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Neurotrophic and Antileukemic Daphnane Diterpenoids from *Synaptolepis kirkii*

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Abstract—Biological assay guided fractionation of a dichloromethane extract of *Synaptolepis kirkii* led to the isolation of four new and five known daphnane-type diterpene orthoesters, whose structure was established by spectroscopic data. Full spectroscopic data of the new and known natural products are reported here for the first time. Pronounced neurotrophic and substantial antileukaemia activities of these compounds were found in in vitro assays.

Introduction

Synaptolepis kirkii Oliv. (Thymelaeaceae) is a traditional medicinal plant endemic to Southeast Kenya and Northeast of Tanzania.[1,2] In Kenya, *S. kirkii* is locally known as Lama (Boni), Mgirambira (Giriama), Mkatu (Swahili), and the roots of the plant are used against snakebite and for the management of epilepsy.[1–3] Plants from the genus *Synaptolepsis* are prolific producers of structurally complex and highly irritant daphnane diterpenoids, some of which show tumor-promoting activity.[4–6] As part of an investigation of African medicinal plants for the discovery of new bioactive compounds, powerful neurotrophic and antileukemic activity in extracts from the roots of *S. kirkii*, was observed. A report is given here on the identification of the compounds responsible for these properties.

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Results and Discussion

A dichloromethane extract of the roots of *S. kirkii* was fractionated by several chromatographic techniques, including preparative high and medium pressure liquid chromatography (HPLC or MPLC), centrifugal partition chromatography (CPC), and thin layer chromatography (TLC) in normal or reversed phase, eventually leading to the isolation of nine bioactive compounds (**1**–**9**).

Daphnetoxin-type diterpene orthoesters

Compound **1** was isolated as the major active component of the root extracts from *S. kirkii* as regarding both the neurotrophic and the antileukaemia activities, and was identified as the synaptolepsis factor K_7 on the basis of its spectroscopic properties. Since the spectroscopic data reported in the literature are incomplete, a full characterization was carried out using modern 2D NMR techniques. A selection of the ^{1}H–^{13}C long range correlation underlying the full assignment of the NMR spectra is depicted in Figure 1. A computer-generated 3D modeling showed that the five-membered ring

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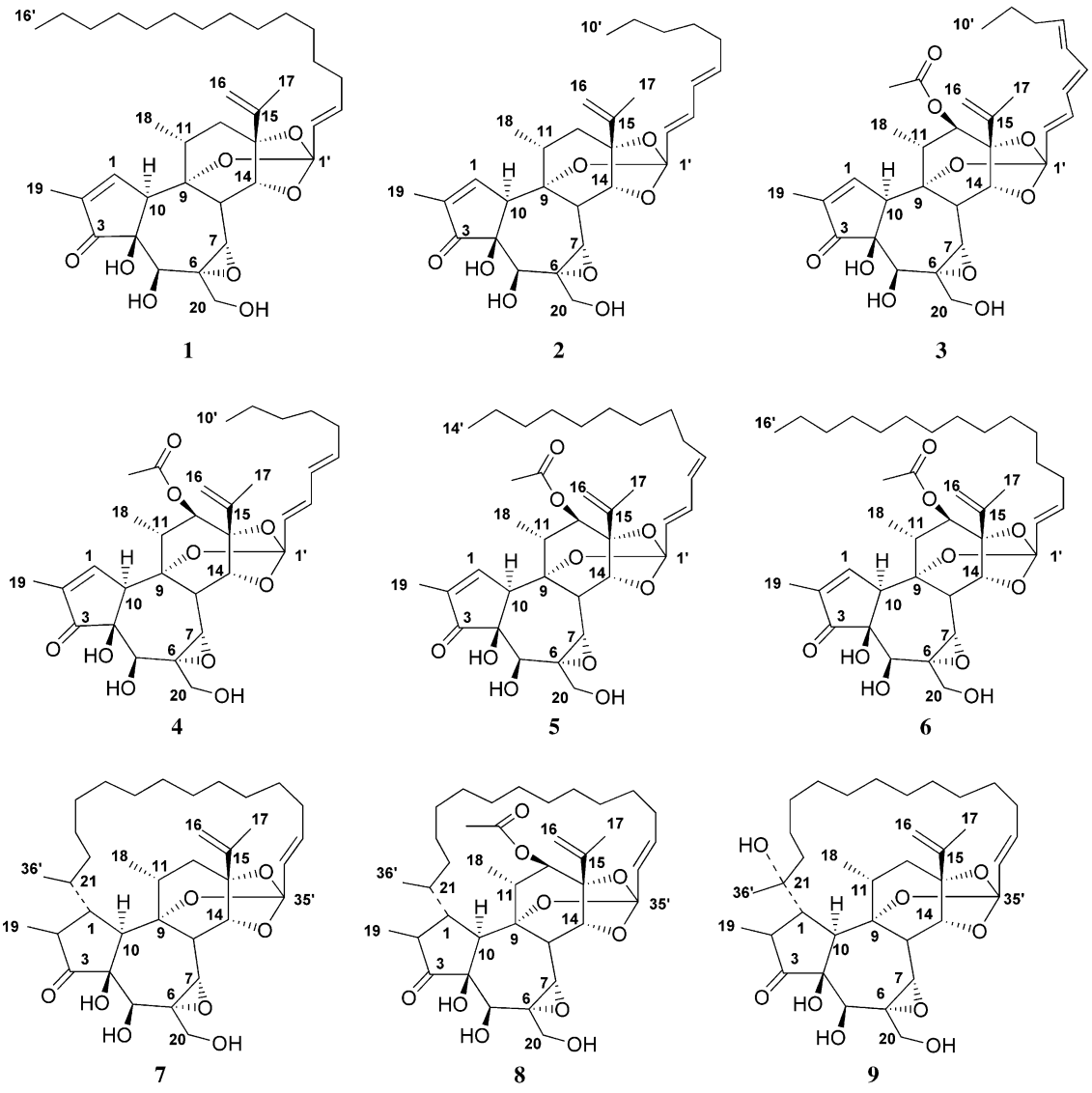

adopts an envelope conformation, while the seven-membered ring is a chair, and the six-membered ring is locked in a boat conformation by the orthoester moiety (see Fig. 2). The orthogonal relationship between H-7 and H-8 easily rationalizes the lack of coupling between these protons.

The spectroscopic assignment of compound **1** furnished a basis for the spectroscopic elucidation of related daphnanes isolated from the extracts. Thus, the spectra of **2** differed from those of **1** only for the presence of a further unsaturation on the alkyl moiety of the orthoester group, expressed as a conjugated *E,E*-dienic system. Compound **2** was therefore identified as excoecariatoxin (**2**), a strong piscicidal agent obtained from both Euphorbiaceous and Thymelaeaceous plants.[7] The spectroscopic data reported in Table 1 correct several wrong assignments originally reported for the ^{13}C NMR spectrum of this compound.[8]

12-Hydroxydaphnetoxin-type diterpene orthoesters

The spectroscopic data of the daphnanes **3**–**5** were very close to those of kirkinine (**6**),[9] their common hallmark being the presence of an acetate on the diterpenoid core (IR absorption bands at ca.1740 cm^{-1}, NMR signals at δ_C 170 ppm and δ_H 1.99). The ^1H NMR spectrum of **3** showed six coupled olefinic protons, suggesting the presence of a conjugated triene system adjacent to the orthoester carbon, the all *E*-configuration being evident from the olefinic couplings. This compound, which was now named kirkinine D, might be identical to the synaptolepis factor K_3[5] and the *Peddiea* factor V_2 from *Peddiea volkensii* (Thymelaeaceae),[10] whose configuration at the triene system was incompletely reported. Compounds **4** and **5** displayed a *E,E*-conjugated diene system bound to the orthoester carbons, and were identified, respectively, as yuanhuadine, a powerful abortificient constituent of the Chinese medicinal plant Yuan

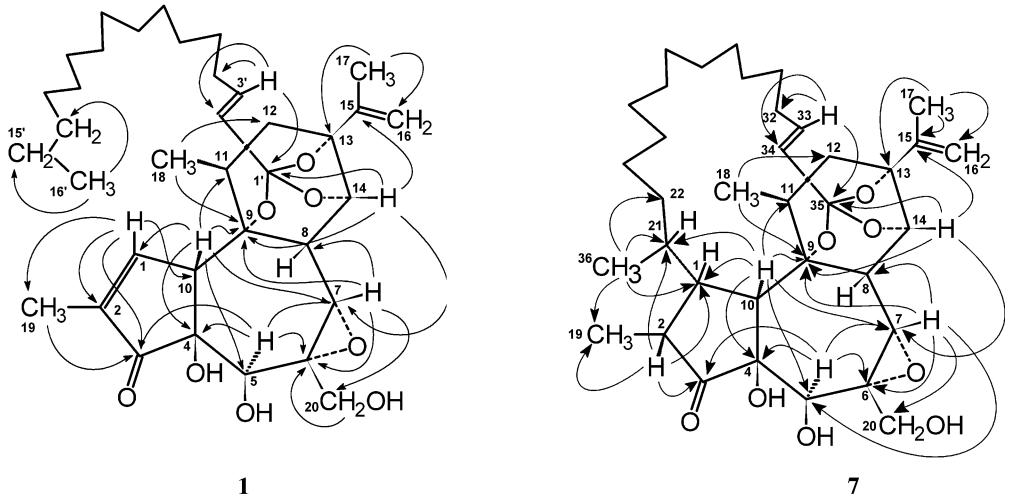

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Figure 1. Selective HMBC of synaptolepis factor K_7 (**1**) and kirkinine B (**7**).

Hua (*Gnidia genkwa*),[11,12] and 12β-acetoxyhuratoxin, previously isolated in several Thymelaeaceous plants.[7] Complete spectral data for these compounds were not available, and are presented in Tables 1–3.

1-Alkyldaphnane-type orthoesters

Compound **7** was a new daphnane, and was named kirkinine B. HRMS suggested a molecular formula $C_{36}H_{54}O_8$ (calcd 614.3818, found 614.3791), showing that compounds **7** and **1** are isomers. The NMR spectra evidenced the saturation of the 1,2-double bond and the presence of a substituent at C-1. These, the lack of the terminal methyl triplet of the alkyl moiety bound to the orthoester carbon, and the downfield shift of the C-3 carbonyl, all strongly suggested that **7** is a 1-alkyldaphnane.

The high value (13 Hz) of $J_{1,10\alpha}$, highly diagnostic of a *trans*-diaxial arrangement, established the configuration at C-1,[13] while the lack of coupling between H-1 and the adjacent proton on the macro bridge (H-21, δ 2.43 ppm) showed an orthogonal relationship between these protons, and suggested an α-configuration for the methyl (36-methyl) at C-21. Inspection of models shows that this arrangement is also the most stable one, since it minimizes the interaction of 36-methyl and those on the diterpenoid core at C-11 and C-2. An *E*-double bond ($J_{33,34}=16$ Hz) was present on the aliphatic moiety, adjacent to the orthoester carbon. Compound **7** is thus related to the synaptolepis factor K_1, whose configuration at the 33,34-double bond was, however, not assessed.[4–6]

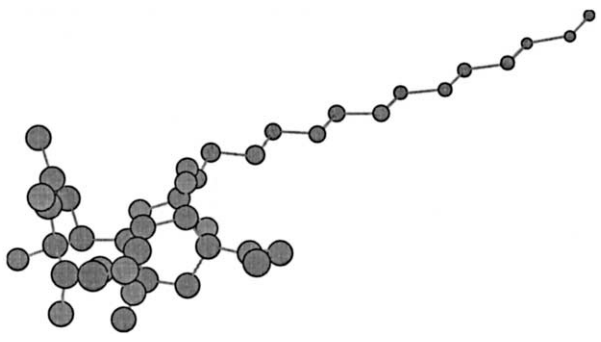

Figure 2. Computer-generated 3D modeling of compound **1**.

HRMS of compound **8** established the same molecular formula ($C_{38}H_{56}O_{10}$) of kirkinine (**6**), and the NMR differences between the two compounds are those expected for a pair of daphnane and 1-alkyldaphnane, as discussed for the structure elucidation of **7**. Thus, compound **8** is the 12-acetoxyderivative of **7**, and it was named now kirkinine C.

Compound **9** showed NMR spectra similar to those of **7**, the main difference being the presence of an extra oxygen atom (MS), a change in the multiplicity of the C-21 methyl, now a singlet at δ 1.33, and a simplification in the multiplicity of H-22a,b in the ^{1}H NMR spectrum. This, and the replacement of the C-21 methine with a quaternary carbon (δ 80.4, s) in the ^{13}C NMR spectra. These observations could be rationalized by a structure where C-21 is hydroxylated, and this new compound was named kirkinine E.

Neurotrophic activity

Neurotrophic factors (NTFs) are endogenous proteins [e.g., Nerve Growth Factor (NGF), Ciliary Neurotrophic Factor (CNTF)] that regulate the development, maintenance and survival of neurons. Recent evidence suggests that NTFs may also be implicated in the normal functional activity of nerve cells and may play a role in processes of neuronal plasticity. Therefore, neurotrophic factors or compounds acting as neurotrophic factors can protect and rescue certain neuronal populations of various neurodegenerative diseases, that is Alzheimer's and Parkinson's diseases.[14,15] Synaptolepis factor K_7 (**1**), kirkinine (**6**) and kirkinine B (**7**) have shown an ultra potential activity in this test, where compounds **1** and **7** have an EC_{50} of 8.8×10^{-9} and 4.5×10^{-8} M (Fig. 3), respectively. The other compounds were relatively less active compared to the above three diterpenes, probably because of the shorter aliphatic chain or more unsaturation (comparison of compound **6** with compounds **2**, **3**, **4**, and **5**). A commercially

Table 1. ^{13}C NMR data of compounds **1**–**9**[a]

C	1	2	3	4	5	6	C	7	8	9
1	161.3	161.2	161.1	160.4	160.5	160.5		49.7	49.7	50.3
2	136.7	136.6	137.7	136.9	136.9	136.8		42.8	42.8	41.3
3	209.8	209.8	210.2	209.5	209.5	209.5		220.4	220.3	220.8
4	72.3	72.2	72.9	72.2	72.2	72.2		75.5	75.4	74.7
5	72.0	72.0	72.8	71.9	72.1	72.0		71.5	71.5	70.8
6	60.4	60.3	61.2	60.5	60.5	60.4		60.6	60.7	60.9
7	64.2	65.0	65.0	64.2	64.3	64.2		64.1	64.3	64.4
8	36.7	36.6	36.2	35.3	35.4	35.3		36.9	35.7	37.0
9	79.4	79.5	78.9	78.1	78.1	78.2		81.6	80.3	81.6
10	48.1	48.1	48.2	47.4	47.5	47.4		44.2	44.0	31.2
11	34.9	34.8	44.7	44.0	44.0	44.0		35.5	43.6	35.6
12	36.4	36.4	79.0	78.2	78.2	78.8		36.9	78.8	35.7
13	84.3	84.4	84.5	83.6	83.7	83.5		83.7	82.8	83.6
14	82.0	81.9	81.2	80.4	80.4	80.4		82.6	80.9	82.5
15	146.2	146.0	143.8	143.1	143.1	143.1		146.4	143.3	146.3
16	111.3	111.3	114.1	113.3	113.4	113.3		111.2	113.2	111.3
17	19.0	18.9	19.4	18.7	18.3	18.7		19.0	18.7	19.0
18	20.4	20.3	19.0	18.2	18.7	18.2		21.5	19.2	22.2
19	9.9	9.9	10.6	9.9	9.9	9.9		14.1	14.0	14.7
20	65.1	65.5	65.8	65.1	65.1	65.1		65.0	65.1	65.4
1′	116.3	116.4	117.7	117.0	117.0	116.7	35	116.0	116.0	116.3
2′	123.3	122.7	124.2	122.2	122.2	122.8	34	124.5	124.0	124.4
3′	136.7	134.7	135.6	135.1	135.1	137.2	33	136.9	137.3	136.5
4′	31.9	128.7	129.1	128.5	139.4	31.9	32	31.8	31.7	31.8
5′	28.4	138.9	137.6	139.4	128.6	28.2	31	26.9[b]	26.9[b]	26.5[b]
6′	29.7[b]	32.6	130.9	32.6	32.7	29.7	30	27.1[b]	27.1[b]	27.0[b]
7′	29.7[b]	29.1	138.0	28.7	29.6[b]	29.7	29	27.3[b]	27.3[b]	27.1[b]
8′	29.7[b]	31.8	35.6	31.3	29.5[b]	29.6	28	27.9[b]	27.9[b]	27.3[b]
9′	29.7[b]	22.6	23.0	22.5	29.3[b]	29.6	27	28.0[b]	27.9[b]	27.3[b]
10′	29.6[b]	14.1	14.4	14.0	29.2[b]	29.5	26	28.2[b]	28.1[b]	27.4[b]
11′	29.5[b]				29.1[b]	29.4	25	28.3[b]	28.3[b]	27.8[b]
12′	29.4[b]				31.9	29.3	24	28.4[b]	28.3[b]	27.9[b]
13′	29.3[b]				22.7	29.3	23	29.7[b]	29.7[b]	28.3[b]
14′	31.9				14.1	31.8	22	38.0	37.8	37.4
15′	22.7					22.7	21	32.0	32.0	80.4
16′	14.1					14.1	36	12.4	12.4	22.8
CH_3CO			170.4	169.7	169.7	169.7			169.8	
$\mathbf{C}H_3CO$			21.9	21.1	21.2	21.1			21.2	

[a]All values, given in ppm downfield from TMS, were determined in $CDCl_3$ at 67.5 (compounds **1**, **3**, **5**, **7** and **9**) or 100 MHz (compounds **2**, **4**, **6** and **8**).
[b]Signal-couples may be internally inverted.

Table 2. ^{1}H NMR data of kirkinine D (**3**), compounds **2** and **4**[a]

H	2	3	4
1	7.65 (1H, *q*, $J_{1,19}=1.31$, $J_{1,10}=2.46$ Hz)	7.58 (1H, *q*, $J_{1,19}=1.41$, $J_{1,10}=2.56$ Hz)	7.58 (1H, *q*, $J_{1,19}=1.28$, $J_{1,10}=2.29$ Hz)
5	4.26 (1H, *s*)	4.26 (1H, *s*)	4.26 (1H, *s*)
7	3.46 (1H, *s*)	3.55 (1H, *s*)	3.55 (1H, *s*)
8	2.95 (1H, *d*, $J_{8,14}=2.54$ Hz)	3.51 (1H, *d*, $J_{8,14}=2.64$ Hz)	3.51 (1H, *d*, $J_{8,14}=2.43$ Hz)
10	3.81 (1H, *d*, $J_{10,1}=2.46$ Hz)	3.82 (1H, *d*, $J_{10,1}=2.56$ Hz)	3.82 (1H, *d*, $J_{10,1}=2.31$ Hz)
11	2.48 (1H, *m*)	2.37 (1H, *d*, $J_{11,18}=7.26$ Hz)	2.37 (1H, *d*, $J_{11,18}=8.14$ Hz)
12a	1.69 (1H, *d*, $J_{12a,12b}=14.30$ Hz)	4.98 (1H, *s*)	4.98 (1H, *s*)
12b	2.24 (1H, *dd*, $J_{12b,11}=8.72$, $J_{12b,12a}=14.30$ Hz)		
14	4.43 (1H, *d*, $J_{14,8}=2.54$ Hz)	4.76(1H, *d*, $J_{14,8}=2.64$ Hz)	4.76(1H, *d*, $J_{14,8}=2.43$ Hz)
16	5.03 (1H, *s*), 4.91 (1H, *d*, $J_{16,17}=2.14$ Hz)	5.02 (1H, *d*, $J=0.66$ Hz), 4.95 (1H,s)	5.01, 4.95 (2H, *ss*)
17	1.80 (3H, *d*, $J_{17,16}=2.14$ Hz)	1.83 (3H, *d*, $J=0.66$ Hz)	1.83 (3H, *s*)
18	1.19 (3H, *d*, $J_{18,11}=7.11$ Hz)	1.29 (3H, *d*, $J_{18,11}=7.25$ Hz)	1.37 (3H, *d*, $J_{18,11}=8.14$ Hz)
19	1.81 (3H, *d*, $J_{19,1}=1.31$ Hz)	1.79 (3H, *d*, $J_{19,1}=1.41$ Hz)	1.79 (3H, *d*, $J_{19,1}=1.28$ Hz)
20	3.83 ± 0.02 (2H, $J_{AB}=12.47$ Hz)	3.85 (2H, *m*)	3.87 (2H, $J_{AB}=12.44$ Hz)
2′	5.71 (1H, *d*, $J_{2',3'}=15.38$ Hz)	5.71 (1H, *d*, $J_{2',3'}=15.26$ Hz)	5.64 (1H, *d*, $J_{2',3'}=15.37$ Hz)
3′	6.71 (1H, *dd*, $J_{3',2'}=15.38$, $J_{3',4'}=10.66$ Hz)	6.70 (1H, *dd*, $J_{3',2'}=15.26$, $J_{3',4'}=10.89$ Hz)	6.66 (1H, *dd*, $J_{3',2'}=15.37$, $J_{3',4'}=10.61$ Hz)
4′	6.06 (1H, *dd*, $J_{4',3'}=10.66$, $J_{4',5'}=15.19$ Hz)	6.12 (1H, *dd*, $J_{4',3'}=10.89$, $J_{4',5'}=14.93$ Hz)	6.06 (1H, *dd*, $J_{4',3'}=10.61$, $J_{4',5'}=15.37$ Hz)
5′	5.70 (1H, *p*, $J_{5',4'}=15.19$, $J_{5',6'}=7.55$, 7.05 Hz)	6.33 (1H, *dd*, $J_{5',4'}=14.93$, $J_{5',6'}=10.57$ Hz)	5.85 (1H, *dt*, $J_{5',4'}=15.36$, $J_{5',6'}=7.21$ Hz)
6′	2.09 (2H, *m*)	6.08 (1H, *dd*, $J_{6',5'}=10.57$, $J_{6',7'}=14.83$ Hz)	2.01 (2H, *q*, $J=7.13$ Hz)
7′	1.39 (2H, *m*)	5.75 (1H, *dt*, $J_{7',6'}=14.83$, $J_{7',8'}=7.59$ Hz)	1.28 (2H, *m*)
8′	1.27 (2H, *m*)	2.09 (2H, *dt*, $J_{8',7'}=7.59$, $J_{8',9'}=7.09$ Hz)	1.62 (2H, *m*)
9′	1.27 (2H, *m*)	1.42 (2H, *m*)	1.28 (2H, *m*)
10′	0.88 (3H, *t*, $J_{10',9'}=6.81$ Hz)	0.90 (3H, *t*, $J_{10',9'}=7.43$ Hz)	0.88 (3H, *t*, $J_{10',9'}=6.77$ Hz)
$\mathbf{C}H_3COO$		1.99 (3H, *s*)	1.99 (3H, *s*)

[a]All values, given in ppm downfield from TMS, were determined in $CDCl_3$ at 270 (compounds **3** and **4**) or 400 MHz (compound **2**).

Table 3. ^{1}H NMR data of synaptolepis factor K_7 (**1**), kirkinine (**6**) and 12β-acetoxyhuratoxin (**5**)[a]

H	1	5	6
1	7.66 (1H, *dd*, $J_{1,19}=1.32$, $J_{1,10}=2.31$ Hz)	7.58 (1H, *t*, $J_{1,19}=0.99$, $J_{1,10}=2.31$ Hz)	7.58 (1H, *q*, $J_{1,19}=1.79$, $J_{1,10}=2.38$ Hz)
5	4.28 (1H, *d*, $J_{5,OH}=1.98$ Hz)	4.26 (1H, *s*)	4.26 (1H, *s*)
7	3.47 (1H, *s*)	3.55 (1H, *s*)	3.55 (1H, *s*)
8	2.96 (1H, *d*, $J_{8,14}=2.64$ Hz)	3.51 (1H, *d*, $J_{8,14}=2.64$ Hz)	3.51 (1H, *d*, $J_{8,14}=2.56$ Hz)
10	3.82 (1H, *d*, $J_{10,1}=2.31$ Hz)	3.82 (1H, *d*, $J_{10,1}=2.31$ Hz)	3.82 (1H, *t*, $J=2.93$ Hz)
11	2.50 (1H, *p*, $J_{11,18}=7.75$ Hz)	2.37 (1H, *q*, $J_{11,18}=7.25$ Hz)	2.37 (1H, *q*, $J_{11,18}=7.32$ Hz)
12a	1.70 (1H, *d*, $J_{AB}=14.19$ Hz)	4.98 (1H, *s*)	4.97 (1H, *s*)
12b	2.26 (1H, *m*)		
14	4.43 (1H, *d*, $J_{14,8}=2.64$ Hz)	4.76(1H, *d*, $J_{14,8}=2.64$ Hz)	4.74(1H, *d*, $J_{14,8}=2.56$ Hz)
16	5.04, 4.93 (2H, *st*, $J_{16,17}=1.32$ Hz)	5.01, 4.95 (2H, *ss*)	5.01, 4.95 (2H, *ss*)
17	1.83 (3H, *d*, $J_{17,16}=1.32$ Hz)	1.83 (3H, *s*)	1.83 (3H, *s*)
18	1.20 (3H, *d*, $J_{18,11}=7.26$ Hz)	1.29 (3H, *d*, $J_{18,11}=7.25$ Hz)	1.29 (3H, *d*, $J_{18,11}=7.32$ Hz)
19	1.82 (3H, *d*, $J_{19,1}=1.32$ Hz)	1.76 (3H, *d*, $J_{19,1}=0.99$ Hz)	1.79 (3H, *q*, $J=1.46$, 1.10 Hz)
20	3.85 (2H, $J_{AB}=12.21$ Hz)	3.87 (2H, $J_{AB}=10.23$ Hz)	3.87 (2H, *dd*, $J_{AB}=12.62$ Hz)
2′	5.69 (1H, *tt*, $J_{2',3'}=15.61$, $J_{2',4'}=1.49$ Hz)	5.64 (1H, *d*, $J_{2',3'}=15.51$ Hz)	5.61 (1H, *d*, $J_{2',3'}=15.55$ Hz)
3′	6.33 (1H, *tt*, $J_{3',2'}=15.50$, $J_{3',4'}=6.60$ Hz)	6.41 (1H, *q*, $J_{3',2'}=15.50$, $J_{3',4'}=10.56$ Hz)	6.27 (1H, *dt*, $J_{3',2'}=15.55$, $J_{3',4'}=6.95$, 6.59 Hz)
4′	2.12 (2H, *m*)	6.05 (1H, *h*, $J_{4',5'}=15.08$, $J_{4',3'}=10.55$ Hz)	2.07 (2H, *h*, $J=6.64$ Hz)
5′	1.44 (2H, *m*)	5.85 (1H, *q*, $J_{5',4'}=14.65$, $J_{5',6'}=7.10$ Hz)	1.40 (2H, *q*, $J=14.27$, 7.32 Hz)
6′	1.27 (2H, *m*)	2.01 (2H, *t*, $J=2.72$ Hz)	1.25 (2H, *m*)
7′	1.27 (2H, *m*)	1.26 (2H, *m*)	1.25 (2H, *m*)
8′	1.27 (2H, *m*)	1.26 (2H, *m*)	1.25 (2H, *m*)
9′	1.27 (2H, *m*)	1.26 (2H, *m*)	1.25 (2H, *m*)
10′	1.27 (2H, *m*)	1.26 (2H, *m*)	1.25 (2H, *m*)
11′	1.27 (2H, *m*)	1.26 (2H, *m*)	1.25 (2H, *m*)
12′	1.27 (2H, *m*)	1.26 (2H, *m*)	1.25 (2H, *m*)
13′	1.27 (2H, *m*)	1.26 (2H, *m*)	1.25 (2H, *m*)
14′	1.27 (2H, *m*)	0.88 (3H, *t*, $J_{14',13'}=6.60$ Hz)	1.25 (2H, *m*)
15′	1.27 (2H, *m*)		1.25 (2H, *m*)
16′	0.90 (3H, *t*, $J_{16',15'}=6.77$ Hz)		0.88 (3H, *t*, $J_{14',13'}=6.95$, 6.59 Hz)
CH_3COO		1.99 (3H, *s*)	1.99 (3H, *s*)

[a]All values, given in ppm downfield from TMS, were determined in $CDCl_3$ at 270 (compounds **1** and **6**) or 400 MHz (compound **5**).

available naturally occurring daphnane diterpene, mezerein, was also tested to possess neurotrophic activity with an EC_{50} 2.4×10^{-8}.

Antileukaemia activity

During the execution of Procedure A (isolation), the antitumour activity test was used to guide the fractionation, step by step. Somehow, positive results were relatively parallel with those obtained from the NGF test. In particular, synaptolepis factor K_7 (**1**) and kirkinine B (**7**) show powerful activity in inhibiting the K562/C1000 human leukaemia cells with still some nice activity even at 3 nM (IC_{50}: 4.1×10^{-9} and 4.4×10^{-9} M, respectively) (Fig. 4 and Table 5). Although less potent, compounds **3**, **4** and **9** also show a good activity in the same cell line (Table 5). This high in vitro antileukaemia activity of the above compounds to the K562/C1000 cell line is reported here for the first time.

Experimental

General

^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC NMR spectra ($CDCl_3$) were obtained with an Avance 400 apparatus (Bruker) or with a JNM-EX270 FT NMR apparatus (JEOL). Mass spectra were measured with a LCT (Micromass) consisting of an Alliance 2690 LC (Waters) and an orthogonal accelerated time-of-flight MS analyser using a Z-spray interface and operated in positive ion electrospray mode and controlled with Masslynx 3.3 software. FAB MS was recorded by use of a Platform II instrument (Micromass). Optical rotations were determined on an AA-10 automatic polarimeter (Optical Active Ltd., UK). IR spectra were measured with a Nicolet Impact 410 FT-IR spectrometer (Thermo Optec, USA). Preparative scale LC was executed with a Prochrom (France) system, packed with Silica gel 60 (20–45 μm, Amicon) at 60 bar. Preparative HPLC was carried out on either a Hypersil BDS C18 (8 μm, 30 × 5 cm) column, controlled by a Gilson UniPoint software to a Waters 3000 pump, a Gilson 402 syringe pump, 233 XL on-line column switching and two Gilson 202 fraction collectors, or a Kontron instrument equipped with two 420 pumps, a 430 UV detector (Kontron, Germany), a Sedex 55 evaporative light scattering detector (LSD) (Sedere, France) (42 °C, 2 bar N_2 flow and split 1/50) and a Retriever II fraction collector. Preparative centrifugal partition chromatography (CPC) was carried out with a CCC-1000High Speed Countercurrent Chromatograph (Pharma-Tech Research Corp., USA), equipped with a SSI 300 pump, a Pharmacia LKB Uvicord S II detector (254 and 280 nm) and a Retriever II fraction collector. Medium pressure liquid chromatography (MPLC) on normal phase (Silica gel 60, 15–40 μm, Merck) or reversed phase (Lichroprep RP-18, 40–63 μm, Merck) was executed with a Büchi system consisting of a 688 chromatography pump, a 687 gradient former, a 684 fraction collector (Büchi, Switzerland) and a Sedex 55 LSD detector. All precoated TLC plates for both normal and reversed phase, and the solvents used for isolation and purification, were from

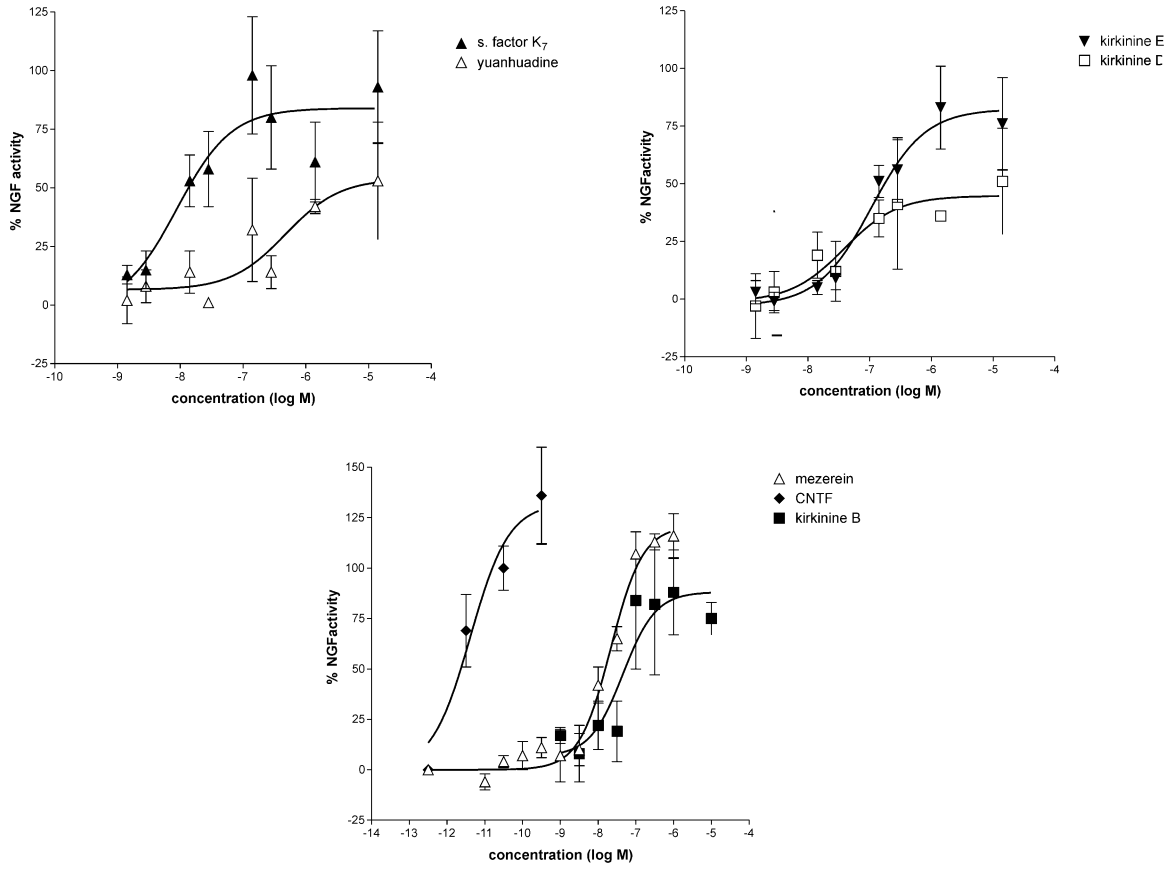

Figure 3. Neurotrophic activity in dissociated cultures of dorsal root ganglion (DRG) for synaptolepis factor K_7 (**1**), kirkinine B (**7**), D (**3**) and E (**9**), yuanhuadine (**4**), mezerein, and ciliary neurotrophic factor (CNTF). Neuronal survival was measured after culturing for 48 h. Synaptolepis factor K_7 (**1**), kirkinine B (**7**), and mezerein showed remarkable activities at about 10 nM. Kirkinine E is less active compared to the above three compounds, having an EC_{50} of 1.1×10^{-7} M. Compounds **3** and **4**, not reaching 50% NGF activity (or reaching it at relatively higher concentration) are considered to have moderate activity. The present results were obtained by using E10 chick embryos.

Table 4. ^{1}H NMR data of kirkinine B (**7**), C (**8**) and E (**9**)[a]

H	7	8	9
1	2.10 (1H, *m*)	2.09 (1H, *m*)	2.30 (*m*)
2	2.29 (1H, *m*)	2.27 (1H, *m*)	2.34 (*m*)
5	4.05 (1H, *d*, $J_{5,OH}=3.96$ Hz)	4.04 (1H, *d*, $J=4.39$ Hz)	4.14 (*s*)
7	3.43 (1H, *d*, $J_{7,10}=1.16$ Hz)	3.50 (1H, *s*)	3.42 (*s*)
8	2.93 (1H, *d*, $J_{8,14}=2.80$ Hz)	3.53 (1H, *d*, $J_{8,14}=2.69$ Hz)	2.95 (*d*, $J=2.64$ Hz)
10	3.19 (1H, *dd*, $J_{10,1}=12.87$, $J_{10,7}=1.16$ Hz)	3.19 (1H, *ds*, $J_{10,1}=12.04$ Hz)	3.59 (*d*, $J=13.19$ Hz)
11	2.56 (1H, *m*, $J_{11,12}=6.77$ Hz)	2.47 (1H, *d*, $J_{11,18}=7.13$ Hz)	2.61 (*m*)
12a	1.70 (1H, *dd*, $J_{12a,11}=2.97$, $J_{12a,12b}=14.36$ Hz)	5.01 (1H, *s*)	1.65 (*m*)
12b	2.24 (1H, *dd*, $J_{12b,11}=6.77$, $J_{12b,12a}=14.36$ Hz)		2.18 (*m*)
14	4.34 (1H, *d*, $J_{14,8}=2.80$ Hz)	4.65 (1H, *d*, $J_{14,8}=2.69$ Hz)	4.35 (*d*, $J=2.64$ Hz)
16	5.03 (*d*, $J=0.66$ Hz), 4.91 (*t*, $J=1.65$, 1.32 Hz)	5.02 (1H, *s*), 4.95 (1H, *s*)	5.03 (*s*), 4.91 (*s*)
17	1.80 (3H, *s*)	1.84 (3H, *s*)	1.80 (*s*)
18	1.27 (3H, *d*, $J_{18,11}=6.93$ Hz)	1.39 (3H, *d*, $J_{18,11}=7.13$ Hz)	1.27 (*d*, $J=7.26$ Hz)
19	1.14 (3H, *d*, $J_{19,2}=6.26$ Hz)	1.13 (3H, *d*, $J_{19,2}=6.53$ Hz)	1.08 (*d*, $J=5.93$ Hz)
20	3.80 (2H, *m*, $J_{AB}=13.20$ Hz)	3.85 (2H, *m*, $J_{AB}=18.03$ Hz)	3.82 ± 0.04 (*dd*, $J_{AB}=12.21$ Hz)
21	2.43 (1H, *m*)	2.36 (1H, *m*)	
22	1.30 (2H, br, *s*)	1.28 (2H, br, *m*)	1.32–1.60 (*m*)
23–31	1.30 (2H, br, *s*)	1.26 (2H, br, *s*)	1.32–1.60 (*m*)
32	2.14 (2H, *m*)	2.15 (2H, *m*)	2.15 (*m*)
33	6.19 (1H, *tt*, $J_{33,34}=15.51$, $J_{33,32}=7.26$, 6.93 Hz)	6.16 (1H, *tt*, $J_{33,34'}=14.88$, $J_{33,32'}=7.67$, 7.16 Hz)	6.22 (*tt*, $J=7.26$, 15.51 Hz)
34	5.61 (1H, *tt*, $J_{34,33}=15.51$, $J_{34,32}=1.32$, 1.22 Hz)	5.55 (1H, *d*, $J_{34,33'}=14.88$ Hz)	5.62 (*d*, $J=15.51$ Hz)
36	0.90 (3H, *d*, $J_{36,21}=6.60$ Hz)	0.91 (3H, *d*, $J_{36,21}=7.51$ Hz)	1.33 (*s*)
CH_3COO		2.01 (3H, *s*)	

[a]All values, given in ppm downfield from TMS, were determined in $CDCl_3$ at 270 (compounds **7** and **9**) or 400 MHz (compound **8**).

Table 5. Antitumour activity of compounds **1**, **3**, **4**, **7** and **9** isolated from *S. kirkii* in inhibiting the K562/C1000 human leukaemia cells (cell proliferation data expressed in% of control)

Concn (M)	S. factor K_7 (**1**)		Kirkinine D (**3**)		Yuanhuadine (**4**)		Kirkinine B (**7**)		Kirkinine E (**9**)	
	Mean[a]	SD[b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1.00×10^{-6}	4.83	3.58	9.31	0.70	8.17	0.23	10.23	4.51	3.19	2.67
1.00×10^{-7}	14.40	10.47	22.16	18.02	27.79	0.01	8.39	2.67	22.98	13.46
3.00×10^{-8}	6.03	2.30	41.78	30.30	62.26	0.08	9.56	6.77	62.54	13.67
1.00×10^{-8}	12.90	3.12	61.13	29.94	77.71	8.36	18.51	15.36	99.22	15.25
3.00×10^{-9}	62.39	18.69	91.81	16.81	97.14	1.08	64.17	13.06	107.80	3.97
1.00×10^{-9}	84.05	16.76	99.50	21.64	106.32	6.39	82.05	12.36	113.55	0.63
3.00×10^{-10}	105.13	8.58	102.05	5.02	99.66	4.32	106.36	5.34	102.68	3.15
IC_{50} (M)	4.1×10^{-9}		1.9×10^{-8}		4.6×10^{-8}		4.4×10^{-9}		4.4×10^{-8}	

[a]Mean values of at least two independent experiments.
[b]Standard deviation.

Merck, Germany. The TLC chromatograms were visualized under UV detection at 254 and 365 nm and sprayed with 10% H_2SO_4 in water/methanol (9:1, v/v) followed by heating at 110 °C for 15 min.

Plant material

Roots of *S. kirkii* Oliv. were collected twice (4 and 42 kg) at the Arabuko-Sokoke forest, Gede, Kenya, in January 1995 and January 1998, respectively. The plant was identified by S. G. Mathenge and F. P. Mudida. The voucher herbarium specimen (No. 839–95) was deposited at the Herbarium of the Department of Botany, Faculty of Sciences, Ghent University. The roots were air-dried and powdered mechanically.

The large quantity of roots (42 kg in Procedure B) was received as air-dried and chopped materials. Due to the toxicity and difficulty in handling in the laboratory, powdering as well as percolation of the plant material with CH_2Cl_2 (2 × 900 L) were performed at OmniChem N.V. (Wetteren, Belgium) by use of their industrial method as described below in Procedure B.

Extraction and isolation

Procedure A. The powder of the roots of *S. kirkii* (4 kg) was successively extracted in a percolator with *n*-hexane (10 × 4 L) and with dichloromethane (10 × 4 L). The extract was filtered and evaporated with a rotavapor under reduced pressure at 35 °C to give 17.1 g of the *n*-hexane (Hex) extract and 11.3 g of the dichloromethane (CH_2Cl_2) extract, respectively. The CH_2Cl_2 extract (11.23 g) was chromatographed over a silica gel in a MPLC system (Büchi), eluted with a Hex–EtOAc–MeOH gradient (column, 460 × 49 mm i.d.; precolumn, 140 × 10 mm i.d.; sample adsorbed on 80 g of silica gel and packed in a 230 × 36 mm i.d. sampling column; flow rate, about 80 mL/min at a back pressure <40 bar; detection, LSD, 42 °C, 2 bar N_2 flow and split 1/50; collection, 30 s per fraction; total run time, 420 min). A total of 32 fractions (F1.1–1.30) was obtained by classification according to the UV-spectrum of detection and TLC profiles. Fractions 1.14–1.18 were pooled (1.72 g) and subjected to a MPLC separation with silica gel (460 × 36 mm i.d., 40 mL/min) in which a gradient elution with CH_2Cl_2–MeOH was performed to give 16 fractions as F2.1–2.16. A Si-MPLC (230 × 26 mm i.d., 40 mL/min) fractionation of the active fraction F2.4 (942.5 mg) was carried out with the upper phase of Hex–EtOAc–MeOH–H_2O (2:1:1:1 and 2:8:5:5) for 30 min to yield fractions F3.1–F3.13 on the base of TLC analysis.

Fraction 3.6 (168.2 mg) was dissolved in 6 mL of upper phase and 6 mL of lower phase of the system Hex–EtOAc–MeOH–H_2O (1:1:1:1), filtered with a glass fiber filter (Acrodisc, 0.45 μm, Gelman) and injected to a CPC system. The isolation was executed by normal phase counter current chromatography with the following configurations: open-tubing column, 120 mL × 3; solvent, Hex–EtOAc–MeOH–H_2O (1:1:1:1); elution mode, head to tail, upper phase as the stationary phase, lower phase as the mobile phase; flow rate, 1 mL/min, resulting in a pressure of <90 psi; revolution speed, 1050 rpm; equilibrium volume, 64 mL; sample loop, 15 mL; detection, UV 254 and 280 nm; sample collection, 4 min per fraction; push out at 550 min with a flow of 4 mL/min, and fractions collected at 16 mL per tube. On the basis of the TLC results, all the eluates were combined into 15 portions (F4.1–4.15). With further purification of F4.14 (25 mg) on preparative RP-HPLC (column, Hyperprep C18HS BDS, 8 μm, 250 × 20 mm i.d., Shandon; gradient elution, CH_3CN–H_2O 80:20–100:0 in 40 min; flow rate, 20 mL/min; detection, UV 230 nm; collection, 0.5 min per fraction), kirkinine B (**7**) (15.0 mg) was obtained from eluates of 28–34 min as a colorless resin after evaporation of the solvent. Because of the very weak UV absorption, compound **7** was analyzed by HPLC with a light scattering detection (LSD) which showed a purity >99% (column, Hypersil BDS-C18, 3 μm, 100 × 4 mm i.d., Hewlett Packard; mobile phase, CH_3CN–H_2O 80:20–100:0 in 10 min, stand for 3 min and return to 80:20 in 1 min; flow rate, 1.2 mL/min; detection, LSD; T_R 6.44 min).

Fractions 3.2–3.5 (301.4 mg) were submitted to a CPC system (Hex–EtOAc–MeOH–H_2O 8:5:5:3, 1.5 mL/min) resulting in 14 fractions (F5.1–5.14) on the basis of TLC profiles. Purification of F5.13 over preparative RP-HPLC (conditions see above) afforded 17.7 mg of compound **1** from the eluates of 35.5 to 40.0 min with a purity of >99% (analytical HPLC: see above; T_R 8.01 min) which was identical with previously reported synaptolepis factor K_7.

Fractions 1.19 and 1.20 were combined (280 mg) and applied to CPC (Hex–EtOAc–MeOH–H_2O 4:6:5:5, 1 mL/min) resulting in 12 fractions (F6.1–6.12). F6.8–6.11 (57.6 mg) was fractionated again by CPC (Hex–EtOAc–MeOH–H_2O 1:1:1:1, 1 mL/min) to yield eight fractions (F7.1–7.8). The last four active fractions (7.5–7.8) were pooled and subjected to a preparative HPLC (conditions see above) which yielded kirkinine C (**8**) (1.7 mg, from the eluates of 37–39 min; T_R 5.40 min in analytical HPLC).

Fractions 1.21–1.27 (1520 mg) were combined and chromatographed over a normal-phase MPLC with gradient elution (Hex–CH_2Cl_2–MeOH, 40 mL/min, total run time 250 min) to yield 15 fractions (F8.1–8.5). Fraction 8.3 was then subjected to a small MPLC eluted with different water-saturated organic phases (upper phase of Hex–EtOAc–MeOH–H_2O 2:1:1:1, 1:1:1:1 and 2:8:5:5, 45 mL/min), a total of 14 fractions (F9.1–9.14) was obtained. Fractions 9.2–9.5 were pooled (86.5 mg) and separated on CPC (Hex–EtOAc–MeOH–H_2O 1:1:1:1, 1 mL/min) to yield F10.1–10.8. The active fraction 10.5 was purified by HPLC over a BDS C18 column (conditions see above) to reveal 3.3 mg of kirkinine D (**3**) (from eluates of 6–7.5 min, T_R 1.79 min in analytical HPLC).

Fractions 10.7 and 10.8 were pooled and fractionated by preparative HPLC over a Hyperprep C18HS BDS column (8 μm, 250 × 20 mm i.d.). The elution was carried out by the use of CH_3CN–H_2O from 90:10 to 100:0 in 40 min with a 20 mL/min flow rate. Fractions were detected by UV 254 nm and collected in 0.8 min per fraction. The eluates from fractions 7–11 (11.1 mg) were combined with the fraction 10.6 and re-chromatographed over the same column. At this time, an isocratic elution with CH_3CN-H_2O 65:35 was used at 20 mL/min. Fractions were automatically collected in 0.5 min. The major peak appearing at 24.4–29.6 min was related to the compound **4** (4.8 mg).

Fractions 8.4–8.6 (336 mg) were pooled and submitted to a MPLC system under the following conditions: column, 260 × 26 mm i.d.; precolumn, 140 × 10 mm i.d.; injection loop, 20 mL; flow rate, 40 mL/min; detection, LSD; collection, 20 s per fraction. Elution was performed by CH_2Cl_2–MeOH (97:3) for 20 min, 96:4 for 25 min and MeOH for 5 min to yield 11 fractions (F11.1–11.11) after the work-out procedure. Fractions 11.1–11.4 (147.3 mg) were submitted to a CPC (Hex-EtOAc-MeOH-H_2O 5:10:5:3, 1.5 mL/min) to obtain 13 fractions (F12.1–12.13) which the active fraction 12.6 (23.9 mg) was purified with preparative HPLC by an isocratic elution (column, Hyperprep C18HS BDS, 8 μm, 250 × 20 mm i.d.; mobile phase, CH_3CN–H_2O (70:30); flow rate, 20 mL/min; detection, UV 254 nm; collection, peak dependent). By drying the eluates between 15.50 and 16.50 min, a pure compound named as kirkinine E (**9**) was obtained (7.2 mg, T_R 3.38 min in analytical HPLC).

Procedure B. Powdered roots (42 kg) were sprayed with 4 L of water for 2 h and extracted with CH_2Cl_2 at room temperature (2 × 900 L). After filtration, removal of the solvent left a waxy residue (350 g, yield 0.83%), which was dissolved in MeOH–H_2O (9:1) and extracted successively with hexane, CH_2Cl_2 and EtOAc as described in Figure 5.

Fraction 14.1, a dark-brown solid was then dissolved in Hex–CH_2Cl_2 (9:1, 2 L) and filtered (P3 glass-sintered funnel). Each one liter of the filtrate was subjected individually to a LC system (column: 530 × 110 mm, i.d.) on silica gel G60 (2 kg) eluting with Hex–CH_2Cl_2–MeOH–EtOH gradient at 500 mL/min and monitored at 254 nm. The eluates were collected every 2 min, so that 31 fractions (F15.1–15.31) were obtained by combination of 170 portions of elutes based on UV absorption. The fractions 15.23 and 15.24 were pooled (12.3 g) and fractionated by reversed phase vacuum-layer chromatography (P2 glass-sintered funnel packed with 90 g of Lichroprep C18, 25–40 μm) which was eluted successively with CH_3CN–H_2O (50:50, 500 mL; 65:35, 500 mL; 80:20, 500 mL), CH_3CN (500 mL), MeOH (200 mL) and THF (300 mL), collected in 100 mL per portion, and resulted in 13 fractions (F16.1–16.13) after combination. Fraction 16.8 (440 mg) was injected to a HPLC system (column, Hypersil BDS C18, 8 μm, 300 × 50 mm, i.d.; mobile phase, CH_3CN–H_2O 80:20 to 100:0 in 40 min, standing at 100:0 for 30 min; flow rate, 40 mL/min; fractions collection, 0.33 min per tube; detection, UV 230 nm; automatic injection, <40 mL) to yield 20 fractions (F17.1–17.20), in which, F17.16 gave 9.9 mg of a pure compound and named as kirkinine (**6**).

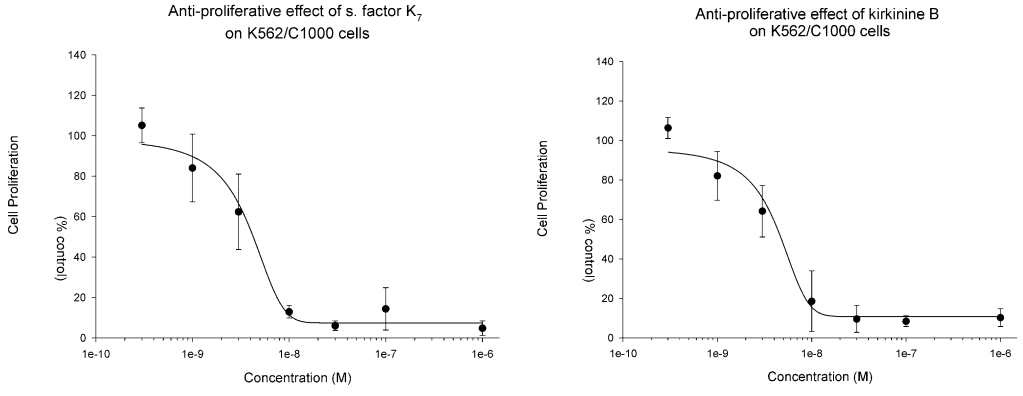

Figure 4. Antiproliferative effect of synaptolepis factor K_7 (**1**) and kirkinine B (**7**) on the K562/C1000 cell line.

Fraction 16.7 (181.7 mg) was submitted to reversed-phase HPLC (multiple injections, Hypersil BDS HS C18, 8 μm, 250 × 20 mm, i.d.; CH_3CN–H_2O 80:20–100:0 in 40 min, 20 mL/min; loop, 5 mL; detection, UV 230 nm and LSD; collection, 1 min/tube), a total of 11 fractions was obtained (F18.1–18.11). Purification of F17.9 (26 mg) and F18.7 (8 mg) by HPLC and by RP-TLC (PLKC 18F, 200 × 200 × 1 mm, Whatman) developed with CH_3CN–H_2O (80:20) afforded 2.7 mg of compound **5** as a colorless resin.

Fractionation of F16.5, F16.6 and F16.10 (680 mg) over HPLC (Hypersil BDS C18, 8 μm, 300 × 50 mm, i.d.) yielded 22 fractions (fraction 20.1–20.22). Fraction 20.8 (17.4 mg) was purified by RP-TLC (PLKC 18F) using CH_3CN-H_2O 80:20 as mobile phase to obtain compound **2** (3.5 mg).

Fraction 17.13 contained compound **7** (42 mg) and F20.17 contained compound **1** (22 mg) with high purity. Purification of F20.5–20.7 with RP-HPLC raised 25 mg of compound **4**. From F16.9 (530 mg), 142.5 mg of compound **1** was obtained directly after a run of preparative RP-HPLC.

5β-Hydroxyresiniferonol-6α,7α-epoxy-9,13,14-*ortho*-2E-hexadecenoate (1). Colorless resin; $[\alpha]_D^{18}$ +0.82° (CH_2Cl_2; *c* 0.8); IR(KBr) ν_{max} 3427 (OH), 2921, 2848, 1702 (C=O), 1632 (C=C), 1455, 991, 844, 753 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 3; HMBC (400 MHz, $CDCl_3$): H-1, C-2, C-3, C-4, C-10, C-19; H-5, C-3, C-4, C-6, C-7; H-7, C-5, C-6, C-8, C-9, C-11, C-14, C-20; H-8, C-6, C-7, C-9, C-11, C-14; H-10, C-1, C-2, C-3, C-4, C-5, C-9; H-11, C-8, C-9, C-10, C-12, C-13, C-18; H-12α, C-8, C-9, C-11, C-13, C-14, C-15, C-18; H-12β, C-11, C-14, C-18; H-14, C-6, C-7, C-9, C-10, C-15, C-1′; H-16α, C-13, C-15, C-17; H-16β, C-13, C-17; H-17, C-13, C-15, C-16; H-18, C-9, C-11, C-12; H-19, C-1, C-2, C-3, C-10; H-20, C-5, C-6, C-7; H-2′, C-1′, C-4′; H-3′, C-1′, C-2′, C-4′, C-5′; H-4′, C-2′, C-3′, C-5′, C-6′; H-5′, C-3′, C-4′; H-16′, C-14′, C-15′; EIMS (70 eV) *m/z* 614 $[M]^+$ (6), 583 $[M-CH_2OH]^+$ (3), 565 $[M-CH_2OH-H_2O]^+$ (2), 360 $[M-RCOOH]^+$ (4), 342 $[M-RCOOH-H_2O]^+$ (8), 301 $[M-RCOOH-H_2O-C_3H_5]^+$ (4), 283 (17), 273 (3), 255 (5); ES–MS *m/z* 613.3 [M−H]; HRES$^+$MS *m/z* 615.3896 $[M+H]^+$ (calcd for $C_{36}H_{54}O_8$ + H, 615.3897).

5β-Hydroxyresiniferonol-6α,7α-epoxy-9,13,14-*ortho*-2E,4E-decadienoate (2). Colorless resin, IR (KBr, film) ν_{max} 3465 (OH), 2929, 2859, 1738 (OC=O), 1697 (C=O), 1621 (C=C), 1374, 1228, 988 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 2; HMBC (400 MHz, $CDCl_3$): H-1, C-4, C-10; H-5, C-3, C-4, C-6, C-7; H-7, C-6, C-8, C-9, C-11, C-14, C-20; H-8, C-6, C-7, C-9, C-11, C-14; H-10, C-5; H-11, C-12, C-13, C-18; H-12α, C-9, C-11, C-13, C-14, C-15, C-18; H-12β, C-11, C-14, C-18; H-14, C-7, C-9, C-15, C-1′; H-16, C-13, C-15, C-17; H-17, C-13, C-15, C-16; H-19, C-1,

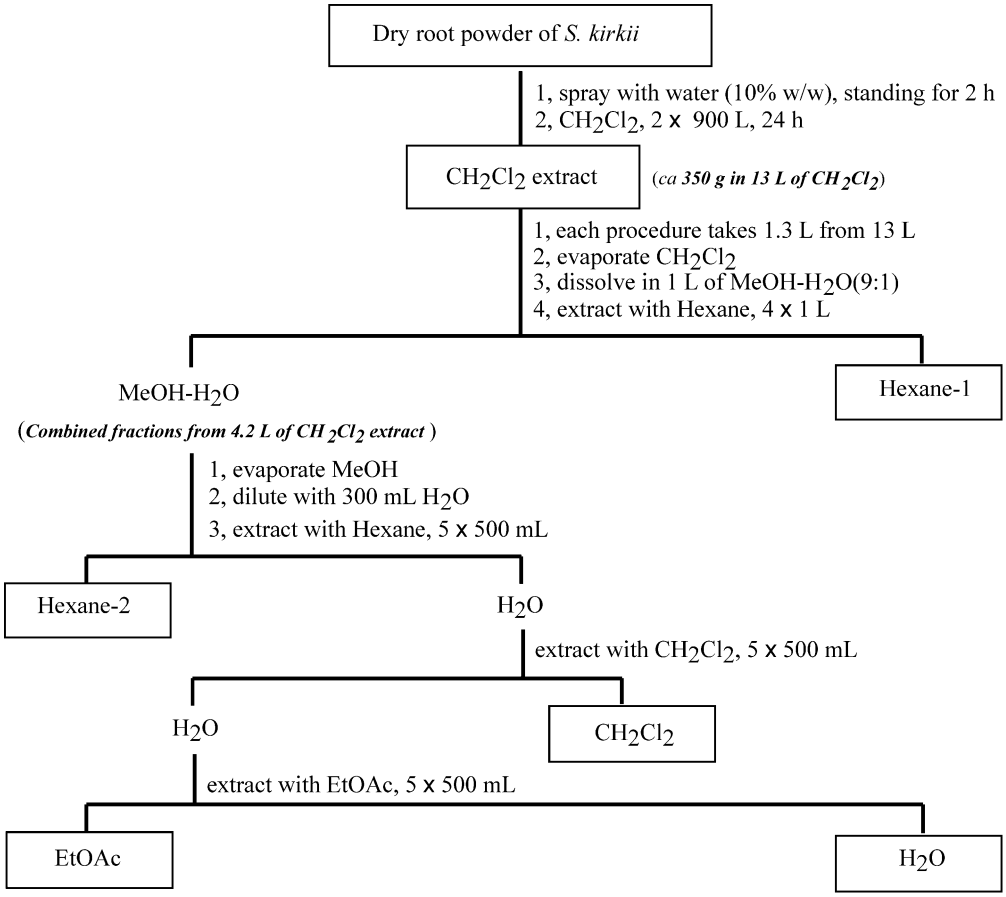

Figure 5. Liquid extraction of the roots of *S. kirkii* to obtain five extracts: F14.1 (hexane-1, 251.3 g), F14.2 (hexane-2, 11.9 g), F14.3 (CH_2Cl_2, 63.5 g), F14.4 (EtOAc, 3.6 g) and F14.5 (H_2O, 15.8 g).

C-2, C-3; H-20, C-4, C-6, C-7; H-2′, C-1′, C-3′, C-4′; H-3′, C-1′, C-2′, C-4′, C-5′; H-4′, C-2′, C-3′, C-5′, C-6′; H-5′, C-3′, C-4′, C-6′, C-7′; H-6′, C-4′, C-5′, C-7′, C-8′; H-7′, C-5′, C-6′, C-8′, C-9′; H-10′, C-8′, C-9′; HRESMS *m/z* 529.2809 $[M+H]^+$ (calcd for $C_{30}H_{41}O_8+H$, 529.2801).

5β-Hydroxyresiniferonol-6α,7α-epoxy-12β-acetoxy-9,13,14-*ortho*-2E,4E,6E-decatrienoate (3). Colorless resin; IR (KBr) ν_{max} 3469 (OH), 2926, 2877, 1741, 1701 (C=O), 1632, 1234, 1031, 994, 936, 755 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 2; EIMS *m/z* 584 $[M]^+$ (20), 317 (18), 281 (18), 269 (21), 149 (100).

5β-Hydroxyresiniferonol-6α,7α-epoxy-12β-acetoxy-9,13,14-*ortho*-2E,4E-decadienoate (4). Colorless resin; $[\alpha]_D^{18}+0.08°$ (CH_2Cl_2; *c* 1.3); IR(KBr, film) ν_{max} 3457 (OH), 2923, 2853, 1738 (C=O), 1698 (C=O), 1230, 1037, 988, 798 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 2; HMBC (400 MHz, $CDCl_3$): H-1, C-4, C-5; H-5, C-4, C-6, C-7, C-20; H-7, C-6, C-8, C-9, C-14, C-20; H-8, C-6, C-7, C-9, C-11, C-14; H-10, C-5, C-6, C-7; H-11, C-9, C-12, C-13, C-18; H-12α, C-9, C-11, C-13, C-14, C-15, $CH_3\boldsymbol{C}OO$; H-14, C-7, C-9, C-12, C-15, C-1′; H-16, C-12, C-14, C-15, C-17; H-7, C-13, C-15, C-16; H-18, C-9, C-11, C-12; H-19, C-1, C-2, C-3; H-20, C-4, C-5, C-6, C-7; H-2′, C-1′, C-4′; H-3′, C-1′, C-2′, C-4′, C-5′; H-4′, C-2′, C-3′, C-5′, C-6′; H-5′, C-3′, C-4′, C-6′; H-6′, C-4′, C-5′, C-6′; H-10′, C-6′, C-9′; $C\boldsymbol{H}_3COO$, $CH_3\boldsymbol{C}OO$; HRES$^+$MS *m/z* 587.2841 $[M+H]^+$ (calcd for $C_{38}H_{56}O_{10}+H$, 587.2856).

5β-Hydroxyresiniferonol-6α,7α-epoxy-12β-acetoxy-9,13,14-*ortho*-2E,4E-tetradecadienoate (5). Colorless resin; IR (KBr, film) ν_{max} 3462 (OH), 2925, 2852, 1742 (OC=O), 1698 (C=O), 1629 (C=C), 1231, 989 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 3; HMBC (400 MHz, $CDCl_3$): H-5, C-3, C-4, C-6, C-7; H-7, C-6, C-8, C-9, C-14, C-20; H-8, C-6, C-7, C-9, C-11, C-14; H-11, C-9, C-13, C-18; H-12α, C-9, C-11, C-13, C-14, C-15, C-18, $CH_3\boldsymbol{C}OO$; H-14, C-7, C-9, C-15, C-1′; H-16α, C-13, C-17; H-16β, C-13, C-15, C-17; H-17, C-13, C-15, C-16; H-18, C-9, C-11, C-12; H-19, C-1, C-2, C-3; H-20, C-7; H-2′, C-1′, C-5′; H-3′, C-1′, C-2′, C-4′, C-5′; H-4′, C-3′; H-5′, C-3′; H-6′, C-4′, C-5′, C-7′; H-14′, C-12′, C-13′; HRESMS *m/z* 643.3305 $[M+H]^+$ (calcd for $C_{36}H_{50}O_{10}+H$, 643.3842).

5β-Hydroxyresiniferonol-6α,7α-epoxy-12β-acetoxy-9,13,14-*ortho*-2E-hexadecenoate (6). Colorless resin; data see Tables 1 and 3.[9]

1,2-Dihydro-5β-hydroxy-21-methyl-6α,7α-epoxy-9,13,14-*ortho*-1α-(33E-pentadecenoate)-resiniferonol-36-oic acid (7). Colorless resin; IR(KBr) ν_{max} 3456 (OH), 2929, 2850, 1736 (C=O), 1457, 909, 728 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 4; HMBC (400 MHz, $CDCl_3$): H-1, C-2, C-9, C-19, C-36); H-2, C-1, C-3, C-19, C-21; H-5, C-4, C-6, C-7; H-7, C-5, C-6, C-8, C-9, C-20; H-8, C-6, C-7, C-9, C-11, C-14; H-10, C-1, C-2, C-4, C-5, C-7, C-9, C-11, C-21; H-11, C-9, C-12, C-13, C-18; H-12α, C-9, C-11, C-13, C-14, C-15, C-18; H-12β, C-11, C-14, C-18; H-14, C-7, C-9, C-15, C-35; H-16β, C-13, C-15, C-17; H-17, C-13, C-15, C-16; H-18, C-9, C-11, C-12; H-19, C-1, C-2, C-3; H-20, C-5, C-6, C-7; H-21, C-2; H-32, C-33, C-34; H-33, C-32, C-34, C-35; H-34, C-32, C-35; H-36, C-1, C-19, C-21, C-22; EIMS (70 eV) *m/z* 614 $[M]^+$, 597, 583, 571, 555, 360; FBMS *m/z* 615 $[M+1]^+$; HRESMS *m/z* 614.3791 $[M]^+$ (calcd for $C_{36}H_{54}O_8$, 614.3819).

1,2-Dihydro-5β-hydroxy-21-methyl-6α,7α-epoxy-12β-acetoxy-9,13,14-*ortho*-1α-(33E-pentadecenoate)-resiniferonol-36-oic acid (8). Colorless resin; IR (KBr) ν_{max} 3465 (OH), 2929, 2855, 1744 (C=O), 1645, 1230, 754 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 4; HRES$^+$MS *m/z* 673.3932 $[M+H]^+$ (calcd for $C_{38}H_{56}O_{10}+H$, 673.3952).

1,2-Dihydro-5β,21α-dihydroxy-21-methyl-6α,7α-epoxy-9,13,14-*ortho*-1α-(33E-pentadecenoate)-resiniferonol-36-oic acid (9). Colorless resin; IR (KBr, film) ν_{max} 3475 (OH), 2927, 2856, 1749 (C=O), 1459, 1384, 1360, 1256, 993, 968 cm^{-1}; ^1H NMR ($CDCl_3$) and ^{13}C NMR data: see Tables 1 and 4; EIMS *m/z* 630 $[M]^+$.

Biological assays

Determination of the antitumor activity. This proliferation assay on different tumour cell lines was carried out for every fraction along with fractionation of the dichloromethane extract of the roots of *S. kirkii* in Procedure A.

Samples. All samples tested were dissolved in DMSO and further dilutions were made in culture medium. Final DMSO concentrations never exceeded 0.1% (v/v) in cell proliferation assays. MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt, PROMEGA, Leiden, The Netherlands] was dissolved at 2 mg/mL in PBS. PMS [3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt, Promega, Leiden, The Netherlands) was dissolved at 2 mg/mL in PBS. PMS (phenazine methosulfate, Serva) was dissolved in PBS at 0.92 mg/mL.

Cell lines and cell culture. The human K562/C1000 leukaemia cell line was a kind gift by Dr. H. Heyligen (Dr. Willems Instituut, Diepenbeek, Belgium) and is a P-gp expressing cell line obtained by culturing the cells at increasing concentrations of colchicine. The cells were kept as a suspension culture at 37 °C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μg/mL gentamicin and 5% fetal calf serum. Stock cultures were diluted twice a week at a 1:30 ratio. All media and supplements were obtained from Life Technologies, Merelbeke, Belgium. Cells were free of mycoplasma contamination as determined using the Gen-Probe *Mycoplasma* Tissue Culture kit (biomérieux, Brussels, Belgium).

Cell proliferation assays. K562/C1000 cells were seeded in Falcon® 96-well culture plates (Life Tech-

nologies) at 5000 cells/well in a total volume of 175 μL. Immediately after seeding, drugs and/or medium were added to a final volume of 200 μL. Following 4 days of incubation, cell growth was assessed using a MTS-based assay.[16] To each well, 25 μL MTS-mix (consisting of a 20/1 v/v mixture of MTS and PMS stock solutions) was added and cells were further incubated for two h. The microtest plates were shaken for 10 min on a microplate shaker and the absorbance at 490 nm was measured using an Emax 96-well spectrophotometer. Data are expressed as inhibitory activity reported as mean ± SD of at least two independent experiments. Within an experiment, the results for each experimental condition are the mean of 6 replicate wells.

Determination of the neurotrophic activity. This assay was carried out for every fraction along with fractionation of the dichloromethane extract of the roots of *S. kirkii* in Procedure A and Procedure B. Fractions were tested as weight concentration series in DMSO, and the pure compounds were in molar concentrations.

Primary culture of chicken dorsal root ganglion (DRG) neurons. Dorsal root ganglia were dissected from White Leghorn chick embryos at embryonic day 10 (E10) as described previously.[17] The ganglia were trypsinised and dissociated by mild trituration in a HBSS buffer supplemented with 0.6% glucose and 0.08% trypsin (Gibco). To remove non-neuronal cells by differential attachment to culture plastic, ganglionic cell suspension was diluted to 2.5×10^5 cells/mL and seeded on tissue culture plastic dishes at 10 mL per 100 mm dish. After 2 h preplating, unattached neurons were collected and resuspended into Basal Eagle Medium containing 10% FCS. To remove cell aggregates, the cell suspension was passed through nylon mesh (50 μM) pore diameter. Neuron-enriched cell suspension was plated at 5×10^4 cells/mL into poly-L-ornithine (100 μg/mL) and laminine (1 μg/mL) coated multiwell 96 plates. NGF (β-Nerve Growth Factor, MW 26400, Peprotech Inc.—SanverTECH, Boechout) and other compounds were diluted in the culture medium and added to cells immediately after plating. Two days later, neuronal viability was assessed with calcein-AM.

HBSS buffer. 8 g NaCl, 0.4 g KCl, 0.35 g $NaHCO_3$, 0.06 g KH_2PO4, 0.048 g $NaHPO_4$ (Merck), dissolved in 1 L of water and filtered, pH 7.4 at 4 °C. *Basal Eagle Medium containing 10% FCS:* BME (Gibco) modified to contain 5.0 g/L D-glucose (Merck), 1 mM sodium pyruvate (Sigma), 10% FCS (Fetal Calf Serum, Bio Whittaker) (heat inactivated, filtered), 2 mM glutamine (Sigma) and 100 IU/mL penicillin GK (Serva) (stock is made in PBS, Ca^{2+} and Mg^{2+} free). *Poly-l-ornithine:* 1 mg/mL stock was made by dissolving poly-ornithine hydrobromide (Sigma) in sterile Borate buffer (150 mM, pH 8.4) and stored at −20 °C. A dilution of 1/10 is prepared with sterile water before use. *Laminin solution:* The stock solution (100 μg/mL) was prepared with laminin (Sigma) and PBS, which was diluted from 1 to 100 with PBS before use.

Neuronal viability assay using calcein-AM. Neuronal viability assay using calcein AM was performed as previously described.[18] For the assay, calcein-AM (Molecular Probes) was diluted in PBS to the final concentration (1 μM). For each experiment aliquot of calcein-AM (1 mg/mL in DMSO stored at −20 °C) was thawed immediately before use. Medium was removed from wells and replaced with the calcein–AM solution. Assay plates were incubated for 1 h at 37 °C in a humidified CO_2 incubator. Following the incubation, reading was done in a Cytofluor II at excitation of 485 nm and emission of 530 nm. Each plate had control wells with no neurotrophic factor added (0% survival) and wells with 10 ng/mL NGF (100% survival). The bioactivity is expressed in term of%NGF activity defined as neuronal survival rate comparing with that of NGF.

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