Platelet Antiaggregating Activity and Chemical Constituents of *Salvia x jamensis* J. Compton

Angela Bisio[a, *], Giovanni Romussi[a], Eleonora Russo[a], Nunziatina De Tommasi[b], Nicola Mascolo[c], Alessio Alfieri[c], Maria Carmela Bonito[c] and Carla Cicala[c]

[a]*Dipartimento di Chimica e Tecnologie Farmaceutiche e Alimentari, University of Genova, Genova, Italy*

[b]*Dipartimento di Scienze Farmaceutiche, University of Salerno, Salerno, Italy*

[c]*Dipartimento di Farmacologia Sperimentale, University of Napoli Federico II, Napoli, Italy*

bisio@dictfa.unige.it

A phytochemical study has been carried out on the surface exudate of *Salvia x jamensis*, which showed a significant platelet antiaggregating activity. The known compounds isopimaric acid (**2**), 14-α-hydroxy-isopimaric acid (**3**), 3β-hydroxy-isopimaric acid (**4**), 7,8β-dihydrosalviacoccin (**5**), betulinic acid (**6**), and ursolic acid (**7**) were isolated together with the new diterpene **1**. The structure of **1** was determined as 15,16-epoxy-cleroda-3-en-7α,10β-dihydroxy-12,17;19,18-diolide on the basis of spectroscopic data analysis. Among all tested compounds, **2** showed a significant concentration-dependent antiaggregating activity when ADP (3 μM) was used as agonist on rat platelets. Conversely, **1** increased ADP–induced platelet aggregation.

Keywords: *Salvia*, diterpenes, triterpenes, platelet aggregation, inhibition.

Platelet aggregation inhibitors are widely used in thromboembolic diseases. Since the currently available compounds have various limitations, it is desirable to find new types of platelet antiaggregating agents, which prevent the initiation of thrombus formation. The surface exudate of *Salvia x jamensis* J. Compton, obtained by rinsing the plant material with methylene chloride, showed a concentration–dependent trend toward platelet antiaggregating activity.

Repeated column chromatography on Sephadex LH-20 and silica gel of the surface exudate of aerial parts of *S. x jamensis* yielded one new diterpenoid (**1**), four known diterpenoids (**2-5**) and two known triterpenoids (**6-7**).

IR absorption bands at 3540 (OH), 1760, 1700 (two lactone rings), and 3140, 1505, 875 (β-substituted furan ring) cm^{-1} and 20 carbon resonances in the ^{13}C NMR spectrum (Table 1) suggested that **1** had an oxygenated clerodane diterpenoid structure. From the TOCSY, HSQC, and HMBC spectral data (CD$_3$OD)
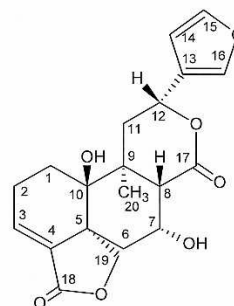

Figure 1: Structure of compound **1**.

the partial structures I: -CH$_2$-CH$_2$-CH=C(CO)-C(CH$_2$)-C- [δ$_C$ = 25.9, 23.8, 136.3, 139.1, 172.0, 48.5, 75.9, 76.7]; II: -CH$_2$-CH-CH-C(CH$_3$)- [δ$_C$ = 31.7, 67.3, 46.8, 43.5, 23.1]; and III: -CH$_2$-CH-C(=CH)-CH=CH- [δ$_C$ = 41.3, 73.0, 125.8, 141.4, 109.8, 145.1] were established. The partial structure I was put in the A and 19,18-olide rings and its carbon signals assigned to C-1, -2, -3, -4, -18, -5, -19, and -10, respectively, on the basis of the following HMBC correlations: δ$_C$ = 76.7 (C-10) / δ$_H$ 1.81 (H$_2$-1), δ$_C$ = 48.5 (C-5) / δ$_H$ = 5.49 (H$_{exo}$-19), 1.81 (H$_2$-1)

Table 1: NMR spectral data for compound **1**: δ values, CD$_3$OD [CDCl$_3$], ^{13}C NMR at 150 MHz, ^{1}H NMR at 600 MHz.

C	^{13}C	DEPT	^{1}H (J in Hz)	HMBC correlations of the C
1	25.9 [26.4]	CH$_2$	1.81 m [1.72 m; 1.82 ddd (3.3, 3.3, 13.4) eq]	2.63, 6.68
2	23.8 [22.4]	CH$_2$	2.35, 2.63 both m [2.54 m]	1.81, 6.68
3	136.3 [133.4]	CH	6.68 m [6.72 dd (3.0, 6.0)]	1.81, 2.35, 2.63
4	139.1 [139.2]	C	-	2.35, 2.63, 5.49
5	48.5 [48.1]	C	-	1.81, 2.01, 5.49, 6.68
6	31.7 [30.9]	CH$_2$	2.01, 2.28 both m [2.16 ddd (2.4, 3.0, 14.4) ax; 2.23 dd (1.7, 14.4) eq]	3.99, 5.49
7	67.3 [65.9]	CH	4.60 br s [4.65 br s]	2.01, 3.79
8	46.8 [45.6]	CH	3.79 d (3.3) [3.58 d (3.1)]	1.19, 1.70, 2.01
9	43.5 [41.5]	C	-	1.19, 1.70, 2.70, 3.79
10	76.7 [76.5]	C	-	1.19, 1.81, 2.70
11	41.3 [40.5]	CH$_2$	1.70 dd (11.5, 13.9) [1.65 dd (11.3, 13.6) ax]	
			2.70 dd (6.1, 13.9) [2.74 dd (6.2, 13.6) eq]	1.19, 3.79
12	73.0 [71.9]	CH	5.44 dd (6.1, 11.5) [5.34 dd (6.2, 11.3)]	1.70
13	125.8 [124.1]	C	-	1.70, 5.44, 6.58, 7.57, 7.65
14	109.8 [108.9]	CH	6.58 br s [6.48 br s]	5.44, 7.57, 7.65
15	145.1 [144.1]	CH	7.57 br s [7.47 br s]	6.58, 7.65
16	141.4 [140.0]	CH	7.65 br s [7.51 br s]	5.44, 6.58, 7.57
17	178.5 [176.3]	C	-	3.79
18	172.0 [168.7]	C	-	5.49, 6.68
19	75.9 [74.6]	CH$_2$	3.99 dd (2.4, 7.1), 5.49 d (7.1) [3.88 dd (2.4, 7.3) endo, 5.49 d (7.3) exo]	2.01, 2.28
20	23.1 [22.9]	CH$_3$	1.19 s [1.21 s]	1.70, 2.70, 3.79

and 6.68 (H-3); δ$_C$ = 172.0 (C-18) / δ$_H$ = 5.49 (H$_{exo}$-19) and 6.68 (H-3). The partial structure II was put in ring B and its carbon signals assigned to C-6, -7, -8, -9, and -20, respectively on the basis of the following HMBC correlations: δ$_C$ = 48.5 (C-5) / δ$_H$ = 2.01 (H$_a$-6); δ$_C$ = 76.7 (C-10) with the signals / δ$_H$ = 1.19 (CH$_3$-20) and 2.70 (H$_a$-11). The carbon signals of the partial structure III were assigned to C-11, -12, -13, -16, -14, and -15 respectively, on the basis of the following HMBC correlations: δ$_C$ = 41.3 (C-11) / δ$_H$= 1.19 (CH$_3$-20), δ$_C$ = 43.5 (C-9) / δ$_H$ = 1.70 and 2.70 (CH$_2$-11), δ$_C$ = 46.8 (C-8) / δ$_H$ = 1.70 (H$_b$-11), δ$_C$ = 125.8 (C-13), 109.8 (C-14), 141.4 (C-16) / δ$_H$ = 5.44 (H-12). The signal at δ$_C$ = 178.5 was assigned to C-17 of a 12,17-olide ring by its HMBC correlation with the signal at δ$_H$ = 3.79 (H-8). The axial orientation of the hydroxyl group at C-7 was indicated by the small J value of H-7. The strong deshielding effect on the *exo* H-19 (δ$_H$ = 5.49) as compared to the *endo* H-19 (δ$_H$ = 3.99 or 3.88 in CD$_3$OD or CDCl$_3$), which showed the expected W coupling (2.4 Hz) with H-6 β axial [1], the downfield shift of CH$_3$-20 (0.36 ppm in CDCl$_3$) in **1** compared to that of bacchotricuneatin A, which lacks the -OH group on C-7 [2], and ROESY correlation (CDCl$_3$) CH$_3$-20 / CH$_2$-19 showed that these last groups and 7-OH are on the same α side. From its coupling constants, H-12 resulted as axial. ROESY correlations H-6 β axial / H-8, and H-8 / H-12 axial showed that these protons are on the same β side. ROESY correlations (CDCl$_3$) of H-1 α-axial with

CH$_3$-20 and H-19 *endo* could be explained with a 5α, 10β *trans*-fused structure and thus with a β orientation of the 10-OH. From these results, **1** is 15,16-epoxy-cleroda-3-en-7α,10β-dihydroxy-12,17;19,18-diolide.

The known compounds **2-6** were identified as isopimaric acid [3], 14α-hydroxy-isopimaric acid, 3β-hydroxy-isopimaric acid [4], 7,8β-dihydro-salviacoccin [5], and betulinic acid [6], respectively by comparison of their physical and spectroscopic data with those published in the literature. Compound **7** was identified as ursolic acid by comparison (TLC, IR) with an authentic sample.

The crude surface exudate from the aerial part of *Salvia x jamensis*, showed, at a concentration of 100 –1000 µg/mL, a trend toward platelet anti-aggregating activity (data not shown). Compound **2** (HPLC analysis: one single peak), at concentrations of 6x10^{-4} and – 10^{-3}M, inhibited significantly ADP (3 µM)–induced platelet aggregation. Compounds **3–4** did not modify platelet aggregation in a significant manner. On the contrary, compound **1** (HPLC analysis: one single peak) increased, at the highest concentration, ADP-induced platelet aggregation (Table 2).

The observed trend toward the platelet anti-aggregating effect of the surface exudate of *Salvia x jamensis* is likely due to the presence of the active compound **2**; however, compound **3 (**HPLC analysis

Table 2: Anti-aggregating activity of the isolated compounds.

Compound	aggregation %		
	(2 x 10^{-4})	(6 x 10^{-4})	(10^{-3})
1	48.1 ± 14.1	29.8 ± 7.1	62.7 ± 8.9 **
2	18.4 ± 2.9	5.4 ± 3.1 **	2.3 ± 1.3 **
3	33.6 ± 9.0	21.0 ± 4.6	16.7 ± 4.0
4	46.7 ± 3.3	34.6 ± 5.1	22.4 ± 3.7

Results are expressed as mean ± S.E. and represent aggregation (%) in response to ADP 3 μM. In brackets are reported concentrations (M) of compounds tested. Vehicle (DMSO) value was 27.9 ± 2.8 %. ** $p < 0.01$ one way ANOVA followed by Dunnett's test vs. vehicle (n = 10).

indicated that the compound was 97% pure) showed an inhibitory trend, although not significant. It is worth noting that compound **3** in comparison with **2** differs by the presence in the molecule of an hydroxyl group; this suggests that the reduced activity could be due to reduced lipophylicity. Compound **4 (**HPLC analysis: one single peak), an isomer of **3**, had lost the observed biological activity. Compounds **5–7** were completely inactive (data not shown). The inactivity of compound **7** on ADP–induced platelet aggregation has already been described in the literature [7]. In a previous paper [8], inhibition of platelet aggregation by diterpenes of the pimarane and abietane groups was also described. All these results may suggest the possibility that pimarane, isopimarane and abietane derivatives may be useful for semisynthesis of new compounds with platelet antiaggregating activity.

Experimental

General experimental procedures: Melting points are uncorrected and were measured on a Tottoli melting point apparatus (Büchi). Sephadex LH-20 (Pharmacia) and silica gel 60 (Merck 230-400 mesh) were used for column chromatography; aluminium sheets of silica gel 60 F$_{254}$ (Merck 0.2 mm thick) with CHCl$_3$/MeOH/HCOOH (10: 0.5: 0.1) as eluent were used for TLC, and the spots were detected by spraying with 50% H$_2$SO$_4$, followed by heating. Analytical HPLC was performed using a Waters model W600 pump equipped with a Rheodyne Waters Delta 600 Injector and a Model 2414 Refractive Index detector (Waters Corporation, Milford, USA); results were analyzed using Waters Empower software.

An injection vol. of 20 μL was used with a Merck LiChrospher 100 RP-18 end-capped column (5 μm, 250 x 4.5 mm). The elution mixture was composed of CH$_3$CN/H$_2$O 75:25. UV spectra were obtained with a diode array HP 8453 spectrophotometer (Hewlett Packard, USA). IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer. ESIMS (positive mode) were obtained from a LCQ Advantage Thermo Electron spectrometer, equipped with Xcalibur software. NMR spectra were recorded on a Bruker DRX 600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C) in CDCl$_3$ using TMS as internal standard. The optical rotation was recorded on a Perkin-Elmer 241MC polarimeter.

Plant material: Aerial parts of *S. x jamensis* J. Compton were obtained from Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga. Italy). The plant was identified by Dr Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).

Extraction and isolation: For the isolation of leaf surface constituents, fresh aerial parts (3.1 kg) were immersed in CH$_2$Cl$_2$ for 20 sec. After filtration the extraction solvent was removed under reduced pressure. The exudate (18 g) was chromatographed in portions of 1.5 g on Sephadex LH-20 columns (60 x 3 cm) using CHCl$_3$/MeOH (7:3) as eluent to give, in order of elution, fractions (3 g) containing waxy compounds (170 mL), fractions (3 g) with very crude **7** (from 170 mL to 220 mL) and fractions (12 g) with a mixture of **1-7 (**from 220 mL to 290 mL). These last fractions groups were chromatographed in portions of 4 g on silica gel columns (40 x 4 cm) eluting with mixtures of *n*-hexane- CHCl$_3$ [50:50 (4.1 L), 40:50 (2.1 L), 33:67 (1.0 L), 25:75 (1.0 L), 10:90 (11.0 L)], then with CHCl$_3$ (8.3 L), and then with CHCl$_3$-MeOH [95:5 (3.4 L)]. Elution with *n*-hexane-CHCl$_3$ 40:50 (from 0.2 L to 2.1 L) and with *n*-hexane-CHCl$_3$ 33:67 and 25:75 afforded fractions with **2** (crystallized from MeOH: 2.05 g). Elution with *n*-hexane-CHCl$_3$ 10:90 afforded first fractions with **6** (from 1.0 to 1.4 L; crystallized from EtOH: 0.2 g), then fractions with **7** (from 1.6 to 2.7 L; crystallized from EtOH: 1.4 g), then fractions with **5** (from 2.7 to 3.0 L; crystallized from CHCl$_3$/MeOH: 0.14 g), then with **1** (from 3.8 to 11.0 L; crystallized from CHCl$_3$/MeOH: 0.58 g). Elution with CHCl$_3$ afforded fractions with **3** (from 3.5 to 6.0 L; crystallized from MeOH/H$_2$O: 0.25 g). Elution with CHCl$_3$-MeOH 95:5 afforded fractions with **4** (from 1.1 to 2.0 L, crystallized from MeOH/H$_2$O: 50 mg).

Compound 1

MP: 229-230ºC (dec.).

[α]$_D^{25}$: -76.0 (*c* 1.00, CHCl$_3$).

Rf : 0.30.
IR (KBr): 3540, 3140, 3010, 1760, 1700, 1600, 1505, 875 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm (log ε): 209 (4.11), 242 (2.97).
^{1}H NMR: Table 1.
^{13}C NMR: Table 1.
ESIMS (positive mode): m/z 375 [M+H]$^+$.
Anal. Calcd. for $C_{20}H_{22}O_7$: C, 64.16; H, 5.92. Found C, 64.21; H, 5.92.

Platelet preparation and aggregation: Blood was withdrawn by cardiac puncture from male Wistar rats (Harlan - Nossan, 200-250 g), slightly anesthetised with enflurane, and anticoagulated with 3.8 % (w/v) trisodium citrate (1:9 v/v). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [9]. Platelet count in PRP was adjusted to 3×10^5 platelets μL^{-1} with autologous PPP. Platelet aggregation was monitored in an Elvi 840 light transmission aggregometer by measuring changes in turbidity of 0.25 mL PRP warmed at 37°C and under continuous stirring. After preliminary experiments, the concentration of 3 μM ADP was chosen and used throughout all the study.

To evaluate the effect of compounds **1-7** on ADP-induced platelet aggregation, 5 μL of each compound tested, was added to PRP 5 min before the ADP (3 μM) at a final concentrations ranging between 2 x 10^{-4} and 10^{-3} M. As control, an equal volume of DMSO (dimethylsulfoxide) was added to PRP.

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