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New Anti-Malarial Flavonol Glycoside from Hydrangeae Dulcis Folium

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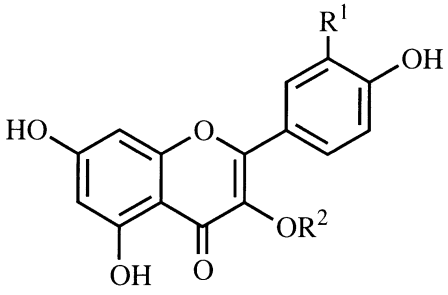
Abstract—Bioassay-guided fractionation of the MeOH extract of Hydrangeae Dulcis Folium resulted in isolation of a new flavonol glycoside and two known congeners as anti-malarial principles. These flavonol glycosides showed characteristic proliferation inhibition of *Plasmodium falciparum* at significantly low concentration without showing any cytotoxicity. In addition, several naturally occurring flavonol glycosides were also shown to exert similar anti-malarial behavior. © 2001 Elsevier Science Ltd. All rights reserved.

The prevalence of malaria in tropical zones worldwide, together with the lack of vaccine and the appearance of a strain of malaria parasite resistant to commercially available anti-malarial drugs, makes the search for new anti-malarials a global demand.[1] This circumstance prompted us to explore new anti-malarial candidates with high selectivity index between the parasite and mammalian host cells from natural sources.[2,3] During the course of executing this project, the MeOH extract of a Japanese traditional crude drug, Hydrangeae Dulcis Folium (processed crude drug from the leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO, Amacha in Japanese), was shown to demonstrate not only in vitro anti-malarial activity against *Plasmodium falciparum*, but also little cytotoxic property against human epidermoid carcinoma KB 3-1 cells. This promising biological outcome encouraged us to undertake bioassay-guided separation of the extract of this crude drug to disclose a new flavonol glycoside **1** together with two known congeners **2** and **3** as active constituents. Furthermore, several naturally occurring flavonol glycosides were assessed for anti-malarial potency. Herein, we describe the anti-malarial property of these flavonol glycosides as well as structure elucidation of the new flavonol triglycoside **1** (Chart 1).



1: R^1= H, R^2= Gal(2-1)Xyl, Gal(6-1)Rha

2: R^1= H, R^2= Glu(6-1)Rha

3: R^1= H, R^2= Gal(2-1)Xyl

4: R^1= OH, R^2= Glu(6-1)Rha

5: R^1= OCH_3, R^2= Glu(6-1)Rha

6: R^1= OH, R^2= Rha(4-1)Glu

7: R^1= OH, R^2= Rha(4-1)Glu, Glu(6)Ac

8: R^1= OH, R^2= Rha

9: R^1= OH, R^2= Glu

10: R^1= OH, R^2= H

11: R^1= H, R^2= H

Gal : β-D-galactopyranosyl Xyl : β-D-xylopyranosyl
Glu : β-D-glucopyranosyl Rha : α-L-rhamnopyranosyl

Chart 1.

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The MeOH extract of Hydrangeae Dulcis Folium was subjected to successive water–AcOEt and water–*n*-BuOH partition. The active *n*-BuOH soluble portion was separated by a combination of normal and reversed-phase column chromatography, Sephadex LH-20 column, and reversed-phase HPLC to furnish a new flavonol glycoside (**1**, 0.001% from crude drug) along with two known flavonol glycosides, kaempferol 3-*O*-β-rutinoside (**2**, 0.003%)[4] and kaempferol 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside (**3**, 0.002%),[5] as active principles.

The molecular formula of **1** was determined as $C_{32}H_{38}O_{19}$ by FAB-HRMS, which showed a quasimolecular ion peak at *m*/*z* 727.2098 $[M+H]^+$. The IR spectrum of **1** showed absorption bands due to the hydroxyl (3312 cm^{-1}) and aromatic-ring conjugated carbonyl (1657 cm^{-1}) groups. The ^1H NMR spectrum of **1** showed the characteristic signals at δ 6.45 (1H, d, $J=7.3$ Hz, Gal-H-1), 5.44 (1H, d, $J=6.7$ Hz, Xyl-H-1), and 5.15 (1H, brs, Rha-H-1) ascribable to three anomeric protons. Additionally, the presence of 1,2,3,5-tetrasubstituted and 1,4-disubstituted aromatic rings was suggested by the ^1H NMR data derived from the aglycon moiety: δ 8.54 (2H, d, $J=8.5$ Hz, H-2′, 6′), 7.21 (2H, d, $J=8.5$ Hz, H-3′, 5′), 6.68 (1H, s, H-6), 6.67 (1H, s, H-8).

A combination of H–H COSY, TOCSY, HMQC, and HMBC experiments allowed unambiguous assignments of all proton and carbon signals of **1** involving the sugar moiety.[6] Based on the chemical shifts as well as the coupling constants of the proton signals in the sugar moiety of **1**, the three sugar residues were deduced to be α-L-rhamnopyranose, β-D-xylopyranose, and β-D-galactopyranose. The HMBC correlations between the anomeric proton and the carbinol carbon (Rha-H-1 to Gal-C-6; Xyl-H-1 to Gal-C-2) clarified connectivity of the sugar residues. Moreover, glycosylation shifts were also observed with respect to the carbons around Gal-C-2 and Gal-C-6.[7,8] The chemical structure of the trisaccharide moiety was, therefore, established. The acid hydrolysis of **1** gave kaempferol. Taking the glycosylation shift observed around C-3 into account, the trisaccharide moiety was elucidated to link to the 3-hydroxyl group of kaempferol.[9] Consequently, the flavonol glycoside **1** was unequivocally elucidated as kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)]-β-D-galactopyranoside.

The anti-malarial potency against *P. falciparum* and the cytotoxic activity against KB 3-1 cells of the three flavonol glycosides **1**–**3** are summarized in Table 1.[10] Each compound exhibited characteristic anti-malarial activity: in particular, approximately 60% of proliferation of the parasite was inhibited even at the concentration of 0.5 ng/mL.[11] On the other hand, these flavonol glycosides have little influence on the growth of KB 3-1 representing the host cell.[12] In order to examine the structure–activity relationship, several flavonol glycosides, rutin (**4**), isorhamnetin 3-β-*O*-rutinoside (**5**), multinoside A (**6**), multinoside A acetate (**7**), quercitrin (**8**), and isoquercitrin (**9**) as well as flavonols, quercetin (**10**) and kaempferol (**11**), were assessed for anti-malarial activity.[13] Despite a slight reduction of activity at the concentration of 0.1 and 0.05 ng/mL of **5**, each flavonol diglycoside showed a similar biological score. In the case of flavonol monoglycosides (**8**, **9**), the anti-malarial activities particularly decreased at 5 and 0.5 ng/mL concentrations in comparison with the other flavonol diglycosides. On the contrary, the two flavonols (**10**, **11**) showed no anti-malarial potency at the concentration of 5 ng/mL. It is curious that most of the flavonol glycosides displayed no definite growth inhibitory activity against *P. falciparum* in a concentration-dependent manner. As a result of preliminary examination, exposure of **1** and **4** at higher concentration (0.5 μg/mL) rather resulted in decline of activity (inhibition ratio, **1**: 39.6±1.2, **4**: 20.2±6.2). Additionally, little growth inhibition was observed at fairly low concentration of **1** and **4** (0.5 pg/mL).

Table 1. Anti-malarial and cytotoxic activities of flavonoid glycosides[a,b]

Compd	Anti-malarial (ng/mL)				Cytotoxicity
	5	0.5	0.1	0.05	5 μg/mL
1	58.9±3.6	68.7±10.2	54.2±6.8	37.6±2.7	ND[c]
2	50.9±1.8	60.1±6.3	49.1±4.0	30.5±12.5	15.2±7.3
3	56.2±5.7	61.9±6.2	43.1±11.0	22.4±5.8	ND
4	56.4±7.4	56.0±9.4	51.6±11.6	38.7±3.3	ND
5	41.5±9.0	44.5±6.6	24.5±4.9	10.8±3.8	9.6±3.2
6	48.1±2.6	43.4±7.1	32.8±5.9	25.0±7.3	2.1±7.6
7	65.4±7.6	52.1±8.9	40.3±5.3	27.4±3.3	ND
8	32.9±4.5	36.0±3.8	39.4±3.3	34.0±1.2	1.4±7.0
9	36.8±6.9	31.7±0.4	27.7±5.9	31.1±5.8	4.2±5.1

[a]Values are expressed as mean±standard deviation. All tests were realized in duplicate.
[b]Quinine was used as a reference agent. Inhibitory ratio on proliferation of *P. falciparum* at 1.0×10^{-7} M concentration of quinine was 45.3±4.4%.
[c]Not detected.

In conclusion, bioassay-guided separation of the MeOH extract of Hydrangeae Dulcis Folium enabled the isolation of three flavonol glycosides as the anti-malarial principles. Although these flavonol glycosides could not accomplish complete inhibition of proliferation of *P. falciparum*, the significant low concentration with no cytotoxic effect gave rise to more than 50% growth inhibition against the malaria parasite. Structure requirement and the mechanism of action in relation to the present interesting anti-malarial property of flavonol glycosides are currently under investigation.

Acknowledgements

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6. Pale yellow powder, $[\alpha]_D$ −64.3° (*c* 0.35, MeOH). IR ν_{max} (KBr) cm^{-1}: 3312, 2937, 1657, 1603, 1086. UV λ_{max} (MeOH) nm (ε): 266 (16,000), 347 (21,000). ^1H NMR (500 MHz, pyridine-d_5) aglycon moiety δ: 6.67 (1H, s, H-8), 6.68 (1H, s, H-6), 7.21 (2H, d, J = 8.5 Hz, H-3′, 5′), 8.54 (2H, d, J = 8.5 Hz, H-2′, 6′), trisaccharide moiety δ: 1.45 (3H, d, J = 5.5 Hz, Rha-H-6), 3.58 (1H, dd, J = 9.0, 9.0 Hz, Xyl-H-5a), 3.90 (1H, dd, J = 10.1, 5.8 Hz, Gal-H-6a), 4.08 (1H, dd, J = 7.6, 5.8 Hz, Gal-H-5), 4.14 (3H, m, Rha-H-4, Xyl-H-3, 4), 4.18 (1H, m, Rha-H-5), 4.21 (1H, dd, J = 7.0, 6.7 Hz, Xyl-H-2), 4.28 (1H, dd, J = 9.0, 4.0 Hz, Gal-H-3), 4.30 (1H, dd, J = 9.0, 4.0 Hz, Rha-H-3), 4.38 (4H, m, Gal-H-4, 6b, Xyl-H-5b, Rha-H-2), 4.80 (1H, dd, J = 9.0, 7.3 Hz, Gal-H-2), 5.15 (1H, brs, Rha-H-1), 5.44 (1H, d, J = 6.7 Hz, Xyl-H-1), 6.45 (1H, d, J = 7.3 Hz, Gal-H-1). ^{13}C NMR (125 MHz, pyridine-d_5) aglycon moiety δc: 94.5 (C-8), 99.7 (C-6), 105.4 (C-10), 116.1 (C-3′, 5′), 122.3 (C-1′), 132.0 (C-2′, 6′), 135.1 (C-3), 157.0 (C-2), 157.6 (C-9), 161.1 (C-4′), 163.0 (C-5), 165.7 (C-7), 178.8 (C-4), trisaccharide moiety δc: 18.5 (Rha-C-6), 66.5 (Gal-C-6), 66.9 (Xyl-C-5), 69.4 (Gal-C-4), 69.7 (Rha-C-5), 70.9 (Xyl-C-4), 72.1 (Rha-C-2), 72.7 (Rha-C-3), 73.9 (Rha-C-4), 75.1 (Xyl-C-2, Gal-C-5), 75.5 (Gal-C-3), 77.5 (Xyl-C-3), 81.0 (Gal-C-2), 100.7 (Gal-C-1), 101.9 (Rha-C-1), 106.1 (Xyl-C-1). FAB-MS m/z: 749 $[M+Na]^+$, 727 $[M+H]^+$. FAB-HRMS m/z: calcd for $C_{32}H_{39}O_{19}$: 727.2086, found: 727.2098.
7. ^{13}C NMR (125 MHz, DMSO-d_6) aglycon moiety δc: 93.5 (C-8), 98.6 (C-6), 103.8 (C-10), 115.0 (C-3′, 5′), 120.8 (C-1′), 130.8 (C-2′, 6′), 132.8 (C-3), 155.5 (C-2), 156.2 (C-9), 159.8 (C-4′), 161.1 (C-5), 163.9 (C-7), 177.3 (C-4), trisaccharide moiety δc: 17.8 (Rha-C-6), 64.8 (Gal-C-6), 65.5 (Xyl-C-5), 67.7 (Gal-C-4), 68.1 (Rha-C-5), 69.3 (Xyl-C-4), 70.3 (Rha-C-2), 70.5 (Rha-C-3), 71.8 (Rha-C-4), 73.7 (Gal-C-5), 73.5 (Gal-C-3), 73.3 (Xyl-C-2), 76.0 (Xyl-C-3), 79.4 (Gal-C-2), 98.4 (Gal-C-1), 99.8 (Rha-C-1), 104.4 (Xyl-C-1).
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10. A strain of *P. falciparum* (FCR3, cycloguanil-resistant from Gambia) was used in sensitivity testing. After synchronization by the sorbitol treatment, 50 μL of parasite culture at ring stage (0.55% parasitemia and 2% hematocrit) was added to each well in 96-well microculture plates. The test samples were dissolved in DMSO and diluted to the appropriate concentration using complete medium, then 50 μL of each sample solution was inoculated. The final concentration of DMSO in the culture was 1%. After incubation at 37 °C for 48 h, the proliferation of *P. falciparum* was assessed by Giemsa-stained smear by observing 10,000 erythrocytes per one thin blood film. Quinine was used as a reference anti-malarial.
11. In this anti-malarial assay, quinine inhibited the proliferation of *P. falciparum* in a concentration-dependent manner with IC_{50} of 40 ng/mL and IC_{90} of 90 ng/mL.
12. Cytotoxic potency was evaluated by colorimetric MTT assay, in which mitomycin C used as a positive control showed the IC_{50} of 0.1 μg/mL. ND means that the growth inhibitory ratio against KB 3-1 is less than 0%.
13. Rutin (**4**)[9] and isorhamnetin-3-*O*-β-rutinoside (**5**)[9] were isolated from *Sophora japonica* (Kaika in Japanese). Multinoside A (**6**)[14] and multinoside A acetate (**7**)[14] were also isolated from *Rosa multiflora* (Eijitsu in Japanese). As for flavonols (**10**, **11**) and its monosaccharides (**8**, **9**), commercially available samples were used.
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