aliquoted serum was then inactivated in a water bath at 56 °C for 50 minutes. The tubes were placed in an upright position at -20 °C overnight and then stored at -70 °C until they were used in the *in vitro* antiplasmodial

Table 3
NMR (400 MHz, $CDCl_3$) data of compound **3**.

Position No.	Compound 3	
	δ_C	δ_H, m, *J* in Hz
1	40.8	1.05-1.09 (m); 2.05-2.08 (m)
2	19.2	1.40-1.51 (m)
3	37.9	1.98 (m); 1.84 (dd, 1.8, 5.0)
4	43.9	
5	57.1	0.98 (dd, 2.2, 9.2)
6	21.9	2.05-2.08 (m); 1.46-1.48 (m)
7	41.3	1.46-1.48 (m)
8	44.3	
9	55.2	1.13 (dd, 2.5, 5.7)
10	39.8	
11	18.5	1.51-1.62 (m); 1.05-1.09 (m)
12	33.2	1.40-1.51 (m); 1.51-1.62 (m)
13	43.8	2.63 (br s)
14	39.7	0.80-0.82 (m);1.88 (d, 7.0)
15	49	2.15 (q, 8.8); 2.02 (d, 8.8)
16	155.8	
17	102.9	4.73 (br, s); 4.79 (br, s)
18	29	1.24 (s)
19	184.4	
20	15.7	0.95 (s)

assays (Trager and Jensen, 1976).
Two-fold dilutions were performed by transferring 25 µL of the drug using a multi-channel micropipette into the 96-well microtitre plates, starting from row A and going down to row G. The last 25 µL from the G wells were discarded. The procedure was replicated twice. Row H wells were used as controls and did not receive any drugs. Consequently, row A wells had an experimental drug concentration of 250 µg/mL, while B wells had a concentration of 125 µg/mL, which was halved down to G with the lowest concentration of 3.90625 µg/mL. The plates were covered and stored at 4 °C. After examining the parasites under a microscope, the parasitemia (P) of the test culture to be added to the wells of pre-dosed plates was adjusted to 0.4%, and the hematocrit (hct) level was adjusted to 1.5% using 50% red blood cells (RBC). The mixture (200 µL) was then added to each well, except for H7 to H12.
The plates were incubated before harvesting. Briefly, after replacing the lids of microtitre plates and gently agitating them, the plates were placed into a gas-tight box. The box contained a damp tissue to maintain a humid atmosphere in the chamber. The gas box lid was put in place, and the airtight box was flushed with a mixture of 92% N_2, 5% CO_2, and 3% O_2. The plates were then incubated at 37 °C. After 48 hours, [G-3H] hypoxanthine (1 µCi/well) was added in 25 µL aliquots to each well, and the plates were incubated for an additional 18 h.
The cells were harvested on glass fiber filters (Skatron®, Norway) for each row (A-H) using a multiple semi-automatic cell harvester (Skartron®, Norway). The filters were then dried at 37 °C overnight, distributed into scintillation vials, and 1 mL of scintillation fluid was added to each vial before being loaded into a liquid scintillation β-counter (1211 Mini Beta, England) (Desjardins et al., 1979). The disintegration of 3H per minute or count per minute (CPM), which represents the incorporation of [G-3H] hypoxanthine into nucleic acids in the parasite, was calculated for each sample. The activity of the samples was calculated based on the CPM values at different concentrations and expressed as the inhibitory concentration fifty (IC_{50}), which is the concentration that inhibits 50% of parasite intake of [3H-hypoxanthine]. Chloroquine and artemisinin were used as standard drugs or positive controls, with anti-plasmodial activity of 0.00116 ± 0.00000 µg/mL and 0.00834 ± 0.00014 µg/mL, respectively. The solvent (DMSO) in the culture medium was used as the neutral control.

2.7. *In vitro* cytotoxicity assay

The *in vitro* cytotoxicity assay was conducted using

Vero 199 kidney epithelial monkey cells, following the colorimetric assay method described by Mosmann (1983). The cells were maintained in minimum essential medium (MEM, GIBCO, Grand Island, New York) containing 10% fetal bovine serum (FBS). Briefly, each sample was dissolved in DMSO at 37 °C to create a stock solution with a concentration of 1 mg/mL. A 96-well microtiter plate with 8 rows (A-H) and 12 columns was seeded with 100 µL of a 2×10^5 cell suspension. The plate was then incubated at 37 °C in 5% CO_2 for 12 h to allow the cells to attach. After this incubation period, various drugs were added to specific columns. Serial dilution of the drug was carried out starting from row B to H, with the highest drug concentration of 100 µg/mL. Row A was used as the control, with wells 3, 6, 9, and 12 serving as blanks, while the remaining eight wells served as negative controls (cells without drugs). The cells were incubated for 48 h at 37 °C in a 5% CO_2. After that, 10 µL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye was added, and the experiment was allowed to continue for another 4 h. The medium was then removed from the wells, and 100 µL of DMSO was added. The plates were read on a scanning multi-well spectrometer (Multi-Scan Ex lassystems, U.K.) at 562 and 620 nm. Podophyllotoxin (PPT) and its semi-synthetic derivative, etoposide, were used as positive controls (Newman et al., 2000). The cell viability (%) was determined at each concentration using the following formula.

$$\%\text{Cell viability } (CC_{50}) = \frac{ODS562 - OD620}{ODC562 - OD620} \times 100 \quad \text{(Eqn. 1)}$$

Where OD represents optical density, and the letters S and C respectively denote the sample and control. The cell viability (%) data was plotted against the concentration of the drug sample using Excel graphic software. The sample concentration that results in 50% cytotoxicity (CC_{50}) was estimated using linear regression analysis.

3. Results and Discussion

In this paper, we report the isolation of *ent*-13-*epi*-manoyl oxide **(1)**, *ent*-3β-hydroxy-13-*epi*-manoyl oxide **(2)** and kaur-16-en-19-oic acid **(3)**. The structures of the compounds are indicated in Fig. 1. In addition, we report the antimalarial and cytotoxic activities of the compounds as shown in Table 4.

3.1. Identification of the isolated compounds

The IR peaks at 1648 (C=C, str) and 3080 (=C-H st) cm^{-1} observed in the spectrum of compound **1** (Fig. 1) suggested the presence of an olefinic group. The signals at δ 6.00 (1H, dd, *J*=18.0, 11.0 Hz), 4.95 (1H, d, *J*=18.0 Hz) and 4.89 (1H, *J* =11.0 Hz) in ^{1}H NMR, ^{13}C-NMR δ 147.7 (=CH-) and 109.4 (=CH_2) and HMQC spectrum, respectively, corroborated the presence of terminal vinyl group. Four distinct singlet peaks were also observed in the ^{1}H NMR between δ 0.71 and 1.21, three of which represented 3 protons each while the fourth peak represented 6 protons thus suggesting the presence of 5 methyl groups attached to quarternary centres in a diterpenoid skeleton. The COSY cross peak showed a correlation between H-15 and H-14. Other important ^{1}H-^{1}H COSY interactions for compound **1** are shown in Fig. 2. The ^{13}C NMR and HMQC data of compound **1** revealed 20 carbon signals, consisting of 5 methyl, 8 methylene, and 3 methine groups plus 4 quaternary centers, indicating the presence of a diterpenoid scaffold. Two quaternary carbons resonating at δ 76.0 and 73.3 in the HMQC spectrum suggested the presence of an oxygen bridge connected to 2 quaternary carbons thus suggesting an ether linkage. The HMBC data for compound **1** showed that δ 6.00 (H-14) exhibited a $^3J_{H,C}$ correlation with δ 32.8 (C-16) and δ 34.9 (C-12). The protons with signals at δ 4.95 and δ 4.89 indicated a $^3J_{H,C}$ correlation with δ 73.3 (C-113) and $^2J_{H,C}$ correlation with δ 147.7 (C-14). The position of C-13 was further confirmed by two bond HMBC correlation between δ 1.12 (H-16) and δ 73.3 (C-13). A $^3J_{H,C}$ correlation was also observed between H-16 and δ 147.7 (C-14) and δ 34.9 (C-12). The epoxide position at C-8 and C-13 was confirmed by both long and short range HMBC correlations between H-12, H-15 and H-16 to C-13 and H-6, H-7, H-9 and H-17 to C-8. The important diagnostic HMBC correlations for compound **1** are shown in Fig. 2. Furthermore, the absence of a hydroxyl peak in the IR spectrum coupled with the presence of the two signals at δ 76.03 and 73.3 indicated the presence of a cyclic ether skeleton in compound **1**. The cyclic ether linkage in **1** was established through further comparison of the ^{13}C NMR data of sclareol in the chemical literature (Abraham, 1994) to that of compound **1**. EIMS gave the highest peak at *m/z* 290 $[M]^+$, corresponding to the molecular formula $C_{20}H_{34}O$. The NMR data were consistent with the literature data (Granados-Garcia et al., 1985; Angelopoulou et al., 2001) and the results obtained by mass spectrometry confirmed the proposed structure of compound **1** to be *ent*-13-*epi*-manoyl oxide.

Subsequently, a simple comparison of the physical, spectroscopic and physical data of compound **1** to that of 13-*epi*-manoyl oxide established that the two data sets are identical (Algarra et al., 1983; Fraga et al., 1999). The compound was therefore confirmed as 13-*epi*-manoyl oxide or 3,4a,7,7,10a-pentamethyl-1,3-vinyldodecahydrobenzo[*f*]chromene.

The ^{1}H and ^{13}C NMR data for the compound **2** (Fig. 1) was largely similar to that of **1** save for a few distinctive differences. As in **1**, the IR absorption bands at 3083 (=C-H, and 1648 (C=C), cm^{-1} in the spectrum of compound **2** suggested the presence of an olefinic alcohol. The distinct presence of an alcohol group was suggested by the band at 3424 (O-H, str) cm^{-1}, in the IR spectrum. ^{1}H NMR indicated the presence of terminal vinyl group at δ 5.99 (^{1}H, ddd, *J* = 1.2, 11.0, 18.0 Hz), 4.96 (^{1}H, dd, *J* = 1.2, 18.0 Hz) and 4.90 (^{1}H, dd, *J* = 1.2, 11.0). The five distinct singlet peaks between δ 0.72 and 1.21 in the ^{1}H NMR spectrum, each representing 3 protons, suggested the presence of 5 methyl groups in **2** attached to quaternary centres as in **1** and confirmed as secondary from the ^{1}H NMR peaks at δ 3.21 (dd, ^{1}H). There was a significant ^{1}H-^{1}H COSY correlation between δ 3.21 (H-3) and δ 1.57 (H-2) in compound **2** as compared to **1**. This confirmed the presence of hydroxyl

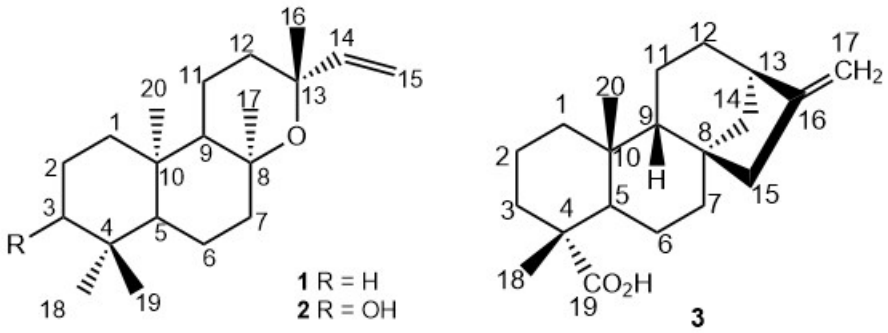

Fig. 1. Chemical structures of compounds **1**, **2** and **3**.

Table 4
In vitro anti-plasmodial activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity (CC_{50}/IC_{50}) of isolated compounds.

Compound	IC_{50} (µM)	CC_{50} (µg/mL)	Selectivity index (CC_{50}/IC_{50})
1	15	≥100	≥23.04
2	7.81	≥100	≥41.8
3	65.4	≥100	≥5.06
CQ	3.63×10^{-6}	77.05	66422.41
PPT		67.35	
Art	2.95×10^{-5}		

CQ: Chloroquine, PPT: Podophyllotoxin, Art: Artemisinin

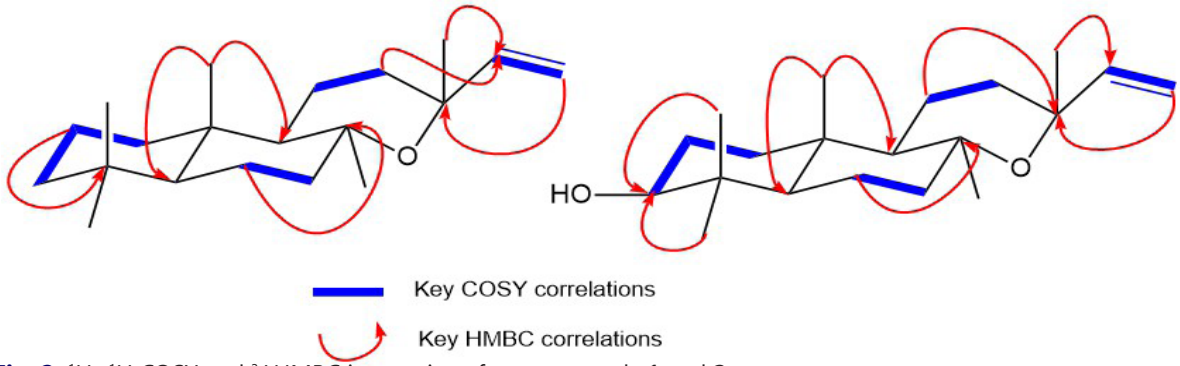

Fig. 2. ^{1}H -^{1}H COSY and ^{3}J HMBC interactions for compounds **1** and **2**.

group on C-3 of compound **2**. The ^{13}C NMR and HMQC spectra of compound **2** revealed 20 signals, consisting of 5 methyl, 7 methylene, and 4 methine groups plus 4 quaternary centers, suggesting a diterpenoid skeleton. ^{13}C NMR signals at δ 147.5 (=CH-) and 109.6 ($=CH_2$) was assigned to olefinic carbons at C-14 and C-15, respectively (Table 2). Two quaternary carbons resonating at δ 75.8 and 73.4 in the ^{13}C NMR and HMQC spectra were attributed to an oxygen bridge connecting to two quaternary centers as in **1**, while the doublet at δ 78.9 which was attributed to the methine group attached to the hydroxyl group (CHOH). Consequently, a close comparison of the ^{13}C NMR of **1** (Table 1) and **2** (Table 2) revealed an additional oxygenated carbon at δ 78.9 in **2** and a reduction in the number of methylene groups by one, with the signal at δ 42.2 (C-3) in **1** being shifted to δ 78.9 in **2**, suggesting oxidation of C-3 to a hydroxyl group. The HMBC $^{3}J_{H,C}$ correlations between H-1, H-5, H-18 and H-19 to C-3 (δ 78.9) further confirmed the presence of oxygenated carbon at C-3 in compound **2** which is missing in compound **1**. Other important HMBC correlations for compound **2** are indicated in Fig. 2. EIMS gave the highest peak at *m/z* 306 $[M]^+$, corresponding to the molecular formula $C_{20}H_{34}O_2$ and confirmed through reported data (Anjaneyulu and Rao, 2000). COSY, HMQC, HMBC and MS data analysis suggested compound **2** to be *ent*-3β-hydroxy-13-*epi*-manoyl oxide. Subsequently, comparison of the physical and spectroscopic data of compound **2** to that of 3β-hydroxy-13-*epi*-manoyl oxide (ribenol) in the

chemical literature confirmed that the two data sets were the identical (Table 2) (Mahato and Kundu, 1994; Konishi et al., 1996). As in **1**, the cyclic ether linkage was established through comparison with the ^{13}C NMR data of 3β-hydroxyscalerol (Kouzi and McChesney, 1990) in the chemical literature and the structure of compound **2** confirmed to be 3β-hydroxy-13-*epi*-manoyl oxide.

Compound (**3**) gave a luminous green color for organic acids with *p*-anisaldehyde under UV light. The characteristic IR absorption bands at 2500-3500 (*br*, CO_2H), 3300, 3100 (w, =C-H *str*), 1734 (C=O str), and 1619 (C=C *str*) cm^{-1} suggested the presence of an olefinic acid. The olefin group was confirmed as terminal by two distinct signals in ^1H-NMR at δ 4.73 (1H) and 4.79 (1H), ^{13}C NMR at δ 155.8 (C=) and 103.0 (=CH_2) ^{13}C NMR spectrum, respectively (Table 3) (Mahato and Kundu, 1994). The presence of a carboxyl group in the compound was confirmed by the peak at δ 184.4 (-CO_2-) in the ^{13}C NMR spectrum. The two distinct peaks at δ 1.24 (s) and 0.95 (s) in the ^1H NMR spectrum, representing 3 protons each, were characteristic of methyl groups in the compound. The ^{13}C NMR spectrum revealed 20 peaks due to 2 methyl, 10 methylene, and 3 methine groups plus 5 quaternary centers. The reduction in the number of methyl groups; and the increase in the number of methylene groups suggested the presence of a rearranged diterpenoid skeleton through oxidation and oxidative cyclization processes. EIMS gave the highest peak at *m/z* 302 $[M]^+$, corresponding to the molecular formula $C_{20}H_{30}O_2$ and confirmed through literature data (De Souza et al., 2010). Subsequently, the physical and spectroscopic data of compound **3** were compared to those of kaur-16-en-19-oic acid in the chemical literature and the structure confirmed the two data sets to be same identical. The compound was therefore confirmed to be *ent*-kaur-16-en-19-oic acid (Fig. 1) (Cross et al., 1963; Fraga et al., 1989; Fraga et al., 1999).

3.1.2. Anti-plasmodial activity, cytotoxicity and selectivity index of isolated compounds

The two manoyl oxides: *ent*-13-*epi*-manoyl oxide (**1**), and *ent*-3β-hydroxy-13-*epi*-manoyl oxide (**2**); and kaur-16-en-19-oic acid were thus evaluated for anti-plasmodial activity *in vitro*. The anti-plasmodial activity for the compounds isolated from *H. forsskaolii* (Vahl) R.Br. are recorded in Table 4 against D6 (CQ-sensitive).

The three compounds exhibited anti-plasmodial activity against the CQ-sensitive *P. falciparum* D6 strain at different levels, as can be discerned from the corresponding IC_{50} values: *Ent*-13-*epi*-manoyl oxide (**1**), *ent*-3β-hydroxy-13-*epi*-manoyl oxide (**2**), and kaur-16-en-19-oic acid (**3**) with IC_{50} values of 15.0 μM, 7.81 μM, and 65.40 μM.

The cytotoxicity data for the compounds isolated from *H. forsskaolii* (Vahl) R.Br. are reported in Table 4. The compounds showed no toxicity, as the cytotoxicity concentration fifty (CC_{50}) for all the compounds was ≥ 100 μg/mL. The selectivity indices for the isolated compounds were determined as a ratio of cytotoxicity to anti-plasmodial activity. The corresponding values are recorded in Table 4.

While *ent*-13-*epi*-manoyl oxide (**1**) is known as the final metabolite of a biosynthetic pathway for many diterpenes in the *Gibberella fujikuroi* fungus (Cross et al., 1963), *ent*-3β-hydroxy-13-*epi*-manoyl oxide (**2**) is a natural product found in the *Sideritis varoi* (Labiatae) plant (Konishi et al., 1996). It has been widely used as a substrate in both chemical and microbial transformations to produce more functionalized manoyl oxide derivatives with various biological activities (Granados-Garcia et al., 1985; Fraga et al., 1989; Fraga et al., 1999; García-Granados et al., 2004). Furthermore, both compounds have been found in the resin (labdan) of Cistus creticus plants and reported to exhibit antican cer activity (Falara et al., 2010). Kaur-16-en-19-oic acid is a common metabolite found in many plants and has previously been isolated from *H. forsskaolii* (Vahl) R.Br. root bark (Cross et al., 1963).

The manoyl oxides exhibited mild to moderate anti-plasmodial activity. Among them, *ent*-3β-hydroxy-13-*epi*-manoyl oxide (**2**) showed moderate activity with an IC_{50} of 7.81 μM against the CQ-sensitive *P. falciparum* D6 strain. Additionally, *ent*-13-*epi*-manoyl oxide (**1**) exhibited mild activity at 15.0 μM, which was significantly better than kaur-16-en-19-oic acid at 65.40 μM. It is worth noting that kaur-16-en-19-oic acid has been reported elsewhere as being inactive against the CQ-sensitive *P. falciparum* D10 strain (IC_{50} 106.5 μM) (Mthembu et al., 2010). Apparently, the addition of a hydroxyl group in *ent*-13-*epi*-manoyl oxide (**1**) to form *ent*-3β-hydroxy-13-epi-manoyl oxide (**2**) increases anti-plasmodial activity and drug selectivity by 100%. The compounds hypoestenonol, verticillarone, and hypoestenone, which were previously isolated from this plant, demonstrated anti-plasmodial activities against the *P. falciparum* K-1 strain. Their IC_{50} values were 18.9 μM, 25.1 μM, and 16.7 μM, respectively (Al Musayeib et al., 2014). *Ent*-kaur-16-en-19-oic acid was previously isolated from *Schefflera umbellifera* (Sond.) Baill (Araliaceae) showed anti-plasmodial activity against the CQ-susceptible *P. falciparum* D10 strain with an IC_{50} value of 106.5 μM (Nogueira and Lopes, 2011).

From the above observations, it appears that the chromene skeleton plays a crucial role in the observed anti-plasmodial activity of the isolated diterpenes, while the hydroxyl group enhances their potency and/or selectivity. The mild to moderate anti-plasmodial activity, low toxicity, and high selectivity suggest that the chromene skeleton is a promising candidate for further studies on structure-activity relationships. These studies aim to enhance all the aforementioned critical parameters in order to identify more potent and effective candidates for anti-malarial drugs.

4. Concluding remarks

Two manoyl oxides, namely ent-13-*epi*-manoyl oxide (**1**), and ent-3β-hydroxy-13-*epi*-manoyl oxide (**2**), are being reported for the first time from *H. forsskaolii* (Vahl) R.Br. root bark, while kaur-16-en-19-oic acid (**3**) has been reported previously. These compounds are being reported for the first time from *H. forsskaolii* (Vahl) R.Br. The three compounds exhibited mild to moderate anti-plasmodial activity against the CQ-

sensitive *P. falciparum* D6 strain, with IC_{50} values of 15.0, 7.81, and 65.40 µM, respectively. The compounds are relatively safe since their cytotoxicity concentration fifty (CC_{50}) values were ≥ 100 µg/mL.

The results partly suggest that the traditional medicinal knowledge and use of the plant by the Marakwet and Kisii communities are confirmed by *H. forsskaolii* (Vahl) R.Br. This substance contains anti-plasmodial compounds with a molecular structure that differs from the currently available commercial anti-malarial medications. Consequently, they reaffirm the importance of providing complementary anti-malarial treatment that is widely available to resource-limited rural pastoral and/or farming communities. They also emphasize the need for more structures to support future studies on structure-activity relationships and drug development programs.

Abbreviations

CC_{50}: Cytotoxicity concentration fifty; **COSY**: Correlation spectroscopy; **CPM**: Counts per minute; **CQ**: Chloroquine; **EIMS**: Electron impact mass spectroscopy; **HMBC**: Hetero multiple bond correlation; **HMQC**: Hetero multiple quantum correlation; **IC_{50}**: Inhibitory concentration fifty; **IR**: Infrared; ***m/z***: Mass to charge; **NMR**: Nuclear magnetic resonance; **TLC**: Thin layer chromatography; **TMS**: Trimethylsilane; **µm**: Micromolar.

Data availability

The findings of this research can be obtained from the corresponding author upon request.

Author contributions

Fred Wanyonyi Sawenja conceived the project. Fred Wanyonyi Sawenja, Ruth Anyango Omole, Peter Gakio Kirira and Hamisi Masanja Malebo performed the experiments. Alex King'ori Machocho, Richard Maveke Musau, and Isaiah Omolo Ndiege supervised the work. All authors evaluated the results and revised the manuscript for publication. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

We thank Prof. Osamu Onamura of Nagasaki University, Japan, and Dr. Samwel Shikuku Libendi of Pwani University, Kilifi, Kenya, for his assistance in the acquisition of NMR data and Mr. Simon Mathenge of the University of Nairobi for plant identification and collection. We are grateful to IUCEA for the financial grants that made this work possible.

Ethical consideration

The Directorate of Studies of Kenyatta University in Kenya approved the research protocol.

References

Abraham, W.R., 1994. Microbial hydroxylation of scareol. Phytochemistry 36, 1421-1424.

Al Musayeib, N.M., Mothana, R.A., Mohamed, G.A., Ibrahim, S.R.M., Maes, L., 2014. Hypoestenonols A and B, new fusicoccane diterpenes from *Hypoestes forskalei*. Phytochem. Lett. 10, 23-27.

Algarra, J., García-Granados, A., de Buruaga, A.S., de Buruaga, J.M.S., 1983. Diterpenoids from *sideritis varo*i. Phytochemistry 22, 1779-1782.

Angelopoulou, D., Demetzos, C., Dimas, C., Perdetzoglou, D., Loukis, A., 2001. Essential oils and hexane extracts from leaves and fruits of *Cistus monspeliensis*. cytotoxic activity of *ent*-13-*epi*-manoyl oxide and its isomers. Planta Med. 67, 168-171.

Anjaneyulu, A.S.R., Rao, V.L., 2000. Five diterpenoids (agallochins A-E) from the mangrove plant *Excoecaria agallocha* Linn. Phytochemistry 55, 891-901.

Atanasov, A.G., Waltenberger, B., Pferschy-Wenzig, E.M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E.H., Rollinger, J.M., Schuster, D., Breuss, J.M., Bochkov, V., Mihovilovic, M.D., Kopp, B., Bauer, R., Dirsch, V.M., Stuppner, H., 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review Europe PMC Funders Group. Biotechnol. Adv. 33, 1582-1614.

Cross, B.E., Galt, R.H.B., Hanson, J.R., Curtis, P.J., Grove, J.F., Morrison, A., 1963. New metabolites of *Gibberella fujikuroi*. Part II. The isolation of fourteen new metabolites. Chem. Soc. 2937-2943.

De Souza, P.A., Rangel, L.P., Oigman, S.S., Elias, M.M., Ferreira-Pereira, A., De Lucas, N.C., Leitão, G.G., 2010. Isolation of two bioactive diterpenic acids from *Copaifera glycycarpa oleoresin* by high-speed counter-current chromatography. Phytochem. Anal. 21, 539-543.

Deletre, E., Martin, T., Duménil, C., Chandre, F., 2019. Insecticide resistance modifies mosquito response to DEET and natural repellents. Parasit. Vectors 12, 1-10.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D., 1979. Quantitative activity semiautomated technique. Antimicrob. Agents Chemother. 16, 710-718.

Falara, V., Pichersky, E., Kanellis, A.K., 2010. A copal-8-ol diphosphate synthase from the angiosperm *Cistus creticus* subsp. *creticus* is a putative key enzyme for the formation of pharmacologically active, oxygen-containing labdane-type diterpenes. Plant Physiol. 154, 301-310.

Fraga, B.M., González, P., Guillermo, R., Hernández, M.G., Rovirosa, J., 1989. The microbiological transformation of some *ent*-13-*epi*-manoyl oxide diterpenes by *Gibberella fujikuroi*. Phytochemistry 28, 1851-1854.

Fraga, B.M., González, P., Hernández, M.G., Suárez, S., 1999. Formation of 2,3-seco-acids in the biotransformation of the diterpene ribenone by *Gibberella fujikuroi*. Tetrahedron 55, 1781-1792.

García-Granados, A., Fernández, A., Gutiérrez, M.C., Martínez, A., Quirós, R., Rivas, F., Arias, J.M., 2004. Biotransformation of *ent*-13-*epi*-manoyl oxides difunctionalized at C-3 and C-12 by filamentous fungi. Phytochemistry 65, 107-115.

Granados-Garcia, A., Martinez, A., Molina, A., Onorato, M.E., Rrco, M., Buruaga, A.S.D.E., 1985. Deterpenoids from *Sidetinis varoi* subspecies *Cuatrecasasii*: ^{13}C NMR of *ent*-13-*epi*-manoyl oxides functionalized at C-12. Phytochemistry 24, 1789-1792.

Harvey, A.L., Edrada-Ebel, R., Quinn, R.J., 2015. The re-emergence of natural products for drug discovery in the genomics era. Nat. Rev. Drug Discov. 14, 111-129.

Iain, D., 2015. The genus *Hypoestes* (Acanthaceae) in Angola. Kew Bull. 70, 1-10.

Kipkore, W., Wanjohi, B., Rono, H., Kigen, G., 2014. A study of the medicinal plants used by the Marakwet Community in Kenya. J. Ethnobiol. Ethnomed. 10.

Konishi, T., Azuma, M., Itoga, R., Kiyosawa, S., Fujiwara, Y., Shimada, Y., 1996. Three new labdane-type diterpenes from wood, *Excoecaria agallocha*. Chem. Pharm. Bull. 44, 229-231.

Kouzi, S.A., McChesney, J.D., 1990. Microbial metabolism of the diterpene sclareol: Oxidation of the A ring by *Septomyxa affinis*. Helv. Chim. Acta 73, 2157-2164.

Kunasol, C., Mayxay, M., Menard, D., Dondorp, A.M., Hlaing, T.M., van der Pluijm, R.W., Day, N.P.J., Suwannasin, K., Smithuis, F.M., Imwong, M., Miotto, O., Rekol, H., Sutawong, K., Tripura, R., White, N.J., Dhorda, M., Tun, K.M., 2017. The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: A molecular epidemiology observational study. Lancet Infect. Dis. 17, 491-497.

Mahato, S.B., Kundu, A.P., 1994. ^{13}C NMR spectra of pentacyclic triterpenoids-A compilation and some salient features. Phytochemistry 37, 1517-1575.

Mohammadhosseini, M., Frezza, C., Venditti, A., Mahdavi, B., 2022. An overview of the genus *Aloysia Paláu* (Verbenaceae): Essential oil composition, ethnobotany and biological activities. Nat. Prod. Res. 36, 5091-5107.

Mojab, F., 2012. Antimalarial natural products: a review. Avicenna J. Phytomedicine 2, 52-62.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55-63.

Mthembu, X.S., Van Heerden, F.R., Fouché, G., 2010. Antimalarial compounds from *Schefflera umbellifera*. S. Afr. J. Bot. 76, 82-85.

Muregi, F.W., Chhabra, S.C., Njagi, E.N.M., Lang'at-Thoruwa, C.C., Njue, W.M., Orago, A.S.S., Omar, S.A., Ndiege, I.O., 2003. *In vitro* antiplasmodial activity of some plants used in Kisii, Kenya against malaria and their chloroquine potentiation effects. J. Ethnopharmacol. 84, 235-239.

Nahar, L., El-Seedi, H.R., Khalifa, S.A.M., Mohammadhosseini, M., Sarker, S.D., 2021. *Ruta* essential oils: Composition and bioactivities, Molecules.

Newman, D.J., Cragg, G.M., Snader, K.M., 2000. The influence of natural products upon drug discovery. Nat. Prod. Rep. 17, 215-234.

Nogueira, C.R., Lopes, L.M.X., 2011. Antiplasmodial natural products. Molecules 16, 2146-2190.

Nsanzabana, C., 2019. Resistance to artemisinin combination therapies (ACTs): Do Not forget the partner drug! Trop. Med. Infect. Dis. 4, 26.

Okaiyeto, K., Oguntibeju, O.O., 2021. African herbal medicines: Adverse effects and cytotoxic potentials with different therapeutic applications. Int. J. Environ. Res. Public Health 18, 1-20.

Richards, S.L., Byrd, B.D., Reiskind, M.H., White, A.V., 2020. Assessing Insecticide resistance in adult mosquitoes: Perspectives on current methods. Environ. Health Insights 14.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science (80) 193, 673-675.