Antiplasmodial Potential of Indonesian Medicinal Plants

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Research

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Abstract

**Background:** Species *A. paniculata* (Burm. f.) Nees known as “Sambiloto” and *P. pellucida* L. Kunth known as “Suruhan” are mainly distributed in Indonesia and their combination was used as a traditional medicine for treating malaria diseases. However, no information appears to have evaluated the antiplasmodial potential of the two plants. This research aimed to evaluate the antiplasmodial activity of the two plants and the species *P. pellucida* L. Kunth alone as a source of antiplasmodial agent.

**Methods:** *In vitro* test of the AP-PP and PP extracts against *Pf D-10* (chloroquine-sensitive) were performed as described by Desjardins *et al.* An *in vivo* test of the PP extract in mice infected with *Pb ANKA* was performed using Peters’ 4-day suppressive test. Parasitemia, growth and inhibition rates were determined via Giemsa-stained smear of blood and analyzed microscopically. Survival was followed up until day 21 post-infection.

**Results:** The increased ratio of the PP extract (20:80) exhibited significant antiplasmodial in contrast to the high ratio of the AP extract (IC$_{50}$, 62.01 mg/mL). Further evaluation of the PP extract alone displayed better antiplasmodial activity with an IC$_{50}$ value of 4.0 mg/mL. Furthermore, an *in vivo* test of the PP extract in BALB/c albino mice infected with *Pb ANKA* exhibited a significant chemosuppressive effect in a dose-dependent manner.

**Conclusion:** The increased ratio of the PP extract exhibited a major contribution for their activity. The PP extract alone showed better antiplasmodial activity than the AP extract and their combination. An *in vivo* test confirmed the efficacy of the PP extract in mouse model.

Introduction

Malaria is a serious global health problem caused by infection of the species *Plasmodium* transmitted to humans by female *Anopheles* mosquitoes [1]. This disease continues to be a world health issue because of the lack of preventative measures [1–3] as well as the increasing resistance to many of the existing antimalarial drugs such as quinine, chloroquine, amodiaquine, mefloquine, piperaquine, lumefantrine, primaquine, artemisinin derivatives and artemisinin-based combination treatments with artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine and artemether-lumefantrine [4]. Currently, there are no commercially available malaria vaccine [1], despite many decades of intense research efforts. Thus, chemotherapeutics using combination drugs appears to be the best option [5] and there is an urgent need to discover new antimalarial compounds. Medicinal plants have commonly been used as a source for new active compounds [6]. For example, Artemisinin, an antimalarial drug which was discovered from *Artemisia annua* L., by You-You Tu in China in the early 1970s [7]. Tropical plants are known to be a rich reservoir of bioactive metabolites, thus they are potential sources of new antimalarial drugs [8].

The family *Acanthaceae* is widely grown in tropical and subtropical regions [9]. Traditionally, this plant has been used to treat acute diarrhea, cough, common cold, inflammation, boils, skin eruptions, and
seasonal fever [10]. Phytochemical screening has revealed the presence of a variety of secondary metabolites with important pharmacological activities [11]. Species A. paniculata known as “Sambiloto” is mainly distributed from the northeast to the south of Indonesia and often consumed as a traditional medicine [12]. Although the antiplasmodial activity of this plant against Plasmodium falciparum strain have been reported [13], but traditional communities believed their combination with P. pellucida would be beneficial for the better antimalarial treatment [14, 15]. Because of P. pellucida use against malaria, we evaluated the activity of combination and different extracts against PfD-10 and the potential of P. pellucida plant as a source of antimalarial agent.

Methods

2.1. Plant Material

Fresh leaves and stems of the species A. paniculata and P. pellucida were collected from north Gorontalo, Gorontalo Province, Indonesia in June 2016. The plant was identified in the Herbarium Biology Laboratorium, Faculty of Mathematics and Natural Sciences, Universitas Negeri Gorontalo and voucher specimen (No. 130/H47.B4.Bio.Lab Bio/LL/2016; for P. pellucida and (No. 131/H48.B4.Bio.Lab Bio/LL/2016; for A. paniculata) were deposited at the herbarium.

2.2. Plant extraction

Dried leaves and stems of P. pellucida (2 kg) and A. paniculata (2 Kg) were macerated with methanol (12 L) at RT for 2 days. After filtrating, the filtrate was evaporated in vacuo to give the crude PP extract (211 g) and AP extract (278 g), respectively.

2.3. Parasite synchronization and maintenance

Malarial parasite PfD-10 strain (chloroquine-sensitive) was obtained from the University of Tokyo, Japan. Parasite PfD-10 was maintained in fresh O⁺ human erythrocytes with a hematocrit of 4% in RPMI 1640 culture medium (Gibco) supplemented with 10% (v/v) human serum, 32 mM NaHCO₃, 25 mM HEPES (N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid, Sigma Aldrich), 45 µg/mL hypoxanthine, and 50 µg/mL gentamicin and incubated at 37 °C under a gas mixture of 5% O₂, 5% CO₂, and 90% N₂. The parasites were synchronized with 5% sorbitol, and after 96 h, synchronized parasites were determined microscopically.

2.4. In vitro antiplasmodial activity

The crude AP-PP extract was prepared at 20 mg/mL stock solutions in DMSO. Chloroquine diphosphate (Sigma, Burlington, MA, USA) stock solution as an antimalarial reference was prepared in water (Milli-Q grade) at 1 mM, while 0.2% DMSO was used as a negative control. Except for CQ, the final solution contained 0.2% DMSO. An in vitro test was performed as described by Desjardins et al [16]. A suspension of 200 µL of synchronized parasites (0.5% parasitemia and 4% hematocrit) were incubated with various ratios of the AP-PP extract ranging from 20:80 to 80:20 in DMSO, CQ (final concentration of 1 µM) and
0.2% DMSO under the same conditions mentioned before. After incubation, a thin blood smear stained with Giemsa was prepared [17, 18]. *In vitro* test of the PP extract was performed at various concentration of 0.01 to 100 µg/mL. Parasitemia, parasite growth and inhibition rates were determined microscopically by calculating the number of infected erythrocytes from 500 erythrocytes. An analysis of dose–response curves was used to determine IC₅₀ values as the mean of three experiments (n = 3).

2.5. *In vivo* antiplasmodial activity

2.5.1. Experimental animals and parasites

Thirty (male) BALB/c albino mice aged 6–8 weeks and weighing 25–28 g were used in this study, provided by the Animal Experimental Development Unit in Gadjah Mada University, Yogyakarta, Indonesia. The mice were maintained RT, with food and water given *ad libitum* at the Animal Laboratory in the Institute of Tropical Disease, Universitas Airlangga. The parasite *Pb* ANKA strain was obtained from the Eijkman Institute for Molecular Biology, Jakarta, Indonesia. The parasite had been maintained at the Institute of Tropical Disease, Universitas Airlangga by a combination of passages in male BALB/c mice and cryoscopic storage.

2.5.2. Experimental design and treatment of mice

An *in vivo* antiplasmodial activity of the crude PP extract in mice infected with *Pb* ANKA was performed as described by Peter [19] with minor modifications. Thirty mice (BALB/c albino) were inoculated with the parasite *Pb* ANKA strain (10⁶ erythrocytes parasitized) intraperitoneally. The volume of inoculum was 200 µL. Mice were divided into five groups of six mice each (three experimental and two control groups). Three experimental groups were treated with the crude PP extract at a concentration of 1, 10 and 100 mg/kg/body in 0.5% CMC-Na (Total volume of 200 µL). A negative control group was treated with 0.5% CMC-Na and a positive control group was treated with chloroquine diphosphate (25 mg/kg/body) intraperitoneally once a day for four days (day-1 to day-4). The animals stopped receiving treatments after 4 days of treatments. On the fifth day, thin blood smears from the tail of the mice were prepared on a slide. After drying and fixing with methanol, the slide was stained with 15% Giemsa-stained solution [17, 18] for 10 min. The slide was finally rinsed with water and dried at RT. The percentage of parasitemia and parasites growth suppression were determined via Giemsa-stained smear of blood and analyzed microscopically. Survival was followed up until day 21 post-infection.

Results And Discussions

Crude methanolic extracts were successfully extracted from fresh leaves and stems of the species *A. paniculata* and *P. pellucida*. With crude extracts in hand, their ability to suppress parasitemia and inhibit parasite growth were evaluated. Following the incubation of synchronized *Pf* D-10 with the AP-PP extract at various ratios (80:20 to 20:80) in DMSO, CQ (1 µM) and 0.2% DMSO, a thin blood smear stained with Giemsa was prepared. Parasitemia, parasite growth and inhibition rates were determined microscopically.
The chemosuppression of parasitemia against the malarial parasite *Pf D-10* was evaluated (Figure 3a). The parasitemia rates decreased when *Pf D-10* were treated with the AP-PP extract. At a ratio of 80:20 to 40:60, the antiplasmodial activity of the AP-PP extract were limited (ca. 2.8 to 3.0 %). Interestingly, parasitemia rate decreased when the ratio of the PP extract was increased. Parasitemia rates at ratio of 30:70 and 20:80 exhibited significant activity (*p* < 0.05). Results of the *in vitro* test in Figure 1b indicate that the growth malarial parasite *Pf D-10* (chloroquine-sensitive) was suppressed by the AP-PP extract. The AP-PP extract exhibited higher parasite growth of *Pf D-10* at a ratio of 80:20 to 40:60, while at a ratio of 20:80 exhibited a significantly killing effect of 1.5 % (*p* < 0.05). The results in Figure 3c indicate that parasite *Pf D-10* was inhibited by the AP-PP extract. Although there is no significant inhibition of *Pf D-10* at ratio of 80:20 to 40:60, but the AP-PP extract at higher ratio of the PP extract (20:80) exhibited significant inhibition effect of 50% (*p* < 0.05). As expected, there was good correlation among the increased ratio of the PP extract and inhibition effects. The antiplasmodial activity of the AP-PP extract was increased in a ratio dependent manner. Furthermore, we determined the IC<sub>50</sub> values of the AP-PP extract at a ratio of 20:80. The extract was tested at a concentration of 0.01, 0.1, 1, 10 and 100 µg/mL and evaluated the inhibition rates after 48 h of incubation. An analysis of inhibition–response curves were used to determine the IC<sub>50</sub> value. The AP-PP extract at a ratio of 20:80 was considered to be marginally potent on the basis of an *in vitro* antiplasmodial activity of plant extract against *Plasmodium falcifarum* strain with an IC<sub>50</sub> value of 62.01 µg/mL [20].

Since the increased ratio of the PP extract exhibited significant activity in contrast to the increased ratio of the AP extract, it is essential to evaluate the PP extract to facilitate the discovery of their biological function and mode of action. We next evaluated the potential of the PP extract alone against parasite *Pf D-10*. The antiplasmodial activity of the PP extract was performed at a concentration ranging from 0.01 to 100 µg/mL. As shown in Fig. 2a, the PP extract possessed promising antiplasmodial activity against *Pf D-10*. At the dilute concentration of 0.01 µg/mL, the antiplasmodial activity of the PP extracts were limited (ca. 4.5%). Parasitemia rates were approximately 3.7%, 3.4%, and 2.4% at a concentration of 0.1, 1 and 10 µg/mL, respectively. Interestingly, at the highest concentration (100 µg/mL) the PP extract exhibited a significantly higher activity in reducing parasitemia of 1.2%. The results in Fig. 2a indicate the inhibition of the *Pf D-10* induced by the PP extract. The PP extract exhibited low suppression of *Pf D-10* at the lowest concentration (0.01 µg/mL), while at a concentration of 10 and 100 µg/mL exhibited significant inhibition effect of 51% and 92%, respectively (*p* < 0.01 and *p* < 0.001). DMSO was used as a negative control showed no antiplasmodial activity, but CQ as an antimalarial reference was more active than the test samples. The results, recorded in Fig. 2c, suggested that the PP extract displayed promising antiplasmodial activity on the basis of plant extract with IC<sub>50</sub> value of 4.0 µg/mL (IC<sub>50</sub> of a promising extract should be less than 10 µg/mL) [20].

A comparison with the results of Mishra *et al*., the *in vitro* antiplasmodial activity of *P. pellucida* L. Kunth possessed a significantly higher activity (IC<sub>50</sub>, 4.0 µg/mL) then *A. paniculata* (Burm. f.) Nees (IC<sub>50</sub>, 7.2 µg/mL). These results clearly show that the increased ratio of the PP extract in the combination of the AP-PP extract provoke better antiplasmodial activity on the basis *in vitro* assay. Although,
andrographolide is a major well known bioactive antimalarial compound from *A. paniculata*, however the phytochemical constituents from *P. pellucida* L. Kunth may provide stronger antiplasmodial activity. Therefore it is important to isolate and identify the compound(s) to facilitate the discovery of their biological function. Unlike the PP extract, the low responses of the AP and AP-PP extracts are presumably due to compound cytotoxicity rather than specific activity against the parasite itself or being negatively influenced by poor pharmacokinetics.

We next evaluated the potential of the PP extract in BALB/c albino mice infected with *Pb ANKA*. The *in vivo* efficacy of the PP extract was evaluated following the procedure described by Peter (a four-day suppressive test) [19]. BALB/c albino mice were inoculated with *Pb ANKA* intraperitoneally. After the fourth days of treatment with 0.5% CMC-Na as a negative control, chloroquine diphosphate as a positive control and the PP extract at a daily dose of 1, 10 and 100 mg/kg/body, Giemsa-stained thin blood smears were prepared on the fifth day for each mice. The parasitemia and parasite growth suppression were determined microscopically (Fig. 3a). The survival of mice was carefully recorded until day 21. The PP extract was considered to be partially active on the basis of an *in vivo* antiplasmodial activity with an ED$_{50}$ value of 12.86 mg/kg/ body weight (Fig. 3b). Clinically available drug quinine against *Pb ANKA* has an [ED$_{50}$] of 34 mg/kg/day and slow clearance [21]. The results in Fig. 3c indicate that at a daily dose of 1, 10 and 100 mg/kg/body after the fourth day treatment, the PP extract exhibited a chemosuppression of parasitemia against *Pb ANKA* in mice. At the lowest dose (1 mg/kg/body), intraperitoneally administration of the PP extract led to 4.4% of parasitemia in contrast to untreated mice (6.4%). Namely, 31% of parasites were killed induced by the PP extract. The rate of parasitemia reduced as the concentration of the PP extract was increased. The parasitemia rates decreased to approximately 3.2% and 2.4% at the PP extract concentrations of 10 and 100 mg/kg/body, respectively. Furthermore, the chemosuppression of parasitemia on day 0 to day 4 was also evaluated (Fig. 3d). Parasitemia rates of untreated mice were approximately 1.0% (day 0), 3.2% (day 1), 4.6% (day 2), 5.7% (day 3) and 7.2% (day 4), indicating that the number of parasites in the blood was consistently increasing after infection on day 0. At a daily dose of 1, 10 and 100 mg/kg/body, the PP extract exhibited a significant chemosuppressive effect against *Pb ANKA* in contrast to untreated mice. The parasite growth inhibition in mice was evaluated (Fig. 3e). The PP extract administered intraperitoneally in mice at a daily dose of 1, 10 and 100 mg/kg/body suppressed *Pb ANKA* in a dose dependent manner. Suppression rates were approximately 64% (100 mg/kg/body), 50% (10 mg/kg/body) and 31% (1 mg/kg/body), respectively (untreated mice defined as 0%). These results clearly demonstrated the parasite killing induced by the PP extract. Survival of infected mice were also increased due to the treatment of the PP extract. Untreated mice succumbed to death after 11 days of infection with *Pb ANKA*, while mice treated with 1, 10, 100 mg/kg/body of the PP extract died on days 13, 16 and 19 after treatment, respectively. A positive control groups treated with chloroquine diphosphate at a daily dose of 25 mg/kg/body all survived up to 21 days, indicating that the infected mice were completely cured of *Pb ANKA*. This is consistent with the results of Fang *et al.*[22]
Although many therapeutic antimalaria have been reported, insufficient efficacy has been a critical issue in treating malaria. Also, antimalarial drug resistance is a major hurdle. Peptides [23, 24] and amino acid-malaria drug conjugated [25–27] were reported for their antiplasmodial activity, however the chemical synthesis of hydrophobic peptide was very challenging [28, 29]. Tropical plants are well known as source of bioactive compounds, thus they are potential for the development of a new class antimalarial agent. Although, the use of the species A. paniculata and their combination with P. pellucida L. Kunth was practically used to treat malaria disease, however there is less information about the beneficial of the species P. pellucida alone for treating malaria disease in humans. Herein we report the potential of species P. pellucida from Indonesia as a source of new antimalaria compounds. This is the first report of the potential of P. pellucida as a source of antiplasmodial agent and we have shown its promising efficacy in an in vivo mouse model. Our results suggest a novel P. pellucida that has the potential as a source of antimalarial agent. We plan to do an in vivo test with the combination of the PP extract and an antimalarial drug artesunate [30] to improve the efficacy of drug action. In addition, we plan to isolate and analyze bioactive metabolites from the species P. pellucida that may be provide more efficacious in antiplasmodial activity and hope to contribute to the development of a new class of antimalarial agent.

Conclusions

In summary, the combination of the species A. paniculata and P. pellucida has been assessed in vitro antiplasmodial activity against Pf D-10. At a ratio of 20:80 exhibited significant antiplasmodial activity. In vitro test of the PP extract alone showed a significantly higher antiplasmodial activity in contrast to the AP extract alone and their combinations. In vivo analysis of the PP extract at a daily dose of 1, 10 and 100 mg/kg/body showed significant chemosuppressive effect in mice infected with parasite Pb ANKA. The raised concentrations of the PP extract exhibited dose-dependent manner.

Abbreviations

A. paniculata, Andrographis paniculata (Burm. f.) Nees; P. pellucida, Peperomia pellucida L. Kunth; AP extract, A. paniculata methanolic extract; PP extract, P. pellucida methanolic extract; Pf D-10, Plasmodium falciparum D-10 strain; Pb ANKA, Plasmodium berghei ANKA strain; DMSO, dimethyl sulfoxide; CMC-Na, Sodium carboxymethyl cellulose; RT, room temperature; IC\textsubscript{50}, the half maximal inhibitory concentration; ED\textsubscript{50}, effective dose in 50%; NC, negative control; CQ, chloroquine.

Declarations

Authors’ contributions

N.B., and B.S designed research; N.B., M.A.M., Y.K.S and A.W performed research; B.S and W.J.A.M analyzed data; J.S and B.S wrote the paper and supervised the project. All authors read and approved the final manuscript.
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Competing interests

The authors declare no competing interests.

Availability of data and materials

All data generated and analyzed are included in this research article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Permission and approval for animal experiments were certified by the Faculty of Veterinary Medicine, Universitas Airlangga, with ethical clearance No: 757-KE/2017.

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Figures

In vitro test of the crude A. paniculata-P. pellucida methanolic extract (AP-PP extract) at various ratios (80:20 to 20:80). (a) Parasitemia, (b) Growth and (c) inhibition rates of untreated cells (0.2% DMSO), cells treated with the AP-PP extract and CQ (1 µM). (d) IC50 value of the AP-PP extract at a ratio of 20:80. An IC50 value was calculated using GraphPad Prism software. The results in Figure 1a indicate parasitemia rates of the AP-PP extract at a ratio of 80:20 (black), 70:30 (herringbone), 60:40 (polka dots), 50:50 (pencil striped), 40:60 (grey), 30:70 (hairline striped), 20:80 (white), NC (grid) and CQ (horizontal). All experiments were performed in triplicate (n = 3). Standard Deviation (SD) is indicated by the error bars. *p < 0.05, **p < 0.01, not significant (ns).
Figure 2

Antiplasmodial activity of P. pellucida methanolic extract (PP extract) against Pf D-10 strain. (a) Parasitemia and (b) inhibition rates of untreated cells (NC), cells treated with the PP extract at a concentration of 0.01 to 100 μg/mL and CQ at a final concentration of 1 μM. (c) IC50 value of the PP extract. An IC50 value was calculated using GraphPad Prism software. The results in Figure 2a indicate parasitemia rates of the PP extract at a concentration of 0.01 μg/mL (black), 0.1 μg/mL (white), 1 μg/mL
(pencil striped), 10 g/mL (herringbone), 100 g/mL (halftone), NC (grid) and CQ (horizontal). All experiments were performed in triplicate (n = 3). Standard Deviation (SD) is indicated by the error bars. *p < 0.05, **p < 0.01, ***p < 0.001, not significant (ns).

Figure 3

In vivo test of the PP extract in BALB/c albino mice infected with Pb ANKA strain. (a) In vivo four-day suppressive test. Infected mice were treated with 0.5% CMC-Na as a negative control (NC), CQ as a
positive control (25 mg/kg/body) and the PP extract at a daily dose of 1, 10 and 100 mg/kg/body. Mice received treatment on day 1 through 4. On day 5, Giemsa stained blood smears were prepared and analyzed microscopically. (b) ED50 value of the PP extract in mice. (c) Parasitemia after four days of treatment. (d) Chemosuppression of parasitemia on day 0 to day 4. (e) Inhibition rates after four days of treatment. (f) Survival rates of parasite Pb ANKA in BALB/c albino mice. Bars in Figure 3c and 3e indicate the parasitemia and parasite growth inhibition rates treated with 0.5% CMC-Na (NC) (grid), CQ (horizontal) and the PP extract at a daily dose of 1 mg/kg/body (pencil striped), 10 mg/kg/body (herringbone) and 100 mg/kg/body (halftone), while in Figure 3b and 3f indicate parasitemia progression and parasite survival rates on day 0 to day 4 treated with 0.5% CMC-Na (pink), CQ (carmine) and the PP extract at a daily dose of 1 mg/kg/body (green), 10 mg/kg/body (blue) and 100 mg/kg/body (red). SD is indicated by the error bars. ***p < 0.001, ****p < 0.0001.