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Triterpenoid profile and bioactivity study of *Oenothera maritima*

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ABSTRACT

Four new triterpenoids, 2α,3α,20β,23-tetrahydroxy-ursa-12,19(29)-dien-28-oic acid (**1**), 2α,3α,20β,23-tetrahydroxy-ursa-12,19(29)-dien-28,20β-lactone (**2**), 2α,3α-dihydroxy-ursa-12,19-dien-28-oic acid 28-O-β-D-glucopyranoside (**3**) and 2α,3α,23-trihydroxy-ursa-12,19(29)-dien-28-oic acid (**4**) together with six known compounds (**5**–**10**), were isolated from the aerial parts of *Oenothera maritima* Nutt. Their structures were elucidated on the basis of spectroscopic data and chemical methods. Compounds **1**,**3**–**10** were evaluated for their *in vitro* thrombin inhibitory activity and their selectivity against factor Xa and trypsin.

1. Introduction

The genus *Oenothera* (Onagraceae), also known as "evening primrose", consists of 145 specie, widely distributed in North and South America as well as in Europe. Some species, in particular *Oenothera biennis*, are the source of evening primrose oil (EPO), which contains a high percentage of unsaturated fatty acids, especially γ-linoleic acid and are widely used in traditional medicine (Hudson, 1984) to treat various diseases. The oil has been shown to have several beneficial effects on human health including antidiabetic, antiinflammatory (Belch and Hill, 2000), and anti-premenstrual (Mahady et al., 2001) activities. Defatted seeds of evening primrose are a waste product of pharmaceutical and cosmetic industries and they were extensively studied for their high content of flavonoids and tannins (Wettasinghe et al., 2002). Antioxidant (Schmidt et al., 2003), antiinflammatory (Kiss et al., 2010), and antitumor proprieties (Dalla Pellegrina et al., 2005; Jaszewska et al., 2009) were ascribed to crude MeOH extracts of defatted seeds.

Reports concerning the chemical composition and bioactivities of extracts prepared from the aerial parts of *Oenothera* species are quite limited and showed that aerial parts contain flavonoids, phenolic acids, and tannins. (Howard et al., 1972; Hatano et al., 1990; Marzouk et al., 2009).

In the course of the multinational collaborative project MAREX, the crude 80% EtOH extracts of *Oenothera maritima* were found to possess good thrombin inhibitory activity. Bioassay guided fractionation of the crude extract of the aerial parts of *O. maritima* led to the isolation of four new (**1**–**4**) and six known (**5**–**10**) triterpenoids (Fig. 1). All isolated compounds, except arjunolic acid (**10**), featured an urs-12-en-28-oic acid skeleton. The compounds **1**–**8** displayed a common 2α,3α,23-trihydroxy substitution and different functionalization of ring E. By comparison of their spectroscopic data with reported values, known compounds **5**–**10** were identified as 2α,3α,23-trihydroxy-ursa-12,19(29)-dien-28-oic acid 28-*O*-β-D-glucopyranoside (**5**) (Yuan et al., 2008), 2α,3α,23-trihydroxy-ursa-12,18-dien-28-oic acid (**6**) (Li et al., 2009), 2α,3α,23-trihydroxy-ursa-12,18-dien-28-oic acid 28-*O*-β-D-glucopyranoside (**7**) (Li et al., 2009), myriantic acid (**8**) (Ojinnaka et al., 1984), 2α,3α,19α-trihydroxy-24-norursa-4(23), 12-dien-28-oic acid (**9**) (Jang et al., 2005), and arjunolic acid (**10**) (Kundu and Mahato, 1993) (Fig. 1).

Despite extensive phytochemical studies of the *Oenothera* genus (Singh et al., 2012), the only previous report of ursane type derivatives was from the whole plant of a western North American collection of *Oenothera cheiranthifolia* (Nakanishi et al., 2007). This paper describes the isolation and structural elucidation of new components and the biological activity of all isolated triterpenoids.

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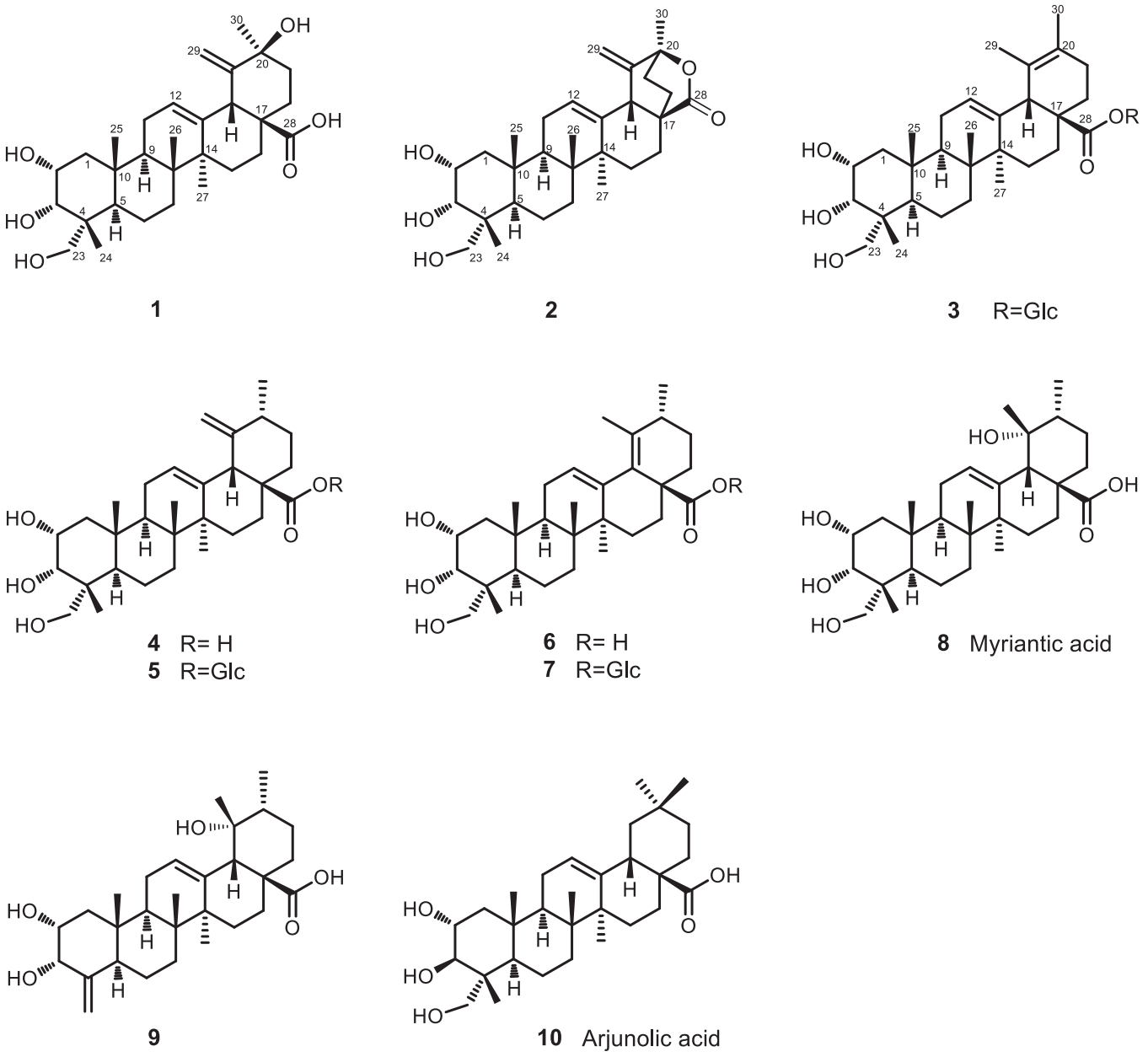

Fig. 1. New (**1**–**4**) and known (**5**–**10**) triterpenoids isolated from *Oenothera maritima*.

2. Results and discussion

The partitioning procedure of the crude MeOH extract *viz*. hexane, CH_2Cl_2, EtOAc, BuOH, and H_2O, afforded five extracts. The CH_2Cl_2-enriched extract was found to possess enzyme inhibitory activity and was further fractionated by silica gel MPLC using a solvent gradient system from CH_2Cl_2 to MeOH followed by reverse phase HPLC to afford ten pure compounds (Fig. 1), including four new ones (**1**–**4**).

2.1. Structural elucidation

Compound **1** was isolated as a white amorphous solid. The molecular formula was established as $C_{30}H_{46}O_6$ by the HR-ESI-MS spectrum. The ^{1}H-NMR spectrum of **1** (Table 1) displayed signals corresponding to five tertiary methyls at δ_H 0.78, 0.91, 1.05, 1.13, and 1.38, and three olefinic protons at δ_H 5.08 (br s), 5.25 (br s) and 5.32 (br t, J = 3.5 Hz).

The ^{13}C NMR spectrum of **1** (Table 2) revealed 30 carbon signals. One olefinic methylene (δ_C 112.4), one olefinic methine (δ_C 128.0), and two olefinic quaternary carbons (δ_C 139.2 and 155.1) indicated the presence of two double bonds, including one exomethylene double bond. The ^{13}C NMR signals at δ_C 128.0 (C-12) and 139.2 (C-13) were characteristic of the double bond of an urs-12-en system and readily distinguished **1** from an isomeric olean-12-ene, in which the chemical shifts of the corresponding pair of signals could be around δ_C 123 and 145, respectively (Mahato and Kundu, 1994; Terreaux et al., 1996).

In addition, the spectrum included one acyl signal at δ_C 182.0, one oxymethylene (δ_C 71.1), two oxymethines (δ_C 67.2 and 78.5), one oxygenated quaternary carbon (δ_C 71.2), and five methyls, eight sp^3 methylenes, three sp^3 methines, and five sp^3 quaternary carbons. The afore mentioned data implied that **1** was a tetrahydroxy-urs-12-en-oic acid derivative.

The analysis of HMBC spectra allowed to localize the exocyclic double bond at C-19(29) position on the basis of diagnostic

Table 1
^{1}H NMR data (CD_3OD) of compounds **1**–**4**.[a]

Position	1[b]	2[b]	3[b]	4[b]
1	1.35 ovl	1.33 ovl	1.30 ovl	1.35 ovl
	1.63 ovl	1.62 ovl	1.66 ovl	1.63 ovl
2	3.90 ddd (2.2, 3.5, 11.6)	3.89 ddd (1.8, 3.5, 11.6)	3.89 ddd (1.8, 3.6, 11.4)	3.89 ddd (2.1, 3.4, 11.5)
3	3.61 d (2.2)	3.60 d (1.8)	3.60 d (1.8)	3.61 d (2.1)
4	–	–	–	–
5	1.55 ovl	1.56 m	1.52 ovl	1.56 m
6	1.37 ovl	1.38 m	1.34 ovl	1.38 ovl
	1.51 m	1.52 m		
7	1.33 ovl	1.33 ovl	1.37 ovl	1.33 ovl
	1.59 ovl		1.62 ovl	1.60 ovl
8	–	–	–	–
9	1.89 m	1.87 ovl	1.63 ovl	1.90 m
10	–	–	–	–
11	2.03 m	2.03 m	1.99 ovl	2.03 m
12	5.32 br t (3.5)	5.32 br t (3.4)	5.49 br t (3.4)	5.28 br t (3.4)
13	–	–	–	–
14	–	–	–	–
15	0.97 m	1.00 m	1.13 m	0.99 m
	1.96 m	1.90 ovl	1.88 m	1.94 m
16	1.60 ovl	1.62 ovl	1.99 ovl	1.60 ovl
			1.78 ovl	
17	–	–	–	–
18	3.88 br s	3.74 br s	3.22 br s	3.48 br s
19	–	–	–	–
20	–	–	–	1.97 m
21	1.60 ovl	1.49 m	1.88 ovl	1.28 m
	1.67 ovl	2.08 m	2.27 m	1.58 ovl
22	1.53 ovl	1.67 m	1.65 ovl	1.80 m
	2.17 m	1.90 ovl	1.82 ovl	
23	3.39 d (10.9)	3.40 d (10.9)	3.39 d (10.8)	3.39 d (10.8)
	3.54 d (10.9)	3.53 d (10.9)	3.52 d (10.8)	3.53 d (10.8)
24	0.78 s	0.78 s	0.77 s	0.80 s
25	1.05 s	1.04 s	1.07 s	1.04 s
26	0.91 s	0.90 s	0.88 s	0.90 s
27	1.13 s	1.12 s	1.00 s	1.16 s
28	–	–	–	–
29	5.08 br s	5.26 br s	1.57 s	4.87 br s
	5.25 br s	5.28 br s		4.94 br s
30	1.38 s	1.40 s	1.64 s	1.11 d (6.4)

[a] NMR data at 700 MHz (**1** and **3**), and 400 MHz (**2** and **4**).
[b] Coupling constants are in parentheses and given in hertz. Ovl: overlapped with other signals.

correlations from exomethylene protons (δ_H 5.08 and 5.25) to C-18, C-19 and C-20. The alternative C-20(30)-double bond was found in a triterpenic acid, named coussaric acid, isolated from *Coussarea brevicaulis* (Su et al., 2003).

The localization of the four hydroxy groups at C-2, C-3, C-20, and C-23 positions followed from the analysis of 2D NMR data. The 2α,3α-dihydroxy configuration was assigned on the basis of the coupling pattern of H-2 (ddd, J = 2.2, 3.5, 11.6 Hz) and H-3 (d, J = 2.2 Hz). The NOE correlations of H-2/H-3, H-2/CH_3-25 and H-3/CH_3-24 confirmed this stereo-assignment and indicated the α-orientation of the hydroxymethyl group at C-4.

The 20β-OH configuration and the α oriented CH_3-30 was assigned by the agreement of the observed values for C-18 (δ_C 49.4) and C-22 (δ_C 32.9) with the calculated ^{13}C NMR γ-effect for C-18 (δ_C 49.3) and C-22 (δ_C 33.7) that were significantly different from those calculated for the 20α-OH epimer (C-18 at δ_C 45.8 and C-22 at δ_C 31.2) (Bruno et al., 1987).

Thus, compound **1** was determined to be 2α,3α,20β,23-tetrahydroxy-ursa-12,19(29)-dien-28-oic acid. The 3β epimer of this compound was isolated from the aerial parts of *Salvia chinensis* (Wang et al., 2009).

The HR-ESI-MS spectrum of compound **2** showed an ion peak at m/z 507.3092 $[M+Na]^+$, corresponding to a molecular formula of $C_{30}H_{44}O_5$, which is 18 u.m.a. less than compound **1** and implying nine degrees of unsaturation.

NMR data of compound **2** were similar to those of compound **1**, whereas only a perturbation in the E ring was observed (Tables 1 and 2) suggesting in accordance with mass data the closure of a δ-lactone ring between the β–OH group at C-20 and the acyl carbon at C-28 (Ouyang et al., 1996).

The presence of a 28,20β-olide moiety was confirmed by the expected upfield shifts for C-17 (δ_C 49.2), C-21 (δ_C 32.4), C-22 (δ_C 32.0) and C-28 (δ_C 179.9) and downfield shift for C-20 (δ_C 82.5), as compared to parent compound, and by HMBC correlation of H_3-30 at δ_H 1.40 with C-20 at δ_C 82.5.

On standing in NMR tube, **2** spontaneously opened to give the corresponding acid **1**. Attempts to reobtain the δ-lactone in different acidic conditions (SiO_2 and TFA) were unsuccessful.

Thus, the structure of compound **2** was determined to be 2α,3α,20β,23-tetrahydroxy-ursa-12,19(29)-dien-28,20β-lactone.

The molecular formula of compound **3** was established as $C_{36}H_{56}O_{10}$. The ^{13}C NMR spectrum showed 36 carbon signals including six methyls, two signals for a trisubstituted double bond at δ_C 128.3 and 138.6 diagnostic for ursane-type triterpene and two signals for a teatrasubstituted double bond (δ_C 124.7, 129.0) a carboxy signal (δ_C 180.2) and one anomeric carbon (δ_C 95.6) (Tables 2 and 3). ^{1}H NMR spectrum of compound **3,** in addition to the signals due to the β-glucopyranosyl moiety (δ_H 5.39 d, J = 8.1 Hz, H-1′), showed two vinylic methyl singlets (δ_H 1.57 and 1.64) suggesting a tetrasubstituted double bond located at C-19/C-

Table 2
^{13}C NMR data (CD_3OD) of compounds **1**–**4**[a].

Position	1	2	3	4
1	42.1	42.1	42.4	42.2
2	67.2	67.1	67.0	67.1
3	78.5	78.5	78.5	78.5
4	42.3	42.2	42.2	42.2
5	44.2	44.2	44.2	44.3
6	18.7	18.8	18.7	18.9
7	33.7	33.7	34.2	33.7
8	40.3	40.4	40.4	40.2
9	48.8	48.7	48.7	48.7
10	39.1	39.1	38.9	39.0
11	24.5	24.5	24.3	24.4
12	128.0	128.0	128.3	128.4
13	139.2	139.0	138.6	139.2
14	43.7	43.9	44.4	43.7
15	29.6	29.5	28.9	29.7
16	26.6	26.4	24.1	26.6
17	51.4	49.2	48.2	51.1
18	49.4	49.5	51.0	53.5
19	155.1	150.7	129.0	155.5
20	71.2	82.5	124.7	38.7
21	35.8	32.4	29.0	31.8
22	32.9	32.0	33.2	38.8
23	71.1	71.0	71.1	71.1
24	17.4	17.4	17.4	17.3
25	17.2	17.2	17.6	17.2
26	17.8	17.8	18.1	17.7
27	26.3	26.3	22.2	26.4
28	182.0	179.9	180.2	181.7
29	112.4	116.2	17.1	109.4
30	29.0	23.5	20.1	19.6

[a] NMR data at 175 MHz (**1**), 100 MHz (**2** and **4**) and 125 MHz (**3**).

Table 3
NMR data for the glucose unit in compound **3**.[a]

Position	δ_H	δ_C
1′	5.39 d (8.1)	95.6
2′	3.33 ovl	73.8
3′	3.40 m	78.0
4′	3.35 ovl	70.9
5′	3.34 ovl	78.5
6′	3.67 dd (3.2, 11.9)	62.3
	3.81 d (11.9)	

[a] CD_3OD; Ovl: overlapped with other signals.

20 (Table 1). The long-range correlations between the methyl groups (δ_H 1.57 and 1.64) and the olefinic carbons (δ_C 124.7 and 129.0) in the HMBC spectrum confirmed this hypotesis. The location of glucosyl linkage was confirmed by the HMBC correlation between H-1′ (δ_H 5.39) and C-28 (δ_C 180.2).

Thus, the structure of compound **3** was determined to be 2α,3α-dihydroxy-ursa-12,19-dien-28-oic acid 28-*O*-β-D-glucopyranoside.

The 3β epimer of this compound was isolated from the roots of *Rubus pinfaensis* (Durham et al., 1996) and *Rubus ellipticus* var. *obcordatus* (Li et al., 2009).

The molecular formula ($C_{30}H_{46}O_5$) of compound **4** was determined by HR-ESI mass spectra and indicated compound **4** as a de-oxygenated derivative of **1**. The ^{1}H and ^{13}C NMR spectra of **4** were similar to those of **1**, except for the presence of signals due to a secondary methyl (δ_H 1.11, d, J = 6.4 Hz; δ_C 19.6, CH_3-30) and a methine (δ_H 1.97 m; δ_C 38.7 CH-20) in **4** that replaced the corresponding signals of a tertiary methyl (δ_H 1.38 s; δ_C 29.0) and an oxygenated quaternary carbon (δ_C 71.2) in **1** (Tables 1 and 2). The α-orientation of the methyl group at C-20 was assigned by the diagnostic NOESY correlation H-20/H-18.

Thus, compound **4**, assigned as 2α,3α,23-trihydroxy-ursa-12,19(29)-dien-28-oic acid, is the 20-deoxy derivative of **1**. The 3β epimer of this compound was isolated from the leaves of *Senecio pseudotites* (De Tommasi et al., 1998).

2.2. Biological activity

According to the thrombin inhibitory activity observed for the CH_2Cl_2 extract obtained by solvent partitioning of the crude MeOH extract (85 % inhibition at 400 μg/mL), the isolated metabolites, with the exception of compound **2**, were tested for their *in vitro* thrombin inhibitory potencies and their selectivity against factor Xa and trypsin and the results are presented in Table 4.

Myriantic acid (**8**) and compound **9** showed moderate inhibitory activity on thrombin, while not showing any inhibitory activity against similar serine protease factor Xa (FXa) and tripsin (Try). Whereas plant triterpenoids with ursane skeleton have been reported to possess a broad range of biological activity, expecially in the antidiabetic (Alqahtani et al., 2013) and anticancer field (Laszczyk, 2009), no previous reports on the antithrombin activity of these metabolites appeared in the literature. A moderate antithrombin activity was found for triterpenes with a lanostane skeleton (Cateni et al., 2010). A comparison of the structure of compounds tested in the present work, indicated that the presence of a 19α-hydroxy group seems to be a requisite essential for the observed biological activity. Interestingly, two ursane derivatives both featuring a 19α-hydroxy group were recently found to inhibit ADP-induced platelet aggregation (Zhou et al., 2013).

3. Experimental

3.1. General experimental procedure

Specific rotations were measured on a PerkinElmer 243 B polarimeter. High-resolution ESI–MS spectra were performed with a Micromass Q-TOF mass spectrometer. ESI–MS experiments were performed on an Applied Biosystem API 2000 triple quadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 400, 500 and 700 NMR spectrometers (^{1}H at 400, and 700 MHz, ^{13}C at 100, 125, and 175 MHz, respectively), equipped with a SUN microsystem ulta5 hardware and recorded in CD_3OD (δ_H 3.31 andδ_C 49.0). Coupling constants (J values) are given in Hertz (Hz), and chemical shifts (δ) are reported in ppm and referred to CHD_2OD. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with mixing times of 200 and 250 ms. HPLC was performed using a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401. Silica gel (200–400 mesh) from Macherey–Nagel Company was used for flash chromatography. The purities of compounds were determined to be greater than 95% by HPLC.

Table 4
Biological activity of triterpenoids from *Oenothera maritima*: inhibition of thrombin, factor Xa, trypsin.[a]

Compound	Ki (Trb)	Ki (FXa)	Ki (Try)
1	>100 μM	>100 μM	>100 μM
3	>100 μM	>100 μM	>100 μM
4	>100 μM	>100 μM	>100 μM
5	>100 μM	>100 μM	>100 μM
6	>100 μM	>100 μM	>100 μM
7	>100 μM	>100 μM	>100 μM
8	20.0 ± 8.8 μM	>100 μM	>100 μM
9	27.0 ± 9.4 μM	>100 μM	>100 μM
10	>100 μM	>100 μM	>100 μM

[a] Compound **2** was not tested, due to its chemical instability.

3.2. Plant material

We purchased aerial parts of *O. maritima* from *SETALG* (Brittany, France), that where collected at Plourin (Brittany, France) on April 2011.

3.3. Extraction and isolation

Dried aerial parts (400 g) were extracted twice with 2.5 L of 60% aqueous EtOH during 3 h at room temperature. The obtained extract (66.0 g) was concentrated under reduced pressure, suspended in 500 mL of H_2O, and then centrifuged at 4000 rpm during 15 min at 20 °C to give a pellet extract (19S084E60P—3.43 g) and a supernatant extract (60.0 g). The supernatant was partitioned against CH_2Cl_2 (4 × 500 mL) to yield a CH_2Cl_2 fraction (0.61 g). The aqueous residue (58.5 g) was partitioned against EtOAc (4 × 500 mL) to yield an EtOAc extract (19S084E60SE—3.87 g) and a final aqueous residue (53.0 g).

The pellet extract (19S084E60P—3.43 g) was fractionated by silica gel MPLC using a solvent gradient system from CH_2Cl_2 to MeOH.

Fraction eluted with CH_2Cl_2:MeOH 95:5 (117.7 mg) was purified by HPLC on a Nucleodur 100-5 C18 column (5 μm, 4.6 mm i. d. × 250 mm) with CH_3CN:H_2O (55:45) and 0.1% of TFA as eluent (flow rate 1.0 mL/min) to give 6.6 mg of compound **9** (t_R = 6.6 min).

Fraction eluted with CH_2Cl_2:MeOH 90:10 (76.7 mg) was purified by HPLC on a Nucleodur 100-5 C18 (5 μm; 10 mm i.d. × 250 mm) with CH_3CN:H_2O (50:50) and 0.1% of TFA as eluent (flow rate 1.0 mL/min) to give 13.4 mg of myriantic acid (**8**) (t_R = 5.7 min).

The EtOAc extract (19S084E60SE—3.87 g) was fractionated by silica gel MPLC using a solvent gradient system from CH_2Cl_2 to MeOH.

Fraction eluted with CH_2Cl_2:MeOH 95:5 (43.1 mg) was purified by HPLC on a Nucleodur 100-5 C18 column (5 μm, 4.6 mm i. d. × 250 mm) with MeOH:H_2O (72:28) as eluent (flow rate 1.0 mL/min) to give 0.4 mg of compound **1** (t_R = 6 min), 1.6 mg of compound **2** (t_R = 10.5 min), 0.7 mg of compound **6** (t_R = 18 min), 1.9 mg of compound **4** (t_R = 27 min).

Fraction eluted with CH_2Cl_2:MeOH 93:7 (85.2 mg) was purified by HPLC on a Nucleodur 100-5 C18 column (5 μm, 4.6 mm i. d. × 250 mm) with MeOH:H_2O (72:28) as eluent (flow rate 1.0 mL/min) to give 5.2 mg of arjunolic acid (**10**) (t_R = 12 min).

Fraction eluted with CH_2Cl_2:MeOH 9:1 (168.7 mg) was purified by HPLC on a Nucleodur 100-5 C18 column (5 μm, 4.6 mm i. d. × 250 mm) with MeOH:H_2O (60:40) as eluent (flow rate 1.0 mL/min) to give 0.8 mg of compound **3** (t_R = 26.1 min), 0.9 mg of compound **7** (t_R = 27.9 min) and 0.8 mg of compound **5** (t_R = 30.6 min).

3.3.1. 2α,3α,20β,23-tetrahydroxy-ursa-12,19-dien-28-oic acid (1)

White amorphous solid; $[\alpha]_D^{25}$ + 39.6 (*c* 0.04, MeOH); ^1H and ^{13}C NMR data in CD_3OD given in Tables 1 and 2; HR–ESI–MS: calcd. for $C_{30}H_{45}O_6$, 501.3216; found 501.3220 $[M-H]^-$.

3.3.2. 2α,3α,20β,23-tetrahydroxy-ursa-12,19-dien-28,20β-lactone (2)

White amorphous solid; $[\alpha]_D^{25}$ + 34.5 (*c* 0.16, MeOH); ^1H and ^{13}C NMR data in CD_3OD given in Tables 1 and 2; HR–ESI–MS: calcd. for $C_{30}H_{44}O_5Na$, 507.3086; found 507.3092 $[M+Na]^+$.

3.3.3. 2α,3α-dihydroxy-ursa-12,19-dien-28-oic acid 28-O-β-D-glucopyranoside (3)

White amorphous solid; $[\alpha]_D^{25}$ + 20.1 (*c* 0.08, MeOH); ^1H and ^{13}C NMR data in CD_3OD given in Tables 1–3; HR–ESI–MS: calcd. for $C_{36}H_{56}O_{10}Na$, 671.3771; found 671.3775 $[M+Na]^+$.

3.3.4. 2α,3α,23-trihydroxy-ursa-12,19-dien-28-oic acid (4)

White amorphous solid; $[\alpha]_D^{25}$ + 23.7 (*c* 0.19, MeOH); ^1H and ^{13}C NMR data in CD_3OD given in Tables 1 and 2; HR–ESI–MS: calcd. for $C_{30}H_{46}O_5Na$, 509.3243; found 509.3251 $[M+Na]^+$.

3.3.5. Known triterpenes

3.3.5.1. Compound 5. White amorphous solid; $[\alpha]_D^{25}$ + 20.2 (*c* 0.09, MeOH); ESI–MS: *m*/*z* 671.4 $[M+Na]^+$.

3.3.5.2. Compound 6. White amorphous solid; $[\alpha]_D^{25}$ + 86.5 (*c* 0.05, MeOH); ESI–MS: *m*/*z* 485.3 $[M-H]^-$.

3.3.5.3. Compound 7. White amorphous solid; $[\alpha]_D^{25}$ + 49.7 (*c* 0.09, MeOH); ESI–MS: *m*/*z* 671.4 $[M+Na]^+$.

3.3.5.4. Myriantic acid (8). White amorphous solid; $[\alpha]_D^{25}$ + 56.3 (*c* 0.14, MeOH); ESI–MS: *m*/*z* 503.3 $[M-H]^-$.

3.3.5.5. Compound 9. White amorphous solid; $[\alpha]_D^{25}$ + 28.6 (*c* 0.33, MeOH); ESI–MS: *m*/*z* 471.3 $[M-H]^-$.

3.3.5.6. Arjunolic acid (10). White amorphous solid; $[\alpha]_D^{25}$ + 49.4 (*c* 0.15, MeOH);ESI–MS: *m*/*z* 487.3 [M − H].

3.4. Enzyme assay for determination of inhibitory activity of serine proteases

Enzyme amidolytic method for determination of inhibitory potency of serine proteases is based on the spectrophotometric determination of absorbance of the product, which is formed after amide bond cleavage in the presence of the enzyme. K_i, which represent the quantitative measure of inhibitor potency, are determined by spectrophotometric measurement of enzyme kinetics without and with the addition of the inhibitor (Cheng and Prusoff, 1973).

Spectrophotometric enzyme tests were performed in microtiter plates in a final volume of 200 μL. Bovine thrombin (Sigma–Aldrich) was tested at final concentration of 0.5 NIH E/mL with the substrate S-2238 (Chromogenix) at 20 and 40 μM final concentration and factor Xa (Chromogenix) at final concentration of 1 mBAEE E/mL with the substrate S-2222 (Chromogenix) at 100 and 200 μM final concentration. Bovine Trypsin (Sigma–Aldrich) was tested at final concentration of 0.5 nkat/mL using the substrate S-2222 (Chromogenix) at 50 and 100 μM final concentration. Inhibitors were dissolved in DMSO (concentration of stock solutions: 10 mmol/L) and the solutions with concentrations from 10 to 100 μM were prepared by dilution with distilled H_2O. The reaction rates in the absence and in the presence of the inhibitor was measured. 50 μM HBSA buffer, 50 μM solution of different inhibitor concentrations (in case of measurement without inhibitor 50 μM HBSA buffer) and 50 μM serine protease solution was pipetted into the microtiter plate. The plate was incubated for 15 min at 25 °C and subsequently 50 μM chromogenic substrate was added. The microtiter plate was put into the spectrophotometer (Synergy H4, Biotek) and the increase of absorbance at 405 nm at 25 °C was measured every 10 s.

Inhibitory constant K_i was determined from the change of absorbance form the initial, linear part of the curve according to the method of Cheng and Prusoff. Measurements were carried out in triplicate with three concentrations of the inhibitors and two concentrations of the substrate. For every combination of concentrations K_i was calculated, and a final result given as their average value.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.07.011.

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