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Flacourtosides A–F, Phenolic Glycosides Isolated from *Flacourtia ramontchi*

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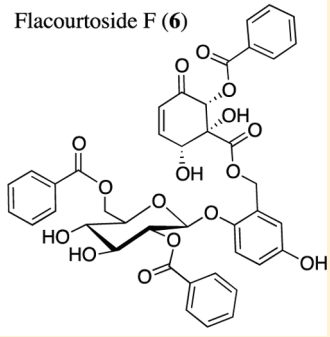
Supporting Information

ABSTRACT: In an effort to identify novel inhibitors of chikungunya (CHIKV) and dengue (DENV) virus replication, a systematic study with 820 ethyl acetate extracts of madagascan plants was performed in a virus-cell-based assay for CHIKV, and a DENV NS5 RNA-dependent RNA polymerase (RdRp) assay. The extract obtained from the stem bark of *Flacourtia ramontchi* was selected for its significant activity in both assays. Six new phenolic glycosides, named flacourtosides A–F (**1**–**6**), phenolic glycosides itoside H, xylosmin, scolochinenoside D, and poliothrysoside, and betulinic acid 3β-caffeate were obtained using the bioassay-guided isolation process. Their structures were elucidated by comprehensive analyses of NMR spectroscopic and mass spectrometric data. Even though several extracts and fractions showed significant selective antiviral activity in the CHIKV virus-cell-based assay, none of the purified compounds did. However, in the DENV RNA polymerase assay, significant inhibition was observed with betulinic acid 3β-caffeate (IC_{50} = 0.85 ± 0.1 μM) and to a lesser extent for the flacourtosides A and E (**1** and **5**, respectively), and scolochinenoside D (IC_{50} values ~10 μM).

Flacourtia ramontchi



Chikungunya virus (CHIKV) and dengue virus (DENV) are two emerging arboviruses. CHIKV recently re-emerged, causing massive epidemics that have moved from Africa throughout the Indian Ocean to India and Southeast Asia. In Europe, clinical cases with this virus have already been reported in Italy, and the number is expected to rise in the near future.[1] In humans, the virus is responsible for an acute disease, characterized by a triad of fever, arthralgia, and maculopapular rash.[2] DENV, which is endemic in most tropical and subtropical regions, affects more than 50 million people annually.[3] Geographical expansion and co-circulation of multiple DENV serotypes, increasing the occurrence of secondary infections that are associated with the more severe dengue hemorrhagic fever and dengue shock syndrome, are the drastic changes noted in the recent epidemiology of this disease.[4] Currently, no specific antiviral therapy is available.

A total of 820 EtOAc extracts, obtained from different parts of 400 plants randomly collected in Madagascar, were evaluated for selective antiviral activity in a virus-cell-based assay for CHIKV, leading to the selection of *Flacourtia ramontchi* L'Hér. for a bioassay-guided purification project.[5] This species, also named Governor's plum in Madagascar, is an evergreen tree native to southern Asia and Madagascar. The genus *Flacourtia*, formerly classified in the Flacourtiaceae family, has now been assigned to the Salicaceae family.[6] The chemical content of Salicaceae plants often includes phenolic glycosides based on hydroxylated derivatives of benzyl alcohol.[7] Phenolic glycosides have been studied extensively for their fundamental role in the interaction of plant species of the Salicaceae family with their natural herbivore enemies.[8] In addition, phenolic glycosides are known for their anti-inflammatory activity by inhibiting COX-2,[9] inhibitory activity on snake venom phosphodiesterase I,[10−13] antimalarial activity,[14] and inhibitory activity on HIV-1 RNase H.[15]

Fruits and seeds of *F. ramontchi* are used in folk medicine for the treatment of rheumatic arthralgia, cholera, and dysentery.[16] Historically, steroids (such as daucosterol and β-sitosterol), the butyrolactone lignan ramontoside,[17] phenolic glycosides (such as flacosides A, B, and C, poliothrysoside, and salirepin), and flavonoids (such as kaempferol 3-rutinoside and quercetin 3-rutinoside)[16] have been isolated from *F. ramontchi*.

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Table 1. NMR Spectroscopic Data (500 MHz, Methanol-d_4) for Flacourtosides A–C (1–3)

	flacourtoside A (1)		flacourtoside B (2)		flacourtoside C (3)	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	148.1, C		128.9, C		129.3, C	
2	146.8, C		154.8, C		154.7, C	
3	118.9, CH	7.07, d (7.5)	121.1, CH	7.11, d (9.0)	121.3, CH	7.10, d (9.0)
4	121.0, CH	6.51, t (7.5)	124.3, CH	6.78, dd (9.0, 2.8)	124.3, CH	6.78, dd (9.0, 2.9)
5	125.0, CH	6.81, t (7.5)	154.6, C		154.9, C	
6	117.3, CH	6.76, d (7.5)	113.3, CH	7.05, d (2.8)	113.2, CH	7.05, d (2.9)
7			192.1, CH	10.35, s	191.9, CH	10.34, s
2-Glc						
1′	104.3, CH	4.74, d (7.8)	104.2, CH	4.82, d (8.2)	104.0, CH	4.85, d (7.8)
2′	75.0, CH	3.49, m	75.1, CH	3.49, t (8.2)	74.0, CH	3.71, m
3′	75.9, CH	3.49, m	78.1, CH	3.46, t (8.2)	88.7, CH	3.63, t (8.7)
4′	72.0, CH	3.41, t (7.8)	72.1, CH	3.40, t (8.2)	70.6, CH	3.49, t (8.7)
5′	77.7, CH	3.74, t (7.8)	75.8, CH	3.69, t (8.2)	76.0, CH	3.67, m
6′	65.5, CH_2	4.41, dd (11.7, 7.8)	65.4, CH_2	4.38, dd (11.4, 8.2)	65.5, CH_2	4.31, dd (11.5, 7.8)
		4.69, dd (11.7, 1.5)		4.65, d (11.4)		4.65, d (11.5)
6′-Bz						
1	129.8, C		131.4, C		131.3, C	
2	130.8, CH	8.02, d (7.6)	130.8, CH	7.94, d (7.3)	131.1, CH	8.05, d (7.5)
3	129.8, CH	7.46, t (7.6)	129.8, CH	7.44, t (7.3)	129.7, CH	7.42, t (7.5)
4	134.5, CH	7.59, t (7.6)	134.5, CH	7.58, t (7.3)	134.5, CH	7.52, t (7.5)
5	129.8, CH	7.46, t (7.6)	129.8, CH	7.44, t (7.3)	129.7, CH	7.42, t (7.5)
6	130.8, CH	8.02, d (7.6)	130.8, CH	7.94, d (7.3)	131.1, CH	8.05, d (7.5)
7	168.4, C		167.9, C		168.4, C	
3′-Glc						
1″					105.7, CH	4.60, d (8.1)
2″					75.4, CH	3.33, t (8.1)
3″					77.7, CH	3.43, t (8.1)
4″					72.0, CH	3.38, t (8.1)
5″					75.4, CH	3.67, m
6″					65.3, CH_2	4.33, dd (11.5, 8.1)
						4.77, m
7″					168.0, C	
6″-Bz						
1					131.1, C	
2					130.8, CH	7.94, d (7.5)
3					129.8, CH	7.45, t (7.5)
4					134.5, CH	7.59, t (7.5)
5					129.8, CH	7.45, t (7.5)
6					130.8, CH	7.94, d (7.5)
7					168.0, C	

The present report describes the bioassay-guided isolation, structure elucidation, and antiviral evaluation of six new phenolic glycosides named flacourtosides A–F (**1**–**6**). The structures of the other compounds, i.e., betulinic acid 3β-caffeate, scolochinenoside D,[10] itoside H,[18] xylosmin,[19] and poliothrysoside,[20] were elucidated through comparison with literature data.

RESULTS AND DISCUSSION

A total of 820 EtOAc polyamide cartridge-filtered extracts were prepared from different parts of 400 Madagascan plant species and were evaluated for selective antiviral activity in a CHIKV virus-cell-based assay starting at a concentration of 100 μg/mL. As measured by MTS readout, 32 samples produced a dose–response curve, indicating cell survival and, thus, inhibition of a virus-induced cytopathic effect. Following microscopic quality control, 13 extracts were selected that, at least at one concentration, completely inhibited the virus from inducing a cytopathic effect without showing any adverse effects on host cell morphology and density as compared to the untreated, uninfected cell control condition. Concomitantly, the extracts were evaluated for inhibitory activity in a DENV polymerase assay using the RdRp domain of the DENV-2 NS5 protein. The EtOAc extract of *F. ramontchi* stem bark was selected for chemical investigation because of clear and selective inhibition of the CHIKV-induced cytopathic effect (EC_{50} = 70 μg.mL^{-1}, CC_{50} > 100 μg.mL^{-1}) and pronounced inhibition of DENV NS5 polymerase activity (89% inhibition at 10 μg.mL^{-1}). Consequently, dried and ground stem bark (1.8 kg) of *F. ramontchi* was extracted by ethyl acetate to yield a crude extract (17.4 g) after evaporation of the solvent. This extract was subjected to silica gel chromatography (CC) to produce 25

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Table 2. NMR Spectroscopic Data (500 MHz, Methanol-d_4) for Flacourtosides D−F (4−6)

	flacourtoside D (4)		flacourtoside E (5)		flacourtoside F (6)	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	128.4, C		128.5, C		127.7, C	
2	149.2, C		149.9, C		149.9, C	
3	119.5, CH	6.94, d (9.0)	120.0, CH	7.04, d (8.6)	120.1, CH	6.68, d (8.8)
4	116.6, CH	6.42, dd (9.0, 3.0)	116.9, CH	6.50, dd (8.6, 2.9)	116.9, CH	6.14, dd (8.8, 3.0)
5	154.4, C		154.4, C		154.8, C	
6	116.7, CH	6.70, d (3.0)	117.3, CH	6.78, d (2.9)	117.6, CH	6.56, d (3.0)
7	63.8, CH_2	4.99, d (13.3)	64.7, CH_2	5.28, m	64.6, CH_2	4.80, d (12.5)
		5.03, d (13.3)				4.86, d (12.5)
2-Glc						
1′	102.2, CH	5.09, d (8.0)	104.2, CH	4.90, d (8.6)	102.5, CH	4.92, d (8.0)
2′	75.9, CH	5.23, dd (9.4, 8.0)	73.5, CH	3.80, brt (8.6)	75.7, CH	5.26, dd (9.7, 8.0)
3′	76.1, CH	3.80, m	79.5, CH	5.28, m	76.2, CH	3.74, brt (9.7)
4′	72.4, CH	3.55, brt (9.4)	70.6, CH	3.73, t (8.6)	72.4, CH	3.56, brt (9.7)
5′	76.1, CH	3.80, m	75.6, CH	3.84, brt (8.6)	76.2, CH	3.74, brt (9.7)
6′	65.4, CH_2	4.44, m	65.3, CH_2	4.45, dd (11.8, 7.0)	65.6, CH_2	4.43, dd (11.8, 7.6)
		4.71, dd (11.7, 1.8)		4.68, dd (11.8, 2.1)		4.70, dd (11.8, 2.1)
6′-Bz						
1	131.3, C		131.4, C		131.1, C	
2	131.1, CH	7.98, d (8.0)	130.8, CH	8.07, d (7.3)	130.8, CH	8.01, m
3	129.8, CH	7.45, m	129.6, CH	7.45, m	129.8, CH	7.42, m
4	134.6, CH	7.56, m	134.5, CH	7.57, t (7.3)	134.6, CH	7.59, m
5	129.8, CH	7.45, m	129.6, CH	7.45, m	129.8, CH	7.42, m
6	131.1, CH	7.98, d (8.0)	130.8, CH	8.07, d (7.3)	130.8, CH	8.01, m
7	167.6, C		167.9, C		167.7, C	
2′- or 3′-Bz						
1	131.3, C		131.8, C		131.1, C	
2	130.8, CH	8.06, d (8.0)	131.0, CH	7.97, d (7.3)	130.8, CH	8.01, m
3	129.8, CH	7.43, m	129.8, CH	7.45, m	129.8, CH	7.42, m
4	134.6, CH	7.56, m	134.5, CH	7.57, t (7.3)	134.6, CH	7.59, m
5	129.8, CH	7.43, m	129.8, CH	7.45, m	129.8, CH	7.42, m
6	130.8, CH	8.06, d (8.0)	131.0, CH	7.97, d (7.3)	130.8, CH	8.01, m
7	168.0, C		168.0, C		167.7, C	
cyclohexanone						
1‴	82.7, C					
2‴	45.5, CH_2	2.46, brt (13.0)				
		2.57, dd (13.0, 6.0)				
3‴	206.1, C					
4‴	78.9, CH	4.44, m				
5‴	70.9, CH	3.81, m				
6‴	77.3, CH	3.98, d (9.0)				
7‴	172.6, C					
cyclohexenone						
1‴			86.3, C		84.3, C	
2‴			77.5, CH	4.48, brs	79.0, CH	5.84, brs
3‴			198.9, C		193.3, C	
4‴			127.3, CH	5.96, dd (10.3, 2.6)	127.6, CH	6.02, dd (10.3, 2.3)
5‴			151.1, CH	6.67, dd (10.3, 2.1)	151.0, CH	6.74, dd (10.3, 2.3)
6‴			72.1, CH	4.91, brs	72.3, CH	4.97, t (2.3)
7‴			172.8, C		171.4, C	
2‴-Bz						
1					130.4, C	
2					131.2, CH	7.78, m
3					129.4, CH	7.22, brt (7.6)
4					134.6, CH	7.38, m
5					129.4, CH	7.22, brt (7.6)
6					131.2, CH	7.78, m
7					166.4, C	

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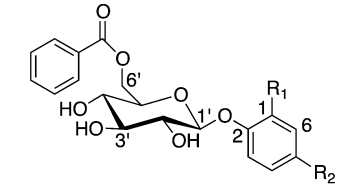
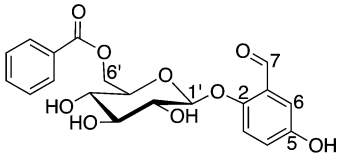


Poliothrysoside: $R_1 = CH_2OH$, $R_2 = OH$
1: $R_1 = H$, $R_2 = OH$



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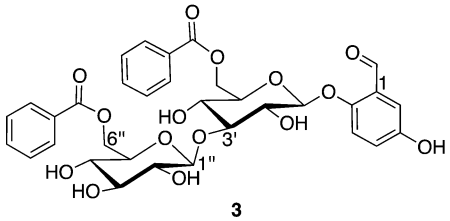

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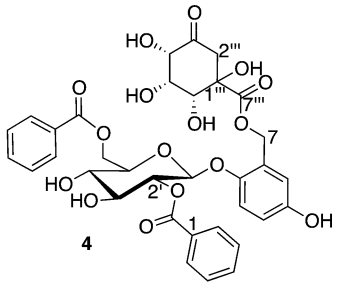

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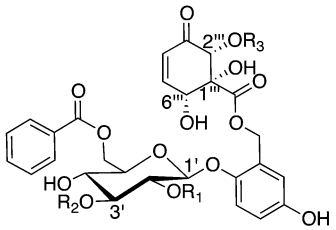
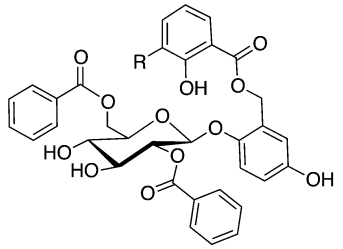


Xylosmin: $R_1 = Bz$, $R_2 = R_3 = H$
5: $R_1 = H$, $R_2 = Bz$, $R_3 = H$
6: $R_1 = Bz$, $R_2 = H$, $R_3 = Bz$



Itoside H: R = H
Scolochinenoside D: R = OH

fractions (F1−F25). Semipreparative and preparative HPLC were used to further fractionate the mixtures. Fractions that showed selective antiviral activity in the chikungunya virus-cell-based assay yielded flacourtosides A, B, E, and F (**1**, **2**, **5**, and **6**), itoside H, and xylosmin. Flacourtosides C and D (**3** and **4**), scolochinenoside D, poliothrysoside, and betulinic acid 3β-caffeate were purified from inactive fractions.

To facilitate comparison of the NMR data of compounds **1**−**6** shown, the carbons were arbitrarily numbered according to the following: the phenolic moiety is denoted from C-1 to C-7, the numbering of the carbons of the first (**1**−**6**) and the second β-glucopyranosyl unit (**3**) and the cyclohexanone (**4**) or cyclohexenone moiety (**5** and **6**) is annotated with single, double, and triple prime symbols, respectively, and a conventional numbering (C-1 to C-7) is used in Tables 1 and 2 for carbons of the remaining benzoyl ester group(s).

Hydrolysis of the ethyl acetate extract produced only D-glucose, and the absolute configuration was identified by UPLC/MS and supercritical fluid chromatography (SFC) analysis, using a chiral analytical column, in comparison with D- and L-glucose as internal references. Hydrolysis of poliothrysoside led to the same result.

Flacourtoside A (**1**) had the molecular formula $C_{19}H_{20}O_8$ on the basis of its quasimolecular ion peak at m/z 399.1064 $[M + Na]^+$ obtained by HR-ESIMS (calcd 399.1056). The IR spectrum revealed absorption bands for OH (3325 cm^{-1}) and conjugated carbonyl (1720 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra (Tables 1 and 2) revealed signals attributable to β-glucopyranosyl, benzoyl, and catechol moieties. The benzoyl moiety was supported by the HMBC correlations from H-2 and H-6 to the ester carbonyl carbon. In addition, the HMBC correlation from H-6′ to the ester carbonyl group indicated that this moiety was attached to the primary alcohol, and an HMBC correlation from H-1′ to C-2 indicated that the catechol moiety was linked through the anomeric carbon of the glucose.

Compounds **2**−**6** are all derivatives of poliothrysoside. Flacourtoside B (**2**) had the molecular formula $C_{20}H_{20}O_9$ as deduced by HR-ESIMS from the pseudomolecular ion peak $[M + Na]^+$ m/z = 427.1009 (calcd $C_{20}H_{20}O_9Na$, 427.1005). Its ^1H NMR spectrum showed peaks characteristic of benzoyl, 2,5-dihydroxybenzaldehyde, and glucose moieties, suggesting a structure similar to that of poliothrysoside.[20] HMBC correlations from H-1′ to C-2 and from H-6′ to the ester carbonyl established the position of the benzoyl and 2,5-dihydroxybenzaldehyde groups on the sugar moiety. The ^1H and ^{13}C spectra revealed the presence of an aldehyde function (H-7/C-7 at δ 10.35 and 192.1). Its location at C-1 was established by HMBC correlations from H-7 to C-2, C-5, and C-6.

The HR-ESIMS of flacourtoside C (**3**) gave a pseudomolecular $[M + Na]^+$ ion peak at m/z 693.1809, indicating the molecular formula $C_{33}H_{34}O_{15}$ (calcd $C_{33}H_{34}O_{15}Na$, 693.1795). Its ^1H, ^{13}C, and HMBC NMR spectra were similar to those of **2**, but with one additional benzoyl group and one additional glucose unit. The glycosidation site was established by HMBC correlations from H-1″ to C-3′ and from H-3′ to C-1″. Thus, the two glucose units were linked via C-3′ and C-1″. The connectivity between the second benzoyl moiety and the second glucose was established by the HMBC correlation from H-6″ to the ester carbonyl carbon. Other 2D correlations were identical to those of compound **2**.

The molecular formula of flacourtoside D (**4**) was established as $C_{34}H_{34}O_{16}$ (by HR-ESIMS). The NMR spectra of **4** suggested a partial structure similar to that of

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poliothrysoside,[20] but with an additional benzoyl moiety and a tetrahydroxylated cyclohexanone moiety. The connectivity between the second benzoyl moiety and the glucose unit was indicated by the HMBC correlation from H-2′ to the ester carbonyl carbon. The cyclohexanone moiety is supported by the presence of a ketone carbonyl signal at δ 206.1 (C-3‴), a quaternary carbon at δ 82.7 (C-1‴), three oxymethines at δ 78.9, 70.9, and 77.3 (C-4‴, C-5‴, and C-6‴, respectively) and a methylene group at δ 45.5 (C-2‴) observed in the ^{13}C NMR and DEPT-135 spectra of **4**. COSY correlations between H-4‴, H-5‴, and H-6‴ and HMBC correlations from H-2‴ to C-3‴, C-4‴, and C-6‴ and from H-4‴ to C-1‴ and C-6‴ confirmed the presence of a 2,3,4,5-tetrahydroxycyclohexanone moiety. Moreover, an HMBC correlation from H-7 to C-7‴ confirmed the ester linkage between the 2′-benzoylpoliothrysoside moiety and the cyclohexanone unit. The relative configurations at C-4‴, C-5‴, and C-6‴ were resolved by analysis of the NOESY spectrum, in which cross-peaks between H-4‴, H-5‴, and H-6‴ indicated that they were all on the same face of the molecule. The configuration at C-1‴ remains unassigned.

Flacourtoside E (**5**) had the molecular formula $C_{34}H_{32}O_{15}$ (by HR-ESIMS). Its ^{1}H and ^{13}C NMR spectra were similar to those of xylosmin,[19] but with a deshielded signal at δ 5.28 for H-3′ and more shielded signals at δ 3.80 and 4.90 for H-2′ and H-1′, respectively, suggesting a different substitution pattern on the glucose unit in **5**. An HMBC correlation from H-3′ to the ester carbonyl carbon established unambiguously the linkage between the glucose unit and the second benzoyl moiety. NOESY correlations between H-2‴ and H-6‴ indicated that they were on the same face of the compound, as shown. The relative configuration of xylosmin reported in the literature[19] indicated that all hydroxy groups were on the same face of the molecule. On this basis and taking into account the identical NMR data for the cyclohexenone moiety of both compounds, flacourtoside E was assigned structure **5** as shown.

The molecular formula of flacourtoside F (**6**) was established as $C_{41}H_{36}O_{16}$. The 1D and 2D NMR data of compound **6** were almost identical to those of scolochinenoside A.[21] The only difference was the absence of an OH on the benzoyl moiety. NOESY correlations between H-2‴ and H-6‴ indicated that they were on the same face of the compound. The orientations of the OH groups at C-1‴ and C-6‴ were assigned arbitrarily as α by comparing the spectral data with those of scolochinenoside A. Thus, flacourtoside F was assigned the structure shown.

It is noteworthy that the chemical constituents from *F. ramontchi* collected in Asia and Madagascar differ in both their nature and quantity, indicating that ecosystems may play a role in the biogenesis of these secondary metabolites.

Unexpectedly and despite the use of a well-controlled bioassay-guided purification process, none of the pure compounds, not even those isolated from bioactive fractions, showed significant anti-CHIKV activity. In an attempt to understand these results, it was decided to reconstruct the parent active fractions by combining the various constituents of each fraction, previously found active, according to their respective peak areas (using ELSD in the final HPLC chromatographic step). However none of the reconstructed fractions produced any significant antiviral effect in the CHIK virus-cell-based assay.

The isolated compounds were also evaluated for inhibitory activity in a DENV NS5 polymerase assay (Table 3). Polymerase activity was assayed by monitoring the incorporation of radiolabeled guanosine into a homopolymeric cytosine RNA template, as previously described.[22] Flacourtosides A (**1**) and E (**5**) and scolochinenoside D displayed moderate inhibitory activity with IC_{50} values of 9.3, 13.4, and 9.5 μM, respectively, and were not found to have an antimetabolic effect on Vero cells. From the available data of the series of phenolic glycosides, it was not possible to derive a structure−activity relationship. Betulinic acid 3β-caffeate was found to exhibit significant inhibitory activity on the polymerase with an IC_{50} of 0.85 μM (3′-deoxy-GTP, IC_{50} = 0.02 μM) and only a moderate antimetabolic effect (CC_{50} = 6.9 μM) on Vero cells. Betulin-derived compounds are well known for their antiviral (HIV,[23] Sindbis virus, Semliki Forest virus,[24] etc.), antimalarial, and anti-inflammatory properties.[23]

Table 3. Biological Evaluation of Compounds on DENV RdRp

compound	polymerase inhibition[a]
1	9.3 ± 2.8
2	71.1 ± 1.2
3	23.8 ± 2.7
4	35.5 ± 3.8
5	13.4 ± 1.9
6	39.8 ± 1.6
itoside H	37.8 ± 3.6
xylosmin	24.3 ± 3.4
scolochinenoside D	9.5 ± 5.0
poliothrysoside	>50
betulinic acid 3β-caffeate	0.85 ± 0.10
3′-deoxy-GTP	0.02

[a]DENV NS5 polymerases: IC_{50} (μM) are mean values ± SD (n = 3).

In conclusion, several new phenolic glycosides have been isolated from the stem bark of *F. ramontchi* using bioassay-guided purification in a CHIK virus-cell-based assay. None of the pure compounds showed selective activity against this virus in this assay. However, modest activity of these compounds was observed in a DENV-2 polymerase assay. In contrast, betulinic acid 3β-caffeate, purified alongside the phenolic glycosides, showed significant inhibition in the latter assay, and it merits further investigation as a selective inhibitor of DENV replication.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined at 24 °C with a JASCO P1010 polarimeter. UV spectra were recorded using a Perkin-Elmer Lamba 5 spectrophotometer. IR spectra were performed on a Nicolet FT-IR 205 spectrophotometer. NMR spectra were recorded in MeOD on a Bruker 500 MHz instrument (Avance 500) with TMS as internal standard. HR-ESIMS data were acquired on a Thermoquest TLM LCQ Deca ion-trap spectrometer. Silica gel 60 (6−35 μm) and analytical plates (Si gel 60F 254) were purchased from SDS (France). Kromasil analytical, semipreparative, and preparative C_{18} columns (250 × 4.5, 250 × 10, and 250 × 21.2 mm; i.d. 5 μm, Thermo) were used for HPLC separations using a Dionex autopurification system equipped with a binary pump (P580), a UV−vis array detector (200−600 nm, Dionex UVD340U), and a PL-ELS 1000 ELSD detector (Polymer Laboratory). The sugar identification was realized by analysis by UPLC and SFC. UPLC analysis was performed with a Waters Acquity Ultraperformance System (UPLC Waters) equipped with a mass detector (TQD Waters). SFC analysis was performed on a Thar Waters SFC Investigator II System using a Waters 2998 photodiode array detector and a Chiralpack analytical IA column (250 × 4.6 mm, 5 μm, Daicel Chemical Industries). All other solvents were purchased from SDS (France).

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Plant Material. Stem bark of *F. ramontchi* was collected in August 2005 from Namakia Toliara in Madagascar and authenticated by Armand Rakotozafy by comparison with an authentic specimen held in the Department of Botany, Parc Botanique et Zoologique de Tsimbazaza, Antananarivo. A voucher specimen (MAD-0055) was deposited at the Institut Malgache de Recherches Appliquées.

Extraction and Isolation. The stem bark (1.8 kg) was successively extracted with EtOAc and MeOH. After concentration, the EtOAc extract (17.4 g) was subjected to silica gel column chromatography (CC) using a gradient of *n*-heptane−acetone−MeOH (1:0:0 to 0:90:10) of increasing polarity, leading to 25 fractions on the basis of TLC. Fraction 18 (286 mg; heptane−acetone, 30:70) was subjected to a semipreparative C_{18} column using MeOH−H_2O (60:40 + 0.1% formic acid) at 3 mL·min^{-1} to afford flacourtoside A (**1**, 3.1 mg), flacourtoside E (**5**, 4.8 mg), flacourtoside F (**6**, 11.3 mg), and itoside H (9.3 mg). Fraction 19 (1100 mg; heptane−acetone, 20:80) was purified on a preparative C_{18} column using MeOH−H_2O (70:30 + 0.1% formic acid) at 21 mL·min^{-1} to afford flacourtoside B (**2**, 7.3 mg), scolochinenoside D (4.2 mg), and xylosmin (11.1 mg). Fraction 20 (908 mg; heptane−acetone, 10:90) was subjected to preparative C_{18} chromatography using MeOH−H_2O (70:30 + 0.1% formic acid) at 21 mL·min^{-1} to afford flacourtoside C (**3**, 1.3 mg) and flacourtoside D (**4**, 3.5 mg). Fraction 21 (4241 mg, heptane−acetone, 0:100) was purified on a preparative C_{18} column using MeOH−H_2O (40:60 + 0.1% formic acid) at 21 mL·min^{-1} to afford poliothrysoside (21.4 mg).

*Flacourtoside A (**1**):* greenish-yellow, amorphous powder; $[\alpha]^{24}_D$ −6 [*c* 0.1, MeOH]; UV [MeOH] λ_{max} (log ε) 229 (3.95), 272 (3.62) nm; IR ν_{max} 3325, 1720, 1453, 1274, 1072, 715 cm^{-1}; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 1; HRESIMS *m*/*z* 399.1064 [M + Na]$^+$ (calcd for $C_{19}H_{20}O_8Na$, 399.1056).

*Flacourtoside B (**2**):* brown, amorphous powder; $[\alpha]^{24}_D$ −21 [*c* 0.1, MeOH]; UV [MeOH] λ_{max} (log ε) 232 (4.16), 257 (4.08), 268 (3.78), 326 (3.58) nm; IR ν_{max} 3442, 1720, 1675, 1450, 1276, 1065, 704 cm^{-1}; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 1; HRESIMS *m*/*z* 427.1009 [M + Na]$^+$ (calcd for $C_{20}H_{20}O_9Na$, 427.1005).

*Flacourtoside C (**3**):* brownish-beige, amorphous powder; $[\alpha]^{24}_D$ −11 [*c* 0.1, MeOH]; UV [MeOH] λ_{max} (log ε) 203 (4.29), 226 (4.37), 256 (3.68) nm; IR ν_{max} 3444, 1724, 1675, 1451, 1276, 1064, 709 cm^{-1}; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 1; HRESIMS *m*/*z* 693.1809 [M + Na]$^+$ (calcd for $C_{33}H_{34}O_{15}Na$, 693.1795).

*Flacourtoside D (**4**):* yellow, amorphous powder; $[\alpha]^{24}_D$ +9 [*c* 0.1, MeOH]; UV [MeOH] λ_{max} (log ε) 229 (4.49), 280 (4.11) nm; IR ν_{max} 3381, 1701, 1457, 1276, 1070, 715 cm^{-1}; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 2; HRESIMS *m*/*z* 721.1734 [M + Na]$^+$ (calcd for $C_{34}H_{34}O_{16}Na$, 721.1744).

*Flacourtoside E (**5**):* brownish-beige, amorphous powder; $[\alpha]^{24}_D$ +8 [*c* 0.1, MeOH]; UV [MeOH] λ_{max} (log ε) 230 (4.41), 274 (3.95), 281 (3.95) nm; IR ν_{max} 3393, 1713, 1455, 1276, 1070, 713 cm^{-1}; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 2; HRESIMS *m*/*z* 703.1641 [M + Na]$^+$ (calcd for $C_{34}H_{32}O_{15}Na$, 703.1639).

*Flacourtoside F (**6**):* brownish-beige, amorphous powder; $[\alpha]^{24}_D$ −14 [*c* 0.1, MeOH]; UV [MeOH] λ_{max} (log ε) 205 (4.57), 228 (4.71), 274 (4.03), 281 (4.03) nm; IR ν_{max} 3444, 1736, 1724, 1453, 1272, 1068, 713 cm^{-1}; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 2; HRESIMS *m*/*z* 807.1909 [M + Na]$^+$ (calcd for $C_{41}H_{36}O_{16}Na$, 807.1901).

Acid Hydrolysis. The EtOAc extract, 487 mg, was heated under reflux in 50 mL of 2 N HCl at 80 °C for 6 h. After removing HCl by evaporation under vacuum, the mixture was diluted with H_2O and EtOAc (3 × 50 mL). The aqueous layer was neutralized with 0.1 M NaOH. The residue containing the sugar was analyzed by UPLC/MS under the following conditions: solvents: (A) ACN−H_2O (30/70) + 0.1% NH_4OH; (B) ACN−H_2O (80/20) + 0.1% NH_4OH, A/B (20/80), flow: 0.17 mL/min, detection: MS. The analysis by SFC was performed under the following conditions: cosolvent MeOH (15%), CO_2 flow rate: 3.4 mL/min, cosolvent flow rate: 0.6 mL/min, total flow: 4 mL/min, detection: ELSD. Identification of D-glucose present in the sugar fraction was carried out by comparison of its retention time with those of authentic samples of L-galactose, D-galactose, D-glucose, and L-glucose.

Chikungunya Virus-Cell-Based Antiviral Assay. Serial dilutions of extract, fractions, or compounds, as well as the reference compound chloroquine, were prepared in assay medium [MEM Rega3 (cat. no. 19993013; Invitrogen), 2% FCS (Integro), 5 mL of 200 mM L-glutamine, and 5 mL of 7.5% sodium bicarbonate] that was added to empty wells of a 96-well microtiter plate (Falcon, BD). Subsequently, 50 μL of a 4× virus dilution in assay medium was added, followed by 50 μL of a cell suspension. This suspension, with a cell density of 25 000 cells/50 μL, was prepared from a Vero cell line subcultured in cell growth medium (MEM Rega3 supplemented with 10% FCS, 5 mL of L-glutamine, and 5 mL of sodium bicarbonate) at a ratio of 1:4 and grown for 7 days in 150 cm^2 tissue culture flasks (Techno Plastic Products). The assay plates were returned to the incubator for 6−7 days (37 °C, 5% CO_2, 95−99% relative humidity), a time at which maximal virus-induced cell death or cytopathic effect (CPE) is observed in untreated, infected controls.

Subsequently, the assay medium was aspirated, replaced with 75 μL of a 5% MTS (Promega) solution in phenol red-free medium, and incubated for 1.5 h. Absorbance was measured at a wavelength of 498 nm (Safire2, Tecan); optical densities (OD values) reached 0.6−0.8 for the untreated, uninfected controls. Raw data were converted to percentage of controls, and the EC_{50} (50% effective concentration, or concentration that is calculated to inhibit virus -induced cell death by 50%) and CC_{50} (50% antimetabolic concentration, or concentration that is calculated to inhibit the overall cell metabolism by 50%) were derived from the dose−response curves. All assay conditions producing an antiviral effect exceeding 50% were checked microscopically for minor signs of CPE or adverse effects on the host cell (i.e., altered cell morphology, etc.). A compound is only considered to elicit a selective antiviral effect on virus replication when, following microscopic quality control, at least at one concentration of compound, no CPE nor any adverse effect is observed (image resembling untreated, uninfected cells). Multiple, independent experiments were performed.

Enzymatic Activity Assay of the Dengue Polymerase. Polymerase activity was assayed by monitoring the incorporation of radiolabeled guanosine into a homopolymeric cytosine RNA template, as previously described.[22] The enzymes were produced and purified as previously described.[22] The determination of the IC_{50} of the pure compounds followed a detailed procedure previously described.[25] IC_{50} was determined using the following equation: % of enzyme activity = $100/[(1 + I^2)/IC_{50}]$, where *I* is the concentration of inhibitor. IC_{50} was determined from curve-fitting using Kaleidagraph (Synergy Software). For each value, results were obtained using triplicates in a single experiment. 3′-Deoxy-GTP was used as the reference.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra for compounds **1**−**6** and chromatograms for determining the sugar are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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