Indazole-Type Alkaloids from Nigella sativa Seeds Exhibit Antihyperglycemic Effects via AMPK Activation in Vitro

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Supporting Information

ABSTRACT: Six rare naturally occurring indazole-type alkaloids including two new compounds, 17-O-β-D-glucopyranosyl)-4-O-methylnigellidine (1) and nigelanoid (2), and four known compounds (3–6) were isolated from a defatted extract of Nigella sativa (black cumin) seeds. 17-O-β-D-Glucopyranosyl)-4-O-methylnigellidine (1) increased glucose consumption by liver hepatocytes (HeptG2 cells) through activation of AMP-activated protein kinase (AMPK). Also, this is the first report of compounds 4 and 6 from a natural source.

AMP-activated protein kinase (AMPK) is an enzyme that plays a key role in cellular energy homeostasis, and the AMPK pathway performs a central function in the regulation of glucose and lipid metabolism. The activation of AMPK can stimulate hepatic fatty acid oxidation and ketogenesis, inhibit cholesterol synthesis, lipogenesis, and triglyceride synthesis, stimulate skeletal muscle fatty acid oxidation and muscle glucose uptake, and modulate insulin secretion by pancreatic beta-cells. Moreover, AMPK has been shown to be a target for antidiabetic drugs, including metformin, and several plant natural products derived from traditional medicines.

Nigella sativa Linn. (Ranunculaceae), commonly known as black cumin, grows in Mediterranean and Middle Eastern countries, South Europe, and Southwest Asia. The seeds of N. sativa have been consumed for centuries and are widely used as a spice and traditional medicine for the treatment of various ailments including diabetes. Previous phytochemical investigations of N. sativa seeds have led to the identification of oils, saponins, flavonoids, and alkaloids. Notably, to date, N. sativa is one of only two Nigella species reported among all natural sources to contain indazole-type alkaloids.

The seeds of N. sativa have been widely studied for their antidiabetic effects, and its most abundant oil constituent, thymoquinone, is implicated as a major bioactive compound responsible for this activity. However, defatted and aqueous extracts of N. sativa seeds, from which the aforementioned rare indazole-type alkaloids were isolated, have also been reported to show antidiabetic effects. Furthermore, the in vivo antidiabetic activity of a N. sativa seed extract was reported to be mediated through the AMPK pathway, but whether indazole-type alkaloids contribute to these effects is not known. Therefore, a defatted extract of N. sativa seeds was investigated to identify bioactive antidiabetic compounds targeting the AMPK pathway. Herein, the isolation and structure elucidation of six rare, naturally occurring indazole-type alkaloids, including two new (1 and 2) and four known (3–6) alkaloids, are reported.

Compound 1, a yellow, amorphous solid, displayed a molecular formula of C_{25}H_{31}N_{2}O_{7}, as determined by 13C NMR data and an HRESIMS ion at m/z 471.2129 [M]+ (calcd for C_{25}H_{31}N_{2}O_{7} 471.2126) with 12 indices of hydrogen deficiency. In the 1H NMR data (Table 1), an AA’BB’ spin system with signals at δ_{H} 7.59 (d, J = 8.3 Hz, H-15, 19) and 7.32 (d, J = 8.3 Hz, H-16, 18), two aromatic protons at δ_{H} 7.17 (brs, H-7) and 6.75 (brs, H-5, 6), and four methylene signals at δ_{H} 4.55 (t, J = 8.3 Hz, H-15, 19) and 4.43 (t, J = 6.0 Hz, H-13), 2.34 (m, H-12) were observed, as well as a methyl signal and a methoxy signal at δ_{H} 2.59 (3H, s) and 3.81 (3H, s), respectively.

The 13C NMR (Table 1) and HSQC data revealed the presence of 25 carbon resonances, comprising two methyl, five methylene, 11 methine (six sp^2 and five sp^3), and three quaternary carbons (C-3, C-6, and C-14), an N-containing tertiary carbon (C-8), an N,N-disubstituted secondary carbon (C-2), and two oxygenated tertiary carbons (C-4 and C-17).
Note

The 1H NMR data of 1 also showed the presence of a β-glucopyranosyl moiety, the anomic proton of which resonated at δH 5.07 (1H, d, J = 7.2 Hz, H-1'). The aforementioned physical data suggested that compound 1 was likely an indazole-type alkaloid, which was supported by the fact that these naturally occurring compounds have been observed only in this genus.8

Analysis of the 2D NMR (including 1H−1H COSY, HSQC, and HMBC) data permitted the construction of the structure of compound 1. After the assignment of all the protons to their bonding carbons by the HSQC data, a hexose moiety (C-1’ to C-6’) and a subunit (C-10 to C-13) (drawn with bold bonds in Figure 1) were established by the 1H−1H COSY data. The HMBC correlation (Figure 1) from H-1 to C-17 (δC 159.8) required that it was connected to nitrogen. The HMBC correlations from H-3 to C-8 suggested that CH2-10 and N-9, were connected based on the requirement of the molecular formula and index of hydrogen deficiency. The p-configuration of the glucopyranosyl moiety was determined by acid hydrolysis. The released glucose was identified by comparison of optical rotation with an authentic sample.

The 13C NMR data of 1 and 2 are given in Table 1. The chemical shifts of two aromatic protons in the 1H NMR spectra (Table 1) of compound 2, compared to 1 in the 1H NMR spectrum, an AA'BB' spin system at δH 7.46 (2H, d, J = 8.7 Hz, H-15, 19)
and 6.97 (2H, d, \( J = 8.7 \) Hz, H-16, 18) and four methylene signals at \( \delta_c 4.51 \) (1H, m, H-10), 4.43 (1H, m, H-10), 4.34 (2H, t, \( J = 6.0 \) Hz, H-13), 2.27 (2H, m, H-11), and 2.17 (2H, m, H-12) indicated the presence of rings A (\( p \)-substituted benzene ring) and B. The HMBC correlations (Figure 1) from m, H-12) indicated the presence of rings A (\( \delta_c 71.3 \), oxygenated tertiary carbon), and C-7 (\( \delta_c 34.2 \)) indicated a consecutive linkage of C-5, C-6, and C-7 and also allowed for the attachment of Me-20 to C-6. A hydroxy group was assigned to linkage of C-5, C-6, and C-7 based on the chemical shift of C-6 (\( \delta_c 71.3 \)) and the fact that it decreased glucose levels by 29% in the cell supernatants by 8 \% as compared to the solvent control (0.1% DMSO). (B) AMPK phosphorylation status in HepG2 cells. HepG2 cells were incubated with metformin (Met) and compounds 1 and 6 (each at 25 and 100 \( \mu \)M, respectively) for 24 h, and then cell lysates were collected. Relative p-AMPK and total AMPK were measured by Western blot.

Figure 2. (A) Effect of the isolates (at 25 \( \mu \)M) on glucose consumption in HepG2 cells. HepG2 cells were acclimated to a low-glucose DMEM overnight and then treated with the isolates or metformin in fresh media. Media was collected 7 h later, and glucose concentration was determined. The values are expressed as the means ± SD of three individual samples. \( * p < 0.05 \) as compared to the solvent control (0.1% DMSO). (B) AMPK phosphorylation status in HepG2 cells. HepG2 cells were incubated with metformin (Met) and compounds 1 and 6 (each at 25 and 100 \( \mu \)M, respectively) for 24 h, and then cell lysates were collected. Relative p-AMPK and total AMPK were measured by Western blot.

for their biological effects with regard to the antidiabetic properties attributed to \( N. \) sativa seeds. In the current study, six indazole-type alkaloids (1–6) were isolated and identified from the defatted extract of \( N. \) sativa seeds. Among them, 17-O-(\( \beta \)-D-glucopyranosyl)-4-O-methylignellidine (1) and nigelanoid (2) are new compounds, and compounds 4 and 6 are being reported from a natural source for the first time. In addition, 17-O-(\( \beta \)-D-glucopyranosyl)-4-O-methylignellidine (1) showed more potent ability to regulate glucose consumption than metformin, which was mediated by activation of AMPK. These studies suggest that naturally occurring indazole-type alkaloids could contribute toward the antidiabetic properties reported for \( N. \) sativa seeds. Also, the compounds identified herein could serve as lead scaffolds for the synthesis of structural analogues with more potent antihyperglycemic activities.

### EXPERIMENTAL SECTION

#### General Experimental Procedures

Optical rotations were measured on an Auto Pol III automatic polarimeter (Rudolph Research, Flanders, NJ, USA) at room temperature. The IR spectra were recorded on a Nicolet 380 FT-IR spectrometer. The UV spectra were measured on a Shimadzu UV-2550 UV–visible spectrophotometer. 1D and 2D NMR data were recorded on a Varian 500 MHz instrument with TMS as internal standard. HRESIMS data were acquired using a synapt G2-S QTOF mass spectrometer (Waters, 

Note

In summary, previous to this study, only four indazole-type alkaloids had been reported from nature (all obtained from the \( \text{Nigella} \) genus\(^{17}\)), but none of these compounds were evaluated
Milford, MA, USA). Semipreparative HPLC separations were performed on a Hitachi Elite LaChrom system consisting of an L2130 pump, an L-2200 autosampler, an L-2455 diode array detector, and a Phenomenex Luna C18 column (250 × 10 mm, 5-μm), all operated by EZChrom Elite software. All solvents were of ACS or HPLC grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was purchased from Milli-Q (Millipore, Billerica, MA). Standards of D-glucose and metformin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant Material. N. sativa seeds were collected and authenticated by matching the macroscopic and microscopic characteristics to internal monograph and botanical reference standards by Verdure Sciences (Nobleville, IN, USA). A voucher specimen (VS-NSSP-001) has been deposited in the Heber-Youkeng Herbarium and Greenhouse, College of Pharmacy, University of Rhode Island.

Extraction and Isolation. The air-dried, ground powder of N. sativa seeds (2.0 kg) was extracted with n-hexane (4 L × 3) by maceration at room temperature (3 days each time) to afford 298.5 g of crude n-hexane extract. The residue was dried and extracted with MeOH (4 L × 3) by maceration at room temperature (3 days each time) to yield 121.1 g of MeOH extract. To further decontaminate, a portion (122.0 g) was reconstituted in MeOH (500 mL) and partitioned with n-hexane (500 mL × 3) to yield n-hexane (123.3 g) and MeOH (110.0 g) extracts, respectively. The MeOH extract (108.0 g) was chromatographed over a column of MCI gel (MeOH: H2O, 50:50 to 100:0, v/v) to yield six subfractions, B2a–B2f. Purification of subfraction B2c (194.4 mg) by semipreparative HPLC, eluting with MeOH: 

### Glucose Consumption Assay

Cells were detached from the culture flasks with a solution of 0.25% trypsin and 1 mM EDTA. Trypsin digestion was stopped by the complete culture medium. The cells were seeded into a 96-well plate at a density of 4.0 × 10^4 cells/well and cultured for 8 h. The cells were incubated with the low-glucose (1 mg/L) detection medium supplemented with 2 mM glutamine and 1% FBS. After overnight incubation in the detection media, the cells were treated for 7 h with metformin (1 μM) or the isolates (25 μM; stock solutions made in DMSO) diluted in the detection medium. The glucose concentration in the medium was determined by a glucose assay kit (Eton Bioscience) as per the manufacturer's instructions. Absorbance was measured at 490 nm, and the assay was performed using 3 replicates per test sample.

### Determination of p-AMPK by Western Blot

The cells were seeded into a 6 well plate for 8 h followed by overnight incubation in low glucose media. After 24 h treatment with metformin (1 μM) or the test compounds 1 and 6 (at 25 and 100 μM), total proteins were isolated using RIPA buffer and quantified by the bichonic assay (Pierce, Rockford, IL). Protein homogenates (20 μg/lane) were electrophoretically separated by 8% SDS-PAGE and then blottedted onto Immobilon PVDF membrane (Millipore EMD Corporation, Billerica, MA). Membranes were blocked in 5% non-fat dry milk followed by incubation with primary antibodies p-AMPK, AMPK and β-actin (Cell Signaling Technologies, Danvers, MA) overnight. Membranes were washed 3 times with tris-buffered saline with 0.1% Tween 20 (TBST) followed by incubation with respective secondary horseradish peroxidase-conjugated antibodies (Sigma-Aldrich, St. Louis, MO) for 1 h. After washing the membranes 3 times with TBST, bands were detected on X-ray films using an ECL chemiluminescence detection kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol.

### ASSOCIATED CONTENT

#### Supporting Information

The NMR and HRMS spectra of compounds 1 and 2, as well as the cell viability data are available free of charge via the Internet at http://pubs.acs.org.

### AUTHOR INFORMATION

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(1) Hardie, D. G. *Diabetes* 2013, 62, 2164–2172. 
(2) Ahmad, A.; Husain, A.; Mujeeb, M.; Khan, S. A.; Najmi, A. K.; Siddique, N. A.; Damanhouri, Z. A.; Anwar, F.; Kishore, K. *Asian Pac. J. Trop. Biomed.* 2013, 3, 337–352.

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### REFERENCES

(1) Hardie, D. G. *Diabetes* 2013, 62, 2164–2172. 
(2) Ahmad, A.; Husain, A.; Mujeeb, M.; Khan, S. A.; Najmi, A. K.; Siddique, N. A.; Damanhouri, Z. A.; Anwar, F.; Kishore, K. *Asian Pac. J. Trop. Biomed.* 2013, 3, 337–352.

(3) Mathur, M. L.; Gaur, J.; Sharma, R.; Haldiya, K. R. *J. Endocrinol. Metab.* 2011, 1, 1–8.
(4) Moretti, A.; D’Antuono, L. F.; Elementi, S. J. Essent. Oil Res. 2004, 16, 182–183.
(5) Taskin, M. K.; Caliskan, O. A.; Anil, H.; Abou-Gazar, H.; Khan, I. A.; Bedir, E. Turk. J. Chem. 2005, 29, 561–569.
(6) Merfort, I.; Wray, V.; Barakat, H. H.; Hussein, S. A. M.; Nawwar, M. A. M.; Willuhn, G. Phytochemistry 1997, 46, 359–363.
(7) (a) Morikawa, T.; Xu, F.; Kashima, Y.; Matsuda, H.; Ninomiya, K.; Yoshikawa, M. Org. Lett. 2004, 6, 869–872. (b) Morikawa, T.; Xu, F.; Ninomiya, K.; Matsuda, H.; Yoshikawa, M. Chem. Pharm. Bull. 2004, 52, 494–497.
(8) (a) Ali, Z.; Ferreira, D.; Carvalho, P.; Avery, M. A.; Khan, I. A. J. Nat. Prod. 2008, 71, 1111–1112. (b) Atta-ur-Rahman, M. S.; Malik, S.; Hasan, S. S.; Choudhary, M. I.; Ni, C.; Clardy, J. Tetrahedron Lett. 1995, 36, 1993–1996. (c) Atta-ur-Rahman, M. S.; Malik, S.; Cun-Heng, H.; Clardy, J. Tetrahedron Lett. 1985, 26, 2759–2762. (d) Liu, Y. M.; Yang, J. S.; Liu, Q. H. Chem. Pharm. Bull. 2004, 52, 454–455.
(9) (a) Fararh, K. M.; Atoji, Y.; Shimizu, Y.; Shiina, T.; Nikami, H.; Takeuchi, T. Res. Vet. Sci. 2004, 77, 123–129. (b) Zaoui, A.; Cherrah, Y.; Alaoui, K.; Hassine, N.; Amarouch, H.; Hassar, M. J. Ethnopharmacol. 2002, 79, 23–26.
(10) (a) Abdelmeguid, N. E.; Fakhoury, R.; Kamal, S. M.; Al Wafai, R. J. Diabetes 2010, 2, 256–266. (b) Pari, L.; Sankaranarayanan, C. Life Sci. 2009, 85, 830–834. (c) Chandra, S.; Murthy, S. N.; Mondal, D.; Agrawal, K. C. Can. J. Physiol. Pharmacol. 2009, 87, 300–309.
(11) (a) Meddah, B.; Ducroc, R.; Faouzi, M. E. A.; Eto, B.; Mahraoui, L.; Benhaddou-Andaloussi, A.; Martineau, L. C.; Cherrah, Y.; Haddad, P. S. J. Ethnopharmacol. 2009, 121, 419–424. (b) Rchid, H.; Chevassus, H.; Nmila, R.; Guiral, C.; Petit, P.; Chokairi, M.; Sauvaire, Y. Fundam. Clin. Pharmacol. 2004, 18, 525–529.
(12) Benhaddou-Andaloussi, A.; Martineau, L.; Vuong, T.; Meddah, B.; Madiraju, P.; Settaf, A.; Haddad, P. S. Evid.-Based Complementary Altern. Med. 2011, DOI: 10.1155/2011/538671.
(13) (a) Cerda-García-Rojas, C. M.; Coronel, A. C.; de Lampasona, M. E. P.; Catalán, C. A. N.; Joseph-Nathan, P. J. Nat. Prod. 2005, 68, 659–665. (b) Sun, Y.; Tian, L.; Huang, J.; Ma, H.; Zheng, Z.; Lv, A.; Yasukawa, K.; Pei, Y. Org. Lett. 2008, 10, 393–396.
(14) Liu, Y.; Yang, J.; Liu, Q. Chem. Pharm. Bull. 2004, 52, 454–455.
(15) Sather, A. C.; Berryman, O. B.; Rebek, J. Jr. Org. Lett. 2012, 14, 1600–1603.
(16) Tariq, M. Saudi J. Gastroenterol. 2008, 14, 105–106.
(17) Blair, L. M.; Sperry, J. J. Nat. Prod. 2013, 76, 794–812.