In vitro antiplasmodial and antioxidant activities of bisbenzylisoquinoline alkaloids from Alseodaphne corneri Kosterm

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ABSTRACT

Objective: To study antiplasmodial and antioxidant activities of the isolation of alkaloids from the active dichloromethane extract of Alseodaphne corneri.

Methods: Phytochemical studies of the crude extract led to the isolation of six alkaloids using recycle high performance liquid chromatography and preparative thin layer chromatography. The antiplasmodial activity of the isolated compounds was evaluated using the histidine-rich protein II assay. The isolated alkaloids were also tested for their antioxidant activity using three different assays; DPPH, ferric reducing ability of plasma and metal chelating assays.

Results: Malaria infection caused the formation of free radicals which subsequently led to oxidative stress and apoptosis. The antioxidant properties of the alkaloids under investigation revealed that in addition to the antiplasmodial activity, the alkaloids could also prevent oxidative stress. (+)-laurotetanine and (+)-norstephasubine exhibited strong antioxidant activity using three different assays; DPPH, ferric reducing ability of plasma and metal chelating assays.

Conclusions: Interestingly, the two most potent compounds that exhibit antiplasmodial activity also exhibit good antioxidant activities. The crude dichloromethane extract and the isolated compounds exert substantial antiplasmodial and antioxidative activities which in turn suppress oxidative stress and cause less damage to the host.

1. Introduction

In modern world, vector-borne diseases still pose a great threat to human health. Those from mosquitoes have killed millions of human beings every year. Malaria is one of the serious vector-borne diseases that people can catch after bitten by Anophelles mosquitoes that are infected with parasites called Plasmodium. Four different types of Plasmodium [Plasmodium falciparum (P. falciparum), Plasmodium vivax, Plasmodium ovale, and Plasmodium malariae] affect humans. According to the World Health Organization [1], malaria is presently considered endemic and has caused approximately 198 million infections each year especially in developing countries, with more than 584,000 deaths in 2013. In Malaysia, 3850 malaria cases have been reported with 14 deaths in the same year [2]. Plasmodium knowlesi followed by P. falciparum are recognized as the main cause of malaria in Malaysia [3,4]. Although the malaria rate has decreased worldwide, however, the resistance to drug therapy has increased. Chloroquine resistant was first reported in Peninsular Malaysia as early as 1963 [2]. Present drugs, artesminin and chloroquine, have become ineffective because of the occurrence detected in 4 countries, Cambodia, Myanmar, Thailand and Vietnam that are resistant to P. falciparum [1].

Plasmodium must acquire nutrients from the environment (host) and convert it into other molecules or energy. This energy will eventually be used for survival and the reproduction of the parasites. The Plasmodium spp. requires amino acids for the synthesis of their proteins from the host or through the digestion of host hemoglobin. Growing substantial evidences have shown that malaria arises as a result of free radical generation from...
reactive oxygen species (ROS). Generation of ROS which induces oxidative stress has played an important role in the development of systematic complication in hosts caused by malaria infection [5,6]. Wide varieties of compounds with synthetic antioxidative properties have been used to reduce ROS formation and protect DNA, lipid and protein damage against oxidative stress. The preference of natural products is becoming significant due to the awareness of the public on the negative side effects caused by synthetic drugs [7]. Therefore, natural products could provide a template molecule which in turn leads to producing effective antimalarial drugs. The combination of drugs that possess different activities, artemisinin (antimalarial) and metalloporphyrins (oxidizing reagents) acting synergistically on strains of *P. falciparum*, has been reported [8]. It is known that this synergism improves the survival rates, reduces the development of resistance and might decrease the transmission of drug resistant parasites [9]. Thus, a single drug that can possess both antiplasmodial and antioxidant activities simultaneously suppressing malaria, might be a better choice compared to treatment with multiple drugs.

The genus *Alseodaphne* belongs to the Lauraceae family which is widely distributed from Yunan to West Malaysia, Sri Lanka and Burma. About 62 species have been identified till present, which include trees or shrubs whereby 20 species are known to occur in Malaysia. *Alseodaphne* is locally known as ‘Medang’. The plants of this genus have been used as furniture, and to build boats and houses. *Alseodaphne corneri* (*A. corneri*) is a small tree, with about 6 m in height. Terminal bud is covered with many glabrous scales. The twigs are stout and gray in color with prominent leaf scars [10]. This genus is a rich source of isoquinoline alkaloids particularly bisbenzylisoquinoline (BIQI) alkaloids that exhibit a wide range of pharmacological activities such as anti-inflammatory, antibacterial and vasorelaxant [11,12]. Most of BIQI have been isolated from families Menispermaceae, Berberidaceae, Monimiaceae and Ranunculaceae plants [13].

Our continuing activities on antiplasmodial screening of Malaysian plants have revealed that dichloromethane (CH2Cl2) bark extracts of *A. corneri* exhibited promising antiplasmodial activity (IC50 = 2.78 μg/mL) [14,15]. Hence, in this study, antiplasmodial and antioxidant activities of the isolation of alkaloids from the active extract were explored.

### 2. Materials and methods

#### 2.1. Instruments

Spectra were recorded using the following instruments; UV, Shimadzu UV-250, UV-Visible spectrometer; IR, Perkin Elmer 1600; NMR, AVN BRUKER with CDCl3 as the solvent to obtain the 400 MHz proton and 100 MHz carbon spectra. Mass spectra were obtained using on Agilent technologies 6530 Accurate-Mass Q-TOF liquid chromatography/mass spectrometry, with 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 were AR grade. Column chromatography separations were conducted by using Merck silica gel 60 (230-400 mesh) and silica gel 60 F254 for thin layer chromatography (TLC) monitoring. Recycle high performance liquid chromatography was performed on LC-908W-C60. Chromatographic analysis and separations were performed on a JAIGEL GS320 (21.5 mm internal diameter, 500 mm L, 13 mm) size exclusion column using methanol (MeOH) as the solvent. Glass and aluminum supported silica gel 60 F254 plates were used for TLC. TLC spots were visualized under UV light (254 nm and 365 nm) followed by spraying with Dragendorff’s reagent for alkaloid detection.

#### 2.2. Plant material

The bark of *A. corneri* Kosterm was collected from Hutan Simpan Kenderong, Gerik, Perak. The plant specimen was identified by Eng and Nor and a voucher specimen (KL5641) of this plant was deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

#### 2.3. Extraction and separation

Plant extraction was carried out by cold percolation. Dried ground bark of *A. corneri* (2.0 kg) was first defatted with hexane (17 L) for three days at room temperature. Then, the hexane extract was filtered and dried on the rotary evaporator. The residues were dried and then moistened with 25% ammonia solution and left for 2 h. The 40.0 g crude CH2Cl2 extract of alkaloids was subjected to column chromatography using silica gel (0.040–0.063 mm) as the stationary phase using mixtures of (CH2Cl2: MeOH) as the eluting solvent (100:0, 99:1, 97:3, 96:4, and 90:1) to obtain eight fractions (F1–F8). Alkaloid was purified from fraction F5 by a recycle high performance liquid chromatography over JAIGEL size exclusion column (21.5 mm internal diameter, 500 mm L, 13 mm) using MeOH as solvent at a flow rate of 4.0 mL/min. Three recycles in a duration of 40 min afforded (+)-gyrolidine with retention times of 35 min. Further purification of fraction F6 by a preparative TLC using CH2Cl2: MeOH with 94:6; v/v, saturated with NH4OH; Dragendorff’s reagent gave alkaloids (+)-O-methyllymacusine, (+)-2-norobabereine, (+)-laurotetanine, (+)-norstephasubine and (+)-stephasubine.

#### 2.4. Antiplasmodial assay

The crude CH2Cl2 extract and the isolated compounds were evaluated for their in vitro antiplasmodial activity against *P. falciparum* strain K1 which was resistant to chloroquine. Chloroquine diphosphate was used as positive controls. The screening was based on the ability to culture *P. falciparum* in human erythrocytes in vitro. It was maintained in continuous culture as described by Trager et al [16] with some modification [17]. The synchronization of the malaria culture to one stage was by Lambros et al [18]. Antiplasmodial activity was evaluated using histidine-rich protein II assay by enzyme linked immunoabsorbent assay [19]. Micro titration techniques were used to measure the activity of samples over a wide range of concentrations. All tests were performed in duplicate. Crude extract was dissolved in DMSO to produce a stock solution of 20 mg/mL. The stock solutions were subsequently diluted with deionized water at 20 concentrations of two-fold dilutions into two 96-well microtiter plates. A total of 10 μL of each concentration was transferred into another 96-well microtiter plates. A total of 200 μL of parasitized red blood cell suspension (1% parasitemia) were added to it. The mixtures were incubated for 24 h at 37 °C and were subsequently cooled at −20 °C to lyse the red blood cells. The plates were allowed at room temperature, and 20 μL of the blood suspension was dispensed into a new microtiter plate containing 100 μL MALSTAT reagent, 20 μL nitroblue tetrazolium and phenazine ethosulfate mixture. Absorbance was measured with an ELISA plate reader at
780 nm. The percentage inhibition at each concentration was determined and the mean of IC50 values of parasite sustainability was calculated using analysis. The antiplasmodial activity of each compound was expressed as an IC50 value defined as the concentration of the compounds causing 50% inhibition of parasite growth relative to untreated control [20,21].

2.5. Antioxidant assay

2.5.1. DPPH assay

The DPPH scavenging activity of pure compounds were tested based on the method previously published [22]. Briefly, 40 µL of purifed compounds at different concentrations were mixed with 200 µL of 50 µM DPPH solution in MeOH. The mixture was immediately shaken and incubated for 15 min in the dark at room temperature. The decrease in absorbance was measured at 517 nm with a microplate reader (Tecan Sunrise, Austria). Ascorbic acid was used as a standard and the control was MeOH. The percentage of inhibition activity of the compounds was calculated (n = 3) and results were presented in Table 1.

2.5.2. Metal chelating activity assay

The ferum ion chelating activity of the purified compounds was determined according to the previously published [22] by measuring the formation of the Fe²⁺-ferrozine complex in the reaction mixture. Briefly, 100 µL of purified compounds or standards (6.25 – 100 µg/mL) were mixed with 120 µL distilled water and 10 µL FeCl₂ (2 mM) in a 96-well microplate and the absorbance was read as blank. Then, 20 µL of ferrozine (5 mM) was added to the mixture to initiate the reaction. The reaction mixture was incubated at room temperature for 20 min and the absorbance at 562 nm was measured. The results were presented in Table 1 and butylated hydroxyanisole was used as the standard reference.

2.5.3. FRAP

The reducing power was determined using the method of Oyaizu [23]. The tested compounds (0.5 mL) dissolved in ethanol at different doses (0, 50, 100, 150, 200 µg/mL) were mixed with phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (0.5 mL, 1%). The mixture was then incubated at 50 °C for 20 min. A total of 0.5 mL trichloroacetic acid (10%) was added to each mixture to stop the reaction and the absorbance was measured at 700 nm. The results were presented in Table 1.

Table 1
Antiplasmodial and antioxidant activities of isolated compounds from A. corneri.

<table>
<thead>
<tr>
<th>Alkaloids</th>
<th>IC₅₀ K1 (µM)</th>
<th>IC₅₀ DPPH (µM)</th>
<th>% Ferric reducing ability of plasma (FRAP)</th>
<th>IC₅₀ ferum metal chelating activity (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Gyrolidine</td>
<td>0.666</td>
<td>280.95</td>
<td>41.32</td>
<td>252.14</td>
</tr>
<tr>
<td>(+)-O-methyllimacusine</td>
<td>1.193</td>
<td>265.09</td>
<td>65.87</td>
<td>263.32</td>
</tr>
<tr>
<td>(+)-2-Norobaberine</td>
<td>0.743</td>
<td>254.95</td>
<td>51.50</td>
<td>351.80</td>
</tr>
<tr>
<td>(+)-Laurotetanine</td>
<td>0.189</td>
<td>131.72</td>
<td>89.22</td>
<td>153.25</td>
</tr>
<tr>
<td>(+)-Norstephasubine</td>
<td>0.116</td>
<td>130.42</td>
<td>74.25</td>
<td>104.58</td>
</tr>
<tr>
<td>(+)-Stephasubine</td>
<td>1.315</td>
<td>233.82</td>
<td>94.01</td>
<td>338.44</td>
</tr>
<tr>
<td>Chloroquine diphosphate</td>
<td>0.090</td>
<td>4.11</td>
<td>4.12</td>
<td>4.13</td>
</tr>
<tr>
<td>Ascorbic acid (standard)</td>
<td>4.14</td>
<td>77.73</td>
<td>4.15</td>
<td>4.16</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (standard)</td>
<td>83.74</td>
<td>4.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butylated hydroxyanisole (standard)</td>
<td>108.74</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Structures of isolated compounds from A. corneri. 1: (-)-gyrolidine; 2: (+)-O-methyllimacusine; 3: (+)-2-norobaberine; 4: (+)-laurotetanine; 5: (+)-norstephasubine; 6: (+)-stephasubine.
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 FeCl₃ (0.1 mL, 0.1%) for 10 min, and then the absorbance was measured at 700 nm using a spectrophotometer. Ethylenediaminetetraacetic acid was used as the standard reference (Table 1).

3. Results

This report communicated the isolation and characterization of five BBQ alkaloids (Figure 1), (--)gyrolidine, (+)-O-methyllylmacusine, (+)-2-norobaberine, (+)-norstephasubine, (++)-stephasubine, and one aporphine alkaloid (++)-laurotetanine from the CH₂Cl₂ bark extract of A. corneri.

Structural elucidation was performed with the aid of spectroscopic methods notably; UV, IR, liquid chromatography/Mass spectrometry, 1D and 2D NMR (COSY, HMBC, HMQC, NOESY). The ESIMS showed a pseudomolecular ion peak m/z at 623.30, 623.30, 609.29, 328.16, 576.23, and 591.13 corresponding to alkaloids (--)gyrolidine, (+)-O-methyllylmacusine, (+)-2-norobaberine, (++)-laurotetanine, (+)-norstephasubine and (++)-stephasubine respectively. All the BBQ alkaloids belonged to type VI that had two diphenyl ether linkages between (C₁₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋˓→

3. Discussion

The importance of antioxidant activity was to reverse or minimize the oxidative damage to the hosts caused by Plasmodium parasite from malaria infection. The iron bound in hemoglobin is in Fe²⁺ form, and upon destruction of the hemoglobin, oxidation of Fe²⁺ to Fe³⁺ occurs, followed by the release of a free electron that eventually generate ROS. The generation of ROS such a hydrogen peroxide, hydroxyl radical and superoxide anion radical will induce oxidative damage [24,25]. Malaria infection induces the generation of the hydroxyl radical in the liver, which will most probably lead to the induction of oxidative stress and apoptosis [8].

It is worthy to note that, Plasmodium parasites from malaria infection invade the host hemoglobin to make their own protein. Upon destruction, free heme (ferum atom) will be released and converted to hemozoin which is important for the survival of Plasmodium parasites. Therefore by having strong ferum chelation, as shown by (+)-laurotetanine and (+)-norstephasubine, the killing of Plasmodium by binding to toxic free heme could prevent the formation of hemozoin [8,26].

The high antioxidant activities of those alkaloids may be due to the hydroxyl group that could donate electron to the free radicals which showed high radical scavenging activities that could suppress the oxidative stress on the host. The hydroxyl group that could donate electron to the notorious free radical could be the reason why it possesses potent radical scavenging activities (DPPH) [27,28].

Malaria infection causes the formation of free radicals which subsequently leads to oxidative stress and apoptosis. The crude CH₂Cl₂ extract and the isolated compounds from the bark of A. corneri exert substantial antiplasmodial and antioxidative effects through the DPPH free radical scavenging FRAP and metal chelating activities which could suppress oxidative stress that cause less damage to the host. The two most potent antiplasmodial alkaloids, (+)-laurotetanine and (+)-norstephasubine, also exhibited potent FRAP and metal chelating antioxidant activities. Therefore, these alkaloids may be good candidates for drug development of potential antimalarial properties possessing antioxidant capability.

Conflicts of interest statement

The authors declare no conflict of interest.

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