EVALUATION OF RENNELLIA ELLIPTICA AS POTENTIAL ANTIPLASMODIAL HERBAL REMEDY

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Abstract

Rennellia elliptica (Rubiaceae) has been used by local Jakun Community in the Endau Rompin State Park for the treatment of jaundice. Previous study has revealed the antiplasmodial activity of the root extract and major anthraquinones isolated from the roots. The present study entails the optimization of extraction methods, qualitative and quantitative analyses of selected marker anthraquinones and in vivo antiplasmodial activity along with toxicity and inhibition of β-hematin in vitro. HPLC profile showed the present of marker compounds as major constituents with content ranging 3-12 µg/g extract. The root extract showed potent antiplasmodial activity against rodent malaria, Plasmodium berghei with ED\textsubscript{50} value of 1.23 µg/ml BW. The major anthraquinones, damnacanthal and nordamnacanthal showed significant inhibition against β-hematin formation via lipids and HRP2 catalyses. However, the root extract is slightly toxic against hepatocyte cell. These data suggests that R. elliptica is a potential herbal remedy for malaria treatment and antiplasmodial of the root extract possibly due to the action of major anthraquinones.

Keywords: Rennellia elliptica, antiplasmodial, anthraquinones, Plasmodium, malaria

Abstrak

Rennellia elliptica (Rubiaceae) digunakan oleh masyarakat Jakun di Taman Negeri Endau Rompin untuk rawatan jaundis. Kajian terdahulu menunjukkan ekstrak akar dan antrakuinon utama daripada ekstrak akar mempunyai aktiviti antiplasmodial. Kajian semasa memperincikan pengoptimuman teknik pengekstrakan, analisis kualitatif dan kuantitatif terhadap sebatian utama, kuantitatif dan aktiviti antiplasmodial in vivo disamping ujian toksik dan hambatan terhadap pembentukan β-hematin secara in vitro. Profil HPLC menunjukkan sebatian utama dengan kandungan antara 3-12 µg/g ekstrak. Ekstrak akar menunjukkan potensi aktiviti antiplasmodial terhadap jangkitan malaria tikus Plasmodium berghei dengan nilai ED\textsubscript{50} 1.23 µg/ml BB. Antrakuinon utama, damnacanthal dan nordamnacanthal menunjukkan aktiviti yang signifikan terhadap pembentukan β-hematin melalui pemangkinan lipid dan HRP2. Walau bagaimanapun, ekstrak akar didapati sedikit toksik terhadap sel hepatosit. Data sedia ada menunjukkan R. elliptica merupakan herba yang berpotensi untuk rawatan anti malaria dan aktiviti antiplasmodial
1.0 INTRODUCTION

Malaria affected 3.2 billion people with 584 000 deaths reported and 78% were young children. The WHO program has seen to make an impressive progress on the reduction of total deaths; however, many are still at potential risk especially in the sub-Saharan Africa where 85% cases and deaths were reported [1]. Irrespective of this fact, only less than 1% of health research was devoted to tropical diseases as most occurrences are in poor countries, and this links to unprofitability. Only 13 new drugs out of 1300 were introduced for parasitic diseases between 1975 and 1999 [2]. The dramatic increase in malarial infection is observed today resulting mainly from the widespread use of insecticide which has led to Anopheles mosquitoes resistance to insecticides as well as the increase in malarial protozoa resistance to the antimalarial drugs such as chloroquine [3, 4] and artesiminin. The parasite resistance against the only affordable drugs for use in resource limited regions such as chloroquine and sulfadoxine-pyrimethamine [5] complicates the efforts of eradicating malarial infection. Many newer drugs were synthetically derived from chloroquine and artesiminin to overcome the parasite resistance against these drugs. However, it was not long before the cross-resistance develops due to their structural similarity with the parent drugs [6].

Many people in low income nations often rely on traditional herbal remedy as first line treatment for malaria [7]. Medicinal plants such as Morinda lucida Benth [8], Newbouldia laevis [9], Bulbine frutescens [10], Cassia siamea [11], Kniphofia foliosa [12], Stereospermum kunthianum [13], Tectona grandis [14], Pentas micrantha [15], Pentas longiflora and Pentas lanceolata [16] are widely used in Africa for treatment of fever or malaria. Investigation on the antiplasmodial activity and active compounds of these plants yielded active metabolites with antimalarial moiety. In South East Asia, about 210 species are listed for treatment of malaria [17].

Rennellia elliptica Korth. is a tropical shrub of about 1-2 m tall and can be found in lowland to hill forest to c. 500m above sea level. R. elliptica is locally known as ‘mengkudu rimba’ or ‘segemuk’ and popularly dubbed as Malaysian ginseng probably due to the appearance of its yellow roots. Among various Malaysian ethnic, this plant is also known as ‘kayu penawar apow’ [Dusun], ‘mengkudu hutan’ [Iban], ‘akar bumi’, ‘urap gandor’ [Sakai], ‘mengkudu gajah’, ‘lempeedu tanah’ and ‘sekenang’ [Jah Hut, Semelai]. R. elliptica is native to South East Asia and widely distributed in Peninsular Malaysia, Southern Thailand, Borneo and Indonesia [18]. The decoction of Rennellia elliptica is traditionally taken for the treatment of jaundice [19] and body aches, as postpartum tonic and as aphrodisiac [20]. During the random screening of selected Malaysian tropical plants for antiplasmodial activity, R. elliptica showed promising activity (4.04 µg/ml) which warranted further investigation. Following the screening program, extensive phytochemical study was carried out on the root extract yielding eleven antrakuinones in which four of them were found to possess strong antimalarial activity with IC50 values of less than 1 µM [21]. In order to establish the use of R. elliptica root extract as a potential herbal drug for the treatment of malaria, optimization of extraction methods, qualitative and quantitative HPLC analyses of the extract as well as the investigation of the extract toxicity and possible mechanism of actions are warranted. The chemotherapeutic targets selected were inhibition against β-hematin formation via lipids and HRP2 catalyses. Thus, this study aims to provide the data required to validate the safety and efficacy of the root extract of R. elliptica as potential antimalarial herbal drug.

2.0 METHODOLOGY

2.1 Plant Material

The roots of Rennellia elliptica Korth. were collected from Endau Rompin State Park, Pahang, Malaysia at an altitude of 165 m above sea level and were identified by Dr Shamsul Khamis of Universiti Putra Malaysia. The voucher specimen (SK1512/08) was deposited at Herbarium of the Institute of Bioscience, Universiti Putra Malaysia. The roots were air dried, cut into small pieces and ground to powder of about 1 mm mesh size using a grinder.

2.2 Optimization of Extraction

The dried root powder (10 g) was subjected to cold and soxhlet extraction using dichloromethane and ethanol. The dichloromethane extract (cold extraction) was used as control to compare the presence of marker antrakuinones with other extraction techniques. The filtrates were concentrated using rotary evaporator and kept in vial at 4°C for further analysis. For accelerated solvent extraction (Dionex ASE 150), 1 g of root powder was used every cycle (10 minutes) by
manipulating composition of ethanol and water at different temperature (H2O: EtOH; 100: 0, 80: 20, 60: 40, 40: 60, 20: 80; Temperature, 60°C, 80°C, 100°C, 140°C) at a fixed pressure of 1000 psi. The filtrates were concentrated using freeze dryer. The percentage yield of root extract for different extraction technique was recorded. The root extracts were subjected to HPLC analyses to determine the presence of marker compounds.

2.3 Quantitative and Qualitative Analysis of Anthraquinones in Rennellia Elliptica

2.3.1 Sample Preparation

The root extract was dissolved in 100 μL CH2Cl2 and 900 μL MeCN:H2O (9:1, v/v) at 10 mg/ml. The sample was passed through SepPak C18 cartridge and Whatman nylon membrane filter (0.45 μM). The marker anthraquinones (nordamnacanthal, damnacanthal, 2-formyl-3-hydroxy-9,10-anthraquinone, 2-methyl-3-hydroxy-9,10-anthraquinone and 1,2-dimethoxy-6-methyl,9,10-anthraquinone) were obtained from extensive chromatographic separation as reported previously [21].

2.3.2 HPLC Analysis

HPLC analysis was executed using a Waters HPLC W600 coupled with 2996 PDA detector system (Waters, USA) equipped with autosampler (2000 μl). Analysis was carried out at room temperature using Sunfire column (C-18 250 mm x 5 μM x 4.6 mm i.d., Waters, USA). The pump was connected to two mobile phases: A: H2O and B: MeCN, and eluted at a flow rate of 1.0 ml/min. Formic acid (0.1 %) was added to the mobile phase. The mobile phase was programmed consecutively in a linear gradient as follows: 0-20 min, 60-35 % A; 21-40 min, 35-5 % A; 41-45 min, 5-0 % A; 46-60 min, 0 % A. The injection volume was 10 μL. The column temperature was maintained at room temperature. The peaks were monitored at spectral window 254-400 nm. The analyses were run in triplicates and standard deviation and coefficient of variance were calculated. The content of the compounds were expressed as microgram per gram of extract (μg/g) by correlating the area of the analyte with the calibration curve built in concentrations of 20-140 mg/L using the generated equations: y = 34904x – 973938 adjusted R² 0.981303 (nordamnacanthal); y = 12626x – 128752 adjusted R² = 0.978949 (2-formyl-3-hydroxy-9,10-anthraquinone); y = 11263 – 3299 adjusted R² = 0.946985 (2-methyl-3-hydroxy-9,10-anthraquinone); y = 11263 – 32993 adjusted R² = 0.946985 (2-formyl-3-hydroxy-9,10-anthraquinone); y = 11263 – 32993 adjusted R² = 0.946985 (2-methyl-3-hydroxy-9,10-anthraquinone); y = 91493x – (1E+6) adjusted R² = 0.961946 (1,2-dimethoxy-6-methyl,9,10-anthraquinone); y = 12641x - 86520 adjusted R² = 0.946985 (damnacanthal).

2.4 Antiplasmodial Activity using Plasmodium berghei in Animal Model

4-day suppressive assay was performed on albino male mice using chloroquine-sensitive Plasmodium berghei as described by Peters (1965) with slight modifications [22]. The mice were maintained on standard animal pellets and water ad libitum. The mice (mean body weight: 20 ± 2g) were infected intraperitoneally (i.p.) with 0.2 ml infected blood in saline containing about 1 x 10⁷ P. berghei parasitized red blood cells at day 0. The mice were divided into groups of five per cage and the mice were administered orally with the sample immediately post infection for four consecutive days with three doses (1 mg/kg BW, 10 mg/kg BW, 100 mg/kg BW). The negative control group was treated with sodium carboxymethyl cellulose while the positive control group was treated with chloroquine diphosphate. On day 4 of the test, thin blood smears after tail blood sampling for each mouse were prepared and the blood film were fixed with methanol. The blood films were stained with Giemsa (Merck) and examined under microscope. The parasitemia was estimated by visual counting of at least 1000 erythrocytes. The antiplasmodial activity of each compound was expressed as an ED₅₀ value, defined as the concentration of the compound causing 50 % inhibition of parasite growth relative to an untreated control. The mice were maintained under institutional animal guidelines at the Department of Pharmacognosy and Phytochemistry, Universitas Airlangga, Indonesia.

2.4 β-Hematin Inhibiton Assay

2.4.1 Preparation of Heme Solution

Hemin chloride (16.3 mg) was dissolved in 1 ml of DMSO. The solution was passed through a 2.0-μm pore membrane filter to remove insoluble particles. The solution can be kept at 4°C up to 1 month as a stock solution [23] and diluted to 50 μM heme with 500mM acetate buffer, pH 4.8, prior to analysis.

2.4.2 Screening for β-hematin Formation Assay

Chloroquine (500 μM) was used as the drug positive control. The compounds were dissolved in 100% DMSO to prepare a stock concentration of 10 mg/ml and 1 mg/ml. 110 μl of heme solution (50 μM), freshly buffered by 500 μM acetate buffer (pH 4.8) was pipetted and added into the microwell plate. Finally, lecithin (2 μg/ml) was added into each well. After incubation at 37°C for 16 h, the plate was read at 405 nm. The fraction (f) of heme converted to β-hematin was calculated as in a previous study [24]:

\[ f = \left( \frac{A_{control} - A_{sample}}{A_{control} - A_{min}} \right) \]
Where $A_{\text{control}}$ is the absorbance of the heme without parasite lysate or lipid extract or an antimalarial at 405 nm, while $A_{\text{sample}}$ represents the absorbance of the heme in the presence of both parasite lysate or lipid extract and plant extracts. $A_{\text{min}}$ is the absorbance of the heme with parasite lysate or lipid extract in the absence of an antimalarial at 405 nm.

Percentage of inhibition of β-hematin by plant extracts was calculated by the following equations:

\[
\% \text{ Inhibition} = \left(1 - \frac{f}{100}\right) \times 100 = 100 \times \frac{A_{\text{sample}} - A_{\text{min}}}{A_{\text{control}} - A_{\text{min}}}
\]

### 2.4.3 HRP2 Assay

Antimalarial assay was carried out using HRP2 (HRP2 Kit Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). Diluted extract solution (100 µL) and final parasite culture (100 µL) were added into the microplate. The plates were then incubated for 72 h at 37°C. They were subsequently frozen-thawed twice to obtain complete hemolysis and stored at 30°C until further processing. Each of the hemolyzed culture samples (100 µL) was transferred to the ELISA plates, which were precoated with monoclonal antibodies against *Plasmodium falciparum* HRP2. The plates were incubated at room temperature for 1 h in humidified chamber. The plates were washed five times with the washing solution (200 µL of each well) and 100 µL of the diluted antibody conjugate was added to each well. After incubation for an additional 1 h in humidified chamber, the plates were washed with washing solution (200 µL) and 100 µL of diluted (1:20) chromogen tetramethyl benzidine (TMB) was added to each well. The plates were then incubated for another 15 min in the dark and 50 µL of the stop solution was added. The optical density values were measured using ELISA microplate reader at an absorbance maximum of 450 nm. The percent inhibition was calculated using the following formula:

\[
\% \text{ Inhibition} = \left(1 - \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{well}}}ight) \times 100
\]

### 2.5 Toxicity Study

The cytotoxicity of the samples was assessed by MTT assay. Hepatocyte, Huh7it cells were treated with serial dilution of the samples in 96-well plates. The condition of the cells was observed after 46 hours incubation and the toxicity was observed under microscope. The medium was removed from 96 well plates and then 150 µL of MTT solution (10%) was added to each well and the plates were incubated for 4 hours at 37°C. MTT solution was removed from each well and 100 µL of DMSO was added to each well prior to shaking for 30 seconds. The absorbance was measured using ELISA microplate reader at 560 and 750 nm.

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**3.0 RESULTS AND DISCUSSION**

Preparation of standardized extract is an authentication of herbal preparation as means of controlling the quality of plant material used for product manufacturing. The standardized extract should have an acceptable content of bioactive metabolites and safe from toxic impurities [25]. The present study included the establishment of the plant metabolites chemical profile of root extract using HPLC analyses. Nordamnacanthal 1, damnacanthal 2, 2-formyl-3-hydroxy-9,10-anthraquinone 3, 2-methyl-3-hydroxy-9,10-anthraquinone 4 and 1,2-dimethoxy-6-methyl-9,10-anthraquinone 5 were selected as marker compounds due to their potent antiplasmodial activity [21]. In order to determine the composition of each biomarker in the root extract, external calibration curves were constructed using five point concentrations. The concentration of compounds 1, 2, 3, 4 and 5 were determined at 3.57, 10.32, 4.47, 12.18 and 4.09 µg/g, respectively, with acceptable standard deviation (SD < 0.2) and coefficient of variance (CV < 5%). It was evident from the chromatogram (Figure 1), the marker anthraquinones present as major compounds in the root extract, thus it is submitted that the antiplasmodial action of the root extract is potentially due to the action of these metabolites.

[Figure 1 HPLC Chromatogram of Dichloromethane Extract of *R. elliptica* Korth]

*The chromatogram was extracted at 276 nm.*

Note: Rubiadin (4.848), Alizarin-1-methyl ether (7.815), 2-Hydroxy-3-methoxy-6-methyl-9,10-anthraquinone (22.477), 1-Hydroxy-2-methoxy-6-methyl-9,10-anthraquinone (23.297), 3-Hydroxy-2-methy-9,10-anthraquinone (24.443), 2-formyl-3-hydroxy-9,10-anthraquinone (28.864), Damcanthal (28.435), Lucidinin-ω-methyl ether (34.406), 1,2-dimethoxy-6-methyl-9,10-anthraquinone (36.066), Nordamcanthal (40.278). The unknown peaks at 10.104, 14.094, 16.547, 19.994 and 30.676 could be due to rennetlanone A and rennetlanone B, scopoletin, 4-hydroxy-3,5-dimethoxybenzaldehyde and 3b-acetateoleanan-13b, 28-tacolane [26].
In the previous study, dichloromethane root extract showed promising antiplasmodial activity [21]. However, dichloromethane is not a suitable extraction solvent for herbal preparation owing to the toxic properties of the solvent. Thus, the extraction of the dried root powder was attempted using ethanol and water in soxhlet and accelerated solvent extraction (ASE). Dichloromethane extract was also prepared as control to compare the presence of selected marker compounds. The extracts were then analyzed for the presence of selected biomarkers using Waters HPLC system. The accelerated solvent extraction (20: 80, H2O: EtOH; 100 °C) gave the comparable amount and quality of marker anthraquinones in the root extract as compared to dichloromethane extract (Table 1). The use of ethanol in cold and soxhlet extraction did not successfully extract the desired biomarkers compounds. The ASE can reduce the polarity of water and ethanol because high pressure and temperature will reduce the dielectric constant of water, which lowers its polarity and assists the extraction of more non-polar compounds [27, 28].

Table 1 Optimization of Extraction of Root Extract of Rennellia elliptica

<table>
<thead>
<tr>
<th>Type of extraction</th>
<th>Solvent/Condition</th>
<th>%Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold extraction</td>
<td>Dichloromethane (3 days)</td>
<td>0.97</td>
</tr>
<tr>
<td>(10 g)</td>
<td>Ethanol (3 days)</td>
<td>2.07</td>
</tr>
<tr>
<td>Soxhlet Extraction</td>
<td>Dichloromethane (2 hours)</td>
<td>0.58</td>
</tr>
<tr>
<td>(10 g)</td>
<td>Ethanol (2 hours)</td>
<td>2.28</td>
</tr>
<tr>
<td>Accelerated solvent extraction</td>
<td>100 : 0 (H2O:EtOH), 60°C, 10 min</td>
<td>0.55</td>
</tr>
<tr>
<td>(1 g)</td>
<td>50 : 50 (H2O:EtOH), 60°C, 10 min</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>20 : 80 (H2O:EtOH), 60°C, 10 min</td>
<td>3.03</td>
</tr>
<tr>
<td></td>
<td>20 : 80 (H2O:EtOH), 80°C, 10 min</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>20 : 80 (H2O:EtOH), 100°C, 10 min</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>20 : 80 (H2O:EtOH), 140°C, 10 min</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The screening of dichloromethane extract for antiplasmodial activity in vitro showed promising activity with IC50 value of 4.04 µg/mL [21]. Crude extracts with IC50 values of less than 50 µg/mL are considered effective as antimalarial agents [29]. Thus, the antiplasmodial activity of the extract was further evaluated in a 4-day suppressive test using P. berghei (ANKA strain) infected mice. The dichloromethane extract showed very strong activity with an ED50 value of 1.23 µg/mL.

The toxicity study was carried out to determine the selectivity of the root extract and marker compounds against the hepatocyte cell. The dichloromethane root extract showed mild toxicity with CC50 value of 318.0 µg/mL (Table 2). For both in vitro and in vivo studies, the selectivity indexes were determined at 78.7 and 258.3, respectively. The selected biomarkers showed no toxicity except 2-formyl-3-hydroxy-9,10-anthaquinone, nordamcanthal and damnacanthal which showed moderate toxicity with CC50 values of 181.34, 908.96 and 338.65 µM, respectively, with moderate selectivity index (Table 2). Thus, traditional preparation of the decoction of the root should be used with caution. Further study is required to assess the safety use of R. elliptica in traditional preparation.

One of the important chemotherapeutic targets in combating malaria infection is its food vacuole. The malaria parasite digests erythrocytes and releases heme [30] along with oxygen [31]. Free heme is toxic owing to its detergent-like properties that destabilizes and lyases membranes [6, 32], as well as inhibits the activity of several enzymes such as cysteine proteases [32] and consequently leads to the death of the parasite. The mechanism of heme detoxification can be broadly classified into two types; primarily via dimerization into hemozoin and secondarily via degradation of heme by glutathione and hydrogen peroxide [33]. Histidine-rich protein II (HRP2) [31, 32] and lipids [33] are proposed to catalyze the reaction but there are other evidences that the hemozoin formation may be spontaneous [34] and autocatalytic [35]. Drugs such as quine and chloroquine which targeted the prevention of β-hematin formation have a longer lifespan of effective use against malarial parasite. The parasite seems to have difficulties in finding alternative processes for haemoglobin utilization and heme detoxification as compared to other chemotherapeutic targets [36].

Table 2 The toxicity, β-hematin and HRP2 Assays of the root extract and selected compounds

<table>
<thead>
<tr>
<th>AQ</th>
<th>Toxicity CC50 µM</th>
<th>Antiplasmodial in vitro IC50 µM</th>
<th>Selectivity index</th>
<th>β-hematin IC50 µM</th>
<th>HRP2 IC50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>908.96</td>
<td>72.46</td>
<td>12.5</td>
<td>67.16 ± 0.2</td>
<td>4.37</td>
</tr>
<tr>
<td>2</td>
<td>338.65</td>
<td>51.28</td>
<td>6.6</td>
<td>5.32 ± 0.2</td>
<td>11.77</td>
</tr>
<tr>
<td>3</td>
<td>181.34</td>
<td>0.63</td>
<td>285.6</td>
<td>158.73 ± 0.2</td>
<td>nt</td>
</tr>
<tr>
<td>4</td>
<td>&gt;3968.3</td>
<td>0.34</td>
<td>12.500</td>
<td>138.65 ± 0.1</td>
<td>nt</td>
</tr>
<tr>
<td>5</td>
<td>&gt;3546.1</td>
<td>1.1</td>
<td>3225</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

| Root   | 318.0†           | 4.04†                         | 78.7             | nt               | nt           |

*Extracted from Osman et al. (2010)
nt- not tested; na- no activity
† - unit µg/mL

In the present study, the biomarkers were probed for their possible mode of action against β-hematin formation. Damnacanthal 1 and nordamcanthal 2 showed significant inhibition against hemozoin formation via HRP2 and lipids catalyses (Table 2). It is interesting to note that the damnacanthal 1 and nordamcanthal 2 showed weaker activity when tested against Plasmodium falciparum (3D7 strain) in vitro as compared to 2-formyl-3-hydroxy-9,10-anthaquinone 3 and 2-methyl-3-hydroxy-9,10-anthaquinone 4 [21]. 2-Formyl-3-hydroxy-9,10-anthaquinone 3 and 2-methyl-3-hydroxy-9,10-anthaquinone 4 showed the strongest antimalarial activity in vitro and their mode of action are yet to be discovered.
4.0 CONCLUSION

Rennelia elliptica is a potential herbal remedy against malarial infection and its activity possibly contributed by the major anthraquinones in the root. One of the identified modes of action is inhibition against β-hematin formation as shown by the marker compounds, nordamcanthal and dammacanthal. The mode of action of other marker compounds is yet to be determined. The root extract showed slight toxicity against normal cell with high selectivity towards Plasmodium falciparum.

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