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Acylated dolabellane-type diterpenes from *Nigella sativa* seeds with triglyceride metabolism-promoting activity in high glucose-pretreated HepG2 cells

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ABSTRACT

Two new acylated dolabellane-type diterpenes, nigellamines B_3 (**9**) and D (**10**), were isolated from *Nigella sativa* (Ranunculaceae) seeds using column chromatography and preparative HPLC. Their structures were determined based on chemical and physicochemical evidence, and confirmed using previously isolated related compounds as reference. Of the seed constituents, nigellamines A_2 (**2**), A_3 (**3**), A_5 (**5**), B_1 (**6**), and B_2 (**7**) had *in vitro* triglyceride metabolism-promoting activities in the high glucose-pretreated human liver carcinoma cell line, HepG2.

1. Introduction

The Ranunculaceae annual plant *Nigella sativa* L. is widely distributed and cultivated in Arabic and Mediterranean countries. The seeds of *N. sativa* (commonly known as "black cumin") are used in many food preparations, in addition to being prescribed by Arabian folk medicine practitioners for the treatment of asthma, flatulence, polio, kidney stones, abdominal pain, *etc.* (El Sayed et al., 2000; Enomoto et al., 2001). Previously, we reported that a methanol extract of the seeds had a lipid metabolism-promoting activity in primary cultured mouse hepatocytes (Morikawa et al., 2004a,b). A re-examination on *N. sativa* to identify new activities detected its triglyceride (TG) metabolism-promoting activity in high glucose-pretreated human liver carcinoma cell line, HepG2. Further separation of the active constituents in the extract allowed us to isolate two new acylated dolabellane-type diterpenes, nigellamines B_3 (**9**) and D (**10**), in addition to the previously reported nigellamines A_1–A_5 (**1**–**5**), B_1 (**6**), B_2 (**7**), and C (**8**). This paper describes the isolation and structure elucidation of the new diterpenes and the TG metabolism-promoting activity of the diterpene constituents in high glucose-pretreated HepG2 cells.

2. Results and discussion

2.1. Effect of the MeOH extract on liver TG content in mice

The seeds of *N. sativa* were powdered and extracted with methanol to give a methanol extract (17.4% from the seeds). As shown in Tables 1 and 2, the methanol extract significantly reduced liver TG content by oral administration at doses of 250 and 500 mg/kg/day for 3 days without affecting food intake, body and liver weights, and with no detectable toxic effects including plasma glucose, TG, and free fatty acid (FFA) levels.

2.2. Effects on TG content in high glucose-pretreated HepG2 cells

Fatty liver is recognized as a significant risk factor for serious liver disease (Bellentani et al., 1994; El-Hassan et al., 1992). There is a strong causal linkage between fatty liver disease and hyperinsulinemic insulin resistance (Marceau et al., 1999; Marchesini et al., 1999). Thus, fatty liver is considered to be highly associated with obesity and type 2 diabetes (Marchesini et al., 1999). During an exploratory study of the novel bioactive functions of the natural medicine in *N. sativa*, we examined the inhibitory effects of its methanol extract on the TG levels in high glucose-pretreated HepG2 cells (Morikawa et al., 2004a,b). The extract significantly reduced the TG levels in hepatocytes (% of control at 86.7 ± 5.2, $p < 0.01$ at 10 μg/mL). The methanol extract

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Table 1
Effects of the methanol extract of *N. sativa* seeds on food intake, visceral fat, liver weight, and liver TG content in mice.

Treatments	Dose (mg/kg/day, p.o.)	*N*	Body weight			Food intake (g/mouse/day)	Liver weight (mg)	Liver TG (mg/g)
			1st day (g)	2nd day (g)	3rd day (g)			
Control	–	7	31.2 ± 0.4	32.1 ± 0.6	32.5 ± 0.7	4.8 ± 0.2	1145 ± 52	56 ± 3
MeOH ext.	250	7	30.9 ± 0.2	32.1 ± 0.4	32.2 ± 0.3	5.0 ± 0.2	1225 ± 28	40 ± 2*
	500	7	30.9 ± 0.4	31.9 ± 0.3	32.0 ± 0.3	5.3 ± 0.3	1236 ± 54	42 ± 1*

Each value represents the mean ± S.E.M.
* Significantly different from the control, $p < 0.01$.

was partitioned into a mixture of EtOAc and water to produce an EtOAc-soluble fraction (10.1%) and an aqueous phase. The aqueous phase was subjected to Diaion HP-20 column chromatography to yield H_2O- and MeOH-eluted fractions (2.6 and 4.7%, respectively). The EtOAc-soluble fraction was active (% of control at 81.8 ± 4.1, $p < 0.01$ at 10 μg/mL), whereas the other fractions lacked activity (H_2O-eluted fraction: % of control at 95.6 ± 2.9 at 10 μg/mL, MeOH-eluted fraction: 93.3 ± 5.5).

2.3. Isolation and structure determination of nigellamines B_3 (**9**) and D (**10**)

The EtOAc-soluble fraction was subjected to SiO_2 and ODS column chromatographies and finally HPLC to furnish acylated dolabellane-type diterpenes nigellamines B_3 (**9**) and D (**10**) together with nigellamines A_1–A_5 (**1**–**5**), B_1 (**6**), B_2 (**7**), and C (**8**) (Fig. 1), and two monoterpenes, carvacrol and thymoquinol. Carvacrol and thymoquinol were identified by comparison of their spectroscopic properties with those of commercially obtained samples. From the MeOH-eluted fraction, two saponins, 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosylhederagenin [28-*O*-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl] ester (Shimizu et al., 1978) and 3-*O*-*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosylhederagenin [28-*O*-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl] ester (Ansari et al., 1988) were isolated by SiO_2 and ODS column chromatographies and finally HPLC (Figure S1).

Nigellamine B_3 (**9**) was obtained as a white powder with positive optical rotation ($[\alpha]_D^{27}$ +33.4 in $CHCl_3$). The positive-ion FABMS showed the quasimolecular ion peak at *m*/*z* 677 $[M+H]^+$, and the molecular formula was determined as $C_{38}H_{48}N_2O_9$ by HRFABMS measurement. The ^1H and ^{13}C NMR (Table 3) spectroscopic properties, which were assigned with the aid of DEPT, ^1H–^1H COSY, HMQC, and HMBC experiments, were quite similar to those of nigellamine B_2 (**7**) (Morikawa et al., 2004a) except for signals due to a *n*-hexanoyl group instead of a benzoyl group at the 15-position: *δ* 0.86 (3H, t, *J* = 7.0 Hz, H_3-6‴), 1.27, 1.30, 1.68 (2H each, all m, H_2-5‴, 4‴, 3‴), 2.45 (2H, t, *J* = 7.0 Hz, H_2-2‴). Treatment of **9** with triphenylphosphine (PPh_3) gave a compound (**9a**) with sixteen units less molecular weight than the reactant (**9**). Characteristic downfield shift of a signal due to C-18 (**9**: δ_C 82.6, **9a**: δ_C 71.6) was observed upon the conversion. Treatment of **9a** with 0.1% sodium methoxide (NaOMe)–MeOH at room temperature furnished **9b**, the spectroscopic properties of which were completely in accord with those of an authentic specimen (Morikawa et al., 2004a), together with methyl nicotinate and methyl *n*-hexanonate (Fig. 2). The positions of the two nicotinic acids and the *n*-hexanoic acid were determined on the basis of HMBC experiments, in which long-range correlations were observed between H-2 and C-7′; H-10 and C-7″; H_2-15 and C-7‴ (Figure S2). Thus the connected positions of these acyl groups in **9** were unambiguously clarified, and the structure of **9** was elucidated.

Nigellamine D (**10**) was also obtained as a white powder with negative optical rotation ($[\alpha]_D^{23}$ −20.5 in $CHCl_3$). The molecular weight (*m*/*z* 666 $[M^+]$) determined by the EIMS was sixteen units more than that of nigellamine A_2 (**2**) (*m*/*z* 650 $[M^+]$), and the molecular formula was determined to be $C_{39}H_{42}N_2O_8$ by the HREIMS. The proton and carbon signals in the ^1H and ^{13}C NMR spectra of **10** were superimposable on those of **2**, except for the signals due to the 20-position [*δ* 4.10 (2H, s)]. In the HMBC experiment on **10**, long-range correlations were observed between the 20-proton and the C-12/C-18/C-19 as shown in Figure S2. The geometric structure of the olefinic parts and relative stereostructure of **10** was characterized by the NOESY experiment as shown in Figure S2. The CD spectrum of **10**, which showed a negative Cotton effect at 261 nm (Δ*ε* −1.73 in MeOH), was similar to that of **2** [256 nm (Δ*ε* −1.42) in MeOH], so that the absolute stereochemistry was proved to be the same as that of **2**.

2.4. Effects on nigellamines (**1**–**9**) on TG content in high glucose-pretreated HepG2 cells

We examined the inhibitory effects of nigellamines (**1**–**9**) on the TG levels in high glucose-pretreated HepG2 cells. As shown in Table 4, **2** (% of control at 10 μM: 89.7 ± 5.7), **3** (62.1 ± 4.5), and **5** (81.0 ± 2.5) significantly reduced the TG levels in hepatocytes and these reductions were equivalent to those using the hypoglycemic medicine, metformin (Bellentani et al., 1994; Marchesini et al., 1999) (82.9 ± 2.4). In addition, **6** (% of control at 30 μM: 82.2 ± 7.4), **7** (75.5 ± 6.1), carvacrol (86.5 ± 1.9), and thymoquinol (84.6 ± 3.8) demonstrated weak activities. The subsequent structural requirements affected the activities of these types of diterpene: (1) those

Table 2
Effects of the methanol extract of *N. sativa* seeds on plasma biochemicals in mice.

Treatments	Dose (mg/kg/day, p.o.)	*N*	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	Free fatty acid (mequiv./L)
Control	–	7	101.8 ± 4.8	127.6 ± 13.8	1.625 ± 0.081
MeOH ext.	250	7	106.1 ± 7.2	133.9 ± 10.3	1.511 ± 0.117
	500	7	104.9 ± 6.5	126.9 ± 14.3	1.491 ± 0.122

Each value represents the mean ± S.E.M.

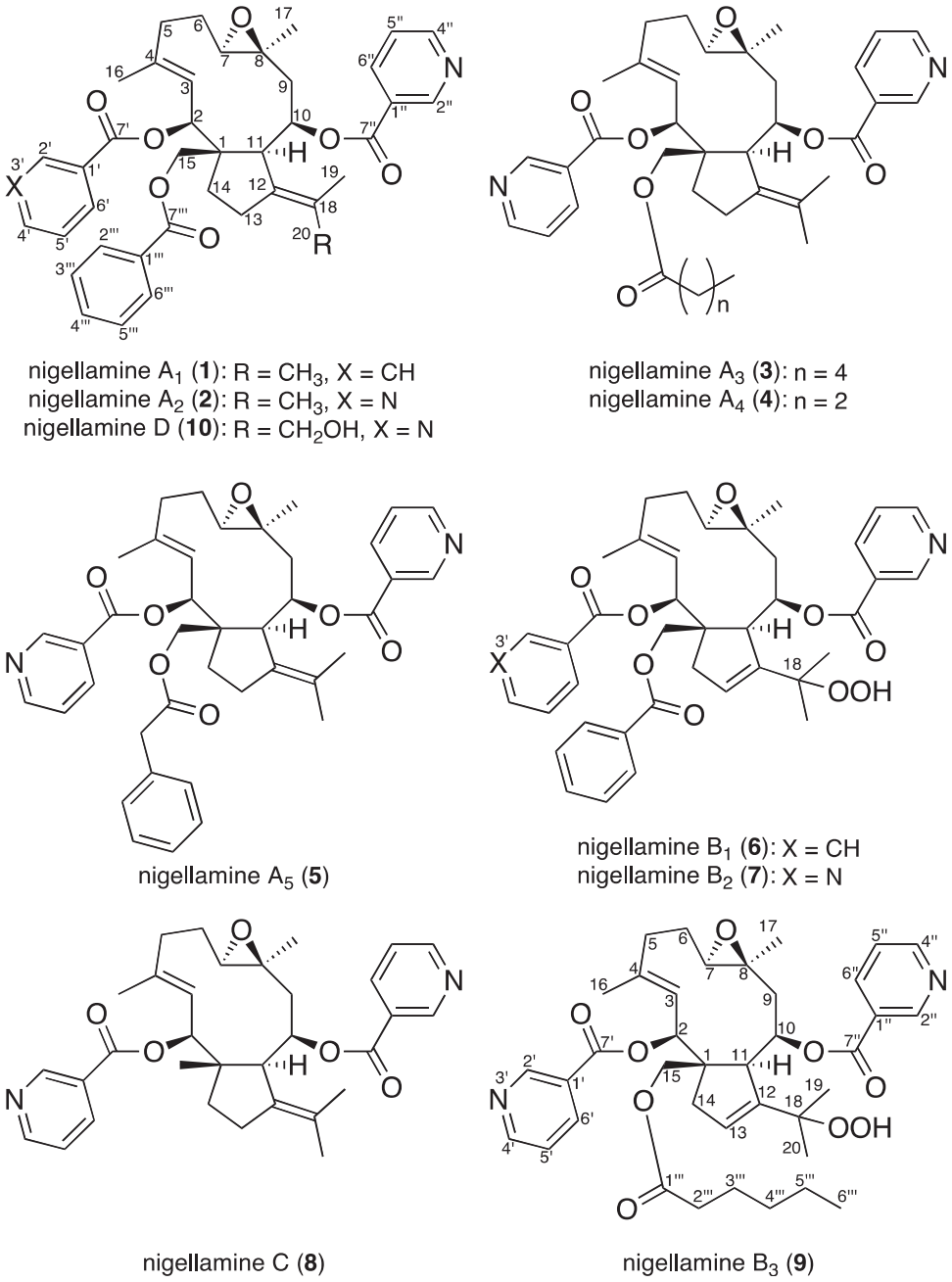

Fig. 1. Structures of nigellamines (**1**–**10**).

with a 2-*O*-nicotinoyl group were more potent than those bearing a 2-*O*-benzoyl group [nigellamine A_2 (**2**) ≫ nigellamine A_1 (**1**); nigellamine B_2 (**7**) > nigellamine B_1 (**6**), respectively]; (2) the 15-*O*-*n*-hexanoyl group enhanced activity [nigellamine A_3 (**3**) > nigellamines A_2 (**2**) and A_5 (**5**) ≫ nigellamine A_4 (**4**)]. Thus, these nigellamines (**2**, **3**, and **5**–**7**) and two known monoterpenes were responsible for the fatty liver preventive effects of the extract.

2.5. Agonistic activity for PPARα

To clarify the mechanism of action of the active nigellamines, the PPARα agonistic activity was examined using a nuclear receptor cofactor assay system. As shown in Table 5, the relative binding activity of the active nigellamines (**2**, **3**, and **5**–**7**) indicated a weak activity. The detailed mechanisms of action of these active nigellamines need to be studied further.

3. Experimental

3.1. General experimental procedures

The following instruments were used to obtain spectral and physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); CD spectra, JASCO J-720WI spectrometer; UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H and ^{13}C NMR spectra, JEOL JNM-LA500 (500 and 125 MHz) spectrometer with tetramethylsilane as an internal standard; EIMS and high-resolution EIMS, JEOL JMS-GCMATE mass spectrometer; FABMS and high-resolution FABMS, JEOL JMS-SX 102A mass spectrometer; HPLC detector, Shimadzu RID-6A refractive index and Shimadzu SPD-10A UV-VIS detectors. HPLC column, YMC-Pack ODS-5-A (YMC Co., Ltd.) columns were used for analytical (250 mm × 4.6 mm i.d.) and preparative (250 mm × 20 mm i.d.) purposes, respectively.

Table 3
^{1}H and ^{13}C NMR data on nigellamines B_3 (**9**) and D (**10**) and related compounds (**9a** and **9b**) in $CDCl_3$.

	9		**10**		**9a**		**9b**	
	δ (J in Hz)	δ_C	δ (J in Hz)	δ_C	δ (J in Hz)	δ_C	δ (J in Hz)	δ_C
1		54.5		56.1		54.4		56.3
2	5.49 (d, 10.4)	73.9	5.47 (d, 10.1)	73.5	5.43 (d, 10.5)	73.9	4.05 (d, 10.7)	72.3
3	5.61 (dd, 0.9, 10.4)	122.0	5.77 (dd, 1.2, 10.1)	123.1	5.61 (dd, 0.9, 10.5)	122.4	5.48 (dd, 0.9, 10.7)	127.6
4		142.1		140.5		141.7		137.3
5α	2.36 (br d, *ca.* 13)	38.1	2.38 (m)	37.8	2.36 (m)	38.2	2.28 (m)	38.1
5β	2.41 (ddd, 4.9, 12.8, 12.8)		2.50 (ddd, 5.2, 12.9, 12.9)		2.43 (ddd, 4.9, 12.9, 12.9)		2.37 (ddd, 4.6, 12.2, 12.2)	
6α	1.66 (m)	22.9	1.71 (m)	22.7	1.67 (m)	22.9	1.61 (m)	23.0
6β	1.99 (m)		2.03 (m)		1.99 (m)		1.92 (m)	
7	2.90 (br d, *ca.* 10)	66.7	3.08 (br d, *ca.* 10)	65.4	2.90 (br d, *ca.* 10)	66.7	2.83 (br d, *ca.* 10)	66.6
8		58.8		58.4		59.1		59.5
9α	2.65 (dd, 5.8, 13.7)	40.9	2.57 (dd, 5.5, 132.8)	42.2	2.47 (dd, 5.8, 13.8)	41.3	2.35 (dd, 5.5, 13.5)	43.3
9β	1.52 (dd, 12.2, 13.7)		1.67 (dd, 12.5, 13.8)		1.64 (dd, 12.5, 13.8)		1.49 (dd, 12.5, 13.5)	
10	5.88 (br dd, *ca.* 6, 12)	73.8	5.79 (br dd, *ca.* 6, 13)	74.6	6.24 (br dd, *ca.* 6, 13)	73.9	4.51 (br dd, *ca.* 6, 13)	70.1
11	2.79 (br s)	51.4	2.75 (br s)	48.7	2.82 (br s)	51.7	2.50 (br s)	52.4
12		146.0		139.7		149.4		149.1
13	5.75 (br s)	128.6	2.43 (2H, m)	27.2	5.49 (br s)	125.1	5.75 (br s)	126.2
14α	2.59 (br s)	38.2	2.29 (m)	31.4	2.40 (br s)	38.2	2.79 (dd, 3.4, 17.4)	36.4
14β	2.59 (br s)		2.17 (m)		2.40 (br s)		2.65 (br d, *ca.* 17)	
15	4.79 (d, 11.0)	65.5	4.92 (d, 11.0)	66.8	4.79 (d, 11.0)	65.5	4.16 (d, 11.0)	63.6
	4.83 (d, 11.0)		5.32 (d, 11.0)		5.07 (d, 11.0)		4.20 (d, 11.0)	
16	1.93 (3H, d, 0.9)	17.3	1.88 (3H, d, 1.2)	16.6	1.95 (3H, d, 0.9)	17.4	1.69 (3H, d, 0.9)	16.8
17	1.50 (3H, s)	17.2	1.55 (3H, s)	18.4	1.49 (3H, s)	17.1	1.33 (3H, s)	17.5
18		82.6		130.8		71.6		71.6
19	1.46 (3H, s)	26.1	2.01 (3H, s)	17.7	1.44 (3H, s)	32.2	1.45 (3H, s)	33.0
20	1.49 (3H, s)	27.2	4.10 (2H, s)	65.2	1.48 (3H, s)	32.7	1.52 (3H, s)	33.2
1′		126.3		125.8		126.5		
2′	9.18 (br s)	150.8	9.09 (br s)	151.0	9.22 (br s)	150.9		
4′	8.80 (br s)	153.6	8.66 (br d, *ca.* 4)	153.4	8.80 (br s)	153.6		
5′	7.40 (dd, 4.9, 8.0)	123.3	6.98 (dd, 4.9, 8.0)	122.9	7.38 (dd, 4.9, 8.0)	123.3		
6′	8.32 (ddd, 1.8, 1.9, 8.0)	137.0	7.94 (ddd, 1.8, 1.9, 8.0)	136.9	8.32 (ddd, 1.8, 1.9, 8.0)	136.9		
7′		164.9		165.2		164.9		
1″		125.8		125.5		125.7		
2″	9.27 (br s)	150.9	9.23 (br s)	150.8	9.27 (br s)	151.0		
4″	8.79 (br s)	153.7	8.79 (br d, *ca.* 4)	153.9	8.79 (br s)	153.8		
5″	7.42 (dd, 4.9, 8.0)	123.6	7.42 (dd, 4.9, 8.0)	123.6	7.42 (dd, 4.9, 8.0)	123.5		
6″	8.30 (ddd, 1.8, 1.9, 8.0)	137.3	8.31 (ddd, 1.8, 1.9, 8.0)	137.2	8.29 (ddd, 1.8, 1.9, 8.0)	137.3		
7″		165.2		164.5		165.2		
1‴		173.8		130.0		173.8		
2‴	2.45 (2H, t, 7.0)	34.5	8.10 (dd, 1.3, 8.5)	129.9	2.44 (2H, t, 7.0)	34.5		
3‴	1.68 (2H, m)	24.8	7.39 (dd, 7.8, 8.5)	128.5	1.67 (2H, m)	24.8		
4‴	1.30 (2H, m)	31.4	7.59 (tt, 1.3, 7.8)	133.3	1.30 (2H, m)	31.4		
5‴	1.27 (2H, m)	22.3	7.39 (dd, 7.8, 8.5)	128.5	1.26 (2H, m)	22.3		
6‴	0.86 (3H, t, 7.0)	13.9	8.10 (dd, 1.3, 8.5)	129.9	0.86 (3H, t, 7.0)	13.9		
7‴				166.4				

The following experimental conditions were used for chromatography: highly porous synthetic resin, Diaion HP-20 (Mitsubishi Chemical Co.); normal-phase silica gel column chromatography (CC), silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh); ODS CC, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh); normal-phase TLC, pre-coated TLC plates with silica gel $60F_{254}$ (Merck, 0.25 mm); reversed-phase TLC, pre-coated TLC plates with silica gel RP-18 F_{254S} (Merck, 0.25 mm); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm), detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

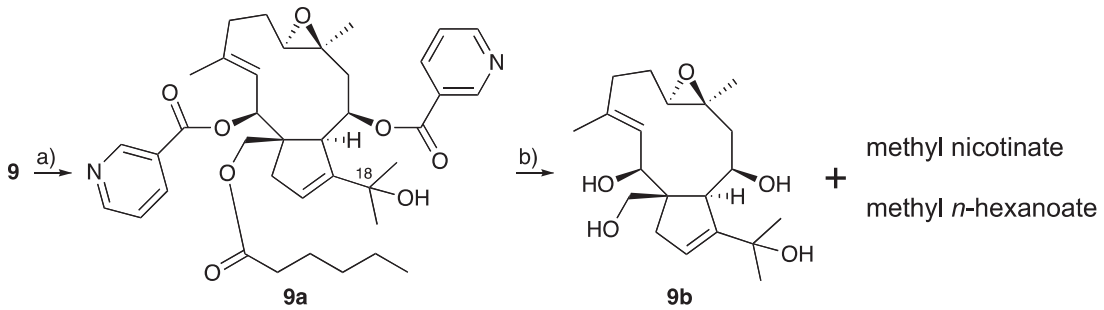

Fig. 2. Absolute stereostructure of **9**. *Reagents and conditions*: (a) PPh_3/CH_2Cl_2, 0 °C, 30 min, 71%; (b) 0.1% NaOMe–MeOH, r.t., 8 h, 90%.

Table 4
Effects of nigellamines (**1**–**9**) on TG contents in high glucose-pretreated HepG2 cells.

	TG/protein (% of control)			
Conc. (μM)	0	3	10	30
Nigellamine A_1 (**1**)	100.0 ± 5.6	94.3 ± 4.8	96.0 ± 6.1	91.9 ± 5.4
Nigellamine A_2 (**2**)	100.0 ± 4.4	103.0 ± 2.4	89.7 ± 5.7**	78.5 ± 8.6**
Nigellamine A_3 (**3**)	100.0 ± 2.1	99.1 ± 3.1	62.1 ± 4.5**	51.2 ± 3.1**
Nigellamine A_4 (**4**)	100.0 ± 4.7	100.4 ± 3.4	104.6 ± 2.1	96.4 ± 1.3
Nigellamine A_5 (**5**)	100.0 ± 4.5	95.6 ± 6.0	81.0 ± 2.5**	83.8 ± 1.1**
Nigellamine B_1 (**6**)	100.0 ± 1.7	97.2 ± 9.3	93.1 ± 1.0	82.2 ± 7.4**
Nigellamine B_2 (**7**)	100.0 ± 5.4	106.3 ± 3.5	95.5 ± 2.1	75.5 ± 6.1**
Nigellamine C (**8**)	100.0 ± 5.2	99.8 ± 2.7	100.9 ± 4.6	113.7 ± 2.5
Nigellamine B_3 (**9**)	100.0 ± 5.2	99.3 ± 2.7	95.7 ± 6.9	95.4 ± 2.5
Carvacrol	100.0 ± 4.9	91.7 ± 8.9	97.2 ± 4.6	86.5 ± 1.9**
Thymoquinol	100.0 ± 4.4	91.2 ± 2.3	90.7 ± 5.6	84.6 ± 3.8**
	TG/protein (% of control)			
Conc. (μM)	0	0.1	1	10
Metformin	100.0 ± 2.0	90.0 ± 2.7*	89.3 ± 3.1**	82.9 ± 2.4**

Each value represents the mean ± S.E.M. ($n = 4$).
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

3.2. Plant material

The seeds of *N. sativa* were purchased in Cairo, Egypt and were identified by one of the authors (M.Y.). A voucher of the plant material is on file in our laboratory (Morikawa et al., 2004a,b).

3.3. Extraction and isolation

The seeds of *N. sativa* (10.0 kg) were finely powdered and extracted three times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a methanol extract (1740 g, 17.4% from the dried material). The methanol extract (1245 g) was partitioned into mixture of EtOAc and water to give an EtOAc-soluble fraction (723 g, 10.1%) and an aqueous fraction (522 g). An aliquot of the aqueous fraction (200 g) was subjected to Diaion HP-20 CC (3.0 kg, $H_2O \rightarrow$ MeOH, twice) to give H_2O- (71.0 g, 2.6%) and MeOH-eluted fractions (129.0 g, 4.7%). The EtOAc-soluble fraction (285 g) was subjected to silica gel CC [3.0 kg, *n*-hexane–EtOAc (20:1 → 10:1 → 5:1 → 2:1 → 1:2) → $CHCl_3$–MeOH–H_2O (20:3:1, lower layer → 6:4:1) → MeOH] to give 12 fractions [Fr. 1 (12.36 g), Fr. 2 (55.91 g), Fr. 3 (74.90 g), Fr. 4 (18.51 g), Fr. 5 (61.82 g), Fr. 6 (2.55 g), Fr. 7 (0.97 g), Fr. 8 (9.68 g), Fr. 9 (6.69 g), Fr. 10 (8.13 g), Fr. 11 (15.82 g), and Fr. 12 (16.57 g)]. The fraction 3 (21.23 g) was subjected to ODS CC [630 g, MeOH–H_2O (80:20, v/v) → MeOH] to give carvacrol (136.0 mg, 0.017%). The fraction 6 (2.55 g) was subjected to ODS CC [80 g, MeOH–H_2O (55:45 → 70:30, v/v) → MeOH] and HPLC [MeOH–H_2O (45:55, v/v)] to give thymoquinol (722.4 mg, 0.026%). The fraction 7 (0.97 g) was subjected to ODS CC [30 g, MeOH–H_2O (50:50 → 70:30 → 85:15, v/v) → MeOH] to give seven fractions [Fr. 7-1 (112.3 mg), Fr. 7-2 (105.0 mg), Fr. 7-3 (68.2 mg), Fr. 7-4 (315.4 mg), Fr. 7-5 (87.8 mg), Fr. 7-6 (152.0 mg), and Fr. 7-7 (120.0 mg)]. The fraction 7-4 (315.4 mg) was further separated by HPLC [MeOH–H_2O (85:15, v/v)] to give nigellamine A_1 (**1**, 270.9 mg, 0.0096%). The fraction 7-6 (152.0 mg) was purified by HPLC [MeOH–H_2O (80:20, v/v)] to give nigellamine B_1 (**6**, 17.5 mg, 0.00062%). The fraction 8 (9.68 g) was subjected to ODS CC [300 g, MeOH–H_2O (50:50 → 75:25, v/v) → MeOH] to give six fractions [Fr. 8-1 (1.466 g), Fr. 8-2 (109.2 mg), Fr. 8-3 (1.920 g), Fr. 8-4 (2.573 g), Fr. 8-5 (748.2 mg), and Fr. 8-6 (2.804 g)]. The fraction 8-2 (109.2 mg) was further separated by HPLC [MeOH–H_2O (80:20, v/v)] to give **6** (15.4 mg, 0.00055%). The fraction 9 (6.69 g) was subjected to ODS CC [200 g, MeOH–H_2O (40:60 → 60:40 → 80:20, v/v) → MeOH] to give 10 fractions [Fr. 9-1 (1.150 g), Fr. 9-2 (943.2 mg), Fr. 9-3 (627.1 mg), Fr. 9-4 (1.312 g), Fr. 9-5 (142.8 mg), Fr. 9-6 (750.5 mg), Fr. 9-7 (150.7 mg), Fr. 9-8 (241.0 mg), Fr. 9-9 (805.0 mg), and Fr. 9-10 (428.0 mg)]. The fraction 9-6 (750.5 mg) was purified by HPLC [MeOH–H_2O (75:25, v/v)] to give nigellamines B_2 (**7**, 101.9 mg, 0.0036%) and D (**10**, 2.1 mg, 0.000075%). The fraction 9-9 (805.0 mg) was purified by HPLC [MeOH–H_2O (80:20, v/v)] to give nigellamines A_2 (**2**, 220.7 mg, 0.0078%) and A_3 (**3**, 13.6 mg, 0.00048%). The fraction 9-10 (428.0 mg) was purified by HPLC [MeOH–H_2O (80:20, v/v)] to give nigellamines A_5 (**5**, 4.3 mg, 0.00015%) and B_3 (**9**, 6.4 mg, 0.00023%). The fraction 10 (8.13 g) was subjected to ODS CC [240 g, MeOH–H_2O (40:60 → 60:40 → 85:15, v/v) → MeOH] to afford eight fractions [Fr. 10-1 (1.422 g), Fr. 10-2 (638.2 mg), Fr. 10-3 (816.4 mg), Fr. 10-4 (1.218 g), Fr. 10-5 (491.5 mg), Fr. 10-6 (921.5 mg), Fr. 10-7 (545.7 mg), and Fr. 10-8 (2.027 g)]. The fraction 10-5 (491.5 mg) was purified by HPLC [MeOH–H_2O (80:20, v/v)] to furnish nigellamines A_4 (**4**, 4.2 mg, 0.00015%) and C (**8**, 8.7 mg, 0.00031%). An aliquot of the MeOH-eluted fraction (20.00 g) was subjected to silica gel CC [600 g, $CHCl_3$–MeOH–H_2O (7:3:1, lower layer → 6:4:1) → MeOH] to give seven fractions [Fr. 1 (300.0 mg), Fr. 2 (1.01 g), Fr. 3 (420.0 mg), Fr. 4 (630.0 mg), Fr. 5 (4.00 g), Fr. 6 (11.60 g), and Fr. 7 (352.6 mg)]. The fraction 6 (11.60 g) was separated by HPLC [MeOH–H_2O (65:35, v/v)] to give 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosylhederagenin [28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (54.8 mg, 0.15%) and 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosylhederagenin [28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (420.2 mg, 1.15%).

Table 5
Effects of nigellamines (**2**, **3**, **5**–**7**) on binding activity for PPARα.

	Relative binding activity (%)[a]
	Conc. 100 μM
Nigellamine A_2 (**2**)	31.3 ± 2.5
Nigellamine A_3 (**3**)	30.7 ± 1.0
Nigellamine A_5 (**5**)	21.4 ± 0.9
Nigellamine B_1 (**6**)	26.4 ± 1.3
Nigellamine B_2 (**7**)	17.6 ± 0.8

Each value represents the mean ± S.E.M. ($n = 4$).
[a] Relative binding activity was calculated with the activity of 500 nM GW4647 as 100%.

*3.3.1. Nigellamine B_3 (**9**)*

A white powder, $[\alpha]_D^{27}$ +33.4 (*c* 0.30, $CHCl_3$); UV [MeOH, nm (log ε)]: 219 (4.31), 264 (3.88); CD [MeOH, nm, (Δε)]: 222 (+5.14), 242 (−1.33); IR (KBr) ν_{max} cm^{-1}: 1717, 1647, 1636, 1593, 1541, 1509, 1420, 1284, 1115, 1024, 941, 743, 702; ^1H and ^{13}C NMR data, see Table 3; positive-ion FABMS *m/z*: 677 $[M+H]^+$; HRFABMS *m/z*: 677.3449 $[M+H]^+$ (calcd for $C_{38}H_{49}N_2O_9$, 677.3438).

*3.3.2. Nigellamine D (**10**)*

A white powder, $[\alpha]_D^{23}$ −20.5 (*c* 0.20, $CHCl_3$); UV [MeOH, nm (log ε)]: 220 (4.50), 264 (3.85); CD [MeOH, nm, (Δε)]: 261 (−1.50); IR (KBr) ν_{max} cm^{-1}: 3410, 1717, 1671, 1647, 1592, 1559, 1509, 1453, 1281, 1111, 1026, 949, 743, 704; ^1H and ^{13}C NMR data, see Table 3; EIMS *m/z*: 666 ($[M^+]$, 2), 648 ($[M^+–H_2O]$, 2), 542 (23), 403 (5), 280 (19), 151 (18), 124 (100), 106 (25), 105 (47); HREIMS *m/z*: 666.2944 $[M^+]$ (calcd for $C_{39}H_{42}N_2O_8$: 666.2941).

*3.4. Triphenylphosphine reduction of **9***

To a solution of **9** (3.7 mg) in CH_2Cl_2 (1.0 mL) was added triphenylphosphine (PPh_3, 8.0 mg) and the mixture was stirred at 0 °C for 30 min. Removal of the solvent under reduced pressure furnished a residue, which was purified by HPLC [MeOH–H_2O (75:25, v/v)] to give **9a** (2.6 mg, 71%).

*3.4.1. Compound **9a***

A white powder, $[\alpha]_D^{25}$ +35.8 (*c* 0.20, $CHCl_3$); UV [MeOH, nm (log ε)]: 218 (4.38), 264 (3.87); CD [MeOH, nm, (Δε)]: 223 (+5.83), 249 (−0.92); IR (KBr) ν_{max} cm^{-1}: 3410, 1725, 1651, 1636, 1592, 1507, 1455, 1285, 1117, 1024, 941, 743, 700; ^1H and ^{13}C NMR data, see Table 3; EIMS *m/z*: 666 ($[M^+]$, 2), 554 (16), 537 (8), 421 (14), 280 (26), 124 (100), 119 (25), 106 (35); HREIMS *m/z*: 660.3413 $[M^+]$ (calcd for $C_{38}H_{48}N_2O_8$: 660.3410).

*3.5. Deacylation of **9a** with 0.1% sodium methoxide (NaOMe)–MeOH*

A solution of **9a** (2.5 mg) in 0.1% sodium methoxide (NaOMe)–MeOH (1.0 mL) was stirred at room temperature for 8 h. By HPLC analysis of the reaction mixture, methyl nicotinate (**i**, t_R 5.58 min) and methyl hexanoate (**ii**, t_R 27.19 min) were identified [detection: RI, mobile phase: MeOH–H_2O (60:40, v/v), flow rate 0.7 mL/min]. The standard samples of methyl nicotinate (**i**) and methyl hexanoate (**ii**) were obtained by diazomethane methylation of commercially available nicotinic acid and *n*-hexanonic acid. The rest of reaction mixture was neutralized over Dowex HCR W2 resin (H^+ form), which was then removed by filtration. The filtrate was concentrated under reduced pressure, and the resulting product was purified by HPLC [MeOH–H_2O (80:20, v/v)] to give **9b** (1.2 mg, 90%).

*3.5.1. Compound **9b***

A white powder, $[\alpha]_D^{24}$ +35.6 (*c* 0.10, MeOH); IR (KBr) ν_{max} cm^{-1}: 3346, 1653, 1559, 1507, 1387, 1262, 1120, 1044, 938, 756, 718; ^1H and ^{13}C NMR data, see Table 3; EIMS *m/z*: 352 ($[M^+]$, 1), 334 ($[M^+–H_2O]$, 1), 286 (6), 138 (17), 120 (100), 95 (35); HREIMS *m/z*: 352.2254 $[M^+]$ (calcd for $C_{20}H_{32}O_5$: 352.2250).

3.6. Effect on food intake, visceral fat, liver weight, liver TG content, and plasma biochemicals in mice

A test sample was administered to ddY male mice (6 week old) once a day (10:00–12:00) for 3 days fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd., Japan). Body weight was measured every day before administration of the test sample. After fasting for 20 h, blood samples (*ca.* 0.2 mL) were collected from an infraorbital venous plexus and put into tubes containing 5 units heparin. Mice were killed by cervical dislocation, and then the liver was removed and weighed. Plasma TG, total cholesterol, and FFA levels were determined using commercial kits (Triglyceride E-test Wako, Cholesterol CII-test Wako, and NEFA C-test Wako, respectively). After removing the liver, *ca.* 100 mg of liver tissue was homogenized with H_2O (5 mL), and the TG concentration in the suspension was determined using Triglyceride E-test Wako.

3.7. Effects on TG content in high glucose pre-treated HepG2 cells

HepG2 cells (RIKEN) were maintained in Minimum Essential Medium Eagle (MEM, Sigma–Aldrich) containing 10% fetal bovine serum, 1% MEM non-essential amino acids (Invitrogen), penicillin G (100 units/mL), and streptomycin (100 μg/mL) at 37 °C under 5% CO_2 atmosphere. The cells were inoculated in 48-well tissue culture plate [10^5 cells/well in 200 μL/well in MEM]. After 20 h, the medium was replaced with 200 μL/well of Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (4500 mg/L) and cultured for 6 days with replacing the medium by a fresh one every 2 days. After accumulation of the lipid, the medium was exchanged to 200 μL/well of DMEM containing low glucose (1000 mg/L) and a test sample, and the cells were cultured. After 20 h, the TG and protein contents in the cells were determined by the same manner as described above. An antidiabetic agent, metformin was used as a reference compound (Muraoka et al., 2009).

3.8. Agonistic activity for PPARα

Agonistic activity for PPARα was examined using a nuclear receptor cofactor assay system (EnBio RCAS for PPARα, EnBioTec Laboratories) according to the manufacturer's instructions. This system is a cell-free assay system using nuclear receptors and cofactors to screen chemicals. The change in absorbance (450 nm) caused by GW4647, a selective PPARα agonist, at 500 nM was calculated as 100%.

3.9. Statistics

Values were expressed as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (*p*) values less than 0.05 were considered significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2013.01.004.

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