Journal of Asian Natural Products Research

ISSN: 1028-6020 (Print) 1477-2213 (Online) Journal homepage: https://www.tandfonline.com/loi/ganp20

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To cite this article: Hanita Omar, Mehran Fadaeinasab, Hairin Taha, Aty Widyawaruyanti, Mohd Azlan Nafiah & Tiah Rachmatiah (2020) Aporphine alkaloids with *in vitro* antiplasmodial activity from the leaves of *Phoebe tavoyana*, Journal of Asian Natural Products Research, 22:1, 52-60, DOI: 10.1080/10286020.2018.1553958

To link to this article: https://doi.org/10.1080/10286020.2018.1553958

Published online: 21 Mar 2019.

JOURNAL OF ASIAN NATURAL PRODUCTS RESEARCH
2020, VOL. 22, NO. 1, 52–60
https://doi.org/10.1080/10286020.2018.1553958

Aporphine alkaloids with *in vitro* antiplasmodial activity from the leaves of *Phoebe tavoyana*

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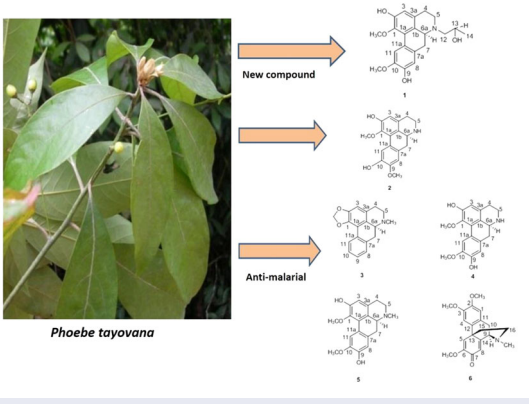
ABSTRACT

One new aporphine named tavoyanine A (**1**), along with four known aporphines laetanine (**2**), roemerine (**3**), laurolitsine (**4**), and boldine (**5**), and one morphinandienone type sebiferine (**6**) were isolated from the leaves of *Phoebe tavoyana* (Meissn.) Hook f. (Lauraceae). The isolation was achieved by chromatographic techniques, and the structural elucidation was performed via spectral methods. This paper also reports the antiplasmodial activity of roemerine (**3**), laurolitsine (**4**), boldine (**5**), and sebiferine (**6**). The results showed that **3**–**6** have a potent inhibitory activity against the growth of *Plasmodium falciparum* 3D7 clone, with IC_{50} values of 0.89, 1.49, 1.65, and 2.76 μg/ml, respectively.



ARTICLE HISTORY
Received 6 June 2018
Accepted 27 November 2018

KEYWORDS
Phoebe tavoyana; Lauraceae; aporphine alkaloid; morphinandienone; antiplasmodial; *Plasmodium falciparum*

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1. Introduction

Malaria is a parasitic disease caused by *Plasmodium* species transmitted from the blood of an infected person and passed to a healthy human by a female *Anopheles* mosquito. Four species of parasites from the genus *Plasmodium* are pathogenic to humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. In recent years, human cases of malaria have also been recorded due to *P. knowlesi*—a species that causes malaria among monkeys, and occurs in certain forested areas of South-East Asia.

Plasmodium falciparum is most prevalent on the African continent, and is responsible for most deaths from malaria [1]. In Malaysia, most malaria cases are caused by *P. falciparum*.

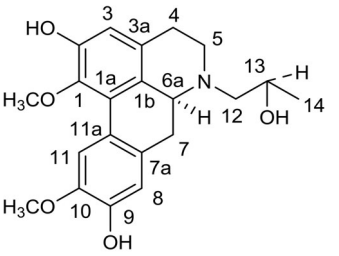
In year 2013, an estimated 198 million cases of malaria occurred worldwide, 82% was in the WHO African Region, followed by the WHO South-East Asia Region (12%) and the WHO Eastern Mediterranean Region (5%). There were an estimated 584 000 malaria deaths worldwide. It is estimated that most (90%) of these deaths were in the WHO African Region, followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%). About 453 000 malaria deaths were estimated to occur in children aged under 5 years, equivalent to 78% of the global total. An estimated 437 000 of deaths occurred in children aged under 5 years in the WHO African Region [1].

Plasmodium falciparum is a major parasitic infection disease in the world and continues to cause morbidity and mortality on a large scale in tropical countries and undermining development in the poorest countries of the world [1]. So, most studies have evaluated the activity of compounds on this species [2–4]. In many parts of the world the parasites have developed resistance to a number of antimalarials such as chloroquine and derivatives, the most widely used treatment for malarial, and so there is an urgent need to discover new compounds with an original mode of action.

In a continuation of our ongoing investigation of traditionally used antimalarial plants of Malay Peninsula, Malaysia, the aim of this study was to evaluate the antiplasmodial activity of *Phoebe tavoyana* (Meissn.) Hook f. (Lauraceae), commonly known as "*medang rungkoi*" (Malaysia) [5,6]. *Phoebe tavoyana* (Meissn.) Hook f. is a tree up to 14 m tall. It is commonly distributed at Langkawi Island, Kedah, Perak, Kelantan and in lowland forests, India, Burma, Indo-China, Thailand and Sumatra [7]. *Phoebe* species have been reported to contain aporphines, proaporphine, oxoaporphines, and morphines [8].

Phoebe tavoyana closely resembles to *P. lanceolata*, the hairy young twigs and perianth help to distinguish them. Some species of *phoebe* are used for treatment of several diseases in China, Indonesia, Indochina, Japan, Philliphine and Malay Peninsula. *Phoebe lanceolata* is an evergreen tree and well reputed in traditional medicine in India. Biological screening on the crude alkaloidal extract of the leaves of *Phoebe grandis* for antiplasmodial activity has shown positive result ($IC_{50} < 8\,mg\,ml^{-1}$) [8]. The crude extract of leaves of *P. scortechinii* showed significant antiplasmodial activity to resistant strain *P. falsiparum*, Gombak A (IC_{50} 6.1067 mg ml^{-1}) and to sensitive strain *P. falsiparum*, D10 (IC_{50} 0.691 mg ml^{-1}) [9].

This paper reports the isolation, structure elucidation, and antiplasmodial activity of a series of alkaloids compounds from CH_2Cl_2 extract of leaves of *P. tavoyana*.



1

Figure 1. Chemical structure of alkaloid **1** isolated from *Phoebe tavoyana*.

2. Results and discussion

The dried ground leaves of *Phoebe tavoyana* was extracted exhaustively with hexane followed by CH_2Cl_2 using Soxhlet extractor for 18 h to give the dark viscous extracts. Chromatographic separation of the extract yielded one new aporphine (**1**) along with five known compounds (Figure 1).

The known compounds were identified as laetanine (**2**) [10], roemerine (**3**) [11], laurolitsine (**4**) [12], boldine (**5**) [13]; and one morphinandienone type, sebiferine (**6**) [14] by comparison of their spectroscopic data with literatures.

The new compound (**1**) was obtained as a dark brown solid, with $[\alpha]_D^{25}$ +0.016 (c 0.06, CH_3OH). The LCMS-ESI electrospray ionization mass spectrometry exhibited an ion peak at *m/z* 372.1776 $[M+H]^+$ giving a possible molecular formula of $C_{21}H_{25}NO_5$. Its ultraviolet (UV) spectrum showed absorption maxima at 283 and 305 nm, thus suggesting a 1, 2, 9, 10-tetrasubstituted aporphine skeleton [15]. In addition, a broad band at 3360 cm^{-1} in the infrared (IR) spectrum indicated the presence of phenolic hydroxyl moiety. Its IR spectrum also showed strong absorptions at 2928 and 2825 cm^{-1} due to the stretching of C-H aromatic, respectively. The absorptions from 1465 to 1510 cm^{-1} indicated aromatic C=C stretching. The strong absorption at 1258 cm^{-1} indicated the presence of the methoxyl groups. Moreover, the UV and IR spectra of alkaloid **1** were typical of an aporphine carrying two hydroxyl groups.

The ^1H NMR spectrum of **1** revealed two aromatic methoxyl singlets at δ 3.85 and 3.50 attached to C-10 and C-1, respectively. Three ^1H singlets were observed at δ 7.80, 6.76, and 6.58. The former signal was assigned to H-11, and the latter was assigned to H-8 on ring D and H-3 on ring A. H-11 showed the highest chemical shift due to the deshielding effect of the ring A. The spectrum also revealed the presence of the -$CH_2CHOHCH_3$ group attached to the nitrogen atom with the occurrence of a doublet at δ 1.12 (J = 6.4 Hz) due to the presence of methyl group (C-14) next to a methine proton (C-13). A multiplet proton resonance at δ 3.88 indicated that C-13 bears a hydroxyl group. Furthermore, four aliphatic protons were observed at δ 3.11 (*dd*, J = 4.1, 11.3 Hz), 2.60 (*d*, J = 4.1 Hz), δ 2.56 (*d*, J = 16.0 Hz), and 2.38 (*dd*, J = 4.1, 11.3 Hz) attributable to H-5β, H-4β, H-4α, and H-5α, respectively. The signal for H-7β and H-7α appeared as doublets at δ 2.94 (*d*, J = 3.6 Hz) and 2.42 (*d*, J = 3.4 Hz). Finally, the remaining signals of one proton as a pair of doublet (*dd*, J = 3.2, 13.7 Hz), was observed at δ 3.17, which can be ascribe to H-6a. The assignment of the aliphatic protons and methylene was also confirmed by analysis of the homonuclear COSY

Table 1. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), DEPT and 2D (HMBC and HMQC) NMR data of tavoyanine A (**1**).

1 in $CDCl_3$					
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	DEPT	HMBC (^{2}J,^{3}J)	HMQC (^{1}J)
1	–	142.2	C_q	–	–
1a	–	126.6	C_q	–	–
1b	–	127.3	C_q	–	–
2	–	148.2	C_q	–	–
3	6.58 (*s*, 1H)	113.3	CH	C_1,C_{1b}, C_2, C_4	H_3
3a	–	130.4	C_q	–	–
4β	2.60 (*d*, *J* = 4.1)	29.4	CH_2	C_{1b}, C_{3a}, C_5.	H_4
4α	2.56 (*d*, *J* = 16.0)			C_{1b}, C_3, C_{3a}.	
5β	3.11 (*dd*, *J* = 4.1, 11.3)	49.4	CH_2	C_{3a},C_4,C_{6a}	H_5
5α	2.38 (*dd*, *J* = 4.1, 11.3)			C_{3a}, C_{6a}	
6a	3.17 (*dd*, *J* = 3.2, 13.7)	60.4	CH	–	H_{6a}
7β	2.94 (*dd*, *J* = 3.7, 12.0)	34.9	CH_2	C_{1a},C_{6a},C_{7a},C_8,C_{11a}	H_7
7α	2.42 (*dd*, *J* = 3.7, 12.0)			C_{1b},C_{6a}, C_8,C_{11a}	
7a	–	130.2	C_q	–	–
8	6.76 (*s*, 1H)	114.3	CH	C_7,C_{10}, C_{11a}	H_8
9	–	145.3	C_q	–	–
10	–	145.8	C_q	–	–
11	7.80 (*s*, 1H)	110.3	CH	C_{1a}, C_{7a}, C_9, C_{11a}	H_{11}
11a	–	123.7	C_q	–	–
12β	2.70 (*dd*, *J* = 10.5, 12.5)	61.5	CH_2	C_5, C_{6a}, C_{13}, C_4, C_5	H_{12}
12α	2.18 (*dd*, *J* = 2.7, 12.6)				
13	3.88 *m*	62.9	CH	–	H_{13}
14	1.12 (*d*, *J* = 6.4)	19.8	CH_3	C_{12}, C_{13}	$3H_{14}$
1-OCH_3	3.50 (*s*, 3H)	60.4	CH_3	C_1	$3H_{1\text{-OMe}}$
10-OCH_3	3.85 (*s*, 3H)	56.4	CH_3	C_{10}	$3H_{10\text{-OMe}}$

δ_H, chemical shift values in ^{1}H-NMR spectrum; δ_C, chemical shift values in ^{13}C-NMR spectrum; Cq, quaternary carbons; *d*, doublet; *s*, singlet; *dd*, doublet of doublet.

data. In the COSY spectrum, H-13 is coupled to H-14; and H-13 is coupled to H-12β. These match with the suggested structure of **1** having aliphatic protons. Whilst, the respective positions of the protons at C-4 and C-5; and C-7 and C-6a were also confirmed by ^{1}H-^{1}H correlations in the COSY spectrum. The analysis showed that H-6a correlated to H-7α; and H-4β correlated to H-5α.

NOE difference spectrum was used to confirm the position of the methoxyl groups on ring A and ring D. Irradiation on C-10 methoxyl (δ 3.85) showed the enhancement of C-11 (δ 7.80) aromatic proton (2.81%) whereas irradiation of C-11 exhibited enhancement of C-10 methoxyl (11.72%); hence, this methoxyl group must be attached to C-10. Irradiation of the H-13 signal at δ 3.88 resulted in NOE enhancement of the methyl signal at δ 1.12 (H-14) and the proton signals at δ 3.11 (H-5β) and δ 2.18 (H-12α). When a proton at H-6a was irradiated, the signal of proton H-7β at δ 2.94 (3.71%) was enhanced. Irradiation of the proton H-12β at δ 2.70 enhanced the proton signal at H-7α and H-5α. The result of NOE-diff experiments supported the finding of the structure of the alkaloid **1**.

The ^{13}C NMR spectrum showed the presence of 21 carbons in the molecule. These 21 carbons belonged to one methyl carbon, four methylene carbons, two methines, three aromatic carbons, nine quaternary carbons, and two methoxyl carbons as indicated by DEPT spectrum of alkaloid **1**.

Direct correlations between carbon and hydrogen were found from the HMQC spectrum, and the results were supported by other data. The structure of alkaloid **1** was finally confirmed by ^{1}H-^{13}C long range correlations as indicated in the HMBC

Table 2. Inhibition growth percentage of *Plasmodium falciparum* and Probit Analysis with SPSS 11.5.

	% inhibition at concentration (μg/ml)					
Sample	10	1	0.1	0.01	0.001	IC_{50} (μg/ml)
Roemerine (**3**)	99.81	99.30	36.10	15.23	0.95	0.89
Laurolitsine (**4**)	100.00	23.44	3.94	0.61	0.00	1.49
Boldine (**5**)	93.12	24.17	6.16	2.28	0.00	1.65
Sebiferine (**6**)	100.00	94.72	6.60	1.05	0.66	2.76
Tavoyanine A (**1**)	–	–	–	–	–	nt
Laetanine (**2**)	–	–	–	–	–	nt

Note: Positive control is chloroquine, $IC_{50} = 0.0069$ μg/ml.

spectrum. The absolute configuration of the asymmetric carbon at C-13 was not determined due to the limited amount of compound available, so alkaloid **1** can be considered a racemic mixture.

Table 1 summarizes the ^{1}H and ^{13}C-NMR chemical shift values and coupling patterns of the proton obtained from various NMR experiments. The compound **1** was characterized as (+)-tavoyanine A or *N*-(2-hydroxypropyl)-norboldine A based on the above spectral data and comparison with literature reports of compound [16,17]. To the knowledge of the author, (+)-tavoyanine A (**1**) is the first report as new naturally occurring compound.

Antiplasmodial activity was conducted on the isolated compounds from CH_2Cl_2 extract of *P. tavoyana* leaves. The isolated compounds were tested for the *in vitro* inhibitory activity against *P. falciparum* 3D7. Only four of the compounds resulted in significant inhibition activity against a chloroquine sensitive strain of *P. falciparum* (3D7); inhibitory concentration ranged from 0.89 to 2.76 μg/ml. They are summarized in Table 2.

Among the compounds, roemerine (**3**) had the most potent inhibitory activity with the IC_{50} value of 0.89 μg/ml. The well-known antimalarial drug chloroquine has the IC_{50} value of 0.006 μg/ml. The enhanced activity of roemerine (**3**) implies that the 1,2-methylenedioxy substituent is required for the expression of inhibitory activity of the aporphine alkaloids. The weak activity of boldine (**5**) or laurolitsine (**4**) may be due to the absence of the 1,2-methylenedioxy or due to polarity which attenuated the ability to inhibit. This results supported by Fadaeinasab *et al.* [18] that most of the aporphine alkaloids displayed significant antimalarial activity. The 1,2-methylenedioxy substituent appears to play a similar role in the oxo-aporphine alkaloids to enhance the antimalarial activity [19].

It is important to notice that compounds boldine (**5**) and norboldine share the same basic skeleton with different substitution of functional group N-Me and N-H. With regard to the structure of these compounds, boldine (**5**) has only one N-Me group; while norboldine with its N-H counterpart, was more active against *P. falciparum* 3D7 as compared to the N-Me derivative with the IC_{50} values of 1.49 and 1.65 μg/ml, respectively. These data also support the previous study of another group of researcher [20], reported that for each pair of 2-*nor*-alkaloid and its di-*N*-Me counterpart, the 2-*nor*-alkaloid which has only one Me group on 2′-*N*, was always more active against both susceptible and resistant strains of *Plasmodium* as compared to the di-N-Me derivative. For example, (+)-2-nor-thalrugosine was more active than those of (+)-thalrugosine [21].

Unfortunately, laetanine (**2**) and tavoyanine A (**1**) were not tested due to insufficient amount to assess the inhibitory *in vitro* activity against *P. falciparum*.

Sebiferine (**6**) was also found active against *in vitro P. falciparum* 3D7, but it was the weakest among the tested compounds. That may be due to the presence of *N*-methyl and more methoxyl groups in the structure of sebiferine (**6**) compared to the others [21].

To the best of our knowledge, there has been no previous phytochemical investigation and medicinal value has been performed on this plant. Interestingly, roemerine (**3**) has no previous record of any antiplasmodial activity. The significant inhibitory activity of these alkaloids supports the traditional use of the *Phoebe* plants as an antimalarial drug.

3. Experimental

3.1.General experimental procedures

Optical rotations data were measured on a JASCO P-1020 Polarimeter (Pennsylvania) with methanol and chloroform as the solvent. UV spectra were obtained using a Shimadzu UV-160 PC Ultraviolet-Visible Spectrometer (Kyoto, Japan). IR spectra were recorded by using a Perkin Elmer Spectrum 2000-FTIR Spectrometer (Massachusetts). The Automass Multi Thermofinnigan was used for the HR-ESI analysis, and EIMS were obtained on a Shimadzu GC-MS-QP2000A Mass Spectrometer 70 eV (Kyoto, Japan). Sometimes, mass spectra were obtained using a Shimadzu LCMS-IT-TOF or LCMS-Q-TOF instrument. The NMR spectra were acquired with either a JEOL FT-NMR (400 and 500 MHz) (Tokyo, Japan) or Bruker AVN (400 and 600 MHz) spectrometer (Faellanden, Switzerland) with tetramethylsilane as an internal standard. Separation and isolation of compounds from the plant crude extract were performed using various chromatographic techniques such as column chromatography (CC) which was packed with silica gel, Silica Gel 60F, 70-230 mesh ASTM (Merck 7734) or Silica Gel 60F, 230-400 Mesh ASTM (Merck 9385), preparative thin layer chromatography, and analytical thin layer chromatography (TLC). TLC was performed on commercially available Merck Aluminium supported silica gel 60 F_{254} TLC sheets (Merck 1.05554.0001) and glass supported silica gel 60 F_{254} TLC plates (Merck 1.05715.0001) (Darmstadt, Germany). Mayer's and Dragendorff's reagents were used for alkaloid screening to identify the presence of alkaloids and alkaloids spotting (TLC).

3.2. Plant material

Leaves of *Phoebe tavoyana* (Meissn.) Hook f. (Lauraceae) were collected from Reserved Forest Chebar Besar, Kedah, Malaysia, and the voucher specimen (KL 5225) was deposited in the Herbarium of Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia and in the Herbarium of the Forest Research Institute, Kepong, Malaysia. The plant (KL 5225) was identified by the botanist and phytochemical survey of Malayan Herbarium, University of Malaya.

3.3. Extraction and isolation

The dried ground leaves 2700 g of *Phoebe tavoyana* were first defatted in hexane for 3 days at room temperature then filtered and air-dried for 24 h, and the solvent evaporated to dryness to give 5.0 g (0.19%) of the hexane extract. After being dried, the leaves samples were sprinkled with 15% ammonia (NH_3) solution and left to soak overnight. They were then exhaustively extracted with dichloromethane (CH_2Cl_2) solvent by a Soxhlet extractor for about 18 h. The CH_2Cl_2 extract was concentrated to about 500 ml by using the rotary evaporator, then re-extracted with 5% hydrochloric acid (HCl) until Mayer's test is negative. The combined extracts were then basified with concentrated ammonia solution to pH 10–11 and re-extracted with CH_2CI_2. The CH_2Cl_2 extracts were washed with distilled H_2O and followed by sodium chloride solution and finally dried over anhydrous sodium sulfate. The solvents were evaporated to dryness to yield 10.5 g of crude alkaloid (0.39% wt/wt). The crude alkaloid fraction was obtained as a dark gummy residue.

The dichloromethane crude extract or crude alkaloids (8.0 g, 0.30%) were fractionated on a column of silica gel 60 (70–230 Mesh, Merck). Elution was done using CH_2Cl_2 gradually enriched with MeOH (100:0, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9, 90:10, 88:12, 80:20, 70:30, 60:40, and 50:50) and finally with pure 100% MeOH. The eluents of 50 ml were collected in a conical flask which was then concentrated. The collected fractions were grouped into a series of fractions, depending on the similar spot on TLC detected under UV light. Each series were then treated separately to isolate and purify its alkaloid by extensive CC followed by preparative TLC.

The fractions collected were grouped into a series of fractions, monitored with TLC. Each series were then treated separately to isolate and purify its alkaloid by extensive CC followed by preparative TLC. The purity of the alkaloids isolated was detected by TLC, using several solvent systems. The yield obtained for pure alkaloids and the solvent system applied for isolation are given, tavoyanine (**1**) (3.1 mg; 0.00011%; $C_{21}H_{25}NO_5$ 99:1), laetanine (**2**) (2.8 mg; 0.00001%; $C_{18}H_{19}NO_4$ 99:1), roemerine (**3**) (8.2 mg; 0.0003%; $C_{18}H_{17}NO_2$ 98:2), laurolitsine (**4**) (8.8; 0.00032%; $C_{18}H_{19}NO_4$ 97:2), boldine (**5**) (7.3 mg; 0.00027%; $C_{19}H_{21}NO_4$ 93:7), and sebiferine (**6**) (4.5 mg; 0.00016%; $C_{20}H_{23}NO_4$ 95:5).

3.3.1. Tavoyanine A (1)

$C_{21}H_{25}NO_5$, dark brown amorphous solid, [α]25 D+0.016 (c 0.06, CH_3OH). Yield: 0.1%. UV (MeOH) λ_{max} (log ε): 283 and 305 nm. IR ν_{max} ($CHCl_3$): ν_{max} 3360, 2928, 2825, 1590, 1510, 1465, 1258, 1080, 995, and 750 cm^{-1}. See Table 1 for ^1H NMR and ^{13}C NMR spectral data. HR-ESI-MS: *m/z* 372.1776 $[M+H]^+$ (calcd for $C_{21}H_{26}NO_5$, 372.1811).

3.4. In vitro antiplasmodial activity assay

Human malaria parasites were cultured according to the method of Trager and Jensen [22]. The antimalarial activity of isolated compounds was determined by the procedure described by Budimulja *et al.* [23]. In brief, stock solutions of the samples were prepared in DMSO (final DMSO concentrations of <0.5% and kept at −20 °C

until used) and were diluted to the required concentration with complete medium (RPMI 1640 supplemented with 10% human plasma, 25 mM HEPES and 25 mM $NaHCO_3$) until the final concentrations of samples in culture plate wells were 10, 1, 0.1, 0.01, and 0.001 μg/ml. The malarial parasite *P. falciparum* 3D7 clone was propagated in a 24-well culture plates. Growth of the parasite was monitored by making a blood smear fixed with MeOH and stained with the Giemsa stain. The antimalarial activity of each compound was expressed as an IC_{50} value, defined as the concentration of the compound causing 50% inhibition of parasite growth relative to an untreated control.

The percentage of growth inhibition was expressed according to following equation:

$$\text{Growth inhibition } \% = 100 - [(\text{test parasitemia}/\text{control parasitemia}) \times 100].$$

Chloroquine: IC_{50} 0.0069 μg/ml.

Acknowledgments

We also like to thank Dr Halo-N and Mr Che Yusof Che Mat for their advice and support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors gratefully acknowledge the financial support provided by PPP Grant (PV091/2011B) and BKP Grant (BKP069-2016) from University of Malaya.

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