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Bioactive kaurane diterpenes and coumarins from *Fortunella margarita*

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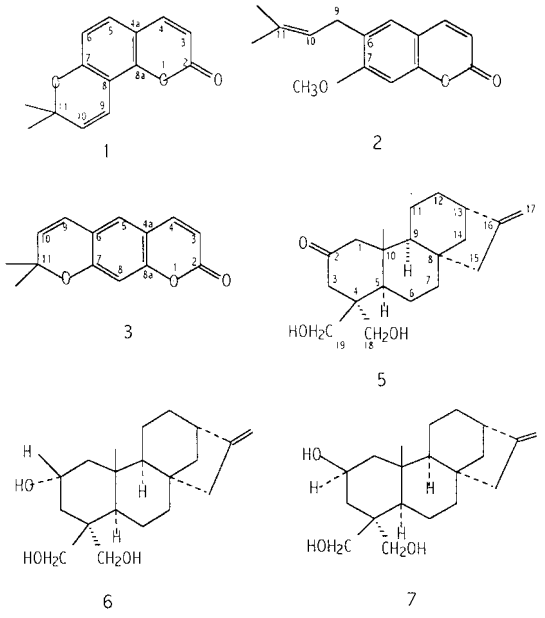
Two bioactive pyranocoumarins **1** (sesselin) and **3** (xanthyletin) and one prenylated coumarin **2** (suberosin), beside three rare kaurene diterpenes **5**–**7** were isolated from the roots of *Fortunella margarita*. Their structures were determined from their spectroscopic data, including $^{1}H/^{13}C$ 2D NMR experiments. The kaurene diterpenes **5**–**7** are reported in Rutaceae for the first time. Diterpene **5** was found to be a potent stimulator of uterine contraction; it also caused stimulation of brain activity.

1. Introduction

The genus *Fortunella* is closely related to *Citrus*, and is represented by only five species [1–4]. *F. margarita* is a shrub or a small tree (3–3.5 m) having elliptical small fruits (2–3 cm in diameter) [1, 2].

Citrus has been the subject of many phytochemical studies resulting in the isolation of numerous bioactive coumarins, alkaloids and flavonoids [5–12], but only a few reports on the chemical constituents of *Fortunella* species are available. These reports were concentrated on the volatile oil composition [3, 4] and evaluation of the aureptene [13].

This paper describes the isolation and identification of some biologically active coumarins (**1**–**3**, reported to have anticancer effects [14]), and three rare kaurene diterpenoids (**5**–**7**). Related diterpenoids exhibited potent uterostimulatory, antihepatotoxic and antibacterial effects [15] as well as anti-HIV activities [16].



Kaurene diterpenes have not been reported before in *Fortunella* or *Citrus*, and the available literature indicated their first occurrence in Rutaceae. The biological activities of the isolated compounds are discussed.

2. Investigations, results and discussion

The dried roots of *F. margarita* were extracted with acetone, and the obtained extract was partitioned between 10% ethanol in water and light petroleum and then chloroform. Repeated CC and PHPLC of the two fractions yielded three coumarins (**1**–**3**) and three kaurene diterpenes (**5**–**7**), as well as two steroids β-sitosterol (**4**) and β-sitosterol-O-glucoside (**8**).

Compound **1** was isolated as colorless needles, m.p. 119 °C ($CHCl_3$/MeOH). Its UV and IR spectra exhibited absorptions typical for 7-oxygenated coumarins [6, 7, 17]. The ^{1}H NMR spectrum showed a pair of doublets at δ 7.57 and 6.20 ($J = 9.5$ Hz), characteristic of H-4 and H-3 in a coumarin nucleus [6, 18]. The pair of doublets at δ 5.70 and 6.86 ($J = 10$ Hz), beside the singlet at δ 1.45 (6 H, *s*) are typical for the dimethylchromene ring. The remaining pair of doublets at δ 7.18 and 6.69 ($J = 8.5$ Hz) was attributed to H-5 and H-6. These data agreed with those reported for sesselin [18]. The MS showed $[M^+]$ at *m/z* 228 and fragments characteristic for sesselin [18, 19]. ^{13}C-NMR assignments (Table 1) based on 2D experiments confirmed that **1** is sesselin.

From the IR and UV it was deduced that **2** is a 7-oxygenated coumarin [6, 7]. The ^{1}H NMR spectrum showed two doublets at δ 6.20 and 7.59 ($J = 9.5$ Hz), and two singlets at δ 6.75 (H-8) and 7.15 (H-5) corresponding to 6,7-disubstituted coumarin [7, 18]. The presence of a doublet at δ 3.28 (2 H, *d*, $J = 7.5$, H-9) coupled with a multiplet at δ 5.26 (1 H, *m*, H-10) and two methyl signals at δ 1.68 and 1.74 indicated a prenyl function. The singlet at δ 3.87 was attributed to the methoxyl group. The MS showed $[M^+]$ at *m/z* 244, a base peak at 229 and a fragmentation pattern similar to that of suberosin [7]. By comparison of the obtained data with those reported for suberosin [7, 18], compound **2** was identified as suberosin. The ^{13}C NMR

Table 1: ^{13}C NMR spectral data* of coumarins 1, 2 and 3 (in $CDCl_3$) and diterpenes 5, 6, and 7 (in DMSO-d6)

Carbon	1	2	3	5	6	7
1	–	–	–	47.42, *t*	35.83, *t*	37.23, *t*
2	160.99, *s*	161.48, *s*	161.00, *s*	213.29, *s*	65.22, *d*	65.22, *d*
3	112.65, *d*	112.72, *d*	112.93, *d*	55.80, *t*	48.16, *t*	45.46, *t*
4	143.87, *d*	143.59, *d*	143.22, *d*	47.60, *s*	39.08, *s*	32.92, *s*
4a	112.58, *s*	127.44, *s*	118.44, *s*	–	–	–
5	127.73, *d*	127.36, *d*	124.71, *d*	44.64, *t*	45.55, *d*	47.89, *d*
6	113.49, *d*	111.85, *s*	112.65, *s*	20.82, *t*	20.38, *t*	26.74, *t*
7	150.08, *s*	154.44, *s*	155.40, *s*	40.39, *t*	40.28, *t*	40.12, *t*
8	109.26, *s*	98.45, *d*	104.27, *d*	43.94, *s*	43.69, *s*	38.88, *s*
8a	156.28, *s*	160.61, *s*	156.78, *s*	–	–	–
9	114.97, *d*	27.75, *t*	120.70, *d*	54.60, *t*	55.56, *d*	52.72, *d*
10	130.72, *d*	121.31, *d*	131.13, *d*	43.56, *s*	41.45, *s*	41.45, *s*
11	77.58, *s*	133.61, *s*	78.31, *s*	18.44, *t*	18.39, *t*	18.62, *t*
11-Me	28.09, *q*	17.72, *q*	28.27, *q*	–	–	–
11-Me	*28.09, q*	*25.78, q*	*28.27, q*	–	–	–
7-OMe	–	55.82, *q*	–	–	–	–
12	–	–	–	32.67, *t*	32.66, *t*	27.99, *t*
13	–	–	–	43.64, *d*	43.17, *d*	35.89, *d*
14	–	–	–	39.08, *t*	38.77, *t*	27.94, *t*
15	–	–	–	48.53, *t*	48.49, *t*	47.07, *t*
16	–	–	–	154.71, *s*	155.04, *s*	151.91, *d*
17	–	–	–	103.59, *t*	103.26, *t*	104.90, *t*
18				65.10, *t*	64.88, *t*	63.82, *t*
19	–	–	–	70.57, *t*	66.98, *t*	66.91, *t*
20-MeOH	–	–	–	19.24, *q*	22.53, *q*	16.95, *q*

* Recorded at 75MHz (**1**, **2**, **3** and **7**) and 100 MHz (**5** and **6**), assignments were confirmed by 2D NMR experiments (HETCOR and COSY) and comparison with similar compounds as mentioned in the text.

spectral data showed 20 carbon signals confirming structure **2**. Their assignments were addressed herein for the first time on the basis of several NMR experiments (DEPT, COSY and HETCOR).

Compound **3** was identified as xanthyletin by comparison with an authentic sample (m.m.p., co-TLC and IR) and with literature data (m.p., IR, UV, MS, ^{1}H and ^{13}C NMR) [6, 7, 17, 18, 20].

Compound **5** was obtained as colorless needles, m.p. 166 °C ($CHCl_3$/MeOH). Its IR spectrum showed absorption bands for carbonyl and hydroxyl groups. The presence of a carbonyl group was supported by the ^{13}C NMR spectra, which exhibited 20 carbon signals, one of them of a ketone (δ 213.29, *s*). Positive ion CI-MS of **5** ($[M + 1]^+$ *m/z* at 319) was in accordance with the molecular formula $C_{20}H_{30}O_3$. These data suggested a diterpene with a ketonic function. The NMR spectra of **5** (Tables 1 and 2) showed signals characteristic of angular methyl (δ_H 1.04, 3 H, *s*, H-20; δ_c 19.24, *q*), exocyclic double bond (δ_H 4.79, 1 H, s, H-17$_D$, δ_H 4.73, 1 H, *br.s*, H-17$_u$; δ_c 103.59, *t*), a hydroxymethyl group (δ_H 3.71, 2 H, *s*, H-18; δ_c 65.10, *t*) and another hydroxymethyl (δ_H 3.82, 1 H, *d*, $J = 10.5$ Hz, H-19$_D$, δ_H 3.49, 1 H, *d*, $J = 10.5$ Hz, H-19$_D$; δ_c 70.57, *t*). All of these data are in accordance with the structure of a kaur-16-ene diterpene [21, 22]. This was confirmed by the ^{13}C NMR data as shown in Table 1. EI-MS of **5** showed a molecular ion *m/z* at 318 [M^+] and a fragmentation pattern similar to that of psiadin a kaurene diterpene isolated from *Psiadia* species [22]. Comparison of the data obtained with those reported for psiadin [22] confirmed structure **5**.

Compounds **6** and **7** were first obtained as a mixture in the form of long colorless needles, showing a single spot on TLC with several solvent systems. However, ^{1}H and ^{13}C NMR analysis revealed the presence of two compounds with very close structures. This suggested the presence of two isomers, which were separated by PHPLC.

Table 2: ^{1}H NMR Data* for diterpenes 5, 6 and 7 (in DMSO-d_6)

Proton	5	6	7
1$_D$	2.62, *d* ($J = 14.5$ Hz)	3.51, *dd* ($J = 11$, 9 Hz)	3.63, *dd* ($J = 11$, 4.5 Hz)
1$_U$	2.34, *d* ($J = 14.5$Hz)	3.41, *dd* ($J = 11$, 6 Hz)	3.51, *m*
2	–	4.50, *m*	4.36, *m*
3$_D$	2.53, *d* ($J = 14$ Hz)	2.0, *m*	2.0, *br.d* ($J = 16$ Hz)
3$_U$	1.93, *d* ($J = 14$ Hz)	1.60, *m*	1.5, *m*
5	2.64, *br.s.***	1.36, *m*	1.45, *m*
6$_D$	1.75, *m*	1.85, *m*	1.36, *m*
6$_U$	1.44, *m*	1.42, *m*	1.36, *m*
7 (2H)	1.56, *m*	1.36, *m*	1.41, *m*
9	1.30, *br.d* ($J = 7.3$ Hz)	1.09, *d* ($J = 8$ Hz)	1.08, *m*
11$_D$	1.62, *m*	1.51, *m*	1.78, *m*
11$_U$	1.49, *m*	1.51, *m*	1.78, *m*
12$_D$	1.62, *m*	1.46, *m*	1.28, *m*
12$_U$	1.50, *m*	1.46, *m*	1.28, *m*
13	2.64, *br.s.***	2.56, *br. s.*	2.19, *br.s.*
14$_D$	1.85, *d* ($J = 11.5$ Hz)	1.93, *m*	1.90, *m* 0.89, *m*
14$_U$	1.08, *m*	0.98, *dd* ($J = 11$,4.5 Hz)	
15	2.06, *br.s.*	1.99, *br.s.*	1.82, *m*
17$_D$	4.79, *s*	4.76, *br.s.*	4.70, *br.s*
17$_U$	4.73, *s*	4.69, *br.s.*	4.50, *br.s.*
18$_D$	3.71, *s*	4.43, *m*	4.35, *m*
18$_U$	3.71, s	4.26, *m*	4.35, *m*
19$_D$	3.82, *d* ($J = 10.5$ Hz)	4.25, *m*	4.03, *m*
19$_U$	3.49, *d* ($J = 10.5$ Hz)	4.05, *m*	4.03, *m*
20-CH$_3$	1.04, *s*	1.18, *s*	1.20, *s*

* Compound **5** run at 300 MHz, and **6** a**n**d **7** run at 400 MHz, using TMS as int. st. Assignments and multiplicity were determined by the aid of 2D NMR experiments (COSY and HETCOR)

** Signals in the sacme column are overlapped. $_U$ and $_D$ means up and down field, respectively

The ^{1}H and ^{13}C NMR data of compound **6** (Tables 1 and 2) suggested a structure similar to **5**. The exocyclic methylene group was deduced from the two broad singlets at δ 4,76 and 4.69 of the two protons at C-17 and the ^{13}C NMR signals at δ 103.26 (*t*, C-17) and 155.04 (*s*, C-16). Signals for the angular methyl and the two hydroxymethyl groups were also observed (Tables 1 and 2). The ketonic carbon signals (observed in **5** at δ 213.29, *q*) was absent, and replaced by an oxygenated methine carbon at δ 65.22, *d* (C-2) in **6**. The IR spectrum confirmed the absence of a C=O group. These data together with the EIMS, which showed a molecular ion [M^+] *m/z* at 320 (two mass units more than that of **5**), suggested that **6** is the 2-hydroxy derivative of **5**. It remained then to decide whether the OH at C-2 is axial or equatorial one. The ^{1}H NMR spectrum of **6** exhibited a pair of double doublets assigned for the H-1 protons at δ 3.51 (*dd*, $J = 11$ and 9 Hz, H-1_{ax}) and δ 3.41 (*dd*, $J = 11$ and 6 Hz, H-1_{eq}). The *J* values of these protons revealed that the OH group at C-2 should be equatorial [23] and hence structure **6** is concluded. Confirmation was achieved through comparison with previously reported data [23] for diterpene **6**.

The MS and NMR data (Tables 1 and 2) of **7** were similar to those of **6**, and suggested an isomer. The *J* values of the H-1 protons at δ 3.63 (*dd*, $J = 4.5$ and 11 Hz, H-1_{ax}) and δ 3.51 (m, H-1_{eq}) indicated that **7** is an epimer of **6** having an axial hydroxyl group at C-2. Comparing the obtained data for **7** with those previously reported for the diterpene **7** [23] confirmed its structure.

Previous biological studies on coumarins proved that sesselin **1** and xanthyletin **3** were potential anticancer agents [14]. The angular pyranocoumarin **1** functions as a DNA-damaging agent, while the linear pyranocoumarin **3** proved to produce cytotoxicity, due to other mechanisms than DNA-damage [14]. Xanthyletin have been isolated in substantial amounts in this study, providing a rich source of this potential anticancer agent for more studies regarding the mechanism of action and structure activity relationships of pyranocoumarins.

On the other hand, kaurane diterpenes having a carbon skeleton close to the isolated diterpenes **5**–**7** were reported to have antibacterial, antihepatotoxic, uterotonic [10, 15] and anti-HIV activities [15]. For example, kaurenoic and grangiflorenic acids are kaurane diterpenes that were isolated from *Aspilia* and *Montanoa* species. These species are employed as remedies for conditions or disorders particular to women (as galactagogue, to alleviate menstrual cramps, to aid childbirth and as abortifacient and inducers of labour). Furthermore, these two diterpenes have exhibited potent stimulation of uterine contraction [15].

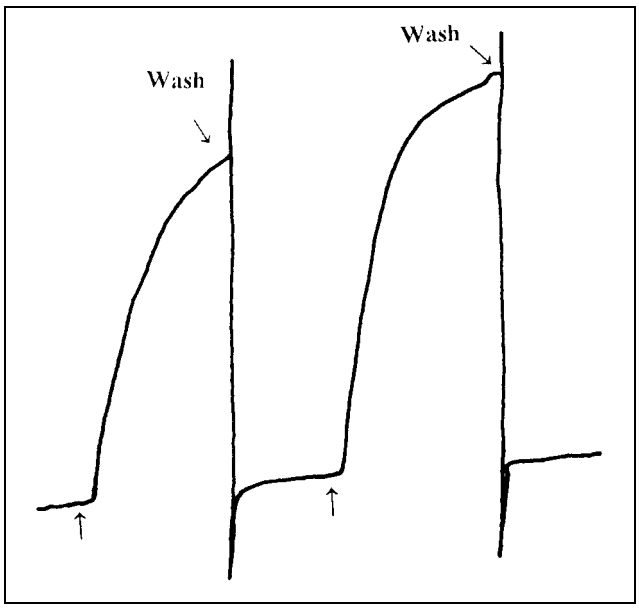


Fig. 1: Response of oestrogenized rat uterine strip to a solution of diterpene **5** at organ bath concentration of: **A**, 40 μl ml^{-1} and **B**, 80 μg ml^{-1}. Arrows indicate introduction of tested compound

Therefore, compound **5**, which is the major diterpene isolated from *F. margarita* in the present study, has been tested *in vitro* for uterotonic effects using female rat uterus. It exhibited uterostimulatory activity at two concentrations (40 and 80 μg/ml), this effect was concentration dependant (Fig. 1). The effect of **5** on rabbit jejunum was also tested and the results indicated stimulation of the contractile activity. The increase in activity was in the amplitude only, but the frequency and the tone of the muscle were not affected.

In addition, the effect of i.v. injection of **5** (1 mg/kg) on the spontaneous EEG pattern and evoked potentials of unanaethetized rabbits was studied. Diterpene **5** caused an increase in the rapid waves (α and β), decrease in slow waves (theta and delta), and increase in the total count (Fig. 2). It also caused reduction in the mean voltage. The

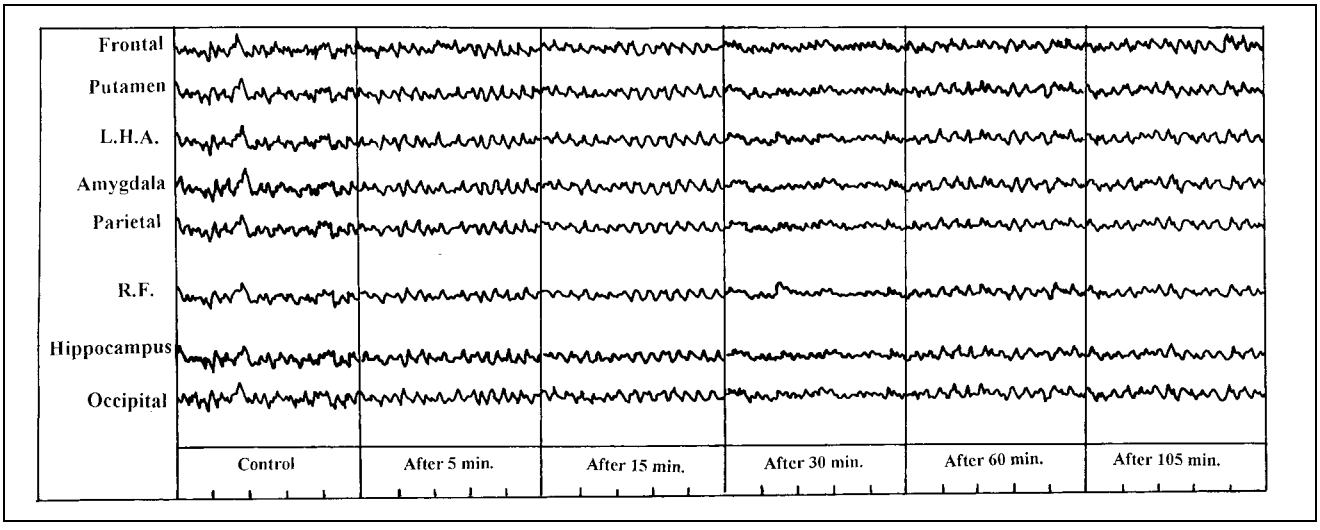


Fig. 2: Spontaneous EEG pattern of conscious rabbits following the intravenous administration of diterpene **5**. L.H.A.: Lateral hypothalamic area; R.F.: Reticular formation

evoked potentials (sonic and photic stimulation) showed a long period of after effect in comparison with control values. This *in vivo* study showed that **5** caused a mild stimulatory effect on the brain activity. This effect may be attributed to an improvement of cerebral circulation rather than elevation of blood pressure.

3. Experimental

3.1. General

IR spectra were recorded in KBr tablets on a PU-9706 IR spectrophotometer (Philips, England). MS were taken with a JMS-700 spectrometer (JEOL, Japan) with a direct inlet system, isobutane was used for ionization in case of PICI-MS. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AM-300 spectrometer using TMS as internal standard, a series of experiments (DEPT, 2D ^{1}H−^{1}H COSY and ^{1}H−^{13}C HETCOR) were performed, to aid structure elucidation. Silica gel 60 (Merck) for CC and precoated TLC plates (Merck) were used. The Waters HPLC machine for PHPLC consisted of a Waters 515 pump, a 2487 dual λ absorbance detector set at 220 nm, a Waters gradient controller and Eurospher-100 C18, 10 μm column (250 × 8 mm).

3.2. Plant material

The roots of *F. margarita* were collected from The Experimental Farm at Moshotohor, Faculty of Agriculture, Zagazig University, Benha branch, in April 2000. Identification was confirmed by Dr. M. G. A. Mogheith, Prof. of Pomology, Department of Horticulture, Faculty of Agriculture, Zagazig University, Benha branch. A voucher specimen is deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.

3.3. Isolation of coumarins and diterpenes

The dry powdered root (6 kg) was extracted twice with acetone (12 l, each) at room temperature. The solvent was evaporated off *in vacuo* and the residue was dissolved in the least amount of EtOH (about 50 ml) and the volume is completed to 1 l with distilled H_2O. The obtained solution was extracted with light petroleum and then with $CHCl_3$.

The light petroleum fraction (103 g) was chromatographed on a column (100 × 6 cm) of silica gel, using increasing percentages of acetone in benzene as eluting solvents, to give three main fractions I–III. Fraction I (eluted with benzene-acetone, 98 : 2.0) gave a mixture of **1** and **2**. Rechromatography on a column (3 × 60 cm) of silica gel, using light petroleum-ethyl acetate (80 : 20) as a solvent in an isocratic mode, gave **1** (650 mg), followed by **2** (180 mg). Fraction II (eluted with benzene-acetone, 96 : 4.0) was freed from the solvents and the residue was washed several times with ethyl acetate and crystallized from a mixture of $CHCl_3$−MeOH to give **3** (6.8 g). Fraction III provided 45 mg of **4** (β-sitosterol).

The chloroform fraction (71 g) was chromatographed on a column (90 × 6 cm) of silica gel, using $CHCl_3$, then mixtures of $CHCl_3$ and MeOH. Fractions eluted with $CHCl_3$−MeOH (96 : 4.0), provided **5** (220 mg). Fractions eluted with $CHCl_3$−MeOH (94 : 6.0), yielded 90 mg of a crystalline material (showing a single spot on silica gel TLC using different solvent systems (R_f 0.56, $CHCl_3$−MeOH, 88 : 12). However. ^{1}H- and ^{13}C NMR analysis revealed two closely related compounds (isomers). They were separated by PHPLC using C_{18} column (250 × 8 mm) and H_2O-MeCN (1 : 1) as a mobile phase at a flow rate of 3.5 ml/min and a UV-detector at 215 nm, to give **6** (22 mg, R_t 11.39 min) followed by **7** (39 mg, R_t 11.98 min). Fractions eluted with $CHCl_3$−MeOH (92 : 8.0) gave 28 mg of **8** (β-sitosterol-o-glucoside).

3.4. Compound 1

Colorless needles, m.p. 119 °C ($CHCl_3$/MeOH). IR ν_{max} (KBr) cm^{-1}: 1710, 1640, and 1590. UV λ_{max} nm (MeOH): 286 (sh), 290, 330 345 (sh). ^{1}H NMR ($CDCl_3$) δ: 7.57 (1 H, *d*, *J* = 9.5 Hz, H-4), 7.18 (1 H, *d*, *J* = 8.5 Hz, H-5), 6.86 (1 H, *d*, *J* = 10 Hz, H-9), 6.69 (1 H, *d*, *J* = 8.5 Hz, H-6), 6.20 (1 H, *d*, *J* = 9.5, H-3), 5.70 (1 H,*d*, *J* = 10 Hz, H-10), 1.45 (6 H, *s*, 11-[CH_3]$_2$). EI-MS *m/z* (rel. ab. %): 228 [M]$^+$ (15), 213 [M-15]$^+$ (100), 185(19), 128 (8), 127 (3), 92 (7). ^{13}C NMR: Table 1.

3.5. Compound 2

Colorless needles, m.p. 90–91 °C ($CHCl_3$/MeOH). IR ν_{max} (KBr) cm^{-1}: 1710, 1620, and 1610. UV λ_{max} nm (MeOH): 245 (sh), 295 (sh), and 330. ^{1}H NMR ($CDCl_3$) δ: 7.59 (1 H, *d*, *J* = 9.5 Hz, H-4), 7.15 (1 H, *s*, H-5), 6.75 (1 H, *s*, H-8), 6.20 (1 H, *d*, *J* = 9.5 Hz, H-3), 5.26 (1 H, *m,* H-10), 3.87 (3 H, *s*, 7-OCH_3), 3.28 (2 H, *d*, *J* = 7.3 Hz, H-9), 1.74 (3 H, *s*, 11-CH_3), 1.68 (3 H, *s*, 11-CH_3). EI-MS *m/z* (rel. ab. %): 244 [M]$^+$ (68), 229 [M-15]$^+$ (100), 201(7), 189 (14), 175 (7), 176 (7), 159 (9), 141(4), 131(7), 115(7), 89(4), 77(8). ^{13}C NMR: Table 1.

3.6. Compound 3

Colorless needles, m.p. 129 °C ($CHCl_3$/MeOH). IR ν_{max} (KBr) cm^{-1}: 1720, 1620. UV λ_{max} nm (MeOH): 265, 304(sh), 348. ^{1}H NMR ($CDCl_3$) δ: 7.52 (1 H, *d*, *J* = 9.5 Hz, H-4), 6.99 (1 H, *s,* H-5), 6.66 (1 H, *s,* H-8), 6.29 (1 H, *d*, *J* = 10 Hz, H-9), 6.15 (1 H, *d*, *J* = 9.5, H-3), 5.64 (1 H, *d*, *J* = 10 Hz, H-10), 1.47 (6 H, *s*, 11-[CH_3]$_2$). EI-MS *m/z* (rel. ab. %): 228 [M]$^+$ (16), 213 [M-15]$^+$ (100), 185 (26), 128 (10), 93 (21). ^{13}C NMR: Table 1.

3.7. Compound 5

Colorless needles, m.p. 166 °C ($CHCl_3$/MeOH). IR ν_{max} (KBr) cm^{-1}: 3350, 1680, and 1630. EI-MS *m/z* (rel. ab. %): 318 [M]$^+$ (31.3), 303 (24.8), 287(9), 257(18.3), 227 (30), 91 (100). PICI-MS *m/z* (rel. ab. %): 319 [M+1]$^+$ (100), 301 [M+1-H_2O]$^+$ (55).

3.8. Compound 6

Colorless needles, m.p. 236–238 °C ($CHCl_3$/MeOH). IR ν_{max} (KBr) cm^{-1}: 3350, 1650. EI-MS *m/z* (rel. ab. %): 320 [M]$^+$ (1.0), 302 (12), 284 (52), 154 (23), 253 (29), 91 (100).

3.9. Compound 7

Colorless needles, m.p. 236–238 °C ($CHCl_3$/MeOH). IR ν_{max} (KBr) cm^{-1}: 3350, 1650. EI-MS *m/z* (rel. ab. %): 320 [M]$^+$ (8.0), 302 (30), 284 (100), 271 (42), 253 (38).

3.10. Pharmacological testing of diterpene 5

3.10.1. Effect on the rat uterus

Adult female rats (150–180 g) received 1.0 mg/kg diethylstilbestrol for two days. Vaginal smear was done to make sure that rats were in estrus stage. Rats were killed by cervical dislocation and the abdomen was opened and both uterine horns were removed, and placed in a Petri dish containing De-Jalon's solution [24]. Each horn was cut longitudinally to be like a sheet of muscle and then divided again longitudinally. A piece of the muscle was mounted in a 25 ml organ bath containing warm (30 °C) aerated De-Jalon's solution. Uterine contractions were recorded on a smoked drum kymograph with very slow speed (Bioscience-England). The tested compound was tested at two dose levels 40 and 80 μg/ml using propylene glycol to assist solubility, a solvent control was considered. The solvent control was inactive.

3.10.2. Effect on the rabbit intestine

White albino rabbits (1.5–2 kg) were used. Segments (2 cm each) of the jejunum were isolated and mounted in a 25-ml organ bath containing warm (37 °C) standard Tyrode solution [24]. Intestinal contractions were measured at organ bath concentrations of 8 and 80 μg/ml of diterpene **5** using the above-described kymograph.

3.10.3. Effect on the electrocephalogram of conscious rabbits [25]

Central action of diterpene **5** was tested on unanaesthetized rabbits. Procaine adrenaline was infiltrated under the skin covering the scalp. The skin and the underlying layer were incised longitudinally. The surface was thoroughly cleaned with 10% H_2O_2. The sites of cortical and subcortical areas under investigation were determined stereotoxically. After implantation and fixation of electrodes, rabbits were allowed to recover for 7 days, and treated with procaine penicillin to prevent postoperative sepsis. Compound **5** was administered i.v. (1 mg/kg). An ink-writing electroencephalogram (Neuroscience-England) was used to record electrical activity of the brain. Control spontaneous EEG patterns were recorded before and after administration of the tested compound. The evoked potentials were studied by subjecting rabbits to photic and sonic stimulations.

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