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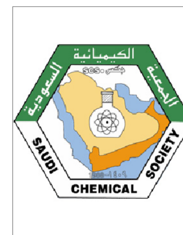

ORIGINAL ARTICLE

Isolation and structure elucidation of acetylcholinesterase lipophilic lupeol derivatives inhibitors from the latex of the Tunisian *Periploca laevigata*

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KEYWORDS

Periploca laevigata;
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Lipophilic lupeol esters;
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Abstract New lupeol long-chain alkanoic ester **1** and lupeol β-hydroxy fatty acid esters **2c**,**d** (laevigatins I and II) in a mixture with the previously isolated procrims a and b (**2a,b**) were isolated together with lupeol **3** and lupeol acetate **4** from the latex of *Periploca laevigata* collected in Tunisia. Their structures were elucidated by extensive spectroscopic methods including 1D (^{1}H, ^{13}C and DEPT 135), 2D-NMR experiments, (^{1}H–^{1}H COSY and NOESY), EI–MS, MALDI-TOF and GC analysis. Anti-acetylcholinesterase activity of most isolated compounds was evaluated and showed that lupeol (**3**) was the best inhibitor of AChE.

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1. Introduction

Plant latex is the milky juice, found in long branching tubes known as latex tubes. This juice is white, yellow or pinkish in color. It is a viscous fluid and colloidal in nature. Accumulated evidences indicate that the latex bearing plants are used in the management to cure various diseases such as diabetes, asthma, dysentery, diarrhea, malaria and skin problems (Nadkarni, 1976). Latex of *Calotropis procera* (Ait.) R.Br. was described for wormicidal activity (Shivkar and Kumar, 2003) and larvicidal activity (Badgujar and Mahajan, 2008) Curcain a proteolytic enzyme isolated from latex of *Jathropha curcas* Linn has been reported for wound healing activity (Nath and Dutta, 1992). *Alstonia scholaris* R. Br. is well-known for various activities, antimicrobial, antiamoebic, antidiarrhoeal, antiplasmodial, hepatoprotective, immunomodulatory, anticancer, antiasthmatic, free radical scavenging, antioxidant, analgesic, anti-inflammatory, antiulcer, antifertility and wound healing activities (Arulmozhi et al., 2007).

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Table 1 ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectral data ($CDCl_3$ solution) of Compounds **1**, **3** and **4**.

Atom	Compound **1**			Compound **3**			Compound **4**		
	δ ^{1}H	J (Hz)	δ ^{13}C	δ ^{1}H	J (Hz)	δ ^{13}C	δ ^{1}H	J (Hz)	δ ^{13}C
1	1.51	m	39.0	1.51	m	38.7	1.51	m	38.1
	1.62	m		1.72	m		1.72	m	
2	1.42	m	27.8	1.50	m	27.8	1.50	m	27.9
	1.56	m		1.70	m		1.70	m	
3	3.18	dd (10.8; 5.3)	79.7	4.48	dd (10.5; 6.6)	81.3	4.48	dd (10.5; 6.6)	80.6
4	–	–	39.2	–	–	38.4	–	–	38.1
5	0.61	m	55.6	0.70	m	55.7	0.70	m	55.4
6	1.23	m	18.6	1.44	m	18.5	1.44	m	18.1
	1.43	m		1.53	m		1.53	m	
7	1.31	m	32.5	1.25	m	34.5	1.25	m	31.9
	1.47	m		1.49	m		1.49	m	
8	–	–	41.2	–	–	41.2	–	–	40.9
9	1.21	m	50.8	1.30	m	50.7	1.30	m	50.4
10	–	–	37.5	–	–	37.4	–	–	37.1
11	1.24	m	21.2	1.25	m	21.3	1.25	m	21.0
	1.39	m		1.47	m		1.47	m	
12	1.46	m	25.5	1.51	m	25.4	1.51	m	25.1
	1.56	m		1.61	m		1.61	m	
13	1.58	m	38.4	1.63	m	38.1	1.63	m	37.8
14	–	–	43.2	–	–	43.2	–	–	42.8
15	1.09	m	26.1	1.13	m	25.4	1.13	m	25.2
	1.60	m		1.41	m		1.41	m	
16	1.35	m	34.6	1.42	m	34.5	1.42	m	34.9
	1.40	m		1.56	m		1.56	m	
17	43.3	43.3	43						
18	1.30	m	48.6	1.56	m	48.3	1.56	m	48.0
19	2.37	dt (11.5; 5.7)	48.3	2.37	dt (11.1; 5.7)	48.3	2.38	dt (11.5; 5.7)	48.3
20	–	–	151.3	–	–	151.4	–	–	150.9
21	0.81	m	30.7	0.87	m	30.2	0.88	m	29.4
	1.21	m		1.08	m		1.08	m	
22	1.33	m	40.3	1.33	m	40.3	1.33	m	40.0
	1.46	m		1.46	m		1.46	m	
23	0.94	s	28.3	0.85	s	28.3	0.88	s	28.0
24	0.79	s	16.5	0.88	s	16.8	0.84	s	16.6
25	0.83	s	16.3	0.88	s	16.5	0.84	s	16.2
26	1.03	s	15.6	1.03	s	16.3	1.03	s	16.0
27	0.96	s	14.8	0.94	s	14.8	0.94	s	14.1
28	0.76	s	18.3	0.79	s	18.3	0.76	s	18.0
29	4.57	m	109.7	4.57	m	109.7	4.57	m	109.4
	4.69	d (2.2)		4.69	d (2.1)		4.71	d (2.2)	
30	1.68	s	19.6	1.68	s	19.6	1.68	s	19.3
1′	–	–	–			171.4			179.7
2′	–	–	–	2.04	–	21.1	2.28	–	34.9
3′-(n′-1)	–	–	–	–	–	–	1.30	–	28–29
n′	–	–	–	–	–	–	0.97	–	14.5

Known ingredients of latex are proteins, alkaloids, tannins, terpenoids, sugars, oils, resins, gums and enzymes (Badgujar and Mahajan, 2008).

Periploca laevigata, source of latex (Asclepiadaceae) is native to the Mediterranean region and widely distributed in the Sahara area (Pottier-Alapetite, 1981). In Tunisia it is predominantly found in the south of the country, especially in the mountains. It is used as a food ingredient (tea) and as a herbal preparation because of its reputed medicinal properties, e.g., for the treatment of headaches and diabetes (Floc'h, 1983). Many chemical compounds have been isolated and identified from this species, such as α- and β-amyrin, lupeol, β-sitosterol and periplocadiol have been isolated from roots of *P. laevigata* (Askri et al., 1989), however the oleanolic acid, masilinic acid (Hichri et al., 2003), 12α-hydroxy-δ-lactone of oleanolic acid, arjunolic acid, Asiatic acid, β-D-glucopyranose and α-D-glucopyranose have been isolated from fruit barks of *P. laevigata* (Hichri et al., 2002).

The richness of this plant in latex whose chemical composition, to our knowledge has not been studied, prompted us to choose it to isolate its components and to study their anti-acetylcholinesterase activity. We describe here the isolation and the structural characterization of new long-chain alkanoic acid ester **1** (lupeol arachidate) and β-hydroxy fatty acid esters in mixture **2a**–**d** of lupeol among which only **2c,d** are new and named laevigatins I and II together with lupeol **3** and lupeol

acetate **4**. The anti-acetylcholinesterase effect of compounds **1**, **3** and **4** was evaluated and discussed.

2. Results and discussion

The chloroform extract from latex of *P. laevigata* was subjected to column chromatography over silica gel, leading to the isolation of three compounds **1**, **2a–d**, **3** and **4**.

Compound 1 was isolated from the chloroform extract of the latex from *P. laevigata*. The comparison of the spectral data of compounds **1** and **3** (Table 1) shows that the two compounds have the same triterpenic skeleton but the ^{1}H-NMR spectrum of **1** shows, moreover, the appearance of a broad signal at δ_H 1.30 attributable to the hydrocarbon chain $(CH_2)_n$. The same spectrum revealed a signal at δ_H 4.48 (1H, dd, J = 10.5; 6.6 Hz) due to the H-3 deshielded by the ester function fixed of the same carbon C-3, a signal at δ_H 2.28 (2H, t, J = 7,7 Hz, H-2') attributable to the methylene in α of the carbonyl group, a triplet at δ_H 0.97 (3H, t, J = 7,7 Hz, H-n') corresponding to the terminal methyl group of the hydrocarbon chain.

All these spectral data were in concordance with the structure of fatty acid ester whose alcohol is the lupeol (compound **3**). The relatively low polarity of compound **1** by comparison with those of compounds **3** and **4** is in agreement with the probable existence of fatty acid ester.

This result was confirmed by the ^{13}C-NMR spectrum showing a signal at δ_C 173.7 ppm attributable to the carbonyl ester function C-1′, a signal at δ_C 80.6 ppm due to the resonance of the methylene carbon C-3 and a signal at δ_C 14.5 ppm corresponding to the CH_3-n' of the hydrocarbon chain.

The ^{1}H and ^{13}C NMR spectra of compound **1** were assigned as shown in Table 1.

To confirm the length and the nature of the hydrocarbon chain of the natural ester **1**, the latter was hydrolysied in a 5% KOH methanolic solution. The analysis of the organic layer by GC-FID allowed to identify the fatty acid as the arachidic acid ($C_{20}H_{40}O_2$).

The analysis established through the 1D NMR, GC-FID and the literature (Boukamcha et al., 2003) allowed to identify compound **1** as lupeol arachidate, a new lipophilic lupeol ester.

Compounds 2a–d in mixture were isolated from the chloroform extract of the latex from *P. laevigata*. The MALDI-TOF mass spectrum of the mixture **2a–d** recorded in the presence of sodium iodide, displayed four ion peaks at m/z 703.48, 731.62, 759.65 and 787.69 which were attributed to the molecules containing sodium ($[M + Na]^{+}$) of four compounds with molecular formula $C_{46}H_{80}O_3$ (680; **2a**), $C_{48}H_{84}O_3$ (708; **2b**), $C_{50}H_{88}O_3$ (736; **2c**) and $C_{52}H_{92}O_3$ (764; **2d**). These data were reinforced by the observation in the same spectrum of ion peaks at m/z 683.21, 711.44, 765.58 and 767.41 corresponding to ($[\mathbf{2a} + 3H]^{3+}$), ($[\mathbf{2b} + 3H]^{3+}$), ($[\mathbf{2d} + H]^{+}$), and ($[\mathbf{2d} + 3H]^{3+}$), respectively. The same spectrum of the mixture **2a–d** showed fragment peaks due to the loss of a water molecule from each protonated compound in mixture, suggesting the presence of a free hydroxyl group. The comparison of the spectral data of compounds **1** and **2a–d** (Tables 1 and 2) shows that they have the same triterpenic skeleton with a long-chain alkanoic acid ester at C-3′. Moreover, the MALDI-TOF mass spectrum in positive mode showed the presence of the peak at m/z 426.18 attributed to the lupeol moiety, this shows that the free hydroxyl group belongs to the fatty acid. The fragmentations presented in Fig. 2 reinforced the above spectral data and the proposal structures.

The ^{1}H-NMR spectrum of **2a–d** shows the appearance of a signal at δ_H 2.52 (2H, dd, J = 16.3; 3.1 Hz, H-2′) attributable to the methylene in α position of the carbonyl group, a signal at δ_H 3.92 (1H, m, H-3′) corresponding to a methine bearing an alcohol function located in β position of the carbonyl group. The ^{13}C-NMR of the same compounds showed the appearance of a signal at δ_C 36.5 ppm (C-2′) and a signal at δ_C 68.2 ppm (C-3′) of the fatty acid chain (Furukawa et al., 2002).

The analysis established through the 1D NMR, mass spectrometry MALDI-TOF in positive mode and the literature (Furukawa et al., 2002) allowed us to propose to compounds **2a,b** the structure of procrims a and b previously isolated from the Alecrim-propolis collected in Brazil (Furukawa et al., 2002) and to compounds **2c,d** the structure of two new lupeol β-hydroxy fatty acid esters named laevigatins I and II (Fig. 1).

Table 2 ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectral data ($CDCl_3$ solution) of Compounds **2a–d**.

	Compounds **2a–d**		
Atom	δ ^{1}H	Multiplicity *J* (Hz)	δ ^{13}C
1	1.54 1.67	m	38.3
2	1.50 1.66	m	28.0
3	4.46	dd (7.5 ; 2.4)	81.4
4	–	–	38.0
5	0.66	m	55.3
6	1.28 1.44	m	18.2
7	1.34 1.51	m	31.9
8	–	–	41.6
9	1 .28	m	50.3
10	–	–	37.0
11	1.32 1.42	m	20.9
12	1.51 1.55	m	27.9
13	1.58	m	37.8
14	–	–	42.8
15	1.09 1.64	m	25.4
16	1.35 1.40	m	35.5
17	–	–	43.0
18	1.42	m	48.3
19	2.38	dt (11.4; 5.4)	48.0
20	–	–	150.9
21	0.91 1.21	m	29.5
22	1.34 1.44	m	40.8
23	0.78	s	29.3
24	0.76	s	16.5
25	0.78	s	16.0
26	0.95	s	16.0
27	0.81	s	14.0
28	0.71	s	18.0
29	4.58 4.70	d (1.2); d (2.1)	109.3
30	1.61	s	19.2
1′	–	–	172.8
2′	2.37; 2.52	m; dd (16.3; 3.1)	36.5
3′	3.92	m	68.2
4′	1.40	m	34.2
5′-(n′-1)	1.18	br s	29–30
n′	0.87	t (2.7)	14.5

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1 R: $CH_3(CH_2)_{18}CO$
2a R: $CH_3(CH_2)_{12}CHOHCH_2CO$
2b R: $CH_3(CH_2)_{14}CHOHCH_2CO$
2c R: $CH_3(CH_2)_{16}CHOHCH_2CO$
2d R: $CH_3(CH_2)_{18}CHOHCH_2CO$
3 R: CH_3CO
4 R: H

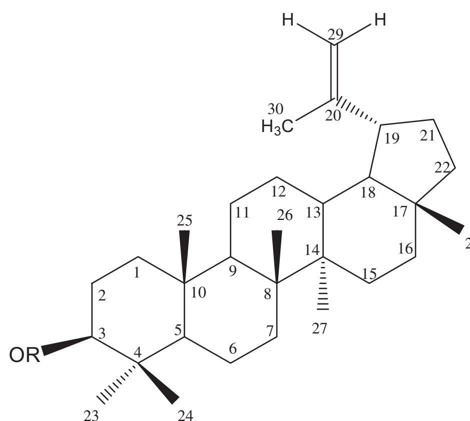

Figure 1 Compounds **1**, **2a–d**, **3** and **4**.

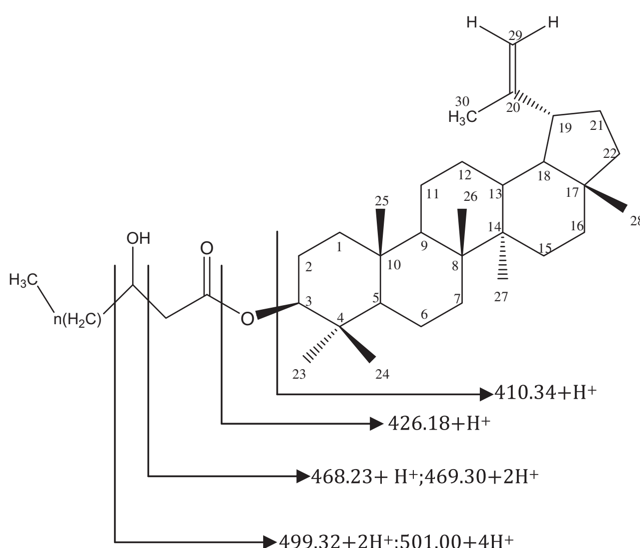

Figure 2 The essential fragment ions for Compounds **2a–d**.

Compounds 3 and 4 were isolated from the chloroform extract of the latex from *P. laevigata*. The structural study of these compounds using spectroscopic methods including 1D-NMR (^{1}H, ^{13}C (Table 1) and DEPT 135), 2D-NMR experiments, (^{1}H–^{1}H COSY and NOESY), EI-MS and the literature (Ghulam et al., 2000; Boukamcha et al., 2003; Jamal et al., 2008) allowed us to propose to compounds **3** and **4** the structure of lupeol and lupeol acetate, respectively.

3. Inhibitor of acetylcholinesterase

The acetylcholinesterase enzymatic activity was measured as described by Falé et al. (2009); briefly, 90 μL of 50 mM Tris–HCl buffer, pH = 8.30 μL of the sample and 7.5 μL of the acetylcholinesterase solution containing 0.26 U/mL were mixed in a microwell plate and left to incubate for 15 min. Subsequently, 22.5 μL of a solution of AChI (0.023 mg/mL) and 142 μL of 3 mM DTNB were added. The absorbance was read at 405 nm when the reaction reached equilibrium. A control reaction was carried out using water instead of sample which was considered 100% activity. The percentage Inhibition ((%) IP) is given as follows:

$$(\%)\text{IP} = 100 - \left(A_{sample}/A_{control}\right) * 100$$

where A_{sample} is the absorbance of the extract containing reaction mixture and $A_{control}$ the absorbance of the reaction. Tests were carried out in triplicate and a blank with Tris–HCl buffer instead of enzyme solution was used. In the case of the standards, a blank with methanol was carried out as these compounds were dissolved in this organic solvent.

The concentration of the extract or of the compound providing 50% of anti-acetylcholinesterase activity (IC_{50}) was obtained by plotting the anti-acetylcholinesterase activity against the concentration of the compound or the extract of plant (Hernandez et al., 2010).

Table 3 Inhibition of AChE of methanol extract, compounds **1**, **3** and **4**.

Samples	IC_{50} (μg/mL)
Methanol extract	60.90 ± 0.39
Compound **1**	439.29 ± 46.17
Compound **3**	38.31 ± 1.30
Compound **4**	142.55 ± 2.12

Table 3 below shows the activity of the methanol extract and that of the isolated compounds (**1**, **3 and 4**) tested with IC_{50} ranging from 38.31 ± 1.30 and 439.29 ± 46.17 μg/mL.

The values given in Table 3 compared with those given in the literature (Falé et al., 2009) of crude extracts and pure products, show that the methanol extract of the latex of *P. laevigata* (IC_{50} = 60.90 ± 0.39 μg/mL) has an interesting activity.

From the results shown in Table 3, lupeol **3** appeared more active than its structural analogues **4** and **1**. These results suggest that the triterpenic skeleton and the free secondary alcohol function at C-3 could be responsible for this activity. The esterification of the alcohol function in **1** and **4** decreases the inhibition of AChE.

We also noted that lupeol arachidate **1** is three times less active than lupeol acetate **4**. This finding could be due to the considerable length of the hydrocarbon chain in **1**.

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4. Experimental

4.1. Plant material

P. laevigata as collected in the region of Sokrine (Monastir, Tunisia) in June 2010. The plant was identified by Prof. Fethia HARZALLAH-SKHIRI in the Laboratory of Vegetal biology and Botanic, High Institute of Biotechnology of Monastir, Tunisia and a voucher specimen (PL-10) was deposited in the same laboratory.

4.2. Extraction and isolation

The latex of the plant (10.71 g) was extracted with methanol for 72 h. the methanolic extract (4,03 g) was dissolved in water and then extracted with chloroform to yield the corresponding extract. The $CHCl_3$ extract (3 g) was subjected to column chromatography over silica gel eluted with (C_5H_{12}/CH_2Cl_2/AcOEt/MeOH) in the increasing order of polarities to afford 11 fractions.

The wash with CH_2Cl_2 several times of fraction 1 (200 mg) led to a pure product **1** (150 mg).

The fraction 4 (670 mg) was purified by column chromatography over silica gel and eluted with (C_5H_{12}/CH_2Cl_2) to afford compounds **2a**–**d** in mixture.

The precipitation of the fraction 6 (150 mg) in CH_2Cl_2 afforded a white solid **3** (30 mg).

The recrystallization of fraction 2 (300 mg) in AcOEt gave a white solid **4** (250 mg).

4.3. Hydrolysis of compound *1*

25 mg of compound **1** was treated at reflux by a solution methalonic of KOH (5%) for 3 h. The chloroform extract of the reaction mixture was washed twice with distilled water and then dried on anhydrous Na_2SO_4.

The organic layer is analyzed using GC-FID.

4.4. Nuclear molecular resonance (NMR)

1H NMR and 13C NMR of compounds **1**, **2a**–**d**, **3** and **4** were measured on a Bruker AM 300 NMR spectrometer, at 300 and 75 MHz, respectively, with CDCl3. The residual solvent resonances were used as the internal references. Coupling constants are given in Hertz. The chemical shifts are expressed in δ ppm. COSY and NOESY spectra were run on a Bruker AM 300 NMR spectrometer.

4.5. GC analysis

The length of the hydrocarbon chain in compound **1** was determined by GC-FID. The analytical GC was carried out on a HP5890-series II gas chromatograph equipped with Flame ionization detectors (FID) under the following conditions: the fused silica capillary columns HP-5 (30 m × 0.25 mm ID, film thickness 0.25 μm). The oven temperature was held at 50 °C for 1 min then programmed to 240 °C at the rate of 5 °C/min and held isothermal for 4 min. The carrier gas was nitrogen at a flow rate of 1.2 mL/min; injector and detector temperature: 250 °C and 280 °C, respectively; the volume injected: 0.1 μL of 1% solution (diluted in hexane). The identification of arachidic acid was done by comparing its retention time to that of an authentic sample whose retention time is indicated in the chromatothec of the device.

4.6. MALDI-TOF

MALDI-TOF spectra were acquired with a Voyager-DE STR (AB Sciex, les Ulis, France) mass spectrometer located at the Institut de Chimie des substances Naturelles 5ICSN, CNRS). Samples were mixed with the matrix solution (2.5-dihydroxybenzoic acid, 10 mg/mL in methanol/chloroform (1/1, v/v)) prior to analysis. Laser power, delay extraction time and grid voltage were optimized to reach the best resolution and sensibility. The mass spectrometer was calibrated using a Pepmix4 standard mixture (LaserBiobs, Sophia Antipolis, France).

4.7. EI-MS

The mass spectra in electron impact (EI) were performed on a mass spectrometer Perkin TURBO MASS. The molecule is bombarded in an ionization source of 150 °C by an electron beam of 70 eV.

5. Conclusion

New lupeol long-chain alkanoic ester 1 and lupeol β-hydroxy fatty acid esters **2c,d** (laevigatins I and II) in mixture with the previously isolated procrims a and b (**2a,b**) were isolated together with lupeol 3 and lupeol acetate 4 from the latex of *P. laevigata* collected in Tunisia. Their structures were elucidated by extensive spectroscopic methods including 1D (1H, 13C and DEPT 135), 2D-NMR experiments, (1H-1H COSY and NOESY), EI-MS, MALDI-TOF and GC analysis. Anti-acetylcholinesterase activity of compounds **1**, **3** and **4** was evaluated and showed that lupeol was the best inhibitor. The results led us to suggest that the triterpenic skeleton and the free secondary alcohol function at C-3 could be responsible of this activity and the esterification of the alcohol function may decrease the inhibition of AChE.

Acknowledgements

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