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Germacranolides from seeds of the endangered Umbelliferae species *Rouya polygama*

Bernard Muckensturm[a,*], Fatih Sen[a], Anna Boulanger[a], Jean-Pierre Reduron[b]

[a]*Laboratoire de Chimie Organique et Bioorganique associé au CNRS, Université de Haute Alsace,*
Ecole Nationale Supérieure de Chimie de Mulhouse, 3 rue Alfred Werner, F-68093 Mulhouse Cedex, France
[b]*Conservatoire Botanique de la Ville de Mulhouse, 2 rue Pierre et Marie Curie, F-68200 Mulhouse, France*

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Abstract

From the etheral extract of *Rouya polygama* Coincy seeds (Umbelliferae) six new germacranolides **1**–**6** were isolated. The structures were elucidated by chemical methods and spectroscopic analysis. Compounds **1** and **4** exhibited activity against *Artemia salina* larvae.

Keywords: *Rouya polygama*; *Thapsia*; Umbelliferae; Chemotaxonomy; Sesquiterpenoids; Rouyolides

1. Introduction

Rouya polygama Coincy is an endangered member of Umbelliferae found in Corsica, Sardinia and the Maghreb, and is protected in Europe. Previous studies (Rasmussen et al., 1981), together with four other *Thapsia* species where *R. Polygama* was referred to *Thapsia polygama* (Fournier, 1961; Quezel and Santa, 1963), resulted only in isolation of scopoletin. As part of our chemotaxonomic studies on European and North African Umbelliferae we investigated its constituents and describe here the isolation and characterization of six new sesquiterpene derivatives for which we propose the names rouyolides **A**–**F** (**1**–**6**).

2. Results and discussion

GC–MS analysis of the different parts of *R. polygama* revealed the presence of several sesquiterpenoids unlisted in our database consisting of the chromatograms of most of the Umbelliferae growing in Europe and North Africa.

The more abundant product isolated from the seeds was a lactone evident from the I.R., containing two angelate or tiglate substituents evident from the M.S. which exhibited signals at m/z 55 and 83 as well as small peaks at M-100 and M-200. The molecular ion at m/z 446 corresponded to the formula $C_{25}H_{34}O_7$ according to the NMR spectra (^{1}H, DEPT and ^{13}C) which indicated seven methyl groups, three methylenes and seven C–H bonds, three of them were attached to an oxygen and three ethylenes, as well as eight quaternary carbons, three of them were carbonyls, three ethylenic and two others bonded to oxygen. Finally HMQC, HMBC and COSY spectra furnished the correlations shown in Fig. 1. The NOESY spectrum gave the relative stereochemistry shown in Fig. 2. Compound **1** was therefore identified as the new 6α,11α-diangeloyloxy-1β,10α-epoxygermacr-4-en-8β,12-olide.

The other constituents had related structures. The least polar was rouyolide **B** (**2**), $C_{25}H_{34}O_6$, where spectral evidence indicated the presence of a double bond in place of the epoxy group. Rouyolide **C** (**3**) was the most polar: the substituent in position 6 was formed by addition of an angelate to a hypothetical epoxyangelate. Rouyolides **D**, **E** and **F** (**4**, **5** and **6**) were minor constituents. Their structures were established by chemical correlations. When treated with a small excess of *m*-chloroperbenzoic acid rouyolide **A** formed rouyolide **D** (95%). Epoxidation of rouyolide **B** gave a mixture of rouyolide **A** (34%), **D** (46%), **E** (15%) and **F** (5%). All structures were established by NMR analysis in the

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* Corresponding author. Tel.: +33-3-89-33-68-62; fax: +33-3-89-33-68-63.
E-mail address: b.muckensturm@uha.fr (B. Muckensturm).

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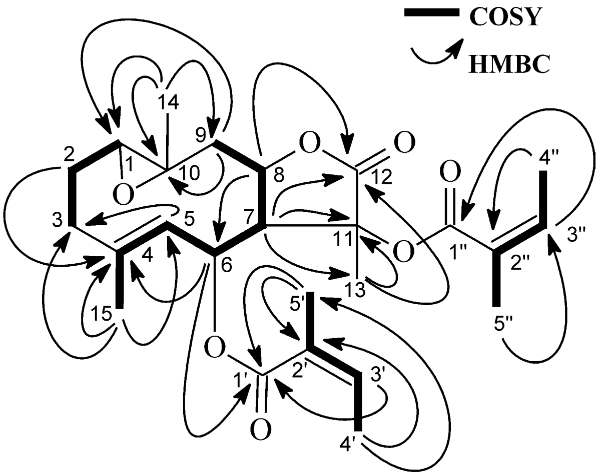


Fig. 1. HMBC and COSY correlations for **1**.

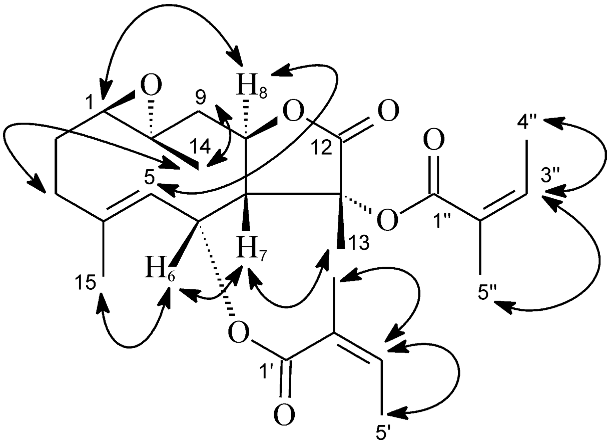


Fig. 2. Important NOE correlations for **1**.

manner described and were also present as natural products in the seed extract (Fig. 3).

The sesquiterpene lactones isolated from members of the Umbelliferae comprise a large group with more than 100 known compounds but very few of them are derived from germacranes (Holub and Budesinsky, 1986; Itokawa, 1986; Gören and Ulubelen, 1987; El-Gamal, 2001). From a chemotaxonomic point of view, our results confirm their separation from the genus *Thapsia* which yields mainly thapsigargins and thapsigargicins, highly active sesquiterpenoids with a guaiane skeleton (Rasmussen et al., 1978; Thastrup et al., 1989; Thastrup, 1990; Lytton et al., 1991), but no germacranolides (Christensen et al., 1997). The only germacrane derivatives isolated from the genus *Thapsia* are related to tovarol and shiromodiol (Pascual Teresa et al., 1985, 1986).

All our compounds were evaluated in the *Artemia salina* toxic assay, a rapid, reliable and inexpensive in-house general bioassay tool (Meyer et al., 1982). Active compounds may then be subjected to more elaborated bioassays for specific pharmacologic activities. Rouyolides **A** and **D** showed a significant activity with a LC_{50} of 1.3 and 1.6 μg/ml respectively, while the other four compounds were inactive (rouyolides **B**, **C**, **E** and **F**, LC_{50} 775, 1573, 40 and 102 μg/ml respectively).

3. Experimental

Column chromatography was carried out with TLC monitoring using Kieselgel 60 (70–230 mesh, Merck). For TLC, glass-supported silica gel 60 plates (0.25 mm layer, F_{250}, E. Merck) were used. Visualization was accomplished by spraying *p*-anisaldehyde-H_2SO_4 reagent followed by heating at 110 °C. Chromatographic conditions: GC–MS (HP 5890A-5970), EI, 70 eV, using a 25m BPX5 capillary (0.15 mm I.D.) GC column with helium as a carrier gas. Then oven temperature was raised from 80 to 310 °C at 3.5 °C/min; ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 400 spectrometer in $CDCl_3$ at 400 MHz and 100 MHz, respectively. Chemical shifts were reported using TMS as the internal standard. Optical rotations were determined with a Perkin Elmer 341LC polarimeter. IR spectra were recorded with a Nicolet FT-IR 20S spectrophotometer.

3.1. Plant material

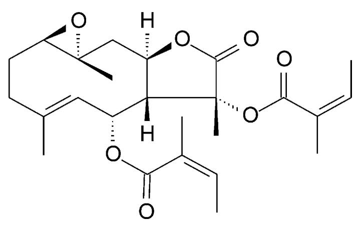
R. polygama was cultivated in the Conservatoire Botanique of Porquerolles (France) and identified by one of us (J.P.R.). A voucher specimen (CBM98174) was deposited in the herbarium of the Conservatoire Botanique of Mulhouse.

3.2. Extraction and isolation of germacranolides **1**, **2** and **3**

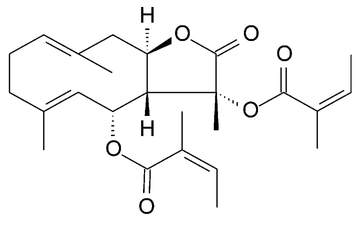
Air dried powdered seeds (900 g) were extracted with Et_2O at room temperature for 2 days. The crude etheral extract (24.8 g) was directly chromatographed over silica-gel eluting with mixtures of pentane–Et_2O of increasing polarity, giving fractions F_1–F_{11}. Successive chromatographic separations of fractions F_6 and F_7, eluted with pentane–Et_2O (70:30), and final preparative TLC on elution with a pentane–CH_2Cl_2–Et_2O (45:45:10) mixture, led to isolation of **2** (8 mg, $R_f = 0.95$). Fractions F_8 and F_9, eluted with Et_2O, were evaporated and submitted to short path distillation at 220 °C (20 mm Hg) to give **1** (25 mg, $R_f = 0.65$). Fraction F_{10}–F_{11}, eluted with Et_2O, was rechromatographed by preparative TLC using a pentane–CH_2Cl_2–Et_2O (45:45:10) mixture, yielding **3** (6 mg, $R_f = 0.31$).

3.3. Identification of **4**, **5** and **6**

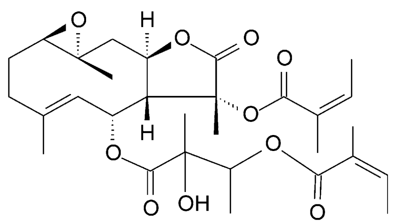
A mixture of **1** (40 mg, 0.089 mmol) and 80% *m*-chloroperbenzoic acid (20 mg, 0.11 mmol) in $CHCl_3$ (20 ml) was allowed to stand at room temperature for 24 h. The mixture was extracted with Et_2O (3×10 ml).



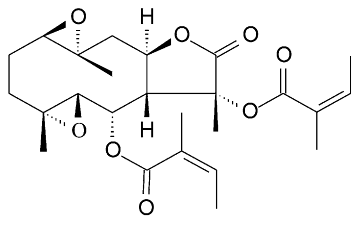
Rouyolide A (1)



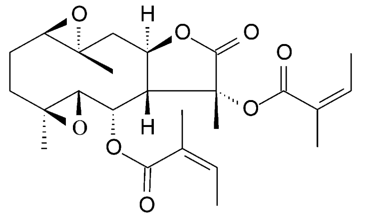
Rouyolide B (2)



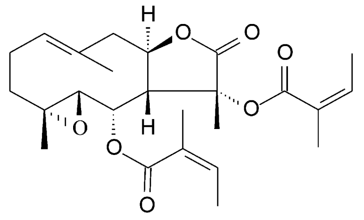
Rouyolide C (3)



Rouyolide D (4)



Rouyolide E (5)



Rouyolide F (6)

Fig. 3. New germacranolides isolated.

Table 1
^{1}H NMR chemical shifts assignments of compounds **1–6** in $CDCl_3$

Position	**1** δ, mult. (*J* in Hz)	**2** δ, mult. (*J* in Hz)	**3** δ, mult. (*J* in Hz)	**4** δ, mult. (*J* in Hz)	**5** δ, mult. (*J* in Hz)	**6** δ, mult. (*J* in Hz)
1	2.81, *dd* (1.5/11.0)	5.01, *bd* (11.4)	2.78, *dd* (1.2/11.6)	2.95, *d* (6.5)	2.75, *dd* (1.0/8.0)	5.09, *d* (7.2)
2	1.50, overlapped 2.08, overlapped	2.20, overlapped 2.40, overlapped	1.45, overlapped 2.00, overlapped	2.10, overlapped	1.20, overlapped 1.55, overlapped	1.60, *m* 2.35, overlapped
3	2.26, (2H) overlapped	2.12, (2H) overlapped	2.17, (2H) overlapped	1.25, overlapped 2.15, overlapped	2.22, overlapped 3.15, overlapped	2.35, (2H) overlapped
4	–	–	–	–	–	–
5	5.21, *dd* (6.1/1.5)	4.90, *d* (4.8)	4.91, *bd* (4.9)	2.89, *dd* (1.0/6.3)	2.70, *dd* (1.0/12.3)	2.50, *dd* (1.0/7.2)
6	5.58, *dd* (2.1/6.1)	5.55, *bs*	5.55, *bd* (4.9)	4.86, *dd* (1.0/6.3)	4.93, *dd* (1.0/12.3)	5.72, *dd* (1.0/7.2)
7	2.96, *dd* (2.1/2.1)	2.77, *bs*	2.95, *bs*	3.12, *bs*	2.81, *bs*	2.81, *dd* (1.0/12.0)
8	4.82, *m*	4.80, *m*	4.75, *m*	4.97, *m*	4.98, *dq* (1.3/8.0)	4.85, *ddd* (1.6/4.8/12.0)
9	2.19, (2H) overlapped	1.90, overlapped 3.00, overlapped	2.17, overlapped 2.22, overlapped	2.20, (2H) overlapped	2.35, (2H) overlapped	2.35, overlapped 2.60, *dd* (4.8/10.0)
10	–	–	–	–	–	–
11	–	–	–	–	–	–
12	–	–	–	–	–	–
13	1.85, *s*	1.85, *s*	1.83, *s*	1.87, *s*	1.79, *s*	1.76, *s*
14	1.40, *bs*	1.75, *s*	1.32, *bs*	1.41, *s*	1.42, *s*	1.42, *s*
15	1.75, *bs*	1.60, *s*	1.69, *bs*	1.58, *s*	1.55, *s*	1.55, *s*
1′	–	–	–	–	–	–
2′	–	–	–	–	–	–
3′	6.10, *qq* (1.5/7.3)	6.09, *qq* (1.0/7.1)	5.23, *q* (6.4)	6.12, *qq* (1.0/7.3)	6.12, *qq* (1.4/7.1)	6.12, *qq* (1.6/7.2)
4′	1.95, *qd* (1.5/7.3) a	1.95, *qd* (1.0/7.1)a	1.23, *bs* a	1.99, *qd* (1.0/7.3)a	1.96, *qd* (1.4/7.1)a	1.96, *qd* (1.6/7.2)a
5′	1.81, *qd* (1.5/1.5) b	1.81, *bs*b	1.18, *bs* a	1.80, *bs*b	1.81, overlapped	1.81, overlapped
6′			–			
7′			–			
8′			6.12, *qq* (1.2/7.0)			
9′			1.99, *qd* (1.2/7.0) b			
10′			1.80, *bs*			
1″	–	–	–	–	–	–
2″	–	–	–	–	–	–
3″	6.18, *qq* (1.5/7.3)	6.18, *qq* (1.0/7.1)	6.20, *qq* (1.2/7.0)	6.20, *qq* (1.0/7.3)	6.18, *qq* (1.4/7.1)	6.18, *qq* (1.6/7.2)
4″	2.01, *dq* (1.5/7.3) a	2.01, *dq* (1.0/7.1)a	2.05, *dq* (1.2/7.0) b	2.05, *dq* (1.0/7.3)a	1.98, *dq* (1.4/7.1)a	1.98, *dq* (1.6/7.2)a
5″	1.85, *dq* (1.5/1.5) b	1.85, *bs*b	1.89, *bs*	1.89, *bs*b	1.81, overlapped	1.81, overlapped

a,b,c,d, Assigments may be interchanged in the same column.

The combined extracts were washed successively with a saturated $NaHCO_3$ aqueous solution (2×20 ml) and a saturated aqueous solution of NaCl (20 ml), dried (Na_2SO_4) and concentrated to give an oily crude product, which was purified by preparative TLC [pentane–CH_2Cl_2–Et_2O (45:45:10)]. Band at $R_f = 0.33$ gave **4** (38 mg, 95%) as an oil. In another experiment, a mixture of **2** (30 mg, 0.07 mmol) and 80% *m*-chloroperbenzoic acid (15 mg, 0.085 mmol) in $CHCl_3$ (20 ml) was allowed to stand at room temperature for 24 h and treated as usual to give an oily crude product which was separated by TLC [pentane–CH_2Cl_2–Et_2O (45:45:10)]. Bands at $R_f = 0.65$, 0.33, 0.38, 0.60 gave, respectively, **1** (10 mg, 34%), **4** (13.4 mg, 46%), **5** (4.3 mg, 15%) and **6** (1.5 mg, 5%).

3.4. Rouyolide **A** (**1**)

Oil $[\alpha]_D^{25}$ +75.3 (c 0.473, $CHCl_3$); IR ν_{max} ($CHCl_3$) cm^{-1}: 3106, 1708, 1230, 1065; ^1H and ^{13}C NMR ($CDCl_3$): see Tables 1 and 2; Retention index: 2905, GC–MS 70 eV, *m*/*z* (rel. int.): 446 $[M]^+$ (1), 363 $[M–Ang]^+$ (1), 347 $[M–OAng]^+$ (1), 263 $[M–Ang–AngOH]^+$ (1), 247 $[347–AngOH]^+$ (1), 83 $[Ang]^+$ (100), 55 $[83–CO]^+$ (30).

Table 2
^{13}C NMR chemical shifts assignments of compounds **1–6** in $CDCl_3$

Position[a]	**1**	**2**	**3**	**4**	**5**	**6**
1	60.1, *d*	130.0, *d*	61.7, *d*	60.6, *d*	59.5, *d*	126.2, *d*
2	24.0, *t*	24.6, *t*	23.9, *t*	23.4, *t*	24.2, *t*	22.6, *t*
3	36.3, *t*	38.8, *t*	35.9, *t*	36.0, *t*	34.0, *t*	31.9, *t*
4	137.5, *s*	137.5, *d*	138.2, *s*	58.8, *s*	58.7, *s*	56.8, *s*
5	126.0, *d*	128.0, *d*	124.4, *d*	64.8, *d*	64.8, *d*	62.1, *d*
6	69.3, *d*	69.4, *d*	71.7, *d*	71.7, *d*	69.8, *d*	68.3, *d*
7	50.5, *d*	50.0, *d*	50.3, *d*	47.6, *d*	50.9, *d*	48.9, *d*
8	76.0, *d*	79.0, *d*	75.6, *d*	75.4, *d*	75.2, *d*	79.6, *d*
9	42.3, *t*	40.0, *t*	42.1, *t*	42.5, *t*	45.1, *t*	46.1, *t*
10	57.0, *s*	129.0, *s*	56.8, *s*	57.1, *s*	56.3, *s*	127.6, *s*
11	77.0, *s*	77.3, *s*	77.5, *s*	77.5, *s*	77.1, *s*	75.7, *s*
12	173.5, *s*	174.0, *s*	173.5, *s*	173.2, *s*	173.1, *s*	173.0, *s*
13	24.8, *q*	25.0, *q*	24.6, *q*	24.4, *q*	25.8, *q*	24.5, *q*
14	21.8, *q*	21.2, *q*	21.8, *q*	16.4, *q*	a16.6, *q*	16.0, *q*
15	17.2, *q*	17.3, *q*	17.2, *q*	21.9, *q*	a16.8, *q*	16.8, *q*
1′	166.0, *s*	166.2, *s*	174.2, *s*	a165.4, *s*	165.3, *s*	165.0, *s*
2′	a127.0, *s*	a128.4, *s*	106.1, *s*	125.5, *s*	b126.4, *s*	128.9, *s*
3′	b140.0, *d*	b140.0, *d*	73.8, *d*	b141.3, *d*	140.8, *d*	140.3, *d*
4′	17.2, *q*	15.6, *q*	13.4, *q*	c15.8, *q*	c15.8, *q*	a15.7, *q*
5′	c20.1, *q*	c20.2, *q*	21.4, *q*	d20.0, *q*	d20.0, *q*	b20.2, *q*
6′	–	–	166.5, *s*	–	–	–
7′	–	–	124.4, *s*	–	–	–
8′	–	–	139.5, *d*	–	–	–
9′	–	–	a16.0, *q*	–	–	–
10′	–	–	20.6, *q*	–	–	–
1″	167.1, *s*	168.1, *s*	167.3, *s*	a165.0, *s*	166.8, *s*	165.4, *s*
2″	a127.1, *s*	a128.3, *s*	127.4, *s*	126.9, *s*	b126.6, *s*	130.5, *s*
3″	b140.1, *q*	b140.5, *d*	141.1, *q*	b141.7, *q*	141.5, *q*	141.3, *d*
4″	16.1, *q*	16.0, *q*	a16.1, *q*	c16.1, *q*	c16.0, *q*	a16.0, *q*
5″	c20.3, *q*	c20.4, *q*	20.6, *q*	d20.3, *q*	d20.2, *q*	b20.4, *q*

a,b,c,d, Assigments may be interchanged in the same column.

[a] Multiplicity was determined using the DEPT sequence.

3.5. Rouyolide **B** (**2**)

Oil $[\alpha]_D^{25}$ +57.3 (c 0.041, $CHCl_3$); IR ν_{max} ($CHCl_3$) cm^{-1}: 3120, 1720, 1250, 1080. ^1H and ^{13}C NMR ($CDCl_3$): see Tables 1 and 2; Retention index: 2770, GC–MS 70 eV, *m*/*z* (rel. int.): 430 $[M]^+$ (1), 331 $[M–OAng]^+$ (1), 247 $[M–Ang–AngOH]^+$ (1), 231 $[331–AngOH]^+$ (1), 83 $[Ang]^+$ (100), 55 $[83–CO]^+$ (30).

3.6. Rouyolide **C** (**3**)

Oil $[\alpha]_D^{25}$ +141.4 (c 0.082, $CHCl_3$); IR ν_{max} ($CHCl_3$) cm^{-1}: 3500, 3117, 1735, 1210, 1180, 1070; ^1H and ^{13}C NMR ($CDCl_3$): see Tables 1 and 2; Retention index: 3283, GC–MS 70 eV, *m*/*z* (rel. int.): 562 $[M]^+$ (1), 463 $[M–OAng]^+$ (1), 363 $[463–AngOH]^+$(1), 83 $[Ang]^+$ (100), 55 $[83–CO]^+$ (30).

3.7. Rouyolide **D** (**4**)

Oil $[\alpha]_D^{25}$ +16.6 (c 0.042, $CHCl_3$); IR ν_{max} ($CHCl_3$) cm^{-1}: 3145, 1720, 1200, 1090; ^1H and ^{13}C NMR ($CDCl_3$): see Tables 1 and 2; Retention index: 3023, GC–MS 70 eV, *m*/*z* (rel. int.): 462 $[M]^+$ (1), 363 $[M–OAng]^+$ (1), 263 $[363–AngOH]^+$ (1), 83 $[Ang]^+$ (100), 55 $[83–CO]^+$ (30).

3.8. Rouyolide **E** (**5**)

Oil $[\alpha]_D^{25}$ +2.95 (c 0.206, $CHCl_3$); IR ν_{max} ($CHCl_3$) cm^{-1}: 3108, 1715, 1230, 1070; ^1H and ^{13}C NMR ($CDCl_3$): see Tables 1 and 2; Retention index: 2931, GC–MS 70 eV, *m*/*z* (rel. int.): 462 $[M]^+$ (1), 363 $[M–OAng]^+$ (1), 263 $[363–AngOH]^+$ (1), 83 $[Ang]^+$ (100), 55 $[83–CO]^+$ (30).

3.9. Rouyolide **F** (**6**)

Oil $[\alpha]_D^{25}$ +85.5 (c 110, $CHCl_3$); IR ν_{max} ($CHCl_3$) cm^{-1}: 3104, 1700, 1235, 1070; ^1H and ^{13}C NMR ($CDCl_3$): see Tables 1 and 2; Retention index: 2857, GC–MS 70 eV, *m*/*z* (rel. int.): 446 $[M]^+$ (1), 363 $[M–Ang]^+$ (1), 347 $[M–OAng]^+$ (1), 263 $[363–AngOH]^+$ (1), 247 $[347–AngOH]^+$ (1), 83 $[Ang]^+$ (100), 55 $[83–CO]^+$ (30).

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References

Christensen, S.B., Andersen, A., Smitt, U.W., 1997. Sesquiterpenoids from *Thapsia* species and medicinal chemistry of the thapsigargins. Fortschritte der Chemie Organischer Naturstoffe 71, 129–167.

El-Gamal, A.A., 2001. Sesquiterpene lactones from *Smyrnium olusatrum*. Phytochemistry 57, 1197–1200.

Fournier, P., 1961. Les Quatre Flores de France. P. Lechevalier Ed., Paris.

Gören, N., Ulubelen, A., 1987. Glechomanolides and eudesmanolides from *Smyrnium perfoliatum*. Phytochemistry 26, 2585–2587.

Holub, M., Budesinsky, M., 1986. Sesquiterpene lactones of the Umbelliferae. Phytochemistry 25, 2015–2026.

Itokawa, H., Matsumoto, H., Mizuno, K., Watanabe, K., Morita, H., Iitaka, Y., 1986. Structures of torilolide and oxytorilolide; two novel germacranolides from *Torilis japonica* (HOUTT.) DC. Chemical Pharmaceutical Bulletin 34, 4682–4686.

Lytton, J., Westlin, M., Hanley, M., 1991. Thapsigargin inhibits the sarcoplasmic or endoplacmic reticulum Ca-ATPase family of calcium pumps. Journal of Biological Chemistry 266, 17067–17071.

Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E., McLaughlin, J.L., 1982. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Medica 45, 31–34.

Pascula Teresa, J., Moran, J.R., Hernandez, J.M., Grande, M., 1985. Tovarol and other germacrane derivatives from *Thapsia villosa*. Phytochemistry 24, 1779–1783.

Pascula Teresa, J., Moran, J.R., Hernandez, J.M., Grande, M., 1986. 12-Hydroxytovarol and derivatives from *Thapsia villosa* var. *minor*. Phytochemistry 25, 1167–1170.

Quezel, P., Santa, S., 1963. Nouvelles Flores de l'Algérie et des Régions Désertiques Méridionales. CNRS Ed., Paris.

Rasmussen, U., Christensen, S.B., Sandberg, F., 1978. Thapsigargin and thapsigargicin, two new histamin liberators from *Thapsia garganica* L. Acta Pharmaceutica Suecica 15, 133–140.

Rasmussen, U., Christensen, S.B., Sandberg, F., 1981. Phytochemistry of the genus *Thapsia*. Planta Medica 43, 336–341.

Thastrup, O., 1990. Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microbial Ca^{2+}-ATPase inhibitor, thapsigargin. Agents and Actions 29, 8–15.

Thastrup, O., Dawson, A.P., Scharff, O., Foder, B., Bjerrum, P.J., Christensen, S.B., Hanley, M.R., 1989. Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents and Actions 27, 17–23.