Biosci. Biotechnol. Biochem., **66** (8), 1727–1730, 2002

Note

Isolation of Antimicrobial Compounds from Guava (*Psidium guajava* L.) and their Structural Elucidation

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Received November 5, 2001; Accepted April 10, 2002

Four antibacterial compounds were isolated from leaves of guava (*Psidium guajava* L.), and the structures of these compounds were established on the basis of chemical and spectroscopic evidence. Two new flavonoid glycosides, morin-3-*O*-α-L-lyxopyranoside and morin-3-*O*-α-L-arabopyranoside, and two known flavonoids, guaijavarin and quercetin, were identified. The minimum inhibition concentration of morin-3-*O*-α-L-lyxopyranoside and morin-3-*O*-α-L-arabopyranoside was 200 μg/ml for each against *Salmonella enteritidis*, and 250 μg/ml and 300 μg/ml against *Bacillus cereus*, respectively.

Key words: *Psidium guajava*; morin glycoside; *Bacillus cereus*; *Salmonella enteritidis*

Antibacterial compounds have been isolated from a large number of plant species throughout the world.[1–3] Many different types of antibacterial compounds play a role in plant defense, polyphenolic compounds being known to have multiple functions. Flavonoids such as naringenin, flavone and flavonol, including kaemferol, morin and quercetin, constitute a large group of secondary plant metabolites that have been reported to have antibacterial activities.[4–6] Guava (*Psidium guajava* L.) is an evergreen growing wild in the torrid zone and subtropics.[1,7] Guava leaves are used for tea. Guava leaves, roots and fruits have also been used for the prevention and treatment of diarrhea[7,8] and diabetes[9,10] as a folk medicine and reportedly had an antimutagenic effect.[11,12] In a preliminary experiment, we screened the antibacterial activities of many kinds of plants used as folk medicine in Okinawa, Japan. A high level of antibacterial activity was detected in guava leaves. The present paper reports the isolation and structural elucidation of the antimicrobial compounds from guava leaves.

Milled dry leaves (130 g) of guava, which had been collected in Okinawa, Japan, were extracted with 90% (v/v) aqueous methanol (1.3 l × 3) at room temperature. The extracts were combined and concentrated under reduced pressure, and the residue was suspended in water. The suspension was successively extracted with *n*-hexane and chloroform to remove the hydrophobic materials. The aqueous phase was extracted with ethyl acetate. The ethyl-acetate soluble fraction was concentrated under reduced pressure, and the resulting residue was dissolved in methanol (400 ml). Chloroform (350 ml) and water (170 ml) were mixed with this methanol solution, the pale yellow clear upper layer being collected and concentrated. The oily materials were dissolved in 25% aqueous methanol containing 0.1% trifluoroacetic acid (TFA). The solution was applied to a Wakogel LP-60C18 column (25 × 100 mm) and eluted with 25%, 50%, and 70% methanol containing 0.1% TFA by stepwise elution. Most of the activity was in the fraction eluted with 50% methanol containing 0.1% TFA. The fractions were concentrated under reduced pressure, and further separated by HPLC in a Cosmosil 5C18-MS column (10 × 250 mm) that was eluted at a flow rate of 2 ml/min with 50% aqueous methanol containing 0.1% TFA.

Four fractions having antibacterial activity were obtained. The antibacterial activity of each fraction was monitored by the paper disk method of Jeongmok *et al.*,[13] using *Bacillus cereus* ATCC11778 and *Salmonella enteritidis* #1 as the test bacteria. Fraction 1 was further purified in the same column with 40% methanol containing 0.1% TFA to give compound I (5.8 mg). Fraction 2 was purified by HPLC with 45% methanol containing 0.1% TFA to give compound II (5.2 mg). Fraction 3 was purified by HPLC with the same solvent to give compound III (12.1 mg, guaijaverin). Fraction 4 was purified by HPLC with 50% methanol containing 0.1% TFA to give compound IV (17.2 mg, quercetin).

The APCI mass spectrum (Hitachi M-1200H liquid chromatograph/APCI mass spectrometer) of compound I showed *m/z* 435 $[M+H]^+$ and 303 $[M+H-132]^+$, and its ^{13}C-NMR spectrum (Bruker DPX-250) showed 20 carbon signals which, according to the DEPT spectrum, represented one methylene, nine methine and ten quarternary carbons corresponding to molecular formula $C_{20}H_{18}O_{11}$ consistent with 12 degrees of unsaturation. The FT-IR

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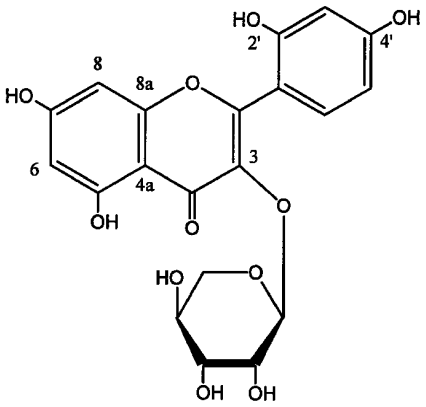
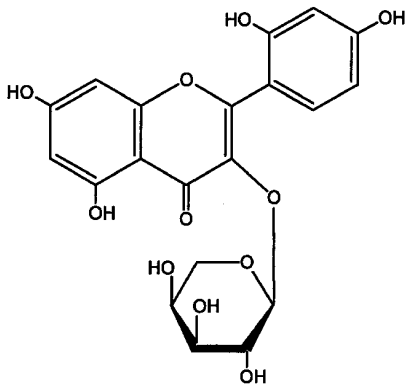


Compound I



Compound II

Fig. 1. Structures of Compounds I and II.

spectrum (Shimadzu FT-IR 8600PC spectrometer) of compound I showed bands of hydroxyl groups (3367 cm^{-1}), α,β-unsaturated ketone (1656 cm^{-1}), and aromatic rings (1606, 1502 cm^{-1}). In the ^{1}H-NMR spectrum (Jeol JNM-GX400 spectrometer) of compound I, a 5,7-dihydroxy A-ring with the flavnoid structure was evident from two *meta*-couplings at δ6.20 ppm (J=2.0 Hz) and δ6.39 ppm (J=2.0 Hz) for H-6 and H-8, respectively (Fig. 1 and Table 1). In addition, three aromatic resonances located at 6.85 (1H, d, J=8.0 Hz), 7.58 (1H, d, J=8.0 Hz) and 7.20 (1H, s) were assigned to H-5′, H-6′ and H-3′, respectively. The δ7.20 signal of compound I had a higher field shift than that of the signal for H-2′ of quercetin (δ7.65). Acid hydrolysis of the glycosides was performed according to the method of Ngounou.[14] Compound I (4 mg) was dissolved in 50% aqueous methanol, before 2 M hydrochloric acid (5 ml) was added. The solution was refluxed for 7 h at 60°C, the reaction mixture then being concentrated under reduced pressure. The residue was dissolved in

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Compounds I and II

Attribution	Compound I		Compound II	
	δ^{13}C	δ^1H	δ^{13}C	δ^1H
2	157.4		157.6	
3	134.5		134.6	
4	178.7		179.0	
4a	104.9		105.1	
5	162.1		162.1	
6	98.5	6.20 d (2.0)	98.8	6.20 d (2.0)
7	165.0		165.0	
8	93.7	6.39 d (2.0)	93.7	6.40 d (2.0)
8a	157.8		158.4	
1′	122.0		122.1	
2′	145.1		145.4	
3′	116.2	7.20 s	115.7	7.13 s
4′	149.0		148.9	
5′	114.5	6.85 d (8.0)	115.4	6.85 d (8.0)
6′	122.3	7.58 d (8.0)	121.9	7.49 d (8.0)
1″	103.6	5.18 d (7.0)	103.1	5.16 d (7.0)
2″	74.2	3.49 m	73.1	3.89 br d (9.6)
3″	76.5	3.39 t (8.6)	71.9	3.64 br d (3.6)
4″	70.0	3.51 m	68.1	3.80 m
5″	66.2	3.77 dd (11.0, 3.8)	65.9	3.81 m
		3.08 dd (11.0, 3.8)		3.43 br d (11.0)

Compounds I and II were dissolved in CD_3OD, and the ^{1}H-NMR (400 MHz) and ^{13}C-NMR (62.5 MHz) data were measured. The chemical shifts are expressed in ppm, and J values in Hz are presented in parentheses. Multiplicity is represented by s for singlet, d for doublet, and m for multiplet. Proton and carbon signals were assigned by H–H COSY and HMBC.

10 ml of water, and the aglycon was extracted with chloroform. The R_f value (0.64) of the aglycon from compound I (RP-18 F_{254} plate in MeOH-H_2O-TFA = 50:50:0.1) was the same as that of morin. The UV spectrum of compound I was also consistent with that of morin [λ_{max} (MeOH) nm (ε): 258 (23,200), 351 (16,400)]. The sugar in the water-soluble portion was compared with standard sugars on a TLC plate (silica gel 60, Merck, Art 5721) by using BuOH-EtOAc-*iso*-PrOH-AcOH-H_2O = 7:20:12:7:6. Spots were detected with the aniline-diphenylamine reagent.[14] The R_f values of arabinose, lyxose, ribose and xylose were 0.38, 0.42, 0.40 and 0.45, respectively. The R_f value (0.42) of the sugar moiety from compound I indicated lyxose, this being confirmed by TLC. The absolute configuration of the lyxose was of L type which was indicated by the retention time (15.1 min) in a chiral column according to the procedure of Tsukamoto *et al.*,[15] and by the optical rotation $[\alpha]_D$ (H_2O) (+13°). Moreover, the cross peak from H-1″ (δ5.18) to C-3 (δ134.6) in the HMBC spectrum of compound I confirmed the presence of a 3-*O*-linkage of the sugar moiety, while the 1″,2″-*trans*-diaxial coupling patterns of compound I confirmed an *O*-α-configuration. It was therefore concluded that compound I was morin-3-*O*-α-L-lyxopyranoside.

Compounds II and III showed similar MS data and DEPT results to those of compound I. Acid hydrolysis resulted in the aglycons of compounds II and III

Table 2. Antibacterial Activities of Compounds I–IV and Morin against *Bacillus cereus* and *Salmonella enteritidis*

Compound	MIC (μg/ml)	
	B. cereus	*S. enteritidis*
Morin-3-*O*-lyxoside (I)	250	200
Morin-3-*O*-arabinoside (II)	300	200
Morin	300	150
Quercetin-3-*O*-arabinoside (III)	350	300
Quercetin (IV)	350	250

Cultures in Mueller-Hinton broth (Difco Laboratory) that had been incubated for 24 h were diluted 1000-fold with the same broth. Aliquots (0.9 ml) of the dilution were mixed with 0.1 ml of a flavonoid solution that had been dissolved or suspended in 10% aqueous dimethyl sulfoxide (DMSO) in sterilized culture tubes. After incubating overnight at 37°C, the growth of each test bacterium was determined by turbidity. MIC is expressed as the minimum concentration of a flavonoid which did not show turbidity.

giving morin and quercetin, respectively. The R_f value (0.38) of the sugar moiety from compounds II and III indicated arabinose. The absolute configuration of arabinose was of L type which was indicated by the retention time (16.6 min) in a chiral column and by the optical rotation $[\alpha]_D$ (H_2O) (+103°). Compounds II and III also had a 3-*O*-linkage, indicating that compounds II and III were morin-3-*O*-α-L-arabopyranoside and quercetin-3-*O*-L-arabinoside (guaijaverin), respectively. Compound IV was also identified as quercetin by the same spectroscopic and chemical analysis. These morin glycosides, compounds I and II, are unique and the first to be reported in plants.

Compound I. Yellow amorphous powder, mp 270–280°C; [α] −40° (c 0.001, MeOH); TLC: RP-18 F_{254} 0.25 mm plate (Merck) in MeOH-H_2O-TFA = 40:60:0.1 (v/v), R_f 0.46; UV λ_{max} (MeOH) nm (ε): 260 (25,700), 352 (18,200). IR γ_{max} (polypropylene) cm^{-1}: 3367, 1656, 1606, 1502, 1355, 1301, 1193, 1081; HRFABMS *m/z* (M^+): calcd. for $C_{20}H_{18}O_{11}$, 434.0849; found, 434.0890; APCIMS *m/z*: 435.02 $[M+H]^+$, 302.95 $[M+H-132]^+$; CD: $[\theta]_{227}$ −15,000, $[\theta]_{252}$ +26,000, $[\theta]_{277}$ −25,000, $[\theta]_{305}$ −1,600, $[\theta]_{350}$ 4,800. The ^{1}H- and ^{13}C-NMR spectral data are shown in Table 1.

Compound II. Yellow amorphous powder, mp 250–260°C; UV λ_{max} (MeOH) nm (ε): 260 (24,500), 352 (17,300); $[\alpha]_D^{25}$ −104° (c 0.001, MeOH); TLC: RP-18 F_{254} 0.25 mm plate (Merck) in MeOH-H_2O-TFA = 40:60:0.1 (v/v), R_f 0.41; IR γ_{max} (polypropylene) cm^{-1}: 3352, 1654, 1604, 1514, 1367, 1317, 1109, 1088; HRFABMS *m/z* (M^+): calcd. for $C_{20}H_{18}O_{11}$, 434.0849; found, 434.0890; APCIMS *m/z*: 435.02 $[M+H]^+$, 303.28 $[M+H-132]^+$; CD: $[\theta]_{227}$ −21,000, $[\theta]_{251}$ +53,000, $[\theta]_{279}$ −58,000, $[\theta]_{308}$ −6,900, $[\theta]_{345}$ −17,500. The ^{1}H- and ^{13}C-NMR spectral data are shown in Table 1.

Compound III (guaijaverin). Yellow needles, mp 250–260°C; UV λ_{max} (MeOH) nm (ε): 265 (31,600), 370 (24,000); IR γ_{max} (polypropylene) cm^{-1}: 3352, 1654, 1604, 1502, 1357, 1301, 1201, 1080; APCIMS *m/z*: 435.11 $[M+H]^+$, 303.34 $[M+H-132]^+$; ^{1}H-NMR (400 MHz in CD_3OD) δ: 7.74 (1H, s, H-2′), 7.57 (1H, d, J=8.0 Hz, H-6′), 6.86 (1H, d, J=8.0 Hz, H-5′), 6.39 (1H, br. s, H-6), 6.19 (1H, br. s, H-8), 5.15 (1H, d, J=7.0 Hz, H-1″), 3.89 (1H, br d, J=9.6 Hz, H-2″), 3.82 (1H, m, H-5″), 3.80 (1H, m, H-4″), 3.64 (1H, br d, J=3.6 Hz, H-3″), 3.43 (1H, m, H-5″); ^{13}C-NMR (62.5 Hz in CD_3OD) δ: 178.5 (C-4), 165.0 (C-7), 162.1 (C-5), 157.7 (C-8a), 157.4 (C-2), 148.9 (C-4′), 145.0 (C-3′), 134.7 (C-3), 122.0 (C-1′), 121.9 (C-6′), 116.5 (C-5′), 115.2 (C-2′), 104.7 (C-4a), 103.6 (C-1″), 98.9 (C-6), 93.7 (C-8), 73.1 (C-2″), 71.9 (C-3″), 68.1 (C-4″), 65.9 (C-5″).

Compound IV (quercetin). Yellow amorphous powder, mp 320–330°C; UV λ_{max} (MeOH) nm (ε): 253 (21,400), 366 (14,800); IR γ_{max} (polypropylene) cm^{-1}: 3342, 3315, 1658, 1612, 1562, 1519, 1444, 1371, 1319, 1207, 1166; APCIMS *m/z*: 303.28 $[M+H]^+$; ^{1}H-NMR (250 MHz in CD_3OD) δ: 7.65 (1H, d, J=2.5 Hz, H-2′), 7.55 (1H, dd, J=10.0, 2.5 Hz, H-6′), 6.80 (1H, d, J=10.0 Hz H-5′), 6.31 (1H, d, J=2.5 Hz, H-6), 6.10 (1H, d, J=2.5 Hz, H-8); ^{13}C-NMR (62.5 Hz in CD_3OD) δ: 178.6 (C-4), 164.6 (C-7), 161.5 (C-5), 157.2 (C-2), 147.8 (C-4′), 146.3 (C-8a), 145.2 (C-3′), 136.2 (C-3), 123.1 (C-1′), 120.7 (C-6′) 115.2 (C-2′), 115.0 (C-5′), 104.7 (C-4a), 98.2 (C-6), 93.4 (C-8).

The antibacterial activities of these four compounds were evaluated as the minimum inhibitory concentration (MIC). Table 2 shows the MIC values for the four separated compounds and for morin by using *B. cereus* and *S. enteritidis* as the test bacteria. The MIC values for morin-3-*O*-lyxoside and morin-3-*O*-arabinoside were 250 μg/ml and 300 μg/ml against *B. cereus*, respectively. *S. enteritidis* was slightly more sensitive than *B. cereus* to these compounds. Morin, quercetin and quercetin-3-*O*-arabinoside have been reported to have antibacterial activity.[4,7] The antibacterial activity of morin-3-*O*-lyxoside and morin-3-*O*-arabinoside seems to have been similar to those of morin and quercetin. The antibacterial activity of the methanol extract of guava was mainly due to flavonoids, especially the morin glycosides, quercetin glycosides and quercetin.

Acknowledgments

We are grateful to Dr. A. Shimizu (Faculty of Agriculture, Kobe University) for providing the bacterial strains. We are also grateful to Dr. T. Ohno for FT-IR measurements, and to Dr. K. Akasaka (Faculty of Science, Kobe University) for allowing us to use the 400-MHz NMR equipment. We thank Sumika Chemical Analysis Service for high-resolution mass measurements.

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