Anti-babesial and Anti-plasmodial Compounds from *Phyllanthus niruri*

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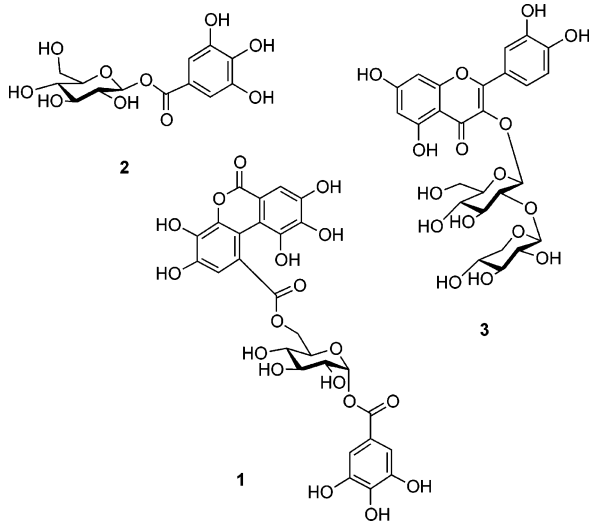
Bioassay-guided fractionation of boiled aqueous extracts from the whole plant of *Phyllanthus niruri* led to the isolation of 1-*O*-galloyl-6-*O*-luteoyl-α-D-glucose (**1**), with IC_{50} values of 4.7 μg/mL against *Babesia gibsoni* and 1.4 μg/mL against *Plasmodium falciparum* in vitro. The known compounds β-glucogallin (**2**), quercetin 3-*O*-β-D-glucopyranosyl-(2→1)-*O*-β-D-xylopyranoside (**3**), β-sitosterol, and gallic acid were also isolated. Structures of these compounds were elucidated on the basis of their chemical and spectroscopic data.

Canine babesiosis is a tick-borne hemolytic disease of dogs caused by intraerythrocytic parasites, *Babesia gibsoni* and *Babesia canis*. The disease occurs frequently in companion animals and has become a serious problem clinically. *Babesia* destroys red blood cells and induces severe clinical symptoms, such as hemolytic anemia, fever, hemoglobinuria, and marked splenomegaly. These symptoms and the mechanisms of infection are very similar to those of malaria. In addition, dogs that have recovered from the disease commonly become chronic carriers and are thereby a source of infection to other dogs.[1−4] Anti-babesial drugs used for the treatment of babesiosis are limited and usually cause pronounced, severe, and drastic side effects.[5−7] In some cases, the drugs are not able to eliminate the parasites completely. Therefore, an alternative chemotherapeutic agent with fewer side effects is urgently needed for the treatment of *B. gibsoni*. One possible source of such affordable treatment lies in the use of plant extracts.

Phyllanthus niruri L. (Euphorbiaceae) is a medicinal plant widely distributed in Indonesia that is often used in folk medicine to treat epilepsy, malaria, hypertension, toothache, diarrhea, fever, tetanus, and urinary calculus.[8] This plant has been intensively studied phytochemically. Constituents such as lignans, alkaloids, flavonoids, phenols, and terpenes have been identified.[9−12] In addition, several pharmacologic experiments have also been reported.[13,14] Despite the many phytochemical and pharmacologic investigations, there are no reports on the anti-babesial activity of this plant. We therefore investigated anti-babesial compounds of this plant and herein present data on a novel polyphenolic glycoside, along with four known related compounds that also possess some anti-babesial activity. Because of similar symptoms and infection mechanisms, anti-plasmodial activity is also discussed.

Results and Discussion

Bioassay-guided fractionations were carried out during the isolation procedure for anti-babesial compounds. Anti-babesial activity was measured using the methods described in the Experimental Section. An extract (boiling water) of the whole plant of *Phyllanthus niruri* (100 g) was cooled and then partitioned using ethyl acetate. Purification of the ethyl acetate extract afforded β-sitosterol (7.6 mg), gallic acid (23.5 mg), and **1** (27.3 mg). The H_2O-soluble layer, using a series of chromatography techniques, gave compounds **2** (34.5 mg) and **3** (34.2 mg).



The isolated compounds, except for **1**, were identified as β-glucogallin (**2**),[15] quercetin 3-*O*-β-D-glucopyranosyl-(2→1)-*O*-β-D-xylopyranoside (**3**),[16] β-sitosterol,[17] and gallic acid,[15] respectively, by comparison of their ^1H and ^{13}C NMR, mass spectral data, and optical rotation values with those of reported data.

Compound **1** was isolated as a yellow powder. Fast atom bombardment mass spectrometry (FABMS) of **1** showed an ion peak $[M - H]^-$ at *m/z* 633, corresponding to the molecular formula $C_{27}H_{21}O_{18}$. The IR spectrum displayed characteristic absorptions for hydroxyl groups (3387 cm^{-1}), carbonyl functions (1719 cm^{-1}), and aromatic rings (1618 cm^{-1}). The ^1H and ^{13}C NMR spectra of **1** were partially similar to those of **2**, particularly the resonances derived from galloyl and pyranose moieties (Table 1). Gas chromatography/mass spectrometry (GC/MS) analysis of the *N*,*O*-bis(trimethylsilyl)acetamide derivative of the methanolysis product of **1** revealed that the pyranose was glucose. The linkage of glucose and galloyl moieties was determined to

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Table 1. ^{1}H NMR (δ) Assignments for **1** and **2** (CD_3OD, 500 MHz)

H	1	2
1	6.37 d (J = 3.7 Hz)	5.59 d (J = 7.8 Hz)
2	3.99 m	3.26−3.44 m
3	4.81 m	3.26−3.44 m
4	4.47 m	3.26−3.44 m
5	4.53 ddd (J = 2.1, 5.0, 11.4 Hz)	3.26−3.44 m
6	4.15 dd (J = 5.0, 11.1 Hz)	3.66 dd (J = 5.1, 11.9 Hz)
	4.96 dd (J = 2.1, 11.1 Hz)	3.83 dd (J = 2.0, 11.9 Hz)
2′, 6′	7.06 s	7.08 s
5″	6.66 *s*	
5‴	6.69 s	

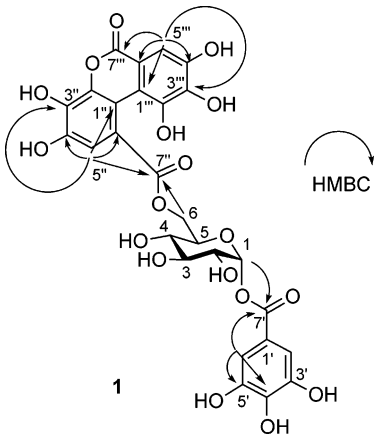

Figure 1. Important heteronuclear multiple bond correlations (HMBC) of compound **1**.

be between the C-1 of the glucose moiety and the C-7′ of the galloyl moiety because of the cross-peak observed in HMBC spectra, and the linkage was determined to be α-glucose due to the J_{C-H} coupling constant value (J = 175.9 Hz).[18] This was also supported by the coupling constant between H-1 and H-2 (J = 3.7 Hz). Because the cross-peaks of the HMBC spectrum appeared, as shown in Figure 1, it was determined that compound **1** had a luteoyl moiety, and the linkage between the glucose and luteoyl moieties was deduced to be between C-6 of the glucose moiety and C-7″ of the luteoyl moiety due to the cross-peak of the HMBC spectrum. To confirm the luteoyl moiety, compound **1** was treated with NaOMe in MeOH followed by treatment with acetic anhydride in pyridine. The usual workup was performed to give a colorless oil, which was purified by PTLC to afford **4** and **5**. Compound **4** was determined to be ellagic acid tetraacetate[19] by comparing its spectroscopic data with those of the acetylated derivative derived from authentic ellagic acid. Compound **5** was determined to be methyl gallate triacetate by comparing its spectroscopic data with those reported.[20] Therefore, compound **1** was determined to be 1-*O*-galloyl-6-*O*-luteoyl-α-glucose.

Since the mode of malarial infection and replication is similar to that of *B. gibsoni*, we also investigated antiplasmodial activities of compounds **1**−**3**, β-sitosterol, and gallic acid. The results of anti-babesial and anti-plasmodial testing of **1**−**3**, β-sitosterol, and gallic acid are given in Table 3. Compound **1** had the strongest activity, with IC_{50} values of 4.7 μg/mL against *B. gibsoni* and 1.4 μg/mL against *P. falciparum*. Diminazene aceturate (product name: Ganazeg)[5−7] and chloroquine[21,22] are effective drugs for babesia and malaria symptoms, respectively, but there are side effects. In the case of chloroquine, the most serious problem is emergence of chloroquine-resistant *P. falciparum*. The isolated compounds were less active relative

Table 2. ^{13}C NMR (δ) Assignments for **1** and **2** (CD_3OD, 125 MHz)

C	1	2
1	94.9	96.0
2	69.3	74.1
3	71.5	78.2
4	62.4	71.1
5	76.1	78.8
6	64.9	62.3
1′	120.5	120.7
2′	110.9	110.6
3′	146.3	146.5
4′	140.4	140.3
5′	146.3	146.5
6′	110.9	110.6
7′	166.7	167.1
1″	116.7[a]	
2″	145.3[b]	
3″	137.6[c]	
4″	145.9[c]	
5″	108.3	
6″	125.4[a]	
7″	170.1	
1‴	117.2[d]	
2‴	145.4[b]	
3‴	138.2[e]	
4‴	145.5[e]	
5‴	110.9	
6‴	125.3[d]	
7‴	168.5	

[a−e] Assignments may be interchanged within each column.

Table 3. Anti-babesial and Anti-plasmodial Activities of Compounds Isolated from *Phyllanthus niruri* against *Babesia gibsoni* and *Plasmodium falciparum* in Vitro[a]

	IC_{50} (μg/mL)	
compound	*Babesia gibsoni*	*Plasmodium falciparum*
1	4.7 ± 0.8	1.4 ± 0.1
2	7.5 ± 0.6	4.6 ± 0.4
3	35.2 ± 1.9	11.0 ± 1.3
β-sitosterol	61.6 ± 2.8	32.0 ± 3.7
gallic acid	13.4 ± 2.0	14.8 ± 1.9
diminazene aceturate	0.6 ± 0.04	
chloroquine		0.04 ± 0.001

[a] Data are expressed as means ± standard deviation in triplicate analyses.

to the standard drugs, diminazene aceturate (IC_{50}: 0.6 μg/mL) and chloroquine (IC_{50}: 0. 04 μg/mL). The host red blood cells were not affected by the compounds at the test concentrations. Parasites of *B. gibsoni* and *P. falciparum* treated with the test compounds demonstrated stagnation in the ring forms, including size reduction of the nucleus and disappearance of the parasite cell cytoplasm. On the other hand, nontreated parasites in the erythrocytes demonstrated typical petaloid and schizont forms after 3 days of incubation with clear cytoplasm and nuclei in the parasite cells. These findings may provide the basis for further understanding of *B. gibsoni* and *P. falciparum* infections and contribute toward the development of new and effective treatments against these parasites.

Experimental Section

General Experimental Procedures. Melting points were measured on a Yazawa micro melting point apparatus. IR spectra were recorded on a Perkin-Elmer 2000 Series FT-IR spectrometer. FABMS and HRFABMS were obtained on a JEOL JMS-AX500 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-500 FT-NMR (500 MHz) and a JEOL JNM-EX 270 FT-NMR (67.5 MHz) spectrometer, respectively. Optical rotations were determined on a JASCO DIP-370 digital polarimeter. GC/MS analysis was

performed using Shimadzu QP-5000. Column chromatography was performed on silica gel 60 (Spherical, 70−140 mesh ASTM, Kanto Chemical), Sephadex LH-20 (Pharmacia), and Diaion HP-20 (Mitsubishi). Silica gel 60 F_{254} precoated plates (Merck) were used for analytical TLC and pTLC. Authentic ellagaic acid was purchased from Wako Pure Chem. Co. Ltd. (Tokyo, Japan).

Plant Material. The whole plant of *Phyllanthus niruri* L. was collected from Palangkaraya District in Central Kalimantan, Indonesia, in July 2000. The plant was identified by Dr. Irawati at the Herbarium Bogoriense, Indonesia, and a voucher specimen (CK.E. 13712) was deposited at the Herbarium.

Anti-babesial Assay. The in vitro assay against *Babesia gibsoni* is given in detail in a previous paper.[23] Standard drug used was diminazene aceturate (Ganazeg).

Anti-plasmodial Assay. Chloroquine-susceptible *P. falciparum* strain FCR-3 was maintained in a culture medium according to a modified method of Trager and Jensen.[24] Parasitized red blood cells (pRBCs) were synchronized with 5% D-sorbitol[25] and then were washed three times with RPMI 1640 medium. The pRBCs were resuspended in RPMI 1640 medium supplemented with 10% human serum, 25 mM Hepes, 25 mM gentamicin, and Na_2CO_3 to achieve 0.25−0.50% parasitemia. The assay was performed in a 24-well culture plate with each well containing 500 μL of synchronous pRBCs suspension and 20 μL of test compound solution. Two wells per plate without test compound served as controls to monitor parasite growth. After 24 h of incubation under a 5% CO_2 atmosphere at 37 °C, the control wells were checked. When a significant percentage (20%) of schizonts appeared, the culture plate was removed from the incubator. Thin smear specimens stained with Giemsa solution were made from each well. The effects of test compounds on parasite growth were expressed as IC_{50}, which were calculated from a simple graph of the inhibition of schizonts against the concentration of test compounds. Chloroquine was used as the standard.

Extraction of Plant Material. Whole plant of *P. niruri* (100 g) was boiled twice with 2 L of H_2O for 30 min. The boiling H_2O was cooled, filtered, and then extracted with EtOAc to give aqueous and EtOAc layers.

Isolation of 1, β-Sitosterol, and Gallic Acid. The EtOAc layer (2.8 g) was chromatographed on a silica gel column, eluted with $CHCl_3$ (500 mL), MeOH−$CHCl_3$ (3:97, 500 mL), MeOH−$CHCl_3$ (1:4, 500 mL), and MeOH (500 mL), successively. The $CHCl_3$ eluate was evaporated to yield a residue (138 mg), which was subjected to column chromatography on silica gel, eluted with MeOH−$CHCl_3$ (1:99) to give five fractions (H−L). Fraction J was purified by PTLC ($CHCl_3$−MeOH, 98:2) to yield β-sitosterol (7.6 mg). The MeOH−$CHCl_3$ (1:4) eluate was evaporated to yield a residue (329 mg), which was subjected to column chromatography on silica gel, eluted with MeOH−$CHCl_3$ (1:4) to give five fractions (M−Q). Fraction O was purified by PTLC using MeOH−$CHCl_3$ (1:49) to yield gallic acid (23.5 mg). Fraction P was purified by PTLC eluted with MeOH−$CHCl_3$ (3:7) to yield compound **1** (27.3 mg).

1-*O*-Galloyl-6-*O*-luteoyl-α-glucose (1): yellow powder; mp 204−205 °C; $[\alpha]_D$ −152.3° (*c* 0.1, MeOH); IR (KBr) cm^{-1} 3387, 2935, 1719, 1618, 1522, 1448, 1350, 1208, 1032; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2; FABMS *m/z* 633 $[M - H]^+$; HRFABMS *m/z* 633.0707 $[M - H]^+$ (calcd for $C_{27}H_{21}O_{18}$ requires 633.0726).

Isolation of Compounds 2 and 3. Bioassay-guided fractionations were carried out for all fractions during the isolation procedure. The aqueous layer (13.1 g) 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. The MeOH−H_2O (3:7) eluate was evaporated to give a residue (194 mg), which was subjected to column chromatography on a Sephadex LH-20 column, eluted with $CHCl_3$−MeOH−H_2O (50:40:10) to give three fractions (A−C). The residue from fraction B was subjected to PTLC ($CHCl_3$−MeOH−H_2O, 50:40:10) to give compound **2** (34.5 mg). The MeOH−H_2O (7:3) eluate was evaporated to give a residue (215 mg), which was subjected to column chromatography on a Sephadex LH-20 column, eluted with $CHCl_3$−MeOH−H_2O (50:40:10) to give three fractions (D−G). Fraction D was purified by PTLC ($CHCl_3$−MeOH−H_2O, 50:40:10) to yield compound **3** (34.2 mg).

GC/MS Experiment. GC/MS analysis of the glycoside part in compounds was performed according to the procedure of Hiradate et al.[26]

Preparation of Ellagic Acid Tetraacetate (4) and Methyl Gallate Triacetate (5). NaOMe (5.4 mg, 0.1 mmol) was added to a stirred solution of **1** (10 mg, 0.015 mmol) in MeOH (2 mL) at room temperature, and the reaction mixture was stirred for 30 min. Excess Amberlite 120B resins were added to the reaction mixture. The resins were filtrated off, and the volatile components of the resulting solution were removed to give an oil. A solution of acetic anhydride (0.5 mL) was added to a stirred solution of the oil in pyridine (1 mL), and the reaction mixture was stirred further for 12 h. The usual workup was performed to give a residue, which was purified by PTLC using MeOH−$CHCl_3$ (3:97) as a solvent to afford **4** (1.2 mg) and **5** (2.0 mg).

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