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CONSTITUENTS OF *ERIOBOTRYA JAPONICA*. A STUDY OF THEIR ANTIVIRAL PROPERTIES

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ABSTRACT.—The $CHCl_3$ extract of *Eriobotrya japonica* from an Italian source was shown to contain four new triterpene esters, namely, 23-*trans*-*p*-coumaroyltormentic acid [**1**], 23-*cis*-*p*-coumaroyltormentic acid [**2**], 3-*O*-*trans*-caffeoyltormentic acid [**3**], and 3-*O*-*trans*-*p*-coumaroylrotundic acid [**4**], in addition to three common ursolic acid derivatives **5**, **6**, and **7**.

An investigation of the antiviral properties of compounds **1–7** revealed that only **3** significantly reduced rhinovirus infection. The compounds were ineffective towards human immunodeficiency virus type 1 (HIV-1) and Sindbis virus replication.

The leaves of *Eriobotrya japonica* Lindl. (Rosaceae), a small tree commonly known as loquat, have been documented for use in folk medicine for the treatment of various skin diseases (24) and diabetes mellitus (25). An alcoholic extract of the leaves has been shown to exhibit anti-inflammatory activity and a significant hypoglycemic effect in rabbits (3). Recently we reported the evaluation of hypoglycemic effects in genetically diabetic mice and normoglycemic rats of sesquiterpene glycosides (2) and triterpenoids (1) isolated from *E. japonica* collected in China (3).

In the present study, using the plant collected in Southern Italy, we have isolated four novel triterpene esters **1–4** and three known triterpenes **5–7** from the $CHCl_3$ extract. The structures of **1–4** have been determined by spectral studies, fabms, nmr, and chemical methods.

All the above-mentioned compounds have been tested in vitro for potential antiviral activity against two positive strand RNA viruses, human rhinovirus (HRV) type 1B and Sindbis virus (SNV), and the human retrovirus HIV-1.

RESULTS AND DISCUSSION

The $CHCl_3$ extract of the leaves of *E. japonica* gave compounds **1–4** after SiO_2 cc and reversed-phase hplc. The molecular formulas, $C_{39}H_{54}O_8$ for **1**, **2**, and **3** and $C_{39}H_{54}O_7$ for **4**, were determined by DEPT ^{13}C nmr, ^{13}C nmr (see Table 1), and fabms analysis in the positive ion mode. The fabms of **1** and **2** showed a quasi-molecular ion at m/z $[M + Na]^+$.

The $\Delta^{12,13}$ structure of the triterpenic moiety was derived from the resonance of sp^2

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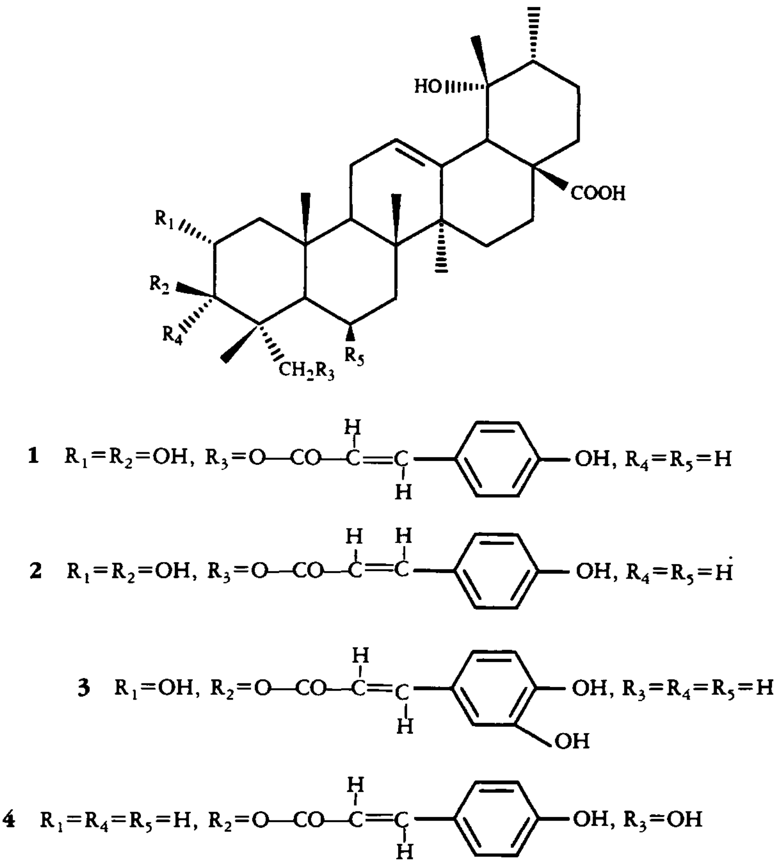

5 R_1=R_4=OH, R_2=R_3=R_5=H

6 R_1=R_3=R_4=H, R_2=R_5=OH

7 R_1=R_2=R_3=OH, R_4=R_5=H

carbons at C-12 (129.4 ppm, CH), which were typical of an urs-12-ene derivative and very different from an olean-12-ene analogue (5).

The ^{1}H-nmr spectrum of **1** in CD_3OD at 250 MHz (see Experimental) showed signals for one olefinic proton at δ 5.25 (1H, t, J = 3.5 Hz), six methyl signals in the region δ 0.76–1.36, one of which was a doublet, and a signal at δ 2.59 (1H, s, H-18) (6) characteristic of a 19α-OH ursolic acid derivative.

The 2α,3β OH substitution of this skeleton was evident from the chemical shift and the J value of two protons ascribable to C-3 (δ 2.95, d, J = 10 Hz) and to C-2 (3.65, ddd, J = 10, 13, 3.5 Hz) and was confirmed by spin decoupling and COSY experiments, which showed a proton sequence H_a-1 (δ 1.77), H_b-1 (δ 0.99), H-2 (δ 3.65), and H-3 (δ 2.95) (Table 2).

Again the ^{1}H nmr spectrum confirmed the presence of a *p*-coumaroyloxy moiety by the signals at δ 6.40 (1H, d, J = 15.8 Hz, H-2′), and δ 7.62 (1H, d, J = 15.8 Hz, H-3′) characteristic of a -CH=CH- trans group, and by the signals at δ 6.90 (2H, d, J = 8.6 Hz, H-2″ and -6″), δ 7.50 (2H, d, J = 8.6 Hz, H-3″ and -5″) requiring a 1-4 disubstituted aromatic ring (4).

The ^{1}H-nmr chemical shifts of C-24 and C-23 in **1** [δ 0.76 (3H, s) and 4.10 (1H, d, J = 10 Hz), 4.68 (1H, d, J = 10 Hz), respectively] and the ^{13}C-nmr shifts (δ 14.06

TABLE 1. ^{13}C-nmr Chemical Shifts of Compounds **1–4**.

Carbon	Compound			
	1	**2**	**3**	**4**
C-1	48.09	47.98	48.61	39.88
C-2	67.00	66.26	66.40	26.22
C-3	78.00	78.37	82.88	82.00
C-4	42.00	43.00	39.85	42.00
C-5	48.21	48.21	54.58	47.92
C-6	18.80	18.71	18.79	17.98
C-7	33.00	32.80	33.36	32.96
C-8	40.11	40.00	40.38	40.80
C-9	46.66	46.50	47.00	46.00
C-10	37.23	37.11	38.00	38.00
C-11	24.80	25.25	24.09	24.80
C-12	129.50	128.00	128.50	129.00
C-13	140.00	138.95	140.08	140.20
C-14	41.00	41.00	41.50	42.90
C-15	28.00	28.79	28.50	28.00
C-16	25.87	25.65	25.50	26.60
C-17	47.00	48.00	48.00	47.80
C-18	55.30	54.86	54.63	55.80
C-19	74.00	73.10	73.50	73.90
C-20	42.88	41.72	42.00	43.10
C-21	26.80	26.50	26.50	26.80
C-22	38.00	37.59	37.90	39.00
C-23	66.40	66.00	29.00	64.00
C-24	14.01	14.31	18.24	15.00
C-25	16.87	17.30	16.81	17.80
C-26	16.94	17.30	17.20	17.30
C-27	23.67	23.67	24.72	23.67
C-28	179.00	179.00	179.50	179.00
C-29	26.18	26.18	27.12	26.16
C-30	16.25	16.15	16.81	16.25
C-1′	167.95	165.95	167.70	167.00
C-2′	115.00	111.07	116.20	115.50
C-3′	145.80	141.60	144.40	145.50
C-1″	125.25	126.71	127.50	125.35
C-2″	130.50	132.75	115.20	130.70
C-3″	116.00	115.00	143.80	116.00
C-4″	160.35	160.44	146.20	160.75
C-5″	116.00	115.00	114.10	116.00
C-6″	130.50	132.75	122.20	130.70

and 66.40 respectively) were indicative of the *p*-coumaric acid moiety linked to the C-23, as deduced by comparison with analogous compounds (7–9). These spectral evidences suggested that **1** was 23-*trans*-*p*-coumaroyltormentic acid.

Compound **2** was obtained in lower yield than its isomer **1**, and the two compounds exhibited closely comparable spectroscopic data, apart from the signals centered at δ 5.90 and 6.92 (J = 13 Hz) corresponding to a cis-conjugated olefinic proton in contrast to the analogous trans-conjugated signals (δ 6.40 and 7.62, J = 16 Hz) observed in the ^{1}H-nmr spectrum of **1**. In addition, the aromatic ring protons at C-2″ and C-6″ occurred at a lower field in the ^{1}H-nmr spectrum of **2** than those of **1** (Table 2). Similar chemical shift and coupling constant differences have been described in the literature of

TABLE 2. ^{1}H-nmr Chemical Shifts (δ) and Coupling Constants (Hz, in parentheses) of Compounds **1–4**.

Proton	Compounds			
	1	**2**	**3**	**4**
H-2β . . .	3.65 ddd (10, 13, 3.75)	3.70	3.94	=
H-3α . . .	2.95 d (10)	3.00 d (10)	4.63 d (10)	4.45 dd (11.5 and 4)
H-23	4.10 d (10)	4.00 d (10)	1.00	3.10 d (10)
	4.68 d (10)	4.58 d (10)		3.40 d (10)
H-24	0.76	0.74	0.90	0.75
H-25	1.00	1.02	1.02	1.02
H-26	0.88	0.88	0.87	0.82
H-27	1.20	1.20	1.22	1.22
H-29	1.36	1.36	1.37	1.38
H-30	0.93 d (6.5)	0.95 d (6.5)	0.96 d (6.5)	0.96 d (6.5)
H-2′	6.40 d (15.8)	5.90 d (13)	6.25 d (16.0)	6.42 d (16.0)
H-3′	7.62 d (15.8)	6.92 d (13)	7.54 d (16.0)	7.60 d (16.07)
H-2″	6.90 d (8.6)	6.74 d (9)	7.09 br s	6.92 d (8.5)
H-3″	7.50 d (8.6)	7.68 d (9)		7.50 d (8.5)
H-5″	7.50 d (8.6)	7.68 d (9)	6.86 d (8)	7.50 d (8.5)
H-6″	6.90 d (8.6)	6.74 d (9)	6.99 br d (8)	6.92 d (8.5)

the cis and trans *p*-coumaroyl esters (4). Therefore, to compound **2** was attributed the structure of 23-*cis*-*p*-coumaroyltormentic acid.

The fabms of **3** in positive ion mode gave a quasi molecular ion species at *m/z* 673 $[M + Na]^+$. The ^{1}H-nmr spectrum of **3** is very similar to that of **1**, thus suggesting the same tormentic-acid skeleton (4, 10). The main differences were the presence of an additional singlet (3H) in the aliphatic region and the absence of the two protons for a 23-CH_2OH group (Table 2). Also, a difference was observed in the chemical shift of H-3 (Table 2) (H-3 δ 4.63, d, $J = 10$ Hz) which was shifted downfield about 1.6 ppm if compared with tormentic acid (4, 10), as expected for an ester bond (11). ^{1}H nmr of **3** also showed the resonances for a 3,4 dihydroxycinnamoyloxy moiety (12). The ^{13}C-nmr chemical shifts of C-2, C-3, and C-4 in **3** (Tables 1 and 2) were indicative of the 3,4-dihydroxycinnamic acid substitution in this compound occurring at C-3 (4). Therefore, compound **3** was assigned as 3-*O*-*trans*-caffeoyltormentic acid.

The fabms of **4**, in the positive ion mode, gave a quasi molecular ion at *m/z* 657 $[M + Na]^+$. The ^{1}H-nmr spectrum confirmed the presence of a 23-CH_2OH group (δ 3.10 and 3.40) (Table 2), of an esterified 3β-OH by signals at δ 4.45 dd ($J = 11.5$ and 4.0 Hz) ascribable to an H-3α, and of a *trans*-*p*-coumaric moiety; the remainder of the ^{1}H-nmr spectrum of **4** was typical of 19α-OH ursolic acid (6, 13) (Table 2). The ^{13}C chemical shifts were indicative of the presence of a rotundic acid skeleton esterified at position C-3 by a *trans*-*p*-coumaric acid (13, 14) (Table 1). Therefore compound **4** was assigned the structure 3-*O*-*trans*-*p*-coumarolyrotundic acid. The compounds **5–7** were identified as 2α,3α,19α-trihydroxyurs-12-en-28-oic acid [**5**], 3β,6β,19α-trihydroxyurs-12-en-28-oic acid [**6**], and 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid [**7**] on the basis of spectral data and comparison with literature data (6, 10, 16).

An in vitro study was performed to determine if compounds **1–7** would offer protection against HIV-1, HRV 1B, and Sindbis virus replication, because antiviral activity has been previously reported for related compounds (17). While none of the compounds displayed any anti HIV-1 activity at noncytotoxic concentrations, slight differences in the cellular cytotoxicities were observed (Table 3). Compound **2** showed the least toxicity; compound **6** was the most toxic. The viral reverse transcriptase is often a

TABLE 3. Anti HIV-1 Activity and Toxicity of Compounds **1–7**.

Compound	Concentration (μg/ml)	% Cell[a]	TC_{50}[b]
1	100	0	30
	20	0	
2	100	0	50
	20	5	
	20	0	10
3	4	1	
4	100	0	30
	20	0	
5	40	0	20
	8	1	
6	4	0	2
	0.8	1	
7	40	0	20
	8	1	
AZT	100	100	>1000
(Zidovudine)	0.016	49	

[a]As determined by the MTT-Formazan method in infected cells as described in the Experimental section. AZT concentrations are given in μM.

[b]Concentration of drug that reduces cell growth by 50%.

key target of antiviral compounds. It has been shown that some natural compounds can inhibit HIV-1 reverse transcriptase but have no antiviral activity in vitro (18); for compounds **1–7** no inhibition of HIV reverse transcriptase activity was observed at concentrations up to 50 μg/ml (data not shown). None of the compounds was found to be effective against infection by SNV when tested at the highest nontoxic concentration (Table 4). In the same experimental conditions compound **3** was found to be active against HRV 1B infection, causing a 50% reduction of CPE at 20μg/ml (Table 4). At a concentration of 4 μg/ml its inhibitory effect was <25%, while a complete loss of activity was observed at 0.8 μg/ml.

Compounds **1–7** were found to have slightly varying cytotoxicities. While the reason for these differences is unclear, it may be attributable to the incubation period used in the assays (5–7 days for HIV-1 vs. 1 day for HRV 1B and SNV) or the different method used to measure cytotoxicity, or it may reflect true differences in the sensitivity of various cell types towards these compounds. This issue was not addressed in the pres-

TABLE 4. In vitro Toxicity and Anti-HRV 1B and Anti-SNV Activity of Compounds **1–7**.

Compound	HRV 1B		SNV	
	Ohio HeLa[a] (μg/ml)	% CPE[b]	HeLa S3[a] (μg/ml)	% CPE
1	50	100	20	100
2	20	100	20	100
3	20	50	4	100
4	20	100	20	100
5	50	100	20	100
6	20	100	20	100
7	50	100	50	100

[a]Maximum nontoxic concentration in μg/ml.

[b]CPE = cytopathic effect.

ent study. Previous results obtained with compounds of related structure, such as quinovic acid glycosides (19) from *Uncaria tomentosa* and *Guettarda platypoda* or oleanolic saponins (20) from *Calendula arvensis*, indicated that they were generally more active against infection by enveloped viruses rather than naked viruses. The presence in *E. japonica* of triterpene or esters of cinnamic acid on the triterpene core instead of sugar residues rendered this kind of molecule ineffective against both enveloped and naked viruses. However, the sugar portion seems to be essential to the activity. Only the caffeic acid derivative **3**, containing two phenolic-OH groups, exhibited a certain antiviral effect against HRV 1B.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The following instruments were used: for nmr, Bruker MW-250 and 500 Spectrospin spectrometers; for fabms, Kratos MS 902 spectrometer equipped with a Kratos fab source; for hplc, Waters Model 6000 A pump equipped with a U6K injector and a 401 refractive index detector; and for optical rotation, Perkin Elmer 241 polarimeter. The fabms spectra and DEPT and COSY experiments were performed as described earlier (1,15).

PLANT MATERIAL.—The plant material was collected in April 1990 at Benevento, Italy and taxonomically examined by Dott. V. De Feo; a sample has been deposited in Dipartimento di Chimica delle Sostanze Naturali, University of Naples.

EXTRACTION AND ISOLATION.—The air-dried leaves (2 kg) were defatted with petroleum ether and extracted with $CHCl_3$ in a Soxhlet apparatus. The dried $CHCl_3$ residue (80 g) was chromatographed on an SiO_2 column by using $CHCl_3$ and increasing MeOH content to 10%. Fractions were checked by tlc on SiO_2 plates in $CHCl_3$-MeOH (9:1) and combined to give three main triterpenic mixtures, A (300 mg), B (139 mg), and C (120 mg). Purification of each fraction was achieved by hplc on a C_{-18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 3 ml/min) using MeOH-H_2O (85:15) as the eluent to afford pure compounds **1** (40 mg, elution time 10 min), **2** (15 mg, elution time 10.5 min), and **3** (30 mg, elution time 4 min) from fraction A, compounds **4** (18 mg, elution time 7 min) and **5** (10 mg, elution time 3.5 min) from fraction B, and compounds **6** (10 mg, elution time 5 min), and **7** (50 mg, elution time 3 min) from fraction C.

Compound **1**.—$[\alpha]^{25}D$ +18.2 (MeOH, c = 1); fabms m/z $[M+Na]^+$ 673; ^1H nmr see Table 2; ^{13}C nmr see Table 1.

Compound **2**.—$[\alpha]^{25}D$ +13.3 (MeOH, c = 1); fabms m/z $[M+Na]^+$ 673; ^1H nmr see Table 2, ^{13}C nmr see Table 1.

Compound **3**.—$[\alpha]^{25}D$ +1.20 (MeOH, c = 1); fabms m/z $[M+Na]^+$ 673; ^1H nmr see Table 2; ^{13}C nmr see Table 1.

Compound **4**.—$[\alpha]^{25}D$ +5.20 (MeOH, c = 1); fabms m/z $[M+Na]^+$ 657; ^1H nmr see Table 2; ^{13}C nmr see Table 1.

Known compounds.—2α,3α,19α-Trihydroxyurs-12-en-28-oic acid [**5**], 3β,6β,19α-trihydroxyurs-12-en-28-oic acid [**6**], and 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid [**7**] were identified by comparison of fabms, ^1H-, and ^{13}C-nmr data with literature values (6,10,16).

CELLS AND VIRUSES.—Ohio and S3 HeLa cells were routinely grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) containing antibiotics (100 UI/ml of penicillin and 100 μg/ml of streptomycin). C8166 cells were grown in RPMI 1640 medium with 10% FCS. The maintenance medium contained only 2% FCS. Rhinovirus type 1B (HRV 1B) and Sindbis virus (SNV) were grown in Ohio HeLa (33 C) and S3 HeLa (37 C) cells, respectively. Cells were infected at a multiplicity of infection (MOI) of 0.1 plaque forming units (PFU) per cell. Virus stocks were harvested when the cytopathic effect (CPE) involved most of the cell monolayer. Cells were frozen and thawed, fluids were clarified by centrifugation at low speed, and the supernatants were stored at −80° until use. The virus titer was estimated by plaque assay (21,22).

ANTIVIRAL COMPOUNDS.—The compounds were dissolved in absolute EtOH at 0.5–1 mg/ml. Suitable dilutions were made in the cell culture medium before use.

DRUG CYTOTOXICITY FOR HELA CELLS AND ESTIMATION OF ANTI-HRV 1B OR SNV ACTIVITY.—To study the cytotoxicity of drugs, the cells were grown to confluence in 96-well tissue culture plates and then treated with different concentrations (100, 50, 20, 4, 0.8 μg/ml) of each compound in

maintenance medium. Triplicate wells were made up for each drug concentration. After 24 h at 37° in 5% CO_2, the cells were inspected microscopically for toxicity. Cell viability was verified by neutral red uptake. To evaluate the antiviral effect of the drugs, cell monolayers in 96-well plates were infected for 1 h with HRV 1B at an MOI of 40 PFW/cell (33°) or SNV at an MOI of 25 PFW/cell (37°). After removing unadsorbed inoculum, the cells were added to maintenance medium containing the indicated concentrations of each compound. After 24 h of incubation (at 33° for HRV 1B and 37° for SNV) the appearance of viral CPE was checked by light microscopy. CPE was graded on a progressive scale of 0 (normal cells) to 100% (complete destruction of the cell layer). At this time virus controls showed 100% CPE.

DRUG CYTOTOXICITY FOR C8166 CELLS AND ANTI-HIV ASSAY.—The anti HIV-1 activities and toxicities of compounds were assessed in C8166 cells infected with HIV III-B strain. Cells (4×10^4 per microtiter plate well) were mixed with 5-fold dilutions of compound prior to addition of 10 $CCID_{50}$ (cell culture infecting dose 50%) units of virus and incubated for 5–7 days. Formation of syncytia was examined from 2 days post-infection. Cell viability of infected cells and cytotoxicity of uninfected cell controls were measured by the MTT-Formazan method as described by Pauwels *et al.* (23).

REVERSE TRANSCRIPTASE ASSAYS.—HIV-1 RT reactions were performed in a volume of 25 μl containing 50 mM Tris, pH 8.0, 100 mM KCl, 6 mM $MgCl_2$, 5 mM DTT, 0.05% Triton X-100, 10 μM [^{3}H] dTTP (4 Ci/mmol) 1.6 μg/ml poly A/oligo (dT), and a final enzyme concentration of 1.4 μg/ml (0.05 U/ml). At the end of the incubation time (20 min), aliquots of the reactions were spotted onto DEAE paper, and dropped immediately into ice-cold 5% TCA, 50 mM sodium pyrophosphate, followed by washing with H_2O and then absolute EtOH. Filters were then air-dried and counted by liquid scintillation.

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