培養中のBabesia gibsoniに対するArcangelisia flavaから得られたprotoberberine alkaloidsと20-hydroxyecdysoneの抗バベシア活性

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Antibabesial Activity of Protoberberine Alkaloids and 20-Hydroxyecdysone from *Arcangelisia flava* against *Babesia gibsoni* in Culture

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ABSTRACT. Bioassay-guided fractionation of the boiled extract from the stems of Arcangelisia flava led to the isolation of palmatine (1), berberine (2), jatrorrhizine (3), dihydroberberine (4) and 20-hydroxyecdysone (5). The chemical structures of these compounds were elucidated on the basis of their chemical and spectral evidence. The isolated compounds were evaluated for their growth inhibiting effects on *Babesia gibsoni* in culture for a week. Compounds (1–4) showed significant inhibitions at concentrations from 100 to 1.0 μg/ml, while compound 5 at a concentration of 100 μg/ml, only.

KEY WORDS: antibabesial activity, *Arcangelisia flava*, protoberberine alkaloid.

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Babesia gibsoni is an intraerythrocytic parasite that causes hemolytic anemia in wild and domestic dogs [1, 17]. It is well known that existing drugs for the elimination of *B. gibsoni* infection are not effective and have associated side effects. Therefore, for the treatment of *B. gibsoni* infected dogs, an alternative chemotherapeutic agent having few side effects is urgently needed. One possible source of such affordable treatment lies in the use of plant extracts.

Arcangelisia flava Merr. (Menispermaceae), a liana plant widely distributed in Central Kalimantan, Indonesia, has been traditionally used by local people for the treatment of malaria, dysentery, fever and as a tonic. The phytochemical investigations of this plant have previously shown the presence of thalifendine, dehydrocorydalmine, jatrorrhizine, pycnarrhine, berberine, palmatine, hydroxyberberine, limacine, homoaromoline and furanoditerpenes [3, 6, 13]. Berberine, a major representative of the protoberberine alkaloids, displays diverse biochemical and pharmacological actions while being relatively non-toxic to humans. The compound has been claimed to be therapeutically useful for the treatment of malaria and as an antimicrobial agent [4, 10, 12]. In addition, protoberberine alkaloids are highly effective as cytotoxic and antileukemic agents against human fibroblast and promyelocytic leukemic cells [7, 9].

Although the chemical constituents and bioactivities of *A. flava* have been studied, the antibabesial potential of their constituents has not yet been evaluated. In this study, we report the evaluation of daily replacement of culture medium containing the isolated protoberberine alkaloids and 20-hydroxyecdysone from *A. flava* on the growth of *B. gibsoni* in culture and compare their effects with an already known *Babesia* chemotherapeutic agent, Ganaseg.

The stems of *A. flava* were collected from Central Kalimantan, Indonesia in July 2000. The plant was identified by a Botanist at the Herbarium Bogoriense, Indonesia. A voucher specimen is deposited at the Research and Development for Biology, Indonesian Institute of Sciences, Bogor. The stems of *A. flava* (50 g) were boiled twice with 1 *l* of water for 30 min. The boiling water was filtered and extracted with EtOAc to give aqueous and EtOAc layers.

To isolate the compounds (1–4), residue from the aqueous layer was chromatographed on a Diaion HP-20 column, eluted with H_2O (1 *l*), MeOH-H_2O (3:7, 1 *l*), MeOH-H_2O (7:3, 1 *l*) and MeOH (1 *l*), successively. Residue from the fraction MeOH-H_2O (7:3) was subjected to column chromatography on a Sephadex LH-20 column, eluted with MeOH-$CHCl_3$ (2:8) to give three fractions (Fr. A-C). Fr. A and B were individually purified by preparative thin layer chromatography (pTLC) (MeOH-$CHCl_3$ 2:8) to give compounds 1 (50.6 mg) and 2 (12.8 mg), respectively. Compound 3 (11.2 mg) was obtained from the Fr. C after being chromatographed on a Sephadex LH-20 column ($CHCl_3$-MeOH-H_2O 80:18:2), and purification by pTLC ($CHCl_3$:MeOH:H_2O 80:18:2). Residue from the fraction MeOH was chromatographed on a Sephadex LH-20 column, eluted with $CHCl_3$:MeOH:H_2O (5:4:1) to give three fractions (Fr. D-F). Compound 4 (11.9 mg) was obtained from Fr. D after purification by pTLC ($CHCl_3$:MeOH:H_2O 5:4:1).

To isolate compound 5, residue from the EtOAc layer was chromatographed on a silica gel column, eluted with $CHCl_3$ (500 m*l*), MeOH-$CHCl_3$ (3:97, 500 m*l*), MeOH-$CHCl_3$ (2:8, 500 m*l*) and MeOH (500 m*l*), successively. Residue from the MeOH fraction was applied to Sephadex

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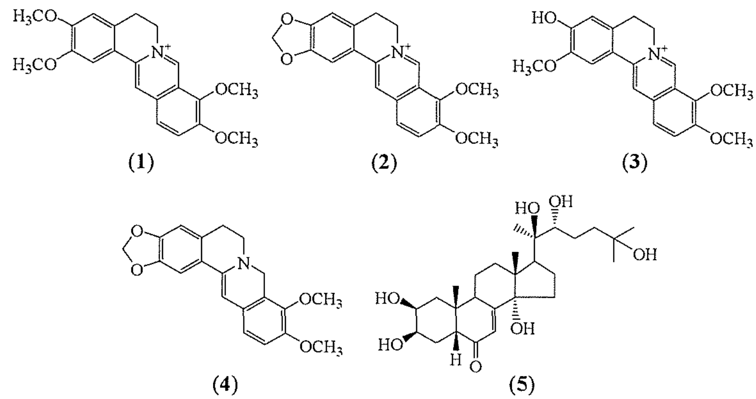

Fig. 1. Chemical structures of palmatine (1), berberine (2), jatrorrhizine (3), dihydroberberine (4) and 20-hydroxyecdysone (5) from *Arcangelisia flava*.

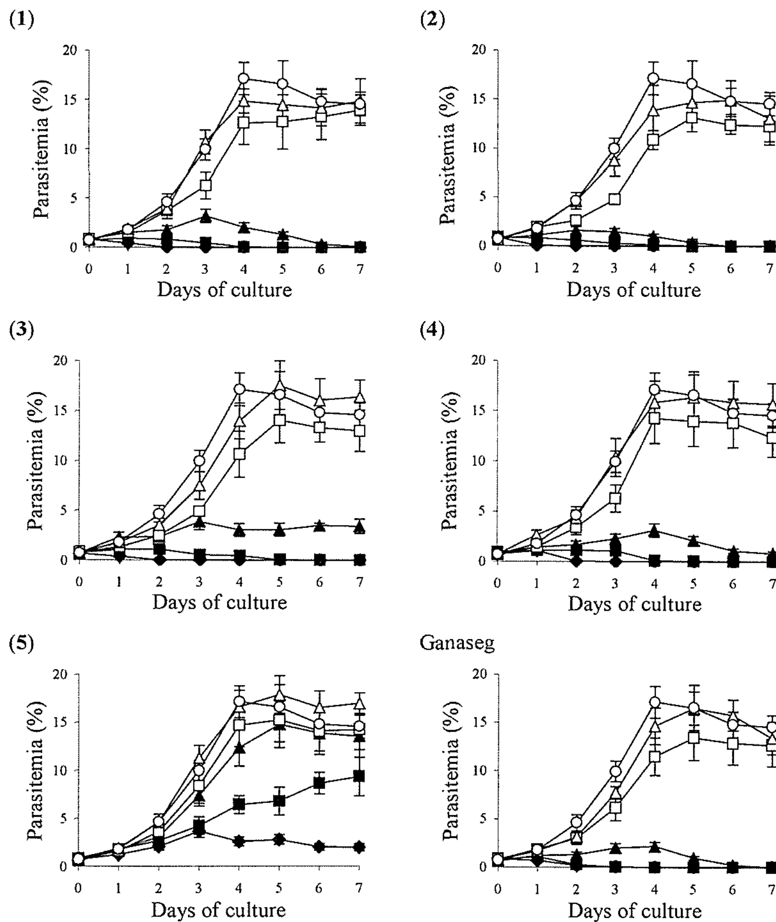

Fig. 2. Effect of palmatine (1), berberine (2), jatrorrhizine (3), dihydroberberine (4), 20-hydroxyecdysone (5) and Ganaseg on the growth of *Babesia gibsoni* in culture. Concentrations of compounds: (◆) 100, (■) 10, (▲) 1, (□) 0.1, (△) 0.01 and (○) 0 μg/m*l*.

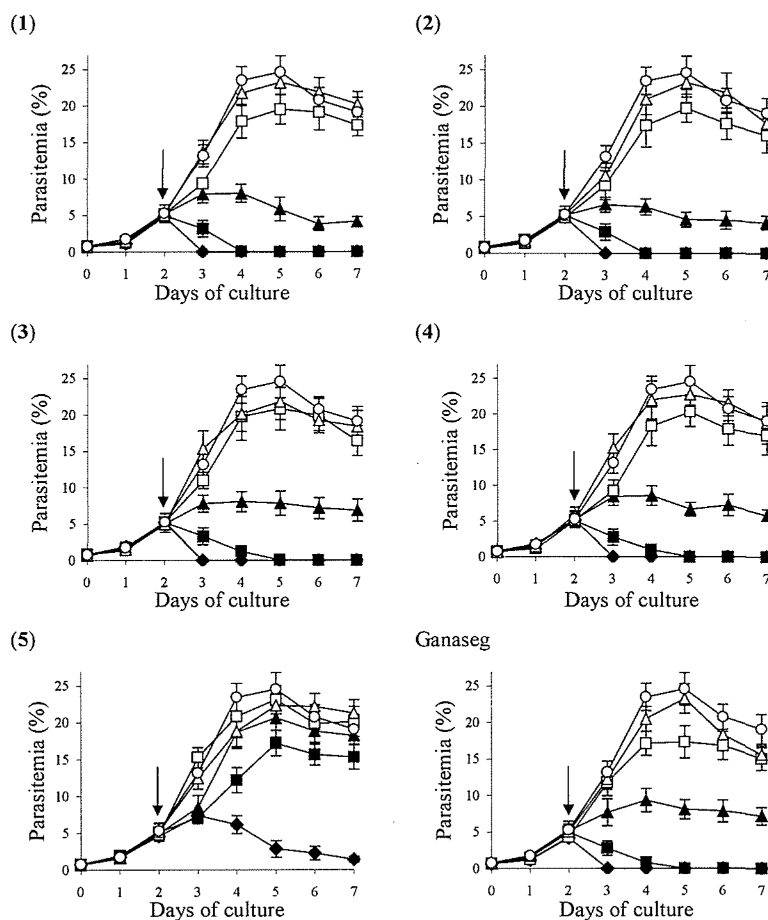

Fig. 3. Effect of palmatine (1), berberine (2), jatrorrhizine (3), dihydroberberine (4), 20-hydroxyecdysone (5) and Ganaseg on the growth of *Babesia gibsoni in vitro* when the tested compounds were added on Day 2 postinfection. Concentrations of compounds: (◆) 100, (■) 10, (▲) 1, (□) 0.1, (△) 0.01 and (○) 0 μg/m*l*. The arrows show the point when the tested compounds were added.

LH-20 column chromatography, eluted with $CHCl_3$:MeOH:H_2O (50:41:9) to give two fractions (Fr. G-H). Compound 5 (13.6 mg) was obtained from Fr. G after purification by pTLC ($CHCl_3$:MeOH:H_2O 50:41:9).

The structures of compounds (1–5) were identified as palmatine [15], berberine [5], jatrorrhizine [2], dihydroberberine [8] and 20-hydroxyecdysone [14], respectively by comparison of their ^{1}H and ^{13}C NMR, mass spectral data and optical rotation values with those of reported data. Compounds (1–3) were already known to be constituents of *A. flava*, while compounds 4 and 5 were isolated as natural products from this plant for the first time (Fig. 1).

B. gibsoni parasites used in this study were maintained in culture medium as previously reported by Yamasaki *et al.* [16]. Heparinized venous blood from a normal dog was washed three times with Vega y Martinez solution [11], and then washed twice with RPMI 1640 supplemented with sodium pyruvate (0.1 mg/m*l*), glutamine (0.3 mg/m*l*), sodium bicarbonate (2 mg/m*l*), penicillin (100 units/m*l*) and streptomycin (100 μg/m*l*). After being washed, erythrocytes were resuspended to a final packed cell volume of 5% in a culture medium consisting of 60% RPMI 1640 medium and 40% normal dog serum. The culture medium was mixed with *B. gibsoni* infected erythrocytes and incubated at 37°C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Every day, 60% volume of the culture supernatant was aspirated and an equal volume of fresh medium was added. An equal volume of uninfected fresh erythrocytes was mixed with infected cultured erythrocytes every 7 days.

To evaluate the antibabesial activity, the isolated compounds were solubilized in a small quantity of dimethyl sulfoxide (DMSO) and further diluted in RPMI 1640 medium. The cultured *B. gibsoni* parasites were mixed with fresh medium to achieve 0.5 to 1.0% parasitemia at the start of incubation. The test was performed in a 24-well culture

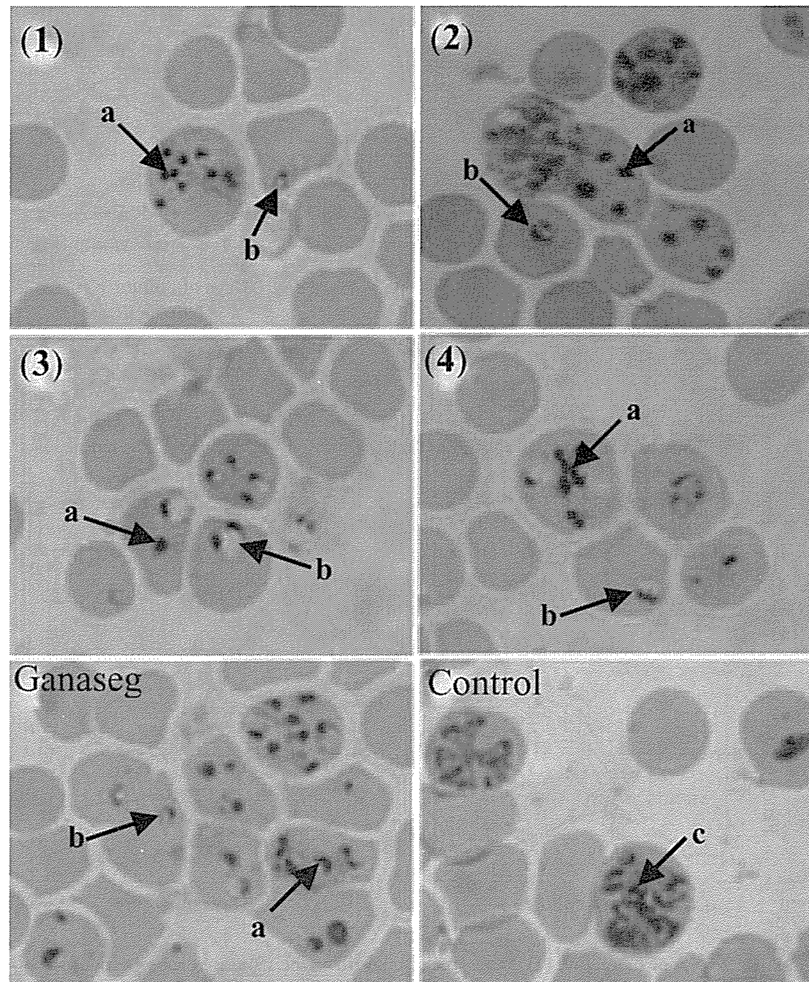

Fig. 4. Morphological changes of *Babesia gibsoni* in the presence of 1 μg/m*l* of palmatine (1), berberine (2), jatrorrhizine (3), dihydroberberine (4), Ganaseg and control after 7 days of incubation. Magnification is 1,000 ×. Oil-immersion field demonstrating cell forms: (a) dead cell, (b) ring and (c) petaloid.

plate, and each well contained 900 μl of parasite-medium suspension and 100 μl of test compound solution. Final concentrations of each test compound were 100, 10, 1, 0.1 and 0.01 μg/m*l*. The plate was incubated at 37°C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 7 days. All tests were performed separately three times. Every day, the medium in each well was aspirated and 1 m*l* of fresh medium containing the appropriate compound concentration was added. A Giemsa-stained thin-smeared specimen was made from each well every day, and the parasitemia level was determined by counting the number of infected cells in 1,000 erythrocytes.

Effects of the isolated compounds (1–5) on the growth of *B. gibsoni* in culture are shown in Fig. 2. Palmatine (1), berberine (2), jatrorrhizine (3) and dihydroberberine (4) showed significant inhibition of the parasite growth at concentrations of 100, 10 and 1 μg/m*l*, similar to those of Ganaseg. On the other hand, 20-Hydroxyecdysone (5) showed significant inhibition at concentration of 100 μg/m*l*, only. The similarity of effects of the protoberberine alkaloids might be due to the similarity of their chemical structures. It appears that the position and type of oxygen substituents on rings A and D had an influence on the inhibition of *B. gibsoni* growth.

To confirm the actual effect of the isolated compounds on the growth of *B. gibsoni*, the parasites were allowed to proliferate for 2 days. After proliferation, the culture medium was aspirated and 1 m*l* of fresh medium containing the appropriate compound concentration was added every day. Compounds (1–4) showed marked effects on the parasite growth, especially for the concentrations of 100 and 10 μg/m*l*. There were no parasites seen between Day 3 and 7 for these concentrations. Parasitemia levels for the concentration of 1 μg/m*l* were relatively stable and by Day 7 they

were between 4.17% and 6.87%. In contrast, the tested compounds at concentrations of 0.1 and 0.01 μg/m*l* did not have any inhibitory effects. Growth inhibitory effects of the compounds (1–4) were similar to those of Ganaseg (Fig. 3).

The host red blood cells were not affected by the concentrations of the tested compounds used as shown by light microscopy (Fig. 4). *B. gibsoni* cells treated with the tested compounds demonstrated stagnation in the ring forms, including size-reduction of the nucleus and disappearance of the parasite cell cytoplasm. On the other hand, the majority of non-treated parasites in the erythrocytes demonstrated petaloid forms with clear cytoplasm and nuclei in the parasite cells after 7 days of incubation.

The mechanism of action of the isolated compounds on the growth of *B. gibsoni* is not yet known. It is possibile that the compounds directly or indirectly affect the parasite invasion into erythrocytes and inhibit the parasite growth. There is a need to determine the precise mechanism of action of these compounds so that their effectiveness can be fully developed.

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