Antiplasmodial and Antioxidant Isoquinoline Alkaloids from *Dehaasia longipedicellata*

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Abstract

The crude extract of the bark of *Dehaasia longipedicellata* exhibited antiplasmodial activity against the growth of *Plasmodium falciparum* K1 isolate (resistant strain). Phytochemical studies of the extract led to the isolation of six alkaloids: two morphinandienones, (+)-sebiferine (**1**) and (−)-milonine (**2**); two aporphines, (−)-boldine (**3**) and (−)-norboldine (**4**); one benzlyisoquinoline, (−)-reticuline (**5**); and one bisbenzylisoquinoline, (−)-*O*-*O*-dimethylgrisabine (**6**). Their structures were determined on the basis of 1D and 2D NMR, IR, UV, and LCMS spectroscopic techniques and upon comparison with literature values. Antiplasmodial activity was determined for all of the isolated compounds. They showed potent to moderate activity with IC_{50} values ranging from 0.031 to 30.40 µM. (−)-*O*-*O*-dimethylgrisabine (**6**) and (−)-milonine (**2**) were the two most potent compounds, with IC_{50} values of 0.031 and 0.097 µM, respectively, that were comparable to the standard, chloroquine (0.090 µM). The compounds were also assessed for their antioxidant activities with di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (IC_{50} = 18.40–107.31 µg/mL), reducing power (27.40–87.40%), and metal chelating (IC_{50} = 64.30 to 257.22 µg/mL) having good to low activity. (−)-*O*-*O*-dimethylgrisabine (**6**) exhibited a potent antioxidant activity of 44.3% reducing power, while di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium and metal chelating activities had IC_{50} values of 18.38 and 64.30 µg/mL, respectively. Thus it may be considered as a good reductant with the ability to chelate metal and prevent pro-oxidant activity. In addition to the antiplasmodial and antioxidant activities, the isolated compounds were also tested for their cytotoxicity against a few cancer and normal cell lines. (−)-Norboldine (**4**) exhibited potent cytotoxicity towards pancreatic cancer cell line BxPC-3 with an IC_{50} value of 27.060 ± 1.037 µM, and all alkaloids showed no toxicity towards the normal pancreatic cell line (hTERT-HPNE).

Abbreviations

BHA:	butylated hydroxyanisole
DCE:	dichloromethane crude extract
DPPH:	di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium
EDTA:	ethylenediaminetetraacetic acid
FRAP:	ferric reducing power assay
ROS:	reactive oxygen species
Rf:	retention time
SOD:	superoxide dismutase
TLC:	thin-layer chromatography

Introduction

Malaria is an important parasitic disease transmitted to humans by the bite of an infected female mosquito. Approximately 219 million people worldwide are affected by malaria and 600 000 deaths in 2010 have been reported in the World Malaria Report [1]. Malaria remains one of the most important infectious diseases in the developing world. In Malaysia, the parasites *Plasmodium falciparum, P. knowlesi*, and *P. vivax* are recognized as the common cause of malaria. Symptoms of malaria include fever, shivering, joint pain, vomiting, and retinal damage [2].

Global warming could increase malaria by expanding the area in which the ambient temperature and climate conditions are suitable. This could lead to the resistance of common antimalarial drugs such as artemisinin-based monotherapies. Parasite resistance to artemisinin has now been detected in 4 countries: Cambodia, Myanmar, Thailand, and Vietnam [1]. Thus, there

is an urgency for research to be done on new antimalarial drugs from natural resources [3].

Antioxidants are widely used as food additives to provide protection against oxidative degradation of food by free radicals [3]. Insufficient levels of antioxidants or inhibition of the antioxidant enzymes can cause oxidative stress. The antioxidant activity is important in the treatment of malaria since oxidative stress is normally synchronized with a malaria infection [4,5]. Antioxidant activity is believed to act in a synergistic way, protecting the body against oxidative stress [6]. In our continuing interest in searching for new and biologically active compounds [7–11], under the framework of the Malaysian-French scientific collaboration, a survey of several crude extracts from Malaysian plants has shown that *Dehaasia longipedicellata* (Ridl.) Kosterm. exhibited promising antiplasmodial activity (IC_{50} = 1.30 µg/mL). Therefore, we have embarked on the investigation of the antiplasmodial and antioxidant activities of compounds isolated from *D. longipedicellata*. Hence, in this study, we communicate the isolation of alkaloids from the active extract and their bioactivities, antiplasmodial and antioxidant.

Dehaasia is a member of the Lauraceae family. It is an evergreen tree of moderate size, with large leaves, found growing in the western parts of Malaysia, China, and the Philipines [12]. About 35 species of *Dehaasia* are spreadout worldwide and 9 species are found in Malaysia [13]. *Dehaasia* is locally known as "gajus hutan" or "pekan", and the timber is durable and used for building houses. *D. longipedicellata* is a small tree with leaves that are apex pointed, blades that are soft and hairy on the undersurface, and are broadly elliptic to obavate. According to the Sakai of the Tapah Hills, the fruit is very poisonous. This is the first report on the antiplasmodial and antioxidant activities of isoquinoline alkaloids from *Dehaasia*.

Results and Discussion

In spite of the large diversity of the compounds which have been studied phytochemically from the *Dehaasia* genus [14–17], this report communicates for the first time the occurrence of (+)-sebiferine (**1**), (−)-boldine (**3**), (−)-norboldine (**4**), (−)-reticuline (**5**), and (−)-*O*-*O*-dimethylgrisabine (**6**) in *D. longipedicellata* (**Fig. 1**). Structural elucidation was performed with the aid of spectroscopic methods, notably UV, IR, LCMS, and 1D and 2D NMR (COSY, HMBC, HMQC, and NOESY).

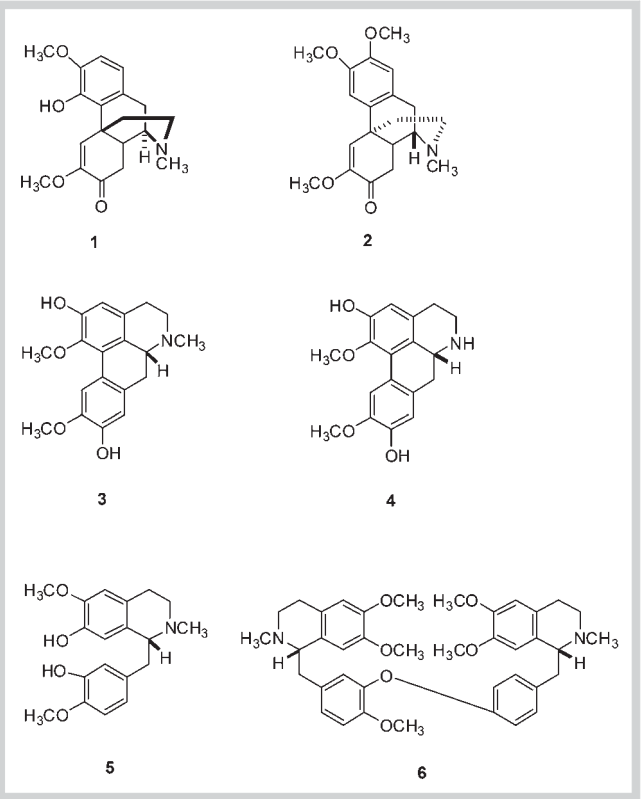

Fig. 1 Structures of isolated compounds from *Dehaasia longipedicellata*.

Based on the potent screening result of the DCE (IC_{50} = 1.30 µg/mL), the compounds isolated from the bark of *D. longipedicellata* were then subjected to *in vitro* antiplasmodial evaluation against a chloroquine-resistant strain of *P. falciparum*, K1 (**Table 1**).

Among the six compounds evaluated for their antiplasmodial activity, (−)-*O*-*O*-dimethylgrisabine (**6**) clearly showed the most potent *in vitro* antiplasmodial activity with an IC_{50} value of 0.031 µM, which was slightly better than the positive control chloroquine. In addition, (−)-milonine (**2**) also displayed a strong inhibition capacity with an IC_{50} value of 0.097 µM, followed by (−)-boldine (**3**), (−)-norboldine (**4**), (+)-sebiferine (**1**), and (−)-reticuline (**5**).

(−)-*O*-*O*-dimethylgrisabine (**6**), a bisbenzylisoquinoline, also showed a high scavenging activity of free radical DPPH with an

Table 1 Antiplasmodial and cytotoxicity activities of isolated compounds from *Dehaasia longipedicellata*.

	IC_{50}†				
Compounds	**K1**	**A549**	**A375**	**BxPC-3**	**hTERT- HPNE**
(+)-Sebiferine (**1**)	22.460	> 200	> 200	93.390 ± 5.564	> 200
(−)-Milonine (**2**)	**0.097**	> 200	> 200	> 200	> 200
(−)-Boldine (**3**)	2.602	117.570 ± 0.067	112.530 ± 3.484	45.500 ± 2.949	> 200
(−)-Norboldine (**4**)	9.284	> 200	82.890 ± 9.699	27.060 ± 1.037	> 200
(−)-Reticuline (**5**)	< 30.400	> 200	97.600 ± 6.561	82.570 ± 0.844	> 200
(−)-*O*-*O*-dimethylgrisabine (**6**)	**0.031**	> 200	82.850 ± 8.735	> 200	> 200
Chloroquine	**0.090**				
Artemisinin	0.002				
Cisplatin		17.520 ± 1.769	35.900 ± 5.199	26.860 ± 0.993	24.670 ± 0.367

* Results expressed as mean ± SE; † results expressed in µg/mL for extracts and µM for pure compounds or standards. **Bold** numbers indicate most active compounds comparable to the standard.

Compounds	IC_{50} DPPH activity (μg/mL)	% FRAP	IC_{50} metal chelating activity (μg/mL)
(+)-Sebiferine (**1**)	107.31	45.69	185.77
(−)-Milonine (**2**)	58.07	27.39	214.35
(−)-Boldine (**3**)	44.84	34.37	257.22
(−)-Norboldine (**4**)	79.97	52.10	157.16
(−)-Reticuline (**5**)	50.72	87.43	107.36
(−)-*O*-*O*-dimethylgrisabine (**6**)	**18.38**	**44.31**	**64.31**
Ascorbic acid (standard)	13.69		
EDTA (standard)		83.74	
BHA (standard)			19.60

Table 2 Antioxidant activities of compounds from the bark of *Dehaasia longipedicellata*.

Bold numbers indicate most active compounds comparable to the standard.

IC_{50} of 18.38 μg/mL and a moderate reducing power of 44.31 %. It also showed high metal chelating activity with an IC_{50} of 64.31 μg/mL. Shi et al. [18] have reported tetrandrine, a bisbenzylisoquinoline, as having antioxidant activity against ROS and SOD. (−)-Milonine (**2**), (−)-boldine (**3**), and (−)-reticuline (**5**) showed moderate DPPH and metal chelating activities. Whereas, (−)-norboldine (**4**) and (+)-sebiferine (**1**) showed low DPPH activity with moderate metal chelating activity (**Table 2**).

Plasmodium parasites from malaria infection are synergistic to high levels of oxidative stress. Oxidative stress will induce the generation of ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and superoxide anion radical ($O_2^{\bullet -}$) [19,20]. The generation of ROS may lead to erythrocyte membrane damage and the amount of hydroxyl radical of infected erythrocytes was twice as much compared with normal erythrocytes [4,21,22]. A study conducted with 100 Gabonese children with severe *P. falciparum* malaria verified the increase of ROS during acute malaria [23].

According to several studies, free radicals and ROS are no longer seen as destructive factors but as messengers, such as for the iron requirement in the host-parasite interaction for oxygen transport, respiration, and enzyme activities [24–26]. *Plasmodium* parasites need iron for survival and replication [27]. Therefore by having an iron chelating agent, the iron needed for the survival of the *Plasmodium* will be chelated and suppressed by the antioxidant compound, which could lead to the killing of the *Plasmodium* parasite [28]. Mabeza et al. [29] have reported on iron chelation therapy with flavonoids for malaria patients. Since (−)-*O*-*O*-dimethylgrisabine (**6**) possess a high chelating activity as part of its antioxidant effect, it could decrease the presence of iron and provide a more effective treatment for malaria. The present study showed that (−)-*O*-*O*-dimethylgrisabine (**6**) exhibited both iron binding and antiplasmodial activity, and the latter is comparable to chloroquine. Thus, the synergism between antimalarials and antioxidants exists through metal chelating activity for malaria treatment [30]. Therefore, (−)-*O*-*O*-dimethylgrisabine (**6**) could be a good candidate for further drug discovery development of potential antimalarial agents possessing antioxidant capability.

All of the isolated compounds showed no potency against lung (A549) cancer cells and weak cytotoxicity against skin (A375) cancer cells with IC_{50} values below 100 μM for (−)-norboldine (**4**) (82.890 ± 9.699 μM) and *(−)-O-O*-dimethylgrisabine (**6**) (82.850 ± 8.735 μM). However, for pancreatic cancer cells (BxPC-3), a great potency was shown by (−)-norboldine (**4**) with an IC_{50} of 27.060 ± 1.037 μM. The same compounds were tested against normal pancreatic cells (hTERT-HPNE) and no cytotoxicity was observed. Therefore, (−)-norboldine (**4**) should be studied further as a potential lead for drug discovery in cancer studies (**Table 1**).

Materials and Methods

General experimental procedures

Spectra were recorded on the following instruments: UV, Shimadzu UV-250, UV-visible spectrometer; IR, Perkin Elmer 1600; NMR, AVN BRUKER with $CDCl_3$ as the solvent. These were used to obtain the 400 MHz for the proton spectrum and 100 MHz for the carbon spectum. Mass spectra were obtained using Agilent Technologies 6530 accurate-mass Q-TOF LC/MS, with a ZORBAX Eclipse XDB-C18 rapid resolution HT 4.6 mm i.d × 50 mm × 1.8 μm column. All solvents, except those used for bulk extraction, are AR grade. Column chromatography separations were conducted using Merck silica gel 60 (230–400 mesh) and silica gel 60 F_{254} for TLC monitoring. Glass and aluminium supported silica gel 60 F_{254} plates were used for TLC. TLC spots were visualized under UV light (254 and 365 nm) followed by spraying with Dragendorff's reagent for alkaloid detection. HPLC was performed on a Waters system equipped with a binary gradient module (Waters 2545), system fluidics organizer (Waters SFO), photoiodide array detector (190–600 nm, Waters 2998), and a sample manager (Waters 2767). Chromatographic analysis and separations were performed on a Chromalith semiprep RP-18 endcapped HPLC column. The samples were eluted at a flow rate of 4 mL/min.

Plant material

The bark of *D. longipedicellata* was collected on 29 February 2009 at Sungai Tekam Reserve Forest, Jerantut, Pahang by the phytochemical group of the Department of Chemistry, Faculty of Science, University of Malaya. The plant specimen was identified by Mr. Teo Leong Eng and Mr. Din Mat Nor. A voucher specimen (KL5634) has been deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

Extraction and separation

Dried, grounded bark of *D. longipedicellata* (2.0 kg) was first defatted with hexane twice for a 3-day period. The hexane extracts were combined and the solvent was evaporated. The plant material was dried up and then soaked with 25 % NH_4OH for 2 hours. It was then macerated twice for a 3-day period with dichloromethane. DCE was finally concentrated to give a crude alkaloid extract of 18.7 g. DCE 10.5 g was subjected to column chromatography

over silica gel (0.04–0.063 mm; 6 × 65 cm) eluting with a mixture of a dichloromethane : methanol solvent in proportions of 100 : 0, 99 : 1, 98 : 2, 97 : 3, 96 : 4, 95 : 5, 94 : 6, 92 : 8, 90 : 10, 80 : 20, 50 : 50, and 0 : 100, v/v, each 1000 mL, in gradient to obtain 7 main fractions (Fr. 1 – Fr. 7) based on their TLC profiles. Further purification of Fr. 4 by preparative TLC (1.00 g using [CH_2Cl_2 : MeOH at a ratio of 97 : 3, v/v], saturated with NH_4OH; Dragendorff reagent) led to the isolation of (+)-sebiferine (**1**) [20 mg, R_f: 0.58, ($[\alpha]_D^{27}$ +92.31, *c* = 0.00013, MeOH)], (−)-milonine (**2**) [17 mg, R_f: 0.42, ($[\alpha]_D^{27}$ − 109.09, *c* = 0.00011, MeOH)], and (−)-boldine (**3**) [10 mg, R_f: 0.33, ($[\alpha]_D^{27}$ − 66.67, *c* = 0.00003, MeOH)]. Preparative TLC of Fr. 5 (80 mg [CH_2Cl_2 : MeOH 96 : 4, v/v] saturated with NH_4OH; Dragendorff reagent) yielded (−)-reticuline (**5**) [10 mg, R_f: 0.38, ($[\alpha]_D^{27}$ − 66.67, *c* = 0.00003, MeOH)] and Fr. 6 (70 mg [CH_2Cl_2 : MeOH 96 : 4, v/v] saturated with NH_4OH; Dragendorff reagent) yielded (−)-*O*-*O*-dimethylgrisabine **6** (5 mg, R_f: 0.83, ($[\alpha]_D^{27}$ −50.00, *c* = 0.00004, MeOH)]. Fr. 7 (200 mg) was further purified by HPLC on a Chromolith semiprep RP-18 endcapped (10–100 mm) Merck kGaA column with the following solvent system: acetonitrile + 0.1% formic acid-water + 0.1% formic acid (90 : 10) at a flow rate of 4.0 mL/min to give (−)-norboldine (**4**) (10 mg, $[\alpha]_D^{27}$ − 100.00, *c* = 0.00010, MeOH). The Rf of (−)-norboldine (**4**) was 2.00 minutes. The degree of purity of the isolated compounds was determined by ^{1}H NMR spectroscopy and was found to be 99% for compounds **1, 2, 3**, and **4**, and 98% for compounds **5** and **6**.

Antiplasmodial assay

The DCE and the isolated compounds were evaluated for their *in vitro* antiplasmodial activity against *P. falciparum* strain K1, which is resistant to chloroquine. Chloroquine (purity 98.0%) and artemisinin (purity 98.0%) were purchased from Sigma Chemicals and used as positive controls. Isolated and standard compounds were maintained in a continuous culture as described by Trager and Jensen [31], with some modifications. The synchronization of the malaria culture to one stage follows the method of Lambros and Vanderberg [32]. Antiplasmodial activity was evaluated using a histidine-rich protein II (HRPII) assay from Noedl et al. [33]. The antiplasmodial 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 the untreated control.

Antioxidant assay

DPPH assay: The DPPH antioxidant assay was determined as described by Shimada et al. [34]. Briefly, DPPH (1 mL, 0.1 mM) dissolved in ethanol was added to an ethanol solution (3 mL) of the tested compounds at different concentrations (0, 50, 100, 150, and 200 µg/mL). An equal volume of ethanol was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance at 517 nm was measured with a UV–VIS spectrophotometer. The percentage of scavenging of DPPH was calculated using the following equation:

$$\text{DPPH scavenging effect } (\%) = \frac{A^\circ - A1}{A^\circ} \times 100$$

Where A° is the absorbance of the control reaction and A1 is the absorbance in the presence of the sample. Ascorbic acid (purity 99.0%) was purchased from Sigma and used as the standard reference.

Ferric reducing power assay (FRAP): The reducing power was determined using the method of Oyaizu [35]. The tested compounds (0.5 mL) dissolved in ethanol at different doses (0, 50, 100, 150, and 200 µg/mL) were mixed with a phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (0.5 mL, 1%). The mixture was then incubated at 50 °C for 20 min. A portion of trichloroacetic acid (0.5 mL, 10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm (1000 g). The upper layer of the solution (0.5 mL) was mixed with distilled water (0.5 mL) and $FeCl_3$ (0.1 mL, 0.1%) for 10 min, and then the absorbance was measured at 700 nm in a spectrophotometer. EDTA (purity 99.0%) was purchased from Sigma and was used as the standard reference.

Metal chelating activity assay: The chelation of ferrous ions was estimated according to the method of Dinis et al. [36]. Briefly, tested compounds dissolved in ethanol (0.95 mL, at different doses of 0, 50, 100, 150, and 200 µg/mL) were added to a solution of $FeCl_2$ (2 mM, 50 µL). The reaction was initiated by the addition of ferrozine (5 mM, 200 µL), and then the mixture was shaken vigorously and left standing at room temperature for 10 min. After equilibrium was reached, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of the ferrozine-Fe^{2+} complex of each sample was calculated according to the following formula:

$$\% \text{ Inhibition} = [(\text{abscontrol} - \text{abssample})/\text{abscontrol}] \times 100$$

where abscontrol = absorbance reading of the control and abssample = absorbance reading of the sample. The IC_{50} value was determined from the graph of the percentage of inhibition against the concentration. BHA (purity 96.0%) was purchased from Acros organic and was used as the standard reference.

Cytotoxic assay

Cytotoxicity of the compounds was evaluated against three types of cancer cell lines: lung (A549), skin (A375), and pancreatic (BxPC-3); and one normal cell line, pancreatic (hTERT-HPNE). Cell lines were cultured in DMEM media supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 50 µg/mL gentamicin, and 10% penicillin-streptomycin (Pen Strep), maintained in a 37 °C humid atmosphere of a 5% CO_2 cell incubator.

Cells were plated into 96-well microplates and maintained in the cell incubator for 24 h. Then, 100 µL of samples were introduced in triplicate to a final concentration of 0.1–200 µM. Drug standards were also introduced to a final concentration of 0.03–2000 µM (cisplatin). Cells were further incubated for 48 h and cell viability was determined using an MTS assay kit (CellTiter 96® AQueous One Solution, Promega) according to the manufacturer's protocol. Microplates were returned to the incubator for 1–2 h and absorbance of the formazan product was read on a microplate reader at 490 nm with 690 nm as the background wavelength (Infinite 200, Tecan). The IC_{50} values of the samples and drug standards were determined using dose-response curves, and statistical analysis using the Student's t-test ($p < 0.05$) was performed in Prism 5.02 software (GraphPad Software, Inc.).

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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