Saponins and Polyphenols from *Fadogia ancylantha* (Makoni Tea)

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Three new saponins (**1**−**3**) and a known saponin, together with four known polyphenolic compounds, have been isolated from the fermented and dried leaves of *Fadogia ancylantha* (Makoni tea). The structures of compounds **1**−**3** were established by analysis of their spectroscopic data. Both an ethanol−water extract of *F. ancylantha* and its phenolic constituents showed significant free-radical-scavenging and antimicrobial activities. No cytotoxicity, as evaluated by analysis of hypodiploid nuclei in HUVEC cells using propidium iodide staining, was observed for either the plant crude extract or its constituents.

Fadogia ancylantha Hiern. (Rubiaceae), commonly known as "makoni" or "marange", is a wild perennial shrub growing in the Eastern Highlands area of Zimbabwe. The leaves have been traditionally used by certain African rural communities as a medicine and beverage to boost the immune system, to tone muscles, and strengthen bones and for the treatment of topical ulcers. The plant is also thought to have antipoisonous and aphrodisiac properties.[1] The leaves and stems when fermented and dried are used for producing a black tea with antioxidant effects,[2] which is caffeine-free and particularly rich in proteins,[1] phenolic compounds,[2] and zinc.[1] It is well recognized that zinc is an essential trace element, influencing growth and reproduction and affecting the development of the immune system and integrity of the skin. Nutritional doses of zinc supplements are able to prevent alteration of the immune system and to improve resistance to infection.[3] Cosmetic compositions comprising *F. ancylantha* extracts are reported to improve skin tone due to antiaging, antibacterial, and anti-free-radical effects on aged, photoaged, stressed, and tired skin, showing simultaneously protective and restorative properties.[4] For centuries, the local population in Zimbabwe has brewed Makoni tea instead of commercial brands, with the tea having grown in popularity and with many commercial products now on the local and global market. Despite its natural nutrients and health benefits, no phytochemical nor biological studies have been reported in the literature on *F. ancylantha.* However, the related species *F. agrestis* has been shown to contain monoterpene glycosides,[5] alkaloids, anthraquinones, and flavonoids[6] and to possess aphrodisiac and antiplasmodial activities.[6,7]

The present study, carried out on the EtOH−H_2O (7:3) extract from a commercial sample of Makoni tea, reports on its triterpenoid saponin and polyphenol composition and its bioactivity. Three new oleanane-type saponins (**1**−**3**) were isolated and their structures elucidated using spectroscopic methods including 1D (^{1}H, ^{13}C, and 1D TOCSY) and 2D NMR (DQF-COSY, ROESY, HSQC, and HMBC) experiments as well as HRESIMS analysis. The in vitro free-radical-scavenging (DPPH test) and antimicrobial (broth microdilution assay) effects of the crude extract and of its major components were also evaluated. Finally, the cytotoxicity of this extract and pure compounds (**1**−**3** and polyphenols) was tested in human umbilical vein endothelial cells (HUVEC) and evaluated as a percentage of apoptotic cells as measured by analysis of hypodiploid nuclei, using a propidium iodide method.

Fermented and dried leaves of *F. ancylantha* were extracted at room temperature with EtOH−H_2O (7:3). The hydroalcoholic extract was dried in vacuo and then suspended in water and lyophilized. A part of this extract was chromatographed over Sephadex LH-20 and by reversed-phase HPLC to yield three new (**1**−**3**) saponins and a known oleanolic saponin from fraction I, two dihydrochalcones from fraction II, a flavone from fraction III, and a flavonol from fraction IV. The known compounds were identified by comparison of their NMR data with those from the literature as 3-*O*-*β*-D-glucopyranosyl-3*β*-hydroxy-olean-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside,[8] confusoside,[9] davidigenin,[10,11] 7-*O*-*β*-D-glucopyranosyl-7,3′,4′-trihydroxyflavone,[12,13] and hirsutrin.[14,15] The concentrations of confusoside, davidigenin, and 7-*O*-*β*-D-glucopyranosyl-7,3′,4′-trihydroxyflavone, as determined by a HPLC direct calibration method (see Experimental Section), were 10.7, 2.7, and 2.2% w/w of the extract, respectively. The total phenolic content of the extract, as determined by the Folin−Ciocalteau method and expressed as a davidigenin equivalent, was 283.0 *μ*g/mg.

The structure elucidation of compounds **1**−**3** proceeded as follows. The HRESIMS of **1** showed a major ion peak at *m*/*z* 949.6000 [M + Na]$^+$, supporting the molecular formula of

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Table 1. ^{13}C and ^{1}H NMR Spectroscopic Data of the Aglycon Portion of Compound **1** in CD_3OD[a]

position	δ_C	δ_H (J in Hz)[b]
1	39.1	0.98, 1.61, m
2	28.8	1.02, 1.76, m
3	90.1	3.17, dd, (4.5, 12.0)
4	39.5	
5	56.3	0.76, m
6	18.4	1.41, 1.55, m
7	31.9	1.58, 1.65, m
8	40.2	
9	48.4	1.57, m
10	37.3	
11	22.6	1.65, 1.87, m
12	122.9	5.25, t (3.5)
13	144.9	
14	42.2	
15	25.1	1.68, 1.92, m
16	23.2	1.65, 2.06, m
17	47.3	
18	41.8	2.81, dd, (3.0, 14.0)
19	46.5	1.31, 1.71
20	30.7	
21	34.0	1.21, 2.33, m
22	33.2	1.34, 1.46, m
23	27.8	1.04, s
24	16.8	0.84, s
25	15.3	0.95, s
26	16.1	0.79, s
27	25.6	1.15, s
28	177.8	
29	32.8	0.90, s
30	23.3	0.93, s

[a] Assignments confirmed by 2D COSY, HSQC, and HMBC experiments. [b] $^{1}H-^{1}H$ coupling constants (Hz) were measured from the COSY spectra.

$C_{48}H_{78}O_{17}$ (calcd for $C_{48}H_{78}O_{17}Na$, m/z 949.6060) and suggesting a triterpene aglycon with two hexoses and one deoxyhexose unit in the molecule. MS-MS analysis of the ion peak showed a fragment ion at m/z 641.2552 $[M - (162 + 146) + Na]^+$, due to the loss of a hexose and a deoxyhexose unit. An intense signal at m/z 331.0273 was also observed, consistent with a sodiated disaccharide chain $[m/z\ (162 + 146) + Na]^+$. The combined NMR data (Tables 1 and 2) indicated that **1** has oleanolic acid[16] as the aglycon and three sugar units (anomeric protons at δ_H 4.31, d, $J = 7.6$ Hz; δ_H 5.44, d, $J = 7.5$ Hz; δ_H 5.37, d, $J = 1.5$ Hz, and a methyl doublet, δ_H 1.25, d, $J = 6.6$ Hz). The glycosylation of the alcoholic function at C-3 (δ_C 90.1) and the esterification of the −COOH group (δ_C 177.8) were indicated by the downfield shift (+11.4 ppm) exhibited by C-3 and upfield shift (−3.5 ppm) shown by C-28 in **1**, when compared with oleanolic acid.[16]

The sugar units were identified as two glucopyranosyls (-I and -II) and one rhamnopyranosyl from the 1D TOCSY and 2D NMR (COSY, HSQC, HMBC, and ROESY) data (Table 2; Supporting Information, Figures S3 to S9). The β-configuration at the anomeric positions of the two glucopyranosyl units and an α-configuration for the rhamnopyranose were suggested by the chemical shifts of key carbons (C-2, C-3, and C-5) and by the coupling constants[16] $J_{H1'-H2'}$ and $J_{H1''-H2''} = 7.6$ Hz and $J_{H1'''-H2'''} = 1.5$ Hz and were confirmed by the NOE effects observed in the ROESY spectra between $H\text{-}1_{eq}'''-H\text{-}2_{eq}'''$, $H\text{-}1_{ax}'-H\text{-}2_{ax}'$, and $H\text{-}1_{ax}''-H\text{-}2_{ax}''$ (Supporting Information, Figure S9). The D-configuration for glucose and L for rhamnose were assigned after hydrolysis of **1** with 1 N HCl and subsequent GC analysis of the trimethylsilylated product. The absence of any ^{13}C NMR glycosidation shift for the carbon resonances of the α-L-rhamnopyranosyl and of the β-D-glucopyranosyl-I moieties suggested that these sugars are terminal units. The chemical shifts of the H-1″ (δ_H 5.44) and C-1″ (δ_C 94.8) signals and the HMBC correlations observed between the H-1″ resonance (δ_H 5.44, glucopyranosyl-II) and C-28 of the aglycon (δ_C 177.8) and between the H-1‴ resonance (δ_H 5.37, rhamnopyranosyl) and C-2″ (δ_C 78.8) of glucopyranosyl-II indicated that this sugar unit is linked to the C-28 carboxylic group by an ester bond[17] and is involved in a (1→2) linkage (C-2″, δ_C 78.8, glycosidation shift + 3.5 ppm by a β-effect) with respect to δ_C 75.3 (C-2′-Glc_I, Table 2). The cross-peak observed in the HMBC spectrum between the H-1′ resonance of the glucopyranosyl-I (δ_H 4.31) and the carbon signal at δ_C 90.1 confirmed the placement of this sugar unit at C-3 of the aglycon. Thus, compound **1** was elucidated as 3-*O*-β-D-glucopyranosyl-3-β-hydroxyolean-12-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl] ester.

Compound **2** gave the molecular formula $C_{47}H_{76}O_{17}$ on the basis of the HRESIMS molecular ion at m/z 935.5305 $[M + Na]^+$ (calcd for $C_{47}H_{76}O_{17}Na$, m/z 935.5364), suggesting it to be a triterpene derivative with two hexose units and one pentose unit in the molecule. MS-MS analysis of the ion peak showed a fragment ion at m/z 641.4288 $[M - (162 + 132) + Na]^+$, indicating the loss of a hexose and a pentose unit. The NMR spectroscopic data of the aglycon moiety of **2** were close to those of **1** (Table 1). The proton coupling network within each of the three sugar residues was established using a combination of 1D TOCSY and DQF-COSY methods; direct evidence for the sugar sequence and their linkage sites was derived from HSQC and HMBC data (Table 2). This indicated that compound **2** differs from **1** only by the presence of a terminal β-apiofuranosyl instead of an α-rhamnopyranosyl unit in the disaccharide chain at C-28. The nature of the terminal sugar unit as β-apiofuranosyl[18] was deduced from the following evidence: the ^{1}H NMR spectrum indicated an anomeric signal at δ_H 5.41 (H-1‴, d, $J = 2.0$ Hz); in the 1D TOCSY experiment, selective excitation of the signal at δ_H 5.41 led to the enhancement only of H-2‴ (δ_H 3.95, d, $J = 2.0$ Hz); the multiplicity of H-2‴ may be derived only from the presence of a quaternary carbon at C-3, characteristic of an apiofuranosyl structure; and in the ROESY spectrum, NOE effects were observed between H-2‴ and the protons of the hydroxymethyl group, and H-2‴ and H-4b‴ (Supporting Information, Figure S13), indicating that H-2‴, the hydroxymethyl group, and H-4b‴ are found on the same face of the ring for this sugar. The sugar configurations were determined to be D-glucose and D-apiose after hydrolysis of **2** with 1 N HCl and GC analysis. The (1→2) linkage between C-1‴ and C-2″ was confirmed by the pertinent correlations observed in the HMBC spectrum. Thus, the structure of **2** was assigned as 3-*O*-β-D-glucopyranosyl-3-β-hydroxyolean-12-en-28-oic acid 28-*O*-[β-D-apiofuranosyl-(1→2)-β-D-glucopyranosyl] ester. The HRESIMS of **3** showed a major peak at m/z 965.5000 $[M + Na]^+$, consistent with the molecular formula, $C_{48}H_{78}O_{18}$ (calcd for $C_{48}H_{78}O_{18}Na$, m/z 965.5050), and suggesting a triterpene aglycon having three hexose units in the molecule. MS-MS analysis of the ion peak showed a fragment ion at m/z 641.4126 $[M - (162 + 162) + Na]^+$, indicating the loss of two hexose units. An intense signal at m/z 347.1020 was also observed, consistent with a sodiated disaccharide chain $[(162 + 162) + Na]^+$. The NMR data (^{1}H, ^{13}C, 1D TOCSY, DQF-COSY, HSQC, HMBC) revealed that compound **3** differs from **1** only in the nature of the disaccharide chain at C-28. The ^{1}H NMR spectrum of the sugar portion of compound **3** showed three anomeric proton signals at δ_H 4.31 (H-1′, d, $J = 7.6$ Hz), δ_H 5.45 (H-1″, d, $J = 7.5$ Hz), and δ_H 4.80 (H-1‴, d, $J = 7.6$ Hz). The assignments of all proton and carbon resonances by 1D TOCSY, 2D DQF-COSY, HSQC, and HMBC experiments (Table 2) as well as hydrolysis and GC analysis of the sugars led to the identification of two terminal β-D-glucopyranosyls (-I and -III) and a β-D-galactopyranosyl glycosylated at C-2. The β-D-galactopyranosyl unit could be located at C-28 on the basis of the chemical shifts of H-1″ (δ_H 5.45) and C-1″ (δ_C 94.5), indicating an ester linkage,[17] and on the basis of the HMBC correlations observed between the resonances of H-1″ (δ_H 5.45) and C-28 (δ_C 177.8). Finally, the cross-peaks observed in the HMBC spectrum between H-1‴ (δ_H 4.80, β-D-glucopyranosyl-III) and C-2″ (δ_C 77.4, β-D-galactopyranosyl unit) and between H-1′ (δ_H 4.31, β-D-glucopyranosyl-I) and C-3

Table 2. ^{13}C and ^{1}H NMR Spectroscopic Data for Glycosyl Moieties of Compounds **1**−**3** in CD_3OD[a]

	1		2		3	
position	δ_C	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)[b]
C-3 Glc-I[d]-1′	105.8	4.31, d (7.6)	106.8	4.31, d (7.6)	106.2	4.31, d (7.6)
Glc_I-2′	75.3	3.19, dd (9.0, 7.6)	75.3	3.18, dd (9.0, 7.6)	75.3	3.18, dd (9.0, 7.6)
Glc_I-3′	77.4	3.34, t (9.0)	77.8	3.33, t (9.0)	77.9	3.32, t (9.0)
Glc_I-4′	70.6	3.29, t (9.0)	71.2	3.27, t (9.0)	71.4	3.28, t (9.0)
Glc_I-5′	76.8	3.24, m	77.4	3.24, m	77.5	3.23, m
Glc_I-6′	61.8	3.68, dd (12.0, 4.0)	62.1	3.66, dd (12.0, 4.0)	62.3	3.66, dd (12.0, 4.0)
		3.84, dd (12.0, 2.5)		3.81, dd (12.0, 2.5)		3.81, dd (12.0, 2.5)
C-28 Gal[e]-1″					94.5	5.45, d (7.5)
Gal-2″					77.4	3.89, dd (7.8, 9.9)
Gal-3″					71.1	3.67, dd (3.2, 9.9)
Gal-4″					70.0	3.37, d (3.4)
Gal-5″					78.1	3.57, m
Gal-6″					61.3	3.68, dd (4.5, 11.5)
						3.79, dd (7.7, 11.5)
C-28 Glc_{II}[d]-1″	94.8	5.44, d (7.6)	94.7	5.39, d (7.5)		
Glc_{II}-2″	78.8	3.55, dd (7.5, 9.0)	79.0	3.54, dd (7.5, 9.0)		
Glc_{II}-3″	78.1	3.39, t (9.0)	76.6	3.38, t (9.0)		
Glc_{II}-4″	70.6	3.39, t (9.0)	73.3	3.36, t (9.0)		
Glc_{II}-5″	77.9	3.32, m	77.1	3.31, m		
Glc_{II}-6″	62.3	3.66, dd (4.5, 12.0)	62.1	3.66, dd (4.5, 12.0)		
		3.79, dd (3.5, 12.0)		3.81, dd (3.5, 12.0)		
C-2″ Api[f]-1‴			110.8	5.41, d (2.0)		
Api-2‴			77.8	3.95, d (2.0)		
Api-3‴			79.8			
Api-4a‴			74.6	3.72, d (10.0)		
Api-4b‴				4.00, d (10.0)		
Api-5‴			64.7	3.54, br s		
C-2″ Rha[g]-1‴	101.6	5.37, d (1.5)				
Rha-2‴	71.5	3.93, dd (1.5, 2.0)				
Rha-3‴	71.6	3.66, dd (2.0, 8.5)				
Rha-4‴	73.3	3.38, t (8.5)				
Rha-5‴	69.8	3.75, m				
Rha-6‴	17.9	1.25, d (6.6)				
C-2″ Glc_{III}[d]-1‴					102.7	4.80, d (7.6)
Glc_{III}-2‴					71.7	3.14, dd (9.0, 7.6)
Glv_{III}-3‴					77.0	3.21, t (9.0)
Glc_{III}-4‴					72.8	3.37, t (9.0)
Glc_{III}-5‴					77.1	3.29, m
Glc_{III}-6‴					62.7	3.62, dd (12.0, 4.0)
						3.90, dd (12.0, 2.5)

[a] Assignments confirmed by 2D COSY, HSQC, and HMBC experiments. [b] $^{1}H-^{1}H$ coupling constants (Hz) were measured from COSY spectra. [c] Glc_I = β-D-glucopyranosyl at C-3. [d] Glc_{III} = β-D-glucopyranosyl at C-2″. [e] Gal = β-D-galactopyranosyl. [f] Api = β-D-apiofuranosyl. [g] Rha = α-L-rhamnopyranosyl.

of the aglycon (δ_C 90.1) led to the structure, 3-*O*-β-D-glucopyranosyl-3-β-hydroxyolean-12-en-28-oic acid 28-*O*-[β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl] ester, for **3**.

Both the EtOH−H_2O (7:3) extract of *F. ancylantha* and its pure phenolic constituents were tested for free-radical-scavenging as well as antibacterial and antifungal activities. In the DPPH test, significant concentration-dependent free-radical-scavenging activity was shown by both the extract and 7-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxyflavone (EC_{50} values 80.4 and 28.4 μg/mL, respectively), with respect to α-tocopherol (EC_{50} 10.1 μg/mL) used as positive control. No antioxidant activity was shown by confusoside and davidigenin (Table S1, Supporting Information). These results confirmed previous observations[19] indicating that mono- and dihydroxylated chalcones and dihydrochalcones possess limited antioxidant activity. The plant extract and its phenolic compounds exhibited a weak antibacterial activity, as tested by a broth microdilution method (Table S2, Supporting Information). The dihydrochalcone davidigenin showed MIC values ranging from 0.12 to 0.25 mg/mL against Gram-positive bacteria and 0.12 mg/mL against *A. niger*. In the case of Gram-negative bacteria and *C. albicans* the MIC values were greater than 1.0 mg/mL. Finally, the cytotoxic activity of the extract, compounds **1**−**3**, and polyphenols was tested in human umbilical vein endothelial cells (HUVEC) as a percentage of apoptotic cells.[20] Cells were incubated as described in the Experimental Section with either vehicle (DMSO) or different concentrations of the crude extract or compounds in a range of concentrations [1.0−100 μg/mL (extract) and 1.0 and 50 μM (compounds)]. None of the tested products produced significant effects on the percentage of hypodiploid cells. Although the leaves of *F. ancylantha* are used widely as a traditional medicinal remedy and beverage, no study on its constituents and active compounds has been reported before. Oleanolic acid saponins and polyphenolic compounds (dihydrochalcones, flavones, and flavonols) are the major characteristic components of *F. ancylantha* leaves. The free-radical-scavenging and antibacterial activity reported and the absence of cytotoxicity suggest that the crude plant extract and its major constituents are good candidates for use as remedies against photoaging skin damage.

Experimental Section

General Experimental Procedures. Melting points were determined using a Mettler-Toledo DSC822[e] apparatus. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell in MeOH solution. For NMR experiments, a Bruker DRX-600 NMR spectrometer was used, operating at 599.2 MHz for ^{1}H and at 150.9 MHz for ^{13}C, and using the UXNMR software package; chemical shifts are expressed as δ (parts per million) values, referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD; coupling constants, J, are in Hz. 1D and 2D NMR experiments were carried out using conventional pulse sequences. ESIMS was performed on a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA) equipped with Xcalibur software. Exact masses (HRESIMS) were measured by a Q-TOF Premier (Waters) triple-

quadrupole orthogonal time-of-flight (TOF) instrument having an electrospray ionization source. HPLC separations were performed with a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, a μ-Bondapak C_{18} column (300 × 7.8 mm i.d.), and a U6K injector. Quantitative HPLC analysis was carried out on an Agilent 1100 series system equipped with a model G-1312 pump, a Rheodyne model G-1322A loop (20 μL), and a DAD G-1315 A detector. Peak areas were calculated with an Agilent integrator. GC analyses were performed using a Dani GC 1000 instrument on a l-Chirasil-Val column (0.32 mm × 25 m).

Plant Material. A commercial sample of fermented and dried (semiprocesed raw leaves in tea bags) *F. ancylantha* was supplied, in October 2007, by Commercio Alternativo Società Cooperativa, Ferrara, Italy. A voucher sample (CIF F.A.1) was deposited at the Herbarium of the Department of Pharmaceutical Sciences, University of Salerno. The authentication of herbal material is supported as a HPLC trace of the crude plant extract (Supporting Information, Figure S1).

Extraction and Isolation. Dried and powdered leaves of *F. ancylantha* (300 g) were extracted at room temperature with EtOH−H_2O (7:3) and dried to give 9.19 g of a residue. A portion (2.6 g) of this extract was chromatographed over a Sephadex LH-20 column (1 m × 3 cm i.d.) using MeOH as eluent (flow rate 0.5 mL min^{-1}). Fractions (8 mL each) were collected and checked by TLC (silica gel; using *n*-BuOH−AcOH−H_2O (60:15:25) and $CHCl_3$−MeOH−H_2O (7:3:0.3) as solvents). Fractions with similar R_f values were combined, giving four major fractions (I−IV), which were further purified by RP-HPLC on a 30 cm × 7.8 mm i.d. μ-Bondapack column (flow rate 2.0 mL min^{-1}). Fraction I (180 mg) was chromatographed with MeOH−H_2O (65:35) as mobile phase to yield compounds **1** (12.6 mg), **2** (11.4 mg), and **3** (20.6 mg) and 3-*O*-β-D-glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (3.4 mg). Fractions II (450 mg) and III (200 mg) were purified using MeOH−H_2O (4:6) as mobile phase, giving confusoside (170 mg) from fraction II and davidigenin (40.7 mg) and 7-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxyflavone (24.2 mg) from fraction III. Fraction IV (100 mg) was purified with MeOH−H_2O (35:65) as the eluent to afford hirsutrin (10.0 mg).

Compound 1: white powder; mp 221 °C; $[\alpha]^{21}_D$ −3.8 (*c* 0.13, MeOH); ^{1}H (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150.9 MHz) data, see Tables 1 and 2; HRESIMS *m/z* 949.6000 $[M + Na]^+$ (calcd for $C_{48}H_{78}O_{17}Na$, *m/z* 949.6060).

Compound 2: white powder; mp 229 °C; $[\alpha]^{21}_D$ −7.3 (*c* 0.20, MeOH); ^{1}H and ^{13}C NMR of the aglycon were superimposable on those of **1**; ^{1}H and ^{13}C NMR of the sugar moieties, see Table 2; HRESIMS *m/z* 935.5305 $[M + Na]^+$ (calcd for $C_{47}H_{76}O_{17}Na$, *m/z* 935.5364).

Compound 3: white powder; mp 233 °C; $[\alpha]^{21}_D$ −3.5 (*c* 0.15, MeOH); ^{1}H and ^{13}C NMR of the aglycon were superimposable on those of **1**; ^{1}H and ^{13}C NMR of the sugar moieties, see Table 2; HRESIMS *m/z* 965.5000 $[M + Na]^+$ (calcd for $C_{48}H_{78}O_{18}Na$, *m/z* 965.5050).

3-*O*-β-D-Glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside: white powder; mp 230 °C; $[\alpha]^{21}_D$ −6.3 (*c* 0.170, MeOH); ^{1}H and ^{13}C NMR data were consistent with those previously reported;[8] HRESIMS *m/z* 819.7172 $[M + Na]^+$ (calcd for $C_{42}H_{68}O_{13}Na$, *m/z* 819.7232).

4′-β-*O*-D-Glucopyranosyl-4,2′,4′-trihydroxydihydrochalcone (confusoside): ^{1}H and ^{13}C NMR data were consistent with those previously reported;[9] ESIMS *m/z* 421 $[M - H]^+$.

4,2′,4′-Trihydroxydihydrochalcone (davidigenin): ^{1}H and ^{13}C NMR data were consistent with those previously reported;[10,11] ESIMS *m/z* 259 $[M + H]^+$.

7-*O*-β-D-Glucopyranosyl-7,3′,4′-trihydroxyflavone: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[12,13] ESIMS *m/z* 433 $[M + H]^+$.

Quercetin 3-*O*-β-D-glucopyranoside (hirsutrin): ^{1}H and ^{13}C NMR data were consistent with those previously reported;[14,15] ESIMS *m/z* 487 $[M + Na]^+$.

Acid Hydrolysis of 1−3. Compounds **1**−**3** (0.8 mg) in 1 N HCl (0.25 mL) were each subjected to hydrolysis, derivatized with 1-(trimethylsilyl)imidazole and pyridine (0.1 mL), and analyzed by GC using an L-Chirasil-Val column (0.32 mm × 25 m), according to a method previously reported.[21] The peaks from the hydrolysate of **1** were detected at 14.72 min (D-glucose) and 5.90 min (L-rhamnose), from the hydrolysate of **2** at 14.74 min (D-glucose) and 11.90 min (D-apiose), and from the hydrolysate of **3** at 14.72 min (D-glucose) and 14.95 min (D-galactose). Retention times for authentic samples (D-glucose, D-galactose, and L-rhamnose from Sigma-Aldrich, Milan, Italy, and D-apiose from Omicrometer Biochemicals, South Bend, IN) in the same experimental conditions were detected at 14.72 min (D-glucose), 14.95 min (D-galactose), 11.90 min (D-apiose), and 5.90 min (L-rhamnose), respectively.

Quantitative HPLC Analysis of the Extracts. Quantitative HPLC was conducted using a 150 × 4.6 mm i.d. particle size 5 μm C_{18} Thermo Electron column. The solvents were HCOOH 0.1% in H_2O (solvent A) and MeOH (solvent B). The elution gradient used was as follows: 0 → 20 min, 20 → 30% B; 20 → 30 min, 30 → 35% B, 30 → 60 min, 35 → 40% B, 60 → 70 min, 40% B. Analysis was carried out in triplicate, at a flow rate of 1.0 mL min^{-1} with a DAD detector set at 277 nm. The reference standard solutions of confusoside, davidigenin, and 7-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxyflavone were prepared at three concentration levels in the range 1.50−6.1 mg/mL for confusoside, 0.45−3.6 mg/mL for davidigenin, and 0.80−3.1 mg/mL for 7-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxyflavone. The standard curves were analyzed using the linear least-squares regression equation derived from the peak area (regression equation $y = 781369x + 11760$, $r = 0.9981$ for confusoside; $y = 1639292x + 7656.2$, $r = 0.9989$ for davidigenin; and $y = 349060x + 1403.3$, $r = 0.9997$ for 7-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxyflavone, where y is the peak area and x the concentration). The peaks associated with the three compounds were identified by retention time and UV spectroscopic and mass spectrometric comparison with the standard compounds and confirmation by co-injection. The EtOH−H_2O (7:3) extract of *F. ancylantha* leaves was dissolved in MeOH and analyzed under the same chromatographic conditions.

Quantitative Determination of Total Phenols. The EtOH−H_2O (7:3) extract of *F. ancylantha* leaves, dissolved in MeOH, was analyzed for its total phenolic content according to the Folin−Ciocalteau colorimetric method.[21] Total phenols were expressed as a davidigenin equivalent (283.0 ± 0.1 μg/mg extract).

Bleaching of the Free-Radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH Test). The antiradical activity of the crude plant extract and the polyphenols under investigation was determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), according to the procedure previously described by Mencherini et al.[21] Briefly, an aliquot (37.5 μL) of the MeOH solution containing different amounts of the extract or compounds from *F. ancylantha* was added to 1.5 mL of DPPH solution (0.025 g/L in MeOH), prepared daily. An equal volume (37.5 μL) of the vehicle alone was added to control tubes. Absorbances at 515 nm were measured on a Shimadzu UV-1601 UV−visible spectrophotometer 10 min after starting the reaction. α-Tocopherol (EC_{50} 10.1 ± 1.3 μg/mL) was used as a positive control in the test. All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC_{50}) were calculated using the Litchfield and Wilcoxon protocol[22] (Supporting Information, Table S1).

Antimicrobial Activity. The extract and polyphenols from *F. ancylantha* were tested for antimicrobial activity using the broth microdilution method in 96-multiwell microtiter plates, in duplicate, as reported by Mencherini et al.[21] and as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2001),[23] using Gram-positive and Gram-negative bacteria, a yeast, and a mold, all from the American Type Culture Collection (ATCC). The lowest concentrations of the products at which microbial growth was inhibited after 24 h (MIC) and at which survival of any microbial cell was not possible after incubation for 48 h (bacteria strains) and 5 days (yeasts and molds) (MBC) were determined (Supporting Information, Table S2).

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were cultured in complete EGM-2 medium containing an EGM-2 bullet kit (Cambrex) with growth factors and 2.5% fetal bovine serum supplied by the vendor. Cells were plated at a density of 1 × 10^5 cells/well (Falcon, BD Bioscience, Bedford, MA) the day before treatment. At the end of the incubation period, cells were processed for flow cytometry analyses. The cells were used up to a maximum of 10 passages.

Analysis of Apoptosis. HUVEC cells were incubated with either vehicle (DMSO) or different concentrations of the extract or compounds (**1**−**3** and polyphenols) in a range of concentrations [1.0−100.0 μg/mL (extract) and 1.0 and 50.0 μM (compounds)]. Hypodiploid DNA content was analyzed using the method of propidium iodide staining and flow cytometry, as described previ-

ously.[20] Briefly, cells were washed in phosphate-buffered saline and resuspended in 500 μL of a solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide (all from Sigma-Aldrich, Milan, Italy). After incubation at 4 °C for 30 min in the dark, cell nuclei were analyzed with a Becton Dickinson FACScan flow cytometer using the Cells Quest program. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on logarithmic scale. The percentage of the cells in the hypodiploid region was calculated. Statistical comparison between groups was made using ANOVA followed by the Bonferroni parametric test.[22] Differences were considered significant if $p < 0.05$.

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Supporting Information Available: 1D and 2D NMR spectra of the new compounds **1** (^{1}H, HSQC, HMBC, DQF-COSY, ROESY, and TOCSY), **2** (^{1}H, ROESY, and TOCSY), and **3** (^{1}H, and TOCSY) as well as Tables S1 (antioxidant activity) and S2 (antimicrobial activity). This information is available free of charge via the Internet at http://pubs.acs.org.

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