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Research Paper

Topical anti-inflammatory activity of quillaic acid from *Quillaja saponaria* Mol. and some derivatives

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Abstract

Objectives Quillaic acid is the major aglycone of the widely studied saponins of the Chilean indigenous tree *Quillaja saponaria* Mol. The industrial availability of quillaja saponins and the extensive functionalisation of this triterpenoid provide unique opportunities for structural modification and pose a challenge from the standpoint of selectivity in regard to one or the other secondary alcohol group, the aldehyde, and the carboxylic acid functions. The anti-inflammatory activity of this sapogenin has not been studied previously and it has never been used to obtain potential anti-inflammatory derivatives.
Methods A series of quillaic acid derivatives were prepared and subjected to topical assays for the inhibition of inflammation induced by arachidonic acid or phorbol ester.
Key findings Quillaic acid exhibited strong topical anti-inflammatory activity in both models. Most of its derivatives were less potent, but the hydrazone **8** showed similar potency to quillaic acid in the TPA assay.
Conclusions The structural modifications performed and the biological results suggest that the aldehyde and carboxyl groups are relevant to the anti-inflammatory activity in these models.
Keywords anti-inflammatory activity; arachidonic acid; phorbol acetate; quillaic acid derivatives; *Quillaja saponaria*

Introduction

Quillaja saponaria Molina D. Don (1831), Quillajaceae, is an indigenous tree of the Coastal Range and the foothills of the Andes in semiarid central Chile, the inner bark (also known as soap bark, Seifenrinde, Panama bark, bois de Panama) of which has long been used to wash hair and wool.[1] Mapuche people, the major ethnic group of south-central Chile, have used it for toothache and against 'inflammation of the respiratory system'.[2] In modern times, the abundance of this tree has rendered it an important commercial source of crude saponins used as foaming, wetting and emulsifying agents, and, in a more purified form, in the preparation of photographic emulsions, cosmetics, vaccines and other medicinal products.[3,4] Quillaja saponins occur as complex mixtures of glycosides and sugar esters, mainly of the pentacyclic triterpene quillaic acid (1, 3β, 16α-dihydroxy-23-oxoolean-12-en-28-oic acid).[5] Quillaic acid has been recognized as the major aglycone of quillaja trees for at least 80 years, long before its structure was clearly established, but recent studies have shown the presence of several other sapogenins sharing its oleanane skeleton.[6]

Many pentacyclic triterpenes are of medicinal interest, both in their natural forms and as hemisynthetic derivatives.[7–13] The anti-inflammatory activity of several of these compounds has been reported, underscoring their interest as ingredients of pharmaceutical and pharmacological composition.[14,15] Since quillaja saponins are currently extracted on an industrial scale, their sapogenins might be inexpensive starting materials for the preparation of derivatives with medical applications. In addition, quillaic acid is a highly functionalised compound that allows a broad range of chemical modifications to modulate the pharmacological activity. The main aim of this study was to demonstrate for the first time the topical anti-inflammatory effects of quillaic acid and several of its derivatives, most of them new, focusing our attention on the more highly

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functionalised rings A and D as characteristic features for structural modification. In spite of the traditional use of saponin-rich aqueous extracts of *Q. saponaria* to treat inflammation, no scientific studies have been published in this regard, and the potential use of their major sapogenin or its derivatives as anti-inflammatory agents has not been explored thus far.

The topical anti-inflammatory activity was evaluated by arachidonic acid (AA) and 12-*O*-tetradecanoyl phorbol-13 acetate (TPA) induced inflammation assays in mouse ears.[16] TPA acts primarily as an activator of protein kinase C and nuclear factor κB (NF-κB), promoting the enhanced expression of proinflammatory enzymes. On the other hand, AA presumably acts as a precursor of inflammatory mediators such as prostaglandins and leukotrienes. In addition, neutrophils that quickly reach the inflamed site release myeloperoxidase and NADPH oxidase as part of the inflammatory response.[17–19]

Materials and Methods

General procedures

Synthesis grade reagents and solvents were purchased from Merck (Darmstadt, Germany). The commercial semi-purified *Q. saponaria* saponin mixture UltraDry 100Q was donated by Desert King Chile (Quillota, Chile). Details of the preparation and purification of quillaic acid and its derivatives are given below. All commercially available chemicals were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 126 MHz or at 400 and 100 MHz, respectively, on commercial spectrometers. Chemical shifts (δ) are reported in ppm relative to the tetramethylsilane signal, using the solvent as internal reference. Mass spectra were obtained using an ESI-IT Esquire 4000 electrospray-ion trap instrument from Bruker Daltonik (Bremen, GFR). Column chromatography (CC) was carried out using Merck silica gel 60 (0.015–0.040 mm) and analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} aluminium foils. Spots were detected by spraying with *p*-anisaldehyde/sulfuric acid reagent, followed by heating for 1 min at 120°C. IR spectra were recorded for samples prepared in KBr discs, using a PerkinElmer 1310 spectrometer. High performance liquid chromatography-diode array detector (HPLC-DAD) determinations were carried out on a Hewlett Packard series 1100 chromatograph (Waldbronn, Germany). Two columns were used: (1) Nova-Pak C18 (300 × 3.9 mm, 4 µm) column (Waters, Milford, MA, USA); and (2) Symmetry C18 (150 × 3.9 mm, 5 µm) column (Waters). The mobile phase was a gradient of 2% aqueous AcOH, MeCN/AcOH/water (20 : 2 : 78) and 100% MeOH. The flow rate was set at 1 ml/min and the UV detector at 280 nm. The injection volume was 20 µl.

The standard work up method was as follows: the organic phase was washed with saturated aqueous $NaHCO_3$ solution (three times) followed by saturated aqueous NaCl solution (three times), then dried over anhydrous Na_2SO_4, and filtered. The filtrate was concentrated *in vacuo* in a rotary evaporator. For the pharmacological studies, TPA and AA were purchased from Sigma (St Louis, MO, USA). Indometacin and nimesulide were donated by Laboratorio Chile (Santiago, Chile).

Isolation and characterization of quillaic acid (1)

UltraDry 100Q (20 g) was refluxed with 9% HCl (500 ml) for 3 h, cooled to room temperature and filtered. The solid was washed with water and dried to obtain a residue weighing 8 g. The residue was subjected to CC on Sephadex LH-20. The fraction eluted with hexane/dichloromethane/methanol (6 : 2 : 1) showed a similar $R_F = 0.52$ on TLC (CH_2Cl_2-EtOAc 1 : 1) to a quillaic acid standard. White amorphous powder (3.5 g; 17.5%); mp 291–295°C; IR (KBr) 3506 (-OH), 2945, 1721 (CO), 1589, 1452 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$, CD_3OD) δ 0.84, 0.88, 0.95, 1.04, 1.10, 1.40 (3H each, s, Me-26, 29, 30, 25, 24, 27), 2.23 (1H, t, $J = 14.2$ Hz, 19-H), 3.01 (1H, dd, $J = 4.1$; 14.2 Hz, 18-H), 3.80 (1H, dd, $J = 5.4$; 9.5 Hz, 3-H), 4.46 (1H, t, $J = 4.4$; 2.9 Hz, 16-H), 5.35 (1H, t, $J = 3.4$ Hz, 12-H), 9.33 (1H, s, 23-H); ^{13}C-NMR (125.6 MHz, CD_3OD) δ 9.40, 16.20, 17.70, 24.88, 26.95, 56.76, 59.75, 72.78, 75.21, 123.1, 145.2, 208.5; HREIMS calculated for $C_{30}H_{45}O_5$: 485.3272481; found: 485.3273220.

Preparation of quillaic acid derivatives

Quillaic acid methyl ester (2)

Methyl iodide (25 µl, 0.41 mmol) was added to a solution of quillaic acid (1, 200 mg, 0.41 mmol) and Cs_2CO_3 (401 mg, 1.23 mmol) in DMF (10.3 ml) at 0°C. The reaction mixture was stirred at 0°C for 45 min, worked up as described above and then purified by silica gel CC (CH_2Cl_2-EtOAc 1 : 1) to afford **2** (190 mg, 95%) as a white solid, $R_F = 0.83$ (CH_2Cl_2-EtOAc 2 : 1); mp 174–177°C; IR (KBr) 3422, 2947, 2363, 1717, 1654 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.73, 0.88, 0.96, 0.99, 1.00, 1.40 (3H each, s, Me-24, 26, 29, 30, 25, 27) 3.01 (1H, dd, $J = 4.2$; 14.1 Hz, 18-H), 3.57 (3H, s, -C*H*$_3$), 3.77 (1H, m, 3-H), 4.44 (1H, m, 16-H), 5.30 (1H, t, $J = 3.3$ Hz, 12-H), 9.29 (1H, s, 23-H). ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 9.40, 16.18, 17.57, 24.82, 27–29, 33.40, 49.90, 52.33, 56.75, 72.78, 75.14, 123.3, 145.0, 179.3, 208.5; HREIMS calculated for $C_{31}H_{48}O_5$: 523.3387280; found: 523.3393957.

Methyl 3β,16α,23-trihydroxyolean-12-en-28-oate (16α-hydroxyhederagenin methyl ester, 3)

$NaBH_4$ (15 mg, 0.39 mmol) was added to a solution of **2** (129 mg, 0.26 mmol) in MeOH (7.0 ml) at 0°C. The reaction mixture was stirred for 2 h at 0°C. Then cold water (70 ml) was added, and the mixture was worked up and the solid residue was purified by silica gel flash CC (CH_2Cl_2-EtOAc 2 : 1) to give **3** (120 mg, 93%), $R_F = 0.36$ (CH_2Cl_2-EtOAc 1 : 1); mp 150–155°C; IR (KBr) 3422, 2947, 2363, 1717, 1654 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.70, 0.76, 0.88, 0.96, 0.98, 1.38 (3H each, s, Me-24, 26, 29, 30, 25, 27), 3.00 (1H, dd, $J = 4.4$; 14.3 Hz, 18-H), 3.29 (1H, d, $J = 11.0$ Hz, 24-H), 3.52 (1H, d, $J = 11.0$ Hz, 24-H), 3.57 (3H, s, CH_3), 3.63 (1H, dd, $J = 5.1$; 11.0 Hz, 3-H), 5.30 (1H, t, $J = 3.3$ Hz, 12-H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 12.75, 16.36, 17.75, 24.84, 27.31, 33.40, 67.30, 73.86, 75.23, 123.5, 145.0, 179.4; HREIMS calculated for $C_{31}H_{49}O_5$: 501.3578930; found: 501.3585483.

3,16-Dihydroxyolean-12-en-23,28-dioic acid 28-methyl ester (4a) and methyl 4-nor-3,16-diketoolean-12-en-28-oate (4b)

To a solution of **2** (100 mg, 0.199 mmol) in acetone (9.7 ml) in an ice bath was added Jones reagent (0.54 ml/mmol substrate, 1.75 ml) dropwise until the colour of the solution changed to pale brown from green. The mixture was stirred

at room temperature for 20 min, and after removal of the acetone, water was added. The aqueous mixture was extracted with EtOAc (3 × 10 ml) and the extract was worked up to give an amorphous solid that was fractionated by silica gel flash CC (CH_2Cl_2-EtOAc 2 : 1) to give **4b** (25 mg, 25%), $R_F = 0.81$ (CH_2Cl_2-EtOAc 2 : 1), and **4a** (10 mg, 10%), $R_F = 0.17$ (CH_2Cl_2-EtOAc 1 : 1). **4a**: mp 180–190°C; IR (KBr) 3447, 2925, 2854, 2361, 1712 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.72, 0.90, 0.97, 1.25, 1.34 (3H each, s, Me-26, 30, 29, 27, 25), 2.04 (1H, s, 24-H), 2.71 (1H, d, $J = 15.2$ Hz, 19-H), 3.35 (1H, dd, $J = 3.8$, 14.4 Hz, 18-H), 3.61 (3H, s, CH_3), 4.30 (1H, t, 16-H), 4.51 (1H, s, 3-H), 5.38 (1H, t, $J = 3.3$ Hz, 12-H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 51.92, 72.24, 74.96, 175.5, 177.4, 122.7, 142.7. **4b**: mp 240–246°C; IR (KBr) 3411, 2956, 2924, 2855, 1719, 1715 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.87, 0.92, 0.93, 1.17, 1.19 (3H each, s, Me-30, 25, 29, 26, 27), 1.00 (3H, d, $J = 6.4$ Hz, 24-H), 2.71 (1H, d, $J = 15.2$ Hz, 19-H), 3.33 (1H, dd, $J = 3.8$; 14.4 Hz, 18-H), 3.67 (3H, s, CH_3), 5.56 (1H, t, $J = 3.3$ Hz, 12-H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 52.44, 124.1, 140.5, 173.3, 208.6, 213.6.

Methyl 3β,16α-dihydroxy-23-(t-butyldimethylsilyloxy)-olean-12-en-28-oate (5)

A mixture of **3** (100 mg), *tert*-butyldimethysilyl chloride (96 mg, 1.3 mmol) and imidazole (170 mg, 2.5 mmol) in anhydrous DMF (0.5 ml) was stirred at room temperature under N_2 for 18 h. Then, hexane (30 ml) was added, and the solution was washed successively with brine (2 × 10 ml) and distilled water (20 ml), and dried with Na_2SO_4. After evaporation of the solvent, the residue was purified by silica gel CC (hexane-EtOAc, 90 : 10) to give **5** (60 mg, 63%), $R_F = 0.80$ (CH_2Cl_2-EtOAc 2 : 1); mp 290–294°C; IR (KBr) 3542, 3467, 2951, 2927, 2856, 1710 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.05 (9H, s, Si-$(CH_3)_3$), 0.72, 0.88, 0.90, 0.96, 1.33 (3H each, s, Me-30, 25, 29, 26, 27), 3.03 (1H, dd, $J = 3.8$, 14.4 Hz 18-H), 3.33 (1H, dd, 2H, -CH_2O), 3.5 (1H, t, 3-H), 3.70 (3H, s, CH_3), 4.50 (1H, s, 16-H), 5.40 (1H, t, $J = 3.3$, 12-H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 11.20, 16.04, 16.98, 23.40, 24.80, 27.05, 52.91, 56.45, 68.85, 72.79, 75.15, 123.7, 145.2, 178.0. HRMS $[M\text{-}Na]^+ = 639.31305$ ($C_{37}H_{64}O_5Si = 616.45230430$).

Methyl 3β,16α-dihydroxy-23-(t-butyldimethylsilyl)-12,13-epoxyolean-28-oate (6)

A mixture of **5** (50 mg, 0.06 mmol), and *m*-chloroperoxy benzoic acid (*m*CPBA) (60%) (43 mg, 0.5 mmol) in CH_2Cl_2 (3 ml) was stirred at room temperature overnight. Then the mixture was diluted with CH_2Cl_2-Et_2O (1 : 2) and was worked up to give a product (65 mg) that was subjected to preparative TLC (hexanes-EtOAc 1 : 5 : 1) to give epoxide **6** as a crystalline solid. An analytically pure, colourless sample was obtained by recrystallization from MeOH, $R_F = 0.60$ (CH_2Cl_2-EtOAc 2 : 1), mp 295–297°C; IR (KBr) 3411, 2948, 2862, 1701, 1627 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 0.55, 0.88, 0.92, 0.96, 1.22 (3H each, s, Me-30, 25, 29, 26, 27), 3.42 (2H, dd, -CH_2O), 4.03 (1H, dd, $J = 3.8$; 14.4 Hz, 18-H),4.34 (1H, s, 16-H), 5.21 (1H, brt, 12-H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 11.45, 16.09, 16.18, 23.35, 26.57, 52.90, 55.47, 56.36, 67.98, 72.24, 75.12, 178.6; HRMS $[M\text{-}Na]^+ = 655.72304$ ($C_{37}H_{64}O_6Si = 632.45623432$).

3β,16α,23-Trihydroxyolean-12-en-28-oic acid (16α-hydroxyhederagenin, 7)

We obtained compound **7** under the same conditions used to obtain compound **3**. $NaBH_4$ (1.15 mg, 0.03 mmol) was added to a solution of 1 (10 mg, 0.02 mmol) in MeOH (1.0 ml) at 0°C. The reaction mixture was stirred for 2 h at 0°C. Then cold water (10 ml) was added, and the mixture was worked up and the solid residue was purified by silica gel flash CC (CH_2Cl_2-EtOAc 2 : 1) to give 7 (8 mg), $R_F = 0.50$, mp 200–204°C; IR (KBr) 3420, 2947, 2370, 1715, 1654 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) 5.50 (1H, t, H-12), 4.45 (H-16), 4.03 (1H, dd, H-3), 3.50 (d, CH_2), 3.30 (d, CH_2 $J = 11.0$ Hz), 3.00 (1H, dd, H-18), 0.70–1.3 (6 signals, 3H each, s).

23-(1-Amino-4-methylpiperazinyl) imino-3β,16α-dihydroxyolean-12-en-28-oic acid (8)

Compound **1** (100 mg, 0.205 mmol), 1-amino-4-methylpiperazine (61.2 mg, 0.6 mmol), absolute ethanol (43 ml) and glacial acetic acid (1.7 ml) were heated under reflux for 24 h. The reaction mixture was concentrated to dryness and the residue was purified by silica gel CC eluting with CH_2Cl_2-EtOAc (1 : 1) to give **8** (35 mg, 35% yield) as a yellow powder; mp 200–210°C; IR (KBr) 3424, 2944, 2862, 2361, 1610 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz) δ 0.77, 0.83, 0.94, 0.95, 0.97, 1.31 (3H each, s, Me-30, 25, 29, 24, 26, 27), 2.41 (3H, s, N-CH_3), 2.63 (4H, m, -CH_2-N-CH_3), 3.04 (4H, m, -CH_2-N-CH_2-), 3.27 (3H, s, CH_3), 3.52 (1H, t, $J = 5.1$ Hz, H-3), 3.67 (1H, dd, $J = 3.8$; 14.2 Hz, H-18), 4.40 (1H, s, H-16), 5.27 (1H, t, $J = 3.5$ Hz, H-12), 6.70 (1H, s, H-23); ^{13}C-NMR (CD_3OD, 100 MHz) δ 11.09, 14.18, 17.03, 19.49, 22.35, 50.70, 51.76, 53.47, 74.25, 75.32, 121.4, 144.2, 152.6, 179.4. HREIMS calculated for $C_{35}H_{57}N_3O_4$: 583.4303220; found: 583.4300958.

Quillaic acid bis-acetate (9)

Compound **1** (100 mg, 0,21 mmol) was treated with Ac_2O (0.5 ml, 0.41 mmol) in dry pyridine (1.5 ml) in the presence of 4-dimethylaminopyridine (25 mg) at room temperature for 12 h. The mixture was worked up to give an amorphous solid, which was purified by silica gel column chromatography eluting with CH_2Cl_2-EtOAc (2 : 1) to give **9** (96 mg, 80% yield); $R_F = 0.85$ (CH_2Cl_2-EtOAc 2 : 1); mp 180–186°C; IR (KBr) 3431, 2950, 2861, 1736, 1656 cm^{-1}; ^{1}H-NMR ($CDCl_3$, Pyr-d_5, 400 MHz) δ 0.70, 0.85, 0.94, 1.00, 1.07, 1.27 (3H each, s, Me-30, 25, 29, 24, 26, 27), 2.07 (6H, s, acetoxy), 3.05 (1H, dd, $J = 3.6$; 14.2 Hz, 18-H), 4.51(1H, s, 3-H), 5.39 (1H, s, 16-H), 5.64 (1H, t, $J = 3.4$ Hz, 12-H); 9.23 (1H, s, 23-H); ^{13}C-NMR ($CDCl_3$, Pyr-d_5, 100 MHz) δ 9.48, 15.65, 16.93, 20.38, 21.96, 23.24, 73.27, 76.25, 123.1, 142.1, 170.0, 170.4, 180.1, 204.5. HRMS $[M\text{-}Na]^+ = 593.3448750$ ($C_{34}H_{50}O_7 = 570.367643025$).

Quillaic acid bis-hemisuccinate (10)

A mixture of **1** (100 mg, 0.21 mmol) and pyridine (138 ml) with succinic anhydride (200 mg, 2.03 mmol) and 25 mg of

4-dimethylaminopyridine was stirred at 90°C for 5 days. Then the mixture was worked up to give a crude solid. The residue was fractionated by silica gel CC eluting with CH_2Cl_2-EtOAc (2 : 1) containing acetic acid (1%) to give **10** (40 mg, 20%); mp: 200–203°C; IR (KBr) 3433, 2929, 2863, 2359, 1708, 1637 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.80, 0.85, 0.90, 0.92, 1.00, 1.10 (3H each, s, Me-30, 25, 29, 24, 26, 27), 2.50 (4H, s, OOC-CH_2-CH_2-COOH), 3.03 (1H, dd, $J = 3.6$; 14.2 Hz, 18-H), 4.31 (s, 3-H), 4.50 (s, 16-H), 5.25 (1H, t, $J = 3.3$ Hz, 12-H), 9.20 (1H, s, 23-H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 8.41, 15.43, 16.85, 17.98, 20.06, 21.60, 22.49, 41.53, 43.72, 53.15, 54.10, 68.88, 73.80, 124.3, 148.5, 171.9, 174.7, 179.2, 205.0.

Animals

Adult male CF-1 mice (20–25 g) derived from a stock maintained at the Public Health Institute (Chile) were used to assess the anti-inflammatory effects of the compounds. All animals were kept on a 12-h light–dark cycle, with water and food provided *ad libitum*; the animals were fasted overnight before the experiments. All animal experiments were performed according to the ethical guidelines of the International Guiding Principles for Biomedical Investigation with Animals promulgated by the CIOMS (1990) and those of the Bioethics Committees of the Chilean Public Health Institute and the Faculty of Chemical and Pharmaceutical Sciences.

Topical anti-inflammatory activity

The topical anti-inflammatory activity was evaluated *in vivo* as described by previously.[16] Briefly, groups of eight animals were treated with a single dose of each test compound (equimolar doses with regard to the reference drugs), dissolved in acetone and applied topically on the inner (10 µl) and outer (10 µl) surfaces of the right ear of the animals of each group. After 5 min, 2 mg of AA or 5 µg of TPA was administered topically on the right ear and acetone on the left ear as a solvent control. The samples were applied before applying either pro-inflammatory agent in order to disturb the absorption of the active principles through the skin as little as possible. Control animals were treated similarly, but they did not receive the samples. Two other groups of eight animals were treated with nimesulide or indometacin, drugs used as references for topical inhibition of inflammatory activity induced by AA or TPA, respectively. After 1 and 4.5 h for AA and TPA, respectively, all the animals were killed by cervical dislocation and a section (6 mm in diameter) of the right and left ears was punched out and weighed. The dermal anti-inflammatory effect (%E) was evaluated according to the following equation:

$$\%E = (W_c - W_s / W_c) \times 100$$

where W_c is the median of the weight differences of the right and left ear sections of controls, and W_s is the median of the weight differences of the right and left ear sections of the treated animals.

Statistical analysis

Data were expressed as median values ± SEM calculated from the weight of the ear disks for treated and untreated animals considering control values as 100% inflammation. A non-parametric method of analysis was used. Statistical significance of more than two groups were evaluated using the Kruskall–Wallis test, followed by Dunnett's multiple test for individual comparisons (Trial version of GraphPad Prism 5 was used).[20] The criterion for statistical significance was set at $P \leq 0.05$.

Results

Quillaic acid (**1**, Figure 1) was isolated by acid hydrolysis of a commercial semi-purified *Quillaja saponaria* saponin mixture followed by chromatography. Compound **1** was

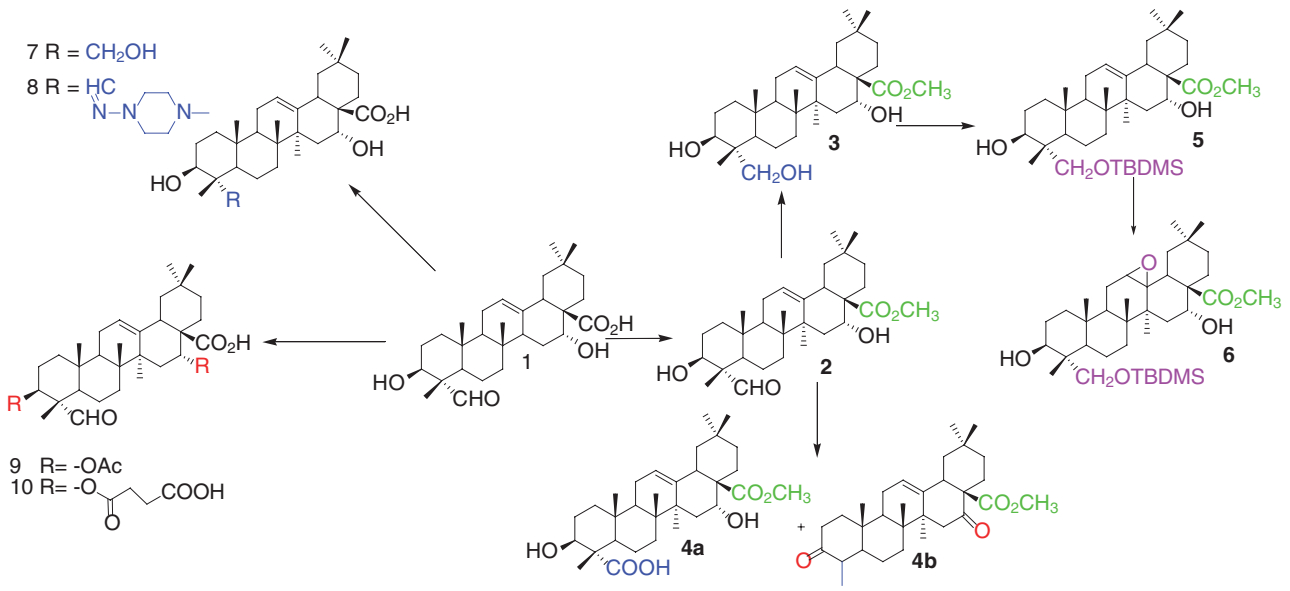

Figure 1 Structure of quillaic acid (**1**), the major aglycone of quillaja saponin, and derivatives.

Table 1 Inhibitory effect of quillaic acid and derivatives against dermal inflammation

Compound	Dose *vs* TPA (mg/20 μl per ear)	Dose *vs* AA (mg/20 μl per ear)	$\%E_{TPA}$	$\%E_{AA}$
1	0.7	1.6	62.2* ± 16.6	92.1* ± 4.2
2	0.7	1.6	38.4*,† ± 9.8	27.3*,† ± 7.4
3	0.9	1.6	47.0*,† ± 17.1	0† ± 9.0
4a	0.5	1.2	32.0*,† ± 10.0	0† ± 5.9
4b	0.5	1.1	24.1† ± 9.2	3.2† ± 18.8
5	0.6	1.5	0† ± 27.5	–6.8† ± 10.2
6	0.9	2.0	9.7† ± 13.7	0† ± 3.4
7	0.7	1.6	31.0*,† ± 7.0	11.4† ± 6.7
8	0.8	1.8	60.0* ± 13.7	–4.5† ± 11.8
9	0.8	1.8	47.3*,† ± 10.5	18.9*,† ± 9.0
10	0.8	1.9	40.3*,† ± 5.9	39.2*,† ± 14.4
Indometacin	0.5	0.5	92.9*,† ± 3.2	0† ± 9.0
Nimesulide	1.0	1.0	5.0† ± 5.6	48.8*,† ± 14.0

$\%E_{AA}$ and $\%E_{TPA}$ refer to the inhibitory effect against dermal inflammation induced by arachidonic acid (AA) and 12-*O*-tetradecanoyl phorbol-13 acetate (TPA), respectively. Each value represents the median ± SEM of the results obtained from eight animals treated with samples or reference drugs (indometacin and nimesulide). $^*P \leq 0.05$, significantly different compared with the control group. $^\dagger P \leq 0.05$, significantly different compared with the group treated with compound **1**.

obtained as a white amorphous powder. The purity (≥95%) of the quillaic acid was established by HPLC. Spectral data were in agreement with published data.[21,22]

As no information was available as to the specific derivatives of quillaic acid that might constitute lead compounds, we focused our attention on the more highly functionalized rings A and D as characteristic features for structural modification, the relevance of which for anti-inflammatory activity might be surmised from the activity of the new compounds (Figure 1). Quillaic acid methyl ester (**2**) was prepared from **1** in order to facilitate CC purification. Spectral data of **2** were in agreement with published data.[3] Some derivatives were prepared from **2**, a somewhat more lipophilic compound that may act as a prodrug if it is a substrate for tissue esterases. Other derivatives (**3**, **5** and **6**) were prepared from **2** to explore the influence of the aldehyde group on ring A and the likely anti-inflammatory activity of the compounds obtained.

Compound **3** (16α-hydroxyhederagenin methyl ester) was obtained by reduction of the aldehyde group of **2** with sodium borohydride in methanol. The primary hydroxyl group of **3** was protected with *tert*-butylchlorodimethylsilane to afford **5**, which by reaction with *m*CPBA gave epoxide **6**. Jones oxidation of **2** gave a mixture of two products, **4a** and **4b**, of which **4b** is the decarboxylation product of the β-ketoacid formed by oxidation of the C-3 secondary alcohol function of **4a**. Their structures were established on the basis of their ^{1}H and ^{13}C NMR spectra, and mass spectra.

In order to extend the range of derivatives with modifications of the C-23 aldehyde function, compounds **7** and **8** were prepared from **1**. Compound **8** incorporates a 4-methyl-1-piperazinylimino group, which has been used to improve the solubility of drugs such as the antibiotic rifampicin. To determine the influence of the secondary alcohol hydroxyl groups at C-3 and C-16, they were esterified, keeping the aldehyde and carboxyl groups intact. Acetylation of **1** with acetic anhydride in pyridine gave diacetate **9**. Finally, quillaic acid *bis*-hemisuccinate (**10**) was obtained in low yield using succinic anhydride in pyridine with 4-dimethylaminopyridine. As is the case for piperazinylimines, hemisuccinates are well known prodrugs, affording improved solubility, but with an opposite charge (pK_a) profile.

Table 1 shows the results of the pharmacological assays for quillaic acid and derivatives, and the reference drugs (indometacin and nimesulide). For the sake of comparison, all compounds were applied at equimolar doses to the reference compounds. Nimesulide was the reference drug against AA-induced inflammation, since this compound is more efficacious than indometacin in this model; indometacin is more efficacious than nimesulide against TPA-induced inflammation, and was used as the reference drug in this model. Dose–response curves were carried out with reference drugs.

Quillaic acid (**1**) showed the strongest topical anti-inflammatory activity in both AA and TPA models, exceeding the efficacy of nimesulide against AA-induced inflammation. Quillaic acid methyl ester (**2**) was considerably less active in both assays. Compounds **3** and **4a** showed significant activity against TPA-induced inflammation, but not against inflammation induced by AA. The nor-compound **4b**, which was formed together with **4a** in the oxidation reaction, was also evaluated pharmacologically, exhibiting an anti-inflammatory effect against TPA which did not reach statistical significance. Compounds **5** and **6** were inactive in both assays. Compounds **7** and **8**, obtained after modifying the aldehyde group of quillaic acid (**1**), only showed significant anti-inflammatory activity against TPA. In particular, compound **8** had the highest activity against TPA-induced inflammation, practically identical to that of **1**. Quillaic acid diacetate **9** and *bis*-hemisuccinate **10** showed significant activity against both TPA- and AA-induced inflammation; this activity was weaker than that exhibited by **1** but over the same range as that of quillaic acid methyl ester (**2**).

Discussion

In-vivo pharmacological studies of quillaic acid (**1**) and a set of derivatives using the topical route allowed us to obtain a preliminary estimate of the potential of these compounds for more advanced work in animals and also in-vitro research into their mechanisms of action. Such knowledge is fundamental if clinical studies are to be undertaken at a later stage. Nonetheless, the use of two inflammatory agents, TPA and AA, has yielded data that provide some insight into the levels at which quillaic acid and its derivatives interfere with the inflammatory cascade.[23]

As to the reference drugs used, indometacin is a potent cyclooxygenase (COX) inhibitor, whereas nimesulide is fairly weak in this regard. Nevertheless, indometacin was much more potent in the anti-inflammatory tests than expected from its ability to inhibit COX, suggesting that it acts largely at an early stage in the cascade of events leading to inflammation. This was borne out by the fact that, in topical assays, similar to those used by us, indometacin was very active against TPA-induced inflammation but was practically inactive in the AA model. In contrast, nimesulide was effective in the latter model and showed little if any activity against inflammation induced by TPA. Inflammation develops much more slowly when induced by TPA than when AA is used, presumably due to the fact that TPA acts primarily as an activator of protein kinase C and NF-κB, promoting the enhanced expression of proinflammatory enzymes such as inducible nitric oxide synthase and COX-2. On the other hand, the action of AA is most probably exerted downstream as a precursor of inflammatory prostanoids.[17,19]

Inhibition of AA- or, more usually, TPA-induced inflammation has been demonstrated over the last two decades for a number of pentacyclic triterpene acids with ursane, oleanane and lupane skeletons. Some of these exhibited potent activity when compared with synthetic non-steroidal anti-inflammatory drugs.[24–27] Older reports had already shown that the anti-inflammatory activity of triterpenoids depends largely on the method used to generate inflammation, with a stronger effect against TPA-induced inflammation.[17] In addition, more recent research has implicated reduced release of a number of early mediators in the activity of natural products against mouse ear inflammation induced by TPA.[23,28] Our study lends support to previous results in the sense that most of our triterpenoids were more active in the TPA model than against AA-induced inflammation, suggesting that they may be acting on early events such as inhibition of NF-κB and/or COX-2 activation. A particularly well studied example of a pentacyclic triterpenoid exerting its anti-inflammatory action by this mechanism is that of α-amyrin, which seems to act by suppressing COX-2 expression via inhibition of ERK, p38 MAPK and PKCα, and blockade of NF-κB activation.[28] Interestingly, however, quillaic acid (**1**) also proved to be very effective in the AA model.

Although less active than quillaic acid, the methyl ester (**2**), diacetate (**9**) and *bis*-hemisuccinate (**10**) were the only other derivatives that exhibited significant activity against AA-induced inflammation. This might be explained by the likely prodrug character of these compounds which could be hydrolysed *in vivo* to release quillaic acid. Pharmacokinetic differences of these derivatives related to their different lipophilicities and rates of hydrolysis point to the possibility of modulating the activity of quillaic acid by rational modification at the C-28 carboxyl and/or the C-3 and C-16 secondary alcohol functions. The decrease of biological activity of **2** with respect to **1** against TPA and AA assays suggests that the carboxyl group must be free in order to obtain a strong anti-inflammatory effect.

Compounds **7** and **3**, 16α-hydroxyhederagenin and its methyl ester, respectively, differ from quillaic acid (**1**) and its methyl ester (**2**) in that the C-23 aldehyde group is reduced to a primary alcohol function. These two reduced derivatives were not only less potent than quillaic acid in the TPA model, but also practically inactive against AA-induced inflammation. Therefore, metabolic reoxidation to afford the aldehydes does not seem to be a likely process. With oxidation of the aldehyde to a carboxyl group, as in **4a**, some activity is also retained in the TPA model and lost against AA. The weak, statistically non-significant activity of **4b** against TPA-induced inflammation is harder to interpret, as not only is the aldehyde group lost, but in addition the two secondary alcohol groups are oxidized to ketone functions. The foregoing results seem to suggest that the presence of an intact aldehyde group at C-23 favours the apparently unusual topical anti-inflammatory activity for compounds **1**, **2**, **9** and **10** in the AA model. In compounds **5** and **6**, with the bulky butyldimethylsilyl group appended to the C-23 oxygen atom, anti-inflammatory activity is completely abolished. At this time we can only speculate about a role for the C-23 carbonyl group in the inhibition of AA-induced inflammation.

The anti-inflammatory activity of compound **8**, with the aldehyde group masked as a hydrazone, is intriguing. This compound has not been reported before and is the first example of an aldehydic pentacyclic triterpene hydrazone. It was as active as quillaic acid against TPA-induced inflammation, but was inactive in the AA model. Considering the latter observation, and also on chemical grounds, hydrolysis of the imino functionality seems unlikely. Nevertheless, in the TPA model, the bulky 4-methyl-1-piperazinylimino group is clearly well tolerated. Therefore, analogous modifications of quillaic acid with other imine-forming reagents might uncover useful structure–activity relationships.

It is important to note that in a previous study we demonstrated that **1**, **2** and **4b** all showed dose-dependent antinociceptive activities in two murine thermal models (tail flick and hot-plate test).[29] These effects were similar to those produced by the reference drug (ibuprofen sodium salt). The fact that these compounds showed anti-inflammatory and analgesic effects could be due to inhibition of COX. Lastly, we want to emphasise that after performing the statistical analysis of the results it can be concluded that **1** is significantly more active as an anti-inflammatory than all its derivatives against inflammation induced by both AA and TPA, with the exception of **8**, which showed an effect against TPA that was not statistically different from the effect of **1**.

Conclusions

This study is the first to demonstrate that quillaic acid is a highly effective inhibitor of in-vivo inflammation induced by

topical application of either TPA or AA. Its apparent dual mode of action is unusual and may prove to be of some clinical relevance, and its maximal effect observed against AA-induced inflammation is particularly attractive when compared with the reference drug nimesulide. Some derivatives of quillaic acid are also active, although generally with a bias toward TPA- rather than AA-induced inflammation. Occurrence of a free aldehyde group at the C-4 seems to be a requirement for activity in the AA model and the free C-28 carboxyl group also appears to be important for both assays (against AA and TPA). Derivatisation of the aldehyde group to the hydrazone formed with 4-methyl-1-aminopiperazine leads to a compound that is as active as quillaic acid in the TPA assay, but inactive against AA-induced inflammation. The results of this screening study together with the availability and low cost of quillaja saponin, the best known source of quillaic acid, are a stimulus for further studies of novel potential anti-inflammatory compounds based on this natural product.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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